Targeted disruption of layer 4 during development increases GABA\textsubscript{\textalpha} receptor neurotransmission in the neocortex

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DURING DEVELOPMENT OF THE mammalian neocortex, immature neurons generated in the germinial zones of the neocortex and ganglionic eminences (GE) migrate in an inside-out pattern to create the six-layered neocortex (Angevine and Sidman 1961; Rakic 1974). This laminar organization is crucial for establishing a balance between excitation and inhibition, leading to proper overall function of the neocortex. Events with adverse impact on neuronal migration lead to cortical dysplasia (CD), a developmental abnormality characterized by aberrant distribution and clustering of neocortical cells, resulting in a pleth-ora of neuropathological disorders, such as epilepsy, schizophrenia, and mental retardation (Calcagnotto and Baraban 2003; Calcagnotto et al. 2002; Choi and Mathias 1987; Colombro et al. 2003; Gleeson and Walsh 2000; Guerrini et al. 2003; Moroni et al. 2005, 2008; Palmini et al. 1991; Ross and Walsh 2001; Tassi et al. 2002; Taylor et al. 1971; Zhu and Roper 2000). A common pathological finding in clinical cases and animal models of CD is an abnormal distribution and grouping of neurons within the neocortex, which alters the microcircuitry and cortical architecture (Benardete and Kriegstein 2002; Colacitti et al. 1999; Ferrer 1993; Jacobs et al. 1999; Moroni et al. 2008; Roper 1998). In addition, abnormal GABA signaling frequently associates with CD, leading to consequent alteration in the balance of excitation and inhibition and disruption of normal cortical function (Benardete and Kriegstein 2002; Brill and Huguenard 2010; Moroni et al. 2008; Roper et al. 1999; Xiang et al. 2006; Zhou and Roper 2010, 2011; Zhu and Roper 2000, Zhu et al. 2009).

We developed a model of CD in ferrets using methylazoxymethanol (MAM; also see Johnston et al. 1982 for initial description). Taking advantage of the pharmacokinetic property of MAM as a short-acting anti-mitotic and the long developmental time course of ferret corticogenesis, we selectively disrupted the birth of layer 4 by delivery on embryonic day 33 (E33) (Noctor et al. 1997, 1999; Palmer et al. 2001). The ferret, as the smallest mammal with a gyrencephalic cortex, is crucial for the study of neocortical development. Because ferrets possess an expanded neocortex with sulci, gyri, and large amounts of white matter, it is distinctive as a research subject. The protracted period of corticogenesis and long period of cell proliferation in the large outer subventricular zone may contribute to the expanded cerebral cortex in the ferret compared with the rodent (Fietz et al. 2010; Martinez-Cerdeño et al. 2006). Distinctive features in the ferret related to neocortical development make it essential to study developmental processes in this animal, since aspects of neurodevelopment differ in mammals with a convoluted vs. lissencephalic cortex (Kriegstein et al. 2006; Poluch et al. 2008).

As a likely result of the loss of layer 4 in our model, thalamic afferents that normally synapse directly in this layer become redistributed to upper and lower cortical layers; the capacity for entrainment and information transfer is also lost within the somatosensory cortex (McLaughlin and Juliano 2005; Noctor et al. 2001; Palmer et al. 2001). Ferrets treated with MAM on E33 additionally show an increased expression of GABA\textsubscript{\textalpha} receptors (GABA\textsubscript{\textalpha}R), which expands to upper cortical layers, and interneurons are disorganized in their laminar positions (Jablonska et al. 2004; Poluch et al. 2008). Changes in the
migratory behavior of GE-derived cells also occur (Abbah and Juliano 2013). These changes are specific to MAM delivery on E33, as administration on different embryonic dates leads to dramatically different results (Gierdalski and Juliano 2003, 2005; Noctor et al. 1999; Poluch and Juliano 2007, 2010). An important question emerging from these observations is whether the loss of layer 4 and subsequent redistribution of GABA<sub>R</sub> and interneurons alters GABA signaling within upper cortical layers. We also assessed whether the emergence of these properties influenced the functional properties of migrating interneurons. Our laboratory's earlier studies in MAM-treated (MAM-Tx) animals show increased expression of GABA<sub>R</sub> within the neocortex, as well as an abnormal pattern of migration and distribution of interneurons leaving the GE (Abbah and Juliano 2013; Jablonska et al. 2004; Poluch et al. 2008). These features prompted us to characterize GABAergic synaptic transmission in interneurons migrating toward the neocortex and evaluate the functional impact of the altered neocortical environment on pyramidal cell responses. We applied whole-cell patch-clamp recording to measure spontaneous GABA<sub>R</sub>-mediated inhibitory postsynaptic currents (sIPSCs) in two types of cells. We studied cells migrating away from the GE relatively early during ferret cortical development postnatal day (P) 0 to P1 and pyramidal cells of layer 2/3 in juvenile ferrets (P28–P38). Choosing these two types of cells for functional analysis allows us to determine the effect of early environmental changes on GABAergic cells as they migrate into the neocortex; and 2) on inhibitory neurotransmission in the somatosensory region of the resulting neocortex. Our results indicate that after E33 MAM treatment, GABAergic synaptic neurotransmission is increased significantly in cells migrating into the cortex from the GE and on cells populating the upper neocortical layers.

