Postsynaptic mGluR5 promotes evoked AMPAR-mediated synaptic transmission onto neocortical layer 2/3 pyramidal neurons during development

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JRG, KMH, and KWL planned and designed experiments. KWL and ABP performed all experiments. KWL analyzed data. JRG and KWL prepared the manuscript.

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Abstract

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, we 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.

Keywords: metabotropic glutamate receptor, synapse, cortex
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 and 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 and 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 and Huber 2010; Nevin and 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 and 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 and Abraham 2002; Kalmbach et al. 2013; Sourdet et al. 2003). 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, we 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. We examined the resulting effects on synaptic input and membrane properties. We 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, we 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

We used floxed Gmr5 mice (Grm5^{flx/flx}) (Xu et al. 2009) 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_{2}PO_{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_{2}PO_{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% O₂ / 5% CO₂. 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 GABA₃R-mediated currents was ~70 mV.

**In utero electroporation**

To delete *Grm5* in a minority of layer 2/3 pyramidal neurons, we 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. We performed a survival surgery following procedures already described (Saito 2006). Briefly, we 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 we 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<sub>A</sub>R 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 intersominal 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<sup>++</sup>] and [Ca<sup>++</sup>] 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 we 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, we locally blocked disynaptic inhibition by positioning a perfusion pipette containing the GABA<sub>A</sub>R 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-mediated currents was in ~-70 mV in our recording conditions. We 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 we 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 \( \text{VAR}_{\text{EPSC1}} - \text{VAR}_{\text{baseline}} \) divided by the \( \text{Mean}_{\text{EPSC1}} \) where \( \text{VAR}_{\text{EPSC1}} \) and \( \text{VAR}_{\text{baseline}} \) are the variance of uEPSC1 and baseline noise respectively, and \( \text{Mean}_{\text{EPSC1}} \) is the mean of uEPSC1 (Faber and 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 (“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\(_A\)R 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. 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 \textit{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 \textit{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 χ²-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

We performed in utero electroporation of E15 embryos to express Cre-GFP in a subset of L2/3 neurons in Grm5^{flx/flx} mice (Fig. 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. 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 and 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, we performed immunocytochemical staining of mGluR5 in dissociated cortical neuron culture (Fig. 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. 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, we found greater mGluR5 expression in cultured UT neurons at ED14 (Fig. 1C).

**Up-regulation of action potential-independent synaptic release**

We 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), we performed simultaneous recordings of untransfected (UT) and Cre-GFP expressing (Cre) pyramidal neuron pairs in L2/3 (Fig. 2A,B). Because we measured mEPSCs at -65 mV and the bath contained a GABA<sub>A</sub>R antagonist (see Methods), mEPSCs were predominantly mediated by AMPARs. Kainate receptors were probably not involved (see Methods). We observed
effects based on both neuron genotype and age (Fig. 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, we pooled the data over all ages (Fig. 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 and Kavalali 2011), we next examined glutamatergic synaptic transmission evoked by presynaptic action potentials. We first examined AMPAR-mediated synaptic transmission onto L2/3 pyramidal neurons originating from presynaptic neurons in layer 4 (L4). We simultaneously recorded from L2/3 untransfected and Cre-expressing neuron pairs, and afferents from layer 4 were stimulated with a metal electrode (Fig. 3A). Local application of a GABAA,R antagonist was applied to decrease contamination by disynaptic inhibition (see Methods). We compared the amplitude of the first EPSC in a 2 pulse train (Fig. 3B), and we observed effects based on both neuron genotype and age (Fig. 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. 3C, right; p<0.05, paired t-test).

We observed no changes in the paired pulse ratio (Fig. 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, we 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. We observed no changes in NMDAR-mediated transmission with Grm5 deletion since both the amplitude and waveform kinetics of the EPSCs were unchanged (Fig. 3E, statistical power = 0.75). For the kinetics, both decay time constant (Fig. 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.

*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. We 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. We performed simultaneous “pre/post” recordings of up to 4 neurons to
examine unitary EPSCs (uEPSCs) from neighboring, single presynaptic pyramidal neurons (Fig. 4A) 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 4B, Neuron 1).

We 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. 4C 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. We 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 and Madison 2007; Patel et al. 2014). Again, postsynaptic Grm5 deletion
decreased connectivity frequency (Fig. 4C 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.

