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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of postsynaptic AMPA receptors in stabilizing presynaptic inputs

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Beth Ann Ripley

Committee in charge:

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The dissertation of Beth Ann Ripley is approved, and
it is acceptable in quality and form for publication on
microfilm:

Chair

University of California, San Diego

2008

DEDICATION

For Nathan Tiffany

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LIST OF ABBREVIATIONS

1. AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
2. NMDA: N-methyl D-aspartate
3. CNS: central nervous system
4. GFP: green fluorescent protein
5. CFP: cyan fluorescent protein
6. Synaptophysin-GFP: Syn-GFP
7. DIV: day in vitro
8. f.o.v.: field of view
9. PSD: postsynaptic density
10. KO: knockout
11. WT: wildtype
12. VGLUT: vesicular glutamate transporter
13. MAP2: microtubule-associated protein 2
14. LGN: lateral geniculate nucleus
15. NMJ: neuromuscular junction
16. AChR: acetylcholine receptor
17. NTD: N-terminal domain
18. CTD: C-terminal domain

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Parts of this thesis will be prepared for submission for publication. The dissertation author will be the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

The Role of Postsynaptic AMPA Receptors in Stabilizing Presynaptic Inputs

by

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Doctor of Philosophy in Neurosciences

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The establishment of functional neuronal circuits relies on the formation of excess synapses, followed by the elimination of inappropriate connections. Although the stabilization of presynaptic inputs is critical for the development of functional circuits, the signals that regulate presynaptic stability are not known. Here we report that synapse formation in cortical cultures is highly dynamic and involves the formation and elimination of synapses at a high rate. During the peak of synaptogenesis, only approximately 50% of putative synapses are stable over an hour. The stability of presynaptic inputs is strongly correlated with the presence of postsynaptic AMPA but not NMDA receptors. Removal of postsynaptic AMPA receptors leads to a decrease in the absolute number of excitatory presynaptic inputs, as well as in the fraction of synaptic contacts that are stabilized. Overexpression of AMPA receptors increases excitatory presynaptic input number and overexpression of AMPA receptors along with neuroligin-

1 in 293T cells is sufficient to stabilize presynaptic inputs onto heterologous cells. The ability of AMPA receptors to stabilize presynaptic inputs is not dependent on receptor activity and instead relies on structural interactions mediated by the N-terminal domain of the AMPA receptor subunit GluR2. These observations indicate that an AMPA receptor-associated signal functions as a retrograde signal to regulate presynaptic stability.

CHAPTER 1: INTRODUCTION

The establishment of functional neuronal circuits relies on the formation of excess synapses followed by the elimination of inappropriate connections. This strategy of synaptic exuberance followed by pruning is maintained from the level of the neuromuscular junction all the way up to sensory processing areas of the cortex. In contrast to a true combinatorial strategy in which each correct pre and postsynaptic partnership is predestined by hard-wired cues, the strategy of sampling several potential partnerships and only maintaining a subset based on ever-changing information shared between those partners allows for great plasticity during wiring. Although it is becoming increasingly clear that patterned activity is integral to synaptic stabilization and elimination, little is known about the molecular events downstream of activity that lead to structural changes in connectivity. Further, it is unclear what the timetable is for stabilization/elimination of individual synapses. Understanding the mechanisms leading to synapse stabilization is crucial to understanding how the brain is wired during development. Failure to stabilize meaningful inputs, as well as failure to eliminate incorrect inputs, is likely to underlie numerous developmental disorders.

1.1 Synapse induction

Synapses are the sites of information transfer in the nervous system. Functional synapses are established through an intricate series of events resulting in the juxtaposition of presynaptic vesicles with appropriate postsynaptic receptors. The presynaptic side of the synapse is composed of scaffolding proteins that make up the active zone as well as synaptic vesicles that contain neurotransmitter. In response to calcium influx triggered by

an action potential, one or a few of these vesicles fuse with the membrane and release neurotransmitter into the synaptic cleft. This neurotransmitter is sensed by a complement of receptors positioned within the membrane of the postsynaptic side, or postsynaptic density (PSD). This leads to influx/efflux of ions in the case of ionotropic receptors or second messenger signaling in the case of metabotropic receptors.

Glutamate receptors underlie the vast majority of transmission at excitatory synapses. There are three main types of glutamate receptors: AMPA, kainite and NMDA. AMPA receptors mediate fast synaptic transmission through the influx of sodium ions, while NMDA receptors serve a modulatory role through the slower influx of calcium, leading to downstream signaling events. Electrophysiological and immunohistochemical experiments suggest that a large percentage of young synapses contain NMDA receptors but lack AMPA receptors (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Li and Zhuo, 1998; Isaac, 2003). These synapses are known as “silent synapses” and are incapable of signaling at the resting potential of cells due to a voltage-dependent magnesium block of NMDA receptors. These synapses are unsilenced by an activity-dependent insertion of AMPA receptors into the PSD.

How are excitatory synapses formed? Growing evidence suggests that synapse induction is a mutual process between presynaptic and postsynaptic partners, rather than an instructive process driven solely by one side of the synapse. This can be inferred from the well-coordinated structure of the pre and postsynaptic side, which change almost in unison in response to activity (Shepherd and Harris, 1998; Kasai et al., 2003; Fox and Umemori, 2006). This suggests that the presynaptic side must form and shape the postsynaptic side and the postsynaptic side must, in turn, form and shape the presynaptic

side. This bi-directional flow of information is likely necessary for target recognition, synapse induction, synapse maturation, and possibly even synapse stabilization and elimination. In this section, I will focus on evidence that trans-synaptic signaling is essential for proper induction of synapses. I will discuss evidence for this type of signaling in two separate synapses, the motor axon to muscle synapse in the neuromuscular junction (NMJ) as well as the neuron to neuron synapse in the cortex.

Postsynaptic induction at the neuromuscular junction

The NMJ has been studied for more than a century, and the bulk of what we understand today about synaptogenesis has been elucidated from studies of this structure (Sanes and Lichtman, 1999). The NMJ is the site where incoming motor axons, target muscle fibers and schwann cells interconnect. Because of its large size, accessibility, and the host of tools developed to image it, the NMJ has yielded extensive knowledge about how the pre and post-synaptic side communicate to form a synapse. Studies in the 1970s demonstrated that incoming motor axons clustered postsynaptic acetylcholine receptors (AChRs) at contact sites (Anderson and Cohen, 1977; Frank and Fischbach, 1979). This finding spurred the search for nerve-derived signals capable of clustering AChRs, and eventually agrin was identified as a major clustering agent (Nitkin et al., 1983; Godfrey et al., 1984). This finding led to the “agrin hypothesis,” which proposes that agrin is a nerve-derived organizer of postsynaptic differentiation (McMahan, 1990).

Agryn was shown to be sufficient for AChR clustering in studies where agrin was presented to denervated muscle fibers (Cohen et al., 1997; Jones et al., 1997; Meier et al.,

1997; Rimer et al., 1997). It was additionally shown to be necessary for clustering in studies of agrin knockout mice, which had an absence of AChR clustering at nerve-muscle contact sites (Gautam et al., 1996; Sanes et al., 1998). Although agrin is expressed by both nerve and muscle, only nerve expresses the agrin splice form α^+ , which is the splice form with AChR clustering capability, confirming that nerve-derived agrin is necessary for postsynaptic differentiation (Ferns et al., 1992; Ruegg et al., 1992; Ferns et al., 1993; Hoch et al., 1993; Gesemann et al., 1995).

What is the agrin receptor? Although no protein has been definitively identified as the receptor for agrin to date, the leading candidate is a transmembrane tyrosine kinase, muscle-specific kinase (MuSK). MuSK colocalizes with AChRs in the postsynaptic membrane (Ganju et al., 1995; Valenzuela et al., 1995). MuSK knockout mice have profound defects in AChR clustering reminiscent of that seen in agrin knockout mice (DeChiara et al., 1996). Further, cultured myotubes from these mice do not form AChR clusters in response to agrin (Glass et al., 1996) and a kinase-dead form of MuSK expressed in myotubes blocks agrin's ability to cluster AChRs (Glass et al., 1997). Agrin and MuSK can be crosslinked on the myotube surface (Glass et al., 1996). However, agrin does not bind to a MuSK-Fc fusion (Glass et al., 1996), and subsequent efforts to demonstrate agrin-MuSK binding have not yielded results. This has led to the hypothesis that MuSK is only one protein in a multiprotein receptor that binds to agrin and transmits its clustering signal.

What are the signals downstream of agrin/MuSK that lead to AChR clustering? Rapsyn is likely to be an important component of this pathway. AChRs and rapsyn are colocalized from early in synaptogenesis and may bind directly (Burden et al., 1983;

Sealock et al., 1984). AChRs expressed in heterologous cells are uniformly distributed but form clusters when coexpressed with rapsyn (Froehner et al., 1990; Phillips et al., 1991). Studies in rapsyn knockout mice demonstrate that AChRs fail to form, as was seen in both agrin and MuSK knockout mice (Gautam et al., 1995) and the addition of agrin to cultured myotubes from these mice is insufficient to rescue the clustering defect, suggesting that rapsyn acts downstream of an agrin-interacting molecule. Thus, these findings together strongly argue for an agrin-MuSK-rapsyn signaling pathway, but additionally suggest that other unknown molecules are part of this pathway and are essential for its signaling.

Presynaptic induction at the neuromuscular junction

Presynaptic terminals can transmit signals to muscles within minutes of contact. Within days, they establish an active zone, acquire synaptic vesicles that are clustered at the active zone, and become polarized. Presynaptic sites only form after contact with muscle, suggesting that presynaptic differentiation in the NMJ is dependent on target-derived factors (Lupa et al., 1990). Further, fixation of the muscle with paraformaldehyde does not disrupt the ability of muscle to induce presynaptic differentiation, arguing strongly for a physical interaction, rather than one mediated by activity. In the NMJ, a basal lamina is positioned between the pre and postsynaptic side. McMahan and colleagues denervated muscle and induced the degeneration of muscle, leading to basal lamina “ghosts.” These “ghosts” are capable of inducing presynaptic differentiation in

regenerating nerves, suggesting that the presynaptic organizing signal was contained within the basal lamina (Sanes et al., 1978; Glicksman and Sanes, 1983).

Fibroblast growth factor (FGF) is one candidate for a presynaptic organizer because it resides in the basal lamina and FGF-coated beads cluster synaptic vesicles in a manner that is dependent on an increase in presynaptic intracellular calcium (Dai and Peng, 1995). A recent report by Umemori and colleagues confirms that FGFs (specifically, FGF 7, 10 and 22) are important for presynaptic vesicle clustering *in vivo* (Fox et al., 2007). Conditional knockout of the receptor for these FGFs led to decreased concentration of vesicles at synaptic sites. This effect was transient, however, and by the third postnatal week vesicle clustering was similar to that in controls.

Another presynaptic organizer candidate is laminin β 2. Laminin β 2 is produced and secreted by muscle and resides in the basal lamina. The LRE domain of laminin β 2 stops outgrowth of neurites (Porter et al., 1995). Laminin β 2 knockout mice have decreased active zones, cannot cluster synaptic vesicles at the presynaptic terminal, and have impaired transmitter release (Noakes et al., 1995; Knight et al., 2003). Recent work from Sanes and colleagues demonstrated that laminin β 2 works by binding to and clustering voltage-gated calcium channels on the presynaptic side (Nishimune et al., 2004). This in turn leads to clustering of presynaptic vesicles. They additionally demonstrated that this interaction is necessary for presynaptic induction and maintenance *in vivo*. Of particular interest is the fact that early synapse formation is intact in laminin β 2 knockout mice (Noakes et al., 1995; Fox et al., 2007). This led to a model in which FGFs and laminin β 2 act sequentially to induce presynaptic differentiation, with FGFs responsible for prenatal synapse induction and laminin β 2 responsible for postnatal

differentiation (Fox et al., 2007). In testing this model, the authors removed both FGFs and laminin $\beta 2$ *in vivo* and assayed presynaptic induction. The persistence of presynaptic induction in this scenario led to the realization that additional molecules induce vesicle clustering in the absence of both FGFs and laminin $\beta 2$, and led in turn to the discovery that collagen IV can also organize presynaptic vesicles. Thus, there are three inducers of presynaptic differentiation in the NMJ to date.

Pre and postsynaptic induction in the CNS

The finding that agrin is a synapse-inducing molecule in the NMJ spurred the search for a similar molecule in the CNS capable of directing synapse formation. Like agrin, this molecule would need to signal trans-synaptically to induce synaptic differentiation. Neural-activity-regulated-pentraxin (Narp) was identified in a screen for activity-induced genes (Tsui et al., 1996). Narp shares properties with agrin in that it is a presynaptic factor that can induce clustering of postsynaptic AMPA receptors (O'Brien et al., 1978; O'Brien et al., 2002). However, Narp does not act universally; its activity is restricted to excitatory synapses on interneurons in the CNS (Mi et al., 2002). Ephrin B is another presynaptically secreted molecule that has been shown to cluster postsynaptic receptors (Dalva et al., 2000). Ephrin B can induce clustering of its receptor, EphB, which resides in the postsynaptic membrane. This, in turn, promotes clustering of NMDA receptors via a specific extracellular interaction between EphB and NMDA receptors. Although it was originally assumed that Ephrin B/EphB signaling was restricted to NMDA receptor clustering, recent work suggests that this receptor-ligand pair may play a

more instructive role in synaptogenesis (Kayser et al., 2006). On the flip side, Wnts and FGFs are target-derived proteins that are secreted by select populations of neurons (Waites et al., 2005). They have been shown to influence axon arborization and vesicle recruitment in areas close to where they are secreted. However, they do not appear to be instructive for presynaptic differentiation. Together, these four types of secreted molecules—presynaptically derived Narp and Ephrin B as well as postsynaptically derived Wnts and FGFs—all control aspects of pre- and postsynaptic synapse formation. However, perhaps the most promising candidate for a master organizer of synapse induction is neuroligin.

Neurexins were identified by Sudhof and colleagues as the binding partner for the black widow spider venom α -latrotoxin, a venom known to cause synaptic vesicle fusion (Ushkaryov et al., 1992). Neuroligin, the binding partner for neurexin, was the first CNS protein shown to be sufficient to induce presynaptic differentiation (Scheiffele et al., 2000). Impressively, neuroligin expressed in non-neuronal cells is capable of inducing presynaptic differentiation in overlying neuronal axons. The ability of neuroligin to induce presynaptic terminal assembly is dependent on interaction with β -neurexin (Scheiffele et al., 2000; Dean et al., 2003). Reciprocally, β -neurexin can induce postsynaptic differentiation via interactions with neuroligin (Graf et al., 2004; Nam and Chen, 2005). An important study demonstrated that the synapses induced by neuroligin are functional. Neuroligin coexpressed with NMDA receptors or GluR4 in HEK 293T cells induced functional currents when co-cultured with cerebellar granule cells (Fu et al., 2003).

Neuroligins influence both excitatory and inhibitory synapse formation.

Neuroigin-1, -3 and -4 are associated with excitatory synapses, while neuroigin-2 is associated with inhibitory synapses (Graf et al., 2004; Prange et al., 2004; Varoqueaux et al., 2004; Levinson et al., 2005). The level of PSD-95 in cells may influence whether neuroigin-2 is targeted to excitatory or inhibitory synapses and thus may control the balance of excitatory and inhibitory inputs onto a cell (Prange et al., 2004; Levinson et al., 2005). Knockdown of neuroligins leads to a decrease in both excitatory and inhibitory synapses, with a more profound reduction in inhibitory synapse function (Chih et al., 2005).

The generation of Neuroigin-1 -2 and -3 triple knockout mice called into question the importance of neuroligins for synapse induction *in vivo* (Varoqueaux et al., 2006). In fact, synapse formation was unperturbed in these mice, as assessed by both immunostaining and EM. Reconciling this finding with previous findings in culture that found decreased synapse formation with neuroigin knockdown by RNAi or dominant-negative neuroligins is difficult (Chih et al., 2005; Nam and Chen, 2005). However, it may be that there are several redundant pathways for synapse induction in the intact animal. This is substantiated by more recent findings of other adhesion molecules that can induce synapse formation, including SynCAMs, EphB receptors, and netrin G ligand 2 (Scheiffele et al., 2000; Biederer et al., 2002; Kayser et al., 2006; Kim et al., 2006).

1.2 Synapse stabilization & elimination

Although the mechanisms leading to synapse induction are increasingly being unraveled, less is known about how synapses are then fated to be stabilized or eliminated. Synapse stabilization and elimination is studied in various areas of the nervous system, including the neuromuscular junction, the retinogeniculate projection, the cerebellum and higher sensory cortex. Although it may seem at first that the problem of pruning axons in the muscle to achieve mono-innervation is completely separate from the problem of pruning synapses in the cortex to achieve learning and memory, lessons from synapse induction have taught us that there is much to be learned by looking for common strategies in disparate synapses (Lichtman and Colman, 2000). However, although a large body of work examining synapse stabilization and elimination in each of these areas exists, it is hard to find comprehensive reviews of the literature that span all of these areas. For this reason, in this section I have tried to touch on important findings regarding synapse stabilization and elimination at each of the synapses in which it is traditionally studied. A common theme that has emerged is the importance of activity in shaping these connections. Although the molecular mechanisms downstream of this activity remain largely elusive at present, trans-synaptic signaling will likely be a key event in these processes.

Synapse elimination in the neuromuscular junction

Muscle fibers of the NMJ are initially innervated by multiple motor neurons, but by adulthood they are singly innervated (Redfern, 1970; Brown et al., 1976). Pruning of excess inputs is usually achieved within the first few weeks of life (Redfern, 1970). This occurs through activity-dependent competition between axons innervating a given muscle fiber. At birth, multiple axons make synapses that grow and come to occupy similarly sized territories on a muscle fiber, suggesting that in this early phase, competition has yet to begin (Balice-Gordon et al., 1993). Shortly after birth, however, terminals of competing axons begin to segregate (Gan and Lichtman, 1998). Dual color labeling of incoming axons has demonstrated that the winning axon can invade and establish synapses in the areas vacated by the losing axon (Walsh and Lichtman, 2003). Interestingly, the size of the presynaptic input does not predict the eventual winner. Sometimes, the axon with the smaller territory eventually emerges as the winner, adding synapses during the process of competition.

At the time of birth, the strength of inputs, determined by the quantal content, is roughly similar between inputs. After several days, however, the strengths of inputs increasingly diverge, with one input becoming progressively stronger as the other becomes weaker (Colman et al., 1997). There are multiple lines of evidence that suggest that the competition leading to synapse elimination is activity-dependent. First, decreasing activity pharmacologically with tetrodotoxin slows down the period of synapse elimination (Thompson et al., 1979; Ribchester, 1993). Second, increasing activity by stimulating incoming axons speeds the transition from poly-innervation to

mono-innervation (O'Brien et al., 1978; Thompson, 1983). However, global manipulations of activity are less likely to foster synapse elimination when compared to asynchronous changes in activity (Busetto et al., 2000). Indeed, in an elegant series of experiments by Lichtman and colleagues, the blockade of a small patch of AChRs led to the removal of the axon overlaying the blocked receptors, while global blockade of AChRs did not induce synapse elimination. The smaller the patch of receptors that were inactivated, the stronger the competitive drive was to eliminate the overlying axon, driving home the point that it is relative differences in the activity of neighboring inputs that govern synapse elimination.

What is the signaling that leads to synapse elimination? The current model suggests that multiple target-derived signals in concert shape synapse stability and elimination. One signal would punish and destabilize inactive inputs, while another signal would protect and reward active inputs (Sanes and Lichtman, 1999; Wyatt and Balice-Gordon, 2003). These signals would have to be highly localized to allow for the selective stabilization and elimination of axons in extremely close proximity to one another. Indeed, loss of terminals closest to a competitor occurs before loss of more distal terminals, suggesting that the signal leading to synapse elimination is most potent at sites near a competitor's terminals (Gan and Lichtman, 1998). It is speculated that punishment signals lead to destabilization of AChRs beneath inactive axons, which would lead to the eventual loss of that axon. At the same time, maintenance/reward signals stimulated by the active axon would serve to protect it from the punishment signal. The identity of these signals is still unknown.

However, a potential stabilizing signal has been recently discovered in the NMJ. Collagen IV was found to have presynaptic differentiation inducing properties, and it was shown that removal of the alpha 3 and 6 chains of collagen IV led to axonal retraction from the postsynaptic membrane during the second month of life, leaving some AChR-rich areas uninervated (Fox et al., 2007). Further investigation to determine if collagen IV serves as a stabilizing signal is warranted.

Synapse elimination and stabilization in the cerebellum

Purkinje cells in the cerebellum are important for both motor coordination and motor learning (Watanabe, 2007). The purkinje cell is initially innervated by several climbing fibers with similar synaptic strengths around birth (Hashimoto and Kano 2003; Hashimoto and Kano, 2005). During the next few weeks one climbing fiber (CF) input becomes much stronger while the other inputs are eliminated, eventually leading to mono-innervation by the third postnatal week. As was seen in the NMJ, the strengthening of one input precedes the elimination of other inputs. Thus, activity-dependent competition also drives synaptic elimination in the cerebellum.

In addition to the CF input, purkinje cells receive inputs from hundreds of thousands of parallel fibers from cerebellar granule cells. Intriguingly, these parallel fiber-purkinje cell inputs appear to be necessary for pruning of CF inputs. Mutant mice lacking parallel fiber synapses, as well as rats in which granule cells are irradiated, fail to prune excess CF inputs (reviewed in Hashimoto and Kano, 2005). How might parallel fiber synapses affect pruning of CF inputs? Parallel fiber inputs restrict CF inputs to

proximal dendrites through competition for distal dendrite territory (Hashimoto et al., 2001; Ichikawa et al., 2002). Additionally, parallel fiber synapses signal via type 1 metabotropic glutamate receptors (mGluR1) localized to those synapses (Baude et al., 1993; Nusser et al., 1994; Lopez-Bendito et al., 2001), and the downstream effects of this pathway lead to CF elimination. Signaling from mGluR1 to PKC γ is necessary for CF elimination, as shown by removal of all intermediate signaling proteins in this pathway (reviewed in Hashimoto and Kano, 2005). However, the molecular mechanisms downstream of PKC γ favoring CF input elimination are unknown.

GluR δ 2 is an orphan receptor channel subunit that shares roughly 14% to 25% sequence homology with AMPA, kainite, and NMDA receptors (Araki et al., 1993; Lomeli et al., 1993). It is specifically localized to the spines of purkinje cells at sites of parallel fiber input in the cerebellum and has been shown to interact with GluR2/3 receptors by EM (Landsend et al., 1997). Knockout of GluR δ 2 leads to a greatly decreased number of parallel fiber inputs (Kashiwabuchi et al., 1995; Kurihara et al., 1997). This was apparent by the second and third postnatal weeks, when knockout mice had roughly 50% less parallel fiber inputs, suggesting that GluR δ 2 is necessary for stabilization of parallel fiber inputs. The idea that GluR δ 2 is involved in stabilization was substantiated by a report that removal during adulthood led to shrinkage of the active zone of parallel fiber inputs (Takeuchi et al., 2005). Pre and postsynaptic elements are mismatched in GluR δ 2 knockout mice, with the PSD often longer than the presynaptic active zone, further arguing that postsynaptic GluR δ 2 signals to the presynaptic side. Recent reports showed that elements of the c-tail of GluR δ 2 are dispensable for the induction/stabilization of parallel fiber inputs, though it was necessary for the GluR δ 2

dependent effects on LTD and restriction of climbing fiber input territory (Uemura et al., 2007; Kakegawa et al., 2008). Thus, it is still unknown how GluR δ 2 affects induction/stabilization of parallel fiber inputs. Because it does not bind glutamate, kainite, or AMPA, and it has no ion channel activity in 293T cells, it is unlikely that activity mediated by this channel is responsible for its stabilization effects (Lomeli et al., 1993; Mayat et al., 1995). Interestingly, it contains an LIVBP domain in its extreme n-terminal domain similar to that found in AMPA and NMDA receptors and it has been proposed to function as an adhesion molecule (Uemura et al., 2007).

Pruning of the retinogeniculate projection

Retinal ganglion cells in the eye relay visual information to the thalamus via axonal projections to the lateral geniculate nucleus (LGN). After an initial period of overlap, these retinal ganglion cell projections are segregated into eye-specific areas in the LGN (reviewed in Huberman, 2007). This segregation is achieved via strengthening synapses in the same eye territory and eliminating synapses in the opposite eye territory. Before receiving visual information, waves of spontaneous activity are present in the retinal ganglion cell layer. This wave-generated activity is necessary for proper segregation of eye-specific inputs, as pharmacological blockade of activity in one eye leads to a diminishment of the territory of that eye and an expansion of the territory of the opposite eye (Penn et al., 1998; reviewed in Torberg and Feller). After gross segregation of RGC projections into eye-specific layers, further synaptic refinement occurs at the level of single LGN neurons. Specifically, in the weeks after eye opening, the number of

inputs onto single LGN neurons is dramatically reduced (Chen and Regehr, 2000). The few remaining inputs are greatly strengthened. Although it was originally hypothesized that this further pruning was dependent on visually mediated activity, recent work suggests that retinal waves may also dictate this aspect of synaptic refinement (Katz and Shatz, 1996; Del Rio and Feller, 2006; Hooks and Chen, 2006). Visual information is important for the subsequent maintenance of these inputs.

One important question in the field has been whether retinal waves are permissive or instructive for pruning of retinogeniculate synapses (Huberman, 2007). Recent studies suggest that aspects of retinal wave activity are indeed instructive for eye-specific segregation (Torborg et al., 2005; Demas et al., 2006). Thus, activity is likely to be essential in determining which inputs are maintained or eliminated. However, the downstream molecular signals resulting in structural changes are not well-characterized. In an attempt to determine molecules downstream of activity responsible for eye-specific segregation, Shatz and colleagues screened for changes in mRNA expression in the LGN in response to retinal wave activity blockade (Corriveau et al., 1998). Surprisingly, Class I MHC molecules were identified in this screen. Class I MHC molecules are now implicated in retinogeniculate refinement, as well as proper induction of LTD, leading to speculation that they may be mediators of synapse elimination downstream of activity (Huh et al., 2000). Interestingly, the complement cascade, another component of the immune system, has also been implicated in synapse elimination (Stevens et al., 2007). Mice lacking C1q or C3 (early mediators of the complement cascade) fail to properly segregate retinogeniculate projections and fail to prune excess inputs onto LGN neurons. The authors speculate that these complement cascade components tag incorrect synapses

for eventual elimination. Finally, neuronal pentraxin knockout mice also have defects in eye-specific segregation of retinogeniculate projections (Bjartmar et al., 2006). Although the identification of these molecules represents significant forward progress towards understanding the molecular mechanisms underlying synapse elimination, the pathways through which these molecules act are still unclear. Molecules necessary for stability also remain elusive.