**MATERIALS AND METHODS**

**Animals.** Handling of animals complied with the Animal Care and Use Committee of the Uniformed Services University of the Health Sciences (USUHS). All procedures involving animals were approved by the USUHS Animal Care and Use Committee. Pregnant ferrets were obtained from Marshal Farms (New Rose, NY) and maintained in the animal facilities of USUHS. On E33, ferrets were anesthetized with isoflurane (1–2%) and injected intraperitoneally with 14 mg/kg of MAM (Midwest Research Institute, Kansas City, MO). Ferrets were closely monitored and allowed to recover from anesthesia before returning to the animal facility to await delivery of their kits. Ferret kits were used for experiments at P0–P1 or P28–P38.

**Organotypic slices.** Preparation of organotypic cultures followed the method described by Palmer et al. (2001). Briefly, P0 ferret kits were anesthetized with pentobarbital sodium (50 mg/kg), and after observing insensitivity to pain, brains were removed and maintained in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> under aseptic conditions in a laminar flow hood. The aCSF is composed of the following (in mM): 124 NaCl, 3.2 KCl, 2.0 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, and 10 glucose. The aCSF was composed of the following (in mM): 125 NaCl, 2.5 KCl, 1.0 CaCl<sub>2</sub>, 2.0 MgCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, and 25 glucose maintained at 28°C for pyramidal cells and 37°C for cells migrating from the GE. Slices were perfused at a rate of 3–4 ml/min. Recordings used a pulled borosilicate pipette filled with an internal solution composed of the following (in mM): 135 Cs-glucosinate, 10 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na<sub>3</sub>GTP, pH 7.3 (280–290 mosM). Each cell was recorded for at least 5 min; 1 min toward the end of the 5 min session was used for analysis. In some experiments, neurobiotin at a final concentration of 0.2% was included in the internal solution for post hoc identification. Whole-cell recordings from the soma of neuronal cells occurred in voltage-clamp mode using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) after a tight seal (1 GΩ) was formed between the recording electrode (with resistance 5–7 MΩ for pyramidal cells and 7–11 MΩ for GE-derived cells) and the cell body. For all recordings, a threshold of 2 kHz was applied to filter signals, which were digitized (Digidata 1322A, Axon Instruments) and recorded using pClamp software (Axon Instruments). IPSCs were analyzed offline using the Mini Analysis Program (Synaptosoft, Leonia, NJ) and detected by manually setting the IPSC threshold (~1.5 times the baseline noise amplitude) and assessing the full width at half maximum. The resistance was monitored on both the mean values presented in bar graphs (using a Student’s t-test) and the cumulative probability distributions using the Kolmogorov-Smirnov test. The access resistance was constantly monitored throughout the recording, and cells that showed fluctuations beyond 15% were excluded from analysis.

