The Importance of Glia-Synapse Interactions in Synaptic Connectivity

Writing assignment

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Research Proposal

Under the supervision of Dr. Rogier Min

Netherlands Institute for Neuroscience

Amsterdam, The Netherlands

Examiner: Dr. Corette Wierenga

Utrecht University

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Teresa Haider

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1 Introduction

Stable synaptic connections between neurons in adulthood provide the basis for brain functioning (Grutzendler et al. 2002). By connecting neurons, synapses perform one of the most vital roles in the organization of the brain, as they allow transfer of signals from one cell to the next. One characteristic of synapses is that they can store information, which they do in multiple ways: The neurons, which the synapses connect, the positioning of synaptic contacts along the axon and dendrite, how synapses are clustered, their strength and their molecular composition all encode information. In this context, information is to be understood as characteristics that have been shaped by the experience of the past of the neuronal circuit, which in turn shape the future behavior of that circuit.

If zoomed out from the synaptic to this circuit level of information storage, it becomes apparent that in order for new information to be stored and old, unused information to be deleted, the characteristics of that neural circuit need to change. Accordingly, the pool of stable synapses is constantly modified, added to and removed from, a process termed synapse turnover. This ability of the synaptic connections of neuronal circuits to change in response to experience is one of the premises that make the brain plastic.

Synaptic connectivity, the pattern of connections between neurons, is established during development and refined in adulthood. Postnatally, an overshoot of synapses is formed and subsequently unfitting synapses are eliminated in an activity-dependent manner (Katz and Shatz 1996). The synapses that persist into adulthood are comparatively stable, yet, even in adult animals there is a basic level of synapse turnover. Dendritic spines are the postsynaptic protrusions from the dendrite, which align with the pressynaptic element to form an excitatory synapse. By tagging the postsynaptic neuron or proteins associated with spines with fluorescent proteins synapse turnover can be quantified over time in imaging studies. These studies have shown that the share of synapses that turn over varies according to the age of the animal and seems to level at around 5% spine turnover per month in the adult mouse (Zuo et al. 2005). Moreover, adult synapse turnover is increased in connection to learning of novel tasks or new movements (O'Malley et al. 2000, Caroni et al. 2012).

In recent years, the role of glial cells in all stages of the synaptic life cycle has become apparent. Not only can glial factors initiate and promote synapse formation, glial cells are also necessary for the proper elimination of weak synapses, which they help tag and engulf via phagocytosis, both during development and in adulthood (Schafer and Stevens 2010, Tasdemir-Yilmaz and Freeman 2014, Chung 2013). Two subtypes of glial cells have been under special investigation: Astrocytes, the glial cells that are in tight contact with neurons through their highly ramified processes which enwrap synapses and thus create the tripartite synapse, and microglia, the resident immune cells of the brain. Microglia have highly motile processes, which sample their surroundings, notably synapses, for immune-relevant substances, as well as cellular debris and weak synapses (Nimmerjahn et al. 2005, Ransohoff and Cardona 2010).

In this thesis, I will review the current state of knowledge of the stages of the life cycle of synapses in regards to how they are defined and shaped by glial cells. Chapter one describes how glia act in synapse formation to promote initial contact and stabilization of newly formed connections. Chapter two focuses on the involvement of phagocytic microglia and astrocytes in the recognition and elimination of weakened synapses. Chapter three delineates first findings on how synaptic ensheathment by astrocytes influences synaptic stability and maturation. Finally, chapter four outlines glia-synapse interactions in synaptic plasticity. At the end of the thesis, I propose a set of experiments, which aim at addressing an important gap in our knowledge of this exciting and growing field of neuroscience: how glial cell signaling can contribute to adult learning.

2 Background

2.1 Synapses

Synapses are specialized structures that allow the transfer of impulses from one neuron to the next. The transfer can occur by multiple means: In chemical synapses electrical activity in the form of propagating ion currents are converted into chemical signals, manifested in the release of neurotransmitters by the presynaptic neuron. These neurotransmitters bind to receptors on the postsynaptic neuron, whose activation elicits the flow of ionic currents and thus passes on the propagating signal. In electrical synapses, on the other hand, gap junctions allow for direct passing of the ionic currents from the presynaptic to the postsynaptic cell. Here, we focus on chemical synapses, as their development, structure and refinement is more complex and thus the current focus of investigation.

Chemical synapses are classified by the effect they have on the excitability of the postsynaptic neuron and by the neurotransmitter they depend on. Excitatory synapses depolarize the postsynaptic cell, thereby raising the probability of it firing an action potential, whereas inhibitory synapses hyperpolarize it, lowering this probability. Based on the observation that most excitatory synapses use glutamate as their neurotransmitter and most inhibitory synapses depend on γ-aminobutyric acid (GABA), this review focuses on these two types of synapses. Far less is known about inhibitory synapses than excitatory synapses. The discrepancy has been mostly due to the lower number of inhibitory compared to excitatory synapses – their ratio varies roughly between 1:20 to 1:3 in different regions of the brain – (Gulyás et al. 1999, Megías et al. 2001) and to the difficulty to biochemically purify their material (Kuzirian 2011).

Both excitatory and inhibitory synapses are formed between a presynaptic axonal bouton and a postsynaptic element, which is mainly located on neuronal dendrites. Axonal boutons are structural bulbs on axons, which can be either formed at the terminals of axonal branches (bouton terminal) or "along the way" of the axonal branch (bouton en passant). Boutons contain vesicles filled with neurotransmitter as well as the machinery to release these vesicles into the synaptic cleft. The structure of excitatory and inhibitory synapses differs in the form and location of their respective postsynaptic specializations. Excitatory synapses form mostly between an axonal bouton and a dendritic spine, which can have one of multiple shapes, such as a thin, mushroom or stubby shape (Bourne and Harris 2008). The dendritic spine contains a specialization of its membrane, called postsynaptic density (PSD; Gray 1959, Siekevitz 1985). Inhibitory synapses form between an axonal bouton and the shaft of a dendrite, cell body or an axon initial segment (Gray 1959, Peters and Palay 1996, Sheng and Kim 2011, reviewed by Kuzirian 2011). When observed in eletron microscopy images, inhibitory synapses appear more symmetrical in comparison to excitatory synapses. The distinct electron-dense structure of excitatory synapses is less pronounced and the organelle-like postsynaptic density is missing (Colonnier 1968, Peters and Palay 1996, Galvan et al. 2004).

Not only the morphology, but also the molecular composition of the postsynaptic element differs between excitatory and inhibitory synapses. In some aspects, the organizing principles of the two synapse types are similar, but the molecules that fulfill the respective roles differ, as is the case of the main postsynaptic scaffolding protein: Postsynaptic density protein 95 (PSD-95) clusters receptors, ion channels and other functional molecules in excitatory synapses, a role that is executed by gephyrin in inhibitory synapses (Colledge and Froehner 1998).

The most abundant group of neurotransmitter receptors found on the postsynaptic element of exitatory synapses are glutamate receptors, namely the ionotropic NMDA, Kainate and AMPA receptors and the metabotropic mGluRs. The main population of neurotransmitter receptors at the postsynaptic site of inhibitory synapses are ionotropic GABA receptors ($GABA_A$ receptors), which are permeable to chloride and whose activation hyperpolarizes the cell and metabotropic GABA receptors ($GABA_B$), which are located extrasynaptically and are activated by GABA spillover (Kulik et al. 2003, Scanziani 2000).

All in all, synapses are highly specialized neuronal structures optimized for a combination of storing and passing on information in the form of chemical signals.

2.2 Glia

In recent decades, glial cells - traditionally viewed as non-excitable, supportive cells - have moved into the focus of research in light of accumulating evidence for their involvement in synapse physiology, plasticity, information processing, connectivity, and rehabilitation after injury. The precise numbers of glia and neurons in the adult human brain are difficult to quantify. Their respective numbers have the same order of magnitude, although the ratio of glia to neurons differs between brain regions (Azevedo et al. 2009). Some post-mortem studies have tried to quantify cellular numbers, as shown in **Table 1**.

Glia is an umbrella term in that it summarizes most cells of the central and peripheral nervous system (CND and PNS) that are not neuronal cells, irrespective of their developmental origin or function. The defining characteristic is that they, unlike neurons, cannot transmit rapid electrical signals, such as action potentials (Kuffler and Potter 1964). Nonetheless, many ion channels and neurotransmitter receptors typical for excitable cells are found in glia (Kuffler and Potter 1964, Kuffler et al. 1966, Orkand et al. 1966, reviewed by Sontheimer 1994, Verkhratsky and Steinhaeuser 2000), enabling them to sense neuronal activity and the environment. In contrast to the common simple dichotomy of neurons versus glia, transcriptome analyses of glia and neurons have revealed that the gene expression profiles of distinct glial subgroups differ from each other as much as they differ from neurons (Cahoy et al. 2008).

The major subgroups of glia in the CNS are oligodendrocytes, astrocytes and microglia. In the PNS, Schwann cells are found in place of oligodendrocytes (Schwann 1839). Some anatomical structures of the nervous system have additional, specialized and individually named glial cell types, including Bergmann glia, a form of astrocytes in the cerebellum, Müller glia, a class of glia in the retina, and ependymal cells, that produce cerebrospinal fluid (Cameron and Rakic 1991). Oligodendrocytes, Schwann cells and astrocytes are collectively referred to as macroglia, in contrast to microglia, that form a developmentally distinct group.

Cell type		**Females**		**Males**	
		Total	Percentage	Total	Percentage
Glia	Oligo-dendrocytes	21.0 billion	75.6%	28.8 billion	74.6%
	Astrocytes	4.8 billion	17.3%	7.8 billion	20.2%
	Microglia	1.8 billion	6.5%	2.0 billion	5.2%
Neurons	Neocortical Neurons	21.4 billion		26.3 billion	

Table 1 | Numbers of glia and neurons in the brain

Data from Pelvig et al 2007. N=37, postmortem study, optical disector. Mean age for females 65.1 years, for males 57.4 years, not corrected for age. The total number of glial cells were estimated to be 27.9 billion in females and 38.9 billion in males. The total number of neurons were estimated to be 21.4 and 26.3 billion in females and males respectively. Percentages missing to 100% in females sic in original publication.

Macroglia develop from ectodermal precursor cells into further specialized subclasses during embryonic development. One such class of precursors are radial glia, which not only give rise to macroglia, but also most neurons of CNS. The precise temporal, spatial and hierarchical lineages of precursor cells, radial glia, astrocytes, oligodendrocytes and neurons have been subject to controversy. In particular, the scientific community was reluctant to accept glial cells to be the precursor of neuronal cells, although this is now generally recognized (Choi et al. 1983, Bentivoglio and Mazzarello 1999, Malatesta et al. 2000, Campbell and Götz 2002, Malatesta et al. 2003, Anthony et al. 2004, Fogarty et al. 2005, Merkle et al. 2004; for historical

review of the controversy Malatesta and Götz 2013). Microglia form an exception as they are derived from primitive myeloid progenitors, early macrophages of the yolk sac (Ginhoux et al. 2010).

In accordance with research in the last three decades on glial involvement in synaptic connectivity, here I will focus on astrocytes and microglia as players in the life cycle of synapses. The main glial cell group that hence will be neglected are oligodendrocytes, the cells that form the insulating myelin sheath around neuronal axons and numerically comprise the biggest group of glia in the brain (Pelvig et al. 2007). The reason for doing so is that they seem to contribute considerably less to the regulation of the synaptic life cycle than astrocytes and microglia. Therefore, the literature on them is limited (for review, see Fields 2005).

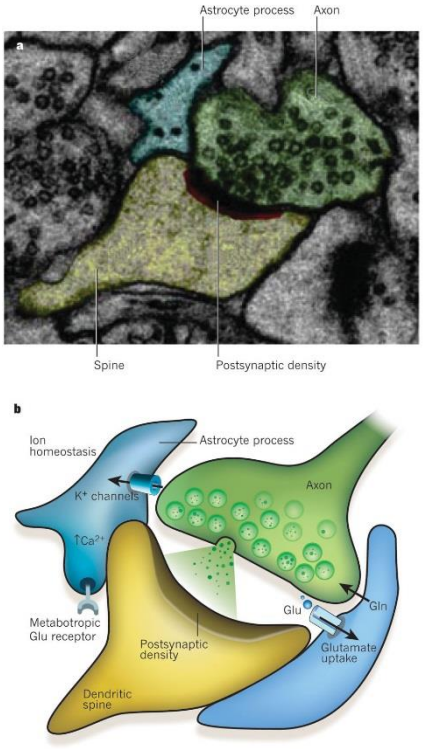

Figure 1 | The tripartite synapse
(Source: Eroglu & Barres 2010)

Above: Electron micrograph showing a tripartite synapse in the hippocampus. The astrocyte process (blue) ensheaths the perisynaptic area. The axon of the neuron is shown in green, with the dendritic spine in yellow and the postsynaptic density in red and black. (In Eroglu & Barres 2010, reproduced from Witcher et al. 2007).

Below: Schematic representation of the image above. Glutamate receptors on astrocytes (such as metabotropic glutamate receptors) sense synaptic glutamate release, which in turn induces a rise in Ca^{2+} concentration in the astrocytes. One of the main functions of glia at the synapse is to maintain ion homeostasis, for example regulating extracellular K^+ concentrations and pH.

Astrocytes

Astrocytes get their name from their stellar shape, as *astron* is the Greek word for star. Their high number of very thin processes extends from the soma and enwraps synapses. Through this tight contact, they are able to directly interact with synapses and fulfill their canonical roles of ion homeostatis, neurotransmitter clearance, energy supply and shielding the synapse from the exterior influences from other synapses. Astrocytes form non-overlapping domains of various shapes, which allows them to influence synapses within their domain in a coordinated fashion (Bushong et al. 2002).

This view of astrocytes as simple housekeeping cells has come under scrutiny, as more and more involvements of astrocytes in functions that have been historically seen as exclusive to neurons are being discovered. The main new role that has been acknowledged is their active shaping of information processing, as is illustrated in the concept of the tripartite synapse (Araque et al. 1999 (**Figure 1**).

Microglia

Even more recently, the concept of synapses has been expanded to a *quadpartite* synapse (Schafer et al. 2013), since microglia are being discovered to play an important role in synaptic development and plasticity. However controversial, the possibility for these conceptual expansions further stresses the paradigm shift from neurons as the sole actor in information processing.

As mentioned earlier, microglia differ from other cells in the brain in that they ontologically form part of the immune system. In their resting state, they sample the neuropil for damages, infection and debris, phagocytosing any undesireable material. If certain events, such as an infection, are detected, microglia can become activated and motive, migrating to the site of insult (reviewed by Lively and Schlichter 2013). Even more, recent evidence suggests that microglia are not only responsive to damages or infection, but that they can also help shape synaptic networks in the healthy brain.

Even though the concept of glia-synapse interaction has become more accepted, the precise molecular mechanisms of interactions have only partially been discovered. What is known and what is yet to be discovered will be outlined in the following chapters.

3 Synapse life cycle

The life cycle of synapses is defined by formation, maturation, plasticity and potentially elimination. These stages should not be seen as temporally distinct, subsequent processes, but their underlying processes co-occur, interact and compete.

A new dendritic protrusion may form a contact with an axonal bouton, after which an astrocyte extends its process partially around it, promoting maturation. Yet concurrently, neighboring synapses might fit the properties of the network better, become relatively stronger and trigger the release of competitive elimination signals that interfere with maturation and lead to the destabilization and eventually elimination of the newly formed, weaker synapse by microglia.

The development and fate of synapses is activity-dependent on an individual level, but should also be seen in the wider framework of energy restraints on an organism. The maintenance of synapses is energetically expensive to an extent where there was evolutionary pressure to reduce the number of synapses required per unit of information processed or stored (Laughlin 2001, Niven and Laughlin 2008). Hence, the selection and refinement of the necessary synapses versus the elimination of the superfluous or inapt synapses are the underlying theme of the synaptic life cycle.

3.1 Synapse formation

Synaptogenesis, the formation of synapses, requires a complex interplay of the pre-, post- and perisynaptic elements to form the tripartite synapse. For an overview of the time course of synaptogenesis, see the **box *Short timeline of synaptogenesis***.

The notion that astrocytes, or glia in general, could be involved in the regulation of synaptogenesis was spurred by observations of their close contact to synaptic elements and the concurrent progression of pre- and postsynaptic assembly and astrocytic ensheathment of the synapse (Pfrieger and Barres 1997, Friedman et al. 2000, Nägler et al. 2001). This temporal coincidence made it conceivable that astrocytes could secrete factors that promote or even induce synaptogenesis. Since these first hints, a multitude of mechanisms by which glia in fact regulate synaptogenesis have been suggested and identified, as reviewed in this section. Of the glial cells in the brain, astrocytes and oligodendrocytes, but not microglia have been shown to promote synaptogenesis (Pfrieger and Barres 1997). Because of the focus of this thesis on astrocytes and microglia, in the rest of this chapter I will therefore only discuss the role of astrocytes in synaptogenesis.

