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available at www.sciencedirect.com www.elsevier.com/locate/brainresrev**BRAIN
RESEARCH
REVIEWS****Review****Role of glial cells in the formation and maintenance of synapses****Frank W. Pfrieger***

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ABSTRACT

Synaptogenesis is a decisive process for the development of the brain, its plasticity during adulthood and its regeneration after injury and disease. Despite tremendous progress during the last decades, it remains unclear, whether neurons can form synapses autonomously. In this review, I will summarize recent evidence that this is probably not the case and that distinct phases of synapse development depend on help from glial cells. The results supporting this view come from studies on the central and peripheral nervous system and on different experimental models including cultured cells as well as living flies, worms and mice. Our understanding of synapse–glia interactions in the developing, adult and diseased brain is likely to advance more rapidly as new experimental approaches to identify, visualize and manipulate glial cells in vivo become available.

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Contents

1. Introduction	40
2. Glial cells in touch with synapses	40
3. Timing of synaptogenesis and glial development	40
4. Formation of glutamatergic synapses in the absence of glia	40
5. Glial cells enhance the number of synapses	41
6. Glial cells promote synapse maturation	42
7. Signals and mechanisms mediating the glial influence on synaptogenesis	42
8. Glial cells affect the turnover of synapses	43
9. Summary and Outlook.	44
Acknowledgments.	44
References	44

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Abbreviations: CEPsh, cephalic sheath; NMJs, neuromuscular junctions; PCs, Purkinje cells; PSCs, perisynaptic Schwann cells; RGCs, retinal ganglion cells; Tgfb1, transforming growth factor beta 1

1. Introduction

Synapses are key elements for brain function, and their formation is a decisive process throughout life. Neurons establish synaptic connections not only during development but also in the adult brain to implement their natural turnover and plasticity-related changes in the neuronal circuitry (Chklovskii et al., 2004; De Roo et al., 2008). The assembly of a synaptic connection requires a cascade of precisely coordinated processes in two partner neurons. Once synaptic partners have found each other, they assemble complex pre- and postsynaptic structures that mediate transmitter-based communication. Subsequently, connections acquire their specific transmission properties. In a last break-up phase, superfluous connections are eliminated. Research within the last decade has greatly advanced our understanding of molecules and mechanisms that mediate synapse development (Waites et al., 2005; Akins and Biederer, 2006; Craig et al., 2006; Fox and Umemori, 2006; Gerrow and El Husseini, 2006; Paukert and Bergles, 2006; Prokop and Meinertzhagen, 2006; Chen et al., 2007b; Dalva et al., 2007; McAllister, 2007; Polleux et al., 2007; Arikath and Reichardt, 2008; Bicker and Schrat, 2008; Biederer and Stagi, 2008; Greer and Greenberg, 2008; Hanus and Ehlers, 2008; Huang and Scheiffele, 2008; Margeta et al., 2008; Salinas and Zou, 2008; Chao et al., 2009). However, a key question remains unanswered: Can neurons form synaptic connections by themselves, or do they require help from neighboring glial cells? In this review, I will focus on recent experimental evidence for the latter. Complementary overviews on this subject can be found elsewhere (Slezak et al., 2006; Barker and Ullian, 2008; Feng and Ko, 2008b; Stevens, 2008; Bolton and Eroglu, 2010). Reviews of glial contributions to axon guidance and growth (Learte and Hidalgo, 2007; Pfrieger, 2009) and global views on astrocyte function (Wang and Bordey, 2008; Barres, 2008) have been published recently.

