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Sculpting the nervous system: glial control of neuronal development

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Glial cells are not passive spectators during nervous system assembly, rather they are active participants that exert significant control over neuronal development. Well-established roles for glia in shaping the developing nervous system include providing trophic support to neurons, modulating axon pathfinding, and driving nerve fasciculation. Exciting recent studies have revealed additional ways in which glial cells also modulate neurodevelopment. Glial cells regulate the number of neurons at early developmental stages by dynamically influencing neural precursor divisions, and at later stages by promoting neuronal cell death through engulfment. Glia also participate in the fine sculpting of neuronal connections by pruning excess axonal projections, shaping dendritic spines, and secreting multiple factors that promote synapse formation and functional maturation. These recent insights provide further compelling evidence that glial cells, through their diverse cellular actions, are essential contributors to the construction of a functionally mature nervous system.

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Introduction

How important are glial cells during nervous system development? Though they are the most abundant cells in the mammalian central nervous system (CNS), glia are considered to be of secondary importance to their neuronal neighbors. But glial cells have intermittently revealed to us the richness of their biological functions, and are steadily making their case as important regulators of nervous system assembly. An extensive body of work has established roles for glial cells in providing trophic support essential for neuronal survival and ensheathing axons to drive nerve formation [1–6]. Numerous studies have also demonstrated key requirements for glial cells in guiding specific steps in axon pathfinding including midline crossing, outgrowth along developing axon scaffolds, and target layer selection within the CNS [7–12]. This review focuses on recent studies that highlight additional mechanisms by which glial cells control neuronal development. Glial cells regulate the spatiotemporal production of neurons by dynamically modulating neural precursor divisions, and later destroy excess neurons to reduce selected neuronal populations. After axon pathfinding, glia sculpt neuronal connections by pruning excess axons, modulating dendrite morphology and secreting factors that promote synaptogenesis. Through this diversity of cellular actions glial cells function as important regulators of nervous system development.

Neural stem cell proliferation is dynamically regulated by glia

Precise control of neural stem cell proliferation is essential for the correct spatiotemporal production of neurons and glia during nervous system development. In the *Drosophila* larval and pupal brain, neuroblasts (NBs) function as neural stem cells and produce the majority of neurons and glia that will populate the adult brain after metamorphosis. These NBs are born at the end of embryonic development but are quiescent until the second larval instar [13]. Glial cells are responsible for keeping these NBs quiescent during early larval stages. Larval brain glia surrounding NBs express the glycoprotein encoded by *anachronism* (*ana*), which when secreted functions to suppress NB divisions. Loss of Ana function relieves this repression and causes brain NBs to precociously enter S phase during the first larval instar rather than during later stages [14].

During later larval stages, when NBs begin generating progeny in earnest, glial cells promote NB divisions. Surface glia surrounding the larval brain are in close contact with proliferating NBs. *Drosophila* E cadherin (DE-cadherin) is expressed in these glial cells, and it is also expressed in NBs and their progeny [15]. Interestingly, selective elimination of DE-cadherin function from glial cells by expression of a dominant negative DE-cadherin construct results in highly reduced NB mitotic activity [16]. Thus, DE-cadherin functions in glial cells to promote local NB proliferation. Taken together, these studies suggest that *Drosophila* larval brain glia provide a niche for NBs, and show that these glia can either negatively or positively regulate NB divisions, depending upon the developmental stage.

Temporal specification of sensory organs is modulated by glia

The zebrafish posterior lateral line (pLL) is an excellent model system in which to dissect neuron–glia interactions