Astrocytes participate in synaptogenesis in three ways: First, in early contact formation by contact-dependent mechanisms, such as the expression of γ-protocadherins or neuroligins. Second, by releasing factors, which, alongside similar factors secreted by neurons, bind to interaction partners on the pre- and postsynaptic site. These interactions often activate factors that then aid in the assembly of synapses. Third, by regulating the extracellular concentration of factors secreted by neurons, such as neurotrophins. In this section I will review, one by one, what is known about these three ways astrocytes are involved in synapse formation.

Box: Short timeline of synaptogenesis

1. Initial contact formation

Synaptogenesis is initiated by contact between a presynaptic axonal bouton and a postsynaptic site. Excitatory synapses almost exclusively form on newly extended dendritic filopodia that later can become stabilized and develop into dendritic spines (Niell et al. 2004). Most inhibitory synapses form at pre-existing crossings of axons and dendritic shafts (Wierenga et al. 2008). Cell adhesion molecules (CAMs) mediate early contact formation. They are pairs of interacting proteins that are found on the pre- and postsynaptic membrane and that help in aligning the two (Fannon and Colman 1996). Both members of the CAM pair consist of three domains: An extracellular domain for the cross-interaction, a transmembrane domain and an intracellular domain that couples the binding of the extracellular domains to intracellular effects, such as signaling cascades or changes in structural proteins (reviewed by Albelda and Buck 1990). For example,

some presynaptic adhesion molecules link to the active zone of the axonal bouton, likewise some postsynaptic adhesion molecules recruit scaffold proteins of the PSD (Irie et al. 1997, Hata et al. 1998, Sheng and Sala 2001, reviewed by Sheng and Kim 2011). In this manner, pre- and postsynaptic CAMs interact to start structural and functional molecular changes that stabilize the contact and enable further assembly of the synapse

2. Synapse assembly

One of the most important early steps in synapse formation is the transport of specific pre- and postsynaptic proteins to the synaptic sites. Time-lapse microscopy indicates that the start of the assembly of the presynaptic bouton often precedes the postsynaptic element's (Friedman et al. 2000). On the presynaptic site, vesicles containing neurotransmitters are recruited and captured by a proteinaceous matrix, termed the presynaptic web (Bloom and Aghajanian 1968, Ahmari et al. 2000, Shapira et al. 2003, Zhai et al. 2001). On the postsynaptic site of excitatory synapses, the postsynaptic density is formed as scaffolding proteins and glutamate receptors arrive, with the former acting as an anchor for the latter (Kim et al. 1996, Kornau et al. 1995, Chevesich et al. 1997, Scannevin and Huganir 2000). Most of the components are delivered by transport vesicles (Prange and Murphy 2001). The actin skeleton connects to the scaffolding proteins and determines the shape of the dendritic spine (Allison et al. 2000). There is accumulating evidence that inhibitory postsynaptic structures are formed in a functionally similar way, but the structural protein complexes that anchor GABA receptors are different from the structural complexes that anchor glutamate receptors (Kirsch et al. 1996, Craig et al. 1996, Lévi et al. 2004).
The regulation of synapse assembly occurs in activity-dependent and activity-independent ways. Activity-dependent signaling pathways often involve molecules sensitive to rises in intracellular calcium, such as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Konur and Ghosh 2005, Saneyoshi et al. 2008). The formation of a synapse takes approximately 30 to 60 min from initial contact to functional cycling of neurotransmitters (Friedman et al. 2000).

3. Synapse stabilization
Once formed, there is a wide spectrum of stability of newly formed synapses. Especially in the developing brain, there is a high turnover of synapses being assembled and disassembled (Ruffolo et al. 1978). The synapses that persist into adulthood are comparatively stable, yet even in adult animals there is a basic level of synapse turnover.
One of the determinants of synaptic stability is the number of neurotransmitter receptors at the postsynaptic membrane. This is conceivable, as only a synapse containing functional neurotransmiter receptors can undergo synaptic potentiation and strengthening. Neurotransmitter receptors constantly cycle between intracellular compartments and the postsynaptic membrane, but can be stabilized in the membrane by interacting with binding partners, such as scaffolding proteins (Gerrow and Triller 2010). One such protein is post synaptic density-95 (PSD-95), one of the most determinant factors of synapse stability (El-Husseini et al. 2000, Taft and Turrigiano 2013). The expression levels of PSD-95 rise in a similar time frame as synapse turnover is reduced (Sans et al. 2000, Sun et al. 2011, Trachtenberg et al. 2002). Combined with the finding that overexpressing of PSD-95 in the developing brain stabilizes excitatory synapses and mimics the adult brain in synaptic stability (Taft and Turrigiano 2013), a causal relationship is suspected. The protein is thought to associate with receptors and cytoskeletal elements at synapses, coordinating the stabilization of AMPA glutamate receptors in the postsynaptic membrane (El-Husseini et al. 2000, Gerrow and Triller 2010).
The correspondent scaffolding protein found at inhibitory synapses, gephyrin, follows the same principle and is required for forming clusters of glycine and $GABA_A$ receptors at the membrane (Alldred et al. 2005). The precise mechanisms of how scaffolding proteins are attracted to the synapse are only starting to be elucidated, with a focus on how the differential recruitment of PSD-95 and gephyrin to excitatory and inhibitory synapses is achieved via CAMs (Giannone et al. 2013).

In summary, they form an essential part of synapse development and provide a possible action site for modulation of synapse stability.

(Reviews of structure and assembly of synapses: For excitatory see Goda and Davis 2003, for inhibitory Kuzirian et al. 2011)

3.1.1 Astrocytic cell adhesion molecules

A transcriptome analysis of CNS cells found astrocytes to express multiple mRNAs coding for synaptic CAMs (Cahoy et al. 2008), notably the mRNAs of the synapse formation-promoting neurexins, neuroligins and cadherins. Neuroligins and neurexins are two interacting groups of synaptic CAMs. According to the classical view, neuroligins are thought to be mainly located at the postsynaptic membrane, whereas neurexins form their counterpart on the presynaptic membrane (Song et al. 1999). Their opposing localization and ability to interact closely guides the alignment of the synaptic specializations during initial contact formation and the subsequent induction of the assembly of pre- and postsynaptic specializations respectively (Dean et al. 2003, Nam and Chen 2005), in excitatory and inhibitory synapses alike (Graf et al. 2004). One way neuroligins/neurexins induce synaptic assembly is by presynaptic neurexins binding postsynaptic neuroligins, which in turn bind to PSD-95, the scaffolding protein responsible for neurotransmitter receptor anchoring in excitatory synapse stabilization (Irie et al. 1997). Conversely, the binding of postsynaptic neuroligins to presynaptic neurexins leads to the clustering of synaptic vesicles in axonal boutons and the assembly of a cytoplasmic scaffold for the exocytotic apparatus (Dean et al. 2003).

Although the function of these molecules in neurons has been studied quite throroughly (reviewed by Lise and El-Husseini 2006), the role of glial neurolignin/neurexin has not been studied to the same extent. In the mouse brain, neuroligin 1 and 2 are able to induce presynaptic specializations even when expressed in non-neuronal cells (Scheiffele et al. 2000). They do so by clustering syntaptic vesicles within axons, congruent with the function of neuronal neuroligins via neurexins. Although there has been no study showing the contribution of astrocytic neuroligins to synapse formation, the fact that they are expressed by astrocytes (Cahoy et al. 2008) and that they have the ability to induce presynaptic specializations (Scheiffele et a. 2000) should inspire further studies into their function.

Another group of astrocytic cell adhesion molecules, which have been shown to be involved in synaptogenesis, are γ-protocadherins. They are a family of CAMs expressed by astrocytes and localized to astrocytic processes. Here, γ-protocadherins mediate the perisynaptic contact with excitatory and inhibitory synapses in the spinal cord and hippocampus (Garrett and Weiner 2009). Their importance for synaptogenesis has been experimentally illustrated by culturing wild-type neurons on top of γ-protocadherins knockout astrocytes. Wildtype neurons cultured on astrocytes from mice wth all γ-protocadherins knocked out have a drastically reduced synapse density at 6 d *in vitro* (DIV). Interestingly, by 9 DIV, the density of excitatory synapses recovers if neurons themselves express γ-protocadherins, showing that the neuronal γ-protocadherins can take over the role of astrocytic γ-protocadherins. However, the density of inhibitory synapses does not recover, which indicates that astrocytic γ-protocadherins in contrast to excitatory synapses are crucial for inhibitory synapse formation. *In vivo*, synapse development observed in γ-protocadherin KOs is delayed in a similar way (Phillips et al. 2003, Garrett and Weiner 2009). The mechanism of action of γ-protocadherins are not fully understood. One member of the γ-protocadherin family, protocadherin-γC5, has been shown to interact with a subunit of the $GABA_A$ receptor in rat brains, promoting the transfer of the subunit to the synaptic cell membrane and the clustering of $GABA_A$ receptors, while having no effect on overall number of inhibitory synapses (Li et al. 2010, 2012). Although only found for one member of the family, this finding indicates γ-protocadherins could either have a function similar to the scaffolding proteins that anchor neurotransmitter receptors to the postsynaptic membrane (see Box *Short timeline of synaptogenesis*, subpoint *3. Synapse stabilization* for background information on scaffolding proteins) or activate them. Consequently, the way in which γ-protocadherins promote synapse formation would be by raising the number of neurotransmitter receptors in the postsynaptic element, hightening the possibility of synaptic activity, which could recruit additional synaptic building blocks and thus stabilize the newly formed synapse.

Taken together, astrocytic CAMs act in similar ways as neuronal CAMs, in that they guide initial contact formation and the assembly of the pre- and postsynaptic specializations. Further research is needed to define the relative contributions of glial and neuronal CAMs and to identify the precise mechanisms of action of glial, synaptogenic CAMs.

3.1.2 Secreted astrocytic factors

After the establishment of initial contact, astrocytes can induce further pre- and postsynaptic differentiation and recruitment of receptors to the postsynaptic membrane. To distinguish the role of astrocytic CAMs from that of astrocyte-secreted factors, one approach is to use astrocyte-conditioned medium, which is a medium containing astrocyte-secreted proteins. Such medium, generated from cultured astrocytes, induces the formation of functional synapses in neuronal cultures (Nägler et al. 2001, Pfrieger and Barres 1997, Ullian et al. 2001). The astrocytic proteins mediating these functions will be discussed in the following two sections on extracellular matric proteins and readily diffusible astrocytic factos. The proteins described are almost exclusively involved in the formation of excitatory synapses. Even though astrocyte conditioned medium increases the number of GABAergic synapses (Hughes et al. 2010), less is known about the responsible factors. Many studies use postsynaptic markers specific to excitatory synapses to identify synapses for analysis and thus do not evaluate the effect of the putative synaptogenic factor under investigation on inhibitory synapses.

The following section covers extracellular matrix components, which are secreted but structurally closer connected to the astrocytic surface, and released factors, which diffuse further away from the cell. Although here divided by their cellular location, the synaptogenic functions of extracellular matrix components and released factors overlap and can be roughly divided into two groups: Those that induce overall synapse formation and those that specifically induce either the formation of excitatory or inhibitory synapses. Factors that fall into the former category often affect the development of the presynaptic bouton. Accordingly, they induce postsynaptically silent synapses that need the interaction with additional factors to gain postsynaptic function. Factors specific to one synapse type are mostly involved in the recruitment, clustering or anchoring of the respective neurotransmitter receptors to the postsynaptic dendritic spine, converting silent synapses into functional ones.

3.1.2.1 Extracellular matrix components of astrocytes

Two groups of factors that astrocytes secrete into the extracellular matrix (EM), where they stay in some proximity of the astrocytic membrane, are matricellular proteins and proteoglycans. Because of the close contact of astrocytes with the synapse, extracellular matrix proteins of astrocytes can interact with neuronal proteins. Unlike most other proteins of the EM, these proteins do not fulfill structural, but modulatory roles (Sage and Bornstein 1991, Bornstein 1995). Matricellular proteins in particular are a class of proteins that modulate cell-cell and cell-matrix interactions. Their early postnatal expression makes them potential regulators of synapse formation (Eroglu 2009, Kucukdereli et al. 2011).

In this section, I will first describe three astrocytic matricellular proteins or protein families (thrombospondins, hevin and SPARC) and then one astrocytic proteoglycan family (glypicans). Collectively, they induce functional synapse formations in two cooperative steps: thrombospondins and the mutually counteracting SPARC and hevin are involved in silent synapse formation (Bornstein 2001, Christopherson et al. 2005, Lively and Brown 2008, Eroglu 2009), and the proteoglycan glypicans in their subsequent activation (Filmus and Selleck 2000, Allen et al. 2012). This sets the stage for further, activity-dependent development of the synapse, which will be discussed in later chapters.

Thrombospondins

Thrombospondins are a family of five calcium-binding glycoproteins whose expression in astrocytes is developmentally regulated (Adams and Lawler 2011). For example, while immature astrocytes express thrombospondins TSP1 and TSP2 (Christopherson et al. 2005), TSP4 is only expressed in mature astrocytes (Cahoy et al. 2008). When added to cultures of retinal ganglion cells (RGCs), TSP1 and TSP2 induce ultrastructurally normal pre- and postsynaptic differentiation, though the thus formed synapses are postsynaptically silent in that they lack glutamate receptors (Christopherson et al. 2005).

How do thrombospondins induce differentiation? Little is known about the precise interaction mechanism or signaling cascade involved. Nevertheless, there are two hints: On the one hand, experiments by Eroglu and colleagues have identified a subunit of the gabapentin receptor α2δ-1 as the receptor responsible for the synaptogenic effect of thrombospondins (Eroglu et al. 2009). Addition of thrombospondins rescues the hyperphosphorylation of protein kinase A (PKA) substrates after astrocyte-deprivation, indicating PKA in the downstream signaling pathways of thrombospondin and α2δ-1 action (Crawford et al. 2012). On the other hand, in experiments where thrombospondin 1 is added to RGC cultures and an increase in the rate of excitatory synapse formation is observed, this increase is blocked by application of the extracellular domain of neuroligin 1 (Xu et al. 2010). It is thus assumed that the synaptogenic effect of thrombospondin 1 depends on neuroligin 1. Neuroligins, as described earlier, aid in the assembly of synapses. Furthermore, thrombospondins interact with a variety of other proteins involved in cell adhesion, synapse architecture and cytoskeletal stability. Their individual roles in thrombospondin-mediated synaptic differentiation have not been established (reviewed by Risher and Eroglu 2012).

The developmental regulation of thrombospondins, taken together with their many interaction partners, make them an interesting object of investigation for comparing developmental with adult synapse formation. An intriguing finding by Rao and colleagues connects amyloid-β, the major component of the plaques associated with Alzheimer's disease in the brain, to the secretion of thrombospondin 1 by astrocytes. When added to cultures of astrocytes, amyloid-β decreased secretion of thrombospondin 1 by astrocytes 1-to-3 fold (Rao et al. 2013). From these astrocyte-amyloid-β cultures the authors produced conditioned media, which, when added to neuronal cultures, led to a loss of synaptophysin and PSD-95 in neurons, for which thrombospondin 1 seemed responsible. Synaptophysin is the most abundant synaptic vesicle protein, involved in their endocytosis during vesicle retrieval (Wiedenmann et al. 1986, Kwon and Chapman 2011). Both synaptophysin and PSD-95 are vital for the assembly and maintenance of synapses, thus their loss has direct consequences on synaptic stability. Accordingly, either the substitution of thrombospondin 1 or the restoration of its release from astrocytes might have therapeutic benefit in Alzheimer's disease (Rao et al. 2013).

On the whole, glial thrombospondins are necessary actors of synapse formation during development and healthy adulthood. The disruption of their function is implicated in Alzheimer's disease and might further be involved in other forms of synapse loss associated with ageing.

Hevin and SPARC

Similar to thrombospondins, the expression of hevin and SPARC (secreted protein, acidic and rich in cysteine) is developmentally regulated, specifically, with their production being restricted to early development. They are expressed by multiple micro- and macroglial cells, which secrete them both *in vivo* and *in vitro*. Notably, astrocytes contain particularly high levels of these proteins (Mendis et al. 1995, Vincent et al. 2008, Cahoy et al. 2008). Hevin induces synapse formation, and analogous to thrombospondins, the synapses induced by hevin are ultrastructurally normal, but postsynaptically silent (Kucukdereli et al. 2011). Hevin-KO mice have less excitatory synapses and the few induced ones have a lower number of presynaptic vesicles, as well as showing other indicators of being less mature. This shows the importance of endogenous hevin for synapse formation. Hevin's mechanism of action might be binding to a surface receptor and mediating clustering of trans-synaptic adhesion molecules. This notion is supported by the observation that hevin-KO mice have larger synaptic clefts, which could be caused by a lack of trans-synaptic adhesion (Kucukdereli et al. 2011).

