The role of the actin-interacting protein DRR1 in shaping neuronal stress resilience

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Zusammenfassung

Vielzählige epidemiologische Langzeitstudien weisen darauf hin, dass Stress und dessen neurobiologische Korrelate bei der Entstehung und dem Verlauf von psychiatrischen Erkrankungen mitwirken. Insbesondere soziale Belastungsfaktoren in frühkindlichen Entwicklungsphasen begünstigen die Entstehung stressbedingter psychischer Störungen, wie beispielsweise Depression. Auch Umwelt-Traumata und variierende Lebensumstände in adulten Jahren erhöhen den Krankheitsdruck. Bei vielen Patienten evoziert Stress Krankheitsperioden aufgrund von genetischer Prädisposition, der sogenannten individuellen Vulnerabilität. Allerdings ist das Wissen über die individuelle Stressanfälligkeit noch unvollständig geklärt. Vor allem neurobiologische Mechanismen, die stressvolle Ereignisse in stressbedingte psychiatrische Mechanismen manifestieren, sind unzureichend erforscht. Dies führt dazu, dass entsprechende Erkrankungen erst spät erkannt und therapiert werden.

Akuter Stress versetzt den Organismus in Alarmbereitschaft („Kampf-oder-Flucht-Reaktion"), indem das autonome Nervensystem aktiviert wird, um die Stresssituation zu bewältigen. Die dabei freigesetzten Neurotransmitter und Hormone lösen (über-) lebensnotwendige Anpassungen in Organen aus. Dadurch werden Energiereserven über die Gluconeogenese speziell zu wichtigen körperlichen Instanzen wie Muskeln oder dem Gehirn mobilisiert. Gleichzeitig setzt sich die zelluläre und humorale Immunabwehr herab. Im zentralen Nervensystem aktiviert Stress die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse), wodurch die Ausschüttung des „Stresshormons" Cortisol reguliert wird. Cortisol wiederum besitzt einen Feedback-Mechanismus zum zentralen Nervensystem, um die Stressreaktion nach der akuten Belastungssituation zu stoppen und den Grundzustand wieder herzustellen. Mineral- und Glucocorticoid-Rezeptoren stellen bei der Rückkopplung der HPA-Achse, die wichtigsten Regulationselemente dar.

Hält Stress über einen längeren Zeitraum an, wird von chronischem Stress gesprochen. Hierbei bleibt eine vollständige Rückregulation der HPA-Achse aus, was zu maladaptiven Anpassungen führt. Neben Belastungen des Herz-Kreislaufsystems, wird der Stoffwechsel gestört und die Immunabwehr beeinträchtigt. Daneben befördern Lern- und Gedächtnisstörungen neuropsychiatrische Erkrankungen. Die Erforschung von Resilienz-Mechanismen, stellt einen neuen wissenschaftlichen Ansatz dar, um präventiv gegen die Entstehung von Depressionen vorzugehen. In diesem Kontext, wird Resilienz als schnelle Erholung oder Aufrechterhaltung mentaler Gesundheit nach Perioden schwerer psychologischer oder körperlicher Belastung definiert. Interessanterweise weisen verschiedene Individuen unterschiedliche Stressresilienz auf.

Heutzutage werden Tiermodelle verwendet, um die zugrundeliegenden neurobiologischen Mechanismen von Stressresilienz genauer zu erforschen. Chronische soziale Ablehnung fungiert dabei als Tiermodell erster Wahl, um Stress resiliente und Stress suszeptible Mäuse zu erzeugen. Unzählige Studien an Nagern zeigten, dass soziales Verhalten mit Stress stark verknüpft ist und die Freisetzung von Glucocorticoiden die Glutamat-Neurotransmission im Präfrontalen Kortex und im Hippocampus beeinflusst, wodurch die kognitive Leistung beeinträchtigt wird. Die dabei einhergehenden Veränderungen in der neuronalen Erregbarkeit stellen einen Kernbefund stressassoziierter mentaler Störungen dar.

Im Gehirn agieren Synapsen als spezialisierte Einheiten, die Signalübertragung zwischen Neuronen ermöglichen. Zur Impulsweiterleitung bilden Nervenzellen ein langes Axon, dass sich am Ende verzweigt und mehrere Dendriten anderer Neurone kontaktiert, um Synapsen zu bilden. Die Informationsweiterleitung im zentralen Nervensystem findet mehrheitlich über chemische Synapsen statt. Auf diese Weise löst ein eingehender Nervenimpuls die Freisetzung von in Vesikel verpackten Neurotransmittern aus der Präsynapse in den synaptischen Spalt aus. Durch den synaptischen Spalt diffundieren diese Neurotransmitter zur postsynaptischen Membran, und werden dort von spezialisierten Rezeptoren gebunden. Schließlich induziert die Aktivierung postsynaptischer Rezeptoren eine Signalkaskade zur Signalweiterleitung. Es herrscht eine Varietät an Nervenzellen, die entweder exzitatorisch oder inhibitorisch wirken. Glutamat stellt den Haupttyp exzitatorischer Neurotransmitter dar, der an verschiedene Rezeptorklassen bindet, unter anderem an AMPA (Englisch: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid) Glutamat-Rezeptoren, die als Tetramer vorliegen. Die vier Untereinheiten werden in GluR1–4 (oder GluA1–4) eingeteilt. Zudem besitzen exzitatorische Neuronen Dornenfortsätze, die sogenannten „spines“ (Englisch für Dornenfortsatz), auf ihren Dendriten. „Spines“ bilden ein Köpfchen auf deren Ende die postsynaptische Dichte mit verschiedenen Rezeptoren liegt, und werden in der Regel durch einen Hals mit dem dendritischen Schaft verbunden. Der „spine“ Kopf bildet ein separates biophysikalisches Kompartiment. Strukturell betrachtet gibt es vier verschiedene „spine“ Klassen, die sich durch ihre individuelle Morphologie in ihrer Funktion unterscheiden. „Mushroom spines“ haben einen kurzen Hals mit sehr großem Köpfchen und stellen die stärkste synaptische Verbindung dar. „Long thin spines“ werden durch einen kleineren Kopf ausgezeichnet und sind weniger stabil als die vorigen. „Stubby spines“ sind unausgereifte Dornenfortsätze, ohne Hals. Und „filopodia“ ohne Köpfchen stellen Vorstufen von „spines“ dar. Durch strukturelle Plastizität sind „spines“ in der Lage ihre Morphologie zu verändern und zwischen den einzelnen Reifegraden zu wechseln und somit die Verbindung und Aktivität zwischen Nervenzellen zu beeinflussen. Nervenzellen, inklusive der Dornenfortsätze, werden von einem Aktin-Zytoskelett aufgebaut. Ausschlaggebend für die Lokalisation und Funktion von AMPA-Rezeptoren und sofern auch für

die glutamaterge Signaltransduktion an Synapsen sind Aktin-Dynamiken. Stressinduzierte Aktin-Dynamiken haben einen relevanten Einfluss auf die Modulation von komplexem Verhalten und Aktin-interagierende Proteine (ABP) sind durch Stress und Glucocorticoide reguliert. Außerdem zeigte sich eine starke Beteiligung von AMPA-Rezeptoren an Mechanismen, die Stressresilienz fördern.

Forschungsinhalt dieser Arbeit war die Untersuchung des Stress- und Glucocorticoid regulierten Gens *Drr1* (Englisch: down regulated in renal cell carcinoma) – auch *Fam107a* – im murinen Gehirn. Bisher war bekannt, dass DRR1 die individuelle Stressempfindlichkeit moduliert und die kognitive Leistungsfähigkeit verbessert. Vermutlich indem es durch seine Interaktion mit Aktinfilamenten die synaptische Wirksamkeit erhöht. Unter Einsatz von chronischem Stress durch soziale Ablehnung (Englisch: chronic social defeat stress (CSDS)) steigt bei Mäusen das *Drr1* mRNA-Level in der hippocampalen CA3-Region. Beim CSDS-Verhaltensparadigma werden männliche C57BL/6J-Mäuse wiederholt und über mehrere Tage als Eindringling in den Käfig eines CD-1 Männchens eingebracht, welches durch aggressives Verhalten den Eindringling bekämpft. Andauernde visuelle und olfaktorische Aussetzung gegenüber dem Aggressor, führen zu chronischem Stress in der C57BL/6J Maus. Die Tiere werden anschließend mithilfe eines sozialen Interaktionstest in stress resiliente und suszeptible Tiere eingeteilt. Somit ist dieses Mausmodell besonders gut geeignet, um die Neurobiologie von Resilienz zu untersuchen. DRR1 scheint insgesamt eine molekulare Komponente darzustellen, die eine Verbindung zwischen stressvollen Ereignissen und langanhaltenden neuronalen Veränderungen herstellt. Erstaunlicherweise, ist der Hippocampus eine besonders stresssensitive Hirnregion mit neuroanatomischen Unterschieden zwischen resilienten und suszeptiblen Tieren. Generell, führt chronischer Stress in dieser Region zu einem Rückgang dendritischer Verzweigungen und einer reduzierten Anzahl als auch morphologisch veränderter „spines“. Außerdem beeinträchtigt Stress hippocampus-abhängige kognitive Vorgänge, die sich durch Adenovirus-vermittelte und regionsspezifische Hochregulation von DRR1 verbessern. Darüber hinaus verändert Überexpression von DRR1 die elektrophysiologischen Eigenschaften von hippocampalen Neuronen, als auch deren Morphologie. Zusammengenommen, ließen uns diese Ergebnisse vermuten, dass DRR1 eine aktive stress-ausgleichende und Resilienz-fördernde Rolle einnimmt.

DRR1 und AMPA-Rezeptoren besitzen das gleiche Promotorprofil und werden co-reguliert und co-exprimiert. Allerdings war bisher noch nicht bekannt, ob DRR1, ähnlich wie andere Aktin-bindende Proteine, direkt den Einbau sowie den Transport von AMPA-Rezeptoren zur postsynaptischen Membran steuern und damit die neuronale Reizbarkeit und Stressresilienz beeinflussen. Aufgrund dessen haben wir in dieser Forschungsarbeit die zentrale Hypothese aufgestellt, dass DRR1 über spezifische Modulation neuronaler Aktin-Dynamiken Resilienz-

fördernde Eigenschaften besitzt, wodurch kognitive Leistungsfähigkeit, als auch synaptische Plastizität, das heißt die neuronale Anpassungsfähigkeit an sich ändernde Umweltbedingungen, verbessert und so das soziale Verhalten stärkt.
Der damit einhergehenden AMPA-Rezeptor Transport, könnte ein effizienter Kern-Mechanismus sein, um neuronale Funktionen und komplexes Verhalten während Stress zu regulieren.

In dieser Arbeit wurde zunächst mittels Immunfluoreszenz die Verteilung des endogenen DRR1 Proteins unter basalen Bedingungen im murinen Gehirn detektiert. Dabei zeigte sich, dass DRR1 flächendeckend vorliegt, jedoch zu größten Teilen im Hippocampus und Cerebellum. Dies ist deckungsgleich mit bisherigen *in-situ*-Hybridisierungsdaten (Allen Mouse Brain Atlas), die *Drr1* mRNA Expression in genau diesen Regionen zeigen. Durch Kombination mit zellspezifischen Antikörpern, wurde ferner die Expression von DRR1 nicht nur in Neuronen, sondern auch in Mikroglia und Astrozyten nachgewiesen. Bei Letzteren findet sich das Protein vor allem in astrozytären Endfüßchen, die den Kontakt zum Hirnblutgefäßsystem herstellen.

Anschließend wurden primäre hippocampale Neuronenkulturen angefertigt, um die **Hypothese** zu testen, dass die DRR1 Expression mit der AMPAR Funktion korreliert, um stressassoziierte Konsequenzen zu modulieren. Hierfür wurde die stressbedingte Hochregulation von DRR1 in den Kulturen durch Transduktion mit einem nicht-pathogenen Adeno-assoziierten viralen Vektors erzeugt. Auf gleiche Weise wurde ein Adeno-assoziierter Virus genutzt, um eine Suppression des Proteins via RNA-Interferenz hervorzurufen. Western Blot Analysen zeigten eine erfolgreiche Suppression bzw. Überexpression des Proteins in neuronalen Kulturen. Im weiteren Verlauf, stellte sich heraus, dass die AMPA-Untereinheit GluR2 mit dem zellulären DRR1-Level korreliert ist. Wobei eine Hochregulierung von DRR1 mit einer Zunahme von GluR2 einhergeht und eine Suppression mit einer Abnahme. GluR2 Untereinheiten sind der bestimmende Faktor bei der Signalweiterleitung, indem sie den Calciumfluss in die Nervenzelle beeinflussen. AMPA-Rezeptoren mit GluR2 Einheiten sind Calcium undurchlässig. Da GluR2 die einwärts gerichteten Calcium-Ströme hemmt, reduzieren GluR2 enthaltende AMPA-Rezeptoren die neuronale Leitfähigkeit. Das führt wiederum zu einer Desensibilisierung der AMPA-Rezeptoren gegenüber des Neurotransmitters Glutamat. Stress suszeptible Mäuse zeigen insgesamt eine erhöhte Anzahl an AMPARs mit einer Untereinheiten Komposition, die eine geringere Rezeptorsensitivität favorisiert. Daher wird eine erhöhte GluR2 zu GluR1 Verteilung allgemein als Depressionsmodell in Mäusen gesehen. Da *in vivo* Experimente zeigen, dass eine spezifische Hochregulation des DRR1 Proteins im Hippocampus zu einer verbesserten kognitiven Leistung führt, ist es sehr wahrscheinlich, dass der Gesamtgehalt der AMPA-Rezeptoren durch eine Hochregulierung von DRR1 beeinflusst wird und nicht nur die Untereinheiten-Komposition, die ansonsten zu einer Verschlechterung der Lernleistung führen würde.

Da unsere Untersuchungen ergaben, dass DRR1 einen direkten Einfluss auf AMPARs hat, wurde durch Zuhilfenahme des „proximity ligation assays“ (PLA) analysiert, ob diese auf molekularer Ebene interagieren. Bei dieser Analyse zeigt die Sichtbarwerdung eines amplifizierten Signals eine Wechselwirkung zwischen zwei Proteinen an, die innerhalb eines 40 nm Abstandes beieinander liegen. In kultivierten primären hippocampalen Kulturen wies das Experiment auf ein starkes Signalvorkommen entlang der Dendriten und vor allem Nahe des Zellkörpers hin. Somit besteht mit hoher Wahrscheinlichkeit eine direkte Wechselwirkung zwischen DRR1 und GluR2 Untereinheiten.

Um die weiterführende **Hypothese** zu testen, dass DRR1 durch seine aktinbindenen Eigenschaften, die Glutamatrezeptor Verteilung und Funktion beeinflusst, wurde der subzelluläre Transport von AMPA-Rezeptoren unter verschiedenen DRR1 Expressionsleveln in neuronalen Kulturen untersucht. Mittel zum Zweck war der „newly inserted AMPAR assay“, der neu eingebauten AMPARs nach Stimulation durch Fluoreszenz-Markierung erfasst. Im Vergleich mit Kontrollgruppen zeigte sich eine Abnahme in neu-eingebauten AMPARs nach neuronaler Stimulation, wenn das DRR1-Protein supprimiert wird. Bei einer Überexpression von DRR1 war das Ergebnis weniger eindeutig. Hauptsächlich wurde kein signifikanter Unterschied gefunden, wobei insbesondere ein Experiment aus der Reihe fiel. In diesem Fall ist eine größere Stichzahl angebracht, um herauszufinden, ob eine erhöhte Anzahl an DRR1 Protein einen negativen Einfluss auf den Oberflächentransport von AMPARs hat, oder das als Ausreißer geltende Experiment anderen Schwankungen wie beispielsweise der Qualität der Zellkultur unterlag.

In dem **zweiten Teil** dieser Arbeit sollten phänotypische Verhaltensweisen der Mäuse, die durch unterschiedliche DRR1-Level im Hippocampus entstehen, mit potentiellen Veränderungen der neuronalen Morphologie korreliert werden. Über virale Transfektion wurde DRR1 in primären hippocampalen Kulturen überexprimiert oder supprimiert. Der adeno-assoziierte Vektor enthielt ein Codon für das grün fluoreszierende Protein (GFP), welches bei erfolgreicher Zell-Transfektion in der kompletten Zelle exprimiert wird. Dadurch ist die gesamte Zellmorphologie sichtbar. Einzelne neurale Zellen verblindet und randomisiert aufgenommen und digital mit dem „Filament-Tracer“ (Imaris) rekonstruiert. Die anschließende Sholl-Analyse diente dazu, die Anzahl der Dendriten innerhalb einzelner Intersektionen um den Zellkörper herum zu erfassen. Die Abstände der Umkreise waren in 5 μm Schritten angelegt. Des Weiteren wurden die Gesamtlänge als auch die Verzweigungspunkte der Dendriten erfasst. Nach Suppression von DRR1 zeigten sich keine signifikanten Veränderungen im Vergleich zur Kontrollgruppe. Allerdings ist zu betonen, dass eine Tendenz zu einer kürzen Gesamtlänge erkennbar wurde. Im Vergleich zur Kontrollgruppe erscheint zudem, dass die Dendriten in Zellkörpernähe in ihrer Anzahl reduziert sind. Unter DRR1

Überexpression konnte eine umgekehrte Korrelation festgestellt werden. Besonders die Sholl-Analyse zeigte eine erhöhte Dendritenzahl mit signifikanten Unterschieden besonders in distalen Dendritenbereichen.

„Spines" unterliegen synaptischer Plastizität und können mit neuronaler Aktivität korreliert werden. Daher haben wir im weiteren Verlauf Anzahl und Morphologie von „spines" in den verschiedenen Konditionen untersucht. Interessanterweise führt eine Abnahme von DRR1 zu einer Abnahme der „spines". Diese Abnahme ist vor allem auf die signifikant reduzierte Zahl der unreifen „filopodia" und „long thin spines" zurückzuführen. Wobei die Anzahl der „mushroom spines" gegenüber der Kontrollgruppe gleichbleibend ist. Bei endogener Zunahme von DRR1 ist die „spine"-Zahl unverändert. Bei genauerer Betrachtung zeigt sich allerdings eine Umverteilung ihrer Komposition, die gegensätzlich korreliert ist als bei DRR1 Suppression. Signifikant geringer ist die Zahl der ausgereiften „mushroom spines". Dies geht mit einer Zunahme an „filopodia" und „long thin" einher, was als kompensatorischer Mechanismus dienen könnte, um die Gesamtzahl auf gleichem Niveau zu halten.

Hoch- und Runterregulation von DRR1 über virale Vektoren stellt einen abgekoppelten Prozess der physiologischen Stressreaktion dar. Um die physiologische Stressreaktion global zu imitieren, wurden primäre hippocampale Neuronenkulturen mit Dexamethason, einem künstlichen Glucocorticoid Agonisten, behandelt. Es zeigte sich, dass Glucocorticoid-vermittelte Stimulation zu einer erhöhten Expression von DRR1 in hippocampalen Neuronen führt und gleichzeitig mit einer Zunahme an GluR2 Untereinheiten korreliert. Dies stimmt mit den Resultaten der viral-vermittelten DRR1 Hochregulation überein und verdeutlich, dass eine Stressreaktion endogenes DRR1 erhöht. Eine zunehmende Insertion von AMPA-Rezeptoren an der Oberflächenmembran nach neuronaler Stimulation konnte nicht nachgewiesen werden, allerdings sind Tendenzen dahingehend zu sehen. Da viral vermittelte DRR1-Zunahme um ein Vielfaches höher ist, als die Glucocorticoid-vermittelte DRR1-Zunahme, könnte ein längerer oder größerer Stressreiz eine erhöhte DRR1 Expression und somit AMPAR-Zunahme verursachen. Mit diesem experimentellen Ansatz konnte gezeigt werden, dass die Adenovirus-vermittelte DRR1 Überexpression, eine geeignete Methode ist, um den physiologischen DRR1 Anstieg unter Stressbedingungen zu imitieren und näher zu untersuchen.

Im Zusammenhang mit der Entstehung von Depressionen, übt chronischer Stress einen starken Einfluss auf die Größe, Anzahl und das Volumen kortikaler und limbischer „spines" aus. Besonders morphologisch plastische Areale, wie der Hippocampus, unterlaufen strukturellen Veränderungen während Stress. Daher wird chronischer Stress in Verbindung mit sozialer Ablehnung als Modell genutzt um depressives Verhalten in Tieren zu erzeugen. Bei Mäusen weisen

30% der Tiere eine Stressresilienz auf. Das Unvermögen adäquat mit stressvollen Lebensumständen umzugehen und das Ausbleiben von biologischen als auch psychischen Adaptionen wird als Prädisposition gesehen neuropsychiatrische Krankheiten zu entwickeln. Der Umfang sozialer Vermeidung wird im Mausmodell als Repräsentation von resilientem und vulnerablem Stressverhalten angesehen. In dieser Studie wurde in Kollaboration mit dem Mueller Labor in Mainz (Klinik für Psychiatrie und Psychotherapie, Universitätsmedizin JGU, Mainz; Leibniz Institut für Resilienzforschung, Mainz) C57Bl/6J Tiere, die GFP unter dem Thy1 Promotor exprimieren mithilfe des modifizierten sozialen Interaktionstests in resiliente ($R^{+/-}$), vulnerable ($R^{-/-}$) und nicht-lernende Gruppen ($R^{+/+}$) eingeteilt, nachdem sie chronischem sozialen Stress ausgesetzt wurden. Unabhängig vom DRR1-Gehalt wurden *in situ* Analysen unternommen, um „spine" Klassen und deren Dichte in den einzelnen Verhaltensgruppen inklusive einer unbehandelten Kontrollgruppe in der CA3- und CA1-Region zu untersuchen. Zusammengefasst war die Gesamtzahl der „spines" in den einzelnen Gruppen in beiden Regionen im Vergleich zu der Kontrollgruppe unverändert. Interessanterweise war die Verteilung der einzelnen Unterklassen in der CA1 Region unterschiedlich, wobei resiliente Tiere einen analogen Phänotyp zu der Kontrollgruppe aufwiesen. Die Gruppen $R^{-/-}$ und $R^{+/+}$ zeigten reduzierte „stubby spines" und gleichzeitig erhöhte „mushroom spines" mit einer Tendenz zu mehr unreifen „spines". Dieses Ergebnis spiegelt unsere *in vitro* Daten, die bei einer DRR1 Erhöhung eine Zunahme in unreifen „spines" zeigten.

Resiliente Tiere unterscheiden zwischen Aggressor und harmlosen Artgenossen. Da keine hippocampale strukturelle Plastizität bei resilienten Tieren in Erscheinung tritt, vermuten wir andere Mechanismen, die den Lernprozess unterstützen, wie beispielsweise die Stärkung synaptischer Verbindungen durch homöostatische Plastizität. Dies Beinhaltet Veränderungen in der Anzahl und Komposition postsynaptischer Glutamatrezeptoren, als auch die feinmaschig regulierte Freisetzung von Neurotransmittern aus der Präsynapse. In den beiden anderen Verhaltensgruppen ($R^{-/-}$ und $R^{+/+}$) deuten die Analysen auf strukturelle Plastizität hin. Mittlerweile ist bekannt, dass Induktion von Stress zu einem Schwund und einer Größenminderung von „spines" führt. Da jedoch unsere Untersuchungen zeigten, dass „mushroom spines" in ihrer Anzahl in der CA1 Region bei den entsprechenden Versuchsgruppen zunehmen, vermuten wir einen kompensatorischen Mechanismus dahinter. Wenn präsynaptische Freisetzung von Neurotransmittern reduziert ist, könnten sich postsynaptische „spine" Köpfchen und damit die postsynaptische Dichte vergrößern, um die Wahrscheinlich einer Signalweiterleitung zu erhöhen. Dies schlägt sich jedoch in maladaptivem Sozialverhalten nieder. LTP-Studien stellten die elektrische Signalweiterleitung in resilienten und vulnerablen Mäusen nach chronischem sozialen Stress gegenüber und fanden reduzierte LTP in vulnerablen Tieren. Unter Einbeziehung unserer Ergebnisse, könnten bei der CA1-CA3 LTP Regulation besonders „stubby spines" eine Rolle

spielen. Um dies weiter zu erforschen wären weitere elektrophysiologische Untersuchungen von großer Bedeutung.

Unser **Hauptziel** ist es die molekularen und zellulären Mechanismen zu verstehen, wie das Aktin- und Glucocorticoid-regulierte DRR1 Protein im Speziellen und neuronale Aktin-Dynamiken und AMPAR Regulation im Weiteren, die Stressantwort regulieren. Dafür wurden im Kontext dieser Arbeit konditionale und hirnregionsspezifische DRR1 knockout Mäuse generiert. In diesen $DRR1^{l/l}$_(CamKIIα)Cre^{+} Mäusen wird DRR1 in der zweiten postnatalen Woche im Vorderhirn und im limbischen System durch Expression der Cre-Recombinase unter dem CamKIIα Promotor inaktiviert. Western Blot Analysen von Hirnlysaten zeigten eine erfolgreiche DRR1 Reduktion um 34–60 Prozent (Abhängig von der Hirnregion) bei DRR1 Mutanten im Vergleich zu Cre-negativen Wurfgeschwistern. Nach eingehender Charakterisierung von DRR1 Mutanten, sollen diese Mäuse chronischem sozialen Stress unterlaufen, um unsere Hypothese zu testen, dass stress-induzierte Hochregulation von DRR1 stress-bezogene negative Konsequenzen im sozialen Verhalten ausbalanciert und so einen Teil zur Stressresilienz beiträgt. Außerdem sollen akute Hirnschnitte von gestressten und ungestressten Mutanten genutzt werden um elektrophysiologische Daten zu generieren und somit die Signaltransmission zu untersuchen. Daneben, können morphologische Analysen und pharmakologische Versuche den Beitrag von DRR1 in struktureller Plastizität bei Stressresilienz aufdecken.

Aufeinanderfolgender **akuter Stress** löst bei Mäusen ebenfalls Beeinträchtigungen in kognitivem Verhalten mit Verlusten an „spines“ aus. Im Vergleich zu chronischem Stress ist der Einsatz von akutem Stress weniger invasiv sowie weniger zeitintensiv. Untersuchungen der akuten Stressantwort könnte neue Einblicke in potentielle Stress-Bewältigungsmechanismen geben. So führt akuter Stress oft zu kurzzeitigen Schäden, welche auf homöostatischem Weg ausgeglichen werden können. In der Wissenschaft ist allgemein bekannt, dass therapeutische Behandlungen von neuropsychiatrischen Erkrankungen bessere Wirksamkeit zeigen, wenn diese früh nach dem Auftreten der Erkrankung erfolgen, solange die Funktionsstörung noch dynamisch veränderbar ist. Um die Entstehung von neuropsychiatrischen Erkrankungen, die mit chronischem Stress assoziiert sind, besser zu verstehen, ist es daher ebenso wichtig die frühe Krankheitsphase zu betrachten. Resilienz-Mechanismen und der Umgang der Organismen mit stressvollen Situationen könnten so besser identifiziert werden. Außerdem könnten früh einsetzende Behandlungen bei Krankheitsausbruch, langfristigen und dysfunktionalen sekundären Erkrankungen vorbeugen. Nach akutem Stress bei Mäusen zeigt sich, dass *Drr1* mRNA bereits nach vier Stunden hochreguliert ist, die Protein Expression ist allerdings erst nach acht Stunden detektierbar. Erst wenn das Protein hochreguliert ist, pendelt sich die Hippocampus-abhängige Kognition wieder

auf Normalniveau ein – dem von ungestressten Tieren gleichend. Da immunhistochemische Analysen in dieser Arbeit zeigten, dass DRR1 unter basalen Bedingungen in astrozytischen Endfüßen vorliegt, und aus weiteren Studien bekannt ist, dass sozialer Stress neurovaskuläre Pathologien auslöst, die zu depressivem Verhalten in Mäusen führen, wurde hier der Zusammenhang von DRR1 und Endothelzellen im Mäusehirn untersucht. Mäusegehirne wurden in diesem Zusammenhang immunhistochemisch nach akutem Stress oder Dexamethason-Injektion untersucht und mit Kontrollgruppen verglichen. Fluoreszenz-Doppelmarkierungen zeigten eine Überlappung von DRR1 und dem Endothelzellmarker Podocalyxin im Hippocampus von Mäusen die mit akutem Stress oder Dexamethason behandelt wurden. Da Tiere acht Stunden nach akutem Stress eine Normalisierung in kognitivem Verhalten zeigen und dies mit einer Zunahme von DRR1 in Endothelzellen korreliert, nehmen wir an, dass das Protein in der Integrität der Blut-Hirn-Schranke impliziert ist.

Zusammengefasst deuten unsere Daten darauf hin, dass DRR1 als Aktin-bindendes und Glucocorticoid-sensitives Protein strukturelle Plastizität von hippocampalen „spines“ beeinflusst. Obendrein interagiert es direkt mit AMPA-Rezeptoren und ist mit hoher Wahrscheinlichkeit am intrazellulären Transport von und zu der dendritischen Oberflächenmembran dieser Glutamat-Rezeptoren beteiligt. Außerdem zeigen unsere Daten, dass DDR1 von weiteren Zellen im zentralen Nervensystem exprimiert wird. Insbesondere ist das Vorkommen von DRR1 in astrozytischen Endfüßchen und Endothelzellen interessant und veranschaulich, dass DRR1 als Integrator der Zell zu Zell Kommunikation agiert und somit stressinduzierte Modifizierungen an der neurovaskulären Einheit integriert. *In vivo* konnte nachgewiesen werden, dass resiliente Tiere in der hippocampalen Region keinen phänotypischen Unterschied zu ungestressten Tieren zeigen und morphologische Veränderungen fast ausschließlich in suszeptiblen und nicht lernenden Tieren zu finden sind. Werden diese Befunde mit vorhandenen Verhaltensdaten integriert, können wir darauf schließen, dass DRR1 Resilienz gegenüber Stress begünstigt, zu diesem Zweck allerdings in einem richtigen Gleichgewicht vorliegen muss.

Summary

A plethora of epidemiological studies points to the fact that stress as well as their neurobiological correlates play a pivotal role in the genesis of neuropsychiatric diseases. Especially social stress factors in developmental stages, traumatizing events, changing life situations and genetic predispositions, known as individual vulnerability, favor incidences of stress induced mental disorders. Because knowledge about individual stress vulnerability and the neurobiological mechanisms involved in the manifestation of stressful events in neuropsychiatric diseases has not been sufficiently explored, these malfunctions are often lately recognized and treated.

Acute stress is identified as a "fight-or-flight' response and shifts the organism on alert, activating the autonomic nervous system to cope with the stressful situation. The released neurotransmitters and hormones cause vital adaptations in the organs. Within the central nervous system, the hypothalamic-pituitary-adrenal (HPA) axis is activated and regulates the release of the stress-hormone cortisol. To establish the basal condition after a stressful event, cortisol operates in a feedback mechanism with mineral- and glucocorticoid-receptors as the main regulatory elements. Upon persistent or chronic stress, the counter-response is lacking, which promotes maladaptation of the entire body system. Negative impact on the brain is manifested in the form of cognitive decline and learning disabilities, and even the onset of neuropsychiatric diseases. Resilience research is a new attempt to find solutions to prevent the development of mental disorders after stress. In this context, resilience is described as the rapid recovery or maintenance of mental health after aversive life events. Interestingly, some individuals show higher stress resilience than others, and researchers aim to dissect the underlying mechanisms. Nowadays, well-established mouse models are used to study resilience mechanisms. Mainly after exposure to chronic social defeat stress (CSDS) and subsequent categorization into stress resilient and stress susceptible animals using a social interaction test. Studies in rodents revealed a strong correlation of altered social interaction after stress exposure and the release of glucocorticoids and enhanced glutamate neurotransmission in the prefrontal cortex and the hippocampus. The accompanying shift in neuronal excitability represents the key component of mental dysfunction.

Signal transmission between neuronal cells occurs at synapses, which are specialized units. Synapses form connections between presynaptic branched axons that contact multiple dendrites of the postsynaptic cell. The predominant type of these units are the chemical synapses. In response to a signal, neurotransmitters are released from presynaptic vesicles into the synaptic cleft. Thereafter they diffuse to the presynaptic membrane, binding to specific receptors to activate a downstream signaling cascade. In the case of excitatory synapses, glutamate is the main excitatory neurotransmitter in the brain. AMPA (*a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic-acid*) receptors

are excitatory, tetrameric glutamate receptors and consist of the GluR1–4 (also referred to as GluA1–4) subunits. Excitatory neurons built their synapses on spines located along dendrites including postsynaptic density with different receptors. Spines are specialized structures that connect their heads to the dendritic shaft via a neck. The head serves as separate biophysical compartment. Spines can be divided in four major classes that differ in morphology and function. Spines are subject to structural plasticity and are thus able to change their morphology and influence neuronal activity. Actin is the main cytoskeletal protein in spines, and actin dynamics are critical for AMPA receptor trafficking, localization, and glutamate-induced neuronal transmission. AMPA-receptors in the hippocampus were found to play a role in stress resilience mechanisms. Moreover, stress-induced actin-dynamics showed a significant impact on the modulation of complex behavior and actin-interacting proteins are additionally affected by stress and glucocorticoids.

Subject of this thesis was to investigate the actin-interacting and glucocorticoid-sensitive protein DRR1 (or Fam107a) and its role in promoting stress resilience in the murine hippocampus. Previous findings identified DRR1 as modulator of the individual stress response. Various stressors increase *Drr1* mRNA in the hippocampus. Further, DRR1 protein was shown to improve cognitive performance. Consequently, this protein constitutes a molecular factor linking stress events to long-lasting changes in neuroanatomical structures. In particular, the hippocampus is an extremely stress- sensitive brain region that exhibits neuroanatomical differences between resilient and susceptible mice. In addition, chronic social stress was found to decrease dendritic arborizations and shrink spine size. Further studies showed electrophysiological alterations when DRR1 was upregulated in hippocampal CA3. These results suggest the hypothesis that DRR1 is a good candidate for modulating stress resilience and may actively facilitate stress resilience through its actin-binding properties. Apart from this, DRR1 is co-expressed with AMPA receptors and shares the same promoter profiles. Interestingly, it is well known that actin-binding proteins directly regulate actin trafficking to the postsynaptic membrane.

We hypothesize that DRR1 specifically modulates neuronal actin dynamics through its actin-binding properties and promotes resilience through synaptic plasticity, leading to improved cognitive performance and social behavior. The accompanying AMPA receptor transport may provide an efficient way to regulate neuronal function and complex behavior during stressful situations.

First, the distribution of DRR1 protein under basal conditions was evaluated in mouse brain by fluorescent immunohistochemistry. The results revealed ubiquitous expression of the protein in the brain, but primarily in the cerebellum and hippocampal CA3 and CA1 regions. Co-

staining with different cell marker proteins showed DRR1 expression in neurons, microglia, and especially astrocytic end-feet that make contact with brain vessels.

To test whether DRR1 and AMPA receptor function correlate to modulate stress-associated consequences, primary hippocampal neuronal cultures were transduced with adeno-associated virus (AAV) vector to introduce additional DRR1 DNA for overexpression of the protein or shRNA for repression of *Drr1* mRNA via RNA-interference. Western Blot analysis of lysed and treated cultures showed a positive correlation between AMPA receptor subunit GluR2 and DRR1 amounts. More specifically, excess of DRR1 expression increased GluR2 amounts, and a suppression of the protein had opposing effects. Further, untreated neuronal cultures were utilized to test direct interaction between AMPARs and DRR1 by applying a proximity ligation assay (PLA). This method is used to visualize the interaction of two proteins by antibody-tagging, rolling circle polymerase chain reaction, and consequent signal amplification. By using antibodies against DRR1 and GluR2, we detected PLA-signals throughout the whole dendritic tree, indicating interaction between DRR1 and AMPARs. To address the question of whether DRR1 affects AMPAR trafficking to the postsynaptic membrane we took advantage of the "newly inserted assay" after virally suppressing or overexpressing DRR1 in primary hippocampal neuronal cultures. This method masks existing GluR2 subunits on the cell surface and fluorescently labels newly inserted receptors after neuronal stimulation. Suppression of DRR1 revealed that fewer new GluR2 subunits were inserted compared with controls. Moreover, when DRR1 was overexpressed, the results indicated no changes in GluR2 insertion.

In the second part, we aimed to correlate the behavioral phenotypes originating from *in vivo* overexpression and suppression of DRR1 in the mouse hippocampus with potential alterations in neuronal morphology. To this end, *in vitro* analysis was performed using AAV-transduced primary hippocampal cultures overexpressing or suppressing DRR1. Synchronously, the viral vector contained a green fluorescent protein (GFP) that was expressed throughout the complete neural cell. GFP staining was used to verify successful transfection and to reconstruct dendrite arbors and dendritic stretches for spine classification. DRR1 suppression tended to result in reduced total dendrite length and fewer dendritic branches. Furthermore, a reduction in endogenous DRR1 is associated with a reduced total number of spines, caused in particular by a reduced number of immature spine classes – namely long thin spines and filopodia. In contrast, mature mushroom spines and stubby spines were not affected. Overexpression of DRR1 in cultured hippocampal neurons tended to increase the total length of dendrites, more branch points and larger dendritic arbor in comparison to controls. The total number of spines was not affected.

However, the number of mature mushroom spines was significantly decreased, but this was compensated by an increased number of immature long thin spines and filopodia.

Viral up- and downregulation of DRR1 represents an uncoupled process of the physiologically stress response. Hence, to mimic a global physiological response, primary hippocampal neuron cultures were treated with the artificial glucocorticoid dexamethasone (DEX). After stimulation with DEX, an increase of endogenous DRR1 was detected, accompanied by increased expression of the GluR2 subunit. An increased in newly inserted AMPARs after neuronal stimulation was not detected. Since viral DRR1 expression is considerably higher and mimics the *in vivo* DRR1 increase after chronic stress induction, we speculated that a higher DEX dose and treatment is necessary to elicit the same response. Nevertheless, these results show that overexpression and suppression of DRR1 is a useful method to unravel the role of DRR1 and study its function in the stress response and resilience mechanisms.

Chronic social defeat stress (CSDS) is widely used in mouse models to study the effects of stress and resilience. A variety of studies have shown that spine numbers, size and volume decrease in rodents following chronic stress, generating depressive-like states in rodents. Interestingly, 30% of animals subjected to CSDS display resilience to stress. In a third part of this study, in collaboration with the Mueller laboratory in Mainz (Department of Psychiatry and Psychotherapy, University Medical Center of the JGU; Leibniz Institute for Resilience Research, Mainz, Germany), we exposed C57Bl/6J mice expressing GFP under the *Thy1* promoter to CSDS and classified them into resilient ($R^{+/-}$), susceptible ($R^{-/-}$) and non-learning ($R^{+/+}$) mice according to a modified social interaction test (MSIT). Thereafter, spines of the apical dendritic trees in CA1 and CA3 pyramidal neurons were analyzed. Compared to untreated control animals, we found no changes in the number of spines. However, CA1 spines showed altered spine compositions, with resilient animals resembling the untreated phenotype. Stress susceptible and non-learning animals displayed reduced numbers of stubby spines with a simultaneous increase in mature mushroom spines. In addition, we observed a tendency for more immature spines in susceptible animals and non-learners, in agreement with our *in vitro* results. Resilient animals discriminate between an aggressor mouse and a harmless conspecific during MSIT. *In vivo* data of chronically stressed mice displayed no phenotypic differences in hippocampal pyramidal neurons from resilient animals compared to unstressed mice. Morphological alterations of spine structures were particularly visible in stress susceptible and non-learning animals. By combining our findings with existing behavioral data, we can conclude that DRR1 plays a role in stress resilience, requiring to be expressed in a tightly controlled homeostatic equilibrium.

Our aim is to identify and understand how DRR1 modulates stress-related consequences and actively promotes resilience in detail. To address this topic, we successfully bred conditional and brain-region specific DRR1 knockout mice (DRR1$^{l/l}$_*Camk2a*-Cre^{+}). In these mice, DRR1 is inactivated in the forebrain and limbic system through the expression of the Cre-recombinase under the *Camk2a* promoter during the second postnatal week. Further, these animals were bred to a *Thy1*-GFP mouse line for future morphological analysis after stress exposure or pharmacological treatment. Prospectively, these DRR1 mutants should be used to first characterize in detail the effects of the absence of neuronal DRR1 under physiological conditions and next to expose these mutant mice to chronic social defeat stress and characterize the resilience response. Those data should be complemented by morphological and electrophysiological analysis.

Finally, in this thesis, we present a different investigative approach. Sequenced acute stress was previously found to impair cognition, including spine loss. Acute stress, unlike chronic stress, is minimally invasive and less time consuming. Furthermore, acute stress often results only in reversable short-term neuronal damages that is easily reversable by intrinsic homeostatic processes. Besides, studies of acute stress responses could give new perspectives on potential stress coping strategies. Scientists agree that neuropsychiatric disorders can be treated most effectively if treatment begins early at the disease onset, as long as the disorder is dynamically modifiable. To better understand the genesis of neuropsychiatric disorders induced by chronic stress, it is equally important to investigate early phases of the disease. This approach could help identify resilience mechanisms. Recent findings showed increases in *Drr1* mRNA four hours after acute stress exposure in mice. Protein expression was visible after eight hours with concomitant cognitive enhancement to basal levels. In combination with our findings on basal DRR1 expression in different cell types of the brain, we wanted to investigate the effects of acute stress on DRR1 levels and its occurrence in these different cell types. In collaboration with the Mueller laboratory in Mainz, we exposed one group of C57Bl/6J mice to acute stress and injected another group with the artificial glucocorticoid DEX. Eight hours post stress, the animals were perfused and brains were subsequently immunobiologically analyzed. We found DRR1 protein levels elevated in the hippocampus of stressed and DEX-treated animals compared to controls. Interestingly, DRR1 was particularly elevated in endothelial cells. This is consistent with our findings in which DRR1was found under basal conditions in astrocytic end-feet. In addition, a previous study revealed that social stress induces neurovascular pathologies due to tight junction impairments that ultimately lead to depressive behavior in mice. After eight hours of acute stress, mice show normal social behavior with concurrent increased DRR1 levels. These results indicate an involvement of DRR1 in blood-brain-barrier integrity.

In summary, our results show that DRR1 is an actin-interacting and glucocorticoid-responsive gene that affects the structural plasticity of hippocampal spines. Moreover, DRR1 directly interacts with AMPA glutamate receptors and presumably is involved in AMPA trafficking to the postsynaptic membrane. In addition, this study could demonstrate that DRR1 is also expressed by other cell types in the brain. Of particular interest is the presence of DRR1 in astrocytic end-feet and endothelial cells, suggesting a role as an integrator of cell-cell communication and, in this context, acting as a modifier of stress-induced consequences at the neurovascular unit.

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1. Introduction

1.1 Stress

1.1.1 History and definition

Ancient philosophers such as Aristotle and Hippocrates were aware of stress and its negative effects. However, the first one to describe possible protective mechanisms against stress in a physiological manner was the French physician, pharmacist, and experimental physiologist Claude Bernard in the 19th century (Fink, 2017). Later, in 1932, the US-American physiologist Walter Bradford Cannon described the concept of homeostasis in terms of physiological processes preserving stable conditions of organisms. In addition, Cannon shaped the expression "fight-or-flight" as animals' response towards danger, representing the acute stress response.

Hans Seyle, a Viennese physician, characterized chronic stress. By performing medical observations and experiments on rats, he found prolonged stress to cause "disease of adaptation" through release of chemicals and hormones from the body. He detected that discharge of those substances cause ulcers in the gastro-intestinal tract as well as high blood pressure. For the first time, stress became attention in the sense of causing diseases and started to be considered having an impact on health. Later, research led to findings of its negative effects on the immune system, involving adrenalin glands (Fink, 2017). An interesting concept explaining homeostasis not only as state of stability maintained by organisms through internal, physical, and chemical balance but also being sustained through change by regulation of the set point which adapts to stressful or challenging situations, was elaborated by Sterling and Eyer in the 1980s and defined as allostasis (Sterling, 1988).

Stressful life events such as severe environmental situations or psychological pressure can trigger a subset of physiological and psychological changes in the body. Nowadays, epidemiological studies, aiming to ascertain stressors affecting health and well-being, show effects of social, workplace, and lifestyle playing a big role as stress factors. Especially work-stress with its high demands and little control is linked to increased cases of heart attacks, obesity, hypertension as well as neuropsychiatric diseases as anxiety, addiction, and depression amongst many others (Sterling, 1988). Early life exposure to stress evokes not only hormonal and epigenetic changes through deoxyribonucleic acid (DNA) methylation, but also impacts on brain plasticity by decreased recruitment of new neurons and an increase of gray matter in the hippocampus (Barnea & Nottebohm, 1994; Barnea et al., 2006; Demetriou et al., 2015) a brain area involved in learning, memory formation and building a spatiotemporal framework.

1.1.2 Stress-disorders and economic effects

The release of stress hormones is the key mechanism and cause for well-orchestrated physiological changes. Generally, stressors as social stress, traumatic life events, physical illness, challenging or changing life conditions are considered as major risk factors for the onset and development of neuropsychiatric disorders (Global Burden of Disease Study 2013 Collaborators, 2015), representing leading causes of disability in our society. Amongst others, anxiety, major depression, chronic pain, addiction, and post-traumatic stress disorder (PTSD) comprise major stress-related disorders (Global Burden of Disease Study 2013 Collaborators, 2015). European epidemiological surveys showed that nearly 30% of the population suffer from above mentioned mental disorders, all connected of being provoked by external stressors as stated at the beginning of this paragraph (Kalisch et al., 2015). The broad spectrum of disorders of the brain, upon which the mentioned neuropsychiatric diseases are included, have a high prevalence entailing short and long-term impairments. Besides high suffering and emotional burdens to the patient, their environment, family, and social life, this includes financial difficulties not only to them but appears to be a trouble for the whole society. This is expressed by a high load to the health-care system plus bringing economic consequences. A study examining direct and indirect economic costs via mathematical data estimation of brain disorders focusing on European countries estimated costs of almost €780 billion in total for the year 2010 (Gustavsson et al., 2011). Whereat, stress-related disorders take up economic cost of almost €200 billion per year (Gustavsson et al., 2011) with indirect economic costs even summing up to €300 billion in Europe only (Global Burden of Disease Study 2013 Collaborators, 2015). Additionally, stress-related disorders have an overall higher contribution to morbidity burden than cardiovascular disease (Wittchen et al., 2011). Taken together, these numbers show the high incidence of stress-related disorders in developed countries with little progress in their treatment and prevention. Thus, it becomes more and more important to promote advances in research of mental health and stress-related disorders.

1.1.3 “Fight or flight”: Biology of the acute stress response

Adaptation of the organism towards a short-term stressor is noticeable in the nervous, cardiovascular, endocrine, and immune system (Schneiderman et al., 2005). These adaptations are reversible when the organism is subjected to this stressor for only a limited period of time (McEwen & Wingfield, 2003) and it helps the organism to successfully cope with aggravating situations, which is beneficial for surviving in the first place. The body reaction during acute stress was first described by Walter Cannon as “fight-or-flight” response, involving the autonomous nervous system, controlled by the brain and engaging neurotransmitters as epinephrin,

norepinephrine, and acetylcholine, that ultimately trigger the release of hormones and chemicals, pushing adaptive organ reactions (Fink, 2017). Firstly, hormones prompt the release of energy stores to make those accessible to the body for direct use, and secondly energy is channeled to tissue and organs that become more active during stress, such as skeletal muscles and the brain. The immune system becomes strongly activated as well and goes into action at regions that require the specific immune response. Vital body functions that are not critical for survival during an aversive state like the digestive system, sexual activity and growth, and gonadal hormones are lowered down (Schneiderman et al., 2005). Stressful environmental events transmit a threat signal to the hypothalamus.

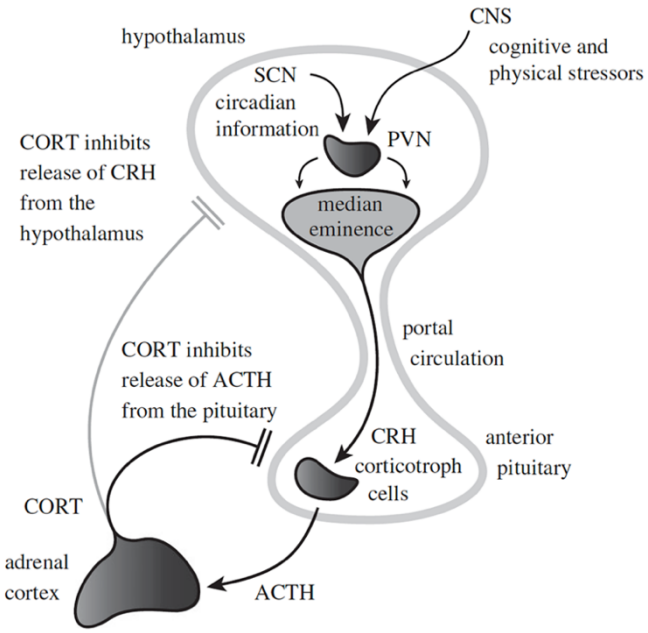

Figure 1.1: HPA-axis activity. Representative model of acute endocrine stress response. Circadian inputs and stressful stimuli from the brain stem and limbic areas activate the hypothalamic suprachiasmatic (SCN) and paraventricular nuclei (PVN). PVN projects to the median eminence, triggering release of the corticotrophin-releasing hormone (CRH) into the portal circulation activating corticotroph cells in the adjacent anterior pituitary, where adreno-corticotropin (ACTH) is unchained from preformed granules. Following, ACTH passes the blood-brain barrier, to the adrenal cortex to activate the synthesis and release of cortisol (CORT) in humans or corticosterone in mice. CORT triggers a feedback loop to inhibit the release of ACTH from the anterior pituitary and CRH from the hypothalamus. From Walker et al., 2010.

Stress hormones are produced by hypothalamic-pituitary adrenocortical (HPA) axis and the sympathetic nervous system (SNS), activating the adrenal medulla which is accountable to produce the neurotransmitters epinephrine and norepinephrine, both representing catecholamines (Figure 1.1). The paraventricular nucleus (PVN) of the hypothalamus produces the corticotropin-releasing factor/hormone (CRF/CRH), which is stored in the median eminence. From there it is released into the portal circulation upon stress (Vuppaladhadiam et al., 2020). This polypeptide stimulates the anterior pituitary to produce adrenocorticotropin (ACTH) which is secreted into the blood stream, traveling through the blood and activating the adrenal cortex to produce and secrete cortisol or glucocorticoids (GC) in mice (Brindley & Rollan, 1989; Dumbell et al., 2016). Collectively, catecholamines and cortisol raise lipolysis, breaking down fats into fatty acids and glycerol, and the conversion of glycogen in glucose to provide usable sources of energy (Brindley & Rolland, 1989).

1.1.4 Chronic stress and its consequences on neurons

Originally, the stress response emerged as adaptive mechanism to maintain a homeostatic internal state upon changing environmental influences. A short and controllable duration of stress comes along with arousal, excitement, and generally intends to provide a survival benefit for the organism. By contrast, severe and prolonged stress causes tissue damage and induces diseases (Selye, 1956; Schneiderman et al., 2005), which goes along with chronically elevated levels of CRH and circulating corticosteroid hormones. Both are believed to promote susceptibility to various diseases, including human affective disorders (Popoli et al., 2012). As shown in primates and mice, chronic stress promotes morphological changes in synaptic plasticity, mainly visible via dendritic spine loss (Pavlides et al., 1996; Sousa et al., 2000; Donohue et al., 2006; Kavushansky et al., 2006; Chen et al., 2008). Yet, it has not been worked out when the stress induced response shifts from protective to detrimental and what are the exact underlying mechanism and down-stream effects causing short and long-term modifications and adaptions of synaptic plasticity (van der Kooij et al., 2016). In particular, the hippocampus is enriched with glucocorticoid (GR) and mineral receptors (MR), making this brain structure vulnerable to stress-induced morphological alterations (Reul & de Kloet, 1985). Primarily, the pyramidal neurons of the CA3 formation within the hippocampus are sensitive to dendritic remodeling, neuronal damage and neuronal cell loss on account of glucocorticoids, increased CRH and social stress (Uno et al., 1989; Uno et al., 1990). Moreover, GRs were found on dendritic spines within the hippocampus, providing evidence that those spines react to glucocorticoids upon acute stress by modifying local spine signaling (Jafari et al., 2012). More details on chronic stress and its effects on the hippocampal brain area are elaborated in chapter 1.3.5.

1.1.5 Social stress: State of the art in rodents

Since decades rodents were used for stress research and started to become a suitable model organism for resilience research as well. Especially, the aspect of social stress was heavily studied in rodents. Social interactions were found to be capable of evoking stress in organisms and influencing the stress response on many levels. Studying social stress in rodents has shed light on the stressor type, the onset, or more specifically the timing of the stressor, and its impact on physiology and behavior (Beery & Kaufer, 2015). The reciprocal influence of stress and social behavior mirrors in the social environment as stressor, stress effects on social behavior, and social backup upon stress experiences (Beery & Kaufer, 2015). Consequences of stressor exposure highly depend on the given circumstances. This includes stressor type, the severity of a stressful event, its duration, but also the sex of the animals, their genetics and the life phase when they are exposed

to stress (early life, adolescence, adulthood, aging), as well as the individual perception, appraisal, and controllability of certain stress loads, which dramatically influence the outcome in sense of resilience and vulnerability (social behaviors due to differing social environments shown in Figure 1.2) (Maier & Watkins, 2005; Amat et al., 2010; Lucas et al., 2014). One physiological measure for immediate stress response is the onset of the HPA-axis with the release of the subsequent stress hormones such as glucocorticoids from the adrenal gland (Bale & Vale, 2004). Other neurochemicals are associated with the stress response, namely brain derived neurotrophic factor (BDNF), serotonin and a variety of neuropeptides (Beery & Kaufer, 2015). Eminently involved in social behavior of rodents are the neuropeptides produced in the hypothalamus oxytocin (OT) and vasopressin (VP). Oxytocin was shown to play a role in maternal behavior, trust, anxiolysis and sexual bonding (reviewed in Ross & Young, 2009; Donaldson & Young, 2008; Neumann, 2008; Carter et al., 2008; Anacker & Beery, 2013). Oppositely, vasopressin is implicated in aggression, anxiety and social behavior (Kelly & Goodson, 2014). Additionally, the dopaminergic and opioid systems play a role in rewarding social behaviors like pair-bonding (Aragona & Wang, 2009; Resendez et al., 2012).

Social behavior reactions of rodents post stress are complex and researchers try to assess them by complex housing areas with enriched environments and caging in social groups (Blanchard et al., 2001; Seney et al., 2006), or even semi-natural enclosures (Ophir et al., 2012). Typical social interaction tests measure the time of animals spend interacting or exploring with conspecifics. Other tests, like social choice tests, are performed in multi-chambered cages where animals have the choice to either interact with conspecifics or investigate non-living stimuli as novel objects or empty restrainers (Moy et al., 2007). These tests can vary in order to observe social habituation or dishabituation to study the recognition for known individuals, or social motivation of the animals, assessing the effort of an animal to reach another animal or the preference for a conditioned environment (Lee et al., 1999; Ferguson et al., 2002; Choleris et al., 2003; Panksepp & Lahvis, 2007). In addition, suitable paradigms detecting social hierarchy, which involve memory and inferences were developed (Cordero & Sandi, 2007; Grosenick et al., 2007). Also, tests for evaluating pro-social behavior were established i.e., in rats, measuring the willingness to release a captured conspecific (Bartal et al., 2011; Bartal et al., 2014).

Numerous examinations of social defeat stress in rodents detected an impact on hippocampal morphology. This includes a reduction of hippocampal volume (Czéh et al., 2001), along with reduced neurogenesis and dendritic reshaping (Magariños et al., 1996; Gould et al., 1998), and a ratio shift of mineralocorticoid to glucocorticoid receptors (Buwalda et al., 2001; Veenema et al., 2003). Even more intriguing is the finding that social stress affects glial cells, including astrocytes, microglia, and oligodendrocytes (Braun et al., 2009; Wohleb et al., 2011; Araya-Callís et al., 2012; Chetty et al., 2014). However, which role glial cells exactly play in the

stress response and which underlying mechanisms are governed by those cell types still needs to be elucidated.

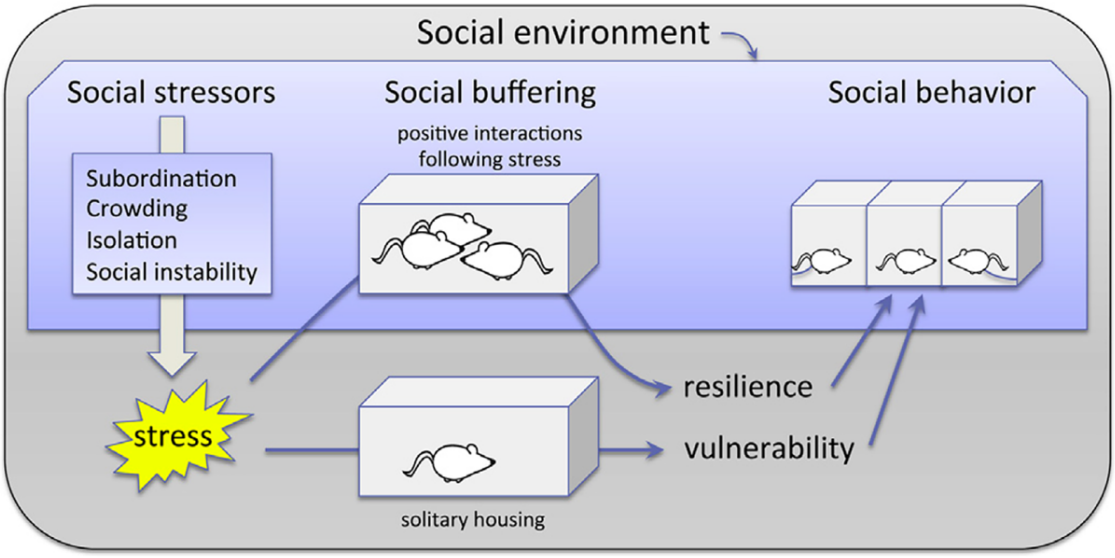

Figure 1.2: Paradigms and effects of social environment.
In rodents, manipulations and changes of the social environment are utilized as potential stressors. Aversive social environments evoke stress responses and are listed on the left. They evoke far reaching physiological responses and influence on social behavior. Social buffering can potentiate these effects and help promoting resilient behavior. From Beery & Kaufer, 2015.

Further, social status accounts for stress throughout a wide range of species. A low social hierarchy, for example well studied in non-human primates, was shown to be accompanied by health problems (Virgin, 1997; Sapolsky, 1989; Sapolsky, 2005; Wu et al., 2014). Also, in humans a low socioeconomic status goes along with a broad range of physical and mental complications, especially when subjectively perceived as such (Adler et al., 1994; Kawachi & Kennedy, 1999; Siegrist & Marmot, 2004).

In rodents, social stress can also be evoked by crowding, isolation and social instability, minded that sex differences exist and male and female animals respond differently to distinct stressors. In mice, high housing densities affect reproduction in a negative way (Christian, 1971). Physiologically, crowding evokes changes in the weight of organs and it manipulates hormone secretion, reactivity of the HPA-axis, pain sensitivity, telomere length, and cardiovascular functioning (Gamallo et al., 1986; Gądek-Michalska & Bugajski, 2003; Kotrschal et al., 2007; Grippo et al., 2010; Tramullas et al., 2012; Puzserova et al., 2012). When female mice are housed in crowded cages during pregnancy, their offspring shows lower birth weight, changes in pubertal development and reproduction (Brown & Grunberg, 1995). Isolation as the other extreme, manifests in reduced body weight, altered blood composition and cardiac function, enhanced pain responsibility (Valzelli, 1973; Späni et al., 2003; Carnevali et al., 2012). These physiological health impairments are accompanied by modifications in aggressive behavior, mating, and learning.

Additionally, neurogenesis and dopamine signaling in different brain regions are affected (Heidbreder et al., 2000; Stranahan et al., 2006; Lieberwirth & Wang, 2012). Similar implications were shown through constant changing of the social environment leading to increased adrenal weight, higher corticosterone secretion, and reduced weight gain (Haller et al., 1999). Besides, social instability leads to reduced food intake and dysfunction of the hypothalamic-pituitary-gonadal (HPG) axis (Herzog et al., 2009).

1.2 Resilience

Resilience is defined as the quick recovery or maintenance of mental health after adversity to severe psychological or physical burdens (Southwick et al., 2005; Bonanno et al., 2011; Feder et al., 2009; Sapienza & Masten, 2011; Southwick & Charney, 2012; Kalisch et al., 2015), accompanied by high stress responses. Resilience research aims to investigate and develop new prevention and treatment strategies regarding stress-related pathologies, representing a paradigm shift in psychological and psychiatric treatment. The main concept of resilience research underlies "appraisal processes in the generation of emotional responses, including responses to potential stressors" (Kalisch et al., 2015). Based on this theorem, positive appraisal of situations is thought to be the key to establish protective mechanisms against stress-associated psychopathologies and promote resilience factors named "positive appraisal style theory of resilience" (PASTOR) (Kalisch et al., 2015). In addition, resistivity towards diseases is the active process of adaptations, requiring a biological process (Russo et al., 2012; Friedman et al., 2014) and the absence of this robustness that creates stress symptoms is a consequence of missing resilience mechanisms (De Kloet, 2008; Holsboer & Ising, 2010; Popoli et al., 2012). For example, a potential resilience mechanism might be the flexible onset and deactivation of the HPA-axis that is triggered by stress (Kalisch et al., 2015), which is postulated to be orchestrated by changes in attention, cognition, subjective experience and activation of the sympathetic system with peripheral, physiological changes, all of which require energy consumption and processing priorities (Kalisch et al., 2015). On the other hand, mechanisms preventing stress related dysfunction by protecting the systems are viewed to promote resilience. Here, all about stands the fine-tuning of the stress response to optimal levels and ideally to exit it, as soon as it is no longer needed for survival. Integral for this process is the flexibility of an organisms to shift to alternative coping strategies, along with an efficient economizing of resources (Kalisch et al., 2015).

Such as acute and chronic stress, resilience mechanisms could be categorized according to the severity and the duration they are activated for. Protection against a single stress-induced syndrome is graded as "dysfunction-specific resilience mechanism", whereas protection against

multiple stress-induced symptoms is categorized into "general resilience mechanisms". Apart from these two differentiations, several stress-induced impairments emerging from a wide range of stressors, are seen as "global resilience mechanisms" (Kalisch et al., 2015). PASTOR is supported by the idea that emotions are evaluated in a context-dependent manner in regard of the relevance of a situation (Arnold, 1969; Cheng, 2001). By implication this means that the evaluation determines a positive (appetitive) or negative (aversive) reaction towards a stimulus (Granger & Johnson, 2013) and that the outcome may evoke different reactions dependent on the circumstances an individual does momentary face. Further, stress is determined by the outcome magnitude, outcome probability, and coping potential (Kalisch et al., 2015). Summing up, appraisal as assessment of a stressful situation is context-dependent, subjective and multidimensional. Additionally, it is a dynamic process, which aims to continuously integrate new information, leading to new adjustments which in turn produce emotional reactions (Kalisch et al., 2015). Also, the appraisal styles influence an individual's management with different situations. They are characterized by beliefs, appraisal habits, cognitive styles, attitudes and interpretative biases and differ from person to person, controlling their aversive or appetitive behavior. A general positive appraisal style, by seeing things positive, is believed to be protective, thus a way to promote resilience (Kalisch et al., 2015). Upon aversive states it is necessary to improve the aversive attitude and change it into less negative appraisals in order to stay resilient. This can happen, as soon as positive situations arise during or after a stressor's onset and lead to a less negative appraisal or replace it by substitution of positive appraisals with the long-term goal of maintaining those.

Extinction research is used to investigate emotion regulation and can be applied as model in both, human and animal research. Moreover, linked with neuronal functions, it represents a model that can transmit resilience research in a cross-species manner (Milad & Quirk, 2012). Previously, it was hypothesized that good extinction memory i.e., adaptive behavior correlates with resilience, such as specific neural mechanisms, like dopamine release in the ventromedial prefrontal cortex (vmPFC) and that latter might promote extinction behavior (Haaker et al., 2013). The neurophysiological findings were shown by comparing resilient versus non-resilient mice post stress (Chaudhury et al., 2013; Friedman et al., 2014). When performing resilience research in animals, an ideal scenario to underline a hypothesis is the manipulation of predicted behavior or neural mechanism with a positive correlation towards resilience. For example, manipulation of extinction in a way that either increases or decreases extinction memory could in turn predict the increase or decrease of resilience in single animals. Similarly, it would be desirable to show that individual differences of dopamine release in the vmPFC affects resilience (Kalisch et al., 2015) and that these two processes are interconnected. Therefore, Kalisch and colleagues suggest a resilience readout R, with a battery of stressors and a behavioral test battery, testing the animals functioning before and after stress exposure (Kalisch et al., 2015). For comparison reasons another

group of animals would be necessary without undergoing the stress battery. Further, to capture a long-term outcome, behavioral testing batteries should be performed weeks after the stress, to avoid capturing acute stress responses (Krishnan et al., 2007; Kalisch et al., 2015). To create an ecological significance and adjust animal studies according to human research, chronic stressors should be chosen to mimic major adversity humans experience. Also, for avoidance of habituation and generating unpredictability, a switch between physical and social stressors is important in animal study designs as well (Kalisch et al., 2015). Also, resilience studies in animals are most expedient when they originate from species-specific behavior, rather than reconstruction of human-like depressive states in animals and analysis should base on vulnerability to disruption of those behaviors after stress (Russo et al., 2012). Different species occupy different niches and therefore developed varying coping mechanisms for challenging situations. In this sense, it is very important to elaborate suiting behavioral read-outs for every species that is used to study stress resilience (Sachser & Richter, 2015). Sensitive markers which can be applied for a broad range of animals include social interactions, reproduction, aversion, sleep and higher cognitive functions (Kalisch et al., 2015).

For reliability reasons, as well as identifying general resilience mechanisms, more than one measure, thus output is necessary. This could include data recordings from behavioral, physiological and molecular mechanisms (Franklin et al., 2012; Russo et al., 2012; Scharf & Schmidt, 2012). Apart from this, scientists developed readouts measuring emotional states in animals by grading differences in judgment biases in rats. They showed that rats who lived in less predictable, quasi stressful housing environments, displayed reduced anticipation of a positive event as compared to control groups and graded their behavior as pessimistic (Harding et al., 2004). These studies were extended to other animal species (mice, dogs, sheep, and macaques), demonstrating that positive and negative appraisal styles can be evaluated in animals compared to humans (Mendl et al., 2010).

Behavioral biologists argue that resilience research in animals should be investigated in an ontogeny perspective as well, since resilience mechanisms are likely to be shaped from prenatal stages throughout adolescence and according mechanisms are shaped by environmental stimuli and genes in sensitive developing stages (Sachser et al., 2013; Sachser & Richter, 2015).

Whether positive appraisal is the only mechanisms facilitating resilience is under debate, since from an evolutionary perspective also other appraisal styles developed and do exist. Which appraisal style comes into account when sustaining mental health seems to heavily depend on environmental situations an individual is located in (Koolhaas et al., 2010). Therefore, animal studies taking a look at different appraisal styles could help shaping the understanding of the contribution of different appraisal styles to mental health (Sweeny & Shepperd, 2010).

1.2.1 Behavioral paradigms for stress vulnerability and resilience in rodents

Stress inoculation describes the attenuation of stress responses in adulthood, after individuals were subjected to mild early life stressors. Later on, this helps to develop resilience towards stress and maintain a good psychological functioning after stressful life events (Rutter, 2006; Macrì et al., 2011). In rodents, early life stress can either be provoked prenatally by subjecting pregnant dams to food deprivation and alternatively via injections with glucocorticoids, or postnatally through maternal separation, glucocorticoid administration to the offspring, or diminished maternal care via stressing the dams (Lupien et al., 2009). The hormonal stress response is reduced in subsequent developmental stages, when the offspring is subjected to mild stress as compared to non-stressed or severely stressed comparison groups. This goes along with cognitive and emotional resilience as well as reduced anxiety-like behavior and better performance in cognitive tasks (Plotsky & Meaney, 1993; Parker et al., 2005; Macrì & Würbel, 2007; Macrì et al., 2009; Lyons et al., 2010).

Application of chronic unpredicted stress over a period of several weeks, aims to prevent habituation and is used to create anxiety-like and depression-like behavior in rodents and is used to investigate the hormonal and neural basis of vulnerability and resilience to stress. To create this state in rodents, mild stressors such as mild foot shocks, physical restraint, disruption of the light and dark cycle, food and water restriction, and changing cage mates are utilized (Willner, 1997; LaPlant et al., 2009). Usually, this evokes anhedonia, measured as decreased sucrose preference, despair, noticeable as increased immobility in the forced-swim task, as well as lowered aggression, grooming, and sexuality (Willner, 1997; Mineur et al., 2006; Krishnan & Nestler, 2011; Feng et al., 2012).

Another animal model revealing susceptible and resilient adult mice is the so-called chronic social defeat stress (CSDS) paradigm (Figure 1.3). It is utilized to investigate the mechanisms underlying depression, since it evokes anxiety-like and depressive-like behaviors in mice, with similarities to human depression, even showing the same biomarkers (Golden et al., 2013; Robison et al., 2013). Here, for ten consecutive days, male C57BL/6J mice are subordinated to larger and more aggressive CD-1 mice. Every physical encounter in the home cage of the larger CD-1 mouse, is followed by a period of sensory contact through separation of the two animals by a transparent and perforated grid. Following the stress period, animals are tested in a social-interaction-task and thereafter divided into susceptible and resilient animals according to their interaction time with the conspecific (Golden et al., 2011). Out of all tested animals usually 2/3 of the animals are detected as susceptible, with anhedonia and social avoidance, metabolic syndrome, weight increase, and insulin insensitivity whereas the other part is viewed as resilient with milder symptoms including corticosterone elevations and increased mild anxiety (Krishnan et al., 2007; Lutter et al., 2008;

Chuang et al., 2010; Donahue et al., 2014). At this point, it is important to mention that the paradigm was recently adapted to distinguish a third group of animals, which are viewed as non-learners and cannot be categorized into the susceptible, neither the resilient group (Ayash et al., 2020).

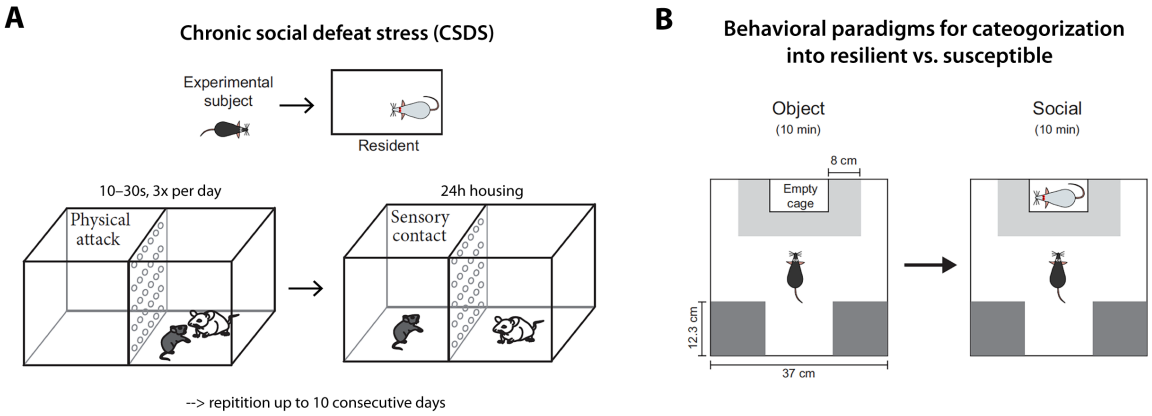

Figure 1.3: Chronic social defeat stress (CSDS) in rodents.
Chronic social defeat stress is used to create a depressive-like behavior in mice. **(A)** After individual housing on day 0, male subjects (C57BL/6) are introduced into the home cage of larger and aggressive CD-1 animal. They encounter physical attacks for 10 seconds followed by a separation through a grid which enables sensory contact. Subjects are housed for 24h together with the resident and physical attacks are repeated 3x a day for up to 10 consecutive days. Social defeat stress is performed up to 10 consecutive days with alternating aggressors. **(B)** Following CSDS mice are tested in different behavioral paradigms, such as novel object recognition tasks or social interaction tasks, to categorize them into resilient versus susceptible groups. Time spend in corners (dark grey) and the interaction zone (light grey) is measured and subsequently used to categorize animals due to their behavior. **(A)** upper part and **(B)** modified from Henriques-Alves & Queiroz et al., 2016, **(A)** lower part modified from Shu & Xu, 2017.

Lastly, to investigate mechanisms of acute stress, creation of learned helplessness is employed in animal models. For that, mice are subjected to repetitive and inescapable foot shocks and subsequently tested in a task, where escape is possible during the shocks. In comparison to resilient animals and control groups, susceptible mice show a longer latency time being capable of escaping or they fail to escape at all (Seligman & Beagley, 1975). Behavioral and physiological changes created by this paradigm include weight loss, HPA-axis disruption, variations in the circadian rhythm and decreased numbers of spines in the hippocampal area (Krishnan & Nestler, 2011). Notably, these effects are short-term, lasting 2–3 days and are reversable by acute administration of antidepressants (Cryan & Mombereau, 2004).

1.2.1.1 Behavioral and neuronal responses and stress resilience in rodents

In rats social defeat stress leads to social avoidance and abandonment from the dominant conspecific (Ruis et al., 1999; Lukas et al., 2011). Chronic social defeat stress is a well-established model to persistently generate susceptible and resilient mice (Krishnan et al., 2007). It induces a

range of negative effects in mice, which can be used as readouts in comparison to an unstressed control group. Amongst them rank social avoidance, altered fear perception and awareness, anhedonia, alterations in neural circuitry and neurotransmission, diminished neurogenesis, and changes in the metabolism (Chou et al., 2014; Donahue et al., 2014). Surprisingly, when giving attention to individual stress responses of a cohort of tested animals, one can see a bimodal distribution of animals affected by stress and others that are not. When testing those cohorts of animals, individually in social interaction tests, it becomes clear that affected animals spend less time interacting with mates in the social interaction zone, as compared to unaffected or "unsusceptible" mice, who rather show similar social interaction as unstressed control animals (Krishnan et al., 2007).

Susceptibility to stress is correlated with induction of BDNF, which is important for the regulation of dopamine release in the nucleus accumbens (NAc). In mice, chronic social defeat stress goes along with permanent BDNF raises within the NAc. Manipulation of BDNF in the ventral tegmental area (VTA) can be obtained via knockdown of this protein factor which in turn leads to decreases in depressive-like behavior and aversive behavior in mice (Berton et al., 2006). Interestingly, only the knockdown of BDNF in the VTA, but not in the NAc, promotes resilience in animals, defined by the absence of BDNF-induced activation of dopaminergic neuronal activity in the VTA (Berton et al., 2006; Krishnan et al., 2007). Generally, unsusceptible animals are not lacking a neural response. They are rather distinguishable from susceptible animals by higher gene expression patterns in the VTA, suggesting that the behavioral unresponsiveness is not a lack of the stress pathology, but an active process.

Gene expression analysis show decreased levels of wingless (WNT)-signaling cascade genes, such as phosphor-GSK3β (glycogen synthase kinase-3β) in the NAc of susceptible mice (Wilkinson et al., 2011). Additionally, susceptible mice show reductions in CRF, and HPA-axis regulations (Elliott et al., 2010; Wood et al., 2010). Moreover, knockdown to reduce CRF levels minimizes stress-induced social avoidance, plus animals that were bred for low anxiety, showed resilient behavior towards subordination (Elliott et al., 2010; Füchsl et al., 2014).

Resilient versus vulnerable animals display differences in the glutamatergic system responses, with different α-amino-3-hydroxy-5-methyl-4-isoxacolepropionic receptor (AMPAR) expression patterns in the dorsal hippocampus and also a different activation pattern of AMPARs during stress responses. Resilient animals seem to be protected by differential AMPARs activation against chronic stress, which subsequently act on physiological, behavioral and neuroendocrine mechanisms (Schmidt et al., 2010). By reducing serotonergic transporters, susceptibility to social avoidance is increased after defeat stress (Bartolomucci et al., 2010). Finally, also the γ-aminobutyric acid (GABA) system is implicated in vulnerability. If the GABAergic system is

knocked down in the prefrontal cortex, animals exhibit a depressive-like behavior. The same accounts for the amygdala after peripubertal stress (Veeraiah et al., 2014; Tzanoulinou et al., 2014).

In general, stress-resilience can develop out of different circumstances. It can be programmed in early life phases through environmental factors, in particular by maternal interactions (Szyf et al., 2007; Lyons et al., 2010; Parker et al., 2011). Second, short-term resilience can evolve out of a response towards an acute and rather mild stressor, protecting against a second stressor (Kirby et al., 2013). Lastly, resilience can be promoted by a positive and supportive environment during stress phases (Virgin & Sapolsky, 1997). In the case of social buffering, positive effects in terms of better functioning of the immune system, the cardiovascular system and longevity were shown in human and animal research (Yee et al., 2008; Holt-Lunstad et al., 2010; Silk et al., 2010; Stanton & Mann, 2012).

1.2.1.2 Endocrinological mechanisms, sex differences and resilience

Social buffering affects endocrine outcomes post stress as well. Animal studies found social housing impacting on HPA-axis responsiveness or CRF stimulation after exposure to stressors. Male rats living in social groups feature lower CORT-levels and adrenocorticotropin (ACTH) post stress relative to separately living rats (Ruis et al., 1999). In prairie volves corticosterone (CORT) and ACTH-level increase upon separation of siblings or partners (Carter et al., 1995; Bosch et al., 2008). In adult rats it was shown that postnatal stress inoculation leads to reduced basal levels of CRF, flatter stress-induced ACTH increase, less corticosterone and CRF secretion and faster recovery of hormonal levels to post-stress situations in comparison to stressed control animals (Meaney & Szyf, 2005). Summing up, the presence of conspecifics can buffer stressful situations and promote stress resilience. Whether social interaction results in positive or negative outcomes depends on the stimulus and environmental setting. Also circulating CORT levels can either be reduced or increased following stress and which CORT-profile occurs depends on timing and chronicity of the stress (Sapolsky et al., 2000; Beery et al., 2012). Further, human and animal studies show similarities when oxytocin and social buffering interact to reduce CORT-levels (Heinrichs et al., 2003). Other physical benefits associated with group housing versus solitary housing in rodents encompass reduced ulcer formation, and reduced adrenal hypertrophy after stress (Conger et al., 1958; Westenbroek et al., 2005). In humans social buffering promotes heart rate and homeostasis of blood pressure after stress exposure (Lepore et al., 1993; Thorsteinsson et al., 1998).

When investigating social behavior, it is important to pay attention to sex differences. Generally, reproductive hormones distinctly serve an important role in the stress response, thus

affect susceptibility and resilience to stress. For example, estrogen is positively correlated with cognitive processes and impacts on catecholamine and monoamine neurotransmission, regulating expression of transcription factors and neurotrophins (Shanmugan & Epperson, 2014). Brain stress circuitries are linked with lower serum estradiol, but higher serum progesterone (Holsen et al., 2011). Oxytocin appears to play a bigger role in the stress response of females. This becomes visible through the calming effect of crowding in females. Oppositely, crowding has a negative effect on males in regard to stress (Kotrschal et al., 2007). Furthermore, testosterone in men is associated with pro-resilience mechanisms, increased mood, and higher social connection (Russo et al., 2012; Pfau & Russo, 2015), whereas lower testosterone levels are found in individuals post stress (Morgan et al., 2000a). Also stress responses have diverse behavioral outcomes in males versus females. For example, classic conditioning is impaired in female rodents, but increased in males post stress (Wood & Shors, 1998). The neuropeptides OT and VP are necessary to take into account, their expression patterns, production and release, as well as densities of their receptors vary between sexes and thus consequent behaviors, relying on those neuropeptides, naturally differentiate (Bales & Carter, 2003; Carter, 2007). Other studies in humans revealed potential pro-resilience markers, namely neuropeptide Y and dehydroepiandrosterone (DHEA), which is released together with cortisol from the adrenal gland antagonizing the effects of glucocorticoids and preventing from PTSD (Yehuda et al., 2006a).

