Translational Study on the Role of Genetic and Prenatal Risk Factors in Neurodevelopmental Psychiatric Disorders

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Dedicated to my family,

for whose unwavering

love, support, and faith in me,

I shall remain eternally grateful.

Посветено на моето семейство,

за чиито непоколебими

обич, подкрепа, и вяра в мен

оставам завинаги благодарна.

SUMMARY

Neurodevelopmental psychiatric disorders (NPDs), such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and schizophrenia (SCZ), and their comorbidities affect millions of people worldwide and have a high burden of disease. Despite recent progress in gaining aetiological, molecular, and morphological insights into NPDs, much remains to be discovered about both their underpinnings and novel therapeutic targets, including the effects of biological sex and age on them.

Research into NPD susceptibility has revealed several risk factors that influence brain development and neuronal signalling, including prenatal inflammation and genetic variation in neurodevelopmental regulatory genes. This dissertation aimed to build upon these findings by combining behavioural, molecular, and neuromorphological investigations in mouse models of such risk factors, i.e. maternal immune activation (MIA), neuron-specific overexpression (OE) of the cytoplasmatic isoforms of the RNA-binding protein RBFOX1, and neuronal deletion of the small Ras GTPase DIRAS2.

Maternal infections during pregnancy pose an increased risk for NPDs in the offspring. While viral-like MIA has been previously established elsewhere, the study "Consequences of MIA for Neuromorphology and the Embryonic and Adult Hippocampal Proteome" was the first in our institution to implement this model. Thus, I validated NPD-relevant deficits in anxiety- and depression-like behaviours, as well as dose- and sex-specific social deficits in mouse offspring following viral-like MIA, induced by poly(I:C) in early gestation. Importantly, analyses of the synaptoneurosomal proteome in embryonic and adult hippocampi (HPC) highlighted novel and known targets affected by prenatal immune challenge. Analysis of the embryonic dataset implicated neurodevelopmental disruptions of the lipid, polysaccharide, and glycoprotein metabolism pathways, important for proper membrane function, signalling, and myelination, for NPD-pertinent sequelae. In adulthood, the observed synaptoneurosomal changes revealed a shift toward disrupted transmembrane trafficking and intracellular signalling, apoptosis, and cytoskeletal organisation pathways. Importantly, 50 proteins altered by MIA in embryonic and mature HPC were enriched in the NPD-relevant synaptic vesicle cycle. Persistently upregulated, NPD-implicated proteins formed a functional network involved in presynaptic signalling, and proteins downregulated in embryos but upregulated in adults by MIA - suggestive of possible compensatory effects - were correlated with

observed social deficits. 49 of 50 genes encoding these proteins were significantly associated with NPD- and comorbidity-relevant traits in human phenome-wise association study data for psychiatric phenotypes. These findings highlight novel, NPD-relevant targets for early intervention in at-risk individuals. Furthermore, MIA-evoked changes in the architecture of sparsely-labelled neurons in the NPD-relevant HPC and prefrontal cortex (PFC) of adult male and female mice were examined. The findings highlighted sex- and region-specific alterations in dendritic and spine morphology, possibly underlining behavioural endophenotypes.

To further investigate risk factors of NPDs, this time of a genetic nature, I performed the study "Expanding on the Role of Cytoplasmatic RBFOX1 in the Brain" based on the implications from human studies of *RBFOX1*'s pleiotropic effects on major NPDs and comorbidities, as well as our previous findings from a mouse model. Cytoplasmatic OE of RBFOX1, which affects the stability and translation of thousands of downstream targets - including those involved in neurotransmission - was used to disseminate the role of this neurodevelopmental regulator in neuromorphology and behaviour. RBFOX1 OE affected the dendritic length and branching profile in the PFC of male mice, also leading to spine alterations in both PFC and HPC, which were extensive in the latter, while having no significant effect on amygdalar morphology.

Due to the previously described ASD-like endophenotypes observed in our *Rbfox1* KO mice, I wanted to probe the interaction effects of cytoplasmatic OE and a lower-dose MIA on the offspring, considering the importance of gene × environment effects on NPD susceptibility, to see if RBFOX1 OE might rescue the MIA phenotype – or indeed, exasperate it. Both RBFOX1 OE alone and especially together with the immune challenge led to increased offspring loss during the perinatal period, probably due to stress caused by construction noise in the holding facility. Another possibility for combined deleterious effects of the interventions, was investigated by assessing known RBFOX1 targets in proteins significantly affected by the low-dose MIA in the embryonic HPC dataset. Nevertheless, preliminary data from behavioural tests suggested that both cytoplasmatic RBFOX1 OE alone and OE × MIA might increase anxiety-like behaviours and affect other NPD-relevant endophenotypes. Morphological changes in the adult male OE HPC and PFC suggested increased spine densities and reduced dendritic complexity. Finally, a small *post-mortem* study in human dorsolateral PFC of older adults did not reveal significant effects of a common genetic risk variant on RBFOX1 abundance, suggesting other avenue of action or such changes occurring earlier in life but not persisting in older age.

To expand upon genetic risk in NPDs, the third study "*Diras2* Expression Levels Exert Subtle Behavioural and Morphological Effects Related to ADHD" was performed. Previously, the Ras GTPase *DIRAS2* has been associated with ADHD in human studies and molecular investigations have implicated the gene in neurodevelopment but its function remains largely unknown. Here, I evaluated the effects of a homo- (KO) or heterozygous (HET) deletion of *Diras2* in a novel, neuron-specific mouse model. In adult animals, there were genotype- and sex-specific effects on behaviour, suggesting more pronounced deficits in NPD-relevant behaviours in the males, which is in keeping with human data. Overall, KO mice seemed to have subtly improved cognitive abilities, while the HET mice exhibited behaviours in line with multiple core symptoms of ADHD, including learning difficulties in females, response inhibition deficits and hyperactivity in males – also in line with findings from ADHD patients, suggesting an NPD-relevant dose-sensitivity and sex-specificity of *Diras2*. The morphological findings from adult males confirmed this profile, with HETs having multiple aberrations in dendritic and spine morphology of the PFC, HPC, and amygdala linked to NPDs, while KO males revealed changes in spine and dendritic morphology exclusively in the PFC – regulating cognitive performance, and largely opposite to those in HETs and NPD-like phenotypes. Furthermore, region- and genotype-specific expressional changes in *Diras2* and its paralog were observed in a targeted molecular analysis of six NPD-relevant brain regions of adult female mice, also revealing differential expression of the major cell survival- and morphology-regulator mTOR in HET and KO mice, which might underlie the observed differences in behaviour and neuronal architecture. Intriguingly, the TOR pathway was also affected by MIA.

In conclusion, the effects of MIA and partial *Diras2* knockdown resembled each other in core, NPD-associated behavioural and morphological phenotypes, while cytoplasmatic RBFOX1 OE and full *Diras2* KO differed in most aspects. My findings suggested a complex dose- and sex-dependent relationship between these prenatal immune and genetic interventions, whose NPD-relevant influences on the brain might converge onto neurodevelopmental processes and molecular pathways. An assessment of such putative overlap, based on available data and insights from the proteomic analyses of embryonic and adult HPC following MIA, suggested that the three models are linked via downstream targets, direct or indirect interactions, and upstream regulators. Future studies should disseminate both the distinct

and shared aspects of MIA, RBFOX1, and DIRAS2 relevant to NPDs and build upon these findings.

Taken together, this dissertation expanded upon the role of prenatal immune challenges and neurodevelopmental genes' dysregulation in NPDs. The behavioural, morphological, and molecular findings offer new translational insights of shared pathways for NPD risk factors and propose novel targets for future aetiological investigations and therapeutic development.

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1. INTRODUCTION

Ὁ βίος βραχύς,	*Vīta brevis,*	*Life is short,*
ἡ δὲ τέχνη μακρή,	*ars longa,*	*art long,*
ὁ δὲ καιρὸς ὀξύς,	*occasio praeceps,*	*opportunity fleeting,*
ἡ δὲ πεῖρα σφαλερή,	*experimentum periculosum,*	*experiment tricky,*
ἡ δὲ κρίσις χαλεπή.	*iudicium difficile.*	*judgement difficult.*

- „*Hippocratis Aphorismi*", Aph. 1.1

„*One should not only prepare oneself to do what one should, but also the patient and those present and the external circumstances […]*" the ancient Greek text, attributed to Hippocrates of Kos and included in the "*Corpus Hippocraticum*" guidelines for physicians, continues. These works included Hippocrates's teachings on psychiatric disorders, how they affect behaviour, and an example of humanity's early understandings of how physiology and environment alike interact with mental health. A lot has changed in both medical practice and science since then, yet a modern variation of this advice from antiquity remains pertinent. Indeed, life is as short as the scientific craft takes long to master, but one should seize opportunity to further one's research field and offer one's best interpretation of those tricky experiments - and, hopefully, others will pick up where one left off.

1.1 NEURODEVELOPMENTAL PSYCHIATRIC DISORDERS (NPDS)

1.1.1 Background and Prominent Examples

Recent insights from large-scale systematic studies position mental disorders as one of the leading causes of overall burden of disease, estimated to account for up to 13% of global disability-adjusted life-years, DALYs (GBD 2019 Mental Disorders Collaborators, 2022; Vigo *et al.*, 2022). The impact, landscape, and prevalence of psychiatric disorders have further solidified as not only age- but also sex- and gender-specific (see Figure 1.1), which experimental study designs and clinical practice have yet to consistently address (GBD 2019 Mental Disorders Collaborators, 2022; Hartung & Lefler, 2019). These differences exist at a variety of different levels, impacting behavioural outcomes, neurite development and morphology, and synaptic form and function (see Figure 1.2 A-D), influenced by sex hormones in important regions like the cortex, hippocampus (HPC), amygdala, and hypothalamus, among others (Barth *et al.*, 2015).

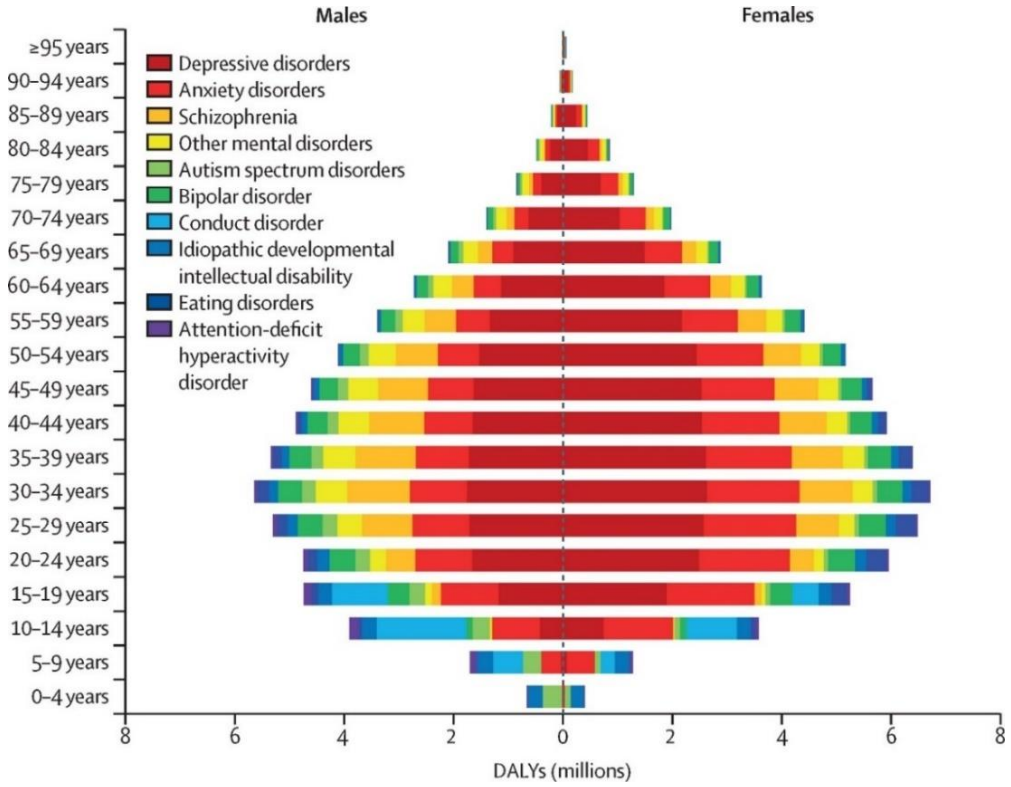


Figure 1.1 Global burden of mental disease, expressed in disability-adjusted life-years (DALYs) by age, sex, and psychiatric disorder. DALYs combine years of life lost and years lived with disability. Disorders of interest for this work are presented in red, orange, green, and purple colours. Figure from GBD 2019 Mental Disorders Collaborators (2022), re-used CC-BY 4.0 licence.

As growing evidence about the pleiotropic nature of mental illness and the major role of brain development therein emerged (Gandal *et al.*, 2018; Lee *et al.*, 2021; Meyer & Lee, 2019; Shohat *et al.*, 2021; Willsey *et al.*, 2018), neurodevelopmental psychiatric disorders (NPDs) have increasingly occupied a more central role in preclinical and translational psychiatric research (Andersen, 2003). Neuropsychiatric disorders with developmental underpinnings often arise from aberrant neuro- and synaptogenesis, and other processes involved in embryonic and early-life brain development, and encompass autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and schizophrenia (SCZ), among others (Dark *et al.*, 2018; Morris-Rosendahl & Crocq, 2020; Owen *et al.*, 2011; Zoghbi & Bear, 2012); see also Figure 1.1, 1.6.

Furthermore, other types of mental illnesses have been proposed, at least in part, to exist on the NPD continuum, i.e. mood disorders like bipolar (BP) and major depressive (MDD) disorder (Ansorge *et al.*, 2007; Kloiber *et al.*, 2020), and anxiety disorders including obsessive compulsive disorder (OCD), to name but a few (Cappi *et al.*, 2016; Leonardo & Hen, 2007). Conversely, some neurodevelopmental disorders (NDDs) not considered psychiatric in nature,

like intellectual disability (ID) and epilepsy, coincide and share genetic candidates with each other and prominent NPDs (Heyne *et al.*, 2018; Snoeijen-Schouwenaars *et al.*, 2021). The following sections will focus on three pervasive NPDs of interest for this dissertation - ASD, ADHD, and SCZ, some prominent risk factors and models, NPD comorbidities, and the role of neuronal and synaptic dysfunction therein.

AUTISM SPECTRUM DISORDER (ASD)

ASD is an NDD with median worldwide prevalence of 1%, which varies significantly across countries and cohorts (e.g. median 0.23 % in South-East Asia, 1-1.3 % in Europe and the Americas, to 2% in Africa), and is more commonly diagnosed in boys with median male-to-female ratio of approx. 4:1 (Zeidan *et al.*, 2022). Individuals with ASD exhibit various degrees of deficits particularly in the domains of social interaction and communication, as well as behavioural inflexibility manifesting as repetitive or pervasive traits (Hirota & King, 2023). The estimated age of onset varies, partly due to difficulties in early diagnoses, but ASD symptoms usually emerge within the first 18 months, while a meta-analysis of studies from the 2010s put the mean age at diagnosis at 3.6-5 years, depending on inclusion criteria (Ozonoff *et al.*, 2008; van 't Hof *et al.*, 2021).

Large-scale genomic studies have identified genetic risk variants in ASD, such as rare *de novo* copy-number variations (CNVs), which might contribute to ASD risk and phenotype to a higher degree in females (Sanders *et al.*, 2015). Affected genes, which can be sex-specific, encode multiple synaptic proteins (see Figure 1.3): for example, adhesion molecules (presynaptic neurexins, NRXN; postsynaptic neuroligins, NLGN) and postsynaptic signalling mediators in the excitatory synapses, including glutamatergic receptors (GluRs) and their downstream partners like SHANK (*SH3 and multiple ankyrin repeat domains*) proteins and SYNGAP1 (*synaptic Ras GTPase activating protein 1*), or inhibitory neurotransmitter gamma-aminobutyric acid (GABA) receptors (Jiang *et al.*, 2022; Sanders *et al.*, 2015). On the other hand, single nucleotide polymorphisms (SNPs) have low individual - but significant additive effects on ASD liability, so that common genetic variants contribute to 40-60% of ASD heritability (Klei *et al.*, 2012). A more recent genome-wide association study (GWAS) revealed genome-wide significant risk loci, encompassing candidate genes involved in synaptic formation, neurotransmission, and splicing such as the polypyrimidine tract binding protein *PTBP2* (Grove *et al.*, 2019); see also Chapter 1.3.1, Section PTBP.

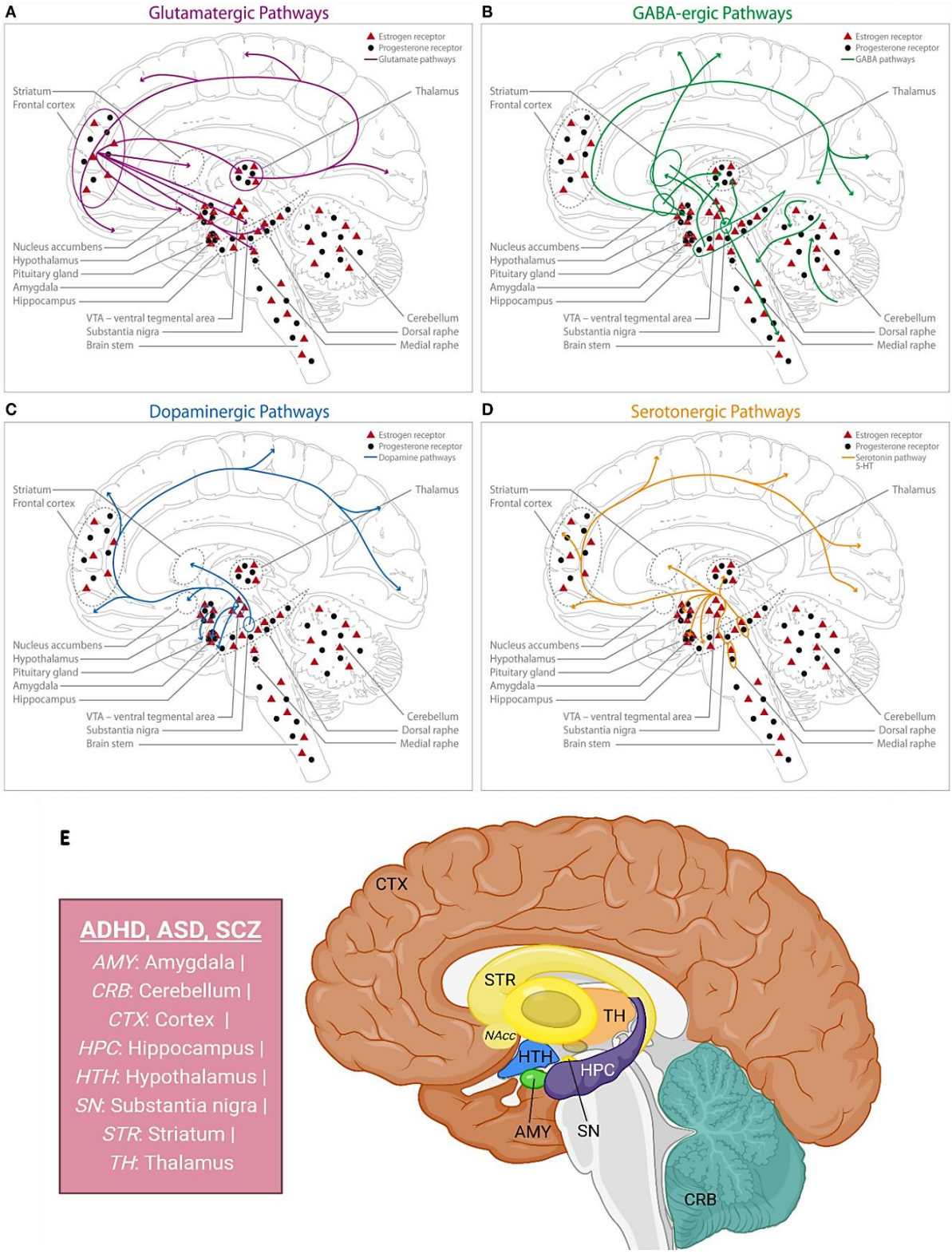


Figure 1.2 Simplified overview of main neurotransmitter (NT) systems and steroid hormone receptors in the brain, and regions with high relevance for neurodevelopmental psychiatric disorders (NPDs). Panels **A-D** from Barth *et al.* (2015) depict selected NT pathways and regional sex hormone (*oestrogen, progesterone*) receptor distribution, re-used under CC-BY 4.0; see reference for details. Many of the NPD-affected structures (**E**) are involved in various behaviours and mental processes, including emotional and stress responses, memory formation, executive and motor functions, attention, and motivation.

Another splicing regulator implicated in ASD, which will be discussed in detail below (see Chapters 1.1.2, 1.3.1.2), is the RNA binding fox-1 homolog *RBFOX1* (Lee *et al.*, 2019; O'Leary *et al.*, 2022). Apart from genetic influence, environmental factors that increase ASD risk include increasing parental age, poor maternal cardiometabolic health and psychiatric medication use during pregnancy (see also Figure 1.6), as well as familial autoimmune disorders (Kim *et al.*, 2019; Wu *et al.*, 2017a).

Multiple brain regions have been implicated in ASD aetiology and symptomology (see Figure 1.2) and overall, brain volume increases during the first year of life were associated with diagnosis and symptom severity in high-risk infants (Hazlett *et al.*, 2017). Cortical thickness aberrations were observed in ASD and the differences peaked in adolescence when compared to healthy subjects, while the volumes of amygdala and basal ganglia subregions were reduced (van Rooij *et al.*, 2018). Interestingly, the amygdala might undergo age-specific changes, i.e. premature enlargement in ASD children in the absence of the usual adolescence-related growth, while HPC volume increases have been observed throughout development (Schumann *et al.*, 2004). Indeed, the HPC plays a central role in deficits of the ASD-associated social and cognitive domains (Banker *et al.*, 2021). Cerebellar subregion deficits in ASD are distinct from those affected in other NPDs and involve connections in the default mode and sensorimotor networks, and those involved in memory, goal-oriented and emotional responses (Stoodley, 2014). In a longitudinal study, toddlers diagnosed with ASD exhibited abnormal cortical growth rate and enlarged grey and white matter, with females more strongly affected than males (Schumann *et al.*, 2010).

Furthermore, reduction in grey matter was found in ASD patients in the hypothalamic region involved in neuropeptide production (Kurth *et al.*, 2011), including that of oxytocin – a target of interest in ASD, social and general anxiety research, among others (Bales *et al.*, 2014; Neumann & Slattery, 2016). Pathomorphological manifestations in ASD further comprise overall brain connectivity disorder, decreased HPC dendritic arborisation, and an increase of immature spines in pyramidal neurons linked to cognitive deficits (Hutsler & Zhang, 2010; Raymond *et al.*, 1995; Rippon *et al.*, 2007).

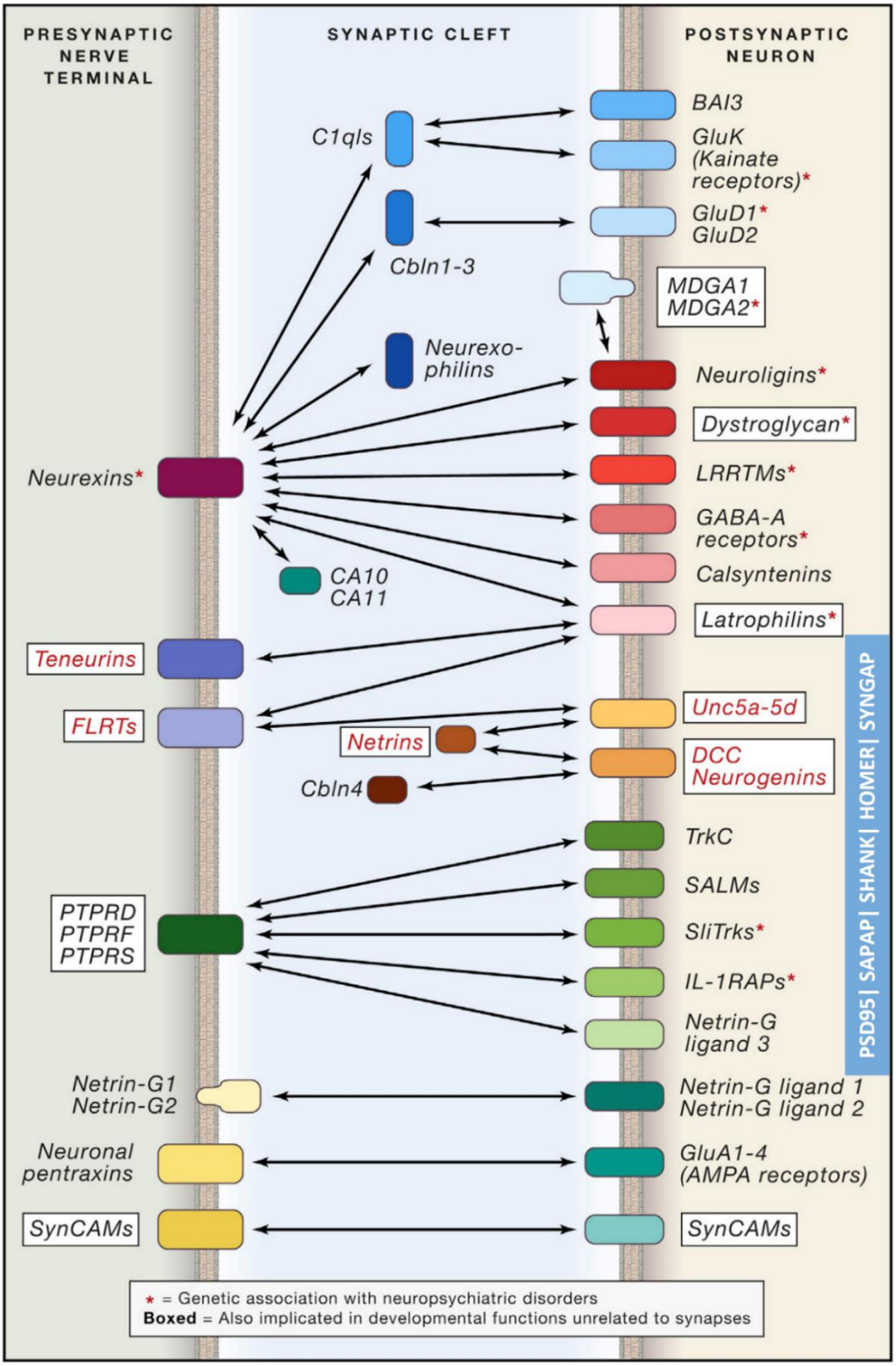


Figure 1.3 Schematic representation of putative synaptic connection molecules, enriched in neuropsychiatric disorder- and developmental genes. Red text indicates uncertainty about pre- vs. postsynaptic protein localisation. Figure from Südhof (2017), re-used with publisher's permission (*licence 5745900584680*); see reference, Chapter 1.3.1, 1.3.2 and Figure 1.10 for more details. The blue box contains selected scaffolding proteins of the postsynaptic density (PSD), also prominently implicated in brain development and dysfunction.

While animal models recapitulating all aspects of NPDs are largely unobtainable, a shift toward specific endophenotypes of psychiatric disorders can offer insights into aetiology and novel treatment options (Gururajan *et al.*, 2019). Despite the difficulty and care needed when aiming to replicate ASD-like phenotypes in rodents (Silverman *et al.*, 2022), core symptoms of this and related NDDs and comorbidities have been observed in translational genetic models (i.e. *Shank2/3*, *Nrxn1/2*, *Nlgn3/4*, *Syngap1, Fmr1* (*Fragile X messenger ribonucleoprotein 1*), knockouts among others; see also Chapter 1.3.1.1) informed by genomic findings in patients (Armstrong *et al.*, 2020; Jiang *et al.*, 2022; Jung & Park, 2022; Lee *et al.*, 2015; Nakajima *et al.*, 2019). Indeed, synaptic transmission and development, and the presynaptic membrane are among the terms enriched in ASD-associated rare CNVs, and respective mouse-model phenotypic enrichment reinforces the overlap of risk variants with ASD-linked behaviours and brain functions (Gai *et al.*, 2012). Deficits along the cortical, basal ganglia (including dorsal striatum, nucleus accumbens, substantia nigra), and thalamus axis can be observed in ASD patient and rodent models alike (Kim *et al.*, 2016a; Woodward *et al.*, 2017); see also Figure 1.2E. Together with the amygdala and HPC, these brain regions are involved in aberrant stereotypic and social behaviours, based on glutamatergic, dopaminergic, GABAergic, and serotonergic pathway dysfunction (Kim *et al.*, 2016a; Muller *et al.*, 2016; Woodward *et al.*, 2017); see Figure 1.2A-D. Furthermore, models utilising gestational exposure to the anti-epileptic valproic acid, pesticides, maternal infection and stress (see also Figure 1.6), given the aforementioned risk factors, have shed more light into environmental risk factors for ASD (Saxena *et al.*, 2020).

ATTENTION-DEFICIT HYPERACTIVITY DISORDER (ADHD)

ADHD is estimated by large-scale, long-term studies to have a worldwide prevalence of between 1.13% (lifelong) and 5.9 and 2.5% for youths and adults, respectively (Cortese *et al.*, 2023; Faraone *et al.*, 2021). The age of onset can vary but mostly coincides with the preadolescent period, i.e. 7-12 years of age, while symptoms can occur earlier and ADHD diagnoses might be received later in life (Franke *et al.*, 2018). ADHD is a very heterogeneous disorder but the core clinical presentation includes hyperactive and impulsive behaviours, especially in children (although symptom severity might decrease throughout development), and inattentiveness that can lead to academic and occupational problems, more commonly observed in adults (Dopfner *et al.*, 2015; Faraone *et al.*, 2021; Grimm *et al.*, 2020).

ADHD persists in two thirds of childhood cases throughout the lifetime with symptom severity, treatment, and comorbidities like conduct disorder (CD) and MDD as predicting factors (Caye *et al.*, 2016; Grimm *et al.*, 2020). Although there is some discussion if adult ADHD represents a separate disorder with its own aetiology (Moffitt *et al.*, 2015), many have suggested that these adult cases might have subthreshold ADHD presentation or other comorbidities masking the NPD during childhood, and that optimal early-life language and intellectual development might delay while trauma later in life might exasperate symptoms, leading to later diagnosis (Franke *et al.*, 2018; Nigg *et al.*, 2020).

ADHD is more commonly diagnosed in males (2:1 male-to-female ratio overall), and is up to seven times more likely to occur in boys for the hyperactive-impulsive disorder subtype, however, related symptoms tend to even out by late adolescence (Faraone *et al.*, 2021; Franke *et al.*, 2018). Additionally, boys with ADHD appear to have more trouble with cognitive flexibility and response inhibition than girls with the disorder, while both sexes present similar other executive function deficits (Loyer Carbonneau *et al.*, 2021). Executive function is the higher-order cognitive ability for reacting to stimuli in a goal-oriented manner, which includes attention, response inhibition, working memory, cognitive flexibility, i.e. changing behavioural responses with rule changes, and planning, among others, and is mediated by the PFC (Logue & Gould, 2014). Early adolescence is a vulnerable period for symptom increases in both sexes (see also Figure 1.6), however, girls exhibit those increases later than their male counterparts, which might delay or obstruct an ADHD diagnosis, leading to undercounting of females with ADHD (Murray *et al.*, 2019).

Brain developmental changes like cortical thickness reduction – opposite to ASD patients, which have thicker cortices (see last section) - have been observed in ADHD (Friedman & Rapoport, 2015). Interestingly, remittent patients, i.e. those that do not meet criteria for the disorder by early adulthood, show slowed down maturation-linked thinning of the cortex, so that they match neurotypical controls by the end of adolescence (Friedman & Rapoport, 2015). Furthermore, enlarged HPC - as observed in ASD patients as well - which was associated with decreased severity of symptoms and suggestive of compensatory mechanisms, and impaired amygdala-to-PFC connectivity have been found in ADHD patients (Plessen *et al.*, 2006), implicating the limbic system in the NPD (see Figure 1.2E). Striatal volume loss has been observed in multiple studies of ADHD patients (Ellison-Wright *et al.*, 2008; Friedman & Rapoport, 2015), similar to ASD patients, in addition to decreases in the thalamic volume and PFC surface area (Batty *et al.*, 2015). ADHD-relevant cerebellar reduction

in children and adolescents has been implicated in disorder aetiology and is ameliorated by systemic stimulant treatment (Ivanov *et al.*, 2014). Overall, multiple brain regions involving the dopaminergic, serotoninergic, GABAergic, and glutamatergic (see Figure 1.2A-D), norepinephrinergic and acetylcholinergic systems have been implicated in ADHD deficits in attention, working memory, response precision and inhibition, and cognitive flexibility (da Silva *et al.*, 2023; Mueller *et al.*, 2017).

Like ASD, ADHD has a strong genetic component - 80% heritability, of which over a quarter is based on SNPs, and rare CNVs involving synaptic cytoskeleton, mRNA lifecycle, GABAergic and metabotropic GluR genes have been significantly associated with the disorder (Grimm *et al.*, 2020; Harich *et al.*, 2020). Overall, large-scale studies have highlighted a polygenic risk score, i.e. small but combinatory effects of single genes, in ADHD (Faraone *et al.*, 2021).

Importantly, among ADHD risk genes, those involved in neuro- and synaptogenesis, synaptic transmission and transmembrane signalling (see Figure 1.3; incl. presynaptic vesicle-, dopaminergic- and noradrenergic-related genes), axon guidance and neurite outgrowth, and nervous system (NS) development are enriched (Bonvicini *et al.*, 2018; Dark *et al.*, 2018; Franke *et al.*, 2018). Indeed, in a polygenic mouse model of ADHD, long-term- and selectively-bred for home cage hyperactivity (Majdak *et al.*, 2016), striatal genes involved in synaptic structure and function were strongly affected, including downregulation of latrophilin 3 (*Lphn3*; see Figure 1.3), which has been associated with ADHD in human studies (Sorokina *et al.*, 2018; Südhof, 2017). Apart from *LPHN3*, genome-wide analyses have highlighted *DIRAS2* (*distinct subgroup of the RAS family member 2*) as a candidate gene in children and adults with ADHD (Bonvicini *et al.*, 2018); see Chapter 1.3.4 for more details. Transgenic mouse models usually target genes involved in neuropeptide signalling, i.e. disrupting transporters or receptors of dopamine, thyroid hormone, neurokinin, among others (Cabana-Dominguez *et al.*, 2023).

Environmental risk factors in ADHD are numerous and encompass pre- and perinatal infection (see also Figure 1.6), exposure to toxic substances both before and after birth, malnutrition, maternal and early-life stress, trauma and adversity, social deprivation, and socioeconomic status – the latter a significant predictor of prevalence, symptoms and impairment later in life, and very akin to ASD (Bock & Braun, 2011; Cheung *et al.*, 2015; Faraone *et al.*, 2021; Rowland *et al.*, 2018). Perinatal stress and stress during vulnerable developmental periods (see Figure 1.6 for respective mouse and human timelines), specifically, has wide-ranging neurobiological implications, affecting brain development,

synaptic density, structure, connectivity, and signalling, e.g. by monoaminergic NTs (Bock & Braun, 2011). Importantly, susceptibility to ADHD is often increased by genes × environment interactions, wherein genetic variation and neurobiological/epigenetic changes caused by e.g., substance use or stress during pregnancy, parental care, psychosocial adversity, exert combinatorial effects, especially on the developing brain and synaptic function (Franke *et al.*, 2018; Todd & Neuman, 2007).

SCHIZOPHRENIA (SCZ)

SCZ is an NPD that affects over 23 million people worldwide, with lifetime prevalence of 0.3-0.4% and has the highest burden of disease in the 30-50 years range (GBD 2019 Mental Disorders Collaborators, 2022; Saha *et al.*, 2005); see Figure 1.1. SCZ encompasses multiple symptomatic domains, i.e. positive (*hallucinations, delusions, disorganised behaviour*), negative (*emotional and social deficits, amotivation and anhedonia*), and cognitive (*executive function, attention, memory, communication, goal-oriented and social cognition*) symptoms (Habtewold *et al.*, 2020; McCutcheon *et al.*, 2020b). There is some uncertainty surrounding difference in the male *vs* female incidence but most sources estimate this at 1.1-1.4; however, males appear to present with earlier SCZ onset, with the median diagnosis age of 20-25 years across genders (Aleman *et al.*, 2003; GBD 2019 Mental Disorders Collaborators, 2022; Saha *et al.*, 2005; Solmi *et al.*, 2022). Earlier onset of SCZ has been further associated with increased risk of treatment-resistant psychosis, which is observed in almost a third of patients on antipsychotics (Legge *et al.*, 2020). An analysis of multiple longitudinal investigations revealed that predictors of cognitive deficits are female gender and younger age, while positive and negative symptoms of SCZ are more strongly associated with the male gender and older age (Habtewold *et al.*, 2020).

Longitudinal neuroimaging studies in patients with the rare childhood-onset SCZ and young adults at high risk of psychosis have provided evidence of subtle morphological changes (Gogtay *et al.*, 2011). These are linked to aberrant brain development in vulnerable time-windows (see Figure 1.6), similar to ASD and ADHD despite SCZ symptoms presenting later in life, and can be observed even prior to diagnosis in the cortex, thalamus, and limbic subregions (Gogtay *et al.*, 2011), similarly to the other two NPDs described here - (see Figure 1.2E). Adolescent, developmentally relevant factors that increase the risk of SCZ include accelerated pruning and blunted myelination (see also Figure 1.6, Figure 1.7), neuronal maturation- and synapse-disrupting cannabis use (Patel *et al.*, 2021). Functional

deficit-linked morphological and connectivity changes in SCZ patients involve multiple regions, including frontal and temporal cortex, thalamus, and striatum (Fornito *et al.*, 2009; Huang *et al.*, 2019), as observed in the other NPDs. SCZ-increased connectivity has been observed in subregions comprising the basal ganglia and limbic system, while decreases are mostly found in cortical regions (Zhuo *et al.*, 2017). Functional imaging studies have revealed that HPC atrophy, specifically, was significantly correlated with aberrant emotional expression via verbal memory deficits in adolescent-onset SCZ patients, as well as progression toward syndromic psychosis in high-risk individuals (Duan *et al.*, 2021; Provenzano *et al.*, 2020), revealing HPC changes as early predictors of SCZ-related sequalae. The opposite, i.e. enlarged HPC, have been observed in ASD and ADHD (see above), but these differences might also be impacted by the respective age of the subjects.

A plethora of evidence has implicated the dopaminergic system in the disorder (see Figure 1.2C), with presynaptic DA function and DA transporter levels consistently increased in SCZ across brain regions (McCutcheon *et al.*, 2020a). Similarly, *in vivo* imaging and *post-mortem* studies have shown that glutamatergic neurons are morphologically and physiologically affected in SCZ (see Figure 1.3), overall glutamine and glutamate levels increased in region-specific manner, and glutamate transport disrupted (McCutcheon *et al.*, 2020a); see Figure 1.2A. Furthermore, presynaptic enzymes that convert glutamate to GABA and membrane GABA transporters are decreased in SCZ, while genes encoding different subunits of the postsynaptic GABA receptors (see Figure 1.3) are significantly down- (dendritic) or upregulated (axonal) in multiple regions of the SCZ brain, including the PFC and basal ganglia (de Jonge *et al.*, 2017); see Figure 1.2B. Finally, the serotoninergic system (see Figure 1.2D) has also been implicated in SCZ aetiology, similarly to ASD and ADHD (see above): multiple preclinical animal models (see below), *in-vivo*, and *post-mortem* studies have found heterogenous alterations in serotonin levels, transporter and receptor genes (Quednow *et al.*, 2020).

Morphologically, spine reduction in multiple regions like cortex, striatum, and the subiculum (the hippocampal formation subregion connecting HPC and cortex) has been consistently described in *post-mortem* investigations in the brains of SCZ patients (Glantz & Lewis, 2000; Glausier & Lewis, 2013; Moyer *et al.*, 2015; Rosoklija *et al.*, 2000). Furthermore, cortical subregion-specific decreases and HPC increases in dendritic arborisation, as well as plasticity-related microtubule aberrations in the HPC and cortical regions have been observed in SCZ (Moyer *et al.*, 2015).

Heritability of SCZ has been estimated at approx. 80% based on data from twin studies and despite recent advances in genomics, only about one third of the variance has been attributed to specific SNPs and rare variants (Owen *et al.*, 2023). Nonetheless, SCZ-associated candidate genes pre-dating and validated by GWAS results involve monoamine and glutamate receptors and synthesising enzymes, neurodevelopmental and immune signalling cascade molecules like *AKT1* (*AKT serine/threonine kinase*), *BDNF* (*brain derived neurotrophic factor*), *TNF* (*tumor necrosis factor*), *NOTCH4* (*notch growth-factor receptor*), *NRG1* (*neuregulin*), and the synaptic *DISC1* scaffold protein (Farrell *et al.*, 2015). As with ASD and ADHD, RBFOX1 has been implicated in SCZ as well (see Chapters 1.1.2, 1.3.2.3 for more details).

Similar to other NPDs, recent insights have provided more evidence for an early neurodevelopment-mediated genetic and epigenetic risk of SCZ and brain architecture outcomes, in line with historical hypotheses (Birnbaum & Weinberger, 2017; Cheng *et al.*, 2022). Single-cell RNA sequencing data combining cell-type analysis with SCZ GWAS results revealed enrichment of common risk variant gene-sets in HPC and cortical pyramidal cells, GABAergic interneurons, and striatal medium spiny neurons, among others (Skene *et al.*, 2018). Furthermore, these specific cell types were independently associated with SCZ and exhibited shared disorder-linked synaptic gene sets as well as other, distinct molecular pathways, i.e. enrichment of neuronal RNA-binding proteins (RBPs, incl. *RBFOX*, *FMR1*, *CELF* (*CUGBP Elav-like family*); see Chapter 1.3.1.2 for more) and dendritic export genes in HPC pyramidal neurons (Skene *et al.*, 2018).

Many of the environmental risk factors relevant for SCZ mimic those associated with ASD and ADHD (see also Figure 1.6): pre- and perinatal infections and complications, early-life nutritional deficits, childhood trauma, poor social adjustment or isolation, lower socioeconomic status, in addition to urban living environment and migrant status (Habtewold *et al.*, 2020; McCutcheon *et al.*, 2020b; Patel *et al.*, 2021; Saha *et al.*, 2005; Vila-Badia *et al.*, 2021). Poor premorbid social adjustment and IQ, often linked to worse educational opportunities, have even been shown to predict treatment resistance in individuals with psychosis (Legge *et al.*, 2020). Data from longitudinal and cross-sectional studies indicate that some risk factors are associated with all symptom domains of the disorder, while others are more strongly linked to specific presentations: e.g., poor social adjustment and living conditions associated with negative and positive symptoms *vs* obstetric complications - with cognitive symptoms (Habtewold *et al.*, 2020).

Furthermore, SCZ polygenic risk scores are significantly higher in a subset of individuals with perinatal complications (McCutcheon *et al.*, 2020b), indicative of gene-by-environment risk interactions, which have also been investigated in rodent models (Moran *et al.*, 2016). Transgenic animal models have utilised disruptions of many of the NT system-, synapse-, and neurodevelopment-related SCZ candidate genes in search of aetiological insights and novel treatments for the disorder (Ayhan *et al.*, 2011; Spark *et al.*, 2022; Winship *et al.*, 2019). In addition to genetic manipulations, pharmacological interventions and regional lesions, as well as environmental risk-based models like maternal immune activation (see Chapter 1.2), substance use, rearing, social isolation and defeat stress have been implemented to study SCZ in rodents (Barnes *et al.*, 2017; Jones *et al.*, 2011).

1.1.2 Complexity and Comorbidity in NPDs

COMORBID RISKS AND PLEIOTROPY IN NPDS

Psychiatric comorbidities, such as other NPDs and NDDs, affective disorders, substance use disorders (SUDs), as well as sleep dysfunction (Freeman *et al.*, 2020; Shelton & Malow, 2021), have been repeatedly observed in multiple NPDs, including ASD (Joshi *et al.*, 2010), ADHD (Reale *et al.*, 2017), and SCZ (Buckley *et al.*, 2009).

Underscoring the shared risk of NPDs and other major mental disorders, initial large-scale studies from the Psychiatric Genomics Consortium found shared genetic effects on ASD, ADHD, SCZ, BP, and MDD over 10 years ago. For example, coheritability was observed between SCZ and BP, MDD, ASD, and between ADHD and MDD (Cross-Disorder Group of the Psychiatric Genomics Consortium *et al.*, 2013), while SNPs in two genes encoding subunits of the L-type voltage-gated calcium channel involved in NT regulation had significant pleiotropic effects across the investigated disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Subsequently, more overlap was identified in a large-scale genomic analysis, including for genes encoding GABAergic receptor, axon guidance, transmembrane signalling, and potassium channel proteins important for neurotransmission and neuronal development (Wu *et al.*, 2020). Expanding on previous GWAS studies, a meta-analysis of the five major psychiatric disorders described above with the addition of OCD, the NDD Tourette's syndrome (TS), and the eating disorder anorexia nervosa (AN), found high intercorrelations between the disorders (see Figure 1.4), as well as 109 genetic loci associated

with at least two disorders, 23 of which were involved in four or more psychiatric disorders (Lee *et al.*, 2019).

A major risk factor for neuropsychiatric disorders and related traits across multiple disorders, is having a parent diagnosed with a psychiatric disorder (Argent *et al.*, 2020). Risk of NPDs does not only confer on genetic factors, but also in the case of environmental exposures (see Figure 1.6A). For example, smoking in pregnancy, which affects neurodevelopment, has been shown to cause epigenetic changes in the offspring (Joubert *et al.*, 2016), which can underlie multiple brain disorders. Adverse pre- and perinatal events, including illness and maternal stress (see also Chapter 1.1.1), have similarly been associated with multiple NPDs (Pugliese *et al.*, 2019). Indeed, immune cytokines have been linked to development and function of neuronal and synaptic circuits, which can have deleterious effects on the brain leading to NPDs and other NS pathology when highly expressed (Zipp *et al.*, 2023).

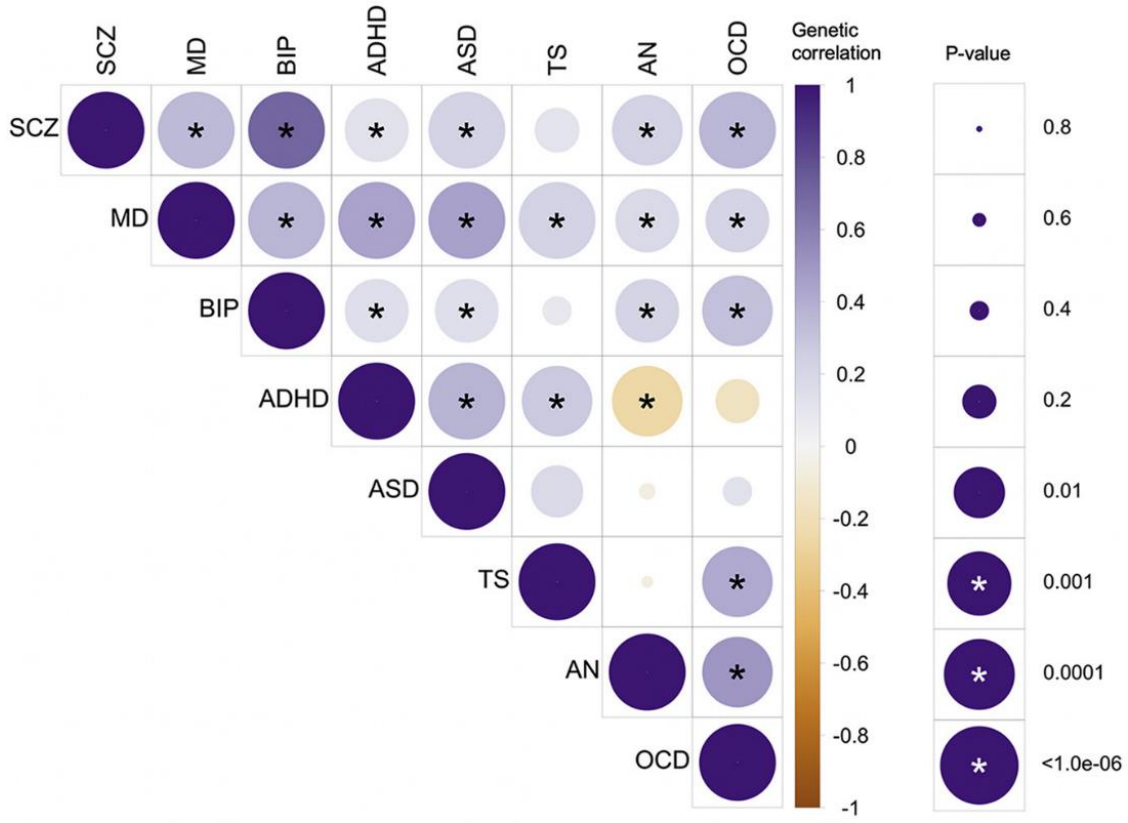


Figure 1.4 Genetic correlation between eight neuropsychiatric disorders, based on meta-analysis of genome-wide association studies. Figure from Lee *et al.* (2019), re-used with publisher's permission (*licence 5745350528144*). Circle size denotes significance (Bonferroni-corrected p-value), darker colour = stronger correlation. *ADHD*, attention-deficit/hyperactivity disorder; *AN*, anorexia nervosa; *ASD*, autism spectrum disorder; *BIP*, bipolar disorder; *MD*, major depression; *OCD*, obsessive-compulsive disorder; *SCZ*, schizophrenia; *TS*, Tourette's syndrome.

NEURODEVELOPMENTAL AND MOLECULAR UNDERPINNINGS

Lee *et al.* (2019) confirmed that brain-enriched pleiotropic risk genes, many of which were mentioned above (see Chapter 1.1.1), are involved in axon guidance and neuronal development (like the netrin 1 receptor, *DCC*), neurotransmission (e.g., glutamate, calcium, and potassium channels), and neuronal post-transcriptional regulation, i.e. *RBFOX1* (second-most significant pleiotropic gene after *DCC*; see Chapter 1.3.2.3). Pleiotropic genes mapped onto risk loci differed from disorder-specific genes in their expression in the brain (high expression during development, especially in the second trimester, and throughout the lifespan), and were enriched in neurons and oligodendrocytes, specifically (Lee *et al.*, 2019); see Figure 1.5A-B. Schork *et al.* (2019) confirmed gene regulation during prenatal neurodevelopment, especially in mid-gestation, as central to NPD risk and added developing cortical glia and interneurons to NPD-relevant cell types. Intriguingly, a persistent, shared neural basis of neuropsychiatric pathology, based on PFC developmental delay leading to executive function deficits has been proposed (Xie *et al.*, 2023).

NPD- and NDD-risk genes have dynamic temporal and regional expression profiles, usually exhibiting the most pronounced expression changes (Li *et al.*, 2018) in the vulnerable early pre- and postnatal periods (see Figure 1.6A-B). Another recent genomic study observed shared rare variant risk between SCZ, and NDDs like ASD, ID, and epilepsy but these genes were often affected by different mutation types, suggesting at a possible mechanism of divergent NPD outcomes despite genetic overlap (Singh *et al.*, 2022). Furthermore, early-life stress differentially affects cell types in NPD-vulnerable periods of development (Rahman & McGowan, 2022), adding to the genetic susceptibility component toward heterogeneity of outcomes (see Figure 1.6).

On a molecular level, NPDs (i.e., ADHD, ASD, SCZ, and X-linked ID) share genes associated with synaptic transmission, inter- and intracellular signalling, metabolic and neural developmental pathways; however, the number of genes involved in each process differs (Cristino *et al.*, 2014). For example, genes associated with synaptic signalling, protein degradation, and G-protein signalling were similar among the NPDs, while other genes were overrepresented in individual disorders, e.g. those involved in vesicle-mediated transport in ADHD, transcription in ASD, and kinase signalling in SCZ (Cristino *et al.*, 2014).

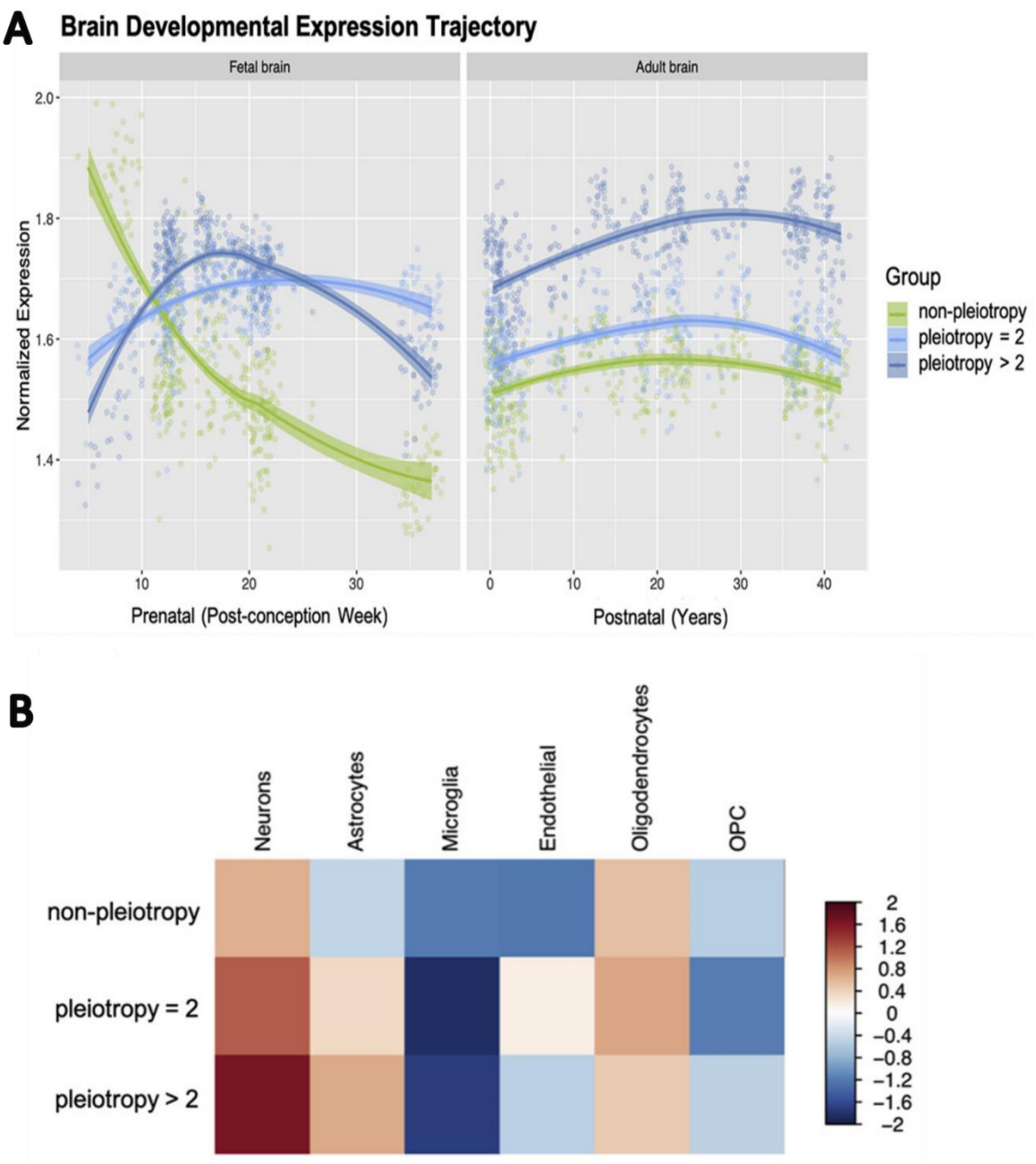


Figure 1.5 Analysis for 146 pleiotropic and disorder-specific risk loci of eight psychiatric disorders. A, brain expression profile of genes mapped onto risk loci, throughout the lifespan: pleiotropic genes were highly expressed in the foetal brain, especially in the second trimester, and those with highest pleiotropic effects were highly abundant in postnatal development as well. **B**, expression in specific neural cell types of pleiotropic genes showed high expression of risk genes, especially those with high pleiotropy, in neurons, oligodendrocytes, and astrocytes. Figure from Lee *et al.* (2019), re-used with publisher's permission (*licence 5745350528144*).

Overlapping cortical gene expression patterns were observed for ASD, SCZ, BP, MDD, and alcohol use disorder, wherein an astrocyte-related gene module was upregulated, while neuronal and mitochondrial modules were downregulated across disorders (Gandal *et al.*,

2018). Shared neurodevelopmental trajectories were also observed on a cortical thickness level for ADHD, ASD, OCD, SCZ, BD, and MDD (see Chapter 1.1.1) and co-expression analyses identified prenatal enrichment for axon guidance genes and a postnatal cluster enriched for synaptic- and plasticity-related genes (Writing Committee for the Attention-Deficit/ Hyperactivity Disorder; Autism Spectrum Disorder; Bipolar Disorder; Major Depressive Disorder; Obsessive-Compulsive Disorder; and Schizophrenia ENIGMA Working Groups, 2021). Indeed, many have implicated aberrant synaptic development, dynamics, and function in neuropsychiatric disorder pathology, positioning the synapse as a central hub for NPD susceptibility (Clifton *et al.*, 2023; Guilmatre *et al.*, 2009; John *et al.*, 2021; Penzes *et al.*, 2011).

The variety of shared and distinct, complex interplays of genetic and environmental factors in NPDs offers an opportunity for multi-dimensional modelling of the disorders in animals and cells, and for better understanding underlying aetiology by incorporating preclinical, multiomic, and clinical insights (Homberg *et al.*, 2016; Khodosevich & Sellgren, 2022).

1.2 Maternal Immune Activation (MIA): A Risk Factor and Preclinical Model

1.2.1 Background

Prenatal immune challenges following maternal infections and stress during pregnancy have long been proposed as important risk factors for neurological, neurodevelopmental, and -psychiatric disorders in the offspring (see Figure 1.6A), and reviewed extensively (Estes & McAllister, 2016; Hall *et al.*, 2023; Han *et al.*, 2021; Scola & Duong, 2017). Epidemiological evidence has confirmed viral infections during gestation as risk factors in multiple NPDs (see Chapter 1.1), specifically, polio and influenza in SCZ (Cheslack-Postava & Brown, 2022; Hall *et al.*, 2023; Mednick *et al.*, 1988; Suvisaari *et al.*, 1999); rubella, herpes simplex, and early/mid-pregnancy or severe generalised infections in ASD (Brown & Derkits, 2010; Casey *et al.*, 2022; Chess *et al.*, 1978; Jiang *et al.*, 2016); fever-inducing, urogenital, and respiratory infections in ADHD (Hall *et al.*, 2022; Walle *et al.*, 2022); influenza in BD (Marangoni *et al.*, 2016; Parboosing *et al.*, 2013), among others.

Recent, large-scale Swedesh cohort-based studies associated severe infections *in utero* with depression as well as ASD (Al-Haddad *et al.*, 2019) and linked maternal impaired immune responses to increased suicidality and psychiatric disorder risk (Isung *et al.*, 2023).

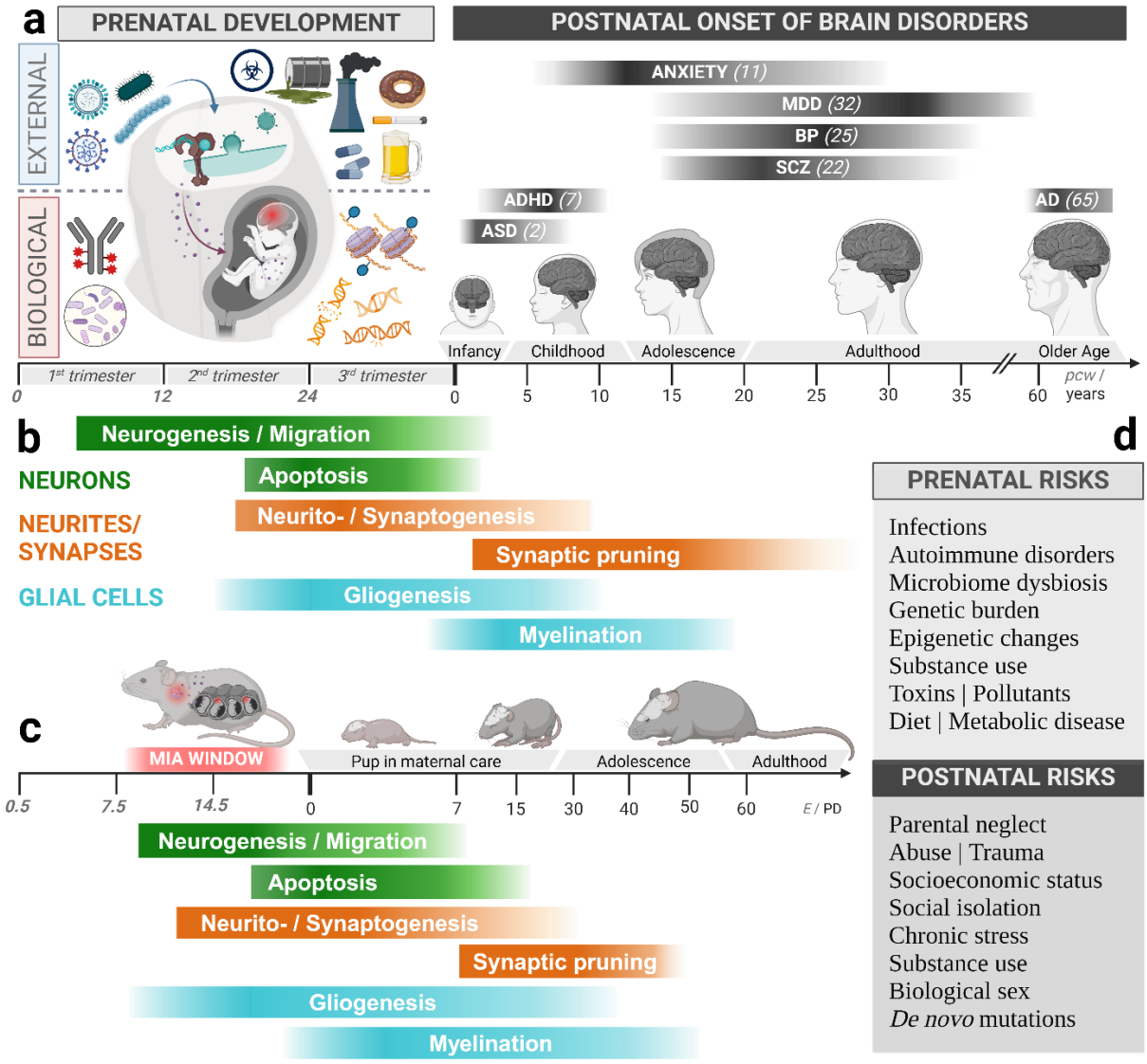


Figure 1.6 Overview of brain development of humans and mice, vulnerable time-windows, NPD onset and risk factors. A, D: External and biological pre- and postnatal risk factors for NPDs, as well as age of onset (see also Chapter 1.1.1). **B:** Timeline of human nervous system development, corresponding to timeline given in panel A. Many processes like neurogenesis and glial-related changes can occur throughout the lifespan, i.e. in the hippocampus. **C:** Mouse development, roughly corresponding to human developmental timeline in A and vulnerable window for maternal immune activation (MIA). Developmental and NPD timelines based on Estes & McAllister (2016), Silbereis *et al.* (2016), Andersen (2003), Marin (2013), Khodosevich & Sellgren (2022), Thion & Garel (2017). AD, Alzheimer's disease; ADHD, attention-deficit/hyperactivity disorder; *ASD*, autism spectrum disorder; *BP*, bipolar disorder; *E*, embryonic day; *MDD*, major depressive disorder; *NPD*, neurodevelop-mental psychiatric disorder; *PCW*, post-conception week; *PD*, postnatal day (*PND elsewhere*); *SCZ*, schizophrenia.

Indeed, autoimmune disorders diagnosed prior to birth in the mother (see Figure 1.6A) were confirmed as developmental delay-, SCZ-, ASD-, and ADHD-associations (adjusted hazard ratios: 1.12-1.35 for different disorders) and provided evidence for increased risk of OCD,

mood and organic mental disorders (adjusted hazard ratios of 1.42, 1.12, and 1.54, respectively) in a Danish population-based study (He *et al.*, 2022).

Interestingly, bioinformatic analyses contrasting GWAS from ASD patients and controls with or without maternal infection during pregnancy indicated that MIA-related ASD might differ in its genetic aetiology from ASD in absence of gestational immune challenge (Nudel *et al.*, 2022), and one of the implicated genes was the RBP *SRRM1* (*serine/arginine repetitive matrix*; see Chapter 1.3.1.1). Unsurprisingly, prenatal neuroinflammation recently gained more prominence as NPD-relevant developmental and social deficits were observed in infants after a maternal SARS-CoV-2 infection during pregnancy (Shook *et al.*, 2022; Wang *et al.*, 2022).

Adding to the compelling findings from human observational studies, a rodent model for studying the effects of prenatal immune challenges on neurobiology, NPD-like behaviours and susceptibility was established over 20 years ago - namely, the maternal immune activation (MIA) model (see Figure 1.6C) utilising both bacterial and viral pathogens (Fatemi *et al.*, 2002; Meyer *et al.*, 2009; Patterson, 2002; Urakubo *et al.*, 2001). Since then, MIA has become a widely used preclinical and translational NPD model of prenatal immune challenges (Cattane *et al.*, 2022), revealing its consequences on embryonic and postnatal brain at pivotal developmental time-points (see Figure 1.6).

MIA leads to elevated maternal expression of pro-inflammatory cytokines such as interleukins (IL) like IL-6, IL-10. IL-17, interferons (INF) and tumour necrosis factors (TNF) – also increased in ASD patients – and can acutely affect the foetal brain by triggering the placental and embryonic inflammatory response (Carter *et al.*, 2022; Hsiao & Patterson, 2011; Jones *et al.*, 2017; Reisinger *et al.*, 2015; Wu *et al.*, 2017b). Indeed, cytokine administration without infection has been shown to induce SCZ- and ASD-like traits in animal models, and cytokines participate in neurodevelopmental processes like neurogenesis and cell fate, as well as regulating synaptic plasticity and function (Bauer *et al.*, 2007; Hsiao & Patterson, 2011; Smith *et al.*, 2007; Wu *et al.*, 2017b; Zipp *et al.*, 2023), with deficits mediated by anti-IL treatment (see Chapter 1.2.4).

Cytokine-induced prenatal immune challenge that leads to neuroinflammation following MIA, might cause latent region-specific brain changes, e.g. in the HPC or frontal cortex, or persistent inflammatory milieu throughout the lifespan (Bergdolt & Dunaevsky, 2019; Garay *et al.*, 2013; Meyer *et al.*, 2011). MIA is often described as a primer, triggering NPD- and psychiatrically-relevant states only after a second adverse event, usually occurring during

childhood or adolescence (Capellan *et al.*, 2023; Estes & McAllister, 2016; Gundacker *et al.*, 2023; Knuesel *et al.*, 2014; Rymut *et al.*, 2020). Understandably, the factors which govern risk versus resilience to neuropsychiatric dysfunction following MIA have also garnered a lot of interest, considering that not all infections during pregnancy lead to NPDs in offspring (Cattane *et al.*, 2020; Estes & McAllister, 2016; Herrero *et al.*, 2022; Massrali *et al.*, 2022; Meyer, 2019; Mueller *et al.*, 2021). One possible hypothesis is the pre- and clinically NPD-implicated neurodevelopmental disruption of neurogenesis, synapse formation and function, among others (see Chapter 1.2.3), which are impacted by MIA and make it an excellent model to study brain disorders.

Crucially, deleterious effects of MIA on offspring are mediated by the timing, pathogen type, dose, and administration procedure, as well as the species or strain of the animal model used in each study (Arsenault *et al.*, 2014; Babri *et al.*, 2014; Ben-Yehuda *et al.*, 2020; Kentner *et al.*, 2019; Meyer, 2014; Morais *et al.*, 2018; Schwartzer *et al.*, 2013; Solek *et al.*, 2018), which is in keeping with the observations in humans described above). The compounds used to induce MIA include attenuated bacteria or bacterial wall particles, i.e. lipopolysaccharides, LPS, viruses like the human influenza H1N1 or the viral mimetic polyinosinic:polycytidylic acid, poly(I:C); or cytokines like IL-6, among others. These can be administered during early-, mid-, or late gestation via different routes and in an acute or repeated fashion in differing dosages, leading to divergent behavioural and morphological phenotypes (Boksa, 2010; Careaga *et al.*, 2017; Guma *et al.*, 2021; Solek *et al.*, 2018). The experimental design heterogeneity is further complicated by basal facility conditions, compound variability, animal strain and vendor suppliers, which impact the findings of each study, thus, diminishing reliability and reproducibility between laboratories (Kentner *et al.*, 2019; Mueller *et al.*, 2018; Mueller *et al.*, 2019). A meta-analysis of 45 studies utilising the poly(I:C) prenatal immune challenge in mice and rats alone showed divergences in cytokine expression in the brain and blood of MIA offspring (Hameete *et al.*, 2021), suggesting degree of persistence of inflammatory processes as one possible mechanism of outcome stratification.

To properly understand and study the NPD-relevant effects of prenatal immune challenges on the offspring, extensive behavioural testing for psychiatric endophenotypes remains crucial, since outcomes differ based on the aforementioned variables. Previous research has elucidated a plethora of behavioural deficits in the MIA animal model, which are discussed below, lending it considerable translational value.

1.2.2 Behavioural Outcomes

Comprehensive reviews of MIA findings show high variability of behavioural outcomes, a consequence of the immense divergence in model type and experimental design, basal conditions, behavioural tests and data analyses (Boksa, 2010; Careaga *et al.*, 2017; Kentner *et al.*, 2019; Meyer, 2014; Patterson, 2009; Reisinger *et al.*, 2015; Solek *et al.*, 2018). The following section will focus on behavioural effects of poly(I:C)-induced MIA in mice (see also Table 1 for gestational day GD9-specific outcomes), as this was the design implemented in the current studies.

When comparing the two most common MIA models – poly(I:C) and LPS – compelling, relatively consistent differences become apparent: while the LPS-induced maternal immune response is more severe with an increased pup mortality rate, poly(I:C) causes more pronounced NPD-like deficits in surviving offspring (Arsenault *et al.*, 2014; Meyer, 2014). The variability in results when comparing early-, mid-, and late-prenatal, viral-like immune challenge might stem from putative differences in maternal cytokine expression following MIA induction at different pregnancy time-points (Meyer *et al.*, 2006; Meyer *et al.*, 2008).

Furthermore, different gestational period-dependent MIA inductions lead to different outcomes based on divergent NPD- and insult-vulnerable windows of brain development (see Figure 1.6). Murine gestational day GD9, which was utilised in this study, corresponds to late first trimester in human brain development when initial neuro- and gliogenesis occurs, while GD15-17 coincides with mid-pregnancy in humans - a period of heightened neuronal migration, neurite outgrowth, and programmed cell death (see Figure 1.6 for references). Depression-like behaviours in offspring, such as increased immobility in the tail suspension or swim tests and decreased sucrose preference in the anhedonia-like test, have been mostly described in offspring after MIA in late gestation and effects seemingly diverge by mouse strains and sex (Babri *et al.*, 2014; Bitanihirwe *et al.*, 2010; da Silveira *et al.*, 2017). Different anxiety-like behavioural phenotypes have also been described for poly(I:C) MIA: systemic administration in mid-to-late gestation had anxiolytic effects, while GD9 acute treatment caused anxiety-like behaviour in adult offspring of treated dams (da Silveira *et al.*, 2017; Meyer *et al.*, 2005; Ozawa *et al.* 2006). In some studies, early viral-like MIA (GD9) caused hyperactivity in offspring of both sexes (da Silveira *et al.*, 2017; Zhu *et al.*, 2014); see also Table 1.

Table 1 Summary of selected behavioural deficits in mouse offspring following viral-like poly(I:C) maternal immune activation (MIA) in early gestation (GD8.5-10.5) and impairments in human neuropsychiatric disorders, corresponding to investigated domains. Based on data from the following – *inbred C57BL6 mice*: 5 mg/kg i.p. - Guma *et al.* (2021); Malkova *et al.* (2012); Ruskin *et al.* (2017); 5 mg/kg i.v. - Meyer and Feldon (2010); Meyer *et al.* (2005); Meyer *et al.* (2006); Meyer *et al.* (2008); Vuillermot *et al.* (2017); 20 mg/kg i.p. - Zhu *et al.* (2014) / *outbred CD1 mice*: 5 mg/kg i.p. - Holloway *et al.* (2013); see references for more details. *Sex annotations*: (/) denotes that only one sex was used in the study, M / F means deficits in both sexes, M+F means deficits in pooled cohort of male (M) and female (F) mice.

Behavioural domain	***MIA-induced deficits***	***Test / Deficit variable***	***Age***	***Sex***	***Domain impaired in psychiatric disorder***
Locomotor activity	Hyperactivity	OF (*spontaneous locomotion*)	adult	M + F	ADHD, SCZ, SUDs
	Increased amphetamine hyperactivity	OF (*systemic amphetamine response*)	adult	M + F	
	Motor deficit	Rotarod (*reduced end speed*)	adult	M (/)	ASD, Psychomotor disorders
Social behaviour	Reduced social preference	Social vs object interaction	adolescent & adult	M (/)	ASD, SCZ, Mood & Panic disorders
	Reduced social approach		adult	M (/)	
	Reduced sociability & social preference	Three-chambre test (*conspecifics*)	adolescent	M (/)	
	Reduced sociability & social preference		adult	M / F M only	
	Impaired social communication	USV (*different contexts*)	infant & adult	M (/)	ASD

Learning & Memory / Cognition	Impaired associative learning	ASST (*compound discrimination stage*)	adult	M / F	ADHD, ASD, SCZ
	Impaired working memory	MWM (*escape latency after 10 min delay*)	adult	M / F	ADHD, ASD, Mood & Panic disorders, OCD, PTSD, SCZ, SUDs
		T-maze alternation (*discrete trial-delay, 40s*)	adult	M (/)	
Anxiety- / Fear-related behaviours	Increased thigmotaxis	OF (*centre entries*)	adult	M + F	ASD, Mood & Panic disorders
	Decreased freezing	FC (*acquisition & CS-cued expression*)	adolescent	M (/)	Anxiety, PTSD
	Impaired latent inhibition	FC (*increased freezing in pre-exposed mice*)	adult	M + F	ADHD, ASD, Mood & Panic disorders, SCZ
Sensorimotor gating / Startle reflex	Decreased pre-pulse inhibition	PPI of ASR	adult	M + F	SCZ, OCD, PTSD
	Diminished habituation of ASR	ASR (*startle amplitude at start vs end*)	adult	M + F	Anxiety, ASD, SCZ
Stereotypic / Repetitive behaviours	Decreased stereotypy	Marble burying	adolescent	M (/)	Anxiety, ASD, OCD, SCZ
	Increased stereotypy		adult	M + F	
	Increased stereotypy	Self-grooming	adult	M + F, M only	

ADHD, attention-deficit/hyperactivity disorder; *ASD*, autism spectrum disorder; *ASR*, acoustic startle reflex; *ASST*, attentional set shifting task; *CS*, conditional stimulus; *FC*, fear conditioning; *MWM*, Morris water maze; *OCD,* obsessive-compulsive disorder; *OF*, open field; *PPI*, pre-pulse inhibition; *PTSD*, post-traumatic stress disorder; *SCZ*, schizophrenia; *SUDs*, substance use disorders; *USV*, ultrasonic vocalisation.

