KU Leuven
Biomedical Sciences Group
Faculty of Medicine
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EXPLORATION OF THE ROLE OF EPHA4 IN NEURODEGENERATIVE DISEASES

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A word of Thank

"My life story is the story of everyone I met"

Jonahan Safran Foer, Extremely loud and incredibly close

Zoals één van mijn favoriete auteurs het al zo correct heeft beschreven, deze laatste acht jaar zouden niet hetzelfde zijn geweest zonder al de fantastische en inspirerende mensen die ik heb ontmoet. Ook al was deze periode gevuld met de typerende 'ups & downs' er stonden altijd mensen klaar voor wetenschappelijk advies, een bemoedigend woord, en een leuk gesprek. Dankzij hen sta ik hier vandaag, en zal ik me de laatste jaren herinneren als jaren boordevol wetenschappelijke en persoonlijke groei, fijne momenten en nieuwe vriendschappen. Ik zou dan ook graag de volgende pagina's aan hen willen opdragen.

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I wish you all the best,

Lindsay

Table of Contents

Abbreviations

Ach	Acetylcholine
AchE	Acetylcholine esterase
AchR	Acetylcholine receptor
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ASO	Antisense oligonucleotide
Aβ	Amyloid-β
AβO	Amyloid-β oligomer
Camk2a	Calcium/calmodulin-dependent protein kinase 2 alpha
CNS	Central nervous system
CPG	Central pattern generator
CRD	Cysteine-rich domain
CST	Corticospinal tract
Ctrl	Control
DlgA	Drosophila discs-large imaginal disc protein
EGF	Epidermal growth factor
EICD	EphA4-intracellular domain
Eph	Human erythropoietin-producing hepatocellular
FN	Fibronectin
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GPI	Glycosyl phosphatidylinositol
GWAS	Genome-wide association study
HLS	Hindlimb suspension test
hnRNP R	Heterogeneous ribonucleoprotein R
Iba1	Ionized calciumbinding adaptor protein 1
IPSC	Induced pluripotent stem cells
IR	Insulin receptor

LBD	Ligand binding domain
LTD	Long term depression
LTP	Long term potentiation
MCI	Mild cognitive impairment
MS	Multiple sclerosis
MWM	Morris water maze
NCALD	Neurocalcin delta
Nck	Non-catalytic region of tyrosine kinase adaptor protein
NF	Neurofilament
NFT	Neurofibrillary tangle
NMJ	Neuromuscular junction
NO	Nitric oxide
NogoR	Nogo receptor
$P75^{NTR}$	P75 neurothropin receptor
PDZ	Drosophila discs-large imaginal disc protein (DlgA) ZO-1 tight junction
PLS3	Plastin-3
PND	Postnatal day
PrPCR	Cellular prion protein receptor
PRS	Polygenic risk score
PSD-95	Post-synaptic density protein 95
PSEN	Presenilin
ROS	Reactive oxygen species
RTK	Transmembrane tyrosine kinase
SAM	Sterile alpha motif
SCI	Spinal cord injury
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron
SNP	Single nucleotide polymorphism
snRNP	Small nuclear ribonucleoprotein
SORLA	Sortilin-related receptor with LDLR class A repeats
SPSN	Sociability/preference for social novelty
SR	Stratum radiatum
TBI	Traumatic brain injury
ThioS	Thioflavin S

TREM2	Triggering receptor expressed on myeloid cells 2
TrkB	Tropomyosin receptor kinase B
tSC	terminal Schwann cell

Chapter I: Introduction

1. Spinal muscular atrophy (SMA)

1.1. A brief history of SMA

In 1891, the Viennese neurologist Guido Werdnig published a report on two brothers with a remarkable combination of pronounced proximal muscle weakness starting around the age of 10 months, delayed motor development with inability to stand or walk and premature death in early childhood (1). Two years later, the German neurologist Johan Hoffmann described seven additional cases with similar symptoms (2). Autopsy revealed degeneration of the anterior horns of the spinal cord and atrophy of the ventral roots and muscles, and these findings resulted in the name of the disease that is still used today: spinal muscular atrophy (1,2).

In general, spinal muscular atrophy is characterized by the degeneration of motor neurons of the spinal cord, resulting in hypotonia, progressive muscle weakness and atrophy, and in the most severe types, paralysis, respiratory failure and death. Muscle weakness is symmetric and proximal muscles are predominantly affected, with additional weakness of axial, intercostal and bulbar musculature (3). As the clinical presentation is very heterogeneous, a classification system based on age of onset and achieved motor milestones was formalized in 1991, including the infantile (type I), juvenile (type II) and adult (type III) forms of SMA (4) (Table 1). A few years later, after identification of the SMA locus, this scheme was modified with the addition of a severe prenatal type 0 and a late-onset type IV (3,5) (Table 1). SMA type 0 is a very severe form with prenatal onset, reduced or absent movements, contractures, and difficulties in swallowing and respiratory failure. Neonates are usually in need of mechanical ventilation support and the disease is fatal before six months of age. In contrast, SMA type IV is at the other side of the spectrum, and is a very mild form with first symptoms in adulthood and a normal life expectancy (3,5).

Table 1 – Classification criteria for spinal muscular atrophy (based on (3,6)**)**

SMA type	Age at onset	Highest function achieved	Natural age of death	SMN2 copy number
Type 0	Birth	Needs respiratory support	< 6 months	1
Type I (severe, Werdnig-Hoffmann disease)	0-6 months	Never sits	<2years	2
Type II (intermediate)	7-18 months	Sits, but never stands	>2years	3
Type III (mild, Kugelberg-Welander disease)	>18 months	Stands and walks	Adult	3-4
Type IV (adult)	Second or third decade	Walks during adulthood	Adult	4-8

SMA is the leading genetic cause of children lethality (3). To date, only a few studies have been performed to investigate the prevalence and incidence of SMA. In an overview of these studies, the incidence is estimated to be around 10 in 100 000 live births, and a prevalence of around 1-2 per 100 000 persons is observed. SMA type I is the most common form, accounting for around 60% of the cases (7).

1.2. Genetics

Already in the first reports, segregation of the disease in families was observed to be in an autosomal recessive manner (1,2). Nevertheless, it took a century to identify the survival motor neuron 1 (*SMN1*) gene on chromosome 5q13.2 as the most important disease-causing gene (8). SMN is ubiquitously expressed and is involved in many important neuronal pathways, including RNA metabolism, actin cytoskeleton dynamics, axonal RNA transport and synaptic vesicle release (9). The majority of SMA patients (92%) have a homozygous deletion of the *SMN1* gene, while in 4% of the patients small loss-of-function mutations are found, usually in combination with an *SMN1* deletion on the other allele (8,10). In rare cases, SMA is caused by mutations in other genes (11).

Humans have one or several copies of a second duplicated gene, the *SMN2* gene. Inefficient splicing due to a single nucleotide substitution in exon 7 of this gene predominantly causes the formation of an unstable, truncated SMN protein lacking exon 7 (SMNΔ7). Full-length SMN protein is only produced at 10 % of the levels encoded by *SMN1* (12,13) (Fig. 1A). These low levels of SMN are enough to prevent lethality, but are not sufficient to compensate for the loss of SMN1. As SMA patients rely on *SMN2* for production of SMN protein, the amount of *SMN2* gene copies is the most important modifying factor for clinical severity. Lower copy numbers are usually found in severe types of SMA, while higher numbers are present in patients with milder forms (6,14,15) (Table 1, Fig. 1B). In addition other (epi)genetic modifiers of the disease exist. A rare single nucleotide polymorphism (SNP) in exon 7 of the SMN2 gene has been described which alters splicing and results in increased exon 7 inclusion (16,17). Moreover, increased levels of Plastin-3 (PLS3) and decreased levels of neurocalcin delta (NCALD) in patients may be protective, possibly via restoration of neuromuscular junction (NMJ) functionality (18–22).

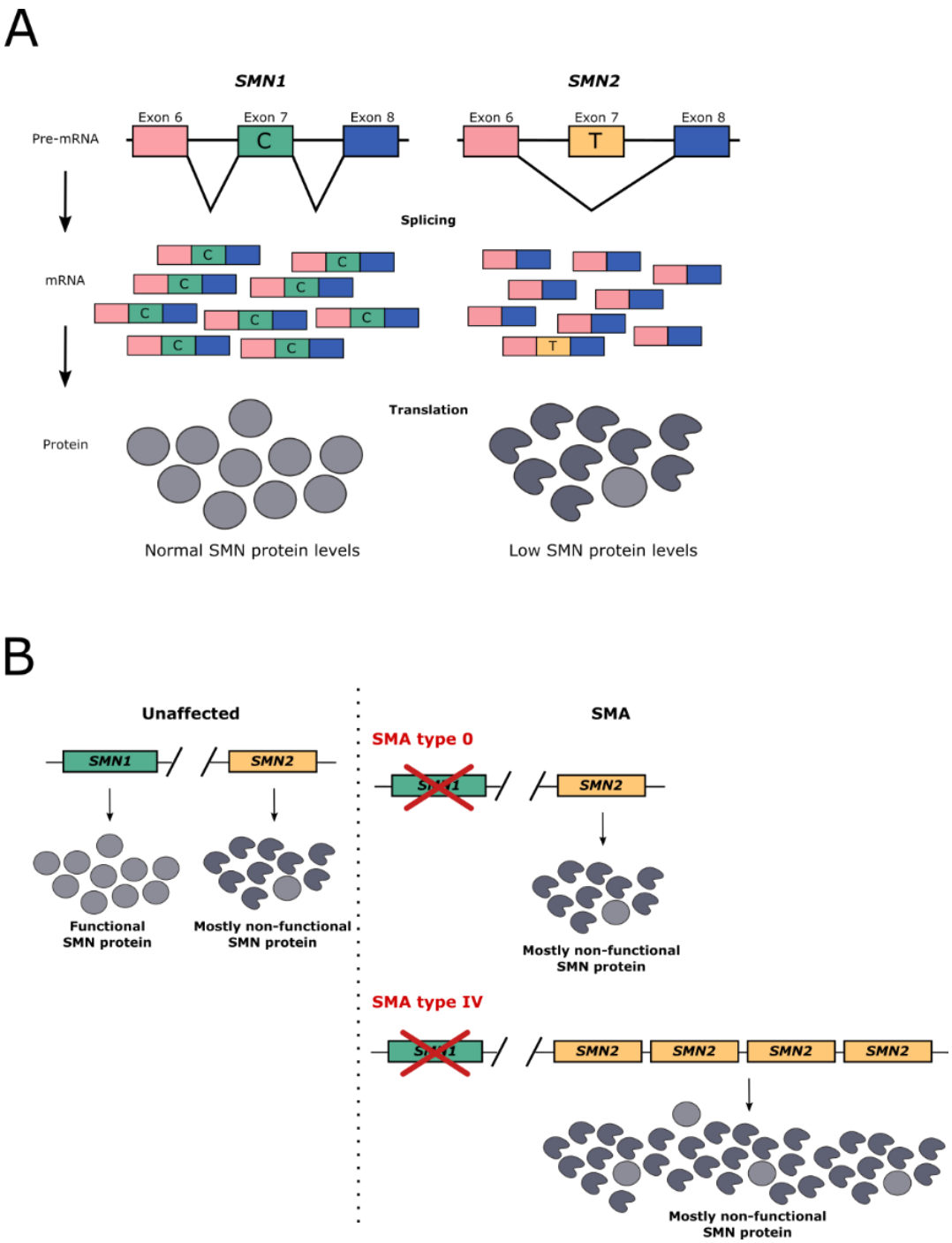

Figure 1 – Genetics of SMA. (A) A single nucleotide change (C-to-T) in exon 7 of *SMN2* disrupts the exonic splicing enhancer (ESE) sequence responsible for inclusion of exon 7 in SMN transcripts. As a result, splicing of *SMN2* is affected and exon 7 is excluded from the majority (~90%) of SMN2-derived mRNA's. A highly unstable, truncated SMNΔ7 protein is produced which is rapidly degraded. However, unaffected SMN2-derived mRNA's still produce small amounts of full-length SMN. (B) Loss of *SMN1* in SMA patients results in reduced SMN levels in most tissues. The number of *SMN2* gene copies determines the residual amount of functional SMN protein and is the most important modifying factor for clinical severity. In general, type 0 SMA patients present with only one copy of *SMN2*, resulting in low levels of functional SMN protein and a severe phenotype. In contrast, type IV SMA patients are expected to have at least 4 copies of *SMN2*, high levels of functional SMN protein and a milder form of the disease. Abbreviations: SMA; spinal muscular atrophy, SMN; survival of motor neuron. Figure based on (23).

1.3. Pathophysiology of SMA

1.3.1. How does a reduction in SMN levels cause specific motor neuron death?

The SMN protein is a 38 kDa protein found in the cytoplasm and nucleus of all cells, raising the intriguing and still unanswered question why motor neurons are (almost) selectively vulnerable to SMN deficiency (24). SMN contributes to numerous cellular processes and pathways, of which the most established function is the regulation of small nuclear ribonucleoprotein (snRNP) biogenesis in the nucleus. SnRNP's are key constituents of the spliceosome that are essential for pre-mRNA processing (25). Hence, SMN deficiency affects snRNP assembly, preferentially in a subset of snRNP's, and pre-mRNA processing, and these defects correlate with severity in mouse models for SMA (26,27). The identification of a number of affected genes involved in synaptic function and NMJ maturation, such as Stasimon and Z+ agrin, could link perturbations in pre-mRNA splicing with the motor system specific pathology in SMA (28,29). However, splicing defects occur late in mouse models and are minor in type I SMA patients, suggesting a role for other functions of SMN in SMA pathogenesis (30,31).

First indications for additional functions of SMN was its localization in dendrites and axons of the developing spinal cord. SMN expression is present in growth cones of developing axons *in vitro*, and in axons and pre-synaptic terminals at the NMJ in developing and adult mice (32–34). As SMA binds heterogeneous ribonucleoprotein R (hnRNP R), HuD and several other mRNA binding proteins, SMA may be involved in the recruitment and transport of RNA into axons and axon terminals (32,35). Indeed SMN knockdown in primary motor neurons reduces mRNA localization in the axonal compartment (35). In addition, SMN has roles in other neuronal processes, such as endocytosis at the NMJ and cytoskeletal dynamics during axonal growth (36–41). Given the multiple roles of SMN, reduction of this protein can affect multiple pathways and functions in cells. Which aberrations are causing the observed motor neuron death in SMA remains largely unknown.

1.3.2. The role of neuromuscular junction dysfunction in SMA

Although it is not clear how loss of SMN causes motor neuron death, increasing evidence suggest a key role for NMJ dysfunctions in the pathogenesis of SMA. Neuromuscular junctions are highly specialized synapses consisting of a pre-synaptic motor neuron axon, a post-synaptic muscle fiber and supporting terminal Schwann cells (tSC's). Motor neuron terminals release acetylcholine (ACh), which activates nicotinic Ach receptors (AChR's) on the muscle fibers and induces muscle contraction. The formation of the neuromuscular junction is a multi-step process. First, small and primitive AChR clusters are formed in the central regions of the myofibers after myogenesis. This process is called pre-patterning and is independent of motor neuron innervation. Once the motor neuron axons arrive in the muscle,

connections with AChR's are formed and non-innervated clusters are dispersed. Finally, during postnatal maturation, plaque-like AChR clusters will convert into pretzel-like shaped AChR clusters and the embryonic γ-subunit of AChR is replaced by the adult ε-subunit. In addition, a specialized post-synaptic composition of proteins and signaling molecules is formed and the cytoskeleton is reorganized. At the pre-synaptic level, poly-innervation of neuromuscular junctions is reduced to mono-innervation via axon withdrawal. Axon terminals differentiate and become perfectly aligned with the AChR clusters and synaptic vesicles containing neurotransmitters are concentrated at the active zone. TSC's are suggested to be indispensable during neuromuscular junction formation, maturation and maintenance (42,43).

In mouse models, several studies identified progressive abnormalities at the NMJ's as the earliest detectable consequence of reduced SMN protein. Pre-synaptically, neurofilament (NF) forms aberrant accumulations in pre-terminal axons and nerve terminals, probably resulting in terminal axonal outgrowth defects as suggested by the poor terminal arborization and, in severe models, axonal sprouting deficits (44–46). Post-synaptically, endplates appear small and immature with plaque-like acetylcholine receptor (AchR) clusters instead of adult perforated pretzel-shaped clusters (44,47,48). In addition, replacement of the foetal γ-subunit by the adult ε-subunit of the AchR's is delayed in several muscles (44,47). The number of terminal Schwann cells tSC's at the NMJ is reduced, resulting in impaired covering of the NMJ (49). These structural defects functionally impair and compromise synaptic transmission, such as synaptic vesicle release (44,47,50) (Fig. 2). Together, these abnormalities suggest an impaired maturation of the NMJ, of which the occurrence and intensity highly correlate with the phenotypic severity of the studied mouse model (44,51). As similar structural and functional defects are also present in patients and in NMJ-like structures generated from patient-derived induced pluripotent stem cells (IPSC's), the view of SMA as a NMJ synaptopathy emerges (44,52–56).

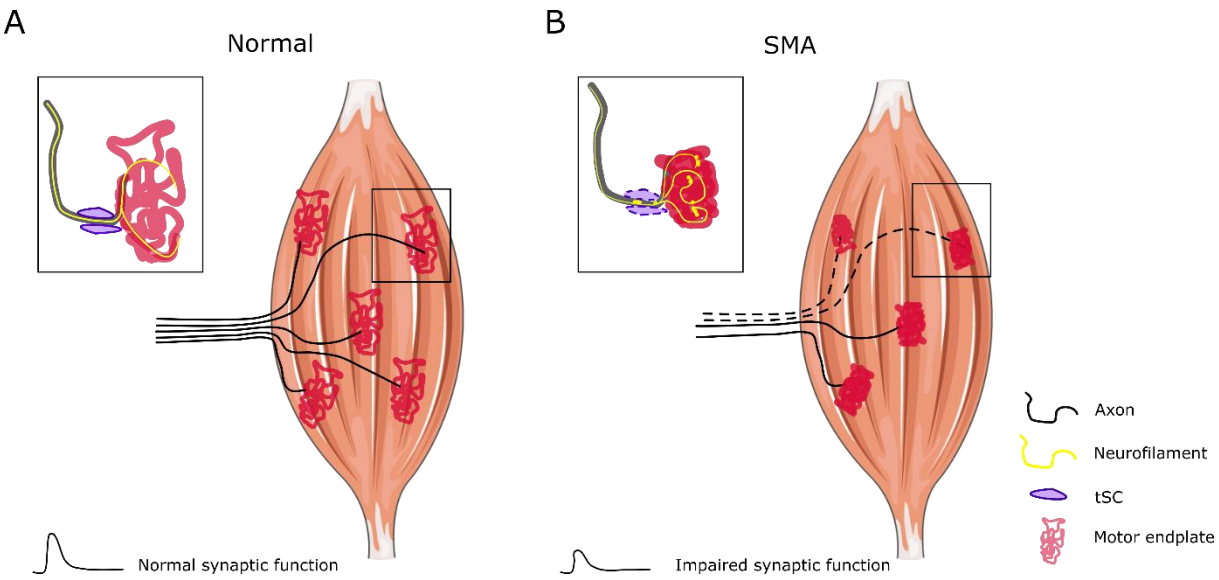

Figure 2 – Overview of NMJ abnormalities in SMA. (A) In normal conditions, NMJ's are mature with a pretzel-like morphology of the motor endplates and AChR's with adult ε-subunits. In addition, NMJ's are innervated by NF-positive axons and normal synaptic activity occurs. (B) In SMA, motor endplates are small and immature with a plaque-like morphology and AChR's expressing foetal γ-subunits. Pre-synaptical NF accumulations pile up in the pre-terminal axons and nerve terminals, ensuing in poor terminal arborization. As a result, synaptic function is impaired and axons degenerate. Abbreviations: NMJ; neuromuscular junction, AChR; acetylcholine receptor, NF; neurofilament, tSC; terminal Schwann cell. Figure based on (51).

While muscle innervation establishes normally during development, impaired NMJ maturation and stabilization probably evokes nerve retraction (Fig. 2), which results in axonal degeneration and motor neuron death via a dying-back mechanism (48,57,58). Indeed, NMJ dysfunction precedes motor neuron degeneration in SMA mouse models (44,51,57). However, it should be mentioned that large differences in muscle vulnerability exist in SMA mouse models and patients, and structural and functional NMJ defects do not always coincide with muscle denervation (57). The underlying reasons for these findings are still unclear.

1.4. Animal models

1.4.1. General introduction

The discovery of the causal *SMN1* gene in SMA allowed researchers to develop animal models to gain mechanistic insights, and to identify diagnostic and treatment strategies. The high degree of evolutionary conservation of the *SMN* gene, permits to model the disease in diverse organisms, such as Caenorhabditis elegans, fruitflies, zebrafish and mice (59–64). Of course these models display phenotypes that differ greatly from those in SMA patients, but C. elegans, fruitflies and zebrafish are well-suited to answer fundamental questions about SMN function and/or to perform high-throughput screening of drug or genetic-knockdown libraries. Similarities in the anatomy and physiology to the

human neuromuscular system in mice, makes the use of mouse models suitable for investigation of SMN biology in the nervous system, SMA pathogenesis and potential therapies (65,66).

In mice, only one *SMN* gene is expressed and homozygous removal of this *SMN* gene is embryonically lethal (67–69). Heterozygous null mice ($smn^{+/-}$) lack a noticeable SMA phenotype, showing that a marked reduction of SMN protein is necessary to induce a phenotype (69). Therefore, additional genetic modifications were integrated in the $smn^{-/-}$ mice to increase functional SMN levels in a range to prevent embryological lethality and recapitulate SMA-related functional defects. As such, a variety of models was generated encompassing different levels of severity, similar to the different types of SMA (for an overview of mouse models mentioned in this doctoral thesis see Table 2) (70).

1.4.2. The $smn^{-/-}$; SMN2 and the SMNΔ7 mouse model

As the SMN2 gene generates about 10% full-length protein, incorporation of two copies of the human SMN2 gene ($smn^{-/-}$; SMN2) increases SMN levels and rescues embryonic lethality in $smn^{-/-}$ mice. Mouse pups present with a severe SMA phenotype with moderate motor neuron loss and severe motor dysfunction, and a lifespan of 5 days on average (71). Addition of a second transgene containing human SMN cDNA lacking exon 7 *(SMNΔ7)*, extends the lifespan to 13 days therefore slightly improving the timeframe in which this mouse model can be studied (72).

SMNΔ7 mice develop morphological and functional abnormalities of the NMJ's, together with neuromuscular junction denervation and weakness of several muscles (44,47,48,57). Muscle fibers are smaller in size and less mature (47,49). The severity and (time of) occurrence of NMJ and muscle pathology highly depends on the muscle, with no clear pattern of vulnerability (57). NMJ pathology is succeeded by modest loss of α-motor neurons in the spinal cord at postnatal day 7 (44,72). As a result, SMNΔ7 mice are smaller than healthy mice and develop progressive motor function impairments from postnatal day 2 (PND2) onwards (72). Consistent with the emerging view of SMA as a multi-system disorder, SMNΔ7 mice also develop cardiac malfunctions (73,74).

Table 2 – Overview of SMA mouse models mentioned in this doctoral thesis.

SMA mouse model	Generation	Most important phenotype	Severity of phenotype	Ref.
$Smn^{-/-}$	Homozygous *SMN* disruption	Embryonic lethality	++++	(69)
$Smn^{+/-}$	Heterozygous *SMN* disruption	- No lethal phenotype - Early loss of ± 28% of spinal cord motor neurons, with slow progressive reduction.	+	(75,76)
Smn-/-; SMN2 (89Ahmb)$^{+/+}$	Introduction of the human *SMN2* transgene	- Lifespan of ± 5 days - ± 35% loss of spinal motor neurons at end stage - Neuromuscular junction denervation, starting during late gestation	+++	(48,71,77)
Smn-/-; SMN2(89Ahmb)+/+; SMNΔ7+/+ (referred to as the SMNΔ7 mouse model)	Introduction of the human *SMNΔ7* transgene to the Smn-/-; SMN2 (89Ahmb)$^{+/+}$ model	- Lifespan of ± 13 days - Modest loss of spinal motor neurons, ± 20% loss by PND9 - Small and immature muscle fibers - Neuromuscular junction immaturity and denervation after birth, together with electrophysiological deficits. - Abnormal NF accumulation in nerve terminals after birth. - Loss of proprioceptive input to motor neurons in the spinal cord - Astrogliosis - Impaired motor function	+++	(44,47,48,57,72)
SMN-/-; SMN2(89Ahmb)+/-; SMN1(A2G) (referred to as the SMNA2G model)	Introduction of a *SMN* transgene carrying a missense mutation (A2G)) to the Smn-/-; SMN2 (89Ahmb)$^{+/+}$ model	- Slightly decreased life span - Spinal motor neuron loss (± 29%) at 3.5 months old - Motor axon loss in lumbar ventral roots at 5 months of age - Mild NMJ defects - Muscle weakness - Reduced activity	+	(44,78)

1.5. Therapeutic strategies

Until recently, clinical care for SMA was exclusively supportive, addressing the respiratory, orthopedic and nutritional consequences of the disease (79,80). In the last years, enormous progress resulted in novel therapeutic strategies for SMA, culminating in the FDA approval of the SMN2 splicing regulator Nusinersen (SPINRAZA, Biogen) by the end of 2016, which was the first approved disease-modifying therapy for SMA. Nusinersen is an antisense oligonucleotide (ASO) developed to promote inclusion of exon 7 in SMN2 gene transcripts. As such, full-length SMN protein expression is increased (81) (Fig. 3). Patients receive an intrathecal loading dose of Nusinersen four times over two months, followed by a maintenance period with injections every four months (82,83). In 2019, the FDA also approved the single-dose intravenous viral delivery of an exogenous SMN1 cDNA (Zolgensma) in SMA infants (84) (Fig.3). Both treatment paradigms result in remarkable benefits, such as improved motor function and reduced risk of death or permanent ventilation although the outcomes are still highly variable (82–85). Clearly, the variability in results is, at least partially, attributable to the age at treatment, as earlier treatments tend to result in better outcomes. Ideally, treatment is started before onset of motor neuron loss or development of clinical symptoms. Neither of the therapies in its current form is a complete cure and all patients will probably manifest some life-long deficits (82–86).