Spine stabilization and elimination in cerebral cortex

Quantification of synapse number in cortical areas of rats, nonhuman primates and humans have revealed that excessive synapse formation during early development is followed by a large decrease in the number of synapses during adolescence (Blue and Parnavelas, 1983; Rakic et al., 1986; Markus and Petit, 1987; Huttenlocher, 1990; Rakic et al., 1994; De Felipe et al., 1997). This suggests that synapse elimination is an important aspect of cortical development. More recently, developmental synapse elimination as assessed by spine turnover has been directly visualized in mice *in vivo*. Recent technical advances now allow for visualization of the dynamics of spines over extended periods of time (from days up to years) through either an opening or a thinned skull in transgenic mice expressing GFP or YFP in a subset of cells (reviewed in Pan and Gan, 2008). Holtmaat et al. showed that spine number decreased during early postnatal development, due to a higher number of eliminating over newly formed spines, but balanced out by 4 weeks of age (Holtmaat et al., 2005). They also found that spines became increasingly stable as animals aged. Zuo et al. also found that elimination of

spines outpaced formation in young animals (1 month old) (Zuo et al., 2005a). The number of filopodia also was significantly decreased during this time. The rate of elimination gradually decreased from 1 to 4 months in age and then remained comparable from 4 to 6 months of age (oldest age imaged). Meanwhile, rates of addition were relatively unchanged across 1 to 4 months of age. Spines were increasingly stable in older animals, with approximately 75% of spines remaining stable for 18 months. These studies confirm that synapse elimination is relevant in the intact animal and that the select stabilization and elimination of subsets of synapses is likely crucial to proper cortical development.

What is the role of activity in cortical synapse elimination during development? A fascinating study from Zuo and colleagues suggests that activity is critical for the elimination of spines in the developing brain (Zuo et al., 2005b), contrary to a number of studies suggesting that increased activity increases spine number (reviewed in Yuste and Bonhoeffer, 2001). The authors found that whisker deprivation from 4 to 6 or 4 to 8 weeks of age led to increasingly diminished spine elimination in mouse barrel cortex *in vivo*. The rate of spine formation was unaltered. The effect of whisker deprivation in preventing spine elimination was much less pronounced in adulthood (> 4months) although there was still a small but significant effect. The negative effect of sensory deprivation on spine elimination was reversible in young animals—if whiskers were allowed to regrow, the spine elimination rate increased relative to controls, thus almost fully compensating for the original decrease in elimination after 2 weeks of recovery. If whiskers were trimmed well into adulthood, however, there was no resulting compensation after whisker regrowth, suggesting that at some point spines stabilize and

become resistant to elimination. Finally, they showed that spine elimination was dependent upon NMDA receptor activation, as blockade of NMDA receptors caused a significant reduction in the rate of spine elimination, and exuberant synapse elimination occurred after withdrawal of NMDA receptor blocking drugs. Therefore, this study strongly suggests that synapse elimination is a crucial part of normal cortical development and additionally proposes a role for NMDAR-dependent activity in spine elimination.

Molecules responsible for synapse stabilization and elimination in the cortex have yet to be identified. Once identified, it will be important to test these molecules by imaging spine and presynaptic bouton dynamics *in vivo* in animals in which these candidate proteins are genetically altered.

1.3 Synapse formation is a dynamic process

Although many have speculated about the mechanisms of synapse stabilization and elimination based on fixed time-point studies, the best way to understand this dynamic process is through time-lapse imaging. Numerous recent advances in imaging now allow us to watch synapses form and change in real time. This has altered the traditional view that synapse formation is a protracted process, occurring over days. In contrast, we now know that synapses can form in minutes to hours and that large-scale structural changes leading to reshaping of circuits can occur over similar time courses (McAllister, 2007).

Dendritic filipodia have been shown by numerous groups to be capable of inducing synaptic contacts with axons (Saito et al., 1992; Fiala et al., 1998; Jontes et al., 2000; Niell et al., 2004). Dendritic protrusions in slice and dissociated cultures are highly motile and turn over extensively within minutes at early ages (Dailey and Smith, 1996; Ziv and Smith, 1996). *In vivo* imaging studies of spines on layer 2/3 neurons in the barrel cortex of rats have demonstrated that spines are highly dynamic on the order of tens of minutes in young animals (Lendvai et al., 2000). Thus, there is the possibility of rapid sampling of axons and induction of synapses within a relatively short period of time. Later in development, this motility is greatly diminished as spines become stable for weeks or months (Grutzendler et al., 2002; Trachtenberg et al., 2002; Zuo et al., 2005a). Thus, the dynamics of spines may be critical to determining the number of synapses being made, and there may be a developmental window in which they achieve this.

Presynaptic terminal precursors also are dynamic early in development (Zhen and Jin, 2004). Transport packets containing essential components of the presynaptic active zone are highly motile before they reach synaptic sites (Shapira et al., 2003). Likewise, synaptic vesicles traffic rapidly along axons before stabilizing at dendritic contact points and are capable of exocytosis as early as one hour after contact (Ahmari et al., 2000). Axonal filopodia are also quite motile, and decrease in motility with development and contact with dendrites (Tashiro et al., 2003). Once a presynaptic terminal comes in contact with a potential postsynaptic partner, appropriate pre and postsynaptic molecules, including glutamatergic receptors, can be recruited to the synaptic site in an hour or less (Friedman et al., 2000; Okabe et al., 2001; Washbourne et al., 2002). Although work by Friedman et al. suggests that elements of the presynaptic side (including bassoon and

synaptic vesicles) are recruited to nascent sites before postsynaptic elements, work by Washbourne et al. suggests that both pre- and postsynaptic elements arrive at the synaptic site at about the same time, or that NMDA receptors may arrive slightly before synaptic vesicles (Friedman et al., 2000; Washbourne et al., 2002). Most recently, Gerrow et al. showed that structural elements of the postsynaptic side can stabilize hours in advance of recruitment of synaptic vesicles to the presynaptic site (Gerrow et al., 2006). Although this suggests that synapses may be assembled within an hour, the signals that regulate synaptic stabilization and elimination remain unknown (see Section 1.2).

1.4 The shaping of neuronal networks in development and disease

Humans are born with immature brain circuitry that is shaped and refined by experience throughout the first two decades of life. Synapses are continually added during the first few years of life and then are eliminated for an extended period stretching into puberty (Huttenlocher, 1990). During this time, the brain appears to be particularly receptive to restructuring and change, as evidenced by the incredible capacity of children to recover from brain injury or radical brain surgeries such as hemispherectomy (Johnston, 2004). It is likely that this plasticity is what endows the brain with the ability to learn and remember.

What happens when this plasticity goes awry? Failures of synapse formation, stabilization or elimination are likely to disrupt the transition of immature circuitry into proper mature circuits, leading to aberrant cortical wiring. Indeed, it is hypothesized that forms of mental retardation are caused by deficiencies in neuronal network connectivity,

which thus leads to abnormal information processing (Vaillend et al., 2008). Altered spine formation and/or abnormal synapse number have been documented in human patients as well as genetic mouse models of mental retardation (Takashima et al., 1981; Comery et al., 1997; Fiala et al., 2002; Dierssen et al., 2003). In fact, almost every aspect of synapse formation, from presynaptic vesicle recycling to postsynaptic organization, is controlled in part by genes that are mutated in forms of mental retardation (Vaillend et al., 2008).

Abnormal cortical connectivity also is implicated in autism (Williams and Minshew, 2007). Considerable attention has been paid of late to neuroligins, postsynaptically located cell-adhesion molecules that trigger presynaptic differentiation (see section 1.1). Mutations in neuroligins and their binding partners, neurexins, are implicated in human cases of autism, again highlighting the importance of proper synapse induction and development for normal brain development (Pardo and Eberhart, 2007). Thus, understanding how subsets of synapses are stabilized and eliminated during development is essential for comprehending both the normal and pathological brain.

1.5 Conclusions and significance of this graduate thesis

Synapse formation is essential for information transfer in the brain, and the mechanisms leading to synapse formation are increasingly being discovered. Equally important for information processing, however, is the stabilization and elimination of subsets of synapses. Although activity has been shown to be critical for these processes, the downstream molecular mechanisms leading to functional stabilization are not known.

Imaging studies suggest that the mechanisms leading to stabilization and elimination are actively at play during synaptogenesis and that these signals are likely to be transmitted and acted upon within the timeframe of minutes to hours. The aim of this thesis was to identify molecules that mediate synaptic stability. To achieve this, we used time-lapse imaging of the presynaptic vesicle protein synaptophysin fused to GFP to characterize the dynamics of synapse stabilization and elimination during synaptogenesis in dissociated cortical neurons. We identified postsynaptic AMPA receptors as a positive regulator of presynaptic input stability. We additionally demonstrated that the ability of postsynaptic AMPA receptors to stabilize presynaptic inputs depended on a structural interaction mediated by its extracellular N-terminal domain.

CHAPTER 2: METHODS

2.1 Primary Cell Culture and Transfections

Cortical neurons were cultured from embryonic day 18 (E18) Long-Evans rats (Charles Rivers, Wilmington, MA) and plated at a density of 85,000/cm² on chamber slides (Nalge Nunc International, Rochester, NY) or glass coverslips coated with poly-d-lysine (0.03 mg/mL final) (Millipore, Billerica, CA) and laminin (0.003 mg/ml final) (BD Biosciences, San Jose, CA). Neurons were cultured in Neurobasal Medium (Invitrogen, Carlsbad, CA) supplemented with 1000 U/ml penicillin G and streptomycin sulfate (Invitrogen), 1x GlutaMAX (Invitrogen), 2% FBS (Invitrogen) and 2x B27 (Invitrogen). Media were refreshed every 3 days. Neurons were transfected using the calcium phosphate method (Threadgill et al., 1997) on the days described in the results section. For drug blockade experiments, neurons were grown in the presence of DNQX (20 μM, Tocris, Ellisville, MO), D-AP5 (50 μM, Tocris), or vehicle (DMSO) from 7-14DIV. Drugs were refreshed daily.

2.2 Plasmids

The Synaptophysin-GFP construct was provided by Dr. Hollis Cline (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The mCherry construct was provided by Dr. Roger Tsien (University of California, San Diego, La Jolla, CA) (Shu et al., 2006). The pECFP-C1 was purchased (BD Biosciences). The CFP:GluR1 c-tail and CFP:GluR2 c-tail constructs were made as described in Shi et al. 2001 (Shi et al., 2001), except that we chose to use CFP instead of GFP so that the Syn-GFP signal would not be interfered

with during live-imaging studies. Stargazin was provided by Dr. Susumu Tomita (Yale School of Medicine, New Haven, CT) and Dr. Roger Nicoll (University of California, San Francisco, CA). Untagged and GFP-tagged GluR1 & GluR2 constructs were provided by Dr. Richard Huganir (Johns Hopkins University, Baltimore, MD). Neuroligin-1 was provided by Dr. Palmer Taylor (University of California, San Diego, La Jolla, CA). The GluR2NTD-pDisplay was made by Megan Williams following the strategy described in Saglietti et al (Saglietti et al., 2007).

2.3 Immunostaining

Neurons were fixed in 4% paraformaldehyde, 4% sucrose in 1X phosphate buffered saline (PBS) for 20 minutes, rinsed 2 x 5 minutes in 1X PBS and blocked in 3% bovine serum albumin, 0.1% Triton-X in 1XPBS for 30 minutes. Primary antibody diluted in blocking solution was applied to neurons for 2 hours, neurons were rinsed 3 times for 5 minutes each in blocking solution, and secondary antibodies were then applied in block for 45 minutes. Neurons were again washed 3 times for 8 minutes each in blocking solution, followed by a final wash in PBS before they were coverslipped. The following primary antibodies were used for this study: Chicken anti MAP2 (1:5000; Abcam, Cambridge, MA), Guinea pig anti VGLUT 1 (1:5000; Millipore) and Guinea pig anti VGLUT 2 (1:1000; Millipore), mouse anti GAD6 (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA), Goat anti GFP (1:3000; Abcam), mouse anti Bassoon (1:1000; Abcam) and rabbit anti Ds-Red to visualize mCherry (1:1000; ClonTech,

Mountain View, CA). Appropriate secondaries raised in donkey were used (1:1000; Jackson ImmunoResearch, West Grove, PA).

For live-labeling experiments, GluR1 (1:10; EMD Biosciences, San Diego, CA) or GluR2 (1:100; Millipore) antibodies were diluted in Opti-MEM (Invitrogen) supplemented with 0.05% sodium azide (to prevent receptor internalization) and added to living neurons for 10 minutes. Neurons were then rapidly washed 4 times in Opti-MEM and fixed. Staining then proceeded as described above. GFP-tagged GluR1 and GluR2 receptors were live labeled using a rabbit anti GFP antibody (Invitrogen; 1:1000).

Images were captured on a Leica SP2 confocal microscope (McBain Instruments, Chatsworth, CA) using a 63X oil-immersion objective at 3X zoom and were analyzed using NIH ImageJ. The number of VGLUT, GAD6, GluR1 or GluR2 positive puncta per 10 μm of dendrite was calculated after images were thresholded (cut-off was 10 times background). For experiments in which MAP2 was used as a dendritic marker, all puncta within 1 μm of the dendrite were included in the data set to account for puncta on spines. All analysis was of proximal dendrites within 200 μm of the cell soma. Inhibitory neurons were identified by GAD6 positive cell bodies and were excluded from the data set. The density of neurons was monitored by MAP2 staining and all captured and analyzed neurons were plated at a standard density. All image capturing and analysis was done blind to condition.

2.4 Live Imaging

1 hour imaging experiments: Cells were plated and grown on glass coverslips, which were transferred into a custom-built holder at the time of imaging. The holder was fitted onto the stage of an Olympus Fluoview 300 upright confocal microscope (Olympus Center Valley, PA) encased in a custom-built plexiglass chamber and the cells were continuously perfused with artificial CSF (124mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.23 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, and 10 mM glucose) bubbled with 95% O₂ / 5% CO₂ for the duration of the imaging experiment. All movies were taken with a 63x water-immersion objective at 3x zoom. After identifying an area of interest containing a Syn-GFP expressing axon, a z-stack was set that began at least 5 μm above and ended 5 μm below the plane of interest. This was essential in order to ensure that eliminated puncta truly disappeared and didn't just fall out of the imaging field within the z-plane. A z-stack (step interval 0.5-1.0 μm) was acquired every 10 minutes over the course of 1 hour. We found that bleaching of signal was negligible even when greater than 14 time points were sequentially acquired, and thus we are confident that bleaching was not an issue across the 7 time points acquired in these experiments. Early dwell time experiments presented in figure 2 were done at room temperature. All other experiments presented in the paper were imaged at 33°C. We did not detect any temperature-dependent difference in dwell times with the 10 minute sampling rate used in these experiments.

6 hour imaging experiments: Cells were plated on glass-bottom dishes (MatTek Corporation, Ashland, MA) and were imaged on an inverted Nikon fluorescent

microscope (Nikon Instruments, Inc., Melville, NY) at 0, 3 and 6 hour time points. Cells were removed from the incubator, imaged, and returned to the incubator until the next imaging time point. For these experiments, a z-stack was set in which the stack began 5 μm above the plane containing the Syn-GFP expressing axon and ended 5 μm below the area of interest to prevent Syn-GFP puncta from falling out of view due to z drift. In a subset of experiments, after the 3rd imaging point, cells were live-labeled for either GluR1 or GluR2 and fixed as described in the immunostaining section of the methods. Another subset of cells was fixed after imaging and stained with anti-NR1 antibodies to visualize the total NR1 content.

Simultaneous imaging of presynaptic axons and postsynaptic dendrites: In these experiments, neurons were plated on small glial islands of 10-60 cells to increase the chance of contacts between neurons. Neurons were transfected at 4DIV with Syn-GFP and again at 7DIV with mCherry (for visualization of dendrites) for experiments presented in Fig 3.8. Neurons were transfected at 4DIV with Syn-GFP and again at 7DIV with mCherry plus either CFP (control) or GluR1 & 2 c-tails for experiments presented in Fig 5.4. Because transfection efficiency is quite low (1 in 100 cells), it was extremely rare for the same neuron to be transfected at both time points. Thus, we were left with a sub-population of neurons expressing Syn-GFP and a separate sub-population expressing mCherry and CFP or c-tails. At 11-12DIV (for experiments in Fig 5.4) or 14DIV (for experiments in Fig 3.8), we located sites where Syn-GFP axons contacted mCherry-expressing cells and we imaged the dynamics of these contacts at 10 minute intervals across an hour. All conditions were exactly as described for the 1 hour imaging experiments. All image acquisition and analysis was done blind to condition.

2.5 Analysis of Imaging Experiments

Z-stacks were collapsed into a maximum projection image in NIH ImageJ and were thresholded (cut-off set at 10 times background). In each case, we confirmed that the z-stack only included the width of the dendrites being imaged to avoid overestimates of colocalization with Syn-GFP. Although X-Y drift was essentially nil in these experiments, all alignments were checked and corrected if necessary using the plug-in ManualAlignerC, downloaded from the ImageJ website. Z-projections for each time point were then assembled into a sequential stack and were analyzed using a custom plug-in for ImageJ written by Tom Maddock. This software parsed through each stack and identified the location of every punctum that appeared at any point during the imaging window. A circular region with a diameter of 2.5 μm was then created around each punctum and it was determined whether a puncta was present or absent from that region of interest for every time point within the experiment. This created a data set that gave the dwell time for each punctum location. If a punctum was present in one image frame, but no punctum existed in the frame preceding or following, the punctum was assigned a dwell time of 0 minutes, the minimum time that it could have existed in that location. If a punctum was present in two consecutive imaging frames, but was absent in the flanking time points, it was assigned a dwell time of 10 minutes. Puncta that were present across the entire imaging period were assigned the designation “stable.” We removed from the data set any puncta that were present at the start of imaging but disappeared by the last frame, as

well as puncta that appeared during the imaging period and persisted through the last imaging period.

2.6 293T cell imaging assay

We cotransfected 293T cells with mCherry (for visualization) and one of the following combinations of plasmids: neuroligin-1, GluR1 & GluR2, neuroligin-1 + GluR1 & GluR2, neuroligin-1 + GluR2NTDpDisplay or vector only. Twelve hours post-transfection, 293T cells were seeded onto 10-11DIV neurons expressing Syn-GFP (transfected at 4DIV). Interactions between Syn-GFP puncta and 293T cells were imaged 18-24 hours post-overlay across 1 hour. Z-stacks were acquired every 10 minutes.

Following imaging, z-stacks were maximum-projected and assembled into 1 hour movies. In each case, we confirmed that Syn-GFP puncta were in direct contact with 293T cells by parsing through optical sections before creating a z-stack. Using an automated analysis program for ImageJ (described above), we determined the ratio of stable (dwell time 60 minutes) to transient (dwell time 0 minutes) Syn-GFP puncta contacting 293T cells expressing the various constructs described above. This gave us a number (stability index) that described the ability of the 293T cell to effectively capture and stabilize trafficking Syn-GFP puncta. Inclusion criteria for 293T cells required that an axon clearly contacted and crossed the 293T cell, as well as that clearly defined Syn-GFP puncta trafficked along this length of axon. Axons additionally were required to have both stable and trafficking puncta at some point along their length within the larger field of view.

2.7 Statistics

Student's t-test was used when comparing 2 conditions. For all multiple condition comparisons, the Kruskal-Wallis Test was used, along with Dunn's multiple comparisons test to determine statistical significance between individual conditions. All data presented in the test are mean \pm SEM.

**CHAPTER 3: DYNAMICS OF PRESYNAPTIC
INPUTS IN CORTICAL CULTURE**

3.1 INTRODUCTION

Presynaptic axons and postsynaptic spines are quite motile during synapse development and have numerous opportunities to interact with one another. At some point, this motility becomes greatly restricted and a subset of contacts between pre and postsynaptic neurons are stabilized—presumably as synapses. Although each contact between a pre and postsynaptic element represents a potential synapse, the number of potential synapses is approximated to be 3-9 fold higher than the number of actual synapses in the cortex (Chklovskii et al., 2004). What is special about the connections that eventually yield synapses? Are there predetermined sites where synapses form, or does each fledging connection have the capacity to become a synapse? What is the sequence of events from first contact to mature synapse and what is the timescale for those events? Once a nascent synapse is formed, can it be unraveled? All of these questions are fundamental to understanding synaptogenesis and all require time-lapse imaging for clear answers.

Imaging studies have revealed that contact between a pre and postsynaptic cell can result in a functional presynaptic input in tens of minutes. Early imaging studies demonstrated that dendritic filopodia are quite dynamic during development, suggesting that they might play a role in actively recruiting presynaptic partners (Cooper and Smith, 1992; Dailey and Smith, 1996). This assumption was tested by Ziv and Smith, and they showed that a developmental decrease in filopodial motility correlated with an increase in the number of spines and synapses (Ziv and Smith, 1996). Interestingly, they were able to image a dendritic filopodium that contacted an axon, stabilized and presumptively

recruited a functional presynaptic puncta by the end of the imaging period (90minutes). Although they did not determine whether the presynaptic puncta was present prior to contact, the small size of the puncta suggested that it was newly formed. The ability of filopodial extensions to recruit functional presynaptic inputs was demonstrated more directly by Friedman and colleagues, who showed that functional presynaptic vesicles, as assessed by FM staining, were capable of accumulating at sites of axo-dendritic contact within 30 minutes of initial contact (Friedman et al., 2000). Ahmari and colleagues also carried out careful observations of axo-dendritic contact. They found that contact could be initiated by extensions from either the axon or the dendrite. Contacts made by a dendrite to areas of axon devoid of the synaptic vesicle marker VAMP-GFP often resulted in consequent trapping of trafficking VAMP-GFP as rapidly as 15 minutes after initial contact. They found that FM-stained vesicles accumulated as soon as 1 hour following dendritic contact, suggesting that functional presynaptic inputs were induced at these sites within an hour.

After initial axo-dendritic contact, the formation of a synapse requires differentiation of both the pre and postsynaptic side. Accumulation of active zone proteins and clustering of synaptic vesicles are hallmarks of presynaptic maturation. Accumulation of glutamatergic receptors signals postsynaptic maturation. Friedman and colleagues were the first to describe the time-course for these events, and based on their studies they proposed that presynaptic differentiation precedes postsynaptic differentiation (Friedman et al., 2000). The authors repeatedly labeled functional presynaptic vesicles in cultured hippocampal neurons with FM dyes to determine lifetimes of presynaptic vesicles. They then fixed the neurons and stained for various pre

and postsynaptic molecules to determine what molecules had accumulated at presynaptic sites of various “ages”. They found that the presynaptic active zone molecule bassoon rapidly accumulated at sites of FM staining, and proposed that it was present at presynaptic sites either before or at the time that those sites acquired the ability to recycle synaptic vesicles at the membrane. PSD-95, a molecule important for organization of the postsynaptic density, was increasingly more likely to colocalize with “older” FM-positive vesicles, suggesting that it was recruited to presynaptic sites later than bassoon. The GluR1 subunit of AMPA receptors and the NR1 subunit of NMDA receptors were found to accumulate at presynaptic sites older than 45 minutes, suggesting that their accumulation at synaptic sites also lagged behind that of bassoon. While this work suggests that presynaptic differentiation precedes postsynaptic differentiation, work by Washbourne et al. suggests the opposite—postsynaptic differentiation precedes or is coincident with presynaptic differentiation (Washbourne et al., 2002). These authors found that NMDA receptors can be recruited to axo-dendritic contact sites within minutes of initial contact, with PSD-95 arriving concomitantly or shortly thereafter. Thus, although it remains to be settled whether pre- or postsynaptic differentiation occurs first, it is clear that elements of a mature synapse can be recruited to potential synaptic sites within minutes to hours.

Are all axo-dendritic contacts equally able to form synapses? A recent report suggests that the answer is no (Sabo et al., 2006). A study looking at the dynamics of synaptic vesicles labeled by VAMP-GFP found that these vesicles “paused” preferentially at certain locations along the axon. Vesicles paused for an average of 1-2 minutes, and this pausing was independent of contact with dendrites. This suggested that

the signals responsible for pausing were intrinsic to the axon and that those signals were not sufficient for the long-term stabilization of presynaptic inputs. When dendritic filopodia contacted axons in these experiments, they only stabilized at pause sites, suggesting that signals at the pause site were capable of communicating with the target cell. This raises the intriguing question of whether the dendrite can signal back to the presynaptic input to translate a short-term “pause” into long-lasting stability.

In this study, we wished to characterize the dynamics of presynaptic vesicles during synaptogenesis with the ultimate goal of understanding the molecular mechanisms controlling the long-term stabilization of presynaptic inputs. Because the accumulation of presynaptic vesicles at the presynaptic active zone is a hallmark of presynaptic development, we imaged Syn-GFP dynamics and determined the fraction of Syn-GFP puncta that stabilized at various developmental time points. We found that the stabilization of presynaptic inputs is developmentally regulated. We also wished to determine whether contact with dendrites was responsible for presynaptic stabilization. While we found that dendrite contact was necessary, we additionally found that puncta only stabilized at a subset of dendritic sites, suggesting that dendrite contact alone was not sufficient for stabilization. Finally, we wished to determine whether stabilized presynaptic inputs retained the ability to be eliminated. During imaging sessions of 5 hour duration, approximately 30% of puncta that were stable for an hour were subsequently eliminated, suggesting that stable presynaptic inputs can indeed be disassembled.