during development of peripheral sensory tissues [17]. During early pLL development a primary neuromast primordium (primI) is derived from cranial placodes; the primI then migrates along the horizontal myoseptum (the future lateral line) and deposits small clusters of cells along its migratory path that will ultimately form primary neuromasts. Neuromasts are mechanosensory structures that detect water movement over the body, providing sensory information to the CNS by synapsing onto pLL neurons, which in turn project to the hindbrain. Formation of the lateral line nerve occurs after primI migration, with axonal growth cones also extending along the horizontal myoseptum. Glial cells closely follow pLL axon growth cones, and are required for proper fasciculation of the pLL nerve [5]. Secondary neuromasts are subsequently generated during zebrafish growth, and are produced from a population of latent precursors that lay adjacent to the pLL nerve [18•]. Secondary neuromasts begin to differentiate only upon migration away from pLL nerve, suggesting that nerve contact suppresses their development. Three mutants were recently identified that possess twice the normal number of neuromasts at early developmental stages; all of these mutants lacked pLL glia. Moreover, blocking or delaying glial migration along the pLL nerve (and, therefore, glial-latent neuromast precursor contact) resulted in the production of excess neuromasts [18•]. Thus, pLL glia appear capable of negatively regulating secondary neuromast differentiation, and thereby controlling the temporal addition of new peripheral sensory organ structures during zebrafish growth.

Glia promote neuronal cell death through engulfment

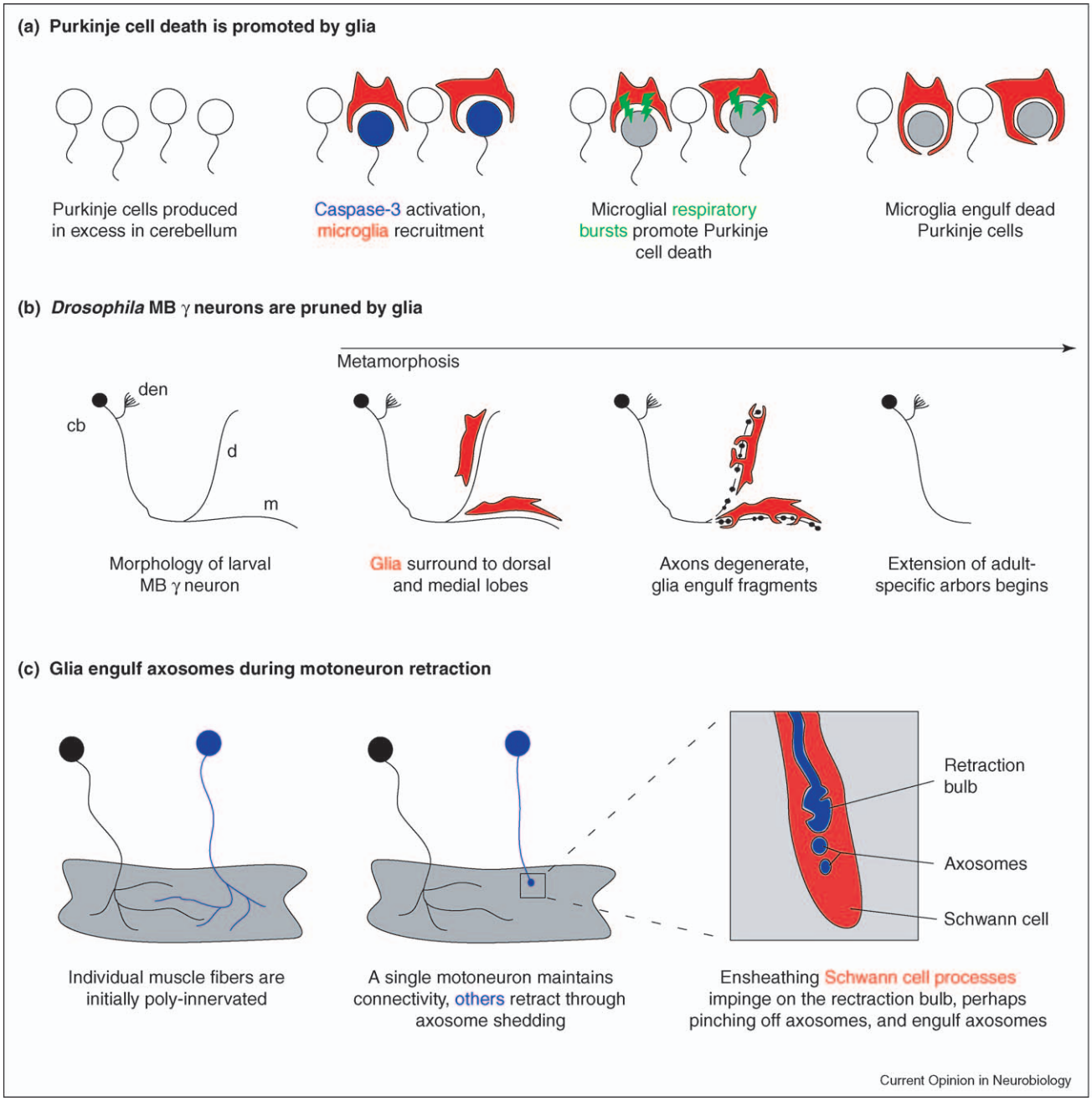
