Effekte von chronischer Hyperhomocysteinämie und spezifischen Mikronährstoff-Interventionen auf die kognitive Leistungsfähigkeit im *App*$^{NL\text{-}G\text{-}F}$ knock-in Mausmodell der Alzheimer-Erkrankung

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„A model is a lie that helps you see the truth“

– Howard Skipper

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Abkürzungen

AD	Alzheimer-Erkrankung
Aβ	Amyloid-β
APP	Amyloid-Precursor-Protein
ApoE	Apolipoprotein E
sAPP	soluble APP
CTF-β	C-terminal fragment β
AICD	APP intracellular domain
HCys	Homocystein
HHCys	Hyperhomocysteinämie
HCA	Homocysteinsäure
NMDA	N-methyl-D-aspartat
ROS	reaktive Sauerstoffspezies
SAM	S-Adenosyl-L-Methionin
SAH	S-Adenosyl-L-Homocystein
PUFA	vielfach ungesättigte Fettsäuren
DHA	Docosahexaensäure
EPA	Eicosapentaensäure
ELISA	Enzyme-linked Immunosorbent Assay
CSF	Cerebrospinalflüssigkeit
WT	Wildtyp
KI	knock-in
IQR	Interquartilsabstand
LC-MS/MS	Flüssigchromatographie mit Tandem-Massenspektrometrie-Kopplung
PAL	Paired Associates Learning Test
ROI	Region of Interest
ZNS	Zentralnervensystem
LLOQ	unteres Quantifizierungslimit
HGF	Hepatocyte Growth Factor
TNR	Tenascin R
CNTN1	Contactin-1
CCL3	C-C Motif Chemokine Ligand 3
GFRA1	GDNF Family Receptor alpha-1
TPP1	Tripeptidyl-peptidase 1

ERBB4	Receptor tyrosine-protein kinase erbB-4
DLL1	Delta-like protein 1
IL1a	Interleukin 1 alpha
CCL2	„C-C Motif Chemokine Ligand 2“
FAS	Tumor necrosis factor receptor superfamily member 6
TNFRSF12A	Tumor necrosis factor receptor superfamily member 12A
TGFBR3	Transforming growth factor beta receptor type 3
DLK1	Protein delta homolog 1
ACVRL1	Serine/threonine-protein kinase receptor R3
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B
FSTL3	Follistatin-related protein 3
WISP1	WNT1-inducible-signaling pathway protein 1

Gruppenbezeichnungen:

C (WT)	Wildtyp-Kontrolle
C (KI)	knock-in-Kontrolle
B-DEF	Vitamin B defizient
B-ENR	Vitamin B angereichert
PUFA-ENR	PUFA supplementiert
B+PUFA-ENR	Vitamin B angereichert und PUFA supplementiert
FC	„Fortasyn® Connect“
B-DEF+PUFA-ENR	Vitamin B defizient und PUFA supplementiert
B-DEF+BET-ENR	Vitamin B defizient und Betain supplementiert

Inhalt

Einleitung

Alzheimer-Erkrankung und -Modelle

Auch nach jahrzehntelanger intensiver Forschung gibt es nach wie vor einen großen medizinischen Bedarf an neuen Interventionen im Bereich der demenzartigen Erkrankungen. Weltweit leben um die 50 Millionen Menschen mit einer Demenz und es wird prognostiziert, dass die Prävalenz aufgrund der älterwerdenden Bevölkerung auf schätzungsweise über 130 Millionen im Jahr 2050 ansteigen wird (Prince *et al.*, 2016). Krankheitsbilder des Überbegriffs Demenz werden gar als größte globale Herausforderung für Gesundheit und Pflege des 21. Jahrhunderts genannt (Livingston *et al.*, 2017). Dabei gehen 2/3 aller Demenz-Fälle auf die neurodegenerative Alzheimer-Erkrankung (AD) zurück, welche sich anfangs insbesondere durch Erinnerungslücken an rezente Ereignisse ausdrückt und mit zunehmendem Fortschreiten auch Symptome umfasst wie Desorientierung, Verwirrung, Stimmungsschwankungen, Sprach- und Schluckschwierigkeiten, Verhaltensveränderungen sowie die zunehmend schwerwiegendere Einschränkung kognitiver Fähigkeiten, was das tägliche Leben der Patienten und ihrer Umgebung substanziell beeinflusst (Winblad *et al.*, 2016).

Die komplexe AD-Pathologie setzt sich aus mehreren prominenten Charakteristika zusammen. Insbesondere sind hier die typischen und seit Jahrzehnten bekannten Amyloid-Plaques und neurofibrillären Tangles im Hirn zu nennen (Masters *et al.*, 1985; Grundke-Iqbal *et al.*, 1986). Die Tangles sind das Produkt einer abnormalen Phosphorylierung des Tau-Proteins, welches physiologisch durch seine Mikrotubuli-Bindung zur axonalen Stabilität beiträgt, wie von Blennow und Kollegen in einem Review zusammengefasst (Blennow, de Leon and Zetterberg, 2006). Bei AD resultiert eine pathophysiologische Hyperphosphorylierung des Tau-Proteins, bedingt durch ein Ungleichgewicht in der Aktivität der beteiligten Kinasen und Phosphatasen, in der Ablösung von den Mikrotubuli und deren Zerfall sowie der damit einhergehenden eingeschränkten axonalen und synaptischen Funktion und schließlich dem Niedergang von Neuronen. Das hyperphosphorylierte Tau-Protein selbst aggregiert zu unlöslichen Fibrillen, den charakteristischen neurofibrillären Tangles. Ähnlich dazu sind auch die Amyloid-β (Aβ) Ablagerungen Ergebnis der Aggregation von Peptiden, die in diesem Fall die AD-typischen extrazellulären Plaques bilden. Aβ ist ein Produkt der Spaltung des transmembranären Amyloid-Precursor-Proteins (APP) mittels β- und γ-Sekretase. Auch im Falle von Aβ ist dessen

auf- und abbauender Metabolismus, bzw. Abtransport, im Rahmen der AD-Pathologie gestört. Fehlerhafte Beseitigung der Peptide ist insbesondere auch auf eine gestörte Apolipoprotein-Funktion (ApoEε4) zurückzuführen, was einen der größten Risikofaktoren für die sporadische AD darstellt (Blennow, de Leon and Zetterberg, 2006; Liu *et al.*, 2013). In der hier vorliegenden Dissertation steht die Amyloid-Pathologie im Fokus, die zum einen bereits sehr früh im zeitlichen Ablauf des Krankheitsgeschehens zu Tage tritt (Bateman *et al.*, 2012) und zum anderen die Grundlage für das hier untersuchte AD-Mausmodell darstellt, wie im Folgenden beschrieben.

Tiermodelle für AD im Speziellen – aber auch generell – simulieren lediglich einen Teil der, in der Regel sehr viel komplexeren, humanen Pathologie (Zahs and Ashe, 2010). Auf der zuvor beschriebenen Amyloid-Pathologie basiert eine Vielzahl unterschiedlicher, hauptsächlich transgener Mausmodelle für AD. Wissenschaftler des japanischen RIKEN-Instituts sehen ein großes Problem der AD-Forschung im Mangel an geeigneten Tiermodellen begründet und haben zu diesem Zweck eine neue Generation von Modellen wie das App^{NL-G-F} knock-in Mausmodell entwickelt mit dem Ziel, regelmäßig auftretenden Problemen der älteren transgenen Modelle zu begegnen (Saito *et al.*, 2014). Diese Probleme umfassen, unter anderem, große auftretende Varianzen in der Expression des Transgens zwischen verschiedenen Mauslinien oder Zeitpunkten, genauso wie Ausschaltung von Genen im Host-Genom aufgrund der Insertion des Transgens in den entsprechenden Gen-Locus. Durch die Vermeidung einer massiven Überexprimierung von APP im KI Modell soll insbesondere die Entstehung von artifiziellen Phänotypen verhindert werden, die durch die Überproduktion von anderen APP-Spaltungsprodukten neben Aβ bedingt sein können (Bsp. sAPP, CTF-β, AICD). Die APP-Sequenz im hier untersuchten App^{NL-G-F} KI Modell weist drei gezielte Mutationen auf: die „Swedish“ (NL)-, „Arctic“ (G)- und „Beyreuther/Iberian“ (F)-Mutation. Auf diese Art soll ein ausgeprägterer Phänotyp induziert werden, dadurch dass die Gesamtmenge an gebildetem Aβ- und der Aβ42/Aβ40-Ratio erhöht wird, sowie pro-inflammatorische Effekte verstärkt werden und so letztendlich Einschränkungen des Erinnerungsvermögens bereits in einem niedrigeren Alter der Tiere auftreten. Durch die Nutzung einer humanisierten Aβ-Sequenz soll zudem die Ausbildung der charakteristischen Plaques in diesem Mausmodell realistischer simuliert werden im Hinblick auf die humane Erkrankung. Der gestörte Aβ Metabolismus im App^{NL-G-F} Modell soll zu einer aggressiven Amyloidose und cerebralen Ablagerungen, beginnend im Alter von zwei Monaten, führen (Saito *et al.*, 2014).

Erhöhte Level von Homocystein (HCys) werden immer wieder in Verbindung mit AD gebracht (Lai *et al.*, 2017) und wurden bereits als ein weiteres Charakteristikum der Erkrankung genannt (Zhao *et al.*, 2018). Die Normalisierung erhöhter HCys-Level wurde daher auch als ein möglicher Ansatzpunkt zur AD-Prävention vorgeschlagen (Oikonomidi *et al.*, 2016). Durch die experimentelle Erhöhung der HCys-Level in der hier vorliegenden Arbeit wurde das Ziel verfolgt, die AD-Pathologie facettenreicher in den KI-Mäusen zu simulieren, indem zwei Ansätze kombiniert wurden: Zum einen bildet der gesteigerte Aβ-Anabolismus ein Charakteristikum der seltener auftretenden, frühen, „familiären" AD-Form ab (Sasaguri *et al.*, 2017), zum anderen tragen erhöhte HCys-Spiegel potentiell zur häufiger auftretenden, späten, „sporadischen" AD-Form bei, da diese insbesondere in älteren Menschen anzutreffen sind und auch mit dem Alter ansteigen (Selhub *et al.*, 1993; Agrawal *et al.*, 2015).

Hyperhomocysteinämie

Die endogene, schwefelhaltige, nicht-proteinogene Aminosäure HCys ist ein zentraler Baustein des Stoffwechsels von molekularen Gruppen mit nur einem Kohlenstoffatom (C1 Metabolismus). Methylierungsreaktionen des C1-Stoffwechsels sind essentiell für vielfältige physiologische Prozesse, wie beispielsweise die Synthese von Nukleinsäuren, Proteinen und Neurotransmittern (Kim *et al.*, 2018). Die Remethylierung von HCys zu Methionin und die Transsulfurierung (siehe Abbildung 1) stellen dabei die hauptsächlichen Abbauwege des HCys dar (Finkelstein, 2000). Im Gegensatz zur Metabolisierung ist die renale Exkretion von unverändertem HCys gering (Guttormsen *et al.*, 1996).

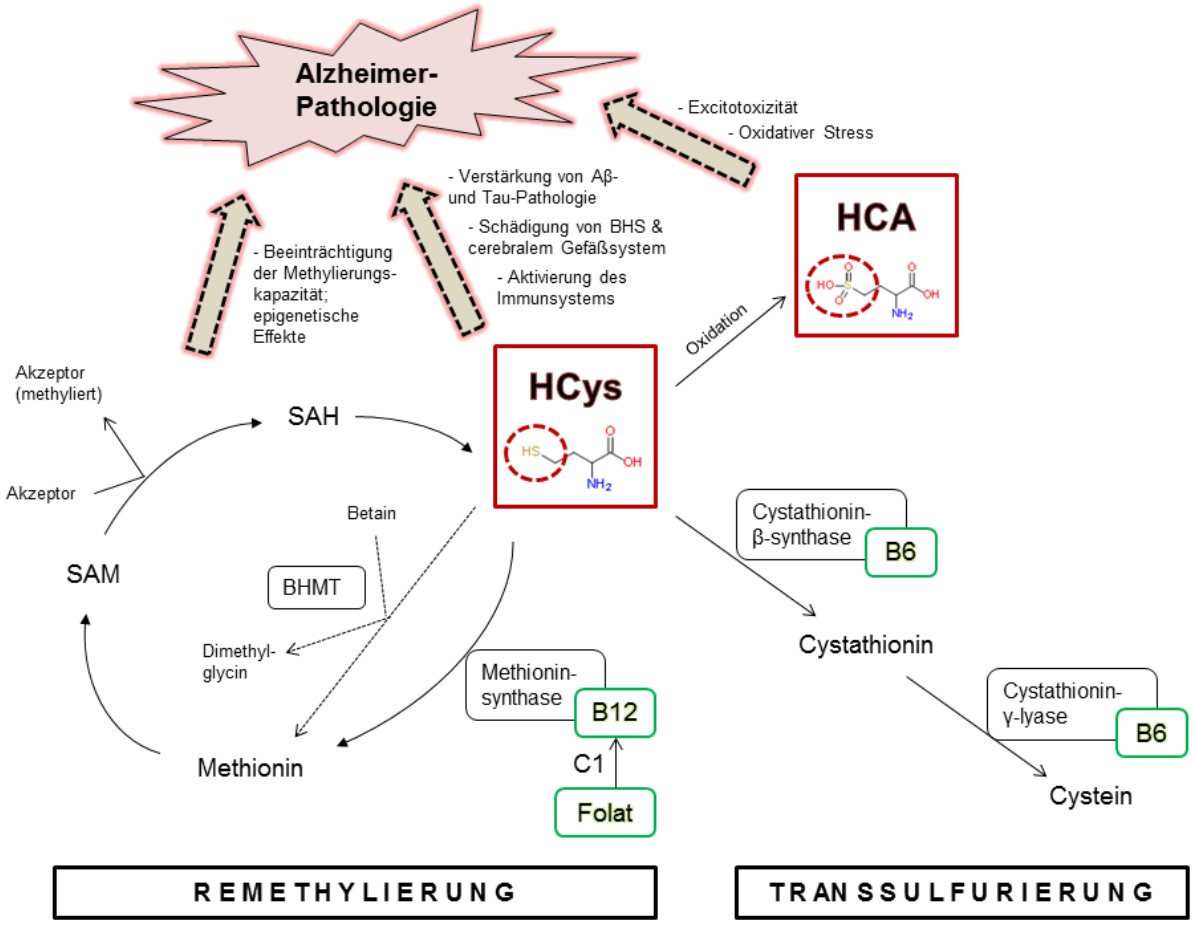

Abbildung 1. Schematisches Schaubild zu Homocystein (HCys) und Homocysteinsäure (HCA): metabolische Rolle und Link zu Alzheimer; Aktivität involvierter Enzyme (dunkle Boxen) abhängig von relevanten B-Vitaminen (grüne Boxen), die als Co-Enzyme und Methylgruppen (C1) Donoren fungieren; BHS: Blut-Hirn-Schranke; SAM: S-Adenosyl-L-Methionin; SAH: S-Adenosyl-L-Homocystein; BHMT: Betain-Homocystein-Methyltransferase (in manchen Geweben vorkommend); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Erhöhte Blutspiegel von HCys (Hyperhomocysteinämie, HHCys) können, genau wie AD, familiär bedingt- oder erworben sein, wie an anderer Stelle zusammengefasst wurde (Kim *et al.*, 2018). Bei der Entstehung der HHCys sind insbesondere in diesem Kontext relevante Vitamine der B-Reihe, vor allem B6, B12 und Folat (siehe Abbildung 1) hervorzuheben, welche in einem antiproportionalen Verhältnis zu HCys stehen (Selhub *et al.*, 1993; Smith *et al.*, 2010). Die verschiedenen experimentellen Induktionsmethoden einer HHCys im Tier sind an die Ursachen für dieser Störung im Menschen angelehnt, eine Thematik, die auch in einem Review adressiert wird, der im Rahmen dieser Arbeit erstellt wurde (Nieraad, Pannwitz, *et al.*, 2021). Neben genetischen Modifikationen, die einen Einfluss auf die Aktivität relevanter Enzyme des C1-Metabolismus wie der Cystathionin-β-Synthase und der Methylentetrahydrofolat-Reduktase haben, stellen, neben der Injektion bestimmter Substanzen, vor allem diätetische Ansätze eine Option zur Induktion einer HHCys im Versuchstier dar. In den dieser Arbeit zugrunde liegenden Studien wurden die HCys-Spiegel in vivo mittels der Fütterung einer Experimentaldiät erhöht, was hauptsächlich auf die Defizienz an Vitamin B6, B12 und Folat zurückgeht und durch den Zusatz von Sulfathiazol zusätzlich verstärkt wurde. Da Mäuse zu Koprophagie neigen, wird das Sulfonamid-Antibiotikum in solchen Studien eingesetzt, um die Folsäureproduktion durch Darmbakterien zu unterbinden (Dayal and Lentz, 2008).

Bei HCys ist der „freie“ Anteil vom Gesamtanteil zu unterscheiden. Ein großer Teil des HCys liegt proteingebunden oder dimerisiert und lediglich ca. 1% mit freier Thiolgruppe in der Seitenkette vor (Isobe *et al.*, 2005). In den durchgeführten Studien im Rahmen der hier vorliegenden Arbeit wurde stets der Gesamtanteil an HCys gemessen, indem ein reduzierendes Agens bei der (prä-) analytischen Methode eingesetzt wurde (genauer im Anhang). Die Gefahr bei Entnahme und Prozessieren von Blut für HCys-Messungen liegt darin, dass durch zu langsames oder inakkurates Vorgehen HCys aus den Erythrozyten freigesetzt wird, was die Plasmaspiegel ansteigen lässt und verfälscht (Nichols, 2017).

Neben HCys ist auch dessen oxidativer Metabolit, die Homocysteinsäure (HCA), zu nennen, welche aus freiem HCys entstehen kann. HCA steht im Verdacht, das neurotoxischere Agens als HCys selbst zu sein (Görtz *et al.*, 2004; Sommer *et al.*, 2004; Vladychenskaya, Tyulina and Boldyrev, 2006), weshalb HCA in den dieser Arbeit zugrundeliegenden in vivo Studien jeweils miterfasst wurde.

Die seit vielen Jahren beforschte HHCys, anfangs hauptsächlich im kardio-vaskulären Bereich (McCully and Ragsdale, 1970), ist mit der Zeit als (Mit)schuldiger bei einer zunehmenden Anzahl an humanen Erkrankungen und vielseitigen Störungen in den Fokus gelangt, so auch im Bereich der kognitiven Störungen und neurologischen Krankheiten wie AD (siehe eigenen Review zur Thematik (Nieraad, Pannwitz, *et al.*, 2021)), ein Zusammenhang, der in dieser Arbeit im Mittelpunkt steht. Frühere Untersuchungen wiesen erhöhte HCys-Level in AD-Patienten auf, bei gleichzeitig erniedrigten Spiegeln an Vitamin B12 und Folat (Clarke *et al.*, 1998; Refsum and Smith, 2003; Isobe *et al.*, 2005); bei Vitamin B6 erscheint die Sachlage etwas unklarer (Lopes da Silva *et al.*, 2014). Dennoch ist die Frage nicht endgültig geklärt, ob auch ein kausaler Zusammenhang besteht.

Ein gestörter HCys Metabolismus (Abbildung 1) steht im Verdacht, über direkt- und indirekt neurotoxische Wirkungen zur AD-Pathologie beizutragen, wie in einem Review zu diesem Thema dargestellt (Smith and Refsum, 2016). Demnach wird die Neurotoxizität über excitotoxische Effekte via NMDA-Rezeptoraktivierung und durch erhöhte Level hochreaktiver Sauerstoffspezies (ROS) ausgeübt. Darüber hinaus ist ein mit der HHCys verbundener Mangel an S-Adenosyl-L-Methionin (SAM), sowie ein Anstieg an S-Adenosyl-L-Homocystein (SAH), assoziiert mit einer reduzierten Methylierungskapazität und der Inhibierung vielfältiger Methylierungsreaktionen, was wiederum über epigenetische Mechanismen zu einer Verschlimmerung der Amyloid- und Tau-Pathologie beitragen könnte. Daneben kann HCys in einer Aktivierung von Immunzellen resultieren und (cerebrale) Gefäße schädigen. Die Blut-Hirn-Schranke scheint es nicht nur zu passieren (Streck *et al.*, 2002), sondern auch zu schädigen (Kamath *et al.*, 2006).

Mit verschiedenen Mikronährstoffen kann möglicherweise Einfluss genommen werden auf HHCys und/oder AD.

Experimentelle Diäten und spezielle Mikronährstoffe

Mehrere hundert gescheiterte Arzneistoffkandidaten im Bereich AD während der letzten Jahrzehnte (Sasaguri *et al.*, 2017) haben den Fokus auch auf Ansätze wie Kombinationstherapien und präventive Optionen gelenkt (Fan and Chiu, 2014). Ein solcher, möglicherweise präventiver Ansatz ist auch Gegenstand der hier vorliegenden Arbeit.

Unter verschiedenen Mikronährstoffen sind insbesondere die weiter oben bereits anmoderierten B-Vitamine (vor allem B6, B12, Folat) aufgrund ihrer Fähigkeit zur Normalisierung eines hyperhomocysteinämischen Zustandes in der Diskussion als potentielle diätetische Präventionsmöglichkeit einer AD, bzw. des Übergangs von einer milderen kognitiven Einschränkung zu AD. Das verdeutlicht die Notwendigkeit eines Tiermodells, das die sehr frühe, präklinische Phase der AD simuliert (Zahs and Ashe, 2010). Folglich ist die Wahl des Modells in der hier vorliegenden Arbeit auf ein Amyloid-basiertes Mausmodell gefallen, da ein gestörter Aβ Metabolismus ein sehr frühes Charakteristikum darstellt, das bereits Jahrzehnte vor den ersten kognitiven Symptomen in den Patienten startet (Bateman *et al.*, 2012). Die Senkung der HCys-Level kann in manchen Geweben des humanen Organismus auch via Betain stattfinden (siehe Abbildung 1). Betain, aufgrund seiner molekulären Struktur auch als Trimethylglycin bezeichnet, stellt eine Vitamin B-unabhängige Option der Methylierung dar und vermag auf diese Weise über das Enzym Betain-Homocystein-Methyltransferase das Homocystein in Methionin umzuwandeln, insbesondere in Leber und Niere (Zhao *et al.*, 2018). Die diätetische Betain-Supplementierung mit dem Ziel der Abmilderung einer HHCys fand sowohl in der hier vorliegenden Arbeit als auch teilweise bereits in früheren Tierstudien Anwendung (Liu *et al.*, 2012; Gupta, Wang and Kruger, 2016).

Im Einklang mit einem internationalen Konsens-Statement finden in der hier vorgestellten Arbeit neben B-Vitaminen auch andere Mikronährstoffe, wie vielfach ungesättigte Fettsäuren (PUFA), Anwendung im Hinblick auf HHCys als einen potentiellen, beeinflussbaren Risikofaktor für Demenz in Älteren (Smith *et al.*, 2018). PUFA, insbesondere DHA und EPA (Docosahexaensäure, Eicosapentaensäure) stehen im Verdacht, vorteilhafte Effekte für die kognitive Funktion generell – und AD-bezogene Pathologie im Speziellen – auszuüben (Janssen *et al.*, 2015; Grimm, Michaelson and Hartmann, 2017). Wie von Grimm und Kollegen zusammengefasst, spielen diese PUFA eine wichtige Rolle im Hinblick auf Neuroinflammation, Membranfluidität und synaptische Plastizität (Grimm, Michaelson and

Hartmann, 2017) und wurden daher in den hier vorgestellten in vivo Studien implementiert (Verhältnis EPA/DHA 1:4).

Da Studien zu Einzelnährstoffen häufig gescheitert sind, könnten Kombinationsansätze die vielversprechendere Option darstellen (Jansen *et al.*, 2013). Beispielsweise weist eine frühere Untersuchung darauf hin, dass eine B-Vitamingabe nur vorteilhaft ist, wenn die Plasmakonzentrationen an PUFA im oberen Normbereich liegen (Oulhaj *et al.*, 2016). Daher wurden die Monodiäten bestehend aus entweder PUFA, Betain oder hochdosierten B-Vitaminen in der vorliegenden Arbeit um verschiedene Kombinationsdiäten ergänzt. Dazu zählt neben einer Kombination von B-Vitaminen mit PUFA auch die komplexere Mikronährstoffmixtur „Fortasyn® Connect“ (Souvenaid®). Fortasyn® Connect (FC) umfasst einige zusätzliche Komponenten wie zum Beispiel Uridin, Selen und Vitamin E, die in dieser Kombination mit einer verbesserten neuronalen Funktionalität in Verbindung gebracht werden und die aufgrund zuvor berichteter positiver Funde bezüglich kognitiver Fähigkeiten hier implementiert wurden (Jansen *et al.*, 2013; Wiesmann *et al.*, 2016).

Abschließend sind im Folgenden (Tabelle 1) die genauen Futterzusammensetzungen aller in dieser Arbeit genutzten Experimentaldiäten aufgeführt.

*Tabelle 1. Zusammensetzung der Experimentaldiäten (AIN93M-basiert); die Spaltenbezeichnungen orientieren sich an den Abkürzungen für die Gruppen in den einzelnen Studien; * bei „PUFA-ENR" ist zu beachten, dass diese Diät für in vivo Studie 3 zusätzlich B-vitamindefizient modifiziert wurde (analog zu „B-DEF"); TBHQ: 2-**tert**-Butylhydrochinon; UMP: Uridinmonophosphat; Tabelle modifiziert nach* (Nieraad *et al.*, 2020).

	Kontrolle	**B-DEF**	**B-ENR**	**PUFA-ENR ***	**B+PUFA-ENR**	**FC**	**B-DEF+ BET-ENR**
Casein	140,0	140,0	140,0	140,0	140,0	140,0	140,0
Kornstärke	355,66	345,69	355,48	355,66	355,48	328,34	335,69
Maltodextrin	155,0	155,0	155,0	155,0	155,0	155,0	155,0
Sucrose	100,0	100,0	100,0	100,0	100,0	100,0	100,0
Dextrose	100,0	100,0	100,0	100,0	100,0	100,0	100,0
Cellulose	50,0	50,0	50,0	50,0	50,0	50,0	50,0
Mineral-Prämix	35,0	35,0	35,0	35,0	35,0	35,0	35,0
Vitamin-Prämix (ohne B-Vitamine)	10,0	10,0	10,0	10,0	10,0	10,0	10,0
Sojabohnenöl	19,0	19,0	19,0	——	——	——	19,0
Kokosnussöl	9,0	9,0	9,0	11,3	11,3	11,3	9,0
Kornöl	22,0	22,0	22,0	18,7	18,7	18,7	22,0
Fischöl (EPA/DHA 1:4)	——	——	——	20,0	20,0	20,0	——
L-Cystin	1,8000	1,8000	1,8000	1,8000	1,8000	1,8000	1,8000
TBHQ	0,0080	0,0080	0,0080	0,0080	0,0080	0,0080	0,0080
Cholinbitartrat, 41%	2,5000	2,5000	2,5000	2,5000	2,5000	2,5000	2,5000
Pyridoxin-HCl	0,0070	——	0,1000	0,0070	0,1000	0,0398	——
Cyanocobalamin, 0.1%	0,0250	——	0,1000	0,0250	0,1000	0,0600	——
Folsäure, 80%	0,0025	——	0,0125	0,0025	0,0125	0,0100	——
Natriumselenit • 5 H_2O, 30%	——	——	——	——	——	0,0036	——
Cholinchlorid, 43%	——	——	——		——	6,9700	——
Ascorbinsäure, 100%	——	——	——	——	——	1,6000	——
DL-a-Tocopheryl-acetat, 50%	——	——	——	——	——	4,6500	——
UMP-Dinatrium (24% H_2O)	——	——	——	——	——	10,0000	——
Sojalecithin	——	——	——	——	——	4,0200	——
Sulfathiazol-Natrium	——	10,0000	——	——	——	——	10,0000
Betain	——	——	——	——	——	——	10,0000
Summe	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**

Ziele der Dissertation

Das primäre Ziel dieser Arbeit ist die Untersuchung von Effekten der langzeitdiätetischen Supplementierung mit bzw. Defizienz in spezifischen Mikronährstoffen auf die kognitive Leistungsfähigkeit im Kontext der AD. Für diese Untersuchungen herangezogen wurde das bis dato noch wenig charakterisierte *App*$^{NL-G-F}$ knock-in Mausmodell, das in einigen Aspekten eine Innovation gegenüber älteren transgenen Modellen darstellt und welches im Zuge der Arbeit weiter charakterisiert werden soll.

Der hauptsächliche Fokus liegt auf der HHCys, induziert durch diätetische Defizienz an Vitamin B6, B12 und Folat, und ihrem potentiell negativen Effekt auf die im Tiermodell simulierte AD-ähnliche Pathologie. Dabei wurde in allen Teilstudien neben HCys auch der oxidative Metabolit HCA untersucht, welcher im Verdacht steht, neurotoxischer zu sein als HCys selbst, eine Hypothese, die in dieser Arbeit ebenfalls untersucht wird.

Darüber hinaus sollte die Funktion von möglicherweise vorteilhaften Mikronährstoffinterventionen, bestehend aus Supplementierungen mit B-Vitaminen, PUFA, FC oder Betain, hinsichtlich kognitiver Performance und Verschlechterung der Tiere bewertet werden.

Die oben genannten Fragestellungen sollten dazu in umfangreichen in vivo Studien untersucht werden mittels einer vielseitigen Verhaltenstestbatterie, die eine umfassende Charakterisierung unterschiedlicher kognitiver Domänen der Tiere erlaubt. Ergänzt wurden die Verhaltensversuche durch diverse ex vivo Analysen. Dazu zählen die Analyse von HCys und HCA in unterschiedlichen biologischen Matrices, die immunhistochemische Visualisierung der Aβ Ablagerungen im Hirn und ELISA-basierte Quantifizierung des löslichen und unlöslichen cerebralen Aβ, eine Vollblutanalyse sowie eine Proteomanalyse im Serum und in der Cerebrospinalflüssigkeit (CSF) der Tiere.

Des Weiteren sollte die Kinetik der genannten Metabolite während Fütterungsperioden mit unterschiedlichen Experimentaldiäten beobachtet werden und im Zuge dessen Fragestellungen beantwortet werden, wie: Gibt es hierbei einen Einfluss des *App*$^{NL-G-F}$ knock-in Genotyps? Ist ein Geschlechter-abhängiger Effekt zu beobachten? Steigen HCys- und HCA-Level auch in cerebralen Geweben an? Korrelieren diese Level zwischen verschiedenen biologischen Matrices?

Bereits publizierte Daten aus früheren Forschungsarbeiten zu dieser Thematik weisen keine konsistenten Effekte von HHCys und interventionellen Diäten im Kontext der neurologischen Erkrankungen auf. Zentrale Fragestellungen sind noch immer unbeantwortet. Somit sollten die in vivo Studien, die dieser Arbeit hier zugrunde liegen, weitere Aufklärung liefern und dadurch möglicherweise den Bedarf an, bzw. Ansatzpunkte für weitergehende Untersuchungen aufzeigen.

Ergebnisse und Diskussion

Im Hauptteil dieser kumulativen Dissertation werden die einzelnen durchgeführten in vivo Studien im Folgenden chronologisch abgehandelt und gegenseitig in Zusammenhang gesetzt sowie im Kontext der Literatur zu der Thematik diskutiert.

In vivo Studie 1

Die Grundfragestellungen dieser Tierstudie spiegeln sich in der Zusammensetzung der experimentellen Gruppen (siehe Tabelle 2) und der im Rahmen der statistischen Auswertung angestellten Gruppenvergleiche wider. Der Gruppenvergleich 2 versus 1 zeigt potentielle Unterschiede zwischen den C57BL6/J Wildtyp (WT) Mäusen und den gleichaltrigen *App*$^{NL\text{-}G\text{-}F}$ knock-in (KI) Mäusen auf, während durch den Vergleich zwischen Gruppe 3 und 2 ein möglicherweise negativer Effekt der im AD-Mausmodell zusätzlich induzierten HHCys sichtbar gemacht werden soll und der Vergleich der Gruppen 4-7, jeweils gegen die KI-Kontrolle (Gruppe 2), auf potentiell vorteilhafte Effekte der diätetischen Interventionen abzielt.

Tabelle 2. Details zu den experimentellen Gruppen (in vivo Studie 1); insgesamt 112 Tiere aufgeteilt auf sieben Gruppen in zwei aufeinanderfolgenden Kohorten, jeweils zur Hälfte männlich und weiblich; Tabelle modifiziert nach (Nieraad *et al.*, 2020).

Gruppe	Genotyp	Experimentaldiät	Abkürzung
1	C57BL/6J Wildtyp	Kontrollfutter	C (WT)
2	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Kontrollfutter	C (KI)
3	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient	B-DEF
4	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B angereichert	B-ENR
5	*App*$^{NL\text{-}G\text{-}F}$ knock-in	PUFA supplementiert	PUFA-ENR
6	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B angereichert und PUFA supplementiert	B+PUFA-ENR
7	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Fortasyn® Connect-haltiges Futter	FC

Das Vorgehen bei der statistischen Analyse war dabei bei allen in vivo Studien in dieser Arbeit gleich. Nach einer initialen Detektion und Exklusion extremer Ausreißer (jenseits des 3-fachen Interquartilsabstands, IQR) wurde mittels Shapiro-Wilk Test auf Normalität der Datensätze geprüft und schließlich, basierend auf dem Ergebnis, der statistische Test ausgewählt. Für darüber hinaus angestellte Korrelationsanalysen wurde der non-parametrische Spearman's Rangkorrelationstest angewandt.

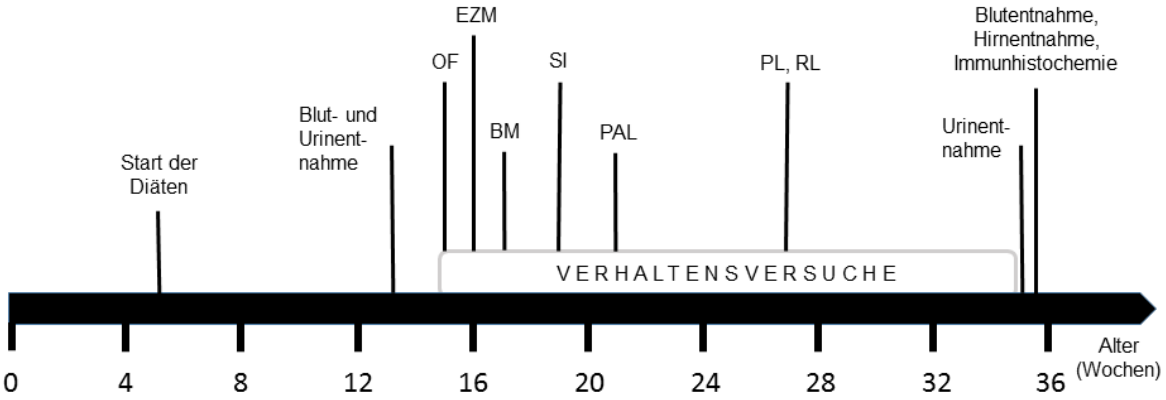

Abbildung 2. Studienverlauf (in vivo Studie 1); OF: Open Field Test, EZM: Elevated Zero Maze, BM: Barnes Maze, SI: Social Interaction Test, PAL: Touchscreen-basierter „Paired-Associates Learning" Test (inkl. Trainingsphase), PL, RL: IntelliCage „Place Learning"- und „Reversal Learning" Test (inkl. Habituierungsperiode); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Abbildung 2 verdeutlicht die zeitliche Abfolge der Teilversuche von in vivo Studie 1. Nach dem Beginn der Fütterung der Experimentaldiäten und einer Blut- und Urinabnahme zwecks Bestimmung von HCys und HCA acht Wochen später, folgten eine vielseitige Verhaltensversuchbatterie sowie – final – weitere Entnahmeschritte für weiterführende Analysen. Die Ergebnisse sind nachfolgend in dieser Arbeit geschlechtergepoolt dargestellt, es sei denn, die Gruppenvergleiche ergaben unterschiedliche Aussagen für männlich und weiblich. In dem Fall sind die Daten geschlechtergetrennt abgebildet.

Vor dem Start der Verhaltensversuche sollte die erfolgreiche Induktion des hyperhomocysteinämischen Zustandes via diätetischer Defizienz an Vitamin B6, B12 und Folat in den entsprechenden Tieren bestätigt werden. Dazu wurde den Mäusen Blut abgenommen via Punktion der Vena facialis, um anschließend HCys und HCA mittels LC-MS/MS zu bestimmen. Die für die oben beschriebenen Gruppenvergleiche erkennbaren Trends in Bezug auf die HCys-Level zeichneten sich dabei ebenso für die HCA ab (Abbildung 3).

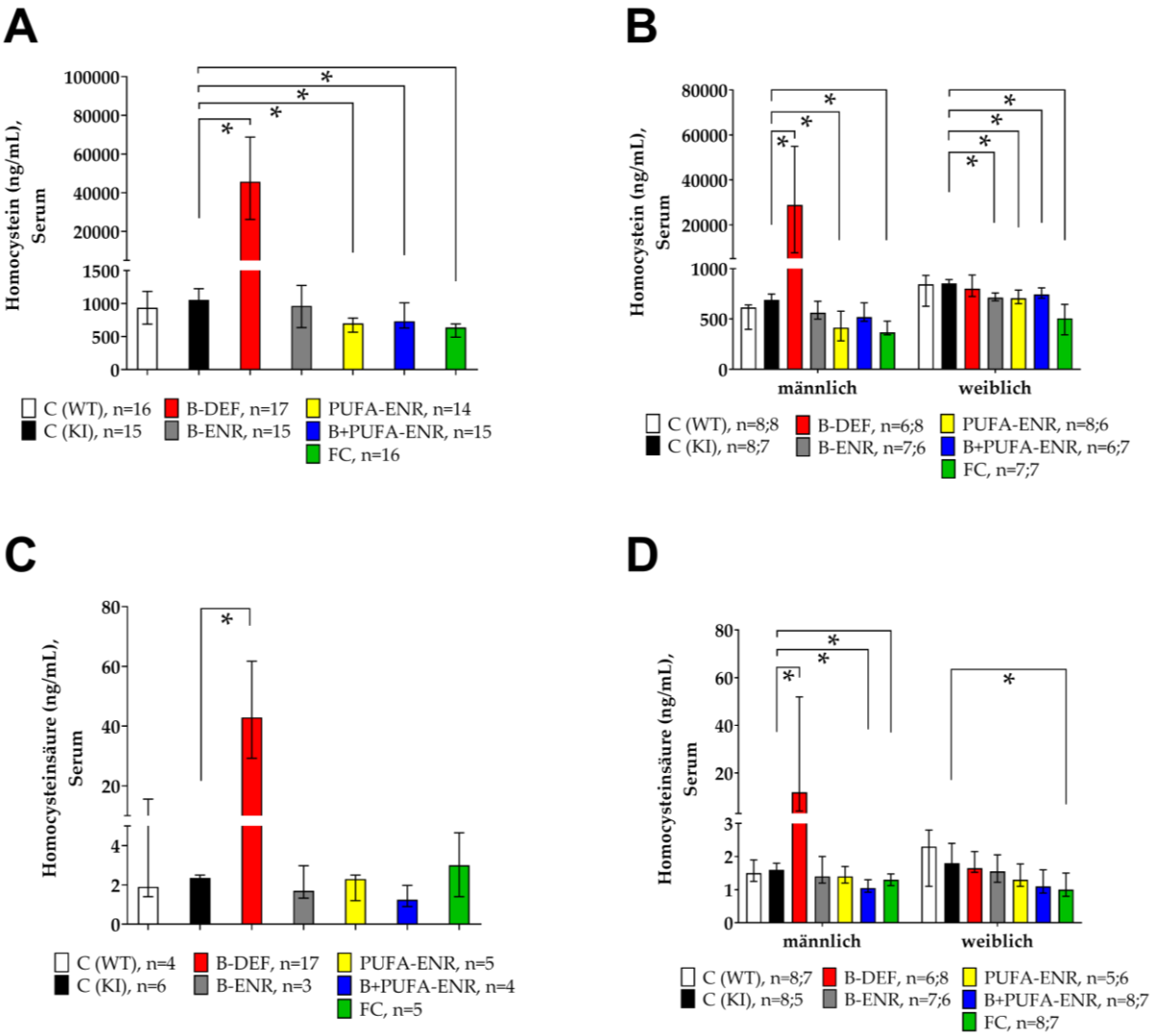

Abbildung 3. Serumspiegel von (A; B) Homocystein und (C; D) Homocysteinsäure in C57BL6/J und App^{NL-G-F} Mäusen im Alter von 13 Wochen (A; C; geschlechtergepoolte Darstellung) und im Alter von 35 Wochen (B; D; geschlechtergetrennte Darstellung); genauere Erläuterungen zu den n-Zahlen bei Homocysteinsäure im Anhang; LC-MS/MS Analyse erfolgte in Kooperation mit der zentralen Analytik des Fraunhofer ITMP am Universitätsklinikum Frankfurt; siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Ein Unterschied zwischen den WT- und KI-Kontrollgruppen, und damit ein Einfluss des AD-Mausmodells, wurde hier nicht sichtbar, ein Punkt, der weiter unten (siehe In vivo Studie 3) noch genauer und im Gesamtkontext betrachtet wird. Die B-vitamindefiziente Diät erzeugte eine schwerwiegende HHCys im Serum der Tiere. Ebenso wie im Fall von HCys selbst, stiegen auch die Spiegel des Metaboliten HCA signifikant an im Vergleich mit der *App*$^{NL-G-F}$ Kontrollgruppe. Translational gesehen stellt Gruppe 3 einen großen Teil der älteren

Bevölkerung dar, der an einer Unterversorgung mit best. B-Vitaminen leidet (Refsum *et al.*, 2004). Die induzierte HHCys war am früheren (Abbildung 3A und C) und am späteren Zeitpunkt (Abbildung 3B und D), wenn auch etwas weniger ausgeprägt, sichtbar. An letzterem war dies allerdings nur noch in den Männchen der Fall. Das ist dadurch zu erklären, dass die Weibchen zu diesem Zeitpunkt versuchsbedingt („IntelliCage“, siehe weiter unten) für einige Wochen Kontrollfutter erhalten haben. Generell gilt, dass bei dieser Studie der Fokus auf dem direkten Vergleich der Gruppen bezüglich der HHCys lag und nicht auf dem longitudinalen Vergleich der frühen versus der späten Messung. Folglich wurden die Proben bald nach der Entnahme vermessen, was den Nachteil hatte, dass die Proben nicht randomisiert über die verschiedenen Entnahmezeitpunkte analysiert werden konnten. Dadurch war jedoch sichergestellt, dass die Analyten nicht langen und wenig vergleichbaren Lagerperioden bei -80°C ausgesetzt waren, was ebenfalls eine kritische Variable im Hinblick auf die Stabilität der Analyten darstellen kann. Die frühere Hypothese zu sekundären metabolischen Effekten, die zu einer Abschwächung der HHCys bei langer defizienter Fütterung führen können (Dayal and Lentz, 2008), scheint sich hier zu bestätigen, kann aber aufgrund der zuvor beschriebenen Unsicherheit bezüglich des longitudinalen Vergleiches nicht endgültig bewertet werden. Abgesehen von der defizienten Diät zeigten die unterschiedlichen interventionellen Diäten in der hier präsentierten Studie im Vergleich zu Kontrollfutter senkende, aber in Summe inkonsistente Effekte auf die Höhe des Serum-HCys.

Parallel zur in vivo Studie wurde eine in vitro Viabilitätsstudie mit primären Zellen gestartet zur Überprüfung der weiter oben anmoderierten Hypothese, dass HCA neurotoxischer ist als HCys. Die Isolation von viablen, hippocampalen Neuronen aus $App^{NL\text{-}G\text{-}F}$ Mäusen zu diesem Zweck konnte allerdings nicht etabliert werden, was zum einen daran liegen könnte, dass die Neuronenisolation aus – in dem Fall – adulten Tieren per se schwerer möglich ist als aus Embryonen oder Neonatalen (Brewer, 1997) und zum anderen, dass eine verschlechterte Überlebensprognose der Neuronen während der Isolation vermutlich darin begründet liegt, dass diese bereits für einige Monate der AD-ähnlichen Pathologie, insbesondere massiver Amyloidose, ausgesetzt waren. Schließlich konnte die oben genannte Hypothese mit Hilfe von kommerziell erworbenen primären Neuronen untersucht und bestätigt werden (hier nicht dargestellt; siehe Anhang).

Nachfolgend sind einige beispielhafte Ergebnisse der Verhaltensversuche der in vivo Studie 1 abgebildet, die in den angehängten Publikationen – auch methodisch – im Detail nachvollzogen werden können.

Der Open Field Test ist neben seiner Bedeutung als Lokomotions- und Ängstlichkeitstest ein Tool zur Messung von Habituierung, welche auch eine Form von Lernen darstellt (Bolivar, 2009). Dazu herangezogen wird hier ein Habituierungs-Ratio, der sich wie folgt definiert (Formel 1):

Formel 1

$$Intrasession - Habituierung = \frac{Aktivität\ (finale\ 5\ min)}{Aktitvität\ (finale + initiale\ 5\ min)}$$

Wie in Abbildung 4 zu erkennen, ist ein statistisch signifikanter Unterschied lediglich für den Vergleich der WT- und KI-Kontrollgruppe und nur für die Weibchen erkennbar, d.h. es zeigte sich ein signifikanter Einfluss des AD-Modells auf die Fähigkeit der Weibchen, zu habituieren.

Genau wie beim Open Field Test zuvor, zeigte sich auch im finalen Test-Durchgang des Barnes Maze ein Unterschied zwischen weiblichen WT- und KI-Tieren (Abbildung 5B). Der Barnes Maze stellt einen gängigen Verhaltensversuch zur Untersuchung von räumlichem Lernen und Erinnern dar (Gawel *et al.*, 2019), wobei eine niedrigere Latenzzeit bis zum Target eine bessere Gedächtnisleistung anzeigt. Der Barnes Maze wurde hier gegenüber dem häufiger verwendeten Morris Water Maze bevorzugt, da er eine weniger aversive Methode für die Mäuse darstellt (Harrison *et al.*, 2006; Attar *et al.*, 2013). Der finale Zeitpunkt der Lernkurve der Mäuse während der Akquisitionsphase (Abbildung 5A, Tag 4) wies keinen signifikanten Unterschied zwischen den verglichenen Gruppen auf.

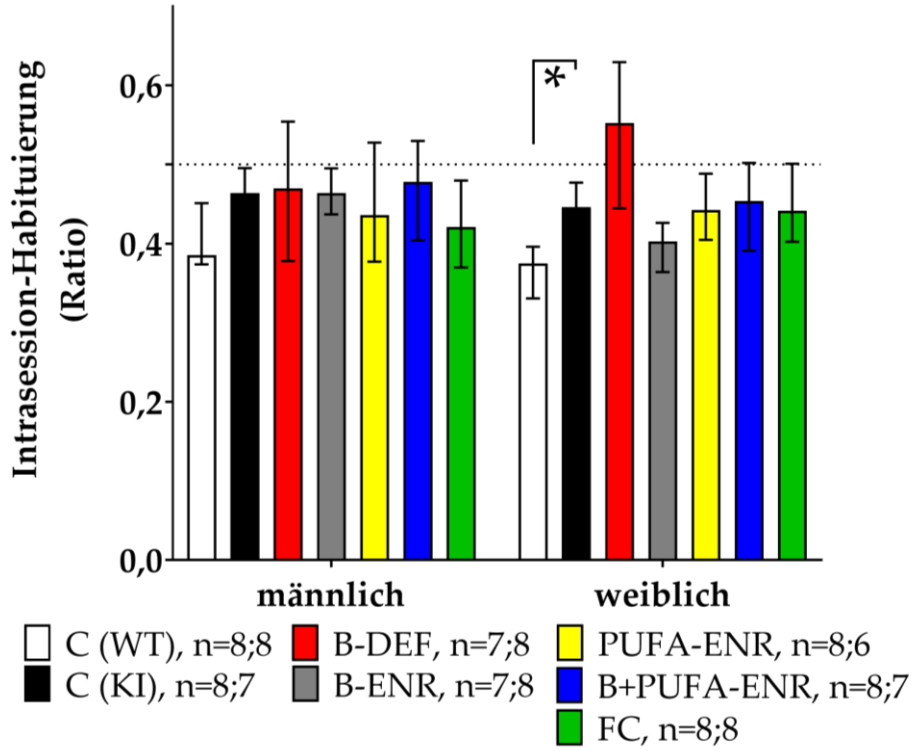

Abbildung 4. Open Field Test, im Alter von 15 Wochen; Intrasession-Habituierung: je niedriger der Ratio, desto größer die Habituierung der Maus während der Session (geschlechtergetrennt); siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

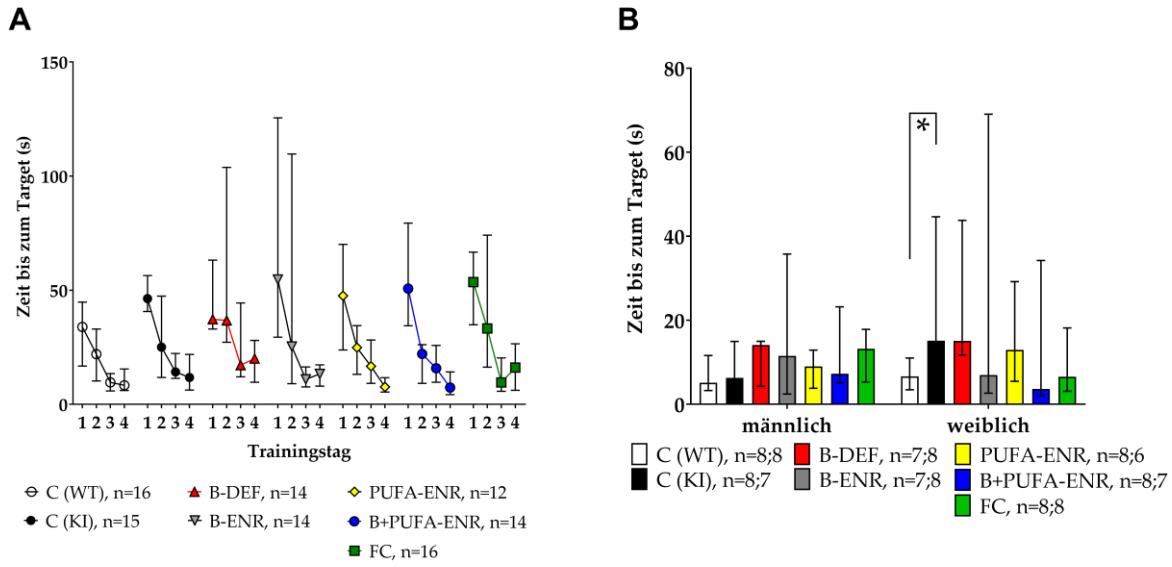

Abbildung 5. Barnes Maze, im Alter von 17-18 Wochen; (A) Zeit, die die Maus bis zum Erreichen des Targets benötigt, Akquisitionsphase, Trainingstage 1-4 (Mittelwert aus jeweils 2 Durchgängen), statistischer Test an Tag 4 (geschlechtergepoolte Darstellung); (B) Zeit, die die Maus bis zum Erreichen des Targets benötigt, finaler Test

an Tag 5 (geschlechtergetrennt); siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Die Abwesenheit von statistisch signifikanten Effekten, d.h. fehlender Einfluss der simulierten AD-ähnlichen Pathologie, insbesondere aber auch der diversen diätetischen Interventionen, zeigte sich in mehreren der durchgeführten Verhaltensversuche. So wurde auch im Test auf soziale Interaktion (Kaidanovich-Beilin *et al.*, 2011) der Mäuse mit unbekannten Stimulus-Mäusen in diesem Verhaltensversuch kein signifikanter Unterschied detektiert (Abbildung 6).

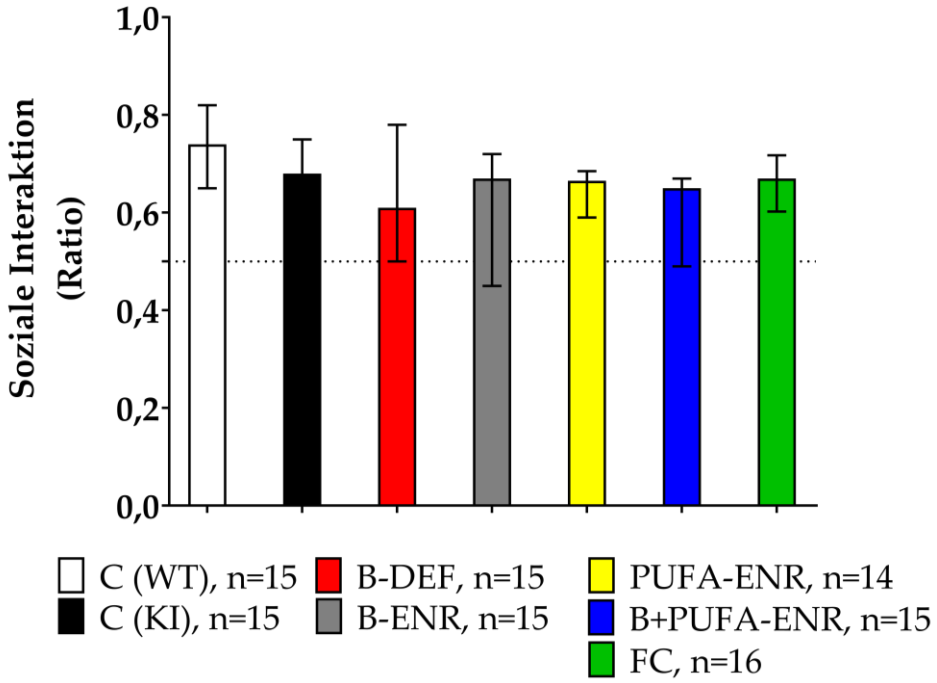

Abbildung 6. Test auf soziale Interaktion, im Alter von 19-20 Wochen; Ratio der Kontaktzeit mit einer Stimulus-Maus und Summe Kontaktzeit Stimulus-Maus + Nicht-Kontaktzeit, d.h. je größer der Ratio, desto größer die soziale Interaktion; siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Abgerundet wurde die Verhaltenstestbatterie durch zwei hoch automatisierte Versuchsanordnungen: Die Weibchen wurden im IntelliCage-System untersucht, was aufgrund großer Gruppengrößen während dieses Versuchs und des aggressiven Verhaltens der Männchen für letztere nicht möglich war und diese daher in einem Touchscreen-basierten Test untersucht

wurden. Der Touchscreen-basierte „Paired Associates Learning“ (PAL) ist ein Test für visuelles, assoziatives Lernen und Wiedererkennen, der auch translational interessant ist, da er in ähnlicher Form in der Klinik Anwendung findet (Talpos *et al.*, 2009; Nithianantharajah *et al.*, 2015). Abbildung 7A zeigt die Quote an korrekten Durchgängen im PAL, bei denen die Männchen die jeweils richtige Kombination aus Symbol und Position auf einem Touchscreen lernen und abrufen müssen. Korrekte Antworten wurden dabei mit gezuckerter Kondensmilch belohnt, falsche Antworten mit hellem Licht bestraft. Die Weibchen hatten zeitgleich im IntelliCage-basierten „Place Learning“- und „Reversal Learning“-Test für räumliches Erinnern und Konditionierung (Krackow *et al.*, 2010; Voikar *et al.*, 2018) die Aufgabe, von den vier Ecken im speziellen IntelliCage-Käfig die jeweils für sie zugeordnete Ecke zu lernen und später, nach einem Wechsel („Reversal“; Abbildung 7B), neu zu lernen. Nur in der jeweils korrekten Ecke konnten die Mäuse, automatisiert über Chip-Erkennung, während zwei ausgewiesener „Trink-Sessions“ Wasser erhalten. Wie teilweise bereits für Parameter der vorherigen Verhaltensversuche beschrieben, offenbarten die Leistungen der Männchen und Weibchen am Touchscreen, respektive im IntelliCage, keine signifikanten Effekte durch Genotyp oder Experimentaldiäten.