**Morphology.** After recording, acute slices were immediately fixed in 4% paraformaldehyde overnight at 4°C. After several washes with phosphate-buffered saline (PBS), slices were incubated with a solu-
tion containing 10% methanol and 0.6% hydrogen peroxide in PBS for 30 min at room temperature. After further washes with PBS, slices were incubated in a solution containing 2% bovine serum albumin and 0.75% Triton X-100 in PBS for 1 h, and then in 2% bovine serum albumin and 0.1% Triton X-100 for 15 min. Slices were then placed in a dilute solution of ABC from an Elite kit overnight at 4°C. After multiple washes, cells were visualized with peroxidase staining using 3,3′-diaminobenzidine (Immpact DAB, Vector Laboratories, Burlingame, CA), and mounted in moviol.

Western blotting. Pieces of the neocortex cut from blocks of the parietal cortex prepared as described above were frozen on dry ice and preserved at −80°C prior to use. Protein samples were prepared first by homogenizing the tissues using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) followed by centrifugation at 14,000 g at 4°C. An estimate of the concentration of protein was obtained through colorimetric assay using the Pierce BCA assay kit. Protein concentration was determined after 30-min incubation at 60°C, followed by spectrophotometry. Separation of proteins in the samples was accomplished by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% Bis-Tris precast gel, and separated proteins were transferred to a polyvinylidene fluoride membranes. A loading volume of 10–15 μl containing 20–40 μg of protein was used for each analysis. To limit nonspecific binding of antibodies, membranes were initially incubated with casein blocking buffer [PBS (0.5 M NaCl) + 3% casein + 0.5% Tween 20] for at least 2 h, followed by affinity purified rabbit polyclonal antibodies directed against GABA_A12 (1:200; ProSci, Poway, CA), GABA_A13 (1:200; Sigma, St. Louis, MO), GABA_A12 (1:400, Sigma-Aldrich, St. Louis, MO), and monoclonal anti-GAPDH (1:6,000, Abcam, Cambridge, MA) for 24 h. After several washes with PBS, protein bands were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:1,000, Jackson Laboratories, West Grove, PA) and horse-radish peroxidase-conjugated anti-mouse (1:6,000, Thermo Scientific, Rockford, IL) and visualized using enhanced chemiluminescence detection. Signal intensities were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). The neocortices of five different brains of each age P28–P35 were used for this analysis. Each brain was run (http://rsb.info.nih.gov/ij/) and visualized using enhanced chemiluminescence. After several washes, cells were visualized with peroxidase staining using 3,3′-diaminobenzidine (Immpact DAB, Vector Laboratories, Burlingame, CA), and mounted in moviol.

### Statistics

The Student’s t-test or Kolmogorov-Smirnov test was applied for all statistical analyses, and differences evaluated at P < 0.05.

**Drugs.** The following drugs were added to the recording buffer to isolate sIPSCs and/or miniature IPSCs (mIPSCs): 50 μM d(-)-2-amino-5-phosphonopentanoic acid (AP-V), 20 μM 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, both N-methyl-D-aspartate receptor antagonists, 20 μM (2S)-(+)-5,5-dimethyl-2-morpholinoneacetic acid (SCH50911), GABA_A antagonist; 10 μM bicuculline methiodide, a GABA_A antagonist; 10 μM 6-cyano-7-nitroquinoxa-line-2,3-dione (CNQX), an AMPA/kainate receptor antagonist (all from Tocris Cookson, Ballwin, MO), 1 μM tetrodotoxin (TTX), a sodium channel blocker (Sigma, St. Louis, MO).

#### Table 1. Passive properties of layer 2/3 pyramidal cells and cells migrating from the GE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Capacitance, pF</th>
<th>R_m, MΩ</th>
<th>R_a, MΩ</th>
<th>V_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>90.97 ± 5.06</td>
<td>110.10 ± 6.65</td>
<td>19.79 ± 1.69</td>
<td>−62.63 ± 1.26</td>
</tr>
<tr>
<td>MAM-Tx</td>
<td>84.87 ± 10.55</td>
<td>118.23 ± 16.00</td>
<td>23.14 ± 2.79</td>
<td>−65.28 ± 1.00</td>
</tr>
<tr>
<td>GE cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>31.76 ± 4.32</td>
<td>645.68 ± 131.00</td>
<td>21.17 ± 3.12</td>
<td>−28.00 ± 1.13</td>
</tr>
<tr>
<td>MAM-Tx</td>
<td>27.15 ± 2.21</td>
<td>608.62 ± 79.75</td>
<td>24.29 ± 2.03</td>
<td>−31.39 ± 2.45</td>
</tr>
</tbody>
</table>

Values are means ± SE, R_m, membrane resistance; R_a, access resistance; V_m, membrane potential; MAM-Tx, methylazoxymethanol-treated; GE, ganglionic eminence.