Activation of programmed cell death in subsets of neurons is a common mechanism for reducing neuronal populations to appropriate levels. Glial cells are responsible for engulfing neuronal cell corpses in the nervous system, and apparently help to drive the destruction of some neurons. In the developing cerebellum a large number of Purkinje cells (PCs) express activated caspase-3 (Figure 1a; [19••]) and undergo apoptotic death [20,21]. Activation of caspase-3 is an early indicator that the apoptotic program of cell destruction has been engaged, but not irreversibly. For example, activation of the *C. elegans* caspase-3 molecule CED-3 leads to morphological changes associated with apoptosis, but if the action of engulfing cells is blocked many CED-3 activated cells can recover and escape death [22,23]. Approximately 64% of caspase-3 activated PCs in the developing cerebellum were found partially engulfed or in close contact with microglial processes. PC-engulfing microglia exhibit respiratory bursts similar to those used by phagocytes to destroy cells targeted for engulfment, suggesting that microglia actively destroy caspase-3 activated PCs. Strikingly, selective ablation of microglia in cerebellar slice preparations led to a strong reduction in overall levels of PC apoptosis [19••]. Thus, in the absence of microglia many PCs that would normally undergo cell death survive. Taken together, these observations argue strongly that engulfing microglia actively promote destruction of caspase-3 activated PCs. Do microglia play an instructive role in determining which PCs will be destroyed? Because one-third of PCs expressing activated caspase-3 were not found in contact with microglial processes, PC initiation of apoptosis might be autonomous and microglial engulfment could function to finalize the destructive event [19••]. However, microglia are highly dynamic cells that continuously extend and retract processes throughout the CNS [24], and transient microglia–PC contact could be sufficient to initiate caspase-3 activation. Future work aimed at imaging dynamic PC–microglial interactions during the thinning of PC populations and identifying the molecular machinery mediating communication between these cell types should help to determine the precise role of glia in PC destruction.

