Synaptic Plasticity and Morphogenesis in the Developing Postnatal Cerebral Cortex

Jacqueline de Marchena Powell

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the
Curriculum in Neurobiology.

Chapel Hill
2010

Approved by,
Franck Polleux, Ph.D; Thesis Co-Advisor.
Benjamin Philpot, Ph.D; Thesis Co-Advisor.
Paul Manis, Ph.D; Committee Chair.
Serena Dudek, Ph.D.
Mark Zylka, Ph.D.

ABSTRACT

Jacqueline de Marchena Powell

Synaptic Plasticity and Morphogenesis in the Developing Postnatal Cerebral Cortex

(Under the direction of Benjamin Philpot, Ph.D. and Franck Polleux, Ph.D.)

Learning how neurons interact, to create functional circuits, is crucial for understanding the basis of cognition and for shedding insight into the underpinnings of neurological disorders. This work will describe how changes in (1) postsynaptic *N*-methyl *D*-aspartate receptor (NMDAR) subunit composition and (2) dendritic spine morphology, influence synaptic transmission and synaptic plasticity in the developing cerebral cortex. Synaptic NMDARs undergo a dramatic activity-dependent change in subunit composition, going from being primarily NR2B-containing, to being increasingly NR2A-containing. Interestingly, this change in synaptic subunit composition correlates with developmental changes in the properties of synaptic plasticity. Using pharmacology, I have elucidated how NR2A- and NR2B-type NMDARs contribute to synaptic plasticity at distinct developmental time points. Using this approach, I demonstrate that the degree of NMDAR activation required for the induction of long term potentiation (LTP) increases with age. This work also focuses on the role of slit-robo GTPase activating protein 2 (srGAP2) on shaping the morphology of postsynaptic specializations, called dendritic spines. While the significance of dendritic spines is highly contentious, it has been shown that they play an important role in modulating the efficacy of glutamatergic synapses.

This work demonstrates that srGAP2 is expressed at the synapse and that it has the ability to induce an elongation of dendritic spine shape, through the synergistic action of both its FBAR and RhoGAP domains. From a physiological perspective, srGAP2 also influences synaptic transmission, by altering the shape and the complement of receptors at the postsynaptic membrane. As a whole, this doctoral work highlights the importance of changes in postsynaptic receptor composition and dendritic spine morphology, in shaping neural circuitry.

In dedication to:

Ashton, for giving me the courage to find my way

Mom, for inspiring me to dream
Dad, for showing me to love learning
Venessa, for keeping it real

Oh, and Nathan, for making it all worthwhile

ACKNOWLEDGEMENTS

Paul Manis, Benjamin Philpot, Franck Polleux, Serena Dudek, Mark Zylka,

Rebekah Corlew, Lindsey Wilfley, Vera Valakh, Maile Henson, Adam Roberts,

Paul Middlebrooks, Koji Yashiro, Janet Berrios, Micheal Wallace,

Dante Bortone, Randal Hand, Sabrice Guerrier, Marie Rougie,

Julien Crochet, Lisa Plummer, Jaeda Coutinho-Budd, Meghan Morgan-Smith,

Scott Hutton, Lorelei Taylor, Stacy Herzer, Eric Chen,

Bruce Herzer, Yukako Yokota, Julie Williams, Brandon Williams,

Venessa Figueroa, Michelle de Marchena, Albert de Marchena Jr., Erika de Marchena,

George & Ivonne de Marchena, Heidi, Hannah, and Heather,

Melanie & Eduardo de Marchena,

Ashley de Marchena, Matthew Shapiro, Eduardo de Marchena Jr., Tiffany de Marchena,

Earl Bryson Powell, Lauren Powell, Franny & Bryce Powell, Ned & Diane Powell,

Mary Powell, Brandon Powell, Becky Powell,

Octavio & Mercedes de Marchena, Clara & Salvador Jaramillo, Zorida Lopez,

Kirk Peppas & MAC swimming, Dr. Graham, Dr. Stone, Dr. Vaida, Mr. Terry,

Mom & Dad

Ashton & Nathan Powell

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AMPA - α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APV - ((2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate)

CNQX - 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate

DIV – Days *in vitro*

FBAR - Fer Cip4 Homolgy (FCH) Domain and Bin/Amphiphysin/Rvs-homology (BAR) Domain

LTD – Long Term Depression

LTP – Long Term Potentiation

mEPSC – mini excitatory postsynaptic current

NMDA - *N*-Methyl *D*-Aspartate

NMDAR – *N*-Methyl *D*-Aspartate Receptor

NR1 – NMDAR 1 Subunit

NR2 – NMDAR 2 Subunit

NR2A - NMDAR 2A Subunit

NR2B - NMDAR 2B Subunit

NVP-AAM077 - (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4 tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid

P - Postnatal day

RhoGAP – Rho Family GTPase Activating Protein

SH3 – SRC Homolgy 3 Domain

srGAP2 – Slit Robo GTPAse Activating Protein 2

TTX - Tetrodotoxin

`

CHAPTER ONE

INTRODUCTION

The brain is anatomically organized in a topographic manner, with distinct segregation of sensory and motor areas. This regionalization allows sensory input from each modality to converge upon a defined subpopulation of neurons, which become specialized in processing discrete types of information. While brain topography is defined by birth, neural connections within these sensory processing areas are refined in an activity-dependent manner during early postnatal development. The focus of this thesis is to better understand the mechanisms that sculpt neural circuitry in the developing cortex. A broad approach, focusing on both synaptic physiology and morphology, has been taken in addressing this multifaceted question.

This body of research first focuses on how the composition of postsynaptic N-Methyl D-Aspartate Receptors (NMDARs) affects the properties of neurotransmission and synaptic plasticity over the course of postnatal development. Because NMDAR activation is required for most types of synaptic plasticity, they are profoundly important for sculpting neuronal circuitry during early postnatal development. While it is well known that NMDARs are critical regulators of synaptic plasticity, relatively little is known about how the distinct properties of different types of NMDARs affect mechanisms of long-term potentiation (LTP) or long-term depression (LTD). NMDAR subunit composition confers distinct properties onto the receptor by tethering the receptor to different intracellular effector molecules, changing the receptor's gating properties, and profoundly influencing the receptor's current kinetics. Interestingly, NMDAR subunit composition is highly regulated over the course of development, in an

activity-dependent manner. Yet, exactly how this affects synaptic plasticity in the developing cortex remains elusive.

While electrophysiology has been used extensively to understand how synaptic function is modulated by the complement of neurotransmitter receptors that are present on the postsynaptic membrane, far less is known about how the morphology of postsynaptic specializations, called dendritic spines, shape neurotransmission. During early postnatal development, dendritic spines are very thin and highly dynamic. But, over the first three postnatal weeks, spines become increasingly stable and assume a more mature "stubby" morphology, with a thin neck and a mushroom-like head. To better understand how the shape of dendritic spines influences synaptic transmission and synaptic plasticity, I focused on a membrane deforming protein called slit-robo GTPase Activating Protein 2 (srGAP2), which is expressed at the synapse. Interestingly, this protein has been shown to directly bind and deform lipid membranes through its FBAR domain. However it can also hydrolyze and inactivate the small G Protein, Rac1, a known regulator of the actin cytoskeleton. Therefore, srGAP2 can synergistically influence membrane dynamics through direct interactions with the cell membrane and indirect interactions through the actin cytoskeleton.

This thesis will first review what is currently known about NMDAR subtypes, their relative expression over the course of postnatal development, and what is currently known about their contribution to synaptic plasticity. This will then be followed by a description of how properties of synaptic plasticity, in the primary visual cortex, have been shown to change over the course of development. The overlap between changing NMDAR subunit composition and concomitant changes in synaptic plasticity will be addressed.

This thesis will also address how structural changes in neuronal morphology play a pivotal role in shaping neural circuitry. How dendritic spine morphology is hypothesized to affect neurotransmission, will be reviewed. Finally, the function of FBAR and RhoGAP containing proteins, like srGAP2, in modulating neuronal morphology will also be discussed.

`

Cortical connectivity and synaptic plasticity

The cerebral cortex is dorsally located within the brain and is subdivided into six layers, which receive input from different brain regions. Each layer then sends information to different brain areas (**Fig. 1A)**. This thesis will focus on the plasticity of synaptic connections made by Layer (L)4 granule cell neurons onto L2/3 pyramidal cell neurons of the developing cerebral cortex. The molecular mechanisms shaping the morphology of dendritic spines on L5 pyramidal neurons will also be discussed.

While the broad topographical organization of the brain is defined prenatally, synaptic plasticity plays an important role in shaping the functional circuitry of the developing postnatal cortex. One cortical region that has particularly well-defined mechanisms of synaptic plasticity is the primary visual cortex. In the binocular region of the primary visual cortex, input from the right and left eye drive activity in defined cortical columns (**Fig. 1B**). However, during an early stage of development known as the “critical period” both eyes compete for the ability to drive the activity of these cortical columns. This is an activity-dependent process that begins at three weeks and ends at five weeks of life in mice (Gordon and Stryker 1996). During this period, if one eye is occluded so that no sensory driven activity can be elicited, the open eye will begin to drive the activity of columns that were previously activated by the occluded eye (Wiesel and Hubel 1965). The relative change in the ability of each eye to drive the activity of cortical columns is called ocular dominance plasticity. This type of plasticity is restricted to the critical period and is very prominent in highly visual species like cats and humans. In mice, there is not a clear anatomical segregation of cortical neurons into discrete columns (Antonini et al. 1999) but the binocular region of the visual cortex still exhibits robust ocular dominance plasticity (Gordon and Stryker 1996).

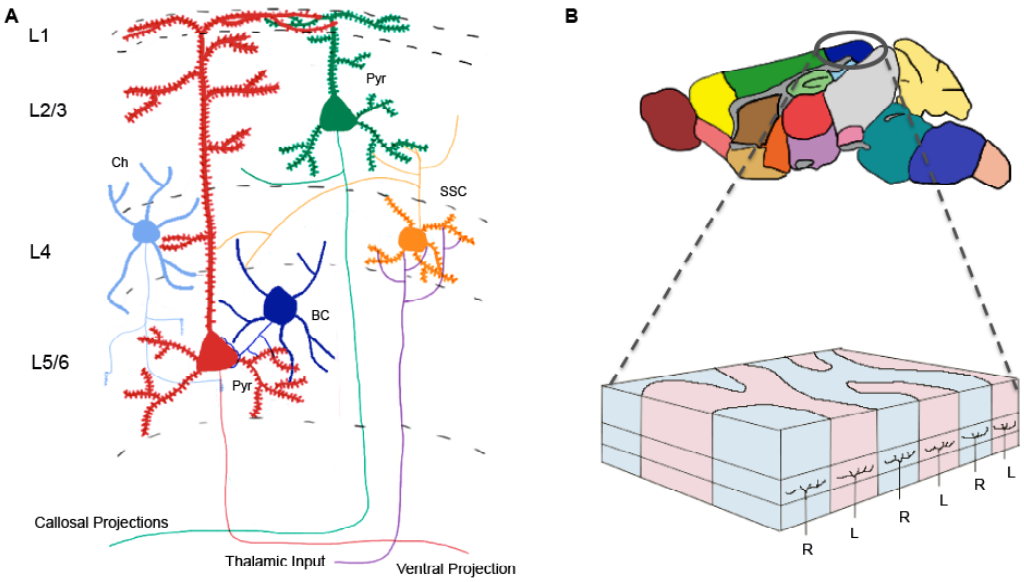

Figure 1: The anatomy of the brain is highly organized, with complex connectivity that can be modified in an activity-dependent fashion. **(A)** The cortex consists of six layers of neurons that receive information from different afferent populations. Sensory information from the thalamus, synapses onto layer (L) 4 spiny stellate neurons (SSC). L4 neurons then synapse onto L2/3 pyramidal neurons (Pyr), which go onto make robust intracortical synaptic connections onto both pyramidal neurons and interneurons, such as basket (BC) and chandelier cells (Ch). However, the axons of L2/3 neurons project collosally to contalateral cortex, while the axons of L5 pyramidal neurons project ventrally, to deeper brain structures. **(B)** Within the demarcated primary visual cortex, specific columns of neurons respond preferentially to sensory information from the right (R) or left (L) eye.

Changing the strength of synaptic connections, in an activity-dependent manner, defines the basis of synaptic plasticity. At its foundation, the concept of synaptic plasticity rests on the premise that repeated stimulation of a presynaptic cell onto a postsynaptic cell will result in a stronger synaptic connection. Conversely, cells that are weakly connected will undergo synaptic weakening (Malenka and Bear 2004). Both excitatory synpases, which use glutamate as a neurotransmitter, and inhibitory synapses that use GABA as a

neurotransmitter (**Fig. 2**), can undergo synaptic strengthening and weakening (Hensch et al. 1998; Scannevin and Huganir 2000; Maffei et al. 2006). However, in this thesis I will focus entirely on the modulation of glutamatergic synapses.

Glutamatergic synapses release glutamate onto a complement of postsynaptic receptors, including AMPA and NMDA receptors. Both depolarize the postsynaptic neuron but AMPA receptors produce large, rapid currents in response to glutamate release, while NMDA receptors have slower current kinetics (Hollmann and Heinemann 1994). NMDA receptors and AMPA receptors that contain the GluR2 subunit, are permeable to calcium (Sprengel and Seeburg 1993). This has important implications for the molecular mechanisms that mediate the expression of synaptic plasticity (Blitzer 2005). A main focus of this thesis is to elucidate how different types of NMDARs contribute to the expression of synaptic strengthening, known as LTP, and synaptic weakening, known as LTD.

Composition and characteristics of NMDARs in the developing cortex

NMDARs allow current influx when presynaptic release of glutamate is coupled to postsynaptic depolarization. Thus, they have been coined “coincident receptors” due to their requirement for temporally paired pre- and post-synaptic activity. This unique property allows NMDARs to play an important role in synaptic plasticity, learning, and memory. NMDARs contain two NR1 subunits and two ancillary subunits (NR2A-D, NR3A-B) that confer unique glutamate-binding and kinetic properties onto the receptor (McBain and Mayer 1994; Flint et al. 1997; Laube et al. 1998). Within the cortex, NR2A and NR2B are the most widely expressed. Thus, the majority of research focusing on the role of NMDAR subunits in synaptic plasticity, learning and memory focus on these subunits.

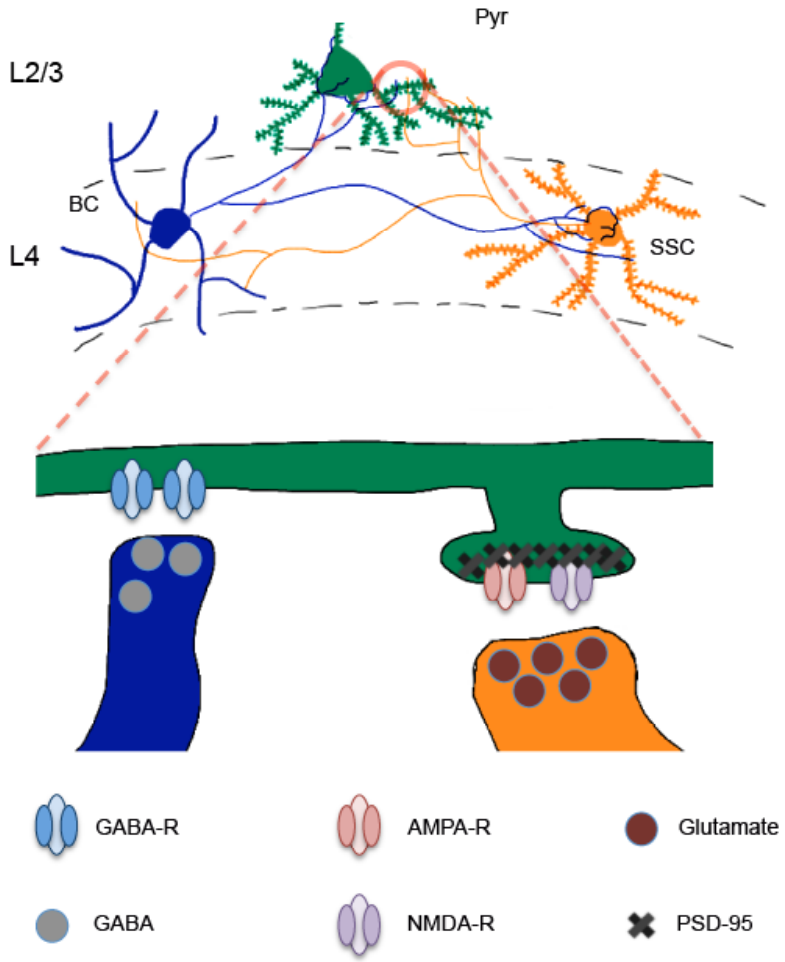

Figure 2: Excitatory glutamatergic and inhibitory GABAergic synapses create the functional connectivity of the cortex. Glutamatergic synaptic contacts are characterized by postsynaptic densities, consisting primarily of PSD-95, that anchor AMPA and NMDA-type glutamate receptors at the synapse. These glutamatergic synapses are subcellularly confined to small dendritic protrusions, called dendritic spines. Glutamate depolarizes neurons by allowing for sodium and calcium influx. Conversely, GABAergic synapses do not have a postsynaptic density and are not exclusively localized at dendritic spines. Furthermore, in the mature cortex, GABA typically hyperpolarizes neurons by promoting the influx of chloride. Thus, excitatory and inhibitory synaptic contacts differ in (1) the neurotransmitter that is released from the presynaptic terminal, (2) the receptor composition on the postsynaptic membrane, (3) the presence of a postsynaptic density, (4) their localization to dendritic spines, and (5) their ability to either depolarize or hyperpolarize the neuron.

`

The exact composition of NMDARs in the developing cortex is difficult to ascertain, with NR1/NR2B dimers, NR1/NR2A dimers, and NR1/NR2B/NR2A triheteromers being difficult to discriminate (Kohr 2006). However, it is well established that the general composition of synaptic NMDARs goes from being primarily NR2B-containing, to increasingly NR2A-containing over the course of development (Monyer et al. 1994; Watanabe et al. 1994)(**Fig 2**). This developmental upregulation of the NR2A subunit is activity-dependent (Quinlan et al. 1999) and is controlled at the transcriptional level (Hoffmann et al. 2000; Yashiro and Philpot 2008). While the significance of this transition is unclear, NR2A and NR2B subunits confer important differences onto the channel properties and intracellular binding partners of NMDARs. NR1/NR2A dimeric receptors have a higher open probability and faster deactivation kinetics than NR1/NR2B receptors (Monyer et al. 1994; Vicini et al. 1998). Functionally, this means that NR1/NR2A dimers have fast current kinetics, while NR1/NR2B dimers have slower currents that result in a greater amount of charge transfer (Erreger et al. 2005). Interestingly, calcium-imaging studies have shown that NR1/NR2B dimers allow the influx of a greater amount of calcium ions per unit of charge than NR1/NR2A dimers (Sobczyk et al. 2005). Because calcium mediates a host of second messenger pathways, this difference in calcium permeability may have important implications for how synaptic plasticity is regulated by these subunits. Not surprisingly, triheteromeric NR1/NR2A/NR2B NMDARs have characteristics that are intermediate to pure NR2A or NR2B dimers (Vicini et al. 1998). One study using serial immunoprecipitation, reported that in adult hippocampus, 2/3 of NMDARs were diheteromeric, while only 1/3 of NMDARs were triheteromeric (Al-Hallaq et al. 2007). However, the relative amount of NMDAR dimers to NMDAR triheteromers is difficult to ascertain due to developmental and region-specific differences in expression.

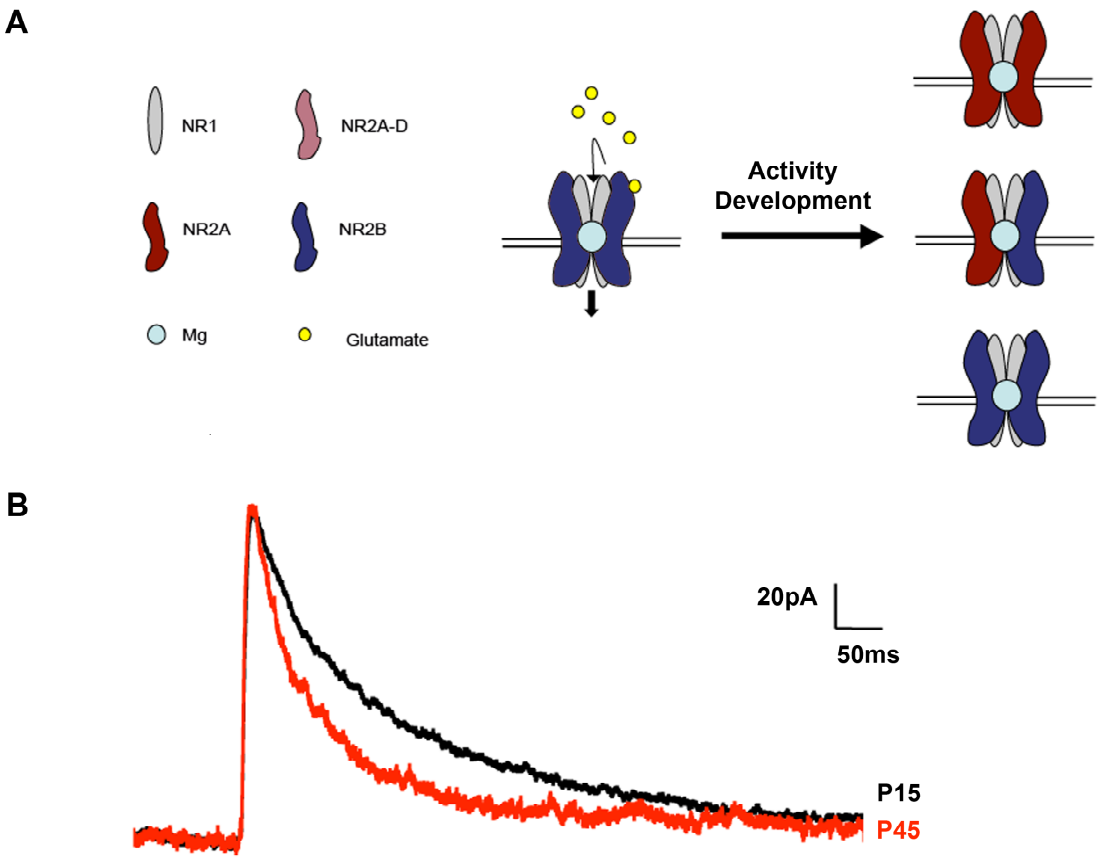

Figure 3: NMDAR subunit composition, at glutamatergic synapses, in the developing cortex. **(A)** NMDARs consist of two obligatory NR1 subunits and two ancillary subunits that can be either NR2A-D or NR3A-B. These secondary subunits confer unique glutamate binding affinities and current kinetics onto the receptor. NDMARs are permeable to sodium and calcium when glutamate is bound to the receptor and when the postsynaptic membrane potential is sufficiently depolarized to release the channel's magnesium block. Because the receptor only allows depolarization to occur when presynaptic release and postsynaptic depolarization are temporally synchronized, the NMDAR has been coined a "coincident receptor" of both pre- and post-synaptic activity. Interestingly, over the first three postnatal weeks, NMDARs undergo a profound activity-dependent shift from being primarily NR2B containing, to largely NR2A containing. **(B)** This developmental change in NMDAR subunit composition strongly affects NMDAR current kinetics. This is because NR2B containing receptors, which are highly expressed at early developmental stages, have slower current kinetics (black trace) than NR2A-containing receptors that have much faster current kinetics (red trace).

`

Subcellular expression of NMDAR subtypes

While it is clear that different NMDAR subunits confer distinct properties onto NMDARs, it is unclear whether different types of NMDARs preferentially populate distinct subcellular regions (synaptic versus extrasynaptic) or whether the composition of NMDARs changes in an input-specific manner. Gaining a better understanding of the where NMDAR subunits are expressed and how their expression changes in response to activity, will shed light onto the physiological importance of different NMDAR subunits.

NR2A-containing NMDARs may preferentially populate the central region of the synapse. This is supported by a study showing that NMDAR mediated mEPSCs have faster current kinetics than evoked NMDAR currents (Dalby and Mody 2003). This suggests that NR2A-containing NMDARs, with relatively fast current kinetics, populate the central region of the synapse that is sensitive to single vesicles of neurotransmitter release. However, upon action potential-evoked release, more peripherally located NR2B-containing receptors that have slower current kinetics are activated, changing the current decay kinetics. This interpretation hinges on the premise that univesicular release is subsaturating at the synaptic cleft, and that only evoked release can stimulate all the NMDARs at the synapse. However, this concept is highly contested and may differ between developmental stages and brain regions (Clements 1996). Yet, the idea that NR2A-containing NMDARs are present in the central portion of the synpase is further supported by the finding that there are no NMDAR components to mEPSCs recorded in NR2A knockout mice (Townsend et al. 2003). There are also a number of studies that demonstrate the presence of extrasynaptic NR2B-containing NMDARs (Stocca and Vicini 1998; Tovar and Westbrook 1999).

Over the course of development, NMDAR subunit composition changes from being largely NR2B-containing, to increasingly NR2A-containing. While it is known that sensory-driven activity is required for this subunit transition to occur (Quinlan et al. 1999), it is still

unknown whether this transition occurs in a synapse-specific manner. However, there are several lines of evidence suggesting that NMDAR composition is regulated on the synaptic level. First, inter- and intra-cortical synapses have different NR2A/NR2B ratios—demonstrating that synapses receiving inputs from different populations of afferents have different complements of NMDARs (Kumar and Huguenard 2003). Also, on the single-cell level, NR2B is differentially expressed on apical and basal dendrites (Kawakami et al. 2003). Additionally, synaptic plasticity appears to change the composition of NMDARs at the synapse. In line with this, the induction of LTP in the CA1 region of the hippocampus causes an input specific increase in the NR2A/NR2B ratio. Therefore, synapse-specific differences in NMDAR subunit composition may explain why different synapses exhibit different degrees of synaptic plasticity (Matsuzaki et al. 2004).

NMDA receptor subunit composition and synaptic plasticity

NMDAR subunit composition may affect plasticity by modulating NMDAR current kinetics and by influencing intracellular binding to distinct signaling molecules. There are a number of reasons to speculate that the NR2B subunit promotes LTP. (1) The NR2B subunit allows for more charge transfer and has greater calcium permeability than the NR2A subunit (Monyer et al. 1994; Vicini et al. 1998; Erreger et al. 2005). (2) NR2B preferentially interacts with CamKII, a molecule known to be important for the induction of LTP (Strack and Colbran 1998; Lisman et al. 2002). (3) Overexpressing the NR2B c-terminus, which limits the intracellular interaction of the NR2B subunit to its endogenous binding partners like CamKII, decreases the magnitude of LTP (Barria and Malinow 2005). (4) Exogenous expression of NR2A, which reduces the relative amount of NR2B at the synapse, attenuates LTP in hippocampus (Barria and Malinow 2005). (5) Genetic loss of NR2A does not affect LTP (Berberich et al. 2005; Weitlauf et al. 2005; Philpot et al. 2007). Taken together, these

lines of evidence provide strong support for the role of NR2B in LTP. However, while NR2B-containing NMDARs are relatively downregulated at the synapse during development, LTP persists at maturity (Kirkwood et al. 1997; Jiang et al. 2007). This suggests that the relative amount of NR2A and NR2B at the synapse may not dictate the polarity of plasticity, as much as modulate the degree of synaptic stimulation required to induce plasticity in the postnatal cortex (Yashiro and Philpot 2008). Because increasing expression of NR2A would limit charge transfer, reduce calcium permeability, and attenuate the amount of CamKII proximal to the synapse, its expression may increase the amount of synaptic stimulation required for LTP.

The idea that NR2A upregulation modulates the amount of stimulation required to induce synaptic plasticity, is supported by studies that manipulate the expression of NR2A in the developing cortex. Using dark rearing to prevent NR2A upregulation in the visual cortex, it has been shown that the frequency of stimulation required to induce LTP and LTD is reduced. This effectively increases the range of frequencies that can be used to evoke LTP in these animals. Therefore, it has been speculated that the developmental change in the NR2A/NR2B ratio causes a shift in the LTP/LTD induction threshold. This would effectively create a lower threshold for LTP induction at early developmental timepoints, when cortical circuitry is being established. Studies done on NR2A knockout animals also provide strong evidence that NR2A upregulation is required for increasing the threshold for LTP, over the course of development.

