DEVELOPMENT OF NEOCORTICAL CIRCUITS: A CELL AUTONOMOUS EXAMINATION OF MGLUR5 AND MEF2C

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DEDICATION

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DEVELOPMENT OF NEOCORTICAL CIRCUITS: A CELL AUTONOMOUS EXAMINATION OF MGLUR5 AND MEF2C

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Development of neocortical circuits requires both genetic programs and sensory experience-dependent modification of synaptic function. The rules that dictate how synapses develop and respond to changing patterns of input influence both the emergence of receptive fields and the capacity for learning. In turn, the factors that determine the rules for synaptic plasticity are defined by the proteins functioning at the synapse. This project investigates two proteins situated to have wide-reaching impacts on synaptic function. One of the challenges in detailing the roles a protein plays in regulating synapses is discerning not only

its acute role on synaptic function, but also its long-term impact on circuit development. Therefore, studying how a protein is engaged by physiological patterns of input *in vivo* over an extended period of time will provide a broader picture of how it influences synaptic function and circuit development.

mGluR5 has previously been implicated in several forms of plasticity that act to directly weaken synaptic function. In this document, I provide evidence that the net-effect of mGluR5 on synaptic function throughout the first few weeks of postnatal development is to promote synaptic input pathway strength, as demonstrated in 2 prominent and well-characterized input pathways to L2/3 pyramidal cells of barrel cortex. Furthermore, I demonstrate a possible role for mGluR5 in a homeostatic mechanism, offsetting the enhanced evoked synaptic input by suppressing both spontaneous transmission and intrinsic excitability.

The transcription factor MEF2 also has established roles in regulating synaptic function. However, much less is known about the synaptic mechanisms through which MEF2 mediates its effects. Here, I implicate MEF2C as the critical MEF2 family member involved in regulating synaptic function in L2/3 pyramidal cells in barrel cortex, and provide potential synaptic and molecular mechanisms by which MEF2C regulates pathway input.

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ADP - Afterdepolarization

AHP - Afterhyperpolarization

AMPAR – α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Arc – Activity-regulated cytoskeleton-associated protein

BDNF – Brain-derived neurotrophic factor

BMI – Bicuculline methiodide

CA – Cornu Ammonis

CB_1R – Cannabinoid receptor type 1

CNS – Central nervous system

CPP - (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid

DAG - Diacylglycerol

DHPG – Dihydroxyphenylglycine

DNQX - Dinitroquinoxaline-2,3-dione

eCB – Endocannabinoid

EMX1 - Empty spiracles homeobox 1

EPSC/P – Excitatory postsynaptic current/potential

ERK 1/2 - Extracellular signal-regulated kinase 1/2

IPSC/P – Inhibitory postsynaptic current/potential

FMRP – Fragile-X mental retardation protein

FXS – Fragile-X Syndrome

GABAR - Gamma-aminobutyric acid receptor

GFP – Green fluorescence protein

GPCR – G-protein coupled receptors

HFS – High frequency stimulation

iLTD – LTD of inhibition

IP3 - Inositol triphosphate

L2/3 – Cortical layer 2/3

L4 – Cortical layer 4

L5 – Cortical layer 5

LFS – Low frequency stimulation

LGN – Lateral geniculate nucleus

LSPS – Laser-scanning photostimulation

LTD – Long-term depression

LTP – Long-term potentiation

KO – Knockout

MADS domain - MCM1, Agamous, Deficiens, and Serum response factor

MEF – Myocyte enhancement factor

mEPSC – Miniature excitatory postsynaptic current

mIPSC – Miniature inhibitory postsynaptic current

mGluR – Metabotropic glutamate receptor

MPEP - 2-Methyl-6-(phenylethynyl) pyridine

MRE – MEF2 resposne element

mRNA – Messenger ribonucleic acid

MSN – Medium spiny neuron

mTOR – Mammalian target of rapamycin

NAc – Nucleus accumbens

NAM – Negative allosteric modulator

NMDAR – *N*-Methyl-D-aspartate receptor

PFC – Prefrontal cortex

PI - Phosphoinositide

PIKE - PI 3-kinase enhancer

PIP_2 - Phosphatidylinositol 4,5-bisphosphate

PKC – Protein kinase C

PLC – Phospholipase C

PP-LFS – Paired-pulse low frequency stimulation

PSD – Postsynaptic density

RT-PCR – Reverse transcription polymerase chain reaction

shRNA – Short-hairpin RNA

SK channel – Small conductance calcium-activated potassium channel

STDP – Spike-timing dependent plasticity

SWD – Spike-wave discharge

TBS – Theta burst stimulation

tLTD – Timing-dependent LTD

tLTP – Timing-dependent LTP

TrkB - Tropomyosin related kinase B

UV – Ultraviolet

UT - Untransfected

WT - Wildtype

CHAPTER ONE
Introduction

NEOCORTICAL CIRCUIT FORMATION AND SYNAPSE DEVELOPMENT

Information processing and neural circuits

The endeavor of understanding thought and memory on a biological level has been an ongoing pursuit of philosophers and scientists dating back millennia. Over time, the individual pursuits of understanding memory and thought have been conflated to a single biological phenomenon represented by the intricate process of disassembling and reassembling information, and today this question can be framed in terms of how the brain functions to process information. In this context, we can approach this problem with specific questions designed to understand information processing on multiple levels, ranging from molecular to cellular to circuit level. From a historical viewpoint, the smallest unit of information processing was considered to be the neurons themselves. However, we now know that it is the configuration and strength of connections between neurons, and by extension the proteins and molecules that influence these connections, which ultimately dictate how information is processed. Therefore, a multi-level approach is necessary to understand the interplay between the molecular forces involved in this process and the dynamic circuitry of the brain.

Information input signals coming into any given brain region can originate both internally, such as in decision making and emotion processing, and externally, such as in the case of sensory perception. By virtue of their intrinsic nature, internally generated input

signals are largely inaccessible to experimental manipulation, whereas externally generated input signals can be precisely regulated and manipulated. Indeed, much of the initial characterization of neural circuits and network organization took place in brain regions involved in sensory processing (Hubel & Wiesel 1959, Mountcastle 1957), and these structures continue to be powerful tools for researchers investigating how information is processed in the brain. Although information signals generated by sensory stimuli are relayed through several brain regions, much of the higher-order processing takes place in neocortical structures. This is in part due to the increased physical space allocated to neocortex which covers the entire cerebral cortex, allowing for more processing units at multiple levels (i.e. synapses and neurons). This enhanced potential for connectivity is not limited to the circuitry within a given neocortical region, but also allows for extensive communication with other brain regions. This high degree of integrative communication results in information being processed in the context of other internally and externally generated information, which ultimately manifests as the complete experience we perceive. Therefore, understanding how perception is constructed begins with understanding the basic unit of neural circuitry, which is the regulation of those synapses within the circuit.

Synapse Dynamics: Formation, Elimination, and Maintenance

One of the most fascinating and unique properties of the brain is its capacity to reorganize the strength and configuration of cell-to-cell connections in response to incoming information. Synaptic plasticity is thought to underlie the functional rewiring of cortical circuits that takes place during development (Holtmaat & Svoboda 2009, Katz & Shatz

1996). The primary locus of this plasticity is at the synapse itself and can be expressed as either a binary (addition/elimination of a connection) or analog (strength change) mechanism (Chklovskii et al 2004). Information processing and memory storage is thought to be encoded through both of the mechanisms. Therefore, factors that regulate synaptic function and gate its capacity to change are a critical component to neural circuitry.

Interestingly, the rules that govern synaptic plasticity are highly dependent upon a number of factors including brain region, cell type, pattern of input activity, and neurotransmission system(s) involved. Furthermore, there is a strong developmental component to synapse regulation. For instance, rules for the initiation of many forms of synaptic plasticity change throughout development, such as in the case of spike-timing dependent plasticity (STDP) at the L4→L2/3 synapse in barrel cortex (Itami & Kimura 2012). Other forms of synaptic plasticity exist only in discrete developmental windows, such as in the case of endocannabinoid-mediated heterosynaptic long-term depression (hetero-LTD) in the hippocampus (Yasuda et al 2008) and visual cortex (Huang et al 2008). Changes in the rules for plasticity are likely due in part to developmental changes in expression and function of proteins involved in synaptic function, such as the developmental switch in subunit composition of the NMDA receptor (Sheng et al 1994, Williams et al 1993) which has profound implications in synapse maturation. Interestingly, this switch is not simply due to a predetermined genetic program, but also requires neural activity and sensory experience (Quinlan et al 1999), highlighting the requirement for external information in the proper development of neural circuits. Some proteins even demonstrate a bidirectional regulation of synaptic function in different developmental periods. This is the case for

FMRP, which promotes synaptic function in hippocampal neurons early in development, but suppresses it in more mature neurons (Zang et al 2013).

Most neocortical circuits develop in a typical manner characterized by discrete periods of changing synaptic dynamics. After neurons migrate to their appropriate layers, they begin sprouting dendritic and axonal arbors and making sparse connections. Over time, these arbors become increasingly complex and the number of connections between cells rises to the extent that an overabundance of connections exists. This is marked by not only large scale restructuring of dendritic and axonal arbors, but also by a burst in formation of small studded structures on the dendrites known as dendritic spines, which are thought to be the locus of nearly all excitatory synapses (Harris & Kater 1994). During this period of highly promiscuous connection formation, new connections are thought to be highly labile, a notion based mostly on the structural observations that spines emerge and disappear at very high rates during this time (Holtmaat et al 2005, Zuo et al 2005a). This dynamic process marked by intense synapse turnover and hyper-connectivity may be necessary for the circuit to test a large number of potential connection paths and refine them to reach the appropriate network configuration (Chklovskii et al 2004).

As this stage of development comes to a close, synapse formation is attenuated, and synapse strengthening becomes less permissible (Itami & Kimura 2012). Meanwhile, synapse elimination rates remain high, resulting in a reduction in both spine number (Holtmaat et al 2005) net pruning of synaptic connections (Chen & Regehr 2000, Patel et al 2014). However, it is important to note that the observation that a net reduction in synapses occurs during this stage is somewhat controversial, as other studies have reported no decerase

in structural synapse number following synaptogenesis (Blue & Parnavelas 1983). This may highlight one of the strong caveats associated with assaying structural synapses, which is that this approach measures synapses from all input pathways, leaving it incapable of probing region- or pathway-specific development of synapses. Eventually, the rates of synapse formation and elimination balance out, resulting in a stabilization of the net number of synapses. These two processes remain active in adulthood in response to neural activity and sensory experience, albeit to a lesser extent.

The role of sensory experience in circuit formation

Circuit formation in both developing and adult animals appear to employ the same strategy: start with a large number of participating neurons and then sculpt from that some optimal network path and strengthen the connections of the remaining neurons (Gdalyahu et al 2012). If the system were to work in the opposite direction and employ a 'generate and test' approach, in which new synaptic connections were formed in response to external stimuli, it would likely run into a significant search problem that would take a much longer time to achieve a similarly efficient network path (Chklovskii et al 2004). This refinement process is dependent on neural activity and sensory experience. Indeed, sensory deprivation during the critical period, which corresponds to the third postnatal week in rodents, specifically reduces spine elimination rates while formation rates remain unaffected (Zuo et al 2005b). This supports the notion that the network encodes experience by pruning unnecessary connections rather than forming *de novo* connections. However, experience-induced synapse elimination is accompanied by a robust strengthening of the remaining

synapses, as sensory enhancement strengthens individual synaptic connections (Clem et al 2008, Wen & Barth 2011). As expected, the summation of these two processes results in an overall enhancement of the response of the network to stimuli, as sensory deprivation also suppresses total functional input-pathway strength (Bureau et al 2008), which is the product of both the number and strength of individual synapses.

Circuit pathology in neurological disease

Several neurological disorders are associated with dysfunctional cortical circuits. For most disorders, the genesis of this dysfunction likely stems from abnormal circuit development, whether genetic or environmental in nature – although some perturbations to circuitry can be acquired in adulthood. Many forms of epilepsy, for example, are characterized by spike-and-wave discharges in the neocortex, and this is understood to be a corruption of the normal circuitry (Beenhakker & Huguenard 2009). Similarly, circuit changes in different areas of prefrontal cortex are associated with obsessive-compulsive disorder and depression (Deisseroth 2014) as well as schizophrenia (Beneyto & Lewis 2011). In sensory cortices, the mouse model of Fragile-X Syndrome, an inherited form of mental retardation and autism, displays a number of circuit abnormalities (Bureau et al 2008, Gibson et al 2008), which may be a common theme among many forms of autism (Dinstein et al 2012)

Somatosensory barrel cortex

Functional organization of sensory cortices

For most sensory modalities, the process of encoding environmental information begins with sensory neurons firing in response to their own specialized sensory input. Each sensory modality will then relay this information over 1 or 2 synapses to its respective area of the thalamus. After some processing, the thalamic neurons then project to their specialized area of neocortex, primarily targeting layer 4 (L4). Though there is some deviation from this canonical pathway (for instance the somatosensory system first projects to the brainstem before relaying to the thalamus), this represents the essential flow of sensory information to the neocortex. Information flow within most sensory cortices then continues with L4 projecting to L2/3, which itself projects to the deeper layers (L5 and L6) which are the main output layers of the neocortex. Similarly, this intracortical circuitry represents a general framework utilized by most primary cortices, but additional and alternative circuits also exist. It is also important to note that this circuitry pertains to the flow of excitatory, glutamatergic transmission, but inhibitory interneurons also contribute in a fundamental way to the circuitry of neocortex, and neuromodulatory innervations exist as well. Furthermore, the population of excitatory neurons that constitutes each layer differs across layers in regards to morphology, function, and molecular identity. Even excitatory neurons within a layer can display great diversity, as with the pyramidal and spiny stellate excitatory neurons of L4. More subtle differences likely exist even among pyramidal cells of the same layer, especially with regards to function (Lubke & Feldmeyer 2007).

While some degree of processing of environmental features clearly takes place within the thalamus (Brecht & Sakmann 2002), this processing is relatively limited and predominantly intrathalamic, aside from some neocortical feedback, and thus the flow of

information through the thalamus is largely linear. Conversely, neocortical processing is much more extensive due the laminar and columnar organization that serves to create a large number of processing compartments. As a result, neocortical processing can extract much more nuanced and detailed sensory information than its subcortical counterparts. Additionally, neocortical processing is heavily influenced by both intercortical input from other neocortical areas (Aronoff et al 2010) as well as neuromodulatory input from subcortical areas (Lee & Dan 2012, Petersen & Crochet 2013). This integrative feature of the non-linear processing in neocortex is thought to underlie the animal's ability to process specific sensory features in the context of other externally and internally generated information (Feldmeyer et al 2013, Petersen 2007). For instance, network synaptic activity of somatosensory cortex is highly dependent on arousal state, which is mediated by subcortical noradrenergic input impinging directly on neocortical neurons (Constantinople & Bruno 2011).

While it may not be appropriate to think of the principle excitatory cells of neocortex as a completely homogenous population, even within a given layer, most of the molecular mechanisms that regulate synaptic function are thought to be largely conserved at glutamatergic synapses. Therefore, considering the relatively standard subcortical processing that takes place, and to some degree the canonical circuitry of neocortex, how do such unique and specialized receptive fields emerge in different sensory cortices? Furthermore, how can synapses that are largely identical in molecular composition engage in different types of plasticity? This is likely the product of both unique firing patterns coding different aspects of

environmental information and the more or less predetermined (i.e. genetically specified) aspects of the circuitry that endow it with the capacity to rewire itself.

Barrel cortex function

The whiskers on the snouts of mice and rats provide crucial information for the animal's interaction with the environment. Rodents use whisker information for spatial navigation, object location, and texture discrimination. This modality have evolved to be highly sophisticated and informative for rodents, who spend much of their lives in environments with very little light and consequently often cannot rely on visual information. The whiskers are arranged on the snout in columns and arcs, which correspond in a one-to-one fashion with the 'barrels' in L4 of somatosensory cortex (one whisker corresponds to one barrel) and match the topographical arrangement of the whisker pad (Feldmeyer et al 2013, Petersen 2007). The cytoarchitecture of the barrels is such that most of the cell bodies of a barrel are arranged to form a barrel wall, which forms an anatomical structures that can be visualized in brain slices. These cell bodies project their dendrites inward to the barrel hollow to contact thalamocortical afferents, and together the barrel wall and hollow represent the receptive field in L4 of one whisker. The entire neocortical receptive field of an individual whisker is defined by the vertical column that spans layers 1-6. The width of this column is approximately 300-500 μm and corresponds to the width of the barrel in L4. The primary organization of the translaminar circuitry is confined to one column. The 6-layer column that exists between barrel walls constitutes the septum, and septal cells generally have broader receptive fields (Bureau et al 2004).

As with most sensory cortices, thalamic input to barrel cortex impinges mostly upon L4. Termed the granular layer, L4 serves to relay thalamic sensory information to the supragranular (L2/3) and infragranular (L5 and L6) layers. The primary projection of L4 is to L2/3, but it also projects more weakly to L5 and L6. The primary functions of the supragranular layers are thought to be to integrate information from other cortical and subcortical brain structures as well as to code specific aspects of whisker-related information. These notions are supported by the high degree of extracolumnar and extracortical input received by L2/3 cells compared to other layers as well as the sparse coding of sensory stimuli and relatively low action-potential firing observed in these cells (Petersen & Crochet 2013). Although the supragranular cells send many projections to other cortical areas, their predominant projection is to the infragranular layers, which are the main output cells of the neocortex targeting their projections to striatum, brainstem, and providing feedback information to the thalamus.

Layer 2/3 circuits

Though each layer processes sensory information in its own specialized way, particular attention has been given to processing occurring in L2/3. This is in part due to the prevalent input from L4, which constitutes the most robust pathway in the cortical column. This synapse displays considerable adherence to columnar boundaries, offering a powerful experimental advantage with regards to sensory stimuli (i.e. whisker deflection). Additionally, this synapse remains plastic into adulthood, unlike the thalamocortical synapses

in L4 (Feldman 2000), and the rules of this plasticity are dependent upon normal sensory experience (Celikel et al 2004).

Furthermore, as alluded to above, coding of many of the specific aspects of whisker information (e.g. angular detection, deflection intensity, and object location) likely occurs primarily in L2/3 (Petersen & Crochet 2013). Interestingly, sparse-network coding in L2/3 may extend beyond processing sensory information, as sparse coding also takes place in L2/3 during an associative learning paradigm involving whisker cues (Gdalyahu et al 2012). The capacity for L2/3 neurons to encode multiple aspects of the whisker information is partly a function of their relatively low average spontaneous and stimulus-evoked firing rates (Zhu & Connors 1999). This allows sparse sub-networks of L2/3 cells to robustly respond to specific attributes of whisker-related information while remaining relatively inactive in the absence of that specific stimulus (Gdalyahu et al 2012).

A number of factors likely dictate the low firing rate of L2/3 cells, such as relatively hyperpolarized membrane potential (Lefort et al 2009) and potential differences in intrinsic membrane properties. However, likely the most critical source of firing suppression is the circuitry of L2/3 itself. Inhibitory interneurons in L2/3 receive input from both nearby L2/3 cells and L4, and upon activation provide feedforward inhibition onto L2/3 pyramidal cells. The high degree of connectivity that exists between pyramidal cells and interneurons in L2/3 (Avermann et al 2012) suggests that this feedforward inhibition is a prominent component of L2/3 circuitry, and is likely largely responsible for suppressing firing rates in pyramidal cells. Interestingly, the inhibitory synapses on pyramidal cells are subject to heterosynaptic LTD in visual cortex (Jiang et al 2010), although this has not been confirmed in barrel cortex. This

regulation of inhibitory drive onto excitatory neurons (I→E) provides a potential mechanism by which specialized receptive fields develop in subnetworks of L2/3 neurons, where input-specific disinhibition allows for a sparse number of pyramidal cells to strengthen their connections and thus enhance their responses to specialized stimuli.

Cortical mGluR5 function

The mGluRs comprise a unique class of glutamate receptors that do not carry ionic conductances but rather signal metabotropically. mGluRs are seven transmembrane G-protein coupled recptors (GPCRs) that consist of 8 types (mGluR1-mGluR8) divided into 3 groups. The different groups vary in function, associated signaling pathways, distribution within the central nervous system (CNS), and subcellular localization (Niswender & Conn 2010). In cortical regions including hippocampus and neocortex, group 1 mGluRs, consisting of mGluR1 and mGluR5, are the most predominately expressed (Ferraguti & Shigemoto 2006) and functionally relevant (Anwyl 2009, Gladding et al 2009, Luscher & Huber 2010).

Group 1 mGluRs are located both postsynaptically and extrasynaptically in cortical neurons, with mGluR5 primarily being located on the periphery of the post synaptic density (PSD) (Lopez-Bendito et al 2002, Lujan et al 1996). Here, they function to modulate cell excitability by regulating ionic membrane conductances including sodium (Carlier et al 2006), calcium, and potassium channels (Anwyl 1999), as well as directly regulating synaptic function (Luscher & Huber 2010). In visual and somatosensory cortex, mGluR5 is the predominant group 1 receptor in terms of both expression (Lopez-Bendito et al 2002) and

function (Bender et al 2006b, Jiang et al 2010), whereas in hippocampus both mGluR1 and mGluR5 have a strong functional presence (Volk et al 2006).

Group 1 mGluR signaling

Group 1 mGluRs consist of mGluR1 and mGluR5, and are canonically coupled to G_q/G_{11} signaling and activation of phospholipase C (PLC). Upon mGluR-induced activation of PLC phosphatidylinositol 4,5-bisphosphate (PIP2) is hydrolyzed to form inositol triphosphate (IP3) and diacylglycerol (DAG), which in turn releases Ca^{++} from internal stores and activates protein kinase C (PKC) respectively. In addition, DAG can be further hydrolyzed by DAG lipase to synthesize the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG), a critical signaling molecule in mGluR5-mediated plasticity in cortex (Castillo et al 2012). mGluR1/5 stimulation in the hippocampus activates the extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) translational regulatory pathways, and both of these pathways are required for hippocampal mGluR-dependent LTD (Gallagher et al 2004, Hou et al 2006). It is important to note, however, that many of these biochemical signaling pathways have been studied in hippocampus, but the extent to which they exist in neocortical cells has not yet been established.

mGluR5 is coupled to many of its signaling pathways through interactions with the long, constitutively active forms of a family of scaffolding proteins called Homers (Shiraishi-Yamaguchi & Furuichi 2007). The activity-inducible short form of Homer, Homer1a, can dissociate mGluR5 interactions with long forms of Homer and thereby disrupt normal

mGluR5 signaling (Ronesi et al 2012). Furthermore, Homer1a-mGluR5 interactions are associated with constitutive, agonist-independent activity of mGluR5 (Ango et al 2001). Interestingly, regulation of mGluR5 by Homer1a may represent an activity-dependent mechanism that can alter mGluR5-mediated regulation of synapses (Hu et al 2010).