In the same model, NPD-relevant repetitive or stereotypic behaviour often manifested in an age- and sex-specific manner (Guma *et al.*, 2021; Malkova *et al.*, 2012; Ruskin *et al.*, 2017). Putative spatial working memory deficits linked to HPC dysfunction usually follow viral-like immune activation during late gestation (Meyer *et al.*, 2006; Meyer *et al.*, 2008; Richetto *et al.*, 2014). However, aberrant spatial memory has also been observed in offspring of early-gestation, poly(I:C)-treated dams (see Table 1), e.g. in the Morris water maze and T-maze alteration tests (Holloway *et al.*, 2013; Meyer *et al.*, 2005). Furthermore, GD9 poly(I:C)-evoked MIA led to impaired associative learning in offspring of both sexes, as reported for the compound discrimination stage of the attentional set shifting task - a correlate of Wisconsin card sorting test and based on rule learning (Guma *et al.*, 2021). Deficits in social behaviour and sensorimotor gating are among the most robust phenotypes observed in adult MIA offspring, the former already manifesting in adolescence - regardless of gestational timing of immune challenge and model species (Careaga *et al.*, 2017; Kentner *et al.*, 2019; Solek *et al.*, 2018); see also Table 1.

Nevertheless, not all studies utilising GD9-induced MIA found effects of prenatal immune activation on pre-pulse inhibition or sociability (Kentner *et al.*, 2019). Thus, even reliable measures of NPD-related deficits following prenatal immune challenges can lead to divergent behavioural outcomes. Indeed, validation of the model via maternal response to MIA and NPD-relevant endophenotypes in the offspring prior to in-depth molecular or morphological investigations within our laboratory was part of the studies performed and presented in this dissertation.

1.2.3 Molecular, Neuronal, and Synaptic Alterations

Prenatal immune activation, like other types of early-life environmental stress (Rahman & McGowan, 2022), has been shown to induce gene expression-regulating epigenetic modifications in the mouse brain as it does in humans (see Chapter 1.1), e.g. dysregulation of cortical and hypothalamic DNA methylation (incl. excitatory and inhibitory synaptic genes), HPC histone acetylation, and glial chromatin accessibility, which might present in an age-specific manner (Basil *et al.*, 2014; Hayes *et al.*, 2022; Reisinger *et al.*, 2016; Richetto *et al.*, 2017b). Importantly, epigenetic neural alterations, together with neuroimmune signalling, shape sex differences in neurodevelopment (McCarthy *et al.*, 2017), possibly underlying

observable sex-specific outcomes following MIA. Notably, while females remain less studied than male offspring, as is common across preclinical studies (Becker *et al.*, 2016), recent advances have been made in incorporating both sexes and gaining valuable insights into sex-specific effects in the MIA model (Braun *et al.*, 2019; Coiro & Pollak, 2019; Solek *et al.*, 2018).

HPC dysfunction has been implicated in multiple behavioural aberrations observed in mental disorders, e.g. ASD and SCZ, including social and spatial memory deficits, novelty response and cognitive impairments (Banker *et al.*, 2021; Gomez-Ocadiz *et al.*, 2022; Ito *et al.*, 2010; Lieberman *et al.*, 2018). Neuroinflammation, especially during gestation, has deleterious effects on HPC morphology, neurogenesis, and function (Couch *et al.*, 2021; Dusedau *et al.*, 2021; Guma *et al.*, 2022; Kim *et al.*, 2016b), particularly at the synaptic level (Andoh *et al.*, 2019; Chugh *et al.*, 2013; de Bartolomeis *et al.*, 2022); see also Figure 1.7. Such developmental disruptions play a central role in NPD susceptibility as shown in both human (Guilmatre *et al.*, 2009; Schork *et al.*, 2019; Trubetskoy *et al.*, 2022) and rodent (Andoh *et al.*, 2019; Block *et al.*, 2022; Cizeron *et al.*, 2020; Coiro *et al.*, 2015) studies (for reviews: de Bartolomeis *et al.* (2022); Estes and McAllister (2016); see also Chapter 1.2.1, Figure 1.6). Sizeable effects of MIA on the HPC have been found in transcriptomic studies in adolescent non-human primates, highlighting synaptic signalling and myelination changes (Page *et al.*, 2021).

Recently, transcriptomic and proteomic studies in male and increasingly in female MIA offspring, have begun to elucidate the effects of prenatal immune activation on the brain, upon which this study aims to expand by adding in-depth synaptic proteome investigation. Gestational transcriptional changes following MIA in rodents have been previously reported in whole embryos or embryonic brains, though these studies did not examine (Baines *et al.*, 2020; Garbett *et al.*, 2012; Oskvig *et al.*, 2012) or identify (Tsivion-Visbord *et al.*, 2020) sex differences. These studies revealed general disruptions in neuroprotective function, neuronal development, metabolism and migration, in addition to the expected neuroinflammatory processes (see Figure 1.7). It should be noted that robust sex differences in microglial function have been observed in mice, including higher pro-inflammatory gene expression and increased microglial activity at baseline in males, as well as a sex hormone-independent neuroprotective effect of microglia from females (Villa *et al.*, 2018).

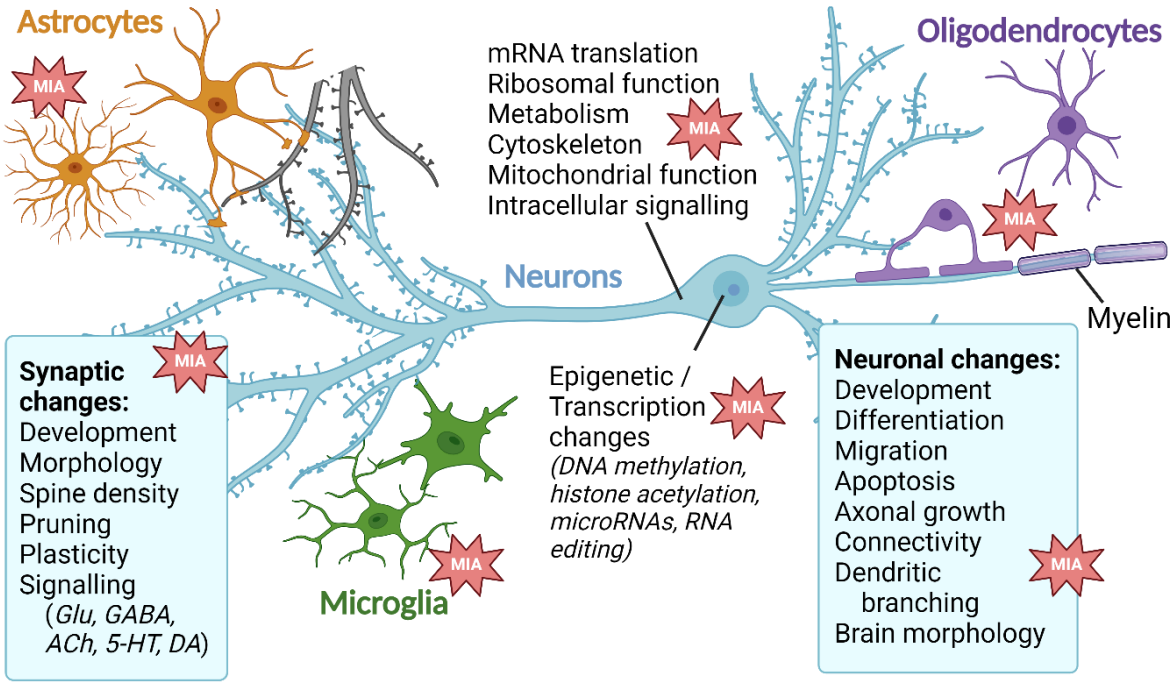


Figure 1.7 An overview of molecular, morphological, and functional changes in the brains of offspring following maternal immune activation (MIA). See Chapter 1.2 for detailed information on MIA effects. *5-HT*, 5-hydroxytryptamine = serotonin; *ACh*, acetylcholine; *DA*, dopamine; *GABA*, gamma-aminobutyric acid; *Glu*, glutamate; *mRNA*, messenger RNA.

Moreover, NT-related measures are altered by MIA in multiple offspring brain regions associated with NPDs (see Figure 1.2) in a partially sex-dependent manner: dopamine (DA) decreases in the striatum, PFC (*metabolites up, some report DA up in males*), and ventral HPC; serotonin (5-HT) increases in the PFC and decreased in the HPC, nucleus accumbens (*males*) and increases in HPC, amygdala, and nucleus accumbens (*female-specific*); decreased glutamate in PFC (*male-specific*) and HPC of both sexes; GABA-A receptor increase in HPC and amygdala, among others (Bitanihirwe *et al.*, 2010; Holloway *et al.*, 2013; Nyffeler *et al.*, 2006; Ozawa *et al.*, 2006; Reisinger *et al.*, 2016; Winter *et al.*, 2009); for a review, see Reisinger *et al.* (2015).

Others have primarily utilised RNA sequencing to probe transcriptomic changes in adult male frontal cortex (Amodeo *et al.*, 2019; Mueller *et al.*, 2021; Richetto *et al.*, 2017a), nucleus accumbens (Richetto *et al.*, 2017a), and amygdala (Mueller *et al.*, 2021; Weber-Stadlbauer *et al.*, 2017) following MIA at different gestational time-points. Neuronal signalling pathway alterations emerged as a common denominator, in addition to changes in myelin and mitochondrial oxidative phosphorylation (see Figure 1.7), among other more subtle region-

specific effects. In more detail, one study combining male and female adolescent mice found increased changes in HPC transcripts after early gestational MIA, disrupting signalling pathways involved in apoptosis, embryo- and synaptogenesis (Guma *et al.*, 2021). A "double-hit" mouse model of MIA and an inflammatory chemokine receptor (*Cx3cr1*) knockout, using transcriptomics in whole adult hippocampi, revealed combinatory sex-specific effects on HPC microglia (hypertrophic in female; microglia increased in density but with blunted reactivity in male), as well as transcriptomic changes in mitochondrial metabolism, apoptosis, and reduced GABA signalling genes in females, specifically (Carrier *et al.*, 2024). Furthermore, poly(I:C)-induced MIA (GD15) has been shown to reduce proliferation of neuronal progenitor cells and negatively affect neuronal maturation, as well as decrease telomere length, typically associated with cellular stress, in murine HPC of adult female offspring (Wolf *et al.*, 2011).

Recent studies implementing single-cell RNA sequencing and chromatin accessibility assays, investigating epigenetic modifications affecting gene transcription, largely focused on the role of microglia and other neuroimmune cells in MIA-linked outcomes, comparing the neuroglial profiles of new-born and adult brains, as well as embryonic cell cultures (Hayes *et al.*, 2022; Matcovitch-Natan *et al.*, 2016). Importantly, Kalish *et al.* (2021) used late-foetal, single-cell transcriptome analyses of cortical tissue to reveal sex-dependent post-transcriptional aberrations after mid-gestational poly(I:C)-evoked MIA (E12.5, 20 mg/kg, i.p.). Expanding upon these major findings, it should be investigated if, and which, perinatal molecular changes persevere into adulthood, which is the crucial question when it comes to the underlying mechanisms of persistent NPDs and, thus, potential treatment targets.

All but three of these molecular studies (*validated in another study*: Garbett *et al.*, 2012; Winter *et al.*, 2009 / *not:* Hayes *et al.*, 2022) incorporated validation of MIA-induced, NPD-like behavioural endophenotypes; however, only two investigations correlated changes in behaviour and gene/protein abundance analyses. Nyffeler *et al.* (2006) revealed a significant correlation in pooled male and female samples between the MIA-affected sensorimotor gating and observed GABA-A receptor subunit abundance in the ventral DG of the HPC of MIA (GD9, 5 mg/kg poly(I:C), i.v.) offspring but not controls, wherein increased protein abundance was associated with decreased PPI. Amodeo *et al.* (2019) reported significant correlations between multiple cortical genes with expression affected by MIA (GD12.5, 20 mg/kg poly(I:C), i.p.) and reversal spatial learning (T-maze) and sociability (mouse vs object

interaction), which were also impaired by the prenatal immune challenge. Among these genes with strong correlations were the glutamate metabotropic receptor *Grm7*, the cell-growth regulating *Rictor* (*RPTOR independent companion of MTOR complex 2*), and multiple ribosomal subunit proteins, which can offer mechanistic explanations of behavioural deficits, i.e. increases in MIA-upregulated *Grm7*/*Rictor* associate with more pronounced deficits, etc. Additionally, correlations between multi-domain behavioural scores (e.g. anxiety-like, sensorimotor gating, social deficits) and functional brain imaging data in mice suggested more pronounced MIA-induced impairments were associated with decreases in HPC volume in offspring, among other regions, especially following early (GD9, poly(I:C) 5 mg/kg, i.p.) MIA compared to a late-gestation (GD17) immune challenge (Guma *et al.*, 2021). These findings suggest that HPC proteomic-behavioural correlation analyses of MIA effects are warranted but lacking to date.

An investigation in male mice found spatial object memory deficits following MIA and concentrated on HPC glutamatergic neurons, providing *in vitro* and *ex vivo* evidence that embryonic HPC synaptogenesis plays a role in MIA-relevant brain aberrations (Mirabella *et al.*, 2021). In this study, the IL-6 administration at GD15 led to increased synaptic punctae density in glutamatergic neurons from embryonic HPC culture, while a similar analysis after GD9.5 poly(I:C)-induced MIA found no effects in glutamatergic primary neurons from adult HPC (Corradini *et al.*, 2018; Mirabella *et al.*, 2021), but the latter did not validate behaviour. A morphological analysis utilising the GD9.5 viral-like (5 mg/kg, i.v.) MIA model and Golgi-Cox staining, found a reduction in spine density of dentate gyrus (DG, see Figure 1.10A) neurons only when combined with *Disc1* mutation in adult transgenic males but not in wild-type murine HPC (Abazyan *et al.*, 2010), in keeping with the lack of MIA-only effects on locomotor, anxiety- and depression-like behaviours, and social preference deficits. Using the same MIA model and staining methodology, an increase in spine density in cortical layer III pyramidal neurons was observed in adult offspring, in addition to decreases in synaptic vesicle and GABA-synthesising enzyme markers, combined with anxiety-like behaviour and a social preference deficit (Soumiya *et al.*, 2011). *In vitro* and *in vivo* immunolabelling of somatosensory cortices from juvenile (P17) mouse offspring, on the other hand, demonstrated MIA-induced reduction in spine density, turnover, and aberrant presynaptic inputs, which led to excitatory (decreased frequency) and inhibitory (increased amplitude)

synaptic signalling dysfunction (Coiro *et al.*, 2015). Decreased cortical spine density in MIA offspring persisted into adulthood and density was, indeed, inversely correlated with stereotypic behaviour in the form of increased marble burying, suggesting functional effects of the enduring morphological changes (Coiro *et al.*, 2015).

Two lower-dose poly(I:C) administrations on GD12.5 and GD17.5 (3 mg/kg, i.p.) caused social and repetitive behavioural deficits as well as lasting increases in density of DG-to-CA3 synapses (see Figure 1.10A) and presynaptic surface area in the HPC of male offspring starting in early adolescence (Andoh *et al.*, 2019) and indicative of aberrant synaptic pruning (see Figure 1.6C). Adding to the complexity of synapse morphology aberrations, LPS-evoked MIA in late gestation has been shown to increase the spine number in HPC granule cells of male offspring – but not of HPC pyramidal neurons or in female HPC, combined with female-specific repetitive behaviours as well as sex-independent social deficits and stereotypy (Fernandez de Cossio *et al.*, 2017).

Thus, comparisons across studies remain difficult due to divergences in MIA timepoints, doses, and immunostimulants used, so more complete investigations encompassing molecular, behavioural, and morphological effects within the same laboratory are needed.

1.2.4 MIA Interactions and Interventions

INTERACTIONS OF MIA, ENVIRONMENTAL AND GENETIC FACTORS

Increasingly, investigations of MIA have addressed the interplay with the mother and offspring microbiome (see Figure 1.6A) - an emerging neuroscientific field of gut-brain axis crosstalk, connecting prenatal stressors and deficits in the postnatal brain, microbiota, and immune development (Romano-Keeler & Weitkamp, 2015; Rotem-Kohavi *et al.*, 2021). Poly(I:C)-induced MIA was shown to impair the intestinal microbial balance, permeability, and motility in offspring – often in a sex- and strain-specific manner (Hsiao *et al.*, 2013; Juckel *et al.*, 2021; Morais *et al.*, 2018; Tartaglione *et al.*, 2022). Prenatal immune challenge via IL-6 administration in mice led to lasting epigenetic changes in gut epithelium of the offspring, which enhanced their immunity to orally-presented pathogens, specifically, but increased the risk of intestinal inflammation later in life (Lim *et al.*, 2021). Furthermore, viral-like MIA has been shown to regulate the behavioural and brain inflammatory responses to an additional,

early-life viral- (PND60, poly(I:C) in pigs) or bacterial-like (PND9, LPS in mice) infections in a partially sex-specific manner (Carlezon *et al.*, 2019; Rymut *et al.*, 2020).

Other postnatal "second hits" that exasperate the effects of MIA on the offspring comprise maternal care aberrations, e.g. maternal separation, decreased grooming and retrieval of pups, among others (Ben-Yehuda *et al.*, 2020; Gundacker *et al.*, 2023; Schwendener *et al.*, 2009), unpredictable or social peripubescent stress (Capellan *et al.*, 2023; Desbonnet *et al.*, 2022; Giovanoli *et al.*, 2013), and chronic adolescent substance use (Guma *et al.*, 2023), among others. Furthermore, adverse environmental conditions (see Figure 1.6A) modelled in rodents like air pollution during pregnancy (Bilbo *et al.*, 2018; Block *et al.*, 2022), maternal malnutrition and iron deficiency as well as gestational diabetes (Harvey & Boksa, 2014; Marques *et al.*, 2015; Money *et al.*, 2018) have been implicated in worse health outcomes for MIA offspring. Ageing has also been proposed as an additive risk factor, beyond age-related increases of mutation burden (see Figure 1.6A), and MIA was shown to elicit AD-like cognitive and neuropathological profiles in old mice (Krstic *et al.*, 2012).

Studies on interactions of MIA and genetic mutations or depletions, especially of genes involved in synaptic function, have expanded upon combinatorial gene × environment effects on brain and behaviour in relation to NPD susceptibility (see Figure 1.6A; see also Chapter 1.2.1). A study combining GD9 poly(I:C) and forebrain neuron-specific expression of mutant human NPD-relevant *DISC1* (*disrupted in schizophrenia 1*), found increased anxiety- and depression-like behaviours along with attenuated 5-HT signalling and decreased granule cell spine density in adult male offspring, following lifelong, but not postnatal-only mutant expression (Abazyan *et al.*, 2010). The mutation alone did not seem to elicit observable deficits without MIA. A point mutation in the same gene (*Disc1-L100P*$^{+/-}$) led to increased MIA sensitivity, so that only 2.5 mg/kg (low-dose in this context) poly(I:C) offspring survived, and exasperated adult MIA-induced deficits in locomotion, PPI, object memory, and latent inhibition, which were ameliorated by acute, intravenous anti-IL-6 treatment at the time of MIA (Lipina *et al.*, 2013). The mutation alone reduced ASR and PPI, MIA alone affected object preference, and both individually impaired fear-based latent inhibition. A heterozygous mutation of the transmembrane domain of another synaptic SCZ risk gene *Nrg1* (see Chapter 1.1), combined with poly(I:C) treatment on GD9 increased aberrations in social novelty preference and sensorimotor gaiting, present in the adult transgenic mice, but reversed

anxiety-like thigmotactic behaviour in the open field in adult offspring of both sexes (O'Leary *et al.*, 2014), suggesting complex interaction of treatments in NPD domains. Heterozygous deletion of the nicotinic acetylcholine receptor alpha 7 subunit (*Chrna7*), which is elevated in the adult HPC and foetal brains of MIA (poly(I:C), GD12.5) mice, caused anxiety-like and PPI deficits in adolescent offspring of both sexes, not observed after poly(I:C) treatment alone (Wu *et al.*, 2015). The genotype alone increased marble burying and PPI at low pre-pulse levels. In the same MIA model, hemizygous deletion of the JNK pathway gene *Map2k7*, involved in glutamatergic and immune signalling, differentially affected placental and brain cytokine and chemokine expression, implicated in GABAergic interneuron development (Openshaw *et al.*, 2019).

Partial or full deletion of the neurexin family ASD-risk gene *Cntnap2* (*contactin-associated protein-like 2*) caused genotype-specific effects on adult female locomotor activity and sex-independent reduction in sociability in heterozygous and in PPI in hemizygous adolescent MIA (poly(I:C), GD9.5) offspring (Haddad *et al.*, 2023), underscoring the importance of age, sex, and genetic dose on MIA-induced deficits. The full deletion alone caused increased startle response, hyperactivity, and moderate PPI reduction (exasperated by MIA) across ages. Combining the same MIA model with the full knockout of the fractalkine receptor *Cx3cr1* (see also Chapter 1.2.2), which regulates the tripartite synapse's composition and pruning, caused sex-specific changes in adult offspring: MIA females exhibited anxiety-like behaviours and hyperactive microglia with decreased GABAergic HPC signature, while in males the prenatal challenge led to stereotypy, reduced sociability and spatial object memory, as well as blunted microglial response in the HPC (Carrier *et al.*, 2024). The study did not present data on effects of the genotype alone and rather compared sex-matched, poly(I:C)-treated wild-type vs poly(I:C) treated knockout mice only. *SHANK* genes, together with the interacting partners of the HOMER family of scaffolding proteins, are major candidates for ASD and SCZ and affect synaptic neurotransmission and formation (Monteiro & Feng, 2017; Soler *et al.*, 2018); see also Figure 1.3. Poly(I:C) treatment (GD12.5) in mice with full exonic deletion of the postsynaptic scaffolding partner *Shank3* exasperated genotype-alone deficits like increases in grooming and more pronounced social deficits in sociability, social preference and communication in adult males, and led to increases in HPC expression of SHANK3 other

glutamatergic postsynaptic proteins, including SHANK2, HOMER1, and mGluR5 (Atanasova *et al.*, 2023).

Taken together, these findings show that viral-like MIA together with genetic manipulations have differential impact on neuronal and synaptic structure and function, especially in the HPC, leading to deleterious changes on the developing brain.

DIETARY AND ENVIRONMENTAL INTERVENTIONS IN THE MIA MODEL

Dietary and environmental interventions in the MIA model have been studied extensively (Bauman & Van de Water, 2020). Nutritional supplementation using vitamin D during pregnancy prevented core ASD-like symptoms, such as marble burying, social approach, and fear conditioning deficits in GD9 poly(I:C)-treated male juvenile offspring (Vuillermot *et al.*, 2017). Metallothionein administration after late pregnancy LPS-induced MIA, causing impairment in astrocytes, increased apoptosis, and inflammation in the embryonic (GD18) brains of offspring, was ameliorated by zinc administration at the time of MIA induction (Chua *et al.*, 2012). Metallothioneins are metal-binding proteins in the cell that are involved in oxidative and inflammatory stress, found in neurons and upregulated in reactive astrocytes, and can inhibit neurite outgrowth (Howells *et al.*, 2010). Metallothioneins can have neuroprotective effects and their expression is differentially impacted by neurodegenerative diseases or injury; during human and mouse brain development, expression starts after mid-gestation and protein overabundance have been proposed to deplete cellular zinc in the foetal brain leading to offspring deficits (Chua *et al.*, 2012; Howells *et al.*, 2010; Suzuki *et al.*, 1994). Systemic selenium administration in pregnant dams injected with poly(I:C) on GD17 rescued sociability deficits in females but not social memory deficits in male offspring, underscoring sex-specific effects (Gillespie *et al.*, 2024).

Similarly, a dietary intervention in juveniles, i.e. a three-week post-weaning ketogenic diet, had positive effects on male social and stereotypic behaviours but did not influence female behavioural outcomes (Ruskin *et al.*, 2017). Choline supplementation throughout pregnancy rescued some deficits, such as stereotypic and anxiety-like behaviours in late adolescence, and reduced MIA-upregulated nicotinic acetylcholine receptor levels in foetal brains of mice prenatally challenged with poly(I:C) on GD12.5 (Wu *et al.*, 2015). Maternal probiotic supplementation in the same viral-like model affected the microbiome and prevented the

MIA-induced anxiety- and depressive-like, stereotypic, and socially deficient phenotypes in young adult males in two independent studies (Hsiao *et al.*, 2013; Wang *et al.*, 2019). In keeping, the H1N1 MIA-evoked (GD9.5) gut microbiome dysbiosis and object memory deficits in adult male offspring, which were recapitulated by microbiota transplant from MIA donors in controls, were rescued by a single-dose antibiotic treatment in prepuberty (Saunders *et al.*, 2020).

Voluntary running exercises in female MIA (GD15, poly(I:C)-treated) offspring in late adolescence to early adulthood (PND50-60; see Figure 1.6C) reversed deleterious effects on sensorimotor gating and HPC neurogenesis (Wolf *et al.*, 2011). Importantly, exercise in the postnatal weeks 4-8 reversed deleterious outcomes of a two-dose poly(I:C) prenatal treatment (GD12.5/17.5) on social preference, stereotypic and anxiety-like behaviours, and reduced MIA-caused synaptic transmission deficits and density increases in adult HPC by stimulating microglial pruning in male offspring (Andoh *et al.*, 2019). Similar positive effects of exercise starting in the vulnerable adolescence time-window (PND21; see Figure 1.6C) on anxiety- and depressive-like behaviours and MIA-affected corticosterone and oxytocin plasma levels were observed in adult male offspring of late-pregnancy LPS-treated dams (Rahimi *et al.*, 2020). These findings suggest sex-independent positive effects of exercise in the adolescent-to-adulthood developmental period on prenatal immune challenge-induced deficits.

Important insights on the effects of environmental enrichment (EE) on MIA-evoked deficits in rodents have been won by researchers in the group of Amanda Kentner. Life-long EE in rats prenatally treated with LPS on GD15 ameliorated placental glucocorticoid metabolism and juvenile social deficits, as well as the MIA-linked male-specific HPC upregulation of the stress gene *Fkbp5*, while EE had opposite effect on the juvenile HPC (Nunez Estevez *et al.*, 2020). In the same model, EE in post-pubertal female rats rescued adult social and object memory deficits, while increasing synaptic plasticity and decreasing stress markers in the amygdala (Zhao *et al.*, 2020). In adult mouse offspring of GD12 poly(I:C)-treated dams, EE rescued dysfunctional social and repetitive behaviours in both sexes and sex-specific, social stress-related transcriptional changes in the ventral HPC, ostensibly by improving maternal care (Zhao *et al.*, 2021a). Another study reported no effects of EE on social novelty and object exploration in male GD9, poly(I:C)-treated MIA offspring (Buschert *et al.*, 2016), but used a

different prenatal challenge timing and outbred CD-1 mice for testing, highlighting possible strain and MIA time-point effects. Finally, Zhao *et al.* (2022) demonstrated that in the same model, MIA caused early puberty initiation in both male and female offspring, altered mechanical pain thresholds in an age-dependent manner, and affected hypothalamic stress-gene expression, with many of those aberrations ameliorated by EE.

PHARMACOLOGICAL INTERVENTIONS IN THE MIA MODEL

Multiple pharmacological interventions in the MIA model have targeted microglia (see Figure 1.7). Systemic treatment with antibacterial compound minocycline in adult male MIA offspring (poly(I:C), GD15) reversed impairments in sensorimotor gating, social and object novelty preference, while restoring phagocytic function, HPC microglia abundance and gene expression (Mattei *et al.*, 2017). Similar ameliorative effects on adult behaviour were observed after minocycline treatment of 3–5-week-old MIA (poly(I:C), GD12) offspring of both sexes, including rescue of HPC GABAergic interneuron numbers and function by modulating microglial activation (Xia *et al.*, 2020). Furthermore, the anti-diabetic medication pioglitazone, which reduces neuroinflammation, when administered during PND21-27 in GD18/poly(I:C)-treated rats rescued spatial working memory and anxiety-like deficits, as well as HPC neurogenesis and microglial aberrations in juvenile MIA males (Zhao *et al.*, 2019).

In male rat offspring of poly(I:C)-treated dams (GD15), systemic treatment with the atypical antipsychotic risperidone in adolescence reversed MIA effects on the expression of SCZ-related, myelin- and extracellular signalling pathway-associated proteins in the PFC (Farrelly *et al.*, 2015). In male mouse offspring prenatally challenged with poly(I:C) on GD12, deficits in social behaviour and inhibitory PFC neurotransmission during adolescence were rescued via acute administration of the GABA-A receptor-agonistic sedative clonazepam (Yang *et al.*, 2021). Similarly, optogenetic decrease in neural activity in the primary somatosensory cortex in male offspring of GD12, poly(I:C)-treated pregnant mice reduced NPD-elevant stereotypic and social impairments (Shin Yim *et al.*, 2017). Crucially, treatment with an 11β-hydroxylase (11βHSD) inhibitor metyrapone targeted (GD15/LPS) MIA-induced overproduction of stress-related hormone corticosterone: it reversed MIA-caused placental reduction of 11βHSD2 in males only and thus, had significant sex-dependent effects (Martz *et al.*, 2023), underscoring the importance of including female subjects preclinical and experimental treatment

studies. The anti-hypercorticolism drug successfully rescued MIA-related social deficits throughout the lifespan and GABAergic, glutamatergic, and dopaminergic gene upregulation in the foetal HPC of male offspring (Martz *et al.*, 2023). Conversely, the intervention did not ameliorate the foetal transcriptomic changes and exasperated juvenile and adult behavioural impairments in the female MIA offspring (Martz *et al.*, 2023).

Despite recent efforts to better understand and treat the deleterious effects of a prenatal immune challenge on the brain, many of the affected synapse-enriched genes and, especially, protein-level consequences of MIA remain elusive, in addition to incomplete studies of morphology and sex-specific differences. Thus, this research will attempt to expand on such MIA-influenced targets, allowing for possible future interventions in individuals at high risk for NPD-relevant sequelae following early gestational neuroinflammation.

1.3 Developmental Regulator Genes in Psychiatric Disorders

1.3.1 RNA-binding Proteins (RBPs)

Apart from environmental early-life stressors like MIA, other factors like RNA-binding proteins (RBPs) play a vital role during prenatal brain formation and possible future impairments throughout the lifespan.

1.3.1.1 RBP Diversity and Function

The heterogeneity in structure, manner of binding, and interaction patterns of the >2000 thus far described RBPs is vast and reflects their intricate role in the proper functioning of the cell (Corley *et al.*, 2020; Ottoz & Berchowitz, 2020; Robinow *et al.*, 1988). Utilising novel and diverse methodologies (Gerstberger *et al.*, 2014; Perez-Perri *et al.*, 2018; Smith *et al.*, 2021; Trendel *et al.*, 2019), recent studies offer novel insights into the diversity of RBPs and their physiological functions (Hentze *et al.*, 2018; Van Nostrand *et al.*, 2020).

RBPs can be classified based on the type of RNA they interact with or regulate, including messenger RNA (mRNA)-binding RBPs, both in the nucleus and cytoplasm, which are involved in alternative splicing (see Figure 1.8), editing, and stability of targets (Gerstberger *et al.* (2014); see Figure 1.9). Functionally, these are involved in a myriad of vital intracellular processes, centred around the biogenesis, transport, stabilisation, and degradation of all RNA

types, comprising the post-transcriptional regulation of the gene-to-protein pathway (Gebauer *et al.*, 2021; Gerstberger *et al.*, 2014).

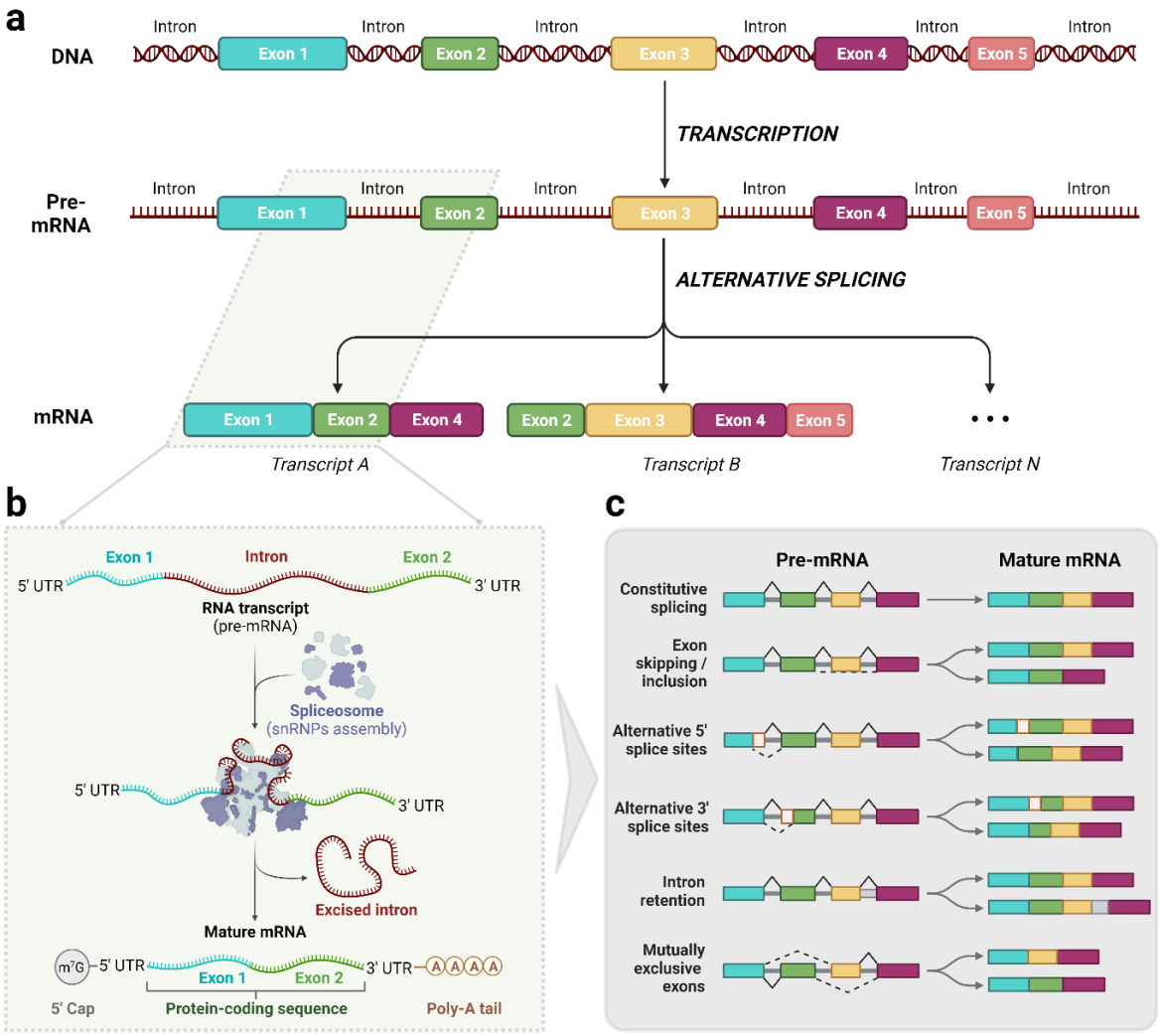


Figure 1.8 Schematic overview of mRNA splicing. At the beginning of the gene expression process, both intronic and exonic DNA information is transcribed into RNA, creating pre-mRNA transcripts (**A**). Subsequently, RNA processing resulting in mature mRNAs occurs: 5′ UTR capping, cleavage and polyadenylation of the 3′ UTR, and splicing (**B**). Splicing usually involves the spliceosome, an RNA-protein complex assembled from snRNPs; however, self-splicing of RNA molecules, as observed for nuclear and cytoplasmatic ribozymes, exists in eukaryotic cells. Similarly, mRNA splicing mostly takes place in the nucleus, yet splicing in the cytoplasm - utilising a separate minor spliceosome pathway and other non-snRNPs - plays its distinct role in cellular function. Alternative splicing is a post-transcriptional modification that can create multiple structurally and functionally different protein isoforms from the same gene via alternative mature mRNA transcripts (**A, C**), regulated in turn by recruitment and modification of different RNA-binding proteins (Fu & Ares, 2014; Glisovic *et al.*, 2008; Marasco & Kornblihtt, 2023). *m^7G*, 7-methylguanylate cap; *mRNA*, messenger RNA; *Poly-A*, adenosine nucleotide chain; *snRNPs*, small nuclear ribonucleoproteins; *UTR*, untranslated region.

This section will focus mainly on the role of RBPs in the context of mRNA regulation and proper protein translation, neuronal, especially HPC, physiology and dysfunction, due to the corresponding functions of the target of this research, RBFOX1 (*RNA binding fox-1 homolog 1*) and the role of HPC in NPDs and lifelong plasticity.

HISTORICAL PERSPECTIVE

In 1961, the RNA synthesis by enzymes that transcribe DNA into a nucleotide chain, mRNA, which serves as the intermediary between the genetic code and the functional translated product, i.e. the protein, was described (Brenner *et al.*, 1961; Gros *et al.*, 1961). By the end of that same decade, the outline of processes and molecules involved in the canonical protein synthesis pathway were identified (Schweet & Heintz, 1966), and links between RNA/protein variability and complex brain functions like learning and memory were emerging (Glassman, 1969). In 1977, alternative splicing (Figure 1.8) was described in eukaryotic cells for the first time, gradually dispelling the prevalent dogma that the expression of one gene always leads to the synthesis of a single protein via one respective RNA sequence (Berget *et al.*, 1977; Chow *et al.*, 1977).

In the 1980s, the spliceosome was discovered - an active protein complex containing small nuclear ribonucleoprotein particles (snRNPs) that can excise introns from precursor mRNAs, re-combine and modify them in a variety of ways during or after transcription (see Figure 1.8), resulting in different RNAs, and thus - multiple protein isoforms (Breitbart *et al.*, 1987; Green, 1986; Padgett *et al.*, 1986). Furthermore, evolutionarily significant RNAs with catalytic functions in and outside of the eukaryotic nucleus, also known as ribozymes, added self-splicing to the diversity of protein machinery outcomes (Altman, 1990; Joyce, 1989; Kruger *et al.*, 1982; Walter & Engelke, 2002). Since then, the minor spliceosome was discovered: a distinct cytoplasmatic, lower-rate functioning counterpart of the nuclear major spliceosome, which cleaves minor-class introns with specific 5′ splice-sites from mRNA in the cytosol, and ostensibly serves an import role during cell proliferation and embryonic development of higher vertebrates (König *et al.*, 2007).

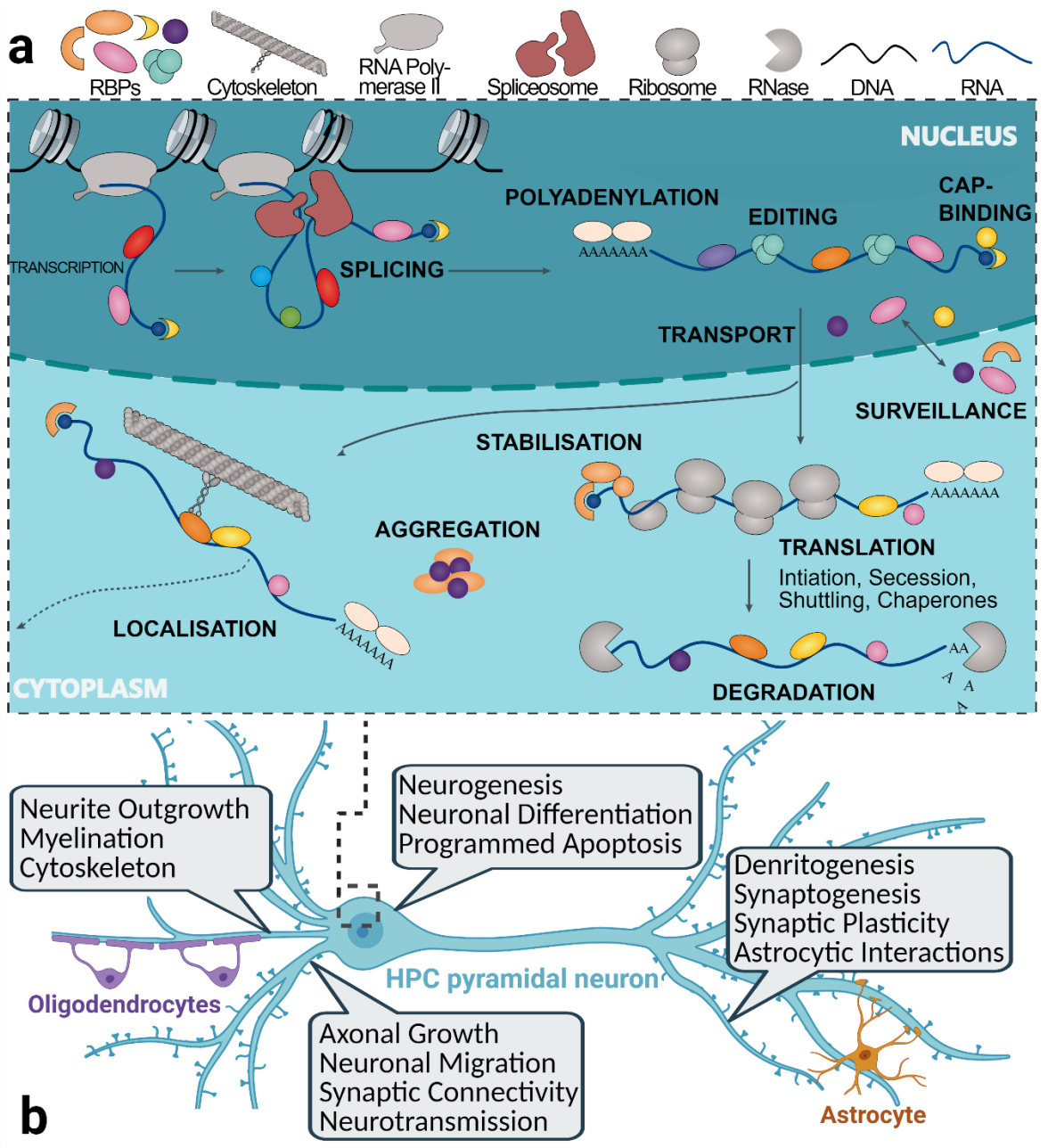


Figure 1.9 Schematic overview of RNA-binding protein (RBP) functions in the neuron. **A**, summary of RBPs' involvement in cellular processes along the protein synthesis pathway. Many post-transcriptional RBP functions, incl. alternative splicing and polyadenylation, mRNA stabilisation and degradation, surveillance, and aggregation can occur both in the nucleus and cytoplasm. Panel A adapted from Gebauer *et al.* (2021) with permission (*publisher's licence 5745540544706*). **B**, Examples of RBPs' role in the development and function of hippocampal (HPC) neurons (*see Chapter 1.3.1 for details*).

ALTERNATIVE SPLICING

Alternative splicing (see Figure 1.8) is guided by the milieu of RNA sequence-mediated splicing signals and splicing regulator elements (*cis*-acting), as well as *trans*-acting splicing factors - enhancers and suppressors, such as serin/arginine-rich (SR) proteins and

heterogeneous nuclear ribonucleoproteins (hnRNPs), respectively (Fu & Ares, 2014; Marasco & Kornblihtt, 2023). Splicing factors can exert opposite effects based on their mRNA-bound position, leading to divergent mature products, while their selective, competitive, and collaborative binding features create complicated interaction and control networks (Fu & Ares, 2014; Marasco & Kornblihtt, 2023).

In neurons, active alternative splicing has been shown in dendrites and synaptoneurosomes (Glanzer *et al.*, 2005), described in more detail in Chapter 1.3.1.3 (see also Figure 1.9, 1.10; Chapter 1.3.2).

OTHER POST-TRANSCRIPTIONAL FUNCTIONS

Other important RBP-regulated processes in eukaryotic cells are cleavage and polyadenylation – alternative modifications of the 3' untranslated region (UTR) or nearby exons and introns (see Figure 1.8), which also influence the diversity of mature mRNA products from the same gene (Zheng & Tian, 2014). These changes often occur in a tissue- and not in a species-specific manner and exhibit distinct patterns in pre- and postnatal mouse development, including in the brain (Ji *et al.*, 2009; Zheng & Tian, 2014). Polyadenylation of pre-mRNA (see Figure 1.8, Figure 1.9), which modulates export, stability, and translation of transcripts, and - like splicing - can occur both in the nucleus and cytoplasm (Glisovic *et al.*, 2008).

RBPs also build complexes with mature mRNAs and play an active role in their transport (see Figure 1.9), i.e. nuclear export and cellular localisation (Kataoka *et al.*, 2000; Müller-McNicoll & Neugebauer, 2013). RBPs further regulate multiple translational processes, including initiation, shuttling of mRNA between active ribosomes, chaperone functions, and importantly – tightly-regulated translational secession (see Figure 1.9), which is vital for proper tissue and embryonic development (Gerstberger *et al.*, 2014; Glisovic *et al.*, 2008). Other RBP subclasses are responsible for stabilising mRNAs across cells and tissues, where they can increase or decrease mRNA stability, which in turn is monitored for degradation by another RBP subset (Masuda *et al.*, 2009; Mukherjee *et al.*, 2011). Indeed, RBPs exhibit substantial involvement in RNA degradation via multiple pathways, including the exosome - an enzymatically active nuclear or cytoplasmatic complex, which exerts quantitative and

qualitative control over mRNA degradation and turnover in the cell (Lykke-Andersen *et al.*, 2009; Statello *et al.*, 2018).

Different types of cytosolic RNA-RBP aggregations, such as RNP granules and amyloid-like structures (see Figure 1.9), constitute mRNA storage or decay reservoirs in physiological and stress conditions, and affect the rate of RBP activity (Garneau *et al.*, 2007; Gebauer *et al.*, 2021; Gerstberger *et al.*, 2014; Kato *et al.*, 2012; Ottoz & Berchowitz, 2020). Post-translational modification of RBPs themselves, such as phosphorylation, methylation, or adenylation, further modulate their localisation, functional behaviour, and efficiency (Corley *et al.*, 2020; Vieira-Vieira *et al.*, 2022). A very important trait of RBPs is that they can auto- (via self-mediated feedback loops) or mutually- (via protein-protein interactions) regulate RBP expression, splicing, and degradation (Fu & Ares, 2014; Ottoz & Berchowitz, 2020; Pervouchine *et al.*, 2019).

The diverse and ubiquitous involvement of RBPs in cellular function and development is consistent with the overrepresentation of RBPs in familial and congenital genetic disorders (Gebauer *et al.*, 2021). The following section will describe important RBPs in the brain and their involvement in developmental and psychiatric aberrations.

1.3.1.2 Role of RBPs in Brain Development, Functionality, and Dysfunction

The existence of a dynamic regulatory framework for proteins throughout brain development, both pre- and postnatally (see Figure 1.11), has been known for many decades (Goertz, 1979; Lerner & Johnson, 1971; McKee *et al.*, 2005; Richter, 1959). In the late 1980s, the role of RBPs in neuronal development, specifically, came into focus through research into the Elav (*embryonic lethal abnormal vision*) protein, which is crucial for lifecycle-dependent neuron maturation and nervous system (NS) formation in the fruit fly (Bandziulis *et al.*, 1989; Robinow *et al.*, 1988; Robinow & White, 1988).

Since then, many more RBPs have been described and implicated in brain development and function, which are too numerous to discuss individually. However, multiple review articles have categorised RBPs based on function, e.g. transport and translational control in neurites (Bramham & Wells, 2007; Dalla Costa *et al.*, 2021), neuronal alternative splicing (Grabowski & Black, 2001; Raj & Blencowe, 2015; Su *et al.*, 2018; Vuong *et al.*, 2016a), and HPC

neurogenesis and synaptic plasticity (Chan *et al.*, 2022; Nishanth & Jha, 2022; Ule & Darnell, 2006). Others have compiled dysfunctions of RBPs in neurological (Bassell & Kelic, 2004; Fernandopulle *et al.*, 2021; Nikom & Zheng, 2023; Nussbacher *et al.*, 2019), neurodevelopmental (Brinegar & Cooper, 2016; Ehninger *et al.*, 2008; Prashad & Gopal, 2021; Schieweck *et al.*, 2021), and psychiatric (Glatt *et al.*, 2011; Klein *et al.*, 2016; Park *et al.*, 2021; Reble *et al.*, 2018) conditions.

This section will highlight a selection of RBPs important for NPDs with a special focus on the HPC due to its role in disordered aetiology and life-long plasticity (see also Chapter 1.2).

FRAGILE X-ASSOCIATED RBPS

The gene encoding the Fragile X messenger ribonucleoprotein (FMRP), *FMR1*, was first described in 1991 and named for its association with the Fragile X syndrome (FXS) - a syndromic NDD (Verkerk *et al.*, 1991). FXS was among the first NDDs to be linked to and molecularly diagnosed by a genetic mutation of the trinucleotide (CGG) repeat expansion type, found in the first exon of *FMR1*, which creates transcripts longer than normal in non-presenting premutation carriers - and longer still in FXS patients (de Vries *et al.*, 1998). ASD is found in up to 30% of FXS patients, and a loss-of-function *FMR1* mutation is observed in a further 3-5% of those diagnosed with ASD, interlinking the two NDD-related conditions and possible dysfunction of synaptic translation (Iossifov *et al.*, 2012; Kelleher & Bear, 2008); see also Figure 1.9B, Figure 1.10C. *FMR1* premutation carriers might also present with ASD, mitochondrial dysfunction, and cumulative RNA toxicity can cause cognitive and psychomotor deficits with age (Hagerman *et al.*, 2010).

Fmr1 abundance in the adult mouse brain is region- and layer-specific, and dynamically expressed throughout development, peaking in the neural tube during mid-gestation at embryonic day E10 (Hinds *et al.*, 1993). In the human HPC, *FMR1* is expressed at robust levels both pre- and postnatally, fluctuating somewhat throughout development and peaking in the first trimester of pregnancy (corresponding to mid-gestation in murine neurodevelopment) and again in periadolescence – both vulnerable time-points for NPDs (see Figure 1.11A, Figure 1.6A).

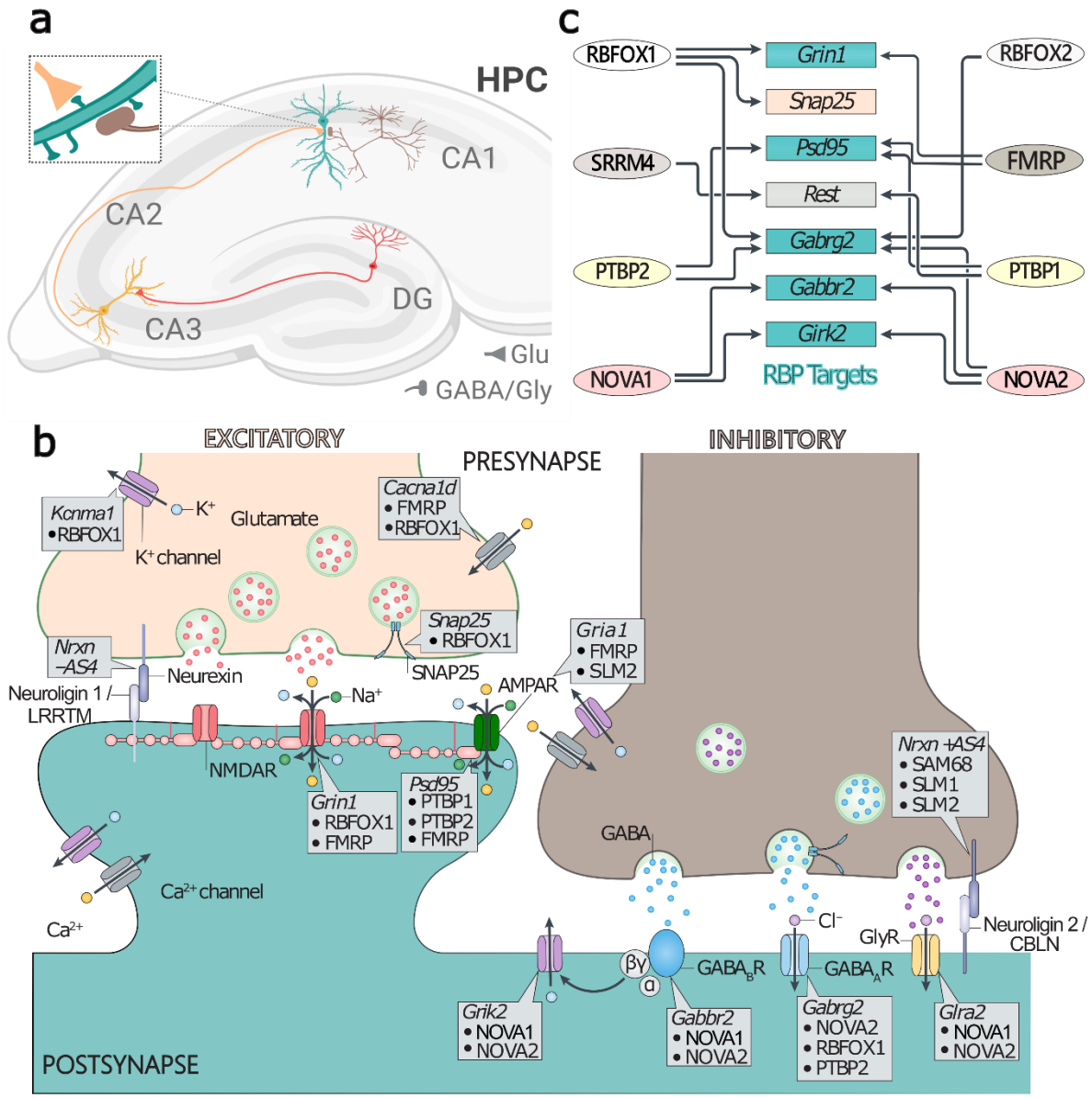


Figure 1.10 Schematic representation of synaptic connections in the hippocampus (HPC) and RNA-binding proteins (RBPs) that influence neurotransmission. A, simplified HPC information flow from granule cells in the dentate gyrus (DG) through pyramidal cells of the cornu ammonis subfield 3 (CA3) to CA1 pyramidal neurons, which also receive inhibitory inputs from local interneurons. **B,** schematic view of selected RBPs and their synaptic targets (*colours as in C*). **C**, excitatory and inhibitory presynaptic inputs on HPC neuron, showing selected RBP regulators of synaptic transmission. *AMPAR*, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptor; *AS4*, alternatively-spliced segment 4; *Cacna1d,* calcium voltage-gated channel subunit alpha 1 D; *FMRP*, Fragile X messenger ribonucleoprotein; *GABA*, gamma-aminobutyric acid; *Gabbr2*, GABA type B receptor subunit 2; *Gabrg2*, GABA type A receptor subunit gamma 2; *Girk2*, G-protein-activated inward rectifier potassium channel Kir 3.2, also known as *Kcnj6*; *Glra2*, glycine receptor alpha 2; *Glu*, glutamate; *GlyR*, glycine receptor; *Gria1*, glutamate ionotropic receptor AMPA type subunit 1; *Grin1*, glutamate ionotropic receptor NMDA type subunit 1; *Kcnma1*, calcium-activated potassium channel subunit alpha 1; *LRRTM*, leucine rich repeat transmembrane neuronal; *CBLN*, cerebellin; *NMDAR*, N-methyl-D-aspartate receptor; *Nrxn*, neurexin; *Psd95*, postsynaptic density protein

95; *PTBP*, polypyrimidine tract binding proteins; *RBFOX1*, RNA-binding protein fox 1 homologue 1; *Rest*, RE1 silencing transcription factor; *Sam68*, SRC-associated in mitosis 68 kDa protein; *SLM*, Sam68-like mammalian protein, also known as *Khdrbs*; *SNAP25*, synaptosomal-associated protein 25; *SRRM4*, serine/arginine repetitive matrix 4. Panels B and C adapted (*see section 1.3.2*) from Vuong *et al.* (2016a) with publisher's permission (*licence 5745540338435*).

FMRP is a multifaceted RBP with thousands of target-RNAs (see Figure 10B-C), vital for e.g. neurite morphology, synaptogenesis, neuronal and astrocytic connectivity, myelination, and synaptic pruning (see Figure 1.9B) in important regions like the HPC and cortex (Richter & Zhao, 2021; Tsai *et al.*, 2012). High-throughput experiments in mouse brain revealed that over 30% and 10% of the RBP's hundreds of targets were postsynaptic and presynaptic (~1/3 of observed presynaptic proteins) transcripts, respectively, and significant overlap between FMRP targets and ASD candidate genes (Darnell *et al.*, 2011). The protein is enriched in the motile processes of the developing HPC and highly associated with the synaptic cytoskeleton and dendritic translational machinery (see Figure 1.9) *in vitro* (Antar *et al.*, 2005). The local production of FMRP in distal dendrites in response to neurotransmitter release, and in dendritic spines during prenatal cortical and HPC development, further strengthens its role in synaptic function (Weiler *et al.*, 1997).

Indeed, loss of *Fmr1* in mice causes aberrations in HPC activity-dependent plasticity by amplifying long-term depression (LTD; see Figure 1.10A,C), specifically via metabotropic glutamatergic receptors, mGluRs (Huber *et al.*, 2002). An RNA binding domain-disrupting mutation of *Fmr1* in a mouse model, which mimicked previous findings in the ASD-like full *Fmr1* knockout (i.e., hyperactivity, increased repetitive behaviours and seizures, and decreased anxiety-like behaviour and startle amplitude), was used to further elucidate the functional role of FMRP (Zang *et al.*, 2009). Zang *et al.* (2009) found that in mutant mice, FMRP lost its RNA-binding and polysome-associative function, and abundance reduction was especially striking at P14 in the NDD-vulnerable, early-life period (see Figure 1.6C). This high expression is also observable in human HPC at twelve months after birth (see Figure 1.11A), indicating conserved functions in infancy.

Notably, high rates of ADHD and related behavioural problems have been reported in children with FXS, especially boys, and an overlap between FXS and anxiety and mood disorders has been described (Hagerman *et al.*, 2010; Lo-Castro *et al.*, 2011; Sullivan *et al.*,

2006). Deficits in prolonged attention, measured in different paradigms of the five-choice serial reaction time task, were recapitulated in a rat model of FXS with a frameshift deletion in its mRNA-binding domain of *Fmr1* in both male and female animals (Golden *et al.*, 2019). Furthermore, a vulnerable cortical expression module, functionally involved in e.g. synaptic signalling and axon guidance (see Figure 1.9B), was enriched for FMRP targets, such as *MTOR*, sodium voltage gated channel *SCN2A*, NMDAR-encoding *GRIN2A*, neurexin 1 (*NRXN1*), as well as known NPC risk genes (Golden *et al.*, 2019). Indeed, alternative splicing of presynaptic neurexins and postsynaptic neuroligins affects glutamatergic signalling (see Figure 1.10C), linked to a myriad of NPDs and comorbidities, including ASD, OCD, SCZ, and ID (Dai *et al.*, 2019; Gomez *et al.*, 2021; Kasem *et al.*, 2018; Oku *et al.*, 2020; Singh & Eroglu, 2013).

Importantly, FMRP dysfunction might play a role in immune responses throughout development: transcriptomic investigations revealed differentially expressed genes in embryonic HPC tissue (E17/18) of *Fmr1* knockout mice, which are involved in multiple inflammatory pathways, such as endocytosis, cytokine receptor interactions, and autoimmune disease (Prilutsky *et al.*, 2015). In line with these findings, a large-scale, phenome-wide association (PheWAS) study in an USA cohort of FXS patients and age-/sex-matched controls, reported a significant increase of infectious disease risk but decrease in risk for a number of autoimmune disorders in FXS, hinting at immune system dysregulation (Yu *et al.*, 2020).

Interestingly, *FMR2*, also known as *AFF2* (*ALF transcription elongation factor 2*) is a transcription activator and a member of another family of RBPs, which is associated with a non-syndromic X-linked ID with a milder phenotype and possibly - NPD comorbidities (Gecz, 2003; Hillman & Gecz, 2001; Melko *et al.*, 2011). Functionally, FMR2 is involved in alternative splicing regulation and in transgenic mouse models, *Fmr2* loss leads to impaired fear conditioning and HPC synaptic strengthening (Gu & Nelson, 2003; Melko *et al.*, 2011). *FMR2* HPC abundance fluctuates in human development, exhibiting expression peaks in vulnerable windows (see Figure 1.6A) – prenatally in each the first and second trimester, in the first year of life, and at the start and end of adolescence (see Figure 1.11A).

FMRP has two autosomal paralogs, FXR1P and FXR2P, which exhibit some redundancy in their polyribosomal translational control, but are not able to compensate for all *FMR1* functions in case of loss (Darnell *et al.*, 2009). Other associated proteins regulate its isoforms' functionality and include the neuronal (NUFIP) and cytoplasmatic (CYFIP) FMRP-interacting proteins, the

latter of which connects to the Rac GTPase pathway and plays a central role in dendritic spine formation (Bardoni *et al.*, 1999; Bonaccorso *et al.*, 2015; De Rubeis *et al.*, 2013; Schenck *et al.*, 2001). FXR1/2P and CYFIP1 exhibit developmental timepoint-specific expression in cortex and cerebellum of mice, decreasing steadily after postnatal P7 (Bonaccorso *et al.*, 2015). Similarly, in the human HPC, *FXR1* and *CYFIP1* are both strongly expressed in early gestation, reduced slightly in mid-pregnancy, with the latter sharply increasing again in the last trimester and greatly reducing its abundance after infancy (see Figure 1.11A). Multiple mutations of the *CYFIP1* gene itself, including chromosomal region deletions and duplications, have been observed in a variety of NPDs, including ASD, SCZ, ID or developmental delays, and obsessive-compulsive behaviour (Abekhoukh & Bardoni, 2014; De Rubeis *et al.*, 2013; Nishimura *et al.*, 2007).

Recent literature reviews of the gene offer further insights into cell-specific effects of FMRP aberrations, modern methods to study its targets and functions (Richter & Zhao, 2021), and FMRP's role in ion channel regulation, channelopathies in FXS, and targeted interventions to restore proper neuronal and synaptic function to ameliorate behavioural deficits (Deng & Klyachko, 2021).

STRESS-RELATED RBPS

FXS-related proteins and other RBP partners have been strongly implicated in the formation of stress granules (see Figure 1.9) in neurites (Akins *et al.*, 2012; Bonaccorso *et al.*, 2015; Darnell *et al.*, 2009), which can locally inhibit translation in HPC dendrites and induce stress-related plasticity changes (Han *et al.*, 2012; Shiina *et al.*, 2005). Another stress granule marker involved in apoptosis, TIA1 (*TIA1 cytotoxic granule associated RNA binding protein*), is highly expressed in the murine embryonic brain, and linked to neurogenesis (Lin *et al.*, 2021; Lowin *et al.*, 1996; Masuda *et al.*, 2009; Monahan *et al.*, 2016).

In mice, it affects fear-related behaviour and memory in a sex-specific manner, i.e. enhanced fear/conditioned odour avoidance and deficits in HPC long-term potentiation (LTP, learning-related synaptic strengthening) in females lacking *Tia1*, ostensibly by activating pro-inflammatory downstream targets involved in HPC synaptic plasticity (Rayman *et al.*, 2019). TIA1 has also been implicated in human neuronal differentiation *in vitro*, in the absence of environmental stressors (Byres *et al.*, 2021). Indeed, *TIA1* is most abundant in the HPC during

pregnancy and in the first year of life (see Figure 1.11B), when programmed perinatal cell death of excessive neurons occurs (see Figure 1.6B). Based on dose- and sex-dependent stress response regulation by TIA1 in mice, underlain by HPC synaptic plasticity-related modulation of fear memory, and a longitudinal human study on *TIA1* common variants, this RBP has been proposed as a possible molecular component of PTSD and anxiety disorder mechanisms (Rayman *et al.*, 2019; Rayman *et al.*, 2020).

Translin (*testis-brain RNA-binding protein*, TSN) is an RBP functionally similar to FMRP that regulates target transport and translation in neurons, especially dendrites, and *Tsn* loss causes multiple sex-specific, NPD-relevant behavioural deficits in a mouse knockout model (Stein *et al.*, 2006). Male mice lacking TSN exhibited spatial learning deficits, reduced startle and increased acoustic pre-pulse inhibition, and decreased HPC 5-HT, while female knockouts had increased fear responses and reduced 5-HT levels in the amygdala (Stein *et al.*, 2006). Furthermore, reduced anxiety-like behaviours and norepinephrine concentration in HPC were observed in both sexes, altogether hinting at sex- and region-specific TSN regulation of monoamine neurotransmitter levels and NPD-like endophenotypes (Stein *et al.*, 2006). Importantly, TSN deletion leads to very pronounced downregulation of GABA-A and glutamate receptor subunits in knockout mouse brains (Chennathukuzhi *et al.*, 2003). Furthermore, a TSN complex might inhibit microRNAs in the murine HPC in an activity-dependent manner, stimulating lasting memory formation through disinhibition of protein kinase signalling after learning (Park *et al.*, 2017a). Loss of this complex causes defects in long-term but not short-term murine HPC object memory in both sexes by impairing learning-related synaptic tagging and LTP in excitatory HPC neurons of mice (Park *et al.*, 2017a). TSN is highly expressed in the human HPC during early pregnancy, at the end of the second trimester, and in periadolescence, hinting at a role during important developmental time-points (see Figure 1.11, Figure 1.6).

NOVA

Among the highly relevant - and the first described - neuron-specific RBPs, are the NOVA proteins, which play a vital role in the development of the nervous system (Buckanovich & Darnell, 1997; Buckanovich *et al.*, 1993; Jensen *et al.*, 2000). In the human HPC, *NOVA1* is expressed at relatively steady levels throughout development, downregulated slightly in late pregnancy and early adolescence (see Figure 1.11B). Conversely, *NOVA2* HPC levels strongly

fluctuate with very high HPC abundance in the first two pregnancy trimesters, infancy, and adolescence, and sharply decreased in the prepubescent HPC (see Figure 1.11B; Figure 1.6). Along with regulation of their own expression, NOVA proteins regulate polyadenylation in the brain (see Figure 1.9A) and alternative splicing of inhibitory receptor subunits, e.g. for neurotransmission via GABA and glycine, causing postnatal lethality in null *Nova1* mice (Dredge *et al.*, 2005; Jensen *et al.*, 2000; Licatalosi *et al.*, 2008). NOVA1 and NOVA2 regulate a large network of target transcripts in murine neurons (see Figure 1.9B, 1.10B-C), and are involved in synaptic function and neurogenesis (Darnell, 2013; Pilaz & Silver, 2015; Ule *et al.*, 2005; Zhang *et al.*, 2010).

NOVA1 haploinsufficiency was previously shown to negatively affect motor inhibition and cognitive development in humans, and *Nova1* deficiency in inhibitory neurons of mice led to increased anxiety and hyperactivity, combined with ribosomal and mitochondrial protein dysregulation (Tajima *et al.*, 2023). Multiple variants of *NOVA2* have been linked to NDD with heterogeneous features, which include ID, ASD traits, ADHD, and psychomotor deficits, underpinned by alternative splicing defects (Mattioli *et al.*, 2020; Scala *et al.*, 2022). In mice, *Nova2* was shown to control the expression of a large number of neurotransmission targets in both cortex and HPC, as summarised in Figure 1.10B-C (Ule *et al.*, 2003; Ule *et al.*, 2005). These were implicated in HPC LTP of the slow inhibitory postsynaptic currents - an inhibition-related LTP that *Nova2* knockout mice lack, suggesting specific long-term changes of synaptic plasticity (Huang *et al.*, 2005). Interestingly, NOVA and RBFOX might work as splicing regulators in a synergistic manner by binding the same mRNA, skipping exons when bound upstream or on target, or including the exon when bound downstream (Raj & Blencowe, 2015; Zhang *et al.*, 2010); see also Figures 8C, Figure 1.10B.

ELAVL

An extensively studied family of RBPs in the brain is ELAVL (*ELAV like RNA binding* proteins), which are also known as Hu antigens in humans (Ince-Dunn *et al.*, 2012). The four ELAVL members are important developmental RBPs, wherein ELAVL2/3/4 (HuB/C/D; also known as neural nELAVL) are expressed in hierarchical tissue- and timepoint-specific manner in neurodevelopment (see also Figure 1.11C), and ELAVL1 is ubiquitous and governs cell fate and stress response (Clayton *et al.*, 1998; Keene, 1999; Mulligan & Bicknell, 2023). ELAVL2 has not been widely studied in rodents due to high mortality rate at weaning following growth

impairments, but has been implicated in memory formation in other vertebrate models (Mulligan & Bicknell, 2023). ELAVL3 upregulation during development promotes cell fate of progenitors of GABAergic inhibitory neurons (see Figure 1.10C), and loss in murine neural stem cells delays neuronal differentiation (Grassi *et al.*, 2018). In keeping, *ELAVL3* abundance in HPC is very high throughout early development and exhibits a rare expression profile of upregulation in mid-pregnancy and strong downregulation in periadolescence (Figure 1.11).

Reduced glutamate synthesis in a mouse knockout model demonstrated that ELAVL3/4 exert control over alternative splicing and transcript stability of glutaminase in neurons (Ince-Dunn *et al.*, 2012). ELAVL1/4 stabilise specific mRNA isoforms of the neurodevelop-mentally and clinically important SYNGAP (*synaptic Ras GTPase activating protein*), leading to impairment in spine maturation and cognitive ability when disrupted (Yokoi *et al.*, 2017). ELAVL4 is localised in dendrites and spines in activity-dependent pattern following NMDAR activation (see Figure 1.10C), implicating it further in synaptic plasticity (Tiruchinapalli *et al.*, 2008). ELAVL4 controls proliferation and differentiation during neuronal development and prenatal downregulation negatively affects dendritic growth in HPC neurons, while causing behavioural changes in adult mice, such as decreased locomotor and anxiety-like behaviour, and impaired spatial memory (Akamatsu *et al.*, 2005; DeBoer *et al.*, 2014). In human HPC, *ELAVL2/4* are expressed in cycles, with high abundance in early pregnancy, end of the second trimester, infancy, periadolesence and early adulthood (see Figure 1.11C), corresponding to NPD-vulnerable windows (Figure 1.6).

RBPs of the nELAVL family exert pleiotropic effects on a multitude of degenerative and psychomotor disorders (Alzheimer's (AD) and Parkinson's (PD) disease, amyotrophic lateral sclerosis, tremor, ataxia), embryogenetic deficits, as well as ASD, SCZ, and related cognitive traits (Mulligan & Bicknell, 2023). These proteins are also relevant in SUDs (Bryant & Yazdani, 2016) and in OCD through upregulation of multiple postsynaptic targets (van de Vondervoort *et al.*, 2016).

PTBP

The PTBP (*polypyrimidine tract binding protein*) paralogs exert tissue-specific, negative control of alternative splicing (see Figure 1.8), resulting in isoform diversity of target proteins (Ashiya & Grabowski, 1997; Modafferi & Black, 1999). The broadly expressed PTBP1, also known as

hnRNPI or PTB, plays a central role in neural progenitor cells during neurogenesis, while the neuron-specific PTBP2 affects maturation and synaptogenesis in development (see Figure 1.9B), especially during the postnatal period (Li *et al.*, 2014a; Licatalosi *et al.*, 2012; Vuong *et al.*, 2016a). This is reflected in their HPC expression profile: while both are highly expressed in early pregnancy, during the last trimester and postnatal periods they have opposite up-/downregulation, with PTBP2 abundance increases during important developmental time-points, i.e. infancy and early adolescence (see Figure 1.11C, Figure 1.6A-B). PTBP1 has been proposed as an antagonistic splicing regulator to SRRM4 and RBFOX proteins (see Figure 1.10B-C), changing its function to exon inclusion or exclusion based on down- or upstream binding, respectively (Raj & Blencowe, 2015). Loss of either PTBP can lead to far-reaching developmental defects and increased perinatal mortality in mice (Vuong *et al.*, 2016a). While they might share similar functions in the forebrain, general neuronal loss of *Ptbp2*, which causes lethality, cannot be rescued by *Ptbp1* and target specificity might be regulated by protein interactions (Vuong *et al.*, 2016b).

Both PTBP paralogs might play a role in addiction through splicing of targets like the dopamine receptor D2 and disruptions in SUD-relevant brain circuits (Bryant & Yazdani, 2016). The two RBPs also regulate the postsynaptic scaffolding protein PSD95 in a maturation stage-dependent manner: *in vitro*, PTBP1 and PTBP2 post-transcriptionally repress PSD95 through alternative splicing and mRNA decay, which is necessary in early development when the RBPs' physiological abundance is high, while their overexpression in mature neurons disrupts PSD95-mediated glutamatergic synapse formation (Zheng *et al.*, 2012). Like many other membrane-associated guanylate kinases in the excitatory synapse, the PSD95 scaffolding protein aids trafficking and stabilisation of NMDA and AMPA receptors, and is strongly implicated in NPDs with mutations associated with SCZ, ASD, and ID (Corley *et al.*, 2020; Zheng *et al.*, 2011). Like ELAVL (Yokoi *et al.*, 2017) and FMRP (Darnell & Klann, 2013), PTBP2 controls the expression of NPD-relevant synaptic targets via splicing and decay regulation (see Figure 1.9A), including the ASD-, ID-, and SCZ-associated SYNGAP1, which plays a role in Ras-GTPase and HPC signalling (see Figure 1.10, Figure 1.16), synaptic strength, and learning (Dawicki-McKenna *et al.*, 2023; De Rubeis *et al.*, 2014; Komiyama *et al.*, 2002; Wu *et al.*, 2022).

CELF

The CELF (*CUGBP Elav-like family*) proteins are RBPs highly expressed the brain, which regulate cell- and tissue-specific alternative splicing, mRNA stability and translation (Dasgupta & Ladd, 2012; Ladd *et al.*, 2001); see also Figure 1.9A. Ladd *et al.* (2001) demonstrated that CELF proteins exhibit abundance profile changes during brain development changes, which appear conserved in embryos across different vertebrate species (Blech-Hermoni *et al.*, 2013), underscoring their neurobiological importance. Indeed, human HPC expression of *CELF2/4/6* is especially high in periods crucial for brain development (see Figure 1.6A-B), such as early- and mid-pregnancy, infancy and before and at the end of adolescence (see Figure 1.11E).

Variants of the *CELF2* gene were described in patients with epilepsy and developmental delays, which might be caused by erroneous cellular localisation signals (Itai *et al.*, 2021). CELF4 and CELF6, which modulate the microtubule-associated protein tau (MAPT) – explaining their relevance for neurodegenerative disorders - as well as respective glutamatergic and serotoninergic neurotransmission-relevant targets, might play a role in addictive behaviours and SUDs (Bryant & Yazdani, 2016; Dasgupta & Ladd, 2012; Wagnon *et al.*, 2012). A rare *CELF4* deletion leads to clinical presentation of NDD phenotypes, comprising ASD, ID, seizures, and behavioural difficulties (Barone *et al.*, 2017; Halgren *et al.*, 2012). Furthermore, CELF4 appears to control translation in prenatal synaptic and neuronal formation in the human and murine neocortex, targeting mRNAs of putative ASD and NDD risk genes (Salamon *et al.*, 2023).

Conditional knockout of *Celf4* in mouse, specific to cortical progenitors and early neurons after E9, caused sex-specific, perinatal synaptic formation deficits (Salamon *et al.*, 2023). GABAergic synapse markers were significantly up- or downregulated in males and females, respectively, while glutamatergic synaptic markers were only upregulated in neonatal male knockouts (Salamon *et al.*, 2023). *Celf6* deletion decreased social communication was observed in mouse pups of both sexes, and behavioural inflexibility was detected in multiple tests in males (Dougherty *et al.*, 2013). In keeping, *CELF6* SNP associations and mutations were found in ASD patients, especially males, underscoring this RBP's gender- and sex-specific involvement in NDD (Dougherty *et al.*, 2013).