2. Glial cells in touch with synapses

The general term “glial cells” is used here for simplicity. In the mammalian nervous system, glial neighbors of synaptic connections are astrocytes and perisynaptic (or terminal) Schwann cells (PSCs). The glial coverage of synapses was first described more than 30 years ago in pioneering ultrastructural studies (Spacek, 1971) and has now been shown in 3D in the cerebellum (Grosche et al., 1999) and the hippocampus, where synapse size appears to correlate with the extent of astrocytic contacts (Witcher et al., 2007). A recent study of fly glia detected astrocyte-like cells near synapses of the antennal lobe indicating that synapse-surrounding glial cells are not limited to the mammalian CNS (Doherty et al., 2009). The intimate vicinity of synapses and astrocytes has led to speculations about the functional implications and inspired the concept of “tripartite synapses”, which regards glial cells as integral elements of synaptic connections (Araque et al., 1999). Information on the steady progress in this field is summarized elsewhere in this issue.

3. Timing of synaptogenesis and glial development

During brain development, most synapses are generated between the first and third postnatal weeks. This happens after the formation of astrocytes (Miller and Gauthier, 2007), which suggests that synaptogenesis requires glia (Pfrieger and Barres, 1996). Fluorescent labeling of individual astrocytes by dye injection followed by immunohistochemical staining revealed morphological changes in astrocytes during postnatal synapse development (Bushong et al., 2004). Notably, the temporal coincidence of synapse development and astrocyte differentiation applies only to glutamatergic connections. GABAergic neurons establish a functional network in the embryonic brain well before astrocytes are generated (Ben-Ari, 2002; Huang and Scheiffele, 2008). Therefore, the formation of GABAergic contacts is likely to proceed independently from the presence of astrocytes, but it may require help from other types of non-neuronal cells.

4. Formation of glutamatergic synapses in the absence of glia

Glia-free cultures allow to test directly whether neurons can form synapses without glia. Such cultures can be prepared from embryonic rodent brains before glial cells are generated or from postnatal brains after active separation from glial cells. This can be accomplished by immunopanning (Barres et al., 1988; Ullian et al., 2004; Steinmetz et al., 2006; Buard et al., 2010) or fluorescence-activated cell sorting (Calof and Reichardt, 1984; Tomomura et al., 2001; Pennartz et al., 2004). Finally, neurons can be generated from stem cells by manipulating their differentiation (Jungling et al., 2003; Berninger et al., 2007; Johnson et al., 2007). These methods reach purities of up to 99.5% and thereby establish virtually glia-free conditions. Studies on such neuronal preparations suggest that there is no absolute requirement for glia: in the absence of glia, some neurons form numerous synaptic connections (Fig. 1A) (Steinmetz et al., 2006), whereas others including retinal ganglion cells (RGCs; Nagler et al., 2001; Ullian et al., 2001), motoneurons (Ullian et al., 2004) and cerebellar Purkinje cells (PCs; Buard et al., 2010) form only very few connections (Fig. 1B). In some of these preparations, synapses may be regarded as artificial, as neurons lack their natural partners. However, few synapses were also observed in glia-free cultures of subplate neurons from embryonic rats or mice, which normally form synapses among each other (McKellar and Shatz, 2009). On the other hand, strong glutamatergic synaptic activity was found in glia- (and serum-) free cultures of neurons from superior cervical ganglia prepared from newborn rats (Perez-Gonzalez et al., 2008), from spinal cords of embryonic mice (Cuevas et al., 2005) and from hippocampi and cerebella of postnatal mice (Steinmetz et al., 2006). Together, these results suggest that the requirement for glia varies with the neuronal cell type and its state of differentiation.

Notably, the competence of neurons to form and to receive synapses may develop independently. RGCs immunisolated from embryonic rats were shown to form, but not to receive, synapses and the latter required contact to astrocytes (Barker

Fig. 1 – Influence of glial cells on synapse formation and maturation. The indicated types of neurons form (A) numerous or (B) only few synapses in the absence of glial cells. Depending on the neuronal cell type, glial cells promote the formation, (C) maturation and (D) elimination of synapses via the indicated molecules. Schematic drawings of synapses derived from electron microscopic images with pre- (white) and postsynaptic elements (gray) and processes of perisynaptic glia (red).

et al., 2008). A reverse sequence was observed in neurons that were generated by genetic re-programming of glial fibrillary acid protein-positive cells from cerebellar cortex of postnatal mice. These neurons could receive glutamatergic inputs, but they could not form presynaptic contacts (Berninger et al., 2007). The discrepancy may be due to differences among the neurons under study.