SPARC specifically counteracts the effect of hevin, presumably because its domain homologous to hevins, which competes with it for an interaction with a molecule that is yet to be identified (Kucukdereli et al. 2011). As shown in a different study, SPARC antagonizes the stabilization of AMPA receptors at the postsynaptic membrane (Jones et al. 2011). Since hevin induces silent synapses, it might interact with one or more different factors, such as scaffolding proteins, which subsequently promote the surfacing and anchoring of neurotransmitter receptors to convert the silent synapses to functional ones. Seen that SPARC counteracts the membrane stabilization of at least one neurotransmitter, the missing link between the opposing effect of the two proteins could be a scaffolding protein, activated by hevin and inhibited by SPARC.

Taken together, astrocytes might be able to control the formation and stabilization of synapses via the counteracting proteins hevin and SPARC, which can serve as an example for the identification of additional control mechanisms of astrocytic, synaptogenic proteins.

Interestingly, SPARC does not block the synaptogenic effect of thrombospondins, suggesting that thrombospondins and hevin act by different pathways (Kucukdereli et al. 2011). This redundancy might be useful if individual proteins are defective, as having multiple, parallel pathways allows for compensation. KO studies of TSP1, TSP2, SPARC and hevin investigated this possibility and found that individual KO organisms do not display severe synaptic abnormalities and that synaptogenic factors indeed might compensate for each other, especially TSP3 and 4 for TSP1 and 2 KOs (Bassuk et al. 1999, Gilmour et al. 1998, Christopherson et al. 2005, Eroglu et al. 2009a, Eroglu 2009b). To estimate the exact potential for compensation, it would be interesting to make combined KOs and hence determine which factors can compensate for each other.

In general, the possibility for compensation stresses the importance for glial factors in synapse formation, as the loss of individual proteins ought to be detrimental enough to the development of neural circuits for the brain to employ additonal energy to maintain partially redundant mechanisms.

Glypicans

The fact that thrombospondins and hevin are only able to induce postsynaptically silent synapses suggests the existence of a complementary factor that leads to the conversion of silent to active synapses by promoting the insertion of glutamate receptors into the PSD. In brief, there are two main ionotropic glutamate receptor families found at excitatory synapses, NMDA and AMPA receptors, which need postsynaptic membrane insertion during synapse formation. NMDA and AMPA receptors differ in their functional roles in that AMPA receptors mediate fast synaptic transmission and NMDA receptors are involved in the slower regulation of synaptic plasticity and learning. Both receptors are composed of multiple subunits. The subunit composition of one receptor can vary, influencing functional properties of the receptor (Monyer et al. 1992, Bochet et al. 1994, Geiger et al. 1995, Yamakura and Shimoji 1999, Isaac et al. 2007).

Assembled AMPA receptors can reach the PSD in two ways: By exocytosis (Gerges et al. 2006, Park et al. 2004, Park et al. 2006) and by lateral diffusion from areas adjacent to the PSD (Heine et al. 2008, Petrini et al. 2009, Makino and Malinow 2009). Different factors, such as scaffolding proteins, stabilize AMPA receptors to the postsynaptic membrane after they have reached it. In additon to neuronal factors, astrocytes can influence the amount of surfaced AMPA receptors by releasing factors that specifically recruit individual AMPA subunits, named GluR1-4, to the synaptic surface (Allen et al. 2012).

Two such factors are the proteoglycan glypicans 4 and 6, which are expressed in postnatal hippocampal astrocytes and promote surfacing and clustering of GluR1 in RGCs (Traister et al. 2008, reviewed by Sarrazin et al. 2011, Allen et al. 2012). The two glypicans are functionally redundant, but might be preferentially expressed at different developmental time periods and in different brain regions (Allen et al. 2012). After the developmental period, glypicans are mainly expressed in neurons (de Wit et al. 2013). Growing RGCs in presence of glypican 4 or 6 increases synapse number threefold. Given the time course of surfacing of AMPA receptors and formation of new synapses through glypicans 4 and 6, it is assumed that they first cluster receptors and then recruit postsynaptic scaffolding molecules, promoting the insertion of AMPA receptors. (Allen et al. 2012)

How is this achieved? In neurons, glypicans, similar to neuroligins and neurexins, participate in so-called trans-synaptic interactions. These interactions are characterized by a pair of proteins, one located presynaptically, one postsynaptically. For both members of the pair the interaction is activating and leads to the proteins in turn activating other factors. For example, glypican 4 interacts with leucine-rich repeat protein 4 (LRRTM4). As part of AMPA receptor complexes, LLRTM4 plays a direct role in AMPA receptor trafficking (de Wit et al. 2013) and knockout of LRRTM4 leads to a decreased recruitment of AMPAR subunit GluR1 to the postsynaptic membrane (Allen et al. 2012). Hence, glypicans can cluster AMPA receptors via the clustering of LRRTM4.

Since astrocytic glypicans 4 and 6, in contrast to neuronal glypicans, are not associated with synaptic elements, it is not clear how the interaction takes place. Nonetheless, it is conceivable that they are secreted into the synaptic cleft by astrocytes, where they interact with their otherwise transsynaptic partner, yet only one side of the trans-synaptic activation would take place. In the case of glypicans 4 and 6, both of which are located presynaptically, this would enable them to promote clustering of GluR1, without affecting the presynaptic element.

AMPA receptors are generally permeable to cations, but the calcium permeability of AMPA receptors is determined by the presence of the GluR2 subunit. If present, the receptor is rendered impermeable to calcium (Hollmann et al. 1991, Traynelis et al. 2010). During development, at first most AMPA receptors lack the GluR2 subunit and only later become impermeable to calcium through incorporation of one or more GluR2s (Kumar et al. 2002). Given that astrocytes are able to influence the surfacing of GluR1 subunits via glypicans, as described above, it would be interesting to investigate whether they are able to do so as well for GluR2 subunits. The ability to turn on or off the calcium permeability of AMPA receptors would make it possible for astrocytes to influence the overall local calcium concentration in dendritic spines, which has an important function in synaptic plasticity, as postsynaptic signaling cascades leading to LTP and LTD are dependent on calcium (Wiltgen et al. 2010). Astrocytes have indeed been found to regulate GluR2 expression in motor neurons (van Damme et al. 2007).

In sum, glypicans are produced in neurons and astrocytes. It is assumed that glial glypicans affect synapse formation via the same interaction partners as neuronal glypicans. Thus, it would be of particular interest to investigate the relative contributions of neuronal and glial glypicans, within and across developmental time-windows, to determine whether they are redundant, or fulfill different developmental roles.

3.1.2.2 Readily diffusible astrocytic factors

In the previous section, I described glial factors that are considered part of the astrocytic extracellular matrix. Even more, apart from these extracellular matrix components, there are also diffusible factors which are released from astrocytes and can diffuse to synaptic sites, and which play a role in synapse formation. In the following section I will focus on these diffusible factors.

TGF-β1 & D-serine

Unlike thrombospondins and hevin, which stimulate the formation of postsynaptically silent synapses, the astrocyte conditioned medium constituent transforming growth factor beta 1 (TGF-β1) induces the formation of functional, postsynaptically active excitatory synapses via the TGF-β signaling pathway in cortical neurons of rodents and humans (Diniz et al. 2012). The mechanism of action depends mostly on the elevation of D-serine in the synaptic cleft. Accordingly, D-serine alone has comparable synaptogenic activity (Diniz et al. 2012).

The synaptogenic activity of D-serine can be blocked by inhibition of NMDA receptors (Diniz et al. 2012), suggesting that this activity comes from an interaction with this receptor. In line with this, it is well established that D-serine acts as an endogenous ligand on the glycine binding site of NMDA receptors (Kleckner and Dingledine 1988, Hashimoto et al. 1992, 1993a, 1993b, Schell et al. 1995, Stevens et al. 2003, Gustafson et al. 2007, Daniels and Baldridge 2009). This binding is important, since in contrast to AMPA receptors, the binding of glutamate is not sufficient to activate NMDA receptors. NMDA receptors act as coincidence detectors in that they need the binding of glutamate, a membrane depolarization and the additional binding of a co-agonist, D-serine or glycine, for their ion channel to open.

In the CNS, endogenous D-serine is chiefly located in glial cells, notably astrocytes (Schell et al. 1995, Mothet et al. 2000, Panatier et al. 2006) and its release, classically, can be stimulated by an AMPA receptor-dependent mechanism (Mothet et al. 2005, Sullivan and Miller 2010). How TGF-β1 induces the rise in D-serine levels in the synaptic cleft is not known. In particular, both a direct interaction with astrocytes or a mechanism in which TGF-β1 interacts with AMPA receptors are possible. The AMPA receptors would then in turn activate D-serine release, as they do in classical triggering of D-serine release.

The requirement of NMDA receptors for the synaptogenic effect of TGF- β1 via D-serine suggests an activity-dependent mechanism, where TGF-β1 raises the levels of D-serine. This in turn causes an increase

in binding or even saturation of the glycine binding site on NMDA receptors. Therefore, this will increase the probability that subsequent glutamate binding paired with a postsynaptic depolarization leads to full NMDA receptor activation. The activation of NMDA receptors can in turn lead to synaptic plasticity (also see the section on synaptic plasticity below), further stabilizing the synaptic contact by recruiting additional glutamate receptors to the PSD (Sullivan and Miller 2010).

There might be species and brain region-specific differences in the effect of TGF- β1. TGF-β1 had a synaptogenic effect in rodent and human cortical neurons (Diniz et al. 2012) and at the neuromuscular junction of *Xenopus* (Feng and Ko 2008), but not in rat RGCs (Christopherson et al. 2005). However, experimental differences might account for some of the differences. In the studies mentioned above, mice cortical neurons were collected from d14 mice embryos, *Xenopus* cells came from stage 21-23 embryos, RGCs were collected from postnatal rats and human cortical cells from adult patients (Christopherson et al. 2005, Feng and Ko 2008, Diniz et al. 2012). Taken into account that the number of days the cultures were incubated with TGF-β1 varied, that the development of synaptogenesis is tightly regulated and that the differences between developmental and adult synaptogenic factors are mostly unexplored, a comparison of the results should be done with caution.

Interestingly, TGF-β1 could possibly link to other synaptogenic, astrocytic factors: It binds to matricellular proteins and it increases the concentration of thrombospondins in astrocytes (Feng and Ko 2008, Ikeda et al. 2010). The effect of TGF-β1 on inhibitory synapses has not been investigated. D-serine is not a necessary ligand for GABA receptors, which makes TGF-β1 induced D-serine increases unlikely to affect inhibitory synapse formation. Nevertheless, other affector molecules of the TGF-β signaling pathway could influence the formation of inhibitory synapses.

Cholesterol

Astrocyte-conditioned medium also contains the synaptogenic lipid cholesterol (Mauch et al. 2001). Cholesterol is needed in both neurons and astrocytes to build membrane surfaces, especially at synaptic sites (Goritz et al. 2005). Moreover, cholesterol is thought to be a limiting factor in synaptogenesis, as neurons only produce enough cholesterol to survive, but depend on the production of glial cholesterol to form mature synapses (Pfrieger 2002). This dependence on glial cholesterol could explain why the formation of most synapses follows the differentiation of macroglial cells (Pamavelas et al. 1983, Skoff et al. 1990, Pfrieger and Barres 1996, Ullian et al. 2001, Pfrieger 2002, reviewed by Pfrieger and Ungerer 2011).

The synaptogenic effect of cholesterol is thought to depend on two roles: First, it binds to membrane-proteins of presynaptic vesicles, facilitating the assembly of these vesicles (Thiele et al. 2000). Second, cholesterol is used as a building material in dendritic differentiation (Horton and Ehlers 2004, Fan et al. 2002, Goritz et al. 2005). When neuronal cultures of rat retinal ganglion cells are supplemented with cholesterol, the frequency of spontaneous excitatory postsynaptic currents increases 12 ± 4–fold compared with glia-free neuronal cultures of the same type (Saito et al. 1987, Mauch et al. 2001), illustrating the crucial role of cholesterol in synaptogenesis.

3.1.3 Astrocytes control the extracellular concentration of neuronal synaptogenic factors

In contrast to the astrocytic mechanisms described in the past sections, which depend on the expression and secretion of proteins by astrocytes, astrocytes can, to a certain extent, control the extracellular levels of specific neuronal proteins. What is interesting about this ability is that astrocytes are thought to endocytose these neuronal proteins and subsequently exocytose them again, thereby controlling their availability. This suggests that astrocytes can act as temporal reservoirs for such neuron-derived proteins.

Brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 ($NT_{4/5}$), both members of the neurotrophin family, are examples for such neuronal proteins whose availability is controlled by astrocytes. The neurotrophin familiy is a family of factors involved in the development and survival of neurons. BDNF, for instance, is secreted into the extracellular space in an activity-dependent manner (Poo 2001) and induces the formation of functional excitatory and inhibitory synapses in cultured hippocampal neurons (Vicario-Abejón et al. 1998). Neurotrophins selectively bind to members of the Trk-receptor family, with BDNF and

$NT_{4/5}$ both binding TrkB. Upon binding, Trk receptors dimerize, autophosphorylate and initiate tyrosine-kinase activation, the first step in multiple signaling cascades, such as the MAP kinase and phospholipase C-γ pathways (reviewed by Ebendal 1992, Meakin and Shooter 1992). Both pathways are implicated in the regulation of synaptogenesis (Huang and Reichardt 2001, 2003, Alonso et al. 2004, Hans et al. 2004, Giachello et al. 2010, Cuesto et al. 2011). Interestingly, the activation of TrkB is necessary to recruit the scaffolding protein gephyrin to GABAergic synapses (Chen et al. 2011).

Astrocytes regulate the extracellular availability of neuronally secreted BDNF and $NT_{4/5}$ by endocytosis and internal storage of these two neurotrophins (Alderson et al. 2000, Bergami et al. 2008). BDNF is not degraded in the internalized reservoirs, but the majority is released back into the extracellular space (Alderson et al. 2000).

While less is known about the mechanism and function of astrocytic control of $NT_{4/5}$ levels, the regulation of BDNF has started to be investigated. In its two secreted forms, pro-BDNF and mature BDNF, BDNF is hypothesized to have opposing functions in such a way that pro-BDNF counteracts the synaptogenic effect of mature BDNF (Lu et al. 2005). If astrocytes can selectively take up and release the two forms, they could be able to use these counteracting factors to influence the promotion of synaptogenesis via neuronal proteins. Indeed, two separate receptors mediate BDNF endocytosis by astrocytes: Mature BDNF binds to a truncated form of the TrkB receptor (tTrkB; Alderson et al. 2000) and pro-BDNF to the pan-neurotrophin receptor p75 (Bergami et al. 2008). After binding, the BDNF molecules are taken up, stored in vesicles and eventually released into the synaptic cleft. Very little is known about this part of the mechanism. Particularly the signal that triggers the exocytosis and subsequent release of BDNF back into the synaptic cleft has only partially been identified. One study showed that a rise in synaptic glutamate levels stimulates the release of BDNF-vesicles from astrocytes (Bergami et al. 2008). Still, this does not explain how two different forms of BDNF could be regulated to be released in varying ratios.

If the signal is a variation on the theme of different synaptic glutamate levels triggering the release of different ratios of the two BDNF forms, it is arguable how *active* the control of astrocytes over exocytosis is. Either way, future studies investigating whether this mechanism is common to many neuronal proteins or specific to a few neurotrophins, will shed more light on its relative importance in the regulation of synaptogenesis.

3.1.4 Conclusion

As already mentioned earlier, the astrocytic factors described here are mostly specific to the formation of excitatory synapses. It has been shown that inhibitory synapse formation does not depend on neurotrophins, thrombospondins or cholesterol (Hughes et al. 2010). It is expected that there would be similar principles of astrocytic involvement in the formation in inhibitory synapses as there are for excitatory synapses, yet the responsible proteins have not been discovered (Kuzirian and Paradis 2011). One study showed that BDNF can increase the number of inhibitory presynaptic terminals, while leaving the postsynaptic compartment unaltered (Elmariah et al. 2005). In summary, on top of the clarification of excitatory mechanisms, substantial additional work is needed to identify the responsible astrocytic proteins involved in the formation of inhibitory synapses.

