CELLS IN EXTRACELLULAR SPACE

We are all shaped and influenced by our environment. The food we eat, the people we meet and events we experience shape how we interpret and respond to the outside world. Cells in an organ as densely packed as the brain behave similarly. Although densely packed, there is space between cells that allow flow of nutrients and extracellular factors. Extracellular factors, which are sensed by receptors protruding from the plasma membrane, or enter cells via passive diffusion or through membrane fusion with extracellular vesicles, can impact the metabolic activity and protein and gene expression of cells. The extracellular space is also filled with a matrix of macromolecules, the extracellular matrix (ECM). The ECM can take many forms, such as bone or teeth, but also softer structures such as tendons, ligaments, and even blood plasma, which is the ECM structure holding together blood cells (Theocharis et al., 2016). It also has many functions. Besides functioning as a passive scaffold, the ECM has an active role in coordinating cell functions such as proliferation, migration and differentiation through interaction with cell-surface receptors or by providing biochemical support (Bonnans et al., 2014). In the brain, where it makes up roughly 20% of the total volume (Nicholson and Sykova, 1998), the ECM also coordinates important aspects of neuronal development, plasticity and pathophysiology (Faissner et al., 2010; Dityatev and Rusakov, 2011; Miyata and Kitagawa, 2017), and the ECM is nowadays considered a critical component of normal brain function in addition to neurons and glia (Song and Dityatev, 2018).

EXTRACELLULAR MATRIX

The ECM as found in the central nervous system (CNS) is rich in proteoglycans decorated with covalently bound glycosaminoglycan chains (GAGs). Proteoglycans can be subdivided in heparan sulfate proteoglycans (HSPGs), chondroitin sulfate proteoglycans (CSPGs), dermanatan sulfate proteoglycans or keratan sulfate proteoglycans, depending on the structure of their GAG chains (Bandtlow and Zimmermann, 2000). CSPGs are the most abundantly expressed in the CNS, carry the highest amount of GAGs and are prominent ECM constituents (Bandtlow and Zimmermann, 2000; Laabs et al., 2005; Zimmermann and Dours-Zimmermann, 2008). GAG chains on CSPGs are large unbranched polymers that due to their negative charge function as a binding site for various other proteins, including growth factors, such as semaphorins (Kantor et al., 2004; Corvetti and Rossi, 2005; De Wit et al., 2005). CSPG-GAG chains are of particular importance since many functions of CSPGs are determined by the number, position and degree of sulfation of...
The localization of ECM and perineuronal nets

ECM is found throughout the adult brain and can be found in roughly three sites: (1) basement membranes surrounding blood vessels, (2) ‘loose’, ‘diffuse’ or ‘interstitial’ ECM, and (3) a highly organized form of ECM called perineuronal nets (PNNs) (Lau et al., 2013). In 1882 Camillo Golgi, who mistook PNNs for a meshwork of cell bodies, was the first one to describe these structures (Golgi, 1898). Fifteen years later, in 1897, Ramón y Cajal initially claimed to be the first one to observe the PNN, stating it was a staining artifact (Ramón y Cajal, 1911), which resulted in a loss of scientific

Figure 1 | Schematic structure of a perineuronal net

Hyaluronan, the PNN backbone, is produced by hyaluronan synthase (HAS), which is located on the plasma membrane of the cell. Lecticans such as aggrecan (ACAN), brevican (BCAN), neurocan (NCAN) and versican (VCAN) bind to hyaluronan via hyaluronan and proteoglycan link proteins (HAPLN). Lecticans are crosslinked by tenascin-R (TNR) and tenascin-C (TNC) at their C-terminal side, creating a net-like structure.
interest in PNNs. It was not until the 1980s that there was a renewed interest in PNNs due to the development of more advanced immunocytochemical techniques (Spreatico et al., 1999).