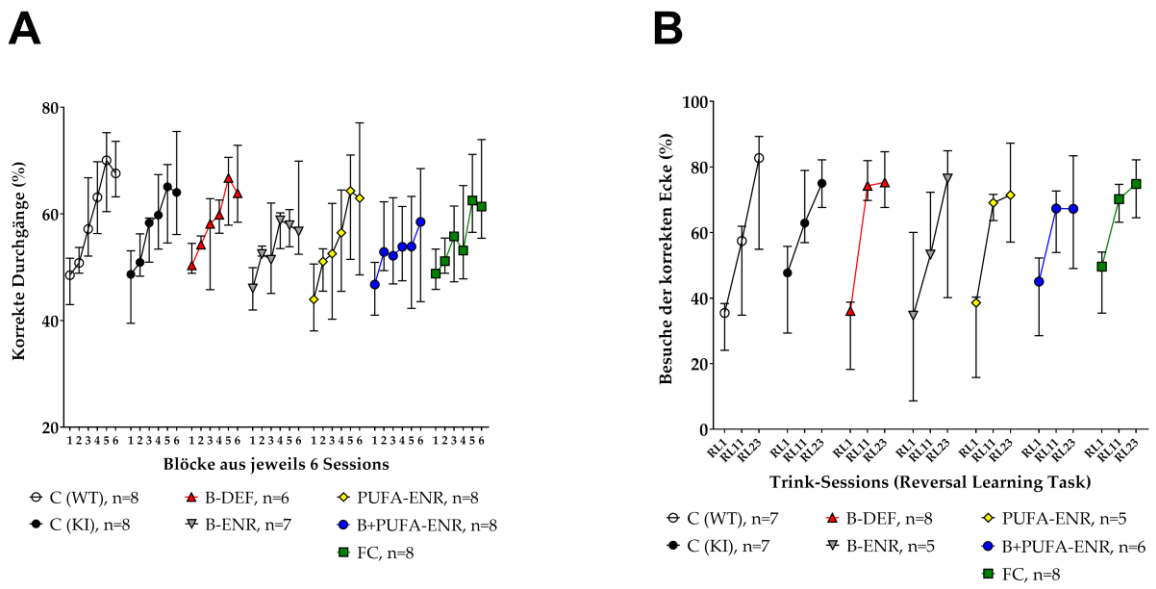

Abbildung 7. Automatisierte Verhaltensversuchsanordnungen zum Lern- und Erinnerungsvermögen, im Alter von 21-34 Wochen (inkl. Trainings-Perioden); (A) Touchscreen-basierter „Paired Associates Learning“ Test der Männchen (Anteil korrekter Durchgänge pro Session), Daten aus jeweils 6 Sessions in einem Block zusammengefasst, statistischer Test am finalen Zeitpunkt; (B) IntelliCage-basierter „Reversal Learning“ Test der

Weibchen (Anteil korrekter Besuche während der ausgewiesenen Trink-Sessions), Daten für 3 Zeitpunkte entlang des Experimentverlaufs abgebildet; statistischer Test jeweils am finalen Zeitpunkt der Versuche; siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Zusammengefasst offenbarte die durchgeführte Verhaltenstestbatterie wenige Unterschiede als Folge des manipulierten Genotyps im *App*$^{NL\text{-}G\text{-}F}$ Mausmodell, sowie die Abwesenheit von statistisch signifikanten Effekten als Folge der experimentellen Diäten. Das verdeutlicht gleichzeitig das Dilemma des AD-Modells an der Stelle: Ein phänotypischer Unterschied soll generiert werden, wobei das Modell ebenso ausreichend subtil sein sowie ein sehr frühes Krankheitsstadium simulieren sollte, um Untersuchungen zu potentiell präventiven Fragestellungen überhaupt zu ermöglichen. Zwei der verschiedenen Verhaltensversuche offenbarten einen milden Einfluss der AD-ähnlichen Pathologie in den Weibchen, ein Fund, der sich in einer späteren Studie zu diesem AD-Mausmodell bestätigte (Kundu *et al.*, 2021). Das ist translational gesehen interessant, da sich AD phänotypisch in Frauen schwerwiegender auszuprägen scheint als in Männern (Perneczky *et al.*, 2007). Neben Berichten zu profunderen Effekten des *App*$^{NL\text{-}G\text{-}F}$ Genotyps, insbesondere den in vivo Studien der Entwickler des Mausmodells (Saito *et al.*, 2014; Masuda *et al.*, 2016; Mehla *et al.*, 2019; Sakakibara *et al.*, 2019), sind zunehmend auch Publikationen erschienen, in denen von allenfalls milden *App*$^{NL\text{-}G\text{-}F}$-assoziierten, kognitiven Einschränkungen berichtet wird (Sakakibara *et al.*, 2018; Whyte *et al.*, 2018; Jacob *et al.*, 2019; Latif-Hernandez *et al.*, 2019). Ein direkter Vergleich der Studien ist indes nicht uneingeschränkt möglich, da nicht in allen Fällen Tiere beiden Geschlechts in die Studien eingeschlossen wurden und der Fokus tendenziell eher auf Männchen liegt. Generell sind weibliche Tiere in der neurowissenschaftlichen Forschung wegen vermeintlich höherer Variabilität unterrepräsentiert (Beery, 2018).

Dass cerebrale Aβ Ablagerungen in den KI Tieren anwesend waren, wurde immunhistochemisch bestätigt. Kurz zusammengefasst wurden die zunächst mittels Formaldehyd fixierten Hirne dehydriert, in Paraffin eingebettet und diese Blöcke schließlich mit einem Mikrotom fein geschnitten. Die Schnitte konnten im Anschluss rehydriert- und gefärbt werden, unter Nutzung eines immunhistochemischen Protokolls. Final wurden die erneut dehydrierten und schließlich eingedeckten Schnitte an einem geeigneten Mikroskop digitalisiert und mit entsprechender Software ausgewertet (genauer im Anhang). Zur Semi-quantitativen Evaluierung der Amyloidose wurde dazu die jeweils von Plaques belegte Fläche

in prä-definierten Hirnarealen (ROI, „region of interest“) bestimmt. Diese ROI befinden sich in für Lern- und Gedächtnisvorgänge relevanten Arealen und wurden daher hauptsächlich in den Cortex und den Hippocampus des murinen Hirns gelegt (siehe Tabelle 3).

Tabelle 3. ROI („region of interest“) für die semi-quantitative Analyse der cerebralen Amyloidose; Tabelle modifiziert nach (Nieraad *et al.*, 2020).

ROI	Hirnregion	Größe (µm)
1	Primärer somatosensorischer Cortex (PSC)	500 x 500
2	Gyrus dentatus (GD)	275 x 275
3	CA1	275 x 275
4	CA3	275 x 275
5	CA2	275 x 275
6	Thalamische Nuclei (TN)	600 x 400
7	Piriformer Cortex (PC)	275 x 275
8	Piriformer Cortex (PC)	275 x 275

Abbildung 8 zeigt die Aβ immunopositive Fläche für alle Gruppen und verdeutlicht die erfolgreiche genetische Induktion der Amyloid-Pathologie in den KI Tieren. Die Frage, warum trotzdem nur sehr milde Einflüsse auf die kognitiven Fähigkeiten der Tiere zu beobachten waren, wird weiter unten genauer diskutiert (siehe In vivo Studie 3).

Trotz schwerwiegend erhöhter Level von HCys und HCA über weite Strecken des Lebens der Mäuse, wurde hier final kein Effekt auf die Plaque-Menge sichtbar. Ebenso wirkten sich die diätetischen Interventionen, bestehend aus hochdosierten B-Vitaminen, PUFA oder der komplexen FC-Diät nicht in einer statistisch signifikanten Weise auf die Plaque-Menge in den Hirnen der entsprechenden Tieren aus. Frühere Hinweise auf positive Effekte durch FC in anderen präklinischen Studien (Jansen *et al.*, 2013; Wiesmann *et al.*, 2016) konnten hier im *App*$^{NL\text{-}G\text{-}F}$ Mausmodell für AD nicht bestätigt werden. Die Funde in letzterem decken sich eher mit den ebenfalls veröffentlichten negativen Berichten zu FC, insbesondere im klinischen Kontext (Shah *et al.*, 2013; Scheltens *et al.*, 2019). Die Evidenzlage zu FC wurde als heterogen zusammengefasst (Rasmussen, 2019).

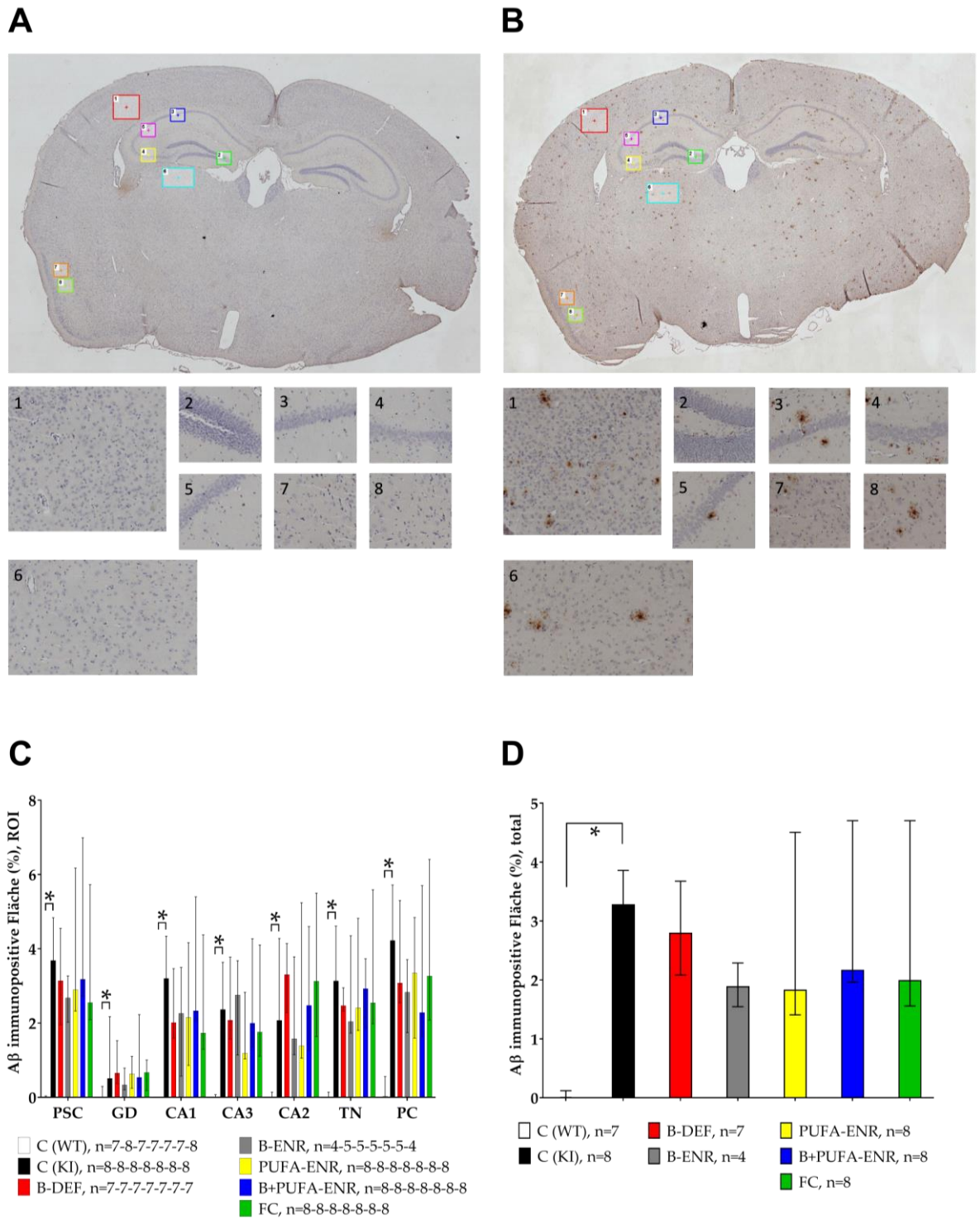

Abbildung 8. Immunhistochemische, semi-quantitative Analyse der Aβ Plaques, im Alter von 35 Wochen (geschlechtergepoolt); (A) repräsentativer gefärbter Hirnschnitt (Gesamthirn und ROI) einer WT-Maus; (B) –und einer KI-Maus; (C) Bestimmung der Aβ immunopositiven Fläche pro ROI („region of interest"); (D) –und für alle ROI gemittelt (total); siehe Tabelle 2 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad *et al.*, 2020).

Insgesamt lassen diese Ergebnisse nicht auf einen Einfluss der HHCys, bzw. der unterschiedlichen hier untersuchten Mikronährstoffe auf kognitive Fähigkeiten oder cerebrale Amyloidose schließen. Mögliche Effekte sollen in älteren Tieren und einem möglicherweise ausgeprägteren $App^{NL\text{-}G\text{-}F}$–induzierten AD-ähnlichen Phänotyp untersucht werden. Die hier vorgestellten Ergebnisse hatten zwei weitere in vivo Studien zur Folge.

In vivo Studie 2

Diese Studie ergab sich, zumindest teilweise, aus Beobachtungen in den Weibchen aus In vivo Studie 1. Bedingt durch den IntelliCage-Versuchsaufbau war die gruppenübergreifende Unterbringung der Mäuse, und damit verbunden die kollektive Gabe von Kontrollfutter für alle experimentellen Gruppen, für die Zeit dieses Verhaltensversuchs erforderlich. In der Folge normalisierten sich die HCys-Serumspiegel der zuvor hyperhomocysteinämischen Weibchen (Gruppe 3; Abbildung 3) während einer verhältnismäßig kurzen Periode unerwartet schnell auf das Niveau der Kontrollgruppen. Die hier präsentierte Kinetikstudie zu HCys und HCA soll den Zusammenhang zwischen den verschiedenen Experimentaldiäten und dem hyperhomocysteinämischen Zustand in den Tieren weiter beleuchten. Details zum Studiendesign sind in Tabelle 4 und Abbildung 9 dargestellt.

Tabelle 4. Details zu den experimentellen Gruppen (in vivo Studie 2); insgesamt 80 Tiere aufgeteilt auf zwei Gruppen, jeweils zur Hälfte männlich und weiblich.

Gruppe	Genotyp	Experimentaldiät
1	C57BL/6J Wildtyp	Vitamin B defizient (8 Wochen)
	C57BL/6J Wildtyp	Vitamin B defizient (8 Wochen) + Kontrollfutter (8 Wochen)
2	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient (8 Wochen)
	App$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient (8 Wochen) + Kontrollfutter (8 Wochen)

Der in Abbildung 9 illustrierte Ablauf dieser Langzeit-Kinetikstudie verdeutlicht gleichzeitig die Zielsetzung: Die frequentierte Entnahme der murinen Proben soll Aufschluss geben über den Anstieg der HCys- und HCA-Level während einer Fütterungsperiode mit B-vitamindefizienter Diät zum einen und über die erwartete Normalisierung der Level nach Rückkehr der Tiere zu Kontrollfutter zum anderen.

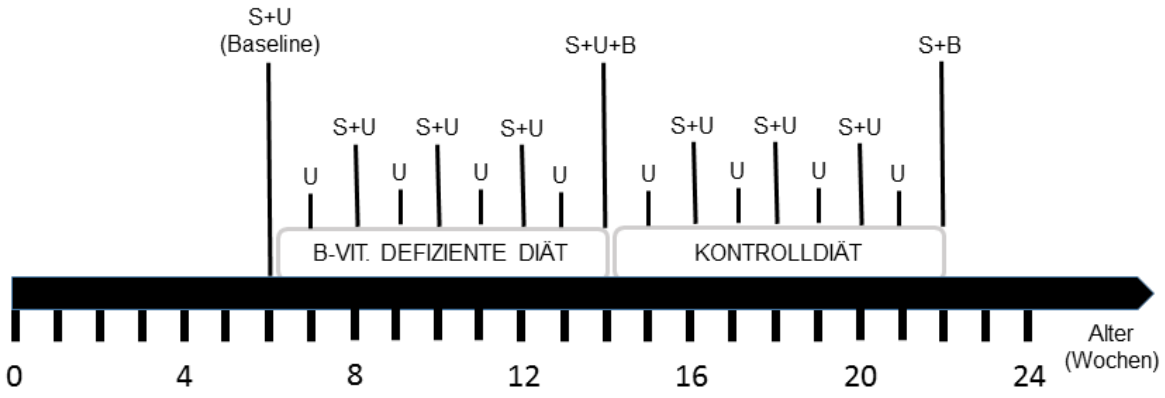

Abbildung 9. Studienverlauf (in vivo Studie 2), Diätenregime und Probenahme: S: Serum, U: Urin, B: Hirngewebe; die Hälfte der Tiere wurde in der Mitte der Studie- die andere Hälfte am Ende der Studie getötet zwecks Hirnentnahme; die Fütterung einer Diät defizient in Vitamin B6, B12 und Folat (8 Wochen) wurde gefolgt von der Gabe von Kontrollfutter (weitere 8 Wochen); Graphik modifiziert nach (Nieraad, de Bruin, *et al.*, 2021).

Darüber hinaus sollen anhand dieser Studie weitere Grundfragestellungen zum Zusammenhang zwischen der AD-ähnlichen Pathologie und HHCys geklärt werden, wie zum Beispiel: Gibt es einen Genotyp-Effekt des *App*$^{NL\text{-}G\text{-}F}$ Mausmodells auf die Ausbildung einer HHCys gegenüber gleichaltrigen WT Tieren? Somit liegt hier, im Gegensatz zu den beiden anderen vorgestellten in vivo Studien, der Fokus auf dem Einfluss der AD-ähnlichen Pathologie auf die HHCys, nicht auf dem Einfluss der HHCys auf die AD-ähnliche Pathologie. In vivo Studie 2 geht in diesem Aspekt einen Schritt weiter als die beiden anderen; hier werden HCys und HCA Werte zwischen WT und KI Kontrolle nicht nur bei Kontrolldiät verglichen, sondern die Ausbildung einer HHCys bei B-vitamindefizienter Diät in beiden Gruppen untersucht. Neben einem möglichen Genotyp-Einfluss fokussiert sich diese Studie auch auf einen potentiellen Geschlechter-Effekt und schließt die Betrachtung von HCys und HCA in cerebralen Geweben mit ein. Ob die Aminosäuren bei einer B-Vitamindefizienz auch im Hirn ansteigen, ist eine essentielle Information im Kontext neurodegenerativer Untersuchungen. Abschließend soll die Kinetikstudie genutzt werden, um mögliche Korrelationen aufzudecken.

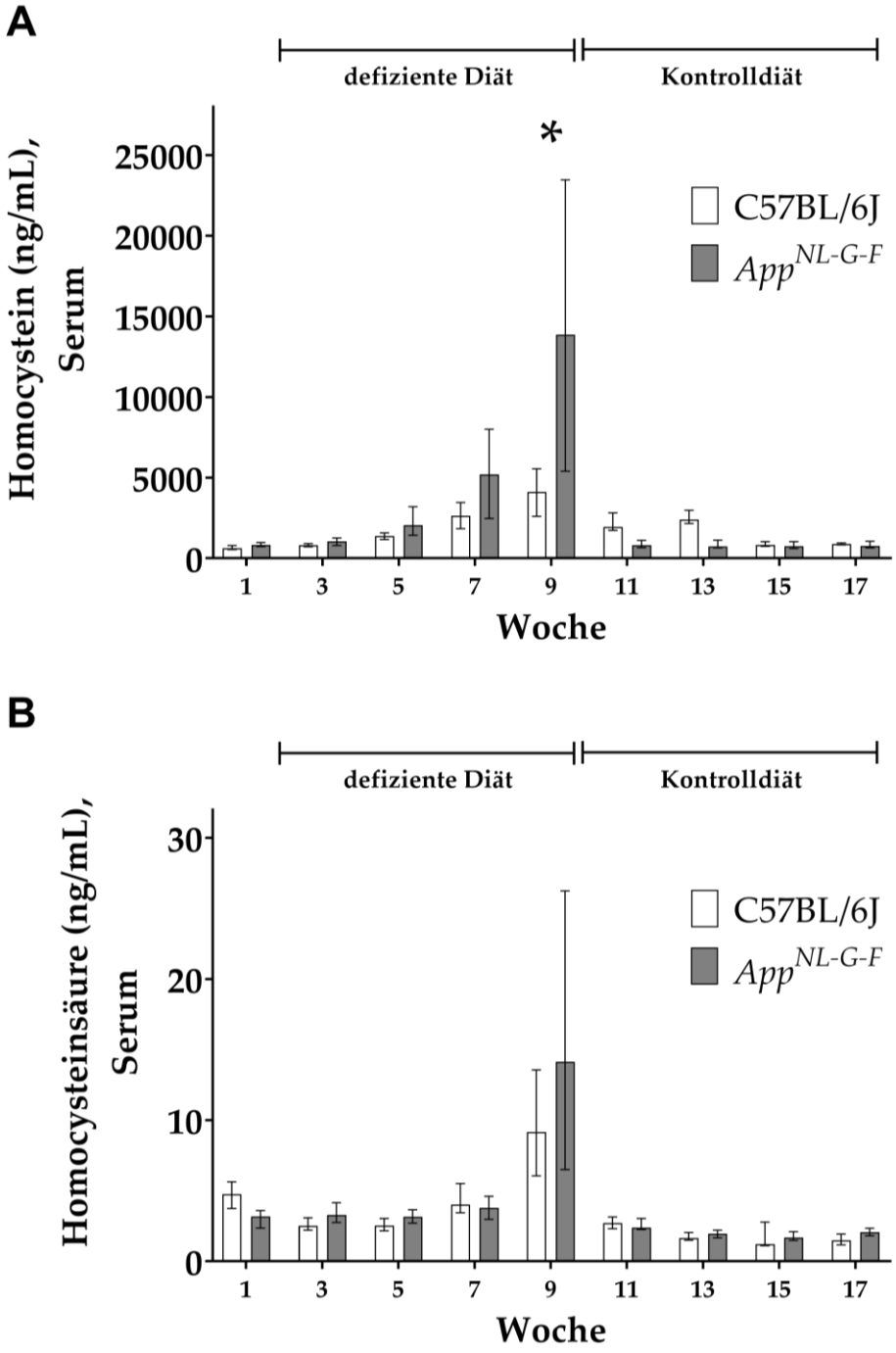

Abbildung 10. Serumspiegel von (A) Homocystein und (B) Homocysteinsäure in C57BL/6J und App^{NL-G-F} Mäusen (geschlechtergepoolt); LC-MS/MS Analyse erfolgte in Kooperation mit der zentralen Analytik des Fraunhofer ITMP am Universitätsklinikum Frankfurt; Anzahl der Tiere (n) hier nicht pauschal angegeben, da durch die Ausreißer-Analyse n zwischen den einzelnen Entnahmezeitpunkten leicht schwanken kann (40 WT- und 40 KI-Tiere wurden initial in die Studie inkludiert); statistischer Test am Zeitpunkt der ausgeprägtesten HHCys (Woche 9); Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Kruskal Wallis-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad, de Bruin, *et al.*, 2021).

Die Blutentnahmen zur Serumgewinnung fanden alle zwei Wochen unter der Verwendung der retrobulbären Methode und Isofluran-Anästhesie statt. Eine wöchentliche Blutentnahme war aus tierethischer Sicht nicht machbar gewesen bei diesem Studiendesign. Für das Serum jedes Entnahmezeitpunktes wurden die Level an HCys (Abbildung 10A) und HCA (Abbildung 10B) bestimmt. Wie der Kurvenverlauf nach der Baseline-Messung (Woche 1) veranschaulicht, resultierte die B-vitamindefiziente Fütterung der Tiere in einem kontinuierlichen Anstieg der Serum-HCys Level. Statistische Testung am hier gezeigten ausgeprägtesten Punkt der HHCys in Versuchswoche 9 offenbarte einen signifikanten Unterschied für HCys zwischen WT und KI, d.h. der AD-ähnliche Genotyp manifestierte sich in der Ausbildung einer schwerwiegenderen HHCys in den *App*$^{NL-G-F}$ Tieren. Ähnliche Auswirkungen von induzierten AD-ähnlichen Pathologiecharakteristika wurden teilweise in früheren Tierstudien mit älteren, transgenen Modellen berichtet (Bernardo *et al.*, 2007; Farkas *et al.*, 2013). Darüber hinaus scheint HCys und Methyldonordefizienz-assoziierter hippocampaler Zelltod in transgenen, nicht aber Wildtyp-Tieren vorzukommen (Kruman *et al.*, 2002). Eine mögliche Erklärung für den zuvor beschriebenen Genotyp-Einfluss auf die Ausprägung der HHCys stellen ROS dar. ROS als Charakteristikum der AD-Pathologie (Butterfield *et al.*, 2002) stehen im Verdacht, mit einer Verringerung der Folat-Spiegel einherzugehen (Fuchs *et al.*, 2001). Letzteres spielt, wie weiter vorne beschrieben, eine wichtige Rolle im Remethylierungszyklus von HCys. Folglich könnte oxidativer Stress in das Auftreten des (ausgeprägteren) hyperhomocysteinämischen Status münden (Hoffman, 2011). Weitere mechanistische Details könnten durch zusätzliche, gezielte Untersuchungen aufgedeckt werden. Ungefähr 0,15% der HCys-Moleküle lagen an diesem Zeitpunkt oxidiert in Form von HCA vor. Bezüglich HCA erreichte der Vergleich zwischen WT und KI Tieren hier knapp keine statistische Signifikanz ($p = 0,065$).

Die erneute Umstellung auf Kontrollfutter hatte anschließend, durch das nun vorhandene Angebot an den relevanten B-Vitaminen, die Normalisierung der HCys- und HCA-Spiegel zur Folge (Abbildung 10), wie aufgrund der Erfahrungen aus in vivo Studie 1 und früheren humanen Plasmadaten (Ubbink *et al.*, 1993) abzusehen war. Unerwartet war jedoch die verhältnismäßig sehr rasche Normalisierung auf Baseline-Niveau, ein Punkt, der weiter unten erneut aufgegriffen wird.

Da kein konsistenter geschlechterbezogener Einfluss auf die Spiegel im Serum und den anderen, im Folgenden vorgestellten, biologischen Matrices detektiert wurde, sind die Daten hier geschlechtergepoolt gezeigt (genauer im Anhang). Die Abwesenheit solcher

geschlechterbezogener Effekte deckt sich mit einigen der früheren präklinischen (Kakimoto *et al.*, 2014) und klinischen Berichten (Seshadri *et al.*, 2002). Auch der Einfluss des Geschlechts auf die AD-Pathologie generell ist ein kontrovers diskutiertes Thema, wie an anderer Stelle zusammengefasst wurde (Mullane and Williams, 2019). Bezüglich der Aβ-Ablagerungen wurde zuvor kein Unterschied zwischen männlichen und weiblichen AppNL-G-F Tieren berichtet (Peters *et al.*, 2018).

In jeder Versuchswoche wurde 24-Stunden-Sammelurin der Tiere in metabolischen Käfigen genommen für die Analyse von HCys (siehe Abbildung 11A) und HCA (Abbildung 11B). Gezeigt sind hier die absoluten Mengen an pro Tag ausgeschiedenem HCys, die mithilfe des Volumens abgegebenen Urins aus der ermittelten Konzentration berechnet wurden. Ähnlich dem zuvor diskutierten Verlauf der Serumwerte entlang des Experiments wurden durch den induzierten B-Vitaminmangel und damit assoziierten hyperhomocysteinämischen Zustand auch kontinuierlich steigende Mengen an HCys renal eliminiert. Auch hier bestätigte der statistische Test für Woche 9 einen signifikanten Unterschied zwischen WT- und KI-Mäusen. Zwar wurden ebenso für HCA die höchsten Werte in den App^{NL-G-F} Mäusen für die experimentellen Wochen 8 und 9 mit einer statistisch signifikant höheren Menge an HCA im Vergleich zu WT in Woche 9 gemessen, allerdings sind hier die Effekte deutlich weniger ausgeprägt als für HCys. An der Stelle soll betont werden, dass die HCA-Analytik im Urin aufgrund von Matrixeffekten in dieser biologischen Flüssigkeit erschwert wurde.

Nach der Futterumstellung zurück zu Kontrolldiät machte sich die Normalisierung der Werte im Urin noch schneller bemerkbar als zuvor für die Blutspiegel dargelegt. Bereits nach einer Woche erreichte insbesondere die HCys-Ausscheidung über den Urin wieder Baseline-Niveau.

Auch im Menschen spielt die Elimination von unverändertem HCys über den Urin nur eine relativ untergeordnete Rolle aufgrund eines hohen Maßes an Reabsorption in den renalen Tubuli (Guttormsen *et al.*, 1996). Die Daten von Guttormsen und Kollegen decken sich mit den Ergebnissen dieser Studie, wonach ein Mangel an essentiellen, HCys-reduzierenden B-Vitaminen zu einer mehr als zehnfach-gesteigerten Ausscheidung von HCys über die Niere sowohl in Experimentalmäusen als auch Menschen führt.

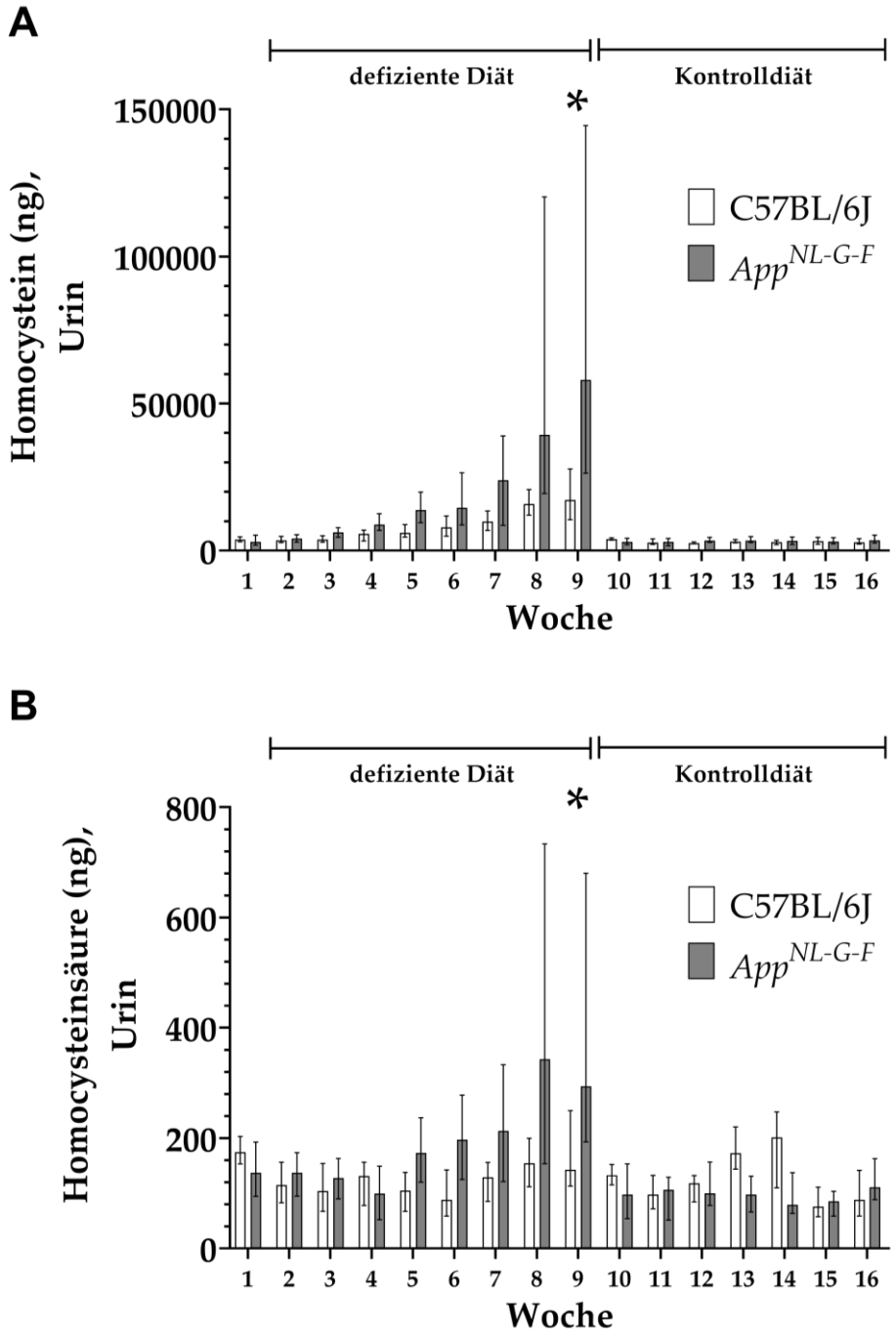

Abbildung 11. Über den Urin-ausgeschiedene Mengen an (A) Homocystein und (B) Homocysteinsäure in C57BL/6J und App^{NL-G-F} Mäusen (geschlechtergepoolt); LC-MS/MS Analyse erfolgte in Kooperation mit der zentralen Analytik des Fraunhofer ITMP am Universitätsklinikum Frankfurt; Anzahl der Tiere (n) hier nicht pauschal angegeben, da durch die Ausreißer-Analyse n zwischen den einzelnen Entnahmezeitpunkten leicht schwanken kann (40 WT- und 40 KI-Tiere wurden initial in die Studie inkludiert); statistischer Test am Zeitpunkt der ausgeprägtesten HHCys (Woche 9); Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Kruskal Wallis-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad, de Bruin, *et al.*, 2021).

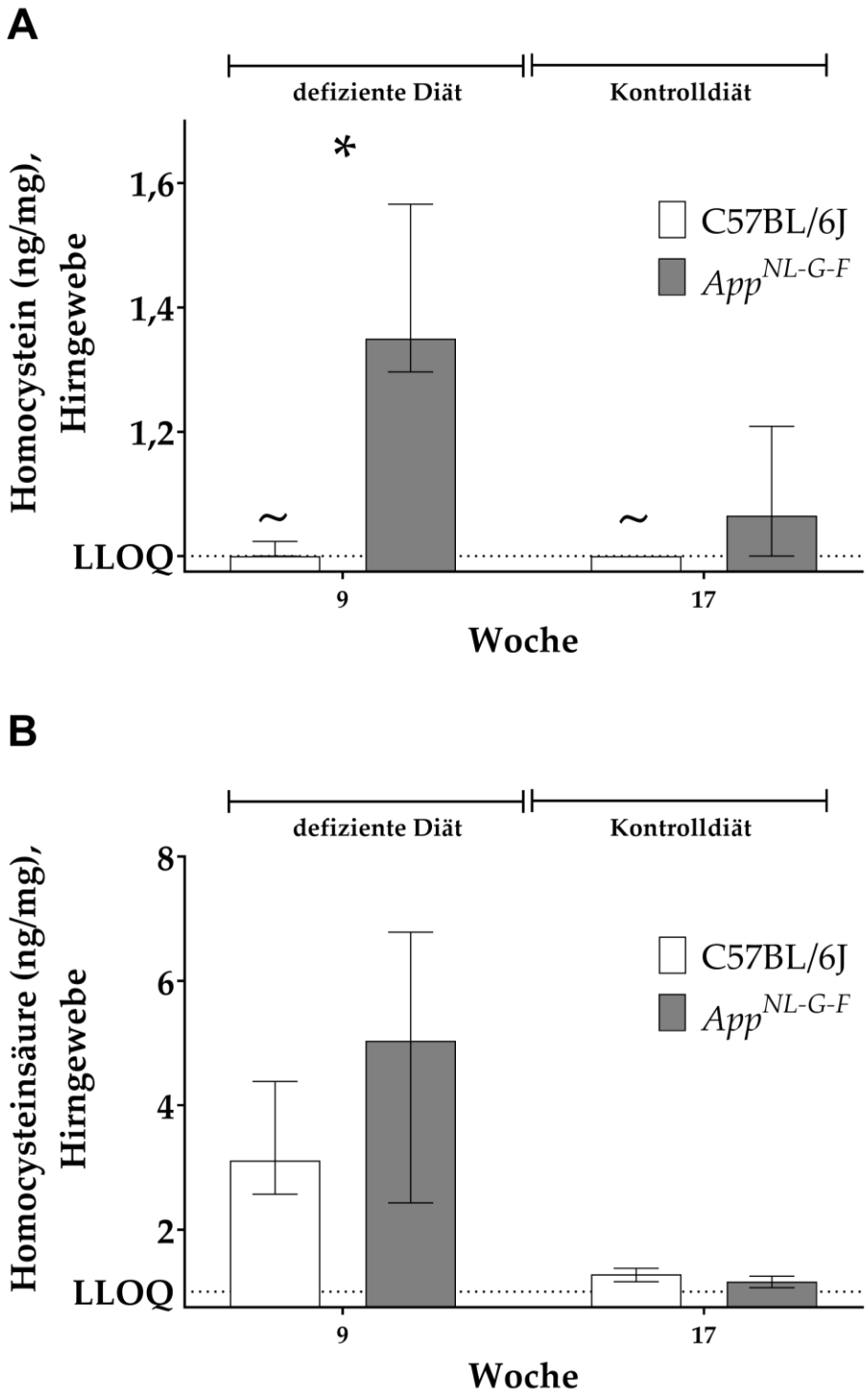

Abbildung 12. Konzentrationen von (A) Homocystein und (B) Homocysteinsäure im Hirngewebe von C57BL/6J und App^{NL-G-F} Mäusen (geschlechtergepoolt); LC-MS/MS Analyse erfolgte in Kooperation mit der zentralen Analytik des Fraunhofer ITMP am Universitätsklinikum Frankfurt; Anzahl der Tiere (n) hier nicht pauschal angegeben, da durch die Ausreißer-Analyse und Werten unter der Quantifizierungsgrenze (lower limit of quantification, LLOQ) n zwischen den einzelnen Entnahmezeitpunkten schwankt (pro Entnahmezeitpunkt wurden die Hirne von jeweils 20 WT- und 20 KI Tieren analysiert); statistischer Test am Zeitpunkt der ausgeprägtesten HHCys (Woche 9); Darstellung als Median ± IQR; ~ bedeutet, dass die Werte teilweise (Woche 9, WT) oder komplett (Woche 17, WT) unterhalb des LLOQ) lagen; p<0.05 (non-parametrischer Kruskal Wallis-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach (Nieraad, de Bruin, *et al.*, 2021).

Bestimmungen im cerebralen Gewebe komplettierten die Analyse von HCys (siehe Abbildung 12A) und HCA (Abbildung 12B) in verschiedenen biologischen Matrices in dieser Langzeit-Kinetikstudie und lieferten gleichzeitig besonders relevante Details im Kontext der neurodegenerativen Erkrankungen. Dazu wurde die Hälfte der Tiere in der Mitte, die andere Hälfte am Ende der Studie getötet zwecks Hirnentnahme für die anschließende Gewebehomogenisierung und HCys-/ HCA-Analytik. Durch dieses Design ergibt sich, dass für diese biologische Matrix keine Baseline-Werte zu Beginn der Studie vorliegen. Als Kontrolle dienen hier die Messwerte vom Ende des Experiments (Woche 17), da sich zu diesem Zeitpunkt die Spiegel, zumindest im Serum, nach Diätumstellung längst wieder normalisiert hatten.

Wie Abbildung 12 verdeutlicht, erwies sich der Unterschied bezüglich HCys zwischen WT und KI als statistisch signifikant am Punkt der ausgeprägtesten HHCys (Woche 9) und deutet somit auf einen Einfluss des *App*$^{NL\text{-}G\text{-}F}$ Modells auch im cerebralen Gewebelysat hin, analog zu den zuvor beschriebenen biologischen Matrices. Ähnliches wurde auch für transgene AD-Modelle berichtet (Modi *et al.*, 2015). Dabei scheint das HCys im Hirn nicht gleichmäßig verteilt zu sein, sondern insbesondere in einigen Strukturen, wie dem für Gedächtnisleistungen wichtigen Hippocampus, angereichert zu sein (Blaise *et al.*, 2007). Für die im Hirngewebe analysierte HCA erreichte der Vergleich zwischen WT- und KI-Gruppe keine statistische Signifikanz. Damit stehen die hier gezeigten Daten im Einklang mit einer früheren Publikation, in der von einem signifikanten Einfluss der AD-Pathologie in einem transgenen Modell auf HCys-, nicht aber auf HCA-Level im Zentralnervensystem (ZNS) berichtet wird (Hasegawa *et al.*, 2005).

Der longitudinale Vergleich zwischen Woche 9 und 17 ist in der hier vorgestellten Studie nur bedingt möglich, da einige HCys-Messwerte unterhalb des Quantifizierungslimits (LLOQ) der analytischen Methode lagen. Semi-quantitativ kann jedoch die Aussage getroffen werden, dass, auch cerebral, die HCys-Level nach wochenlanger Gabe von B-vitamindefizientem Futter erhöht waren gegenüber der Messung nach einigen Wochen Kontrollfuttergabe, da bei den niedriger erwarteten Werten von Woche 17 weitaus mehr Messungen unterhalb des LLOQ lagen als in Woche 9. Das gilt für die WT-Tiere, die per se niedrigere Spiegel aufwiesen als die KI-Tiere (siehe oben). HCys war auch im Median höher in Woche 9 (Abbildung 12). Für die *App*$^{NL\text{-}G\text{-}F}$ Tiere war der Vergleich der Zeitpunkte zudem statistisch signifikant. Signifikant fiel auch der longitudinale Vergleich für die HCA aus, für welche kaum Messwerte unterhalb des Quantifizierungslimits lagen: Sowohl die WT-Tiere als auch für die KI-Tiere offenbarten

signifikant erhöhte HCA-Konzentrationen im Hirngewebe als Folge des B-vitamindefizienten Futters.

Tabelle 5. Korrelationsanalyse in den hyperhomocysteinämischen Mäusen (Versuchswoche 9; Spearman's Rangkorrelationstest); Tabelle modifiziert nach (Nieraad, de Bruin, *et al.*, 2021).

Korrelation	**Korrelationskoeffizient**	**Signifikanz**
Serum – Urin (HCys)	0,771	< 0,001
Serum – Hirngewebe (HCys)	0,735	< 0,001
Serum – Urin (HCA)	0,675	< 0,001
Serum – Hirngewebe (HCA)	0,521	0,001
HCys – HCA (Serum)	0,660	< 0,001
HCys – HCA (Urin)	0,860	< 0,001
HCys – HCA (Hirngewebe)	0,142	0,509

Wie in Tabelle 5 zusammengefasst, wurden durch die HCys- und HCA-Messungen in den verschiedenen biologischen Matrices der hyperhomocysteinämischen Mäuse diverse Korrelationen detektiert. Serum-HCys korrelierte dabei stark mit jeweils der über den Urin ausgeschiedenen HCys-Menge und der cerebralen HCys-Konzentration. Wie zu erwarten, korrelierte HCys ebenfalls positiv mit seinem oxidativen Metaboliten HCA in den verschiedenen Matrices, mit Ausnahme der cerebralen Level, wobei hier bedacht werden muss, dass die HCys-Messwerte teilweise unterhalb des LLOQ lagen. HCA selbst zeigte ähnlich wie HCys, wenn auch mit einem niedrigeren Korrelationskoeffizienten, einen positiven Zusammenhang zwischen Serum- und Urinwerten sowie Hirngewebekonzentrationen.

Zusammenfassend lässt sich festhalten, dass in vivo Studie 2 zusätzlich negative Auswirkungen der induzierten AD-ähnlichen Pathologie auf die durch diätetische B-Vitamindefizienz ausgelöste HHCys-Ausbildung verdeutlicht. Eine weitere Erkenntnis ist die verhältnismäßig sehr schnelle Normalisierung der schwerwiegend erhöhten HCys-Werte in verschiedenen Körperflüssigkeiten bei einer Rückkehr der Tiere zu Futter, das die relevanten Vitamine B6, B12 und Folat enthält. Das unterstreicht die mögliche praktische Durchführbarkeit einer B-Vitaminintervention als potentielle AD-präventive Option. Neben der prinzipiellen Aussage zur

Praktikabilität einer solchen Anwendung von B-Vitaminen, trifft die hier vorgestellte Kinetikstudie keine Aussagen zur tatsächlichen Wirksamkeit der Gabe. Dieser Fragestellung gehen die beiden anderen durchgeführten Tierstudien in dieser Arbeit nach, insbesondere die im Folgenden vorgestellte in vivo Studie 3.

In vivo Studie 3

Die chronologisch gesehen dritte Tierstudie zur Thematik ist als logische Weiterführung der oben beschriebenen „in vivo Studie 1“ zu verstehen und soll die zuvor erhaltenen Ergebnisse in älteren *App*$^{NL\text{-}G\text{-}F}$ Tieren mit eventuell ausgeprägterem Phänotyp bestätigen, bzw. erweitern. Die Gruppenvergleiche und statistische Analyse werden dabei analog zur Vorläuferstudie durchgeführt. Bei der Gruppen-, bzw. Diätenzusammensetzung sind hier zwei Veränderungen vorgenommen worden: Die PUFA-supplementierte Gruppe 5 erhält nun ebenfalls B-vitamindefizientes Futter und Gruppe 7 erhält mit dem Methylgruppendonor Betain angereichertes, B-vitamindefizientes Futter (Tabelle 6). Somit soll in dieser Studie in nun drei Gruppen eine chronische, diätetisch-induzierte HHCys in vivo induziert werden, auf die der Einfluss der genannten Mikronährstoffen untersucht werden kann.

Tabelle 6. Details zu den experimentellen Gruppen (in vivo Studie 3); insgesamt 84 Tiere aufgeteilt auf sieben Gruppen, jeweils zur Hälfte männlich und weiblich; Tabelle modifiziert nach: siehe Anhang.

Gruppe	Genotyp	Experimentaldiät	Abkürzung
1	C57BL/6J Wildtyp	Kontrollfutter	C (WT)
2	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Kontrollfutter	C (KI)
3	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient	B-DEF
4	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B angereichert	B-ENR
5	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient and PUFA supplementiert	B-DEF+PUFA-ENR
6	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B angereichert und PUFA supplementiert	B+PUFA-ENR
7	*App*$^{NL\text{-}G\text{-}F}$ knock-in	Vitamin B defizient and Betain supplementiert	B-DEF+BET-ENR

Die hier vorgestellte Studie ist aus drei Zeitblöcken mit wiederkehrenden Blutentnahmen und Verhaltensversuchen aufgebaut und wird durch die finale Entnahme verschiedener biologischer Matrices für ex vivo Analysen komplettiert (Abbildung 13). Bei den im Folgenden dargestellten Ergebnissen liegt der Fokus auf dem dritten, finalen Zeitblock (genauer im Anhang). Eine Limitierung der in vivo Studie 3 ist, dass, im Gegensatz zur oben gezeigten ersten Studie, die Versuche nicht in zwei separaten aufeinanderfolgenden Kohorten durchgeführt wurden, was aufgrund der geringeren statistischen Power keine geschlechtergetrennte Analyse und Darstellung analog zur ersten Studie erlaubte.

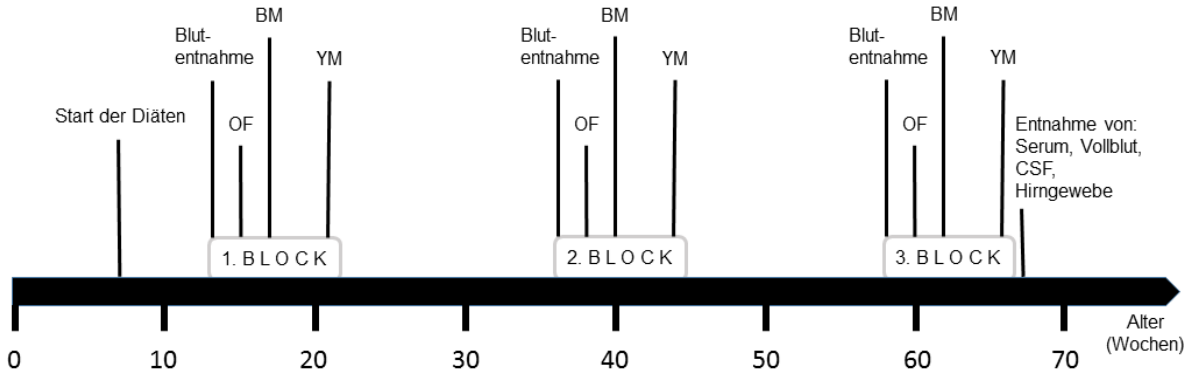

Abbildung 13. Studienverlauf (in vivo Studie 3); OF: Open Field Test, BM: Barnes Maze, YM: Y-Maze, CSF: Cerebrospinalflüssigkeit; Graphik modifiziert nach: siehe Anhang.

Auch in dieser Studie wurde die experimentelle Steigerung der HCys und HCA Level vor jedem der durchgeführten Verhaltensversuchblöcke via LC-MS/MS-Analytik bestätigt. Abbildung 14 zeigt die Ergebnisse der kardialen Blutentnahme am finalen Entnahmezeitpunkt (genauer im Anhang), welche die erfolgreiche Induktion der HHCys in allen drei B-vitamindefizient gefütterten Gruppen (3, 5 und 7) verdeutlichen und nachfolgend diskutiert werden.

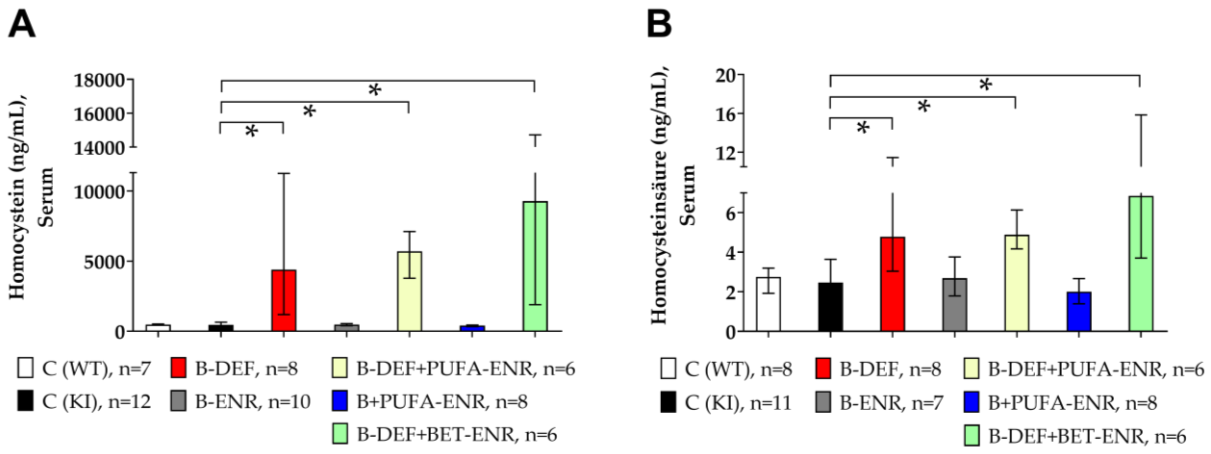

Abbildung 14. Serumspiegel von (A) Homocystein und (B) Homocysteinsäure in C57BL6/J und App^{NL-G-F} Mäusen, finaler Entnahmezeitpunkt (geschlechtergepoolte Darstellung); LC-MS/MS Analyse erfolgte in Kooperation mit der zentralen Analytik des Fraunhofer ITMP am Universitätsklinikum Frankfurt; siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

Die HCys- und HCA-Serumdaten in dieser Studie zeigten keinen konsistenten Unterschied zwischen WT und KI Tieren auf Kontrolldiät. Auch bei einer statistisch gesehen noch stärkeren, studienübergreifenden Auswertung, bei der alle vergleichbaren HCys und HCA Werte (bei Kontrollfutter) der drei in vivo Studien gepoolt betrachtet wurden, wurde hier kein signifikanter Einfluss des hier untersuchten App^{NL-G-F} Genotyps sichtbar. Somit kann, zumindest für dieses Krankheitsmodell, HCys/HCA in dem Zusammenhang nicht als Biomarker betrachtet werden, ein Begriff, der für HCys bei AD zuvor genannt wurde (Montecinos-Oliva *et al.*, 2020). Die gezeigten Ergebnisse stehen wiederum im Einklang mit früheren Berichten, laut denen HCys-Blutspiegel in (älteren, transgenen) murinen AD-Modellen- (Santiard-Baron, Aupetit and Janel, 2005) und in Patienten mit der frühen, familiären AD-Form nicht erhöht waren (Nilsson, Gustafson and Hultberg, 2002). Darüber hinaus ergab die durchgeführte Korrelationsanalyse keinen Zusammenhang zwischen HCys oder HCA und cerebralem Aβ.

Zusätzliche Supplementierung des Futters mit B-Vitaminen und/oder PUFA (Gruppen 4 und 6; gleiche Diätzusammensetzung wie bei in vivo Studie 1) zeigte keinen konsistenten Einfluss auf die Höhe der HCys- oder HCA-Spiegel im Vergleich mit der KI Kontrolle. Ebenso zeigte die zusätzliche Gabe von PUFA keinen vorteilhaften Einfluss auf den Schweregrad des hyperhomocysteinämischen Status (Gruppe 5), ein Zusammenhang, zu dem in der Vergangenheit mehrdeutige Funde gemacht wurden (Huang, Wahlqvist and Li, 2010; Martínez-Vega *et al.*, 2015). Grundüberlegung der diätetischen Kombination der B-Vitaminrestriktion mit Betain-Supplementierung (Gruppe 7) war es, eine Gruppe mit normalisierten HCys-Spiegeln bei gleichzeitiger B-Vitamindefizienz untersuchen zu können, um so zwischen potentiell schädlichen Einflüssen von HHCys einerseits und zugrundeliegendem B-Vitaminmangel andererseits unterscheiden zu können. Betrachtet man alle Entnahmezeitpunkte dieser Studie (genauer im Anhang) so wird deutlich, dass die diätetische Kombination von B-Vitaminrestriktion und Betain keine HCys-Senkung, sondern, in der Tendenz, paradoxerweise sogar eine zusätzliche Erhöhung der HCys-Serumspiegel zur Folge hatte. Die Ursache dafür könnte sein, dass das Betain-haltige Futter aus unbekannten Gründen (z.B. einer Aversion der Mäuse gegen diese Diät) von den Tieren schlechter angenommen und dadurch auch das im Futter vorhandene Cholin vermindert aufgenommen wurde. Cholin stellt eine Vorstufe des Methylgruppendonors Betain dar, dessen Funktion weiter oben bereits anmoderiert wurde (siehe Einleitung). In dem Fall hätte eine zusätzliche Analyse von Betain in Serum oder Lebergewebe Aufschluss über die tatsächliche Bioverfügbarkeit in

den Mäusen gegeben. Die gewählte Betain-Dosis von 1% im Futter orientierte sich an früheren Untersuchungen (Liu *et al.*, 2012). Die Frage, ob ein B-Vitaminmangel oder HCys selbst potentiell schädliche Effekte ausübt, kann also an der Stelle nicht endgültig beantwortet werden und stellt eine Limitierung der Studie dar. Bei möglichen kommenden Untersuchungen könnte man diesen Fallstrick umgehen, indem man eine nichtdiätetische Administration von Betain wählt. Interessanterweise wurde allerdings vereinzelt andernorts bereits berichtet, dass die perorale, aber auch die subkutane Betain-Gabe nicht zu einer Senkung, bzw. sogar zu einer Erhöhung von HCys geführt haben (Kunisawa *et al.*, 2015; Ahmad *et al.*, 2019).

Nebeneffekt der Langzeit-Einnahme des schwerwiegenden diätetischen Eingriffs war eine Verschlechterung des allgemeinen Gesundheitszustandes (unter frequentiertem Monitoring), ein geringeres Körpergewicht, Hautprobleme und auch der Verlust von Tieren in den drei hyperhomocysteinämischen Gruppen. Um die Ausfälle zu begrenzen – und auch aus tierethischen Gründen – wurden wenn nötig kurze Regenerationsperioden mit Kontrollfutter für die entsprechenden Mäuse implementiert. Trotzdem wurde darauf geachtet und sichergestellt, dass die Tiere über den größten Teil ihres Lebens ihre adäquate Experimentaldiät erhielten und dadurch eine signifikante HHCys aufwiesen.

Auch in dieser Studie wurden die kognitiven Fähigkeiten der Mäuse in verschiedenen Verhaltensversuchen bewertet, für die im Folgenden jeweils die wichtigsten Parameter im finalen Zeitblock abgebildet sind. Dabei wurden in Analogie zu in vivo Studie 1 der Open Field Test (Abbildung 15) und der Barnes Maze (Abbildung 16) durchgeführt. Zusätzlich dazu wurde hier der Y-Maze implementiert (Abbildung 17), um einen direkten Vergleich zur ursprünglichen $App^{NL\text{-}G\text{-}F}$-bezogenen Publikation der Entwickler des Mausmodells herzustellen (Saito *et al.*, 2014). Dort wurde eine signifikante kognitive Einbuße für dieses Mausmodell im Alter von sechs Monaten berichtet. Ein solcher Effekt im Y-Maze wird mittlerweile allerdings deutlich kontroverser diskutiert (Whyte *et al.*, 2018; Izumi *et al.*, 2020; Narukawa *et al.*, 2020; Uruno *et al.*, 2020; Degawa *et al.*, 2021). Der Y-Maze erlaubt die Bewertung des Arbeitsgedächtnisses (Prieur and Jadavji, 2019), wobei gilt: Je größer die spontane Alternierung der Mäuse im Maze, desto größer die Arbeitsgedächtnisleistung (siehe Formel 2; genauer im Anhang).