Does synaptogenesis require glial cells in living animals? This can only be addressed in selected invertebrate and vertebrate species, but not in mammals, because genetic ablation of astrocytes in developing and adult mice damages neurons massively (McCall et al., 1996; Cui et al., 2001) thus precluding conclusions about their relevance for synaptogenesis. In *Caenorhabditis elegans*, whose cells are inventoried, four of its 50 glia-like cells, the so-called cephalic sheath (CEPsh) glia, are in contact with dendrites of sensory neurons and with synaptic connections in the nerve ring (Shaham, 2006). Their ablation by optical and genetic methods perturbs the morphology of associated sensory neurons (Yoshimura et al., 2008) and impairs chemotactic behavior (Bacaj et al., 2008) without affecting neuronal survival. Still, it remains unclear, whether these changes are due to reduced synapse formation. Selective and efficient ablation of PSCs in developing and adult frogs has been accomplished by a PSC-specific antibody combined with complement-mediated cell lysis. These experiments revealed that PSCs are required

for the formation and growth of neuromuscular junctions (NMJs) in developing frogs in vivo (Reddy et al., 2003).

5. Glial cells enhance the number of synapses

The aforementioned coincidence of synaptogenesis and glia development implies a causal relation. Direct evidence that glial cells increase the number of glutamatergic synapses was obtained in cocultures of frog spinal cord neurons and myocytes (Peng et al., 2003; Feng and Ko, 2008a) and in cultured rat and mouse RGCs (Pfrieger and Barres, 1997; Ullian et al., 2001; Nagler et al., 2001; Steinmetz et al., 2006), PCs (Buard et al., 2010), motoneurons (Ullian et al., 2004; Feng and Ko, 2008a), neurons derived from human embryonic stem cells (Johnson et al., 2007) as well as cortical (Hu et al., 2007; McKellar and Shatz, 2009) and hippocampal neurons (Tournell et al., 2006; Boehler et al., 2007) from embryonic rats (Fig. 1C). Previous studies showed that astrocytes enhance the number of inhibitory connections among cultured hippocampal neurons from embryonic rats by enhancing axon length and branching (Elmariah et al., 2005) via secreted proteins (Hughes et al., 2010). However, this effect may depend on the specific culture preparation, since the formation of inhibitory synapses in cultures of hippocampal neurons purified from

postnatal mice was not affected by glia (Steinmetz et al., 2006).

It should be noted that glial cells can enhance synaptic connectivity indirectly, by promoting neuronal survival. A glia-induced increase in the number of surviving neurons can explain the enhanced level of synaptic activity (Boehler et al., 2007). Other studies have excluded this possibility (Pfrieger and Barres, 1997; Ullian et al., 2004; Elmariah et al., 2005; Steinmetz et al., 2006; McKellar and Shatz, 2009). Moreover, glial signals may enhance the neuronal ability to receive synaptic inputs by promoting the growth or differentiation of axons and dendrites. Effects on dendrites have been observed in stem cell-derived neurons (Johnson et al., 2007), in RGCs (Goritz et al., 2005) and in PCs (Buard et al., 2010).

Finally, glial cells have been shown to impede synapse formation, for example by preventing physical contact between neurons. A shortening of Bergmann glia processes by expression of dominant negative forms of Rac1 or RhoG reduced the glial ensheathment of PCs and enhanced the number of parallel fiber inputs (Lippman et al., 2008). A similar effect was observed after experimentally induced retraction of Bergmann glia processes due to overexpression of a specific glutamate receptor subunit: this caused an increase in the number of climbing fiber inputs to PCs (Iino et al., 2001).