Today we know that PNNs are composed of a highly organized linkage of four ECM protein families: (1) hyaluronan, the membrane-bound backbone to which other PNN constituents bind (Toole, 2004), (2) CSPG lecticans aggrecan (ACAN), brevican (BCAN), neurocan (NCAN) and versican (VCAN) (Yamaguchi, 2000; Galtrey and Fawcett, 2007), (3) a family of the hyaluronan and proteoglycan link proteins (HAPLN₁, -2, -3 and -4), which stabilize the binding of CSPGs onto the hyaluronan backbone, and (4) tenascin-R and tenascin-C (TNR and TNC), which form trimers that crosslink lecticans within PNNs (Zimmermann and Dours-Zimmermann, 2008; Morawski et al., 2014; Sorg et al., 2016). Lecticans bind non-covalently through their G₁ domain to the hyaluronan backbone that is protruding from the plasma membrane. HAPLN₁s stabilize the CSPGs link to the hyaluronan backbone, which makes them an essential stabilization factor of PNNs (Spicer et al., 2003; Oohashi et al., 2015). Lectican proteins also have a G₃ domain, allowing them to bind tenascins. TNR forms homotrimers and is therefore able to crosslink up to three lecticans. TNC can form a complex of six proteins thus creating a so-called hexabrachion. Replacement of TNR by TNC doubles the amount of lecticans being crosslinked, thus increasing the density of the PNN (Zimmermann and Dours-Zimmermann, 2008) (FIG 1).

Even though all lecticans, tenascins and HAPLN₁s are found in PNNs (Deepa et al., 2006; Galtrey and Fawcett, 2007), not all are essential for normal PNN formation. Studies showed that animals with selective removal of either HAPLN₁ (Carulli et al., 2010), ACAN (Giamanco et al., 2010), BCAN (Brakebusch et al., 2002) or TNR (Brückner et al., 2000; Morawski et al., 2014) show attenuated PNN formation. In contrast, animals in which the NCAN (Zhou et al., 2001) or TNC genes have been deleted (Irinchev et al., 2005) show structurally normal PNNs. This indicates the differential importance of PNN components for the formation and maintenance of PNN structures.

**ECM synthesis**

ECM components are not exclusively synthesized by the cells they may ensheath but can also originate from other cell types (Carulli et al., 2006; Song and Dityatev, 2018). HAPLN₁, HAPLN₄, ACAN and NCAN are mainly produced by neurons (Engel et al., 1996; Carulli et al., 2006; Mcrae et al., 2007; Galtrey et al., 2008) BCAN, on the other hand, is primarily produced
by astrocytes (Carulli et al., 2006; John et al., 2006; Giamanco and Matthews, 2012), and VCAN by oligodendrocytes (Carulli et al., 2006). TNR can be produced by both neurons and glial cells (Carulli et al., 2006). After synthesis and release, individual ECM components combine to form a PNN structure attached to the neuronal plasma membrane. There have been many theories on how PNNs organize themselves around neurons. The current consensus states that PNNs attach to neurons through hyaluronan synthases (HAS). HAS are anchored in the plasma membrane where they synthesize hyaluronan and keep it docked at the plasma membrane (Toole, 2004; Carulli et al., 2006; Galtrey et al., 2008; Kwok et al., 2010). Hereby they allow lecticans and HAPLNs to bind to the hyaluronan backbone and offer a structural link to the plasma membrane. Other ECM receptors, such as CD44 and RHAMM, are also able to bind hyaluronan, but since in the CNS CD44 is only expressed in astrocytes (Asher and Bignami, 1992) and RHAMM is localized intracellularly (Carulli et al., 2006), it seems unlikely that they play a role in PNN anchoring (Galtrey et al., 2008).

**PNN function**

For a long time it was thought that the main function of the ECM was structural support of the cells in their environment, but in the last decades it became clear that ECM also creates microenvironments through which it regulates neuronal activity and development. For instance, PNNs support ion homeostasis around active neurons (Härtig et al., 1999; Morawski et al., 2015), are neuroprotective for the neurons they enclose (Morawski et al., 2004; Suttkus et al., 2012) and limit synaptic plasticity.