CPEB

CPEB (*cytoplasmic polyadenylation element binding;* see Figure 1.9, Figure 1.10) are major regulators of activity-dependent synaptic plasticity and all four known paralogs are found in vertebrate neurons (Darnell & Richter, 2012; Richter, 2007). In the HPC postsynapse, loss of the NMDAR-activated CPEB1 dampens the LTP-inducing effects of growth hormone and its downstream transduction pathway JAK/STAT, involved in learning and memory (Zearfoss *et al.*, 2008a). Furthermore, CPEB1-deficient mice exhibited impairment of HPC-dependent spatial and fear memory extinction, absent other behavioural deficits (Berger-Sweeney *et al.*, 2006). CPEB2 is found primarily in the dendrites of inhibitory and excitatory HPC neurons of juvenile and adult mice, and in juvenile astrocytes, as well as midbrain dopaminergic neurons (Turimella *et al.*, 2015). CPEB3 and CPEB4 are expressed in a region-specific manner, both co-occurring with presynaptic markers and highly enriched in the postsynaptic density of HPC neurons (Huang *et al.*, 2006). Furthermore, CPEB3 regulates the local translation of an AMPA receptor subunit, strengthening the link between CPEB proteins and synaptic plasticity via glutamatergic regulation (Huang *et al.*, 2006). Increased expression of CPEB in human HPC can be generally observed in late first and second trimester of pregnancy, as well as in infancy, early and late adolescence (see Figure 1.11F).

CPEB4 has been proposed to increase NPD risk through shortening of poly(A) tails (see Figure 1.8, Figure 1.9) and downregulation of relevant targets when mis-spliced (Parras *et al.*, 2018). Mice overexpressing a deadenylating *Cpeb4* isoform recapitulated downstream modifications and behavioural, morphological, and electrophysiological ASD phenotypes (Parras *et al.*, 2018). The exon-missing *Cpeb4* isoform is upregulated and targets are downregulated in untreated SCZ patients, and its overexpression mimics correlated PPI, social deficits, and grooming phenotypes (Olla *et al.*, 2023). Dysfunction of another alternative polyadenylating RBP, hnRNPK, which regulates the serotonin transporter 5-HTT, can influence the risk of anxiety and panic disorders (Yoon *et al.*, 2013). HPC hnRNPK is decreased in MDD patients and the corresponding social chronic stress mouse model, which led to parallel changes in ERK pathway targets like PSD95 (Zhuang *et al.*, 2023); see Figure 1.10C.

STAR

The STAR protein family includes RBPs implicated in neural splicing regulation and embryonic brain development, including QKI (*quaking*), SAM68 (*Src-associated in mitosis, 68 kDa*), SLM1 (*Sam68-like mammalian protein*), and SLM2, also known as T-STAR (Ehrmann *et al.*, 2016; Galarneau & Richard, 2009; Wang *et al.*, 2017). QKI paralogs are major regulators of oligodendrocyte differentiation and abundance of SCZ-altered myelin-associated proteins - thus, affecting myelin sheath formation (see Figure 1.9B), together with other RBPs like FMRP (Grabowski & Black, 2001; Martins-de-Souza, 2010; McInnes & Lauriat, 2006; Zearfoss *et al.*, 2008b). QKIs play a role in maturation and cytoskeletal composition of human astrocytes, furthering their involvement in glial function (Radomska *et al.*, 2013; Sakers *et al.*, 2021).

SAM68, similar to its paralogs SLM1 and SLM2, has been shown to produce multiple neurexin isoforms (see Figure 1.10), and if depleted causes motor and synaptic vesicle aberrations in mice (Iijima *et al.*, 2011). Through neuronal activity-dependent alternative splicing, SAM68 affects membranal or cytoplasmatic localisation of targets that can lead to NPD-relevant deficits in excitatory and inhibitory synapses (Darwish *et al.*, 2023). Furthermore, SAM68 splice targets include the cytokine IL1 receptor-associated protein (IL1RAP), which is involved in presynaptic organisation, cellular inflammatory response, and postsynaptic receptor activation (Iijima *et al.*, 2019).

SLM1 and SLM2 have subregion- and interneuron subtype-specific expression in the murine HPC (Iijima *et al.*, 2014). In *Slm2* knockout mice, synaptosomal abundance of an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) subunit was increased, leading to LTP-mediated plasticity deficits in the HPC, in the absence of spatial memory deficits (Ehrmann *et al.*, 2013; Traunmüller *et al.*, 2016). Targeted depletion of SLM2 from HPC somatostatin-type GABAergic interneurons increased synapse numbers and short-term facilitation, leading to impaired object recognition and revealing a vital role of SLM for cell-specific specialisation (Traunmuller *et al.*, 2023). In the human HPC, all three paralogs have different expression profiles (see Figure 1.11G-H) but are highly abundant in neurodevelopmentally-relevant windows (see Figure 1.6A-B), i.e. early/mid-pregnancy, infancy, and periadolescence.

ZINC FINGER AND OTHER SYNAPTIC RBPS

Generally, zinc finger RBPs are central to immune responses of the cell, and some are directly involved in neuronal formation and maturation (Fu & Blackshear, 2017). *ZNF804A* is a major translational and neuronal migration regulator and highly expressed throughout the murine and human embryonic brain (Hess & Glatt, 2014; Squassina *et al.*, 2019; Zhou *et al.*, 2018). Its abundance in the human HPC peaks in the second trimester, followed by upregulations at important postnatal timepoints (see Figure 6B), i.e. in infancy, before and after the adolescent period (see Figure 1.11D). *ZNF804A* has been implicated in cognitive function and brain connectivity, and numerous SNPs have been significantly associated with one, multiple, or all of the five major neuropsychiatric disorders (SCZ, BD, ADHD, ASD, and MDD) in individual or cross-disorder studies across different populations (Hess & Glatt, 2014; Squassina *et al.*, 2019). Interestingly, ZNF804A interacts with other prominent RBPs like RBFOX1 and CELF3/4 to regulate NDD-relevant and synaptic targets (Chapman *et al.*, 2019).

JAKMIP1 (*Janus kinase and microtubule interacting protein*, also known as MARLIN1) was found to associate with and repress the GABA receptor subunits GABBR1 and GABBR2, respectively, in rat HPC neurons (Couve *et al.*, 2004). In ASD patients, a reduction of this inhibitory receptor complex was discovered in cortical areas, relevant for social, emotional, and cognitive processes (Oblak *et al.*, 2010). Significant hypermethylation of *JAKMIP1* was observed in female but not male *post-mortem* cortices of SCZ and BP patients, highlighting gender-specific epigenetic effects in psychosis (Mill *et al.*, 2008). JAKMIP1 is expressed most strongly around the second week of postnatal cortical development in mice, during neurite and synaptic growth (see Figure 1.6C), and associates with FMRP in transport and translational control of ASD-implicated postsynaptic targets (Berg *et al.*, 2015). Similarly, in the human HPC, *JAKMIP1* expression is at its peak in infancy, as well as mid-pregnancy, early and late adolescence, i.e. neurodevelopmentally relevant time-points (see Figure 1.6A-B, Figure 1.11D). *Jakmip1* knockout mice exhibit multiple ASD-related behavioural traits, including motor stereotypies and social impairments, which might stem from disrupted NMDAR-scaffolding-dependent signalling (see Figure 1.10C) in the postnatal period (Berg *et al.*, 2015).

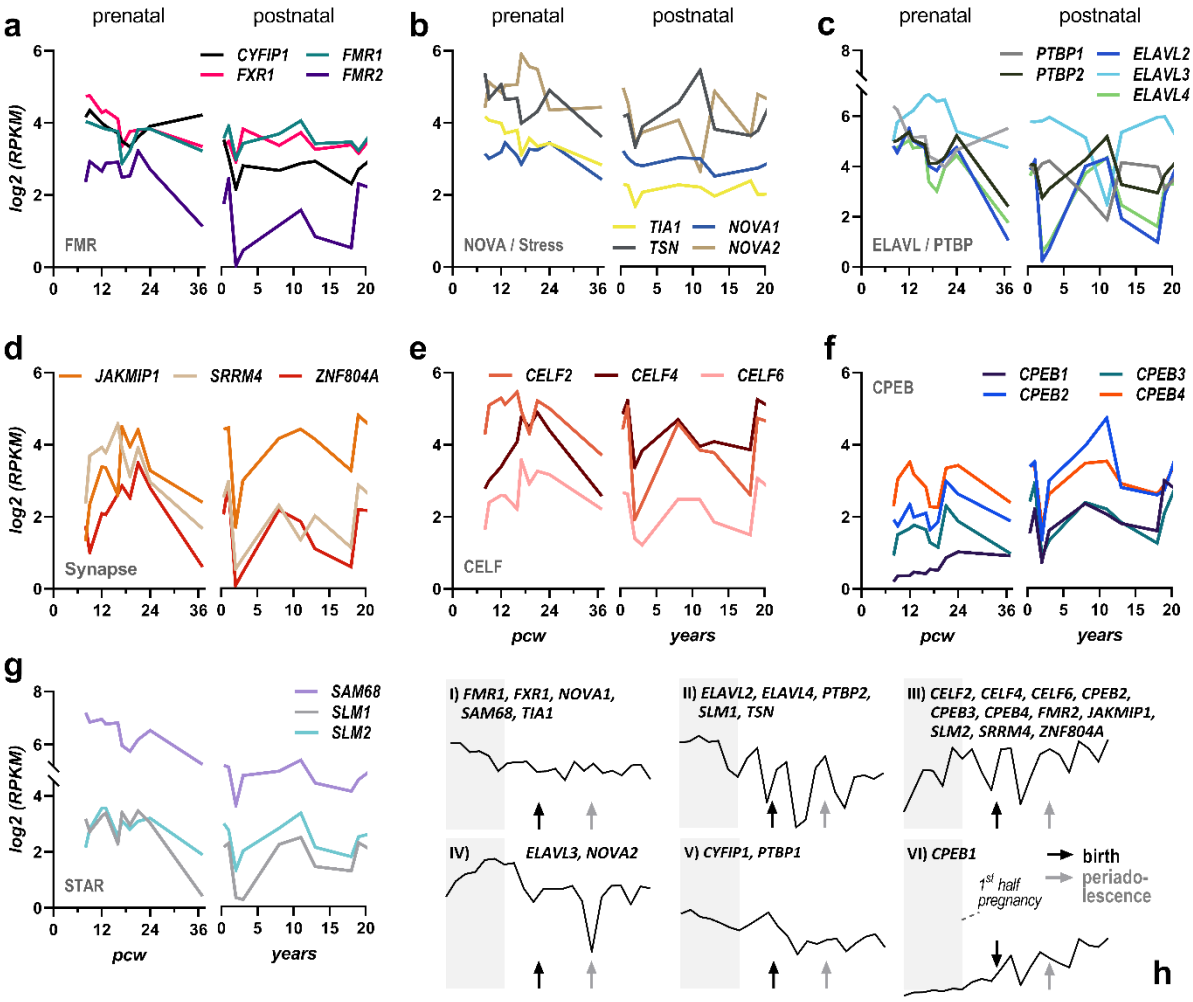


Figure 1.11 Hippocampal expression of RNA-binding proteins (RBPs) with relevance for neuropsychiatric disorders across development. A-G, pre- and postnatal transcriptomic profile for RBPs in human hippocampus (HPC), sorted by protein family or function (*grey text*; *see section 1.3.2 for more on the proteins*). **H,** example expression outlines of RBPs grouped by *transcriptomic profile as relating to vulnerable developmental time-windows (see also Figure 1.6A-B). RNA sequencing* data obtained for HPC from Allen Human Brain Atlas: BrainSpan (Atlas of the Developing Brain; www.brainspan.org/) for all available donors and averaged if multiple data/time-point. *pcw*, weeks post-conception; *RPKM*, reads per kilobase/million mapped reads.

As part of the SR protein family, SRRM (*serine/arginine repetitive matrix*) proteins control alternative splicing events in the brain, implicated in neurodevelopmental processes and dysfunctions (Reble *et al.*, 2018).

Accordingly, HPC upregulation of the neuronal-specific *SRRM4* coincides with NPD-relevant time-windows (see Figure 1.6A-B), such as early gestation, infancy, childhood, early and late adolescence (see Figure 1.11D). SRRM4 is decreased in brains of ASD patients, and reduced abundance in a mouse model leads to synaptic excitatory-inhibitory disbalance and ASD-

reminiscent behaviours in both sexes, such as startle response increase and sensorimotor gating impairment (Quesnel-Vallieres *et al.*, 2016; Reble *et al.*, 2018). Furthermore, male mice revealed more pronounced or sex-specific deficits in overall sociability and social novelty preference, respectively, adding to the causal association between SRRM4 dysfunction and NPD symptoms presentation (Quesnel-Vallieres *et al.*, 2016). Together with SRRM3, SRRM4 might govern the developmental GABAergic switch, which transforms GABA signalling from excitatory to inhibitory, and double-mutation in mice causes impaired splicing of synaptic and cytoskeletal targets in the embryonic (E19) brain, which is lethal (Nakano *et al.*, 2019). Interestingly, early viral-like MIA offspring exhibit a similar delay in the GABAergic excitation-to-inhibition switch in HPC neurons (Corradini *et al.*, 2018). Notably, other NDD-like manifestations of excitation-inhibition disbalance such as epilepsy and seizures have been linked to both RBPs (e.g., RBFOX1, FMRP, CELF4, ELAVL3) and MIA (Corradini *et al.*, 2018; Ince-Dunn *et al.*, 2012; Nussbacher *et al.*, 2015; Tolino *et al.*, 2012).

In summary, RBPs play a vital role in both neurodevelopment and nervous system homeostasis, and their dysfunction can lead to increased risk of NPDs, comorbid psychiatric conditions or other brain disorders. Among RBPs, the RBFOX family of brain-enriched proteins has emerged as a leading research target due to its manifold regulatory function in the brain – both in health and disease – and will be explored in the next chapter.

1.3.2 RBFOX

1.3.2.1 The Multifunctional RBFOX Family

The RBFOX family of RBPs contains three paralogs with highly conserved RNA recognition motifs (RRMs) binding to UGCAUG nucleotide sequences (see Figure 1.12B), which perform vital roles in the development and physiological function of a variety of cells and tissues in arthropods and vertebrates alike (Auweter *et al.*, 2006; Conboy, 2017; Mukherjee & Nongthomba, 2023). RBFOX1 was first described by Shibata *et al.* (2000) and named A2BP1 (*ataxin-2 binding protein 1*) due to its interactions with the protein linked to the movement disorder spinocerebellar ataxia type 2 (see Figure 1.12A). The study revealed the existence of multiple isoforms as well as human tissue- and cell organelle-specificity, including in the neuronal cytoplasm and the skeletal and heart muscles, and suggested homologous expression in multiple organisms (Shibata *et al.*, 2000). Like its paralog (Jin *et al.*, 2003),

RBFOX2 (also known as RBM9) is found in the brain and muscles and undergoes tissue-specific and cellular localisation-specific alternative splicing that affects the regulation of downstream targets, as originally reported by Nakahata and Kawamoto (2005) *in situ* in mice and *in vitro* in human cells. RBFOX3, more commonly known as NeuN (*neuronal nuclei*), has been used as a marker for mature neurons since the early 1990s (Duan *et al.*, 2016). It was proposed as the third paralog in humans and mice by Kuroyanagi (2009), and simultaneously experimentally identified as a product of the *RBFOX3* gene with neuronal, cell-specific splicing function (Kim *et al.*, 2009).

A large network of human RBFOX1/2 splicing targets - including many other RBPs - was proposed together with an evolutionary role for mutations of RBFOX RNA-binding sites (Sun *et al.*, 2012; Zhang *et al.*, 2008). More modern approaches have expanded upon the RBFOX regulatory network, suggesting clinical relevance of all family members in NDDs, as evident by their downregulation in ASD brains (Weyn-Vanhentenryck *et al.*, 2014). In a recent study, Ye *et al.* (2023) employed high-throughput analyses to reveal that the incredibly specific RRM of RBFOX proteins binds consensus sequences (5'-GCAUG) with high affinity and other RNA sequences with lower affinity. This conformation change-induced specificity was impaired by RRM mutations, which increased their affinity toward other RNA variants (Ye *et al.*, 2023), indicating possible molecular mechanisms of disfunction in NDDs and NPDs linked to *RBFOX* mutations (see Chapter 1.3.2.3 for more).

In a mouse model of Rett syndrome, a severe NDD that affects primarily females, mutants did not properly form protein complexes and condensates involving RBFOX *in vitro*, and negatively affected splicing of cortical synaptic targets like neurexins and neuroligins was observed (Jiang *et al.*, 2021).

Since RBFOX1 represents one of the main investigation targets for this work, a brief overview of its paralogs will be given first, followed by an in-depth description of the RBP itself and its role in brain development, function, and dysfunction.

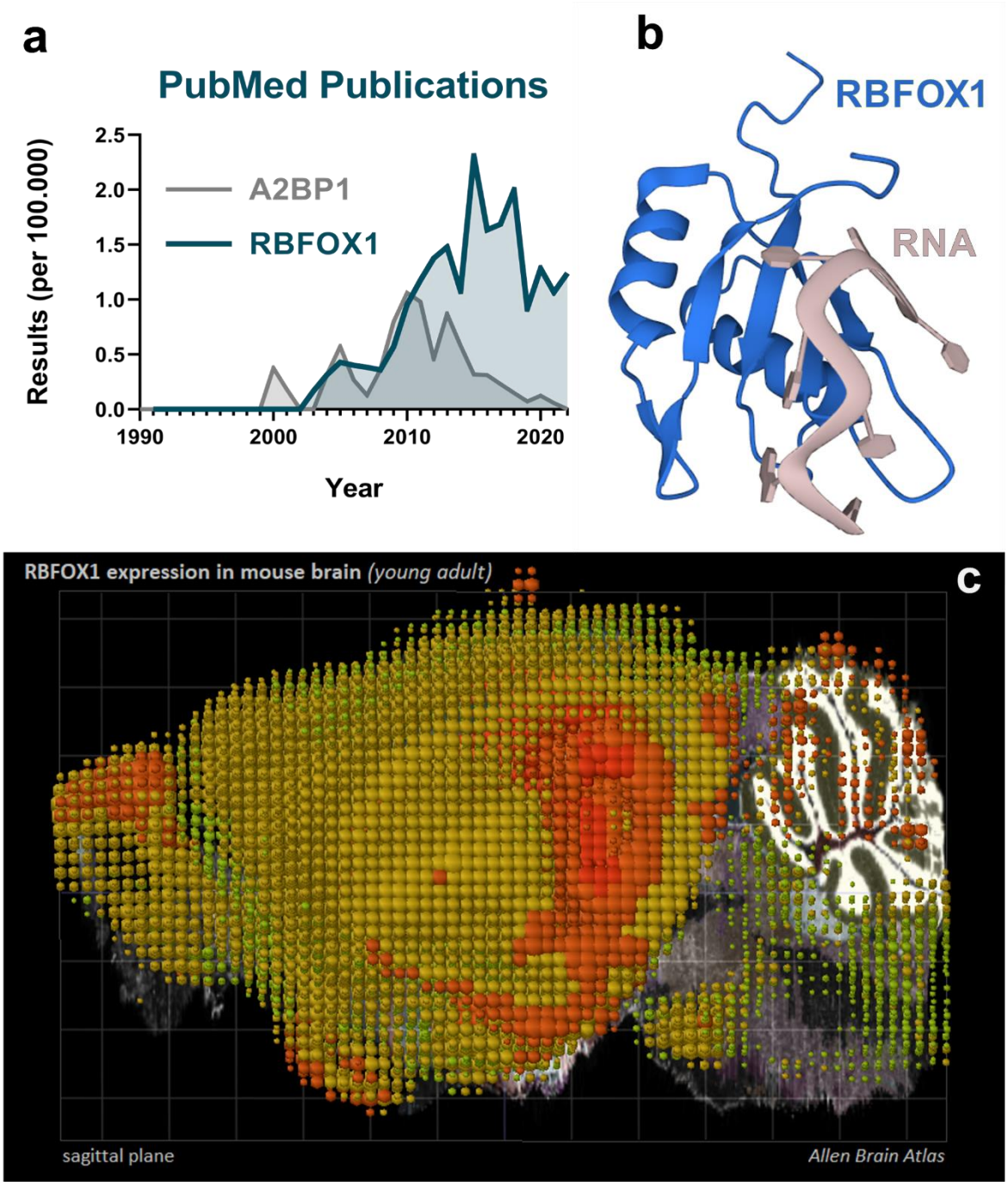


Figure 1.12 Figure 12. RBFOX1 publication history, structure, and murine brain expression profile. A, publication record of RBFOX1, previously known as A2BP1, measured in number of search term publications per 100.000 articles published each year, showing relative research interest (*tool:* http://esperr.github.io/pubmed-by-year/). **B**, crystal structure of the human RBFOX1 protein, shown in its RNA-bound state, wherein RNAs' UGCAUGU sequence (nucleotide residues shown), binds to the RNA recognition motif of RBFOX1. Provided by the Protein Data Bank in Europe Knowledge Database (https://www.ebi.ac.uk/pdbe/pdbe-kb/). **C**, *Rbfox1 in situ* hybridisation-based expression pattern in adult mouse brain, according to the Allen Brain Atlas (*P56 male samples, Brain Explorer 2;* https://mouse.brain-map.org/static/brainexplorer). RNA signal levels (*green=lower, red=higher*), mapped onto the sagittal plane of the reference image (Lau *et al.*, 2008).

RBFOX2

A team led by Douglas L. Black asserted a role of RBFOX1/2 as splicing enhancers of neuronally-regulated target exons in human and murine cells (Underwood *et al.*, 2005). The two proteins, however, do not exhibit fully redundant functions: at least *in vitro*. RBFOX2 was abundant at all developmental stages of differentiation of human induced pluripotent stem cells (iPSCs) into neurons and its depletion increased neural proliferation, while RBFOX1 was only present in the later neuronal stages (Chen *et al.*, 2016b). Underwood *et al.* (2005) also revealed differential expression of the two RBPs in E14 mouse embryos and that *in vitro* overexpression of both RBFOX paralogs leads to increases in target exon inclusion, while silencing of *RBFOX2* has the opposite effects.

RBFOX2 has been implicated, not only in mouse (Cibi *et al.*, 2019), but also in human embryogenesis, and might play a role in differentiation of placental progenitors (Goldman-Wohl *et al.*, 2020). Furthermore, *RBFOX2* knockdown in embryonic stem cells revealed the RBP to be vital for cell survival in a dose-dependent manner, and possibly binding nearly 10% of the human protein-coding genome in developing cells (Yeo *et al.*, 2009). In the human HPC, *RBFOX2* starkly increases its expression in early gestation and peaks in the second trimester and once again in infancy and the pre- and postadolescence period – time-windows connected to important brain changes (see Figure 1.13, Figure 1.6A-B).

Besides splicing, RBFOX2 can also repress transcription by chromatin binding, regulated via feedback by newly-formed RNAs, indicating targeted control of gene expression (Wei *et al.*, 2016). Finally, RBFOX2, together with its two paralogs, was shown to participate in the formation of cytoplasmatic stress granules in human cells *in vitro*, hinting at its role in controlling translational repression under stress conditions (Park *et al.*, 2017b).

Recent findings further implicate RBFOX2 in insulin regulation through splicing targets involved in exocytosis, and pancreas-specific *Rbfox2* mutant male mice exhibit impaired glucose tolerance and insulin secretion (Moss *et al.*, 2023). Intriguingly, *Rbfox1* and *Rbfox2* silencing in rat insulinoma beta cell line led to upregulation of insulin secretion following glucose stimulation, which was dose-dependent and based on actin remodelling changes in *Rbfox1* knockdown (Juan-Mateu *et al.*, 2017).

RBFOX3

Damianov and Black (2010) showed that RBFOX proteins self-regulate their splicing behaviour to produce isoforms with incomplete RRMs in adult mouse tissues - thus, decreasing their products' RNA-binding affinity and suppress splicing by other RBPs and isoforms via competitive binding. *In vitro*, full (but not partial) functional RRM-containing *Rbfox1/3* isoforms induced excision of the exon carrying the RRM subregion of *Rbfox2*, confirming the RBFOX autoregulatory network (Damianov & Black, 2010). Additionally, *Rbfox3* has multiple neuronal and cytoplasmatic alternative transcripts, and regulates splicing of *Rbfox2*, with nuclear isoforms driving increased *Rbfox2* mRNA decay *in vitro* (Dredge & Jensen, 2011).

In mice, RBFOX3's abundance pattern is region-specific and increases from the late prenatal to early postnatal period, peaking at the end of week 2-3, when it decreases again to stabilise at a high level (Lin *et al.*, 2016). Heterozygous knockout of *Rbfox3* caused impairments in cold sensitivity and novel object recognition in male mice, while a full knockout impaired HPC adult neurogenesis and synaptic transmission, apparent as reduced LTP and increased number of excitatory synapses (Lin *et al.*, 2016). During development of the human HPC, *RBFOX3* levels fluctuate, exhibiting highest prenatal expression mid-pregnancy and, similarly to mice, is most abundant in early postnatal window with additional peaks in the childhood, adolescence, and early adult periods (see Figure 1.13,). In line with its proposed role in brain development, RBFOX3 has been linked to epilepsy subtypes (Lal *et al.*, 2013), NDDs (Utami *et al.*, 2014), and the rare genetic Prader-Willi syndrome with neurodevelopmental implications (Coulson *et al.*, 2018). Multiple SNPs of *RBFOX3* were significantly associated with sleep latency – often disturbed in children with NDD (Shelton & Malow, 2021) – in a large European-cohort GWAS, and the RBP was predicted to be involved in dysfunctional NT (GABA, dopamine, serotonin, glutamate) release cycles (Amin *et al.*, 2016).

1.3.2.2 RBFOX1, the Developmental and Splicing Regulator

Shortly after its first identification in human tissue (see Figure 1.12B), RBFOX1 was characterised in mice and found to closely match its ortholog in chromosomal location, overall and RBP-relevant binding site sequence, and tissue availability (Kiehl *et al.*, 2001). Furthermore, developmentally-regulated neuronal RBFOX1 was detected starting at E12,

while in adult murine samples (see Figure 1.12C), cell- and regional expression selectivity as well as tissue specificity of isoforms were observed, including NS-specific isoforms (Kiehl *et al.*, 2001). While discussing RBFOX1 in the brain here, it should be noted that the RBP and its paralog RBFOX2 are expressed in the periphery as well, and this might lead to phenotypic and other differences in animal models based on the design of genetic manipulation (see below and Chapters 1.3.2.1, 1.3.2.3).

EXPRESSION PROFILES

Hammock and Levitt (2011) later reported that RBFOX1 was present in mouse embryos in postmitotic cells of the neural tube on E11.5 and increased its expression during postnatal (P14) development in discrete areas. RNA sequencing data have suggested that *Rbfox1* might be present in the murine neural tube as early as E8.5 (Yu *et al.*, 2017). Mouse cortex's *Rbfox1* levels have been shown to increase sharply at postnatal weeks 1 and 3 and different nuclear-localisation isoforms exhibit divergent expression patterns based on developmental stage and region, highlighting potential regulatory mechanisms via alternative splicing of *Rbfox1* itself (Casanovas *et al.*, 2020).

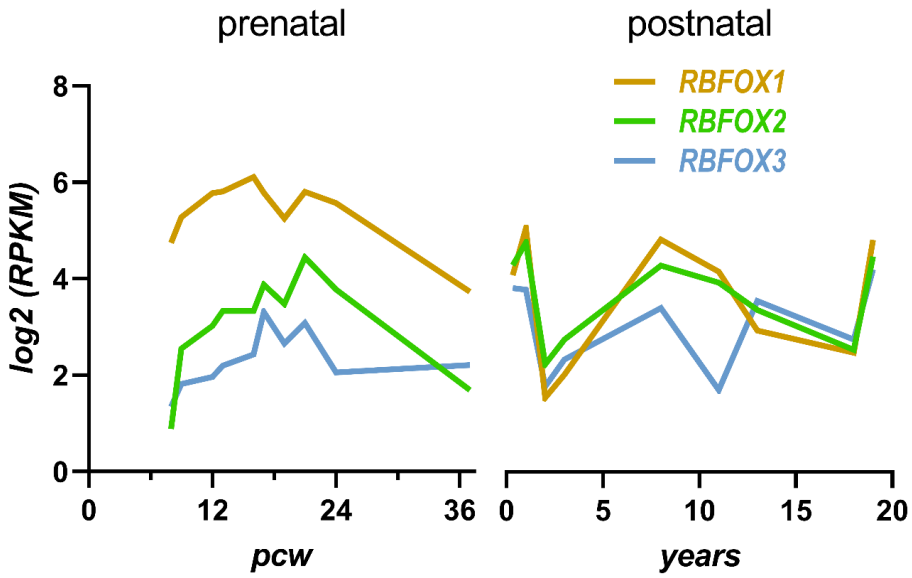


Figure 1.13 Hippocampal expression of RBFOX transcripts, relevant for brain development and neuropsychiatric disorders. The pre- and postnatal RNA expression profile for *RBFOX1-3* in human hippocampus (HPC) during vulnerable developmental time-windows (*see also Figure 1.6*). RNA sequencing data obtained for HPC from Allen Human Brain Atlas: BrainSpan (Atlas of the Developing Brain; www.brainspan.org/) for all available donors and averaged if multiple data/time-point. *pcw*, weeks post-conception; *RPKM*, reads per kilobase / million mapped reads.

Hamada *et al.* (2013) asserted the lack of RBFOX1 in proliferating progenitors but pronounced expression in murine HPC neurons with nuclear and diffuse cytoplasmatic localisation. Additionally, in immature rat HPC neurons, cytoplasmatic RBFOX1 was distributed throughout the soma, dendrites and axons, but in the mature HPC neurons it was observed in neurites as punctae near labelled synapses *in vitro* (Hamada *et al.*, 2013). Findings from a zebrafish model indicate that *rbfox1* is expressed in embryonal neurons, starting early (28 h post-fertilisation) in specific, discrete regions throughout the adult brain (Anton-Galindo *et al.*, 2023), hinting at a largely conserved mechanism of developmental expression programmes throughout evolution. Indeed, in the human HPC, *RBFOX1* is highly expressed throughout gestation, especially in the first two trimesters, and similarly to murine brain development, peaks in infancy as well as the pre- and post-adolescent periods (see Figure 1.13), which are linked of increased synaptic pruning and myelination events and NPD-vulnerable time-windows (see Figure 1.6A-B).

RBFOX1'S ROLE IN THE BRAIN

RBFOX1 encompasses a plethora of vital functions in the brain and its dysfunction causes pervasive deficits, which have been extensively studied *in vitro*, in humans and animal models. Therein, neuronal nuclear and cytoplasmatic RBFOX1 isoforms differ in some of their regulatory functions, i.e. primarily splicing versus localisation, stability, and translation of targets, respectively, which has implications for outcomes following their genetic manipulations (see next section for more).

During neuronal differentiation of multipotent mouse embryonic cells, *Rbfox1* was shown to be highly expressed in the neural stage and to regulate the developmental stage-specific alternative splicing of synaptic formation-implicated *Mef2c* target *in vitro* (Hakim *et al.*, 2010). In the developing cortex, RBFOX1 plays a role antagonistic to that of PTBP1 (see Chapter 1.3.1.2, Section PTBP), promoting differentiation of progenitor cells by enhancing the splicing of an isoform of the cytoskeletal protein ninein, which is excluded from the centrosome to reduce proliferation and increase neuronal maturation (Zhang *et al.*, 2016). Confirming the global developmental splicing changes under RBFOX control, the overall number of exons included in targets interrogated in this study is higher in adults than embryos (n=100 vs 30), whereas repression of exon inclusion is more pronounced in embryonic than in adult brains (Weyn-Vanhentenryck *et al.*, 2014). For example, the physiologically relevant, progressively

increasing inclusion of exon 33 and exclusion of partial exon 9 in transcripts of the voltage-gated calcium channel (*CACNA1C*) is regulated by embryonic stage-specific increases in RBFOX1/2 (Tang *et al.*, 2009).

An extensive investigation of the regulatory function of RBFOX1 in mouse cortical development (E18-PND22) found that dynamic RBFOX1-mediated alternative splicing in different GABAergic interneuron subtypes controls circuit building (Wamsley *et al.*, 2018). Full GABAergic neuron-specific deletion of *Rbfox1* in the mouse embryonic forebrain caused lethality before reaching adulthood, possibly due to seizures, and impaired inhibition in the form of reduced frequency and amplitude of inhibitory post-synaptic currents of pyramidal neurons in PND17-18 pups (Wamsley *et al.*, 2018). Finally, the study expanded upon target isoform regulation by RBFOX1, highlighting those enriched in the presynapse and in ASD (e.g. synaptosomal-associated protein *Snap23*, sodium voltage-gated channel *Scn8a*, semaphorin *Sema6d*) and the cell type-specific alternative splicing in cortical development of inhibitory interneurons (Wamsley *et al.*, 2018).

RBFOX1 has been put forward as a network regulator in human neuronal development, i.e. *in vitro* knockdown in foetal neural progenitor cells caused an aberrant splicing pattern and downstream gene expression changes, overlapping with NDD- and ASD-relevant candidates and pathways (Fogel *et al.*, 2012); see also Chapter 1.3.2.3. Li *et al.* (2020b) proposed *RBFOX1* as a hub regulator gene in the early-brain transcriptomic network and a role in hominin brain evolution, based on its human-specific, rapidly evolved non-coding regions. Intriguingly, Forastieri *et al.* (2022) showed that in higher primates exclusively, RBFOX1 possesses the ability to functionally splice a transcriptional corepressor (*lysine specific demethylase*, *LDS1*) and an epigenetic modulator involved in environmental stress response via transcriptional regulation.

The role of RBFOX1 as a functional stress regulator was further described in human iPSC-derived neurons, where a cytotoxic compound strongly increased the partially reversible aggregation of the RBP in nuclear and cytosolic granules, as well as its cytoplasmatic localisation (Kucherenko & Shcherbata, 2018). This was possibly achieved by controlling *Rbfox1* expression of specific isoforms upon stress-dependent modulation of the upstream *miR-980* (Guven-Ozkan *et al.*, 2016). In adult fruit flies, *miR-980* inhibition enhanced odour-induced learning and memory by increasing neuronal excitability -

recapitulated by *Rbfox1* overexpression and reversed when downregulated, indicating the *Rbfox1*-dependency of these effects (Guven-Ozkan *et al.*, 2016).

Genome-wide gene expression analysis of male murine HPC after contextual fear conditioning revealed downregulation of *Rbfox1* and a non-coding RNA *miR-219*, specifically after memory retrieval but not acquisition (Peixoto *et al.*, 2015). Furthermore, the expression of *miR-219* is regulated by an RBFOX1 splicing target – the synaptic transmission- and plasticity-relevant *Grin1* (Gehman *et al.*, 2011; Kocerha *et al.*, 2009), suggesting downstream epigenetic effects of the memory-dependent RBFOX1 decrease. Another micro-RNA, *miR-129-5p*, which might modify excitability via synaptic membrane and cytoskeletal targets' abundance regulation, was observed by Rajman *et al.* (2017) to benefit HPC synaptic downscaling following chronic neuronal overexcitation *in vitro* and *in vivo*. This miRNA also downregulated HPC *Rbfox1* expression during the homeostatic downscaling process to further counterbalance the RBP's positive regulation of many synaptic genes that increase excitability (Rajman *et al.*, 2017). Synaptic downscaling is a process of reduction in spine activity and size that assures homeostasis of neuronal cells during prolonged activity, which might otherwise affect their proper function as is the case in excitation overdrive, e.g. in epilepsy (Rajman *et al.*, 2017). Downscaling was further proposed as a memory consolidation mechanism during sleep, thus, highlighting the importance of RBFOX1 in functional synaptic maintenance in the HPC.

Conversely, overexpression of RBFOX1 in mice was also shown to affect plasticity by impairing BDNF-mediated hippocampal LTP via synaptosomal increase of truncated tropomyosin receptor kinase B (TrkB), a kinase function-deficient isoform of the neurotrophin receptor (Tomassoni-Ardori *et al.*, 2019) - a transmembrane protein that mediates antidepressant action (Casarotto *et al.*, 2021). Interestingly, a transcriptomic analysis of the murine HPC overexpressing RBFOX1 compared to those from knockout HPC revealed a largely independent set of differentially expressed genes, suggesting divergent mechanisms in *Rbfox1* loss- and gain-of-function (Tomassoni-Ardori *et al.*, 2019). In line with this hypothesis, heterozygous knockdown of *Rbfox1* in mice has no effect on TrkB levels but significantly increased HPC RBFOX2 expression, which was in turn linked to increased truncated TrkB production *in vitro*; meanwhile, RBFOX1 overexpression did not change its paralogs' abundance but directly increased truncated TrkB expression (Tomassoni-Ardori *et al.*, 2019).

RBFOX1's involvement in excitatory-inhibitory balance of the brain was demonstrated convincingly in the seminal paper by Gehman *et al.* (2011), which utilised an *Rbfox1* knockout mouse model to reveal subsequent hyperexcitability of the brain, especially in the HPC and amygdala, in absence of gross morphological changes. Furthermore, the lack of RBFOX1 affected the splicing of multiple targets important for synaptic transmission, such as receptors for GABA and glutamate, calcium and potassium ion channels, synaptic vesicle fusion proteins, among others, which play a role in neuronal function and when disrupted - in dysfunction like epilepsy (Gehman *et al.*, 2011). In animal models, epileptic seizure risk was significantly associated with intronic SNPs in whole-genome analysis of primates (Kos *et al.*, 2021), while the nervous system-specific deletion of *Rbfox1* using *Nestin-Cre* driver in mice led to both spontaneous seizures and pharmacologically induced ones by overexcitation following an ionotropic GluR agonist administration (Gehman *et al.*, 2011). Wen *et al.* (2015) observed significantly increased RBFOX1 abundance in the temporal cortices of individuals with treatment-resistant epilepsy or malformations of cortical development. Furthermore, *Rbfox1* and epilepsy-linked targets like *Grin1* and *Scn8a* were upregulated *in vitro* in rat cortical neurons and implicated in the observed hyperexcitability (Wen *et al.*, 2015).

Taken together, the evidence of RBFOX1's role in synaptic transmission, neuronal development and excitability is substantial. While much of the function of the RBP described above is linked to splicing regulation performed mainly by nuclear isoforms, cytoplasmatic RBFOX1 has other distinct post-transcriptional roles in the brain, which are important for this study and will be outlined next.

FUNCTION OF NUCLEAR AND CYTOPLASMATIC RBFOX1 ISOFORMS

The localisation and balance of nuclear and cytoplasm RBFOX1 variants play an important role in brain physiology and possible pathology. Lee *et al.* (2009) showed that potassium ion-induced chronic depolarisation in differentiated mouse neurons *in vitro* caused a slow activation-dependent splicing regulation and decreased cytoplasmatic localisation of *Rbfox1* by skipping of exon 19, which was recovered after the membrane milieu was normalised. Furthermore, the authors suggested that this mechanism counteracts the deleterious depolarisation effects of a calcium/calmodulin-dependent protein kinase, which represses exonic splicing of targets like the ionotropic NMDAR subunit *Grin1,* by enhancing RBFOX1's nuclear function of alternative splicing (Lee *et al.*, 2009). In another mouse study, the *in-utero*

downregulation of the predominant nuclear *Rbfox1* isoform caused deficits in cortical neuronal migration, possibly by impairing nucleokinesis – the cytoskeleton-mediated nuclear movement - and impaired axon growth, dendritic length and arborisation at PND3 (Hamada *et al.*, 2016). Moreover, the silencing of the same isoform in a murine HPC neuron culture recapitulated the observed morphological dendritic-tree and axonal deficits, in addition to decreased spine density and mature mushroom spine ratio; all impairments were at least partially ameliorated by re-expression of the silenced *Rbfox1* variant (Hamada *et al.*, 2016).

In a complimentary study, Hamada *et al.* (2015) interrogated the role of the predominant neuronal cytoplasmatic isoform of the RBP in corticogenesis. The silencing of cytoplasmatic *Rbfox1 in vitro* and *in utero* recapitulated the same deficits in neuronal migration via impaired nucleokinesis, axon elongation, decreases in spine density and mature spine ratio, albeit to a slightly lesser degree than mediated by the nuclear transcript (Hamada *et al.*, 2015; Hamada *et al.*, 2016). Additionally, an increase in the developmentally-common stubby spines was observed in HPC neuronal cell culture, which all together suggests an overlap but not complete redundancy in morphological regulation or function by nuclear and cytoplasmatic RBFOX1 during cortical development (Hamada *et al.*, 2015). In human cells, an upstream regulatory protein kinase (WNK3) was demonstrated to phosphorylate RBFOX1 and thus, impair alternative splicing of RBFOX1 targets by shifting its localisation towards cytoplasmatic *in vitro*, which may explain the implication of WNK3 in membrane transport and excitability (Lee *et al.*, 2012). In human brain samples, cytoplasmatic RBFOX1 is enriched in parvalbumin GABAergic interneurons of the PFC (Chung *et al.*, 2024).

In the influential paper by Lee *et al.* (2016), the authors showed that cytoplasmatic RBFOX1 predominantly increases target expression by regulating stability and translation by binding to the 3′ UTR of mRNAs, while the neuronal isoforms govern mostly splicing events. Furthermore, the study showed that decreased RBFOX1 abundance between birth and postnatal week 3 in the mouse HPC and cortex was influenced by the cytoplasmatic forms and that *in vitro* targets are enriched in synaptic transmission, cortical development, and ASD genes (Lee *et al.*, 2016). Building upon these findings, Vuong *et al.* (2018) examined the brain transcriptome of adult *Rbfox1* knockout mice and identified the inhibitory neuron-specific, vesicle exocytosis-relevant synaptobrevin (*vesicle associated membrane protein 1*, *Vamp1*) among the most downregulated targets in both protein and mRNA form in the HPC and

cortex. In HPC cell culture, cytoplasmatic *Rbfox1* re-expression rescued VAMP1 at both levels, while the observed deficits in HPC inhibitory synaptic transmission in animals lacking *Rbfox1* were rescued by the re-expression of *Vamp1*, suggesting a mechanism of excitation-to-inhibition balance impairments seen in ASD and epilepsy (Vuong *et al.*, 2018). On the other hand, *in vitro* triple knockout of *Rbfox1/2/3* in ventral spinal cord neurons led to splicing-induced impairment of the initial cytoskeletal axon segment important for action potential generation - and of the electrophysiological embryonic-to-adult developmental switch, which normally increases motor neuron excitability (Jacko *et al.*, 2018). *Rbfox* deletion further led to global target splicing changes in synaptic and membrane genes including *Bin1* (*bridging integrator 1*) and multiple potassium, calcium, and sodium channels (Jacko *et al.*, 2018).

Due to the implication of RBFOX1 as central neuronal regulator and its multifaceted function in brain development, maintenance, and dysfunction, the RBP has attracted scientific interest as an NPD target. Much of the corresponding evidence came from clinical and large-scale genomic studies of psychiatric disorders from the past 20 years and lead to translational investigations of *RBFOX1*, which will be presented in the next section.

1.3.2.3 RBFOX1, the Major Neuropsychiatric Disorder Target

Bhalla *et al.* (2004) first described *de novo* translocations of a chromosome 16 region within the RBFOX1 gene in two boys exhibiting epilepsy, ID, developmental and behavioural problems. These findings were independently reproduced by Martin *et al.* (2007), finding a different translocation as well as decreased *RBFOX1* levels in a female patient with ASD, ID, and epilepsy, but found no significant association of individual SNPs in a small sample of ASD children and their parents. In a study, using multiple validation methods, and affirming *de novo* copy number mutations' association with ASD, linked a rare deletion variant of *RBFOX1* with the disorder (Sebat *et al.*, 2007). CNVs, primarily involving intragenic deletions, were also found in multiple patients with NDD phenotypes and comorbidities in a separate Chinese study (Zhao, 2013). Notably, all these studies were performed in peripheral blood samples and not in nervous tissue. A transcriptomic approach in human brains from controls and ASD individuals revealed neuronal marker-enriched network modules of mostly downregulated NDD- and synaptic genes: *RBFOX1* was identified as a network hub and a number of its alternatively spliced targets observed in ASD brains (Voineagu *et al.*, 2011).

These findings of strong associations of rare, usually loss-of-function, deleterious *RBFOX1* CNVs with ASD has been replicated in multiple genomic studies in US (Griswold *et al.*, 2015; Turner *et al.*, 2016) and Finnish (Kanduri *et al.*, 2016) cohorts. Adding to the bioinformatic and genome-derived data, Parras *et al.* (2018) showed that alternative splicing of *CPEB4* (see Chapter 1.3.1.2, Section CPEB) is regulated by RBFOX1 and an increase of isoforms missing exon 4 can be observed in ASD cortex. RBFOX1 reduction was observed on the protein level in both cortices from young idiopathic ASD patients and in the striatum of adolescent animals of the ASD-like transgenic mouse model *CPEB4Δ4*, strengthening the link between aberrations in alternatively spliced RBFOX1 targets and NPDs (Parras *et al.*, 2018).

Findings from a small-sample, USA-based study documented multiple causative missense mutations, i.e. base pair substitutions, in the RRM of *RBFOX1* in patients with diverse NDD phenotypes, which in all cases included developmental delay and behavioural problems (Li *et al.*, 2024). A case study reported a 16p13.3 microdeletion, encompassing the exon 2-and partial intron 1/2-containing region of *RBFOX1*, in a female patient with a Tourette-like syndrome (Murgai *et al.*, 2018). Cortical organoids from patients with the NDD-relevant, chromosome 16p11.2 region-spanning deletion revealed a concomitant, transcriptional- and proteomic-level expression downregulation of its neighbouring RBFOX1 and target genes (Kostic *et al.*, 2023), suggesting molecular pathway overlaps.

RBFOX1 has been implicated in NPDs other than ASD and ID/developmental delay disorders, i.e. ADHD, BD, and SCZ. In a large study encompassing patients from seven European countries, an SNP of *RBFOX1* (r*s12921846*, risk allele: T) was identified as a marker of ADHD with conduct disorder (Anney *et al.*, 2008), and the authors asserted previous association of this polymorphism in BP (Wellcome Trust Case Control, 2007). In a GWAS of ADHD children aged 6-16, deficits in delayed visuospatial working memory (WM, assessed using the Rey Complex Figure Test Delayed Component) were significantly associated with five *RBFOX1* SNPs (Zhong *et al.*, 2023). Habituation learning deficits, including in the visual domain, were affected in larval zebrafish lacking *rbfox1*, possibly due to isoform changes in synaptic vesicle cycle-governing synaptosomal associated protein *snap25a* (Zhong *et al.*, 2023), suggesting a conserved regulatory mechanism for RBFOX1 in WM deficits observed in ADHD.

Not only common mutations, but also rare *RBFOX1* variants have been linked to ADHD. In a US-based, case-control genetic study in children (ages 6-18), Elia *et al.* (2010) demonstrated

an enrichment of *RBFOX1* ADHD-linked CNVs, both duplications and intronic hemizygous deletions, in autism and schizophrenia candidates. Furthermore, the authors highlighted the glutamate metabotropic receptor 5 (*GRM5*) as an ADHD candidate gene, which is a prominent target of RBFOX1 (Elia *et al.*, 2010). A large-scale analysis of 11 studies combined over 30 000 ADHD and control subjects and multiple bioinformatic approaches to identify 26 consistently high-priority genes, based on ADHD CNVs, of which *RBFOX1* was one (Harich *et al.*, 2020). Additionally, Harich *et al.* (2020) described a common variants' significant association with ADHD in available GWAS meta-analysis data and cross-disorder GWAS.

In a Northern European-ancestry cohort of BD patients and controls, a rare intronic *RBFOX1* deletion was discovered and validated in a case-control expression study (Noor *et al.*, 2014), which also revealed RBFOX synaptic targets (Lee *et al.*, 2016; Weyn-Vanhentenryck *et al.*, 2014) like neurexins (*NRXN1/3*) and glutamate ionotropic AMPAR subunit (*GRIA3*) among the BD-linked genes. Rare *de novo RBFOX1* copy number gain was also shown to significantly associate with SCZ in a South African cohort used in a genomic study of sporadic schizophrenia (Xu *et al.*, 2008). Furthermore, both nuclear and cytoplasmatic RBFOX1 expression was reduced in PFC parvalbumin-type (PV) interneurons of SCZ patients (Chung *et al.*, 2024). This cytosolic reduction reduced the transcript abundance of the RBP's target synaptic vesicle-regulating *VAMP1*, investigated using immunohistochemical analyses (Chung *et al.*, 2024). This is in keeping with the findings of Vuong *et al.* (2018) in *Rbfox1* knockout mice (see Chapter 1.3.2.1), which showed that cytoplasmatic *Rbfox1* downregulation, specifically, reduces *Vamp1* levels in murine PFC and HPC inhibitory neurons, thus, highlighting the importance and validity of RBFOX1 mouse models for the study of NPDs.

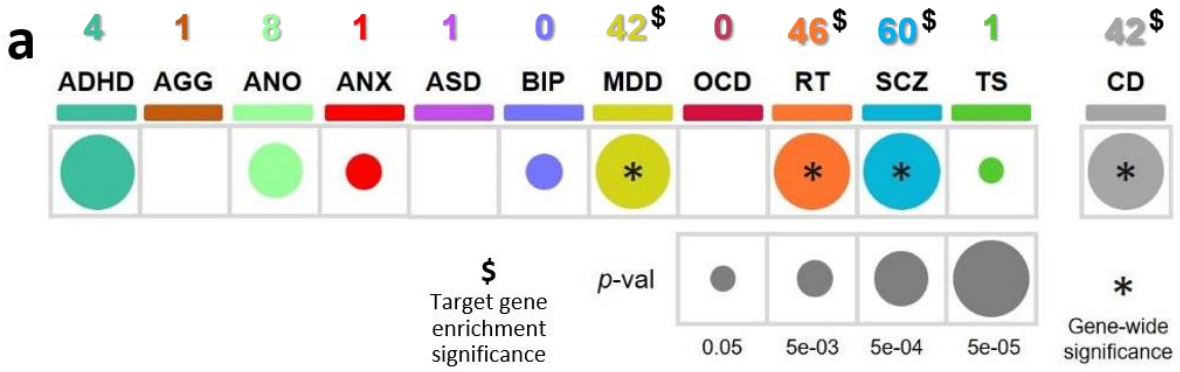
A meta-analysis of GWAS for NPD-relevant personality traits using data of over 120 000 individuals from large genetic databases implicated an intronic *RBFOX1* SNP (*rs7498702*, effect allele: C) in extraversion, which was itself genetically correlated with ADHD and BD (Lo *et al.*, 2017). In the seminal meta-analysis of genome-wide data from over 230 000 psychiatric patients across eight psychiatric disorders (ASD, ADHD, SCZ, BP, OCD, MDD, Tourette syndrome, and anorexia nervosa) and over half a million controls, Lee *et al.* (2019) identified *RBFOX1* as one of the main pleiotropic genes (see also Chapter 1.1.2). The lead intronic SNP (*rs7193263*) was significantly associated with all disorders but anorexia and shown to gradually increase its expression prenatally (Lee *et al.*, 2019). Furthermore, the suggested

pleiotropic risk genes - among them *RBFOX1* (risk locus 16p13.3) - were enriched in the brain, neural development regulation, neuronal formation and differentiation, as well as ASD, SCZ, BPD and neuroticism (Lee *et al.*, 2019). Taken together, the evidence of RBFOX1 as neurodevelopmental regulator and central gene in NPD risk motivated us to conduct a translational study of its neuropsychiatric role in humans and mice. First, we established via bioinformatic analysis gene-based associations of common *RBFOX1* variants and target enrichment in multiple psychiatric disorders and traits (Figure 1.14A), while rare variants were involved in early-onset NPDs (Figure 1.14B) and significantly enriched in ASD patients (O'Leary *et al.*, 2022).

Through functional imaging analysis, we showed that carriers of a common *RBFOX1* SNP (*rs6500744*, risk allele: T) displayed brain and behavioural differences: healthy risk-carriers had higher emotional reactivity and reduced cognitive control response, while enhanced fear expression and increased avoidance behaviour was observed in panic disorder risk-carriers (O'Leary *et al.*, 2022). We performed extensive phenotyping in *Synapsin1*-Cre-driven, neuronal-specific *Rbfox1* knockout male mice, which provided construct and face validity as an animal model of ASD-like endophenotypes.

Specifically, full knockout males displayed hyperactivity and stereotypy in the absence of anxiety-like behaviours (see Figure 1.15A-C, F), as well as impaired cued fear-specific learning (Figure 15G), as described by O'Leary *et al.* (2022). Furthermore, neuronal *Rbfox1* loss led to social deficits and lack of aggression (Figure 1.15D-E), and diminished startle response with intact sensorimotor gating (Figure 1.15H-I), mimicking multiple impairments linked to neuropsychiatric disease. Deficits, observed both in our investigation of *Rbfox1*-deficient mice and in NPD patients, were largely replicated in zebrafish with intronic point mutation or exonic deletion of *rbfox1* (Anton-Galindo *et al.*, 2023), strengthening the hypothesis of the RBP's conserved brain function. The Anton-Galindo *et al.* (2023) team involving our collaborators revealed increased locomotor activity and anxiety-like behaviour, decreased freezing and sociability in mutant fish, further validating the translational value of RBFOX1 animal models.

a



b

Disorder	Total N		*RBFOX1* CN gain		*RBFOX1* CN loss		Ratio *RBFOX1* CNVs cases *vs* controls
	Cases	Controls	Cases	Controls	Cases	Controls	
ADHD	1 072	5 668	3	8	5	4	3.5 : 1
ASD	5 923	10 327	2	0	18	7	5.0 : 1$
SCZ	4 611	3 335	0	0	16	9	1.3 : 1
TS	3 980	7 013	3	4	7	9	1.4 : 1
ALL	17 404	29 273	10	12	47	29	2.3 : 1

Figure 1.14 Genetic *RBFOX1* variation in neuropsychiatric disorders and traits. Data mining and bioinformatic analysis revealed significant gene-wide significance of common *RBFOX1* variants in MDD, RT, SCZ, and the cross-disorder meta-analysis (**A**). Targets of the RBP were also enriched among significantly disease-associated genes (*number of target genes above disorder/trait name in corresponding colours*). Rare *RBFOX1* variants like copy number (CN) losses and gains were increased in patients with early-onset NPDs like ADHD, ASD, SCZ, and TS (**B**) – and significantly enriched in ASD individuals. Data previously published in O'Leary *et al.* (2022). *ADHD*, attention-deficit/hyperactivity disorder; *AGG*, childhood aggression; *ANO*, anorexia nervosa; *ANX*, anxiety; *ASB*, antisocial behaviour; *ASD*, autism spectrum disorder; *BIP*, bipolar disorder; *CD*, cross-disorder meta-analysis; *CNV*, copy number variant; *MDD*, major depressive disorder; *OCD*, obsessive-compulsive disorder; *SCZ*, schizophrenia; *RT*, risk tolerance; *TS*, Tourette syndrome.

RBFOX1 has been implicated in mental disorders and comorbidities, other than classical NPDs. Based on a review of genetic association studies, Kasap and Dwyer (2021) put *RBFOX1* forward as part of a network of risk genes, which overlap for PD and neuropsychiatric disorders, i.e. BD and MDD. Indeed, in a large-scale, meta-analysis GWAS of MDD patients and controls, two common *RBFOX1* variants (*rs8063603*, *rs7198928*) were significantly associated with major depression risk (Wray *et al.*, 2018). Additionally, a GWAS for anxiety sensitivity in a cohort of female twins demonstrated six associated *RBFOX1* SNPs (*rs13334105* reached genome-wide significance), while the analysis of monozygotic twins with phenotypic

discordance highlighted nine more polymorphisms, of which *rs116206041* was significant (Davies *et al.*, 2015).

Multiple lines of evidence, including those from various GWAS and animal model studies (Zhang-James *et al.*, 2019), suggest that *RBFOX1* variants are involved in aggressive behaviour - a comorbidity in a variety of NPDs – and were reviewed by Fernandez-Castillo *et al.* (2020). In a small European cohort, *RBFOX1* SNPs were associated with personality traits like neuroticism and extraversion, relevant for mood and anxiety disorders, as well as risk for alcohol use disorders (Vaht *et al.*, 2020). Furthermore, *RBFOX1* belongs to a group of pleiotropic genes, associated with shared genetic liability for SCZ and nicotine dependence, as suggested by GWASs in European decedents (Chen *et al.*, 2016a). In line with RBFOX1's putative role in SUD susceptibility, repeated cocaine exposure in mice caused a large number of splicing changes, increased nuclear RBFOX1 and affected target expression in the addiction-relevant brain region nucleus accumbens (Feng *et al.*, 2014).

In the NDD- and NPD-related sleep domain, the *RBFOX1* SNP *rs1478693* was significantly associated with sleep time in both males and especially females in an Australian twin cohort (Byrne *et al.*, 2013). Moreover, *rbfox1* exerted effects on night sleep duration and the morphology of sleep-regulating brain regions in fruit flies in a gene × environment-dependent manner, together with early-life nutrition status (Olivares *et al.*, 2023).

In line with the hypothesis of aberrant *RBFOX1* expression leading to an excitatory-inhibitory disbalance in the brain, a plethora of genetic evidence in clinical and animal studies have linked mutations of the gene to different forms of epilepsy. Human data encompasses SNPs with SCZ risk association (Karadag *et al.*, 2023) or rare microdeletions in patients with generalised genetic (Lal *et al.*, 2015b) and lateral temporal (Fanciulli *et al.*, 2014) epilepsies, rare exon variants and microdeletions in idiopathic focal childhood epilepsy (Lal *et al.*, 2013), and exonic deletions in sporadic focal epilepsy (Lal *et al.*, 2015a). Finally, increased RNA editing of *RBFOX1* and a number of its binding partners was described in inhibitory *vs* excitatory neurons and a large number of differentially edited *RBFOX1* sites were observed in ASD patients (Ansell *et al.*, 2021), suggesting another possibly reversable mechanism of neuronal excitation disbalance.

Interestingly, RBFOX1 appears to play a role in neurodegeneration as well as neurodevelopment. *In vitro* overexpression of RBFOX1 in human cell lines induced exon 7-skipping alternative splicing events of its target APP (Alam *et al.*, 2014).

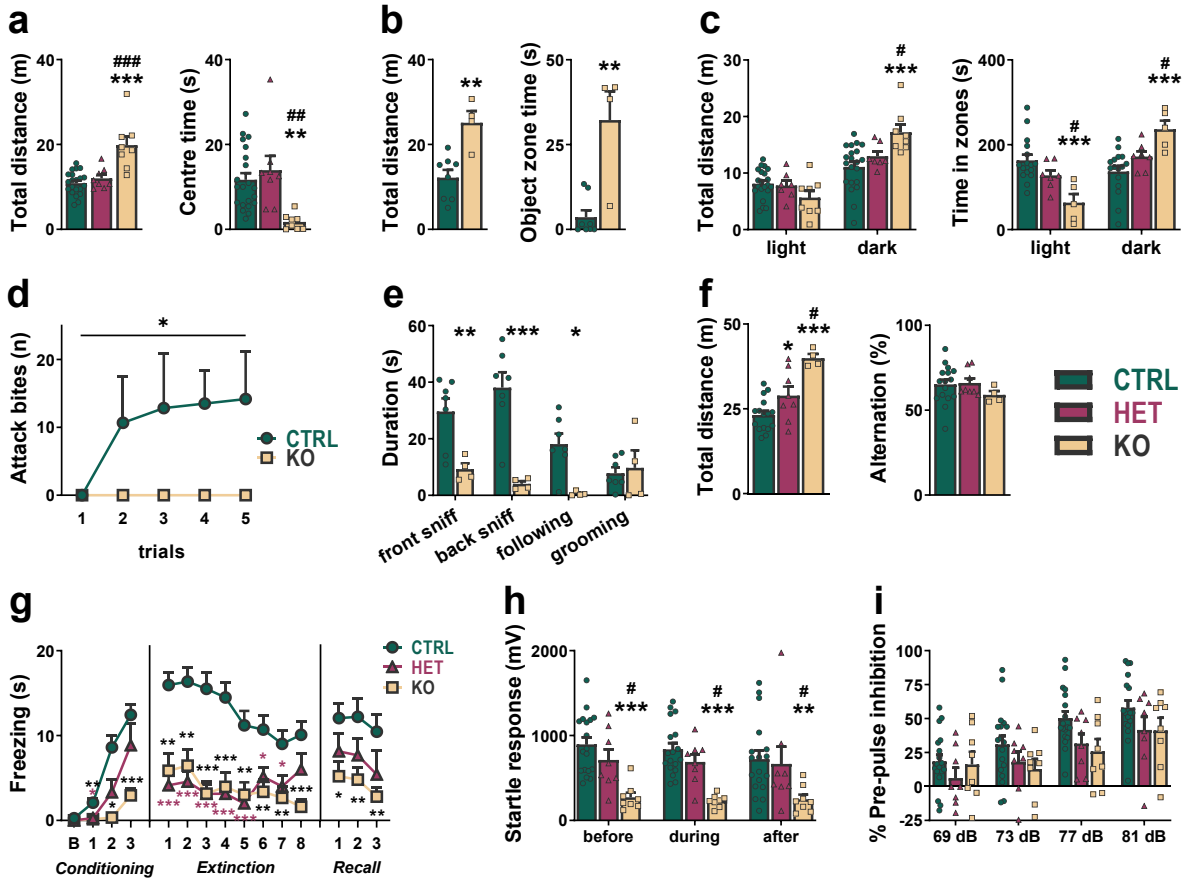


Figure 1.15 Neuronal knockout of *Rbfox1* caused behavioural alterations in male mice, reminiscent of ASD-related phenotypes. Neuron-specific *Rbfox1* knockout (KO) and knockdown (HET) mice were generated from *Rbfox1*$^{fl/f}$ and *Synapsin1*-Cre animals on a *C57Bl/6J* background. *Rbfox1* KO animals were hyperactive in an open field (OF) test (**A**), the dark compartment of a light-dark box (LDB, **C**), and the Y-maze (**F**). KO males also displayed anxiety-like behaviours in open and brightly lit spaces in OF and LDB (**A**, **C**). Aged animals were also hyperactive in OF and spent longer investigating a novel object (**B**). *Rbfox1* KOs completely lacked aggression (**D**) and exhibited striking social deficits (**E**). In the auditory fear conditioning test (**G**), both acquisition and extinction learning were significantly diminished in KO males, while *Rbfox1* HETs had impaired fear extinction, alone. KO mice had deficient acoustic startle response compared to both control (CTRL) and HET males (**H**), but intact sensorimotor gating (**I**). Adult male mice were tested in four cohorts [n=8-21 per genotype (A, C, F-I), n=4-8 per genotype (B, D, E)] at ages 9-14 weeks, except for B (aged mice: 8 months old). Significance annotations: *p<0.05, **p<0.01, ***p<0.001 vs CTRL; #p<0.05, ###p<0.001 vs HET. Data previously published in O'Leary *et al.* (2022).

Indeed, this specific isoform was only produced under regulatory control by RBFOX1 and to a lesser extent RBFOX2 - but not other RBPs like NOVA, CELF, PTBP (see Chapter 1.3.1.2) - which implies a link to AD, since exon 7-containing isoforms are much more common in patients than controls (Alam *et al.*, 2014; Matsui *et al.*, 2007). In keeping with these findings, RBFOX1 co-localises with tau tangles surrounding amyloid *β* plaques in dystrophic AD neuronal processes, and downregulation of *RBFOX1* in *post mortem* prefrontal cortex samples of elderly adults with and without AD is significantly correlated with higher amyloid *β* abundance, impaired cognition, and steeper cognitive decline (Raghavan *et al.*, 2020). Furthermore, *RBFOX1* SNPs were significantly associated with increased amyloid *β* levels in the brains of aging adults in a meta-analysis GWAS and amyloid functional imaging data (Raghavan *et al.*, 2020). Intriguingly, functional imaging of cellular glucose uptake in a European descendants' cohort containing elderly healthy controls, AD and mild cognitive impairment patients, revealed genome-wide association between impaired brain glucose metabolism and the *RBFOX1* SNP *rs12444565* (Kong *et al.*, 2018), in line with the previously described role of *Rbfox1 and Rbfox2* in insulin regulation outside the brain (see Chapter 1.3.2.1). These findings are of importance due to the newly established associations between insulinopathies, brain inflammation, and NPDs and comorbidities (Fanelli *et al.*, 2022; Wimberley *et al.*, 2022).

Taken together, the extensive evidence of RBFOX1's role in brain development and function and its association with NPDs and comorbid disorders motivate further investigation of the effects of the less-studied RBFOX1 overexpression, alone and in combination with prenatal immune-system intervention, on brain and behaviour.

1.3.3 The RAS Family

1.3.3.1 RAS GTPases and Their Function

The research into the RAS (Rat sarcoma) family of proteins began in the 1960s, leading to the discovery of a variety of human *RAS* genes in the next two decades, which were ultimately found to play a role in cancer as proto-oncogenes (Malumbres & Barbacid, 2003). Around that time, the RAS superfamily was identified as small guanosine triphosphatases (GTPases), meaning they function as guanine-nucleotide exchange proteins (Wittinghofer, 2014). Thus, RAS proteins are enzymes that hydrolyse GTP to guanosine diphosphate (GDP), upon which

they change their conformation to an active state (see Figure 1.16), working as molecular switches in the cell (Wennerberg *et al.*, 2005). From the 1990s onward, researchers found multiple distinct regulatory upstream binding partners and downstream effectors as well as molecular pathways (see Figure 1.16), by which RAS GTPases functionally regulate signal transduction and cell fate (Bos, 1998; Cox & Der, 2003; Dhillon *et al.*, 2007; Downward, 2003; Molina & Adjei, 2006; Simanshu *et al.*, 2017).

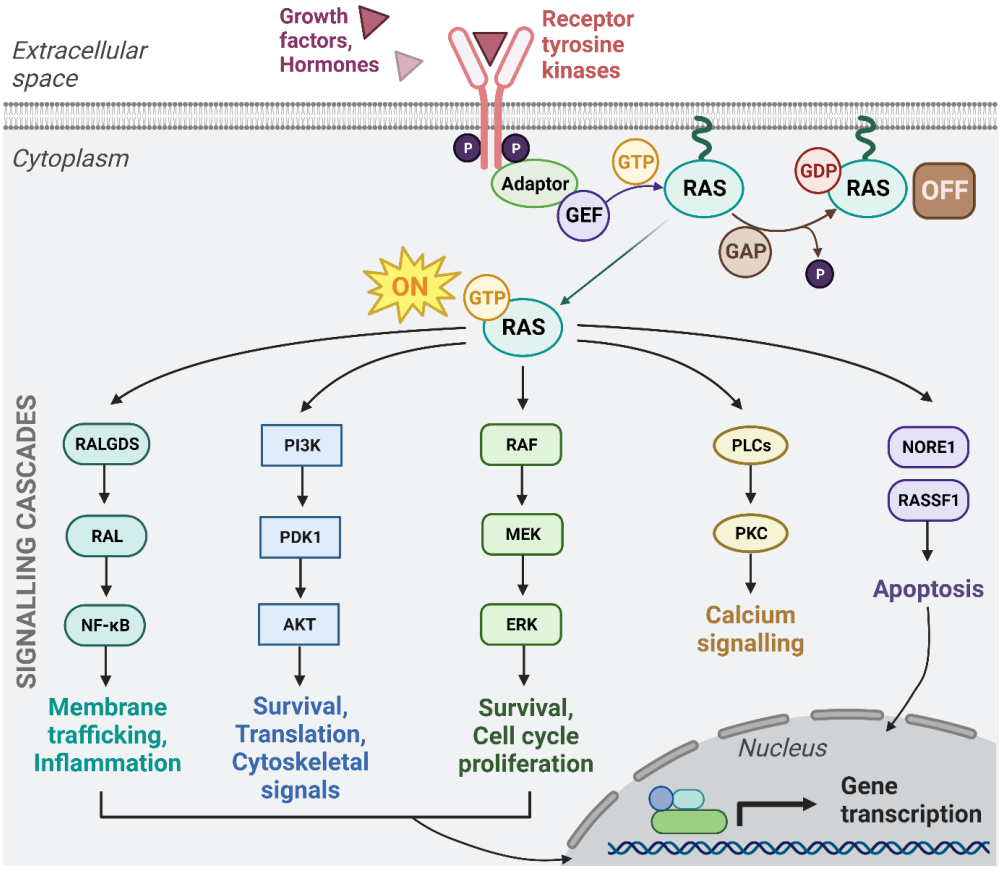


Figure 1.16 Simplified overview of signal transduction by the RAS family within the mammalian cell. RAS superfamily proteins work as cellular signal relays by transducing extracellular information, delivered by molecules like growth factors or hormones via receptor tyrosine kinases, using distinct molecular response pathways. Upon interaction with receptor adaptor proteins (CRKL, GRB2, SHC, among others), upstream guanine-nucleotide exchange factors (GEFs, like SOS, CDC25) can increase the catalytically active, GTP-bound form of RAS. GTPase-activating proteins (GAPs, e.g. RasGAP, ARHGAP, SynGAP) on the other hand accelerate the enzymatic activity of RAS, i.e. GTP hydrolysis and phosphate (P) dissociation, and leaving the protein in its inactive, GDP-bound state. This cycle renders the RAS proteins, which can interact with downstream effectors when active and elicit different molecular cascades, as switches of inter- and intracellular signalling. Depending on the targeted transduction pathways, RAS signalling can dictate cell transformation and fate by either effecting downstream extranuclear post-translational modification or calcium signalling, or inducing transcriptional changes in the cell (Bos, 1998; Cox & Der, 2003; Dhillon *et al.*, 2007; Downward, 2003; Molina & Adjei, 2006; Simanshu *et al.*, 2017).

Depending on the downstream signalling cascade, elicited by extracellular messengers - usually growth factors and hormones - and modulated by other transduction pathways, RAS activation can lead to different outcomes (see Figure 1.16). These include vesicle transport or inflammation (RAL/NF-κB pathway); cell survival, translation, cytoskeletal reformation (PI3K/AKT pathway); survival and cell cycle proliferation (MAPK/ERK pathway); cell death (NORE/RASSF1 pathway). Another function of RAS affects calcium signalling (PLC/PKC pathway), often achieved by direct intracellular action outside nuclear transcription (see Figure 1.16). Meanwhile, upstream guanine-nucleotide exchange factors (GEFs) can increase the amounts of GTP-bound, active RAS molecules that can interact with targets (see Figure 1.16), while GTPase activating proteins (GAPs) intensify the hydrolysis activity of RAS - innately a relatively slow process for this superfamily – and thus, inactivating them (Colicelli, 2004). Importantly, RAS proteins can undergo many post-translational modifications, e.g. by lipids, which increases their diversity of action and membrane targeting function (Wennerberg *et al.*, 2005).The RAS superfamily comprises a great number of GTPases and is represented in all eukaryotic organisms by evolutionary and functionally divergent forms (Fernandez-Medarde *et al.*, 2021; Rojas *et al.*, 2012). There are five major classified protein subfamilies, namely Ras, Rho, Rab, Arf, and Ran, with Ras purportedly containing up to 39 distinct genes (or at least, protein isoforms) in humans, while 36 Ras orthologues have been found in mice (Rojas *et al.*, 2012; Wennerberg *et al.*, 2005). Important examples of family members involved in development, dendritic and spine morphology, and function, especially from the Ras and Rho subfamilies, will be provided in the next section.

Importantly, RAS signalling has been directly (Haddad *et al.*, 2003; Johnson & Chen, 2012; Zhang & Dong, 2005) or indirectly – via interlinked molecular pathways (Beck *et al.*, 2009; Rawlings *et al.*, 2004) – implicated in innate and adaptive immune functions, including in stress responses, chronic inflammation, and diabetes (Kyriakis & Avruch, 2001). In the brain, downstream RAS pathways, such as MAPK, appear to play a major role in pro-inflammatory processes in ischemia (Wang *et al.*, 2004) and cortical microglia- and astrocyte-mediated response to injuries (Dusaban *et al.*, 2013; Koistinaho & Koistinaho, 2002).

Discoveries from the past 35 years, stemming from animal models of aberrant RAS signalling, have not only elucidated the essential cellular function and involvement in tumorigenesis of these proteins, but also revealed their central role in development (Fernandez-Medarde *et al.*,

2021; Iroegbu *et al.*, 2021; Malumbres & Barbacid, 2003). Deficits in RAS signal transduction can affect viability of the offspring due to embryonic or placental dysfunction or lead to syndromic conditions known as RASopathies, which include neurodevelopmental delays (Hebron *et al.*, 2022; Malumbres & Barbacid, 2003; Simanshu *et al.*, 2017), and will be discussed in the next section.

1.3.3.2 RAS in Brain Development and RASopathies

RAS AND THE BRAIN

In neurons, Ras signalling can be activated by Ca^{2+}, which is important for neuronal functions like neurotransmitter release, dendritic and synaptic formation and function, and survival (Farnsworth *et al.*, 1995). Notably, RAS/ERK signal transduction processes regulate cortical progenitor genes that can change neuronal-glial cell fate *in vitro* (Li *et al.*, 2014b). Furthermore, in the spines of pyramidal HPC neurons, Ras is an extremely sensitive sensor for neuronal activation, having both rapid downstream action to guide neuronal response, as well as a function in plasticity (LTP, specifically) by eliciting Ca^{2+}-mediated, local signalling cascades (Harvey *et al.*, 2008; Yasuda *et al.*, 2006); see also Figure 1.17.

The titular H-, N-, and K-RAS GTPases are membrane-bound and differ in their subcellular localisation, downstream targets, and functional aspects, including in the NS, while sharing a high degree (~85 %) of sequence homology (Bender *et al.*, 2015). Neural stem cell-specific activation of K-RAS (but not H- or N-RAS) leads to increased astrogliogenesis in the brain stem of juvenile (PND18) mice, while not affecting astrocytic differentiation *in vitro* (Bender *et al.*, 2015). Finally, Bender *et al.* (2015) showed that activated K-RAS but not its paralogs further increased proliferation of the neural stem cells *in vitro*, ostensibly by upstream regulator inhibition, mediated by another RAS GTPase – RAF1 proto-oncogene, serine/threonine kinase.