6. Glial cells promote synapse maturation

Once a synaptic contact is established, it probably undergoes a maturation phase, during which it attains its characteristic transmission properties (Fig. 1C). So far, the mechanisms of this process are largely unknown, but there is evidence that it is influenced by glial cells. In single-cell microcultures of neurons from rat superior cervical ganglia, Schwann cells were dispensable for the formation of cholinergic connections, but they enhanced the frequency of action potential-independent release and paired-pulse depression (Perez-Gonzalez et al., 2008). This points to a glial enhancement of presynaptic release efficacy, which has also been observed in cultured RGCs (Nagler et al., 2001; Christopherson et al., 2005; Goritz et al., 2005) and PCs (Buard et al., 2010). In addition, glial cells appear to augment the postsynaptic sensitivity of neurons. They increased the size of miniature excitatory postsynaptic currents in cultures of purified hippocampal neurons (Steinmetz et al., 2006), cortical subplate neurons (McKellar and Shatz, 2009), RGCs (Nagler et al., 2001; Ullian et al., 2001; Steinmetz et al., 2006) and spinal cord motoneurons (Ullian et al., 2004). How can glia affect the postsynaptic sensitivity to glutamate? Recent studies start to provide answers: Transcript profiling of cultured neurons revealed that glial factors upregulate mRNAs encoding for glutamate receptor subunits and for components that regulate their clustering and stability (Goritz et al., 2007; McKellar and Shatz, 2009).

7. Signals and mechanisms mediating the glial influence on synaptogenesis

Within the last years, we have learnt more about the molecules and mechanisms that mediate the influence of glial cells on synapse development (Fig. 1).

So far, several molecules have been identified that mediate the glial influence on synapse development. A genetic screen for abnormal synapse distribution in *C. elegans* revealed that contact establishment between interneurons requires the netrin receptor/UNC-40 on axons of the presynaptic cell and its ligand netrin/UNC-6 on CEPsh glia (Colon-Ramos et al., 2007). A netrin-dependent function of CEPsh glia as guideposts may explain why growth and branching of axons and dendrites fail following the elimination of these cells (Yoshimura et al., 2008).

A candidate approach uncovered transforming growth factor beta 1 (Tgfb1) as one of the factors by which Schwann cells promote the formation of NMJs in vitro (Feng and Ko, 2008a), albeit indirectly. The authors showed that Tgfb1 mediates the previously reported increase in the neuronal agrin level (Peng et al., 2003), which in turn controls the expression and clustering of nicotinic acetylcholine receptors. Tgfb1 did not mimic the potentiation of spontaneous transmitter release from motoneuron terminals observed after acute application of Schwann cell-conditioned medium (Cao and Ko, 2007) indicating the involvement of additional factors. Schwann cell-derived neuregulin 2 has been shown to stimulate transcription of nicotinic acetylcholine receptors by activation of the ErbB4 receptor (Rimer et al., 2004; Ponomareva et al., 2006).

In the CNS, thrombospondin was identified as an astrocyte-derived matrix component that promotes the formation of ultrastructurally normal, but postsynaptically silent synapses in cultured RGCs. Transgenic mice lacking thrombospondins showed a reduced density of immunohistochemically identified synapses in the cortex (Christopherson et al., 2005). Recently, the Barres group identified the receptor mediating the synaptogenic effect of thrombospondin as alpha2/delta 1 protein (Cacna2d1), an accessory subunit of voltage-activated calcium channels (Eroglu et al., 2009). The involvement of this protein in synapse development is further confirmed by recent data on *Drosophila*, where its absence disturbs the formation of synaptic boutons by motoneurons (Kurshan et al., 2009). Recent studies also suggest that thrombospondins promote neurite outgrowth in cultured RGCs and enhance their expression of synaptic and dendritic components (Wang et al., 2009), possibly by acting as matrix organizers (Dunkle et al., 2007). Parallel studies on cultured RGCs identified glia-derived cholesterol as a component that promotes different aspects of synapse development (Mauch et al., 2001) including the differentiation of dendrites and the efficacy and stability of presynaptic transmitter release (Goritz et al., 2005). Our finding that neurons produce cholesterol less efficiently than glial cells (Nieweg et al., 2009) reinforces the idea that they depend on import of cholesterol via lipoproteins (Pfrieger, 2003). Neurons may convert glia-derived cholesterol to steroids, which then promotes synapse formation. A recent study on organotypic hippocampal cultures shows that cholesterol or testosterone, but not estradiol enhanced the density of spine synapses by 40%, whereas pharmacologic inhibition of steroid synthesis reduced synapse numbers (Fester et al., 2009). Other studies on cultured cells imply that astrocytes produce and release estradiol, which in turn enhances neurite growth and promotes synapse number and function (Chen et al., 2007a; Hu et al., 2007). The discovery of