Formel 2

$$Spontane\ Alternierung\ (\%) = \frac{Anzahl\ Alternierungen\ im\ YM}{(Anzahl\ betretener\ YM\ Arme - 2)} * 100$$

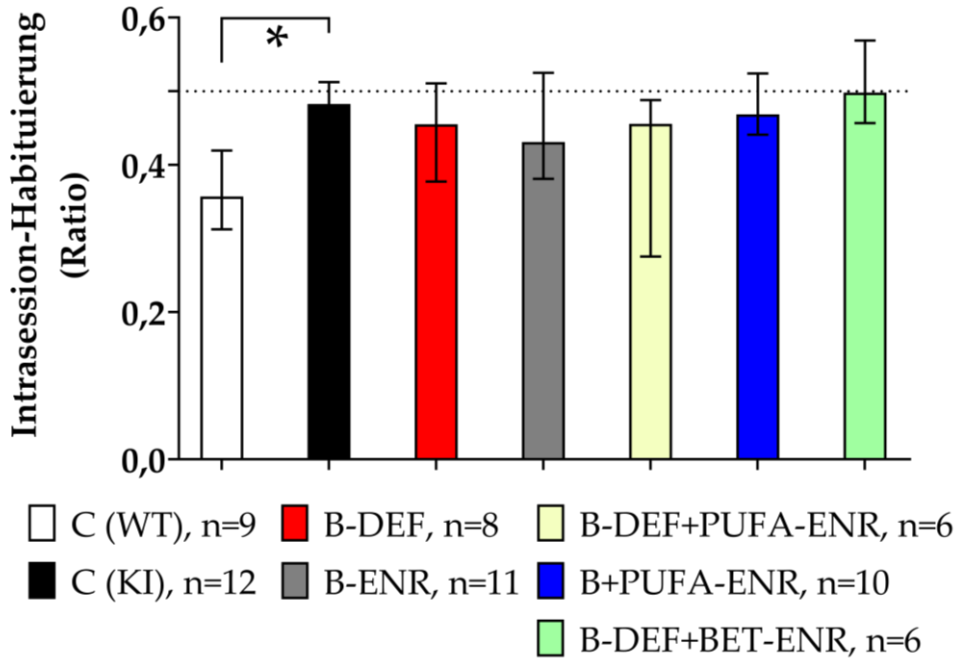

Abbildung 15. Open Field Test, finaler Entnahmezeitpunkt; Intrasession-Habituierung: je niedriger der Ratio, desto größer die Habituierung der Maus während der Session (geschlechtergepoolt); siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

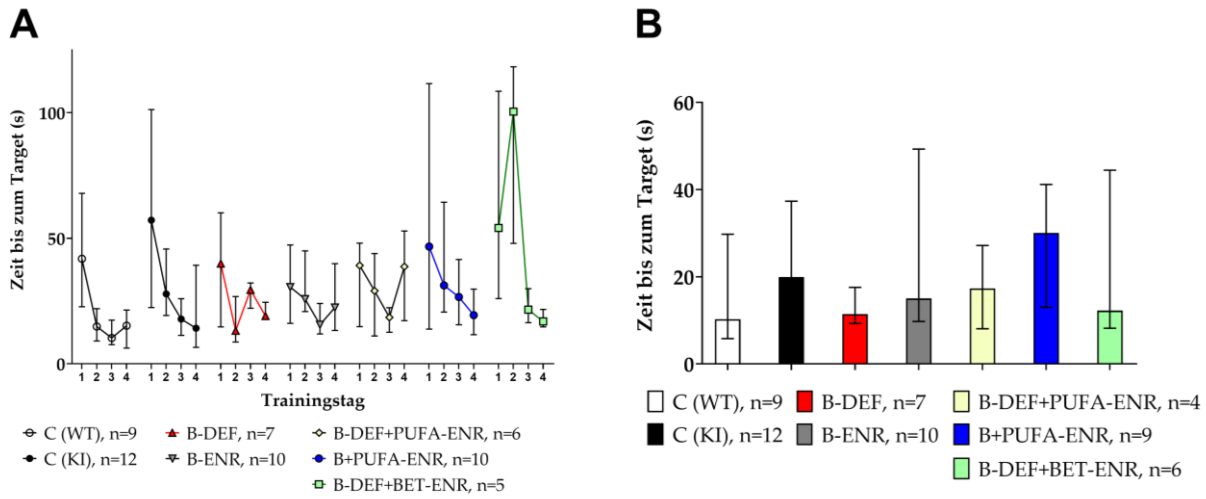

Abbildung 16. Barnes Maze, finaler Entnahmezeitpunkt; (A) Zeit, die die Maus bis zum Erreichen des Targets benötigt, Akquisitionsphase, Trainingstage 1-4 (Mittelwert aus jeweils 2 Durchgängen), statistischer Test an Tag 4 (geschlechtergepoolte Darstellung); (B) Zeit, die die Maus bis zum Erreichen des Targets benötigt, finaler Test an Tag 5 (geschlechtergetrennt); siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

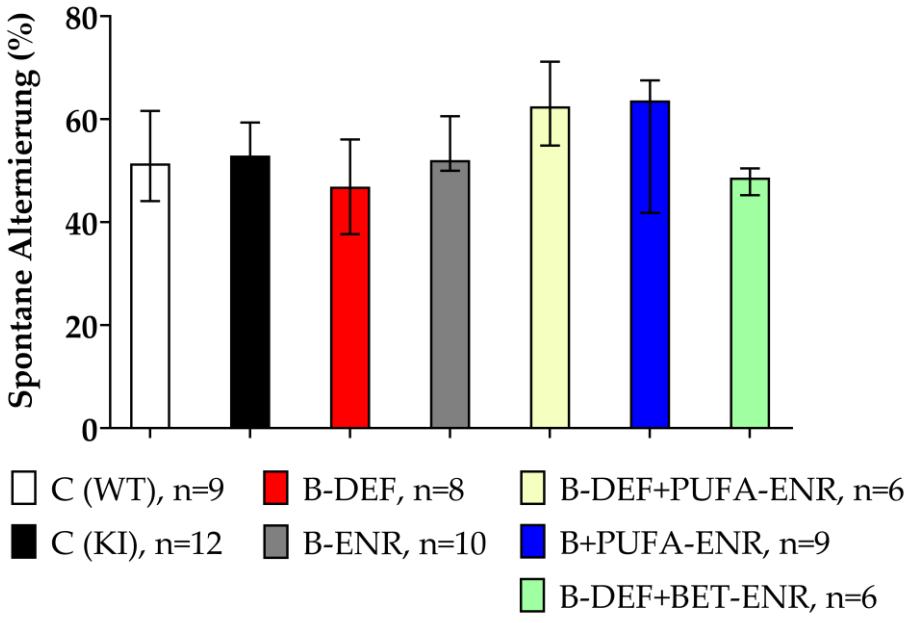

Abbildung 17. Y-Maze, finaler Entnahmezeitpunkt; je größer die spontane Alternierung, desto größer die Arbeitsgedächtnisleistung der Maus (geschlechtergepoolt); siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; p<0.05 (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

Auf die Testabhängigkeit der Genotyp-Effekte in Verhaltensversuchen wurde kürzlich auch in einer anderen *App*$^{NL\text{-}G\text{-}F}$-bezogenen Publikation hingewiesen (Kundu *et al.*, 2021). In der hier vorgestellten Studie offenbarte das AD-Modell einen, in Summe, subtilen Einfluss auf den Phänotyp der Tiere. So wurde ein statistisch signifikanter Einfluss auf das Habituierungslernen deutlich (Abbildung 15), nicht aber auf das räumliche Lernen und Erinnern (Abbildung 16) oder das Arbeitsgedächtnis (Abbildung 17). Ebenso wurde kein Effekt auf das ängstlichkeitsbezogene Verhalten sichtbar, welches ebenfalls im Open Field Test bewertet wurde mittels der Zeit, die die Maus im Zentrum der Arena verbringt (hier nicht dargestellt; genauer im Anhang). Insbesondere die fehlenden Defizite in räumlichem Lernen und Abrufen im Barnes Maze decken sich mit rezenten, vergleichbaren Tierstudien, in denen dieses KI Mausmodell für AD untersucht wurde (Sakakibara *et al.*, 2018; Hongo *et al.*, 2020). Die Entwickler des Mausmodells sind unterdessen dabei, das *App*$^{NL\text{-}G\text{-}F}$ Modell weiterzuentwickeln und zu optimieren, d.h. noch ausgeprägtere AD-Charakteristika- und somit einen definierteren Phänotyp zu induzieren (Sato *et al.*, 2021).

Trotz signifikant erhöhter Level von HCys und HCA konnte keine eingeschränkte kognitive Performance gegenüber der KI Kontrolle in den entsprechenden Tieren festgestellt werden, was die vorherigen Ergebnisse aus in vivo Studie 1-, sowie teilweise Funde in anderen Tierspezies aus vergleichbaren Studien (z.B. (Algaidi *et al.*, 2006; Ahmad *et al.*, 2019)) bestätigt. Wie kürzlich in einem eigenen Review analysiert und zusammengefasst (Nieraad, Pannwitz, *et al.*, 2021), weist die Mehrzahl an bisherigen präklinischen Studien in diesem Feld eher auf einen Einfluss von HHCys auf kognitiven Abbau hin. Wir denken, das ist zu einem gewissen Teil auf einen „Publication Bias“ zurückzuführen, welcher (ganz generell) ein gängiges Phänomen darstellt und durch den der analysierte Querschnitt durch die Evidenz an sich gewissermaßen verzerrt ist (Shineman *et al.*, 2011; ter Riet *et al.*, 2012; Wieschowski *et al.*, 2019). Etwas anders verhält sich die Situation bei den humanen Studien in diesem Feld, wo die Evidenzlage deutlich kontroverser ausfällt. Der Publication Bias wird hier vermutlich durch den stärkeren Gebrauch von Maßnahmen wie die Präregistrierung von Studien abgeschwächt (Song *et al.*, 2010; Mlinarić, Horvat and Šupak Smolčić, 2017). Initiativen wie „European Quality in Preclinical Data“, EQIPD (Bespalov *et al.*, 2021), in der wir als Forschungsgruppe partizipieren, haben das Ziel, solche Maßnahmen auch zunehmend im präklinischen Umfeld zu etablieren und ermutigen zur Publikation von nicht ausschließlich „positiver“ Ergebnisse. Zusätzlich wurden

in den hier gezeigten Studien weitere Maßnahmen wie Randomisierung und teilweise Verblindung angewandt mit dem Ziel, weitere Arten von Bias möglichst gering zu halten.

Zusammenfassend muss für die in vivo Studien, auf denen die hier vorliegende Arbeit basiert, festgehalten werden, dass trotz signifikant-erhöhter HCys und HCA Spiegel über den größten Teil des Lebens der Tiere keine kausale Verschlimmerung des AD-ähnlichen Phänotyps stattgefunden hat. Das gilt zumindest für das hier untersuchte *App*$^{NL\text{-}G\text{-}F}$ KI Mausmodell, welches, wie die meisten anderen AD-Mausmodelle, die familiäre Form der Erkrankung simuliert. Tatsächlich sind aber die meisten AD-Fälle der sporadischen Form zuzuordnen, bei der die HHCys womöglich eher eine Rolle spielen könnte (siehe auch Einleitung) und für die stärkere Bestrebungen zur Entwicklung geeigneter Mausmodelle sinnvoll sein könnten.

Wie zuvor diskutiert, findet man für die Effekte der HHCys im Bereich der Demenz, vor allem aber auch für potentiell vorteilhafte diätetische Ansätze, eine kontroverse und teils widersprüchlich diskutierte Evidenz vor. Bezüglich des Einsatzes von Betain gibt es frühere klinische und präklinische Hinweise auf möglicherweise positive Auswirkungen auf die Kognition (Sun *et al.*, 2017; Ibi *et al.*, 2019), die im hier untersuchten Krankheitsmodell nicht bestätigt werden konnten. Passend dazu wurde von Zhao und Kollegen geschlussfolgert, dass die Rolle von Betain widersprüchlich bleibt (Zhao *et al.*, 2018). Ergebnisse aus den Gruppen 5 und 6 zeigen, dass ebenfalls der Einsatz von PUFA keinen Benefit gegenüber den KI Kontrolltieren hervorbrachte, was im Einklang mit Negativergebnissen aus ähnlichen, früheren Untersuchungen zum Einsatz von PUFA (Arendash *et al.*, 2007; Quinn *et al.*, 2010), bzw. Hinweisen auf limitierte und mehrdeutige Evidenz (Bos *et al.*, 2016) steht. Im Hinblick auf einen möglichen Benefit durch die Gabe von B-Vitaminen konnte in der hier vorliegenden Studie kein konsistenter Effekt auf das Lern- und Erinnerungsvermögen in den Mäusen gezeigt werden (Gruppe 4), was im Einklang mit den Ergebnissen einer kürzlich publizierten Humanstudie steht (Kwok *et al.*, 2020). Insbesondere bei diesem Thema ist die Literatur allerdings gespalten: Es sind rezente systematische Reviews und Meta-Analysen verfügbar, die Aussagen pro und contra eines therapeutischen Nutzens von B-Vitaminen treffen, bzw. darauf verweisen, dass weitere gut designte Studien dazu vonnöten sind (McCleery *et al.*, 2018; Ford and Almeida, 2019; Behrens *et al.*, 2020; Wang *et al.*, 2021). Ein möglicher Diskussionspunkt dabei ist der Startzeitpunkt der diätetischen Interventionen. Hier ist allerdings anzumerken, dass diese in der vorliegenden Studie translational gesehen bereits sehr früh gestartet wurden.

Abgesehen von den fehlenden kognitiven Resultaten, wurde in dieser Studie jedoch ein Einfluss in verschiedenen ex vivo Analysen in den hyperhomocysteinämischen Tieren deutlich. Ein Beispiel dafür war die finale Analyse des Vollbluts der Tiere, welche mehrere Veränderungen im Hinblick auf Erythrozyten-bezogene Parameter in den hyperhomocysteinämischen Gruppen im Vergleich zur KI Kontrolle offenbarte. So waren die Hämoglobinkonzentration und der Hämatokrit in diesen Gruppen statistisch signifikant erniedrigt sowie die Erythrozytenverteilungsbreite erhöht (Abbildung 18).

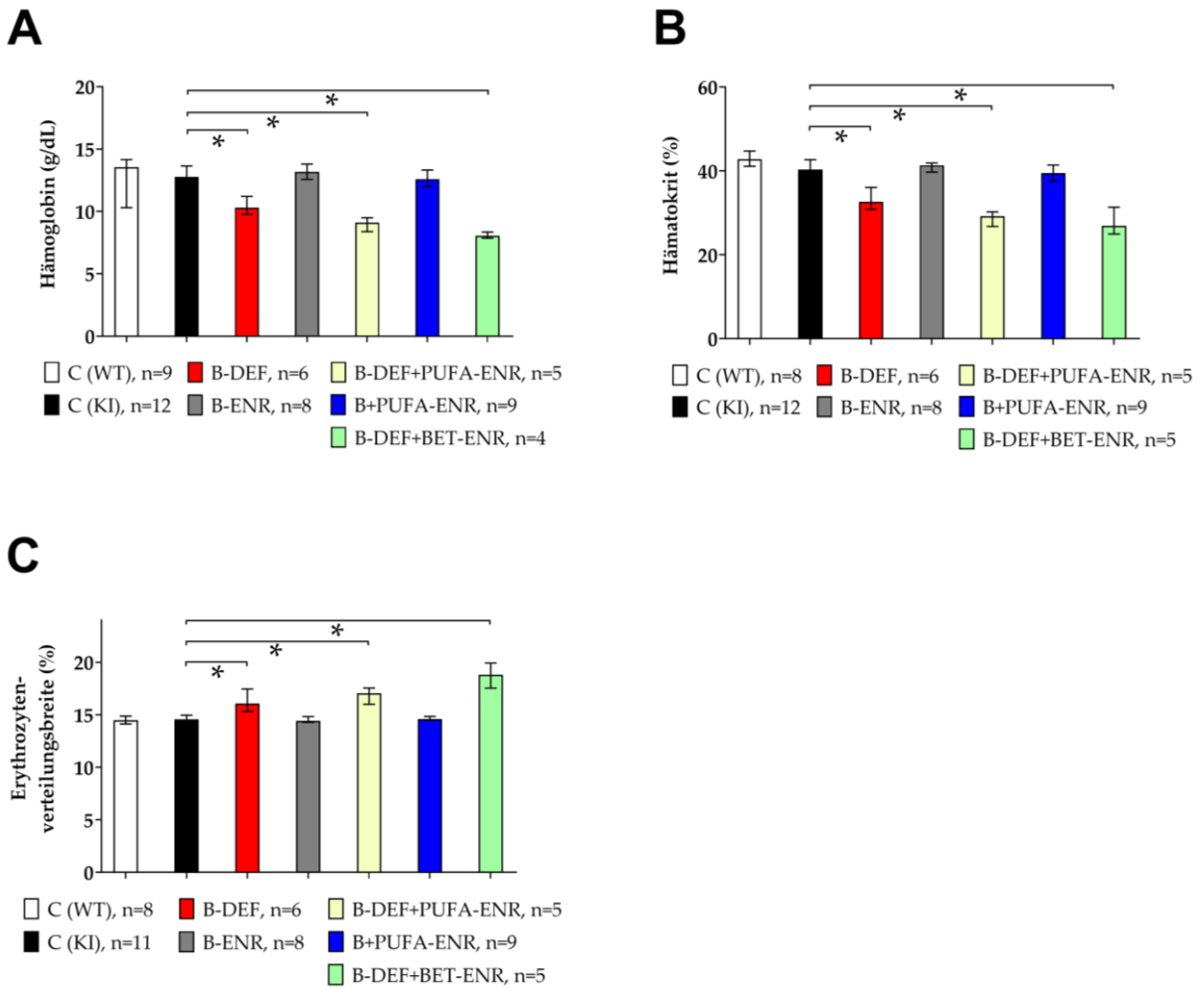

Abbildung 18. Hämatologische Parameter, Untersuchung des Vollbluts am finalen Entnahmezeitpunkt (geschlechtergepoolte Darstellung); (A) Hämoglobin-Konzentration; (B) Hämatokrit; (C) Erythrozytenverteilungsbreite; siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

Es ist weithin bekannt, dass ein Mangel an B-Vitaminen, insbesondere B12 und Folsäure, eine Anämie hervorrufen kann. Die Daten dieser Studie geben einen Hinweis darauf, dass neben der B-Vitamindefizienz auch erhöhte Spiegel von HCys selbst zu diesem Effekt beitragen könnten. Bei der Betrachtung aller Entnahmezeitpunkte (genauer im Anhang) wird deutlich, dass die Tiere der Gruppe 7 (B-vitamindefizient, betainsupplementiert) tendenziell die höchsten HCys-Spiegel und gleichzeitig die ausgeprägtesten Effekte in der Vollblutanalyse aufwiesen, bei gleicher B-Vitamindefizienz (genaue Zusammensetzung der Experimentaldiäten in Tabelle 1). Die durchgeführte Korrelationsanalyse deutete auf eine, wenn auch schwache, negative Korrelation zwischen HCys und Hämoglobin sowie dem Hämatokrit hin (genauer im Anhang), was auch im Einklang mit einer rezenten klinischen Studie steht (Hymavathi, Shukla and Madhuri, 2020). Dasselbe gilt für die positive Korrelation zwischen HCys und der Erythrozytenverteilungsbreite, unabhängig von der B-Vitamindefizienz (Peng and Pan, 2017). Wenngleich sich diese Effekte offenbar als nicht stark genug erwiesen, um in phänotypischen kognitiven Defiziten im Verhaltensversuch zu münden, so weisen die Funde dennoch auf eine vaskuläre Wirkungsweise der HHCys hin, die durch ein vermindertes Sauerstoffangebot, unter anderem im Hirn, ihren Beitrag zu einer Demenz-zugrundeliegenden Pathologie leisten könnte. Darüber hinaus erklären diese hämatologischen Funde wahrscheinlich den insgesamt schlechteren Allgemeinzustand der Tiere in den hyperhomocysteinämischen Gruppen. Interessant sind diese Ergebnisse insbesondere in Kombination mit den ergänzenden Daten zu HHCys, die in der hier vorgestellten Proteomanalyse erhoben wurden.

Wie bereits für die hämatologische Analyse berichtet, brachte auch „Olink Proteomics“ signifikante Unterschiede zwischen den Gruppen hervor. Dabei handelt es sich um ein Hochdurchsatzanalyseverfahren für die relative Quantifizierung von 92 Proteinbiomarkern in jeweils 1 µL Probenvolumen, das auf dem „Proximity Extension Assay“ beruht (Assarsson *et al.*, 2014) und in der hier vorliegenden Studie sowohl in Serum als auch CSF (erhalten durch Punktur der Cisterna Magna) angewandt wurde. Einige der insgesamt 92 Proteinmarker des Olink Exploratory Mouse Panels erwiesen sich als signifikant dereguliert (siehe Tabelle 7), entweder durch den Einfluss der AD-ähnlichen Pathologie oder der zusätzlich induzierten HHCys. Im Folgenden stehen bei dieser Auswertung die Gruppen 1-3 im Vordergrund. Gruppen 5 und 7 wurden bereits vor der praktischen Durchführung ausgeschlossen, da der Olink-Chip nur eine limitierte Kapazität bot. Gruppen 4 und 6 werden hier nicht weiter diskutiert aufgrund weniger und lediglich schwacher Effekte in Serum oder CSF.

Tabelle 7. Einfluss von AD-ähnlicher Pathologie und zusätzlicher HHCys auf Proteine, die im Rahmen der Proteomanalyse als statistisch-signifikant (p<0,05) hoch- oder herunterreguliert detektiert wurden; Effektstärke berechnet als $|r| = z / \sqrt{N}$; Tabelle modifiziert nach: siehe Anhang.

***App*$^{NL-G-F}$ knock-in Genotyp** (Gruppe 1 versus 2)						**Hyperhomocysteinemia** (Gruppe 2 versus 3)					
Protein		**Matrix**	**N**	**P-Wert**	**Effektstärke r**	**Protein**		**Matrix**	**N**	**P-Wert**	**Effektstärke r**
CCL3	↑	Serum	19	0,016	0,549	CCL2	↓	Serum	19	< 0,001	0,777
CLMP	↑	Serum	19	0,041	0,474	CXCL9	↓	Serum	19	0,007	0,606
ERBB4	↑	Serum	19	0,007	0,606	DLL1	↓	Serum	18	0,012	0,586
GFRA1	↑	Serum	19	0,033	0,493	EDA2R	↓	Serum	19	0,016	0,549
IGSF3	↓	Serum	8	0,036	0,791	EPCAM	↑	Serum	14	0,038	0,563
NOTCH3	↑	Serum	19	0,026	0,511	FAS	↓	Serum	19	0,007	0,606
VEGFD	↑	Serum	19	0,033	0,493	FSTL3	↓	Serum	19	0,026	0,511
CCL3	↑	CSF	19	< 0,001	0,833	GFRA1	↓	Serum	19	< 0,001	0,833
CNTN1	↑	CSF	19	0,041	0,474	IGSF3	↑	Serum	9	0,032	0,735
ENO2	↑	CSF	19	< 0,001	0,833	IL1a	↑	Serum	19	0,026	0,511
HGF	↑	CSF	18	< 0,001	0,838	IL23R	↓	Serum	19	0,001	0,701
RGMA	↓	CSF	17	0,002	0,710	LGMN	↓	Serum	19	0,007	0,606
TNR	↑	CSF	18	< 0,001	0,800	MATN2	↓	Serum	18	0,034	0,503
TPP1	↑	CSF	19	0,005	0,625	S100A4	↓	Serum	19	0,007	0,606
						TGFBR3	↓	Serum	19	0,003	0,663
						TNFRSF11B	↓	Serum	19	0,001	0,720
						TNFRSF12A	↓	Serum	19	0,003	0,663
						TPP1	↓	Serum	19	0,001	0,701
						VSIG2	↓	Serum	17	0,025	0,544
						WISP1	↓	Serum	19	0,012	0,568
						ACVRL1	↓	CSF	13	0,035	0,594
						DLK1	↓	CSF	13	0,030	0,609

Da an dieser Stelle nicht alle gezeigten deregulierten Marker im Detail beleuchtet und diskutiert werden können, soll hier der Fokus auf einer Auswahl liegen, die insbesondere durch große Effektstärken und einen direkten Bezug zu neuronaler Funktionalität oder (neuro-) inflammatorischen Prozessen zustande kommt. Abbildung 19 veranschaulicht solche Beispiele für die beiden anmoderierten Gruppenvergleiche.

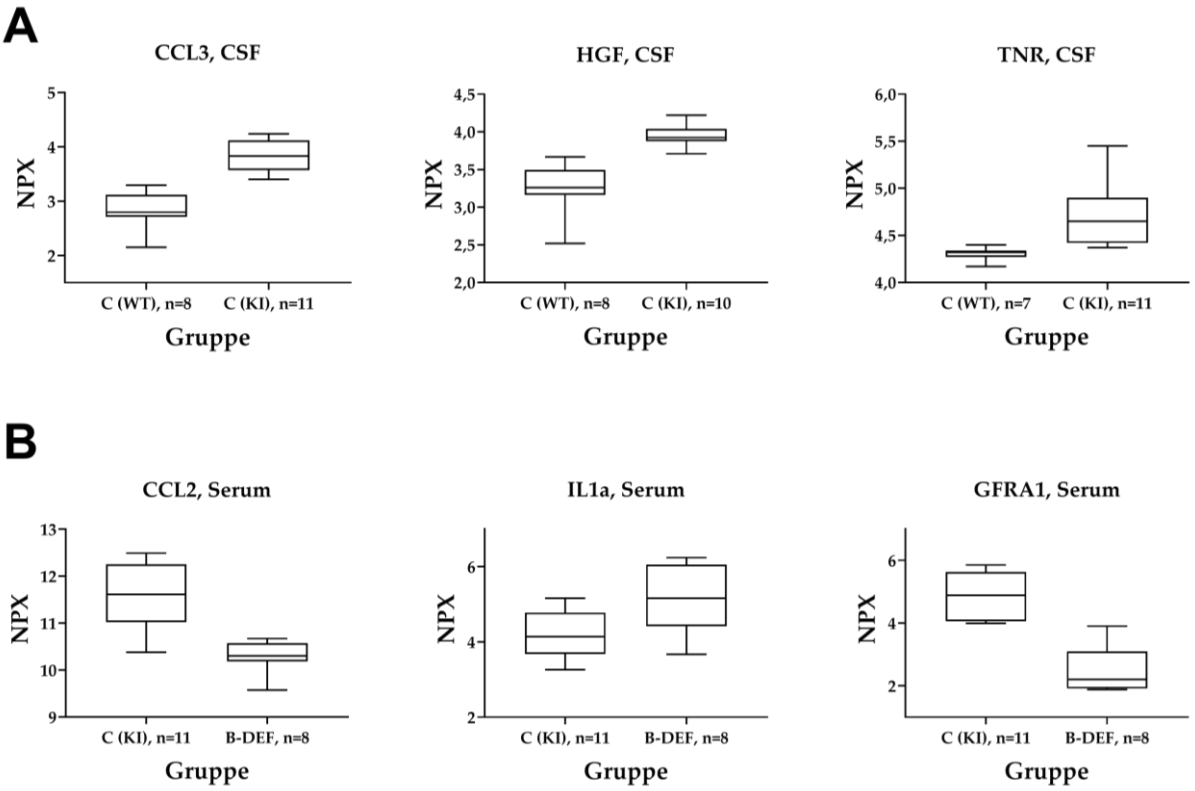

Abbildung 19. Auswahl deregulierter Proteinbiomarker mit neuronalen oder (neuro-) inflammatorischen Funktionen, finaler Entnahmezeitpunkt; (A) App^{NL-G-F} KI versus C57BL/6J WT; (B) HHCys versus App^{NL-G-F}-Kontrolle; geschlechtergepoolte Darstellung als NPX (normalized protein expression): eine von Olink-eingesetzte willkürliche Einheit mit log2-Skala; je höher NPX, desto höher die jeweilige Proteinkonzentration; Darstellung als Median ± IQR; alle hier gezeigten Beispiele erreichten statistische Signifikanz ($p<0.05$; non-parametrischer Mann-Whitney U-Test); Graphik modifiziert nach: siehe Anhang.

Die Verteilung der hoch- oder herunterregulierten Marker auf Serum oder CSF ist gruppenabhängig. Die hauptsächlichen effektstarken Funde im CSF, einer relevanten Matrix im Kontext der neurodegenerativen Erkrankungen, entfielen auf den Gruppenvergleich zwischen WT und KI und damit auf einen Einfluss des AD-Modells, der auch im Zusammenhang mit der Einschränkung im Habituierungslernen im Open Field Test stehen könnte. Dabei waren insbesondere Marker mit Bezug zu neuronaler Funktionalität, wie der „Hepatocyte growth factor“ (HGF) oder „Tenascin R“ (TNR) hochreguliert. HGF stellt einen neurotrophen Faktor dar (Ko *et al.*, 2018), der in diesem Fall vermutlich hochreguliert wurde, um der Schädigung des ZNS durch die induzierte cerebrale Amyloidose in diesem Modell zu begegnen. Translational interessant ist dabei, dass HGF ebenfalls in AD-Patienten erhöht zu sein scheint (Tsuboi *et al.*, 2003; Zhu *et al.*, 2018). Die Funktion von TNR ist stark abhängig von seinem jeweiligen molekularen Interaktionspartner (Probstmeier, Braunewell and Pesheva,

2000). Insbesondere in Kombination mit „Contactin 1“ (CNTN1), welches im CSF der *App*$^{NL-G-F}$ Tiere ebenfalls hochreguliert war, inhibiert es die Bildung von neuronalen Auswüchsen (Pesheva *et al.*, 1993; Apostolova, Irintchev and Schachner, 2006). Darüber hinaus wurde eine Hochregulierung des pro-inflammatorischen Chemokins „C-C Motif Chemokine Ligand 3“ (CCL3) sowohl im Serum, als auch im CSF der *App*$^{NL-G-F}$ Tiere sichtbar. CCL3 steht für neuronale Schädigung und destruktive Effekte auf die synaptische Plastizität und Lernfähigkeit durch Inflammationsprozesse im ZNS, die es hervorruft, indem es die Migration von T-Lymphozyten ins Hirn vereinfacht (Marciniak *et al.*, 2015; Zenaro and Constantin, 2017; Martin and Delarasse, 2018). (Neuro-) inflammatorische Prozesse sind ein weiteres in diesem Modell induziertes AD-Charakteristikum, wie von den Entwicklern des Modells in einem Review zusammengefasst wurde (Saito and Saido, 2018). Interessanterweise zeigte die durchgeführte Korrelationsanalyse eine mittelstarke positive Korrelation zwischen der cerebralen Amyloidose (Aβ42 Level) und den zuvor beschriebenen CSF Markern auf (genauer im Anhang). Das ergänzt einen früheren Bericht zur Korrelation zwischen Amyloidose und Neuroinflammation in diesen Tieren (Castillo *et al.*, 2017).

Im Gegensatz zum Einfluss der *App*$^{NL-G-F}$ Pathologie betraf die zusätzlich induzierte HHCys kaum Proteinmarker im CSF, insbesondere keine deregulierten neuronalen CSF-Marker mit großer Effektstärke, wie das für die KI Tiere im Vergleich zur WT Kontrolle der Fall war. Wohl aber resultierte die HHCys in der Deregulierung einer Vielzahl von Proteinmarkern im Serum (siehe Tabelle 7). Darunter waren auch Marker, die unter anderem neuronenbezogene Funktionen ausüben, wie zum Beispiel „GDNF family receptor alpha-1“ (GFRA1); (Sarabi *et al.*, 2003). Dass diese Deregulierungen nur im Serum und nicht im CSF stattfanden, deutet darauf hin, dass Auswirkungen auf Neuronen weniger im Vordergrund stehen als die zahlreichen spezifischen Funktionen, die diese Proteine im Organismus ausüben (können). Das wiederum steht im Einklang mit der Abwesenheit jeglicher phänotypischer Effekte in den Verhaltensversuchen in den hyperhomocysteinämischen Tieren. Speziell GFRA1 (Golden *et al.*, 1999), sowie weitere aus der Liste der deregulierten Proteine, spielen ohnehin (hauptsächlich) während früher Entwicklungsstadien eine Rolle (z.B. TPP1, ERBB4, DLL1 und andere; genauer im Anhang). Daher kommen diese in dem fortgeschritten Alter der Mäuse in dieser Studie weniger oder nicht mehr zum Tragen. Neurogenese findet zwar zu einem gewissen Teil und in wenigen Hirnregionen auch noch in Adulten statt, allerdings ist der Prozess primär auf die pränatale Periode beschränkt (Apple, Fonseca and Kokovay, 2017).

Es fällt auf, dass (neuro-) inflammatorische Prozesse durch HHCys hier in beiden Richtungen betroffen zu sein schienen: Neben einer Hochregulierung der Serumlevel des pro-inflammatorischen Interleukins IL1a (Dinarello, 2018) zeigten sich andere wie zum Beispiel „C-C Motif Chemokine Ligand 2“ (CCL2) (Martin and Delarasse, 2018) herunterreguliert im Vergleich zur KI Kontrollgruppe. Demnach ist unklar, ob letztendlich die pro- oder antiinflammatorischen Effekte überwiegen und in wie weit ein Einfluss auf die Immunantwort besteht. In Summe könnte das Fehlen kognitiver Beeinträchtigungen auch unter anderem dadurch erklärbar sein, dass sich diese im Hintergrund ablaufenden, gegenläufigen Prozesse gegenseitig neutralisierten im Hinblick auf eine phänotypische Ausprägung in den Tieren. Des Weiteren muss auch an der Stelle berücksichtigt werden, dass es sich hier um eine experimentell induzierte HHCys in den Tieren handelt und die Ergebnisse womöglich nicht eins zu eins mit einer HHCys im Menschen oder auch mit anderen Induktionsmethoden wie der genetischen, chemischen und anderen (Nieraad, Pannwitz, *et al.*, 2021) vergleichbar ist.

Abseits des Fokus auf neurotoxische oder (neuro-) inflammatorische Wirkungen ermöglichte das Olink Mouse Panel die Aufdeckung von Effekten, die nicht in direktem Bezug zur primären Forschungsfrage stehen, was eine Stärke des explorativen Charakters dieser Proteomanalyse darstellt. So wurde die Herunterregulierung mehrerer pro-angiogener Proteine in Serum (FAS, TNFRSF12A, TGFBR3 (Wiley *et al.*, 2001; Lambert, Landau and Desbarats, 2003; Ambartsumian, Klingelhöfer and Grigorian, 2019; Wang *et al.*, 2019)) und CSF (DLK, ACVRL1 (Urness, Sorensen and Li, 2000; Huang *et al.*, 2018)) detektiert (genauer im Anhang). Die verminderte Angiogenese könnte hier einen weiteren Aspekt der vaskulären Wirkungsweise von HCys und einen möglichen Beitrag zu kognitivem Niedergang darstellen, insbesondere in Kombination mit dem zuvor beschriebenen Auftreten einer Anämie in diesen Gruppen. Ein verminderter Hämoglobin- und Sauerstoffgehalt und ein weniger dichtes kapilläres Netz bewirkten dann auf verschiedene Weisen eine verminderte Versorgung der Neuronen mit Sauerstoff, ein Fund, der in anderen Tiermodellen, bzw. Humanstudien validiert werden sollte. Eine verringerte kapilläre Dichte im Hirn und ein dadurch verringerter cerebraler Blutfluss sowie Angebot an Sauerstoff und Glucose stehen im Verdacht, zur Einschränkung von synaptischer Funktionalität und kognitiver Leistungsfähigkeit in Älteren und AD-Patienten beizutragen (Katsimpardi *et al.*, 2014; Ambrose, 2015). Auch der Blick in die Literatur gibt Hinweise auf eine vornehmlich vaskuläre Wirkung von HCys, ein Link der translational gesehen relevant ist, da vaskuläre Demenzen mit AD in etwa 40% der Patienten co-inzident ist

(Sudduth *et al.*, 2013). Laut einer früheren klinischen Studie sind HCys Spiegel nicht bei familiärer AD, wohl aber bei vaskulärer Demenz erhöht (Nilsson, Gustafson and Hultberg, 2002) und auch eine rezente Meta-Analyse kam zu dem Ergebnis, dass HCys eine größere Relevanz für vaskuläre Demenz als für AD hat (Wang *et al.*, 2021).

Ebenfalls abseits der eigentlichen Forschungsfrage offenbarte die explorative Proteomanalyse eine Deregulierung diverser Proteinmarker durch HHCys im Hinblick auf verringerte Knochenmineralisierung, verringerten Knochenanabolismus und gesteigerten Knochenkatabolismus (TNFRSF11B, TGFBR3, FSTL3, WISP1, IL1a; genauer im Anhang). Dass HHCys in der Diskussion steht, zu erhöhter Knochenbrüchigkeit und einer Osteoporose-ähnlichen Pathologie beim Menschen beizutragen, wurde zum Beispiel kürzlich in einem Review zusammengefasst (Azzini, Ruggeri and Polito, 2020). Dahingehend sind die Funde dieser in vivo Studie auch translational gesehen möglicherweise relevant. Darüber hinaus könnte das leichtere Knochengerüst in diesen Tieren, neben dem geringeren Körperfettanteil, auch eine Erklärung für das signifikant geringere Gewicht in den hyperhomocysteinämischen Gruppen sein.

Eine Limitierung der Studie ist, dass nicht endgültig bestimmt werden kann, ob oder in wie weit die gesehenen Effekte von der HHCys selbst oder der zugrundeliegenden Defizienz an Vitamin B6, B12 und Folat abhängen. In letzterem Fall würde die hier gewählte diätetische Induktionsmethode einen Bias darstellen.

Abschließend fand auch in dieser Studie eine finale Bewertung der cerebralen Amyloidose statt. Dabei wurden lösliche und unlösliche Aβ-Anteile erfasst durch die Homogenisierung von Hirngewebe in Guanidin-Hydrochlorid-haltigem Lysepuffer und via ELISA quantifiziert, was einen Unterschied zur Vorläuferstudie darstellt (siehe In vivo Studie 1). Quantifiziert wurden hier spezifischerweise Aβ42-Peptide, da diese Aβ-Spezies zum einen insbesondere im App^{NL-G-F} Modell überexprimiert ist (Saito *et al.*, 2014) und zum anderen aufgrund seiner Hydrophobizität und hohen Neigung zur Aggregation eine übergeordnete Rolle bei der Plaquebildung spielt (Grimm, Michaelson and Hartmann, 2017).

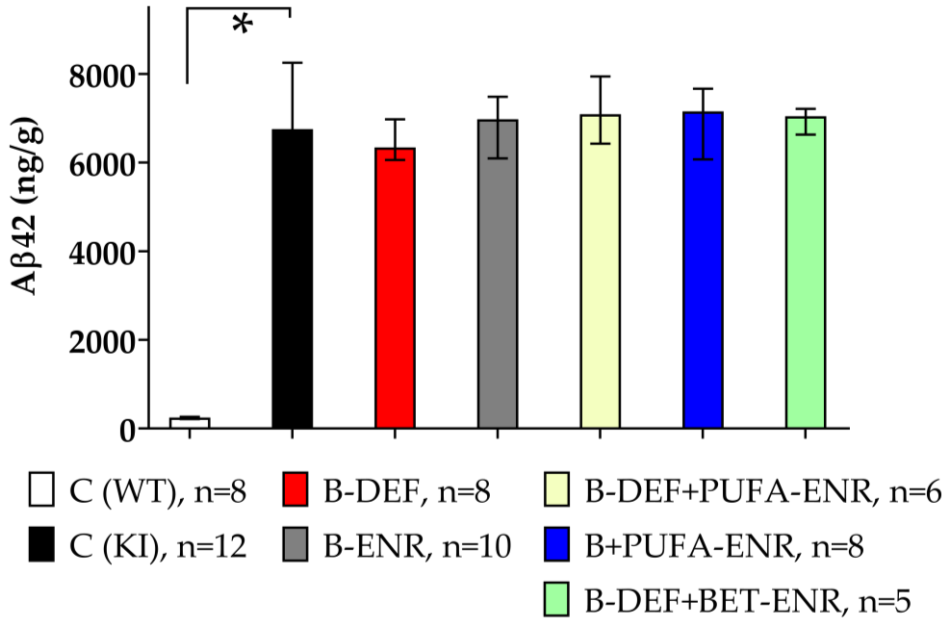

Abbildung 20. Quantifizierung der Aβ42-Peptide pro Gramm Hirngewebe (Nassgewicht) in einem 100 mg Querschnitt via ELISA am Ende der Studie (geschlechtergepoolt); Erfassung löslicher und unlöslicher Aβ-Anteile durch den Einsatz von Guanidin-Hydrochlorid im Lyse-Puffer; siehe Tabelle 6 für Gruppenbeschreibungen; Darstellung als Median ± IQR; $p<0.05$ (non-parametrischer Mann-Whitney U-Test) als statistisch signifikant angesehen (*); Graphik modifiziert nach: siehe Anhang.

Abbildung 20 bestätigt die Ausbildung der induzierten Amyloid-Pathologie im *App*$^{NL-G-F}$ Modell in dieser Studie und steht im Einklang mit den zuvor berichteten Trends (siehe In vivo Studie 1), d.h. die cerebralen Aβ-Level sind in den KI Tieren erwartungsgemäß hochsignifikant erhöht im Vergleich zur WT Kontrolle, während HHCys und supplementierte Mikronährstoffe keine statistisch signifikanten Effekte darauf ausübten. Auch die zusätzliche Korrelationsanalyse ergab keinen Zusammenhang zwischen der Höhe der Aβ-Konzentration im Hirn und den Serumspiegeln von HCys oder HCA, wobei diese im Falle einer HHCys auch cerebral erhöht sind (siehe In vivo Studie 2). Ein Einfluss von HCys auf die Plaquebildung wurde bereits in früheren Studien und anderen Tiermodellen kontrovers diskutiert (Pirchl, Ullrich and Humpel, 2010; Zhuo and Praticò, 2010a, 2010b; Kovalska *et al.*, 2018).

Dass trotz massiver cerebraler Amyloidose nur ein subtiler Einfluss auf die kognitive Leistungsfähigkeit in den KI Tieren im Vergleich zum WT detektiert wurde, spricht nicht für die „Amyloid-Hypothese“, laut der ein gestörter Aβ-Metabolismus die zentrale Säule der AD-Pathologie ist, die die weiteren pathologischen Prozesse bedingt (Selkoe and Hardy, 2016). Es ist bekannt, dass auch substanzielle Plaque-Ablagerungen nicht immer mit demenzähnlichen

Symptomen in Zusammenhang stehen (Aizenstein *et al.*, 2008). Aufgrund mangelnder therapeutischer Optionen gegen die Erkrankung trotz jahrzehntelanger Forschung, insbesondere im Kontext der Amyloid-Pathologie, wurde vorgeschlagen, dass alternative Ansätze (ZNS-Inflammation, Tau-Pathologie u.a.) stärker in den Fokus gerückt werden sollten (Panza *et al.*, 2019). Für die kausale Beteiligung von Aβ an der Erkrankung sprechen jedoch rezente Daten zum therapeutischen monoklonalen Antikörper Aducanumab, der kürzlich (Sommer 2021) in den USA zugelassen wurde, dessen Wirkung allerdings nicht unumstritten ist, da die Daten aus den zugehörigen klinischen Studien nicht vollends konsistent waren (Kaplon *et al.*, 2020). Es wird spannend zu sehen sein, wie sich die Datenlage zu Aducanumab, auch nach weiteren erfolgten Zulassungen, entwickeln wird. Die Amyloid-Hypothese selbst ist und bleibt ein wiederkehrender und kontroverser Diskussionspunkt in der Pathologie der Alzheimer-Erkrankung.

Zusammenfassung und Ausblick

Kurz zusammengefasst waren die Ziele dieser Arbeit, die in vivo Untersuchung einer Hyperhomocysteinämie und spezifischer diätetischer Mikronährstoffe im Kontext der Alzheimer-Erkrankung. Zu diesem Zweck wurden zwei Krankheitsmodelle in den Mäusen induziert. Zum einen wurde eine Alzheimer-ähnliche Pathologie genetisch simuliert durch den Einsatz des neuen *App*$^{NL\text{-}G\text{-}F}$ knock-in Modells, das im Zuge dieser Arbeit auch weiter charakterisiert wurde. Zum anderen wurde eine chronische Hyperhomocysteinämie in den Tieren induziert via Langzeit-Fütterung einer Spezialdiät, die defizient an den Vitaminen B6, B12 und Folat war, was sich durch erhöhte Werte der Aminosäuren Homocystein und Homocysteinsäure in verschiedenen biologischen Matrices der Mäuse wie Serum, Urin und Hirngewebe, bemerkbar machte. Durch die Kombination der Krankheitsmodelle wurden sowohl Aspekte einer familiären Alzheimer-Erkrankung (verstärkter Amyloid-β-Anabolismus im knock-in Modell) als auch ein potentielles Charakteristikum der sporadischen Form der Krankheit (erhöhte Homocystein-Spiegel) simuliert. Auswirkungen des *App*$^{NL\text{-}G\text{-}F}$ Genotyps, einer zusätzlichen Hyperhomocysteinämie und potentiell vorteilhafter, oder gar präventiv wirksamer Mikronährstoffe wurden dabei mit Hilfe von diversen Verhaltensversuchen und ergänzenden ex vivo Analysen bewertet.

Trotz massiver cerebraler Amyloidose war lediglich ein milder Einfluss auf die kognitive Leistungsfähigkeit der *App*$^{NL\text{-}G\text{-}F}$ Tiere im Vergleich zur gleichaltrigen Wildtyp-Kontrolle detektierbar. Dies weist zum einen auf die Subtilität des Mausmodells hin und zum anderen befeuert es die kontroverse, häufig geführte Diskussion um die zentrale Bedeutung der „Amyloid-Hypothese“ im Rahmen der komplexen Alzheimer-Pathologie. Die kognitiven Fähigkeiten der entsprechenden Mäuse verschlechterten sich auch nicht bei gleichzeitig signifikant erhöhten Homocystein- und Homocysteinsäurespiegeln, d.h. die Hyperhomocysteinämie hat in diesem Modell für familiären Alzheimer nicht kausal zur Verschlimmerung der induzierten Pathologie beigetragen sowohl hinsichtlich der kognitiven Leistung in diversen Verhaltensversuchen als auch hinsichtlich dem Schweregrad der cerebralen Amyloidose.

Zur Hyperhomocysteinämie, vor allem aber auch zur Rolle bestimmter diätetischer Interventionen in dem Kontext, findet man eine heterogene, teilweise konträre Literatur vor, insbesondere im klinischen Kontext. Die untersuchten diätetischen Ansätze in dieser Arbeit,

bestehend aus hochdosierten B-Vitaminen, mehrfach ungesättigten Fettsäuren, Betain und einer komplexeren Mikronährstoffkombination, zeigten ebenfalls keinen konsistenten Effekt auf Phänotyp und Amyloid-β-Menge in den Hirnen der Tiere. Die Ergebnisse der durchgeführten Studien legen daher, zumindest in diesem Krankheitsmodell, keinen Wert als potentiell präventiven Ansatz der kognitiven Verschlechterung bei Alzheimer nahe.

Da die dieser Arbeit zugrundeliegenden in vivo Studien keine per se erhöhten, *App*$^{NL\text{-}G\text{-}F}$-assoziierten Homocysteinspiegel offenbarten, zeigte sich Homocystein nicht als Biomarker, zumindest für die in diesem Mausmodell simulierten Aspekte der komplexen Alzheimer-Pathologie. Neben den zuvor beschriebenen fehlenden Effekten der Hyperhomocysteinämie, konnten in dieser Arbeit jedoch auch statistisch signifikante Einflüsse sichtbar gemacht werden. Wie in der durchgeführten Kinetikstudie gezeigt, resultierte die Alzheimer-ähnliche Pathologie in einem signifikant höheren Schweregrad der ausgebildeten Hyperhomocysteinämie in den *App*$^{NL\text{-}G\text{-}F}$ Tieren im Vergleich zur gleichaltrigen Wildtyp-Kontrolle. Folglich übte der gestörte Amyloid-β-Metabolismus, neben der B-vitamindefizienten Diät, einen zusätzlich verstärkenden Effekt auf den hyperhomocysteinämischen Status aus. Sowohl für knock-in-, als auch Wildtyp-Tiere konnte gezeigt werden, dass bei Beendigung der Karenz an Vitamin B6, B12 und Folat, die erhöhten Homocystein- und Homocysteinsäurespiegel innerhalb kurzer Zeit wieder auf Baseline-Niveau normalisiert werden können.

Weitere signifikante Effekte wurden detektiert bezüglich Erythrozyten-bezogener Parameter wie den Hämoglobingehalt im Blut der hyperhomocysteinämischen Tiere. Ein reduzierter Sauerstofftransport und die damit einhergehende verringerte Versorgung der Neuronen mit Sauerstoff in den entsprechenden experimentellen Gruppen deuten auf eine vornehmlich vaskuläre Wirkung hin im Hinblick auf Homocystein-bezogene Pathomechanismen, die potentiell zu einer Demenz beitragen. Solche Effekte können zusätzlich verstärkt worden sein durch die, in der durchgeführten Proteomanalyse gezeigte, Herunterregulierung angiogener Marker im Serum und in der Cerebrospinalflüssigkeit dieser Tiere. Eine Verringerung der kapillären Dichte im Hirn und ein verringerter cerebraler Blutfluss haben ein zusätzlich reduziertes Angebot an Sauerstoff und Glucose zur Folge und stellen einen Link zu eingeschränkter kognitiver Leistungsfähigkeit in Älteren und Alzheimer-Patienten dar. Translational relevant ist eine vaskuläre Wirkung von Homocystein auch dadurch, dass vaskuläre Demenz und Alzheimer in etwa 40% der Fälle koinzident sind und Homocystein in

früheren Humanuntersuchungen eine größere Bedeutung bei der vaskulären Demenz im Vergleich zur Alzheimer-Erkrankung nahelegte.

Auch wenn in Summe die beschriebenen Effekte der Hyperhomocysteinämie nicht groß genug waren, um sich in phänotypischen Einschränkungen in den Tieren auszudrücken, so konnten in der hier vorliegenden Arbeit dennoch Details zur Rolle erhöhter Homocysteinspiegel für verschiedene biologische Prozesse aufgeklärt werden. Insbesondere die Funde der explorativen Proteomanalyse in Serum und CSF könnten Ansatzpunkte für weitergehende Untersuchungen darstellen und sollten in anderen präklinischen Krankheitsmodellen und/oder einer Humanstudie validiert werden.

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Anhang

Publikationen

Publikationsliste

Peer-Review Publikationen im Rahmen der Promotion

Nieraad, H. et al. (2020) 'Impact of Hyperhomocysteinemia and Different Dietary Interventions on Cognitive Performance in a Knock-in Mouse Model for Alzheimer's Disease', Nutrients, 12(11), p. 3248. doi: 10.3390/nu12113248.

Nieraad, H., de Bruin, N., et al. (2021) 'Effects of Alzheimer-Like Pathology on Homocysteine and Homocysteic Acid Levels—An Exploratory In Vivo Kinetic Study', International Journal of Molecular Sciences, 22(2), p. 927. doi: 10.3390/ijms22020927.

Nieraad, H., Pannwitz, N., et al. (2021) 'Hyperhomocysteinemia: Metabolic Role and Animal Studies with a Focus on Cognitive Performance and Decline—A Review', Biomolecules, 11(10), p. 1546. doi: 10.3390/biom11101546.

Nieraad, H., de Bruin, N., Arne, O., Hofmann, M. C. J., Pannwitz, N., Resch, E., Luckhardt, S., Schneider, A., Trautmann, S., Schreiber, Y., Gurke R., Parnham, M. J., Till, U. and Geisslinger, G. 'The Roles of Long-term Hyperhomocysteinemia and Micronutrient Supplementation in the App^{NL-G-F} Model of Alzheimer's Disease' (unveröffentlichtes Manuskript)

Poster

Nieraad, H. et al. (2020) 'P.759 Impact of hyperhomocysteinemia and different dietary interventions on cognitive performance in a knock-in mouse model for Alzheimer's disease', European Neuropsychopharmacology, 40 (Supplement 1), p. S429. doi: 10.1016/j.euroneuro.2020.09.557.

Anteile des Autors und der Co-Autoren an den Publikationen

Nieraad, H. et al. (2020) 'Impact of Hyperhomocysteinemia and Different Dietary Interventions on Cognitive Performance in a Knock-in Mouse Model for Alzheimer's Disease', Nutrients, 12(11), p. 3248. doi: 10.3390/nu12113248.

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- Takashi Saito und Takaomi Saido: Entwicklung und Bereitstellung des $App^{NL\text{-}G\text{-}F}$ knock-in Mausmodells
- Robert Gurke und Dominik Schmidt: Entwicklung / Anwendung der LC-MS/MS Methodik zur Analyse von HCys, HCA
- Uwe Till: Studiendesign, fachliche Beratung, insbesondere zu den experimentellen Diäten und zur Induktion der Hyperhomocysteinämie in vivo
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Nieraad, H., Pannwitz, N., et al. (2021) ‘Hyperhomocysteinemia: Metabolic Role and Animal Studies with a Focus on Cognitive Performance and Decline—A Review’, Biomolecules, 11(10), p. 1546. doi: 10.3390/biom11101546.

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- Michael Parnham: Supervision, fachliche und sprachliche Verbesserung des Manuskripts
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Publikationsmanuskripte

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MDPI

Article

Impact of Hyperhomocysteinemia and Different Dietary Interventions on Cognitive Performance in a Knock-in Mouse Model for Alzheimer's Disease

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Abstract: Background: Hyperhomocysteinemia is considered a possible contributor to the complex pathology of Alzheimer's disease (AD). For years, researchers in this field have discussed the apparent detrimental effects of the endogenous amino acid homocysteine in the brain. In this study, the roles of hyperhomocysteinemia driven by vitamin B deficiency, as well as potentially beneficial dietary interventions, were investigated in the novel App^{NL-G-F} knock-in mouse model for AD, simulating an early stage of the disease. Methods: Urine and serum samples were analyzed using a validated LC-MS/MS method and the impact of different experimental diets on cognitive performance was studied in a comprehensive behavioral test battery. Finally, we analyzed brain samples immunohistochemically in order to assess amyloid-β (Aβ) plaque deposition. Results: Behavioral testing data indicated subtle cognitive deficits in App^{NL-G-F} compared to C57BL/6J wild type mice. Elevation of homocysteine and homocysteic acid, as well as counteracting dietary interventions, mostly did not result in significant effects on learning and memory performance, nor in a modified Aβ plaque deposition in 35-week-old App^{NL-G-F} mice. Conclusion: Despite prominent Aβ plaque deposition, the App^{NL-G-F} model merely displays a very mild AD-like phenotype at the investigated age. Older App^{NL-G-F} mice should be tested in order to further investigate potential effects of hyperhomocysteinemia and dietary interventions.

Keywords: hyperhomocysteinemia; vitamin B deficiency; Alzheimer's disease; amyloid beta-peptides; disease models; animal; memory and learning tests

1. Introduction

After decades of research, there is still a huge unmet medical need for novel interventions to treat dementia-like disorders. In 2019 more than 50 million people were affected by dementia and the number could increase to about 152 million by 2050 [1]. Alzheimer's disease (AD) is the most common type of dementia, accounting for 2/3 of all cases [2]. More than 400 failures in drug development during the last decades [3] have led to the consideration of alternative intervention options, e.g., repurposing, combinatory approaches and preventive treatments [4].

The complex pathology of the disease is characterized by several hallmarks, such as prominent extracellular amyloid plaques [5,6]. According to the amyloid cascade hypothesis, an alteration of amyloid-β (Aβ) metabolism is the central pillar of AD pathology and crucially influences and initiates other hallmarks [7]. In AD, initial pathologic processes progress decades before the first cognitive symptoms appear in patients, a stage entitled preclinical Alzheimer's [8]. Disruptions in amyloid metabolism, as one of the first chronological hallmarks, potentially represent a relevant target for preventive interventions in AD.

In order to further elucidate disease mechanisms and identify novel treatment options, the group of Takaomi Saido at the RIKEN Center for Brain Science has developed a new generation of AD mouse models. These knock-in (KI) mice provide advantages compared to transgenic models, which are based on massive amyloid-β protein precursor (AβPP) overexpression with the result of artificial phenotypes due to overproduction of other AβPP fragments aside from Aβ. In the App^{NL-G-F} model, the murine AβPP sequence is humanized and three mutations are introduced. Swedish (NL), Arctic (G) and Beyreuther/Iberian mutations (F) increase the total amount of Aβ and the Aβ42/Aβ40 ratio, show pro-inflammatory effects and finally result in a three times faster memory impairment [9].

Elevated levels of the endogenous amino acid homocysteine (HCys), called hyperhomocysteinemia, have been described as another hallmark of AD [10]. HCys is increased significantly in AD patients, whereas levels of different B-vitamins are reduced compared to controls [11,12]. A remaining question is whether hyperhomocysteinemia is merely a marker or whether it contributes causally to AD pathology, thereby providing options for therapeutic intervention. Some authors describe the role of plasma HCys as an independent risk factor for memory deficits and AD [13,14]. Consequently, B-vitamin supplementation as a HCys-modifying intervention was proposed previously [15]. According to Smith et al., B-vitamins lowered HCys levels and subsequently slowed the rate of brain atrophy and cognitive decline in patients [16,17]. However, a causal link between hyperhomocysteinemia and Alzheimer's disease, called the "homocysteine hypothesis", has been a source of controversy for years. Kennedy teased out the equivocal results of numerous studies in detail [18]. Several studies neither support an association of HCys with AD nor an improvement of cognitive performance by B-vitamin treatment [19,20]. Meta-analyses were conducted to assess this topic, challenging the homocysteine hypothesis and amelioration of cognitive functions by the use of folate and other B-vitamins [21,22]. In the context of an international consensus statement, researchers assessed the homocysteine hypothesis as being plausible and considered hyperhomocysteinemia a modifiable risk factor for dementia. Furthermore, they recommended considering polyunsaturated fatty acids (PUFAs) in addition to B-vitamins for future trials [23]. PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are also suggested to be linked to AD pathology and HCys metabolism [24,25], i.e., elevated HCys impairs the formation of PUFAs and leads to a lower availability of PUFAs in the brain. B-vitamin treatment might only be successful when PUFA plasma concentrations are in the upper normal range [26]. A more recent systematic review points out that the evidence for nutrient supplementation remains limited and indicates that more research is needed to assess preventive measures in dementia [27].