On the other hand, a neuronal-specific, constitutive activation of H-RAS in a transgenic mouse model (Syn-Ras mice) led to many changes in pyramidal neurons of the somatosensory cortex: significantly increased neuronal somata volume, spine and synaptic densities, dendritic length and basal dendrite number, axonal diameter, and thus -

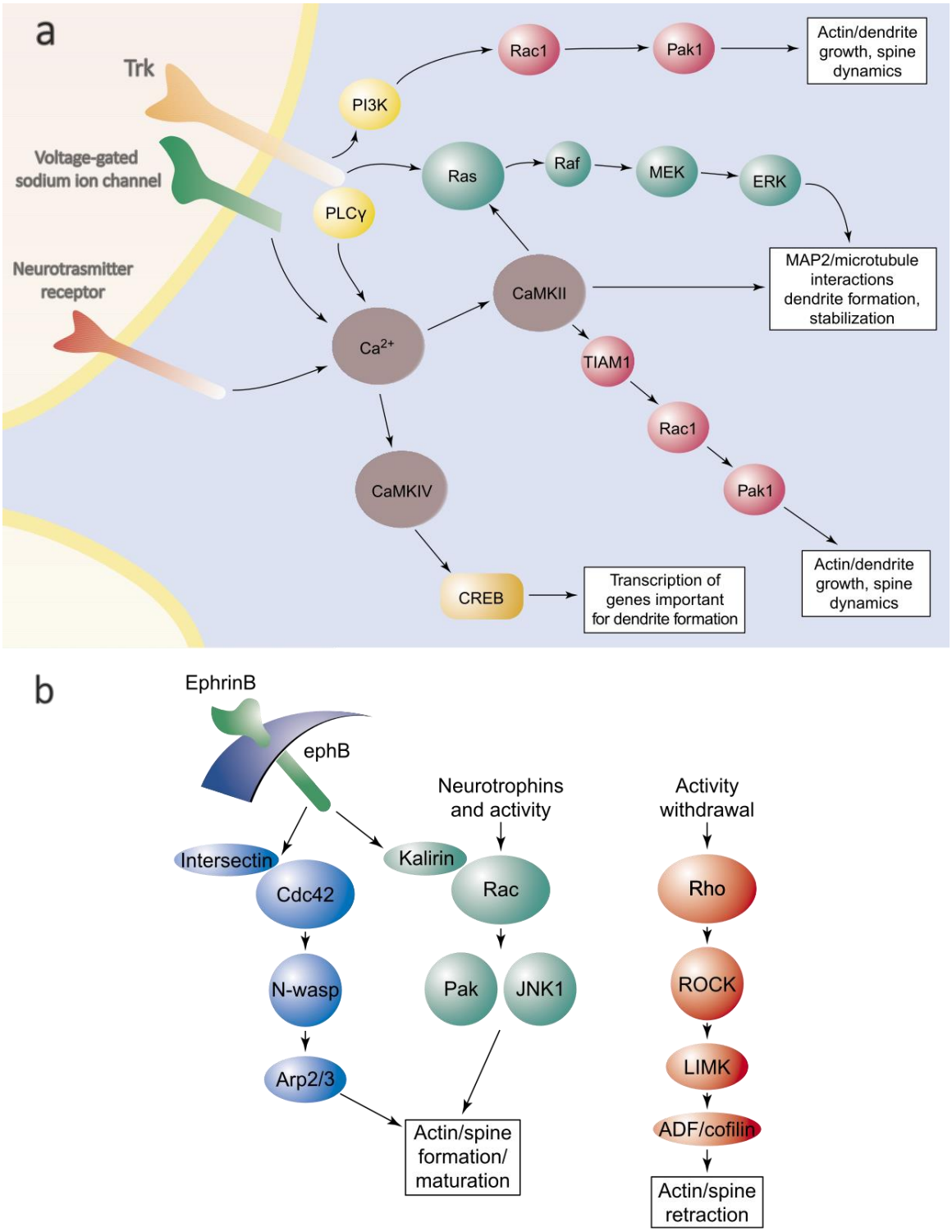


Figure 1.17 Simplified overview of RAS GTPase-regulated outcomes for neuronal dendrites and synapses. Adapted from Miller and Kaplan (2003) and re-used with publisher's permission (*licence 5770151468701*). **A**, neuronal activity and neurotrophin signalling (e.g. through Trk receptor) elicit signal cascades along multiple pathways involving RAS GTPases and calcium signals to differentially regulate dendritic morphology. **B**, regulation of the actin cytoskeleton by Ras and Rho GTPases and effectors in response to extracellular signals leads to divergent outcomes for dendrite and spine formation and maturation. For more information, see Miller and Kaplan (2003) and main text.

– increased global and glutamatergic synaptic transmission and enhanced LTP in the investigated cortical region (Arendt *et al.*, 2004). N-RAS is mostly known as a cancer target, including in brain tumours, but it has been shown that it shares functional redundancies with H-RAS in axon growth, i.e. constitutive activation of N-RAS led to the formation of multiple axons *in vitro* in HPC neurons from a mouse model, as was the case for activated H-RAS (Fivaz *et al.*, 2008). Crucially, RAS proteins in the brain and their modulators are involved in behavioural and neuronal plasticity through synaptic signalling regulation during memory processes (Nourbakhsh & Yadav, 2021; Ye & Carew, 2010). For example, the Ras homolog neurofibromin NF1 regulates presynaptic GABA release, while the Ras protein-specific guanine nucleotide releasing factor RasGRF, localised in spines, modulates AMPA receptor dynamics and neuronal excitability (Ye & Carew, 2010).

Disruptions in Ras and Rap signalling – both over- and under-activation – impair glutamatergic receptor trafficking linked to dysfunctions in adaptive learning, as observed in a number of psychiatric, neurodevelopmental, and neurological disorders (Stornetta & Zhu, 2011). For example, *SYNGAP1* - a regulator for multiple RAS GTPases and a major ASD/NPD candidate gene - has been shown to both control dendritic/synaptic morphology and network connectivity in human neuronal development and to regulate synaptic plasticity in the mouse HPC (Araki *et al.*, 2023; Araki *et al.*, 2020; Komiyama *et al.*, 2002; Llamosas *et al.*, 2020). In another mouse model of ASD, inhibiting the RAS/MAPK/ERK pathway rescued motor learning inflexibility by reducing cortical dendritic spine clustering caused by overstabilisation of experience-mediated synaptic formations (Ash *et al.*, 2021).

In the brainstem of rats, Rho GTPase pathways, which include RAC, RHO, and CDC42 (*cell division cycle 42*) proteins play a central role in norepinephrine neurite growth and arborisation via actin remodelling following stress-mediated signals from corticotropin-releasing factor (Swinny & Valentino, 2006). Different members of the RAS family have divergent roles therein: RAC1 – also known to cause activity-dependent enlargement of spines related to memory formation - stimulates growth and branching, while RHOA inhibits dendritic outgrowth and arborisation (Costa *et al.*, 2020; Swinny & Valentino, 2006); see also Figure 1.17. Upon activation in the HPC, RHOA acts to inhibit (e.g. cypin) or activate (e.g. ROCK, *Rho associated coiled-coil containing protein kinase*) downstream targets which leads to reduced dendritic number and arborisation, while blockade leads to spine density reduction

and length increases (Chen & Firestein, 2007; Tashiro & Yuste, 2004). Conversely, RAC1, which also targets ROCK, promotes maturation and stability of spines, and when deactivated increases the abundance of immature spine protrusions (Soriano-Castell *et al.*, 2017; Tashiro & Yuste, 2004). Similarly, CDC42 is a positive regulator of dendritic length and number, and its activity is needed for proper neuronal tree branching and spine formation (Miller & Kaplan, 2003; Scott *et al.*, 2003).

Importantly, upstream regulators of Rho GTPases have vital and distinct roles in dendritic spine development, as shown in cultured neurons from embryonic (E19) rat HPC (Martin-Vilchez *et al.*, 2017). In the study, the researchers showed that early in developing neurons, an actin-polymerisation activator RAC1 GEF induces spine formation, while CDC42 GEF and RhoA GAP drive precursor elongation. On the other hand, later in neuronal development, the RAC1-regulator ARHGAP23 and Rho guanine dissociation inhibitors inactivate dynamics of the spine cytoskeleton to increase the stability of mature spines, highlighting the complex role of Rho GTPases in HPC neurodevelopment (Martin-Vilchez *et al.*, 2017). In keeping, the Rho subfamily has been implicated in affecting neurodevelopment in ASD and related syndromes through signalling and synaptic morphology alterations connected to neuronal scaffolding proteins (Reichova *et al.*, 2018; Wu *et al.*, 2022).

Transgenic models (e.g. neuronal expression of activated H-RAS) and optogenetic tools (e.g., sustained activation of RAS triggers mTOR and MAPK pathway responses) have been successfully employed to investigate and manipulate RAS signalling to gain insights into neurodegenerative conditions (Heumann *et al.*, 2000; Schoneborn *et al.*, 2018; Toettcher *et al.*, 2013), in line with their role as neuronal differentiation and survival switches. Importantly, R-Ras subfamily proteins might control and coordinate myelination processes through oligodendrocyte development, which in turn govern proper brain signalling propagation (Alcover-Sanchez *et al.*, 2020). The RAS/MAPK/ERK pathway further appears involved in sensory neuron differentiation and neuropathic pain propagation through Raf GTPases (Zhong, 2016). Aberrant RAS signalling has been strongly implicated in epilepsy, further strengthening its role in regulating neurodevelopment (Sran & Bedrosian, 2023).

While the functions of RAS GTPases in neuronal formation, dendritic, axonal, and spine formation are too numerous to list individually and beyond the scope of this study, some

noteworthy examples are mentioned here and shown in Figure 1.17 (see review by Miller and Kaplan (2003) for more).

RASOPATHIES

RASopathies comprise disorders of RAS/MAPK signalling, usually caused by germline mutations in pathway genes, their modulators and effectors (Gelb & Tartaglia, 2006; Tajan *et al.*, 2018) and combined prevalence of 1:1000 births (Jindal *et al.*, 2015). These clinically defined, distinct syndromes exhibit phenotypic overlap, including different degrees of musculoskeletal, tumour-related, cardiovascular, as well as neurological and cognitive developmental impairments, observed in humans and animal models alike (Aoki *et al.*, 2016; Grant *et al.*, 2017; Tajan *et al.*, 2018). Nervous system development is affected on a cellular level in RASopathic conditions, with genetic mutations in *HRAS*, *KRAS*, *PTPN11* (*protein tyrosine phosphatase non-receptor type 11*), and *NF1*, among others, influencing neuronal and glial progenitors during differentiation and maturation (Kim & Baek, 2019). Interestingly, *NF1* suppresses glial proliferation and is a negative regulator of the RAS/MAPK pathway, while the others are positive regulators (Kim & Baek, 2019). Aberrations in the RAS pathway can lead to a plethora of divergent neurological, cognitive, neurotransmission, and psychiatric-like endophenotypes, as shown by a large body of evidence from mouse models (Kang & Lee, 2019). Furthermore, patients with RASopathies typically display specific personality trait deficits in agreeableness, extraversion, and openness, among others, when compared to unaffected siblings, thus, revealing sociability difficulties (Bizaoui *et al.*, 2018).

Interestingly, ASD, which has a high prevalence in RASopathy patients, has similar symptomatic profile across the different syndromes regardless of developmental differences, suggesting a common underlying mechanistic link (Geoffray *et al.*, 2020). When comparing children with the behaviourally defined, idiopathic ASD and children with RASopathies, the latter displayed more resilience in the domain of empathy relative to other social skills, as well as a negative association between emotional challenges and social competence (Foy *et al.*, 2022). Although research of psycho-behavioural dysfunctions across the RASopathy spectrum is somewhat limited, generalised findings single out social impairments as common NPD-related symptoms and further highlight anxiety- and depression-, and to a lesser extent, aggression-related problems occurring in patients with disordered RAS signalling (Walsh *et al.*, 2020). Deficiencies in attention and behaviour, similar to those observed in ADHD, have

been described in children with Noonan syndrome – one of the most common, (~1:1500, (Jindal *et al.*, 2015), prevalence sex-balanced), and best documented RASopathies – and initial results suggest that ADHD-like parental training programmes might have similarly positive therapeutic effects on Noonan patients (Montanaro *et al.*, 2022). A recent structural brain imaging study in such children and controls revealed RASopathy-related changes in striatal and cortical anatomy, possibly affecting inhibition functions via frontostriatal circuits, similar to observations in ADHD (Rai *et al.*, 2023).

In summary, RAS signalling plays a crucial role in brain development on molecular and cellular level, and dysfunctions therein can lead to syndromic impairments that include NPD-related cognitive and neuropsychological deficits with links to ASD and ADHD, among other mental disorders and NDDs (see review by Borrie *et al.* (2017) for more). A Ras GTPase that gained traction due to findings from our group (Grünewald *et al.*, 2016) as an ADHD candidate is DIRAS2, which is somewhat unusual based on its MAPK activity, and is the focus of next section.

1.3.4 DIRAS2

1.3.4.1 The DIRAS Subfamily

DIRAS2 (*distinct subgroup of the RAS family member 2*) is a small RAS-like GTPase, first described by Kotani et al. (2002; Figure 18A-B). DIRAS2 and its paralog DIRAS1 were shown to localise in cellular membranes and were able to induce morphogenesis *in vitro*. Furthermore, these proteins appeared to predominantly exist in GTP-bound form and differ from other family members in their apparent inability to directly activate the MAPK or AKT downstream signalling pathways, due to inefficiency in binding to the RAS-specific domains of Raf and PI3K, respectively (Kontani *et al.*, 2002). However, their high expression profiles in the brain, as well as their cell morphogenic properties, hinted at a neurodevelopmental role of these distinct small GTPases. A later study into the properties of DIRAS1/2 proteins revealed intrinsic, RAS-typical high nucleotide affinity and exchange rate, low GTP hydrolysing activity, and catalytic regulation by RAP1GAPs in the absence of shared GEFs or effector proteins (Gasper *et al.*, 2010). A study by Ogita *et al.* (2015) further uncovered that a protein encoded by *RAP1GDS1* (*Rap1 GTPase-GDP dissociation stimulator 1*) which acts as a GEF for other GTPases from the Rap- and Rho-families, bound DIRAS2 in the rat brain cytoplasm, leading to

its decreased guanine nucleotide affinity but increased stability. Taken together, these findings suggested a yet undiscovered mechanism of cellular signal transduction by the DIRAS subfamily members, separate from those of other Ras- and Rap-proteins.

A third suggested paralog, *DIRAS3* (also known as *ARHI*, *Aplasia Ras homolog member I*), is a purported tumour suppressor gene that decreases oncogenic RAS clustering and Ras/Raf binding, resulting in MAPK and PI3K pathway inhibition in human cells (Sutton *et al.*, 2019). While this gene shares sequence homology with the other two *DIRAS* genes, some have argued that its divergent structural and functional properties might place it as a member of a separate Ras subgroup but not a paralog to *DIRAS1/2* (Gasper *et al.*, 2010; Kontani *et al.*, 2002). Interestingly, there is no *DIRAS3* orthologue in mice or rats, which might be the result of breakpoint-associated gene deletion, following a divergent evolutionary chromosome rearrangement in rodents (Fitzgerald & Bateman, 2004).

DIRAS1, similarly to *DIRAS2*, is a common target of cancer research. Outside of its putative tumour-suppressing function (see below) and findings from studies looking at both paralogs together, not much has been revealed about DIRAS1. Nevertheless, a *DIRAS1* mutation was shown to cause increased expression of the protein in cholinergic neurons across the brain of a naturally-occurring, canine model of genetic generalised epilepsy with early onset, i.e. in juvenile dogs, corresponding to <10 years-of-age in humans (Wielaender *et al.*, 2017). Importantly, DIRAS1 has been shown to affect post-translational modification and localisation of RAC1 *in vitro* (see Figure 1.17), by inhibiting binding of RAP1GDS1 to RAC1 – along with the binding of RHOA and KRAS (Koehn *et al.*, 2023) – thus, possibly indirectly affecting neuronal morphology. This is supported by findings in the zebrafish model, suggesting that *diras1* positively regulates neurite growth and axon guidance by increasing Rac1 protein levels and activity, while downregulating RhoA expression (Yeh & Hsu, 2016). Taken together, these findings suggest a cross-species role of *DIRAS1* in brain development, neuromorphology, and function.

As previously mentioned, the *DIRAS* family members have gained attention in the field of cancer research, due to their presumed function as tumour suppressors. Multiple studies have shown that DIRAS1 and DIRAS2 play a role in tumorigenesis via divergent downstream signalling pathways (Gonyo *et al.*, 2017; Rao *et al.*, 2020; Sutton *et al.*, 2018; Ying *et al.*, 2022). Some have reported that downregulation of *DIRAS2* is associated with poorer prognosis in

cancer patients (Cao *et al.*, 2022; Rothhammer-Hampl *et al.*, 2021; Sutton *et al.*, 2018; Ying *et al.*, 2022), while others argue for detrimental effects of its upregulation on survival rates (Rao *et al.*, 2020; Zhang *et al.*, 2022), further suggesting distinct functional mechanisms in different cancer types. Although DIRAS2 is highly expressed in the human brain (see next section), these studies suggest its expression in cancer cell lines and tumours in non-NS human tissues, e.g. ovaries, thymus, intestines. Importantly, recent literature purports a link between DIRAS2 and inflammation and immune function in both cancer (Qi *et al.*, 2019; Xue *et al.*, 2022; Ying *et al.*, 2022) and virally induced encephalitis (Selinger *et al.*, 2022).

1.3.4.2 Neuronal *DIRAS2*: Location and Function

When first described by Kontani *et al.* (2002), *DIRAS2* was proposed to be selectively expressed in the human brain, with higher RNA abundance profiles in cortical, orbitofrontal, and cerebellar tissue samples. Later studies from our colleagues (Grünewald *et al.*, 2018) extended these findings to show strong expression of *DIRAS2* in the HPC. Furthermore, they examined various NPD-relevant regions in the mouse brain, using both RNA and protein measures, and reporting the highest *Diras2* levels in the frontal cortex and HPC of male mice (see Figure 1.18D). This study revealed additional notable localisation of *Diras2* in the basolateral amygdala, striatum, and nucleus accumbens. In the posterior cortex, *Diras2* was most strongly visible in the layers II/III and V/VI, as well as the dentate gyrus (DG), CA1 and CA3 fields of the murine HPC (Grünewald *et al.*, 2018).

Furthermore, results from the same study using primary HPC culture suggested that *in vitro*, DIRAS2 is mainly located in the distal branches of neuronal axons and dendrites and less so in dendritic spines. Interestingly, DIRAS2 was visible to some degree in glial cells, strengthening the previously described inflammation link and suggesting a possible role in synaptic signalling modulation (Vallejo *et al.*, 2010). Importantly, Grünewald and colleagues found significant increases of *Diras2* RNA expression during early developmental stages, i.e. from the prenatal (E11-15) to early (P0-7) and then to later (P9-15) postnatal periods, and unchanged abundance in the mouse brain starting from adolescence and into early adulthood (see Figure 1.18C).

To further elucidate the molecular function and neurodevelopmental role of *DIRAS2*, Grünewald *et al.* (2021) analysed the effects of the gene's downregulation on murine hippocampal cell culture.

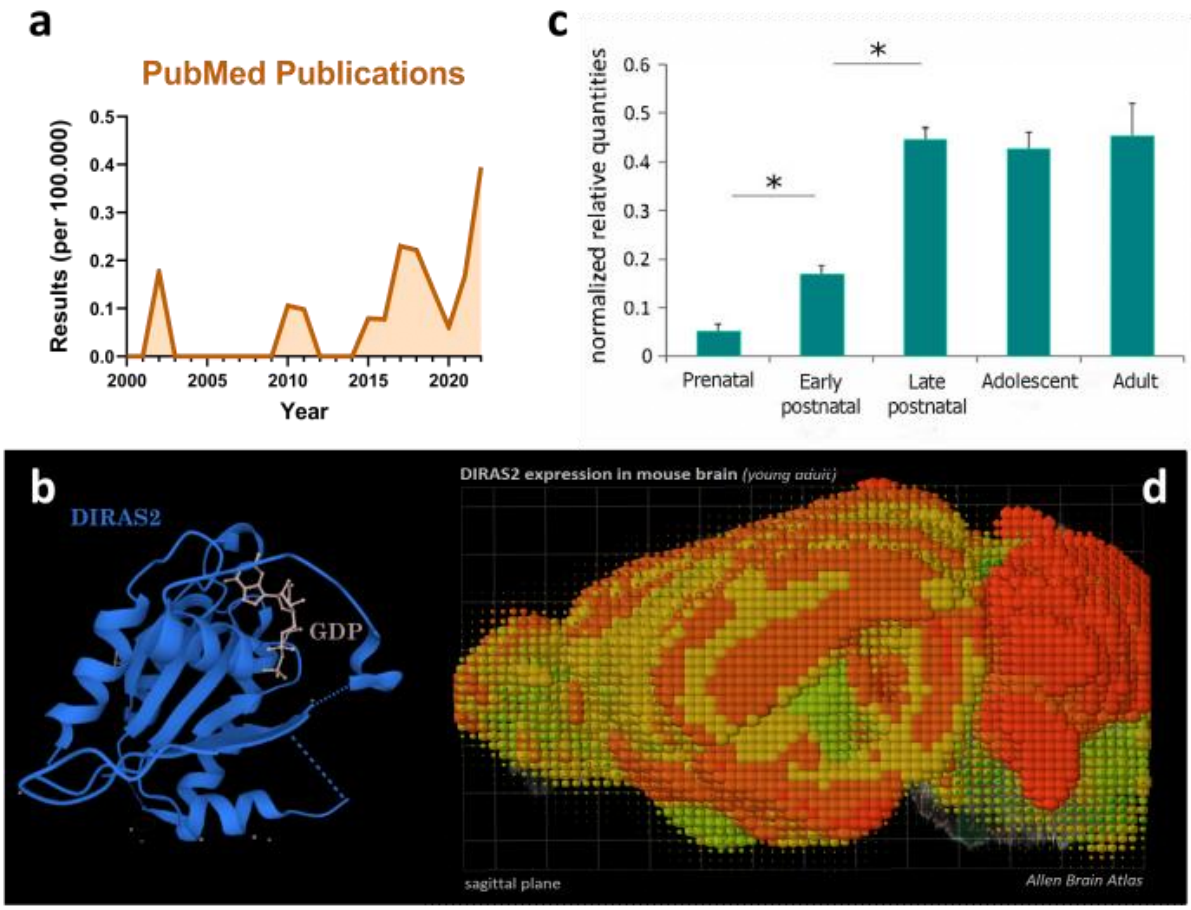


Figure 1.18 DIRAS2 publication history, structure, and murine brain expression profile. **A,** publication interest record of *DIRAS2*, measured in number of search term publications per 100.000 PubMed articles published each year (tool: *http://esperr.github.io/pubmed-by-year/*). **B**, crystal structure of the human DIRAS2 protein, shown in its inactive GDP-bound state, provided by the Protein Data Bank in Europe Knowledge Database (by EMBL-EBI: *https://www.ebi.ac.uk/pdbe/pdbe-kb/*). **C**, *Diras2* mRNA transcript profile across mouse brain development, according to Grünewald et al. (2018). **D**, *Diras2 in situ* hybridisation-based expression pattern in adult mouse brain, according to the Allen Brain Atlas (P56 male samples, from Brain Explorer 2: *https://mouse.brain-map.org/static/brainexplorer*). RNA signal levels (*green=lower, red=higher*), mapped onto the sagittal plane of the mouse brain (Lau et al., 2008).

The authors found that knocking down *Diras2* caused expressional changes in over 1500 genes *in vitro*, which were enriched in neurodevelopmental processes and molecular pathways. Validation of *Diras2*-influenced genes in the same study revealed that a number of

these targets are implicated in neuronal and spine development. These included *Clasp2* (*cytoplasmic linker associated protein 2*), *Ctdsp2* (*CTD small phosphatase 2*), *Eif2c2* (also known as *Ago2, Argonaute RISC catalytic subunit 2*), *Ppp1r9a* (*protein phosphatase 1 regulatory subunit 9A*)) and survival, especially via the mammalian target of rapamycin (mTOR) pathway (e.g., *mTOR*, *Rictor, Mmp5* (*matrix metallopeptidase 5*, now *Mmp24*), among others) - another important kinase signalling route.

1.3.4.3 *DIRAS2* in Disordered Brain Aetiology

Mounting evidence for the involvement of *DIRAS2* in ADHD and related cognitive processes infers causative links between perturbations in the gene's expression or function and disordered neurodevelopment. Building upon previous genome-wide association research, such as linkage studies for chromosomal region and GWAS for genetic variant identification, Reif *et al.* (2011) investigated *DIRAS2* as a candidate gene for ADHD. In this study, the authors found significant associations between adult ADHD (aADHD) and four *DIRAS2* single nucleotide polymorphisms (SNPs), as well as a haplotype (ACGCTT) comprising six common variants in a German cohort. Furthermore, an increased risk of aADHD was shown in pooled samples from four different Western European populations for one of these SNPs (*rs1412005*, risk allele: T) and the ACGCTT haplotype that includes the *rs1412005* T allele, both of which relate to the *DIRAS2* promoter region (Reif *et al.*, 2011). The risk haplotype's association with the disorder was also found in a German cohort of children with ADHD (cADHD), in addition to a familial over-transmission of a neighbouring promoter SNP (*rs7848810*, risk allele: C). Reif and colleagues (2011) found multiple *DIRAS2* common variant associations in adults with comorbid impulsive disorders, i.e. bipolar and personality disorders, and related behavioural traits including neuroticism, novelty seeking, and harm avoidance.

In a follow-up study, the molecular and systemic function of the *rs1412005* SNP was probed further, revealing that the T allele increased the gene's expression *in vitro* (Grünewald *et al.*, 2016). Moreover, cADHD patients across genders who were risk allele carriers were shown to have significantly impaired NoGo-anteriorisation in a continuous performance test (CPT), measured by electroencephalogram. This type of anteriorisation, pertaining to motor response inhibition based on the presented stimulus, has been identified as a reliable measure of cognitive control (Fallgatter & Strik, 1999), suggesting *DIRAS2* polymorphisms

might cause executive function deficits in ADHD (Grünewald *et al.*, 2016). This causal relationship is also supported by the disorder-relevant temporal and regional expression patterns of *Diras2* in the brain (Grünewald *et al.*, 2018), as well as the gene-wise enrichment of multiple downstream targets in ADHD (Grünewald *et al.*, 2021). *In situ* data from Grünewald *et al.* (2018) revealed that on a cell type-specific level, *Diras2* is predominantly localised in glutamatergic and catecholaminergic neurons, which have both been implicated in playing a role in ADHD (Miller *et al.*, 2013). Functional imaging analysis during auditory and visual emotional tasks in adults demonstrated that aADHD patients who do not carry the *DIRAS2 rs1412005* risk allele exhibit a lower activation of the right thalamus compared to both healthy controls and to an even larger extent - aADHD T carriers, hinting at a possible compensatory mechanism in absence of emotional recognition deficits (Hillmann *et al.*, 2022).

Further evidence for the role of DIRAS2 in brain and synaptic function came from a cross-species study on alcohol consumption, which included mice, nonhuman primates, and humans (Nimitvilai *et al.*, 2017). Exploratory synaptome as well as follow-up experimental and bioinformatic analyses revealed that DIRAS2 expression in the frontal cortices of male monkeys and mice of both sexes was predictive for alcohol intake. Thus, Nimitvilai *et al.* (2017) proposed *DIRAS2*, in line with existing evidence from human studies, as a possible biomarker for heavy drinking behaviour, observed in SUD, which are comorbid with NPDs like ADHD (see also Chapter 1.1.2).

Interestingly, in a recent study, lower expression of *DIRAS2* was found in all samples of patients with different types of brain cancer (gliomas), when compared to healthy tissue (Rothhammer-Hampl *et al.*, 2021). The authors posed that epigenetic control of *DIRAS1/2* was causal to their downregulation in glioma tissue and demonstrated that overexpressing the genes *in vitro* increased the susceptibility of cancerous cells to alkylating chemotherapy, suggesting a role in DNA damage repair.

Taken together, the evidence suggests that while the exact molecular function of DIRAS2 remains unknown, it appears to play an important role in neurodevelopment, brain function and dysfunction, related to NPDs and comorbidities. Thus, further study of the behavioural, molecular, and morphological effects of neuronal *Diras2* in a mouse model remains warranted.

2. AIM AND OBJECTIVES

As outlined above, the interaction between genetic and environmental factors, especially when occurring early in life, represent strong risk factors for the development of NPDs. These include prenatal immune activation due to infections, as well as functional disruptions of genes shaping neurodevelopment, especially those affecting neural formation and transmission.

Thus, in my dissertation I aimed to build upon this evidence and body of literature to provide novel and further insights into how specific genetic targets (i.e., RBFOX1 and DIRAS2) and maternal immune activation (MIA) shape the behavioural, molecular, and morphological underpinnings of NPDs. In particular, I aimed to study whether these specific factors may determine potential sex-differences in a wide-range of readouts based on the relative dearth of studies examining multiple levels in both sexes at the same time.

To achieve this, I conducted three major studies: all of which combined behavioural, neuromorphological, and molecular experimental techniques. While separate, each was designed to inform on one another and to reach the overall aim of the dissertation.

In the first study "Consequences of MIA for Neuromorphology and the Embryonic and Adult Hippocampal Proteome", I was to establish and validate an early-gestation maternal immune activation (MIA) mouse model, which mimics infection during pregnancy – a known environmental risk factor for NPDs like autism spectrum disorder (ASD), schizophrenia (SCZ), and attention deficit/hyperactivity disorder (ADHD). To validate the model, I assessed NPD-linked endophenotypes in the adult offspring subjected to MIA. Subsequently, I performed a novel interrogation of the synaptoneurosomal proteome of both embryonic and adult hippocampi (HPC), a region of great importance for disordered aetiology and linked to the observed behavioural deficits, in MIA offspring and control mice. This was aimed at examining NPD-relevant dynamic and persistent changes at the level of the synapse, due to its role in brain function and dysfunction. These insights could benefit the search for targets for early interventions in individuals at higher risk of NPDs following prenatal immune activation, and separate those that are life-long from those that occur early or later in life following MIA. Having determined the consequence of MIA on the proteome, I wanted to perform a morphological readout to determine how these changes related to the

dendritic architecture and spine morphology. To do so, I sparse-labelled neurons of the HPC and the prefrontal cortex (PFC), based on its role in cognition and susceptibility to MIA- and NPD-related changes, and assessed those in MIA offspring, building upon previous morphological studies in the model. Throughout this study, sex-specific differences were highlighted due to their relevance in NPD symptomatology and prevalence.

The second study, "Expanding on the Role of Cytoplasmatic RBFOX1 in the Brain" comprised an investigation of neuronal-specific overexpression (OE) of the cytoplasmatic form of the neurodevelopmental regulator and RNA-binding protein RBFOX1. The *RBFOX1* gene was highlighted by large-scale, human genomic studies as one of the main pleiotropic genes in major psychiatric disorders, including ASD, SCZ, and ADHD. Furthermore, previous studies from our department have demonstrated its functional role in cognitive and emotional processes, as well as presented evidence for ASD-like endophenotypes in male mice with neuronal *Rbfox1* downregulation. Thus, I performed a targeted interrogation of RBFOX1's role in a transgenic mouse model of neuron-specific, cytoplasmatic *Rbfox1* OE. This included protein abundance validation and dendritic- and spine-morphology analyses in the HPC and PFC of male mice, with the addition of the amygdala due to its role in emotional and fear responses, which are affected in multiple NPDs and comorbidities, and by known *RBFOX1* risk variants. Next, an experiment combining the validated MIA and cytoplasmatic RBFOX1 OE mouse models sought to uncover gene × environment interaction effects on maternal care and NPD-relevant behaviours of adult male and female offspring. Finally, a molecular investigation in *post-mortem* samples from the dorsolateral PFC of older, psychiatrically-healthy adults was used to probe the effects of a common *RBFOX1* polymorphism on protein abundance.

The third study titled "Diras2 Expression Levels Exert Subtle Behavioural and Morphological Effects Related to ADHD" probed the effects of neuronal-specific, homo- and heterozygous deletion of the ADHD-candidate gene *Diras2* – a Ras kinase involved in neurodevelopment and cellular signalling – in a mouse model. Here, an evaluation of NPD-relevant behavioural outcomes of the partial and full *Diras2* knockout in adult male and female mice was performed, using unpublished data from our laboratory. Next, dendritic and spine architecture were assessed, as described above, in the HPC, PFC, and amygdala of adult males from both *Diras2* genotypes and controls. Finally, in order to gain insights into the *in*

vivo effects of *Diras2* downregulation, mRNA quantification was performed in the brains of adult female mice. Expression of *Diras2* and its paralog *Diras1*, as well as downstream targets with neurodevelopmental relevance shown to be affected by the gene's deletion *in vitro*, was analysed in NPD-relevant brain regions, i.e. HPC, PFC, amygdala, nucleus accumbens, hypothalamus, and cerebellum.

In summary, this dissertation aimed to demonstrate and compare observed behavioural, molecular, and morphological alterations in the NPD-like mouse models utilising prenatal immune challenge and transgenic manipulations of the neurodevelopmentally-relevant genes *Rbfox1* and *Diras2*. At its core, this work's aim is to expand upon our knowledge of NPD aetiology and offer preclinical findings with the translational purpose of informing future, improved treatment opportunities.

3. METHODS

3.1 Study 1: "Consequences of MIA for Neuromorphology and the Embryonic and Adult Hippocampal Proteome"

3.1.1 Animals

All experiments were performed with wild-type *C57BL/6J* mice (*Mus musculus*), obtained from a commercial vendor (Janvier Labs, Le Genest-Saint-Isle, France) or their F1 offspring, bred in-house. Experimental mating, immune activation procedure, behavioural testing, and morphological analyses was performed in adult mice above 12 weeks of age with the exception of stimulus mice used for social interaction, which were juvenile conspecifics (5 weeks old at testing) obtained from the same vendor. A minimal period of 7 days post-arrival was allowed for acclimation to the new environmental conditions (Obernier & Baldwin, 2006). For a full list with animal numbers, please see Table 2, Supplementary Tables S1-S2. Standard food pellets and autoclaved drinking water were provided ad *libitum*. The animals were housed in same-sex groups of 3-5 mice in individually ventilated cages (IVCs) with a microbiological filter (Tecniplast, Buguggiate, Italy), containing wood chip bedding and enriched with naturalistic nesting material. The mice were housed in the central research facility of the University Hospital Frankfurt under controlled environmental conditions: constant room temperature of 21±1°C, air humidity levels of 55±5%, and a 12 h light/dark cycle (light phase: 7:00-19:00 h). All experiments were conducted in accordance with institutional guidelines, current national legislation (*TierSchG, TSchV*), and the EU directive regarding the use of experimental animals animals for scientific purposes (*86/609/EEC*). Ethical approval was obtained from the local authorities (Regierungspräsidium Darmstadt, licence *AZ: FK/1101*).

3.1.2 Maternal Immune Activation (MIA)

MATING AND PREGNANCY DETERMINATION

An overview of the timeline and design is provided in Figure 3.1. Naïve *C57Bl/6J* dams aged 12±2 weeks were introduced to a cage with males in a 2:1 female-to-male ratio, separated in the morning, and left undisturbed for a week. The males were age-matched with the first mating cohort and reused as experienced sires for an additional cohort (up to 5 months old).

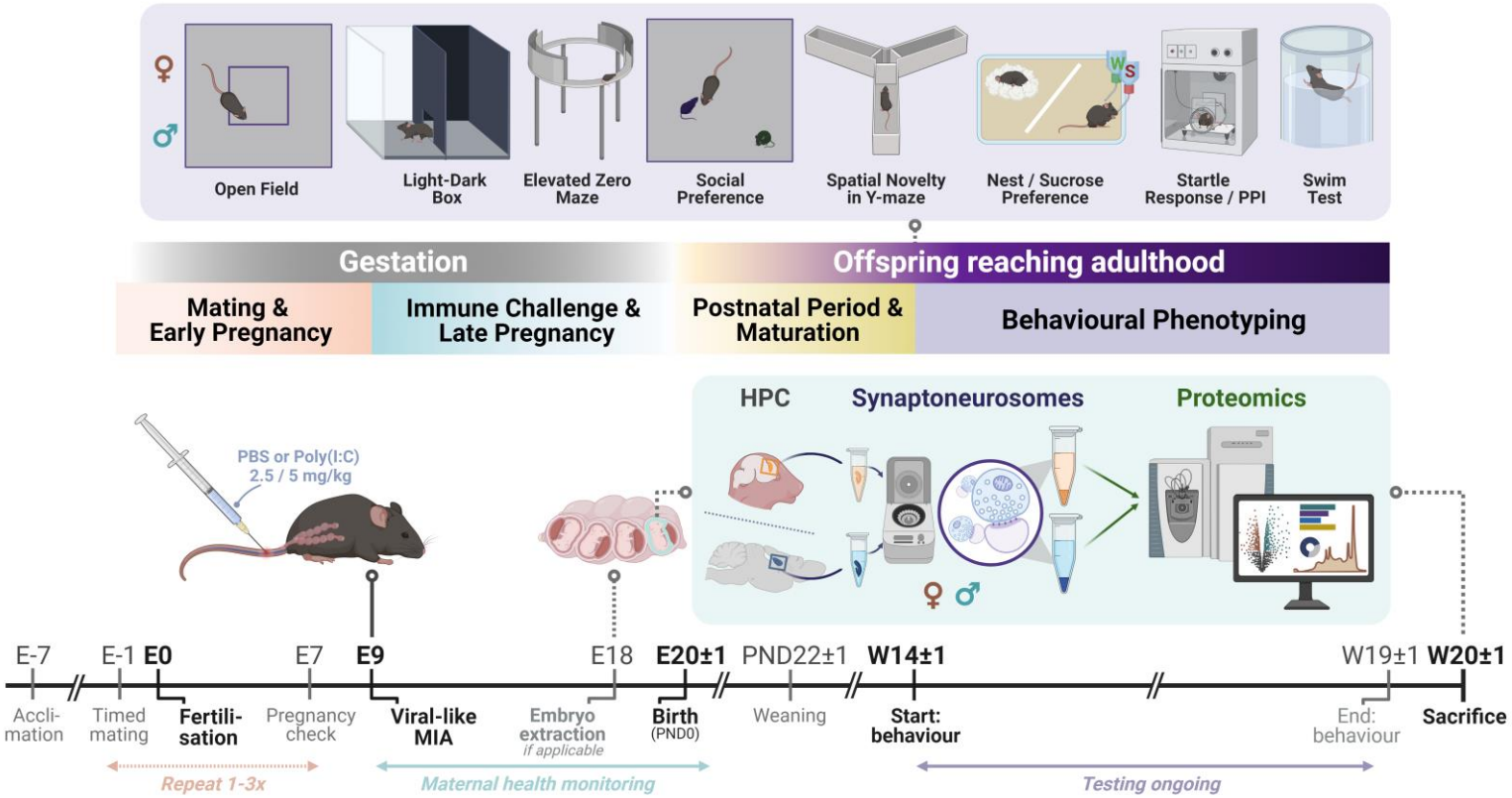


Figure 3.1 Timeline and summary of experimental design. Maternal immune activation (MIA) was induced by 2.5 or 5 mg/kg viral-like immunostimulant poly(I:C) i.v. injection in *C57BL/6J* mice during early pregnancy (E9). An equivalent vehicle injection of phosphate-buffered saline (PBS) was used for controls and all dams' health was closely monitored. In four separate cohorts, male and female offspring were either extracted before birth (E18, n=92) for proteomic analysis in hippocampal (HPC) synaptoneurosomes or allowed to reach adulthood. Adult offspring (n=83) was tested via a behavioural test battery for psychiatrically relevant endophenotypes. We assessed locomotion, exploration, and anxiety-like behaviour (Open Field, Light-Dark Box, Elevated Zero Maze), social memory and preference, spatial novelty response and memory (Y-maze), acoustic startle reflex and sensorimotor gating (pre-pulse inhibition, PPI), and depressive-like behaviours (Nesting, Sucrose Preference, Swim Test). Mouse brains were collected, and SN proteomics or morphological analyses (see Chapters 3.1.4-3.1.5) were performed. See also Table 2, Figure 3.2, Figure 3.3.

Bedding from the female home cage was used to encourage mating behaviour in the males through olfactory cues. The mice were placed together 2 h before their active dark phase (Braden, 1957) and returned to their home cages the following morning. At separation, all females were examined for successful copulation and oestrous cycle phase utilising visual inspection of the vaginal opening and presence of a vaginal plug (Byers *et al.*, 2012). Initial microscopic investigation of vaginal cytology for determination of the reproductive cycle and for spermatozoa (Cora *et al.*, 2015) via a vaginal smear on embryonic day 0 (E0, defined as the light cycle immediately after mating) while establishing this model proved unreliable, resulting in a number of false positive and negative pregnancies. Serendipitous discovery of a viscous mucification indicative of oestrus cycle cessation in the vaginal canal of pregnant dams on E7, clearly identifiable by a simple visual inspection, eliminated false negatives and decreased false positives five-fold (ese factors combined with maternal weight on E9 immediately before injection, as to keep disturbances to a minimum (Heyne *et al.*, 2015), were used for definitive pregnancy determination, after which MIA induction commenced.

Only primiparous females were used for this experiment. However, to adhere to the strict animal number guidelines, non-pregnant females were mated for up to two additional cycles, a week after the initial mating and the last vaginal swabs occurred. This period of undisturbed state should be enough to overcome diapause, in mouse first occurring at day 3-4, considering the lack of strong environmental and other stress stimuli or postpartum hormone disbalance and lactation (Fenelon *et al.*, 2014; Renfree & Fenelon, 2017). The described mating and testing procedures were repeated up to two times with all females identified as not pregnant. Information on mating, immune challenge procedure, and experimental design are provided in accordance with established guidelines (Kentner *et al.*, 2019).

MIA PROCEDURE

MIA was induced on E9 of the putative pregnancy according to Meyer *et al.* (2005) by administration of the viral mimetic polyinosinic:polycytidylic acid (poly(I:C), a synthetic RNA immunostimulant that binds to toll-like receptor 3 (TLR3) and induces transient infectious-like states by cytokine pathway activation. Dams were injected with vehicle (phosphate-buffered saline, PBS 1x) or equivalent parts 2.5 or 5 mg/kg poly(I:C) solution, prepared from poly(I:C) potassium salt (Sigma-Aldrich, Schnelldorf, Germany) aliquoted in PBS.

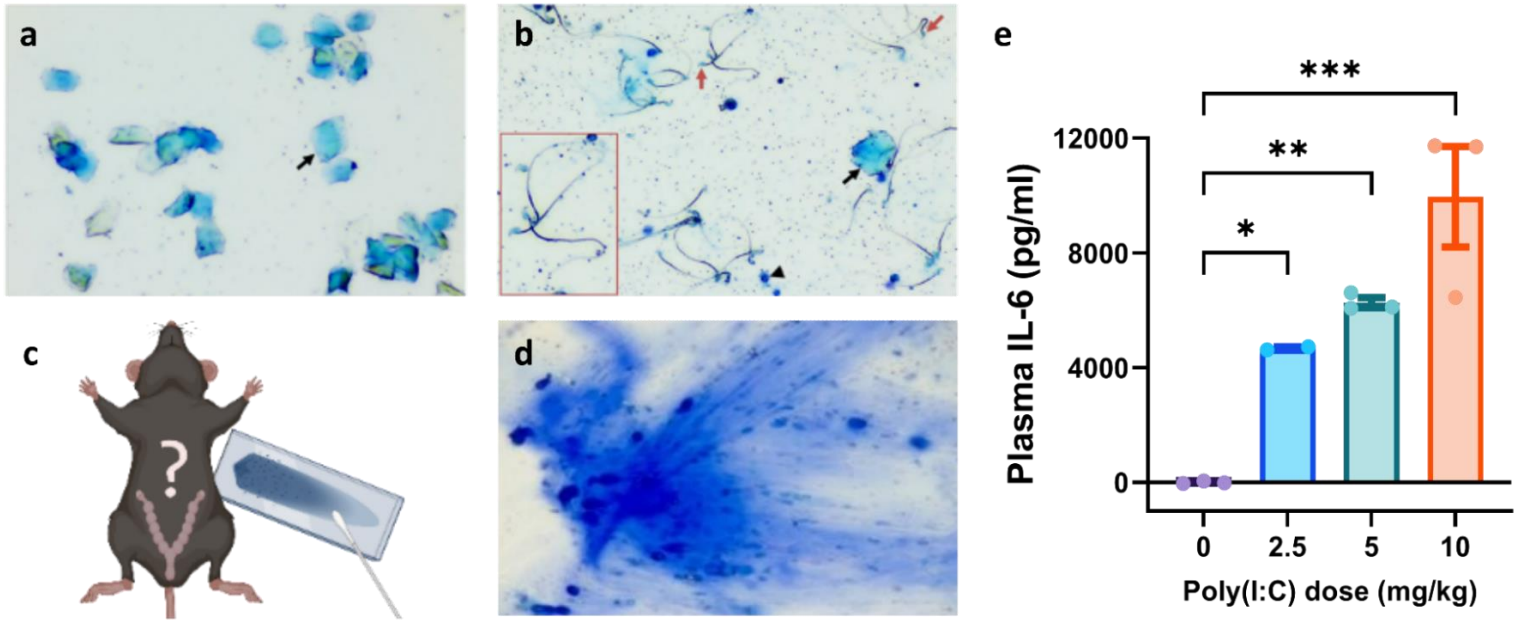


Figure 3.2 Tools for pregnancy determination and maternal immune activation (MIA) model validation. Visual determination of mating success was performed first by visual inspection of the vaginal opening and the presence of a vaginal plug. Additionally, embryonic day E9 maternal weight and cytological analysis of intravaginal secretion samples on day E0 (light phase after mating, *pilot data*) or E7 (*dams of offspring used here)* were used. Vaginal smears were stained with methylene blue solution and investigated microscopically (Axio Observer Z1, Carl Zeiss Microscopy; 25x magnification) for determining the female oestrus cycle stage (**A-C**). During the fertilisation-receptive oestrus (**A**), the samples comprised large anuclear, keratinised epithelial cells (*black arrows*) and cell debris. Additionally, spermatozoa (**B;** *red arrows*, *zoom-in in left-corner red-framed box*) were occasionally observed, distinguished by their characteristic hooked heads and long flagella. E0 cytology-informed pregnancy decisions led to a high number of both false positives and negatives, so later pregnancy determination was aided by the serendipitously discovered vaginal mucification on E7. This secretion indicated oestrus cycle cessation and birth canal changes (**D**, 10x magnification), and was clearly identifiable by naked eye. For validation of the MIA model, levels of the pro-inflammatory cytokine IL-6 were measured in blood plasma (1 h after MIA) of a separate cohort of pregnant dams (n=2-3 biological & technical replicates each), using a sensitive enzyme-linked immunosorbent assay (ELISA MAX). Panel **E** shows significant immune responses to the treatment with poly(I:C) (one-way ANOVA, $p<0.001$; *mg/kg*: *2.5 v 0,* *$p=0.04$; *5 v 0,* **$p=0.006$; *10 v 0*, ***$p<0.001$, *post-hoc*), thus validating the implemented immune stimulation procedure. Data presented as mean ± SEM.

The solutions were randomly administered at 5 ml/kg body weight intravenously (i.v.) in the lateral tail vein with a 27-gauge needle, using a low-stress, reduced restrain tail injection platform (In Low, AIMS Lab Products; *coloured in red*). The severity of induced MIA state was assessed by monitoring the dams' activity, spontaneous behaviour, overall, and weight over a period of 2 weeks using an animal licence-approved scoring system at multiple time-points (6 times within 72 h, at day 7 and 14 post-procedure).

The success of immune activation was further validated in blood plasma collected from a separate cohort of MIA dams (n=3, except 2.5 mg/kg n=2 biological replicates; n=2 technical replicates each) 1 h after poly(I:C) injection, showing a significant, dose-dependent increase in the relevant pro-inflammatory cytokine interleukin IL-6 (Hsiao & Patterson, 2011) using a sensitive enzyme-linked immunosorbent assay (ELISA MAX, BioLegend, Koblenz, Germany; Figure 3.2E). Treatment dose had a significant, dose-dependent effect on plasma IL-6 concentration ($F_{(3,7)}$ = 19.2, p<0.001; multiple comparisons, *0 v 2.5 mg/kg*: 0.04, *0 v 5 mg/kg*: p=0.006, *0 v 10 mg/kg*: p<0.001. Preliminary tests showed that poly(I:C) doses of 7.5 and 10 mg/kg (maternal plasma IL-6 tested for the latter, see Figure 3.2E) poly(I:C) led to complete foetal loss, indicating these doses were too high for our institution.

In total, 93 mice (n=64 dams, 22 sires) were successfully mated and used for MIA in three cohorts, producing 30 surviving litters (see Table 2). All resulting offspring (n=4-7 litters/group) were utilised for either behavioural, morphological, or embryonic and adult molecular analyses. Delivered offspring were weaned at postnatal day PND22±1, group housed according to sex (n=2-5 per cage), and allowed to mature for behavioural phenotyping and brain tissue collection in adulthood.

3.1.3 Behavioural Experiments

Adult offspring (n=83, 40 males, 43 females from 5±1 litters/group; see Table 2) was phenotyped using a battery comprising nine behavioural tests in order of increasing aversiveness, starting at 14±1 weeks (see Figure 3.3A). In a few cases, data was not available for individual animals in a test due to technical issues, e.g. if mice left the test apparatus, so numbers of individual animals for each test are presented in Supplementary Table S1. Locomotion and exploration, anxiety- and depressive-like behaviours, sensorimotor gating, sociability, and memory in different contexts were assessed in a sex-dependent manner.

These were adapted from experimental designs published previously (Freudenberg *et al.*, 2021; O'Leary *et al.*, 2022). All experiments were performed in the light phase under controlled environmental conditions (RT=22±1°C; humidity=45±5%). The mice were acclimated to the experimental room and ambient conditions for at least 45 min before testing and a resting period of min. 1-3 days was allowed between tests, based on severity. The experimental apparatuses were thoroughly cleaned with 20 % aqueous solution of ethanol between trials. The behavioural experiments lasted 6±1 weeks and the test animals were left undisturbed for at least a week before sacrifice for organ collection.

Table 2 Animal numbers according to experimental design, sex, and dosage of immunostimulant challenge on embryonic day E9 (*Ctrl*: control, PBS injected, *Low*: 2.5 mg/kg poly(I:C), *High*: 5 mg/kg poly(I:C), intravenous injection). Females (*F*) in the pregnancies category correspond to injected dams and males (*M*) to respective sires. For more information on animals used for each experiment, see Supplementary Tables S1-S2.

Experimental Cohorts	MIA PROCEDURE				MIA OFFSPRING					
	Mated mice #		Pregnancies #		Litters # *(average size ± SEM)*			Tested animals # *(F / M)*		
	F	M	F	M	Ctrl	Low	High	Ctrl	Low	High
Adult behaviour, morphology, and proteomics	64	22	44	18	5 *(7.4±0.2)*	6 *(6.2±0.5)*	5 *(3.4±0.8)*	34 *(17/18)*	33[1] *(18/15)*	15 *(8/7)*
Embryonal proteomics	28	10	22	9	6 *(5.7±1.4)*	4 *(8.5±0.6)*	4 *(7.5±0.3)*	30 *(14/16)*	32 *(16/16)*	30 *(14/16)*
TOTAL	124		93		32			174		

[1]low concentration animals used only for phenotyping, the rest of adults for both behaviour and proteomics.

ELEVATED ZERO MAZE (EZM)

The EZM setup (Stoelting Europe, Dublin, Ireland) consists of a circular platform (inner Ø 50 cm, lane W 5 cm, H 50 cm) under an infrared (IR) LED illuminator (Sygonix GmbH, Nürnberg, Germany; see Figure 3.3B). The walkway comprises four quadrants: two opposing enclosed quadrants with IR-transparent, black acrylic walls (H 15 cm), and two corresponding open ones. Illumination was measured at 30±1 lux in the closed arms and at 140±5 lux in the open arms using a luxmeter (Conrad Electronic SE, Hirschau, Germany). In the beginning of the test, the mice were placed in the same enclosed compartment. Their movement was recorded for 10 min via an IR-sensitive digital camera (The Imaging Source Europe GmbH,

Bremen, Germany; *used for all subsequent tests if not stated oth*erwise). Behaviour was analysed using automated tracking software (ANY-maze v6.0, Stoelting Europe). Line crossings and time spent in the open arms were used as measurements of exploratory and anxiety-related behaviour.

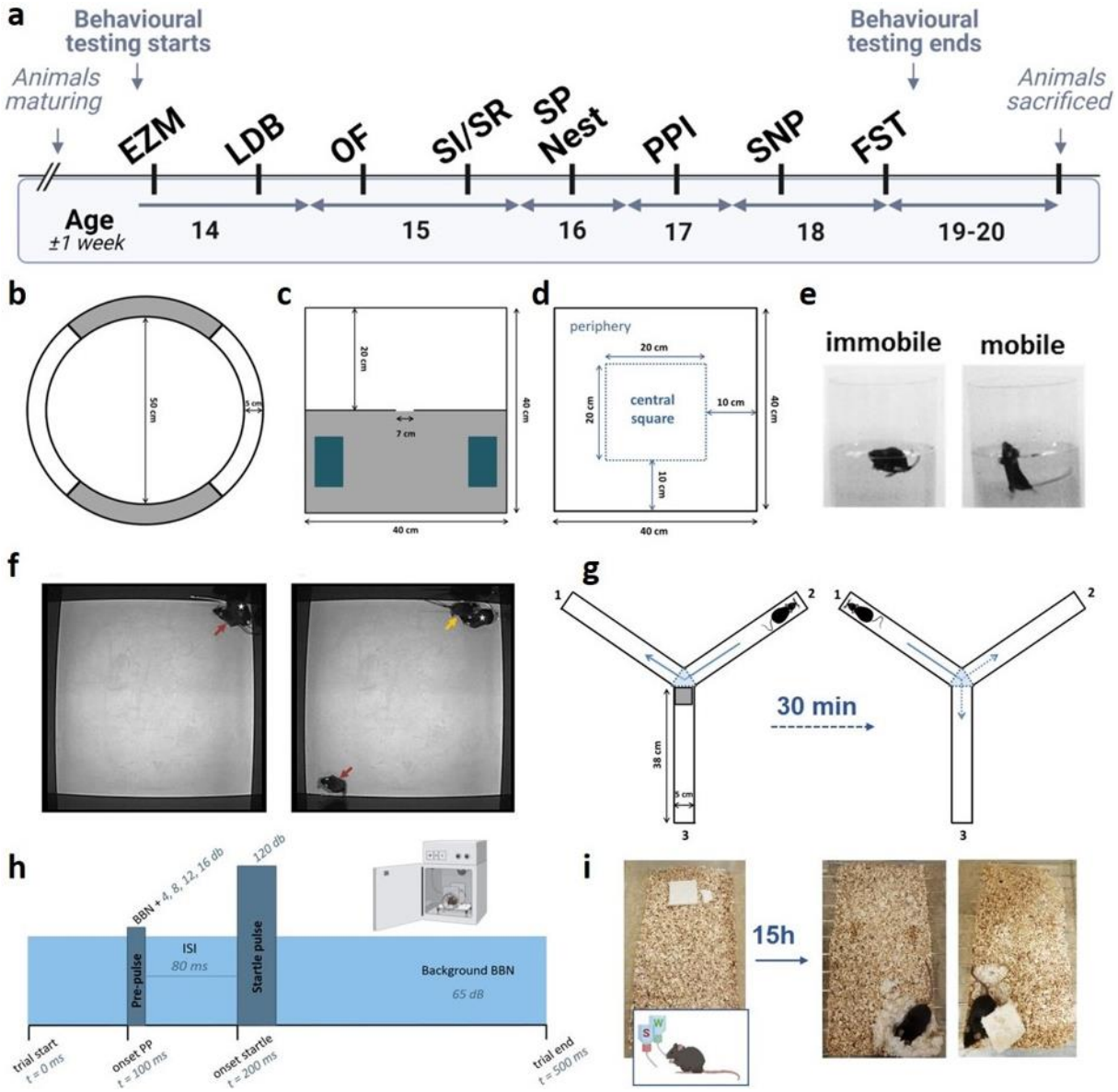


Figure 3.3 Behavioural experimental design and setup. The behavioural battery comprised nine behavioural tests for psychiatrically relevant endophenotypes, depicted in chronological order (**A**). Spontaneous exploration and anxiety-like behaviours were measured in an EZM (**B**), LDB (**C**), and OF (**D**) tests over 10-15 min (*grey shading*: dark, enclosed spaces). Social deficits were assessed in a SI/SR experiment (**F**): the test mouse (*white asterisk*) could freely interact with a sex-matched juvenile conspecific (*red arrow*) in an initial SI trial. After 30 min, the now familiar conspecific was reintroduced together with a novel conspecific (*orange arrow*) to assess social interaction and memory (SR), using novelty preference. *Continued.*

Continued. Spatial working memory was tested via SNP in a Y-maze (**G**). On five consecutive trials (*left panel*), performed with randomised, alternating staring arms and one blocked arm (*shown*: start arm 2, novel arm 3), mice were allowed to explore freely for 2 min after entering the other open arm (max. 4 min). In the sixth test trial (*right panel*) 30 min later, all arms were open for exploration and SNP was assessed as time spent in the novel arm compared to time spent in all arms, disregarding the time in the centre (*blue shaded area*). Sensorimotor gating was examined via PPI in an ASR chamber (**H**). Here, a schematic of an example pre-pulse and startle pulse trial is shown, a variation of which was pseudorandomly delivered for assessment of PPI. A PP-trial consisted of background BBN with 20 ms PP (either 69-, 73-, 77-, or 81-dB SPL, 10x each), preceding a 40 ms long 120 dB SPL startle pulse. Additionally, 10x each PP-, startle-, and BBN-only trials were presented, as well as six startle-only trials at the start and end of the session to test ASR habituation. Anhedonic and self-neglect behaviours were modelled by SP and nesting tests (**I**), respectively. SP assessed consumed volumes of 2% sucrose and drinking water over 48h (position of bottles reversed after 24h), allowing an assessment of innate rodent reward sensitivity. Overnight nest building was appraised by the weight of unused material and nest integrity on a 1-5 scale. Shown here (**I**) are nests of a control (*left*, score 4) and a treated (*right*, score 2) male. Finally, depressive-like states and habituation learning were evaluated using a two-day FST, assessing immobility time and latency (**E**). After the end of behavioural testing, mice were allowed to recover for 1-2 weeks (**A**), after which their brains were collected and stored at -80°C for future molecular analyses. *ASR*, acoustic startle response; *BBN*, broadband noise; *EZM*, elevated zero maze; *FST*, forced swim test; *ISI*, inter-stimulus interval; *LDB*, light-dark box; *OF*, open field; *OI/OR*, object interaction and recognition; *PP*, pre-pulse; *PPI*, pre-pulse inhibition; *SI/SR*, social interaction and recognition; *SNP*, spatial novelty preference in a Y-maze; *SP*, sucrose preference.

LIGHT-DARK BOX (LDB)

The LDB (Stoelting Europe) comprises two compartments (W 20 x L 40 x H 35 cm each, opening between chambers: 7 x 7 cm): one brightly lit (400±10 lux) with clear acrylic walls and the other - a closed-off dark chamber (3±0.2 lux) with IR-transparent, black acrylic walls, equipped with two IR-illuminators on top (Instar, Hünstetten, Germany; see Figure 3.3C). The mice were placed in the dark compartment and allowed to explore both chambers freely for 10 min. Behavioural tracking and analysis was automatically performed as described for the EZM. The distance travelled and the time spent in each compartment were assessed as activity- and anxiety-related measures.

OPEN FIELD (OF) TEST

The OF was used to assess spontaneous locomotion, exploration drive, and anxiety-like behaviour. The OF comprised a square arena (Stoelting Europe) with dark acrylic walls (W 40 x L 40 x H 35 cm, 120-140 lux illumination; see Figure 3.3D), equipped with an IR-sensitive digital camera and ANY-maze tracking software. The total area of the arena was digitally

divided in two fields: the central square had an area of 20 x 20 cm, and the periphery compounded the surrounding area within 10 cm of the walls. Mice were placed in one corner of the OF arena and activity was recorded for a period of 15 min. The total distance travelled and time spent in the centre, used for measuring anxiogenic effects, were automatically analysed with ANY-maze.

SOCIAL INTERACTION AND RECOGNITION (SI/SR)

The established SI/SR was used as a tool to identify aberrant social behaviours and/or memory, which are hallmarks of multiple psychiatric disorders. For the SI trial, the mouse was placed in the corner of the OF arena for 1 min of habituation, followed by an introduction of an unfamiliar, juvenile conspecific for a 5 min free interaction (see Figure 3.3F, left panel). 30 min after the trial ended, the test mouse was returned to the OF arena to explored freely for 1 min, after which the familiar SI and a novel juvenile stimulus mouse were introduced to the arena for 5 min (see Figure 3.3F, right panel). Sex-matched, 5±1 week-old mice of the same strain were used as stimulus mice. Juvenile mice were chosen to avoid fighting following social provocation, specifically between males (Winslow, 2003). The social interactions, defined as active sniffing or following, were recorded digitally and the first 3 min analysed offline, using a semiautomated tracking protocol (ANY-maze). Data on the number and total duration of interactions was obtained for each novel and familiar conspecific, and social novelty preference was calculated as a percentage of total time or number of social interactions.

SUCROSE PREFERENCE (SP)

Modelling anhedonia, expressed in rodents as attenuated sensitivity to reward, has long been established as a depressive-like endophenotype and can be measured experimentally by decreased SP in mice (Willner *et al.*, 1987). During the SP experiment the animals were individually housed over a period of 48 h and presented with two identical drinking bottles with ca. 100 ml 2% sucrose (Sigma-Aldrich) solution and water, respectively (see Figure 3.3I, left panel). To minimise the effects of preference of one side over the other, the position of the bottles was switched after 24 h. The full bottles were weighed before the test and again at 24 and 48 h, and SP (%) was calculated as follows: *[(sucrose solution intake [g]) / (total liquid intake [g])] x 100*.

NESTING BEHAVIOUR

In parallel to the SP test, nesting behaviour was assessed as a measure of self-neglect, since hippocampal impairments have been associated with deficits in nesting of mice(Deacon, 2006). For the nesting experiment 3 g of pressed cotton (Nestlets, Ancare Corp., Bellmore, NY, USA) were placed in the otherwise nesting material-free cage shortly before the dark phase and assessed after approx. 15 h (see Figure 3.3I, right panel). The full nest was assessed after the dark phase both quantitatively and qualitatively by weighing any remaining unused cotton material (pieces > 0.1 g) and using the scale previously published by Deacon (2006). 24 h after the first test, the experiment was repeated with fresh cotton material to examine possible susceptibility to effects of single-housing on nesting behaviour (Hohlbaum *et al.*, 2022). There were no significant differences observed, so data presented here is averaged from the two trials.

PRE-PULSE INHIBITION (PPI)

Animal models of psychiatric disorders exhibit central deficits in sensorimotor gating, i.e. abnormal PPI of the robust acoustic startle response (ASR), which can be reliably measured and compared across species (Swerdlow & Geyer, 1998). This experimental design has been previously described elsewhere (Freudenberg *et al.*, 2021; O'Leary *et al.*, 2022). Briefly, ASR experiments were conducted with a SR-LAB startle response system (San Diego Instruments Inc., San Diego, CA, USA; see Figure 3.3H). A test session lasted approx. 35 min per animal, during which time background broadband noise (BBN) was played at 60 dB SPL in the experimental room to minimise startle habituation of yet untested mice. Following 5 min for acclimation in the chamber to a background BBN (65 dB SPL), 82 pseudorandomised trials of 500 ms each were delivered as follows: 6 startle pulse-only trials every 10 s (startle pulse level=120 dB SPL, t=40 ms, onset=200 ms) were presented at each the beginning and end of a session. 10 pre-pulse (PP) and stimulus trials each with PP levels of 69, 73, 77, 81 dB SPL (PP t=20 ms, onset=100 ms), as well as 10 of each startle pulses, PPs alone (81 dB SPL), and no-stimulus trials with inter-trial intervals (ITIs) of 20-30 s were further presented (see Figure 3.3H). ASR of pulse-only trials were averaged for each animal, measuring startle amplitude at the beginning and end of a session to assess habituation. PPI was calculated using the formula *PPI = 100 × ([startle-only units − (pre-pulse + startle units)] / (startle-only units))*.

SPATIAL NOVELTY PREFERENCE (SNP)

Unconditioned spatial novelty preference, used to assess the integrity of hippocampal function as a neural correlate of spatial working memory, and was performed here in a Y-shaped maze. The Y-maze comprised 3 equivalent arms (W 5 x L 38 x H 10 cm, Stoelting Europe): one start arm, another open arm, and a blocked novel arm (see Figure 3.3G, left panel). 6 trials were performed for each mouse – 5 trials with the same novel arm blocked, where the mouse was put in alternating start arms after 1 min retention periods, and a test trial, in which all 3 arms were freely accessible (see Figure 3.3G, right panel). The duration of each trial was set at 2 min after the mouse has entered an arm other than the start arm, or a maximum of 4 min. The spatial novelty preference experiment was recorded and digitally tracked as described above using ANY-maze tracking software. Relevant data from the test trial included distance and time spent in each arm and novel arm preference - a calculation of the time spent in the novel arm as percentage of total time in all arms.

SWIM TEST (FST)

To assess depression-like symptoms, mice were tested in a FST paradigm. Immobility, defined as motionless floating or minimal movement to keep the mouse above water (Slattery *et al.*, 2012), was used as main output (see Figure 3.3E). Mice were placed in cylindrical glass containers (Ø 17 cm, H 33 cm) filled with warm water (t=25±0.5°C, approx. 5.5 L) and digitally recorded (The Imaging Source Europe) for 6 min each. Immobility was analysed offline in a blinded, semiautomated manner to assess immobility rate: *100* × (*time immobile / total time*).

3.1.4 Proteomics and Bioinformatics

TISSUE PREPARATION

Adult mice were anaesthetised with isoflurane gas (Isofluran-Piramal, Piramal Critical Care, Hallbergmoos, Germany), sacrificed via decapitation, their brains swiftly extracted, and snap-frozen. Some brains were used for morphological analysis (see Chapter 3.1.5). The brains were dissected on a cold plate (t=4°C) and the hippocampi (HPC) stored at -80°C for synaptoneurosome preparation. For the embryonic (E18) sample cohort, dams were sacrificed in the same manner, foetal brains and hippocampi were extracted in ice-cold cold dissection medium (Hank's balanced salt solution, HBSS, Thermo Fisher Scientific) and stored liquid-free

at -80°C. Additionally, embryonic tail clippings were used to determine the sex by performing a simplex PCR assay (KAPA2G Fast HotStart Genotyping Mix, Thermo Fisher Scientific) as described elsewhere (Clapcote & Roder, 2005). Accordingly, the X-chromosome-specific gene *Jarid1c* (Ensembl Transcript ID: ENSMUST00000082177) and the Y-specific *Jarid1d* (Ensembl Transcript ID: ENSMUST00000055032) were used for sex determination. Tissue extraction shortly before birth allowed for an examination at a time after supportive cells involved in immune and cellular responses, i.e. astrocytes and oligodendrocytes, have differentiated (La Manno *et al.*, 2021), but before the perinatal excitatory-to-inhibitory GABA switch, which is affected by MIA (Corradini *et al.*, 2018), is completed.

SYNAPTONEUROSOME (SN) EXTRACTION

For SN preparation, a filtration method (Hollingsworth *et al.*, 1985) was used, similar to previous descriptions (Chang *et al.*, 2012). HPC samples were mechanically homogenised in ice-cold SN buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.53 mM KH_2PO_4, 212.7 mM glucose, 1 mM kynureic acid, 0.5mM ethylene glycol tetraacetic acid (EGTA), 5 mM HEPES, protease inhibitors, 1mM DTT, adjusted to pH=7.4) and filtered through a PTFE membrane (Mitex 5 µm, Merck Millipore, Darmstadt, Germany). The homogenate was centrifuged at 1000 x *g* (1 min, 4°C) to remove the nuclei. The supernatant was then centrifuged at 14 000 x *g* (4 min, 4°C). The resulting SN-enriched pellets containing myelin and mitochondria were resuspended in buffer phosphate-free buffer (118mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.5mM EGTA, 5 mM HEPES, protease inhibitors, 1 mM DTT, adjusted pH=7.4) and protein measured. After another centrifugation (14 000 x *g*, 4 min at 4°C) and protein measurement, the pellet was resuspended (50 mM Tris, 1% SDS, pH=8.0 buffer) at 65°C under agitation and used for proteomic analysis. In total, four replicates of each control and high concentration poly(I:C) offspring were used for proteomic analyses (E18 samples: 14 F, 16 M and 4 replicates per group, n=7-8 embryos/ replicate; adult samples: 2 M, 2 F and 4 replicates per group, replicates represent individual animals; see also Table 2).

PROTEIN DIGESTION AND MASS SPECTROMETRY ANALYSIS

Mass spectrometry analysis was performed at the Biocenter Finland-supported Turku Proteomics Facility (University of Turku and Åbo Akademi University). 25 or 50 µg of protein for embryos and adults, respectively, were taken for in-solution digestion and subjected to

filter-aided sample preparation (FASP), according to the facilities protocol (Wisniewski *et al.*, 2009). Samples were suspended in urea buffer (8 M Urea, 50 mM Tris-HCl, pH 8.0) and proteins were reduced with 10 mM DTT for 30 min at 37°C, then added to Microcon YM-10 Centrifugal Filter Units (Merck Millipore) and centrifuged for 15min at 14 000 x *g*. Proteins were alkylated with 40 mM iodoacetamide for 30 min in the dark at room temperature. Samples were centrifuged for 30 min at 14 000 x *g*, followed by three washes with 50 mM Tris-HCl, pH 8.0, with centrifugation for 15 min at the same speed each. Proteins were digested with a 1:25 (enzyme:protein) ratio of trypsin (Trypsin Gold, Promega, Walldorf, Germany) for 18 h at 37°C. The resulting peptides were recovered by centrifugation at 14 000 x *g* for 40 min, followed by elution with 50 µL of 500 mM NaCl, and centrifuged again for 20 min. Combined eluates were acidified using trifluoroacetic acid (TFA) to a final concentration of 1 %. After digestion, peptides were desalted with a Sep-Pak C18 96-well plate (Waters, Milford, MA, USA), evaporated to dryness, and stored at -20°C. Digested peptide samples were dissolved in 0.1 % formic acid and peptide concentration was determined with a NanoDrop device (Thermo Fisher Scientific). For data-independent acquisition (DIA) analysis, 600 ng (adults) or 800 ng (embryos) of peptides were injected and analysed in random order. Wash runs were submitted between each sample to reduce potential carry-over of peptides.

Samples were analysed by a DIA liquid chromatography with tandem mass spectrometry (LC-MS/MS) method on a nanoflow high-performance liquid chromatography HPLC system (Easy-nLC1200, Thermo Fisher Scientific) coupled to a high-resolution, accurate-mass Orbitrap Fusion Lumos (adults) or Q Exactive HF (embryos) mass spectrometer (Thermo Fisher Scientific), equipped with a nano-electrospray ionisation source. Peptides were first loaded on a trapping column and subsequently separated inline on a 15 cm C18 column (ReproSil-Pur C18-AQ - 3 µm/120 Å for embryos; 5 µm/200 Å for adults, Dr. Maisch, Ammerbuch-Entringen, Germany). The mobile phase consisted of water with 0.1 % formic acid (solvent A) or acetonitrile/water (80:20 v/v) with 0.1 % formic acid (solvent B). A 120 min gradient was used to elute peptides (70 min 5-21 % solvent B, 40 min 21-36 % solvent B, followed by ramping solvent B concentration up to 100 % in 5 min and 5 min washout with 100 % solvent B / 120 min gradient 7-32 % solvent B for adult samples). MS data was acquired automatically by using Xcalibur 4.1 software (Thermo Fisher Scientific). In the DIA method, a duty cycle contained one full scan (resolution 120 000, AGC target 3e6, max.

injection time 50 ms, 400-1000 m/z) and 40 DIA MS/MS scans (resolution 30000, AGC target 1e6, automatic max. injection time), covering the mass range 400-1000 with variable width isolation windows.

PROTEIN IDENTIFICATION, QUANTIFICATION, AND CLUSTERING ANALYSIS

Data was processed by Spectronaut (v18.0.2, Biognosys, Schlieren, Switzerland) software and analysis consisted of protein identifications and label-free quantifications of protein abundances. Direct DIA approach was used to identify proteins and label-free quantifications were performed with the intensity determination and normalization procedure MaxLFQ. Main data analysis parameters were: (I) Enzyme: Trypsin/P; (II) missed cleavages: 2; (III) fixed modifications: carbamidomethyl (cysteine); (IV) variable modifications: acetyl (protein N-terminus) and oxidation (methionine); (V) protein database: *Mus musculus* Swiss-Prot reference proteome (UniProt release 2023_01); and (VI) normalisation: Global normalisation. Differential abundance analysis was performed using in-built functionality of the Spectronaut software on *log2*-transformed protein quantity values.

The resulting detected protein lists were then cleaned by removing histones and keratins considered impurities (n=24 in adult, 29 in embryonic data set), and used for all subsequent analyses. All data sets were filtered at biologically relevant thresholds of 20 % absolute change (1.2>fold change, FC >0.8) and multiple testing corrected significance values $q<0.1$ (uncorrected $p<0.05$). The detection of a larger number of proteins for the embryonic dataset (n=7 723 vs 4 744 for adults) could result from the immature state of the synapse influencing the synaptoneurosomal composition and isolation, or the marginally higher sensitivity of mass spectrometry equipment used. To explore the comparability of data further, we randomly selected five (8.7%) of the proteins only detected in adults, for which or whose paralogs data was available in the Allen Brain Atlas (Thompson *et al.*, 2014) for the developing mouse brain. We compared the expression profiles between the late embryonic (E15.5-18.5) and postnatal (P14-28) brain. For the five probed proteins (GRIN2A, LRRTM1/2, NEFH, SCN1A, VAMP1) corresponding gene expression in the telencephalic vesicle, giving rise to the HPC, was significantly higher after birth. This suggests that the differences in the number of identified proteins might be rooted in physiological changes in expression during brain development.

For visualisation, abundance differences were calculated as *log2*(FC) and significance as negative *log10*(q-value). The heat map and hierarchical clustering of the differentially regulated proteins common to the adult and embryonic datasets was generated using the *pheatmap* R package. The colour scale represents *log2*(FC) in protein expression in synaptoneurosomes ranging from downregulated (*blue*) to upregulated (*orange*).

ENRICHMENT AND NETWORK ANALYSES

Gene symbols corresponding to significantly changed protein sets were compared between groups and visualised using InteractiVenn, an online tool for set analyses, identifying overlapping and novel abundance differences (Heberle *et al.*, 2015). Enrichment was assessed using the web-based toolkit WebGestalt (*https://www.webgestalt.org/*, v2019), selecting *Mus musculus* as organism of interest (Liao *et al.*, 2019). First, over-representation analysis (ORA) was performed to identify enrichment ratio (number observes vs expected proteins) of experimentally relevant proteins against the protein-coding reference list to reveal their functionality (min. 3 genes/category; ranking by multiple-corrected false discovery rate (FDR)<0.05, then selecting the non-redundant categories with lowest FDR, NCBI Entrez gene ID used for mapping). Enriched gene ontology (GO) terms from Biological Process (BP) and Molecular Function (MF), pathway (KEGG), and phenotype (Mammalian Phenotype Ontology) enrichment were identified and visualised.

The online platform SynGO (*https://www.syngoportal.org/*; v1.2) for synapse research based on published experimental evidence (Koopmans *et al.*, 2019) was utilised to assess MIA-induced changes in synaptic proteins, specifically. This allowed for a more accurate identification and a higher-resolution, experimental data-based assessment of synaptic ontology for a subset of results. Annotated proteins for the SynGO terms cellular component (CC) and biological process (BP) were visualised, summarising the location and function of MIA-affected proteins at synaptic level, and respective enrichment analyses were performed (min. 3 genes/category, automated FDR threshold <0.05). Protein network analysis was performed by STRING (*https://string-db.org/*; v12.0, input UniProt IDs), using the full STRING network (Szklarczyk *et al.*, 2019). Functional enrichment in the networks was assessed using the respective, cleaned background reference list for embryonic and adult datasets. The meaning of network edges was set to confidence, displaying the strength of data support in

the database, and minimum required interaction score to 0.4. Here, the strength of functional enrichment terms corresponds to *log10*(enrichment ratio).

PHENOME-WIDE ASSOCIATION STUDY (PHEWAS) ANALYSIS

PheWAS was performed for all genes corresponding to persistently changed proteins (n=50), using the genome-wide association study (GWAS) ATLAS database (*https://atlas.ctglab.nl*; v20191115), based on publicly available data resources (Watanabe *et al.*, 2019). Associations of these genes and a total of 399 GWAS-acquired traits across two relevant domains (Psychiatric, n=321; Cognitive, n=78) were probed and significant correlations were extracted ($p<0.05$, Bonferroni-corrected).