**RESULTS**

**Experimental population.** In this study, two populations of cells from the parietal cortex were used. 1) We assessed cells leaving the GE and migrating toward the neocortex in normal and MAM-Tx organotypic cultures. These were labeled by electroporation at P0 and allowed to migrate for several days. At this point we identified the migrating cells and recorded responses. We previously determined that MAM-Tx brains have a specific deficit in interneuron migration (Abbah and Juliano 2013; Poluch et al. 2008); it is important to understand distinctions of the functional properties of MAM-Tx interneurons as they migrate into the neocortex. 2) We additionally recorded from layer 2–3 pyramidal cells of parietal cortex from animals 4–5 wk old. We also know that the distribution of interneurons in the mature cortex is altered, most likely reflecting the migratory deficits. We concluded that the most effective way of determining the effect of the altered distribution was to record from the targets of these cells, the pyramidal cells. We also know from our previous studies that MAM treatment leads to increased GABA_A expression; it is important to determine the effect of this increased expression in relation to the migrating interneuron and also in relation to the more mature pyramidal cells that function in the environment of increased GABA_A.

The passive properties of the layer 2/3 pyramidal cells at 4–5 wk of age and the cells migrating away from the GE were determined and are presented in Table 1. No significant differences between the normal and MAM-Tx cells in either condition were observed that could account for altered response properties.

**Exposure to MAM increases GABA_A neurotransmission in cells originating from the GE.** Migrating cells leaving the GE are influenced by a variety of signaling molecules including GABA. Our laboratory previously demonstrated that the normal pattern of migration and orientation of GE-derived neurons is altered in MAM-Tx animals, in part due to altered GABA_A-mediated activity (Poluch et al. 2008). To determine whether migrating interneurons are influenced by increased GABA_A activity, we evaluated GABA_A-mediated synaptic neurotransmission in cells leaving the GE by recording sIPSCs. Migrating cells were prior labeled with a plasmid that codes for RFP using electroporation directed into the GE and identified under fluorescent microscopy. Labeled cells were presumed to be migrating if they expressed RFP, crossed the corticostriatal junction, and displayed the morphology of a migrating cell (exhibited a leading process). An example can be seen in Fig. 1E. Whole-cell patch-clamp recording occurred on cells leaving the GE after they crossed the corticostriatal boundary and were...
positioned close to the parietal neocortex (Fig. 1E). sIPSCs were isolated using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid (SCH50911; 20 μM), and D-(-)-2-amino-5-phosphonopentanoic acid (AP-V; 50 μM). A: representative traces of sIPSCs recorded in a cell leaving the GE in normal cortex shown over different periods of time and when blocked with bicuculline. B: sIPSCs in a representative MAM-Tx cell leaving the GE, which were also blocked by bath application of 10 μM bicuculline. C: the cumulative probability of the amplitude shows a shift to the right that is significantly greater in the cells leaving the GE in the MAM-Tx animals (red line) compared with the controls from untreated animals (black line). D: the cumulative probability plot of sIPSC frequency shows a leftward shift following treatment with MAM (red line), indicating a reduction in interevent interval and significant increase in frequency. E: a schematic diagram of an organotypic slice illustrating the positioning of the red fluorescent protein construct in a pipette and the migratory path of the cells exiting the GE. The small black dots in the left diagram indicate the site of electroporation, while the larger black dot in the right diagram indicates the approximate position of cells at the time of recording. The white dotted line indicates the position of the corticostriatal junction. Also shown in E is an image of a cell migrating away from the GE during whole-cell patch-clamp recording. CTX, cortex. F: the mean amplitude of the sIPSCs is significantly increased in MAM-Tx animals relative to control. G: the mean frequency is also significantly increased in the MAM-Tx cells. **P < 0.0001, Komolgorov-Smirnov test. *P < 0.05, Student’s t-test. Error bars = standard error of mean. See Table 2 for sample sizes.