Involvement in neurological disease

mGluR5 has been implicated in the pathology and treatment of a number of neurological diseases. Interestingly, the etiology of many of these diseases, and the involvement of mGluR5 therein, manifests as alterations in synaptic function and aberrant neural circuitry. For instance, withdrawal from cocaine self-administration leads to synaptic changes in MSN neurons of the NAc, which is mediated in part my mGluR5 (Scheyer et al 2014). Additionally, activation of mGluR5 in NAc is required for alcohol binge-drinking behavior (Cozzoli et al 2009) and negative allosteric modulators (NAMs) of mGluR5 are effective in ameliorating addictive behaviors in mice (Pomierny-Chamiolo et al 2014). Some genetic forms of temporal-lobe epilepsy, a disorder defined by circuit dysfunction, are associated with alterations in mTOR function (Goldberg & Coulter 2013), which is a downstream effector of mGluR5 signaling (Hou & Klann 2004). Consistent with this, MPEP, an mGluR5 antagonist, reduces spike-and-wave discharges (SWDs), an electrophysiological hallmark of epilepsy (Ngomba et al 2011). An emerging hypothesis in understanding the pathophysiology underlying many aspects of schizophrenia is the hypoglutamatergic theory, in which NMDAR function is thought to be abnormally low in critical schizophrenia-related circuits. Consistent with observations that mGluR5 activation

potentiates NMDAR function, positive allosteric modulators (PAMs) of mGluR5 have proven to be effective in treating both pathophysiological and behavioral abnormalities in mouse models of schizophrenia (Lecourtier et al 2007, Nickols & Conn 2014). mGluR5 dysfunction is also strongly implicated in FXS, a genetic cause of several forms of intellectual disability, where it contributes to a number of synaptic and circuit level phenotypes (Bear et al 2004, Hays et al 2011). Interestingly, mGluR5 dysfunction may be a common deficiency in several forms of autism, and is thus a highly appealing drug target for treatment (D'Antoni et al 2014).

In targeting mGluR5 for pharmacological therapeutic intervention, it is important to consider the diverse functions of mGluR5, and the precise nature of its dysfunction in each disease. As mentioned above, drugs designed to both inhibit and enhance mGluR5 function are being investigated in different diseases. Therefore, understanding the mechanism of action of a given drug on mGluR5 function, trafficking, and signaling must be examined in the context of the disease being investigated. This of course requires a detailed understanding of how mGluR5 behaves under normal conditions – an understanding which is far from complete. For instance, in FXS, mGluR5 is abnormally coupled to its canonical signaling pathways, rendering it defective in both its activation by glutamate and its ability to signal properly (Ronesi et al 2012). As a consequence, simply inhibiting mGluR5 activation through pharmacological intervention may not be as effective as a treatment that also restores the mechanisms which couple mGluR5 to its typical signaling pathways.

Role in learning and memory

Understanding the role mGluR5 plays in the pathology of different neurological diseases provides some insight as to its function. Reciprocally, elucidating the direct role of mGluR5 in normal physiology will guide how we view its role in disease states. Additionally, examining the physiological function of mGluR5 will be useful in uncovering the biological underpinnings of learning and memory, which mGluR5 is heavily implicated in. For instance, mGluR5-LTD in CA1 is thought to be induced by novelty and consequently play a role in the recognition of familiar objects (Jakkamsetti et al 2013). Fear extinction requires insertion of calcium-permeable (CP) AMPARs in neurons of infralimbic cortex. Interestingly, administration of MPEP inhibits both behavioral performance in a fear extinction task and extinction-induced insertion of CP-AMPARs (Sepulveda-Orengo et al 2013). In paradigms probing adaptive learning, mGluR5 KO mice display learning deficits while PAMs targeting mGluR5 enhance such behaviors (Xu et al 2009, Xu et al 2013). Similarly, both working memory and reference memory are disrupted when MPEP is administered immediately prior to training (Manahan-Vaughan & Braunewell 2005). Taken together, these data demonstrate that normal mGluR5 function is required for several forms of learning.

Regulation of cell excitability

The ability of a neuron to fire an action potential is dependent on the membrane excitability of that cell. Membrane excitability is a highly regulated feature, and enhancing or suppressing excitability can have a profound impact on both the output of the cell, via alterations in spike rate, and the inputs to the cell, via the dependence of plasticity rules on

temporal spiking dynamics and membrane potential. Interestingly, there is ample evidence that mGluR5 regulates membrane excitability. For instance, the enhanced mGluR5 signaling in FXS is responsible for increased neocortical UP state duration, a measure of circuit excitability (Hays et al 2011). Additionally, acute application of MPEP disrupts theta and gamma rhythms in dentate gyrus (Bikbaev et al 2008). At a cellular level, mGluR5 activation transiently attenuates AHP and increases input resistance in CA1 pyramidal cells, resulting in a net increase in excitability as observed by enhanced action potential firing (Ireland & Abraham 2002). Similarly, in L5 of sensorimotor cortex, activation of mGluR5 leads to potentiation of intrinsic excitability by downregulating calcium-activated postassium (SK) channels and thereby diminishing AHP, although this appears to be a longer lasting effect (Sourdet et al 2003b). In L5 of PFC, group 1 mGluR activation has differential effects on 2 different classes of projection neurons, presumably through regulation of I_h (Kalmbach et al 2013). Interestingly, comparative analysis of mGluR-mediated effects on these 2 different cell populations demonstrated that mGluR activation could elicit similar, preferential (affecting one cell type but not the other), or bi-directional effects depending on the physiological property assayed. This suggests that mGluR5 can influence intrinsic membrane properties through a number of mechanisms, and the consequence of mGluR5 activation is highly dependent on the molecular composition of a given cell. Consistent with this notion, mGluR5 activation has a bidirectional effect on burst firing in 2 distinct classes of pyramidal neurons in CA1 (Graves et al 2012).

Homosynaptic plasticity

Synaptic plasticity refers to the ability of neurons to change the strength of their connections in response to correlated activity. This can be manifested as either a strengthening (long-term potentiation, or LTP) or weakening (long-term depression, or LTD) of synaptic function. Plasticity is one aspect of circuit rewiring and is thought to be critical for learning and memory. The direction and magnitude of plasticity is influenced by a number of factors, including the pattern of input activity and subsequent postsynaptic calcium wave, molecular composition of the synapse, and previous synaptic experience. Additionally, some synapses can undergo similar forms of plasticity through different expression mechanisms. For instance, LTD in hippocampal CA1 neurons can be mediated by mGluR or NMDAR. Furthermore, plasticity can occur at the synapse being activated (homosynaptic plasticity) or at an inactive synapse neighboring the synapse being activated (heterosynaptic activity). Interestingly, mGluR5 regulates several forms of plasticity in many different brain regions, making it a key protein of interest for understanding how synaptic function is regulated (Luscher & Huber 2010).

In hippocampus, a form of homosynaptic LTD can be induced in CA1 neurons by either application of the group 1 agonist, DHPG, or by (paired-pulse low frequency stimulation) PP-LFS of the Schaffer collateral. This plasticity requires activation of both mGluR1 and mGluR5, but is NMDA-independent (Volk et al 2006). Although NMDAR activation can also induce LTD at this synapse, the induction and expression mechanisms appear to be distinct from those associated with mGluR-LTD (Huber et al 2001). Whereas LFS (1 Hz for 15 min) is sufficient to induce NMDA-LTD, mGluR-LTD requires paired pulses (50 ms paired-pulse interval) delivered at similar frequencies. This may reflect the

positioning of mGluR5 at the periphery of the PSD (Lopez-Bendito et al 2002, Lujan et al 1996), as increased glutamate release would be expected with paired pulses. This provides an explanation for how 2 glutamate receptors can differentially engage 2 separate forms of plasticity, but the physiological distinction of these 2 stimulation protocols (LFS vs PP-LFS) remains unclear.

mGluR5-LTD is dependent on local translation of existing dendritic mRNAs, such as Arc (Park et al 2008, Wilkerson et al 2014), and endocytosis of post synaptic AMPARs (Waung et al 2008). While induction of Arc-mRNA in dendrites occurs in response to brief novelty, presumably through an mGluR5-independent mechanism, functional synaptic changes only occur in response to repeated exposure (Jakkamsetti et al 2013). These functional changes are likely mediated by mGluR5 activation, as repeated novelty exposure occludes mGluR5-LTD. This data provides a biological substrate for a learning paradigm in which repeated, but not brief exposure to novelty, leads to learning on the behavioral level and concurrently mGluR5-mediated synaptic changes on the cellular level.

In neocortex, mGluR5 mediates another form of homosynaptic plasticity, spike-timing dependent plasticity (STDP). STDP can be expressed as a strengthening (timing-dependent LTP, or tLTP) or weakening (timing-dependent LTD, or tLTD) of synaptic strength) In this form of plasticity, the precise timing of the presynaptic spike and the postsynaptic spike determine the magnitude and direction of plasticity. When the presynaptic spike leads the postsynaptic spike by less than 20 ms, as would occur in the sequence of events of a feedforward signal, LTP occurs. However, if the order of spiking is reversed (i.e. post before pre), LTD takes place. In this way, STDP is Hebbian because when

the presynaptic spike contributes to the firing of the postsynaptic cell the connection is strengthened, but when the spikes are uncorrelated the connection is weakened (Feldman 2012). At the L4→L2/3 synapse in barrel cortex, tLTD and tLTP require distinct detection and expression mechanisms. Whereas tLTP requires activation of postsynaptic NDMARs, tLTD requires activation of postsynaptic mGluR5. Activation of mGluR5 in turn generates eCBs that diffuse retrogradely to activate presynaptic CB_1Rs and thereby decrease presynaptic function (Feldman 2012).

Evidence for mGluR5-mediated homosynaptic LTP is less convincing. Although this type of plasticity has been observed in at least one pathway (Wang & Daw 2003) and under nonphysiological circumstances in more well characterized pathways (Clem et al 2008), a common role for a direct potentiating effect of mGluR5 on synaptic function remains controversial. For instance, standard LTP induction protocols fail to induce an mGluR5-dependent LTP in hippocampus, whereas stronger stimulations do require mGluR5-dependent mechanisms, but may not represent physiological patterns of activity (Anwyl 2009). Furthermore, in studies where mGluR5-dependent LTP has been observed, synaptic function has been assayed by measuring field potentials and population spike (Neyman & Manahan-Vaughan 2008, Tsanov & Manahan-Vaughan 2009), which cannot distinguish between homosynaptic and heterosynpatic changes.

Heterosynaptic plasticity

As mentioned above, mGluR5 activation induces the synthesis and release of eCB to retrogradely activate CB_1R. In the case of STDP in barrel cortex, the coincident activation of

presynaptic NMDARs is also required in order to trigger a reduction in presynaptic function, conferring synapse specificity to this form of LTD (Rodriguez-Moreno & Paulsen 2008). However, other forms of mGluR5 and eCB-dependent LTD can impact synapses in a non-specific, heterosynaptic manner. In hippocampus, HFS stimulates mGluR5-induced eCB release which targets CB_1Rs on the presynaptic terminals of GABAergic neurons that are in the immediate vicinity of the synapse being activated. This leads to a sustained reduction of presynaptic release of GABAergic transmission, termed LTD of inhibition (iLTD). iLTD in turn facilitates glutamatergic transmission as excitatory inputs are disinhibited, potentiating EPSP-to-spike coupling (Chevaleyre & Castillo 2003). This is considered to be a form of metaplasticity, as sustained disinhibition results in a persistent reduction in the threshold for LTP. In visual cortex, stimulating the L4→L2/3 pathway with TBS causes mGluR5 and eCB-mediated heterosynaptic LTD of both excitatory (hetero-eLTD) (Huang et al 2008) and inhibitory transmission (hetero-iLTD) (Jiang et al 2010). This may suggest a dual role for mGluR5 in which its activation enhances strongly activated inputs to a cell via hetero-iLTD mediated disinhibition and weakens common input onto surrounding cells via hetero-eLTD. In this model, mGluR5 would function to sharpen receptive fields by enhancing the signal-to-noise ratio of an individual cell's response through these 2 mechanisms.

Metaplasticity

Any form of synaptic regulation that leads to a sustained change in the rules governing plasticity is referred to as metaplasticity. As mentioned above, mGluR5-mediated iLTD results in a metaplastic alteration to the induction of LTP in hippocampus. Similarly,

the observed mGluR5-mediated heterosynaptic iLTD in visual cortex is also likely to have metaplastic influences on L4→L2/3 excitatory synapses, although the nature of this metaplasticity has yet to be described. Additionally, mGluR5 may contribute to other forms of metaplasticity. For instance, strong synaptic activity drives the developmental NMDAR subunit switch in hippocampus in an mGluR5-dependent manner (Matta et al 2011). This switch has been shown to alter the gating of LTP as the different NMDAR subunits confer distinct properties regarding channel conductance and coupling to intracellular signaling pathways (Kohr et al 2003, Monyer et al 1994). This was also observed in visual cortex, where both synaptic activity and visual experience drove the subunit switch in an mGluR5-dependent manner. Additionally, although not considered to be a conventional form of metaplasticity, the previously mentioned mGluR5 regulation of intrinsic membrane currents likely has a significant impact on rules governing plasticity. This has particular relevance in STDP, where changes in firing properties would disrupt the normal timing dynamics that are critical to the direction and magnitude of plasticity (LTD vs LTP).

Synapse elimination

Changes in synaptic strength are a major component of experience induced alterations to circuit wiring, but a second type of circuit rewiring can also be achieved by the removal or addition of entire inputs. In neural circuitry, synapse elimination and formation can contribute to both of these circuit changes – eliminating a fraction of the inputs from one cell (or adding an input from an already connected cell) would be analogous to a weight change, whereas elimination/formation that changes the status of a cell from connected to not

connected, or vice versa, would represent the second type of circuit rewiring. Synapse formation and elimination are very active forces in the development of neocortical circuits of young animals, but also likely play a role in circuit refinement that takes place during learning in adult animals (Holtmaat et al 2005, Zuo et al 2005a, Zuo et al 2005b). Furthermore, synaptic pruning seems to target synapses from common inputs, as the number of connected cells decreases during development (Patel et al 2014). mGluR5 likely plays a role in both forms of circuit rewiring, as previous evidence has implicated it in synapse elimination. In CA1 pyramidal cells, artificial activation of the transcription factor MEF2 induces synapses elimination in an mGluR5-dependent manner (Wilkerson et al 2014). Interestingly, this form of elimination was observed as an alteration in mEPSC frequency but not amplitude, suggesting that synapses were entirely eliminated rather than weakened in a linear way. This implies that mGluR5 induces synapses elimination by either triggering a molecular switch to functionally mute them or weakening them to the point of elimination on a very rapid timescale. In support of the latter, mGluR5 activation in hippocampus induces a rapid (within 30 min.) reduction in spine size (Oh et al 2013), and repeated mGluR5-LTD is associated with a reduction in spine number (Shinoda et al 2005). Whether mGluR5-mediated synapse elimination can also occur by mechanisms other than repeated LTD is unclear.

Developmental role in formation of circuits in barrel cortex

In somatosensory cortex, mGluR5 is critically implicated in several aspects of circuit development. As mentioned above, mGluR5 activation is required for tLTD at the L4→L2/3

synapse. Perhaps unsurprisingly, it may also be required for tLTD at the L2/3→L2/3 synapse, demonstrating a common role in regulating synaptic function in barrel cortex (Zilberter et al 2009). Both brain-wide and cortex-specific deletion of mGluR5 results in disrupted barrel patterning in somatosensory cortex (Ballester-Rosado et al 2010, Wijetunge et al 2008). Additionally, functional receptive fields may also be impaired, further suggesting abnormal circuitry in these mice (She et al 2009). However, the synaptic mechanisms underlying these potential circuit changes are unclear. One study reports reduced spine density and expression of synaptic proteins in L4 of mGluR5 KO mice with no changes in dendritic morphology (Wijetunge et al 2008), suggesting a role for mGluR5 in promoting synaptic function. In contrast, another group has demonstrated that mGluR5 deletion enhances dendritic complexity and increases mEPSC frequency in L4 neurons, implicating mGluR5 as a negative regulator of synaptic function (Ballester-Rosado et al 2010). Paradoxically, when mGluR5 deletion was restricted to excitatory cortical cells, no change in mEPSC frequency was observed, although dendritic complexity was still enhanced. Lastly, when spine density was examined in older mice (45 days vs ~22 days), brain-wide deletion of mGluR5 resulted in an increased spine density (Chen et al 2012). Taken together, these data demonstrate a clear role for mGluR5 in the development of neural circuits in somatosensory cortex. However, the potential for indirect network effects that arises from global or large-scale deletion strategies, along with the inconsistencies between studies and deletion strategies used (i.e. global vs cortex-specific), confound any interpretation at how mGluR5 might be regulating synaptic function.

MEF2

Another likely key regulator of activity-dependent, postnatal neocortical circuit refinement is the Myocyte Enhancer Factor-2 (MEF2) family of activity-dependent transcription factors. The MEF2 family is comprised of four genes– *Mef2a, b, c,* and *d* – all of which are expressed in the brain with MEF2C being the predominantly expressed gene in neocortex, constituting ~75% of total MEF2 expression in that region (Lyons et al 1995, Lyons et al 2012b, Potthoff & Olson 2007). Conserved among all MEF2 members are a transcriptional activation domain and a MEF2/MADS domain, which facilitates dimerization and DNA binding (Wu et al 2011). Neuronal depolarization and calcium influx through L-type calcium channels and NMDA receptors stimulates calcineurin and dephosphorylation which activates MEF2 (Flavell et al 2006, Mao et al 1999). Activated MEF2 then binds to genes with MEF2 response elements (MREs) in their promoter region and regulates transcription of target genes, generally by promoting transcription (Flavell et al 2008).

Role in normal and pathological brain function

As a transcription factor, MEF2 influences expression of a large number of genes and, accordingly, mediates a wide range of cellular functions including neuron survival and migration (Akhtar et al 2012, Li et al 2008, Mao et al 1999, McKinsey et al 2002). However, among the vast number of cellular processes associated with MEF2, particular attention has been given to its role in learning and memory. This is in part due to its observed regulation of a number of genes involved in synaptic function (Flavell et al 2008). Accordingly, there is a strong interplay between MEF2 activity and learning and memory. For instance, learning

paradigms known to robustly induce memory formation cause a phosphorylation of MEF2 (Cole et al 2012), which results in suppression of MEF2-meditaed transcription (Flavell et al 2006). Conversely, driving MEF2 transcriptional activity by overexpressing a constitutively active form of MEF2 blocks certain types of memory formation. Furthermore, brain-specific deletion of the *Mef2c* gene results in a disruption of hippocampal-dependent learning in mice (Barbosa et al 2008).

Consistent with a role in learning and memory, MEF2 has been implicated in cognitive disorders and epilepsy in both humans and animal models. Genome-wide analysis studies in humans have revealed that mutations in the *Mef2c* gene are linked to a host of neurological disorders including autism-spectrum disorders (Novara et al 2010), mental retardation and epilepsy (Le Meur et al 2010, Zweier et al 2010) along with several variable minor abnormalities. Additionally, animal studies manipulating MEF2 activity have implicated its involvement in neurological disorders. For instance, MEF2-induced synapse elimination is deficient in hippocampal neurons in the mouse model of FXS (Pfeiffer et al 2010). Here FMRP, an RNA-binding protein that regulates translation of mRNAs, is thought function downstream of MEF2 to regulate its target transcripts, such as Arc. Thus understanding how MEF2 regulates synaptic function and connectivity will help to identify its target genes with critical involvement in synapse regulation and thereby assist in the development of treatments for this and other diseases.

Regulation of synapses

MEF2 is most commonly observed to have a role in suppressing excitatory synapse

number, likely by promoting elimination of established synapses (Barbosa et al 2008, Flavell et al 2006, Pfeiffer et al 2010, Pulipparacharuvil et al 2008) A role for MEF2 in suppression of synapse number *in vivo* has been demonstrated using shRNA-mediated knockdown of MEF2A and MEF2D in the nucleus accumbens (Pulipparacharuvil et al 2008) and overexpression of a constitutively active MEF2 in anterior cingulate cortex (Vetere et al 2011). However, a role for MEF2 in promoting synaptic function has also been demonstrated. In hippocampus, MEF2C deletion results in a reduced fEPSP response to extracellular stimulation (Li et al 2008). Additionally, LV cells of neocortex display an increased mEPSC frequency in the MEF2C KO mice. Taken together, these data suggest that MEF2 may regulate synaptic function through a number of mechanisms that have varying outcomes. Therefore, a detailed analysis of member subtype and cell-type specific roles of MEF2 is needed.

Constitutive embryonic and brain-wide deletion of *Mef2c* increases the number of functional synapses and spines in hippocampal granule cells, suggesting an *in vivo* role for MEF2C in promoting synapse elimination or suppressing synapse formation (Barbosa et al 2008). While cell-autonomous knockdown of MEF2A and MEF2D increases functional and structural synapse number in cultured hippocampal and in nucleus accumbens neurons *in vivo* (Flavell et al 2006, Pulipparacharuvil et al 2008), embryonic and brain-wide deletion of *Mef2a* and *Mef2d* has no detectable effect on dendritic spine number or population EPSPs of hippocampal CA1 neurons (Akhtar et al 2012). One possible explanation for these disparate results is that *Mef2* deletion in a sparse number of neurons reveals the cell-autonomous role of MEF2 in synaptic pruning, whereas constitutive, brain-wide deletion of *Mef2* causes

circuit adaptation to maintain normal

synapse number via MEF2-independent mechanisms.

Although a clear role has been demonstrated for MEF2 in regulating synapses, the molecular mechanisms underlying this regulation are largely unknown. Recently, 2 potential cellular mechanisms by which MEF2 mediates the elimination of excitatory synapses in hippocampus have been identified. First, MEF2 activation drives transcription of protocadherin-10 (PCDH10) - also an autism candidate gene - which mediates dendritic spine elimination by facilitating proteasomal degradation of ubiquitinated PSD-95 (Tsai et al 2012). Second, MEF2-induced synapse elimination in hippocampus requires both Arc and mGluR5 activation (Wilkerson et al 2014). Taken together, these data identify 2 distinct mechanisms by which MEF2 regulates synaptic function in hippocampal neurons. However, virtually nothing is known about how MEF2 regulates synaptic function in neocortex.