3.1.5 Morphological Analyses

SLICE PREPARATION AND GOLGI-COX STAINING PROCEDURE

For the neuromorphological analysis, sparse-labelling of neurons was achieved by Golgi-Cox staining of the brains from high-dose (5 mg/kg) poly(I:C) and control male (n=3-4 control, n=4-5 MIA) and female (n=3-4 control, n=4-5 MIA) offspring (see Figure 3.4A, Supplementary Table S2 for full numbers). Immediately upon extraction, brains were fixed with 4% formaldehyde solution (Roti Histofix, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 3 h and stained using the FD Rapid GolgiStain Kit (FD Neuro Technologies, Inc., Columbia, MD, USA), in accordance with the vendor's protocol (Du, 2019). After the impregnation and staining, the brains were stored at -80°C and brought up to -20°C for equilibration before cutting - overnight or min. 1 h. The brains were embedded in cryo-gel (Surgipath, Leica Biosystems, Wetzlar, Germany) and cut in 100 µm sagittal sections using a cryostat (CM3050S, Leica Biosystems), set to -22°C chambre / -20°C specimen holder. The sections were placed on microscopy slides (SuperFrost Plus, Thermo Scientific) with a drop of FD Rapid GolgiStain solution C and stored for drying in a horizontal position in a dark box to dry for 48–72 h. The slices were processed according to vendor's instructions (Du, 2019). Briefly, slides were washed twice for 4 min in 4°C ice-cold, double-distilled water (Milli-Q IQ 7000, Merck, Germany; replaced after each wash), incubated in a mixture of solution D, E, and water (0.5: 0.5: 1) for 10 min, washed twice for 4 min, and then placed for 4 min each in ethanol

solutions with increasing concentration (50%, 75%, 90%, and ≥ 99.8%, the latter repeated a second time). Lastly, the slides were placed twice in pure xylene for 4 min and fixed with Histofluid (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and thin glass coverslips on top. The slides were stored in the dark until dry (usually 60-72 h) and cleaned with water for microscopy in the next step.

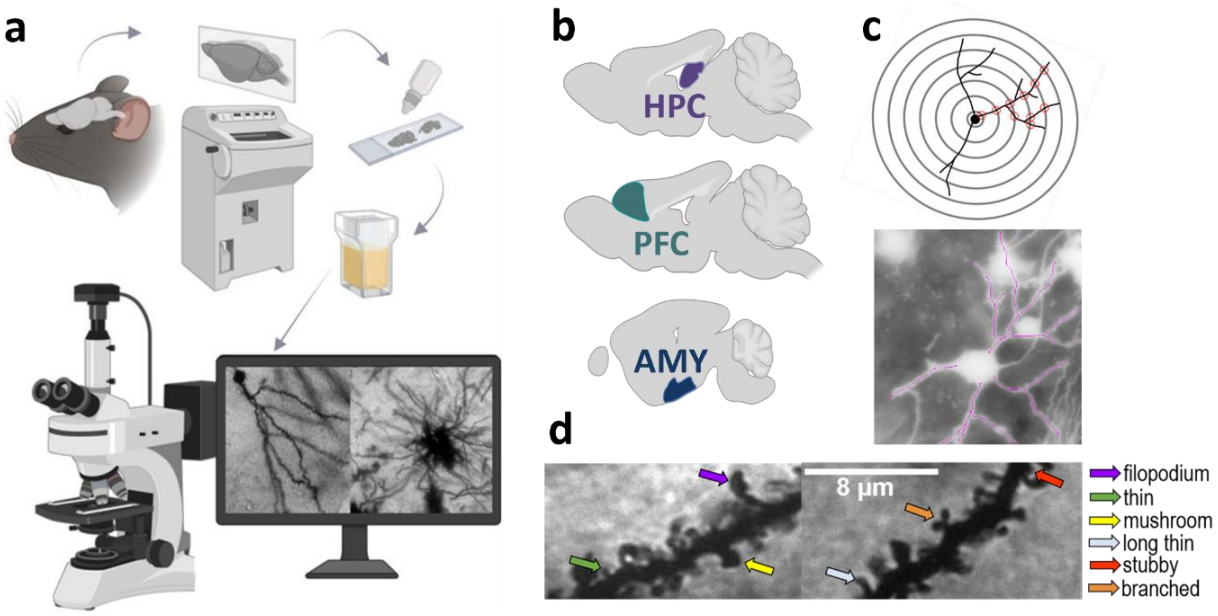


Figure 3.4 Schematic representation of morphological analysis steps.A, mouse brains were extracted, impregnated, cut (100 µm sagittal), and stained using the Golgi-Cox method. Regions of interest (**B**), i.e. hippocampus (HPC), prefrontal cortex (PFC), and amygdala (AMY, *added in the following two studies*) were located and microscopically imaged. Morphological analyses of dendritic length, number, and branching (**C**; *top shows the principle of Sholl analysis, i.e. intersections / radius from soma on an imagined neuron; bottom is an example tracing*) and semiautomated spine analysis (**D**, *with examples of spine types assessed*) of density and type were performed subsequently.

IMAGING, DENDRITIC, AND SPINE ANALYSES

Microscopy imaging of neurons was performed as described previously (Freudenberg *et al.*, 2021). Briefly, the brain regions of interest (HPC and PFC) were located with the help of an Allen Mouse Brain Atlas at 2.5x (0.085 numerical aperture) magnification on an upturned Axio Observer Z1 microscope with an Axiocam 506 mono (Carl Zeiss Microscopy GmbH, Jena, Germany). Images of pyramidal neurons were taken with 20x objective (EC Plan-Neofluar Ph M27, 0.5 numerical aperture) and secondary dendritic segments (30±3 µm, no more than two/neuron) were imaged with 40x (EC Plan-Neofluar Oil M27, 1.3 numerical aperture)

objective combined with 1.6x Tubelens Optovar. The former images were used for dendritic tree arborisation and Sholl morphometric analyses and the latter – for dendritic spine analyses. ZEN Pro software (v2012 blue edition, Zeiss) was used to create z-stack images (section interval 0.33 µm, bright-field contrast).

Dendritic morphology was assessed by semimanually tracing the dendrites using the Simple Neurite Tracer (Longair *et al.*, 2011) and the open-source image processing software package Fiji (*https://imagej.net/software/fiji/*, v1.53c) based on ImageJ2 (Schindelin *et al.*, 2012). For the HPC, n=53-67 neurons/condition from 4-5 brains were traced and for the PFC – n=31-48/group from 3-5 brains (see Supplementary Table S2 for full numbers). It should be noted that neurons are both labelled and selected randomly withing the regions based on availability and quality (including intact dendritic tree), so no further subtype analysis was possible, except for the selection of pyramidal neurons in each region. Information about the dendritic number, length, and dendritic order was obtained and analysed. Morphometric Sholl analysis (Binley *et al.*, 2014) was performed to assess the branching profile of neurons (at incremental radii, 5 µm steps; see Figure 3.4C) and data on the enclosing radius (=ending radius, r at which the last branch is measured; gives data on area occupied by neurites) and the number of branches (=intersections) at each radius.

Dendritic spine analysis was performed according to Risher *et al.* (2014) using the freely available software Reconstruct (*https://synapseweb.clm.utexas.edu/*, v1.1) and ImageJ. With this software, z-stack images were used to trace the length and width of spines, classifying them accordingly (see Figure 3.4D) as filopodia (spine precursors), thin, long-thin, stubby, mushroom, and branched spines – the latter three classified as mature here (Stratton & Khanna, 2020). While branched spines were assessed, they were excluded from the analysis die to very low numbers and inconsistencies between scoring sessions. The only changes made to the original protocol was a small adaptation of the formula used in Microsoft Excel for spine-type classification, namely:

IF(ISNUMBER(SEARCH("branch";E#);"branch";IF(G#>2;"filo";IF(AND(F#>0.6;G#>1);"mush";
IF(G#>1;"long_thin";IF(H#>1;"thin";"stub")))))

This was done due to previous observation in our laboratory that mushroom spines needed to be better defined for more accurate assessment (letters = columns in Excel template; G: length, F: width, H: length-to-width ratio, LWR), so in addition to width>0.6 µm, a length

criterion >1 µm was defined. In the HPC n=56-71 dendritic segments/condition from 4-5 brains and in the PFC n=37-58 /condition from 3-4 brains were analysed (see Supplementary Table S2 for full numbers). Data on the average (per dendrite) LWR and spine density (spines/10 µm) for all and individual spine-types, collated as immature and mature spines here, was gathered and presented.

3.2 Study 2: "Expanding on the Role of RBFOX1 Overexpression in the Brain"

3.2.1 Human *post-mortem* study

To examine the effects of a common genetic risk variant implicated in NPDs and relevant deficits on RBFOX1 expression in the dorsolateral PFC, a quantitative, near-infrared fluorescent Western Blot was performed.

SAMPLES BACKGROUND, GENOTYPING, AND TISSUE PREPARATION

All post-mortem samples were obtained from the NeuroBioBank of the National Institute of Health (NIH NBB; Request ID #613) in accordance with institutional and ethical guidelines. Age-matched samples from the dorsolateral PFC of psychiatrically healthy, older male and female subjects from five biorepositories (see Table 3) were genotyped for the *RBFOX1* SNP.

Table 3 Sample characteristics for *post-mortem* study. Included are single-nucleotide polymorphism (SNP) genotype (*rs6500744, C: risk allele*), biological sex, cohort, and age.

SNP Genotype	Sample Size *(% male)*	Cohort Breakdown[a]					Age *Mean ± SEM*	Age: Statistics
		MTS	HBS	PIT	HRV	MIA		
CC	**10** *(50.0)*	2	2	4	1	1	61.5 ± 6.9	$F_{(2,28)}$=0.43
CT	**15** *(53.3)*	4	3	2	3	3	68.2 ± 4.7	p=0.65
TT	**6** *(83.3)*	-	-	1	3	2	62.8 ± 5.8	*n.s.*

[a] *Cohorts = biorepositories from the brain bank network. MTS = Mount Sinai Brain Bank; HBS = Human Brain and Spinal Fluid Resource Center; PIT = Brain Tissue Donation Program at the University of Pittsburgh; HRV = Harvard Brain Tissue Resource Center; MIA = University of Miami Brain Endowment Bank.*

For the determination of the SNP genotype, Kompetitive Allele-Specific PCR (KASP) assay was performed according to the vendor's instructions (LGC Genomics, Berlin, Germany). Briefly, a custom-made *RBFOX1 rs6500744* SNP-specific KASP assay mix (see Table 4) was used according to the wet DNA extraction method for a 384-well plate as recommended by the vendor. Thermal cycling was performed according to a 61-55°C touchdown protocol on a LightCycler 480 Instrument II (Roche Life Science, Basel, Switzerland).

SNP allele carriers were designated by fluorescent signal-based sample clustering, implementing fluorescence resonant energy transfer (FRET) cassettes, containing FAM (C allele, excitation-emission wavelength 465-510 nm) and HEX (T allele, 533-580 nm) fluorophore dyes. Water was used for negative control and analysis performed according to manufacturer instructions (Roche).

Table 4 Information about the custom-made KASP assay (LGC Genomics) used for SNP-specific genotyping of *post-mortem* samples.

Assay ID Aliquot ID	FAM Allele	HEX Allele	Thermal Cycling Protocol	Sequence
rs6500744-Rbfox1 1217845236	C	T	61-55°C *touchdown*	CACTTAATTTAGGTTTTCCAGTGTGGAGCATATCAGGAAGAGAAATGGC AGACATATTACTTGTCTCAAGCATTATCCATCATTAGCGTCTGTTCTCT TTCTCCGACACACACACACACACACACACACACACACACACACACAC CCCCTTATATTTTTCCCCTAGTACCTTCCTCACTGATCATATTGTTCTG TTAACACCCACCACCAAAACTACATCCTGGGTCCTCTCCACACTGCAGC TCTCAAGACGATCGTTTACTCTATTATCAAGCTTACCATTTATTTTATT TTCAGG**[C/T]**GGTTGTATTCATTATAATGCCATTAGCATGAGAAGTGG GGTCCTGTCACCAGGGCAACCAGGAGCACAGAGTGCACATTCCCTCCTT AGCTGATGTCTTGTTCCCTCTTGGGTGGACTCCAACTGTGTCTTTGATT CATTCCATTAGGTGGTCACTGGGATGTAGATGCTTGACAGCCTCCTTGT CCTTAAGTTGTCTGTCTTTATATCCTTTACTGGCCAAGATTTCTCTCTC CAGAATTCATTAATTTATTACCTTTTATTTCTGTAAGCATAACACATAT AATATTTCCAAGCATAT

All age-matched samples with sufficient protein amount were prepared for Western Blotting (n=31/40 tested; see Chapter 5.2.1 for more). The PFC tissue previously stored at -80°C was suspended in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 25 mM Tris base (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulphate (SDS), 1% Nonidet P 40 substitute (NP-40), 1 mM dithiothreitol (DTT), and 1 mM protease and phosphatase inhibitor cocktail (Halt, EDTA-Free, Thermo Scientifoc). The lysates were homogenised on ice with a microtube homogeniser system (Bel-Art Products Inc., Wayne, NJ, USA) and centrifuged at 17,000 × *g* for 10 min at 4°C. Protein concentration was

measured in the collected supernatant using a colorimetric protein assay (Pierce™ 660nm Protein Assay Kit, Thermo Scientific) and a bovine serum albumin standard curve (molecular biology grade BSA, VWR Chemicals, Darmstadt Germany). Samples were subsequently heated to 95°C for 10 min in 4x lithium dodecyl sulphate (LDS)-containing loading buffer (Thermo Scientific), vortexed, and centrifuged at maximum speed for 10 min at 23°C.

QUANTITATIVE WESTERN BLOT PROCEDURE AND PROTEIN ABUNDANCE ANALYSIS

5 µg of protein from each sample were loaded onto a 4-12% Bis-Tris SDS-PAGE gel (Thermo Scientific) for a total of three technical replicates. The loading of the three technical replicates was optimised for diminishing positional variation effects. A pre-stained protein ladder (Chameleon Duo, Li-Cor Biosciences GmbH, Bad Homburg, Germany) was used for a two-colour near-infrared (NIR) detection of molecular band size. Gel electrophoresis for molecular weight-dependent protein migration was performed at 200 V for 50 min utilising a 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer (pH 7.3, Thermo Scientific). The proteins were transferred overnight at 16 V onto a nitrocellulose membrane (0.2 µm, Thermo Scientific).

Total protein staining procedure was performed as described by the manufacturer (Revert 700 Total Protein Stain, Li-Cor). The membranes were then blocked for 1 h with a commercial Tris-buffered saline (TBS)-based blocking buffer (Li-Cor). The blots were incubated under agitation with the monoclonal primary antibody anti-RBFOX1 (0.5 µg/ml, mouse IgG2b κ, BioLegend; 862701) overnight at 4°C. After washing thoroughly with TBST (0.1% Tween-20 in TBS [20 mM Tris, pH 7.6, 150 mM NaCl]), the blots were incubated for 1 h in a fluorescent secondary antibody (1:20000, IRDye 800CW goat anti-mouse IgG, Li-Cor) on a shaker at room temperature, protected from light. Finally, the blots were washed thoroughly in TBST under agitation, rinsed in TBS, and air-dried in the dark for NIR fluorescent imaging. Two-channel NIR fluorescent imaging was implemented for protein visualisation on an Odyssey Fc Imaging System (Li-Cor). Image acquisition and pre-processing was performed with Image Studio Software (Li-Cor; v5.2) and quantitative expression analysis - with the Empiria Studio Software (Li-Cor; v1.3), allowing for automatic lane detection and background subtraction.

Total protein amount was used as internal loading control (Aldridge *et al.*, 2008; Eaton *et al.*, 2013), using a consistent, representative molecular weight range of 30-260 kDa for

normalisation (Kirshner & Gibbs, 2018); see Figure 3.5. Samples with technical replicates' coefficient of variation (CV) above 20% were excluded from further analysis and the averaged signal was used for further analyses. Linear dynamic range determination of the total and target protein abundance, as well as the validation of the antibody, including a dilution curve for target protein linearity, were performed prior to sample analysis to optimise the quantitative validity (Ghosh *et al.*, 2014). The results were presented as normalised signal (see Chapter 5.2).

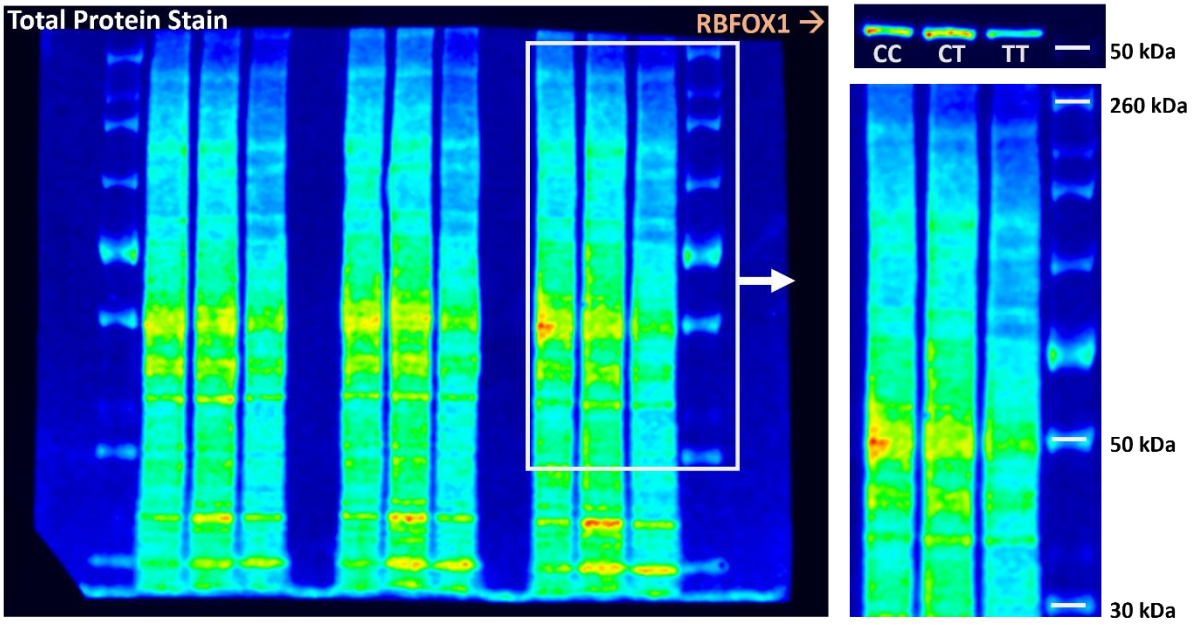


Figure 3.5 Example of total protein stain and RBFOX1 abundance in *post-mortem* PFC samples. On the left is a full blot stained for total protein (700 nm channel) with n=3 replicates each from one CC-, CT-, and TT-allele carrier of the SNP *rs6500744*. On the left are the respective measured RBFOX1 (800 nm channel; one 52-53 kDa band) and the blot portion (30-260 kDa) used for normalisation in the automated Empiria Studio software.

3.2.2 Cytoplasmatic Overexpression of RBFOX1 in Mice

3.2.2.1 Animals

For this study, adult (8-18 weeks) neuronal-specific cytoplasmatic RBFOX1 overexpressing (OE) mice with *Synapsin*-Cre expression driver on a *C57BL/6* background (C57BL/6-Gt(Rosa)26Sortm1$^{(Rbfox1\text{-}IRES\text{-}eGFP)}$Geno x B6.Cg-Tg(Syn1-cre) 671Jxm/j; see O'Leary *et al.* (2022) for more) and controls lacking the knock-in construct or Syn-Cre (designated as wild-type) were used. Importantly, only Syn-Cre-expressing females were used for breeding, as there is a

known possibility for off-target expression of Cre in males mice, i.e. expression in the testes, which can be inherited by the offspring as recombination (Rempe *et al.*, 2006). The mice were housed in the animal facility as described in Chapter 3.1.1. For the morphological analysis, 5-8 brains per genotype from adult males (see Supplementary Table S2 for details). For the MIA × RBFOX1 OE experiments, n=5-6 primiparous dams and the same number of sires were used (licence number FK/1101). The surviving offspring was tested in for behaviour after reaching adulthood (see Chapters 3.2.2.4, Supplementary Table S1). Additional dams (n=4/group) from a different study I performed, which implemented the same MIA model in *C57BL/6J* mice (licence number FK/1134), were used to supplement the groups for maternal care observations.

3.2.2.2 Morphological analysis

Morphological analyses of the male HPC (n=6 CTRL, 6 OE) and PFC (n=8 CTRL, 7 OE) were performed as described in Chapter 3.1.5. Additionally, the amygdala of adult male mice was assessed in the same manner (n=6 CTRL, 5 OE). For the analyses of dendritic architecture 30-80 neurons per group were assessed. It should be noted that the HPC in this study was used for the initial optimisation of this method, so data on the branching profile from the morphometric Sholl analysis was not available. For the spine analysis, 64-120 dendritic segments / genotype were analysed. The detailed list of animal, cell, and dendritic segment numbers can be found in Supplementary Tables S2.

3.2.2.3 RBFOX1 OE Validation

For the cytoplasmic RBFOX1 validation, a semiquantative Western Blot analysis of protein abundance in the HPC and PFC was used. The regions were dissected from frozen brains (-80°C) on a cold plate (4°C). The procedure was performed as described in 3.2.1 with the following exceptions, since am electrochemiluminescence (ECL) was used here based on availability. 35 µg of PFC and 50 µg of HPC total protein each from n=3 male mice / group was loaded with pre-stained protein standard (SeeBlue Plus2, Thermo Scientific). The blocking of the blots was achieved with 5% skim milk powder solution in TBST buffer, the primary antibody was used (1:500, mouse monoclonal IgG2 aκ, Anti-Fox1, clone 1D10, Merck Millipore, MABE985) was diluted in 5% BSA-TBST (2 mM sodium azide). The secondary antibody was prepared in 5% milk-TBST (1:20 000, Amersham ECL Mouse IgG, HRP-linked

whole Ab (sheep), GE LifeSciences, NA931-1ML). For the ECL imaging, blots were covered with ECL West Dura detection solution (Thermo Scientific) and incubated in the dark at room temperature under agitation for 5 min. Images were taken with MyECL Imager (Thermo Scientific) at the software-recommended exposure times. The blots were stripped (Restore Western Blot Stripping Buffer, Thermo Scientific) according to manufacturer's instructions and re-probed in the same manner with β-actin primary (1:2000, rabbit polyclonal IgG, Anti-beta Actin, Thermo Scientific, PA1-183) and secondary (1:20.000, Amersham ECL Rabbit IgG, HRP-linked whole Ab (donkey), GE LifeSciences, NA934-1ML) antibodies. The β-actin was used for normalisation and protein abundance was assessed with the Fiji ImageJ2 Gels analysis tool as described elsewhere (Stael *et al.* (2022); *https://imagej.net/ij/docs/menus/analyze.html#gels*).

3.2.2.4 MIA × RBFOX1 OE

MIA PROCEDURE AND MATERNAL BEHAVIOUR

The procedure was performed as described in 3.1.2 using the low-dose (2.5 mg/kg poly(I:C), MIA group) or PBS vehicle (VEH) on E9. The five control (CTRL) and six cytoplasmatic RBFOX1 OE dams were separated on E17 and daily birth checks were performed at 17:00 h. If the mother gave birth before that time (postnatal day PND0), maternal behaviours were observed in the home cage on the next two days (PND1-2, 8:00-9:00 h) without disturbing the mother and pups. The scoring was performed by noting down the observations from a range of behaviours every 2 min for 60 min (adapted from previously published protocols, i.e. Bosch *et al.* (2007), Priestnall (1973), and discussion with D.A. Slattery, *personal communication*). Arched back nursing, blanket posture nursing, licking/grooming pups, carrying pups, self-grooming, nest building, digging, locomotion, feeding and drinking, as well as their location in relation to the nest were assessed. Some behaviours were not or rarely (<3 times across all mothers) observed, i.e. pup carrying and digging, and were excluded from further analyses. The surviving offspring was allowed to reach adulthood (8-10 weeks) and was tested in an NPD-relevant behavioural battery.

OFFSPRING BEHAVIOUR

In total, 24 non-overexpressing controls (n=17 VEH, 7 MIA) and 5 RBFOX OE (n=3 VEH, 2 MIA) mice of both sexes were tested. For more information, see Supplementary Table S1 and Chapter 5.1.1.3.

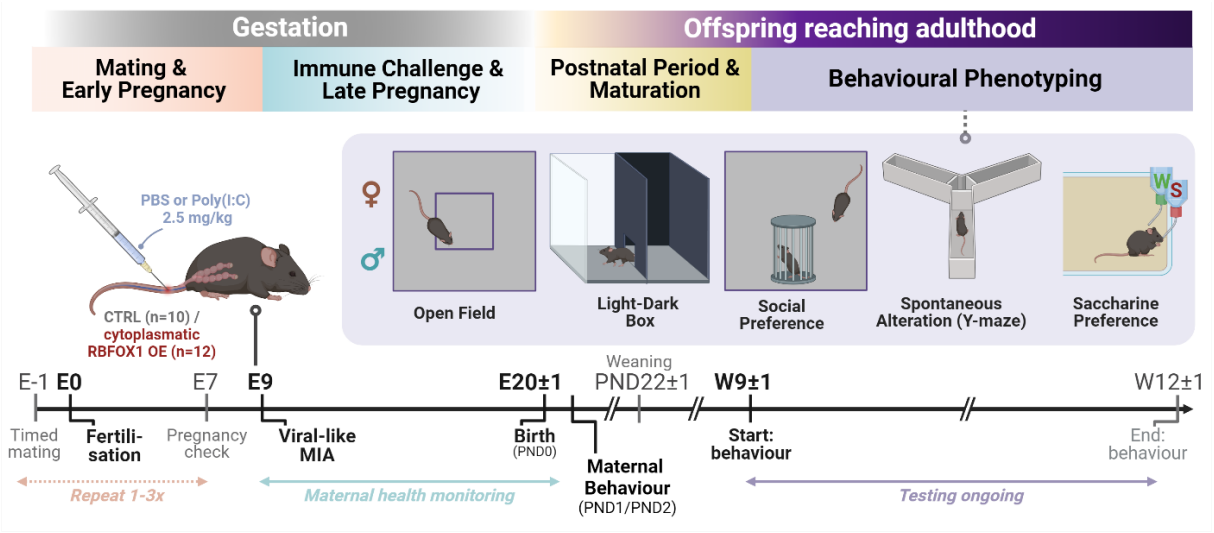


Figure 3.6 Timeline of Maternal Immune Activation (MIA) and behavioural testing of offspring in the cytoplasmatic RBFOX1 overexpression (OE) mouse model. For more information, see Figure 3.1 and Chapters 3.1.1. Maternal behaviour was added, and paradigm changes were made to the original behavioural battery, which be found in 3.1.3. *E*, embryonic day; *PBS*, 1x phosphate buffered saline (vehicle); *PND*, postnatal day; *Poly(I:C)*, polyinosinic: polycytidylic acid (viral mimetic for MIA); *W*, week (age).

Offspring was tested for NPD-relevant behaviours, adapted from the previous study (see Chapter 41.3, D. A. Slattery, *personal communication*), after reaching adulthood, i.e. 8-10 weeks, and lasted for 3-4 weeks (see Figure 3.6). There was a minimum of 2-3 days between tests to reduce the effects on subsequent investigations. Adaptations of the tests are described below, for more details see Chapter 3.1.3.

The open field and light-dark box tests were performed as described in the first study, but the duration was reduced to 5 min. The social preference test was performed immediately after the open field test by adding a small empty cage (ø 8 cm, H: 10 cm) in the middle of one wall of the arena (see Figure 3.6) and an interaction zone (L (*wall to centre*): 14 cm, W (*along wall*): 20 cm) around it was designated for assessment. The mice were allowed to explore freely for 3 min and the duration of investigating the object, i.e. amount of time the head of the animal was detected in the interaction zone, was assessed. Next, an age-, genotype-, and sex-matched novel conspecific was placed in the cage and another equivalent 3 min trial was recorded. The social preference index was calculated by contrasting the time spent investigating the social stimulus versus the total time spent investigating both the social and object stimulus. Normal preference values for this strain usually range from 0.4 to 0.8 but there is natural variability of behaviour outside of those (Rein *et al.*, 2020).

For the spontaneous alteration (SA) test, a Y-maze (see Chapter 3.1.3) was used, and animals were placed in one arm (A) and allowed freely for 5 min, as described previously (O'Leary *et al.*, 2022). The total arm entries were calculated was the sum of entries in all three arms (A, B, C). Furthermore, the software calculated the total spontaneous alterations, i.e. when the animal visited different arms on each of the last three arm entries, as the sum of all sequences (alteration patterns ABC, ACB, BCA, BAC, CAB, CBA). Finally, the spontaneous alteration percentage was calculated as *SA% = [Spontaneous Alterations / (total arm entries – 2)] × 100*. All tracking and automated analyses for the aforementioned tests were performed with ANY-Maze (see Chapter 3.1.3).

Finally, the anhedonia-like saccharin preference test was performed as described in Chapter 3.1.3, except for switching the sucrose used in the previous study to account for possible differences in caloric intake need between the groups. The 2 % saccharine solution was prepared in warm water (same as used for the water bottle in the test; poorer aqueous solubility than sucrose), using a magnetic stirrer in the morning of the first test to ensure it reached room temperature before the test. Liquid intake was measured, and saccharine preference was calculated as described in Chapter 3.1.3.

3.3 Study 3: "*Diras2* Expression Levels Exert Subtle Effects on Behaviour and Morphology Related to ADHD"

3.3.1 Animals

The *Diras2* knockout mice were created through Cre-LoxP recombination: the *Diras2* gene was flanked by two flox sequences and *Diras2*$^{fl/fl}$ mice were crossed with *Synapsin*-Cre tagged recombinase animal to achieve neuronal-specific deletion (see O'Leary *et al.* (2022) for more). Animals were maintained in-house on a *C57Bl/6J* background. For more on housing and experimental conditions, please refer to Chapter 3.1.1. For the investigations, adult male and female hetero- (HET, *Diras2*$^{+/-}$) and homozygous (KO, *Diras2*$^{-/-}$) knockout mice and controls (CTRL, *Diras2*$^{+/+}$) were used. All testing was performed in accordance with European and local legislation (licence number FK/1121, see Chapter 3.1.1 for more) and institutional guidelines.

For behavioural testing, n=8-24 CTRL, 4-20 HET, and 4-13 KO males and females were used in three cohorts (see Supplementary Table S1 for more details). For morphological analyses brains from adult males (n=5-8 CTRL, 5-8 HET, 4-7 KO, see Supplementary Table S2 for more

details) and for molecular expression analyses all available brains from adult females (n=8 CRTL, 4 HET, 5 KO) were used. Behavioural, morphological, and molecular tests were performed on as many animals as possible based on availability of in-house bred offspring and the need to keep the females for breeding purposes (A. O'Leary, D. A. Slattery, *personal communication*; see Chapter 3.2.2 for more).

3.3.2 Behavioural Testing

Experiments were performed by others and data provided (see Chapter 3.5 for more). More information on equipment and individual tests can be found in Chapters 3.1.2, 3.2.2.4.

EXPLORATION, ANXIETY-LIKE BEHAVIOUR, AND SENSORIMOTOR GATING

The open field and light-dark box test (5 min each) were performed in adult control, *Diras2* HET and KO male and female mice as described in Chapter 3.2.2.4 and sensorimotor gating was assessed via pre-pulse inhibition (PPI) of the acoustic startle reflex in adult males as described in Chapter 3.1.2 (see Figure 3.7A; for detailed numbers see Supplementary Table S1). Additionally, three other behavioural tests were performed and will be briefly described below.

BARNES MAZE

The Barnes maze (BM) was used to assess spatial learning and memory in adult neuronal-specific *Diras2* HET and KO females, and controls (O'Leary & Brown, 2012). The mouse Barnes maze (Stoelting Co.) comprises an elevated arena (ø 91 cm, H: 90 cm) with 20, ø 5 cm holes around the edge (see Figure 3.7A). For training, one of the holes is equipped with a deep, long escape chamber (metal box) and the other 19 holes are secured with shallow, "false escape" boxes. Different visual cues comprising black, white, and grey patterns were placed along the walls to help with orientation. The setup was recorded with a wide-angle camera on top (The Imaging Source) and tracked with the automated software ANY-Maze as described above.

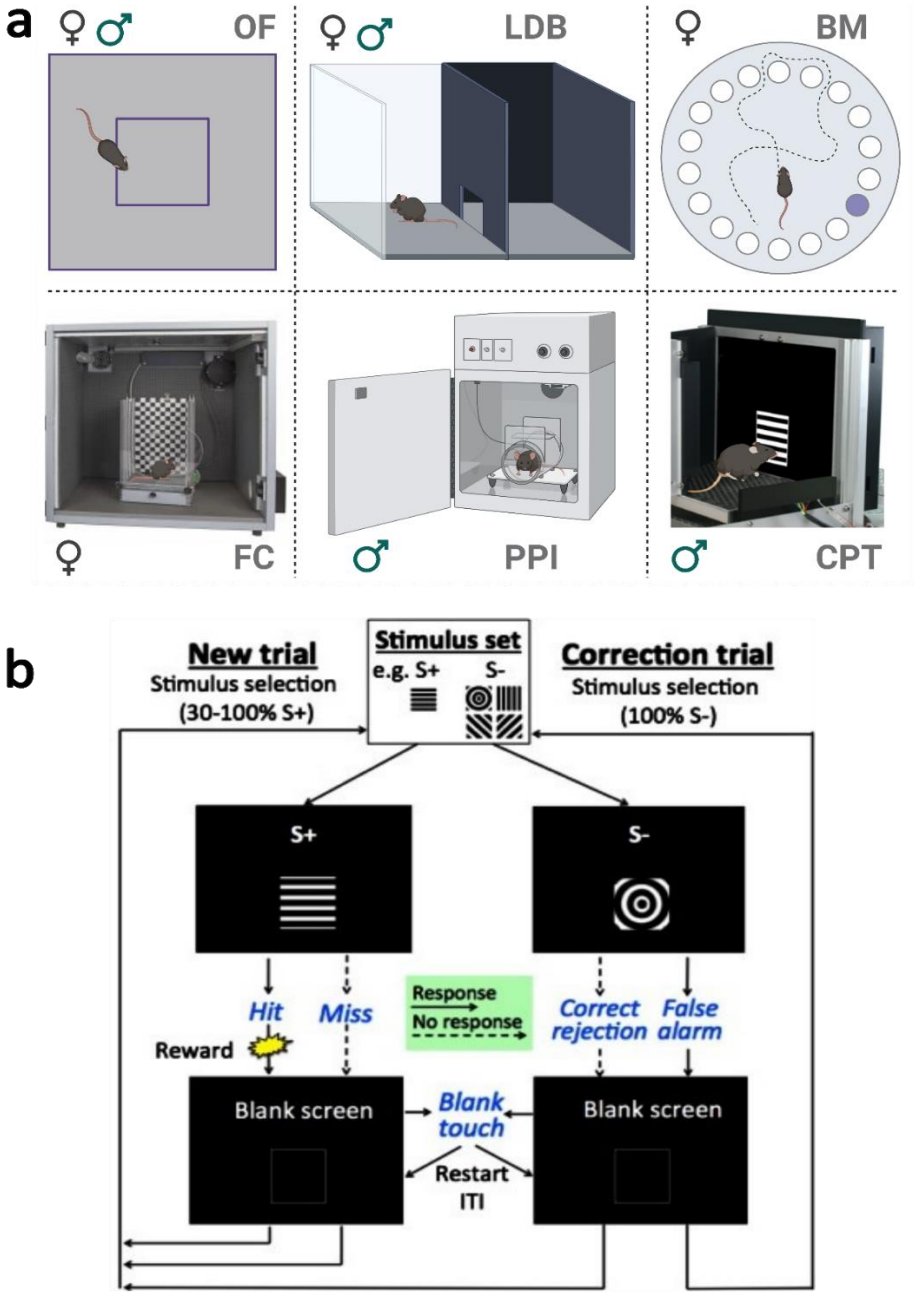


Figure 3.7 Schematic overview of the behavioural tests performed in adult *Diras2* knockout mice and controls. A, the open field (OF) and light-dark box (LDB) tests for locomotion and anxiety-like behaviours were performed in both adult male and female mice. The Barnes maze (BM) test for spatial learning and memory, and the fear conditioning (FC) test assessing fear-based learning were performed exclusively in adult females. Sensorimotor gating was investigated via the pre-pulse inhibition (PPI) of the acoustic startle response in males. Higher-cognitive abilities, including attention and response inhibition, were assessed in the rodent continuous performance test (CPT) in adult males. **B**, flowchart of the CPT by Kim *et al.* (2015), re-used under CC BY 4.0 licence. Visual discrimination and selection between a target (S+) and non-target (S-) stimuli can lead to a reward (strawberry milk) for the mouse if correctly selected, while non-responses lead to a new trial after the inter-trial interval (ITI). Incorrect selection of the S- led to correction trials, where another S- was presented with certainty (see reference for more). See Chapters 3.1.3, 3.3.2 for more details and Supplementary Table S1 for animal numbers.

The day before training started, the animal was habituated to the arena: each mouse was allowed to visually orientate itself while in a transparent polyacrylic cylinder (ø 15 cm) placed in the middle of the arena for 1 min. The mouse was guided slowly with the cylinder to the escape hole, allowed to explore there for one more minute, then gently guided in the escape box, the hole was closed and the mouse kept in the box for 1 min.

The females were trained on five consecutive days (2 trials/day, 10 min inter-trial interval (ITI); averaged for analysis). Each animal was trained twice a day with an inter-trial interval of 10 min. The mouse was placed in the middle of the arena and allowed to explore for up to 3 min: if the mouse did not escape during that period, it was gently guided into the escape box. Since this was a pilot trial for our laboratory, it was noticed during training that the females were largely not entering the deep, dark box. So, instead of escape latency (full-body escape), the latency to first escape attempt (head and shoulders of the mouse inside the escape box) was recorded for spatial learning assessment. Later, clean sawdust (as found in the home cages) was added to the box, while still leaving space for the animals to enter and explore inside, which improved the test for future studies (A. O'Leary, *personal communication and observations*).

In the probe trials, the escape box was replaced with a box identical to the ones attached to other 19 holes. The probes were 90 s long and were performed 24 h and 7 days after the last training session to test shorter- and long-term spatial memory. Here, in addition to monitoring distance travelled and visits to the different maze zones, the first escape attempt was also recorded.

FEAR CONDITIONING

The auditory fear conditioning test was used to assess fear learning as described elsewhere (O'Leary *et al.* (2022); see Figure 3.7A). Briefly, a fear conditioning system (Ugo Basile SRL, Gemonio, Italy) was used to train the animal to associate a conditional auditory stimulus (CS) with an unpleasant sensation and test learning and memory. On the first day 3 min habituation to the apparatus, was followed by three trials (80 dB-, 1000-Hz CS, 30 s; 0.4 mA scrambling foot-shock at the end: 2 s, ITI: 2 min). On the second day, the chamber was covered with black-and-white panels on the walls and a grey one on the floor to cover the electric grid. After 3 min habituation, fear extinction was performed by 16 CS presentations

(30 s, ITI: 5 s). Fear extinction recall was assessed on the third day: the chamber set-up was left as the previous day. After habituation (3 min), three CS (30 s, ITI: 5 s) were presented. Freezing behaviour was used for assessing conditioned fear, automatically-tracked and quantified with ANY-Maze freezing detection module (threshold: 1000 ms).

CPT

The rodent CPT is analogous to a human test used to assess deficits in the cognitive, attentional, and impulsivity domains in multiple psychiatric disorders, including ADHD. The CPT presented here was performed as described by Kim *et al.* (2015), investigating vigilance and response inhibition in mice (see Figure 3.7). Food-restricted adult males (CTRL: n=18, HET: n=6, KO; n=13) were habituated to the reward (strawberry milk) and environment in one of the available operant touchscreen chambers (Bussey-Saksida Touch Screen Chamber for Mice, Campden Instruments Ltd, London, UK), equipped with ABET II Software for Touch Screens running on a Whisker control system (Lafayette Instrument Company, Lafayette, IN, USA) with Microsoft 2010. The mice were food-restricted and fed for 1 h per day *ad libitum* to ensure optimal motivation and their weight monitored.

The males were trained in four stages at individual learning pace until reaching a set criterion as described by Kim *et al.* (2015); see reference for more information. Briefly, in the first stage for reward seeking / consumption training, a white square is presented in the middle of the screen (stimulus duration, SD: 10 s, ITI: 2 s) and if correctly touched (“hit”), the mice receive a reward and have 2 s to consume it during which time the reward is illuminated (see Figure 3.7). If the screen is not touched (omission error “miss”), the ITI is initiated and if a “blank” touch occurs during that time, the ITI is restarted, prolonging the wait time. 60 consumed rewards are needed for progressing in the test and the session is ended after 45 min/100 rewards. The second stage presents the target stimulus S+ (white and black stripes) and repeats the first stage training but reduces the SD to 2 s and adds 5 s for consuming the reward. In the third, two-choice visual discrimination stage, an additional non-target stimulus S- is introduced (snowflake patter) randomly in half of the trials. A commission error (“false alarm”) triggers the ITI and subsequent correction trials present S- until the correct rejection is made (see Figure 3.7), to dissuade non-selectivity of responses and sensitivity threshold was set; for more on criteria see Kim *et al.* (2015).

The last training stage was identical except for the non-target stimulus S- now representing one of four previously unknown stimuli (see Figure 3.7) and the last trials were used to assess baseline CPT performance.

After successfully completing the training, four probes were conducted to test performance under more cognitively-challenging conditions. Probe 1 was conducted over two consecutive days and presented stimuli with varying duration (SD: 0.25, 0.5 s, 0.75 s, 1 s, 1.5 s, 2 s) randomly, mimicking the human CPT to increase attentional load – and translational value. Probe 2 decreased the contrast of both S+ and S- (12.5%, 25%, 50%, 100%). SC) to assesses performance under visually- and attentionally-demanding conditions. Probe 3 added two increased ITIs (5 s, 10 s) to the standard 2 s ITI to lower the event rate and challenge sustained attention. In the last, fourth probe, two distractor patterns were shown on the left and right side of the stimulus, to test tests whether these "flanker distractors" decrease attentional performance with increased demand of distraction inhibition, i.e. executive function. The distractors are randomly selected by the software from S+/S- pattern pool and one of three options are presented: non-distractor trials (*no flankers*); congruent trials (*all three stimuli presented simultaneously are similar – all S+ or all S-*); non-congruent trials (*S+ in the centre, two identical S- flankers - or S- in the centre, two identical S+ flankers*). For more information, see Kim *et al.* (2015), where the same probes were conducted in a different order (*1. variable SD here*: probe 2; *2. variable contrast here*: probe 3; 3. *long ITI here*: probe 6; *4. flanker distractor here*: probe 4).

The hit rate, HR [*hits / (hits+misses)*]; false alarm rate, FAR [*false alarm / (false alarm+correct rejection)*], sensitivity index, SI *[*$(HR-FAR)$ / $(2(HR+FAR) - (HR+FAR)^2)$]; responsivity index, RI [$(HR+FAR-1)$ / $(1-(HR-FAR)^2)$] were calculated. A high FAR indicates response inhibition deficits, a high SI represents vigilance and good visual discrimination (S+ vs S-) ability, while RI measures response bias - lower RI, i.e. less responsivity, means higher, more conservative response bias (Bhakta & Young, 2017; Kim *et al.*, 2015).

3.3.3 Morphological Analyses

Morphological analyses of the male HPC (n=7-8 mice/genotype), PFC (n=4-5/genotype), and amygdala (n=7-8/genotype) were performed as described in Chapter 3.1.5. For the analyses of dendritic architecture 49-77 neurons per group and for the spine analyses, 40-83 dendritic

segments / genotype were analysed. The detailed list of the numbers of animals, neurons, and dendritic segments analysed for each region can be found in Supplementary Table S1-S2.

3.3.4 Expression Analyses

Quantitative real-time polymerase chain reaction (qPCR) was used in this study to assess gene expression of *Diras1* and *Diras2*, as well as previously reported *in-vitro* targets of interest *mTOR*, *Clasp2*, *Ctdsp2*, *Eif2c2*, *Fktn*, *Mpp5* (Grünewald *et al.*, 2021). These were assessed in the brains of female CTRL (n=8), HET (n=4), and KO (n=5) adult females, used in the behavioural analysis (>2 weeks before sacrifice). Brains were extracted, immediately frozen, and regions of interest (HPC, PFC, amygdala, nucleus accumbens, hypothalamus, cerebellum; see Chapter 1.1.1) isolated as described in Chapter 3.1.5.

The PCR procedure was performed as described elsewhere (Freudenberg *et al.*, 2021). Briefly, RNA isolation was performed in accordance with the vendor's protocol, using a MagJET RNA kit (Thermo Scientific) and a pipetting robot (Biomek NXP, Beckman Coulter GmbH, Krefeld, Germany). The samples (100 ng each) were reverse-transcribed into cDNA with an iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) and random oligodeoxy-ribonucleotide primers and hexamers in a thermocycler (Mastercycler, Eppendorf SE, Hamburg, Germany; 5 min 25°C, 20 min 46 °C, 1 min 95°C, Hold on 10 to 20°C).

The qPCR was performed in 10 µl reaction solution containing AMPLIFYME SG No-ROX Mix (QIAGEN Gdańsk, Poland), target-specific primer pairs (0.3 µM final concentration; custom-made: Eurofins Scientific, Luxemburg), and synthesised cDNA (final dilution 1:160). Duplicates of each sample were pipetted by the robot in a 384-well plate (primaPLATE, Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany), sealed with foil, and the qPCR was performed in a LightCycler 480 Instrument II (Roche; 3 min at 95°C, 5-s 45 cycles at 95°C, 10 s at 60°C, 10 s at 72°C). Crossing point (Cp) values were calculated by the LightCycler's software (v1.5.1.62). The forward and reversed primer sequences used for this study are described in detail in Table 4. For normalisation, the housekeeper genes *Sdha1* and *Pgk1* were used based on a standard curve assessment (Normfinder function in analysis software GenEx6 v3.1.3 by MultiD Analyses AB, Sweden), as well as previous validations in our laboratory (F. Freudenberg, *personal communication*).

Table 5 Oligonucleotide primer pairs used for qPCR. All genes for *Mus musculus*. Standard curve slope (m)/intercept (b) equation: y=mx+b; r^2: coefficient of determination, measure of goodness-of-fit; *E:* efficiency

Gene symbol	NCBI Entrez GeneID	**Official gene name**	**Primer sequences** *5′ → 3′* (*F: forward, R: reverse*)	**Standard curve HPC** [a]	**Standard curve PFC** [b]
Clasp2	76499	CLIP associating protein 2	F: GAATCCTGTCAGCAGTGCAA R: CTCCAGCCCCTCCTTCTTTA	y = -3·81x+23·2 r^2 = 0·991 | E = 0·829	y = -4·01x+23·1 r^2 = 0·980 | E = 0·775
Ctdsp2	52468	CTD small phosphatase 2	F: AACACCATCGCCAAGTCC R: GGTCCCAGGGATCTGATAAAA	y = -4·06x+23·7 r^2 = 0·969 | E = 0·762	y = -3·77x+24·0 r^2 = 0·992 | E = 0·841
Diras1	208666	DIRAS family, GTP-binding RAS-like 1	F: TACCGTGTGGTCGTGTTCG R: TCCTCTATGGTAGGGATGTAGGT	y = -3·69x+23·0 r^2 = 0·968 | E = 0·865	y = -3·75x+23·3 r^2 = 0·977 | E = 0·849
Diras2	68203	DIRAS family, GTP-binding RAS-like 2	F: GAGCTGCGCCTGGAGACCTG R: CCGCCACCCGGTAGTCGTTG	y = -3·77x+22·3 r^2 = 0·992 | E = 0·841	y = -3·60x+22·2 r^2 = 0·993 | E = 0·900
Eif2c2	239528	argonaute RISC catalytic subunit 2 *(Ago2)*	F: GCAACGCCACCATGTACTC R: ATATCCTGGGATGGGTGATG	y = -3·69x+25·2 r^2 = 0·985 | E = 0·866	y = -4·06x+24·9 r^2 = 0·980 | E = 0·763
Fktn	246179	fukutin	F: CGAGTGCAGGTATAAGGAAGC R: GACCATAGCATCCACAGTATTATCA	y = -4·45x+24·7 r^2 = 0·989 | *E = 0·678*	y = -3·37x+26·5 r^2 = 0·961 | E = 0·982
Mpp5	56217	protein associated with LIN7 1, MAGUK family member (*Pals1*)	F: TGATTCCTAGTCAACAGATCAAGC R: GTCAAAATGAGCTTTCACATGG	y = -3·65x+25·4 r^2 = 0·974 | E = 0·878	y = -3·66x+26·5 r^2 = 0·991 | E = 0·875
Mtor	56717	mechanistic target of rapamycin kinase	F: ACCGGCACACATTTGAAGAAG R: CTCGTTGAGGATCAGCAAGG	y = -3·43x+25·8 r^2 = 0·975 | E = 0·957	y = -3·49x+25·7 r^2 = 0·993 | E = 0·933
Pgk1	18665	phosphoglycerate kinase 1	F: CCAGTTGCTGCTGAACTCAA R: CCACACAATCCTTCAAGAACAG	y = -4·22x+22·2 r^2 = 0·994 | E = 0·727	y = -4·07x+23·0 r^2 = 0·930 | E = 0·761
Sdha	66945	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	F: GGACAGGCCACTCACTCTTAC R: CACAGTGCAATGACACCACG	y = -4·00x+21·9 r^2 = 0·992 | E = 0·779	y = -3·30x+23·4 r^2 = 0·938 | E = 1·011

[a] HPC standard curve based on hippocampi from n=6 wild-type mice, used for HPC, amygdala, hypothalamus targets.

[b] PFC standard curve based on prefrontal cortices from n=6 wild-type mice, used for PFC, nucleus accumbens, cerebellum targets.

The standard curves for the housekeepers and targets were prepared in dilution 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 (RNA pooled from each HPC and PFC of three adult *C57Bl/6J* female mice). The results of the standard curve analyses for all genes, including primer efficiency and goodness-of-fit are presented in Table 4.

Relative gene expression levels were analysed with GenEx6 v3.1.3 (MultiD Analyses AB, Sweden) by first efficiency-correcting the Cp values based on the standard curve ran on the plate (see Table 4) and imputing missing datapoints (<2/region and genotype; excluded here from further analyses: *Eif2c2*, *Fktn*, *Mpp5*). Relative gene expression was calculated based on *Sdha* and *Pgk1* expression by the software, and the results were normalised to the average of the control genotype group (fold-change, FC). Results were transformed and presented as *log2*(FC).

3.4 Statistical Analyses

In the first study, proteomic data were analysed for differential abundance using unpaired t-tests and multiple-testing correction of the false discovery rate with the Storey-Tibshirani method (q-values; significance accepted $p<0.05$, $q<0.1$) provided by the Spectronaut software. All tested treatment and sex groups were contrasted against each other in dose- and sex-specific proteomic analyses – thus, correction procedures were stringent, increasing q-values. The full statistical analyses of proteomic data can be found in the shared repository (see Data Availability below), the comparisons of males and females presented here concern MIA effects stratified by sex. Enrichment of protein abundance differences was calculated with the pertinent online software (Koopmans *et al.*, 2019; Liao *et al.*, 2019); Fisher's exact test for ORA/SynGO analyses; Benjamini-Hochberg corrected q-values**.** Significant gene-trait associations in PheWAS were Bonferroni-corrected.

For the *post-mortem* RBFOX1 abundance study, a factorial analysis of covariance was performed to test the effects of genotype and sex on the normalised protein signal, whilst controlling for age, using the IBM SPSS Statistics software (v29, IBM Corp., Armonk, NY, USA).

All other statistical analyses were performed using Prism (v9.4.1, GraphPad Software Inc., San Diego, CA, USA). Data were analysed using t-tests and one-, two-, or three-way ANOVA with repeated measures as necessary, followed by software-recommended *post-hoc* tests (Šídák's test for simple two-way ANOVA to compare means within each variable level when

appropriate; Tukey multiple comparison test for the rest if not specified otherwise). Normality (D'Agostino-Pearson omnibus) and homogeneity of variances (Brown-Forsythe) were probed and if assumptions were violated, the appropriate tests were performed (Mann Whitney U or Kruskal-Wallis with Dunn's multiple comparisons test, and Welch's t-test or Welch's ANOVA with Dunnett's *post-hoc*, respectively). Statistical significance was accepted for $p<0.05$, trends denoted at $p<0.08$. All values are presented as mean ± standard error of the mean (S.E.M.) if not stated otherwise.

3.5 Data Availability and Additional Information

The mass-spectrometry proteomics data have been deposited to the ProteomeXChange Consortium via the PRIDE partner repository (Perez-Riverol *et al.*, 2022) with the dataset identifier PXD043094. Access to this raw data and the synaptic proteome analysis files used for this dissertation will be made available on Figshare (CC-BY 4.0) upon acceptance for publication by a scientific journal and accessed under the following DataCite DOIs:
embryonic analysis datasets: doi.org/10.6084/m9.figshare.22581628; *adult analysis datasets:* doi.org/10.6084/m9.figshare.22581766. This dissertations' reviewers and examination committee members were granted access. Blots from Study 2 (*upon journal publication*) here, DOI: 10.6084/m9.figshare.25970728. Other data supporting this studies' findings is presented either in the main text or supplementary data. Additional data can be granted upon request.
Behavioural phenotyping of the *Diras2 KO* model was performed, and data kindly provided by Dr A. O'Leary, assisted by V. Beck, M. Luckow, and S. Waas. Subsequent analyses and visualisation were performed by me. Preparation of samples, imaging, and data acquisition for the Golgi-Cox experiments were performed in part by students under supervision (L. Burger, A. Fuß, P. Getty, J. Huang, B. Kurt, F. Schultze-Seemann, L. Kassel, M. Kögler, M. Luckow, L. Pieper, M. Stegemann, S. Waas) and our laboratory's staff technicians (S. Stanzel, T. Töpner). Genotyping of human samples for the RBFOX1 study (T. Töpner) and sex determination of the embryonic samples for the MIA study (J. Auer) were also performed upon instruction by the technicians. Synaptoneurosomal preparation and Spectronaut protein analysis were performed by Dr L. Li (Turku, Finland) with my involvement. Clustering analysis of proteins in the MIA study was performed by Dr A. O'Leary. Support with timed mating, MIA induction and maternal scoring, and sample extractions in the studies involving MIA was kindly provided by Dr. F. Freudenberg, E. Sehir, S. Waas, M. Melch, and P.-L. Tsai. All other

experiments and analyses were performed by me in accordance with best scientific practices and institutional guidelines.

A previous version of the first study on MIA has been made publicly available as a preprint (PMID: 37461513). Some figures were created with BioRender (*https://www.biorender.com/*). For re-use of previously published figures, the publisher's permission was obtained (*licence number given in respective legend*).

4. CONSEQUENCES OF MIA FOR MORPHOLOGY AND THE EMBRYONIC AND ADULT HIPPOCAMPAL PROTEOME

As stated previously (see Chapter 1.2), MIA represents a validated animal model to assess the effect of inflammation as a risk factor for NPDs. However, few studies have assessed both sexes at a range of behavioural, functional protein-level, and morphological readouts, especially in sex-specific manner (see Chapter 3.1 for Methods). Here, I present the results of such a study performed for this dissertation and novel findings on changes of the HPC synaptic proteome following MIA.

4.1 RESULTS

4.1.1 Behavioural Outcomes

To confirm the validity of the prenatal immune activation model, mouse offspring of both sexes were generated following MIA via administration of the viral mimetic poly(I:C) in two concentrations (*low*, 2.5 mg/kg and *high*, 5 mg/kg; *control (ctrl)*, 0 mg/kg vehicle, PBS 1x) and extensively tested for NPD-relevant behaviours (Figure 3.1-3.3), using tests already validated in the laboratory (Candemir *et al.*, 2023; Freudenberg *et al.*, 2021; O'Leary *et al.*, 2022). The dose-dependent maternal immune response was verified by measuring plasma levels of the pro-inflammatory cytokine IL-6 1 h post injection (Figure 3.2E).

High-dose MIA offspring of both sexes exhibited reduced anxiety-like behaviours, increased distance travelled, and time spent in open and brightly lit environments, specifically (Figure 4.1B-C, Figure 4.2B-C), while retaining normal locomotor function (Figure 4.1A; detailed statistical analysis results are provided in Table 6 and figure legends). The anxiolytic effect of the prenatal high-dose poly(I:C) treatment was more pronounced in females than males in multiple tests (Figure 4.1B-C, Figure 4.2B-C). No differences were observed in the open-field test for the high-dose group, which was used to assess general locomotor activity (Figure 4.1A, Figure 4.2A). Additionally, the low and high viral mimetic dosages led to divergent spatial novelty preference outcomes in MIA male offspring: increasing preference in the high- versus low-dose MIA males but not controls (Figure 4.1D).

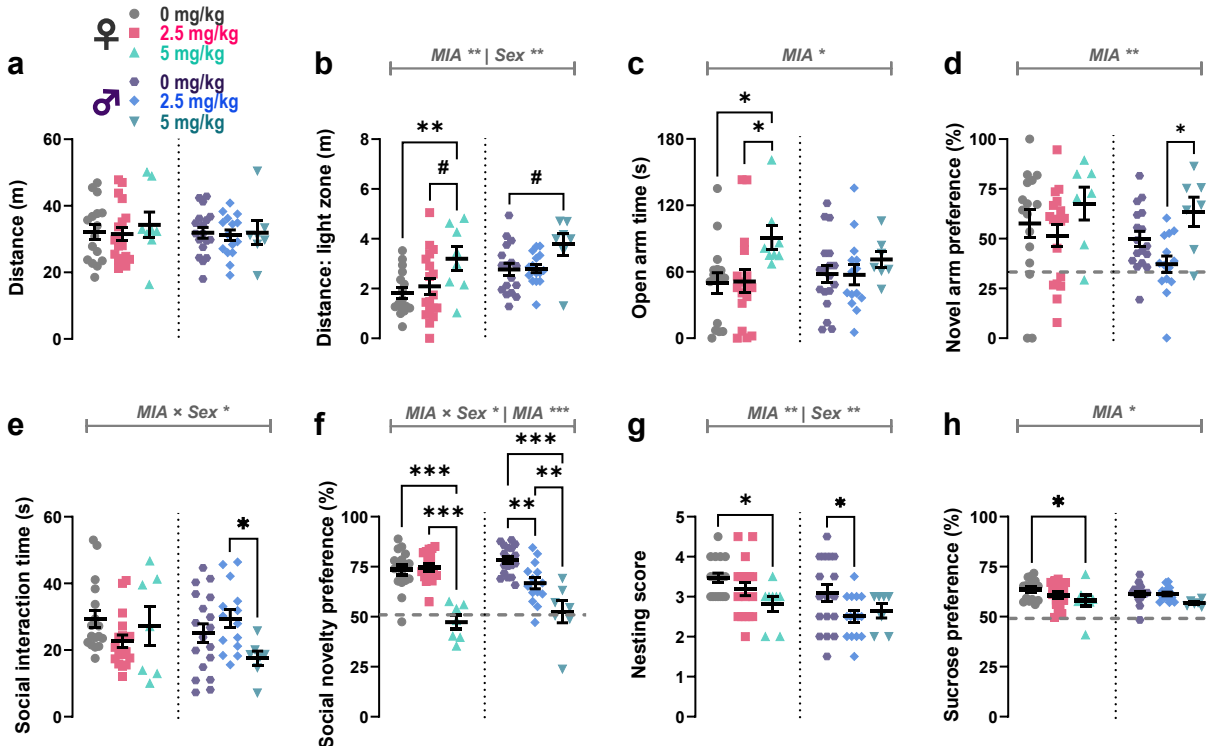


Figure 4.1 Early maternal immune activation (MIA) alters relevant behavioural endophenotypes in adult male and female mice. A, no differences in locomotion were observed in an Open Field test (two-way ANOVA). **B,** males and females of the 5 mg/kg MIA-exposed group displayed hyperactivity in the brightly lit compartment of the Light-Dark Box. **C,** a stronger prenatal immune challenged (5 mg/kg) increased the time spent in the open arm of an Elevated Zero Maze, a measure of reduced anxiety-like behaviour, more robustly in females. **D,** different dosage of the prenatal poly(I:C) treatment have divergent impact on the spatial novelty response in a Y-maze test, stronger in male offspring. **E,** when presented with a novel and familiar conspecific of the same sex in a social test, the time experimental animals spent socially interacting was impacted by MIA in a sex-specific manner, with poly(I:C) dose affecting males only. **F,** pronounced deficits in social preference toward the novel mouse were present in offspring of the 5 mg/kg MIA group for both sexes, while only the males of the 2.5 mg/kg group displayed social memory deficits. Social interactions were measured as interaction by the experimental animal alone. **G,** both treatment and sex affected nesting score, wherein female offspring of 5 mg/kg poly(I:C)-challenged dams and males from the 2.5 mg/kg offspring displayed a significant decrease of nest building quality in a two-day nesting test, indicative of self-neglect. **H,** the 5 mg/kg poly(I:C)-treated group exhibited an anhedonia-like decrease in sucrose preference, which was more pronounced in the female offspring. Grey dashed lines were used to denote chance levels of in choice tests. *Ctrl:* n=15-18 F, 15-18 M; *Low*: n=10-18 F, 14-15 M; *High*: n=7-8 F, 6-7 M. For more details on statistical analysis and animal numbers, see Tables 2 and 6, and Supplementary Table S1. Significance denoted as follows: two-way ANOVA main effects (grey) and Tukey *post-hoc* (black), *p<0.05, **p<0.01, ***p<0.001, #p<0.08. All data presented as mean ± SEM.

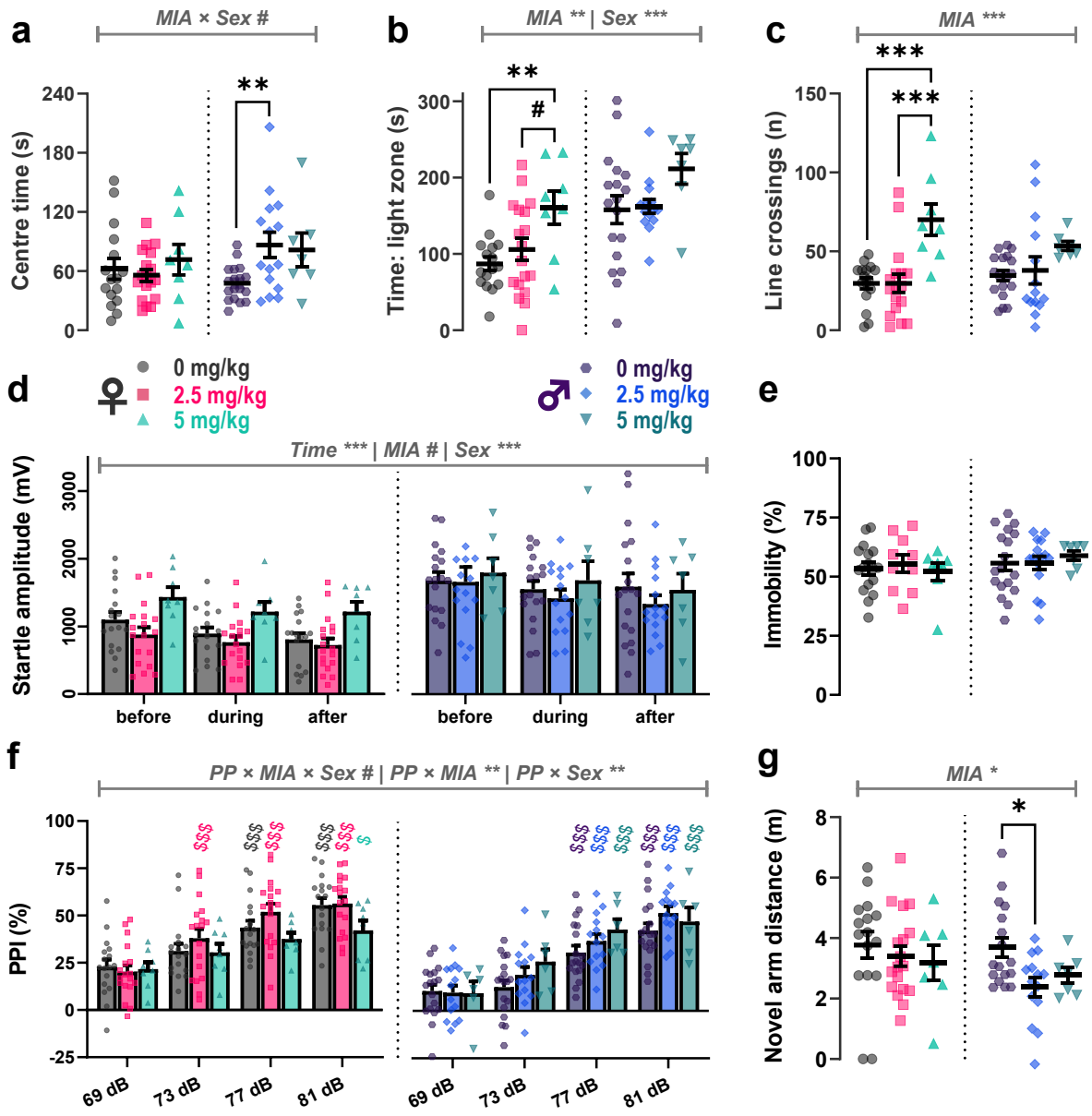


Figure 4.2 Differences in behavioural profiles of adult maternal immune activation (MIA) mouse offspring of both sexes. A, MIA slightly affected the time spent in the centre of an Open Field in a sex-dependent manner, with only males of the 2.5 mg/kg offspring spending significantly more time in the centre, indicating sex-specific anxiolytic effects of this poly(I:C) treatment dose. **B,** a stronger prenatal immune challenge (5 mg/kg) led offspring to spend significantly more time in the brightly lit compartment of the Light-Dark Box – an effect more pronounced in the females, indicative of reduced anxiety-like behaviour. **C,** the 5 mg/kg poly(I:C) treatment had a similar anxiolytic effect on offspring, which was also more significant in females, when tested in an Elevated Zero Maze. **D,** MIA had only a slight effect on the acoustic startle reflex, somewhat visible in the females, but did not yield significant differences in a multiple-comparison *post-hoc* test (three-way RM ANOVA, MIA: p=0.065). Sex affected startle amplitude due to animal size differences (three-way ANOVA, Sex: p<0.001), while habituation remained intact (three-way RM ANOVA, Time: p<0.001). **E,** no differences in immobility, as percentage of time, were found for any of the MIA offspring in a swim test. *Continued next page.*

Continued. **F,** sensorimotor gating measured via pre-pulse inhibition (PPI) of the startle response was generally intact. However, there was a trend toward sex-specific as well as a significant overall interaction effect of treatment and pre-pulse level, which manifested in a deficit in the 5 mg/kg females, unable to reliably inhibit the startle reflex. **G**, in a Y-maze spatial memory and preference test, MIA affected novel arm distance, where males of the 2.5 mg/kg poly(I:C) group were significantly hypoactive in the novel arm, indicating aberrant novelty response. For additional information, see also Figure 4.1. *Ctrl:* n=15-18 F, 15-18 M; *Low*: n=10-18 F, 14-15 M; *High*: n=7-8 F, 6-7 M. For more details on statistical analysis and animal numbers, see Tables 2 and 6, and Supplementary Table S1. Significance denoted as follows: ANOVA main effects (grey) and multiple-corrected Tukey *post-hoc* (black): *p<0.05, **p<0.01, ***p<0.001, #p<0.08 (A-E, G), in F: $ p<0.05, $$ p<0.01, $$$ p<0.001 for Tukey *post-hoc* vs respective group baseline (PPI at 69 db SPL pre-pulse level, RM ANOVA). All data presented as mean ± SEM.

The low-dose poly(I:C) treatment showed moderate effects on some behavioural deficits in males but not females, including increased centre time in the open field, and hypoactivity in a novel environment in the Y-maze (Figure 4.2A, G).

Interestingly, prenatal immune activation had detrimental sex-dependent effects on social interaction and novelty preference (Figure 4.1E-F), as measured by the duration the experimental animal interacted with a juvenile conspecific. Impairment of the social novelty preference was highly significant in both sexes following the high-dose treatment. The low-dose poly(I:C) treatment, on the other hand, significantly reduced social novelty preference in the absence of social interaction deficits in male MIA offspring, specifically (Figure 4.1E-F).

Furthermore, depressive-like behaviours in high-dose offspring manifested as self-neglect, as assessed in the nest building test (Figure 4.1G), and anhedonia (Figure 4.1H), assessed in the sucrose preference test – both more pronounced in females. The low-dose poly(I:C) MIA again only affected male offspring, manifesting in decreased nesting score (Figure 4.1G). However, no significant behavioural deficits were observed in the FST (Figure 4.2E).

The ASR revealed a slight trend (Table 6) towards MIA-induced differences, but overall startle response remained intact (Figure 4.2D). Expected differences in ASR between the sexes due to the higher weight of males were observed in control and low-dose MIA offspring but were abolished by high-dose MIA (Table 6). Simultaneously, subtle deficits in sensorimotor gating, especially at the high pre-pulse levels, as measured by PPI of the ASR, were observable in female MIA offspring (Figure 4.2F).

Taken together, these behavioural and immunological findings substantiate the overall MIA model validity as established by me within our institution, and further support its relevance for NPD-associated behaviours, while revealing some sex- and dose-specific effects.

Table 6 Detailed statistical analyses of behavioural data from high- and low-dose MIA and control offspring. Simple two-way ANOVA was performed for all tests, measuring main effects of sex, treatment, and their interaction, except for ASR (repeated-measure, two-way ANOVA) and PPI (repeated-measure, three-way ANOVA, additional variable: pre-pulse, PP level). For corrected multiple comparisons, a Tukey post-hoc test was performed. See Chapter 3.1.3 for more information on behavioural testing parameters and Supplementary Table S1 for animal numbers, see Figure 4.1-4.2 for visuals. *MIA*, maternal immune activation; *OF*, open field; *EZM*, elevated zero maze; *LDB*, light dark box; *SI/SR*, social interaction/social recognition; *SP*, sucrose preference; *FST*, swim test; *ASR*, acoustic startle reflex; *PPI*, pre-pulse inhibition; *PP*, decibel (dB) pre-pulse level.

Test	**Parameter**	**Variable**	*Main effects*		**Multiple comparisons** *(relevant results)*
			Statistics	**p-value**	
OF	Distance (m)	Interaction	F (2,76) = 0.094	P=0.911	n.a.
		Sex	F (1,76) = 0.251	P=0.618	
		MIA	F (2,76) = 0.245	P=0.784	
	Centre time (s)	Interaction	F (2,76) = 3.111	P=0.050	n.a.
		Sex	F (1,76) = 0.940	P=0.335	
		MIA	F (2,76) = 2.452	P=0.093	
EZM	Open arm time (s)	Interaction	F (2,74) = 0.860	P=0.427	*High v Ctrl*: p=0.044 * *High v Low*: p=0.0498 * [*F*: * / *M*: n.s.]
		Sex	F (1,74) = 0.038	P=0.847	
		MIA	**F (2,74) = 3.496**	**P=0.0354**	
	Line crossings (n)	Interaction	F (2,74) = 1.781	P=0.176	*High v Ctrl*: p=0.0001 *High v Low*: p=0.0003 [*F*: *** / *M*: n.s.]
		Sex	F (1,74) = 0.034	P=0.854	
		MIA	**F (2,74) = 10.79**	**P<0.0001**	
LDB	Distance in light (m)	Interaction	F (2,77) = 0.127	P=0.881	*M*: *High v Ctrl*: p=0.056 # *F: High v Ctrl*: p=0.007 ** *High v Low*: p=0.055#
		Sex	**F (1,77) = 7.720**	**P=0.0069**	
		MIA	**F (2,77) = 7.493**	**P=0.0011**	
	Time in light (s)	Interaction	F (2,77) = 0.356	P=0.7020	*High v Ctrl*: p=0.001 ** *High v Low*: p=0.013 * [*F*: * / *M*: n.s.]
		Sex	**F (1,77) = 20.32**	**P<0.0001**	
		MIA	**F (2,77) = 7.079**	**P=0.0015**	
Y-maze	Novel arm distance (m)	Interaction	F (2,73) = 0.945	P=0.393	*Ctrl v Low*: p=0.049 * [*F*: n.s. / *M*: *]
		Sex	F (1,73) = 0.888	P=0.349	

		MIA	**F (2,73) = 3.212**	**P=0.046**	
	Novel arm preference (%)	Interaction	F (2,73) = 0.333	P=0.718	*High v Low*: p=0.008 ** [*F*: n.s. / *M*: *]
		Sex	F (1,73) = 2.827	P=0.097	
		MIA	**F (2,73) = 4.988**	**P=0.0093**	
SI/SR	Social interaction (s)	**Interaction**	**F (2,75) = 3.836**	**P=0.0259**	*M*: *High v Low*: p=0.044 * *F:* n.s.
		Sex	F (1,75) = 0.976	P=0.326	
		MIA	F (2,75) = 1.046	P=0.357	
	Social novelty preference (%)	**Interaction**	**F (2,75) = 4.266**	**P=0.0176**	*M*: *High v Ctrl*: p<0.001*** *Low v Ctrl*: p=0.0028** *High v Low*: p=0.004 ** *F: High v Ctrl*: p<0.0001*** *High v Low*: p<0.0001***
		Sex	F (1,75) = 0.064	P=0.801	
		MIA	**F (2,75) = 39.20**	**P<0.0001**	
SP	Sucrose preference (%)	Interaction	F (2,70) = 0.541	P=0.585	*High v Ctrl*: p=0.0112 * [*F*: *. / *M*: n.s.]
		Sex	F (1,70) = 0.442	P=0.508	
		MIA	**F (2,70) = 4.416**	**P=0.0156**	
Nest	Nesting score	Interaction	F (2,76) = 0.886	P=0.417	*High v Ctrl:* p=0.02 * *Low v Ctrl*: p=0.02 * [*F*: *High v Ctrl:* p=0.035* / *M*: *Low v Ctrl:* p=0.048*]
		Sex	**F (1,76) = 7.430**	**P=0.008**	
		MIA	**F (2,76) = 5.461**	**P=0.0061**	
FST	Immobility (%)	Interaction	F (2,69) = 0.381	P=0.685	n.a.
		Sex	F (1,69) = 1.333	P=0.252	
		MIA	F (2,69) = 0.075	P=0.928	
ASR	Startle amplitude (mV) during	Interaction	F (2,77) = 0.249	P=0.780	*High v Low*: p=0.045 * [*F*: # / *M*: n.s. / *M v F (high):* n.s., *M v F (low & ctrl):* ***]
		Sex	**F (1,77) = 27.95**	**P<0.0001**	
		MIA	***F (2,77) = 2.972***	***P=0.057***	
PPI	Pre-pulse inhibition (%)	**PP level**	**F (3,225)=196.5**	**P<0.001**	*PPI vs baseline (69 dB):* *M:* Ctrl / Low / High – all: *69 v 77, 81* *** *F*: Ctrl – *69 v 77, 81* *** Low – *69 v 73, 77, 81* *** High – *69 v 81 */ rest:* n.s.
		PP × Sex	**F (3,225) = 4.26**	**P=0.006**	
		PP × MIA	**F (6,225) = 3.09**	**P=0.006**	
		PP × Sex ×MIA	***F (6,225)= 1.99***	***P=0.068***	

4.1.2 Synaptoneurosomal Proteomic Analysis

ADULT HIPPOCAMPAL SYNAPTOME CHANGES FOLLOWING MIA

To interrogate the functional molecular consequences of MIA in the HPC, this study investigated the HPC synaptoneurosomal proteome using mass spectrometry in the high-dose MIA group, as it resulted in a more discernible behavioural phenotype (see Chapter 4.1.1). The functional relevance of the observed proteomic changes was compared to control mice by enrichment analyses in the protein-coding genome and at the synapse, specifically. Of the 4 744 detected proteins, 593 were significantly altered ($p<0.05$, $q<0.1$, $|change|>0.2$), the large majority (75.9%) of which were upregulated in the adult MIA-exposed offspring (Figure 4.3G-H; see Chapter 3.5).