other synaptogenic signals from glia may be facilitated by a recent study, which identified proteins secreted by astrocytes using shotgun proteomics (Dowell et al., 2009).

There is also evidence that contact between neurons and astrocytes promotes synapse development. A recent study revealed that γ -protocadherins are located in perisynaptic processes of astrocytes in the spinal cord of neonatal and adult mice. Absence of γ -protocadherins in astrocytes led to a transient decrease in synapse density among spinal cord neurons in vitro and in vivo without affecting neuronal survival (Garrett and Weiner, 2009). Moreover, contact of cultured hippocampal neurons to astrocytes increased the number of synapses possibly by activation of integrins and protein kinase C (Hama et al., 2004). As described above, RGCs from embryonic day 17 rats were shown to receive synaptic inputs, when contacted by astrocytes, possibly by changes in the subcellular distribution of neuroligins (Barker et al., 2008). In both cases, however, the astrocytic signals that initiate these changes remain unknown.

Unexpected mechanisms of glia-induced synapse development have been uncovered by transcriptional profiling of purified neurons. Three recent reports have proven the validity of this approach (Goritz et al., 2007; Stevens et al., 2007; McKellar and Shatz, 2009). Studies on cultured RGCs revealed glia-induced upregulation of components that had not been discussed in a neuronal context. This included a component of the complement system, which contributes to synapse elimination (see below; Stevens et al., 2007) and matrix gla protein (Mgp; Goritz et al., 2007), a component of the extracellular matrix, which may regulate signaling by members of the Tgf family (Moon and Birren, 2008). So far, the glial signals that mediate transcriptional changes remain unknown.

An exciting idea is that the ability of astrocytes to promote synapse development is controlled by neurons. Although the evidence supporting this view comes mainly from studies on the growth-promoting activity of astrocytes, similar mechanisms may apply to their synaptogenic action. Activation of purinergic receptors in cultured astrocytes appeared to control the expression and release of synapse-promoting thrombospondin (Tran and Neary, 2006) and the release of protease nexin-1, which stimulates neurite growth, was regulated by G protein-coupled receptors (Giau et al., 2005). Progesterone treatment enhanced the astrocytic production of agrin (Tourneil et al., 2006) and lysophosphatidic acid (Guizzetti et al., 2008), whereas acetylcholine (de Sampaio et al., 2008) enhanced the ability of astrocytes to promote neuronal differentiation and growth. A study on hippocampal cultures from embryonic rats reported that blocking spontaneous calcium transients in astrocytes reduced axonal and dendritic growth, possibly by reducing astrocytic levels of the cell adhesion molecule N-cadherin (Kanemaru et al., 2007). Notably, cultured astrocytes lacking MeCP2 failed to support neuronal differentiation compared to wild-type cells (Ballas et al., 2009) suggesting that transcription factors control the growth-promoting effects of astrocytes.

Neurons may harbor signals that modulate astrocyte properties, but non-neuronal cells may also participate in this regulation. An example has been reported recently in vitro (Au et al., 2007). The authors showed that olfactory ensheath-

ing glia prepared from embryonic mice released osteonectin/secreted protein acidic rich in cysteine. This factor in turn enhanced the ability of Schwann cells to promote neurite outgrowth from explants of dorsal root ganglia from mouse embryos via secretion of Tgfb. These data provide a first glimpse that the influence of astrocytes on neuronal differentiation probably requires cooperative actions of multiple signals, whose cellular sources and molecular targets remain to be clarified.