Organization of ECM components into PNNs marks the closure of critical periods. A critical period is the time window during which experience shapes the correct formation of synaptic connections and thus the normal development of neuronal circuits (Hensch, 2005; Oohashi et al., 2015). The closure of critical periods is well studied in regions such as the visual cortex, where it is tightly regulated and strongly depends on visual experience (Berardi et al., 2003). In a normal developing visual cortex both eyes drive an equal amount of neurons and thus contribute equally to a visual representation. However, if one eye is occluded during the critical period, the non-deprived eye will dominate the input onto neurons in the visual cortex. In other words, monocular deprivation leads to a shift in ocular dominance, following the principles of Hebbian plasticity (Wiesel and Hubel, 1963; Berardi et al., 2003, 2004). More recently, the important role of the ECM in critical period closure has been demonstrated. The expression of
Figure 2 | Plasticity is restricted by pNNS via three proposed mechanisms

(A) PNNs can function as a physical barrier and thereby prevent establishment of new synaptic connections. ChABC disrupts PNN structures, thereby attenuating the barrier and thus allowing formation of new synaptic connections. (B) A second way PNNs can prevent formation of new synaptic connections, is through selective binding chemorepulsive guidance cues. ChABC removes GAG chains of CSPGs, which may lead to release of inhibitory molecules. (C) PNNs may prevent lateral diffusion of receptors in and out of the synapse by acting as a scaffold on plasma membranes. ChABC disrupts PNN structures, allowing for example exchange of AMPA receptors (Frischknecht et al., 2009). Adopted from Sorg et al. (2016) with permission.

development (Carulli et al., 2010). Moreover, dark rearing of animals prevents PNN formation and extends the critical period (Pizzorusso et al., 2002). Finally, it was shown that plasticity can be reinstated after the closure of the critical period by degradation of PNNs with chondroitinase ABC (chABC), allowing recovery of equal visual contribution from both eyes after monocular deprivation (Pizzorusso et al., 2002). ChABC is an enzyme derived
from the Proteus vulgaris bacteria (Yamagata et al., 1968), which cleaves and thus removes the GAG chains of CSPGs (Prabhakar et al., 2005). Taken together, these findings show that PNN formation is activity-dependent and leads to closure of the critical period whereby network plasticity becomes markedly reduced. Similar processes have been shown to regulate the development of sensory cortex (M Crae et al., 2007; Nowicka et al., 2009), cerebellum (Carulli et al., 2007; Foscarin et al., 2011), spinal cord (Galtrey et al., 2008) and hippocampus (Dityatev et al., 2007).

Several mechanisms have been proposed through which PNNs limit plasticity (Fig 2). The first is for PNNs to function as a physical barrier through which they can prevent establishment of new synapses onto the neurons they ensheath (De Vivo et al., 2012; Wang and Fawcett, 2012). A second proposed mechanism is that PNNs prevent formation of new synaptic connections because they selectively bind chemorepulsive guidance cues such as semaphorin 3A (De Wit et al., 2005; Dick et al., 2013; Vo et al., 2013) and semaphorin 5A (Kantor et al., 2004). These theories are supported by the fact that treatment with chABC enhances axonal sprouting (Corvetti and Rossi, 2005; Massey et al., 2006). A third mechanism is through PNNs acting as a scaffold on plasma membranes, thus limiting the mobility of receptors in and out of the synapse (Frischknecht et al., 2009).

Given that PNNs are commonly associated with parvalbumin-expressing (PV+) γ-aminobutyric acid (GABA)ergic inhibitory interneurons, it seems likely that their plasticity-restricting properties arise from modulating the activity of these neurons. Hence, in the visual cortex it was shown that the closure of the critical period relies on GABAergic transmission. Inhibition of GABAergic transmission delays critical period closure and enhancing GABAergic inhibition accelerates critical period closure (Hensch, 2005). Several proteins have been associated with this process such as brain-derived neurotrophic factor (BDNF), of which increased levels promote critical period closure (Huang et al., 1999). More recently, loss- and gain-of function of the orthodenticle homeobox 2 (OTX2) homeoprotein has also been associated with promotion or inhibition of critical period closure, respectively (Sugiyama et al., 2008). Specifically, persistent internalization of OTX2 by PV+ interneurons was shown necessary to maintain critical period closure, since blockage of OTX2 internalization reinstated plasticity beyond the critical period (Beurdeley et al., 2012). Furthermore, dark rearing reduces both BDNF (Huang et al., 1999) and OTX2 (Sugiyama et al., 2008) expression, thus extending the closure of the critical period. Digestion of PNNs with chABC reduced intracellular localization of OTX2 (Sugiyama et
al., 2008) and increased PV+ interneuron firing rates, rendering them more excitatory (Dityatev et al., 2007).