It turns out that maternal care produces early life stress resilience, as shown in behavioral rat studies, with long lasting positive effects regarding individual gene expression patterns and subsequently attached neuroendocrine stress response (Liu et al., 1997; Pfau & Russo, 2015). Those pro-resilience effects were long lasting into adulthood with a negative correlation on circulating plasma ACTH and corticosterone after stress induction, most likely due to increased expression of GRs which in turn leads to higher glucocorticoid sensitivity, and negatively feeds back on CRF and arginine vasopressin (AVP), basically attenuating the HPA-axis response to stress (Liu et al., 1997; Kappeler & Meaney, 2010). Also, handling of postnatal rats promotes resilience with enhanced GR expression mediated by a subset of increased mechanisms such as thyroid hormone secretion, turnover of serotonin in the hippocampus and expression of the cyclic adenosine monophosphate (cAMP)-inducible transcription factor nerve growth factor-inducible protein A (NGFI-A), which binds to the GR promotor (Meaney et al., 2000; Weaver et al., 2004; Meaney & Szyf, 2005). Furthermore, adult female rodents show better cognitive resilience after chronic stress as compared to male rodents, which in turn show better emotional resilience (Luine, 2002; Conrad et al., 2003; Kitraki et al., 2004). The underlying mechanisms can be ascribed to sex-characteristic changes in hippocampal morphology after stress. This includes atrophy of apical *cornu ammonis* 3 (CA3)-dendrites in males, but not females, plus increased immunoreactivity of

hippocampal GRs and MRs in females and opposite effects in males (Galea et al., 1997; Kitraki et al., 2004).

Parental stress has particular effects on the offspring. Thereby, it specially depends which parent was subjected to what kind of stress and it can have disparate effects on male or female descendants (Pfau & Russo, 2015). Experiments with mice revealed effects only on male offspring when mothers have been subjected to chronic unpredictable stress (CUS) in early pregnancy stages with elevated CRF expression in the amygdala and reduced hippocampal GR occurrence determining in epigenetic changes in regard to gene methylation (Mueller & Bale, 2008). Later, the same group showed a link between male's susceptibility to prenatal stress and stress-induced placental onset of pro-inflammatory genes, such as Interleukin-6 (IL-6) (Bronson & Bale, 2014). Interestingly, also paternal exposure to CUS in puberty and young adulthood has effects on their male offspring with measurable HPA-axis hypoactivity, accentuated by modified sperm micro-ribonucleic acid (miRNA) expression patterns of the fathers (Rodgers et al., 2013).

Another factor impacting on social behavior is anxiety. Social interaction tests performed with rodents reveal increased exploration time with conspecifics, when time in an open arm field is spent mostly in the center of the field or when more time is spent in the light area of the dark-light box paradigm (File & Hyde, 1978; Starr-Phillips & Beery, 2014). Also, maternal grooming behavior has long-lasting effects on the offspring anxiety behavior with decreased anxiety bearing in pups, when care was provided intensively (Gonzales, 2001; Beery & Francis, 2011). This is accompanied by lowered stress-responsiveness and increased glucocorticoid receptor expression in the hippocampal area of the offspring (Liu et al., 1997; Weaver et al., 2004), again minimizing negative feedback to the HPA-axis (Sapolsky et al., 1985; Liu et al., 1997).

1.2.1.3 Differences in neuroanatomy and genetic imprints in resilient vs. stress susceptible animals

Anacker and colleagues (2016) investigated neuroanatomical differences via MRI (magnet resonance imaging) and DTI (diffusion tensor imaging) of stress susceptible, resilient and control C57BL/6 mice after chronic social defeat stress and their respective outcome in a social avoidance paradigm post stress. They searched for functional neuroplasticity and its correlation with changes of the gray matter with special attention to brain regions with implications in stress susceptibility and resilience. Especially the reward-system including the VTA, NAc, amygdala, and prefrontal cortex (PFC) are known to be involved in the neural circuitry mediating differences between stress susceptible and resilient animals (Fekete et al., 2009; Narayanan et al., 2011; Chaudhury et al., 2013). Activity from VTA neurons to NAc neurons boosts susceptibility and firing of VTA

towards PFC promotes resilience (Chaudhury et al., 2013). On the other hand, hippocampal to hypothalamic circuits inhibit the activity of HPA-axis through inhibition of glucocorticoid release (Anacker et al., 2011; Anacker & Pariante, 2011; Anacker et al., 2014). In stress susceptible animals, researcher found smaller volumes of cingulate cortices, with larger VTAs and vice versa in resilient mice. Interestingly, Anacker and colleagues found social avoidance predicting a positive correlation with hippocampal and hypothalamic volume (Anacker et al., 2016). These findings are contrary to previous observations of smaller hippocampi in depressed patients and hippocampal atrophy post stress (Gould et al., 1991; Watanabe et al., 1992; Magariños et al., 1996b; Sheline et al., 1996; Lupien et al., 1999; MacQueen et al., 2003). On the downside, other studies have found a link between increased hippocampal volume and the number of stressful life events in elderly, nondepressed patients (Zannas et al., 2013). Specially men, with larger hippocampi display a more prominent cortisol arousal in response to social stress (Pruessner et al., 2007). Due to these findings, the group hypothesized that hippocampal volume might be associated with higher stress reactivity and HPA-axis hyperactivity (Anacker et al., 2016) It still remains elusive whether volume differences are the basis or result of stress vulnerability. In addition, Anacker and colleagues showed no correlation of social avoidance with brain volume in control animals, hypothesizing, that differing anatomy and connectivity within the brain is a consequence of stress exposure (Anacker et al., 2016). Besides, a longitudinal study suggests that prior to stress exposure, susceptible animals already show higher hippocampal volumes as compared to resilient individuals (Tse et al., 2014). Specifically, the hippocampal CA3-region seems to have a volume covariance with the hypothalamic region in stressed and control mice, with particularly increased CA3 volume in stress susceptible animals and oppositely in resilient mice, sensitizing susceptible animals to develop HPA-axis abnormalities, leading to a social avoiding behavior (Anacker et al., 2016).

Genetic research is performed on a large scale to identify so called susceptibility genes that enhance stress vulnerability of individuals after adverse life events. Those studies performed in the field of psychiatry aim to link Gene x Environment interactions (GxE). Discovery of a polymorphism in the monoamine oxidase A (*Maoa*) gene promoter, which leads to reduced MAOA expression is likely induced by childhood abuse and affects vulnerability to environmental stress (Caspi et al., 2002). Recent studies showed GxE interactions throughout the whole life span, involving MAOA alleles after exposure to abuse, and antisocial behavior (Fergusson et al., 2012). Biologically, monoamine oxidases are located within the mitochondrial membrane, serving as enzymes for oxidative deamination, thus playing an important role in degradation of toxic substances. Additionally, there is evidence of a higher risk of susceptibility to stress when a polymorphism is present in the promoter of the serotonin transporter SERT (or 5-HTT) (Caspi et al., 2003; Rutter et al., 2009), which is also a target molecule of serotonin reuptake inhibitors, treating depressions. Many studies investigating polymorphisms in the corticotropin-releasing

factor receptor-1 gene showed links to suicidal thoughts and behavior, depression, panic, and fear (Ben-Efraim et al., 2011; Ishitobi et al., 2012; Wasserman et al., 2008a/2009a/2010; Weber et al., 2015). A functional deletion variant of the gene ADRA2B, encoding the alpha 2b-adrenergic receptor, is responsible for elevated emotional and traumatic memory (Rasch et al., 2009). Functional magnetic resonance imaging (fMRI) and genetic studies found increased connectivity in brain areas processing emotional memory (Rasch et al., 2009; De Quervain et al., 2007). Other studies suggest epigenetic changes through glucocorticoid-induced DNA methylation, after accumulated life stress (Zannas et al., 2015).

1.2.1.4 Immunity and resilience

The underlying assumption of the "cytokine hypothesis of depression" (Maes et al., 2009), established in the 1990s, postulates that peripheral cytokines are released upon external or internal stress, or due to chronic diseases, partially causing central nervous system distortions, such as enhanced HPA-axis activity, neurodegeneration, disruption of neurogenesis, oxidative stress, and dysfunction of the serotonergic system, all of which are typical for the depressive phenotype (Pfau & Russo, 2015). Growing evidence from human and animal studies links pro-inflammatory cytokine-release from leukocytes with stress, depression and anxiety-like behavior. Since cytokines are soluble proteins, they are able to pass the blood-brain barrier (BBB) and thus can act on the central nervous system, activating sickness behaviors, like appetite loss or social drawback, which in part overlap with depressive states (Dantzer et al., 2008). Interestingly, chronic inflammatory diseases, like multiple sclerosis, rheumatoid arthritis, and asthma, raise the likelihood to develop a depression by the factor of six. In this line, also depressed patients show inflammatory markers with soluble cytokines in cerebrospinal fluids, mostly with increased IL-6 (Maes et al., 1997; Moussavi et al., 2007; Dowlati et al., 2010). In rodents, chronic stress elevates inflammatory serum- and brain marker, predominantly IL-6 and interleukin-1β (IL-1β) (Koo & Duman, 2008; Sukoff Rizzo et al., 2012; Voorhees et al., 2013). In both, animals and humans, pro-inflammatory cytokines initiate anxiety-like behavior and depression (Bonaccorso et al., 2001/2002; Sakic et al., 2001; Anisman et al., 2002). Social disruption stress (SDR), evoked by hierarchy disturbance through introduction of a dominant intruder into a social mouse group, activates the HPA-axis with proinflammatory cytokine release, which elevates integrin alpha-M (CD11b+) and stimulates glucocorticoid resistance in immune cells (Engler et al., 2008; Avitsur et al., 2009). These effects are predominantly observable in mice with subordinative behavior post SDR (Avitsur et al., 2001). Furthermore, glucocorticoids and cytokines induce stress-associated hyperinflammation and increased leukocyte numbers lead to increased release of the Tumor Necrosis Factor α (TNF-α)

and IL-6 (Avitsur et al., 2005; Stark et al., 2001). Taken together, in susceptible mice the adaptive immune response provoked by stress-infused corticosterone is compromised in comparison to non-stressed or resilient animals (Pfau & Russo, 2015). Fascinatingly, vulnerability to CSDS is predictable via higher peripheral blood leucocyte numbers and their higher release of IL-6 in response to immune challenges in mice which became susceptible to stress by contrast to resilient animals (Hodes et al., 2014). Also, increased spleen and blood levels of CD11b+ monocytes are risks factors for susceptibility of CSDS, since stress promotes proliferation and emission of immature, pro-inflammatory myeloid cells from bones with striking expression of the surface marker lymphocyte antigen 6 complex (Ly6C) and dependent from β-adrenergic signaling (Powell et al., 2013). Intriguingly, sympathetic nervous system mediated leukocytosis is linked to stress vulnerability as well (Heidt et al., 2014). Interestingly, resilient and control mice lack peripheral markers which are detrimental in stress susceptible mice.

In the central nervous system immune signals can either derive through microglia, locally producing cytokines or latter can enter the brain from the periphery through the blood brain barrier (Dantzer et al., 2008; Wohleb et al., 2013). In addition, the brain is capable of receiving peripheral immune responses via stimulation of vagal nerves and brainstem nuclei (Quan, 2008; Pavlov & Tracey, 2012). Inflammatory signals within the central nervous system cause behavioral changes, set off by connected HPA-axis activation, by glucocorticoid-induced neuronal atrophy, and excitatory synaptic plasticity (Boersma et al., 2011; Christoffel et al., 2011; Iwata et al., 2013). Above all, IL-1β has a prominent role in the inflammatory brain response, most likely through release of the microglial NLRP3 (Nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3) inflammasome, which is built via stress induced stimulation of the cytosolic pattern recognition receptor NLRP3 and the pro-caspase-1. The complex then cleaves pro-IL-1β into IL-1β, which is subsequently released from microglial cells (Farrar et al., 1987; Iwata et al., 2013). Specially, the hippocampus has vast amounts of microglial cells and consequently upon acute stress NLRP3 inflammasome is highly activated in this region (Iwata et al., 2013). Blockage of IL-1β receptors reverses anhedonia in rats (Koo & Duman, 2008), suggesting IL-1β as important player in stress vulnerability and resilience. Through down-stream mechanisms IL-1β activates HPA-axis and inhibits hippocampal neurogenesis (Koo & Duman, 2008; Koo et al., 2010). Moreover, the activation of IκK–NFκB promotes CSDS induced structural changes in glutamatergic neurons of the NAc, and inhibition promotes resilience (Christoffel et al., 2011). Further, upon acute stress, thin spines were formed in the NAc and drove social avoidance behavior (Christoffel et al., 2012) were formed in the NAc and drove social avoidance behavior.

1.2.1.5 Molecular and cellular resilience mechanisms in the brain

Mesocorticolimbic circuitry is involved in the reward system, ensuring survival through focusing on rewards and was heavily studied in resilience mechanisms (Russo & Nestler, 2013). The mesocorticolimbic system includes neurons from the medial prefrontal cortex (mPFC), hippocampus, NAc, amygdala, VTA, and lateral hypothalamus (Pfau & Russo, 2015). Dopaminergic neurons of the VTA project onto GABAergic neurons in the NAc and fire in response to reward, and sometimes aversive stimuli (Pfau & Russo, 2015). NAc neurons signal back to the VTA in a reciprocal way. It was shown in humans that this pathway is compromised in depressive and anxiety patients and that the volume of these brain regions is reduced in older people (Husain et al., 1991; Drevets et al., 1992; Krishnan et al., 1992).

Analysis of CSDS stressed mice, revealed epigenetic changes in the NAc of resilient animals involving the upregulation of the factor ΔFosB (delta FosB proto-oncogene) (Vialou et al., 2010), which downstream targets the AMPAR subunit GluA2 (glutamate ionotropic receptor AMPA type subunit) and Sparc-like 1 (SC1). Resilient animals show higher expression of the GluA2 subunit in the NAc, ultimately resulting in decreased AMPAR function since GluA2 containing receptors are Ca^{2+}-impermeable, leading to lower conductance and less inwardly rectifying currents. SC1 on the other hand, localizes to the PSD and is important for the proper assembly of synapses, plus it is overexpressed in animals resilient to CSDS (Pfau & Russo, 2015). Additionally, Golden and colleagues (2013) showed an influence of the Rac1 (ras-related C3 botulinum toxin substrate 1) Rho (rhodopsin) GTPase (Guanosine triphosphate hydrolase enzyme) in resilience and susceptibility to chronic stress via epigenetic changes. Rac1 acts on cofilin 1, an actin interacting protein, mainly involved in synaptic plasticity and it is upregulated after CSDS in resilient mice and oppositely downregulated in stress susceptible animals (Golden et al., 2013a). Upregulation is achieved through reduced methylation within the *Rac1* promoter and resilient behavior most likely results due to structural changes in spine structure of the NAc.

Further, mice susceptible to CSDS show enhanced burst firing of dopaminergic neurons in VTA, due to enhanced hyperpolarization cation currents, but resilient animals are spared from these events (Krishnan et al., 2008; Cao et al., 2010). Another factor implicated in enhanced neuronal activity of the VTA is the protein kinase B (AKT), which targets GABAergic inhibitory neurons. This event produces a reduction of inhibitory tone on VTA dopaminergic neurons which eventually guides social avoidance behavior (Krishnan et al., 2008). It was also found that stress-induced phasic firing of dopaminergic VTA neurons projecting on NAc neurons is sufficient to provoke depressive-like behavior in mice (Chaudhury et al., 2013). In resilient animals, normal dopaminergic neuron firing rates and AKT signaling is preserved before and after stress exposure. Interestingly, the hyperpolarization cation current is even more enhanced as compared by

susceptible mice, but is compensated by an enhancement of potassium (K^+) channels, which revert the firing rate to a normal level in a homeostatic way (Friedman et al., 2014).

The NAc receives additional glutamatergic innervations from the amygdala, hippocampus, PFC, and thalamus (Pfau & Russo, 2015). Animal studies revealed decreased PFC activity after chronic stress, with significant atrophy and synapse loss on glutamatergic PFC neurons (Christoffel et al., 2011; Duman & Li, 2012; McEwen & Morrison, 2013). Apart from this expression of some immediate early genes (IEG) such as *Arc* and *Egr1* – a representation of neuronal activity – was diminished in the ventromedial PFC of stress susceptible mice (Pfau & Russo, 2015). In addition, burst firing in mPFC neurons, seen as resilience favoring event, goes along with increased IEG expression c-*Fos* (Pfau & Russo, 2015). In summary, inhibition of PFC projection is pro-susceptible and might be a suitable target for promoting resilient behavior towards stress.

1.3 The hippocampus

1.3.1 Historical perspective on the hippocampus

Intensive documentations of the hippocampus over the past decades evidenced this brain region as key player in memory formation, learning, synaptic plasticity and computation (Andersen et al., 2007). Already early observations in the second half of the 19th century, and the first half of the 20th century, carried out on animals and patients with an impaired or lesioned temporal lobe suggested a role in emotion processing and attention control for the hippocampus together with its integration into the olfactory system (Ferrier, 1877; Papez, 1995; Brodal, 1947; Green & Arduini, 1954). Anatomically advanced studies from the 1980s proved (Shipley & Adamek, 1984; Amaral et al., 1987). Many of those proposed functions for the hippocampus were under debate. Hippocampal function was condensed to the same result – its implication in memory formation (Andersen et al., 2007). Chiefly, the hippocampus started to become focus of attention with the famous case of the patient H.M. in 1953. Through the attempt to treat his epileptic seizures by the removal of major parts of his hippocampus, the patient lost the ability to form a declarative memory by showing no impairments in intellectual and perceptive functions at the same time (Scoviille & Milner, 1957). From this point on, H.M. and similarly treated patients became subjects of memory research (Squire, 2009). Following up these observations, behavioral studies and neuroanatomical work on the hippocampus in human and animal research helped to shape the understanding of learning and memorization skills, plus pushed advances in memory research (Squire, 2009). Consequently, the hippocampus with its *cornu ammonis* (CA)-fields, the dentate gyrus, and the subicular complex together with its connectivity to the neighboring entorhinal,

perirhinal and parahippocampal cortices, were identified in building declarative memory (Squire & Zola-Morgan, 1991; Suzuki & Amaral, 1994; Burwell et al., 1995). In particular, declarative memory is characterized as recollection or upbringing memories into consciousness for assessment of the external world (Squire, 2009; Knierim, 2015). It is subdivided into episodic and semantic memory. Where episodic memory describes the recollection of memories bound to a specific time and location, semantic memory refers to one's general knowledge about the environment (Knierim, 2015). Especially the hippocampus is involved in forming and sustaining the episodic memory (Squire, 2004; Knierim, 2015). Over the years it became evident that the hippocampus is not only susceptible to damage from epilepsy, but also hypoxia, ischemia and encephalitis (Knierim, 2015). Associated with the vulnerability of the hippocampal region is the evidence that plaques and tangles start to occur initially in the entorhinal cortex upon Alzheimer's disease (Knierim, 2015).

The discovery of hippocampal place cells in rats in 1970s by O'Keefe and Dostrovsky uncovered the processing of spatial information within the nervous system (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978). In detail, pyramidal neurons of the CA1-, CA2-, and CA3-field as well as granule cells of the DG are activated, showing selective firing potentials when the animals occupy a distinct location in a given environment (Dostrovsky, 1971; Knierim, 2015). Aside from place cells, grid cells, located in the medial entorhinal cortex (MEC) and other extrahippocampal areas, participate in computing a spatial position through processing of self-motion and calculation of environmental boundaries via close communication with the hippocampus (Fyhn et al., 2004). After many years of research, the importance of the hippocampus and its adjacent structures such as the entorhinal cortex (EC) were unveiled as main structures in the brain encoding position, distance, movement and time (MacDonald et al., 2011; Derdikman & Knierim, 2014). With advancing hippocampal research, the idea of its involvement in emotions, anxiety and stress was well established (Bannerman et al., 2014) with accessory proof of its processing of odor-guided learning and social memory integrating information from olfactory and limbic systems (Eichenbaum & Otto, 1992; Eichenbaum et al., 1996; Petrulis et al., 2005). It is important to point out that the hippocampus integrates sensory and cognitive information from multiple sources to facilitate memory (Squire, 2004; Neves et al., 2008). Hippocampal synaptic long-term plasticity (LTP) is required for memory formation in rodents and is stimulated through inputs from the auditory, visual and olfactory systems that process spatial experiences (Kemp & Manahan-Vaughan, 2004; André & Manahan-Vaughan, 2013; Dietz & Manahan-Vaughan, 2017). Especially electrophysiological recordings showed that associative, odor-dependent memory formation is an intrinsic processing mechanism of the hippocampus (Rangel et al., 2016). Different hippocampal functions are associated with distinct hippocampal areas along the longitudinal axis (Moser, 1997). Memory function and spatial navigation are attributed pre-dominantly to the dorsal (rodents) or posterior (primates) hippocampus, whereas regulation of anxiety-related behaviors is

traced back to the ventral (primates) or anterior (primates) hippocampus (Strange et al., 2014). This functional division of the hippocampus is debated, since genomic analysis, together with electrophysiological and anatomical investigations, illustrate a gradient with superimposed functional occupations along the long axis of the hippocampus (Strange et al., 2014). Another fascinating quality of this brain structure is the ability of adult neurogenesis as characteristic of granule cells (Andersen et al., 2007) which are the principal cells of the dentate gyrus. Exploration of the life-long renewal of granule cells shields light into neuronal repair and substitution mechanisms, thus opens therapeutic opportunities regarding the treatment of neuronal loss (Andersen et al., 2007).

Some aspects of hippocampal function, such as explicit cellular mechanisms and network, microcircuits as well as its detailed role as inter-regional integrator of various cortical brain regions to allow memory-guided behavior, raise open questions and need to be pursued in the future (Lisman et al., 2017; Moser et al., 2017). Other aspects of hippocampal function are currently investigated. Above all, the finding of its implication in the stress response via inhibitory connections to the hypothalamus (Andersen et al., 2007) are of special interest for our research facing stress resilience.

1.3.2 Hippocampal structure

The hippocampus is a three-layered subcortical structure, allocated ventrally beneath the medial temporal lobe with an anterior-posterior orientation in primates and humans (Figure 1.4(A)) (Strange et al., 2014). Amongst mammals the hippocampus appears to be a highly conserved region and it is subdivided into several subregions (Figure 1.4(C)) including the dentate gyrus (DG), the CA-subfields 1-3 and the subiculum (SUB) (Andersen et al., 2007; Strange et al., 2014). A broader anatomical definition includes adjacent brain structures, which show a neocortical six-layered structure, such as the highly interconnected presubiculum (pre) and parasubiculum (para), and the EC into the hippocampal formation. These regions are often referred to as parahippocampal formation (Figure 1.5(A)) (Andersen et al., 2007, van Strien et al., 2009; Strange et al., 2014).

The hippocampus presents as long and elongated structure, owing its name by its sea horse-shape (genus *Hippocampus*) (Strange et al., 2014; Knierim, 2015). In rodents the hippocampus is a relatively large structure, allocated beneath the neocortex (Figure 1.4(B)) and it can be separated anatomically, along the longitudinal axis into the dorsal, more rostral part and the ventral part where it curves in more caudal levels (Figure 1.6(A)) (Knierim, 2015). The hippocampal cortex is organized in three layers (Figure 1.5(B)). While the deepest layer harbors afferent and efferent fibers and interneurons named hilus in the DG and *stratum oriens* (so) in the CA-fields, the surface

layer *stratum pyramidale* (sp) above is the substantial cell layer comprised of interneurons and granule cells in the DG and pyramidal neurons in the CA-subfields, lying beneath the third and most superficial molecular layer or *stratum moleculare* (sm), (van Strien et al., 2009). In the pyramidal subfields the molecular layer is subdivided into the *stratum lucidum* (sl), receiving inputs from the DG, the *stratum radiatum* (sr), with apical dendrites of the cells comprising the pyramidal layer, and the superficial *stratum lacunosum-moleculare* (slm) with the apical tufts of those apical dendrites (van Strien et al., 2009). Notably, the *stratum lucidum* is missing in the CA1- and CA2 area (van Strien et al., 2009). Within the dentate gyrus, the molecular layer is subdivided into three sublayers, the inner (iml), middle (mml) and outer molecular (oml) layers, with approximately the same width (Andersen et al., 2007; van Strien et al., 2009). Also, the parahippocampal formation is separated into several layers as outlined at the beginning. The layers II-III and V to VI represent four dense layers with the sparse layer IV and I in between separating them from other structures (Figure 1.5(C)) (Andersen et al., 2007; van Strien et al., 2009).

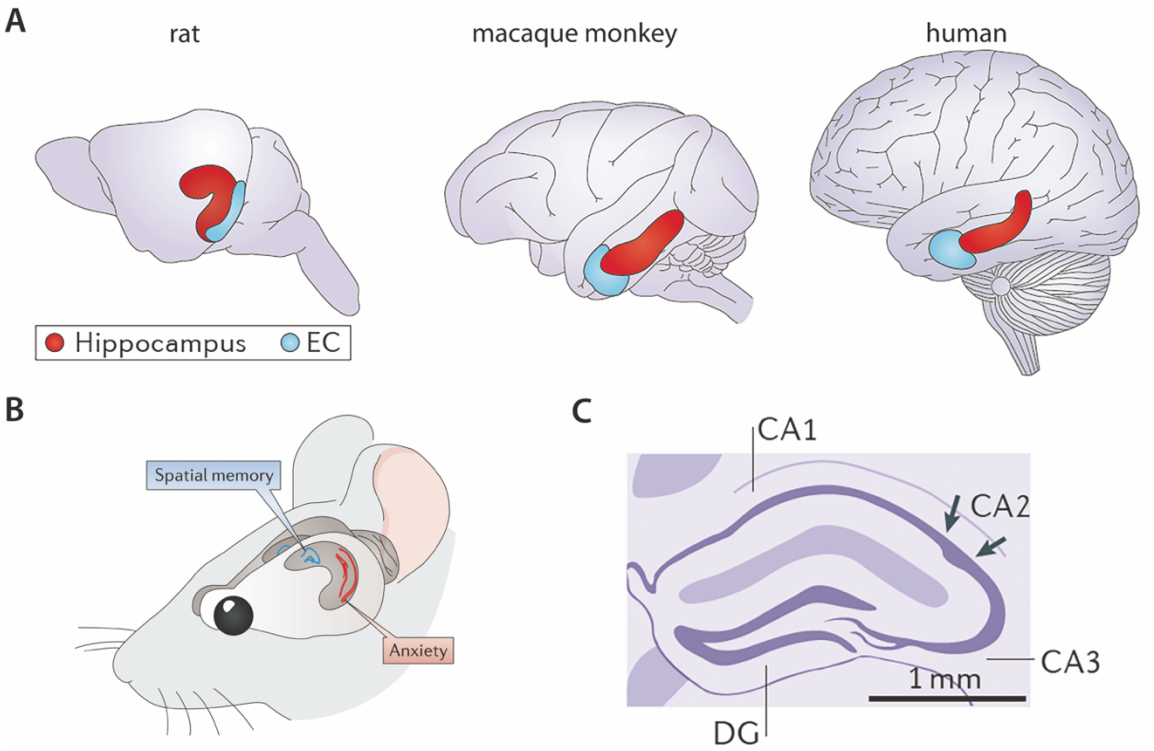

Figure 1.4: Anatomical position of the hippocampus and its structural subdivision.
(A) Schematic and cross-species comparative illustration of rat, macaque monkey and human brains in an anterior-posterior orientation with plotted hippocampi (red) and adjacent entorhinal cortices (EC; blue) along their long axis respectively. **(B)** Subregional differences of the rodent hippocampus were revealed through behavioral experiments. Spatial memory is processed in the dorsal hippocampus (blue), whereas the ventral hippocampus is important for anxiolytic functions (red) from Bannerman et al. 2014. **(C)** Illustrations of a Nissl-stained brain section depicting the murine hippocampus, showing principal hippocampal structures: Dentate gyrus (DG) and *cornu ammonis* fields CA1-CA3. Panels **(A)-(C)** modified from Strange et al. 2014.

The hippocampal CA-fields mainly project to the dense layers of the parahipocampal formation with different activity patterns (Witter et al., 2017; Böhm et al., 2018; Simonnet & Fricker, 2018). Recent investigations aim to distinguish classes and functional groups of principal neurons within the hippocampus for better understanding of connections between functional, molecular, anatomical and physiological differences of those cells by analyzing variations in gene expression, cell morphologically, electrophysiology and connectivity (Khalaf-Nazzal & Francis, 2013; Cembrowski et al., 2016; Soltesz & Losonczy, 2018).

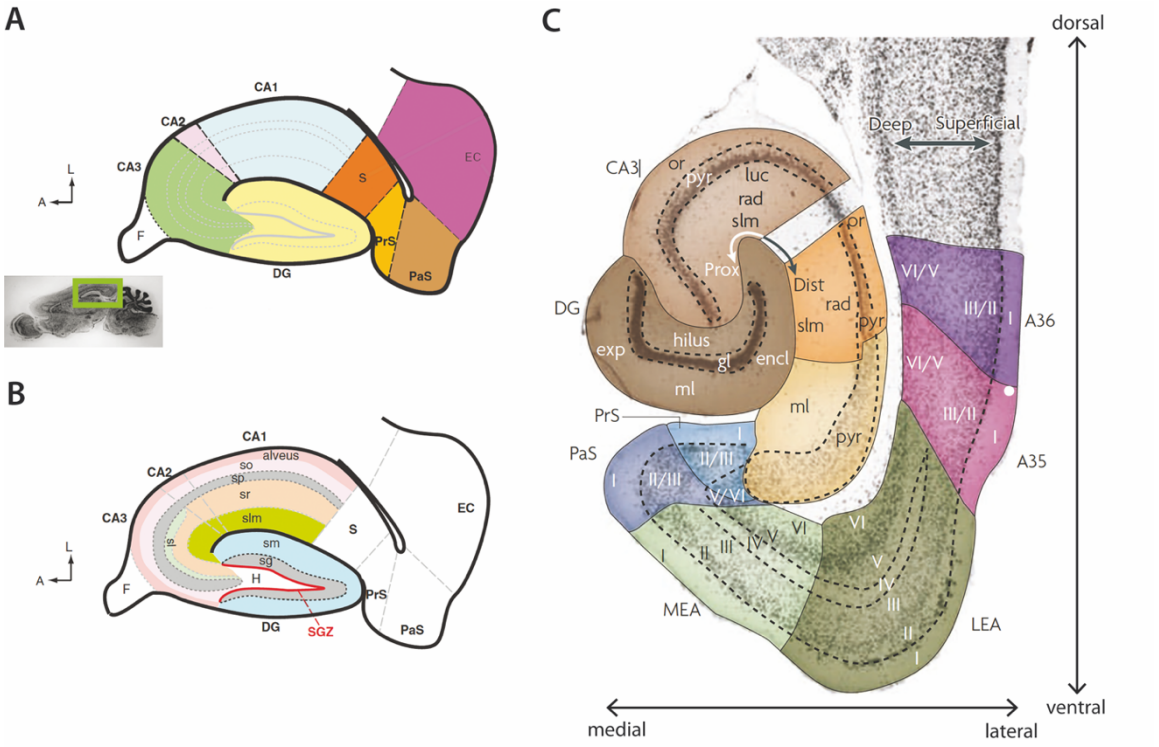

Figure 1.5: Hippocampal structure and lamination.
(A) Schematic illustration of the hippocampal formation within the murine brain, depicting the area surrounded by the green box in the sagittal mouse brain section presented in the left bottom corner. The scheme shows the position of distinct hippocampal and parahippocampal structures: Dentate gyrus (DG; citron), CA3 (green), CA2 (rose), CA1 (light blue), subiculum (S; orange), presubiculum (PrS; yellow), parasubiculum (PaS; brown), entorhinal cortex (EC; lilac). **(B)** Schematic overview of hippocampal layering: The innermost layer of the dentate gyrus is called hilus (H) or polymorphic cell layer with projections from mossy fibers. Above lies the granule cell layer or *stratum granulosum* (sg) with the subgranular zone (SGZ) in between. The sg is bordered superficially by the *stratum moleculare* (sm) or molecular layer with fibers from the hilus and EC. The principal cell layer of the CA-subfields is the *stratum pyramidale* (sp). Adjacent to sp in the CA3 area is the *stratum lucidum* (sl) containing mossy fibers from the DG. Above sl in CA3 and sp in CA1 and CA2 boarders the *stratum radiatum* (sr) with Schaffer collateral connections from CA3 or self-associated fibers at the CA3 area. Adjoining and most superficial is the *stratum lacunosum-moleculare* (slm) with projections from the EC via the perforanth path. Apical dendrites of pyramidal neurons extend into the *stratum oriens* (so), a structure with a dense net of inhibitory interneurons. Beneath it lies the alveus, containing pyramidal cell axons. **(C)** Horizontal and nissl-stained murine section of the hippocampus. Superimposed are distinct hippocampal structures with adjoining representations of parahippocampal formation and three-dimensional axes. Cortical layers are labeled with roman numbers (I-VI). The entorhinal cortex is divided into the lateral (LEA) and medial (MEA) part. The perirhinal cortex is represented by the Brodmann areas A 35 and A 36 along the dorsoventral axis. Hf, hippocampal fissure; gcl, granule cell layer; ml, molecular layer; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; SUB, subiculum. **(A-B)** modified from Li & Pleasure, 2014; **(C)** modified from van Strien et al. 2009.

1.3.3 Hippocampal connectivity

There are three major fiber bundles connecting the hippocampus with other brain areas. The angular bundle is known as the fiber path between the entorhinal cortex and the hippocampus (Andersen et al., 2007). Connections between hippocampus and the forebrain, hypothalamus and brain stem are named fimbria-fornix pathway and the last major bundle is carrying the dorsal and ventral commissures, connecting the hippocampi of both brain hemispheres with each other (Andersen et al., 2007).

The hippocampal connectivity is described as "trisynaptic loop" (Figure 1.6(B)) due to its three major synaptic circuits (Knierim, 2015). Most of the inputs to the hippocampus are provided by the entorhinal cortex with the strongest projections to the dentate gyrus (DG) via the perforant path, which traverses the subiculum (Ramón y Cajal, 1995; van Groen et al., 2003; Andersen et al., 2007; Knierim, 2015). In rodents, the EC is composed of two parts. First, there is the medial entorhinal cortex (MEC), linked with brain areas important for spatial processing and second, the lateral entorhinal cortex (LEC) associated with brain areas processing high order object recognition (van Groen et al., 2003; van Strien et al., 2009; Knierim, 2015). Both regions do have feedback projections to each other and receive inputs from the olfactory brain system and the prefrontal cortex (Andersen et al., 2007; Knierim, 2015). Axons from neurons of the DG project via their mossy fibers to the *stratum lucidum* reaching pyramidal CA3 neurons through the so-called mossy-fiber pathway (Spruston, 2008), and CA3 neurons connect to *stratum radiatum* and *stratum oriens* of the CA1-area by the Schaffer collateral pathway (Figure 1.6(B)) in an ipsi- and contralateral way (Amaral et al., 2007; Andersen et al., 2007; Shinohara et al., 2012). To close the loop, CA1 neurons project back to deep layers of the EC and the subiculum (Böhm et al., 2018; Köhler, 1986). Likewise, the deep layers of the EC project back to cortical brain areas which originally projected on the hippocampus, making it an area merging sensory input, processed information and subsequent output (Köhler, 1986). Aside from the trisynaptic loop, CA3-neurons build collateral synaptic connection with other CA3-neurons (Le Duigou et al., 2014). Additionally, intense experimental studies performed on the hippocampus revealed more complex signaling within the hippocampal structures, finding feedback circuits and parallel processing circuits (Figure 1.6(C)). For example, the entorhinal cortex was found to directly connect to CA3 and CA1-regions as well (van Groen et al., 2003; Andersen et al., 2007; van Strien et al., 2009; Witter et al., 2017) and CA3-neurons are able to project in a feedback loop to the DG via excitatory mossy fiber cells of the hilus, mainly innervating inhibitory neurons (Scharfman, 2007). Inputs from layer II of the MEC and LEC are similarly sent to CA3 and DG in the transverse axis and do lap over. In contrast, projections to CA1-neurons are more regionally segregated with the MEC mainly projecting to CA1 close to CA2 and LEC mainly projects to CA1-areas close to the subiculum.

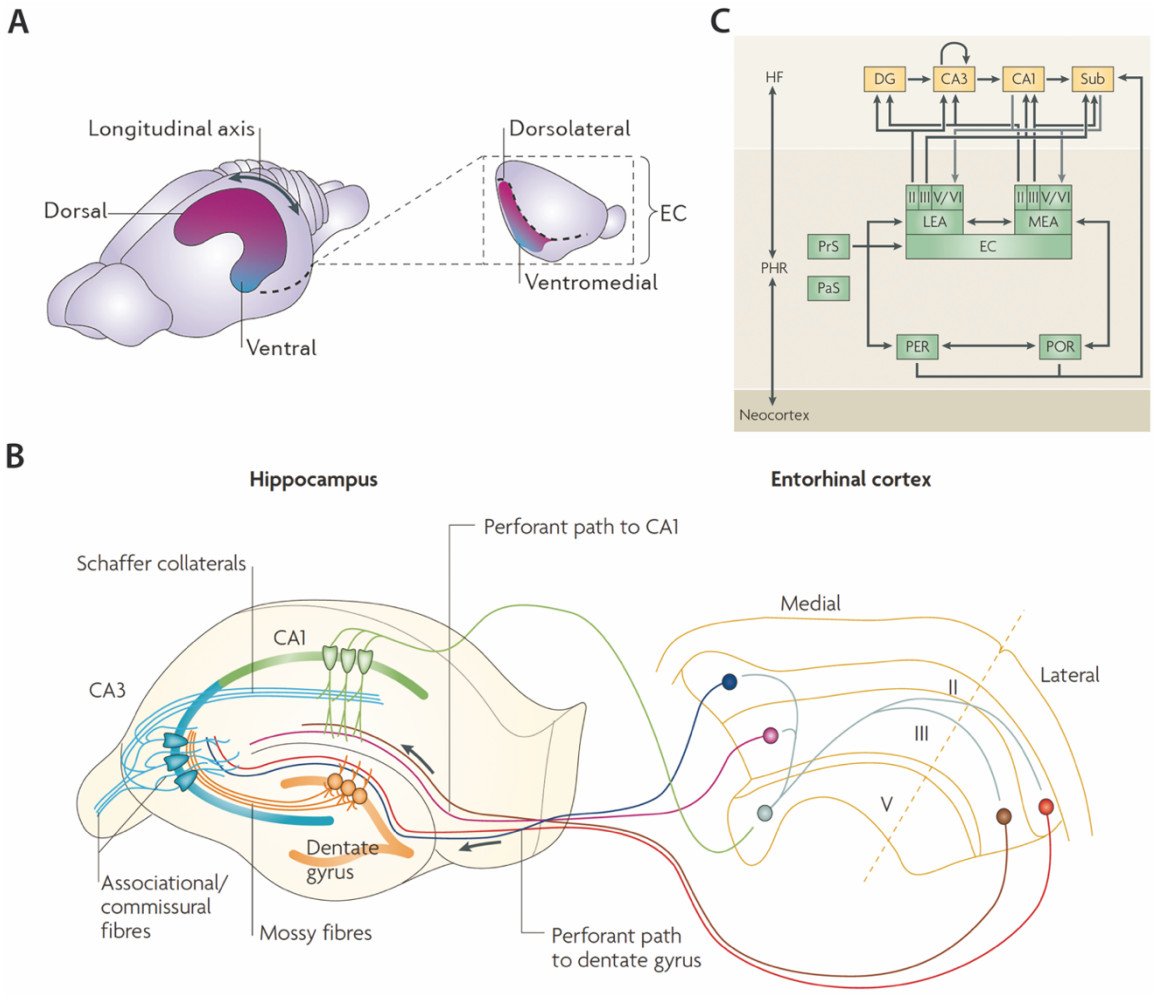

Figure 1.6: Hippocampal connectivity patterns
(A) Representation of the murine hippocampus and EC with topographical arrangements of their connectivity pattern along the longitudinal axis. The magenta-shaded dorsolateral portion of the entorhinal cortex (EC) is primarily connected with the dorsal part of the hippocampus, whereas more ventral parts (purple to blue) are increasingly connected with the ventral part of the hippocampus (modified from Strange et al. 2014). **(B)** The basal fiber pathways within the hippocampus are referred to as trisynaptic loop. Most sensory inputs arrive from layer II of the EC via its axons (perforant path) to the DG, where they build synaptic contacts with dendrites of the outer and middle third of granule cells. Granule cells connect with their axons via the mossy fiber tract with pyramidal CA3 neurons and they in turn project ipsilaterally to CA1 neurons via Schaffer collaterals and contralateral to CA3 and CA1 cells through the commissural fiber tract. CA3 cells build interconnections within themselves and additionally receive direct inputs from layer II of the EC. Distal apical dendrites of CA1 neurons receive inputs from the EC as well, coming from layer III. Axons from CA1 neurons project back to layer V of the EC (modified from Neves et al. 2008). **(C)** Circuitry overview of the hippocampus and parahippocampus. The parahippocampal (PHR) regions receives neocortical projections and transfers inputs to the hippocampal formation (Hf). The perirhinal (PER) cortex projects to the lateral entorhinal (LEA) cortex, the postrhinal (POR) cortex projects to the medial entorhinal (MEA). The EC matches inputs from PER and POR and receive additional inputs from the presubiculum (PrS). As described in **(B)**, the trisynaptic loop is depicted as connectivity pattern originating from the EC and Hf interconnections are mapped as well. The subiculum (Sub) connects to the deep layers of the EC (modified from van Strien et al. 2009).

The EC inputs to CA1 originate from layer III prevailingly (van Groen et al, 2003; Andersen et al., 2007; van Strien et al., 2009; Knierim, 2015; Witter et al., 2017). Deeper layers of the EC do receive feedback inputs from the hippocampus. The inputs from the EC to the hippocampus are critically

affected by the interconnections established by distinct layers of the EC. As result entorhinal interconnections directly affect neural processing of hippocampal inputs (Knierim, 2015).

Shortly summarized, the hippocampus also receives inputs from other cortical and subcortical brain areas such as the perirhinal and postrhinal cortices, the medial septum, the locus coeruleus, the raphe nucleus, the nucleus reuniens, and the amygdala (Knierim, 2015). On the other hand, most of the CA1 and CA3 outputs leave through the fornix (Figure 1.6(B)) to the lateral septum. In addition, CA1 neurons project to the nucleus accumbens of the ventral striatum, the amygdala, and the prefrontal cortex. In addition, recent studies award the CA2-area a separate and in part unattached function from the CA1- and CA3-areas (Jones & McHugh, 2011). Most CA2 neurons receive afferents from DG granule cells and CA3 in the *stratum radiatum* and from the EC in *stratum lacunosum-moleculare* (Benoy et al., 2018; Carstens & Dudek, 2019), while projecting ipsilaterally to CA1-neurons (Shinohara et al., 2012). The dorsal hippocampus builds connections especially with the MEC involved in spatial processing and the ventral hippocampus connects primarily with the amygdala and the prefrontal cortex (Knierim, 2015). Many studies worked out computational difference between species. It was indicated that the posterior hippocampus in primates functionally correlates to the dorsal hippocampus in rodents. Equally the primate anterior hippocampal part corresponds to the ventral hippocampus in rodents, highlighting computational heterogeneity between distinct hippocampal parts.

1.3.4 The development of the hippocampus

The hippocampus derives from the medial lobe of the telencephalic vesicle (Andersen et al., 2007). It starts to develop out of the cortical hem, positioned at the medial edge of the telencephalon and expressing morphogens such as the Wingless-related (WNT) signaling molecule (Hébert et al., 2002; Shimogori et al., 2004). In the murine brain, first hippocampal cells appear at embryonic day (E) 10 (Andersen et al., 2010) with CA3 pyramidal neurons being generated first having their peak on day E17 and CA1 neurons one day later (Angevine 1965; Grove & Tole, 1999; Danglot et al., 2006) with lasting generation until early postnatal days (Altman & Bayer, 1990). Granule cells begin to be generated at the same time as pyramidal cells, with a much longer production lasting until a late postnatal period and even with a remaining neurogenic zone, known as the subgranular zone (SGZ), in adulthood (Ming & Song, 2011; Fuentealba et al., 2012; Paridaen & Huttner, 2014). The stem cells for both, pyramidal neurons and granule cells, derive from the ventricular neuroepithelial layers situated below the ventricular wall next to the CA1 subfield (Altman & Bayer, 1990). At the same time gliogenesis starts at late embryonic stages and continues in postnatal stages with widely production of astrocytes and oligodendrocytes throughout the brain

in adulthood (Gallo & Deneen, 2014; Guérout et al., 2014). At birth, the pyramidal cell layer in the rat hippocampus consists of 6–10 rows or neuronal cell bodies yet becomes thinner during postnatal stages due to pyramidal cell rearrangements and volumetric enlargement under contribution of glial cells and Cajal-Retzius cells (Czurkó et al., 1997; Andersen et al., 2007). Concurrently, 15% of granule cells are generated before birth in rats and continue to be formed during their lifespan (Altman & Bayer, 1975). When reaching the hippocampal fissure during embryonal development, neural progenitor cells build a center of proliferating cells in the so-called tertiary matrix, generating the granule cell layer originating from the matrices. In late postnatal stages neurogenesis becomes more restricted in the DG, finally bound to the SGZ (Urbán & Guillemot, 2014). Morphologically, the DG consists of two blades with the suprapyramidal blade occurring first before the infrapyramidal blade starts to form (Andersen et al., 2007). Distinct hippocampal fields are distinguished by specific cell types but also molecular markers characteristic for the hippocampal subfields (Tole et al., 1997; Khalaf-Nazzal & Francis, 2013). Since this work does not focus in embryonic development of the hippocampus, genetic patterning during embryonic stages won't be explained in detail here. Fascinatingly, microcircuits of principal neurons in the hippocampus can be subdivided into preferential connections formed at the same developmental and synaptogenic time-points while expressing similar genetic markers (Deguchi et al., 2011).

Taking a look at extrahippocampal connectivity formation, studies showed that entorhinal afferents start to innervate the hippocampus at embryonic day 15 (E15) and the DG at E18/19 (Super & Soriano, 1994). In mice, intrahippocampal connections start to reach out after principal cells arrived at their final destination shortly before birth by building axons and dendrites (Bayer, 1980). Commissural fibers begin much later in embryonic development to connect with the contralateral hippocampus starting at E18 until P2 by originating from CA3-neurons and hilar mossy fibers (Altman & Bayer, 1990; Andersen et al., 2007). Finally, synaptic contacts occur when axons and dendrites come into close proximity with growing spine density until sexual maturation in rodents (Andersen et al., 2007). Moreover, the hippocampal network continues to grow postnatally, including the creation of CA1 dendrites until postnatal day 90 in rats (Pokorny & Yamamoto, 1981). Only after this timepoint the hippocampus and the rest of the whole brain is considered to have reached the mature stage (Dumas & Foster 1995; Steward & Falk, 1991).

1.3.5 Stress and the hippocampus

In 1982 the hippocampus was first proposed to show an involvement in stress and anxiety circles, commonly known as the "septo-hippocampal" theory by Gray, proposing a hippocampal role in a behavioral inhibition system (BIS). Thereafter, a period started to elucidate the hippocampus's role in processing stressful events and its engagement, maintaining a homeostatic state (Andersen et al., 2007). Anxiolytic functions are ascribed to the amygdala and septo-hippocampal brain region (McNaughton & Gray, 2000; Andersen et al., 2007). Scientists, performing lesion experiments in animals, discovered solely the involvement of the ventral hippocampus affecting unconditioned fear and anxiety related behaviors (Kjelstrup et al., 2002; Bannerman et al., 2004). The presence of corticosteroid receptors supports the idea of the hippocampus being implicated in stress-responses and regulation of the HPA-axis (McEwen et al., 1968). In fact, persistent stress causes magnificent structural changes in some hippocampal regions and the highest density of mineralocorticoid receptors in the mammalian brain is found in the hippocampus (McEwen et al., 1980). Further research points to the fact that HPA-axis related release of cortisol (humans) or corticosterone (rodents) feeds back to the hypothalamus, but also other brain regions including the hippocampus (Andersen et al., 2007). Supporting allostasis results in physiological but also cognitive and behavioral adjustments to favor an organism's survival (McEwen, 2002). Inclusive of these adaptations may be enhanced memory functions to consolidate a reaction to stressful life events (Andersen et al., 2007). Sustaining stressful reactions lead to the so-called allostatic load with dragging negative effects like maladaptation and tissue damage (McEwen, 2000). Primary studies recommended a hippocampal inhibitory effect on glucocorticoid levels (Feldman & Conforti, 1980; Fischette et al., 1980; Wilson et al., 1980; Sapolsky et al., 1984/1990; Lupien & Lepage, 2001). Further, it was found that neurotoxic CA3 lesions in rats cause a stress-induced hypersecretion of corticosterone (Roozendaal et al., 2001). In the rat hippocampus corticosterone binds to two different types of GC receptors, namely the high-affinity type I MR with a ten-fold higher affinity to corticosterone as compared to the type II and lower-affinity GR (Joëls, 2001). Other differences are displayed by their binding properties, distribution and intracellular mechanisms, as for example the GRs are widely distributed throughout the mammalian brain reflected by their presence in the limbic system, the cerebellum and brain stem (Reul & de Kloet, 1985; de Kloet et al., 1999; de Kloet et al., 1999; Helm et al., 2002; de Kloet, 2004). MRs in the hippocampus can lead to an inhibition of the hypothalamic PVN upon ending of a stressful event, limiting the duration of a stress-response (Andersen et al., 2007). It is important to note that various systems do interplay and exert a tonic inhibition of the HPA-axis function, thus the absence of hippocampal influence on this matter could be compensated by other signaling pathways (Andersen et al., 2007). In animals, stress research is conducted applying diverse extrinsic

stressors before testing their behavioral outcomes in distinct paradigms. Exposure to mild stress was shown to facilitate increased spatial memory function (Sandi & Rose, 1994/1999), contextual fear conditioning (Cordero et al., 2003) and eye blinking condition (Shors, 2001), whereas durative stress impairs spatial memory function (Diamond et al., 1996; de Quervain et al., 1998; Conrad et al., 1999; Diamond & Park, 2000), recognition memory (Baker & Kim, 2002) and contextual fear conditioning (Figure 1.7) (Pugh et al., 1997; Rudy et al., 1999). Tests in humans revealed that stress or application of cortisol impair declarative memory, while on the contrary administration of low cortisol doses, mimicking mild stress, improved memory recall functions, like the recall of emotionally arousing pictures (Becker & Olton, 1980; Kirschbaum et al., 1996; Newcomer et al., 1999; de Quervain et al., 2000; Buchanan & Lovallo, 2001).

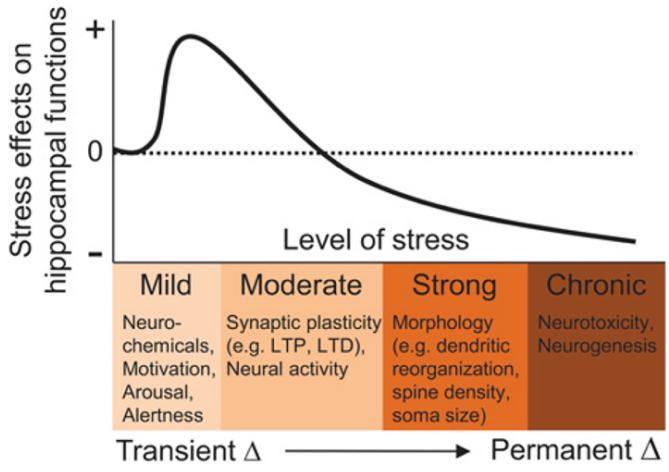

Figure 1.7: Effects of stress on the hippocampus.
Upon persistent stress alterations of hippo-campal functions do increase and positive effects evoked by stress do change into harmful adaptations within the hippocampus, including shifts in neurochemicals, synaptic plasticity, neural activity, morphological changes, and neurogenesis. In consequence these changes affect behavioral and cognitive functions and favor the onset of psychopathologies. Y-axis: (+) represents the positive and (-) negative hippocampal functions. X-axis: from left to right increasing and enduring stress with adding malfunctions occurring in the hippocampus (From Kim et al., 2015).

Early effects of stress on the hippocampus might be carried out via the amygdala which is activated by adrenalin. Additionally, the activation of GRs can provoke changes of receptor molecules in the cytoplasm (de Kloet, 2004). Other nongenomic and fast reactions were revealed influencing the MRs. Within 20 minutes after stress exposure, GABA-transmission is affected and CA1 neurons show an increased frequency in miniature excitatory postsynaptic currents (EPSCs), most likely by insertion of additional MRs from the nucleus and cytoplasm to the surface membrane (Karst et al., 2005). On the other hand, delayed effects of corticosteroids involve gene expression. *In vitro* experiments from Joels and colleagues (2001) examined delayed responses after GC administration revealing decreased granule cell turnover, which diminished afterhyperpolarization (AHP), smaller Ca^{2+}-currents, and milder responses to serotonin (5-HT) (Joëls, 2001). Activity-dependent synaptic plasticity is also impaired after prolonged exposure to stress, as reflected in impaired LTP, while simultaneously induction of long-term depression (LTD) increases in DG granule cells and CA1 pyramidal neurons (Foy et al., 1987; Diamond et al., 1992; Kim et al., 1996; Garcia et al., 1997; Xu et al., 1997; Mesches et al., 1999; Diamond & Park, 2000).

Interestingly, low serum levels of corticosteroids and acute small doses of glucocorticoid agonists help facilitate LTP and are positively correlated (Pavlides et al., 1994; Bennett et al., 2013). These findings led researchers formulate the idea of hippocampal "metaplasticity", defining it as physiological range of endogenous plasticity without necessarily inducing LTP and which is triggered by stress and adrenal steroids, concurrently reversible and bi-phasically modulated (De Kloet et al., 1999; Kim & Diamond, 2002; Abraham, 2004). The "MR/GR balance" hypothesis attempts to explain the stress level by GC dose-dependent and biphasic effects on synaptic plasticity and memory. This hypothesis implies that predominantly MRs are occupied and active upon basal circulatory corticosteroid levels, due to their higher affinity to GCs, which is mainly the case during non-stressful phases and the circadian rhythm. Once corticosteroid levels rise, GRs become steadily occupied as well until both receptor types are entirely activated. Over a short period of time, it maintains the information processing of the hippocampus with a tonic effect on the HPA axis. However, strong and persisting stress goes along with GR saturation and weakening of excitatory inputs by implication of negative effects on synaptic plasticity (de Kloet et al., 1999; Joëls, 2001). Transcription-dependent mechanism exert a delayed modulation of hippocampal cells after stress-exposure (de Kloet et al., 1998; Joels, 2001) and are happening in two ways. For one, GRs translocate as homodimers to the nucleus, binding to glucocorticoid response elements (GRE) in target genes such as *N*-methyl-D-aspartate (NMDA) receptors (Weiland et al., 1997) and serum- and glucocorticoid-inducible kinase (*Sgk*) (, a kinase involved in memory consolidation in rodents. Second, GR complexes can alter gene expression through protein-protein interaction with transcription factors such as AP1 (Activator protein 1), NFκB and CREB (cAMP response element-binding protein) (Auphan et al., 1995). Generally, exposure to chronic stress with long-lasting high levels of stress hormones is associated with structural changes in the hippocampal formation (Andersen et al. 2007).

In the 1980's Sapolsky formulated the "glucocorticosteroid cascade hypothesis" postulating that adrenal steroids have an impact on aging in hippocampal neurons, resulting in neuronal damage and dysfunction (Sapolsky et al., 1986). In particular, extended stress leads to sustained HPA-axis activity and thereby structural modifications in the hippocampus (McEwen, 1999; Anderson et al., 2007). For example, in rat experiments it was observed how moderate exposure to stress leads to a reversible atrophy of apical CA3 dendrites, granule cell neurons and altered synaptic structure (Woolley et al., 1990; Magariños et al., 1996b; Magariños et al., 1997), all going along with reversible impairments in spatial learning (Luine et al., 1994). Chronic stress on the other hand was shown to cause complete loss of CA3 and CA1 pyramidal neurons in rats as well as in primates (Sapolsky et al., 1985; Kerr et al., 1991; Mizoguchi et al., 1992). In addition, a longitudinal MRI study on rats that underwent chronic stress, demonstrated hippocampal volume loss in comparison to prestress conditions (Lee et al., 2009). Strikingly, adult neurogenesis is

negatively affected by chronic stress, through reduction of granule cell proliferation in the hippocampal neurogenic niche, the SGZ (Cameron & Gould, 1994; Gould et al., 1997). Investigations revealed this influence of corticosteroids on granule cell precursor cells as secondary mechanism, most likely via NMDA receptors, since they do not express MR and GR receptors themselves (Kim & Diamond, 2002). Further negative effects of long-term stress show increased susceptibility of hippocampal cell death after neurological damage through hypoxia, ischemia and seizures mediated via GC-induced reduction of glucose transport and raised Ca^{2+}-influx (Andersen et al., 2007). Early life stress affects prenatal development and associated malfunction can persist throughout adult life (Andersen et al., 2007). During fetal development GCs play an important role, whereas an excess of GCs leads to fetal growth impairment and tissue maturation deficits. Administration of GCs to pregnant rats caused reduced body weight in their offspring after birth and cardiovascular, metabolic, and neuroendocrine maladaptation, observable throughout the offspring's life-span (Seckl & Meaney, 2004). Stress exposure in later developmental prenatal stages, as demonstrated in various mouse studies, impairs cognitive function in adult stages due to permanent HPA-axis activity and reduced levels of MRs and GRs in the hippocampus (Andersen et al., 2007).

Apart from the hippocampus, additional higher brain regions are sensitive to stress as well, and partially signal to the hippocampus plus work together in a synergistic manner. For instance, it is known that the amygdala mediates emotional arousal effects on memory consolidation and that adrenalin such as glucocorticoids modulate long-term memory consolidation regarding fear and emotions (Cahill & McGaugh, 1998; McGaugh, 2000; McGaugh & Roozendaal, 2002). The reciprocal amygdala-hippocampus circuitry is speculated to qualify important experiences through emotional arousing events (Richter-Levin & Akirav, 2003; Majak & Pitkänen, 2003). Another HPA-axis regulatory brain region is the prefrontal cortex (Diorio et al., 1993) with its HPA-axis feedback loops (Lupien & Lepage, 2001). It is interesting to notice that apart from the hormonal involvement, HPA-axis regulation is mainly mediated by cognitive processing areas including learning, planning, decision-making, and memory (Andersen et al., 2007).

After all, there is strong indication that stress steroids initiate changes in hippocampal excitability and modifications in synaptic plasticity (Andersen et al., 2007). Acute, short-term stress leads to internal physiological, but also behavioral adaptations to serve short-term coping strategies for the organism's survival, eventually triggering fight, flight or freezing (Andersen et al., 2007). The arising questions why stress can be both, negative and positive for an individual is explained by the theory that even the negative effects help creating an adaptive mechanism, focusing only on storage of beneficials information that primarily subserve survival (Diamond et al., 2004; Andersen et al., 2007). A good example is shown by behavioral rat experiments, which revealed memory impairment when animals are subjected to stressors coming after learning (Diamond et al., 2004).

The scientists behind this study suggests a disbalance in synaptic strength and amplitude (synaptic weight) shifting into LTP-like plasticity in the hippocampus, yet caused by stress. This effect in turn represents the stressful event itself invoking an overwriting of the preceding learning, even if completely unrelated (Diamond et al., 2004). Further, the context of stressors seems to play in important role on positive or negative effects that stressors can evoke. Releasing the same corticosteroid levels, strong, aversive stimuli usually lead to memory impairment, whereas mild within-context stress can be beneficial, most likely by corticosteroid levels meeting the optimum performance levels in a certain situation (Sandi et al., 1997; Akirav et al., 2001/2004). Stress, with strong corticosteroid release pushes an organism especially to its limits when unique, life-threatening, and traumatic events occur, often going along with a persistent clinical condition – the PTSD. Studies show an implication of GRs in the hippocampus with a higher sensitivity to GCs and an increased number of GRs (Yehuda et al., 1995). Besides, imaging studies point to hippocampal atrophy in PTSD-patients with impaired declarative memory, decreased concentration and attention (Bremner et al., 1995; Gurvits et al., 1996; Bremner, 2001; Villarreal et al., 2002; Elzinga & Bremner, 2002; Sala et al., 2004).

To conclude, acute stress has a dual and phasic effect on synaptic plasticity and cognition, chronic stress on the contrary is maladaptive and destructive and one of the factors that damage the hippocampus throughout life (Andersen et al., 2007).

1.4 Neurons: Function, structure and morphology

The mammalian brain consists of billions of neurons, building functional units and cumulating into neuronal circuits that finally serve to shape our thoughts, behavior, learning, memory, emotions and dreams (Hotulainen & Hoogenraad, 2010). Communication between neurons within neuronal circuits is passed through specialized junctions – the so-called synapses (Hotulainen & Hoogenraad, 2010). Chemical synapses administer electric communication between neurons. Shortly, electric information arrives at presynaptic axon terminals and triggers neurotransmitter release through vesicle exocytosis into the synaptic cleft. On the opposite postsynaptic region neurotransmitters bind to postsynaptic receptors and induce a bunch of downstream signaling mechanism to further forward the electric signal. As a whole, concise connectivity and development of synapses are crucial for proper network connectivity and function which is the substantial basis for regular brain function (Hotulainen & Hoogenraad, 2010). Gross of excitatory neuronal synapses within the mammalian brain are formed at dendritic spines (Bourne & Harris, 2008). Spine morphology has an influence on synaptic function and the storage of information. Spine morphology, number and distribution are important for synapse

formation or elimination (Yuste & Bonhoeffer, 2001; Kasai et al., 2003). Dynamical changes of synaptic structure are known to build the basis of learning and memory in mammalian brains (Holtmaat & Svoboda, 2009; Kasai et al., 2010).

1.4.1 Pyramidal neurons

Pyramidal neurons exhibit a unique cellular architecture and are omnipresent in mammalian brain regions (Elston, 2003) where higher cognitive function is taking place, namely the cerebral cortex, the hippocampus and the amygdala (Ramón y Cajal, 1995). Their existence in birds, reptiles and fish shows a preserved function and indicates a crucial and adaptive role in the nervous system (Nieuwenhuys, 1994). Above all, these cells were studied especially in the hippocampal CA1 region and the layer V of the neocortex (Spruston, 2008). They have a recognizable appearance with a pyramidical shape of their soma with basal and apical ramifications of their dendrites, forming characteristic dendritic trees, descending from the apex and the base of the cell soma respectively (Spruston, 2008). Pyramidal neurons expose characteristic morphological appearance in different brain regions (Figure 1.8). Functional studies revealed the dendritic trees to be segmented in specific dendritic domains, all subserving distinct processes like synaptic inputs, excitability, modulation and plasticity jointly coordinating action potential generation (Spruston, 2008).

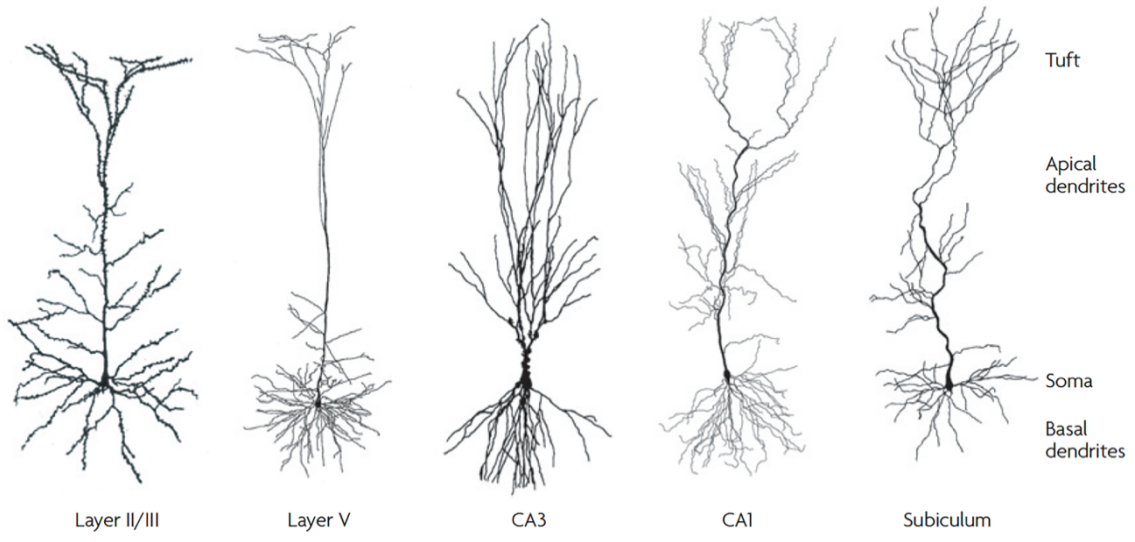

Figure 1.8: Representative structures of pyramidal neurons from different brain areas.
All pyramidal cells exhibit similar morphologies with apical and basal dendrites originating from the cell soma. Apical dendrites branch into apical tufts. The first two cells on the left show pyramidal cells from cortical layers II/III (rat) and layer V (rabbit), followed by hippocampal pyramidal cells of areas CA3 and CA1 (both from rat). The right cell is representative for the subiculum (rat). Apical dendrites of layer V cells are longer and less oblique compared to layer II/III cells. In comparison to CA1 cells, CA3 cells branch closer to the cell soma, whereas CA1 cells have a strong main dendrite and apical tuft. In addition, CA3 cells show a higher density of large spines within the first 100 μm of apical dendrites. From Spruston 2008.

1.4.1.1 Dendritic structure of pyramidal neurons

As common morphological feature, all pyramidal neurons show relatively short basal dendrites, with a long apical dendrite often bifurcating into twin dendrites which both further branch in oblique neurites with various angles (DeFelipe & Fariñas, 1992; Bannister & Larkman, 1995; Ito et al., 1998). This basal structure varies in its appearance between different brain regions (Kasper et al., 1994; Gao & Zheng, 2004). For example, comparison of cortical regions in the visual cortex of macaques points out simpler basal dendrites with diminished spine densities in layer II/III in contrast to higher visual regions as well as areas of the prefrontal cortex (Elston, 2003). Differences of pyramidal cell structures within same brain regions were also found in between species. Such as more elaborated pyramidal neurons with higher spine numbers in the prefrontal cortex of humans compared to macaques (Elston, 2003). Structural differences, thus distinct molecular composition of pyramidal neurons is postulated to exhibit specialized functions (Spruston, 2008).

1.4.1.2 Synaptic inputs and spines of hippocampal pyramidal neurons

Pyramidal cells receive inhibitory GABAergic inputs at the somatic region as well as the axon, whereby excitatory signals and their processing is happening at dendrites. In latter, attention should be paid that according to their position, dendrites receive inputs from different locations. That way, proximal dendrites are stimulated by collaterals from the same or adjacent area, whereas connections from more distant brain areas are made with apical parts of the dendrites, the so-called tuft (Cauller & Connors, 1994). Integration of inputs from different parts can either happen by coinciding arrival of proximal and distant signals or responsiveness of proximal parts is controlled by excitation of tuft regions (Larkum et al., 2004). Also, the characteristic structure of basal and apical dendrites suggests that arriving signals might be integrated in a different way accordingly. Nevertheless, studies showed that distinct dendritic domains of pyramidal neurons get specific synaptic inputs as it was shown in hippocampal neurons. Here, CA1 cells receive inputs to the distal tuft from the entorhinal cortex and yet the remainder get inputs from adjacent CA3 neurons via the Schaffer collateral. Furthermore, the proximity of CA3 neurons to CA1 is correlated to their projection. CA3 neurons distant to CA1 neurons mainly project to apical dendrites and the closer they are in relation to CA1 neurons projections are mainly found towards basal dendrites of CA1 neurons (Ishizuka et al., 1990; Li et al., 1994) Besides, a structural particularity of pyramidal neurons is represented by the thousands of dendritic glutamatergic spines (Figure 1.9(A)), which represent the postsynaptic side of synapses (Spruston, 2008). Notably, spine

numbers do highly differ between distinct brain regions and especially species (Elston & DeFelipe 2002; Ballestero-Yáñez et al., 2006). In chapter 1.5 spines are described in more detail.

Electric currents travel from dendrites, via the soma to the axon, thus synapses distant from the soma have less impact on action potential initiation and maintenance in the axon due to loss of charge (Magee & Carruth, 1999; Stuart et al., 1997; Stuart & Spruston, 1998; Golding et al., 2005). This is the reason why integration of incoming excitatory signals varies according to their location on the dendrite (Spruston, 2008). Synaptic scaling counterbalances conductance. For this purpose, distant synapses on small diameter dendrites show high input resistance with large synaptic potentials generating huge local depolarizations (Spruston, 2008). Different synaptic function according to synapse location on dendrites becomes evident when looking at hippocampal CA3 neurons. Synapses formed between mossy fibers, projecting from the dentate gyrus and CA3 pyramidal neurons exhibit large synaptic boutons and spines with several synapses on each spine and are situated on proximal pyramidal dendrites, emphasizing large excitatory signal incomes (Chicurel & Harris, 1992; Gonzales et al., 2001). On the other hand, synapses formed by neurites from the entorhinal cortex and CA3 neurons are found on distal apical dendrites (Amaral et al., 2007; Spruston, 2008). These observations suggest a different unification of signals. A view on CA1 neurons shows a different synapse composition. There are more perforated spines on apical tufts, but with less AMPAR densities as compared with mid apical dendrites, where AMPARs show high deposits (Nicholson et al., 2006). Furthermore, the apical tuft exhibits more synapses on the dendritic shaft directly (Megías et al., 2001).

Distinct classes of GABAergic interneurons have specialized dendritic target regions on pyramidal neurons to form synapses, resulting in different inhibition patterns, respectively (McBain & Fisahn, 2001; Kawaguchi & Kondo, 2002; Somogyi & Klausberger, 2005). For instance, interneurons like Basket cells, targeting the perisomatic region, reduce firing probability of all activated synapses, whereas interneurons from the outermost layer (OLM) of the hippocampus contact the apical tuft and have a more local inhibitory effect on the neuron (Silberberg & Markram, 2007). Interneurons play an important part in activity dependent synaptic inhibition via calcium (Ca^{2+}) spike limitation (Kim et al., 1995; Miles et al., 1996; Pérez-Garci et al., 2006) and in this way prevent hyperexcitability of neurons. This is important for a smooth processing and guidance of information in a dynamic way and maintain neuronal circuits (Spruston, 2008; Fröhlich, 2016).

There are two different functional ways of synaptic inhibition. One is in a feedforward manner (Figure 1.9(B)), where afferent excitatory fibers activate inhibitory and excitatory neurons simultaneously, generating action potentials in both populations. The activity of excitatory neurons is limited also in duration of synaptic excitation and the sum of incoming excitatory signals by the synaptic activation it receives from simultaneously activated inhibitory neurons (Spruston, 2008;

Fröhlich, 2016). The second form of inhibition is the so-called feedback inhibition (Figure 1.9(C)), where activated excitatory neurons also excite an inhibitory neuron population, which signals back to them and in this way directly controls excitation and limits sustained neuron firing (Spruston, 2008; Fröhlich, 2016). Inhibitory neuronal circuits have been extensively studied in CA1-neurons of the hippocampus. Here, two types of inhibition were found. The onset-transient inhibition rapidly responds to activation of CA1 neurons, terminates fast and is evoked by synapses close to the soma and proximal dendrites. The more sustainable late-persistent inhibition takes longer to be evoked and is occurring in distal dendrites (Pouille & Scanziani, 2004).

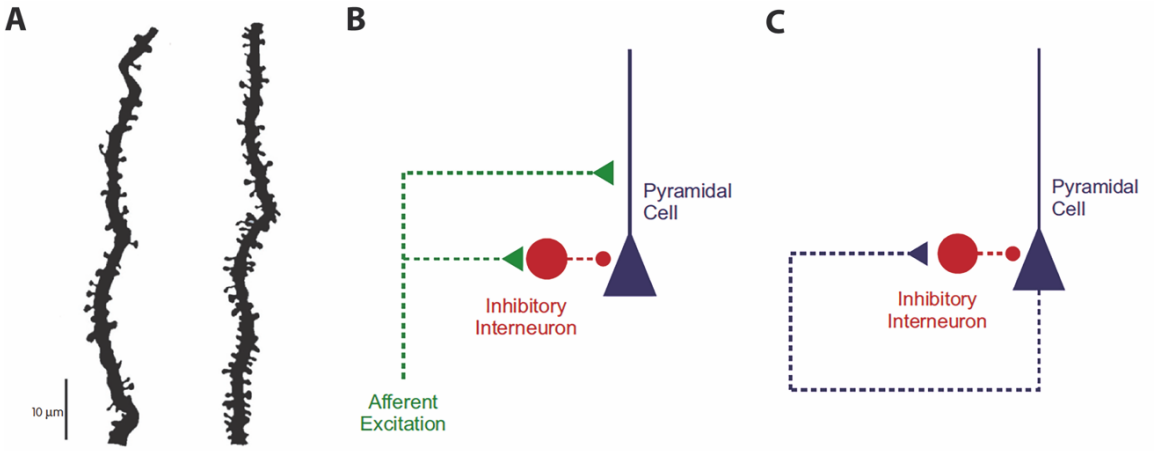

Figure 1.9: Representation of dendritic spines and synaptic inhibition.
(A) Sections of two dendrites of stained CA1 pyramidal neuron with different spine densities (from Spruston, 2008). **(B)** Feedforward inhibition. An action potential is triggered in the inhibitory interneuron by afferent excitation. Thereupon the pyramidal neuron receives an excitatory input, rapidly followed by an inhibitory input. **(C)** Feedback inhibition. Activity of the pyramidal cell leads to excitation of the inhibitory interneurons, eventually inhibiting the pyramidal cell. (**B, C** from Fröhlich, 2016.

Alternations in firing patterns regarding intrinsic burst firing or spike-frequency adaptations reflect the diversity of pyramidal neuron function (Connors & Gutnick, 1990; Staff et al., 2000). Normally, action potentials in pyramidal neurons are ensued by afterdepolarization, which is mediated by voltage-gated Na^+(sodium)- and Ca^{2+}-channels and the quick closure of K^+-channels to end the depolarization (Staff et al., 2000).

The strength of action potentials and afterdepolarization is affected by ongoing pre-activity and neurotransmitter-release (Zhang & Linden, 2003; Yue & Yaari, 2006). Contribution to intrinsic bursting is added by dendritic channels via current promotion at the local spike initiation zone (Larkum et al., 1999; Magee & Carruth, 1999; Williams & Stuart, 1999; Lemon & Turner, 2000; Yue and Yaari, 2006; et al., 2007), more by excitatory synapses as by current injection in the soma (Pinsky & Rinzel, 1994; Mainen & Sejnowski, 1996; Bastian & Nguyenkim, 2001). The distribution of voltage-gated channels along dendrites can either be uniform as it is mainly the case for Na^+-channels, or vary between pyramidal cell types and between channel types. This way ion-

gated channels have a huge impact on the integration of synaptic potentials (Johnston et al., 1996; Hoffman et al., 1997; Häusser et al., 2000; Korngreen & Sakmann, 2000). There are various types of dendritic spikes. So-called Na^+-spikes appear brief in contrast to large-scale Ca^{2+}-spikes (Schwartzkroin & Slawsky, 1977; Johnston et al., 1996; Schiller et al., 1997; Stuart et al., 1997; Golding et al., 1999; Spruston et al., 2008). Also, NMDA receptors can contribute to dendritic spikes after a voltage-dependent release of the magnesium (Mg^{2+}) blockage (Schiller et al., 2000; Rhodes, 2006), but are stopped when they reach areas where glutamate release took place (Schiller & Schiller, 2001). Where one or the other type of spikes occurs, depends heavily on the dendritic region as NMDA-spikes and Na^+-spikes are observed in basal dendrites, but not Ca^{2+} initiated spikes (Ariav et al., 2003; Nevian et al., 2007), suggesting a different excitability of dendrites depending on their location (Spruston, 2008). Fast, as well as long lasting potentiation are formed through the contribution of Na^+- and Ca^{2+}-channels and NMDA-receptors (Schwindt & Crill, 1997; Oakley et al., 2001; Wei et al., 2001; Cai et al., 2004; Milojkovic et al., 2004; Milojkovic et al., 2007). Spike initiation requires strong and synchronous activation of multiple synapses, motoring dendritic spike propagation towards the soma. The threshold to evoke a spike is lower when multiple synapses along a dendrite are sequentially activated, in contrast to clustered synapse activation on a dendrite (Figure 1.10(A)).

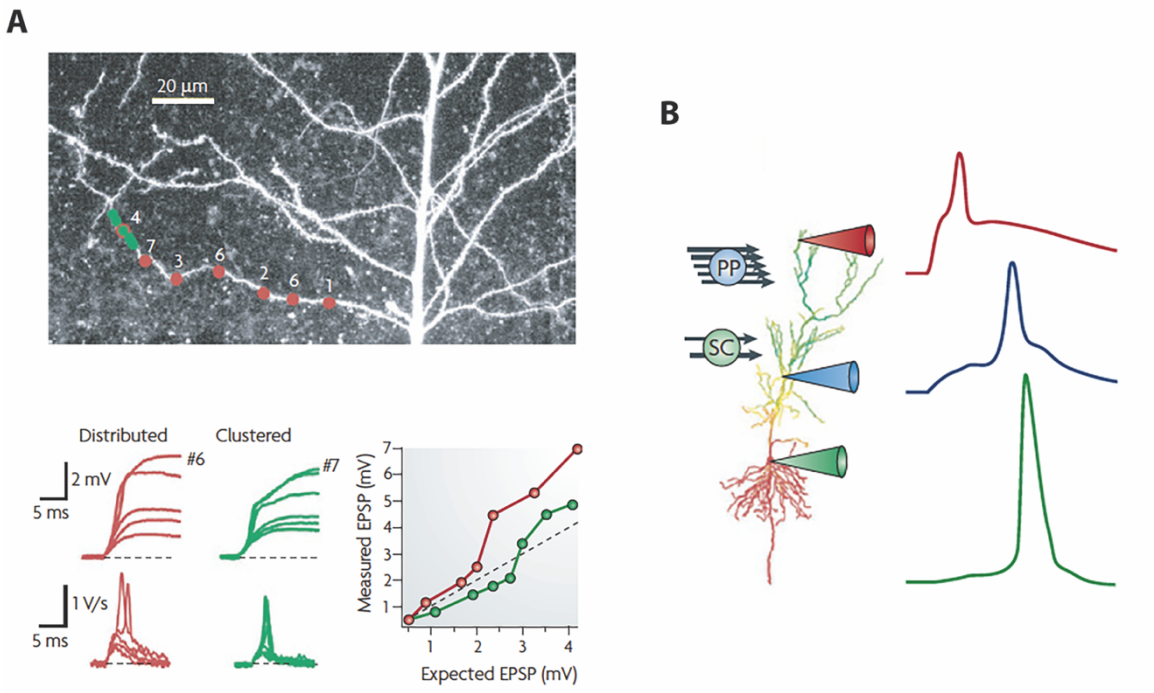

Figure 1.10: Pyramidal neurons – dendritic excitability and coincidence detection.
(A) Glutamate uncaging at several spots of a pyramidal CA1 neuron. Red for distributed and green for clustered input. A nonlinear increase in voltage responses was measured in the soma (lower panel), when multiple spots were activated in a rapid sequence. Threshold to evoke a dendritic spike was lower for distributed inputs than for clustered inputs. EPSP, excitatory postsynaptic potential. **(B)** Simulation of a CA1 pyramidal neuron by perforanth path (PP) synapses and Schaffer-collateral (SC) synapses. Activation through both fiber tracts lead to a successful spike propagation along the apical dendrite to the soma and axon. From Spruston et al., 2008.

However, clustered inputs on single dendrites, provide higher probability of spike initiation compared to inputs coming from branched dendrites (Mel, 1993; Poirazi et al., 2003). Pyramidal neurons operate as coincident detectors (Figure 10.1(B)), through summation of a sufficient number of small spikes from individual dendritic branches to reach an action potential threshold (Spruston, 2008). Spikes from CA1 apical tufts can propagate and influence action potential firing when Schaffer collaterals and perforant-path synapses are simultaneously activated (Jarsky et al., 2005; Nicholson et al., 2006). On the other hand, inhibition of synapses in apical regions prevent propagation of dendritic spikes (Jarsky et al., 2005).

Dendritic excitability and especially dendritically evoked spikes constitute a strong influence on synaptic plasticity, whereby the relative timing of an excitatory postsynaptic potential (EPSP) and the evoked action potential are crucial. Long-term-potentiation (LTP) and depression (LTD) are so called spike-timing-dependent plasticity forms and require dendritically evoked spikes in response to strong postsynaptic signals or pairing of EPSPs with postsynaptic bursts (Yuste & Denk, 1995; Pike et al., 1999; Golding et al., 2002; Holthoff et al., 2004; Kampa et al., 2006; Letzkus et al., 2006; Wittenberg & Wang, 2006; Remy & Spruston, 2007). In addition, synaptic integration can be modulated by a subset of neurotransmitters such as dopamine, serotonin, noradrenaline and acetylcholine (Spruston, 2008). Those neuromodulators target voltage gated channels and in this way fine-tune cellular functions like firing rates, synaptic strengths, gene expression and dendritic excitability (Spruston, 2008). Interestingly, synaptic plasticity can evoke long-lasting changes in subcellular domains as LTP induction shows in CA1 pyramidal neurons where long-term excitability is evoked via K^+-channel modulation, but only in branches close by the activated synaptic zone (Frick et al., 2004).