8. Glial cells affect the turnover of synapses

Once formed, synapses last for a limited time period. In vivo two-photon imaging of fluorescently labeled neurons revealed that the lifetime of spines, and probably their associated synapses, ranges from minutes to hours during postnatal development and from days to months in the adult brain (Bhatt et al., 2009). Whereas controlled synapse turnover is required for brain development and plasticity, uncontrolled synapse loss may spearhead pathological changes in brain diseases (Walsh and Selkoe, 2007; Knobloch and Mansuy, 2008).

Recent studies suggest that glial cells influence the stability of synaptic connections (Fig. 1D). Simultaneous time-lapse imaging of fluorescently labeled astrocytes and dendritic spines in organotypic hippocampal cultures revealed that contact to astrocytes enhances the lifetime of dendritic protrusions and promotes their conversion to spines. This effect was possibly mediated by astrocytic ephrin-A3 and dendritic EphA4 receptors (Nishida and Okabe, 2007). Along this line, ablation of PSCs in adult frogs in vivo caused long-term loss of NMJs (Reddy et al., 2003) indicating that the maintenance of these connections also depends on the glial sheath. There is also evidence for the opposite effect, namely that glial cells promote synapse elimination (Fig. 1D). A striking mechanism has been uncovered in cultures of purified RGCs (Stevens et al., 2007). Coculture with glial cells strongly enhanced the neuronal level of C1q, a component of the complement cascade (Perry and O'Connor, 2008), which appeared to be present at synapses. Mice lacking C1q or C3, another complement factor, showed defects in the eye-specific segregation of synaptic inputs to the lateral geniculate nucleus (Stevens et al., 2007). These observations suggest that astrocytes command the onset and extent of synapse execution.

Synapse destruction involves removal of axonal branches that form obsolete connections. In the peripheral nervous system, motoneurons form supernumerary NMJs, most of which are destroyed postnatally (Sanes and Lichtman, 1999). Repeated imaging of NMJs in living transgenic mice combined with electron microscopy revealed that axon remnants, so-called axosomes, end up in PSCs (Bishop et al., 2004). Moreover, elimination of unwanted NMJs and climbing fiber inputs to cerebellar PCs was accompanied by enhanced lysosomal activity in glial cells (Song et al., 2008). Whether glial cells play an active or passive role in this process remains unclear.

Direct evidence for a glial contribution to axon—and thereby synapse—removal comes from studies on the

olfactory system of flies, which undergoes substantial remodeling during metamorphosis (Jefferis et al., 2002). Selective labeling of neurons and glial cells in the mushroom body and genetic disturbance of glial membrane function revealed that the pruning of axons is mediated by glial cells expressing draper and related receptors (Awasaki et al., 2006; Hoopfer et al., 2006; MacDonald et al., 2006; Kurant et al., 2008; Doherty et al., 2009; Fig. 1D).

9. Summary and Outlook

The experimental results summarized above support the idea that neurons require help from glia to form and maintain proper synaptic connections. The evidence still relies largely on in vitro studies, and it remains important to determine whether and how glial cells instruct synapse development in vivo. This will require the identification of the signaling pathways that mediate the glial influence on synapses and their cell-specific manipulation in vivo. In this review, I have focused on how glial cells influence synapses. A key question to be addressed by future studies is whether the reverse is true, i.e., whether synapses instruct the differentiation of the glial processes surrounding them. Given recent technological advances research on glia–synapse interactions will progress on three “techno-tracks” (Pfrieger, 2009). First, advanced genetic and proteomic approaches will divulge the molecular setup of glial cells in contact with synapses. Second, new imaging techniques enable the visualization of glia–synapse interactions and third, new transgenic animals allow for temporally controlled manipulation of glia in vivo. Armed with this arsenal of new tools, we should now be able to find out whether the coincidence of glia development and synaptogenesis is mere chance or bare necessity.

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