**PNNs and memory**

PNNs are also found in the hippocampus. The hippocampus has relatively low PNN numbers, with the CA2 area expressing the highest PNN density. Even though in the CA2 area some PNNs have been found around pyramidal neurons (Carstens et al., 2016; Morikawa et al., 2017), the majority of hippocampal PNNs ensheaths PV+ interneurons (Härtig et al., 1992; Morris and Henderson, 2000; Miyata et al., 2005; Lensjø et al., 2017). This opens up the possibility that PNNs not only regulate critical period plasticity, but also adult plasticity underlying learning and memory. Indeed, direct inhibition of hippocampal PV+ neurons or chABC treatment both enhanced learning in mice, suggesting that PNNs regulate learning and memory by controlling inhibitory transmission in the hippocampus (Donato et al., 2013).

**EXTRACELLULAR VESICLES**

Clearly, the extracellular space is not a static environment in which cells are kept in place. In addition to the ECM, which turns the extracellular space into a highly organized system that regulates neuronal and synaptic functions, cells also communicate through secretion of molecules or vesicles into the extracellular space. Extracellular vesicles (EVs), play an important role in cell-to-cell communication, as they mediate transfer of cytosolic proteins, lipids and RNA between cells (Chivet et al., 2013). In addition to non-coding RNAs, EVs can contain many coding RNAs, thus enabling the emitting cell to modulate protein expression in the recipient cell (Valadi et al., 2007). EVs can be released from various cell types such as microglia (Prada et al., 2013), astrocytes (Proia et al., 2008), oligodendrocytes (Krämer-Albers et al., 2007; Fitzner et al., 2011) and neurons (Faure et al., 2006; Lachenal et al., 2011) and they can be transferred between different cell types (Krämer-Albers et al., 2007; Frühbeis et al., 2013a).

**Biogenesis of EVs**

EVs can be subdivided into two classes based on their subcellular origin: microvesicles and exosomes. Microvesicles (ectosomes) are formed through outward budding and shedding of the cells’ plasma membrane and are between 100–200 nm in diameter (Budnik et al., 2016). Exosomes are with 40–100 nm in diameter smaller than microvesicles and are initially formed intracellularly within multivesicular bodies (MVBs) through inward budding
Figure 3 | Biogenesis and release of microvesicles and exosomes

Microvesicles and exosomes are released via two independent pathways. Microvesicles are formed through outward budding and shedding of the plasma membrane. Exosomes are formed within multivesicular bodies (MVBs) through inward budding of the endosomal membrane, thus releasing intraluminal vesicles (ILVs). MVBs can fuse with the plasma membrane, thereby releasing exosomes into the extracellular space. Adopted from Meldolesi et al. (2018) with permission.

of the endosomal membrane, forming so-called intraluminal vesicles (ILVs). The formation of ILVs is driven by cellular sorting machineries that can work in an ESCRT-dependent or -independent manner (FIG 3) (Colombo et al., 2014).

The ESCRT (endosomal sorting complexes required for transport)-dependent machinery was first discovered at the beginning of this century and can be subdivided into 4 subcomplexes; ESCRT-o, -I, -II and -III (Katzmann et al., 2001; Hurley and Hanson, 2010; Colombo et al., 2013). These four complexes play distinct roles in the formation of a vesicle. ESCRT-o recognizes and recruits ubiquitylated cargo, ESCRT-I and -II facilitate bending of the membrane and the final budding and shedding of the vesicle are mediated by ESCRT-III (Wollert et al., 2009; Hurley and Hanson, 2010; Henne et al.,
2011). Recently, studies identified MVB biogenesis pathways that do not rely on the ESCRT machinery, hence giving rise to the discovery of ESCRT-independent sorting machineries. It was shown that in oligodendroglial cells exosome release was markedly reduced after ceramide depletion (Trajkovic et al., 2008). Furthermore, in human MNT-1 cells, CD63 has been implicated in ILV formation (Van Niel et al., 2011), and CD9 has also been linked to MVB sorting and subsequent exosome release (Buschow et al., 2009). These findings were further supported by the fact that MVBs are formed in the absence of ESCRTs (Stuffers et al., 2009).