3.2 RESULTS

Development of presynaptic inputs in cortical culture

To determine whether synapse elimination occurs in cortical cultures, we established a time course for the number of presynaptic inputs onto dendrites at 6, 10, 14 and 18 DIV, a time window spanning synaptogenesis in these cultures (Hall et al., 2007). Neurons were stained with an excitatory presynaptic marker, vesicular glutamate transporter (VGLUT) 1 & 2, as well as the dendritic marker microtubule-associated protein 2 (MAP2), and the number of presynaptic inputs per 10 μm of dendrite was quantified (Fig 1.1). Presynaptic inputs onto postsynaptic neurons were seen as early as 6 DIV, although the number of inputs was quite small (1.59 ± 0.33 inputs/10 μm of dendrite; mean \pm SEM) (Fig 1.1 A,E). Inputs increased by 10 DIV (7.79 ± 1.54 inputs/10 μm of dendrite) (Fig 1B,E), reached a peak at 14 DIV (13.6 ± 1.69 inputs/10 μm of dendrite) (Fig 1C,E), and decreased at 18 DIV (5.84 ± 0.68 inputs/10 μm of dendrite) (Fig 1D,E) to levels comparable to those observed at 10 DIV. This suggested that a subset of the inputs observed at 14 DIV either failed to stabilize or were eliminated by 18 DIV. This decrease in input number was not caused by the death of neurons between 14-18 DIV (29 ± 2.93 cell bodies per f.o.v. at 14 DIV, 28.5 ± 2.12 at 18 DIV, $p=0.89$) (Fig 1.2A), nor was it because of a significant change in dendritic length between these time points (1540 ± 101 μm of total dendrite length per f.o.v. at 14 DIV, 1789 ± 135 μm of total dendrite length at 18 DIV, $p=0.14$) (Fig 1.2 B).

Dynamics of Syn-GFP puncta in cortical culture

To characterize the dynamics of presynaptic vesicles during cortical development, we imaged synaptophysin-GFP (Syn-GFP) puncta in living neurons (Fig 3.3A). Synaptic vesicles are a key component of presynaptic terminals, and GFP fusions of synaptic vesicle proteins (such as Syn-GFP) have been widely used to study the development and dynamics of presynaptic terminals. The stability of presynaptic vesicle protein-containing puncta is a good indicator of functional presynaptic terminals (Ahmari et al., 2000; Zhai et al., 2001; Sabo et al., 2006). We therefore used imaging of Syn-GFP as a marker for presynaptic input dynamics in these experiments. We additionally confirmed that transfection of Syn-GFP did not lead to over-expression relative to endogenous synaptophysin in our experiments (17 ± 5.3 Syn-GFP puncta per 100 μm of axon, 24 ± 3.9 endogenous synaptophysin puncta per 100 μm of axon, $p=0.25$). Neurons were transfected at 4 DIV with Syn-GFP and imaged at various developmental time points (11, 14 and 17 DIV). The majority of Syn-GFP puncta observed were between 0.4 and 1.4 μm in diameter, and thus each Syn-GFP punctum likely represents an aggregate of several vesicles (Ahmari et al., 2000).

We imaged Syn-GFP puncta dynamics at 11, 14 and 17 DIV at 10-minute intervals over an hour and quantified the fraction of puncta that were stable (persisted for the entire 1 hour of imaging), new (appeared during imaging and persisted for at least 2 consecutive imaging frames) or eliminated (present for at least 2 consecutive imaging frames but then disappeared) (Fig 3.3). We also characterized the absolute number of stable, new and eliminated puncta as a function of axon length (results are similar to

those obtained from analyzing percent distribution, and thus we will only discuss the percentage data here; Fig 3.4). At 11 DIV, the majority of puncta were either new ($45 \pm 2.0\%$) or eliminated ($41 \pm 1.8\%$) (Fig 3.3B). Additions were more frequent than eliminations, consistent with an increase in presynaptic input number during this point in synaptogenesis. Only $14\% (\pm 2.0)$ of Syn-GFP puncta were stable across the hour. At 14 DIV, the fraction of stable puncta increased to $24\% (\pm 4.3)$, and the fraction of additions ($39 \pm 5.3\%$) still slightly outweighed that of eliminations ($37 \pm 1.6\%$). By 17 DIV, additions ($20 \pm 2.8\%$) fell slightly below eliminations ($21 \pm 1.9\%$). Strikingly, the fraction of stable puncta was dramatically increased ($59 \pm 1.5\%$) by this developmental age. This suggested that stabilization of presynaptic inputs is developmentally regulated, with a significant increase in stability between 11 and 17 DIV.

We additionally determined the average lifetimes of Syn-GFP puncta at 14 DIV, a time when puncta are being increasingly stabilized. Initial experiments to determine the optimal sampling rate were carried out with either a 10 minute sampling rate for one hour (Fig 3.5) or a 2 minute sampling rate for twenty minutes (Fig 3.6). Because a central goal of this work was to study longer term stabilization of presynaptic puncta, we chose to use a 10 minute sampling rate for our experiments. Using automated analysis software, each imaged punctum within a given experiment was assigned a dwell time (see methods for detailed description) (Fig 3.5A). Puncta that were present in only one imaging frame were assigned a dwell time of 0 minutes (actual dwell time is > 0 min but < 10 min). Puncta that appeared in two to five consecutive imaging frames were assigned a dwell time of 10 to 40 minutes, respectively. Puncta with dwell times from 0-40 minutes were termed “transient”. Puncta that were present throughout the duration of the imaging

experiment were designated as “stable” (dwell time > 60 min). We found that distinct populations of transient ($63 \pm 9.9\%$) and stable ($37 \pm 9.8\%$) Syn-GFP puncta exist (Fig 3.6B). Stable puncta were, on average, much larger than transient puncta (stable puncta area: $0.93 \pm 0.55 \mu\text{m}^2$, area of puncta with dwell time 10-40 minutes: $0.27 \pm 0.03 \mu\text{m}^2$, area of puncta with dwell time of 0 minutes: $0.15 \pm 0.01 \mu\text{m}^2$, mean \pm SEM; Fig 3.7A). Further, larger Syn-GFP puncta were much more likely to colocalize with bassoon, a component of the active zone (Fig 3.7B). These results demonstrate that both stable and dynamic populations of presynaptic puncta are present at 14 DIV, a time when we observe peak synaptogenesis in culture (Fig 3.1, see also Hall et al., 2007).

Presynaptic puncta preferentially stabilize at dendritic sites

We hypothesized that the stable puncta observed in the previous experiments were in contact with dendrites and the transient puncta were in areas lacking dendrites. To address this, we transfected a subset of neurons with Syn-GFP and a separate subset with mCherry to visualize dendrites. We then imaged puncta contacting mCherry positive neurons at 10 minute intervals across an hour at 14 DIV (Fig 3.8). We separated puncta into two groups: Syn-GFP puncta contacting dendrites (n=155 puncta) and Syn-GFP puncta not contacting dendrites (n=267 puncta). We observed numerous examples of stable Syn-GFP puncta in contact with dendrites (white arrows in Fig 3.8A-C). In fact, 61 out of the 76 stable puncta observed in these experiments were in direct contact with dendrites, suggesting that a contact-dependent signal stabilizes presynaptic vesicles. Dwell time analysis revealed that 38% (± 4.4) of puncta contacting dendrites were stable

across the hour (Fig 3.8D, E). As expected, the majority (94%) of puncta not in contact with dendrites were transient (Fig 3.8D). What we did not expect, however, was that a significant portion (62%) of Syn-GFP puncta that contacted dendrites were also transient (Fig 3.8D). Thus, while contact with a dendrite may be a prerequisite for stabilization, this contact alone is not sufficient for synaptic stability. This finding argues for a dendrite-derived stabilizing factor that is found only at select sites along the dendrite.

Stable presynaptic vesicles can still be eliminated

Little is known about the time window in which nascent synapses are either stabilized or eliminated. It is possible that information is transferred within minutes of synapse formation that leads to maintenance or disassembly, or it may take hours or days for the process of elimination to be carried out. Further, it is unknown whether a stabilized synapse can then be disassembled later in life. We wondered whether presynaptic puncta that were stable for at least 1 hour could subsequently be destabilized or eliminated. To address this, we lengthened our imaging window to 5 hours (10 minute sampling rate). In these experiments we imaged a total of 24 puncta that were stable for the first hour; of these puncta, 15 remained stable for the entire 5 hours while 9 were eliminated (Fig 3.9). This argues strongly that puncta that are stable for at least 1 hour retain the ability to be eliminated. It also demonstrates that at the peak of synaptogenesis in culture (14 DIV), a subset of presynaptic puncta are stable for at least 5 hours.

3.3 CONCLUSIONS

The developing brain is wired during development by forming excess connections that are pruned before the developmental period ends. This necessitates the elimination of inappropriate inputs and the stabilization of appropriate inputs. This also suggests that structural plasticity is essential to proper cortical development. In this study, we found that the number of VGLUT positive excitatory presynaptic inputs onto dendrites in cortical culture increases during synaptogenesis (from 6 to 14 DIV). By 18 DIV, the number of inputs was decreased relative to that at 14 DIV, suggesting that a subset of inputs was eliminated. We further found that the dynamics of presynaptic inputs are developmentally regulated. At 11 DIV, only 14% of Syn-GFP puncta are stable. By 14 DIV, 24% of puncta are stable and by 17 DIV, that number is increased to 59%, suggesting that presynaptic vesicle stabilization is developmentally regulated. A recent study utilizing *in vivo* imaging in mice found that the stability of spines is also developmentally regulated (Holtmaat et al., 2005). They found a steady developmental increase (from P16 to P175) in the number of persistent spines, defined as spines lasting for > 8 days. At P16-25, 35% of spines were stable, while by PND 35-80, that number had reached 54%. Our findings, together with those of Holtmaat et al., suggest that both pre- and postsynaptic elements are increasingly stabilized during development, perhaps reflecting the closing of plasticity windows. Implicit in this fact is the finding that a portion of synapses is quite dynamic during early development. We found that a large number of presynaptic puncta were formed and eliminated at 11 and 14 DIV, with additions outnumbering eliminations. Additionally, dwell time analysis at 14 DIV

revealed that a significant population (63%) of puncta had lifetimes of only 0-40 minutes. Strikingly, stable puncta were capable of maintaining stability for at least 5 hours (the longest imaging period in our experiments), suggesting that at least 2 distinct mechanisms govern the lifetime of transient versus stable inputs.

We asked whether contact with a dendrite was necessary for stabilization of Syn-GFP puncta. In support of this assumption, we found that stable puncta overwhelmingly were in contact with dendrites (80%). This argues strongly that a stabilization signal resides within the dendritic compartment. However, a significant fraction of puncta (62%) that came into contact with dendrites did not stabilize, suggesting that contact with a dendrite is not sufficient for stabilization. One potential explanation is that the stabilization signal is found at only a subset of dendritic sites. This is consistent with recent work from El Husseini and colleagues that proposes that the location of “preformed complexes” of postsynaptic proteins, including PSD-95, GKAP and Shank, dictate sites of potential synapses and may help to recruit presynaptic elements (Gerrow et al., 2006).

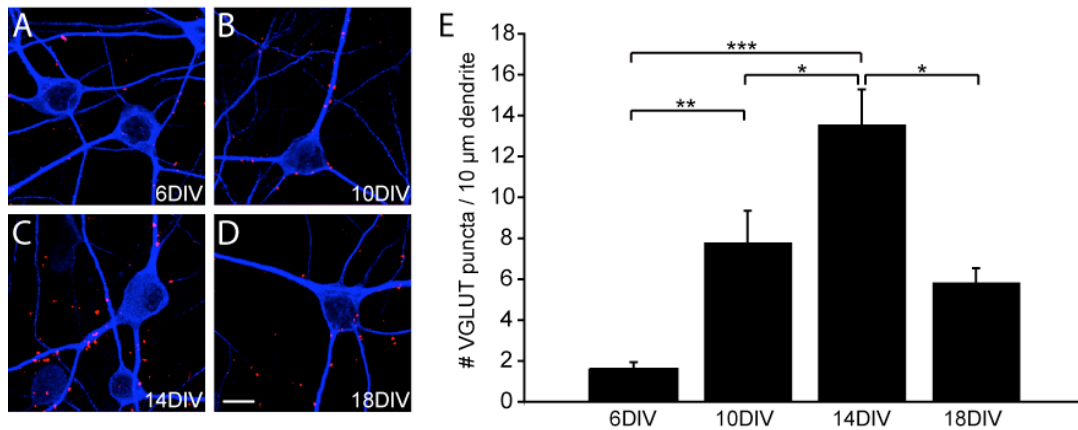


Figure 3.1 The number of presynaptic inputs increases over time

A-D. Development of excitatory presynaptic inputs in cortical cultures. Cortical neurons were stained for the excitatory presynaptic markers VGLUT 1 & 2 (red) and the dendritic marker MAP2 (blue) at 6, 10, 14, and 18DIV. Scale bar =10 μm.

E. Quantification of excitatory inputs (the number of VGLUT 1 & 2 positive puncta per 10 μm of dendrite) for each neuronal age. Few excitatory inputs were seen at 6DIV. The number peaked at 14DIV, then decreased by 18DIV, suggesting that only a subset of presynaptic inputs observed at 14DIV were stabilized. n=8-20 f.o.v. for each time point. Data are from 3 separate cultures. *** p < 0.001, ** p < 0.01, * p < 0.05. Error bars represent SEM.

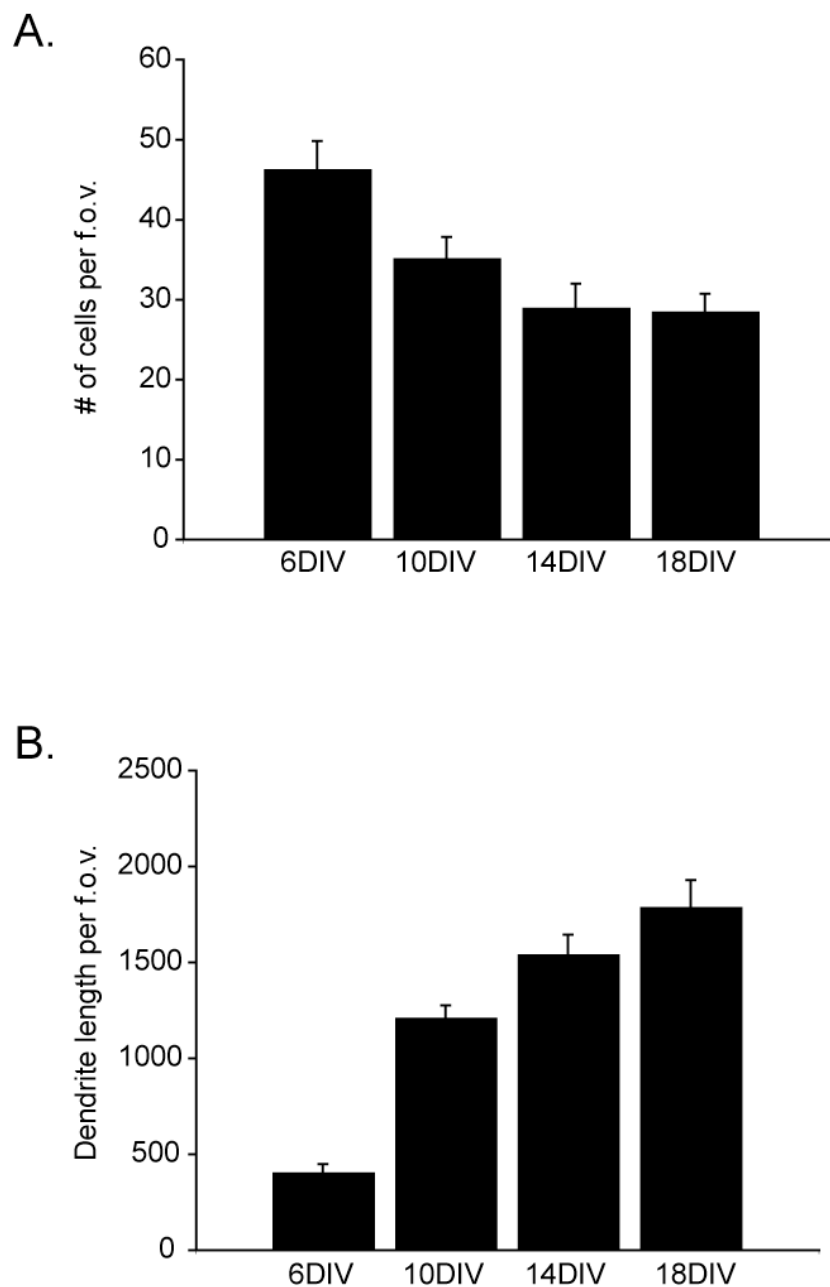


Figure 3.2 Cell survival and dendrite length as a function of number of days in culture.

A. Quantification of the number of cells per field of view (f.o.v.) at 6-, 10-, 14- and 18DIV. n=10 f.o.v. each developmental age, 2 separate cultures.

B. Quantification of total dendrite length per f.o.v. at 6-, 10-, 14- and 18DIV. n=15-40 f.o.v per developmental age, 3 separate cultures.

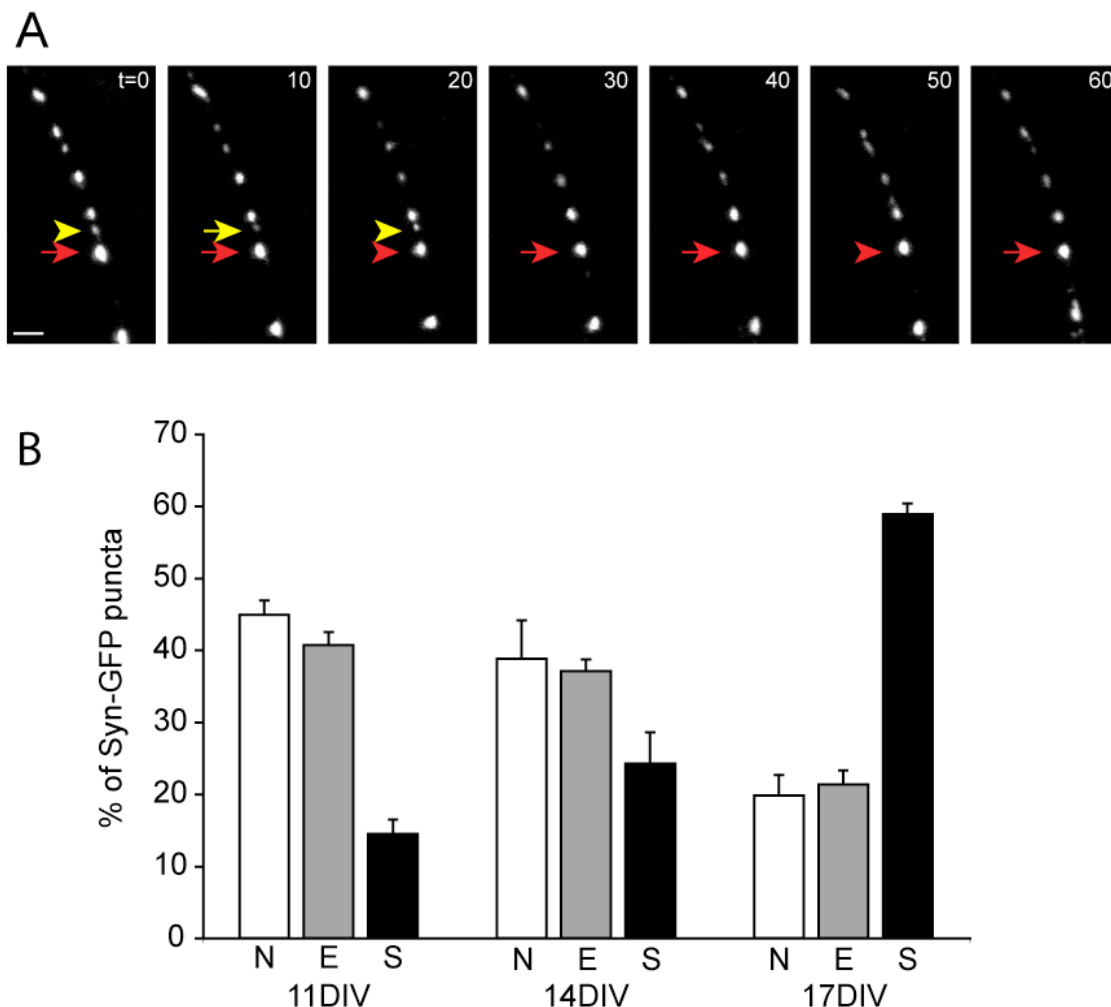


Figure 3.3 The fraction of stable presynaptic inputs increases over time

A. An example of an axon segment from a neuron expressing Syn-GFP (transfected at 4 DIV and imaged at 14 DIV) imaged at 10 minute intervals over the course of an hour. The red arrow points to a Syn-GFP positive puncta that was stable for at least 60 minutes. The yellow arrow points to a puncta that was present in the first 3 imaging frames but disappeared by the 4th frame. Scale bar = 2 μ m.

B. Quantification of the fraction of new (N), eliminated (E), and stable (S) puncta observed at 11, 14 and 17 DIV. New puncta appeared during the hour-long imaging period and persisted for at least 2 consecutive frames. Eliminated puncta were any puncta present for at least 2 consecutive frames that disappeared before the end of the hour imaging period (see panel A, yellow arrow for an example). Stable puncta were present before the imaging window began and persisted throughout the entire hour (see panel A, red arrow for an example). Data are from 2 separate cultures, 4 fields of view, 200-400 puncta each developmental age. Error bars are SEM.

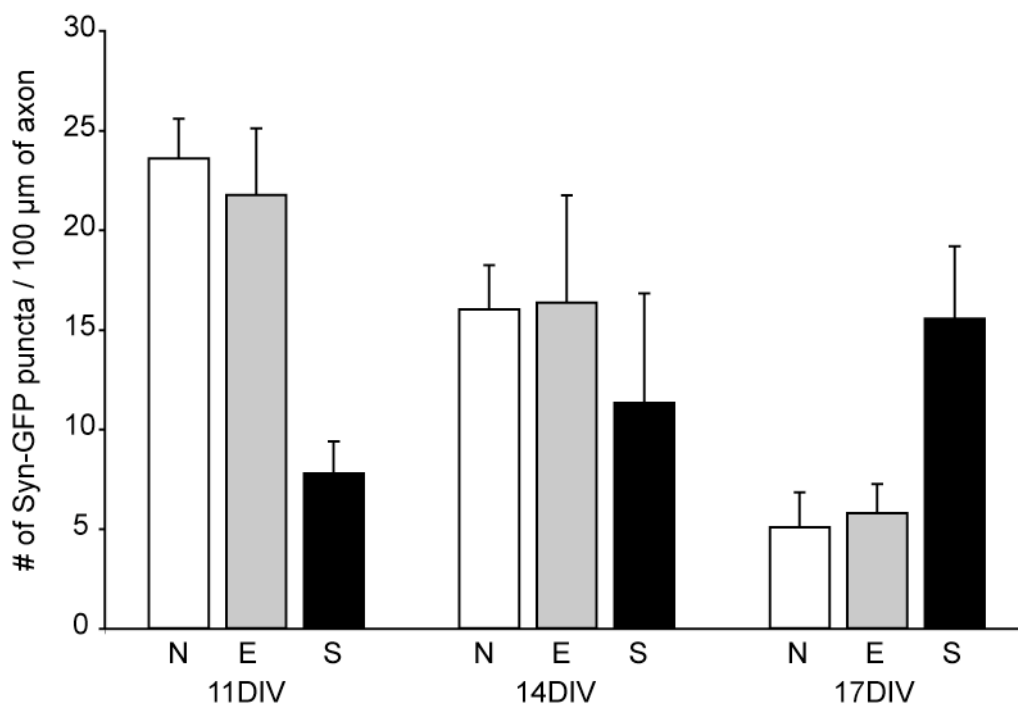


Figure 3.4 The number of stable presynaptic inputs increases over time

A. Quantification of the number of new (N), eliminated (E), and stable (S) puncta per 100 μm of axon observed at 11, 14 and 17 DIV. New puncta appeared during the hour-long imaging period and persisted for at least 2 consecutive frames. Eliminated puncta were any puncta present for at least 2 consecutive frames that disappeared before the end of the hour imaging period (see Figure 3.3 panel A, yellow arrow for an example). Stable puncta were present before the imaging window began and persisted throughout the entire hour (see Figure 3.3 panel A, red arrow for an example). Data are from 2 separate cultures, 4 fields of view, 200-400 puncta each developmental age. Error bars are SEM.

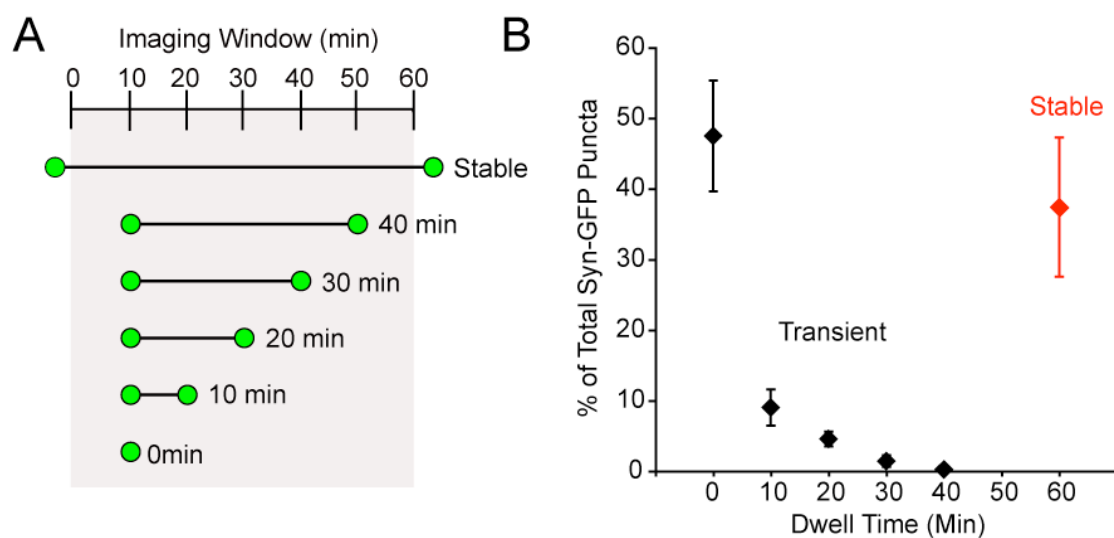


Figure 3.5 Dwell times of presynaptic inputs at 14DIV

A. Schematic of the imaging paradigm. Z-stacks were acquired every 10 minutes across 1 hour. Syn-GFP puncta were assigned a dwell time that corresponded to the shortest time they could have remained in a given location. Puncta that were present in only 1 imaging frame and were absent from frames immediately preceding and following were assigned a dwell time of 0 minutes. Puncta present in 2 frames were assigned a dwell time of 10 minutes, puncta present in 3 frames were assigned a dwell time of 20 minutes and so forth. Puncta that were present during the entire imaging experiment were labeled as stable. Puncta that were present before imaging began but disappeared during imaging (see Fig 3.3A, yellow arrow) or puncta that appeared during imaging and persisted throughout the remainder of the experiment were excluded from analysis since it was impossible to determine an accurate dwell time.