Transsulfuration and re-methylation are major metabolic pathways for HCys (Figure 1), being dependent on an adequate supply of B-vitamins, particularly B6, B12 and folate [28]. As illustrated, the relevant B-vitamins play key roles in intrinsically decreasing HCys levels and therefore correlate negatively with HCys. Vitamin B12 and folate are crucial in providing methyl groups in the context

of the re-methylation cycle, whereas the transsulfuration pathway depends on vitamin B6 as an essential enzymatic cofactor. Disturbed HCys metabolism (Figure 1) is likely to be linked to AD pathology by direct and indirect neurotoxic pathways [24]. Neurotoxicity is caused by excitotoxicity via N-methyl-D-aspartate receptor (NMDA) activation and by increased levels of reactive oxygen species promoting oxidative stress. Furthermore, excess HCys and subsequently a lack of methionine and S-Adenosyl-L-methionine (SAM), as well as elevated S-Adenosyl-L-homocysteine (SAH), are associated with a reduced methylation capacity and the inhibition of methylation reactions, which is suggested to exacerbate amyloid and tau pathologies in AD. Moreover, HCys results in an activated immune system, damages cerebral vessels and disrupts the blood-brain-barrier [24,29]. Both homocysteine and its oxidative metabolite homocysteic acid (HCA) are considered neurotoxic [30,31], but HCA is suggested to be the more potent species [32–34] and might contribute to dementia through oxidative stress and excitotoxicity by NMDA activation. Both mechanisms have been considered relevant for AD pathology [5,24].

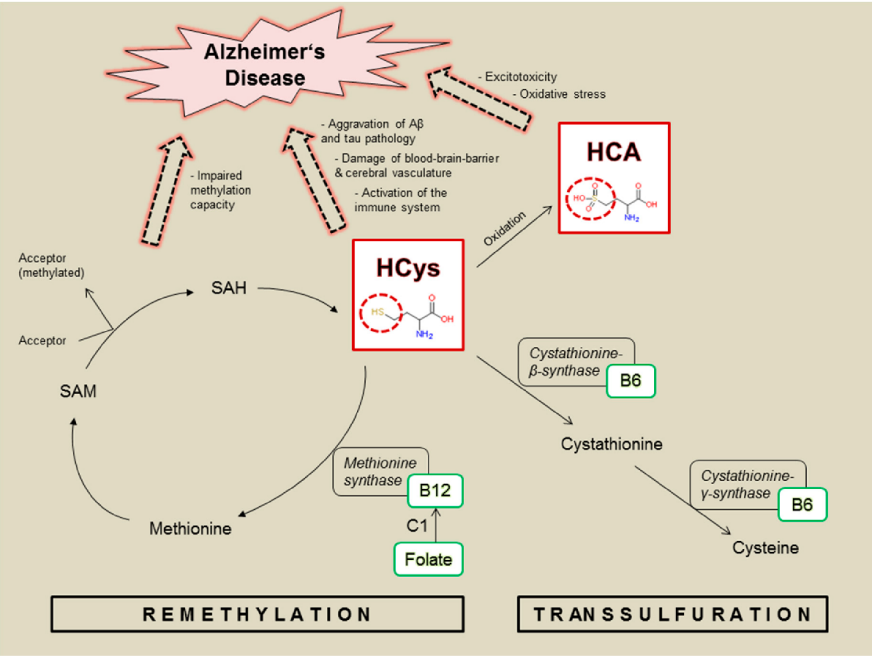

Figure 1. Homocysteine (HCys) and homocysteic acid (HCA): metabolic role and link to Alzheimer's disease; involved enzymes (black boxes) linked to relevant B-vitamins (green boxes) functioning as coenzymes or methyl donor (C1); SAM = S-Adenosyl-L-methionine; SAH = S-Adenosyl-L-homocysteine.

The present exploratory animal study concentrates on the role of hyperhomocysteinemia, driven by vitamin B deficiency, in the context of AD. Therefore, we used the novel and not yet fully characterized App^{NL-G-F} knock-in mouse as a model of the disease. The App^{NL-G-F} mouse is expected to display a mildly impaired phenotype, simulating the very early preclinical period of AD pathology and thus should provide the possibility of assessing preventive interventions adequately. A versatile behavioral test battery should firstly assess potential deterioration of cognitive performance by hyperhomocysteinemia. Secondly, behavioral testing should clarify whether special diets enhance cognition and potentially

could serve as preventive measures for AD. Here, we compared B-vitamins and PUFAs with a more complex micronutrient mixture similar to Fortasyn® Connect [35]. HCys and HCA levels were measured in urine and serum using a validated LC-MS/MS method (liquid chromatography-tandem mass spectrometry) and the quantity of Aβ plaques in the brains was assessed.

2. Materials and Methods

A detailed description of all experimental procedures including the single behavioral testing systems, analytical methodologies and quality parameters of the current study can be found in Appendix A.

2.1. Animals and Experimental Diets

All experimental procedures were carried out in compliance with the '3R' and in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication no. 86-23, revised 1985), the DIRECTIVE 2010/63/EU and the regulations of GV-SOLAS and were approved by the local Ethics Committee for Animal Research in Darmstadt, Germany (approval number: F152/1011; approval date: 31.07.2017). In the current study, 16 C57BL/6J wild type mice (WT) and 96 homozygous App^{NL-G-F} knock-in (KI) mice, consisting equally of males and females, were included.

AIN93M chow served as a basis for the experimental diets and was modified, defining the different groups of App^{NL-G-F} mice (Table 1). The exact composition of the diets is summarized in Table A1. Each mouse received four grammes of diet per day, except for the period of food restriction for males during the touchscreen PAL-task. Water was available ad libitum, except for the period of temporally conditioned water access for females during the IntelliCage experiment.

Table 1. Details of the experimental groups.

Group Number	Genotype	Diet	Abbreviation
1	C57BL/6J wild type	Control	C (WT)
2	App^{NL-G-F} knock-in	Control	C (KI)
3	App^{NL-G-F} knock-in	Vitamin B deficient	B-DEF
4	App^{NL-G-F} knock-in	Vitamin B enriched	B-ENR
5	App^{NL-G-F} knock-in	PUFA supplemented	PUFA-ENR
6	App^{NL-G-F} knock-in	Vitamin B enriched and PUFA supplemented	B+PUFA-ENR
7	App^{NL-G-F} knock-in	Fortasyn® Connect-like	FC

2.2. Behavioral Testing

The testing battery we conducted consisted of diverse behavioral tests investigating different domains of cognition in the animals (Figure 2). At the age of 15 weeks, resp. 10 weeks on diet, the mice were first tested in the open field, followed by the elevated zero maze, Barnes maze and social interaction test. Finally, males were tested in a touchscreen task and females in the IntelliCage system.

Outcomes of every behavioral experiment were assessed automatically by camera or transponder detection. All experiments were performed between 8 a.m. and 3 p.m. during the light phase. After each trial, testing systems were cleaned with 70% ethanol to remove odors in the devices and to achieve comparable conditions for each animal.

2.3. Sample Collection

As illustrated in Figure 2, serum and 24-h urine of the mice were sampled after 8 and 30 weeks on experimental diets, resp. 13 and 35 weeks of age. The biological matrices were stored at −80 °C for subsequent analysis of HCys and HCA. At the end of the study, we euthanized all animals at the age of 35 weeks in order to harvest the brains. Brains were removed and post-fixed in 4% paraformaldehyde, followed by a stepwise dehydration, and embedding in paraffin. Ten µm thick sections were cut and mounted on glass slides for subsequent immunohistochemical analysis.

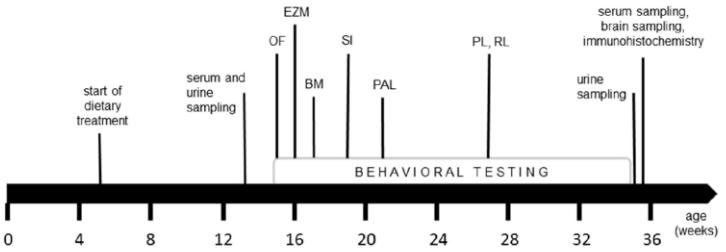

Figure 2. Time line of the study course; open field test (OF), elevated zero maze (EZM), Barnes maze (BM), social interaction test (SI), touchscreen paired associates learning (PAL) inclusive training phase, IntelliCage place learning task (PL) and reverse learning task (RL) inclusive habituation period; see Appendix A for detailed explanations of the single tests.

2.4. Biochemical and Immunohistochemical Analyses

The determination of HCA was performed as previously described in detail [36] using a combination of protein precipitation and solid phase extraction for sample preparation followed by an LC–MS/MS analysis applying a combination of a HILIC separation and tandem mass spectrometry. HCys was analyzed using protein precipitation in combination with reversed phase chromatography and tandem mass spectrometry.

Brain sections were immunohistochemically stained for amyloid-β peptides (Aβ) using an ABC/DAB protocol that is described in detail in Appendix A. After digitization of the sections, we analyzed the resulting images for the area of Aβ plaques in several regions of interest (ROI; Table A2), using ImageJ software.

2.5. Statistical Analyses

All experiments were statistically analyzed using IBM SPSS Statistics 25 (Ehningen, Germany). For each test, we conducted an outlier analysis in order to exclude extreme outliers (more than three times the interquartile range). Shapiro Wilk tests revealed whether Gaussian distribution could be assumed or not. Because of several data sets, which did not show a normal distribution, testing of statistically significant differences was computed by non-parametric Mann-Whitney-U-tests (comparison 1: C57BL/6J (group 1) versus $App^{NL\text{-}G\text{-}F}$ control (group 2); comparison 2: $App^{NL\text{-}G\text{-}F}$ control (group 2) versus $App^{NL\text{-}G\text{-}F}$ on special diets (groups 3–7)). A p value lower than 0.05 was considered statistically significant. Results were expressed as median ± interquartile range (IQR). Where applicable, medians were further compared to hypothetical medians using the non-parametric one-sample Wilcoxon signed rank test.

Graphical presentation was performed using GraphPad Prism 7 software (San Diego, CA, USA).

3. Results

3.1. Homocysteine and Homocysteic Acid

LC-MS/MS analysis was performed in order to measure HCys and its oxidative metabolite HCA in serum and urine samples. Vitamin B deficiency resulted in an elevation of both HCys and HCA serum levels in males and females after 8 weeks on experimental diet (HCys male $p < 0.001$, female $p = 0.001$; HCA (pooled) $p < 0.001$) (Figure 3A,C). A consistent statistically significant difference between C57BL/6J wild type (WT) and $App^{NL\text{-}G\text{-}F}$ knock-in (KI) mice was not observed. Dietary interventions resulted in decreased serum levels of HCys (PUFA-ENR male $p = 0.001$, female $p = 0.005$; B+PUFA-ENR male $p < 0.001$, female 0.026; FC male & female $p < 0.001$). Serum samples had to be pooled for an adequate analysis of HCA because of low sample volumes obtained by vena facialis puncture (Figure 3C).

Because of the resulting decreased number of observations, data are not depicted separately for males and females in this case. After 30 weeks on the diet, vitamin B deficient males remained significantly hyperhomocysteinemic (*HCys* $p = 0.001$; *HCA* $p = 0.001$), although to a lower extent, compared to 8 weeks on the diet, whereas females returned to baseline level due to the maintenance chow they received during the IntelliCage tasks. Analysis of 24-h urine samples delivered data that were largely comparable to the results from the serum samples. After 8 weeks on the diets (Figure 3E,G), both urinary HCys and HCA were significantly elevated because of the vitamin B deficient chow (HCys male & female $p < 0.001$; HCA male $p = 0.001$, female $p = 0.035$), whereas a genotype effect was not detectable. Experimental diets resulted in decreased amounts of HCys (PUFA-ENR female $p = 0.014$) and HCA (B-ENR female $p = 0.001$; PUFA-ENR female $p = 0.022$; FC female $p = 0.040$) in the urine compared to KI control mice. After 30 weeks on diets (Figure 3F,H), males deficient in vitamin B6, B12 and folate displayed elevated urinary amounts of HCys ($p = 0.001$) and HCA ($p = 0.003$), but to a lower extent compared to that after 8 weeks on the diets. Vitamin B deficient females showed equal quantities to the control groups due to the maintenance chow they had received during the IntelliCage tasks.

3.2. Open Field

This behavioral test aimed to evaluate locomotion, anxiety, and habituation behavior of the mice during a 30-min session in the open field boxes. The total distance moved revealed no statistically significant differences (Figure 4A). Consequently, locomotion activity was not influenced by genotype or dietary intervention. The time the animals spent in the inner zone of the box, an indicator of anxiety, was not affected by genotype or diet (Figure 4B). As a third parameter, the amount of intrasession habituation was expressed by a habituation ratio (Equation (1)):

$$\text{ratio intrasession habituation} = (5\ \text{min(final)})/((5\ \text{min(final)} + 5\ \text{min(initial)})) \quad (1)$$

A ratio lower than 0.5 indicates habituation; a ratio of 0.5 means no change in activity, i.e., that no habituation occurred as in the case of groups 2–6 in males and groups 3 and 5–6 in females. Females fed with a vitamin B deficient chow displayed the least tendency to habituate; however, effects of experimental diets did not reach statistical significance in comparison to the KI control group. Female $App^{NL\text{-}G\text{-}F}$ control mice displayed a significantly lower level of habituation compared to the C57BL/6J WT control ($p = 0.009$), indicating an impact of the genotype (Figure 4C).

3.3. Elevated Zero Maze

We tested anxiety behavior of each mouse for a session duration of 5 min. C57BL/6J WT and $App^{NL\text{-}G\text{-}F}$ KI control mice moved equal distances in the maze; only male $App^{NL\text{-}G\text{-}F}$ mice fed with a vitamin B and PUFA enriched diet moved less than $App^{NL\text{-}G\text{-}F}$ controls ($p = 0.003$) and thus displayed lower locomotion activity (Figure 5A). The time spent in the open corridors of the maze was an index for open space-induced anxiety in mice (Figure 5B). No genotype effect was observed between C57BL/6J and $App^{NL\text{-}G\text{-}F}$ mice, whereas different dietary interventions showed a reduction of cumulative time in open corridors. Particularly, male mice fed with the combination of PUFA and vitamin B enriched chow as well as with FC-like, spent significantly less time in the open corridors (*B+PUFA-ENR* $p < 0.001$; *FC* $p = 0.021$) and thus displayed increased anxiety. In females, a reduced time in the open corridors was observed in the vitamin B deficient group ($p = 0.040$) compared to KI control mice.

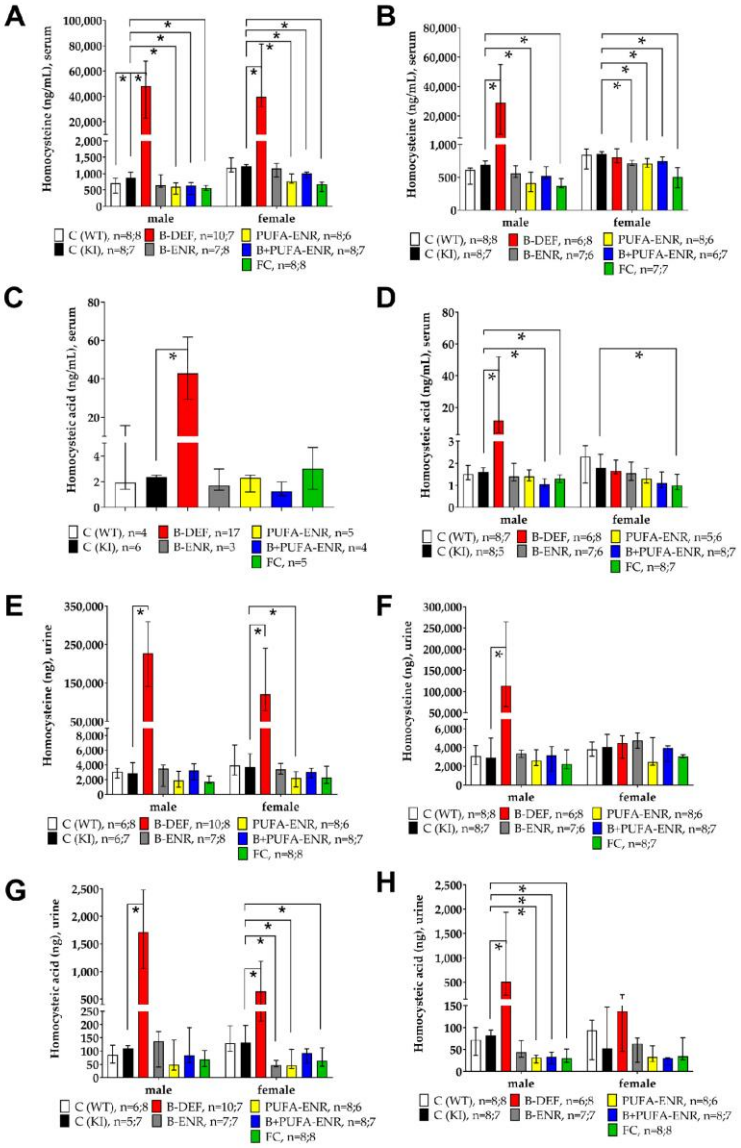

Figure 3. Homocysteine (HCys) and homocysteic acid (HCA) urine and serum levels in 13 and 35 weeks old C57BL/6J and $App^{NL\text{-}G\text{-}F}$ mice; 8 resp. 30 weeks on experimental diet; all samples analyzed by LC-MS/MS; data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*). (**A**) HCys serum levels; 8 weeks on diet. (**B**) HCys serum levels; 30 weeks on diet. (**C**) HCA serum levels; 8 weeks on diet (males and females pooled); samples pooled for analytical method due to low volumes obtained by vena facialis puncture. (**D**) HCA serum levels; 30 weeks on diet. (**E**) HCys urine levels; 8 weeks on diet. (**F**) HCys urine levels; 30 weeks on diet. (**G**) HCA urine levels; 8 weeks on diet. (**H**) HCA urine levels; 30 weeks on diet.

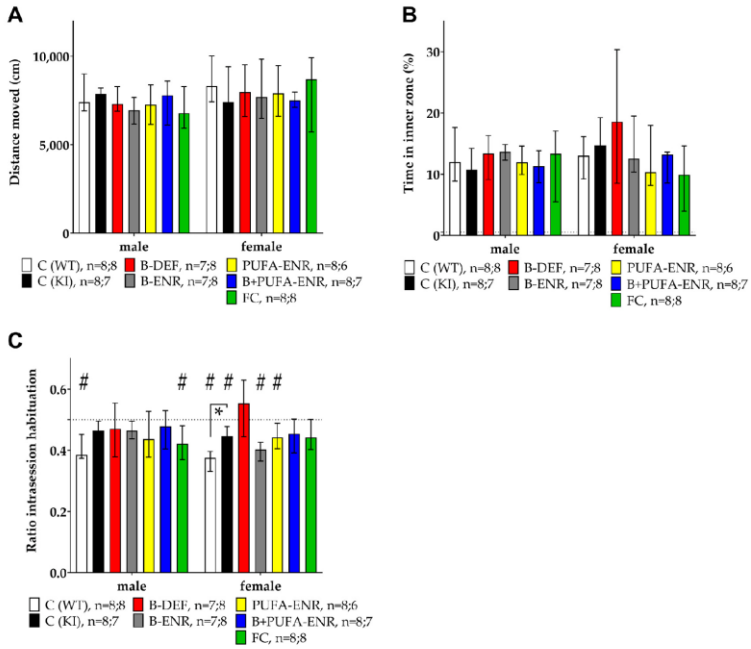

Figure 4. Open field test (30 min) in 15 weeks old C57BL/6J and App^{NL-G-F} mice; 10 weeks on experimental diet; data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*). (**A**) Total distance moved. (**B**) Percentage of time spent in inner zone. (**C**) Intrasession habituation expressed as ratio between the distance moved in the final time block divided by the sum of the final and the initial time block. ($^{\#}$) Ratio different from 0.5 (one-sample Wilcoxon signed rank test).

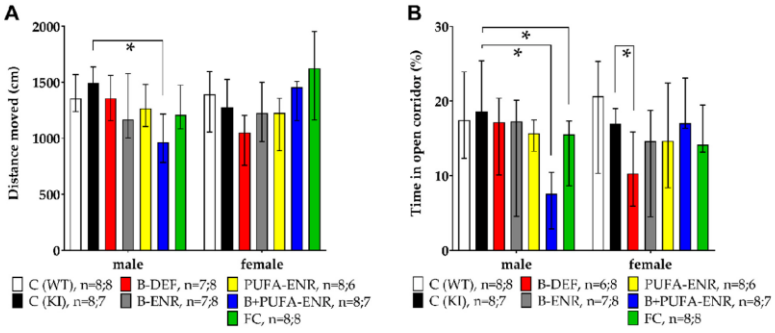

Figure 5. Elevated zero maze test (5 min) in 16 weeks old C57BL/6J and App^{NL-G-F} mice; 11 weeks on experimental diet; data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*). (**A**) Total distance moved. (**B**) Percentage of time spent in open corridors.

3.4. Barnes Maze

To investigate spatial memory and learning, the Barnes maze test was implemented in this study. In the first part of the test, the acquisition phase, the mice had to learn and remember the location of the escape box at the target hole. Figure 6A shows the latencies the mice needed to reach the target hole on subsequent days of training in the acquisition phase. The graph indicates a learning curve in every group. Tests on statistical significance were carried out for day 4 and revealed no differences at this stage of the test. In the probe trial on day 5 (Figure 6B), the reference memory of the previously learned target hole was tested. At this time, female $App^{NL\text{-}G\text{-}F}$ controls needed significantly longer to reach the target hole compared to the C57BL/6J WT control animals ($p = 0.016$). Vitamin B deficiency and corresponding hyperhomocysteinemia did not result in a worse performance at any stage of the Barnes maze test.

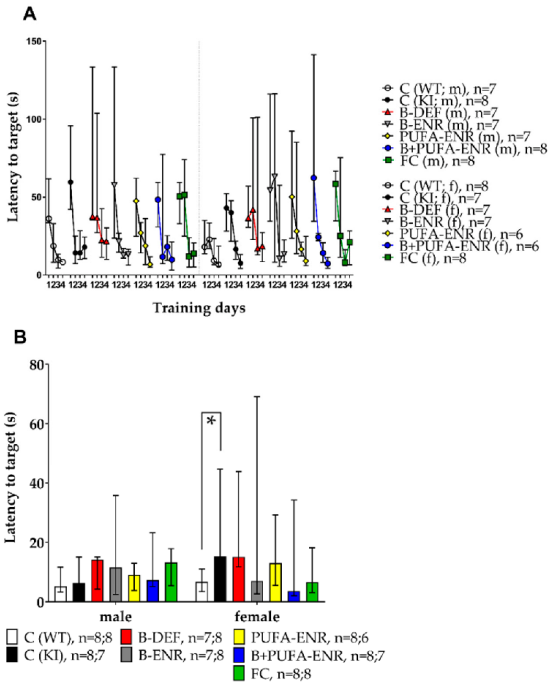

Figure 6. Barnes maze test in 17–18 weeks old C57BL/6J and $App^{NL\text{-}G\text{-}F}$ mice; 12–13 weeks on experimental diet; data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*). (**A**) Latency to target hole; training days 1–4; 180 s per trial; acquisition phase; test on statistical significance carried out for day 4. (**B**) Latency to target hole; day 5; 90 s per trial; probe trial.

3.5. Social Interaction Test

Testing social behavior proceeded in two subsequent phases. At first, we assessed sociability, describing the curiosity of the animals towards the stimulus mouse in the testing system (Equation (2)) (Figure 7A).

$$\text{ratio sociability} = (\text{time social cage})/((\text{time social cage} + \text{time empty cage})) \quad (2)$$

No statistically significant difference was observed between C57BL/6J WT and App^{NL-G-F} control animals. Experimental diets also had no impact on the social ability of the mice. Medians were statistically unequal to 0.5 except for group 2, 4 and 6 (males) and group 2 and 3 (females). A ratio of 0.5 means that contact times with the conspecific stimulus mouse and the empty cage were equal.

In the second phase of the test, we assessed the social recognition performance of the animals (Equation (3)) (Figure 7B).

$$\text{ratio social recognition} = (\text{time novel animal})/((\text{time novel animal} + \text{time familiar animal})) \quad (3)$$

As for sociability, neither genotype nor experimental diets had an influence on social recognition in the different experimental groups. In neither phase of the test did hyperhomocysteinemia aggravate the cognitive performance of the mice. Except for group 1 (males) and group 5 and 6 (females), medians of the other groups did not differ significantly from 0.5.

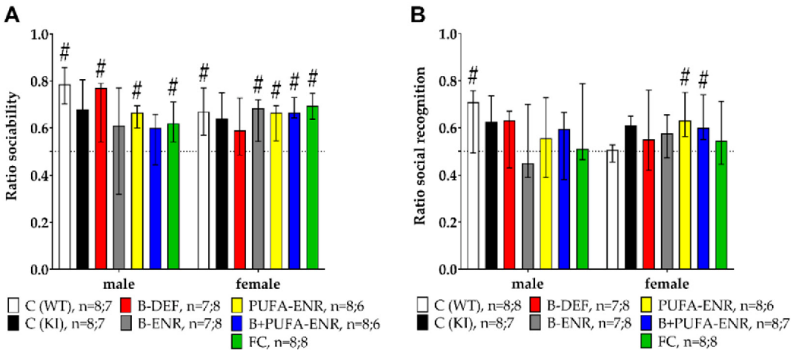

Figure 7. Social interaction test (25 min) in 19–20 weeks old C57BL/6J and App^{NL-G-F} mice; 14–15 weeks on experimental diet; data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*); (#) ratio different from 0.5 (one-sample Wilcoxon signed rank test). (**A**) Sociability expressed as the ratio between the contact time with the stimulus mice and the sum of the contact times with the stimulus mice and the empty cage. (**B**) Social recognition expressed as the ratio between the contact time with novel stimulus mice and the familiar ones.

3.6. Paired Associates Learning (PAL) Task

The touchscreen PAL was used to assess potential cognitive impairment of the male mice (about five to eight months of age). Both the session duration and the number of trials completed per session, as well as the percentage of correct trials per session were analyzed (Figure 8). The resulting learning curves revealed no statistically significant difference in these parameters between C57BL/6J WT mice and App^{NL-G-F} KI mice in the final phase of the test (block 6). Hyperhomocysteinemic App^{NL-G-F} mice did not perform worse than App^{NL-G-F} control mice. Other experimental diets also had no benefit on the cognitive abilities of App^{NL-G-F} mice at this age. The C57BL/6J WT group showed a smaller variability in the touchscreen chambers in comparison to the App^{NL-G-F} KI groups. This effect was particularly observed in the parameter trials completed (Figure 8B). Vitamin B deficient animals showed a tendency to perform better at the beginning of the test (trials completed, block 1) and thus did not display a learning curve like that of App^{NL-G-F} control mice. However, no effects reached statistical significance in block 6. Animals fed with a vitamin B and PUFA combination diet did not reach the maximum number of trials per session. Therefore, the session duration scarcely also decreased over time in this group. The proportion of correct and incorrect trials was not affected.

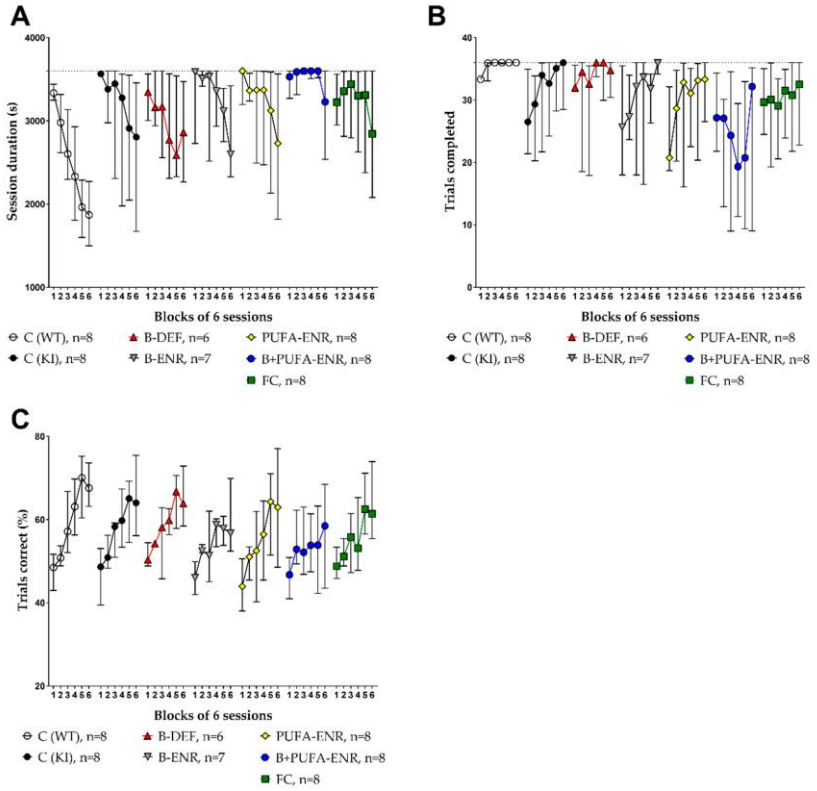

Figure 8. Touchscreen paired associates learning test (PAL) in 21–34 (incl. training phase) weeks old C57BL/6J and $App^{NL\text{-}G\text{-}F}$ mice; 16–29 weeks on experimental diet; only males; data summarized in blocks of 6 sessions and presented as median ± IQR; outliers beyond threefold IQR removed; test on statistical significance carried out for block 6. (**A**) Session duration: time (s) needed to complete 36 trials per session; maximum 3600 s. (**B**) Amount of trials completed per session; maximum 36 trials. (**C**) Percentage of correct trials per session.

3.7. Place Learning (PL) and Reversal Learning (RL) Task

Learning and memory performance of the females at the age of about six to eight months was finally tested using two tasks in the IntelliCage system. We detected the visits of the mice to the drinking corners and analyzed the percentage of correct visits during the drinking sessions in the place learning (PL) and the reversal learning (RL) tasks. Three points in time along the course of the tasks are illustrated in Figure 9. Statistical analysis of the late phase of this course in both (Figure 9A) PL and (Figure 9B) RL (session 31; resp. 23) revealed no significant differences between $App^{NL\text{-}G\text{-}F}$ and age-matched C57BL/6J mice. In comparison to the $App^{NL\text{-}G\text{-}F}$ KI control group, none of the groups fed with experimental diets showed improved or impaired memory abilities.

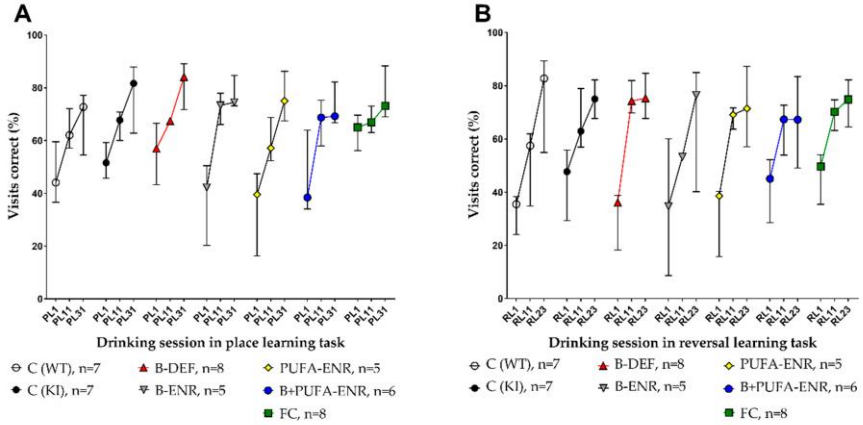

Figure 9. IntelliCage place learning (PL) and reversal learning (RL) task in 27–34 (incl. habituation period) weeks old C57BL/6J and *App*$^{NL\text{-}G\text{-}F}$ mice; 22–29 weeks on experimental diet; only females; data are shown for three points in time along the course of the task and presented as median ± IQR; outliers beyond threefold IQR removed; test on statistical significance carried out for the final point in time. (**A**) Percentage of correct visits during drinking sessions in the PL. (**B**) Percentage of correct visits during drinking sessions in the RL.

3.8. Immunohistochemical Analysis

Brain sections of all animals were immunohistochemically stained and analyzed in order to semi-quantify the amount of amyloid plaques. For this purpose, we assessed the area (percentage) occupied by plaques in images of several regions of interest (ROI). The positions of the different cortical and hippocampal ROI (Table A2) are marked in Figure 10.

Figure 10 illustrates examples of brain sections of a C57BL/6J WT mouse and an *App*$^{NL\text{-}G\text{-}F}$ KI mouse. Aβ plaques, indicated by characteristic brown staining, occurred abundantly and diffusely in the brain sections of the KI animals (Figure 10B), whereas WT mice did not show any signs of Aβ deposition at all (Figure 10A). The differences in the Aβ burden between the C57BL/6J and *App*$^{NL\text{-}G\text{-}F}$ genotype, as well as a potential impact of the experimental diets, were further analyzed using ImageJ software. Semi-quantification of the Aβ burden confirmed a significant difference between WT and KI control groups (Figure 11) in all ROI ($p < 0.001$; $p = 0.002$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$).

There was no statistically significant difference in the plaque area between the diet groups and the *App*$^{NL\text{-}G\text{-}F}$ control group in the single ROI and in total. However, the immunohistochemical results indicate prominent plaque formation in all *App*$^{NL\text{-}G\text{-}F}$ groups at about 8 months of age.

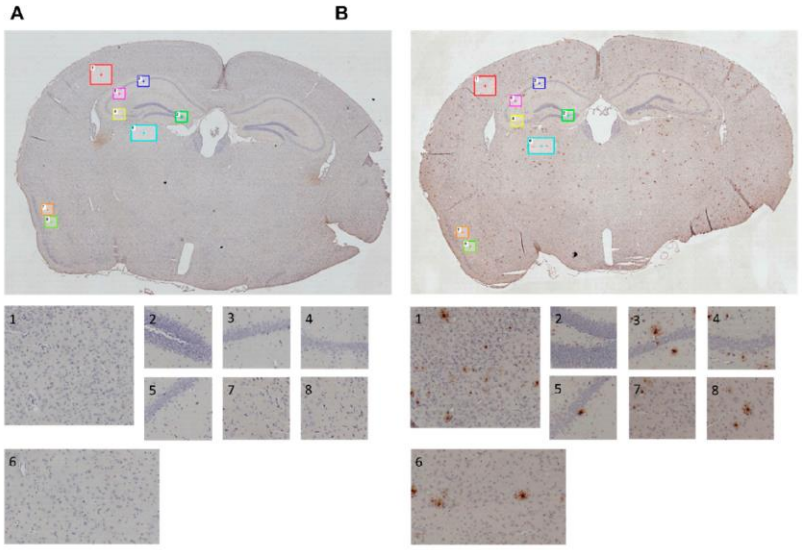

Figure 10. Immunohistochemically stained sections of mouse brains; regions of interest (ROI) in cortical and hippocampal areas are marked in whole brain images (100× magnification) and depicted separately for further semi-quantification of amyloid plaques; 35 weeks of age, 30 weeks on experimental diet. (**A**) Exemplary section of a C57BL/6J wild type animal. (**B**) Exemplary section of an *App*$^{NL\text{-}G\text{-}F}$ knock-in animal.

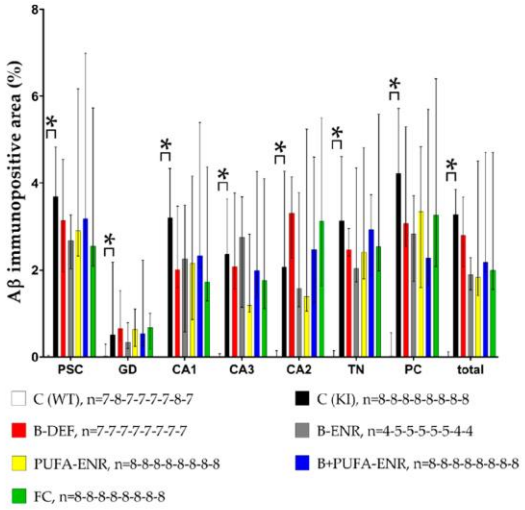

Figure 11. Semi-quantitative analysis of amyloid-β (Aβ) in immunohistochemically stained brain sections; results are shown for single regions of interest (ROI) and in total; 35 weeks of age, 30 weeks on experimental diet (males and females pooled); data presented as median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

4. Discussion

The current preclinical study investigated the impact of an induced hyperhomocysteinemia in the App^{NL-G-F} knock-in mouse model for AD, as well as potentially preventive benefits of different micro-nutritional interventions. In order to characterize the phenotypes of the mice, we conducted a versatile behavioral test battery, accompanied by an analysis of HCys-/HCA levels and of the Aβ plaque burden. However, despite successful induction of prominent cerebral plaque deposition and hyperhomocysteinemia, merely subtle impairments were observed in the App^{NL-G-F} mice.

C57BL/6J mice, a frequently studied mouse strain and background strain of the App^{NL-G-F} knock-in (KI) model, served as an age-matched wild type (WT) control group in this study. Hence, results in these mice indicated a reference behavior and enabled subsequent assessment of the App^{NL-G-F} genotype in the KI mice. In the open field, we focused on the intrasession habituation of the mice, which is one form of learning. Intrasession habituation describes a decreasing level of exploration of a new environment over time in a single session which can typically be detected in C57BL/6J mice [37]. This is in accordance with our finding that the habituation ratio in C57BL/6J was significantly lower than 0.5 and therefore indicated intrasession habituation. As expected, C57BL/6J mice demonstrated spatial learning and memory ability on consecutive days of training in the Barnes maze [38]. In a test for sociability and social recognition [39], C57BL/6J mice preferred to spent time with a conspecific (ratio sociability > 0.5) [40]. However, they did not prefer the novel conspecific in the second part of the test (ratio social recognition = 0.5). In the touchscreen PAL [41], male WT animals completed the maximum number of all 36 trials per session, accompanied by decreasing session duration. The increase in the percentage of correct trials is in accordance with observations in a similar study [42]. In the IntelliCage setup [43,44], learning curves indicated a constant learning effect in the female WT animals.

For several reasons, we decided to use an AβPP-based KI mouse model for AD in this study. Firstly, the novel KI models provide the advantage of not overexpressing AβPP in comparison to the more established transgenic models. Consequently, artificial phenotypes due to an overproduction of AβPP fragments besides the Aβ peptide should be avoided [9]. Secondly, an increased anabolism of Aβ levels is primarily a hallmark of hereditary- or early-onset AD [3]. Hyperhomocysteinemia, which is especially prominent in older people [45], is supposed to be a risk factor for AD [24]. Therefore, elevated HCys and HCA have been regarded as a hallmark of sporadic- or late-onset AD. The late-onset form affects the vast majority of AD patients [3]. By combining both the increased Aβ anabolism as a feature of hereditary AD and the detrimental effects of excess HCys as a feature of sporadic AD, we attempted to simulate cognitive decline more comprehensively. Thirdly, in order to investigate preventive treatments, it is mandatory to use a model displaying subtle phenotypes corresponding to a very early stage of the disease. According to a review by Zahs and Ashe, AβPP-based mouse models simulate the early phase of AD and thus are adequate for preventive interventions [46]. In the current study, a very subtle phenotype, i.e., very mild cognitive deficits, was observed. For each analysis, we compared C57BL/6J WT animals with App^{NL-G-F} KI control animals. Both groups received the same control diet. KI mice displayed an impaired habituation behavior in the open field. Male mice of the two control groups habituated equally to the new environment, whereas females differed significantly. Data from the probe trial in the Barnes maze confirmed this finding: App^{NL-G-F} KI mice needed longer to locate the former target hole than WT mice. As in the open field, this effect reached statistical significance only in females. Previous clinical studies suggest that a reduced cognitive reserve in women might explain the female vulnerability to develop a more severe phenotype of AD, a disorder affecting more women than men [47,48]. Other behavioral tests did not reveal differences caused by the KI genotype. However, data from the PAL test indicated an increased variance of results (higher IQR) in the App^{NL-G-F} versus WT mice. WT animals showed a clearer performance curve with regard to the session duration and the number of trials completed along the course of the test, meaning that WT mice did not need as long as the KI mice to fulfil the 36 trials in a 1-h session. This enhanced efficiency might be the result of a higher motivation of the WT animals. Nevertheless, effects at the final stage of the test (block 6) did not indicate a significant impact of the genotype.

Other groups reported similar findings in App^{NL-G-F} mice, indicating a very subtle phenotype. Two recent publications summarized these findings in tabular overviews, considering also sex and age of the mice of the included studies [49,50]. Latif-Hernandez and colleagues showed that the behavior of App^{NL-G-F} mice was largely unaffected at the age of 3–10 months [51]. Similarities with our study can also be found in a publication by Whyte et al., who observed no differences between C57BL/6J and App^{NL-G-F} mice in different cognitive tests at the age of 6 months [52]. Sakakibara and colleagues tested App^{NL-G-F} mice at a higher age (15–18 months) and reported an intact learning ability but also recommended App^{NL-G-F} as an AD model for preventive studies [53]. One year later, Jacob et al. observed neither consequences on cognitive performance in a touchscreen task nor age-dependent changes in a phase-amplitude coupling analysis, which was used as a measure of neurophysiological functioning, in 4.5 month old App^{NL-G-F} mice. In accordance with our findings in the App^{NL-G-F} model, these mice displayed a higher variability than WT control mice [42].

The question remains whether the KI mice were too young to display clear impairments. Further investigations are required to test the combination of the App^{NL-G-F} genotype with our experimental diets in older mice. However, other groups detected significant cognitive deficits in the App^{NL-G-F} model [9,49,50,54]. As summarized elsewhere [49], the majority of studies in the field investigated only male animals. Hence, a 1:1 comparison of these studies with our results comprising both sexes is difficult. Furthermore, a review of the topic described a relatively high level of variability in AβPP KI models between different laboratories [55]. Staining results of App^{NL-G-F} brain sections showed prominent plaque deposition throughout the brain, as previously reported in similar studies [49,52], and thus indicate amyloid pathology as a central hallmark of early AD.

In order to investigate potentially detrimental effects of elevated HCys and HCA levels, one group of App^{NL-G-F} mice received a special diet deficient in vitamin B6, B12 and folate. The resulting hyperhomocysteinemic state was confirmed in serum and urine prior to the start of behavioral tests. Our behavioral testing data obtained in the social interaction test, PAL and in the IntelliCages revealed no deficits in hyperhomocysteinemic mice and therefore do not support previous findings (e.g., [56]). The open field test and Barnes maze indicated subtle deficits in habituation behavior and spatial learning and memory, but these effects did not reach statistical significance. Only the elevated zero maze revealed an increased anxiety in hyperhomocysteinemic females. This observation might be of translational relevance, because anxious behavior is also one aspect of the AD phenotype [57]. Various preclinical studies in the field indicate a significant impact of hyperhomocysteinemia on plaque burden [58,59]. Other groups reported no such effects, which is in accordance with our immunohistochemical results in the App^{NL-G-F} model [60,61]. In conclusion, despite severely elevated levels of HCys and HCA over a longer period of their life span, App^{NL-G-F} mice showed neither a modified plaque burden nor significant cognitive deficits due to hyperhomocysteinemia. A majority of preclinical data published in the field indicate behavioral deficits in animal models caused by increased HCys (e.g., [56,59,62]). However, we assume that the evidence might be biased to some extent. On the one hand, behavioral data obtained in transgenic models based on massive AβPP overexpression might be somewhat artificial because of an overproduction of other AβPP fragments aside from Aβ [9]. It should also be considered that negative results are often not published, although equally important as positive results. The publication bias, meaning the reduced publishing of negative or null results, is not restricted to the field of AD research, but is rather a general problem [63].

Hyperhomocysteinemia is referred to as a hallmark of AD [10], but its impact on the disease is still under discussion. From a translational point of view, this experimental group simulates the portion of elderly people who are deficient in B-vitamins [64]. Preclinical evidence [65] and clinical evidence [45] confirm an age-related elevation of HCys levels. An impaired vitamin status is one reason amongst others for hyperhomocysteinemia in the elderly [66]. In the present study, the lack of vitamin B6, B12 and folate in combination with 1% sulfathiazole sodium to inhibit bacterial folate synthesis in the gut [58], led to a "severe" hyperhomocysteinemic state, according to a classification used in other publications [67]. Consequently, our vitamin B deficient mice displayed high HCys serum

concentrations (45,760 ng/mL ≈ 339 µmol/L) in comparison to our App^{NL-G-F} KI control (1054 ng/mL ≈ 8 µmol/L) and in comparison to elevated HCys levels in similar studies (e.g., [56,62,68]). Fuso and colleagues also reached high plasma total HCys (>400 µmol/L ≈ 54,000 ng/mL) in their study with TgCRND8 mice and explained the relatively high levels by not fasting the mice before sacrifice and by inhibiting both the re-methylation and the transsulfuration pathway [58].

Vitamin B deficient chow resulted in ~50 fold higher serum and urinary HCys and ~10–20 fold higher serum and urinary HCA compared to animals fed with control diet for 8 weeks. About 0.1% of HCys molecules were oxidized to HCA in serum (42.9 ng/mL ≈ 0.23 µmol/L) and excreted in urine (1184 ng) in 24 h. Only free HCys can be oxidized to HCA, which is suggested to be the main neurotoxic species [32–34]. In the current study, we did not measure the free form but the levels of total HCys by adding a reduction step (TCEP-solution) in the analytical method. In vivo, most HCys molecules are protein-bound or dimerized; only about 1% are available in the free thiol form [12]. Hasegawa et al. reported cognitive impairment in transgenic 3xTg-AD mice, triggered by elevated HCA in the brain [69].

We also investigated other experimental diets besides the vitamin B deficient chow discussed above. Group 4 received a vitamin B enriched diet containing a particular high content of folate, B6 and B12 compared to both the control diet and the FC-like diet. The goal of this diet was to investigate whether an additional increase, specifically of B-vitamins, in comparison to the FC-like diet could provide further benefits in the outcome of the study. Therefore, the difference in B-vitamin contents should simulate a potentially different effectiveness between FC (Souvenaid®) and existing higher dosed vitamin B preparations as human treatment options. In accordance with a recent international consensus statement [23], PUFAs (DHA + EPA) have been suggested to be beneficial for cognitive functioning in general and might be additionally linked to AD pathology [25,70]. Because single nutrient intervention studies often failed to show beneficial effects on cognitive function, it has been suggested that it might be important to investigate combinatory approaches [35]. For this purpose, we combined the high content vitamin B enrichment with the supplementation of PUFAs (group 6). Finally, group 7 received the FC-like diet, a complex mixture of ingredients (Table A1), which we implemented due to positive previous findings (e.g., [35,71]).

Supplementation of B-vitamins and PUFAs, as well as combinatory approaches and the FC-like mixture, were capable of lowering HCys and HCA below the levels of the App^{NL-G-F} control mice fed with a standard rodent chow. However, by taking both sampling points (8 and 30 weeks on diet) as well as behavioral testing data into consideration, results appear inconsistent. In the open field, anxiety-related behavior did not differ between groups fed with B-vitamins, PUFAs or a mixture and App^{NL-G-F} control animals. However, the elevated zero maze revealed increased anxiety in males fed with the combination diets. Especially the mice supplemented with both B-vitamins and PUFAs were more anxious and stayed in the closed corridors of the zero maze, but it has to be emphasized that these mice also displayed a reduced locomotion activity during the test. In the Barnes maze, experimental chow did not affect latencies to target at day 4 of training. Other researchers too did not observe benefits of PUFA-supplementation in cognitive tasks [72]. We confirmed the lack of dietary effects on cognitive performance in the social interaction test, the IntelliCage and PAL. Although not significant in the final block of 6 sessions, the session duration and trials completed indicate a worse learning curve for group 6 (B+PUFA-ENR) in the PAL test. This might be due to a lack of motivation in these mice receiving a high number of vitamins and PUFAs, which possibly lowered their affinity to the milk reward in the PAL task. One reason could be that the food restriction was not strict enough for this group. The FC-like diet did not prove beneficial in any test in comparison to the control chow. This is in accordance with some clinical studies, which do not support the benefit of the FC diet and thus indicate equivocal evidence [73,74]. In conclusion, the beneficial tendencies we observed did not mostly reach statistical significance in behavioral tests and biochemical-/immunohistochemical analyses and consequently do not suggest a clear beneficial effect of B-vitamins or PUFAs in this mouse model at the investigated age and diet duration. It is important to question here whether it is

possible to observe amelioration through dietary intervention when merely a subtle behavioral deficit is induced in the KI mouse model.

Overall, this mouse model, simulating amyloid pathology without AβPP overexpression, merely displays a very mild phenotype despite massive cerebral Aβ deposition at the age of 35 weeks. The amyloid hypothesis has been questioned frequently because of the disappointing track record in clinical trials of drugs that target Aβ despite decades of extensive research in the field [7,75]. In addition, in some cases, substantial plaque deposition does not even cause dementia-like symptoms [76]. However, the window for potentially preventive measures is limited to an early stage of AD, where cerebral amyloidosis remains the central hallmark of the pathology [3]. Despite all criticism of the amyloid hypothesis, beneficial effects were recently observed using the human anti-Aβ monoclonal antibody aducanumab [77], confirming a causal role of Aβ in AD pathogenesis.

5. Conclusions

The current study only indicates a mild hyperhomocysteinemia-driven exacerbation of the AD-like phenotype, simulated in the $App^{NL\text{-}G\text{-}F}$ knock-in mouse model. Dietary interventions consisting of B-vitamins and/or PUFAs as well as the FC-like diet as a complex micronutrient mixture were unable to modify cognitive performance in this mouse model for AD. Neither the B-vitamin deficient diet, resulting in elevated HCys and HCA levels, nor the potentially beneficial diets affected the amount of plaque deposition in the brain. In comparison with the age-matched C57BL/6J wild type control group, $App^{NL\text{-}G\text{-}F}$ control mice displayed merely subtle behavioral deficits at the investigated age. Further investigations should clarify whether the $App^{NL\text{-}G\text{-}F}$ genotype and the experimental diets have an impact in older animals.

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Appendix A

Animals

Wild type (WT) mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany), whereas the knock-in (KI) mice were kindly provided by the RIKEN Center for Brain Science (Saitama, Japan) on a C57BL/6J background and further bred at mfd Diagnostics GmbH (Wendelsheim, Germany). After their arrival at our facility at the age of four weeks, the animals were chipped with subcutaneous transponders to facilitate identification and to enable the IntelliCage task. Furthermore, additional genotyping via polymerase-chain-reaction analysis was carried out to ensure the adequate genetic background of each animal. Their allocation to the home cages was in a randomized order. All animals were housed in groups of two mice per cage (Green Line, Tecniplast, Hohenpeissenberg, Germany). In the maintenance room, constant temperature (mean: 22.7 °C) and humidity (mean: 48.6%) conditions as well as a 12/12 h dark/light cycle were provided. The pathogen-free status of the maintenance room was regularly monitored using sentinel mice. After an acclimatization phase of one week, the mice were allocated randomly to the experimental groups based on different diets. Body conditions scores were monitored, and the mice were weighed every week.

Experimental Diets

The composition of the FC-like diet was oriented towards the work of Jansen et al. All diets containing PUFAs were stored at −20 °C to minimize oxidation [35]. Due to coprophagia (the ingestion of fecal matter) in mice, the vitamin B deficient diet additionally contained the antibiotic sulfathiazole sodium (Sigma-Aldrich, Taufkirchen, Germany) to prevent bacterial folate synthesis in the gut [58]. All experimental diets were purchased from Ssniff-Spezialdiäten GmbH (Soest, Germany).

Table A1. Composition of the experimental diets.

	Control	B-DEF	B-ENR	PUFA-ENR	B+PUFA-ENR	FC
Casein	140.0	140.0	140.0	140.0	140.0	140.0
Corn starch	355.6575	345.6920	355.4795	355.6575	355.4795	328.3386
Maltodextrin	155.0	155.0	155.0	155.0	155.0	155.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Dextrose	100.0	100.0	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Mineral premix	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin pre-mix (w/o B-vitamins)	10.0	10.0	10.0	10.0	10.0	10.0
Soybean oil	19.0	19.0	19.0	——	——	——
Coconut oil	9.0	9.0	9.0	11.3	11.3	11.3
Corn oil	22.0	22.0	22.0	18.7	18.7	18.7
Fish oil (eicosapentaenoic acid/docosahexaenoic acid = 1:4)	——	——	——	20.0	20.0	20.0
L-Cystine	1.8000	1.8000	1.8000	1.8000	1.8000	1.8000
Tert-butylhydroquinone	0.0080	0.0080	0.0080	0.0080	0.0080	0.0080
Choline bitartrate, 41%	2.5000	2.5000	2.5000	2.5000	2.5000	2.5000
Pyridoxine-HCl (Vit. B6)	0.0070	——	0.1000	0.0070	0.1000	0.0398
Cyanocobalamin, 0.1% (Vit. B12)	0.0250	——	0.1000	0.0250	0.1000	0.0600
Folic acid, 80%	0.0025	——	0.0125	0.0025	0.0125	0.0100
Sodium selenite • 5 H_2O, 30%	——	——	——	——	——	0.0036
Choline chloride, 43% Choline	——	——	——		——	6.9700
Ascorbic acid, 100% (Vit. C)	——	——	——	——	——	1.6000
DL-a-tocopheryl acetate, 50% (Vit. E)	——	——	——	——	——	4.6500
Uridine monophosphate disodium (24% H_2O)	——	——	——	——	——	10.0000
Soy lecithin	——	——	——	——	——	4.0200
Sulfathiazole sodium	——	10.0000	——	——	——	——
Sum	1000	1000	1000	1000	1000	1000

Open Field

Besides its value as a test for locomotor activity and anxiety, the open field task provides information on habituation as a form of learning [37]. For this purpose, each mouse was placed into the center of a 28.5 × 29.8 cm box (in-house manufactured, Fraunhofer IME, Schmallenberg, Germany). Animals were allowed to explore the new environment for 30 min. Total distance moved and percentage of time spent in the inner zone of the box were automatically detected by camera tracking and corresponding EthoVision XT 13 software (Noldus, Wageningen, The Netherlands). Data were analyzed additionally for time blocks of 5 min.

Elevated Zero Maze

To investigate anxiety related behavior [78], we placed each animal into the open corridor of a 60 cm diametric elevated zero maze (Ugo Basile SRL, Gemonio, Italy) for a duration of 5 min. The maze consisted of two open and two closed 5 cm wide corridors. Besides the time spent in the open corridors, the total distance moved by the mice was automatically detected by camera tracking and corresponding EthoVision XT 13 software.

Barnes Maze

The Barnes maze test is a common tool to measure spatial learning and memory [38] in AD mouse models, based on the aversion of mice to bright open spaces. We particularly preferred the Barnes maze over the Morris water maze, since it presents a less aversive alternative [79]. The apparatus (Ugo Basile SRL, Gemonio, Italy) consisted of a circular surface (diameter 100 cm) with 20 holes at the edge and an escape box positioned below one of the holes. There were four different visual cues positioned around the maze. The task required the mouse to localize the escape hole and enter the box. Initially, we transported each animal to the center of the maze in an opaque vessel to prevent an orientation before the start of the trial. The procedure was divided into two phases. First, in the acquisition phase, each mouse was subjected to two trials per day for four days (3-min limit per trial; inter-trial interval 15–30 min). The trials ended when either the mouse entered the escape box or when a duration of 180 s was over. On day 5, animals were subjected to a probe trial (90 s). During this phase, the escape box was not available anymore. Latencies to the target hole (acquisition & probe) were automatically detected by camera tracking and corresponding EthoVision XT 13 software.

Social Interaction Test

This method enables the assessment of sociability and social recognition in mice [39]. For this purpose, a three-chamber cage consisting of a central chamber and two lateral compartments (Noldus, Wageningen, Netherlands) was used. The lateral compartments included sex-matched stimulus mice in separate acrylic rod cages, which allowed social interaction without direct contact. Test animals explored the setup during three consecutive phases. During the first time block of 5 min, the mice were allowed to explore only the middle chamber. As a next step, we opened the dividers to the lateral compartments and placed a stimulus mouse into one of the rod cages (social cage). The second rod cage remained empty. The experimental mouse had a period of 10 min to explore the whole three-chamber cage and to interact with the unknown stimulus mouse. For the next 10 min, we placed an additional unknown stimulus mouse into the second rod cage. The cumulative contact time with the familiar and non-familiar conspecific was automatically detected by camera tracking and corresponding EthoVision XT 13 software.

Paired Associates Learning (PAL) Task

The ability of visuospatial associative learning was tested in males in the touchscreen PAL (touchscreen and corresponding Abet II Touch 18.7.6 software: Campden Instruments, Loughborough, UK and Lafayette Instrument Company, Lafayette, IN, USA). The task requires a lot of training, but is also a valuable tool in terms of translational cognitive research due to its similarities with the human CANTAB [41,80]. Based on the Bussey-Saksida method, animals initially were habituated to the touchscreen chambers during different pre-training phases. After completion, mice were introduced to the proper PAL task. Here, two objects were shown in two spatial locations on the screen. In each trial, only one correct association of object and location was presented, and the animal had to detect it via nose poke. As a result, a reward was delivered automatically (sugared condensed milk, 7 µL, Hochwald Foods GmbH, Thalfang, Germany). Incorrect responses were followed by an aversive light stimulus (5 s time-out period). After an inter-trial interval (20 s), the next trial was initiated by the mouse. A session ended when either 36 trials were completed, or 60 min ran out. The animals were

food restricted through the whole experiment with the aim of reducing body weights to about 90% of the baseline weight before the test. This should enhance the motivation of the mice to collect the reward after each correct trial. Animal weights were monitored three times a week. For the assessment of the 36 sessions of the PAL task, the parameters' session duration, trials completed, and percentage of correct trials were analyzed. The procedure was highly standardized and the closed touchscreen chambers reduced variability due to the experimenter to a minimum.