Overall, there is no coherent theory on how astrocytic factors coorperate with neuronal factors to induce synapse formation, let alone how their relative contribution is. This chapter therefore stressed what is known about the individual factors, mentioning cross-interactions where they are known (An overview of the factors mentioned in this chapter is given in **Table 2**). Yet, there are some common themes for astrocytic synaptogenic factors: Most, directly or indirectly, promote the assembly of the synapse by interacting with neuronal scaffolding proteins, which anchor synaptic structures, such as neurotransmitter receptors, to the membrane. Many of the factors are developmentally regulated, appearing only in a restricted postnatal time-window or at higher levels during adulthood. It is widely accepted that synapse formation decreases after an initial burst, yet it is now known how this reduction in synaptogenesis is orchestrated and which role glial factors play in this developmental regulation. Future studies need to address this issue, possibly by assessing the synaptic interactome with special focus on glial factors. Once binding interactions are known, combining

multiple knock outs *in vivo* could clarify interactions, parallel mechanisms and relative timing of action.

Similarly to thrombospondins, the developmental and adult actors seem to be akin and possibly come from the same protein family. This indicates only subtle differences in their function. Still, very little is known about the difference between developmental and adult synapse formation. For example, the time-windows for the expression of astrocytic synaptogenic factors are elusive and it is not known whether the majority is even expressed in adulthood.

In my research proposal, I design experiments that would help to fill this knowledge gap by creating a conditional knockout mouse for thrombospondin-4. Transcriptome studies have shown this glial factor to be expressed in mature astrocytes (Cahoy et al. 2008), in contrast to other members of the thrombospondin protein family. This model would allow for a disruption of the protein function specifically in astrocytes of the mature brain. My proposed experiments connect this genetic manipulation to behavioral readouts of learning and the direct imaging of synapse turnover *in vivo*.

<table>
<tr><th colspan="3">Glial factor</th><th>Mode of action</th></tr>
<tr><td colspan="2" rowspan="2">CAM</td><td>γ-protocadherins</td><td>Increase synapse formation rate, clustering of neurotransmitter receptors</td></tr>
<tr><td>Neurexins/neuroligins</td><td>Promote neurotransmitter receptor anchoring, clustering of synaptic vesicles, assembly of cytoplasmic scaffold (neuronal function, astrocyte-derived function to be determined)</td></tr>
<tr><td rowspan="5">Secreted factors</td><td rowspan="3">Extracellular matrix components</td><td rowspan="2">Matricellular proteins</td><td>Thrombospondins</td><td>Induce silent synapse formation</td></tr>
<tr><td>Hevin & SPARC</td><td>Hevin: Induces silent synapse formation, clusters trans-synaptic adhesion molecules; SPARC: Counteracts Hevin and the stabilization of AMPARs to the postsynaptic membrane.</td></tr>
<tr><td>Proteoglycans</td><td>Glypicans</td><td>Support surfacing and clustering of AMPARs.</td></tr>
<tr><td colspan="2" rowspan="2">Readibly diffusible</td><td>TGF-β 1</td><td>Raises D-serine levels, promoting NMDAR receptor activation</td></tr>
<tr><td>Cholesterol</td><td>Facilitation of vesicle assembly, building material for dendritic differentiation</td></tr>
<tr><td colspan="2" rowspan="2">Endocytosed neuronal proteins</td><td>BDNF</td><td rowspan="2">Signalling molecules, multiple synaptogenic functions, e.g. recruitment of scaffolding molecules.</td></tr>
<tr><td>$NT_{4/5}$</td></tr>
</table>

Table 2 | Astrocytic factors that promote synaptogenesis

3.2 Synapse elimination

Of the many neuritic branches and synaptic contacts that are formed during development, few undergo maturation, become established and survive. The majority is not used, weakened and finally eliminated in the process of forming functioning neural circuits. Glia play an essential role in this synapse elimination: Not only are astrocytes involved in the provision of essential building blocks on which synapses depend, glia also detect the level of activity of individual synapses and mark them accordingly. Finally phagocytic glia engulf fragmented neurites and those synapses that are deemed unnecessary or inappropriate. This section examines these processes, with a focus on the elimination of individual synapses by glia. After a general introduction to synapse elimination, I will first describe the elimination by microglia, then recent evidence for the elimination by astrocytes. For the mechanisms of the removal of bigger structures by glia, such as axonal and dendritic branches, see recent reviews (general review: Corty and Freeman 2013; for axons: Luo and O'Leary 2005; for astrocyte involvement: Tasdemir-Yilmaz and Freeman 2013).

It is generally assumed that synapse elimination is the way by which inappropriate synapses are removed in order to make neuronal circuits more specific and apt for their functions or, in short, more *mature* (Katz and Shatz 1996). The notion is that synapses that fit the properties of the circuit are strengthened because the pattern of activity of their pre- and postsynaptic neurons leads to synaptic plasticity that strengthens the synapse, such as spike-timing or pattern dependent LTP. Synapses that do not fit the circuit's firing properties are not strengthened, or even weakened, by mechanisms that work in the opposite direction of strengthening, such as spike-timing or pattern-dependent LTD (Wiegert and Oertner 2013). Phagocytic glia sense such weak synapses, engulf them and digest the internalized synaptic molecules.

There are three principal time-windows of synapse elimination: First, substantial, widespread elimination following overshooting synapse formation during early development, second, basic turnover that occurs throughout the lifetime of an organisms and third, the elimination that follows local and short-lived synapse formation linked to learning (Puro et al. 1977, Trachtenberg et al. 2002). The latter has in outlines been studied in different learning tasks, where the learning leads to a heightened synapse turnover in the respective cortex or hippocampus (Moser et al. 1994, O'Malley et al. 1998, 2000, Klintsova and Greenough 1999, Knafo et al. 2001), followed by synaptic pruning (Wolff and Missler 1993).

Similarly to other stages of the synaptic life cycle, more is known about excitatory than inhibitory synapses, one reason being that immunocytochemistry is mostly performed with antibodies against PSD95, a postsynaptic scaffolding proteins found in glutamatergic synapses. Another important difficulty is that while excitatory synapses are postsynapticallly formed with a relatively easily visible dendritic protrusion called a spine, inhibitory synapses are formed with dendritic shafts or other less distinct neuronal structures. Consequently, structural imaging studies are much easier to conduct on excitatory than inhibitory synapses.

The main open questions in the realm of glial influences on synapse elimination are: Which glia cells are phagocytic? How do glia locate synapses (*find me*-signal) and how do they subsequently differentiate between strong and weak synapses (*eat me/don't eat me*)? How are potential *eat me* and *don't eat me* signals placed on the synapse? Which glial receptors interact with the *eat me/don't eat me* signals? Which signaling cascades are subsequently activated within glia to initiate the molecular changes necessary for phagocytosis?

Among phagocytic glia, research initially focussed on the phagocytic activity of microglia. In addition, recently a contribution of astrocytes to this process been reported (Tasdemir-Yilmaz and Freeman 2013). Interestingly, according to one report from the LGN, astrocytes seem to contribute more to overall synapse elimination than microglia, although individual contributions might vary over developmental periods and brain areas (Chung et al. 2013).

The following two sections on microglia and astrocytes individually describe the activity and engulfment signals, glial receptors and corresponding signaling cascades responsible for the engulfment of inappropriate synapses. Across the different brain areas and species investigated in past studies, there are some common themes, but also notable differences.

3.2.1 Microglia

Microglia locate synapses via ATP-mediated chemotaxis

Microglia have at least two states of activation: A non-activated, resting and an activated, reactive state. The classical function of the activated state is response to neuronal injury or infection (Gonzalez-Scarano and Baltuch 1999, Rock et al. 2004, Chew et al. 2006). Changes between states might also play a role in the elimination of synapses, as phagocytosis is more prominent in reactive microglia (Chew et al. 2006). Microglia have been shown to engulf synapses or other neuronal elements. The limitation of *in vitro* studies is that the disruptive nature of brain slice preparation can activate microglia and transform them into their reactive state, making it difficult to study their function in the healthy brain (Davalos et al. 2005). Even many *in vivo* preparations require some surgical intervention, such as injection of a virus for gene delivery or installation of a cranial window for imaging, and can cause similar reactivity (Marker et al. 2010). In the resting state, microglia are resident glia in that their cell bodies and main branches of their processes are stationary. In contrast, their finer processes are highly motile and reach away multiple µm from the soma (Davalos et al. 2005, Nimmerjahn et al. 2005). Small processes appear, extend and retract on a timescale of seconds to minutes, as shown in **Figure 2**. With those processes, microglia contact the pre- and postsynaptic elements of close-by synapses approximately once per hour for an average duration of 5 min (Wake et al. 2009). Overall, the entire brain parnchyma is sampled by resting microglia once every few hours (Nimmerjahn et al. 2005), which makes the term ‚resting' rather misleading.

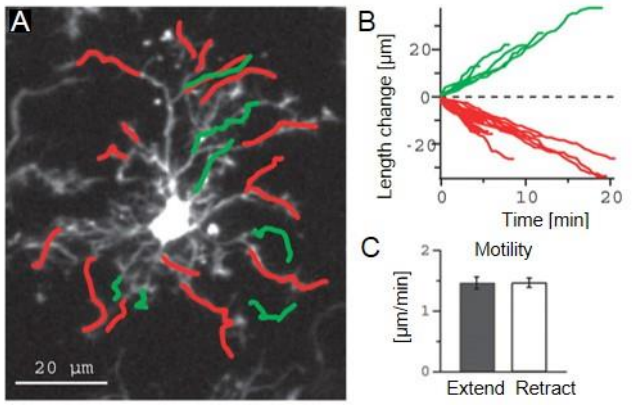

Figure 2 | Motility of microglial processes (adapted from Nimmerjahn et al. 2005)

(A) Extensions (green) and retractions (red) of microglial processes over the time course of 20 min.
(B) Length changes of the processes shown in (A) as a funtion of time.
(C) Mean motility values in µm/min for extensions and retractions.

One of the most vital questions in this area is how microglia locate synapses (*find me*-signal) and how they recognize which ones to phagocytose (*eat me*-signal). One proposed mechanism for synapse localization is the chemotaxis of microglial processes depending on local elevations of extracellular ATP and ADP, sensed by microglial P2Y receptors (Honda et al. 2001, Inoue 2002, Davalos et al. 2005, Wu et al. 2007). P2Y receptors are metabotropic and activate a potassium current as well as the phosphatidylinositide 3-kinase (PI3K) pathway, which in turn invokes the actin reorganization necessary for process motility and, in some cases, phagocytosis (Wu et al. 2007).

Why is ATP elevated around synapses? ATP is coupled to neuronal activity, a notion that is based on findings that it is diminished when action potentials are blocked by TTX. Furthermore, neuronal depolarization induces the release of ATP from synapses in cultured chick retinal neurons (Santos et al. 1999, Stevens and Fields 2000). The ATP sensed by microglia most likely comes from two sources: First, ATP is the extracellular messenger released by astrocytes to coordinate calcium waves (Guthrie et al. 1999, reviewed by Fellin et al. 2006). Second, it can be co-released from the presynapse via vesicles, either alone or with other neurotransmitters, such as glutamate and GABA, or reach the synaptic cleft via channels permeable to ATP (Jo and Schlichter 2000, Lazarowski et al. 2003, reviewed by Pankratov et al. 2006).

Regardless of the exact release mechanism, it is hypothesized that synaptic transmission leads to an increase in local ATP, which attracts microglial processes via chemotaxis and activated pathways that induce a reorganization of the actin cytoskeleton. The essential paradox of this theory is that strong, active synapses are likely to have higher levels of ATP and thus attract microglia more effectively. Though, since microglia are on the lookout for weak, destabilized synapses, which need to be pruned, ATP would attract them to

the wrong synapses. There is a possibility that microglia don't need particular signals from weak synapses, as they could encounter them by chance during their regular sampling activities. Conversely, if we assume that both strong and weak synapses are sampled by microglia equally, one question that remains open is how microglia recognize weak synapses for phagocytosis while leaving strong synapses unaffected.

The differentiation between strong and weak synapses is not straightforward. Historically, there have been two competing hypotheses: One states that strong synapses compete with others by secreting a short-range, protective signal and a longer-range punishment signal that promotes the elimination of nearby weaker synapses (Jennings 1994, Hua and Smith 2004). Another possibility is that weak synapses secrete the *eat me*-signal themselves and that the secretion of this signal is somehow disrupted in stronger synapses (**Figure 3**).

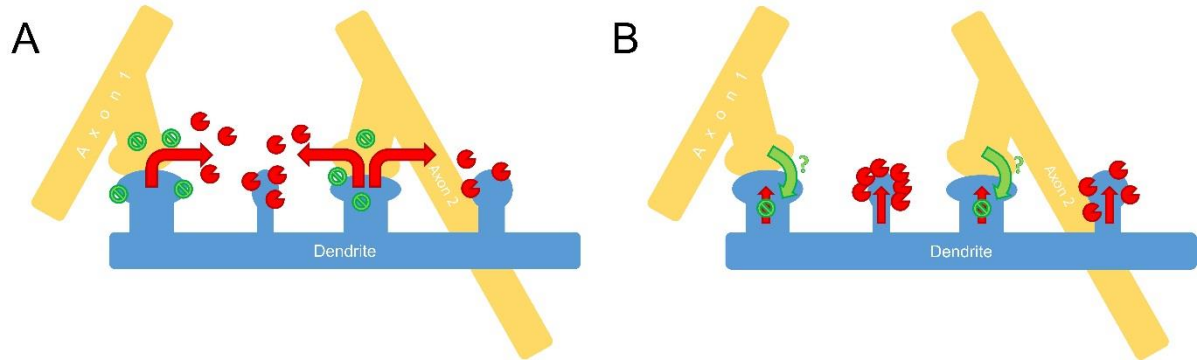

Figure 3 | Eat me & Don't eat me-signals

Illustration of two alternative hypotheses on synapse tagging for elimination

(A) Strong synapses secrete two signals: An *eat me*-signal, which diffuses to other, nearby synapses, and a *don't eat me*-signal, which protects strong synapses against the *eat me*-signal.
(B) Weak synapses secrete an *eat me*-signal whose secretion is somehow suppressed in strong synapses.

Legend: *eat me*-signal *don't eat me*-signal

Most likely, synaptic strength is not determined in absolute terms, but relative to the surrounding synapses. Studies have tried to address this question by blocking of all neuronal firing alltogether in a certain area. The hypothesis was that if there is an absolute, not a relative, threshold for elimination, the blockage should lead to widespread synaptic degeneration and elimination. This is not the case, as demonstrated in cells that project to the LGN, a popular model system for developmental circuit refinement. Instead, if spontaneous activity in all neurons projecting to the LGN is blocked by TTX, too many projections and synapses of similar strength persist into adulthood, leading to dysfunctional circuits (Kalil et al. 1986, Sretavan et al. 1988). This is the contrary of what would be expected in response to general weakening, according to the absolute threshold-hypothesis. In addition, if spontaneous activity of only one eye is blocked specifically, the projections of the cells coming from that eye are selectively weakened and their synapses eliminated (Schafer et al. 2012). This finding supports the relative threshold-hypothesis, which would predict the artificially weakened synapses to be outcompeted by the stronger ones.

However, when interpreting these findings, there are some additional mechanisms to be taken into account. One is synaptic scaling, a mechanism by which neurons try to keep the level of overall firing constant by modulating their synaptic inputs. This mechanism interferes with experiments utilizing total activity blockage, as mentioned before, because the reduced activity leads to neurons strengthening their excitatory inputs to stay at the same level of firing (Turrigiano 2008). Consequently, the persistence of too many synapses in the above mentioned experiment could be explained by a lack of competition, but possibly also by scaling mechanisms. With this in mind, the *competition* of synapses seems to drive the selective elimination of synapses, in particular, stronger synapses provoking the elimination of weaker ones, but it is not the only mechanisms coordinating synaptic strength across multiple synapses.

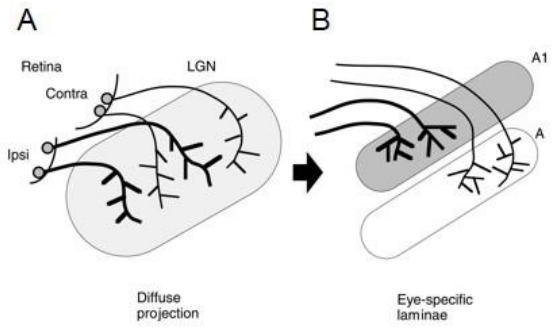

Figure 4| Axon segregation in the visual system (adapted from Sanes and Yamagata 1999)

(A) Initially, axons from both eyes form rudimentary arbors in overlapping territories in the lateral geniculate nucleus.
(B) Axons from the left and right eyes then withdraw inappropriate branches and extend appropriate branches to form distinct eye-specific laminae.