B. Quantification of dwell times. For each imaging experiment, the total number of puncta to which dwell times could be assigned was determined and the percentage of that number that dwelled for 0-60 minutes was graphed. The graph shows mean values \pm SEM for puncta from 3 separate cultures (a total of 566 puncta imaged across 19 fields of view).

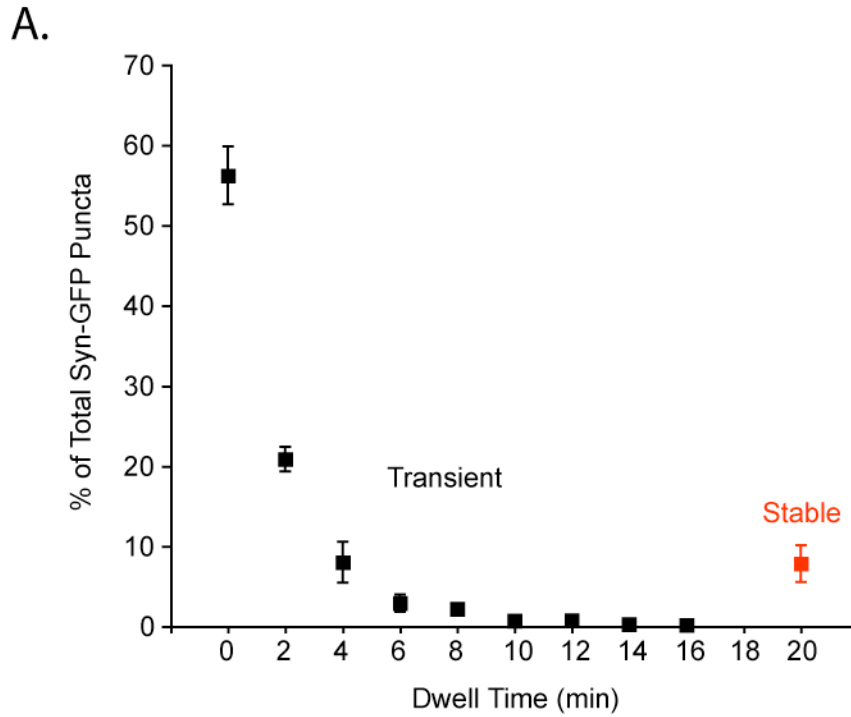


Figure 3.6 A faster sampling rate reveals that a majority of presynaptic inputs are transient at 14DIV

A. Quantification of dwell times for puncta imaged across 20 minutes with a 2 minute sampling rate at 14DIV. The graph shows mean values \pm SEM for puncta from 3 separate cultures (a total of 1256 puncta).

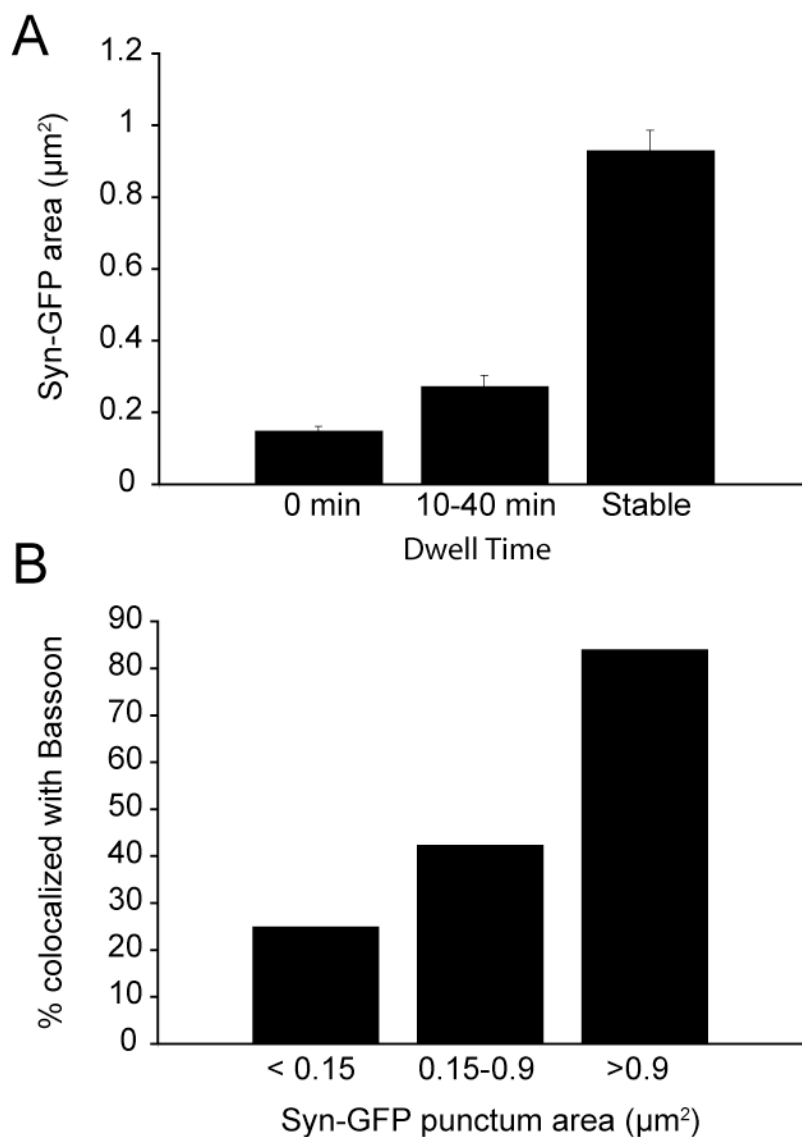


Figure 3.7 Syn-GFP punctum size is a good indicator of stability of presynaptic vesicles

A. Quantification of Syn-GFP size as a function of punctum dynamics. In general, the longer the dwell time, the larger the Syn-GFP punctum was. N=288 stable puncta, 189 puncta with dwell time of 0 minutes, and 78 puncta with dwell time of 10-40 minutes. Error bars represent SEM.

B. Colocalization of Bassoon and Syn-GFP as a function of Syn-GFP size. Syn-GFP puncta were separated into 3 size groups: $< 0.15 \mu\text{m}^2$, $0.15-0.9 \mu\text{m}^2$, and $> 0.9 \mu\text{m}^2$, which corresponded with average dwell times of 0, 10-40 or > 60 minutes (see A). The fraction of Syn-GFP puncta within each group that colocalized with the presynaptic terminal marker bassoon is graphed here.

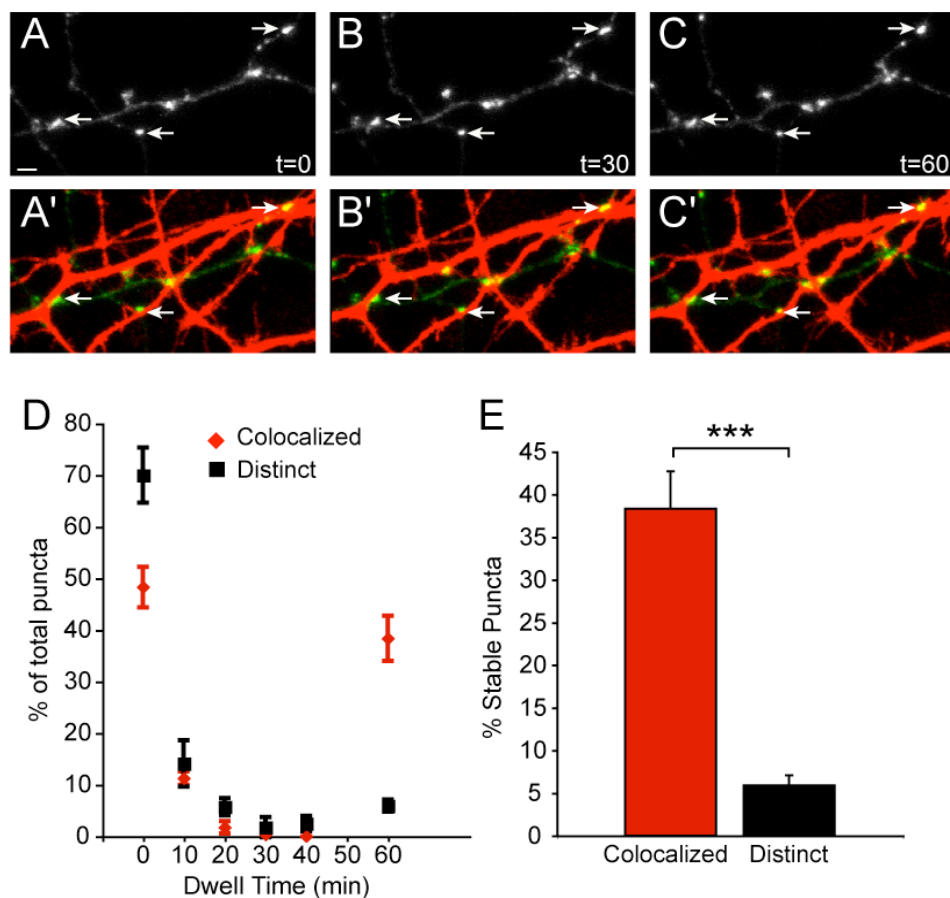


Figure 3.8 Stable puncta are associated with dendrites

Time-lapse imaging of presynaptic Syn-GFP puncta interacting with postsynaptic dendrites. A subset of neurons were transfected with Syn-GFP (green) at 4DIV and a separate subset were transfected at 7DIV with mCherry (red) to visualize dendrites (A-C). Sites where Syn-GFP puncta contacted mCherry positive dendrites were located and imaged at 10 minute intervals across 60 minutes at 14DIV. A-C show axons expressing Syn-GFP puncta at time 0-, 30- and 60-minutes, respectively. A'-C' are the same images, now merged with the red channel (mCherry positive dendrites). White arrows point to examples of puncta that were stable across 60 minutes. Note that these were all in contact with dendrites, suggesting that contact with a dendrite is necessary for presynaptic stabilization. Scale bar = 2 μ m. D. Distribution of Syn-GFP dwell times at and away from sites of dendritic contact. Syn-GFP puncta were divided into a population colocalized with dendrites (red diamonds, n=155 puncta) and a population distinct from dendrites (black squares, n=267 puncta) and were assigned dwell times. We confirmed by DIC imaging and/or retrospective immunostaining that no unlabeled dendrites were in the imaging field. A greater percentage of puncta were trafficking in the distinct from dendrites vs. colocalized with dendrites group. Data are from 5 separate cultures. Graph shows mean values \pm SEM. E. Percentage of puncta in the “colocalized” and “distinct” groups that were stable for 60 minutes (n=5 fields of view). *** p<0.001.

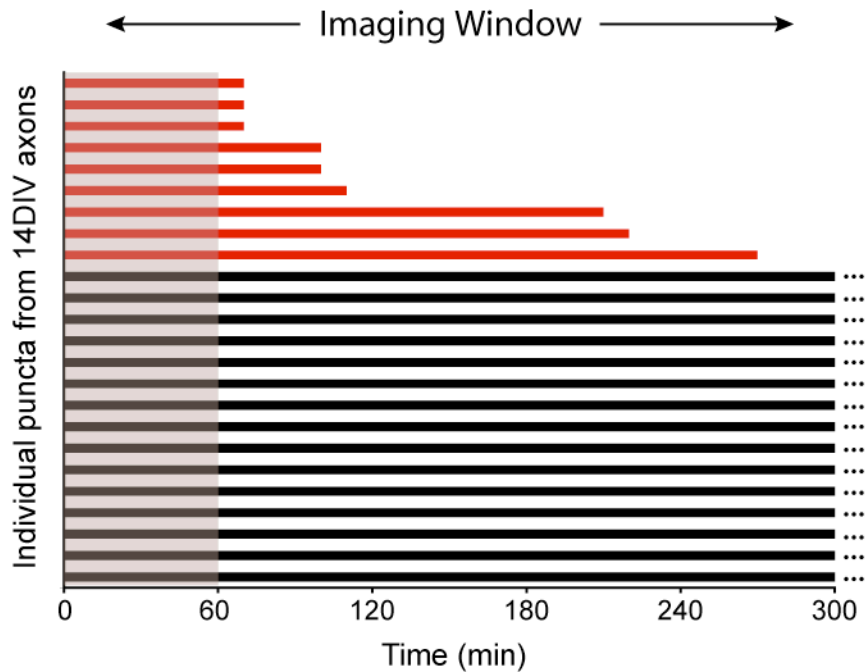


Figure 3.9 Presynaptic puncta can remain stable for at least 5 hours

A. We imaged Syn-GFP dynamics for 5 hours with a 10 minute sampling rate to determine how long puncta could remain stable at 14DIV. Here, we graphed the lifetimes of 24 puncta that maintained stability for the 1st hour of imaging (shaded gray box). 15 of these puncta remained stable for the full 5 hours of imaging while 9 puncta were eventually eliminated. This suggests that a portion of puncta can remain stable for long periods of time. It also suggests that puncta that are stable for at least 1 hour can still be eliminated.

**CHAPTER 4: RECRUITMENT OF AMPA
RECEPTORS TO THE SYNAPSE**

4.1 INTRODUCTION

It is widely thought that the hallmark of a mature synapse is the incorporation of AMPA receptors into the postsynaptic density. AMPA receptors are ionotropic glutamate receptors composed of tetrameric combinations of the subunits GluR1 through GluR4 (Hollmann and Heinemann, 1994). Electrophysiological and immunohistochemical experiments suggest that a large percentage of young synapses contain NMDA receptors but lack AMPA receptors (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Li and Zhuo, 1998; Isaac, 2003). Over the course of synaptogenesis, AMPA receptors are gradually recruited to postsynaptic sites, resulting in an increase in the AMPA/NMDA ratio at these synapses (Crair and Malenka, 1995; Isaac et al., 1997; Hsia et al., 1998; Lu et al., 2001; Ye et al., 2005). This recruitment is dependent on NMDA receptor-mediated activity (Hayashi et al., 2000; Zhu et al., 2000).

GluR1/2 and GluR2/3 heteromers are assembled in the endoplasmic reticulum (ER) and are differentially trafficked to the synapse. GluR1/2 receptors quickly leave the ER, while a large portion of GluR2/3 receptors are actively retained in the ER (Greger et al., 2002). Trafficking of GluR subunits is determined, in part, by their c-terminal domains (c-tails). The PDZ domain of GluR2 interacts with several proteins, including PICK1, and this interaction is necessary for its trafficking out of the ER (Song and Huganir, 2002). The GluR2 c-tail additionally interacts with GRIP1. GRIP1 interacts with several microtubule motor proteins and is important for trafficking of AMPARs to the synapse (Dong et al., 1997; Setou et al., 2002). The GluR1 c-tail interacts with SAP97

and while it is not known whether this interaction is necessary for ER export, it is clear that this interaction is important for trafficking into synapses (Hayashi et al., 2000).

The insertion of GluR1/2 and GluR2/3 receptors into the synaptic membrane is differentially regulated. While GluR2/3 containing receptors are constitutively recycled into and out of the membrane independent of activity (Passafaro et al., 2001; Shi et al., 2001), the insertion of GluR1/2 containing receptors is dependent on phosphorylation events mediated by NMDAR-dependent activity (Hayashi et al., 2000; Esteban et al., 2003). Removal of GluR2-containing receptors is mediated by PICK1 and AP2, and reinsertion is dependent on interactions with NSF (Chung et al., 2000; Perez et al., 2001; Hanley et al., 2002). Trafficking of GluR1/2 containing receptors is controlled by the GluR1 c-tail, while the trafficking of GluR2/3 containing receptors is controlled by the GluR2 c-tail (Shi et al., 2001).

In addition to c-tail mediated trafficking, AMPA receptors are trafficked via interactions with a family of proteins known as transmembrane AMPAR regulatory proteins, or TARPs (reviewed in (Ziff, 2007)). Four TARPs, γ -2 (a.k.a. stargazin), -3, -4 and -8, have been shown to regulate AMPAR surface expression and synaptic targeting (Tomita et al., 2003). They additionally modulate AMPAR current (Yamazaki et al., 2004). In contrast to c-tail mediated trafficking, TARP-mediated trafficking does not appear to be differentially regulated based on subunit composition, and may represent a more global trafficking paradigm (Ziff, 2007). It does appear critical for trafficking, however, as knockout of different TARPs abolishes AMPAR currents in the neurons normally expressing those TARPs (Hashimoto et al., 1999; Chen et al., 2000; Rouach et al., 2005).

AMPA receptors are traditionally thought of as calcium impermeable and non-rectifying, in contrast to NMDA receptors. The majority of AMPA receptors contain a GluR2 subunit. RNA editing of the GluR2 subunit from a neutral glutamine to a positively charged arginine at position 586 within the pore region causes receptors containing this subunit to be resistant to calcium and non-rectifying (Hollmann and Heinemann, 1994). Recent reports have shown that AMPA receptors lacking GluR2 exist transiently during development (Pickard et al., 2000; Kumar et al., 2002; Ho et al., 2007). This receptor type is unique in two ways—it fluxes calcium and exhibits inward rectification. This is likely of great relevance, given the role of calcium as a second messenger during development (Redmond and Ghosh, 2005). Thus GluR2 lacking AMPA receptors may contribute to synapse formation or plasticity during development.

We presented data in Chapter 3 that suggests that stabilization of presynaptic inputs is developmentally regulated and is critically dependent on a postsynaptic signal present at a subset of dendritic sites. As a complement to that body of work, here we examined the maturation of synapses from the postsynaptic side. Specifically, we characterized the recruitment of GluR1 and GluR2 containing AMPA receptors to the membrane surface and to synapses during synaptogenesis, as well as the relative complement of GluR1 and GluR2 subunits at synapses as assessed by immunohistochemistry.

4.2 RESULTS

AMPA receptor surface expression is developmentally regulated

We determined a developmental time course of AMPA receptor insertion into the postsynaptic membrane (Fig 4.1). To achieve this, we live-labeled GluR1 and GluR2 AMPAR subunits with monoclonal antibodies raised against their extracellular N-terminal domains (NTDs). Live-labeling receptors allowed us to exclusively visualize receptors on the membrane surface. The majority of AMPA receptors in cortical culture are either GluR1/GluR2 containing or GluR2/GluR3 containing, so by staining for GluR1 and GluR2 we were able to visualize most AMPA receptors (data not shown). We found that the number of surface-expressed GluR1, GluR2 and total GluR (GluR1 and/or GluR2) puncta per dendrite length was developmentally regulated. At 6 DIV, there was a modest number of receptor puncta per 10 μm of dendrite (2.8 ± 0.42 GluR1 puncta, 1.2 ± 0.48 GluR2 puncta, 3.7 ± 0.84 total [GluR1 and/or GluR2] puncta, all per 10 μm of dendrite) (Fig 4.1A, E). The number of surface-expressed receptors increased by 10 DIV (6.1 ± 1.3 GluR1 puncta, 7.7 ± 0.72 GluR2 puncta, 12 ± 1.4 total puncta) (Fig 4.1B, E). At 14 DIV, the number of surface receptors was similar to that seen at 10 DIV (7.4 ± 0.69 GluR1 puncta, 7.1 ± 1.0 GluR2 puncta, 12 ± 1.4 total puncta) (Fig 4.1C, E). Interestingly, the number of receptors expressed on the surface at 18 DIV was lower than that seen at either 10 or 14 DIV (3.0 ± 0.23 GluR1 puncta, 3.8 ± 0.46 GluR2 puncta, 5.6 ± 0.40 total puncta) (Fig 4.1 D, E). Together, these results demonstrate that the surface targeting of GluR1 and GluR2 containing AMPA receptors is developmentally regulated, with the

highest levels coinciding with a time of active synaptogenesis in cortical culture (10-14 DIV; see Fig 3.1).

AMPA receptors are present at presynaptic input sites

To determine the association of AMPA receptors with presynaptic terminals, we live-labeled neurons with antibodies against the NTDs of the AMPA receptor subunits GluR1 and GluR2. We then fixed neurons and additionally stained for VGLUT 1 & 2 (an excitatory presynaptic input marker) and MAP2 (a dendritic marker). We quantified the number of GluR1 and GluR2 puncta that colocalized with VGLUT per 10 μm of dendrite length across the period of synaptogenesis (6, 10, 14 and 18 DIV) (Fig 4.2). We found that there were few AMPA receptor-containing synapses at 6 DIV (0.25 ± 0.05 GluR1/VGLUT puncta, 0.16 ± 0.05 GluR2/VGLUT puncta, 0.36 ± 0.10 Total [GluR1 and/or GluR2]/VGLUT colocalized puncta per 10 μm dendrite) (Fig 4.2A, E). These numbers increased by 10 DIV (0.78 ± 0.17 GluR1/VGLUT puncta, 1.8 ± 0.40 GluR2/VGLUT puncta, 2.17 ± 0.41 Total/VGLUT puncta) (Fig 4.2 B, E). The number of synapse-localized receptors was highest at 14 DIV (2.1 ± 0.28 GluR1/VGLUT puncta, 2.4 ± 0.31 GluR2/VGLUT puncta, 3.45 ± 0.40 Total/VGLUT puncta) (Fig 4.2C,E) and decreased by 18 DIV (0.89 ± 0.18 GluR1/VGLUT puncta, 1.2 ± 0.18 GluR2/VGLUT puncta, 1.73 ± 0.30 Total/VGLUT puncta per 10 μm) (Fig 4.2D,E). Thus, recruitment of AMPA receptors to synaptic sites is also developmentally regulated across synaptogenesis.

Heterogeneity of AMPA receptors during development

While the majority of AMPA receptors are composed of GluR1/2 heteromers or GluR2/3 heteromers, recent reports describe the presence during development of a third species of AMPA receptor that lacks the GluR2 subunit (Pickard et al., 2000; Kumar et al., 2002; Ho et al., 2007). The combination of receptor subunits present at individual synapses is likely to strongly influence ligand affinity and ion conductance properties, and may activate different types of signaling pathways, allowing for unique information transfer on a single synapse level (Hollmann and Heinemann, 1994). To determine whether different combinations of receptor subunits are present at developing cortical synapses, we assessed the degree of colocalization of GluR1 and GluR2 at individual VGLUT positive synapses across development. We additionally assessed the composition of all receptors on the surface, regardless of synapse status. This data is summarized in Table 4.1. Our findings suggest that a large percentage of AMPA receptors on the membrane surface and in synapses are either GluR1 or GluR2 lacking during synaptogenesis. Immunostaining data are qualitative and thus we cannot say for sure that the absence of fluorescent signal translates to a true absence of receptor subunit at synapses. Rather, the data suggests that the relative levels of these subunits are different at different developmental ages and at different synapses within a given age. Immunocytochemistry (EM) would shed light on the absolute receptor subunit contribution at individual synapses. Still, the data argue for a heterogeneous population of synapses with varying levels of GluR1 and GluR2 present at the postsynaptic density during synaptogenesis.

4.3 CONCLUSIONS

To begin to answer the question of how synapses are eventually stabilized, we must first understand the processes of synapse maturation, as maturation is likely to be a prerequisite for long-term stabilization. The experiments presented here address the defining characteristic of postsynaptic maturation—AMPA receptor incorporation into the synaptic membrane. We quantified the number of GluR1 and GluR2 containing AMPA receptors that were targeted to the surface and to synapses during synaptogenesis (6-18 DIV). We found that both surface and synaptic targeting were tightly regulated during development. Few receptors were present on the membrane surface and in synapses at 6 DIV, but by 14 DIV the number had increased 3-fold in the case of surface expression and 10-fold in the case of synaptic incorporation. This impressive increase suggests that mechanisms for AMPA receptor synaptic targeting are actively at work and are upregulated during synaptogenesis. Indeed, in a separate series of experiments, we identified two mechanisms that act as positive and negative regulators of AMPA receptor incorporation into the synapse during development (Ince-Dunn et al., 2006; Hall et al., 2007). These experiments are described in Appendices 1 and 2.

At 14 DIV, a period of dynamic turnover for presynaptic puncta, the 3.5 AMPA receptor/VGLUT colocalized puncta per 10 μm of dendritic length (Fig 4.2) fell short of the 12 VGLUT clusters per 10 μm of dendrite we observed earlier (Fig 3.1). Thus, only 30% of presynaptic inputs were apposed to GluR1 or GluR2 subunits at this age. Data presented in Chapter 3 demonstrated that presynaptic inputs stabilized at only a subset of

dendritic contact sites (Fig 3.8). It is possible that the 30% of presynaptic inputs colocalized with AMPA receptors represent the population of presynaptic inputs that are stable. The remaining 70% of VGLUT puncta negative for GluR1/GluR2 could either be in the process of trafficking to synaptic sites or could be located at nascent synaptic sites. This raises the possibility that AMPA receptors provide the dendrite-derived stabilization signal proposed in Chapter 3 (this hypothesis is discussed in detail in Chapter 5).

It is possible that each synapse contains roughly the same complement of GluR1 and GluR2 subunits. However, it is well known that GluR1 and GluR2 subunits are differentially trafficked and maintained at synapses (Song and Huganir, 2002). GluR1 and GluR2 subunits have different ligand affinities and conductance properties and potentially signal via different downstream signaling pathways. Further, GluR2 lacking AMPA receptors are calcium permeable, allowing for unique calcium-mediated signaling at the synapses in which they are present. Thus, different complements of GluR1 and GluR2 at synapses would allow for heterogeneous signaling at synapses. We demonstrate here that subsets of synapses have expression levels of GluR1 or GluR2 that are below the detection limit of immunostaining and that the proportion of these receptors changes across development. This raises the possibility that these synapses have different signaling in response to activity or may participate in different structural interactions with other synapse-associated proteins during development. Further experiments addressing these possibilities are warranted.