Place Learning (PL) and Reversal Learning (RL) Task

The start of the IntelliCage experiment (IntelliCage and IntelliCagePlus 3.2.8 software: New Behavior, TSE Systems, Bad Homburg, Germany) in female mice was scheduled around the time when male mice entered the proper PAL task. Thus, males and females were largely age-matched during the last phase of the behavioral test battery (27 weeks old, resp. 22 weeks on diet). We chose not to test males in the IntelliCage setup, because males are more prone to show aggressive behavior and hierarchical fighting, potentially resulting in injuries due to the housing of male mice in large groups. The IntelliCage tasks of learning ability cover a broad cognitive spectrum by combining the analysis of spatial memory with operant conditioning [43] and provide the advantage of being both home cage and behavioral test during the time of the experiment. Animals from all experimental groups lived together in the special cage for the period of about 7 weeks. Due to this mixed group housing, the experimental diets were substituted by standard maintenance chow (ad libitum) for the duration of this behavioral test. Each apparatus had the capacity to house and detect up to 16 mice simultaneously. The experiment started with a habituation period of 1 week, followed by a pre-training phase on nose poke behavior in corners for water access for 1–2 weeks. During the following week, the animals were habituated to the two defined drinking sessions per day (5–7 a.m.; 7–9 p.m.). In the PL, only one corner per mouse yielded water access in response to nose pokes during drinking sessions (~2 weeks). Motorized doors, controlled by radio-frequency identification (RFID) transponders, opened when a mouse was detected in its adequate corner. In the RL, a different corner was designated as correct (2 weeks). Visits to the correct corners were analyzed for PL and RL. We did not weigh the animals for the duration of the experiment to avoid interference with the automated behavior recording; instead, we visually observed the mice for any sign of deficiency. The IntelliCage enabled a high throughput cognitive investigation of mice, while stress due to human intervention was reduced to a minimum.

Sample Collection

Blood was taken by carrying out a puncture of the facial vein using 5 mm Goldenrod animal lancets (MEDIpoint, Mineola, NY, USA). A maximum volume of 170 µL per 25 g mouse according to animal welfare guidelines (GV-SOLAS) was collected in serum tubes containing a clotting factor to accelerate coagulation in the subsequent 15–30 min (Sarstedt Microvette 200 Z, Nümbrecht, Germany). The tubes were centrifuged at 3200× *g* for another 15 min at 4 °C and subsequently frozen on dry ice. For 24-h urine sampling, mice were placed into metabolic cages (Tecniplast, Hohenpeissenberg, Germany). Absolute urine volumes were documented for subsequent calculations. In order to harvest the brains, the animals were deeply anaesthetized by injecting a mixture of 200 mg/kg (body weight) ketamine (Vétoquinol GmbH, Ismaning, Germany) and 10 mg/kg (body weight) xylazine (Bayer Health Care, Leverkusen, Germany) intraperitoneally. After cessation of reflexes, blood was taken cardially and treated as described before. Mice were then perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (Medite, Burgdorf, Germany). Brains were removed and postfixed in the same fixative for another three days followed by a stepwise dehydration in increasing ethanol concentrations (Medite) and xylene steps (Medite). Brains were then embedded in paraffin (Medite) in a heated embedding station (Thermo Fisher, Frankfurt am Main, Germany) and cut with a microtome (Thermo Fisher). 10 µm thick sections were retrieved from three different positions of the animals' brains: −1.2, −1.7 and −2.2 posterior to bregma [81]. Data of the three positions were

pooled because of absent statistically significant differences. Finally, the sections were mounted on glass slides (Klinipath, Typograaf, Netherlands).

Biochemical Analysis

The determination of homocysteic acid (HCA) was performed as recently described in detail [36] with minor modifications, as the method was originally validated for the analysis of human serum and urine. Briefly, HCA was determined in murine serum and urine using a combination of protein precipitation and solid phase extraction for sample preparation followed by an LC–MS/MS analysis using a combination of a HILIC separation and tandem mass spectrometry. Samples were processed as previously described [36] by adding formic acid followed by protein precipitation using cooled acetonitrile. Samples were vortexed, centrifuged and loaded onto conditioned tables (Strata X AW SPE columns (33 µm, 30 mg / 1 mL, Phenomenex, Aschaffenburg, Germany) using the automated sample preparation system Extrahera (Biotage, Uppsala, Sweden). After washing the cartridges using water, methanol and a mixture of acetonitrile and aqueous ammonium hydroxide solution, HCA was eluted using two times a mixture of methanol and aqueous ammonium hydroxide solution. The eluate was dried and reconstituted by adding ammonium acetate solution and acetonitrile separately. Afterwards, the samples were injected into the LC-MS/MS system. The LC-MS/MS system consisted of a triple quadrupole mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray source operated in negative electrospray ionization mode and an Agilent 1290 Infinity LC-system with binary HPLC pump, column oven and autosampler (Agilent, Waldbronn, Germany). The chromatographic separation was performed using a Luna 3 µm HILIC 200 Å 100 × 2 mm column in combination with a KrudKatcher in-line filter (both Phenomenex, Aschaffenburg, Germany). Data acquisition was done using Analyst Software 1.6.3 and quantification was performed with MultiQuant Software 3.0.2 (both Sciex, Darmstadt, Germany), employing the internal standard method. Calibration curves were calculated by linear regression with 1/x weighting. Acceptance criteria and quality assurance measures have been applied as previously described [36].

The determination of homocysteine (HCys) was performed using protein precipitation in combination with LC–MS/MS. Briefly, 20 µL of serum or urine was pipetted to a polypropylene tube and 20 µL of 15 mg/mL aqueous TCEP-solution (tris(2-carboxyethyl)phosphine), 40 µL IS working solution (500 ng/mL HCys-d4 in methanolic TCEP solution, 1 mg/mL) and 40 µL methanolic TCEP solution, 1 mg/mL were added. Afterwards, samples were vortexed, centrifuged, transferred into another polypropylene tube, and dried using nitrogen. The dried samples were reconstituted using 50 µL of water containing 10 mM ammonium acetate buffer and 10 mM acetic acid, centrifuged again and injected into the LC-MS/MS system. The same LC-MS/MS-system and acceptance criteria as described for HCA were used. However, positive electrospray ionization mode was applied and a Luna Omega 1.6 µm Polar C18 100 × 2.1 mm column in combination with a respective pre column (both Phenomenex, Aschaffenburg, Germany) was used.

Immunohistochemical Analysis

A stepwise rehydration of the brain sections was conducted, followed by a heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) including 0.05% Tween-20 (Sigma-Aldrich, Taufkirchen, Germany). After rinsing, sections were incubated for 5 min in 0.6% H2O2 (Sigma-Aldrich) in PBS (0.1 M; pH = 7.3) in order to block endogenous peroxidases. Sections were rinsed and incubated for 30 min in PBS containing 1% bovine serum albumin (PBS-B) and 5% normal goat serum (NGS, Sigma-Aldrich) to prevent unspecific binding of the antibody. After subsequent rinsing, sections were incubated overnight at 4 °C in PBS-B containing 1% NGS and the primary antibody (anti-human Aβ 82E1 mouse IgG MoAb 1:1000, IBL international, Hamburg, Germany). Rinsing was followed by an incubation with goat anti-mouse IgG H&L Biotin (1:1000, Abcam, Berlin, Germany) in PBS-B containing 1% NGS for one hour. Sections were rinsed followed by a 1-h incubation with avidin-biotin conjugate in PBS (ABC; Vectastain Elite ABC HRP Kit, Linaris, Dossenheim, Germany). After another rinsing step, sections were

treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) in water (0.2 mg/mL; pH = 7.6) for 10 min. The immunostaining was then developed by adding 50 µL H_2O_2 to a final concentration of 0.006%, incubating for another 10 min. The reaction was stopped by rinsing in ice-cold distilled water followed by a counterstaining using Mayer's hematoxylin (Morphisto, Frankfurt am Main, Germany). Sections were finally dehydrated and covered with Pertex (Medite). We digitized appropriate sections using a Nicon Eclipse Ni-E microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands). Whole brain images were taken at a final magnification of 100x and the area occupied by plaques in several regions of interest (ROI; Table A2) was analyzed using the color segmentation plugin (Daniel Sage, Biomedical Imaging Group, EPFL, http://bigwww.epfl.ch/sage/soft/colorsegmentation/) for ImageJ software (National Institute of Health, Bethesda, MD, USA). Only animals of the first cohort were immunohistochemically investigated in this study.

Table A2. Regions of interest (ROI) in different cortical and hippocampal areas.

ROI	Brain Area	Height (µm)
1	Primary somatosensory cortex (PSC)	500 × 500
2	Gyrus dentatus (GD)	275 × 275
3	CA1	275 × 275
4	CA3	275 × 275
5	CA2	275 × 275
6	Thalamic nuclei (TN)	600 × 400
7	Piriform cortex (PC)	275 × 275
8	Piriform cortex (PC)	275 × 275

Preclinical Quality Parameters

Several aspects were considered to ensure the quality of the applied methodologies and resulting data. These points are in accordance with initiatives such as EQIPD ("European quality in preclinical data"; https://quality-preclinical-data.eu/). The aim of EQIPD is broadly to implement various quality improving measures in order to enhance the reproducibility of preclinical data [63]. In the present study, we performed a power calculation to estimate the needed group size (http://www.biomath.info/power/). The resulting total amount of 112 animals was tested in two consecutive cohorts. Nine animals were lost during the course of the whole study. In terms of translatability, we have decided to include both male and female animals in the experiments, since Alzheimer's disease affects both sexes in the clinical context, with a higher rate in women than in men [48]. In general, female animals are largely underrepresented in neuroscience research [82]. Randomization was applied at several stages along the study course. Mice were initially allocated to the home cages according to a random list (https://www.random.org/) and target holes in the Barnes maze were set randomly. Besides, drinking corners in the IntelliCages as well as the stimulus mice in the social interaction test were also assigned randomly. A within-cage randomization between groups was not applicable in this case because every mouse matched strictly to its adequate experimental diet. All animals were regularly pre-handled and transferred to the experimental rooms at least half an hour before behavioral analysis. Blinding of the experimenter in order to prevent detection bias was not performed here, because in all behavioral tests automated outcome assessment was applied (via EthoVision XT, IntelliCage and Touchsreen software). However, blinding was performed during the immunohistochemical analysis. Here, a second experimenter marked the ROI in the images without being aware of animal ID or experimental group. Furthermore, an automated animal management software as well as an electronic lab-book were used throughout the study. Standard operating procedures had been written prior to the experimental procedures.

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Effects of Alzheimer-Like Pathology on Homocysteine and Homocysteic Acid Levels—An Exploratory In Vivo Kinetic Study

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Abstract: Hyperhomocysteinemia has been suggested potentially to contribute to a variety of pathologies, such as Alzheimer's disease (AD). While the impact of hyperhomocysteinemia on AD has been investigated extensively, there are scarce data on the effect of AD on hyperhomocysteinemia. The aim of this in vivo study was to investigate the kinetics of homocysteine (HCys) and homocysteic acid (HCA) and effects of AD-like pathology on the endogenous levels. The mice received a B-vitamin deficient diet for eight weeks, followed by the return to a balanced control diet for another eight weeks. Serum, urine, and brain tissues of App^{NL-G-F} knock-in and C57BL/6J wild type mice were analyzed for HCys and HCA using LC-MS/MS methods. Hyperhomocysteinemic levels were found in wild type and knock-in mice due to the consumption of the deficient diet for eight weeks, followed by a rapid normalization of the levels after the return to control chow. Hyperhomocysteinemic App^{NL-G-F} mice had significantly higher HCys in all matrices, but not HCA, compared to wild type control. Higher serum concentrations were associated with elevated levels in both the brain and in urine. Our findings confirm a significant impact of AD-like pathology on hyperhomocysteinemia in the App^{NL-G-F} mouse model. The immediate normalization of HCys and HCA after the supply of B-vitamins strengthens the idea of a B-vitamin intervention as a potentially preventive treatment option for HCys-related disorders such as AD.

Keywords: hyperhomocysteinemia; vitamin B deficiency; alzheimer disease; disease models; animal

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1. Introduction

The endogenous and non-proteinogenic amino acid homocysteine (HCys) has obtained increasing attention during the last decades due to the potential contribution to versatile pathologies. It is part of the one-carbon metabolism, which physiologically enables diverse methylation reactions such as during the synthesis of nucleic acids, proteins, neurotransmitters [1]. Besides its remethylation to methionine, that is dependent on the adequate supply of vitamin B12 and folate, HCys is metabolized to cysteine in a vitamin B6-dependent manner in the transsulfuration pathway [1,2]. In contrast to its metabolization, the urinary excretion of unchanged HCys is minimal [3]. Lack of the aforementioned B-vitamins as important enzymatic cofactors causes tissues to export HCys into plasma [3]. Elevated levels of HCys in the blood are referred to as hyperhomocysteinemia. As summarized by Cohen and colleagues, hyperhomocysteinemia can arise for different reasons, including a lack of relevant B-vitamins [4]. At present, a hyperhomocysteinemic state is

suggested to contribute to and exacerbate different human disorders, covering the whole range of cardiovascular diseases, cancer and neurologic disorders such as Alzheimer's disease (AD) [1,5]; the latter is also the focus of the current study. Dementia affects more than 50 million people today [6]. Especially AD, which accounts for most dementia cases [7], still lacks appropriate medical interventions to treat or prevent the ongoing neurodegenerative processes. In the current study, a knock-in mouse model for AD was used, which simulates amyloid-β (Aβ) pathology that is one of the most prominent hallmarks of the complex disease [8,9]. Saido and colleagues generated the App^{NL-G-F} knock-in (KI) model and overcame problems of transgenic AD models based on massive amyloid precursor protein (APP) overexpression [10]. The disturbed Aβ metabolism in these mice results in aggressive Aβ amyloidosis and deposition in the brain from 2 months on. The early-stage plaque deposition has also been confirmed recently by others [11].

HCys is thought to be linked to the pathology of AD through direct and indirect neurotoxic mechanisms, as reviewed recently [12]. Hyperhomocysteinemia might not only be associated with AD [13,14], but also causally linked to AD pathology [15–20] and vascular contributions to dementia [20]. B-vitamin treatment has been shown to slow cognitive decline in subjects with elevated HCys levels [21] and therefore, may provide a preventative approach [22,23]. However, because of ambiguous findings in different studies [24,25], especially the role of B-vitamins as a potential preventative approach in cognitive disorders, there is still a lack of agreement on this topic [26–29]. In the context of neurodegenerative investigations, it is crucial that not only blood HCys but also cerebral levels are taken into consideration. HCys reaches cerebral tissues by crossing the blood-brain barrier [30] and also disrupts it [31,32]. Detrimental effects on the blood-brain barrier are exerted, for example, by excitotoxic species [33,34] such as homocysteine and homocysteic acid (HCA). The oxidative metabolite HCA is suggested to be more neurotoxic than HCys itself [35–37], and therefore, we assessed HCA levels as well.

In previous work from our group, we observed normalization of HCys and HCA serum levels in App^{NL-G-F} mice after they had received control chow for only a short period of time in the IntelliCage behavioral testing system [38]. The current exploratory study attempts to provide better comprehension of the long-term kinetics, i.e., increase in HCys and HCA in mice fed a special diet deficient in B-vitamins and the expected normalization after the animals return to a normal diet. By frequently collecting mouse samples and analyzing these via LC-MS/MS, we aimed to elucidate the following central questions:

1. Is there a genotype effect in the hypothesized elevation of HCys and HCA levels in the App^{NL-G-F} knock-in mouse model for AD compared to C57BL/6J wild type mice?
2. Are there sex-specific differences between male and female mice?
3. What is the availability of HCys and HCA in cerebral tissues?
4. Are there correlations in HCys and HCA between the different biological matrices?

2. Results

Body condition scores were not affected by genotype or sex. Differences were observed in the weights: male mice weighed significantly more than female mice and KI mice weighed slightly more in comparison to WT mice.

2.1. Serum Analysis

During the course of the study, serum was sampled and analyzed using a validated LC-MS/MS method. The resulting data for HCys and HCA are depicted in Figures 1 and 2. Consumption of the B-vitamin deficient diet caused steadily increasing serum HCys in both males and females over eight weeks of dietary intake, resulting in about 4400 ng/mL (≈33 µmol/L) mean serum HCys (male + female) in WT and 15,400 ng/mL in KI (≈114 µmol/L) at week 9. App^{NL-G-F} KI mice developed significantly higher HCys levels on a B-vitamin deficient diet compared to C57BL/6J WT animals (week 9: $p < 0.001$). There were no sex-related effects detected in HCys levels between males and females after the onset of hyperhomocysteinemia (week 9: $p = 0.070$). Merely at baseline levels at the

start (week 1: $p < 0.001$) and at the end of the study (week 17: $p = 0.002$), HCys levels were slightly higher in females in comparison to males. The return to control chow after eight weeks on the deficient diet (week 9) resulted in a rapid normalization of serum HCys. These levels took slightly longer to decrease to baseline in females. This delayed normalization was more obvious in WT mice (week 11 and 13).

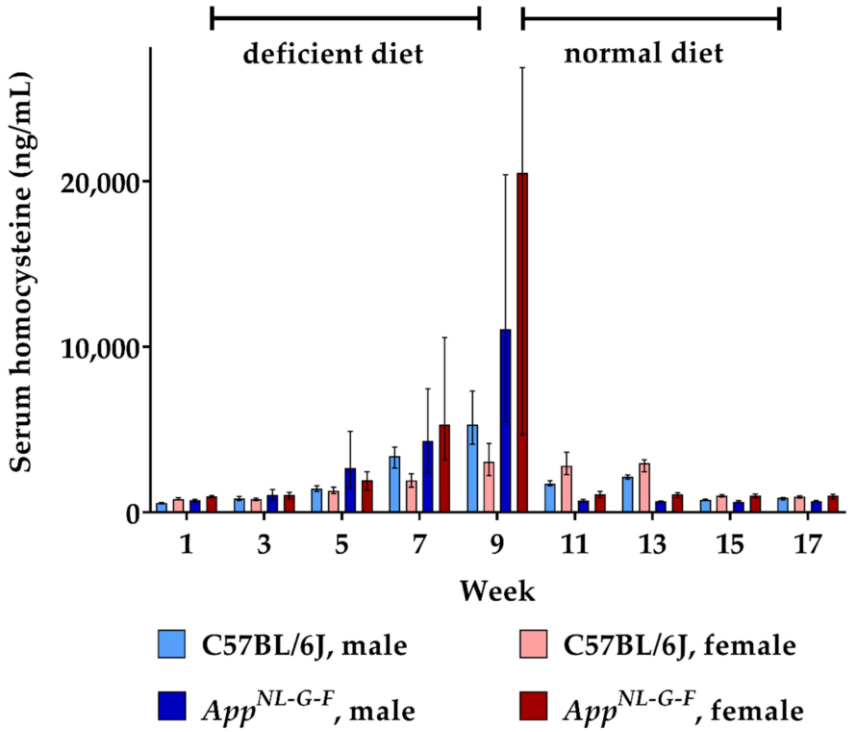

Figure 1. Serum homocysteine levels in C57BL/6J wild type and $App^{NL\text{-}G\text{-}F}$ knock-in mice, data are depicted for males and females separately for experimental week 1-17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all serum samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded.

Similar to HCys, serum levels of its metabolite HCA also increased due to the lack of vitamin B6, B12 and folate over eight weeks. At week 9, approximately 0.15% of HCys molecules had undergone oxidation to HCA, reaching a mean serum level of about 15 ng/mL. There were no consistent differences between WT and KI mice, nor between males and females regarding the increase in HCA levels. Following the return to the balanced control diet, serum HCA had normalized to baseline levels already at the subsequent sampling point (week 11).

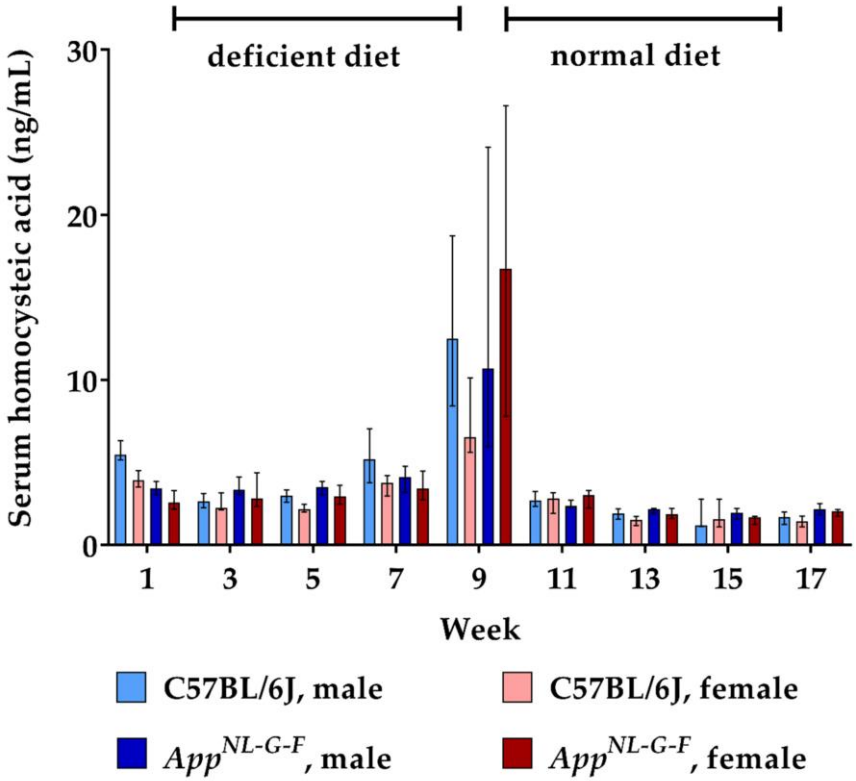

Figure 2. Serum homocysteic acid levels in C57BL/6J wild type and $App^{NL\text{-}G\text{-}F}$ knock-in mice, data are depicted for males and females separately for experimental week 1–17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all serum samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded.

2.2. Urine Analysis

24 h urine collections were sampled every week in order to assess the renal excretion of HCys and HCA. The amount of excreted HCys steadily increased in all animals during the 8-week-long period on B-vitamin deficient chow (Figure 3). At week 9, mean urinary HCys (male + female) of about 20.3 µg (≈0.15 µmoL) was excreted by WT mice and about 97.2 µg (≈0.72 µmoL) by the KI animals in 24 h. Similar to the findings in the serum, $App^{NL\text{-}G\text{-}F}$ KI mice displayed significantly higher urinary HCys amounts compared to WT (week 9 (male + female): $p = 0.001$). However, at baseline level and after normalization, HCys excretion did not differ between WT and KI animals (week 1: $p = 0.228$; week 10: $p = 0.095$). Furthermore, no sex-related effect was observed. Normalization of HCys excretion was achieved immediately after the return to control chow. Baseline levels were restored within one week.

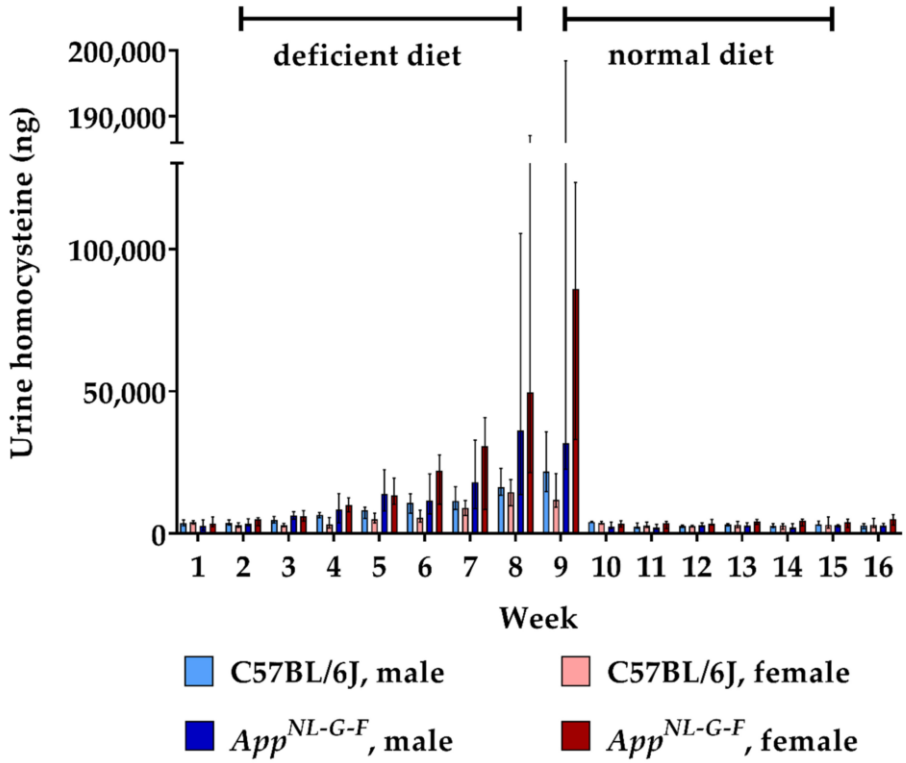

Figure 3. Urinary homocysteine levels in C57BL/6J wild type and App^{NL-G-F} knock-in mice, data are depicted for males and females separately for experimental week 1-17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all urine samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded.

Feeding the deficient diet also resulted in an increased urinary excretion of HCA in App^{NL-G-F} KI mice, but not in WT control mice (Figure 4). Consequently, urinary HCA amounts were significantly higher in hyperhomocysteinemic KI mice (week 9: $p = 0.002$). As for HCys, we did not detect a consistent sex-dependent impact on the renal clearance of HCA. The conversion from B-vitamin deficient chow to normal chow decreased HCA excretion in the KI animals to baseline level within two weeks. Overall, dietary impact on urinary HCA was not as high as in the case of HCys.

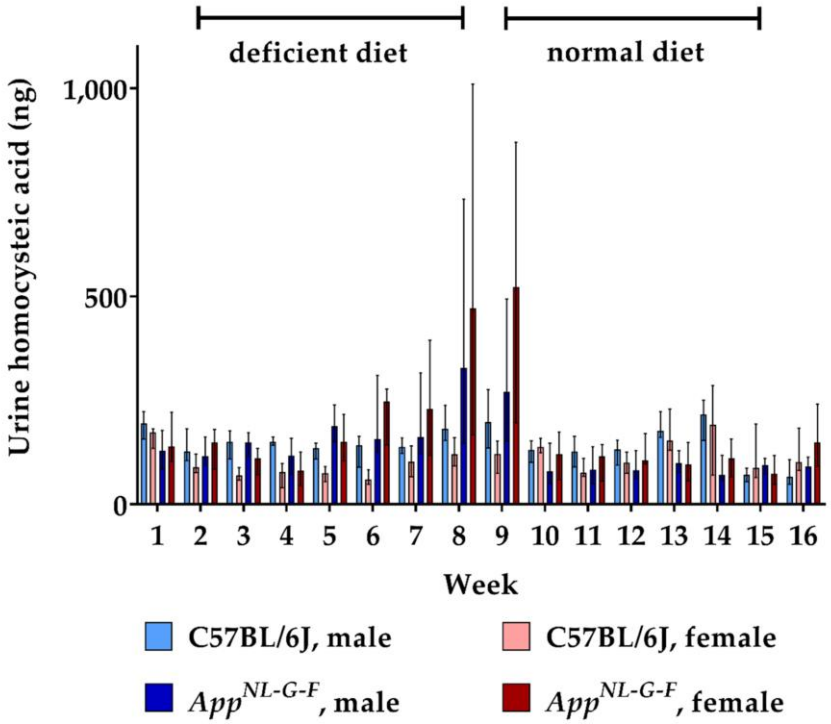

Figure 4. Urinary homocysteic acid levels in C57BL/6J wild type and $App^{NL\text{-}G\text{-}F}$ knock-in mice, data are depicted for males and females separately for experimental week 1–17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all urine samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded.

2.3. Brain Tissue Analysis

Murine brains were analyzed for HCys and HCA at week 9 and week 17 of the experiment. As no animals were sacrificed at the beginning of the study, there were no cerebral baseline measurements for HCys and HCA. Data obtained after feeding control chow for eight weeks (week 17) therefore served as a control in this case. Feeding with the B-vitamin deficient diet for eight weeks resulted in higher cerebral HCys in both WT and KI mice (week 9) compared to control levels (week 17). Hyperhomocysteinemic $App^{NL\text{-}G\text{-}F}$ mice displayed significantly higher levels than the C57BL/6J control (*week 9*; $p = 0.001$). There was no statistically significant sex-dependent impact on cerebral HCys at week 9. Although cerebral HCys was reduced in all groups after the control chow period (week 17), male KI mice still displayed relatively high levels compared to females and wild type mice. Results for HCys in brain tissues are presented, in part, in a descriptive manner, because all measured WT samples after eight weeks on control chow (week 17) had very low levels, below the lower limit of quantification (<LLOQ) of our LC-MS/MS method, which is 1.000 ng/mg tissue (Figure 5).

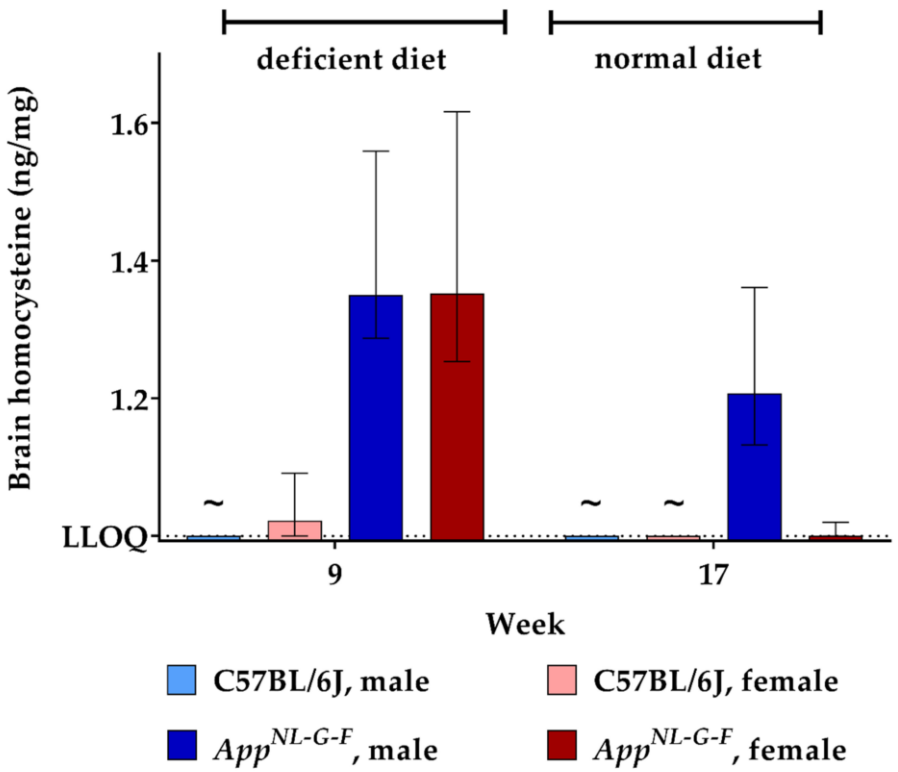

Figure 5. Homocysteine levels in brain tissue in C57BL/6J wild type and App^{NL-G-F} knock-in mice, data are depicted for males and females separately for experimental week 1–17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all brain samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded; ~indicates levels below the lower limit of quantification (LLOQ).

Similar to what we found for brain HCys levels, feeding the deficient diet increased brain HCA (week 9) compared to control levels (week 17), as illustrated in Figure 6. The difference between KI and WT mice did not reach statistical significance here (week 9). Also, no sex-related differences were observed between males and females. Another eight weeks on control chow decreased cerebral HCA concentrations to about 1.2 pg/mg tissue in the mice. In the case of HCA, we were able to assess cerebral levels at both week 9 and week 17 quantitatively, due to a more sensitive method reaching an LLOQ of 1.000 pg/mg tissue.

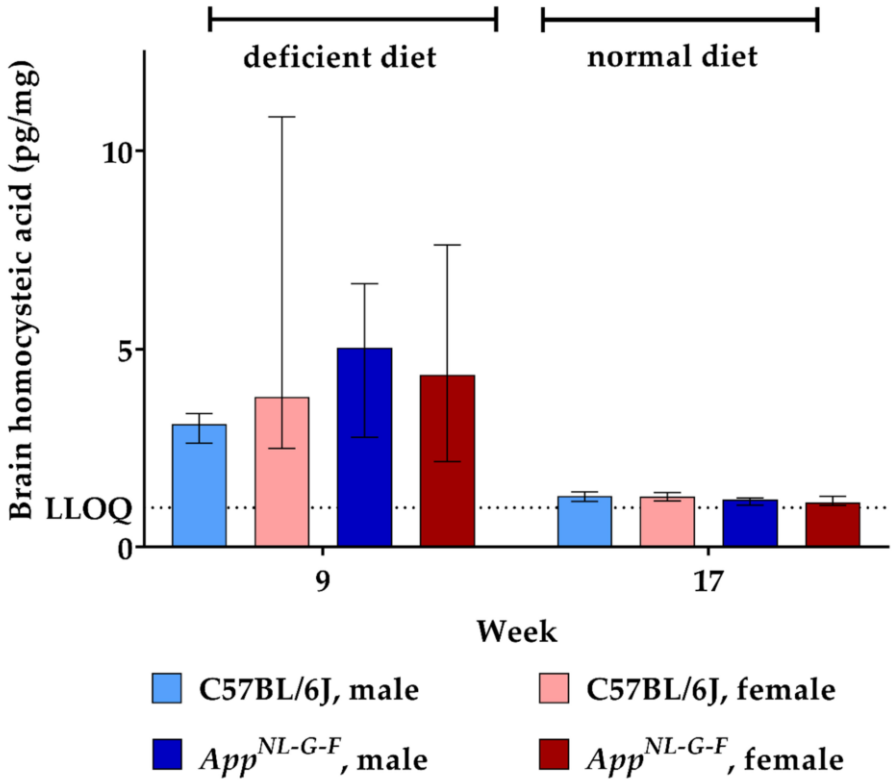

Figure 6. Homocysteic acid levels in brain tissue in C57BL/6J wild type and App^{NL-G-F} knock-in mice, data are depicted for males and females separately for experimental week 1–17: baseline measurement (w. 1), diet deficient in vitamin B6, B12 and folate (for 8 w.), balanced normal chow (for 8 w.); all brain samples were analyzed using a combination of liquid chromatography with tandem mass spectrometry and statistically tested non-parametrically using the Kruskal-Wallis test; data are presented as median ± interquartile range (IQR); extreme outliers beyond 3xIQR have been excluded.

2.4. Correlation

Murine samples from different biological matrices were collected during the course of the current study in order to assess bioavailability and kinetics of HCys and HCA. After the induction of a hyperhomocysteinemic state through B-vitamin deficiency (week 9), we detected a significant positive correlation for HCys between its serum and urine levels (Table 1). Serum HCys levels were also positively correlated with HCys levels in cerebral tissues. For HCA, serum and urine correlation data indicated the same type of effects as for HCys (Table 1). Serum HCA also correlated positively with cerebral HCA concentrations. Furthermore, serum HCys correlated positively with serum HCA and urinary HCys correlated positively with urinary HCA. However, no significant correlation was found between cerebral HCys and cerebral HCA. In summary, we observed the overall relation that low HCys and HCA at baseline level (at the beginning or the end of the study) did not show any correlation, whereas a positive correlation was found after the onset of hyperhomocysteinema.

Table 1. Spearman's rank correlation analysis of hyperhomocysteinemic mice at week 9 (after 8 weeks on deficient diet).

Correlation	Correlation Coefficient	Sig. (2-Tailed)
Serum-urine (HCys)	0.771	<0.001
Serum-brain (HCys)	0.735	<0.001
Serum-urine (HCA)	0.675	<0.001
Serum-brain (HCA)	0.521	0.001
HCys-HCA (serum)	0.660	<0.001
HCys-HCA (urine)	0.860	<0.001
HCys-HCA (brain)	0.142	0.509

3. Discussion

In the current in vivo kinetic study, frequent sampling of murine matrices and subsequent LC-MS/MS analysis of HCys and HCA was carried out to clarify whether the AD-like genotype in the App^{NL-G-F} KI mouse model had an impact on the hyperhomocysteinemic state. Furthermore, potential sex-specific differences, the bioavailability of HCys and HCA in the brain and correlations between serum, urine and cerebral tissues were investigated. In similar previous experiments, hyperhomocysteinemia was mostly induced chemically and kinetic parameters were investigated within a maximum of 24 h [30,39–42]. In contrast, we conducted a long(er)-term kinetic study by inducing chronically elevated levels of HCys and HCA using a dietary approach and investigating both increases and decreases in these endogenous metabolites over 17 weeks.

3.1. Genotype Effects

Amyloid pathology is a crucial and initial hallmark of AD, occurring many years before the onset of cognitive symptoms [43,44]. We used the novel App^{NL-G-F} KI mouse model in order to simulate the AD-like amyloid pathology more realistically, compared to APP-based transgenic models [10]. Numerous studies in the field report an exacerbation of Aβ accumulation and deposition induced by elevated levels of HCys [45–50] or HCA [51,52]. As the majority of in vivo studies focused on HCys levels and its consequences for AD hallmarks, vice versa, the impact of amyloid pathology on HCys levels has been poorly investigated. In our study, we observed a B-vitamin deficiency-triggered elevation of HCys and HCA, which proved to be significantly higher in App^{NL-G-F} KI mice in comparison to C57BL/6J WT mice. This is in accordance with previous findings by Bernardo and colleagues [53], who investigated HCys plasma levels in female, transgenic APP-overexpressing mice. An impact of the AD-like genotype on HCys metabolism was proposed, because it obviously induced higher HCys in both the control diet group and the methyl-donor deficient diet group [53]; hippocampal cell death was reported in transgenic but not in WT mice [53,54]. A similar genotype effect was observed in ArcAβ transgenic mice [55], where a diet-induced hyperhomocysteinemia occurred in the transgenic mice in contrast to WT control mice. This genotype effect might possibly be explained by reactive oxygen species that are part of the amyloid pathology in AD [56]. Oxidative stress is suggested to deplete folate [57], which plays an essential role in the context of the HCys remethylation cycle. Consequently, oxidative stress and the subsequent lack of folate could be the reason for the occurrence of the hyperhomocysteinemic state [58]. In order to elucidate mechanistical details, future experiments will be helpful. As summarized by Hasegawa and Ukai, the oxidative metabolite HCA is also suggested to be increased by oxidative stress via amyloid pathology [59]. However, our data do not confirm a significant elevation of serum HCA compared to the WT control. Also, the HCA/HCys ratio was not increased in the App^{NL-G-F} KI model.

3.2. Sex-Specific Effects

WT males tended to display higher HCys and HCA in serum and urine than females after eight weeks on the deficient chow. This is translationally in accordance with the

observation that men display higher HCys levels than women [60,61], a difference that is suggested to be the result of hormonal effects [62,63]. As summarized by Cohen and colleagues [4], a lower transsulfuration rate and higher creatine metabolism in men might also lead to the sex-related divergence in HCys levels. However, the aforementioned tendency fades out by also taking the findings in the KI animals into account. In total, no consistent sex-dependent effect was detected during the course of our study. According to this, other clinical [15] and preclinical [40] findings do not confirm significant sex-related effects. Others even detected higher HCys in female mice than in males [64], so all things considered, the evidence is equivocal in this topic. A limitation of our study is that no quantification of the amyloid pathology in the KI mice has been undertaken. Previous investigations in the App^{NL-G-F} model did not indicate a sex difference in the Aβ plaque load [65].

3.3. Brain HCys and HCA

Levels of HCys and HCA, especially the bioavailability in the brain, which is essential information in the context of neurodegeneration, have not yet been characterized in the novel App^{NL-G-F} KI mouse model for AD. Data for HCys and HCA were reported previously in human CSF [51,66–68]. However, we sampled cerebral tissues in the current study, because murine CSF would not have delivered enough volume to apply our LC-MS/MS methods. We detected HCys levels at about 1 ng/mg tissue in the App^{NL-G-F} brains and thus, within the range of 0.135 ng/mg and 44 ng/mg that has been reported previously [30,69–72]. However, levels are hardly directly comparable between the in vivo studies, because different rodent models, as well as varying strategies to induce hyperhomocysteinemia, were used. Currently, we do not know the reason for the lower decrease in HCys in male KI mice on control chow (week 17). In combination with the poor correlation, we observed between HCys and HCA in cerebral tissues, this indicates that the underlying HCys metabolism in the brain, including the formation of HCA, might differ from the other matrices. HCA levels in our mice were detected at about 5 pg/mg tissue (week 9), which is not in accordance with one of the rare previous publications on HCA [52]. Hasegawa and colleagues reported 2000-fold higher HCA values for cerebral tissues and, in a subsequent publication, considered these relatively low [73]. Nevertheless, we agree with Hasegawa that AD pathology seems to significantly elevate HCys, but not HCA levels, in the CNS compared to non-AD controls [51].

3.4. HCys and HCA in Other Biological Matrices

HCys serum concentrations beyond 15 µmoL/L (≈2030 ng/mL) were considered high and referred to as hyperhomocysteinemia [74]. By feeding a diet deficient in vitamin B6, B12 and folate, we induced elevated levels of HCys beyond 15 µmoL/L and therefore, a hyperhomocysteinemic state in our mice. HCA concentrations were much lower than HCys, because oxidation of the latter is only possible in the free thiol form, which accounts for merely 1% [14].

In this study, we also focused on the correlation between HCys and HCA, as well as on the levels in different biological matrices. There was a significant correlation between elevated serum HCys and HCA in the mice after eight weeks on deficient chow, but not for baseline levels at the beginning or at the end of the study. This lack of correlation was also previously reported for plasma levels and might be explained by different levels of oxidative stress and its impact on the spontaneous oxidation of HCys to HCA [36,75,76]. Hasegawa reported an inverse correlation between blood HCA and urinary HCA and suggested that a reduced renal excretion results in elevated blood levels and subsequent cognitive impairment in patients [73]. Our murine HCA data, indicating increasing urinary excretion at increasing serum levels, did not confirm this relation and were detected at lower levels [52,73]. Recently, lower levels were also measured in human serum by conducting LC-MS/MS analytics [68,77]. In humans, urinary excretion of unchanged HCys is considered minimal due to a high extent of reabsorption in renal tubules [3];

about 6 µmoL (≈811 µg) HCys are excreted per day [78]. According to our results, lack of essential HCys-reducing B-vitamins led to a more than 10-fold elevated excretion of HCys in the kidney in both experimental mice and humans [3].

4. Materials and Methods

4.1. Animals, Diets and Study Design

All animal experiments were carried out according to the DIRECTIVE 2010/63/EU and the regulations of GV-SOLAS, approved by the local Ethics Committee for Animal Research in Darmstadt, Germany (approval number: F152/1011; approval date: 31.07.2017) and based on the ARRIVE-Guidelines.

A total of 80 mice were included in this study, equally consisting of C57BL/6J wild type (20 males, 20 females) and age-matched App^{NL-G-F} knock-in mice (20 males, 20 females; C57BL/6J background). Wild type animals (WT) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany) and knock-in (KI) animals were kindly provided by the RIKEN Center for Brain Science (Saitama, Japan) and further bred at mfd Diagnostics GmbH (Wendelsheim, Germany). All animals were allocated to their home cages (Green Line, Tecniplast, Hohenpeissenberg, Germany) according to a randomization list (https://www.random.org/) and were housed pairwise at constant temperature (mean: 22.7 °C) and humidity (mean: 55.4%) conditions under a 12/12 h dark/light cycle (lights on at 7:00 am).

After baseline serum and urine sampling, all mice received a special diet deficient in vitamin B6, B9 (folate) and B12 for eight weeks. Subsequently, half of the animals were euthanized in order to collect the brain tissue. The other half of the animals returned to a balanced control chow (normal diet) for another eight weeks in order to investigate a potential normalization of the levels of HCys and HCA. Potential side effects that might be induced by over-supplementation with B-vitamins are not further investigated or described here [79–85]. Sampling steps were carried out frequently in order to collect different biological matrices. The exact study course is illustrated in Figure 7.

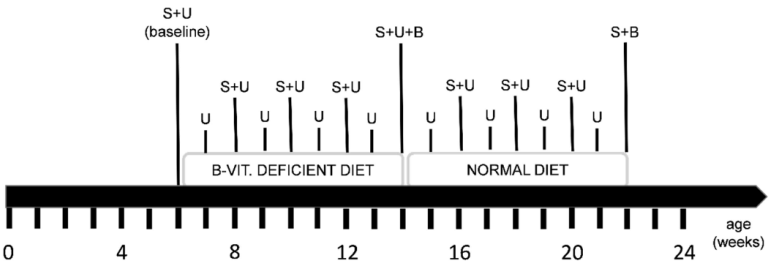

Figure 7. Time line of the study course, diet regimen and sampling points: S = serum sampling, U = 24-h urine sampling in metabolic cages, B = brain sampling: one half of the animals was euthanized in the middle (experimental week 9) and the other half at the end of the study (experimental week 17); feeding a diet deficient in vitamin B6, folate (B9) and B12 for 8 weeks was followed by a normal diet period for another 8 weeks.

Because mice are prone to coprophagia, the antibiotic sulfathiazole sodium (Sigma-Aldrich, Taufkirchen, Germany) had been added to the deficient diet in order to prevent folate synthesis by gut bacteria [45]. Both the B-vitamin deficient and the control diet were obtained from (Ssniff-Spezialdiäten GmbH, Soest, Germany). Table 2 indicates the exact composition of these diets, based on the AINM93M chow. Each animal received four gram of diet per day and water ad libitum. All mice were weighed every week and scored for body condition twice a week in order to monitor the nutritional status.

Table 2. Exact composition of the control (normal) diet and the B-vitamin deficient diet.

	Control	Deficient
Casein	140.0	140.0
Corn starch	355.6575	345.6920
Maltodextrin	155.0	155.0
Sucrose	100.0	100.0
Dextrose	100.0	100.0
Cellulose	50.0	50.0
Mineral premix	35.0	35.0
Vitamin premix (w/o B-vitamins)	10.0	10.0
Soybean oil	19.0	19.0
Coconut oil	9.0	9.0
Corn oil	22.0	22.0
L-Cystine	1.8000	1.8000
Tert-butylhydroquinone	0.0080	0.0080
Choline bitartrate, 41%	2.5000	2.5000
Pyridoxine-HCl (Vit. B6)	0.0070	——
Cyanocobalamin, 0.1% (Vit. B12)	0.0250	——
Folic acid, 80%	0.0025	——
Sulfathiazole sodium	——	10.0000
Sum	1000	1000

4.2. Sample Collection

Before the start of the experimental diet, serum and urine were collected from the mice at the age of 6 weeks in order to assess baseline levels of HCys and HCA. In accordance with the national animal welfare guidelines of the GV-SOLAS, we collected blood (170 µL per 25 g mouse) every two weeks during the study course. For the purpose of blood collection, we anaesthetized the mice using isoflurane (Piramal Critical Care, Hallbergmoos, Germany) and subsequently punctured the retrobulbar vein with a glass capillary (Brand GmbH + Co KG, Wertheim, Germany). Blood was collected in serum tubes containing a clotting factor (Sarstedt Microvette 200 Z, Nümbrecht, Germany) and coagulated for 15–30 min. Subsequent centrifugation (3200 g; 4 °C; 15 min) provided serum. Additionally, urine from each mouse was sampled every week using metabolic cages (Tecniplast, Hohenspeissenberg, Germany). Serum and urine were immediately frozen on dry ice and stored at −80 °C for further biochemical analysis. Volumes of the urine samples were documented in order to determine the absolute excretion of HCys and HCA in 24 h. At the age of 14 weeks, resp. after 8 weeks on B-vitamin deficient diet, half of the animals was euthanized by cervical dislocation and brains were harvested. After the removal of cerebellum and olfactory bulbs, the hemispheres were divided, weighed and frozen in liquid nitrogen for subsequent analysis of HCys and HCA. Afterwards, the remaining animals returned to control chow for 8 weeks. At the end of the study, the remaining animals were euthanized, and brains were sampled and processed as described before.

4.3. Biochemical Analysis

The determination of HCA in serum and urine samples was performed as described in detail [38,77], using a combination of protein precipitation (ice-cold acetonitrile) and solid phase extraction (tabless Strata X AW SPE columns (33 µm, 30 mg/1 mL, Phenomenex, Aschaffenburg, Germany) and the automated sample preparation system Extrahera (Biotage, Uppsala, Sweden)) for sample preparation followed by an LC–MS/MS analysis applying a combination of a HILIC separation (Luna 3 µm HILIC 200 Å 100 × 2 mm column in combination with a KrudKatcher in-line filter (both Phenomenex, Aschaffenburg, Germany)) and tandem mass spectrometry. As the concentration of HCA in murine samples is higher than in human samples, lower sample volumes were used for murine samples and PBS was added to achieve a total sample volume of 200 µL. Thereafter, the samples were processed as described [77]. HCys in serum and urine samples was analyzed using

protein precipitation (methanolic TCEP solution) in combination with reversed phase chromatography (Luna Omega 1.6 µm Polar C18 100 × 2.1 mm column in combination with a respective pre column (both Phenomenex, Aschaffenburg, Germany)) and tandem mass spectrometry as described in detail [38]. As we had not analyzed brain samples for HCA or HCys in our lab before, the established methods were adapted to the new matrix. The first step was the homogenization of the brain samples using a weight-dependent volume of a mixture of water and ethanol (*v*:*v*–75:25) and a swing mill (Mixer Mill MM 400, Retsch, Haan, Germany) generating a homogenate with a tissue concentration of 0.2 mg/mL. A sample volume of 200 µL homogenate was used for the determination of HCA using the same sample preparation protocol and LC-MS/MS method as for serum and urine. The determination of HCys in brain tissue homogenate using reversed phase chromatography was not possible as interferences in the chromatogram occurred making the quantification of HCys in these samples difficult. Therefore, a hydrophilic interaction liquid chromatography (HILIC) method was applied using the same column as for HCA and a mixture of water, acetonitrile, and 0.1 M ammonium acetate solution (88:10:2, *v*/*v*/*v*) as solvent A, and acetonitrile containing 0.1% formic acid as solvent B. For separation, a gradient program was used at a flow rate of 0.75 mL/min. The initial buffer composition 3% (A)/97% (B) was held for 0.25 min and then within 2.25 min, linearly changed to 50% (A)/50% (B) and held for 1.00 min. Subsequently, the composition was linearly changed within 0.1 min to 3% (A)/97% (B) and then held for another 3.4 min. The total running time was 7 min, and the injection volume was 2.5 µL. The sample preparation protocol was identical to the procedure for the determination of HCys in serum and urine.

The LC-MS/MS system for the determination of HCA in serum, urine and brain tissue as well as for the determination of HCys in serum and urine consisted of a triple quadrupole mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray source and an Agilent 1290 Infinity LC-system with binary HPLC pump, column oven and autosampler (Agilent, Waldbronn, Germany). The LC-MS/MS system for the determination HCys in brain tissue consisted of a triple quadrupole mass spectrometer QTRAP 5500+ (Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray source and an Agilent 1200 LC-system with binary HPLC pump, column oven (Agilent, Waldbronn, Germany) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). Data acquisition was done using Analyst Software 1.7.1 and quantification was performed with MultiQuant Software 3.0.3 (both Sciex, Darmstadt, Germany), employing the internal standard method. Calibration curves were calculated by linear regression with 1/x weighting. Acceptance criteria and quality assurance measures have been applied as previously described [77].

4.4. Statistical Analysis

Prior to the study, a statistical power calculation was conducted in order to estimate the adequate number of mice for the experiments (http://www.biomath.info/power/). For the statistical analysis of the data, we used IBM SPSS Statistics 26 (Ehningen, Germany). We conducted an outlier analysis to detect extreme outliers (more than threefold the interquartile range). Shapiro-Wilk test indicated that a Gaussian distribution could not be assumed for various data sets. Consequently, the non-parametric Kruskal–Wallis test (+ Bonferroni adjustment for *p*-values) and Spearman rank correlation test were applied. A p value lower than 0.05 was considered statistically significant. Data were depicted as median ± interquartile range (IQR), separately for males and females, using GraphPad Prism 7 software (San Diego, CA, USA).

5. Conclusions

1. In this exploratory in vivo kinetic study, we observed a significant genotype effect on HCys, but not HCA, in hyperhomocysteinemic $App^{NL\text{-}G\text{-}F}$ KI mice compared to the age-matched C57BL/6J WT control and therefore, confirmed a potential causal contribution of the AD-like pathology to hyperhomocysteinemia.

2. Our data did not indicate a consistent sex-related effect on the hyperhomocysteinemic state and therefore did not meet the expectation that levels are generally higher in males.
3. HCys and HCA were detected in cerebral tissues of WT and KI mice, which is a crucial condition for the potential contribution to pathologies in the context of neurodegenerative disorders. The consumption of a B-vitamin deficient diet resulted in increased cerebral levels of HCys and HCA.
4. In hyperhomocysteinemic mice, serum HCys and HCA levels correlated positively with both cerebral concentrations and urinary amounts.

To our knowledge, this is the first in vivo kinetic study that considers HCA, which might be the actual culprit in terms of neuronal damage, additionally to HCys. The observed genotype impact in the App^{NL-G-F} KI mouse model for AD suggests that AD pathology affects HCys levels and indicates a synergistically exacerbating effect of B-vitamin deficiency and AD pathology on hyperhomocysteinemia. The immediate normalization of HCys and HCA after the supply of relevant B-vitamins in a balanced diet, strengthens the idea of a B-vitamin intervention in order to interrupt HCys-triggered pathologic processes. Additional studies will have to be performed to corroborate the previously suggested potential as a preventative treatment option for AD, which might be effective and easily feasible.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	Alzheimer's disease
HCys	Homocysteine
HCA	Homocysteic acid
WT	Wild type
KI	Knock-in
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
HPLC	High-performance liquid chromatography
HILIC	Hydrophilic interaction liquid chromatography
LLOQ	Hydrophilic interaction liquid chromatography
GV-SOLAS	Gesellschaft für Versuchstierkunde / Society of Laboratory Animal Science

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Review

Hyperhomocysteinemia: Metabolic Role and Animal Studies with a Focus on Cognitive Performance and Decline—A Review

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Abstract: Disturbances in the one-carbon metabolism are often indicated by altered levels of the endogenous amino acid homocysteine (HCys), which is additionally discussed to causally contribute to diverse pathologies. In the first part of the present review, we profoundly and critically discuss the metabolic role and pathomechanisms of HCys, as well as its potential impact on different human disorders. The use of adequate animal models can aid in unravelling the complex pathological processes underlying the role of hyperhomocysteinemia (HHCys). Therefore, in the second part, we systematically searched PubMed/Medline for animal studies regarding HHCys and focused on the potential impact on cognitive performance and decline. The majority of reviewed studies reported a significant effect of HHCys on the investigated behavioral outcomes. Despite of persistent controversial discussions about equivocal findings, especially in clinical studies, the present evaluation of preclinical evidence indicates a causal link between HHCys and cognition-related- especially dementia-like disorders, and points out the further urge for large-scale, well-designed clinical studies in order to elucidate the normalization of HCys levels as a potential preventative or therapeutic approach in human pathologies.

Keywords: hyperhomocysteinemia; vitamin B deficiency; dementia; disease models; animal

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1. Introduction

The metabolism of molecular groups with only one carbon atom (C1 metabolism) is part of the basic equipment of cells. Its products function as building blocks or as links in regulatory chains and are essential for the synthesis or completion of an enormous number of larger molecules. The large number and variety of products show that disorders of C1 metabolism can also lead to numerous symptoms and diseases. The present review aims to list disease groups or particular, mostly common diseases, for which the involvement of C1 metabolism and underlying pathological mechanisms are substantiated by relevant evidence, especially clinical studies including interventions. For this purpose, both the human and animal parts of the current review focus on the contribution of C1 metabolic disorders and hyperhomocysteinemia (HHCys) on cognitive performance and decline. In order to facilitate understanding, we prefixed a critical, general part with the subsequent five sections.

2. C1 Metabolism and HHCys

2.1. Reactions of the C1 Metabolism and Its Main Products

Figure 1 shows the reactions through which all C1 compounds are generated and it serves as a building block for the subsequent figures.

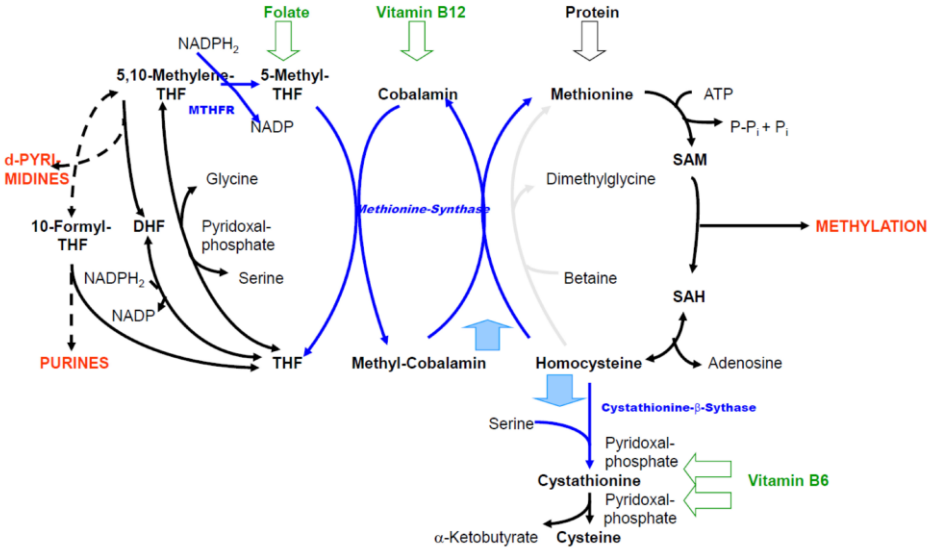

Figure 1. All essential reactions of the C1 metabolism: On the left side, the folic acid cycle is depicted, showing formyl and methenyl groups bound to tetrahydrofolate (THF), which is involved in purine synthesis (DNA, RNA), and methylene, involved in deoxythymidylate synthesis (DNA) and methyl groups that are required for the re-methylation of homocysteine (HCys) to methionine. On the right side, the methylation cycle is illustrated, including S-adenosylmethionine (SAM) methylations: nucleic acids, proteins, phospholipids, neurotransmitters, hormones, creatine and others. Histone protein, DNA and RNA methylations cause epigenetic regulation [1,2]. The vast majority of methylations originate from SAM [3]. More than 200 SAM-dependent methyltransferases are encoded in the human genome [4]. Red: products, intermediate reactions are omitted (dashed arrows); blue: reactions with enzymes for which genetic defects frequently occur or which catalyze reactions that can be reduced; green: necessary B-vitamins that cannot replace each other. The reaction catalyzed by methionine synthase needs two vitamins as cofactors at the same time; two light blue arrows: re-methylation of HCys (upwards), transsulfuration of HCys (downwards); MTHFR: 5,10-methylene tetrahydrofolate reductase, THF: tetrahydrofolate, DHF: dihydrofolate, SAM: S-adenosyl methionine, SAH: S-adenosyl homocysteine.