Motivation for studies and summary of research

mGluR5 and MEF2 have been implicated in a number of neurological diseases. Furthermore, pathological conditions can emerge from either defective forms of these proteins or through a disruption in their normal functions caused by mutations in any one of hundreds of proteins they interact with either directly or indirectly. Therefore, due to the permeating influence that both of these proteins exude on a vast array of cellular functions, it is likely that their dysfunction plays a common role in a number of clinically relevant diseases. Consequently, pursuing both the synaptic mechanisms and underlying molecular

mechanisms regulated by these two proteins could offer several therapeutic targets for a number of diseases.

mGluR5 and MEF2 may regulate synapses through common mechanisms. In hippocampus, mGluR5 signals to regulate the phosphorylation state of FMRP, which in turn influences LTD (Narayanan et al 2007, Niere et al 2012). Interestingly, MEF2-induced synapse elimination requires FMRP, and conversely FMRP function is dependent on MEF2 transcriptional activity (Pfeiffer et al 2010, Zang et al 2013). Further demonstrating a link between mGluR5 and MEF2, mGluR5 activation is required for MEF2-dependent elimination of structural and functional synapses (Wilkerson et al 2014), with Arc-induced AMPAR endocytosis implicated as the common mechanism. Furthermore, my data demonstrates striking parallels between the consequences of mGluR5 and MEF2C deletion on neocortical circuitry in barrel cortex.

Although elucidating mechanisms by which these 2 proteins regulate synaptic function and circuit formation will provide insight into their involvement in, and potential treatment of, several neurological diseases, understanding how they function will also further a broader pursuit in understanding how information is processed in the brain. Understanding any biological function is an iterative process, and although molecular mechanisms are usually detailed after the discovery of a physiologically phenomenon, often exploring these mechanisms results in a refinement of our understanding of biology. Accordingly, there is great potential for gleaning knowledge about information processing on a more macro level from the examination of specific proteins. For instance, mGluR5 has long been considered to be one of the coincidence detectors in different forms of synaptic plasticity due to both its

unique activation profile as well as the dependence of its signaling on postsynaptic calcium transients. Furthermore, its ability to mediate both homosynaptic and heterosynaptic plasticity of both excitatory and inhibitory inputs situates mGluR5 in a position to have a profound effect on circuitry in a number of nuanced ways. Therefore, examining how patterns of neurotransmission that occur *in vivo* engage mGluR5 in shaping circuitry will also further our understanding of the rules by which circuits are formed and refined.

Dissecting the direct role of any protein in regulating synaptic function poses a number of challenges. One of these challenges is discerning not only the acute role in synaptic function, but also the long-term impact on circuit development. In a simple model, the long-term role would be an accumulation of all of the acute functions, although this does not take into consideration the cascade of events triggered by an acute role that are independent of the protein of interest. Nevertheless, an understanding of the long-term impact guides hypothesese about the acute roles. In the case of mGluR5, a long-term role in circuit development has been examined by deleting *Grm5*, the gene coding for mGluR5, in either the entire brain or a large population of neurons. While these studies do indeed implicate mGluR5 as a critical protein involved in circuit development (Ballester-Rosado et al 2010, She et al 2009, Wijetunge et al 2008), it is difficult to interpret any direct roles mGluR5 has on synaptic function due to the high potential for mGluR5-independent network compensation that influences measures of synaptic function. Strong evidence for indirect network effects has been demonstrated by the observations that 1) brain-wide deletion of mGluR5 resulted in increased mEPSC frequency in excitatory cells of L4 barrel cortex, whereas deletion restricted to cortical excitatory cells displayed no such change, and 2)

mGluR5 deletion in cortical excitatory cells resulted in a decreased mIPSC frequency in excitatory cells of L4 of barrel cortex (Ballester-Rosado et al 2010). Taken together, these data highlight another obstacle in studying the direct, long-term roles in synapse function, which is the challenge of examining function within a cell autonomous context. To overcome these 2 challenges, I have employed a paradigm in which permanent deletion of either mGluR5 or MEF2C early in development can be restricted to only a small population of neurons, thereby allowing a KO cell to develop *in vivo* in otherwise WT tissue and circumventing any compensatory network effects. Although this technique can largely mitigate network compensation, it is important to note that the chronic deletion strategy I employ may result in compensatory mechanisms on the cellular level that may influence my observations. Moreover, another added benefit of this paradigm is that it offers a within-experiment control, eliminating the between-experiment variability that is so prevalent in acute slice electrophysiology.

CHAPTER TWO

Results

POSTSYNAPTIC MGLUR5 PROMOTES EVOKED AMPAR-MEDIATED SYNAPTIC TRANSMISSION ONTO NEOCORTICAL LAYER 2/3 PYRAMIDAL NEURONS DURING DEVLEOPMENT

Summary

Both short- and long-term roles for the group I metabotropic glutamate receptor number 5 (mGluR5) have been examined for the regulation of cortical glutamatergic synapses. But how mGluR5 sculpts neocortical networks during development still remains unclear. Using a single-cell deletion strategy, I examined how mGluR5 regulates glutamatergic synaptic pathways in neocortical layer 2/3 during development. Electrophysiological measurements were made in acutely prepared slices to obtain a functional understanding of the effects stemming from loss of mGluR5 *in vivo*. Loss of postsynaptic mGluR5 results in an increase in the frequency of action potential-independent synaptic events, but paradoxically, results in a decrease in evoked transmission in 2 separate synaptic pathways providing input to the same pyramidal neurons. Synaptic transmission through AMPARs, but not NMDARs, is specifically decreased. In the local L2/3 pathway, the decrease in evoked transmission appears to be largely due to a decrease in cell-to-cell connectivity and not in the strength of individual cell-to-cell connections. This decrease in

evoked transmission correlates with a decrease in the total dendritic length in a region of the dendritic arbor that likely receives substantial input from these two pathways, thereby suggesting a morphological correlate to functional alterations. These changes are accompanied by an increase in intrinsic membrane excitability. Our data indicate that total mGluR5 function, incorporating both short- and long-term processes, promotes the strengthening of AMPAR-mediated transmission in multiple neocortical pathways.

Introduction

Group I metabotropic receptors (Gp1 mGluRs) consist of the two subtypes, mGluR1 and mGluR5. Over the first 4 weeks of postnatal development, their expression and function is strong (Dudek & Bear 1989, Jia et al 1995). The mGluR5 subtype is expressed in all layers of the rodent somatosensory cortex and peaks in expression during the first 3 weeks of life (Munoz et al 1999, Wijetunge et al 2008). During this time, extensive proliferation of synapses is occurring, and networks are being built and refined (Micheva & Beaulieu 1997). Therefore, Gp1 mGluRs are likely a crucial part of network formation during development.

Gp1 mGluR-dependent regulation of cortical circuitry has mainly been studied in the short-term. At synapses, Gp1 mGluRs are most commonly observed to acutely induce long-term depression (Bender et al 2006b, Luscher & Huber 2010, Nevian & Sakmann 2006, Zilberter et al 2009), but in some cortical pathways and circumstances, they induce long-term potentiation (Anwyl 2009, Clem et al 2008). They also play a modulatory role in plasticity by priming and enhancing NMDA receptor (NMDAR) mediated potentiation (Cohen & Abraham 1996). Because Gp1 mGluRs, and specifically mGluR5, exists predominantly

postsynaptically on the edge of the synaptic specialization (Lopez-Bendito et al 2002, Wijetunge et al 2008), it is likely that their synapse regulation is postsynaptic in origin. In addition to synapses, intrinsic membrane currents are also acutely regulated by mGluRs (Carlier et al 2006, Ireland & Abraham 2002, Kalmbach et al 2013, Sourdet et al 2003a). These roles for Gp1 mGluRs at synapses and membrane channels are based on *in vitro* techniques where conditions are not identical to that *in vivo*. Therefore, while Gp1 mGluRs are found to potentially play a role in many acute processes, the most prominent long-term functional roles *in vivo* remain unclear.

By genetically removing mGluR5, the long-term role *in vivo* can be examined, but use of this approach has been limited. Both global deletion and cortex specific deletion of *Grm5* (the mGluR5 gene) result in less defined cytoarchitectonic layer 4 barrels in the somatosensory cortex (Ballester-Rosado et al 2010, She et al 2009, Wijetunge et al 2008). Global deletion impairs NMDAR mediated synaptic transmission in CA1 hippocampal neurons (Lu et al 1997) and impairs the developmental subunit switch from GluN2B to GluN2A in cortical neurons (Matta et al 2011). The effects on AMPA receptor (AMPAR) mediated transmission remain unclear (Ballester-Rosado et al 2010). Finally, these widespread deletion approaches may result in indirect, or secondary, effects that are not directly related to cell-autonomous function of mGluR5 (Ballester-Rosado et al 2010, She et al 2009, Wijetunge et al 2008).

To obtain a clearer picture of how mGluR5 regulates cortical network development, I deleted mGluR5 in a minority of layer 2/3 pyramidal neurons in an otherwise normal neocortex so that a direct, cell-autonomous role could be studied. I examined the resulting

effects on synaptic input and membrane properties. I examined 2 glutamatergic input pathways - the layer 4 to layer 2/3 (L4→L2/3) pathway and the local interconnectivity among layer 2/3 neurons – the L2/3→L2/3 pathway. In both of these pathways, acute activation of Gp1 mGluRs is known to induce long-term depression (Bender et al 2006b, Zilberter et al 2009), and for the L4→L2/3 pathway, this acute process may be linked to pathway weakening in the long-term (Bender et al 2006a). Therefore, I hypothesized that the long-term role for mGluR5 *in vivo* was to weaken synaptic pathways, and upon loss of mGluR5, the pathways would be strengthened. Surprisingly, evoked transmission mediated by AMPARs in both pathways was weakened with loss of mGluR5 suggesting that mGluR5 can function to enhance the strength of these pathways – perhaps by additional mGluR5-dependent processes that are independent of acutely induced long-term depression.

Materials and Methods

Mice

Floxed *Gmr5* mice ($Grm5^{flx/flx}$) (Xu et al 2009) were maintained on a C57Bl6 background strain. All experimental procedures using these mice in this study were approved by the Institutional Animal Care and Use Committee at UT Southwestern.

Electrophysiology

Acute brain slices were prepared from $Grm5^{flx/flx}$ mice (P9-P30) with the following procedure. Mice were anesthetized with Ketamine (125 mg/kg)/Xylazine (25 mg/kg) and the brain removed. For ages greater than P21, mice were perfused with cold dissection buffer before brain removal, and the dissection buffer for perfusion and slicing contained 1 mM

kynurenate. Coronal slices, 250 μm thick, were cut at ~4° C in dissection buffer, placed in ACSF at 35° C for 30 minutes, and slowly cooled to 21° C over the next 30 minutes. Only slices containing the visible cytoarchitectonic barrels of somatosensory cortex were used. Whole-cell recordings were performed in layer 2/3 centered above a barrel hollow, and cells were targeted with IR-DIC optics in an Olympus FV300 confocal microscope. Recordings were performed at 21° C. Data were collected with a 10 kHz sampling rate and a 3 KHz Bessel filter. Neurons with mGluR5 deleted were identified by GFP fluorescence (see *in utero electroporation* below).

Electrophysiology Solutions

ACSF contained (mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 25 dextrose, and 2 $CaCl_2$. All slices were prepared in the following dissection buffer (mM): 75 sucrose, 87 NaCl, 3 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 20 dextrose, and 0.5 $CaCl_2$. All solutions were pH 7.4. ACSF was saturated with 95% O_2 / 5% CO_2. Unless stated otherwise, the pipette solution consisted of (mM): 130 K-Gluconate, 6 KCl, 3 NaCl, 10 HEPES, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, 14 phosphocreatine-Tris, 10 sucrose. This was adjusted to pH 7.25 and 290 mOsm. The junction potential was ~10 mV and was not corrected. Therefore, actual membrane potentials are ~10 mV lower than that stated. With this pipette solution, the reversal potential for $GABA_A$R-mediated currents was ~-70 mV.

In utero electroporation

To delete *Grm5* in a minority of layer 2/3 pyramidal neurons, I expressed Cre-recombinase fused to EGFP (Cre-GFP) (Ho et al 2006) through *in utero electroporation* (Saito 2006) at embryonic day 15 (E15) in *Grm5*$^{flx/flx}$ mice. I performed a survival surgery following procedures already described (Saito 2006). Briefly, I initially anesthetized an E15 pregnant dam in an induction chamber perfused with 3% isofluorane at 1.5L/minute and then immediately fitted a nose-cone to maintain the isofluorane anesthesia at 1.5%. Then I prepared the abdomen for a sterile procedure which included hair removal. A 2-3 cm vertical incision along the midline was made and multiple pups in the uterus were temporarily removed from the abdominal cavity keeping the uterus intact. For each pup, a solution containing FUGW plasmid DNA expressing a Cre-GFP fusion protein under the ubiquitin promoter (Ho et al 2006) (0.9 mg/ml) was injected into the lateral ventricle (1-2µL/embryo). This was followed by electroporation across the head to inject the DNA into radially migrating excitatory neurons (5 pulses, 45V, 50ms on, 100ms off). After repeating this procedure for all pups, the uterus was reinserted into the abdominal cavity, and the incision was sutured. Buprenorphine (0.05 mg/kg) was administered by I.P. injection immediately before the first incision and into the abdominal cavity immediately before suturing. It was also injected I.P. the following morning (0.1 mg/kg). The antibiotic, Baytril (2.5mg/kg), was administered directly into the abdominal cavity immediately preceding suturing.

Miniature EPSCs

Experiments were performed with the voltage-dependent sodium channel blocker, TTX (1 µM, Sigma), and the $GABA_AR$ antagonist, picrotoxin (100 µM, Sigma). These

compounds together with the -65 voltage clamp effectively isolated AMPAR-mediated currents. Miniature EPSCs (mEPSCs) were detected using a minimum amplitude and area that were derived from baseline noise measured in a 2 second period (5x for amplitude, and 7.5x for area).

Evoked AMPAR-mediated transmission in the layer 4 to layer 2/3 pathway

Simultaneous recordings of adjacent L2/3 pyramidal neuron pairs (< 50 µm intersomal distance) were performed where one neuron was GFP-negative, untransfected (UT) and the other was GFP-positive, Cre-expressing. A 2-conductor cluster stimulating electrode (FHC, Inc.) was positioned in layer 4 to stimulate afferents of the layer 4 to layer 2/3 (L4→L2/3) pathway (stimulation intensities ranged from 1-20uA). Biphasic pulses (200 µs, 1-10 µA) were applied to induce excitatory postsynaptic currents (EPSCs) in L2/3 neurons. To reduce the occurrence of polysynaptic responses, $[Mg^{++}]$ and $[Ca^{++}]$ were raised to 4mM in the ACSF. In all our experiments, kainate receptors were probably not involved based on their developmental profile and on the faster kinetics of the mEPSCs and EPSCs that I observed (Contractor et al 2011) (see Results).

To isolate AMPAR-mediated transmission (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP, 5 µM, Sigma), an NMDAR antagonist, was added to the bath. To better isolate the monosynaptic EPSCs, I locally blocked disynaptic inhibition by positioning a perfusion pipette containing the $GABA_AR$ antagonist, bicuculline methiodide (BMI, 5 mM), 5-15 µm above the slice surface at the location of the recordings. The tip of the perfusion pipette was 2-5 µm in diameter and 0.1-0.2 lbs/in² of positive pressure was applied

using a pressure gauge. This has been done in previous studies examining this same L4→L2/3 pathway (Bender et al 2006b). The efficacy of the blockade was assessed by the observation of IPSCs while holding the cell at -45 mV and increasing stimulation intensity. IPSCs could be easily observed as outward currents at -45 mV since the reversal for $GABA_A$R-mediated currents was in ~-70 mV in our recording conditions. I collected data when no IPSC was observed at -45 mV with a stimulation intensity that was 1.5x the threshold intensity for evoking EPSCs in both neurons – this generally resulted in responses where EPSCs were observed >50% of stimulation trials in both neurons. Inhibition was never completely blocked, but this procedure dramatically improved isolation and measurement of monosynaptic EPSCs. Two stimulation pulses with a 50 ms interval were applied for each trial, and each trial was separated by 4 seconds. Only EPSCs collected at -45 mV holding potential were analyzed in an effort to constantly monitor IPSC contamination. At this potential, baseline current was very stable and individual EPSCs clearly resolved.

Unitary EPSCs

Simultaneous "pre/post" recordings of up to 4 L2/3 pyramidal neurons were performed to examine cell-to-cell, or "unitary", connections among these neurons. An action potential was evoked in one neuron at a time and unitary EPSCs (uEPSCs) examined in the other neurons. Only a minority of possible unitary connections actually displayed a functional connection. A "connection" refers to a one-way, cell-to-cell connection and was detected when the average uEPSC amplitude was >1.6 pA. Connectivity frequency was the

percent of detected connections out of all possible connections examined. "Failures" in individual trials were included in all average uEPSC calculations. Unitary EPSCs were measured at a holding potential of -65 mV, and therefore, were mainly AMPAR-mediated. When action potentials were evoked in a presynaptic neuron, a train of 4 with a 50 ms interval was applied. Individual presynaptic action potentials were elicited with an 8 ms depolarizing current step (generally 600 pA). No effort was made to collect data by age groups. Instead, data was sampled continuously over the age range (P15-30). Unless stated otherwise, analysis was performed on data pooled across all ages so that I could resolve 5% to 10% differences in connectivity frequency (Chi-square analysis). PSCs were confirmed to be uEPSCs since they did not reverse polarity between -80 mV and -45 mV – they were inward currents at both potentials. On the other hand, IPSCs in our recording conditions would be expected to change from an inward to an outward current with this membrane potential change.

Analysis of evoked AMPAR-mediated EPSC

The Coefficient of Variation (CV) was calculated as the square root of (VAR_{EPSC1} - $VAR_{baseline}$) divided by the $Mean_{EPSC1}$ where VAR_{EPSC1} and $VAR_{baseline}$ are the variance of uEPSC1 and baseline noise respectively, and $Mean_{EPSC1}$ is the mean of uEPSC1 (Faber & Korn 1991, Markram et al 1997). Only average responses >10 pA and > 5 pA were used to avoid significant contamination by noise for L4 and L2/3 input, respectively. Short-term plasticity (STP) analysis for L2/3 input was performed when the average uEPSC was >1.6 pA. No amplitude criterion was required for paired pulse measurements of L4 input. An

experiment was analyzed if the postsynaptic recording had a series resistance less than 30 MΩ. The duration of EPSCs was measured by the width at half-height of the average EPSCs that were >9 pA and > 2 pA for L4 and L2/3 input, respectively.

Evoked NMDAR-mediated EPSCs

Simultaneous recordings of L2/3 pyramidal neuron pairs were performed as described above (see "*Evoked AMPAR-mediated transmission in the layer 4 to layer 2/3 pathway*"), but with the following modifications: 1) no perfusion pipette was used, 2) CPP was not included, and 3) the ACSF contained the AMPAR antagonist, DNQX (20 µM, Sigma), and the $GABA_AR$ antagonist, picrotoxin (100 µM, Sigma). Single EPSCs were collected at +40 mV separated by 15 second intervals. For analysis, responses were filtered with a 15 point box average (±1.4 ms window) – this had no detectable affect on EPSC amplitude on these slower NMDAR-mediated responses. Tau decay values (Fig. 2.3E) were obtained by the time between response peak and the first point at which the response was 1/e of peak.

Immunocytochemistry

Dissociated cultures of cortical neurons were prepared from electroporated P0-1 *Grm5*$^{flx/flx}$ pups as described previously (Niere et al 2012). Staining was performed at equivalent day (ED) 8 and 14, which equals the number of days since the birth of the pups (p0). Antibodies were for GFP (1:500, chick, Aves lab, #GFP-1020; secondary: Alexa488, Invitrogen, #A11039) and mGluR5 (1:1000, rabbit, Millipore, #AB5675; secondary: Alexa555, Invitrogen, #A21430). Fluorescence images were acquired on a Nikon TE2000

microscope with a cooled CCD camera (CoolSnap HQ; Roper Scientific) and quantified with Metamorph software (Molecular Devices). mGluR5 signal was measured in 50 µm sections of proximal dendrites (3 dendrites averaged per cell) and a total mGluR5 signal was quantified by the following sequence: 1) thresholding the signal by either 1.5X (ED8) or 2X (E14) the background, 2) obtaining the average intensity of the detected puncta, and 3) multiplying this average by the total area covered by all the puncta area. Untransfected and Cre-GFP expressing neurons were imaged in the same cultures and each culture was imaged entirely in one session. Imaging parameters remained constant within each imaging session.

Dendritic morphology

Whole-cell recordings of single L2/3 neurons were performed as described except that biocytin (4 mg/ml, Sigma) was added to the pipette solution. Only Cre-GFP expressing cells in wild-type (WT) or *Grm5*$^{flx/flx}$ mice were examined. Recordings lasted 10-20 minutes to allow the biocytin to perfuse into the dendritic tree, and subsequently, slices were incubated in a recovery chamber containing ACSF for at least 30 minutes. Slices were then put into cold 4% paraformaldehyde for 12-18 hours. After processing of tissue as described (Gibson et al. 1999), neurons were traced and projected into a single plane, and from this, a Sholl analysis was applied to measure branch crossings every 10 µm (Jin et al. 2003; Sholl 1956). Dendritic length was measured using Neuron J. Total dendritic length was measured in both entire compartments (basal and apical) and within a 40 µm annulus beginning 30 µm from the center of the soma. The annulus measurement was only applied to the basal compartment of the dendritic arbor.

Statistics

All statistics were performed using Graphpad Prism software (Systat Software, Inc.), and statistical significance was $p<0.05$. A paired t-test was applied to all comparisons of 2 groups unless stated otherwise. All comparisons involving greater than 2 groups were made using 2-factor ANOVAs with the genotype factor (UT vs. Cre) a repeated measures dimension unless stated otherwise. For connectivity frequency, comparisons were made with a χ-square test. F-I curves were compared using 2-factor ANOVAs with repeated measures in both genotype and current step dimensions. For the morphology data, dendritic complexity was compared using a 2-factor ANOVA with repeated measures in the distance dimension, and total dendritic length was compared using an unpaired t-test. Measurement error is ±SEM except for connectivity frequency which is 95% confidence interval (CI) (Clopper-Pearson method).

Results

Grm5 deletion in individual L2/3 neurons with Cre-expression

I performed *in utero* electroporation of E15 embryos to express Cre-GFP in a subset of L2/3 neurons in *Grm5*$^{flx/flx}$ mice (Fig. 2.1A). By inspection during experiments, all somas had the size and shape of pyramidal neurons. All Cre-expressing neurons displayed a regular spiking firing pattern (McCormick et al 1985), and when they were presynaptic in a cell-to-cell connection to any other neurons that were simultaneously recorded (23 times out of a total of 341 potential connections tested, see Fig. 2.4C), the connection was always

excitatory (see Methods, "*Unitary EPSCs*"). This observation together with previous studies demonstrating that the *in utero* electroporation technique selectively targets excitatory neurons (Adesnik & Scanziani 2010, Saito 2006) indicated that all Cre-expressing neurons in this study were excitatory. The untransfected (UT) neurons in our experiments had the same electrophysiological properties indicating that they were predominantly excitatory neurons as well.