1.4.1.3 Action potential

During their resting state neuronal plasma membranes preserve a transmembrane electrical potential difference created by help of different ion concentrations inside and outside the cell. This leads to an electric difference between the intracellular membrane surface and the outer membrane with approximately 70–80 mV of difference with the intracellular side being negatively charged. This electric gradient is maintained through different ion concentrations inside and outside the cell with characteristic permeabilities for distinct ions. K^+ ions flow according to their gradient from inside to outside the cell, whereas lower concentrations of Na^+, Ca^{2+}, and Cl^- (chloride) ions are found inside the cell plasma compared with the outer space. By producing action potentials (AP), neuronal membranes ensue excitability as rapid and fast responses to external stimuli, creating a transmembrane electrical potential difference. Within the initial phase of AP, the membrane

becomes highly permeable for Na^+ ions, which start to explosively travel from outside to inside the cell, according their concentration gradient. Upon this very quick, only millisecond lasting event, the membrane is depolarized, turning the membrane potential into a positive value and repolarized by voltage-gated Na^+- and K^+-channels. Action potentials in neurons are all- or none events and self-propagating along the membrane with a quick restoration of the cell excitability (Fletcher et al., 2011) Forward propagation of APs is found along the axon towards presynaptic terminals after being started at the initiation zone region of the soma, which has the lowest threshold for AP initiation (Stuart et al., 1997). Backpropagation of action potentials is opposing to this event and occurring after AP rapidly invaded the soma and next propagate back into the dendrites. In contrast to forward propagating AP, backpropagating action potentials (bAP) are not all-or-none events, meaning they can change in amplitude and declines along the dendrite (Gasparini & Migliore, 2014). Limiting factors for bAP are distributions of voltage-gated channels along dendrites, neuronal morphology, and neuronal activity (Gasparini & Migliore, 2014).

1.5 Synapses and dendritic spines

1.5.1 Spine structure and plasticity

As part of excitatory synapses, spines are small, actin-rich protrusion on the postsynaptic side, allocated on neuronal dendrites and receiving inputs from single presynaptic terminals (Sorra & Harris, 2000). Spines mainly serve as proxy to study excitatory synaptic function, nevertheless a subset of spines (14%) in cortical pyramidal neurons was found to respond to inhibitory signals as well (Chen et al., 2012). Their main function in the brain is neurotransmission and information storage, which they put into place by changing their morphological appearance regarding shape and size in a plastic way (Sorra & Harris, 2000) through their highly dynamic actin content to strengthen synaptic transmission and excitation (Hotulainen & Hoogenraad, 2010). Underlying mechanisms, eventually building memory and learning, highly depend on actin cytoskeleton regulation, managing formation, maturation and plasticity of spines (Hotulainen & Hoogenraad, 2010). In this manner spines can change their shape *in vivo* in response to variations in neuronal signal transmission i.e., due to new experiences (Hooltmat et al., 2005/2006).

Depending on neuronal type and location, spine densities can vary between 1–10 spines per micrometer at dendritic stretches. For example, hippocampal neurons present one of the highest spine prevalence within the mammalian brain (Sorra & Harris, 2000). The morphology of spines is composed of three main parts which consist of a delta-shaped base build at the dendritic shaft, a spine neck and a bulbous head touching the axons (Hotulainen & Hoogenraad, 2010).

Moreover, they appear in different shapes and size variations between 0.2 to 2 μm and a volume range of 0.001 to 1 μm^3. Spine function is determined by the relative length and width of their head and neck, whereby head size is in relation to the number of neurotransmitter receptors situated at the surface. The width and length of the neck regulates the diffusion barrier between spine head and dendritic shaft. By implication this means that neck appearance influences the transmission of downstream signaling cascades of the subsequently activated receptors at the spine head (Bertling & Hotulainen, 2017). Observations over the past years show a positive correlation between spine head size and the number of surface receptors (Ashby et al., 2006; Noguchi et al., 2011). Spines are categorized into four main classes (Figure 1.11(A)) which display distinct structural forms according to their grade of maturation (Segal, 2017; Parajuli et al., 2017): filopodia appear with a long neck and no clear head, thin spines are filopodia-like protrusions with a small spine head, stubby spines lack a clear spine neck and mushroom spines feature a neck with a large bulbous head (Bourne & Harris, 2008; Rochefort & Konnerth, 2012). Notably, spines are part of plastic synaptic structures showing enormous adaptation capacities in regard of structural modifications due to signal transduction and the current need of strengthening or selective renaturation of synaptic connections even in the adult brain (Grutzendler et al., 2002; Trachtenberg et al., 2002). *In vitro* and *in vivo* studies using live imaging revealed spine modifications upon neuronal activity (Matsuzaki et al., 2004; Holtmaat et al., 2006; Roberts et al., 2010). The magnification of spine heads was identified during LTP, a process that occurs during learning and memory formation, consequently showing that spine morphological changes are key regulators for proper cognitive function (Yuste & Bonhoeffer, 2001; Kasai et al., 2003). The majority of dendritic spines are shaped out of dendritic filopodia during early postnatal development, with increasing densities until the third week of life, followed by pruning and decreasing spine turn over until adulthood (Harris et al., 1992; Holtmaat & Svoboda, 2009; Yoshihara et al., 2009; Hotulainen & Hoogenraad, 2010). However, new spines can also be formed during adult neuroplasticity (Holtmaat & Svoboda, 2009). Together with their high motility rate and their fragile shape with long outgrowth, filopodia are presumed to palpate the environment for potential presynaptic contacts (Figure 1.11(B)). Only upon successful contact with axons, they differentiate into mature spines and build proper synapses, elsewise they regress (Bertling & Hotulainen, 2017; Parajuli et al., 2017). However, spines can also form without filopodia stages (Kwon & Sabatini, 2011; de Roo et al., 2008).

A detailed view shows spines being composed of a postsynaptic density (PSD) including postsynaptic glutamate receptors, a high enrichment of actin molecules building the stabilizing actin cytoskeleton and membrane-bound organelles such as mitochondria, smooth endoplasmic reticulum and endosomes (Sheng & Hoogenraad, 2007). As represented in Figure 1.11(C), the PSD lies at the spine tip, thus represents the direct postsynaptic membrane of the pre-synapse.

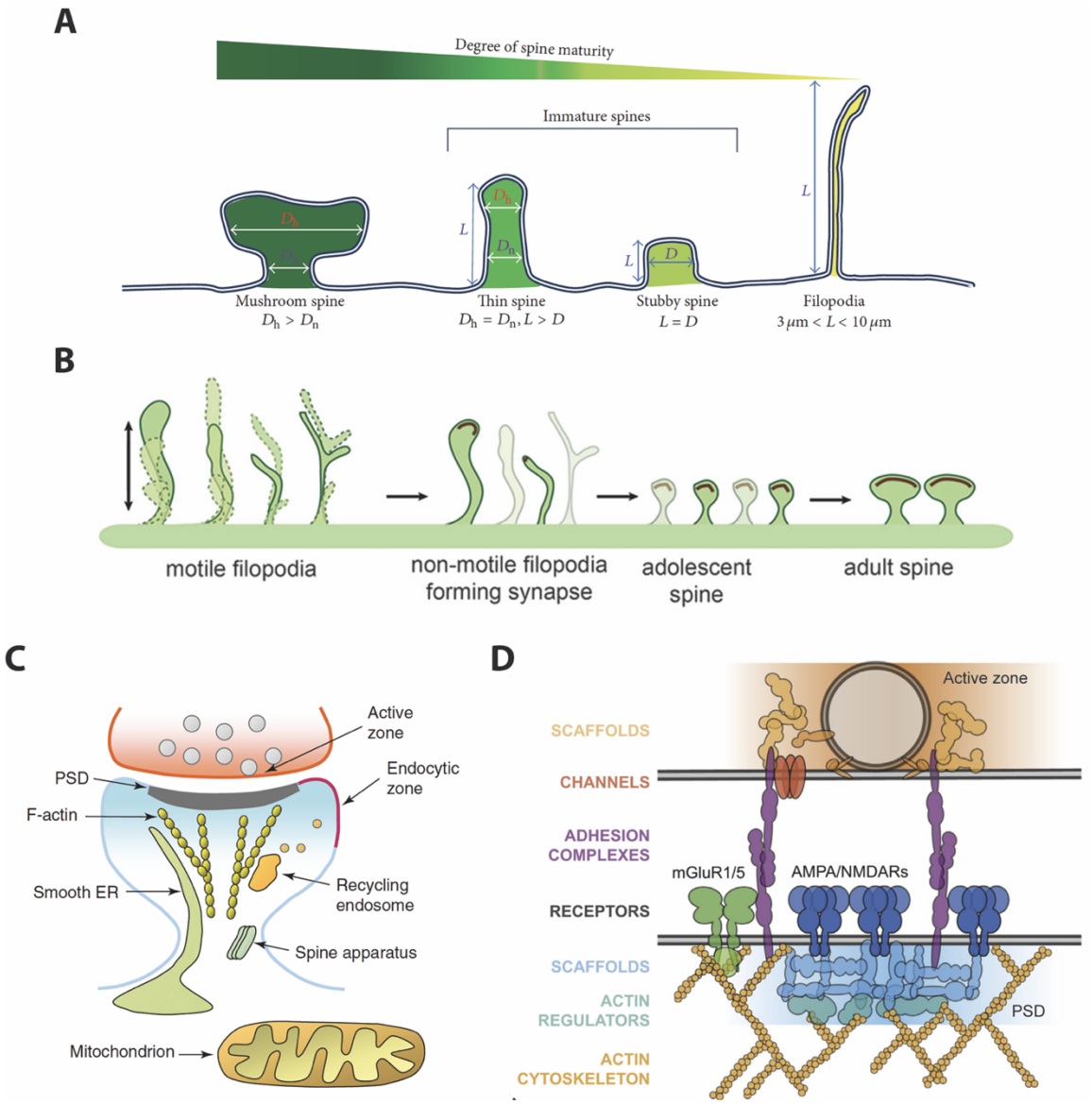

Figure 1.11: Spine subtypes and spine structure.
(A) Dendritic spines are categorized into mushroom, thin, and stubby spines, according to their grade of maturation. Filopodia represent a precursor form of dendritic spines. Length of spine (*L*), diameter of spine head (*D*h), and diameter of spine neck (*D*n). From Qiao et al., 2016. **(B)** Filopodia, displayed on the right, are motile dendritic protrusion, palpating the environment for presynaptic contacts. They become immobile when making first synaptic contacts. Filopodia with weak connections get eliminated during the maturation and refinement process and a portion morph into spines. From Sudarov et al., 2013. **(C)** The postsynaptic density (PSD) lies opposite the active zone and is attached to spinal F-actin. The PSD is mounted laterally by the endocytic zone. Spines are comprised of smooth endoplasmatic reticulum (ER), the spine apparatus, and recycling endosomes. Mitochondria are located in the dendrite. From Kim & Sheng, 2009. **(D)** Close-up of the synaptic active zone and PSD. Scaffold proteins of the PSD interact with each other and other proteins. They form a network for assembly of membrane proteins and signaling molecules. From Scheefhals & MacGillavry, 2018.

The PSD includes receptors, adhesion molecules and channels and it congregates a variety of signaling molecules (Figure 1.11(D)) (Kennedy et al., 2005; Renner et al., 2008). Endocytosis of postsynaptic receptors – a clathrin-dependent mechanism – is taking place at the endocytic region adjacent to the PSD to recycle and gather AMPA receptors in a postsynaptic pool (Blanpied et al., 2002; Lu et al., 2007; Rácz et al., 2007; Petrini et al., 2009). There are two main mechanisms after

synaptic activation that are linked to modifications in spine shape. Firstly, mobilization and turnover of AMPARs and other postsynaptic receptors adjust postsynaptic membrane size in an activity-dependent manner. For AMPARs this is documented during stimulation upon which their transport into the recycling endosomal compartment results in enlargement of spines (Park et al., 2004/2006). Secondly, downstream signaling pathways act on the actin dynamics affecting the spine size as well (Hotulainen & Hoogenraad, 2010).

Generally, a large majority of nascent spines disappears within a couple of hours and the stabilization of continual spines depends on the formation of PSD linked to locating a proper presynaptic connection (Yuste & Bonhoeffer, 2004; Parajuli et al., 2017). Nevertheless, some postsynaptic formations from denervated granule cells form without presynaptic partners and also hippocampal spine formation and maintenance seems to be normal in organotypic slice cultures missing neurotransmitter release, suggesting that spine formation might in parts rely on predetermined cell-intrinsic factors, on which activity-dependent spinogenesis build up (Perederiy et al., 2013; Sigler et al., 2017).

In vivo spines appear to be very stable as it was found especially in the adult brain in mature circuits. The exact lifetime of spines is variable depending on the brain region they appear, but persistent spines remain a month and even longer (Trachtenberg et al., 2002; Holtmaat & Svoboda, 2009; Gu et al., 2014). A positive correlation is present between spine head size and their persistency, indicating mushroom spines as the most stable form and a turnover rate of a couple of days appears with decreasing spine head size (Trachtenberg et al., 2002; Holtmaat & Svoboda, 2009). Functionally, thin spines reinforce learning processes and larger, more stable spines support storage of memories (Kasai et al., 2003, Bourne & Harris, 2007). Enduring activation of thin spines induces their growth and higher responsibility towards glutamate (Matsuzaki et al., 2004) and on the other hand low level activation of AMPARs sustains stability of spines (Matus, 2000) which depicts the variable roles of glutamate receptors upon distinct activation in structural plasticity. This shows the constant fluctuation of spine head size in an activity-dependent manner (Yasumatsu et al., 2008; Minerbi et al., 2009). Mushroom spines with their broad heads are considered to build the strongest synapses (Rochefort & Konnerth, 2012). Most of the spines are contacted by one pre-synapse and represent weak, even silent synapses, whereas only few large and more stable spines show multiple release-sides with perforated PSDs and high densities of AMPARs with increased synaptic strength (Cohen et al., 1977; Cohen & Siekewitz, 1978; Harris & Stevens 1989; Geinisman, 1993; Sorra & Harris, 1993; Matsuzaki et al., 2001; Yankova, 2001; Ganeshina et al., 2004b; Ganeshina et al., 2004a). Those larger and fully functional spines arise in an activity-dependent manner out of small, possibly silent spines (Geinisman, 2000). Nevertheless, new models suggest that memory storage is primarily promoted by inhibitory networks with more

stability and most likely between two whole neurons or even the whole network rather than through individual synapses (Mongillo et al., 2017).

In mature spines, actin's most relevant role is to stabilize postsynaptic proteins and regulate the structure of spine heads in response to synaptic signaling (Allison et al., 1998; Fischer et al., 2000; Star et al., 2002; Okamoto et al., 2004; Kuriu et al., 2006; Renner et al., 2009). During cell migration and prolongation, the cell plasma membrane is pushed towards lamellipodia and filopodia shaped appearances through the growing or so-called barbed ends of actin filaments (Pollard & Borisy, 2003). Lamellipodia consist of short and branched actin filaments, whereas filopodia are composed of long and unbranched actin filaments organized as tight and parallel bundles (Le Clainche & Carlier, 2008). Generally, mature spines consist of a mix of branched and linear actin filaments. Whereby the ratio of both differs according to the spine compartment, with the highest levels of branched actin filaments at the tip of the spine heads where actin treadmilling allows a dynamic turnover of actin filaments, thus rapid change of size and shape (Star et al., 2002; Honkura et al., 2008; Korobova & Svitkina, 2010). Longitudinal arrangements of actin filaments are found in spine necks and filopodia-shaped spines (Korobova & Svitkina, 2010). Interestingly, actin filaments are polymerized from both ends of dendritic filopodia, the base and the tip and they can show mixed polarity, whereas the predominant form is pointing towards the tip (Hotulainen et al., 2009; Korobova & Svitkina, 2010). Also, they contain myosin-II which acts as actin-motor for contraction and binding of actin-filaments (Korobova & Svitkina, 2010). To maintain or rearrange a certain spine morphology, actin dynamics are regulated by actin-binding proteins (ABPs). One is the actin-related protein (Arp) 2/3 complex, important for actin nucleation and the assembly of the spine head (Hotulainen & Hoogenraad, 2010; Bertling & Hotulainen, 2017). Other actin dynamics regulating factors act on cell surface receptors and are described in detail in Chapter 1.8.

1.5.2 The two synapse types – chemical and electrical

The transmission of information by neurons happens in two basic ways – a fast and direct transmission via synapses and a wide-spread and slower way through messenger signaling like neuropeptides, endocannabinoids and monoamines (Südhof, 2018). There are two procedures of how this information transduction takes place, namely though chemical and electrical synaptic transmission, often interplaying, and both present in the developing and the adult brain (Pereda et al., 2014). Electrical synapses (Figure 1.12(B)) connect the cytoplasm of two neighboring cells via cluster of intercellular channels, the so-called gap junctions and thus allow a more direct way of communication (Shimizu & Stopfer, 2013; Pereda et al., 2014; Nagy et al., 2018). In vertebrates,

those gap junctions are formed by two vis-à-vis hexameric hemichannels which consist of connexin subunits. Ions and small metabolites can pass through gap junctions (Shimizu & Stopfer, 2013). Both forms of synapses coexist and occur in most organisms and brain structures. Electrical synapses mostly interact through axon-axon or dendrite-dendrite connections, but they can also adjoin axons to somas or to dendrites (Pereda, 2014; Nagy et al., 2018). They are mostly known for coupling inhibitory and GABAergic neurons to larger functional brain networks in vertebrates to enhance input sensitivity and synchronize network activity (Vervaeke et al., 2010; Shimizu & Stopfer, 2013). Predominately they are found in developing brains, appear before chemical synapses are formed early in developmental stages and are important for network formation, widely spread throughout the mammalian brain (Shimizu & Stopfer, 2013). By localizing close to chemical synapses, they can form combined synapses, regulating neuronal signal transmission by the interplay of chemical and electrical signal transduction (Pereda, 2014; Nagy et al., 2018). Another features of gap junctions, is their functionality as adhesion molecules, serving for attachment of migrating neurons to guiding radial fibers (Shimizu & Stopfer, 2013). Besides, connexins have a critical role in neural differentiation and neurite outgrowth (Shimizu & Stopfer, 2013). Electrical synapses infuse currents into coupled cells and measure changes in membrane potential of the subsequent cell (Shimizu & Stopfer, 2013). One interesting feature of electrical synapses is the bidirectional passage of currents, forwarding not only action potentials but also subthreshold responses, like synaptic potentials or spontaneous oscillations (Connors & Long, 2004; Placantonakis et al., 2006; Zsiros et al., 2007; Nagy et al., 2018). Another conspicuous property of electrical synapses is their function as low pass filter depending on membrane properties and geometries of the coupled cell. In this way they can differentially transmit information between neurons (Nagy et al., 2018). Finally, electrical synapses are extremely plastic, changing their properties and coupling strength according to recent physiological conditions (Landismari & Connors, 2005; Haas et al., 2011; O'Brien, 2014; Haas et al., 2016). Through the action of neuromodulators, such as dopamine or through glutamatergic synapses, they are modifiable and capable of activity-dependent plasticity (Rovainen, 1974). The hemichannel composition of electrical synapse also influences their properties. Two hemichannels with the same connexin-composition built homomeric gap junctions, whereas different connexin composition is accountable for heterotypic gap junctions (Shimizu & Stopfer, 2013). Besides, each hemichannel can vary in their individual composition of various types of connexins, thus constituting homo- or heteromeric hemichannels (Shimizu & Stopfer, 2013). As for heterotypic hemichannels pre- and postsynaptic sides functionally differ from each other, they are associated with different electrical resistances and for example admit current flow only unidirectional (Pereda, 2014). Also, phosphorylation of connexins and interplay with nearby chemical synapses was shown to modify

their conductance properties, for instance by allowing the passage for distinct ions types only (Shimizu & Stopfer, 2013).

Chemical synapses (Figure 1.12(A)) are specialized intercellular junctions standing out by the release of neurotransmitters from the pre-synapse of one neuron that is recognized by the post-synapse of an adjacent cell and transmits the information via the synaptic cleft and they are the most prevalent type of synapses in the adult mammalian brain (Südhof, 2018).

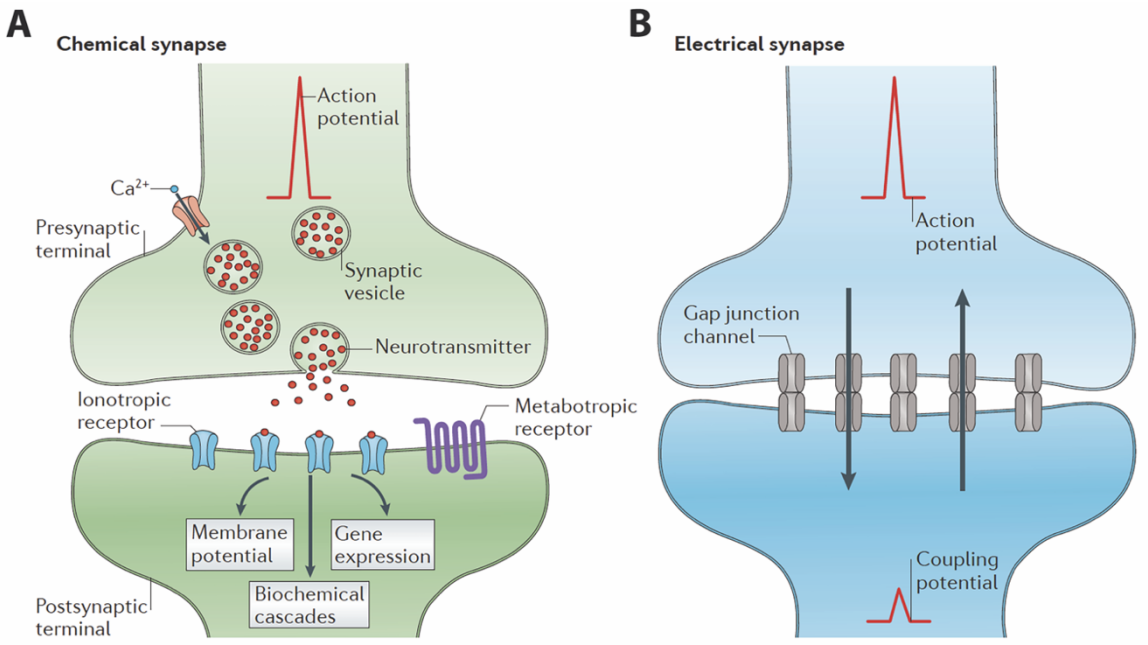

Figure 1.12: Two main types of synapses and synaptic neurotransmission.
(A) Chemical synapse: Upon depolarization of the presynaptic terminal through arrival of an action potential, voltage-gated calcium channels are activated. The postsynaptic machinery consists of ionotropic and metabotropic receptors binding presynaptic neurotransmitters and translating them into various events, such as changes in resting membrane potential or gene expression, amplifying the presynaptic signal. **(B)** Electrical synapse: Gap junction represent clusters of intercellular junctions connecting the two adjacent cells and their inside. Via this connection the direct and bidirectional passage of electrical currents carried by ions and intracellular messengers and small metabolites is enabled. From Pereda, 2014.

Usually, chemical synapses are found at special sides of neurons through connection of synaptic terminals along the axon and dendrites or the soma of another neuronal cell, and also muscle or gland cells (Peredea et al., 2014). Classically, chemical synapses are formed between a presynaptic axon and a postsynaptic dendrite with a high diversity of properties caused through different neurotransmitter types, release probabilities, postsynaptic receptor types, and short- and long-term plasticity (Südhof, 2018). The presynaptic active zone is composed of voltage-gated calcium channels, enabling fast and release/synchronous coupling, it contains vesicles packed with neurotransmitters and houses transsynaptic cell-adhesion molecules (CAMs) to align oppositely lying pre- and postsynaptic specialized zones (Südhof, 2012). Intrinsic proteins at the active zone help prime and dock vesicles containing neurotransmitter to the plasma membrane. When action

potentials reach the presynapse, voltage-gates Ca^{2+}-channels trigger calcium-influx and Ca^{2+}-sensitive vesicles dock to the plasma membrane, fuse and release neurotransmitters via exocytosis into the synaptic cleft. Next, released neurotransmitters bind to specialized receptors at the postsynapse, and upon their activation downstream signaling cascades are elicited (Südhof, 2018). The molecular composition of synapses is highly diverse and depends on the developmental stage, brain regions, neuronal types and subcellular expression, creating very specialized functioning of each synapse (Henley & Wilkinson, 2016; Südhof, 2018; Barberis, 2020). Strikingly, chemical and electrical synapses can differentially influence neighboring neurons by variable coupling with electrical synapses to induce network desynchronization (Vervaeke et al., 2010).

1.5.2.1 Synaptogenesis

Most synapses merge during pre- and postnatal development in humans and roughly half of them is pruned within the following two decades (Petanjek et al., 2011). Figure 1.13(A) depicts the distinct stages of synaptogenesis. Most of the synapses that were pruned in adolescence maintain stable in adulthood, however synapses continue to be formed and eliminated throughout life (Südhof, 2018). It is outstanding that synapses formation is largely activity-independent, whereas pruning requires activity (Südhof et al., 2018). During development neurons reach a certain maturation which enables them to form synapses. The spatio-temporal window regulating synaptogenesis is evoked by secretion factors such as WNT and fibroblast growth factors (FGFs) family members (Kurshan & Shen, 2019). The selection of proper targets with which neurons can build synapses is guided by several signaling molecules that in parts also mediate axon guidance (Jabeen & Thirumalai, 2018). This includes calcium-dependent cell-adhesion molecules like cadherins and protocadherins, mediating cell adhesion via homophilic interaction and enabling target recognition and stabilization of contact sites (Waites et al., 2005; Hayashi-Takagi et al., 2015; de Wit & Ghosh, 2016). After the first contact is made, synaptic cell adhesion molecules (SynCAM) and neuroligins/neurexins come into account for synapse formation (Figure 1.13(B)). Neuroligins are found on postsynaptic membranes, making contact with presynaptic neurexins (Jabeen & Thirumalai 2018). Neuroligins prompt vesicle clustering and assembly of other presynaptic components, whereas neurexins induce insertion of NMDA receptors and PSD-95 at post-synapses in excitatory neurons and $GABA_A$ receptors and Gephyrin at inhibitory post-synapses (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004; Dean & Dresbach, 2006). The formation of a fully functional active zone is further promoted by the secretion proteins WNT, FGFs, NARP (neuronal pentraxin 2), EFNB (EphrinB, family of receptor tyrosine kinases) (Dalva et al., 2000; Scheiffele, 2003; Lee et al., 2017). Further maturation of nascent synapses involves increases in

synaptic vesicle and postsynaptic receptor amounts, happening in a coordinated manner of involved cell adhesion molecules (Waites et al., 2005; Jabeen & Thirumalai, 2018).

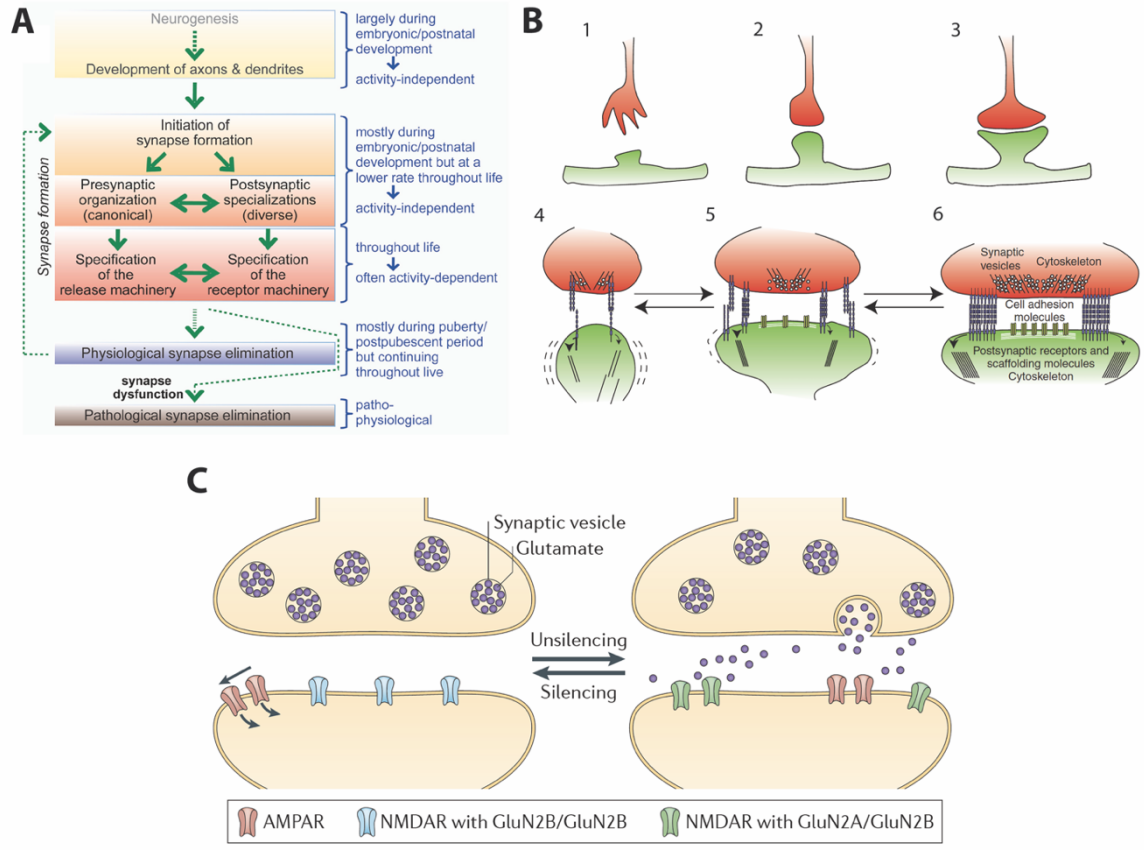

Figure 1.13: Synaptogenesis.
(A) Diagram for synapse formation. During development neurogenesis is taking place, including cell migration with axon formation and dendritic growths. Although synapse formation occurs throughout life, most synaptic contacts between axons and dendrites are established during development and early postnatal stages. First synaptic contacts initiate the formation of pre- and postsynaptic specializations. Synapses formation is an activity-dependent process, continuously taking place for most synapses through restructuring via synaptic plasticity. Additionally, synapse elimination occurs during early life phases, but can also take place in adult stages. Pathological synapse elimination happens during neurodegenerative events. From Südhof, 2018. **(B)** Exemplified stages of synapse formation. (1) Target selection, (2) Synapse assembly, (3) Synapse maturation and stabilization. (4-6) Cell adhesion molecules in synapse formation is illustrated by N-cadherin and catenins in coordinating the morphology and strength of dendritic spines. (4) Early stage: Dendritic spines (postsynaptic) are elongated from motile structures "searching" presynaptic partners. (5) Contacts between presynaptic and postsynaptic compartments are stabilized by cell adhesion molecules. Adhesional interactions activate downstream mechanisms and modulate the cytoskeleton inclusive organization or pre- and postsynaptic apparatuses. (6) Cell adhesion complexes are stabilized by increased synaptic activity. Expansion of the dendritic spine head and spine maturation, that depend on synaptic plasticity. From Giagtzoglou et al., 2009. **(C)** Model of a glutamatergic neuron with pre- and postsynaptic areas. Clustering and incorporation of AMPARs at the postsynapse refers to unsilencing, whereas silencing refers to loss of synaptic AMPARs through endocytotic processes and lateral receptor diffusion. From Hanse et al., 2013).

Overall, functional properties of developing synapses do change during maturation, including release probability of neurotransmitters, quantal size changes, changes in the PSD proteome, and postsynaptic receptor composition. For example, silent excitatory synapses, which conduct only via NMDARs can become un-silent through insertion of AMPARs and receptor

subunit composition can be adjusted resulting in different postsynaptic cell responses (Petralia et al., 1999; Law et al., 2003; Waites et al., 2005; Cline & Haas, 2008; Jabeen & Thirumalai, 2018). Underlying mechanism of silent synapses is the fact that purely NMDARs cannot be activated at resting membrane potentials. Through a change of AMPAR and NMDAR subunit composition, NMDAR-mediated correlated activity, and a switch in PSD scaffold proteins AMPAR-silent synapses are stabilized (Kerchner & Nicoll, 2008; Hanse et al., 2013). The switch from synapse-associated protein (SAP) to PSD-95 is relevant for glutamatergic synapse activation (Hanse et al., 2013). Unsilencing of AMPAR containing synapses (Figure 1.13(C)) occurs after the second postnatal week in mice and is achieved through substitution of GluA4 homomers that are calcium-permeable (CP) by calcium-impermeable (CI) GluA2 subunits in an NMDAR-dependent way (Henley & Wilkinson, 2016). The maintenance of CP-AMPARs specially on GABAergic neurons in the adult cerebellum, upon resting potential, and their exchange with CI-AMPARs upon stimulation, is an important modulator of inhibitory synapses (Henley & Wilkinson, 2016). Further, neuronal activity via neurotransmitter release provokes dendritic spinogenesis and GABA or AMPA receptor clustering in inhibitory or excitatory synapses, which in turn is a critical factor for activity-dependent synaptogenesis evoked by elevated calcium levels through activation of these receptors (Kwon & Sabatini, 2011). Later in development, activity-dependent signaling becomes critical for fine-tuning of neural circuits through synaptic maintenance, pruning or elimination of synapses (Parajuli et al., 2017; Jabeen & Thirumalai, 2018).

So far little is known about the formation of electrical synapses. They require the transport of innexin or connexin proteins to the junctional area of two neurons and are primarily necessary for the formation of neural circuits (Jabeen & Thirumalai, 2018). Therefore, they occur early in development, even before chemical synapses are formed and steadily decrease afterwards until reaching a steady state in adulthood. In addition, they show an inverse relationship to the formation of glutamatergic synapses (Belluardo et al., 2000; Jabeen & Thirumalai, 2018). Electrical synapses are involved in the regulation of cell proliferation and neural circuit formation as it was observed in neural progenitors where electrical synapses create an electrical and chemical link between joined neurons, supporting synchronization of cell cycles and calcium waves (Pereda, 2014; Shimuzu & Stopfer, 2013). When strong chemical synapses originate from same radial glia cells as electrical synapses, latter can disappear. Same wise, electrical synapses are necessary for the formation of strong chemical synapses (Yu et al., 2012).

1.6 Neuronal plasticity

Synaptic plasticity is defined as the potential of neurons to adapt to changing external or internal stimuli adjusting their preexisting synaptic connections in response (Gispen, 1993). Experiences, such as learning or stressful events, have the capacity to modify certain brain circuits through neuronal activity. An underlying mechanism is the activity-dependent modification of strength and efficacy of synaptic transmission – effectively synaptic plasticity (Citri & Malenka, 2008). Many forms of synaptic plasticity have been described. The major forms are referred to as Hebbian and homeostatic plasticity. These forms of plasticity change without excessive modifications brought by physiological and morphological alterations at the synapse directly, including changes in receptor number and types, and dendritic structure and spine size (Citri & Malenka, 2008; Keck et al., 2017). Morphological alterations are studied under the term structural plasticity (Forrest et al., 2018). Besides, transient and brief plasticity forms without changes in signaling pathways are predominantly promoted by presynaptic changes and are called short-term plasticity (Zucker & Regehr, 2002). Further, intrinsic plasticity comes into account when neurons change their own excitability which affects their global response to given stimuli and can either be destabilizing or homeostatic (Zhang & Linden, 2003). Lastly, prior experiences can alter the threshold of neurons engaging into behavior-induced plasticity. This so-called metaplasticity happens when neurons perform differently to the same stimulus due to previous network activity levels (Li et al., 2019).

Neuronal plasticity is found throughout all brain regions and amongst all neuronal types, including excitatory and inhibitory neurons, existing on both synaptic sides. As AMPAR trafficking is one of the foremost procedures in excitatory glutamatergic synapses and the base for Hebbian and homeostatic plasticity, this will be the focus in the following section together with ensuing structural changes.

1.6.1 Hebbian Plasticity

Hebbian Plasticity was first characterized by the psychologist Thomas Hebb in 1949 and is nowadays widely established as mechanism to encode and retain information in the mammalian brain, as it appears to happen during learning and memory storage and retrieval (Sweatt, 2016). The overall principle is the coordinated activity of neuronal assemblies which strengthen their reciprocal connections during memory coding and thus form a functional microcircuit.

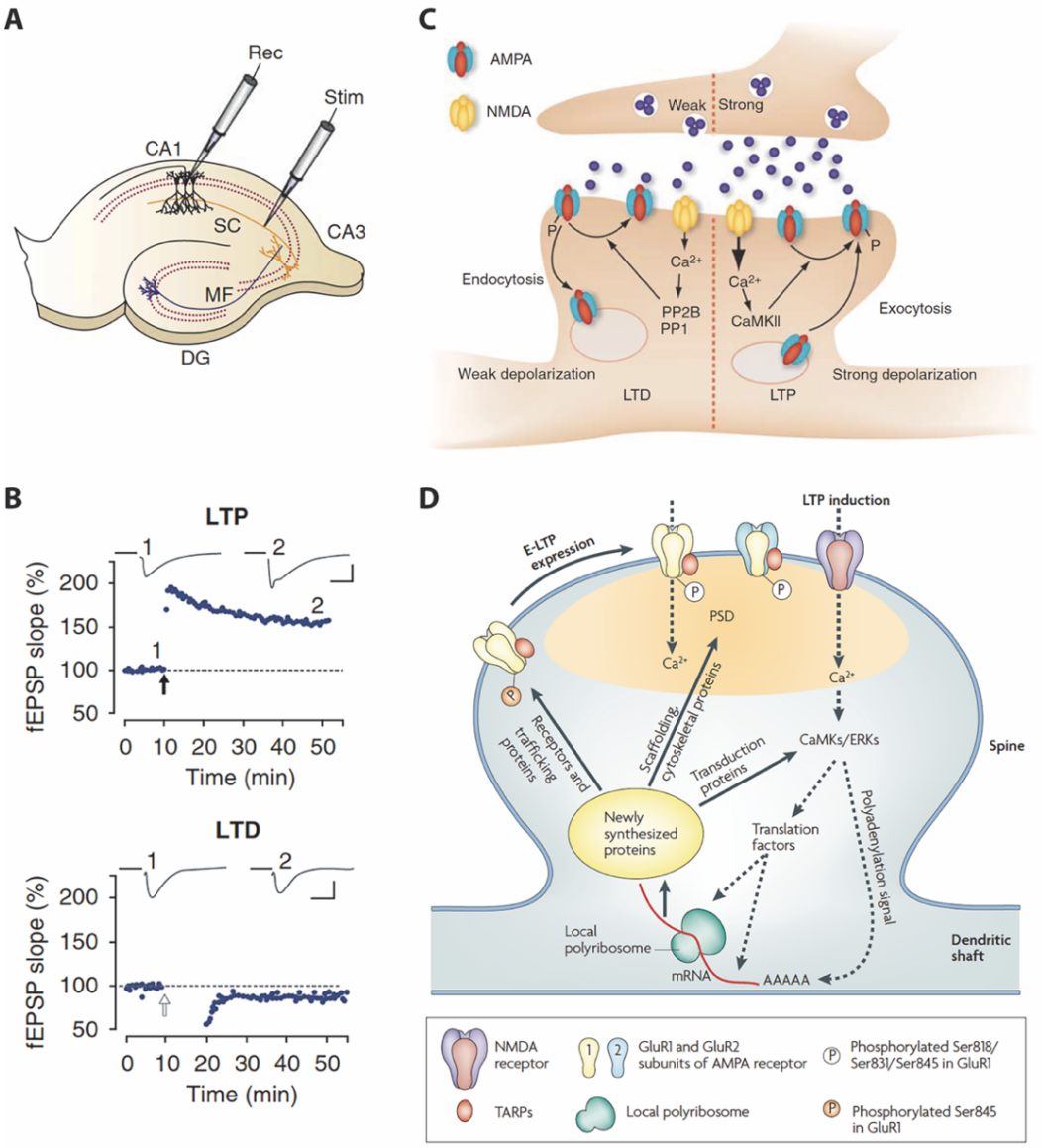

Figure 1.14: Hebbian plasticity.
Hippocampal synaptic plasticity is typically studies in hippocampal slices with electrodes stimulating Schaffer collaterals and recording CA1 synapses **(A)**. Stim: stimulating electrode; Rec: recording electrode; SC: Schaffer collateral; MF: mossy fiber; DG: dentate gyrus. **(B)** Representative experiments illustrating LTP and LTD in the CA1 region of the hippocampus. The recording electrode is capturing a subset of CA1 neurons that are activated simultaneously and defined as synaptic strength. The initial slope of the field excitatory postsynaptic potential (fEPSP; normalized to baseline) is plotted against time and represents the synaptic strengths. Upper recording shows LTP, elicited by high frequency tetanic stimulation. Lower panel demonstrates LTD elicited by low-frequency stimulation. For each condition two traces are shown above the graph, recorded at two given time points (indicated by subsequent numbers in the graph). The second trace is bigger after LTP and smaller upon LTD induction as compared to forgoing 10 min baseline recording (scale bar: 0.5mV, 10ms). Dark arrow: high frequency recording; open arrow: low-frequency recording. **(A, B)** From Citri & Malenka, 2008. **(C)** Postsynaptic LTP and LTD mechanisms. Weak presynaptic activation triggers postsynaptic depolarization and calcium influx via NMDA receptors. Next, AMPA receptors are dephosphorylated by phosphatases leading to receptor endocytosis, resulting in LTD. Strong depolarization triggers LTP through CaMKII, receptor phosphorylation and exocytosis. From Lüscher et al., 2012. **(D)** Maintenance of synaptic strength induced by activity and dendritic protein synthesis. Right: LTP induces CaMKII and extracellular signal-related kinases (ERKs) in dendritic spines. Those kinases phosphorylate and activate translation factors to stabilize and translate local mRNAs resulting in local protein synthesis. The feedforward mechanism increases receptor numbers and receptor trafficking. In addition, synthesized scaffolding and cytoskeleton proteins assist surface expression, later diffusion and stabilization of AMPARs, simultaneously potentiating synapses. E-LTP, early phase LTP; PSD, postsynaptic density; TARPs, transmembrane AMPAR regulatory proteins. From Derkach et al., 2007.

Hebbian plasticity and its implication in associative learning, spatial memory and adaptive behavioral changes was largely studied in the amygdala, cerebral cortex and hippocampus of mammals (Sweatt, 2016). Most of our understanding regarding learning and memory comes from hippocampal studies and the finding of long-lasting synaptic plasticity (Sweatt, 2016). Especially, the NMDAR-dependent LTP and LTD in the Schaffer collaterals on the hippocampal CA1-region was well studied (Figure 1.14(A, B)). LTP occurs upon strong presynaptic activity and postsynaptic membrane depolarization with large EPSPs. This initial induction phase decays within a few minutes and is categorized under short-term potentiation (STP) and is followed by early (E-LTP) and late (L-LTP) stages during which synaptic responses increase and are maintained. E-LTP lasts between 60–90 min, whereas the term L-LTP is used when activity dependent gene transcription and protein synthesis occurs and endures for days or even weeks *in vivo* (Park et al., 2018). During E-LTP the postsynapse is depolarized and through removal of the Mg^{+} block of NMDARs (Figure 1.14(C)), Ca^{2+} can enter the cell and bind to calmodulin, activating a subset of enzymes most notably calcium/calmodulin-dependent kinase II (CaMKII) and the protein kinases A (PKA) and C (PKC). These events enhance the recruitment of AMPARs to the postsynaptic side which then enhances biochemical transmission. Additionally, they elicit the phosphorylation of synaptic targets including GluA1 and TARPs (Transmembrane AMPA receptor Regulatory Proteins) (Figure 1.14(D)) (Lüscher et al., 2012; Lisman, 2017; Park et al., 2018; Baltaci et al., 2019). Further details on the function of Hebbian plasticity is described in Chapter 1.7 examining the role of AMPARs.

1.6.2 Homeostatic Plasticity

Homeostatic plasticity describes several mechanisms and permanent synaptic tuning by which a neuron maintains its firing rate set points. Practically this happens by offsetting excessive excitation or inhibition through changes in synaptic strength (Pozo & Goda, 2010; Fox & Stryker, 2017; Turrigiano, 2017). Several investigations evidenced that homeostatic plasticity occurs over a number of spatial scales from individual synapses to cell population levels (Slomowitz et al., 2015; Hengen et al., 2016). Compared with Hebbian plasticity, homeostatic plasticity is relatively slow to prevent unstable feedback systems and oscillations that could overshoot the set point (Zenke & Gerstner, 2017). Hebbian plasticity is a positive feedback process, becoming clear through the process of LTP, since induction of LTP leads synapses to become more excitable with lower thresholds for further LTP induction. Conversely, homeostatic plasticity acts as negative feedback process to prevent, for example, runaway excitation (Figure 1.15(A)), by constraining activity levels and preserving network stability (Turrigiano, 2008; Pozo & Goda, 2010). For the moment, homeostatic plasticity is divided into four forms which cannot always be separated in their plain

form, but often interact together to maintain homeostasis (Fox & Stryker, 2017). It is distinguished between firing rate homeostasis, synaptic scaling, inhibitory feedback and plasticity of intrinsic membrane properties (Fox & Stryker, 2017). The firing rate is a parameter by which cells can monitor the activity to create an error signal to induce homeostasis. Also, firing rates can be restored by inhibitory feedback, intrinsic membrane properties or changes of the synaptic weight (Turrigiano, 2017).

Synaptic scaling occurs when neuron globally and bidirectionally adjusts its synaptic weights due to a chronic network activity shift (Turrigiano et al., 1998). Thereby, it scales synapses up or down in strength to stabilize neuronal firing. In practice, this means an increase in weights upon prolonged reduction of activity and inversely scaling down upon long-lasting high activity (Turrigiano, 2008) as compensatory changes (Figure 1.15(B)) to bring perturbed network activity back to normal baseline levels (Turrigiano et al., 1998).

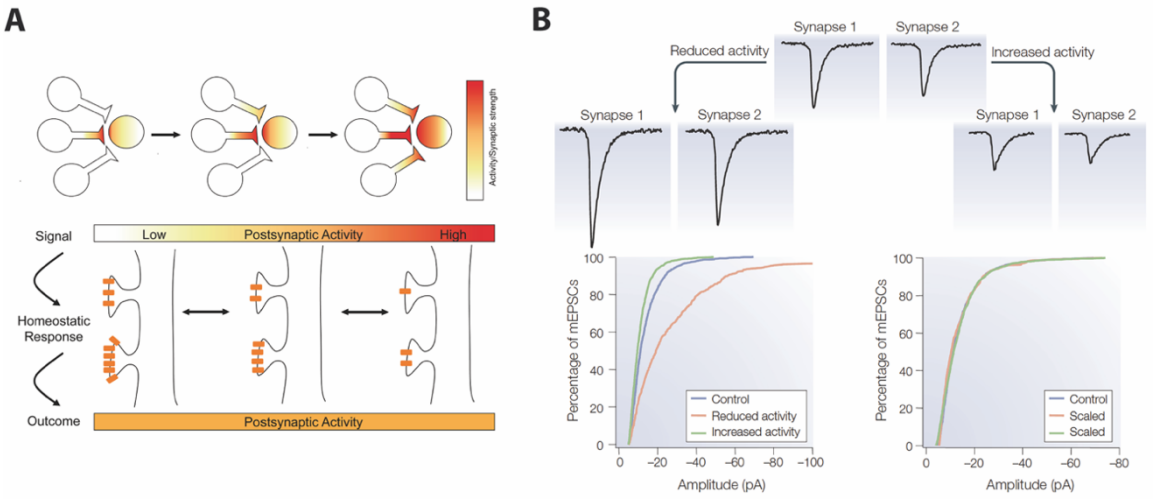

Figure 1.15: Homeostatic plasticity.
(A) Upper part illustrates runaway excitation and Hebbian plasticity. As soon as postsynaptic firing rates increase, other synapses undergo LTP and the postsynaptic neuron loses the capacity to store information in its synaptic weights. Lower part demonstrated homeostatic plasticity. Upon long-term changes in synaptic activity synaptic scaling occurs. The strength of incoming synaptic inputs is proportionally reduced until firing rates reach baseline levels. The relative strength of the potentiated synapses remains the same. Synaptic scaling is characterized by multiple surface AMPAR changes at the synapse. From Watt & Desai, 2010. **(B)** The distribution of synaptic weights is changed through synaptic scaling, represented by miniature excitatory postsynaptic currents (mEPSCs). Increased activity reduces the amplitude of mEPSCs on pyramidal neurons. The opposite effect occurs upon decreased activity. Entire distributions are proportionally affected (left) and fit the baseline distribution after synaptic scaling (up or down (right). This indicates that prolonged excitatory activity changes onto pyramidal neurons are scaled up or down in a multiplicative way. From Turrigiano & Nelson, 2004.

Artificially induced changes of activity provoked by pharmacological manipulations evoke bidirectional compensation of glutamatergic synapse strength. This effect can be measured when recording miniature excitatory postsynaptic currents (mEPSCs) reflecting the postsynaptic response to release of individual vesicles of neurotransmitters and expresses the unit strength of a synapse (Turrigiano, 2008). Synaptic scaling allows neurons to normalize firing without changing

the relative strength of synaptic inputs conserving the initial relative differences in efficacy between synapses. As LTP potentiates synapses, they will become stronger compared to non-potentiated synapses, leading to alteration of the total excitatory strength of a neuron (Turrigiano & Nelson, 2004; Turrigiano, 2008). By means, the relative strength of individual synapses maintains the same although the global excitatory input is shifted (Citri & Malenka, 2008). There are indications of a preferential range of firing rates neurons gain to keep (Keck et al., 2017; Turrigiano, 2017) by sensing their own firing rates most likely via a calcium sensor as activity indicator (Turrigiano, 2008).

Homeostatic plasticity is expressed through altering of presynaptic neurotransmitter release and postsynaptic receptor abundance control (Pozo & Goda, 2010). Synaptic scaling itself is mediated by changes in the numbers and subunit composition of AMPARs and NMDARs. They coordinatively increase or decrease upon scaling up or down, respectively (Turrigiano, 2008). In addition, homeostatic plasticity is mediated by transcriptional events and local protein synthesis at dendritic trees, translating already present mRNAs at dendritic trees (Steward and Schumann 2003; Pozo & Goda, 2010). Briefly summarized, scaling up is achieved by reduced activity of CaMKIV through reduced calcium influx and characterized by a specific gene expression profile. Amongst translated genes are the immediate early genes FBJ osteosarcoma oncogene (c*Fos*) and activity-regulated cytoskeleton associated protein (*Arc*) (Ibata et al., 2008; Schaukowitch et al., 2017). Scaling down occurs after an increase in calcium influx which triggers activation of CaMK kinase (CaMKK) and CaMKIV (Goold & Nicoll, 2010). In addition, secretion of specific proteins promotes scaling up or down. Factors that are required for scaling up include TNFα, GRIP1 (glutamate receptor-interacting protein 1), PICK1 (protein interacting with protein kinase C, alpha 1), and PSD95, whereas scaling down shows to be dependent on PSD95 or PSD93 and EFNA4, mGluRs (metabotropic glutamate receptor 2) and HOMER1 (homer scaffolding protein 1) (Pozo & Goda, 2010; Fernandes & Carvalho, 2016).

1.6.3 Structural Plasticity

Structural modifications of synapses arise after repeated neuronal activation to enable groups of neurons to process information and reproduce activity-patterns attained through experiences. Structural synaptic rearrangements include enlargement, growth, pruning and elimination of synapses and involve spines, dendrites and even astrocytic processes, which are often found at synaptic sides. In addition, morphological changes take place in different time scales from minutes to days (Bernardinelli et al., 2014). These structural changes of synapses appear to be long-lasting hence are hypothesized to sustain long-term memory (Figure 1.16(C)) and portray

synaptic plasticity (Carasatorre et al., 2016). Decades of research revealed a positive correlation between improved learning skills and new experiences with increased dendritic branching as well as structural synaptic changes (Rosenzweig & Bennett, 1996). Moreover, mice studies early in the 1990s showed that learning of new motor skills produced anatomical changes in neural networks with increased synaptic contacts in the cerebellum and cerebral cortex (Greenough and Anderson, 1991). Synaptic plasticity was especially studied in the hippocampus since it is a key area of processing learning and memory. After the above finding, researchers proved that LTP induction of perforant path stimulation elevates the number of synaptic contacts with dentate granule cells (Geinisman, 2000). Following studies showed increased numbers of mossy fiber boutons in the *stratum oriens* of CA3 after inducing LTP in the mossy fiber (Escobar et al., 1997). Many subsequent studies that were performed on rats, doubtlessly revealed that hippocampal-dependent spatial experiences emanate in heightened mossy fiber boutons contacting CA3 pyramidal cells and increase spine densities in CA1 dendritic subgroups (Moser et al., 1994/1997; Rusakov et al., 1997; Ramírez-Amaya et al., 2001; Stewart et al., 2005). Especially spines have been studied in activity-dependent context, detecting stable fluctuations of spine sizes within a specific range and finding that spine size correlates with AMPAR packing at the PSD (Figure 1.16(B)) (Yasumatsu et al., 2008; Ziv & Brenner, 2018). Activity-induced plasticity increases sensitivity to glutamate, stabilization of spines and spine turnover in both directions – elimination and formation (Matsuzaki et al., 2004; Nägerl et al., 2007; Honkura et al., 2008, Bernardinelli et al., 2014). Fluctuations in spine size highly dependent on actin cytoskeleton reorganization (see section 8.1.3). Many observations unveiled new spines preferentially building up in a non-random way, but rather in close proximity to activated synapses (Figure 1.16(C)), resulting in a sort of clustering (Dubos et al., 2012; Bernardinelli et al., 2014).

Clustered spines supposedly have a functional role in influencing neighboring synapses as LTP or LTD induction was shown to leak out from individual spines, i.e., reducing the threshold for LTP induction of closely located spines or elimination of depressed synapses in close proximity following LTD (Harvey & Svoboda, 2007; Wiegert & Oertner, 2013). Synaptic clustering can also reflect input sharing when it receives signals from one shared presynaptic axon. Alternatively, adjacent synaptic input can sum non-linearly to reflect each dendritic compartment of a neuron. In this way the information storage of a single neuron can be increased (Fiala et al., 2002; Govindarajan et al., 2006; Larkum & Nevian, 2008; Kasthuri et al., 2015).

Structural changes like spine enlargement and increased spine density upon LTP or conversely spine shrinkage and removal during LTP (Forrest et al., 2018) are crucial for learning and memory (Hayashi-Takagi et al., 2015). During late LTP, enlargement of spine head sizes is boosted by a raise of AMPAR synaptic content and is referred to as structural LTP (sLTP) (Bosch et al., 2014).

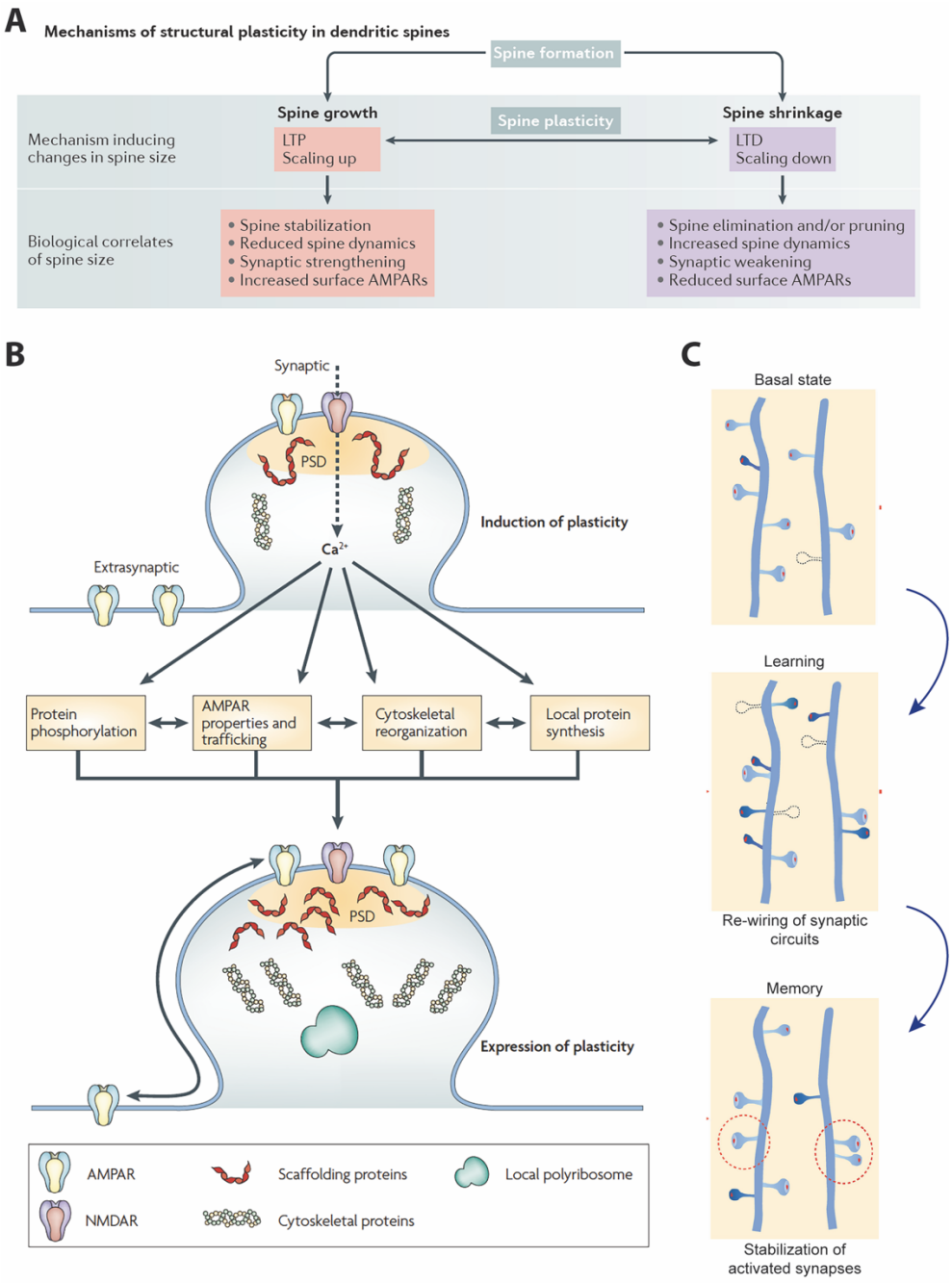

Figure 1.16: Structural plasticity.
(A) Summarized processes regulating spine development and plasticity. Spine growth can be induced by LTP or scaling up and drives spine stability linked with reduced spine dynamics, increased synaptic strength, and increased surface AMPARs. Shrinkage of spines is induced by LTD or scaling down associated with spine destabilization which can lead to elimination of spines, spine weakening, and reduced surface AMPSARs. From Forrest et al., 2018. **(B)**. Postsynaptic plasticity causes interconnected processes of signal transduction pathways contributing to activation of kinases and phosphatases regulating receptor trafficking, cytoskeletal organization and protein synthesis. These mechanisms affect AMPAR properties such as subunit composition and trafficking. Reorganization of actin modulates spine morphology. Local protein synthesis is triggered in spines and dendrites. Bottom spine scheme outlines bidirectional synaptic plasticity (for example LTP/LTD) involving AMPAR trafficking between extrasynaptic and synaptic sites as well as upregulation of scaffolding proteins and changes in cytoskeletal proteins. Altogether this triggers spine morphology. From Derkach et al., 2007. **(C)** Structural plasticity is mediated by activity. Synaptic networks undergo a constant process of growth (dark blue) and elimination (dotted line) of spines. Upon learning basic spine turnover enhances with increased spine development and elimination driving remodeling and adaptation of network connectivity. Formation of spine clusters is favored when activated synapses are in close proximity. Stabilization of newly formed spines maintains important functional connections and occurs during memory formation. Modified after Bernardinelli et al., 2014.

Structural LTP is initiated through CaMKII-mediated activation of Rho GTPases which induce actin polymerization und subsequent extension of the spine head cytoskeleton (Chapter 8). Interestingly, some GTPases diffuse into adjacent spines promoting sLTP there as well (Bosch & Hayashi, 2012; Nakahata & Yasuda, 2018). Persistent sLTP grounds on local protein synthesize particularly actin and actin-interacting proteins to increase and sustain grown PSD area (Lisman, 2017; Nakahata & Yasuda, 2018).

1.7 Glutamate receptors

Glutamate is the primary excitatory neurotransmitter in mammalian brains and unfolds its effects through two glutamatergic receptors families which are able to function via crosstalk (Reiner & Levitz, 2018). These are ionotropic glutamate receptors (iGluRs) that are ligand-gated ion channels producing glutamate-evoked currents, and also metabotropic glutamate receptors (mGluRs), which act as G-protein-coupled receptors (GPCRs), controlling intracellular processes via G-protein signaling cascades (Figure 1.17) (Reiner & Levitz, 2018).

Glutamate receptors are widely spread in the central nervous system and their subtype composition is variable in different brain regions and multiple receptor types can be expressed by one cell (Figure 1.17(C)) (Ferraguti & Shigemoto, 2006; Hadzic et al., 2017). Metabotropic GluRs hold eight family members (mGluR1–8), belong to family C GPCRs and assemble as constitutive dimers (Doumazane et al., 2011; Levitz et al., 2016). They are characterized by a huge extracellular ligand binding domain (LBD), linked to a 7-helix pore-forming transmembrane domain (TMD) (Figure 1.17(A)). Metabotropic GluRs are split into three subfamilies (I–III), whereas group I is preferentially G_q-coupled and group II and III primarily couple with $G_{i/o}$ (Ferraguti & Shigemoto, 2006; Niswender & Conn, 2010).

Ionotropic GluRs assemble as tetramers and create non-selective cation-channels (Traynelis et al., 2010). Each individual subunit possesses one extracellular amino-terminal domain (ATD), a LBD, a TMD, and an intracellular C-terminal domain (CTD) (Figure 1.17(A)) (Reiner & Levitz 2018). The subunits GluR (or GluA) and GluK (Glutamate ionotropic receptor kainate type subunit) form homo- or heterotetrametric receptors, activated in a quick way by glutamate binding to the LBD site that simultaneously evokes their desensitization (Contractor et al., 2011; Greger et al., 2017). The main subfamilies of iGluRs are AMPA, NMDA, (Figure 1.17(B)) and kainite (KA) receptors (Hollmann & Heinemann, 1994). NMDARs are heterotetramers consisting of two GluN1 (glutamate ionotropic receptor NMDA type) subunits, binding glycine or D-serine and two GluN2 subunits binding glutamate (Paoletti et al., 2013). Latter can be substituted by two GluN3 receptors, binding glycine or D-serine and allow the formation of triheteromeric GluN1/2/3

receptor types (Pérez-Otaño et al., 2016). In sum, NMDA receptors require the binding of two agonists for activation and membrane depolarization and the removal of the external magnesium ion block (Figure 1.17(B)). This makes them voltage- and ligand-gated channels subserving as coincidence detectors (Hansen et al., 2018).

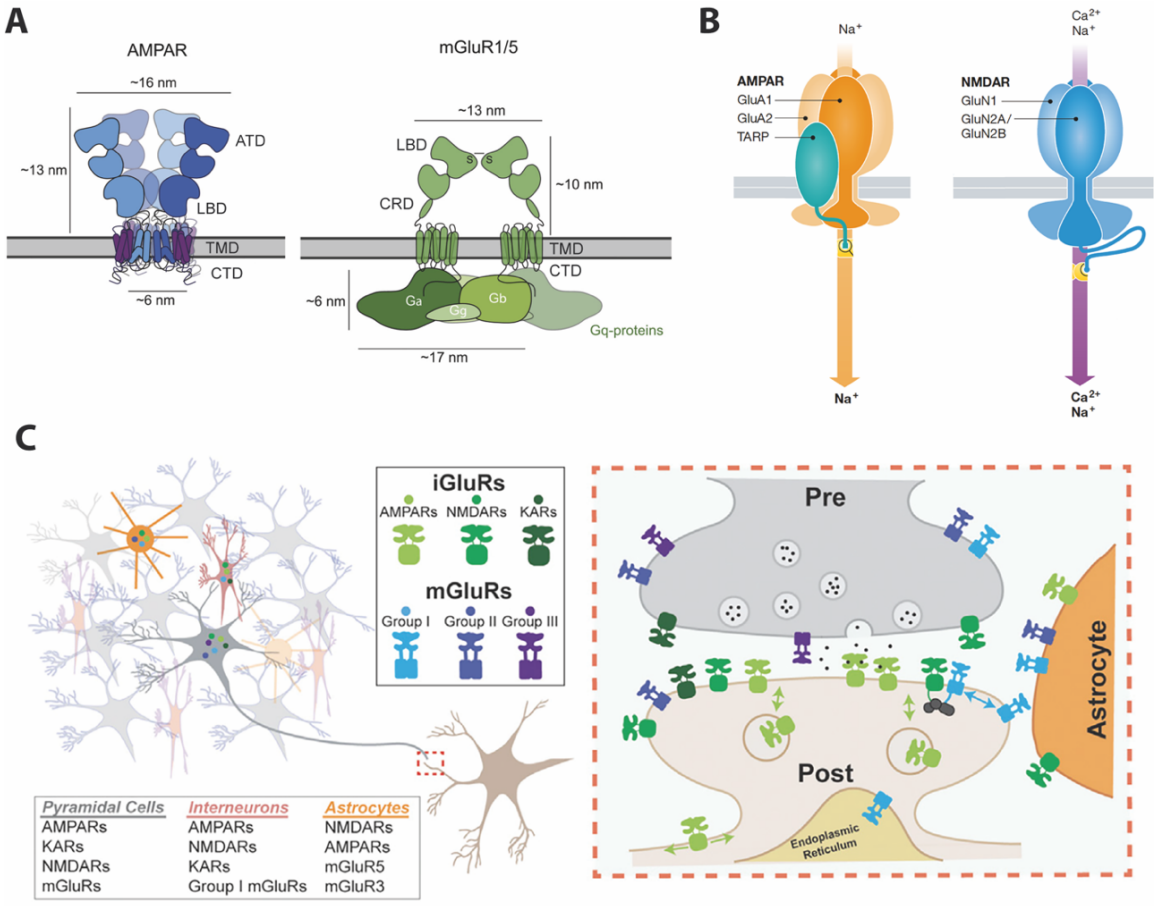

Figure 1.17: Glutmate receptors.
(A) Structural organization as side view of iGluRs represented by AMPARs (blue) and mGluR1/5-type receptors (green) coupled to cognate G_q-proteins via the C-terminal domain (CTD). The tetrameric AMPAR (GluR2 homomer) shows its membrane position in a Y-shape confirmation. The mGluR dimer is shown in closed-closed resting confirmation. Distinct domains are labeled. Glutamate and orthosteric ligands bind in the ligand binding domains (LBDs). Ions bind in the inter-LBD interface in both classes or the amino terminal domain (ATD) of iGluRs. Allosteric modulators of mGluRs bind in the transmembrane domain (TMD). In case of iGluRs allosteric modulators bind in the ATD or LBD, and pore blockers bind in the ion channel-forming TMD. Models are roughly scaled in nm. Modified from Scheefhals & MacGillavry, 2018. **(B)** Postsynaptic AMPARs mostly consists of two GluR1 and two GluR2 subunits (yellow) und TARP subunits (turquoise) which mediate postsynaptic localization. NMDARs mostly consist of two GluN1 and GluN2 subunits respectively. AMPARs are Na^+ and K^+ permeable, whereas NMDARs also conduct Ca^{2+}. Modified from Patriarchi et al., 2018. **(C)** United GluR signaling at synapses. GluR types are listed on the left side with respective color codes. Prevalence and overlapping expression patterns of iGluRs and mGluRs in pyramidal cells, interneurons and astrocytes are shown in the table on the bottom left. Close-up scheme on the right side of representative synaptic compartments including presynaptic terminals, PSD, perisynaptic regions and astrocytic processes. iGluRs and mGluRs are present in all compartments with different compositions. Their precise localization characterizes timing and concentration of glutamate-induced activation and affects downstream signaling and potential interaction. From Reiner & Levitz, 2018.

AMPARs and KARs assemble from four subunits, GluR1-4 or GluK1-5 respectively and assemble in homo- or heterotetramers (Traynelis et al., 2010). The diversity of glutamate receptors is increased through different subunit compositions, but also by alternative ribonucleic acid (RNA) splicing and editing, leading to diverse expression patterns and functioning according to subsequent isoforms (Niswender & Conn, 2010; Traynelis et al., 2010; Doumazane et al., 2011; Levitz et al., 2016). In case of AMPARs, alternative splicing generates two isoforms of the LBD named flip and flop, controlling desensitization, deactivation, and sensitivity to allosteric modulators in different ways (faster desensitization in case of the flip isoform) (Traynelis et al., 2010).

In case of GluR2 subunit most of the RNA is edited post-transcriptionally, by conversion of a glutamine residue to arginine by adenosine deaminase in the ion-conducting pore. This change confers Ca^{2+} impermeability to the GluR2 subunit and by implication making all GluR2 containing AMPARs calcium impermeable (Traynelis et al., 2010). While iGluRs and mGluRs have distinct functions and properties, they simultaneously display overlapping glutamate sensitivity and kinetics. Within iGluRs AMPARs and KARs have the fastest kinetics upon high glutamate concentrations as they open in less than one millisecond to produce fast excitatory currents. Similarly, they deactivate or desensitize very fast, i.e., to glutamate drop downs or persisting elevated levels of glutamate (Traynelis et al., 2010).

On the other hand, NMDARs have a high sensitivity towards glutamate, concurrently a slower response towards glutamate, but also higher Ca^{2+} permeabilization which makes them more appropriate to elicit signaling cascades (Traynelis et al., 2010). Metabotropic GluRs show slower responses towards glutamate due to the G-protein coupled machinery, though the effects are more long-lasting (Reiner & Levitz, 2018).

1.7.1 AMPA receptors

Activity-dependent changes in excitatory synapses build the cellular basis for plasticity of neuronal networks, triggered by cognitive function (Derkach et al., 2007). AMPAR-glutamate receptors are the main transducers of rapid excitatory neuronal transmission in the mammalian brain and their regulations promote synaptic strength by induction of cellular signaling cascades (Derkach et al., 2007). Cognitive functions such as learning and memory and their maintenance induce long-lasting changes in glutamatergic synapses promoting synaptic strength through AMPAR trafficking and phosphorylation (Rioult-Pedotti et al., 2000; Dragoi et al., 2003; Lee et al., 2003; Takahashi et al., 2003; Gruart et al., 2006; Pastalkova et al., 2006; Whitlock et al., 2006). Most of the AMPARs are composed of four glutamate subunits ranging from GluR1 to GluR4 and vary

in their composition depending on the brain region. Decisive for functional shifts of AMPARs are the carboxyl (C) termini with most structural and functional differences in their regulatory domains that are targeted by intracellular signaling pathways (Derkach et al., 2007). Furthermore, C-termini interact with scaffolding proteins such as phosphatases and kinases as well as cytoskeletal proteins such as actin proteins (Collingridge et al., 2004; Kim & Sheng, 2004; Nicoll et al., 2006). The diversity given by the distinct domains of the different glutamate receptor subunits and their various compositions impact trafficking, gating and stabilization of AMPARs at synapses (Collingridge et al., 2004; Terashima et al., 2004; Lu & Ziff, 2005; Nicoll et al., 2006; Tomita et al., 2006). Neuronal activity determines local synthesis of AMPARs and their deposition in synapses (Ju et al., 2004; Sutton et al., 2006) and developmentally regulated expression and interaction with specific partners in distinct brain regions (Derkach et al., 2007).

1.7.2 AMPA receptors in CA1 and CA3 pyramidal neurons

AMPARs in mature hippocampal synapses are predominantly composed of GluR1/2 subunits and sometimes of GluR2/3 subunits (AMPAR structure is depicted in Figure 1.18). The GluR2 subunit underlies RNA editing by which the glutamine residue 607 can be replaced by arginine and in this way modify the permeability for Ca^{2+}-permeability, channel conductance, kinetics, glutamate affinity and receptor assembly (Geiger et al., 1995; Jonas & Burnashev, 1995; Swanson et al., 1997; Kask et al., 1998; Dingledine et al., 1999; Mansour et al., 2001; Greger et al., 2003; Oh & Derkach, 2005). On top of that, presence or absence of GluR2 subunits drastically influences synaptic transmission. GluR2-containing receptors exhibit a small channel conductance with poor Ca^{2+}-permeability, low open probability and a lack of rectification, by means of a voltage-dependent block (Burnashev et al., 1992; Swanson et al., 1997; Bowie et al., 1998; Oh & Derkach, 2005; Burnashev, 2005).

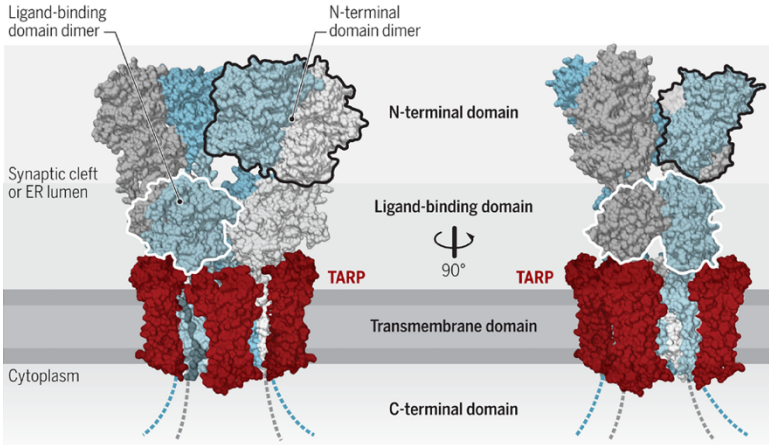

Figure 1.18: AMPAR structure: Four subunits that are functionally and conformationally distinct form tetrameric AMPARs. Grey: pore-proximal subunits; blue: pore-distal subunits. Each subunit consists of an extracellular N-terminal, a ligand binding domain, an integral membrane domain and an intra-cellular C-terminus. The large extra-cellular domain faces projects towards the synaptic cleft. TARPs (red) interact with the receptor at four positions at the transmembrane domain. From Buonarati et al., 2019.

Furthermore, the composition of AMPARs affects their phosphorylation and thus regulates its functionality and membrane trafficking, since it affects receptor properties (Derkach et al., 1999; Banke et al., 2000). For example, serine residues (Ser) 831 and Ser845 in GluR1 subunits act as important regulatory phosphorylation sites. Missing of those residues causes malfunctions during LTP and LTD and memory deficits in spatial learning shown in mice (Lee et al., 2003). After phosphorylation of Ser831 through calcium/calmodulin-dependent protein kinase II (CaMKII), binding of glutamate and coupled single-channel conductance, which accompanies LTP, is increased in monomeric GluR1 AMPARs (Barria et al., 1997; Derkach et al., 1999; Lee et al., 2000). In CA1 neurons CaMKII-mediated phosphorylation is part of an early phase LTP (E-LTP) (Poncer et al., 2002; Derkach et al., 2007). Additionally, phosphorylation of Ser845 by PKA enhances the opening probability of monomeric GluR1 channels, regulates trafficking of AMPARs to the surface in synapses and is implicated in synaptic plasticity, thus learning processes (Banke et al., 2000). Per contra, phosphorylation of heteromeric GluR1/2 receptors, constituting the major part of GluR1-containing AMPAR under basal conditions, are differently regulated (Wenthold et al., 1996; Holman et al., 2007; Derkach et al., 2007). GluR2 subunits silence basal channel conductance of GluR1 and even prevent enhancement of channel conductance upon phosphorylation of Ser831 in GluR1, which normally occurs during LTP (Barria et al., 1997; Lee et al., 2000; Whitlock et al., 2006). E-LTP is accompanied by newly insertion of AMPARs lacking GluR2 subunits (Plant et al., 2006). This changes the properties of synaptic AMPARs at the postsynaptic surface and influx plus phosphorylation by CaMKII emerges, which enables higher channel conductance as studied in CA1 hippocampal LTP (Benke et al., 1998; Poncer et al. 2002; Lüthi et al., 2004). Several studies found a link between activity-dependent strengthening of hippocampal synapses or experiences-dependent strengthening of neocortical synapses and increased contribution of GluR2-lacking AMPARs (Ju et al., 2004; Thiagarajan et al., 2005; Clem & Barth, 2006). Subunit re-composition of surface AMPARs might ubiquitously impact synaptic plasticity throughout different areas of the brain, since also activity-dependent depression of synaptic strength was found in cerebellar stellate cells when the amount of AMPARs lacking GluR2 decreases (Liu & Cull-Candy, 2000/2002). Reverse effects were found in cultured hippocampal slices, with significantly lower amounts of GluR2-containing AMPARs under basal conditions as compared to acute slices. In addition, GluR2 numbers were increased upon pre- and postsynaptic stimulation (Bagal et al., 2005).

The AMPA receptor density at the plasma membrane is dynamically regulated via endo- and exocytosis in an activity-dependent manner (Ehlers, 2000; Passafaro et al., 2001; Kim & Sheng, 2004; Park et al., 2004). GluR2/3 subunit containing AMPARs undergo constant cycling in the absence of plasticity-inducing signaling (Nishimune et al., 1998; Passafaro et al., 2001; Shi et al., 2001). Upon stimulation, Ca^{2+}-influx through NMDA receptors initiates AMPAR trafficking and

cycling with changes in AMPAR subunit composition (Passafaro et al., 2001; Horton & Ehlers, 2004; Park et al., 2004).

The composition of AMPAR subunits is an important adjuster for trafficking, plasticity and phosphorylation of even those (Derkach et al., 2007). It was evidenced in different brain areas, that activity alters postsynaptic properties and synaptic strength by changes in AMPAR amounts and compositions (Liu & Cull-Candy, 2000/2002; Ju et al., 2004; Thiagarajan et al., 2005; Clem & Barth, 2006; Plant et al., 2006; Derkach et al., 2007). Studies of hippocampal neurons revealed insertion of AMPARs at CA3-CA1 synapses upon LTP with GluR1 subunit contributing to the delivery of AMPARs (Hayashi et al., 2000; Lu et al., 2001; Pickard et al., 2001; Shi et al., 2001). Similarly, GluR2 subunits contribute to AMPAR endocytosis during LTD, by interacting with the activator protein 2 (AP2)-hippocalcin complex, a sensor for Ca^{2+} (Lüthi et al., 1999; Lee et al., 2002; Palmer et al., 2005). Generally, GluR1 homomers or heteromers are transported to the synapse upon activity and GluR2/3 heteromers assist integral and activity-dependent replacement of existing receptors (Shi et al., 2001). CA3-CA1 synapses show different mechanisms for LTP induction. For one, synapses can change their single channel-conductance through subunit re-composition and CaMKII-depended regulation of AMPARs with increased GluR1 contribution to postsynaptic currents or secondly, by increasing the numbers of postsynaptic AMPARs (Fukunagas et al., 1993; Barria et al., 1997; Benke et al., 1998; Hayashi et al., 2000; Liu & Cull-Candy, 2000/2002; Ju et al., 2004; Lüthi et al., 2004; Thiagarajan et al., 2005; Clem & Barth, 2006; Plant et al., 2006). A second re-composition of AMPARs appears after LTP-induction and a latency of approximately 25 minutes, whereafter GluR1-containing AMPARs are replaced by GluR2-containing receptors during the maintenance phase of LTP (Plant et al., 2006). Interestingly both, conductance and receptor number LTP mechanisms seem to depend on phosphorylation of AMPA receptors (Lee et al., 2000; Lüthi et al., 2004).

1.7.3 Multistep trafficking of AMPARs to synapses

Upon inactivated states, AMPARs traverse a constitutive recycling between synapses and cytosol, sorted for degradation or reinsertion (Figure 1.19(A)) (Ehlers, 2000; Passafaro et al., 2001). On the other hand, during induction of LTP, exocytosis and endocytosis of AMPARs occur at extrasynaptic membrane sides wherefrom AMPARs laterally travers along to and from synaptic PSDs (Blanpied et al., 2002; Petralia et al., 2003; Park et al., 2004). Stabilization of surface AMPARs occurs upon synaptic potentiation and lateral diffusion is correlatively reduced. Typically, AMPARs are anchored to PSD-95 proteins which reduces their mobility within synapses (Borgdorff & Choquet, 2002; Triller & Choquet, 2005). GluR1-containing receptors are first

dispatched to extrasynaptic sides upon LTP before they integrate into synapses which is ultimately mediated by NMDA activation and associated Ca^{2+}-influx (Derkach et al., 2007).