It seems that cargo and cell type together determine through which sorting machinery ILVs are formed; accordingly, cells can contain multiple types of MVBs. However, it remains elusive whether different sorting mechanisms can act concomitantly in one MVB or that they function in distinct MVBs (Van Niel et al., 2018). Nevertheless, the mere existence of different sorting mechanisms already indicates that exosomes are not a homogenous type of vesicle, and there is heterogeneity within exosome populations (Kowal et al., 2016; Willms et al., 2016). Proteins ALIX and TSG101 are associated with ESCRT-dependent and -independent machinery and are retained on the exosome membrane (Baietti et al., 2012). As a result, they are ubiquitous exosome markers.

The mechanisms underlying the biogenesis of microvesicles are less well understood. In contrast to exosomes, microvesicles are formed directly on the plasma membrane of the cell and require local accumulation of cargo at specialized plasma membrane microdomains. Subsequently, formation of a vesicle requires outward budding and shedding of the plasma membrane. The fluidity and the thereof resulting blebbing of the membrane are enhanced by rearrangements of the inner and outer layers of the phospholipid bilayer by enzymes such as floppase, flippase and scramblase (Piccin et al., 2007; Colombo et al., 2014). Additionally, calpain causes a restructuring of the cytoplasmic cytoskeleton, thus facilitating microvesicle shedding (Piccin et al., 2007). Moreover, in glial cells it was shown that acid sphingomyelinase (A-SMase) plays a crucial role. A-SMase is recruited to the plasma membrane where it rapidly hydrolyses sphingomyelin (SM), thus increasing the fluidity of the plasma membrane which enhances membrane blebbing and subsequent microvesicle shedding (Bianco et al., 2009). The ESCRT machinery and TSG101 are both involved in microvesicle shedding, indicating potential mechanistic overlaps between microvesicle and exosome biogenesis (Henne et al., 2011).
Release of EVs

While the release of microvesicles directly follows cargo recruitment, membrane budding and shedding from the plasma membrane, exosome release requires translocation of MVB to and fusion with the plasma membrane. Despite this difference, both microvesicles and exosomes are released in an activity-dependent manner. Specifically, exosomes release is triggered by glutamatergic activation and opening of NMDA receptors (Faure et al., 2006; Lachenal et al., 2011; Chivet et al., 2014), and microvesicle release is triggered upon stimulation with glutamate (Antonucci et al., 2012) and Ca2+ (Bianco et al., 2005). Even though exosome and microvesicle release both rely on glutamatergic activation, there may be a difference in release latency. Microvesicles synthesis takes place at the plasma membrane and can therefore be more readily released than exosomes, as more steps are required for their release.

MVBs can either fuse with lysosomes, which leads to their degradation, or they can fuse with the cells’ plasma membrane, thus releasing ILVs into the extracellular space, which from then on are called exosomes (Von Bartheld and Altick, 2011; Budnik et al., 2016). What determines this difference in destination remains largely unknown, although some factors have been identified that either permit or avert lysosomal degradation. It has been shown that involvement of syndecan-synthenin-ALIX protein complex in MVB biogenesis favors fusion with the plasma membrane instead of the lysosome (Baietti et al., 2012). In contrast, ESCRT machinery component TSG101 fates MVBs for lysosomal degradation (Edgar et al., 2015). Targeting of MVBs to the plasma membrane is facilitated by various proteins from the RAB family, e.g. RAB7 (Baietti et al., 2012), Rab11 (Savina et al., 2002), Rab27 (Ostrowski et al., 2010) and Rab35 (Hsu et al., 2010; Frühbeis et al., 2013b). Subsequent fusion of MVBs with the plasma membrane is enabled by the SNARE complex (Wei et al., 2017).