To determine how effective this approach was at removing mGluR5 (the protein product of *Grm5*) from individual neurons, I performed immunocytochemical staining of mGluR5 in dissociated cortical neuron culture (Fig. 2.1B). The dissociated culture preparation was used because the clear mGluR5 antibody staining enabled quantification in individual neurons, which was not possible in brain sections. Using this method, I observed that removal of mGluR5 occurred in Cre-GFP expressing cells by equivalent day 8 (ED8, corresponding to 8 DIV). *In vivo*, mGluR5 expression has previously been shown to increase until it peaks around P14 (Wijetunge et al 2008), and similarly, I found greater mGluR5 expression in cultured UT neurons at ED14 (Fig. 2.1C).

Up-regulation of action potential-independent synaptic release

I first determined if glutamatergic synaptic function was affected by *Grm5* deletion by examining miniature EPSCs (mEPSCs) – action potential-independent, spontaneously occurring EPSCs. At three different ages (P9-11, P14-17, P20-24), I performed simultaneous recordings of untransfected (UT) and Cre-GFP expressing (Cre) pyramidal neuron pairs in L2/3 (Fig. 2.2A,B). Because I measured mEPSCs at -65 mV and the bath contained a

$GABA_AR$ antagonist (see Methods), mEPSCs were predominantly mediated by AMPARs. Kainate receptors were probably not involved (see Methods). I observed effects based on both neuron genotype and age (Fig. 2.2C,D; 2-factor ANOVA, $p<0.05$ for main effect of both cell genotype and age). Most notably, Cre-expressing neurons displayed increased mEPSC frequency but unchanged mEPSC amplitude. Changes were not resolved within a single age group. As an alternative analysis, I pooled the data over all ages (Fig. 2.2C,D, *right*) which revealed a 31% increase in mEPSC frequency with Cre-expression ($p<0.05$, paired t-test). In control experiments performed in WT P14-20 mice, no changes in mEPSC frequency (1.33±0.42 vs. 1.34±0.78 Hz, UT vs. Cre, N=26 pairs) or input resistance (183±20 vs. 218±20 MΩ, N=26 pairs) were observed indicating that Cre-expression alone had no detectable effects. While it is not clear whether the increase in frequency represents an increase in synapse number or in presynaptic release probability, this finding suggests that mGluR5 may function to suppress action potential-independent glutamatergic synaptic function.

Down-regulation of evoked transmission in the L4→L2/3 synaptic pathway

Because mEPSCs and action potential-dependent synaptic transmission may be independent processes (Ramirez & Kavalali 2011), I next examined glutamatergic synaptic transmission evoked by presynaptic action potentials. I first examined AMPAR-mediated synaptic transmission onto L2/3 pyramidal neurons originating from presynaptic neurons in layer 4 (L4). I simultaneously recorded from L2/3 untransfected and Cre-expressing neuron pairs, and afferents from layer 4 were stimulated with a metal electrode (Fig. 2.3A). Local

application of a $GABA_AR$ antagonist was applied to decrease contamination by disynaptic inhibition (see Methods). I compared the amplitude of the first EPSC in a 2 pulse train (Fig. 2.3B), and I observed effects based on both neuron genotype and age (Fig. 2.3C, *left*; 2-factor ANOVA, $p<0.05$ for main effect of both cell genotype and age). Unlike mEPSCs, evoked transmission in the L4→L2/3 pathway was decreased by *Grm5* deletion as observed by decreased EPSC amplitude in Cre-expressing neurons. No amplitude change was resolved within the individual age groups. Pooling the data over all the age groups revealed a 24% decrease in EPSC amplitude (Fig. 2.3C, *right*; $p<0.05$, paired t-test).

I observed no changes in the paired pulse ratio (Fig. 2.3D) or in CV (0.28 ± 0.03 vs. 0.29 ± 0.02; UT vs. Cre, n=40) indicating that presynaptic release probability is not greatly altered in this pathway. No change in EPSC kinetics was observed (width at half-height: 9.5±0.5 vs. 8.9±0.6 ms, UT vs. Cre; n=33; p = 0.08; decay time constant: 15.8±0.6 vs. 15.0±0.5 ms, UT vs. Cre; n=33; p=0.15).

In a separate set of experiments performed at P14-16, I measured NMDAR-mediated synaptic currents in complete isolation from AMPARs and $GABA_ARs$ since the antagonists DNQX (20 µm) and picrotoxin (100 µm) were included in the ACSF. I observed no changes in NMDAR-mediated transmission with *Grm5* deletion since both the amplitude and waveform kinetics of the EPSCs were unchanged (Fig. 2.3E, statistical power = 0.75). For the kinetics, both decay time constant (Fig. 2.3E) and width at half-height (157±7 vs. 169±6 ms, UT vs. Cre; n=43; p=0.18) were unchanged. In summary, deletion of *Grm5* resulted in the selective decrease in evoked AMPAR-mediated synaptic transmission, but not in NMDAR-mediated transmission.

I next determined if any changes in feedforward inhibition accompany the decrease in excitation in order to better understand the network impact of mGluR5 regulation. It is important to note that the site of regulation for disynaptic IPSCs would be in the recorded excitatory neuron since inhibitory neurons are not transfected with our *in utero* electroporation. I performed the same experiment except did not perfuse the $GABA_AR$ antagonist onto the recorded cells. Neurons were voltage-clamped at -45 mV and evoked postsynaptic currents were biphasic with an early downward component followed by a later upward component (Fig. 2.4A). The early downward peak represented the EPSC, but probably was altered due to superposition with disynaptic IPSCs. The later upward peak represented the disynaptic IPSC with less contribution from the EPSC since our experiments with $GABA_AR$ antagonist perfusion indicated that approximately 25% of the EPSC remained at the latency for this second later peak. For each experiment, I measured each peak and calculated the I:E ratio for both the UT and Cre neuron (Fig. 2.4B). On average, the ratio increased for Cre neurons (Fig. 2.4C, $p<0.05$). While I cannot resolve whether this was due to any changes in IPSC amplitude, it suggests that our observed decrease in EPSCs is accompanied by a shift in the balance in this pathway that favors disynaptic inhibition.

Down-regulation of evoked transmission in the local L2/3→L2/3 pathway

Perhaps the discrepancy between the upregulation of AMPAR-mediated transmission with *Grm5* deletion observed with mEPSCs and the down-regulation of L4→L2/3 evoked transmission can be explained by the L4→L2/3 pathway being uniquely down-regulated while evoked transmission in other synaptic pathways onto the same L2/3 neurons are up-

regulated. I tested this possibility by examining the effects of *Grm5* deletion on local AMPAR-mediated input from neighboring L2/3 pyramidal neurons – the local L2/3→L2/3 pathway. I performed simultaneous "pre/post" recordings of up to 4 neurons to examine unitary EPSCs (uEPSCs) from neighboring, single presynaptic pyramidal neurons (Fig. 2.5A) in the P15-30 age range. These uEPSCs were most likely AMPAR-mediated (see Methods). Unless stated otherwise, analysis was performed on data pooled across all ages. For each trial, a train of 4 presynaptic action potentials (50 ms interval) was applied (Fig. 2.5B, Neuron 1).

I first examined connection frequency which is the percent of one-way functional connections observed out of all possible one-way connections (see Methods). When plotted as a function of pre- and postsynaptic Cre-expression (Fig. 2.5C *left*), any connection involving a Cre-expressing neuron had a decreased connection frequency compared to UT pairs. Most notably, just as postsynaptic Grm5 deletion weakened the L4→L2/3 projection, postsynaptic *Grm5* deletion also weakened the local L2/3→L2/3 pathway through a connectivity frequency decrease. I also performed an additional analysis of these connectivity frequencies where the 4 genotypic pre/post combinations were sorted into 2 groups based on pre- or postsynaptic Cre-expression. This type of merging has been performed in previous studies using pre/post recordings (Hanson & Madison 2007, Patel et al 2014). Again, postsynaptic *Grm5* deletion decreased connectivity frequency (Fig. 2.5C *right*). Therefore, both analysis protocols show a clear effect of postsynaptic *Grm5* deletion. A similar grouping based on presynaptic expression was not significant ($p=0.16$) although a similar decreasing trend with Cre-expression was observed.

I detected no changes in uEPSC amplitude (Fig. 2.5D, uEPSC1 in the train) or duration (width at half-height: 9.1 ± 0.5 vs.11.7 ± 1.4 ms, UT vs. Cre; n=27,18; p = 0.06). Evidence of an increase in release probability was shown by more short-term depression with postsynaptic *Grm5* deletion (Fig. 2.4E, *left*, 2-factor ANOVA with main effect of postsynaptic genotype), but with an alternative analysis based on pooled data, this effect was not significant (Fig. 2.5E, *right*, paired t-test, Bonferroni correction=2). Therefore, even though uEPSC1 amplitude is unchanged, it is possible that increased release probability offsets a slight amplitude decrease. Coefficient of variation was not detectably altered (0.34 ± 0.04 vs. 0.31 ± 0.05; postsynaptic UT vs. Cre; n=20,14).

In summary, postsynaptic *Grm5* deletion in the local L2/3→L2/3 synaptic pathway resulted in a similar down-regulation of AMPAR-mediated transmission like that observed for the L4→L2/3 pathway suggesting that this decrease in evoked transmission is nonspecific and that the up-regulation observed with mEPSC frequency might be due to another, independent mGluR5-regulated process. Our data indicate that long-term mGluR5 function promotes the strength of evoked AMPAR-mediated transmission in multiple glutamatergic input pathways.

Decreased dendritic arbor with Grm5 deletion

To determine if the above changes in synaptic transmission possibly involved morphological changes, I examined how the dendritic tree of L2/3 pyramidal neurons was altered with *Grm5* deletion (Fig. 2.6A). I compared the morphology between Cre-GFP expressing neurons in WT versus *Grm5*$^{flx/flx}$ mice (P13-17, average age P15 and P15.3,

respectively). A Sholl analysis was performed to determine if the complexity of the dendritic tree was altered, and found a decrease in complexity with *Grm5* deletion (2-factor ANOVA, genotype x distance). To determine the locus for this decrease, I then split the Sholl analysis between basal and apical dendritic compartments. This revealed a decrease in dendritic complexity in the basal compartment in the 30-70 μm distance range from the soma (multi-comparison test, Fig. 2.6B,C). Interestingly, both the L4→L2/3 and L2/3→L2/3 synapses likely have strong overlap with this dendritic region (Feldmeyer et al 2006, Feldmeyer et al 2002) (see Discussion). To correlate this decrease in complexity with a possible reduction in available dendritic membrane, I measured the total dendritic length in basal and apical dendritic compartments. I observed no detectable alteration (Fig. 2.6D), but when I limited our analysis to the 30-70 μm range in the basal compartment where a decrease in complexity was observed, I did observe a length decrease with *Grm5* deletion (Fig. 2.6E). While the link between this decrease in dendritic arbor and our observed changes in evoked synaptic transmission is unclear, these data suggest that there is less dendritic membrane for synapses originating from the L4→L2/3 and L2/3→L2/3 pathways to make contact.

Increased Excitability with Grm5 deletion

Group I mGluRs are also known to regulate intrinsic membrane currents which, depending on the study, results in either increases or decreases in excitability (Carlier et al 2006, Ireland & Abraham 2002, Kalmbach et al 2013, Sourdet et al 2003a). Therefore, it was unclear how loss of mGluR5 might affect excitability. This was important to investigate since changes in excitability may be related to the synaptic alterations that I observe. To

measure excitability, I sequentially applied current steps (400 ms duration) of increasing amplitude under resting potential conditions and counted the number of action potentials evoked (Fig. 2.7A). From this I obtained curves of firing frequency as a function of current step amplitude (F-I curves). At all age groups examined, I found that Cre-expressing neurons were more excitable since their average F-I curves were significantly above that for UT neurons (Fig. 2.7B). This excitability increase may have been due to changes in suprathreshold currents since the subthreshold properties of input resistance and resting membrane potential were mostly unchanged (Fig. 2.7C,D). Therefore, synaptic changes are accompanied by clear increases in excitability, and these data suggest that mGluR5 activation normally functions to decrease membrane excitability.

Discussion

With chronic, cell-autonomous loss of mGluR5, AMPAR-mediated transmission in glutamatergic synaptic pathways is weakened when assayed with evoked transmission. This occurred in 2 separate pathways providing input to L2/3 neurons. On the other hand, NDMAR-mediated transmission was not affected. This indicates that the final outcome of all mGluR5-dependent processes (incorporating both acute and long-term processes) during postnatal development is to strengthen AMPAR-mediated synaptic transmission across multiple input pathways. Interestingly, excitability increased with loss of mGluR5 suggesting that mGluR5 regulation of nonsynaptic membrane currents may act to decrease excitability. These results indicate that mGluR5 may have a dual functional role in

maintaining normal excitability during development by having opposite effects on processes that impact excitability at synapses versus nonsynaptic ion channels.

The decrease in AMPAR-mediated transmission was somewhat surprising considering that acute Gp1 mGluR activation, with a strong contribution from mGluR5, is commonly observed to weaken synaptic pathways (Luscher & Huber 2010), particularly in the ones that I examined – L4→L2/3 (Bender et al 2006b) and L2/3→L2/3 (Zilberter et al 2009). As opposed to the short-term acute weakening examined in these earlier studies, our results suggest that there may be a longer term process *in vivo* involving mGluR5 that functions to strengthen synaptic pathways. But acute activation of mGluR5 may be involved as well. Acute strengthening, or long-term potentiation (LTP), by Gp1 mGluRs is observed in other cortical glutamatergic pathways (Anwyl 2009). In the L4→L2/3 pathway that I examined, mGluR5-mediated LTP has only been observed under special conditions where sensory input is removed by whisker trimming (Clem et al 2008) , but it is unclear how these conditions apply to our study.

Our findings may be considered analogous to that found for cell-autonomous loss of NMDARs (Adesnik et al 2008, Gray et al 2011). These glutamatergic receptors have many roles in plasticity and development, but one most notable role is in LTP. Therefore, NMDAR loss may be expected to weaken glutamatergic synaptic pathways. On the contrary, a prominent strengthening occurs suggesting that these receptors suppress synaptic pathway strength in the long-term. In our study, it appears that long-term mGluR5 function may paradoxically strengthen glutamatergic pathways by increasing AMPAR-mediated transmission. This paradox for mGluR5 is not entirely without precedent since a longer-term

process involving mGluR5 in the strengthening of layer 5 connectivity has been observed (Le Be & Markram 2006).

Our ability to effectively observe weakening in single pathways with postsynaptic *Grm5* deletion was made possible by our cell-autonomous approach and simultaneous recording of test and control. This and other observations in our study are consistent with some somatosensory cortex data obtained from the global *Grm5* KO mouse (Wijetunge et al 2008). In this previous study, spine number in L4 neurons were decreased in the *Grm5* KO mouse suggesting that glutamatergic pathways onto these neurons may be weakened in some form. Moreover, glutamatergic synaptic proteins obtained from cortical tissue were also decreased. Our paradoxical observation of increased mEPSC frequency has been observed in the *Grm5* KO mouse (Ballester-Rosado et al 2010).

On the other hand, some of our findings were not consistent with more global *Grm5* deletion. While I observe a decrease in dendritic length and complexity, other studies examining L4 neurons observed an increase or no change in the same properties (Ballester-Rosado et al 2010, Wijetunge et al 2008). I also did not observe alterations in NMDAR mediated synaptic transmission reported in the *Grm5* KO (Lu et al 1997, Matta et al 2011, She et al 2009). Therefore, some of these previously observed changes could stem from indirect effects of *Grm5* deletion. Alternatively, differences in our data could simply reflect the different cell types and synaptic pathways examined.

While evoked AMPAR-mediated transmission was weakened, it remains unclear how direct a role the loss of mGluR5 played. Just as with deletion of NMDARs in earlier studies, insight into synaptic specific function of these glutamate receptors is confounded both by

their clear affects on membrane potential and their established role in regulating nonsynaptic ion channels (Carlier et al 2006, Ireland & Abraham 2002, Johnston et al 2003, Kalmbach et al 2013, Sourdet et al 2003a). I find that loss of mGluR5 resulted in an intrinsic excitability increase, and previous studies have demonstrated that increases in chronic firing can result in depressed glutamatergic transmission through homeostatic processes (Turrigiano & Nelson 2004). Moreover, intrinsic excitability changes could alter evoked glutamatergic transmission through changes in spike-timing-dependent plasticity known to occur in the pathways I examined (Bender et al 2006b). Therefore, it is possible that the synaptic pathway weakening was caused by membrane excitability changes and not by loss of mGluR5 function at the synapse.

In the context of AMPAR-mediated transmission, it was interesting that action potential-*independent* mEPSCs were regulated in the opposite direction of that observed for action potential-*dependent* evoked transmission. This may be due to mEPSCs being regulated differently from evoked release (Ramirez & Kavalali 2011). While I prefer to stress the effects on evoked transmission since I think this would have a more direct impact on cortical processing, this opposite regulation of mEPSCs may impact more local, homeostatic processes that may also indirectly impact cortical processing (Sutton & Schuman 2009).

The substrate for the specific decrease in AMPAR-mediated, but not NMDAR-mediated, transmission with *Grm5* deletion is unclear. I observed no change in paired pulse ratio in the L4→L2/3 pathway and more short-term depression in the L2/3→L2/3 pathway (Fig. 2.3D and 4E). This suggests that a decrease in release probability does not underlie the

decreased responses. The receptor subtype composition appeared unaltered based on the lack of change in EPSC kinetics of both AMPAR- and NMDAR-mediated responses. Among many possibilities, I consider 3 scenarios: 1) synapse number is unchanged, but there is a decrease in the content of AMPARs at each synapse, 2) synapse number is unchanged, but there are a higher proportion of NMDAR-only, or "silent", synapses, and 3) there is a decrease in synapse number, but a compensatory increase in NMDARs at each synapse. The likelihood of scenario one is diminished since quantal amplitude of AMPAR-mediated mEPSCs was unaltered (Fig. 2.2D). The decrease in connectivity in the L2/3→L2/3 pathway with *Grm5* deletion is more consistent with scenarios 2 and 3 (Fig. 2.4C). If our observation of decreased dendritic length in the 30-70 μm range reflects a decrease in synapse number due to reduced membrane availability for synapses, this observation would be consistent with scenario 3. But there is no compelling evidence at this point to argue for a single one of these scenarios, and future study is needed to resolve this issue.

Mechanisms shaping dendritic and synaptic development are likely intertwined (Cubelos et al 2010, Xu et al 2011). Therefore, our observation of decreased dendritic length in the 30-70 μm distance range of the basal dendritic tree may be related to the decrease in AMPAR-mediated synaptic transmission. This particular basal dendritic region substantially overlaps with the region shown to contain the synapses originating from both the L4→L2/3 and L2/3→L2/3 pathways - 67±34 and 91±47 μm, respectively (mean±sd) (Feldmeyer et al 2006, Feldmeyer et al 2002). While these previously reported synaptic target regions are skewed to longer distances on the basal dendrites compared to our zone of decreased dendritic length, these previously reported data are based on older age rats (P17-23).

Therefore, the reported distances may scale down in the younger mice that I employ (P13-17) resulting in even stronger overlap between regions of synaptic targeting and dendritic length decrease.

Based on the sparse-cell deletion strategy used in this study and mGluR5's known functions at excitatory synpases, it is likey that the effects I observed on baseline evoked transmission are influenced at least in part by mGluR5's direct action on these synapses. However, mGluR5 has also been shown to be involved in a form of heterosynaptic plasticity affecting GABAergic transmission (Huang et al 2008). Interestingly, mGluR5 deletion may alter the L4-L2/3 microcircuit by enhancing inhibitory tone, as I observed an increase in the I:E ratio. Furthermore, the predominant form of synaptic plasticity at the L4-L2/3 synapse is spike-time-dependent plasticity (Feldman 2012). As precise timing of pre and post synaptic spikes is crucial to both the direction and magnitude of changes to synaptic function, expression of this form of plasticity is likely strongly influenced by both inhibitory control and membrane excitability. Therefore, while mGluR5's regulation of evoked transmission reflects in part its homosynaptic function, it is important to consider the contribution of its heterosynaptic and non-synaptic functions to this regulation as well.

Interestingly, delaying deletion of mGluR5 until later in postnatal development by injecting AAV-Cre-GFP into p1 *Grm5*$^{flx/flx}$ mice (see Chapter 3 Methods) seems to have very little effect on synaptic transmission (Appendix A). Based on previous reports using a similar deletion strategy, loss of protein product may not be complete until approximately p13 (Gray et al 2011). By staining for mGluR5 in dissociated cortical cultures generated from previously injected p0 pups, I observed that mGluR5 signal was virtual undetectable in

GFP expressing cells by p10 (data not shown). Therefore, I estimate that complete loss of mGluR5 occurs around p9. Although there was no detectable effect on spontaneous or evoked transmission when probed at either 2 or 3 weeks, a trend was observed in mEPSC frequency later in at 3 weeks ($p = 0.118$, Appendix A), suggesting that delaying removal of mGluR5 may simply delay the disruption of synaptic function.

Both the etiology and proposed treatments of a number of neurological disorders involve chronic, long-term alterations in mGluR5 function (D'Antoni et al 2014, Dolen et al 2007, Nickols & Conn 2014, Pomierny-Chamiolo et al 2014, Yin & Niswender 2014). Interestingly, our unexpected finding that AMPAR-mediated evoked transmission is weakened with *Grm5* deletion in light of the known role of mGluR5 in LTD is analogous to a paradox in synaptic phenotypes in the Fragile X model mouse – the *Fmr1* KO. In this mouse, there is enhanced acutely induced Gp1 mGluR-dependent long-term depression, but at the same time, there is increased synapse number and connectivity in the *Fmr1* KO (Bagni & Greenough 2005, Bear et al 2004, Huber et al 2002, Patel et al 2014). These contradicting phenotypes may both be due to enhanced mGluR5 signaling (Bear et al 2004, Dolen et al 2007). Our data support this speculated role for mGluR5 alterations in the *Fmr1* KO since long-term mGluR5 function promotes pathway strengthening, but at the same time, mediates acutely induced long-term depression.