Findings suggest that AMPAR trafficking consists of a two-step expiration (Figure 1.19(B)). The first step is associated with a PKA-mediated Ser845 phosphorylation, driving receptors to extrasynaptic sides. Upon stimulation, this is followed by a NMDA-dependent Ca^{2+}-influx, enhancing lateral diffusion of receptors into the synapse and thus increasing synaptic potentiation (Esteban et al., 2003; Derkach et al., 2007). The stabilization of newly incorporated receptors might require phosphorylation of Ser818 in GluR1 by PKC and CaMKII. In contrast, during LTD Ca^{2+}-influx leads to activation of calcineurin and subsequent endocytosis of AMPARs with decreasing levels of Ser845 phosphorylation (Boehm et al., 2006). The first step with newly delivered AMPARs at extrasynaptic sides represents the reserve pool of primed receptors which upon stimulation and NMDA-dependent Ca^{2+} rise can rapidly be incorporated into synaptic sites for synaptic strengthening (Hayashi et al., 2000; Boehm et al., 2006).

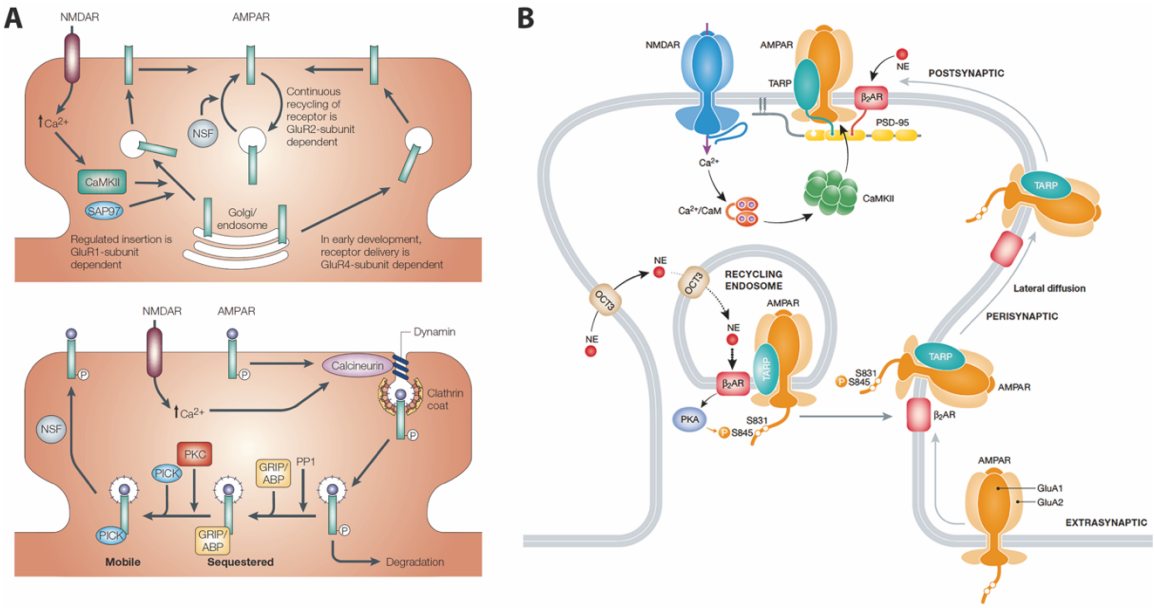

Figure 1.19: AMPAR Cycling and insertion.
(A) Upper image shows AMPAR insertion into the synaptic membrane. During LTP CaMKII is activated and GluR1-containing AMPARs are delivered to the membrane surface. Continuing LTP maintains final levels of AMPARs at the synapse, requiring GluR2-containing AMPARs. During development activity-dependent insertion of newly synthesized receptors requires GluR4 subunits. Lower image demonstrates AMPAR endocytosis during LTD in the hippocampus. Upon NMDAR activation cytosolic Ca^{2+} levels rise and stimulates calcineurin activating the clathrin endocytic machinery. Consequently, AMPARs are internalized and degraded or intracellularly stabilized through GRIP interaction after dephosphorylation of S880. Protein phosphatases (PP) are responsible for dephosphorylation. PKC-dependent phosphorylation of S880 mobilizes AMPARs returning them back to the membrane surface. From Carroll et al., 2001. **(B)** AMPAR surface trafficking and lateral diffusion to the postsynaptic site. A part of AMPARs is synthesized in the ER of dendrites. Intracellular norepinephrine (NE) signaling promotes surface insertion via recycling endosomes (RE). Possibly, NE is transported from the extracellular space via the OCT3 (organic cation transporter 3) transporter into the cytosol and later into the lumen or REs. NE is transported into the Golgi apparatus activates b_2 ARs (adrenergic receptor) which are associated with GluR1 in the RE. S845 phosphorylation by PKA triggers AMPAR insertion into the perisynaptic membrane. Next, AMPARs diffuse laterally into the postsynaptic density. AMPASRs are trapped through Ca^{2+} induced CaMKII activation. From Patriarchi et al., 2018.

Endocytosis of AMPA receptors depends on initial lateral diffusion of receptors in a NMDA-dependent manner which in turn leads to a decrease in synaptic strength (Beattie et al., 2000; Derkach et al., 2007).

Different PSD located proteins like PICK 1 and GRIP interact with C-termini of various AMPAR subunits and by this, they impact on receptor trafficking and properties (Figure 1.19(A)) (O'Brien et al., 1998; Liu & Cull-Candy, 2000; Lu and Ziff 2005). For instance, increased interaction of PICK1 with GluR2 in hippocampal synapses causes reduction of surface GluR2 at the synapses and as a result strengthens synapses (Terashima et al. 2004). Overall, this interaction is motored by PKC and CaMKII (Terashima et al. 2004). Transmembrane AMPA receptor regulatory proteins (TARPs) represent γ-subunits of Ca^{2+}-channels and were found to assist as complementary subunits for AMPARs (Burgess et al., 1999; Bredt & Nicoll, 2003; Fukata et al., 2005; Nakagawa et al., 2005). Phosphorylation of TARPs promotes trafficking of hippocampal AMPARs and LTP whereas their dephosphorylation through calcineurin and phosphatase 1 is a prerequisite for LTD (Tomita et al., 2005; Nicoll et al. 2006).

1.8 The actin protein

Within eukaryotic cells, actin is the most common protein and thereto highly conserved amongst vertebrates (Dominguez & Holmes, 2011). More than any other protein, actin seems to be included in a variety of protein-protein interactions, interplaying with ions, actin-binding proteins and controlling nucleotide hydrolysis. It exists in two forms, the globular and monomeric actin (G-actin) and the filamentous, asymmetric and double-stranded helical actin (F-actin), between which it can switch through polymerization and disassembly (Cingolani & Goda, 2008; Dominguez & Holmes, 2011). Non-covalent and thus weak interactions between G-actin molecules facilitate prompt assembly and disassembly of F-actin (Figure 1.20(A)). Upon stable conditions, actin polymerizes at the barbed end, whereas disassembly of G-actin occurs at the pointed end. The interplay between assembly and disassembly at both ends can vary and ultimately leads to a specific net turnover of actin filaments in an activity-dependent manner. This allows the actin protein to be very dynamic and flexible. Thus, it is no wonder that it was found to be involved in numerous cell processes such as motility, cell division, cell morphogenesis, intracellular protein trafficking and transcription (Cingolani & Goda, 2008; Dominguez & Holmes, 2011). All of those processes are possible through assistance of a subset of actin-binding proteins (ABPs), which lead to dynamic alterations of actin filaments or interlink them in order to fabricate cytoskeletal proteins and networks (Figure 1.20(B, C)) (Dos Remedios et al., 2003; Pollard and Borisy, 2003; Revenu et

al., 2004). Ultimately, dynamic adaptions of actin towards synaptic activity result in morphological changes of spines.

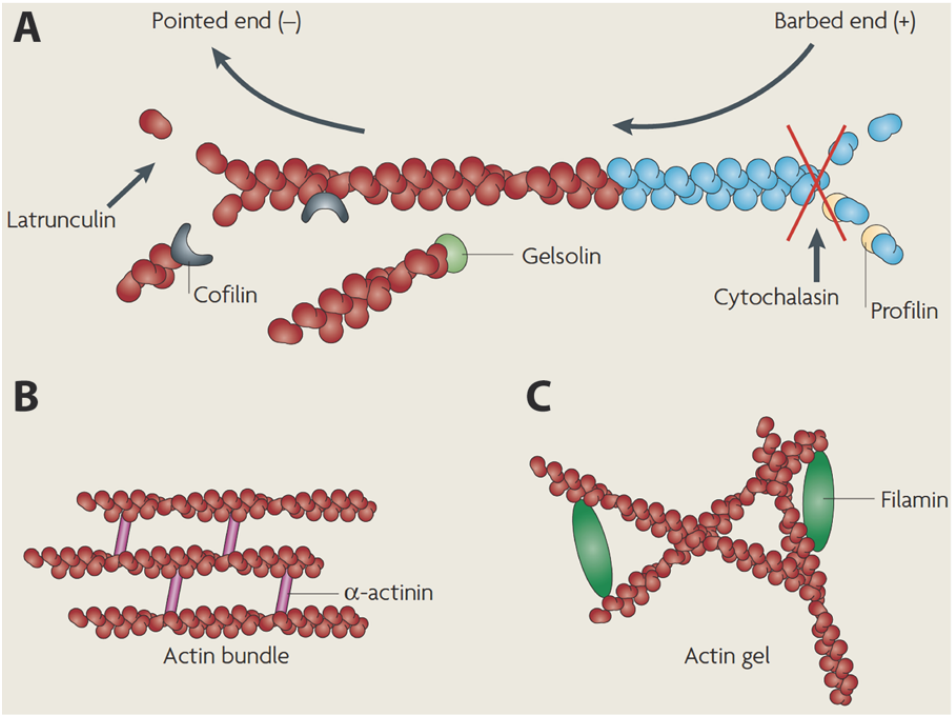

Figure 1.20: Actin protein and dynamics.
(A) Monomeric, globular actin (G-actin) is polymerized into filamentous actin (F-actin). ATP-bound G-actin (blue) spontaneously associates into weak interactions. The polarity of both ends differs due to constituent actin monomers that are ADP-bound on the other end (red). Polimerization mostly occurs at the barbed end over the pointed end. Latrunculin promotes F-actin depolymerization, whereas Cytochalasin prevents addition of G-actin. ABPs affect the structure of the actin cytoskeleton. F-actin depolymerization and severing are promoted by ADF/cofilin and gelsolin. Polymerization is prompted by profilin. Cross-linking proteins such as α-actinin or filamin **(B, C)** help arranging actin filaments into networks, bundles and gels. From Cingolani & Goda, 2008.

1.8.1 Actin dynamics and actin-dependent synaptic activity

Actin proteins compose the cell cytoskeleton, subserving as scaffold proteins and shaping the cell. As a highly modulative structure, actin provides guidance for neuron formation, extension and branching of neurites and synaptogenesis, whereas in mature neurons, actin is mainly located at pre- and postsynaptic sides and is especially concentrated at dendritic spines, which are protoplasmic, postsynaptic protrusions mediating excitatory neurotransmission (Landis et al., 1988; Hirokawa et al., 1989; Matus, 2000; Capani et al., 2001; Bloom et al., 2003; Yuste & Bonhoeffer, 2004).

Pre- and postsynaptic mechanisms are involved in shaping synaptic efficacy, including actin dynamics for structural plasticity such as LTP where spine size is increased and new synapses are formed (Matus, 2003; Dillon, 2005; Cingolani & Goda, 2008, Neves et al., 2008). The effectiveness of synaptic transmission is dynamically modulated by a use-dependent manner over

a wide range of timescales to enable the brain computational, learning and information storage features (Zucker & Regehr, 2002; Silberberg et al., 2005; Kim & Linden, 2007; Neves et al., 2008). Efficacy of synaptic transmission is accompanied by structural plasticity at pre- and postsynaptic sides as well (Cingolani & Goda, 2008). This accounts specifically for LTP, characterized as long-lasting enhancement, in terms of hours to days, of synaptic activity between neurons, resulting in increased spine size and newly formed synaptic connections (Cingolani & Goda, 2008). Moreover, the constant modulation and turnover of actin filaments contributes to junctional scaffolding and the trafficking of essential parts of the synaptic apparatus under involvement of a diverse set of helper proteins (Dillon et al., 2005).

Being the major structural component of synapses and spines, with high accumulations at presynaptic terminals and spine formations, F-actin shows high dynamical changes by being assembled and disassembled in order to help adapt and modulate the efficacy of synapses, facilitating neurotransmission (Cingolani & Goda 2008).

1.8.2 Actin-dependent presynaptic mechanisms

Axonal endings build presynaptic terminals with an enriched pool of synaptic vesicles containing neurotransmitters that are released upon activity into the synaptic cleft by a process called exocytosis. Distinguishing for this process is the fusion of vesicles with the presynaptic active zone membrane resulting in a synaptic vesicle cycle. Firstly, synaptic vesicles that are enriched in the center of axonal endings need to be transported to the active zone, a process defined as docking. This is followed by priming, a highly complex step, leading to assembling and maturation of the membrane fusion machinery. Through arrival of an action potential, Ca^{2+}-influx triggers final exocytosis. Finally, released neurotransmitters undergo a re-uptake for reuse in the presynapses through endocytosis (Ceccarelli et al., 1973; Heuser & Reese, 1973; Murthy & De Camilli, 2003; Sudhof, 2004). According to their state, vesicles are classified into three different groups. The readily-releasable pool, which consists of primed vesicles at the active zone, the reserve pool, representing vesicles within the core region of synaptic boutons and the recycle pool, characterized by vesicles that are not necessarily primed, yet play an active role in the synaptic vesicle cycle (Rizzoli & Betz, 2005; Fernández-Alfonso & Ryan, 2006). There is evidence of distinct vesicle pools being spatially separated (Schikorski & Stevens, 2001; Rizzoli & Betz, 2004). As actin is universally present in presynapses (Figure 1.21) it serves as scaffolding protein for the transfer of vesicles between the different pools according to their current need (Dillon & Goda, 2005).

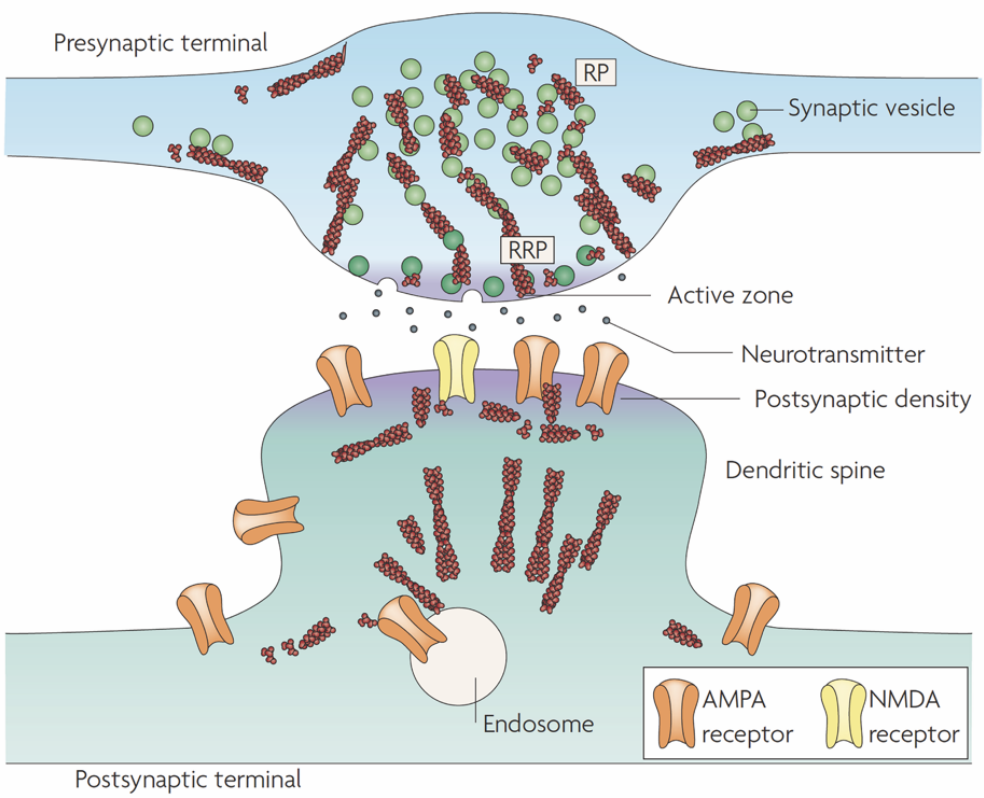

Figure 1.21: Synaptic actin.
Schematic view of an excitatory synapse. Actin is involved in synaptic vesicle transport at the presynaptic terminal. Dark green vesicles belong to the readily releasable pool (RRP) docked to the active zone. Here they undergo exocytosis to release neurotransmitter into the synaptic cleft. The reserve pool (RP) of vesicles (light green) is located at the center of the presynapse. Short actin filaments (red) interlink RP vesicles. Opposite the active zone lies the PSD inheriting AMPARs (orange) and NMDARs (yellow). Scaffolding proteins which set up the PSD are interlinked through a submembraneous actin network. Actin filaments constitute the whole spine, defining its shape and regulating receptor diffusion and their exo- and endocytic trafficking. From Cingolani & Goda, 2008.

At the core region of axonal boutons, actin is associated with synapsin, which is in turn connected to the vesicles, subserving as reserve pool. Incoming signals drive phosphorylation of synapsin, eventually breaking the meshwork of actin-synapsin-vesicles. This causes mobilization of the reserve-vesicles and restocks the readily-releasable pool which undergoes exocytosis upon neuronal activity. Depending on the synapse type and the active state of the synapse actin can act as promoter at the active zone by guiding arriving vesicles and facilitating docking. Simultaneously, its presence acts as physical and molecular barrier for the priming reaction to prevent unlabored fusion of vesicles. Beneath regulatory proteins, ABPs could influence the underlying mechanisms and the dual actin function to modulate neurotransmitter release. As consequence, intrusion of actin dynamics alters vesicle mobilization as shown by studies in primary hippocampal neuronal cultures, where F-actin depolymerization goes along with increased vesicle mobility (Jordan et al., 2005; Shtrahman et al., 2005). On the contrary, there were no effects upon actin depolymerization on vesicle transport in axonal boutons with fundamentally low vesicle movement (Gaffield et al., 2006; Gaffield & Betz, 2007). Further, experiments on neuromuscular junctions (NMJ) of

Drosophila melanogaster revealed F-actin to be essential for the recruitment of synaptic vesicles to the side of the readily releasable pool as F-actin depolymerization leads to declines of F-actin thus vesicle mobilization at boutons. This mechanism involves the protein *N*-ethylmaleimide-sensitive factor (NSF) which interplays with the SNARE-complex (Soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors complex) for vesicle fusion (Sudhof, 2004; Jahn & Scheller, 2006; Nunes et al., 2006). Additionally, vesicles are transported along F-actin through help of myosin-V, which eventually is captured at the active zone by syntaxin to couple to the SNARE-complex (Watanabe et al., 2005). This is promoted by cellular calcium-influx triggered upon neuronal activity. As a result, according to the synapse type actin depolymerization, it can constrict neurotransmitter release by hindering rehabilitation of the released vesicle pool (Cole et al., 2000; Sakaba & Neher, 2003) or it does not have a major effect on vesicle mobilization as it is the case for small synapses at hippocampal neurons (Morales et al., 2000; Schnell & Nicoli, 2001; Tokuoka & Goda, 2006).

Endocytosis of the vesicle cycle is facilitated by actin and the minus-end-directed protein myosin VI (Bloom et al., 2003; Shupliakov et al., 2002; Yano et al., 2006). During synaptic vesicle recycling, actin is enriched around and prolongs from the active zone of endocytosis (Bloom et al., 2003; Shupliakov et al., 2002; Sankaranarayanan et al., 2003). Recycled vesicles are then transported to the functional pool of neighboring boutons where they undergo repeated exocytosis (Darcy et al., 2006). Naturally, this mechanism requires constant actin-turnover. Shared vesicle pools between synapses are presumably important components of synaptic plasticity, allowing for adaptation to incoming signals at individual synapses to facilitate fast enlargement or downscaling of vesicle amounts (Cingolani & Goda, 2008). The brain derived neurotropic factor (BDNF) is involved in activity-dependent synaptic plasticity (McAllister et al., 1999; Turrigiano, 2007) and notably responsible for removal of actin from available synapses for the usage in newly formed synapses, which is accompanied by new vesicle clusters (Bamji et al., 2006). Actin-dependent activation and maturation of silent boutons as well as developing of new boutons were revealed by several investigations (Shen et al., 2006; Yao et al., 2006). Vital for this transformation is the repetitive incoming of signals and activation of synapses (Yao et al., 2006). Hereby, actin-dependent un-silencing of boutons is a rapidly happening mechanism with actin polymerization serving as impulse for signal transduction and causing restructuring of the presynaptic matrix for induction of efficient release and recycling of preexisting vesicles (Zhang & Benson, 2001; Shen et al., 2006). Lying ahead of activity-dependent actin polymerization is the upstream signaling of BDNF and Cdc42 (cell division control protein 42) (Shen et al., 2006).

1.8.3 Actin and postsynaptic mechanisms

Post-synapses accommodate the PSD at distal tips of spine heads where neurotransmitter receptors are massed. The PSD is located opposite the active zone of presynapses and determines synaptic strength by receptor amount and diversity (Sheng & Hoogenraad, 2007). Postsynaptic strength is modified by dynamic change of surface receptors at the PSD caused by lateral diffusion of receptors. In this way, a constant change of receptor quantity or receptor types paves the way for progressive adaptations towards activity (Choquet & Triller, 2003; Groc & Choquet, 2006; Shepherd & Huganir, 2007). Actin is highly concentrated at postsynaptic sides (Figure 1.22) and encounters a multiplex role. Interacting with a bundle of scaffolding proteins, it is responsible for the removal and addition of receptors via lateral diffusion which synaptic strength as well as anchoring of glutamatergic AMPA and NMDA receptors at excitatory neurons (Allison et al., 1998; Kuriu et al., 2006). Both receptors show differential sensitivity to depolymerization of F-actin going along with distinct ranges of receptor resources after activity at the postsynaptic side (Malinow & Malenka, 2002; Morishita et al., 2005; Shepherd & Huganir, 2007). Actin occurrence in miscellaneous forms at the post-synapse in regard of its arrangements and stability states in means of its turnover rate (Halpain et al., 1998; Star et al., 2002; Rostaing et al., 2006; Honkura et al., 2008). Sub-plasmalemmal actin as anchoring regulator of receptors, might plastically remodel the spine head size, whereas stable and longitudinal filaments within the core of postsynaptic spines are providing stability to spines and act as guides for trafficking of receptors upon exo- and endocytosis (Cingolani & Goda, 2008). Studies on hippocampal neuron cultures revealed internalization and lateral diffusion of AMPARs when actin is depolymerized, evoking a situation alike the glutamate elicited NMDAR-dependent internalization of AMPARs (Zhou et al., 2001). Coherently, when stabilization of F-actin filaments is induced, AMPARs are prevented from internalization (Zhou et al., 2001). Simultaneously, stable actin filaments at the core region of synapses are not depolymerized during this event as they are needed for the myosin VI involving endocytosis mechanism (Osterweil et al., 2005). Interestingly, investigations on myosin VI knockout mice show reduced dendritic spine numbers in hippocampal neurons accompanied by morphological abnormalities resulting in short spine lengths (Osterweil et al., 2005). These findings point out the importance of myosin VI in AMPAR trafficking along F-actin and its role in transforming spine morphology. Spines morphologically differ in shape and size (see Chapter 1.5) (Hering & Sheng, 2001). The head size of spines is correlated with the PSD area and the number of glutamate receptors at the synaptic membrane surface which means in turn that spine head size and synaptic efficacy are positively correlated with each other (Cingolani & Goda, 2008). Although microfilaments of actin protrude from the PSD, its enrichment at the PSD is relatively low (Cingolani & Goda, 2008). Experiments with immune-fluorescently labeled actin in alive neurons

found the protein to be remarkably dynamic with an exchange rate of approximately 85% within two minutes (Star et al., 2002; Honkura et al., 2008). Furthermore, it was shown, that long term plasticity conducts rapid and long-lasting re-organization of actin in spines. In case of LTP, spine volume rises and the ratio of F-actin to G-actin shifts in favor of the first, while in case of long-term depression (LTD) spines shrink and the ratio shift in favor to G-actin (Fukazawa et al., 2003; Okamoto et al., 2004; Lin et al., 2005). Importantly, this affects both, structural as well as functional plasticity (Figure 1.22), since actin provides an anchor for signaling and structural proteins such as neurotransmitter receptors, CaMKII, and ARC (Allison et al., 1998; Kim & Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003; Okamoto et al., 2004; Osterweil et al., 2005, Okamoto et al., 2007; Huang et al., 2007). At this point, it is very important to note that structural changes of actin in dendritic spines are not necessarily sufficient to trigger functional plasticity, since those signaling pathways are separable from each other. This was specially shown in studies of LTD indicating an uncoupling of synaptic depression and spine shrinkage (Zhou et al., 2004; Morishita et al., 2005; Wang et al., 2007).

By now, it is known that NMDA receptor activity is essential to induce rearrangements of actin (Fukazawa et al., 2003; Zhou et al., 2004). Notably, investigations at cerebral Purkinje cell synapses uncovered that reduction of AMPARs independent of NMDA receptor signaling is not accompanied by spine shrinkage (Sdrulla & Linden, 2007). Conversely, redemption of spines did not show a decline in synaptic currents (Sdrulla & Linden, 2007). Supplementary, AMPAR trafficking in hippocampal neurons to extrasynaptic areas does not go along with morphological changes of spines (Wang et al., 2007). In summary, spine shape changes are not necessarily associated with decreases of AMPAR, but do depend on NMDA when it comes to LTD. Also, in case of LTP, structural and functional mechanisms can be uncoupled. For example, before insertion of the AMPAR-subunit GluR1 is taking place at the PSD, spine head size enlargement is forgoing by approximately 4 minutes (Kopec et al., 2006). Moreover, a rise of the scaffolding postsynaptic density protein 95 (PSD95) can enhance the GluR1 numbers at the spine surface with increasing synaptic strength, without changing spine size (El-Husseini et al., 2001; Ehrlich & Malinov, 2004). This is especially prevented when transfer of GluR1 carboxy-terminals into the PSD are restrained (Kopec et al., 2006). Though, by inducing LTP, dendritic spines grow in size but without showing any synaptic strength (Ehrlich & Malinov, 2004; Kopec et al., 2007). Taken together, all studies propose that increase in spine size involves an NMDA-dependent mechanism upon activation during LTP as well as ADF (actin depolarizing factor)/cofilin and actin reorganization (Fukazawa et al., 2003).

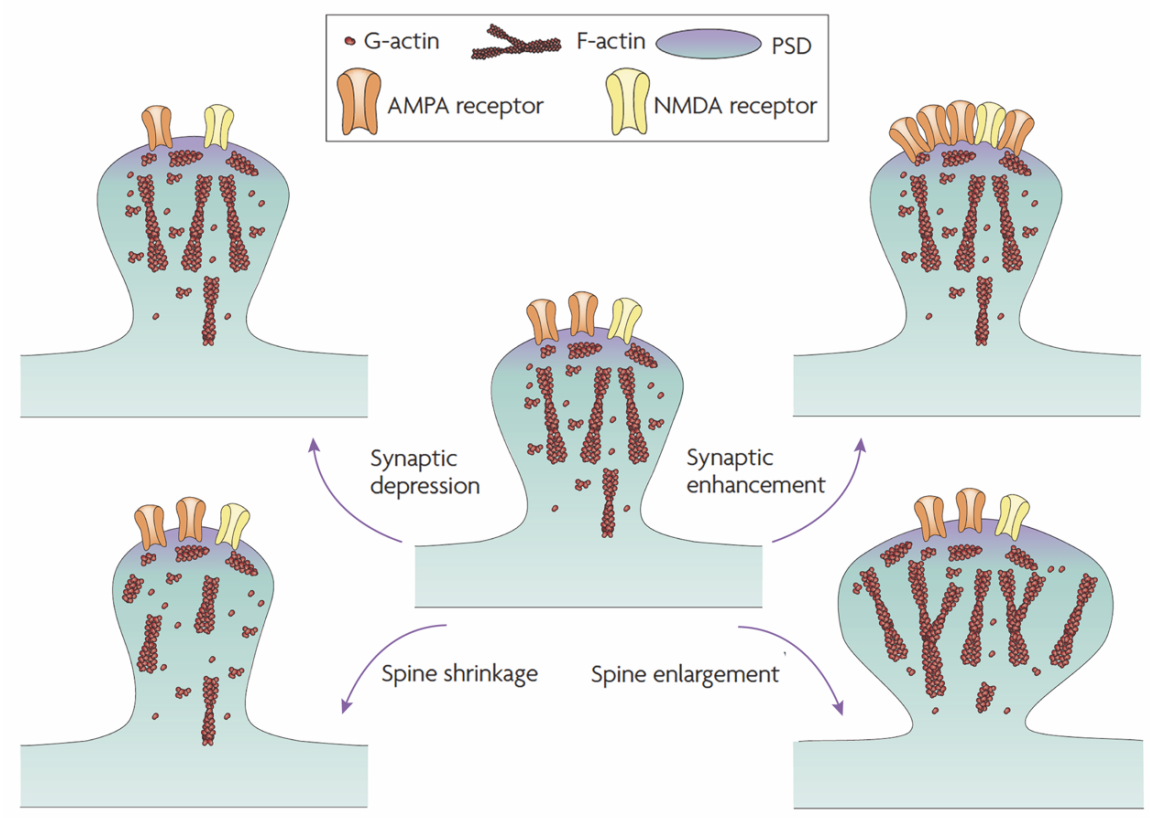

Figure 1.22: Functional role of actin in spine plasticity.
Alterations in actin dynamics modulate and affect the efficacy of synaptic transmission and synaptic plasticity. Synaptic depression is characterized by the reduction of AMPARs at the membrane surface without spine shrinkage, whereas spine shrinkage is not necessarily associated with changes synaptic currents. Synaptic potentiation is defined by new insertion of AMPARs occurring independently of spine size changes. Contrary spine enlargement can occur without potentiation of synaptic transmission. Modified from Cingolani & Goda, 2008.

1.8.4 Actin-binding proteins

Mass spectrometry analysis of postsynaptic spines revealed big resources of actin-binding and actin-crosslinking proteins within the PSD area, including CaMKIIβ, cortactin, drebrin A, and neurabin I (Cheng et al., 2006). A jointly effect during downregulation of these individual proteins is the reduction of maturation and formation of spines which is linked to memory formation and synaptic plasticity (Hering & Sheng, 2003; Terry-Lorenzo et al., 2005; Okamoto et al., 2007; Wu et al., 2008; Ivanov et al., 2009; van Woerden et al., 2009; Kojima et al., 2010).

Important for the nucleation of new actin filaments is the Arp2/3 complex, consisting of seven conserved subunits including the actin-related proteins Arp2 and Arp3 (Hotulainen & Lappalainen, 2006). The Arp2/3 complex was found to be predominantly present in spine heads necessary for their formation by jointing the sides of existing actin filaments thus promoting net formation and actin branching (Goley & Welch, 2006; Rácz & Weinberg, 2008; Wegner et al., 2008; Hotulainen et al., 2009). Not only its depletion, but also the knockdown of its activators, namely

cortactin, ABI2 (abI interactor 2), WAVE-1 (WASP-family verprolin homology protein-1), N-WASP (neural Wiskott-Aldrich syndrome protein), and ABP1 (actin-binding protein 1) alter the number and morphology of hippocampal spines and have a negative impact on hippocampal-dependent learning and memory (Hering & Sheng, 2003; Kim et al., 2006; Soderling et al., 2007; Haeckel et al., 2008; Wegener et al., 2008).

Profilins are ubiquitously present G-actin-binding proteins. Although profilin-I is also present, specially, profilin-II is the main isoform within the brain (Witke et al., 2001) and associated with postsynaptic spine stabilization during fear-conditioning and synaptic plasticity (Lamprecht et al., 2006). Profilin-II knockout mice show hyperactivity, evoked through presynaptic cytomatrix and polymerization impairments, resulting in enhanced neurotransmitter release which in turn leads to enhanced excitation and hyperstimulation of striatal neurons (Ackermann & Matus, 2003; Lamprecht et al., 2006; Boyl et al., 2007). Via changing actin nucleotides from adenosine diphosphate (ADP) to adenosine triphosphate (ATP) profilins promote the barbed-end polymerization of actin molecules (Pollard et al., 2000). Interestingly, a resting state of hippocampal neurons houses low amounts of profilin-II in spines, whereas numbers increase during chemical or electrical stimulation of neurons (Ackermann & Matus, 2003; Neuhoff et al., 2005). Fear-conditioning experiments in rats revealed a re-localization of actin filaments from the dendritic shaft into spines, pointing towards an activity-dependent actin modulation (Ackermann & Matus, 2003; Lamprecht et al., 2006).

Another player in actin modulation is the capping protein that is present throughout the whole spine (Korobova & Svitkina, 2010) and binds to the barbed end of actin filaments to keep the protein limited in size. In this way the actin monomer pool is kept available for fast re-organization and polymerization of actin filaments limited to areas where it is required (Pollard & Borisy, 2003). Without the presence of capping protein actin filaments grow long and form filopodia-like structures instead of lamellipodia-like protrusions (Mejillano et al., 2004). This also accounts for Eps8 (epidermal growth factor receptor pathway substrate 8) protein which turned out to have actin-capping functions as well (Menna et al., 2009). On one hand, depolarizing factors like ADF/cofilins help inducing the breakdown of actin molecules from the pointed end to sustain the G-actin pool (Kiuchi et al., 2007; Hotulainen et al., 2009). Specifically in neurons cofilin-I is required for actin-turnover and correct morphological formation of spines (Hotulainen et al., 2009). On the other hand, LIM kinase 1 (LIMK-1) constrains ADF/cofilin function which drives diversifications in spine morphology and synaptic function (Meng et al., 2002). Experiments with LIMK-1 knockout mice revealed structural spine abnormalities with enhanced LTP going along with behavioral alterations regarding spatial memory and fear responses (Meng et al., 2002).

Elongation of actin filaments is induced by formin DRF3/mDia2 (human Diaphanous-related formin 3/mouse Diaphanous 2) polymerizing straight actin filaments and resulting in

malformation of spine necks and reduction of filopodia-like spines when absent (Hotulainen et al., 2009; Paul & Pollard, 2009). Other than that, Ena/VASP (enabled/vasodilator-stimulated phosphoprotein) proteins anti-cap polymerizing tails of branched actin-filaments promoting elongation and were found to be important for filopodia formation and neuritogenesis in the developing cortex (Dent et al., 2007; Kwiatkowski et al., 2007).

1.8.5 Actin signaling in dendritic spines

In dendritic spine structures, actin signaling is controlled by excitatory synaptic NMDA receptors as well as AMPA glutamate-receptors (Fischer et al., 2000). NMDA receptors influence actin-dynamics in two ways. One is triggered by Ca^{2+}-influx, acting on the activity of actin-binding proteins like CaMKIIβ and gelsolin, whereas the other way is by directly binding actin-binding proteins, i.e., CaMKII, α-actinin and myosin regulatory light-chain (Wyszynski et al., 1997; Lisman et al., 2002; Bajaj et al., 2009; Nag et al., 2009; Raveendran et al., 2009). In addition, receptor tyrosine kinases (TRK) like ephrin family members, BDNF and adhesion molecules are essential for actin regulation in spines (Schubert & Dotti, 2007; Menna et al., 2009; Yoshihara et al., 2009). GTPases constitute major regulators in spine morphogenesis including RhoA, which leads to actin filament stabilization resulting through cofilin inactivation and as outcome leading to LTP. On the other hand, the GTPases Rac and Cdc42 promote spine head enlargement by activating Arp2/3 complex-induced nucleation by blockage of actin depolymerization through cofilin and filopodia formation is driven by the GTPase Rif (Rho in filopodia) (Irie & Yamaguchi, 2002; Wegner et al., 2008; Hotulainen et al., 2009; Rex et al., 2009). Ras (rat sarcoma virus) family GTPases, their downstream signaling and GTPase activators and inhibitors were shown to have elementary influence on spine morphology and neuronal function (Kennedy et al., 2005). The postsynaptic Ras signaling inhibitor SynGAP (synaptic GTPase activating protein) is strongly deposited in the PSD area and its depletion leads to spine head enlargement towards mushroom spines (Vazquez et al., 2004). Distinct spine phenotypes can be explained by crosstalk of the various signaling pathways of GTPases. More interestingly, mutations in GTPase activators and inhibitors were linked to mental retardation (Newey et al., 2005).

During developmental stages synaptic contact formation including signal transmission between motile filopodia and axons, can lead to transformation into more stable forms of spines (Craig et al., 2006; Arikkath & Reichardt, 2008; Yoshihara et al., 2009). Formation of filopodia emerges from preexisting small lamellipodia or branched actin accumulations at the dendritic shaft (Andersen et al., 2005; Korobova & Svitkina, 2010) and is initialized by presynaptic glutamate-release (Tashiro et al., 2003; Andersen et al., 2005) which triggers spine elongation by Ena/VASP-

induced anti-capping of actin filaments. Also, several findings propose DRF3/mDia2 formin (Hotulainen et al., 2009) and microtubule-associated actin filament nucleators or actin-microtubule crosslinking factors being important for proper filopodia growth (Tada et al., 2007; Korobova & Svitkina, 2010). Once filopodia has made an axonal connection, its motility reduces and stabilization of the spine advances through pre- and postsynaptic segments (Craig et al., 2006; Arikkath & Reichardt, 2008; Yoshihara et al., 2009). For further development spine head growth is initiated by accurate coordination of the Arp2/3 complex (Grove et al., 2004; Kim et al., 2006; Soderling et al., 2007; Wegner et al., 2008; Hotulainen et al., 2009), possible by a switch from DRF3/mDia2 actin-polymerization towards an Arp2/3 complex-based actin polymerization (Beli et al., 2008; Hotulainen et al., 2009). In addition, intrusion of microtubule into spine heads elicits head size and protrusion, most likely via distinct signaling cascades triggered by the microtubule plus-end tracking protein EB3 (end-binding protein 3) and its binding partner p140Cap (Cas-associated protein) (Jaworski et al., 2009), finally guiding branched actin-networks into more stable forms up to development into mushroom-like spines (Hering & Sheng, 2003). In order to form a spine head, three-dimensional organization of actin filaments is required. Activity and inhibition of myosin-II generates contractility and allows spine heads to change in shape (Zhang et al., 2005; Ryu et al., 2006). Further actin-crosslinking proteins including CaMKIIβ, neurabin I, and debrin A participate in spine head structuring (Terry-Lorenzo et al., 2005; Okamoto et al., 2007; Ivanov et al., 2009). Synaptic plasticity such as happening during LTP requires fast and persistent re-organization of the actin cytoskeleton (Cingolani & Goda, 2008). Interestingly, actin polymerization is needed for LTP, as thereafter following spine head enlargement, facilitated by Arp2/3 complex and cofilin, actin filaments are stabilized in spine heads (Ramachandran & Frey, 2009). This process is driven by inactivation of cofilin and involvement of the actin crosslinking protein CaMKII (Fukazawa et al., 2003; Chen et al., 2007; Fedulov et al., 2007; Okamoto et al., 2007). As part of spine stabilization and the contribution of synaptic strength, morphological adjustments of spine necks are controlled by myosin-II to couple the shaft with the head (Bloodgood & Sabatini, 2005). Studies show similar spine-alike protrusions in central nervous system neurons of *Drosophila melanogaster*. Further investigations and the dissection of genetic actin cytoskeleton in the mushroom body system of flies showed that re-organization of the actin cytoskeleton involving Rac leads to memory loss (Shuai et al., 2010).

It has become clear that actin rearrangement and synaptic function are tightly joined together, by showing correlations between actin dynamics, spine morphology and synaptic plasticity. Notably, actin polymerization in spines is essential, but not sufficient by itself for functional LTP (Okamoto et al., 2004; Hotulainen & Hoogenraad, 2010).

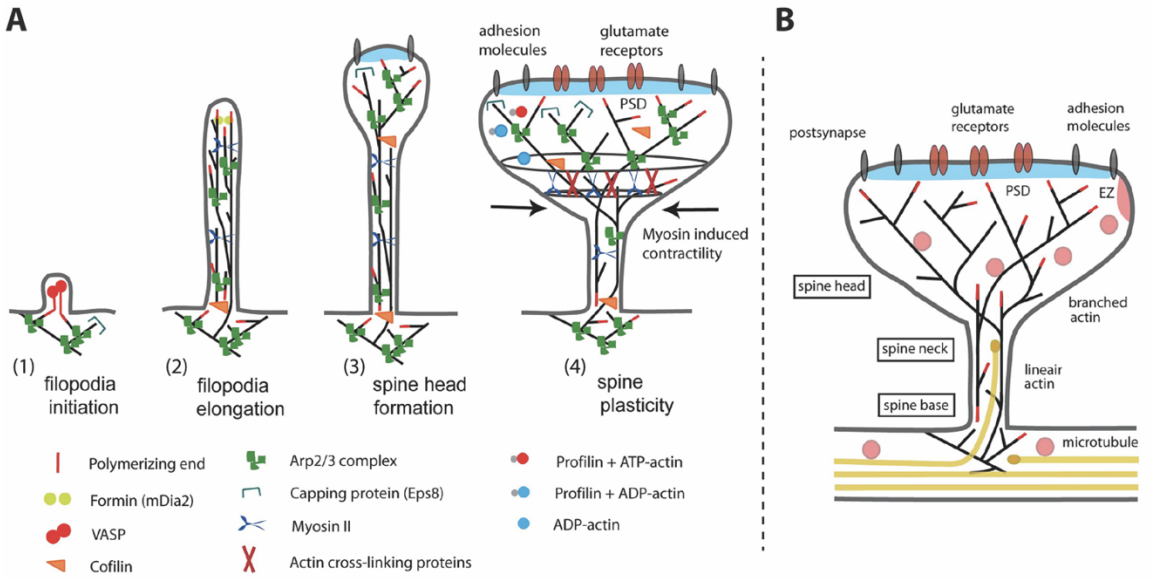

Figure 1.23: Actin organization and actin regulatory mechanisms in spines.
(A) 1-4 shows distinct steps of spine development. (1) Filopodia initiation and elongation. This process can be blocked by Eps8 through its capping activity. Ena/VASP proteins could induce branching of actin filaments by anti-capping of barbed ends. (2) At filopodia tips mDia2 promotes actin filament polymerization. (3) Actin branching occurs at the filopodium tip and a spine head starts to form. Actin assembly is increased and Arp2/3-nucleated actin network promotes enlargement of the spine head. ADF/cofilins control the length of actin filaments preventing them from abnormal protrusions. (4) Mature spines remain dynamic induced by small Arp2/3 complexes on the surface of the spine head. The shape of the spine head is further changed by myosin-II-dependent contractility and the cross-linking of actin filaments. During prolonged LTP activity cofilin is reduced, whereas the activity of other ABPs such as Arp2/3, profilin, actin crosslinking proteins, myosin-II, and actin filament capping proteins is enhanced. **(B)** Scheme of a mushroom spine. Blue: PSD; gray: adhesion molecules; red: glutamate receptors; black: actin; yellow: microtubule; pink: recycling endosomes. The endoplasmatic reticulum (ER) is positioned at the extrasynaptic region. Mature spines show a network of straight and branched actin filaments. At the spine base the actin-network is spread and gets constricted in the spine neck. At the neck-head junction the network starts to branch and finally stays highly branched at the spine head. Red lines indicate the actin polymerizing barbed ends. Stabilizing microtubules are mainly found in the spine shaft. A proportion of dynamic microtubule emanate from the shaft and curve towards the spine before they enter the spine structure. Plus-ends of microtubules are represented in yellow. Modified from Hotulainen & Hoogenraad, 2010.

It is evident that proper spine formation requires accurate composition and dynamic changes in the actin cytoskeleton. By now, many interrelations are known between neuropsychiatric disorders and alterations in spine numbers or impaired morphology of spines (Blanpied & Ehlers, 2004). For example, autism spectrum disorder is associated with mutations in SH3 (multiple ankyrin repeat domains 3), a synaptic scaffolding protein and neuroligin-3 and 4 both adhesion molecules (Südhof, 2008) and also various memory disorders are linked to dysfunctional actin cytoskeletal reorganizations (Newey et al., 2005). The schizophrenia risk factor DISC1 (disrupted in schizophrenia 1) regulates dendritic spine morphology via Rac1 (Hayashi-Takagi et al., 2010) mutations in the cofilin kinase PAK3 (RAC1 activated kinase 3) gene lead to X-linked mental retardation (Allen et al., 1998) and decreased levels of PAK3 are associated with Alzheimer's diseases (Kreis & Barnier, 2009). Fittingly, murine studies showed cofilin pathology and memory impairment in mice with PAK3 inhibition (Zhao et al., 2006).

Various genetic studies identified a subset of mutations impacting on spine actin regulations and as consequences motor synapse dysfunctions. Variations in actin dynamics ultimately caused by mutations of actin-binding proteins or signaling proteins can lead to neurological disorders, thus having an impact on cognitive and behavioral function (Bardoni & Mandel, 2002; Ramakers, 2002; Blanpied & Ehlers, 2004; Newey et al., 2005; Zhao et al., 2006).

1.9 DRR1 (down regulated in renal cell carcinoma) protein

The *DRR1* (downregulated in renal cell carcinoma) gene is also termed *TU3A* (Tohoku University cDNA clone A on chromosome 3) and *Fam107A* (*Family* with sequence similarity 107, member A). DRR1 is a multifunctional protein and highly conserved amongst vertebrates (Schmidt et al., 2011) showing a 90% homology among humans and mice (Masana et al., 2014). The *Drr1* gene was first isolated and characterized by Yamato and colleagues (Yamato et al., 1999) after previously identifying a 700 kb allelic region at the short arm of chromosome 3 (3p14.3→14.2). A region with frequent genetic aberrations associated with diverse types of human cancers appearing in kidney, lung, breast, uterine cervix and ovary as well as head and neck (Kholodnyuk et al., 2006). The gene *TU3A* or *DRR1*, encoding a 144 amino acid sized protein, was found and isolated from that region (see Figure 1.23). It consists of four exons and three introns. Further, northern analysis revealed in 1999, that the gene is expressed in most tissues, except peripheral blood cells (Yamato et al., 1999). *In vitro* analysis performed in renal cell carcinoma cell lines, outlined the loss of expression in most of the lines, suggesting the gene playing a role in renal cell carcinogenesis, possibly by epigenetic modifications or transcriptional regulations (Yamato et al., 1999). Additional work by Wang and colleagues (Wang et al., 2000) identified solid deletion of DNA sequences in various types of human tumors refining its position to the chromosomal region 3p21.1 (Wang et al., 2000). In search of tumor suppressor genes within the chromosomal region 3p14. – 3p21.1 these researchers cloned a gene, naming it downregulated in renal cell carcinoma (*DRR1*) (Wang et al., 2000). Their work revealed the gene to occupy 10 kb and the final transcript to be 3.5 kb in size with an open reading frame. The mature protein shows nuclear localization, a coiled coil domain and total molecular weight of 18 kDa.

DRR1 was found to be expressed in many normal tissues, as heart, brain, lung, liver, kidney, pancreas and skeletal muscle, with the strongest expression profile in the brain (Wang et al., 2000, Kholodnyuk et al., 2006). In addition, it was found to show loss of expression in all eight analyzed renal cancer cell lines and other tested cancer cell lines for ovarian cancer, cervical cancer, gastric and non-small-cell lung cancer lines. Mutational analysis of the lines detected base substitutions and gain of function experiments by transfecting the *DRR1* gene into *DRR1*-negative

cell lines showed retardation of tumoral cell growth and apoptosis (Wang et al., 2000, Liu et al., 2009). Since DRR1 is located mainly in the nucleus it was hypothesized being involved in regulating gene transcription due to its coiled coil domain or interacting with other proteins regulating signal transduction (Wang et al., 2000). *DRR1* is a conserved gene and was found to operate as tumor antagonizing gene in human chromosome 3-mouse fibrosarcoma 9 microcell hybrid (MCH) cell lines (Kholodnyuk et al., 2006). Whereas in 2006 van den Boom and colleagues found (van den Boom et al., 2006) reduced *DRR1* in glioblastomas, leading to tumor progression. Later, a study demonstrated epigenetic modification of human *DRR1* through promoter hypermethylation, being the cause for the onset of cancer (Awakura et al., 2008). Overall, those studies didn't elucidate the function of the gene product. To elucidate the biological role of the DRR1 protein, gene function studies were performed in the model organism *Xenopus laevis* (Zhao et al., 2007). *xDRR1* shares a high sequence similarity with 70% homology with human and 66% with murine *Drr1* gene. Primary analysis showed *xDRR1* localizing in the nuclei of transfected cells, playing an important role in embryo development, mainly present in heart and brain, and cell growth regulation. Later was demonstrated by introducing the protein to cancer cell lines lacking DRR1 and suppressing cell growth (Zhao et al., 2007). Following studies showed loss of *DRR1* mRNA in 6 of 7 human cancer cell lines and lack of DRR1 protein when immunostaining specimens of human non-small cell lung cancer (Liu et al., 2009). Murine *in vitro* and *in vivo* studies revealed reduction of cell growth and apoptosis when introducing DRR1 into DRR1-negative-expressing A549 cell lines and injecting *Drr1* cDNA (complementary deoxyribonucleic acid) into mice with A549 xenograft tumors (Liu et al., 2009). Through development of DRR1-specific polyclonal antibodies, examination of the expression pattern was possible for the first time in a large scale (Asano et al., 2010). Expanding research evaluated the occurrence of DRR1 protein in carcinogenesis and embryogenesis using rats as model organism. They found the protein to be primarily expressed in the nervous system, peripheral as well as central nervous system, during embryogenesis. More precise analysis of primary cultured cortical neurons showed DRR1 appearing especially in neurites and axons (Asano et al., 2010) whereas RNAi-induced suppression of DRR1, diminished the survival of those cultured neurons. Transgenic mice, serving as glioblastoma models, revealed downregulated expression of DRR1 during neuroblastoma carcinogenesis (Asano et al., 2010). Up taking studies aimed to figure out underlying function of DRR1. That way researchers found DRR1 being highly expressed in invasive gliomas, while absent in normal glial cells, by associating with actin- and microtubular cytoskeletons, which makes them co-responsible for focal adhesion (FA), disassembly and cell invasion (Le et al., 2010) and pointing DRR1 out as cytoskeletal crosslinker and important for cell movement. Following studies revealed a new mechanism of brain cancer invasion involving DRR1, which is highly expressed in glioblastomas and recruits AKT (protein kinase B) to FA. A major driver for glioblastoma invasion is the epidermal growth receptor

(EGFR)/phosphatidylinositol 3-kinase (PI3K) AKT pathway (Fan & Weiss, 2010). When AKT is activated through phosphorylation, it translocates to the cell nucleus initiating downstream signaling cascades that impact on cell survival, proliferation, invasion and metabolism (Song et al., 2005; Manning & Cantley, 2007; Chin & Toker, 2009). DRR1 induces the relocation of AKT which leads to its activation and invasion (Dudley et al., 2014). By reducing the expression of DRR1 in glioblastomas utilizing oligonucleotide therapeutics in mouse models *in vivo*, researchers found reduced glioblastoma invasion (Dudley et al., 2014).

1.9.1 DRR1 and Stress

A decade ago, a microarray study identified the murine orthologue *Drr1* showing strong basal mRNA expression in brain regions such as the cerebellum and limbic areas like the hippocampal CA3-region and the septum (Liebl et al., 2009; Schmidt et al., 2011). Further, they showed *Drr1* strongly up-regulated upon maternal separation as stress factor within the neonatal mouse brain (Liebl et al., 2009; Schmidt et al., 2011). Interestingly, precedent gene-expression profiling analysis discovered that transcription of DRR1 depends on GR dimerization (Frijters et al., 2010). Continuing studies from Schmidt and colleagues in 2011 showed a strong increase of *Drr1* mRNA in stress relevant regions, specifically the PVN and the hippocampal CA3 region after 24 h of maternal separation (Schmidt et al., 2011). Food deprivation, a stress stimulus similar in intensity to maternal separation in neonatal mice, revealed the same outcome in adult mice. Further, Schmidt and colleagues showed strong basal *Drr1* mRNA and protein expression in the septum, the neocortex, the hippocampal CA3 region, and the cerebellum. To verify a glucocorticoid-dependent, thus stress-related increase of DRR1 expression, they injected the artificial and selective glucocorticoid receptor agonist dexamethasone (DEX) subcutaneously (s.c.) into mice. In comparison to a vehicle-treated control group, they found increased levels of *Drr1* mRNA and protein within the hippocampal CA3-region and the PVN 8 h post-injection. Furthermore, administration of GR antagonist RU487 (mifepristone) abolished the expression of DRR1 in mice after maternal separation or food deprivation in the PVN. In addition, they identified glucocorticoid responsive elements (GREs) in conserved regulatory regions (promoter, intron 1 and intron 3' untranslated region (UTR) of DRR1, showing binding to GR *in vitro*. By this, they could show that DRR1 is a glucocorticoid-regulated gene. Apart from that, extensive molecular investigations on DRR1, demonstrated its interaction with β-actin, colocalizing with filamentous Actin (F-actin) and enriching those structures upon enhanced DRR1 expression. More specifically, DRR1 governs actin dynamics through polymerization of globular actin to F-actin. Furthermore, it has stabilizing effects on actin filaments via actin capping as well as bundling

capabilities (Figure 1.23(B)) (Schmidt et al., 2011; Kretzschmar et al., 2018). The analysis of DRR1 truncation and point mutants indicated the both termini (C- and N-terminus) as actin binding domains. The middle region, containing the coiled coil motif, was determined to promote homodimerization (Figure 1.23(B)) (Kretzschmar et al., 2018). In a next step the researchers first analyzed neuron-specific cell localization of the DRR1 protein in rat primary hippocampal and cerebral cell cultures, revealing a punctuate pattern along neurites, especially localizing to outgrowing protrusions, colocalizing with actin and partially with synapsin, a synaptic marker. Electron microscopy identified DRR1 to mainly localize presynaptic and to a smaller extend postsynaptic. Since actin is crucial for neurite outgrowth, and DRR1 was shown to interact with actin, the group further assessed the effects of elevated DRR1 levels on cell morphology via ectopic expression of DRR1 in a neuroblastoma cell line Neuro2a (N2a), susceptible to changes of actin dynamics (Schmidt et al., 2011). Induced differentiation of the cells combined with increased DRR1 levels led to reductions in neurite outgrowth, and higher accumulations of F-actin. When they transfected primary hippocampal neurons ahead of cultivation with DRR1 they similarly showed impaired outgrowth of protrusions. Additionally, they could not show defective effects of increased DRR1 levels on neurites already developed (Schmidt et al., 2011). This led to the argumentation that DRR1 manipulates actin-dependent organization of the cytoskeleton. To put the effects of DRR1 level changes into context with the overall stress response, they enhanced endogenous DRR1 levels specifically in the hippocampus of adult mice by region-specific injection of an adeno-associated viral vector carrying the *Drr1* gene. Subsequent morphological analysis showed decreased spine density on apical dendrites of CA1 and CA3 pyramidal neurons compared to control mice. Previous findings reported an increased probability of neurotransmitter release at excitatory synapses as consequence to depolymerization of presynaptic actin (Morales et al., 2000). Since DRR1 was found to promote actin polymerization and is mainly present in presynaptic structures, Schmidt and colleagues hypothesized increases in DRR1 protein to have opposing effects on neurotransmitter release. Electrophysiological analysis in acute brain slices of mice, overexpressing DRR1, revealed a reduced release probability of synaptic neurotransmitters, shown by field excitatory postsynaptic potential (fEPSP) recordings. Precisely, fEPSP amplitudes were smaller in DRR1 overexpressing specimens compared to controls resulting in increased paired-pulse facilitation at CA3-CA1 synapses. At the same time, the magnitude of LTP was decreased in mice with higher levels of DRR1 compared to controls. Finally, the study was brought to a close, by performing hippocampus-dependent learning tasks with animals overexpressing DRR1 in the hippocampal region, eventually showing that DRR1 improves cognitive flexibility (Schmidt et al. 2011).

Another region showing high basal DRR1 levels within the murine brain is the lateral septum, constituting a brain region highly involved in the stress response (Singewald et al., 2011)

emotional processing (Calfa et al., 2006/2007) and social behavior (Litvin et al., 2011). Most exciting is the fact that the lateral septum receives major inputs from the hippocampus (Risold & Swanson, 1996) the prefrontal and entorhinal cortex, both regions important for cognitive function as well as the hypothalamus and amygdala which process affections (Sheehan et al., 2004). Thereon, Masana and colleagues elucidated the function of DRR1 in forming complex behavior. Upon acute stress and GR activation *Drr1* mRNA expression is elevated in the septum mainly in neurons, but also astrocytes (Masana et al., 2014). By region-specific adenovirus-associated mediation of DRR1 they overexpressed the protein within the septum to mimic stress-induced upregulation of *Drr1* mRNA and protein. Various behavioral experiments performed with mice overexpressing the protein specifically in the septum, pointed higher sociability compared to control animals (Masana et al., 2014). Simultaneously, nor cognitive, anxiety-like or anhedonic behavior were altered in animals transfected with adeno-associated virus (AAV), carrying DRR1 (Masana et al., 2014). Thus, the researcher considered *Drr1* expression in the lateral septum as protective mechanism to outbalance negative effects of stress exposure on social behavior (Masana et al., 2014).

Summarized, both above-described studies exposed *Drr1* as glucocorticoid, thus stress-regulated gene. In addition, increased DRR1 protein levels modulate actin-dynamics in neurons along with improvement of cognitive performance and social behavior (Masana et al., 2014; Schmidt et al., 2011). Taken together, it was suggested that DRR1 promotes stress-resilience.

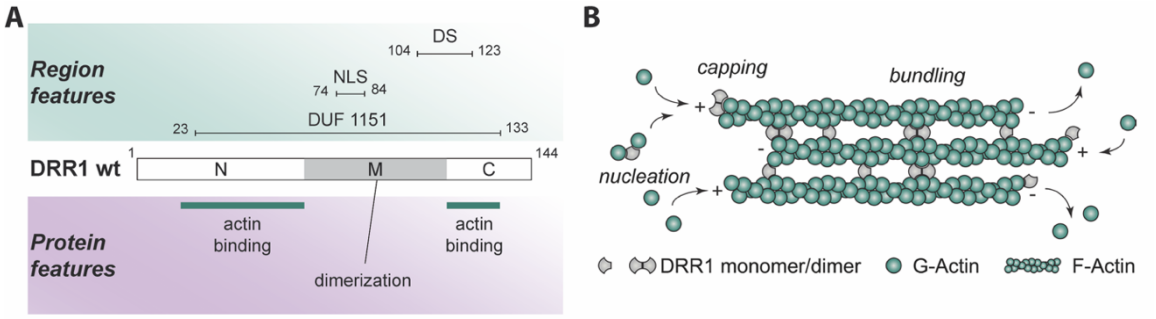

Figure 1.24: DRR1: Domain structure, actin interaction, and filopodia development.
(A) Murine DRR1 consists of 144 amino acids (aa). It contains the domain of unknown structure 1151 (DUF 1151) from aa 23–133, the nuclear localization signal (NLS) from aa 74–84, and a disordered sequence from aa 104–123, generating intrinsically disordered regions (IDRs). IDRs and structured domains increase the function al versatility of proteins. Distinct domains exhibit specific functions. The C-terminal (C) and the N-terminal (N) are actin-binding regions. The middle region (M) includes a coiled coil domain, potentially mediating homo-dimerization. **(B)** Scheme of DRR1's function as monomer and dimer on actin dynamics: capping, nucleation and bundling. Modified from: Kretzschmar et al., 2015 (Dissertation) and Kretzschmar et al., 2018.

A more recent study examined *Drr1*, especially as stress-response gene, being implicated in pathways of various neuro-degenerative diseases, such as amyotrophic lateral sclerosis (ALS) (Shin et al., 2016) involving low-complexity domains (LCD) of RNA-binding proteins. This was specifically shown by investigation of the RNA-binding protein CPEB4 (cytoplasmic polyadenylation element-binding protein 4) mainly expressed in brain and spinal cord tissue. Mice

only expressing the CPEB4 LCD display defective neuronal development caused by reduction of motor axon branching as well as malformation of neuromuscular junctions (Shin et al., 2016). *Drr1* mRNA is elevated in the spinal cord CPEB4$^{GT/GT}$ mutant mice, which serve as model for functional analysis of CPEB4 LCD, reducing neurite outgrowth. Later was shown build nucleolar aggregates, leading to cell toxicity, instead of showing a cytoplasmatic localization as it is the case for the full-length protein (Shin et al., 2016). The researchers hypothesize that the actin-binding protein DRR1 is a downstream factor in CPEB4 LCD-mediated cytotoxicity, triggering a stress response, which upregulates the expression of DRR1. Combined with other molecular changes this contributes to inhibition of neurite outgrowth due to blockage of F-actin polymerization leading to neuronal dysfunction in the above-mentioned mutant mouse model. Screening of post-mortem human brain tissue of ALS, Huntington's disease (HD) and Parkinson's disease (PD) patients, identified the *Drr1* gene being altered, amongst 243 other genes (Li et al., 2014). Additionally, *Drr1* is highly expressed in human outer radial glia during cortical development (Pollen et al., 2015). Collectively, these propose that by regulating actin-dynamics, DRR1 might play an important role in neurodevelopment, maintenance and repair of neurons (Shin et al., 2016).

Moreover, analysis of human tissue unfolded DRR1 levels to be increased in the dorsolateral prefrontal cortex of schizophrenic and bipolar patients (Shao & Vawter, 2008). Supporting the idea that DRR1 might be involved in the pathophysiology of neuropsychiatric disorders.

1.10 Aims of this study

Previously, DRR1 was suggested to promote resilience towards stress (Schmidt et al., 2011). Moreover, DRR1 was shown to affect AMPAR subunits after stress exposure in mice (Schmidt et al., 2010). Taken altogether we hypothesized that DRR1 induced modulation of actin dynamics and AMPA receptor trafficking during stress exposure is a way to regulate neuronal function and behavior in response to stressful environmental stimuli. This could constitute an active stress-regulatory resilience mechanism.

In vitro studies have the benefit of tightly controlled chemical and environmental settings. This allows to investigate molecular and cellular mechanisms of one specific system unbiased from other influences and factors, hence dissecting the role of specific actors for the particular framework. Therefore, primary hippocampal neuron cultures represent an excellent way to investigate the newly discovered glucocorticoid regulated and actin-binding protein DRR1 and neuronal actin dynamics and AMPAR trafficking in fine-tuning stress-related consequences in a way that minimizes negative effects of stress. As described above, chronic stress often leads to

morphological and structural changes in neurons, especially in the hippocampal region. This implicates modifications in actin dynamics, resulting in disrupted signal transmission in neurons which comprises differed receptor trafficking and receptor functioning at postsynaptic sites. Ultimately, this often concludes in cognitive and behavioral impairments and neuropsychiatric diseases such as major depression. The conceptual link between stress and neural actin dynamics is of particular relevance to reinforce the understanding of neurobiological resilience mechanisms.

Based on the previous data suggesting a resilience promoting role for DRR1, we aimed to examine the neurobiological pathways from DRR1 to resilience. We tested the hypothesis that DRR1, via its actin-interacting properties regulates glutamate receptor distribution at the synapse. Accordingly, we first investigated basal DRR1 levels in the adult murine brain via immunohistochemical qualitative analysis. Next, we used primary hippocampal neuron cultures and performed a subset of well-established assays (Essmann et al., 2008; Sawamiphak et al., 2010; Bissen et al., 2021) after inducing stress to the cultures. Alternatively, DRR1 was directly overexpressed or suppressed in the cultures utilizing an rAAV-mediated approach. To visualize the newly insertion of AMPARs at the postsynaptic membrane we applied an assay to mask the initial amount of AMPARs at the surface of the membrane and further detected incorporated receptors upon chemically-induced (KCl) neuronal activity. Further, the molecular interaction of DRR1 and AMPAR was investigated by applying the proximity ligation assay (PLA) in untreated wildtype cultures. The assays principle is based on the hybridization of two complementary oligonucleotide probes that are attached to species-specific secondary antibodies, forming a circle when the labeled proteins are in close proximity. Rolling circle amplification creates a distinct fluorescent spot.

Additionally, we tested the influence of DRR1 on structural and functional consequences of stress *in vitro*. In primary hippocampal neuron cultures, we either increased or decreased DRR1 levels through rAAV-mediation and analyzed the dendritic arborization and spine number and morphology in a semi-automatic way with the Imaris filament tracer and spine classifier software. To characterize the role of DRR1 in modulating stress-related consequences, we further successfully generated conditional DRR1 mutant mice with hippocampus-specific deactivation of DRR1 after the second postnatal week. Hereby, expression of the Cre recombinase under the CaMKII*α* excised functional regions of the *DRR1* gene in DRR1$^{lox/lox}$ mice. Future plans aim to use DRR1 mutant mice to perform structural and morphological analysis combined by electrophysiological and behavioral analysis following chronic social defeat stress to specifically asses the role of DRR1 on shaping resilience. The experimental designs should challenge the hypothesis that DRR1 attenuates negative consequences of stress on neuronal morphology and behavior.

In a second part of the study, we planned to focus merely on morphological spine analysis of stress resilient versus stress susceptible mice after chronic social defeat stress in the hippocampal region. By utilizing a new classification paradigm, namely the modified social interaction test (MSIT), we planned to ensure a more accurate and more sensitive division of animals into the above-mentioned groups. The MSIT prevents acquisition of animals that show neither resilient nor susceptible behavior. These mice are called non-learners and built their own category. This experiment pioneers future analysis of DRR1 expression in the subsequent groups, linking potential morphological and/or electrophysiological changes with DRR1 to help assess its role in shaping resilience.

In a third part we intended to investigate the involvement of DRR1 in the attenuation of acute stress responses in mice. Previously, a correlation between DRR1 elevation and cognitive improvements in mice after acute stress was discovered (Jene et al., 2018). Moreover, our preceding findings and a huge amount of existing data evidenced the expression of DRR1 in most of the existing cell types within the murine brain. Another study revealed blood brain barrier permeability in stress susceptible but not resilient animals (Menard et al., 2017). Thus, we performed immunohistochemical analysis labeling DRR1 protein in the hippocampus of mice which were subjected to acute social defeat stress or were injected with dexamethasone – a synthetic glucocorticoid – inducing an acute stress response. We aimed to explore potential DRR1 protein level changes in the hippocampal region after acute stress and simultaneously determining the cell types with occurring DRR1 level changes.

2. Results

In order to correctly adapt to external stimuli and stressors that individuals conceive from the environment, the neuronal interplay and plasticity of the central nervous system must be in a homeostatic state.

Postsynaptic glutamatergic AMPA receptors play a central role in excitatory signal transmission by trafficking to and from postsynaptic densities, thus initiating neuronal downstream mechanisms important for neuronal plasticity. The resulting synaptic strength and transmission of information at synapses show adaptations to external and intrinsic variations, ultimately leading to behavioral responses.

The efficacy of synaptic neurotransmission is formed in a use-dependent manner over a wide range of time-scales to enable computational tasks as well as learning and memory formation. These processes only become feasible through dynamic pre- and postsynaptic mechanisms which permanently are accompanied by structural alterations, such as increases in spines size or formation and elimination of spines. A key player in the modulation of shape size associated with synaptic plasticity is the cytoskeletal protein actin. In addition, actin organizes components of the junctional scaffold, thus assisting in the trafficking of the synaptic machinery.

Along with molecular alterations, there are morphological synaptic changes. The stabilization and morphological modifications of synapses and in particular postsynaptic spines, feature plastic adaption of neurons to distinct signals and heavily rely on proper and dynamic assembly and disassembly of actin proteins which arrange the cell cytoskeleton and build a scaffold for receptor transport from and to the side of action. Important helper proteins are so-called actin-binding proteins which interact with actin to facilitate fast and accurate remodeling of the existing actin-nets to quickly adapt to stimuli with corresponding morphological shape changes of spines as relevant response (Revenu et al., 2004). This process allows postsynaptic densities to shrink or grow in size, change receptor availability at the region of signal input at the PSD and thus helping modify synaptic strength.

The actin-binding and glucocorticoid-regulated gene *Drr1* was identified as an important player in the stress response, particularly in resilience to stress (Liebl et al., 2009; Schmidt et al., 2011, Masana et al., 2014). Additionally, *Drr1* and AMPARs show similar promoter profiles (Chong et al., 2007). In search of a molecular mechanism that promotes resilience, previous investigations from the Müller lab in Mainz showed an involvement of AMPARs in shaping resilience to stress (Schmidt et al., 2010). Moreover, AMPA receptor potentiators appeared to be highly effective in supporting resilience to stress. Furthermore, their findings on DRR1 showed, that it has the ability

to improve memory performance and attenuate stress-related aversive behavior (Schmidt et al., 2011) by modulating actin dynamics, thereby impacting on synaptic efficacy and synaptic plasticity (Schmidt et al., 2011; Kretzschmar et al., 2018). Analysis of the DRR1 domains uncovered multiple mechanisms that influence actin dynamics i.e., by shifting the actin network towards more, thicker, and shorter F-actin filaments in neurons (Kretzschmar et al., 2018).

With the work of this thesis, we aimed to investigate whether DRR1 directly engages in AMPA receptor trafficking and insertion at synaptic spines and whether it is involved in modulating the stress response by dissecting the underlying mechanisms of DRR1, neuronal acting dynamics and AMPAR trafficking during stress signals. We hypothesized that DRR1-driven modulations of actin-dynamics and AMPAR-interaction during stress exposure might be an efficient way to regulate neuronal function in response to stressful environments. Moreover, these mechanisms might constitute an active, stress-regulatory resilience mechanism.

2.1 Hippocampal DRR1 – preliminary findings

DRR1 was previously found to be ubiquitously expressed throughout the whole mammalian body. It plays a crucial role in the central nervous system by being involved in actin polymerization (Kretzschmar et al., 2018), which is important for spine development, axon formation and neurotransmitter release. Moreover, *Drr1* is a glucocorticoid sensitive gene (Liebl et al., 2009, Schmidt et al., 2011, Masana et al., 2014), thus implicated in the stress response. These both features make DRR1 an interesting candidate to study potential mechanisms of stress resilience in detail. The RNA-sequencing transcriptome database from Zhang and colleagues reveals *Drr1* RNA prevalence in cortical brain areas from seven days old mice within all major cell types of the murine brain (Zhang et al., 2014). According to this database, highest *Drr1* RNA levels exist in astrocytes, oligodendrocytes, and endothelial cells. Smaller amounts are seen in microglia. In neuronal cells only sparse *Drr1* RNA levels are found (Zhang et al. 2014; see Figure 2.1). Preliminary work showed DRR1 expression in neurites and axons of cultured hippocampal neurons (Schmidt et all. 2011). Upon stress DRR1 mRNA is highly upregulated in the stress sensitive CA3 region of the hippocampus. Besides, the finding of glucocorticoid responsive domains within the gene sequence as well as the ability to bind GRs *in vitro* illustrate the stress associated regulation of DRR1. Additionally, modified DRR1 protein levels affect neuronal morphology in CA1 and CA3 displaying decreased spine numbers, an increased neurotransmitter release probability and elevated cognitive flexibility, as behavioral read out linked to the hippocampus (Schmidt et al., 2011).

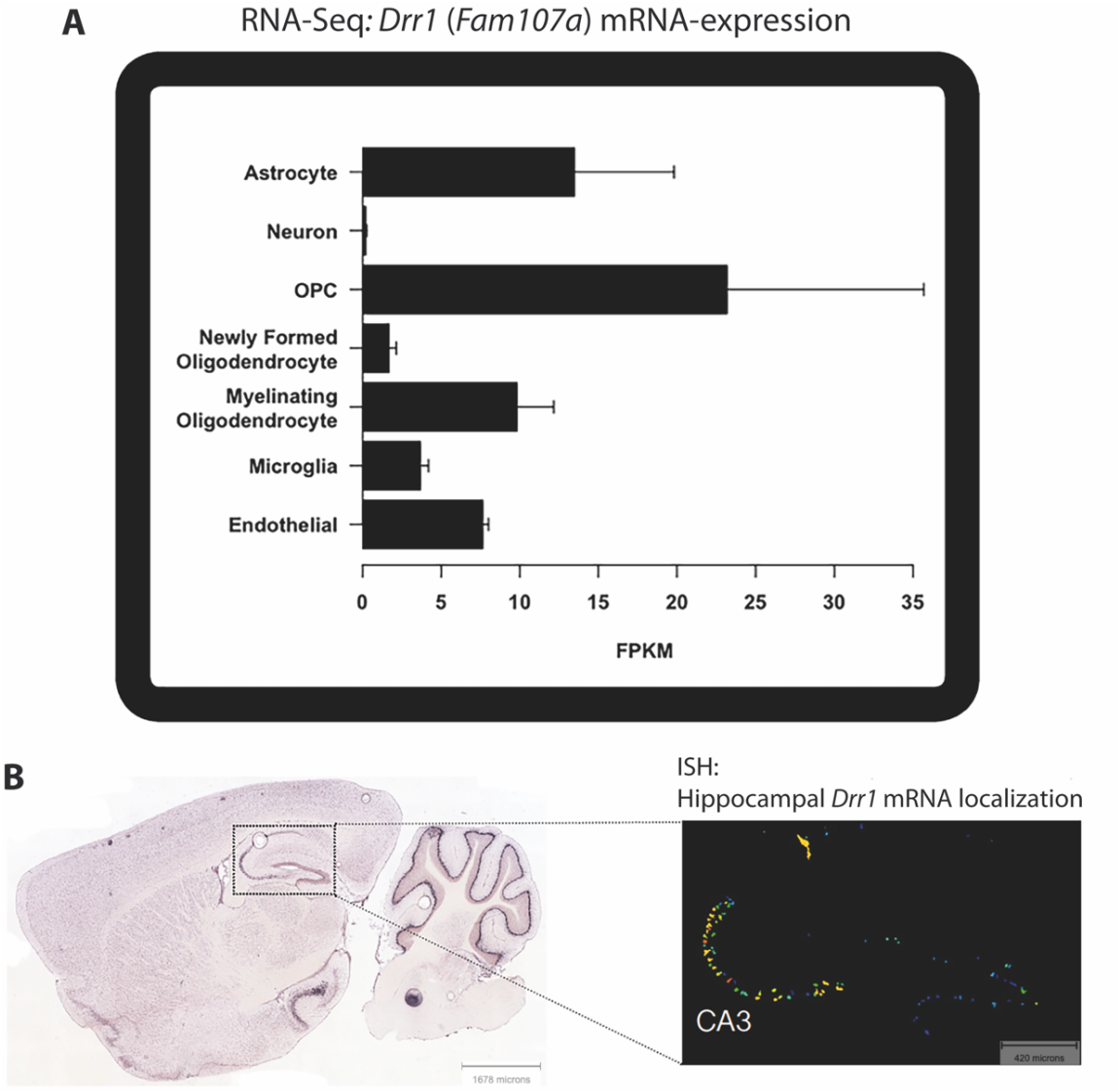

Figure 2.1: *Drr1* mRNA expression in *mus musculus*.
(A) Cortical single cell RNA sequencing data from P7 mice. *Drr1* mRNA is prevalent in all cell types. Highest proportions were measured in astrocytes and oligodendrocyte precursor cells (OPC). Cortical neurons show lowest *Drr1* gene expression. Expression level measured as fragments per kilobase of transcript sequence per million mapped fragments (FPKM). FPKM is a gene length normalized expression unit. The higher the FPKM for a gene, the higher the expression of that gene. From Zhang et al., 2014, web.stanford.edu/group/barres_lab/brain_ rnaseq.html (2019) and brainrnaseq.org (2022). **(B)** ISH reveals expression pattern of *Drr1* mRNA in the adult mouse brain. CA3 area as immunofluorescent close-up on the right. Scale bar left image: 1 678 µm; right image: 240 µm (25× zoom). From the Allen Mouse Brain Atlas, mouse.brain-map.org/gene/show/92564 (2022).

As actin-interacting protein DRR1 promotes neurodevelopment and the repair of neurons (Shin et al., 2016). Yet, upon cell stress and neurotoxicity DRR1 is upregulated and disturbs F-actin assembly, and thus neurite outgrowth. Therefore, we hypothesized that DRR1 needs to be expressed in a balanced amount in neurons to prevent stress-induced malformation of neuronal dendrites and spines, thereby maintaining proper neuronal function and consequent related behavior. We aimed to find out whether DRR1 can even display a resilience mechanism on the cellular and molecular level in the brain.

For that, we first investigated whether different DRR1 protein-levels, that deviate from basal levels have an impact on neuronal morphology. We first checked existing *in situ* hybridization (ISH) data from the Allen Mouse Brain Atlas regarding basal levels of *Drr1* RNA in the adult murine brain. As shown in Figure 2.1 highest RNA expression is found in the cerebellum and the anterior part of the hippocampal CA1-CA3 areas (Allen Mouse Brain Atlas). Adding to this data, pre-existing results (Schmidt et al., 2010), showed increases of DRR1 in the hippocampal CA3 area of adult mice upon elevated glucocorticoid levels after chronic stress. To connect this with behavioral analysis, mice overexpressing DRR1 in the CA3-region were tested in hippocampal specific learning tasks, showing improved spatial memory and cognition (Schmidt et al., 2011). Other studies using structural magnetic resonance imaging (MRI) and diffusion tensor imaging revealed neuroanatomical differences in hippocampal CA3-regions between stress susceptible and stress resilient mice after chronic stress (Anacker et al., 2016).

2.2 Endogenous DRR1 in the hippocampal subfield of adult mice

To validate and connect *Drr1* RNA expression patterns in the adult murine brain with DRR1 protein levels under normal conditions, we performed an immunohistochemical fluorescent staining against DRR1 in fixed, 50 μm thick coronal sections of wildtype C57BL/6J mice 8–12 weeks of age. Firstly, to find the best antibody for further qualitative *in situ* studies concerning DRR1, we tested two antibodies for their efficiency. A commercially available polyclonal antibody from Sigma-Aldrich (α-Fam107a from rabbit) and a self-designed and manufactured antibody (MUE-ab) from our collaborative laboratory (Müller laboratory, Mainz, Germany). Apart from PFA fixated tissue, we additionally tested the antibodies in TCA fixed brains of 30-day-old mice to determine the most suitable fixative for DRR1 antibody-staining.

To compare intensity, quality and specificity of both antibodies we applied one staining protocol (described in Materials and Methods) using the two distinct antibodies in parallel experiments utilizing brain sections of same animals. As previously described, this study focused on the hippocampal region, due to its sensitivity to stress and the high basal expression of DRR1 in the anterior part of the CA3 region. An important area for spatial memory formation, thus easy to test and link with behavioral outcomes. Additionally, the hippocampus is a glucocorticoid-sensitive region involved in the stress response and potentially forming resilience to stress. Since we aimed to study the hippocampus *in situ*, we used the CA3-area as representative region to compare both antibodies.