In addition to SMN-targeted therapies, several non-SMN therapies are moving towards clinical development, such as neuroprotective (Olesoxime) and muscle-supporting (CK-2127107 and SRK-015) strategies. These strategies could provide additional support for patients, or would be of importance for patients that are intolerant, not responsive to or excluded from SMN-targeting therapies (87–90)

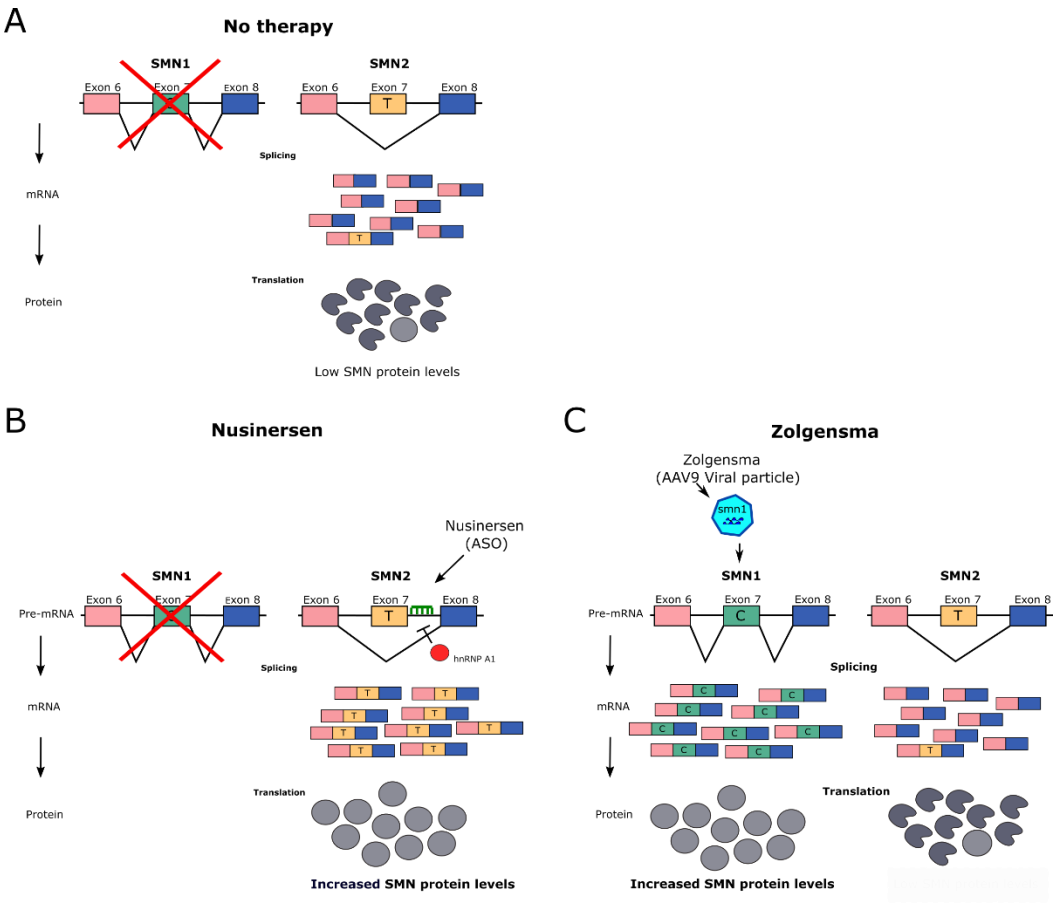

Figure 3 – Approved disease-modifying therapies in SMA. (A) Loss of the SMN1 gene in SMA patients results in reduced SMN levels. Residual SMN protein is solely originating from the SMN2 gene which produces only 10% of the levels encoded by SMN1. (B) Nusinersen is an ASO that binds a splicing silencer region on SMN2 pre-mRNA and inhibits binding of the splicing repressor protein hnRNP. As a result, inclusion of exon 7 in SMN2 gene transcripts is promoted and full-length SMN production is increased. (C) Viral delivery of exogenous SMN1 cDNA (Zolgensma) replaces the SMN1 genes and increases SMN1-dependent SMN expression. Abbreviations: SMN; survival motor neuron protein, ASO; antisense oligonucleotide, hnRNP; heterogeneous ribonucleoprotein.

2. Alzheimer's disease

2.1. Clinical presentation of Alzheimer's disease (AD)

In the beginning of the 20th century, the German neuropsychiatrist Dr. Alois Alzheimer described his long-term study of Auguste Deter, a 50- year-old demented woman. At her relatively young age, she presented with progressive memory and language problems in combination with sleep and psychiatric disorders. After her death, Alzheimer performed a brain autopsy and identified two unusual histopathological alterations later known as neuritic plaques and neurofibrillary tangles, currently the two major pathological hallmarks of Alzheimer's disease (91–93).

When Alzheimer presented the case of Auguste Deter at a scientific meeting of German psychiatrists in 1906, stressing the interesting connection between the histopathological findings and clinical symptoms, the response of the scientific community was very disappointing (91). However, more than 100 years later, Alzheimer's disease is known as a devastating neurodegenerative disorder,

representing the underlying cause of 60-70% of the dementia cases. AD is characterized by progressive decline in memory, together with multiple cognitive impairments such as executive dysfunctions, language problems, visuospatial difficulties and personality changes, all compromising the patient's daily life (94). A common first symptom of the disease is difficulty to remember recent events, names or conversations. Thereafter patients will present with additional symptoms such as disorientation, confusion and impaired communication. In later stages more severe memory loss occurs, often in combination with behavior changes (aggression, mood swings and paranoia), and difficulties with speaking, swallowing, and walking (95). These symptoms increase the risk of serious life-threatening conditions, such as pneumonia which is the most common cause of death in patients with Alzheimer's disease (96).

2.2. Genetics

2.2.1. Familial AD

In very rare cases (<1% of patients), AD is inherited in an autosomal dominant manner within families (97). These cases are categorized as familial AD and onset of clinical disease is usually between 30-65 years of age. Molecular genetic investigation of affected family pedigrees, resulted in the identification of mutations in three genes encoding proteins involved in Amyoid-β (Aβ) generation; *Amyloid precursor protein (APP)*, *Presenilin 1 (PSEN1)* and *Presenilin 2 (PSEN2)* (98–100). APP is the Aβ precursor protein, while PSEN1 and PSEN2 function as the catalytic subunit of the γ-secretase complex. Mutations in *PSEN1* are most common (>150 mutations), and carriers develop the most severe forms of AD with very early disease onset (101).

2.2.2. Sporadic AD

In more than 99% of the patients, the etiology of AD is considered multifactorial with genetic background, environment and increasing age as important contributors to the risk of disease (97,102). In these sporadic patients, clinical symptoms predominantly develop after the age of 65 years (97). The most well-known and strongest genetic risk factor is the apolipoprotein E (APOE) ε4 allele, which increases AD risk 3- or 12-fold in the presence of one or two alleles respectively. In contrast the APOEε2 allele is protective, while the APOEε3 allele has no contribution to disease risk (103). In the last 10 years, more than 40 additional genetic risk factors have been identified in genome-wide association (GWAS) and next generation sequencing studies (104). Mutations in the microglial associated protein triggering receptor expressed on myeloid cells 2 (TREM2) were identified as the second most important Alzheimer genetic risk factor. A rare mutation (p.R47H) in TREM2 increases Alzheimer's disease risk by two-or threefold (105,106). Moreover, thousands of SNP's associated with AD risk, but without

genome-wide significance are incorporated in polygenic risk scores (PRS's). These PRS's have a predictive value in estimating an individual's risk to develop AD (107). In addition PRS's illuminate a range of biological processes contributing to AD.

2.3. Pathological hallmarks

Macroscopically, AD is characterized by atrophy of the hippocampal cortex and neocortex of the brain (108). At the microscopic level, key pathological features of AD are the occurrence of extracellular amyloid plaques, intraneuronal neurofibrillary tangles (NFT's), neuro-inflammation and extensive synapse and neuronal loss (Fig. 4) (109).

Amyloid plaques are accumulations of, predominantly, insoluble forms of Aβ peptide, a small peptide derived from the APP protein after sequential cleavage by β- and γ-secretases. Aβ peptides can self-associate into oligomers and protofibrils, and further accumulation into fibrils precedes the formation of insoluble amyloid plaques (110). As the exact cleavage site of γ-secretase is variable, peptides ranging from 37-49 amino acids are produced (111). Most (90%) of the produced fragments is Aβ40, while the more aggregation-prone Aβ42 and Aβ43 peptides account for a much smaller fraction. Interestingly, the ratio of Aβ42 over Aβ40 in the brain, rather than the total amount of Aβ, seems important as it defines the aggregation rate and correlates with the age of onset in Alzheimer patients and with the amount of plaques in mouse models (112–115). Interestingly, causal mutations in the APP and PSEN genes affect the Aβ profile (ratio of longer versus shorter peptides), the production rate or the aggregation capacity (116–118). Amyloid plaques are major sites of neuroinflammation as they are surrounded by reactive astrocytes and microglia. The role of neuroinflammation in AD pathogenesis will be further discussed in section 2.6.

NFT's are aggregates composed of misfolded and hyperphosphorylated forms of the microtubule-associated tau protein. In healthy brains, tau is most abundantly located in axons and stabilizes microtubules. In AD, tau is hyperphosporylated, dissociates from the microtubules and translocates from the axons to the neuronal cell bodies and dendrites where it can aggregate and form NFT's (119). As a result of these pathological events, synapses and neurons are gradually lost during the disease, and patients will present with progressive cognitive impairments (120–122).

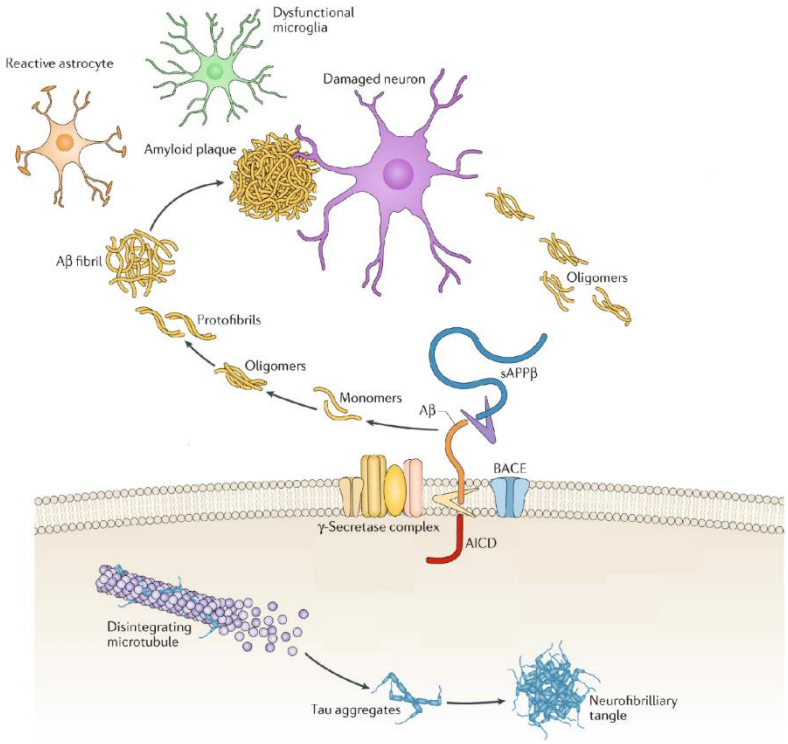

Figure 4 – Overview of the pathological hallmarks of Alzheimer's disease. Sequential cleavage of the APP protein by β- and γ-secretase releases Aβ-peptides in the extracellular space. Longer aggregation-prone monomers of Aβ (mainly Aβ42 and Aβ43) accumulate into oligomers, protofibrils and eventually Aβ fibrils which form the extracellular Aβ-deposits. Amyloid plaques are surrounded by reactive astrocytes and microglia. Inside the neurons, hyperphosphorylation of microtubule-associated tau protein, disentangle tau from the microtubules and induces formation of tau aggregates and NFT's. As a result, synapses become dysfunctional and synapses and neurons are gradually lost during the disease. Abbreviations: BACE; β-secretase, Aβ; amyloid-β, NFT; neurofibrillary tangle. Figure adapted from (123).

2.4. The amyloid cascade hypothesis

The relationship between the two major pathological hallmarks, Aβ plaques and NFT's, and the disease process has been topic of debate since a long time. The discovery of mutations in the *APP* and *PSEN* genes provided a strong genetic framework for the amyloid cascade hypothesis, which is a prominent theory in the field. This hypothesis proposes that disturbances in Aβ metabolism are central to AD pathogenesis, and initiate a sequence of events that ultimately lead to neurofibrillary tangle formation, synapse dysfunction, neuronal death and cognitive impairments (124,125). Despite scientific progress important gaps remain and extensive research focuses on understanding how Aβ precedes tau pathology and how this in turn leads to neuronal death (126). It is striking that neuronal loss and dementia only establish late in the disease, decades after the start of Aβ and tau deposition (Fig. 5) and the amyloid cascade hypothesis does no fully clarify this delay. This resulted in the hypothesis of a "cellular phase" characterized by responses of different cell types such as microglia and astrocytes in an effort to cope with the biochemical alterations. Clinical symptoms may only become apparent when these homeostatic mechanisms fail (127).

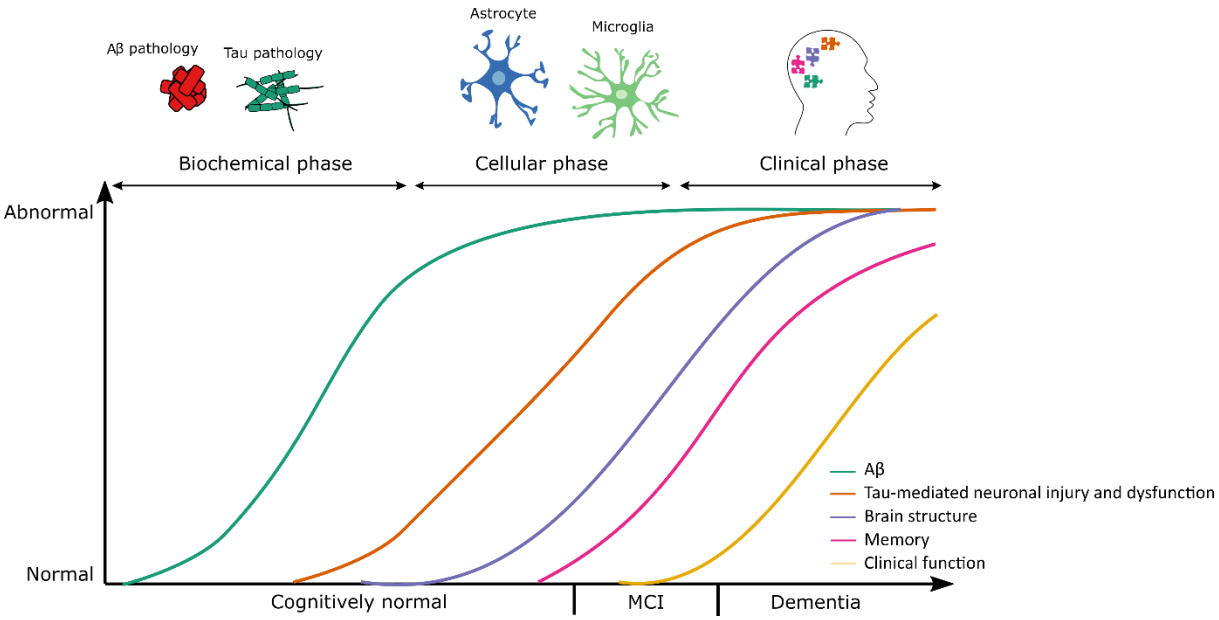

Figure 5 – Alzheimer's disease progression. The disease progression of Alzheimer's disease is characterized by three main clinical phases. First, an asymptomatic phase in which Aβ pathology accumulates without any neurological symptoms. In a second phase, tau pathology and neurodegeneration result in MCI, characterized by early episodic memory impairments not meeting the criteria for dementia yet. In the last phase, neurons and neuronal circuits are eliminated in an irreversible manner with symptoms of dementia as multiple cognitive domains are affected and the daily life of the patient is compromised. Disease progression is very slow and can take decades before patients present with dementia symptoms. Therefore, it is suggested that the biochemical phase with Aβ and Tau pathology is separated from the clinical phase by a cellular phase. During the cellular phase, different cell types such as astrocytes and microglia respond to the biochemical alterations in an effort to cope with the induced stress. Clinical symptoms might initiate when this cellular response fails and becomes pathological. Abbreviations: Aβ; amyloid-β, MCI; mild clinical impairment. Figure based on (127–129).

2.5. The emerging view of Alzheimer's disease as a synaptic disorder

Synapse dysfunction and loss precede cell death in AD, and correlate even more strongly with cognitive impairments than other neuropathological hallmarks. These synaptic changes are also present in many AD transgenic mouse models in combination with impairments in learning and memory (130–133). This resulted in the hypothesis that AD is a synaptic disorder in which synapse dysfunction and loss are sufficient to drive AD-related cognitive decline (134–136).

Both in human postmortem brain and in mouse models, synapse loss is most pronounced in close proximity to the amyloid plaques in the brain (132,133,137–140). For long time, amyloid plaques were supposed to be the toxic substance in AD, though, the number of these plaques does not correlate with the severity of cognitive impairments. The *in vitro* identification of soluble Aβ oligomers (AβO's), and their subsequent extraction from brain tissue of patients shed a new light on Aβ as an important player in disease pathology (141–143). AβO's are soluble intermediates formed during self-association of Aβ monomers to the insoluble fibrils found in plaques. They present in different forms ranging from small dimers and trimers to larger spherical oligomers composed of 12 to 24 monomers, and localize both intra- and extracellularly (144,145). Levels of AβO's correlate better with severity of disease than amyloid plaques, which is supported by many studies that suggest a more potent role for AβO's in the

disturbance of synaptic functions and neuronal network activity in comparison to amyloid plaques (146–148).

Addition of high concentrations (nanomolar range) of AβO's to neuronal cultures and/or brain slices induces loss of synaptic NMDA receptor expression, dendritic spine retraction, synapse dysfunction and loss, long term potentiation (LTP) inhibition, long term depression (LTD) facilitation and neuronal death (146,149–153). Notably, similar deficits are observed after injection of AβO's into the brains of rodent models, with additional learning and memory impairments (147,154).

One of the proposed mechanisms for oligomeric synaptotoxicity comprises the binding of AβO's to post-synaptic receptors (Fig. 6). These interactions can trigger signaling cascades that compromise, amongst others, AMPA and NMDAR signaling, Ca^{2+} signaling, actin cytoskeleton assembly and tau phosphorylation. Many binding partners have been described, including the NMDAR, AMPAR, insulin receptor (IR), cellular prion protein receptor (PrPCR), Nogo receptor (NogoR), erythropoietin-producing hepatocellular B2 receptor (EphB2 receptor) and the EphA4 receptor (155–163). However, the specificity and relative contribution of these interactions to synaptotoxicity remain to be determined (164,165).

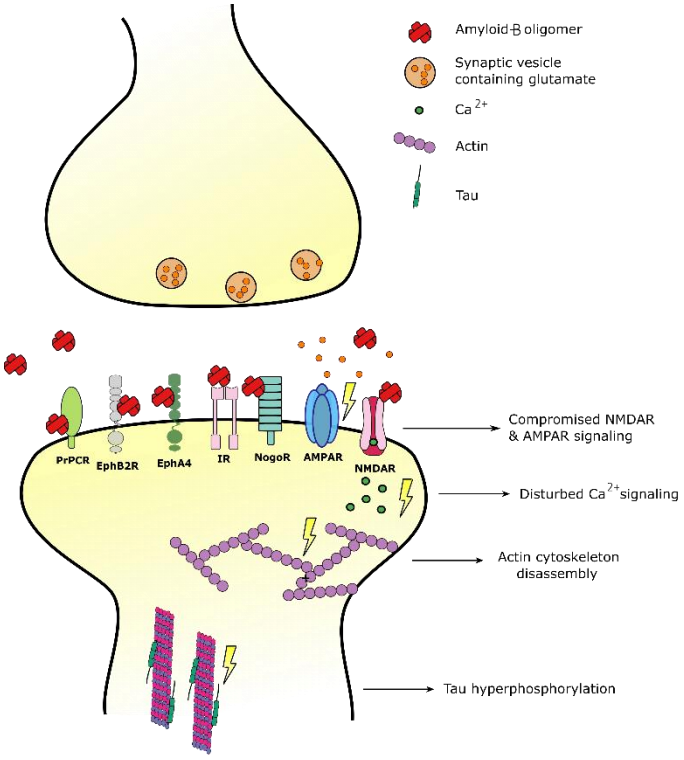

Figure 6 – Aβ oligomer-induced toxicity at the synapse. Overview of mechanisms contributing to synaptotoxicity induced by binding of AβO's to various post-synaptic receptors. Abbreviations: NFT; neurofibrillary tangles, Aβ; amyloid-β, IR; insulin receptor, PrPCR; cellular prion protein receptor, NogoR; Nogo receptor. Figure based on (166).

AD-related pathological modifications of tau, such as hyperphosphorylation, are also suggested to contribute to synapse pathology. As described before, hyperphosphorylated tau dissociates from the axonal microtubules and translocates to the somatodendritic compartment, including the spines, where it can aggregate into NFT's (119). However, similar to Aβ, evidence indicates that intermediate soluble oligomeric tau species, rather than NFT's are toxic and interfere with synaptic function (167–171). Some proposed mechanisms for tau-mediated synaptotoxicity are the disruption of microtubule-based transport of mitochondria and receptors to the synapse, NMDAR-dependent excitotoxicity and synaptic calcium dysregulation (172–177).

2.6. Neuroinflammation

A neuroinflammatory response is present in AD, revealed by changes in the morphology, activation and distribution of microglia and astrocytes, as well as increased expression of inflammatory modulators (178–180). Microglia are the resident immune cells of the brain and are involved in physiological housekeeping and host defense against pathogens and central nervous system (CNS) disease (181). These cells constantly scan the surrounding environment (182) and monitor neuronal activity and modulate synaptic plasticity, for example via synaptic pruning and remodeling (183–185). Other housekeeping functions are the maintenance of myelin homeostasis and the phagocytosis of dead or dying cells (186–188). During stressors, such as infection, brain injury and neurological diseases, microglia become activated and initiate a neuroinflammatory reaction to clear harmful agents and maintain brain homeostasis (189). Astrocytes are the most abundant glial cells in the brain and perform several functions including the provision of nutritional support to neurons, regulation of extracellular ion and neurotransmitter concentrations, modulation of synaptic transmission and synaptic plasticity, and regulation of synaptic pruning during development and adulthood (190–192).

Neuroinflammation was previously considered a rather secondary event in AD, but the search for genetic risk determinants in sporadic AD has highlighted the central role of non-neuronal cells in the pathogenesis of this disease. Most of the genes associated with the more than 40 risk loci identified via GWAS and next generation sequencing studies are expressed in astrocytes and microglia, including TREM2 and APOE (193–197).

However, the exact mechanisms by which astrocytes and microglia contribute to AD pathogenesis are not fully understood yet. Based on many studies in AD mouse models and brain tissue of AD patients, Shi and Holtzman (198) suggested a theoretical model in which neuroinflammation in Alzheimer's disease can be either protective or harmful, depending on the disease stage and the biological targets of microglia.

In the early stage, before or right after initial plaque deposition, microglia appear to facilitate Aβ seeding (199,200). First, microglia bind Aβ seeds and secrete specific inflammatory factors, called the '*apoptosis-associated speck-like protein containing a caspase recruitment domain*' (ASC) specks. ASC specks rapidly bind and cross-seed Aβ, promoting Aβ aggregation (201). Second, microglia might also take up extracellular Aβ, which aggregates inside the cell and induces microglial cell death. As dying microglial cells subsequently release the aggregated Aβ clusters, this process is thought to contribute to plaque growth (200,202) (Fig. 7A).

During the mid-stage of the disease, plaques accumulate and attract activated microglia, which cluster around plaques (203,204). Plaque-associated microglia show alterations in their RNA expression profile resulting in the reduction of homeostatic genes and an upregulation of inflammatory genes and/or genes involved in the phagocytosis of dead neurons, injured neurons and amyloid plaques (205,206). It is hypothesized that these plaque-associated microglia are initially beneficial and implicated in plaque trimming and compaction via phagocytosis, which helps to restrict plaque growth. In addition, these microglia might constitute a physical barrier around amyloid plaques to prevent plaque-induced neuronal toxicity (207–209) (Fig. 7B).

The late stage of AD is characterized by the accumulation of tau pathology and during this phase microglial activation might be harmful and precede intracellular tau phosphorylation and tau pathology via the activation of several neuronal tau kinases (210–214). In addition, microglia might actively contribute to tau spreading in the brain via uptake and release of extracellular tau (215–217) (Fig. 7C). Continuous microglial activation drives the progress of microglia into highly inflammatory, phagocytic and deleterious cells, which will actively contribute to neurodegeneration (206) via phagocytosis of stressed, but still viable neurons and synapses (130,131,218–221) (Fig. 7D). These cells can also secrete high amounts of reactive oxygen species (ROS) and nitric oxide (NO), toxic to neurons. In addition, microglia can contribute to the transformation of neuroprotective A2 astrocytes into harmful A1 astrocytes. These astrocytes lose their neurotrophic functions and are harmful to neurons and synapses (222–224) (Fig. 7E).

Interestingly, many of these neuroinflammatory processes are modulated by AD risk factors, including TREM2 and APOE (198). In addition, GWAS studies identified thousands of SNP's without genome-wide significance of which many are also involved in inflammatory pathways (107,225). While individual effects of these various SNP's are probably minor, studies suggest that combination of many of these subtle alterations affect microglial function and determine whether a pathological response is induced to Aβ pathology.

In summary, the exact functions of microglia in AD pathogenesis, whether these functions are protective or detrimental and how genetic alterations in microglia contribute to the disease process

are not fully known. However, the described genetic and biological evidence suggests that microglia might be an important contributor in the previously described 'cellular phase', in which their genetic makeup determines whether Aβ deposition is linked to pathological neuroinflammation, tau pathology, neuronal loss and dementia (127,225–227).