Functionally-enriched pathways included those involved in autophagy, receptor-mediated phagocytic response, cell growth and morphogenesis, mitochondrial function and metabolic regulation, cytoskeletal organisation, and plasticity-related signal transduction (Figure 4.3I; Figure 4.5A). Closer inspection of synaptic mechanisms affected by MIA highlighted synapse organisation and transport, postsynaptic specialisation, and synaptic vesicle membrane and trafficking, which is central to neurotransmission (Figure 4.4D, G).

Considering the role of sex in animal behaviour highlighted by the findings of this study (see previous section), as well as prevalence differences of NPDs and comorbid psychiatric conditions, this study set out to further examine possible sex-dependent effects of MIA on the synaptic proteome. Indeed, 59.2% of significantly up- or downregulated proteins in the adult pool were sex-specific (Figure 4.3A, D, Figure 4.4A).

Moreover, some of the proteins changed in both male and female MIA offspring (e.g., NNT, SNCA) were regulated in opposite directions, while other protein abundance differences were exclusive to either females or males (n=546 and 406, respectively; Figure 4.3A, A6D, Figure 4.4A). In females, 50.2% of the 930 significantly-altered synaptoneurosomal proteins were downregulated, while in males a large majority (n=522 of 713) were upregulated (Figure 4.3B, E).

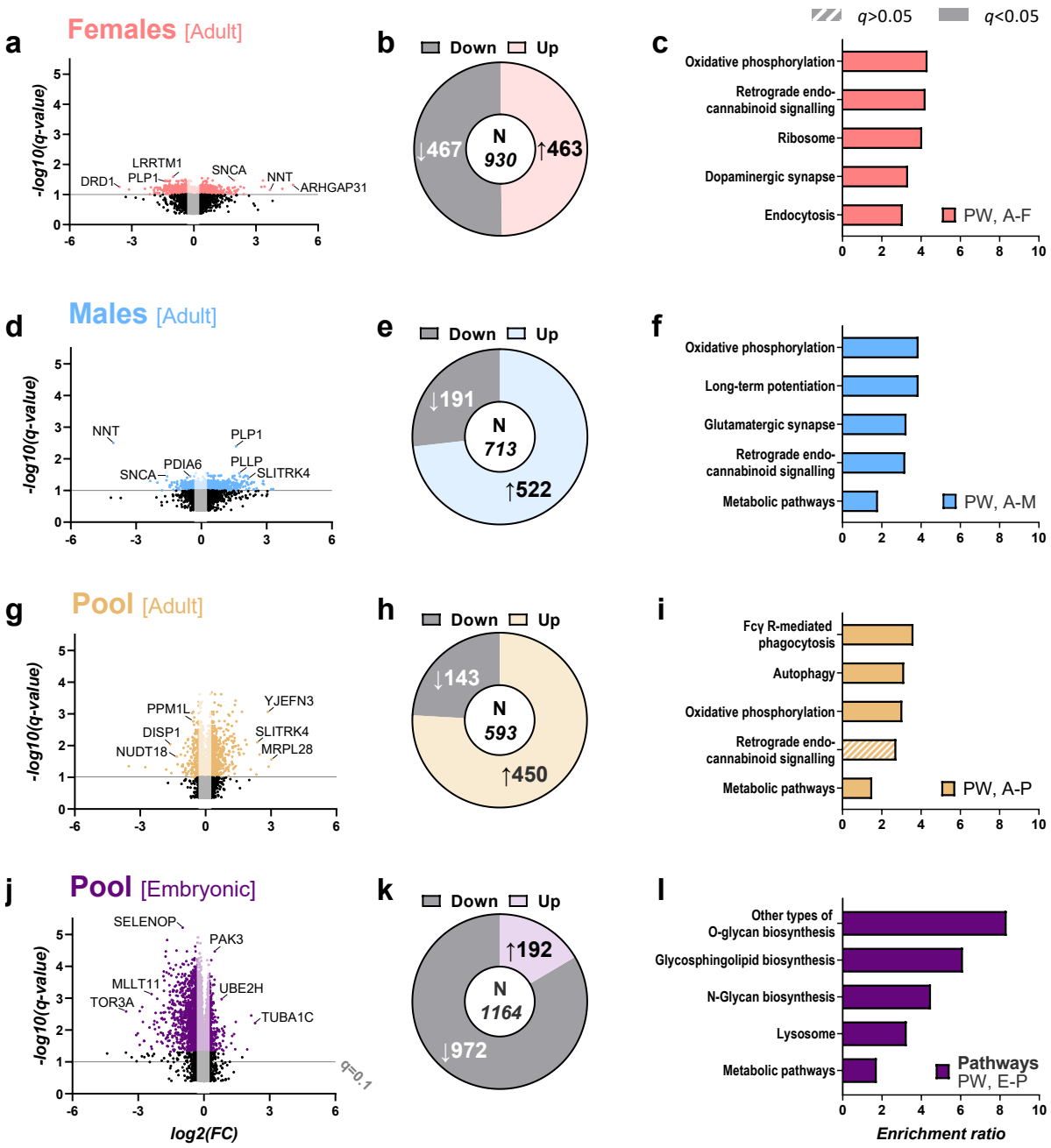


Figure 4.3 Dynamic alterations of the embryonic and adult synaptic proteome follow maternal immune activation (MIA). Volcano plots of proteomic results from synaptoneurosomes of early MIA mice (poly(I:C), 5 mg/kg) and controls. *Continued. Continued*. **A**, volcano plot adult females (AF); **D**, adult males (AM); **G,** adult pool (AP); **J,** embryonic pool (EP, E18; adults: n=2 M, 2 F per group, 4 AP replicates per group; embryos: n=16 M, 14 F each control and treated animals; 4 EP replicates per group). These show all detected proteins, based on the significance (*q*-value) and fold-change (FC) in hippocampi of MIA offspring when compared to controls. Selected proteins shown by name in the volcano plots exhibit a combination of high significance, FC, and biological relevance. Colour denotes significant changes (q<0.1, *t*-tests, *all volcano plots*), shaded boxes indicate minor changes in regulation (absolute FC below 0.2). Of those meaningfully altered synaptoneurosomal proteins, upregulation was observed in adults for nearly half in females (**B**) and most in males and pool (**E, H**), while most changes in the embryonic pool were downregulations (**K**).

Continued. The functional involvement of prenatal immune challenge-influenced synaptoneurosomal proteins in molecular pathways was investigated further for all groups (*right column*). The KEGG pathway (PW) enrichment ratios for MIA-altered proteins of adult females (**C**) and males (**F**) indicated partially sex-specific activation of distinct cellular responses to MIA immune challenge, such as overrepresentation of dopaminergic synapses and endocytosis in females, and glutamatergic synapses and metabolism in males. Enriched terms for the pooled adult samples mirrored some pathway categories from the male and female-specific analyses, including mitochondrial and general metabolism, and plasticity-regulating synaptic signalling (**I**). Here, more generalised effects of MIA manifested in the form of the pathogen response-related phagocytosis and cellular self-degradation. The dynamic nature of the synaptoneurosomes' proteomic alterations following MIA is reflected in the embryonic data, which lacked significant sex differences before birth. The altered proteins were involved in highly enriched cell membrane-building glycoprotein and lipid synthesis, as well as lysosomal pathways (**L**). Significance threshold for pathway analysis, using Benjamini-Hochberg multiple-test corrected *p*-value (q), was set at p/q=0.05 (Fisher's exact test, *all pathways*).

Overrepresented molecular pathways in the females included actin cytoskeleton and synaptic organisation, synaptic vesicle and endocytic processes, and the ribosome (Figure 4.3C, Figure 4.5B). In males, specifically, enriched pathways were involved in synaptic plasticity via long-term potentiation, multiple metabolic processes, gliogenesis, and myelination (Figure 4.3F, Figure 4.5C). In both sexes, pathways related to retrograde endocannabinoid signalling, mitochondrial oxidative phosphorylation, and intracellular signalling via GTPases were affected by MIA (Figure 4.3C,F, Figure 4.5B-C).

Importantly, prenatal MIA exposure caused enrichment of proteins associated with dopaminergic synapses in females and glutamatergic synapses in males, which play different roles in NPDs (Figure 4.3C, F). Classical NPD-relevant targets like the HOMER and SHANK postsynaptic density adaptor proteins were also affected by MIA (see Chapter 1.2.4). While HOMER2/3 were substantially downregulated in MIA synaptoneurosomes, HOMER1 was increased in male – in line with previous findings (Atanasova *et al.*, 2023) - and decreased in female HPC samples (see Chapter 3.5, Data Availability). In line with sex-specific MIA alterations, SHANK1/2 were significantly decreased only in males and SHANK3 – only in females.

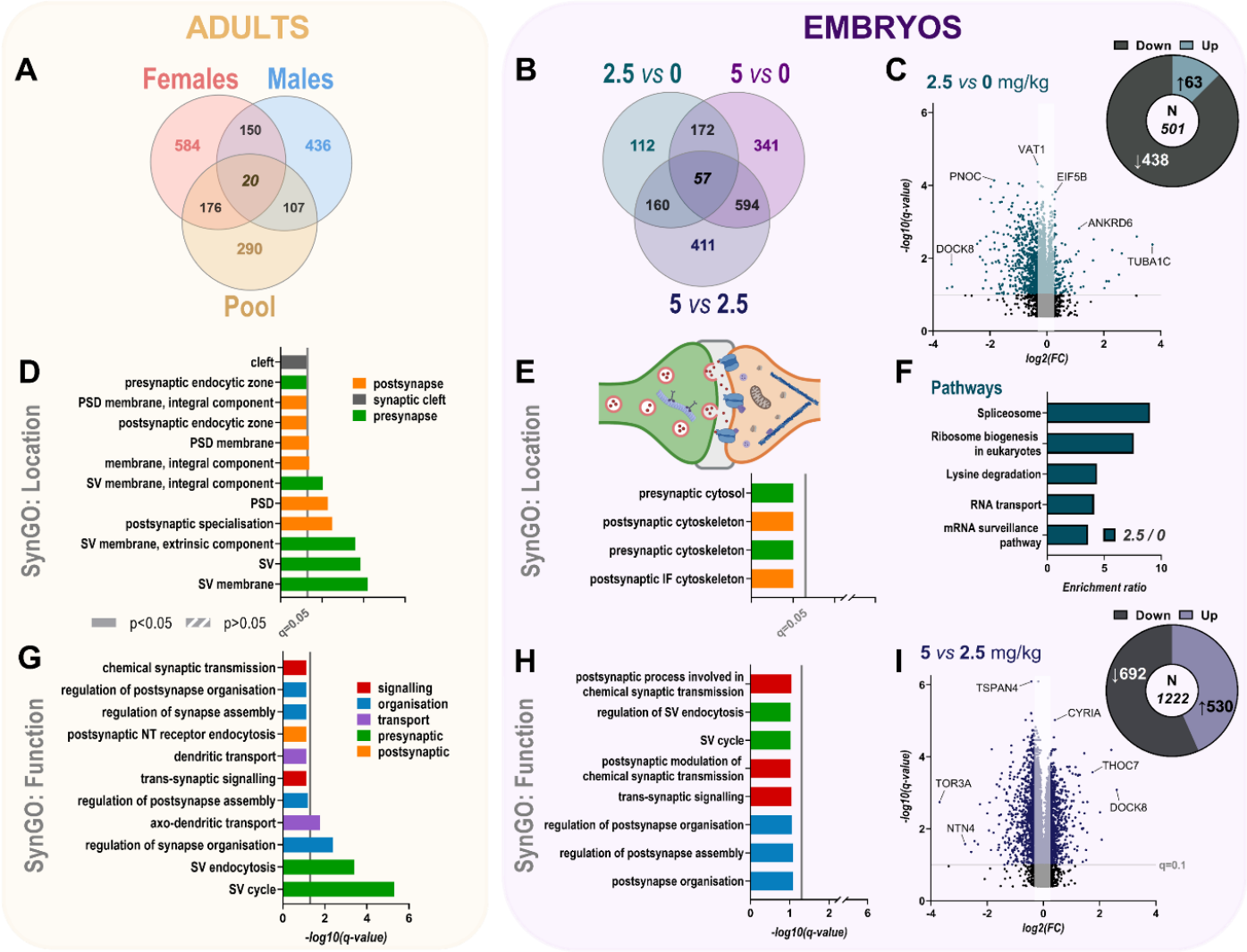


Figure 4.4 Synaptic proteome changes in adult and embryonic hippocampi of prenatally immunostimulated mice. *Continued.*

Continued. **A**, overlap of significantly and relevantly changed proteins between adult female, male, and pooled adult hippocampal synaptoneurosomes (SN) of early-gestation (E9) immune challenged mice (viral-like mimetic poly(I:C), 5 mg/kg). Synaptic enrichment (SynGO) for the adult pool, revealed the central role of the synaptic vesicle membrane and the postsynapse (**D**), as well as functional synaptic organisation and synaptic transport (**G**) in long-lasting proteome changes following prenatal immune activation. **B**, overlap of significantly and relevantly changed proteins between embryonic (E18) hippocampal SN of early-gestation immune challenged mice (poly(I:C), 5 or 2.5 mg/kg) and controls of both sexes. Here, SynGO terms for proteins changed in 5 mg/kg poly(I:C) offspring were the synaptic cytoskeleton (**E**, *bar chart legend and colours of synaptic components as shown in **D, G***), and functionally - postsynaptic organisation, synaptic transmission, and the vesicle cycle (**H**), but did not cross the multiple-correction significance threshold here. Volcano plots for embryonic SN proteome changes of the lower-intensity prenatally immune challenged mice (poly(I:C), 2.5 mg/kg), compared to controls (**C**) and high concentration offspring (**I**). Embryonic samples were pooled due to lack of sex differences. Shown are all detected proteins, based on the significance (q-value) and fold-change (FC) and selected proteins shown by name in the volcano plots exhibit a combination of high significance, FC, and biological relevance. Colour denotes changes above q=0.1, shaded boxes indicate small change in regulation (absolute change below 0.2). A less severe immune challenge (2.5 mg/kg) led to alterations of proteins, involved in the RNA and amino acid metabolism and enriched protein-coding genome pathways were centred around ribosome biogenesis and posttranscriptional processes like splicing, mRNA transport and surveillance (**F**). When comparing the effects of the two poly(I:C) doses directly, the highest number of significant changes were observed, indicating divergent effects of MIA on HPC development beyond simple dose-dependency (**I**). Comparison revealed glycan and ribosome biosynthesis, mRNA splicing, and focal adhesion as main affected pathways (*not shown*). Significance threshold for proteomic changes (*t*-test; $p<0.05$, *volcano plots*) was set at $q=0.1$, calculated via the Storey-Tibshirani false discovery procedure, and at q=0.05 for all subsequent analyses using the Benjamini-Hochberg multiple-test correction method (Fisher's exact test, $p<0.05$, *pathways/synaptic enrichment*). *IF,* intermediate filament; *NT,* neurotransmitter; *PSD,* postsynaptic density; *SV,* synaptic vesicle.

ALTERATIONS OF THE PRENATAL SYNAPTIC PROTEOME

Assessment of late embryonic (E18) HPC synaptoproteome of high-dose MIA offspring and controls revealed 1164 proteins significantly altered by MIA, most (83.5%) of which were downregulated (Figure 4.36J-K). Interestingly, HOMER3 (-8.3%) and SHANK3 (-12%) were slightly but significantly downregulated by MIA (see Chapter 3.5). Synapse-level enrichment revealed nominal significance for pre- and postsynaptic structure and organisation, modulation of chemical transmission, and the synaptic vesicle cycle, though these associations did not pass the significance threshold after correction (Figure 4.4E, H).

Affected protein-coding genome pathways include lysosomes and mitochondria, as well as modifications related to proper biomembrane development and environmental stimuli response function, such as sialic acid, membrane lipid, and glycoprotein metabolism (Figure 4.3L, Figure 4.5D). MIA appears to prominently affect the embryonic lipid and polysaccharide metabolism at the synapse, i.e., the synthesis of glycosphingolipids and glycans, essential for nervous system development and functionality.

Notably, no MIA-induced sex-specific differences in protein abundance crossed the significance threshold in the embryonic tissue when compared to controls, except one (SSX2IP), in male vs female low-dose MIA offspring.

Next, the poly(I:C) dose-dependent effects on prenatal synaptic neurodevelopment were investigated instead. Indeed, 501 proteins were significantly influenced in the lower-dose MIA offspring when compared to controls, of which 87.4% were downregulated (Figure 4.4C). Here, significantly affected pathways centred around the regulation of RNA metabolism and localisation, especially post-transcriptional processes like splicing, transport, and surveillance, indicating more transient synaptic responses to the low-dose immune challenge (Figure 4.4F, Figure 4.5E).

The majority of the observed changes (54.3%) comprised proteins different from those altered by a high-dose treatment compared to controls (Figure 4.4B). Indeed, when contrasting the synaptoneurosomal proteome composition between the two poly(I:C) treatments directly, 1 222 proteins differed significantly, of which 56.6% were downregulated in the high-dose MIA-exposed offspring (Figure 4.4B, I).

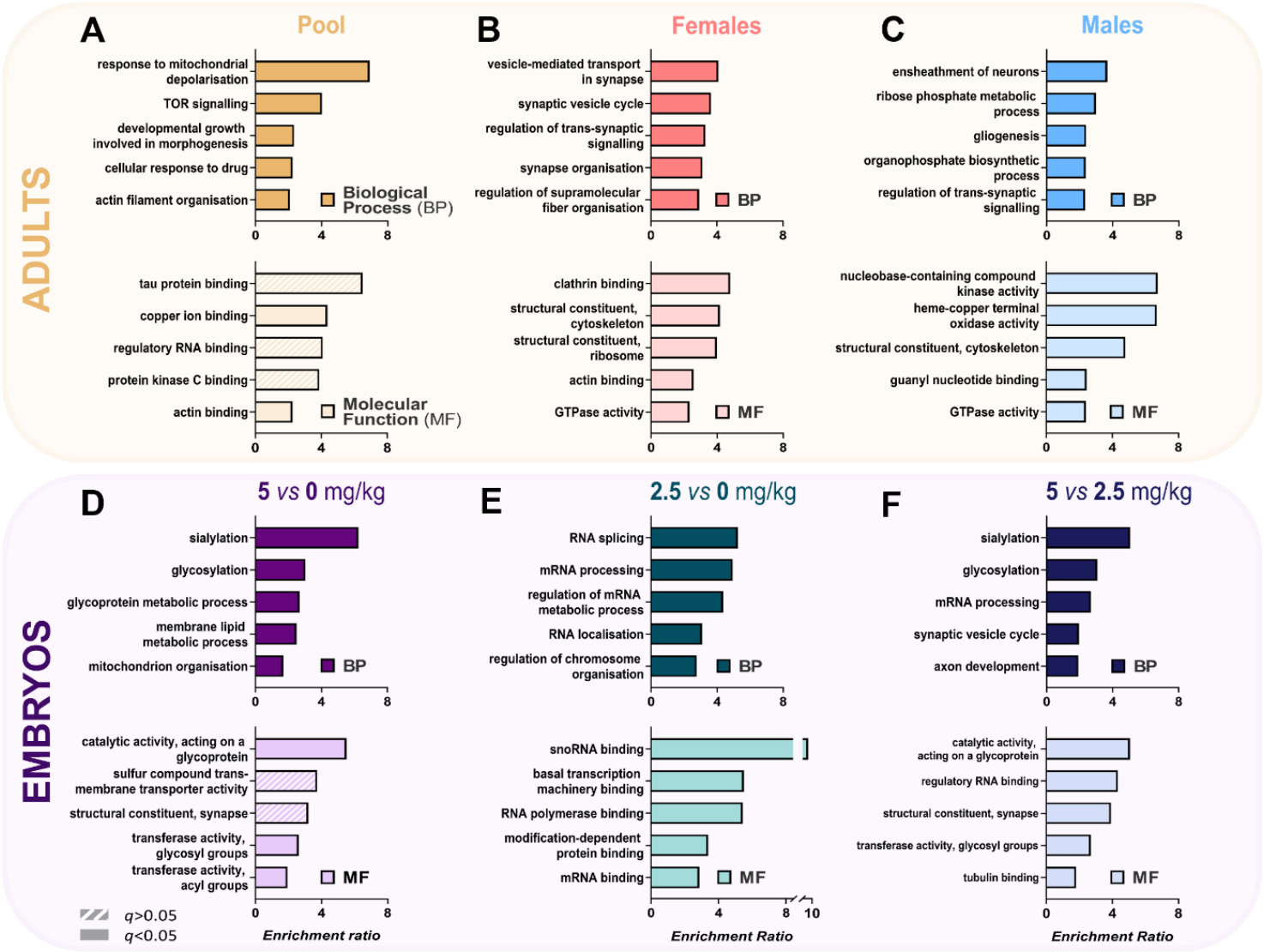


Figure 4.5 Functional enrichment of adult and embryonic MIA-induced changes in the protein-coding genome based on sex and treatment, respectively. Proteins for analysis were meaningfully altered ($p<0.05$, $q<0.1$, absolute fold-change>0.2) when compared to their respective control groups. In adults (**A-C**), only offspring of 5 vs 0 mg/kg poly(I:C)-treated dams was used, in embryos (**D-F**) males and females were pooled for the respective treatment comparisons in absence of sex-specific effects on the developing synapse. Analysis was performed with a publicly available online tool (*www.webgestalt.org*, $p<0.05$). q, Benjamini-Hochberg, false discovery rate-adjusted p-value.

Functional differences involved regulatory RNA processing and binding, membrane surface modification, synaptic structure and vesicle cycle, and axonal development (Figure 4.5F). 411 of those proteins differentially altered by the high and low poly(I:C) treatment were not affected for either prenatally challenged group compared to controls, which might underlie some of the observable phenotypic divergences in low- versus high-dose MIA offspring.

Collectively, my results show significant modifications of the embryonic synaptic proteome after MIA during a relevant developmental time-window in late gestation, which are dose- but not sex-dependent.

SYNAPTONEUROSOMAL PROTEINS CHANGED AT BOTH AGES AND NPD RELEVANCE

Subsequently, the influence of early gestational MIA on the developing and mature synaptic proteome was explored. While most of the identified proteomic changes in the HPC following high-dose MIA exposure were age-specific, 50 affected proteins discovered in the embryos were common to the adult pool (Figure 4.6A). Hierarchical clustering of these common proteins revealed four major clusters, namely two downregulated in adults and mostly in embryos (n=12 proteins), one upregulated at both ages (n=19), and one decreased in embryos but increased in adults (n=19; Figure 4.6D).

Synaptic-level analyses revealed these proteins to be especially enriched in proteins of the presynapse and synaptic vesicle membrane, and that their functional role was related to synaptic vesicle endocytosis and cycling (Figure 4.6B-C).

Interrogation of protein-protein-interactions using the STRING database revealed a network of significantly interconnected proteins in the fourth, persistently upregulated cluster of 19 proteins (interaction enrichment $p=6.48 \times 10^{-8}$ in embryonic set, $p=6.28 \times 10^{-5}$ in adult set; Figure 4.6D-E). A subset of proteins (including BIN1, DPYSL2, SNCB, MAPT, SYN1) within this network was strongly represented in the identified functional pathways (Figure 4.6E-F). Functional enrichment in the embryonic dataset highlighted cytoskeletal protein binding, while component enrichment confirmed the axonal terminus and the synaptic vesicle membrane as subcellular structures significantly converging with this upregulated cluster (Figure 4.6E-F).

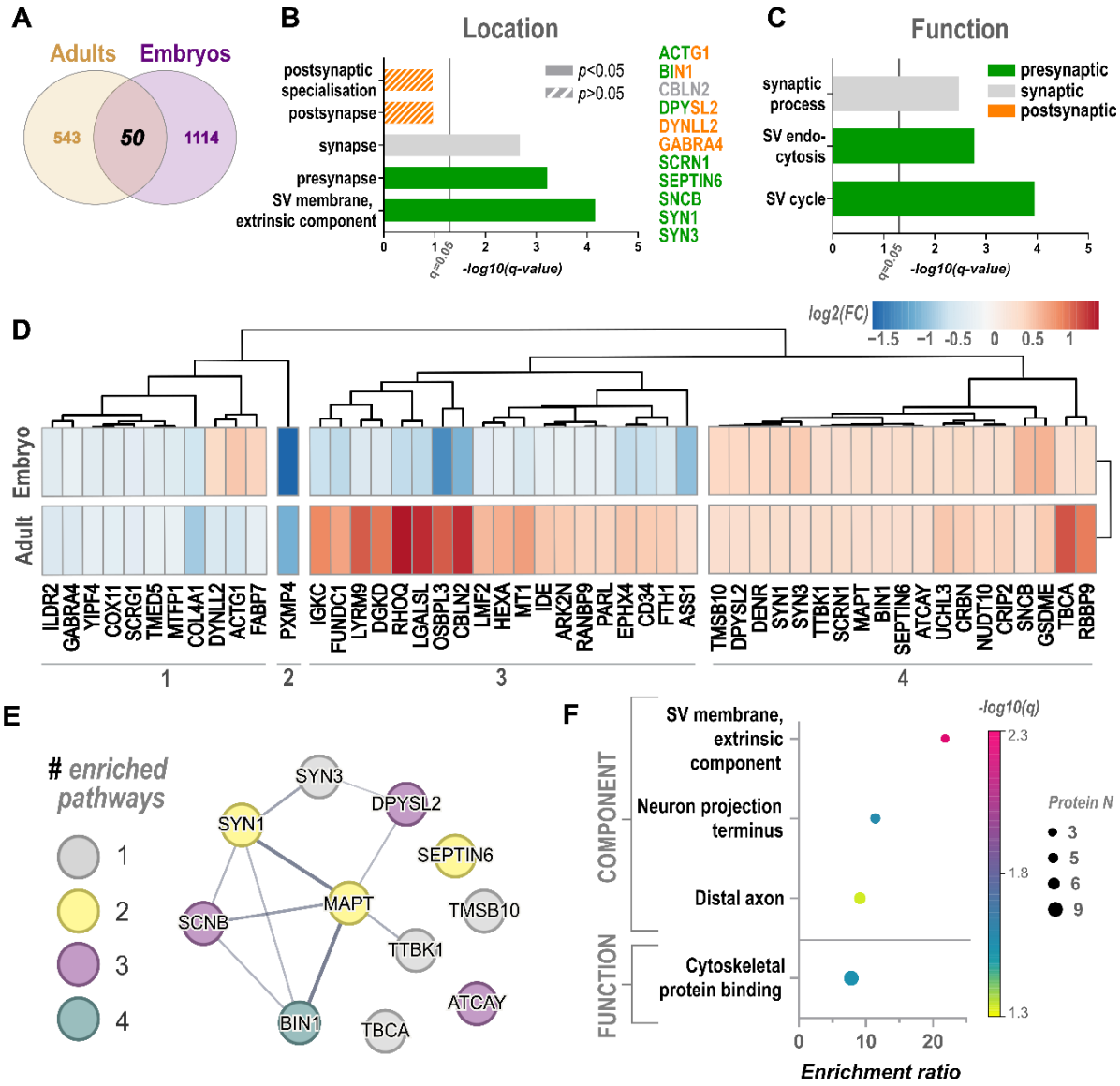


Figure 4.6 Maternal immune activation (MIA)-induced protein changes shared in embryonic and adult hippocampal (HPC) synaptoneurosomes involve neuronal signalling via the synaptic vesicle cycle and cytoskeletal dynamics. Long-term effects of MIA are revealed by overlap of proteins altered in SNs of both embryonic and adult HPC (**A**). *Continued.* The 50 overlapping proteins were enriched at the presynapse and the synaptic vesicle (SV) membrane (**B**). Functionally, these proteins are significantly involved in the SV cycle and endocytosis (**C**). Colours for overrepresented proteins in **B** match the respective enriched term category (legend in C, *multiple colours= protein included in multiple terms*). Hierarchical clustering of the respective average fold-change (FC) revealed four clusters (**D**), namely downregulated in adults (Cluster 1, n=11; Cluster 2, n=1), downregulated in embryos and upregulated in adults (Cluster 3, n=19), and persistently upregulated in both (Cluster 4, n=19 / *replicate numbers as in Figure 4.3*). Cluster 3 formed a highly significant interaction network of proteins (p=6.48 x 10^{-08} embryos, p=6.28 x 10^{-05} adults; **E**). Pathway enrichment underscored the involvement of this network's embryonic brain-enriched common proteins in synaptic signalling via SV, the neurite terminus and distal axon, and cytoskeletal dynamics (**F**). Additionally, the extrinsic SV membrane component was overrepresented in the adult dataset (*Enrichment ratio: 19.2, n=3, -log10(q)=1.62*). q<0.05 for all (Benjamini-Hochberg corrected *p*).

To investigate the extent of association between MIA-induced, long-term synaptic changes with NPDs, I analysed PheWAS data for the genes encoding the 50 proteins that overlapped between the embryonic and adult proteomes. All but one of these genes (immunoglobulin kappa constant, IGKC) were significantly associated with psychiatric or cognitive traits. Apart from NPDs in a narrower sense, i.e. ADHD, ASD, SCZ, and BP, the MIA-linked genes were associated with known comorbid conditions like conduct disorder, OCD, PTSD, SUDs, anxiety- and depression-related traits (Figure 4.7, Table 7; see also Chapter 1.1.2). Memory, higher cognitive functions, and mild intellectual disability as well as temperament and personality traits were similarly among the phenotypic categories significantly associated with many of the probed genes (Table 7).

Finally, the correlation between adult social novelty preference and HPC abundance of the 19 proteins downregulated in embryos and upregulated in adults by MIA, hinting at postnatal compensatory mechanisms, was examined (Figure 4.8). A pattern became apparent, wherein all MIA-upregulated proteins were negatively correlated with performance in the social task. Five proteins were significantly correlated (CBLN2, DGKD, MT1, ARK2N, FTH1) and three more revealed a trend (IDE, RANBP9, ASS1), further strengthening the association of proteins changed in both embryonic and adult HPC synaptoneurosomes and behavioural NPD-relevant phenotypes.

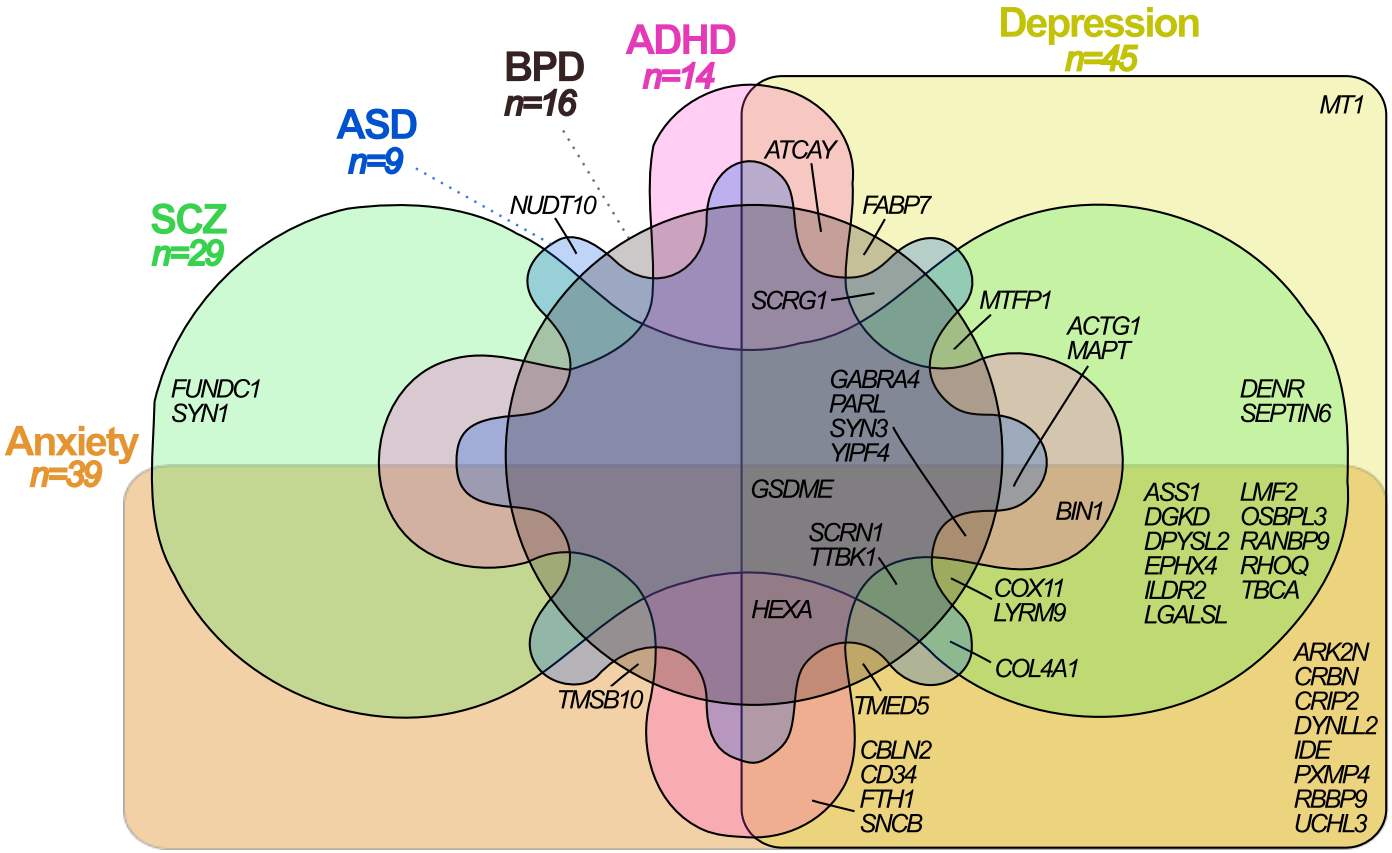


Figure 4.7 Proteins altered in the hippocampi of both the prenatal and adult mice following maternal immune activation (MIA) correspond to genes significantly associated with neurodevelopmental psychiatric disorders and comorbid phenotypes. Genes exhibiting pleiotropic effects and correlated with most of the six interrogated psychiatric conditions were *GSDME* (alias *DFNA5*, n=6) and *ACTG1, GABRA4, HEXA, MAPT, PARL, SCRN1, SYN3, TTBK1, YIPF4* (n=5) - a multitude of which play a role in neuronal cell fate, inflammatory response, and signal transmission. Additional information on the PheWAS results can be found in Table 7. Significance threshold for gene-trait associations: $p<0.05$, Bonferroni-corrected. *ADHD*, attention deficit hyperactivity disorder; *ASD*, autism spectrum disorder; *BPD*, bipolar disorder (*BP elsewhere*); *SCZ*, schizophrenia.

Table 7 Genes, encoding proteins significantly changed in both before birth and in adult prenatally immune-challenged offspring, are strongly correlated with psychiatric conditions and mental functions. A publicly available phenome-wide association study (PheWAS) database was used to interrogate the causal links of persistently changed genes and NPDs and comorbidities of interest. For visual representation of the directionality of changes in our age- and sex-specific datasets, see Figure 4.7.

Condition or Function	**Significantly correlated genes** PheWAS analysis summary
ADHD	*ACTG1, BIN1, CBLN2, CD34, FABP7, FTH1, GABRA4, GSDME, HEXA, MAPT, PARL, SNCB, SYN3*
CD	*FTH1, PARL, SEPTIN6*
Anxiety* | Neuroticism	*ACTG1, ARK2N, ASS1, BIN1, CBLN2, CD34, COL4A1, COX11, CRBN, CRIP2, DGKD, DPYSL2, DYNLL2, EPHX4, FTH1, GABRA4, GSDME, HEXA, IDE, ILDR2, LGALSL, LMF2, LYRM9, MAPT, OSBPL3, PARL, PXMP4, RANBP9, RBBP9, RHOQ, SCRN1, SNCB, SYN3, TBCA, TMED5, TMSB10, TTBK1, UCHL3, YIPF4*
ASD	*ACTG1, COL4A1, GSDME, HEXA, MAPT, NUDT10, SCRG1, SCRN1, TTBK1*
BP	*ATCAY, COX11, FABP7, GABRA4, GSDME, HEXA, LYRM9, MTFP1, PARL, SCRG1, SCRN1, SYN3, TMED5, TMSB10, TTBK1, YIPF4*
Depressive Disorders**	*ACTG1, ARK2N, ASS1, ATCAY, BIN1, CBLN2, CD34, COL4A1, COX11, CRBN, CRIP2, DENR, DGKD, DPYSL2, DYNLL2, EPHX4, FABP7, FTH1, GABRA4, GSDME, HEXA, IDE, ILDR2, LGALSL, LMF2, LYRM9, MAPT, MT1, MTFP1, OSBPL3, PARL, PXMP4, RANBP9, RBBP9, RHOQ, SCRG1, SCRN1, SEPTIN6, SNCB, SYN3, TBCA, TMED5, TTBK1, UCHL3, YIPF4*
Memory | Cognition***	*ACTG1, ARK2N, ASS1, ATCAY, BIN1, CBLN2, CD34, CRBN, CRIP2, DENR, DPYSL2, DYNLL2, FABP7, FTH1, FUNDC1, GABRA4, GSDME, HEXA, IDE, ILDR2, LGALSL, LMF2, LYRM9, MAPT, MT1, MTFP1, OSBPL3, PARL, PXMP4, RANBP9, RBBP9, RHOQ, SCRG1, SCRN1, SEPTIN6, SNCB, SYN3, TBCA, TMED5, TMSB10, TTBK1, UCHL3, YIPF4*
OCD	*FTH1, PARL, SEPTIN6*
PTSD	*CBLN2, MAPT, OSBPL3, RANBP9*
SCZ | SCZ vs BP	*ACTG1, ASS1, BIN1, COL4A1[$], COX11, DENR[#], DGKD[#], DPYSL2, EPHX4[$], FUNDC1[#], GABRA4[$], GSDME[&], ILDR2, LGALSL[#,&], LMF2[#], LYRM9[&], MAPT, MTFP1, OSBPL3, PARL, RANBP9, RHOQ[#], SCRN1, SEPTIN6[&], SYN1[#], SYN3[&], TBCA[$], TTBK1[&], YIPF4[$]*

*Anxiety includes traits containing "Anxiety", "Anxious", Neuroticism comprises "Neuroticism" and "Worry". **Depressive Disorders includes "Depressive Episode", "Recurrent Depressive Disorder", and "Bipolar Affective Disorder/Depressive Episode" subchapters, as well as "Depressive Symptoms" and "Depressive Affect" traits, ***Memory comprises "Memory Functions" and "Dementia in Alzheimer's Disease" subchapters, Cognition corresponds to "Higher-Level Cognitive Functions", "Mild Mental Retardation" (*updated to Mild Intellectual Disability in text*), "Attention Functions", and "Mental Functions of Language". [#]SCZ only, [$]SCZ vs BP only, [&]incl. Psychiatric Genomics Consortium (PGC) cross-disorder. *ADHD*, Attention Deficit Hyperactivity Disorder; *CD*, conduct disorder; *ASD*, Autism Spectrum Disorder; *BP*, Bipolar Disorder; *OCD*, Obsessive Compulsive Disorder; *NPD*, Neurodevelopmental Psychiatric Disorder; *PTSD*, Posttraumatic Stress Disorder; *SCZ*, Schizophrenia.

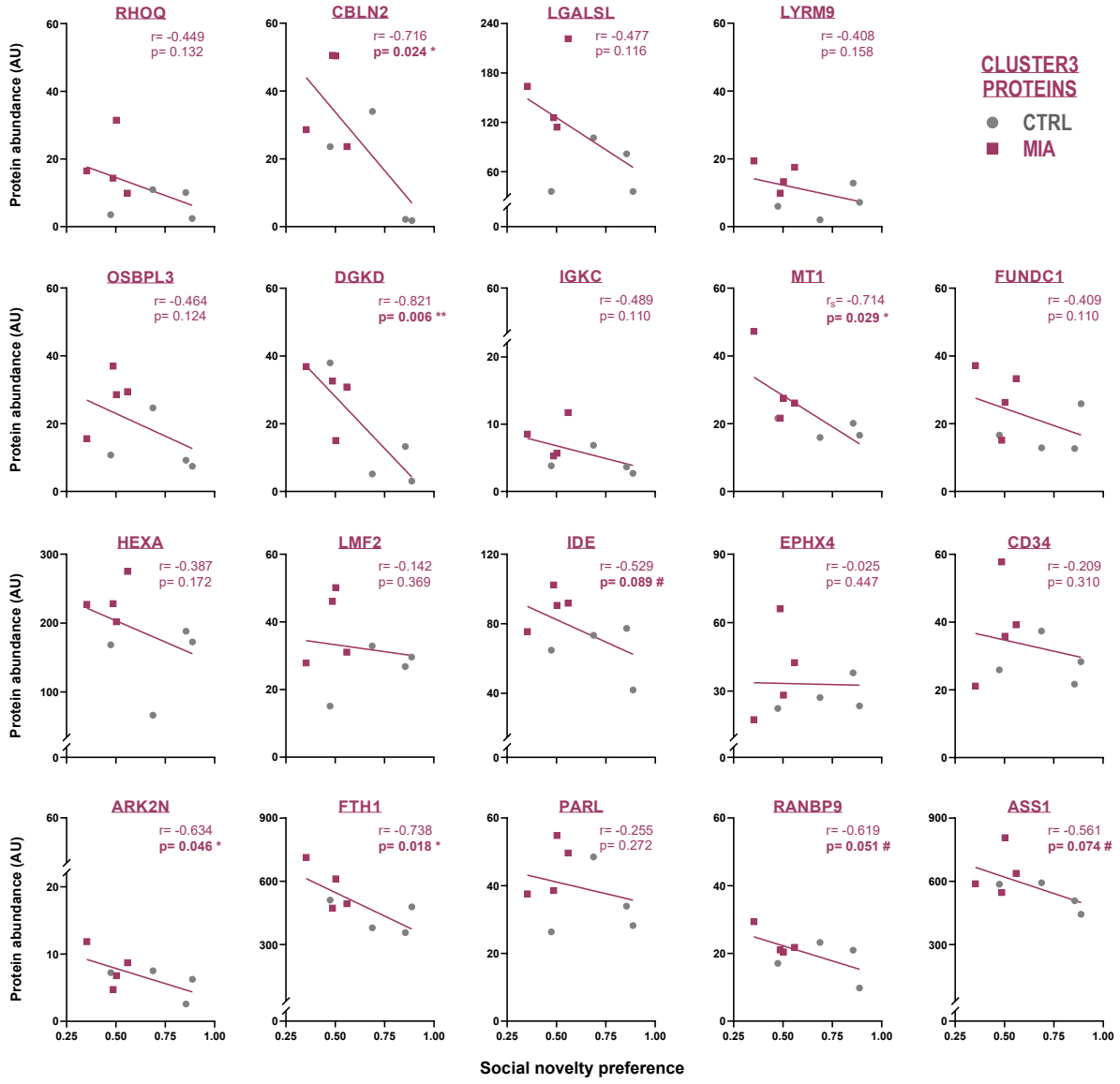


Figure 4.8 Social novelty preference is negatively correlated with abundance of the 19 MIA-affected proteins, which were downregulated in embryonic and upregulated in adult hippocampi. All these maternal immune activation (MIA)-upregulated proteins were negatively correlated with social novelty preference, hinting at a functional association between synaptoneurosomal changes and behaviour of adult offspring (n=4 CTRL, 4 MIA). The five significantly correlated proteins (CBLN2, DGKD, MT1, ARK2N, FTH1) have been liked to hippocampal and/or synaptic development and dysfunction. Social novelty preference index ranges between 0 and 1 (0-100% time spent interacting with a juvenile novel *vs* familiar conspecific); relative protein abundance was obtained from mass spectrometry data and presented in arbitrary units (AU). Pearson correlation coefficient *r* (linear) ranges from -1 to 1, with values close to 0 indicating weaker correlations (Spearman, non-normal distributed data: r_s; only MT1). Significance denoted as **p<0.01, *p<0.05, #p<0.1.

4.1.3 Morphological Investigations

In order to determine the morphological consequences that coincide with the behavioural and proteomic alterations, I next wanted to assess the neuronal architecture of high-dose MIA and control offspring in the PFC and HPC regions - selected for their central role in NPDs and observed MIA-evoked deficits in the mouse model, especially those reported in this study (see previous sections; Chapters 1.2.1, 4.1.1). Considering the importance of neuronal connectivity and transmission for brain function and NPD-related traits, as well as the synaptoneurosomal protein changes following MIA (see Chapter 4.1.2), morphological dendritic and spine analyses were performed in the HPC and PFC of high-dose MIA offspring and controls. For more detailed information on number of brains and cells, please refer to Supplementary Table S2.

DENDRITIC TREE ANALYSES

In the PFC, an interaction effect of prenatal treatment and sex was observed for the average number of dendrites ($F_{(1,174)}$=4.12, p=0.044; Figure 4.9A), wherein MIA impacted male and female offspring differently and led to a reduction of dendrites in MIA females compared to males (p=0.049), specifically (Figure 4.9B). Neither sex nor MIA influenced PFC dendritic length (Figure 4.9B). The morphometric Sholl analysis of neuronal arborisation revealed significant differences in dendritic architecture. The mean enclosing radius – a measure of the area occupied by neuronal branches – was affected by a MIA × sex interaction ($F_{(1,174)}$=9.56, p=0.002): MIA caused a reduction in male and an increase in female offspring's PFC dendritic tree span (p<0.001, Figure 4.9C). The Sholl profile measuring branching at incremental distance away from the cell body revealed significant influence of MIA on dendritic branching - in a distance-specific but sex-independent manner (Figure 4.9D; three-way RM ANOVA, *MIA* × *radius*: $F_{(23,2742)}$=2.45, p<0.001).

In HPC pyramidal neurons, neither MIA nor sex influenced dendritic number (p>0.18; Figure 4.9F, H) and enclosing radius (p>0.62; Figure 4.9H). However, there was a trend toward a MIA × sex effect on average dendritic length ($F_{(1,240)}$=3.55, p=0.061), wherein female (p=0.022) but not male MIA offspring had shorter dendrites than controls (Figure 4.9G). Three-way RM ANOVA revealed main effects of both sex ($F_{(1,6745)}$=7.36, p=0.007) and immune challenge ($F_{(23,6745)}$=5.78, p=0.016) on dendritic arborisation profiles (*interaction*: p>0.13; Figure 4.9H).

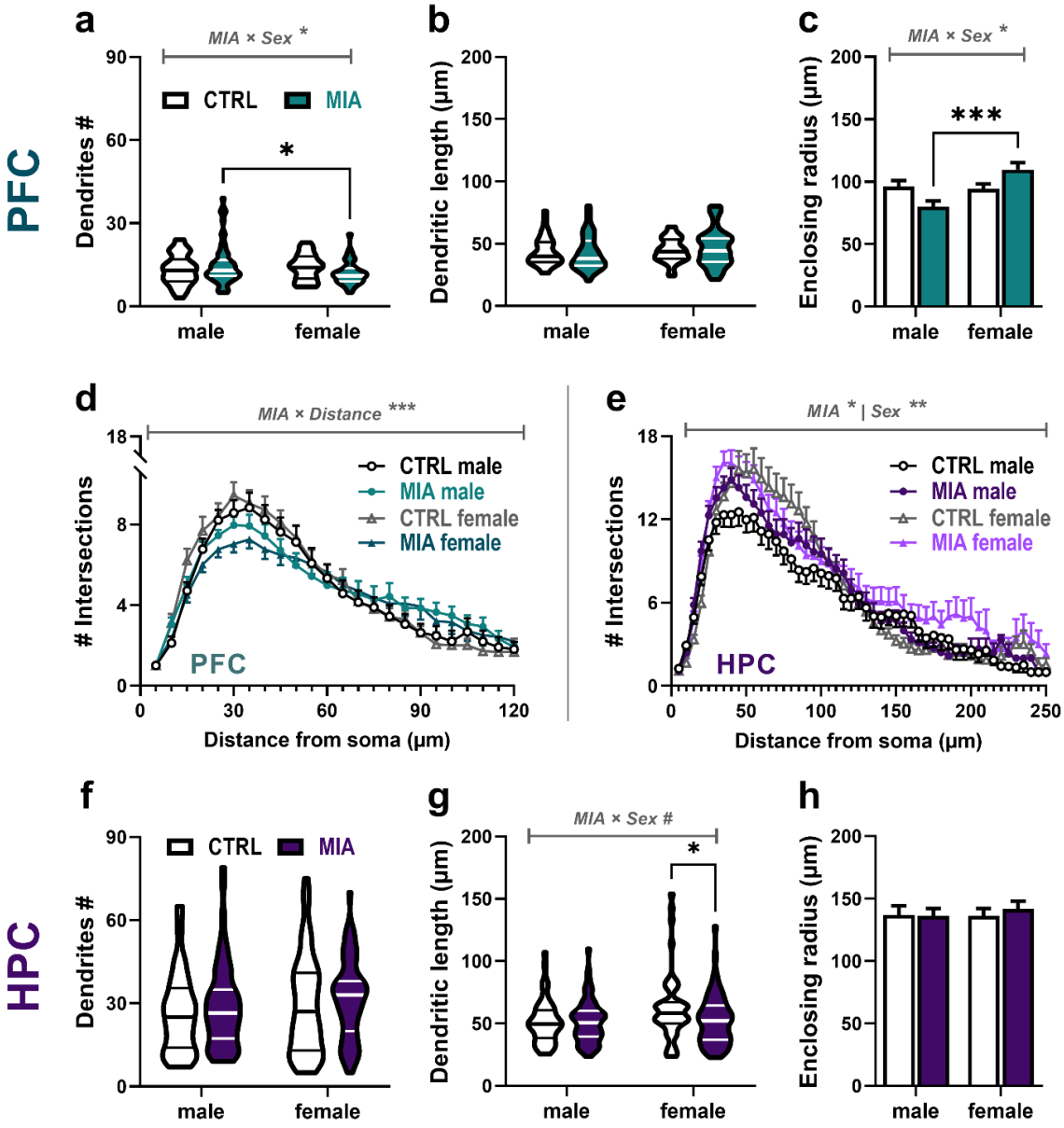


Figure 4.9 Effects of high-dose MIA on the dendritic architecture of pyramidal neurons in the prefrontal cortex (PFC) and hippocampus (HPC) of male and female adult offspring. Average dendritic number (**A**) but not length (**B**) was revealed an interaction effect of MIA and sex in the PFC. Sholl morphometric analysis was used on neurite traces to investigate the branching profile of the dendritic tree at 5 µm increments. Like dendrite number, MIA had sex-specific consequences for the average area occupied by the dendritic trees (**C**) in the PFC measured as enclosing radius, i.e. last intersection/branch's distance from the soma. MIA affected PFC dendritic arborisation in a distance- but not sex-dependent manner (**D**), leading to lower branching in the proximal and higher branching in the distal dendrites of the PFC. In the HPC, dendritic number (**F**) and enclosing radius (**H**) did not differ. Dendritic length revealed a trend of sex-specific MIA effects (**G**) and reduced average length in females. In a three-way ANOVA, both MIA and sex independently influenced branching (**E**). *M*: n=3-4 CTRL M, 4-5 MIA; F: n=3-4 CTRL, 4-5 MIA brains (see Supplementary Table S2 for full numbers. *Significance*: grey for main ANOVA effects, black for post-hoc tests: *p<0.05, **p<0.01, ***p<0.001. Violin plots show data distribution, median, and quartiles; rest mean ± SEM.

SPINE ANALYSES

Average PFC length-to-width ratio (LWR), which describes spine proportions, was not significantly affected by MIA or sex (Figure 4.10A). However, there was a significant main interaction effect of sex × MIA on overall ($F_{(1,179)}$=4.15, p=0.043; Figure 4.10A), immature ($F_{(1,179)}$=6.48, p=0.012; Figure 4.10A), and mature ($F_{(1,179)}$=5.05, p=0.03; Figure 4.10A) spine density. While MIA male offspring displayed no significant spine alterations except for a trend toward higher density of immature spines (p=0.076; Figure 4.10C) than control males, female MIA offspring had reduced total (Figure 4.10B) and mature spine densities (Figure 4.10D) in the PFC, compared to female controls.

In the HPC, high-dose MIA caused a sex-independent change in LWR ($F_{(1,237)}$=10.99, p=0.001;), so that both male (p=0.047) and female (p=0.032) MIA offspring had a lower average LWR than controls (Figure 4.10E). In contrast to the PFC, the overall (p=0.9; Figure 4.10F) and mature (p=0.094; Figure 4.10H) spine densities in the HPC were not influenced by MIA. However, there was a significant effect of MIA causing a decrease in immature spine density ($F_{(1,237)}$=7.44, p=0.007), which was more pronounced in females (*MIA vs CTRL*: p=0.016; Figure 4.10G).

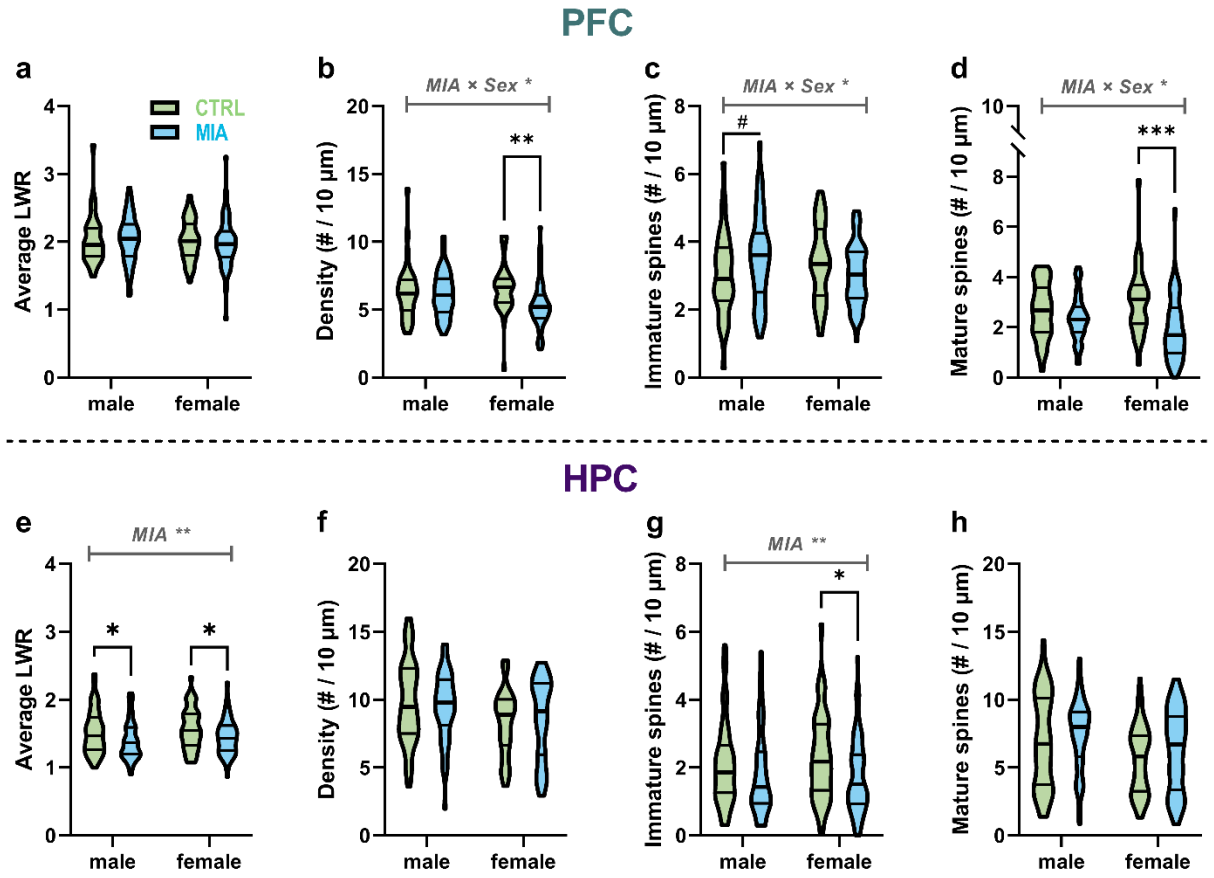


Figure 4.10 Effects of high-dose prenatal immune challenge on dendritic spine morphology and density in the prefrontal cortex (PFC) and hippocampus (HPC) of adult offspring. A, Average PFC length-to-width ratio (LWR) describing spine shape was not significantly affected by MIA. The interaction of sex and MIA had a significant effect on overall (**B**), immature (**C**), and mature (**D**) spine density. While female MIA offspring had reduced total and mature spine densities in the PFC, males displayed no significant spine alterations following prenatal immune challenge. In the HPC, MIA significantly decreased average LWR (**E**) irrespective of sex. While prenatal immune challenge did not influence overall (**F**) or mature (**H**) spine density, MIA had a significant effect on immature spine density (**G**), which was more pronounced in female MIA offspring. Significance, grey for main ANOVA effects, black for post-hoc tests: *p<0.05, **p<0.01, ***p<0.001. Plots show data distribution, median, and quartiles.

4.2 DISCUSSION

In the current study, the consequences of early, viral-like high-dose MIA on sex-specific, NPD-relevant behaviours and dendromorphology were assessed in adulthood, while proteomic changes were examined and directly compared in embryonic and adult MIA offspring of both sexes. The findings revealed sex-specific effects in adult-, but not embryonic-, HPC proteome of MIA-exposed mice. Importantly, a subset of 50 proteins, which were consistently affected by MIA in mature and prenatal HPC synaptoneurosomes were associated with NPD- and

comorbidity-related phenotypes and synaptic signalling. One cluster of upregulated proteins at both timepoints, which created a functional network enriched in the synaptic vesicle membrane and cytoskeletal binding, and another cluster of proteins downregulated in embryos and upregulated in adults and negatively correlated with social deficits, were especially intriguing. The identified proteins may represent targets for tailored approaches for early treatment in individuals at high-risk or after early diagnosis of NPDs. Furthermore, dendritic and spine analyses revealed sex-specific effects of MIA on neuronal architecture and spine abundance, especially in the HPC, which might be associated with observed divergences in NPD-relevant behaviours.

MIA during gestation increases the risk for developmental perturbations, which can lead to subsequent neuropsychiatric disorders in offspring (Isung *et al.*, 2023). The optimal doses of the viral mimetic poly(I:C) have to be determined for each laboratory environment and I initially demonstrated that while 2.5 and 5 mg/kg were viable in our facility, higher doses that have been previously used by others, i.e. 7.5 and 10 mg/kg, led to complete litter loss (Mueller *et al.*, 2018; Mueller *et al.*, 2019). Both viable poly(I:C) doses led to NPD-relevant behavioural endophenotypes (see Figure 4.1, Figure 4.2), such as social deficits, which represent one of the more robust MIA-evoked impairments (Zhao *et al.*, 2021a), and decreased nest quality – a measure of self-neglect, which together with anhedonia can be used to model negative symptoms in SCZ in mice (Pedersen *et al.*, 2014). However, the higher (5 mg/kg) dose caused more robust behavioural manifestations, and the lower dose only led to behavioural changes in male offspring. Taken together, these findings suggest that male offspring are more susceptible to a low-dose MIA exposure, as previously shown (Block *et al.*, 2022; Braun *et al.*, 2019; Haida *et al.*, 2019), with the behavioural consequences only observed in both sexes at the higher (5 mg/kg) poly(I:C) dose. Therefore, the high-dose group was selected to compare the behavioural findings with alterations in the HPC synaptoneurosomal proteome and neuromorphology in the HPC and PFC, considering their role in NPD symptomology (see Chapter 1.1).

When selecting a brain region for molecular investigations, I considered the observed MIA endophenotypes and the role of the HPC in learning and memory, NPD-relevant social behaviours (Banker *et al.*, 2021; Hitti & Siegelbaum, 2014; Lagace *et al.*, 2010; Zhao *et al.*, 2021a), nest-building behaviour (Jirkof, 2014), and novelty response (Gomez-Ocadiz *et al.*,

2022), and its life-long plasticity. Among the most reproducible manifestations of early-to-mid pregnancy viral-like MIA are social deficits, while HPC-dependent memory and novelty responses are more variable (Kentner *et al.*, 2019; Solek *et al.*, 2018). Previous studies have examined HPC changes after poly(I:C)-induced immune activation in early gestation in adolescent (PND38) and adult (Guma *et al.*, 2021; Nakamura *et al.*, 2022) mice of in the same strain, and mechanistically implicated the region in dysfunction following MIA (Couch *et al.*, 2021; Ito *et al.*, 2010; Mirabella *et al.*, 2021). The present study focused on enriched synaptic proteome of MIA offspring given the emerging central role of the synapse in neuroinflammation and psychiatric illness (Coiro *et al.*, 2015; de Bartolomeis *et al.*, 2022; Estes & McAllister, 2016; Han *et al.*, 2021; Hayes *et al.*, 2022; Lombardo *et al.*, 2018; Parenti *et al.*, 2020; Sanacora *et al.*, 2022; Trubetskoy *et al.*, 2022), as well as the scarcity of hypothesis-free, proteomic investigations in MIA, especially in a sex-specific manner.

The presented findings of extensive prenatal synaptic proteome modifications in offspring of 5 mg/kg poly(I:C)-treated dams (see Figure 4.3, Figure 4.5) implicated lipid, polysaccharide, and glycoprotein metabolism pathway disruptions during a relevant neurodevelopmental time-window for possible lasting NPD-pertinent sequelae (Schneider *et al.*, 2017). Further changes in mitochondrial organisation and the lysosome, as well as impaired glycan and glycosphingolipid metabolism, which are important for cell signalling and myelination (Hannun & Obeid, 2008), were observed. Moreover, MIA-induced changes in proteins involved in post-translational modifications by glycans and sialic acid, which affect membrane bio-properties and proteins controlling the secretory pathway, intercellular interactions, and developmental timing were also observed (Hayes & Melrose, 2018; Lee *et al.*, 2020; Li & Ding, 2019). Interestingly, a study of the transcriptome of stimulated microglia in an adulthood following early-gestational MIA found enrichment of fatty acid metabolic and protein localisation pathways in poly(I:C)-treated offspring (Hayes *et al.*, 2022). The changes in embryonic HPC of the high-dose MIA offspring (see Figure 4.3, Figure 4.5) are largely in keeping with previous findings, highlighting pathway enrichment for cellular and immune responses, transmembrane signalling, and axon guidance (Baines *et al.*, 2020; Oskvig *et al.*, 2012), networks of neuronal development and metabolic/energy processes (Tsivion-Visbord *et al.*, 2020), and cytoplasmatic translation (Kalish *et al.*, 2021).

The present study further revealed novel dose-dependent alterations in foetal synaptic protein composition with the lower viral mimetic dose influencing alternative splicing and RNA metabolism (see Figure 4.4, Figure 4.5), which might constitute more transient neuronal changes. The low-dose MIA caused upregulation of 55 and downregulation of 233 proteins compared to controls that were not changed in the same way in any other comparison, including brain disorder-relevant synaptotagmin SYT7 and synaptopodin SYNPO in the former and alpha-synuclein SNCA, neurotrophic factor neuritrin (NRN1), and septin SEPT4 in the latter. The number of differentially abundant proteins in embryonic HPC of low-dose MIA compared to controls was lower than those caused by high-dose MIA when compared to controls (501 and 1164, respectively). Furthermore, comparison of the proteomic profile of low- and high-dose MIA embryonic samples directly (see Figure 4.4) revealed the most extensive changes in any comparison, with proteins exclusively upregulated in high *vs* low MIA included NPD-relevant SYN2 and signalling kinases (MAP2K, PAK1) and exclusively downregulated contained neurodevelopmental regulators like netrin NTN1, DS cell adhesion molecule DSCAM, and NOTCH1 (see Chapter 3.5). This suggested that different degrees of MIA might have differential effects on the development of the embryonic HPC proteome, underlying divergent NPD risk, which is in line with behavioural observations in this study but more investigations, including in adults, are needed in the future.

In line with, and building upon, previous findings in MIA models (Amodeo *et al.*, 2019; Farrelly *et al.*, 2015; Guo *et al.*, 2018; Gyorffy *et al.*, 2016; Lombardo *et al.*, 2018; Mirabella *et al.*, 2021; Mueller *et al.*, 2021; Richetto *et al.*, 2014; Richetto *et al.*, 2017a; Weber-Stadlbauer *et al.*, 2017; Woods *et al.*, 2021), this study found that high-dose MIA-induced changes in pooled male and female, adult HPC synaptoneurosomes had largely shifted to divergent transmembrane and cytoskeletal trafficking, energy metabolism, cell fate-governing signals and processes, and plasticity-related endocannabinoid signalling (see Figure 4.4, Figure 4.5). Intriguingly, the latter has been shown to have effects of HPC excitability through disinhibition of dendritic signalling in pyramidal cells, and HPC plasticity and excitability disbalance have been linked to manifestations of anxiety and fear (Ghasemi *et al.*, 2022). Thus, endocannabinoid pathway changes might underlie the observed anxiolytic effects of MIA on offspring in both sexes, which can be examined in follow-up studies.

The 50 proteins altered by high-dose MIA in both the embryonic and adult pool were enriched for those of the presynaptic terminal and synaptic vesicle (see Figure 4.7), in line with previous studies (Coiro *et al.*, 2015; Gyorffy *et al.*, 2016; Hayes *et al.*, 2022; Ito *et al.*, 2010; Oh-Nishi *et al.*, 2010). While previous transcriptomic studies of the MIA model have repeatedly linked immune challenge-caused gene dysregulation to NPDs (Han *et al.*, 2021; Hayes *et al.*, 2022; Lombardo *et al.*, 2018; Mueller *et al.*, 2021; Patterson, 2009), the degree of phenotypic association and number of proteins with pleiotropic effects in this dataset remains compelling (see Table 7, Figure 4.6). Thus, the association of the proteomic changes with psychiatric and cognitive traits was probed (Bonnycastle *et al.*, 2021; John *et al.*, 2021; Marchisella *et al.*, 2016; Waites & Garner, 2011; Zhao *et al.*, 2015). Importantly, the proteins common to the embryonic and adult HPC were significantly associated with psychiatric disorders and contain a cluster of persistently upregulated proteins that were enriched in a functional network (see Figure 4.6). These proteins, including BIN1, DPYSL2, SNCB, MAPT, SYN1 are involved in synaptic plasticity, cognition, and neuro-inflammatory, -degenerative, and -developmental psychiatric conditions (Biundo *et al.*, 2018; De Rossi *et al.*, 2020; Desprez *et al.*, 2023; Maphis *et al.*, 2015; Mohaupt *et al.*, 2022; Parenti *et al.*, 2022; Pham *et al.*, 2016; Sudwarts *et al.*, 2022). Furthermore, a negative correlation between the 19 proteins downregulated in embryonic and upregulated in adult HPC, and observed social novelty preference inferred a role of these proteins in MIA-induced social deficits (see Figure 4.8). Indeed, many of the most strongly correlated proteins - CBLN2 (Seigneur & Sudhof, 2018; Tao *et al.*, 2018), DGKD (Barber & Raben, 2020; Lu *et al.*, 2020), MT1 (Mamdani *et al.*, 2022; West *et al.*, 2008), FTH1 (Mazare *et al.*, 2020; Otero-Garcia *et al.*, 2022), RANBP9 (Kootbodien *et al.*, 2023; Wang *et al.*, 2014) - are implicated in neuronal function, synaptic transmission, and neuropsychiatric deficits. Interestingly, metallothioneins like MT1 (upregulated in embryonic / downregulated in adult MIA HPC), have been shown to exasperate MIA-induced deficits and importantly - upregulation during gestation indicates glial activation and might deplete cellular zinc stores (see Chapter 1.2.4). Thus, the resulting developmental zinc deficiency might affect morphology in the developing HPC (Adamo & Oteiza, 2010) with possible life-long consequences regardless of adult downregulation (see below). Zinc has been proposed as a neuroprotective agent with possible anti-depressant effects (Mlyniec *et al.*, 2015), while zinc depletion has been shown to rescue fear extinction deficits in a PTSD-like mouse model by reducing zinc-dependent

neuronal activation in the cortico-amygdalar circuit mediating this behaviour (Whittle *et al.*, 2010).

Finally, this study suggests that significant sex-specific, synaptic-level MIA effects emerge later in life, possibly following known influences such as chromatin remodelling, environmental, and hormonal regulation (Gegenhuber *et al.*, 2022; McCarthy *et al.*, 2017), which add to the available data regarding MIA-induced changes in the brain (Herrero *et al.*, 2022; Kalish *et al.*, 2021; Nakamura *et al.*, 2022; Tsivion-Visbord *et al.*, 2020).

The observed alterations imply that adult males and females differ in their structural synaptic response to the early prenatal immune challenge. This hypothesis is in line with the sex-dependent innate immune response (Klein & Flanagan, 2016) and whole-cell transcriptomic results from previous studies in HPC of male and female offspring from a comparable MIA model (Guma *et al.*, 2021; Nakamura *et al.*, 2022). As mentioned previously (see Chapter 1.2.4), MIA has been shown to recapitulate or exasperate ASD-relevant behavioural and HPC molecular changes in *Shank3*-deficient male mice (Atanasova *et al.*, 2023). Thus, the presented findings that MIA prenatally downregulated SHANK3, which was substantially decreased in adult females as opposed to males, in which SHANK1/2 were significantly downregulated instead, might offer more insights into age- and sex-specificity of NPD-pertinent synaptic changes.

Protein alterations exclusive to males were enriched in pathways related to gliogenesis (Braun *et al.*, 2019; Page *et al.*, 2021; Woods *et al.*, 2021) and myelination (Farrelly *et al.*, 2015; Richetto *et al.*, 2017a; Zhang *et al.*, 2020), in concurrence with previous experiments (see Figure 4.4, Figure 4.5). The pathways selectively changed in the females are more involved in endocytosis and the ribosome, as well as actin binding and filament, organisation; which are possibly linked to oestrogen effects (Babayan & Kramar, 2013). Furthermore, previous studies in mice utilising poly(I:C) in early-to-mid pregnancy (E9/12) have highlighted different molecules in adult PFC and amygdala as well as adolescent HPC along the converging signalling cascades in cellular kinase pathways (Amodeo *et al.*, 2019; Estes & McAllister, 2015; Guma *et al.*, 2021; Mueller *et al.*, 2021; Weber-Stadlbauer *et al.*, 2017), such as the ERK and mTOR pathways – the latter of which is enriched in our adult female MIA offspring - that are heavily implicated in NPDs and treatment responses (Dai *et al.*, 2021; Kumar *et al.*, 2005; Soriano *et al.*, 2021).

Importantly, enrichment of proteins involved in divergent, NPD-relevant signalling pathways (see also Chapter 1.1) – namely, dopaminergic (Dunlop & Nemeroff, 2007; Hasbi *et al.*, 2020) in female and glutamatergic (Javitt, 2004; Mohn *et al.*, 1999) in male MIA offspring – might further underlie differences in behavioural outcomes, such as the observed more pronounced depressive-like behaviours in female and social interaction deficits in males. Similar effects of a prenatal immune challenge have been observed in both glutamatergic (Amodeo *et al.*, 2019; Block *et al.*, 2022; de Bartolomeis *et al.*, 2022; Mirabella *et al.*, 2021; Nakamura *et al.*, 2022) and dopaminergic (Basil *et al.*, 2014; Hayes *et al.*, 2022; Luchicchi *et al.*, 2016) signalling - even across multiple generations (Weber-Stadlbauer *et al.*, 2017; Weber-Stadlbauer *et al.*, 2021). Thus, these pathways may represent targets to negate the effect of MIA and can be explored in future studies.

Neuroarchitecture investigation in the HPC high-dose MIA adult male and female offspring revealed an overall change in the average spine shape, corresponding to a more mature spine profile (see Figure 5.1), in line with enrichment of proteins involved in cytoskeletal processes (see above). This change might represent overstabilisation of spines, which reduces plasticity – a phenomenon observed in cortical pyramidal neurons of an ASD-like mouse model (Ash *et al.*, 2021). Importantly, male MIA offspring lacked other architectural changes, while female MIA mice exhibited decreases in dendrite length and immature spine abundance (see Figure 4.9). These findings might be explained in part by long-lasting, sex-specific synaptoneurosomal protein changes. On one hand, beta-tubulin proteins (e.g., TUBB2/4) forming microtubules that govern dendritic length in neurons even in adulthood (Nanda *et al.*, 2020), were significantly downregulated in female MIA HPC (local decrease of microtubules correlates with branch termination, decreases length). They were also upregulated in male MIA offspring, suggesting at possible underlying mechanisms (see Chapter 3.5). Furthermore, the downstream Rho pathway kinase ROCK2 implicated in anxiety-like behaviour, negatively affecting dendritic branching as well as mature spine formation (Nakayama *et al.*, 2000; Weber *et al.*, 2021) was increased in female but not male MIA HPC, while CDC42, which has the opposite effect on dendrites (Miller & Kaplan, 2003), was upregulated exclusively in male HPC following MIA (see also Chapter 1.3.2). The increased branching in female MIA HPC might also be underlined by mechanisms compensating length deficits, or the observed increase in branching might be a consequence of the female-

specific, MIA-induced upregulation of guanine deaminase (GDA, or cypin) – a known effect of this downstream RhoA target in the HPC (Chen & Firestein, 2007); see also Chapter 1.3.2. Interestingly, HPC is one of the main regions governing nesting behaviour in mice, which is disrupted by synaptic and neurotransmission aberrations in models of NDDs and neurodegenerative disorders (Jirkof, 2014). The here observed morphological changes might, therefore, affect neurotransmission in addition to the MIA-induced changes on synaptic level and explain the more pronounced deficits in nest quality observed in MIA females in this study, but more investigation of this hypothesis is needed on a physiological level.

While no significant differences in MIA mice compared to controls were observed in the PFC, the morphological analysis revealed effects of the prenatal immune activation based on sex (see Figure 4.9), with male MIA offspring having more numerous dendrites than MIA females, while opposite changes in branching area could represent compensatory mechanisms by the brain to normalise function. These findings could be correlated with subtle as well as pronounced sex differences in behaviours modulated by the PFC, such as anxiety- and anhedonia-like behaviours and sensorimotor gating (Alam *et al.*, 2015; Keedwell *et al.*, 2005). For example, reduced PPI in rodents, which was observed in MIA female but not male offspring (see Figure 4.2), have been correlated with decreased neuronal activity in the PFC – a consequence of reduction in dendrites or spines, also exclusively observed in PFC of MIA females in this study (see Figure 4.10). Such decrease in spine density has been observed in PFC of SCZ patients (Glantz & Lewis, 2000) and previously reported in cortical pyramidal neurons of adult MIA mice (Coiro *et al.*, 2015), and matches the phenotype of MIA offspring. Conversely, increases in immature spines, which have been described in the cortices of individuals with ASD and animal models of NPDs like FXS, together with abnormal dendritic branching (Hutsler & Zhang, 2010; Martinez-Cerdeno, 2017), were revealed by this study in male MIA offspring, specifically. These findings are intriguing, considering the gender prevalence differenced in NPDs like ASD (see Chapter 1.1), and the implications of the immature spine profile of the PFC in social deficits in mice (Medendorp *et al.*, 2018). Both social deficits and an increase in immature spines of the PFC in MIA offspring compared to controls were observed in this study, specifically in males.