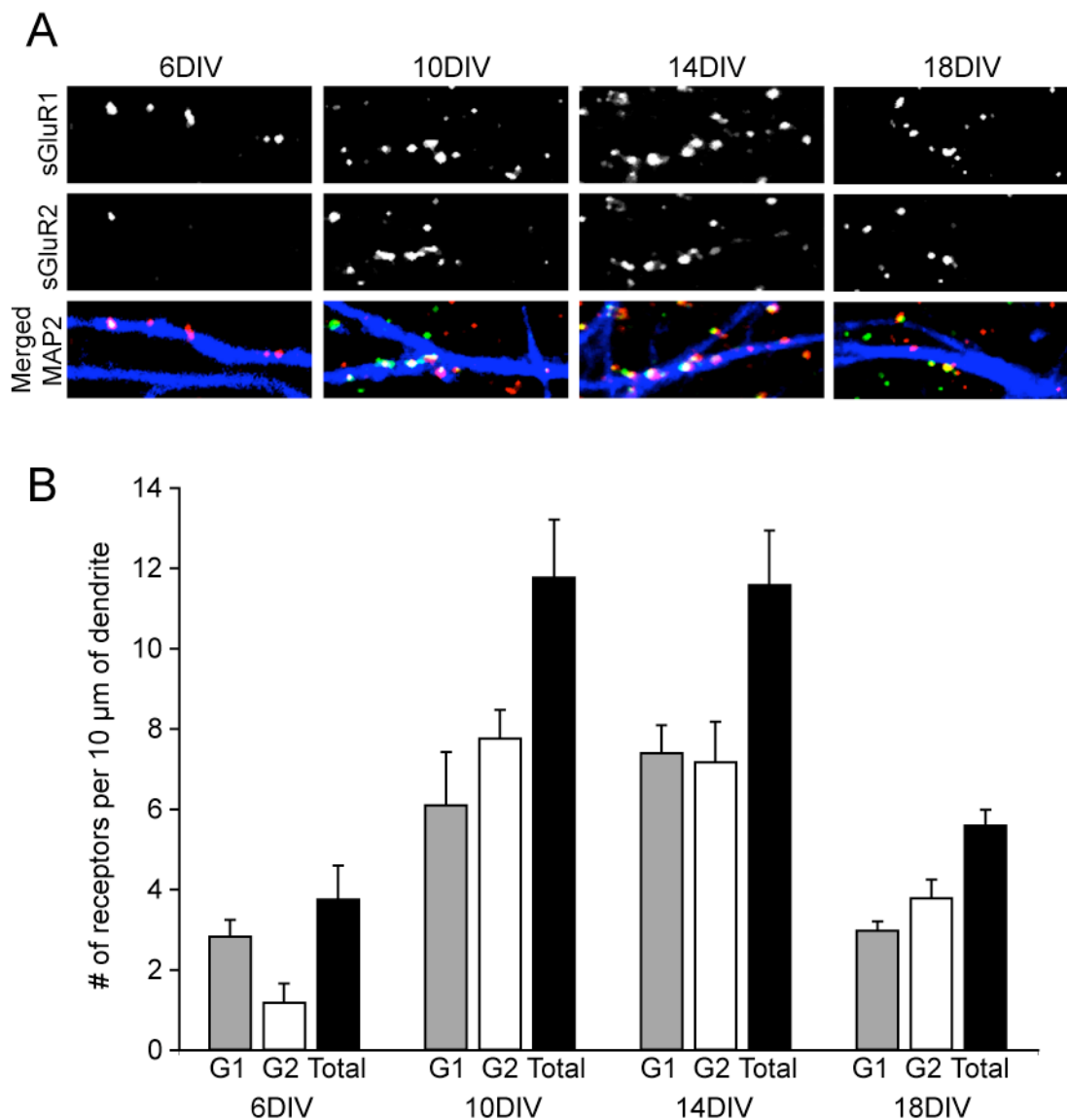


Figure 4.1 AMPA receptor surface expression increases over time

A. Examples of AMPA receptor staining at 6, 10, 14 and 18DIV. Surface GluR1 (red), surface GluR2 (green) and MAP2 (blue; dendritic marker) are depicted together in the merged images (bottom panels).

B. Quantification of the number of surface-expressed receptor clusters per 10 μm of dendrite across synapse development (6-18DIV). Surface GluR1 (G1), surface GluR2 (G2) and total number of AMPA receptor puncta (Total; GluR1 and/or GluR2 positive) are graphed here. (n=8-15 f.o.v. for each time point, 3 separate cultures). Error bars are SEM.

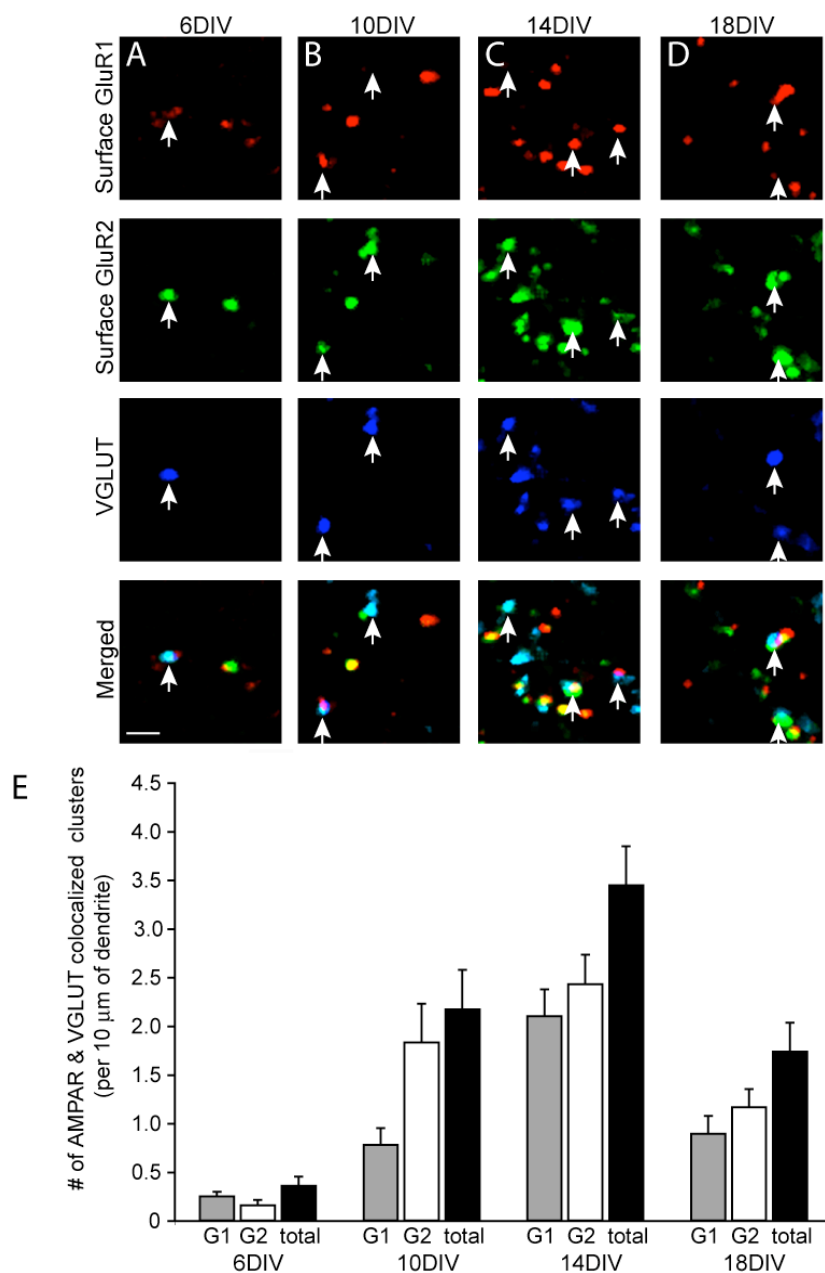


Figure 4.2 AMPA receptor recruitment to synapses increases over time

AMPA receptor staining at 6, 10, 14 and 18DIV (A-D). Surface GluR1 and GluR2 are depicted in red and green, respectively. VGLUT 1 & 2 staining is shown in blue. White arrows point to examples of AMPA receptor clusters colocalized with VGLUT 1 & 2. Scale bar = 1 μm. E. Quantification of the number of AMPA receptor puncta colocalized with VGLUT puncta per 10 μm of dendrite. Surface GluR1 (G1), surface GluR2 (G2) and total number of AMPA receptor puncta (total; GluR1 and/or GluR2 positive) are graphed here (n=8-15 f.o.v. for each time point, 3 separate cultures).

Table 4.1 Colocalization of GluR1 and GluR2 subunits

SYNAPTIC (V-GLUT POSITIVE)	10 DIV	14 DIV	18 DIV
% GluR1 positive for GluR2	63 ± 6.3%	51 ± 2.8%	46 ± 6.6%
% GluR2 positive for GluR1	26 ± 3.8%	46 ± 4.1%	30 ± 2.4%
TOTAL SURFACE RECEPTOR	10 DIV	14 DIV	18 DIV
% GluR1 positive for GluR2	46 ± 6.2%	40 ± 2.6%	39 ± 6.7%
% GluR2 positive for GluR1	26 ± 3.3%	46 ± 5.0%	29 ± 2.4%

**CHAPTER 5: THE ROLE OF AMPA RECEPTORS
IN PRESYNAPTIC STABILIZATION**

5.1 INTRODUCTION

Contact between an axon and a dendrite is not sufficient to induce a stable synapse. Indeed, only a small percentage of axo-dendritic contact sites result in a mature synapse, suggesting that additional signals are necessary for synapse stability (Chklovskii et al., 2004). What are the likely criteria for a stabilization signal? This signal should be present at only a subset of axo-dendritic contact sites, it should be able to signal trans-synaptically to ensure proper stabilization of both pre and postsynaptic inputs, and it should be easily removed from inappropriate contacts and maintained at correct contacts. Key proteins such as neurexins/neuroligins, SynCAMs, Ephs/EphB receptors, and netrin G ligand 2/netrin G2 are known to signal trans-synaptically to induce recruitment of necessary pre and postsynaptic components (Scheiffele et al., 2000; Biederer et al., 2002; Kayser et al., 2006; Kim et al., 2006). However, they are unlikely to play a role in synapse stability because their promiscuous nature precludes the requirement for a signal present at only a subset of nascent synapses. It also is unclear whether they are easily trafficked into and out of synapses.

A central goal of this work was to determine the nature of the dendrite-derived signal that regulates presynaptic input stability (described in Chapter 3). AMPA receptors were an intriguing candidate because several nascent synapses contain NMDA receptors but not AMPA receptors (Isaac, 2003), the AMPA/NMDA ratio increases over development and AMPA receptor trafficking is tightly regulated (Bolton et al., 2000) (Ehlers et al., 2007). Further, data presented in Chapter 4 demonstrate that AMPA receptor trafficking to the surface and to synapses is developmentally regulated.

While AMPA receptors are most famous for their role as the major excitatory ionotropic receptor in the brain, they are gaining recognition for their potential role in aspects of synapse formation separate from neurotransmission. Recent studies suggest that overexpression of the GluR2 subunit of AMPA receptors can increase the size and number of postsynaptic spines (Passafaro et al., 2003; Saglietti et al., 2007). This effect is mediated by the extracellular NTD of GluR2, and in fact, the NTD alone is sufficient to increase spine size and number. The NTD of GluR2 can also influence the number of presynaptic inputs onto cells (Saglietti et al., 2007). Another potential role for AMPA receptors is in the shaping of dendritic arbors. Work from Cline and colleagues demonstrated that interfering with AMPA receptor targeting to synapses via dominant negative c-tail constructs led to significant decreases in dendritic arbor complexities in *Xenopus* optic tectal neurons (Haas et al., 2006). This was due in part to the fact that new dendritic branches were less likely to stabilize in neurons with decreased AMPA receptor levels. Additionally, presynaptic inputs onto these neurons were found by EM to be less mature, as assessed by decreased accumulation of synaptic vesicles. Thus there appears to be a role for AMPA receptors in proper development of dendrites, spines and potentially even synapses.

In this study, we hypothesized that reverse signaling by an AMPA receptor-associated complex regulates presynaptic input stability. We demonstrated previously using time-lapse imaging of Syn-GFP puncta that a portion of presynaptic puncta are stabilized during the period of synaptogenesis while another portion are eliminated. We also demonstrated that puncta preferentially stabilized at sites of dendritic contact. Here

we show that stable puncta were associated with AMPA receptor subunits a majority of the time. AMPA receptors were absent from sites where presynaptic puncta were eliminated, suggesting that a lack of AMPA receptors at the synapse leads to presynaptic elimination. Indeed, removal of AMPA receptors from the surface of postsynaptic cells via dominant negative constructs led to a dramatic decrease in the number and stability of presynaptic inputs onto these cells. Overexpression of AMPA receptors increased the number of inputs onto neurons. Finally, the NTD of GluR2 expressed along with neuroligin-1 in 293T cells was sufficient to stabilize presynaptic inputs onto heterologous cells. All of these observations support a role of postsynaptic AMPA receptors in regulating the number and stability of presynaptic inputs.

5.2 RESULTS

Correlation between presynaptic stability and postsynaptic AMPA receptors

Our previous studies suggested that presynaptic inputs were stabilized by a signal that was developmentally upregulated and present at a subset of dendritic locations (Chapter 3). We additionally showed that the surface expression and synaptic targeting of AMPA receptors were greatly upregulated during synaptogenesis and that AMPA receptors were present at only a subset of synaptic sites (Chapter 4). This suggested that AMPA receptors were well positioned to provide a retrograde stabilization signal to the presynaptic input. To determine whether presynaptic inputs co-localized with AMPA receptors were indeed more likely to be stabilized, we imaged the dynamics of Syn-GFP

puncta across 6 hours, and then live-labeled surface GluR1 or GluR2 receptors for 10 minutes at the end of the imaging period before fixing and staining neurons (Fig 5.1). We also stained for total NR1 in a subset of experiments. We found that 52.5% (± 5.69) of stable puncta were GluR1 positive and 66.7% (± 10.7) were GluR2 positive (Fig 5.1A, D). In contrast, only 29.7% (± 16.5) of sites where Syn-GFP puncta were eliminated were GluR1 positive and 27.9% (± 7.39) were GluR2 positive (Fig 5.1B, D). It is important to note that NR1 puncta were present at both stable and eliminated sites 60.0% (± 8.88) and 65.3% (± 12.2) of the time (Fig 5.1 C, D), ruling out the possibility that the postsynaptic site was completely disassembled or was never present at sites of presynaptic terminal elimination. Thus, there is a strong correlation between presynaptic elimination and the absence of postsynaptic AMPA receptors.

Knockdown of AMPA receptors leads to a decrease in presynaptic inputs

Because of the retrospective nature of the AMPA receptor staining in the previous experiments, it was impossible to determine whether AMPA receptors left nascent synaptic sites before the presynaptic terminal disengaged, or whether the presynaptic terminal withdrew prior to AMPA receptor internalization. Thus, we decided to actively remove AMPA receptors from the postsynaptic surface to determine whether a loss of AMPA receptors leads to a decrease in the number or stability of presynaptic inputs onto that neuron. To achieve AMPA receptor knockdown, we created fusion proteins in which the entire c-tail of either GluR1 or GluR2 was fused to CFP. These constructs have previously been shown to disrupt synaptic targeting of GluR1 and GluR2 (Shi et al.,

2001; Haas et al., 2006), likely by competing for endogenous proteins involved in the normal trafficking of these receptors. To confirm AMPA receptor knockdown, we tested these c-tail constructs in cortical neurons in culture (Fig 5.2). We found that the constructs were expressed and efficiently trafficked to dendrites and spines and that cells expressing c-tails were morphologically similar to control-transfected neurons (CFP alone) (Fig 5.2A-B). Whole cell voltage clamp recordings revealed that c-tail expressing neurons had decreased AMPA receptor-mediated currents in response to focal application of AMPA (B. Hall, personal communication) (data not shown). Additionally, live-immunostaining for surface expressed GluR1 and GluR2 revealed a significant reduction in the number of GluR1 and GluR2 puncta per dendrite length for c-tail transfected neurons when compared to CFP-transfected controls (control neurons: 1.00 ± 0.08 GluR1 puncta, c-tails: 0.23 ± 0.05 GluR1 puncta, $p < 0.0001$; control neurons: 1.00 ± 0.19 GluR2 puncta, c-tails: 0.41 ± 0.17 GluR2 puncta, $p < 0.05$; surface GluR puncta number per dendrite length normalized to controls) (Fig 5.2 C-F).

We transfected neurons with either GluR1 and GluR2 c-tails or CFP alone at 7 DIV and assessed the effects on VGLUT 1 & 2 (excitatory) and GAD6 (inhibitory) inputs at 14DIV. Strikingly, c-tail expressing neurons had a roughly 50% decrease in the number of excitatory inputs received (control neurons: 1.00 ± 0.08 VGLUT puncta, c-tails: 0.46 ± 0.06 VGLUT puncta, p value < 0.0001 ; VGLUT puncta per dendrite length normalized to control values) (Fig 5.3A-C), consistent with the idea that AMPA receptors are necessary for presynaptic terminal stabilization. Importantly, the number of inhibitory inputs onto these neurons was not altered (control neurons: 1.00 ± 0.11 GAD6 puncta, c-tails: 0.86 ± 0.19 GAD6 puncta, $p=0.51$; GAD6 puncta per dendrite length normalized to

control values) (Fig 5.3D) indicating that loss of AMPA receptors selectively affects excitatory presynaptic terminals.

In the next set of experiments, we directly examined the role of postsynaptic AMPA receptors in presynaptic stability by time-lapse imaging. We transfected a subset of neurons with the presynaptic vesicle marker Syn-GFP at 4DIV and a separate population with mCherry (to visualize neurons) and either CFP or GluR1 & 2 c-tails at 7DIV. At 11-12DIV, we identified areas where Syn-GFP expressing axons contacted mCherry positive dendrites (also expressing either CFP or c-tails) and imaged the dynamics of those axo-dendritic interactions at 10 minute intervals across 1 hour (Fig 5.4). Numerous Syn-GFP puncta contacted both control and c-tail expressing dendrites during each imaging period (average of 42 puncta/neuron for control neurons, 66 puncta/neuron for c-tail expressing neurons). We found that 21% (± 4.7) of Syn-GFP puncta contacting control neurons were stable across an hour (Fig 5.4A, C). This number is slightly lower than the number of stable puncta contacting dendrites seen in Figure 3.8, most likely due to the earlier age of imaging (11-12DIV vs. 14DIV). Strikingly, Syn-GFP puncta in contact with GluR c-tail expressing cells showed a marked decrease in stability when compared to controls ($9.3 \pm 1.5\%$ stable puncta, $p < 0.05$) (Fig 5.4B, C). This represents a greater than 50% decrease in stable inputs onto c-tail expressing neurons over the course of only one hour and strongly supports the hypothesis that AMPA receptors are necessary for presynaptic stabilization.

Overexpression of AMPA receptors increases excitatory presynaptic inputs

To determine if AMPA receptor overexpression leads to an increase in the number of presynaptic inputs, we transfected neurons with GFP-tagged GluR1 and GluR2 subunits at 7DIV and fixed and stained for VGLUT 1 & 2 and GAD6 inputs at 14DIV (Fig 5.5). To facilitate trafficking of AMPA receptors to the synapse, we additionally co-transfected neurons with stargazin (Ziff, 2007). In each set of experiments, we confirmed overexpression of AMPA receptors by live labeling a subset of transfected neurons with an antibody against the N-terminally located GFP tag on GluR1 and GluR2 (Fig 5.6). Neurons transfected with stargazin and GluR1 & 2 had a significantly increased number of VGLUT inputs per length of dendrite when compared to vector or vector + stargazin transfected controls (GluR1 & 2 / stargazin co-transfected neurons: 1.56 ± 0.11 VGLUT inputs per dendrite length vs. 1.13 ± 0.09 VGLUT inputs for stargazin transfected neurons [$p < 0.01$] and 1.00 ± 0.07 VGLUT inputs for controls [$p < 0.001$], all values normalized to controls) (Fig 5.5 A-C, G). This increase was selective for excitatory inputs, as there was no difference in the number of GAD6 positive inputs per dendrite length between the 3 conditions (GluR1 & 2 / stargazin co-transfected neurons: 0.95 ± 0.15 GAD6 inputs; stargazin transfected neurons: 1.15 ± 0.13 GAD6 inputs; vector transfected neurons: 1.00 ± 0.10 GAD6 inputs, GAD6 inputs per dendrite length normalized to control values; $p=0.24$) (Fig 5.5 D-F, H). These observations indicate that increased surface delivery of AMPA receptors in neurons leads to an increased number of excitatory presynaptic inputs.

Coexpression of AMPA receptors and neuroligin-1 in 293T cells is sufficient to stabilize Syn-GFP inputs onto heterologous cells

In the next set of experiments, we asked whether AMPA receptors could induce presynaptic input stability in a reduced heterologous culture system. Schieffele et al. first demonstrated that co-culturing neurons with neuroligin-1-expressing non-neuronal cells promotes the induction of presynaptic inputs onto those non-neuronal cells (Schieffele et al., 2000). We modified this assay into an imaging assay to test whether 293T cells expressing AMPA receptors are capable of stabilizing Syn-GFP inputs from co-cultured neurons. We cotransfected 293T cells with mCherry (for visualization) along with one of the following combinations of plasmids: neuroligin-1, GluR1 & GluR2, neuroligin-1 + GluR1 & 2, or vector (see Fig 5.7 for confirmation of expression). We then overlaid these 293T cells onto 10-11DIV neurons previously transfected with Syn-GFP (at 4DIV) and imaged interactions between 293T cells and Syn-GFP puncta 18-24 hours later.

We imaged Syn-GFP puncta dynamics at 10 minute intervals across an hour and determined a stability index for each 293T cell, defined as the fraction of stable (dwell time 60 minutes) to trafficking (dwell time 0 minutes) Syn-GFP inputs contacting that cell (Fig 5.8). This index is a good measure of the ability of the 293T cell to effectively trap and stabilize trafficking Syn-GFP puncta. Vector-transfected control 293T cells were not very efficient at stabilizing presynaptic inputs (stability index 0.23 ± 0.05) (Fig 5.8A-E, K). Neuroligin-1 expressing 293T cells had a slightly increased stability index (0.40 ± 0.05) (Fig 5.8 K), as did 293T cells expressing AMPA receptors only (stability index 0.31 ± 0.08) (Fig 5.8K) but neither were significantly different from controls. However, 293T

cells expressing neuroligin-1 in combination with AMPA receptors had an almost 4 fold increase in stability index relative to control cells (stability index: 0.87 ± 0.14 ; $p < 0.001$) (Fig 5.8F-J, K). This result strongly implies that a molecular interaction mediated by AMPA receptors themselves or by an AMPA receptor associated molecule endogenous to 293T cells can stabilize presynaptic inputs. It also further suggests that a synapse-inducing molecule (in this case, neuroligin-1) is required for AMPA receptor-mediated stabilization.

Activity blockade of AMPA receptors leads to an increase in presynaptic inputs

AMPA receptors are responsible for the majority of fast, excitatory neurotransmission in the brain. Activity through AMPA receptors could lead to downstream signaling events that could then lead to synthesis or recruitment of stabilizing molecules to the postsynaptic density (see model, Fig 5.9). To test whether the decrease in presynaptic input number seen with AMPA receptor knockdown (Fig 5.3) was due to a loss of AMPA receptor-mediated activity, we decided to pharmacologically block AMPA receptors. We treated neurons with DNQX (20 μM) to selectively block AMPA receptors, APV (50 μM) to selectively block NMDA receptors, or a vehicle control (DMSO) from 7-14DIV and then fixed and stained neurons with antibodies against VGLUT and MAP2. We found that selective blockade of AMPA receptors led to a significant increase in VGLUT inputs, rather than a decrease (DNQX treatment: 2.1 ± 0.21 VGLUT inputs per dendrite length vs. 1.0 ± 0.13 VGLUT inputs for APV treatment

[$p < 0.001$] and 1.00 ± 0.10 VGLUT inputs for vehicle treatment [$p < 0.001$], all values normalized to controls) (Fig 5.10A-D).

DNQX treatment also caused an increase in the surface expression of GluR subunits (DNQX treatment: 2.7 ± 0.46 GluR1 puncta per dendrite length vs. 1.2 ± 0.46 GluR1 puncta for APV treatment [$p < 0.01$] and 1.0 ± 0.25 GluR1 puncta for vehicle treatment [$p < 0.01$]; DNQX treatment: 1.8 ± 0.29 GluR2 puncta per dendrite length vs. 1.2 ± 0.24 GluR2 puncta for APV treatment and 1.0 ± 0.15 GluR2 puncta for vehicle treatment [$p = 0.059$]; surface GluR puncta number per dendrite length normalized to controls) (Fig 5.10E-F). Together, these results demonstrate that loss of AMPA receptor-mediated activity cannot explain the decrease in excitatory input number seen with receptor knockdown.

The N-terminal domain of GluR2 is sufficient for AMPA receptor-dependent presynaptic stabilization

AMPA receptors have a large extracellular N-terminal domain (NTD) that could potentially interact with presynaptic proteins across the synaptic cleft (see model, Fig 5.11). Past work has shown that the NTD of GluR2 is sufficient to induce postsynaptic spines; it additionally increases staining for various presynaptic markers and increases the frequency of mEPSCs (Passafaro et al., 2003; Saglietti et al., 2007). Therefore, we asked whether the GluR2 NTD plays a role in presynaptic stability. We subcloned the first 400 amino acids of GluR2 into the Invitrogen pDisplay vector. This resulted in a GluR2 NTD

that had an Ig K-chain leader sequence fused to its N-terminal side and a platelet derived growth factor receptor (PDGFR) transmembrane domain fused to its c-terminal side. When expressed in cells, the GluR2 NTD was targeted and inserted into the membrane surface with the GluR2 NTD located in the extracellular space (Fig 5.12A, B). We transfected GluR2 NTD-pDisplay into 293T cells along with neuroligin-1 and assessed the ability of these co-transfected 293T cells to stabilize Syn-GFP puncta relative to control 293T cells transfected with neuroligin-1 only (+ pDisplay vector). The experiment was exactly as described above. Remarkably, GluR2-NTD in combination with neuroligin-1 led to an almost 2 fold increase in stability over neuroligin-1 alone (stability index of 0.57 ± 0.09 for pDisplay vector/neuroligin-1 transfected 293T cells, stability index of 1.0 ± 0.09 for GluR2-NTD/ neuroligin-1 cotransfected 293T cells, $p < 0.01$) (Fig 5.13). This demonstrates that the GluR2-NTD in combination with neuroligin-1 is sufficient to induce long-term (>1 hour) stabilization of presynaptic inputs onto non-neuronal cells.