2.1.1. Thermodynamic Features

(a) The reaction catalyzed by 5,10-methylenetetrahydrofolate reductase (MTHFR) proceeds almost completely unidirectional to 5-methyl-THF under normal metabolic conditions [5]. There is a reason for the so-called folic acid trap [6,7]: if there is a pronounced vitamin B12 deficiency, there is no re-methylation of HCys via methionine synthase (Figure 1). Even with sufficient folic acid intake, it accumulates as 5-methyl-THF potentially resulting in a deficiency in C1 compounds of the folic acid cycle.

(b) The reaction of S-adenosyl homocysteine (SAH) to HCys (by the SAH hydrolase) tends towards SAH formation [8]. In this way, the cellular HCys concentration is kept low under normal metabolic conditions.

2.1.2. Special Features of the Enzyme Equipment and Kinetics

(a) In Figure 1, the re-methylation of HCys to methionine by betaine (betaine homocysteine methyl transferase) is marked in light gray, because the enzyme is only expressed in few tissues, such as liver and kidneys [6,9].

(b) The cellular concentrations of total HCys for most organs are 2–7 nmol/g wet weight [10]. Calculating with approximately 70% cell water results in concentrations of 3–10 µM. The K_M values for HCys of the initiating enzymes of re-methylation (methionine synthase) and transsulfuration (cystathionine-β-synthase) are 0.06 mM and approximately 10 mM [11] and thus, differ by three orders of magnitude. Serine, the second substrate of cystathionine-β-synthase (CBS), also has a high K_M value of 2 mM [12]. From this, it can be concluded that if there is an adequate supply with folic acid, vitamin B12 and B6, HCys is predominantly re-methylated.

(c) Transsulfuration is not possible in some tissues, because there is no expression of CBS (heart, vessels, lungs, adrenal gland, spleen, testes) or cystathionase (brain, adipose tissue) [11].

(d) The availability of sufficient SAM as a substrate for the majority of methylation reactions is a crucial function of C1 metabolism. In humans, 6–8 g SAM are synthesized daily [13]. Its synthesis is largely ensured by the effector functions of SAM and, at the same time, HCys metabolism is influenced, since SAM inhibits the MTHFR [14] and activates the CBS [15]. In cells that express both enzymes, when SAM levels rise (e.g., due to an abundant supply of methionine) this is irreversibly removed via transsulfuration. Owing to the high K_M value of CBS (cf. above) enhanced flux rate through transsulfuration is accompanied by an increase in cellular HCys concentration. When there is a deficiency of SAM, re-methylation of HCys is stimulated.

(e) SAH is a potent inhibitor of most SAM-dependent methylation reactions [16]. However, the consequences are different for individual methylations, as will be explained later.

(f) A special kind of methylation cycle arises from the ability of the methionine synthase to catalyze also protein-bound HCys, as in the case of the D4 dopamine receptor (D4). Stimulation of D4-bound methionine leads via D4-bound SAM to methylation of membrane phospholipids [17].

2.2. Principal Causes of C1 Metabolic Disorders

As illustrated in Figure 2, insufficient clearance with simultaneously constant production leads to increases in the levels of intermediates (HCys, SAH) and consequently, to the formation of new products, such as homocysteine thiolactone and homocysteic acid.

The following list of causes for disorders in C1 metabolism does not take into account genetic defects with prevalence ≤1:10,000, because they are too rare as disease triggers. Frequent causes can essentially be assigned to four groups (Table 1).

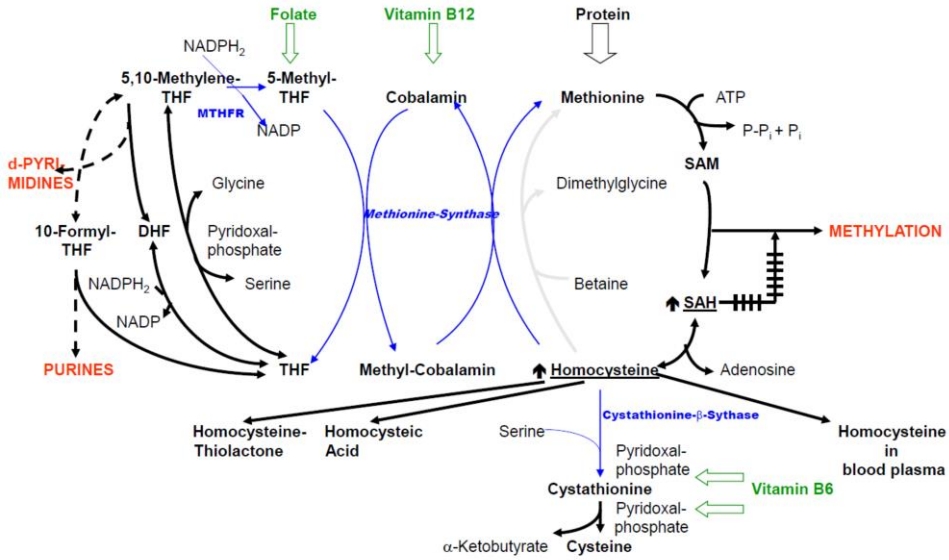

Figure 2. Adaption of the previous chart (colors and abbreviations: see Figure 1 caption): C1 metabolism in the case of vitamin deficiency or genetic enzyme variants; reductions in supply or turnover due to various causes are marked by thinner arrows; inhibitory effects are marked by the crossed-out arrow.

Table 1. Frequent causes of C1 metabolic disorders.

Cellular deficiency in one or more of the vitamins B6, B12 and folate:
(a) Insufficient intake via food:
1. Vitamin B12 deficiency in vegetarians and vegans, who do not supplement vitamin B12 [18,19].
2. All three vitamins in elderly subjects, especially in nursing homes [18,20].
3. Pronounced folic acid deficiency in industrialized countries around the world [21,22]. It is the main reason for folic acid supplementation in more than 70 countries [23].
(b) Loss due to inadequate preparation, especially for folic acid [24].
(c) Increased need during pregnancy, lactation and hemodialysis.
(d) Insufficient intestinal absorption: unspecific in celiac disease, inflammatory bowel diseases and resections, specific for B12 with intrinsic factor deficiency or auto-antibodies against parietal cells [25,26].
(e) Intracellular, metabolic causes, e.g., accumulation of 5-methyl-THF in the case of pronounced B12 deficiency ("folic acid trap"), leading to a deficiency of THF-dependent C1 compounds, despite adequate folic acid intake [6,7].
(f) Side effects of pharmaceuticals on absorption or metabolism of particular vitamins, e.g., anticonvulsant drugs, levodopa, metformin [27].

Table 1. *Cont.*

Common genetic variants in C1 metabolism: (a) MTHFR: C677T point mutation in homozygous form (TT) in 12–15% of the European population, which can be compensated by adequate folic acid intake [28]. Combined occurrence of the heterozygous form (CT) with the heterozygous form (AC) of another point mutation—A1298C—is relatively common (approximately 25%) and can be associated with various disturbances [29,30]. (b) CBS: About 230 known mutations that are rarely homozygous. In the heterozygous form, they potentially occur in around 1% of the European population [31].
Lifestyle factors (the underlying mechanisms are often not clear or multifactorial and usually linked to their effect on plasma HCys level): (a) Acquired reductions in the activity of enzymes, e.g., methionine synthase due to acetaldehyde in alcoholics [32]. (b) Cigarette smoking appears as an independent determinant of HCys levels, with an increase in approx. 1% per cigarette smoked [33]. (c) Relatively large amounts of coffee consumption are necessary to increase HCys [34]. Oxidative stress: Particularly nitric oxide inhibits methionine synthase directly, as well as by binding cobalamin [35]. Consequently, increase in plasma HCys is accompanied by that of markers of NO formation, e.g., citrulline. In addition, methylmalonic acid strongly increases as NO inhibits cobalamin transport from cytosol into mitochondria [36].

2.3. HCys as a Diagnostic Measurable Biomarker of Disorders

Numerous laboratory parameters for measuring individual causes of HHCys, such as molecular-genetic analyses, vitamin level measurements and others, only measure one parameter at a time and rarely provide information about the extent and severity of the disorder. They are not dealt here within this context.

Under consideration of Figure 2, it is clear that each of the causes discussed above must lead to an increase in cellular HCys. Concomitantly, this results in the increased formation of two secondary metabolites of HCys: homocysteine thiolactone and homocysteic acid. On the other hand, all human cell types investigated so far have transport systems that remove accumulated cellular HCys into the extracellular space, partly also against a concentration gradient [37–39]. Most of the elimination from plasma occurs in the kidneys. The clearance rate in healthy kidneys is remarkably constant, regardless of the plasma HCys level [39].

Plasma HCys is therefore a sensitive parameter for quantifying disorders in C1 metabolism. Compared to other parameters, it usually has higher sensitivity: plasma concentrations of HCys, folic acid, vitamins B12 and B6 were measured in more than 1000 participants in the Framingham Heart Study. For all three vitamins, the HCys level rose in the lower half of their reference range and was around 35% higher at the lower end [40]. Such studies were repeated several times with the (same) result of recommending HCys measurement, because the measurement of the three B-vitamins in plasma only allowed limited conclusions to be drawn about their cellular availability [27].

Plasma HCys levels show differences dependent on age and gender (measured in populations without folic acid supplementation) [27]: The values reach 10 µM at the age of 50 and 60 years in men and women and an increase to approximately 12 µM up to 80 years. The increase in older subjects is apparently also due to the lack of availability of the B-vitamins, since parenteral vitamin substitution lowers the level to that in middle age [41]. Depending on the responsible institution in European countries and in the USA, a level of 10–12 µM has been considered a threshold value. Moderate increase above these threshold values up to approximately 30 µM mark the range for human diseases listed in this part of the present review. These HCys levels are generally considered as a biomarker of disease and/or having a pathogenic effect. Some of the following incomplete findings from older studies, however, should be restrictively considered:

(a) Because HCys is transported out of the cells, the concentrations in the extracellular space and plasma do not have to correspond to those in the cells, which are responsible for the increased production. The liver is the main organ for HCys formation [39]. However, when HCys formation and export are stimulated, the cellular concentration in the liver remains relatively constant [10]. Cultured endothelial cells continuously export HCys into the medium and keep the cellular concentration at a significantly lower level [38]. In contrast, the addition of HCys to the medium (100 μM) leads to absorption and increases the intracellular concentration [38]. It can, therefore, be assumed that endothelial cells have only a small capacity to re-utilize HCys. Increase in HCys levels in plasma and extracellular space is not only effective at, but also in endothelial cells.

(b) Only free HCys is reactive. The ratio of free to protein-bound HCys is different intracellularly than in blood plasma [10]. For example, approximately 4.5 and 3 nmol/g wet weight for free and bound HCys were measured in rat liver, which exchange with a half-life in the range of seconds. The quotient of free/bound HCys is 1.47 for rat liver. For cerebrum and cerebellum, it is 2.72 and 17.81, respectively. Free HCys is exported [10].

Consequently, liver cells might keep constant HCys levels by exporting it to the plasma as a consequence of vitamin deficiency, whereas other cells take it up and highly increase their cellular concentration of free, reactive HCys.

For laboratory diagnostics, total HCys is measured in blood plasma. The major part is bound to albumin (approximately 80%), followed by disulfides with itself or cysteine (cf. Figure 4D). Free, reduced HCys makes up less than 2% [27]. If the level changes in the range of a normal or moderate increase in total HCys concentrations, this equilibrium between the fractions is restored very quickly [42]. With increased cellular HCys formation, free, reduced HCys is exported. Adjustment of the equilibrium in plasma inevitably forms reactive oxygen species (ROS). As shown in Figure 4E, every release of HCys is associated with the formation of radicals.

The use of plasma HCys as a marker of the detection and extent of a C1 metabolic disorder is only possible in patients without significant impairment of their kidney function. The kidneys play a major role in the elimination of HCys from the blood—not through excretion in the urine, which only accounts for about 1% of the daily amount of HCys produced [43]. This is only higher with extremely high HCys levels in plasma, for example with homocystinuria [44]. The renal clearance of HCys occurs mainly through re-methylation and transsulfuration [45,46]. Both pathways are reduced in renal insufficiency [46]. The kidneys are also the major organs for the clearance of SAH from plasma, both by filtration and by metabolism [47]. Kidney damage is typically accompanied by an increase in plasma HCys level. It not only affects the end stages of renal insufficiency, but also reflects the entire course of the disease: a meta-analysis of 41 studies with 2700 test subjects revealed a highly significant correlation between plasma HCys and the reciprocal value of the glomerular filtration rate [48]. It affects the entire range of the glomerular filtration rate [49]. Regardless of its cause, plasma HCys concentrations of 20–80 μM are measured in terminal kidney failure [50]. The increase in plasma HCys is caused by the kidney disease itself. The influence of confounders such as body mass index, plasma lipids, hypertension, smoking, diabetes mellitus and others could be excluded [51]. HHCys caused by renal insufficiency is the only form that mainly arises from reduced clearance of HCys and therefore differs primarily from all other forms of HHCys that result from increased HCys production as a result of disorders in cellular C1 metabolism.

2.4. Principal Pathological Mechanisms with Morbid Effects in C1 Metabolic Disorders

Based on the previous charts, Figure 3 additionally highlights different pathogenic consequences of the disorders, which are subsequently discussed further.

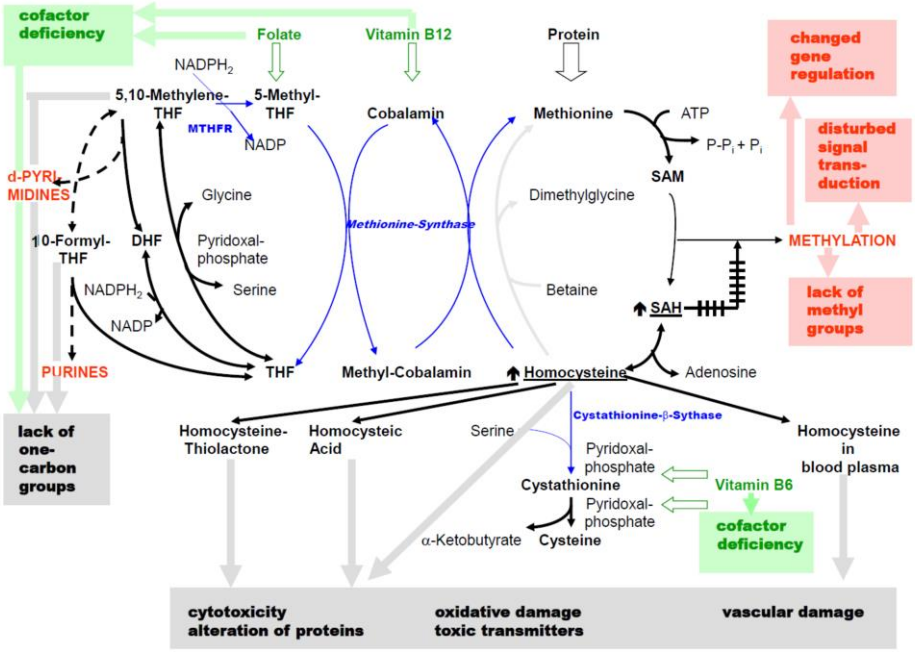

Figure 3. Adaption of the previous chart (colors and abbreviations: see Figure 1 caption): pathogenic effects of metabolites or products in the context of disorders in C1 metabolism.

Deficiency in one or more of vitamin B6, B12 and folate affects cofactors derived from them:

(a) The supply of C1 compounds from THF metabolites is reduced due to folic acid deficiency. This results in lack of nucleotides in energy metabolism and impairment of DNA and RNA synthesis. There is also impairment of mitotic rate.
(b) In addition to reduced HCys transsulfuration, vitamin B6 deficiency causes inhibition of numerous pyridoxal phosphate-dependent reactions in amino acid metabolism.
(c) Vitamin B12 deficiency leads to the accumulation of HCys.

The genetic variants for MTHFR and CBS, as well as acquired causes of inhibition of these enzymes, also lead to accumulation of HCys. The increase in cellular HCys levels also leads to the accumulation of SAH, which is a strong inhibitor of methylation reactions [16]. Consequences are: lack of methyl groups for syntheses, altered methylation of DNA and histones leading to disturbed epigenetic gene regulation, impairment of signal transduction when particular elements, e.g., kinases and phosphatases, are regulated via methylation [52].

As described above (cf. Section 2.1.2), the SAM/SAH as substrate/inhibitor quotient could regulate the overall activity of numerous methyltransferases, meaning increased or decreased methylation of all substrates. However, the regulating effect of the SAM/SAH quotient is differentiated by various mechanisms:

(a) The K_M values for SAM and the K_I values for SAH are different for individual methyltransferases and differ between the various enzymes by almost three orders of magnitude [52,53]. A changed SAM/SAH quotient can either do nothing at all, e.g., if the enzyme continues to work in the V_{Max} range, or result in changes in methylation.

(b) There are "buffer reactions" without metabolic effects, such as the methylation of glycine to sarcosine by glycine-N-methyltransferase, which regulates the SAM concentration [3].

(c) The cellular concentrations of SAM and SAH respond to changes in the intake of vitamin B6, B12 and folate, but evidently vary in different tissues and vary in the individual developmental stages of the organs [52,54].

In addition to being a biomarker of disorders, the increase in HCys levels can also have direct cytotoxic effects (Figure 4).

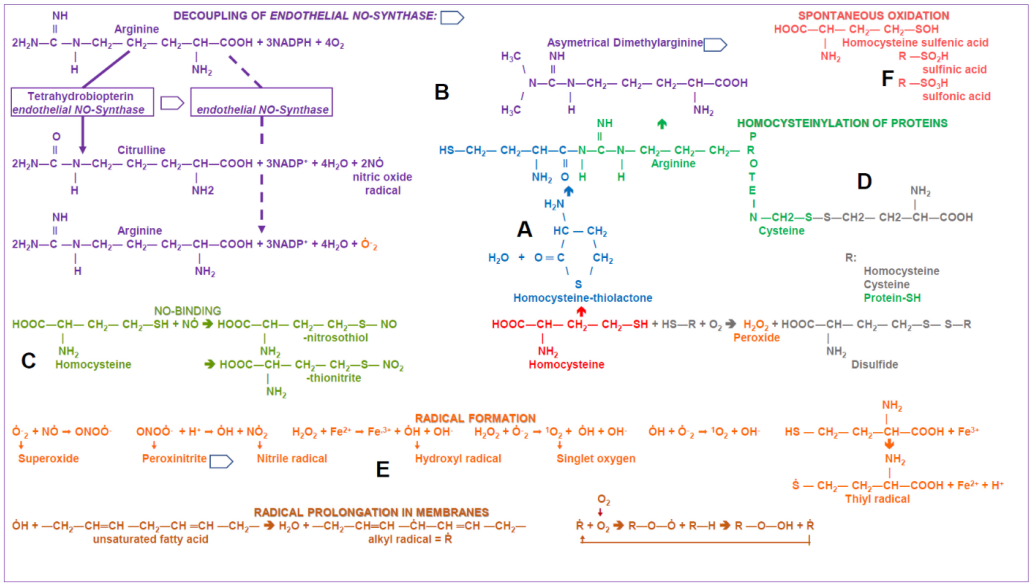

Figure 4. Homocysteine pathology; presentation of the most important possible reactions of HCys resulting in pathological effects; color differentiation and capital letters allow assignment to the different mechanisms and their effects: (**A**) In contrast to cysteine, HCys has a highly reactive sulfhydryl group, e.g., it can form a ring shape in the case of homocysteine thiolactone, which accounts for approximately 1/10 of the free HCys in blood plasma and can bind to lysine or arginine residues of proteins via a peptide bond (N-homocysteinylation) [55]. (**B**) In the latter case, asymmetrical dimethylarginine is released during the destruction of the protein, which decouples the endothelial nitric oxide (NO) synthase, so that it produces the superoxide anion instead of NO [56]. NO and the superoxide anion form peroxynitrite, which further contributes to decoupling of the enzyme complex [56]. (**C**) HCys itself can bind NO and thus inactivate it [57]. (**D**) HCys forms mixed disulfides with cysteine residues of proteins, called S-homocysteinylation, which can lead to functional impairments [58]. (**E**) HCys generates hydrogen peroxide via disulfide formation, from which all important ROS and radicals can arise [59]. (**F**) The spontaneous oxidation of the sulfhydryl group results in homocysteic acid (homocysteine sulfonic acid) with an agonistic effect on N-methyl-D-aspartate (NMDA) receptors [60].

The increase in plasma HCys leads to vascular damage through covalent binding to proteins, formation of ROS and, as described before, inactivation of the vasodilator NO [57,61].

Experimental Use of Methionine or HCys

When working with cell cultures, in animal experiments and in humans, HCys is often applied directly, as further described in the second part of the review (cf. Section 4.1). The same applies to methionine, which is used in exercise tests in humans to temporarily increase plasma HCys concentration [62–64]. Physiologically, both amino acids occur only

as L-enantiomers. The additives from commercial batches are, unless stated otherwise, racemates from L- plus D-form. Their effects are usually equated with those of the physiological enantiomers. This is not justified, because enzymatic reactions or receptors can be stereospecific and therefore, spatial orientation might play an important role:

(a) In humans, D- and DL-methionine show only 30% and 65% effectiveness, respectively, compared with L-methionine regarding the nitrogen balance [65].
(b) In chicks, D-HCys is only re-methylated to methionine to about 25% via methionine synthase, compared with L-HCys [66].
(c) In a methionine-deficient diet, L-HCys can replace 65% of the growth-promoting effect of L-methionine via this reaction, but D-HCys only 7% [67].
(d) Of the spontaneous oxidation products of HCys (Figure 4F), only L-homocysteine sulfonate and D-homocysteine sulfinate are selective activators of NMDA receptors, but not the D or L-enantiomers of the two acids [60].

2.5. Homocystinuria as a Result of an Existing Homozygous Defect in CBS—Witness of HCys Pathology

The pathogenic effects of disorders in C1 metabolism are usually complex. As stated earlier, HCys, resp. HHCys, has a biomarker function of these disorders. It remains to be seen, however, whether HCys is only a biomarker of disease or whether it is causally involved. For common disease groups, such as atherosclerosis and cerebral diseases, a causal involvement in the pathogenesis is plausible. Clinical intervention studies, however, frequently showed heterogeneous findings and resulted in assigning HCys only the role of a biomarker. The ambiguity of the study results is often due to study design, which is unsuitable for clarification of the question about causality. We, therefore, analyzed the studies from this point of view (cf. Section 3). In the following, however, an attempt will first be made to look at the pathological effects and symptoms of a disease with obviously isolated HHCys—without significant other disorders in C1 metabolism. These should then be referred back to HCys as a pathogenic agent. Corresponding conclusions can be drawn from analogies to common diseases. At CBS defect, primarily only the transsulfuration of HCys fails (Figure 2), with the consequence of excessive increase in plasma HCys of more than 100 µM and concomitant homocystinuria [44]. HCys is apparently the decisive pathogenic agent in this disease:

(a) There are no other causes of disorders in C1 metabolism, such as a vitamin deficiency.
(b) A suspected defect-related deficiency in cysteine or glutathione cannot be proven. Concentrations in plasma and urine correspond to those of controls [68,69].
(c) CBS also catalyzes the formation of hydrogen sulfide (H_2S), which may be diminished. There are, however, two further enzymes that catalyze H_2S formation from cysteine: cystathionine-γ-lyase and 3-mercaptopyruvate sulfurtransferase [70]. Moreover, HCys was found to upregulate cystathionine-γ-lyase in cardiomyocytes and also in vivo (*Cbs*+/− mice), the enzyme was upregulated [71]. Furthermore, even in the absence of pyridoxal-5′-phosphate, brain homogenates of CBS-knockout mice produced H_2S levels from cysteine similar to those of wild-type mice by 3-mercaptopyruvate sulfurtransferase in combination with cysteine aminotransferase [72].

If left untreated, the disease is therefore to be regarded as a "key witness" for frequent HCys-associated diseases, because most of the organs and clinical symptoms affected are also found in common diseases, that are listed subsequently and for which a prophylactic or therapeutic lowering of HCys level is recommended (Table 2).

Table 2. Clinical symptoms associated with a homozygous CBS defect in analogy to common HHCys-related diseases (green).

Vessels: - Arteries: intimal thickening, media destruction, fibrous plaques, thrombosis →cf.: atherosclerosis and its complications - Veins: deep leg vein thrombosis, embolism →cf. thrombosis, embolism If left untreated, most patients die in childhood or adolescence from consequences of vascular damage: arterial and venous thrombosis, embolism, myocardial infarction and stroke
Central nervous system: - Mental retardation, epilepsy →cf. cognitive impairment, dementia, depression
Skeleton: - Marfanoid habit with arachnodactyly, bone deformities, osteoporosis →cf. increased fracture rate
Eyes: - Lens dislocation and severe myopia, also possible cataract, optic atrophy and retinal degeneration →cf. retinopathies and macular degeneration

Since HCys must primarily have an effect on the disease in this defect, the analogies to the common diseases result in clear indications of a function of HCys as a pathogenic agent and thus a high level of plausibility for a causal role of this risk factor in the development of the disease. It affects a relatively large number of common diseases, which is probably due to the (untreated) lifelong effects of high HCys levels, including the developmental years.

3. Diseases in which C1 Metabolic Disturbances and HHCys Are Significantly Involved in the Pathogenesis

One aim of this review is to give an overview of human disorders that are discussed to be causally affected by elevated HCys levels. As the current review focusses on the impact of C1 metabolic disturbances, especially HHCys, on cognitive performance and decline, findings of relevant human studies are subsequently outlined (Table 3). The table mainly provides a summary of other, already existing reviews on this particular topic. Nevertheless, we also reviewed evidence on other indication areas, which are summarized in Appendix A.

Table 3. Cognitive decline and dementia; left column: relevant HCys-associated pathomechanisms; right column: correlation analyses and information on clinical studies; citation of other reviews or meta-analyses is marked as Rev [citation], followed by the reported findings, without individual quotations.

Hypo-methylation—Rev [52]; also see Figure 3: HCys ↑ → SAM/SAH ↓ → hypo-methylation of the presenilin 1 gene → increased β-amyloid formation. Hypo-methylation of the enzyme protein phosphatase 2A → loss of activity for phosphate cleavage of protein tau → accumulation of over-phosphorylated protein tau in neurofibrils → deposition of neurofibrillary tangles.	**Clinical studies—Rev** [73–75]: Plasma HCys negatively correlates with the thickness of the medial, inferior temporal lobe in normal subjects; equally in Alzheimer's patients (already lower baseline values). Meta-analysis (77 case-control studies, 33 prospective studies, 46,000 subjects):

Table 3. *Cont.*

Neurotoxicity—Rev [73]; also see Figure 4E,F: HCys and oxidation products (homocysteic acid) activate NMDA receptors → excitotoxicity (cellular Ca^{2+} increase → activation of proteases and radical formation → cell death = neuronal degeneration). Increased formation of ROS → activation of NFκB → inflammatory reaction.	Plasma-HCys ≥15 µM ≥14 µM Of approx. 10 placebo-controlled intervention studies with the three B-vitamins (B6, B12, folate), only five meet the decisive criteria: primary preventive approach, increased HCys starting level, study duration of at least two years, adequate vitamin dosage, proven decline in cognitive parameters in the placebo group. Significant results of these studies in favor of the vitamins: reduction of the brain atrophy rate, mainly gray matter, significantly better values for dementia status, MMSE (mini mental state evaluation) and learning test. Positive influence of plasma omega-3 fatty acid level on the effect of the B-vitamins [76]. Patients in the Alzheimer's prodromal stage benefit from multi-nutrients with B-vitamins and omega-3 fatty acids: significantly better dementia status. The effect correlates directly with the baseline MMSE value [77] → importance of early start of prevention!	Risk 3-fold for cognitive impairment 2-fold for Alzheimer's dementia

Brain tissue: no HCys transsulfuration and no re-methylation of HCys to methionine by betaine (cf. Section 2.1) high sensitivity to folic acid and vitamin B12 deficiency.

4. Animal Studies on HHCys—Literature Search Results and Discussion

Based on the relevance of HHCys in humans, which has been teased out in the previous sections, this review indicates the need for adequate animal models for HHCys. Due to the diversity of HCys-related pathologies in humans and the large number of investigations in experimental animals, also this second part of the review focusses on cognition-related investigations of HHCys. Our goal was to collect, summarize and assess findings of various relevant animal studies in order to finally answer the question: "What is the current consensus of preclinical evidence on the impact of hyperhomocysteinemia on cognitive performance and decline?".

In general, adequate animal models attempt to simulate a human disorder as comprehensive as possible. However, there is always a gap between the model and the human pathology, which is in most cases far more complex. Several strategies have been applied in order to induce HHCys in animals, providing pros and cons regarding the simulation of a disturbed HCys metabolism. The choice of an appropriate model should depend on the particular research question and the increase in HCys levels that the researcher aims to induce. With respect to animal research on HHCys, the following two sections focus on the analysis and discussion of the results from our systematic literature search. In Appendix B, all included animal studies are summarized (Tables A13 and A14) and more information on the literature search strategy, the analysis of average HCys blood levels, as well as behavioral cognitive-related outcomes are provided.

4.1. HHCys Induction Methods in Animal Models

Different factors have been determined as culprits in terms of the development of a hyperhomocysteinemic state in humans and therefore, served as targets for artificial manipulation in experimental animals (illustrated in Figure 5).

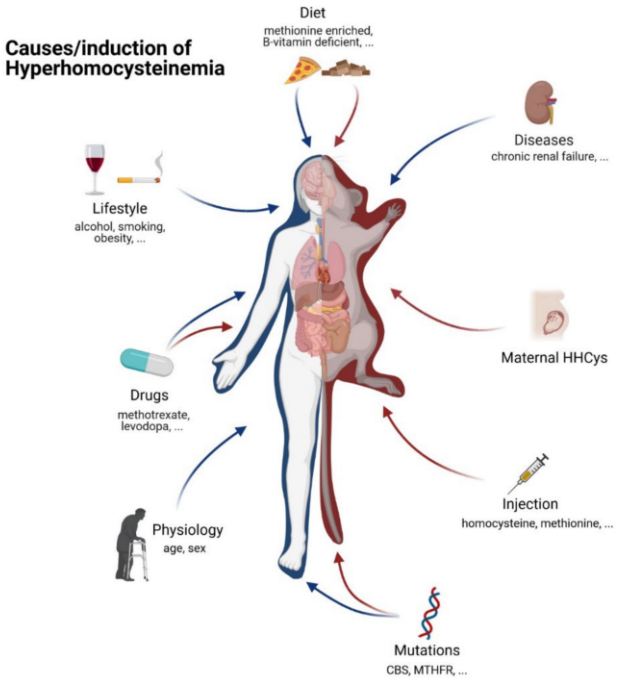

Figure 5. The most common causes of HHCys in humans (blue) and induction methods in animal models (red); created with BioRender.com.

As discussed in the first part of the review, HHCys is the result of either increased formation or decreased degradation of HCys, as well as decreased elimination due to impaired renal function. However, renal failure does not play a relevant role as an induction strategy of HHCys in animals, as it would display an unspecific method probably resulting in phenotypical artifacts. Relevant HHCys induction strategies, resulting from the literature analysis we conducted, are depicted in Figure 6C and subsequently described in-depth. As expected, rodent species played a pivotal role in animal experimentation towards HHCys (Figure 6A). In the reviewed animal studies, HCys levels in various biological matrices, such as blood, cerebrospinal fluid (CSF) and urine, as well as different tissues, e.g., brain and liver tissue, have been reported (Figure 6B). Average blood HCys levels (plasma, serum) are depicted in Figure 6D in order to show the HCys increase for each induction method.

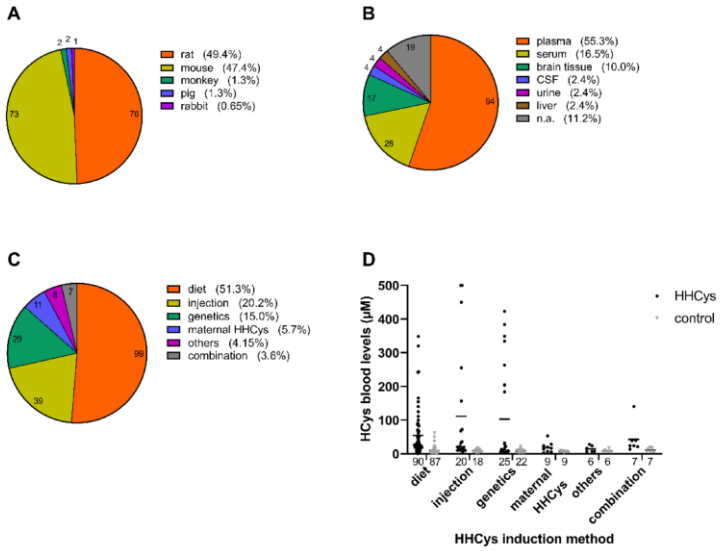

Figure 6. Average HCys levels and prevalence of different parameters, resulting from the analysis of the reviewed animal studies (numbers in the pie charts and at the bottom of the bars indicate the absolute amount of underlying studies): (**A**) animal species (154 cases in 154 studies in total); (**B**) biological matrices (170 cases in 154 studies); (**C**) HHCys induction methods (193 cases in 154 studies); (**D**) HCys elevation per induction method (every included study is considered as n = 1); since this is no meta-analysis according to the PRISMA guidelines, it should be considered semi-quantitatively; due to the large variation in individual studies, this panel should be used as a reference only; further methodological details are provided in the appendix of this review; created with GraphPad Prism 8 (San Diego, CA, USA).

4.1.1. Dietary Induction

The most prominent strategy to induce HHCys in animals (cf. Figure 6C) is the dietary manipulation of different "players" in the C1 metabolism. On average, diets are fed for approximately three months. However, the duration of intake to build up HHCys is strongly dependent on the exact experimental diet. A feeding period of eight weeks is the most common duration in the reviewed studies. As indicated in Figure 1, a pivotal role belongs to several vitamins of the B series, affecting both the transsulfuration pathway and re-methylation of HCys. For that reason, diets deficient in B-vitamins, especially B6, B12 and folate, are a common option among the dietary HHCys induction methods (e.g., [78]). Additional supplementation with a sulfonamide antibiotic may further increase plasma HCys by inhibiting microbial folate synthesis in the gut [79,80]. In few trials, riboflavin (B2) and choline were also depleted from the chow (e.g., [81]). Vitamin B2 contributes to the catalytic functionality of the MTHFR and choline, a precursor of betaine and formerly known as vitamin B4, is another important methyl donor and therefore, also relevant for the homeostasis of HCys levels [82].

Additionally, plasma levels can also be elevated by excess consumption of L-methionine (e.g., [83], cf. Section 2.4). As the supplementation of chow or drinking water with methionine is another reliable method to induce HHCys in animals, this method was applied equally often as B-vitamin restriction. Interestingly, a combination diet of both B-vitamin deficiency and methionine supplementation did not additionally increase plasma HCys, but even lowered the levels compared to a diet with normal methionine content and a lack of B-vitamins [84]. According to the authors, this attenuation might be explained by an

allosteric activation of the enzyme CBS by SAM [85]. However, similar investigations did not confirm this finding [86].

HCys levels can also be elevated by directly feeding the animals with HCys itself (e.g., [87]) or methyl group acceptors, interfering with the C1 metabolism. One example is guanidinoacetic acid, which is methylated to creatine and, for that reason, consumes a large portion of methyl groups provided by SAM [88]. In consequence, higher levels of SAH, and subsequently HCys, are built in the re-methylation cycle. Rarely, also nicotinic acid was used to elevate HCys levels [89]. Figure 6D shows that the entirety of the aforementioned dietary induction methods resulted in a mean blood HCys level of about 54 μM (versus 8 μM; control), which might be classified as a moderate HHCys.

4.1.2. Parenteral Induction

The parenteral administration route is an alternative to diets in order to induce HHCys in experimental animals. In most cases, HCys is injected subcutaneously or intraperitoneally (e.g., [90]). In contrast to ad libitum dietary approaches, where the special chow is permanently offered to the animals, the frequency of injections is a major variable additionally to the dosage with respect to the chronicity of the resulting HHCys. The issue of separate injections was overcome by some researchers, who made use of osmotic minipumps in order to constantly infuse HCys [91,92]. However, since a major part of the reviewed trials reported acute HCys data, our analysis revealed a high mean level of about 111 μM (versus 8 μM; control), which indicates a severe HHCys (Figure 6D). Instead of directly administering HCys itself, few studies reported the administration of its metabolites homocysteine thiolactone [93] or homocysteic acid (HCA) [94], as well as the injection of drugs or L-methionine in order to increase HCys levels [95]. Despite of differing administration routes, the mechanisms underlying the induction of HHCys do not differ between peroral and parenteral protocols.

4.1.3. Genetic Induction

Genetic animal models for HHCys are based on mutations in genes encoding for different enzymes that play central roles in the C1 metabolism (cf. Section 2). One of the most prominent enzymes in this context is CBS, which catalyzes the first step of the transsulfuration pathway in a vitamin B6-dependent manner. Reduced CBS functionality is responsible for decreased degradation of HCys to cystathionine and hence HCys elevation. Severe phenotypes were observed in experimental animals harbouring homozygous mutations in the *Cbs* gene, often leading to early death due to extremely high HCys levels (e.g., [96]). Due to limitations, especially lethality, in the investigation of homozygous (*Cbs*−/−) models, heterozygous (*Cbs*+/−) models were introduced (e.g., [97]) in order to enable comparison of biochemical and behavioral effects of less severe HHCys with wild type control (*Cbs*+/+).

Another prominent enzyme in the metabolism of HCys is the MTHFR, which enables an essential preliminary working step for the subsequent re-methylation of HCys by the vitamin B12-dependent methionine synthase. Because of the relevance of mutations in human pathology, genetic manipulation of the *Mthfr* gene has equally been utilized to introduce homozygous (*Mthfr*−/−) and heterozygous (*Mthfr*+/−) animal models for HHCys (e.g., [98]). In comparison to CBS-based models, MTHFR mutations only result in a mild to moderate elevation of HCys levels, which is also translationally relevant as human data, likewise, show higher HCys in the case of impaired CBS function than impaired MTHFR function [99].

In addition to CBS and MTHFR, other enzymes are summarized below, which are directly or indirectly involved in HCys homeostasis. Since these enzymes only play a minor role as HHCys induction method in animals, we summarized them as "others" in the literature analysis (Tables A13 and A14). One example is cystathionine-γ-lyase (CTH), also known as cystathionase, which is involved in the transsulfuration pathway by catalyzing the conversion of cystathionine to cysteine in a vitamin B6-dependent manner.

Although mutations in the *Cth* gene might even be more prevalent than in the *Cbs* gene in humans [100], CTH-based models are scarcely utilized in animal research so far, although *Cth*−/− proved to highly elevate serum and CSF HCys levels [101]. Another example is the betaine homocysteine methyl transferase (BHMT), of which homozygous (*Bhmt*−/−) and heterozygous (*Bhmt*+/−) forms were used in order to induce mild HHCys [102]. BHMT is involved in the reduction of HCys levels in a vitamin B12 and folate independent manner, by re-methylating HCys to methionine using betaine. Similar to BHMT, genetic modification of the methionine synthase reductase (encoding gene: *Mtrr*), which is responsible for the activation of the methionine synthase, resulted in slightly increased plasma HCys [103]. The average HCys level for genetic induction methods (Figure 6D) was approximately 103 μM (versus 7 μM; control), which might be classified as severe HHCys and therefore, equally to the human context, genetically-induced HHCys reflects higher levels than dietary-induced HHCys.

4.1.4. Impact of Maternal HHCys

About 6% of the reviewed trials were summarized under the term "maternal HHCys impact" (e.g., [104]), comprising all the studies that focused on HCys levels in newborn pups. For several reasons, this is a special strategy for the induction of HHCys in animals. The primary HHCys induction is not applied in the pups, but in the dams, using one of the methods described in the paragraphs above and it can be applied even before pregnancy, during pregnancy or until weaning. Thus, it is possible to expose the offspring to elevated HCys levels via trans-placental transmission, resp. lactation, even during the very early stages of development. An average blood HCys of about 19 μM (versus 6 μM; control) was measured in pups (Figure 6D).

4.1.5. Combinatory and Other Induction Methods

A small percentage of the reviewed studies utilized combinatory approaches of the aforementioned methods to induce HHCys, mainly by combining dietary and genetic models. In addition, researchers made use of more "exotic" strategies, such as the manipulation of parameters that are termed "lifestyle factors" in the human context. Xu and colleagues induced obesity by feeding a high fat diet und measured significantly elevated HCys in the hippocampi of the mice [105]. An elevation of plasma HCys of approximately 60% has been reached by the application of chronic unexpected mild stress to the animals [106]. Both of the aforementioned effects were apparently driven by a reduced CBS activity. An even higher increase in plasma HCys was observed in mice, fed with alcohol for several weeks [107]. This increase probably resulted from the interaction of ethanol with essential enzymes in re-methylation cycle of HCys [108].

Equally, physiological parameters, especially age, have been shown to contribute to elevated HCys in plasma and brain tissue [97,109]. Furthermore, a heterogeneous compilation of different animal treatment options has been proven to lead to HHCys. These range from inhalative N_2O [110], peroral $AlCl_3$ [111,112] and γ radiation exposure [113] to mechanical olfactory bulbectomy [114]. Of particular interest in the context of HHCys and dementia-like disorders is the potential impact of amyloid-β (Aβ) on HCys levels, as shown by infusing Aβ in rats [115]. Aβ pathology is a central hallmark of Alzheimer's disease, which is the leading cause for dementia, accounting for approximately two thirds of all cases [116]. In a recent kinetic study in an amyloid-based mouse model for Alzheimer's disease, we confirmed a significant effect of Aβ pathology on HHCys [117]. Finally, HHCys can also be pharmacologically induced in animals, e.g., by using the folate antagonist methotrexate ([118]). A comprehensive list of HCys level-modifying drugs has been provided in a previous review [27].

A limitation of the literature analysis in this review is that the assessment of HHCys has to be considered semi-quantitative (Figure 6D) and not as a quantitative meta-analysis, since not all aspects of the PRISMA guidelines for systematic reviews are fulfilled, as further explained in Appendix B.

4.2. HHCys Impact on Cognition in Animal Models

Based on the potential relevance of C1 metabolism disturbances for human health (cf. Section 3) and the variety of different animal models of HHCys, a huge amount of preclinical evidence has been accumulated by now. For that reason, the current review particularly focusses on the potential association of HHCys and impaired cognitive performance, resp. cognitive decline and dementia. In contrast to the early stages of HCys research, mainly focusing on cardio-vascular phenomena, HCys research in the context of neurodegeneration and cognitive abilities mainly gained increasing importance during the past 20 years.

Regarding the available literature until 2020, preclinical evidence suggests a causal link between HHCys and cognitive performance and cognitive decline. As summarized in Table A13, the vast majority of the reviewed animal studies (approx. 9 of 10 studies) revealed an impact of HHCys, meaning that, in these studies, at least one of the conducted behavioral tests showed significant effects following an elevation of HCys levels. In order to enable a consistent analysis, the plethora of behavioral tests in the reviewed studies was summarized in different cognitive domains (Figure 7). Further methodological information is provided in Appendix B. Interestingly, not all of the investigated behavioral domains were equally affected by HHCys: spatial memory, recognition memory and anxiety were affected in about 80–90% of the reviewed studies, whereas only approximately half of the relevant trials reported an impairment of working memory or psychomotor abilities through increased HCys. A potential explanation is an altered susceptibility of the different underlying brain areas to HCys-related damage. As shown previously, hippocampal structures are more vulnerable to HCys [119] and HCA [120] than cortical structures. Explorative behavior and psychomotor abilities, which are primarily associated with brain areas such as cerebellum and different cortical regions, indicated a lower susceptibility to HCys-related damage than spatial learning and memory, which is primarily associated with the hippocampus [121]. The hippocampal formation is implicated in both (spatial) working memory (short-term memory), and spatial learning and memory (long(er)-term memory). However, short-term working memory appears to be less affected by HCys-driven damage (Figure 7).

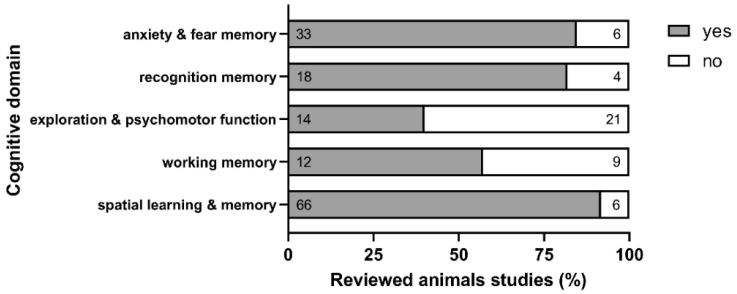

Figure 7. Impact of HHCys on cognitive performance; data resulting from the analysis of the reviewed animal studies (numbers in the bars indicate the absolute amount of underlying studies); different cognitive domains were analyzed: anxiety and fear memory (39 studies), recognition memory (22), exploration and psychomotor function (35), working memory (21), spatial learning and memory (72); in total, cognitive tests were performed in 102 of the reviewed animal studies; further methodological details are provided in the appendix of this review; created with GraphPad Prism 8 (San Diego, CA, USA).

With regard to the frequently used HHCys induction method via B-vitamin deficient diet and its resulting effects on behavioral outcomes, the central question remains, whether the impairment of cognitive performance is actually a consequence of HHCys or an artifact

due to the lack of essential B-vitamins. To elucidate this topic, we separately assessed all the reviewed studies, in which an elevation of HCys levels was reached only by parenteral administration of HCys itself (e.g., [122,123]). In these studies, no further manipulation of the C1 metabolism of the animals was undertaken. Nearly all of them reported a cognitive deterioration induced by the injection of HCys. Consequently, HHCys might be considered as a stand-alone risk factor for cognitive decline, independent of a restriction of B-vitamins.

HCys as a potential risk factor for Alzheimer's disease (AD) in particular, was the subject in numerous of the reviewed studies. In the case of AD, most of the available preclinical models are based on genetic modifications relevant in amyloid metabolism and rather simulate the early onset form of the disease, which accounts for only 1% of AD patients [124]. The simulation of a more comprehensive AD-like phenotype inspired by the more prevalent late onset form of AD, might be reached by an additional induction of HHCys in these mouse models, as elevated HCys levels are common in the elderly [125].

The vast majority of research articles and review papers in the field focus on potentially detrimental effects of HCys itself. However, it should be emphasized that metabolites of HCys, such as HCA, might be the actual culprits. Previous findings indicated that higher concentrations of HCys are needed to suppress the activity of neuronal circuits to the same extent as HCA does [126]. Not HCys itself, but HCA seems to be responsible for toxic calcium influx into neurons [127]. Furthermore, investigations at ionotropic NMDA and metabotropic glutamate receptors revealed that ROS are produced to a higher extent by HCA than by HCys [128]. In a recent human study, Hasegawa and colleagues considered HCA as an early diagnostic marker of mild cognitive impairment and as more relevant than HCys in this context [129]. Earlier, it has been shown that treatment with an anti-HCA antibody attenuated cognitive impairment in the 3xTg-AD mouse model [130]. Interestingly, despite the aforementioned findings in humans and animals, HCA merely played a role in few of the reviewed trials, which are highlighted in Tables A13 and A14.

Finally turning back to evidence derived from human trials, findings are not as clear as described for animal studies at the beginning of this section. This topic was not further addressed in our literature search, as there are other recent reviews available, concentrating on the equivocal role of HHCys and B-vitamins in the context of cognitive abilities in humans [75,131–133]. Both data pro [134–144] and contra [145–151] a causal link have been reported, systematically assessed and discussed through many years. We have the impression that, also in the clinical context, the sum of human studies predominantly strengthens the aforementioned causality, however, there is a larger portion of articles reporting negative results, compared to the field of animal studies. The design of some of these studies has been under criticism [152]. The gap in ambiguity between the clinical and preclinical field can probably, at least in parts, be explained by a publishing bias. Publishing bias is a common phenomenon, particularly in preclinical research, meaning that studies yielding positive results are more likely to be published than negative or null results [153–155]. Furthermore, overstatement of findings can occur due to lack of procedures such as randomization, blinding and appropriate power calculation [156–158]. Approaches such as pre-registration, which can help in reducing publishing bias, are more commonly applied in human studies than in animal research [155]. At present, there are preclinical initiatives attempting to raise awareness of complying with consistent quality parameters in order to struggle bias and attenuated reproducibility [159,160].

5. Summary and Conclusion

In the first part of this review, we outlined and critically discussed the metabolic role and effects of C1 metabolism disturbances, especially HHCys. These seem to contribute to versatile human disorders of diverse indication areas, ranging from the metabolic and vascular area to psychological and cognitive disorders, as well as the reproduction system, bone fracture rate and others. Based on this, the need for adequate animal models for HHCys becomes clear, as they are crucial to better understand basic processes and pathomechanisms. With the help of a systematic literature search and a focus on the link

between HHCys and cognition, we summarized studies of the aforementioned animal models and analyzed the findings with the aim to assess, whether the majority of animal studies indicates a tendency pro or contra a causative role of HHCys in cognitive decline. Regarding the entirety of the reviewed preclinical evidence, the vast majority of included studies (approx. 9 of 10 studies) reported an impact of HHCys on cognitive outcomes and therefore underpinned a potential role of HCys in this context. With respect to the clinical situation, this means that it is firmly recommended to conduct additional large-scale and well-designed human studies to elucidate, whether the normalization of HCys levels represents a valuable preventative or therapeutic approach in terms of HCys-related pathologies in humans.

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Appendix A

Due to the large number of diseases, in which C1 metabolic disturbances and HHCys are potentially involved in the pathogenesis, these are subsequently presented in concise tables (Tables A1–A12) with two columns: on the left, we summarized relevant HCys-associated pathomechanisms and on the right, correlation analyses and information on clinical studies are provided. Diseases with a comparable pathogenesis are grouped and facts are briefly communicated with reference to previous sections of the review. Where available, recent reviews or meta-analyses are cited, marked as Rev [citation], followed by the reported findings, without individual quotations.

Table A1. Atherosclerosis and its consequences: cardiac ischemia, peripheral occlusion, cerebral ischemia.

Disease course over years or decades without symptoms. Elevated HCys values are pathogenetically involved. Clinical symptoms first become prevalent through complications of atheromatous plaques with a different pathogenesis, in which B-vitamins and HCys hardly play a role. **Initial endothelial cell damage—Rev** [61,161]; also see Figure 4A–C: Reduced formation and efficacy of NO → inadequate vasodilatation in response to atherosclerosis-promoting stimuli. After coronary angiographic localization of such functional restrictions, vascular constrictions can be found after years in patients with acute coronary syndrome [162]. HCys causes increased formation of ROS (see Figure 4E) → cell activation with increased formation of adhesion molecules and pro-inflammatory cytokines; cell damage; apoptosis. S-homocysteinylation of endothelial proteins → loss of function (see Figure 4D). N-homocysteinylation by HCys-thiolactone → cytotoxicity (see Figure 4A). With HCys, the cellular SAH concentration increases → altered DNA or RNA methylation (see Figure 3) → reduced expression of enzymes with an antioxidant effect. Decrease in the thromboresistance of the endothelial surface by promoting coagulation and inhibiting anticoagulant and fibrinolytic mechanisms. **Plasma lipids and white blood cells—Rev** [161]: Oxidation of LDL → uptake by white blood cells → promotion of foam cell formation. Increase in chemotactic motility of white blood cells. **Smooth muscle cells—Rev** [61]: Oxidative stress → activation of the transcription factor NFκB → proliferation. **Platelets—Rev** [61]: Increase in thromboxane A_2 synthesis → promotion of reactivity and aggregation.	**Studies on the influence of plasma HCys concentration on atherosclerosis consequences:** Recording of the period until the onset of symptoms. Elimination of conventional risk factors. Meta-analysis from >70 case-control studies as well as prospective studies with >20,000 subjects: An increase in HCys of 5 µM results in a 33% increase in risk for ischemic heart disease and 59% for ischemic stroke [163]. Meta-analysis from 12 prospective studies with >9000 subjects: Lowering HCys by 3 µM results in an 11% decrease in risk for ischemic heart disease and 19% for ischemic stroke [164]. Peripheral occlusion: case-control studies, with significantly higher levels of HCys than controls [165]. Renal insufficiency: high HCys levels (cf. Section 2.3). Greatly increased risk for all consequences of atherosclerosis, which are the main causes of death [166]. **Prospective intervention studies—Rev** [167,168]: A total of 15 studies had the effect of at least one of vitamin B6, B12 or folate was compared with placebo. 11 of these studies were secondary preventive—*after* a clinical event such as myocardial infarction—the remaining 4 studies in renal insufficiency requiring dialysis → no primary prevention overall. Results: heterogeneous/controversial. For ischemic stroke only, 25% risk reduction with the three B-vitamins. Only one primary preventive intervention study: >20,000 subjects with hypertension received folic acid + ACE inhibitors (Enalapril) versus only ACE inhibitors for 5 years: 34% reduction in ischemic stroke, 20% reduction in the combination of stroke, myocardial infarction, cardiovascular death. Renal insufficiency: meta-analysis of a total of 3886 patients: Monotherapy with folic acid resulted in a significant reduction in cardiovascular endpoints by 15%; in patients without (additional) dietary folic acid fortification by 20% [169].

Table A2. Metabolic syndrome and type 2 diabetes mellitus.

With a comparable HCys level, oxidative stress is stronger than in controls [61], directly detectable in myocardial fibrils (bypass surgery): H_2O_2 production ↑, antioxidants ↓ [170]. Metformin therapy inhibits intestinal absorption of vitamin B12 and folic acid [171].	Plasma HCys level as in controls, but higher if nephropathy is present. HCys is more strongly associated with atherosclerosis and its consequences than in non-diabetes controls [172]. Primarily preventive, folic acid supplementation lowers HCys and insulin levels, reduces insulin resistance, normalizes glucose homeostasis and atherogenic plasma lipid profile [173]. Metformin therapy lowers B12 levels, increases HCys levels and intensifies diabetic neuropathy [174].

Table A3. Thrombophilia: venous thrombosis, embolism.

HCys causes a decrease in the thromboresistance of the endothelial surface by promoting coagulation and inhibiting anticoagulatory and fibrinolytic mechanisms [61].	**Meta-analyses of clinical studies**—[175,176]: An increase in HCys of 5 µM increases the risk of deep vein thrombosis by 60% (case-control studies) or 27% (prospective studies). Patients with deep vein thrombosis and pulmonary embolism have significantly reduced plasma folic acid and/or vitamin B12 levels. Intervention studies so far unsatisfactory. The combination of HHCys and factor V (Leiden) is multiplicative [177].

Table A4. Depression.

Reduced transmitter formation—**Rev** [52]; also see Figure 3: Folic acid and SAM necessary for the synthesis of serotonin, noradrenaline and dopamine, both directly as well as via the synthesis of tetrahydrobiopterin. Significant changes in patients with depression: increase in HCys (plasma), decrease in folic acid (plasma, erythrocytes, liquor), SAM (liquor) and metabolites of the 3 transmitters (liquor)	Case control studies: Plasma HCys >10 µM → doubling the risk of depression [178]. Intake of vitamin B6, B12, folate correlates negatively with the occurrence of depression (12 years observation period) [179]. Intervention studies: The three B-vitamins versus placebo in patients at risk of depression for 7 years: significantly lower frequency [180]. Therapy with antidepressants in combination with folic acid, 5-methyl-tetrahydrofolate or SAM: better effect than antidepressants alone [52].

Brain tissue: no HCys transsulfuration and no re-methylation of HCys to methionine by betaine (cf. Section 2.1) high sensitivity to folic acid and vitamin B12 deficiency.

Table A5. Autism—**Rev** [75].

HCys and oxidation products (HCA) activate NMDA receptors → excitotoxicity (cellular Ca^{2+} increase → activation of proteases and radical formation → cell death = neuron degeneration)—see Figure 4F	Case-control studies: significant deviations in plasma levels in autistic children: HCys ↑; vitamin B6, B12, folate ↓. Interventional study: folic acid supplementation lowers plasma HCys levels and reduces deficits in cognition, communication and social behavior.

Brain tissue: no HCys transsulfuration and no re-methylation of HCys to methionine by betaine (cf. Section 2.1) high sensitivity to folic acid and vitamin B12 deficiency.