Targeting and function of EVs in the CNS

For a long time it was assumed that EV release is merely a mechanism for cells to dispose of unwanted proteins, until two studies showed that exosomes mediate immune responses and cell-cell communication in the immune system (Raposo et al., 1996; Zitvogel et al., 1998). EVs released into extracellular space can influence other cells in different ways. They can interact with receptors on target cells, thus activating intracellular signaling cascade, they can fuse with target cells, releasing their content into the cytoplasm of the target cell, or they can be endocytosed and form
new MVBs within the target cell (Meldolesi, 2018; Van Niel et al., 2018). Target specificity of EVs seems to rely on adhesion molecules located on the membranes of EVs and of the recipient cell, such as tetraspanins (Morelli et al., 2004) and fibronectin (Leiss et al., 2008; Sung et al., 2015).

The role of EVs in the CNS appears to be very diverse, and new studies keep revealing novel mechanisms and functions. As mentioned previously, exosomes are hypothesized to mediate communication between different cell types. However, the effects that exosomes have on recipient cells depend both on their origin and the identity of the receiving cell. For example, oligodendrocyte-derived exosomes that are taken up by neurons protect recipient neurons against oxidative stress (Frühbeis et al., 2013a; Fröhlich et al., 2014). In contrast, when taken up by microglia, no apparent change in microglial function is measured (Fitzner et al., 2011). Finally, when taken up by oligodendrocytes, they inhibit differentiation and myelin formation (Bakhti et al., 2011). Other cell types in the CNS likely release exosomes and regulate CNS functions in an equally complex manner. For instance, glutamatergic activity triggers exosome release from neurons and glial cells, which can be blocked by AMPA or NMDA receptor antagonists (Lachenal et al., 2011; Frühbeis et al., 2013a; Chivet et al., 2014). Additionally, Park et al. showed that local fusion of endosomes in spines following LTP induction, contributes to spine enlargement due to membrane fusion (Park et al., 2006). These data and the fact that glutamate triggers exosome release, suggest a role for exosomes in synaptic plasticity. However more research is needed to further elucidate this.

**ALZHEIMER’S DISEASE: AN EXTRACELLULAR VIEW**

**Alzheimer’s disease**

In 1906, Aloïs Alzheimer reported on memory loss, cognition and language impairment in August D, the first patient diagnosed with Alzheimer’s disease (AD). In the last 100 years AD became the most prevalent form of dementia, and even though substantial research has been performed into the mechanisms underlying AD pathogenesis much of these remain elusive to date. With age being the most important risk factor for AD, and an increasing life expectancy of the world population, the number of AD patients is expected to increase dramatically in the coming decades (Brookmeyer et al., 2007).

AD is characterized by progressive episodic memory impairment. Over time
other cognitive functions, such as language and orientation, and motor function, such as swallowing and walking are also attenuated. The symptoms of AD typically develop around 20 years before clinical diagnosis (Bateman et al., 2012). The most characteristic neuropathological hallmarks of AD are neuritic plaques, which are large extracellular aggregates mainly composed of the peptide amyloid-beta (Aβ) (Masters et al., 1985), and neurofibrillary tangles, which are intracellular aggregates of hyperphosphorylated tau protein.

Aβ is the product of proteolytic cleavage of the single-pass transmembrane amyloid precursor protein (APP) (Kang et al., 1987; Seubert et al., 1993). APP undergoes sequential cleavage by either α- or β- and γ-secretases, causing APP processing through either the non-amyloidogenic or the amyloidogenic pathway, of which the latter produces Aβ. Non-amyloidogenic processing of APP occurs via the α-secretase pathway, which cleaves APP at its C-terminus, close to the membrane, generating a peptide named C83. Importantly, α-secretase cleaves APP within the Aβ sequence, thus making Aβ production impossible. The membrane-retained C83 peptide is subsequently cleaved by γ-secretases (Haass et al., 1993), an enzyme complex consisting of presenilin 1 and 2 (PS1 and PS2), which form the catalytic core of the complex (Nunan and Small, 2000; Laferla et al., 2007; De Strooper et al., 2010). APP processing through the amyloidogenic pathway is mediated by β-secretases, which cleaves APP further away from the membrane, outside the Aβ sequence, generating a larger C-terminal fragment named C99. As in the non-amyloidogenic pathway, this peptide is cleaved by γ-secretases thereby releasing Aβ40 and Aβ42 peptides (Sisodia et al., 1990; Laferla et al., 2007; De Strooper et al., 2010). Aβ42 aggregates more readily than Aβ40 due to its more hydrophobic properties (Jarrett et al., 1993).