Also, dark rearing does not reduce the threshold for LTP induction in these knockouts, suggesting that NR2A is required for activity-dependent changes in the synaptic plasticity threshold (Philpot et al. 2007). Furthermore, the LTP threshold in NR2A knockout mice is greatly diminished, with 1Hz stimulation being sufficient to drive LTP (Philpot et al. 2007).

While the NR2B subunit of the NMDAR has been shown to be important for LTP, less is known about how NR2A subunits directly contribute to synaptic plasticity. While

NR2A upregulation increases the stringency for LTP induction, by diluting the amount of NR2B at the synapse, it does not prevent the expression of LTP all together. Yet, a direct association between NR2A and known LTD signaling pathways, like the PP1/PP2B (calcineurin pathway) (Yashiro and Philpot 2008), has yet to be shown. Because of this, it does not appear that each subunit is coupled to LTP or LTD in a binary fashion. Rather, it seems that the developmental transition in NMDAR subunit composition regulates plasticity over the course of postnatal development by changing the amount of stimulation required to induce LTP, in an activity-dependent fashion.

Using pharmacology to assess subunit-specific functions

One approach to better understand how current through distinct subtypes of NMDARs contribute to synaptic plasticity, is to use pharmacological antagonists that selectively block particular subunits. While much can be gained from these types of studies, it should be re-emphasized that NMDARs in the cortex heterogeneous—and this can strongly influence their susceptibility to NMDAR antagonists. As stated earlier, it has been estimated that 2/3 of cortical NMDARs are dimeric NR1/NR2A or NR1/NR2B-containing receptors, while 1/3 of cortical NMDARs are triheteromeric NR1/NR2A/NR2B- containing NMDARs (Al-Hallaq et al. 2007).

Ifenprodil is a well-characterized antagonist for NR2B-containing NMDARs. It binds the amino terminus of the NR2B subunit in a non-competitive fashion (Gallagher et al. 1996; Perin-Dureau et al. 2002; Mosley et al. 2009). Performing whole-cell recordings, in the presence of ifenprodil, it has been demonstrated that more current is carried by NR2B-containing NMDARs at early developmental timepoints when compared to later developmental timepoints. This is completely congruent with its biochemically defined expression pattern (Monyer et al. 1994; Stocca and Vicini 1998; de Marchena et al. 2008). However, it binds with very low affinity to triheteromeric NMDARs that contain both NR2B

and NR2A subunits (Kew et al. 1996; Tovar and Westbrook 1999). This makes it impossible to assess the contribution of trihetomeric NMDARs, which may represent as much as 1/3 of the total NMDAR population (Al-Hallaq et al. 2007). It has also been reported that the efficacy of ifenprodil is strongly influenced by the concentration of glutamate at the synaptic cleft. Ifenprodil has actually been shown to potentiate NMDAR currents at low glutamate concentrations (Kew et al. 1996). However, the broad applicability of this phenomenon to cortical synapses is unclear.

More recently, an antagonist was developed to specifically block NR2A-containing NMDARs. This antagonist, NVP-AAM077, was initially reported to be 100 times more selective for NR2A, over NR2B-containing receptors in a cell line that was exogenously expressing human NMDARs (Auberson et al. 2002). However, subsequent reports found that NVP-AAM077 was only 6-12 times more specific for NR2A, when rodent NMDARs were expressed (Feng et al. 2004). Furthermore, it was found that NVP-AAM077 blocks a significant amount of NMDAR current in brain preparations obtained from NR2A knockout animals (Neyton and Paoletti 2006; de Marchena et al. 2008).

Yet, because the drug was initially reported to have such strong specificity for NR2A-containing NMDARs, many investigators began use NVP-AAM077 alongside ifenprodil to probe for NR2 subunit-specific contributions to synaptic plasticity. Interestingly, two reports emerged demonstrating that plasticity in the hippocampus and in the perirhinal cortex was strongly dependent on subunit-specific activation (Liu et al. 2004; Massey et al. 2004). In these studies, LTP was blocked by the NR2A-specific antagonist NVP-AAM077 and LTD was blocked by the NR2B-specific antagonist ifenprodil. These findings suggested that LTP was dependent on NR2A-containing NMDARs and LTD was dependent on NR2B-containing NMDARs. However, three separate laboratories were unable to replicate these findings, reporting that LTD in hippocampus was completely insensitve to ifenprodil application. LTP was also found to persist in the presence of 50nM NVP, a concentration believed to have

the highest possible specificity for NR2A-containg NMDARs (Berberich et al. 2005; de Marchena et al. 2008).

However, if properly interpreted, pharmacology can be used to better understand how different types of NMDARs contribute to synaptic plasticity. The work described in Chapter Two of this thesis employs pharmacology to carefully assess how the properties of synaptic plasticity change over the course of development. In this work, we found that NVP-AAM077 only attenuates LTP at late developmental time points. Using a subsaturating concentration of APV, a pan-NMDAR antagonist, we mirrored the differential effect of NVP-AAM077 on LTP. We propose that this is the result of a developmental increase in the amount of NMDAR activation needed to elicit LTP (de Marchena et al. 2008).

Synaptic morphology and the development of dendritic spines

While this introduction has focused on the role of postsynaptic NMDARs, the morphology of synapses is also very important in shaping neural circuitry. While the postsynaptic element of synapses cluster neurotransmitter receptors, its general shape affects its response to neurotransmitter and its ability to drive dendritic depolarization. Dynamic changes in the shape of the synapse and the width of the synaptic cleft are also thought to underlie elements of synaptic plasticity (Engert and Bonhoeffer 1999; Yuste and Bonhoeffer 2001; Hofer et al. 2009).

90% of glutamatergic synapses are located on dendritic protrusions, known as dendritic spines (Nimchinsky et al. 2002). These spines have a characteristic morphology that undergo dynamic changes during development (Dailey and Smith 1996; Ziv and Smith 1996; Matus 2000; Yuste and Bonhoeffer 2004). The spine contains an electron dense region, known as the postsynaptic density, where the majority of glutamate receptors and accompanying signaling molecules are clustered (Gray 1959; Parnavelas et al. 1977). This is in contrast to GABAergic synapses that are located throughout the somatodendritic

region. These inhibitory synapses do not form dendritic spines or contain a postsynaptic density (DeFelipe and Farinas 1992). However, the biological purpose of dendritic spines and the reason why they are specific to glutamatergic synapses, remains debated.

Dendritic spines begin to form during the first week of postnatal life and increase in density until the fourth postnatal week (Nimchinsky et al. 2002). At this point, there is a gradual reduction in the number of dendritic spines. Therefore, there is an initial overproduction of dendritic spines, which is followed by elimination. Early in development thin, long filopodial-like protrusions emerge from dendrites. The terminal filopodial-like protrusions that extend from the ends of developing dendrites, guide the formation of branches on the dendritic tree (Luo 2002). However, in CA1 of the hippocampus, collateral filopodia have been shown to extend from the middle of dendritic processes and make putative synaptic contacts (Fiala et al. 1998). These axo-dendritic contacts then go on to form dendritic spines (Fiala et al. 1998).

Dendritic spines are conventionally subdivided into three categories: stubby, thin, and mushroom-like (Peters and Kaiserman-Abramof 1970). Developmentally, dendritic spines transition from being stubby, to thin, and then finally to mushroom-shaped (Yuste and Bonhoeffer 2004). However, dynamic filopodia play an important role in initiating synaptic connections that have the potential to form dendritic spines. Because developing neocortical neurons have been reported to form as many as 50,000 new filopodial protrusions per day (Portera-Cailliau et al. 2003), these extensions are uniquely able to probe the extracellular environment for appropriate synaptic connections. This active process allows developing neurons to create functional synaptic circuitry. However, while several models have been proposed, the exact mechanism that induces the formation of dendritic spines is still an area of active debate (Yuste and Bonhoeffer 2004).

One conceptualization, coined the Sotello Model, posits that dendritic spines form in a cell-autonomous fashion (i.e. independent of the presence of pre-synaptic axons). Most of

the evidence for this model comes from the cerebellum, looking at synaptic connections between granule and Purkinje cells. In mutants where granule cells are missing, resulting in a loss of presynaptic innervation onto Purkinje cells, dendritic spine formation is unperturbed (Mariani et al. 1977). Purkinje neurons in these knockouts even have normal postsynaptic specializations without pre-synaptic apposition. Similar findings have been reported using X-irradiation to ablate granule cells in the cerebellum (Altman and Anderson 1972). It also appears that dendritic spines, at least in the cerebellum, emerge before the presynaptic specializations can be visualized in the proximity of the spine. These spines are referred to as "naked spines" and their presence suggests that spines can form independently of presynaptic specializations. However, the rules that govern spinogenesis may differ across different neuronal sub-types, as naked spines are rarely observed in the neocortex (Arellano et al. 2007).

Another theory on spine formation is that filopodia are spine precursors that directly seek out presynaptic afferents (Ziv and Smith 1996; Yuste and Bonhoeffer 2004). Once a functional synaptic contact is made, dendritic spines are thought to mature from these elongated protrusions. However, two lines of evidence suggest that this is an unlikely scenario. First, filopodial protrusions are made by many neurons that do not go on to form dendritic spines (Mason 1983; Wong et al. 1992; Linke et al. 1994). Therefore, while dynamic filopodial protrusions may play a role in dendritic spine formation, they must serve other functions that are unrelated. Secondly, the idea that filopodia are precursors to dendritic spines is contrary to the ontogeny of dendritic spine development in the neocortex and cerebellum, which progress from being stubby, to being longer and mushroom-like (Harris et al. 1992; Yuste and Bonhoeffer 2004).

The most likely mechanism guiding spinogenesis in the neocortex is the Miller/ Peters hypothesis, where axonal contacts induce the formation of dendritic spines through direct contact (Yuste and Bonhoeffer 2004). Serial electron microscopy conducted over a

range of developmental time points, in the CA1 region of the hippocampus, provides convincing evidence that synaptic connections precede the formation of dendritic spines (Fiala et al. 1998). These early synaptic contacts do not exhibit any type of postsynaptic protrusions and are referred to as shaft synapses. These contacts go on to form stubby spines, before ultimately making mature mushroom-shaped spines. However, the extensive number of filopodia that are generated during the early postnatal period probably act to provide cellular surface area for the formation of synapses (Fiala et al. 1998). Therefore, while filopodia appear to play an important role in allowing dendrites to make contacts with axons, spine formation itself seems to be coincide with the presence of a presynaptic contact.

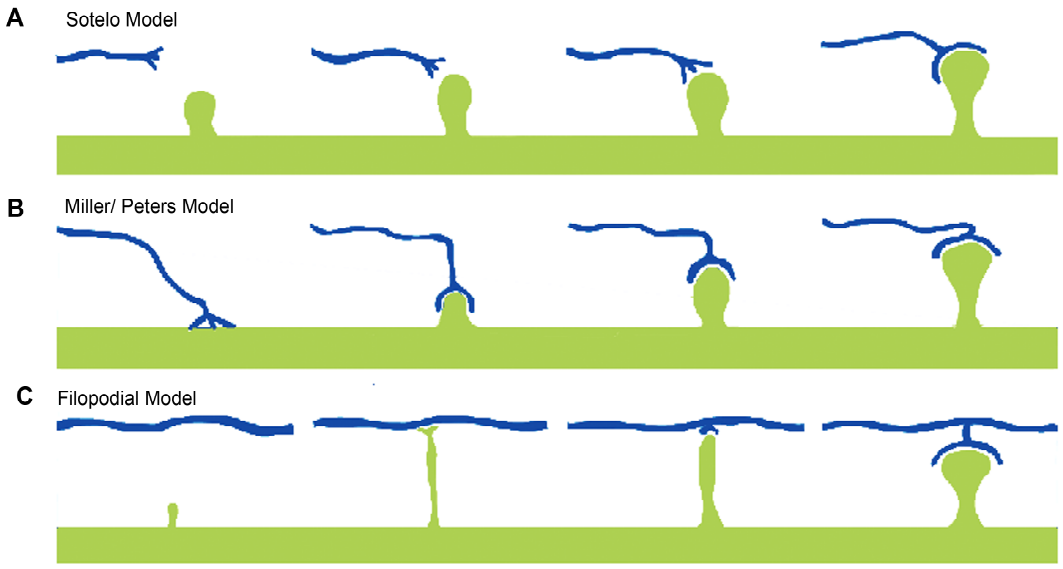

Figure 4: Genesis and maturation of dendritic spines in the postnatal cortex (Yuste et al. 2004). **(A)** The Sotelo Model of dendritic spine formation, suggests that dendritic spines form in a cell autonomous fashion, independent of cues from presynaptic axons. **(B)** The Miller/Peters Model claims that the axon induces dendritic spine formation. In this model, the axon dictates where a spine is formed and how it moves along the dendritic shaft. **(C)** The filopodial model proposes that both the dendrite and the axon play a complementary role in the formation of mature dendritic spines. The axon may provide specific cues that allow the dendrite to seek out a synaptic connection, through the formation of filopodial-like protrusions.

Functional significance of dendritic spines

Although dendritic spines are prominent features of glutamatergic synapses, how they influence neural circuitry is just beginning to be understood. Spines are highly dynamic structures that can change in shape, appear de novo, or disappear altogether. Although changes in spine morphology and turnover are more robust during early postnatal development, the fact that this phenomena persists into adulthood suggests that morphological changes in spines continue to play an important role in sculpting neural communication throughout life (Grutzendler et al. 2002; Trachtenberg et al. 2002).

Live imaging experiments on fluorescently labeled neurons in vivo reveal a developmental decrease in spine dynamics (Grutzendler et al. 2002; Trachtenberg et al. 2002). During early postnatal development there is rapid addition and retraction of filopodial and spine-like processes. Interestingly, as development proceeds, the overall density of dendritic spines decreases and the spines that remain are increasingly persistent (Grutzendler et al. 2002). This suggests that there is an early phase of dynamic connectivity that is followed by a process of refinement. This progressive elimination of dendritic spines reflects the pruning of synaptic connections that occurs through input competition, a process known to play a central role in neural development (Lendvai et al. 2000).

Spines have been shown to change their dynamics in an activity dependent fashion. Globally preventing neural activity, by perfusing tetrodotoxin (TTX), causes a robust increase in spine density (Bravin et al. 1999). A similar increase in spine density was found subsequent to monocular deprivation in the binocular region of the primary visual cortex (Hofer et al. 2009). This may reflect a homeostatic mechanism that acts in response to decreased levels of synaptic activity. Interestingly, the change in spine density that follows monocular deprivation persists and repeated deprivation fails to produce any additional changes in spine density. Therefore, it appears that the spines underlying an alternative pattern of synaptic connectivity can exist in a dormant state until they are once again

recruited to respond to a change in the sensory environment. Whisker trimming also affects the protrusive motility of dendritic spines in the barrel cortex (Lendvai et al. 2000). It should be noted that this change in spine motility is restricted to the critical period, with no effect observed for sensory deprivation occurring before or after this developmental window. High frequency stimulation protocols that are typically used to induce LTP in organotypic slices have also been shown to increase spine density in CA1 of the hippocampus (Engert and Bonhoeffer 1999). Therefore, it appears that directly manipulating activity with drugs, stimulation protocols, and natural sensory-evoked activity can all influence spine dynamics.

The shape of dendritic spines also plays an important role in modulating postsynaptic characteristics. Elongated spine necks are thought to create a diffusional bottleneck that impedes the flow of calcium and important intracellular signaling molecules from the proximity of the synapse (Yuste et al. 2000). Spine specific increases in calcium concentration have been visualized, with calcium sensitive dyes, following synaptic stimulation (Denk et al. 1996). Diffusional exchange has also been measured using fluorescence recovery after photobleaching (FRAP) experiments—confirming the anatomical assumption that spines can act as semiautonomous microdomains (Svoboda et al. 1997). However it is also hypothesized that spine shape is capable of not only influencing its diffusional characteristics, but also its electrical properties (Nimchinsky et al. 2004; Tsay and Yuste 2004; Spruston 2008). Specifically, the length of the spine neck may act to limit how depolarization, at the spine head, translates into depolarization of the dendritic shaft. This is important because voltage sensitive calcium channels may restrict calcium entry to this small microdomain, if depolarization is largely restricted to the spine head. This physical restriction of calcium diffusion could underlie input-specific mechanisms governing plasticity. The ability of spines to restrict depolarization is also important because it influences how synaptic inputs are integrated at the dendritic shaft. However, the ability of the spine to

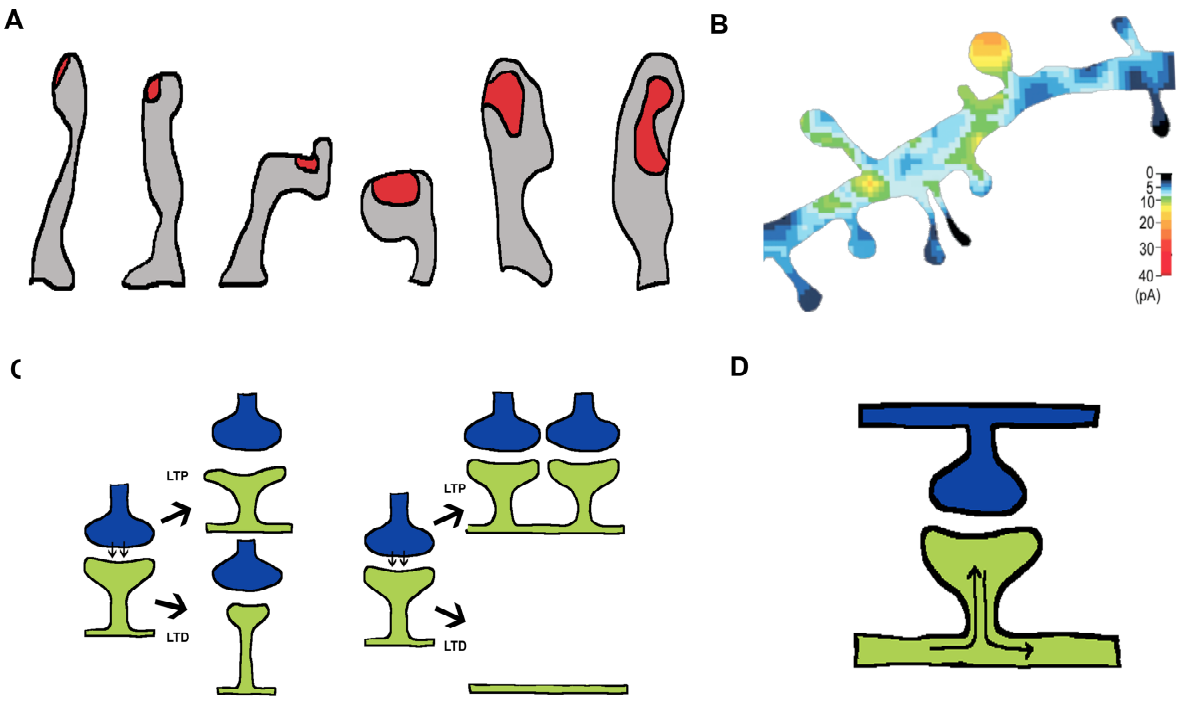

Figure 5: The physiological significance of dendritic spines **(A)** There is a strong correlation between dendritic spine shape and the size of the postsynaptic density (PSD), illustrated in red. The three spines on the left have an elongated, thin, filopodial-like shape and they have relatively small PSDs. However, the three spines on the right have a stubby, bulbous morphology and concomitantly larger PSDs (Arellano et al. 2007). **(B)** Stubby spines are also more responsive to uncaged glutamate. Thus, it appears that the large PSDs found in stubby spines correspond with the presence of large postsynaptic regions of glutamatergic receptors (Matsuzaki et al. 2004). **(C)** Dendritic spines have also been speculated to play an important role in structural plasticity. The shape of the dendritic spine can be modified to increase or decrease the density of postsynaptic receptors (as illustrated on the left) in response to either long-term potentiation (LTP) or long-term depression (LTD). The gross number of dendritic spines along the shaft can also change in response to synaptic plasticity (as illustrated on the right). **(D)** Dendritic spines have also been speculated to play a role in limiting the diffusion of calcium and signaling molecules, keeping them proximal to the synapse. Also, the length of the dendritic spine neck influences its resistivity. This affects the ability of a local postsynaptic potential, generated at the spine head, to translate into a depolarization of the dendritic shaft.

restrict depolarization is dependent on the resistivity of the spine neck, which is highly coupled to spine length and width.

While spine neck resistivity has been estimated to be as high as 150 MΩ, this value is not predicted to be high enough to significantly modulate synaptic currents (Svoboda et al. 1997). However, several lines of evidence suggest that spine head volume and spine neck length can both affect somatic depolarization. Using glutamate uncaging to map the glutamate sensitivity of individual dendritic spines, a direct correlation was found between spine head volume and the degree of depolarization that was measured at the soma (Matsuzaki et al. 2001). Therefore, larger, more mushroom-shaped spines solicited a greater depolarization at the soma than longer more filopodial-like spines. Although differing densities of AMPA receptors at differently shaped spines could account for this finding, subsequent studies have found a direct correlation between spine neck length and degree of depolarization at the soma (Araya et al. 2006). Therefore, while biophysical evidence suggests that spines cannot act as electrically isolated units, experimental evidence provides functional evidence that they do.

In summary, dendritic spines are highly dynamic structures that act to probe the neural environment and modulate synaptic connectivity. While Ramon y Cajal was the first to describe their structure and postulate their function in the nervous system,a renewed interest in their dynamics came when it was discovered that spines were rich in actin (Bonhoeffer and Yuste 2002). The presence of actin in dendritic spines was considered strong evidence for their motility, before live imaging experiments were able to demonstrate this definitively (Fischer et al. 1998). Now molecular mechanisms that influence the actin cytoskeleton, in order to shape spine dynamics and morphology, have been explored in detail.

Actin cytoskeleton and dendritic morphology

Both immunohistochemistry and live imaging of GFP-actin fusion proteins have shown that actin is highly enriched in dendritic spines (Fischer et al. 1998; Hotulainen et al. 2009). Using pharmacology to manipulate actin dynamics has demonstrated that the actin cytoskeleton plays an important role in mediating not only spine motility, but also spine formation. Application of Cytochalasin D, a potent inhibitor of actin polymerization, arrests motility in mature dendritic spines (Fischer et al. 1998; Dunaevsky et al. 1999), suggesting that spine dynamics are critically dependent on modulation of the actin cytoskeleton. Additionally, strong tetanic stimulation has been shown to induce actin remodeling, in pre- and post-synaptic terminals (Colicos et al. 2001). Interestingly, at earlier developmental time points, inhibiting actin polymerization completely prevents the formation of dendritic spines. This indicates that actin dynamics are also important for dendritic spine formation. However, as spines mature, their gross morphological structure becomes increasingly independent of actin dynamics (Zhang and Benson 2001). Therefore, while the actin cytoskeleton may not play a critical role in modulating the gross morphology of mature spines, it appears to be important in mediating structural adaptations to neural stimulation.

Rho-GTPases are known to be potent regulators of the actin cytoskeleton. They act as intracellular switches that go from an inactive GDP bound state to an active GTP bound state (Fig6). In their active state, they modulate downstream effectors, like Arp2/3, WASP, and cofilin, which directly regulate the actin polymerization (Hall 1998). The three best characterized Rho GTPases are RhoA, Rac1, and cdc42 (Luo 2002). In cultured fibroblasts, expression of RhoA induces the formation of stress fibers, expression of Rac causes the formation of lamellipodia, and expression of cdc42 induces the formation of filopodia (Hall 1998). However, the binary relationship between these Rho GTPases and the phenotype they produce does not hold in neurons, with Rac1 and cdc42 inducing the formation of both

filopodia and lamellapodia (Luo 2002). Therefore, a clear understanding of exactly how Rho GTPases affect the actin cytosketon of neurons remains elusive.

However, Rho GTPases clearly regulate spine formation and spine morphology. These effects are likely mediated by the influence these proteins have on the actin cytoskeleton. In transgenic mice that overexpress a constitutively active form of Rac1, there is an increase in spine number (Luo et al. 1996). Interestingly, biolistically inducing Rac1 expression in wild-type neurons that had already undergone synaptogenesis revealed a similar increase in spine number (Nakayama and Luo 2000). This suggests that Rac1 is not only important for synaptogenesis at early developmental time points, but is also important for regulating spine number at maturity. Interestingly, RhoA was found to have the opposite effect on dendritic spine density. Therefore, while Rac1 expression is sufficient to increase spine number, RhoA expression dramatically decreases spine number. Rac1 and RhoA also have opposite effects on the length of spine necks. Rac1 expression induces the formation of elongated necks, while RhoA expression causes a dramatic reduction in neck length (Tashiro et al. 2000). Therefore, Rac and RhoA appear to influence spine number and morphology in a reciprocal fashion.

Mutation of RhoGAP and RhoGEF proteins that regulate the activity of Rac1 and RhoA have both been shown to result in mental retardation syndromes (Chelly and Mandel 2001; Ramakers 2002). Therefore understanding how these upstream effectors are modulated will be important in understanding the types of intra- and extracellular cues that are able to solicit changes in spine morphology. Proteins containing RhoGEF and RhoGAP domains have been shown to interact with receptor mediated signaling pathways that influence spine morphology (Yamashita et al. 1999; Li et al. 2002). Therefore, RhoGAPs and RhoGEFs may be the intermediaries that allow neurons to respond to cues from the extracellular environment, like presynaptic stimulation. This may allow receptor activation to be translated into action. While much attention has been paid to the role of GAP or GEF

domains, in proteins that are known to modulate spine dynamics, other domains that directly interact with the cell membrane may be underappreciated in their ability to shape spine morphology.

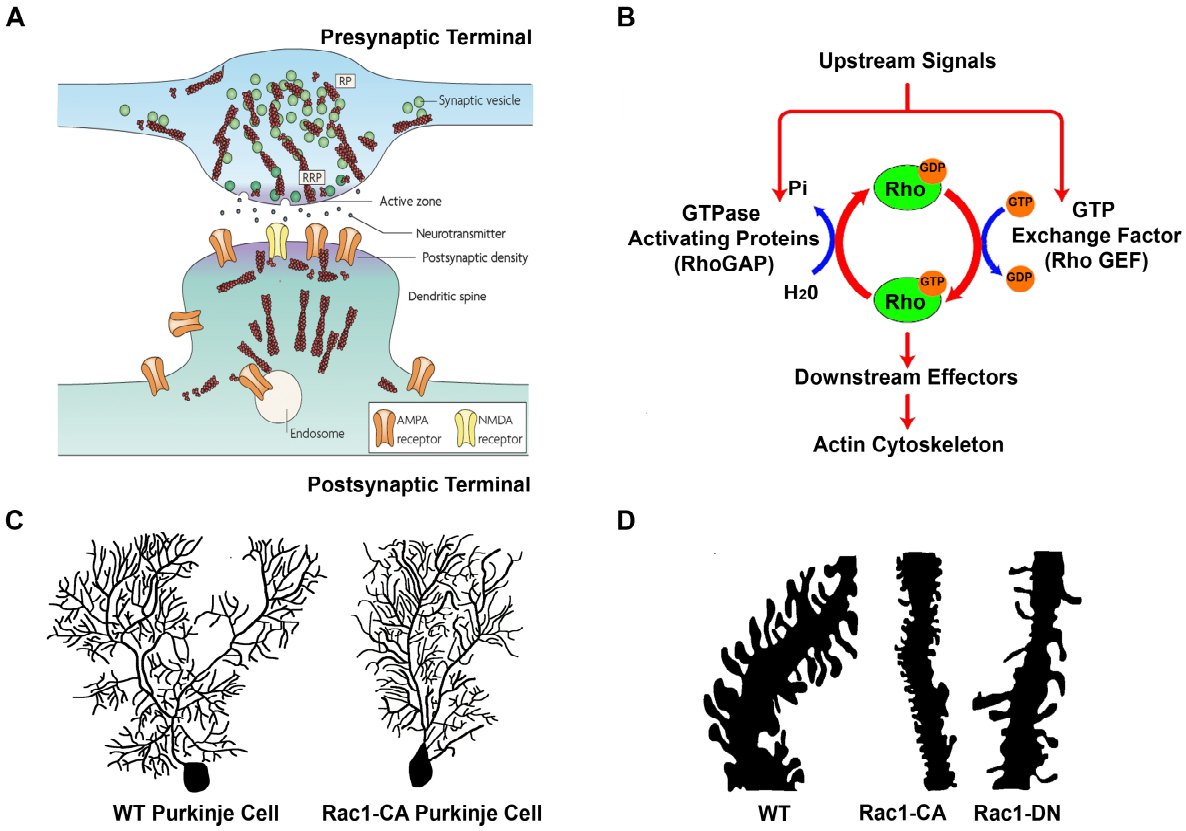

Figure 6: The actin cytoskeleton and small G-proteins play a critical role at the synapse. **(A)** Actin plays an important role both pre- and post-synaptically, regulating vesicle docking and recycling. This ultimately affects both presynaptic neurotransmitter release, and the composition of receptors at the postsynaptic membrane. **(B)** The Rho family of small G-proteins plays an important role in regulating the actin cytoskeleton. These proteins cycle between being in an active GTP bound state, which is catalyzed by a guanine exchange factor (GEF) or an inactive GDP bound state, facilitated by a GTPase activating protein (GAP). The active G-protein activates a cascade of downstream signaling molecules, which ultimately influence the actin cytoskeleton (Luo et al. 2002). **(C)** In Purkinje cells, expressing a constitutively active form of a Rho family G-protein, Rac1, causes a reduction in dendritic branching (Luo et al. 2002). **(D)** Expressing constitutively active Rac1 also causes a robust increase in dendritic spine density, accompanied by a shortening of spine length. However, expressing a dominant-negative form of Rac1 causes a reduction of dendritic spine density and an elongation of spine length (Luo et al. 2002). Thus, Rac1 plays an important role in shaping dendritic spine density and morphology.