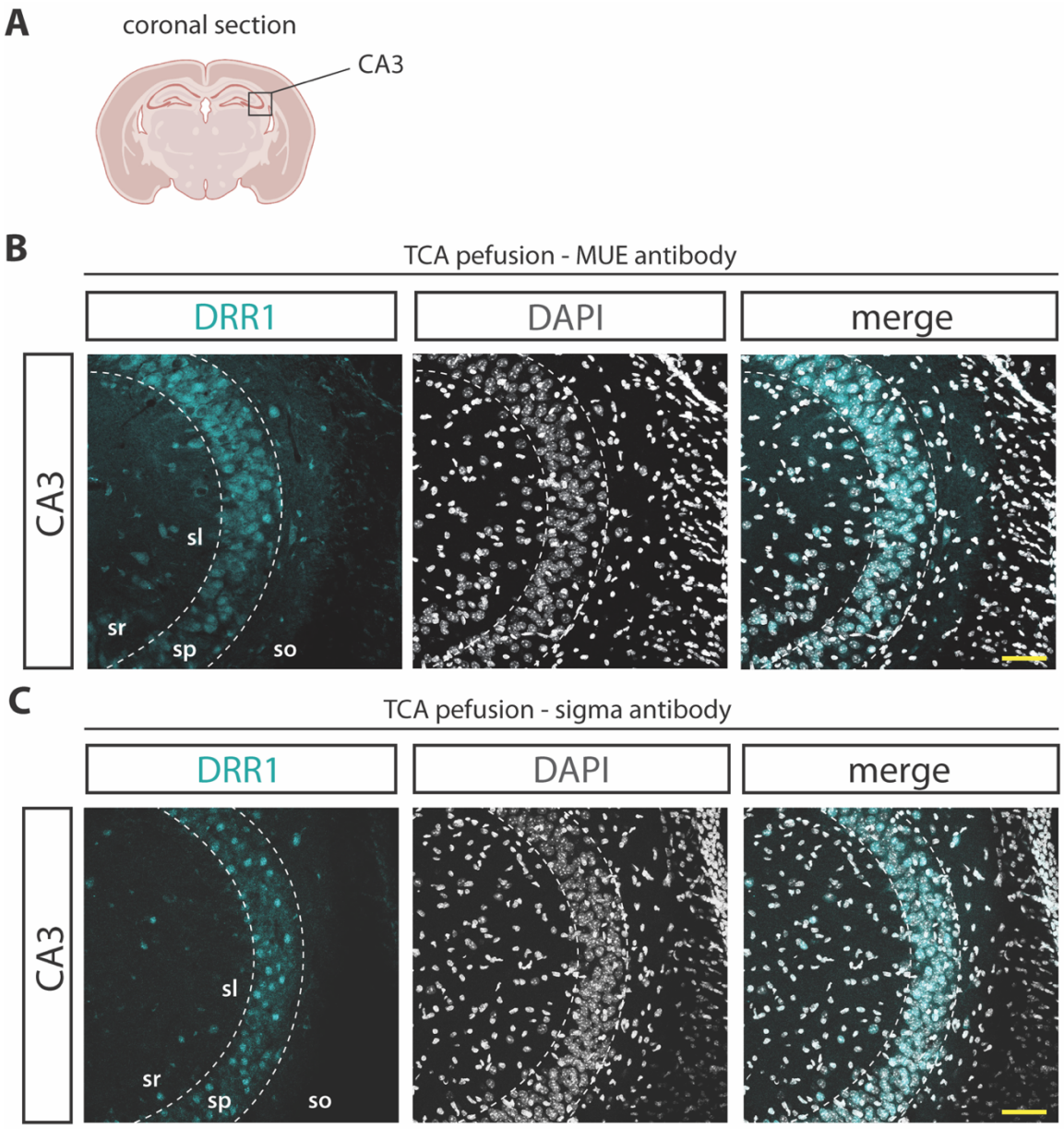

Figure 2.2: *In situ* DRR1 protein expression in hippocampal CA3 region (TCA fixation).
The images show the CA3-area, a section of the hippocampus **(A)**. 80 µm thick coronal sections of 30-day-old mice, perfused with TCA and fluorescently stained for DRR1 (cyan) and DAPI (white). Overlaps shown on the right (merged). **(B)** Fluorescent staining with self-manufactured antibody from the Müller lab (MUE-ab) combined with DAPI staining. **(C)** Staining performed with commercially available antibody from Sigma-Aldrich (see Materials and Methods). Both antibodies for DRR1 reveal protein localization in the cytosol. Partial overlap with DAPI nuclei staining shows DRR1 localization in the nucleus of CA3 cells as well. Acronyms: *stratum lucidum* (sl); *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so). Scale bars in yellow: 50 µm; 40× oil immersion.

As shown in fluorescent images of the CA3-area in Figure 2.2, TCA perfusion is suitable for both antibodies against DRR1, showing a substantial staining of the protein, which reveals good binding capabilities. Although, the MUE-ab shows deeper and more intense coverage of DRR1 (Figure 2.2(B)), both antibodies reveal localization of DRR1 especially in the cytosol and partially in the nucleus of cells within the CA3 area, which we could show by combining fluorescent staining against DRR1 with 4'6-diamidino-2-phenylindole (DAPI) nuclear stain. Similarly, we tested both

antibodies in PFA fixated brains of 8-week-old mice. As shown in Figure 2.3 both antibodies show a substantial staining for DRR1 within the hippocampal CA3-region. DRR1 *in situ* staining of PFA fixed brains show the same staining pattern as found in TCA fixated tissue.

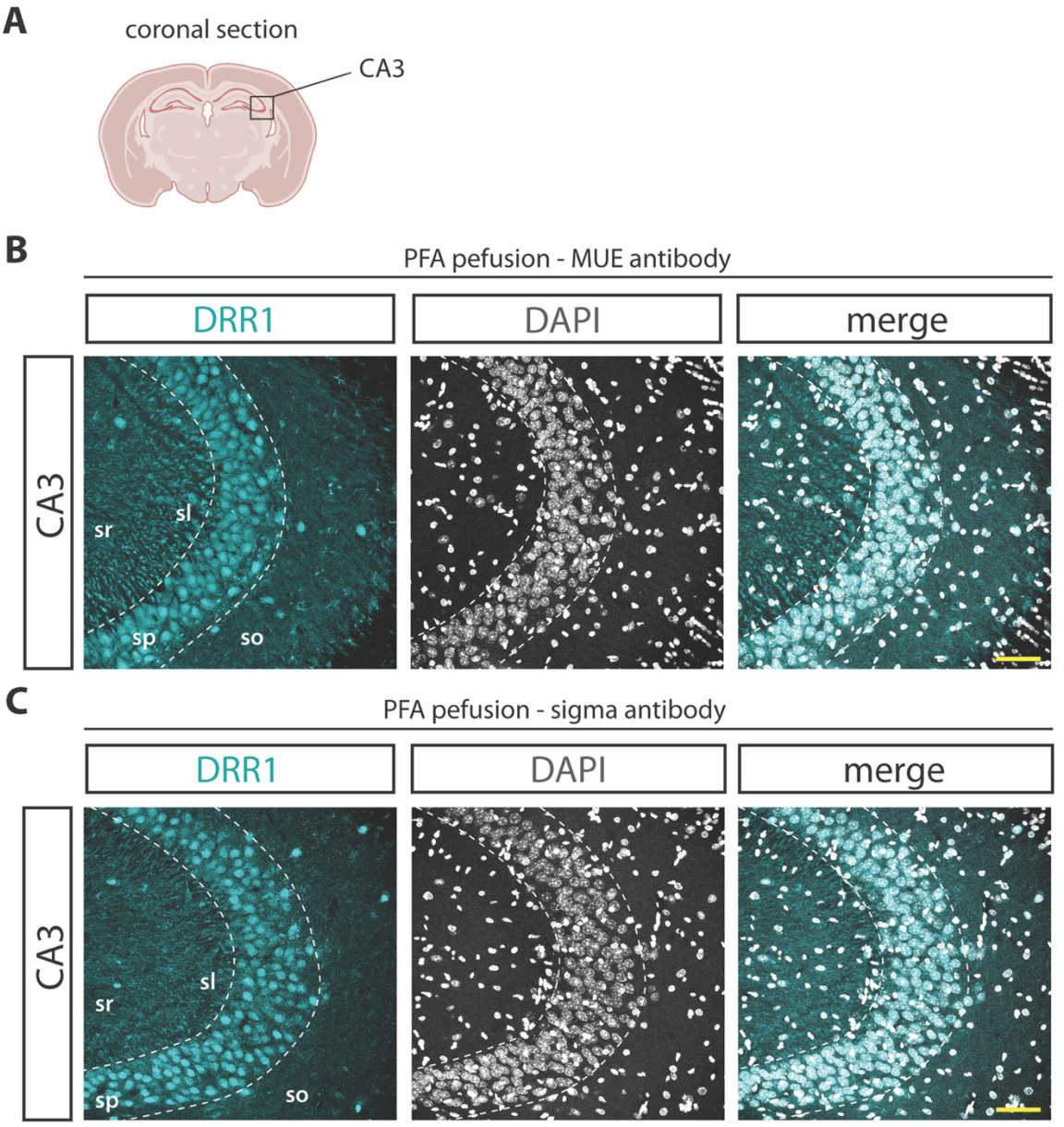

Figure 2.3: *In situ* DRR1 protein expression in hippocampal CA3 region (PFA fixation).
The images show the CA3-area, a section of the hippocampus **(A)**. 50 µm thick coronal sections of 8-week-old mice, perfused with PFA and fluorescently stained for DRR1 (cyan) and DAPI (white). Overlaps shown on the right (merged). **(B)** Fluorescent staining with self-manufactured antibody from the Müller lab (MUE-ab) combined with DAPI staining. **(C)** Staining performed with commercially available antibody from Sigma-Aldrich (see Materials and Methods). Both antibodies for DRR1 reveal protein localization in the cytosol and ramifications of cells. Partial overlap with DAPI nuclei staining shows DRR1 localization in the nucleus of CA3 cells as well. Acronyms: *stratum lucidum* (sl); *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so). Scale bars in yellow: 50 µm, 40× oil immersion.

Also here, DRR1 is primary found in the cytosol of cells including partial overlaps with DAPI staining, which reveals its occurrence in cell nuclei. It becomes apparent that DRR1 staining

appears stronger and more intense in tissue from PFA perfused animals. Thereby, both antibodies show similar outcomes. In addition, also cell ramification show appearance of DRR1 in PFA perfused tissue staining. Due to better staining quality and a broader capturing of DRR1, we decided to use PFA perfusion for subsequent experiments.

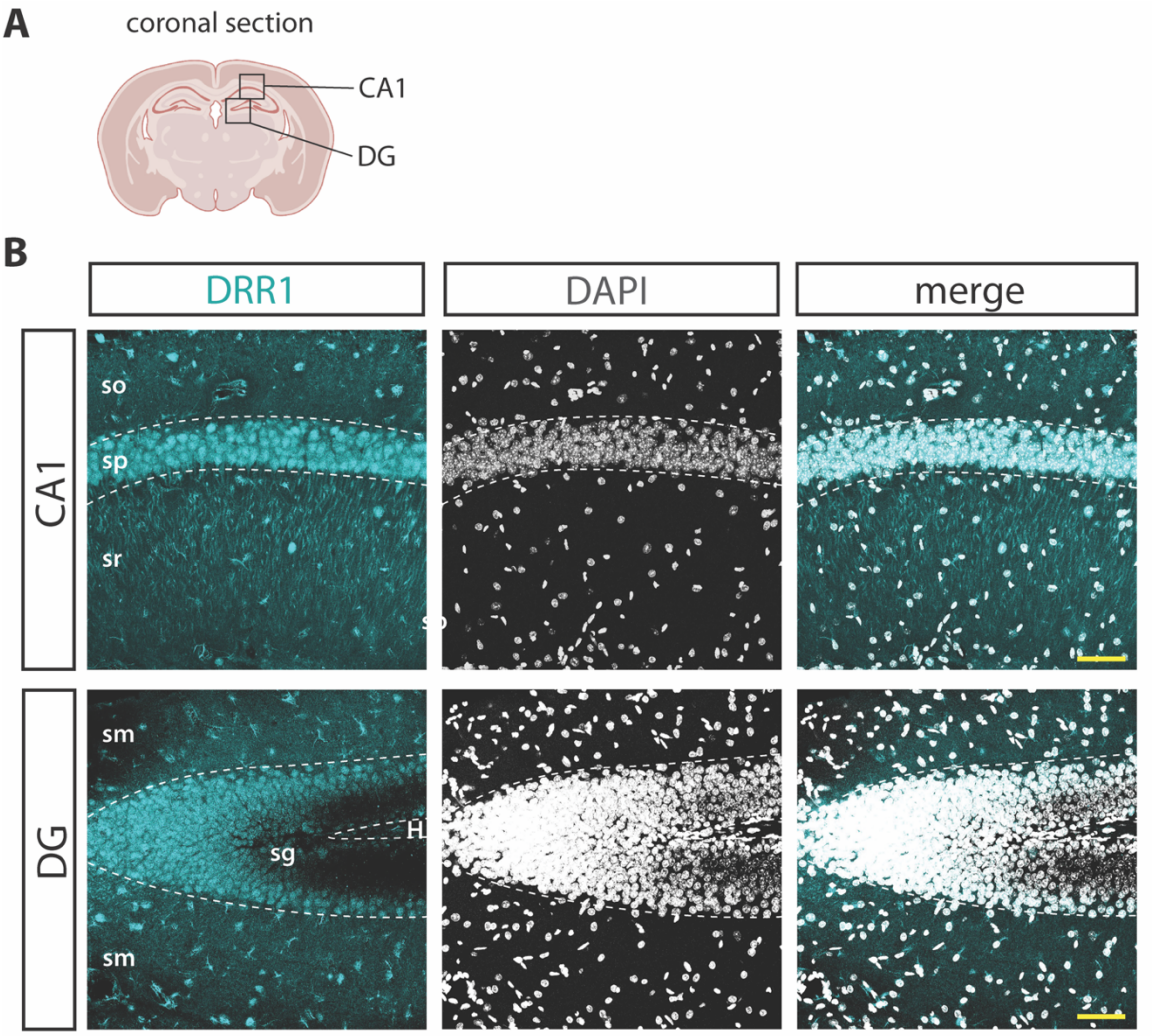

Figure 2.4: *In situ* DRR1 protein expression in hippocampal CA1 region and DG (PFA fixation).
(A) Scheme of coronal murine brain section with examined brain regions. **(B)** The upper panel shows an extract of the hippocampal CA1 region fluorescently stained for DRR1 (cyan), DAPI (white), and overlap on the right (merged). Lower panel shows the dentate gyrus separating into dorsal and ventral blades. Staining was performed in 50 µm thick coronal sections of 8-week-old mice with the MUE-ab. DRR1 overlaps with DAPI-staining and is pred-dominantly found in the granule cell layer of the DG and the pyramidal cell layer of the CA1-area. DRR1-staining reveals protein deposits in a sub-portion of surrounding cells and neurites of CA1. Acronyms: *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so); *stratum granulosum* (sg); *stratum moleculare* (sm); hilus (H); dentate gyrus (DG). Scale bars in yellow: 50 µm, 40× oil immersion.

Within the hippocampus CA1 to CA3 areas, as well as the dentate gyrus show high DRR1 expression as revealed by qualitative immunostaining, shown in Figure 2.4.

We found DRR1 protein ubiquitously expressed in diverse brain areas of unstressed wildtype mice. As expected by the RNA expression pattern for *Fam107a* depicted by the ISH database of the Allen Mouse Brain Atlas, the qualitative fluorescent protein staining against DRR1 showed highest

protein prevalence in the cerebellum, cortical area and the limbic system including the hippocampus (see Figure 2.5). Interestingly, within the cerebellum, DRR1 occurs specially at the boarder of molecular and granule layer, staining cells characteristic for Purkinje cells (Figure 2.5(B), upper panel).

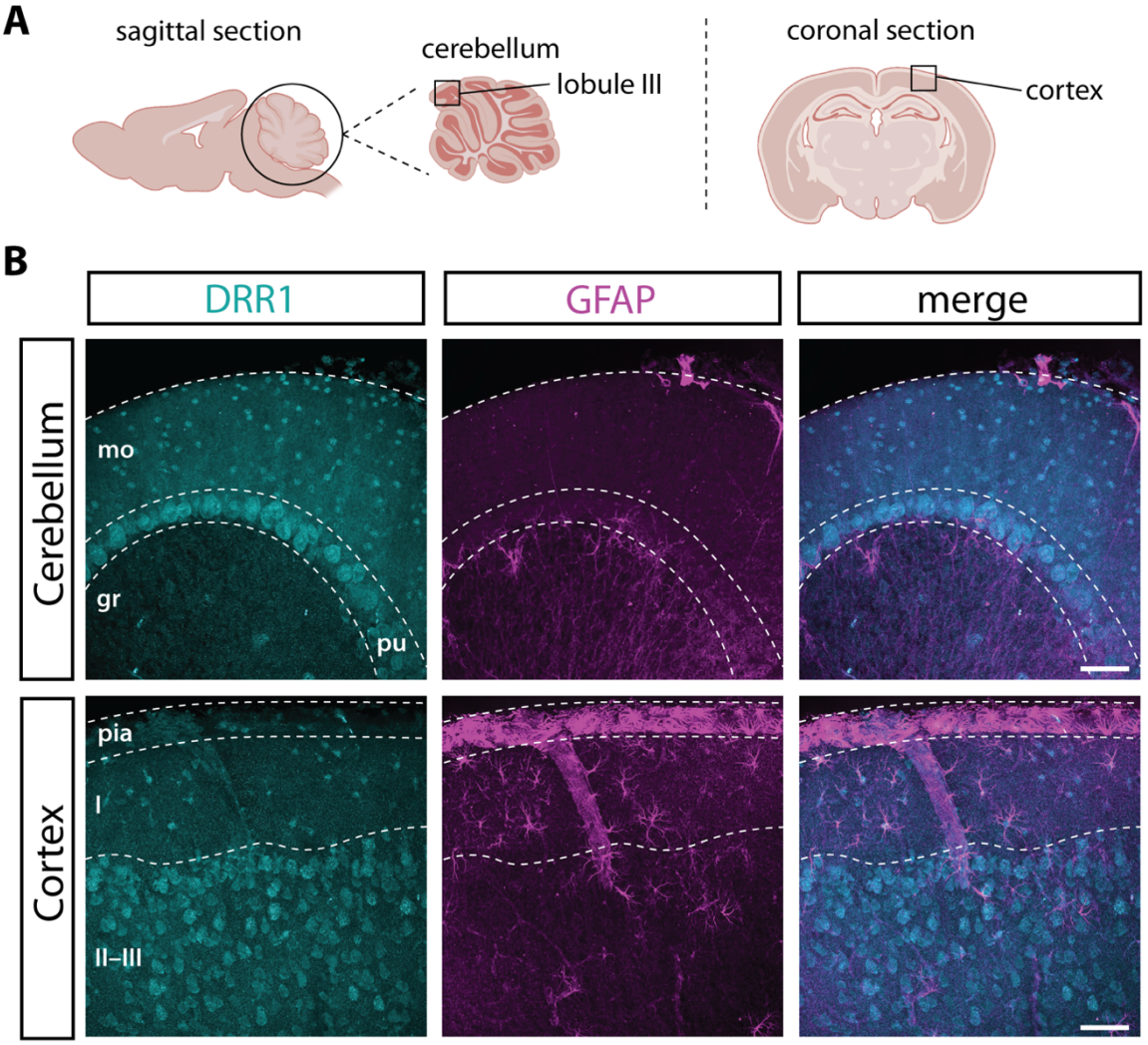

Figure 2.5: *In situ* DRR1 protein expression in cerebellum and cortex.
(A) Schemes of murine brain sections with examined brain regions. **(B)** The upper panel shows an extract of cerebellar lobule III stained for DRR1 (cyan), GFAP (magenta), and overlap on the right (merged). Staining was performed in 40 µm thick sagittal sections of 8-week-old mice with the MUE-ab. DRR1 overlaps with GFAP-staining and is predominantly found at the boarder of molecular and granule layer, marking a single cell layer characteristic for Purkinje cells. Co-staining with GFAP (magenta) reveals overlap with surrounding Bergman glia in the cerebellum and astrocytes in the cortex. Lower panel shows an extract of the cortex. DRR1 occurs in many cell bodies and is co-expressed in GFAP-positive cells. Acronyms: molecular layer (mo); granule layer (gr); Purkinje cell layer (pu); *pia mater* (pia); cortical layer I (I); cortical layer II–III (II–III). Scale bars in yellow: 50 µm, 40× oil immersion.

Moreover, DRR1 antibodies seemed to stain in a much lesser extent, also other cell types, that appeared as cortical astrocytes and cerebellar Bergman glia. Since the RNA sequencing database of Zhang and colleagues (Zhang et al. 2014) shows *Fam107a* RNA expression in cortical brain of P7 aged mice mainly in other cell types than neurons, we started to check for an overlap

of distinct cell type specific markers and DRR1, in adult brains. First, we combined neuronal nuclei (NeuN) antibody – a protein localized to nuclei and perinuclear cytoplasm in most neuronal cell types (Gusel'nikova & Korzhevskiy, 2015) – with DRR1 antibody.

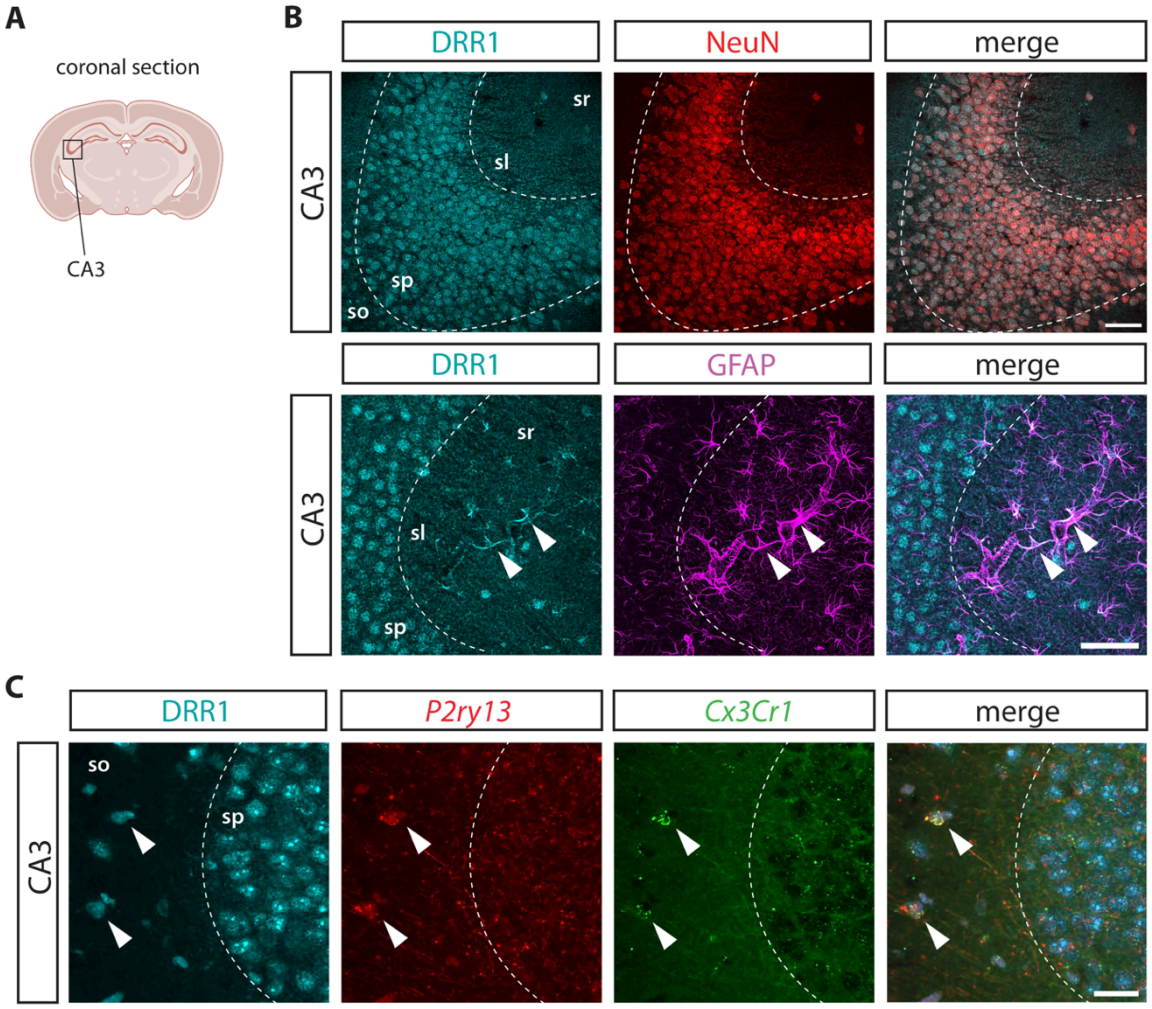

Figure 2.6: Co-localization of DRR1 with other cell-type specific markers.
The images show a selection of the hippocampal CA3-area **(A)**. Immunohistochemistry was performed in 50 µm thick coronal sections of 8-week-old mice, perfused with PFA. **(B)** DRR1 (cyan), NeuN (red), GFAP (magenta) and overlaps (merge). DRR1 strongly co-localizes with the neuronal nuclei marker NeuN (upper panel) and partially with GFAP in astrocytic end-feet and ramifications (lower panel). White scale bars: 50 µm, 40× oil immersion. **(C)** IHC against DRR1 (cyan) combined with ISH with mRNA probes for *P2ry13* (red) and *Cx3Cr1* (green), both microglial markers in 15 µm thick sagittal sections of 8-week-old C57BL/6J mice. Acronyms: *stratum lucidum* (sl); *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so). DRR1 staining overlaps with microglial RNA-probes. Scale bar: 25 µm, 63× water immersion.

Qualitative immuno-histological analysis as depicted in Figure 2.6 exemplify the strong overlay of NeuN protein and DRR1 in the hippocampal CA3 and CA1 region, identifying the major part of DRR1 being expressed by neurons in the hippocampal subfield.

In addition, our staining confirmed the findings of Schmidt and colleagues that DRR1 is predominantly localized in cell nuclei and cell bodies. However, some of the DRR1 protein did not localize with the NeuN marker, letting us hypothesize, that this protein might also be expressed

by other cell types in the adult murine brain. This is why we performed immune-histological co-localization analysis with other cell-type specific markers such as glial fibrillary acidic protein (GFAP) for astrocytic cells and *in situ* hybridization with the mRNA probes *Cx3Cr1* and *P2ry13* specific for microglia, which we combined with a DRR1 protein staining. Previous investigations from our laboratory verified the purinergic receptor $P2RY_{13}$ being localized to microglia (Stefani et al., 2018), which identifies it as a microglial marker. Qualitative images as shown in Figure 2.6 illustrate the co-localization of DRR1 and the cell type specific markers GFAP and *Cx3Cr1* together with *P2ry13*. In sum, the results reveal a co-localization of DRR1 with microglial markers, demonstrating that DRR1 protein is also expressed by microglial cells within the hippocampus. Also here, the protein seems to mainly localize to the cell body, presumably nuclei, rather than in microglial ramifications. Noticeably, DRR1 also co-localizes with a fraction of astrocytic cells in different brain areas, such as the hippocampus and cortex. Whereas the strongest DRR1 expression occurs not only in cell bodies but primarily in astrocytic end-feet contacting vessels (Figure 2.6). Within the cerebellum GFAP-antibody stains Bergman glia without notable co-localization with DRR1.

2.3 DRR1 in primary hippocampal neuron cultures: Impact on AMPARs and neuronal morphology

After qualitatively analyzing the endogenous distribution of the DRR1 protein *in situ* in C57BL/6 wildtype mice, we found that the protein is highly present in hippocampal neurons, especially within the CA3 area and the granule cell layer. Together with the findings of Schmidt and colleagues, showing that upon stress DRR1 is upregulated in the hippocampal subfield, we aimed to investigate morphological changes *in vitro*, when manipulating the amount of DRR1 protein in primary hippocampal neurons. In this way we aim to mimic upregulation of DRR1 as seen after stress or to investigate the effects of suppressing the protein on neuronal cells. Before manipulating DRR1 protein levels *in vitro*, we first planned to verify basal levels and distribution of endogenous DRR1 in primary mouse hippocampal neurons in comparison to results made in primary rat hippocampal cultures (Schmidt et al., 2011).

Primary hippocampal neurons were extracted from E17/18 mouse embryos as described in materials and methods and cells were cultured for 14–21 days before fixing them with PFA and performing cyto-immunohistochemistry to visualize DRR1. In addition, the cell cytoskeleton was visualized via immuno-staining actin protein. In primary mouse hippocampal neuron cultures DRR1 is consistently present within the whole cell. Strongest fluorescent signal is found in the cell bodies with a punctuated pattern in dendritic neurites. Figure 2.7 exhibits a close-up view at one

neuronal cell *in vitro* with staining against DRR1, MAP2 to visualize the cell structure, and DAPI as nuclear marker.

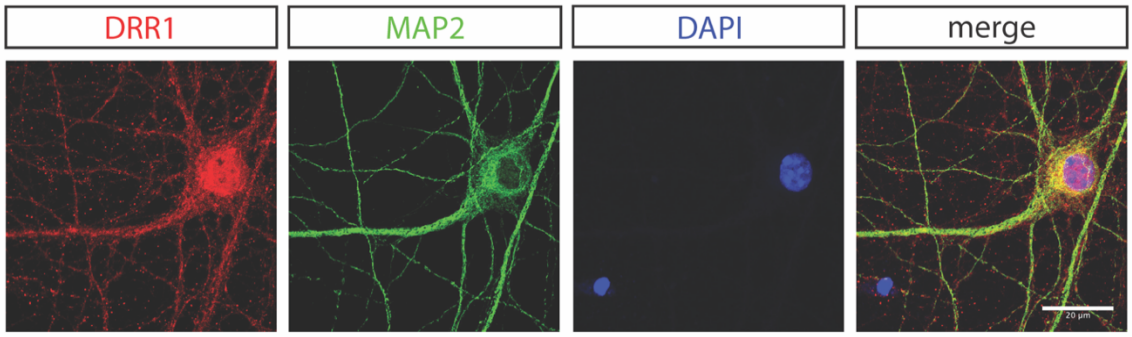

Figure 2.7: DRR1 patterning in primary hippocampal neuron culture.
The panel depicts a PFA fixed primary hippocampal neuronal culture with immunostaining against DRR1 (red), MAP2 (green), DAPI (blue) and an overlap (merge). Strongest DRR1 signal is found in the cell nuclei (DAPI staining) and cytosol. DRR1 is distributed in a punctuated pattern along dendrites (visualized through MAP2 staining). White scale bars: 20 µm, 63× oil immersion; 2× zoom.

2.3.1 In vitro manipulation of DRR1 protein expression in primary hippocampal neurons using rAAV vectors

Viral vectors present a functional approach of delivering genes to specific target sites. Since decades, genome sciences and structural biology conducts research in this field utilizing viral vectors to pass therapeutic genes for gene delivery as targeted therapies to treat the root of diseases (Kay et al., 2001; Breyer et al. 2006; Crystal, 2014; Lee et al. 2017). Amongst different viral vectors, adeno-associated virus is one of the most established and efficient gene transmission systems in view of applicability in multiple tissue and cell types, its clearly defined biology, genetic stability, high efficacy of gene transduction, fast gene expression and the easiness of large production (Breyer et al. 2006; Crystal 2014; Seymour & Fisher, 2011). Adenovirus is characterized by its non-enveloped linear double DNA-strand of about 26 kb to 45 kb and six subgroups of serotypes, which show diversity in tropism for different cell types, mostly specified by targeted cell surface receptors (Rauschhuber et al., 2012; Nathanson, 2014). The best characterized serotypes are type 2 (Ad2) and type 5 (Ad5) showing the lowest pathogenicity in humans (Walther & Stein, 2000). The gene of interest is usually inserted in the deleted early gene 1 (*E1A*) originally suiting for replicating the vector (Walther & Stein 2000). Adenoviral DNA is flanked by two hair-pin like inverted terminal repeats (ITRs), serving as self-primer and enabling the integration into the host genome.

We used recombinant adeno-associated viral vectors (rAAV) to manipulate endogenous DRR1-protein level expression in primary hippocampal neuron cultures through transgene delivery specifically into neuronal cells. As small and helper-dependent virus, AAV is capable of

small cargo gene delivery transducing dividing and non-dividing cells mostly with episomal transgene products (Walther & Stein 2000; Waehler et al., 2007; Nayak & Herzog, 2010; Samuski & Muzyczka 2014, Balakrishnan & Jayandharan, 2014). Hence, to suppress or overexpress the DRR1 gene for morphological analysis in primary hippocampal neuronal cultures *in vitro*, transduction with AAV for gene delivery is, apart from mild immune-toxicity, a perfect cell-preserving way to change gene expression patterns. We tested the customized recombinant adeno-associated viral vectors (rAVE™) which were kindly provided by the Müller laboratory (Department of Psychiatry and Psychotherapy, University Medical Center of the JGU; Leibniz Institute for Resilience Research, Mainz, Germany). Several viral vectors were designed by this company to specifically infect neurons in the murine brain to silence the *DRR1* gene either by siRNA interference or by infiltrating additional DRR1 transgenes for overexpression of the protein. The rAAV vector was effectively utilized in *in vivo* studies of Schmidt and colleagues (Schmidt et al., 2011) to locally manipulate the expression of DRR1 specifically in the CA3-area of the hippocampus by injecting the viral vector into this brain area in adult male mice (Schmidt et al., 2011). For our purpose we first tested the different rAAV vectors and their transfection efficacy in murine primary hippocampal neuron cultures *in vitro*. To assess the transfection efficacy of the viral vector, we observed the expression of the incorporated reporter gene GFP by evaluating the fluorescent intensity. At the beginning we used different titers with MOIs of 100, 1000 and 10 000, monitoring the peak of fluorescent intensity, cell death and the number of transfected neuronal cells over a course of 14–21 DIV. We transfected the cells seven days after plating and found that cells start to express the green fluorescent protein after three days post-transduction, reaching a peak of fluorescence after seven days post-transduction. Cell death was qualitatively evaluated by microscopy and cell counting. Here, we found that the higher the viral titer, the higher the cell mortality. A MOI of 1000 showed most effective transfection with the virus, with a barrable amount of dying cells. With this understanding we continued to perform transfection of primary hippocampal neurons with a MOI of 1000 for further experiments.

In a next step, we checked the protein amounts of DRR1 in primary hippocampal cells after 14 DIV via Western Blot, to ascertain sufficient silencing or overexpression of DRR1. As shown in Figure 2.8, we detected a stable and partial suppression of endogenous DRR1 with rAAV mediated gene silencing (shDRR1), which acts at the post transcriptional level targeting *Drr1* mRNA, inducing RNA interference and cleavage. Thereby, we compared two conditions. As control condition, we used a scrambled sequence rAAV (shSCR) and transfected primary hippocampal cell cultures with either shDRR1, shSCR, or left the cultures untreated (procedure see Materials and Methods). By default, cultures were prepared out of one embryonic litter thus similar hippocampal brain cells were cultured. Hippocampal neurons were plated on individual 6 cm ∅ culture plates. Cultures were transfected after seven DIV with a MOI of 1000 each. In

addition, cells were seeded with same cell densities, which varied amongst experiments between 850 000 and 1 million cells. In this way it was guaranteed that all conditions were housed and incubated with exactly the same environmental factors, including medium. In addition, by applying an identically built viral vector to the control condition, we aimed to mimic the disturbances that are caused by the viral infection machinery, such as minor toxicity and cell death, but without manipulating the expression of the *DRR1* gene in neurons. In this way, we could constitute a good model for comparing DRR1 silencing and a suitable control. SDS-page gel electrophoresis was performed by applying previously transfected cell lysates on the same gel with protein concentrations of 100–150 µg. After harvesting the cells and performing Western Blot, we used a chemiluminescent procedure, applying antibodies against the 17 kilodalton (kDa)-sized DRR1 protein and the reference protein β-actin with a size of ~42 kDa.

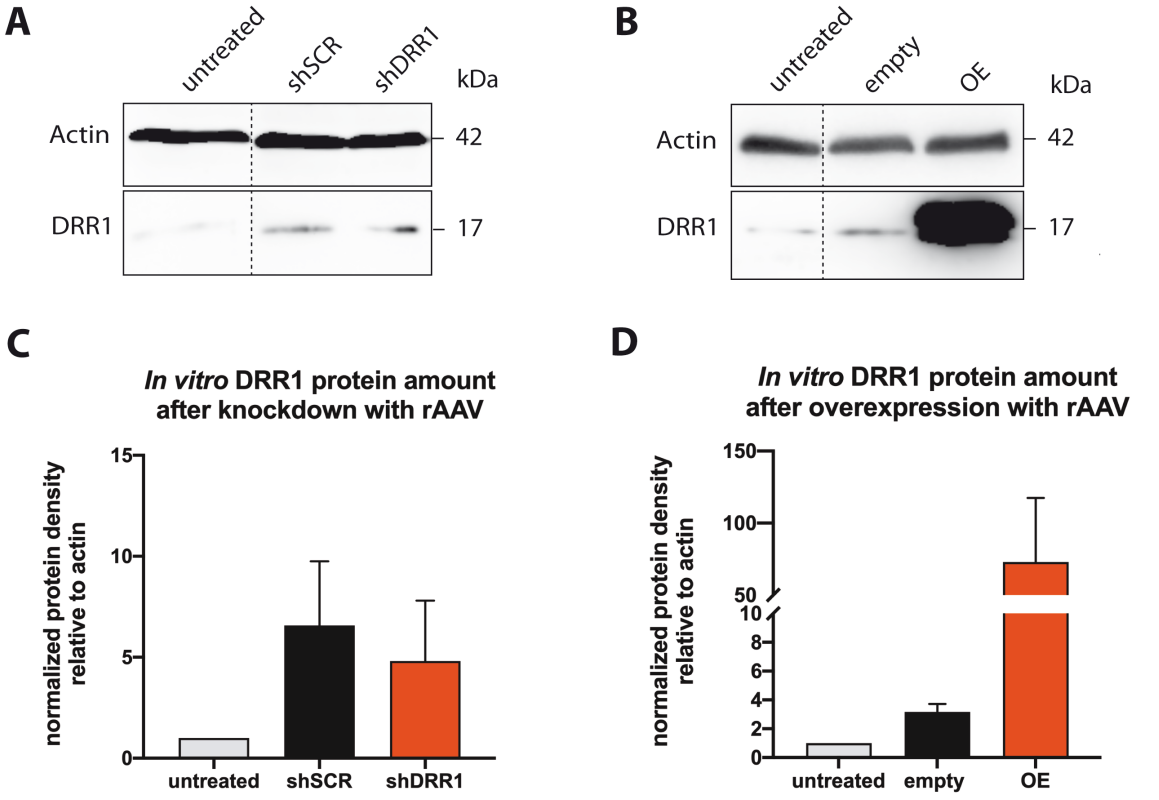

Figure 2.8: DRR1 protein amounts in cultured hippocampal neurons after transduction with rAAV vectors.
(A, B) Representative Western Blots of primary hippocampal cell lysates after 14 DIV and 7 days post transduction with untreated cultures, siRNA targeting *Drr1* mRNA (shDRR1), respective control (shSCR), overexpression (OE) and its empty control (empty). **(C, D)** Respective boxplots show average protein densities from three individual experiments for suppression and two individual experiments for overexpression. Values shown with ± SEM and normalized to actin. Untreated condition is plotted in gray, rAAV-transduced control shSCR or empty control in black, and rAAV-transduced shDRR1 for endogenous DRR1 suppression or OE construct for overexpression in red. **(C)** Average decrease of DRR1 between shSCR and shDRR1 is 26.717% (three independent experiments). **(D)** DRR1 increase in OE conditions is 23-fold higher as compared to empty condition (two independent experiments). Untreated condition serves as sample control and is normalized to 1. Protein densities represent arbitrary numbers measured as percentage per area. Multiple students t-test performed as statistical analysis. No significant differences between experiments, when $p < 0.5$.

The protein bands were visualized by a chemical reaction using horse radish peroxidase and capturing images with a chemiluminescent sensitive camera and the program LASAF4. For Quantitative analysis, we converted the images to an eight-bit image and utilized the "gel-analysis" tool, measuring absolute band densities in Fiji. For calculating the relative density of the individually measured proteins, we divided the values of the untreated culture by the silenced condition (shDRR1) or control (shSCR). In a final step we calculated the adjusted densities, to scale the DRR1 relative densities by dividing their relative values with the relative values of the actin loading controls. Representative Western Blot images in Figure 2.8(A), display a visible decrease of the DRR1-protein in lysates of shDRR1 treated cultures as compared to their shSCR control condition visualized via protein bands. It is also noticeable that the visualized actin-bands, suiting as loading control, do not notably differ between conditions, indicating that all lysates contained the same protein amount. Quantitative analysis as displayed in Figure 2.8(C) of three repeated experiments with different hippocampal neuronal cultures showed a stable decrease of DRR1 by 26.7% in average shDRR1 transfected cultures compared to the control conditions that were transfected with shSCR (Fig 2.8). Due to high variations of band intensities between experiments, result showed no significant difference. In comparison to the untreated condition, transfection with rAAV seems to elevate basal levels of DRR1, evident through more than fivefold higher protein densities in the shSCR treated condition. Nevertheless, treatment with siRNA for knockdown, seems to decrease this initial protein level. The boxplots in (B) represent average values ± SEM of three experiments per condition, normalized to actin and showing the relative densities of the proteins with arbitrary numbers.

The efficiency of the viral overexpression of DRR1 was tested in equivalent experiments, utilizing identical viral vectors with the same serotype. In this case the viral vectors contained an additional gene sequence for DRR1. The viral control did contain the same expression cassette without a transgene, hence characterized as an empty control. Also in this case, both conditions were cultured and virally transfected in the same way as for the silencing experiments. A representative Western Blot gel image is shown in Figure 2.8(B). As for the silencing experiments antibody-staining of β-actin was performed to suit as reference and loading control. The protein band of DRR1 overexpressing cell lysate reveals a very strong and over-saturated band. Simultaneously this condition seems to show a relatively similar actin band in comparison to the untreated reference and the empty control. This points out that the protein load ranges around similar levels in all conditions. Further, Western Blot analysis show a very strong increase of DRR1 in neuronal cultures that were transfected with the DRR1 overexpressing viral vector (OE) as compared to the transfection of the empty control (empty) vector (Fig 2.8). The protein gain is twenty-three-fold higher in OE lysates as compared to empty control lysates. Also, here the basal

level of DRR1 is already elevated in conditions transduced with the empty control when comparing with untreated conditions.

2.3.2 GluR2 subunit amount in primary hippocampal neuronal cultures after overexpression and silencing DRR1 with rAAV

Previous co-expression and co-regulation studies found DRR1 and AMPARs co-expressed plus showing same promoter profiles (Chong et al., 2007). Further, unpublished data from our collaborator in Mainz (Müller laboratory, under preparation) revealed increased mRNA of GluR1 and GluR2 AMPAR-subunits in the CA1 and CA3 area of the hippocampus after silencing DRR1 specifically in the CA3-area in murine brains *in vivo*. Hereby, they used the same rAAV constructs and controls as described in the chapter before. The brains were analyzed four weeks post viral injection. However, functional data on the impact of DRR1 on AMPA receptor localization and function are still missing. To validate the link between the glucocorticoid sensitive gene *Drr1* and changes in the neurotransmission shaping AMPA receptor expression, we analyzed the protein levels of the AMPAR-subunit GluR2 via Western Blot after DRR1 silencing or overexpression *in vitro*.

For that, we cultured primary hippocampal neurons for 14 days in 6 cm ∅ culture plates and transfected them after 7 DIV with the distinct rAAV vectors and respective controls to silence or overexpress DRR1. After harvesting the cultures, we performed Western Blots to detect GluR2 and DRR1 protein levels in the same protein lysates comparing experimental conditions with the respective controls as described in the previous chapter. As reference and house-keeping protein β-actin was utilized. Blots were visualized and analyzed as described in the foregoing chapter. The results exhibit decreased GluR2 protein levels from lysates of primary hippocampal cultures treated with shDRR1 in comparison to shSCR treated control conditions. Boxplots show average values ± SEM of three individual experiments. As described before, actin was used as loading control and reference protein. DRR1 protein levels were measured to link changes of DRR1 protein amounts with GluR2 protein amounts. For analysis percentage of DRR1 and GluR2 protein band densities were normalized to actin. The control condition shSCR served as standard, was normalized to one and used to calculate relative densities. Analysis and comparison between both conditions reveals a correlation between GluR2 and DRR1 protein amounts. Low GluR2 protein densities go along with low DRR1 densities as it is found by suppressing the protein with shDRR1. A significant difference in the protein level was identified for DRR1 between conditions with a 72% decrease of DRR1 in shDRR1 in average. In case of GluR2 no significant difference was found between conditions. However, we detected a drop in GluR2 protein amount after silencing with shDRR1

by 24.5% in average (Fig 2.9). In case of DRR1 overexpression, we performed two individual experiments, detecting an increase of DRR1 protein amounts of 718.9% percent in average. Due to low sample size and high variability between experiments, the Student's t-test did not reveal a significant difference in DRR1 protein amount between OE and empty control. On the other hand, upon DRR1 overexpression also GluR2 levels raised in comparison to the empty control. Here, the percentual differences lies around 20.6% percent and statistical analysis reveal a significant difference with p = 0.0246.

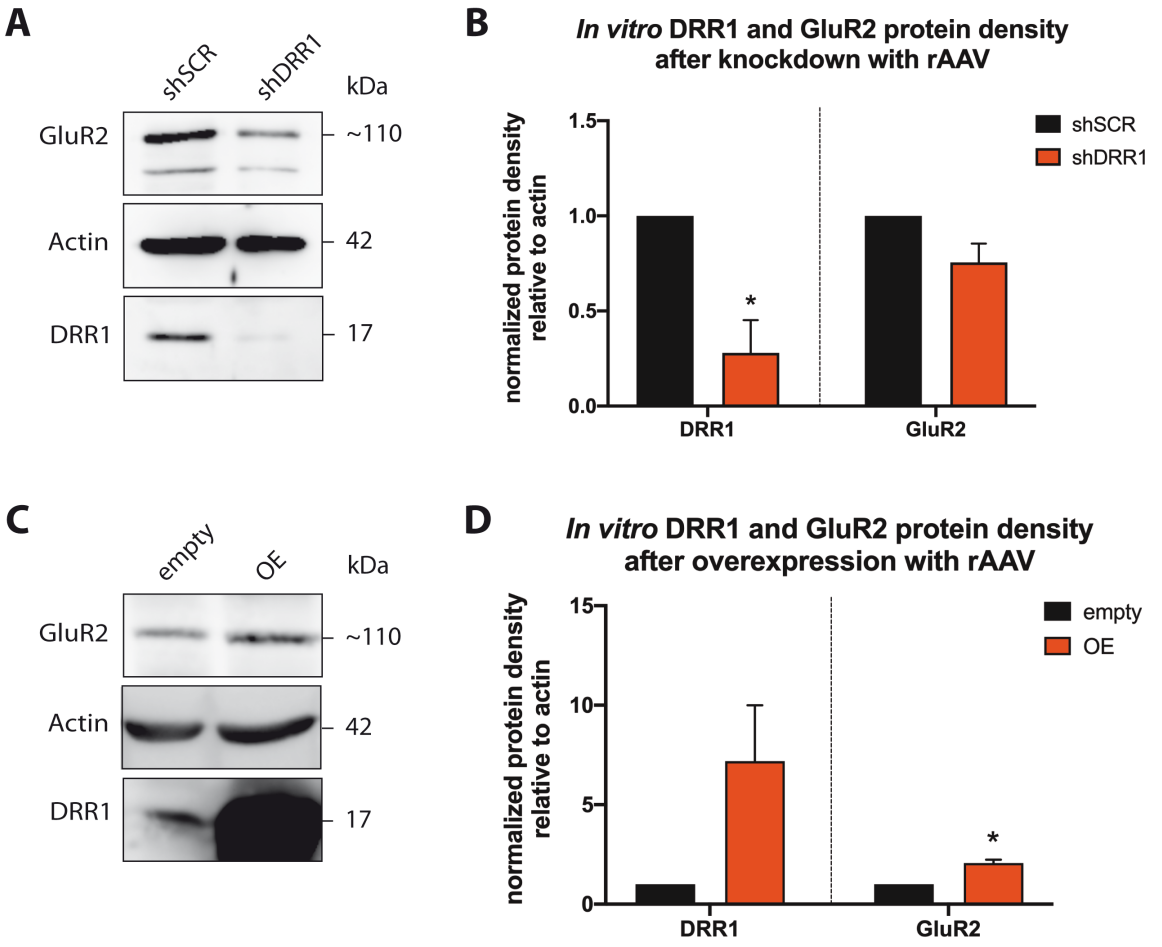

Figure 2.9: Co-expression of DRR1 & GluR2 in cultured hippocampal neurons after transduction with rAAV.
(A, C) Representative Western Blots of primary hippocampal cell lysates after 14 DIV and 7 days post transduction with shSCR as control and shDRR1 for DRR1 knockdown and rAAV overexpression (OE) construct and empty control. **(B, D)** Boxplots show average protein densities from three individual experiments suppressing DRR1 in cultures with rAAV and evaluating DRR1 and GluR2 protein amounts through Western Blot. For overexpression experiment two individual experiments were performed. Controls served as sample control and were normalized to 1. Protein densities represent arbitrary numbers measured as percentage per area. Students t-test performed as statistical analysis. Significant difference of DRR1 between shSCR and shDRR1 conditions; p = 0.014, Significant difference between empty and OE in GluR2 amount p = 0.0246.

2.3.3 Identification of DRR1 and GluR2 protein-protein interaction via proximity-ligation assay (PLA) assay

An elegant way to identify a potential interaction of two proteins either *in situ* or *in vitro* is the proximity ligation assay (PLA). Through this method, two distinct proteins are labeled with primary antibodies each from different species. A pair of oligonucleotide-labeled secondary antibodies is next applied to target primary antibodies. If the PLA probes are in close proximity (less than 40 nm) to each other, added hybridizing connector oligonucleotides can link the PLA probes together and an applied ligase can form a closed circle DNA template. The DNA template serves for rolling-circle amplification (RCA) and the PLA probe acts as primer for a DNA polymerase, that generates tandem repeat DNA during RCA process. The emerging signal is still attached to the PLA probe, multiplying the signal up to 1000-fold. The signals appear as good identifiable dot and allows to localize the signal. These spots can be detected by fluorescent microscopy with following image analysis.

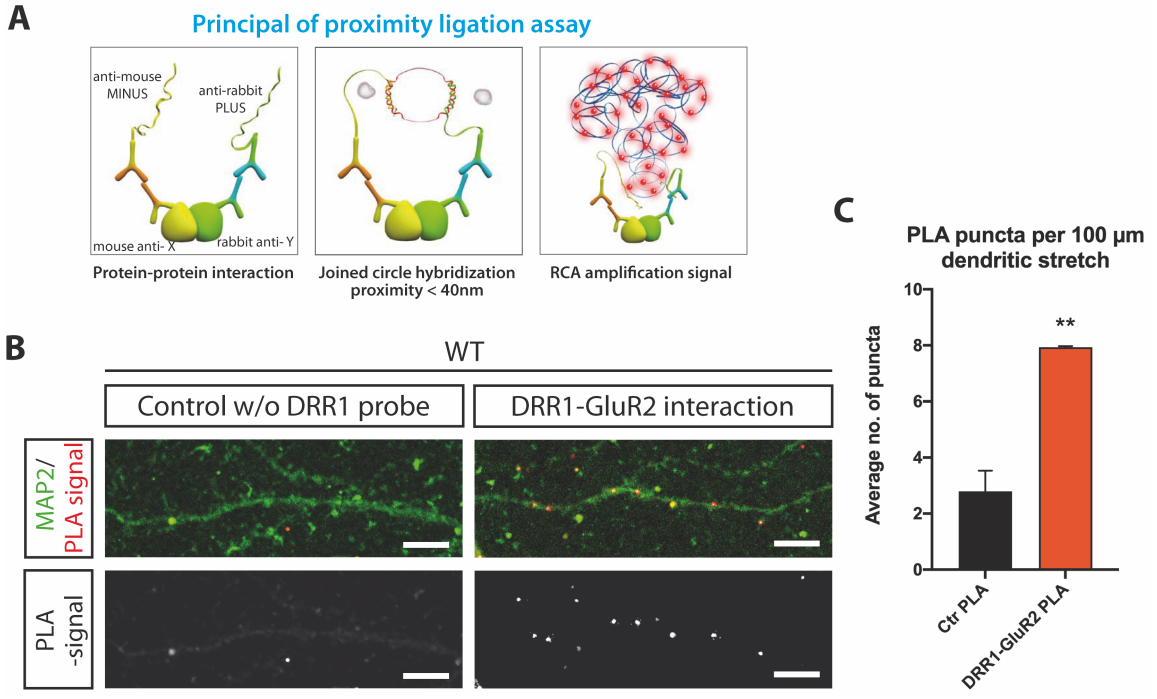

Figure 2.10: PLA to identify protein-protein interaction between DRR1 and GluR2.
(A) Principle of proximity ligation assay (PLA). From left to right: 1. Proteins labeled with different primary antibodies; PLA-probes bind to respective antibodies, 2. When proteins are in close proximity below 40 nm, probes with oligodendrocytes hybridize a joined circle, 3. Rolling circle amplification (RCA) for signal amplification. **(B)** Representative fluorescent pictures of dendritic stretches from primary hippocampal neuron cultures from C57BL/6J mice for negative control without DRR1 primary antibody and PLA-probes to identify DRR1-GluR2 protein-protein interaction. PLA signal is visible through orange/red amplification signal. Upper panel shows PLA-signal combined with MAP2 staining (green). Lower panel shows plain PLA-signal in black and white. **(C)** Average number of PLA-signal as puncta per 100 µm dendritic stretches. Three individual cultures à 5–10 traced cells per experiment. Data represent average values per cultures. Students t-test performed as statistical analysis. High significant differences between experiments, with $p = 0.0023$. Scale bars: 10 µm. Image in A modified from Sigma User Guide Duolink® In Situ Fluorescence.

We applied this method to identify a potential protein-protein interaction between DRR1 and surface GluR2 proteins in wildtype primary hippocampal neuron cultures. The PLA assay was performed *in vitro* for better visualization of PLA puncta location in neuronal cells. Therefore, we cultured hippocampal neurons for 14 days in 24-well plates on coverslips, fixed them with 4% PFA afterwards and thereafter performed the PLA assay according to the manual instructions. For the negative control condition, we out labeling of DRR1 protein. Subsequently, neurons were imaged with a confocal microscope and dendritic stretches of individual neurons were analyzed with Fiji. Therefore, dendritic stretches were circled and puncta within region of interest (ROI) were automatically counted with the plug-in "Find Maxima". The noise for all experiments was set equally to 50, catching all puncta. The ratio between encircled dendritic length and the number of puncta was calculated and extrapolated to a dendritic length of 100 µm for better comparison between groups. As for the negative control, almost none puncta were found along dendrites. The few visible spots were ascribed as unspecific reaction or remaining precipitates of reaction reagent. In contrast, PLA probes specifically binding to DRR1 and GluR2 show high amounts of PLA-signal along dendrites, suggesting a protein-protein interaction between DRR1 and GluR2. Representative pictures of dendritic sections comparing both conditions are found in Figure 2.10. Statistical analysis reveals highly significant difference between conditions with $p = 0.0023$.

2.3.4 Changes in DRR1 expression impact on synaptic scaling

Various changes in synaptic strength maintain a neuron's set point upon activity and environmental changes. These multifaceted mechanisms are known as homeostatic plasticity and serve as compensatory adjustments in response to diverse network activities (Turrigiano, 2008). Synaptic scaling involves changes in surface AMPAR content (Turrigiano, 2008; Forrest et al., 2018). Previous findings associate DRR1 with cell growth regulation and neuronal outgrowth (Mu et al., 2017; Lu et al., 2021; Chen et al., 2022). More importantly, DRR1 was found sharing similar promoter profiles with AMPARs and is co-expressed and co-regulated with AMPAR-subunits (Chong et al., 2007; Schmidt et al., 2011). As actin-interacting protein DRR1 is likely to modulate AMPAR location and function (Revenu et al., 2004, van der Kooij et al., 2015). Schmidt and colleagues (Schmidt et al., 2010) showed a critical role for hippocampal AMPARs in modulating resilience to stress. The combination of its actin-binding properties and regulation upon stress, let us hypothesize that DRR1 could directly regulate localization and function of AMPARs at the synapse. For that, we first analyzed new insertion of surface GluR2 after stimulating cultured primary hippocampal neurons with potassium chloride (KCl).

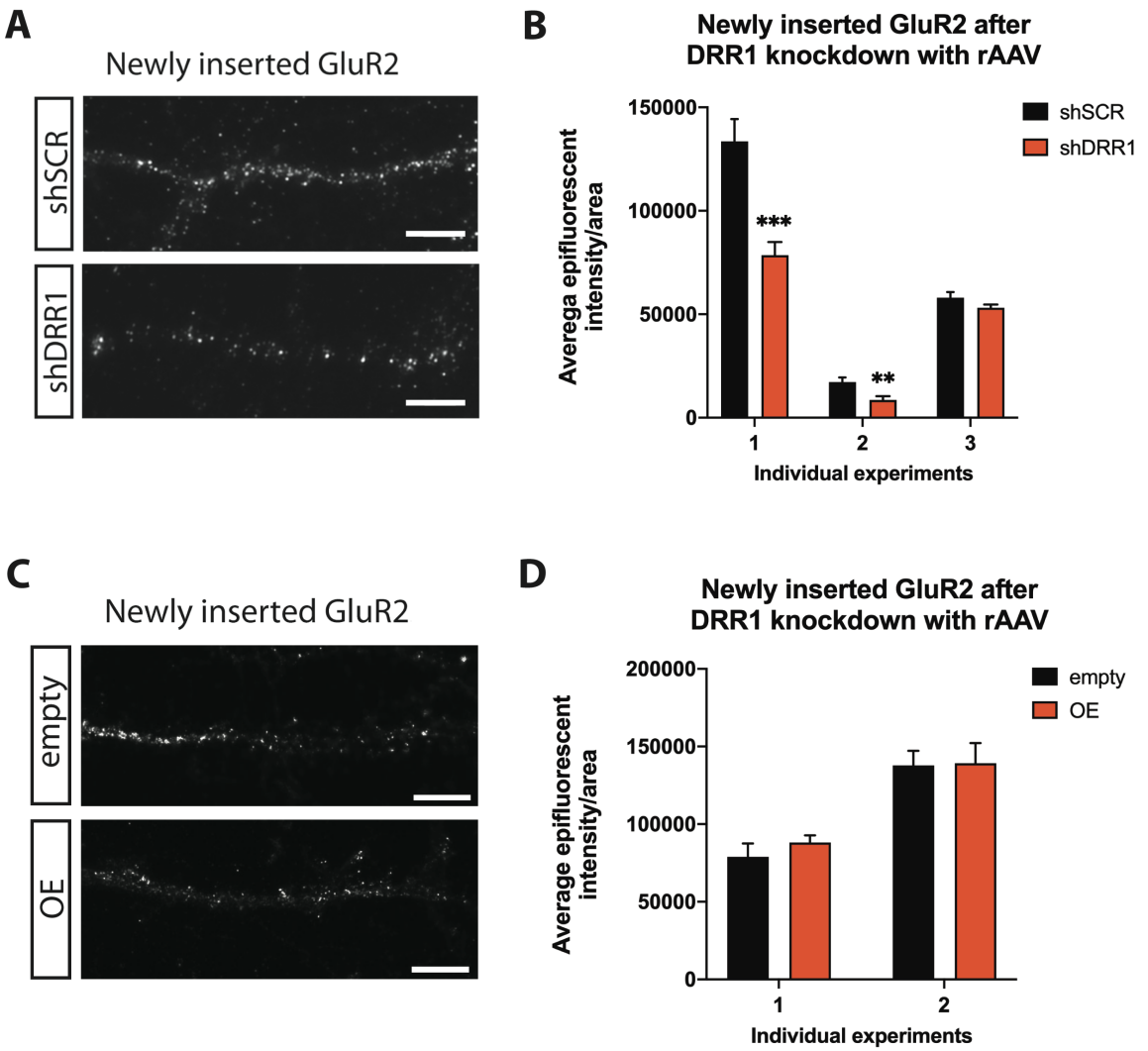

Figure 2.11: Newly inserted surface GluR2 subunits after DRR1 overexpression or knockdown.
(A, C) Representative fluorescent images of dendritic stretches from different experimental conditions. Surface GluR2 is depicted as white dots along the dendrites. **(B, D)** Analysis of individual primary hippocampal cultures per condition. Student's t-test was performed to compare DRR1 OE or shDRR1 with respective controls. Chi-square test was executed to test for independence between experiments. **(B)** T-test Exp1: $p = 0.00003$; Exp.2: $p = 0.0034$, Exp.3: $p = 0.156$. Chi-square: $p < 0.0001$. **(D)** Exp.1: $p = 0.358$; Exp.2: $p = 0.943$. Chi-square: $p < 0.0001$. Boxplots represent average values between cells ± SEM. Per condition 10–41 cells were analyzed. Scale bars: 10 μm.

Beforehand, primary hippocampal neurons were cultured for 14 days and transduced with rAAV after 7 days to either overexpress or suppress DRR1. Prior to stimulation, surface GluR2 proteins were masked and newly inserted antibodies were visualized via immunocytochemistry post-stimulation. In total primary hippocampal cultures from three different embryonic litters were analyzed for newly inserted GluR2 upon overexpression and suppression of DRR1 each. For each condition several secondary dendritic stretches of minimum ten pyramidal cell were analyzed and integrated intensity was measured and normalized per encircled area. Upon DRR1 knockdown, less GluR2 puncta appeared as compared to shSCR. Students t-test revealed significantly decreased integrated intensity for shDRR1 in two experiments and a tendency for a decrease in the third

experiment (Figure 2.11(A)). On the basis of these independent experiments the Chi-square test was executed, showing a highly significant statistical p-value ($p < 0.0001$). This evinces that the differences we observed regarding GluR2 subunit-insertion between conditions do not show different proportions between individual experiments. Representative images, demonstrating a dendritic stretch with surface GluR2 are shown in Figure 2.11(A). Upon DRR1 overexpression results were not significantly different between OE and empty control. (Figure 2.11(D)). Representative images are shown in Figure 2.11(C). It is important to note, that one experiment was discarded as outlier. Due to poor quality of the primary hippocampal culture with high amounts of dead cells, proper cell development of surviving cells was not given within that culture. We wanted to prevent false results through malfunctioning cell mechanisms and GluR2 trafficking by excluding the culture from analysis.

2.4 The influence of DRR1 on the morphology of hippocampal pyramidal neurons *in vitro*

2.4.1 Effects of DRR1 level changes on hippocampal pyramidal cell morphology

DRR1 was found to interact with actin serving as crosslinker protein, important for cell movement (Le et al., 2010). Besides, previous *in vivo* studies suggested an implication for DRR1 in diverse processes requiring the cytoskeleton, such as spine formation and axonal outgrowth (Schmidt et al., 2011, Masana et al. unpublished data). Also, acting-interacting proteins are important to control neuronal actin dynamics through a broad range of actions (Revenue et al., 2004). In addition, DRR1 expression is increased upon stress within the hippocampal area (Schmidt et al., 2011). Interestingly, some actin-interacting proteins are specifically regulated by stress and glucocorticoids (van der Kooij et al., 2015). Increasing evidence exists that they promote cytoskeletal rearrangements to modulate resilience to stress (Golden et al., 2013). Therefore, we aimed to study the morphology of pyramidal neurons upon different intracellular protein levels of DRR1, investigating a potential role for this protein in neurite outgrowth and spine development due to stress. To evoke overexpression of DRR1, mimicking a stress situation, and DRR1 knockdown as opposing effect, we transfected primary hippocampal neuron cultures *in vitro*, utilizing rAAVs.

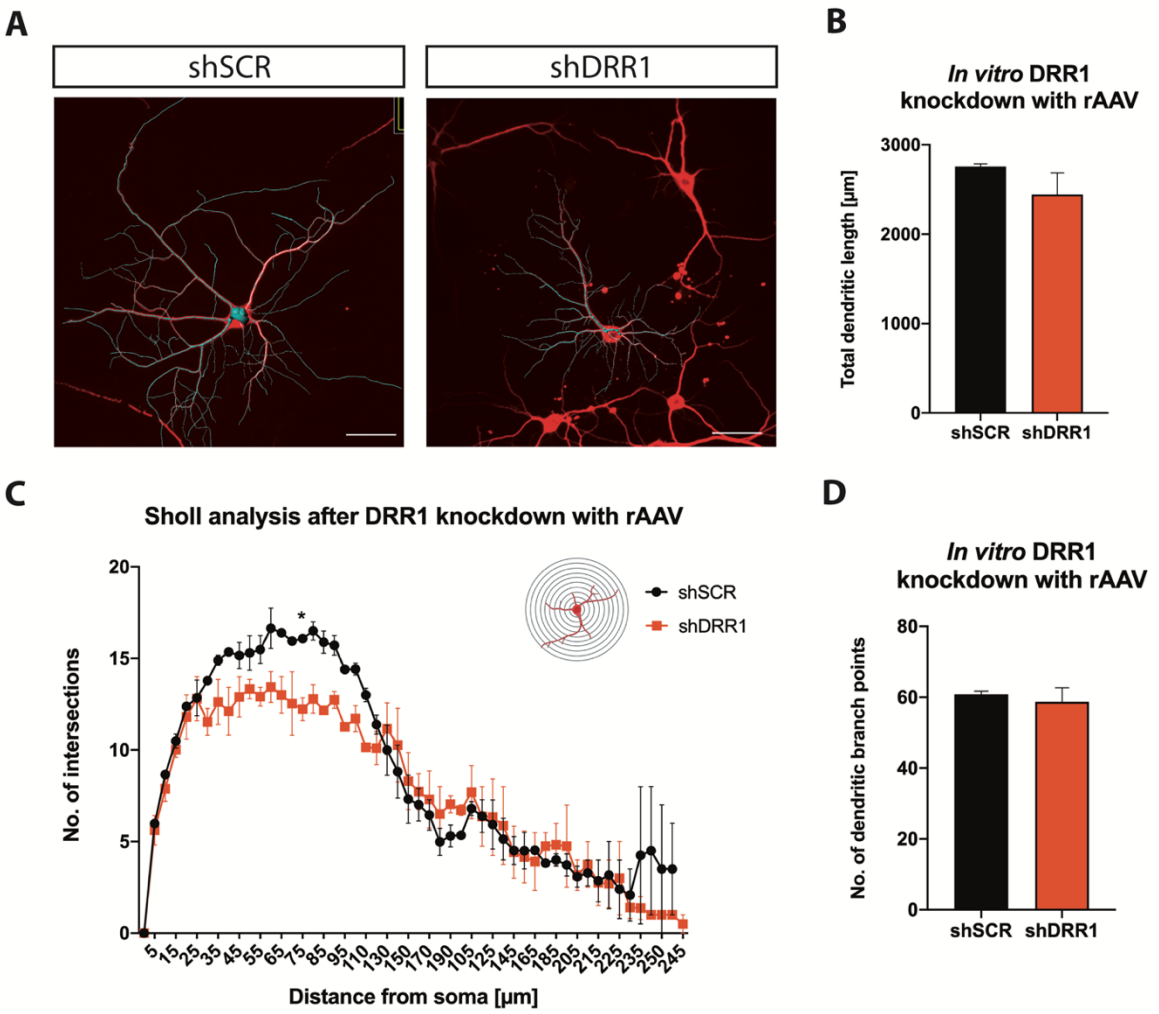

Figure 2.12: Morphological analysis of dendritic trees from primary hippocampal neurons after DRR1 knockdown.
(A) Representative fluorescent images of single neurons cultured for 14 DIV and transfected with shSCR (left) and shDRR1 (right) after 7 DIV. Neurons express GFP throughout the whole cell (red channel). Semi-automated neuronal reconstructions with Imaris are visible in cyan. **(B)** Total dendritic length of both conditions was measured and analyzed with no significant difference between conditions. Boxplots represent average values of two experiments à 6–11 cells. **(C)** Sholl analysis comparing both conditions with number of intersections per 5 µm distance from soma. **(D)** Boxplots show average numbers of dendritic branch points of same cells analyzed in (B). Statistical analysis performed with Student's t-test. Scale bars: 20 µm.

Firstly, we cultured primary hippocampal neurons for 14 DIV with rAAV infection after 7 DIV. The viral constructs each contained a green fluorescent (GFP) cassette, which was expressed throughout the whole neuronal cells when successfully transfected with the virus. In this way, we could identify the complete structure of all transduced cells, either overexpressing DRR1 or suppressing endogenous DRR1. Afterwards cells were fixed with PFA and subsequently DRR1, MAP2, and GFP were fluorescently labeled, before images were obtained using an epifluorescent microscope. Four to eleven cells from two separate experiments were imaged for each condition and semi-automatically traced with Imaris filament tracer tool (details see Materials). Afterwards, Sholl analysis was performed. Thereby, the MAP2-signal was used to quantitatively assess

morphological characteristics of dendritic trees of pyramidal neurons. Sholl analysis counts the number of dendritic intersections with increasing concentric circles originating from the middle of the cell body with a gradual increase (5 µm steps). Figures 2.12 and 2.13 show representative primary hippocampal neurons stained against MAP2 with overlaid reconstructions and tracings for the different conditions. The cells were previously transduced with rAAV, expressing GFP as transfection control. No significant differences in total dendritic length or number of branch points were detected when overexpressing or suppressing DRR1 in comparison to respective controls.

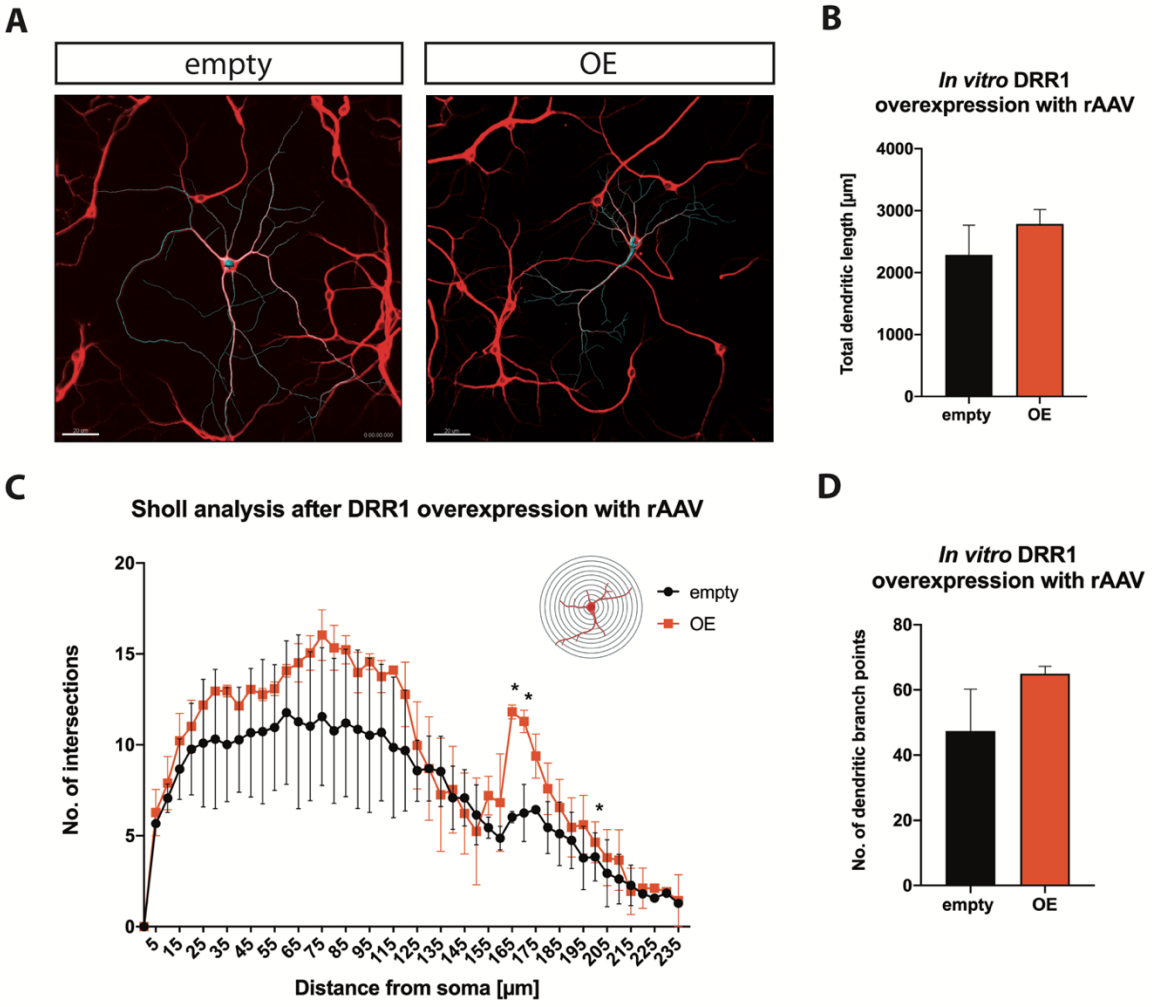

Figure 2.13: Morphological analysis of dendritic trees from primary hippocampal neurons after DRR1 overexpression.
(A) Representative fluorescent images of single neurons cultured for 14 DIV and transduced with empty control (left) and OE (right) after 7 DIV. Neurons express GFP throughout the whole cell (red channel). Semi-automated neuronal reconstructions with Imaris are visible in cyan. **(B)** Total dendritic length of both conditions was measured and analyzed with no significant difference between conditions. Boxplots represent average values of two experiments à 4–11 cells. **(C)** Sholl analysis comparing both conditions with number of intersections per 5 µm distance from soma. Significant differences at 165 µm, 170 µm, and 195 µm from soma with $p = 0.03$, $p = 0.019$, and $p = 0.026$ respectively. **(D)** Boxplots show average numbers of dendritic branch points of same cells analyzed in (B). Statistical analysis performed with Student's t-test. Scale bars: 20 µm.

Nonetheless, there is a tendency for decreased dendritic length and branch points upon DRR1 knockdown (Figure 2.12) and a tendency for increased dendritic length and number of branch points when the protein is overexpressed (Figure 2.13). Further, Sholl analysis of dendritic trees revealed decreased numbers of intersections in the case of shDRR1 transduced cells in comparison to shSCR control (Figure 2.12(C)). Conversely, number of intersections is increased in DRR1 overexpressing cells as compared to controls. These results suggest a tendency for dendritic outgrowth to reduce when DRR1 is suppressed, whereas dendritic outgrowth increases when DRR1 is overexpressed (Figure 2.13(C)).

2.4.2 Effects of DRR1 level changes on spine morphology in hippocampal neurons *in vitro*

It is commonly known that synaptic plasticity depends on actin-based growth and re-modeling of dendritic spines, ultimately leading to lasting consequences on synaptic efficacy and complex behavior. DRR1 is interacting with actin as well as GluR2 AMPAR subunits as previously shown in Chapter 2.3. In addition, Schmidt and colleagues found a decreased LTP signal within the hippocampus upon local overexpression of DRR1 *in vivo*. Additionally, DRR1 was found to control assembly and disassembly of actin filaments (Kretzschmar et al., 2018). Also, a positive correlation exists between spine shape and synaptic strength. Moreover, studies revealed an involvement of cytoskeletal rearrangements involving the actin-binding protein cofilin-1 in modulating stress resilience. The study revealed stress induced negative effects in non-resilient animals, increasing the formation of immature spines through redistribution of synaptic cofilin-1 (Golden et al., 2013).

Since spines are specially actin-rich structures, we investigated spine formation in primary hippocampal neuron cultures consequent to different DRR1 levels that were reached through transduction with rAAV.

Special attention was given to investigate spine density and the composition of distinct spine classes of neurons exhibiting changed endogenous DRR1 protein expression in comparison to subsequent control groups. Later were transduced with an empty vector in case of the control group for DRR1 overexpression or shSCR vector in case of DRR1 suppression. Thus, the control groups underwent the same mechanism of viral transduction with equal toxicity, but without manipulating the endogenous amount of DRR1 protein.

This experiment ought to answer the question whether DRR1's interaction with actin filaments and most importantly its different intracellular levels impact on the assembly and disassembly of actin filaments on the level of dendritic spines, changing its morphology and proper

formation and development. To distinguish transduced versus not transduced cells, the rAAV construct included an incorporated GFP sequence, which was simultaneously expressed upon successful viral DNA integration and protein translation within the cells. After 14 DIV and 7 days post-transduction (dpt), the cells were fixed, immuno-stained against GFP, DRR1 and MAP2 (for details see Materials and Methods), a neuron-specific intracellular protein associated with microtubules, mainly present in dendrites. For tracings of dendritic arborization MAP2 staining was utilized as visualization of neurite formations. The ubiquitous cell expression of GFP was utilized for quantitative spine analysis. First, GFP was tagged with an Alexa 488 fluorophore via immuno-staining to increase its immunofluorescent signal. After imaging three neurons per condition with an epifluorescence microscope, approximately 30 µm dendritic stretches of secondary dendrites per cell were chosen for quantitative spine analysis using filament tracer and spine classifier plug-in in Imaris. Dendrites were imaged as z-stack and displayed as three-dimensional (3D) image within Imaris, whereas the images shown in Figure 2.14(A) and Figure 2.15(A) present two-dimensional (2D) cutouts. The total number of spines per dendritic stretch was normalized and the average number was calculated throughout one condition and between three experimental replications. The spine densities are depicted in average numbers per 1 µm dendritic stretch, whereas the classification in different spine subtypes is outlined in average number of spines per 30 µm stretch.

Figure 2.14 illustrates representative dendritic stretches of GFP-positive, thus successfully transduced neuronal cells. The left panel in (A) shows the plain GFP staining of dendritic stretches. The left upper panel features the shSCR control condition. Herein, a rich density of spines is visible as well as different spine sizes and formations upon the depicted dendritic stretch. In comparison, the left lower panel shows the shDRR1 knockdown condition, where spine density seems to be lower. The right lower and upper panels show Imaris reconstructed dendritic stretches together with reconstructed spines for each condition. Additionally, spines were categorized with the spine classifier corresponding to their known properties within primary hippocampal cultures (such as spine head size, neck length and total length) into four distinct subtypes. A red color code illustrates stubby spines, lilac color pictures long thin spines, blue demonstrates long thin spines and green illustrates mushroom spines. Analysis of both groups determined a significantly reduced spine density in shDRR1 compared to shSCR dendritic stretches (Figure 2.14(B)). Moreover, statistical comparison between groups (Figure 2.14(C)) revealed significant differences between individual spine classes, with a clear reduction in number of long thin and filopodia spines in shDRR1 compared to shSCR treated cells. Filopodia spines are long protrusions that upon repeating signal transduction mature into proper spines. However, upon missing signal transduction filopodia can retract again. Long thin spines are fully developed spines with a clear neck and head domiciling a proper postsynaptic density.

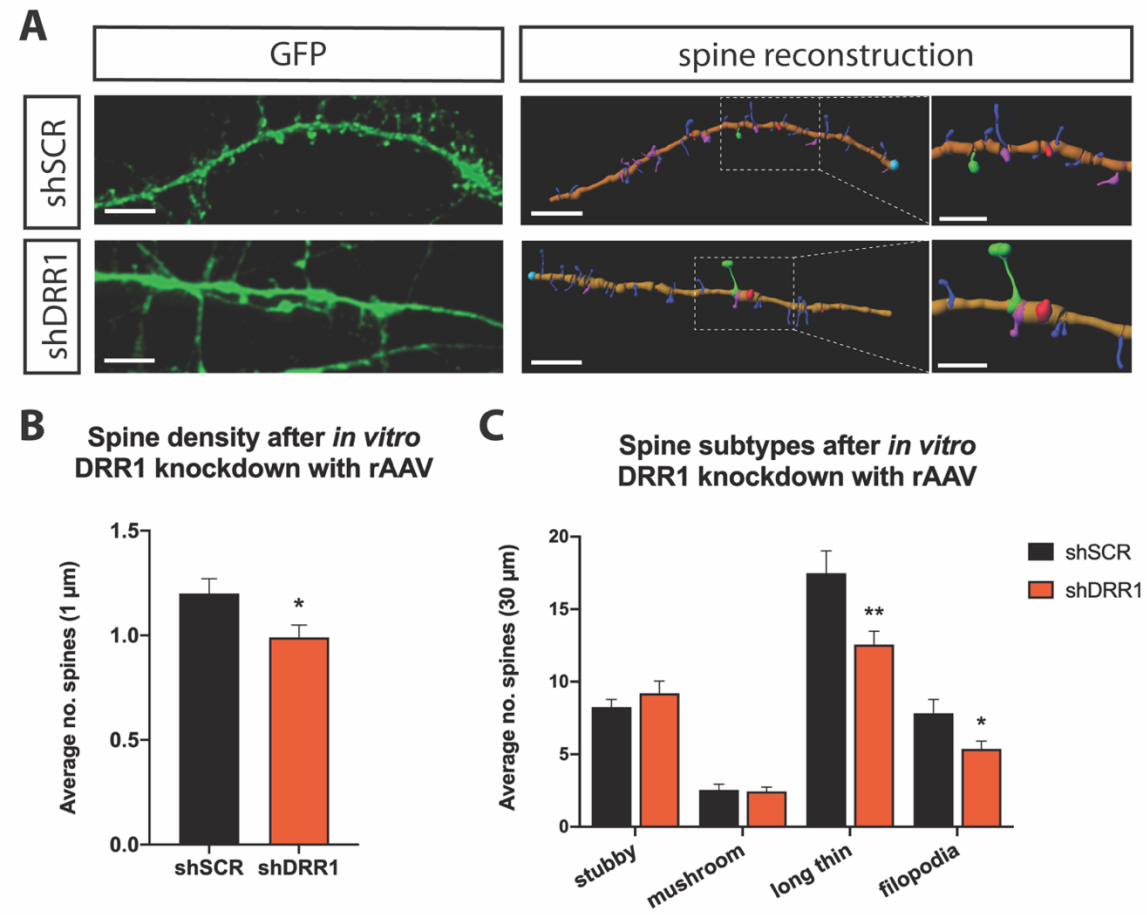

Figure 2.14: Silencing DRR1 reduces spine density and the number of immature spines.
(A) Representative images of GFP-positive dendritic neuronal stretches of primary hippocampal neuron cultures treated with rAAVs containing either shDRR1 for DRR1 knockdown or shSCR as control. Before (left) and after (right) tracing and spine re-construction with Imaris. Images show GFP signal (green). Close-ups of the re-constructed spines on the right side. **(B)** Quantification of total spine numbers and different spine classes per 1 µm dendritic stretch. **(C)** Differentiation between the major spine types from left to right: Mushroom (mature, with big spine head and large postsynaptic density; green), stubby (without a neck; red), long thing (with a neck and a small head; blue), filopodia (developing spine without a head, magenta). Scale bars: 4 µm (left and middle panels in A), 2 µm (right panels in A). Per conditions n=3 experiments; n=3 analyzed neuronal cells with 3–9 dendritic stretches per cell; averaged numbers per condition, normalized to 1 µm; unpaired t-test ns > 0.05; *p < 0.05; **p < 0.01; ***p < 0.01. In (B) p = 0.02; in (C) p-values for long thing p = 0.0057; filopodia p = 0.022.