Glia sculpt axonal connections through developmental axon pruning

A common feature in the development of neural circuits is the excessive innervation of target cells, followed by the selective elimination of excess synapses or axon branches to establish an appropriate final map of connectivity. Such mechanisms enable the refining of neuronal connections after initial axon pathfinding has taken place. In some situations process elimination entails the simple 'resorption' of axon projections or synapses, in more extreme cases entire axon branches undergo fragmentation. For example, *Drosophila* mushroom body (MB) γ neurons form larval-specific dorsal and medial branches that are pruned during early metamorphosis [25]. During pruning, dorsal and medial branches of MB γ neurons become physically separated from other portions of the axon and undergo extensive fragmentation [26]. Similar types of fragmentation-based axon pruning have been described in the mammalian CNS [27], and have recently been observed using *in vivo* two-photon time-lapse microscopy of GFP-labeled transgenic mice [28]. It is not currently known how particular axon branches are specified for pruning, nor which mammalian cell types engulf pruned axons.

In *Drosophila*, glial cells have a central role in developmental axon pruning events. Both high resolution electron microscopic (EM) studies with a genetically encoded marker for MB γ neurons [29••] and confocal analysis with fluorescence-based markers [30••] revealed that glial cells invade the region of the brain harboring degenerating MB γ neuron axons, and glia engulf fragmenting axonal material (Figure 1b). Selectively blocking glial endocytosis suppresses glial infiltration into this brain region and dorsal and medial branches of MB γ neurons are not removed [30••]. It has been proposed that *Drosophila* glia play an active role in driving axon fragmentation, rather

Figure 1



Glial cells can reduce neuronal populations during development and help to shape axonal projections after initial wiring. **(a)** Glial cells promote Purkinje cell (PC) death in the developing cerebellum. Purkinje cells are initially produced in excess, but a subset activate caspase-3 (blue) and are associated with microglia (red). Microglia exhibit respiratory bursts (green) that are thought to promote PC destruction. Microglia subsequently engulf PC corpses thereby reducing the total number of PCs in the cerebellum. **(b)** Glial cells engulf pruned mushroom body γ neuron axons in *Drosophila*. Dorsal (d) and medial (m) axon branches of mushroom body (MB) γ neurons are pruned during metamorphosis. Glial cells (red) are recruited to mushroom body axons, where the axons subsequently undergo fragmentation, and glial cells engulf the axonal debris. Later in metamorphosis adult-specific axonal projections are articulated. Abbreviations: cb, cell body; den, dendrites. **(c)** Axosomes shed at the neuromuscular junction are engulfed by Schwann cells. Muscle fibers are initially poly-innervated at early developmental stages, but only a single axon maintains connectivity and excessive axonal processes (blue) undergo retraction. Retraction occurs through axosomal shedding, a process by which small organelle-rich membrane bound vesicles pinch off from the distal end of the retraction bulb. Shed axosomes are engulfed by Schwann cell processes, but also might be generated by Schwann cells as they impinge heavily on the retracting axon.

than acting as simple scavengers that engulf axonal debris [30,31]. Consistent with this idea, glial processes are recruited to MB γ neuron axons before their fragmentation [29••,30••], and glial invasion is not absolutely dependent upon axon fragmentation [29••]. In addition, inhibiting glial endocytosis has been reported to suppress the fragmentation of axons normally targeted for pruning [30••]. However, entire axons bundles were imaged in these studies and it, therefore, remains unclear whether the dorsal and medial axon branches of MB γ neurons remain intact when glial endocytosis is blocked, or if they indeed fragment with only their engulfment by glia being suppressed. Dorsal and medial branches remaining intact would suggest that glial signals are essential to initiate axon destruction, whereas if only glial-engulfment were suppressed it would suggest that axon destruction is cell autonomous. Incisive experiments in which glial engulfment of axons is blocked while axon morphology is visualized with single-cell resolution will be essential to resolve these issues. Nevertheless, these studies highlight an essential role for glial cells in developmental axon pruning.

Glia engulf axosomes shed at the neuromuscular junction

Selective elimination of axons is also essential for the fine sculpting of synaptic connections at the vertebrate neuromuscular junction (NMJ). This type of axon elimination appears morphologically distinct from the degeneration-based pruning observed in the CNS of *Drosophila* and mammals [27,28], but glial cells are again important for axon retraction. When motoneurons first project axons to developing muscle fields, individual muscle fibers normally receive inputs from multiple neurons. During early postnatal development these axons compete for synaptic space on the muscle fiber. Ultimately, only a single axon wins this battle and maintains connectivity while the remaining axons are eliminated [32,33]. The axon to be eliminated forms a characteristic retraction bulb, and the predominating view has been that retracting axons simply withdraw and shuttle their resorbed contents to other axonal branches. However, Bishop *et al.* [34••] have recently shown, using an elegant combination of time-lapse imaging of fluorescently labeled axons and serial electron microscopic (EM) reconstructions, that axon retraction entails a novel mechanism whereby axons shed small organelle-rich membrane bound remnants termed 'axosomes' (Figure 1c; [34••]). Interestingly, Schwann cells might be a driving force behind the formation of axosomes. Axosomes always form distal to the retraction bulb, and small non-fluorescent fingers impinge on retraction bulbs and cause dramatic shape changes that might underlie the 'pinching off' of new axosomes. These processes are GFP-negative (and hence non-neuronal), but positive for S100, a marker for Schwann cells. Serial EM reconstructions confirmed the identity of retraction bulb- and axosome-surrounding processes as those from Schwann cells. Future experiments in which specific glial functions such as engulfment are blocked should unravel the precise role for glia in axosome formation and metabolism, and axon retraction at the NMJ.