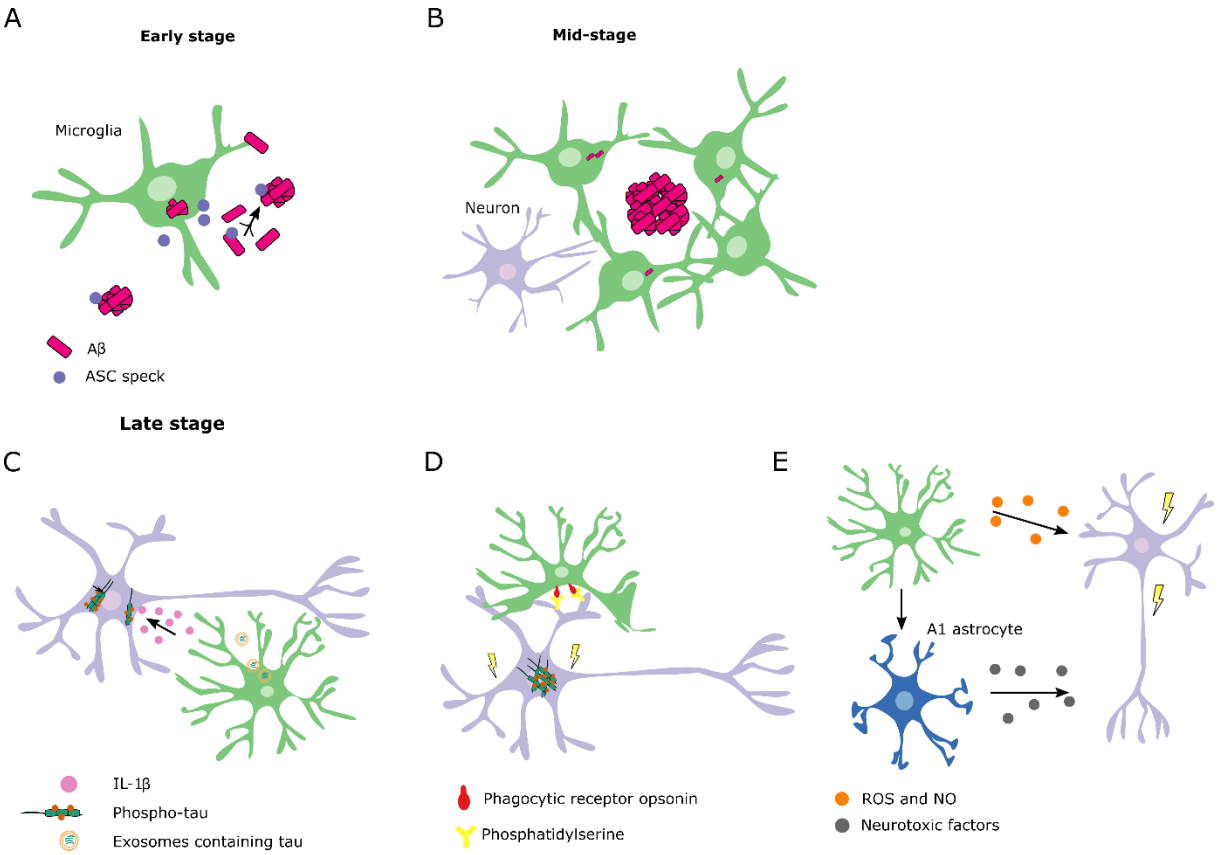

Figure 7 – Roles of neuroinflammation during the different stages of Alzheimer's disease. (A) In the early stage, microglia might be harmful by facilitating Aβ seeding via release of ASC specks that bind and cross-seed Aβ. In addition, microglia might capture Aβ and induce Aβ aggregation intracellularly. (B) In the mid-stage of the disease, microglia gather around Aβ plaques where they might be beneficial via trimming and compaction of the plaque, which helps to restrict plaque growth. In addition, the physical barrier around the plaque formed by microglia prevents toxicity to neurons. (C-E) During the late stage of AD, microglia might be harmful in several ways. (C) Secretion of IL-1β by microglia activates several neuronal tau kinases and results in increased tau phosphorylation. In addition, microglia appear to take up and release tau via exosomes, which might contribute in the spread of tau during the disease. (D) Interaction of aberrantly expressed phosphatidylserine on stressed neurons and the opsonin receptor on microglia, might trigger phagocytosis of viable neurons by microglia. (E) Microglia can contribute to the transformation of astrocytes to harmful A1 astrocytes that secrete neurotoxic factors. In addition, microglia themselves can secrete high amounts of toxic ROS and NO. Abbreviations: ASC speck; apoptosis-associated speck-like protein containing a caspase recruitment domain, IL-1β; interleukin-1β, ROS; reactive oxygen species, NO; nitric oxide. Figure based on (198).

2.7. Transgenic animal models for AD

2.7.1. General introduction

As wild-type mice do not develop Aβ plaques or NFT's, the majority of animal models used in AD research are transgenic mice. Over the past 20 years, overexpression of human *APP* and/or *PSEN* genes

containing familial AD - associated mutations, resulted in a numerous amount of animal models with plaque pathology and varying amounts of additional AD related pathology. Plaque-associated gliosis, synaptic and cognitive impairments are frequently detected. Major limitations of these models are the lack of NFT's and, in most models, prominent neuronal loss and brain atrophy (228). In order to study the pathological effects of tau aggregation, mice expressing mutant tau, whether or not in combination with mutant *APP* and/or *PSEN* genes, have been generated. These mice do develop NFT'S, although only at old age (229). The translation of findings in these models to humans is compromised by the lack of similar mutations in AD patients.

These experimental models provide insights, but also have limitations. Expression constructs are randomly inserted without knowledge on the integration site and copy number, which might result in integration artefacts (229). Moreover, overexpression of APP and PSEN1 can induce artificial phenotypes, for example via increased production of APP fragments, which could affect their physiological functions (129,230). In order to tackle this issue, a new generation of transgenic models with physiological expression of mutated APP was generated, the APP knock-in mouse model. These APP knock-in mice express a humanized Aβ sequence with one or multiple APP mutations. Dependent on the identity and the number of expressed mutations, APP knock-in models can develop plaque formation, concomitant Alzheimer-like pathology (except for NFT formation and neuronal loss) and memory impairments (231,232).

As with all transgenic models in neurodegeneration based on mutations in familial cases, the relevance for the sporadic form remains speculative. The use of familial AD-associated mutations implies that these models might not accurately represent the more common, sporadic forms of the disease. This might impair the translatability of observations to human clinical trials (233). To tackle this problem, the US National Institute on Aging initiated the formation of a research consortium called "MODEL-AD" aiming to model sporadic forms of the disease, for example via the implementation of risk factors for sporadic AD (234).

2.7.2. The APPPS1-21 mouse model

In the experiments described in the following chapters, the APPPS1-21 mouse model, further on referred to as APPPS1 mice, was used. APPPS1 mice overexpress human APP protein containing the Swedish double mutation (KM670/671NL) and human PSEN1 protein with a L166P mutation specifically in neurons under the control of a Thy1 promotor. These mice develop Aβ pathology at the age of 2-3 months in the frontal cortex, followed by the hippocampus and other brain regions. Plaque

pathology is accompanied by astro- and microgliosis and loss of dendritic spines in close vicinity to the plaques (132,235). LTP is impaired from 8 months onwards (236), which coincides with the first signs of cognitive decline from 7-8 months on (235,237). More specifically, defects in spatial learning, recognition memory and contextual fear learning are observed in APPPS1 mice (235,237,238).

2.8. Therapeutic strategies

So far, no disease-modifying treatment for AD exists and current treatments are symptomatic and focused on the restoration of neurotransmitter imbalances in the brain. As such, two classes of cognition-enhancing drugs have been approved for use in AD, the acetylcholinesterase inhibitors (e.g. rivastigmine, donepezil and galantamine) and the NMDA receptor antagonist, memantine. Acetylcholinesterase inhibitors are used to prevent enzymatic breakdown of acetylcholine and enhance cholinergic signaling, while memantine is administered to decrease glutamate excitotoxicity (239–241). However, the efficacy of these drugs is limited and patients might suffer from side effects such as gastrointestinal problems, dizziness and headache (242).

Considering the immense epidemiological and socioeconomic burden of this disease, the need for disease-modifying therapies able to prevent, delay or slow the progression of the disease by targeting the primary pathological mechanisms is pressing (243). Based on the amyloid hypothesis, therapies aiming to ameliorate Aβ pathology are of high interest to the field (123). As the β-secretases and γ-secretases play a fundamental role in cleavage of the APP protein and Aβ formation, strategies to inhibit and/or modify their function are investigated in preclinical and clinical studies for AD. As a result, total Aβ levels or even the ratio of longer over shorter Aβ peptides might be reduced (244,245). In addition, considerable research efforts have been focused on the development of selective anti-Aβ antibodies. These antibodies might destabilize Aβ plaques, fibrils or oligomers, increase microglia-mediated Aβ phagocytosis, or enhance Aβ clearance from the brain (123,246).

Aβ-targeted therapies show high theoretic and preclinical potential, but clinical trials have unfortunately not convincingly show benefit of this novel treatment options for AD patients (247). A first explanation for this low success rate is the high toxicity of the γ-secretase and β-secretase inhibitors, due to low substrate specificity and many off-target effects (244,247). In addition other variables may have contributed to this disappointing results: clinical heterogeneity of AD patients included in the trials, suboptimal outcome measurement and too late stage of disease to initiate the treatment (247). In many trials, patients were treated in a symptomatic stage, which might be too late to reverse the progression of neurodegeneration as significant synapse and neuronal loss might have been established (123,244,247). In response, an increasing amount of trials is now targeting patients in preclinical or asymptomatic stages of AD, and cognitively healthy subjects at risk of developing AD

(248). In addition, other promising approaches targeting neuroinflammation, tau aggregation and neuroprotective pathways are currently finding their way to clinical trials (248).

3. The ephrin system

3.1. Introduction

The human erythropoietin-producing hepatocellular (Eph) receptors comprise the largest family of the superfamily of transmembrane tyrosine kinase receptors (RTK's). Eph receptors were named after the human carcinoma cell line in which they were initially discovered over 30 years ago (249). This triggered a vast number of studies, revealing a wide range of functions in many biological processes. Due to its unique properties, the Eph/ephrin system is involved in many short distance cell-to-cell communication events that trigger fast changes in cell morphology, adhesion, movement, proliferation, survival and differentiation (250). Consequently, this system plays a role in many developmental processes such as axonal guidance, cell positioning and tissue patterning (251). Further, the ephrin system is involved in specialized cell functions including synaptic plasticity, epithelial development and homeostasis, bone remodeling, insulin secretion, stem cell renewal and immune responses during adult life (252–257). Imbalance of the system might contribute to several pathological conditions, such as cancer, neurodegeneration and post-traumatic injury of the nervous system (258–260).

3.2. Protein structure of Eph receptors and ephrin ligands

Eph receptors are classified into EphA (EphA1-8, EphA10) and EphB (EphB1-4, EphB6) subfamilies, depending on sequence similarity and ligand affinity. EphA receptors preferentially bind ephrin-A (ephrin-A1-5) ligands, while EphB receptors bind ephrin-B (ephrin-B1-3) ligands. As an exception, EphA4 also binds ephrin-B ligands and EphB2 binds also ephrin-A5 (261–263). Similar to all RTK's, Eph receptors are transmembrane proteins consisting of an extracellular region, a transmembrane domain and an intracellular region (Fig. 8). The extracellular region contains a globular ligand-binding domain (LBD), adjacent to a cysteine-rich domain (CRD) with Sushi and epidermal growth factor (EGF)-like motifs and two fibronectin (FN) type III repeats. The intracellular region comprises a tyrosine kinase domain, followed by a sterile alpha motif (SAM) and a post-synaptic density protein 95 (PSD-95) Drosophila discs-large imaginal disc protein (DlgA) ZO-1 tight junction (PDZ)-binding motif (264).

Both classes of ephrin ligands have a receptor binding globular domain, but are distinct in the mode of cell membrane attachment (Fig. 8). Ephrin-A ligands are anchored to the plasma membrane via a

glycosylphosphatidylinositol (GPI) anchor, while ephrin-B ligands contain a transmembrane domain and a cytoplasmic tail including a PDZ-binding motif (260).

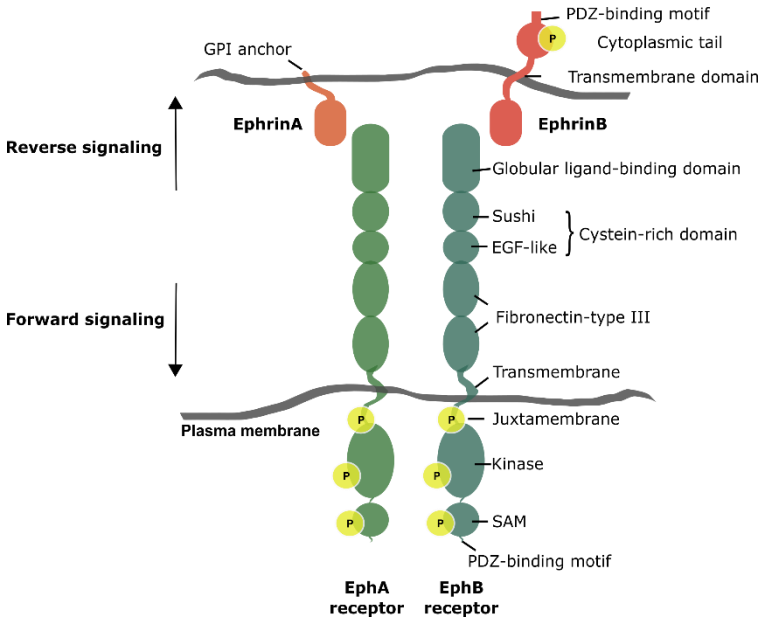

Figure 8 – Protein structure of Eph receptors and ephrin ligands. Domain structures and phosphorylation sites of Eph receptors and ephrin ligands are indicated in the figure. Ephrin-EphR signaling can occur in both directions, referred to as forward and reverse signaling.

3.3. Eph-ephrin signaling mechanisms

As both the receptors and ligands are bound to the plasma membrane, the ephrin system has two unique properties. First, cell interaction is typically essential for signaling to occur. Second, different signaling directions are possible. In the classical signaling model, ephrins act as *in trans* ligands for Eph receptors, which is called forward signaling. In addition, reverse signaling can also ensue, in which Eph receptors act as *in trans* ligands for ephrins. Both signaling directions can also be induced simultaneously, which is called bidirectional signaling. In addition, Eph receptors can interact *in cis* with ephrins on the same cell, and attenuate Eph forward signaling (265). Occasionally, Eph receptors can induce signaling cascades independently of their ligands and ephrins can induce signaling cascades independently of their receptors (266).

Unique to this family of RTK's is that typically only membrane-bound or artificially clustered ligands can induce signal activation (267). Non-clustered, monomeric or dimeric forms of recombinant ephrins generally act as antagonists, although some exceptions exist (268–270). Ephrin clusters form through a seeding mechanism which starts with the binding of Eph receptors and ligands on opposing cell sufaces and the formation of heterodimers (271,272). Subsequently, these heterodimers can tetramerize into heterotetramers, which form a ring structure in which each receptor interacts with

two ligands and each ligand interacts with two receptors (273). These tetramers assemble into higher-order clusters via lateral expansion (274). Non-ligand bound Eph receptors can also be recruited in the clusters (275). The size of the clusters correlates with the strength of the signal and can affect downstream signaling pathways and biological effects (274,276).

The close and accurate organization of Eph receptors induced by clustering, stimulates extensive phosphorylation of intracellular tyrosine residues via transphosphorylation and receptor-associated Src-family kinases (277). Phosphorylation of two tyrosine residues in the juxtamembrane domain induces a conformation change, releasing this domain from its interaction with the kinase domain. This allows the kinase domain to convert into its active state (278–280). In addition, phosphorylation of a tyrosine residue in the activation segment of the kinase domain favors substrate binding (278). Once the receptor is activated, signaling and adaptor molecules like Src family kinases, non-catalytic region of Tyr kinase adaptor protein 1 (Nck1) and Nck2, Vav2, Vav3, α-chimaerin and ephexins associate with the receptor and transmit signals into the cell (281–283).

Reverse signaling in the ligand bearing cell differs between ephrin-A and –B ligands. Similar to Eph receptors, tyrosine phosphorylation of ephrin-B ligands by Src kinases, regulates the binding of several signaling molecules (284,285). As ephrin-A ligands lack a cytoplasmic domain, it is less well understood how they propagate cell signaling. Evidence suggests the interference of associated co-receptors such as the p75 neurotrophin $P75^{NTR}$, tropomyosin receptor kinase B (TrkB) and Ret tyrosine kinase receptors (286,287).

Important effector proteins of both forward and reverse signaling are the Ras and Rho (RhoA, Rac and Cdc42) family of GTPases, and the Abl kinase, involved in cytoskeletal dynamics via assembly and disassembly of actin filaments (281,288–294). In general, ephrin/EphR signaling will induce repulsion of cells and cellular processes, although occasionally cell attraction and/or adhesion may occur. In order to allow cell separation and repulsive effects, the ephrin/EphR complex needs to dissociate via proteolytic cleavage of ephrins and/or Eph receptors or is trans-endocytosed in one of the interacting cells (295–298).

3.4. EphA4 in nervous system development

EphA4 is highly expressed in the developing nervous system, where it is involved in many important processes such as guidance of axons essential for corticospinal tract (CST) formation, the organization

of central pattern generators (CPG's) and hindlimb innervation. In addition, evidence suggests a role for EphA4 in the formation and function of neuromuscular junctions.

3.4.1. Axon guidance during CST formation, organization of CPG's and hindlimb innervation

During development of the nervous system, axons travel through highly organized pathways to form connections with distant neurons. This neural wiring is a very complex task achieved by interaction of the axonal growth cone with a variety of guidance cues in the environment, such as the semaphorins, the slits, the netrins and the ephrins. These cues can trigger adhesive, attractive or repulsive responses and guide the growth cone through a series of decision-making steps before reaching their synaptic targets (297). Due to their intrinsic properties, ephrins will mainly function as axonal repellents. EphR/ephrin interaction will induce regional differences in actin dynamics within the growth cone resulting in growth cone turning and the guidance of a particular axon trajectory (298). EphA4 signaling prevents midline crossing of growing axons during the formation of the CST and the organization of CPG's (301–309). The latter are spinal circuits on each side of the spinal cord important for the generation of rhythmic movements without the use of sensory or descending inputs, such as walking and swimming (310). In addition, EphA4 is indispensable for proper dorsal hindlimb innervation as it navigates spinal motor neurons axons to the dorsal hindlimb, while repelling them from the ventral hindlimb (311–313).

Insights in the role of EphA4 in the developing central nervous system can be obtained via study of mice with reduced EphA4 levels. Mice with a complete loss of EphA4 protein (EphA4-KO mice), are viable and fertile. Although as a result of abberant CST formation, CPG organization and dorsal limb innervation, hesitation to initiate locomotion, a rabbit-like hopping gait and an abnormal hindlimb position (club-foot) are observed. Mice with a heterozygous loss of EphA4 ($EphA4^{+/-}$ mice) have no robust phenotype although mild deficits are described (302,314).

3.4.2. Formation and function of neuromuscular junctions

In the further development, after the innervation of the hind limb, EphA4 remaind highly expressed in embryonic muscle and in neonatal and adult neuromuscular junctions and is suggested to be involved in neuromuscular junction formation and/or stabilization (315–318) (Fig. 9). Although evidence is still sparse and specific mechanisms are still unraveled. First, EphA4 is suggested to be involved in the cluster formation of AchR's via changes in the actin dynamics through interaction with cortactin (316,319,320) (Fig. 9A). In addition, adhesive interaction between pre-synaptic EphA4 and post-synaptic ephrin-A5 is hypothesized to mediate stability of the NMJ (318) (Fig. 9B). EphA4 may also play a role in the functioning of the NMJ by regulation of acetylcholinesterase (AchE) and

neurotransmission via the Jak/Stat pathway (317) (Fig. 9C). Surprisingly, adult EphA4-KO mice do not show defects in NMJ innervation (321), although more subtle alterations such as AchR cluster formation and NMJ function were not investigated.

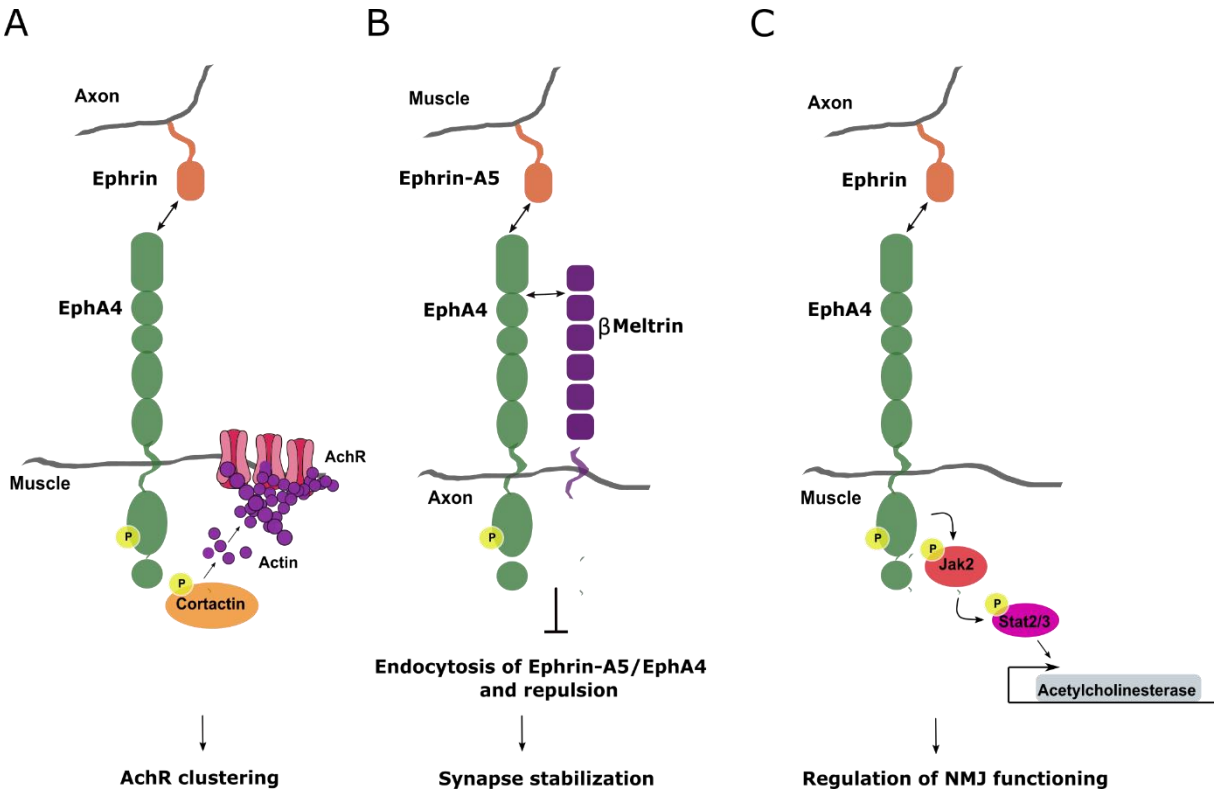

Figure 9 – Suggested mechanisms of EphA4-mediated neuromuscular junction formation and functioning. (A) EphA4-mediated cortactin phosphorylation and activation at the post-synaptic region might alter actin dynamics, resulting in the sequestration of AchR's and cluster formation. (B) EphA4-expressing axons interact with ephrin-A5 at the post-synaptic region. Subsequent interaction of EphA4 with Meltrinβ inhibits endocytosis of the Ephrin-A5/EphA4 complex in order to avoid axon repulsion and maintain synapse stability. (C) EphA4 signaling at the post-synaptic region might drive acetylcholinesterase gene expression via the Jak/Stat pathway. Hence, EphA4 signaling might affect neurotransmission and NMJ function. Abbreviations: AchR; acetylcholine receptor, NMJ; neuromuscular junction. Figure based on (315–318).

3.5. EphA4 is involved in synapse formation and function

In the brain, EphA4 is present during development and is still extensively expressed in high-plasticity regions of the adult brain, such as the cortex and hippocampus (322,323). In these structures, EphA4 localization gradually shifts from the cell bodies to synapse-associated axon terminals, dendritic spines and astrocyte processes during postnatal development, suggesting diverse roles in brain synapses (324–326). Post-synaptic EphA4 in dendritic spines interacts with astrocytic ephrin-A3 and is involved in the cell-contact dependent communication between astrocytes and neurons via several signaling routes, evoking functional alterations in both cell types (Fig. 10) (327,328).

3.5.1. EphA4 forward signaling

EphA4 forward signaling induces several signaling cascades in the spines, resulting in dendritic spine remodeling. Dendritic spines are highly specialized protrusions on dendrites that are the primary sites of innervation by pre-synaptic excitatory nerve terminals. They have been categorized by shape in stubby, thin, mushroom and filopodia spines, although this classification probably underestimates the heterogeneity in spine morphology (329,330). Live-imaging studies have revealed that dendritic spines are highly dynamic structures, changing density and morphology within minutes via alterations in actin dynamics (331,332). The morphology of spines affect their functionality and spines with larger heads are more sensitive for glutamate, form stronger synapses and are suggested to have different functions in memory than spines with smaller heads. Therefore, spine remodeling might change the physiology of synapses, and together with alterations in density, this is of importance during cognitive processes such as learning and memory (333–336).

EphA4 activation induces spine retraction, resulting in a reduction in spine length, spine head width and density (327,337–339). These effects are caused by actin remodeling via activation of RhoA GTPases, inhibition of Ras GTPases, regulation of cofilin activity, and disruption of the function of the cell adhesion receptor, β-integrin (337–340) (Fig. 10A). The growth-inhibiting property of EphA4 is suggested to counter-act growth-promoting signals, and supports a balanced system in which spines can remodel without changing their overall organization. In accordance, spines in EphA4-KO mice look irregularly shaped and disorganized, are longer, and higher in number (327).

EphA4 forward signaling is capable of changing the composition of excitatory synapses. During periods of persistent synaptic activity, EphA4 mediates the downregulation of AMPA receptors at the post-synaptic membrane via proteasome-dependent degradation of its GluR1 subunit (Fig. 10B). As such, neurons can adapt their excitability in response to changes in the strength and number of synapses, during the process of homeostatic scaling (341). This is important for neurons to maintain their activity in a physiological range, which is essential for normal synaptic function (342).

3.5.2. EphA4 reverse signaling

Reverse signaling via Ephrin-A3 on the astrocytes changes astrocyte properties. EphA4/ephrin-A3 reverse signaling reduces the expression levels of the hippocampal and cortical glial glutamate transporters glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST), resulting in reduced astrocytic glutamate influx and increased synaptic glutamate levels (328,343) (Fig. 10C). As such, glial glutamate transporter and synaptic glutamate levels can be prevented to reach non-physiological levels, promoting normal synaptic activity and plasticity. Interestingly, post-synaptic EphA4 is indispensable at the CA3-CA1 synapse for LTP, as observed in EphA4-KO mice and mice with

specific loss of EphA4 in CA1 neurons (328,344). Loss of EphA4 impairs performance in hippocampus-dependent tasks, such as spatial novelty detection and contextual fear conditioning (345,346). Although, LTP promotion by EphA4 can be mediated by alterations in spine modelling as well as changes in synaptic glutamate levels, only evidence for the latter exists (328,344).

3.5.3. Ephrin-independent signaling

Less studied is the ephrin-independent cleavage of EphA4 by γ-secretase, which generates a cytosolic fragment, the EphA4-intracellular domain (EICD). In contrast to Ephrin-A-dependent EphA4 signaling, this EICD stimulates formation of dendritic spines via regulation of the Rac-GTPase pathway (Fig. 10D). EphA4 processing is stimulated by synaptic activity, and might be involved in synaptic activity-dependent spinogenesis (295,347,348).