`

BAR-domain containing proteins and membrane dynamics

Because small GTPase signaling shapes actin dynamics and because mutations in a number of proteins that regulate small GTPases result in mental retardation syndromes, tremendous focus has been placed on understanding how perturbations in these pathways affect neuronal function. Interestingly, proteins like oligophrenin1 and srGAP3, which are linked to mental retardation syndromes, have a RhoGAP domain and bin-amphiphysin-Rvs (BAR) domain (Itoh and De Camilli 2006). However, the functional importance of proteins with BAR domains is just beginning to be understood.

BAR domains were first characterized as highly homologous N-terminal sequences in amphiphysins, a large family of proteins known to be important regulators of endocytosis (Zhang and Zelhof 2002). These domains are subdivided into three subtypes: BAR, F-BAR, and I-BARs, which induce the formation of narrow, wide, and inverted membrane tubules, respectively. BAR domains are alpha helical dimers that interact with cellular membranes through electrostatic interactions between their positively charged ends and the negatively charged membrane (McMahon and Gallop 2005; Itoh and De Camilli 2006; Zimmerberg and Kozlov 2006). These domains oligomerize to cooperatively form rigid scaffolds that force membrane bending (Itoh and De Camilli 2006). Many BAR domains bind to actin in addition to the membrane and are therefore ideally positioned at the interface between membrane and actin cytoskeleton (Itoh and De Camilli 2006).

Because neurons have elaborate shapes that that are tightly coupled to their function, BAR-containing proteins that directly modulate membrane deformation are hypothesized to be important for achieving mature axodendritic morphology. In fact, BAR-containing proteins have been found to influence dendritic spine shape and density (Choi et al. 2005; Khelfaoui et al. 2007). They have also been implicated in the formation of dendritic arborization patterns (Sawallisch et al. 2009) and in the modulation of synaptic plasticity (Khelfaoui et al. 2009; Kim et al. 2009). While classically defined I-BARs can form

protrusions that can guide dendritic outgrowth, BARs and F-BARs can induce invaginations that are important for endocytosis of surface receptors (Khelfaoui et al. 2009). However, most of proteins that have been studied, which contain these domains, have focused on their ability to regulate small GTPase signaling. Therefore, a thorough understanding of how the BAR domains of these proteins directly regulate neuronal morphology, has yet to be elucidated.

IRSp53 is an I-BAR containing protein that is highly expressed in the postsynaptic density and is known to link activated Rac1 and cdc42 to downstream effectors for actin regulation (Choi et al. 2005). Because I-BAR domains mediate the formation of membrane protrusions, it would be reasonable to speculate that they might influence the dynamics of filopodial-like structures in neurons, such as dendritic spines. In accordance with this, overexpression of IRSp53 in cultured neurons results in increased spine density, whereas knockdown of IRSp53 results in decreased spine density (Choi et al. 2005). This ties in well with electrophysiological data showing that overexpression of IRSp53 causes an increase in mEPSC frequency (Hori et al. 2005). This increase in mEPSC frequency suggests that the new spines formed by overexpression of IRSp53, are physiologically functional. IRSp53 has also been shown to translocate to dendritic spines upon NMDAR activation (Hori et al. 2005). This translocation is dependent on phosphorylation of the N-terminal I-BAR domain of IRSp53, by Protein Kinase C (PKC). Therefore, it appears that the phosphorylation of the I-BAR domain in IRSp53 regulates its intracellular localization. Interestingly, NMDAR-mediated mEPSCs are reduced in the IRSp53 knockout. Exactly how this reduction of NMDAR-mediated mEPSCs is related to the fact that NMDAR activation influence IRSp53 localization is unclear. However, the link between IRSp53 and NMDARs may help explain two independent observations that genetic loss of IRSp53 results in enhanced LTP (Kim et al. 2009; Sawallisch et al. 2009).

`

Oligophrenin 1 is a BAR domain containing protein that is ubiquitously expressed throughout the brain, during embryonic and postnatal stages of development. Mutation of this gene results in a nonspecific X-linked mental retardation syndrome in humans. Because this protein contains a BAR domain, it is hypothesized to play an important role in vesicle endocytosis. However, it appears that oligophrenin1 may play distinct, seemingly contradictory, roles through the actions of its BAR and RhoGAP domains. Yet, a thorough functional dissection of the BAR and RhoGAP domains of this protein remains to be done.

Several lines of evidence suggest that oligophrenin1 plays an important role in vesicle recycling. Paired pulse facilitation in the hippocampus of oligophrenin1 knockout mice reveals an increase in the probability of neurotransmitter release (Khelfaoui et al. 2007). This suggests that oligophrenin1 mediates vesicle recycling at the presynaptic terminal. Additional experiments, measuring FM1-43 internalization, reveal that genetic loss of oligophrenin1 results in diminished endocytosis (Khelfaoui et al. 2009). Specifically, it appears that loss of oligophrenin1 results in an impairment of AMPA receptor internalization, a process critically regulated by endocytosis. Two functional measures that could be explained by this impairment in endocytosis are (1) diminished LTD (Khelfaoui et al. 2009), which is critically dependent on the postsynaptic internalization of AMPARs, and (2) poor performance on spatial learning tasks, like the Morris Water Maze (Khelfaoui et al. 2007). However, because oligophrenin1 is knocked out in all neurons it is difficult to separate its pre- and post-synaptic function. For example, could the effect of oligophrenin1 on presynaptic neurotransmitter release be secondary to the protein's postsynaptic role in AMPAR endocytosis? Another issue that confounds the interpretation of these results is the potentially overlapping function of the RhoGAP and BAR domains of oligophrenin1. The RhoGAP domain of oligophrenin1 downregulates RhoA/ROCK signaling, which inhibits vesicular endocytosis (Fauchereau et al. 2003; Govek et al. 2004; Khelfaoui et al. 2009). Therefore, in the absence of oligophrenin1 there would be an increase in RhoA/ROCK

signaling, which would attenuate endocytosis. However, oligophrenin1 may also affect endocytosis directly through the action of its BAR domain. Yet this possibility remains entirely unexplored.

Oligophrenin1 also influences spine formation, stabilization, and morphogenesis. Genetic deletion of oligophrenin1 causes a decrease in dendritic spine density along the apical dendrite of CA1 neurons (Khelfaoui et al. 2007). Furthermore, the morphology of dendritic spines becomes elongated in knockout animals but shorter and stubbier upon knockdown *in vitro* . This suggests that oligophrenin1 positively regulates the formation of dendritic spines and that it somehow influences spine morphology. Interestingly, the activity of the RhoGAP domain of oligophrenin1 is inconsistent with the observed decrease in spine density. This is because oligophrenin1 decreases RhoA activity, which is important for increasing spine density and promoting an elongated filopodial-like morphology (Tashiro et al. 2000). Therefore, in the absence of oligophrenin1, RhoA activity should be increased, resulting in a concomitant increase in spine density. However, it is possible that the effect of Oligophrenin1 on spine density is secondary to its better characterized influence on AMPA receptor internalization (Khelfaoui et al. 2009). While it is unclear how Oligophrenin1 modulates dendritic spine number and morphology, a better understanding of exactly how its BAR and RhoGAP domains influence membrane dynamics will help elucidate the complex role of this protein.

Pacsin1 (also called Syndapin1) is an F-BAR containing protein that is important in regulating the activity-dependent internalization of NR3A-containing NMDARs (Perez-Otano et al. 2006). This function of Pacsin1 is entirely consistent with the fact that it contains an F-BAR domain, which has been classically defined as a protein domain that solicits the formation of membrane invaginations and thereby regulates endocytosis. Interestingly, Pacsin1 internalizes a specific type of NMDAR, with spatiotemporal specificity. More recent work demonstrates that Pascin1 can also induce filopodia-like protrusions and regulate

dendritic branching in neurons by not only coordinating WASP-mediated actin polymerization through its SH3 domain, but also by deforming the membrane directly through its F-BAR domain (Dharmalingam et al. 2009).

srGAP3 is an F-BAR containing protein that also has a RhoGAP domain. srGAP3 has been shown to directly interact with WAVE1, which can influence cytoskeletal dynamics by activating Arp2/3 (Soderling et al. 2007). Interestingly, transgenic mice that have a WAVE1 mutation that abolishes its interaction with srGAP3, display enhanced LTP (similar to the IRSp53 knockouts)(Soderling et al. 2007). The molecular mechanism that drives this enhancement in LTP remains unknown. Additionally, the biological function of the F-BAR domain of srGAP3 remains to be explored.

Although speculative, BAR containing proteins may influence dendritic spine morphology by forming dense scaffolds that promote their stabilization. Similar to BAR containing proteins, septins can polymerize into oligomers that form microscopic ring-like structures close to the plasma membrane. Septins are also GTPases that have structural similarity to the Ras family (Kinoshita 2003). Therefore, they can also modulate downstream effectors of the actin cytoskeleton. Interestingly, Septin7 localizes to the base of dendritic spines and to the branch points in dendritic arbors (Tada et al. 2007; Xie et al. 2007). Knocking down Septin7 results in an elongation of dendritic spine morphology and a decrease in dendritic branching (Xie et al. 2007). Overexpressing septin7 causes an increase in spine density and increases dendritic branching (Tada et al. 2007). Thus, it appears that complex protein heteromers can localize in a spatially restricted manner to influence dendritic morphology. Therefore, it will be interesting to investigate whether similar complexes of BAR-containing proteins function in a similar manner.

srGAP2 is an F-BAR-containing protein that regulates neuronal morphology (Guerrier et al. 2009). This protein may act similar to septins by forming complex oligomers that can directly deform neuronal cell membranes. Similar to septin7, which can

heterodimerize with septin5 and septin11 (Xie et al. 2007), srGAP2 may heterodimerize with other srGAP family members to achieve its effect. However, unlike other proteins that contain both a BAR-like and a GTPase domain, the influence of each domain has been examined independently. Interestingly, while F-BAR domains are structurally predicted to form membrane invaginations, the F-BAR domain of srGAP2 induces the formation of filopodia-like membrane protrusions (Guerrier et al. 2009). Overexpression of srGAP2 or its F-BAR domain only causes increased branching in developing neurons. However, how srGAP2 affects the membrane at later developmental time points, subsequent to neuronal migration and synaptogenesis, is unexplored. Yet, because srGAP2 can potently induce the formation of filopodial-like protrusions, it is reasonable to speculate that the protein may regulate the formation and morphology of dendritic spines. Therefore Chapter Three of this thesis will describe our effort to determine how srGAP2 affects dendritic spine morphology, synaptic transmission, and synaptic plasticity.

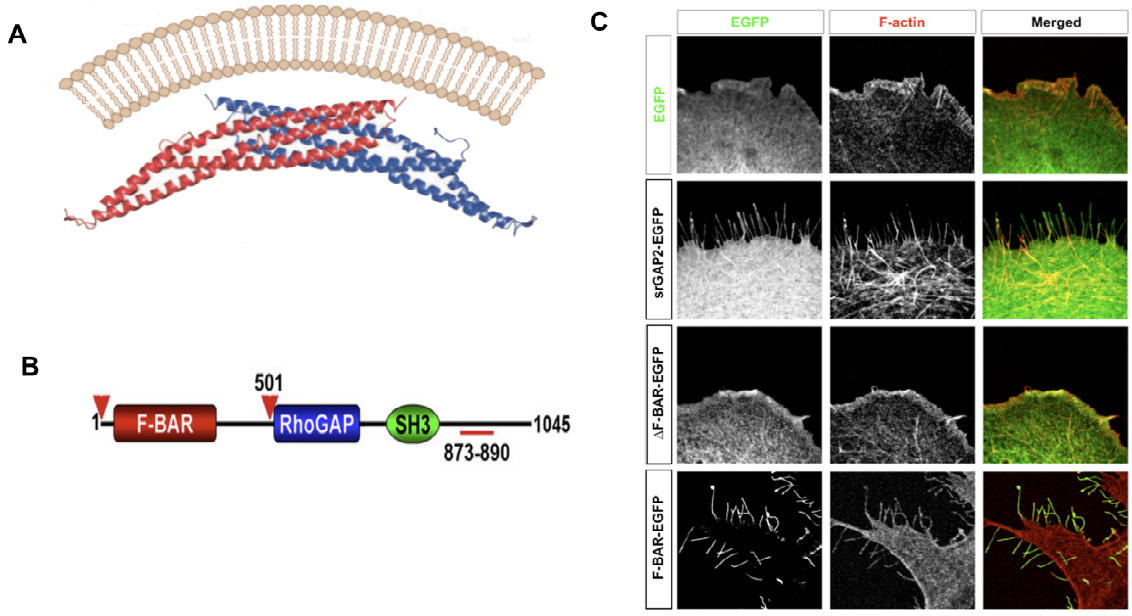

Figure 7: BAR containing proteins can sculpt cellular morphology through direct interactions with the lipid membrane. **(A)** BAR domains are alpha-helical dimers that directly bind the cell membrane through hydrostatic interactions. These hydrostatic interactions have the ability to directly deform the membrane, independently of the actin cytoskeleton (McMahon et al. 2005) . **(B)** Slit-Robo GTPase Activating Protein 2 (srGAP2) has an FBAR, RhoGAP, and SH3 domain (Guerrier et al. 2009). **(C)** srGAP2 induces filopodial formation in an FBAR-dependent manner. Expressing a srGAP2-GFP fusion protein in cos-7 cells induces the formation of filopodial-like protrusions. However, the formation of filopodia is FBAR dependent, because expressing a form of srGAP2 that lacks the FBAR domain (ΔFBAR) does not induce filopodial formation, while expressing the FBAR domain alone is sufficient to induce the formation of filopodia

BIBLIOGRAPHY

Al-Hallaq RA, Conrads TP, Veenstra TD, Wenthold RJ (2007) NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. J Neurosci 27(31): 8334-8343.

Altman J, Anderson WJ (1972) Experimental reorganization of the cerebellar cortex. I. Morphological effects of elimination of all microneurons with prolonged x-irradiation started at birth. J Comp Neurol 146(3): 355-406.

Antonini A, Fagiolini M, Stryker MP (1999) Anatomical correlates of functional plasticity in mouse visual cortex. J Neurosci 19(11): 4388-4406.

Araya R, Jiang J, Eisenthal KB, Yuste R (2006) The spine neck filters membrane potentials. Proc Natl Acad Sci U S A 103(47): 17961-17966.

Arellano JI, Espinosa A, Fairen A, Yuste R, DeFelipe J (2007) Non-synaptic dendritic spines in neocortex. Neuroscience 145(2): 464-469.

Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R et al. (2002) 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. Bioorg Med Chem Lett 12(7): 1099-1102.

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48(2): 289-301.

Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH et al. (2005) Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. J Neurosci 25(29): 6907-6910.

Blitzer RD (2005) Teaching resources. Long-term potentiation: mechanisms of induction and maintenance. Sci STKE 2005(309): tr26.

Bonhoeffer T, Yuste R (2002) Spine motility. Phenomenology, mechanisms, and function. Neuron 35(6): 1019-1027.

Bravin M, Morando L, Vercelli A, Rossi F, Strata P (1999) Control of spine formation by electrical activity in the adult rat cerebellum. Proc Natl Acad Sci U S A 96(4): 1704-1709.

Chelly J, Mandel JL (2001) Monogenic causes of X-linked mental retardation. Nat Rev Genet 2(9): 669-680.

Choi J, Ko J, Racz B, Burette A, Lee JR et al. (2005) Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. J Neurosci 25(4): 869-879.

Clements JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 19(5): 163-171.

Colicos MA, Collins BE, Sailor MJ, Goda Y (2001) Remodeling of synaptic actin induced by photoconductive stimulation. Cell 107(5): 605-616.

Dailey ME, Smith SJ (1996) The dynamics of dendritic structure in developing hippocampal slices. J Neurosci 16(9): 2983-2994.

Dalby NO, Mody I (2003) Activation of NMDA receptors in rat dentate gyrus granule cells by spontaneous and evoked transmitter release. J Neurophysiol 90(2): 786-797.

de Marchena J, Roberts AC, Middlebrooks PG, Valakh V, Yashiro K et al. (2008) NMDA receptor antagonists reveal age-dependent differences in the properties of visual cortical plasticity. J Neurophysiol 100(4): 1936-1948.

DeFelipe J, Farinas I (1992) The pyramidal neuron of the cerebral cortex: morphological and chemical characteristics of the synaptic inputs. Prog Neurobiol 39(6): 563-607.

Denk W, Yuste R, Svoboda K, Tank DW (1996) Imaging calcium dynamics in dendritic spines. Curr Opin Neurobiol 6(3): 372-378.

Dharmalingam E, Haeckel A, Pinyol R, Schwintzer L, Koch D et al. (2009) F-BAR proteins of the syndapin family shape the plasma membrane and are crucial for neuromorphogenesis. J Neurosci 29(42): 13315-13327.

Dunaevsky A, Tashiro A, Majewska A, Mason C, Yuste R (1999) Developmental regulation of spine motility in the mammalian central nervous system. Proc Natl Acad Sci U S A 96(23): 13438-13443.

Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399(6731): 66-70.

Erreger K, Dravid SM, Banke TG, Wyllie DJ, Traynelis SF (2005) Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. J Physiol 563(Pt 2): 345-358.

Fauchereau F, Herbrand U, Chafey P, Eberth A, Koulakoff A et al. (2003) The RhoGAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain. Mol Cell Neurosci 23(4): 574-586.

Feng B, Tse HW, Skifter DA, Morley R, Jane DE et al. (2004) Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl) piperazine-2,3-dicarboxylic acid. Br J Pharmacol 141(3): 508-516.

Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. J Neurosci 18(21): 8900-8911.

Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. Neuron 20(5): 847-854.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H (1997) NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J Neurosci 17(7): 2469-2476.

Gallagher MJ, Huang H, Pritchett DB, Lynch DR (1996) Interactions between ifenprodil and the NR2B subunit of the N-methyl-D-aspartate receptor. J Biol Chem 271(16): 9603-9611.

Gordon JA, Stryker MP (1996) Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. J Neurosci 16(10): 3274-3286.

Govek EE, Newey SE, Akerman CJ, Cross JR, Van der Veken L et al. (2004) The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. Nat Neurosci 7(4): 364-372.

Gray EG (1959) Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. Nature 183(4675): 1592-1593.

Grutzendler J, Kasthuri N, Gan WB (2002) Long-term dendritic spine stability in the adult cortex. Nature 420(6917): 812-816.

Guerrier S, Coutinho-Budd J, Sassa T, Gresset A, Jordan NV et al. (2009) The F-BAR domain of srGAP2 induces membrane protrusions required for neuronal migration and morphogenesis. Cell 138(5): 990-1004.

Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279(5350): 509-514.

Harris KM, Jensen FE, Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. J Neurosci 12(7): 2685-2705.

Hensch TK, Fagiolini M, Mataga N, Stryker MP, Baekkeskov S et al. (1998) Local GABA circuit control of experience-dependent plasticity in developing visual cortex. Science 282(5393): 1504-1508.

Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2009) Experience leaves a lasting structural trace in cortical circuits. Nature 457(7227): 313-317.

Hoffmann H, Gremme T, Hatt H, Gottmann K (2000) Synaptic activity-dependent developmental regulation of NMDA receptor subunit expression in cultured neocortical neurons. J Neurochem 75(4): 1590-1599.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17: 31-108.

Hori K, Yasuda H, Konno D, Maruoka H, Tsumoto T et al. (2005) NMDA receptor-dependent synaptic translocation of insulin receptor substrate p53 via protein kinase C signaling. J Neurosci 25(10): 2670-2681.

Hotulainen P, Llano O, Smirnov S, Tanhuanpaa K, Faix J et al. (2009) Defining mechanisms of actin polymerization and depolymerization during dendritic spine morphogenesis. J Cell Biol 185(2): 323-339.

Itoh T, De Camilli P (2006) BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta 1761(8): 897-912.

Jiang B, Trevino M, Kirkwood A (2007) Sequential development of long-term potentiation and depression in different layers of the mouse visual cortex. J Neurosci 27(36): 9648-9652.

Kawakami R, Shinohara Y, Kato Y, Sugiyama H, Shigemoto R et al. (2003) Asymmetrical allocation of NMDA receptor epsilon2 subunits in hippocampal circuitry. Science 300(5621): 990-994.

Kew JN, Trube G, Kemp JA (1996) A novel mechanism of activity-dependent NMDA receptor antagonism describes the effect of ifenprodil in rat cultured cortical neurones. J Physiol 497 (Pt 3): 761-772.

Khelfaoui M, Pavlowsky A, Powell AD, Valnegri P, Cheong KW et al. (2009) Inhibition of RhoA pathway rescues the endocytosis defects in Oligophrenin1 mouse model of mental retardation. Hum Mol Genet 18(14): 2575-2583.

Khelfaoui M, Denis C, van Galen E, de Bock F, Schmitt A et al. (2007) Loss of X-linked mental retardation gene oligophrenin1 in mice impairs spatial memory and leads to ventricular enlargement and dendritic spine immaturity. J Neurosci 27(35): 9439-9450.

Kim MH, Choi J, Yang J, Chung W, Kim JH et al. (2009) Enhanced NMDA receptor-mediated synaptic transmission, enhanced long-term potentiation, and impaired learning and memory in mice lacking IRSp53. J Neurosci 29(5): 1586-1595.

Kinoshita M (2003) Assembly of mammalian septins. J Biochem 134(4): 491-496.

Kirkwood A, Silva A, Bear MF (1997) Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice. Proc Natl Acad Sci U S A 94(7): 3380-3383.

Kohr G (2006) NMDA receptor function: subunit composition versus spatial distribution. Cell Tissue Res 326(2): 439-446.

Kumar SS, Huguenard JR (2003) Pathway-specific differences in subunit composition of synaptic NMDA receptors on pyramidal neurons in neocortex. J Neurosci 23(31): 10074-10083.

Laube B, Kuhse J, Betz H (1998) Evidence for a tetrameric structure of recombinant NMDA receptors. J Neurosci 18(8): 2954-2961.

Lendvai B, Stern EA, Chen B, Svoboda K (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. Nature 404(6780): 876-881.

Li Z, Aizenman CD, Cline HT (2002) Regulation of rho GTPases by crosstalk and neuronal activity in vivo. Neuron 33(5): 741-750.

Linke R, Soriano E, Frotscher M (1994) Transient dendritic appendages on differentiating septohippocampal neurons are not the sites of synaptogenesis. Brain Res Dev Brain Res 83(1): 67-78.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3(3): 175-190.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y et al. (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304(5673): 1021-1024.

Luo L (2002) Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annu Rev Cell Dev Biol 18: 601-635.

Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY et al. (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 379(6568): 837-840.

Maffei A, Nataraj K, Nelson SB, Turrigiano GG (2006) Potentiation of cortical inhibition by visual deprivation. Nature 443(7107): 81-84.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44(1): 5-21.

Mariani J, Crepel F, Mikoshiba K, Changeux JP, Sotelo C (1977) Anatomical, physiological and biochemical studies of the cerebellum from Reeler mutant mouse. Philos Trans R Soc Lond B Biol Sci 281(978): 1-28.

Mason CA (1983) Postnatal maturation of neurons in the cat's lateral geniculate nucleus. J Comp Neurol 217(4): 458-469.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW et al. (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. J Neurosci 24(36): 7821-7828.

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H (2004) Structural basis of long-term potentiation in single dendritic spines. Nature 429(6993): 761-766.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M et al. (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 4(11): 1086-1092.

Matus A (2000) Actin-based plasticity in dendritic spines. Science 290(5492): 754-758.

McBain CJ, Mayer ML (1994) N-methyl-D-aspartic acid receptor structure and function. Physiol Rev 74(3): 723-760.

McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 438(7068): 590-596.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12(3): 529-540.

Mosley CA, Myers SJ, Murray EE, Santangelo R, Tahirovic YA et al. (2009) Synthesis, structural activity-relationships, and biological evaluation of novel amide-based allosteric binding site antagonists in NR1A/NR2B N-methyl-D-aspartate receptors. Bioorg Med Chem 17(17): 6463-6480.

Nakayama AY, Luo L (2000) Intracellular signaling pathways that regulate dendritic spine morphogenesis. Hippocampus 10(5): 582-586.

Neyton J, Paoletti P (2006) Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. J Neurosci 26(5): 1331-1333.

Nimchinsky EA, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. Annu Rev Physiol 64: 313-353.

Nimchinsky EA, Yasuda R, Oertner TG, Svoboda K (2004) The number of glutamate receptors opened by synaptic stimulation in single hippocampal spines. J Neurosci 24(8): 2054-2064.

Parnavelas JG, Sullivan K, Lieberman AR, Webster KE (1977) Neurons and their synaptic organization in the visual cortex of the rat. Electron microscopy of Golgi preparations. Cell Tissue Res 183(4): 499-517.

Perez-Otano I, Lujan R, Tavalin SJ, Plomann M, Modregger J et al. (2006) Endocytosis and synaptic removal of NR3A-containing NMDA receptors by PACSIN1/syndapin1. Nat Neurosci 9(5): 611-621.

Perin-Dureau F, Rachline J, Neyton J, Paoletti P (2002) Mapping the binding site of the neuroprotectant ifenprodil on NMDA receptors. J Neurosci 22(14): 5955-5965.

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127(4): 321-355.

Philpot BD, Cho KK, Bear MF (2007) Obligatory role of NR2A for metaplasticity in visual cortex. Neuron 53(4): 495-502.

Portera-Cailliau C, Pan DT, Yuste R (2003) Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. J Neurosci 23(18): 7129-7142.

Quinlan EM, Philpot BD, Huganir RL, Bear MF (1999) Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat Neurosci 2(4): 352-357.

Ramakers GJ (2002) Rho proteins, mental retardation and the cellular basis of cognition. Trends Neurosci 25(4): 191-199.

Sawallisch C, Berhorster K, Disanza A, Mantoani S, Kintscher M et al. (2009) The insulin receptor substrate of 53 kDa (IRSp53) limits hippocampal synaptic plasticity. J Biol Chem 284(14): 9225-9236.

Scannevin RH, Huganir RL (2000) Postsynaptic organization and regulation of excitatory synapses. Nat Rev Neurosci 1(2): 133-141.

Sobczyk A, Scheuss V, Svoboda K (2005) NMDA receptor subunit-dependent [Ca2+] signaling in individual hippocampal dendritic spines. J Neurosci 25(26): 6037-6046.

Soderling SH, Guire ES, Kaech S, White J, Zhang F et al. (2007) A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. J Neurosci 27(2): 355-365.

Sprengel R, Seeburg PH (1993) The unique properties of glutamate receptor channels. FEBS Lett 325(1-2): 90-94.

Spruston N (2008) Pyramidal neurons: dendritic structure and synaptic integration. Nat Rev Neurosci 9(3): 206-221.

Stocca G, Vicini S (1998) Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. J Physiol 507 (Pt 1): 13-24.

Strack S, Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor. J Biol Chem 273(33): 20689-20692.

Svoboda K, Denk W, Kleinfeld D, Tank DW (1997) In vivo dendritic calcium dynamics in neocortical pyramidal neurons. Nature 385(6612): 161-165.

Tada T, Simonetta A, Batterton M, Kinoshita M, Edbauer D et al. (2007) Role of Septin cytoskeleton in spine morphogenesis and dendrite development in neurons. Curr Biol 17(20): 1752-1758.