Figure 2.1. *Grm5* deletion results in loss of the protein product, mGluR5, in cortical neurons

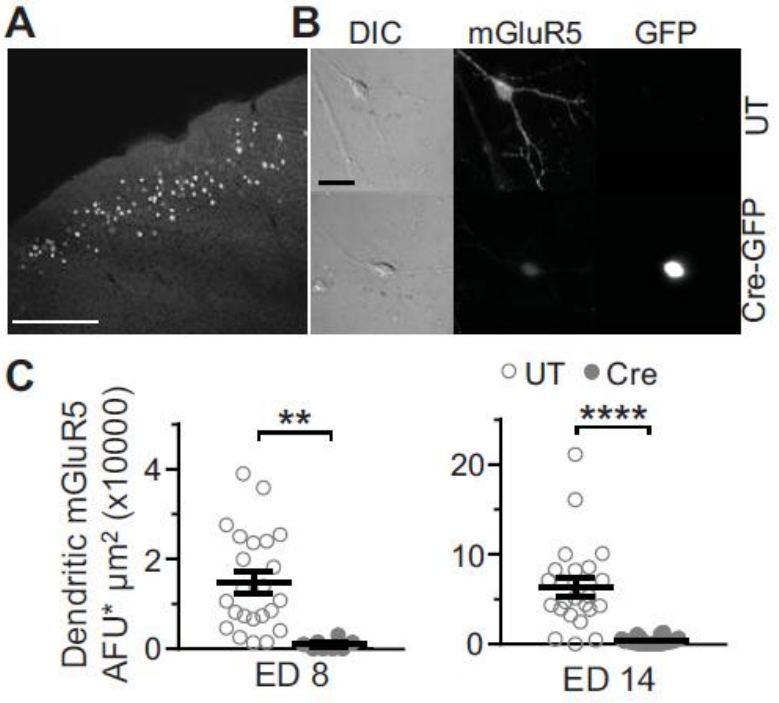

Figure 2.1. *Grm5* deletion results in loss of the protein product, mGluR5, in cortical neurons. A) Fluorescent image of a P15 acute slice showing Cre-GFP expression in a subset of L2/3 neurons (scale bar: 250 µm). B) Immunocytochemical staining for mGluR5 and GFP in dissociated neocortical cultures show removal of mGluR5 with Cre-GFP expression in individual neurons (ED8, scale bar = 20 µm). C) At both ED8 (*left*) and ED14 (*right*), mGluR5 expression is virtually undetectable with Cre expression in the population of neurons examined. ***, $p<0.001$.

Figure 2.2. mEPSC frequency is increased with *Grm5* deletion.

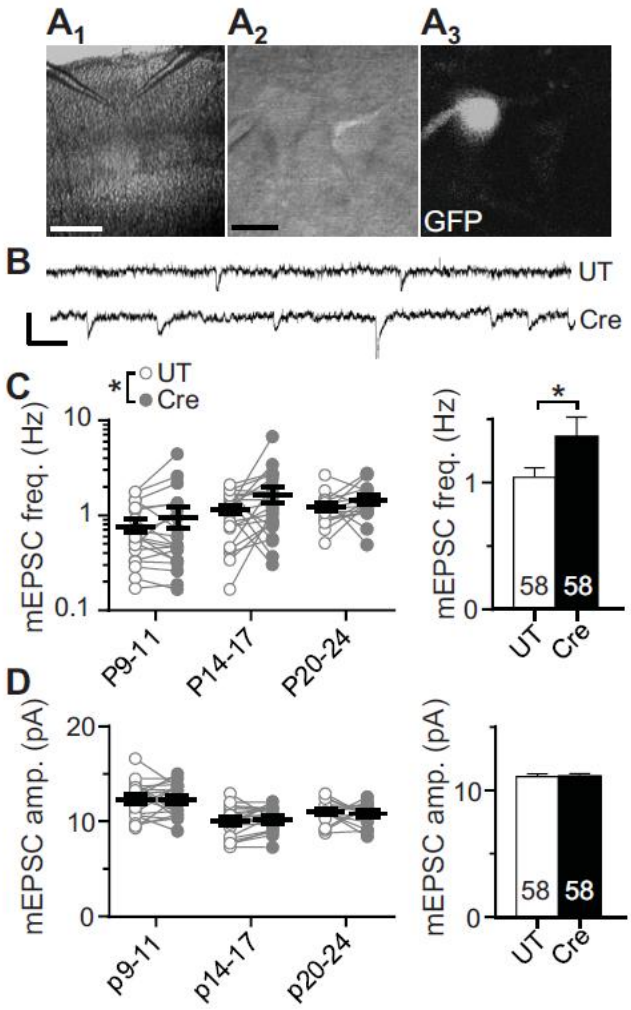

Figure 2.2. mEPSC frequency is increased with *Grm5* deletion. A) Images of the simultaneous recording of an untransfected (UT) and Cre-expressing (Cre) pair of L2/3 pyramidal neurons: 1) low power DIC, 2,3) high power DIC and GFP fluorescence, oriented with pia below cell pair. Scale bars are 200 µm (*white*) and 10 µm (*black).* B) Example traces of mEPSCs. Scale bar: 20 pA/50 ms. C) mEPSC frequency in recorded pairs in a scatterplot ordered by age (*left*) and pooled over all ages in a bar graph (*right*). In the scatter plot, statistical significance is derived from a 2-factor ANOVA with a main effect of cell genotype and in the bar graph, from a paired t-test. *, $p<0.05$. D) mEPSC amplitude in a scatterplot ordered by age (*left*) and pooled over all ages in a bar graph (*right*). For each age group, N ranges from 16-22. For bargraphs, total cell sample number is indicated inside the bars. For scatter plots, mean±SEM is in bold black.

Figure 2.3. Evoked transmission in the L4→L2/3 pathway is weakened with *Grm5* deletion.

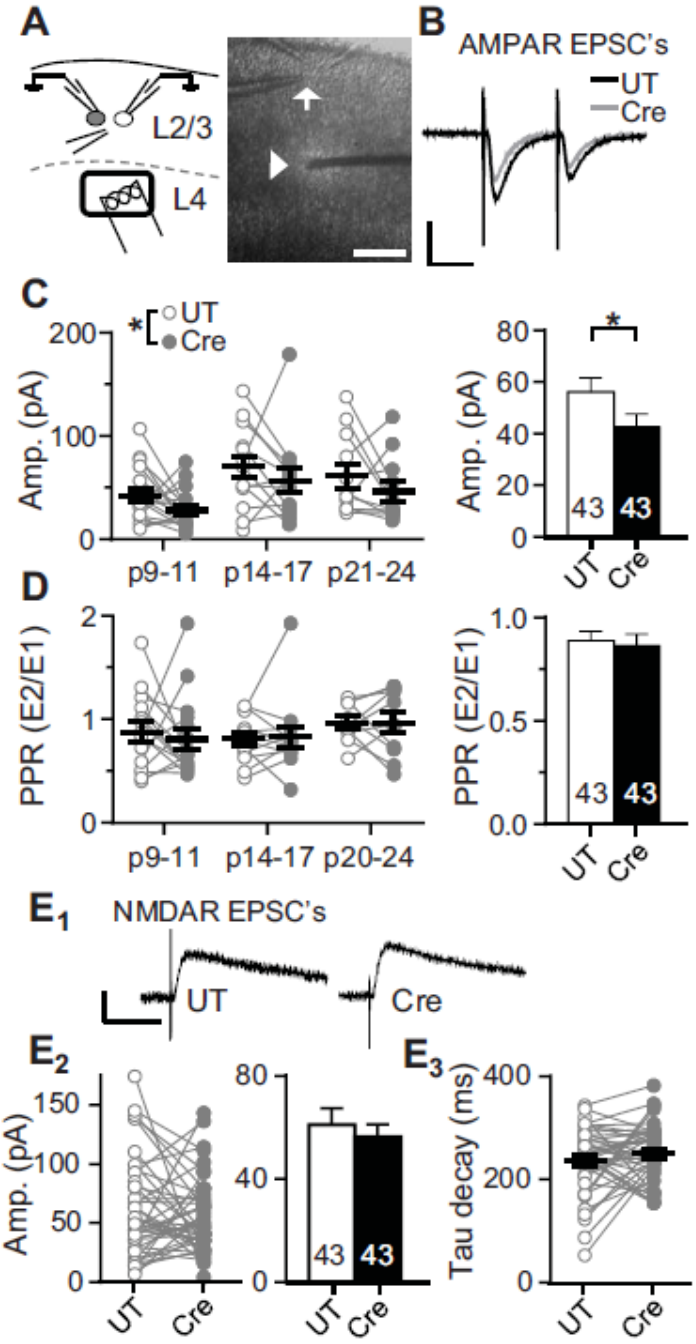

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Figure 2.3. Evoked transmission in the L4→L2/3 pathway is weakened with *Grm5* deletion. A) Cartoon schematic and DIC image of the experiment configuration with a stimulating electrode in L4 (*horizontal arrow*), and a perfusion pipette applying the $GABA_AR$ antagonist, picrotoxin, (*vertical arrow*), and 2 recording pipettes (*unmarked;* scale bar: 200 µm) in L2/3. B) Example traces of AMPAR-mediated evoked EPSCs with 2 pulses of stimulation (scale bar: 50 pA/50 ms). C,D) AMPAR-mediated EPSC amplitude (C) and pair pulse ratio (D) plotted by age in a scatterplot (*left*) and pooled over all ages in a bar graph (*right*). Statistics identical to that performed in Figure 2C. *, $p<0.05$. E) Sample trace averages from individual experiments examining NMDAR-mediated EPSCs (E1, Scale bar: 25 pA/100 ms) and scatter plots of their amplitude (E2; averages depicted in the bar graph) and tau (E3) decay showing no change with *Grm5* deletion. For bargraphs, total cell sample number is indicated inside the bars. For scatter plots, mean±SEM is in bold black.

Figure 2.4. Relative feed-forward inhibition is enhanced with *Grm5* deletion.

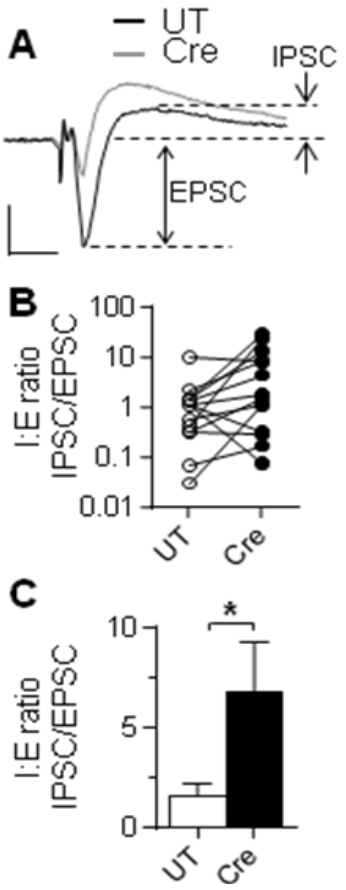

Figure 2.4. Relative feed-forward inhibition is enhanced with *Grm5* deletion.
A) Example traces of L4-evoked, biphasic responses consisting of an EPSC (early, downward component) and a disynaptic, feed-forward IPSC (late, upward component) in L2/3 cell pairs. B) Ratio of inhibition to excitation (IPSC/EPSC) for each cell plotted to show the relationship between each UT and Cre expressing cell pair (averaged in *C*). *, $p<0.05$.

Figure 2.5. Evoked transmission in the local L2/3→L2/3 pathway is weakened with Grm5 deletion.

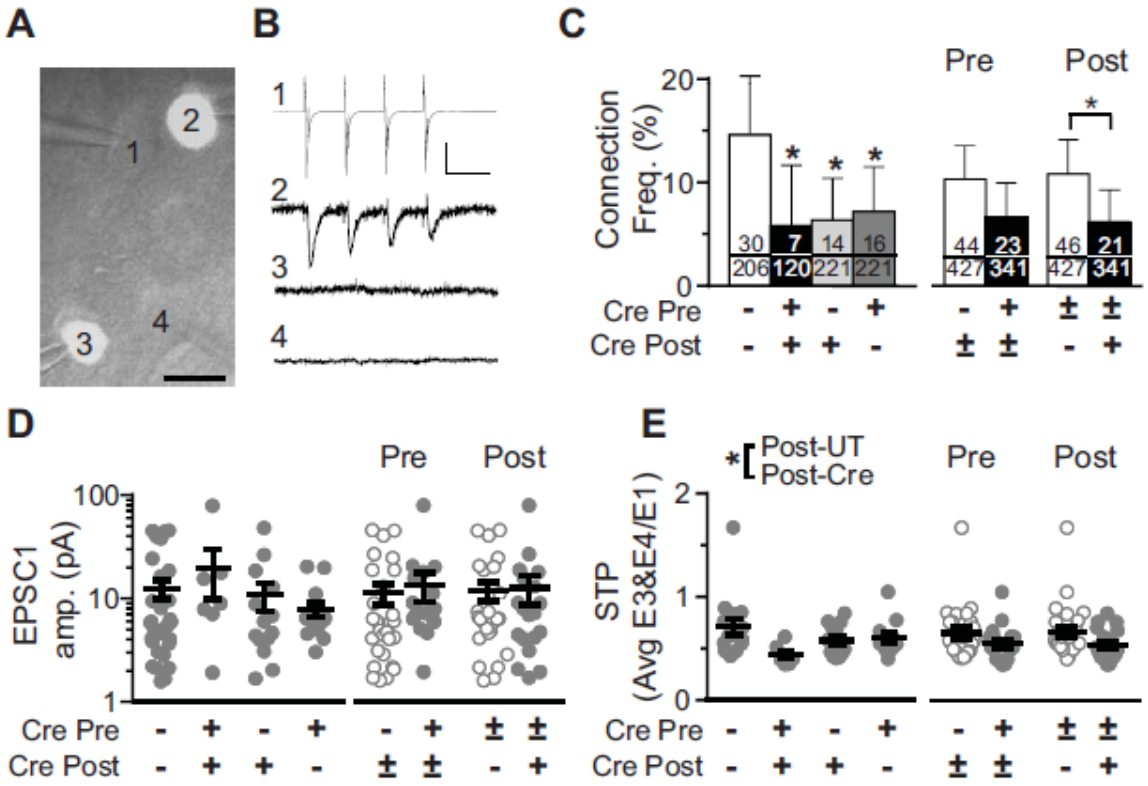

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Figure 2.5. Evoked transmission in the local L2/3→L2/3 pathway is weakened with *Grm5* deletion. A) Overlaid DIC and fluorescent image of the simultaneous recording of 4 L2/3 pyramidal neurons (scale bar = 10 μm). B) Sample traces obtained from the neurons in (A) where action potentials were evoked in neuron 1 and only neuron 2 displayed a functional unitary connection. Traces are averages except for that of neuron 1 which is a single trace. Scale bars: B1=1600 pA/50 ms, B2-4=10 pA/50 ms. C) Connection frequency plotted as a function of the genotypic combination of the pre- and postsynaptic neurons (*left*) and as a function of merged presynaptic and postsynaptic groups (*right*). Genotypic identity is indicated by the presence (+) or absence (-) of Cre in the pre- and postsynaptic cells (pooled data sets indicated with ±). Significance in left graph is based on chi-square comparisons to -/- combination with Bonferroni correction of 3. Significance in right graph based on chi-square with a Bonferroni correction of 2. D) uEPSC amplitude is unchanged based on either pre- or postsynaptic groupings. N's for genotype combination are (from left to right) 30, 7, 14, & 16 and for merged presynaptic and postsynaptic groups are 47, 23, 43, & 21. E) Short-term plasticity (STP, average amplitude of EPSC3 and 4 divided by EPSC1 amplitude) as a function of genotypic combination (*left;* N's (from left to right) are 14, 7, 12, & 13) and merged presynaptic and postsynaptic groups (*right*; N's (from left to right) are 26, 19, 27, & 18). In E, statistical significance is based on a 2-factor ANOVA with a main effect of postsynaptic genotype (*left*). *, $p<0.05$.

Figure 2.6. Complexity of the basal dendrites of L2/3 pyramidal neurons is decreased with *Grm5* deletion.

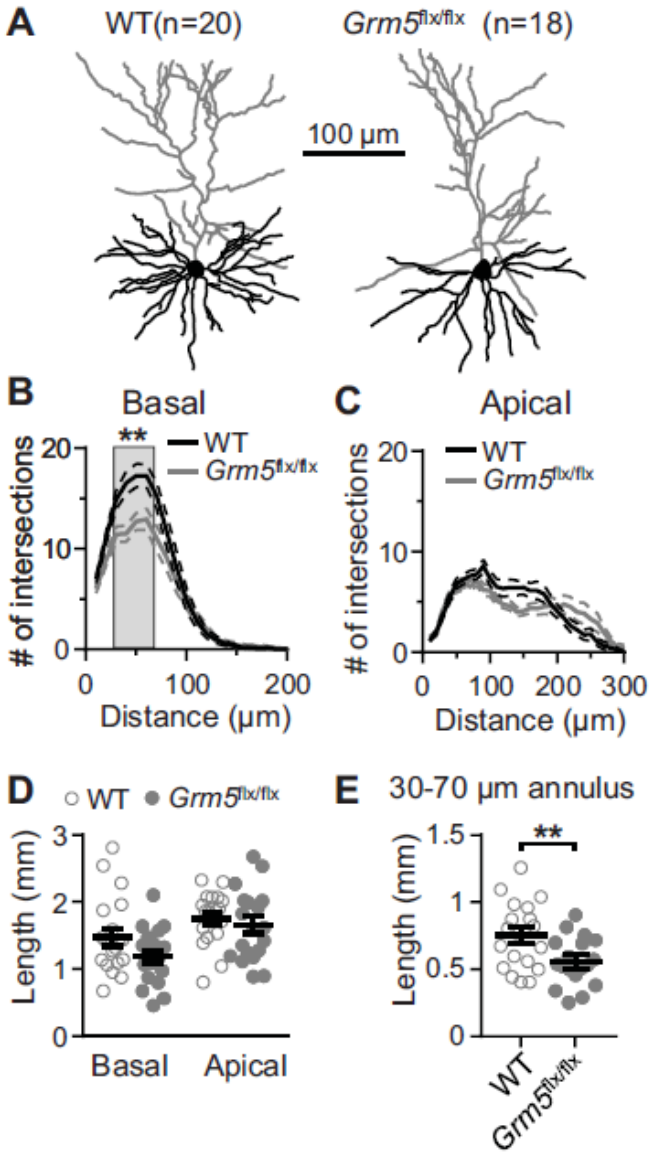


Figure 2.6. Complexity of the basal dendrites of L2/3 pyramidal neurons is decreased with *Grm5* deletion. A) Example tracings of filled Cre-expressing L2/3 pyramidal neurons in WT and *Grm5*$^{flx/flx}$ mice. The dendritic arbors are divided into basal (black) and apical (gray) compartments. B,C) Applying a Sholl analysis to assay dendritic complexity, a decrease in intersection number is observed with *Grm5* deletion in a limited region of the basal dendritic tree (B). 30, 40, 50, 60 and 70 µm distances from the soma had significant differences (indicated by shaded box). D) No differences in total dendritic length in basal or apical were resolved. E) Restricting length analysis of the basal compartment to an annulus 30-70 µm from the soma revealed a significant decrease. **, $p<0.01$.

Figure 2.7. Intrinsic excitability is increased with *Grm5* deletion while subthreshold membrane alterations are less pronounced.

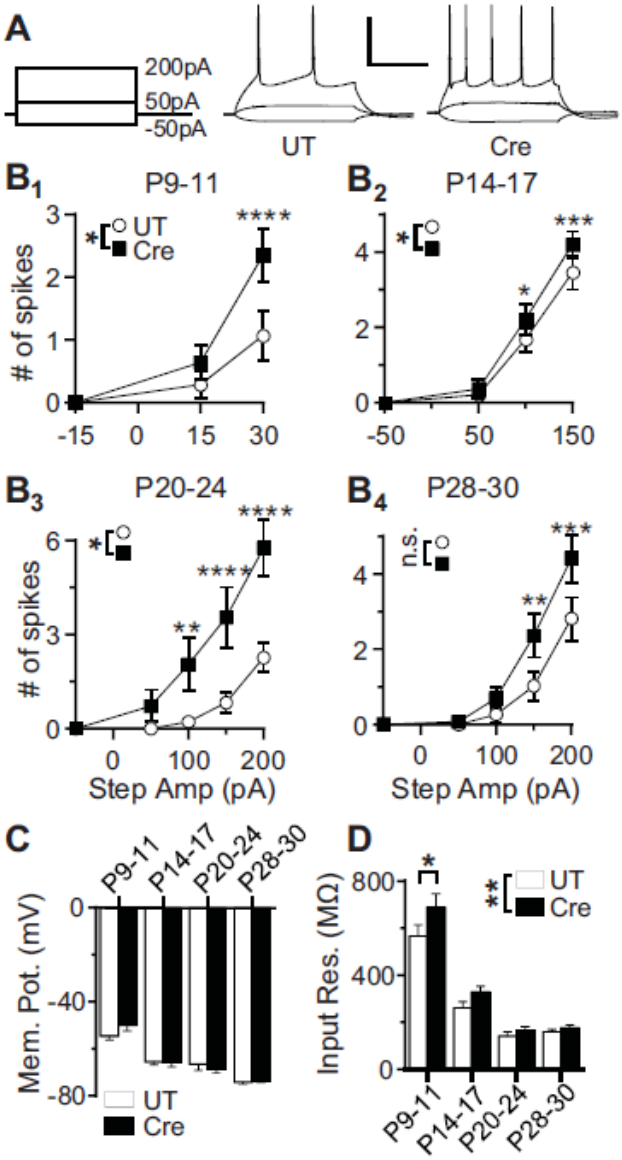


Figure 2.7. Intrinsic excitability is increased with *Grm5* deletion while subthreshold membrane alterations are less pronounced. A) Example traces (*middle, right*) of action potential firing in response to varying current steps (*left*) in a pair of UT and Cre expressing neurons. Scale bars: 50 mV/200 ms. B) F-I curves showing the number of spikes evoked with increasing current step amplitudes at the four timepoints tested (B_1-B_4). N's for B_1-B_4 are, respectively: 14, 19, 18, & 13. C) Average resting membrane potential ordered by age. D) Average input resistance ordered by age. ***, $p<0.001$, **, $p<0.01$, *, $p<0.05$.

CHAPTER THREE
Results

POSTSYNAPTIC MEF2C PROMOTES EVOKED GLUTAMATERGIC SYNAPTIC TRANSMISSION ONTO NEOCORTICAL LAYER 2/3 PYRAMIDAL NEURONS DURING DEVLEOPMENT

This chapter describes a project that was studied in collaboration with Kacey Rajkovich, who collected much of the data discussed herein. Contributions to data collection are noted in the figure legends with mine (KL) and/or Kacey's (KR) initials.

Introduction

Proper development of neural circuits is critical for an organism's capacity for learning and memory. One question in the development of circuits is how genetically programmed cell functions interact with sensory experience to result in mature circuits that are relatively consistent across animals. What set of genetically defined rules would result in a relatively standard circuitry considering the variability in sensory experiences among individual animals? Considering the remarkable similarities in circuit arrangement of certain brain areas even across species, it seems that the dictum set forth by the genetic code is descriptive enough to orchestrate the assembly of neural circuits, yet offers enough flexibility with which neural circuits can be adapted by experience without losing their general framework. Furthermore, this flexibility is important beyond the initial organization of neural circuits, as ongoing learning and memory throughout an animal's life requires circuit adaptation.

It is therefore of critical importance to identify and characterize the genetic factors that set up this remarkably adaptive set of rules. While identifying transcription factors involved in early components of development, such as differentiation and migration, is one critical piece of this puzzle, investigating transcription factors that are engaged by sensory experience to shape and modify circuit formation and synaptic function will offer additional insight to mechanisms of learning and memory that persist into adulthood. One intriguing candidate is the activity-dependent transcription factor MEF2. A role for MEF2 in regulating synaptic function in response to neural activity has been previously demonstrated (Flavell et al 2008). The nature of this regulation is unclear and likely contains both subtype and cell-type specific components, as different circumstances reveal roles for both MEF2-mediated suppression (Barbosa et al 2008, Wilkerson et al 2014) and enhancement (Li et al 2008) of synaptic function.