In contrast to mushroom spines with a bigger head to neck ratio, long thin spines are less stable and less persistent. The number of mushroom spines between knockdown and control group appears to be unchanged and the stubby spines that don't depict a head are slightly increased in number within the shDRR1 group, but without showing a significant tendency. Altogether, silencing of DRR1 seems to impact on the formation of new spines, expressing itself in diminished overall spine density along secondary dendrites and the decline of spine subtypes that represent immature states of these synaptic connections. Figure 2.15(A) shows representative tracings of 30 µm long dendritic stretches of primary hippocampal neurons *in vitro* after transfection with rAAV constructs for overexpressing DRR1 or respective empty control vector. Left panels show GFP signal of successfully transduced neuronal cells for OE or empty conditions.

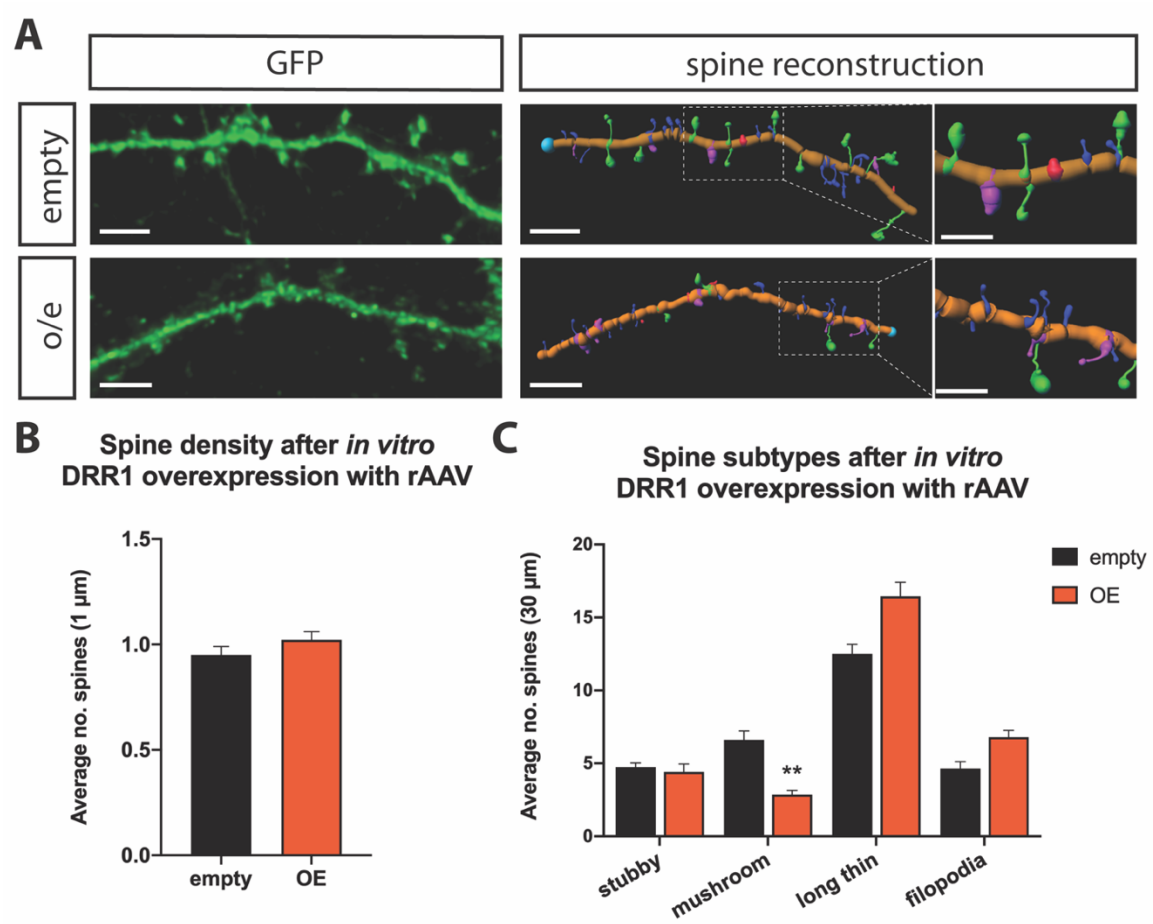

Figure 2.15: Silencing DRR1 reduces spine density and the number of immature spines.
(A) Representative images of GFP-positive dendritic neuronal stretches of primary hippocampal neuron cultures treated with rAAVs containing additional *DRR1* gene for overexpression or empty vector as control. Before (left) and after (right) tracing and spine re-construction with Imaris. Images show GFP signal (green). Close-ups of the re-constructed spines on the right side. **(B)** Quantification of total spine numbers and different spine classes per 1 µm dendritic stretch. **(C)** Differentiation between the major spine types from left to right: Mushroom (mature, with big spine head and large postsynaptic density; green), stubby (without a neck; red), long thing (with a neck and a small head; blue), filopodia (developing spine without a head, magenta). Scale bars: 4 µm (left and middle panels in A), 2 µm (right panels in A). Per conditions n = 2 experiments; n = 3 analyzed neuronal cells with 2–4 dendritic stretches per cell; averaged numbers per condition, normalized to 1 µm; unpaired t-test ns > 0.05; *p < 0.05; **p < 0.01; ***p < 0.01. In (C) p-values for mushroom p = 0.0055.

The left upper panel features the empty control condition. Notably, bigger spines are visible in the control condition in comparison to OE. The right panels represent spine reconstructions of dendritic stretches for each condition. The color code is similar to Figure 2.14. Analysis displays no significant differences in spine density between conditions (Figure 2.15(B)). However, statistical comparison between groups (Figure 2.15(C)) indicates a significant difference of mushroom spine amounts, with a decrease in DRR1 overexpressing neurons. Thereto, immature filopodia spines and long thin spines seem to increase in number when DRR1 is overexpressed, although they don't show significant differences between groups. When pulling these classes of spines together, a significant difference appears with p=0.015. In summary, DRR1 overexpression appears to create a shift of spine classes by reducing the development of mature mushroom spines, while increasing

the number of small and immature spines. Ultimately, the same spine density as under normal conditions is maintained.

2.5 Stressed hippocampal neuron cultures and DRR1

Clear evidence for a glucocorticoid-dependent regulation was shown for DRR1 in forgoing *in vivo* studies (Liebl et al., 2009; Schmidt et al., 2011; Masana et al., 2014). Together with the fact that DRR1 expression requires GR homo-dimerization (Schmidt et al., 2011) this already indicates that DRR1 is directly involved in the stress-response system. Furthermore, stress-induced GR activation leads to elevated DRR1 protein amounts in the hippocampus (Schmidt et al., 2011). Interestingly, GR-mediated mechanisms are characteristic for late-phase responses to stress (De Kloet et al., 2005; Yau & Seckl, 2012). On top, local overexpression of DRR1 in the hippocampal CA3-area of mice reduced local spine densities and hippocampal LTP, while cognition and cognitive flexibility were improved (Schmidt et al., 2011). Those findings indicate a temporal segregation of morphological features and behavioral outcomes, in support of the belief that spines provide neuroprotective qualities rather than being integrally implicated in learning processes (Segal, 2010).

By now there are numerous studies pointing detrimental effects evoked by stress on the morphology of neurons, especially spine loss (Pavlides et al., 1996; Sousa et al., 2000; Donohue et al., 2006; Kavushansky et al., 2006; Chen et al., 2008). The hippocampus displays a particularly stress-sensitive region enriched with glucocorticoid receptors (Reul & de Kloet, 1985). Its vulnerability to stress reflects in dendritic remodeling and neuronal cell damage and loss (Uno et al., 1989/1990).

It is intriguing to study actin-binding proteins and their link with stressful environmental events changing neuronal morphology, plasticity and the resulting complex behavior. Synaptic neuroplasticity requires reorganization of the actin-cytoskeleton (Cingolani & Goda, 2008; Hotulainen & Hoogengraad, 2010; Lamprecht, 2014) which are governed by ABPs including DRR1. Dexamethasone (DEX) is a potent synthetic agonist of the glucocorticoid receptor. It is well established to mimic stress situations in rodents via subcutaneous (s.c.) injections. *In vivo* DEX injections elevated *DRR1* mRNA throughout the whole murine brain 8 h post-injection (Masana et al., 2018). Significant elevations were especially found in brain areas with high basal DRR1 expression such as the hippocampal CA3-region (Masana et al., 2018).

2.5.1 Stress induced by DEX leads to increased DRR1 and GluR2 protein levels *in vitro*

We wanted to examine a correlation between DEX application and the function of DRR1 *in vitro*. DEX treatment served as global stress induction, mimicking physiological stress conditions. For that, we applied DEX onto primary hippocampal cultures after 12–13 DIV for either 24 h or 48 h. Subsequently, cells were lysed at DIV 14 and Western Blot analysis were performed to evaluate DRR1 and GluR2 protein amounts. Previous *in vivo* experiments utilized a concentration of 10 mg/kg body weight (Masana et al., 2018).

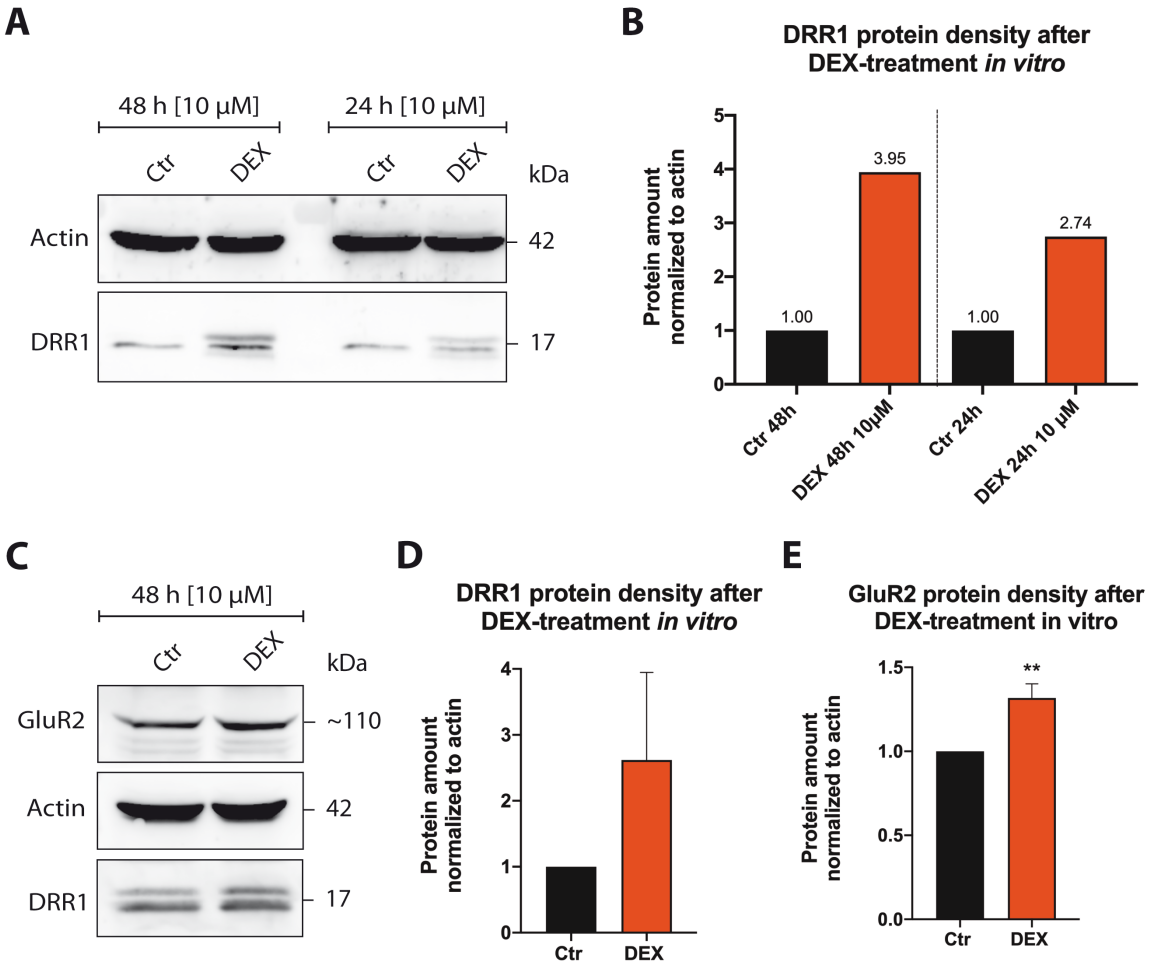

Figure 2.16: DEX application on primary hippocampal neuron cultures.
(A) Representative Western Blot images of test experiment for duration of primary hippocampal cultures with 10 µM DEX treatment. Actin served as house-keeping protein. DRR1 shows stronger bands in DEX treated conditions. **(B)** Quantification of DRR1 protein amount after DEX treatment shows increases after 24 h and 48 h (n=1). **(C)** Representative Western Blots for GluR2, actin and DRR1 proteins in primary hippocampal cultures after 48 h DEX application to the culture. GluR2 and DRR1 bands show slightly stronger protein density as compared to control. **(D)** Protein bands from two individual cultures after 48 h DEX were analyzed and averaged. DEX treated condition shows increased DRR1 amount (n = 3). **(E)** Independent experiments with same experimental conditions reveal significantly increased GluR2 protein (n = 3, p = 0.003). Unpaired t-test ns > 0.05; *p < 0.05; **p < 0.01; ***p < 0.01.

Literature reveals an apoptotic effect after DEX-treatment on chondrocytes starting at 25 μM concentrations after 48 h of application (Chrysis et al., 2005). Other sources report a delay in apoptosis when human and rat hepatocytes were treated with 50 μM DEX (Bailly-Maitre et al., 2001). To avoid cell death due to DEX toxicity, we performed preliminary experiments testing duration of DEX-treatment with 10 μM concentrations. Since dexamethasone was solved in EtOH we used controls containing EtOH without DEX. Both, 24 h as well as 48 h of DEX treatment increased DRR1 protein levels, whereas longer treatment evoked higher DRR1 increases as compared to the respective control (Figure 2.16(B)). Representative Western Blots are shown in Figure 2.16(A). Next, we wanted to elucidate, whether a DEX-induced stress response in cultured neurons also impacts on GluR2 protein levels. We performed two individual experiments, applying DEX for 48 h and measured the protein levels of DRR1 and GluR2 via Western Blot. Analysis of the Blots revealed a significant increase in GluR2 levels after DEX treatment in comparison to the control condition (Figure 2.16(E)). As expected from the forgoing experiment, also DRR1 protein levels were increased after increasing the sample size (Figure 2.16(D)). The results confirm the link between the stress-induced protein DRR1 and glutamate receptors in form of elevated GluR2-subunbnits upon activation of a physiological stress cascade via treatment with the artificial glucocorticoid DEX. Further, the results confirm that the effects of DRR1 overexpression indeed mirror a stressed situation, where DRR1 and GluR2 proteins are elevated (Chapter 2.3).

2.5.2 DEX-treatment shows no impact on newly inserted GluR2 subunits in primary hippocampal neuron cultures

We showed before that overexpression and suppression of DRR1 in primary hippocampal neuron cultures has consequences on the new insertion of the AMPA receptor subtype GluR2 upon stimulation. However, overexpression and suppression of DRR1 via rAAV imitate a detached mechanism of the stress-reaction. DEX application resembles a more physiological activation of the stress response as it represents a glucocorticoid receptor agonist. Consequently, DEX-treatment represents a more physiologic manner of inducing DRR1 increases and a neater way of testing whether this impacts on AMPARs. In the prior chapter we showed that DEX is able to elevated DRR1 as well as GluR2 levels in primary hippocampal neuron cultures. To investigate the role of DRR1 coupled to the whole stress-response, we analyzed the trafficking of GluR2 to the membrane surface after stimulation of primary hippocampal cultures, applying the newly inserted AMPAR assay.

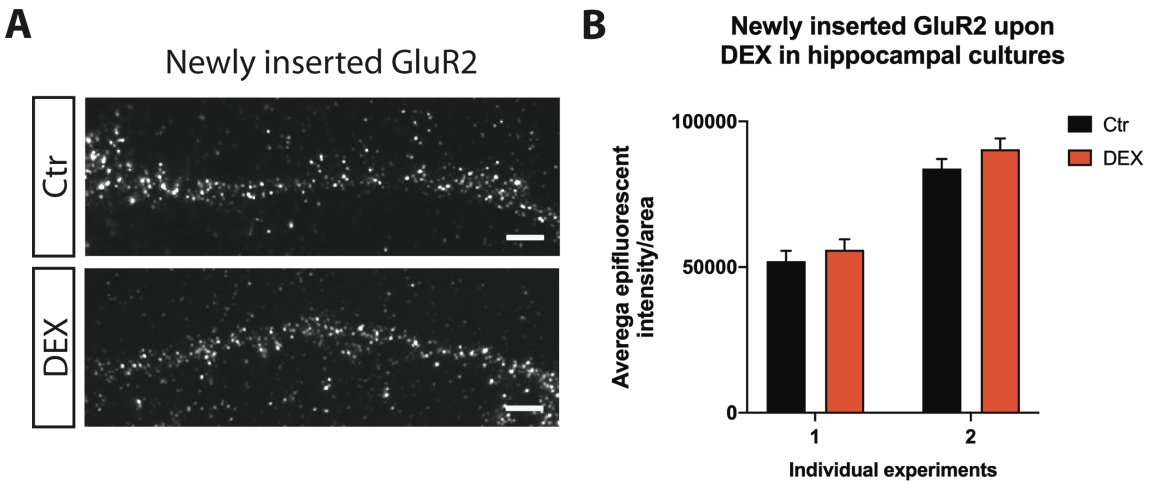

Figure 2.17: Newly inserted surface GluR2 after stress-induction with DEX.
(A) Representative fluorescent images of dendritic stretches from different experimental conditions. Surface GluR2 is depicted as white dots along the dendrites. **(B)** Analysis of two individual primary hippocampal cultures per condition. Student's t-test was performed to compare DEX-treated cultures and control. No significant differences between conditions. Boxplots represent average values between cells ± SEM. Per condition 20–24 cells were analyzed. Scale bars: 10 μm.

As before, surface GluR2 subunits were masked in cultures at DIV 14 after 48 h of DEX treatment and prior to stimulation with KCl. Afterwards, cells were cultured for another ~3 h and newly inserted GluR2 subunits were fluorescently labeled after PFA-fixation. Fluorescent intensity of 2–3 dendritic stretches per 20-24 neuronal cells were measured per experiment and compared to the control condition without DEX treatment. Representative dendritic stretches with surface GluR2 staining are represented in Figure 2.17 as black and white images for both conditions. In total two experiments were performed and values were averaged and analyzed. Similar to DRR1 overexpression experiments, no significant differences in surface GluR2 were detected between DEX-treated and control cells (Figure 2.17 (B)). The results suggest that mechanisms exist to counterbalance the DRR1-triggered effects on GluR2 trafficking.

2.6 The consequences of chronic social defeat stress on hippocampal spines of stress-resilient versus stress-susceptible mice

Long lasting stress promotes changes in synaptic plasticity through dendritic alterations and spine loss (Sousa et al., 2000; Donohue et al., 2006; Kavushansky et al., 2006; Chen et al., 2008). Stress is known to affect hippocampal LTP as well as hippocampal morphology and cognitive performances negatively (Garcia et al., 1997; McEwen, 1999; Diamond & Park, 2000; Diamond et al., 2004; Lee et al., 2009). Alterations in dendritic spine densities seem to play a role

in stress-induced depression-like behavior in mice. An integral study, revealed differences in spine densities in several brain regions between resilient and susceptible mice after CSDS (Qu et al., 2018). Strikingly, decreased spine numbers were specially located in the hippocampal CA3-region and DG of susceptible mice in comparison to control animals, whereas resilient mice showed no changes in spine densities (Qu et al., 2018).

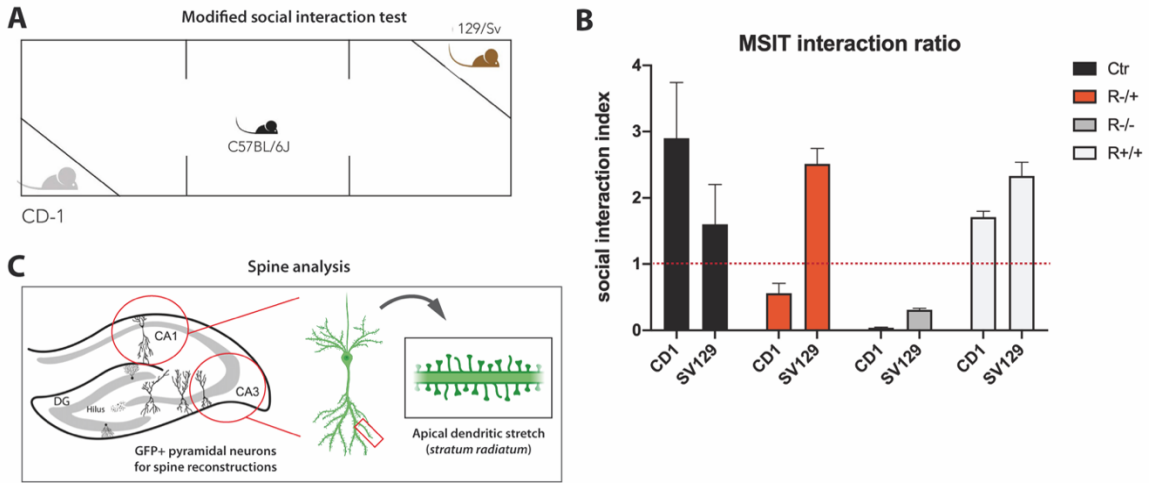

Figure 2.18: Modified social interaction test (MSIT); behavioral classification; procedure of spine analysis.
(A) Experimental setup for MSIT. Sociability arena is divided into three equal parts (60 × 40 cm) which are separated by transparent plastic walls with openings to allow movement in between. Each end of the outer sides occupies a mesh enclosure where either a novel CD-1 mouse is placed or a brown mouse of the 126/Sv strain matching the sex, size and age of the CD-1 mouse. CSD stressed *Thy1*-GFP male mouse (C57BL/6J background) was introduced in the middle part of the arena after CSDS twice for 2.5 min. Whereby the first time, served for habituation without a CD-1 mouse. The interaction time of the mouse of interest with enclosed mice was measured. **(B)** In total ten animals underwent CSDS and were tested in the MSIT to categorize them into stress susceptible ($R^{-/-}$), non-learners ($R^{+/+}$), and resilient ($R^{-/+}$). Additionally, five animals served as controls with handling only. Animals showing abnormal behavior, where excluded for following analysis. Animal no.: four controls (black), three $R^{-/+}$ (red), three $R^{-/-}$ (dark gray), and two $R^{+/+}$ (light gray). MSIT and analysis of results were performed by Dr. Esin Candemir (Müller laboratory, Mainz). Social interaction indices presented as mean ± SEM. Social interaction index: time [%] spent exploring each strain during test phase divided by average of time [%] exploring the two empty mesh enclosures during habituation phase. Classification into resilient (index ≥ 1) or susceptible (index ≤ 1) based on their interaction time with CD-1 mouse (previously defined by Golden et al., 2011). **(C)** Schematic overview of region of interest. Dendritic stretches of GFP^+ CA1 and CA3 pyramidal neurons in *stratum radiatum* were acquired and reconstructed using Imaris software. Left: Modified from Conrad et al., 2017. Right: templates adapted by BioRender.com (2023). Retrieved from: https://app.biorender.com/biorender-templates.

So far, the detailed morphological differences in regard of distinct spine classes were not elucidated between resilient versus susceptible individuals. Hence, we performed CSDS with male C57BL/6 (Thy1)-GFP positive mice and analyzed spine density in combination with spine classification in CA1- and CA3-areas. The Thy1-promoter is specific for neuronal cells. Using (Thy1)-GFP positive animals we assured GFP expression throughout whole pyramidal neuron cells, including spine structures to ease the reconstruction with Imaris of dendritic stretches and spines. To classify the animals in resilient and susceptible, we performed the modified social interaction test offering the animals a simultaneous choice between an unknown mouse of the

aggressor's strain or an unknown mouse of a different strain (Ayash et al., 2020). The paradigm was designed to distinguish three behavioral categories with more sensitivity characterizing resilient and susceptible animals, excluding mice who fall through the categorization. Resilient animals compare with so-called "learners", who avoid the aggressor's strain, but interact with the unknown strain normally ($R^{-/+}$). Mice are viewed as susceptible when they avoid both strains ($R^{-/-}$) and "non-learners" are animals that do not learn from the CSDS paradigm and still do interact with both strains ($R^{+/+}$). The behavioral experiments were performed in collaboration with Dr. Esin Candemir from the Mueller laboratory at the Institute for Molecular Biology in Mainz. In total, fifteen (Thy1)-GFP male mice subjected to the behavioral experiments. Five animals served as control and were introduced in a new cage without an aggressor. Remaining ten animals underwent CSDS with subsequent classification through MSIT (modified social interaction test) paradigm. Eventually, according to their individual MSIT interaction ratio, three animals were categorized into $R^{-/+}$ and $R^{-/-}$ respectively and two animals could be categorized into the $R^{+/+}$ group (Figure 2.18). In the process, three animals were excluded from analysis due to abnormal behavior in the MSIT. Next, mice were sacrificed two hours after MSIT paradigm with subsequent PFA perfusion. Afterwards coronal brain sections were prepared and immunohistochemistry was performed against GFP and DRR1. We reconstructed 30 µm dendritic stretches of secondary apical dendrites from either CA1 or CA3 pyramidal neurons on confocal images utilizing Imaris filament tracer tool. Beforehand, images were deconvolved utilizing Huygens software, to reduce unspecific signal and background signal. Afterwards, spines were classified using the spine classifier. Spine classes were programmed according to typically characteristic features of pyramidal spines in the hippocampus known from the literature. We included spine head sizes, length of neck and head/neck ratio as distinctive parameters.

Representative pictures of stretches from apical secondary dendrites located in the *stratum radiatum* and corresponding 3D-reconstructions including spines are depicted in Figure 2.19 for CA1 region and in Figure 2.20 for CA3 region. For each animal, 5–7 dendritic stretches from different pyramidal cells located in diverse coronal sections were analyzed. Statistical analysis revealed no significant differences in total spine densities between resilient, susceptible and non-learners compared to the control group, nor in CA1 and neither in CA3 region. However, spine classification in CA1 determined differences in spine classes in different groups (Figure 2.19(C)). In comparison to the control group, resilient animals show almost the same phenotype regarding distinct spine classes with no significant differences except for filopodia, where numbers were increased. A significant difference in filopodia also manifest when comparing non-learners ($R^{+/+}$) with resilient mice ($R^{-/+}$), whereby in the $R^{+/+}$ group numbers of filopodia are lower. Long thin spines seem to be unaffected within the different conditions, provided that there are no statistical significances. However, numbers of long thin spines are elevated in average in $R^{+/+}$ and $R^{-/-}$ groups.

Interestingly, stubby spines were considerably lower in $R^{+/+}$ and $R^{-/-}$, whereas numbers of mushrooms spines were significantly elevated in those groups in comparison to the control. Reconstructions of dendritic stretches and spines within the CA3 area, were performed by Maximilian Ken Kracht (PhD-Student) from our laboratory (AG Acker-Palmer). Interestingly, no morphological differences were detected in spine classes between groups within the CA3 area (Figure 2.20(B)/(C)).

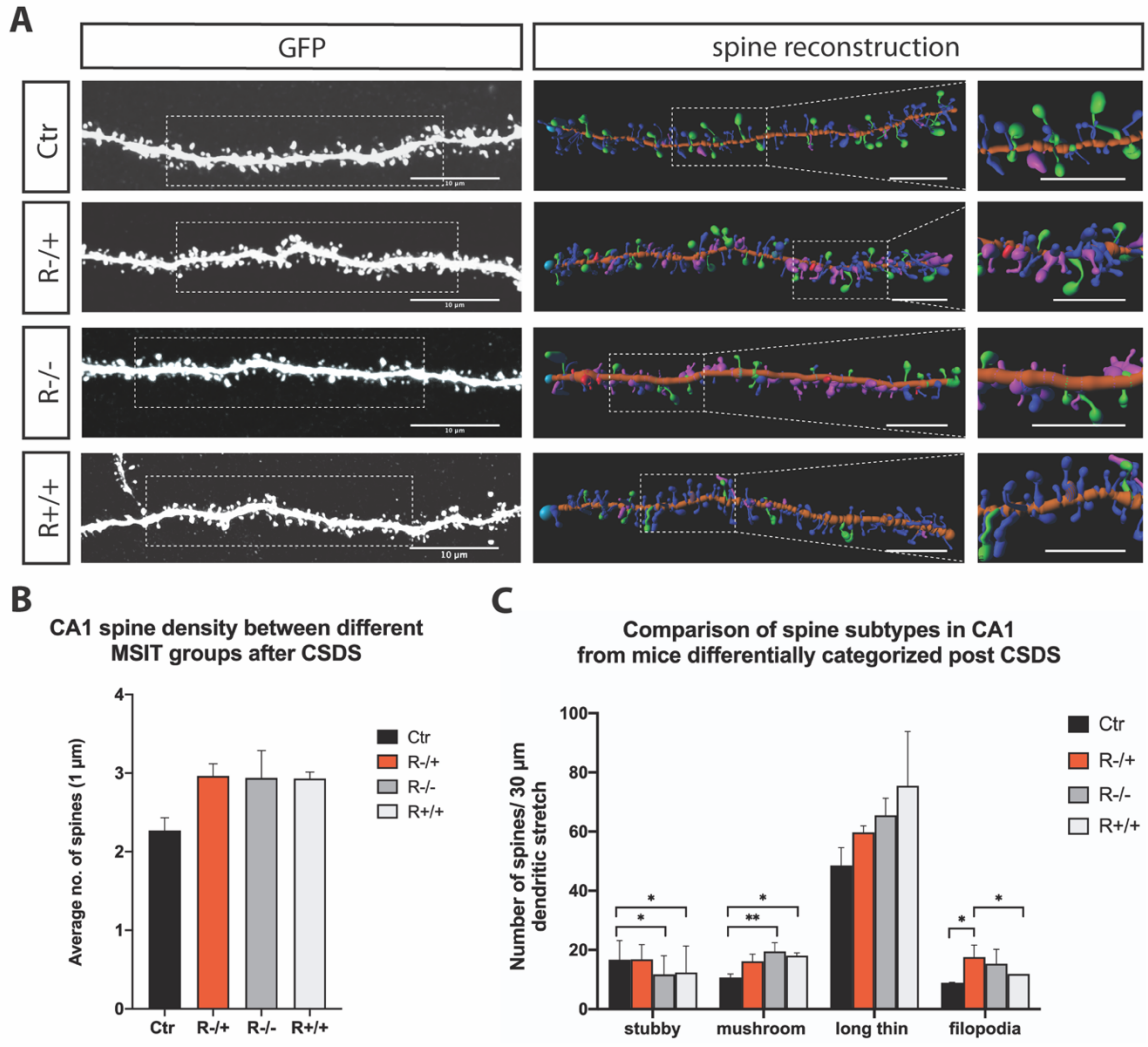

Figure 2.19: CA1 spine morphology after chronic social defeat stress.
(A) Representative fluorescent images (2D, z-stack) of dendritic stretches in *stratum radiatum* from CA1 pyramidal neurons of resilient ($R^{-/+}$), susceptible ($R^{-/-}$), non-leaners ($R^{+/+}$) and control animals. Dendrite and spine reconstructions (3D) of rectangular section represented on the right with magnified parts. **(B)** No significant differences in spine densities between different groups. Boxplots represent average values of 2–3 animals per group ± SEM. Six to seven dendritic stretches from different cells were analyzed. **(C)** Evaluation of spine types from different behavioral groups in CA1. $R^{-/-}$ and $R^{+/+}$ animals show significant difference in stubby (decrease) (respectively $p = 0.015$ and $p = 0.047$) and mushroom (increase) (respectively $p = 0.0023$ and $p = 0.0145$) spines compared to the control group. Resilient ($R^{-/+}$) mice have significantly increased filopodia compared to control ($p = 0.0137$), and $R^{+/+}$ ($p = 0.0185$). Students t-test as statistical analysis. Scale bars: 10 µm in 2D images; 5 µm in 3D reconstructions.

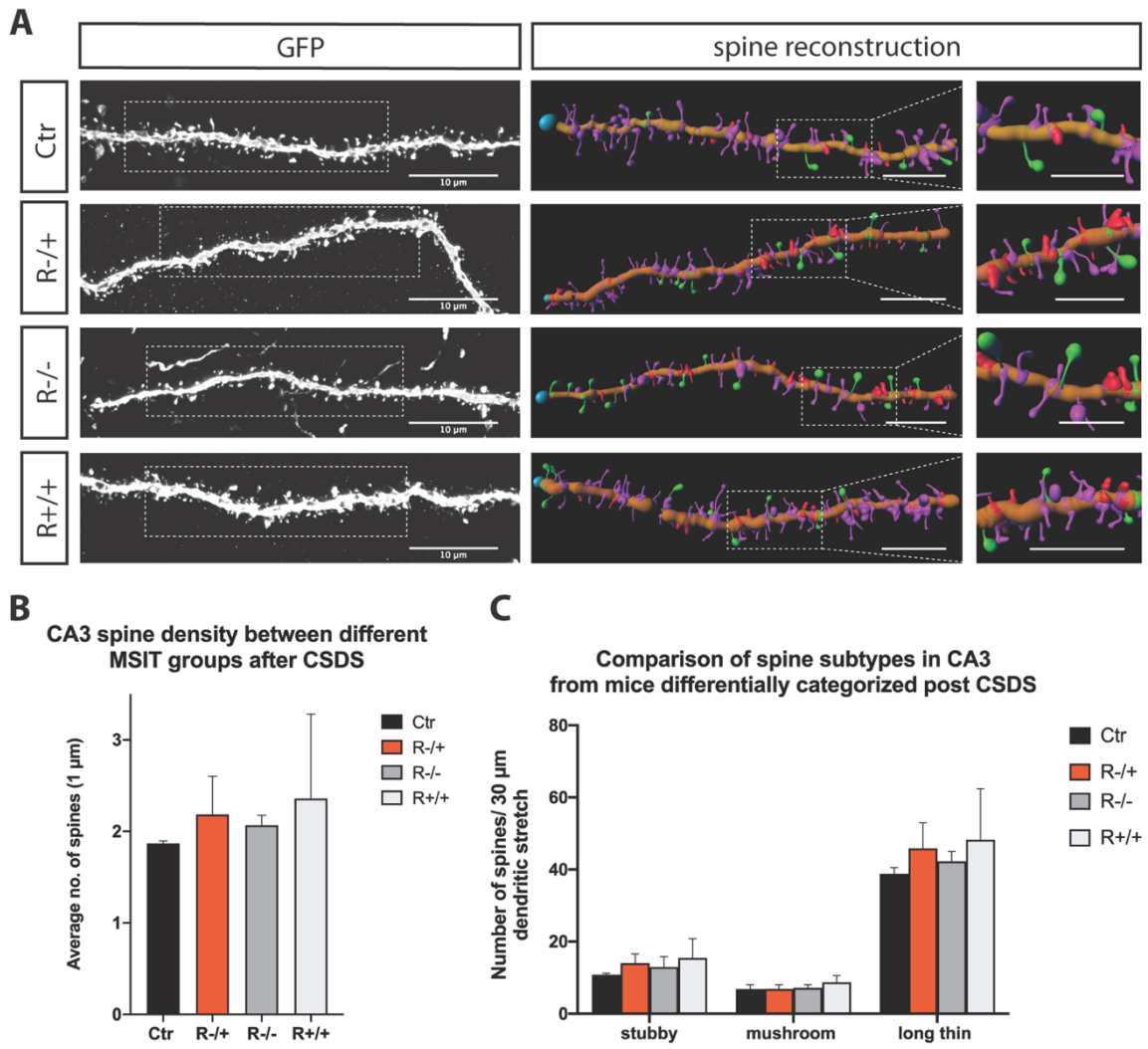

Figure 2.20: CA3 spine morphology after chronic social defeat stress.
(A) Representative fluorescent images (2D, z-stack) of dendritic stretches in *stratum radiatum* from CA3 pyramidal neurons of control, resilient ($R^{-/+}$), susceptible ($R^{-/-}$) animals and non-leaners ($R^{+/+}$). Dendritic reconstructions (3D) inclusive spines of rectangular section are represented on the right. Magnifications of reconstructed stretches are shown aside. **(B)** No significant differences in spine densities between different groups. Boxplots represent average values of 2–3 animals per group ± SEM. Per animal 5–6 dendritic stretches from different cells in different planes were analyzed. **(C)** Evaluation of spine types from respective behavioral groups in CA3. No significant differences between groups. Students t-test as statistical analysis. Scale bars: 10 µm in 2D images; 5 µm in 3D reconstructions. Reconstructions of dendritic stretches and spines within the CA3 area, were performed by Maximilian Ken Kracht.

2.7 Generation of conditional, neuron-specific DRR1 knockout mice

To study the gene function of *Drr1*, we generated neuron-specific DRR1 knockout animals. Conditional $DRR1^{l/wt}$ mice were generated at Ingenious Targeting Laboratories Inc., USA. We further generated homozygous $DRR1^{l/l}$ animals and breed them to animals expressing the Cre recombinase under control of the calcium/calmodulin-dependent protein kinase II alpha

(CaMKIIα) promoter to postnatally delete neuron-specific DRR1 in the forebrain and limbic system, including the hippocampus. The Cre protein translocates to the nucleus, where it recognizes the *loxP* sites flanking the DRR1 coding exon 4, ultimately excising this region. The missing exon prevents proper DRR1 translation in tissue containing the *Camk2a* promoter. We validated the gene knockout by DNA isolation and subsequent polymerase chain reaction (PCR) for the presence of both *loxP*-flanked *Drr1* alleles and the presence of Cre recombinase. All animals containing the *Camk2a*-Cre showed a band at approximately 600 bp. To control for homo- or heterozygous floxed *Drr1*, we used primer pairs upstream and downstream of both *loxP* sites. An amplified product for the proximal *loxP* site appears at 546 bp. The respective wildtype band is 410 bp in size. Heterozygous animals show both bands, whereas homozygous mice only show the *loxP* band (Figure 2.21(C)). Likewise, the distal *loxP* site was verified with a wildtype product at 450 bp and a product with 524 bp upon presence of *loxP* site. DRR1 knockdown only appears in animals with homozygous *loxP*-flanked *Drr1* alleles and simultaneous Cre expression. DRR1$^{1/1}$ animals negative for *Cre*, served as control animals.

The generated DRR1$^{1/1}$_*Camk2a*-Cre mouse line (Figure 2.21(A)) is useful in examining DRR1-dependent synaptic plasticity and LTP in the hippocampal network. Little is known about the function of DRR1 in the adult nervous system or about DRR1-specific modulation of baseline behavior. Therefore, we planned to first characterize DRR1$^{1/1}$_*Camk2a*-Cre mutants with respect to baseline behavior with special focus to stress-related aspects as well as hippocampus-dependent cognitive performance. Following those analysis, we intended to analyze behavior after CSDS to assess the impact of DRR1 on shaping resilience and to test the hypothesis that DRR1 attenuates stress-related negative consequences on behavior and neuronal morphology.

For future morphological and electrophysiological analysis of neuronal cells lacking DRR1 within in the hippocampus, we crossed the generated DRR1$^{1/1}$_*Camk2a*-Cre animals with a DRR1$^{1/1}$_*Thy1*-GFP mouse line (Figure 2.21(B)) expressing GFP in a subset of cerebral and hippocampal pyramidal neurons (Feng et al., 2000). The genotype of individual mice was verified through genotyping (Figure 2.21(C–E)). Thereby, GFP-positive animals revealed an amplified product with 350 bp in size. Using those animals, spine morphology and dendritic arborization was planned to be analyzed in mutant animals in comparison to control mice. Further structural analysis can be performed in mutants post CSDS. Besides, GFP-expressing DRR1 nKO (neuronal knockout) animals present an attractive model for electrophysiological studies or *ex vivo* studies such as organotypic slice culture applying several stimulation paradigms for investigation of dendritic dynamics and spine remodeling.

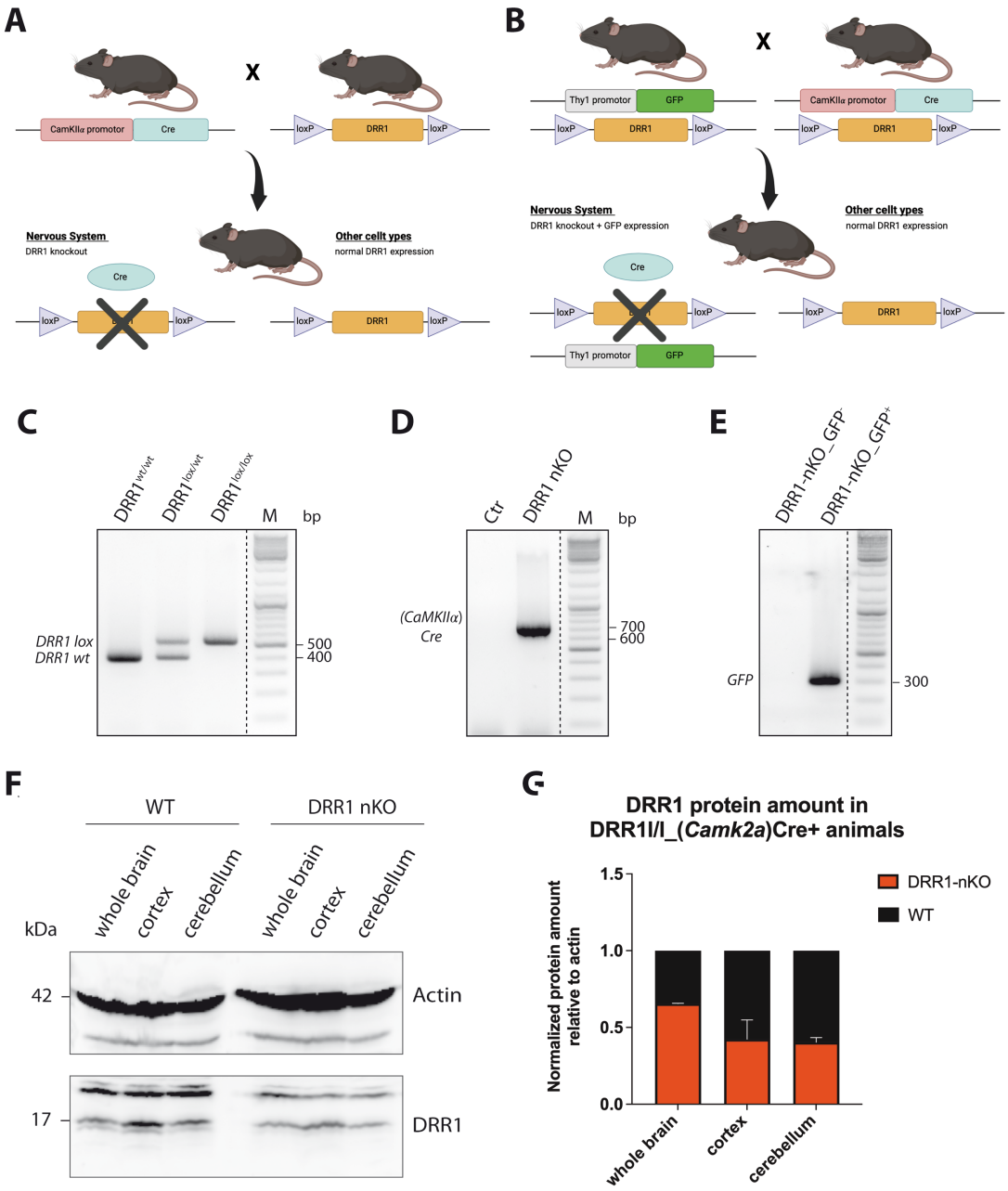

Figure 2.21: Generation of DRR1 knockout (KO) mice.
(A) Breeding scheme to generate DRR1 knockout mice expressing Cre under the *Camk2a* promoter. *Camk2a*-Cre animals were crossed to homozygous DRR1 floxed animals until F2, F3 generation with homozygous DRR1 deletion. **(B)** Schemata shows the generation of DRR1$^{l/l}$_*Camk2a*-Cre_*Thy1*-GFP mice. Mice expressing GFP under the *Thy1* promotor were crossed to generated DRR1$^{l/l}$_ *Camk2a*-Cre animals. In both cases DRR1 is deleted in the central nervous system, but remains intact in all other cells. **(C-E)** Representative images of agarose gel electrophoresis after PCR for different genes. Marker for base pair sizes ist represented on the right side. **(C)** Gel band shows distal *loxP* side with 546 bp in size (*DRR1 lox*) and allele without *loxP* with 450 bp (*DRR1 wt*). From left to right: DRR1$^{wt/wt}$ with wildtype alleles; DRR1$^{lox/wt}$ represents heterozygous floxed animals; DRR1$^{lox/lox}$ shows homozygous animals. **(D)** Gel band shows DRR1$^{lox/lox}$ animals that are Cre positive animals. Band is visible at 600 bp size (right). Band doesn't appear in control animals without expressing Cre recombinase (left). **(E)** EGFP positive animals have a gene product with 350 bp in size. DRR1$^{l/l}$_*Thy1*-GFP^{-} (DRR1-nKO_GFP^{-}) mouse on the left, DRR1$^{l/l}$_*Thy1*-GFP^{+} (DRR1-nKO_GFP^{+}) animal expressing GFP is depicted on the right. **(F)** Western Blot analysis from brain lysates of DRR1$^{l/l}$_ *Camk2a*-Cre^{+} (DRR1 nKO) and wildtype (WT) mice serving as controls. Protein measure was performed from whole brain lysates and from cortex and cerebellar lysates only. Actin served as reference protein. **(G)** Quantification of protein bands shows 34% decrease in DRR1 protein amount in whole brains lysates and 58% and 60% in cortex and cerebellum respectively. Measures were performed in brains from two individual animals per brain region.

The Cre recombinase is expressed under the *Camk2a*-Cre promoter, excising DRR1 in cells existed in all cells expressing as depicted in Figure 2.21(F) whole brain lysates served as probes for Western Blot analysis to verify the knockdown of DRR1 protein through measurement of the protein amount in DRR1$^{1/1}$_ *Camk2a*-Cre animals. We compared whole brain lysates, lysates from cerebellum und lysates from cortex of KO animals with wildtype C57BL/6 animals. Thereby, we detected a decrease of total DRR1 protein amount of 34% in whole brain lysates in comparison to controls. The decreases of DRR1 in cortex only is 58% and in cerebellum only even 60%. Our results depict a successful knockdown of DRR1 in DRR1$^{1/1}$_*Camk2a*-Cre $^{+}$ animals and makes it a suitable mutant for our future analysis.

2.8 Acute stress and DEX treatment show elevated DRR1 protein in the hippocampal formation *in situ*

Even acute stress already modifies local spine signaling (Jafari et al., 2012). However, moderate exposure to stress and its consequences measurable in dendritic atrophy and spine loss in the CA3-region were shown to be reversable (Woolley et al., 1990; Magarinos et al., 1996; Magarinos et al., 1997). Chronic stress can cause total loss of some hippocampal neurons (Sapolsky et al., 1985; Kerr et al., 1991; Mizoguchi et al., 1992).

Treatment of stress-related disorders may be more effective at earlier and more dynamic stages of mental disfunctions. Just like the attempt to identify potential resilience factors. The understanding of coping mechanisms of an organism at early stages post stress, could bring insight into processes underlying chronic stress-induced sequel (Krishnan et al., 2007; Jene et al., 2018). Exposure to acute social defeat (ASD) stress is a stress model of high translational value (Jene et al., 2018). Previous findings proved single restrain stress and multiple concurrent acute stresses initiating loss of spines in the hippocampus and impairing learning and memory (Maras et al., 2014; Chen et al., 2016). Jene and colleagues investigated the role of hippocampal DRR1 in response to ASD in mice *in vivo*. They showed an increase in *Drr1* mRNA within the CA3-region after 4 h and 8 h post stress, whereas DRR1 protein was elevated only 8 h after stress (Jene et al., 2018). Cognitive impairments were seen at 4 h post ASD, but were abolished after 8 h, emphasizing resilience promoting effects of DRR1. GR-mediated mechanisms come into account in late phase responses to stress, involved in homeostatic processes (De Kloet et al., 2005; Yau & Seckl, 2012). *DRR1* gene expression is increased in stress-relevant brain regions including the hippocampus after GR activation (Liebl et al., 2009; Schmidt et al., 2011, Masana et al., 2014; Stankiewicz et al., 2014). Additionally, mouse studies with *in vivo* DEX injections revealed increases of its expression in membrane-rich structures (Masana et al., 2015).

Together with the awareness that DRR1 is expressed in radial glia during development (Pollen et al., 2015) and later also in other cell types, such as astrocytes, neurons and oligodendrocytes (Cahoy et al., 2008; Masana et al., 2014; Hochgerner et al., 2018) within the murine brain, we wanted to investigate its upregulation in other cell types beneath neurons upon stress as well. Thereby, we wanted to emphasize special attention to blood vessels, since unlike other brain cells, little was known about the occurrence of DRR1 in endothelial cells within the murine brain. Hereby, an interesting study by Menard and colleagues from 2017 was very inspiring. The scientists found an increased blood-brain barrier (BBB) permeability upon chronic social defeat stress due to reduced expression of the tight-junction protein claudin-5 (Cldn-5) in stress susceptible mice. However, these deficits were not detected in stress-resilient animals (Menard et al., 2017). Increased peripheral inflammation occurs upon BBB permeability and is known to cause the onset of major depressive disorder (Menard et al., 2017).

We used two approaches to increase circulating glucocorticoids in mice *in vivo*. One approach was the sub-cutaneous injection of DEX. A method utilized in previous studies (i.e., Masana et al., 2015) to increase GR activation and *Drr1* expression in stress-sensitive regions. The second approach was the exposure to an acute social defeat paradigm, which increases blood corticosterone levels, evoking stress. Both experiments were performed with wildtype mice together with our collaborator Tanja Jene (Mueller laboratory) at the Institute of Molecular Biology in Mainz. Six hours (when DRR1 protein-levels start to increase) after DEX injections or acute stress, mice were sacrificed and perfused with PFA.Brains were collected and brought back to Frankfurt for further processing. Coronal brain sections were produced and used to fluorescently label DRR1 and podocalyxin (Pdx), a sialoglycoprotein expressed on endothelial cells and important to maintain the BBB function during acute inflammation (Cait et al., 2019). Three wildtype C57BL/6 male mice underwent acute stress and another three animals were subjected to handling only, serving as control. DEX-injections were administered to another three male wildtype mice and a vehicle control was administered to the control group. Confocal fluorescent images of the hippocampal CA3 region showed an increased fluorescent intensity of DRR1 in the area of granule cells, supposedly neuronal cells, of DEX treated animals as compared to the control group. Representative images are shown in Figure 2.22. The fluorescent signal was especially increased in the cell body and nuclei. In the DEX group, but not the control group, we detected a DRR1 signal overlapping with the Pdx-signal of endothelial cells labeling blood vessels. Arrowheads in Figure 2.22(B) indicate the DRR1 signal overlapping with Pdx-staining. Close-ups of the separate channels are visualized in the bottom panel. Interestingly, an even stronger DRR1 appearance is visible in endothelial cells from animals that underwent acute social defeat stress, but not in respective control animals (Figure 2.23).

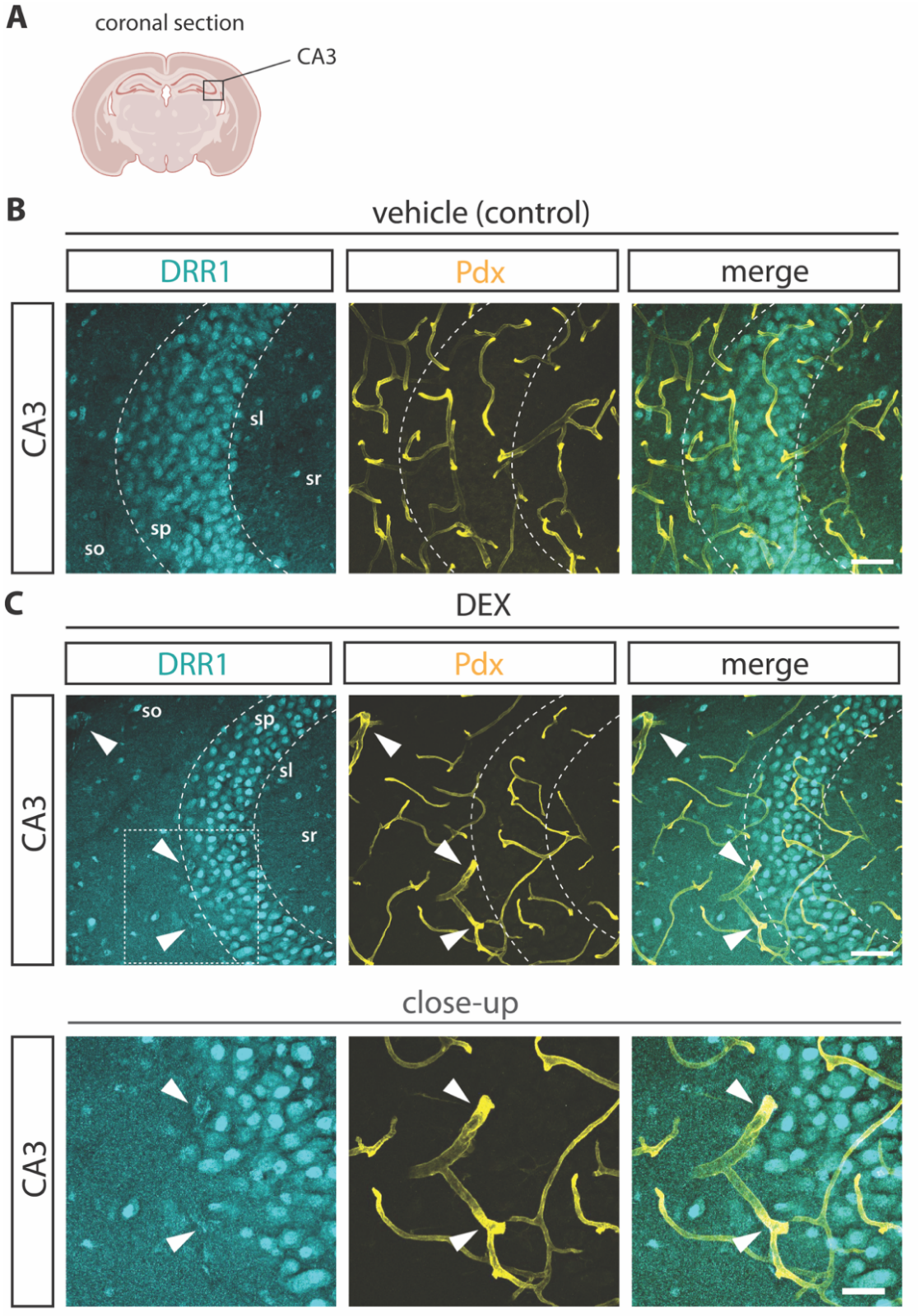

Figure 2.22: Immunohistochemical staining of DRR1 and Podocalyxin in CA3 of DEX-injected animals. CA3-area **(A)** stained for DRR1 (cyan), Podocalyxin (Pdx, yellow), and overlap on the right (merge). Staining was performed in 200 µm thick coronal sections of 8 to 12-week-old mice after DEX- or vehicle-injection. Animals were perfused 6 h post-injection. **(B)** No visible overlap of DRR1 and Pdx-staining in vehicle injected animals. **(C)** DRR1 overlaps with Pdx staining. Arrow-heads indicate double-labelled vessel structures. Lower panel shows close-up of one prominent co-localization. Scale bars: 50 µm, 40× oil immersion. Acronyms: *stratum lucidum* (sl); *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so). Scale bar for close-up: 25 µm, 2× zoom. Z-stack: 60–75 µm.

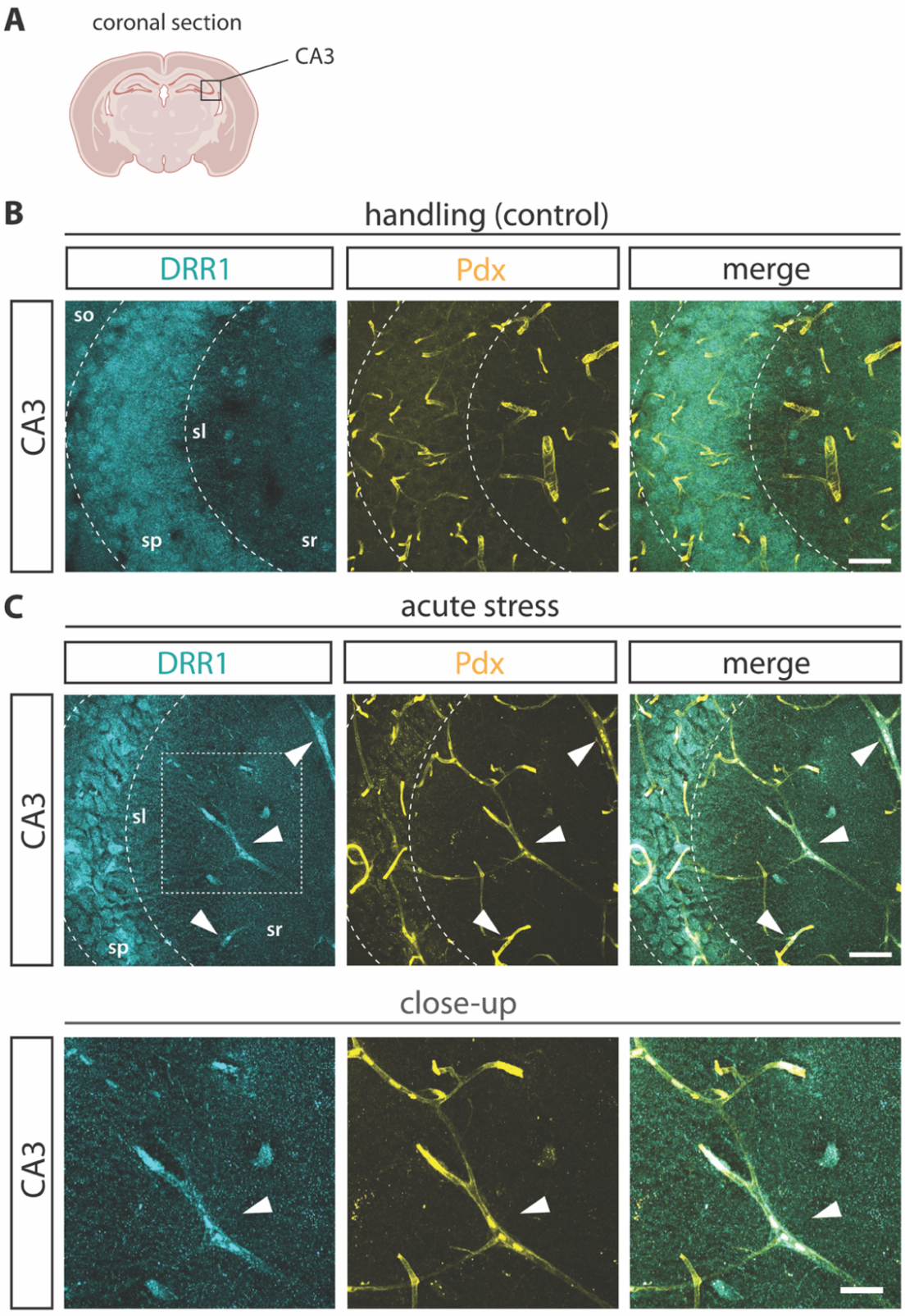

Figure 2.23: Immunohistochemical staining of DRR1 and Podocalyxin in CA3 of acute stressed animals. CA3-area (A) stained for DRR1 (cyan), Podocalyxin (Pdx, yellow), and overlap on the right (merge). Staining was performed in 200 µm thick coronal sections of 8 to 12-week-old mice after acute stress or handling. Animals were perfused 6 h post treatment. (B) No visible overlap of DRR1 and Pdx-staining in handled animals. (C) DRR1 partially overlaps with Pdx staining. Arrow-heads indicate double-labelled vessel structures. Lower panel shows close-up of one prominent co-localization. Acronyms: *stratum lucidum* (sl); *stratum pyramidale* (sp); *stratum radiatum* (sr); *stratum oriens* (so). Scale bars: 50 µm, 40× oil immersion. Scale bar for close-up: 25 µm, 2× zoom. Z-stack: 60–75 µm

3. Discussion

Stress has a global impact on organisms initiated by hormonal changes which act at multiple sites of the body. The disruption of a homeostatic balance in organs upon chronic stress can lead to multiple maladaptation and the onset of diseases (Schneiderman et al., 2005; McEwen, 2012; Chou et al., 2014; Donahue et al., 2014; Fink, 2017). Concurrently, resilience mechanisms are able to attenuate severe consequences of stress, leading to the maintenance of mental health. A deeper understanding of functionality and the reasons for the onset or absence of resilience could help to design new prevention strategies against stress-induced psychiatric disorders (Kalisch et al., 2015).

There are several stress-sensitive regions in the brain, including the hippocampus with its glucocorticoid receptors (GRs) (Reul & de Kloet, 1985), detrimentally reacting to prolonged stress. In case of the hippocampal brain region, chronically elevated CRH levels lead to spine loss and dendrite shrinkage (Chen et al., 2008; Donohue et al., 2006; Kavushansky et al., 2006; van der Kooij et al., 2016), ultimately changing the neuronal transmission (Jafari et al., 2012) and affecting complex behavior (Krishnan & Nestler, 2011; Schmidt et al., 2011; Feng et al., 2012; Golden et al., 2013). At worst, this can trigger onset of neuropsychological diseases i.e., major depression (Fink, 2017). The shift in neuronal plasticity via locally modified spine signaling is triggered by changes in actin dynamics and AMPAR-trafficking (Derkach et al., 2007; Cingolani & Goda, 2008). Neuronal plasticity is mediated by a wide range of mechanisms involving a plentitude of molecular players. Consequently, there are diverse degrees of neuroplasticity that are highly interlinked with each other. The complexity of the interplay between physiological and structural mechanisms such as Hebbian, homeostatic and structural plasticity remains elusive in many parts (Turrigiano et al., 2017). However, a key component of excitatory neural plasticity are synaptic surface AMPARs. They highly respond to activity and in this way modulate transmission. Through interaction with supporting proteins such as GRIP1, PICK1 or TARPs their trafficking to and at the synaptic site is well orchestrated (Liu & Cull-Candy 2000; Lu & Ziff 2005). Beyond that, dynamic changes of the synaptic actin cytoskeleton are indispensable for structural plasticity and therefore shaping synaptic efficacy on both sites of the synapse (Cingolani & Goda, 2008, Neves et al., 2008). Apart from presynaptic mechanisms such as vesicle transport and neurotransmitter-release, it builds a scaffolding net for receptors at the postsynaptic side supporting the PSD (Sudhof, 2004; Cingolani & Goda, 2008). Additionally, F-actin guides receptor transport from and to the postsynaptic side. To ensure a smooth synchrony of temporal and spatial remodeling of the actin-cytoskeleton as basis of cellular functions and structural plasticity, ABPs are inevitable (Hotulainen & Hoogenraad, 2010).

Numerous observations revealed reduced synapse numbers and dendritic remodeling especially in the hippocampal CA3 area after stress exposure (Fuchs et al., 2006). Additionally, shifts in actin dynamics influence synaptic function (Cingolani & Goda, 2008). However, so far it remained largely unknown, which molecular players are involved in translating stressful environmental stimuli into adaptive changes in neuronal function and synaptic plasticity.

The stress-responsive protein DRR1 was found to interact with actin, serving as ABP. Intensive analysis of DRR1 truncation and point mutants, helped defining the functionality of the protein (Chapter 1.9.1). For example, the C-terminus and the N-terminus of the 144 amino acids long protein binds to actin proteins, whereas the coiled coil domain within the middle part initiates a homodimerization of DRR1 proteins (Kretzschmar et al., 2018). DRR1 displays a couple of functions on F-actin such as capping, bundling, and nucleation. Moreover, DRR1 is sensitive to glucocorticoids and upregulated upon stress i.e., in the hippocampal region amongst others (Masana et al., 2014). Stress-induced DRR1 upregulation is dependent on GR-activation and its expression requires GR homodimerization (Schmidt et al., 2011; Frijters et al. 2010). Experiments with mice, locally overexpressing DRR1 within the hippocampus mimicked an aspect of the stress-response. Those experiments found reduced hippocampal LTP and reduced release probability of presynaptic neurotransmitters in pyramidal CA1 and CA3 cells (Schmidt et al., 2011). At the same time, hippocampus dependent cognitive performance was increased in examined animals (Schmidt et al., 2011). These findings opened the prospect of DRR1 constituting a protein with resilience promoting properties, able to counterbalance negative effects of stress on hippocampus-dependent functions.

Even more intriguing is the finding that DRR1 shares similar promoter and expression profiles with AMPARs (Chong et al., 2007). Co-expressed genes are often transcriptionally co-regulated suggesting complemental functions within a specific operative context or system (Chong et al., 2007). Therefore, DRR1 represents a strong candidate for further studies, elucidating its impact on AMPARs and investigating its potential role as molecular link translating stressful environmental stimuli into synaptic adaptations via its impact on actin dynamics and AMPAR co-regulation.

A correlation between DRR1 and AMPARs has been demonstrated (Schmidt et al. 2011) but no direct interaction was shown so far. Furthermore, molecular processes driven by DRR1 and its linkage with resilience mechanisms were open for clarification. Therefore, in the present thesis we first investigated the impact of DRR1 level changes in primary hippocampal neuronal cultures in respect to AMPAR GluR2 subunit interaction and structural plasticity. Furthermore, we analyzed the spine morphology in resilient versus susceptible animals post CSDS with the assertion that DRR1 expression differs in the subsequent groups. We found that distinct DRR1

protein expression levels, evoked through overexpression or suppression of the endogenous protein, affected total GluR2 subunit amounts as well as surface GluR2 loads in contrast to respective controls. We detected abnormalities in neuronal morphology *in vitro* after manipulation of DRR1 protein expression. Apart from that, examination of spines in pyramidal CA1 and CA3 neurons from resilient, susceptible and non-learning mice, exposed a disbalance of spine subtypes, without affecting the total number of spines. We could correlate these findings to our preceding *in vitro* experiments, suggesting that a disbalance of DRR1 might indeed play an important key role in the stress response and resilience to stress. Additionally, experiments of this thesis revealed the expression of DRR1 in other cell types within the brain. Especially *in vivo* stress induction through acute social defeat stress and the application of the artificial glucocorticoid DEX elevated the expression of DRR1 in endothelial cells in the hippocampal area, showing that the protein is a versatile player. Taken together, our results suggest that DRR1 interacts with AMPAR receptors, presumably participating in AMPAR surface trafficking evoked by stress.

3.1 Distribution of endogenous DRR1 in the adult murine brain

Initially, *DRR1* was identified as putative tumor suppressor gene appearing in most tissues of the human body, inter alia, the brain (Yamato et al., 1999; Wang et al., 2000). Expanding investigation in human and rodents linked its biological function to cell growth and movement processes, embryogenesis, actin interaction, and cell invasion in gliomas while absent in neuroblastoma carcinogenesis (Asano et al., 2010; Le et al., 2010; Schmidt et al., 2011). Interestingly, studies detected a mechanism of cell cancer invasion involving DRR1. Hereby, it participates in the recruitment of phosphorylated AKT to the cell nucleus, where later this triggers downstream signaling cascades activating cell survival, proliferation, invasion and metabolism (Song et al., 2005; Manning & Cantley, 2007; Chin & Toker, 2009; Dudley et al., 2014). Following microarray analysis showed strongest constitutive *Drr1* mRNA signal in the neocortex, the CA3 region, the lateral septum and the cerebellum (Liebl et al., 2009, Masana et al., 2018).

Fundamental requirement for studies decoding the detailed role of DRR1 in shaping stress-related behavior and potential resilience mechanisms, we have performed extensive neuroanatomical analysis of constitutive DRR1 protein expression in the adult mouse brain in the first place. To ascertain the exact basal expression of DRR1 within the murine brain, we first searched existing databases displaying *Drr1* mRNA prevalence. The Allen Mouse Brain Atlas exhibits profound in situ hybridization data for genes in adult C57BL/6 mice. In case of *Drr1* mRNA the screening data from the Allen Brain Institute (Allen Mouse Brain Atlas) displays

strongest basal expression within the hippocampus, the cerebellum as well as the olfactory bulb region and the subventricular zone (Figure 2.1(B)). For the present thesis the expression within the hippocampus was of most interest, since we focused on DRR1 analysis in this region. We further looked up an RNA sequencing database (Zhang et al., 2014) which provides data on mRNA of 7 days old mice in different cortical cell types (Figure 2.1(A)). Interestingly, in the cortex of young postnatal mice, *Drr1* mRNA is mostly expressed in astrocytes, oligodendrocytes, microglia and endothelial cells. The smallest proportion was found in neurons. Since profound data on DRR1 protein expression throughout the adult mammalian brain is missing, we first performed immunohistochemistry in coronal sections of adult male C57BL/6 mice to elucidate basal DRR1 protein expression. We started by comparing available antibodies against murine DRR1 to find a proper antibody for further analysis. Namely, the commercially available antibody from sigma Aldrich (sigma) and a self-manufactured antibody from our collaboration partner in Mainz (MUE). Both antibodies are polyclonal. Coherently, also suitable fixation methods were assayed for the distinct antibodies. By qualitative comparison of TCA versus PFA fixation, we found PFA suiting better for DRR1 antibody staining with stronger signal to background ratio (Figure 2.2 and Figure 2.3). Besides, the sigma antibody showed a stronger DRR1 signal together with a slightly broader signal coverage. We focused on the hippocampal CA3-region, since above mentioned research and previous publications (Schmidt et al., 2011) demonstrated basal DRR1 levels highest in this brain area apart from the fact that the hippocampal CA3-region is one of the most stress sensitive regions, consequently the focus of our investigations. Qualitative analysis of the same immunostaining further showed strong DRR1 occurrence in cell bodies located in the granule cell layer of the dentate gyrus and the *stratum pyramidale* of the CA1-region. Also here, DRR1 staining strongly overlapped with DAPI cell nuclei labelling (Figure 2.4), confirming previous studies describing DRR1 occurrence in the cell nucleus (Mu et al., 2017). Interestingly, a certain proportion of surrounding cell bodies was positive for DRR1 as well. This observation together with mRNA sequencing data from Zhang and colleagues conducted us to perform double immunostaining using protein markers against DRR1 and the major cell types – neurons, astrocytes and microglia (Figure 2.6). As expected, we detected a high overlap between DRR1 and neuronal cell bodies, especially in the hippocampal pyramidal cell layer and the granule cell layer. A vast majority of astrocytic cells showed an overlap with DRR1 proteins, particularly visible in the cell body and astrocytic end-feet as demonstrated in the CA3-region in Figure 2.6(A) combination of DRR1 immunostaining and *in situ* hybridization with microglia specific RNA-probes exposed signal overlay mostly in the cell nucleus of microglial cells.

To complement the DRR1 expression analysis we examined brain regions with strongest *Drr1* mRNA appearance. Figure 2.5 shows DRR1 staining together with GFAP as astrocytic

marker protein in a posterior lobe cerebellum, where DRR1 barely overlaps with astrocytes and mainly stains the Purkinje cell bodies. The lower panel of Figure 2.5 demonstrates a fluorescent image of the posterior parietal association area with a vessel situated in this section. The vessel is surrounded by astrocytes making contact via astrocytic end-feet. The DRR1 protein partially overlaps with astrocytic cells mainly locating to cell bodies and scarcely at rising ramifications starting from the cell body.

With qualitative immunohistochemistry analysis we showed for the first time, distribution of endogenous basal expression levels of DRR1 protein in the adult murine brain. The analysis complements existing mRNA data on *Drr1* expression demonstrating its omnipresent occurrence throughout the whole brain, but most strongly in the cerebellum and the hippocampus. Thereby, we confirmed protein translation in areas with *Drr1* mRNA expression. Additionally, we visualized that DRR1 is expressed by several cell types, including astrocytes, neurons and microglia. Notably, DRR1 seems to be differently expressed by the same cell type depending on the brain area as shown by the lack of overlap with astrocytes in the cerebellum. Unlike the RNA-sequencing data from Zhang and colleagues corresponding to postnatal brain, we found DRR1 being expressed by a major part of cells throughout the pyramidal cell layer of adult mice in the cortical area, overlapping with the neuronal marker NeuN (see appendix Figure 6.1), suggesting a modulation of its expression overtime. Yet, strongest DRR1 signal is seen in granule cells and pyramidal cells in the hippocampal area.

The innate immune system is relevant for genetic factors that are associated with stress resilience. Within the mammalian brain microglia constitute resident immune cells fundamentally responsible for stress responsiveness (Rimmerman et al., 2017). Researcher showed emotional and cognitive stress resilience in animals with depletion of the *Cx3cr1* gene, which is exclusively expressed by microglia (Rimmerman et al., 2017). Compared to WT mice, CX3CR1$^{-/-}$ animals exhibit larger microglial somas. Interestingly, upon chronic unpredictable stress reduced microglial densities and processes length in the hippocampus were found to be similar to WT mice. Differences were observed in gene transcripts downstream the estrogen receptor signaling which were altered in CX3CR1$^{-/-}$ only (Rimmerman et al., 2017). The findings suggest that microglia and especially CX3CR1 signaling are implicated in resilience.

Other investigations showed BBB permeability and infiltration of peripheral Il-6 into the brain parenchyma upon CSDS in mice. This was accompanied by depressive-like behavior (Menard et al., 2019). The study discriminated stress-susceptible and resilient animals. The comparison of these animals considerably showed reduced expression of the tight-junction protein Claudin-5 and

altered blood vessel morphology in the NAc of susceptible animals. These findings emphasize the impact of CSDS on BBB integrity and the onset of depressive-like behavior.

Collectively it appears that CSDS has negative consequences on the major cell types in the mammalian brain. Whether or not DRR1 is involved in the stress-response cascade in microglia, blood vessel and astrocytes is an intriguing research question. Even further, DRR1 might even connect resilience promoting actions through cell-to-cell communication.

3.2 Manipulation of DRR1 expression levels via rAAV transduction in primary hippocampal neurons

Our DRR1 immunohistochemical analysis as well as earlier investigations detected strongest DRR1 expression in the murine hippocampal CA3 subfield (Schmidt et al, 2011; Masana et al., 2015). As outlined in the introductory section 1.3.5 of this thesis, the hippocampal CA3-region is a particular key target for stress-related consequences and effects of glucocorticoids. Additionally, the CA3 area modulates important facets of cognitive performance requiring synaptic plasticity. Preceding experiments managed to mimic stress within the hippocampus of adult mice through region-specific injections of adeno-associated viruses (AAV) carrying the *Drr1* sequence (Schmidt et al., 2011). Animals overexpressing DRR1 showed improved cognitive flexibility and simultaneously a reduction of hippocampal LTP (Schmidt et al., 2011). In order to investigate a physiological role of DRR1 in the brain, our collaborators (AG Mueller, Department of Psychiatry & Psychotherapie, Johannes-Gutenberg University Medical Center, Mainz) performed loss of function experiments via viral *in vivo* AAV transfection with viral capsules carrying an shRNA (shDRR1) construct to suppress the endogenous gene (unpublished data). The viral transfection partially suppressed the endogenous *Drr1* gene within the hippocampal area. Animals with shDRR1 injections were impaired in fast learning of spatial information and showed changes in pre- and postsynaptic function such as increased hippocampal LTP (unpublished data Mueller lab).

In order to study the effects of hippocampal DRR1 on neuronal morphology and its impact on AMPARs at the postsynaptic interface, we desired to replicated DRR1 overexpression and suppression *in vitro* using the same rAAV constructs as previously applied *in vivo*. Using recombinant AAVs as vectors for gene delivery at specific target sites is an elegant way (Lee et al., 2017) to study functional roles of specific genes. Furthermore, AAVs constitute efficient and easy applicability with low pathogenicity (Breyer et al., 2006; Seymour & Fisher, 2011). We utilized the central nervous system optimized and chimeric AAV with serotype 1 and 2 designed from

Gene.Detect.com Ltd to transduce primary hippocampal neuron cultures. An included GFP construct served as transfection control, expressed by cells effectively transfected by rAAV. For DRR1 overexpression (OE) rAAVs contained an inserted *Drr1* sequence, whereas vectors carrying shDRR1 sequence were applied for suppression of DRR1 acting via RNA interference. Preliminary experiments served for detection of a suitable virus titer to transfect a high number of neuronal cells with least toxicity effects. We determined MOI (multiplicity of transfection) of 1000 (details see methods section) as most effective application.

Consecutive Western Blot analysis of DRR1 protein amount from cultures transfected with either OE or shDRR1 and the comparison with respective controls revealed a prosperous overexpression and partial suppression of DRR1 in cultured hippocampal neurons. Through fruitful rAAV transduction we conferred DRR1 protein level manipulation from *in vivo* experiments *in vitro*. Additionally, cyto-histochemical staining of fixated primary hippocampal cultures demonstrated ubiquitous expression of DRR1 throughout the entire neuronal cell including dendrites, cell body, and nuclei.

3.2.1 Surface GluR2 subunits correlate with intracellular DRR1 ratio in hippocampal neuron cultures

A major risk factor for the onset of stress-related psychiatric disorder is vulnerability to aversive behaviors. However, the molecular bases of stress vulnerability or oppositely stress resilience are still to be elucidated. The glutamatergic system is widely implicated in the pathogenesis of affective disorders, such as major depression (Sanacora et al., 2008; Hashimoto, 2009; Kadriu et al., 2021). More correlative is the relation between high plasma glutamate levels and the severity of depressive systems (Mitani et al., 2006). Studies on the ionotropic glutamate receptor NMDA demonstrated robust anti-depressive effects in patients with severe depression when activated by the noncompeting antagonist ketamine (Zarate et al., 2006). Additionally, AMPAR activation is suspected to induce ketamine's antidepressant effects (Maeng & Zarate, 2007; Maeng et al., 2008). Moreover, indications point to a critical involvement of AMPARs in mood disorders (Alt et al., 2006; Bleakman et al., 2007). In animals chronically treated with antidepressants hippocampal expression levels of GluR1 subunits and phosphorylation of AMPARs were increased (Du et al., 2004; Svenningsson et al., 2007). And acute treatment with an AMPAR potentiator elevated antidepressant-like behaviors in rodents (Li et al., 2003). Novel drugs acting on the glutamatergic system show successful outcomings regarding antidepressant effects (Sanacora et al., 2008; Machado-Vieira et al., 2009). Taken together, over the past decades, the glutamatergic system received considerable attention as approach for treatment of depressive

disorders (Alt et al., 2006; Skolnick et al., 2009; Nisenbaum & Witkin, 2010. The antidepressant effects induced by ketamine are linked to fast improvements in synaptic plasticity in executive brain areas (Duman et al., 2016; Ionescu et al., 2018; Rantamäki & Kohtala, 2020). In rodents, ketamine was shown to restore dendritic arborization and spine densities which were reduced as consequences due to chronic stress (Li et al., 2011; Moda-Sava et al., 2019). Moreover, its antidepressant effects were much more rapid compared to classical antidepressants (Li et al., 2011; Moda-Sava et al., 2019). The underlying mechanisms are thought to include enhanced AMPAR throughput relative to NMDAR activation. As consequence this leads to increased BDNF release at synapses and activation of mTOR (Zhou et al., 2014) which in turn activates local synthesis of synaptic proteins (Li et al., 2010; Moda-Sava et al., 2019). Especially acute elevation in excitatory neurotransmission triggered by AMPARs is resulting in long-term adaptions displayed by increased GluR1 and GluR2 subunits, seem to exert antidepressant effects (Zanos et al., 2016; Shaffer et al., 2019).

Individual stress vulnerability is decisively for the etiology of affective disorders. Schmidt and colleagues were able to show differences in AMPARs expression and function in the dorsal hippocampus of stress resilient versus vulnerable mice (Schmidt et al., 2010). This is in line with preclinical data reporting altered AMPAR expression in dorsal hippocampus of stress susceptible individuals (Fanselow & Dong, 2010). Moreover, AMPAR dependent behavior, expressed by spatial short-term memory is predictive for stress vulnerability (Schmidt et al., 2010). Interestingly, genetic variations in the AMPAR GluR1 subunit were found in stress vulnerable animals as well. This fits to preceding findings associating low GluR1 while high GluR2 expression with psychiatric disorders (Meador-Woodruff et al., 2001). Consequently, mice with relatively higher GluR2-to-GluR1 ratio became a model of depression (Chourbaji et al., 2008). Beyond that, antidepressants have been shown to affect AMPAR expression and function through modulation of AMPAR mRNA modulation, mRNA editing and receptor phosphorylation (Barbon et al., 2006; Svenningsson et al., 2007; Sawada et al., 2009). Interestingly stress hormones seem to have primary an impact on AMPAR trafficking and mobility (Martin et al., 2009).