Stevens and colleagues raised the possibility that the punishment signal during development could be of immune origin. They showed that a KO model of complement components C1q and C3, which are traditionally part of the immune system (see below), led to a failure of axonal segregation in the lateral geniculate nucleus (LGN; Stevens et al. 2007). Axonal segregation, as it occurs in the LGN, is a developmental process in which axons become segregated into eye-specific and other specialized sublayers (White and Sur 1995), as illustrated in **Figure 4**. A failure of axon segregation means the persistence of the unspecific projections formed during development into adulthood, without inapt connections being weakened and degraded. Later studies found that KO models of C1q and C3 had significantly more synapses in adult animals and defects in synaptic connectivity (Schafer et al. 2012). These findings make complement components promising candidates for the *eat me*-signal.

Complement cascade

The complement cascade in its traditional function forms part of the innate immune system. It is a cascade in that some of its small protein components get activated by molecular triggers, such as binding of an antibody, and in turn activate other components of the cascade, and so forth. Collectively, they opsonize the target cell or debris, leading to its destruction, while attracting phagocytic cells to the opsonized body through chemotaxis. Like macrophages, microglia express multiple receptors for complement components: For C1q (C1qRp; Webster et al. 2000), for C3 (CR3; Graeber et al. 1988) and for cleavement fragments of C3 (Gasque et al. 1998, Webster et al. 2000, Chiu et al. 2009). The signaling of these receptors commonly induce or enhance phagocytosis.

C1q localizes to synapses via an unknown binding partner

In order to show that complement components can act as *eat me*-signals at neuronal synapses, a first step is to show that these components can be localized at synaptic sites. In the brain, immunocytochemical studies and other structural analyses have shown that C1q can be localized at synapses. More specifically, it has been found close to weak synaptic structures, such as non-matched pre- and postsynaptic elements (Stevens et al. 2007). C1q can bind synapses by a yet to be identified mechanism or receptor. Possible candidates are neuronal pentraxins and the prion protein Prpc (Stevens et al. 2007). Neuronal pentraxins are 20-30% homologous to pentraxins of the immune system, which bind C1q and enhance microglial phagocytic activity (Bottazzi et al. 1997, Gershov et al. 2000, Nauta et al. 2003a, Nauta et al. 2003b, Jeon et al. 2010). A KO of neuronal pentraxins yields a similar defect of LGN axonal segregation as C1q KOs, although this defect is only transient (Bjartmar et al. 2006). Prpc is anchored in the membrane and activates C1q when it is in an abnormal conformation (Mitchell et al. 2007, Erlich et al. 2010).

The localization of C1q to weak synapses makes it a likely candidate for the *eat me*-signal. It is currently not known whether C1q can bind to all synapses and stronger ones can "shake it off" while weaker ones are left to phagocytosis, as proposed in the hypothesis by Jennings, or whether it selectively binds to weaker synapses. Future studies will have to investigate this important question.

Complement has multiple sources

What is the origin of synaptic C1q? C1q is expressed in postnatal, but not in adult retinal ganglion cells (RGC), the cells that innervate the LGN. This expression can be mimicked by the exposure of RGCs to

immature astrocytes, which are thus thought to induce the expression of C1q through an unknown signal molecule (Stevens et al. 2007). The time-windows of synaptic pruning and development of immature astrocytes coincide, opening the possibility that immature astrocytes could be the endogenous trigger for the expression of C1q in neurons. Similarly, activated microglia express C1q, C3 and other complement proteins, although notably, not only during development but also during adulthood (Veerhuis et al. 1999, Stevens et al. 2007 and Cahoy et al. 2008). The current model, as proposed by Stephan et al. (2012), suggests that the expression of C1q is triggered by a signal of unknown identity secreted by immature astrocytes, which acts upon neurons and microglia to produce and release C1q and possibly other complement components. In order for the C1q to be specific to weak synapses, the binding of C1q would have to somehow be coupled to the stability state of the synapse.

At this point, there are no studies showing the relative contribution of neuronal and microglial C1q to synapse elimination. Based on the expression of C1q in microglia during adulthood, it is compelling to think that they could be responsible for the baseline supply of complement during learning and adult maintenance, while astrocyte-triggered neuronal C1q could be responsible for the early postnatal peak in synaptic pruning. Summarizing the current findings, a possible model for the timecourse of C1q functioning is proposed in **Figure 5**. In order to experimentally determine the relative contributions of neuronal and microglia C1q to synapse elimination in the mature brain, a conditional knockout study of C1q in adult animals is neccessary. Such a study would evade the developmental deficits connected to a complete knockout, while disrupting the protein in the adult.

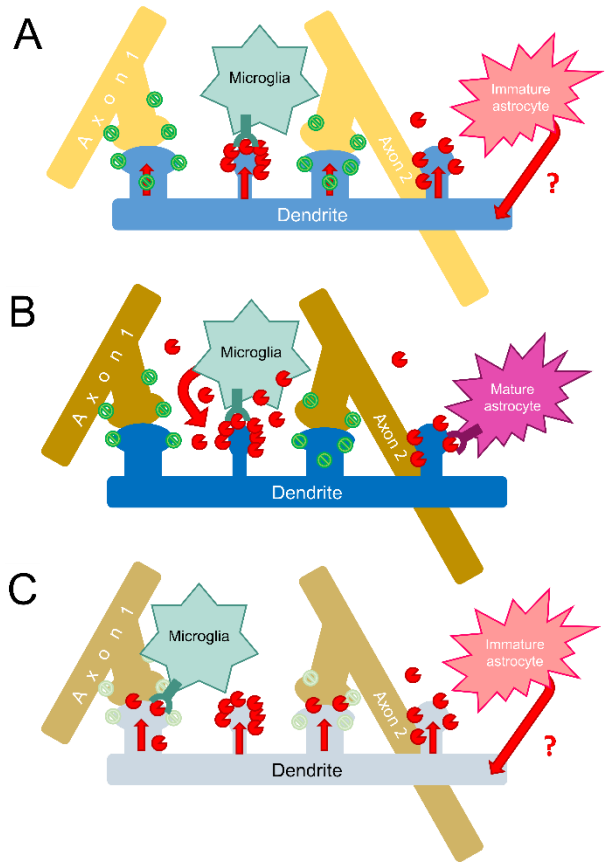

Figure 5| Tagging of synapses with complement during development, adulthood and disease

Based on findings presented in the text, the following timecourse of C1q expression is possible:

(A) Development: Immature astrocytes trigger the neuronal expression of C1q by an unknown signal. Subsequently, C1q localizes to weak synapses, where it is recognized by phagocytic glia (here microglia shown).
(B) Adulthood: Microglia express C1q, which localizes to weak synapses, which are subsequently phagocytosed by microglia and astrocytes.
(C) Neurodegenerative disease: Astrocytes are reverted to an immature state, become reactivated and trigger the expression of C1q. By an unknown mechanism, C1q also localizes to strong, functional synapses, which are phagocytosed along with weak synapses.

The complement signaling cascade in synaptic might not only play a role in development and refinement of the healthy brain. Importantly, it is hypothesized that in neurodegenerative diseases, mature astrocytes could revert back to an immature state, thereby triggering inappropriate release of C1q from neurons. This could increase synaptic binding of C1q and lead to synapse loss (Stephan et al. 2012; Figure 5C). For example, such elevated binding of C1q to synapses has been proposed to form part of the mechanisms of increased synapse loss in Alzheimer's disease (Fischer et al. 1995, Fonseca et al. 2004, Tenner and Fonseca 2006).

Additional mechanisms

Even in mice with KO of complement, some synaptic refinement still takes place. CR3 and C3 KO mice have 50% of engulfment levels of WT littermate controls (Schafer et al. 2012). This lack of complete abolishment of synapse elimination indicates that there must be additional mechanisms for synapse elimination. There are some hints to which molecules could be involved.

Mice deficient of the immune major histocompatibility complex I (MHC-I) show a similar phenotype as complement-deficient mice and MHC-I and C1q are closely associated at RGN synapses, which raises the possibility that they could interact in synapse elimination (Corriveau et al. 1998, Huh et al. 2000, Oliveira et al. 2004, Datwani et al. 2009, Tyler and Boulanger 2012). This notion is supported by findings of raised expression of MHCI in brain regions that have high activity-dependent remodeling (Shatz 2009). Activated microglia express MHC-I, also during normal development (Neumann et al. 1998, Biber et al. 2007).

In addition, the neuromuscular junction (NMJ) can offer some hints to alternative processes, as synapse elimination there is not affected in a KO model of C1q (Stevens et al. 2007). In spite of these interesting earlier findings, recent studies have pointed at neuronal signals, such as BDNF, playing a role in NMJ refinement (Je et al. 2012, 2013). Therefore, it could be that the remaining part of synapse elimination in the brain is also neuronal in origin.

Taken together, microglia sample the brain parenchyma and locate weak synapses by an unknown mechanisms. The most promising candidate, C1q, was first discovered in the immune system, where it has a defined role in opsonization of unwanted debris. Although C1q has been linked directly to synapse elimination through KO studies, its main mode of action in synapse elimination, in particular its triggering signal and interaction partners, are yet to be identified. The synapses that have been tagged for elimination are subsequently phagocytosed, either by microglia, or by astrocytes, as discussed in the following paragraphs.

3.2.2 Astrocytes

In comparison to the synapse-sampling microglia, astrocyte-neuron contacts are stable, as many central synapses are permanently ensheathed by astrocytes. It has been shown that 57% of synapses in the hippocampus show such ensheathment (Xu-Friedman et al. 2001). Thus, the localization and contacting of synapses by astrocytes happens indepently of synapse elimination. However, one recently published study by Chung and colleagues (2013) suggests that astrocytes, like microglia, can also play a role in synapse pruning. By tagging astrocytes and synaptic elements with fluorescent markers, Chung and colleagues observed that synaptic elements localized within astrocytes. Based on these findings, they concluded that astrocytes engulf synaptic elements, or even whole synapses including the pre- and postsynaptic element. The authors found that this engulfment is dependent on extracellular molecules contained in astrocyte-conditioned media and that neuronal activity in the LGN greatly increased astrocytic engulfment. Similar to microglia, synaptic engulfment by astrocytes is developmentally regulated and peaks in the murine LGN around P6. Astrocytic phagocytosis of synapses is dependent on MEGF10 and MERTK, two receptors that activate phagocytic pathways. (Curiously, while MERTK is also expressed in microglia, a KO of the protein led to a transient increase in microglial engulfment (Chung et al. 2013).

In double KO of MERTK and MEGF10, eye-specific segregation in the LGN failed, similar to phenotypes of KO complement (Chung et al. 2013). MEGF10 is an ortholog of *Drosophila* Draper and *C. elegans* CED-1, receptors that have been implicated in phagocytosis and synapse elimination as well (Zhou et al. 2001, Ziegenfuss et al. 2008). MEGF10 and MERTK are activated by *eat me*-signals such as phosphatidylserine, which is expressed on dead cell debris, but has not been shown to be expressed on synapses (Neumann et al. 2008). Other, synapse-specific interaction partners of these receptors are yet to be identified. They lead to the engulfment of weak synapses (Chung et al. 2013), but how they distinguish weak from strong synapses is not understood. The activation of MERTK in turn activates integrin pathway, which regulates CrKII/DOCK180/Rac1, molecules involved in the rearrangements of the actin cytoskeleton related to phagocytosis (Wu et al. 2005). MEGF10, on the other hand, requires GULP1 and ABCA1 (Hamon et al. 2006). Both signaling pathways are evolutionarily conserved phagocytic signaling pathways (Kinchen and

Ravichandran 2007).

What the authors fail to address is the discrepance of the activity-dependent increase in synapse engulfment by astrocytes reported in their study and the generally accepted notion that an increase in neuronal activity leads to an increase in astrocytic ensheathment of the activated, strengthened synapses (discussed in later section, Wenzel et al. 1991, Filosa et al. 2009). Thus, based on these first findings, which established the phagocytic ability of astrocytes, future research will have to determine how astrocytes switch between the stable ensheathment and the engulfment of synapses. In addition, similar quesitons as for microglia will have to be addressed: How do astrocytes differentiate between synapses of different strength? What are the precise mechanisms that lead to engulfment? Are there common *eat me/don't eat me*-signals for astrocytes and microglia?

3.2.3 Common themes of astrocytes and microglia in synaptic pruning

Both microglia and astrocytes have non-overlapping domains in which they contact synapses (Halassa et al. 2007, Nimmerjahn et al. 2005). The borders of microglial domains are dynamic, yet individual processes repel each other (Nimmerjahn et al. 2005, Tremblay et al. 2010a for changes in association of microglia to dendritic spines upon changes in visual sensory experience in the juvenile visual cortex)

Chung and colleagues were the first ones to compare the relative contributions of microglia and astrocytes to synaptic pruning during development. Although astrocytes engulfed less debris per unit cell volume in an LGN model (Chung et al 2013), they outnumbered microglia by 10 to 4-fold between P5 and P9, in sum contributing more to engulfment. Especially in light of how little is known about synaptic pruning in the juvenile and mature brain, it will be of special interest to examine relative contributions of different glia types at later developmental stages. First conclusions can be drawn from studies in *Drosophila*, where the disruption of the classical phagocytic cascades mentioned in the section on astrocytes did reduce early, but not adult pruning (Tasdemir-Yilmaz and Freeman 2013), which suggests that microglia are the prime pruning cells in the adult brain.

3.2.4 Conclusion

Taken together, these findings form the basis of disentangling the roles of glial cells in synaptic elimination. Future studies should expand our knowledge about this process, with a special focus on elimination during adulthood, as there have been almost no studies describing it. Knowledge in most part is limited to the conclusion that the KO of molecular actors of developmental synapse elimination, such as complement, does not have an effect on mature pruning. This area of investigation is particularly topical, as aberrant synapse elimination is being implicated in neurodegenerative diseases, such as Alzheimer's disease, and one possible cause for the exessive elimination observed in these disorders could be the reversion to developmental synapse pruning at inapproriate, later stages of an organism's life.

In my research proposal, I address this issue by suggesting an experiment using a mouse with a conditional knockout for C1q. This construct would make it possible for the mouse to develop normally until adulthood, when C1q expression can be selectively disrupted in microglia. Using a motor learning task and *in vivo* imaging in the motor cortex, the proposed experiments would show if the loss of C1q in the mature brain would have an effect on the animal's learning abilities in that specific part of the cortex.

3.3 Synapse maturation & maintenance

The synapses that sufficiently fit the properties of the circuit to successfully evade elimination need to undergo additional maturation in order to be fully functional. This maturation has two elements: First, the internal maturation of the pre- and postsynaptic elements, in the form of addition of transmitter receptors, ion channels and structural molecules to the synapse. This stage and its glial affectors have been covered in the section on synapse formation. A second part of maturation can be the ensheathment of the synapse by a perisynaptic astrocytic process to form the tripartite synapse. Importantly, emsheathment has a direct effect on synapses in that the presence of an astrocytic contact directly regulates the development of the pre- and postsynaptic compartments. The ways in which astrocytes do this is subject of this short chapter, in which I first discuss the stabilization of young, dendritic protrusions and then the balance between homeostatic coverage in the mature brain and plastic, activity-dependent changes in coverage.

By ensheathing synapses, astrocytes form the close contact necessary for many of their housekeeping roles, such as neurotransmitter clearance and ion homeostasis. The presence of these functions is important for synapses, as they are necessary for some normal synaptic functioning, as well as some forms of synaptic plasticity (Min and Nevian 2012). The motility of astrocytic processes is high in the young, developing brain (Nishida and Okabe 2007), but limited in the mature brain (Nimmerjahn et al. 2005). During development, there seem to be two categories of astrocytic contact: Longer contact, which develops into the stable contact of the mature brain, and shorter contacts (Nishida and Okabe 2007). The function of these shorter contacts has not been described, but this could be a form of sampling before the appropriate synaptic partner is identified.

3.3.1 Stabilization of young dendritic protrusions

The contact formation of astrocytes with synapses can already begin before the pre- and postsynaptic elements are apposed, when immature dendritic protrusions emerge from the postsynaptic dendrite (Nishida and Okabe 2007). During postnatal development, dendritic protrusions appear and disappear on a timescale of minutes to a few hours and accordingly turn over fast. This fast turnover is attenuated in mature slices. Of the new protrusions, those that form a contact with astrocytic processes, either transiently or stably, had a longer lifetime and were more likely to mature to spines (Nishida and Okabe 2007). Astrocytic processes turn over in a similar manner, but at a higher rate, and either transiently or stably can be tangent to individual dendritic protrusions or synapses (Hirrlinger et al. 2004, Nishida and Okabe 2007). Possibly, the contact of dendritic protrusions with astrocytic protrusions is mutually stabilizing.