As a transcription factor, MEF2 regulates a multitude of cellular functions, the extent of which is currently unknown. Therefore, in an effort to circumvent potential network compensatory effects from globally deleting MEF2 genes in a large number of cells, I employ a sparse-population deletion strategy in which only a fraction (5-20%) of cells are manipulated by injecting a Cre-GFP AAV into neonates from either the *Mef2C*$^{flx/flx}$ or the *Mef2A/D*$^{flx/flx}$ background. Furthermore, this technique bypasses any potential perturbation of early developmental processes such as cell differentiation or migration as previous studies using this technique have observed that protein expression is not completely absent until p13 (Gray et al 2011). Therefore, this deletion strategy allows for the investigation of the cell-

autonomous roles of different MEF2 genes after early developmental events have already taken place.

Here, MEF2C is identified as the critical MEF2 genes in regulating synaptic function in somatosensory cortex, consistent with MEF2C being the most predominately expressed MEF2 family member in cortex (Lyons et al 2012a). In agreement with previous reports examining pyramidal cells of hippocampus and neocortex (Li et al 2008), I report a decrease in synaptic input in MEF2C deleted cells, suggesting MEF2C has a normal role in facilitating synaptic transmission. Furthermore, I establish a link between sensory experience and MEF2C-mediated regulation of synaptic function.

Materials and methods

Mice

EMX1-Cre mice (Gorski et al 2002), floxed *Mef2C* mice (*Mef2C*$^{flx/flx}$) (Arnold et al 2007) and floxed *Mef2A/D* mice (*Mef2A/D*$^{flx/flx}$) (Kim et al 2008, Naya et al 2002) were maintained on a mixed C57Bl6 and 129/SvEv background strain. EMX1-Cre mice express Cre in all cortical glia and excitatory neurons from E10.5 onward. For sensory deprivation experiments, mice were subjected to daily unilateral whisker trimming from p9-p18 where all whiskers on one side of the facial pad were maintained at a length of < 2 mm. The brain hemisphere corresponding to the 'deprived' facial pad was contralateral to the trimmed facial pad, as these sensory inputs predominately innervate the opposite hemisphere. All experimental procedures using these mice in this study were approved by the Institutional Animal Care and Use Committee at UT Southwestern.

Electrophysiology

Acute brain slices were prepared from *Mef2C*$^{flx/flx}$ mice (P20-P25) with the following procedure. Mice were anesthetized with Ketamine (125 mg/kg)/Xylazine (25 mg/kg) and the brain removed. All mice were perfused with cold dissection buffer before brain removal, and the dissection buffer for perfusion and slicing contained 1 mM kynurenate. Coronal slices, 250 μm thick, were cut at ~4° C in dissection buffer, placed in ACSF at 35° C for 30 minutes, and slowly cooled to 21° C over the next 30 minutes. Only slices containing the visible cytoarchitectonic barrels of somatosensory cortex were used. Whole-cell recordings were performed in layer 2/3 centered above a barrel hollow, and cells were targeted with IR-DIC optics in an Olympus FV300 confocal microscope. Recordings were performed at 21° C. Data were collected with a 10 kHz sampling rate and a 3 KHz Bessel filter. Neurons with MEF2C deleted were identified by GFP fluorescence (see *AAV postnatal injection* below).

Electrophysiology Solutions

ACSF contained (mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 25 dextrose, and 2 $CaCl_2$. All slices were prepared in the following dissection buffer (mM): 75 sucrose, 87 NaCl, 3 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 26 $NaHCO_3$, 20 dextrose, and 0.5 $CaCl_2$. All solutions were pH 7.4. ACSF was saturated with 95% O_2 / 5% CO_2. Unless stated otherwise, the pipette solution consisted of (mM): 130 K-Gluconate, 6 KCl, 3 NaCl, 10 HEPES, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, 14 phosphocreatine-Tris, 10 sucrose. This

was adjusted to pH 7.25 and 290 mOsm. The junction potential was ~10 mV and was not corrected. Therefore, actual membrane potentials are ~10 mV lower than that stated. With this pipette solution, the reversal potential for $GABA_AR$-mediated currents was ~-70 mV.

AAV postnatal injection

All neonatal stereotaxic injections were performed according to a previously described procedure (Adesnik et al 2008) with some modifications. Briefly, postnatal day 1 (P1) $Mef2C^{flx/flx}$ mice were anesthetized on ice and then fixed in a custom head mold prior to a single unilateral stereotaxic injection of AAV2.9-Cre-GFP (15 x 69-nL pulses @ 10^{12} titer, University of Pennsylvania Vector Core & Gene Therapy Program) targeting the lateral ventricle at a depth of approximately 1.2 mm into the brain with a beveled virus-filled pipette fastened to a Nanoject™ injector (Drummond Scientific, Inc.). Mice quickly recovered on a heating pad until regaining mobility and were then returned to the home cage until needed for electrophysiological experiments at age P20-P25.

Miniature IPSCs

Experiments were performed with the voltage-dependent sodium channel blocker, TTX (1 µM, Sigma). To block fast glutamatergic transmission, antagonists of NMDARs (CPP; 5 µM, Sigma) and AMPARs (DNQX; 20 µM, Sigma) were also added. These compounds together with the -65 voltage clamp effectively isolated GABAR-mediated currents. To further facilitate detection of GABAR-mediated currents, the chloride concentration of the internal solution was raised to 54 mM, thus driving chloride out of the

cell upon GABAR opening resulting in downward-deflecting responses in a -65 mV voltage clamp configuration. Miniature IPSCs (mIPSCs) were detected using a minimum amplitude and area threshold of 7 pA and 10 pA respectively.

Evoked AMPAR-mediated transmission in the layer 4 to layer 2/3 pathway

Simultaneous recordings of adjacent L2/3 pyramidal neuron pairs (< 50 µm intersomal distance) were performed where one neuron was GFP-negative, untransfected (UT) and the other was GFP-positive, Cre-expressing. A 2-conductor cluster stimulating electrode (FHC, Inc.) was positioned in layer 4 to stimulate afferents of the layer 4 to layer 2/3 (L4→L2/3) pathway (stimulation intensities ranged from 1-20uA). Biphasic pulses (200 µs, 1-10 µA) were applied to induce excitatory postsynaptic currents (EPSCs) in L2/3 neurons. To reduce the occurrence of polysynaptic responses, $[Mg^{++}]$ and $[Ca^{++}]$ were raised to 4mM in the ACSF. In all our experiments, kainate receptors were probably not involved based on their developmental profile and on the faster kinetics of the EPSCs observed (Contractor et al 2011) (see Results).

CPP was added to the bath to isolate AMPAR-mediated transmission. To better isolate the monosynaptic EPSCs, disynaptic inhibition was locally blocked by positioning a perfusion pipette containing the $GABA_AR$ antagonist, bicuculline methiodide (BMI, 5 mM), 5-15 µm above the slice surface at the location of the recordings. The tip of the perfusion pipette was 2-5 µm in diameter and 0.1-0.2 lbs/in² of positive pressure was applied using a pressure gauge. This has been done in previous studies examining this same L4→L2/3 pathway (Bender et al 2006b). The efficacy of the blockade was assessed by the observation

of IPSCs while holding the cell at -45 mV and increasing stimulation intensity. IPSCs could be easily observed as outward currents at -45 mV since the reversal for $GABA_AR$-mediated currents was in ~-70 mV in our recording conditions. I collected data when no IPSC was observed at -45 mV with a stimulation intensity that was 1.5x the threshold intensity for evoking EPSCs in both neurons – this generally resulted in responses where EPSCs were observed >50% of stimulation trials in both neurons. Inhibition was never completely blocked, but this procedure dramatically improved isolation and measurement of monosynaptic EPSCs. Four stimulation pulses with a 50 ms interval were applied for each trial, and each trial was separated by 4 seconds. Only EPSCs collected at -45 mV holding potential were analyzed in an effort to constantly monitor IPSC contamination. At this potential, baseline current was very stable and individual EPSCs clearly resolved.

Evoked NMDAR-mediated EPSCs

Simultaneous recordings of L2/3 pyramidal neuron pairs were performed as described above (see "*Evoked AMPAR-mediated transmission in the layer 4 to layer 2/3 pathway*"), but with the following modifications: 1) no perfusion pipette was used, 2) CPP was not included, and 3) the ACSF contained the AMPAR antagonist, DNQX (20 μM, Sigma), and the $GABA_AR$ antagonist, picrotoxin (100 μM, Sigma). Single EPSCs were collected at +40 mV separated by 15 second intervals. For analysis, responses were filtered with a 15 point box average (±1.4 ms window) – this had no detectable affect on EPSC amplitude on these slower NMDAR-mediated responses. Tau decay values (Fig. 3.2B) were obtained by the time between response peak and the first point at which the response was 1/e of peak.

In similar set of experiments investigating NMDAR subunit composition, ifenprodil (3 μM, Tocris) was washed into the bath after collecting a sweep of baseline NMDAR-EPSCs. Following a 20 minute wash-in period, a second sweep of NMDAR-EPSCs was collected.

Results

MEF2C is the critical MEF2 subtype in promoting synaptic function in the L4→L2/3pathway

The use of the Cre-GFP fusion protein facilitated identification of MEF2-deleted cells during electrophysiological experiments. Previous work using the same deletion strategy has demonstrated that Cre-GFP expression in cortical lysates from Cre-GFP-AAV injected mice is associated with MEF2 truncation (Appendix B) and consequent transcriptional inactivation (Arnold et al 2007). To more precisely characterize how different subtypes of MEF2 influence synaptic function, I probed evoked transmission at one of the most well-characterized synapses in barrel cortex, the L4→L2/3 synapse. While stimulating with an extracellular electrical stimulation electrode, responses were simultaneously recorded from Cre- (UT) and Cre-GFP expressing (Cre) cell pairs in mice from either the floxed *Mef2C* ($2C^{flx/flx}$) or floxed *Mef2A/D* ($2A/D^{flx/flx}$) background (Fig. 3.1A). This technique has 2 strong advantages over between-slice comparisons by 1) eliminating the variability introduced by factors such as slice angle and health, electrode placement, and column preservation and, 2) allowing optimal stimulation intensities to be used that elicit sizeable EPSCs while avoiding recruitment of polysynaptic activity. Additionally, a third pipet was added near (within 20 μm) the recorded L2/3 cell pair that locally applied the GABAAR

antagonist bicuculline to block GABAergic feedback inhibition and thereby isolate excitatory transmission.

The MEF2A and MEF2D subtypes do not contribute to the development of this synapse, as there was no effect of deletion of these 2 subtypes on EPSC amplitude or STP, a measure of presynaptic function (Fig 3.1B, C). Conversely, deletion of the MEF2C gene robustly reduces the strength of this input pathway. This regulation appears to be localized to the postsynaptic structure, as EPSC amplitude was reduced by 54% whereas STP was unaffected (Fig 3.1B, C). This subtype specific regulation of synaptic function is consistent with the previously observation that MEF2C deletion increased the frequency of mEPSCs events in the same cells, whereas deletion of MEF2A/D had no effect (data not shown). However, I was surprised to find that deletion of MEF2C results in decreased synaptic input, suggesting a role for MEF2C in promoting synaptic function. This finding is supported by the observation that MEF2C-deleted cells have reduced synaptic input from nearly all of the neocortical input pathways into L2/3, as demonstrated by synaptic input maps generated by uncaging glutamate with laser-scanning photostimulation (Figure 3.1D, E). Taken together, MEF2C is the critical MEF2 subtype in regulating synaptic function in L2/3 of barrel cortex, where it plays a role in promoting synaptic function.

MEF2C deletion down-regulates NMDAR-mediated transmission and subunit composition

I next explored the possibility that the reduced input from L4 reflects more 'silent', synapses, or synapses containing only NMDAR's with no AMPAR's, in MEF2C-KO cells, which would be more consistent with a deficit in synapse maturation than a reduction in

synapse number. To examine NMDAR-mediated synaptic responses, I pharmacologically isolated NMDAR-mediated EPSCs by applying antagonists of AMPAR's and GABAR's (DNQX and picrotoxin, respectively) and recorded responses to L4 electrical stimulation in simultaneously recorded L2/3 WT and MEF2C-KO cell pairs. Similar to the weakened AMPAR-mediated input, MEF2C-KO cells display a 59% reduction in NMDAR-EPSC amplitudes compared to WT (Fig 3.2A; paired t-test, $p < 0.0001$). This suggests that the reduced AMPA-mediated input observed in MEF2C-KO cells likely reflects a reduction in synapse number as opposed to a selective deficit in AMPAR-mediated transmission. Interestingly, I also observed changes in the kinetics of the NMDAR-mediated responses, such that responses in MEF2C-KO cells have faster decay constants on average (Fig 3.2B; 266.2 ± 9.878 ms vs 225.7 ± 14.5 ms, WT vs KO; $p < 0.01$). In the normal development of postsynaptic NMDARs in mouse barrel cortex, the subunit composition of the receptors change at the end of the first postnatal week from slower-decaying, predominately NR2B-containing receptors to faster-decaying, NR2A-containing receptors (Gray et al 2011). To determine if the faster kinetics observed in MEF2C-KO cells is indicative of a shift in the subunit composition of the NMDARs, I conducted a similar set of experiments with the addition of ifenprodil, an antagonist that specifically blocks NR2B-containing NMDARs. Wash-in of ifenprodil reduced the NMDAR-EPSC amplitude in WT cells by ~25% (2-factor ANOVA, significant effect of ifenprodil in WT by multiple comparison; $p < 0.01$), whereas wash-in had no significant effect on MEF2C-KO cells (Fig 3.2C). This supports the conclusion that the faster kinetics observed in MEF2C-KO cells reflects a shift in subunit composition such that MEF2C-KO cells have more NR2A containing receptors. In

summary, MEF2C deletion appears to result in a reduction of functional synapse number, as the reduction in AMPAR-mediated transmission is accompanied by a similar reduction in NMDAR-mediated transmission. Interestingly, MEF2C deletion may also alter some aspects of synapse maturation, as NR2A contributes more to NMDAR function in MEF2C-KO cells, although the consequence of this is unclear.

MEF2C reduces GABAergic transmission through a cell non-autonomous mechanism

Due to its implication in diseases involving network excitability, I considered the possibility that MEF2C is also impacting inhibitory tone. Experiments conducted by Kacey Rajkovich demonstrated that sparse-population deletion of MEF2C reveals no change in mIPSC frequency or amplitude compared to UT cells, suggesting MEF2C does not contribute to postsynaptic GABAR development (Fig 3.3B). However, although sparse-population deletion allows for a more precise examination of postsynaptic function that is free of compensatory network effects, it is less relevant to the disease state and may be a less effective strategy at probing retrograde regulation of presynaptic function. This type of regulatory role can be described as both cell-autonomous, highlighting the direct action of the cell being investigated, and second-order, pertaining to the necessary participation and modulation of a presynaptic partner. Because of this second-order dimension, manipulating just one cell may be insufficient to cause an observable change in the presynaptic cell, since other nearby WT cells may be able to deliver the putative retrograde signal and thereby compensate for the cell-autonomous perturbation. In this instance, manipulating a large number of postsynaptic cells may be necessary to drive observable changes in synaptic

function of the presynaptic cell. Furthermore, although probing evoked transmission at specific synapses can address these potential functions of MEF2C, this approach overlooks many other input pathways and cell types that may be differentially regulated.

To address this specific set of challenges, EMX1-Cre mice were crossed with *Mef2C*$^{flx/flx}$ mice to delete MEF2C in all excitatory cortical neurons (Gorski et al 2002) and subsequently WT inhibitory inputs onto L2/3 excitatory neurons were examined. Interestingly, MEF2C deletion results in an increase in both mIPSC frequency and amplitude at 2 weeks of age (+39% freq; +16% amp; Fig. 3.3D) that becomes more drastic at 3 weeks of age (+142% freq; +27% amp; Fig. 3.3D).

Taken together, these data suggest that MEF2C functions to regulate excitatory and inhibitory transmission in opposite directions by influencing different synaptic mechanisms. MEF2C promotes glutamatergic neurotransmission in a cell-autonomous manner that is specifically postsynaptic, while also suppressing GABAergic transmission, possibly by inhibiting presynaptic function through a retrograde mechanism.

MEF2C deletion occludes sensory deprivation induced weakening of the L4→L2/3 synapse

Sensory experience is critically involved in shaping circuit formation and synaptic function in all sensory cortices. In somatosensory cortex, depriving information by trimming the whiskers on the facial pad results in reduced L4 input onto L2/3 neurons in corresponding barrel columns (Bureau et al 2008). Considering that MEF2C transcriptional activity can be induced by neural activity (Flavell et al 2008), I tested the possibility that sensory experience strengthens synaptic input through a MEF2C-mediated mechanism. Again, EPSC's were

recorded in a simultaneously recorded WT and MEF2C-deleted cell pair of L2/3 in response to extracellular stimulation in L4 with a stimulation electrode while BMI was locally applied in L2/3. The contribution of sensory experience was tested by trimming all of the whiskers on one side of the facial pad daily from ages p9-p18. L2/3 responses were recorded in both hemispheres – one receiving normal sensory input (spared) and one receiving disrupted input from the corresponding trimmed whisker pad (deprived). Surprisingly, MEF2C deletion significantly weakened EPSC amplitudes recorded in both spared and deprived hemispheres (Fig3.4B), although this reduction was smaller in pairs from the deprived hemisphere on average (41% and 28% reduction in pairs from spared and deprived hemispheres, respectively). This result challenged my hypothesis that deprivation regulates the strength of this pathway through a MEF2C-dependent mechanism. However, due to the variability in stimulation between experiments that is introduced by factors such as electrode placement, depth, and impedance, the effect of deprivation on pathway strength could not be adequately examined in either WT or MEF2C-deleted cells. To overcome this variability, a similar set of experiments was conducted with one modification: instead of electrical stimulation with an electrode, cell bodies were stimulated by uncaging glutamate specifically in L4 with a UV laser. By setting a constant laser power for all experiments, this technique has the advantage of a standard stimulation intensity that remains constant across experiments, allowing for comparison of EPSC amplitudes both within and across experiments. Using this paradigm, sensory deprivation induced a detectable reduction in the average EPSC amplitudes of WT neurons. However, deprivation had no effect on the average EPSC amplitudes of MEF2C-deleted cells. Furthermore, MEF2C-deleted cells displayed a reduction in EPSC amplitude

compared to their paired WT cell in pairs from spared hemispheres, but not in pairs from deprived hemispheres (Fig 3.4C, D). This suggests that MEF2C is the signal through which sensory experience strengthens synaptic input. Therefore, when this signal is removed by deprivation of sensory experience, the pathway is weakened. However, in MEF2C-deleted cells, where the signal has already been removed, sensory deprivation has no effect.

Discussion

MEF2C is the critical MEF2 subtype that promotes excitatory synaptic function in neocortex

At the RNA level, MEF2C is the predominant MEF2 subtype expressed in the neocortex (Lyons et al 2012b). Consistent with this observation, MEF2C deletion profoundly affects synaptic function in somatosensory cortex, whereas the contribution MEF2A and MEF2D is insignificant. Interestingly, MEF2C seems to be promoting synaptic function, as postsynaptic deletion results in a weakening of the L4→L2/3 pathway. The locus of this regulation appears to involve changes in the postsynaptic compartment as presynaptic function is unaffected by deletion. Whether this decreased input involves a reduction in synapse number, a weakening of individual synapses, or a combination of the two remains unclear. Additionally, as AMPAR-mediated and NMDAR-mediated transmission were weakened to a similar degree in MEF2C-deleted cells (54% and 59% reduction in AMPAR and NMDA EPSC amplitudes, respectively), a role in silencing/unsilencing of synapses is unlikely.

A role for MEF2C in promoting synapse number has been previously demonstrated in brain-wide deletion models. Here, deletion is associated with a reduction in synapse number

in pyramidal cells of both hippocampus and neocortex (Li et al 2008). Conversely, granule cells of dentate gyrus display an increase in structural and functional synapse number, suggesting MEF2C is involved in suppressing synaptic function in these cells (Barbosa et al 2008). These bi-directionally effects likely highlight the variation in MEF2C function that is dependent upon the molecular and epigenetic composition among different cell types.

NMDAR prevalence and subunit composition is altered in MEF2C-deleted cells

In addition to decreased EPSC amplitude in NMDAR-mediated response, changes in kinetics were also observed, suggesting NMDAR subunit composition is regulated by MEF2C. Importantly, AMPA-mediated currents were unaffected by deletion (width at half-height for UT vs Cre = 6.802 ± 0.992 vs 6.715 ± 1.977, $p = 0.865$), eliminating the possibility that the observed kinetic changes in NMDAR-EPSC are due to altered spatial distribution of synapses along the dendritic arbor. The nature of this subunit change suggests that MEF2C normally either suppresses NR2A expression or promotes NR2B expression, as the faster kinetics and relative insensitivity to ifendprodil observed in the KO cells are consistent with a subunit composition biased towards NR2A. While the consequence of this type of shift in subunit composition on synaptic function is unclear, the developmental subunit switch is a critical step in the synapse maturation process, and proper stoichiometry of NR2A and NR2B is thought to be critical for the rules dictating plasticity induction (Liu et al 2004). Therefore, determining if NR2B or any associated genes are transcriptional targets of MEF2C will be an interesting avenue of research.

MEF2C down-regulates GABAergic transmission

When MEF2C is ablated in a sparse-population of neurons using AAV-mediated deletion, mIPSCs are unaffected. Among other things, this indicates that MEF2C does not directly regulate postsynaptic strength of inhibitory synapses onto pyramidal cells. While this finding may also seem to suggest that postsynaptic MEF2C has no role in retrogradely modulating presynaptic function, the possibility of a retrograde signal being compensated for by neighboring WT cells cannot be eliminated. In consideration of this possibility, one interesting target gene of MEF2 is BDNF (Flavell et al 2008), a gene whose transcriptional regulation is most strongly associated with the MEF2C subtype (Lyons et al 2012b). BDNF has been shown to transfer from postsynaptic to presynaptic compartments in an activity dependent manner (Kohara et al 2001). Furthermore, postsynaptic compartments of hippocampal pyramidal cells release BDNF to weaken inhibitory transmission by retrogradely suppressing presynaptic function of GABAergic terminals (Wardle & Poo 2003). Similarly, BDNF plays a critical role in heterosynaptic plasticity at the L4→L2/3 synapse in visual cortex via retrograde activation of presynaptic TrkB receptors (Huang et al 2008). Based on these converging lines of evidence, I propose a hypothetical model in which neuronal activity drives MEF2C-mediated transcription of BDNF in the postsynaptic cell, which travels retrogradely from the postsynaptic compartment to activate TrkB receptors on presynaptic terminals of inhibitory neurons and consequently suppresses GABA release from these terminals. In MEF2C-deleted cells, this retrograde signal is absent, resulting in a loss of this negative regulation of GABAergic transmission. In the AAV-mediated sparse-population deletion model, this form of regulation is intact in the majority of

excitatory cells. The prevalence of this retrograde signal from the WT cells in this circumstance may be able to compensate for the deficient signal in the KO cells, which would explain why mIPSCs are unaltered in this model. However, in the case where MEF2C is deleted in all excitatory cells, the retrograde signal is irrevocably lost and consequently GABAergic transmission cannot be normally suppressed. Further experiments are needed to validate this model (see Chapter 4).