The majority of all AD cases occurs on a seemingly sporadic basis, with specific genes adding to risk (Bertram et al., 2010; Jansen et al., 2019), however, in approximately 2% of the cases AD is caused by mutations in the APP (Goate et al., 1991; Citron et al., 1992), presenilin 1 (PSEN1) or presenilin 2 (PSEN2) gene (Levy-Lahad et al., 1995; Sherrington et al., 1995; Tanzi and Bertram, 2005; Winblad et al., 2016). These mutations lead to an increase in Aβ42/Aβ40 ratios, which promotes aggregation of Aβ42 peptides (Haass et al., 1995; Borchelt et al., 1996). Mutations in APP, PSEN1 or PSEN2 cause familial forms of AD (fAD) or early-onset AD. Additionally, mutations in APP have been identified that protect against cognitive decline and AD (Jonsson et al., 2012). These findings, combined with the fact that people with Down’s syndrome inevitably develop AD due to trisomy of
chromosome 21, on which the APP gene is located (Glenner and Wong, 1984; Patterson et al., 1988), led to a strong focus on Aβ being the causative factor in AD pathogenesis and the postulation of the amyloid cascade hypothesis (Selkoe, 1991; Hardy and Higgins, 1992). The amyloid cascade hypothesis states that accumulation of the Aβ peptide due to increased production or reduced clearance is the causative agent for AD pathology and dementia. This hypothesis assumes that familial and sporadic AD, though caused by different mechanisms, manifest as the same disease, as Aβ pathology is morphologically comparable between the two.

**Extracellular processes in AD**

Extracellular accumulation of Aβ is one of the hallmarks of AD and many studies have been devoted to unraveling which extracellular factors influence Aβ aggregation and which render neurons more or less vulnerable for Aβ neurotoxicity. Over the years ECM and later also EVs have been associated with AD.

Several studies have reported protective effects of ECM against extracellular toxicity (Morawski et al., 2004; Suttkus et al., 2012; Cabungcal et al., 2013) and a inverse correlation between ECM level and AD pathology (Brückner et al., 1999; Morawski et al., 2010a). Miyata and colleagues showed in an *in vitro* experiment that specifically PNN-positive neurons are protected against Aβ toxicity and that removal of PNNs with chABC attenuates these protective properties (Miyata et al., 2007). Thus it seems that ECM can protect against Aβ toxicity and AD pathology. In AD patients ECM levels are decreased (Baig et al., 2005) and in a transgenic mouse model for AD (Tg2576) ECM levels are unaffected (Morawski et al., 2010b). However, there are also studies reporting increased ECM levels in transgenic mouse models (Howell et al., 2015) and AD patients (Hondius et al., 2016). These contradicting findings may be due to the sensitivity of GAG side chains to decomposition that is inevitable in cases with a long post mortem delay (Morawski et al., 2012). Nevertheless, since ECM may serve as a platform for Aβ plaque formation (DeWitt et al., 1993) and ECM has protective properties, it can be hypothesized that neurons and glia increase secretion of ECM proteins in AD. Because condensation of ECM proteins into PNNs markedly reduces synaptic plasticity, this may be a causative factor in AD-characteristic problems with learning and memory.