We detected no changes in uEPSC amplitude (Fig 4D, 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. 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 4E, 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 \textit{Grm5} deletion in the local L2/3\textarrow L2/3 synaptic pathway resulted in a similar down-regulation of AMPAR-mediated transmission like that observed for the L4\textarrow 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.

\textit{Decreased dendritic arbor with Grm5 deletion}

To determine if the above changes in synaptic transmission possibly involved morphological changes, we examined how the dendritic tree of L2/3 pyramidal neurons was altered with \textit{Grm5} deletion (Fig. 5A). We compared the morphology between Cre-GFP expressing neurons in WT versus \textit{Grm5}\textsubscript{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 \textit{Grm5} deletion (2-factor ANOVA, genotype x distance). To determine the locus for this decrease, we 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. 5B,C). Interestingly, both the L4\textarrow L2/3 and L2/3\textarrow 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, we measured the total dendritic length in basal and apical dendritic compartments. We observed no detectable alteration...
(Fig. 5D), but when we limited our analysis to the 30-70 µm range in the basal compartment where a decrease in complexity was observed, we did observe a length decrease with Grm5 deletion (Fig. 5E). 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 and Abraham 2002; Kalmbach et al. 2013; Sourdet et al. 2003). 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 we observe. To measure excitability, we sequentially applied current steps (400 ms duration) of increasing amplitude under resting potential conditions and counted the number of action potentials evoked (Fig. 6A). From this we obtained curves of firing frequency as a function of current step amplitude (F-I curves). At all age groups examined, we found that Cre-expressing neurons were more excitable since their average F-I curves were significantly above that for UT neurons (Fig. 6B). 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. 6C,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 and Huber 2010), particularly in the ones that we 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 we 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 and 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 we 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). We 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 and Abraham 2002; Johnston et al. 2003; Kalmbach et al. 2013; Sourdet et al. 2003). We 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 and Nelson 2004). Moreover, intrinsic excitability changes could alter evoked glutamatergic transmission through changes in spike-timing-dependent plasticity known to occur in the pathways we 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 and Kavalali 2011). While we prefer to stress the effects on evoked transmission since we 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 and Schuman 2009).

The substrate for the specific decrease in AMPAR-mediated, but not NMDAR-mediated, transmission with Grm5 deletion is unclear. We 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. 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, we 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. 2D). The decrease in connectivity in the L2/3→L2/3 pathway with Grm5 deletion is more consistent with scenarios 2 and 3 (Fig. 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 we employ (P13-17) resulting in even stronger overlap between regions of synaptic targeting and dendritic length decrease.
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 and Conn 2014; Pomierny-Chamiolo et al. 2014; Yin and 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 and 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 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.** 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.
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 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\(_A\)R 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 4. 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 5. 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 Grm5flx/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 6. 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 (B1-B4). N’s for B1-B4 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.
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Figure 1

A

B

DIC mGluR5 GFP

UT

R

5

00)

C

Cre-GFP

Cre

Dendritic mGluR5

AFU* μm² (x10000)

ED 8

ED 14

UT

Cre

**

****
**Figure 2**

A1, A2, A3

B

C

D
Figure 3

A

B

AMPAR EPSC's
UT
Cre

C

D

PPR (E2/E1)

UT Cre

E1

NMDAR EPSC's
UT Cre

E2

Amp. (pA)

0 100 200

0 1 2

E2

E1

P9-11 P14-17 P20-24

PPR (E2/E1)

0 0.5 1

0 0.5 1

UT Cre

UT Cre

E3

Tau decay (ms)

0 200 400

0 200 400

UT Cre

UT Cre
Figure 4

A

B

C

D

E

Connection Freq. (%)

Cre Pre
Cre Post
Pre
Post

Pre-UT
Post-Cre

Post-UT
Post-Cre

Cre Pre
Cre Post
Cre Pre
Cre Post
Figure 5

A. WT(n=20)  Grm5^{flx/flx} (n=18)  100 μm

B. Basal

C. Apical

D. WT  Grm5^{flx/flx}

E. 30-70 μm annulus

**
Figure 6

A  

B1  

B2  

B3  

B4  

C  

D