Whether stability attracts astrocytes or astrocytes stabilize protrusions cannot be concluded from these results. Nevertheless, manipulations suppressing contact events by reducing astrocytic mobility decreased maturation of dendritic protrusions. These findings are a first indication that the contact by astrocytes is not only indicative, but also causative to maturation (Nishida and Okabe 2007). Future studies are needed to fully understand how astrocytes are involved in maturation of dendritic protrusions.

3.3.2 Balancing homeostatic and plastic astrocytic synapse coverage in the mature brain

Synapse coverage by astrocytes is thought to differ between brain areas. However, it has only been determined for few of them. For example, 57% of synapses in the hippocampus are ensheathed by astrocytes (Xu-Friedman et al. 2001). Even in the mature brain, astrocytic processes are motile and can undergo morphological changes on a time scale of minutes (Theodosis et al. 2008). Already small changes can lead to substantial motifications of the geometry and diffusional properties of synapses, as well as their ion homeostasis. It is important to recognize that even ensheathed mature synapses are often not completely isolated, but only partially ensheathed (Olmos et al. 1989, Kuroda and Price 1991). These holes in astrocytic coverage allow for some spillover of neurotransmitters, which allows for important cross-interaction between multiple synapses located closely to each other (Isaacson 2000).

Coverage changes can be elicited by multiple means: Enriched sensory input and learning, but also reproductive status. More specifically, long term potentiation of a synapse can increase ensheathment in an

activity-dependent manner (Wenzel et al. 1991, Filosa et al. 2009). The degree of astrocytic coverage is also directly related to behavioral processes. For example, being reared in a complex environment increases coverage, as does behavioral conditioning, whereas conditioned inhibition has the opposing effect (Jones and Greenough 1996). In the mouse barrel cortex, repeated stimulation of whiskers led to an increase in astrocyte coverage of excitatory synapses (Genoud et al. 2006). For review, see Theodosis et al. 2008.

The molecular mechanisms that regulate the change in astrocytic coverage have not been described, but are most likely linked to factors involved in synaptic plasticity, in order to link activity-dependent changes in the synapse to its perisynaptic partner.

3.4 Synaptic plasticity

In an intriguing study, which highlights the significance of astrocytic contributions to synaptic plasticity, Han and colleagues grafted human embryonic glial precursor cells into neonatal mice and investigated the physiological and behavioral differences between these chimeric mice and wild-type conspecifics (Han et al. 2003). Their study showed that human astrocytes in mice kept their human characteristics, such as faster propagating calcium waves and a more complex architecture. The mice had enhanced basal excitatory transmission in the hippocampus, as well as accentuated LTP. In behavioral tasks, the chimeric mice performed better in hippocampus-dependent tasks, such as contextual fear conditioning and the Barnes maze. The authors' suggest TNFα as a possible molecular actor responsible for the differences in synaptic plasticity, as it is expressed in human astrocytes but not in mice and the inhibition of TNFα reversed the enhancement seen in chimeras.

Most likely, there are many more yet-to-be identified mechanisms that distinguish human astrocytes and their capacities, in turn augmenting the computational power of human neuronal networks. Studies like these into the differences in synaptic transmission and synaptic plasticity between humans and non-human mammals will possibly be able to identify some of them, but there are strong limitations, as a complete replacement of one species' cell type for another's is difficult to impossible to accomplish and changes in development, due to differences in molecular signalling cascades, cannot be ruled out completely. Overall, astrocytes are a promising candidate for explaining some of the differences in computational power between species. This chapter highlights general principles and recent findings of how exactly glial cells participate in synaptic plasticity. Since almost all studies so far have been conducted on astrocytes, this chapter similarly focuses on them.

From the very beginning of synapse formation, synapses are subject to activity-dependent modifications. Even when an established synaptic contact has evaded elimination and reached a certain level of maturity, it does not stop to be influenced by the activity patterns of the neurons it connects. Broadly, weakening and subsequent elimination of synapses can be seen as one extreme of a continuum of processes that interact and counteract each other to dynamically determine the strength of an individual synapse. These processes are what makes a synapse *plastic*. Specifically, synaptic plasticity is an umbrella term for molecular changes at the pre- and/or postsynaptic elements that change properties of the synapse (for an overview of synaptic plasticity, see the box **Forms of synaptic plasticity**). The characteristics by which synapses are most commonly described are synaptic efficacy, the capacity of the presynaptic input to influence the postsynaptic site (measured at the mean peak amplitude of all responses including failures), synaptic potency (the mean peak amplitude of successes) and probability of release (the ratio between number of successes and total number of stimuli). In this context, a success is considered the triggering of an excitatory or inhibitory postsynaptic potential or current. Common targets of modulation of synaptic strength are the number, size and neurotransmitter content of presynaptic vesicles, the number, composition and surface-ratio of postsynaptic receptors and the clearance of neurotransmitters from the synaptic cleft. For a recent review on the molecular mechanisms of synaptic plasticity, see Ho et al. 2011.

Box: Forms of synaptic plasticity

Synaptic plasticity has two valences and two timescales: On the level of valence, a synapse can either be weakened or strengthened. Both strengthening and weakening can either be temporary (short-term plasticity) or persistent (long-term plasticity). The valence of plasticity is determined by multiple characteristics of pre- and postsynaptic activation and the precise mechanisms that distinguish the triggering of either are vivid area of research.

Subforms of plasticity have been classified according to multiple principles. Most commonly, their valence and important induction characteristics are used, such as *spike-timing dependent* long-term *potentiation* for long-term potentiation invoked by the adequate timing of pre- and postsynaptic action potential firing. Another way of classifying plasticity is by comparing it to Hebb's postulate. Accordingly, a strengthening process based on timed firing is termed *Hebbian*. *Non-Hebbian* and *anti-Hebbian* forms of plasticity depend on different mechanisms, such as an interaction of multiple synapses in synaptic scaling or the hyperpolarization of the

postsynaptic neuron, respectively (Urban and Barrionuevo 1996, Kullmann and Lamsa 2008).

Within long-term potentiation, a distinction is made between early and late-phase potentiation. Late-phase potentiation differs in that it depends on novel protein synthesis and in that it outlasts early potentiation, which roughly lasts 2-4 hours.

Plasticity has been traditionally observed with electrophysiological means, by determining synaptic strength with either extracellular field potential recordings or whole-cell patch-clamp recordings. In such a way properties of synaptic strength, such as synaptic potency, efficacy and the probability of neurotransmitter vesicle release, can be accurately determined.

With modern imaging techniques, it is possible to observe multiple characteristics of synapses without having electrophysiological access to the cell. On one hand, synapses as a whole can be marked with fluorescent proteins and observed under a microscope. Specific areas of a neuron are identified and imaged with high resolution, in order to make individual synaptic elements visible. During analysis, both the number and the location of synaptic protrusions can be determined. Of particular value is that animals can be implanted with chronical cranial windows, which allow for imaging of synapses over days and weeks, making it possble to track structural changes in synapses in the living animal or in cultured slices. First studies employing this technique have directly liked behavioural tasks, such as a motor learning task, to an increase in synapse turnover in the primary motor cortex in mice (Xu et al. 2009).

On the other hand, even more subtle changes in synaptic strengh are starting be be tracked with imaging techniques. Long-term potentiation and depression can be indirectly observed via structural changes (reviewed by Holtmaat and Svoboda 2009). First structure-function studies of synapses and dendritic spines describe such relationships, which are not always straightforward (Segal 2010). Once structural characteristics can be reliably used as a means to track relative weight of synapses without electrophysiological access to a cell, they will allow for a direct analysis of synaptic plasticity using visual determinants.

3.4.1 Astrocytes' role in synaptic plasticity

At the tripartite synapse astrocytic processes are tightly connected to the synaptic cleft in a way to allow them to clear neurotransmitter from excitatory synapses and to control the local ionic composition. Classically, these roles have been seen as "routine" housekeeping tasks of astrocytes, while neurons were ascribed the active and sophisticated role of synaptic plasticity. In recent years, the view that astrocytes participate in synaptic plasticity in a way that has been described as more "active", in contrast to the "passive" housekeeping roles, has gained some acceptance. According to this newer view, astrocytes are thought to influence synaptic plasticity via at least three pathways: release of gliotransmitters, neurotransmitter clearance and ion homeostasis & supply of energy. It is important to note that these processes are not physiologically distinct, but closely tied to each other. In the following sections I describe what is known about these three pathways and give some hints on how they interact.

3.4.1.1 Gliotransmitters

Gliotransmitters, analogous to neurotransmitters, are molecules released from glia following physiological stimuli, which rapidly activate responses in neighboring cells (Parpura and Zorec 2009). In brief, gliotransmitters are released by glia cells into the synaptic cleft, where they interact with receptors on the presynaptic and/or postsynaptic element. Gliotransmitters can directly influence synaptic plasticity and they have been shown to do so in various brain regions, among which the hippocampus, cortex, thalamus, cerebellum and retina (reviewed by Araque et al. 2014). Araque and colleages define three important differences between glio- and neurotransmitters: First, they act on distinct spatial and temporal scales. While neurotransmitter responses occur fast and locally, glial transmission is slower and might rely on slow-desensitizing receptors in astrocytes and neurons. Second, through the domain-like structure of astrocytes, they have the ability to influence multiple synapses within one domain in a coordinated manner. Astrocytes indeed participate in multi-synaptic forms of plasticity, such as D-serine increasing synaptic scaling in the amygdala and TNF doing the same in the hippocampus (Stellwagen and Malenka, 2006). Third, the varied

involvements of glia in other brain functions besides information processing, e.g. vascular and immune, enables them to integrate environmental information and adapt their transmitter output accordingly (Araque et al. 2014).

Although subject to some controvery, it is generally accepted that the release of gliotransmitters, mostly through exocytosis, is triggered by Ca^{2+} elevations in astrocytes. As an exception to this principle, some additonal Ca^{2+}-independent mechanisms have been identified (Hamilton and Attwell 2010).

The gliotransmitters on which studies have focussed are ATP/adenosine, glutamate and D-serine. Less studied, there is evidence that GABA and tumor necrosis factor (TNF) are released by glial cells as well (Angulo et al. 2008, Beattie et al. 2002).

ATP/adenosine

One of the earliest identified gliotransmitters is ATP. ATP can be released by both neurons and glial cells (Stevens and Fields 2000, Pankratov et al. 2006, Hamilton et al. 2008) and ATP signaling has been described as bidirectional (Verderio and Matteoli 2011). ATP can interact with receptors both pre- and postsynaptically. Its main interaction partner are purinergic receptors, such as P2X. Once in the synaptic cleft, ATP is often degraded to adenosine by ectonucleotidases. Adenosine can act as a gliotransmitter as well, binding to adenosine receptors on both neurons and glia. Of the two dozen of studies reported, the majority implicates ATP in the depression of basal synaptic transmission, especially in the hippocampus, and the regulation of certain forms of plasticity, such as LTP (Araque et al. 2014). One exception is a study by Gordon and colleagues, which connects ATP released by astrocytes in the hypothalamus to binding of postsynaptic P2X receptors, which increases neuronal excitability and the amplitude of miniature excitatory postsynaptic currents induced by the insertion of additional AMPA receptors (Gordon et al. 2005).

Glutamate

Astrocytic release of glutamate has been linked to both synaptic potentiation and depression. The determinant of valence for synapses seems to be an interplay of relative activation of the pre- and postsynaptic element, certain proteins found in these compartments that act as molecular timers, and the specific timing of release of glutamate from astrocytes, which orchestrates the neuronal reaction to the activation of different receptors. Retrograde messengers and even the extracellular matrix have also been shown to play a role (Dityatev and Rusakov 2011). On one hand, in spike-timing dependent depression in the developing barrel cortex, astrocytes have been suggested to be a necessary and sufficient link between activation of cannabinoid receptors and presynaptic NMDA receptors via their intracellular Ca^{2+} signaling (Corlew et al. 2008, Min and Nevian 2012), leading to a presynaptic synaptic depression.

In contrast to the aforementioned example, a special form of long-term potentiation, namely cholinergic-induced LTP, is also dependent on astrocytic released glutamate and its interaction with presynaptic mGluRs. More specifically, evoking an increase in astrocytic calcium by uncaging in the presence of acetylcholine in the synaptic cleft transiently increased the probability of neurotransmitter release in the CA3-CA1 synapses (Perea and Araque 2007). If they paired the increase with a mild postsynaptic depolarization, the increase in probability became permanent. They determined the process to depend on the release of glutamate from astrocytes and its subsequent activation of metabotropic glutamate receptors (Perea and Araque 2007). Building on their work in vitro, the same group used first investigations in the hippocampus *in vivo* to support the notion that glial-released glutamate can play a vital role in some forms of plasticity. They show that cholinergic LTP also in vivo depends on Ca^{2+}-signaling in astrocytes that induces the release of glutamate, which in turn activates metabotropic glutamate receptors, and a postsynaptic depolarization evoked by choline (Navarrete et al. 2012). The site of plasticity in this form of LTP is most likely presynaptic, as the transmitter release probability is increase, but the amplitude of the postsynaptic response remains unchanged (Navarrete et al. 2012).

These findings establish glutamate as a gliotransmitter *in vivo*, ruling out artefacts of *in vitro* studies. This has been an important step in the field, as gliotransmitter release as a widespread phenomenon has been subject to some criticism, particularly since experimental findings by Agulhon and colleagues that show hippocampal short and long-term plasticity to be independent of astrocytic Ca^{2+}-signaling (Agulhon et al.

2010). Nedergaard and Verkhratsky similarly argue against ascribing too much importance to gliotransmitters and stress the role of astrocytes as providing an "astroglial cradle", isolating the synapse from its surroundings and creating a compartment that makes individual synaptic plasticity possible in the first place (Nedergaard and Verkhratsky 2012). Most probable, both opinions have their place and time, as it is likely that astrocytic involvement differs between brain areas and possibly developmental time periods. Thus, it is especially important to test *in vitro* reports of gliotransmitter release in living animals.

D-serine

D-serine, as described in the chapter on synapse formation, is a gliotransmitter released by astrocytes that is a co-agonist for NMDA glutamate receptors. Via NMDA receptors, D-serine is mainly involved in the modulation of long-term plasticity, such as the LTP in the hippocampus (Yang et al. 2003, Henneberger et al. 2010) and LTP/LTD in the cortex (Takata et al. 2011, Fossat et al. 2012).

TNFα

One of the less studied gliotransmitters is tumor necrosis factor α. It is released by astrocytes and has the ability to enhance synaptic efficacy by increasing the surfacing of AMPA receptors at excitatory synapses (Beattie et al. 2002).

In summary, these studies show that the actions of gliotransmitters are just as varied as the actions of many neurotransmitters, and their effect on synaptic plasticity depends on brain region and context of release (Araque et al. 2014). Future research has to address the controversy around the release mechanisms of gliotransmitters and further conceptualize how glia can complement mechanisms of neuronal synaptic plasticity by covering a wider temporal and spatial range.

3.4.1.2 Neurotransmitter clearance

Astrocytic glutamate transport increase is necessary for late-phase LTP (l-LTP)

In the CA1 of the hippocampus, both early and late-phase LTP of excitatory synapses occur simultaneously to an increase in glutamate uptake, but the respective increases the depend on distinct mechanisms. The early increase is based on a translocation of the neuronal glutamate transporter EAAC1 to the plasma membrane (Levenson et al. 2002), whereas the late-phase increase is mostly, if not solely, dependent on the astrocytic glutamate transporter GLT-1 (Pita-Almenar et al. 2006). In their 2012 study, Pita-Almenar and colleagues showed that the increase in astrocytic GLT-1 glutamate transport is necessary for the induction of l-LTP. When they inhibited astrocytic GLT-1, no l-LTP was observed. L-LTP was restored when a glutamate scavenger was used to mimic increased glutamate uptake. (Pita-Almenar et al. 2012)

Taken together, they show that astrocytic glutamate uptake does not only parallel synaptic plasticity, but that it is essential for the induction of LTP that lasts hours to days or even longer.

3.4.1.3 Ion homeostasis & Energy supply

One example of interacting pathways is the astrocytic uptake of glutamate from the synaptic cleft, which is tied to the cotransport of Na^+ for some glutamate transporters (Stephens et al. 1992, Rose and Ransom 1996). These processes link ion homeostasis, neutrotransmitter clearance and energy supply to neurons.