MEF2C regulates synaptic function in response to sensory experience

The relationship between sensory experience and proper circuit formation has been well-established. Consistent with this notion, disrupting either MEF2C or sensory experience reduces the strength of the L4→L2/3 pathway, as demonstrated by the laser-scanning photostimulation (LSPS) maps. These experiments also demonstrated that MEF2C is necessary for sensory experience-mediated enhancement of this pathway, as deprivation in both WT and MEF2C-deleted cells reduced pathway strength to the same level as MEF2C-deleted cells in a spared context. This question could not be addressed using electrical stimulation experiments, as pathway strength could not be compared between spared and deprived contexts.

On the other hand, whether MEF2C can promote pathway strength through an additional, sensory experience-independent mechanism remains unclear. Data obtained from the LSPS maps suggests that MEF2C regulates pathway strength exclusively in response to sensory experience, as MEF2C-deletion had no effect in a deprived context. Furthermore, both sensory deprivation and MEF2C-deletion reduce pathway strength to the same degree

compared to WT spared cells, consistent with these 2 manipulations functioning in a mutually exclusive manner. However, using electrical stimulation to probe the L4→L2/3 pathway revealed that MEF2C-deletion can reduce pathway strength even in the absence of sensory experience, suggesting MEF2C also regulates pathway strength, at least in part, through sensory experience-independent mechanisms. Taken together, these experiments demonstrate a clear requirement for MEF2C activity in sensory experience-mediated pathway strengthening. However, whether MEF2C can function through additional experience-independent mechanisms remains unclear.

These disparate findings may reflect subtle differences in the two techniques used. The primary difference is the blockade of GABAergic transmission in experiments using electrical stimulation. Sensory deprivation has been linked with changes in inhibitory circuitry in both visual and somatosensory cortex (Kuhlman et al 2013, Li et al 2009). Although the consequence of these changes is unpredictable in the deprivation paradigm used here, if sensory deprivation leads to a strengthening of inhibitory drive onto WT cells preferentially, then blocking GABAergic transmission would be expected to enhance EPSC amplitude in these cells, which is consistent with the technique-specific effects we observe. Furthermore, potential deprivation-induced changes in the excitability of L4 neurons that alters their firing properties may also introduce technique-specific effects. For instance, if deprivation raises the threshold to fire an action potential, the trial-to-trial incidence of eliciting a unitary connection might be expected to increase. This increased incidence would be more prominent when using a constant stimulation intensity, as is done with UV uncaging of glutamate, but could be circumvented in experiments using electrical stimulation where

stimulation intensity is determined experimentally. Therefore, if MEF2C mediates its effects on synaptic function by increasing the number of connections rather than the individual strength of each connection, this difference might only be observed with an experimental paradigm that strongly activates the input pathway (i.e. electrical stimulation).

Figure 3.1. MEF2C is the critical MEF2 subtype in promoting synaptic input onto L2/3 neurons.

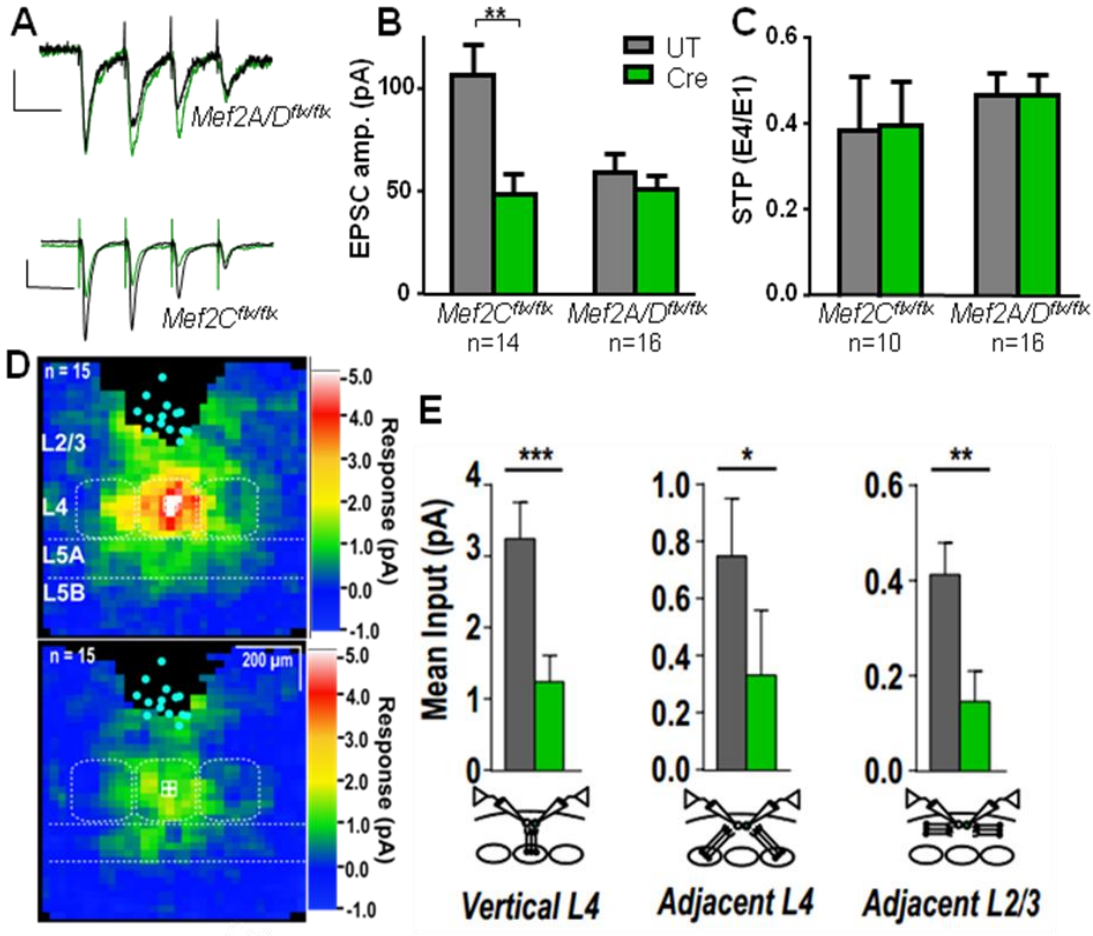


Figure 3.1. MEF2C is the critical MEF2 subtype in promoting synaptic input onto L2/3 neurons. EPSCs recorded in L2/3 neurons in response to extracellular stimulation. MEF2C-deleted cells display decreased EPSC amplitude compared to a simultaneously recorded UT cell in response to electrical stimulation in layer 4, whereas no change is observed in MEF2A/D-deleted cells (**B**; example traces in **A**); scale bars in **A** are 20pA x 50ms (*top*) and 50pA x 50 ms (*bottom*). STP is unaffected by deletion of any subtype (**C**). Using UV-LSPS of caged glutamate, response maps were constructed for L2/3 cell UT and Cre-expressing cell pairs (**D**), demonstrating reduced input from several pathways onto Cre-expressing cells (**E**). Electrical stimulation data collected by KL; LSPS (**D,** E) data collected by KR

Figure 3.2. MEF2C deletion weakens NMDAR-mediated synaptic transmission at L4→L2/3 inputs and alters the kinetics and subunit composition of NMDAR EPSCs.

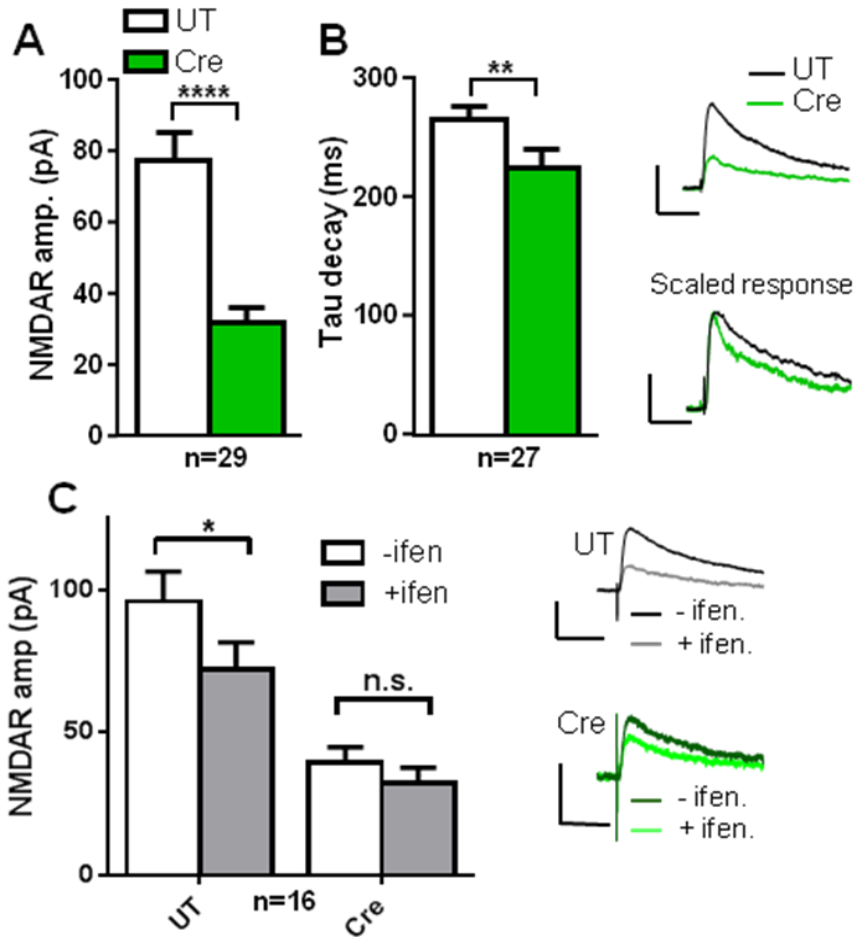


Figure 3.2. MEF2C deletion weakens NMDAR-mediated synaptic transmission at L4→L2/3 inputs and alters the kinetics and subunit composition of NMDAR EPSCs. **A.** NMDAR-mediated EPSC amplitudes are decreased in L2/3 Cre-expressing cells compared to untransfected (UT) cells in response to L4 stimulation (example trace on right, *top*; scale bar = 50pA x 100ms). **B.** Decay time constants of NMDA-responses are faster in Cre-expressing cells (scaled example trace to the right, *bottom*; scale bar = 50% x 100ms). **C.** Ifenprodil sensitivity of UT (left) and Cre-expressing cells (right). The NMDA-mediated response is reduced by ~25% after ifenprodil wash-in (gray bar; baseline=white bar) in UT cells, whereas there is no significant effect of ifenprodil on NMDA currents in Cre-expressing cells. (example traces to the right; scale bars are 50pA x 100ms and 20pA x 100ms for UT and Cre, respectively). All data from these experiments collected by KL.

Figure 3.3. MEF2C regulates inhibition through a potentially retrograde mechanism

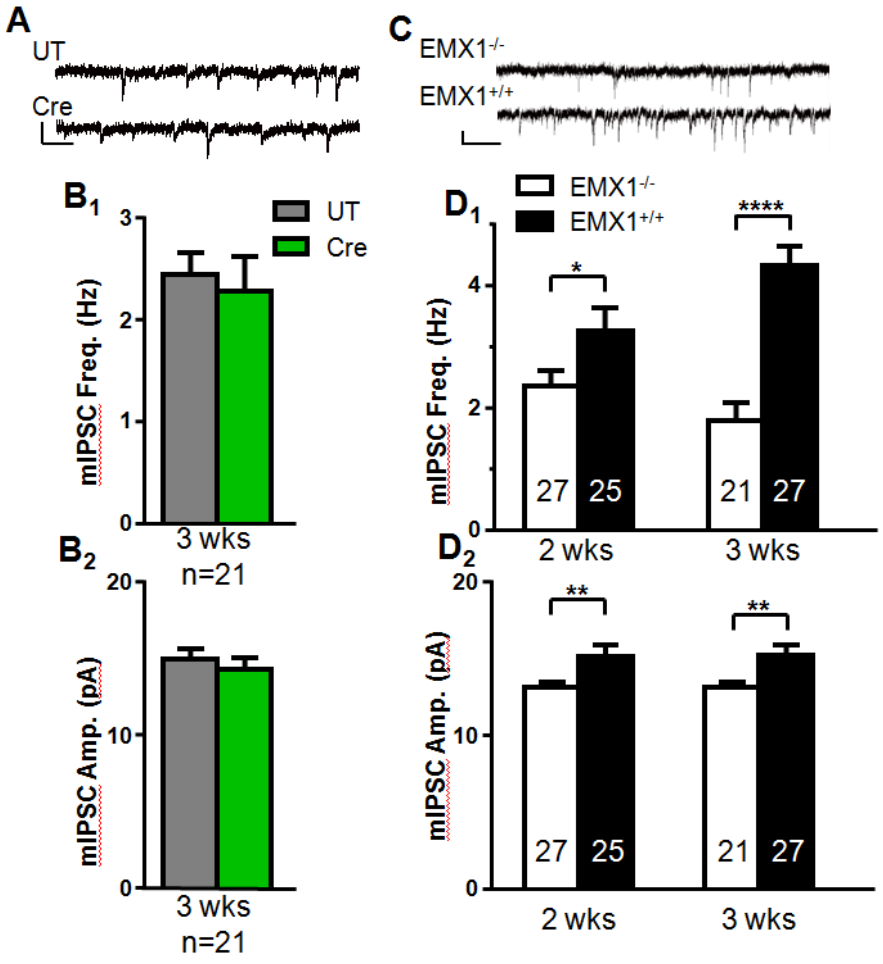


Figure 3.3 MEF2C regulates inhibition through a potentially retrograde mechanism. mIPSCs in L2/3 pyramidal cells are unaffected (**B_1 & B_2**) by MEF2C deletion when deletion is restricted to a sparse population of L2/3 neurons (example traces in **A**; scale bar is 10pA x 200ms). Conversely, mIPSC frequency (**D_1**) and amplitude (**D_2**) are increased when MEF2C is deleted in all excitatory cortical cells ($EMX1^{+/+}$) (example traces in **C**; scale bar is 10pA x 500ms).

Figure 3.4. Sensory deprivation weakens the L4→L2/3 pathway through a MEF2C-dependent mechanism

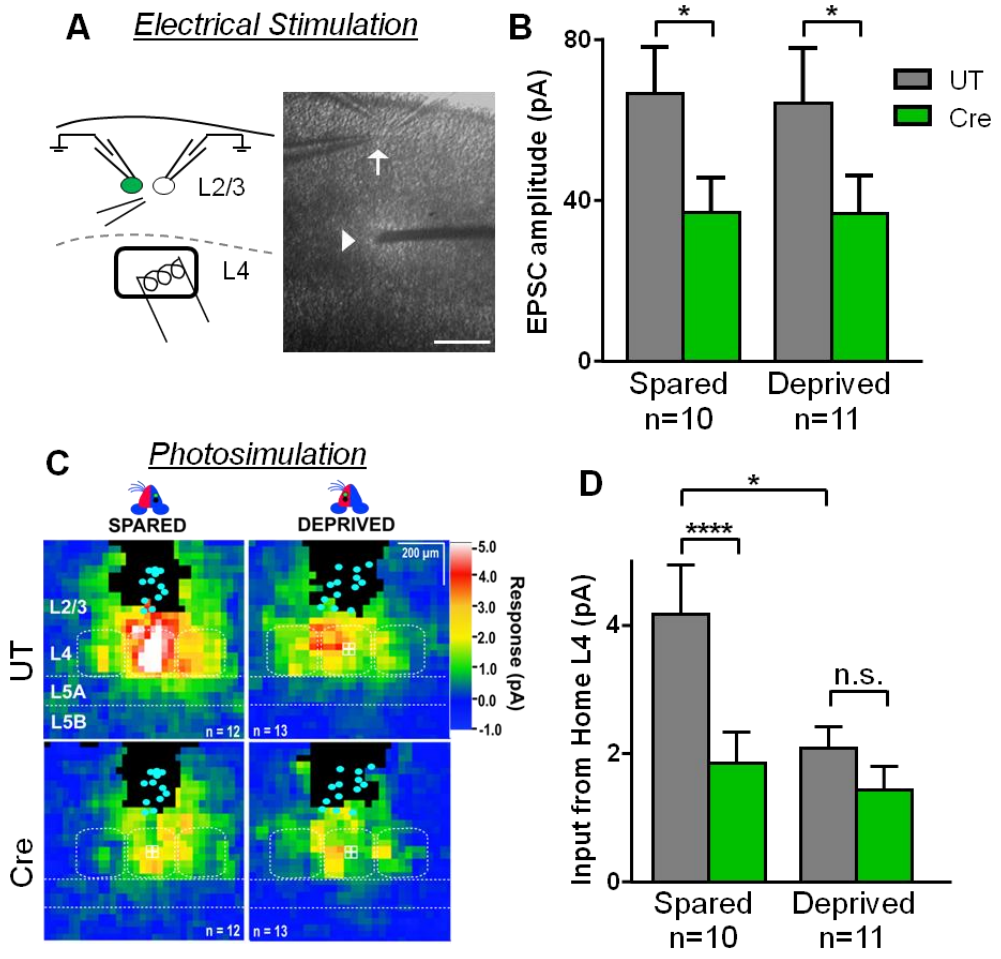


Figure 3.4. Sensory deprivation weakens the L4→L2/3 pathway through a MEF2C-dependent mechanism. Experimental configuration (cartoon and DIC image) of electrical stimulation of the L4→L2/3 pathway (**A**) showing the stimulating electrode in L4 (*horizontal arrow*), a pipet locally perfusing bicuculline (*vertical arrow*), and the simultaneously recorded UT and Cre-expressing cell pair in L2/3. Electrical stimulation reveals a reduced EPSC amplitude in MEF2C-KO (Cre+) cells in both spared and deprived hemispheres (**B**). **C,** Spatial response maps constructed from LSPS experiments demonstrating that sensory deprivation can only weaken pathway input in the presence of MEF2C; quantified in **D**. Electrical stimulation data collected by KL; LSPS data collected by KR

CHAPTER FOUR
Discussion

CONCLUDING REMARKS AND RECOMMENDATIONS FOR FUTURE STUDIES

Summary of findings

Acute patterns of activity have been shown to recruit mGluR5 to suppress synaptic function at a number of different synapses in various brain regions. Although some patterns of acute activity can promote synaptic function in an mGluR5-dependent manner, the mechanisms involved in these forms of synaptic strengthening are not a direct consequence of mGluR5 function (i.e. metaplasticity). My work provides evidence that the cumulative, long-term role of mGluR5 throughout the first 2-3 weeks of postnatal development is to promote synaptic function in 2 of the major input pathways to L2/3 neurons of barrel cortex. It is my contention that this result does not challenge the previously demonstrated roles of mGluR5 in suppressing synaptic function, but rather suggests that there are likely some currently unknown roles of mGluR5 that have yet to be identified. Consistent with this notion, mGluR5 may regulate spontaneous and evoked transmission differently, implicating a novel role for mGluR5 in regulating action potential-independent transmission.

My data also implicate MEF2C in promoting synaptic function in L2/3 neurons of barrel cortex. This is consistent with a previously demonstrated role for MEF2C-sepcific enhancement of synaptic function (Li et al 2008). These observations bear striking similarity with the phenotypes observed with mGluR5 deletion, including the puzzling bidirectional regulation of spontaneous and evoked transmission. This is consistent with previous

observations that mGluR5 and MEF2C regulate synaptic function through common mechanisms. For instance, both mGluR5 and MEF2C suppress synaptic function in hippocampus in an Arc-dependent manner (Waung et al 2008, Wilkerson et al 2014). Direct evidence that these two proteins function through the same pathway to regulate synaptic function was demonstrated by the observation that MEF2-induced synapse elimination is blocked by genetically or pharmacologically silencing mGluR5 (Wilkerson et al 2014). Moreover, MEF2C deletion has a more profound effect on synaptic function than mGluR5 deletion, likely reflecting the pervasive impact on many aspects of cell function expected form a transcription factor influencing thousands of genes.

Postsynaptic mGluR5 promotes synaptic function at 2 major input pathways to L2/3

Based on previous studies, I initially hypothesized that mGluR5 would play a role as a negative regulator of synaptic function, and thus mGluR5 deletion would result in enhanced synaptic function. Therefore, I was surprised to find that deletion of postsynaptic mGluR5 consistently weakened evoked synaptic transmission in 2 prominent synaptic pathways. While this may implicate a novel role for mGluR5 in directly promoting synaptic function, I argue that this observation reflects the long-term outcome of the combination of previously demonstrated roles for mGluR5.

As outlined in Chapter 1, mGluR5 suppresses synaptic function through a number of mechanisms at both excitatory and inhibitory synapses. In the subsequent sections, I put forth a number of scenarios in which different combinations of these established functions of mGluR5 could result in the effects observed in my work, and what these scenarios might

suggest about the overarching role of mGluR5 in coordinating circuit dynamics and shaping receptive fields.

mGluR5 influences receptive field formation by regulating several forms of plasticity

Global deletion of mGluR5 results in a loss of barrel patterning in layer 4, indicative of ill-defined receptive fields (Wijetunge et al 2008). In line with this, the temporal dynamics of L4 responses to primary and surround whisker deflection are altered in KO mice compared to WT, further demonstrating altered receptive fields.

To interpret my findings in the context of a putative role for mGluR5 in shaping receptive fields, I will briefly summarize 3 previously established mGluR5 functions that are of relevance. First, mGluR5 is required for homosynaptic tLTD of excitatory transmission at the L4→L2/3 synapse in barrel cortex (Bender et al 2006b). Second, presynaptically expressed heterosynaptic-LTD of both inhibitory (hetero-iLTD) (Jiang et al 2010) and excitatory (hetero-eLTD (Huang et al 2008) L2/3 transmission in response to HFS stimulation of L4 in visual cortex requires postsynaptic mGluR5. This HFS also activates homosynaptic NMDARs which release BDNF to retrogradely activate TrkB receptors and effectively shield active and correlated presynaptic inputs from mGluR5-eCB-mediated LTD in a synapse-specific manner, thus conforming to Hebbian rules. Although this type of heterosynaptic plasticity has not been investigated at the L4→L2/3 synapse in barrel cortex, both of these synapses (i.e. L4→L2/3 of both barrel and visual cortex) contain the implicated molecular mechanisms (e.g. mGluR5-eCB-CB1R signaling pathway) and generally share very similar circuitry. Third, in hippocampus, a mechanistically similar mGluR5-mediated

heterosynaptic LTD of inhibitory inputs triggers a metaplastic state that lowers the threshold for LTP induction, thereby facilitating synaptic strengthening (Chevaleyre & Castillo 2003). Based on a combination of these plasticity mechanisms, I describe the following model in which mGluR5 responds to two broadly defined patterns of input activity to modulate pathway strength both directly and indirectly at individual L2/3 neurons. The physiological correlate of these activity patterns, that is, the types of sensory information coded by these specific firing rates, is unclear. However, I will assume that they represent fundamentally different types of information and simply refer to them by their operationally defined induction protocols, STDP and HFS.