5.3 CONCLUSIONS

A major goal of this work was to identify a molecular signal responsible for stabilization of presynaptic inputs. Here we describe evidence that postsynaptic AMPA receptors can positively influence presynaptic stability through interactions mediated by their NTD.

We found that GluR1 and GluR2 subunits were absent a majority of the time from sites where Syn-GFP positive presynaptic inputs had been eliminated. This suggested that

removal of AMPA receptors from the postsynaptic membrane could lead to the destabilization and elimination of inputs. It is also possible that AMPA receptors were never recruited to these synaptic sites. Of note was the fact that NMDA receptors were still present at elimination sites, demonstrating that elements of the postsynaptic density were still intact following presynaptic input removal.

Removal of AMPA receptors from the membrane surface by dominant negative c-tail constructs selectively decreased excitatory input number, with no effect on inhibitory input number. The fact that inhibitory input number was unaltered argues that cells with decreased AMPA receptors were alive and capable of receiving presynaptic inputs. The decrease in excitatory input number raised the possibility that there was decreased stabilization of these inputs over time. Indeed, neurons with decreased AMPA receptor surface expression had a greater than 50% decrease in the number of stable (>1 hour) Syn-GFP puncta contacting their dendrites relative to control neurons. This argues strongly for a positive role of AMPA receptors in stabilizing presynaptic inputs.

This finding led us to ask whether overexpression of AMPA receptors can increase stabilization of inputs. As predicted, overexpression of AMPA receptors in neurons led to a 1.5 fold increase in excitatory inputs, with no change in inhibitory inputs, strengthening the assertion that AMPA receptors positively influence presynaptic stability. Further, overexpression of GluR1 and 2 or just the NTD of GluR2 along with neuroligin-1 was sufficient to induce stabilization of Syn-GFP puncta onto non-neuronal 293T cells. Thus, neuroligin-1 and the NTD of GluR2 represent a minimal protein complement that can positively influence presynaptic input stabilization.

What is the role of neuroligin-1 in stabilizing presynaptic inputs? Neuroligin-1 is known for its ability to induce synapses onto non-neuronal cells (Craig and Kang, 2007). AMPA receptors, in contrast, are incapable of inducing synapses in heterologous culture assays (Megan Williams and B.R., unpublished data). It is therefore possible that the requirement for neuroligin-1 in Syn-GFP stabilization is actually an induction requirement (see Figure 5.14 for a model). In this scenario, neuroligin-1 would recruit Syn-GFP positive axons and induce transient vesicle accumulation at contact sites. In the absence of AMPA receptors, these vesicles would disperse after minutes. This would explain a slightly increased stability index as a portion of trafficking Syn-GFP puncta were temporarily recruited to neuroligin-1 positive sites. The addition of AMPA receptors to those sites would then transform nascent accumulations of Syn-GFP puncta into stable ones. AMPA receptors thus would depend on the presence of neuroligin-1 and would be poor stabilizers on their own. Of course, it is also possible that neuroligin-1 possesses stabilization properties as well, and that those properties depend on the presence of AMPA receptors. This scenario is discussed in detail in Chapter 6.

AMPA receptor activity does not appear to be responsible for stabilizing presynaptic inputs. In fact, blockade of activity greatly enhanced excitatory input number. This coincided with an increase in surface-expressed AMPA receptors, which has been observed previously with chronic AMPA receptor blockade (Turrigiano, 2007). One intriguing possibility is that the increase in presynaptic input number seen with AMPA receptor blockade is due to the increased physical presence of AMPA receptors on the surface in response to this blockade. Experiments in which cells expressing c-tail

constructs are exposed to chronic drug blockade may answer whether the homeostatic effect described here is dependent on structural AMPA receptor interactions.

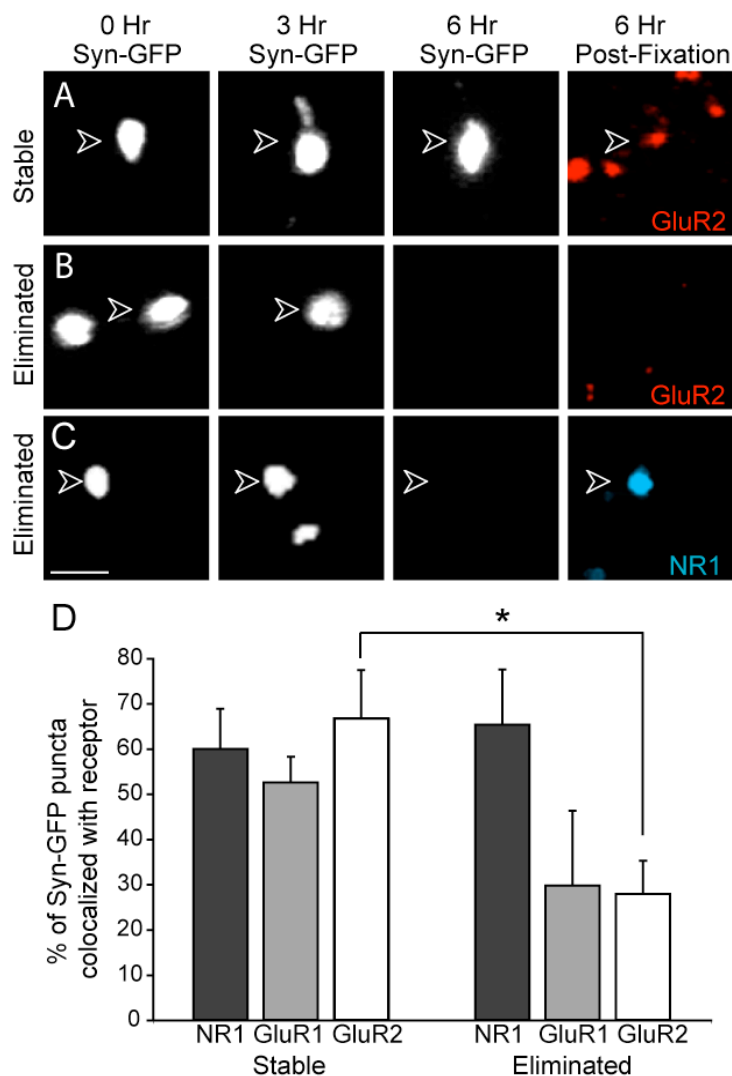


Figure 5.1 Correlation between presynaptic stability and the presence of synaptic AMPA receptors

A-C. Examples of stable and eliminated Syn-GFP puncta with retrospective staining for surface-expressed GluR2 and total NR1. A. Example of a stable Syn-GFP punctum. Live-labeling at the 6 hour mark revealed the presence of a GluR2 punctum that colocalized with the Syn-GFP punctum. B. Example of an eliminated Syn-GFP punctum. No staining for GluR2 was seen at the elimination site. C. Another example of an eliminated punctum. NR1 staining is present at the elimination site, suggesting that elements of the postsynaptic site remain after presynaptic elimination. Scale bar = 1 μ m.

D. Quantification of the fraction of stable and eliminated Syn-GFP puncta that colocalized with NR1, GluR1, or GluR2 receptor subunits. Data describing colocalization with GluR1 are from 5 f.o.v. across 3 separate cultures (34 stable, 26 eliminated Syn-GFP puncta), data for GluR2 are from 4 f.o.v. across 2 separate cultures (63 stable, 35 eliminated Syn-GFP puncta), and data for NR1 are from 9 f.o.v. across 4 separate cultures (133 stable, 33 eliminated Syn-GFP puncta).

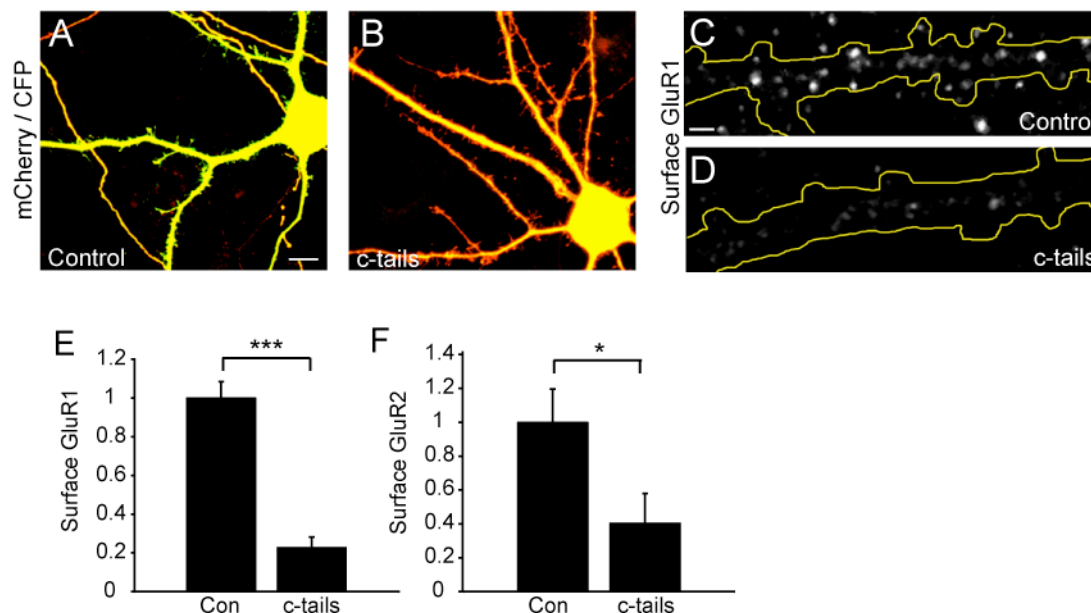


Figure 5.2 GluR1 & GluR2 c-tails decrease AMPA receptor surface expression

A. Example of a control neuron expressing mCherry (red) and CFP (visualized in green).

B. Example of a neuron expressing mCherry (red) and CFP:GluR1 & 2 c-tails (green). Neurons expressing c-tails looked morphologically similar to CFP controls. Scale bar = 5 μ m.

C-D. Examples of dendrite segments (outlined in yellow) expressing either CFP (control, C) or CFP:c-tails (c-tails, D) that were live-labeled with anti-GluR1 antibodies. Scale bar = 1 μ m.

E. Quantification of surface GluR1 levels in CFP and CFP:GluR1 & 2 c-tail expressing neurons. C-tail expressing neurons had a 5-fold reduction in the number of surface-expressed GluR1 puncta per length of dendrite relative to controls (values normalized to controls; n=31-32 neurons each condition, p < 0.0001).

F. Quantification of surface GluR2 levels in CFP and GluR1 & 2 c-tail expressing neurons. There was a 2.5 fold reduction in the number of surface GluR2 puncta per length of dendrite on c-tail expressing neurons (values normalized to controls; n=14 neurons each condition, p < 0.05).

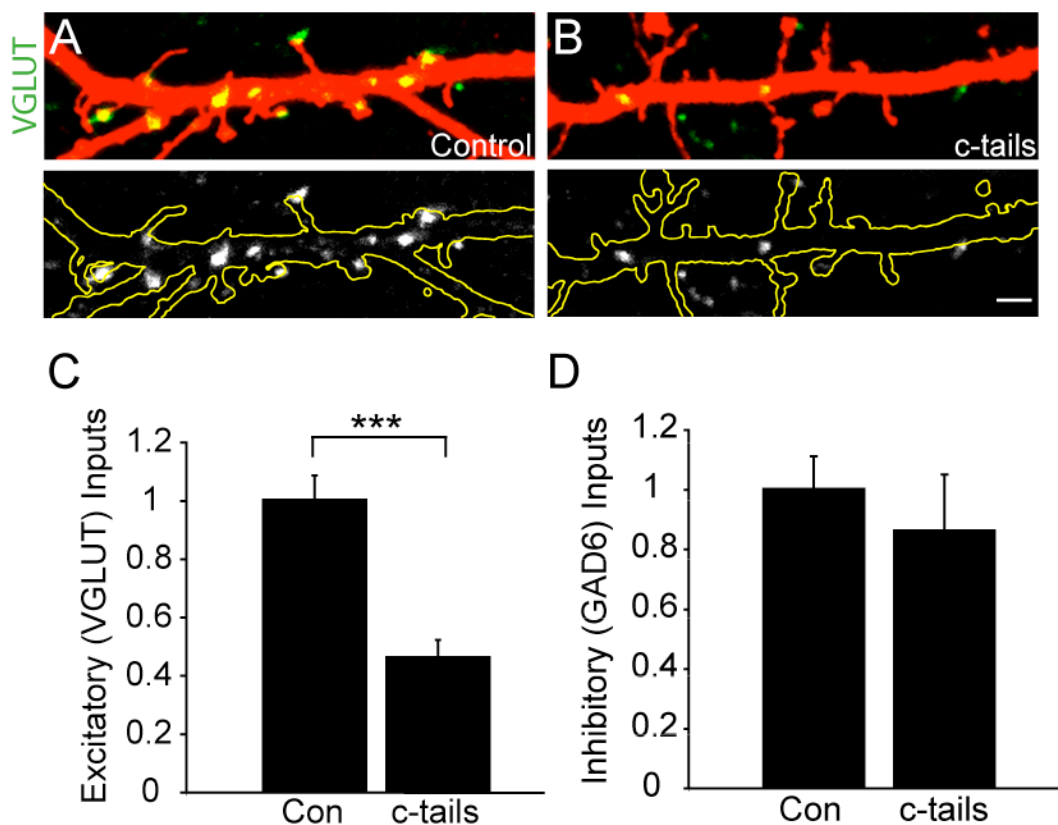


Figure 5.3 AMPA receptor knockdown decreases the number of presynaptic inputs

A-B. Examples of endogenous VGLUT 1 & 2 staining onto control (A) or GluR1 & 2 c-tail expressing neurons (B). The upper panels show VGLUT inputs (green) onto mCherry filled dendrites (red). The lower panels show the same VGLUT inputs in white with an outline of the dendrite in yellow. Scale bar = 2 μ m.

C. Quantification of excitatory VGLUT inputs onto control and c-tail expressing neurons at 14DIV. There was over a 50% decrease in the number of excitatory inputs onto c-tail expressing neurons relative to controls (VGLUT inputs per dendrite length normalized to controls; $n=35-37$ neurons each condition, $p < 0.0001$).

D. Quantification of inhibitory GAD6 inputs onto control and c-tail expressing neurons at 14DIV. There was no significant difference in the number of inhibitory inputs onto control vs. c-tail expressing neurons (GAD6 inputs per dendrite length normalized to controls; $n=16-22$ neurons each condition, $p=0.51$).

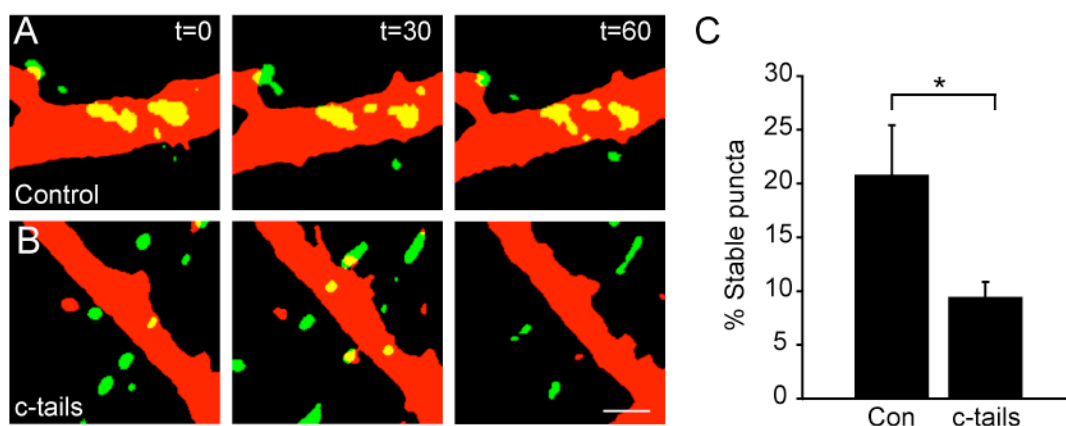


Figure 5.4 AMPA receptor knockdown decreases the stability of presynaptic inputs

A-B. Example pictures from imaging experiments in which Syn-GFP puncta (green) contacted mCherry (red) expressing dendrites that were also expressing CFP or GluR1 & 2 c-tails. A. Example pictures from an imaging experiment in which Syn-GFP puncta (green) contacted an mCherry (red) expressing dendrite that was also expressing CFP (control). The panels show time points at 0, 30 and 60 minutes. Several Syn-GFP puncta were stabilized on the control neuron for the duration of 1 hour, while other puncta either appeared or disappeared. B. Example pictures from the same imaging experiment as in A, only in this case Syn-GFP puncta contacted an mCherry expressing dendrite that was also expressing GluR1 & 2 c-tails. The panels show time points at 0, 30 and 60 minutes. Although several Syn-GFP puncta contacted the c-tail expressing dendrite, no puncta were stabilized across the hour. At the 30 minute time point (middle panel) the c-tail expressing dendrite extended small protrusions that contacted Syn-GFP puncta, but these were not stabilized (see right panel). Scale bar = 2 μ m.

C. Quantification of the fraction of Syn-GFP inputs contacting control or c-tail expressing dendrites that were stable across 1 hour. There was a greater than 50% decrease in stable inputs onto c-tail expressing neurons (n=6-7 neurons, 3 imaging experiments, $p < 0.05$). Error bars show SEM for all graphs.

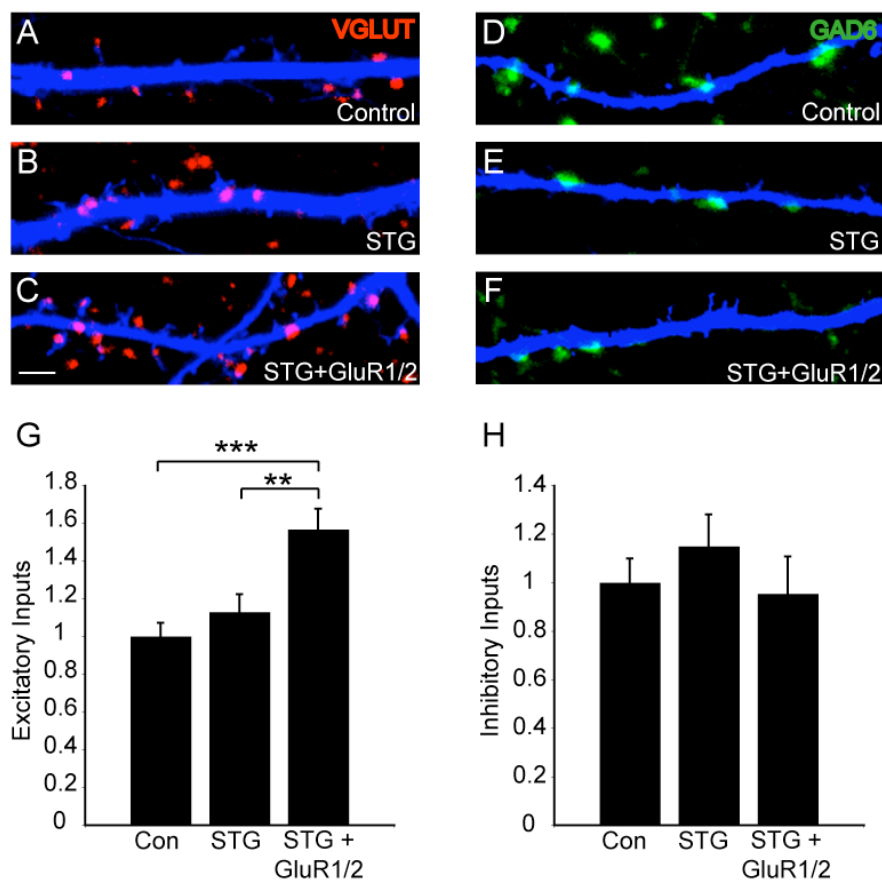


Figure 5.5 Overexpression of AMPA receptors increases excitatory presynaptic input number

A-C. Representative images of VGLUT 1 & 2 positive inputs (red) onto 14DIV neurons transfected at 7DIV with vector (A), stargazin (STG; B), or stargazin + GFP-GluR1 & 2 (C), along with mCherry for visualization of dendrites (pseudocolored blue). Scale bar = 2 μ m.

D-F. Representative images of GAD6 positive inputs (green) onto 14DIV neurons transfected with vector (D), stargazin (E), or stargazin + GFP-GluR1 & 2 (F), along with mCherry for visualization of dendrites (pseudocolored blue).

G. Quantification of the number of excitatory (VGLUT 1 & 2 positive) inputs per length of dendrite, normalized to control values. N=47-52 neurons per condition. *** $p < 0.001$, ** $p < 0.01$.

H. Quantification of the number of inhibitory (GAD6 positive) inputs per length of dendrite, normalized to controls. There was no significant difference between the three conditions ($p = 0.24$). N=23-24 neurons per condition.

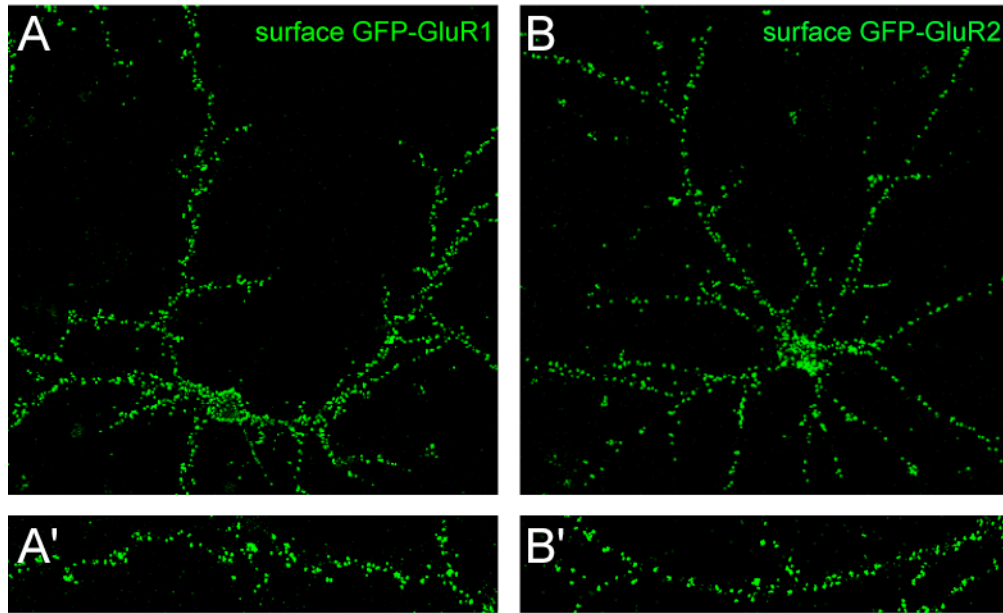


Figure 5.6 Overexpression of GFP-GluR1 and GFP-GluR2

Transfection of neurons with GFP-tagged GluR1 and GluR2 along with stargazin leads to robust surface expression of these constructs.

A. Example of a neuron transfected with GFP-GluR1. Surface expression of GFP-GluR1 was visualized by live-labeling with anti-GFP antibodies targeted against the extracellularly located GFP. A magnified view of a dendrite segment from panel A is shown in panel A'.

B. Example of a neuron transfected with GFP-GluR2. Surface expression of GFP-GluR2 was visualized by live-labeling with anti-GFP antibodies targeted against the extracellularly located GFP. A magnified view of a dendrite segment from panel B is shown in panel B'.

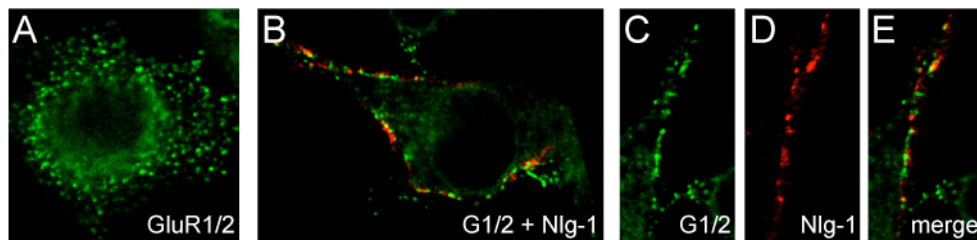


Figure 5.7 Confirmation of construct expression in 293T cells

A. Example of a 293T cell transfected with GluR1 and GluR2. AMPA receptors make it to the surface and are clustered.

B-E. B. Example of a 293T cell cotransfected with GluR1 and 2 (G1/2; green) and flag-neuroigin-1 (Nlg-1; red). Both constructs are expressed and are targeted to the membrane. C-E Magnified view of a portion of the cell in B, demonstrating partial colocalization of AMPA receptors and neuroigin-1 at the membrane surface.