Table A6. Pharmacotherapy of neurodegenerative diseases: Parkinson's disease, epilepsy.

Levodopa is degraded by methylation → significantly higher HCys levels than in untreated Parkinson's patients → increased risk of stroke, coronary artery disease, dementia and peripheral neuropathy [181]. **Anticonvulsants**, especially valproate, influence the metabolism of folic acid (inhibition of cellular receptors), vitamin B6 (increased degradation) and reduce betaine uptake → lowering of plasma level of the two vitamins and increase in HCys [182]—see Figure 2 Particular risk: carrier of the TT variant of the C677T mutation of the MTHFR—see Table 1	Substitution with vitamin B6, B12, folate lowers HCys levels [183]. Significantly more femoral neck and vertebral fractures [184] and brain atrophy **Rev** [185]. Pregnancy: 10-fold increased risk of abortion, malformations in 6–11% of newborns, often cognitive deficits [186]. Improvement after supplementation with folic acid and B6 [184] or folic acid and B12 [186].

Brain tissue: no HCys transsulfuration and no re-methylation of HCys to methionine by betaine (cf. Section 2.1) high sensitivity to folic acid and vitamin B12 deficiency.

Table A7. Peripheral neuropathy.

Most frequent cause: damage to the peripheral myelin protein-22 [187]. HHCys → methylation disorders (see Figure 3): Hypo-methylation of Arg_{107} of the basic myelin protein → loss of binding for acidic lipids → disrupted lamellar formation.	HCys ↑ causally affects neuropathies in: Parkinson's disease under levodopa therapy, type 2 diabetes mellitus (especially with metformin therapy), chronic alcoholism. Supplementation with vitamin B6, B12, folate improves the symptoms [170].

Table A8. Pregnancy and childbirth.

Pregnancy changes laboratory parameters for assessing C1 metabolism: U-shaped course with a lowering of vitamin B6, B12, folate and HCys in plasma in early pregnancy. Only holotranscobalamin remains constant [188]; only HCys levels in the interval are meaningful. Threshold for women of childbearing age: 9 µM	Evaluation of >14,000 pregnancies: HCys level in the interval >8.9 µM → significantly increased risk of preeclampsia, premature birth, stillbirth, low birth weight, neural tube defects [189].

Pregnancy complications: preeclampsia—Rev [190]**, abortion**
Preeclampsia and cardiovascular diseases are essentially **one** entity—women with 2–3 preeclampsia episodes: significantly elevated laboratory parameters (HCys, von Willebrand factor, fibrinogen, insulin, total cholesterol, VLDL, triglycerides).

Significantly increased risk of hypertension, coronary artery disease, stroke, venous thromboembolism, type 2 diabetes mellitus, cardiovascular mortality

Table A8. *Cont.*

Preeclampsia: decoupling of the endothelial NO synthase (see Figure 4B) → significant reduction in endothelium-dependent vasodilatation. **Abortion:** HCys level >18 µM: significantly reduced vascularization of the villi placentae.	Intervention study, three B-vitamins versus placebo (3000 pregnancies, periconceptional onset to end of pregnancy): plasma folic acid ↑, plasma HCys ↓, 63% fewer preeclampsia Risk classification for early abortions: HCys ≥9.9 µM → 2-fold; ≥12.3 µM → 4-fold; ≥15.3 µM → 7-fold [191]. Intervention study: 25 nullipara, MTHFR C677T-TT carriers, HCys > 12 µM, 3-5 early abortions; 5 mg folic acid and 750 mg vitamin B6 per day for 3 months → 22 women became pregnant without complications [192].
Neural tube defects and other malformations—Rev [193]	
High HCys levels: → Hypo-methylation of genomic DNA in the brain → Post-translational hypo-methylation of cytoskeletal proteins (see Figure 3) → Homocysteinylation of histone proteins (see Figure 4D) → disruption of gene expression. Recommended prevention with 0.4 mg folic acid/day → it takes 3 months to reach ≥900 mM erythrocyte folate [194].	Neural tube defects correlate directly with HCys and indirectly with folic acid and vitamin B12 levels in plasma as well as erythrocyte folate; increased risk in case erythrocyte folate <900 mM. MTHFR C677T-TT carriers → increased risk. Interventional studies with periconceptional folic acid substitution (0.4 mg/day)—meta-analysis: 72% reduction [195] → Interventional study with 0.8 mg folic acid, 4 µg B12, 2.6 mg B6/day versus placebo: >90% reduction and approx. 80% reduction in other defects (heart malformations, pyloric stenoses, ureteral obstruction) [196].
Vitamin B deficiency in the mother → permanent disorders in the children—Rev [197–201] Vitamin B12 ↓ → birth weight ↓, insulin resistance → visceral obesity, type 2 diabetes mellitus → metabolic syndrome, atherosclerosis and consequences	

Table A8. *Cont.*

Cause of the sequence listed above: B12 ↓ → HCys ↑ → methylation disorders with epigenetic effects in children (see Figure 3) B12-deficient diet (periconceptional until the end of pregnancy) in rats → offspring after one year: liver methylome with 190 differently methylated genes; liver proteome with 38 differently expressed proteins of lipid, carbohydrate and amino acid metabolism → atherogenic plasma lipid pattern (triglycerides ↑, HDL ↓) [202]. B12- and folic acid-free diet (periconceptional and during pregnancy and lactation) in rats → offspring after 80 days: pyramidal cell layer thickness (hippocampus) ↓, memory deficits [203]. High energy versus standard food with the same vitamin intake (3 years) in monkeys: high energy food results in more body fat and a lower birth weight of the offspring, plasma B12 ↓, atherogenic plasma lipid pattern	Meta-analysis of 20,000 women: plasma HCys in >90 percentile → 50% increased risk for children with reduced birth weight. Increased insulin resistance in 6-year-olds in case mothers had significantly low B12 levels during pregnancy.

Table A9. Infertility—**Rev** [201,204].

Plasma concentrations of vitamin B6, B12, folate and HCys are similar to those in seminal fluid. Every alteration of C1 metabolism associated with HCys ↑ leads to DNA fragmentation, telomere shortening, a different methylation pattern (see Figure 3) and radical formation (see Figure 4E) in sperm and oocytes. Hypomethylation of IGF2_H19 locus in men correlates with infertility. In vitro fertilization—quality of the embryo: Positive correlation with B12 content and negative correlation with HCys concentration in plasma and follicular fluid	Prospective study—approx. 20,000 women, 8 years: Infertility correlates negatively with daily folic acid intake. MTHFR C677T-TT carriers: more often infertile. Significantly higher levels of HCys in spermatozoa in infertile men. In vitro fertilization—intervention: Supplementation with the three B-vitamins reduces DNA fragmentation in sperm, doubles the pregnancy rate and triples the birth rate.

Table A10. Vision loss: exudative macular degeneration, diabetic retinopathy—**Rev** [205,206].

Human retinal cell culture: HCys induces production of VEGF (vascular endothelial growth factor). Plasma HCys correlates with VEGF-concentration in vitreous humor	Direct correlation between plasma HCys level and risk of macular degeneration. Significantly higher plasma HCys levels in exudative macular degeneration than in dry macular degeneration and controls. Intervention study: three B-vitamins versus placebo for 7 years in 5000 subjects: 34% less macular degeneration. Diabetes mellitus: in patients significantly higher HCys levels in serum, vitreous humor and retina.

Table A11. Increased fracture rate in old age—**Rev** [75].

S-homocysteinylation (see Figure 4D) of collagen fibrils hinders the regular formation of the bone matrix → increased fragility with mostly unchanged bone density.	Prospective studies: on average about twice the risk of femoral neck fractures with plasma HCys ≥15 µM. MTHFR C677T-TT carrier: significantly increased fracture rate. Interventional studies—three B-vitamins versus placebo: negative if related to bone density and plasma bone turnover parameters; mostly positive when it comes to fracture rates.

Table A12. Chronic fatigue syndrome, fibromyalgia—**Rev** [207].

Chronic stress: (1) Increased formation of ROS (see Figure 4E) → peroxynitrite anion ↑ in the respiratory chain → irreversible inhibition of cytochrome C oxidase → cellular energy production ↓. (2) Leukocytes from patients with fibromyalgia: hypo-methylation and increased mRNA formation (see Figure 3) of genes with sensory, adrenergic and immunological functions. Cobalamin acts as an intracellular antioxidant in high concentrations.	Plasma vitamin B12 and HCys levels correlate positively/negatively, with exhaustion, comprehensive psychopathological rating scale, pain and memory ability. Therapy with high doses of vitamin B12 (1–2 mg/day) and folic acid (1–5 mg/day).

Appendix B

The second part of the present (narrative) review might be categorized as systematized, but not as a systematic review, since it does not fulfill all aspects of the PRISMA guidelines, such as a comprehensive risk of bias assessment. Nevertheless, several characteristics of systematic reviews, e.g., a structured literature search, pre-defined exclusion criteria and the revision by a second investigator, have been considered.

Appendix B.1 Literature Search Strategy

We systematically searched PubMed/Medline using a structured literature search filter. For that purpose, the animal-specific search filter template by Hooijmans and colleagues [208] has been adapted to the topic of this review with the aim to detect, as far as possible, all animal studies regarding HHCys in the context of cognitive decline, especially dementia-like disorders. In addition to the systematic record of studies, we manually included several hand-searched references from other sources for different reasons, such as the assessment of HCys in more exceptional biological matrices.

Overall, 154 studies have been included: the structured search resulted in 523 hits up to the year 2020, whereof titles and abstracts were screened. In the case of 19 hits, especially of the years 2018–2020, full texts were not available to us. 152 studies were selected for full text analysis. In this step, another 27 studies were excluded. Criteria for the inclusion of preclinical studies in the current review were the application of a method aiming to elevate HCys levels in animals. Furthermore, HCys levels and/or cognitive behavioral outcome should be reported by the authors. In addition to the systematically searched results, 29 additional references were included manually.

Appendix B.2 Literature Analysis

All reviewed animal studies are listed in Tables A13 and A14, serving as a basis for further analyses that are subsequently described in-depth.

Although this is no meta-analysis, we considered it interesting to provide a semi-quantitative impression of HCys levels depending on the different HHCys induction methods in order to enable a classification of the resulting hyperhomocysteinemic state in the animals. Therefore, the exact numbers of observations (n) in the single studies were not totaled here, but each study was considered as n = 1. A strict quantification of HCys levels was, furthermore, hardly possible because of limited comparability between the included studies due to a high diversity in the HHCys induction protocols:

Examples for this diversity were the type, duration and grade of deficiency in dietary induction methods, as well as in some cases a fasting period prior to the sampling step and the sampling method itself. The umbrella term "B-vit. def." (B-vitamin deficiency) that we used (Tables A13 and A14), mainly comprised folate (sometimes referred to as B9), B12 and to a lesser extent B6. Although it might be regarded as an independent HHCys inducing diet, we attributed lowered choline levels to the "B-vit. def." category, since, in most of the reviewed studies, the impact of choline deficiency was investigated in combination with a lack of B-vitamins. In general, trials largely varied in the dietary restriction of vitamins (and vitamin-like substances), resulting in either mild, moderate or severe HHCys according to a frequently used classification system [209]. In the case that injections were used to elevate HCys levels, studies varied with respect to the time interval between injection and sampling step, which plays an important role in the assessment of (acute) HCys levels. Plasma sampling immediately after a single injection yields acutely higher HCys (e.g., [210]) than sampling after a longer period of time (e.g., [211]), due to excretion and degradation processes in the meantime. For several articles, an assignment to the aforementioned options was not possible because of an insufficient reporting. In the case of the common genetic induction methods, both homozygous and heterozygous models were subjects of investigation. A specialty was the maternal HHCys induction method, as it must be distinguished between dams and pups. In the case that a trial reported HCys levels for both dams and pups, the dam-related data were assigned to the

primary HHCys induction method (in most cases a dietary regimen) and the pup-related were analyzed as "maternal HHCys impact". Sex and age of the animals were additional sources of variance in HCys data between the studies. Last but not least, the same applied for the applied analytical method [209].

Several of the reviewed animal studies reported varying HCys levels, related to varying experimental conditions. In the case that HCys levels in a study were reported for wild type (WT) and transgenic (Tg) animals (e.g., [212]), only data derived from WT animals were included in the analysis for reasons of comparability. Data derived from Tg animals were only included in the analysis, if the transgenic model was a primary method to induce HHCys (e.g., deficiency of CBS, MTHFR . . .), but not if it was a transgenic model for another purpose (e.g., a model for early-onset Alzheimer's disease). In the case that varying HCys levels in a single study derived from varying HHCys induction strategies, all of the reported levels were analyzed. Where applicable, data for males and females, as well as for different background strains in WT animals were pooled for reasons of comparability. In the case that an article reported data of more than one sampling step during a dietary HHCys induction period, only the full-length data were included in the analysis (e.g.,[213]). Since some of the reviewed studies did not report exact values for HCys levels in the text, levels were estimated from the related graphs in this case and marked in the tables. Others required a recalculation to μM in order to enable comparability with the other studies. In the case that recalculation to μM was not applicable (e.g., reporting of percentages or unfeasible units), levels of these studies were excluded from analysis. Insufficient reporting of HCys levels such as "*<5 μM*" or "*below quantification limit*" was also excluded from further analyses and led to divergent amounts of underlying studies for HHCys induction strategies (Figure 6C,D).

With respect to the analysis of behavioral experiments, we assigned the versatile cognitive tests of the reviewed studies to six cognitive domains: e.g., Morris water maze to "*spatial learning and memory*", e.g., Y-maze to "*working memory*", e.g., open field test to "*exploration and psychomotor function*", e.g., novel object recognition test to "*recognition memory*", e.g., elevated plus maze to "*anxiety and fear memory*". Few tests that did not fit to one of these categories were grouped as "*others*", but without being depicted in a separate graph due the high heterogeneity of this category. In some cases, transition between the categories is fluent, as e.g., the T-maze test might be attributed to working memory but also to explorative behavior. It was also challenging to assign particular behavioral tests to either "pure" motor function or cognitive performance. For example, locomotion in the open field test shows the distance moved by the animal and, therefore, is a marker of physical activity on the one hand. However. on the other hand, regarded over a period of time, locomotion is a parameter for habituation behavior, which is a form of learning [214]. The umbrella term "psychomotor" that we use in Table A13 is meant to comprise only behavioral testing with a relation to cognition such as exploration, sensorimotor testing or coordination tasks. Tests for motor function without a relation to cognitive performance such as testing of "pure" muscle function, e.g., paw grip endurance test, were not included in the analysis of cognitive outcomes. The assignment of behavioral tests to cognitive domains is in parts a subjective decision and might have been made differently by others.

Table A13. Reviewed animal studies derived from the systematic literature search of PubMed/Medline, which served as a basis for further analysis and indicates potential treatment options for elevated HCys levels or related symptoms; abbreviations: WT: wild type, KI: knock-in, KO: knock-out, Tg: transgenic, B-vit. def.: deficiency in B-vitamins (and related substances), Met suppl.: supplementation of L-methionine, CBS: cystathionine β-synthase, MTHFR: methylenetetrahydrofolate reductase, CTH: cystathionine γ-lyase.

		Strategy to Induce HHCys												Investigated Biological Matrix						Impact on Cognitive Performance	
		Diet/Drinking Water				Injection		Genetic Manipulation			Maternal HHCys Impact	Others									
Publication	Animal Species	B-vit. def.	Met suppl.	HCys suppl.	Others	HCys	Others	CBS	MTHFR	Others			Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[83]	rat		■										28.8 vs. 6.3		■					spatial learning & memory (+)	ozagrel
[215]	rat		■										2.3 vs. 0.9 [1]	■						spatial learning & memory (+)	edaravone
[80]	mouse	■											348.2 vs. 7.7 [2;3;4]		■			■		exploration (-); anxiety (-); spatial learning & memory (-) ; recognition memory (-); others (-)	B-vitamins, PUFA, Fortasyn® Connect-like diet
[216]	rat	■									■		5.65 vs. 4.85 (offspring)	■						offspring:working memory (+)	mild transient neonatal hypoxia
[217]	mouse	■	■										19.0 vs. < 5 (WT); 14.7 vs. < 5 (KI)	■						n.a.	n.a.
[218]	rat		■										11.22 vs. 7.08	■						n.a.	n.a.
[104]	rat		■								■		27.3 vs. 7.9 (dams); 19.5 vs. 6.3 (offspring)	■						offspring: exploration (+); anxiety (+); psychomotor function (+); working memory (+); spatial learning & memory (+);others (+)	sodium hydrosulfide
[219]	rat					■							n.a.							exploration (-); spatial learning & memory (+); fear memory (+)	synthetic tricyclic sulfonamide PP2A activators

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (µM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[220]	mouse									■	■		n.a.			■				spatial learning & memory (+)	maternal choline supplementation
[123]	rat					■							10.1 vs. 6.1	■						exploration (-);recognition memory (+); spatial learning & memory (+)	emodin
[221]	rat		■										11.38 vs. 7.15	■						n.a.	n.a.
[222]	mouse	■	■										71.5 vs. 4.9	■						spatial learning & memory (+)	n.a.
[223]	mouse							■					423 vs. < 16	■						anxiety (n.a.); exploration (n.a.); others (+)	methionine restriction, enzyme replacement
[224]	mouse	■	■										140.50 vs. < 5	■						spatial learning & memory (+); others (-)	n.a.
[225]	mouse					■						■	22 vs. 17 (injection); 24 vs. 17 (age) [1]		■					recognition memory (-); fear memory (+); spatial learning & memory (+)	B-vitamins, SAM
[101]	mouse							■		■			263 vs. 13 (CBS); 184 vs. 13 (CTH)		■		■			n.a.	n.a.
[226]	rat	■											13.13 vs. 8.5	■						spatial learning & memory (-); recognition memory (-); anxiety (-)	betaine
[227]	mouse	■	■										82.93 vs. 5.89 (WT); 84.67 vs. 6.34 (KO)	■						n.a.	n.a.
[105]	mouse				■								n.a.			■				exploration (+); anxiety (+); recognition memory (+); spatial learning & memory (+)	methionine restriction

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (µM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[122]	rat					■							20 vs. 9 [1]	■						spatial learning & memory (+)	liraglutide
[228]	mouse	■							■				13.97 vs. 8.55 (genetic); 18.93 vs. 8.55 (diet, WT); 38.87 vs. 13.97 (diet, Tg)	■						recognition memory (+); working memory (-); exploration (-); anxiety (+)	n.a.
[229]	rat	■									■		24 vs. 8 [1] (offspring)	■						offspring: sensorimotor function (+); spatial learning & memory (+)	n.a.
[230]	rat					■							17.5 vs. 8	■						n.a.	B-vitamins
[231]	rat	■	■										22 vs. 8 (Met suppl.); 62 vs. 8 (B-vit. def. + Met suppl.)		■					exploration (+); anxiety (+)	statins
[232]	mouse	■											n.a.			■				working memory (-); spatial learning & memory (+)	n.a.
[233]	rat					■							n.a.							spatial learning & memory (+)	Moringa oleifera extract
[113]	rat											■	28 vs. 10 [1]	■						n.a.	epigallocate-chin-3-gallate
[90]	rat					■							255.15 vs. 7.15 (acute); 16.64 vs. 7.15 (chronic)	■						n.a.	n.a.

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[234]	mouse	■	■										52 vs. 22 [1]	■						recognition memory (+)	n.a.
[235]	rat		■										0.59 vs. 0.3 [1]		■					spatial learning & memory (+)	caffeine
[236]	mouse	■											22.01 vs. 14.43		■					anxiety (+); spatial learning & memory (+)	n.a.
[237]	rat	■									■		22 vs. 10 [1] (dams)	■						offspring:sensorimotor function (+); spatial learning & memory (+)	folate
[238]	rat					■							n.a.							exploration (-); spatial learning & memory (+);fear memory (+)	Ginkgo biloba extract
[239]	rat					■							n.a.							working memory (+); anxiety (+)	hydrogen sulfide
[118]	rat						■						n.a. [2]				■			exploration (-); anxiety (-); spatial learning & memory (+); recognition memory (+)	n.a.
[111]	rat				■								9 vs. 4.5 [1]		■					working memory (+)	Vitis vinifera leaves polyphenols
[102]	mouse									■			29 vs. 10 (homozygous); 11 vs. 10 (heterozygous) [1]	■						spatial learning & memory (+); working memory (+); psychomotor function (-)	n.a.
[240]	rat					■							n.a.							recognition memory (+); fear memory (+)	creatine
[241]	rat					■							36 vs. 4 [1]	■		■				spatial learning & memory (+); anxiety (+); exploration (-); psychomotor function (-)	hydrogen sulfide

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[115]	rat		■				■						22 vs. 7 (diet); 12 vs. 7 (injection); 24 vs. 7 (diet + injection) [1]		■					spatial learning & memory (+); recognition memory (+)	bosentan
[242]	mouse	■											n.a.			■				working memory (-); fear memory (-)	genetic absence of ALOX5
[243]	mouse	■											n.a.			■				working memory (+); fear memory (+); spatial learning & memory (+)	ALOX5 inhibition (zileuton)
[244]	rat		■										153.79 vs. 62.21 [3]		■					working memory (+);spatial learning & memory (+)	fisetin
[245]	rat		■										165.48 vs. 49.64 [3]		■					working memory (+);spatial learning & memory (+)	hesperidin
[246]	rat					■							n.a.							spatial learning & memory (+); recognition memory (+)	hydrogen sulfide
[247]	mouse	■	■										67.40 vs. <detection range (WT); 70.29 vs. <detection range (Tg)	■						spatial learning & memory (+)	anti-Aβ immunotherapy
[106]	rat		■									■	8.18 vs. 4.43 (diet); 7.37 vs. 4.43 (stress)	■						exploration (+); recognition memory (+); fear memory (+); spatial learning & memory (+)	B-vitamins, betaine

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[248]	rat	■									■		26 vs. 15 (dams); 53 vs. 7 (offspring) [1]	■						n.a.	maternal vitamin B6 supplementation
[249]	mouse					■							n.a.							recognition memory (+); fear memory (+)	hydrogen sulfide
[250]	mouse		■										n.a.							fear memory (+); spatial learning & memory (+)	n.a.
[251]	mouse									■			n.a.			■				spatial learning & memory (+); working memory (+); recognition memory (+)	cinnamon
[252]	mouse				■								46.1 vs. 4.6	■						spatial learning & memory (+)	Brazilian propolis extract
[253]	mouse			■									n.a. [5]			■				working memory (+); fear memory (+)	betaine
[254]	mouse	■							■		■		22 vs. 14 (dams) [1]; 28.4 vs. 9.8 (offspring)							offspring: recognition memory (+); working memory (-)	n.a.
[110]	rat											■	n.a. [5]		■					exploration (+); others (+)	n.a.
[255]	rat					■							n.a.							spatial learning & memory (+)	atractylenolide III
[256]	mouse	■											18 vs. 13 (WT); 26 vs. 14 (Tg) [1]	■						spatial learning & memory (+); psychomotor function (-); anxiety (+)	n.a.

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[257]	rat				■								16.7 vs. 16.3		■					n.a.	zinc
[258]	rat					■							n.a.							fear memory (+); spatial learning & memory (+)	n.a.
[114]	rat											■	16 vs. 7 [1]	■						spatial learning & memory (+); fear memory (+)	fatty acids
[259]	rat					■							n.a.							spatial learning & memory (+)	combination: acetyl-cholinesterase inhibitor + calcium channel blocker
[260]	rat	■									■		n.a.			■			■	offspring: exploration (+); anxiety (+); fear memory (+)	n.a.
[261]	mouse		■										22 vs. 6 [1]	■		■				locomotion (-); recognition memory (-)	n.a.
[262]	mouse							■					n.a.							recognition memory (+)	ablation of MMP9 gene
[103]	mouse									■			13 vs. 3 (homozygous); 5 vs. 3 (heterozygous) [1]	■						recognition memory (+); working memory (+)	n.a.
[263]	rat					■							n.a.							spatial learning & memory (+); recognition memory (+)	n.a.
[81]	rat	■											48 vs. 7 [1]	■						exploration (-); anxiety (+); others (-)	n.a.
[264]	mouse	■											n.a.			■				working memory (-); fear memory (+); spatial learning & memory (+)	n.a.

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[265]	rat					■							10 vs. 6 [1]	■						spatial learning & memory (+)	hydroxysafflor yellow A
[94]	rat						■						n.a. [2]				■			recognition memory (+)	memantine
[112]	rat				■								9.2 vs. 3.8	■						spatial learning & memory (+)	rivastigmine (liposomal)
[97]	mouse							■				■	7.5 vs. 5.5 (age); 11 vs. 5.5 (genetic, adult); 13.5 vs. 7.5 (genetic, old) [1]	■						spatial learning & memory (+)	n.a.
[266]	mouse	■	■										26 vs. 8 (WT); 54 vs. 9 (Tg) [1]	■						n.a.	n.a.
[267]	mouse	■	■										82.93 vs. 5.89	■						spatial learning & memory (+); psychomotor function (-)	n.a.
[268]	rat					■							n.a.							spatial learning & memory (+)	betaine
[269]	rat		■										19.16 vs. 5.21	■						spatial learning & memory (+)	resveratrol
[270]	rat			■									n.a.							spatial learning & memory (+)	n.a.
[98]	mouse								■				n.a.	■						psychomotor function (+); exploration (+); anxiety (+); recognition memory (+); working memory (+)	n.a.
[271]	rat		■										21.2 vs. 6.16		■					spatial learning & memory (+)	diethyl dithio carbamate trihydrate, folacin

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[272]	rat		■								■		21 vs. 7.4 (dams)	■						offspring: spatial learning & memory (+)	ginkgo biloba extract
[273]	rat		■										5.1 vs. 3.2 [1]		■					spatial learning & memory (+)	n.a.
[274]	rat		■										52.3 vs. 6.96	■						exploration (+); anxiety (+); others (+)	n.a.
[275]	mouse	■											90.68 vs. 2.04 (WT); 118.75 vs. 0.41 (Tg)	■						fear memory (-); spatial learning & memory (-)	SAM
[276]	rat		■										21.2 vs. 6.16		■					spatial learning & memory (+)	pioglitazone; rosiglitazone
[277]	mouse	■	■										100 vs. 8 (Met suppl.); 70 vs. 8 (B-vit. def.) [1]	■		■				working memory (-); fear memory (-)	n.a.
[278]	rat					■							n.a.							spatial learning & memory (+); fear memory (+)	acetyl-L-carnitine
[279]	rat						■						n.a. [2]				■			recognition memory (+); spatial learning & memory (+)	dextrome-thorphan
[78]	mouse	■											111 vs. 5 (WT); 76.4 vs. 3.8 (Tg)	■						n.a.	SAM
[280]	pig											■	6.88 vs. 5.45		■					exploration (+); psychomotor function (-); working memory (-); others (+)	folate
[109]	rat											■	n.a.			■				n.a.	N-acetyl cysteine + α-lipoic acid + α-tocopherol

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (µM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[281]	rat					■							n.a.							fear memory (+); exploration (-)	curcumin
[282]	mouse	■	■										n.a.							spatial learning & memory (+)	n.a.
[210]	rat					■							~500 µM vs. n.a.	■		■				n.a.	n.a.
[283]	mouse										■	■	2.39 vs. 2.37 (offspring)	■						offspring: exploration (-); anxiety (-)	n.a.
[87]	rat			■									26.7 vs. 10.4	■		■				spatial learning & memory (+)	n.a.
[130]	mouse	■											n.a. [2]			■		■		spatial learning & memory (+)	anti-HCA antibody
[213]	mouse		■										16.8 vs. 3.4	■						fear memory (+)	n.a.
[284]	mouse	■	■										155 vs. 5 [1]	■						n.a.	n.a.
[285]	mouse	■											30 vs. 6 [1]	■						n.a.	n.a.
[286]	mouse		■										35.4 vs. 6.33	■						others (+)	n.a.
[287]	rat					■							n.a.							psychomotor function (-); fear memory (+)	folate
[288]	rat	■									■		16.5 vs. 6.8 (offspring)	■		■				offspring:sensorimotor function (+); spatial learning & memory (+); others (-)	short-term neonatal hypoxia
[289]	rat					■							10.2 vs. 6.2	■						spatial learning & memory (+)	B-vitamins
[92]	mouse					■							32.1 vs. 11.6	■						n.a.	n.a.
[290]	mouse	■											67 vs. 8.5 (WT); 49.9 vs. 9.6 (Tg)		■					exploration (+); spatial learning & memory (+); anxiety (+); others (+)	n.a.
[96]	mouse							■					257–365 vs. 15.4–25.4 (diff. strains)		■					exploration (-); anxiety (-); fear memory (+)	n.a.

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[291]	rat		■								■		24.8 vs. 6.8 (dams)	■						offspring:spatial learning & memory (+)	melatonin
[292]	rat	■	■										31.3 vs. 4.2 (B-vit. def.); 31.2 vs. 4.2 (B-vit. def. + Met suppl.)	■						spatial learning & memory (+); psychomotor function (-)	methionine
[293]	mouse	■	■										28.7 vs. 5.2 (B-vit. def.); 13.9 vs. 5.2 (Met suppl.)	■						spatial learning & memory (+); psychomotor function (-)	n.a.
[79]	mouse	■											320 vs. 0.2 (WT);450 vs. 1 (Tg) [1]	■						spatial learning & memory (-)	n.a.
[294]	mouse	■											7.3 vs. 4.0	■						exploration (+); anxiety (+); working memory (-); psychomotor function (+); spatial learning & memory (-)	n.a.
[295]	rat					■							10.2 vs. 6.2 [1]	■						n.a.	B-vitamins
[296]	rat		■								■		26 vs. 6 (dams)	■						offspring:spatial learning & memory (+)	n.a.
[203]	rat	■									■		13.3 vs. 6.8 (offspring)	■		■				offspring: sensorimotor function (-); anxiety (+); spatial learning & memory (+)	n.a.
[212]	mouse	■		■									101 vs. 37 (WT); 178 vs. 103 (Tg)	■						working memory (-); spatial learning & memory (+)	n.a.
[84]	mouse	■	■										243.7 vs. 5.1 (B-vit. def.); 86.7 vs. 5.1 (B-vit. def. + Met suppl.)	■						spatial learning & memory (+);psychomotor function (-); exploration (-)	B-vitamins

Table A13. *Cont.*

Publication	Animal Species	Strategy to Induce HHCys: Diet/Drinking Water: B-vit. def.	Diet/Drinking Water: Met suppl.	Diet/Drinking Water: HCys suppl.	Diet/Drinking Water: Others	Injection: HCys	Injection: Others	Genetic Manipulation: CBS	Genetic Manipulation: MTHFR	Genetic Manipulation: Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Investigated Biological Matrix: Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue	Impact on Cognitive Performance: Cognitive Domain & Reported Effects of HHCys yes (+) or no (-)	Investigation of Potential Treatment Option
[297]	mouse							■					12.6 vs. 7.9		■					n.a.	n.a.
[211]	rat					■							4.5 vs. 2.9 [1]	■						spatial learning & memory (-)	n.a.
[298]	rat		■										20 vs. 7.5 [1]	■						fear memory (+); spatial learning & memory (+)	melatonin
[299]	mouse	■						■					205 vs. 3.9	■		■				n.a.	n.a.
[300]	rat	■			■								26.2 vs. 6.5	■						n.a.	folate
[301]	rat					■							400–500 vs. 10	■		■				spatial learning & memory (+); working memory (+); exploration (-)	n.a.
[302]	mouse	■		■									25 vs. 2 (WT); 27 vs. 3 (Tg) [1]		■					n.a.	n.a.
[303]	mouse								■				5.3 vs. 3.25 (heterozygous); 32.3 vs. 3.25 (homozygous)	■						n.a.	n.a.
[304]	mouse	■											125 vs. 9		■					others (+)	n.a.

[1]: Estimated from graph; levels not exactly reported in the study; [2]: HCA is also considered in the study; [3]: data converted to μM; [4]: for reasons of comparability with other studies: reporting of mean; not median as in the original manuscript; [5]: transformation of data to μM not applicable.

Table A14. Additional hand-searched animal studies; abbreviations: WT: wild type, Tg: transgenic, B-vit. def.: deficiency in B-vitamins (and related substances), Met suppl.: supplementation of L-methionine, CBS: cystathionine β-synthase, MTHFR: methylenetetrahydrofolate reductase, GAA: guanidinoacetate.

		Strategy to Induce HHCys												Investigated Biological Matrix					
		Diet/Drinking Water				Injection		Genetic Manipulation											
Publication	Animal Species	B-vit. def.	Met suppl.	HCys suppl.	Others	HCys	Others	CBS	MTHFR	Others	Maternal HHCys Impact	Others	Blood Levels (μM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue
[305]	mouse						■				■		6.5 vs. 5.1 [1] (offspring)		■				
[85]	mouse	■	■										243.7 vs. 4.6 (B-vit. def.); 86 vs. 4.6 (B-vit. def. + Met suppl.)	■					
[306]	mouse							■					349 vs. n.a.		■				
[307]	pig					■							72.33 vs. 10.53	■					
[308]	rat	■											34.1 vs. 15.1	■					■
[309]	mouse							■					383.6 vs. n.a.		■				
[310]	mouse	■							■				19 vs. 10 (genetic); 16 vs. 10 (diet, WT); 40 vs. 19 (diet, Tg) [1]	■					
[311]	rat		■		■								45 vs. 15 (Met suppl.); 65 vs. 15 (GAA) [1]	■					■
[312]	mouse	■											9 vs. 1.5 [1]	■					
[313]	mouse		■	■									51.8 vs. 3.0 (Met suppl.); 21.4 vs. 3.0 (HCys suppl.)	■				■	
[95]	rat		■				■						140 vs. 20 (diet); 68 vs. 15 (injection) [1]	■					■
[314]	mouse		■					■					3.8 vs. 3.7 (genetic); 40.7 vs. 3.7 (diet, WT); 140.3 vs. 6.8 (diet, Tg)	■					
[315]	mouse	■	■										23.5 vs. 4.1	■					
[209]	mouse	■											4.0 vs. 3.38		■				
[86]	mouse	■	■						■				4.5 vs. 3 (genetic); 4.4 vs. 3 (Met suppl., WT); 8.4 vs. 3 (B-vit. def., WT); 9.5 vs. 3 (Met suppl. + B-vit. def., WT) [1]	■					
[316]	rabbit	■				■							20.3 vs. 12.3	■					
[317]	mouse	■	■					■					242 vs. 13		■				
[318]	mouse								■				8.2 vs. 4.0	■					
[319]	mouse		■	■									53.6 vs. 9.46 (Met suppl.); 51.4 vs. 9.46 (HCys suppl.)	■					
[107]	mouse				■								24.5 vs. 2.6	■					
[320]	rat	■											19.5 vs. 6.15	■					

Table A14. *Cont.*

		Strategy to Induce HHCys												Investigated Biological Matrix					
		Diet/Drinking Water				Injection		Genetic Manipulation											
Publication	Animal Species	B-vit. def.	Met suppl.	HCys suppl.	Others	HCys	Others	CBS	MTHFR	Others	Maternal HHCys Impact	Others	Blood Levels (µM): ↑HCys vs. Control/Baseline Data (Where Applicable)	Plasma	Serum	Brain Tissue	CSF	Urine	Liver Tissue
[321]	rat					■							500 vs. n.a.	■		■			
[322]	mouse		■					■					8.3 vs. 5.0 (genetic); 17.2 vs. 5.0 diet, WT); 21.2 vs. 17.2 (diet, Tg)	■					
[323]	mouse		■					■					6.3 vs. 4.1 (genetic); 13.0 vs. 4.1 (diet, WT); 23.9 vs. 6.3 (diet, Tg)	■					
[88]	rat				■								15.5 vs. 10.5 [1]	■					
[89]	rat				■								23.6 vs. 11.0	■				■	
[324]	monkey	■	■										10.6 vs. 4.0	■					
[325]	mouse							■					13.5 vs. 6.1 (heterozygous); 203.6 vs. 6.1 (homozygous)	■					
[326]	monkey					■							157 vs. 1	■					

[1]: Estimated from graph; levels not exactly reported in the study.

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The Roles of Long-term Hyperhomocysteinemia and Micronutrient Supplementation in the *App*$^{NL-G-F}$ Model of Alzheimer's Disease

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Abstract: A causal contribution of hyperhomocysteinemia to cognitive decline and Alzheimer's disease (AD), as well as potential prevention or mitigation of the pathology by dietary intervention, have frequently been subjects of controversy. In the present in vivo study, we attempted to further elucidate the impact of elevated homocysteine (HCys) and homocysteic acid (HCA) levels, induced by dietary B-vitamin deficiency, and micronutrient supplementation on AD-like pathology, which was simulated using the amyloid-based *App*$^{NL-G-F}$ knock-in mouse model. For this purpose, cognitive assessment was complemented by analyses of ex vivo parameters in whole blood, serum, CSF and brain tissues from the mice. Furthermore, neurotoxicity of HCys and HCA was assessed in a separate in vitro assay. In confirmation of our previous study, older *App*$^{NL-G-F}$ mice also exhibited subtle phenotypic impairment and extensive cerebral amyloidosis, whereas dietary manipulations did not result in significant effects. As revealed by proximity extension assay-based proteome analysis, the *App*$^{NL-G-F}$ genotype led to an up-regulation of AD-characteristic neuronal markers. Hyperhomocysteinemia, in contrast, indicated mainly vascular effects. Overall, since there was an absence of a distinct phenotype despite both a significant amyloid-β burden and serum HCys elevation, the results in this study did not corroborate the pathological role of amyloid-β according to the "amyloid hypothesis", nor of hyperhomocysteinemia on cognitive performance. Nevertheless, this study aided in further characterizing the *App*$^{NL-G-F}$ model and in elucidating the role of HCys in diverse biological processes. The idea of AD prevention with the investigated micronutrients, however, was not supported, at least in this mouse model of the disease.

Keywords: hyperhomocysteinemia; vitamin B deficiency; Alzheimer disease; amyloid beta-peptides; memory and learning tests; proteomics

1. Introduction

A causal contribution of the endogenous amino acid homocysteine (HCys) and its metabolites to neurodegenerative diseases has been discussed for years. Alzheimer's disease (AD), especially sporadic AD which is the highly prevalent and dominant late-onset form [1], has previously been shown to be associated with increased HCys levels [2]. One hallmark of sporadic AD is a deficiency in vitamin B12 [3]. As pointed out in previous reviews, B-vitamin deficiency, especially lack of vitamin B6, B12 and folate, is one factor amongst others that results in increased HCys levels, called hyperhomocysteinemia (HHCys) [4,5]. Consequently, the reduction of HCys levels using B-vitamin intervention offers an interesting option and has been discussed frequently with respect to potential mitigation of AD-related pathology. In order to test HCys-lowering as a possibly valuable preventative approach, it is crucial to examine the role of B-vitamin intervention at a very early stage of the disease. The initial phase of AD pathology is typically characterized by disrupted amyloid metabolism, a central hallmark of this neurodegenerative disease [6,7]. Amyloid-β (Aβ) peptides aggregate and build up characteristic

extracellular plaques, which can be detected many years before the onset of the first cognitive symptoms [8]. Soluble Aβ oligomers have been suggested to be the major detrimental Aβ species, particularly at an early stage of the disorder, also entitled preclinical AD [9,10].

In terms of animal research, the long asymptomatic phase of AD can be simulated by animal models based on modifications of the amyloid-β precursor protein (AβPP) [11]. Many AβPP-based mouse models are available, however, transgenic models often pose the risk of artificial phenotypes due to an overexpression of AβPP. This pitfall has been overcome by the introduction of knock-in mouse models such as *App*$^{NL-G-F}$ [12]. Humanization of the AβPP sequence and implementation of three targeted mutations (NL-Swedish, G-Arctic, F-Beyreuther/Iberian) generates distinct amyloidosis that resembles human AD and simulates aspects of neuroinflammation [13].

Additional HHCys can be induced in experimental animals, e.g. by feeding special diets, which are deficient in specific micronutrients that are essential in the context of the remethylation or transsulfuration of HCys [14]. Besides supplementation with B-vitamins, HCys remethylation may also proceed independently of vitamins with betaine in some tissues [14]. Dietary betaine supplementation with the goal of mitigation of HHCys, has also been the subject of former animal studies [15,16]. According to an international consensus statement, also other micronutrients, such as polyunsaturated fatty acids (PUFA), should be taken into account in terms of controlling plasma HCys as a modifiable risk factor for dementia in the elderly [17]. PUFA might play a role in slowing cognitive decline, however, evidence is not consistent on this topic [18]. It has been shown that PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are required for an adequate effect of B-vitamin treatment in cognitive decline and for preventing HCys-related deterioration of cortical Aβ load [19,20]. Equally, B-vitamin status is thought to be important for PUFA-associated effects on cognition and therefore, future studies on the link between these micronutrients and cognitive outcomes were imperative [21].

The research questions raised in the present in vivo study were mostly oriented towards our previous work on this topic [22] and a potential contribution of experimentally induced HHCys to the AD-like pathology in the *App*$^{NL-G-F}$ model was the focus of the study. Additionally to HCys, we also considered its oxidative metabolite homocysteic acid (HCA), which has been suggested be the actual neurotoxic culprit, as summarized recently [14]. This hypothesis was tested and is reported in the in vitro part of the present article. Aside from potentially detrimental effects of HHCys, we were interested in effects by the *App*$^{NL-G-F}$ model itself and investigated dietary effects of different micronutrients on various read-outs, comprising behavioral cognitive outcomes, as well as ex vivo parameters in whole blood, serum, CSF and brain tissues from the mice. In our previous study, we detected almost no aggravation of AD-like pathology by HHCys in *App*$^{NL-G-F}$ KI mice, which proved to be a very subtle model for the disease. Consequently, for the present long-term animal study, several experimental details have been adapted, including the diets to some extent, procedures and outcomes. Most of all, the duration of the study was nearly doubled, enabling the examination of WT and KI mice at a higher age of approximately 70 weeks, with the aim to examine the experimental variables on a more distinct AD-like pathology. Overall, the current exploratory study can be seen as a continuation and extension of our previous study on this topic [22].

2. Materials and Methods

2.1. Cell viability in vitro assay

Examination of cell viability after compound treatment was performed using the luciferase-based CellTiter-Glo assay (Promega, Walldorf, Germany). Prior to the assay, primary cortical neurons (rat brain, R-CX-500, Lonza, Basel, Switzerland) were plated into a poly-D-lysine coated opaque 96-well plate (Corning, Wiesbaden, Germany). Therefore, counting of viable cells in the cell suspension was done with the help of trypan blue staining (Sigma-Aldrich). Approximately 10,000 cells were plated per well. Cultivation of the cells on the plate proceeded in Neurobasal medium, supplemented with B27 (minus antioxidants), L-glutamine and penicillin-streptomycin (ThermoFisher, Frankfurt am Main, Germany) at 37°C, 5% CO2. To increase cell survival and reduce debris, the first 4 hours of cell culture was done in a medium containing 5% fetal calf serum (ThermoFisher), which was subsequently replaced by serum-free medium. First neurite networks became visible by day 3. At day 4, 50% of the medium was changed. Finally, after 5 days of cell culture, the actual assay was performed on two consecutive

days. Either the test compounds L-homocysteine or L-homocysteic acid (Sigma-Aldrich, Taufkirchen, Germany), celecoxib as a frequently used positive control for this purpose (Biomol, Hamburg, Deutschland) or vehicle were pipetted into the plate. Compound treatment wells (cells + test compound in different concentrations), positive control (cells + 10 mM celecoxib), negative control (cells + vehicle) and blank wells (medium without cells + vehicle) were fully randomized over the 96-well plate. After an incubation period of 24 hours, an equal amount of CellTiter-Glo reagent was added to each well, shaken and read out after 10 minutes at a luminometer (Perkin Elmer, Hamburg, Germany). The luminescence signal (relative light units) of blank wells was subtracted from all treatment and control wells. The signal of the negative control was defined as 100% cell viability. A luciferase-based assay was chosen, since other common testing systems based on water soluble tetrazolium or comparable substances were not applicable due to the redox properties of HCys, which would lead to an interaction with the assay reagent. We performed the present in vitro assay in n=3 and furthermore used triplicates on each plate.

2.2. Animals, experimental groups and diets

All animal experiments were carried out in accordance with the '3R', the DIRECTIVE 2010/63/EU and the regulations of GV-SOLAS. Experimental procedures were approved by the local Ethics Committee for Animal Research in Darmstadt, Germany (approval number: F152/1011; approval date: 31.07.2017) and based on EQIPD and the ARRIVE-Guidelines [23].

In total, 84 mice were examined in the present study, equally consisting of males and females. Twelve age-matched C57BL/6J wild type (WT) animals were obtained from Charles River Wiga GmbH (Sulzfeld, Germany) and 72 homozygous *App*$^{NL-G-F}$ knock-in (KI) animals were bred at mfd Diagnostics GmbH (Wendelsheim, Germany). The *App*$^{NL-G-F}$ knock-in model was kindly provided by Dr. Saido and colleagues from RIKEN Center for Brain Science (Saitama, Japan). All animals were chipped with subcutaneous transponders in order to minimize the risk of erroneous animal allocation. Genotyping at our lab, using polymerase-chain-reaction analysis, confirmed the adequate genetic background of each animal. The mice were randomly assigned to their home cages (Green Line, Tecniplast, Hohenpeissenberg, Germany). All randomization steps in the current study were based on randomization lists (https://www.random.org/). Animals were housed pairwise at constant temperature (mean: 22.7 ◦C) and humidity (mean: 53.6%) conditions under a 12/12 hour dark/light cycle (lights on at 7:00 am with twilight phases starting at 6:30 am and 18:30 pm). The use of sentinel mice enabled monitoring of the hygiene status of the colony room.

One week after arrival, the mice were allocated to 7 experimental groups (Table 1).

Table 1. Experimental groups.

Group Number	Genotype	Diet	Abbreviation
1	C57BL/6J wild type	Control	C (WT)
2	*App*$^{NL-G-F}$ knock-in	Control	C (KI)
3	*App*$^{NL-G-F}$ knock-in	Vitamin B deficient	B-DEF
4	*App*$^{NL-G-F}$ knock-in	Vitamin B enriched	B-ENR
5	*App*$^{NL-G-F}$ knock-in	Vitamin B deficient and PUFA supplemented	B-DEF+PUFA-ENR
6	*App*$^{NL-G-F}$ knock-in	Vitamin B enriched and PUFA supplemented	B+PUFA-ENR
7	*App*$^{NL-G-F}$ knock-in	Vitamin B deficient and betaine supplemented	B-DEF+BET-ENR

The seven groups were defined by the animals' genotype and the different experimental diets that the KI animals received. Compositions of the diets were based on the AIN93M chow and modified as shown in detail in Table A 1. In comparison to our previous study [22], adaptions have been made in groups 5 and 7. All diets were purchased from Ssniff-Spezialdiäten GmbH (Soest, Germany) and stored at refrigerator temperature or, in case of polyunsaturated fatty acid (PUFA) containing diets, at -20°C in order to minimize oxidation [24]. Due to the long duration of the study, diets were obtained in consecutive batches, thus avoiding expiry. Each animal received tap water ad libitum and a daily amount of four gram of diet. Moreover, each animal was weighed weekly and scored for body condition twice a week in order to monitor its nutritional status. Feeding the experimental diets started at the age of six weeks and proceeded for the entire course of the study, except for short regeneration periods due to poor body condition and animal loss in the B-vitamin deficiently fed groups.

2.3. Sample collection

The timeline of the in vivo study course is illustrated in Figure 1, highlighting all sampling steps as well as the schedule of behavioral testing.

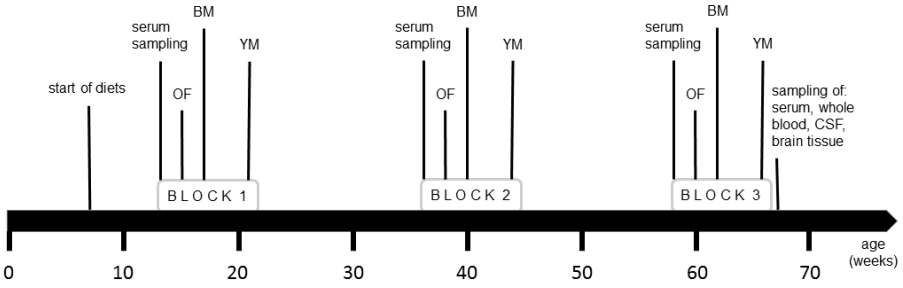

Figure 1. Timeline of the in vivo experiments; abbreviations: open field test (OF), Barnes maze (BM), Y-maze (YM).

In the first serum sampling steps, puncturing the facial vein with 5 mm Goldenrod animal lancets (MEDIpoint, Mineola, NY, USA) enabled blood collection (max. 170 µL per 25 g mouse, according to GV-SOLAS animal welfare guidelines). Further processing to serum is described below. At the end of the study, at the age of nearly 70 weeks, different biological matrices were sampled for subsequent analyses. For that purpose, the mice were deeply anaesthetized using 200 mg/kg (body weight) ketamine (Vétoquinol GmbH, Ismaning, Germany) and 10 mg/kg (body weight) xylazine (Bayer Health Care, Leverkusen, Germany). After the cessation of reflexes had been confirmed, we sampled cerebrospinal fluid (CSF) by cisterna magna puncture. Therefore, we shaved the back of the mice' head and made a small incision. Tissues were gently removed as much as needed using extra fine graefe forceps (FST, Heidelberg, Germany) and pulled borosilicate glass capillaries (WPI, Friedberg, Germany) were utilized to carry out the puncture with the help of a surgical stereoscope (Zeiss, Oberkochen, Germany). Approximately 10 µL of clear CSF were obtained per mouse, frozen on dry ice and stored at -80°C for further use. Subsequently, blood was taken cardially and animals were killed by cervical dislocation and brains were harvested. The blood was transferred into tubes, containing either a clotting factor or EDTA (Sarstedt, Nümbrecht, Germany). In the serum tubes, blood was allowed to fully coagulate for 15-30 minutes before it was centrifuged (3200 g; 4°C) for 15 minutes. Whole blood was immediately analyzed, whereas serum was frozen on dry ice and stored at -80°C for subsequent analyses. For the brains, a cross sectional part of about 100 mg tissue posterior to bregma was removed using razor blades and a brain matrix (WPI), weighed, frozen in liquid nitrogen and stored at -80°C. Cross sections were taken independently of specific brain structures, as the App^{NL-G-F} previously proved to exhibit amyloidosis throughout the whole brain [22,25]. The order of animals during sampling steps and euthanasia was defined according to a randomization list.

2.4. Biochemical analysis of HCys and HCA

Quantification of HCys and HCA in serum samples using liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) was performed as described in detail in previous publications of our group [22,26,27].

2.5. Behavioral testing

In the present study, the animals' cognitive performance was tested in three different settings and in three time blocks across the course of the study (15-21, 38-44 and 60-66 weeks of age). All outcomes in the open field test, Barnes maze and Y-maze were automatically detected by an over-head camera and analyzed with the corresponding EthoVision XT 13 software (Noldus, Wageningen, the Netherlands). Between the trials, testing systems were cleaned with 70% ethanol in order to remove

odor cues. Every experiment was performed between 8 a.m. and 1 p.m. during the light phase and the animals were allowed to acclimatize to the experimental room 30 minutes before the start of behavioral testing. The order in which the animals were tested, was defined according to a randomization list.

2.5.1. Open field

Habituation and anxiety-related behavior of the mice were evaluated using an open field apparatus (Hugo Sachs Elektronik, March-Hugstetten, Germany). Procedures were conducted as described recently [22], using the habituation ratio as an indicator for intrasession habituation (Equation 1). The position in the arena and the distance moved by the mice was continuously recorded and further analyzed for the total duration of 30 minutes and for different time bins.

$$habituation\ ratio\ = \frac{activity\ (final\ 5\ min)}{actitvity\ (final\ 5\ min + initial\ 5\ min)} \quad \text{Equation 1}$$

2.5.2. Barnes maze

Procedural details for the acquisition phase and probe trial in the Barnes maze were previously reported in depth [22]. Briefly, spatial learning and memory performance of the animals were assessed by measuring the time the mice needed to escape from an aversive, bright and open space, while orienting to different visual cues positioned around the maze. The test consisted of two consecutive phases: in the acquisition phase, the mice needed to learn the position of an escape box (target) in 3 minute sessions on four subsequent days of training, and escape latencies were recorded. In the probe trial on day five, the mice were subjected to the maze after removal of the escape box for 1.5 minutes. The exact position of the target in the maze was randomly assigned per animal and it was ensured that when repeating the test in time blocks two and three, reversal settings were applied for each mouse.

2.5.3. Y-maze

As a testing system for working memory, we used a Y-maze apparatus (Hugo Sachs Elektronik) to further characterize cognitive abilities. For this purpose, each mouse was placed into the center of the maze and explored the three symmetrical arms for a period of 8 minutes under reduced light (30 lux). Sequence and number of arm entries were automatically recorded and used for the calculation of spontaneous alternation (Equation 2). Thus, an alternation indicates that sequential entry into all three different arms was completed (e.g. A, B, C).

$$spontaneous\ alternation\ (\%) = \frac{number\ of\ alternations}{(total\ number\ of\ arm\ entries - 2)} * 100 \quad \text{Equation 2}$$

2.6. Hematology

Immediately after cardiac puncture, 10 µL whole blood from each mouse were measured in a Celltac α MEK-6500K automated hematology analyzer (Nihon Kohden, Rosbach v.d.H., Germany) in order to examine potential effects of AD-like genotype, HHCys or dietary interventions on various hematological parameters.

2.7. Olink proteomics

Protein analysis of 92 mouse biomarkers was performed using the Olink mouse exploratory panel (Olink, Uppsala, Sweden). A list comprising all proteins in this panel and their relation to diverse biological processes is provided by the supplier (https://www.olink.com/products/target/mouse-exploratory/). We used both serum and CSF from the animals, from which volumes of 1 µL were required for the measurement. Due to limited capacity on the plate, animals of the groups 1, 2, 3, 4 and 6 (resp. n=8; 11; 8; 9; 8) were included, whereas groups 5 and 7 were excluded from this analysis. The total number of observations in the proteome analysis was limited by animal loss or failure of appropriate sampling volumes. Plate loading proceeded randomly and the pipetter was blinded to the experimental groups at this stage of the experiment. All quality control parameters were applied according to the supplier's instructions. The Olink proteomics technology is based on the proximity extension assay [28] and results in relative quantification of protein biomarkers in the Normalized Protein eXpression (NPX) unit, which is an arbitrary unit on log2 scale. Thereby, higher NPX values

indicate higher protein concentrations. Resulting data were further analyzed for enrichment with the help of Geneontology (GO) and PANTHER classification system (http://geneontology.org/).

2.8. Quantification of Aβ

The amounts of cerebral Aβ peptides, i.e. the humanized and modified Aβ42 from the App^{NL-G-F} KI mice, were determined in this study using ELISA (KHB3441, Invitrogen, ThermoFisher). The Aβ extraction protocol reported here is similar to that in other studies using App^{NL-G-F} KI mice [29,30]. Initially, brain tissue sections of about 100 mg were thawed on ice in 1 mL of pre-cooled extraction buffer, which consisted of 5 M guanidine hydrochloride (GuHCl), 50 mM TRIS and 1x protease inhibitor cocktail (Sigma-Aldrich), adjusted to pH=8. With the help of GuHCl, not only the soluble but also the insoluble portion of Aβ could be determined. One-step mechanical lysis was done in a FastPrep-24 5G homogenizer (MP Biomedicals, Eschwege, Germany) using the mouse brain program (6 m/s; 40 s) and homogenates were shaken (300 rpm) afterwards for 3.5 hours at room temperature. After 1:10 dilution with cold PBS, including protease-inhibitor, the homogenates were centrifuged (20,000 g; 20 min; 4 °C) and supernatants were further diluted 1:200 in standard diluent buffer. Loading GuHCl in this dilution did not pose the risk of disintegrating the ELISA plate. Plate loading and absorption measurement then proceeded according to the supplier manual. Peptide concentrations were finally derived from absorption data via four parameter logistic curve fit model, and under consideration of the dilution factor, were normalized to the initial weights of the tissue sections.

2.9. Statistical analyses

Initially, the number of mice per group had been estimated through statistical power calculation (http://www.biomath.info/power/). SPSS Statistics 26 (IBM, Ehningen, Germany) was used for all statistical analyses in the present study. Prior to data analysis, extreme outliers (> 3x interquartile range) were excluded, followed by normality testing using Shapiro Wilk test. As Gaussian distribution could not be assumed in most data sets, we mainly performed non-parametric Mann-Whitney-U tests for the pairwise comparisons (group 1 versus 2 and group 2 versus 3-7). A p value (two-tailed exact sig.) lower than 0.05 was considered statistically significant. Furthermore, non-parametric (group-unspecific) Spearman rank correlation tests were applied. Data were expressed as median ± interquartile range, pooled for males and females, using GraphPad Prism 7 (San Diego, CA, USA). Merely for the cell viability assay, normality testing indicated Gaussian distribution and therefore, parametric analysis (t test) was performed and results are depicted as means ± standard error of the mean.

3. Results

3.1. Cell viability in vitro assay

Luciferase-based testing showed a concentration-dependent effect of HCys and HCA on the viability of primary neurons (Figure 2). In relation to the vehicle control (100% viability), increasing concentrations of each compound were associated with increasing levels of cell death. At a measured concentration of at least 2.5 mM, the difference in neurotoxicity between HCys and HCA became significant (*2.5 mM $p<0.001$; 5.0 mM $p=0.011$; 10.0 mM $p=0.005$*). Celecoxib (1 mM), as a positive control, decimated almost all cells in the respective wells (not shown).