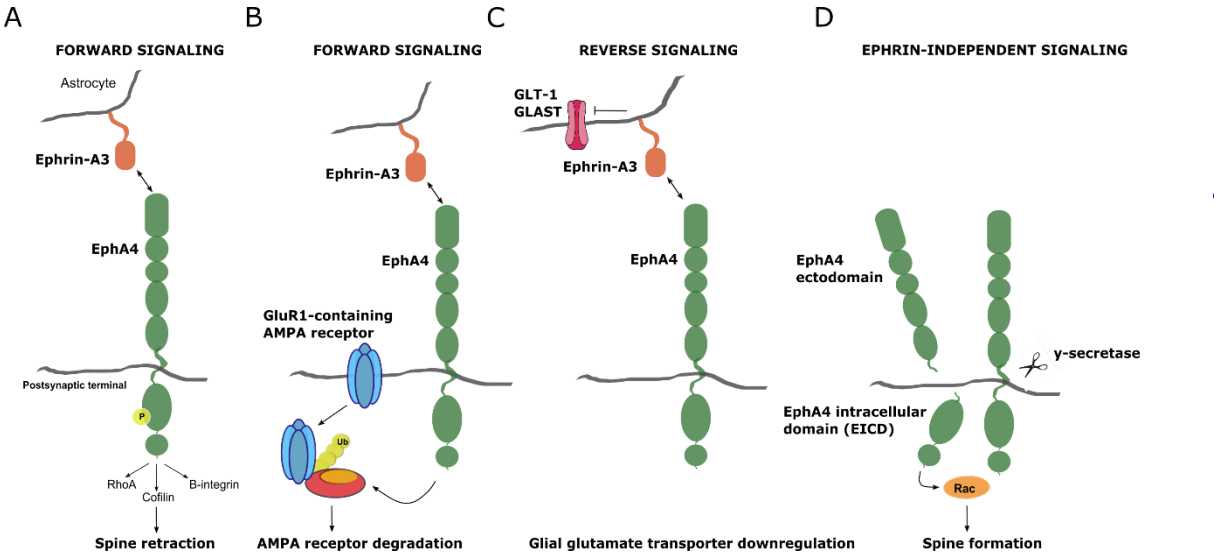

Figure 10 – EphA4 is involved in synapse formation and function. (A-C) Post-synaptic EphA4 in dendritic spines interacts with astrocytic ephrin-A3 and is involved in synapse formation and function via several pathways. (A) Forward signaling induces spine retraction via RhoA activation, regulation of cofilin activity and disruption of B-integrin signaling. (B) Forward signaling contributes to homeostatic scaling via proteasome-dependent degradation of the GluR1 subunit of AMPA receptors at the post-synaptic membrane. (C) Reverse signaling into the astrocyte reduces astrocytic glutamate influx and increases synaptic glutamate levels via downregulation of GLT-1 and GLAST. (D) Ephrin-independent cleavage of EphA4 by γ-secretase generates an EICD, which stimulates formation of dendric spines via activation of Rac-GTPases. Abbreviations; GLT-1; glutamate transporter 1, GLAST; glutamate aspartate transporter, EICD; EphA4-intracellular domain. Figure based on (322).

In conclusion, EphA4 has very diverse, and even opposing, roles in synapse formation, activity and plasticity. Further research is needed to elucidate the spatial and temporal characteristics of the different EphA4-induced changes and how they might work together to ensure proper brain function. According to current evidence, the outcomes of EphA4 stimulation depend on the availability of ephrins and γ-secretases, and the presence of synaptic activity (320).

3.6. EphA4, an interesting target in neurological disorders

Due to its high expression and plethora of functions in the nervous system, EphA4 has been extensively studied in several acute and degenerative neurological disorders. A short overview will be given in the next paragraphs.

3.6.1. Spinal cord injury (SCI), traumatic brain injury (TBI) and ischemic stroke

The adult CNS has the plasticity to promote axonal regeneration after injury. However, the majority of axons will fail to regenerate beyond the lesion site, due to the non-permissive inhibitory environment (349). Axons encounter inhibitory myelin-associated proteins in damaged myelin sheets and a physical barrier mainly formed by reactive astrocytes, the glial scar (350,351). In addition, axon regeneration is restricted by the presence of repulsive axon guidance cues, such as ephrins and EphR's (299).The therapeutic potential of EphA4 inhibition is studied in models for SCI, TBI and ischemic stroke.

SCI is characterized by damage to the spinal cord resulting from an external physical impact (traumatic) or from disease or degeneration (non-traumatic). Patients can suffer from severe motor, sensory and autonomic dysfunction (352).

In spinal cord injury models, EphA4 expression is increased in axon stumps and in peri-lesional astrocytes after injury (353–356). Loss of EphA4 and inhibition of EphA4 signaling improves axonal regeneration and functional recovery (353,357,358). *In vitro* and *in vivo* experiments suggest several roles for EphA4 in spinal cord injury. First, communication of axonal and/or astrocytic EphA4 with ephrin ligands on the astrocyte or axon, might suppress axonal outgrowth (353,354). Second, EphA4 signaling in the astrocytes may induce astrocyte proliferation, astrocyte reactivity and formation of the glial scar (353,358). Although evidence for the latter is inconclusive, since as loss of EphA4 signaling is not always associated with reduced astrocyte reactivity in these models (359). Last, EphA4 can regulate some additional inflammatory pathways. Subtle alterations in the neuroinflammatory response are observed in EphA4 KO mice after spinal cord injury, of which the proportional reduction in Arginase 1-positive macrophages/microglia is the most obvious one. How this might be beneficial in SCI is unclear (360,361).

TBI refers to structural and/or physiological disruption of brain function as a result of an external force (362). Also in this condition, alterations in EphA4 levels are observed. In human patients and in non-human primates, EphA4 is upregulated in peri-lesional reactive astrocytes after cortical injury (363,364). Similar as to spinal cord injury, EphA4 is thought to stimulate astrocytic gliosis and the formation of the glial scar (363). Rat and mouse models for cortical injury did not confirm these findings

since EphA4 levels are even slightly decreased in the cortex and hippocampus (365,366). Mice with reduced EphA4 levels in the forebrain have unaltered reactive astrogliosis, axonal sprouting and cognitive function in these experimental TBI models (366). Therefore, currently EphA4 reduction after TBI does not seem an interesting target and further research in different animal models for TBI seems necessary.

Ischemic stroke is caused by an interruption of focal blood flow in the brain, resulting in focal neurological dysfunction (367). Post-stroke, expression of EphA4 and its ligands is increased in affected regions (368–371), and modifying EphA4 seems an interesting target to improve stroke outcome. Indeed some studies report that genetic reduction of EphA4 improves neuronal survival (368) and enhances functional recovery (368,372). Interfering with EphA4 signaling following stroke had no effect on outcome, although levels of inhibition may have been too low (371). Similar to SCI and TBI, EphA4 is thought to exert its detrimental effect in stroke via inhibition of axonal regeneration. However, several other mechanisms are investigated in mouse models and/or *in vitro* models for ischemic stroke and are suggested to underlie the potential harmful effect of EphA4, such as glutamate toxicity due to decreased glial glutamate transporter expression, BBB damage and microglial activation (365-367,369).

3.6.2. Multiple sclerosis (MS)

MS is an auto-immune disorder characterized by demyelination and axonal degeneration (373). EphA4 is present in inflammatory cells, reactive astrocytes, macrophages and axons around active lesions in MS patients (374). In a mouse model for MS, EphA4 is expressed in astrocytes and reduction of EphA4 results in a reduction of axonal degeneration. In addition, pharmacological inhibition of EphA4 delays disease onset and attenuates disease progression. Currently insight in the exact mechanism of action, anti-inflammatory or effect on degeneration, is lacking (375).

3.6.3. Amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA)

ALS is a neurodegenerative disease, affecting both the upper and lower motor neurons in the brain and spinal cord (376). EphA4 is identified as an ALS disease modifier in zebrafish, rodent models and patients (321). When EphA4 function was genetically ablated or pharmacologically inhibited in mutant SOD1 rodent models, disease onset and/or progression was slowed down. Moreover, the age of onset and survival inversely correlated with mRNA expression levels of EphA4 in blood of sporadic ALS patients.

Interestingly, in ALS patients and mouse models different types of motor neurons exhibit diverse patterns of vulnerability, which is determined by EphA4 expression levels. Spinal motor neurons with the highest EphA4 levels (fast-twitch fast fatigable, FF) are most vulnerable in the disease and degenerate first, while motor neurons with lower EphA4 levels (slow, S) are more resistant (321,377,378) (Fig. 11). Deletion of EphA4 in the SOD1 mouse, results in increased survival of these FF motor neurons. These data suggest a role for EphA4 in determining the vulnerability of motor neurons for degeneration in ALS. In addition, loss of EphA4 enhances the innervation of neuromuscular junctions in this mouse model. Further evidence supports a role for EphA4 in modifying the regeneration capacity of motor axons as the re-innervation of neuromuscular junctions after sciatic nerve axotomy in mice lacking EphA4 is increased (321) (Fig. 11).

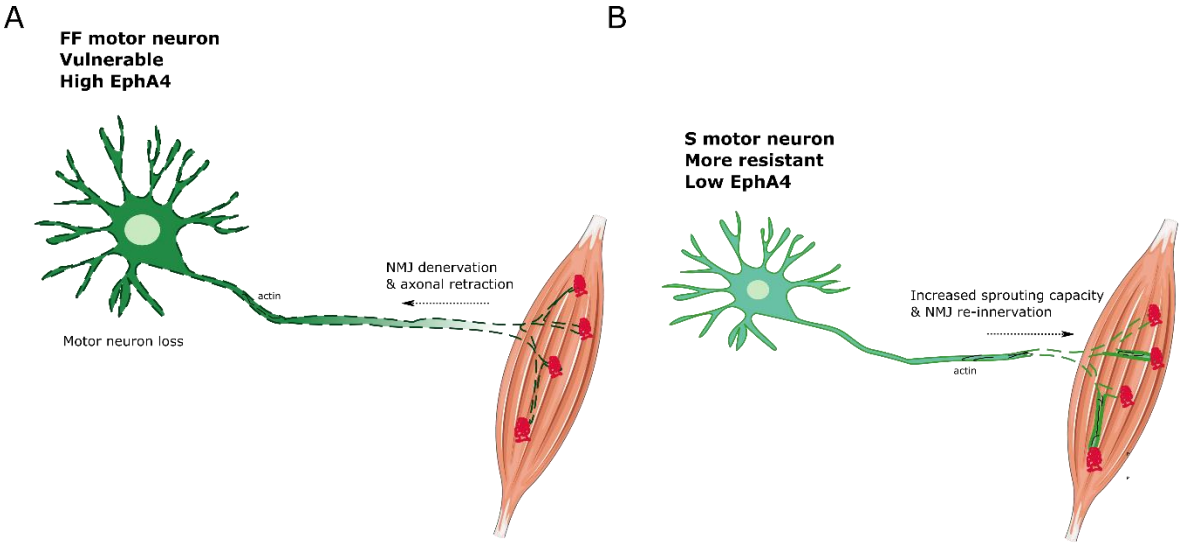

Figure 11 – EphA4 determines motor neuron vulnerability and re-innervation capacity in ALS. (A) FF motor neurons express high EphA4 levels and are highly vulnerable for degeneration in ALS. In addition, EphA4 might hinder compensatory sprouting events, further enhancing NMJ denervation and axonal retraction. (B) S motor neurons express low EphA4 levels and are more resistant to degeneration in ALS. Low EphA4 levels boost compensatory sprouting events and NMJ re-innervation. Abbreviations: ALS; amyotrophic lateral sclerosis, FF; fast-twitch fast fatigable, S; slow, NMJ; neuromuscular junction.

Follow-up studies have investigated the therapeutic potential of EphA4 using genetic approaches, ASO's targeting EphA4 and EphA4 antagonists. These studies reveal a very complex nature of EphA4 targeting as results seem dependent on the time that treatment is started, route of administration and on the amount of residual EphA4 expression and activity (379–381). In addition, both EphA4 agonist and EphA4-ligand antagonist approaches extend survival in rodent models for ALS (379,382), adding another level of complexity to therapeutic targeting of EphA4 and endorsing the need for more in depth study to unravel the mechanisms of action.

Interestingly, knockdown of EphA4 also rescued the axonal deficits in a zebrafish model for SMA (321), suggesting that the neuroprotective effect of EphA4 inhibition could be independent of the cause of degeneration.

3.6.4. Alzheimer's disease and depression

Due to its significant contribution to synapse formation and function in the cortex and hippocampus (322), EphA4 has become an interesting target for disorders characterized by synaptic dysfunction, such as depression or AD.

Postmortem studies in human AD patients reveal interesting connections between EphA4 and AD pathology. While total EphA4 protein levels are not altered, higher EphA4 levels are found in synaptosomes and in neuritic amyloid plaques and tangles of patients (383–385). Moreover, increased activation of EphA4 and reduced EICD levels are observed in brains of patients (385,386).

Postsynaptic EphA4 activation is directly or indirectly triggered by AβO's and is suggested to mediate the synaptotoxic effects caused by these oligomers via c-Abl signaling and alterations in actin dynamics (160,385,386). Indeed, reduction or pharmacological blockage of EphA4 rescues Aβ-induced dendritic spine loss, neuronal death and LTP deficits in cultured hippocampal slices and primary hippocampal cultures (160,386) (Fig. 12). In addition, reversal of Aβ-dependent memory impairment in a sortilin-related receptor with LDLR class A repeats (SORLA)-overexpressing mouse, is suggested to be associated with decreased EphA4 activation (385). Whether EphA4 acts as a direct receptor for AβO's needs further investigation, as current evidence is inconsistent (165,166,386).

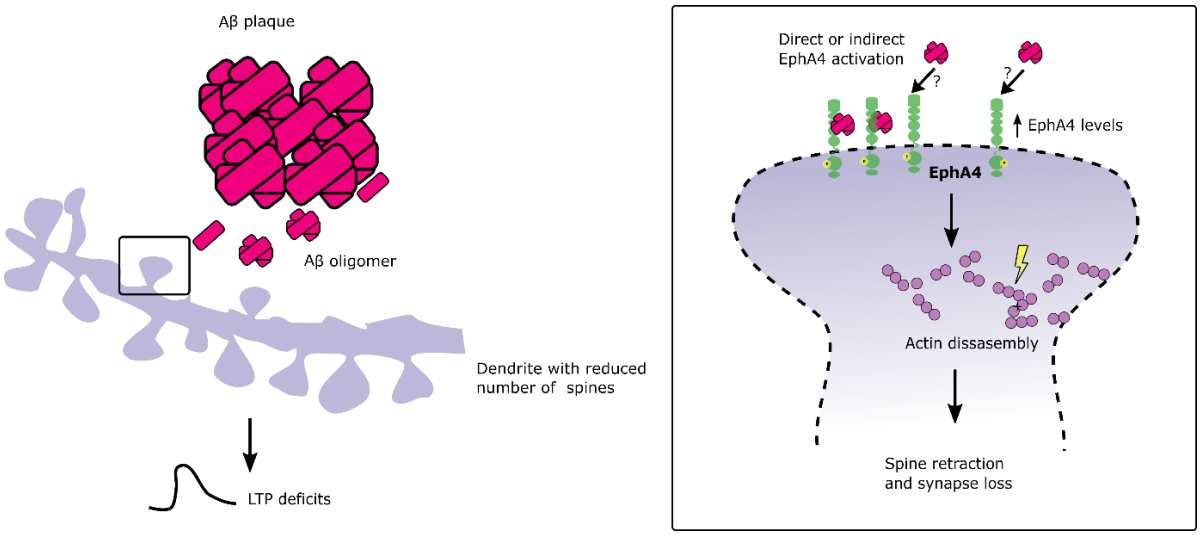

Figure 12 – EphA4 mediates Aβ-mediated synaptotoxicity in AD. EphA4 expression is upregulated in AD brains. Direct or indirect activation of EphA4 by AβO's stimulates cellular pathways that trigger actin disassembly, spine retraction, synapse loss and subsequent LTP deficits. Abbreviations: AD; Alzheimer's disease, AβO; amyloid-β oligomer, LTP; long term potentiation.

However, other and yet unexplored signaling pathways might link postsynaptic EphA4 activation to synapse dysfunction and loss in AD.

First, the co-appearance of EphA4, Fyn and tau in dendritic spines might hint towards a common involvement of these proteins in AD pathogenesis. Dendritic tau targets Fyn to the postsynaptic membrane where it is involved in synaptic plasticity via enhanced NMDAR stability (387). However, increased AβO-dependent Fyn activation induces aberrant NMDAR stimulation and excitotoxicity resulting in spine loss (157). In addition, Fyn might contribute to AD pathology via local dendritic translation and phosphorylation of tau (388,389). As EphA4 has been described to modulate and even stimulate Fyn signaling in several conditions (340,390,391), it could be hypothesized that increased EphA4 signaling in AD could contribute to Fyn-induced excitotoxicity and tau pathology.

Second, EphA4 might contribute to AD pathology mediated by the proline-rich tyrosine kinase 2 (Pyk2/PTK2B) protein. GWAS studies in sporadic AD patients revealed Pyk2 as one of the risk factors for LOAD (392,393). Pyk2 is a non-receptor tyrosine kinase similar to focal adhesion kinase (FAK) and is highly expressed in forebrain neurons and implicated in synaptic plasticity via several pathways, such as modulation of NMDAR function and spine dynamics (339,394–398). Interestingly, the latter is suggested to be, among others, influenced by EphA4 signaling as Pyk2 inactivation by EphA4 might contribute to β-integrin-mediated spine shortage and loss. These data suggest that EphA4-mediated Pyk2 inactivation might be of importance in AD-related synapse loss. However, contrarily, evidence suggests a role for aberrant Pyk2 activation in AD pathogenesis. Pyk2 activation has been thought to mediate Aβ-induced synaptic dysfunction and loss via activation of Fyn and/or RhoA (399,400). In addition, Pyk2 was identified as a tau kinase (401). Nevertheless, the role of Pyk2 is still controversial as both strategies to block and enhance Pyk2 signaling ameliorate synapse loss and cognitive dysfunction in two different mouse models for AD (400,402).

Last, the tau kinases c-Abl and cdk5 are downstream regulators of EphA4-mediated synaptic plasticity (160,337,403–405). As such, aberrant EphA4 activation in AD might enhance tau phosphorylation and aggregation, and consequently contribute to disease pathogenesis (166).

Although, *in vitro* experiments have shown that processing of EphA4 by γ-secretase is impaired by mutations in PS1 that are linked to familial AD, the precise mechanisms underlying the changes in EICD levels in a cohort of sporadic AD patients are unclear (295,406). Nevertheless, low EICD levels in brains of AD patients correlate with lower levels of active Rac1 and PSD95, suggesting that decreased EICD levels contribute in the synapse loss observed in the disease (406).

Besides its potential impact on synapse dysfunction and loss in AD, *in vitro* evidence hints towards a role for EphA4 activation in Aβ production via reduced proteasomal degradation of APP fragments (407).

Increased activation of EphA4 has been observed in the cortex of patients with depression and bipolar disorder, suggesting an involvement of EphA4 in these conditions. Changes in spine density in the prefrontal cortex and hippocampus contribute to the neurobiology of depression, rendering EphA4 an interesting target for these diseases. In support, blockage of EphA4 signaling has an anti-depressant-like effect in a mouse model for depression, associated with a normalization in spine density (391).

Chapter II: Research objectives

EphA4 is a tyrosine kinase receptor of the ephrin system which is highly expressed in the nervous system. In the host lab, EphA4 was identified as a modifier of ALS in both zebrafish and rodent models. Inhibition of EphA4 signaling slows down disease progression and improves motor function in rodent models for ALS by reducing the motor neuron vulnerability and enhancing neuromuscular junction innervation. Interestingly, knockdown of EphA4 also rescues axonal deficits in a zebrafish model for SMA, suggesting that the neuroprotective effect of EphA4 inhibition could translate to other motor neuron diseases. Therefore, **the first aim** of this doctoral thesis was to further investigate the modifying potential of reducing EphA4 levels in NMJ innervation, motor neuron survival, motor function and survival in a mouse model for SMA.

Furthermore, EphA4 is a mediator of spine morphology and plasticity, and of glial reactivity and inflammation, which are pivotal processes in the pathophysiology of AD. *In vitro*, inhibition of EphA4 signaling rescues Aβ-induced dendritic spine loss and LTP deficits. In addition, reversal of Aβ-dependent memory impairment via SORLA overexpression in a mouse model, is suggested to be associated with decreased EphA4 activation. Therefore, **the second aim** of this doctoral thesis was to investigate whether reduced EphA4 signaling could improve cognitive function in a mouse model for AD via alterations in spine number and morphology, and via attenuation of neuroinflammation.

Chapter III: Materials & Methods

1. Animal housing and study approval

Mice were housed in the KU Leuven animal facilities with a 12 h light-dark cycle at a temperature of 20°C. Animals were given free access to standard rodent chow and water. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH publications No. 8023, revised 1978). Experiments were designed to minimize animal discomfort and were approved by the Ethical Committee for Animal Research of the University of Leuven, Belgium (P097/2013, P178/2013 and P003/2019).

2. Origin and breeding of mice

2.1. SMA

Frozen sperm of SMNΔ7 mice (FVB.Cg-*Grm7*$^{Tg(SMN2)89Ahmb}$ *Smn1*^{tm1Msd} Tg(SMN2*delta7) 4299Ahmb/J; stock number: 005025) was kindly provided for revitalization by Dr. Achsel (University of Lausanne, Switzerland). Mice have only one *SMN* gene of which removal is embryonically lethal (67–69). Therefore, in addition to homozygous deletion of the murine *SMN* gene caused by insertion of a β-galactosidase cassette, SMNΔ7 mice carry homozygous transgenes for the human *SMN2 (hSMN2)* gene and cDNA of *SMN1* lacking exon7 *(hSMNΔ7)* to extend the lifespan to approximately two weeks (72). Control (Ctrl) mice were wild-type (smn$^{+/+}$) or heterozygous (smn$^{+/-}$) for the murine *SMN* allele and homozygous for *hSMN2* and *hSMNΔ7*.

To heterozygously delete EphA4 in SMNΔ7 mice, we crossbred these mice with EphA4$^{-/-}$ mice (302), which were kindly provided by Dr. Turnley in a C57/Bl6J background (University of Melbourne, Victoria, Australia). EphA4$^{-/-}$ mice were backcrossed to FVB/N background for more than 10 generations, before being intercrossed with SMNΔ7 mice, to obtain experimental mice in pure FVB/N background. The following experimental groups were obtained: Ctrl-EphA4$^{+/+}$ (smn$^{+/+}$-EphA4$^{+/+}$ and smn$^{+/-}$-EphA4$^{+/+}$), Ctrl-EphA4$^{+/-}$ (smn$^{+/+}$-EphA4$^{+/-}$ and smn$^{+/-}$-EphA4$^{+/-}$), SMA-EphA4$^{+/+}$ (smn$^{-/-}$-EphA4$^{+/+}$) and SMA-EphA4$^{+/-}$ (smn$^{-/-}$-EphA4$^{+/-}$). The day of birth was defined as postnatal day 0 (PND0). Both male and female mice were included, and due to the frequent birth of only one SMA pup per litter, it was not feasible to use littermate controls in each litter. All experiments were conducted by a researcher blinded for the genotype.

2.2. Alzheimer's disease

We crossbred APPPS1 mice with EphA4$^{flox/flox}$ (EphA4$^{tm1.1Bzh}$/J; stock number: 012916, The Jackson Laboratory) (408) and Camk2aCre (B6.Cg-Tg(Camk2a-Cre)T29-1Stl/J; stock number: 005359, The

Jackson Laboratory) mice (409) to generate APPPS1 mice with a profound loss of EphA4 in the forebrain from the first postnatal weeks on (Fig. 13). All mice were maintained in a C57/Bl6J background. All experiments were performed with mixed cohorts containing similar numbers of male and female mice and by a researcher blinded for the genotype.

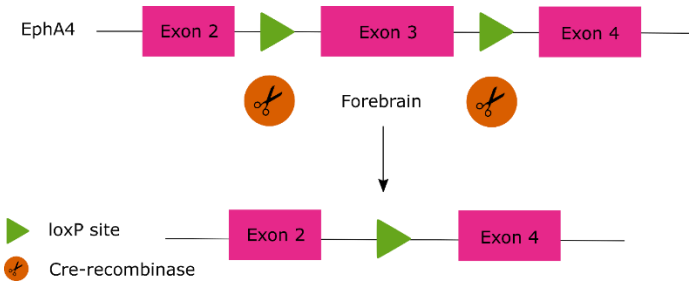

Figure 13 – Cre/lox-mediated loss of EphA4 in the forebrain of APPPS1 mice. EphA4$^{flox/flox}$ mice contain *EphA4* alleles in which exon 3 is flanked by two loxP sites. Cre-recombinase mediated excision of exon 3 induces splicing of exon 2 to exon 4 and a frameshift in the downstream coding sequence, resulting in a null allele. As Cre-recombinase is under control of the Camk2a promotor, this null allele is predominantly created in the forebrain. Abbreviations: Camk2a; calcium/calmodulin-dependent protein kinase II alpha.

3. Genotyping of mice

3.1. SMA

Mice were genotyped using tail biopsies obtained at PND1. Both *SMN* and *hSMN2* genotypes were determined via regular PCR using the following primer pairs (Table 3): Primer pair 1 to amplify a region in the murine smn gene, primer pair 2 to amplify a region of the β-galactosidase cassette, primer pair 3 to amplify the inserted SMN2 gene and primer pair 4 to amplify the non-inserted region. The *hSMNΔ7* transgene was genotyped using qPCR using primer pair 5 together with a 5'-CTTCTGGACCACCAATAATTCCCCCACC-3' probe. Digital droplet PCR (ddPCR) was used to genotype for EphA4 using a commercially available Taqman copy number assay (Mm00530479_cn, Thermo Scientific). A commercially available copy number assay targeting *AP3B1* was used as a reference gene for both qPCR and ddPCR (10031245, Bio-Rad).

Table 3 – Primer pairs used for genotyping of SMNΔ7 mice.

Primer pairs	Primer 1	Primer 2
1	5'-GTGTCTGGGCTGTAGGCATTG-3'	5'-GGCTGTGCCTTTTGGCTTATCTG-3'
2	5'-GCCTGCGATGTCGGTTTCCGCGAGG-3'	5'-CCAGCGCGGATCGGTCAGACG-3'
3	5'-CTGACCTACCAGGGATGAGG-3'	5'-GGTCTGTTCTACAGCCACAGC-3'
4	5'-CTGACCTACCAGGGATGAGG-3'	5'-CCCAGG TGGTTTATAGACTCAGA-3'
5	5'- TGCTGGCTGCCTCCATTT-3'	5'- GCATCATCAAGAGAATCTGGACAT-3'

3.2. AD

Mice were genotyped via regular PCR using tail biopsies obtained at weaning. The following primer pairs were used (Table 4): Primer pair 1 to amplify a region in the human *PS1* gene, primer pair 2 to amplify a region of the Cre-recombinase, primer pair 3 to differ between wild-type and floxed *EphA4* alleles.

Table 4 – Primer pairs used for genotyping of AD mice with conditional deletion of EphA4 in the forebrain.