Bishop *et al.* [34••] also noticed that axon-derived GFP was found within the Schwann cell cytoplasm, suggesting that axosome engulfment might lead to the contribution of axosome components directly into the glial cell cytoplasm. Such an event would provide a new mechanism for neuron to glia signaling through cytoplasmic transfer, and potentially enable glia that had interacted in this way with neurons to acquire new cellular machinery from neurons [34••].

Glia promote synapse formation and functional maturation

A final step in assembling neural circuits is the formation of synaptic contacts. How do neurons decide when and where to form synapses? During embryonic development of the mammalian brain, neurons are first generated in large numbers but they form very few synapses. Subsequently, during early postnatal development, massive numbers of astrocytes are produced throughout the brain and a major wave of CNS synaptogenesis follows. Accumulating evidence suggests that an underlying cause of this delay in synapse production until postnatal stages might be a lack of glial-derived synaptogenic factors.

Significant insight into the role of glia in synaptogenesis has come from studies of neuron–glia interactions in tissue culture (Table 1). Purified retinal ganglion cells

Table 1

Secreted glial factors regulate synapse formation, functional maturation and efficacy.

Condition or glial factor	Effect
Astrocyte feeding layers	Promote assembly of pre- and post-synaptic terminals, promote acquisition of post synaptic AMPA receptor-mediated responses and enhance synaptic efficacy [39,40••,44]
Astrocyte-conditioned medium	Promotes assembly of pre- and post-synaptic terminals, and post-synaptic AMPA receptor-mediated responses are absent [44]
Thrombospondins (TSP)	Promote assembly of pre- and post-synaptic terminals, and post-synaptic AMPA-receptor mediated responses are absent [44]
Cholesterol	Enhances synaptic efficacy [44,47]

(RGCs) cultured below an astrocyte feeding layer produce ~7-fold more functionally mature synapses than RGCs cultured in the absence of glia [35–37]. The lack of contact between the RGCs and the glial feeding layers indicates that soluble glial-derived factors promote the formation of functionally mature synapses. Similarly, acutely isolated rat spinal motorneurons form few synapses unless cultured in the presence of Schwann cells or astrocytes [38], and Schwann cell-conditioned medium can promote acetylcholine receptor clustering at neuromuscular junctions when *Xenopus* spinal motoneurons are co-cultured with muscle cells [39]. Thus, glial cells can promote synaptogenesis in diverse neuronal cell types, and might, therefore, exert broad control over synapse formation in the nervous system.

Christopherson *et al.* [40••] compared astrocyte conditioned medium (ACM) and astrocyte feeding layers in terms of their ability to promote synaptogenesis. ACM was found to induce RGC synapse formation at levels similar to those at which glial feeding layers could induce RGC synapse formation, and through an elegant series of fractionation experiments the authors identified thrombospondins (TSPs) as the glial-derived synaptogenic factor. Purified TSP1 was shown to mimic the ACM-induced increase in synapses in RGC cultures, and immunodepletion of TSP2 from ACM blocked its synapse-promoting activity [40••]. Using an antibody that recognizes both TSP1 and TSP2 (TSP), TSP immunoreactivity was found on astrocytic processes throughout the developing brain during the window of postnatal development when synapses form in large numbers. However, TSP levels are significantly lower in the adult brain when neurons have a reduced capacity to generate new synapses. Strikingly, loss of TSP function reduces CNS synaptogenesis *in vivo*: day 8 postnatal mice carrying null mutations in both TSP1 and TSP2 show a 40% reduction in synaptic puncta compared with the number in wild type controls. These data indicate that TSPs are the synaptogenic molecule present in ACM, and are the first to demonstrate an *in vivo* role for glial secreted factors in CNS synaptogenesis. Taken together, this work suggests that astrocytic TSP expression might define a window of developmental time during which CNS neurons can robustly produce synapses.