In conclusion, I observed dynamic alterations at the HPC synapse following MIA that were age-specific and varied by sex or immunostimulant dose in the adult and embryonic brain, respectively. The more abundant protein alterations in embryonic HPC might be based on the temporal closeness to the *in-utero* assault, whereas the long-term affected proteins in adult samples could represent more permanent effects of MIA on the brain. These long-lasting effects correspond to morphological changes in HPC and PFC, which were largely sex-specific, and might underlie divergence in behavioural endophenotypes. Future studies are warranted to further assess a causal link between the MIA-evoked changes on a proteomic and neuromorphological level and functional outcomes. Importantly, a smaller, but highly relevant subset of MIA-evoked embryonic synaptoneurosomal changes in the HPC, which persisted into adulthood, could represent targeted approaches in individuals at high-risk or with early diagnosis of NPDs. These findings, taken together with previous transcriptomic research into changes caused by poly(I:C)-evoked MIA, add to the puzzle of how prenatal immune challenges affect the brain across the lifespan.

5. EXPANDING ON THE ROLE OF RBFOX1 OVEREXPRESSION IN THE BRAIN

5.1 OVEREXPRESSION (OE) OF CYTOPLASMATIC RBFOX1 IN A MOUSE MODEL

Based on the importance of RBFOX1 as a neuronal developmental regulator, its role in NPDs, and our previous insights into the behavioural consequences of neuronal *Rbfox1* deletion (see Chapter 1.3.2.1), a transgenic neuron-specific, cytoplasmatic *Rbfox1* overexpression (OE) mouse model was developed and then bred in-house. The cytoplasmatic RBFOX1 isoform, rather than the nuclear isoform, or full RBFOX1 OE, was selected to investigate how increased abundance of the RBP affects behaviour (*unpublished data, personal communication A. O'Leary*) and dendritic morphology through its wide-ranging and dynamic posttranscriptional regulator functions relating to its thousands of downstream targets. Given that *Rbfox1* KO mouse data as well as *RBFOX1* mutations and reduced protein expression in human brains are linked with NPDs, I planned to determine whether OE of cytoplasmatic RBFOX1 could offset some of the negative consequences of MIA. Thus, a "double-hit" mouse model, combining RBFOX1 OE and early, viral-like MIA, was used to test for genotype × environment effects on NPD-relevant phenotypes.

5.1.1 Results

5.1.1.1 Protein Overexpression Validation

Overexpression of RBFOX1 was validated in HPC and PFC of adult male OE and wild-type (WT=CTRL) mice (Figure 5.1). RBFOX1 was visible as two protein bands (Figure 5.1A). Of those two bands, the lower molecular-weight band (~45 kDa) is putatively dominated by nuclear protein isoforms and the larger one (~51 kDa) predominantly represents cytoplasmatic isoforms (Lee *et al.*, 2016; Vuong *et al.*, 2018). Indeed, the overall higher RBFOX1 abundance (*HPC:* genotype $F_{(1,12)}=16.61$, p=0.002, *OE vs WT*: p=0.012; *PFC*: $F_{(1,12)}=12.11$, p=0.005, *OE vs WT*: p=0.032) in OE males was driven entirely by the overexpression of the cytoplasmatic band in both the HPC (p=0.024) and PFC (p=0.020; Figure 5.1B). Conversely, the low molecular-weight band did not differ between the OE and WT group (p>0.98). The full blots are shown in Supplementary Figure S3.

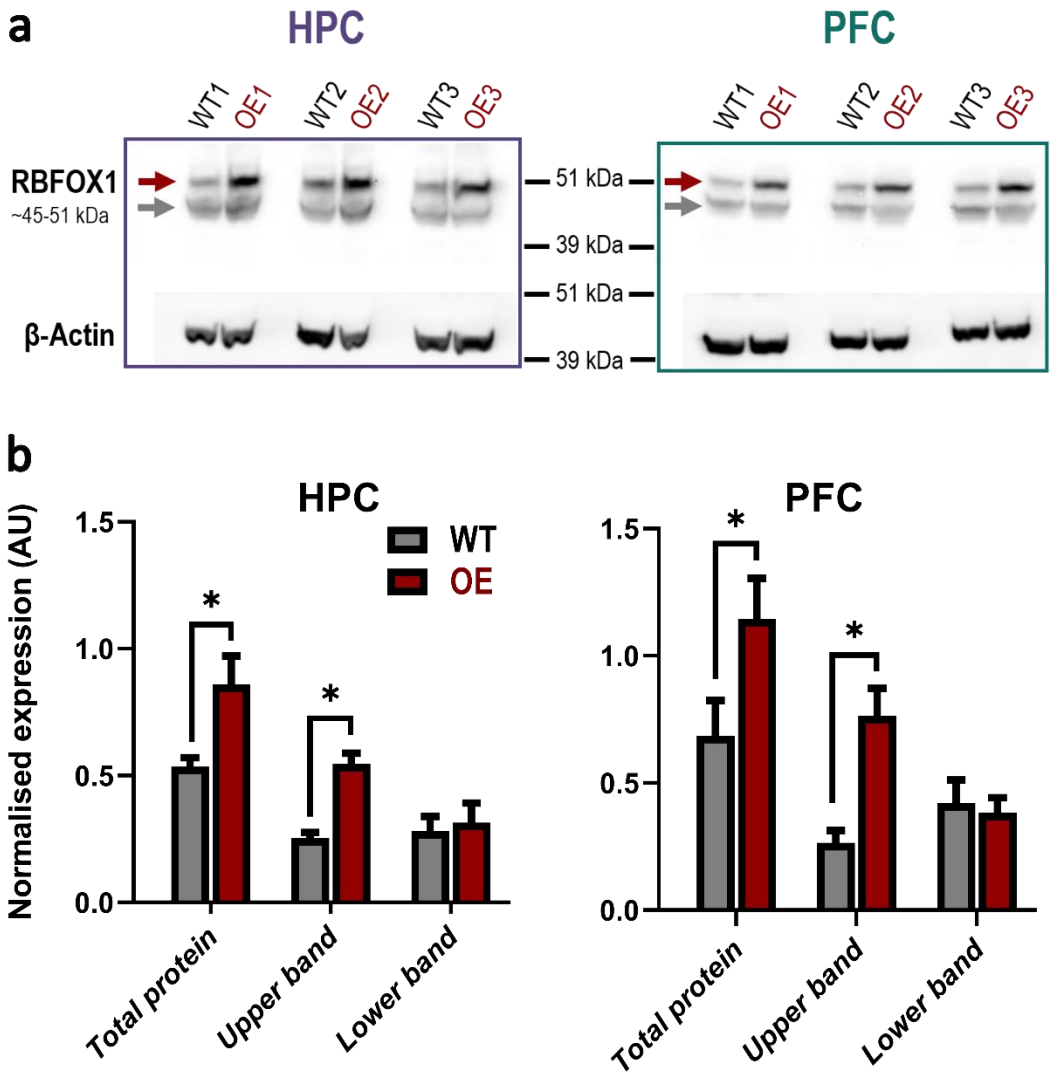


Figure 5.1 Protein validation in the hippocampus (HPC) and prefrontal cortex (PFC) of adult males from a cytoplasmatic RBFOX1-overexpression (OE) mouse line. A, semi-quantitative chemiluminescent immunoblots were used to validate increased RBFOX1 expression in the HPC (*left*) and PFC (*right*) in OE males compared to wild-type (WT) conspecifics (n=3/genotype). β-Actin was used for normalisation. RBFOX1 manifested as two protein bands, the lower-molecular weight one is dominated by nuclear isoforms (*grey arrow*) and the larger one (*red arrow*) represents cytoplasmatic isoforms. **B**, higher normalised RBFOX1 abundance in OE males was driven by the overexpression of the putative cytoplasmatic, larger protein band. *Šídák's post-hoc test*: *p<0.05. Data: mean ± SEM. Full blots available in Supplementary Figure S3.

5.1.1.2 Neuromorphological Analysis

DENDRITIC ARCHITECTURE ANALYSIS

In the PFC, the *Rbfox1* genotype did not affect the average dendritic number ($t_{(149)}$=0.173, p=0.86) but significantly decreased the dendritic length in OE males compared to controls (Mann-Whitney U=2092, p=0.005; Figure 5.2A). In the morphometric Sholl analysis of

dendritic arborisation, the enclosing radius – as a measure of average neuronal span area – was significantly decreased in the PFC of OE mice (U=2272, p=0.034; Figure 5.2B).

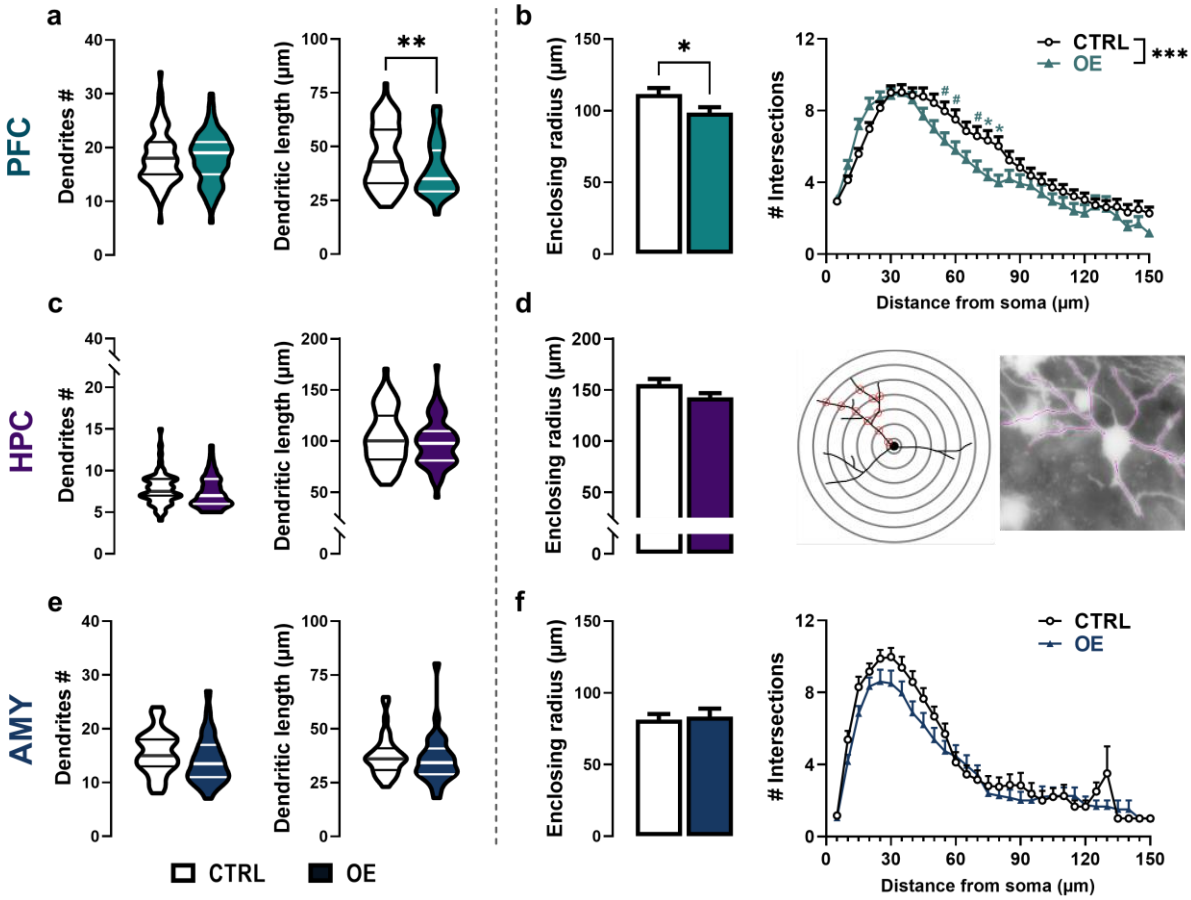


Figure 5.2 Dendromorphological investigation of neurons in the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMY) of adult control (CTRL) and cytoplasmatic RBFOX1 overexpressing (OE) male mice. A, average PFC dendritic number (*left*) was not changed by genotype. Dendritic length (*right*) was significantly decreased in the PFC of RBFOX1 OE males. **B**, Sholl morphometric analysis of dendritic arborisation revealed a significant decrease in enclosing radius (*left*), as well as overall and distance-specific branching (*right*) in the PFC of OE male mice. **C**, in the HPC, dendrite number (*left*) and length (*right*) were unaffected by genotype. **D**, the average area of the HPC dendritic branching field was not changed by cytoplasmatic RBFOX1 OE (*left*). but did not cross the significance threshold. Schematic representation of Sholl morphometric analysis (*right*). In the AMY, cytoplasmatic RBFOX1 OE had no discernible effects on dendritic number (**E**, *left*), length (**E**, *right*), tree span (**F**, *left*), or arborisation (**F**, *right*). *HPC*: n=6 CTRL, 6 OE males; *PFC*: n=8 CTRL, 7 OE; *AMY*: n=6 CTRL, 5 OE; full information can be found in Supplementary Table S2. Statistical significance denoted as *p<0.05, **p<0.01, ***p<0.001, #p<0.08 trend. Violin plots show distribution, median, and quartiles, remaining data is presented as mean ± SEM.

Furthermore, the analysis revealed an interaction effect of genotype and the distance from soma on number of intersections ($F_{(29,3050)}$=2.41, p<0.001), so that OE males displayed a less complex branching profile, especially in the more distal dendritic segments (*distance 55, 60, 70, 75, 80 μm*: p<0.08, Figure 5.2B).

Investigation of HPC dendrites revealed no significant effects of cytoplasmatic RBFOX1 OE on dendritic number (U=1501, p=0.11) or length (U=1843, p=0.27; Figure 5.2C). The morphometric Sholl analysis of dendritic arborisation demonstrated no differences in the average area of the neuronal branching trees (Figure 5.2C). Since this region was part of the pilot study for morphological analysis, full intersection-per-distance profiles were not available (see Chapter 3.2.2.2 for more details).

Similarly to HPC, the average amygdalar dendrite number ($t_{(64)}$=1.4, p=0.17) and length (U=474, p=0.4) did not appear to be affected by the *Rbfox1* genotype (Figure 5.2E). Furthermore, Sholl analysis revealed no significant changes in average dendritic tree span (U=531.5, p=0.92) or branching profile ($F_{(1,1020)}$=2.56, p=0.11) in the amygdala of cytoplasmatic RBFOX1 OE male mice.

DENDRITIC SPINE ANALYSIS

Next, spine shape and density analyses were performed in the same brain regions of adult male cytoplasmatic RBFOX1 OE and control mice, i.e. PFC, HPC, and amygdala. In the PFC, there were no significant genotypic effects on the average spine shape (U=2363, p=0.94) or density of specific spine types ($F_{(1,680)}$= 2.77, p=0.10; Figure 5.3A). However, RBFOX1 OE mice exhibited a significant increase in overall PFC spine density ($t_{(136)}$=2.78, p=0.006; Figure 5.3A).

More pronounced effects of cytoplasmatic RBFOX1 OE were present in the HPC of adult male mice. Here, average HPC spine shape was altered in the form of reduced LWR in OE mice (U=5854, p=0.012; Figure 5.3B). Moreover, spine density was significantly higher in the HPC of OE males (U=6123, p=0.044; Figure 5.3B). A more detailed investigation of the differential abundance of specific spine types (*genotype* × *spine type*: $F_{(4,1190)}$=13.21, p<0.001) in RBFOX1 OE animals further revealed a strong decrease in spine precursors, i.e. filopodia (p<0.001), paired with an increase in immature thin (p=0.002) as well as the more mature, stubby spines (p=0.002; Figure 5.3B).

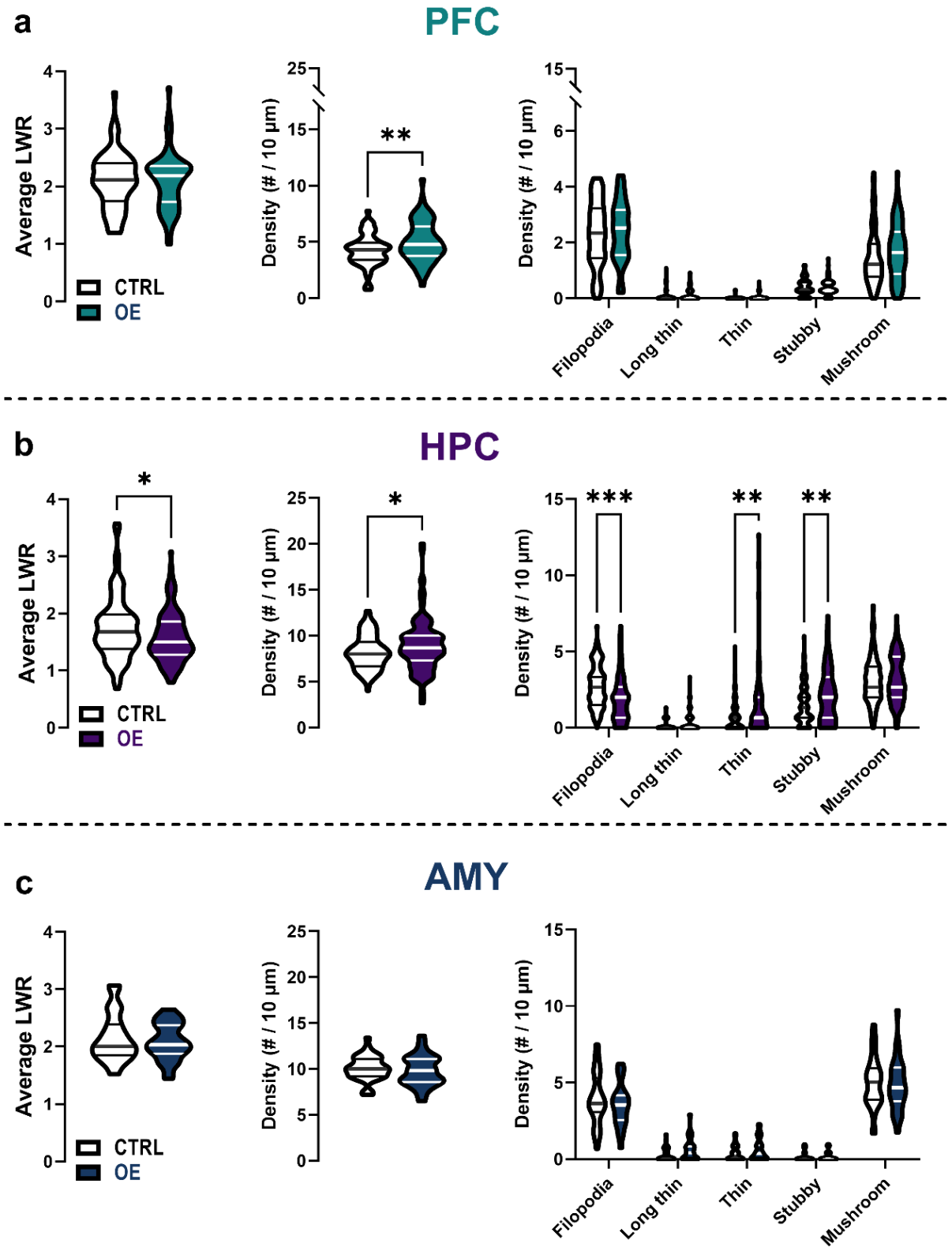


Figure 5.3 Analyses of dendritic spine density and morphology in the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMY) in a neuron-specific, cytoplasmatic RBFOX1 overexpression (OE) mouse model and controls (CTRL). A, RBFOX1 OE did not affect spine length-to-width ratio (LWR, *left*) or the density of specific spine types (*right*) in the PFC. OE males did, however, exhibit a significant increase of overall PFC spine density (*middle*). **B**, in the adult HPC, the RBFOX1 OE led to a robust decrease in LWR (*left*) and

Continued. increase in overall spine density (*middle*). The HPC spine type-specific densities (*left*) of immature thin and mature stubby spines were significantly increased, while the filopodia-type protrusion density was reduced by RBFOX1 OE. **C**, in the AMY, neither spine LWR (*right*), nor overall (*middle*), nor spine type-specific (*left*) density was impacted by the RBFOX1 OE. See Supplementary Table S2 for more information on group sizes; *HPC*: n=6 CTRL, 6 OE males; PFC: n=8 CTRL, 7 OE; AMY: n=6 CTRL, 5 OE. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, #$p<0.08$ trend. Violin plots show distribution, median, and quartiles.

In the amygdala, on the other hand, *Rbfox1* genotype had no observable differential impact on either average spine LWR ($U=541$, $p=0.83$), or the overall ($t_{(65)}=0.84$, $p=0.4$) and specific spine-type density (*genotype* × *spine type*: $F_{(4,325)}=0.99$, $p=0.41$, *genotype*: $p=0.96$; Figure 5.3C).

5.1.1.3 Interaction of MIA and Cytoplasmatic RBFOX1 OE in Mice

In order to investigate the interactive effects of MIA and RBFOX1 OE (see Chapters 1.1.1, 1.2.1, 1.2.4), I induced MIA with poly(I:C) on GD9 (2.5 mg/kg) in pregnant RBFOX1 OE and non-OE (CTRL) dams (PBS 1x vehicle, VEH was used for MIA control). This immunostimulant dose was selected because it caused more subtle, sex-specific changes in behaviour and increased survival of offspring as opposed to the high, 5 mg/kg poly(I:C) dose (Chapter 4.1.1).

MIA INDUCTION AND PRODUCED OFFSPRING

Timed mating was performed for up to three consecutive weeks, equivalent to Chapter 3.1.1, 4.1.1 (see timeline in Figure 3.1), in two cohorts (5 and 6 dams/genotype, respectively; n=11 total CTRL and OE each). Of these, 63.6% (n=7) of CTRL and 45.4% (n=5) of RBFOX1 OE dams became pregnant and were intravenously injected on GD9 with either poly(I:C) solution (*MIA*: 4 CTRL, 3 OE) or an equivalent volume of the vehicle (*VEH*: 3 CTRL, 2 OE; Figure 5.4A, C). One of the MIA-CTRL and none of the VEH-CTRL dams experienced offspring loss through spontaneous abortion (Figure 5.4C). Furthermore, 80-100% of offspring of CTRL dams survived into adulthood and litter sizes at birth were comparable regardless of MIA status ($t_{(4)}=2.14$, $p=0.1$; Figure 5.4C).

While both pregnancies in the OE-VEH group resulted in births, offspring survival rate was only 20 and 50% per litter (Figure 5.4D). Furthermore, litter size at birth was affected by genotype ($F_{(1,6)}=9.20$, $p=0.023$), with cytoplasmatic RBFOX1 OE leading to significantly less pups born to VEH-treated OE dams compared to VEH-CTRL ones ($p=0.035$, Figure 5.4C, D).

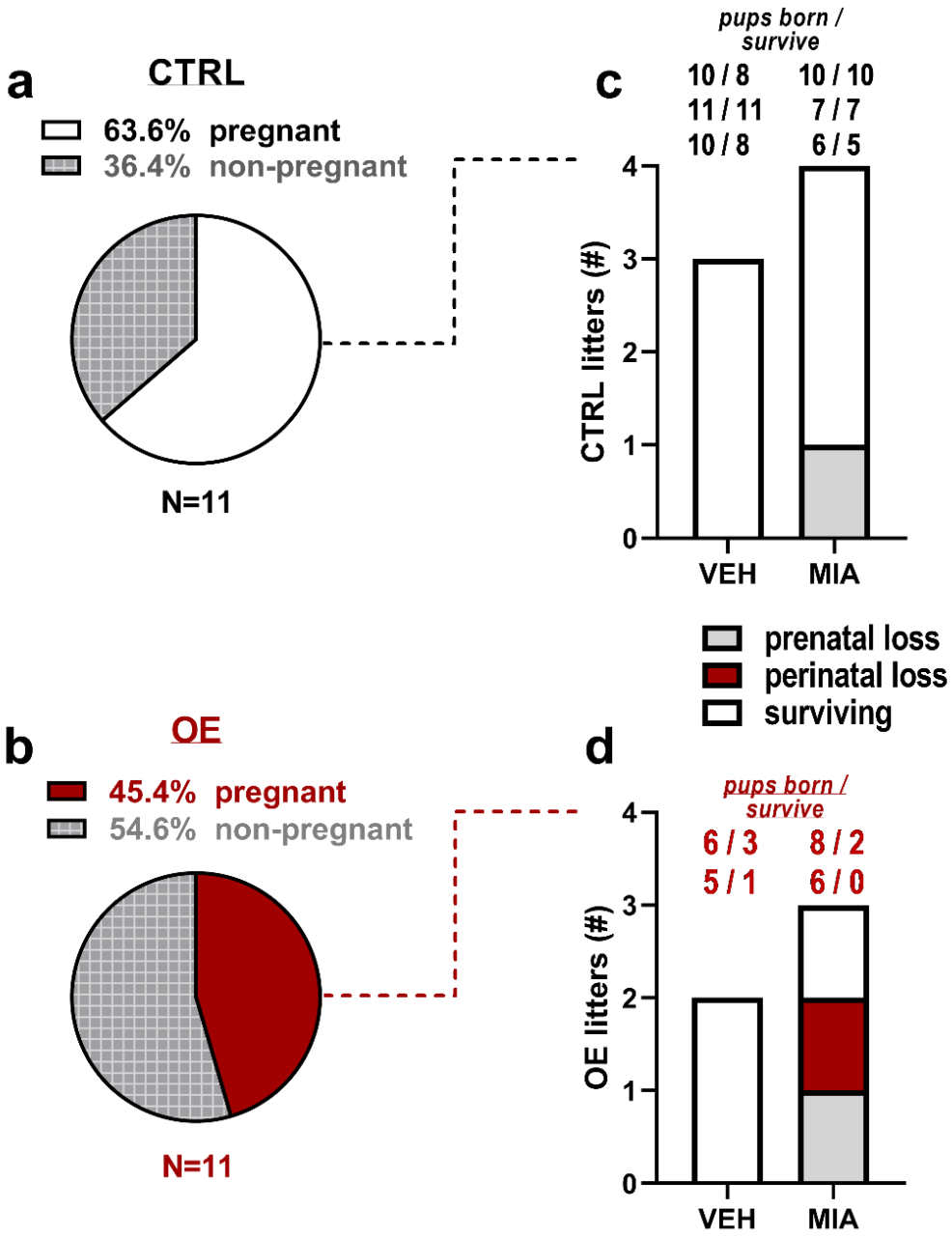


Figure 5.4 Observed pregnancy and offspring survival outcomes following maternal immune activation (MIA) of cytoplasmatic RBFOX1 overexpressing (OE) and non-OE control (CTRL) mice. 63.6% (n=7/11) of CTRL (**A**) and 45.4% (n=5/11) of RBFOX1 OE (**B**) dams became pregnant after three rounds of timed mating (5 and 6 females/genotype in *Continued*. Cohort 1 and 2, respectively). These were intravenously injected on the ninth gestational day with either 2.5 mg/kg poly(I:C) solution (*MIA*: 4 CTRL, 3 OE) or an equivalent volume PBS 1x vehicle (*VEH*: 3 CTRL, 2 OE) solution. One of the MIA and none of the VEH control dams experienced prenatal offspring loss (**C**). 80-100% of offspring of CTRL dams survived into adulthood and litter sizes at birth were comparable regardless of MIA status (**C**). In OE dams (**D**), MIA led to one each pre- and perinatal (PND1-3) loss of pups (2/3 of successful pregnancies). In OE mice of the VEH group, offspring survival rate was 20 and 50% per litter, while in the double-hit OE-MIA group, only 0-25% of the offspring reached adulthood. Furthermore, litter size at birth was significantly reduced by RBFOX1 OE when compared to controls (C *vs* D, p=0.023).

In OE dams, on the other hand, MIA led to spontaneous abortion in one - and perinatal loss (before PND3) of pups in another of the three successful pregnancies, resulting in offspring from only one viable litter (Figure 5.4D). In the "double-hit" OE-MIA group, only 25% (n=2/8) of the offspring from the single remaining litter reached adulthood.

Consequently, the continuation of the experiment was suspended in accordance to animal welfare and legislative rules. Surviving offspring from the first cohort that reached adulthood was tested in a pilot study of NPD-relevant behaviours.

MATERNAL CARE

Maternal behaviour, which was also assessed in the initial cohort as MIA has been shown to affect it (see Chapter 1.2.1), was supplemented with additional wild-type VEH and MIA animals to investigate MIA effects on maternal care (n=7 CTRL, 7 MIA in total, Figure 5.5I). A range of behaviours, including arched-back and blanket-nursing, self- or pup-grooming, nest-building, feeding and drinking, and locomotion, were scored on PND1 and PND2.

Extensive analysis of individual behavioural measures revealed nominal differences in maternal self-care and activity but no significant effects of MIA (see Supplementary Figure S1 for more), so the variables were combined into nursing and other behaviours within the nest, as well as activity outside the nest (Figure 5.5I). Statistical analyses confirmed neither MIA nor any interaction with time-point and type of behaviour ($p>0.48$, three-way RM ANOVA) had influence on maternal care, suggesting that deficits observed in the offspring are not caused by early-life parental neglect - at least pertaining to the earliest neonatal period.

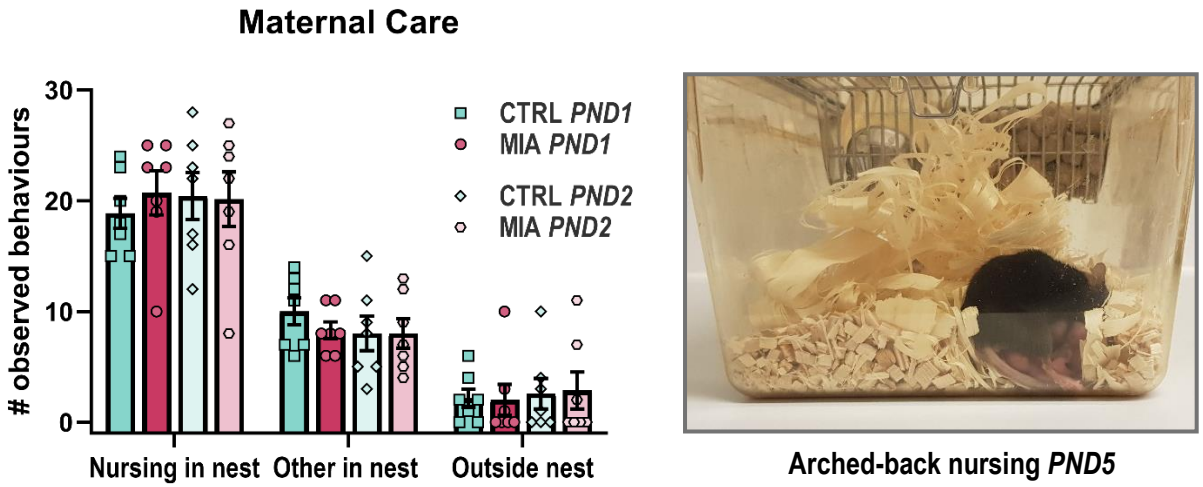


Figure 5.5 Maternal care assessment during the early neonatal period in CTRL and MIA mice. There were no significant effects of MIA on any type of scored behaviours at both assessed time-points (postnatal days PND1/PND2). On the right, an example of arched-back nursing posture on PND5. Dams: n=7 / group. Data presented as mean ± SEM. For more information, see Supplementary Figure S1 for more.

BEHAVIOURAL ASSESSMENT

Relevant behaviours, including spontaneous activity, anxiety- and anhedonia-like behaviours, sociability and social preference, and spatial working memory were investigated in adult male and female offspring of VEH- and MIA-treated CTRL and RBFOX1 OE dams (Figure 5.6). Considering the low sample sizes of the surviving offspring (*CTRL-VEH*: n=17, 6 female / 11 male; *CTRL-MIA*: n=7, 3 female / 4 male; *OE-VEH*: 2 female, 1 male; *OE-MIA*: n=2, female only), no statistical analysis of genotype nor its interaction with MIA, or sex-specific investigations were feasible due to the low sample size. Thus, only pairwise comparisons of the CTRL-VEH and CTRL-MIA groups (t-test or Mann-Whitney U, based on normality) were performed.

In the open-field test, no differences in spontaneous activity or centre time were apparent (Figure 5.6A), including between the offspring of VEH- and MIA-treated controls (p=0.17 centre time, p=0.19 for distance). In the light-dark box test, there was no difference in distance travelled in either the dark ($t_{(22)}$=1.27, p=0.22) or light ($t_{(22)}$=0.77, p=0.45) or time spent ($t_{(22)}$=1.61, p=0.12) in each compartment between the CTRL-VEH and CTRL-MIA offspring (Figure 5.6C). There was an indication of reduced time spent in the brightly-lit compartment as well as increases in dark-box activity in the RBFOX1 OE mice, but variability was high and sample size too low for a conclusive result (Figure 5.6C).

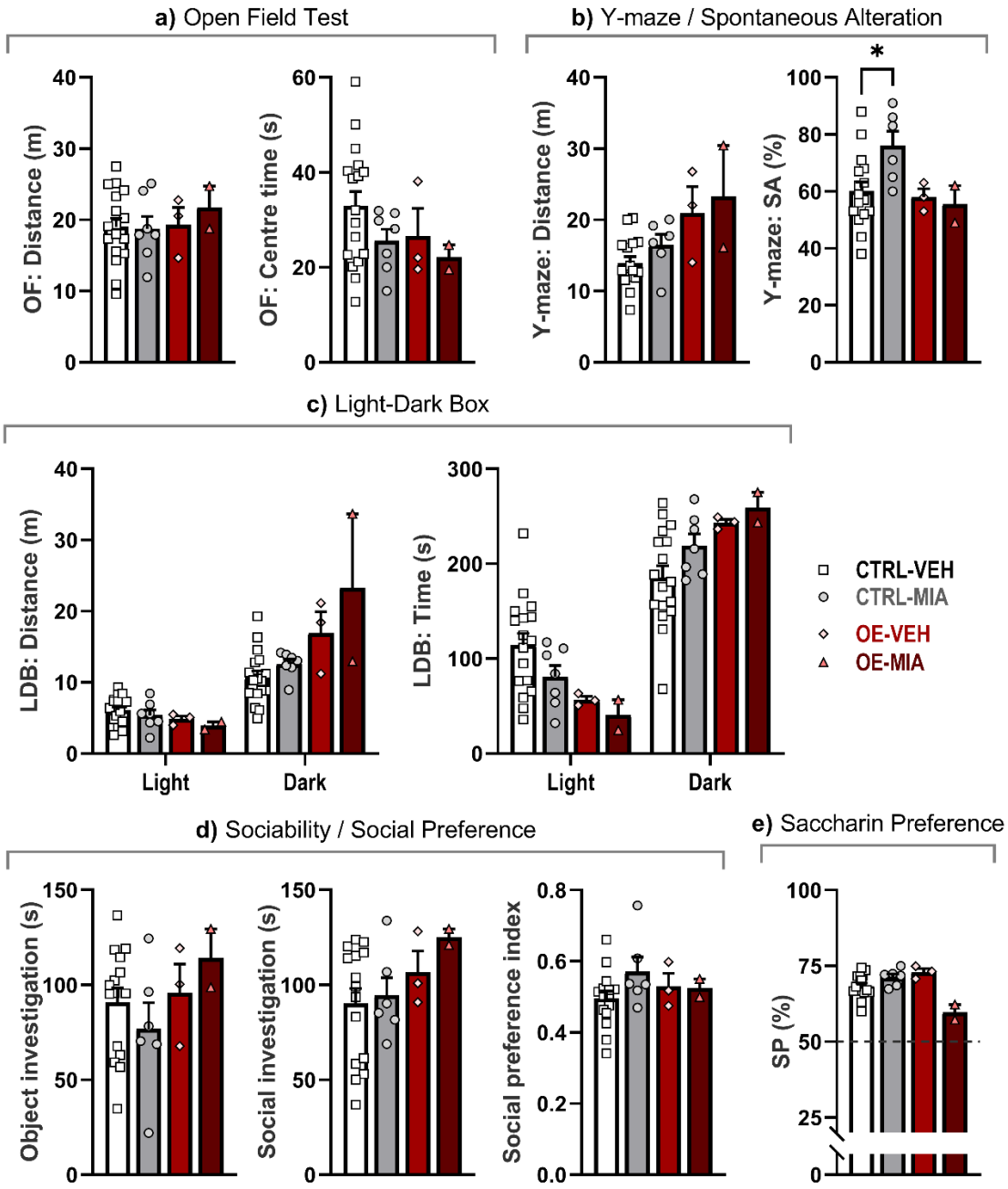


Figure 5.6 Behavioural investigations of environment × gene interaction effects, using early, viral-like maternal immune activation (MIA) and neuronal-specific, cytoplasmatic RBFOX1 overexpression (OE) mouse models. A, locomotor activity (*left*) and time spent in the centre (*right*) of an open field (OF) arena. **B**, distance travelled (*left*) and spontaneous alteration (SA, *right*) percentage during a single, untrained Y-maze trial. **C**, Distance travelled (*left*) and time spent (*right*) in each compartment of a light-dark box (LDB). **D**, time spent investigating an empty cage (*left*) and a sex- and genotype-matched, novel conspecific (*middle*) during two consecutive 3 min trials in the OF arena, also presented as a social preference index (*right*; investigation time of social *vs* object stimulus). **E**, saccharin preference (SP), representing the ratio of sweetened vs plain water consumed on average over two consecutive days. Animal numbers: *CTRL-VEH*: n=17 (except B: n=16; D, E: n=15); *CTRL-MIA*: n=7 (except B, D, E: n=6); *OE-VEH*: 3; *OE-MIA*: 2. Unpaired t-test *CTRL-VEH vs CTRL-MIA*: *p<0.05. Data: mean ± SEM.

In the Y-maze, mice of the RBFOX1 OE groups seemingly travelled more distance than controls but were comparable in their spatial working memory performance (Figure 5.6B). In CTRL offspring, MIA did not affect general activity ($t_{(20)}$=1.49, p=0.15) but increased spontaneous alteration ($t_{(20)}$=2.62, p=0.017), indicative of robust spatial working memory, attention, and possibly - increased spatial novelty preference (Figure 5.6B; no data available for one CTRL-VEH and one CTRL-MIA animal due to hardware error).

A test of sociability and social preference measured time spent exploring an empty cage (object investigation) in the open-field arena and time spent exploring the same cage containing a novel, sex- and genotype-matched conspecific (Figure 5.6D).

Two CRTL-VEH and one CTRL-MIA mice were excluded from the analyses, because they spent less than 1% (<18 s) of the trial duration investigating the cage during the initial, object-investigation trial. While the CTRL-MIA mice appeared somewhat less interested in investigating the cage in the initial trial than controls of the VEH group, this difference was not significant ($t_{(19)}$=0.99, p=0.34) - and neither was the time spent investigating the novel conspecific ($t_{(19)}$=0.76, p=0.31; Figure 5.6D). Observed behaviours in the RBFOX1 OE groups hinted at slightly increased sociability and object investigation time but these results remain inconclusive (Figure 5.6D).

The social preference index, which assesses the propensity of mice to spend more time investigating a social versus inanimate, i.e. object, stimulus - and is considered an NPD-relevant measure - was not significantly affected by MIA (*CTRL-VEH vs CTRL-MIA*: $t_{(19)}$=1.82, p=0.09) or visibly by RBFOX1 OE (Figure 5.6D).

Finally, the anhedonia-like measure of saccharin preference (SP), which gives each individual mouse's ratio of sweetened versus plain drinking water consumed over 48 h, was assessed in all groups (Figure 5.6E). Saccharin was used instead of sucrose (see Chapter 4.1.1) to ensure that possible difference in caloric intake needs would not affect the outcomes. Two CRTL-VEH and one CTRL-MIA mice were excluded from the analyses because of water bottle malfunctions. MIA did not significantly influence SP in control offspring ($t_{(19)}$=1.63, p=0.12) and seemingly, neither did cytoplasmatic RBFOX1 OE alone (Figure 5.6E). While nominal reduction of SP in the OE-MIA offspring could be observed, this group only contained two female mice and thus, is not sufficient to draw conclusions.

5.2 COMMON *RBFOX1* VARIANT'S EFFECT ON *POST-MORTEM* PROTEIN LEVELS

A molecular investigation in *post-mortem* samples from the dorsolateral PFC of older, psychiatrically-healthy adults (see Chapter 3.2 for more) was used to probe the effects of the common polymorphism *rs6500744* on *RBFOX1*'s abundance on a protein level. This SNP was selected based on previous insights from our laboratory (O'Leary *et al.*, 2022); see also Chapter 1.3.2.3.

RESULTS

There was no statistically significant interaction between sex and allele type on RBFOX1 protein abundance, whilst controlling for age ($F_{(2,26)}$=1.78, p=0.194, partial η^2=0.064) in a factorial analysis of covariance. Sex ($F_{(1,26)}$=0.99, p=0.33) similarly did not independently influence RBFOX1 levels, so males and females were pooled. While the average RBFOX1 protein signal was nominally higher in C-carriers (1.69±0.09 AU) compared to the TT-carriers (1.48±0.29 AU), there was no significant difference in RBFOX1 fold-change (Welch's $t_{(5.49)}$=0.76, p=0.24; Figure 5.7B), and the TT-carrier group exhibited high coefficient of variation (45.3% vs 25.8% in C-carriers).

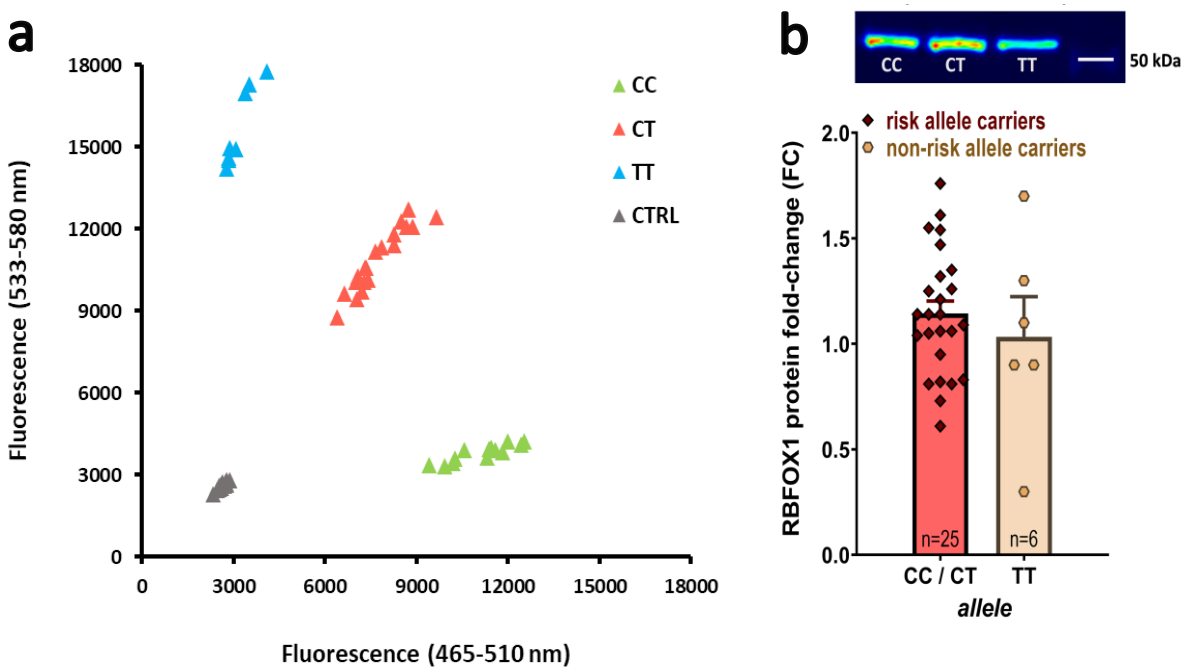


Figure 5.7 Effects of the common polymorphism *rs6500744* on RBFOX1 protein abundance in *post-mortem* dorsolateral prefrontal cortex (PFC) of older, psychiatrically healthy adults. *Continued.*

A, endpoint fluorescence scatter plot of allele-specific PCR of *post-mortem* samples (n=40). Homo- (CC, TT) and heterozygous (CT) allele carriers' designation was performed by fluorescence signal clustering, based on the measured signal with excitation-emission wavelengths of 465-510 nm (x-value; risk C-allele) and 533-580 nm (y-value; non-risk T-allele). Water was used for negative control (CTRL). **B**, near-infrared Western Blot (*top panel for example*) analysis was used for quantification of RBFOX1 abundance in the dorsolateral PFC of psychiatrically healthy older adults (58.1% male; age mean ± SEM: 65.0 ± 3.3). The nominal increase in fold-change in C-carriers was not statistically significant, and age and sex did not affect RBFOX1 levels. Replicates with coefficient of variation above 20% were excluded from the analysis (n=25 C-carriers, n=6 TT-carriers). For full information on samples, including age and sex, see Table 3.

5.3 DISCUSSION

The investigation of neuronal-specific, cytoplasmatic RBFOX OE in mice was performed to assess morphological effects in the brain and if its lifelong upregulation might interact with a low-dose poly(I:C)-induced MIA. Finally, the effect of a common polymorphism of interest, which has been associated in humans with ADHD, cognitive and emotional function, and fear-related behavioural aberrations, on RBFOX1's abundance in *post-mortem* samples of the dorsolateral PFC was investigated.

Protein validation in adult murine OE HPC and PFC confirmed RBFOX1 overexpression and OE was present in the putative cytoplasmatic but not nuclear protein band (see Figure 5.1), indicating that compensation by nuclear RBFOX1 isoforms did not occur – at least in adult males. Importantly, the mouse line utilised in this study implemented synapsin (Syn) instead of nestin (Nes) as a Cre driver, in order to specifically target neurons and not all CNS and neural progenitor cells - in contrast to previous *Rbfox1* KO and OE studies in mice (e.g. Gehman *et al.*, 2011; Tomassoni-Ardori *et al.*, 2019); see Chapter 1.3.2.2. Nestin is a marker for neural progenitor cells, while RBFOX1 associates with neurons and according to data from the Allen Brain Mouse Atlas (*https://mouse.brain-map.org/experiment/ivt?id=100145385,100146145*; *accessed 15.04.2024*), RBFOX1 abundance, at least in the adult murine HPC, is much higher than that of Nestin. Furthermore, *NES* expression is higher – and at its peak - earlier during gestation (post-conception week 9) in the human developing HPC than RBFOX1 (see Figure 1.13), which combined with the epileptic phenotype observed in Nes-Cre mice (Gehman *et al.*, 2011), suggests a different role in neurodevelopment and divergent consequences with NPD relevancy for the two models. While *Rbfox2/3* expression was not

assessed in this study, our previous findings in the complimentary *Rbfox1* Syn-Cre KO mouse model (see Figure 1.15) and those from others using Nes-Cre OE mice did not find compensatory changes in the paralogs (O'Leary *et al.*, 2022; Tomassoni-Ardori *et al.*, 2019).

The findings from the dendromorphological analyses in adult male mice hinted at regions-specific effects of cytoplasmatic RBFOX1 overexpression on the dendritic architecture of the murine brain, which were discernible as a decrease in dendritic length, area occupied, and arborisation in the PFC of adult RBFOX1 OE males (see Figure 5.2). These findings interestingly matched previous findings in the cortex of cytoplasmatic *Rbfox1* KO mice, where downregulation was induced by silencing the gene *in utero* at E14.5 (Hamada *et al.*, 2015). This could be explained by either the region-specificity of each study (dorsal vs prefrontal cortical areas), or the fact that RBFOX1 OE was active during early-to-mid-gestation and the KO occurred afterwards. However, the most compelling explanation falls in line with the hypothesis posed by Rice and McLysaght (2017) that like many other neurodevelopmental and NPD-relevant genes (e.g. *NRXN1/3*, *QKI*, *SHANK3*), *RBFOX1* is a "Goldilocks gene", i.e. dose-sensitive, and can lead to pathological outcomes when both down- and upregulated. This is further supported by findings of both up- and downregulation of RBFOX1 in patients with both NPDs and NDDs, especially in the cortex, and both copy-number gains and -losses are linked to these disorders (O'Leary *et al.*, 2022; Wen *et al.*, 2015). It should be noted that none of those studies focused on cytoplasmatic versus nuclear RBFOX1 isoforms but rather investigated the total gene or protein expression.

Conversely, the changes in HPC spine density and spine types in RBFOX OE males observed in this study (see Figure 5.2) were largely opposite to those described *in vitro* in HPC neuronal culture following cytoplasmatic *Rbfox1* silencing (Hamada *et al.*, 2015), as would be expected. The reduction in filopodia and increases in thin spines, in line with a decreased overall LWR, were novel observations, which might suggest impaired spine dynamics that possibly affect synaptic function. Importantly, the overall PFC and HPC spine density was increased in RBFOX1 OE adult males, which is in line with the observed aberrations in ASD patients and multiple ASD- and NPD-like models (Hutsler & Zhang, 2010; Penzes *et al.*, 2011). The lack of changes in the amygdala – examined for its relevance in RBFOX1-affected fear and avoidance behaviour in patients with panic disorders (O'Leary *et al.*, 2022) - underscored the region-specificity of the overall more subtle changes caused by cytoplasmatic RBFOX1 manipulation

compared to nuclear RBFOX1 or other prenatal ASD-like models (Bringas *et al.*, 2013; Hamada *et al.*, 2016). Female RBFOX1 OE mice were not used for analyses of brain tissue for practical reasons, including their implementation for the MIA × RBFOX1 OE experiment (see below). As previously mentioned, only Syn-Cre expressing females were used to create OE mice to avoid male germline recombination leading to full-body overexpression in the offspring (Rempe *et al.*, 2006); see also see Chapter 3.2.2.1).

Evaluation of the gene × environment influence of MIA and neuronal-specific, cytoplasmatic RBFOX1 OE on behavioural endophenotypes in adult mice of both sexes (see Figure 5.6) revealed no significant or wide-ranging NPD-relevant deficits in wild-type mice as a whole, but should be interpreted with caution due to the low sample size in the CTRL-MIA group and since analysis of sex-specific effects was not possible. While in the initial investigation on the effects of low-dose MIA in wild-type mice, some subtle deficits, e.g. in spatial novelty preference and nesting, were observed specifically in male offspring (see Figure 4.1), none were apparent here. However, nest quality was not assessed in this study and the tests performed in the Y-maze, which indicated somewhat opposing effects of low-dose MIA on spatial working memory, had different designs and measured different variables. Namely, novel-arm preference (decreased in MIA male offspring) after forced-choice training trials, which involved repeated handling by the experimenter 30-60 min before the probe and might negatively affect novel environment exploration in mouse models (Sensini *et al.*, 2020) - versus unconditioned, spontaneous alteration with limited handling (increased in MIA offspring). Interestingly, poly(I:C)-induced MIA has been shown previously to positively affect cognitive performance in the CPT, i.e. visual discrimination and reversal learning, in offspring (Zhao *et al.*, 2021b). In other, more comparable tests, the endophenotypes of MIA offspring, i.e. lack of activity alterations, anhedonia- or anxiety-like behaviours, matched between the two studies. Another possibility is that the environmental enrichment of the home cages, including mouse igloo and more varied nesting material, which was centrally implemented in the animal facility between the two experiments, improved spatial memory in MIA offspring - as environment enrichment has been shown to ameliorate poly(I:C) MIA-induced behavioural aberrations (see Chapter 1.2.4 for more). While some data hinted that RBFOX1 OE, both alone and in combination with MIA, might increase anxiety-like behaviours and cause anhedonia-like behaviour when combined with MIA, there

were not enough mice in either condition to test these hypotheses (see Chapter 7 for more on study limitations). These changes could be theoretically affected by the demonstrated morphological alterations in the dendritic and spine architecture of the PFC and HPC in adult RBFOX1 OE mice (see Figure 5.1, Figure 5.2), as both regions have been implicated in influencing these behaviours in NPD-like animal models (Birnie *et al.*, 2022; Cunniff *et al.*, 2020) but further investigation is needed due to the lack of low-dose and combined MIA × OE morphological data. Interestingly, the behavioural profile of OE offspring in the light-dark box (reduced time in light compartment) and Y-maze (increased activity absent memory deficits) closely resembled those previously reported in neuron-specific *Rbfox1* KO males from our laboratory (O'Leary *et al.*, 2022).

Notably, OE dams became pregnant at a somewhat lower rate and experienced more pronounced loss of embryos or neonates even in the vehicle-treated group (see Figure 5.4). This is most probably an effect of the ongoing construction in the animal facility during the period of this experiment. Construction noise has been previously shown to significantly increase the number of stillborn pups in mice exposed to noise at all times of the pregnancy and reduce litter sizes in dams exposed to the noise at the time of embryo implantation (Rasmussen *et al.*, 2009). While this was not so much the case for wild-type controls, all other in-house bred transgenic lines were affected by the construction (D. A. Slattery, *personal communication*). Indeed, breeding and survival of offspring were negatively affected in all lines, including the one used in the *Diras2* knockout study (see Chapter 6.2), which have previously had no such issues. These stressful changes in basal conditions might also explain the moderate reduction in litter size and some inconsistencies in behaviour in the wild-type MIA group (see previous paragraph), since the results both in the previous MIA study and a follow-up study I performed using the same mouse strain and MIA design matched closely. There are other possible explanations, such as increased parental age, or other, unknown basal stressors in the holding facility, to which the RBFOX1 OE mice might be more susceptible. Oxidative stress has been reported to cause cytoplasmatic RBFOX1 granule formation in immune cells of the fruit fly model (Kucherenko & Shcherbata, 2018), which might affect proper function, while both viral- and bacterial-like MIA has been shown to induce expressional changes in oxidative stress-pathway genes (Guma *et al.*, 2021). Indeed, oxidative phosphorylation was among the pathways, enriched for proteins significantly

affected by high-dose MIA in both male and female adult HPC synaptoneurosomes in the first study (see Figure 4.3). Together, these findings add to the possible explanations of why even a low-dose MIA led to an increased neonatal loss of offspring in RBFOX1 OE mice, including the 100% lethality rate for OE-MIA males. Another possibility of lethal, combined impact of the prenatal immune activation and RBFOX1 OE lies in the consequences for specific targets, revealed to be changed by MIA in the late-embryonic HPC during the perinatal period (see Chapter 7 for more information). Others using early-/mid-gestation, poly(I:C)-induced MIA in transgenic animals of NPD-relevant genes have also reported high loss of offspring in the MIA-treated group (Atanasova *et al.*, 2023) or have implemented mouse lines with either exogenous mutations or hetero-/hemizygous deletions (Abazyan *et al.*, 2010; O'Leary *et al.*, 2014; Openshaw *et al.*, 2019).

The *post-mortem* investigation assessed the influence of the common polymorphism *rs6500744*, which led to higher emotional reactivity and reduced executive functioning during cognitive performance task in healthy (C-allele) risk-carriers, on RBFOX1 abundance (see Figure 5.7). The study in the dorsolateral PFC of older, psychiatrically-healthy adults did not reveal significant genotype effects. However, the number of TT-carriers was rather low, and since RBFOX1 is more highly expressed in neurodevelopmentally-relevant periods, the time-point of measurement might be too late for differences to be noticeable. Mutations on non-coding regions, especially of a single nucleotide might not be enough to elicit abundance changes – although introns might affect transcription rate and splicing events, specifically when close to the gene's promoter region (Shaul, 2017). Indeed, the SNP of interest is positioned on the first *RBFOX1* intron (Fernandez-Castillo *et al.*, 2020) but the present findings suggest that its effects in older adults might not regulate transcriptional or translational rates, but rather influence splicing, i.e. isoform ratios which could not be measured in human samples via Western Blot, or post-transcriptional events, such as *RBFOX1* mRNA stability or transport. It is also possible that the effects are exerted in other brain regions, e.g. dorsal anterior cingulate cortex, where SNP-related functional changes were also observed (O'Leary *et al.*, 2022). Finally, an interaction with other common (polygenic risk) or rare variants might cause the observed RBFOX1 dysregulation in cortices from NPD patients – usually associated with the larger CNVs (O'Leary *et al.*, 2022).

Taken together, the findings of this study suggest that neuronal-specific, RBFOX1 OE causes region-specific alterations in dendritic and spine morphology in the PFC and HPC of adult male mice, which might be linked to suspected behavioural changes in the model. Furthermore, OE of this developmental regulator protein strongly decreased survival of offspring, especially males, following an early viral-like, low-dose MIA, which could be caused by basal stressors or combined effects of the immune challenge and RBFOX1 on vital synaptic proteome targets, e.g. oxidative stress genes, during the vulnerable perinatal period (see Chapter 7 for more information).

6. *DIRAS2* EXPRESSION LEVELS EXERT SUBTLE BEHAVIOURAL AND MORPHOLOGICAL EFFECTS RELATED TO ADHD

As stated in Chapter 1.3.4.2-1.3.4.3, previous human studies, especially from our department, have provided evidence for a role of *DIRAS2* as an ADHD candidate gene. Thus, in this study, I made use of wild-type control (CTRL), homo- (KO) and heterozygous (HET) mice from a transgenic, neuron-specific *Diras2* knockout line to determine the behavioural and morphological consequences of lifelong alteration in *Diras2* expression. This was done to build upon the findings from predominantly human, genetic risk studies (for more details, see Chapter 1.3.4.2 and 3.3.1).

6.1 RESULTS

6.1.1 Behavioural Outcomes

FEMALES

In adult females, *Diras2* expression affected spontaneous locomotor activity ($F_{(2,15)}$=5.205, p=0.02). In detail, HET females were hypoactive compared to CTRL (Tukey's multiple comparisons test, p=0.07) and especially - KO (p=0.02) female mice (Figure 6.1A), whereas no difference was observed between CTRL and KO females. When comparing locomotion in the open field, differences based on genotype ($F_{(2,30)}$=2.965, p=0.067) were more pronounced in the periphery (*HET vs KO*: p=0.017), while there was no significant divergence in time spent in the centre (p=0.53; Figure 6.1A. Indeed, there were also no genotype-based differences in the distance travelled (p=0.17) or time spent (p=0.87) in the light zone, or transitions made between the compartments (p=0.71, see Supplementary Figure S2) in a light-dark box, which is more commonly used to assess anxiety-like behaviour.

Spatial learning and memory, assessed in the Barnes maze by measuring latency to the first escape attempt, suggested that *Diras2* downregulation might impair learning in a dose-specific manner (*trial* × *genotype*: $F_{(12,84)}$=2.039; p=0.030; Figure 6.1B). Indeed, on the second day of training, KO females were faster to attempt escape than both the CTRL (p=0.012) and HET (p=0.045) groups. Conversely, HET females had significantly higher latency to first escape attempt than both CTRL (*training day 5*: p=0.0137) and KO (*day 4*: p=0.049, *day 5*: p=0.0156)

mice. Simultaneously, no effects of *Diras2* were observed on short- or long-term spatial memory in the 24 h- and 7 day-probe trials (Figure 6.1B). Notably, the latency to escape itself was not affected by genotype, however, all female mice were hesitant to fully enter the escape chamber, so the latency to the first escape attempt, defined as partial entry into the escape chamber, was used instead.

Fear-based learning and memory was assessed via the auditory fear conditioning paradigm, i.e. time freezing in the acquisition, extinction, and extinction recall trials of the test (Figure 6.1C). Females of all genotypes were able to learn the paradigm and trial progression reliably affected fear expression across conditions ($F_{(13,182)}$=21.99, p<0.001). The only significant multiple-comparison between genotypes was during the last conditioning cue (Figure 6.1C), where KO females froze longer than HET mice (p=0.014), suggesting intact fear-based memory and learning in females with neuronal *Diras2* downregulation. All behavioural, previously unpublished data was provided by Dr O'Leary, assisted by M. Luckow and S. Waas.

MALES

Neither partial nor full deletion of neuronal *Diras2* had effects on spontaneous activity (p=0.84 total, p=0.83 by zone) and centre time (p=0.9) in adult male mice, as measured in the open field test (Figure 6.1D). In the light-dark box, total distance travelled was similarly unaffected (p=0.16), but there was a marginal effect of genotype on activity in each compartment ($F_{(2,102)}$=2.48, p=0.089, Figure 6.1F). A subsequent multiple comparison-corrected test was revealed a significantly increased locomotion by HET males compared to KO mice (p=0.027) in the dark compartment.

Similarly, the number of transitions between compartments (*genotype*: $F_{(2,51)}$=3.38, p=0.04) was higher in HET than CTRL male mice (p=0.03), hinting at context-specific hyperactivity in more complex environments (Figure 6.1F). Furthermore, the time spent in each compartment was impacted by genotype × zone ($F_{(2,102)}$=5.81, p=0.004) with KO males spending slightly more time in the light zone (p=0.059, Figure 6.1F), indicative of subtle anxiolytic effects of the stronger *Diras2* downregulation in males.

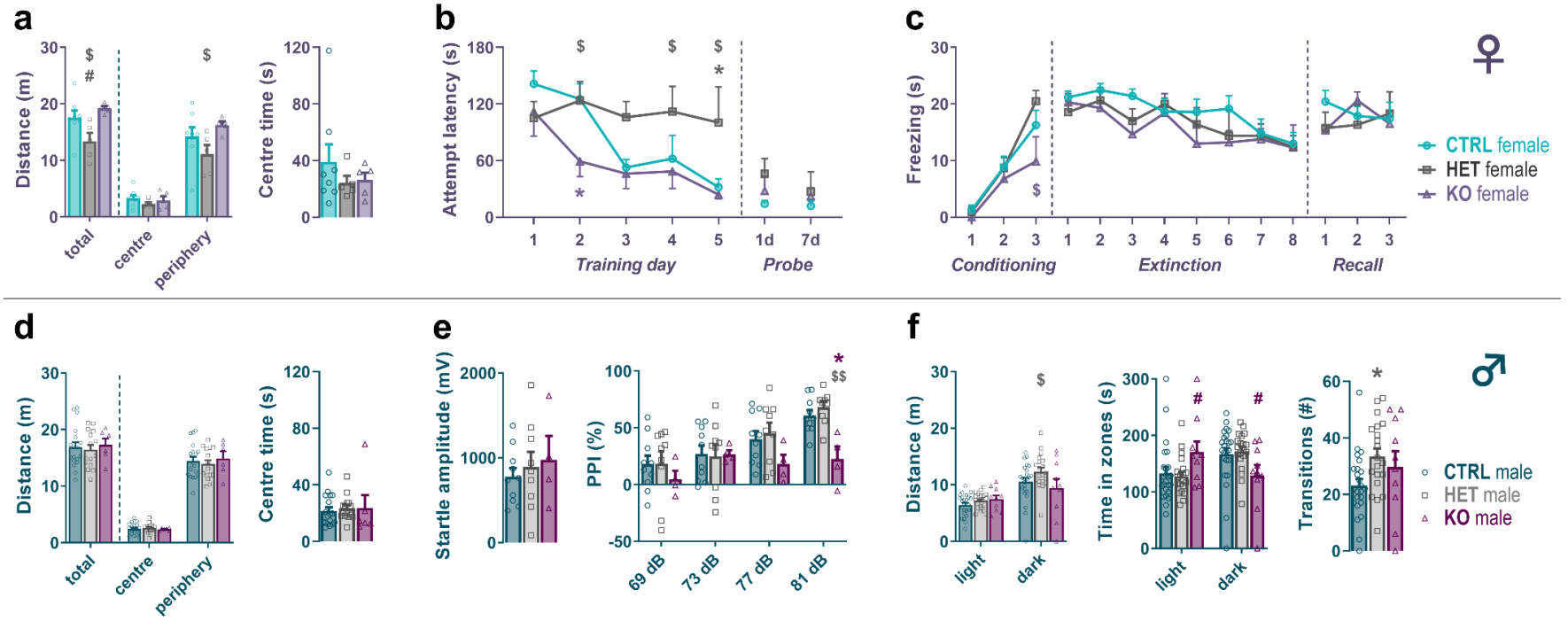


Figure 6.1 Behavioural effects of partial (HET) and full (KO) neuronal *Diras2* downregulation in adult female and male mice. A, open-field (OF) locomotion in females was affected by genotype, so that partial *Diras2* deletion caused hypoactivity in HET females, more pronounced in the periphery (*left*). There were no observable anxiogenic effects of *Diras2* on centre time or activity in females (*right*). **B**, latency to first escape attempt in a Barnes maze was used to assess spatial learning and memory in females. While spatial short- and long-term memory was intact across genotypes, there was a dose-specific effect of *Diras2* on training, with KO females learning faster and HET females generally having longer latencies than the other groups. **C**, the cued fear conditioning test revealed intact fear expression and memory across conditions, with KO freezing more than HET females only during the last fear acquisition cue. **D**, adult males did not differ in baseline locomotor activity (*left*) or time spent in the centre of the OF arena (*right*). **E**, *Diras2* deletion had no effect on the acoustic startle reflex of male mice (*left*) but significantly impaired pre-pulse inhibition (PPI) at high pre-pulse levels (*right*). **F**, the LDB test revealed nominal increase in distance travelled by HET vs KO males in the dark (*left*). A combined zone × genotype effect manifested as increased time spent in the light by KO males (*middle*) and transitions between zones were higher in HET than in CTRL males (*right*). See Supplementary Table S1 for full information on animal numbers (n=8-24 CTRL, 4-20 HET, and 4-13 KO). *Tukey post-hoc:* *p<0.05, #p<0.08 *vs CTRL*; $p<0.05, $$p<0.01 *HET vs KO*; all data mean ± SEM.

The startle amplitude of the acoustic startle reflex (ASR) was not affected by *Diras2* downregulation (p=0.73) in adult males (Figure 6.1E). However, there was an interaction main effect (*pre-pulse level* × *genotype*: $F_{(6,60)}$=2.8, p=0.017) on sensorimotor gating (Figure 6.1E). PPI was impaired in KO males, wherein pre-pulses (PP) did not inhibit the startle response at any PP level (p>0.15), the way they did in CTRL and HET (*77 dB / 81 dB:* p<0.02) mice. Moreover, PPI at the highest PP level was significantly lower in KO animals than both CTRL (p=0.0193) and HET (p=0.005) males (Figure 6.1E).

CPT

To test the effects of *Diras2* downregulation on attention and impulsivity, a CPT was performed in adult male mice (*unpublished data*, provided by A. O'Leary and V. Beck). Baseline behavioural parameters were comparable between the treatment groups, including number of sessions to criterion, response latency, average schedule length, number of screen touches during inter-trial intervals (ITIs), number of hits and mistakes (*data not shown*), suggesting that all animals were equally able to learn the paradigm. Only subthreshold effects, such as reduced number of stimuli missed and of correct rejections made, were observed in *Diras2* HET males (*data not shown*).

During the stimulus duration (SD) probe, SD significantly affected performance (Figure 6.2A), reliably increasing hit rate (HR) with longer duration ($F_{(5,170)}$=54.17, p<0.001). While no significant main effect of *Diras2* genotype (p=0.2) on HR was observed, simple comparisons at each SD condition revealed subtle differences (Figure 6.2A). At longer durations, HET males had higher hit rates than control (CTRL) animals (*1.5 s*: p=0.062; *2 s:* p=0.058). At the lowest SD, HET males further exhibited higher false alarm rates (FAR) than both CTRL (p=0.027) and *Diras2* knockout (KO; p=0.01) mice, but genotype did not overall effect FAR across stimuli (p=0.21; Figure 6.2E).

Sensitivity, i.e. stimulus discrimination ability measured by the non-parametric sensitivity index (SI; Figure 6.2I), significantly increased with higher SD ($F_{(5,170)}$=28.24, p<0.001), as expected. Here, knockout and knockdown animals were more sensitive than controls when the visual stimulus was presented for longer (*1.5 s*: *KO vs CTRL* p=0.046; *2 s: HET vs CTRL* p<0.001*, KO vs CTRL* p=0.018). Response bias, representing the animal's willingness to respond to stimuli and shown here as a non-parametric responsivity index (RI; Figure 6.2M, lower responsivity corresponding to higher bias), was also significantly correlated with SD.

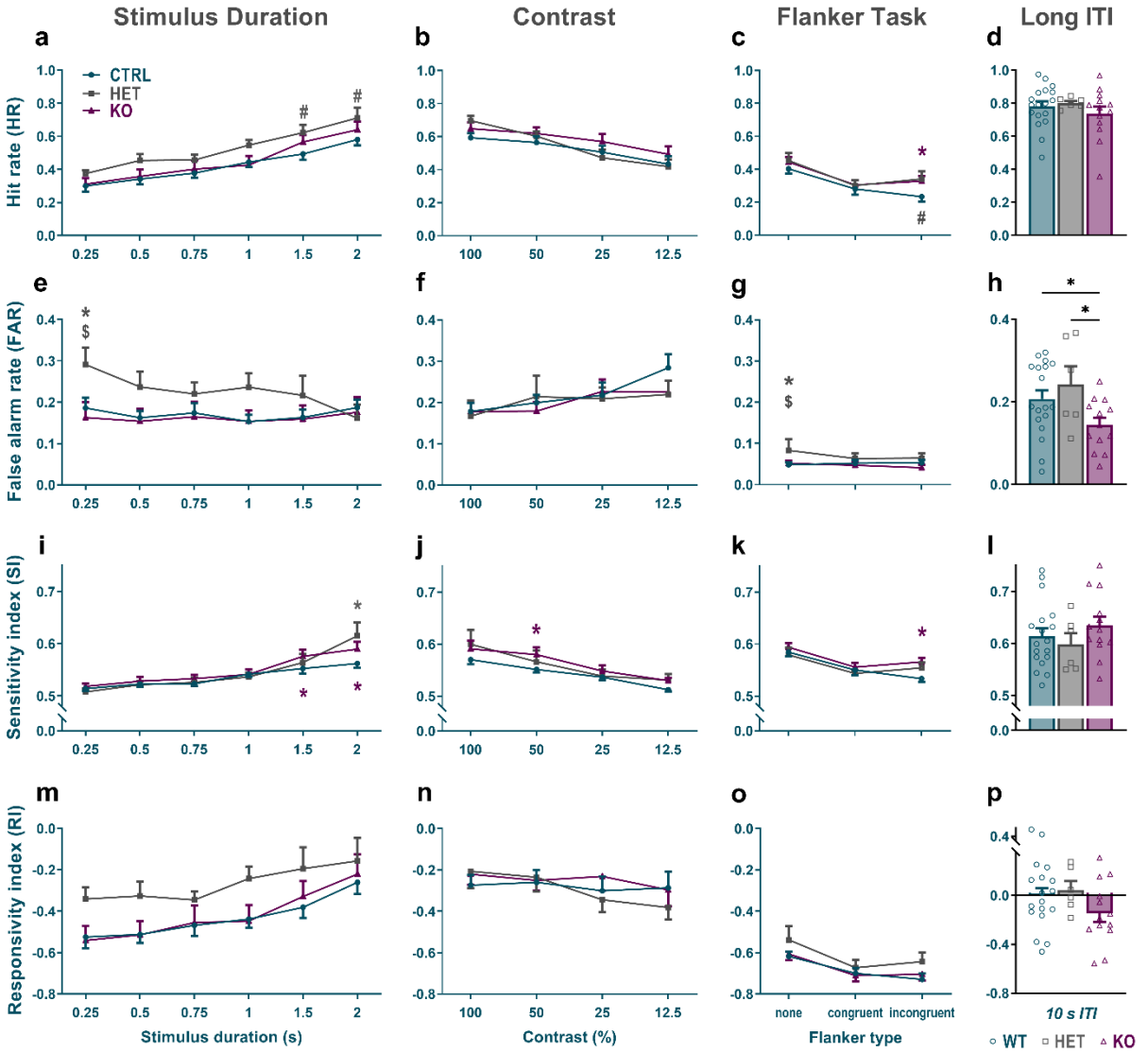


Figure 6.2 Neuronal *Diras2* deletion had subtle stage-specific effects on cognition and attention in adult male mice during the continuous performance task (CPT). The effects of stimulus duration increase on hit rate (HR, **A**), false alarm rate (FAR, **E**), sensitivity index (SI, **I**), and responsivity index (RI, **M**) were investigated in control (CTRL, *Diras2*$^{+/+}$), heterozygous knockdown (HET, *Diras2*$^{+/-}$), and homozygous knockout (KO, *Diras2*$^{-/-}$) males. Genotype did not significantly affect any of those, however, subtle differences were observed as slightly increased sensitivity in KO and HET (**I**) mice and marginally higher HR in HETs (**A**) at longer SD. Decreasing stimulus contrast negatively impacted HR (**B**), FAR (**F**), and SI (**J**) but not RI (**N**) and genotype had no significant effects on performance in these trials. The cognitively demanding flanker task revealed significant effects of genotype × distractor on HR (**C**) and SI (**K**), especially in the challenging incongruent condition, manifesting in improved performance in KO males. The FAR was not affected by distractor type (**G**) but simple pairwise comparisons suggest higher impulsivity in HET mice during the distractor-free trials. The long inter-trial interval (ITI) did not affect HR (**D**), sensitivity (**L**) or responsivity (**P**) but significantly decreased FAR (**H**) in KO male mice. CTRL: n=18, HET: n=6; KO: 13. *Fisher LDS, uncorrected multiple comparison test*: *p<0.05, #p<0.08 *vs CTRL*; $p<0.05, *HET vs KO*; all data presented as mean ± SEM.

Here, response bias decreased with increasing duration ($F_{(5,170)}=14.32$, $p<0.001$; Figure 6.2M). While responsivity in HET males was nominally increased compared to both other groups, especially in the SD range 0.25-1 s, significance was not reached (*main effect:* $p=0.21$; *pairwise comparisons:* $0.1>p>0.08$).

The contrast stage of the CPT was used to investigate performance under increasingly more challenging visual conditions, i.e. reduced stimulus contrast. Overall, decreasing contrast had significant negative effects on HR (Figure 6.2B, $F_{(3,102)}=29.11$, $p<0.001$), FAR (Figure 6.2F, $F_{(3,102)}=4.83$, $p=0.004$), and SI (Figure 6.2J, $F_{(3,102)}=29.27$, $p<0.001$) but did not impair responsivity (Figure 6.2N, $F_{(3,102)}=2.14$, $p=0.1$). Genotype did not strongly affect performance in any of the test. However, simple exploratory analysis revealed a subtle difference of SI at 50% contrast, wherein KO males had better discrimination ability than CTRL animals (Figure 6.2J, $p=0.031$).

The more challenging, long ITI (10 s) did not overall decrease performance in any of the paradigms tested, compared to the default (5 s) ITI ($p>0.3$, *data not shown*). However, when directly comparing stimulus responses in the long ITI trials between genotypes, FAR was significantly lower in KO males ($F_{(2,34)}=3.46$, $p=0.043$; Figure 6.2H) compared to both CTRL ($p=0.048$) and HET ($p=0.024$) animals. *Diras2* downregulation did not affect HR ($p=0.52$, Figure 6.2D), SI ($p=0.46$, Figure 6.2L), or RI ($p=0.2$, Figure 6.2P).

The most cognitively demanding flanker task used visual distractors (congruent: the flankers matched presented stimulus; incongruent: they did not match stimulus) to test if these negatively affect attention, as top-down inhibition of distractions is required to maintain performance. The distractor condition and *Diras2* downregulation had a significant interaction effect on HR (Figure 6.2C, $F_{(4,68)}=3.21$; $p=0.0178$), especially visible in the most challenging, incongruent trials. KO ($p=0.033$) males had significantly higher HR, and HET ($p=0.06$) males trended toward a higher HR, than CTRLs when the distractors did not match the stimulus, indicative of better attentional performance in the transgenic males. Similarly, stimulus discrimination ability measured by SI was somewhat affected by genotype × distractor type ($F_{(4,68)}=2.47$, $p=0.053$; Figure 6.2K) and the KOs performed better than CTRL ($p=0.008$) mice in the incongruent trial. Interestingly, FAR was not significantly influenced by either distractor type ($p=0.28$) or genotype ($p=0.2$, Figure 6.2G). However, simple exploratory comparisons revealed subtle differences in the distractor-free probe, which mimics training conditions (Figure 6.2E). Here, HET animals displayed lower

response inhibition that both KO (p=0.049) and CTRL (p=0.023) males, similar to their performance in the low stimulus-duration SD probe (Figure 6.2E). RI was influenced by the distractor type as expected ($F_{(2,68)}$=61.2, p<0.001, Figure 6.2O), but there was no impact of genotype on responsivity, though HETs had nominally lower response bias under the distractor-free and incongruent conditions.