Primer pairs	Primer 1	Primer 2
1	5'-CAGGTGCTATAAGGTCATCC-3'	5'-ATCACAGCCAAGATGAGCCA-3'
2	5'-GCGGTCTGGCAGTAAAAATATC-3'	5'-GTGAAACAGCATTGCTGTCACTT-3'
3	5'- GCA CAC TTA GCA ATT CAG TGT GGG -3'	5'-CCT GCA AAT TAA GGG CAG GAA GAG-3'

4. Determination of weight, motor function and survival in SMA mice

Weight was measured from PND1 on. From PND2 onwards, motor function was assessed using the righting reflex test every other day and the hindlimb suspension (HLS) test daily. During the righting reflex test, pups were placed on a flat surface on their backs, and the time to flip back to an upright position with all paws touching the bench was measured (with a cutoff of 60 s). The average of three consecutive trials with a 5-min resting period in between was calculated. The HLS test was performed using a 50 ml conical tube filled with cotton wool at the bottom and positioned upright in a tube holder. Pups were placed inside the tube with the hind paws over the rim of the tube and facing down. The time spent hanging before falling down in the tube, the number of pulls (attempts to get out of the tube using the hind-limb muscles) and the hind-limb score (a score based on the position of the hind-limbs and tail of the animal) were measured as previously described (El-Khodor *et al.*, 2008). In addition, a quantitative HLS test score (HLST score) was calculated via insertion of previous parameters in the following equation: $HLST = [(time\ spent\ hanging) + 10(\#\ of\ pulls)]\ x\ \frac{(HLS+1)}{4}$, as described by Heier and DiDonato, 2009. For all parameters, averages of two consecutive sessions with a 5-min resting period were calculated. In control mice, both tests were conducted only until PND8, as they were already strong enough to right immediately and escape the tube. For survival analysis, mice were monitored daily until found dead.

5. Quantification of SMN and EphA4 levels in SMA mice

To determine SMN and EphA4 protein levels, pups were euthanized with an overdose of Dolethal (20 mg/ml) on PND8. Whole spinal cords were collected, snap frozen in liquid nitrogen and stored at -80°C.

Samples were homogenised in RIPA buffer (Sigma-Aldrich, R0278) with protease (cOmplete; Roche, 11697498001) and phosphatase (phosSTOP; Roche, 4906845001) inhibitors using the MagNaLyser oscillator (Roche). Protein concentration was determined with the Pierce BCA protein assay kit (Thermo Scientific, 23225). For electrophoresis, we used 4-20 % precast acrylamide gels (Mini-PROTEAN® TGX™; Bio-rad, cat#456-1096) and 20 µg of protein were loaded for each sample. Proteins were transferred to Immobilon-P (PVDF) membrane (Millipore, IPVH00010) and subsequently blocked with 5% nonfat-dry milk (Blotting-Grade Blocker; Bio-Rad, cat#170-6404) and 5% bovine serum albumin (BSA; Serva Electrophoresis GmbH, 1193003) in Tris-buffered saline with 0.001% Tween® (TBS-T) for 1h at room temperature (RT). Membranes were incubated with the following primary antibodies in TBS-T with 1% BSA: C-terminal mouse anti-EphA4 (1/500; Invitrogen, 37-1600), mouse anti-SMN Clone 8 (1/5000; BD Biosciences, 610646) and mouse anti-β-actin (1/5 000; Sigma, A54411). An anti-mouse-HRP antibody (1/5 000, DAKO) was used as secondary antibody and was diluted in TBS-T containing 5% nonfat-dry milk. ECL or FEMTO ECL (Thermo Scientific, 32106 and 34095) was used as a substrate and the signal was detected using LAS4000 (GE Healthcare). Band optical density was quantified with the ImageQuantTL software (EG Biosciences).

6. Determination of neuromuscular junction innervation in SMA mice

Pups were euthanized with an overdose of Dolethal (20 mg/ml) on PND11. Splenius and longissimus muscles were dissected and fixed for 20 min in 4% PFA at RT. Muscles were quenched for 30 min in 0.1M glycine in PBS and subsequently incubated in Alexa 555-conjugated α-bungarotoxin (α-BTX; 1/250, Thermo Fisher Scientific, B35451) in PBS for 10 min to visualize the post-synaptic endplates. Next, muscles were incubated for 5 min in methanol at -20°C and blocked in 2% BSA diluted in PBS with 0.3% TritonX-100 (blocking solution) for 1h and subsequently incubated overnight with primary antibodies in blocking solution at 4°C. The following primary antibodies were used to visualize axons and the pre-synaptic terminal respectively: Alexa 488-conjugated rabbit anti-neurofilament-L C28E10 (1/500, Cell signaling, 2837S) and rabbit anti-synaptophysin YE269 (1/200, Abcam, ab32127). Alexa 647-labeled anti-rabbit antibody (1/500, Life Technologies) was used as a secondary antibody and muscles were incubated for 2h in blocking solution containing this antibody. Muscles were extensively washed in PBS after each step and all steps were performed at RT unless described otherwise. Image z-stacks were taken at sequential focal planes 1 µm apart for a total depth of ± 30 µm with a Leica TSC SP8 confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH) with a HC PL APO CS2 20x/0.75 dry lens. The innervation status of individual post-synaptic endplates was evaluated based on the co-localization of synaptophysin and α-BTX. Fully innervated NMJs were defined by a complete overlap of the endplate with synaptophysin, while partially innervated NMJs were incompletely

covered with synaptophysin. Fully denervated NMJs were lacking any pre-synaptic labeling. Illustrated images are maximum Z-projections created using the ImageJ software by Wayne Rasband (National Institutes of Health). For each smn genotype, EphA4$^{+/-}$ mice were normalized to EphA4$^{+/+}$ mice.

7. Determination of motor neuron survival in SMA mice

Pups were euthanized with an overdose of Dolethal (20 mg/ml) on PND11. Lumbar spinal cords were dissected and homogenized in TRIzol (Thermo Fisher Scientific, 15596026) using the MagNaLyser oscillator. Total RNA was precipitated with isopropanol, of which 1µg was used to prepare cDNA with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, 18080051). Quantitative PCRs were performed with the TaqMAN Fast Universal PCR Master Mix 2X (Thermo Fisher Scientific, 4364103) using 1/10 diluted cDNA and the following Taqman assays (IDT): Chat (Mm01221882_m1), Gapdh (Mm.PT.39a.1) and Polr2a (Mm.PT.58.13811327). PCR reaction was performed in a StepOnePlus instrument (Life Technologies) and relative gene expression was analysed with the Qbase+ software (Biogazelle).

8. Tissue collection in AD mice

After cognitive assessment, all mice were anesthetized with 10% Nembutal (Ceva chemicals). For immunoblot and Aβ extractions, mice were transcardially perfused with phosphate buffered saline (PBS) and the brain was microdissected to collect hippocampi and cortices. Samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis. For RNA *in situ* hybridization, amyloid plaque analysis and Golgi-Cox staining, mice were transcardially perfused with PBS and 4% paraformaldehyde (PFA). For RNA *in situ* hybridization and amyloid plaque analysis, brains were further fixated by overnight incubation in 4% PFA and cryoprotected in subsequently 10%, 20% and 30% sucrose gradients. Brains were frozen in ice cold isopentane and stored at -80°C until further analysis. For Golgi-cox staining, brains were processed as described below.

9. Quantification of EphA4, APP, PS1, GFAP and Iba1 levels in AD mice

Mouse hippocampi were homogenised in T-PER® Tissue protein extraction reagent (Thermoscientific, 78510) with protease (cOmplete; Roche, 11697498001) and phosphatase (phosphostop; Roche, 4906845001) inhibitors using the MagNaLyser (Roche). Protein concentration was determined with the Pierce BCA protein assay kit (Thermo Scientific, 23225). For electrophoresis, we used 4-20 % precast acrylamide gels (Mini-PROTEAN® TGX™; Bio-rad, cat#456-1096) and fifteen or twenty micrograms of protein were loaded for each sample. Proteins were transferred to Immobilon-P (PVDF)

membrane (Millipore, IPVH00010) and subsequently blocked with 5% nonfat-dry milk (Blotting-Grade Blocker; Bio-Rad, cat#170-6404) and 5% bovine serum albumin (Serva Electrophoresis GmbH, 1193003) in Tris-buffered saline with 0.001% Tween® (TBS-T) for 1h at room temperature. Membranes were incubated with the following primary antibodies: C-terminal mouse anti-EphA4 (1/500; Invitrogen, 37-1600), mouse anti-GAPDH (1/10 000; Thermo Scientific, AM4300), rat anti-PS1 (1/500; Millipore, MAB1563), an in-house made rabbit anti-APP (B63, 1/5 000), rat anti-GFAP (1/1000; Invitrogen, 2.2B10) and rabbit anti-Iba1 (1/1000; Abcam, 178847). Anti-mouse-HRP, anti-rat-HRP and anti-rabbit-HRP (all 1/5 000, DAKO) were used as secondary antibodies. ECL or FEMTO ECL (Thermo Scientific, 32106 and 34095) was used as a substrate and the signal was detected using LAS4000 (GE Healthcare). Band optical density was quantified with the ImageQuantTL software (EG Biosciences).

10. RNA in situ hybridization for EphA4 in AD mice

Cryosections of 30 µm thickness were post-fixated, dehydrated and dried. In situ hybridization was performed using the commercially available RNAscope® Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics), as stated in the manufacturer's instructions. Slides were incubated overnight with a probe specific for *EphA4* (Mm-EphA4-C1, ACD Diagnostics) and the signal was amplified using TSA Plus Cyanine 3 (1/500, Perkin Elmer). Cell nuclei were stained with Hoechst and slides were mounted using Prolong® Gold antifade mountant (Thermo scientific, P36934). Image z-stacks were taken every 2 µm for a total depth of 8 µm with a Leica TSC SP8 confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH) with an HC PL APO CS2 20x/0.75 dry lens and a pinhole of 0.6 Airy Units.

11. Behavioral testing in AD mice

11.1. Open field test

Exploration and anxiety were studied in the open field exploration test. Mice were dark-adapted for 30 min before being placed in the open field arena (50x50 cm^2). After one minute of habituation in the arena, exploratory behavior was recorded for 10 min using Anymaze software (Stoeltus) and total distance was measured as a parameter for locomotor activity. As mice will typically spend more time in the 'protected' periphery of the arena, and increased exploration of the 'unprotected' center of the field demonstrates anxiolytic behavior, the time spent in the open field center and in the small periphery were also measured.

11.2. Morris water maze (MWM) test

The MWM test was performed to study spatial learning and memory capacity. The standard hidden-platform acquisition of the Morris water maze was used (238,412). The maze consisted of a large circular pool (diameter 150 cm) filled with water (26°C) to a depth of 16 cm. Water was made opaque with non-toxic white paint to prevent animals from seeing the platform. The pool was divided in four imaginary quadrants and a circular platform (diameter 15 cm) was hidden 1 cm beneath the water surface at a fixed position. The pool was localized at the center of a room with various fixed cues (e.g. posters, computers, tables). The experimenter always sat in the same place. Mice were trained for 10 days to find the hidden platform during four trials per training day with a trial interval of 15 min. Mice were placed in the pool in one of the four quadrants and the starting quadrant was alternated during a training day. When mice were not able to find the platform within 100 s, they were guided to the platform and had to stay on it for 10 s, before being returned to their cages. Escape latency (average duration to find the platform during the four trials per day) was recorded with Ethovision software (Noldus). After the fifth and tenth learning day, mice had two days of rest followed by a probe trial to evaluate spatial retention memory. During this first and second probe trial, the platform was removed, and the time spent in each quadrant was measured for 100 s.

11.3. The sociability/preference for social novelty (SPSN) test

The SPSN test was performed in a large transparent Plexiglas box divided into three compartments by transparent Plexiglas walls with small square openings as described previously (413). Briefly, a holding cage was placed in the middle of the two outer-most compartments and the procedure consisted of three consecutive steps. First, mice were acclimatized in the middle compartment for 5 min (acclimatization phase). In a second phase (sociability trial), an unfamiliar mouse of the same sex (novel mouse) was introduced in one of the holding cages in one outer compartment, while the other holding cage remained empty. Exploratory behavior towards the novel mouse and the empty holding cage was measured for 10 min. In the third phase (social memory trial), another unfamiliar mouse of the same sex (novel mouse) was introduced in the other compartment. During this last phase, exploratory behavior towards the familiar and the novel mouse was recorded for 10 min. Exploratory behavior was defined as sniffing time towards a holding cage (with or without a mouse in it). The location of the novel and familiar mouse was counterbalanced across testing animals and the apparatus was cleaned thoroughly with water after each mouse and with ethanol when a mouse of a different gender was tested. Behavior was recorded using Anymaze software (Stoeltus) and sniffing times (ST) were measured manually by watching the video recordings. We calculated preference ratio during the

sociability trial as ST novel mouse/(ST novel mouse + ST empty cage), and recognition ratio during the social memory trial as ST novel mouse/(ST familiar mouse + ST novel mouse).

12. Golgi-Cox staining and spine analysis in AD mice

Brains were stained using the FD Rapid GolgiStain kit (FD NeuroTechnologies, PK401) according to manufacturer's instruction. In brief, brains were immersed in a 1:1 mixture of FD Solution A and B for two weeks at room temperature in the dark. Next, brains were transferred to FD Solution C for 48 h at 4°C in the dark. After the first 24 h, Solution C was renewed. Brains were frozen and kept at -80°C until further processing. Coronal cryosections of 100 µm thickness were cut with a CryoStar NX70 cryostat (ThermoFischer Scientific). Slices were transferred to small droplets of FD Solution C on gelatin coated slides (FD NeuroTechnologies, P0101). Sections were dried for at least three hours at room temperature before staining. Further staining was performed as described in the product manual. For dendritic spine analysis, images of apical dendrites from ventral CA1 pyramidal neurons of the hippocampus were taken using a Leica TSC SP8 confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH) with a HC PL APO CS2 63x/1.40 oil lens. A transmitted light detector was used to mimic bright-field imaging. Image z-stacks were obtained every 0.2 µm with a 2048x2048 pixel resolution. Dendritic segments of approximately 20 µm in length from two regions of the CA1 stratum radiatum (SR) were imaged: 30-120 µm from the soma (proximal SR) and 120-300 µm from the soma (distal SR). Minimum Z-projections were created using ImageJ by Wayne Rasband (National Institutes of Health) and were loaded into the Neurolucida 360 software to trace dendritic segments and quantify dendritic spine numbers, length and head thickness. Approximately six segments (of which not more than two segments from the same neuron) per region per mouse were included in the study.

13. Immunohistochemistry for GFAP and Iba1 in AD mice

Free-floating coronal sections of 30 µm thickness were cut with a CryoStar NX70 Cryostat (ThermoFisher Scientific). Sections were blocked in 10% normal donkey serum (Sigma) in PBS-0.1% TritonX-100 (PBS-T) for 1h at room temperature before overnight incubation of the primary antibody in 5% normal donkey serum in PBS-T at 4°C. The following primary antibodies were used: mouse anti-GFAP (1/500, Sigma, G3893), rabbit anti-Iba1 (1/250, Wako, Cat.#019-19741) and rabbit anti-PU.1 (1/75, Cell signaling, #2258). Nuclei were stained with Hoechst. Slices were mounted with Prolong® Gold antifade mountant (Thermo scientific, P36934). Image z-stacks were taken every 2 µm for a total depth of 8 µm with a Leica TSC SP8 confocal laser scanning microscope (Leica Microsystems Heidelberg

GmbH) with an HC PL APO CS2 20x/0.75 dry lens and a pinhole of 0.6 Airy Units. The total area of GFAP and Iba1 positive staining was quantified using ImageJ by Wayne Rasband (National Institutes of Health). The number of microglia was determined by counting the nuclei positive for the microglial transcription factor PU.1. For each staining, two brain slices per animal were stained and two images of the CA1 region were taken per slice. Average numbers of these four images were considered as representative for the whole CA1 region.

14. Amyloid plaque load quantification in AD mice

Mouse brains were cut in free-floating series of 30 mm thick coronal sections using a CryoStar NX70 Cryostat (ThermoFisher Scientific). Every ninth section was assigned to one series. Consequently, every series is representative for the whole selected brain area. Sections were stored in PBS with 0.02% sodium azide at 4°C. Slices were immersed in 0.015% Thioflavin S (ThioS) solution for 10 min and washed in PBS-T before incubation with TO-PRO®-3 staining solution (Thermo Scientific, T3605) for 30 min. Slices were mounted with Prolong® Gold antifade mountant (Thermo scientific, P36934). Fluorescent images of one series per animal were made with a Leica DMI 6000B inverted microscope. Images covering the whole hippocampus in one section were made using a 10x objective and all these images were merged to one mosaic picture. An average of 13 mosaic pictures were made per animal, depending on the rosto-caudal extension of the hippocampus. Plaque number and plaque load (% of hippocampal area positive for ThioS) was quantified using ImageJ software by Wayne Rasband (National Institutes of Health). In brief, particle analyzer was used and a threshold was set to only detect plaques. Particles larger than 75 µm² were considered as plaques. The average of plaque densities and plaque burden (the percentage of hippocampal area covered with ThioS-positive amyloid plaques) for all pictures per animal was considered as representative for the whole hippocampus.

15. Quantification of Aβ levels in AD mice

Mouse hippocampi were homogenized in T-PER® tissue extraction reagent (Pierce) supplemented with protease (cOmplete; Roche, Vilvoorde, Belgium) and phosphatase (phosphostop; Roche, Vilvoorde, Belgium) inhibitors using the MagNaLyser (Roche, Vilvoorde, Belgium). Homogenised hippocampi were centrifuged for 1 h at 4°C at 100 000g and supernatant was used for ELISA to measure TBS (tris-buffered saline) –soluble Aβ levels. For the GuHCl soluble Aβ levels, pellets were dissolved via sonication in a 6M GuHCl extraction buffer and centrifuged for 20 min at 4°C at 130 000g. Supernatant was diluted 1/12 to reduce the concentration of GuHCl and was used for ELISA. Aβ40 and Aβ42 levels

were determined using commercially available ELISA kits from Wako Chemicals (290-62601 and 294-64701).

16. Statistical analysis

For SMA, a power analysis was performed. Based on previous results with other modifying treatments in SMNΔ7 mice (414,415), we estimated a sample size of 13 animals per group to detect a relevant 20% difference in survival with 80% power at an α=0.05. Similarly, a sample size of 3 animals per group was estimated to detect a relevant 20% difference in fully innervated NMJs with 80% power at an α=0.05. As researchers were blind for genotype at the time of muscle extraction and analysis, a total of 27 splenius muscles and 25 longissimus muscles were analyzed to ensure a sufficient number of animals in each experimental group.

Due to differences in survival between mice, last weight, righting reflex test and HLS test observations in SMA mice were carried forward to enable repetitive measures statistical analysis. Survival was analyzed using the Log-rank Mantel-Cox test.

Unpaired two-tailed Student's t-test was used for the comparison of two means. One-way, two-way and two-way with repeated measures ANOVA tests were used for multiple group analysis. Data were tested for normality using D'Agostino and Pearson's or, in case of small sample sizes, the KS normality test. Kruskal-Wallis and Mann-Whitney U tests were used when the data was not normally distributed. Student's t-tests, one-way, two-way ANOVA, Kruskall-Wallis, Mann-Whitney U and Log-rank Mantel-Cox tests were performed using GraphPad Prism software version 7 (GraphPad software Inc), while two-way with repeated measures ANOVA tests were performed with IBM SPSS Statistics 25 software (IBM). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, **** (comparison of different genotype groups). $p \leq 0.0001$, $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{++++}p \leq 0.0001$ (comparison of performance in the Morris water maze in one genotype group compared to chance level). All data represents mean ± SEM.

Chapter IV: Lowering EphA4 does not ameliorate disease in a mouse model for severe spinal muscular atrophy

The work presented in this chapter is published in the following scientific paper:

Poppe L, Smolders S, Rué L, Timmers M, Lenaerts A, Storm A, Schoonaert L, de Boer A, Van Damme P, Van Den Bosch L, Robberecht W, Lemmens R. Lowering EphA4 does not ameliorate disease in a mouse model for severe spinal muscular atrophy. Frontiers in Neuroscience. 2019; 13:1233.

1. Introduction

SMA affects spinal motor neurons within the central nervous system and patients present with muscle weakness and paralysis resulting from NMJ dysfunction and denervation caused by reduced levels of the SMN protein (3,52,53). Nusinersen and Zolgensma are the only approved drugs for treatment of SMA patients, and both increase the production of functional SMN protein (81,84). Still, other neuroprotective therapies could provide additional support for patients, or would be of importance for patients that are intolerant, not responsive to or excluded from these SMN-targeting therapies (416). One interesting candidate is EphA4, a tyrosine kinase of the ephrin system which functions as an important axonal repellent cue during formation of the nervous system (417). In rodent models for ALS, another motor neuron disorder, inhibition of EphA4 signaling attenuates motor neuron degeneration and improves motor function. Loss of neuronal EphA4 reduces the vulnerability of motor neurons for degeneration and is thought to enhance neuromuscular junction innervation, probably via increased sprouting and re-innervation capacity of the axons (321). Interestingly, knockdown of EphA4 also rescued the axonal deficits in a zebrafish model for SMA (321), suggesting that the neuroprotective effect of EphA4 inhibition is independent of the cause of degeneration. In this study, we aimed to further investigate whether the modifying potential of EphA4 could translate to a mouse model for SMA (Fig. 14).

We made use of the SMNΔ7 mouse model for severe SMA. SMNΔ7 mice have an average lifespan of approximately two weeks and mice show severe motor function abnormalities together with modest loss of motor neurons in the anterior horn of the spinal cord, functional and morphological abnormalities at the neuromuscular junctions, muscle denervation and muscle atrophy (44,47–49,57,72). We heterozygously deleted EphA4 in this mouse model as removal of one EphA4 allele was sufficient to improve the disease phenotype in an ALS mouse model (321). Moreover, mice with a full deletion of EphA4 develop a ‘hopping gait’ phenotype, and show low birth rates and reduced body weight during the first postnatal weeks (302,307,321). In order to explore whether heterozygous deletion of EphA4 could ameliorate the disease phenotype, we aimed to investigate NMJ innervation, motor neuron survival, motor function and survival in this mouse model for SMA.

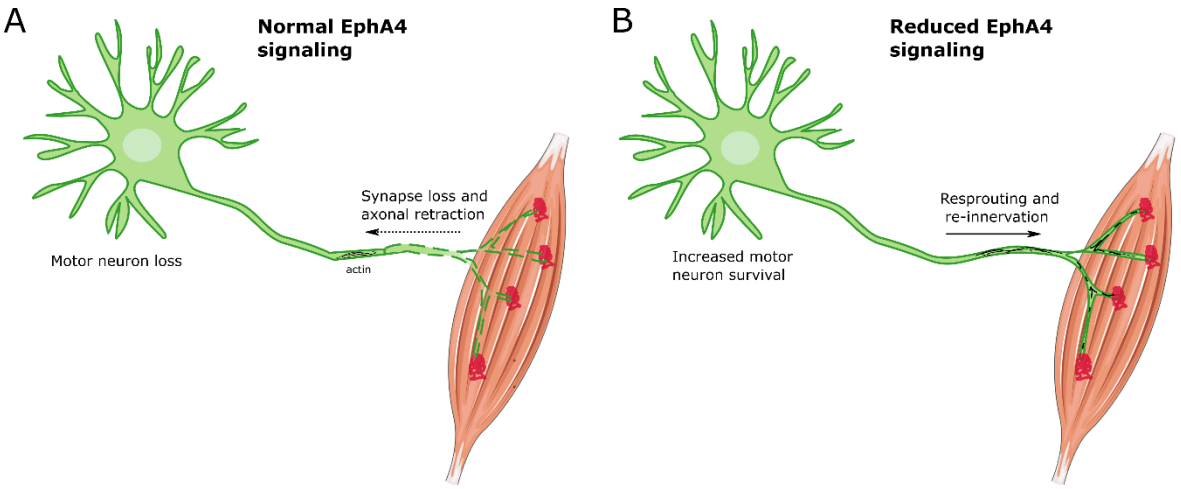

Figure 14 - Hypothesized beneficial effect of EphA4 signaling reduction in SMA. (A) SMA is characterized by synapse loss and axonal retraction at the NMJ's, resulting in motor neuron degeneration and loss. (B) Reduced EphA4 signaling might stimulate axonal re-sprouting and re-innervation of NMJ's, and prevent motor neuron loss. Abbreviations: SMA; spinal muscular atrophy, NMJ; neuromuscular junction.

2. Results

2.1. Loss of EphA4 does not improve motor function or survival in SMNΔ7 mice

Crossbreeding of EphA4$^{+/-}$ mice with SMNΔ7 mice, reduced EphA4 protein levels by ± 50% in Ctrl-EphA4$^{+/-}$ (51.7 ± 12.7%) versus Ctrl-EphA4$^{+/+}$ (100 ± 27.2%) mice, and in SMA-EphA4$^{+/-}$ (45.1 ± 13.7%) versus SMA-EphA4$^{+/+}$ (100 ± 24.4%) mice as confirmed by Western blotting of spinal cord lysates at PND8 (Fig. 15A,B). Reduction of SMN protein was similar in mice with normal *versus* lower EphA4 expression levels (Fig. 15A,C).

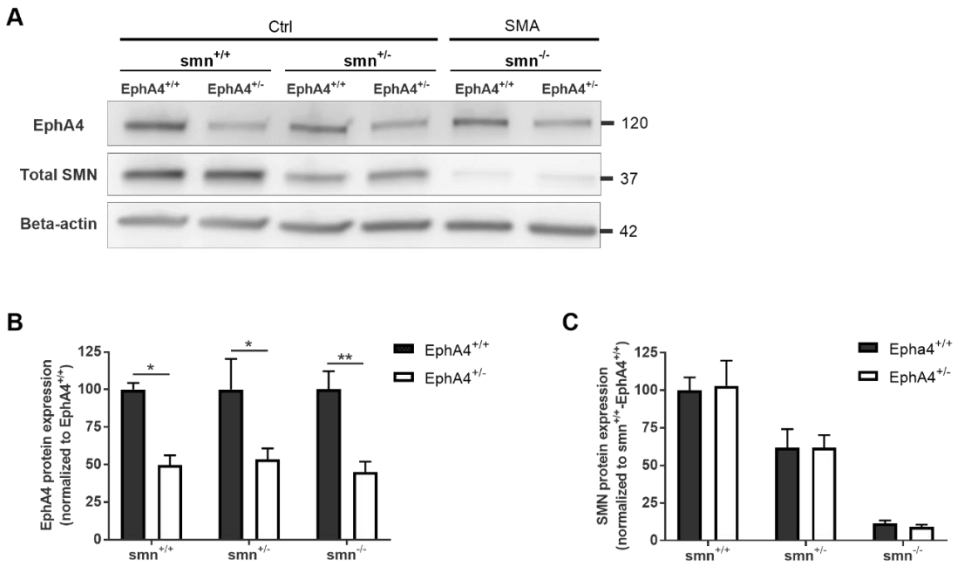

Figure 15 – EphA4 levels are reduced in the spinal cord of SMNΔ7 mice, without affecting SMN protein levels. Representative images (A) and quantifications (B, C) of a Western blot analysis of EphA4 and SMN levels in spinal cord lysates of Ctrl (smn$^{+/+}$ and smn$^{+/-}$) and SMA (smn$^{-/-}$) pups are shown. Beta-actin protein levels were used as a loading control (two-way ANOVA with Sidak's multiple comparison test, n=4-5 mice/group). *p ≤ 0.05, **p ≤ 0.01. Abbreviations: Ctrl; control.