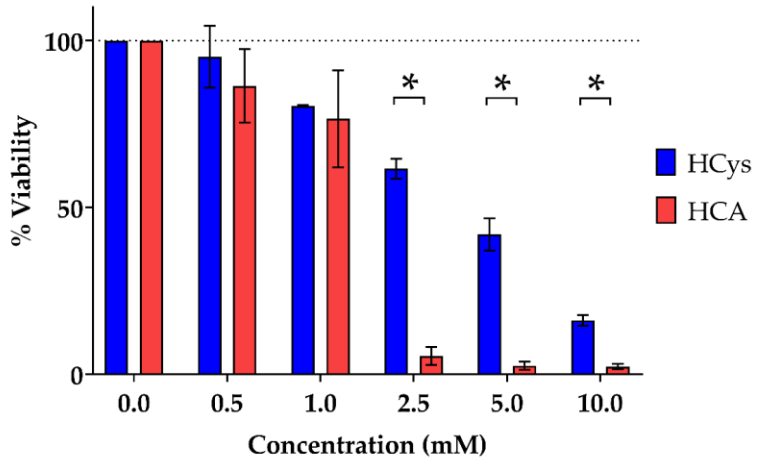

Figure 2. Luciferase-based cell viability measurement of primary cortical neurons (10,000 per well) after 24 hours of incubation with homocysteine (HCys) or homocysteic acid (HCA); mean ± SEM (n=3); $p < 0.05$ (t test) considered statistically significant (*).

3.2. Biochemical analysis of HCys and HCA

In vivo serum levels of the amino acids HCys and HCA were analyzed several times longitudinally during the study course using LC-MS/MS analysis (Figure 3). Consequent to the dietary B-vitamin restriction, HCys levels were shown to be significantly elevated in time block 1 (*B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*), time block 2 (*B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*), time block 3 (*B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*) and at the end of the experiment (*B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*). A difference between WT and KI control mice just reached significance in time block 1 (*p=0.043*) and was not further confirmed at any other sampling step. We did not detect a statistically significant impact of B-vitamin and/or PUFA supplementation on serum HCys compared to KI control mice.

Also, HCA serum levels were elevated in all three B-vitamin deficient groups at every sampling step (time block 1: *B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*; time block 2: *B-DEF p<0.001; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*; final sampling step: *B-DEF p=0.041; B-DEF+PUFA-ENR p=0.001; B-DEF+BET-ENR p=0.005*). For time block 3, no HCA data were obtained due to sample loss while sample preparation. No effect of the App^{NL-G-F} KI genotype on HCA levels was detected. The diet supplemented with B-vitamins and PUFAs resulted in significantly different HCA compared to KI control, however, only in time block 1 (*p=0.026*).

Overall, hyperhomocysteinemic mice supplemented with betaine tended to build up higher serum levels of both HCys and HCA than animals from the other hyperhomocysteinemic groups.

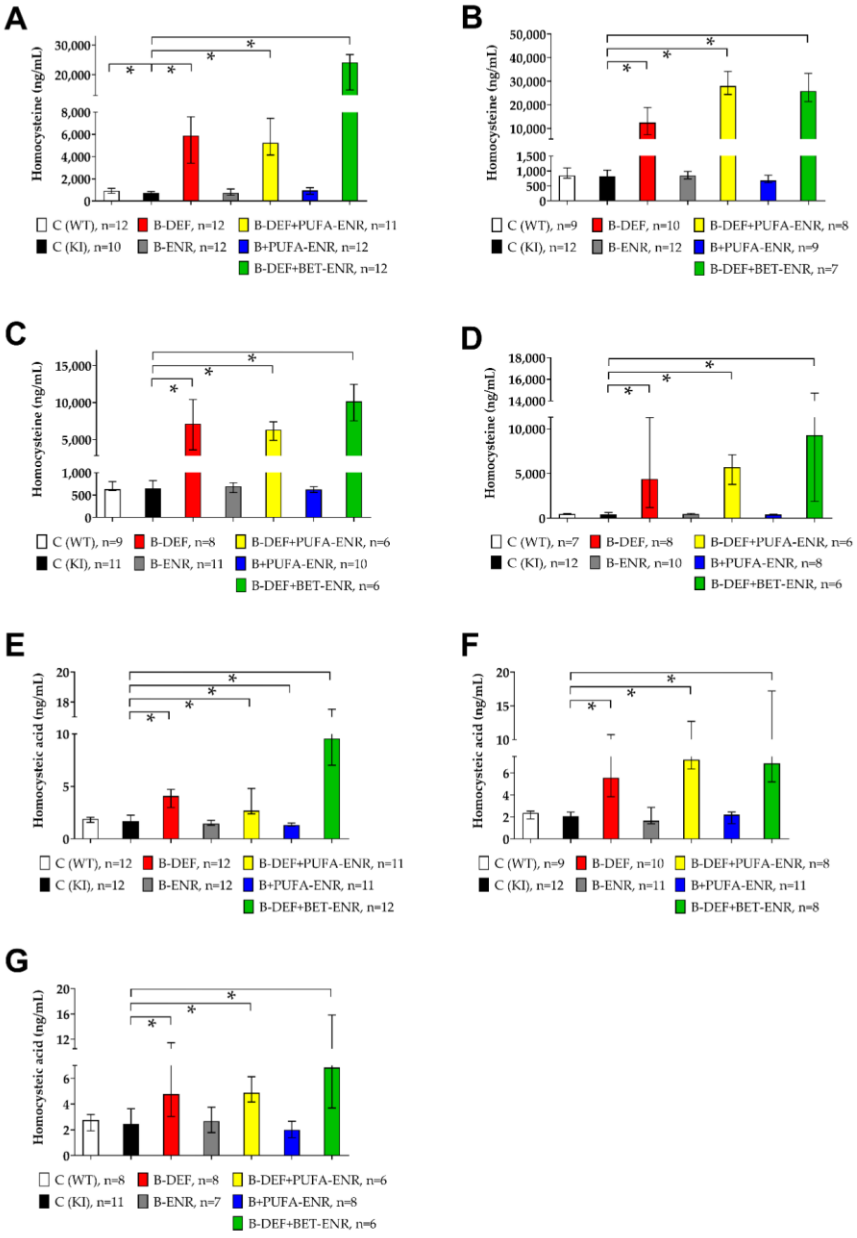

Figure 3. In vivo serum levels of homocysteine (HCys) and homocysteic acid (HCA); (A) HCys, time block 1; (B) HCys, time block 2; (C) HCys, time block 3; (D) HCys, final sampling step; (E) HCA, time block 1; (F) HCA, time block 2; (G) HCA, final sampling step; no data obtained for homocysteic acid in time block 3 due to sample loss while sample preparation; all samples analyzed via LC-MS/MS; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

3.3. Behavioral testing

In the open field test, two different behavioral domains were investigated. Firstly, calculation of the habituation ratio, based on the distance moved by the mice in different time bins (Equation 1), enabled assessment of habituation learning, whereby a lower ratio is associated with a higher level of intrasession habituation. Figure 4 (A; C; E) depicts habituation ratios for all three time blocks. In the first time block, hyperhomocysteinemic KI mice supplemented with betaine showed a lower level of intrasession habituation compared to KI control animals ($p<0.001$). However, this effect did not prove to be consistent when taking the subsequent time points into consideration. A difference between WT and KI control mice became statistically significant in time block 2 ($p=0.012$) and was confirmed in time block 3 ($p=0.007$), also at a higher effect size r. Comorbid AD-like pathology and HHCys did not further impair habituation learning compared to KI control mice.

Secondly, the time spent in the inner zone of the arena was used as an indicator for anxiety-related behavior: the lower the time in the inner zone, the higher is the level of anxiety. For this parameter, no differences between the experimental groups were detected (Figure 4 B; D; F).

Data derived from the Barnes maze and Y-maze did not reveal consistent differences between the experimental groups and can be found in the appendix.

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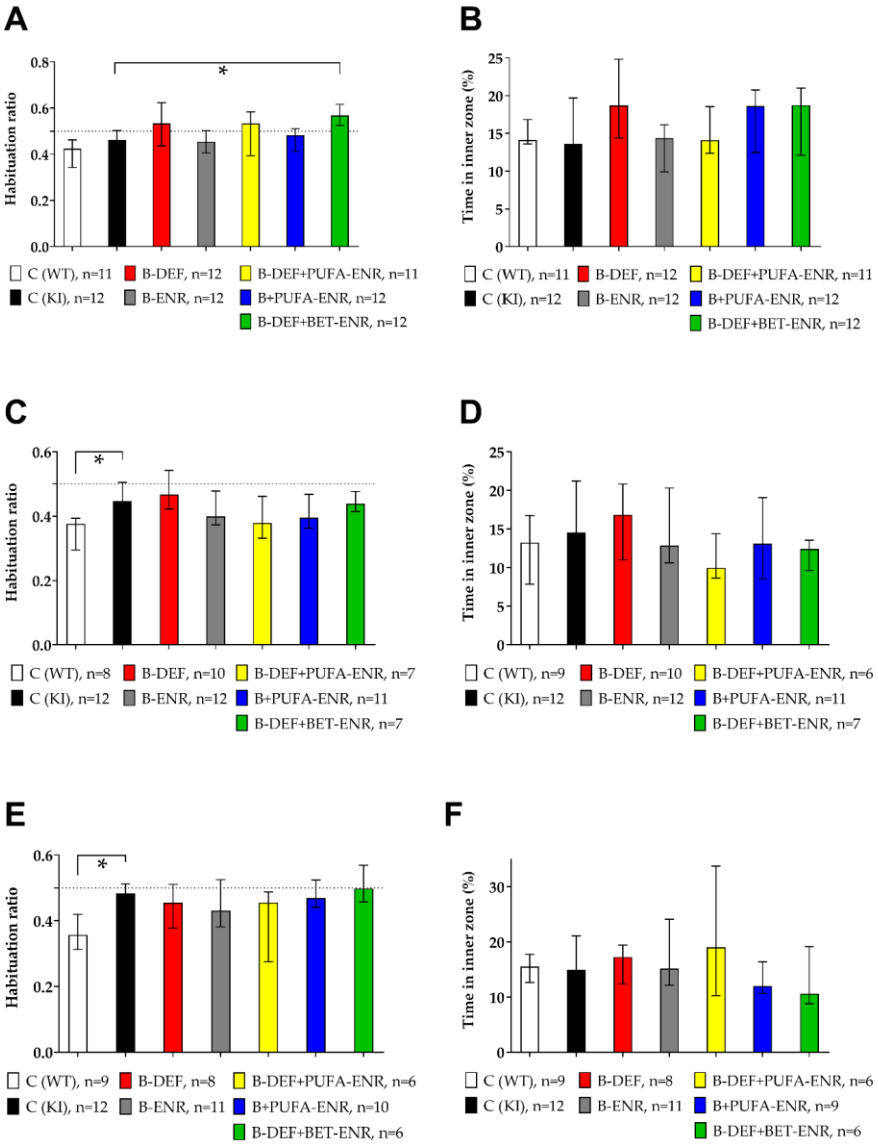

Figure 4. Open field testing in time block 1-3: (A; C; E) intrasession habituation (lower habituation ratio meaning a higher level of intrasession habituation) and (B; D; F) anxiety-related behavior; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

3.4. Hematology

For the evaluation of several hematological parameters, whole blood from the mice was taken and measured at the final sampling step. As illustrated in Figure 5, we detected significant group differences in parameters related to red blood cells. In all three hyperhomocysteinemic groups (group 3; 5; 7), decreased hemoglobin levels (*B-DEF p=0.039; B-DEF+PUFA-ENR p=0.018; B-DEF+BET-ENR p=0.013*)

were measured, as well as reduced hematocrit (*B-DEF p=0.007; B-DEF+PUFA-ENR p=0.002; B-DEF+BET-ENR p=0.001*). Furthermore, a higher red cell distribution width, meaning an increased range between smaller and larger erythrocytes, was detected in these groups (*B-DEF p=0.002; B-DEF+PUFA-ENR p<0.001; B-DEF+BET-ENR p<0.001*).

As indicated by non-parametric Spearman rank correlation test, HCys negatively correlated with hemoglobin (r_s= *-0.369; p=0.006*) and hematocrit (r_s= *-0.388; p=0.004*) and positively correlated with red blood cell distribution width (r_s=*0.340; p=0.012*).

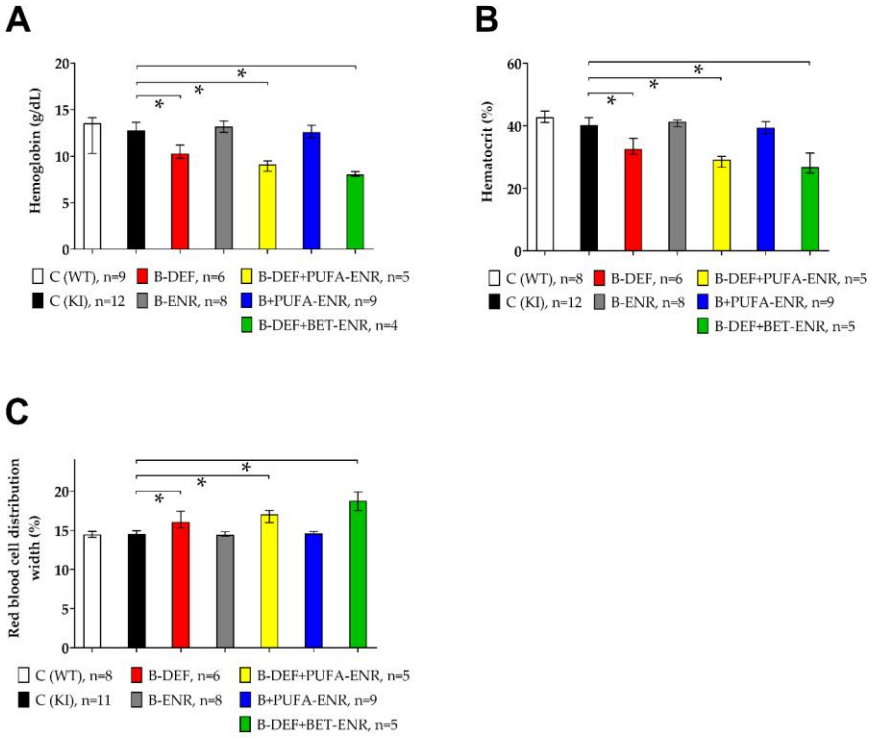

Figure 5. Hematological parameters in whole blood at the age of 67-68 weeks; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*); (A) hemoglobin concentration; (B): hematocrit; (C) red blood cell distribution width coefficient of variation.

3.5. Olink proteomics

Proteome analysis revealed significant up- or downregulation of various protein biomarkers in serum and CSF. Only data from groups 1-3 are depicted here due to the absence of effects of dietary supplementation with B-vitamins and/or PUFA. These merely had a low effect-sized impact on a few markers, did not result in findings in both serum *and* CSF and, finally, did not reveal any enrichment for particular biological processes at all in the GO-analysis. Consequently, in this section we focus on effects of the App^{NL-G-F}-related AD-like pathology on the one hand and additionally induced HHCys on the other hand.

Details on the de-regulation of all affected proteins, as well as effect sizes and other parameters are summarized in Table 2. Compared to WT, we detected 14 proteins, for which concentrations differed significantly in KI mice. Especially differences in CSF markers revealed large effect-sizes for this group comparison. B-vitamin deficiency and concomitant elevation of HCys affected 22 different proteins,

mainly in serum of the animals. Hyperhomocysteinemic mice expressed merely few differences in CSF in comparison to KI mice fed with control chow.

Table 2. Impact of the AD-like genotype and HHCys on proteins that were found to be significantly up- or down-regulated in the Olink proteomics analysis; effect size was calculated as $|r| = z / \sqrt{N}$.

App^{NL-G-F} knock-in genotype (group 1 vs. 2)						Hyperhomocysteinemia (group 2 vs. 3)					
Protein		**Matrix**	**N**	**P value**	**Effect size r**	**Protein**		**Matrix**	**N**	P value	Effect size r
CCL3	↑	Serum	19	0.016	0.549	CCL2	↓	Serum	19	< 0.001	0.777
CLMP	↑	Serum	19	0.041	0.474	CXCL9	↓	Serum	19	0.007	0.606
ERBB4	↑	Serum	19	0.007	0.606	DLL1	↓	Serum	18	0.012	0.586
GFRA1	↑	Serum	19	0.033	0.493	EDA2R	↓	Serum	19	0.016	0.549
IGSF3	↓	Serum	8	0.036	0.791	EPCAM	↑	Serum	14	0.038	0.563
NOTCH3	↑	Serum	19	0.026	0.511	FAS	↓	Serum	19	0.007	0.606
VEGFD	↑	Serum	19	0.033	0.493	FSTL3	↓	Serum	19	0.026	0.511
CCL3	↑	CSF	19	< 0.001	0.833	GFRA1	↓	Serum	19	< 0.001	0.833
CNTN1	↑	CSF	19	0.041	0.474	IGSF3	↑	Serum	9	0.032	0.735
ENO2	↑	CSF	19	< 0.001	0.833	IL1a	↑	Serum	19	0.026	0.511
HGF	↑	CSF	18	< 0.001	0.838	IL23R	↓	Serum	19	0.001	0.701
RGMA	↓	CSF	17	0.002	0.710	LGMN	↓	Serum	19	0.007	0.606
TNR	↑	CSF	18	< 0.001	0.800	MATN2	↓	Serum	18	0.034	0.503
TPP1	↑	CSF	19	0.005	0.625	S100A4	↓	Serum	19	0.007	0.606
						TGFBR3	↓	Serum	19	0.003	0.663
						TNFRSF11B	↓	Serum	19	0.001	0.720
						TNFRSF12A	↓	Serum	19	0.003	0.663
						TPP1	↓	Serum	19	0.001	0.701
						VSIG2	↓	Serum	17	0.025	0.544
						WISP1	↓	Serum	19	0.012	0.568
						ACVRL1	↓	CSF	13	0.035	0.594
						DLK1	↓	CSF	13	0.030	0.609

In order to determine biological processes for which the markers were enriched, GO analysis was conducted. Figure 6 illustrates the top 5 enriched pathways for the focussed group comparisons. For both cases, especially pathways in the neuronal context seemed to be affected: the strongest enrichments were found for the regulation of axon regeneration in *App*$^{NL-G-F}$ control mice and dendrite regeneration in hyperhomocysteinemic *App*$^{NL-G-F}$ mice, respectively. As the presented GO analysis has to be recognized purely as an *association* analysis, the assessment of the single de-regulated markers is mandatory and the respective biological matrix (serum, CSF) must be taken into consideration. An in-depth discussion of all affected proteins is provided subsequently (see discussion, chapter 4.3.).

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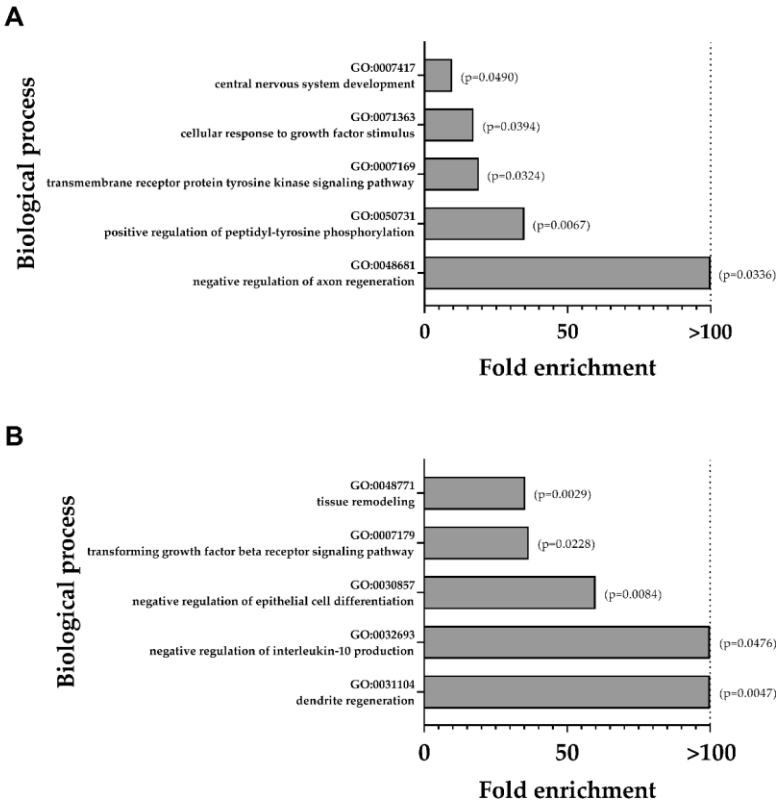

Figure 6. Enrichment analysis was performed using geneontology.org (GO); top 5 of the most strongly enriched biological processes are depicted for (A) App^{NL-G-F} knock-in versus C57BL/6J wild type mice and (B) hyperhomocysteinemic versus control App^{NL-G-F} knock-in mice; p values are expressed in brackets for each enriched biological process.

Some selected protein markers are depicted separately and highlighted in Figure 7, mainly because of large effect-sizes (cf. Table 2). Protein markers referring to neuronal function or (neuro-) inflammation were the focus of this study and proved to be de-regulated especially in CSF of KI control mice (e.g. ENO2, HGF, RGMA, TNR). With respect to a hyperhomocysteinemic impact, Figure 7 B equally highlights neuron and inflammation-related parameters – however, mostly in serum - but also parameters of relevance apart from the neurodegenerative context (e.g. TGFBR3), as discussed further below (see discussion, chapter 4.3.). Despite apparently strong effect sizes, we did not separately depict and further discuss IGSF3 due to a decreased number of observations and therefore, a higher level of uncertainty in the proteome analysis.

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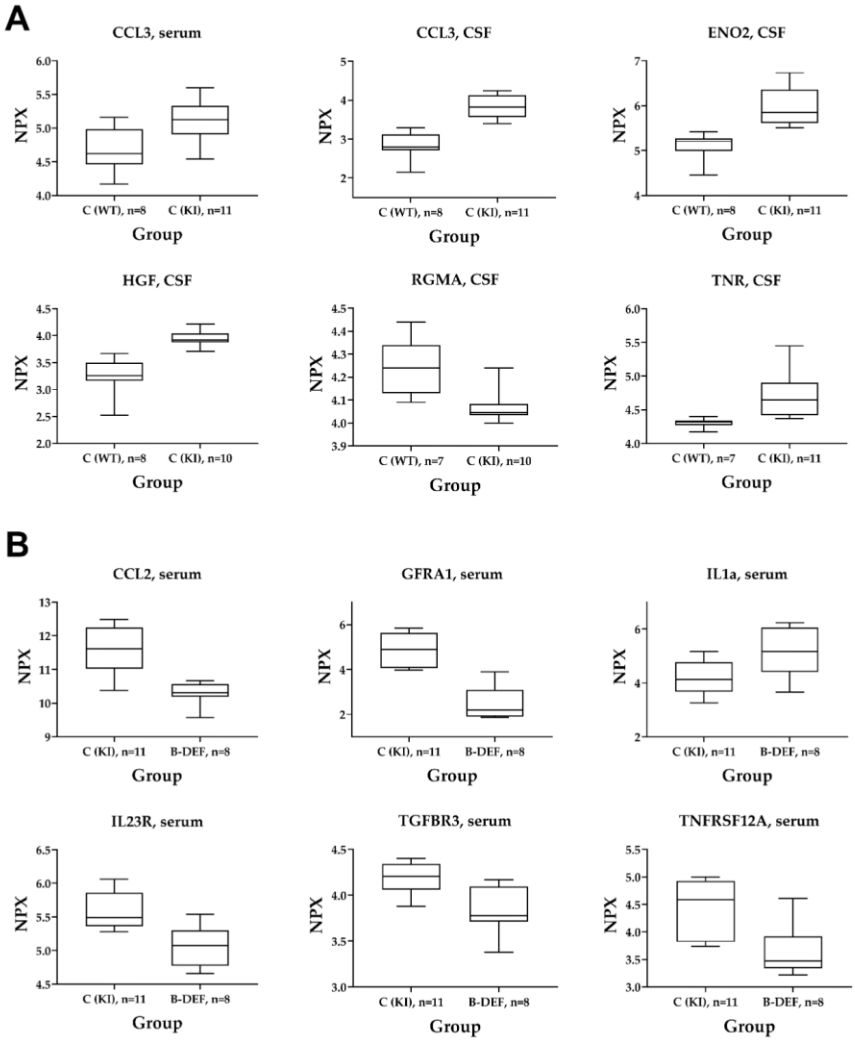

Figure 7. Selected protein biomarkers that were differentially regulated in (A) *App*$^{NL-G-F}$ knock-in versus C57BL/6J wild type mice and (B) hyperhomocysteinemic versus control *App*$^{NL-G-F}$ knock-in mice; age 67-68 weeks; differences in the protein quantities depicted here did all reach statistical significance (p values < 0.05); NPX (normalized protein expression) is an arbitrary unit on log2-scale; high NPX means high protein concentration.

3.6. Quantification of Aβ

Analysis of brain section homogenates using ELISA revealed cerebral Aβ42 levels that are depicted in Figure 8. The difference in Aβ levels between the different genotypes was highly significant (*p<0.001*), whereas none of the other groups showed a statistically significant difference in comparison to KI control mice.

Correlation analysis revealed positive correlation between cerebral Aβ42 and CSF protein markers, e.g. CCL3 (r_s*=0.476; p=0.001*), ENO2 (r_s*=0.505; p=0.001*), HGF (r_s*=0.507; p=0.001*) and TNR (r_s*=0.491; p=0.001*).

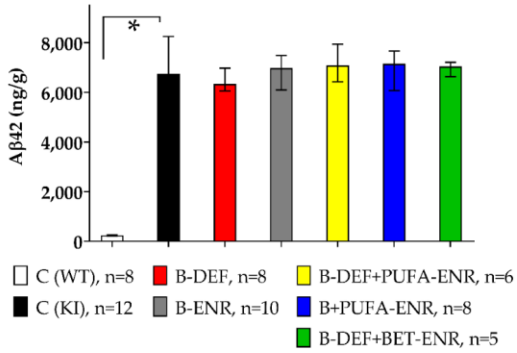

Figure 8. Total Aβ42 level (GuHCl-soluble) per gram wet brain tissue in a 100 mg cross-section; age 67-68 weeks; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

4. Discussion

The primary goal of this study was to examine the effects of long-term B-vitamin deficiency or dietary supplementation with specific micronutrients on cognitive performance in the *App*$^{NL-G-F}$ KI mouse model for AD. We were particularly interested in elucidating a potential aggravation of AD-like pathology by the amino acids HCys and HCA. Additionally to the phenotypic characterization of the mice using different behavioral tests, we performed analyses of serum HCys/HCA, hematological parameters and cerebral Aβ burden, as well as serum and CSF proteomics. A second aim was to further characterize the *App*$^{NL-G-F}$ model for AD. The results of the presented behavioral tests, as well as the assessment of plaque burden, mostly confirmed the findings of our previous in vivo study at a higher age of the animals in this long-term experiment. However, previous findings have been extended especially by the ex vivo experiments consisting of hematological and proteome analyses, but also by revelations through group and diet adaptions, as well as the findings of the in vitro assay performed in the presented study. These aspects are subsequently discussed in more detail.

4.1. Homocysteine and homocysteic acid

The in vitro results of the present study confirmed the hypothesis that HCys is actually less neurotoxic than its oxidative metabolite HCA. In both cases, marked cytotoxicity was detected at concentrations beyond 1 mM, which is higher than in vivo relevant levels of these compounds. However, these values in isolated cells might not be directly translatable to animal or human studies, but have to be discussed in the context of the setting of the in vitro assay. Firstly, despite using isolated primary rat cortical neurons that are biologically more relevant than artificial cell lines, it has to be emphasized that other types of neurons such as hippocampal neurons might have been more vulnerable to HCys and HCA than cortical neurons that were used in the present study [31,32]. Secondly, (higher) neurotoxicity of HCys and HCA may synergistically derive from a combination with hallmarks of AD, e.g. amyloid pathology [33], and vice versa, also HCys and HCA can potentially deteriorate toxicity of other AD hallmarks [34]. Thirdly, incubation of cells with the compounds occurred for merely 24 hours in this short-term assay and is, therefore, much shorter than chronic exposure in vivo. Moreover, neurotoxicity exerted by the NMDA receptor is dependent on the height of glycine levels, due to different HCys-binding sites at the NMDA receptor: higher glycine levels in the brain are suggested to correlate with higher HCys-driven NMDA agonism and consequently higher toxicity occurs already at lower HCys concentrations [35,36]. In accordance with our results, neurotoxicity of HCys has been reported > 1 mM, whereas HCA is supposed to be more potent [37]. We consequently also determined the level of HCA in addition to HCys in serum in the animals in the present in vivo study.

As revealed by LC-MS/MS analysis, HHCys was successfully induced in *App*$^{NL-G-F}$ mice by feeding a B-vitamin restricted diet (group 3), which was additionally supplemented with either PUFA (group

5) or betaine (group 7). The absence of vitamin B12 and folate is suggested to result in a disrupted remethylation cycle and chronically elevated serum HCys; additionally enhanced by the lack of vitamin B6, which an essential cofactor in the transsulfuration pathway of HCys [14]. As recently reviewed, one carbon metabolism disturbance, especially decreased S-adenosylmethionine and increased S-adenosylhomocysteine and concomitantly reduced methylation capacity, affects numerous metabolic pathways [14]. Epigenetic regulation, as a consequence of diminished supply with methylation groups, might also explain our findings in the present proteome analysis, i.e. the de-regulation of several protein biomarkers.

Dayal and Lentz hypothesized that long-term dietary treatment might be associated with secondary metabolic effects, leading to varying HCys levels between different sampling points throughout the study course [38]. In our study, variability between the time blocks might, to some extent, also result from an absent randomization of samples over the different LC-MS/MS runs during the analysis procedure. This was not possible, because of long-term sample storage duration, in this case due to the long-term study design. However, significant HHCys (groups 3; 5; 7) was clearly confirmed prior to each block of cognitive testing. In the current study, we were more interested in the comparison between different experimental groups at each particular time point than in longitudinal comparisons. No consistent impact on the height of HCys and HCA levels was detected, neither for the *App*$^{NL-G-F}$-related amyloid pathology nor for B-vitamin treatment. In the case of PUFA, researchers reported findings pro [39] and contra [40] beneficial effects on HCys levels. This effect proved to be absent in the present mouse study, as revealed by group 5 (hyperhomocysteinemic + PUFA-supplemented), which had been modified compared to our previous in vivo study [22]. With group 7 (hyperhomocysteinemic + betaine-supplemented), another adaption was implemented in the current study: we attempted to mitigate HHCys in a B-vitamin-independent manner in order to differentiate between potentially detrimental effects of HCys itself and the underlying B-vitamin deficiency. However, this question could not be answered, since serum HCys was not normalized by the betaine-supplemented diet, but elevated compared to *App*$^{NL-G-F}$ control animals. Interestingly, it has also been reported previously that betaine did not lead to a decrease in HCys or even further increased levels [41,42]. A reason for this might be reduced food intake by the mice due to a potential aversion against the betaine-containing chow and, as a consequence, even lower intake of dietary choline and additionally decreased methylation capacity. Overall, pathologically elevated levels of serum HCys and HCA severely affected the general health condition and resulted in decreased body weight, skin problems and a higher death rate in the respective groups of mice.

In hyperhomocysteinemic mice, erythrocyte-related parameters proved to be affected, as revealed by whole blood analysis. However, the question remains whether these findings resulted from elevated HCys or from the underlying deficiency in vitamin B6, B12 and folate, a lack of which is known to be responsible for the onset of anemia. Nevertheless, there are also hints that elevated levels of HCys itself might modulate severity of the reported effects. Mice of group 7 (hyperhomocysteinemic + betaine-supplemented), which showed the highest serum HCys of all three hyperhomocysteinemic groups (Figure 3), also expressed the most distinct effects on red blood cell-related parameters (Figure 5), although B-vitamin deficiency was not different for all three groups. Correlation analysis indicated (weak) negative correlation between HCys and hemoglobin and hematocrit, as well as positive correlation between HCys and red blood cell distribution width (RDW) according to a human study [43]. This potential contribution of HCys to vascular aspects of cognitive impairment is further addressed below in the proteomics section (see discussion, chapter 4.3.). The induced anemia and the consequent reduced supply of oxygen likely explains the poorer general condition of the hyperhomocysteinemic mice compared to control animals.

4.2. Aβ pathology and behavioral outcome

The results of the cerebral Aβ42 analysis indicated successful induction of the AD-like amyloid pathology in the *App*$^{NL-G-F}$ model, which furthermore, is in accordance with the immunohistochemical data of our former in vivo study [22]. Also in agreement with our previous study [22], cerebral amyloidosis was not affected by different dietary micronutrients or by elevated serum HCys/HCA due to vitamin-B deficiency. In contrast to our previous study, here, we measured both the insoluble and soluble portion of Aβ42, which is overexpressed in the *App*$^{NL-G-F}$ mouse model [12] and plays a major role in amyloid pathology [44]. Brain tissue levels of Aβ42 lay within ranges that have been reported for

App^{NL-G-F} in similar investigations [12,29], however, absolute values might not be exactly comparable with other trials, as different ELISA systems and different antibodies have been used. Also, antibodies may exhibit varying binding affinity in the case of the WT control group compared to the KI mice, which contained an altered Aβ sequence due to humanization and insertion of the Arctic mutation. Nevertheless, in the current study, an *absolute* quantification of Aβ was not required and the method adequately enabled semi-quantification of Aβ, i.e. a comparison between the different groups with the *App*$^{NL-G-F}$ control mice.

Despite massive cerebral amyloidosis, only one of three behavioral tests revealed a significant impact of the AβPP-based KI mouse model. This further fuels the controversial discussion about the role of Aβ in neurodegeneration, which is frequently debated [45]. The combination of disturbed Aβ metabolism with other typical hallmarks of AD, e.g. the characteristic Tau pathology, might model the complex pathology more comprehensively, as it has been attempted in older transgenic models such as the 3xTg-AD mouse model. However, it might be valuable to combine the versatility of such a model with the aforementioned advantages of the knock-in strategy in terms of model development.

The assessment of working memory performance in the Y-maze [46], was implemented in the current study in order to enable comparability with previous investigations, which indicated ambiguous findings in this test [12,47–51]. The apparent absence of an effect of the AD-like genotype in our KI mice might, however, be the result of the sub-optimal performance of the WT control mice in this test (approx. 55% spontaneous alternation versus approx. 70% in similar studies). In keeping with previous examinations, negative findings were obtained for spatial learning in the Barnes maze [52,53], whereas the open field test indicated *App*$^{NL-G-F}$-related deterioration of habituation learning (cf. [22]). Test dependency of genotype effects of *App*$^{NL-G-F}$ in behavioral tests has recently been emphasized [54]. Meanwhile, the originators of the model have developed a 3rd generation AD mouse model, attempting to increase plaque pathology and neuroinflammation [55].

Similar to our former in vivo study [22], long-term HHCys did not exhibit exacerbation of cognitive performance, despite of significantly elevated levels of HCys and HCA over the major part of the lives of the mice. Therefore, our data do not reinforce the "homocysteine hypothesis" – at least in the present mouse model for the familial AD. It has to be emphasized that most AD models, comprising also the *App*$^{NL-G-F}$ model, simulate the early-onset (familial) form of the disease and the very prominent and well-described hallmarks such as Aβ plaques and neurofibrillary tangles, whereas the more prevalent late-onset (sporadic) AD and the less-extensively investigated characteristics of the multi-facetted AD pathology might actually be the more valuable approach [45]. Polis and Samson entitled the late-onset form a "brain expression of a systemic complex metabolic disorder".

Micronutritional interventions comprising dietary supplementation with B-vitamins (B6, B12, folate), PUFA or betaine did not consistently improve learning and memory in the respective KI mice. The absence of B-vitamin effects is in accordance with a recent human trial [56]. Previous clinical and preclinical hints on potentially beneficial effects of betaine on cognition were, however, not confirmed in the disease model that we used [57,58]. As concluded by Zhao et al., findings on the role of betaine remain contradictory [59]. With respect to effects of PUFA, our data (groups 5 and 6) are in accordance with previous publications that indicated negative results [60–62] or laid emphasis on the limited or ambiguous level of evidence [63].In general, evidence on this topic is conflicting and equivocal, as elucidated previously [64–68]. Recently, we summarized available human and animal literature on C1 metabolic disturbances, especially the metabolic role and pathomechanisms of HHCys, in the context of cognitive decline [14].

As a limitation of the current study, significant but inconsistent dietary effects in behavioral tests, which are typically associated with variability to some extent, might in part be explained by decreased statistical power due to the death of several animals as a consequence of the long study duration and severe dietary restrictions.

4.3. Olink proteomics

Of the 92 protein biomarkers in the mouse exploratory kit, many proved to be significantly affected by either AD-like pathology or comorbid amyloidosis with HHCys. In terms of enrichment analysis, it has to be emphasized that no "weighting" of the involved markers occurred at this stage, meaning that all proteins were included and treated equally in the GO analysis, independent of effect size or sampling

matrix. As the GO analysis is purely an association analysis, the following section discusses effects of the individual de-regulated protein markers on related biological processes.

Analysis of CSF, which is a relevant matrix in terms of neurodegenerative processes, mainly revealed effects that were related to the *App*$^{NL-G-F}$ genotype rather than to HHCys and, therefore, indicated an impact of the AβPP-based AD mouse model. This is in accordance with our findings in the open field test, where KI mice showed impaired habituation learning. Particularly neuronal markers were de-regulated with large effect-sizes, such as HGF, ENO2 or TNR. The upregulation of HGF, for instance, is translationally relevant, since HGF has also been described to be elevated in AD patients [69,70]. It functions as a nerve repair-promoting growth factor [71] and is therefore, most probably, up-regulated as a consequence of neuronal damage due to cerebral amyloidosis in AD patients or in the animal model. Similarly, the neurotrophic factor ENO2, a marker for neuronal damage, has been related to neuroinflammation and neurodegeneration [72]. The function of TNR, another neuronal marker that proved to be up-regulated in *App*$^{NL-G-F}$ mice, is strongly dependent on interacting molecules [73]. Particularly in combination with CNTN1, which was also elevated in CSF, it is known to inhibit neurite outgrowth [74,75]. Complex and opposite effects on regulation of neuronal survival have been proposed to be exerted by RGMA, as reviewed recently [76]. The down-regulation of RGMA might be recognized as an attempt to repair the induced brain damage [77], as described above for HGF and ENO2. De-regulation of other markers at a lower effect-size was found in the serum proteome. Hence, markers (ERBB4, NOTCH3, GFRA1, VEGFD) that have also been associated with neuronal maintenance [78–80], are probably of less relevance than the aforementioned CSF markers, at least in this mouse model. However, particularly VEGFD has previously been described as an inflammatory serum biomarker that correlates with AD [81]. Furthermore, up-regulation of the pro-inflammatory chemokine CCL3 was detected in both CSF and serum of *App*$^{NL-G-F}$ compared to WT mice. CCL3 is said to exert neuronal damage and deleterious effects on synaptic plasticity and learning performance by contributing to CNS inflammation by promoting migration of T-lymphocytes into the brain [82–84]. The induction of (neuro-) inflammatory processes is one hallmark of the AD-like pathology in *App*$^{NL-G-F}$ KI mice, as summarized by the developers of the model [13]. Interestingly, we also found a positive correlation between cerebral amyloidosis and CSF markers such as CCL3, ENO2, HGF and TNR.

On the other hand, the absence of findings in CSF of hyperhomocysteinemic KI mice - especially of pronounced changes in neuronal parameters - might explain the lack of cognitive aggravation in all three behavioral tests. Elevated HCys, however, proved to de-regulate various proteins in serum of the animals. Serum levels of different markers related to neuronal functionality were down-regulated, such as GFRA1, MATN2 or the two members of the tumor necrosis factor receptor superfamily TNFRSF12A and FAS [85–88]. In contrast, a decreased serum concentration of LGMN was detected with the opposite result compared to the above proteins, i.e. deletion of LGMN is associated with the alleviation of synapse loss [89]. However, a recent human trial did not confirm previous reports, according to which LGMN was a driver of the process of AD development [90]. Interestingly, the present proteome analysis revealed that (neuro-) inflammatory pathways were affected by HHCys in both directions: aside from the elevation of pro-inflammatory IL1a serum level [91], concentrations of other protein markers were decreased in comparison to KI control mice (IL23R, CCL2, CXCL9, S100A4, WISP1, EDA2R), indicating also anti-inflammatory effects [84,92–95]. In other studies using different animal models, researchers found HCys-related elevations of mainly pro-inflammatory markers [96–98]. Rarely, anti-inflammatory effects or no effects on inflammation parameters at all have also been reported for HHCys [99,100]. It is obvious that the studies are not completely comparable, since HHCys-induction varies between genetic, parenteral and dietary methods, as well as between different durations of assessment. In terms of neuroinflammation, glial cells, particularly microglia, play an important role as key regulators [13]. Prominent glia-related proteins such as TREM2 or ApoE that are frequently discussed in the context of AD pathology, were not part of the current proteome analysis, which therefore is a limitation of this study. Here, we cannot comprehensively review the complex interactions and all functions of the single inflammation markers in-depth. However, in sum, the absence of phenotypic impairment of the outcome of the behavioral tests might be explained by a potential abrogation of the inflammation-related effects, which are in part mutually counteractive and might also derive from an adaptive response of the organism to restore homeostasis.

As another HHCys-related effect, reduction in angiogenesis results from the down-regulation of pro-angiogenic factors in serum (FAS, S100A4, TNFRSF12A and TGFBR3; [93,101–103]) and CSF (DLK1

and ACVRL1; [104,105]), which might display an interesting mechanistic aspect in terms of vascular contribution to cognitive decline and dementia. HCys is known to exhibit a vascular impact, i.e. multiple deleterious effects on endothelial cells [14]. Endothelial dysfunction is, in turn, considered a contributor to oxidative stress in the brain, chronic cerebral hypoperfusion and AD development [45]. As accordingly suggested in a recent meta-analysis, HCys plays a more important role in vascular dementia than in "pure" AD [67]. In consideration of our hematological data, effects are probably aggravated as a result of the combination with reduced hemoglobin in the hyperhomocysteinemic mice, due to which neuronal exposure to oxygen is additionally diminished. Although it was apparently not sufficient to trigger phenotypic impairment in this study, it has previously been hypothesized that decreased capillary density in the brain and, therefore, decreased cerebral blood flow and supply of oxygen and glucose, contributes to the impairment of synaptic function and cognitive performance in elderly people as well as AD patients [106,107]. Ambrose considered this approach underrated and it should be regarded, not in contrast to, but in addition to the amyloid hypothesis.

Apart from the actual research question of the current analysis, the detected combination of de-regulated biomarkers also indicates other effects of HHCys. For instance, down-regulation of TNFRSF11B, TGFBR3, FSTL3 and WISP1 and up-regulation of IL1a in serum are associated with diminished bone mineralization, decreased bone anabolism or increased bone catabolism [108–112]. Accordingly, HCys has previously been suggested to be involved in osteoporosis-like pathology in patients, as reviewed earlier [113]. A potentially lighter skeletal structure, besides reducing appetite due to B-vitamin deficiency, is in accordance with the lower weights of the hyperhomocysteinemic mice in our study.

For both group comparisons, proteome analysis revealed de-regulation of some neuron-related markers such as TPP1, ERBB4, NOTCH3, ENO2, RGMA, GFRA1 or DLL1, which are associated with developmental processes [79,80,114–118]. As the mice were already about 70 weeks old when we performed the analysis, neuronal developmental processes most probably did not play an important role any more, although neurogenesis proceeds to some extent in specific brain regions also in adult individuals [119]. For that reason, other functions of these markers should probably be considered more relevant in older mice. In the case of the remaining de-regulated markers, neither initial literature research using the uniprot database ([120]; https://www.uniprot.org/) nor enrichment analysis indicated a direct connection with neuronal maintenance function or (neuro-) inflammation at all (EPCAM, VSIG2, FST, CLMP).

A limitation of the present study setting relates to the HCys-elevation method we applied, meaning that the extent to which the observed effects are potentially biased by the dietary B-vitamin deficiency cannot be conclusively determined. Nevertheless, an advantage with the explorative setup of the Olink mouse panel is to highlight topics that were originally not the focus of the study and may open up new research ideas.

5. Conclusions

In this study, the induction of in vivo disease models for AD-like pathology and HHCys have revealed findings that confirm and extend the results of our previous animal study. The amyloid-based *App*$^{NL-G-F}$ KI model proved to be associated with subtle changes in the present cognitive assessment, despite massive amyloidosis in the brain. This further challenges the role of Aβ plaque deposition as the sole or main culprit for AD, according to the frequently discussed amyloid hypothesis. A combination with additional characteristic AD hallmarks, such as Tau pathology, might also be a valuable approach in the context of knock-in mouse model development. Placing our findings in the light of the literature on HHCys, which is heterogeneous and in parts equivocal, it must be emphasized that elevated serum HCys and HCA did not cause aggravation of the induced amyloid pathology in this mouse model of familial AD. Although HHCys was not associated with impaired cognitive performance, a significant impact on erythrocyte-related parameters and angiogenesis was found, potentially indicating a mainly vascular contribution of HCys to cognitive decline by impairing the supply of neurons with oxygen and nutrients. Dietary supplementation with different micronutrients did not reveal improvement compared to *App*$^{NL-G-F}$ control mice in terms of Aβ burden or behavioral outcome and therefore, did not indicate prevention of cognitive decline in this AD mouse model. Our data on the explorative Olink mouse panel further characterize the *App*$^{NL-G-F}$ model and clarify to some extent the role of HCys in diverse biological processes. Therefore, proteome analysis data might present

a springboard for subsequent investigations and should be validated in other preclinical models and/or human trials.

Acknowledgments: We are very grateful to MEDICE Arzneimittel Pütter GmbH & Co. KG for funding the present in vivo study. We also wish to thank RIKEN Center for Brain Science for providing the App^{NL-G-F} knock-in mouse model.

Appendix A

Barnes maze data

In the first part of the Barnes maze test (acquisition phase), spatial learning and memory, expressed as the latency the mice needed to locate the target hole, was tested twice a day, with a short interim period of 15 minutes. Mean values are illustrated for each of the four training days in Figure A 1. Testing for statistically significant effects, which was performed on day 4, did not reveal differences between experimental groups. After applying reversal settings in time block 2 and 3, we additionally evaluated latencies to the previous target position on day 1 each time in order to examine potential differences in long-term memory of the mice to their respective target at former time points. However, no effects were detected for any group or time block (data not shown).

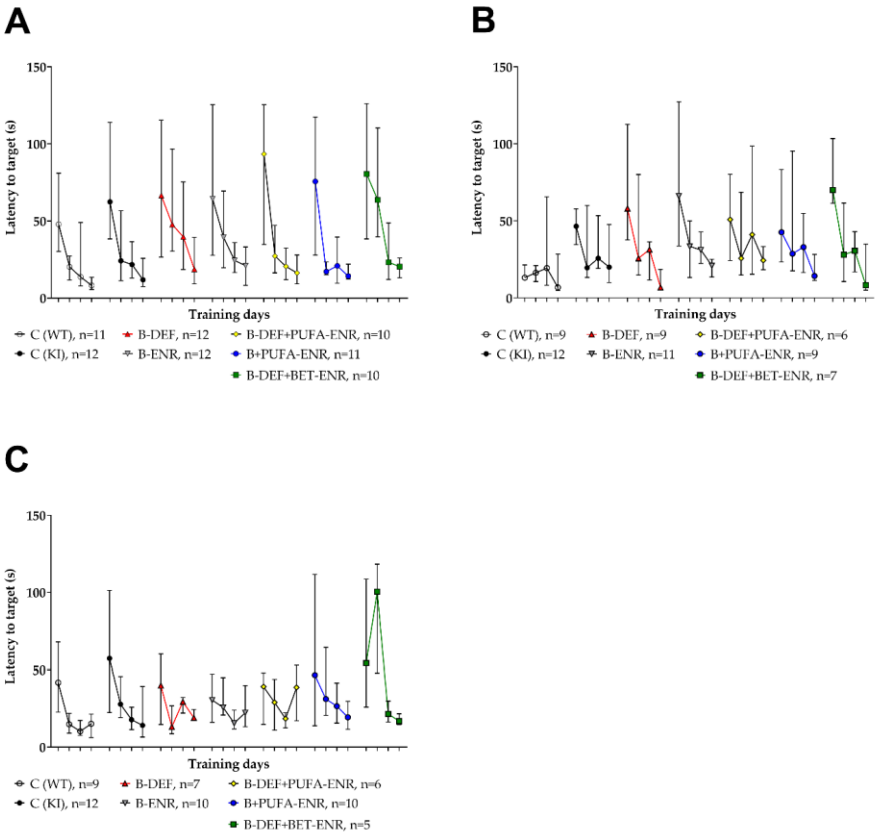

Figure A 1. Barnes maze acquisition phase: (A-C) latency to target hole in time block 1-3 (4 subsequent days of training each time: average of 2 sessions per day); statistical testing for differences on day 4; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

In the second part of the test (probe trial, day 5), memory performance was tested by measuring the latency to the target hole in all three time blocks (Figure A 2 A; C; E). Additionally, the time the mice spent in the target quadrant of the Barnes maze arena was determined (Figure A 2 B; D; F). In time block 2, hyperhomocysteinemic mice supplemented with PUFA needed significantly longer to reach the target position that had previously been learned in the acquisition phase (*p=0.013*). However, this finding might be biased by an unknown factor, since the effect was not consistently detected in the other time blocks and therefore, appears to be biologically irrelevant. An impact of the AD-like pathology or other dietary interventions on spatial memory was not observed in this test. Also, the cumulative time at target, meaning the persistence of a mouse at the position where the escape box had been located during the acquisition phase, did not significantly differ between the groups.

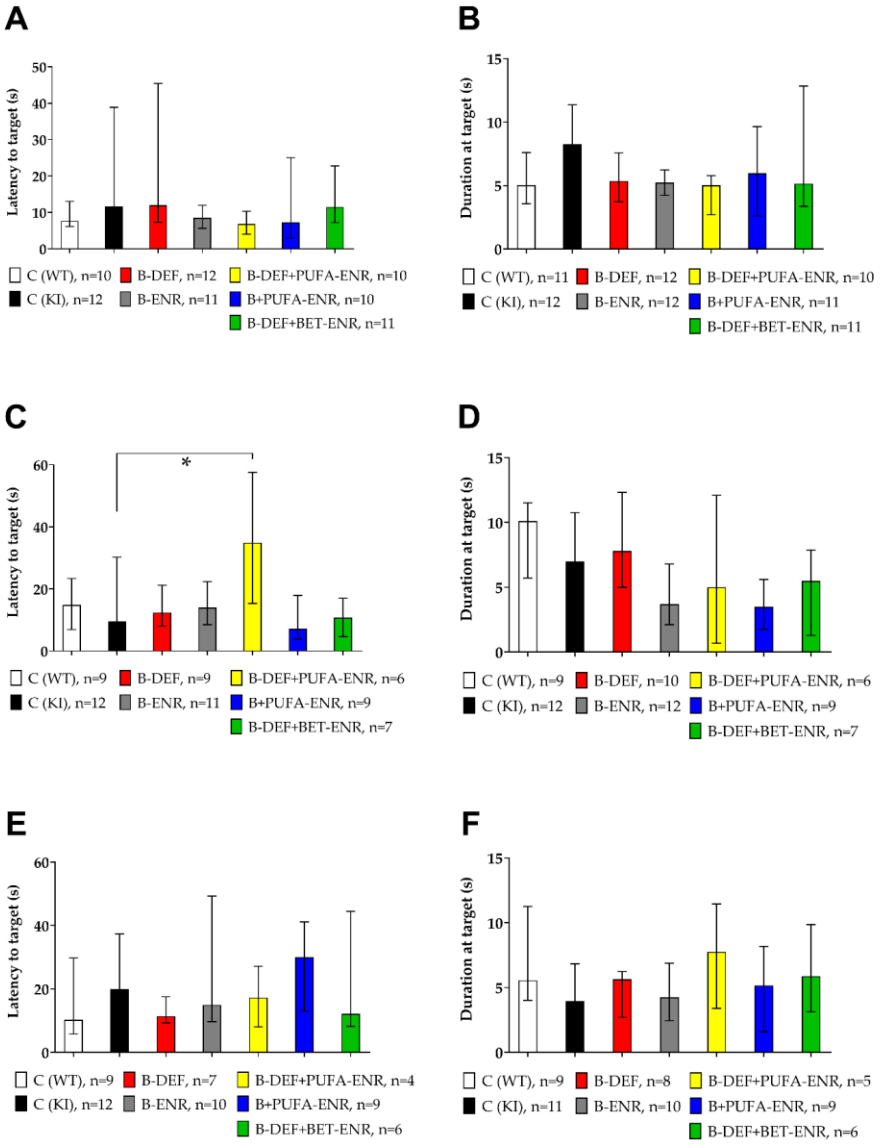

Figure A 2. Barnes maze probe trial in time block 1-3: (A; C; E) latency to target hole and (B; D; F) cumulative duration at target hole; median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

Y-maze data

Working memory was assessed in the Y-maze (Figure A 3) by measuring the spontaneous alternation of the mice, a natural behavior that is hypothesized to be disrupted as a consequence of cognitive deterioration (Equation 2). In time block 2, for unknown reasons, the KI control mice performed poorly, with the result that comparisons with some of the dietary treatment groups became

significant (*B-DEF p=0.040; B-ENR p=0.028; B-DEF+BET-ENR p=0.026*). It has to be emphasized that these effects were not sustained and should therefore not be overestimated. Overall, no consistent effects were related to either the AD-like genotype or treatment with different experimental diets.

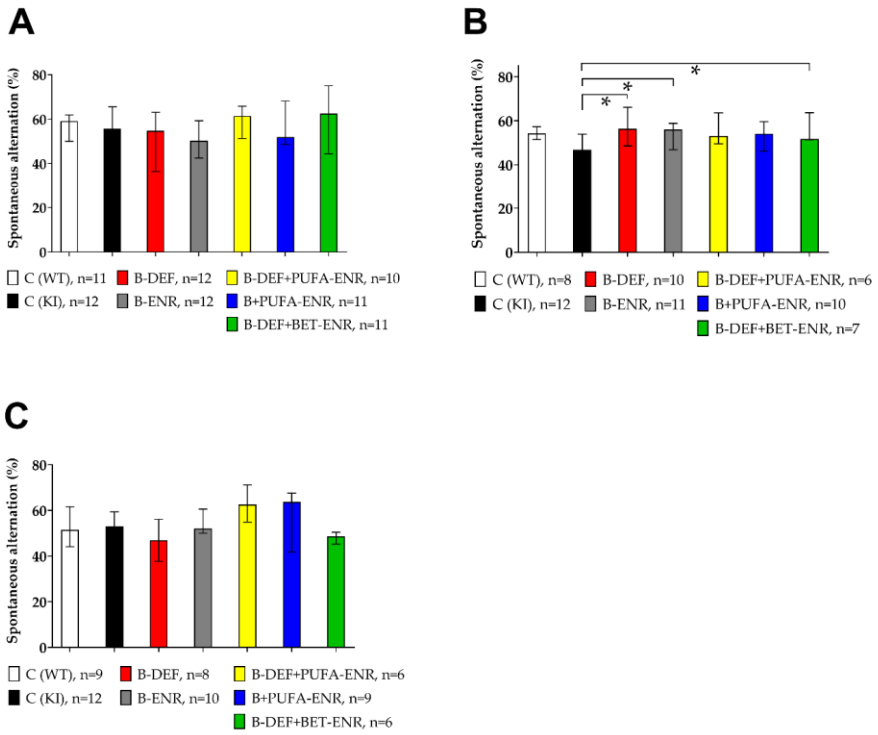

Figure A 3. Y-maze, spontaneous alternation rate in time block 1-3 (A-C); median ± IQR; outliers beyond threefold IQR removed; $p < 0.05$ (Mann-Whitney-U-test) considered statistically significant (*).

Appendix table

Table A 1. Experimental diets.

	Control	B-DEF	B-ENR	B-DEF+PUFA-ENR	B+PUFA-ENR	B-DEF+BET-ENR
Casein	140.0	140.0	140.0	140.0	140.0	140.0
Corn starch	355.6575	345.6920	355.4795	345.6920	355.4795	335.6920
Maltodextrin	155.0	155.0	155.0	155.0	155.0	155.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Dextrose	100.0	100.0	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Mineral premix	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin pre-mix (w/o B-vitamins)	10.0	10.0	10.0	10.0	10.0	10.0
Soybean oil	19.0	19.0	19.0	– –	– –	19.0
Coconut oil	9.0	9.0	9.0	11.3	11.3	9.0
Corn oil	22.0	22.0	22.0	18.7	18.7	22.0

Fish oil (eicosapentaenoic acid/docosahexaenoic acid = 1:4)	−−	−−	−−	20.0	20.0	−−
L-Cystine	1.8000	1.8000	1.8000	1.8000	1.8000	1.8000
Tert-butylhydroquinone	0.0080	0.0080	0.0080	0.0080	0.0080	0.0080
Choline bitartrate, 41%	2.5000	2.5000	2.5000	2.5000	2.5000	2.5000
Pyridoxine-HCl (Vit. B6)	0.0070	−−	0.1000	−−	0.1000	−−
Cyanocobalamin, 0.1% (Vit. B12)	0.0250	−−	0.1000	−−	0.1000	−−
Folic acid, 80%	0.0025	−−	0.0125	−−	0.0125	−−
Sulfathiazole sodium	−−	10.0000	−−	10.0000	−−	10.0000
Betaine	−−	−−	−−	−−	−−	10.0000
Sum	1000	1000	1000	1000	1000	1000

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