Tashiro A, Minden A, Yuste R (2000) Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and Rho. Cereb Cortex 10(10): 927-938.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. J Neurosci 19(10): 4180-4188.

Townsend M, Yoshii A, Mishina M, Constantine-Paton M (2003) Developmental loss of miniature N-methyl-D-aspartate receptor currents in NR2A knockout mice. Proc Natl Acad Sci U S A 100(3): 1340-1345.

Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR et al. (2002) Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420(6917): 788-794.

Tsay D, Yuste R (2004) On the electrical function of dendritic spines. Trends Neurosci 27(2): 77-83.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH et al. (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. J Neurophysiol 79(2): 555-566.

Watanabe M, Mishina M, Inoue Y (1994) Distinct spatiotemporal expressions of five NMDA receptor channel subunit mRNAs in the cerebellum. J Comp Neurol 343(4): 513-519.

Weitlauf C, Honse Y, Auberson YP, Mishina M, Lovinger DM et al. (2005) Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. J Neurosci 25(37): 8386-8390.

Wiesel TN, Hubel DH (1965) Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. J Neurophysiol 28(6): 1029-1040.

Wong RO, Yamawaki RM, Shatz CJ (1992) Synaptic Contacts and the Transient Dendritic Spines of Developing Retinal Ganglion Cells. Eur J Neurosci 4(12): 1387-1397.

Xie Y, Vessey JP, Konecna A, Dahm R, Macchi P et al. (2007) The GTP-binding protein Septin 7 is critical for dendrite branching and dendritic-spine morphology. Curr Biol 17(20): 1746-1751.

Yamashita T, Tucker KL, Barde YA (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron 24(3): 585-593.

Yashiro K, Philpot BD (2008) Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. Neuropharmacology 55(7): 1081-1094.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 24: 1071-1089.

Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: insights from ultrastructural and imaging studies. Nat Rev Neurosci 5(1): 24-34.

Yuste R, Majewska A, Holthoff K (2000) From form to function: calcium compartmentalization in dendritic spines. Nat Neurosci 3(7): 653-659.

Zhang B, Zelhof AC (2002) Amphiphysins: raising the BAR for synaptic vesicle recycling and membrane dynamics. Bin-Amphiphysin-Rvsp. Traffic 3(7): 452-460.

Zhang W, Benson DL (2001) Stages of synapse development defined by dependence on F-actin. J Neurosci 21(14): 5169-5181.

Zimmerberg J, Kozlov MM (2006) How proteins produce cellular membrane curvature. Nat Rev Mol Cell Biol 7(1): 9-19.

Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17(1): 91-102.

CHAPTER TWO

NMDA receptor antagonists reveal age-dependent differences in the properties of visual cortical plasticity

Jacqueline de Marchena[1], Adam C. Roberts[2, 3], Paul G. Middlebrooks[3], Vera Valakh[4], Koji Yashiro[3], Lindsey R. Wilfley[3], and Benjamin D. Philpot[1,2,3]

[1] Curriculum in Neurobiology, [2] Neuroscience Center, [3] Department of Cell and Molecular Physiology, [4] Summer Undergraduate Research Experience (S.U.R.E) Program, University of North Carolina, Chapel Hill, 105 Mason Farm Road, Chapel Hill, NC 27599

Running head: Age-dependent effects of NMDAR antagonists on plasticity

Corresponding author: Benjamin D. Philpot
5109E Neuroscience Research Building, Campus Box #7545
115 Mason Farm Road
Chapel Hill, NC 27599-7545
E-mail: bphilpot@med.unc.edu

`

SUMMARY

The suggestion that NMDA receptor (NMDAR)-dependent plasticity is subunit specific, with NR2B-types required for long-term depression (LTD) and NR2A-types critical for the induction of long-term potentiation (LTP), has generated much attention and considerable debate. By investigating the suggested subunit-specific roles of NMDARs in the mouse primary visual cortex over development, we report several important findings that clarify the roles of NMDAR subtypes in synaptic plasticity. We observed that LTD was not attenuated by application of ifenprodil, an NR2B-type antagonist, or NVP-AAM007, a less selective NR2A-type antagonist. However, we were surprised that NVP-AAM007 completely blocked adult LTP (postnatal day (P) 45-90), while only modestly affecting juvenile LTP (P21-28). To assess whether this developmental transition reflected an increasing role for NR2A-type receptors with maturity, we characterized the specificity of NVP-AAM007. We found not only that NVP-AAM007 lacks discernable subunit specificity, but also that the effects of NVP-AAM077 on LTP could be mimicked using subsaturating concentrations of APV, a global NMDAR antagonist. These results indicate that the effects of NVP-AAM077 on synaptic plasticity are largely explained by non-specific blockade of NMDARs. Moreover, our findings are the first to reveal a developmental increase in the sensitivity of LTP to NMDAR antagonism. We suggest that discrepant reports describing the effect of NVP-AAM077 on LTP may be partially explained by this developmental shift in the properties of LTP. These results indicate that the degree of NMDAR activation required for LTP increases with development, providing insight into a novel underlying mechanism governing the properties of synaptic plasticity.

INTRODUCTION

N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity is critical for learning and memory (Morris 1989; Moser et al. 1998), as well as experience-dependent modifications that have been particularly well defined in the visual cortex, such as ocular dominance plasticity and orientation selectivity (Bear et al. 1990; Ramoa et al. 2001; Roberts et al. 1998). Because of the heterogeneity of NMDAR subtypes (Cull-Candy et al. 2001; Dingledine et al. 1999), it has been suggested that different subpopulations of NMDARs may mediate unique aspects of synaptic plasticity. The primary visual cortex is an ideal model system to delineate the roles of NMDAR subunits, because it undergoes a natural developmental transition in NMDAR expression (Quinlan et al. 1999a). These changes in NMDAR composition occur across a period of developmental (Jiang et al. 2007; Kirkwood et al. 1997) and experience-dependent (Kirkwood et al. 1996; Philpot et al. 2007; Philpot et al. 2003) modifications in the properties of synaptic plasticity.

NMDAR composition is modulated over development in an experience-dependent manner (Quinlan et al. 1999b). While all NMDARs contain two obligatory NR1 subunits, these must dimerize with a combination of two NR2A-D or NR3A-B subunits to form a functional receptor (Laube et al. 1998; Mayer and Westbrook 1987; McBain and Mayer 1994; Perez-Otano and Ehlers 2004). These secondary subunits confer distinct functional properties onto NMDARs by influencing current kinetics, glutamate affinity, and the milieu of intracellular signaling proteins proximal to the synapse (Barria and Malinow 2005; Chatterton et al. 2002; Flint et al. 1997; Vicini et al. 1998). The predominant NMDAR subtypes in the postnatal neocortex are NR2A and NR2B (Flint et al. 1997; Monyer et al. 1994; Watanabe et al. 1994). Over development, neocortical NMDARs transition from being primarily NR2B-containing (NR2B-type) to primarily NR2A-containing (NR2A-type) in an

experience-dependent manner (Quinlan et al. 1999b). Because NR2B-type NMDARs have slower current kinetics than NR2A-type receptors, NMDAR currents become progressively faster with age (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992; Vicini et al. 1998). Changes in the composition and function of NMDARs have been tied to changes in the properties of synaptic plasticity (Carmignoto and Vicini 1992; Nase et al. 1999; Philpot et al. 2007), adding to the speculation that different NMDAR subunits contribute to distinct aspects of synaptic plasticity.

The suggestion that LTP and LTD induction were mediated, respectively, by NR2A and NR2B subtypes generated great excitement (Liu et al. 2004; Massey et al. 2004). However, while subsequent studies, using a variety of different stimulation protocols, have uncovered a similar subunit-specific trend (Fox et al. 2006; Gerkin et al. 2007), other studies have failed to do so (Bartlett et al. 2007; Berberich et al. 2005; Morishita et al. 2007; Toyoda et al. 2006; Weitlauf et al. 2005). Although the possibility of NR2-specific plasticity is appealing, we suggest that it must be considered in the context of several important factors. First, it is important to assess the specificity of subunit-specific antagonists used in these plasticity studies, as the selectivity of these drugs may change between brain regions due to differences in synaptic cleft glutamate (Frizelle et al. 2006), the composition of NMDARs, or even the proportion of triheteromeric (NR1-NR2A-NR2B) NMDARs at the synapse (Kew et al. 1998; Neyton and Paoletti 2006). Because most regions of the brain, including the visual cortex, display a profound developmental shift in NMDAR subunit composition (Chen et al. 2000; Hestrin 1992; Quinlan et al. 1999a; Ramoa and McCormick 1994), characterizing the efficacy of an antagonist over development (within one region) can be a helpful way of discerning its specificity. Second, the effect of a subunit-specific antagonist should be compared to the effect of a global NMDAR antagonist that similarly attenuates NMDAR currents. Such an approach will help delineate whether deficiencies in plasticity can be

attributed to a particular NMDAR subunit or to an overall reduction in NMDAR-mediated currents.

In an effort to elucidate whether visual cortical plasticity depends on NMDAR subunit-specific functions, our data reveal that the induction of LTD and LTP is not directed by distinct NMDAR subtypes. Instead, our findings unexpectedly provide compelling evidence to suggest a developmental increase in the sensitivity of LTP to disruption by NMDAR antagonism. This developmental change in plasticity could also reconcile the wide range of results that have been reported using NVP-AAM007, a purported NR2A-selective antagonist.

`

RESULTS

Developmental changes in NMDAR subunit expression and function in mouse visual cortex

To understand the roles of different NMDAR subunits in plasticity, we focused our studies on the primary visual cortex, a region that exhibits a developmental upregulation of NR2A-type relative to NR2B-type receptors. While this subunit transition in NMDARs has been well-described in several species (Chen et al. 2000; Quinlan et al. 1999a; Roberts and Ramoa 1999; Sheng et al. 1994), we wanted to characterize the specific nature of this trend in murine visual cortex. By enriching for proteins associated with the postsynaptic density (PSD), over a range of developmental time points, we were able to temporally reveal the graded expression of postsynaptic NMDAR subunits. Because this preparation enriches for postsynaptic proteins, it is well-suited to discern the composition of receptors mediating synaptically-evoked currents. Pools of visual cortex samples from P8, P16, P26, and P62 animals were run in triplicate, averaged, and compared. Consistent with observations in other species, we found a dramatic developmental increase in NR2A between P8 and P62, with only a modest upregulation of NR2B (Fig. 1A-1B) (NR2A subunit levels relative to P8: P16, 3.66 ± 1.13; P26, 7.97 ± 0.92; P62, 9.79 ± 0.81; $p < 0.001$) (NR2B subunit levels relative to P8: P16, 1.43 ± 0.33; P26, 2.18 ± 0.15; P62, 2.33 ± 0.07; $p < 0.01$).

To further characterize the nature of this developmental subunit transition, we pharmacologically isolated whole-cell NMDAR currents in layer (L) 2/3 pyramidal cells over the same age range. Since NR2A-type receptors have faster decay kinetics than NR2B-type receptors (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992; Monyer et al. 1992; Stocca and Vicini 1998; Vicini et al. 1998), this allowed us to corroborate our

biochemical data with a physiological measure. As expected, an ANOVA revealed a significant decrease in τ_w over development (Fig. 1C), demonstrating that NMDAR currents become progressively faster with age (Average: P12-18, 196.9 ± 11.8, *n* = 15; P21-28, 131.3 ± 16.0, *n* = 12; P45-90, 96.2 ± 11.0, *n* = 16; *p* < 0. 0001).

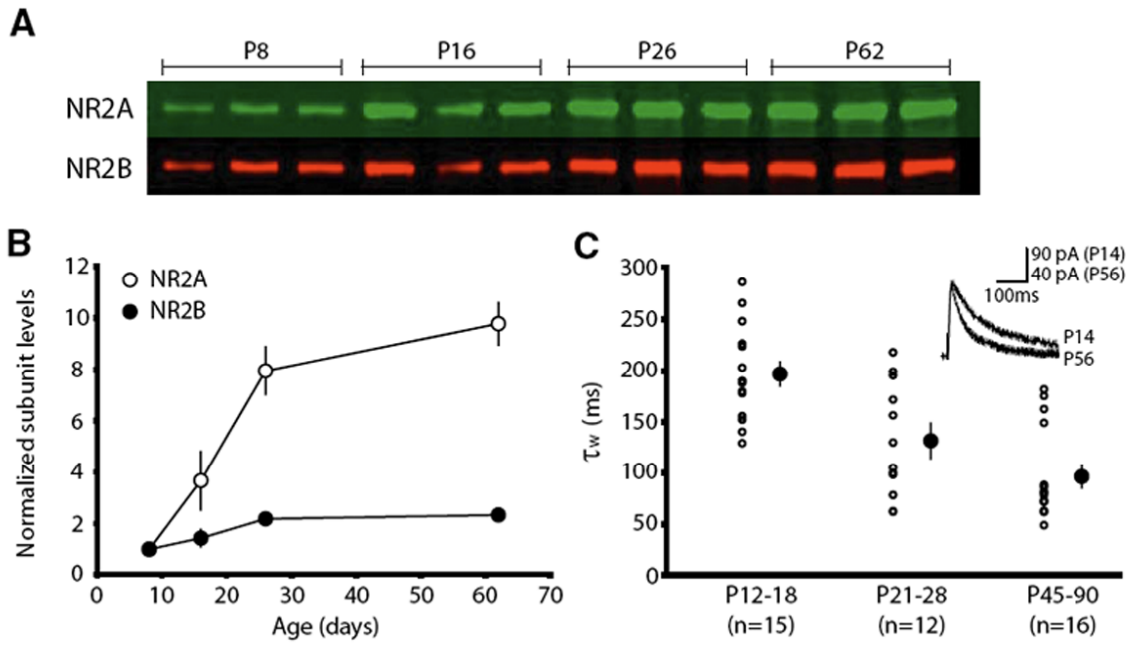

Figure 1: The functional expression of synaptic NR2A/NR2B increases over development in the mouse visual cortex. **(A)** Postsynaptic density fractions, enriched for synaptic proteins, show the relative expression of NR2A and NR2B at four developmental time points. Samples were obtained by pooling together visual cortices from 3-10 brains (see Methods). **(B)** NMDAR subunit expression normalized to P8 values. NR2A expression increases dramatically over development, while there is only a modest developmental increase in NR2B expression. Note that some error bars are obscured by the overlying symbol. **(C)** Shortening of NMDAR current durations over development reflects an increase in the relative level of NR2A expression. Using whole-cell voltage clamp recordings, NMDAR currents were isolated at three age groups from L2/3 pyramidal cells following extracellular L4 stimulation. Cells were held at +40mV in the presence of DNQX (see Methods). Open circles represent averages of individual cells, while filled circles reflect the age group's average ± SEM. A double exponential fit of the deactivation current revealed a decrease in τ_w, corresponding to faster current kinetics over development. Stimulation artifacts were blanked in this and all subsequent figures for clarity.

By measuring NR2 expression and NMDAR current kinetics at different ages, we show: (1) the expression of NR2A relative to NR2B increases with development; (2) the

`

most rapid upregulation of synaptic NR2A occurs prior to P30; (3) there is a functional correlate to changes in subunit expression at the synapse. With this information, we were able to evaluate synaptic plasticity at discrete developmental stages, using the natural process of NR2A upregulation as a mechanism to address the potentially unique roles of NR2A and NR2B-type NMDARs in synaptic plasticity.

LFS-LTD is developmentally sensitive to NMDAR antagonism

We began by evaluating whether low frequency stimulation (LFS)-LTD in the primary visual cortex was NR2B dependent, as had been reported previously in other regions of the brain (Liu et al. 2004; Massey et al. 2004). To isolate the roles of different NMDAR subunits, we conducted these experiments in the presence of ifenprodil, an NR2B-type antagonist (Williams et al. 1993), or NVP-AAM007, a low-affinity NR2A-type antagonist (Auberson et al. 2002; Feng et al. 2004). In young animals (P12-18), we found that neither ifenprodil nor NVP-AAM007 had an appreciable effect on the magnitude of LFS-LTD (Fig. 2A) (Average: Control, 79.0% ± 3.5% of baseline response, n = 13; 50 nM NVP-AAM077, 81.8% ± 2.0% n =10; 3 μM ifenprodil, 83.8% ± 4.2%, n =13; p = 0.6334). However, in juvenile (P21-28) animals, both antagonists modestly attenuated the level of LFS-LTD (Fig. 2B) (Average: Control, 79.1% ± 2.7%, n = 15; 50 nM NVP-AAM077, 88.7% ± 2.1% n =16; 3 μM ifenprodil, 85.3% ± 1.8%, n =17; p < 0.05). Taken together, it appears that both subpopulations of pharmacologically-sensitive NMDARs can contribute to LFS-LTD expression, but neither is absolutely required for its induction. This is similar to previous observations using subsaturating concentrations of APV, a pan-NMDAR antagonist, to

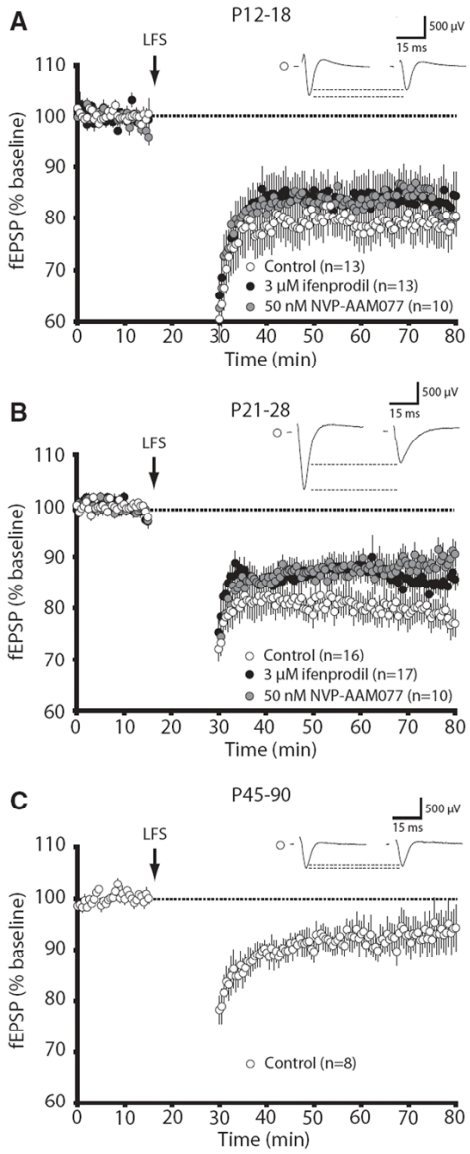

Figure 2: NVP-AAM077 and ifenprodil have developmentally specific effects on the expression of LFS-LTD in the visual cortex. LTD was induced with 1 Hz stimulation (15 min) of L4 (downward arrow). Data represent averages ± SEM. Representative waveforms show field potentials before and after 1 Hz stimulation under control conditions. **(A)** In young animals (P12-18), LTD is insensitive to either 3 µM ifenprodil or 50 nM NVP-AAM077. **(B)** In juvenile animals (P21-28), LTD is modestly attenuated by either 3 µM ifenprodil or 50 nM NVP-AAM077. **(C)** In adult animals (P45-90), only a small level of LTD could be induced by 1 Hz stimulation under control conditions.

attenuate the magnitude of LTD induced by 1 Hz stimulation (Philpot et al. 2003). Consistent with previous observations that 1 Hz LFS produces less robust LTD with age (Jiang et al. 2003; Jiang et al. 2007; Kirkwood et al. 1997), we observed that the magnitude of 1 Hz LFS-LTD decreases substantially with age (Fig. 2C). Therefore, in adult (P45-90) animals, the small degree of plasticity evoked with 1 Hz stimulation (Average: Control, 95.4% ± 4.5) precluded us from investigating the effect of antagonist application on this form of LTD. However, we do not suggest that LTD is absent in adult mice, as previous studies

`

have used different stimulation protocols, such as a paired-pulse LFS stimulation, to induce LTD in the adult cortex (Jiang et al. 2003; Lee et al. 2003).

NR2B-type NMDARs not required for expression of chemically-induced LTD

Because extrasynaptic NR2B-type NMDARs have been suggested to play a critical role in the induction of LTD (Massey et al. 2004), we chose to globally stimulate both synaptic and extrasynaptic NMDARs by bath application of NMDA. This protocol has been shown to induce a chemical form of LTD (chemLTD) that is mediated by rapid dephosphorylation of AMPA receptors and is occluded by homosynaptic LTD, suggesting that chemLTD and LFS-LTD share common mechanisms (Lee et al. 1998). We found chemLTD to decrease in magnitude with age, mirroring the developmental profile observed with LFS-LTD (Fig. 3A) (Average: P12-28, 44.7% ± 2.5%, n = 5; P45-90, 86.4% ± 3.9% n =5; p < 0.0001). We also confirmed that this form of LTD is NMDAR-dependent by completely blocking it with 100 μM D,L-APV (Fig. 3B) (APV, 105%, n = 1). Yet, chemLTD is not attenuated by antagonizing ifenprodil-sensitive, presumably NR2B-type, NMDARs (Fig. 3B) (Average: Control, 44.7% ± 2.5%, n = 5; 3μM ifenprodil, 44.6% ± 4.5%, n =5). Thus, both our synaptic and extrasynaptic stimulation paradigms fail to suggest a critical role for NR2B-type NMDARs in the induction of LTD in the mouse visual cortex.

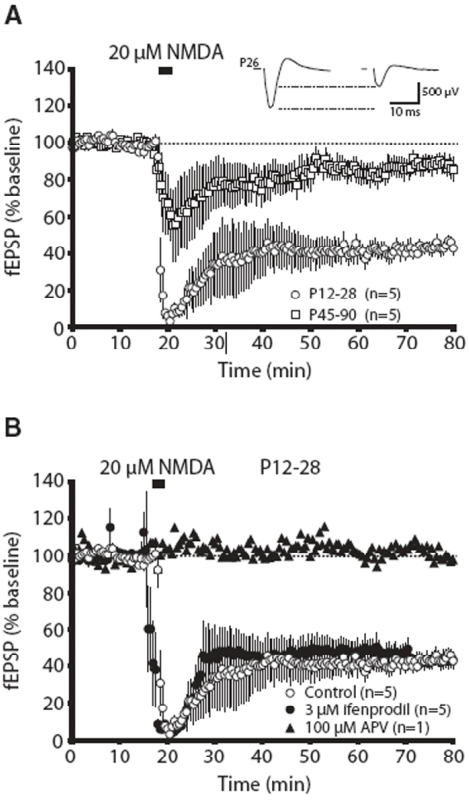

Figure 3: Chemically-induced LTD (chemLTD) is insensitive to ifenprodil application. **(A)** The magnitude of chem-LTD induced by brief application of NMDA (20 µM) is greater in the visual cortex of young (P12-18) animals than adult (P45-90) animals. Representative traces are shown from a P26 recording. **(B)** Ifenprodil (3 µM) fails to reduce the magnitude of chem-LTD, while APV (100 µM) prevents its induction.

`

NVP-AAM077 completely blocks LTP in adult, but not juvenile, animals

In an attempt to investigate the discrete roles of NR2A and NR2B-type NMDARs over development, we bath applied ifenprodil and NVP-AAM077 at different developmental time points. We observed that juvenile LTP was significantly attenuated, but not blocked, in the presence of either antagonist (Fig. 4A) (Average: Control, 116.8% ± 3.1%, *n* = 12; 50 nM NVP, 108.0% ± 2.5%, *n* = 9; 3μM ifenprodil, 105.7% ± 2.7% *n* = 7; $p < 0.05$). However, we were surprised that the induction of adult LTP was completely prevented by NVP-AAM077, while entirely unaffected by ifenprodil (Fig. 4B) (Average: Control, 120.5% ± 3.7%, *n* = 6; 50 nM NVP, 101.4% ± 2.7%, *n* = 6; 3μM ifenprodil, 116.9% ± 3.9%, *n* = 5; $p < 0.01$). To further examine why ifenprodil and NVP-AAM077 had different effects on synaptic plasticity at different developmental time points, we characterized the fidelity of these NMDAR antagonists in our slice preparation.

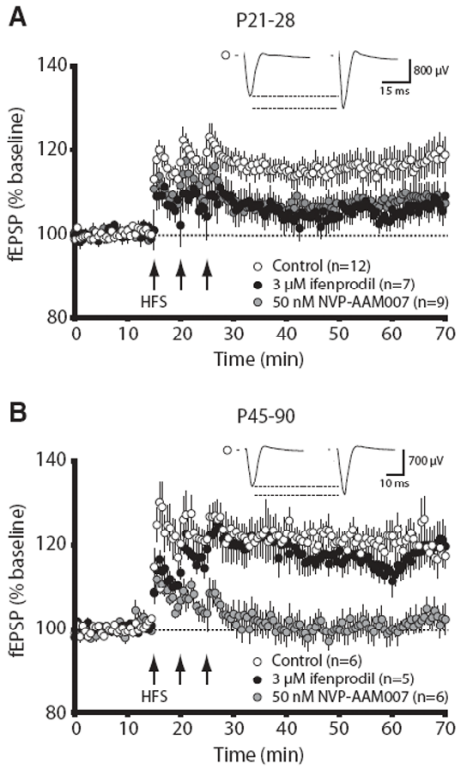

Figure 4: The ability of ifenprodil or NVP-AAM077 to block LTP in the visual cortex is age-dependent. **(A)** In juvenile animals (P21-28), LTP is significantly attenuated by either ifenprodil or NVP-AAM077. **(B)** In mature animals (P45-90), 50 nM NVP-AAM077 completely blocks LTP, whereas 3 μM ifenprodil has no effect on its magnitude. In both panels, the traces shown above each plot are averages of the 15 min baseline and the last 15 min post-stimulation of a representative recording under control conditions.

The developmental effect of NVP-AAM077 is not the result of NR2A-type specificity

Pharmacological antagonists, with varying degrees of subunit specificity, have been used to parse the role of different NMDAR subtypes in synaptic plasticity (Bartlett et al. 2007; Berberich et al. 2007; Berberich et al. 2005; Fox et al. 2006; Hrabetova et al. 2000; Massey et al. 2004; Morishita et al. 2007; Sheng et al. 1994; Toyoda et al. 2006; Weitlauf et al. 2005). While the NR2B-specific antagonist, ifenprodil, has been well characterized (Williams et al. 1993), the recently developed NR2A-selective antagonist NVP-AAM077 has been the subject of controversy. Initial reports assessing the specificity of NVP-AAM077 for human NR2A-containing NMDARs indicated that NVP-AAM077 had a 110-fold preference for human NR2A over NR2B (Auberson et al. 2002). To date, over 100 studies have been performed and interpreted under the assumption that NVP-AAM077 is highly selective for NR2A-containing NMDARs in rodents. However, several recent studies examining the specificity of NVP-AAM077 suggest that the drug has a much lower subunit preference in rodent compared to human NMDARs and that the drug cannot reliably distinguish between NR2A- and NR2B-type receptors (Feng et al. 2004; Frizelle et al. 2006; Neyton and Paoletti 2006).

Although NVP-AAM077 is reported to have only modest selectivity for rodent NR2A-type receptors (Feng et al. 2004; Frizelle et al. 2006), we wanted to test whether it was sensitive enough to discriminate the developmental upregulation of NR2A relative to NR2B that we had established both biochemically and electrophysiologically (Fig 1A-C). To probe the specificity of these antagonists, NMDAR current durations were quantified by a weighted time constant (see Methods) and measured before and after drug application. Because NR2B-type receptors have slower decay kinetics than NR2A-types, we reasoned that

`

selective antagonism of NR2B-type receptors with ifenprodil should increase NMDAR decay kinetics and cause a decrease in current decay time (e.g. a decrease in τ_w) relative to its pre-drug application level. We expected this effect would be most pronounced in young animals that express high levels of NR2B. Similarly, blockade of faster NR2A subtypes would be expected to prolong NMDAR decay kinetics, and this effect would be most profound during adulthood, when NR2A-containing NMDARs predominate at the synapse.

As predicted, ifenprodil significantly shortened NMDAR decay kinetics in young but not adult animals (Average (in ms): P12-28 baseline, 164.5 ± 14.3; P12-28 3 μM ifenprodil, 117.2 ± 11.6, n = 14; p < .05; P45-90 baseline, 92.4 ± 18.2; P45-90 3 μM ifenprodil 86.4 ± 15.4, n = 6), validating that the developmental change in relative NR2 expression can be pharmacologically discerned with a reliable subunit-specific antagonist (Fig. 5A-B). However, we observed that NVP-AAM077 did not alter the duration of isolated NMDAR current kinetics at either developmental stage (Fig. 5C-D) (Average in ms: P12-28 baseline, 171.2 ± 18.6; P12-28 50 nM NVP, 149.1 ± 11.3, n = 13; P45-90 baseline, 98.4 ± 14.4; P45-90 50 nM NVP 101.1 ± 14.9, n = 10). Thus, NVP-AAM077 failed to selectively antagonize the fast NR2A component of the NMDAR current, even in adulthood when the relative levels of NR2A are highest. These data are summarized in Fig. 5E.