The composition of AMPA receptor subunits highly affects its functionality. Hereby, the GluR2 subunit is a rate-limiting factor for calcium influx post activation accountable for desensitization of GluR2-containing AMPARs (Isaac et al., 2007). Stress vulnerable mice show a higher total number of AMPARs with a subunit composition favoring lower receptor sensitivity (Schmidt et al., 2010). Despite the higher number of AMPARs in stress vulnerable animals, their signaling might be reduced due to lower receptor sensitization. Potentiation of AMPA receptors displays increase of cell proliferation and cell survival in the hippocampus (Su et al., 2009) normally affected by stress induction. In the past years a variety of drugs acting on AMPARs was investigated. Positive allosteric modulators enhance the function of AMPARs function in the

presence of glutamate, without activating the receptors on their own (Arai & Kessler, 2007). Moreover, ampakines potentiate the receptors and were found to carry out antidepressant effects (Li et al., 2001; Bleakman et al., 2007) via enhancement of synaptic excitation (Gates et al., 2001; Miu et al., 2001; Vandergriff et al., 2001; Quirk & Nisenbaum, 2002). Interestingly, Schmidt and colleagues additionally indicated that above mentioned drugs are effective in reducing individual stress vulnerability and even promote stress resilience and faster recovery (Schmidt et al., 2010). Since increased GluR2 mRNA is correlated with impairments in spatial working memory (Simões et al., 2007), individual GluR2 mRNA-levels could portray a biomarker for individual vulnerability (Mannie et al., 2010). Most interestingly, Schmidt and colleagues demonstrated a polymorphism in mouse GluR1 gene to significantly correlate with individual stress vulnerability concomitant with a lower hippocampal GluR1 expression. In the current thesis we merely investigated the AMPAR GluR2-Subunit. However, due to the above-mentioned findings it would be beneficial to observe the modulation of GluR1 with DRR1 to better understand its impact on AMPARs and their modulation during stress.

There is increasing evidence that inflammation is implicated in the etiology of major depression (Kappelmann et al., 2016; Miller & Raison, 2016). The bioactive sulfated polysaccharide flucoidan exerts anti-inflammatory activity and was found to exhibit antidepressant effects in mice showing depressive-like behavior after subjected to chronic stress (Li et al., 2019). Flucoidan promotes hippocampal BDNF-CREB pathway leading to phosphorylation of Ser845 of AMPAR GluR1 subunits. This in turn stabilizes surfaces AMPARS and correlates in antidepressant like behaviors. Most likely hippocampal glutamatergic neurotransmission is enhanced via inhibition of the caspase-1-IL-1β inflammation pathway (Li et al., 2019).

Research from the past years demonstrated a strong implication of the glutamatergic system in stress susceptibility and depressive-like behavior. Whether glutamate carries out protective effects on neurons or causes neuronal damage is highly dose dependent (Rubio-Casillas & Fernández-Guasti, 2016). For example, low glutamate levels activate adaptive stress responses with expression of protective proteins against stress. On the other hand, particularly high glutamate levels cause neuronal atrophy and promote the onset of depression (Rubio-Casillas & Fernández-Guasti, 2016). Excessive glutamate levels emerge through high release or insufficient glutamate removal. With regard to depression provoked by chronic stress, the glutamate release is heightened. This overactivated NMDA receptors and as consequence impaired AMPA receptor activity (Rubio-Casillas & Fernández-Guasti, 2016). Through antidepressant application plasma glutamate levels are lowered and NMDAR activity is reduced by decreased expression of its subunits. In this connection AMPAR neurotransmission is reinforced (Rubio-Casillas & Fernández-Guasti, 2016).

Several in vivo studies identified coregulation and co-expression of *Drr1* and AMPARs (Chong et al., 2007; Schmidt et al., 2011). With our work we intended to identify an interaction between the actin-interacting and glucocorticoid regulated protein DRR1 with AMPA glutamate receptors. For this purpose, we localized and detected DRR1 and GluR2 protein on a western blotting membrane after separating the proteins from primary hippocampal cell lysates by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Neuronal cultures were previously infected with rAAV to either overexpress or suppress DRR1. Our results show a correlation between DRR1 and GluR2 protein amounts (Chapter 2.2.2). Increased intracellular DRR1 protein levels are positively correlated with higher protein amount of GluR2. Oppositely, we found decreased GluR2 levels upon DRR1 suppression. Our *in vitro* results mirror findings from the Mueller lab, showing the same correlation between DRR1 and AMPAR subunits in the mouse CA3- and CA1-region after region specific DRR1 overexpression and suppression (Schmidt et al. 2011; unpublished data Mueller lab). For example, Masana and colleagues detected a decrease in hippocampal CA3 GluR1 and GluR2 mRNA after local shDRR1 transduction (unpublished data).

Under basal conditions synaptic AMPARs in hippocampal pyramidal neurons predominantly contain GluR2 subunits (Bagal et al., 2005). GluR2-containing AMPARs are calcium impermeable or at least profoundly decrease Ca^{2+} passage drastically (Hollmann et al., 1991) and represent a linear current–voltage relationship. Calcium-permeable AMPARs are strongly inwardly rectifying and play an important role in synaptic plasticity (Jonas & Burnashev, 1995; Bagal et al., 2005; Plant et al., 2006). Increasing evidence shows the importance for Ca^{2+} permeable AMPARs in synaptic plasticity, learning and diseases (Wright & Vissel, 2012). At the same time the presence of the GluR2 subunit affects the stabilization and trafficking of AMPARs to the plasma membrane after assembly in the ER (Malinow & Malenka, 2002).

By overexpressing DRR1 we found an increase in GluR2 subunits. This fits the model of depression with a higher GluR2 amount, showing that DRR1 elevations are sufficient to mimic a stressful condition without the application of glucocorticoids. This observation implies that DRR1 is implicated in the glucocorticoid signaling cascade. Furthermore, our data shows that DRR1 has an influence on the number of GluR2-containing surface AMPARs. DRR1 might control the insertion of AMPARs through its actin-binding properties. The actin cytoskeleton is an important scaffold for the passage of AMPA receptors to and from the postsynaptic site. DRR1 was found to act as ABP important for the assembly and disassembly of the actin cytoskeleton in synapses. An increase of DRR1 could promote local actin dynamics which in turn establish a stable scaffold for AMPAR trafficking. Further studies need to elucidate if the total number of AMPARs is elevated or decreased upon DRR1 overexpression or suppression, respectively, or whether the

ratio of GluR2-to-GluR1 is changed. Nevertheless, in both cases DRR1 would have an effect on AMPAR trafficking. Our immunohistological DRR1 specific-staining revealed high DRR1 occurrence within the cytosol and nucleus of diverse cell types in the murine brain. Whether DRR1 might have regulatory gene functions, directly intervening in expression of AMPARs would be an interesting research topic.

3.2.2 Identification of DRR1 and GluR2-containing AMPARs interaction

Other actin-interacting proteins have the ability to directly regulate the insertion and trafficking of AMPARs (Rust et al., 2010). Therefore, we hypothesized that DRR1 might exert similar functions. To dissect the neurobiological pathways from DRR1 to stress resilience mechanisms *in vitro* we used primary hippocampal wildtype cultures and investigated the potential interaction of DRR1 and the AMPA glutamate receptor subunit GluR2 at the protein level. By using the proximity ligation assay (PLA) we could confirm the molecular interaction of DRR1 and AMPA receptors. The PLA is able to detect the proximity of two proteins with a distance under 40 nm, displaying their synergy. This technique enabled us to verify the colocalization of DRR1 and GluR2 in cultured neurons (Figure 2.10). The appearance of PLA puncta throughout neuronal dendrites and most strikingly at synaptic spines, proposes that DRR1 might be directly involved in AMPAR transport.

3.2.3 Intrinsic changes of DRR1 expression impact on synaptic scaling *in vitro*

A neuron maintains its set point upon activity or environmental changes through adaptation of synaptic strength. The underlying multifaceted mechanisms are known as homeostatic plasticity (Turrigiano, 2008). Synaptic scaling involves changes in surface AMPAR content (Turrigiano, 2008; Forrest et al., 2018). As actin-interacting protein DRR1 is likely to modulate AMPAR location and function (Revenue et al., 2004; van der Kooij et al., 2015). Schmidt and colleagues (Schmidt et al., 2010) showed a critical role for hippocampal AMPARs in modulating resilience to stress. The combination of its actin-binding properties and regulation upon stress, let us hypothesize that DRR1 could directly regulate localization and function of AMPARs at the synapse.

Previous findings from our collaboration partner in Mainz (AG Mueller, Department of Psychiatry & Psychotherapie, Johannes-Gutenberg University Medical Center, Mainz) provided

evidence that AMPA receptors play a notable role in modulating resilience to stress (Schmidt et al., 2010). So far, via the virus-mediated DRR1 knockdown and overexpression approach we were able to show that DRR1 expression is positively correlated with GluR2 levels. Further investigations revealed a direct protein-protein interaction between DRR1 and GluR2. In a next step we wanted to dissect the molecular and functional role of the DRR1–GluR2 interaction. To dissect the molecular pathway of DRR1 and AMPAR interaction we employed the newly inserted *in vitro* assay (Essmann et al., 2008; Sawamiphak et al., 2010), by which the new insertion of surface GluR2 is measured after stimulation with KCl. Neuronal depolarization is triggered by KCl application which then leads to activation of voltage-gated ion channels and expression of immediate-early genes (Bading et al., 1993; Michod et al., 2012). We combined the newly inserted assay with AAV transfection of cultured neurons to overexpress or suppress DRR1 beforehand. Using this approach, we aimed to imitate a disbalance of DRR1 as for example found in stress situations (Masana et al. 2014, Jene et al., 2018; Masana et al., 2018). We detected a significant decrease of newly inserted GluR2 subunits upon DRR1 knockdown. Since DRR1 serves as ABP (Schmidt et al., 2011; Kretzschmar et al., 2018) an intracellular reduction could affect the assembly of actin filaments and therefore disrupt the proper morphological development of dendritic spines. As consequence, the reduction of proper assembled actin cytoskeleton withdraws a proportion of the track for AMPAR trafficking to the site of action, thus reduces the amount of newly inserted GluR2 subunits. At this point it is important to emphasize the limitations of this experiment, especially in regard of the time component. The treatment with shDRR1 was performed after 7 days of primary hippocampal neurons in culture. During this time proper cell development was feasible under neuron specific conditions *in vitro* and cells were able to form a proper dendritic tree with normal spine development and AMPAR insertion at the PSD. Treatment with rAAV fabricates a mild cytotoxic environment wherefore we chose a suitable control condition by transfecting cultures with shSCR creating the same cytotoxicity for the controls. Cell toxicity was generally reduced through decreasing the MOI ascertained via preliminary experiments. Additionally, profound translation of virally delivered genes or shRNA occurs at day 3 post infection with a peak after 7 days post-infection. By means of this, in our model reliable changes in DRR1 expression could only be established after 14 days of primary hippocampal cells *in vitro*. Adequate neuronal cell development appeared until 7 DIV during which AMPARs could normally insert into the plasma membrane of spines. On one hand we could show, that DRR1 reduction affects AMPAR trafficking to the PSD. However, we did not investigate so far to what extend the AMPAR amount was reduced already before stimulation as compared to controls and whether this impacts on the new insertion on AMPARs or not.

In contrast to DRR1 knockdown, its overexpression could not reveal significant differences in the new insertion of GluR2 subunits as compared to controls. An indistinguishable

amount of surface GluR2 turnover between neurons treated with rAAV for OE and control, points to a stabilizing effect of DRR1 equivalent to earlier findings where presence of DRR1 heightened the amount of net F-actin (Schmidt et al., 2011).

Despite the difference in observations, we demonstrated that DRR1 is involved in AMPAR trafficking to the post-synapse, most likely through its actin-binding properties. Furthermore, different DRR1 expression levels have direct effects on AMPA receptor numbers. Specifically, suppression of DRR1 alters the insertion and trafficking of GluR2-subunits, whereas overexpression has no effect. A decrease of DRR1 could affect local actin dynamics, which in turn disrupt the stable scaffold for AMPAR trafficking. The findings suggest that DRR1 is necessary for AMPA-trafficking. However, excess of DRR1 does not alter the system, presumably due to saturation. Our results may be indicative of the differences observed *in vivo*, showing altered LTP in mice with locally overexpressed DRR1 in the hippocampus. Local overexpression of DRR1 in the hippocampal CA3 field decreases CA3-CA1 LTP (Schmidt et al., 2011).

3.3 The influence of DRR1 on the morphology of primary hippocampal neurons *in vitro*

Following our findings showing a functional correlation between DRR1 and GluR2 we questioned whether reduction of newly inserted surface GluR2 after DRR1 knockdown might not only appear due to dysfunctional AMPAR trafficking but rather correspond to changes in neuronal morphology, especially in regard to dendritic spines or dendrites themselves. These changes could include malformations, reduction of spine numbers or simply a shift in spine subtypes, eventually downsizing the PSD and preventing insertion of new AMPARs even upon activity. Under normal conditions glutamatergic neurotransmission leads to recruitment of new glutamate receptors to the PSD and continuing activity induces diverse downstream signaling cascades to trigger structural plasticity for stabilization and strengthening of synaptic connections (details see introduction Chapter 1.7). Concurrently we aimed to find out if DRR1 expression manipulation has an effect on spine morphology, hence acting as resilience promoting player.

3.3.1 DRR1 impacts on dendritic complexity in primary hippocampal neuron cultures

Neurite outgrowth requires the reorganization of actin filaments. Since DRR1 has the potency to modulate actin dynamics, we hypothesized that dendrite growth and spine development might be affected by DRR1. By overexpressing DRR1 in the murine neuroblastoma cell line Neuro2a – a commonly used cell line to study actin dynamics – Schmidt and colleagues showed an impairment in the development of neurites and a decrease in neurite length compared to control conditions (Schmidt et al., 2011). At the same time the researchers could detect an accumulation of F-actin, suggesting that high DRR1 negatively influence on actin organization. Further experiments with DRR1 overexpression in developing primary hippocampal cultures (36 h after cultivation) were able to show the accumulation of DRR1 in outgrowing protrusion in normal conditions but not in transfected cells overexpressing DRR1. Interestingly, transfection of older cultures with already developed neurites did not show an effect on neurites (Schmidt et al., 2011).

We wanted to expand the present findings by utilizing our viral *in vitro* model manipulating DRR1 levels in primary hippocampal neuron cultures in combination with morphological analysis. Therefore, we used the customized rAAV vectors tagged with GFP, which serves as reporter molecule for gene expression. Neuron-specific GFP features the benefit that it is expressed throughout neuronal cell bodies and dendritic trees. The fluorescent GFP signal enabled us to capture the complete cell architecture of interest before performing morphological reconstructions with subsequent structural analysis. Additionally, we stained the neurons with anti-microtubule associated (MAP2) antibodies to visualize the complete structure of dendritic trees for a better tracing and digital reconstruction. MAP2a/b isoforms are only expressed in the perikaryon and dendrites of neuronal cells (Soltani et al., 2005). To investigate the significance of DRR1 protein on dendritic development of hippocampal neurons we performed Sholl-analysis after rAAV transfection thus manipulation of DRR1 expression. During development cell-specific dendritic arborizations are determined in a strongly regulated process. When these regulatory processes are aberrant or interfered for example at the time of diseases or injury, the shape of dendritic tress can be altered which in turn disturbs the neural circuitry (Kulkarni & Firestein, 2012; O'Neill et al., 2015). Sholl analysis (Sholl, 1953) is utilized to analyze dendritic arbor morphology by identifying changes as a whole. Characteristic for Sholl-analysis is the intersection profile by counting the number of dendritic branches at fixed distances from the soma in concentric circles. In this way, the method reveals the complexity of dendritic trees based on the number of branches, the geometry of the branches and the branching pattern of the neurons (Caserta et al., 1995). Besides dendritic branching, we captured the domain spanned by dendritic arbors and the total dendritic length.

Our investigations revealed no significant differences between controls and conditions where DRR1 was either suppressed or overexpressed. Nevertheless, we found tendencies diverging from control conditions in both cases. A decrease of intracellular neuronal DRR1 showed a trend towards reduced dendritic length; at the same time unchanged numbers in branch points (Chapter 2.4.1; Figure 2.12). Also, the number of intersections in closer proximity to the soma (from 25–130 μm) was lower for shDRR1 treated cultures in comparison to shSCR controls, which explains the decrease of total dendrite length in shDRR1 cultures. With increasing distance from the soma up to 250 μm the number of intersections between control and shDRR1 cannot be discriminated. More experiments are needed to show whether this outcome is reproducible and if a higher N might show significant differences. By now, we can speculate that DRR1 abundance has an effect on the morphology of neurites. By contrast, an overexpression of DRR1 in primary hippocampal neurons shows an increase in the total dendritic length as well as in the number of dendritic branch points. The difference is notable in the increased count of intersections especially from 15–125 μm from the cell soma. Significant peaks of intersection amounts are visible at distance of 170 μm and 180 μm in neuros with increased DRR1 in comparison to controls. Our results show opposing fallouts in dendritic complexity when DRR1 amounts are decreased or increased indicating a relevant role for DRR1 in dendritic plasticity. Unlike Schmidt and colleagues, we investigated the influence of different DRR1 level changes in primary hippocampal neurons at later developmental time points after a big proportion of dendritic neurites was already established. Similar to their findings we did not detect a neurite-destructive activity of DRR1 upon overexpression, but rather an effect on actin-dependent reorganization in cells, even affecting dendritic complexity.

In our *in vitro* Sholl-analysis, we considered the whole dendritic tree and did not distinguish between basal and apical dendrites due to the challenge of discrimination between the two subtypes in culture. However, murine *in vivo* studies indicate a difference in stress induced dendritic retraction of basal and apical dendrites with basal dendrites not being affected (Tata & Anderson, 2010; Conrad et al., 2017a). Also, for our *in vitro* analysis we dissected the whole hippocampus culturing all subtypes of hippocampal neurons such as CA1 and CA3 pyramidal cells as well as granule cells. In vivo, hippocampal granule cells and pyramidal cells develop morphologically distinguishable dendritic arbors with some common features (Wu et al., 2015). While basal dendrites are only formed by pyramidal cells, both cell types from long apical dendrites originating from the apex of the cell soma (Wu et al., 2015). In culture, both cell types show similar dendritic patterning with a single principal dendrite with several minor dendrites making them hard to distinguish by appearance. Thereto, both cell types start to emerge simultaneously in mice by E10 (Angevine Jr., 1965; Bayer, 1980; Wu et al., 2015). Moreover, retraction of the basal dendrites of granule cells requires external matrix cues which are provided *in vivo* but not in *in vitro* models thus

regression of the basal dendrite is incomplete in culture (Wu et al., 2015). In culture these cell types are merely distinctive through molecular marker expression. The final principal dendrite designation in pyramidal neurons is obtained at DIV 10. Taking this into consideration, we selected cultured neurons with most elaborated dendritic trees and a clear and big basal dendrite to minimize capturing granule cells into our analysis. Taken together, it would be of need to perform *in vivo* studies to investigate dendritic morphology in hippocampal pyramidal neurons upon DRR1 overexpression and knockdown, since *in vitro* models can't completely display *in vivo* situations.

Generally, dendritic arbors are very plastic structures that have the ability to expand and retract according to incoming stimuli. Through postsynaptic signaling those structures are maintained (Dailey & Smith, 1996; Cline, 2001; Weerasinghe-Mudiyanselage et al., 2022). Alterations in dendritic structure involve reorganization of the actin cytoskeleton leading to changes in dendritic patterning, fragmentation, and retraction (Parrish et al., 2007). Branching of dendrites requires the interplay of a large number of cellular factors, encompassing microtubules, microtubule regulatory proteins, neurotransmitters, glucocorticoids, growth factors (Parrish et al., 2007), and presumably DRR1 through its action as actin-binding protein. Depressive disorder induces neurogenerative conditions leading to hippocampal dysfunction and cognitive impairments ascribed to reductions in dendritic complexity with lowered dendritic length, dendrite numbers, and dendrite crossings (Weerasinghe-Mudiyanselage et al., 2022). When chronic stress is robust and endured long enough, dendritic retractions can be observed in hippocampal neurons (Conrad, 2006) first occurring in dentate granule cells, CA1 pyramidal neurons and basal CA3 dendrites before also CA3 apical dendrites show a reduced dendritic complexity (Conrad et al., 2017), similar to our observations when DRR1 is endogenously reduced, hypothesizing that DRR1 elevation might have protective effects on dendritic tree stabilization and preservation. Moreover, reduced CA3 apical dendrite complexity coincides with impairments in hippocampal-dependent cognition like spatial learning and memory (Conrad et al., 2017). However, when chronic stress periods end, a post-stress recovery is able to improve atrophied dendritic arbors and stress-induced cognitive impairments even if those still don't reach states of non-stressed controls (Conrad et al., 2017). Multiple studies explored how glucocorticoid administration and chronic stress affect subregional dendrites of pyramidal cells in the hippocampus. For instance, MRI imaging revealed a reduction of the neuropil in CA regions after chronic stress (Swanson-Park et al., 1999) excluding cell soma regions. All studies unitedly report that stress-induced dendritic retraction particularly appears in apical dendrites of CA3 pyramidal neurons (Ren & Dubner, 2007; Conrad et al., 2017; Esmaeili-Mahani et al., 2021) arranging the sl, sr, and slm implicating inputs from the EC pathways and commissural/association pathways. Moreover, the majority of stress-induced dendritic pruning was spotted in middle and most distal ends of apical dendrites (Vyas et al., 2002; Eiland

& McEwen, 2012; Orlowski et al., 2012; McCall et al., 2013). Proximal dendrites receive excitatory inputs from local sources, i.e., collaterals from adjacent areas, whereas distal apical tufts get inputs from more distant thalamic regions (Spruston et al., 2008). When looking at the changes of dendritic tree morphology that we observed *in vitro*, mainly the middle parts of dendrites were affected. Our results are consistent with the findings made *in vivo* where middle and distal apical dendrites were shown to be mainly affected by chronic stress effects.

3.3.2 Divergent DRR1 protein levels alter spine morphologies in opposing ways

Interestingly, Schmidt and colleagues found increased cognitive function in mice with stability viral-mediated DRR1 overexpression in the CA3-region. This is supported by our *in vitro* observations of dendritic complexity in neurons with elevated DRR1. Contradictory, electrophysiological measurements in similarly treated animals displayed significant reductions of spines on apical dendrites of CA1 and CA3 neurons. This was accompanied by reduced probability of hippocampal synaptic neurotransmission release indicated by field excitatory postsynaptic potential (fEPSP) recordings in acute brain slices from DRR1 overexpressing mice (Schmidt et al., 2011). Coherent with this finding, elevated DRR1 levels enhanced paired-pulse facilitation at CA3-CA1 synapses and led to reduced LTP magnitudes in comparison to respective controls (Schmidt et al., 2011).

Reductions in hippocampal spine numbers were reported in rodents and humans after stress exposure (Chen et al., 2008; Soetanto et al., 2010). Since elevated DRR1 levels were especially found in the CA3 region of mice following stress exposure, it was suggested to play a role in the stress response (Schmidt et al., 2011). The use of a viral expression system allows to mimic the stress-induced increase of DRR1 in specific brain regions or *in vitro* and thereby distinguish its effects from the multitude of other stress induced factors (Joëls & Baram, 2009). Moreover, the rAAV approach can be used to study the role of DRR1 abundance on neuronal morphology and function.

With the acquainted findings, we aimed identifying the effects of DRR1 on the formation and stability of spines, a process that is important for learning and memory (Xu et al., 2009; Yang et al., 2009; Roberts et al., 2010). Using our established rAAV *in vitro* model, we investigated the influence of DRR1 level changes on spine morphology focusing on differences in spine subtypes and spine numbers. For this purpose, we reconstructed dendritic stretches of 30 µm length from cultured hippocampal neurons transfected with shDRR1 and the viral overexpression construct

for DRR1. The reconstructions included dendritic spines as well. Afterwards we compared total spine numbers and spine subtypes with respective controls.

Upon DRR1 suppression, we discovered significantly decreased total spine numbers compared to hippocampal neuron cultures transduced with a virally delivered shSCR control. After analyzing the spine subtypes, we ascertained a significant decrease in both, filopodia and long thin spines in shDRR1 treated cultures in comparison to controls (Chapter 2.4.2, Figure 2.14). Numbers of mushroom stubby spines showed no differences. Hence the decrease in total spine numbers can be solely ascribed to a reduction of immature spines. Filopodia represent a precursor stage that scans the environment for synaptic contacts and retracts in the absence of them (Bertling & Hotulainen, 2017; Parajuli et al., 2017). Thin spines represent most immature spine types with a small PSD. Our findings suggest that formation of new spines is disrupted when DRR1 levels are lowered, most likely due to reduced F-actin bundling. However, stabilization of already formed spines represented by mushroom spines and stubby spines does not seem to be affected by reduced DRR1 levels. This suggests that DRR1 is an important component especially in spine formation when actin-dynamics are higher and when it involves actin assembly for spine growth processes of the spine neck (Hotulainen et al., 2009; Yoshihara et al., 2009). Overexpression of DRR1 on the other hand did not change total spine numbers in our experimental model. Nevertheless, we evidenced changes in the composition of spine types with mushroom spines being significantly decreased. The total spine number is not affected due to compensatory raise in the numbers of filopodia and long thin spines. Also, these results show the importance of DRR1 in formation of new spines. It seems that is especially plays a role in the elongation of spine structures, whereas stability of spine heads seems to be disrupted upon high DRR1 amounts.

Elongation of filopodia depends on polymerization of actin filaments at the tips. When spines start to develop out of filopodia they require branching and spine head formation involving actin assembly at filopodium tips (Hotulainen & Hoogenraad, 2010). Dynamics and stability of mature spine heads are supported by actin crosslinking proteins. In order to guarantee a dynamic change in shape, for example upon activity and LTP, myosin-II-dependent contractility is critical (Hotulainen & Hoogenraad, 2010). Furthermore, when LTP is persistent activity of ABPs such as Arp2/3, profilin and actin-crosslinking proteins is enhanced. The mentioned proteins promote actin polymerization and net formation. On the other hand, cofilin activity is reduced in spines when LTP occurs (Hotulainen & Hoogenraad, 2010). As depolarizing factor cofilin induces the breakdown of actin filaments (Hotulainen & Lappalainen, 2006; Kiuchi et al., 2007) and its dampening promotes stabilization.

A surplus of DRR1 seems to inhibit the net formation and actin branching in spine heads. One explanation could be a distortive effect in joining the sides of existing actin filaments.

Stabilization of spine heads requires increased activity of capping proteins. Our results let us speculate that the identified polymerizing properties of DRR1 (Kretzschmar et al., 2018) affect primary straight actin filaments that are patterned during filopodia growth, since decreases in DRR1 minimize filopodia and long thin spines, whereas upon overexpression these spine types are increased. Nucleation of actin filaments initiates the formation of an actin nucleus consisting of three actin monomers and thereby initiates the formation of filopodia. Since DRR1 was found to promote nucleation (Kretzschmar et al., 2018) it fits our findings of higher numbers of filopodia and long thin spines in DRR1 OE conditions. Moreover, it is known that anti-capping of actin filaments promotes formation of filopodia (Mejillano et al., 2004). Thus, the increase in filopodia and immature long thin spines after DRR1 OE can be explained by its capping properties (Kretzschmar et al., 2018). The formation of new spines is reduced when DRR1 is not present, most likely to reduced actin capping. More work needs to be done on finding interaction partners of DRR1 that could explain the destabilization of mature spine heads upon overexpression. Potentially, excess of DRR1 leads to early capping of actin nets in spine heads preventing them from increasing in size. Previous findings subscribed DRR1 diminishing effects on actin-treadmilling, actin filament elongation and cell spreading (Kretzschmar et al., 2018) which as consequence could take action in preventing spine head growth. Contrary, the enhancing effect of DRR1 on actin bundling, cellular F-actin content might support filopodia outgrowth. DRR1 shift the neuronal actin network towards more, thicker, and shorter actin filaments which might be useful for elongation but not the creation of a stable actin network with long filaments as necessary in spine heads.

Putting our findings together, we could show that excess of DRR1 promotes initiation of new spines as well as dendritic arborization most likely due to its actin polymerizing and bundling properties. Suppression of DRR1 leads to decreased dendritic complexity and a reduction in spine numbers. There is strong evidence that actin dynamics in synapses are critical for localization and function of AMPARs (Allison et al., 1998; Kim & Lisman, 1999; Zhou et al., 2001), which our findings underpin. Structural changes evoked by changing DRR1 levels can be linked to different amounts of new inserted surface GluR2 subunits. Reduced new insertion of GluR2 subunits in neurons with suppressed DRR1 could be an indirect result of lowered total spine numbers and dendritic complexity because the area of postsynaptic densities is automatically reduced. The non-differentiating numbers of GluR2 subunits in DRR1 OE and empty conditions could be explained by reduced numbers of mature mushroom spines but simultaneous increase in immature spines and a gain in dendrite numbers as compensatory balance eventually leading to equal GluR2 amounts.

Research on actin-binding proteins that physically interact with AMPARs starts to shed light on the underlying mechanisms involved (Gu et al., 2010; van der Sluijs & Hoogenraad, 2011).

Such ABPs either regulate actin dynamics and in this way modulate mechanical forces on AMPAR containing membranes or associate with actin-filaments to transport or target vesicles containing AMPARs to specific subcellular regions where the receptors can exert their functions. Moreover, actin-regulatory proteins that do not interact with AMPARs can still influence receptor trafficking by regulating the local actin environment in synaptic spines.

3.4 Stress induction in primary hippocampal cultures and DRR1

Precedent gene-expression profiling analysis discovered that transcription of DRR1 depends on GR dimerization (Frijters et al., 2010). Further, the increase of *Drr1* expression upon stress in the glucocorticoid sensitive CA3-region of the hippocampus proved a regulation through glucocorticoids (Schmidt et al., 2011; Masana et al., 2015). Acute and chronic stress was shown to release glucocorticoids which induce changes in glutamate neurotransmission in the prefrontal cortex and the hippocampus with negative influences on cognitive performance (Schmidt et al., 2010; Propoli et al., 2012).

We reproduced a glucocorticoid-dependent, thus stress related increase of DRR1 expression (Masana et al., 2018) in a physiologically analogous manner, by stimulating primary hippocampal cultures for 24–48 h with the artificial glucocorticoid dexamethasone (DEX). In this way we wanted to draw a bigger picture on the function of DRR1 not segregated from other cellular stress response pathways. Similar to our *in vitro* DRR1 overexpression analysis we could find an increase of DRR1 protein amount in correlation with elevated GluR2 subunits upon DEX treatment. This underlines previous *in vivo* findings of DRR1 increases in glucocorticoid sensitive regions upon stress induction (Schmidt et al., 2010; Popoli et al., 2012) and confirms that DRR1 overexpression can be used as a stress model to dissect the function and analyze the role of DRR1 unaffected from other stress-response pathways. Applying the newly inserted GluR2 assay in combination with DEX treatment we could not identify any differences in newly inserted GluR2 subunits after KCl stimulation between control conditions and DEX treated cultures. The results mirror our outcomes from DRR1 overexpression experiments where new insertion of surface Glur2 were not detectable.

Our results show that neuronal DRR1 is indeed regulated by glucocorticoids and that this process acts directly on hippocampal neurons without the necessity of signal transmission from other cell types. It also shows that glucocorticoids alone elevate DRR1 expression which affects GluR2 insertion at membrane surfaces. We can speculate that the processes are similar to our observations in neuronal cultures with virally overexpressed DRR1 (Chapter 3.3.2). These findings are important to pioneer an experimental approach of studying the action of DRR1 *in vitro* through

stress application. An advantage of this approach is the leave of invasive techniques such as viral delivery systems to manipulate DRR1 levels and thereby represents a milder and more physiological manipulatory system.

Utilizing this approach, one could induce stress in primary hippocampal cells for a longer period of time, thus mimicking a chronic stress response. Beyond that, DEX application can be subjected on control and DRR1 knockout neurons to study the differences in their AMPAR trafficking and neuronal morphology upon stress induction.

3.5 Resilient, susceptible and non-learning mice show differences in hippocampal spine compositions after chronic social defeat stress

Humans present a broad variation in their response to psychological stress. Multiple studies suggest that stress resilience is mediated by changes in diverse neural circuits involving plenty of neurotransmitters and molecular pathways (Feder et al., 2009; Russo et al., 2012). The underlying molecular mechanisms that facilitate stress resilience are mainly unexplored. Concurrently, the identification of such molecular players and mechanisms is key for the development of therapeutic treatments of psychiatric disorders including major depression. The development of new drugs is of importance since stress-related disorders have detrimental effects on the brain structure with long-lasting consequences on behavior. Multiple lines of evidence demonstrated that chronic stress impacts on the size, volume and spine numbers in cortical and limbic brain regions implicated in depression (Nestler & Hyman, 2010; Duman & Duman, 2015; Ohgi et al., 2015). Especially the morphologically plastic regions such as the prefrontal cortex, the hippocampus, the amygdala and the nucleus accumbens undergo structural changes upon stress (Radley et al., 2006; Nestler & Hyman 2010). In rodents chronic social defeat stress (CSDS) is widely used as model of depression. Thereby approximately 30% of the animals show stress resilient behaviors (Nestler & Hyman 2010; Qu et al., 2018). Multiple studies applied this model of depression in mice and demonstrated abnormalities in spine densities in stress susceptible animals within the CA3 region and the DG of the hippocampus amongst the other above mentioned brain regions (Yang et al., 2015; Ma et al., 2016; Yao et al., 2016; Dong et al., 2017). Changes in spine densities within the mPFC, hippocampus, NAc, and VTA are believed to be involved in the pathogenesis of depression (Nestler & Carlezon, 2006; Duman & Duman, 2015; Ohgi et al., 2015; Zhang et al., 2016). One important study examined spine densities in these crucial and stress-sensitive brain areas of stress-resilient mice (Qu et al., 2018). In comparison to control

animals, they identified significantly decreased spine densities in CA3 pyramidal neurons and granule cells of the DG from stress susceptible animals but not stress resilient mice, where spine numbers corresponded unstressed controls (Qu et al., 2018). Interestingly, there were no differences in spine numbers within the hippocampal CA1 region (Qu et al., 2018). The study provides an indication of the structural homeostatic effects occurring in resilient mice to counterbalance negative stress effects.

Social avoidance induced through chronic social defeat stress in male mice represents a feature of stress-related mental dysfunction. The absence of social avoidance post CSDS is viewed as a proxy of stress resilience (Ayash et al., 2020). The individual ability to cope with stress involves adequate biological and psychological adaptation whereas the inability of adequately coping with stressful situations can predispose neuropsychiatric disorders. Surprisingly, mammals and humans have an immense capacity for resilience which is demonstrated by the fact that a big proportion of individuals does not develop mental dysfunctions after acute or chronic stress phases (Russo et al., 2012). The mechanism of fear conditioning in humans was proposed to play a role in the individual stress response and the development of mental disorders, but also the determination of the level of coping abilities (Mahan & Ressler, 2012). Two aspects of conditioned fear learning in humans were identified in promoting resilience to stress. One is the ability to discriminate between stimuli and extinguish aversive memories when the opportunity of re-learning is given (Banerjee et al., 2017). Over the past years these findings were translated into resilience research in mice (Berton et al., 2006; Krishnan et al., 2007; Chaudhury et al., 2013; Dias et al., 2014; Friedman et al., 2014; Tse et al., 2014; Li et al., 2017; Focking et al., 2018) and the scope of social avoidance is now considered as representation of susceptible and resilient states (Krishnan et al., 2007) assigning subgroups of rodents post CSDS into non-avoidant and avoidant or rather resilient and susceptible, respectively (Berton et al., 2006; Krishnan et al., 2007; Russo et al., 2012). Due to contradictory reports of behavioral and physiological phenotypes in resilient and non-resilient mice after categorizing them according their social interaction post stress-exposure (Krishnan et al., 2007; Dias-Ferreira et al., 2009; Chou et al., 2014), a new measure was developed. Prior social interaction tests left the opportunity to CSD mice to either interact with an unknown conspecific from the aggressor's strain only or not interact with the presented mouse at all (Hammels et al., 2015). The interaction time was measured and by threshold application mice were subsequently categorized. The modified social interaction test (MSIT) advanced the classification of animals into resilient and susceptible groups and helped deepen the characterization of the social avoidance phenotype (Ayash et al., 2020). It was shown that conditioned learning is involved in CSD-induced social avoidance and that it can be reversed through extinction learning (Ayash et al., 2020). The MSIT is performed in a sociability chamber divided into three compartments. In the outer

compartments mice from different strains are placed. Hereby, one corner is occupied by a mouse of the aggressor's strain and the other by an unknown and harmless strain (Ayash et al., 2020). Mice that underwent CSDS specifically avoided conspecifics with the aggressor's phenotypic characteristics whereas interaction with mice from an unfamiliar strain still took place. Control animals that were not exposed to social defeat showed similar interaction times with both strains (Ayash et al., 2020). The results proves that social avoidance induced by chronic social defeat stress is strain specific. The MSIT further revealed susceptible mice to generally interact less with conspecifics, regardless of the strain, but mostly and significantly lower with conspecifics of the aggressor's strain. Resilient animals behave differently. They show avoidance towards the aggressor's phenotype but normally interact with the mouse from the unknown strain (Ayash et al., 2020). Moreover, the MSIT paradigm unraveled a third behavioral group of mice that underwent CSDS. These animals did not avoid interaction with the aggressor nor with animals of the unknown strain. Hence, they were categorized into a new group as non-learners.

We investigated stress related outcomes on the spine composition and morphology in CA1 and CA3 pyramidal neurons of mice subjected to CSDS. Our investigations discern from previous studies, where spine densities were measured and compared between resilient, susceptible and unstressed mice (Qu et al., 2017). However, the studies lacked a categorization into spines subtypes potentially missing out an important shift in spine morphology. From our observations *in vitro* we speculated that there might be a potential shift in spine subtypes, promoting stress resilient rather than a simple stabilization of spine numbers. To broaden our understanding of the underlying cellular processes in stress resilient individuals we therefore classified the spine subtypes in resilient, control and susceptible animals for the first time. Moreover, we classified the animals into the distinct groups applying a new paradigm which enabled us to distinguish not only between stress resilient ($R^{-/+}$) and susceptible animals ($R^{-/-}$), but also a third group, namely non-learners ($R^{+/+}$). Non learners are not able to discriminate between an inoffensive mouse strain and the aggressor's strain, socially interacting with both for the same period of time (Ayash et al., 2020).

We traced 30 µm long dendritic stretches of hippocampal CA1 neurons and reconstructed the dendritic stretch including their spines by utilizing filament tracers and spine classifier in Imaris (Bitplane). For comparison reasons we solely selected secondary dendrites situated in the *stratum radiatum* of the hippocampal CA1 area. To ease the tracings, wildtype C57BL/6 mice expressing GFP under the Thy1 promoter were subjected to CSDS before undergoing MSIT with subsequent categorization into the distinct stress response groups according to their behavioral outcome. Under the Thy1 promotor the GFP protein is expressed in a subset of CNS neurons including pyramidal neurons of the hippocampus (Feng et al., 2000). As a result, the whole cellular structure

of GFP positive neurons is perceivable, making it easy to analyze the morphological cell structure. An additional fluorescent antibody-labeling increased the signal and alleviated the tracing.

In average the total number of spines was not changed between the distinct subgroups ($R^{-/+}$; $R^{-/-}$; $R^{+/+}$) in CA1 dendrites post chronic social defeat stress in comparison to non-stressed controls (Ctr) (Chapter 2.5). This result mirrors existing studies which showed no differences of spine numbers in stress-resilient versus stress susceptible animals, concluding that CA1 is less stress sensitive as the CA3 region were spine densities are found to be lower in susceptible animals (Qu et al., 2017). Also, these studies did not discriminate the third behavioral group $R^{+/+}$ as it was done through our paradigm and these mice rather slipped into the category "resilient" or "susceptible" or were even excluded from analysis.

Nevertheless, we further analyzed potential shifts in distinct spine subclasses. Here, we detected quite some discrepancies and significant differences between groups. Resilient animals appeared to have the most analogous spine phenotype like non-stressed controls with significant differences only catched in filopodia spines where numbers were elevated. $R^{-/+}$ mice also showed significantly more filopodia compared to non-learners ($R^{+/+}$). Interestingly, stubby spines were decreased in susceptible ($R^{-/-}$) and non-learning ($R^{+/+}$) animals in relation to controls. On the other hand, same behaving animals were found to possess significantly higher mushroom spines. The decrease in stubby spines and increase in mushroom spines could outweigh the total amount of spines equalizing them to a wildtype phenotype. Due to high variances, we couldn't detect any differences in long thin spines between groups. However, there are tendencies towards higher amounts in long thin spines in the $R^{-/-}$ and $R^{+/+}$ groups.

The shift in spine subtypes in differently reacting mice groups regarding social interaction as individual stress response underlines our *in vitro* findings. Elevated glucocorticoid levels were shown to increase total DRR1 levels in the hippocampus and especially in the CA3 area (Schmidt et al., 2011; Masana et al., 2014; Masana et al., 2018). Notably, the major-fold change expression after stress is observed in the hippocampal CA1 area (unpublished data Mueller lab). Therefore, we utilized DRR1 OE *in vitro* as stress model and showed a decrease in mature mushroom spines with simultaneous increases in long thin spines and filopodia. The partial suppression of DRR1 decreased immature spine numbers. When looking at CA1 spine subtype distribution *in situ* from stress resilient animals, we do not find a reduction in mature spines as proposed in our stress model. At the same time the number of filopodia is increased and could represent a coping mechanism in regards to stressful situations. It shows that structural plasticity is taking place presumably due to elevated synaptic activity upon stress. The formation of new spines is crucial for learning mechanisms (Hayashi-Takagi et al., 2015). Resilient animals learn how to discriminate between an aggressor and another mouse whiteout cultivating a general social avoidance towards conspecifics. This shows that learning processes have taken place in conjunction with a relatively

normal spine phenotype similar to unstressed animals. Since structural plasticity is not heavily taken place in resilient animals, other plasticity mechanism presumably come into account to strengthen synaptic connections. Inter alia this might arise from homeostatic plasticity, preserving network stability through i.e., changes in the glutamatergic synapse strength (Turrigiano et al., 2008; Pozo & Goda, 2010). This involves changes in number and composition of postsynaptic glutamate receptors, but also tightly regulated release of neurotransmitters from the presynapse (Pozo & Goda, 2010). Differently are the findings in stress susceptible and non-learning mice. In both cases we detected a decline in stubby spines with concurrent increases in mushrooms spines and tendencies of elevated long thin spines. These observations show that a clear structural plasticity is taking place in those two behavioral subgroups. A decrease in stubby spines fits the common knowledge of spine shrinkage upon stress-induction (van der Kooij et al., 2015). Contradictory is the finding of elevated mushroom spines. We hypothesize that presynaptic neurotransmitter release is reduced. The observed enlargement of spine heads could represent a compensatory mechanism to outbalance neurotransmission. This compensatory mechanism creates maladaptive behavior resulting in social avoidance or non-learning effects. A recent study investigated LTP in resilient and susceptible mice after CSDS. They found impaired LTP in susceptible mice (Lee et al., 2021). Combining these findings, it could reflect the necessity of stubby spines for CA1-CA3 LTP regulation.

In contrast to the CA1 region, we could not find any differences between spine subtypes of the behavioral groups in CA3 dendrites. Consequently, also the total spine numbers were unchanged after CSDS in resilient, susceptible and non-learning mice in comparison to the control group. Moreover, we could not detect any filopodia in analyzed dendritic stretches.
Regarding the CA3-area our results do not fit previous findings showing spine differences in resilient compared to susceptible animals (Qu et al., 2017). At this point it is important to mention that the amount of GFP expressing CA3 neurons was extremely low in our experimental animals. Hence it was difficult to locate and detect suiting dendritic stretches with high quality for our analysis. Because of these difficulties, we analyzed primary and secondary dendritic stretches located throughout the whole apical dendritic tree. These variations could have contributed to our findings and a repetition of the experiment is of importance.

When taking previous findings into account showing declined spine numbers in CA3 apical dendrites, our results in CA1 neurons could indeed reflect a compensatory mechanism. Reduced synaptic connections in CA3 could lead to reduced electric signal transmission via Schaffer collaterals to CA1 neurons, with consequently reduced presynaptic neurotransmitter release. Additionally, the inhibitory feedback loop on CA1 pyramidal neurons could be impaired

leading to less LTD, lowering the threshold of incoming signals and increasing hypersensitivity of these neurons. Eventually, this could promote spine growth in existing synaptic connections through increased actin dynamics. Missing neuronal inhibition, maladaptation in the CA1 network and resulting interference onto downstream neural circuits could provoke the behavioral phenotype observed in susceptible and non-learning mice. These speculative presumptions could be taken into account for future perspectives.

In a next step we aim to correlate endogenous DRR1 levels to the different behavioral groups ($R^{-/+}$, $R^{+/+}$; $R^{-/-}$, Ctr), by dissecting hippocampal area-specific brain tissue through tissue punching with subsequent protein measurements. By doing this we expect to clarify the question of DRR1's involvement in shaping spine morphology and its resilience-promoting effects.

3.6 Conditional and neuron-specific DRR1 knockout mice

The overall aim of our ongoing investigations is to dissect the molecular and cellular mechanisms determining how the stress and glucocorticoid regulated protein DRR1 in particular and neuronal actin dynamics and AMPAR trafficking in general modulate and fine-tune stress responses. Thereby, we are particularly interested in the minimization of the long-term damaging effects of stress.

Our previous data in combination with the data from our collaborating laboratory (AG Mueller, Department of Psychiatry & Psychotherapie, Johannes-Gutenberg University Medical Center, Mainz) emphasized resilience promoting properties to DRR1 in the context of stress (Schmidt et al., 2011; Masana et al., 2015; Masana et al. 2018). To characterize the detailed role of DRR1 in modulating stress related consequences and a speculated function in promoting resilience, we generated conditional and brain-region specific DRR1 knockout mice. In those $DRR1^{l/l}$_*Camk2a*-Cre^{+} mice the DRR1 protein is inactivated after the second postnatal week in the forebrain and limbic system through the expression of the Cre under the control of the *Camk2a* promoter (see Chapter 2.6 for detailed description).

To validate the inactivation of DRR1 in the brain of adult mutant mice, we lysed whole brains, the cortex and cerebellum of mutant and control mice and performed Western Blot analysis with the dissected tissue. Beforehand, we discerned $DRR1^{l/l}$_ *Camk2a*-Cre^{+} from $DRR1^{l/l}$_ *Camk2a*-Cre– control littermates through genotyping involving tissue lysis and PCR (Chapter 2.6). We compared control brains with our mutants and ascertained a decrease in total DRR1 protein amount in whole brain lysates as well as the respective brain fragments by 34–60%.

The knowledge about the function of DRR1 in adult nervous system and its implication in baseline behavior is missing. Hence, it is essential to characterize the baseline behavioral phenotype of DRR1 nKO mice. In collaboration with the Mueller laboratory, we planned to test the animals for normal motor and sensory skills, which are prerequisite for further behavioral tasks. Afterwards, hippocampus-dependent learning tasks can be addressed. This includes non-aversive paradigms for example, the novel object recognition test, where animals are tested whether they can discriminate between a novel and a familiar object. Also, social interaction and anxiety-like behavior can be assessed. If cognitive impairments are recognized, they can be deeper analyzed via the Morris water maze test or contextual fear conditioning. After extensive characterization of DRR1 knockout animals, their behavior following CSDS will help to assess the hypothesis that stress-induced upregulation of DRR1 attenuates stress-related negative consequences on (social) behavior (Masana et al, 2014) and takes a part in shaping resilience and might neuronal morphology.

We further aim to explore the brains of DRR1 nKO animals and control animals with *in situ* studies after CSDS exposure. To enable structural and morphological analysis of neurons, we crossed the DRR1 KO mice to a *Thy1*-GFP line, to ease the visualizations of hippocampal neurons. We could successfully breed first animals comprising the DRR1$^{l/l}$_*Camk2a*-Cre^{+}_*Thy1*-GFP^{+} phenotype (Chapter 2.6). This mouse line enables us to analyze the spine morphology and dendritic arbors in mutants and controls under baseline conditions and stress exposure. Furthermore, the mutants can be used to generate organotypic slice cultures for morphological analysis upon exogenous stress induction or stimulation with corticosterone or norepinephrine (Karst et al., 2000; Hu et al., 2007) which essentially increase DRR1 expression. Also, dendritic remodeling and spine dynamics can be recorded by time-lapse microscopy through induction of chemical LTP via glycine or TEA application. The combination of the above-mentioned experiments allows to correlate behavioral phenotypes with changes in neuronal morphology induced by inactivation of DRR1 in adult stages and chronic stress exposure.

3.7 *In situ* DRR1 protein levels are elevated in endothelial cells within the hippocampal formation of mice that underwent acute stress and DEX treatment

Stress acts on brain and behavior in various ways and the stress impact is influenced by multiple factors such as environment, the stressor type as well as the timing and duration of stress (Joëls & Baram 2009; Lupien et al., 2009). Formerly, it was shown that single restraint stress with

a number of following acute stresses lead to hippocampal dendritic spine loss and accompanied behavioral and cognitive impairments (Chen et al., 2010; Maras et al., 2014; Chen et al., 2016). In particular social stress was found to affect dendritic reorganization and the morphology of spines (Kole et al., 2004; Iñiguez et al., 2016). The stress response depends on multiple mediators like neurotransmitters, neuropeptides like CRH, or steroid hormones like corticosteroids (Joëls & Baram 2009).

The identification of resilience promoting-proteins which sustain neuroplasticity and synaptic function are important to broaden our understanding of the mechanisms of stress regulation. One resilience promoting candidate is BDNF (Krishnan et al., 2007). As reviewed in Chapter 1.6 and Chapter 1.8 synaptic neuroplasticity requires reorganization of the actin cytoskeleton with ABPs playing an important part. In addition, ABPs are inevitable for hippocampus-dependent memory function (Fischer et al., 2004; Nelson et al., 2012; Lamprecht, 2014). Glucocorticoid receptors bind corticosterone and may regulating local actin dynamics by genomic and non-genomic action (Jafari et al., 2012; Stournaras et al., 2014). Certain ABPs are directly stress regulated upon which DRR1 enqueues (van der Kooij et al., 2016) emphasizing the role of ABPs and actin dynamics in psychiatric disorders (Zhao et al., 2015).

In contrast to chronic stress exposure, acute stress in mice is a less invasive and time-consuming strategy to investigate neurobiological mechanisms underlying the stress response. Moreover, the response to acute stress may provide novel insight into potential stress coping mechanisms. Acute stress can cause short-term impairments which can outbalance in a homeostatic way when long-term sequelae is absent. Chronic stress however often causes compromised cognitive function, social behavior and can even lead to psychiatric disorders (Lupien et al., 2009). It is commonly agreed that therapeutic treatment of mental diseases is more effective at earlier stages of diseases onset when dysfunction is still dynamically variable. To identify resilience mechanisms (Krishnan et al., 2007) and to know how an organism copes with stressful situations it might be important to get insights from early stages to understand the genesis of chronic-stress induced impairments. When treatment is positioned in early stages of diseases onset, it could prevent long-term dysfunctional sequelae (Jene et al., 2018).

Jene and colleagues designed a behavioral test battery to evaluate numerous behavioral scopes within a restrained time window to capture acute stress responses and its temporal profile (Jene et al., 2018). After acute social defeat circulating corticosteroid levels are highest four hours (4 h) later and return to basal levels after eight hours (8 h) post stress in mice. Acute social defeat (ASD) stress had negative effects on hippocampus-dependent cognition 4 h (ASD-early) post stress, whereas after 8 h (ASD-late) no impairments were longer measurable (Jene et al., 2018).

They also tested the capability of DRR1 to confine stress-related consequences and showed that DRR1 mRNA expression is heightened in ASD-early and ASD-late compared to controls, while protein levels were only increased in ASD-late. This led to the hypothesis that absence of DRR1 protein upregulation in ASD-early caused the associated cognitive impairments. To test whether this phenotype can be rescued they overexpressed DRR1 in ASD-early mice beforehand, but could not improve the behavioral outcome. It is suggested that a global response in actin dynamics is necessary to protect from stress-induced detrimental effect and this might include other stress-sensitive ABPs which interact synergistically (Jene et al., 2018).

The findings of elevated DRR1 protein levels in ASD-treated mice motivated us to undertake immunohistochemical analysis in those mice to locate the exact area of DRR1 elevation. The Muller laboratory provided us with brain samples of ASD-late and DEX injected animals and respective controls. All animals were sacrificed 6 h after stress exposure. As expected, we could detect highest DRR1 increases in the glucocorticoid-sensitive CA3 region comparable to DRR1 protein elevations in mice after CSDS (Schmidt et al., 2011). Since our previous DRR1 localization analysis under basal conditions revealed expression of DRR1 in astrocytic end-feet we performed co-staining with the blood vessel marker podocalyxin (Pdx). Surprisingly we could not detect DRR1 protein in vessels of control mice but an appreciable colocalization of Pdx and DRR1 in the CA3-area of ASD-late and DEX-treated animals.

Interestingly, a study performed in 2017 by Menard and colleagues, evidenced social stress causing neurovascular pathology and the promotion of depression in mice (Hodes et al., 2014; Menard et al., 2017). They specifically investigated the effects of chronic social defeat stress on the permeability of the blood brain barrier (BBB) since raised peripheral inflammation upon chronic stress was reported to contribute to the pathogenesis of depressive disorder (Dantzer, 2009; Powell et al., 2013; Hodes et al., 2015; Weber et al., 2015; Miller & Raison, 2016). most likely through infiltration of peripheral immune signals in brain tissue (Hodes et al. 2014; Menard et al., 2017). Previous acute stress experiments in rodents suggested negative effects on BBB integrity (Esposito et al., 2001; Sántha et al., 2016). Menard and colleagues found abnormal blood vessel morphologies in NAc of susceptible but not resilient animals. Moreover, susceptible animals showed reduced expression of the tight junction protein claudin-5 (Cldn5) – one of the major cell adhesion molecules in brain endothelial cells (Günzel & Yu, 2013). This complies with findings in mice and in depressed human patients with decreased *CLDN5* expression (Agren & Niklasson, 1988; Nitta et al., 2003; Ménard et al., 2016). Rescue experiments through chronic application of antidepressants were able to revert the phenotype and promote resilience (Menard et al., 2017). Contrary downregulation of *Cldn5* via an AAV-mediated approach was sufficient to induce a depressive-like phenotype in mice (Menard et al., 2017). Hereby, peripheral cytokines like

interlekin-6 (Il-6) infiltrated into the brain parenchyma. An important note is that the BBB is formed by endothelial cells sealed by tight junction proteins, pericytes and astrocytes preventing potentially harmful signals from the blood entering the brain (Menard et al., 2017).

In conclusion, we hypothesize that DRR1 is involved in the adjustment of BBB integrity during stressful events, since the time point at which we detected elevated DRR1 levels in the vessels coincide with the behavioral normalization to basal cognitive performance (Jene et al., 2018). DRR1 could not only have resilience promoting effects on neurons but also other cell types in the brain. To test the effect of DRR1 level changes on endothelial cells, we started preliminary experiments on endothelial cells in culture (see appendix, Figure 6.2). After inducing stress for 48 h on endothelial cell cultures (bEnd.3 cell line) using DEX application, we performed protein measurements using Western Blot analysis. First experiments showed an elevation of DRR1 similar to *in vivo* conditions. To ascertain whether DRR1 impacts on tight junction proteins we measured CLDN5 levels and found a correlation between DRR1 increase and CLDN5 elevation. Moreover, qualitative immunocytochemistry analysis (performed by Eva Peterson during her Bachelor thesis in 2019/20 in the laboratory of Prof. Acker-Palmer) indicated stronger fluorescent signals for DRR1 and CLDN5 in DEX-treated cells as compared to controls. Additionally, cell morphology of bEnd.3 cells seemed affected within the DEX-treated condition with more, closer, longer and slimmer endothelial cells (see appendix, Figure 6.3). These preliminary findings point to a strengthening of tight junctions upon stress-induced DRR1 increase. To verify our preliminary finding, more experiments will be necessary in the future.

3.8 Concluding remarks and Outlook

Neuropsychiatric disorders affect millions of people worldwide and pose a major burden on individuals (Kessler et al., 2003). Stress is a causative factor in many mental disorders. Acute stress evokes a variety of molecular and cellular events that optimize an organism's biological fitness. At the same time, prolonged stress can have damaging effects on the brain (de Kloet et al., 2005). Stress associated cognitive maladaptation's are particularly associated with the hippocampal region, including network and morphological changes (Airan et al., 2007; Lupien et al., 2009). Underlying research on diseases such as major depressive disorder focuses mainly on the monoamine hypothesis of depression (Charney, 1998), with antidepressants increasing monoamine signaling in the brain (Schmidt et al., 2010). Yet a substantial fraction of patients does not respond adequately to the pharmacological treatment that currently exists on the market (Huynh & McIntyre, 2008). One explanation for this is the lag phase of antidepressant effects,

which suggests that downstream adaptations are more relevant than the increase in monoamine levels themselves (Schmidt et al., 2010). This shows how relevant a shift towards alternative neurotransmitter systems becomes. Increasing evidence over the past years showed involvement of the glutamatergic system in affective disorders (Sanacora et al., 2008; Hashimoto, 2009).

The molecular players that translate stressful environmental stimuli into adaptive synaptic changes and neurotransmission leading to changes at the behavioral have been largely unknown. Our studies demonstrated that DRR1 is not only a stress- and glucocorticoid sensitive protein (Liebl et al., 2009; Schmidet et al. 2011), but also affects hippocampal spine morphology in an adaptive mode upon stress. Previous findings identified DRR1 as linker protein between actin-dependent processes and stress (Schmidt et al., 2011). Behavioral experiments showed improved hippocampus-dependent cognitive flexibility upon DRR1 overexpression within the CA3-region (Schmidt et al., 2011) and improved social behavior when DRR1 was elevated in the hippocampus (Masana et al., 2014). Unpublished data from the Mueller lab, demonstrated reverse effects on hippocampus dependent learning when DRR1 was virally suppressed in the CA3-region. These studies suggest that DRR1 is important for cognitive performance. Furthermore, first evidence shows improved cognitive performance in animals subjected to CSDS after viral overexpression of DRR1 in the hippocampal CA3-region.

3.8.1 Main findings

To investigate potential resilience promoting properties of DRR1, we investigated the molecular and cellular actions of DRR1. Thereby we found, that DRR1 specifically modulates spine density and maturation in cultured murine hippocampal neurons. By virally overexpressing and suppressing DRR1 in cultured neurons, we showed that DRR1 has the ability to modulate spine morphology and dendritogenesis. Mechanistically, we proved the interaction of DRR1 and AMPA receptors and showed that DRR1 influences AMPA receptor trafficking at the synapse.

Inspired by these findings, we assessed spine morphologies of hippocampal CA1 and CA3 pyramidal neurons in mice that had previously undergone CSDS and were subsequently categorized into resilient, susceptible and non-learning groups according to their behavior in the modified social interaction test. Strikingly, we identified the least difference in spine morphology between resilient animals compared to non-stressed controls. Our findings revealed the largest difference in spine morphologies between controls and stress susceptible and non-learning animals. The similarity of resilient and control animals regarding cognitive strength (reviewed in Chapter 1.2 ff.) can be attributed to the unchanged spine morphologies in stress-resilient mice.

In addition, we found DRR1 being expressed by other central nervous cell types, such as endothelial cells and astrocytic end feet. This means that DRR1 could act as an integrator of cell-to-cell communication connecting stress-induced modulation of the neurovascular unit.

3.8.2 Future perspective

Future studies could include our DRR1 knockout model with the relevant behavioral paradigm for chronic stress, as well as biochemical and cellular functional assays, including high-resolution confocal imaging, live cell imaging and behavioral tests. Application of this conceptual link between stress and neural actin dynamics could shed light on the neurobiological resilience mechanisms and the role of DRR1 in shaping resilience. Therefore, it would be important to first examine behavioral aspects of DRR1 in the adult nervous system by characterizing DRR1$^{l/l}$_*Camk2a*-Cre mice in stress-relevant behavioral domains. This could include assessment of hippocampus-dependent cognition by the novel object recognition tasks, anxiety-like behavior, and social interaction. Afterwards these behaviors should be investigated after CSDS to specifically screen the impact of DRR1 on shaping resilience. In addition to the proposed behavioral analysis, hippocampal neurons could be imaged from DRR1 mutants crossed to *Thy1*-GFP mice under basal and CSDS conditions. Complexity of neurons can be measured by Sholl-analysis paired with spine analysis utilizing Filament tracer and spine classifier in Imaris (as described in Materials and Methods Section 4.7.5).

Our morphological studies pave the way for electrophysiological analysis, aiming to show a link between DRR1 and neuronal functionality in terms of transmission and plasticity, ultimately explaining cognitive adaptations of stress-related DRR1 increases (Schmidt et al., 2011). This can be studied by investigating the synaptic connections between the association/commissural (A/C) – CA3 connections in the generated DRR1$^{l/l}$_*Camk2a*-Cre^{+} mouse line by activating the A/C fibers combined with electrophysiological recordings from the dendritic layer of the *stratum radiatum* in the CA3-region after theta-burst stimulation inducing persistent LTP. In addition, field recordings in the CA1-region could give information on synaptic transmission in DRR1 mutants. Synaptic responses could be evoked through stimulation of the Schaffer collaterals between CA3 and CA1 to measure fEPSPs in the *stratum radiatum* of CA1. Moreover, input-output measurements are a good way to determine whether basal synaptic transmission is altered in DRR1 mutants. Here, field-EPSP responses are recorded at increasing stimulation intensities before plotting the postsynaptic response against presynaptic fiber volley amplitude. The latter correlates with the

number of activated axons. Additionally, paired-pulse measurements are able to show the presynaptic release probability.

To investigate the contribution of DRR1 to functional changes induced by a stress response, comparison of stress resilient versus non-resilient animals is of importance. DRR1$^{l/l}$_*Camk2a*-Cre^{+} animals would be subjected to CSDS and compared with Cre- littermates to ascertain potential DRR1 related differences in neuronal excitability regulation in association to resilience.

Since the data of this dissertation point to the fact that DRR1 interacts with AMPA receptors at the dendritic membrane, future experiments could investigate the regulation of this interaction in detail. For example, using PLA assays after dexamethasone treatment. Furthermore, assays quantifying activity-induced new insertion of AMPA receptors at the membrane (Pfennig et al., 2017) – a methods performed in this thesis – can be also applied for this purpose.

To evaluate the impact of DRR1 in the regulation of stress-induced modulation of AMPAR trafficking and insertion at the membrane, *in vitro* studies as stated in this dissertation can be performed in primary hippocampal cells isolated from DRR1 mutant mice. These experiments can be broadened by subjection of stress conditions via glucocorticoids. Subsequent rescue experiments can be performed by virally overexpressing DRR1 in mutant cultures.

Moreover, generation of organotypic slice cultures from DRR1 mutant mice allows analysis of spine morphologies upon stress inducing stimulation paradigms through treatment with artificial glucocorticoids in an *ex vivo* model. Induction of chemical LTP via glycine or TEA allows screening of dendritic dynamics and spine remodeling utilizing time-laps microscopy. Further, expansion microscopy in organotypic slices cultures helps to quantitatively assay the distribution of endogenous AMPARs in different spine subtypes. Using this method, one could quantify the AMPAR distribution alteration among the distinct spine subtypes following stress via DEX treatment. Methodologically, GluR2 is stained using antibodies against this AMPAR subunit labeling endogenous AMPA receptors in slices from *Thy1*-GFP mice, where GFP labeling allows the reconstruction of the whole dendritic tree in a subset of neurons. After expansion of the slices, the GluR2 labeling is reconstructed using Imaris (Bissen et al., 2022). The level of resolution allows to selectively quantify the intracellular versus the surface staining. Those analysis can also be performed in mutant mice following CSDS. Comparison of Cre+ and Cre- mice could help to find out DRR1's involvement in regulating AMPAR dynamics and neural excitability regulating resilience *in vivo*.

Our *in vitro* investigations of DRR1's influence on spine development could be followed up, by generating primary hippocampal neuron cultures from mutant animals. Altogether these

experiments could finally allow to correlate behavioral phenotypes with changes in neuronal morphology in regard to DRR1.

Our findings showed DRR1 being expressed by endothelial cells and astrocytic end feet, especially upon acute stress and DEX treatment. The results suggest that DRR1 could be involved in regulating cell-to-cell contacts at the neurovascular unit (NVU). Studies suggest that BBB defects are associated with stress susceptibility to chronic social defeat, whereby stress resilient animals maintain BBB integrity (Menard et al., 2017). Hence, we hypothesize that DRR1 plays a role in BBB integrity due to its stress responsiveness and that it even might maintain the BBB integrity upon basal levels. Characterisation of DRR1 expression levels following CSDS would help to better understand the function of DRR1 in these cell types in regard to stress resilience. One could inspect the BBB leakage by injecting in the blood circulation tracers of different sizes. Leakage mechanisms can be studied via immuno-staining of tight junctions (claudin-5, occludin, ZO1) or by identification of transcytotic vesicles through scanning electron microscopy. The first method displays tight junction disruptions, while the second shows increased intracellular transport through transcytosis (Segarra et al., 2018).

In addition, *in vitro* assays could support analysing the mechanistic and cell-specific role of DRR1 in BBB function. A combination of endothelial and astrocytic cultured cells allows to study BBB permeability with the benefit of easy pharmacological interventions. DRR1 loss-of-function could be achieved by crossing tamoxifen-inducible DRR1$^{l/l}$ mice to endothelium and astrocytes Cre deleting mice. In order to address the contribution of DRR1 and endothelial cells as well as astrocytes, these mice can be subjected to CSDS and classified in resilient versus non-resilient mice after MSIT.
Altogether these methods help defining the molecular mechanisms that keep a functioning BBB in resilient animals.

So far it is unrecognized which downstream targets are regulated by DRR1. To identify genes that are regulated in their transcriptional regulation by DRR1 could uncover potential targets through which DRR1 unfolds its potentially resilience promoting properties and mechanisms. The identification of downstream or upstream targets of DRR1 further allows the development of new therapeutic strategies against stress-related neuropsychiatric diseases by actively strengthening resilience mechanisms redirecting treatment towards new concepts. Transcriptome analysis (RNA sequencing) could be performed in mice overexpressing DRR1 in regions of interest and compared to control animals or animals in which DRR1 is knocked down.

3.8.3 Limitations of this study

Depression is a multifractional diseases comprising a substantial genetic proportion in the range of 30–50% (Kendler et al., 1995), emerging out of a combination of sex, the individual stress vulnerability, and severe environmental challenges such as chronic stress exposure. To dissect effective prevention strategies and treatments, neural and molecular mechanisms underlying stress vulnerability and resilience are studied in animal models subjected to chronic social stress. The widely used behavioral model CSDS evokes chronic stress and is solely applied on male mice using aggression as basis (Furman et al., 2022). This model appeared to be difficult to implement in female animals (Harris et al., 2017). Since this model is well established and was successfully used for many years, we utilized it in our thesis as well when performing behavioral experiments stated in this thesis. However, increasing evidence suggests sex differences not only in the prevalence of stress-induced diseases, but also sex differences in the stress response and even in the responsiveness of treatments. Interestingly, even DRR1 was shown to be an androgen-regulated gene (Quartier et al., 2018), thus pointing to potential differences between sexes. In this means, it is necessary to include and expand investigations on chronic stress on female-based protocols in parallel to the well-established CSDS models. Recently, researchers started to inaugurate female mouse models of chronic social stress alongside with new male CSDS models, enabling comparable examinations of short-term and long-term consequences of chronic stress such as stress vulnerability and coping mechanisms. Since females react to social crowding more intensively in contrast to males, this is a good paradigm to create chronic social stress in them. First data showed female mice more vulnerable to weight loss and hyperactive anxious behavior following chronic social stress as compared to male conspecifics (Furman et al., 2022). A modified version of the social defeat paradigm was also shown to evoke social stress in females. Here, male odorants are applied to females increasing aggressive behavior in resident males which in turn leads to exposure of aggressive behavior towards females (Harris et al., 2017). The differential focus on both sexes could help develop treatment profiles in a sex-appropriate manner.

4. Materials and Methods

4.1 Materials

4.1.1 Chemicals, reagents, commercial kits

All chemicals, consumables and commercial kits employed in this thesis are stated in the following lists.

Table 4.1: List of chemicals, reagents and commercial kits

Name	Supplier	Product Number
β-Mercaptoethanol	Sigma	M7522
0.9% Sodium chloride	B. Braun	3820084
Acetic Acid	Roth	3738.4
Acrylamid/Bisacrylamid (29:1) 30%	AppliChem	A0951
Agarose Low Melt	Roth	6351.2
Ammonium chloride (NH_4Cl)	Sigma	A4514
Ammonium persulfate (APS)	BioRad	161-0700
Aqua-Poly/Mount	Polysciences Europe	18606
B-27 supplement	Invitrogen (Life Technologies)	17504-044
Boric Acid	Merck	203667
Borax	Sigma	B3545
Bovine serum albumin (BSA)	Sigma	A7906
Bromophenol blue sodium salt	Roth	A512.1
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	Roth	HN04.1
Complete EDTA-free (Protease inhibitor cocktail tablets)	Roche	11873580001
DAPI (4',6-Diamidino-2-phenylindol Dihydrochlorid) > 98% p.a.	Roth	6335.1
DC Protein Assay Reagent Kit	BioRad	500-116
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs	N0447S
Dexamethasone	Sigma-Aldrich	D4902-100 mg
Dimethylsulfoxide (DMSO), anhydrous	Sigma	276855
di-Sodium hydrogen phosphate (Na_2HPO_4)	AppliChem	A6292,0250
Distilled Water (Membrane filtered and endotoxin screened)	Gibco (Life Technologies)	15230-089

1,4-Dithioerythritol, minimum 99% (DTT)	Roth	6908.2
Dulbecco's modified Eagle medium (DMEM)	Gibco (Life Technologies)	61965-059
Duolink Detection Reagents Orange	Sigma	Duo 92007-100rxn
Duolink® In Situ PLA® Probe Anti-Rabbit PLUS	Sigma	Duo 92002-100rxn
Duolink® In Situ PLA® Probe Anti-Mouse Minus	Sigma	Duo 92004-100rxn
Donor Horse serum	Sigma	H1138
DMEM	Invitrogen (Life Technologies)	61965-059
DPBS – (1X) [- Ca^{2+}, - Mg^{2+}]	Invitrogen (Life Technologies)	14190-169
DPBS – (1X) [+ Ca^{2+}, + Mg^{2+}]	Invitrogen (Life Technologies)	14040-091
ECL Western blotting detection reagents	GE Healthcare	RPN2106
Ethylenediaminetetraacetic acid (EDTA)	Roth	X986.2
EDTA disodium (0.5 M, pH 8.0)	Calbiochem	1032456
Ethanol ≥ 99,5%	Roth	5054.1
Ethidium bromide (EtBr)	Roth	2218.1
Fetal bovine serum (FBS)	Sigma	F7524
Fluorescent mounting medium	DAKO	S3023
Formaldehyde, 10%, methanol free, ultrapure	Polyscience	04018-1
Gene Ruler DNA Ladder Mix	Thermo Scientific	SM0331
(D-)Glucose	Sigma	G7021
GlutaMax	Life Technologies/Gibco	35050-038
(L-)Glutamine (200 mM)	Life Technologies/Gibco	25030-024
Glycerol	Sigma	G2025
GoTaq Green Master Mix, 2x	Promega	M7823
HBSS (1X) [+ Ca^{2+}, + Mg^{2+}, + phenol red]	Invitrogen (Life Technologies)	24020-091/24020-133
HEPES Buffer Solution 1 M	Invitrogen (Life Technologies)	15630-056
HNO3 (Salpetersäure) 65%	Sigma	84381-1L
Hydrochloric Acid (HCl) (4N)	Roth	N076.1
Isopropanol	Roth	7343.2
Ketamin 10% (10 mL)	Medistar	13690.00.00
Laminin	Sigma	L2020
6X Loading Dye Solution	Thermo Scientific	R0611
$MgSO_4.7H_2O$	AppliChem	A6414.0500

Methanol (Molecular Biology)	Roth	7342.2
Milk powder (blotting grade)	Roth	T145.2
Neurobasal Medium	Invitrogen (Life Technologies)	21103-049
Nitric acid (HNO_3)	Roth	X898.1
Normal donkey serum	JIR/dianova	017-000-121
Normal goat serum	JIR/dianova	005-000-121
Paraformaldehyde (PFA)	Roth	0335.3
Penicillin/Streptomycin	Gibco (Life Technologies)	15140-122
Poly-D-Lysine hydrobromide	Sigma	P0899
Potassium dihydrogen phosphate (KH_2PO_4)	Roth	3904.1
Potassium chloride (KCl)	Roth	HN02.2
ProLong Antifade Kit	Molecular Probes	P7481
ProteinLadder Page Ruler Prestained	Thermo Fisher Scientific	26616
Proteinase K	Roche	03115828001
Saline	Fresenius Kabi	14KM31
SDS (Sodium lauryl sulfate), 20%	Ambion	AM9820
Sodium azide	Sigma	71289
Sodium bicarbonate ($NaHCO_3$)	Roth	HN01.1
Sodium chloride (NaCl)	Roth	HN00.2
Sodium deoxycholate ($C_{24}H_{39}NaO_4$)	AppliChem	A1531
Sodium hydroxide (NaOH)	Roth	P031.2
Sodium fluoride (NaF)	Sigma	S-1501-100G
Sodium pyrophosphate (NaPP)	Sigma	221368-100G
Sodium orthovanadate (NO_3VO_4)	Sigma	S-6508
Sucrose	AppliChem	A3935.1000
Taq DNA Polymerase	BioLabs	M0267X
Tetramethylethylenediamine (TEMED)	Roth	2367.3
ThermoPol® Reaction Buffer	New England Biolabs	B9004S
Trans-Blot Turbo 5X Transfer Buffer	BioRad	10026938
Tricholoroacetic acid	Sigma	T6399
Tris Base	Roth	4855.2
Tris-HCl	Roth	9090.3
Tween 20	Sigma	P1379
Triton® X-100	Merck	9036-19-5
Trypan Blue Solution 0.4%	Life Technologies/Gibco	15250-061

Western Chemiluminiscent HRP Substrate	Millipore	WBKLS0050
Xylene cyanol FF	Sigma	X4126
Xylazine (20 mg/mL, 10 mL)	WDT	794-765
0.05% Trypsin – EDTA (1x)	Life Technologies	25300-054

4.1.2 Consumables

Table 4.2: Index of utilized consumables

Name	Supplier	Product number
6-, 12-, 24-, 96-Well plate	Corning	353046/353043/353047/353072
8-strip opt. clear flat caps (PCR)	Sarstedt	651.998.400
12-Well Chamber, removable (sterilized microscopy glass slide)	Ibidi	81201
96-Well Clear Microplate (PCR)	Falcon	353072
10 cm Petri dishes (sterile)	Falcon	
96-Well PCR plate without skirt	Sarstedt	72.1978.202
Aluminum foil	Roth	
Autoclavable micropistilles	Eppendorf	0030 120.973
Biopsy punch with plunger (2 mm)	Integra	33-31-P/25
Blotting paper sheets	Hartenstein	2.520.085120 N
Cell scraper	Sarstedt	833.951
Cover slips rectangular (24 × 60 mm, 1.5H)	Marienfeld	
Cover slips (Ø 12/13 mm)	Marienfeld	01-115-30
Combi-tips (0.5/5/10 mL)	Eppendorf	0030089421/0030089456/0030089464
Disposal nitrile gloves, powder-free	VWR	112-2755
Embedding molds	18646A	PolySciences
Falcon Tube (15/50 mL)	Corning	352096/352070
Filter paper	A.Hartenstein	
Filtered pipette tips (10/40/200/1000 µL)	Biozym	701021/701041/701071/701081
Heidelberg extension 75 cm	Henry Schein	988-3619
Hypodermic needle	Henry Schein	30GX1/2” 9003630
Instant adhesive	Permabond	102

Nitrocellulose Blotting Membraine	GE Healthcare	10600002
Paint brush	Rico design art school	7267.003
Parafilm	Bemis	PM-996
Petri dishes (sterile), 60 mm	Greiner	627 160/628 160
Pipette tips (10/200/1000 µL)	Sarstedt	70.1130/70.760.002/70.762
Plastic pasteur pipettes (2.2/3.5/6.2 mL)	Roth	
Plastic pipettes (2/5/10/25/50 mL)	Becton Dickinson	
Plastic pipettes (Cellstar) (5/10/50 mL)	Greiner	
Razor blade	Wilkinson Sword	7005115S
Reaction tube, safe lock (1.5/2 mL)	Eppendorf	30121589/30121597
Serological pipettes (2/5 mL)	Sarstedt	86.1253.001/86.1254.001
Stainless steel blades	Campden Instruments	7550/1/SS/50
Stericup-GP, 0.22 µm, polyethersulfone, 150 mL, radiosterilized	Millipore	SCGPU01RE
Stericup-GP, 0.22 µm, polyethersulfone, 500 mL, radiosterilized	Millipore	SCGPU05RE
Storage box 50 slides	Roth	N953.1
Superfrost Plus™ Adhesion Microscope Slides	Thermo Fischer scientific	J1800AMNZ
Surgical disposable scalpels	Braun	BA210/BA211/BA222
Syringe 0.3 mL 30 G	Braun	324826
Syringe 1 mL Omnifix – F Luer Solo	Braun	01.06.24
Syringe 1 mL 30 Gx1/2”	Braun	9161502
Syringe filters (0.22 µm)	Roth	P666.1
Syringes for filtering (50 mL)	Becton Dickinson	300865
Tiffen Lens Cleaning paper	Tiffen	
Trans-Blot Turbo Nitrocellulose	BioRad	170–4271
Tubes (1.5 mL)	Sarstedt	72.960
Tubes (2 mL)	Eppendorf	0030 120.094
Type F Immersion liquid (10 mL)	Leica	11513859

4.1.3 Equipment

Table 4.3: List of utilized general equipment

Equipment	Supplier	Model
Automated Cell Counter	BioRad	TC-20
Cell culture hood	Thermo Scientific	Maxisafe 2020
Centrifuge	Eppendorf	5424 R
Centrifuge	Eppendorf	5418 R
Centrifuge	Hettich	Rotanta 460 R
Centrifuge	Fisherbrand	GT 2
Centrifuge	Eppendorf	5424 R
CO_2 Incubator with copper chambers	Thermo Fischer Scientific	Heracell™ 240i, Heracell TM 150
Confocal Microscope	Leica Instruments	TCS SP5
Digital camera	Visitron systems	
Dissection tools	Fine Science Tools	
Electrophoresis power supply	PowerPac HC	PowerPac HC
Electrophoresis power supply	Amersham pharmacia biotech	EPS 301
Epifluorescence microscope	Zeiss	Imager M1
Fine balance	Denver Instrument	
Freezer -20 °C	Liebherr	Comfort and ProfiLine
Freezer -80 °C	Thermo Scientific	CryoCube
Fridge 4 °C	Liebherr	TP 1760
Gel documentation system	Analytik Jena	UVP Gel Studio PLUS
Heat block	Techne	DRI-BLOCK DB 2D
Heat plate stirrer	Stuart	CB162
Horizontal flow hood	Thermo Scientific	Hera guard III
ImageQuant	LAS 4000	GE Healthcare
Magnetic stirrer	IKA	IKAMAG RCT
Microscope	Olympus	CKX31
Microwave	Severin	700
Multi-channel pipette 0.5–10 µl	Eppendorf	H49604G
Multi-channel pipette 20–200 µl	Brand	
Osmometer	Slamed, 800CL	
pH meter	SI Analytics	Lab 855
Perfusion pump	Hardvard Apparatus	PHD Ultra

Perfusion pump	Gilson	Minipuls 3, peristaltic pump
Pipette girl	Integra Biosciences	155 021
Pipettes (P2, P20, P200, P1000)	Gilson	
Plate reader	BioRad	iMark TM Microplate Reader
Precision balance	Denver Instrument	Si-234
Printer (Compact Digital Monochrome)	Mitsubishi	P95DW
Rotator	Stuart	SB2
Scale	Kern	EMB 1000-2
Scale	Denver Instrument	Si-203
Shaker	neoLab	DRS-12
Stereo microscope	Olympus	SZX10
Table centrifuge (small)	Biozym	Sprout
Therapeutic pad	FIR	MHP-E1220
Thermomixer Compact	Eppendorf	
Thermoshaker	FIR	TS1 96 x 0.2 mL MTP
Thermocycler	Analytik Jena	Biometra Advanced
Thermocycler	Biozym	Biometra Advanced
Trans-Blot transfer system	BioRad	Turbo system
Vibratome	Leica	VT1200S
Vortex	Fisher bioblock scientific	Top-Mix 11118
Water bath	Fisher Scientific,	Polystat24
Water bath	Benning GFP	00159
Water bath	Julabo	Corio CD

4.1.4 Software

Table 4.4: List of employed software

Software	Version	Supplier
Adobe Illustrator	26.0.1–27.0	Adobe Systems, San Jose, USA
Adobe Photoshop	26.0.1–27.0	Adobe Systems, San Jose, USA
Allen Brain Mouse Atlas		
BioRender	2022/2023	https://app.biorender.com
Fiji (Image J)	2.0.0-rc-68/1.52e	National Institutes of Health (NIH),

		Bethesda, USA
GraphPad Prism for MacOS	8.3.0	GraphPad Software Inc., San Diego, USA
Huygens Professional	17.04 –19.10	Scientific Volume Imaging (SVI), Hilversum, The Netherlands
Image Quant TM LAS 4000	1.2	GE Healthcare Bio-Sciences
Imaris	9.0–9.8	Bitplane, Zürich, Switzerland
Leica application suite X (SP5)	3.5.2.18963	Leica Microsystems, Wetzlar, Germany
Matlab		Mathworks, Natick, USA
MetaMorph		Molecular Devices, San Jose, USA
Mendeley	1.19.8	George Mason University
Microplate Manager 6 Software	6	BioRad
Microsoft® Office for Mac (2019)	16.65	Microsoft, Redmond, USA
PyRat (Python based Relational Animal Tracking)	4.4.1–443	Scionics Computer Innovation
VisionWorks	9.1.20063.7760	Analytik Jena, Jena, Germany

4.1.5 Antibodies

Table 4.5: List of primary antibodies

Target	Host	Supplier	Product number	Dilution
α-β-Actin	rabbit	Sigma	A-2066	1:1000
α-DRR1	rabbit	Mueller laboratory (Mainz, Germany)	-	1:500
α-Fam107a	rabbit	Sigma	SAB2108568	1:500
a-GFAP (Glial fibrillary acidic protein)	mouse	Sigma	G3893	1:500
α-GFP	chicken	Abcam	ab13970	1:500
α-GluR2	rabbit	Millipore	AB1768-I	1:500
α-GluR2, extracellular epitope	mouse	Millipore	MAB397	1:500
α-MAP2	rabbit	Millipore	AB5622	1:1000
α-Podocalyxin	mouse	R&D Systems	AF1556	1:1000

Table 4.6: List of secondary antibodies

Target	Host	Supplier	Product number	Dilution
α-chicken Alexa 488	donkey	Dianova	703-546-155	1:500
α-goat Alexa 647	donkey	Molecular Pobes	A21447	1:500
α-mouse Alexa 488	donkey	Molecular Probes	A21202	1:200
α-mouse Alexa 647	donkey	Molecular Probes	A31571	1:500
α-Mouse	donkey	Dianova	715-005-150	1:200
α-mouse IgG Cy3	donkey	Dianova	715-165-151	1:200
α-rabbit Alexa 555	donkey	Molecular Probes	A31572	1:500
α-rabbit Alexa 647	donkey	Molecular Probes	A31573	1:500
α-rabbit IgG Cy3	donkey	Dianova	711-165-152	1:500
α-rabbit IgG HRP (rabbit TrueBlot®)	rabbit	Rockland	18-8816-33	1:1000

4.1.6 Oligonucleotides

Oligonucleotide sequences were supplied as lyophilized powder from Eurofins Genomics. A number of oligonucleotides were used as primers (Table 4.7).