We evaluated motor function in control and SMA mice with normal versus reduced EphA4 levels with the righting reflex test and the HLS test at regular time points during disease progression. Ctrl-EphA4$^{+/+}$ and Ctrl-EphA4$^{+/-}$ pups showed normal development of motor function, as reflected in the time to right during the righting reflex test (Fig. 15A) and in the increased hanging time, number of pulls, and HLST score (Fig. 15B-E) during the HLS test. Behavioral analysis of SMA-EphA4$^{+/+}$ pups revealed a compromised righting ability and performance during the HLS test which did not improve by reducing the expression of EphA4 (Fig. 16A-E). We monitored weight as an evaluation of general health and muscle mass. While Ctrl-EphA4$^{+/+}$ and Ctrl-EphA4$^{+/-}$ mice showed a continuous gradual increase in body mass, weight gain stagnated in SMA-EphA4$^{+/+}$ and SMA-EphA4$^{+/-}$ mice (Fig. 16F) with no difference between the two genotypes. Finally, we investigated whether loss of EphA4 extended lifespan in SMA mice. The average lifespan of SMA-EphA4$^{+/+}$ mice (13.9 ± 2.4 days) was similar to SMA-EphA4$^{+/-}$ mice (13.4 ± 2.7 days) (Fig. 16G).

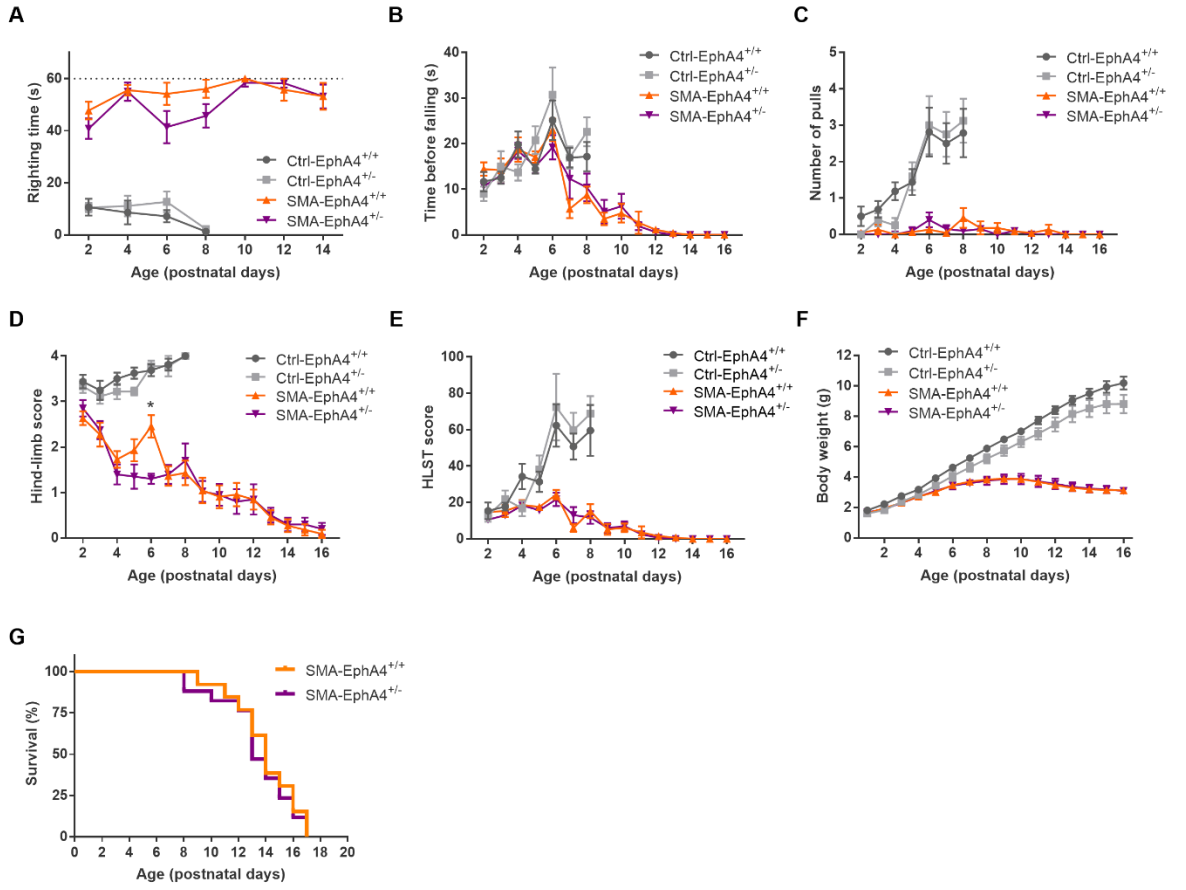

Figure 16 –Decrease of EphA4 does not alter motor function and survival in SMNΔ7 mice. Motor performance as assessed in the righting reflex test (A) and HLS test (B-E), and body weight (F) were monitored during disease progression in SMA-EphA4$^{+/+}$ (n = 11) and SMA-EphA4$^{+/-}$ (n = 10) pups. Ctrl-EphA4$^{+/+}$ (n = 8) and Ctrl-EphA4$^{+/-}$ (n = 9) pups were included as controls. Two-way repeated measures ANOVA with Sidak's multiple comparison test was used to compare Ctrl-EphA4$^{+/+}$ versus Ctrl-EphA4$^{+/-}$ pups and SMA-EphA4$^{+/+}$ versus SMA-EphA4$^{+/-}$ pups. Survival analysis (G) in SMA-EphA4$^{+/+}$ (n = 13) and SMA-EphA4$^{+/-}$ (n = 17) pups (Log-rank Mantel-Cox test). *$p \leq 0.05$. Abbreviations: Ctrl; control, HLS; Hind-limb score.

2.2. Decrease of EphA4 does not increase neuromuscular junction innervation in SMNΔ7 mice

Since lowering EphA4 did not improve motor function, nor survival in SMNΔ7 mice, we confirmed the lack of a modifying role for EphA4 by evaluating innervation status of NMJs of two severely affected axial muscles in the SMNΔ7 mouse model, the splenius and longissimus capitis muscles (415). At PND11, all NMJs were fully innervated in Ctrl-EphA4$^{+/+}$ and Ctrl-EphA4$^{+/-}$ mice in both muscles (Fig. 17A,B). In contrast, a profound denervation of NMJs occurred in SMA-EphA4$^{+/+}$ mice, with only 61% and 34% of NMJs remaining fully innervated in the splenius and longissimus muscles respectively (Fig. 17A,B). Loss of EphA4 did not affect the innervation status of the NMJs in both muscle types (Fig. 17A,B). As SMNΔ7 mice also present with a modest loss of motor neurons in the spinal cord (72), we additionally evaluated expression levels of the motor neuron marker gene Chat in the lumbar spinal cord of Ctrl and SMA mice at PND11. As expected, we observed a reduction of Chat mRNA levels in SMA-EphA4+/+ mice in comparison to Ctrl mice. Loss of EphA4 in SMA mice (SMA-EphA4+/-) did not alter Chat mRNA levels, suggesting no effect of reducing EphA4 expression on motor neuron survival in SMNΔ7 mice (Fig. 17C).

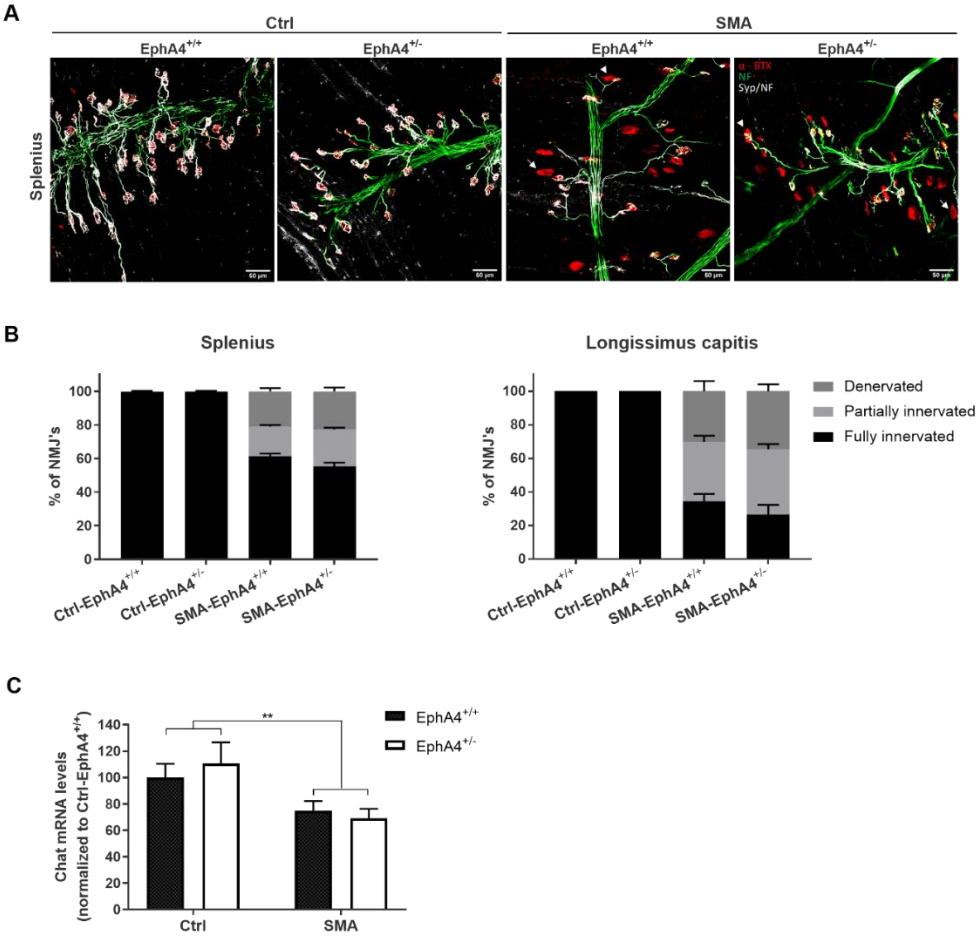

Figure 17 – Decrease of EphA4 does not improve neuromuscular junction innervation in SMNΔ7 mice. Innervation status of NMJs in affected muscles was determined in pups at PND11 via immunohistochemical labeling with antibodies specific for NF-L and synaptophysin. Alpha-bungarotoxin was used to label the motor endplates. Representative images (A) and quantifications (B) of fully innervated, partially innervated (arrowhead) and denervated (arrow) NMJs in the splenius and longissimus capitis muscles of Ctrl-EphA4$^{+/+}$ (n = 6), Ctrl-EphA4$^{+/-}$ (n = 4), SMA-EphA4$^{+/+}$ (n = 9-10) and SMA-EphA4$^{+/-}$ (n = 6-7) pups are shown (two-way ANOVA with Sidak's multiple comparison test). (C) Quantification of a quantitative PCR analysis of Chat mRNA expression levels in the lumbar spinal cord of Ctrl-EphA4$^{+/+}$ (n = 5), Ctrl-EphA4$^{+/-}$ (n = 4), SMA-EphA4$^{+/+}$ (n = 8) and SMA-EphA4$^{+/-}$ (n = 7) pups at PND11 (two-way ANOVA with Sidak's multiple comparison test). Expression data was normalized to Gapdh and Polr2a. $^{**}p \leq 0.01$. Scale bar = 50µm. Abbreviations: NF-L; neurofilament-L, Ctrl; control.

3. Conclusions

In order to study the modifying role of EphA4 in SMA, we heterozygously deleted EphA4 in SMNΔ7 mice as removal of one EphA4 allele was sufficient to improve the disease phenotype in an ALS mouse model (321). Similar to other findings, SMA-EphA4$^{+/+}$ pups showed a compromised motor function in the righting reflex and HLS test, and an average lifespan of ±13 days. In addition, modest loss of spinal motor neurons was observed, in combination with severe denervation of NMJ's of the splenius and lonigissimus capitis muscles (72,410,414,415). Loss of EphA4 (SMA-EphA4$^{+/-}$) did not improve motor function and survival of SMA pups, which was in accordance with the lack of improvements in lumbar motor neuron survival and neuromuscular junction innervation.

The results in this chapter demonstrate that the modifying effect of EphA4 in rodent models for ALS, does not translate to the SMNΔ7 mouse model for severe SMA. This differential effect could be explained by several clinical and pathological dissimilarities between these diseases and mouse models, such as differing disease severity, NMJ re-innervation capacity and pathology, and motor neuron vulnerability (3,44,45,47,57,72,376,377,418,419).

Chapter V: EphA4 loss improves social memory performance and alters dendritic spine morphology and microglial phenotype in a mouse model of Alzheimer's disease

The work presented in this chapter is partially published in the following scientific paper:
Poppe L, Rué L, Timmers M, Lenaerts A, Storm A, Callaerts-Vegh Z, Courtand G, de Boer A, Smolders S, Van Damme P, Van Den Bosch L, D'Hooge R, De Strooper B, Robberecht W, Lemmens R. Alzheimers Research & Therapeutics; 2019: 11(1): 102.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder, characterized by progressive decline in memory, together with multiple cognitive impairments compromising the patient's daily life (94). AD is the underlying cause of 60-70% of the dementia cases. Pathological features observed in AD brains are the extracellular amyloid depositions of Aβ peptides into senile plaques and the intraneuronal neurofibrillary tangles, composed of hyperphosphorylated tau protein (109). Moreover, neuroinflammatory features, such as astrogliosis and microgliosis are present in brains of AD patients and appear to be important drivers of the disease (194,225,226,420,421). Dysfunction and loss of synapses are early events in AD and correlate with cognitive impairments in patients suggesting synaptic dysfunction as the underlying cause of cognitive deficits in patients (135,422–424). So far, no cure for AD exists and current treatments generally concentrate on symptomatic care (239–241). Meanwhile, research mainly focuses on targeting the amyloid and tau pathology present in the brains of AD patients (244,425,426). However, therapies aimed at neuroinflammation and synapse dysfunction are valid alternative strategies (127).

Interestingly, EphA4 is highly expressed in the adult cortex and hippocampus, and functions as a crucial mediator of synapse morphology, synaptic functionality and plasticity. Interaction of postsynaptic EphA4 with ephrin-A3 in hippocampal slices induces spine retraction, a process involved in synapse pruning (323,327,337). In the context of AD, postsynaptic EphA4 activation is suggested to be directly or indirectly triggered by AβO's. Genetic reduction or pharmacological blockage of EphA4 rescues Aβ-induced dendritic spine loss and LTP deficits in cultured hippocampal slices and primary hippocampal cultures (160,386). In addition, reversal of Aβ-dependent memory impairment in a SORLA-overexpressing mouse, is suggested to be associated with decreased EphA4 activation and redistribution to the post-synaptic densities (385). Finally, EphA4 is also a modulator of neuroinflammation, as shown in mouse models of SCI, where loss or inhibition of EphA4 reduces reactive astrocytosis and alters the microglial and neuroinflammatory gene expression profiles at the glial scar (353,358,360). In this study, we investigated whether the beneficial effects of reduced EphA4 signaling on Aβ-induced spine pathology and neuroinflammation, would translate into a mouse model of AD, and ultimately ameliorate cognitive performance (Fig. 18).

We specifically reduced EphA4 levels in neurons of the forebrain of the APPPS1 mouse model from the first postnatal weeks on. APPPS1 mice develop Aβ pathology early in life accompanied by astro- and microgliosis, dendritic spine loss, and cognitive decline from 9 months on [189,235, 237]. In these mice, we investigated cognitive function together with dendritic spine density and morphology, neuroinflammation and amyloid pathology.

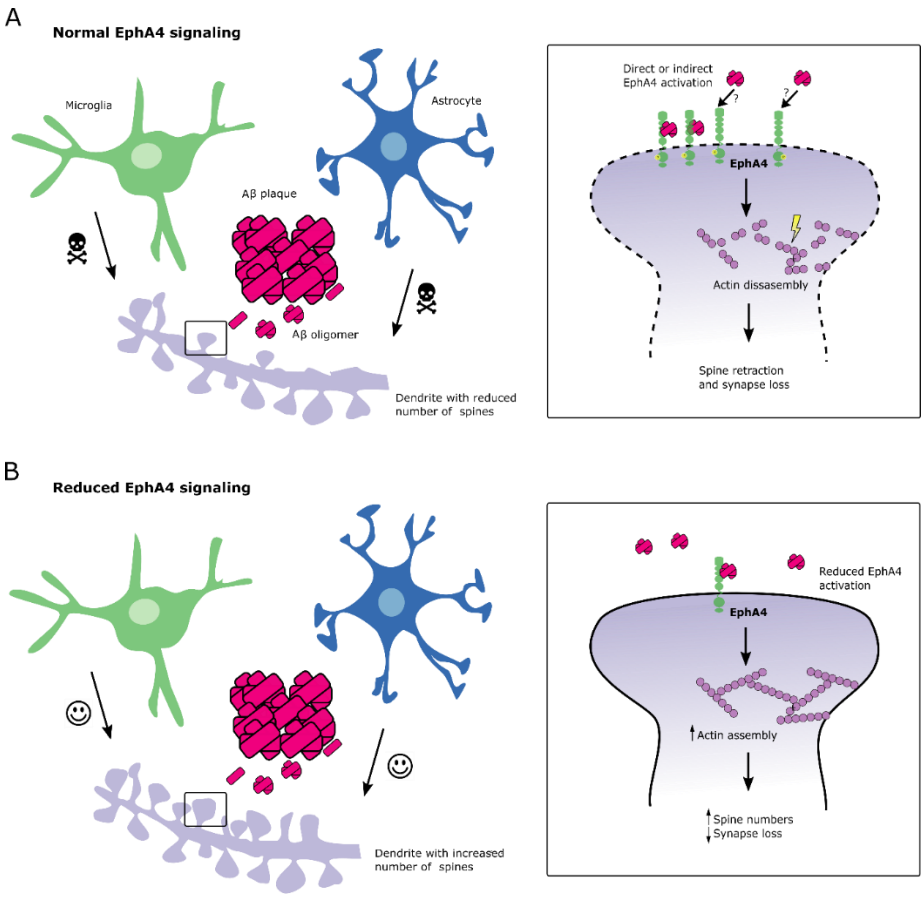

Figure 18 – Hypothesized beneficial effects of reduced EphA4 signaling in AD. (A) AD is characterized by the aggregation of Aβ into toxic oligomers and extracellular plaques. Direct or indirect activation of EphA4 receptors in dendritic spines by Aβ O's disturbs actin assembly, resulting in spine retraction and synapse loss. In addition, Aβ plaques are surrounded by reactive astrocytes and activated microglia, which might be neurotoxic and contribute to synapse loss. (B) Reduced EphA4 signaling might ameliorate the disease via attenuation of Aβ-induced spine loss and synapse loss. In addition, harmful neuroinflammation might be tempered via a decrease in reactive astrocytosis and an alteration in the microglial phenotype. Abbreviations: AD; Alzheimer's disease, Aβ; amyloid-β; AβO, amyloid-β oligomer.

2. Results

2.1. Generation of APPPS1 mice with loss of EphA4 protein in the forebrain

We crossbred APPPS1 mice with EphA4flox/flox and Camk2aCre mice to specifically decrease EphA4 expression in neurons of the forebrain in APPPS1 mice. Western blotting confirmed a strong reduction in cortical and hippocampal EphA4 protein in EphA4$^{flox/flox}$ x Camk2aCre (EphA4-KO) versus EphA4$^{flox/flox}$ (Ctrl) mice and EphA4$^{flox/flox}$ x Camk2aCre x APPPS1 (AD;EphA4-KO) versus EphA4$^{flox/flox}$ x APPPS1 (AD) mice (Fig. 19A-D). To investigate the regional recombination efficiency in the hippocampus of the Camk2aCre mouse, we performed in situ hybridization using RNA scope with specific probes for *EphA4*. *EphA4* mRNA levels were low in the dentate gyrus (DG) and CA3 regions of the hippocampus and almost absent in the CA1 region (Fig. 19E). In AD;EphA4-KO mice, hAPP and hPS1 expression was similar compared to AD mice, as determined by Western blot (Fig. 19F-G).

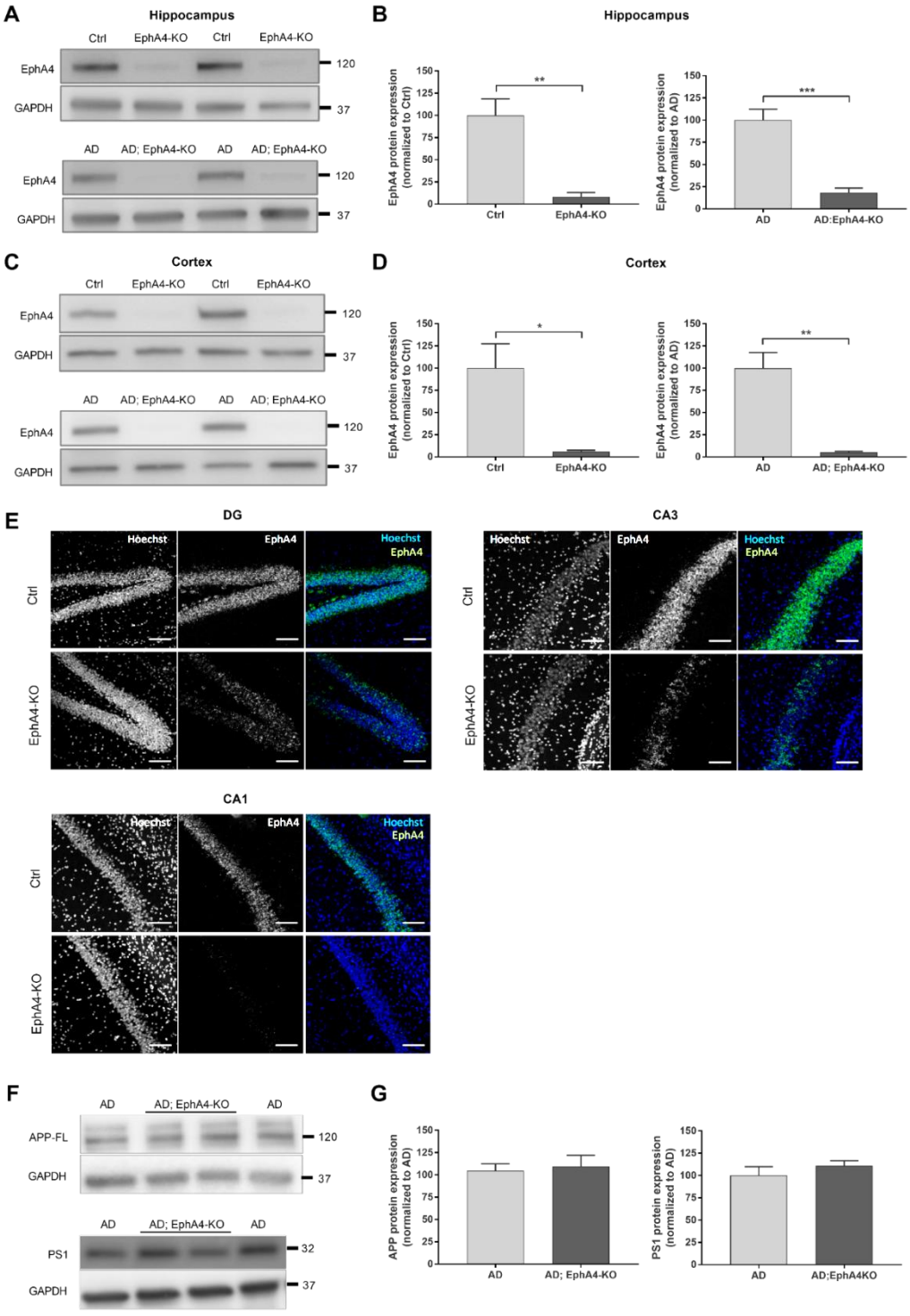

Figure 19 – Reduction of EphA4 protein and mRNA levels in the hippocampus and cortex of APPPS1 mice. Representative images (A) and quantification (B) of a Western blot analysis of hippocampal EphA4 protein levels in Ctrl and EphA4-KO mice (upper blot), and AD and AD;EphA4-KO mice (lower blot) with GAPDH protein levels as a loading control (unpaired t-test and Mann-Whitney test respectively, n=4-8 mice/group). Representative images (C) and quantification (D) of a Western blot analysis of cortical EphA4 protein levels in Ctrl and EphA4-KO mice (upper blot) and AD and AD;EphA4-KO mice (lower blot). GAPDH protein levels were assessed to control for equal loading (unpaired t-test, n=4 mice/group). (E) Representative images of RNA scope with specific probes for EphA4 in the DG, CA3 and CA1 region of the hippocampus of Ctrl and EphA4-KO mice. Hoechst was used to stain cell nuclei. Representative images (F) and quantifications (G) of Western blot analysis with antibodies specific for human APP and PS1 in AD and AD;EphA4-KO mice (unpaired t-test, n=8-10 mice/group). *p ≤ 0.05, **p ≤ 0.01. If no * is shown in the graph, this implies no significance. Scale bar = 100µm. Abbreviations: Ctrl; control.

2.2. EphA4 knock-down improves social memory, but not spatial memory in APPPS1 mice

To determine whether EphA4 knock-down ameliorates the hippocampus-dependent cognitive memory deficits observed in the APPPS1 mouse model, we assessed spatial learning and memory with the Morris water maze test. Ctrl and EphA4-KO mice efficiently learned the location of the hidden platform as reflected in the gradual reduction in time to reach the platform (escape latency) (Fig. 20A). During the probe trials, Ctrl and EphA4-KO mice spent more time in the target quadrant compared to the chance level, showing that they were able to retrieve the information previously learned (Fig. 20B,C). In contrast, AD mice had reduced learning capacities as illustrated by increased time to reach the platform and inability to retrieve information from the previous training sessions in the probe trials (Fig. 20B,C). Loss of EphA4 in AD mice (AD;EphA4-KO) did not affect spatial learning and memory performance in this test (Fig. 20A-C).