To provide an additional gauge of the subunit specificity of these drugs, we evaluated their degree of antagonism with consideration to the amount of subunit expression over development (Fig1A-B). As shown previously, we expected ifenprodil-induced antagonism of NMDAR currents to be greatest in young animals that express relatively high levels of NR2B-type receptors (Vicini et al. 1998; Yoshimura et al. 2003). Conversely, assuming a modest degree of selectivity, NVP-AAM077 should attenuate a progressively greater proportion of NMDAR currents over development, as expression of the NR2A subunit increases. To evaluate the ability of NMDAR antagonists to block NMDAR-mediated currents, we measured their effects on current amplitude. As predicted, ifenprodil

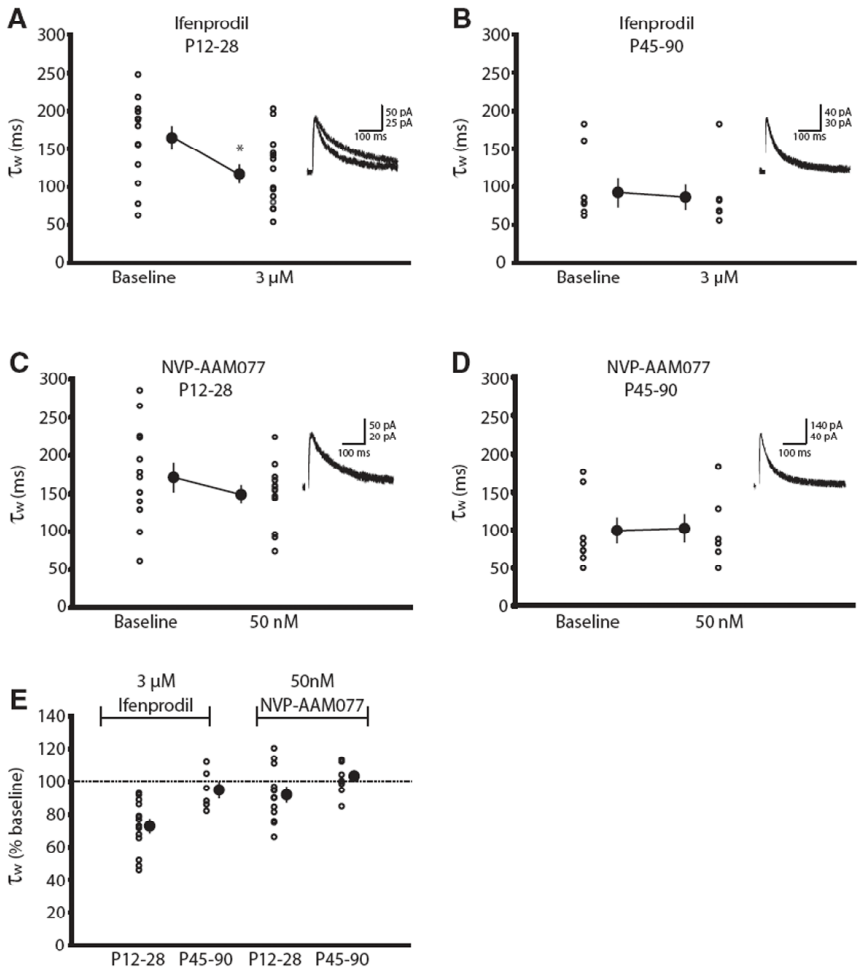

Figure 5: Unlike ifenprodil, NVP-AAM077 fails to alter NMDAR decay kinetics in a manner consistent with subtype specificity. **(A)** Perfusion of ifenprodil results in faster NMDAR current kinetics early in development (P21-P28), reflected as a decrease in τ_w, suggesting ifenprodil selectively blocks long-duration currents from NR2B-containing NMDARs. Open circles represent raw data points and filled symbols indicate means ± SEM before and after drug application. Traces to the right are representative of NMDAR currents before and after ifenprodil application. *$p<0.05$ from baseline. **(B)** Ifenprodil does not alter NMDAR decay kinetics in pyramidal cells from older mice (P45-P90), likely reflecting the small proportion of NR2B-containing NMDARs at this age. **(C)** NVP-AAM077 fails to alter NMDAR current decay kinetics in cells from young mice. **(D)** NVP-AAM077 also fails to alter NMDAR decay kinetics in older mice (P45-P90), when NR2A-containing NMDARs are expected to predominate at the synapse, suggesting that the drug lacks specificity for NR2A-containing NMDARs. **(E)** Data are normalized and summarized.

discriminated the developmental transition in synaptic NMDAR subunit expression by antagonizing significantly less NMDAR current in adult animals (Fig. 6A) (% baseline amplitude: P21-28, 40.4% ± 3.5% n = 6; P45-90, 69.5% ± 4.5%, n = 5; $p < 0.001$). However, NVP-AAM077 failed to exhibit selectivity for NR2A subtypes, as it attenuates NMDAR EPSCs to a similar extent across development (Fig. 6B) (% baseline amplitude: P21-28, 50.6% ± 5.3% n = 4; P45-90, 52.0% ± 3.9%, n = 10) (similar effects were seen on charge transfer, data not shown), suggesting that NVP-AAM077 largely lacks specificity for NR2A subtypes in mice. To additionally test whether NVP-AAM077 lacked selectivity for NR2A subtypes in the mouse visual cortex, we tested the effect of the drug on NMDAR currents from mice lacking NR2A. Further verifying that NVP-AAM077 lacks specificity for NR2A subtypes, the drug significantly blocked NMDAR currents in the visual cortex of NR2A knockout mice, across a broad developmental spectrum. (Fig. 6B) (% baseline: NR2A KO P12-90, 68.6% ± 4.3%, n = 8; $p < 0.001$).

In summary, we found evidence that NVP-AAM077 has limited specificity for NR2A-type receptors, in mice. However, it is difficult to reconcile how a non-selective NMDAR antagonist can have discrete effects on plasticity at different developmental time points. The developmental effect of NVP-AAM077 cannot be attributed to increased NMDAR current antagonism, since the drug blocks a comparable amount of NMDAR current at both ages (Fig. 6B). Therefore, it seemed reasonable to postulate that NVP-AAM077 has a greater affect on LTP, not due to its action on NR2A-type receptors, but due to a developmental increase in the amount of NMDAR activation required for plasticity.

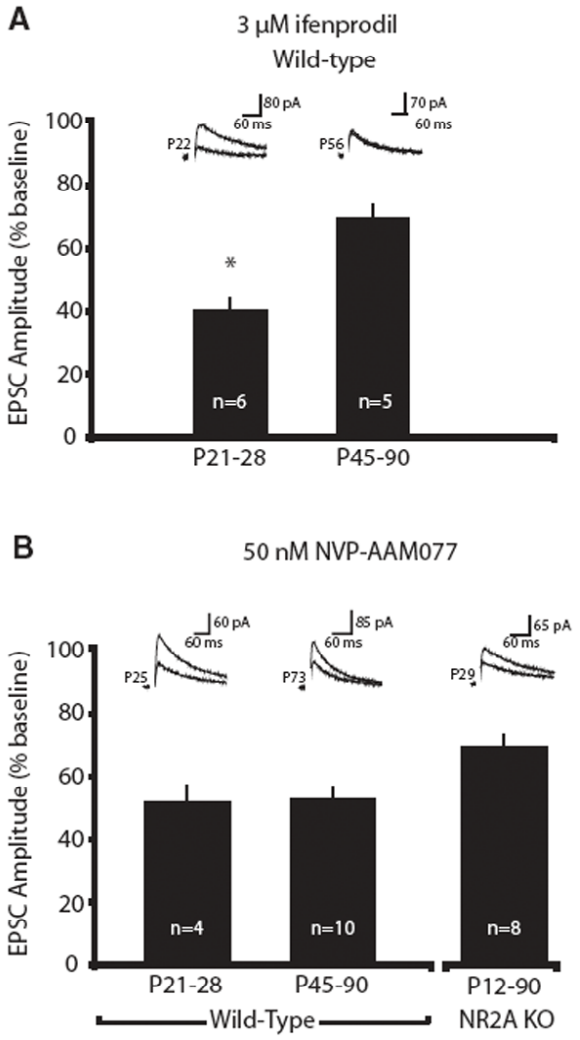

Figure 6: Block of NMDAR currents by ifenprodil, but not NVP-AAM077, correlates to developmental changes in NR2A/NR2B expression. **(A)** Antagonism of NMDAR currents by ifenprodil is reduced over development, reflecting the developmental decrease in relative NR2B expression. **(B)** NVP-AAM077 similarly antagonized NMDAR currents over the same developmental time frame, as the expression of NR2A increased. NVP-AAM077 also blocked a significant proportion of the NMDAR current in mice lacking NR2A. Taken together these data indicate that NVP-AAM077 lacks the ability to discriminate between NR2A and NR2B types in the mouse visual cortex.

`

Developmental differences in the requirements for LTP can explain why NVP-AAM077 has a more profound effect on adult LTP

An age-dependent shift in the amount of NMDAR activation needed to induce LTP could underlie the developmental change in response to NVP-AAM077. That is, the induction of LTP in adult cortex may be more sensitive to disruption by a partial block of NMDAR currents than juvenile cortex.

To measure whether there are age-dependent differences in the ability to disrupt LTP with NMDAR antagonists, we first compared the amount of NMDAR current blocked by NVP-AAM077 during juvenile and adult stages of development (Fig. 6B). It is clear that the total amount of NMDAR current remaining after NVP-AAM077 application is comparable at both developmental stages. However, the same degree of NMDAR antagonism at these two developmental time points caused distinct effects on LTP (Figs 4A-B). These data encouraged us to test whether other relatively non-subunit selective NMDAR antagonists had similar age-dependent consequences on LTP induction.

Because the threshold for plasticity has been shown to vary with experience-dependent regulation of NMDAR current (Philpot et al. 2003), we postulated that there might also be age-dependent changes in NMDAR requirements for the induction of LTP. To investigate this possibility, we used a subsaturating concentration of APV, a pan-NMDAR blocker. While there are some indications that APV has a slight preference for recombinant NR2A-type receptors (Buller and Monaghan 1997), this has not been demonstrated in endogenously expressed receptors (Christie et al. 2000). In our preparation, 1 μM APV failed to attenuate more current with maturity and did not prolong NMDAR current kinetics (data not shown), as would have been expected if the drug had a higher affinity for NR2A-type receptors. These data are consistent with previous observations that APV cannot

distinguish differences in the relative expression of NMDAR subtypes in rodent visual cortex (Quinlan et al. 1999b).

If the developmental effect of NVP-AAM077 on LTP could be attributed to a shift in NMDAR requirements needed to induce plasticity and not a subunit-specific effect, subsaturating concentrations of APV should mimic the age-dependent effects of NVP-AAM077. Through empirical observations, we found that 1 μM APV and 50 nM NVP-AAM077 antagonized a similar amount of NMDAR current in juvenile and adult animals (Fig. 7A) (amplitude % baseline: P21-28: NVP, 50.6 ± 5.3, *n* = 4; APV, 53.9 ± 6.4, *n* = 6; P45-90: NVP, 52.0 ± 3.9, *n* = 10; APV 55.8 ± 9.2, *n* = 4). Excitingly, 1 μM APV failed to antagonize LTP in the juvenile visual cortex, while it strongly attenuated LTP in adults (Fig. 7B-C) (Average (P21-28): Control, 119.1% ± 4.1%, *n* = 8; 1 μM APV, 115.8% ± 5.5% *n* = 9; Average (P45-90): Control, 128.9% ± 3.6%, *n* = 4; 1 μM APV, 107.2% ± 1.5% *n* = 5; *p* = 0.001). Thus, much like NVP-AAM077, APV blocked a similar magnitude of the NMDAR current in juvenile and adult animals, yet differentially blocked the magnitude of LTP.

While the effect of NVP-AAM077 (Fig. 4A-B) and APV (Fig. 7B-C) are not identical, they both demonstrate the same developmental trend. This strongly suggests that the degree of NMDAR activation required for LTP increases with development. Although the induction of plasticity has been shown to be sensitive to partial NMDAR blockade (Berberich et al. 2007; Cummings et al. 1996; Nishiyama et al. 2000; Philpot et al. 2003), our study provides the first indication that LTP sensitivity to NMDAR antagonism changes with development.

The developmental effect of NMDAR antagonism on LTP could reflect a developmental shift in the postsynaptic response to the tetanizing LTP stimulus. This is particularly relevant because the neocortex undergoes a significant postnatal maturation of inhibitory networks (Hensch 2005; Rozas et al. 2001) and presynaptic release properties (Reyes and Sakmann 1999) that could influence short-term synaptic dynamics. To begin to

`

address this, the relative field potential amplitude of the first 28 pulses in the100 Hz tetanus were analyzed (Fig. 8A-D). The rate of decay of the field response was quantified by fitting the normalized data with a triple exponential defined as: $I(t)= I_1*\exp(-t/\tau_1) + I_2*\exp(-t/\tau_2) + I_3*\exp(-t/\tau_3) + C$, where I_x is the peak amplitude of the field response, τ_x is the corresponding decay time constant, and C is the plateau potential of the response. For quantification, the three decay constants were combined into a weighted time constant as follows: $\tau_w=\tau_1 * [I_1/(I_1+I_2+I_3)] + \tau_2 * [I_2/(I_1+I_2+I_3)] + \tau_3 * [I_3/(I_1+I_2+I_3)]$. No significant differences were observed

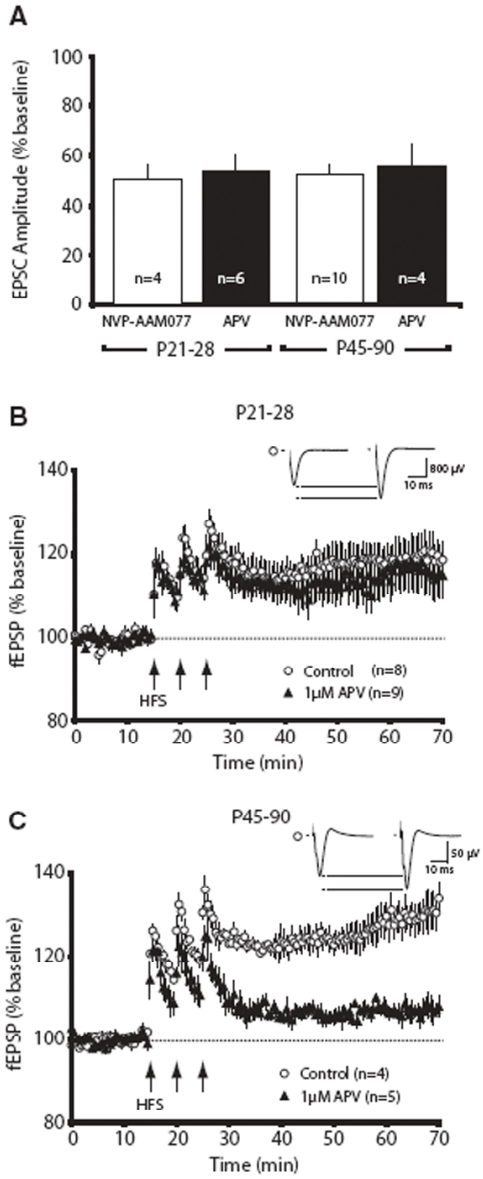

Figure 7: There is a developmental increase in the ability to disrupt LTP with a non-subunit- selective NMDAR antagonist. **(A)** In juvenile (P21-28) and adult (P45-P90) mice, subsaturating concentrations of APV (1 µM) and NVP-AAM077 (50 nM) block a similar degree of NMDAR current amplitude. NVP-AAM077 data are replotted from Fig. 6B for the purposes of comparison. (**B)** Subsaturating APV fails to attenuate the induction of LTP in the visual cortex of young mice (P21-28). **(C)** Subsaturating APV dramatically reduces the magnitude of LTP in adult mice (P45-90).

between the two ages of interest, as both the weighted time constant and the plateau potential of the response decay during the 100 Hz tetanus were similar at both developmental stages (P21-P28 τ_w=13.0±1.4 ms, P45-P90 τ_w=15.6±1.5 ms, p=0.22; P21-P28 C=0.26±0.03, P45-P90 C=0.20±0.03, p=0.21). These findings are consistent with previous observations showing that there are minimal developmental changes in the L4-L2/3 paired-pulse response (Rozas et al. 2001), although there are much greater age-dependent effects on the L2/3 paired-pulse response when stimulation is evoked from white-matter (Ramoa and Sur 1996; Rozas et al. 2001). In addition to observing a similar decay in the field potential response during the 100 Hz stimulation, we also observed that the half-maximal amplitude of the postsynaptic response was similar at both developmental ages (Average fEPSP amplitude: P21-28, 1023.7 ± 118.4 μV, *n* = 10; P45-90, 1121.8 ± 149.0 μV, *n* = 6; *p* = 0.6166). In addition, using immunogold electron microscopy, it appears that the size of the postsynaptic density and the number of synaptic NMDARs is similar between juvenile and adult stages of development (RJ Corlew & RJ Weinberg, personal communications). These results, taken together, suggest that the increasing developmental sensitivity to NMDAR antagonism is not a reflection of a developmental change in our ability to drive postsynaptic L2/3 activity with L4 stimulation. Although our data suggest that the degree of NMDAR activation required for the expression of 100 Hz LTP is increased in adulthood at the L4-L2/3 synapse, we cannot rule out the possibility that age-dependent differences in the susceptibility to NMDAR antagonists might also arise from changes in the way extracellular stimulation recruits neocortical microcircuits.

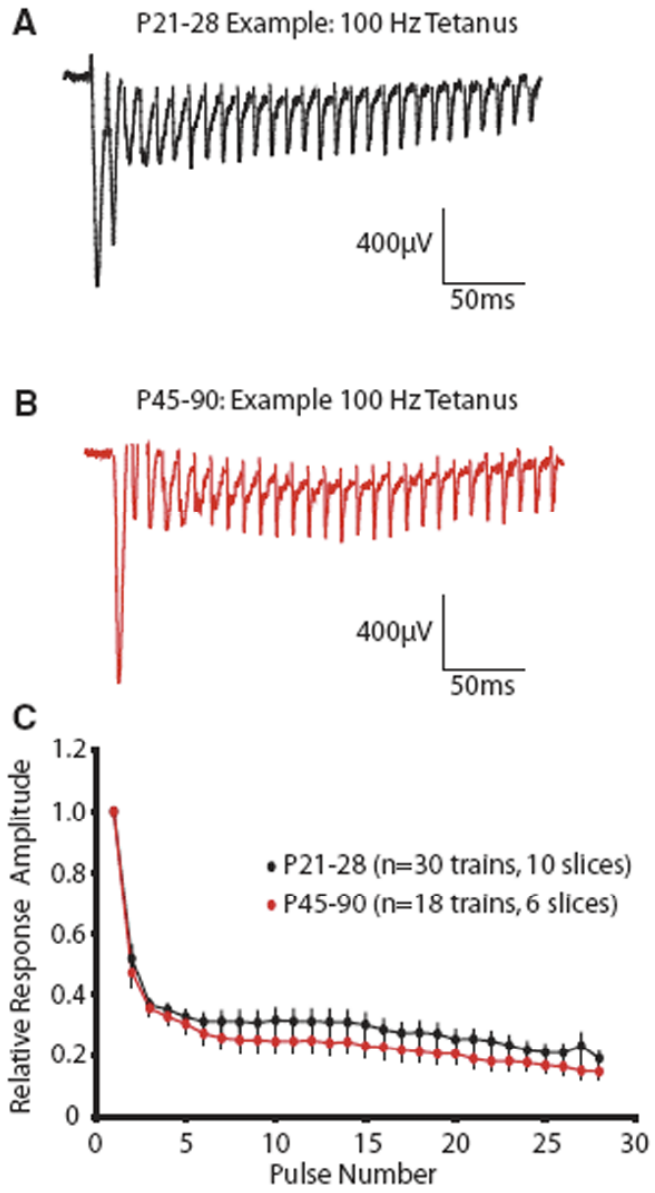

Figure 8: There are no apparent differences between juveniles (P21-28) and adults (P45-90) in the L4-L2/3 field potential responses generated by 100 Hz stimulation. Example field potential traces from **(A)** juvenile and **(B)** adult stages of development. The first 28 pulses of a 100 Hz stimulus are depicted, and stimulus artifacts are clipped for clarity. **(C)** The normalized field potential amplitude of the first 28 pulses of a 100 Hz stimulus does not differ between juvenile and adult animals.

DISCUSSION

The work described here uses a pharmacological approach, over the course of development, to determine whether NMDAR subtypes have specific roles in synaptic plasticity. A major finding of our study is that ifenprodil-sensitive and NVP-AAM077-sensitive NMDARs have overlapping roles in synaptic plasticity. Both populations can contribute to the expression of LTD and LTP. Using the same NMDAR antagonists, our conclusions are in striking contrast to previous studies that suggest opposing roles for NR2A and NR2B in synaptic plasticity. We help resolve this controversy by showing it can be explained, at least in part, by the non-specific effects of a purported NR2A-selective antagonist and by the age-dependent differences in the properties of synaptic plasticity. Specifically, we demonstrate that NVP-AAM077, a commonly employed antagonist, lacks strong selectivity for NR2A-containing NMDARs in rodents. This is highlighted by showing that the age-dependent effects of NVP-AAM077 on LTP can be largely replicated using a non-selective NMDAR antagonist (APV) at subsaturating concentrations. These studies also demonstrate that expression of synaptic plasticity is more easily disrupted by NMDAR antagonists in the adult, compared to the juvenile, cortex. The implications of this are not yet known, although we speculate that the more robust synaptic plasticity in early development is important for shaping cortical networks during early critical periods.

Activation of NMDARs is required for the induction of LTD in the visual cortex (Kirkwood and Bear 1994). However, this LTD is not critically mediated by synaptic or extrasynaptic NR2B-type receptors that are sensitive to ifenprodil. While these findings contrast with previous reports in the hippocampus and perirhinal cortex (Liu et al. 2004; Massey et al. 2004), our results are in agreement with a recent report in the hippocampus conducted across three separate laboratories (Morishita et al. 2007). Interestingly, we did

observe a subtle, but significant, developmental increase in the disruption of LTD by NVP-AAM077 and ifenprodil. This suggests that there is an age-dependent increase in the degree of NMDAR activation required for the full expression of LTD. This observation may help explain why it is more difficult to induce LTD, if it can be induced at all, in many parts of the adult brain (Kirkwood et al. 1996; Kirkwood et al. 1997).

NMDARs are also required for the induction of LTP in the visual cortex (Kirkwood and Bear 1995). Similar to what we observed for LTD, we show that neither NVP-AAM077 nor ifenprodil-sensitive populations of NMDARs are required for the induction of LTP. Interestingly, ifenprodil has no effect on adult LTP, although it attenuates LTP in juvenile mice. We suggest that this maturational change may simply be a consequence of the fact that ifenprodil blocks a greater proportion of the total NMDAR current in juvenile mice. In contrast to our observations using ifenprodil, we observed that NVP-AAM077 more effectively blocks LTP in adult cortex compared to juvenile cortex. This observation is in agreement with recent findings, at the single-cell level, in cortical neurons (Le Roux et al. 2007). While we initially hypothesized that the increasing ability of NVP-AAM077 to block LTP with age was due to an increasing role for NR2A in LTP with development, we discarded this notion for two reasons. First, we used convergent approaches to demonstrate that NVP-AAM077 lacks appreciable specificity for blocking NR2A-containing NMDARs. Second, because NVP-AAM077 blocks the same amount of NMDAR current at both developmental stages studied, we reasoned that a more plausible interpretation of our data is that the degree of NMDAR activation required for plasticity increases with age. In support of this idea, we show that a sub-saturating concentration of APV, a non-subunit selective NMDAR antagonist, mimics the age-dependent consequences of NVP-AAM077 on LTP. Taken together, our data provide convincing evidence that the developmental effect observed with the NMDAR antagonists NVP-AAM077 and APV reflects an increase in the degree of NMDAR activation required for LTP.

The underlying mechanism regulating this developmental shift in the properties of LTP are likely multifold and are currently unknown. Although we demonstrate that NR2A and NR2B subtypes do not have polarized roles in synaptic plasticity, developmental changes in the properties of synaptic plasticity are likely to still be mediated, at least in part, by differences in the NMDAR signaling complex. For example, a developmental change in the relative proportion of diheteromeric (e.g. NR1-NR2B) and triheteromeric (NR1-NR2A-NR2B) NMDARs could alter the requirements for synaptic plasticity. In fact, our pharmacological data suggest that the relative amount of triheteromeric receptors may increase between juvenile and adult stages of development. While our findings show that the NR2A/NR2B ratio changes only modestly between P26 and P62, the NR2B antagonist ifenprodil blocks significantly more NMDAR current at the younger developmental time point. This is revealing because several studies have shown that ifenprodil can better antagonize diheteromeric NR2B-containing NMDARs than triheteromeric receptors, containing both NR2A and NR2B subunits (Kew et al. 1998; Tovar and Westbrook 1999). Therefore, we postulate that the developmental decrease in ifenprodil sensitivity may reflect an increased presence of triheteromeric NMDARs, which have been documented in both the cortex and hippocampus (Al-Hallaq et al. 2007; Luo et al. 1997; Sheng et al. 1994). While a developmental change in the relative amount of triheteromeric receptors has not been demonstrated in hippocampus (Al-Hallaq et al. 2007), our data, and that of others (Kew et al. 1998), hint that there may be a developmental increase in cortical triheteromeric NMDARs. While the decay kinetics of triheteromeric NMDARs are intermediate to diheteromeric (NR1/NR2A and NR1/NR2B) NMDARs that contain only one type of NR2 subunit, it is unclear how these triheteromeric NMDARs influence plasticity. The intracellular signaling of triheteromeric receptors may differ from that of diheteromeric receptors since signaling cascades, linked to specific NR2 subunits may be affected by oligomerization. However, the NR2B subunit, which is more likely to be in a diheteromeric form during

`

juvenile stages of development, has several attributes that favor its involvement in the expression of LTP. First, NR2B-type receptors have been shown to allow more calcium influx per unit charge than NR2A-type receptors (Sobczyk et al. 2005). In addition, NR2B subunits recruit CaMKII, a critical modulator of LTP, to the synapse (Barria and Malinow 2005; Lisman et al. 2002). Taken together, a developmental decrease in NR2B-like properties, which may parallel the increase in triheteromeric NMDARs, would be less permissive for calcium influx and NR2B-associated signaling cascades at the synapse. As such, LTP may be more easily induced, and less easily disrupted, when diheteromeric NR2B-type NMDAR expression is high (Philpot et al. 2007). Thus, a developmental change in NMDAR subunit composition may be one factor, in addition to other developmental changes in the synaptic milieu (Berardi et al. 2004; Hensch 2005), that increases the sensitivity of LTP to NMDAR antagonism.

If the composition of NMDARs at young synapses allows LTP to occur at a lower threshold, thereby making LTP less easily disrupted by partial NMDAR blockade, it might be expected that the amplitude of LTP should be greater in young animals. However, previous findings indicate that LTP induction and expression may be regulated by separate mechanisms. Indeed, synapses may compete for “plasticity factors” that limit the expression of LTP (Fonseca et al. 2004). Further support for this hypothesis comes from observations of plasticity in NR2A knockout animals, where the threshold for inducing LTP is lowered without an appreciable change in the magnitude of the LTP expressed (Philpot et al. 2007). Thus, one set of “plasticity factors” might control the threshold for modifying synapses, while a second, likely overlapping, set of molecules might regulate the magnitude of synaptic modifications.

Parsing out the mechanisms underlying the developmental increase in the degree of NMDAR activation required for plasticity will require further investigation. For example, it is already clear that the maturation of inhibitory circuitry is of profound importance (Corlew et

al. 2007; Hensch and Fagiolini 2005; Huang et al. 1999; Maffei et al. 2006; Steele and Mauk 1999; Yoshimura et al. 2003), while its role in regulating the properties of synaptic plasticity continues to be more fully defined. Although we provide evidence that our ability to drive L4-L2/3 activity is similar between juvenile and adult stages of development, we cannot preclude the possibility that enhanced cortical inhibition, changes in presynaptic release probability, or differences in synaptic NMDAR content may play a role in shifting the ability of NMDAR antagonists to disrupt LTP over development.