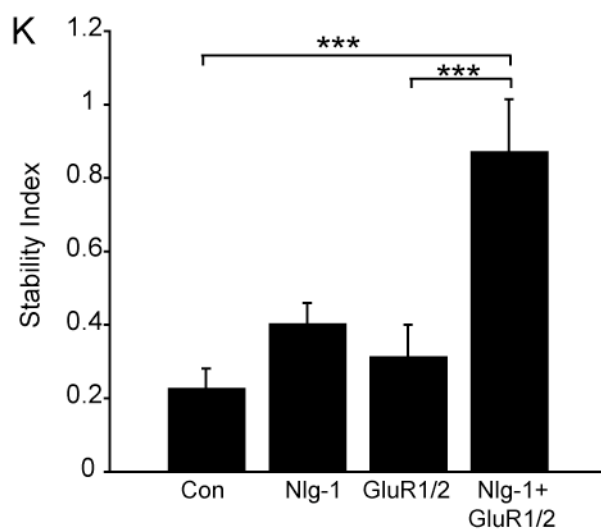
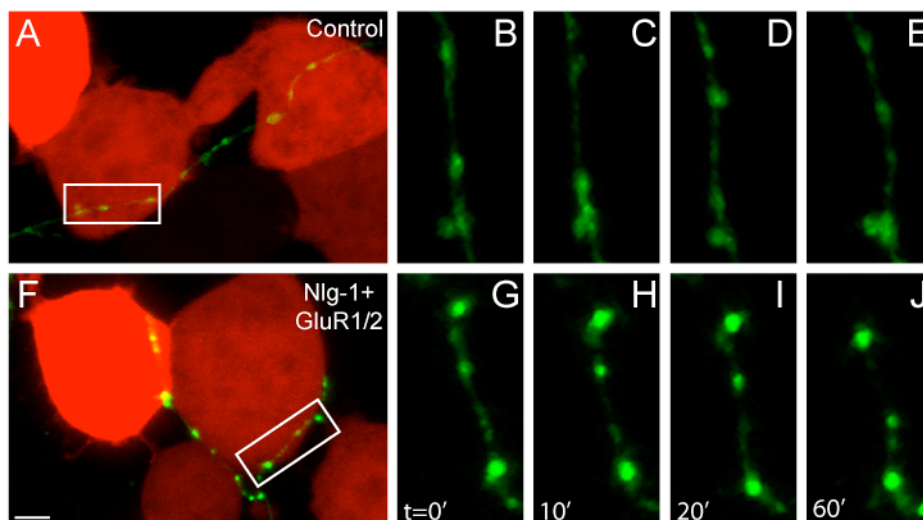


Figure 5.8 Overexpression of AMPA receptors and neuroligin-1 in non-neuronal cells is sufficient to increase presynaptic stability

A-J. Examples of live-imaged control (A-E) and Neuroligin-1 (Nlg-1) + GluR1 & 2 (F-J) expressing 293T cells (red) contacted by Syn-GFP expressing axons (green) from co-cultured neurons (11-12DIV). 293T cells were overlaid onto neurons 18-24 hours before imaging. Interactions between Syn-GFP puncta and 293T cells were imaged every 10 minutes for 1 hour. The white rectangle in A & F demarcates an area of Syn-GFP axon that is magnified in the panels on the right (control: B-E, Nlg-1 + GluR1 & 2: G-J). A large fraction of Syn-GFP puncta contacting control 293 cells were trafficking. In contrast, a large fraction of Syn-GFP puncta contacting Nlg-1 + GluR1 & 2 expressing 293 cells were stable, and less trafficking puncta were seen. Scale bar = 5 μ m.

K. The ratio of stable to trafficking Syn-GFP puncta contacting individual 293T cells (stability index) was determined for 24-26 cells for each condition (vector, Nlg-1, GluR1 & 2, or Nlg-1 + GluR1 & 2; 7 imaging experiments). *** $p < 0.001$.

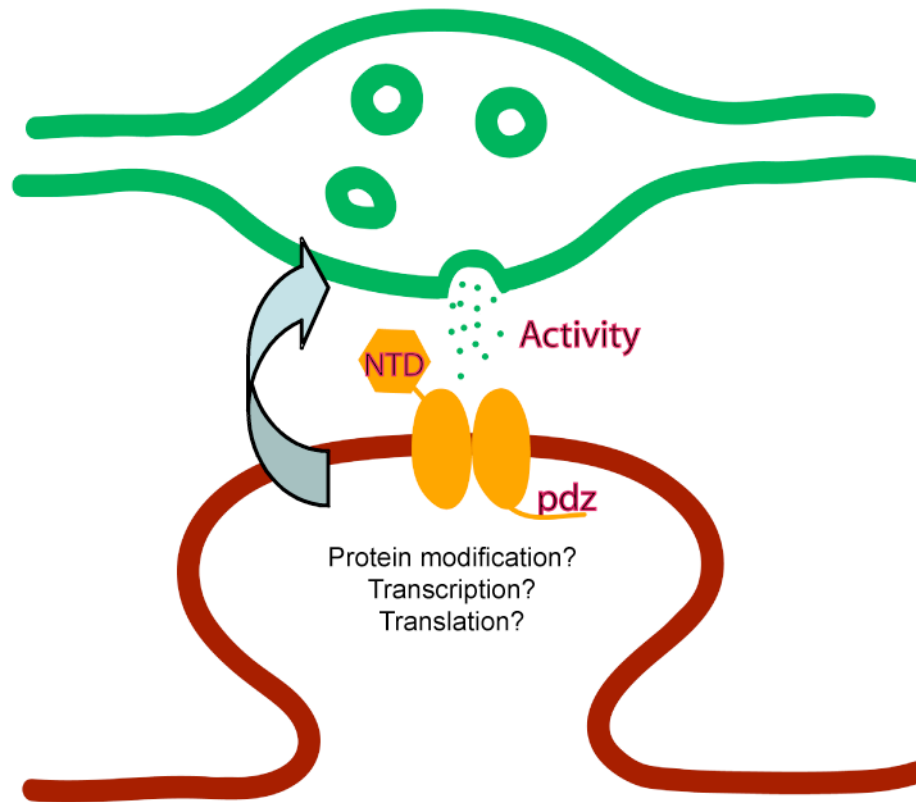


Figure 5.9 Model of how activity might be responsible for AMPA receptor-mediated presynaptic stabilization

Activity through AMPA receptors may lead to downstream events such as modification of existing proteins or modification of transcription and/or translation. These modified proteins or newly synthesized proteins then could signal back to the presynaptic input to influence stabilization.

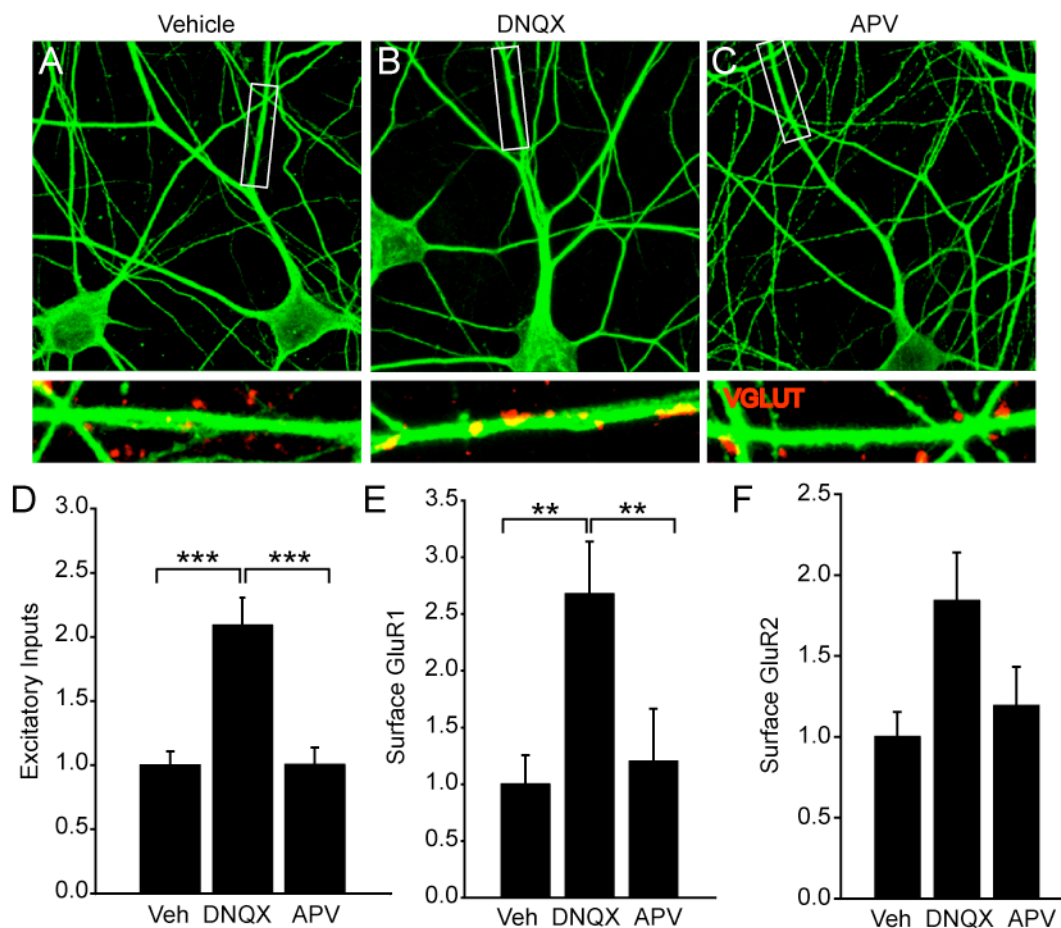


Figure 5.10 DNQX blockade increases the number of excitatory presynaptic inputs.

A-C. Examples of 14DIV neurons stained with MAP2 (green). Neurons were treated from 7-14 DIV with either vehicle (A), DNQX (B) or APV (C). The inset panels show MAP2 staining (green) along with VGLUT 1 & 2 staining (red).

D. Quantification of excitatory (VGLUT) inputs onto vehicle, DNQX and APV treated neurons. The number of VGLUT positive puncta per length of dendrite is normalized to control values. N=46-50 neurons for each condition. *** $p < 0.001$.

E. Quantification of surface GluR1 number per length of dendrite (normalized to controls). N=13-16 neurons for each condition. ** $p < 0.01$.

F. Quantification of surface GluR2 number per length of dendrite (normalized to controls). N=10 neurons for each condition.

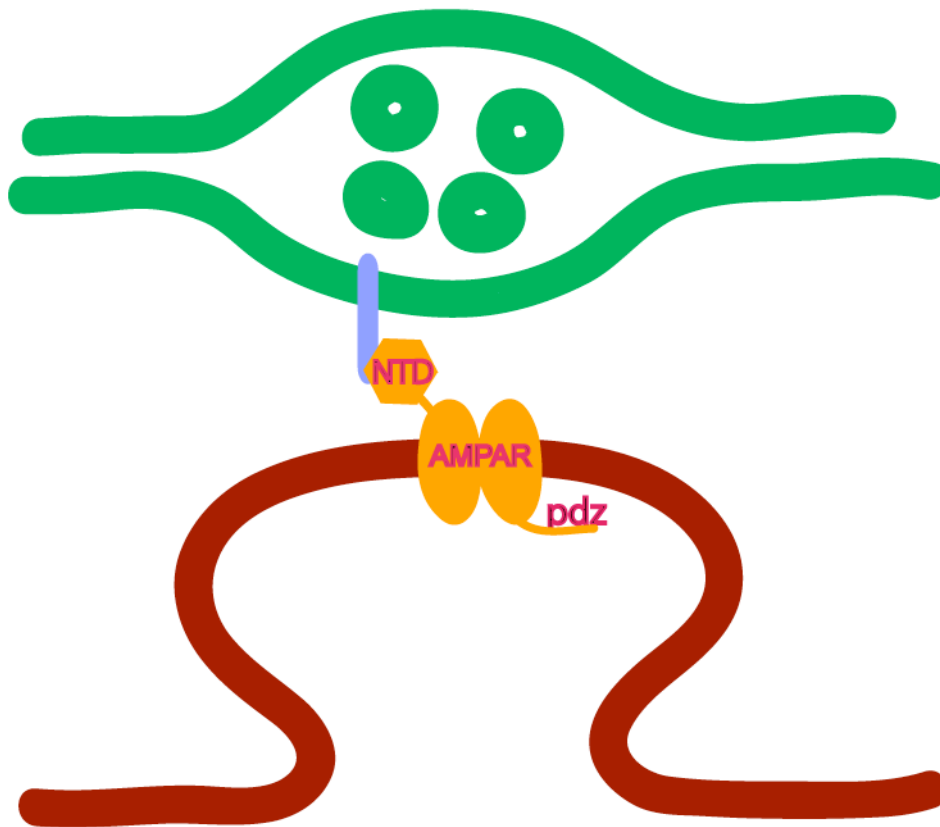


Figure 5.11 Model of how structural interactions might be responsible for AMPA-receptor mediated presynaptic stabilization

AMPA receptors interact with many molecules via their c-tails and NTDs. This model proposes that structural interactions between the AMPA receptor NTD and transmembrane molecules expressed on the presynaptic input (blue oval) lead to the stabilization of presynaptic inputs.

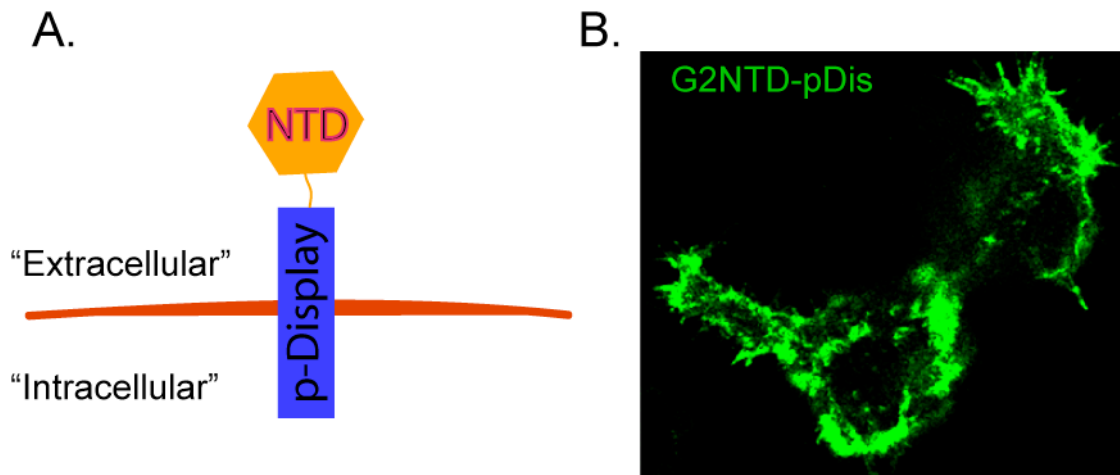


Figure 5.12 Confirmation of GluR2NTD-pDisplay expression in 293T cells

A. Cartoon showing the GluR2NTD-pDisplay construct. The platelet derived growth factor receptor (PDGFR) transmembrane domain is fused to the NTD of GluR2, leading to its insertion into the membrane and presentation to the extracellular surface.

B. Example of a 293T cell transfected with GluR2NTD-pDisplay (G2NTD-pDis). Note the membrane localization of the protein.

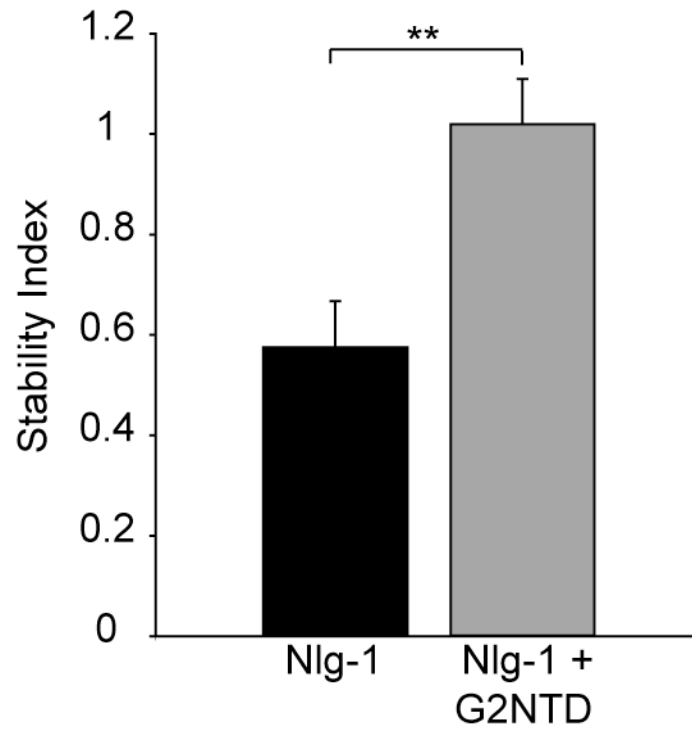


Figure 5.13 GluR2NTD in concert with neuroligin-1 is sufficient to stabilize presynaptic inputs

A. Quantification of the stability index (stable over trafficking Syn-GFP puncta) for 293T cells expressing neuroligin-1 only (+ pDisplay vector) (n=15 cells) or neuroligin-1 + GluR2NTD-pDisplay (n=12 cells). 5 experiments, ** p<0.01.

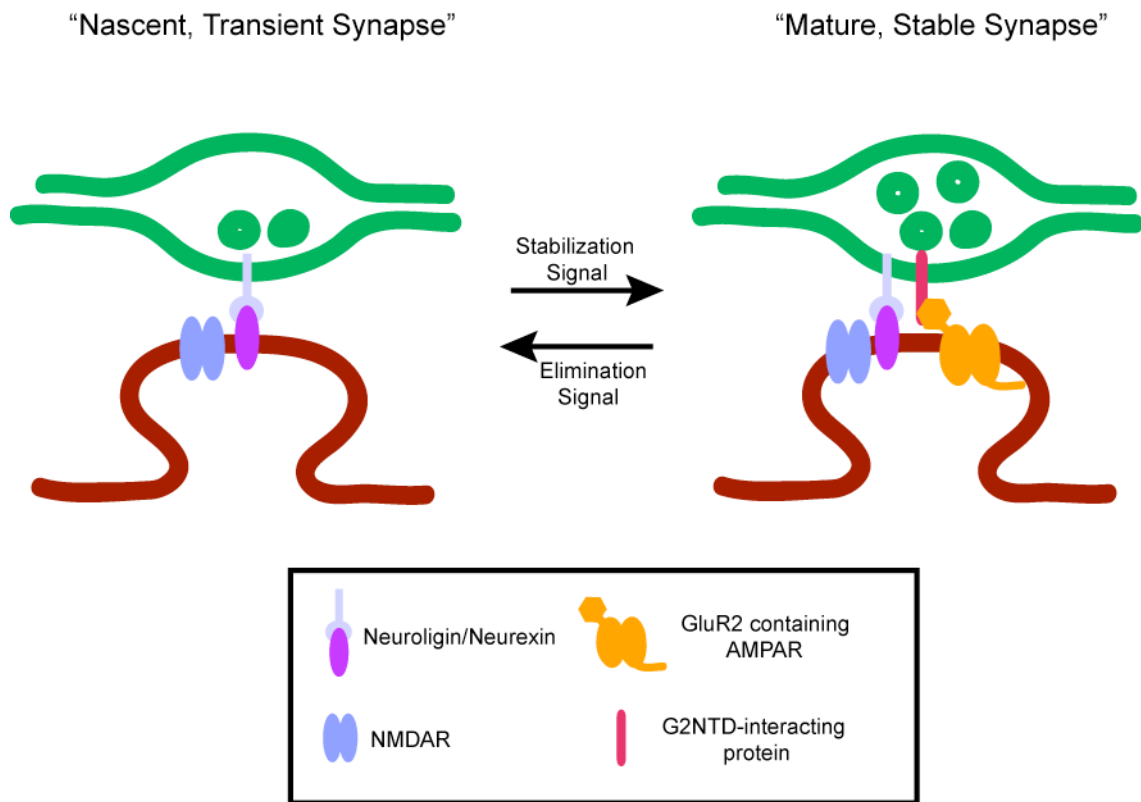


Figure 5.14 Diagrammatic representation of how AMPA receptors may orchestrate a retrograde signal that stabilizes presynaptic inputs

CHAPTER 6: DISCUSSION

During development, it is essential that proper synaptic connections be stabilized while incorrect connections are eliminated. However, almost nothing is known about how synapse stabilization is achieved. Here, we show that AMPA receptors play a critical role in regulating presynaptic stability. Given the postsynaptic localization of AMPA receptors, we propose that AMPA receptors themselves or an AMPA receptor-associated protein complex function as a retrograde signal to stabilize synaptic inputs.

6.1 Synapse elimination occurs in cortical culture

The number of synapses initially formed during cortical development is much larger than the number that is maintained (Blue and Parnavelas, 1983; Rakic et al., 1986; Markus and Petit, 1987; Huttenlocher, 1990; Rakic et al., 1994; De Felipe et al., 1997). We show here that this pattern of excess synapse formation followed by elimination is preserved in cortical culture, allowing for molecular characterization of this phenomenon. Synapses are first observed in culture at 6DIV. Synapse number peaks at 14DIV and is decreased by 18DIV, a time when network-level activity appears to be established (Hall et al., 2007). Our imaging experiments demonstrate that there is considerable turnover of a subset of presynaptic inputs at 14DIV, while a separate population of inputs remains stable for hours (1-5 hours). This suggests that active processes for synapse stabilization and elimination are at play at this developmental time point.

6.2 Presynaptic puncta are dynamic during synaptogenesis

To make correct connections, axons must project across long distances to target regions and identify appropriate postsynaptic partners within that region. One way to identify correct partners would be for each axon-dendrite pair to have a unique molecular cue that would signify an appropriate connection; another possibility is that patterns of activity between the neurons signify the correct choice. Both of these options require at the least, an axo-dendritic contact, and in the case of activity, the assembly of a nascent synapse. The incredibly dynamic turnover of presynaptic vesicles that we describe here would allow for widespread sampling of a target region within a short period of time. Across a typical hour-long imaging experiment at 14DIV, we observe 16.1 presynaptic puncta per 100 μm of axon. Of these, roughly 5.6 are trafficking, 2.3 are transient (dwell on the order of tens of minutes), and 8.2 are stable across the hour. This means that within an hour, an axon 100 μm in length has the opportunity to sample 16.1 axo-dendritic contacts, and potentially has the ability to sample activity at 10.5 transient or stable sites. One cubic millimeter of neocortex contains up to 4 km of axon (Braitenberg & Schuz, 1991). Thus, if the dynamics we observe here translate *in vivo*, there would be 64.5 million axo-dendritic contacts made and 42 million transient connections sampled per cubic millimeter of cortex in just one hour.

6.3 Presynaptic input stability is developmentally regulated

Our imaging experiments revealed separate populations of dynamic and stable Syn-GFP puncta during synaptogenesis. We found that the fraction of stable Syn-GFP puncta increased dramatically (14% to 59%) from 11-17DIV, coinciding with the end of synaptogenesis and the establishment of network level activity in cortical culture (Fig 3.1, (Hall et al., 2007)). Additionally, the number of stable Syn-GFP puncta per 100 μm of axon doubled (8 puncta to 16 puncta) over the same timeframe. We imaged a subset of Syn-GFP puncta at 14DIV for 5 hours and we found that ~60% of puncta that were stable for the first hour of imaging persisted throughout the entire imaging session. This suggests that once stabilized, presynaptic inputs can maintain stability for long periods of time. In fact, it is possible that past a critical time period, presynaptic inputs lose their ability to turn over, suggesting the closure of a plasticity window. Recent work addressed that assumption by repeatedly imaging presynaptic boutons in Layer 1 of adult mice *in vivo* (De Paola et al., 2006). The authors found that axons arising from neurons in different cortical layers or from the thalamus had presynaptic boutons with different kinetics. Presynaptic boutons on putative thalamic axons could persist for up to 9 months (85% persisting for 1 month), suggesting that they may be able to last for the lifetime of an animal. Boutons on putative Layer 6 axons were more labile (40% persisting for 1 month). Serial section EM confirmed that new presynaptic boutons were synapses. Thus, while a majority of presynaptic inputs are stabilized past development, some retain the ability to be eliminated or added, suggesting that stabilization and elimination of presynaptic inputs is still relevant in adult cortex. Thus, identification of a signal that

controls the stabilization of inputs may be important not only for understanding cortical development but for understanding adult plasticity as well.

6.4 Synapses are preferentially stabilized at dendritic sites

In this study we show that presynaptic inputs are preferentially stabilized on dendrites. In fact, only 4% of puncta not in contact with dendrites were stable across 1 hour. However, contact with a dendrite is not sufficient for stabilization, suggesting that an additional dendrite-derived signal is necessary. Indeed, almost 60% of Syn-GFP puncta contacting dendrites failed to stabilize. This argues strongly that only certain areas of the dendrite are capable of stabilizing puncta. This finding is particularly intriguing in light of two recent findings describing local hotspots for synapse formation in dendrites and axons (Gerrow et al., 2006; Sabo et al., 2006).

Work by Gerrow and colleagues suggest that elements of the postsynaptic density are able to recruit presynaptic inputs (Gerrow et al., 2006). The authors describe complexes of PSD-95, GKAP and Shank that colocalize even in the absence of presynaptic inputs. This fact, coupled with the fact that these elements can traffic together along dendrites, led the authors to conclude that these three proteins are part of a “preformed complex of postsynaptic proteins”. They went on to show that FM 4-64 positive and Syn-DsRed positive presynaptic inputs can be recruited to a subset of these preformed complexes over the course of a few hours. Neuroligin-1 colocalized with preformed complexes and the ability of neuroligin-1 to recruit Syn-GFP puncta appeared to be related to its colocalization with PSD-95. Knockdown of PSD-95 led to a

concurrent decrease in GKAP and SHANK clusters and a decrease in excitatory VGLUT inputs, but did not affect the number of neuroligin-1 clusters. The authors conclude that preformed clusters of postsynaptic proteins containing PSD-95 and neuroligin-1 may determine eventual synaptic sites by recruiting and stabilizing (> 15 min) presynaptic components. It is possible that these preformed complexes are part of the dendrite-derived signal that was implied from our imaging studies (see Chapter 3). Our own results suggest that AMPA receptors are necessary for presynaptic stabilization (see Chapter 5 and additional discussion below). It may well be that AMPA receptors are also part of the “preformed complexes” described by Gerrow et al. Indeed, overexpression of Shank leads to increased recruitment of AMPA receptors to spines (Roussignol et al., 2005). Thus, it is plausible that the ability of “preformed complexes” to recruit and stabilize presynaptic puncta may depend, in part, on recruitment of AMPA receptors to these complexes. Unfortunately, it was not determined in the Gerrow study whether these complexes were also positive for AMPA receptors (GluR1 and GluR2 subunits) at the time of presynaptic recruitment.