We next studied the ability to remember social interactions, another hippocampus-dependent memory function, with the SPSN test. During the sociability trial, all groups showed normal social behavior as revealed by the preference to explore a novel mouse in comparison with an empty cage (Fig. 20D-F), although this trend did not reach statistical significance for the preference ratio in AD ($p=0.085$) and AD;EphA4-KO mice ($p=0.095$). After the introduction of a novel mouse in the social memory trial, Ctrl and EphA4-KO mice preferred to explore the novel mouse in comparison to the familiar mouse. AD mice spent similar time sniffing the novel and the familiar mouse indicative of impaired social memory. This impaired social memory was no longer present in AD;EphA4-KO mice since they showed more interest in the novel mouse (Fig. 20G-I).

As alterations in activity and anxiety levels might affect the performance in the memory tasks, the open field exploration test was used to assess these parameters for the different groups. EphA4-KO mice did not differ from Ctrl mice in activity (total distance covered in the field) and anxiety levels (time spent in the small periphery and center of the field) (Fig. 20J-L). AD mice were more active than Ctrl and EphA4-KO mice with no changes in anxiety (Fig. 20J-L). Loss of EphA4 in AD mice (AD;EphA4-KO) did not affect these parameters (Fig. 20J-L).

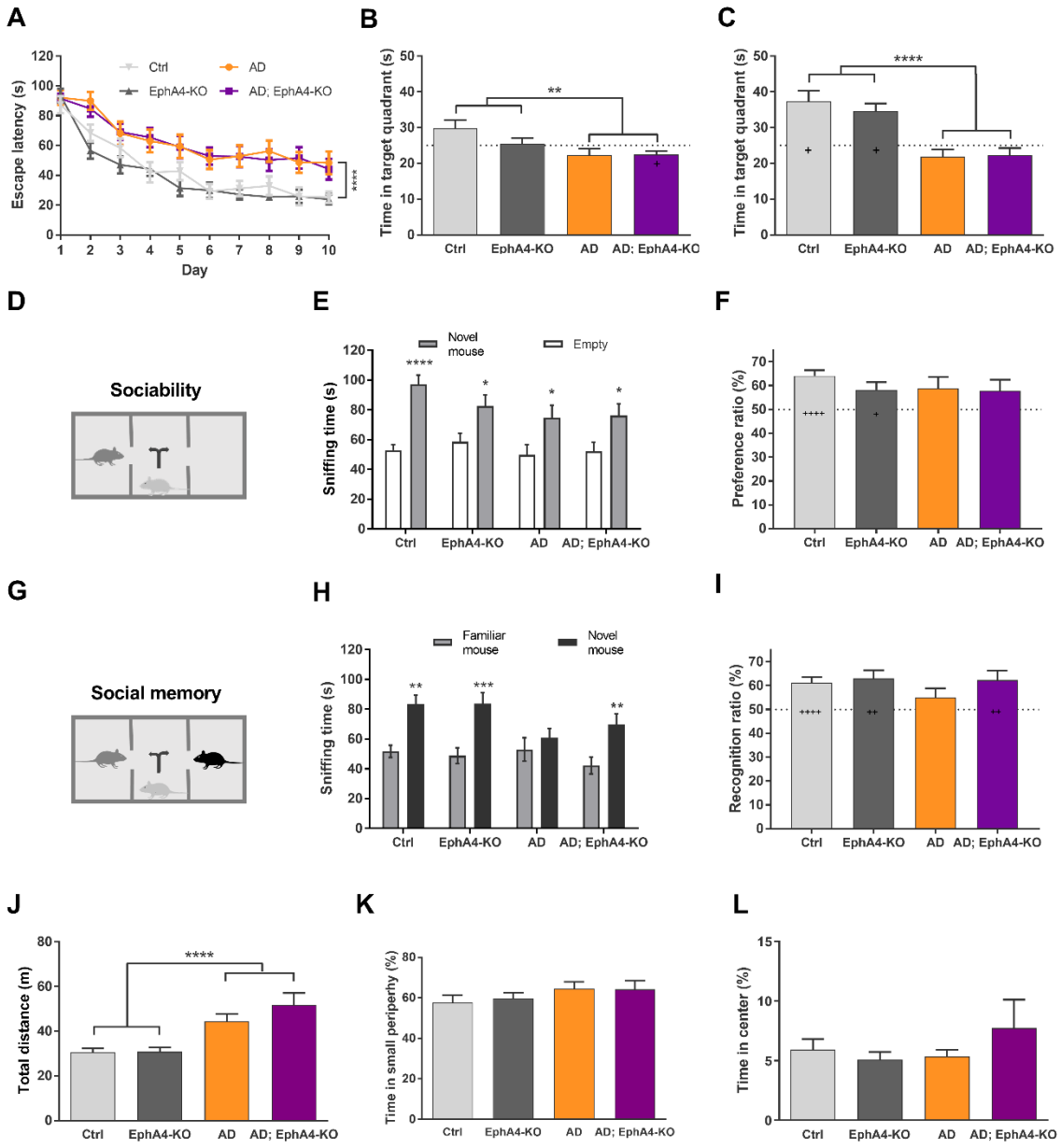

Figure 20 – EphA4 loss ameliorates social memory in APPPS1 mice. At 9 months of age, mice were subjected to different cognitive tests to assess memory performance. Escape latency over 10 training days (A), time spent in the target quadrant in probe trial 1 (B) and probe trial 2 (C) during the MWM test (two-way RM ANOVA and two-way ANOVA with Tukey's multiple comparison test, and unpaired t-test to compare to chance level). Sniffing time to the empty cage *versus* the novel mouse and preference ratio (D-F) in the sociability trial (unpaired t-test or Mann-Whitney U test and unpaired t-test compared to chance level, respectively). Sniffing time to the familiar *versus* the novel mouse and recognition ratio (G-I) in the social memory trial of the SPSN test (unpaired t-test or Mann-Whitney U test and unpaired t-test compared to chance level, respectively). Total distance crossed (J), time spent in the small periphery (K) and in the center (L) of the open field exploration test (two-way ANOVA with Tukey's multiple comparison test). N=22-28 mice/group. In panel B, C and J, significant group effects (AD *versus* non-AD) are indicated as follows: $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. In panel E and H, significant effects between social subjects (novel mouse *versus* empty or familiar mouse) are indicated as follows: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Performance above chance levels (panel B, C, F, and I) are indicated as follows: $^{+}p \leq 0.05$, $^{++}p \leq 0.01$, $^{++++}p \leq 0.0001$. If no * or $^{+}$ is shown in the graph, this implies no significance. Abbreviations: MWM; Morris water maze, SPSN; sociability/preference for social novelty.

2.3. EphA4 knock-down alters hippocampal spine morphology in the stratum radiatum

In order to find out the underlying mechanism responsible for the observed improvement in social memory, we explored alterations in dendritic spine density and/or morphology. Golgi-Cox staining was used to visualize Ctrl, AD and AD;EphA4-KO mice pyramidal neurons in the ventral CA1 region, an area important for the storage of social memory (427). Spine density and morphology were measured in dendritic segments derived from apical dendrites in two regions of the stratum radiatum (SR), the proximal SR (30-120 µm from the cell soma) and the distal SR (120-300 µm from the cell soma (428–430) (Fig. 21A). Spine density and length were similar in AD mice compared to control mice in both proximal and distal apical dendrites (Fig. 21C-E, H-J). EphA4 loss did not alter spine density, but spine length in the proximal SR was longer in AD;EphA4-KO mice versus AD and Ctrl mice (Fig. 21C-E). This increase in spine length in mice with loss of EphA4 was also present in the distal SR when comparing AD;EphA4-KO mice versus Ctrl mice (trending compared to AD mice) (Fig. 21H-J). As increased head width of spines correlates with improved synapse strength, we also measured the head width of the spines (330,334). The spine head width did not differ between AD versus control mice in both the proximal and distal SR (Fig. 21F,G,K,L). EphA4 knock-down increased spine head width in AD mice in the proximal SR, while in the distal SR we only observed this difference in comparison to Ctrl mice (Fig. 21F,G,K,L), similar to the findings on spine length.

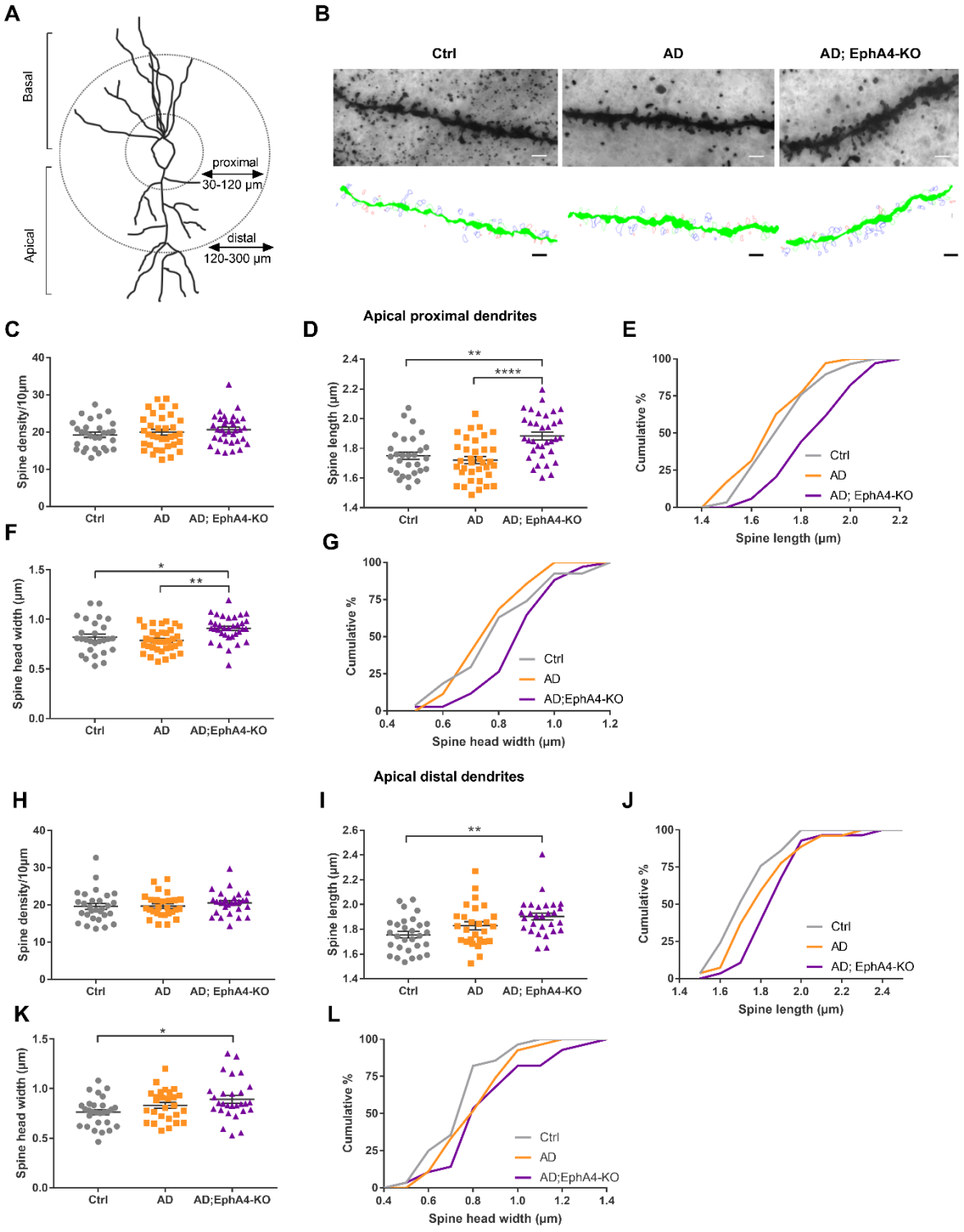

Figure 21 – Loss of EphA4 increases dendritic spine length and changes spine morphology in ventral CA1 region. (A) Apical dendritic segments of pyramidal neurons of the ventral CA1 region were randomly chosen and imaged in mice of 10-11 months old. A distinction was made between proximal (30-120 µm from the soma) and distal (120-300 µm from the soma) segments. (B) Representative images and Neurolucida 360 reconstructions of proximal apical dendritic segments of Ctrl, AD and AD;EphA4-KO mice. Quantifications of spine density (C), spine length (D,E) and spine head width (F,G) of segments of apical proximal dendrites (one-way ANOVA with Tukey's multiple comparison test, N=5 mice/group, n=29-35 dendritic segments/group). Quantifications of spine density (H), spine length (I,J) and spine head width (K,L) of segments of apical distal dendrites (Kruskall-Wallis test with Dunn's multiple comparison test, N=5 mice/group, n=27-29 dendritic segments/group). *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$. If no * is shown in the graph, this implies no significance. Scale bar = 2 µm.

2.4. EphA4 knock-down alters the microglial phenotype in the hippocampus

As EphA4 also mediates neuroinflammation in the nervous system, two processes involved in the pathophysiology of AD (353,358,375,431,432), we investigated alterations in astrocytic and microglial activity in the APPPS1 mouse model with normal versus reduced levels of EphA4. Therefore, we quantified hippocampal protein levels of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba1) by Western blot. GFAP and Iba1 levels increased in AD mice compared to Ctrl mice (Fig. 22A,B). Astrocytosis, as characterized by increased GFAP expression, was similar in AD mice with normal versus reduced levels of EphA4. The upregulation of Iba1 was stronger in AD;EphA4-KO compared to AD mice (Fig. 22A,B). To confirm these observations, we analyzed the GFAP and Iba1 positive areas by immunohistochemistry in the SR of the CA1 region in AD mice compared to AD;EphA4-KO mice (Fig. 22C-F). The GFAP positive (GFAP+) area was similar in both AD and AD;EphA4-KO mice (Fig. 22C,D), but the Iba1 positive (Iba1+) area was increased by 28.1 % (Fig. 22E,F). We excluded microglial proliferation since the number of microglia (as measured by the number of PU.1 positive nuclei) was similar in AD;EphA4-KO mice (Fig. 22G,H) and hypothesize an alteration in microglial phenotype to cause this increased expression of Iba1.

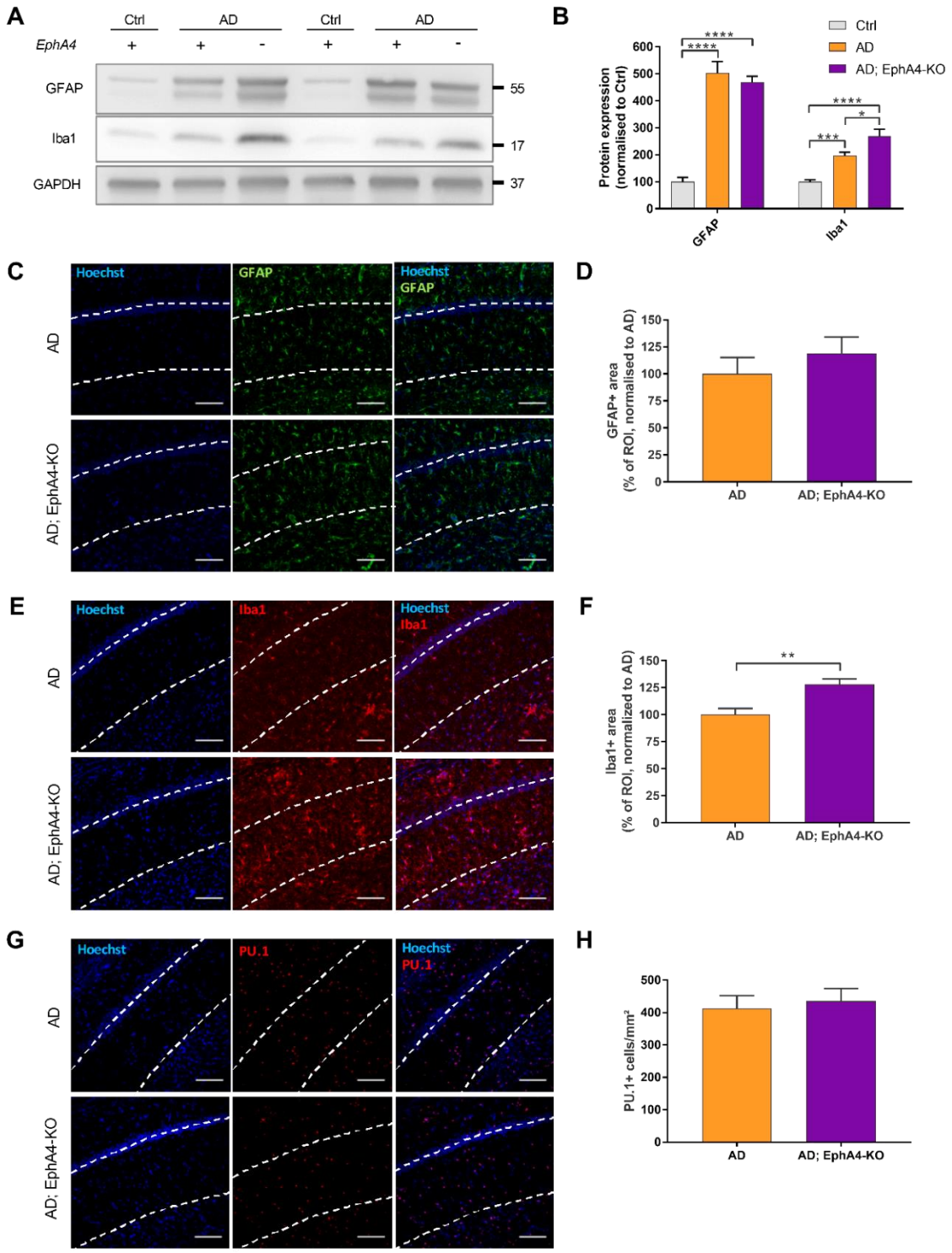

Figure 22 – Loss of EphA4 alters the microglial phenotype in the hippocampus of APPPS1 mice. Representative images (A) and quantifications (B) from Western blotting of hippocampal lysates with antibodies specific for GFAP and Iba1. GAPDH protein levels were used as a loading control (one-way ANOVA with Tukey's multiple comparison test, n=7-9 mice/group). Representative images (C) and quantifications (D) of immunohistochemistry with an antibody specific for GFAP in the CA1 region of AD and AD;EphA4-KO mice (unpaired t-test, n=6 mice/group, 2 brain slices/mouse, 2 regions/slice). Representative images (E,F) and quantifications (G, H) of immunohistochemistry with antibodies specific for Iba1 and PU.1 in the CA1 region of AD and AD;EphA4-KO mice (unpaired t-test, n=6 mice/group, 2 brain slices/mouse, 2 regions/slice). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Scale bar = 100µm and dashed lines outline the quantified area of the CA1. Abbreviations: GFAP; glial fibrillary acidic protein, Iba1; ionized calciumbinding adaptor molecule 1.

2.5. EphA4 knock-down does not alter hippocampal β-amyloid pathology

Microglia are involved in trimming and compaction of amyloid plaques in the brain (207–209). As alterations in microglial phenotype are often associated with changes in function (433–435), we investigated whether there was an association between the observed microglial alterations in AD;EphA4-KO mice and amyloid pathology in the hippocampus. In addition, EphA4 is suggested to mediate Aβ production *in vitro* (407). First, plaque density and plaque burden were determined (Fig. 23A-D). Amyloid deposits were highly abundant in AD mice and loss of EphA4 did not modify plaque density or plaque burden. The distributions of plaque sizes were similar between AD and AD;EphA4-KO mice (Fig. 23D). Finally, TBS-soluble and GuHCl-soluble hippocampal Aβ40 and Aβ42 levels were measured. EphA4 knock-down did not change Aβ40 and Aβ42 levels (Fig. 23E,F), nor the Aβ42/Aβ40 ratio's (Fig. 23G).

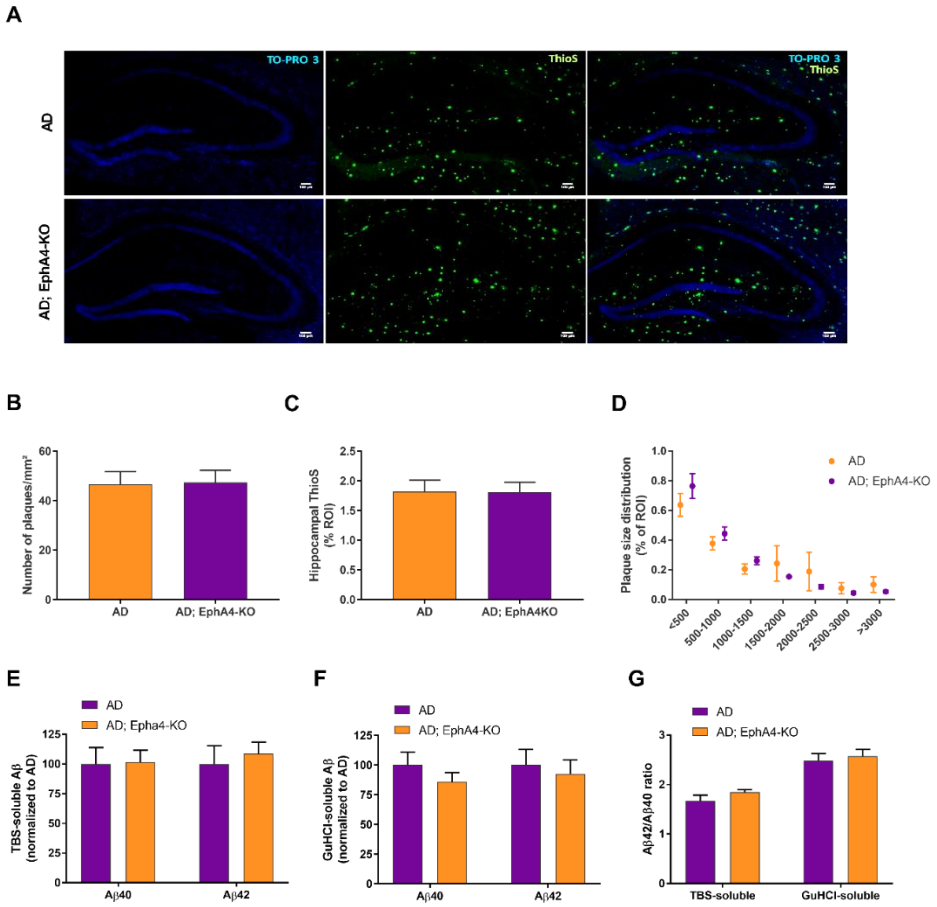

Figure 23 – Loss of EphA4 does not alter hippocampal plaque load in APPPS1 mice. (A) Representative images of a Thioflavin S staining to determine hippocampal plaque load in AD and AD;EphA4-KO mice at 10-11 months of age. TO-PRO3 was used to stain cell nuclei. Quantification of the number of plaques/mm² (B) and the percentage of the hippocampus positive for ThioS (C) in AD *versus* AD;EphA4-KO mice (unpaired t-test, n=10-13 mice/group). (D) Quantification of the plaque size distribution (in µm²) in the hippocampus of AD and AD;EphA4-KO mice (two-way RM ANOVA, n=10-13 mice/group). Quantification of the levels of TBS-soluble (E) and GuHCl-soluble (F) Aβ40 and Aβ42 levels (unpaired t-test, n=11 mice/group). (G) Quantification of the Aβ42/Aβ40 ratio in TBS-soluble and GuHCl-soluble extracts (unpaired t-test, n=11 mice/group). If no * is shown in the graph, this implies no significance. Scale bar = 100 µm. Abbreviations: ROI; Region of interest, ThioS; Thioflavin S.

3. Conclusions

In this study, we have shown that lowering neuronal EphA4 levels by at least 80% in the cortex and hippocampus of AD mice, specifically improved social memory without alterations in spatial memory. These effects were independent of explorative behavior as activity and anxiety were not affected by reduced EphA4 expression levels.

As inhibition of EphA4 signaling ameliorates Aβ-induced spine loss *in vitro* (160,386), we measured dendritic spine loss and morphology in the stratum radiatum of the ventral CA1 region of the hippocampus, a region important for the storage of social memory (427). Surprisingly, we did not detect any alterations in spine density and morphology in AD mice compared to Ctrl mice. In addition, not spine density, but spine morphology was altered upon EphA4 loss as both spine length and spine head width were increased.

Next, we investigated hippocampal inflammation as this process is affected by EphA4 inhibition in a mouse model for SCI and is pivotal in the pathophysiology of AD (198,353,358,360,363). Loss of EphA4 did not affect astrocytosis, as measured by GFAP expression levels and the GFAP positive area in the hippocampus of AD mice. However, EphA4 knock-down increased Iba1 expression levels. In addition, the Iba1 positive area was increased, while the number of microglia was similar, hinting towards an alteration in microglial morphology and activity. Hippocampal plaque density and burden were not affected, as well as the TBS-soluble and GuHCl-soluble Aβ40 and Aβ42 levels, suggesting that the altered microglial phenotype is not associated with increased Aβ clearance.

Chapter VI: General discussion & Conclusions

EphA4 is a tyrosine kinase receptor of the ephrin system, which mediates axon repulsion during formation of the nervous system and as an important mediator of spine morphology and plasticity in the adult brain (323,327,328,337,341,417). Due to its specific properties and functions, EphA4 has been shown to be an interesting target in several neurological disorders (160,321,353,357,358,368,372,391,436). In this doctoral thesis, we investigated the disease modifying potential of EphA4 loss in mouse models for SMA and AD.

1. Lowering EphA4 does not ameliorate disease in a mouse model for severe spinal muscular atrophy

EphA4 was identified as a disease modifier of ALS in both zebrafish and rodent models for this disease, and in patients (321). Interestingly, knockdown of EphA4 also rescued the axonal outgrowth deficits in a zebrafish model for SMA (321), suggesting that the neuroprotective effect of reduced EphA4 signaling could be independent of the cause of degeneration. In this study we aimed to validate the disease modifying potential of reduced EphA4 protein levels in the SMNΔ7 mouse model for severe SMA by evaluating neuromuscular junction innervation, motor neuron loss, motor function and survival. Loss of one EphA4 allele in this mouse model did not enhance any of these parameters despite clear reduction of EphA4 protein levels.

Both in ALS and SMA patients as well as in mouse models, motor neurons are degenerating via a 'dying-back mechanism' in which pathology starts at the neuromuscular junction and progresses towards the cell body along the axon (48,377,437,438). However, our findings in the SMNΔ7 mouse model are in contrast with previous results in the mutant $SOD1^{G93A}$ mouse model for ALS in which similar reduction of EphA4 levels ameliorated motor neuron disease progression (321). Clinical and pathological dissimilarities between these diseases and mouse models could explain the differential effect of EphA4 modulation on disease outcome.

First, disease severity varies significantly in ALS versus SMA. ALS is an adult onset disease resulting in death 3 to 5 years after symptom onset. In contrast, clinical onset in severe SMA patients starts shortly after birth and rapidly progresses to death before the age of 2 years (3,439). In mouse models, this variation is reflected in the NMJ pathology where first signs are observed during adult stages in the mutant $SOD1^{G93A}$ ALS mouse model, while this already happens in the neonatal phase in the SMNΔ7 mouse (44,47,48,57,377,440). Moreover, disease progression is much faster in the SMNΔ7 mouse model and results in death within two weeks, while mutant $SOD1^{G93A}$ mice survive for a few months following disease onset (72,418). Therefore, the period during which compensatory re-innervation of

NMJs could occur, could have been too short and therefore insufficient to modify SMA pathology and phenotype.