Although the properties underlying the mechanisms of synaptic plasticity are complex, our work contributes several important findings that increase our understanding of developmentally-regulated plasticity and distinct NMDAR subunit functions. First, our data indicate that ifenprodil-sensitive NR2B-type NMDARs are not critical for the induction of either LTD or LTP. Second, NVP-AAM077 is an unreliable NR2A-type antagonist in the mouse visual cortex. Thus, the myriad of studies that have been interpreted with the assumption that NVP-AAM077 is a highly subunit-selective antagonist must be re-evaluated. Third, we reveal a developmental increase in the degree of NMDAR activation required for the induction of LTP. These observations not only elucidate an important mechanism that regulates plasticity over development, they may also reconcile discrepant results concerning NR2 subunit-specific roles in plasticity. The roles of NR2A and NR2B may be better understood in the future through careful consideration of the limitations of subunit-selective antagonists, the age of the animal, the contribution of triheteromeric NMDARs, and the brain region and species being examined.

`

EXPERIMENTAL PROCEDURES

Biochemical Fractions:

Each biochemical fraction was prepared using visual cortices pooled from 3-10 brains (brains per pooled sample; P8, *n* = 10; P16, *n* = 5; P26, *n* = 5; P62, *n* = 3), as previously described (Yashiro et al. 2005). Samples were homogenized in HEPES-buffered sucrose (4 mM HEPES, 0.32 M sucrose, pH 7.4) using a motor-driven dounce homogenizer. Post-nuclear supernatant (PNS) fractions were prepared by centrifuging homogenates twice at 1,000 x *g* for 10 min to eliminate nuclei. PNS fractions were centrifuged at 10,000 x *g* for 20 min yielding crude synaptic pellets, which were then suspended in HEPES-buffered sucrose and centrifuged. The resulting pellets were lysed in a hypoosmotic buffer (4 mM HEPES, pH 7.4) using a motor-driven dounce homogenizer and mixed constantly for 30 min. Lysates were centrifuged at 25,000 x *g* for 20 min and pellets were suspended in HEPES-buffered sucrose to obtain lysed synaptosomal membrane (LSM) fractions. LSM fractions were subjected to density centrifugation (150,000 x *g*, 2 hrs) using a gradient consisting of 0.8 M, 1.0 M, and 1.2 M sucrose in 4 mM HEPES (pH 7.4). Synaptic plasma membrane (SPM) fractions were collected at the 1.0-1.2 M interface, diluted with 4 mM HEPES, and pelleted (150,000 x *g*, 30 min). These pellets were resuspended in 50 mM HEPES (pH 7.4) containing 0.5% Triton X-100, rotated for 15 min, and centrifuged at 32,000 x *g* for 20 min. The resulting pellets were resuspended in a 0.5% Triton-containing buffer, rotated for 15 min, and centrifuged at 200,000 x *g* for 20 min to obtain postsynaptic density (PSD) fractions, which were suspended in 50 mM HEPES containing 0.2% SDS. Complete protease inhibitor cocktail tablets (Roche, Germany), pepstatin 10 µg/ml, and phosphatase inhibitor cocktail 1 & 2 were added to all buffers. The above procedures were carried out on

ice or in a cold room and fractions were stored at -80^{o}C. Protein concentrations were measured using Coomassie Plus reagent (Pierce, IL).

Immunoblot Analysis

PSD fractions (5 µg) were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Both blotting and imaging with the Odyssey system (LI-COR, NE) were carried out following the manufacturer's protocols. The primary antibodies used were rabbit anti-NR2A (1:500, sc-9056, Santa Cruz, CA) and goat anti-NR2B (1:10,000, sc-1469, Santa Cruz, CA). The employed secondary antibodies were Alexa Fluor 680-labeled anti-goat IgG (1:5,000, Molecular Probes, OR) and IRDye 800-labeled anti-rabbit IgG (1:3,000, Rockland, PA). All protein quantification was based off the average of three separately loaded lanes. Because the PSD fraction is enriched for synaptic proteins, commonly employed loading controls that remain constant over the course of development are not available.

Pharmacological Agents

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO).

Statisitics

Data were expressed as means ± SEM. Statistical comparisons were performed using InStat3 software (GraphPad Software Inc., San Diego, CA). For multiple group comparisons, analyses of variance (ANOVAs) were first performed, followed by between-group comparisons with Student-Newman-Keuls (SNK) tests. Significance was placed at $p < 0.05$. All reported levels of statistical significance represent two-tailed values.

`

ACKNOWLEDGMENTS

We thank Yves Auberson of the Novartis Institutes for Biomedical Research (Basel, Switzerland) for the generous gift of NVP-AAM077, and we are indebted to Paul Manis for useful suggestions and for assistance in generating offline analyses. We also thank Rebekah Corlew, Richard Weinberg, and Maile Henson for technical support and for editing of the manuscript.

Grants: This work was funded by a Helen Lyng White Fellowship (ACR), a NICHD grant T32-HD40127 (ACR), the Whitehall Foundation (BDP), and a grant from the National Eye Institute RO1 EY018323 (BDP).

Disclosures: The authors declare no potential conflicts of interest.

BIBLIOGRAPHY

Al-Hallaq RA, Conrads TP, Veenstra TD, and Wenthold RJ. NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. *J Neurosci* 27: 8334-8343, 2007.

Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R, and Schmutz M. 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett* 12: 1099-1102, 2002.

Barria A and Malinow R. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48: 289-301, 2005.

Bartlett TE, Bannister NJ, Collett VJ, Dargan SL, Massey PV, Bortolotto ZA, Fitzjohn SM, Bashir ZI, Collingridge GL, and Lodge D. Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology* 52: 60-70, 2007.

Bear MF, Kleinschmidt A, Gu QA, and Singer W. Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. *J Neurosci* 10: 909-925, 1990.

Berardi N, Pizzorusso T, and Maffei L. Extracellular matrix and visual cortical plasticity: freeing the synapse. *Neuron* 44: 905-908, 2004.

Berberich S, Jensen V, Hvalby O, Seeburg PH, and Kohr G. The role of NMDAR subtypes and charge transfer during hippocampal LTP induction. *Neuropharmacology* 52: 77-86, 2007.

Berberich S, Punnakkal P, Jensen V, Pawlak V, Seeburg PH, Hvalby O, and Kohr G. Lack of NMDA receptor subtype selectivity for hippocampal long-term potentiation. *J Neurosci* 25: 6907-6910, 2005.

Buller AL and Monaghan DT. Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in Xenopus oocytes. *Eur J Pharmacol* 320: 87-94, 1997.

Carmignoto G and Vicini S. Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science* 258: 1007-1011, 1992.

Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, and Zhang D. Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* 415: 793-798, 2002.

Chen L, Cooper NG, and Mower GD. Developmental changes in the expression of NMDA receptor subunits (NR1, NR2A, NR2B) in the cat visual cortex and the effects of dark rearing. *Brain Res Mol Brain Res* 78: 196-200, 2000.

Christie JM, Jane DE, and Monaghan DT. Native N-methyl-D-aspartate receptors containing NR2A and NR2B subunits have pharmacologically distinct competitive antagonist binding sites. *J Pharmacol Exp Ther* 292: 1169-1174, 2000.

Corlew R, Wang Y, Ghermazien H, Erisir A, and Philpot BD. Developmental switch in the contribution of presynaptic and postsynaptic NMDA receptors to long-term depression. *J Neurosci* 27: 9835-9845, 2007.

Cull-Candy S, Brickley S, and Farrant M. NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* 11: 327-335, 2001.

Cummings JA, Mulkey RM, Nicoll RA, and Malenka RC. Ca2+ signaling requirements for long-term depression in the hippocampus. *Neuron* 16: 825-833, 1996.

Dingledine R, Borges K, Bowie D, and Traynelis SF. The glutamate receptor ion channels. *Pharmacol Rev* 51: 7-61, 1999.

Dudek SM and Bear MF. Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus. *J Neurosci* 13: 2910-2918, 1993.

Feng B, Tse HW, Skifter DA, Morley R, Jane DE, and Monaghan DT. Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl) piperazine-2,3-dicarboxylic acid. *Br J Pharmacol* 141: 508-516, 2004.

Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, and Monyer H. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. *J Neurosci* 17: 2469-2476, 1997.

Fonseca R, Nagerl UV, Morris RG, and Bonhoeffer T. Competing for memory: hippocampal LTP under regimes of reduced protein synthesis. *Neuron* 44: 1011-1020, 2004.

Fox CJ, Russell KI, Wang YT, and Christie BR. Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus in vivo. *Hippocampus* 16: 907-915, 2006.

Frizelle PA, Chen PE, and Wyllie DJ. Equilibrium constants for (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquino xalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) acting at recombinant NR1/NR2A and NR1/NR2B N-methyl-D-aspartate receptors: Implications for studies of synaptic transmission. *Mol Pharmacol* 70: 1022-1032, 2006.

Gerkin RC, Lau PM, Nauen DW, Wang YT, and Bi GQ. Modular Competition Driven by NMDA Receptor Subtypes in Spike-Timing-Dependent Plasticity. *J Neurophysiol*, 2007.

Gordon JA and Stryker MP. Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. *J Neurosci* 16: 3274-3286, 1996.

Hensch TK. Critical period plasticity in local cortical circuits. *Nat Rev Neurosci* 6: 877-888, 2005.

Hensch TK and Fagiolini M. Excitatory-inhibitory balance and critical period plasticity in developing visual cortex. *Prog Brain Res* 147: 115-124, 2005.

Hestrin S. Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. *Nature* 357: 686-689, 1992.

Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE, Monaghan DT, and Sacktor TC. Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction. *J Neurosci* 20: RC81, 2000.

Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, and Tonegawa S. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell* 98: 739-755, 1999.

Jiang B, Akaneya Y, Hata Y, and Tsumoto T. Long-term depression is not induced by low-frequency stimulation in rat visual cortex in vivo: a possible preventing role of endogenous brain-derived neurotrophic factor. *J Neurosci* 23: 3761-3770, 2003.

Jiang B, Trevino M, and Kirkwood A. Sequential development of long-term potentiation and depression in different layers of the mouse visual cortex. *J Neurosci* 27: 9648-9652, 2007.

Kadotani H, Hirano T, Masugi M, Nakamura K, Nakao K, Katsuki M, and Nakanishi S. Motor discoordination results from combined gene disruption of the NMDA receptor NR2A and NR2C subunits, but not from single disruption of the NR2A or NR2C subunit. *J Neurosci* 16: 7859-7867, 1996.

Kew JN, Richards JG, Mutel V, and Kemp JA. Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. *J Neurosci* 18: 1935-1943, 1998.

Kirkwood A and Bear MF. Elementary forms of synaptic plasticity in the visual cortex. *Biol Res* 28: 73-80, 1995.

Kirkwood A and Bear MF. Homosynaptic long-term depression in the visual cortex. *J Neurosci* 14: 3404-3412, 1994.

Kirkwood A, Rioult MC, and Bear MF. Experience-dependent modification of synaptic plasticity in visual cortex. *Nature* 381: 526-528, 1996.

Kirkwood A, Silva A, and Bear MF. Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice. *Proc Natl Acad Sci U S A* 94: 3380-3383, 1997.

Laube B, Kuhse J, and Betz H. Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci* 18: 2954-2961, 1998.

Le Roux N, Amar M, Moreau A, and Fossier P. Involvement of NR2A- or NR2B-containing N-methyl-D-aspartate receptors in the potentiation of cortical layer 5 pyramidal neurone inputs depends on the developmental stage. *Eur J Neurosci* 26: 289-301, 2007.

Lee HK, Kameyama K, Huganir RL, and Bear MF. NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. *Neuron* 21: 1151-1162, 1998.

Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, and Huganir RL. Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* 112: 631-643, 2003.

Lisman J, Schulman H, and Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* 3: 175-190, 2002.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, and Wang YT. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science* 304: 1021-1024, 2004.

Luo J, Wang Y, Yasuda RP, Dunah AW, and Wolfe BB. The majority of N-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). *Mol Pharmacol* 51: 79-86, 1997.

Maffei A, Nataraj K, Nelson SB, and Turrigiano GG. Potentiation of cortical inhibition by visual deprivation. *Nature* 443: 81-84, 2006.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, and Bashir ZI. Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *J Neurosci* 24: 7821-7828, 2004.

Mayer ML and Westbrook GL. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J Physiol* 394: 501-527, 1987.

McBain CJ and Mayer ML. N-methyl-D-aspartic acid receptor structure and function. *Physiol Rev* 74: 723-760, 1994.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, and Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12: 529-540, 1994.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, and Seeburg PH. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science* 256: 1217-1221, 1992.

Morishita W, Lu W, Smith GB, Nicoll RA, Bear MF, and Malenka RC. Activation of NR2B-containing NMDA receptors is not required for NMDA receptor-dependent long-term depression. *Neuropharmacology* 52: 71-76, 2007.

Morris RG. Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist AP5. *J Neurosci* 9: 3040-3057, 1989.

Moser EI, Krobert KA, Moser MB, and Morris RG. Impaired spatial learning after saturation of long-term potentiation. *Science* 281: 2038-2042, 1998.

Nase G, Weishaupt J, Stern P, Singer W, and Monyer H. Genetic and epigenetic regulation of NMDA receptor expression in the rat visual cortex. *Eur J Neurosci* 11: 4320-4326, 1999.

Neyton J and Paoletti P. Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. *J Neurosci* 26: 1331-1333, 2006.

Nishiyama M, Hong K, Mikoshiba K, Poo MM, and Kato K. Calcium stores regulate the polarity and input specificity of synaptic modification. *Nature* 408: 584-588, 2000.

Perez-Otano I and Ehlers MD. Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals* 13: 175-189, 2004.

Philpot BD, Cho KK, and Bear MF. Obligatory Role of NR2A for Metaplasticity in Visual Cortex. *Neuron* 53: 495-502, 2007.

Philpot BD, Espinosa JS, and Bear MF. Evidence for altered NMDA receptor function as a basis for metaplasticity in visual cortex. *J Neurosci* 23: 5583-5588, 2003.

Quinlan EM, Olstein DH, and Bear MF. Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. *Proc Natl Acad Sci U S A* 96: 12876-12880, 1999a.

Quinlan EM, Philpot BD, Huganir RL, and Bear MF. Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. *Nat Neurosci* 2: 352-357, 1999b.

Ramoa AS and McCormick DA. Enhanced activation of NMDA receptor responses at the immature retinogeniculate synapse. *J Neurosci* 14: 2098-2105, 1994.

Ramoa AS, Mower AF, Liao D, and Jafri SI. Suppression of cortical NMDA receptor function prevents development of orientation selectivity in the primary visual cortex. *J Neurosci* 21: 4299-4309, 2001.

Ramoa AS and Sur M. Short-term synaptic plasticity in the visual cortex during development. *Cereb Cortex* 6: 640-646, 1996.

Reyes A and Sakmann B. Developmental switch in the short-term modification of unitary EPSPs evoked in layer 2/3 and layer 5 pyramidal neurons of rat neocortex. *J Neurosci* 19: 3827-3835, 1999.

Roberts EB, Meredith MA, and Ramoa AS. Suppression of NMDA receptor function using antisense DNA block ocular dominance plasticity while preserving visual responses. *J Neurophysiol* 80: 1021-1032, 1998.

Roberts EB and Ramoa AS. Enhanced NR2A subunit expression and decreased NMDA receptor decay time at the onset of ocular dominance plasticity in the ferret. *J Neurophysiol* 81: 2587-2591, 1999.

Rozas C, Frank H, Heynen AJ, Morales B, Bear MF, and Kirkwood A. Developmental inhibitory gate controls the relay of activity to the superficial layers of the visual cortex. *J Neurosci* 21: 6791-6801, 2001.

Rumbaugh G and Vicini S. Distinct synaptic and extrasynaptic NMDA receptors in developing cerebellar granule neurons. *J Neurosci* 19: 10603-10610, 1999.

Santos-Sacchi J. Voltage-dependent ionic conductances of type I spiral ganglion cells from the guinea pig inner ear. *J Neurosci* 13: 3599-3611, 1993.

Sheng M, Cummings J, Roldan LA, Jan YN, and Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* 368: 144-147, 1994.

Sobczyk A, Scheuss V, and Svoboda K. NMDA receptor subunit-dependent [Ca2+] signaling in individual hippocampal dendritic spines. *J Neurosci* 25: 6037-6046, 2005.

Steele PM and Mauk MD. Inhibitory control of LTP and LTD: stability of synapse strength. *J Neurophysiol* 81: 1559-1566, 1999.

Stocca G and Vicini S. Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. *J Physiol* 507 (Pt 1): 13-24, 1998.

Tovar KR and Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* 19: 4180-4188, 1999.

Toyoda H, Zhao MG, and Zhuo M. NMDA receptor-dependent long-term depression in the anterior cingulate cortex. *Rev Neurosci* 17: 403-413, 2006.

Trachtenberg JT, Trepel C, and Stryker MP. Rapid extragranular plasticity in the absence of thalamocortical plasticity in the developing primary visual cortex. *Science* 287: 2029-2032, 2000.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, and Grayson DR. Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol* 79: 555-566, 1998.

Watanabe M, Mishina M, and Inoue Y. Distinct spatiotemporal distributions of the N-methyl-D-aspartate receptor channel subunit mRNAs in the mouse cervical cord. *J Comp Neurol* 345: 314-319, 1994.

Weitlauf C, Honse Y, Auberson YP, Mishina M, Lovinger DM, and Winder DG. Activation of NR2A-containing NMDA receptors is not obligatory for NMDA receptor-dependent long-term potentiation. *J Neurosci* 25: 8386-8390, 2005.

Williams K. Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol* 44: 851-859, 1993.

Williams K, Russell SL, Shen YM, and Molinoff PB. Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. *Neuron* 10: 267-278, 1993.

Yashiro K, Corlew R, and Philpot BD. Visual deprivation modifies both presynaptic glutamate release and the composition of perisynaptic/extrasynaptic NMDA receptors in adult visual cortex. *J Neurosci* 25: 11684-11692, 2005.

Yoshimura Y, Ohmura T, and Komatsu Y. Two forms of synaptic plasticity with distinct dependence on age, experience, and NMDA receptor subtype in rat visual cortex. *J Neurosci* 23: 6557-6566, 2003.

`

CHAPTER THREE

srGAP2 regulates dendritic spine morphogenesis and synaptic transmission through the synergistic action of its F-BAR and RhoGAP domains

Jacqueline de Marchena[1,2], Takayuki Sassa[1], Jaeda Coutinho-Budd[1,2], Sabrice Guerrier[1,3], Janet Berrios[1], Marie Rougie[1], and Franck Polleux[1,3]

[1] Neuroscience Research Center, [2] Neurobiology Curriculum, [3] Department of Pharmacology, School of Medicine
University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Running head: srGAP2 modulates dendritic spine morphogenesis and synaptic transmission

Corresponding author: Franck Polleux
E-mail: fpolleux@med.unc.edu

SUMMARY

RhoGAP containing proteins have been shown to play a critical role in neural development, with a number of mutations leading to severe mental retardation syndromes. However, many RhoGAP containing proteins also contain other signaling motifs, including lipid-binding domains, such as BAR, F-BAR, or I-BAR domains that can directly deform membrane. Yet, the relative contribution of membrane-deforming domains for the function of these physiological important proteins remains entirely unexplored. We have recently shown that the F-BAR domain of srGAP2 induces filopodia-like protrusions in non-neuronal and neuronal cells. Here, we show that the F-BAR domain of srGAP2 is required for the localization of srGAP2 to the synapse. By isolating the function of the F-BAR domain and its Rac1-specific RhoGAP domain, we demonstrate that both parts of the protein act synergistically to promote changes in dendritic spine morphology. srGAP2 is also an important postsynaptic regulator of glutamatergic neurotransmission that may negatively regulate the prevalence of silent synapses. These results show (1) the F-BAR domain is essential for the proper subcellular targeting of srGAP2 to the synapse, (2) the F-BAR and Rac1-GAP domains of srGAP2, are directly capable of modulating dendritic spine morphology, and (3) full-length srGAP2 regulates postsynaptic AMPARs.

`

INTRODUCTION

Throughout development, cortical neurons undergo dramatic changes in morphology underlying their migration, polarization, axo-dendritic differentiation and synaptogenesis. During late postnatal development, neurons continue to exhibit profound morphological changes (Fischer et al. 1998; Engert and Bonhoeffer 1999; Lendvai et al. 2000; Yuste and Bonhoeffer 2001; Bonhoeffer and Yuste 2002) that are restricted to dendritic spines, which are specialized membrane protrusions containing the postsynaptic machinery of glutamatergic synapses (Gray 1959; Parnavelas et al. 1977). Spines are highly motile during the first postnatal month, as synapses are being established and neuronal circuitry undergoes refinement (Dailey and Smith 1996; Yuste and Bonhoeffer 2004). Following this period, spine density becomes more stable and the rate of spine addition and retraction decreases dramatically (Ziv and Smith 1996). However, the morphology of mature dendritic spines remains dynamic, with spine heads undergoing considerable changes in shape. These structural changes are thought to underlie mechanisms of learning and memory (Engert and Bonhoeffer 1999; Yuste and Bonhoeffer 2001; Hofer et al. 2009) and require coordinated cytoskeletal dynamics, regulated by a myriad of intracellular effectors.

Signaling through small G-proteins is important for regulating of the shape of actin-rich dendritic spines (Matus et al. 1982), which have been roughly subdivided into three categories: stubby, thin, and mushroom-shaped. Several Rho-family of small GTPases have been shown to regulate spine morphology: (Peters and Kaiserman-Abramof 1970). For example, Rac1 promotes a mushroom-shaped dendritic spine morphology, while RhoA, make spines thinner by reducing head size (Tashiro et al. 2000). Interestingly, a number of proteins containing Rho-GAP domains, which inactivate small GTPases by promoting their rate of GTP hydrolysis, have been implicated in mental retardation syndromes Accordingly, the majority of studies on these proteins have focused on their RhoGAP activity (Billuart et

al. 1998; Chelly and Mandel 2001; Endris et al. 2002). However, the majority of these proteins contain a myriad of other signaling domains, including lipid binding domains of the BAR superfamily, which are capable of directly deforming cell membranes (McMahon and Gallop 2005; Zimmerberg and Kozlov 2006; Scita et al. 2008). Yet, how these membrane-deforming properties contribute to the function of RhoGAP and other types of proteins, remains poorly understood.

The BAR superfamily contains three main subfamily domains: (1) the Bin-amphiphysin-Rvs (BAR) domain, (2) the extended Fes-cip4 homology (EFC) or F-BAR domain, and the (3) Inverted-BAR or I-BAR domain (Scita et al. 2008). Members of the BAR superfamily are capable of directly binding the membrane through the positively charged residues that are located at the ends of their dimeric α-helical structure (Scita et al. 2008). Structural analysis of BAR domains suggests they are curved and that they can oligomerize to form a tubulated scaffold capable of deforming the membrane through direct electrostatic interactions, in *vivo* and in *vitro* (Masuda and Mochizuki). While classically defined BAR and F-BAR domains are predicted to induce the formation of membrane invaginations, I-BAR domains are predicted to have a structure that induces the formation of membrane protrusions (Scita et al. 2008). However, the structure and activity of each domain should be considered on a protein-by-protein basis, as sequence predicted F-BAR domains have been shown to result in membrane protrusions, as well as invaginations (Shimada et al. ; Guerrier et al. 2009). Additionally, these domains are located within the large proteins, which can contain a number of functional domains. Therefore, it is important to discern the function of BAR-like domains in the context of full-length proteins.

Oligophrenin1 and IRSp53 are two proteins implicated in mental retardation syndromes, which contain both BAR superfamily and RhoGAP domains (Billuart et al. 1998; Chelly and Mandel 2001; Ramakers 2002; Itoh and De Camilli 2006). Interestingly, they have been shown to be important modulators of dendritic morphology and glutamatergic

synaptic transmission. Oligophrenin1 is a BAR containing protein that also contains a RhoGAP domain that inactivates RhoA (Fauchereau et al. 2003; Govek et al. 2004; Khelfaoui et al. 2009). Knockdown of this protein in cultured neurons causes increased spine density and length, making dendritic spines more filopodial-like (Govek et al. 2004). Because loss of oligophrenin1 increases RhoA activity, which positively regulates spine density and length (Tashiro et al. 2000), this finding is completely consistent with what is known about its RhoGAP domain. Interestingly, oligophrenin1 has also been shown to play an important role in vesicular recycling and in the internalization of AMPA-type glutamate receptors (Khelfaoui et al. 2009). While this work suggests that oligophrenin1 positively regulates AMPA receptor internalization through inactivation of the RhoA/ROCK pathway (Khelfaoui et al. 2009), the contribution of oligophrenin1's BAR domain to vesicular endocytosis remains unknown. This is an important consideration because similar BAR domains present in amphiphysin are known to be important regulators of endocytosis (Zhang and Zelhof 2002). IRSp53, an I-BAR containing protein with a RhoGAP domain, has also been shown to play an important role in the regulation of glutamatergic synaptic transmission. However, IRSp53 appears to regulate dendritic spine density (Choi et al. 2005) and the presence of NMDA-type receptors at the synapse (Hori et al. 2005; Sawallisch et al. 2009). IRSp53 knockouts exhibit augmented NMDA receptor currents and have elevated charge transfer through NMDA-type glutamate receptors during theta-burst stimulation, resulting in enhanced long-term potentiation (LTP) (Kim et al. 2009; Sawallisch et al. 2009). Interestingly, synaptic localization of IRSp53 is induced by NMDA receptor activation (Hori et al. 2005). Therefore, it appears that there is a dynamic interplay between NMDA receptors and IRSp53. While NMDA receptor activation sequesters IRSp53 to the synapse, IRSp53 in-turn down regulates NMDA receptors at the synapse. Intriguingly, N-terminal phosphorylation of IRSp53 on the region corresponding to its I-BAR domain appears to direct the NMDA receptor-induced translocation of IRSp53 (Hori et al. 2005).

However, exactly how the IBAR domain of IRSp53 contributes to its function still remains to be elucidated.

Therefore, several proteins containing both RhoGAP and BAR superfamily domains have been shown to play an important role in modulating dendritic spine morphology (Govek et al. 2004) and in regulating the trafficking of postsynaptic glutamatergic receptors (Khelfaoui et al. 2007). However, the focus of these studies has been on determining how the RhoGAP domain contributes to the function of these proteins and the relative contribution of the BAR domain (for Oligophrenin-1) or I-BAR domain (for IRSp53) is currently unknown. In the present study, we highlight the importance of both the F-BAR and RhoGAP domains of slit-robo-GTPase Activating Protein 2 (srGAP2) in modulating dendritic spine morphology, neurotransmission, and synaptic plasticity. Our results demonstrate that the F-BAR domain of srGAP2 is required for the synaptic localization of the protein and that both domains act synergistically to promote a thin, elongated spine shape. We also demonstrate a role for srGAP2 in modulating glutamatergic neurotransmission and, possibly, synaptic plasticity.

RESULTS

To begin our study of the role of srGAP2 in dendritic spine morphogenesis and synaptic plasticity, we focused on establishing its pattern of expression. Expanding upon previous work (Mattar et al. 2004; Yao et al. 2008; Bacon et al. 2009; Guerrier et al. 2009), we found that srGAP2 is not only expressed throughout embryonic and early postnatal development (**Fig 1A-B**), but that it is also associated with the synapse at postnatal day (P) 18. This was revealed by performing a western blot for srGAP2, on synaptosomal membrane fractions (**Fig 1C**). Also, in transfected neurons expressing low levels of a C-terminal srGAP2-EGFP fusion protein, we found that srGAP2 is enriched in dendritic spines (**Fig 1D**). However, expressing a truncated form of srGAP2 that does not contain the N-

terminal FBAR domain (ΔFBAR-EGFP) disrupts the synaptic targeting of the protein (**Fig 1D**). Therefore, the FBAR domain of srGAP2 is required for synaptic enrichment of srGAP2, while the RhoGAP and SH3 domains are dispensable in this process (**data not shown**).