While we found that Syn-GFP puncta selectively stabilize (>1 hour) at dendritic sites, Sabo et al. demonstrated that another mechanism controls short-term “pausing” (1-2 minutes) of presynaptic vesicles completely independent of dendrite contact (Sabo et al., 2006). These authors demonstrated that VAMP:GFP puncta pause at predefined sites along an axon in the absence of a postsynaptic contact, suggesting that axons are capable of stabilizing inputs at only a subset of sites along their length (Sabo et al., 2006). Interestingly, vesicles repeatedly visited the same site and multiple vesicles paused at the same site, arguing strongly for a fixed signal at those points. The average length of

pausing was roughly 1 minute, however, suggesting that additional signals are necessary for the long-term stabilization of presynaptic puncta. We found that puncta could be stabilized for hours when in contact with a postsynaptic dendrite. Almost never was a puncta stabilized for an hour in the absence of a dendrite (4% of puncta). While we did not specifically define pause sites nor determine whether stable puncta occurred at pause sites, it is very interesting to speculate that these pause sites contain the signaling partner to the dendritic signal we describe here. The fact that these pause sites may be capable of releasing glutamate further argues for cross talk between the presynaptic terminal and postsynaptic sites that contain AMPA receptors.

6.5 Lack of AMPA receptors at sites of presynaptic elimination

We find that the majority of stable Syn-GFP puncta are apposed to a GluR1 (50%) or GluR2 (65%) subunit (Fig 5.1). This likely underestimates the number of stable excitatory inputs positive for AMPA receptors, since 10% of Syn-GFP puncta are inhibitory inputs and several AMPA receptor containing synapses at this age are selectively positive for either GluR1 or GluR2 (Chapter 4, Table 4.1). Presynaptic elimination sites were much less likely to be positive for surface GluR1 (30%) and GluR2 (25%) receptors, while association with total NR1 puncta remained roughly the same (55% of stable sites and 60% of elimination sites). This suggests that a selective removal of AMPA receptors occurs at elimination sites or that AMPA receptors are not recruited to those sites in the first place.

6.6 Effect of manipulating AMPA receptors on the number and stability of presynaptic inputs

Perhaps the most important conclusion of this study is that postsynaptic AMPA receptors regulate the number and stability of presynaptic inputs. Removal of AMPA receptors led to a 2-fold reduction in the number of excitatory inputs onto dendrites. Interestingly, the number of inhibitory inputs onto these neurons was not altered. The decrease in input number could be explained by a decrease in the stability of presynaptic terminals. This is strongly supported by our observation of a 50% decrease in stable Syn-GFP puncta in contact with neurons lacking surface AMPA receptors compared to controls (Fig 5.4). This decrease in stable inputs is not due to a loss of axo-dendritic contacts. In fact, neurons lacking surface AMPA receptors were contacted by roughly the same number of puncta as control neurons (average of 42 puncta/neuron for control neurons, 66 puncta/neuron for c-tail expressing neurons). These neurons even retain the ability to extend filopodia to contact Syn-GFP puncta (see middle panel of Fig 5.4B) but they fail to stabilize those puncta.

We additionally found that overexpression of AMPA receptors led to a 1.5 fold increase in the number of excitatory inputs onto cells (Fig 5.5). Further, overexpression of AMPA receptors along with neuroligin-1 in 293T cells led to an almost 4-fold increase in the ability of 293T cells to stabilize Syn-GFP inputs across 1 hour (Fig 5.8). The AMPA receptor-mediated increase in stability was dependent on the presence of neuroligin-1, as AMPA receptors themselves were unable to stabilize Syn-GFP inputs. One interpretation of this experiment is that synapse inducing signals (via neuroligin-1)

must precede synapse stabilizing signals (via AMPA receptors) (see model, Fig 5.14).

Another possibility is that AMPA receptors and neuroligin-1 work in concert to stabilize inputs (See section 6.10 for an in depth discussion on this topic). Either way, the ability of AMPA receptors to stabilize inputs onto 293T cells strongly suggests that AMPA receptors themselves or an AMPA receptor associated protein endogenous to 293T cells may structurally interact with the presynaptic input to influence its stability.

6.7 The positive effect of AMPA receptors on presynaptic stability is independent of receptor-mediated activity

AMPA receptors could potentially influence presynaptic stability by signaling directly via their N terminal domain (NTD) to presynaptic surface proteins or by C terminal domain (CTD) interactions with other postsynaptic proteins that could then communicate with the presynaptic terminal. Alternatively, AMPA receptor-mediated activity could trigger downstream signaling that results in the transcription, translation, or recruitment of proteins that then provide a retrograde signal to the presynaptic terminal (see model, Fig 5.9). An elegant series of studies by Misgeld and colleagues demonstrated that acetylcholine released from the nerve terminal can destabilize acetylcholine receptors at the NMJ (Misgeld et al., 2005). Along these lines, experiments carried out by De Paola and colleagues showed that application of AMPA caused presynaptic Syn-GFP puncta to be converted from stable to dynamic in 40DIV hippocampal organotypic slice cultures (De Paola et al., 2003). Thus, there is precedence for neurotransmission to directly influence synapse stabilization—in these cases, in a

negative manner. However, we believe neurotransmission is not responsible for the AMPA receptor-mediated stabilization described in this thesis. Chronic (7-14DIV) blockade of AMPA receptor signaling by DNQX increased the number of excitatory presynaptic inputs onto neurons (Fig 5.10). This is in direct contrast to the loss of excitatory inputs we observed with AMPA receptor knockdown (Fig 5.3). This demonstrates that the physical removal of the receptor has an effect that is distinct from the resulting loss of activity due to receptor removal. Our finding of increased excitatory input number and increased number of surface AMPA receptors in response to DNQX is likely to be due to homeostatic mechanisms (reviewed in Turrigiano, 2007). Thus there appear to be multiple independent pathways, including homeostatic mechanisms, which influence synapse input number. Clearly, however, activity is unlikely to be responsible for the mechanism that we have been describing here, namely the increase in presynaptic input number and stability positively mediated by AMPA receptors. Thus, we propose that AMPA receptors play a structural role in the physical stabilization of synapses.

6.8 Structural interactions mediated by the GluR2 NTD are sufficient to increase presynaptic stability

AMPA receptors contain a large extracellular NTD of approximately 400 amino acids that is distantly related to bacterial periplasmic amino acid binding proteins (O'Hara et al., 1993). Beyond involvement in the initial subunit-specific assembly of receptors into dimers, the importance of this NTD is largely unknown (Ayalon and Stern-Bach, 2001; Pasternack et al., 2002; Ayalon et al., 2005). An exciting series of studies from

Passafaro and colleagues was the first to address whether the NTD of AMPA receptors played a role in signaling (Passafaro et al., 2003; Saglietti et al., 2007). They found that the NTD of GluR2 is sufficient to increase the length, size and number of spines, as well as the number of functional inputs when overexpressed in neurons. Here, we demonstrate that the influence of the GluR2 NTD extends across the synapse to the presynaptic side, where it positively regulates the long-term stabilization of presynaptic vesicles. We found that overexpression of the GluR2 NTD fused to pDisplay along with neuroligin-1 in non-neuronal cells was sufficient to stabilize Syn-GFP puncta expressed in co-cultured neurons for at least one hour. This is the first identification and confirmation by time-lapse imaging, to our knowledge, of a protein both necessary (Fig 5.4) and sufficient (Fig 5.8, Fig 5.13) to induce presynaptic stabilization in the CNS.

6.9 What is the signaling partner for the NTD of GluR2?

How might the NTD of GluR2 signal to the presynaptic membrane to positively regulate presynaptic input stability? The NTD of GluR2 interacts with N-cadherin in both *cis* and *trans*, and N-cadherin is necessary for the GluR2 NTD-mediated effect on spine growth and miniature EPSC frequency described previously (Saglietti et al., 2007). N-cadherin is present at both pre- and postsynaptic sites prior to synapse formation, and it is rapidly clustered at synapses during formation (Benson and Tanaka, 1998). It is only retained at a subset of excitatory synapses and is absent from inhibitory synapses, strengthening the possibility that it plays a role in excitatory synapse stability. N-cadherin interacts with a protein complex that includes β -catenin and p120 catenin, and loss of β -

catenin in the presynaptic neuron leads to a reduction in the number of docked vesicles (Bamji et al., 2003). Finally, pan-cadherin blockade does not alter synaptogenesis in neurons older than 10DIV, but alters the size, vesicle recycling and frequency of spontaneous activity, suggesting a role in maturation or stability (Bozdagi et al., 2004). Thus, it will be interesting to explore whether a trans-synaptic GluR2-N-cadherin interaction is involved in regulating presynaptic stability. Experiments to address this possibility are ongoing.

The AMPA receptor NTD also interacts with several members of the pentraxin family, including neuronal activity-regulated pentraxin (NARP), neuronal pentraxin 1 (NP1), and neuronal pentraxin receptor (NPR) (O'Brien et al., 1999; O'Brien et al., 2002; Xu et al., 2003; Sia et al., 2007). These proteins are localized to excitatory inputs, are predominantly expressed presynaptically, and have all been shown to cluster AMPA receptors with varying potency. However, whether these proteins play a role in presynaptic synapse induction or stability remains to be explored.

6.10 Role of neuroligin-1 in presynaptic stabilization

We demonstrated that the GluR2 NTD in concert with neuroligin-1 represents a minimal complex of proteins that is sufficient to stabilize presynaptic inputs. What role does neuroligin-1 play in this stabilization? While overexpression of AMPA receptors in concert with neuroligin-1 in 293T cells led to a 4 fold increase in Syn-GFP stabilization over control values, overexpression of AMPA receptors alone or neuroligin-1 alone did not significantly increase Syn-GFP stability. However, each did lead to a modest increase

in stabilization, raising the possibility that both AMPA receptors and neuroligin-1 act as weak stabilizers in parallel. Neuroligin-1 is well-known for inducing presynaptic differentiation via β -neurexin (Scheiffele et al., 2000; Dean et al., 2003). Recent work has shown that a neuroligin-PSD-95 complex can trans-synaptically influence presynaptic release probability, suggesting that neuroligin-1 plays an additional role in maturation of presynaptic inputs (Futai et al., 2007). Indeed, a study of neuroligin-1, -2, and -3 triple knockout mice suggests that neuroligins are not necessary for initial synapse formation and instead are responsible for functional aspects of excitatory and inhibitory synaptic transmission (Varoqueaux et al., 2006). Thus it is possible that neuroligin-1 plays a role independent of AMPA receptors in stabilization of presynaptic inputs. In this additive scenario, the increased stability of neuroligin-1 alone or AMPA receptors alone should sum to the stability resulting from coexpression of the two proteins. Control transfected 293T cells had a stability index=0.23. The increased stability due to neuroligin-1 in excess of control values was 0.18, and that due to AMPA receptors was 0.09, which sums to 0.27. The increase in excess of control values for coexpression of neuroligin-1 and AMPA receptors was 0.64, suggesting a synergistic rather than additive effect.

Another interpretation of the data is that neuroligin-1 and AMPA receptors act sequentially rather than in parallel. In this model, neuroligin-1 provides a synapse induction signal that is a prerequisite for an AMPA receptor-mediated stabilization signal (see model, Figure 5.14). Two independent reports demonstrate that clustering of neuroligin-1 does not recruit AMPA receptors to new postsynaptic sites, even though PSD-95 and NMDA receptors are recruited to those sites (Graf et al., 2004; Nam and Chen, 2005) (see left side of model, Fig 5.14). The omission of AMPA receptors from a

newly induced synapse may serve to hold that newly formed synapse in a plastic state (i.e. unstable state) until appropriate signals recruit AMPA receptors to that synapse and thus transition it to a permanent state (i.e. stable state) (see right side of model, Fig 5.14). If this model is correct, it should be possible to replace neuroligin-1 with another synapse-inducing molecule such as Syn-CAM or netrin G ligand 2, and still get increased stabilization with AMPA receptor co-transfection in the 293T imaging assay. Experiments testing this are ongoing.

6.11 The Role of NMDA receptors in recruiting AMPA receptors to the synapse

A recent study from our laboratory suggests that NR2B-containing NMDA receptors negatively regulate AMPA receptor incorporation into synapses during early synaptogenesis (Hall et al., 2007) (also see Appendix 1). NR2B containing NMDA receptors are the predominant NMDA receptor species during early development (Monyer et al., 1994; Sheng et al., 1994). As synapses mature, NR2A containing NMDA receptors increasingly accumulate in synapses. Given its negative regulation of AMPA receptor synaptic localization, one role of NR2B may be to maintain young synapses in an unstable, plastic state by actively restricting a presynaptic stabilization signal (in this case, AMPA receptors). Activity at a synapse may override the 2B-mediated restriction of AMPA receptors, leading to increased synaptic AMPA receptors and thus stabilization of the presynaptic input providing the activity. Whether NR2B-containing NMDA receptors signal bi-directionally and can actively recruit AMPA receptors given the right

activity level remains to be determined. Another possibility is that NR2A containing NMDA receptors positively recruit AMPA receptors. This is being actively investigated.

6.12 Is there a critical window for synapse elimination?

Although synapses are quite dynamic early in development, later on they maintain stability for weeks, months or perhaps even years (Grutzendler et al., 2002; Trachtenberg et al., 2002). The importance of this stability is highlighted by recent reports that the disassembly of synapses late in life may be linked to Alzheimer's disease (Selkoe, 2002). In fact, recent work suggests that beta amyloid-induced loss of AMPA receptors may lead to this late-stage destabilization of synapses (Hsieh et al., 2006). Even though we raise the possibility that proper trafficking of AMPA receptors to synapses mediates cortical wiring during development, it will be equally important to determine whether their prolonged presence at synapses is essential for stabilizing synaptic inputs in adult life and old age.

**APPENDIX 1: NR2B IS A NEGATIVE REGULATOR
OF AMPA RECEPTOR RECRUITMENT TO
SYNAPSES**

A popular view of synapse maturation suggests that AMPA receptors are recruited to young synapses in a manner that is dependent upon NMDA receptor activation. Several lines of evidence seem to support this conclusion. First, there is a developmental increase in the AMPA/NMDA receptor ratio, suggesting that AMPA receptors are increasingly incorporated into synapses across time (Crair and Malenka, 1995; Isaac et al., 1997; Hsia et al., 1998; Lu et al., 2001; Ye et al., 2005). Further, electrophysiological and immunohistochemical studies have documented the existence of “silent synapses” which contain NMDA receptors but not AMPA receptors during early development, suggesting that newborn synapses are AMPA receptor lacking (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Li and Zhuo, 1998; Isaac, 2003). Finally, trafficking of GluR1/2 containing receptors into the synapse has been shown to require NMDA receptor-mediated activity (Liao et al., 1995; Shi et al., 1999; Hayashi et al., 2000; Heynen et al., 2000).

NMDA receptors are composed of obligate NR1 subunits in combination with NR2 subunits (2A-D) (Meguro et al., 1992; Monyer et al., 1992). During early development, the predominant subunit is NR2B, and levels of NR2A are developmentally upregulated to a point at which the predominant NMDA receptor species is NR1/NR2A during late development and adulthood (Monyer et al., 1994; Sheng et al., 1994; Vicini et al., 1998; Barria and Malinow, 2005). We asked whether NR2B-mediated signaling was necessary for AMPA receptor recruitment into synapses during development. To answer this question, we visualized AMPA receptor distribution in cortical cultures from embryonic day 16-18 NR2B knockout (KO) mice (Hall et al., 2007).

We live-labeled GluR1 and GluR2 subunits of AMPA receptors in cortical neurons from NR2B KO mice and wildtype littermates at DIV14. By live-labeling these subunits with an antibody raised against an extracellular domain of these proteins, we were able to stain specifically for surface-expressed receptor. We then fixed neurons and additionally stained for the dendritic marker MAP2, as well as the excitatory presynaptic markers VGLUT 1 & 2. We found that surface expression of GluR1 was significantly increased in neurons from NR2B KO animals when compared to controls (WT neurons: 2.99 ± 0.26 GluR1 puncta per $10 \mu\text{m}$ of dendrite; NR2B KO neurons: 5.07 ± 0.350 GluR1 puncta; $p=1.8 \times 10^{-5}$) (Fig A1.1 A-C). There was no change in GluR2 surface expression (WT neurons: 3.91 ± 0.56 GluR2 puncta per $10 \mu\text{m}$ of dendrite; NR2B KO neurons: 4.92 ± 0.36 GluR2 puncta, $p=0.128$) (data not shown).

We next asked whether synaptic targeting of AMPA receptors was affected in NR2B KO neurons. We assessed the intensity of GluR1 and GluR2 staining in regions of interest that were positive for VGLUT 1 & 2 (anatomically defined synapses). We found that intensities of both GluR1 and GluR2 were significantly increased at VGLUT positive synapses in NR2B KO neurons when compared to controls (WT GluR1 3.3 ± 0.24 arbitrary units, KO GluR1 4.6 ± 0.20 ; $p=0.013$; WT GluR2 3.7 ± 0.24 , KO GluR2 4.5 ± 0.20 ; $p=0.009$). This is consistent with the finding that the amplitude of AMPA receptor mediated miniature EPSCs is increased in NR2B KO neurons relative to controls (Hall et al., 2007).

Together, these results suggest that NR2B acts as a negative, rather than as a positive, regulator of AMPA receptor surface and synaptic recruitment during development. This is contrary to the model proposed above which suggests that NMDA

receptors are positive regulators of AMPA receptor synaptic incorporation during development.

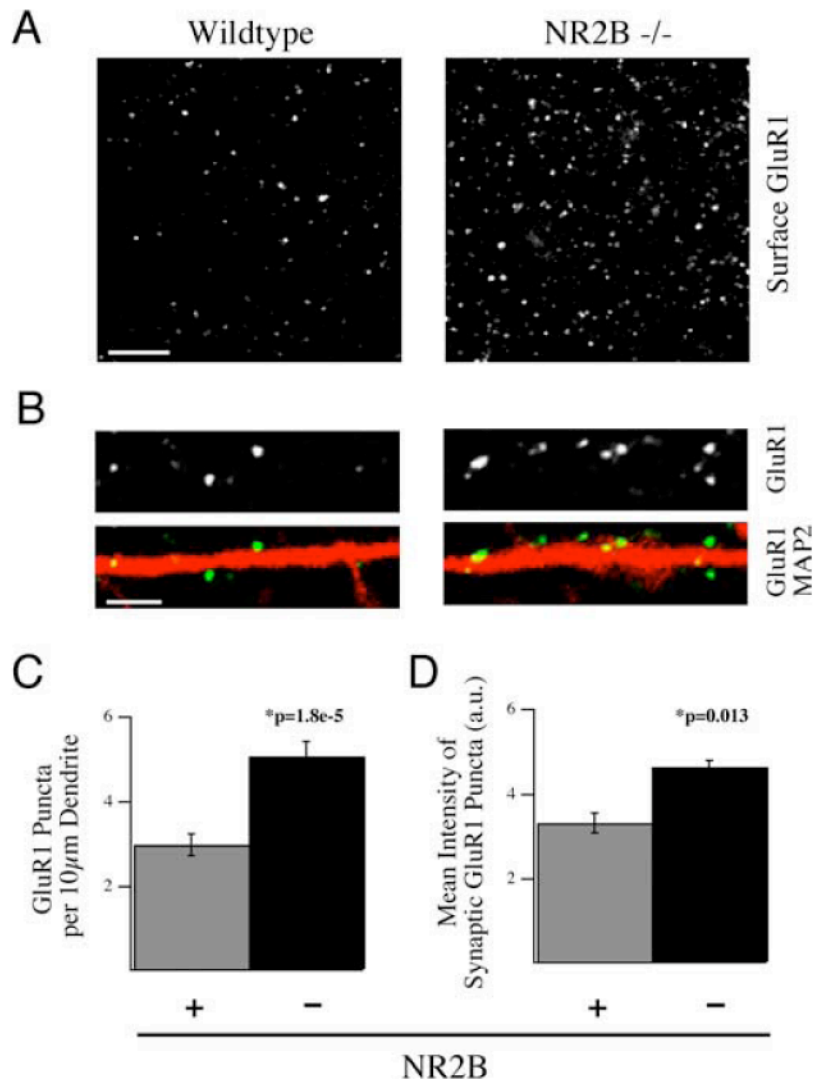


Figure A1.1 NR2B negatively regulates surface and synaptic localization of the AMPAR subunit GluR1

A-B. Cultured cortical neurons from NR2B knockout animals (right) and WT littermates (left) were live-labeled with anti-GluR1 antibodies at 14DIV and subsequently fixed and stained for MAP2 at 14DIV. These data show the dramatic increase in surface localization of this receptor subunit in the absence of NR2B. Scale bar = 5 µm in (A) and 2µm in (B).

C-D. Cultures at 14DIV were live-labeled for GluR1 and then fixed and stained for MAP2 and the synaptic marker VGLUT1/2. C. Quantification of the immunostaining data showing the surface expression of GluR1 puncta per length of dendrite was increased in NR2B knockout neurons compared with WT neurons. D. The integrated pixel density of GluR1 staining in VGLUT positive synapses was also significantly increased in NR2B knockout neurons versus control neurons (350 synapses per animal). All histograms show mean ± SEM. Figure from Hall et al., 2007.

**APPENDIX 2: NEURO-D2 IS A POSITIVE
REGULATOR OF AMPA RECEPTOR
RECRUITMENT TO SYNAPSES**

NeuroD2 was recently identified in a screen for calcium-activated transcription factors in cortical neurons (Aizawa et al., 2004; Ince-Dunn et al., 2006). NeuroD2 expression is developmentally upregulated during the first postnatal week in cortical layers II-VI, and shows particularly strong expression in Layer IV somatosensory barrel cortex. Strikingly, barrels fail to form in NeuroD2 knockout mice, suggesting a disruption in the development of this thalamocortical circuit. Indeed, thalamocortical axons did not segregate properly in these mice and postsynaptic barrel organization was similarly disrupted.

Also prominent in the NeuroD2 knockout mouse was a failure of proper synaptic maturation. During development, there is an increase in the AMPA to NMDA receptor ratio in several areas of the brain, including cortex (Crair and Malenka, 1995; Isaac et al., 1997; Hsia et al., 1998; Lu et al., 2001; Ye et al., 2005). In NeuroD2 null animals, there was a failure of this ratio change, which could potentially be due to either increased NMDA receptor-mediated current or decreased AMPA receptor-mediated current. Pharmacological blockade of NMDA receptors revealed a predominantly AMPA receptor-mediated current at +70 mV in barrel cortex neurons in response to evoked stimulation of white matter in heterozygous animals. However, outward current was totally blocked in slices from NeuroD2 knockout mice, suggesting that AMPA receptor-mediated responses are decreased in these animals. Pharmacological dissection of the relative contributions of NMDA, AMPA and kainite receptors in heterozygous and knockout animals further suggested that AMPA receptor currents are decreased in knockout animals.

To determine whether AMPA receptor trafficking was disrupted in NeuroD2 knockout animals, we live-labeled GluR1 and GluR2 subunits with antibodies directed against the extracellular domain of these proteins. Surface GluR1 and GluR2 levels were significantly decreased in NeuroD2 knockout animals in comparison to control littermates, in agreement with the findings just described. Thus, taken together, the data suggest that NeuroD2 acts as a positive regulator of AMPA receptor trafficking to the surface and synapses of neurons. In light of our recent findings describing a role for postsynaptic AMPA receptors for stabilization of presynaptic inputs, it is interesting to speculate on whether the failure of segregation of thalamocortical afferents into barrels is due in part to a failure of synaptic stabilization. More experiments are needed to explore this prediction.

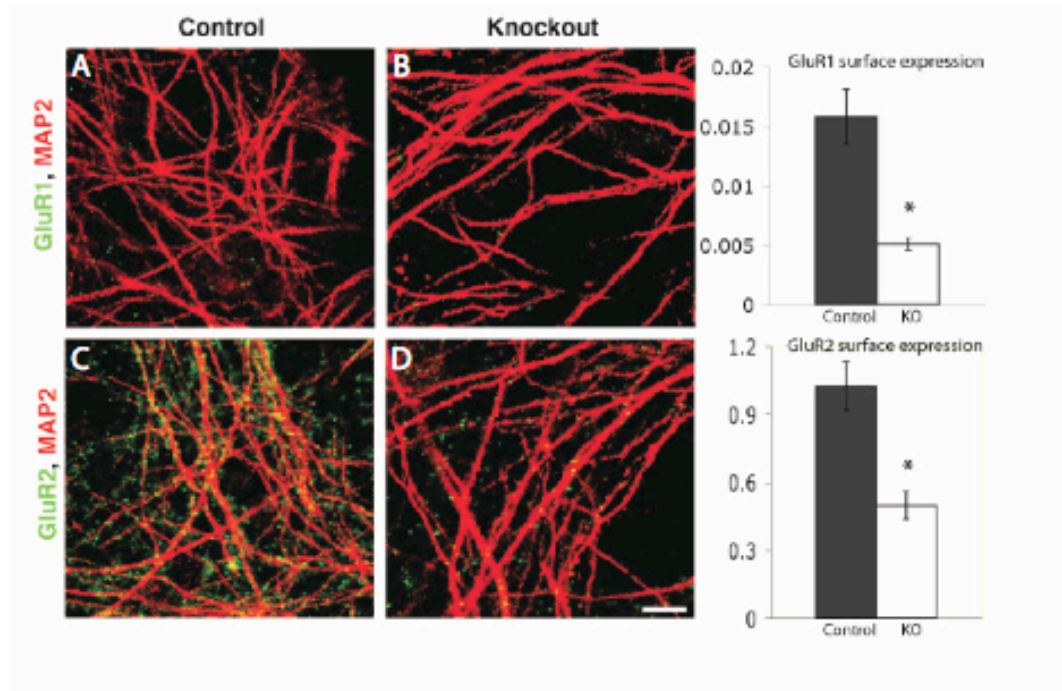


Figure A2.1 GluR1 & 2 surface expression is decreased in NeuroD2 knockout neurons.

A-B. 21DIV cultured neurons from NeuroD2 knockout mice (B) and control littermates (A) were live-labeled with anti-GluR1 antibodies (green) and subsequently fixed and stained for MAP2 (red). The graph to the right demonstrates that there was a significant decrease in the number of surface-expressed GluR1 puncta per micron of dendrite in NeuroD2 knockout animals. N=2 animals each condition. * $p < 0.05$

C-D. 21DIV cultured neurons from NeuroD2 knockout mice (D) and control littermates (C) were live-labeled with anti-GluR2 antibodies (green) and subsequently fixed and stained for MAP2 (red). The graph to the right demonstrates that there was a significant decrease in the number of surface-expressed GluR2 puncta per micron of dendrite in NeuroD2 knockout animals. N=2 animals each condition. Scale bar = 10 μm . * $p < 0.05$. Figure from Ince-Dunn et al., 2006.

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