Synaptic activity, glutamate uptake and its metabolism by astrocytes are tightly linked via a processed termed metabolic coupling. Glutamate taken up by astrocytes is glycolysed into lactate and released into the synaptic cleft to provide the extra energy to neurons that is spent on synaptic transmission (Magistretti 2006). The additional energy won by glycolysis within the astrocyte is used to fuel a Na^+/K^+ ATPase, which exchanges three intracellular Na^+ for two extracellular K^+ while consuming ATP for this work (Pellerin and Magistretti 1997). In general, this ion exchange ensures ionic homeostasis. However, changes in extracellular K^+ have a direct effect on the excitability of the neighboring neuron, as K^+ is a determinant of membrane potential and even small changes in concentration can influence excitability. Although this change in K^+ does not have a direct implication in synaptic plasticity, overall changes in neuronal excitability can have an effect on the likelihood of a cell untergoing potentiation or depression.

Metabolic coupling can undergo plasticity in parallel to synaptic plasticity. It follows the principle that certain

molecules related to neuronal activity, such as adenosine, noradrenaline and some cytokines, can change the expression of astrocytic genes involved in the energy supply to neurons, such as glycogen metabolism (Megistretti 2006). A form of this plasticity is observed in the hippocampus, where metabolic plasticity takes place during learning (Magistretti 2006).

The plasticity of metabolic coupling is particularly interesting because it involves the plastic changes of astrocytes themselves, an area that has occasionally been investigated, but leaves many open questions (Shao and McCarthy 1994, Theodosis and Poulain 2008).

3.4.2 Conclusion

Glial cells, particularly the astrocytes discussed in this section, are active participants in the modulation of synaptic plasticity. They complement neuronal mechanisms of plasticity in that they cover a wider temporal and spatial range. Glial cells influence synaptic plasticity in at least three ways: They release gliotransmitters that directly affect synaptic plasticity, they alter neurotransmitter clearance in parallel to synaptic plasticity and they change their energy supply to synapses according to altered needs during plasticity.

3.5 The bigger picture & Open questions

In the past sections, I have described the many ways in which glia are indispensable partners of synapses in information processing. They initiate synapse formation, promote their stabilization, provide the niche necessary for local alterations of concentrations in the synaptic cleft and remove weak, unstable synapses. In addition, glia actively modulate synaptic plasticity, a complex process, which until recently, was conceptually reserved for neurons.

Even though, or possibly even because there has been a gigantic rise in interest in glia-synapse interactions, multiple open questions remain. On one hand, these questions lie on a mechanistic level, such as the identification of individual factors in synapse formation, the attribution of expression of *eat me*-signals or the ambiguity of functions of gliotransmitters. But more importantly, there is a big need to conceptualize the individual, qualitative findings in a quantitative, comprehensive way. Few studies address precise relative contributions of glia and neurons or define the relative developmental time-windows for certain mechanisms. Of course, there is a inherent restriction on quantification in biology, as pathways overlap, counter-balance and compensate for each other. Additionally, the plethora of model systems makes it difficult to directly compare studies. Consequently, initiatives that focus on the transcriptome and interactome of different glial cells in different organisms are of high value, and the few existant studies have been of great help in making sense of the detailed, isolated molecular findings that are often recorded.

The identification of precise astrocytic contributions also remains difficult because of limited experimental means to specifically record from or disrupt astrocytic activity. In addition, glial cells, as regulators of homeostasis, tend to compromise their own health for support of their surrounding. Hence, acute slice preparations, as well as current requirements for many *in vivo* preparations, such as cranial windows, inherently pose some restrictions on how much can be deducted from glia observed in this manner. Developments of *in vivo* methodology will most likely aid in observing glia in a less disrupted, reactive state.

The review of the literature has brought one specific aspect to my attention, which I think has not been sufficiently explored to the present day: The difference between developmental synapse turnover and synapse turnover in the mature adult. Particulary, I am interested in how specific learning situations can affect synapse turnover. In hope to address this issue with a proposal for further research, which I present in the last chapter of this text. In the proposal, I try to take into account common experimental drawbacks of past studies, such as problematic activation of disrupted glia, the lack of *in vivo*-evidence, the early onset of knockouts, which disrupt normal development and the missing link between molecular and behavioral effects.

4 Research proposal

The structure of this research proposal has been based on grant proposals to the NWO, the Netherlands Organisation for Scientific Research.

1. Title of research proposal

The contribution of glial factors to adult synaptic connectivity in vivo

2. Summary of research proposal

Stable synaptic connections of neurons in adulthood provide the basis for brain functioning (Grutzendler et al. 2002). By connecting neurons, synapses perform one of the most vital roles in the organization of the brain. This connectivity is established during development, where a period of heightened synapse formation is followed by synaptic pruning, until the adult brain with its connectivity is established. After this time period, axonal and dendritic branches are mostly stable. Nevertheless, there is a persistent addition and removal of synapses from the synaptic pool even in adulthood, a process termed synapse turnover. In connection with learning tasks, this turnover rate is considerably increased, which reflects the learning process. In recent years, it has become clear that glia play an essential role in the life cycle of synapses, participating in synapse formation, elimination, maturation, maintenence and plasticity. This suggests that glia could play an essential role in learning. Additionally, it has become apparent that the considerable loss of synapses during neurodegenerative diseases could be associated with a dysfunction of the formation, maintenence and elimination of synapses necessary for learning and memory. Despite a growing share of the ageing population facing such diseases, very little is known on how exactly synapses are lost – and how they could be rescued.

To the contrary, previous research has focussed the role of glia in synapse formation and elimination during development. A growing number of glial factors that are directly involved in these processes are being identified, yet albeit growing research interest in this field, little is known about the contribution of glia to synapse turnover associated with adult learning. In addition, most experiments have been conducted in *in vitro* model systems and few studies have combined molecular modifications and structural imaging studies with *in vivo* learning tasks and chronical imaging concurrently, which allows to draw direct connections between the structural and functional consequences of glial factor deficiencies with behavioral outcomes.

Here, I propose to investigate the contributions of two glial factors to synaptic connectivity in respect to motor learning. To this end, I will use genetically modified mice carrying conditional knockouts for glial factors involved in synapse formation and elimination and imaging techniques to track synapse turnover in motor cortex as well as learning performance throughout a motor learning task.
The findings will provide the first insights into glial contributions to synapse-turnover during adult learning, both structurally as well as behaviorally. The behavioral read-out is a particular strength of this research, as no data is available that directly links the disruption of structural plasticity with learning in the behaving, adult animal.

3. Keywords

Glia, synaptic connectivity, synapse formation, synapse elimination, in vivo, imaging, neurons, astrocytes, microglia, motor learning

4. Scientific/Scholarly quality

a. Background

The adult brain is a finely tuned structure of trillions of synapses, which form the very basis of brain functioning. None of the higher functions - perception, memory or emotions - would be possible without the precise synaptic wiring of the underlying neuronal circuits. Synaptic connectivity is established during development, when an overshoot of synapses is formed. Subsequently, synapses that do not fit the properties of the neuronal network they form part of are weakened by plasticity mechanisms, destabilized

and finally eliminated by phagocytic glia (Katz and Shatz 1996, Wiegert and Oertner 2013). The synapses that persist into adulthood are comparatively stable, yet even in adult animals there is a basic level of synapse turnover. The share of synapses that turn over varies according to the age of the animal and seems to level at around 5% spine turnover per month in the adult mouse (Zuo et al. 2005). Dendritic spines are the postsynaptic protrusions that subsequently align with the presynaptic element to form an excitatory synapse. How this level of synapse turnover is achieved and which molecules are responsible for it, is not known.

The learning of new skills through repetitive practice leads to an increased turnover and persistent higher number of synapses in the respective area of the brain (reviewed by Caroni et al. 2012). This is also true for new motor skills and the primary motor cortex, where a single-seed reaching task has been previously used sucessfully to increase synapse turnover and stabilization (Xu et al. 2009). Notably, the persistence of synapses is related to the retention of the learned task (Luft and Buitrago 2005, Kami et al. 1995, Rioult-Pedotti et al. 2007).

Consequently, there are two principal time-windows of synapse formation and elimination: First, substantial, widespread elimination following overshooting synapse formation during early development and, second, the increased turnover that follows local and short-lived synapse formation linked to learning (Puro et al. 1977, Trachtenberg et al. 2002). The latter has in outlines been studied in different learning tasks, where the learning leads to a heightened synapse turnover in the respective cortex or hippocampus (Moser et al. 1994, O'Malley et al. 1998, 2000, Klintsova and Greenough 1999, Knafo et al. 2001), followed by synaptic pruning (Missler et al. 1993). However, the details of learing-related synapse turnover remain elusive.

In recent years, the role of glial cells in all stages of the synaptic life cycle has become aparant. Not only can glial factors initiate and promote synapse formation, glial cells are also necessary for the proper elimination of weak synapses, which they help tag and engulf via phagocytosis (Schafer and Stevens 2010, Tasdemir-Yilmaz and Freeman 2013, Chung 2013). Two subtypes of glial cells have been under special investigation: Astrocytes, the glial cells that are in tight contact with neurons through their highly ramified processes which enwrap synapses and thus create the tripartite synapse, and microglia, the resident immune cells of the brain. Microglia have highly motile processes, which sample their surroundings, notably synapses, for immune-relevant substances, as well as cellular debris and weak synapses (Nimmerjahn et al. 2005, Ransohoff and Cardona 2010).

The proposed experiments focus on two periods of the synaptic life cycle: Synapse formation and elimination. The following section will outline what is known about these stages, while focussing on two glial factors: thrombospondin-4, which has synaptogenic properties, and C1q, an immune molecule involved in the tagging of weak synapses for phagocytosis. I chose these two factors because they fulfill opposing roles: One promotes the formation, one the elimination of synapses. In respect to synapse turnover, these contrary roles are highly relevant, as there must be a allostatic mechanism controlling the total level of synapse turnover in a certain region at a certain time. Dispite their functional differences, there are principal similarities between thrombospondin-4 and C1q: (1) Their expression is developmentally controlled, but it is not known how, (2) they are prime candidates for adult synapse turnover regulation, while close structural relatives participate in developmental regulation, and (3) only complete KOs no conditional KOs during adulthood are available.

Here, I will describe what has been found so far, as well as put the proposed experiments into the context of what is yet to be explored.

Glial promotion of synapse formation: Astrocytic thrombospondin-4 as promoter of adult learning?

Multiple classes of glial factors, which have an effect on parts of the synaptic life cycle, have been identified using *in vitro* and *in vivo* studies. For example, the glial factors that promote synaptogenesis can be categorized according to multiple systems: Whether they are extracellular matrix components or secreted proteins, or whether they induce functioning or silent synapses. Of the factors identified, on a handful is expressed beyond development and in the adult organism. One of these factors is thrombospondin-4, a member of the thrombospondin protein family, which consists of at least four members. When added to cultures of retinal ganglion cells, all thrombospondins induce ultrastructurally normal pre- and postsynaptic differentiation. However, the thus formed synapses are postsynaptically silent in that they lack glutamate receptors (Christopherson et al. 2005, Eroglu et al. 2005). It is thought that thrombospondins induce differentiation via a subunit of the $\alpha_2\delta$-1 voltage-gated calcium channel subunit, integrins, neuroligins and PKA, among other mechanisms involving cell adhesion, synapse architechture and cytoskeletal stability proteins (**Figure 6**, Eroglu et al. 2009, Crawford et al. 2012, reviewed by Risher and Eroglu 2012, Clarke and Barres 2013). Thrombospondin-4 is only expressed in mature astrocytes (Cahoy et al. 2008), and is thus thought to promote synaptogenesis in the mature animal, which makes it a prime candidate for influencing the increased synapse formation associated with learning. Hence, I propose here to selectively disrupt thrombospondin-4 in the adult animal, in order to define its role in the mature brain.

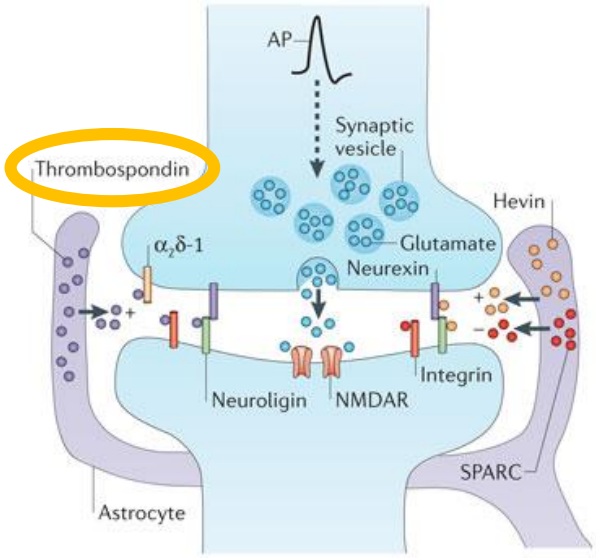

Figure 6 |Astrocytes instruct structural synapse formation by the secretion of thrombospondins (adapted from Clarke & Barres 2013)

Astrocytes (purple) release multiple factors that induce structural excitatory synapse assembly, among which thrombospondins (purple circles). Thrombospondins bind to receptors on the pre- and postsynaptic membrane, promoting the formation of postsynaptically silent synapses.

The dashed arrow indicated action potential (AP) propagation, which induces glutamate release from synaptic vesicles. NMDAR = NMDA receptor.

(Original figure text adapted from Clarke & Barres 2013).

Phagocytic glia in synapse elimination: C1q – a possible *eat me*-signal secreted by microglia?

Among the many open questions concerning the realm of glial influences on synapse elimination, the identification of an *eat me*-signal has received most attention. More specifically, researchers are trying to identify the molecular signal that distinguishes strong synapses from weak ones. From the proposed molecules, a component of the immune complement cascade, C1q, is the strongest candidate (Stevens et al. 2007, Schafer et al. 2012). C1q localizes to weaker synapses and non-matched pre- and postsynaptic elements (Stevens et al. 2007). KO models of C1q and another component of the complement cascade, C3, showed a significant increase in synapse number in adult animals in comparison to wild type and additional deficits in synaptic connectivity (Schafer et al. 2012). C1q is expressed in postnatal, but not adult retinal ganglion cells (RGC), the cells that innervate the lateral geniculate nucleus (LGN). This expression can be mimicked by the exposure of RGCs to immature astrocytes (Stevens et al. 2007). The time-windows of synaptic pruning and immature development coincide. Similarly, activated microglia express C1q, C3 and other complement proteins, although notably, not only during development but also during adulthood (Veerhuis et al. 1999, Stevens et al. 2007 and Cahoy et al. 2008). The current model, as proposed by Stephan et al. 2012, suggests that the expression of C1q is triggered by a signal of unknown identity secreted by immature astrocytes, which acts upon neurons and microglia to produce and release C1q and possibly other complement components. C1q can bind synapses by a yet to be identified mechanism or receptor. Possible candidates are neuronal pentraxins and the prion protein Prpc (Stevens et al. 2007). In neurodegenerative

diseases, they hypothesize, mature astrocytes could reverse to an immature state, triggering inappropriate release of C1q and synapse loss.

At this point, there are no studies showing the relative contribution of neuronal and microglial C1q to synapse elimination. Based on the expression of C1q in microglia during adulthood, it is compelling to think that they could be responsible for the baseline supply of complement during learning and adult maintenance, while astrocyte-triggered neuronal C1q could be responsible for the early postnatal peak in synaptic pruning. Summarizing the current findings, a possible model for the timecourse of C1q functioning is proposed in **Figure 7**. In order to experimentally determine the relative contribution, a conditional knockout study of C1q in adult animals is neccessary. Such a study would evade the developmental deficits connected to a complete knockout, while disrupting the protein in the adult.

Accordingly, the here proposed conditional knockout of C1q in adult microglia will determine its contribution to adult synapse elimination.

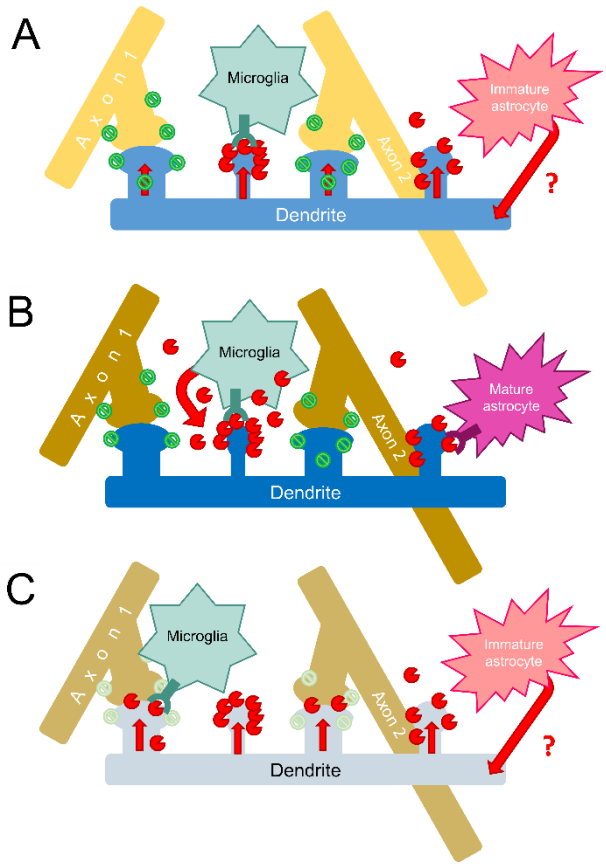

Figure 7 | Tagging of synapses with complement during development, adulthood and disease

Based on findings presented in the text, the following timecourse of C1q expression is possible:

(D) Development: Immature astrocytes trigger the neuronal expression of C1q by an unknown signal. Subsequently, C1q localizes to weak synapses, where it is recognized by phagocytic glia (here microglia shown).
(E) Adulthood: Microglia express C1q, which localizes to weak synapses, which are subsequently phagocytosed by microglia and astrocytes.
(F) Neurodegenerative disease: Immature astrocytes become reactivated and trigger the expression of C1q. By an unknown mechanism, C1q also localizes to strong, functional synapses, which are phagocytosed along the weak synapses.