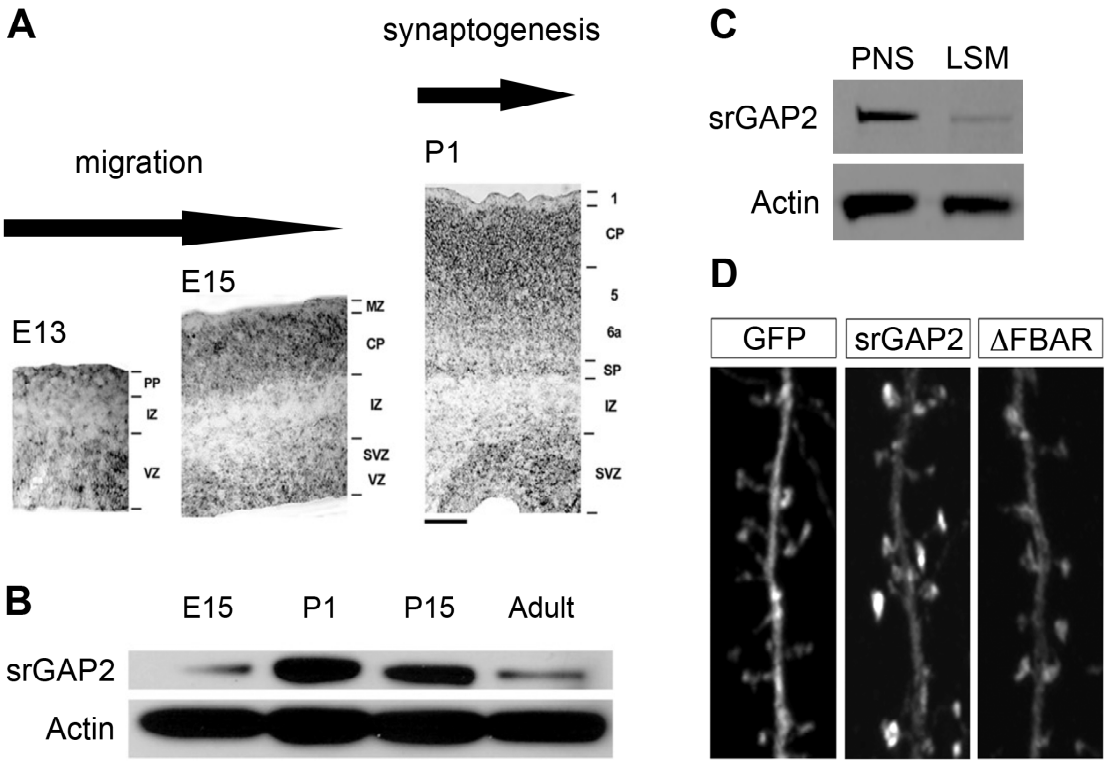

Figure 1: srGAP2 is expressed throughout embryonic and postnatal stages of development and the F-BAR domain of srGAP2 is required for its synaptic localization. **(A)** In-situ hybridizations and **(B)** western blots for srGAP2 indicate that the protein is expressed throughout the cortex at embryonic and postnatal stages of cortical development (Guerrier et al. 2009). **(C)** SrGAP2 is present in lysed synaptosomal membrane (LSM) fractions that are enriched for synaptic proteins, in addition to the postnuclear supernatant (PNS), at P18 indicating that srGAP2 is present at the synapse. **(D)** DIV 21 neurons were transfected at DIV18 to express low-levels of the EGFP, srGAP2-EGFP, and truncated form of srGAP2 that lacks the F-BAR domain, Δ F-BAR-EFP. Analysis of the fluorescence in neurons expressing srGAP2-EGFP reveal an enrichment of the protein at dendritic spines, when compared to EFGP expression alone. However, neurons expressing Δ F-BAR-EFP do not show an enrichment of GFP at dendritic spines, suggesting that the F-BAR domain of srGAP2 is important for the synaptic localization of the protein.

To directly examine how srGAP2 affects dendritic spine morphogenesis, subsequent to the early phase of synaptogenesis, we emoployed a long-term culture system. Dissociations of embryonic day (E) 18 cortices were performed to ensure that a sufficient number of astrocytic progenitors were present. Dissociated neurons were then plated in the presence of fetal bovine serum (FBS) for the first seven days, to allow astocytes to proliferate and form a confluent layer over the bottom of the glass coverslips. This glial layer was cultivated and maintained in our cultures because they secrete important synaptogenic factors, like thrombospondin and TNFα, that increase the number and efficacy of synaptic connections made in-*vitro* (Christopherson et al. 2005; Stellwagen and Malenka 2006). At 7 days in-*vitro* (DIV) glial proliferation was arrested (see methods), leaving a confluent layer of astrocytes in direct contact with the dissociated neurons. Cultures were then maintained until 18 DIV, when they were transfected with srGAP2-EGFP fusion proteins. How expression of these proteins influenced dendritic spine morphology was evaluated three days later, at 21DIV.

Expressing srGAP2, following synaptogenesis, causes dendritic spines to adopt an elongated, thin morphology (**Fig 2**). Interestingly, this effect on dendritic morphology can be recapitulated by expressing just the F-BAR domain of srGAP2 (**Fig 2**). A quantitative analysis of spine length and width reveals that srGAP2 and F-BAR expression results in elongated, thin dendritic spines (**Fig 2B**). These observations are entirely consistent with previous findings from our laboratory demonstrating that the F-BAR domain of srGAP2 can induce the formation of membrane protrusions (Guerrier et al. 2009). However, we were initially surprised to find that expression of a mutated form of srGAP2, containing a RhoGAP domain that cannot hydrolyze GTP, results in dendritic spines that have a short and stubby mushroom-like morphology (**Fig 2A-B**). Yet, the RhoGAP domain of srGAP2 is specific for Rac1 (Guerrier et al. 2009) which, in its active GTP-bound form, is considered to promote the formation of mushroom-shaped spines (Luo et al. 1996; Tashiro et al. 2000). Therefore,

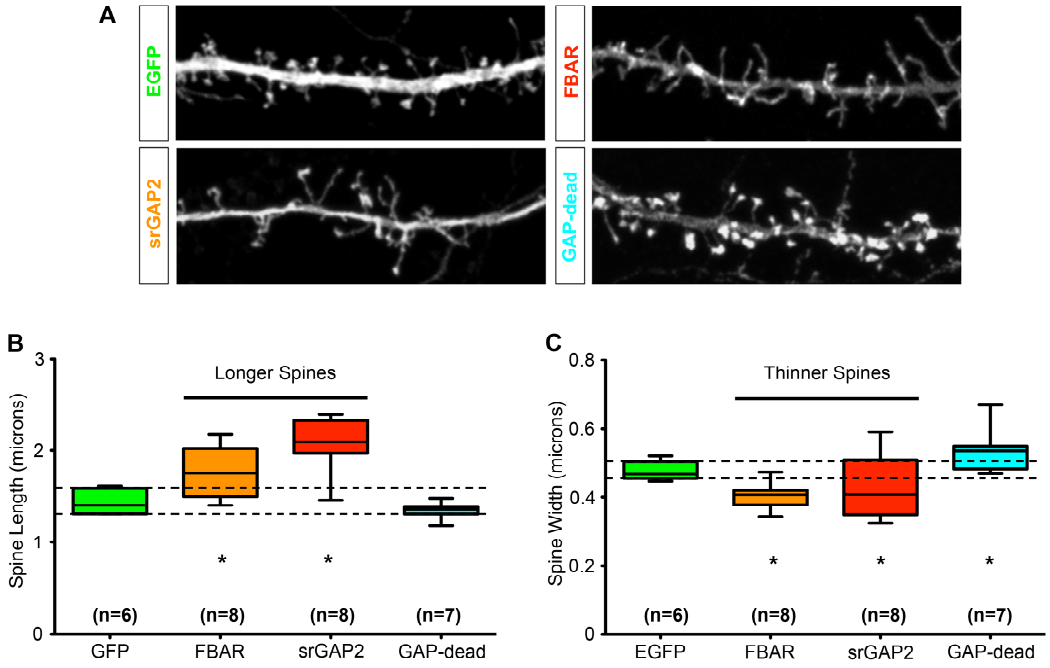

Figure 2: Both the F-BAR and the Rho-GAP domains of srGAP2 function to elongate dendritic spine morphologies. **(A)** Expression of srGAP2 and the F-BAR domain of srGAP2 alone result in elongated dendritic spines. However, expression of a mutated form of srGAP2 which has a RhoGAP domain that cannot hydrolyze GTP (GAP-dead) results in dendritic spines that are more mushroom-like. A quantitative assessment of dendritic spine length **(B)** and width **(C)** demonstrate that overexpression of F-BAR and srGAP2 induces the expression of significantly longer and thinner dendritic spines. Yet the expression of the GAP-dead form of srGAP2 resulted in significantly wider dendritic spines that tended to be shorter in length. The box plots denote data points that fall within the 10^{th}-90^{th} percentile, with the whiskers depicting the minimum and maximum values. The horizontal line in the center of the boxes corresponds to the mean.

when expressing a form of srGAP2 that is incapable of hydrolyzing GTP, there is no way to prevent Rac1 from inducing cytoskeletal rearrangements that promote the formation of mushroom-like spines. Intriguingly, expressing this GAP-dead form of srGAP2 results in a transition to mushoom-like spines even though the F-BAR region of srGAP2, which has an elongating effect, is also expressed as part of the protein. Therefore, the GTP binding activity of srGAP2 may exert a more potent effect on dendritic spine morphology than the

membrane deforming F-BAR domain. Yet, regardless of the relative influence of the two domains on spine morphology, it is clear that both domains act to promote an elongated dendritic spine shape. The F-BAR domain, through its ability to directly bind and deform the membrane, promotes elongation of dendritic spines and the RhoGAP domain, which inhibits the activity of Rac1, actively suppresses the formation of a mushroom-like morphology.

We then turned to assess the physiological role of srGAP2 at the synapse. Using our long-term culture system, we transfected neurons with EGFP, srGAP2-EGFP, and F-BAR-EGFP at 18 DIV. Then, at 21 DIV, we recorded mini excitatory postsynaptic currents (mEPSCs) from fluorescently labeled transfected neurons (**Fig 3**). We took care to backfill each neuron so that we could post-hoc confirm, with immunostaining for the layer-specific marker CTIP2, that each neuron included in our analysis was a L5 pyramidal neuron. While expression of F-BAR and srGAP2 induces the same elongating effect on dendritic spines (**Fig 2B**), their impact on synaptic physiology differs (**Fig 4A-B**). F-BAR expression causes a robust decrease in mEPSC frequency, while expression of srGAP2 has no effect (**Fig 4A**). F-BAR expression also decreases mEPSC amplitude, unlike expression of srGAP2 (**Fig 4B**). Because expression of these constructs does not affect dendritic spine density (**Fig 4C**), these results suggest that srGAP2 influences the efficacy and not the number of glutamatergic synapses. We posit that srGAP2 regulates the expression of postsynaptic AMPA-type receptors, which drive the large fast component of mEPSC currents. When F-BAR is expressed, it acts as a dominant-negative to endogenous srGAP2, thereby inhibiting the ability of srGAP2 to direct AMPARs to the postsynaptic membrane. This is consistent with the function of another BAR superfamily member, IRSp53, in regulating NMDAR-mediated neurotransmission (Kim et al. 2009). These results also imply that while the F-BAR domain alone is capable of inducing morphological changes to dendritic spines, it is not sufficient for regulating the postsynaptic expression of glutamate receptors. Therefore, the

RhoGAP, SH3, or combination of all three domains must be important for carrying out this srGAP2-related process.

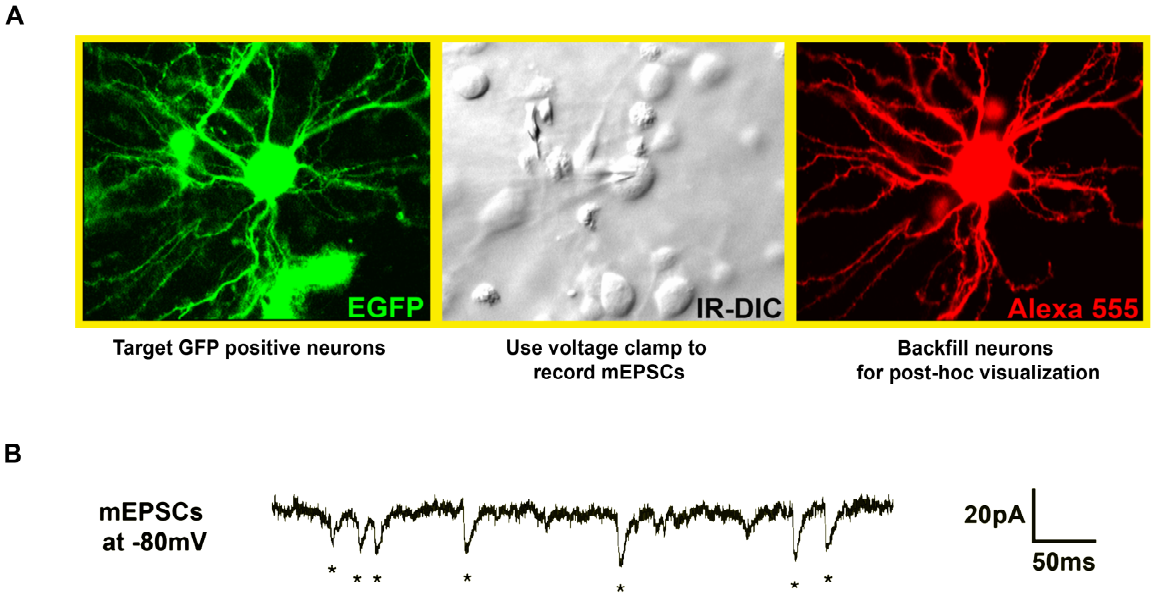

Figure 3: Genetically modified Layer 5 pyramidal neurons can be fluorescently targeted for electrophysiological recordings. **(A)** Cultured cortical neurons were transfected with srGAP2-EGFP fusion constructs at 18 DIV and then recorded from at 21 DIV. Over the course of the electrophysiological recording, neurons were backfilled with Alexa 555 hydrazide so that they could be post-hoc identified. IR-DIC optics were used to position electrodes for obtaining whole-cell recordings. **(B)** Example traces from mEPSC recordings obtained in voltage clamp at -70mV.

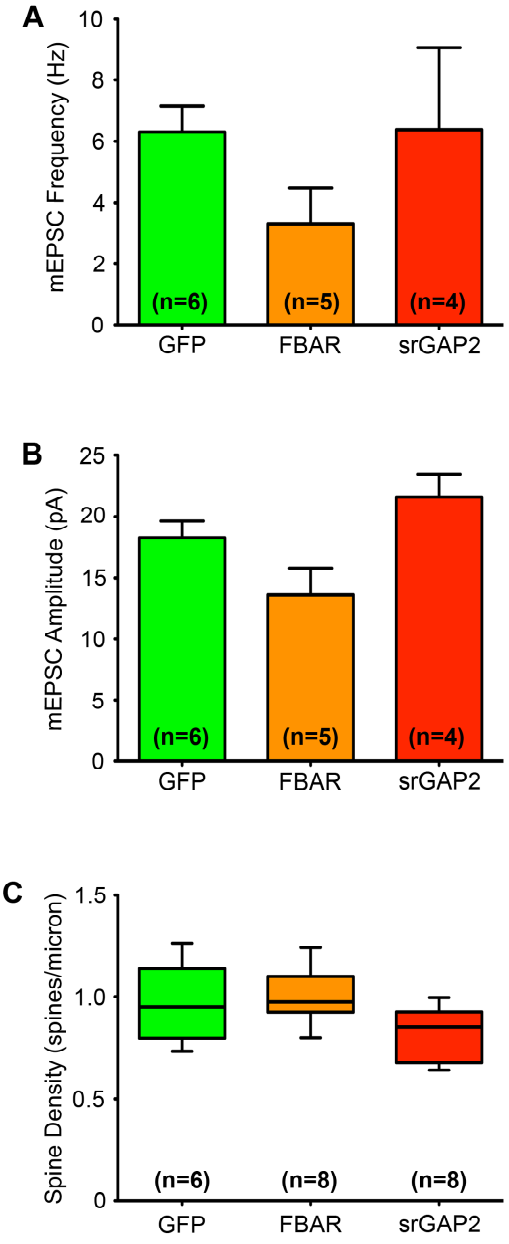

Figure 4: Expressing the F-BAR domain of srGAP2 reduces mEPSC frequency and amplitude **(A)** Expressing the F-BAR domain of srGAP2, but not the full-length protein, results in a decrease in mEPSC frequency. **(B)** The same trend is observed for mEPSC amplitude **(C)** The decrease in mEPSC frequency seen in neurons overexpressing the F-BAR domain cannot be explained by a decrease in the number of dendritic spines. The graphical boxes denote data points that fall between the 10^{th}-90^{th} percentile and the whiskers depict the minimum and maximum values. The horizontal line in the center of the boxes corresponds to the mean.

Further evidence to support the role of srGAP2 in mediating glutamatergic synaptic transmission comes from mEPSC recordings performed in knockout animals. The frequency of mEPSCs in knockout L5 pyramidal neurons is significantly reduced, compared to wildtypes (**Fig 5A**). However, this finding could reflect either a pre-synaptic change in neurotransmitter release or post-synaptic change in synapse number or glutamatergic receptor expression. Yet, a pre-synaptic role for this protein seems unlikely because paired pulse facilitation (PPF), a measure of presynaptic release probability, is unchanged in

knockout animals **(Fig 5C**). Interestingly, unlike cultured neurons expressing the F-BAR domain of srGAP2, knockout neurons do not show a decrease in mEPSC amplitude (**Fig 5B**). Therefore, the observed reduction in mEPSC frequency is either a reflection of (1) a reduced number of synaptic connections or (2) an increased number of "silent" synaptic connections, which do not contain AMPA receptor currents.

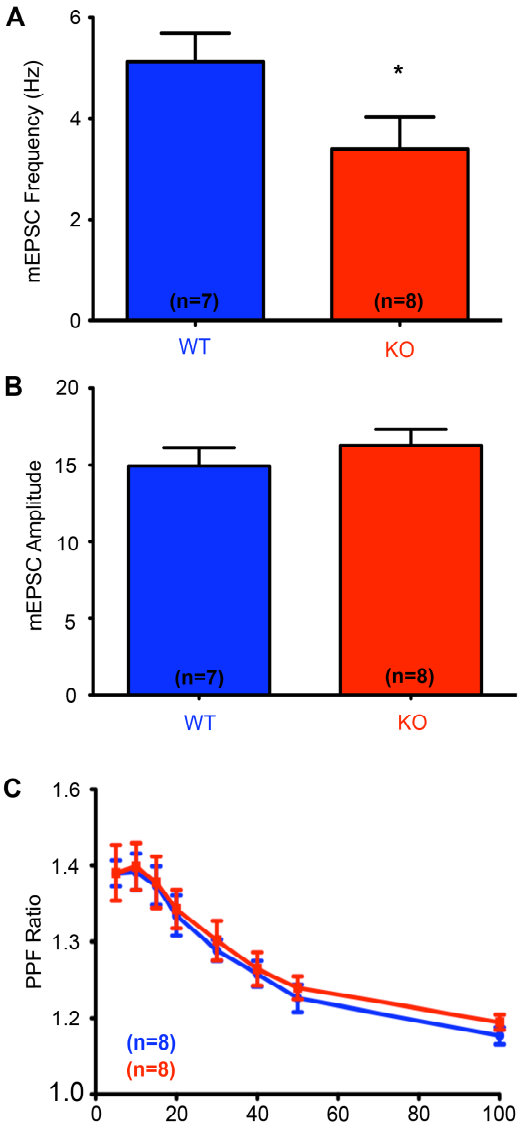

Figure 5: srGAP2 knockout Layer 5 pyramidal neurons exhibit a significant reduction in mEPSC frequency. **(A)** mEPSC frequency but not **(B)** mEPSC amplitude is reduced in srGAP2 knockout neurons. **(C)** However, this change in mEPSC frequency is unlikely to be the result of a presynaptic change in release probability as paired-pulse facilitation (PPF) in CA1 of the hippocampus is not different in srGAP2 knockout animals.

Spine analysis of srGAP2 knockout neurons suggests that the reduction in mEPSC frequency can be explained by an increase in the number of silent synapses. This is because knockout neurons display a robust increase in the number of dendritic spines along

the apical dendrite of L5 pyramidal neurons (**Fig 6A**). This increase in dendritic spine density can also be recapitulated in dissociated cortical knockout neurons (**Fig 6C**). Provocatively, this increase in dendritic spine density appears to be specific to the apical dendrite, as it does not apply to spine density of basal dendrites (**Fig 6B**).

Furthermore, a trend suggesting enhanced LTP in srGAP2 knockout animals (**Fig 7**) provides additional proof that srGAP2 normally limits the expression of silent synapses, which are more easily potentiated due to the complement of postsynaptic receptors they express (Matsuzaki et al. 2004; Sobczyk et al. 2005).

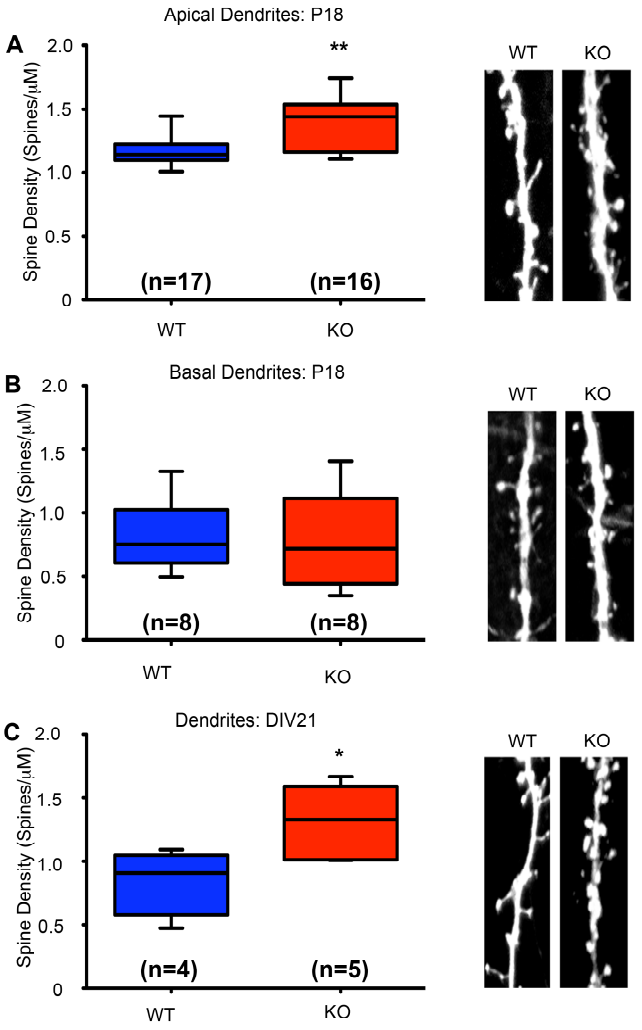

Figure 6: Dendritic spine density is significantly increased along the apical dendrite of srGAP2 knockout animals. **(A)** Dendritic spine density is significantly increased in Layer 5 pyramidal neurons of srGAP2 knockout animals labeled with a Thy1-EFP reporter. **(B)** However, the density of basal dendrites is not different between wild-type and knockout animals. **(C)** The increase in dendritic spine density, seen in the apical dendrite of srGAP2 KO neurons, is recapitulated in cultured 21 DIV neurons. The box plots denote data points that fall within the 10^{th}-90^{th} percentile, with the whiskers depicting the minimum and maximum values. The horizontal line in the center of the boxes corresponds to the mean.

Therefore, we suggest that loss of srGAP2 affects the expression of AMPA receptors at postsynaptic sites. When srGAP2 is functionally knocked-down in cultured neurons by expression of its F-BAR domain, AMPA receptor mediated currents are attenuated and there is a reduction in the number of functional synaptic contacts. These findings are corroborated by a reduction in mEPSC frequency that is observed, despite an increase in dendritic spine density, in srGAP2 knockout animals. Enhanced LTP is a predicted outcome of an increase in silent synapse number because silent synapses contain a higher density of the LTP associated NR2B-containing NMDA receptors (Barria and Malinow 2005; Sobczyk et al. 2005), and are more readily potentiated (Matsuzaki et al. 2004).

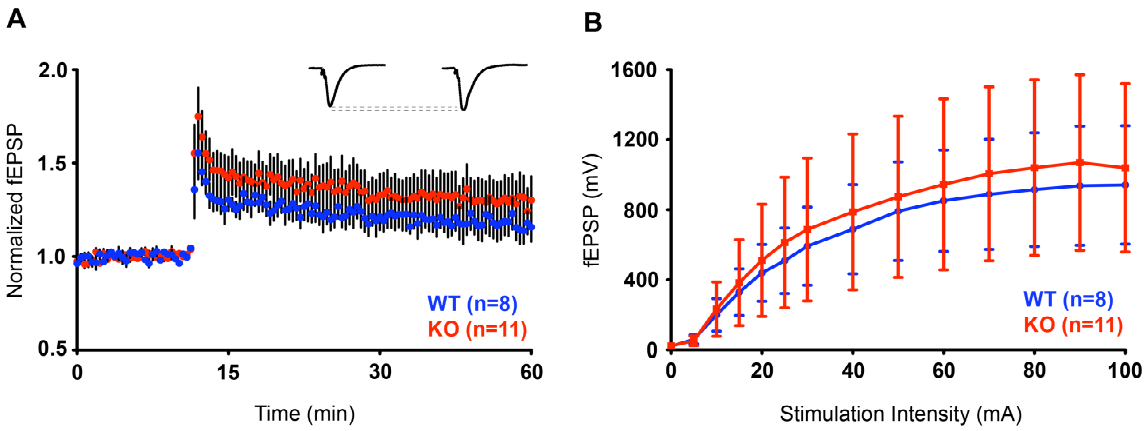

Figure 7: CA1 hippocampal LTP may be augmented in srGAP2 knockout animals. **(A)** The magnitude of LTP induced by a 3 X 100Hz stimulation paradigm is slightly increased in srGAP2 knockout neurons. However, this observation can only be considered a trend, as $p > 0.05$. **(B)** Any difference in the ability to evoke LTP in the hippocampus is not a reflection of changes in synaptic drive onto CA1 pyramidal neurons because there is no difference between wild-type and knockout animals in the relationship between synaptic stimulation intensity and evoked postsynaptic responses.

The observed change in dendritic spine morphology, with srGAP2 knockouts displaying a more elongated shape, does not directly reflect the biological function of its F-BAR and RhoGAP domains on neuronal cell membranes. However, we suggest that the more filopodial-like shape of dendritic spines in srGAP2 knockout animals is not a reflection of the direct role of srGAP2 on membrane dynamics, but rather the result of compensatory changes that occur in response to a change in AMPA receptor expression at the synapse.

SUPPLEMENTARY DATA

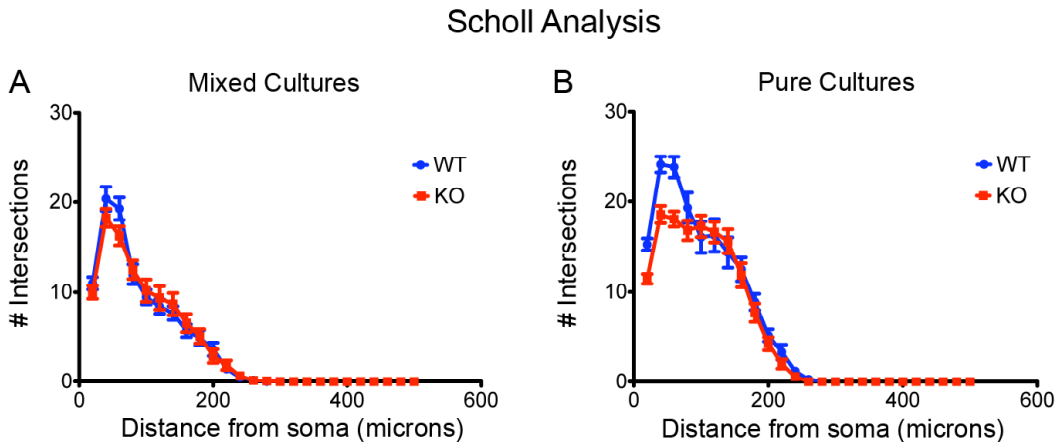

Figure 8: Genetic loss of srGAP2 does not affect dendritic branching patterns in DIV 21 cultures where **(A)** eighty percent of the neurons are wild-type (n=17) and 20 percent are srGAP2 knockout (n=15) or **(B)** all the neurons are either wild-type (n=20) or srGAP2 KO (n=20).