In addition to TSPs, other factors are released by astrocytes that enhance efficacy or promote the functional maturation of synapses. In contrast to the functionally mature synapses found when RGCs are cultured with glial feeding layers, TSP- and ACM-induced RGC synapses are presynaptically active, but post-synaptically silent, lacking their normal AMPA receptor-mediated response [40••]. Therefore, an additional unidentified factor is released by glial feeding layers that promotes acquisition of postsynaptic AMPA receptor-mediated responses. Similar AMPA 'silent synapses' are found in abundance in the neonatal brain [41,42]. Do glial factors normally drive their functional maturation *in vivo*? Additionally, long-term potentiation (LTP) induces an AMPA receptor-dependent increase in synaptic strength [43,44]. This raises an intriguing question; could glial-derived factors play a role driving postsynaptic changes in AMPA receptor levels during LTP or learning and memory?

Glial control of dendritic spine morphology

Glia can exert strong inhibitory control over postsynaptic structures. In the adult mouse hippocampus postsynaptic dendritic spines are highly dynamic elements [45] and are the major site of innervation by excitatory presynaptic terminals. The receptor tyrosine kinase EphA4 is expressed on the dendritic spines of hippocampal pyramidal neurons. Ephrin-A3, a ligand for this receptor, is expressed on surrounding glial processes that delineate spine boundaries. EphA4 is not constitutively associated with the ligand, but receptor activation drives spine retraction, and inhibiting EphA4 interactions with ligands results in disorganized spine morphology [46]. Thus, dendritic spine morphology might be established, and dynamically regulated, by local repulsive interactions between spines and surrounding glia.

Conclusions

Interest in glial cells has increased dramatically in recent years and we are working our way towards a deeper understanding of the essential roles of this enigmatic cell type in the developing nervous system. The closer we look at glia, the more surprised we become by the richness of their biology, and the many ways this 'neuronal support cell' can potently influence neuronal development and function. Though glial cells are emerging as essential participants in nervous system morphogenesis, it remains unclear whether glial cells act instructively, or simply respond to neuronal developmental programs. Do glia influence which axons will be pruned? Do glia direct synapse formation, or simply secrete permissive factors? We are also largely ignorant of the molecular pathways mediating most neuron–glia interactions. How do glia recognize caspase-3 activated PCs, or axons targeted for pruning? What molecular pathways drive glial engulfment of pruned axons? How do glial TSPs drive synaptogenesis? Delineating the molecular mechanisms underlying neuron–glia interactions will be essential to answer these questions, and could eventually provide new insights into nervous system disease. For example, could glial cells promote neurodegenerative disease by inappropriately pruning axons? Finally, although the studies discussed above provide compelling evidence that glial cells regulate neuronal development in important ways, it remains unclear how broadly these neuron–glia interactions occur throughout the developing nervous system. Future work aimed at generating new tools to manipulate glial subtypes or specific cellular functions, and the use of these tools to look broadly at neuron–glia

interactions during nervous system development, is essential. If the past is any indicator, we can expect that such approaches will reveal additional unexpected roles for this dynamic cell type in nervous system morphogenesis.

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This seminal work shows that glial cells secrete multiple factors that drive the stepwise assembly of a functionally mature synapse. Thrombospondins (TSPs) are identified as glial secreted proteins that drive the formation of morphologically normal, but post-synaptically silent synapses *in vitro*. Double mutants lacking TSP1 and TSP2 function exhibit reduced synapse formation *in vivo*, and compelling evidence is provided that TSPs define a window in developmental time when synapse formation can occur. A second (yet to be identified) factor secreted by glial feeding layers is also shown to be essential for postsynaptic acquisition of AMPA receptor-mediated responses.

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