Synapse turnover in the primary motor cortex during motor learning

As mentioned above, the learning of new tasks is directly tied to an increase in synapse turnover. Even more, the rise in synapse formation elicited by a novel task, combined with the refining elimination of unnecessary synapses, is the structural basis of learning. It is thus particularly surprising, that no studies are available that attempt to solve the puzzle of how exactly this rise in turnover is orchestrated on a molecular level.

To observe the structural synaptic changes during learning, I have chosen a motor learning task – a simple seed-reaching task - and the primary motor cortex for the proposed experiments. These are very suitable for the experimental set-up for three reasons: First, the motor cortex is easily surgically accessible, which is important for chronical *in vivo* imaging. Second, the learning of the task is relatively simple for the animal, making it easier to train them and to train multiple animals. Third, the system is established, and results can directly be brought in context with earlier publications (**Figure 8**).

By comparing images of structural plasticity with the learning curves of the animals during the seed-reaching task, I would be the first researcher to describe the connection between the disruption of glial synapse-affecting factors, structural plasticity and learning.

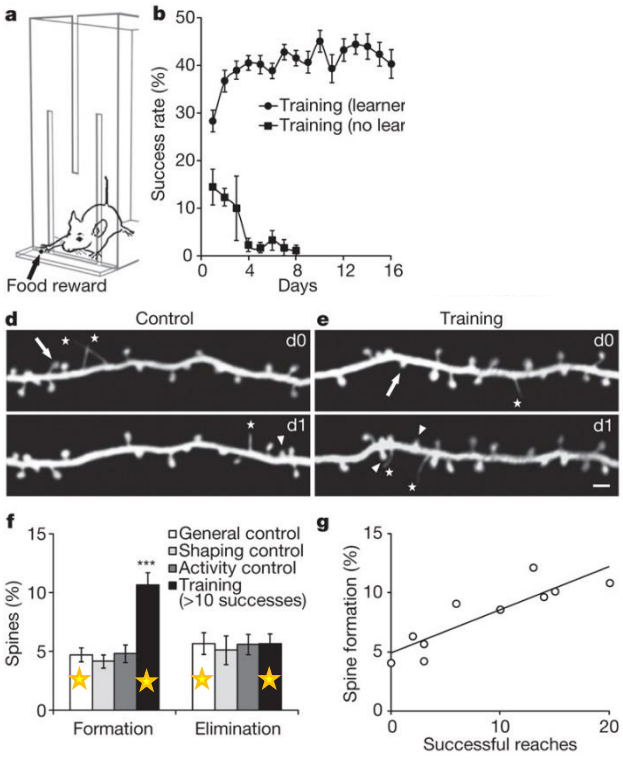

Figure 8 | A motor learning task increases synapse formation (adapted from Xu et al. 2009)

a, A cartoon of motor training. **b**, Average success rates during training for learning and non-learning mice (mean ± s.e.m., 42 learners and 5 no learners). **d**, **e**, Repeated imaging of the same dendritic branches over one-day intervals reveals spine elimination (arrows) and formation (arrowheads), and filopodia (asterisks) in a general control (**d**) and a trained (**e**) mouse. Scale bar, 2 μm. **f**, Percentage of spines formed and eliminated under various control and training conditions immediately following the first training session (mean ± s.d., ***P < 0.001). The data that would be collected in this study has been highlighted with stars. **g**, The degree of spine formation observed following the first training session is linearly correlated with the number of successful reaches during this session (r^2 = 0.77). (Original figure text adapted from Xu et al. 2009).

b. Research aims

The aim of the research is to provide *in vivo* evidence for the functional importance of glia in the synaptic life cycle of mature animals. More specifically, the behavioral and structural effect of a conditional knockout of two glial factors that have been shown to be involved in the synaptic life cycle *in vitro* will be investigated *in vivo*. To this end, I will compare performance in a motor learning task to synapse turnover in the respective cortical area.

i. Aim 1: How are learning and synapse turnover compromised in mice lacking astrocytic thrombospondin 4 in adulthood?
ii. Aim 2: How are learning and synapse turnover affected in mice lacking microglial C1q in adulthood?

c. Approach

Animals

For these experiments I will use mice who will genetically modified in order to fulfill two criteria: First, genes coding for thrombospondin-4 and C1q ('glial factors') can be conditionally knocked out in astrocytes and microglia, respectively, in order to allow mice to develop normally into adulthood and avoid any delopmental abnormalities connected to KO of these glial factors, as well as to be sure that any effects come from a lack of expression in glia and not neurons. Second, neurons express a fluorescent marker, allowing for structural imaging of synapses.

I will utilize two mouse lines in which the genes coding for thrombospondin-4 and Cq1a have been modified in order to be principally knocked out, but can be selectively turned on using the FLP-FRT system and subsequently turned off using the Cre-lox system. They are termed $Thbs4^{tm1a(KOMP)Wtsi}$ and $C1qa^{tm1a(EUCOMM)Wtsi}$ respectively, have been generated previously and are readily available live via The

Jackson Laboratory (Bar Harbor, ME, USA). C1qa is a major constituent of the C1q complex and its KO is sufficient to abolish C1q formation (Petry et al. 2001)

Since the genetic modification of these lines leads to a default knockout, which is not desireable in the proposed experiments, these mice will be crossed with mice carrying Flippase in all somatic cells in order to reinstate protein expression from early development on. For this trait I will use Flpo deleter mice (C57BL/6-Tg(CAG-Flpo)1Afst/Ieg), a strain of mice carrying a more efficient form of Flippase, which is readily available live from the Mutant Mouse Regional Resource Center (Davis, CA, USA). The resulting mice will thus have a simple conditional knockout potential of the glial factors via the Cre-lox system. Circumventing the default knockout via this additional breeding step is deemed less labourous than creating a conditional knockout line directly, which is not available through suppliers. The utilization of a deleter line, as proposed here, is a routine procedure when using mouse lines created by consortia, as the lines used in these experiments.

The thus bred mice will be crossed with a line of mice expressing yellow fluorescent protein (YFP) under the thy1 promoter, which is specific to neurons and optimized for imaging, as not all neurons are labelled with fluorescent protein and it strongly marks axons. This strain is readily available via The Jackson Laboratory (Bar Harbor, ME, USA) as B6.Cg-Tg(Thy1-YFP)16Jrs/J.

The delivery of transgenes to specific cell groups of the brain by viral vectors has been recently described (Meriennce et al. 2013,). In order to introduce Cre into astrocytes or microglia and subsequently knock out the expression of the glial factors thrombospondin-4 and C1qa in the adult mice, I will inject the Thbs4(cKO)-YFP and C1qa(cKO)-YFP mice with either a viral vector with tropism for astrocytes or microglia. For Thrombospindin-4 KOs, I will utilize a AAV5-gfa2-Cre vector, which is tropic for astrocytes (Drinkut et al. 2012), as described previously. For C1qa-KOs, I will similarly use a micro-RNA-9 regulated lentiviral vector specific to microglia and expressing Cre (Brown et al. 2007, Åkerblom et al. 2013). This vector uses the fact that all cells of the brain express micro-RNA-9 except microglia. Thus, the expression of vector genes is disrupted in all other brain cells, while microglia specifically express the gene carried by the vector – in this case Cre. This way of Cre expression in microglia is superior to coupling Cre to microglial promoters, as this approach avoids also targeting macrophages. Viral vectors can be ordered costum-made from the Vector Core of the University of Pennsylvania and shipped worldwide.

In consequence, Cre will be specifically expressed in either astrocytes or microglia, excising thrombospondin-4 and C1qa and stopping its expression. At the same time, their expression remains intact in all other cells of the CNS.

The expression of thrombospondin-4, C1qa, Flippase and Cre will be verified by genotyping before the start of the experiments and immunohistochemical stainings of brain tissue against thrombospondin-4 and C1aq will check for the right location of expression.

Imaging preparation

To preparte the animals for imaging, a thin-skul window will be placed above the primary motor cortex, as described previously (Marker et al. 2010). This technique is less disruptive than traditional open skull and thinned skull preparations and correspondingly leads to less activation of reactive microglia, which is favorable for studies of the endogenous, non-disease related funtion of microglia.

Motor learning task

I will use a single-seed reaching task, with which mice will be trained daily over a period of 4 days. There will be 5 experimental groups:

Wt - sham injected - motor learning
Thbs4/sham injected, no Cre - motor learning
Thbs4/Cre/KO - motor learning
C1qa/sham injected, no Cre - motor learning
C1qa/Cre/KO - motor learning

Imaging

Imaging will be performed transcranially in anaesthetized animals with a two-photon microscope, as has been described previously (Grutzendler et al. 2002).

Animals will be imaged on days 0 (day before training), 1-4 and, after the end of the training period, on days 6, 8 and 16, anologous to previous experiments (Xu et al. 2009, Fu et al. 2012). More specifically, the same dedritic segments will be identified and the number, location and size of dendritic spines before the beginning of the learning task will be determined and compared to the same parameters on later days. The resolution of the images allows for the tracking of individual spines, hence the fate of every imaged spine can be followed over the duration of the experiment and the formation and elimination of individual and overal spines reported. For identification of spines, protrusions and filopodia, the same criteria as previously reported will be used in order for the data to be comparable to the existing literature (Grutzendler et al. 2002).

The acquired images will be analyzed using ImageJ (NIH, USA).

Immunihistochemical controls

After the last imaging session on day 16, animals will be sacrificed, their brains removed, sliced and stained with antibodies against thrombospondin-4 or C1qa (both commercially available) in order to determine expression levels in and around the imaged area.

Hypothesis

In accordance with the above mentioned previous studies, I hypothesize that this motor learning task will lead to an increase in synapse formation as well as elimination during the learning period in control animals, with a persistent increase in synapse number outlasting the training period.

In animals lacking the synaptogenic astrocytic thrombospondin-4, I hypothesize less formation of new spines and, since less new spines are formed, also less elimination of the newly formed spines. In addition, I expect a less steep learning curve compared to controls. Beyond the training period, I expect less spines to persist and that the animal will perform worse than controls because of impaired learning.

In contrast, I hypothesize that the animals carrying a knockout for microglial C1qa, a factor involved in synapse elimination, will have synapse formation at similar levels to controls, but reduced synapse elimination, leading to a higher number of spines remaining after the training period. To elucidate this further, I will determine the strength of the persistent spines, estimated by their size (Grutzendler et al. 2002) and compare their mean size to spines of the control animals. As elimination is perturbed, I expect weaker, inapt spines that would be eliminated in control animals to persist in C1qa KO animals and decrease the mean size of spines recorded. Behaviorally, I initially do not expect an effect on learning compared to controls. Further into the training period, I expect impaired improvement compared to controls, but better performance than mice with thrombospondin-4 KO, as synapse formation should not be impaired.

5. Originality/Innovative elements

These experiments touch new ground in three ways: First and foremost, most current knowledge of glial factors in the synaptic life cycle has been generated from *in vitro* model systems, such as retinal ganglion cell cultures. Here I propose an *in vivo* paradigm, employing a recently developed, low-invasive imaging technique, where the role of glial cells, especially microglia, can be observed in a more physiological, undisturbed fashion. The verification of *in vitro* findings in the living animal has become increasingly important. These experiment take this approach one step further, in that this *in vivo* paradigm concurrently combines behavioral training and imaging, which will make it possible to directly link genetically modified synapse turnover with learning. Second, it explores glial involvement in adult synapse turnover. The majority of previous studies have focussed on developmental synapse formation and elimination and little is known about the role of glia in adult, learning-related synapse turnover. These experiments will be the first ones employing conditional, glial cell type specific knockouts for studying synaptic connectivity under the influence of glia. The state-of-the-art viral vectors provide high glial and temporal knockout specificity,

allowing the animals to develop normally and only show deficits when they are experimentally relevant. Third, investigation will focus on the cortex as a site for glial involvement in synaptic connectivity, while most studies have been conducted in the lateral geniculate nucleus and the hippocampus.

6. Appropriateness of candidate

The proposed experiments will involve four main areas of expertise: (1) Breeding, handling and training of transgenic animals, (2) design and ordering of viral vectors and genotyping, (3) conducting surgery for virus injection and skull modifications for imaging and (4) *in vivo* imaging on a two photon microscope.

(1) I have experience breeding and handling transgenic animals from the major internship during my masters, where I bred mice to carry a certain GFP-variant and removed another allele through outbreeding. Throughout the internship, I maintained and handled my own mice on a daily basis. This experience makes me confident that I will learn how to train the mice for the paradigm quickly.
(2) Through two internships in molecular biology and biochemistry and various laboratory courses during my bachelors in molecular biology, I have the knowledge and experience in molecular biology and genetics to design the viral vectors needed in accordance with the requirements of the producing institution. During a previous internship, I genotyped mice and will thus be able to perform the required procedures.
(3) So far I have not conducted surgery on animals myself. However, in order to acquaint myself with the procedures, I have assisted my former supervisor with virus injection surgeries in mice.
(4) I have participated in a 4-week Live Cell Imaging course at the VU University Amsterdam. As part of the course, I gained theoretical knowledge on imaging techniques, as well as hands-on experience with two-photon imaging. This course will form the basis for learning to image independently.

7. Time schedule

Task	Year 1				Year 2				Year 3			
Breeding of required transgenic mouse line	■	■	■	■								
Virus ordering & production	■	■	■									
Surgical training			■									
Injection, Surgery & Recovery				■	■							
Animal training & imaging					■	■	■	■				
Immunocytochemistry				■	■	■	■	■				
Analysis						■	■	■	■	■	■	■

8. Knowledge utilization

The proposed experiments would contribute to the understanding of how glial cells influence synaptic turnover in adult mammals. This is not only of interest for fundamental research, but also has implications for potential treatment of medical conditions.

Both factors on which this proposal focusses are implicated in the mechanisms of synapse loss connected to Alzheimer's disease (AD). Thrombospondin-4 is associated with β-amyloid containing plaques in AD cases and some control cases (Cáceres et al. 2007). A closely related glial factor, thrombopsondin-1, is abnormally retained in astrocytes exposed to amyloid-β and it is possible that a similar dysfunction could be found for thrombospondin-4. Additionally, the elevated binding of C1q to synapses has been proposed to form part of the mechanisms of increased synapse loss in AD (Fischer et al. 1995, Fonseca et al. 2004,

Tenner and Fonseca 2006). Although it is thought that the rise of C1q during this disease is a reinstatement of developmental neuronal expression, it is of interest to understand the relative contributions of neuronal and microglial C1q to adult learning to be able to estimate the effect of potential pharmacological or genetical interferences with C1q.

Taken together, these two factors, no matter how opposing their effects, are both possibly involved in the pathology of AD. Understanding their precise mechanism will aid to being able to develop therapeutical interventions for the resue of synapse loss in neurodegenerative diseases like Alzheimer's disease.

Interestingly, the mRNA coding for thrombospondin-4 is one of the mRNAs whose expression most upregulated in human cerebral cortex in comparison with other primates). It could be one of the factors not only responsible for part of the added synaptic complexity of humans, but also for our vulnerability to neurodegenerative diseases (Cáceres et al. 2007). The findings of this research would add insights to the function of thrombospondin-4 *in vivo* and thus contribute to the understanding of its mechanisms of action.

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10. Cost estimates & Budget

Item	Year		
	2015	2016	2017
Personnel (PhD)	€ 51 000	€ 51 000	€ 51 000
Disposables	€ 6 000	€ 7 000	€ 4 000
Animals	€ 10 000	€10 000	€ 5 000
Equipment	€ 1 000	€ 2 000	€ 2 000
Sum (yearly)	**€ 68 000**	**€ 70 000**	**€ 62 000**
Sum (total)s	**€ 200 000**		

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