Table 4.7: List of primers for genotyping

Amplified sequence	Primer	Sequence (5'-3')	amplicon (bp)
distal *loxP*-site	SC1_fwd	CAC CAC GAT GGA AAA CAG CTT	524
	SDL2_rev	GGC AGG TGC AGT ACA ACA GGT GAG	(450 WT)
loxP-FRT	NDEL1_fwd	TTC ATA GGT GGG AGG GTC TGG G	546
(*neo*-deletion)	NDEL2_rev	AAT GGG GAA AGC CTG GGT TGG	(410 WT)
***Flp* transgene**	FLP1_fwd	CAC TGA TAT TGT AAG TAG TTT GC	725
	FLP2_rev	CTA GTG CGA AGT AGT GAT CAG G	
***Thy1*-GFP**	Thy1_fwd	TCT GAG TGG CAA AGG ACC TTA GG	350
	Thy1_rev	CGC TGA ACT TGT GGC CGT TTA CG	
***Cre*-transgene**	Cre_fwd	GCC TGC ATT ACC GGT CGA TGC AAC GA	650
	Cre_rev	GTG GCA GAT GGC GCG GCA ACA CCA TT	

4.1.7 Recombinant adeno-associated Virus (rAAV)

For *in vitro* DRR1 overexpression and suppression in primary neuronal cultures or within the hippocampal brain region *in vivo*, a recombinant adeno-associated viral (rAAV) approach was used as vector to carry the genomic material into neuronal cells. Utilized viral vectors are listed in the table below.

Table 4.8: List of recombinant AAVs used for viral transduction (rAVE gene delivery reagent; GeneDetect)

Product	Vector description and titer (genomic copies; GP)
ddRNAi mouse ***Fam107a - shRNA***	Chimeric (CNS-optimized) AAV-1/2 viral vector: U6 -- ***Mouse Fam107a shRNA*** -- terminator CAG-***EGFP***-WPRE-BGH-polyA Titer > 1.2 x 10^{12} GP/mL
ddRNAi control ***SCR-shRNA_EGFP***	Chimeric (CNS-optimized) AAV-1/2 viral vector: U6--***GeneDetect SCR shRNA*** -- terminator CAG-***EGFP***-WPRE-BGH-polyA Titer > 1.2 x 10^{12} GP/mL
Over-express ***(Fam107a)_(MPIP101)***	Chimeric (CNS-optimized) AAV-1/2 viral vector: CAG---***Fam107a (MPIP101)***---IRES---***EGFP***--WPRE-BGH-polyA Titer > 1.2 x 10^{12} GP/mL
Control ***Transgene=Null/Empty***	Chimeric (CNS-optimized) AAV-1/2 viral vector: CAG---***Null/Empty***------IRES-----***EGFP***---WPRE-BGH-polyA Titer > 1.2 x 10^{12} GP/mL

4.1.8 Standard solutions

Table 4.9: List of standard solutions for regular use

Solution	Composition
1X Phosphate buffered saline (PBS)	137 mM NaCl 2.7 mM KCl 8.0 mM Na_2HPO_4 1.5 mM KH_2PO_4 Adjusted to 1L dH_2O, pH 7.4
1X PBS-Tween	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4

	1.8 mM KH_2PO_4 0.1% Tween 20
1X Tris buffered saline (TBS)	0.15 M NaCl 0.1 M Tris-HCl pH 7.5
Wash buffer A for PLA	10 mM Tris, pH 7.4 150 mM NaCl 0.05% Tween Filtered through 0.45 μm and stored at 4 ˚C
Wash buffer B for PLA	200 mM Tris, pH 7.5 100 mM NaCl Filtered through 0.45 μm and stored at 4 ˚C
Tissue cryoprotectant solution (TCS)	250 mL Glycerol 300 mL Ethylene glycol 450 mL 0.1 M Sodium phosphate buffer pH 6.7
Saline sodium citrate (SSC) buffer for antibody retrieval	0.3 M NaCl 0.03 M Sodium citrate 0.5% Tween 20 pH 7.0

4.1.9 Genotyping: Reagents and solutions

Table 4.10: List of solutions and reagents used for genotyping

Solution	Composition	Amount
1.5 M Tris-HCl pH 8.8	Tris-HCl dH_2O pH adjusted with 10 M NaOH	61.8 g 500 mL
50X Tris-acetate-EDTA (TAE) buffer	Tris Base Acetic acid 0.5 M EDTA (pH 8.0) Adjusted to 1L dH2O, pH to 8.3	242 g 57.1 mL 100 mL
Agarose gel (2%)	Agarose 0.1 μg/mL Ethidium bromide 1X TAE	6 g 15 μL 300 mL
DNA size marker	Gene Ruler DNA Ladder Mix	100 μl

	6X Loading Dye solution	100 µl
	dH_2O	400 µl
Loading buffer	6X Loading Dye solution	
Lysis buffer for tails	50 mM NaOH	1 g NaOH in 500 mL dH2O
Neutralization buffer for tails	1.5 M Tris-HCl (pH 8.8)	
Running buffer (1X TAE)	50X TAE	40 mL
	dH_2O	1960 mL

4.1.10 Western Blot: Solutions and buffers

Table 4.11: List of solutions for Western Blots

Solution	Composition	Amount
LBA buffer for storage at 4 ºC	1 M Tris, pH 7.5	25 mL
	5 M NaCl	15 mL
	0.5% Triton X-100	2.5 mL
	Adjusted to 500 mL dH2O	
LBA lysis buffer	LBA Buffer	5 mL
	1 mM Sodium orthovanadate (Na_3VO_4)	50 µL
	10 mM sodium pyrophosphate (NaPP)	0.022 g
	20 mM sodium fluoride (NaF)	0.004 g
	Complete protease inhibitor tablet	50 µL
RIPA lysis buffer	150 mM NaCl	3 mL
	1% Triton X-100	1mL
	0.5% Sodium deoxycholate	5 mL
	1% SDS	7 mL
	1 M Tris-HCl	5mL
	Adjusted to final Volume of 100 mL, protected from light, stored at 4 ºC	
4X SDS sample buffer	8% SDS	2.4 g
	200 mM Tris-HCl, pH 6.8	4 mL, 1.5 M Tris
	400 mM DTT	1.85 g
	0.4% Bromophenol Blue	0.12 g
	40% Glycerol	12 mL 99% Glycerol
SDS separating gel (12%, 10 mL)	30% Acrylamid/bisacrylamid	4 mL
	1.5 M Tris-HCl, pH 8.8, 0.4% SDS	2.6 mL
	H_2O	3.35 mL

	10% APS	50 µL
	TEMED	5 µL
SDS stacking gel (4%, 5 mL)	30% Acrylamid/bisacrylamid	0.65 mL
	0.5 M Tris-HCl, pH 6.8, 0.4% SDS	1.3 mL
	H_2O	3.05 mL
	10% APS	50 µL
	TEMED	5 µL
5X Laemmli electrophoresis buffer	100 mM Tris Base	15.45 g
	100 mM Glycine	72.1 g
	3.5 mM SDS	5 g
	Adjusted to 1000 mL dH2O	
Transfer buffer	Transblot Turbo 5X	200 mL
	Distilled H_2O	600 mL
	20% Ethanol	200 mL

4.1.11 Primary hippocampal neuron cultures: Media and buffers

Table 4.12: List of solutions and chemicals for primary hippocampal neuron cultures. All media stored at 4˚C.

Solution	Composition	Amount
Borate buffer	Boric acid	3.1 mg/mL
	Borax in H_2O	4.75 mg/mL
	Adjusted to 1l dH_2O, pH to 8.5	
Dissection medium (DM)	HBSS w/o Phenol red (+ Ca^{2+}, +Mg^{2+})	500 mL
	Penicillin/Streptomycin	5 mL
	HEPES	5 mL
	GlutaMax	5 mL
Neurobasal medium + (NB^+)	Neurobasal Medium	500 mL
	GlutaMax	5 mL
	D-Glucose	7 g
Neurobasal medium ++ (NB^{++})	NB+	50 mL
	B-27 supplement (1:50)	1 mL
Serum medium (SM)	DMEM	500 mL
(Sterile filtered)	10% FBS	50 mL

4.2 Molecular biology

4.2.1 Genomic DNA extraction and genotyping PCR

For identification of distinct genotypes within litters ear punches of weaned and three-week-old mice were collected. A standard animal identification earmark pattern was applied for animal distinction when collecting tissue via ear punches. DNA was extracted by boiling the tissue for 60–90 min in 80–100 µL of 50 mM NaOH at 95 °C with subsequent neutralization by adding 8–10 µL of 1.5 M Tris-HCl pH 8.8. Samples were vortexed and spined down to remove tissue residues and condensates from the reaction tubes. Polymerase chain reactions (PCR) was performed in 96-well plates using a total of 25 µL reaction solution and 1.5 µL of lysate. The master mix used for individual genes is described in Table 4.13 with corresponding primers and PCR reactions in Table 4.14.

Table 4.13: PCR master mix per reaction

Genotype	***Drr1$^{lox/lox}$; Drr1$^{wt/lox}$; Drr1$^{wt/wt}$; Drr1-Flp Thy1-Gfp***	***Camk2a-Cre***
DNA	1.5 µL	2 µL
Distilled H_2O	11 µL	19.55 µL
GoTaq green master mix	12.5 µL	
10x Taq pol buffer (NEB)	-	2.5 µL
dNTP (25 mM each)	-	0.2 µL
Primer	0.25 µL	0.25 µL
Taq polymerase (NEB)	-	0.125 µL

Table 4.14: Genotyping PCR programs

Genotype / **Cycle step**	***Drr1 loxP-FRT***		***Drr1 distal loxP***		***Drr1-Flp***	
	Temp [°C]	**Time**	**Temp [°C]**	**Time**	**Temp [°C]**	**Time**
Initiation	94	2 min	94	2 min	94	2 min
Denaturation	94	30 s	94	30 s	94	30 s
Annealing	64 (30 cycles)	30 s	55 (30 cycles)	30 s	55 (30 cycles)	30 s
Extension	72	1 min	72	1 min	72	1 min
Final extension	72	4 min	72	4 min	72	4 min
Final hold	4	∞	4	∞	4	∞

Genotype / Cycle step	*Thy1-GFP*			*Cre*-recombinase		
	Temp [°C]		**Time**	**Temp [°C]**		**Time**
Initiation	95		2 min	94		3 min
Denaturation	95	35 cycles	30 s	94	35 cycles	1 min
Annealing	60		30 s	67		1 min
Extension	72		40 s	72		1 min
Final extension	72		5 min	72		5 min
Final hold	4		∞	4		∞

4.2.2 Agarose gel electrophoresis

To separate DNA fragments by size, electrophoresis of an agarose gel was administered. During gelation agarose polymers form bundles creating pores. Higher gel concentration creates finer pore size. The application of an electrical field allows movement of negatively charged DNA through the gel matrix towards the anode. Fragment size as well as number of negative charges define speed and distance of migrations of DNA fragments separating them by size. During electrophoresis, electric conductivity is provided by the Tris-acetate-EDTA (TAE) buffer. To obtain a 2% agarose gel corresponding amount of agarose powder was dissolved in 1x TAE buffer via heating in a microwave. After cooling down to ~60 °C, 0.05 µg/mL ethidium bromide (EtBr) was added. EtBr intercalates between bases of nucleic acids. Exposure to ultraviolet (UV) light (~590 nm) allows detection of DNA bands. Next, the mix was poured into a gel chamber with combs to create loading pockets for the samples. After solidification the gel was placed into a running chamber and covered with TAE buffer. Depending on the master mix 20 µL volume per sample was loaded either directly or mixed with loading dye (1:6) prior to loading. In addition of joining color and density to the sample, loading dyes enable samples to move in standard rates through the gel, allowing an accurate estimation of migration and fragment size. For determination of fragment sizes, 7 µL of DNA ladder mix containing pre-determined sizes of DNA fragments was applied. The gel was run for 45–50 min at 200 mV. The DNA bands were visualized by UV light and images were acquired using a gel documentation system.

To screen heterozygous and homozygous allele deletion in DRR1$^{1/1}$ animals the primer pair NDEL1 and NDEL2 was used to detect the *loxP-FRT* side, and Neo-cassette deletion simultaneously, resulting in a 546 base-pair (bp) product and a 410 bp product for the wildtype condition. To detect the distal *loxP* side the primer pair SC1 and SDEL1 was utilized, creating a 450 bp product in the absence of the *loxP* and a 524 bp product while present. After Neo-cassette deletion, one *Flp* (flipase) side remains, resulting in a 725 bp product, when using the primer pair

FLP1 and FLP2. The *Thy1-Gfp* gene sequence was identified by a 350 bp DNA band, presence of the *Camk2a-Cre* recombinase gene by a band sized 600 bp.

4.3 Cell Biology

4.3.1 Primary hippocampal neuron cultures

4.3.1.1 Preparation of cover slips and plates

For neuronal cultures, cover slips (12–13 cm in diameter) were inserted in single wells of 24-well plates. To roughen the surface of cover slips for better attachment of neurons, they were incubated overnight either in nitric acid or hydrochloric acid at room temperature (RT). Thereafter, they were washed three times for 30 min in water on a shaker to remove excessive acid. To dry, cover slips were separately placed on filter paper using forceps and afterwards collected in a glass container which was baked over night at 184 ºC for sterilization. Subsequently, one cover slip per well was placed in 24-well plates. For better cell adhesion, through refinement of surface tension, both, 6 cm Ø dishes and 24-well plates containing cover slips, were treated with the synthetic compound poly-D-lysin (PDL). PDL was dissolved in borate buffer (table 4.9) to reach a concentration of 1 mg/ mL and sterile filtered through a 22 µm filter. The dishes were incubated with 2 mL of PDL in borate buffer and 24-well plates with 400 µL per well. Both were incubated over night at 37 °C and 5% CO_2, before they were washed three times with distilled water and dried for 20–30 min under a cell culture hood. The dishes were filled with 6 mL of 1X phosphate buffered saline (PBS) and kept in the incubator with above mentioned settings until usage. To enhance neuronal growth, 24-well plates were further coated with the extracellular matrix component laminin. They were incubated with sterile filtered 5 µg/mL laminin in PBS over night or minimum 5 h at 37 °C and 5% CO_2. Extra amount of laminin was washed of three times with 1X PBS. Plates were filled with 1X PBS and stored in the incubator until usage.

4.3.1.2 Isolation of primary hippocampal neurons from murine embryos

Primary hippocampal neurons were cultivated from E16.5–18.5 wildtype (C57Bl/6J) mouse embryos. That for, pregnant dams were sacrificed by cervical dislocation followed by quick decapitation. Embryos were removed from the abdomen, put on ice and removed from the placenta before likewise decapitation. Embryonic brains were removed from the head, after removing the skin and opening the skull, and stored in dissection medium on ice. Utilizing a

binocular microscope, cortices were separated from the brain stem, meninges were detached, and hippocampi were isolated from diencephalic structures in ice cold dissection medium (HBSS 1X (+ $MgCl_2$, + $CaCl_2$) containing Pen Strep, 1 M HEPES and L-Glutamine (200 mM, 100X) diluted 1% each (all reagents from Gibco)). Hippocampi were collected and kept in a 15 mL falcon containing 2 mL of dissection medium on ice, until all brains were processed. For dissociation of dissected hippocampal tissue, the medium was replaced by 1 mL pre-warmed 0,05% Trypsin-EDTA 1X (Gibco) and incubated for 15 min in a water bath at 37 °C. After the mild tissue digestion, Trypsin-EDTA was removed and cells were washed twice with 1 mL prewarmed serum medium (DMEM (Gibco) containing 10% horse serum), followed by two washes with 1 mL of pre-warmed NB^+ medium (neurobasal medium (Gibco) including 0.5 mM L-Glutamine). Afterwards cells were gently triturated 30–40 times with a fire-polished Pasteur pipette and centrifuged for 5 min at 71 × g. Supernatant was removed and cells were resuspended in 1–2 mL of pre-warmed NB^{++} medium (neurobasal medium supplemented with B27 (Invitrogen) containing 0.5 mM Glutamine). Counting of living cells was performed by mixing 10 µL of cell suspension with 10 µL of Trypan Blue, pipetting 10 µL of the mixture in a cell counter slide and utilizing an automated cell counter for quantification. 35 000 (24-well plates) or 7.5^6–10^6 (6 cm Ø plates) neurons were plated on single coverslips coated with poly-D-lysin (1 mg/mL, Sigma) and laminin (5 µg/mL) or tissue culture plates coated with poly-D-lysin solely, respectively. Neurons were grown in Neurobasal medium containing supplements (1:50) and glutamine (1:400) for 14 days *in vitro* (DIV), stored in an incubator at 37 °C and 5% CO_2. Or otherwise, on DIV 7 neurons were transfected with rAAVs and correlating controls for endogenous knockdown or overexpression of DRR1. Cells were either fixed and stained, stimulated for newly inserted assay on DIV 14 or lysed for Western blot experiments.

4.3.2 AAV-induced DRR1 knock-down and overexpression in primary hippocampal neuron cultures

For endogenous overexpression and suppression of the DRR1 protein in primary hippocampal neuron cultures, cells were virally transfected with custom-made (rAVE™, GeneDetect), non-pathogenic (S1), chimeric and CNS-optimized recombinant adeno-associated virus (rAAV) shown in table 4.12. The viral vectors contain an EGFP sequence, which is translated by cells, indicating the efficacy of the virus.

For viral overexpression (OE) of DRR1 CNS-optimized bi-cistronic AAV1/2 vectors were used containing an enhanced GFP (EGFP) expression cassette (expression cassette: CAG-DRR1-IRES-EGFP-WPRE-BGH-polyA) or EGFP (expression cassette: CAG-Null/Empty-

IRES-EGFP-WPRE-BGH-polyA) alone as control condition (empty). Knock-down of DRR1 was obtained by application of rAAV containing a short hairpin RNA against DRR1 (shDRR1) or a scrambled version as control (shSCR), both containing an EGFP expression cassette (Table 4.8) as well (all rAAV constructs supplied by GeneDetect, New Zealand; titers: > 1.2×10^{12} genomic particles/mL). Neuronal cultures were virally transfected on DIV 7 with a multiplicity of infection (MOI) of 1000 and a titer of 1.2×10^{10} genomic copies (GP) per mL (1:100 dilution of original stock). MOI refers to the number of virions per cell added via infection. Hereby, the probability of each cell being infected by different MOIs is described by the Poisson distribution, which was used to determine a suitable MOI besides qualitative assessment of transfected cells after 7 days post-transfection (dpt). Depending on the cell density of neuronal cultures, the volume of seeded viral vectors was calculated as follows to reach the desired transfection rate:

(Number of transfected cells × MOI) ÷ AAV titer = Volume seed virus

Example for 35 000 cells per well of a 24-well plate:

- Number of transfected cells × MOI 1000
  → $3.5 \times 10^4 \times 1000 = 3.5 \times 10^7$
- rAAV titer = 1.2×10^{10} GP/mL
- Volume seed virus → $3.5 \times 10^7 \div 1.2 \times 10^{10}$ GP/mL = 0.00291 mL (≙ 2.91 µL)

The virus was kept at -80 °C and dilutions were defrozen on ice before usage. Cultures were transfected with subsequent rAAV constructs and rAAV controls using the calculated volume. Afterwards, cultures were directly put back into the incubator at 37 °C and 5% of CO_2 and kept for 7 dpt, until they reached DIV 14/15. Next, cultures were either lysed, fixed or stimulated before immunocytochemistry.

4.3.3 Dexamethasone treatment of primary hippocampal neuron cultures

To mimic an acute glucocorticoid mediated stress response in primary hippocampal neuron cultures, the artificial glucocorticoid Dexamethasone (sigma) was applied on DIV12/13 for either 24 or 48 h, before cells were further processed on DIV14/15 for either Western Blot analysis or newly inserted AMPAR GluR2 assay. To dissolve Dexamethasone (DEX), 1 mL of ethanol was added per 1 µg Dexamethasone (392.46 g/mol). Per milliliter of ethanol, 49 mL of NB^{++} were added to obtain a working solution of 20 µg/mL and a concentration of 51 µM. The working solution was used to make dilutions of 0.2 mM and 0.1 mM DEX in NB^{++}, which were further used to apply on cultures resulting in an end concentration of 10 µM and 50 µM. For that,

the corresponding volume of growing medium was withdrawn and supplemented with the same amount of NB^{++} working solution, before cells were put back into the incubator for another 24–48 h.

4.4 Biochemistry

4.4.1 Cell and tissue lysis

Mouse brains were dissected and either processed as whole or separated in cortices and cerebelli. The tissue was lysed on ice in a glass tube using a glass homogenizer in 0.5–1 mL of chilled lysis buffer, depending on the amount of tissue. Afterwards, lysates were transferred into 2 mL Eppendorf tubes. In case of DIV 14 primary hippocampal neuron cultures, cultured in a 6 cm Ø dish and treated with either DEX or transduced with distinct rAAVs (as previously described in Section 4.3.2), cells were first washed with 1X PBS on ice, before adding 100–120 µL of LBA lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.4% Triton X, dH_2O, 1 mM Na_3VO_4, 10 mM NaPPi, 20 mM NaF, 1 protease inhibitor tablet for 5 mL buffer). HUVEC and bEnd.3 cells were cultured in 10 cm Ø dishes were lysed when they reached 90% coverage density with 150 µL RIPA lysis buffer at RT. Using a cell scraper, cells were harvested and collected within a 1.5 mL Eppendorf tube and directly put in a spinning wheel at 4 °C for 20–30 min. Next, lysates were centrifuged at 21 130 g at 4°C for 15 min to clear lysates from remaining debris. Afterwards, supernatants were put into a fresh Eppendorf tube and either directly processed or frozen in liquid nitrogen and stored at -20 °C.

4.4.2 Protein measurement

The BioRad DC Assay kit was used to determine the protein concentration. A BSA-standard ranging between concentrations of 0–20 mg/mL (0, 1, 2, 4, 10, 20) served as control. Lysates were measured applying 2 µL of the samples. Based on the standard the protein amounts per sample were determined and equal protein concentrations were used within one Western blot between different conditions (100–150 µg protein/ condition).

4.4.3 SDS polyacrylamide gel electrophoresis

Protein samples were applied on a 12–15% SDS page with a 4% stacking gel (1,5 mm thickness) and subsequently transferred to a nitrocellulose membrane using a semi-dry, fast transfer chamber (BioRad). Two layers of Whatman-Paper and the membrane were incubated in Trans-Blot Turbo 5x Transfer Buffer from BioRad (20% buffer, 20% EtOH, 60% dH_2O) before putting the membrane in between. The transfer took place for 7 min at 25 V. Membrane was incubated in blocking solution (5% milk in PBS-T) for 1h at room temperature before cutting the membrane horizontally to separate different protein sizes for actin (42 kDa), GluR2 (~110 kDa) and DRR1 (17 kDa). The different membrane slices were each incubated with their specific primary antibodies (rabbit anti-FAM107A 1:500, mouse anti-GluR2 1:1000, rabbit anti-actin 1:1000) according to their protein sizes in 5% milk PBS-T over night at 4 °C. Afterwards the membrane was washed 3X for 20 min at room temperature with PBS-T, before applying host specific secondary antibodies coupled to horseradish peroxide for 2 h at room temperature. Subsequently, the membrane was washed again 3X for 10 min with PBS-T. Protein bands were visualized by applying enhanced chemiluminescence system (Merck, Millipore) in a concentration of 1:1 on the membrane. Chemiluminescence was measured with ImageQuant LAS 4000 by taking images in different intervals and exposure times. Comparison of densities between different protein bands were analyzed using the Gel Analysis method in Fiji.

4.4.4 Immunocytochemistry

Primary hippocampal neuron cultures, cultured on coverslips in 24-well plates were placed on an ice tray and first washed twice with 1X PBS before fixation with 4% paraformaldehyde (PFA) and 4% sucrose in PBS for 12 min. Afterwards, PFA was removed, cells were washed two times with cold PBS to remove extant PFA and incubated with 50 mM NH_4Cl in PBS for 10 min. Cells were rinsed again twice with cold PBS and permeabilized for 5 min with 0.1% Triton X-100 in PBS. For GluR2 surface staining in newly inserted assay, cells were not permeabilized. Coverslips were rinsed three times with PBS, before blocking for 1h at RT in blocking solution (2% bovine serum albumin, 4% donkey serum in PBS). Coverslips were incubated with primary antibodies (chicken anti-GFP 1:1000, goat anti-MAP2 1:500 and mouse anti-GluR2 1:500) in blocking solution either overnight at 4 °C or for 90 min at RT. After washing three times with PBS for 5 min each, cells were incubated with secondary antibodies (AlexaFluor488 anti-chicken, Cy3 anti-mouse, AlexaFluor647 anti-goat; 1:200 each) in blocking solution for 60 min at RT. In the end, coverslips were washed again for three times, each 5 min with PBS, dipped in dH_2O, mounted

on slides using the ProLong Antifade kit (Invitrogen) and stored at 4 °C until imaging with an Epifluorescence microscope (Axio Imager M1, Zeiss) using the imaging program VisiView.

4.4.5 Proximity Ligation Assay

For the proximity ligation assay the Duolink II Fluorescence Kit was used to investigate a putative interaction between the protein DRR1 and the AMPAR subunit GluR2 via a polymerase chain reaction and consecutive signal enhancement when in close proximity (≤ 40 μm) within the cell. Therefore, DIV 14 WT primary hippocampal neuron cultures, seeded on cover slips, were fixed as described in Section 4.4.4, skipping permeabilization with Triton X-100. After 3 washes with 1X PBS, coverslips were transferred into a humidified cuvette and treated with blocking solution (2% BSA and 4% donkey serum in PBS) for 30 min at RT. Afterwards cells were incubated with primary antibody (rabbit anti-DRR1; 1:500, mouse anti-GluR2; 1:500 and goat anti-MAP; 2:1000) in blocking solution for 30 min at 37°C in a pre-heated oven. Next, the primary antibody solution was removed, and cells were washed two times for three minutes with 1X Duolink washing buffer A (Table 4.9). In the meantime, PLA probes were mixed and diluted in a 1:5 concentration within the blocking buffer as described in the user manual of Duolink II Fluorescence. A 40 μL reaction was used per coverslip, composed of 8 μL PLA probes each and 24 μL blocking solution. PLA probe MINUS (mouse) and PLA probe PLUS (rabbit) consist of secondary antibodies conjugated with oligonucleotides. After washes were performed and washing buffer was removed, the PLA probes mixtures was added and cells on coverslips, placed in a humidified chamber were incubated for 1 h in a pre-heated oven at 37 °C. Thereafter, mixture was removed, and cells were washed once for two times à three minutes with Duolink washing buffer A. In the next step, the Ligation solution was put on top of the cells. Here, when adding two oligonucleotides together with ligase, the underlying principle is hybridization of the PLA probes, creating a joined circle, when in near proximity to each other. For this, Duolink ligation buffer was diluted 1:5 in dH_2O, ligase was added 1:40 (for 40 μL amount per coverslip 8 μL of 5X ligation mix, 31 μL dH_2O and 1 μL of Ligase were mixed) right before usage, added to the cells, which were then incubated for 30 min at 37 °C. Once again, cells were washed two times two minutes wish wash buffer A, before starting the amplification process, which requires the Amplification solution, consisting of nucleotides and fluorescently labeled oligonucleotides together with polymerase to start a rolling-circle amplification (RCA), generating a repeated-sequence product. Labeled oligonucleotides hybridize to the RCA product and amplified signal is visible as fluorescent dot. For this step, Duolink Orange Amplification buffer was mixed 1:5 with dH_2O and 1:80 of Polymerase (for 40 μL reaction mix per coverslip, 8 μL of 5X Amplification stock, 31.5 μL

dH_2O and 0.5 µL Polymerase were mixed) and Amplification-Polymerase solution was added to the cells still within the humidified chamber, which were subsequently incubated for 100 min at 37 °C. After, Amplification mix was removed and cells were washed for three min with 1X Duolink washing buffer B, before incubating with DAPI (1:1000) in 1X washing buffer B for 10 min at RT. Next, cells were washed once more with washing buffer B for 5 min. Buffer was decanted, and coverslips were dipped in VE water, before mounted upside down on glass-slides using Antifade kit (XY) and stored at 4 °C before image acquisition with Confocal laser scanning microscope (Leica).

4.4.6 Newly inserted AMPA receptor membrane Assay

To investigate the insertion of new AMPA receptors into the dendritic membrane of DEX-stimulated or AAV-transfected primary hippocampal neurons, pre-existing surface membrane GluR2 receptor subunits were first masked by placing the cultures of a 24-well plate onto an ice tray, collecting the subsequent media containing either stimulants or controls and immediately applying mouse anti-GluR2 antibody (Millipore, 1:500) in NB^{++} with 2% BSA and 4% donkey serum for 30 min on ice to prevent cell death. Conditioned media was kept at RT, divided into two portions, whereas one half was mixed with 1 M KCl (1:100) for stimulation. Afterwards, neurons were washed three times with cold NB^{+} and incubated with unconjugated anti-mouse secondary antibody (1:200) in NB^{++} for 30 min on ice. After repeating the washing step as described above, neurons were stimulated with conditioned medium containing 10 µM KCl in total for 10 min at 37 °C within the incubator. Subsequently, the medium was removed, and original unconditioned medium was added to neurons. Cells were placed into the incubator at 37 °C for 2 h and 50 min. For fixation, cultures were placed on ice, washed two times with 1X PBS and fixed for 12 min with 4% sucrose in 4% PFA. Following, neurons were stained as for immunofluorescence, images of dendritic branches were acquired using an epifluorescence microscope (Zeiss), single plane, 63X magnification. Cells were selected for imaging, showing intact nuclei by DAPI staining and few to less MAP2 signal. Signal intensity was quantified using MetaMorph.

4.5 Histology

4.5.1 Intracardial perfusion for brain fixation

For brain fixation, animals were cardiac perfused with 4% paraformaldehyde (PFA). Cardiac perfusion allows preservation of tissue without the onset of hypoxia and consequential changes in tissue and cell morphology. For morphological analysis of pyramidal neurons in the hippocampus, male *Thy1*-GFP animals aged 9–12 weeks, were perfused after undergoing the modified social interaction test (MSIT) and subsequent stratification in distinct behavioral groups. Male animals that underwent acute stress or were injected with DEX were aged 9–11 weeks when perfused. Wildtype female and male mice as well as DRR1$^{l/l}$_*Camk2a*-Cre and DRR1$^{l/l}$_*Camk2a*-Cre_*Thy1*-GFP animals had an age of 8–12 weeks upon perfusion. For perfusion animals received a lethal dose of anesthesia via intraperitoneal injection. Either 100 mg/kg Ketamine, 10 mg/kg Xylazine or when performed after social stress paradigms Burpenorfin/Pentobarbital (for 25 g body weight 170–200 µL) provided by collaborators in Mainz. When animals reached a state were reflexes disappeared, they were fixed at their extremities and abdominal cavity and thorax were cut open, after they reached the asphyxia-state with agonal respiration. The heart was exposed, extricated from the pericardium and the perfusion needle was injected caudally into the left ventricle without bruising the septum. Immediately an incision was made in the right atrium to avoid increased blood pressure and generating an efflux of the blood. Animals were first pre-perfused with ice-cold 0.9% NaCl for 1 min to rinse the blood from the system, before perfusion with 100–150 mL ice-cold 4% PFA for 17–19 min. Perfusion solutions were infused using a perfusion pump (Gilson) with a flow rate of 7 mL/min mimicking the physiological blood pressure of the animals. Immediately after, the heads were removed, and brains were carefully dissected by removing the skin first before cutting the skull open with small scissors. Thereafter, skulls were completely opened using forceps and brains were separated from the head and put into ice cold 4% PFA in 15 mL falcons for 3–5 h post-fixation at 4 °C. Afterwards, brains were washed in 1X PBS and kept in 1X PBS at 4 °C until further processing.

4.5.2 Brain sectioning

To prepare brains for immunohistochemical staining and fluorescent microscopy, coronal brain sections were produced. Therefore, brains were dried and embedded in a 4% agarose gel. Brains were serially cut (Twelver series) at 0.75 mm amplitude and 65 Hz frequency into coronal free-floating sections, either of 250 µM thickness for whole cell and spine analysis or of 50 µM

thickness for qualitative immunostaining. Sections were conveyed into a 24-well plate in 1X PBS and stored in TCS at -20 °C until further processing for immunohistochemistry.

4.5.3 Brain extraction

To verify DDR1 protein levels in wildtype C57BL/6J, DRR1$^{l/l}$_*Camk2a*-Cre^{+} and DRR1$^{l/l}$_ *Camk2a*-Cre^{-} animals, brains were extracted without preceding tissue fixation to generating whole brain lysates for Western Blot analysis. Mice were sacrificed by cervical dislocation and heads were quickly amputated with scissors. Following, the skin was removed from the skull with scissors and brains were removed from the head as described in Section 4.5.1 (after intracardial perfusion). Brains were placed in 15 mL falcons and kept at -20 °C until whole brain lysis (see Section 4.4.1).

4.5.4 Immunohistochemistry

Exploiting antigen antibody interaction, immunohistochemistry was applied for fluorescent labeling of DRR1 and various proteins characteristic for distinct cell types for their discrimination or for signal amplification of GFP in *Thy1*-GFP^{+} animals. Per animal every third coronal section of the prepared Twelver series was used for standard immunostaining. Sections of individual animals were placed in a cell strainer placed in a 6-well plate in 1X PBS and washed three times for 10 min. Subsequently, sections were placed in 2 mL Eppendorf tubes filled with sodium citrate buffer (Table 4.9) and incubated at 80 °C in a heating block for 30 min to achieve antigen retrieval. Consecutively, sections were poured back into individual cell strainers and washed with 1X PBS for three times. Next, sections were placed in a 24-well plate and incubated in 500 µL of 5% bovine serum albumin (BSA) and 0.5% Triton X-100 in 1X PBS for 1h at RT on a shaker. Primary antibodies (for concentrations see table 4.5) were diluted in 1% BSA and 0.1% Triton X-100 in 1X PBS and sections were incubated over night at 4 °C in 24-well plates, containing 500 µL of primary antibody solution and placed on a shaker. For sections with a thickness of 250 µM, sections were incubated in primary antibodies for three nights at 4 °C. Afterwards, sections were put back into cell strainers and rinsed with 1X PBS for three times 10 min. Sections were placed back into fresh wells of a 24-well plate into 500 µL of secondary antibody solution, consisting of secondary antibodies conjugated to fluorophores, directed against the host animal of primary antibodies and DAPI for intercalation with the DNA and visualization of nuclei. Incubation was performed for 2 h, at RT on a shaker and in case of 250 µM sections for 4 h, at RT on a shaker. After three washes à 10 min with 1X PBS in cell strainers, sections were mounted

on glass slides with Aqua Poly/Mount or Fluorescent mounting medium (DAKO) and kept at 4 °C until confocal laser scanning microscopy.

4.6 Mouse work and breeding

4.6.1 Animals

All animal experiments were approved by the local government and performed under veterinarian supervision in accordance with European regulations. Male C57Bl/6J mice (Charles River Laboratories, Germany and Janvier, France; > 8 weeks old) were used for *in vivo* and *ex vivo* experiments performed in Mainz (12/12 h light/dark cycle, 23 ± 2 °C). Behavioral experiments were performed in the animal facility of the Institute of Molecular Biology (IMB) at the Johannes Gutenberg University Mainz, Germany. *In vitro* experiments were performed in primary hippocampal neuron cultures at the Goethe-University in Frankfurt am Main, using E17.5–18.5 wildtype embryos from C57Bl/6J female mice that were bred in house. Parental mice were kept in breeding cages (1:1 or 1:2 male to female ratio) with food and water supply *ad libitum* and a 12/12 h day-night cycle. All Mouse lines were bred and housed at the animal facility of the Goethe University Frankfurt am Main, Campus Riedberg. Wildtype (C57BL/6J background) *Thy1*-GFP mice were generated in Frankfurt and shipped to Mainz for behavioral experiments. All efforts were made to minimize animal suffering.

Table 4.15: Animal strains

Mouse line	Symbol	Supplier	MGI ID	Reference
Wildtype (WT)	C57BL/6J	Jackson Laboratories #000664	-	-
***Camk2a*-Cre**	Tg(Camk2a-cre)#Kln	Ruediger Klein (MPI for Neurobiology, Martinsried)	3835518	Minichiello et al., 1999
***Thy1*-GFP**	Tg(Thy1-EGFP)MJrs/J	Jackson Laboratories #007788	3766828	Feng et al., 2000
DRR1$^{wt/lox}$	B6-*Drr1^{tm1Gu}*	Ingenious targeting laboratory #1745	-	-

4.6.2 Strategy for generating DRR1 knockdown animals

Wildtype (WT) mice were obtained from C57BL/6J breedings. Mutant strains used in this thesis were bred in the C57BL/6J background. DRR1$^{wt/lox}$ mice were crossed with conspecifics of the same background to obtain DRR1$^{lox/lox}$ (or DRR1$^{l/l}$) animals to achieve homozygous animals with *loxP* flanking exon 4 of the *Drr1* gene. DRR1$^{l/l}$ animals were further crossed with *Camk2a*-Cre^{+} animals to generate constitutive and brain-region-specific neuronal knockout mice (DRR1$^{l/l}$_*Camk2a*-Cre). To obtain DRR1$^{l/l}$_*Camk2a*-Cre^{+}_*Thy1*-GFP^{+} mice, Cre-positive DRR1$^{l/l}$ animals were first crossed to male *Thy1*-GFP^{+} mice to achieve DRR1$^{l/l}$_ *Thy1*-GFP animals. GFP-positive DRR1$^{l/l}$ mice were further crossed to DRR1$^{l/l}$ Cre-positive mice.

4.6.3 Behavioral experiments

4.6.3.1 Acute social defeat (ASD) paradigm

Adult male C57Bl/6J mice (8–9 weeks) and CD-1 retired breeders were obtained from Janvier Labs (France) and stress exposure experiments were performed in Mainz at the Institute of Molecular Biology (IMB) in collaboration with Dr. Tanja Jene (Translational Psychiatry, Department of Psychiatry and Psychotherapy & Focus Program Translational Neuroscience (FTN) Johannes Gutenberg University Medical Center Mainz, Germany; German Resilience Center (DRZ), Johannes Gutenberg University Medical Center Mainz, Germany).

Wildtype C57Bl/6J mice were subjected to 10 s aggressive encounters with CD-1 male. Thereby, the intruder was sequentially introduced for three times in the home cage of three different, unknown, and single housed CD-1 residents. Following each encounter, the mice were separated by a perforated metal grid, allowing sensory contact and simultaneously preventing physical contact. The paradigm was designed in a way to increase the psychological aspect of the ASD. Control animals underwent handling and were introduced in a new cage for the same time but without an aggressive encounter with CD-1 mice. After 6 h post-stress, when corticosterone levels start to return to basal levels (Jene et al., 2018), animals were perfused and brains were dissected for further immunohistochemical experiments.

4.6.3.2 Chronic social defeat (CSD) paradigm

To chronically stress male adult wildtype and *Thy1*-GFP expressing mice of the C57Bl/6J strain, animals underwent exposure to an older, larger and retired male of the CD-1 strain for, serving as aggressor as described in Golden et al. (2011). While putting the mice together for 30 s, the C57Bl/6J mouse was physically attacked and defeated before a mesh was placed in the middle of the cage separating the animals for 24 h, still enabling sensory and visual contact. This procedure was repeated 10 consecutive days, whereby the conspecific including the home cage changed every other day. All animals undergoing chronic social defeat (CSD) experienced defeat from the same group of CD-1 mice. Mice in the control group were same age and maintained in same conditions. Animals were split in non-defeat and defeated groups in a randomized manner. Control animals were put in empty cages for 1.5 min before putting them back into their home cages divided by a mesh in half. Before modified social interaction test (MSIT) and subsequent perfusion 2 h afterwards, mice were kept for resting in individual, new cages on the last days of the CSD.

4.6.3.3 Subcutaneous dexamethasone injections

As potent synthetic agonist of glucocorticoid receptors, dexamethasone (Merck Pharma GmbH, Germany) was diluted using 0.9% saline to a final concentration of 2 mg/mL and injected subcutaneously (s.c.) with a single dosage of 10 mg/kg body weight into adult (8–9 weeks) male mice of the C57BL/6J strain, obtain from Janvier Labs (France). Vehicle-treated animals served as control mice and were injected with the same volume of 0.9% saline. After 6 h post-injection, mice were sacrificed via PFA-perfusion and brains were dissected for further immunohistochemical analysis. Experiments were performed at IMB in Mainz in collaboration with Dr. Tanja Jene (affiliation see Section 4.6.3.1).

4.6.3.4 Modified social interaction test

In total 15 male *Thy1*-GFP adult mice, aged 12–14 weeks were subjected to chronic social defeat stress as previously described (Section 4.6.3.2). Out of this group, 5 animals were picked in a randomized manner to serve as non-defeated control group. After 24 h, CSD-stressed mice and controls were subjected to the modified social interaction test (MSIT), to stratify the animals into three groups as described in Ayash et al. (2020): social discriminators, equivalent to resilient mice, social avoiders as susceptible mice and non-learners. Here, selectivity CSD-induced social avoidance in male mice was tested towards the aggressor mouse strain CD-1 and a brown mouse

from the 129/Sv serving as control strain. The test was performed in a specifically designed sociability arena (see scheme Figure 2.18) which is divided in three equally sized parts (60 × 40 cm) by transparent plastic walls. The walls displayed openings to allow animals to walk in between the parts. At each end of the outer parts a mesh was placed housing a novel mouse of the CD-1 strain in one side and a 129/Sv mouse on the other side. The mice had a matching size, age, and sex. First, *Thy1*-GFP mice were placed for two times in the middle of the arena for habituation without other mice in the mesh enclosures. Directly afterwards novel mice were placed inside the mesh enclosures for testing social interaction. Each trial continued for 6 min and the time percent interaction (time%) of each mouse presented was measured and analyzed. Interaction was considered, when *Thy1*-GFP mice halted in the interaction zone that was defined 1 cm apart from the boundaries of the enclosure. The social interaction index was calculated by dividing the time [%] exploring each mouse from the different strains by the average of time [%] exploring the meshes without counter mice during the habituation phase. Based on their interaction with CD-1 mice, animals were classified into resilient when the index was ≥ 1 and into susceptible when index was < 1 based on their interaction.

4.7 Data acquisition and analysis

4.7.1 Image acquisition

Images of neuron cultures were acquired using a digital camera (SpotRT; Diagnostic Instruments) attached to an epifluorescence microscope (Zeiss) equipped with 40×, 63×, and 100× oil immersion objectives (Plan-Apochromat; Zeiss). Vibratome sections and PLA cultures were acquired using a Leica SP5 multi-channel confocal laser scanning microscope equipped with a 20×/0.5 NA dry objective as well as a 40×/1.3 NA and a 63×/1.4 NA Plan Apo oil immersion objective for high resolution imaging. Fluorescent signal was detected with bi-directional scanning using two PMT detectors. Laser speed was set to 400 Hz and image size to 1024 × 1024 pixels. Single z-planar (1 ± 0.05 μm) images were exported as TIF files and modified for figure composition. Identical exposure times were chosen between experimental conditions and respective controls. Brightness and contrast were adjusted with Fiji-ImageJ software or Adobe Photoshop. Represented structures are indicated in the figure legends.

4.7.2 Analysis of new AMPAR insertion assay

Following the newly inserted AMPAR assay, the neuronal cultures were imaged with a digital camera (Spot Pursuit, Diagnostic Instruments) attached to an epifluorescence microscope (Zeiss Axio Imager.M1) using a 63× objective (Zeiss). Neurons with a bright MAP2 staining indicated a breakdown of membrane integrity with a high likelihood of intracellular GluR2 staining and were thus excluded from the analysis. The background fluorescence was downregulated by applying a fixed threshold to all pictures of a given experiment. Afterwards, MetaMorph (Molecular Devices) was used to quantify the GluR2 staining intensity. Hereby, dendritic stretches of the 16-bit images were manually defined (~100 μm length) with the "polygon" tool and the integrated fluorescent intensity per stretch was calculated by dividing the measured GluR2 staining intensity by the area of the dendritic stretch. Per neuron several stretches were analyzed wherefor the average was calculated. These averages were used to calculate the average per condition across all independent experiments (2–3).

4.7.3 Analysis of PLA puncta

For colocalization analysis, single plane images were acquired using a confocal microscope (Leica TCS SP5, 63× objective; 2× zoom). Colocalization was quantified using ImageJ by counting the PLA puncta in dendritic ROIs of ~100 μm length. At least 10 cells with on average three dendritic stretches were analyzed per condition and experiment.

4.7.4 Quantification of Western Blots

Western Blot signals were quantified using ImageJ. The signal intensity of the analyzed protein (GluR2, DRR1) was divided by the signal of the control. Signals were normalized to loading control (β-actin). A minimum of three experiments was used to calculate the average of the relative signal intensities (n = 3).

4.7.5 Dendritic branching and Sholl analysis

For analysis of dendritic branching after rAAV treatment *in vitro*, neuronal cultures were imaged using an epifluorescence microscope (Zeiss Axio Imager.M1) with a digital camera (Spot Pursuit, Diagnostic Instruments) and a 40× objective (Zeiss). Images were obtained as stacks of 10–12 μm thickness with a z-stack size of 0.1 μm. Dendritic trees were reconstructed in 3D utilizing

Imaris Filament Tracer (Bitplane). The MAP2 fluorescent immunocytochemical staining served as channel for reconstruction, since it stains the whole dendritic tree, including distal dendrites. Automated Sholl analysis in Imaris was employed to indicate the intersections of concentric spheres around the cell body of traced dendrites at 10 μm distances from the soma. Additionally, dendritic parameters as total dendritic length and the number of branch points was assessed.

4.7.6 Dendritic spine analysis *in vitro*

For spine analysis of virally induced DRR1 level changes in primary hippocampal neuron cultures, the cultures were imaged after PFA fixation using a digital camera (Spot Pursuit, Diagnostic Instruments) attached to an epifluorescence microscope (Zeiss Axio Imager.M1) with a 100× objective (Zeiss). Healthy neurons with a consistent GFP signal and extended dendritic tree were chosen for image acquisition. The background fluorescence was downregulated by applying a fixed threshold to all pictures of a given experiment. Afterwards, the GFP staining was used to trace and reconstruct dendritic stretches of approximately 30 μm length with the Filament Tracer module in Imaris (Bitplane). Dendritic stretches were automatically reconstructed using the Autopath mode in the "Filament Tracer" and the treshold was manually adjusted to fit the diameter of the stretch being analyzed. Subsequently, spines were added using the same module by rebuilding the dendritic diameter and adjusting the threshold to spot all visible spine heads with a seed point. Reconstructed spines were manually corrected by adding missed spines, removing incorrectly added spines or by retracing the spine neck. Finally, spines were classified using the "Classify Spines" Matlab extension in Imaris with specified morphological criteria: mushroom spines: maximal width of spine head ≧ mean width of neck *1.5 and maximal spine head width of ≧ 0.4; stubby spines: spine length < 0.5; long thin spines: mean width of spine head ≧ mean of spine neck; filopodia: spine length > 3–10 μm or remaining spines. For each dendritic stretch, total spine numbers and spine numbers per category were counted.

4.7.7 Dendritic spine analysis *in situ*

For spine analysis of resilient, susceptible and mice categorized as 'non-learners' secondary dendritic stretches of apical pyramidal CA1 and CA3 dendrites (located in the *stratum radiatum*) were imaged after PFA fixation using a 63×/1.4 NA Plan Apo oil immersion objective attached to Leica SP5 multi-channel confocal laser scanning microscope (4× digital zoom, image size 1024 × 256 pixels, z-step-size 0,02 μm) images were exported as TIF files and deconvoluted utilizing Huygens software. GFP expressing cells were chosen for image acquisition. The GFP staining was

used to trace and reconstruct dendritic stretches of approximately 30 μm length with the Filament Tracer module in Imaris (Bitplane). Dendritic stretches were automatically reconstructed using the Autopath mode in the "Filament Tracer" as described before (Section 4.7.4). Spines were classified using the "Classify Spines" Matlab extension in Imaris with specified morphological criteria: mushroom spines: maximal width of spine head ≧ mean width of neck *1.5 and mean width of head ≧ 0.3; stubby spines: mean width of neck ≧ mean width of spine head and spine length < 0.75; long thin spines: mean width of spine head ≧ mean width of spine neck; filopodia: spine length ≧ 3–10 μm or remaining spines. For each dendritic stretch, total spine numbers and spine numbers per category were counted.

4.7.8 Statistical analysis

Statistical analysis was performed on the number (n) of individual experiments and is expressed as mean ± SEM. For all experiments, statistical significance was determined by the two-tailed Student's t test in GraphPad Prism. Statistical significance was assumed when $p < 0.05$. In figures, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The Chi-square test was applied on all individual experiments of the newly-inserted assay to verify the reliability of the observed phenotype.

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6. Appendices

6.1 Supplementary figures and preliminary results

6.1.1 DRR1 expression in cortical neurons of adult mice

As discussed in Chapter 3.1 Figure 6.1 shows DRR1 protein expression in cortical neurons of adult C57BL/6J mice. Those cortical cells were labeled with the neuronal marker NeuN and strongly overlapped with fluorescent DRR1 immunostaining.

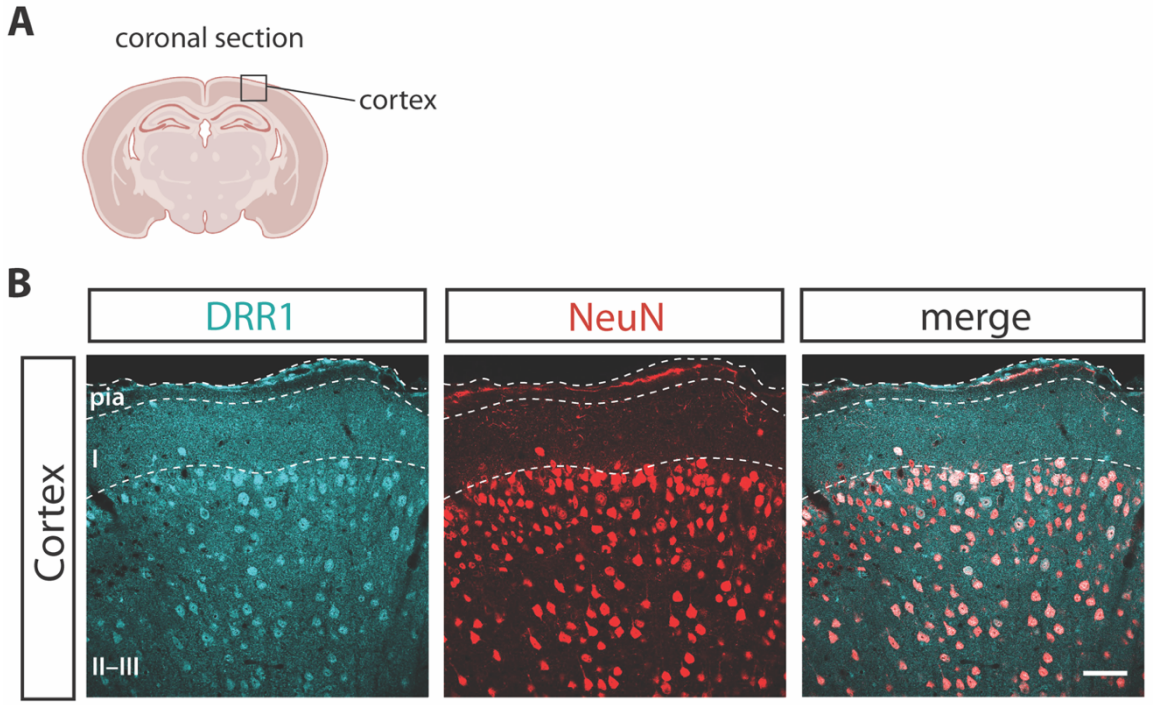

Figure 6.1: *In situ* DRR1 and NeuN expression within the murine cortex.
(A) Scheme of murine brain section with examined cortical brain region. **(B)** Brain section stained for DRR1 (cyan), NeuN (red), and overlap on the right (merged). Staining was performed in 50 µm thick sagittal sections of 8-week-old mice with the MUE-ab. DRR1 overlaps with NeuN-staining and is predominantly found in cortical layer II–III. Acronyms: *pia mater* (pia); cortical layer I (I); cortical layer II–III (II–III). Scale bar: 50 µm, 40× oil immersion.

6.1.2 DEX application in bEnd.3 cells elevates DRR1 and CLDN5 expression

As mentioned in section 3.7, preliminary Western Blot analysis of DEX-treated bEnd.3 cultures show elevated DRR1 as well as CLDN5 protein levels as depicted in Figure 6.2. In addition, qualitative immunocytochemistry analysis (performed by Eva Peterson, Bachelor student, AG Acker-Palmer) of DEX treated bEND.3 endothelial cells is visualized in Figure 6.3 (Images

modified from Bachelor Thesis of Eva Peterson, Title: "The influence of the stress inducible protein DRR1 on the blood-brain-barrier *in vitro*"). Images indicate stronger fluorescent signals for DRR1, CLDN5 and less actin accumulation in DEX-treated cells in comparison to control condition. Figure 6.3(B) indicates changed cell morphology in DEX treated cells towards elongated and thinner endothelial cells (VE-cadherin staining).

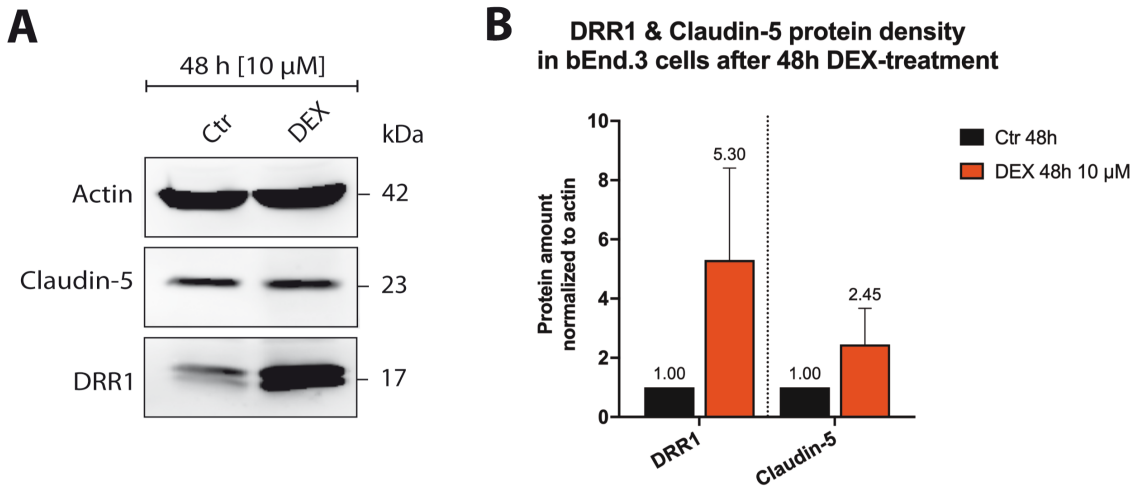

Figure 6.2: Expression of DRR1 & CLDN5 in bEnd.3 cell cultures after DEX application.
(A) Representative Western Blot of bEnd.3 cell lysates after 48 h treatment with DEX. Cells were stressed with artificial glucocorticoid DEX, when reaching confluency of 80%. Blots show DRR1, CLDN5 and actin (house-keeping protein). **(B)** Boxplots show average protein densities from three individual experiments after acute stress through artificial glucocorticoid (DEX) treatment. DRR1 protein levels and Claudin-5 levels are elevated after DEX treatment in comparison to respective controls. Controls served as sample control and were normalized to 1. Protein densities represent arbitrary numbers measured as percentage per area. Students t-test performed as statistical analysis. No significant differences between conditions.

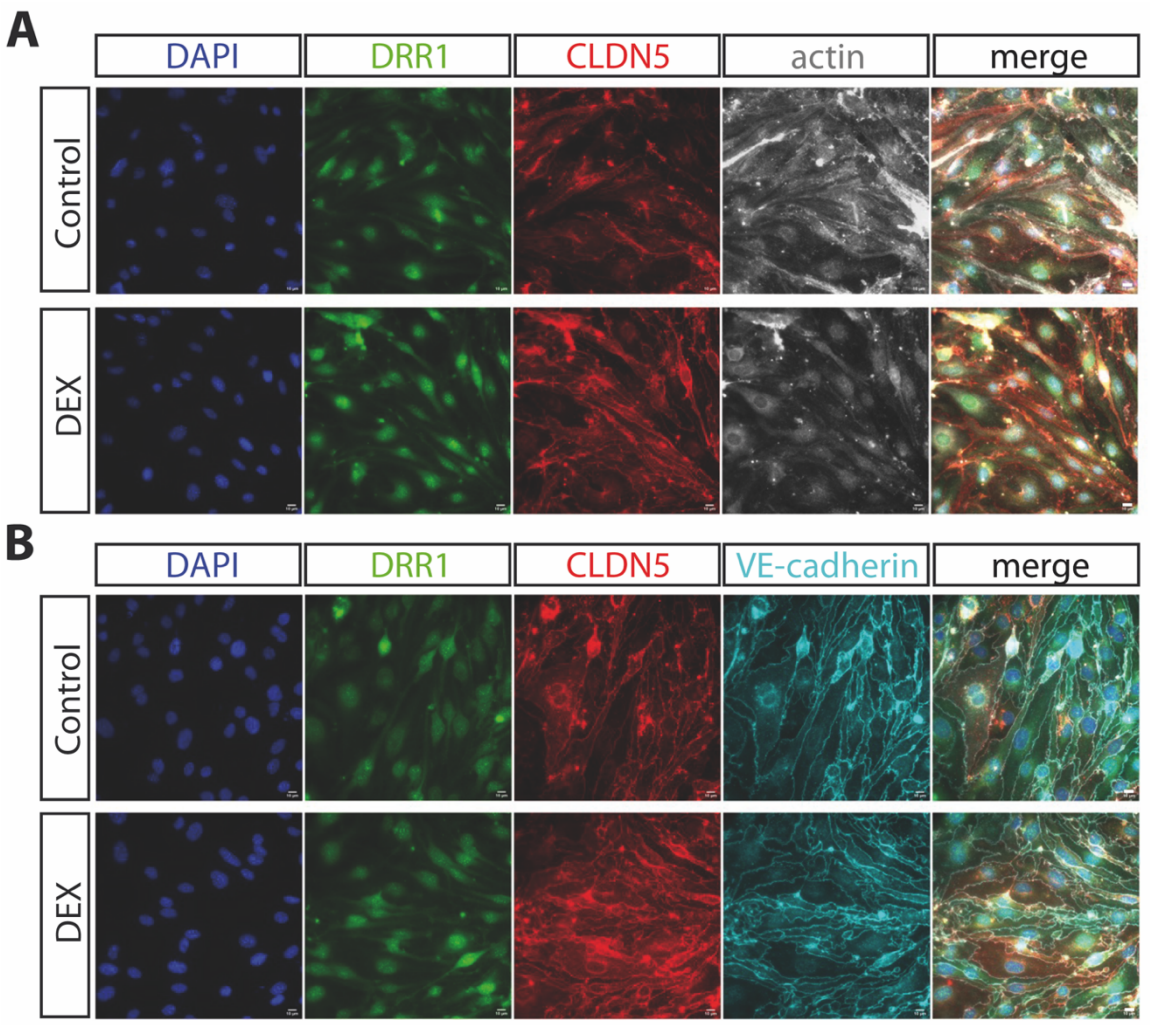

Figure 6.3: *In vitro* immunocytochemistry in bEnd.3 cell cultures after DEX application.
(A) Representative fluorescent images of bEnd.3 cell cultures after 48 h treatment with 10 µM DEX. Cells were stressed with artificial glucocorticoid DEX, when reaching confluency of 80% and approximately 50 000 cells per 24-well plate. Cells were fixated with 10% TCA solution before fluorescent antibody staining was performed. Images show DAPI-staining (blue), DRR1 (green), Claudin-5 (CLDN5, red), actin (grey), VE-cadherin (cyan), and a merged image on the right in **(A–B)**. DEX-treated cells seem to grow more closely together with elongated and thinner cell bodies as compared to control **(B)**. DRR1 as well as Claudin-5 amounts appear to be higher in DEX-treated bEnd.3 cells compared to controls **(A–B)**. Scale bar: 10 µm; 64× oil immersion. Modified from Bachelor Thesis of Eva Peterson 2020, AG Acker-Palmer, Goethe-University.

6.2 Supplementary methods: bEnd.3 cell culture

Brain microvascular endothelial cells build the microanatomical side of the blood brain barrier (BBB). Therefor immortalized cells, such as the murine-derived endothelial cell line bEnd.3 are widely used for *in vitro* BBB models. The bEnd.3 cell line descends from BALB/c mice. It offers consistency in culturing and is well characterized for functional molecular and cellular assays (Sun et al., 2022).

The results described in Section 6.4.2–6.4.3 of this thesis were performed from the 19th passage of the bEnd.3 cell line. Among other established cell lines, bEnd.3 cells express highest

levels of Claudin-5 (Watanabe et al., 2012), making it a good model to study the effects of stress on the tight junction protein.

6.2.1 bEnd.3 maintenance and experimental procedure

bEnd.3 cells (CLR-2299$_{TM}$, ATCC) were culture in 10 cm diameter petri dishes with DMEM containing GlutaMax and supplemented with 10% fetal bovine serum (FBS), and 1% PenStrep at 37 °C and 5% CO_2. Medium was changed every 48 h. When cells reached confluency of 90%, they were split 1:4. One culture served for further maintenance and three dishes were used for experiments.

Splitting was performed by removing the culture medium and washing the cells with DPBS (Dulbecco's Phosphate-Buffered Saline). Thereafter, 1 mL of 0.05 Trypsin was applied for 10 min until cells started to detach from the bottom of the culture plate. Right after, 4 mL of DMEM were added to stop the reaction. Cells were carefully scratched from the bottom and transferred into an Eppendorf tube. After centrifuging the cells for 5 min at 300 × g the supernatant was discarded and cells were resuspended in fresh 5 mL DMEM. 1 mL of the resuspended cell solution was added to 6 mL of DMEN in fresh petri dishes and carefully distributed by moving the plate. Cells were put back into the incubator at 37 °C until further experiments were performed.

To mimic an acute glucocorticoid mediated stress response in bEnd.3 cells, the artificial glucocorticoid Dexamethasone (sigma) was applied on the cells upon 80% confluency for 48 h. Next cells were further processed for Western Blot analysis. To dissolve Dexamethasone (DEX), 1 mL of ethanol was added per 1 µg Dexamethasone (392.46 g/mol). Per milliliter of ethanol, 49 mL of DMEM were added to obtain a working solution of 20 µg/mL and a concentration of 51 µM. The working solution was used to make dilutions 0.1 mM DEX in DMEM, which were further used to apply on cultures resulting in an end concentration of 10 µM. For that, the corresponding volume of growing medium was withdrawn and supplemented with the subsequent amount of DMEM working solution, before cells were put back into the incubator for another 48 h. Control solution contained EtOH only without dissolved DEX. After 48 h culture plates containing bEnd.3 cells were put on ice and first washed with DPBS for two times. Thereafter cells were lysed using 120 µL RIPA-buffer by applying the buffer for 5 min to reach cell detachment from the plate. Cells were harvested with a cell scraper and transferred into a 1.5 mL Eppendorf tube. Thereafter lysates were treated as described in Section 4.4.1–4.4.3 in Material and Methods.

For immunohistochemistry cells were cultivated in 12-well chamber (Ibidi) glass microscope slide with removable walls. The bEnd.3 cell density was set to 30 000 cells per well. After cell splitting the cell density of resuspended cells (as described in the section above) was measured using an automated cell counter (BioRad). For cell counting 10 µL of resuspended cells were mixed with the same amount of Trypan Blue (Gibco) and transferred into a counting chamber before inserting into the cell counter. To calculate the volume of resuspension needed for the desired cell number, 30 000 was divided by the counted living cells. The appropriate volume was transferred into each well and filled up with DMEM including supplements until reaching a total volume of 250 µL/well. The cells were left to settle for 24 h before the medium was exchanged for DMEM containing 10 µM DEX or EtOH control solution. After 48 h of incubation the cultures were washed once with DPBS and subsequently treated with 200 µL of 10% Trichloroacetic acid (TCA) in DPBS on ice for 15 min for fixation. Next cells were washed twice with DPBS before adding 200 µL of 0.3% Triton X-100 in DPBS for 5 min. After two rinses with DPBS the cells were incubated for 1 h in blocking solution (4% donkey serum, 2% bovine serum albumin in DPBS). Immunocytochemistry was performed as described in Section 4.4.4 against the proteins depicted in Section 6.4.2. For mounting the removable walls on the microscope slide were stripped off and a cover slip was mounted with mounting solution (DAKO). Cells were monitored using an epifluorescence microscope (Zeiss) with a 64× oil objective.

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6.5 Abbreviations

ΔFosB	delta FosB proto-oncogene
%	Percent
ø	Diameter
∞	Infinite
~	Approximately equal
2D	Two-dimensional
3D	Three-dimensional
5-HT	Serotonin
5-HTT	5-hydroxytryptamine transporter
AAV	Adeno-associated virus
ABI	abI interactor
ABP	Actin-binding protein
ACTH	Adrenocorticotropin hormone
Ad	Adenovirus serotype
ADF	Actin depolarizing factor
ADP	Adenosine diphosphate
AG	Working group ("Arbeitsgruppe")
AHP	Afterhyperpolarization
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxacolepropionic receptor
AP	Action potential
AP1/2	Activator protein 1/2
APS	Ammonium persulfate
AR	Adrenergic receptor
Arc	Activity-regulated cytoskeleton-associated protein
Arp	Actin-related protein
ASD	Acute social defeat
ATD	Amino-terminal domain
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
bAP	Backpropagating action potentials
BBB	Blood brain barrier

BDNF	Brain derived neurotrophic factor
bEnd.3	Mouse brain endothelial cell line derived from BALB/c mice
BGH	Bovine growth hormone gene
BIS	Behavioral inhibition system
bp	Base pair
BSA	Bovine serum albumin
Ca	Calcium
CA	*Cornu ammonis*
CAM	Cell-adhesion molecules
CaMK	Calcium/calmodulin-dependent kinase
cAMP	Cyclic adenosine monophosphate
Cap	Cas-associated protein
CD11b+	Integrin alpha-M
Cdc42	Cell division control protein homolog 42
cDNA	Complementary deoxyribonucleic acid
CI	Calcium-impermeable
Cl	Chloride
Cldn-5	Claudin-5
cm	Centimeter(s)
CNS	Central nervous system
CO_2	Carbon dioxide
CORT	Cortisol
CP	Calcium-permeable
CPEB4	Cytoplasmic polyadenylation element-binding protein 4
CREB	cAMP response element-binding protein
CRF	Corticotrophin-releasing factor
CRH	Corticotrophin-releasing hormone
CSD	Chronic social defeat
CSDS	Chronic social defeat stress
CTD	C-terminal domain
Ctr	Control
CUS	Unpredictable stress
Cx3Cr1	CX3C motif chemokine receptor 1
DAPI	4'6-diamidino-2-phenylindole
ddRNAi	DNA-directed RNA interference

DEX	Dexamethasone
DG	Dentate gyrus
*D*h	Diameter (of spine head)
dH_2O	Distilled water
DHEA	Dehydroepiandrosterone
DISC1	Disrupted in schizophrenia 1
DIV	Day(s) *in vitro*
DM	Dissection medium
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
*D*n	Diameter (of spine neck)
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
DPBS	Dulbecco's phosphate-buffered saline
dpt	Day(s) post-transfection
DRF3	Diaphanous-related formin 3
DRR1	Downregulated in renal cell carcinoma 1
DTI	Diffusion tensor imaging
DTT	1,4-Dithioerythritol
DUF	Domain of unknown structure
E	Embryonic day
E-LTP	Early long-term potentiation
E1A	Adenovirus early Region E1A Protein
EB3	End-binding protein 3
EC	Entorhinal cortex
EDTA	Ethylenediaminetetraacetic acid
EFNA4	Ephrin A4
EFNB	EphrinB, family of receptor tyrosine kinases
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth receptor
Egr1	Early growth response protein 1
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein)
Eps8	Epidermal growth factor receptor pathway substrate 8
EPSCs	Excitatory postsynaptic currents
EPSP	Excitatory postsynaptic potential

ER	Endoplasmatic reticulum
ERK	Extracellular signal-related kinase
EtBr	Ethidium bromide
F-actin	Filamentous actin
FA	Focal adhesion
Fam107A	*Family* with sequence similarity 107, member A
FBS	Fetal bovine serum
fEPSP	Field excitatory postsynaptic potential
ff.	Following pages
FGF	Fibroblast growth factor
Fig.	*Figure*
FLP	Flipase
fMRI	Functional magnetic resonance imaging
Fos	Fos proto-oncogene
FPKM	fragments per kilobase of transcript (sequence) per million (mapped fragments)
FRT	Flippase recognition target
FTN	Focus Program Translational Neuroscience (Johannes Gutenberg University Medical Center Mainz, Germany)
G-actin	Globular actin
GABA	Gamma-aminobutyric acid
GC	Glucocorticoid
gcl	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GKAP	Guanylate kinase-associated protein
GluA	Glutamate ionotropic receptor AMPA type subunit
GluK	Glutamate ionotropic receptor kainate type subunit
GluN	Glutamate ionotropic receptor NMDA type subunit
GluR	Glutamate receptor
GmbH	Gesellschaft mit beschränkter Haftung (Englisch: KOMM., limited liability company)
GP	Genomic copy(/ies)
GPCR	G-protein-coupled receptors
GR	Glucocorticoid receptor

gr	Granule layer
GRE	Glucocorticoid responsive elements
GRIP	Glutamate receptor-interacting protein
GSK3β	Glycogen synthase kinase-3β
GTPase	Guanosine triphosphate hydrolase enzyme
GxE	Gene x Environment
h	Hour(s)
H	Hilus
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hf	Hippocampal fissure
HOMER	Homer scaffolding protein 1
HPA	Hypothalamic-pituitary adrenocortical
HPG	Hypothalamic-pituitary-gonadal
HRP	Horseradish peroxidase
HUVEC	*Human umbilical vein endothelial cells*
Hz	Hertz
IBM	Institute of Molecular Biology (Mainz, Germany)
ID	Identification (number)
IDR	Intrinsically disordered region
IEG	Immediate early genes
IgG	Immunoglobulin G
iGluR	Ionotropic glutamate receptors
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iml	Inner molecular layer
IRES	Internal ribosome entry site
ISH	*In situ* hybridization
ITR	Inverted terminal repeat
Iκ*K*	Inhibitor of Kappa-B kinase
JGU	Johannes Gutenberg-University (Mainz)
K^+	Potassium ion
KA	Kainite

KAR	Kainite receptor
KCl	Potassium chloride
kDa	Kilodalton
L	Liter(s)
L	Length (of spine)
L-LTP	Late long-term potentiation
LBA	Lysis buffer A
LBD	Ligand binding domain
LCD	Low-complexity domains
LEA	Lateral entorhinal area
LEC	Lateral entorhinal cortex
LIMK-1	LIM kinase 1
LoxP	Locus of crossover in P1
LTD	Long-term depression
LTP	Long-term potentiation
Ly6C	lymphocyte antigen 6 complex
m	Milli (as prefix)
m	Meter(s) (as suffix)
M	Molar(s)
MAOA	Monoamine oxidase A
MAP2	*Microtubule-associated protein 2*
MCH	Microcell hybrid
mDia	Mouse Diaphanous
MEA	Medial entorhinal area
MEC	Medial entorhinal cortex
mEPSC	Miniature excitatory postsynaptic current
Mg	Magnesium
mGluR	Metabotropic glutamate receptor
min	Minute(s)
miRNA	Micro ribonucleic acid
ml	Milliliter
ml	Molecular layer
mm	Millimeter
mM	Millimolar
mml	Middle molecular layer

MOI	Multiplicity of infection
mPFC	Medial prefrontal cortex
MR	Mineral receptor
MRI	Magnet resonance imaging
MSIT	Modified social interaction test
MUE	Mueller laboratory (AG Mueller, Department of Psychiatry & Psychotherapie, Johannes-Gutenberg University Medical Center, Mainz)
mV	Millivolt
n	Sample size (number)
N-WASP	Neural Wiskott-Aldrich syndrome protein
N2a	Neuro2a cell line
Na	Sodium
NA	*Numerical aperture*
NAc	Nucleus accumbens
NARP	Neuronal pentraxin 2
NB	Neurobasal medium
NB	Neurobasal medium
NE	Norepinephrine
NeuN	Neuronal nuclei
NFκB	Nuclear factor kappa-B
NGFI-A	Nerve growth factor-inducible protein A
nKO	Neuronal knockout
NLRP3	Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3
NLS	Nuclear localization signal
nm	Nanometer(s)
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
NMJ	Neuromuscular junctions
NSF	N-ethylmaleimide-sensitive factor
ºC	Degrees Celsius
ºC	Degree Celcius
OCT3	Organic cation transporter 3
OE	Overexpression (of DRR1 construct)
OLM	Outermost layer of the hippocampus

oml	Outer molecular layer
OPC	oligodendrocyte precursor cell
OT	Oxytocin
p	Propability
p140Cap	Cas-associated protein
P2RY13	Purinergic Receptor P2Y13
PAK3	RAC1 activated kinase 3
para/ PaS	Parasubiculum
PASTOR	Positive appraisal style theory of resilience
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDL	Poly-D-lysin
Pdx	Podocalyxin
PER	Perirhinal
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PHR	Parahippocampal
PI3K	Phosphatidylinositol 3-kinase
PICK	Protein Interacting With Protein Kinase C
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Proximity Ligation Assay
POR	Postrhinal
PP	Perforant path
PP1	Protein phosphatase 1
pre/prS	Presubiculum
PSD	Postsynaptic density
PTSD	Post-traumatic stress disorder
pu	Purkinje layer
PVN	Paraventricular nucleus
$R^{-/-}$	Stress susceptible mice
$R^{-/+}$	Stress resilient mice
$R^{+/+}$	Non-learners (behavior of mice after chronic social defeat stress)
rAAV	Recombinant adeno-associated virus

Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma virus
rAVE™	Customized recombinant adeno-associated viral vectors
RCA	Rolling-circle amplification
RE	Recycling endosome
Rho	Rhodopsin
Rif	Rho in filopodia
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNAseq	RNA-sequencing
ROI	Region of interest
RP	Reserve pool
RRP	Readily releasable pool
RT	Room temperature
RU487	Mifepristone – glucocorticoid receptor antagonist
s	Second(s)
s.c.	Subcutaneously
SAP	Synapse-associated protein
SC	Schaffer-collateral
SC1	Sparc-like 1
SCN	Suprachiasmatic nucleus
SCR	Scrambled
SDR	Social disruption stress
SDS	Sodium lauryl sulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
Ser	Serine residue
SERT	Serotonin transporter
sg	*Stratum granulosum*
Sgk	Serum- and glucocorticoid-inducible kinase
SGZ	Subgranular zone
SH	Multiple ankyrin repeat domains
SHANK	Multiple ankyrin repeat domains protein
shDRR1	Short hairpin ribonucleic acid (directed against *Drr1* RNA)
shRNA	Short hairpin ribonucleic acid

shSCR	Short hairpin ribonucleic acid (scrambled version)
sl	*Stratum lucidum*
slm	*Stratum lacunosum-moleculare*
sLTP	Structural LTP
sm	*Stratum moleculare*
SM	Serum medium
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
SNS	Sympathetic nervous system
so	*Stratum oriens*
sp	*Stratum pyramidale*
sr	*Stratum radiatum*
SSC	Saline sodium citrate
STP	Short-term potentiation
SUB/ Sub	Subiculum
SynCAM	Synaptic cell adhesion molecules
SynGAP	Synaptic GTPase activating protein
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TARP	Transmembrane AMPA receptor regulatory protein
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TCS	Tissue cryoprotectant solution
TEMED	Tetramethylethylenediamine
Temp	Temperature
TIFF	Tag Image File Format
TMD	Transmembrane domain
TNF-α	Tumor Necrosis Factor α
TRK	Tyrosine kinases
TU3A	Tohoku University cDNA clone A on chromosome 3
UTR	Untranslated region
UV	Ultraviolet
V	Volt
vmPFC	Ventromedial prefrontal cortex
VP	Vasopressin

VTA	Ventral tegmental area
WAVE-1	WASP-family verprolin homology protein-1
WNT (Wnt)	Wingless
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
WT	Wildtype
X	Times
ZO1	Zonula-occluding-1
α	Anti-
µ	Mikro (as prefix)
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
μm^3	Cubic micrometer

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7 Acknowledgments

8 Curriculum vitae

PERSONAL DATA

EDUCATION

WORK EXPERIENCE AND COMMUNITY INVOLVEMENT

ADDITIONAL SCIENTIFIC TRAINING

RESEARCH SUPERVISION

CONFERENCES AND MEETINGS (Selection)

AWARDS

LANGUAGES

9 Publication

Stefani J., **Tschesnokowa O.***, Parilla M.*, Robaye B., Boeynaems J.-M., Acker-Palmer A., Zimmermann H., Gampe K. (2017): "Disruption of the Microglial ADP-Receptor $P2Y_{13}$ Enhances Adult Hippocampal Neurogenesis". Frontiers in Cellular Neuroscience. DOI: 10.3389/fncel.2018.00134.

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