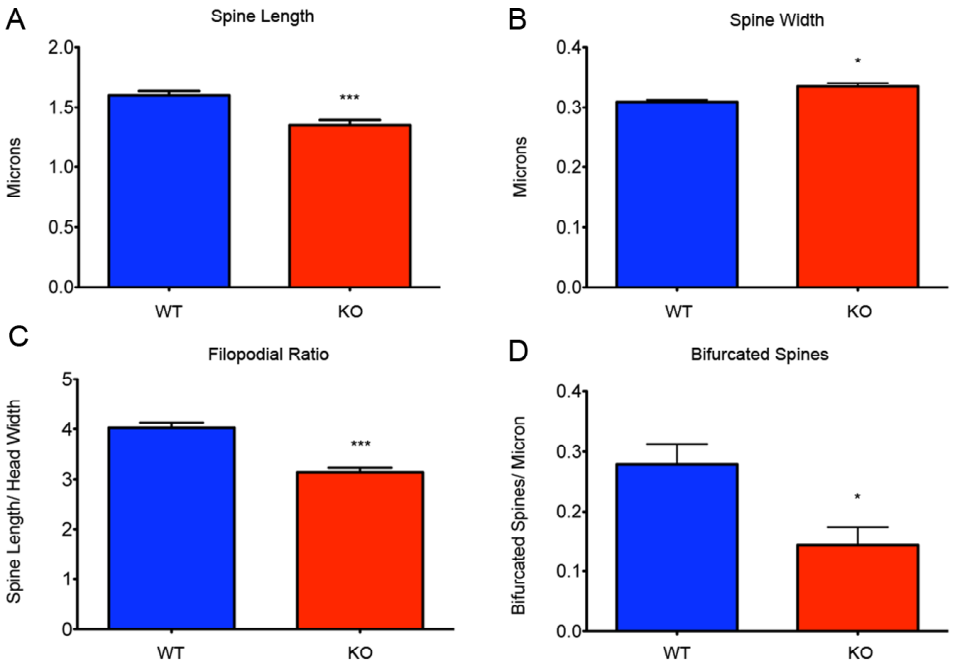

Figure 9: srGAP2 KO neurons are shorter **(A)** and wider **(B)** than WT neurons, making them less filopodial-like at DIV14 **(C)**. There is also a dramatic reduction in the number of bifurcated dendritic spines in srGAP2 KO neurons **(D)**. Combined with live-imaging experiments, this suggests that srGAP2 KO neurons display significantly less protrusive motility (data not shown).

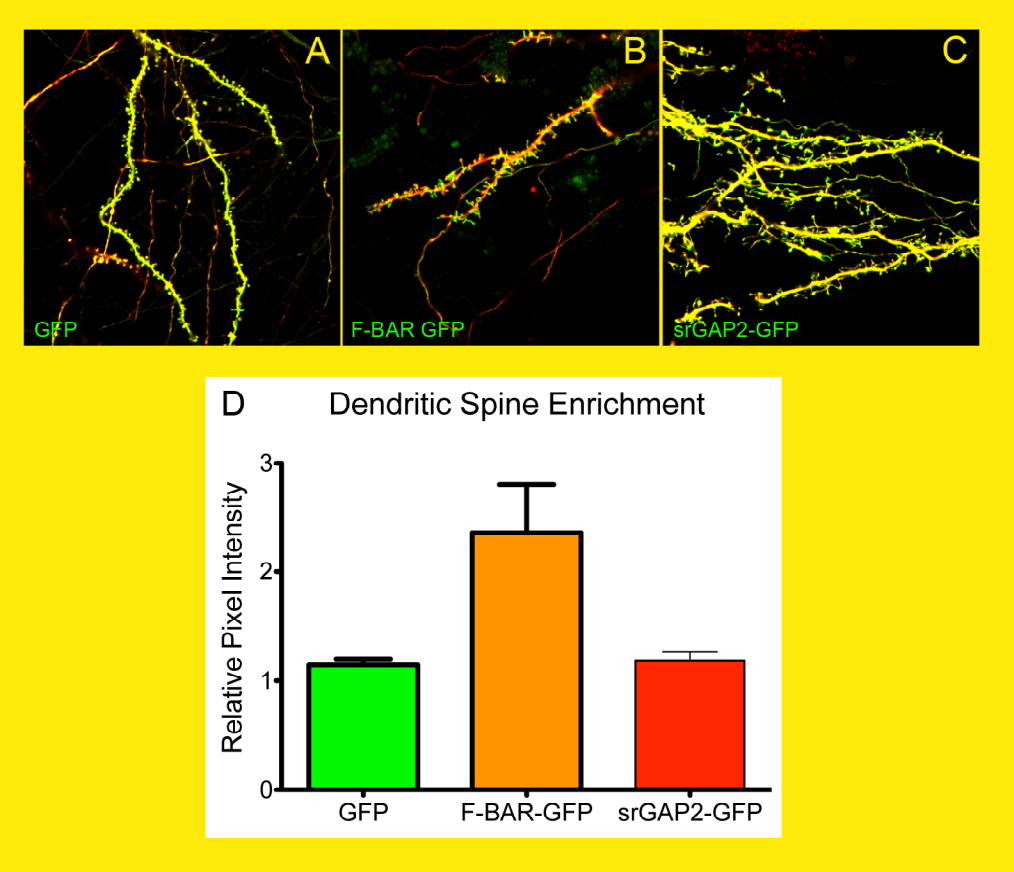

Figure 10: The F-BAR domain of srGAP2 is enriched in dendritic spines. **(A)** GFP, **(B)** F-BAR-GFP, and **(C)** srGAP2-GFP were cotransfected at DIV18 with RFP and imaged three days later, at DIV21. **(D)** While GFP and srGAP2 are present throughout the dendritic shaft and spine, the F-BAR domain is significantly enriched in dendritic spines.

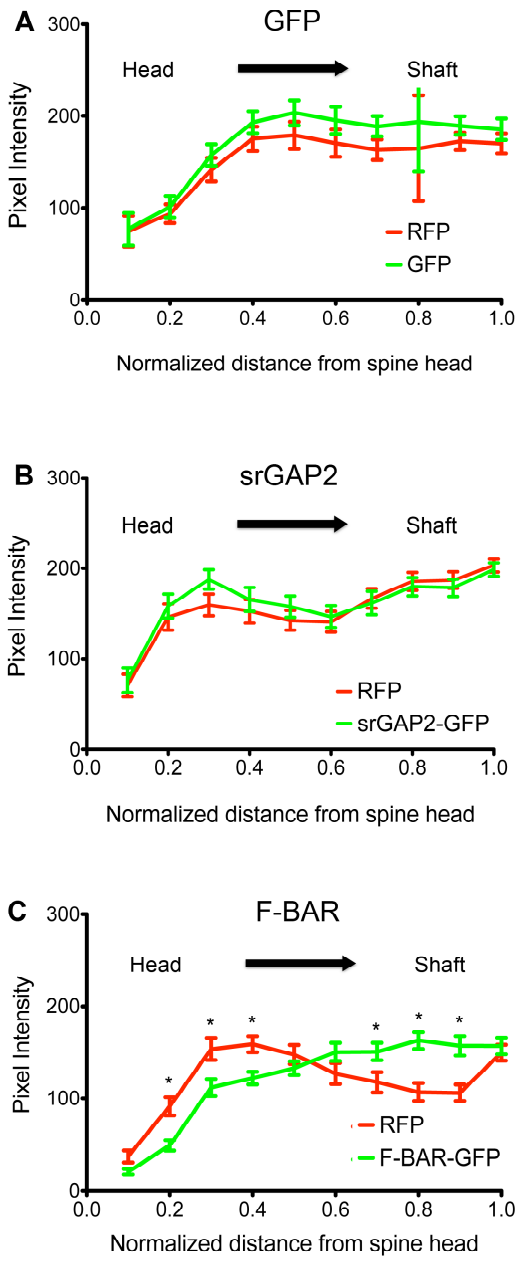

Figure 11: The F-BAR domain of srGAP2 is specifically enriched along the dendritic spine shaft, relative to the dendritic spine head. **(A)** GFP and **(B)** srGAP2-GFP are localized to the dendritc spine head and shaft, equally. **(C)** However, F-BAR GFP is relatively absent from dendritc spine heads, while it is enriched along the shaft of dendritic spines.

DISCUSSION

Membrane associated proteins that interact with the cytoskeleton, have been shown to play an important role in shaping the morphology of actin-enriched dendritic spines (Govek et al. 2004; Choi et al. 2005). Here we provide evidence that srGAP2 is enriched at the synapse, through the membrane binding activity of its F-BAR domain. Additionally, we demonstrate that the F-BAR and the RhoGAP domains of srGAP2 act in concert, to promote elongated dendritic spine morphologies. Our data also suggests that, similar to other BAR family proteins (Khelfaoui et al. 2009; Kim et al. 2009), srGAP2 modulates glutamatergic synaptic transmission, in a postsynaptic manner.

However, it is surprising that both overexpression of srGAP2 and genetic loss of srGAP2 induce lengthening of dendritic spines. We suggest that this reveals two overlapping functions of srGAP2 at dendritic spines: (1) srGAP2 can directly modulate dendritic spine morphology through the activity of its F-BAR and RhoGAP domains and (2) srGAP2 can indirectly modulate dendritic spine morphology by regulating the localization of AMPA receptors at the postsynaptic membrane. If srGAP2 acts to positively regulate AMPA receptors at the cell surface, it would increase the size of the postsynaptic density, which would in-turn produce more mushroom-like dendritic spines.

Strong evidence in support of srGAP2 regulating AMPA receptors at the postsynaptic membrane comes from electrophysiological mEPSC recordings performed in L5 pyramidal neurons of srGAP2 knockouts. Despite the fact that these neurons have an increased number of dendritic spines along their apical dendrite, they exhibit reduced mEPSC frequency. This suggests that there is an increased number of “silent synapses,” which contain NMDA-type glutamate receptors but not AMPA-type glutamate receptors (Isaac et al. 1999).

srGAP2 may keep AMPA receptors at the cell surface by either mediating the exocytosis of AMPA receptor vesicles or by actively antagonizing the endocytosis of AMPA

receptors at the cell surface. While membrane invagination has been shown to be critical for exocytosis (Hui et al. 2009), the role of proteins that induce membrane protrusions—like srGAP2— remains unexplored. However, it has recently been shown in drosophila that an I-BAR containing protein actively inhibits endocytosis of cell surface receptors (Quinones et al.). Therefore, it is possible that srGAP2 similarly inhibits the endocytosis of AMPA receptors from the postsynaptic membrane. However, in its absence, there is less inhibition of AMPA receptor endocytosis, which results in a decreased number of AMPA receptors at the postsynaptic membrane.

Because expression of the F-BAR domain causes a reduction in mEPSC frequency that is similar to what is seen in srGAP2 knockout animals, we propose that the F-BAR domain can act as a dominant-negative to endogenous srGAP2 function. This would suggest that the non-FBAR component of the protein is critical for regulating the presence of AMPA receptors at the postsynaptic membrane. While the F-BAR domain of srGAP2 appears to be required for the synaptic localization of srGAP2, the RhoGAP and SH3 domain are required for stabilizing AMPA receptors at the postsynaptic membrane.

Our electrophysiology and morphological data also suggest that srGAP2 is acting upon a discrete subset of synaptic connections onto L5 pyramidal neurons. First, the loss of srGAP2 results in an increase in spine density that is restricted to the apical dendrite. Similar observations are not found along the basal dendrites. Furthermore, our electrophysiological recordings reveal a decrease in mEPSC frequency without a concomitant decrease in mEPSC amplitude. This suggests that srGAP2 acts to modulate AMPA receptors at a subset of synapses. If srGAP2 were globally acting to modulate AMPA receptors at all synapses there would have been a decrease in mEPSC amplitude, which is a reflection of the density of AMPA receptors at the postsynaptic membrane.

While we provide evidence that srGAP2 regulates glutamatergic synaptic transmission, other BAR family proteins with RhoGAP domains have been similarly

implicated in this process. Oligophrenin1, a BAR containing protein with a RhoGAP domain that is specific for RhoA, has been shown to play a direct role in the internalization of AMPA receptors (Khelfaoui et al. 2009). Additionally, the IBAR containing protein IRSp53, has been shown to modulate the composition of postsynaptic receptors at glutamatergic synapses (Sawallisch et al. 2009). IRSp53 kockouts have augmented NMDA receptor mediated currents and display enhanced LTP (Kim et al. 2009).

In summary, we provide evidence that srGAP2 modulates glutamatergic synaptic transmission. The F-BAR domain of srGAP2 is required for its synaptic localization and both the F-BAR and RhoGAP domains of srGAP2 are important in promoting an elongated filopodial-like dendritic spine morphology. The full-length protein plays an important role in regulating postsynaptic AMPA receptors, with the expression of the F-BAR domain alone acting as dominant-negative.

`

BIBLIOGRAPHY

Bacon C, Endris V, Rappold G (2009) Dynamic expression of the Slit-Robo GTPase activating protein genes during development of the murine nervous system. J Comp Neurol 513(2): 224-236.

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48(2): 289-301.

Billuart P, Bienvenu T, Ronce N, des Portes V, Vinet MC et al. (1998) Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. Nature 392(6679): 923-926.

Bonhoeffer T, Yuste R (2002) Spine motility. Phenomenology, mechanisms, and function. Neuron 35(6): 1019-1027.

Chelly J, Mandel JL (2001) Monogenic causes of X-linked mental retardation. Nat Rev Genet 2(9): 669-680.

Choi J, Ko J, Racz B, Burette A, Lee JR et al. (2005) Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. J Neurosci 25(4): 869-879.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW et al. (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120(3): 421-433.

Dailey ME, Smith SJ (1996) The dynamics of dendritic structure in developing hippocampal slices. J Neurosci 16(9): 2983-2994.

Endris V, Wogatzky B, Leimer U, Bartsch D, Zatyka M et al. (2002) The novel Rho-GTPase activating gene MEGAP/ srGAP3 has a putative role in severe mental retardation. Proc Natl Acad Sci U S A 99(18): 11754-11759.

Engert F, Bonhoeffer T (1999) Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature 399(6731): 66-70.

Fauchereau F, Herbrand U, Chafey P, Eberth A, Koulakoff A et al. (2003) The RhoGAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain. Mol Cell Neurosci 23(4): 574-586.

Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. Neuron 20(5): 847-854.

Govek EE, Newey SE, Akerman CJ, Cross JR, Van der Veken L et al. (2004) The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. Nat Neurosci 7(4): 364-372.

Gray EG (1959) Electron microscopy of synaptic contacts on dendrite spines of the cerebral cortex. Nature 183(4675): 1592-1593.

Guerrier S, Coutinho-Budd J, Sassa T, Gresset A, Jordan NV et al. (2009) The F-BAR domain of srGAP2 induces membrane protrusions required for neuronal migration and morphogenesis. Cell 138(5): 990-1004.

Hofer SB, Mrsic-Flogel TD, Bonhoeffer T, Hubener M (2009) Experience leaves a lasting structural trace in cortical circuits. Nature 457(7227): 313-317.

Hori K, Yasuda H, Konno D, Maruoka H, Tsumoto T et al. (2005) NMDA receptor-dependent synaptic translocation of insulin receptor substrate p53 via protein kinase C signaling. J Neurosci 25(10): 2670-2681.

Hui E, Johnson CP, Yao J, Dunning FM, Chapman ER (2009) Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion. Cell 138(4): 709-721.

Isaac JT, Nicoll RA, Malenka RC (1999) Silent glutamatergic synapses in the mammalian brain. Can J Physiol Pharmacol 77(9): 735-737.

Itoh T, De Camilli P (2006) BAR, F-BAR (EFC) and ENTH/ANTH domains in the regulation of membrane-cytosol interfaces and membrane curvature. Biochim Biophys Acta 1761(8): 897-912.

Khelfaoui M, Pavlowsky A, Powell AD, Valnegri P, Cheong KW et al. (2009) Inhibition of RhoA pathway rescues the endocytosis defects in Oligophrenin1 mouse model of mental retardation. Hum Mol Genet 18(14): 2575-2583.

Khelfaoui M, Denis C, van Galen E, de Bock F, Schmitt A et al. (2007) Loss of X-linked mental retardation gene oligophrenin1 in mice impairs spatial memory and leads to ventricular enlargement and dendritic spine immaturity. J Neurosci 27(35): 9439-9450.

Kim MH, Choi J, Yang J, Chung W, Kim JH et al. (2009) Enhanced NMDA receptor-mediated synaptic transmission, enhanced long-term potentiation, and impaired learning and memory in mice lacking IRSp53. J Neurosci 29(5): 1586-1595.

Lendvai B, Stern EA, Chen B, Svoboda K (2000) Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. Nature 404(6780): 876-881.

Luo L, Hensch TK, Ackerman L, Barbel S, Jan LY et al. (1996) Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature 379(6568): 837-840.

Masuda M, Mochizuki N Structural characteristics of BAR domain superfamily to sculpt the membrane. Semin Cell Dev Biol 21(4): 391-398.

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H (2004) Structural basis of long-term potentiation in single dendritic spines. Nature 429(6993): 761-766.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M et al. (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 4(11): 1086-1092.

Mattar P, Britz O, Johannes C, Nieto M, Ma L et al. (2004) A screen for downstream effectors of Neurogenin2 in the embryonic neocortex. Dev Biol 273(2): 373-389.

Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K (1982) High actin concentrations in brain dendritic spines and postsynaptic densities. Proc Natl Acad Sci U S A 79(23): 7590-7594.

McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 438(7068): 590-596.

Parnavelas JG, Sullivan K, Lieberman AR, Webster KE (1977) Neurons and their synaptic organization in the visual cortex of the rat. Electron microscopy of Golgi preparations. Cell Tissue Res 183(4): 499-517.

Peters A, Kaiserman-Abramof IR (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am J Anat 127(4): 321-355.

Quinones GA, Jin J, Oro AE I-BAR protein antagonism of endocytosis mediates directional sensing during guided cell migration. J Cell Biol 189(2): 353-367.

Ramakers GJ (2002) Rho proteins, mental retardation and the cellular basis of cognition. Trends Neurosci 25(4): 191-199.

Sawallisch C, Berhorster K, Disanza A, Mantoani S, Kintscher M et al. (2009) The insulin receptor substrate of 53 kDa (IRSp53) limits hippocampal synaptic plasticity. J Biol Chem 284(14): 9225-9236.

Scita G, Confalonieri S, Lappalainen P, Suetsugu S (2008) IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions. Trends Cell Biol 18(2): 52-60.

Shimada A, Takano K, Shirouzu M, Hanawa-Suetsugu K, Terada T et al. Mapping of the basic amino-acid residues responsible for tubulation and cellular protrusion by the EFC/F-BAR domain of pacsin2/Syndapin II. FEBS Lett 584(6): 1111-1118.

Sobczyk A, Scheuss V, Svoboda K (2005) NMDA receptor subunit-dependent [Ca2+] signaling in individual hippocampal dendritic spines. J Neurosci 25(26): 6037-6046.

Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440(7087): 1054-1059.

Tashiro A, Minden A, Yuste R (2000) Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and Rho. Cereb Cortex 10(10): 927-938.

Yao Q, Jin WL, Wang Y, Ju G (2008) Regulated shuttling of Slit-Robo-GTPase activating proteins between nucleus and cytoplasm during brain development. Cell Mol Neurobiol 28(2): 205-221.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu Rev Neurosci 24: 1071-1089.

Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: insights from ultrastructural and imaging studies. Nat Rev Neurosci 5(1): 24-34.

Zhang B, Zelhof AC (2002) Amphiphysins: raising the BAR for synaptic vesicle recycling and membrane dynamics. Bin-Amphiphysin-Rvsp. Traffic 3(7): 452-460.

Zimmerberg J, Kozlov MM (2006) How proteins produce cellular membrane curvature. Nat Rev Mol Cell Biol 7(1): 9-19.

Ziv NE, Smith SJ (1996) Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. Neuron 17(1): 91-102.

`

CHAPTER FOUR

DISCUSSION

This thesis addresses how postsynaptic modifications influence synaptic plasticity and neurotransmission. A broad repertoire of approaches was used to address this complex topic. Consideration was given to both the complement of receptors expressed at the postsynaptic membrane and to the morphology of postsynaptic specializations. This work (1) provides evidence for a developmental change in the mechanisms underlying LTP in the primary visual cortex (2) elucidates a role for the BAR superfamily protein, srGAP2, in modulating dendritic spine shape and glutamatergic neurotransmission.

Developmental changes in LTP

Chapter Two of this thesis describes how LTP is differentially sensitive to NMDAR antagonism at different developmental stages. We provide evidence to support that a higher degree of NMDAR activation is required to induce LTP at later developmental time points, when the relative amount of NR2B-containing NMDARs at the synapse is reduced. However, we did not directly test whether the developmental upregulation of NR2A-containing NMDA receptors was required to shift the degree of NMDA receptor activation needed for LTP. Performing similar experiments in NR2A knockout animals would be helpful in determining the requirement of NR2A for this developmental transition.

Recent evidence also suggests that the underlying mechanism for inducing LTP becomes less reliant on NR2B-containing NMDA receptors (Foster et al.). Similar to the

results described in Chapter Two, LTP in organotypic hippocampal cultures goes from being completely abolished by the NR2B specific antagonist Ro-25-6981, to far less sensitive at later developmental stages. This implies that the current going through NR2B-containing NMDA receptors is less important for the expression of LTP at later developmental timepoints. Interestingly, this group found that the contribution of the NR2B subunit to LTP was not related to the current kinetics conferred by the NR2B subunit. They find that chimeric proteins consisting of the NR2A channel attached to the NR2B intracellular domain, evokes wild-type levels of plasticity in organotypic hippocampal cultures. This, combined with other findings implicating the importance of the intracellular domain of the NR2B subunit in evoking LTP (Barria and Malinow 2005), suggest that the current kinetics of NR2A- and NR2B-containing NMDARs are not the determining factors in modulating plasticity. However, the ability of NR2B to directly interact with CamKII (Strack and Colbran 1998) is likely to be the most important factor affecting LTP. Yet, the fact that NR2B-containing NMDARs constitute a smaller proportion of NMDA receptors in mature synapses is likely to have important implications.

We suggest that the upregulation of NR2A is important for modulating the ability of synaptic connections to undergo plasticity, in a manner that corresponds to its history of neural activity (Philpot et al. 2007). Because NR2A is upregulated in an activity-dependent fashion (Quinlan et al. 1999), it is likely that its expression at the synapse hinders synaptic connections from undergoing further potentiation. This concept is known as metaplasticity, and the importance of NR2A-containing NMDA receptors in this process has been well established.

However, there are many developmental changes that occur alongside the shift in NMDA receptor subunit composition. The contribution of more developed inhibitory GABAergic circuitry, which is developing over the same developmental timeframe (Jiang et al. 2005), is not explored in this body of work. Although we show that that our ability to drive

synaptic connections between L4 and L2/3 remains the same at both young and mature time points, this work does not directly assess the contribution of developing GABAergic synaptic connections to the expression of LTP.

srGAP2 at the synapse

While Chapter Three begins to describe the role of srGAP2 in modulating dendritic spine morphology and glutamatergic synaptic transmission, several experiments can be done to increase our understanding of this important protein.

(1) Does srGAP2 play a role in synaptogenesis?

Because srGAP2 has been shown to play an important role in inducing neuronal membrane protrusions, it may play a role in extending filopodia that are important for neurons during synaptogenesis. Live imaging of wildtype and knockout neurons in dissociated cultures, during synaptogenesis, will provide insight into the role of srGAP2 in this dynamic process. Also, using our EGFP fusion proteins, we can dissect the function of the F-BAR and RhoGAP domain of srGAP2 in modulating the dynamics of filopodial-like protrusions that play an important role in synapse formation.

(2) Is srGAP2 recruited to the synapse in an activity-dependent manner?

While similar proteins, like oligophrenin1, have been shown to be recruited to the synapse in response to NMDA receptor activation, it is unknown whether the localization of srGAP2 is affected by neuronal activity. The action that srGAP2 has upon the membrane, both directly through the action of its F-BAR domain and indirectly through its RhoGAP domain, could be important in mediating activity-dependent changes in dendritic spine morphology that accompany synaptic plasticity. By applying TTX, KCl, NMDA, and AMPA to dissociated cultures and then performing synaptosomal membrane

preparations, the activity dependent recruitment of srGAP2 to the synapse can be determined.

(3) While Chapter Three provides evidence that genetic loss of srGAP2 upregulates the prevalence of silent synapses, this needs to be directly tested.

Although srGAP2 knockouts exhibit a reduced mEPSC frequency despite an increase in apical dendritic spine density, we have not directly assessed the presence of silent synapses. To do this, we could determine the AMPA/NMDA ratios in wild-type and srGAP2 knockout animals. If srGAP2 knockouts have more silent synapses they should have enhanced NMDA receptor currents, corresponding to a decrease in the AMPA/NMDA ratio.

(4) Does genetic loss of srGAP2 influence endocytosis?

Although overexpression of the FBAR domain of srGAP2 does not completely inhibit endocytosis in cell lines (Guerrier et al. 2009), some evidence suggests that BAR family proteins that induce membrane protrusions can actively inhibit endocytosis (Quinones et al.). Additionally, BAR family proteins oligophrenin1 and IRSp53 play an important role in modulating the surface expression of AMPA and NMDA receptors, respectively. Furthermore, our own data suggests that srGAP2 plays a role in keeping AMPA receptors at the synapse (inhibiting the expression of silent-synapses) and it may be functioning in this manner by actively preventing the internalization of AMPA receptors. Therefore, it is important to assess the role of srGAP2 in endocytosis by performing transferring uptake assays in neuronal cultures and also by specifically examining AMPA receptor internalization.

(5) If srGAP2 acts to prevent AMPA receptor internalization, does it negatively regulate LTD?

If srGAP2 plays a role in antagonizing the internalization of AMPA receptors, it is reasonable to suspect that it may inhibit the expression of LTD.

(6) Is the function of srGAP2 cell-autonomous?

While srGAP2 knockouts display an increased number of dendritic spines along the apical dendrite and a reduced mEPSC frequency, it is unclear whether this is a reflection of a cell-autonomous (postsynaptic) function of the protein. We are currently performing "mixed" culture experiments, where 10% of the neurons are GFP labeled knockout neurons and the remaining 90% are RFP labeled wild-type neurons. Examining dendritic spine density and morphology in knockout neurons that have been placed in a predominately wild-type context will provide insight into the protein's cell autonomous function.

(7) Does srGAP2 play an important role in modulating presynaptic release onto L5 pyramidal neurons?

While PPF experiments have been performed in CA1 of the hippocampus, single cell recordings examining PPF in L5 pyrmaidal neurons are necessary. These experiments would definitively demonstrate that srAP2 does not play a role in the exocytosis of presynaptic glutamatergic vesicles.

(8) Does srGAP2 modulate dendritic spine morphology by modulating the activity of Rac1?

While previous work from our laboratory suggests that the RhoGAP domain of srGAP2 is specific for Rac1 (Guerrier et al. 2009), we do not provide direct evidence to demonstrate this specificity in dendritic spines. To more convincingly show an interaction between srGAP2 and Rac1, in the context of dendritic spine morphogenesis, we should co-transfect the GAP-dead form of srGAP2 and a dominant-negative form of Rac1. If this ameliorates the effect of the GAP-dead form of srGAP2 on dendritic spine morphology, this would provide further evidence that Rac1 is downstream of srGAP2.

`

BIBLIOGRAPHY

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48(2): 289-301.

Foster KA, McLaughlin N, Edbauer D, Phillips M, Bolton A et al. Distinct roles of NR2A and NR2B cytoplasmic tails in long-term potentiation. J Neurosci 30(7): 2676-2685.

Guerrier S, Coutinho-Budd J, Sassa T, Gresset A, Jordan NV et al. (2009) The F-BAR domain of srGAP2 induces membrane protrusions required for neuronal migration and morphogenesis. Cell 138(5): 990-1004.

Jiang B, Huang ZJ, Morales B, Kirkwood A (2005) Maturation of GABAergic transmission and the timing of plasticity in visual cortex. Brain Res Brain Res Rev 50(1): 126-133.

Philpot BD, Cho KK, Bear MF (2007) Obligatory role of NR2A for metaplasticity in visual cortex. Neuron 53(4): 495-502.

Quinlan EM, Philpot BD, Huganir RL, Bear MF (1999) Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat Neurosci 2(4): 352-357.

Quinones GA, Jin J, Oro AE I-BAR protein antagonism of endocytosis mediates directional sensing during guided cell migration. J Cell Biol 189(2): 353-367.

Strack S, Colbran RJ (1998) Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor. J Biol Chem 273(33): 20689-20692.