**AD and EVs**

Beginning of this century Takahashi and colleagues showed that Aβ
accumulates in MVBs, indicating a potentially important role for EVs in the spreading of Aβ (Takahashi et al., 2002a). A few years later it was shown that Aβ and APP c-terminal fragments can be released in association with EVs (Rajendran et al., 2006; Sharples et al., 2008; Perez-Gonzalez et al., 2012), which is not surprising considering that APP cleavage takes place in endosomes (Haass et al., 1992; Huse et al., 2000; Ehehalt et al., 2003). Until now it remains unclear whether EVs contribute to the spreading or clearance of Aβ. Aβ can increase release of EVs, which subsequently leads to apoptosis. Blockage of EV release prevents apoptosis (Wang et al., 2012) and reduces plaque load (Dinkins et al., 2014). Additionally, EVs from AD have shown to be neurotoxic and can spread their content to other cells (Agosta et al., 2014; Joshi et al., 2014; Sardar et al., 2018). Collectively, these data indicate that release of Aβ in association with EVs may aid the progression of AD, comparable to prion proteins that also spread from cell to cell through exosomes (Fevrier et al., 2004). In contrast to this, other studies show exosomes hinder Aβ spreading through sequestration of Aβ on their surface via glycosphingolipids (GSLs) (Yuyama et al., 2014) and cellular prion protein (PrPc) (An et al., 2013). Through GSLs exosomes stimulate Aβ fibril formation and uptake by microglia. Also, the scavenging of Aβ by exosomes also rescues Aβ-mediated LTP impairments in vivo. All in all these studies illustrate a relation between ECM, EVs and AD, though the precise role of ECM and EVs in the pathogenesis and progression of AD remains elusive.

AIMS AND OUTLINE OF THIS THESIS

The primary aim of this thesis is to gain insight into the role of the ECM in early cognitive deficits in AD. For this we use the APPswe/PS1dE9 mouse model, which harbors two human genes with mutations that both lead to an increased Aβ production: the Swedish APP mutation K670N/M671L (APPswe), and a mutation in PSEN1 that results in a deletion of exon 9 (PS1dE9). Each gene is driven by a prion protein promoter (Jankowsky et al., 2001, 2003, 2004). APP/PS1 mice show accelerated Aβ deposition (Holcomb et al., 1998), which aggregate into amyloid plaques around 6-7 months-of-age (Jankowsky et al., 2004; Reiserer et al., 2007). As a result of this APP/PS1 mice develop LTP induction deficits at 3 months-of-age (Trinchese et al., 2004) and different ages have been reported on when the first cognitive deficits occur (Hsiao et al., 1996; Holcomb et al., 1998; Trinchese et al., 2004; Reiserer et al., 2007). In these mice, we enzymatically manipulate hippocampal ECM levels and study the effects at the behavioral, cellular and molecular levels.
In **chapter 2** we show that rising Aβ levels in 3 months old APP/PS1 mice correlate with an increase in hippocampal ECM levels and with early learning and memory deficits. Enzymatic digestion of hippocampal ECM with chABC rescued these early learning and memory deficits, suggesting a crucial role for the ECM in memory deficits in the early stages of AD.

**Chapter 3** describes the effects of local hippocampal chABC treatment on discrimination learning in the CognitionWall and spatial learning in the Morris water maze. When applied immediately prior to the task, chABC treatment at 4 months of age rescued discrimination learning but not spatial learning. However, no beneficial effects of chABC were observed anymore 4 weeks later. Additionally, chABC treatment of APP/PS1 mice at 9 months of age did not rescue discrimination or spatial learning deficits.

In **chapter 4** we address the question whether there is a causal link between Aβ42 levels and ECM upregulation in APP/PS1 mice. We treated APP/PS1 mice with two different doses of LY2886721, a potent BACE1 (β-secretase) inhibitor. Only with a single high dose of LY2886721 we were able to reduce brain Aβ42 levels and rescue learning and memory deficits. However, this did not result in a decrease in ECM levels. The data is discussed in light of the current debate on the validity of the amyloid cascade hypothesis.

In **chapter 5** we aim to elucidate the molecular mechanisms underlying chABC-mediated rescue of learning and memory in APP/PS1 mice. For this we designed a new procedure to isolate and identify ECM-bound proteins that are released upon chABC treatment. The resulting proteomic data showed a chABC-enhanced release of EVs and a differential regulation of EV protein content between APP/PS1 transgenic and wildtype mice. These data are discussed in the light of the upcoming role for EVs in the pathogenic spreading of brain diseases.

The results from the presented studies are reviewed and discussed in **chapter 6** to provide a general model for the role of the ECM and EVs in the early stages of AD. Additionally, future perspectives are presented focusing on the relevance of the obtained results.