STDP engages postsynaptic mGluR5 to weaken L4→L2/3 excitatory, homosynaptic connections. In response to strong synaptic stimulation (HFS), mGluR5 weakens heterosynaptic excitatory and inhibitory input. Concurrent NMDA-dependent, mGluR5-independent LTP is also elicited by HFS. Thus, HFS both induces LTP by engaging NMDA-mediated LTP and facilitates LTP induction by lowering the threshold via mGluR5-mediated disinhibition while simultaneously attenuating similar input to neighboring excitatory cells. Thus, any postsynaptic cell receiving strong enough input to retrogradely activate TrkB receptors (through postsynaptic NMDA-mediated release of BDNF) would not be subject to heterosynaptic LTD, and would be strengthened in a given receptive field, while cells receiving weak, uncorrelated input would be weakened and ultimately be excluded from a receptive field. The culmination of both of these mGluR5-mediated effects (STDP and TBS) would be to sharpen the receptive field by 1) weakening mistimed inputs (STDP), 2) weakening weakly activated L2/3 cells (hetero-eLTD) and 3) promoting strongly activated

L2/3 cells (hetero-iLTD). This model is supported by my data in a number of ways: 1) I see an increase in inhibitory drive onto KO cells, 2) presynaptic release probability is enhanced in KO cells while uEPSC amplitude is unaffected (consistent with mGluR5 activation retrogradely attenuating release probability), and 3) pathway strength is stronger in WT cells relative to KO cells, consistent with WT cells maintaining the capacity for hetero-eLTD while KO cells are incapable of 'tuning down' their neighboring (WT) cells.

L2/3 neurons respond to whisker stimulation with a relatively low firing rate compared to L4 neurons. It has been suggested that this sparse-coding of L2/3 neurons reflects a large number of sharply tuned receptive fields that each code a unique aspect of whisker deflection (e.g. deflection intensity, deflection direction, object location vs. texture discrimination) (Petersen & Crochet 2013). While aspects of this information must be encoded to L4 responses to some degree, the sparse-cell coding of L2/3 likely allows for sharpening response acuity and associative learning (Gdalyahu et al 2012). To implicate mGluR5 in receptive field dynamics, an experiment directly probing specific aspects of sensory inputs is required. To this end, I propose an experiment combining *in vivo* calcium imaging of whisker responses with the sparse-population deletion model I have employed throughout my project. This experiment would require creating a cranial window and delivering a calcium sensitive dye to the appropriate barrel column to image calcium signals in response to sensory deflection (Gdalyahu et al 2012). Whiskers would then be deflected under varying parameters (i.e. increasing intensities and varying directions) and response strength and frequency would be compared between WT and mGluR5-KO (identified by GFP expression) cells in the corresponding column *in vivo*. If the above hypothesis is

correct, I would expect that mGluR5-KO cells would respond to more types of sensory input compared to their WT counterparts, reflecting the inability of KO cells to eliminate inappropriate inputs. Furthermore, I would expect the strength of each response to be reduced in KO cells compared to WT cells, indicative of KO cells being incapable of suppressing inhibitory inputs (via hetero-iLTD) and therefore unable to induce the form of metaplasticity which facilities LTP. These findings would indicate that the various forms of mGluR5-mediated synaptic regulation culminate to contribute to the sparse-coding of L2/3 by shaping cellular receptive fields that are both fine-tuned and high fidelity.

Differential effects of mGluR5 deletion on spontaneous and evoked transmission

The finding that mGluR5 deletion results in both an increase in mEPSC frequency and a decrease in evoked EPSC amplitude was surprising. Unfortunately, little is known regarding the extent to which mEPSCs represent synapses mediating evoked vs spontaneous transmission, which in itself highlights the lack of understanding of a broader inquiry of distinguishing the differences between these 2 forms of neurotransmission (Chung & Kavalali 2006, Ramirez & Kavalali 2011). For instance, it is not known if mGluR5 can be differentially targeted by spontaneous and evoked transmission, or conversely whether mGluR5 can signal to differentially modify these modes of transmission. Nevertheless, I will outline 3 broad possibilities to explain these interesting findings.

One possibility is that postsynaptic mGluR5 directly regulates spontaneous and evoked transmission differently. Unfortunately, there is no direct evidence to argue for or against a role for mGluR5 in directly regulating spontaneous release of neurotransmitter.

However, any postsynaptically generated signal with the capability of modulating spontaneous release would have to act retrogradely. Indeed, mGluR5 regulates evoked release by retrogradely signaling through eCBs and CB1Rs to diminish presynaptic release in response to multiple patterns of input activity (Chevaleyre & Castillo 2003, Feldman 2012). CB1Rs are expressed predominately on the presynaptic terminal and are also GPCRs coupled to a number of signaling pathways (Turu & Hunyady 2010), leaving open the possibility that activation of these receptors could impact presynaptic function in a number of discrete ways. Therefore, it is possible that mGluR5 bidirectionally affects both spontaneous and evoked release of neurotransmitter through a CB1R-dependnet mechanism. If this is the case, then a similar sparse-population deletion of CB1R might be expected to result in similar phenotypes that I observed with sparse-population deletion of mGluR5.

Another possibility is that mGluR5 has a direct role on synaptic function in response to evoked neurotransmission specifically. In this case, the reduced evoked input observed in mGluR5-deleted cells would be a direct consequence of mGluR5 ablation, and the increase in mEPSC frequency is merely a homeostatic, non-Hebbian response of the cell that is mGluR5-independnet. This type of homeostatic plasticity has been previously demonstrated, where treating cortical cell cultures with TTX for 2 days to suppress network activity leads to increased amplitudes of both mEPSCs and evoked responses. Furthermore, this plasticity is bidirectional, as ‘down-scaling’ of synaptic strength occurs when network culture activity is enhanced with bicuculline (Turrigiano et al 1998). Little is known about the induction of homeostatic plasticity, although there are at least some examples of homeostatic mechanisms

that are distinct from Hebbian plasticity induction mechanisms (Hu et al 2010, Turrigiano & Nelson 2004).

A third possibility is that mGluR5 has a direct role in both Hebbian plasticity and homeostatic plasticity. (One could argue that the converse of this situation is also possible, in which mGluR5 has a direct role in regulating spontaneous transmission and the effects I observed when probing evoked transmission were simply a homeostatic response, but considering the previously established roles for mGluR5 in regulating evoked transmission I consider this to be unlikely.) This possibility is supported by the observation that mGluR5 mediates homeostatic down-scaling of mEPSC amplitudes in response to pharmacological enhancement of network activity in hippocampal cultures (Zhong et al 2012). Furthermore, in a similar homeostatic plasticity paradigm, regulation of group 1 mGluRs by the immediate early gene (IEG) Homer1a was demonstrated to be critical for mGluRs to scale down synaptic strength (Hu et al 2010). In this study, bicuculline treatment elevated Homer1a expression and reduced both synaptic strength and surface expression of AMPAR subunits, consistent with a down-scaling of synapses. Interestingly, this downscaling was absent with either Homer1a deletion or application of inverse agonists, but not neutral antagonists, of group 1 mGluRs. Taken together, these data suggest that high levels of neural activity can induce Homer1a which binds to group 1 mGluRs, evoking agonist-independent signaling that results in AMPAR endocytosis and consequent homeostatic reduction of synaptic strength. Furthermore, reduction in surface AMPAR subunits was associated with phosphorylation of these subunits but was independent of Arc, providing 2 potential mechanisms by which group 1 mGluRs can distinctly regulate synapse-specific vs. cell-wide synaptic strength.

Such a distinct, homeostatic role for mGluR5 may explain my observations that mGluR5 deletion differentially affects mEPSCs and evoked pathway strength. To test this, one could design an experiment to manipulate Homer-mGluR5 interactions in a similar, cell-autonomous manner as I have done with mGluR5 through sparse-population deletion/knockdown of Homer1a or replacement of mGluR5 with the mGluR5$^{F/R}$ mutant which cannot bind to long forms of Homer. If this interaction does indeed represent a distinct mechanism by which mGluR5 mediates homeostatic but not Hebbian plasticity, then I would expect for such a manipulation to result in an increased mEPSC frequency with no change in evoked pathway strength.

mGluR5 directly and bidirectionally regulates both synaptic strength and intrinsic excitability

Based on previous reports demonstrating that mGluR5 regulates both intrinsic excitability and synaptic function on acute timescales, I believe that my observations further demonstrate direct roles for mGluR5 on these 2 cellular functions. Interestingly, mGluR5 seems to regulate intrinsic and synaptic excitability in opposite directions, suppressing the former and promoting the latter. This may suggest that mGluR5 serves as a balancing mechanism to ensure that the cell maintains some standard level of action potential firing. This could allow for a scenario in which enhancement of synaptic input is offset by suppressed intrinsic excitability to prevent excessive action potential firing while experience-dependent circuit modification is occurring.

However, due to the long time-scale of mGluR5 deletion used in my thesis project, it is difficult to disentangle mGluR5-dependent effects from homeostatic, mGluR5-independnet effects. For instance, mGluR5 may have a direct action solely on suppressing intrinsic excitability. Therefore, intrinsic excitability is increased in mGluR5-deleted cells, which triggers a homeostatic reduction in synaptic input, preventing the cell from firing action potentials at an increased level that would metabolically exhaust it. An experiment to determine if the reduced synaptic inputs I observed reflect a direct action of mGluR5 on synaptic function would be to test the capacity of mGluR5-deleted cells to engage in tLTD, a form of plasticity which has previously been shown to require acute activation of mGluR5. If mGluR5 has a direct and acute role on synaptic function that is involved in this form of plasticity, I would expect to be able to induce tLTD in WT cells but not mGluR5-KO cells. However, if mGluR5 exclusively regulates intrinsic excitability, this form of plasticity would still be intact in KO cells. Furthermore, by voltage clamping cells throughout the course of an experiment to a similar degree, any differences in excitability between WT and KO cells would not influence the cells ability to undergo tLTD. Additionally, observing that tLTD cannot be induced in mGluR5-KO cells would confirm that long-term manipulation of mGluR5 (i.e. deletion early in development) does not result in compensation for this function of mGluR5 by mGluR1, or any other mechanism, that would influence the interpretation of my findings.

Postsynaptic MEF2C promotes synaptic function at the L4→L2/3 synapse

MEF2C regulates multiple aspects of NMDAR-mediated transmission

Consistent with MEF2C promoting synaptic function, I observed a decreased NMDAR-mediated EPSC amplitude in MEF2C-deleted cells. However, I was surprised to find that subunit composition of NMDARs was also altered such that KO cells had a larger contribution from NR2A than WT cells. This observation is inconsistent with KO cells having delayed or absent synapse maturation, as deficit in maturation would be reflected by a larger contribution of NR2B, not NR2A. Therefore, I believe that rather than MEF2C playing a role in one of the critical developmental steps required for this subunit switch, MEF2C is instead directly promoting transcription of NR2B or a gene associated with its trafficking and/or integration into NMDARs. To test this possibility, I propose conducting single cell RT-PCR of WT and MEF2C-deleted cells and probing for these mRNA transcripts. If MEF2C is indeed regulating one or several of these candidate transcripts, I would expect to see decreased, or potentially increased, mRNA levels in MEF2C-deleted cells.

MEF2C mediates sensory experience-dependent strengthening of synapses

As described in Chapter 3, technical differences may be able to account for the disparate findings regarding the dependency of MEF2C on sensory experience to regulate synaptic function. One potentially critical difference that can be addressed experimentally is the pharmacological blockade of inhibition used in the electrical stimulation experiments but not the LSPS experiments. If deprivation-induced increase in inhibition is driving the difference in experimental effects as suggested earlier, then leaving inhibition intact may reveal no effect of deletion in deprived slices in response to electrical stimulation.

Conversely, locally applying bicuculline in the LSPS experiments may dramatically enhance WT response amplitudes and reveal a difference between WT and KO cells in deprived slices.

Another experiment that would further establish a link between MEF2C and sensory experience is to drive sensory experience by persistently deflecting single whiskers. One advantage to this paradigm is that compensatory network effects can be minimized by using a more precise and local manipulation as compared to the broad perturbation caused by removing every whisker on one side of the facial pad. Furthermore, by conducting these experiments in mice that express a reporter of neural activity, such as Arc-GFP or Fos-GFP, the cortical column corresponding to the manipulated whisker(s) can easily be identified during the experiment. The expectation would be that WT cells in columns corresponding to deflected whiskers would display enhanced pathway input compared to WT cells columns corresponding to whiskers receiving normal sensory experience. Conversely, if sensory experience requires MEF2C, I would expect that enhanced sensory experience would have no effect on MEF2C-deleted cells.

MEF2C regulation of inhibition

As outlined in Chapter 3, the discrepancy between the effect of global-deletion and sparse-population deletion of MEF2C on mIPSC frequency may implicate a role for MEF2C in retrogradely influencing GABAergic terminals. In this section, I will lay out a series of possible experiments to test this assertion.

As previously mentioned, the absence of any effect on mIPSC in the sparse-population deletion model may be due to the large number of WT neurons capable of supplying the necessary retrograde signal that is deficient in KO cells in a cell non-autonomous way. I argue that this effect can therefore be detected by deleting MEF2C in all excitatory neurons since there aren't any WT cells available to provide the signal. Important to this explanation is the consideration of the spatial component of retrograde signaling. Postsynaptic compartments of active glutamatergic synapses can only diffuse a retrograde signal to neighboring synapses. Therefore, in order for the retrograde signal to be supplemented in a KO cell, the inhibitory to excitatory (I-E) synapse onto this cell must be near an E-E synapse between to WT cells. In the sparse-population deletion model, where the majority of the cells are WT, the probability of this occurrence is high. Therefore, one way to experimentally bolster this argument would be to use a deletion strategy that is in between sparse-population and global deletion. One such strategy would be to use a similar technique to transfect MEF2C in a sparse-population of neurons in EMX1-Cre x *Mef2C*$^{flx/flx}$ mice. Under this paradigm, a retrograde signal would only be provided by a small number of MEF2C expressing pyramidal cells, thereby reducing the occurrence of postsynaptic KO I-E synapses being in proximity of WT E-E synapses and allowing detection of deficiency of this mechanism in KO cells. A similar experiment could be conducted to express MEF2-VP16, a constitutively active form of MEF2, in a WT background using a similar sparse-population transfection strategy. Under my hypothesis, this would result in MEF2-VP16 expressing neurons displaying a reduced mIPSC frequency compared to WT neurons.

Another experiment to probe how postsynaptic MEF2C regulates inhibitory inputs would be to test unitary I-E connections, where the presynaptic inhibitory cell is always WT and the postsynaptic excitatory cell is either WT or MEF2C-KO. This paradigm would allow for a probing of different aspects of synaptic transmission, including connection frequency, unitary strength, and short-term plasticity. A caveat to this approach is that MEF2C may specifically regulate certain classes of inhibitory cells, of which L2/3 contains a heterogeneous population. One approach to circumventing this problem would be to illicit heterosynaptic iLTD by stimulating L4 with protocol similar to that used in visual cortex (Jiang et al 2010) and assaying whether this type of plasticity can be induced in MEF2C-deleted cells. This form of plasticity has not been investigated in barrel cortex and must therefore be demonstrated in this context in order for this experiment to be conducted. However, in the event that such a form of plasticity does exist in this pathway, not only could the effect of deletion on regulating inhibitory inputs be probed, but downstream molecular mechanisms could also be investigated. For instance, if MEF2C-deleted cells are indeed incapable of inducing iLTD, then one could attempt to rescue the deficiency by applying BDNF during the plasticity induction, thereby assessing the aforementioned possibility that BDNF is the retrograde signal generated by MEF2C.

Concluding remarks

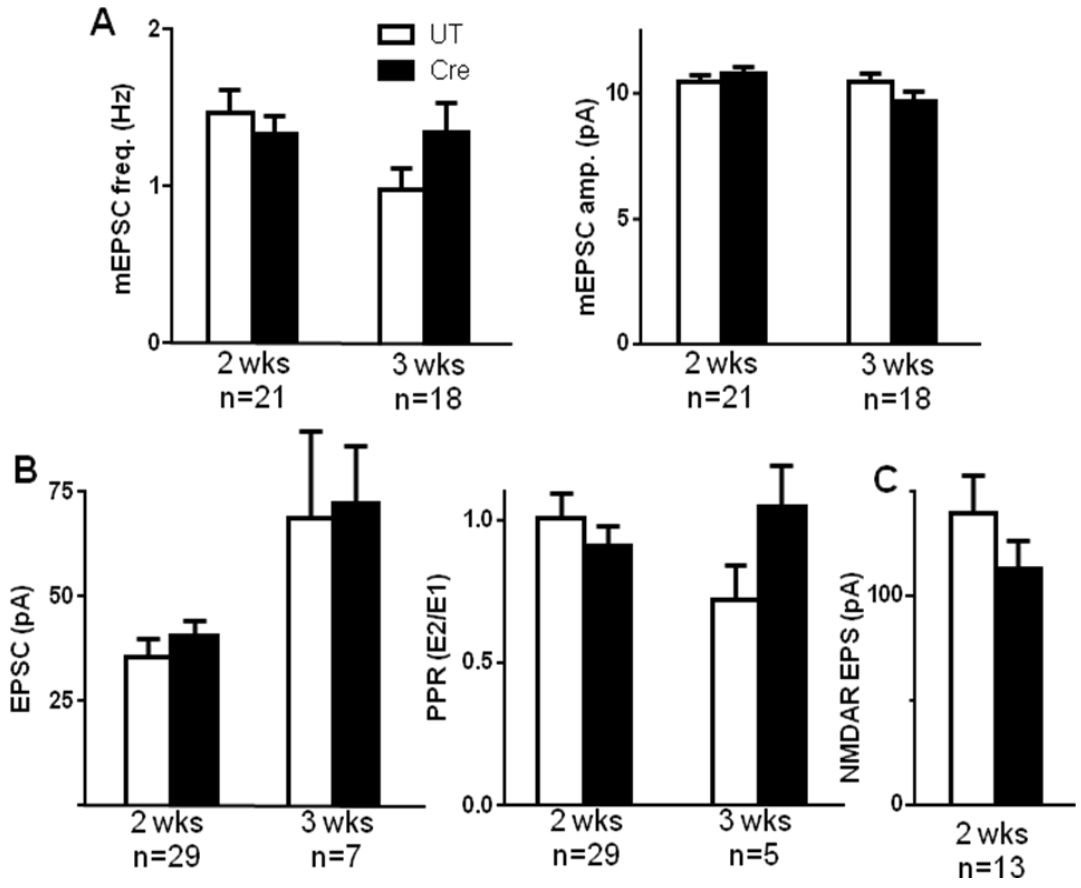
My data represent a critical first step in understanding the cumulative roles of these 2 proteins, mGluR5 and MEF2C, on synapse regulation and circuit development on a cell-

autonomous level. This, in turn, will guide investigations into the direct synaptic functions these proteins have on a more acute timescale. Furthermore, consideration of how dysfunction of these proteins impacts disease states requires the understanding of their long-term roles in physiological contexts. My findings regarding mGluR5 function strongly highlight the importance of this type of approach. Previous studies have provided overwhelming evidence that mGluR5 has a direct role as a negative regulator of synaptic function. However, my data shows mGluR5 deletion weakens evoked synaptic input, which is inconsistent with its previous notions of how mGluR5 regulates synapses. I do not believe this challenges previous work, but rather suggests that there are currently undefined mechanisms by which mGluR5 can acutely regulate synaptic function, and that the net effect of integrating all of these functions results in an outcome that is more complex than a simple summation of all its acute functions. Similarly, my findings that deleting MEF2C during postnatal development results in a net decrease in synaptic input will guide future studies aimed at investigating acute mechanisms by which this protein regulates synaptic function.

My findings that both mGluR5 and MEF2C are positive regulators of synaptic function in L2/3 neurons also support the notion that these two proteins regulate synapses through common mechanisms. Interestingly, dysfunction of both of these proteins is attributed to synapse and circuit deficiencies in many of the diseases they are implicated in. Therefore, advancing the understanding of what molecular mechanisms these proteins work through to impact synaptic function may lead us to common therapeutic targets, thereby providing more reliable treatments for disease.

APPENDIX A

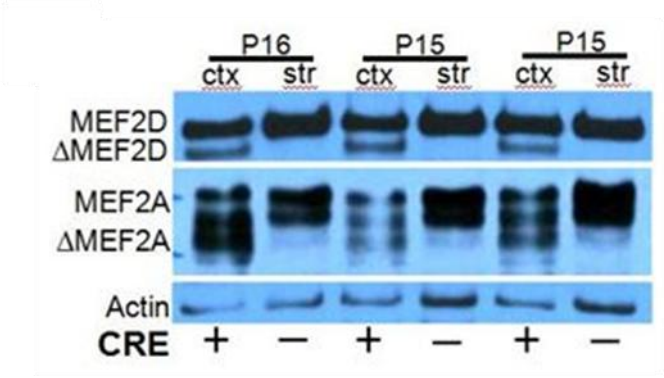
Appendix 1. mGluR5 plays a more critical in the 1st 2 weeks of postnatal development.



Appendix 1. Using AAV-Cre-GFP mediated deletion of *Grm5*, delaying mGluR5 ablation to approximately p9 (see Chapter 2 Discussion), results in no change of mEPSC frequency or amplitude (**A**). Neither AMPAR-mediated (**B**) or NMDAR-mediated (**C**) evoked transmission was affected in L2/3 neurons of barrel cortex at either 2 or 3 weeks. Virus pseudotypes for these experiments are as follows: 2 and 3 wk mEPSCs = AAV2.1 & 2.9; 2 wk evoked = AAV2.1; 3 wk evoked = AAV2.1 & 2.9.

APPENDIX B

Appendix B. Truncation of MEF2 proteins using AAV-Cre-GFP mediated gene excision



Appendix B. Excision of exon 2 by introducing an AAV expressing Cre-GFP at p1 in floxed MEF2 mice results in a truncated protein (ΔMEF2A and (ΔMEF2D), which is associated with transcriptional inactivation (see Chapter 3 Results). The lack of truncated protein product in striatum reflects the cortex specific expression of Cre-GFP associated with this method of AAV delivery. Data collected by KR

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