Second, compensatory sprouting and re-innervation is limited in SMA patients and mouse models for severe SMA, while these mechanisms are well-established phenomena in ALS patients and in the mutant $SOD1^{G93A}$ mouse model (45,46,377,419,440–442). Pre-synaptic neurofilament accumulations and instability of the NMJs due to immaturity and decreased numbers of terminal Schwann cells, contribute to the observed sprouting and re-innervation deficiencies in severe SMA (44,47–49,443). Hence, even if the axonal intrinsic sprouting capacity would increase via EphA4 modulation in severe SMA mice, this might not be sufficient to restore NMJ innervation, as the NMJs are severely affected. In addition, evidence suggests a possible role for neuronal and muscular EphA4 in NMJ formation and stability (315–318). Loss of EphA4 might even further destabilize the already weak NMJ connections.

Third, in ALS patients and mouse models different types of motor neurons exhibit diverse patterns of vulnerability, which is determined by EphA4 expression levels. Spinal motor neurons with the highest EphA4 levels (fast-twitch fast fatigable, FF) are most vulnerable and degenerate first, while motor neurons with lower EphA4 levels (slow, S) are more resistant (321,377,378). In SMNΔ7 mice, no clear correlation between motor neuron type and vulnerability exists, as muscles innervated by both FF and S motor neurons can be equally affected (57). Therefore, EphA4 might not contribute to motor neuron vulnerability in SMA, limiting its therapeutic potential in this disease.

Further research will need to clarify whether loss of EphA4 might be beneficial in mouse models representing milder forms of the disease with a broader therapeutic window for intervention, such as $Smn^{+/-}$ and SMN A2G transgenic mice. In addition to a slower disease progression, sprouting events have been reported in these models (78,444), possibly enabling a greater potential for beneficial effects from reduced EphA4 levels as well. Moreover, it might be interesting to investigate the potential of reducing EphA4 levels in combination with other therapeutic disease-ameliorating strategies. Encouraging data come from two studies in which administration of Plastin-3 (PLS3) or suppression of Neurocalcin Delta (NCALD) failed to fully modify the severe SMA phenotype, while combining these strategies with an SMN protein increasing compound had additional benefit (20,445). Therefore, a similar strategy might be useful for EphA4 knockdown in the SMNΔ7 mouse model.

The present study was limited by the partial 50% reduction of EphA4. Although removal of one EphA4 allele ($EphA4^{+/-}$) was sufficient to improve the disease phenotype in an ALS mouse model (321), this could be insufficient to modify the severe SMA phenotype and a more dramatic reduction of EphA4

might be required. However, this is unfeasible to investigate as EphA4$^{-/-}$ mice show very low birth rates and develop a 'hopping gait' phenotype, limiting the chance of obtaining SMA-EphA4$^{-/-}$ mice and the assessment of motor function (302,307,321).

In conclusion, in contrast to previous positive results in a zebrafish model for SMA and in the mutant SOD1^{G93A} mouse model for ALS, our work demonstrates that loss of one EphA4 allele is not sufficient to improve the innervation of the NMJs, motor neuron survival, motor function and survival in the SMNΔ7 mouse model for severe SMA. We hypothesize that this is due to either a too severe clinical phenotype in the SMNΔ7 mouse model with a too short time window for possible re-innervation, or to a limited therapeutic potential of (at least partially) reduced EphA4 levels in SMA.

2. EphA4 loss improves social memory performance and alters dendritic spine morphology in a mouse model of Alzheimer's disease

We studied the modifying role of reduced expression of EphA4 in the forebrain on memory function in the APPPS1 mouse model of AD via alterations in spine density and morphology, and neuroinflammation. Our results show that loss of EphA4 improves social memory performance in association with alterations in dendritic spine morphology and microglial phenotype.

EphA4 knock-down in APPPS1 mice selectively improved social recognition memory, but did not beneficially influence spatial learning and memory. This effect was independent of explorative behavior since activity and anxiety were similar in APPPS1 mice with normal versus reduced EphA4 expression. In constitutive full EphA4-KO mice (345) cognitive deficits are present which is in contrast to our findings in EphA4$^{flox/flox}$ x Camk2aCre mice which revealed no impairments in activity, anxiety, spatial and social recognition memory. There are several possible explanations for the variation in these results. First, different test paradigms to assess activity, anxiety, spatial and recognition memory were used. Second, in constitutive EphA4-KO mice, EphA4 is absent during development *versus* reducing EphA4 levels from the third postnatal week on in EphA4$^{flox/flox}$ x Camk2aCre mice thereby circumventing developmental deficits occurring in constitutive EphA4-KO mice (327,409). Third, the mice used in our study have preserved +/-10% of the physiological EphA4 protein levels in the cortex and hippocampus which might be sufficient for normal cognitive function as LTP is not affected in mice with similar EphA4 levels (328).

The specific improvement of social memory, but similar spatial memory performance in APPPS1 mice with EphA4 loss is of interest. One possible explanation for these differences is the differential test sensitivity. Mice rely on the sense of olfaction for social recognition, which is extremely sophisticated in contrast to vision in mice (446,447). Hence, we argue that the formation of social recognition memory is an easier task in comparison to the formation of a spatial map based on visual cues. Therefore, effects of loss of EphA4 in the forebrain might be mild and only sufficient to improve social memory, but not spatial memory in the more difficult Morris water maze test. Moreover, various aspects of learning and memory rely on different regions of the brain. In the hippocampus, spatial memory involves preferentially dorsal CA1 and CA3 regions (448–450), while social memory is dependent on ventral CA1 and CA2 activity (451,452). In addition, the amygdala has a significant role in the acquisition of social memory (453). As Cre-recombinase activity in the amygdala of the Camk2aCre mouse was reported to reach similar levels as in the hippocampus and cortex (454), considerable loss of EphA4 levels in this region might contribute to the specific improvement in social memory.

Consecutively, we measured spine density, length and head width in the ventral CA1 region since the reduction of EphA4 levels was most pronounced in the CA1 region and ventral CA1 activity is indispensable for social memory (451). Loss of spines is reported to be more pronounced in close proximity to the amyloid plaque in the cortex of APPPS1 mice, as well as in several other mouse models and in patients (132,137–140,455). As a substantial plaque load was present in the stratum radiatum of the ventral CA1, a region in which projections implicated in social memory terminate, we focused spine analysis on this region (452). Segments from both the proximal and distal stratum radiatum were analyzed as these regions can be selectively affected in AD mouse models (429). However, we were not able to detect spine loss on apical dendrites in APPPS1 mice, which is in accordance with a previous study (456).

Loss of EphA4 did not increase spine density in the hippocampus. Although previous studies reported the normalization of spine numbers in an *in vitro* AD model upon reduced EphA4 signaling, spine density was not increased upon reduced EphA4 signaling in control conditions with normal spine densities (160,386). Absence of spine loss in our AD mice model hampers the study of the modifying role of EphA4 expression on spine density. Interestingly, loss of EphA4 induced changes in spine morphology as both spine length and spine head width were increased in the proximal SR. These spine alterations induced by EphA4 loss might underlie social memory improvements in AD mice as increased spine length has been described in individuals with cognitive resilience to AD pathology, and is believed to extend the reach of spines to form new synaptic connections or to connect with degenerating axons (457). In addition, we assume that the synapses in the proximal SR are stronger in mice with reduced

EphA4 levels, as the size of the spine head is directly correlated with synaptic strength (332). In accordance, a previous study linked reduced EphA4 signaling in non-Aβ conditions to an increased number of mushroom-shaped spines, which are characterized by thin necks, large heads and the formation of strong and stable synapses (338,459).

In order to explore the potential role for EphA4 in neuroinflammation in the APPPS1 mouse model, we investigated the effect of EphA4 loss on the hippocampal astrocytic and microglial phenotype. Although EphA4 loss diminishes astrocytic reactivity in vitro and in a mouse model of spinal cord injury (353,358,460,461), we found that astrocytic reactivity was not affected by loss of EphA4 in APPPS1 mice. Microglial activation was altered by loss of EphA4 in APPPS1 mice as shown by the increased Iba1 expression and Iba1 positive area. As microglial density remained the same, we hypothesize that these alterations could signify changes in microglial morphology such as increased ramification or cell body area, which might be correlated with a modified function (462). Interestingly, inhibition of a downstream effector of EphA4, Rho kinase, altered microglial ramification and function in a mouse model of amyotrophic lateral sclerosis (ALS) (463).

In Alzheimer's disease, accumulating evidence supports the view of microglia as important drivers of disease progression. However, the exact functions of microglia are not fully understood yet and are probably dependent on the disease stage (198). Several studies support a role for plaque-associated microglia in amyloid plaque trimming and compaction, and methods to increase clearance capacity have shown to improve cognitive function in mouse models for AD (207–209,464–466). Furthermore, EphA4 is suggested to mediate Aβ production *in vitro* (407). As APPPS1 mice present with robust amyloid pathology, we investigated whether the improvement in social memory upon EphA4 loss was associated with reduced amyloid pathology (235). Amyloid plaque burden and Tris-soluble and GuHCl soluble Aβ levels did not differ in AD mice with normal versus reduced EphA4 levels. These results suggest that altered microglial activation is not linked to a modified clearance function and/or that EphA4 inhibition does not reduce Aβ production in this model.

Although loss of EphA4 is associated with altered spine morphology and microglial phenotype, further research will need to clarify how these alterations contribute to improved social memory.
First, the current work was limited by the inability to measure spine density in close proximity to the beta-amyloid plaques, as the combination of Golgi-Cox staining and plaque visualization was technically not feasible. Novel techniques have recently been developed to combine these techniques and await validation (467). It would be interesting to examine if spine loss can be detected near beta-

amyloid plaques in the APPPS1 mouse model and, when affirmative, if the improvement in social memory is associated with a specific amelioration in plaque-associated spine loss.

Second, further investigation of spine subtypes, synapse formation and synapse electrophysiology could provide more insight in how increased spine length and spine head width underlies the observed improvement in social memory.

Third, examination of spine morphology and density in other brain regions such as the amygdala could be of importance to estimate the involvement of other brain regions, and to explore possible mechanisms for the specific improvement of social memory upon EphA4 loss, while spatial memory was unaffected.

Last, as altered microglial activation was not associated with increased amyloid clearance, other microglial functions might be affected by loss of EphA4. Although microglial activation is often deleterious in neurodegenerative disorders, several beneficial effects are suggested. Microglia constitute a physical barrier around amyloid plaques to prevent formation of Aβ toxic hot-spots and to protect neurons from these toxic species (207–209). Moreover, microglia mediate synaptic circuit modelling via trogocytosis ('nibbling') of presynaptic terminals and induction and reorganization of dendritic spines, which facilitates the formation of new connections (468,469). These latter findings even raise the intriguing question whether microglia are involved in spine morphology alterations upon EphA4 loss in AD mice. We believe that further in-depth analysis of the aforementioned processes in combination with single cell microglial transcriptomics in AD mice with normal *versus* reduced EphA4 levels will provide more insights in the altered microglial activation and the potential role of these changes on improving cognitive function.

In conclusion, our work demonstrates that loss of EphA4 in the forebrain ameliorates the social memory deficit observed in APPPS1 mice, in association with alterations in spine morphology and microglial activation. We hypothesize that changes in spine morphology could enhance the formation of connections between spines with axons and might be associated with enhanced synaptic strength and connectivity. Alterations in microglial activation can modify microglial function resulting in improvement of synaptic and/or neuronal function. Further studies will need to clarify how these alterations contribute to improved social memory.

3. Is EphA4 still an interesting therapeutic target in SMA and AD?

In this doctoral thesis, we have explored the modifying potential of EphA4 in mouse models for SMA and AD. These data provide insights in the therapeutic potential of EphA4 targeting for these neurological disorders.

First, we investigate whether reducing EphA4 levels could modify disease pathology in a mouse model for SMA. Our results did not show any beneficial effect of lowering EphA4 levels on motor function, survival, NMJ innervation and motor neuron survival in the SMNΔ7 mouse model. Although these results hint towards a limited therapeutic potential for EphA4, the severe clinical phenotype and fast clinical progression in SMNΔ7 mice might impede beneficial effects of EphA4 loss on re-innervation of NMJ's and motor neuron survival (72,418). Therefore, we believe it would be of interest to explore the modifying potential in mouse models representing milder forms of the disease.
In addition, EphA4 might be an interesting candidate for supportive treatment in SMA patients on SMN-targeting therapies, as these therapies might not be sufficient to fully suppress SMN symptoms, especially in severe cases. Hence, it would be interesting to explore whether loss of EphA4 in combination with SMN-targeting therapies could ameliorate disease pathology in a mouse model for SMA. While EphA4 reduction on its own might not be potent enough to improve NMJ innervation and motor neuron survival in models for severe SMA, it might be capable to support SMN-targeting strategies and attain additional benefits on disease progression, as was shown before for NCALD and PLS3 targeting (20,445).

The second aim of this doctoral thesis was to examine whether reducing EphA4 levels could affect spine pathology and neuroinflammation in a mouse model for AD. Our results show that loss of EphA4 specifically improved social recognition memory in APPPS1 mice, together with alterations in spine morphology and microglial phenotype. From a therapeutic perspective, we need to consider whether the observed cognitive improvement in this AD mouse model is of value for patients, as loss of EphA4 only improved social memory, while spatial memory was unaffected. This selective enhancement is probably not enough to improve cognitive impairments in AD patients. However, our work was limited by the inability to detect spine loss in the hippocampus of APPPS1 mice. As a result, it needs to be explored whether spine loss is completely absent or rather specifically distributed around plaques.

If specific spine loss in proximity to plaques is present in APPPS1 mice, it should be explored whether plaque-associated spine loss is ameliorated by loss of EphA4. Lack of effect on spine density could explain the limited cognitive improvement, and would suggest that a reduction of EphA4 levels by 80-

90% in the forebrain soon after birth is not adequate to ameliorate spine loss in APPPS1 mice. However, residual EphA4 levels might be too low, compromising EICD generation and consequent EICD-induced spine formation (406). Hence, it is possible that an optimal level of EphA4 inhibition is required to evoke a net positive effect on total spine numbers. Indeed, a mild reduction of EphA4 activation by ±20%, was suggested to mediate the complete reversal of Aβ-induced spatial memory impairments in SORLA-overexpressing mice (385). In addition, it would be of interest to target EphA4 later in the disease, as loss of EphA4 from 3 weeks after birth on might cause developmental defects. Although we did not observe cognitive impairments in EphA4$^{flox/flox}$ x Camk2aCre mice, other groups have found LTP deficits and impaired performance in hippocampus-dependent tasks in mice with constitutive and/or early hippocampal loss of EphA4 (328,344–346). To address these issues, it could be an interesting strategy to use EphA4 inhibiting peptides (e.g. APY-d3) or nanobodies to explore different levels of EphA4 inhibition later in disease (470,471).

Although this scenario would be in contrast to other findings, a lack of severe spine loss in proximity to plaques could limit the therapeutic potential and hence cognitive improvement of lowering EphA4 in this mouse model since the consequent inability to modify spine loss by EphA4 knock-down (132). Another AD mouse model with confirmed spine loss should be carefully selected to further investigate our hypothesis.

Summary

EphA4 is a tyrosine kinase receptor of the ephrin system, which is highly expressed in the nervous system. During development of the nervous system, EphA4 mediates axon repulsion during the formation of the corticospinal tract and the innervation pattern of the hindlimbs. In amyotrophic lateral sclerosis (ALS), a motor neuron degenerative disorder, EphA4 has been identified as an interesting disease modifier in zebrafish models, rodent models and in patients. Inhibition of EphA4 signaling slowed down disease onset and/or progression, and improved motor function in rodent models for ALS by reducing the vulnerability of motor neurons and enhancing the neuromuscular junction innervation. The latter is most likely caused by increased sprouting and re-innervation capacity of motor axons upon EphA4 loss. Interestingly, knockdown of EphA4 also rescued the axonal deficits in a zebrafish model for spinal muscular atrophy (SMA), suggesting that the neuroprotective effect of EphA4 inhibition could translate to other motor neuron diseases.

SMA is a neurodegenerative disorder characterized by the degeneration of motor neurons of the spinal cord, resulting in hypotonia, progressive muscle weakness and atrophy, and in the most severe cases paralysis, respiratory failure and death. The diseases segregates in an autosomal recessive manner and is caused by reduced levels of survival of motor neuron protein (SMN) resulting from deletions or loss-of-function mutations in the SMN1 gene. Nusinersen and Zolgensma are the only approved therapies and both increase the production of functional SMN protein. However, they do not cure the disease and disease outcomes remain variable. Therefore, non-SMN therapies could provide additional support to patients, or could be of importance for patients that are intolerant, not responsive to or excluded from SMN-targeting strategies.

In the first part of this doctoral thesis, we investigated whether the modifying potential of EphA4 in a zebrafish model could translate to a mouse model for SMA. We heterozygously deleted EphA4 in the SMNΔ7 mouse model for severe SMA and showed that this strategy did not ameliorate disease progression. Although these results hint towards a limited therapeutic potential for EphA4, the severe clinical phenotype and fast clinical progression in SMNΔ7 mice might impede beneficial effects of EphA4 loss and further studies need to clarify whether EphA4 might still be an interesting target in mouse models of milder forms of the disease or in combination therapy.

In the adult nervous system, EphA4 is highly expressed in high-plasticity regions, such as the cortex and hippocampus, where it is a critical mediator of synapse morphology, functionality and plasticity. In addition, EphA4 modulates neuroinflammation as shown in mouse models for spinal cord injury (SCI). Hence, EphA4 is an interesting target for diseases characterized by synaptic dysfunction and

neuroinflammation such as Alzheimer's disease (AD). Interestingly, inhibition of EphA4 signaling rescues Amyloid-β (Aβ)-induced dendritic spine loss and long-term potentiation deficits *in vitro*.

AD is a neurodegenerative disorder, representing the underlying cause of 60-70% of the dementia cases. AD is characterized by progressive memory loss, together with multiple cognitive impairments that compromise the quality of life for patients and their relatives. The key pathological features of AD are the extracellular amyloid depositions of the Aβ peptide into senile plaques and the intraneuronal aggregates of misfolded hyperphosphorylated tau protein. In addition, several neuroinflammatory alterations are present, including changes in astrocyte and microglial reactivity, which appear to be important drivers of disease pathophysiology. Synapse dysfunction and loss occur early in AD and are suggested to be the underlying cause of cognitive deficits in patients. Current treatments are the acetylcholinesterase inhibitors and an NMDA receptor antagonist, which are symptomatic and aim to enhance cognitive function in patients. Unfortunately the efficacy of these drugs is limited. As the socioeconomic burden of the disease is high and still increasing, the need for disease-modifying therapies is urging. Several research strategies exist, designed to ameliorate Aβ and tau pathology, but therapies focused on neuroinflammation and synapse dysfunction are also of interest.

In the second part of this doctoral thesis, we studied whether the beneficial effects of EphA4 reduction on Aβ-induced spine pathology and on neuroinflammation, would translate to a mouse model for AD, and improve cognitive function. In order to do so, we profoundly reduced EphA4 levels in the forebrain of the APPPS1 mouse model for AD. Our work demonstrates that loss of EphA4 selectively improves social memory, in association with alterations in spine morphology and microglial phenotype. However, further studies are needed to clarify how these changes contribute to the observed social memory improvement and whether these results are of interest for the development of therapeutic strategies.

In conclusion, we explored the modifying potential of EphA4 in two neurodegenerative disorders, SMA and AD. We have shown that loss of EphA4 did not improve the disease in a mouse model for severe SMA, while a similar strategy could partially improve cognitive function in a mouse model for AD.

Nederlandstalige samenvatting

EphA4 is een tyrosine kinase receptor behorende tot het efrinesysteem en komt in hoge mate tot expressie in het zenuwstelsel. Tijdens de ontwikkeling van het zenuwstelsel functioneert EphA4 als een repulsieve factor voor axonen tijdens de vorming van de corticospinale baan en de innervatie van de achterste ledematen. EphA4 werd reeds geidentificeerd als een opmerkelijke factor die het ziekteverloop van zebravismodellen, muismodellen en patienten met amyotrofe laterale sclerose (ALS) kan beïnvloeden. Inhibitie van EphA4 signalisatie in muismodellen voor ALS, vertraagde de start en/of het verloop van de ziekte, en verbeterde de motorische functie door reductie van de vatbaarheid voor degeneratie van de motorneuronen en door stimulatie van de innervatie van de neuromusculaire junctie. Dit laatste wordt hoogstwaarschijnlijk veroorzaakt door een verhoogde sprouting en re-innervatiecapaciteit van de motorneuronen bij verlies van EphA4. Een zeer interessante bevinding was dat een verlaging van de EphA4 expressie ook axonale defecten in een zebravismodel voor spinale musculaire atrofie (SMA) kon herstellen. Dit suggereert dat het neuroprotectieve effect van EphA4 inhibitie zich ook vertaalt naar andere motorneuronaandoeningen naast ALS.

SMA is een neurodegeneratieve aandoening die wordt gekenmerkt door het afsterven van motorneuronen in het ruggenmerg. Dit resulteert in hypotonie, progressieve spierzwakte en atrofie, en in de meest ernstige gevallen verlamming, falen van de ademhaling en overlijden. De aandoening wordt overgeërfd volgens een autosomaal recessief patroon en wordt veroorzaakt door verlaagde expressie van het ‘survival of motor neuron’ eiwit (SMN) door deleties of loss-of-function mutaties in het SMN1 gen. Tot op heden zijn Nusinersen en Zolgensma de enige goedgekeurde therapieën en beide verhogen de productie van functioneel SMN eiwit. Echter, deze therapieën genezen de ziekte niet en de resultaten zijn zeer variabel. Therapieën die niet gefocust zijn op SMN zouden bijgevolg extra steun kunnen geven aan de patiënten. Bovendien kunnen ze van belang zijn voor patiënten die geen significante verbetering of ernstige bijwerkingen vertonen na behandeling met Nusinersen of Zolgensma, of patiënten die niet voor deze therapievorm in aanmerking komen.

In het eerste deel van deze thesis, hebben we onderzocht of het ziektemodificerende effect van EphA4 in een zebravismodel voor SMA kan vertaald worden naar een muismodel voor deze ziekte. We hebben EphA4 expressie met 50% verlaagd in het SMNΔ7 muismodel voor een ernstige vorm van SMA, maar hebben aangetoond dat deze strategie geen effect had op het ziekteverloop. Hoewel deze resultaten suggereren dat EphA4 maar een beperkt therapeutisch potentieel heeft in SMA, bestaat de mogelijkheid dat het SMNΔ7 muismodel een te ernstig fenotype en een te snel ziekteverloop kent. Deze zouden de mogelijke positieve effecten van EphA4 verlies kunnen verhinderen en verdere studies zijn nodig om na te gaan of EphA4 toch een mogelijk doelwit kan zijn bij behandeling van muismodellen met mildere vormen van SMA of bij combinatietherapie.

In het volwassen zenuwstelsel komt EphA4 sterk tot expressie in regio's met hoge plasticiteit zoals de cortex en hippocampus. In deze regio's is EphA4 belangrijk voor de morfologie, functionaliteit en plasticiteit van de synapsen. Daarnaast moduleert EphA4 ook inflammatie zoals werd aangetoond in muismodellen met ruggengraatletsels. Bijgevolg is EphA4 een interessant doelwit voor de behandeling van ziekten die worden gekenmerkt door dysfunctie van de synapsen en neuroinflammatie zoals de ziekte van Alzheimer (AD). Een interessante bevinding was dat inhibitie van EphA4 signalisatie het verlies van 'dendritic spines' en defecten in lange termijn potentiatie *in vitro* kon voorkomen.

AD is een neurodegeneratieve aandoening die in 60-70% van de patiënten de oorzaak is van dementie. De ziekte wordt gekenmerkt door een progressief verlies van het geheugen, samen met meerdere cognitieve problemen die de levenskwaliteit van de patiënten en hun dierbaren ondermijnen. De belangrijkste pathologische verschijnselen zijn de extracellulaire amyloide afzettingen van het Amyloid-β (Aβ)-peptide in senile plaques en de aggregatie van fout gevouwen en gehyperfosforyleerd tau eiwit in de neuronen. Daarnaast zijn verschillende neuroinflammatoire veranderingen aanwezig, o.a. een reactiviteit van de astrocyten en microglia die belangrijke drivers zijn van de ziekte. Dysfunctie en verlies van synapsen komen reeds vroeg voor in de ziekte en veroorzaken hoogstwaarschijnlijk de cognitieve problemen bij patiënten. Huidige behandelingen zijn de toediening van acetylcholinesterase inhibitoren en NMDA receptor antagonisten. Deze werken enkel symptomatisch en hebben als doel om de cognitieve functie van patiënten te verbeteren, maar helaas is het effect van deze medicijnen gelimiteerd. Aangezien de sociale en economische druk op de maatschappij hoog is én nog steeds stijgt, wordt de nood voor therapieën die de oorzaken van de ziekte aanpakken hoger. Verschillende strategieën worden momenteel verkend met de focus op Aβ en tau pathologie, maar therapieën gefocust op neuroinflammatie en dysfunctie van de synapsen zijn ook zeer interessant.

In het tweede deel van deze thesis hebben we onderzocht of de voordelige effecten van EphA4 inhibitie op het verlies van 'dendritic spines' en neuroinflammatie konden worden vertaald naar een muismodel voor AD. Hiervoor hebben we EphA4 expressie sterk verlaagd in de voorhersenen van het APPPS1 model voor AD. We demonstreren dat dit verlies aan EphA4 specifiek het geheugen voor sociale gebeurtenissen verbetert. Bijkomend werden ook veranderingen in de morfologie van de 'dendritic spines' en in het fenotype van de microglia waargenomen. Verdere studies zijn echter nodig om na te gaan hoe deze veranderingen bijdragen tot de verbetering in het sociale geheugen en of deze resultaten van belang zijn voor de ontwikkeling van therapeutische strategieën.

Tot slot, we hebben in deze doctoraatsthesis de modificerende eigenschappen van EphA4 bestudeerd in twee neurodegeneratieve aandoeningen, SMA en AD. We hebben aangetoond dat verlies van EphA4

geen invloed had op het ziekteverloop in een muismodel voor SMA, maar dat een gelijkaardige strategie de cognitieve functie deels kon herstellen in een muismodel voor AD.

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Conflict of interest statement

The authors report no conflicts of interest.

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Curriculum Vitae

LINDSAY POPPE

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PERSONAL PROFILE

During my PhD, I investigated therapeutic strategies in mouse models for neurological diseases. During this study, I had the opportunity to improve my managerial and communication skills. I would enjoy to pursue my career by working on applicable strategies in a clinical or public health setting to help patients and improve general health and well-being.

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PUBLICATIONS

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