While there were subtle statistical differences in CPT performance observed between groups when using exploratory, multiple-comparison uncorrected tests, none of these survived multiple-testing correction (Tukey post-hoc; remaining *trends*: FAR / long ITI (Figure 6.2H), *HET vs KO*: p=0.06; FAR / Flanker-no distractor (Figure 6.2G), *HET vs CTRL*: p=0.059), suggesting discrete, dose-specific and often subthreshold effects of *Diras2* downregulation on response inhibition in cognitively-demanding conditions, manifesting as increased FAR in HET males.

6.1.2 Morphological Investigations

To test the effects of partial and full, neuronal-specific *Diras2* knockout on cell morphology, dendritic architecture was assessed in adult male PFC, HPC, and amygdala, due to their relevance for observed behaviours and NPDs of interest (see Chapters 1.1.1, 6.1.1).

DENDRITIC ARCHITECTURE ANALYSES

In the PFC, the number of dendrites was not affected by genotype ($F_{(2,136)}$=1.28, p=0.28) but *Diras2* deletion had an effect on the dendritic length (Kruskal-Wallis H=72.64, p<0.001; Figure 6.3A). Dendrites were longer on average in KO males compared to control and HET animals (Dunn's post-hoc, p<0.001).

Similarly, a Sholl morphometric analysis revealed that neurons in PFC of KO mice occupy a larger area (H=11.01, p=0.004; Figure 6.3B) than those of HET (p=0.03) and CTRL (p=0.005) animals. Dendritic arborisation in the PFC was also affected by genotype, both as a whole ($F_{(46,2037)}$=7.93, p<0.001) and in interaction with distance from soma ($F_{(46,2037)}$=1.88, p<0.001; Figure 6.3B). Here, full neuronal-specific *Diras2* deletion led to a significantly increased number of intersections compared to both the CTRL (p=0.003) and HET (p=0.007) group, which was most pronounced in the middle dendritic segment (Figure 6.3B).

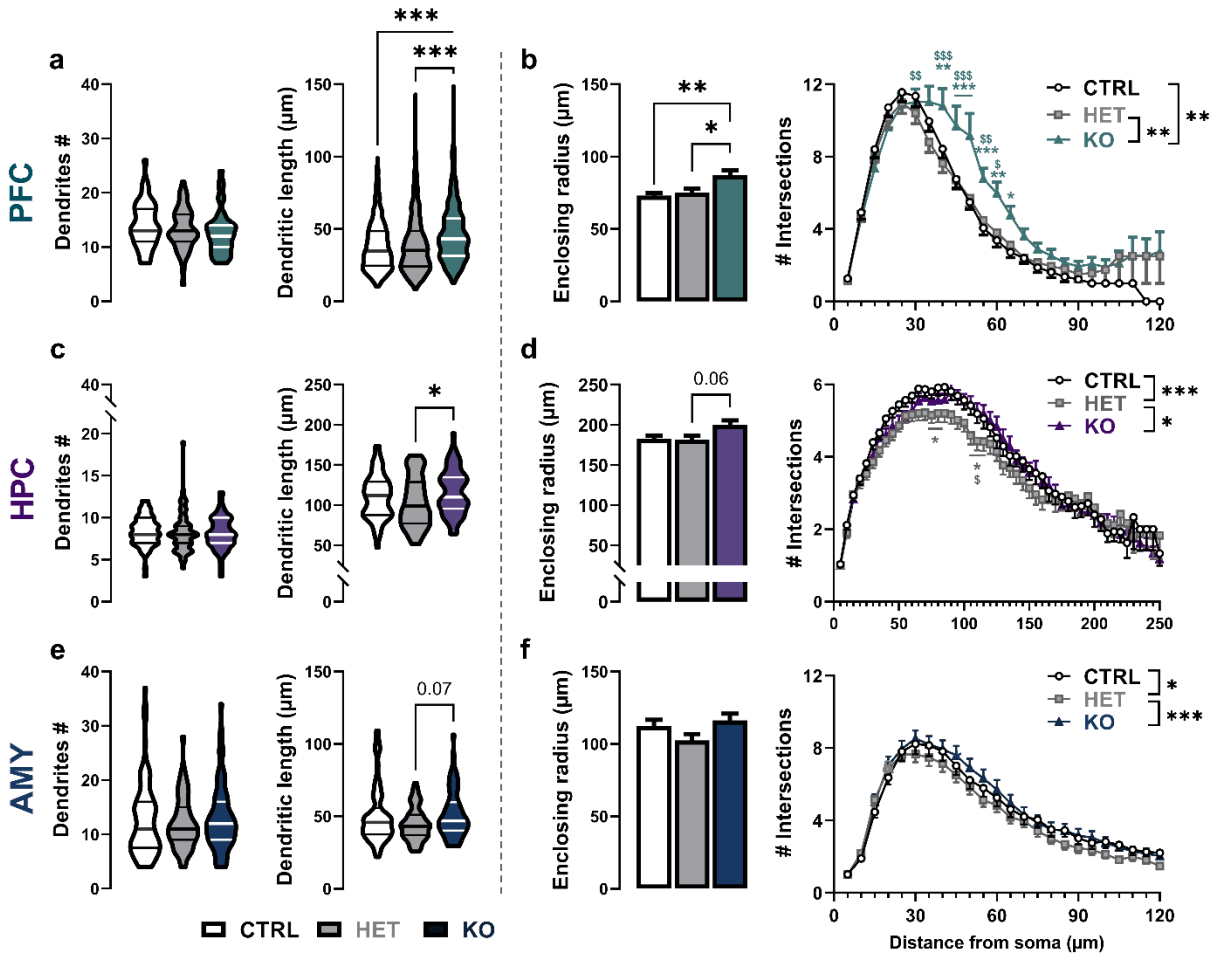


Figure 6.3 Dendritic morphology of neurons in the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMY) of adult control (CTRL), hetero- (HET), and homozygous (KO) *Diras2* knockout males. A, average dendritic number (*left*) and length (*right*) of PFC neurons, the latter of which was significantly increased in KO males. **B**, Sholl morphometric analysis of dendritic arborisation showed increased enclosing radius (*left*), as well as overall and distance-specific branching (*right*) in the PFC of KO male mice. C, in the HPC, dendrite number (*left*) was unaffected by genotype, while dendritic length (*right*) was mildly decreased in HET males compared to KOs. Similarly, the average area of the HPC neuronal field was higher in KO than HET but not CTRL mice (**D**, *left*). The dendritic arborisation was affected by *Diras2* deletion in the HPC (**D**, *right*), with HET animals having significantly less-branched profiles than the other groups. In the AMY, *Diras2* genotype did not affect the average number of dendrites (**E**, *left*) and had subthreshold effects on dendritic length (**E**, *right*) and enclosing radius (**F**, *left*) in HET *vs* KO brains. Neuronal-specific *Diras2* downregulation had a significant effect on AMY dendritic arborisation (**F**, *right*), with HET males having less branching than CTRL and KO males on average. n=5-8 CTRL, 5-8 HET, 4-7 KO males/region, see Supplementary Table S2 for more details. *Significance of post-hoc tests*: *p<0.05, **p<0.01, ***p<0.001, p-values for trends given as numbers. For pairwise comparisons at individual distances (in B, D, F): *p<0.05, **p<0.01, ***p<0.001 *vs CTRL (colour-coded)*; $p<0.05, $$p<0.01, $$$p<0.001 *KO vs HET*. Violin plots show distribution, median, and quartiles of data, rest of data presented as mean ± SEM.

In the HPC, dendritic number was not influenced by genotype (H=1.99, p=0.37) but the degree of *Diras2* deletion had a modest effect on average dendritic length (H=5.99, p=0.05): here, HET mice had on average shorter dendrites than KO animals (p=0.044; Figure 6.3C). Similarly, neurons in the HPC of HET males tended to occupy a smaller area than in the HPC of controls (*genotype*: H=6.13, p=0.05; *post-hoc*: p=0.06; Figure 6.3D).

Furthermore, genotype strongly affected the dendritic branching profile ($F_{(2,7123)}$=8.40, p<0.001; Figure 6.3D), with HET animals having reduced HPC arborisation compared to both CTRL (p<0.001) and KO (p=0.026) males.

In the amygdala, similar to the HPC, *Diras2* deletion did not influence dendritic number (H=0.59, p=0.74; Figure 6.3E). Here, genotype only marginally affected dendritic length (H=5.16, p=0.08) and neuronal area (H=5.01, p=0.08) between HET and KO groups (*length*: p=0.07; Figure 6.3E; enclosing radius: p=0.09; Figure 6.3F). However, *Diras2* knockdown had a significant effect on the arborisation of HPC dendritic trees ($F_{(2,4046)}$=9.11, p<0.001), wherein branching was overall reduced in the HPC of HET males compared to both CTRL (p=0.011) and KO (p<0.001) mice (Figure 6.3F).

Overall, neuronal-specific *Diras2* deletion affected dendritic length and branching profile in a brain region-specific way, leading to robust changes in the PFC of KO male mice and more subtle aberrations in the limbic regions, i.e. HPC and amygdala, of HET males.

DENDRITIC SPINE ANALYSES

Next, morphological spine analyses were performed in the same regions of interest for control, *Diras2* knockdown and knockout male mice. In the PFC, genotype affected neither LWR (H=0.31, p=0.86), nor overall spine density (H=0.61, p=0.74; Figure 6.4A). However, genotype had a subtle effect on the density of different spine types ($F_{(2,710)}$=2.73, p=0.066).

Here, *Diras2* HET male mice had a lower density of spine precursors, i.e. filopodia, than KO males (p=0.014), as well as lower density of mature, mushroom-type spines than both other genotypes (*HET vs CTRL*: p=0.26; *HET vs KO*: p=0.11; Figure 6.4A).

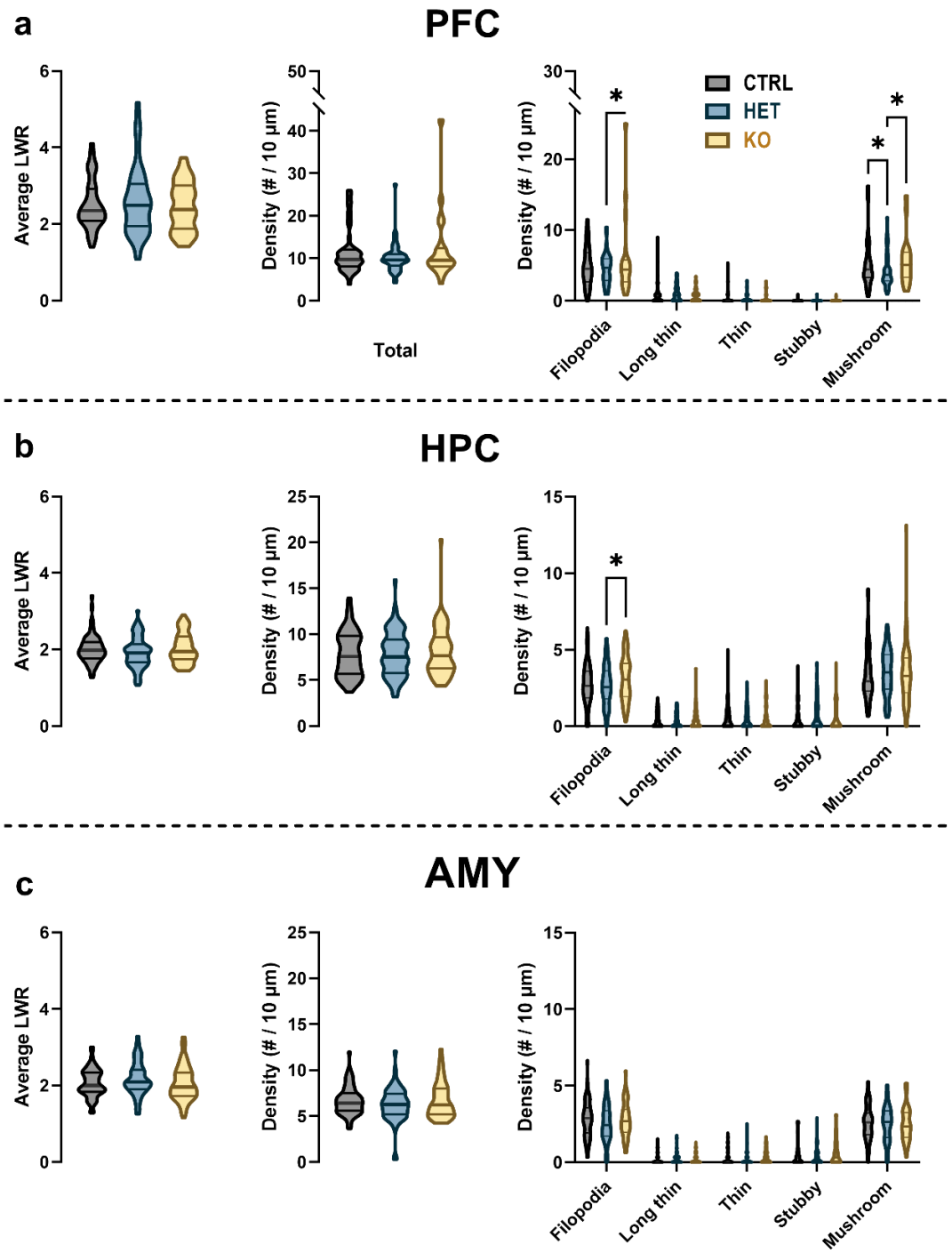


Figure 6.4 Spine density and morphology in the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMY) of adult control (CTRL), hetero- (HET), and homozygous (KO) *Diras2* knockout male mice. A, neuron-specific *Diras2* deletion did not have an effect on spine length-to-width ratio (LWR, *left*) or overall spine density (*middle*) in the PFC. However, genotype had a modest effect on density of different PFC spine types, observable in HET males as lower spines precursor (filopodia, *vs KO*) and mature (mushroom, vs CTRL and KO) spine density (*right*). **B**, in the HPC, partial and full *Diras2* knockout did not impact spine LWR (*left*), overall dendritic spine density (*middle*), or the density of any specific spine type (*right*). **C**, similarly, in the AMY, genotype had no effect on spine LWR (*left*), overall density (*middle*), or specific spine-type abundance (*right*). n=5-8 CTRL, 5-8 HET, 4-7 KO males/region, see Supplementary Table S2 for more information.

In the HPC, genotype similarly did not produce alterations in spine LWR (H=2.55, p=0.28) or overall density (H=0.31, p=0.86; Figure 6.4B). Here, *Diras2* deletion had no significant effect on different spine-type densities ($F_{(2,1155)}$=0.29, p=0.75; Figure 6.4B). This was also the case in the amygdala (Figure 6.45C), where genotype did not have an observable impact on spine LWR ($F_{(2,189)}$=1.65, p=0.20), density (H=1.35, p=0.51), or spine-type abundance ($F_{(2,945)}$=1.24, p=0.29).

6.1.3 Expression Analysis

Expression of *Diras2*, paralog *Diras1*, and known *in-vitro* targets like *mTOR*, *Clasp2*, *Ctdsp2*, *Eif2c2*, *Fktn, Mmp5* was assessed in multiple regions of interest, relevant for NPDs including ADHD, i.e. HPC, PFC, amygdala, nucleus accumbens, hypothalamus, and cerebellum of adult female mice (Figure 6.5; see Chapter 1.3.2.3.2). The latter three genes did not exhibit differences based on genotype in any of the regions of interest (p>0.1, *data not shown*), indicating possible divergences between *in vitro* and *ex vivo* analyses or type of *Diras2* downregulation.

In the HPC, a dose-dependent reduction in *Diras2* expression based on genotype was confirmed (Figure 6.5A; $F_{(2,14)}$=35.54, p<0.001; *HET vs CTRL*: p=0.045, *KO vs CTRL*: p<0.0001, *KO vs HET*: p=0.0009). Additionally, genotype impacted HPC *mTOR* abundance ($F_{(2,14)}$=6.19, p=0.01) with mRNA levels higher in the HPC of KO compared to both other groups (p<0.03; Figure 6.5A). In the amygdala (Figure 6.5B), there was similarly a strong, dose-dependent decrease of *Diras2* ($F_{(2,14)}$=107.8, p<0.001; *all pairwise tests*: p<0.001), in addition to a *Diras1* downregulation trend ($F_{(2,14)}$=2.98, p=0.08) in KO compared to CTRL animals (p=0.07). Furthermore, there was a trend ($F_{(2,14)}$=3.33, p=0.07) toward differential effects of partial- compared to full-*Diras2* deletion (p=0.054) on *mTOR* expression (Figure 6.5B).

Diras2 expression in the hypothalamus was affected by genotype ($F_{(2,14)}$=23.3, p<0.001; *HET vs CTRL/KO*: p=0.02, *KO vs CTRL*: p<0.001; Figure 6.5C), similarly to the observed effects in HPC and amygdala (Figure 6.5A-B). Here, significant effects of *Diras2* downregulation on its paralog *Diras1* ($F_{(2,14)}$=4.41, p=0.03) were additionally observable as a *Diras1* reduction in KO versus CTRL mice (p=0.027, Figure 6.5C). In the hypothalamus, *mTOR* ($F_{(2,14)}$=3.13, p=0.075) and *Clasp2* ($F_{(2,14)}$=2.85, p=0.091) expression was marginally downregulated by genotype,

especially in HET brains (*mTOR*: p=0.079, *Clasp2*; p=0.1) compared to CTRL, but did not quite reach significance.

PFC expression of *Diras2* was also impacted by genotype ($F_{(2,14)}$=14.05, p<0.001) but only significantly downregulated in KO (p<0.001 *vs CTRL*, p=0.014 *vs HET*) - and not HET (p=0.43) females compared to controls (Figure 6.5D). This region of interest appeared more resilient to other expressional changes as well – none of the other investigated genes were affected by either the partial or full *Diras2* knockout (p>0.25).

Similar effects were observed in the nucleus accumbens for *Diras2* abundance (*genotype:* $F_{(2,14)}$=23.12, p<0.001, Figure 6.5E) with significant downregulation in KO (p<0.001 *vs both*) but not HET (p=0.62) animals. Here, an additional trend ($F_{(2,14)}$=3.65, p=0.053) for upregulation of *Diras1* was revealed in the HET group (p=0.071 *vs both*), which might play a compensatory role for *Diras2* changes, e.g. in other regions of the limbic system (Figure 6.5A-C).

In the cerebellum (Figure 6.5F), genotype affected *Diras2* expression only in a subtle manner ($F_{(2,14)}$=2.96, p=0.085; *KO vs CTRL*: p=0.076). It did, however, significantly impact the abundance of *Diras1* ($F_{(2,14)}$=4.24, p=0.036), which was downregulated in KO (p=0.029) compared to CTRL females' cerebellum. Furthermore, genotype-effected reduction in mRNA levels of *Clasp2* ($F_{(2,14)}$=6.19, p=0.01) and *Ctdsp2* ($F_{(2,14)}$=5.28, p=0.02) was found in the cerebellum of KO versus CTRL mice (p=0.015/0.06, respectively; Figure 6.5F). Interestingly, effects on cerebellar *mTOR* abundance ($F_{(2,14)}$=3.91, p=0.045) manifested as a reduction, specific to the HET group when compared to CTRL animals (p=0.042, Figure 6.5F).

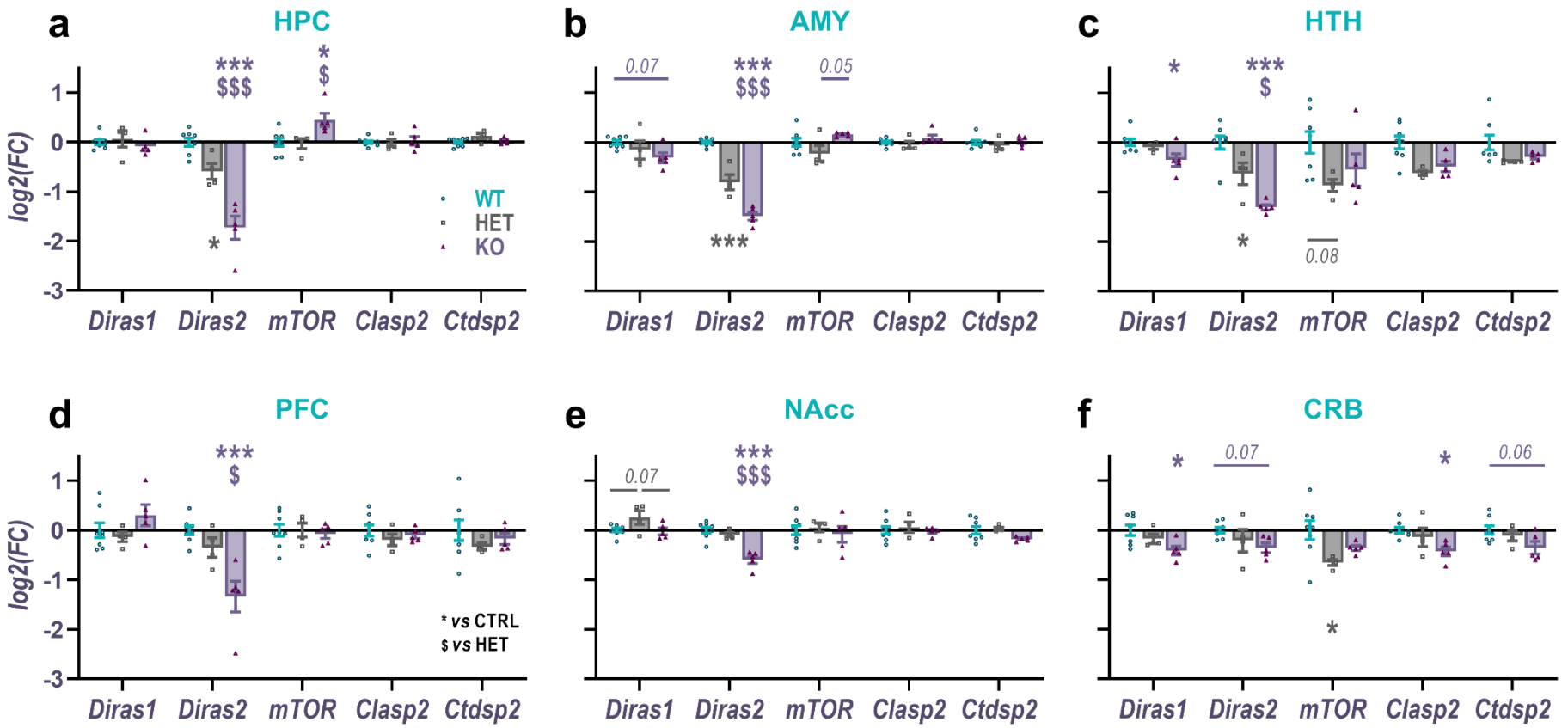


Figure 6.5 Quantitative PCR analysis of *Diras2* genotype effects on gene transcript expression in multiple brain regions of adult female mice. Full neuronal-specific *Diras2* knockout (KO, n=5) led to significantly decreased mRNA expression in hippocampus (HPC, **A**), amygdala (AMY, **B**), hypothalamus (HTH, **C**), prefrontal cortex (PFC, **D**), nucleus accumbens (NAcc, **E**), and a trend in cerebellum (CRB, **F**) of adult female mice. *Diras2* was also less expressed in the HPC, AMY, and HTH of knockdown (HET, n=4) females (**A**-**C**) in a dose-specific manner. *Diras1* was slightly (**B**) or significantly (**C**, **F**) downregulated in the AMY and HTH/CRB of KO mice, respectively. Expression of *mTOR* was notably higher in HPC of KOs and downregulated in AMY, HTH, and CRB (**B**, **C**, **F**) of HET females. *Clasp2* and *Ctdsp2* mRNA was nominally reduced in the HTH of HET mice (**C**) and reached or approached significance, respectively, in the CRB of KO females (**F**). *FC*, fold-change *vs* controls (CTRL, n=8). *Tukey post-hoc significance*: *p<0.05, ***p<0.001 *vs CTRL*, $p<0.05, $$$p<0.001 *vs HET*; trends given as respective p-value; data as mean ± SEM.

6.2 DISCUSSION

Neuronal-specific deletion of *Diras2* had sex- and dose-specific effects on locomotor and anxiety-like behaviours (see Figure 6.1). It also had a subtle impact on performance during the continuous performance task (see Figure 6.2): stimulus sensitivity, i.e. visual discriminability, was higher in KO males than controls, and the false alarm rate was increased in HET males in multiple probes compared to controls and KO males. Furthermore, impaired sensorimotor gating and spatial- but not fear-related learning or memory were observed in adult KO males and HET females, respectively (see Figure 6.1). *Diras2* genotype affected dendritic length and branching profile in a brain region-specific manner (see Figure 6.3F), leading to robust changes in the PFC of KO male mice and more subtle aberrations in the limbic regions, i.e. HPC and amygdala, of HET males. Subtle changes were also observed in the PFC of adult HET males – specifically, a reduction in mature mushroom spines compared to controls and full *Diras2* KO mice (see Figure 6.4).

In female adult mice, expressional effects of neuronal-specific partial or full *Diras2* deletion on NPD-relevant regions-of-interest were dose-, region-, and target-specific. Namely, *Diras2* was decreased in all brain regions in KO females, in addition to *Diras1* in hypothalamus and cerebellum, and *mTOR* was increased in the HPC (see Figure 6.5). In HET females, *Diras2* was decreased in the limbic regions of HPC, amygdala, and hypothalamus, while *mTOR* was decreased in the hypothalamus and cerebellum. Together, the findings of this study hinted at possible ADHD-like effects of heterozygous *Diras2* deletion underlying genotype-linked behavioural, morphological, and subtle molecular differences.

Considering the proposed role of *DIRAS2* as a candidate gene for ADHD in humans and the findings of this study, a comparison with other rodent models of the disorder is pertinent. In ADHD-like, dopamine transporter knockout models, hyperactivity and cognitive dysfunction is usually present and correlated with reduction in BDNF levels and TrkB activation in the PFC (see Chapter 1.3.2.1); however, not all dopaminergic models of ADHD (e.g., D4 receptor knockout mice) exhibit hyperactivity (Leo *et al.*, 2018; Regan *et al.*, 2022). Since HET males exhibited novel-environment hyperactivity and subtle deficits in the response inhibition-domain of PFC-mediated executive function but full *Diras2* KOs did not, it is possible to assume that unknown compensatory mechanisms in *Diras2* KO males (El-Brolosy & Stainier,

2017) might lead to the observed increased branching and length profiles linked to behavioural output. For example, local BDNF release in the brain can cause increases in dendritic branching and length (Horch & Katz, 2002) and can improve cognitive performance (Piepmeier & Etnier, 2015), which was suggested for the KO males in CPT test (subtle improvement in vigilance during Flanker task) and females in the Barnes maze (faster spatial learning in early-stage escape training); see Figure 6.1, Figure 6.2. As all available females were used for molecular analyses after breeding disruptions (A. O'Leary, D.A. Slattery; see Chapter 5.3 for more), morphology was not assessed in the female brains. However, *mTOR* was significantly upregulated in the HPC - and neuronal BDNF has been shown to activate mTOR causing plasticity-related protein synthesis, affecting dendritic and spine architecture (Moya-Alvarado *et al.*, 2023). Increased BDNF-mTOR signalling might underlie morphological and behavioural changes in KO mice of both sexes, alleviating negative consequences of *DIRAS2* downregulation on ADHD-like endophenotypes by compensatory mechanisms but future studies need to evaluate this hypothesis. While male KO mice exhibited SCZ-associated, sensorimotor gating deficits, PPI does not appear to be a core deficit in ADHD patients, especially adults (Feifel *et al.*, 2009; Schulz-Juergensen *et al.*, 2014), so that other NPD-related neurobiological pathways (Geyer, 2006) might be affected in adult male mice by full deletion of neuronal *Diras2*. This deficit might also stem from PFC changes in neuronal architecture, leading to changes in function, as distinct firing rates in neurons of this region have been linked to PPI mechanisms (Toth *et al.*, 2017).

In the CPT (Figure 6.2), ADHD-relevant cognitive control, impulsivity, and attention (Bari & Robbins, 2013), can be measured in both humans and rodent models (Bhakta & Young, 2017; Cope & Young, 2017; Fallgatter *et al.*, 2005; Kim *et al.*, 2015). Children's performance in the CPT changes with age, with response inhibition and attention to stimuli improving and stabilising as early as 5-8 years of age, which correlates with vulnerable time-windows for ADHD (see also Chapter 1.1.1). Thus, the investigation in adult mice needs to be supplemented in the future with performance tests at earlier, NPD-relevant ages. This test was especially pertinent for this study as deficits in executive function, as measured in the CPT, are among the core deficits in ADHD (Willcutt *et al.*, 2005; Wodka *et al.*, 2007) and a common risk SNP of *DIRAS2* has been previously shown by colleagues from our department to negatively affect response inhibition in children with ADHD (Grünewald *et al.*, 2016). In this study, HET males had subtle deficits in response inhibition in the form of higher false alarm rate, lacking

other deficits, including in the domains of attention or vigilance. These findings were paired with decreases in mature spine density in the PFC and dendritic arborisation in the amygdala and HPC in adult HET males, suggesting that partial neuronal-specific *Diras2* deletion might affect neurotransmission in vital brain regions (see above, Chapter 1.1.1) and lead to the observed behavioural aberrations. For example, pyramidal neurons in the rodent PFC projecting to the basal ganglia have been implicated in inhibitory control during goal-directed behaviour (Li *et al.*, 2020a), which might be affected by the spine alterations by affecting signal input processing. Furthermore, a metabolic challenge in neonate rats using homocysteine, proposed as an ADHD-like model, similarly caused decreases in mushroom spines in the PFC in adulthood, among other changes (De la Torre-Iturbe *et al.*, 2022), so further assessments are warranted.

The performance of HET males in the CPT is in line with one hypothesis that inhibitory control issues are only present in some ADHD patients, i.e. those with hyperactivity or combined but not inattentive subtype of the disorder (Milich *et al.*, 2001). Together with observed hyperactivity, i.e. increased light-dark box transitions, these findings indicate that *Diras2* HET males might represent a distinct ADHD-like phenotype. This is in keeping with some common or rare variants in NPDs affecting only one of the genetic copies rather than missing both, which is the case in full KO mice, as well as partial knockdowns of other NPD-relevant genes, e.g. *Snap25* (Dark *et al.*, 2018), eliciting ADHD-like behaviours.

Interestingly, female HET mice did not exhibit hyperactivity but rather a subtle reduction in the spontaneous locomotion, mostly compared to the KO rather than CTRL group. While only subtle, possibly due to the low group sizes, this is consistent with findings in humans that girls with ADHD more rarely fit the hyperactive/impulsive disorder phenotype (Franke *et al.*, 2018), and needs further investigation in the future. This observation was paired with learning but not memory impairment in a spatial task, manifesting as increased escape attempt latency throughout training, which could be affected by inattentiveness, commonly observed in both male and female ADHD patients. These findings might be connected to the significant decreases of *Diras2* in the HPC, amygdala, and hypothalamus and a trend toward a decrease of *mTOR* in HET female cerebellum, specifically – regions involved in spatial memory, escape behaviour, and locomotion (Albergaria *et al.*, 2018; Lammers *et al.*, 1988; Nithianantharajah & Murphy, 2009; Terburg *et al.*, 2018). *Diras1* was not significantly upregulated in HET or KO females in any region, suggesting that such compensatory effects

either occur earlier in life, at times when strong increases in the murine *Diras2* expression are usually present, or might be compensated in adults by other genes. One such possibility is changes in other RAS GTPases, since a strong interaction network between different RAS protein members and very high sequence homology between DIRAS1/2 and H-/K-/N-RAS (>60%), as well as other Ras family members (see Figure 1.17), have been reported (Kiel *et al.*, 2007; Rezaei Adariani *et al.*, 2021); see also Chapter 1.3.4.1. Furthermore, early-life compensatory events could be affected by glial cells, which also express the gene, while the mouse model targeted neuronal-specific *Diras2*. Subtle downregulation of *Diras1* in amygdala, hypothalamus, and cerebellum can be similarly affected by changes in other connected genes, or *DIRAS1* abundance outside the brain, as well as off-target downregulation by Cre recombinase due to sequence similarities between the two paralogs (Kontani *et al.*, 2002).

It should be noted that some deficits and ADHD symptomology have been shown to decrease over time and indeed, completely disappear in a subpopulation of adults diagnosed with the NPD as children (Faraone *et al.*, 2006); see also Chapter 1.1.1. Thus, while some ADHD-linked behaviours were not observed in the *Diras2* model, such as pronounced impulsivity or inattentiveness, they might be present in younger mice, which should be investigated in the future (see Chapter 1.1.1). Furthermore, ADHD is a highly heterogenous NPD and symptoms depend not only on age but also biological sex or gender, environmental factors, and testing paradigms (see Chapter 1.1.1). Finally, human studies have shown that an ADHD risk SNP within the *DIRAS2* promotor region upregulates rather than downregulates expression of the gene (Grünewald *et al.*, 2016), suggesting that overexpression of *DIRAS2* might also play a causal role in the NPD. However, since subtle ADHD-relevant behaviours were observed by the gene's neuronal-specific deletion in this study, this suggests a dose-sensitivity of *DIRAS2* (see Chapter 7).

Taken together, these findings indicate that hetero- and homozygous *Diras2* deletion affect neuromorphology and gene expression in a region-specific manner, and have partially sex-dependent effects on behaviour in adult mice. The findings from *Diras2* HET animals, especially males, support the implication of *DIRAS2* in ADHD aetiology and invite further in-depth investigations in this model, while adding to the body of evidence on the role of DIRAS2 in the brain as relating to NPDs.

7. GENERAL DISCUSSION

This dissertation encompassed studies investigating the effects of genetic and environmental interventions with neurodevelopmental underpinnings in mice. While the three studies addressed somewhat different aspects of behaviour, molecular expression, and neuromorphology, they offer an opportunity to compare the consequences of each manipulation within these domains and its relevance for NPDs (see Table 8).

Early viral-like MIA and neuronal-specific *Diras2* deletion had robust anxiolytic effects in mice (see Table 8), which indicates a possible shared mechanism of function. Reduced anxiety-like behaviours have been reported previously in ADHD-like rodent models in combination with hyperactivity, along with behaviours related to depression-like measures, which were present in the two investigated models. Such ADHD-like mouse models include the cortical deletion of the nuclear hormone receptor (*Nr2f1*) regulating HPC and PFC development, forebrain overexpression of the neurotransmission regulator casein kinase (*Cnsk1d*), and the global exon deletion in the ADHD-associated cryptochrome circadian regulator (*Cry1*) – the latter two targeting the dopaminergic system and altering DA receptor signalling (Contesse *et al.*, 2019; Liu *et al.*, 2023; Zhou *et al.*, 2010). This is especially interesting, since in the high-dose MIA females, which best fit the above-described behavioural profile, affected HPC synaptoneurosomal proteins were enriched in the dopaminergic pathway, with dopamine transporter SLC6A3 as well as receptors DRD1 and DRD5 significantly downregulated in the adult MIA HPC. Intriguingly, D1/5 receptor antagonist (SCH23390) has been shown to reduce responsivity and differentially affect CPT performance in females of the same background strain, suggesting relevance of these targets and DA for cognitive and attentional performance in this paradigm (Klem *et al.*, 2023). Thus, it would be interesting to see the effects of MIA and *Diras2* downregulation on CPT outcomes in female mice.

Crucially, DIRAS2 was significantly upregulated and DIRAS1 nominally downregulated in the adult MIA proteomic pool, especially in the females, strengthening the link between the two models, NPDs, and immune function. Considering the opposite direction of protein changes in the two models, overlapping effects of *Diras2* downregulation and MIA on behaviour and dendritic and spine morphology might either be affected through unknown compensatory mechanisms in the genetic model, up- or downstream effectors, or a common pathway.

Table 8 Summary of findings from models of maternal immune activation (MIA), cytoplasmatic RBFOX1 OE, hetero- (HET) and homozygous (KO) *Diras2* knockout. (M) or (F) denotes that experiment was performed only in the respective sex; M+F denotes pooled males and females. If not stated explicitly otherwise, tests were performed in adult mice and changes given compared to respective controls. [changes] = exploratory analysis- or nominal changes rather than significant effects.

Domain	Model	Genotype / Treatment	Sex	Changes
BEHAVIOURAL CHANGES	**MIA**	Low *poly(I:C)*	M	• subtle anxiolytic effects • ↓ spatial novelty preference (vs *high*) • pronounced ↓ social novelty preference • self-neglect-like ↓ nest quality • ↓ activity in a novel environment
			F	n.s.
		High *poly(I:C)*	M	• subtle anxiolytic effects • subtle ↓ social interaction (vs *low*) • pronounced ↓ social novelty preference • anhedonia-like ↓ sucrose preference • subtle context-dependent hyperactivity
			F	• pronounced anxiolytic effects • pronounced ↓ social novelty preference • self-neglect-like ↓ nest quality • subtly impaired sensorimotor gating (↓ PPI) • context-dependent hyperactivity
	RBFOX1	*Rbfox1* OE	M + F (M*)	[OE: nominal anxiogenic effects] *HET*: anxiogenic effects / ↓ PPI*
	RBFOX1 × MIA	*Rbfox1* OE + Low *poly(I:C)*	(F)	[nominal anxiogenic- / anhedonia-like effects]
	DIRAS2	*Diras2* HET	M (M)	• context-dependent ↑ activity • subtle response-inhibition deficits (↑ FAR) • [liberal response strategy]
			F (F)	• Subtle ↓ spontaneous activity • Impaired spatial learning / intact memory • Normal conditioned-fear learning
		Diras2 KO	M (M)	• Subtle anxiolytic effects • Impaired sensorimotor gating (↓ PPI) • [increased vigilance]
			(F)	• Improved early-stage spatial learning • Normal conditioned-fear learning

Domain	Model	Genotype / Treatment	Sex	Changes
MORPHOLOGICAL CHANGES	**MIA**	High *poly(I:C)*	M	PFC: ↑ dendritic number (vs *F*) ↓ dendritic branching area (vs *F*) ↓ proximal branching, ↑ distal branching Subtle ↑ immature spine density HPC: ↓ spine LWR
			F	PFC: ↓ dendritic number (vs *M*) ↑ dendritic branching area (vs *M*) ↓ proximal branching, ↑ distal branching ↓ total / mature spine density HPC: ↓ dendritic length ↑ distal branching ↓ immature spines ↓ spine LWR
	RBFOX1	*Rbfox1* OE	(M)	PFC: ↓ dendritic length ↓ dendritic branching area ↓ branching ↑ total spine density HPC: ↓ spine LWR ↑ total spine density ↑ thin / stubby spines ↓ spine precursors AMY: n.s.
	DIRAS2	*Diras2* HET	(M)	PFC: subtle ↓ mature spine density HPC: subtle ↓ dendritic length (vs *KO*) subtle ↓ dendritic branching area (vs *KO*) ↓ branching AMY: subtle ↓ dendritic length (vs *KO*) ↓ branching
		Diras2 KO	(M)	PFC: ↑ dendritic length ↑ dendritic branching area ↑ branching subtle ↓ spine precursor density HPC: n.s. AMY: n.s.

Domain	Model	Genotype / Treatment	Sex	Changes
MOLECULAR CHANGES	**MIA****	Low *poly(I:C)*	M + F *embryos*	RNA metabolism / localisation / surveillance / splicing / transcription | ribosomal biogenesis
		High *poly(I:C)* / High *poly(I:C)*	M + F *embryos*	lysosomes | mitochondrial organisation | sialic acid / membrane lipid / glycoprotein metabolism
			M + F	TOR signalling | phagocytosis | morphogenesis | mitochondrial function | metabolic function | cytoskeletal organisation | signal transduction *Synaptic enrichment:* postsynaptic specialisation | synapse organisation | SV membrane / trafficking
			M / F	retrograde endocannabinoid signalling | mitochondrial oxidative phosphorylation | GTPase signalling
			M	long-term potentiation | glutamatergic synapses | metabolism | gliogenesis | myelination
			F	cytoskeletal organisation | dopaminergic synapses | SV / endocytic processes | ribosome
	RBFOX1	*Rbfox1* OE	(M)	↑ total / cytoplasmatic RBFOX1 (PFC / HPC)
		rs6500744 humans	M + F	[nominal ↑ RBFOX1 in risk C- vs TT-allele carriers]
	DIRAS2	*Diras2* HET	(F)	PFC: ↓*Diras2* HPC: ↓*Diras2* AMY: ↓*Diras2* / [↓*mTOR* - vs *KO*] HTH: ↓*Diras2* / [↓*mTOR*] / [↓*Clasp2*] NAcc: [↑*Diras1*] CRB: ↓*mTOR*
		Diras2 KO	(F)	PFC: ↓*Diras2* HPC: ↓*Diras2* / ↑*mTOR* AMY: ↓*Diras2* / [↓*Diras1*] HTH: ↓*Diras2* / ↓*Diras1* NAcc: ↓*Diras2* CRB: [↓*Diras2*] / ↓*Diras1* / ↓*Clasp2* / [↓*Ctdsp2*]

* Includes unpublished data (provided by A. O'Leary) from adult cytoplasmatic-RBFOX1 HET male mice.
** Enrichment analyses summary shown. Abbreviations: *AMY*, amygdala; *CRB*, cerebellum; *FAR*, false alarm rate; *HPC*, hippocampus; *HTH*: hypothalamus; *LWR*, length-to-width ratio; *NAcc*, nucleus accumbens; *PFC*, prefrontal cortex; *PPI*, pre-pulse inhibition; *SV*, synaptic vesicle.

Diras2 might therefore – similarly to *Rbfox1* (see Chapter 5.3) – represent a dose-sensitive gene, which elicits aberrations regardless of the direction of change (Rice & McLysaght, 2017). Additionally, shared phenotypic outcomes might be induced by transient abundance changes that are not measured at these fixed time-points, by protein and gene modifications not influencing expression, or molecules not enriched in synaptoneurosomes – and thus, not measured in this study.

Regarding possible interaction mechanisms, adult HPC synaptoneurosomal proteins were significantly enriched in the GTPase activity category, with different morphology-regulating RAS-family proteins changed in male and females MIA offspring (see Chapter 4.2). Furthermore, there was significant upregulation of the K-RAS-activator RASGRP2 (see Chapter 1.3.3.2) in both embryonic and adult MIA HPC pools, which can lead to disruptions in neuronal growth and synaptic transmission (Ryu *et al.*, 2020). K-RAS and both DIRAS1/2 also share a common upstream regulator (Garcia-Torres & Fierke, 2019), RAP1GDS1 (SmgGDS), which was moderately upregulated in both the embryonic and adult MIA HPC and is a putative target upregulated by cytoplasmatic RBFOX1 (Lee *et al.*, 2016). Another line of evidence of the common pathway theory is the strong MIA-induced downregulation of GTPase-activating proteins in adult HPC. Multiple Rho GTPase activators (ARHGAP) and the ASD-associated Ras GTPase activator - and common RBP target - SYNGAP1 (see Chapter 1.3.1.2, 1.3.3.2) were decreased by MIA in the adult set, suggesting effects on cellular signalling and downstream GTPase transmission. Indeed, the Rho GTPase RHOG, which stimulates neurite outgrowth (Katoh *et al.*, 2000), was strongly downregulated in adult HPC synaptoneurosomes, suggesting a link to the observed changes in morphology, i.e. reduction in females but not males due to the compensatory upregulation of CDC42 in the latter (see also Chapter 4.2). The connection between the two NPD models also extends to shared ADHD-associated targets, e.g. the proteostasis-regulating ubiquilin-1, which was upregulated in embryonic and adult MIA HPC and downregulated by *Diras2* knockdown in HPC neurons *in vitro* (Grünewald *et al.*, 2021).

Importantly, small GTPases and associated proteins were among the 50 proteins affected by MIA at both the prenatal and adult time-points, strengthening the association between the NPD models. These were RHOQ and RANPB9 in the downregulated in embryos and upregulated in adults cluster, and SEPTIN9, which was upregulated at both ages. RHOQ,

known as TC10, is required for membrane dynamics and axonal outgrowth, and the protein's local neurite synthesis is mediated by mTOR (Dupraz *et al.*, 2009; Gracias *et al.*, 2014) - a target of DIRAS2, demonstrated here to be upregulated in the HPC of *Diras2* KO mice. Deletion of RANBP9 leads to neonatal lethality and negatively impacts HPC and cortical growth, while overexpression in adulthood is linked to AD pathology and synaptic loss (Palavicini *et al.*, 2013a; Palavicini *et al.*, 2013b), mimicking the course of MIA-induced protein changes and highlighting this RAS-binding protein as an important target for future studies. SEPTIN9 is a GTPase associated with the cytoskeleton that mediates neurite outgrowth and polarity, as well as directed axo-dendritic transport (Radler & Spiliotis, 2022). In the embryonic rat HPC-derived neurons it localises in proximal dendrites to affects the polarity of dendritic and synaptic protein transport, while *Sept9* deletion causes neonatal lethality and affects the localisation of PSD95 and SYN1 (also MIA-upregulated overlapping protein), among others (Karasmanis *et al.*, 2018). These findings further underscore the importance of GTPases in neuronal function, which appear to be affected by viral-like MIA, and shared NPD-relevant pathways for the two interventions studied here.

The neuron-specific, cytoplasmatic RBFOX1 OE changed the neuroarchitecture of the PFC and HPC (reduced length and branching, overall increased spine density) in a manner different from the morphological profile of the *Diras2* and MIA models (see Table 8). This is in line with the preliminary findings that OE might have anxiogenic effects, opposite to the other two investigated models, and the fact that the knockout was previously shown to induce ASD-like endophenotypes, including anxiety-like behaviours (O'Leary *et al.*, 2022). It would be therefore interesting to see if neuron-specific *Rbfox1* KO might better recapitulate the dendritic and spine changes of the other NPD-like models, or if nuclear RBFOX1 overexpression might reveal different effects.

While preliminary data from low-dose MIA × neuronal cytoplasmatic RBFOX1 OE mice suggested changes in anxiety- and anhedonia-like behaviour (see Table 8), the combined intervention led to increased abortion and neonate mortality rate. While this was most probably the result of construction noise and also the case for RBFOX1 OE without immune activation (see Chapter 5.3), it is still warranted to examine what possible molecular changes might have interacted on a cellular level. In the embryonic HPC protein set, there were 19 proteins, which are known cytoplasmatic RBFOX1 targets (Lee *et al.*, 2016) and of

those - 12 were affected by MIA and RBFOX1 in the same direction. Of those, two proteins were significantly changed (>30%): COL1A1, downregulated by both MIA and cytoplasmatic RBFOX1, has been shown to cause perinatal death in humans with a deletion and embryonic lethality in knockout mice (Löhler *et al.*, 1984; Symoens *et al.*, 2017). COL1A1 is an interesting target, because it was also one of the proteins, which were downregulated by high-dose MIA in both adult and embryonic HPC synaptroneurosomes. The other common protein, the neuropeptide proenkephlin (PENK, upregulated by both interventions) has been shown to increase in the rat HPC following specific, lethal viral infections, and to mediate SUD-linked behavioural deficits after adolescent cannabis exposure – an NPD- and comorbidities-risk factor (Fu *et al.*, 1993; Tomasiewicz *et al.*, 2012). Apart from these two hypotheses, there also remain the following possibilities on a molecular level: the changes that caused lethality occurred in the nucleus or in proteins outside of the synaptoneurosomal compartment; there are still unknown targets of the cytoplasmatic RBFOX1; or multi-target interaction effects caused perinatal loss.

Although RBFOX1 was not among the detected MIA-affected targets in the HPC datasets, this might be due to the timing of the measures, i.e. during periods of lower HPC expression (see Chapter 1.3.2), and the fact that especially in immature neurons, RBFOX1 is enriched outside of the synaptic fraction (Hamada *et al.*, 2013). Since RBFOX1 expression should peak around the time of the investigated prenatal immune challenge (early-/mid-gestation) and in the neonatal and adolescent periods of NPD vulnerability (see Figure 1.6), a consideration of a possible role for RBFOX1 in MIA onsequences remains pertinent. Indeed, of the 50 MIA targets changed in both the prenatal and adult HPC, there were 11 targets, the expression of which is regulated by either cytoplasmatic or total RBFOX1, as well as one (OBSPL3) alternatively spliced target (Lee *et al.*, 2016). Of the 11 proteins, the predicted or experimentally observed RBFOX1-mediated upregulation matched the changes in the embryonic dataset for eight proteins: CBLN2, CD34, DPYSL2, FTH1, IDE, MAPT, SNCB, TTBK1, which were all associated with NPDs and comorbidities and in the case of CBLN2, FTH1, and IDE – with observed social deficits (see also Chapter 4.1.). The presence of the insulin-degrading enzyme IDE, which has been associated with AD and SCZ (Ambrozova *et al.*, 2023), provides another line of evidence for the connection between brain disorders, insulin signalling, and inflammation (see Chapters 1.2.1, 1.3.2). These insights suggest that a transient RBFOX1 change might have occurred subsequently to MIA that may have led to

long-lasting consequences on the synaptic proteome and NPD-relevant behavioural endophenotypes.

Furthermore, RBFOX1 has been shown to downregulate DIRAS1 and upregulate DIRAS2 in neurons *in vitro* (Lee *et al*., 2016), matching the observed MIA-induced changes in HPC synaptoneurosomes, which were most pronounced in the adult females (see Chapter 3.5). This suggests that following MIA, RBFOX1 might affect the two paralogs in a sex-specific manner postnatally, e.g. during the neonate, prepubertal, or postadolescent period when its expression peaks (see Chapter 1.3.2.2). Furthermore, *Diras2* overexpression might be an interesting model for future investigations, in line with the presented *Diras2* KO behavioural and previously published findings from ADHD patients (see Chapter 6.2).

Considering the dynamic interactome of RBFOX1 with other RBPs, it is important to discuss the MIA-caused changes in NPD-relevant RBPs (see Chapter 1.3.1.3) in the HPC proteome. Effects on RBPs of interest included a moderate MIA-induced upregulation of CYFIP2 and a downregulation of FXR1 and multiple paralogs of CELF, ELAVL, and CPEB at both investigated time-points. Furthermore, the NPD-associated, RBFOX1-binding partners FMR1 and PTBP1 were exclusively downregulated by MIA in the embryonic HPC dataset, in line with their neurodevelopmental roles (see Chapter 1.3.1.3 for more). These observations are in keeping with transcriptomic findings from murine embryonic cortices at 2-4 days after a mid-gestation poly(I:C) immune challenge (E12.5): Kalish *et al.* (2021) described MIA-induced, wide-spread dysregulation of genes affecting protein synthesis, including aberrant cytoplasmatic translation, extended here to the synapse-enriched proteome of the foetal HPC.

Apart from downstream and direct interactions between the MIA-affected, RBFOX1, and DIRAS2 proteins, it would be pertinent to look for shared upstream regulators with NPD relevance to address in future studies. Using the Harmonizome database and ENCODE Transcription Factor Targets dataset (Rouillard *et al.*, 2016; The ENCODE Project Consortium, 2011), it was revealed that RBFOX1 and DIRAS2 share 20 common transcription regulators – of those, tow were promising targets for future investigation. One, the chromodomain helicase DNA-binding protein CHD7, is vital for embryonic and brain development and is linked to multiple NDDs and ASD (Feng *et al.*, 2017). CHD7 was downregulated >30% following both low-and high-dose MIA in the embryonic HPC samples,

suggesting a possible link between immune activation and regulation of the investigated genetic targets. The other transcription regulator of interest, histone lysine methyltransferase complex subunit RBBP5, binds all RBFOX and DIRAS paralogs as well as genes encoding shared pathway targets, e.g. BDNF, BIN1, and dozens of Ras / Rho GTPases and associated proteins, according to the Harmonize database. *RBBP5* has been implicated in SCZ risk (Takata *et al.*, 2014) and as a novel NDD-causative gene in humans, while preliminary findings indicate that it disrupt embryonic and brain development in a dose-sensitive manner in the fruit fly model (Huang *et al.*, 2024). While only a nominal (~20%) MIA-induced downregulation of RBBP5 was detected in embryonic HPC, its binding partner RBBP9 was among the proteins significantly upregulated by MIA at both ages and was phenotypically associated with anxiety and depression in the performed PheWAS analysis. Furthermore, significant downregulation of RBBP9 has been demonstrated in HPC of AD-like mice in early adulthood, suggesting a neurodegenerative link (Mori *et al.*, 2023). These hypothetical upstream regulators of RBFOX1, DIRAS2, and MIA-affected proteins warrant further investigations in human populations and animal models.

LIMITATIONS AND OUTLOOK

While the studies presented in this thesis expand upon our knowledge of NPD-relevant prenatal and genetic risk factors using mouse models, there are some limitations to consider. Animal models of NPDs are a valuable tool for the study of psychiatric disorders, especially since the field has been moving toward a more translational mindset. These changes include the use of endophenotypes and more natural environments (Gururajan *et al.*, 2019; Shemesh & Chen, 2023; Silverman *et al.*, 2022; von Mucke-Heim *et al.*, 2023), as well as more encompassing disorder spectrums rather than fixed or granular symptomology considering shared risk and molecular pathways (Adam, 2013; Lee *et al.*, 2019). However, it should be noted that rodents cannot recapitulate all aspects of mental disorders and might have a different response to genetic, environmental, stress, and pharmacological manipulations than humans, so one needs to consider the predictive validity and the reliability of observed core phenotypes to ensure reproducibility (Gururajan *et al.*, 2019).

In the studies performed for this dissertation, group sizes - especially those used to examine the sex-specific effects of MIA on the adult HPC proteome and behavioural deficits in the

RBFOX1 and DIRAS2 transgenic models - should be built upon in the future to increase statistical power. Furthermore, while care was taken to minimise litter effects by careful planning and using all available offspring from as many litters as possible for each condition, these possibilities were limited by German and European animal welfare legislation, as well as the breeding and pup mortality constraints.

To expand upon the current findings, future investigations should address the sex-specific effects in the two genetic models, i.e. extend the morphological analysis, molecular, and behavioural tests to males and females, which were either performed in males or only partially overlapped between the sexes. For the cytoplasmatic RBFOX1 OE study, it might be beneficial to perform more tests on the heterozygous mice to disseminate the RBPs' effects on brain and behaviour. Moreover, a more in-depth molecular investigation of RBFOX1 paralogs, related RBPs, and novel targets highlighted in these studies would be of interest. Similarly, expanding the gene expression analysis performed in *Diras2* females to both sexes, as well as to targets from the other two studies, including GTPases and associated proteins of interest, should be considered.

Since RBFOX1 OE led to too high perinatal lethality when combined even with the low-dose MIA, possible future avenues would be to introduce the immune challenge in heterozygous dams – or to choose a different NPD-relevant, early-life risk factor, such as maternal or early-postnatal stress (e.g., maternal separation, social disruption, enrichment-deprived environment, etc.). Furthermore, in the DIRAS2 study, it might be beneficial to probe the effects of MIA on *Diras2* HET and KO offspring, which exhibited divergent NPD-relevant phenotypes. I further propose a transgenic model of *Diras2* overexpression - or one incorporating a known NPD-relevant human mutation, to help elucidate the role of the gene in psychiatric disorders, considering the presented findings.

Overall, adapting the behavioural test battery for future investigations to include targeted translational tests, e.g. more-challenging cognitive tests such as the continuous performance test and the attentional set-shifting task, can increase the translational value of the findings. However, such paradigms can be time-consuming and require careful planning to ensure consistency, especially regarding the animals' age. Notably, the behavioural studies were performed in mice after reaching adulthood. Considering the outlined role of the investigated manipulations in neurodevelopment and during early-life

vulnerable time-windows, it is advisable to assess possible behavioural, morphological, and molecular changes in the postnatal and adolescent periods, as well as later in life to probe the consequences of aging on the phenotypes.

The MIA study, which encompassed the most in-depth analyses of all three models, might still benefit from follow-up investigations. For example, extending the HPC synaptoneurosomal proteomic and neuromorphological analyses to male and female adult offspring from the low-dose MIA group would allow for a deeper understanding of the dose-dependency of age-specific and -independent changes resulting from the immune challenge. Furthermore, disregarding the financial constraints, adding other relevant time-points, e.g. neonatal, adolescent, and aging, to the proteomic analysis could facilitate further novel insights into the effects of MIA, relevant for treatment target discovery and further elucidating the aetiology of NPDs. It is important to state that the observed findings in the MIA proteomic study do not capture proteins that are enriched outside of the synaptoneurosomes - or in immune brain cells, which have been previously investigated by others (see Chapter 1.2.3).

I propose that early pharmacological interventions and subsequent study of the proteomic and behavioural effects in the high-dose MIA model, including dopaminergic, glutamatergic, and GABAergic receptor-targeting agents could be beneficial, considering the sex-specific and -independent effects of the prenatal immune activation on these pathways. Furthermore, early-life *in vivo* manipulation of targets, selected from the proteomic findings based on NPD-relevance, abundance profile, and interactions with RBFOX1 and DIRAS2 (see above), can benefit novel insights. This can be achieved by multiple different routes, including using viral vectors to manipulate expression, or administering known and novel compounds through local delivery to the brain (Davidson & Breakefield, 2003; Yoon *et al.*, 2022), among other possibilities. Furthermore, investigating upstream regulators common to the three models, such as the transcription regulators described in the previous section, might shed more light on their shared and diverging roles in NPDs.

Expanding each investigation to other relevant brain regions could be beneficial; however, a subregion- or cell-specific analyses might also be considered for follow-up studies building upon the more holistic investigations of these models presented here, which were either novel - or previously untested within our institution. Finally, it should be noted that the

presented findings provide evidence of the effects of each NPD-relevant manipulation on molecular and morphological level, but only infer their functional consequences. Dendritic, spine, gene and protein abundance analyses are static snapshots in time, rather than visualising the dynamic processes of the brain. Thus, others might want to address the functional, electrophysiological, and neural circuit outcomes in each of the presented models. Classic *in vivo* methodologies like functional imaging using two-photon microscopy, PET, and fMRI, or microelectrode arrays and optogenetic probes have been – or are currently being - extended to freely-moving animals. Simultaneously, cutting-edge technologies like chemogenically-activated synthetic receptors and miniature, long-term implanted neuronal probes (Roth, 2016; Steinmetz *et al.*, 2021) can be used to elaborate on the complex functional consequences of prenatal and genetic interventions relevant for NPDs.

CONCLUSION

In conclusion, the effects of MIA and partial *Diras2* knockdown resembled each other in core NPD-associated behavioural and morphological phenotypes, while cytoplasmatic RBFOX1 OE and full *Diras2* KO differed in most aspects. My findings suggested a complex dose- and sex-dependent relationship between these prenatal immune and genetic interventions, whose NPD-relevant influences on the brain might converge onto neurodevelopmental processes and molecular pathways. An assessment of such putative overlap, based on available data and insights from the proteomic analyses of embryonic and adult HPC following MIA, suggested that the three models are linked via downstream targets, direct or indirect interactions, and upstream regulators. Future studies should disseminate both the distinct and shared aspects of MIA, RBFOX1, and DIRAS2 relevant to NPDs and build upon these findings.

Taken together, this dissertation expanded upon the role of prenatal immune challenges and neurodevelopmental genes' dysregulation in NPDs. The behavioural, morphological, and molecular findings offer new translational insights of shared pathways for NPD risk factors and propose novel targets for future aetiological investigations and therapeutic development.

8. BIBLIOGRAPHY

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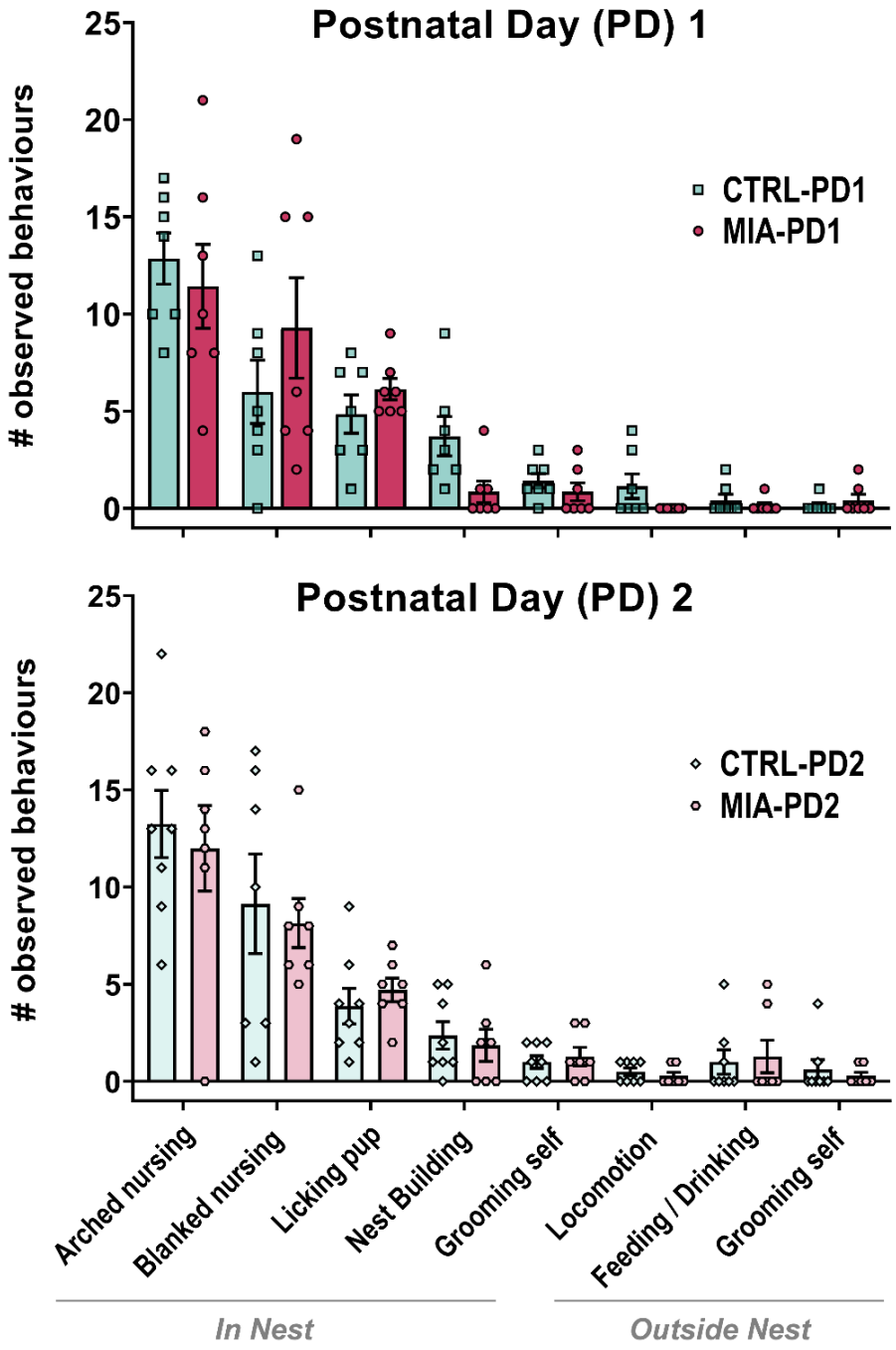
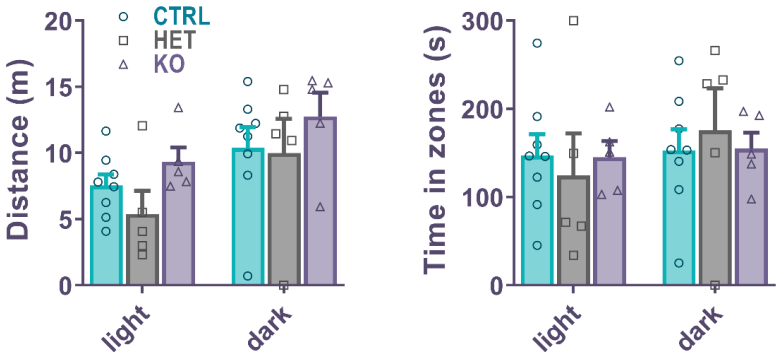
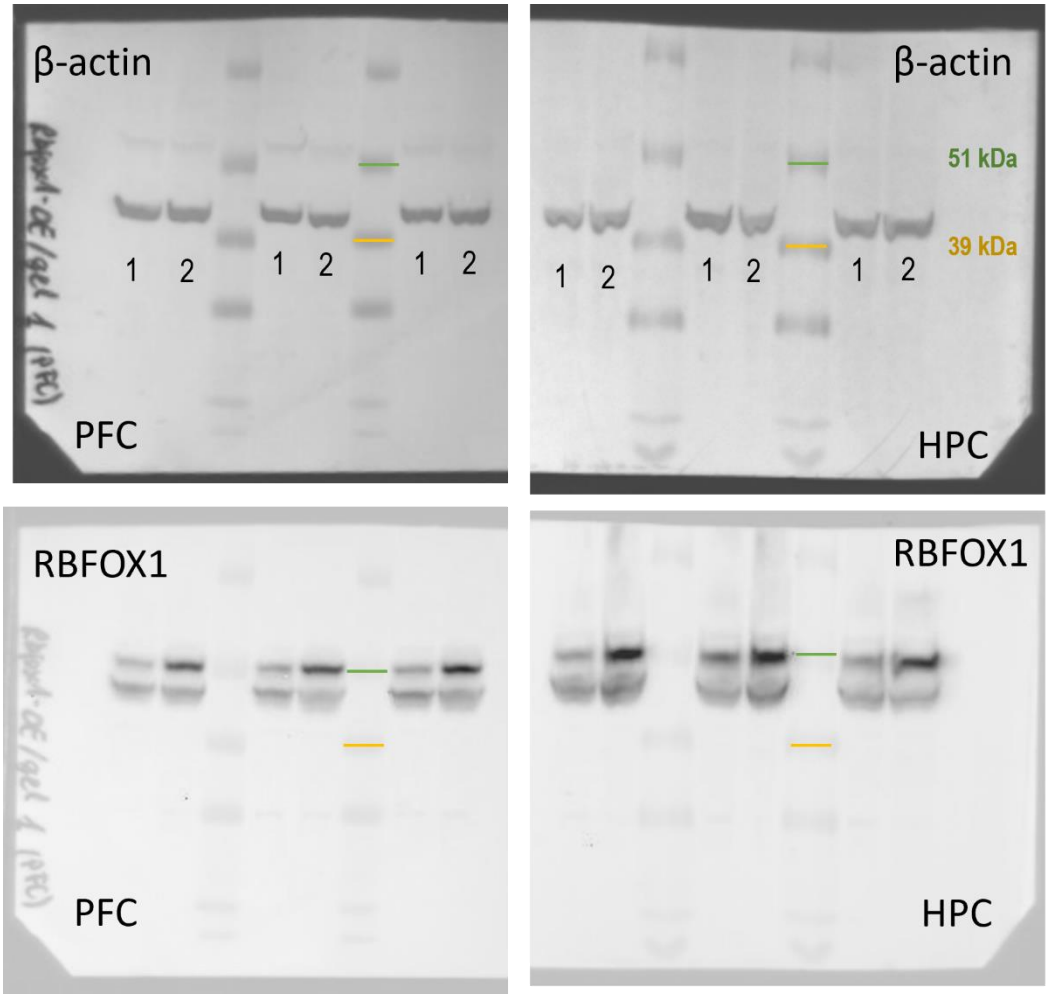
SUPPLEMENTARY DATA



Supplementary Figure S1. Full results from maternal behaviour observations in maternal immune activation (MIA)-exposed and control mice. Observations were performed on the first two postnatal days every 2 min for 60 min (30 observations each) in wild-type mothers, in which MIA was induced on gestational day 9 (2.5 mg/kg poly(I:C) i.v. injection), and controls (PBS 1x vehicle-injected). Although nominal differences were observed between the groups for nest building and locomotor activity outside the nest on the first day, no significant effects of MIA were found (two-way ANOVA, Day 1: *MIA*: p=0.75, *MIA* × *behaviour*: p=0.2; Day 2: *MIA*: p=0.98, *MIA* × *behaviour*: p=0.66; n=7/group). PD as *PND* elsewhere in text. Data presented as mean ± SEM.



Supplementary Figure S2. Results from the light-dark box (LDB) test for neuronal-specific, heterozygous (HET) and homozygous (KO) *Diras2* knockout adult females. The adult mice did not significantly differ in their propensity to spend time in the light compartment or distance travelled based on genotype (two-way ANOVA, *distance*: p=0.17, *time*: p>0.99; CTRL, controls: n=9, HET: n=5, KO: n=5).



Supplementary Figure S3. Full blots for RBFOX1 overexpression validation. Adult hippocampi (HPC, right) and prefrontal cortices (PFC, left) were used for validation from n=3 males/group. Cut lanes on the sides were used for other experiments. In the software, the chemiluminescent image (presented in results) is superimposed on an automatic capture of the visible image to show the molecular weight markers (relevant sizes marked in colour). 1= controls; 2= overexpression mice.

Supplementary Table S1. Mouse numbers for behavioural experiments. *Left*: Maternal immune activation (MIA); *CTRL*: 0 mg/kg poly(I:C), *LOW*: 2.5 mg/kg poly(I:C), *HIGH*: 5 mg/kg poly(I:C). *Right, top*: *Diras2* deletion, heterozygous (*HET*) and knockout (*KO*). *Right, bottom*: cytoplasmatic RBFOX1 overexpression (*OE*) and wild-type (*CTRL*); *VEH*: 0 mg/kg poly(I:C), *MIA*: 2.5 mg/kg poly(I:C); male (*m*), female (*f*).

TEST	MIA	SEX	N
Open field, Nesting	CTRL	m	18
		f	16
	LOW	m	15
		f	18
	HIGH	m	7
		f	8
Light-dark box, Sensorimotor gating	CTRL	m	18
		f	17
	LOW	m	15
		f	18
	HIGH	m	7
		f	8
Elevated-zero maze	CTRL	m	16
		f	18
	LOW	m	14
		f	17
	HIGH	m	7
		f	8
Social interaction	CTRL	m	18
		f	17
	LOW	m	14
		f	18
	HIGH	m	7
		f	7
Y-maze	CTRL	m	17
		f	16
	LOW	m	14
		f	18
	HIGH	m	7
		f	7
Succrose preference	CTRL	m	15
		f	15
	LOW	m	14
		f	18
	HIGH	m	6
		f	8
Swim test	CTRL	m	18
		f	17
	LOW	m	15
		f	10
	HIGH	m	7
		f	8

TEST	DIRAS2	SEX	N
Open field	CTRL	m	18
		f	8
	HET	m	15
		f	5
	KO	m	6
		f	5
Light-Dark box	CTRL	m	24
		f	8
	HET	m	20
		f	5
	KO	m	10
		f	5
Sensorimotor gating	CTRL	m	10
	HET	m	9
	KO	m	4
Continuous performance test	CTRL	m	18
	HET	m	6
	KO	m	13
Fear conditioning, Barnes maze	CTRL	f	8
	HET	f	4
	KO	f	5

TEST	RBFOX1	MIA	N all	m	f
Open field, Light-dark box	CTRL	VEH	17	11	6
		MIA	7	4	3
	OE	VEH	3	1	2
		MIA	2	0	2
Y-maze	CTRL	VEH	16	10	6
		MIA	6	3	3
	OE	VEH	3	1	2
		MIA	2	0	2
Social preference	CTRL	VEH	15	10	5
		MIA	6	4	2
	OE	VEH	3	1	2
		MIA	2	0	2
Saccharine preferencce	CTRL	VEH	15	10	5
		MIA	6	4	2
	OE	VEH	3	1	2
		MIA	2	0	2

Supplementary Table S2. Mouse brain, neurons, and dendritic segment numbers for morphological analysis of the prefrontal cortex (PFC), hippocampus (HPC), and amygdala (AMY) across all experiments. In the MIA study, three additional brains were used for dendritic *vs* for spine analysis (*given in brackets)*. CTRL = controls. MIA = offspring of the 5 mg/kg poly(I:C) group.

Brain Region	***Treatment / Genotype***	***Sex***	***N brains***	***N neurons*** *(for dendrites)*	***N dendrites*** *(for spines)*
Maternal Immune Activation (MIA) Model					
PFC	CTRL	m	3 *(4)*	35	37
		f	3 *(3)*	31	39
	MIA	m	4 *(5)*	48	49
		f	4 *(5)*	44	58
HPC	CTRL	m	4	57	57
		f	4	53	57
	MIA	m	5	68	56
		f	5	67	71
Neuron-specific, Cytoplasmatic RBFOX1 Overexpression (OE) Model					
PFC	CTRL	m	8	80	70
	OE	m	7	71	68
HPC	CTRL	m	6	60	120
	OE	m	6	60	120
AMY	CTRL	m	6	36	72
	OE	m	5	30	64
Neuron-specific, Hetero- (HET) and Homozygous (KO) DIRAS2 Knockout Model					
PFC	CTRL	m	5	49	53
	HET	m	5	50	52
	KO	m	4	40	40
HPC	CTRL	m	8	68	77
	HET	m	8	70	83
	KO	m	7	62	74
AMY	CTRL	m	8	77	71
	HET	m	7	63	60
	KO	m	7	69	61

SELECTED ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
aADHD	Adult ADHD
Ach	Acetylcholine
AD	Alzheimer's disease
ADHD	Attention-deficit/hyperactivity disorder
AKT	serine/threonine kinase (a.k.a. Protein kinase B (PKB), RAC GTPase)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism spectrum disorder
ASR	Acoustic startle response
BD	Bipolar disorder
CA	*cornu Ammonis*, hippocampal subfield designation
cADHD	Childhood ADHD
CD	Conduct disorder
CNV	Copy-number variation
CPT	Continuous performance test
DA	Dopamine
DG	Dentate gyrus of the hippocampus
DIRAS	DIRAS (*distinct subgroup of the RAS*) family GTPase
ERK	Extracellular signal-regulated kinase, encoded by different *MAPK* genes
Ex	Embryonic day X
EZM	Elevated zero maze
FAR	False alarm rate in CPT
FST	Swim test
FXS	Fragile X Syndrome
GABA	Gamma-aminobutyric acid
GAP	GTPase activating protein
GD	Gestational day
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide exchange factor
Glu	Glutamate
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GWAS	Genome-wide association study
HET	Heterozygous
HPC	Hippocampus
HR	Hit rate in CPT
ID	Intellectual disability
ITI	Inter-trial interval
LDB	Light-dark box

LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
LTP	Long-term potentiation
LWR	Length-to-width ratio
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MIA	Maternal immune activation
miRNA	Micro-RNA
mRNA	Messenger RNA
MTOR	mammalian target of rapamycin
NDD	Neurodevelopmental disorder
NMDA	N-methyl-D-aspartate
NPD	Neurodevelopmental psychiatric disorder
OCD	Obsessive-compulsive disorder
OE	Overexpression
OF	Open Field
pcw	Post-conception weeks
PD	Parkinson's disease
PFC	Prefrontal cortex
PheWAS	Phenome-wide association study
PheWAS	Phenome-wide association study
PI3K / PIK3	Phosphoinositide 3-kinase
PNDx	Postnatal day X
Poly(I:C)	Polyinosinic:polycytidylic acid
PPI	Pre-pulse inhibition
PTSD	Post-traumatic stress disorder
RAS	Rat sarcoma protein family
RASopathy	Syndrome caused by gen. mutation along RAS/MAPK signalling pathway
RBFOX1	RNA Binding Fox-1 Homolog 1
RBP	RNA-binding protein
RI	Responsivity index in CPT
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCZ	Schizophrenia
SI	Sensitivity index in SPT
SNP	Single-nucleotide polymorphism
SP %	Sucrose preference
SUD	Substance use disorder
tRNA	Transfer RNA

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