Treatment with MAM increases the amplitude of sIPSCs in layer 2/3 pyramidal cells of parietal cortex. Following treatment with MAM on E33, the width of layer 4 in ferret somatosensory cortex diminishes, leading to reduction in the overall cortical thickness (Noctor et al. 2001). Additionally, the distribution of GABA_A subunits is expanded and increased in the upper cortical layers; several subtypes of interneurons are also abnormally positioned (Jablonska et al. 2004; Poluch et al. 2008). To examine the functional implication of the increased expression of GABA_A subunits on the inhibitory tone within the upper cortical layers, we performed whole-cell patch-clamp recording on pyramidal cells that resided in the parietal cortex. sIPSCs were isolated using CNQX (10 μM), SCH50911 (20 μM) and AP-V (50 μM) and recorded at a
holding potential of $-70$ mV. In P28–P38 MAM-Tx animals, the amplitude of sIPSCs was significantly increased (40.08 ± 5.61 pA; $n = 22$ cells/6 animals) relative to control animals (29.45 ± 2.31 pA; $n = 40$ cells/7 animals) (Fig. 2, A–C). Our data also indicate that the frequency of sIPSCs was significantly altered following treatment with MAM when tested on the cumulative frequency curves, and a nonsignificant trend toward an increase occurred when tested on the mean values (MAM-Tx animals: 125.09 ± 18.01 Hz; control: 101.65 ± 10.69 Hz; $P = 0.05$, Fig. 2, D and H). The increased amplitude of sIPSCs (Fig. 2, C and G) in MAM-Tx animals with less change in frequency (Fig. 2, D and H) suggests that the altered responsiveness results from enhanced GABAAR expression in the postsynaptic membrane, probably due to the observed increase in expression of GABAAR. To further evaluate factors responsible for these alterations, we evaluated the kinetics of GABAAR. First, we analyzed the 10–90% rise time and compared control with MAM-Tx values. The 10–90% rise time provides spatial information about key synaptic elements and potential alterations due to changes in presynaptic terminal number and positioning (Alberto and Hirasa 2010; Kobayashi and Buckmaster 2003; Wierenga and Wadman 1999; Xie et al. 1997). In our study, the 10–90% rise time of the sIPSCs (MAM-Tx: 6.00 ± 0.25 ms; control: 6.82 ± 1.27 ms) and mIPSCs (MAM-Tx: 6.34 ± 0.44 ms; control: 5.66 ± 0.24 ms) was not significantly different between normal and MAM-Tx animals (Fig. 4, AI, BI, DI, and EI). This parameter was significantly reduced in cells migrating from the GE in our model animals during the recording of these cells in organotypic cultures (4.94 ± 0.41 ms and 6.86 ± 0.86 ms, MAM-Tx and control, respectively, Fig. 4, CI and FI). We next evaluated the decay time constant. In MAM-Tx animals, the decay time constant of sIPSCs (MAM-Tx: 19.20 ± 1.00; control: 9.04 ± 0.90) and mIPSCs (MAM-Tx: 4.17 ± 0.29; control: 3.54 ± 0.24) in the pyramidal cells was significantly prolonged (Fig. 4, AI, BI, DI, and EI). This is also true for the migrating cells originating from the GE (9.08 ± 0.82 ms and 6.53 ± 0.72 ms, MAM-Tx and control, respectively, Fig. 4, CI and FI). Analysis of the charge transfer (measured as area) also reveals a significant increase in this parameter for the sIPSCs of the pyramidal cells in layer 2/3 (537.34 ± 77.38 pA × ms and 345.95 ± 42.09 pA × ms MAM-Tx and control, Fig. 4, AI, and DI) and the cells originating in the GE (371.14 ± 61.44 pA × ms and 226.11 ± 28.07 pA × ms MAM-Tx and control, respectively, Fig. 4, CI, and FI). This was also true for the recorded mIPSCs (MAM-Tx: 133.40 ± 16.62 pA × ms; control: 72.76 ± 4.57 pA × ms; Fig. 4, BI and EI).

Treatment with MAM increases the expression of GABAAR within the parietal cortex of ferrets. The electrophysiological results presented so far support the idea that GABAAR-mediated activity is increased following treatment with MAM. To further validate the aberrant elevation of GABAAR activity in our model animals, we performed Western blot analysis to measure the level of expression of different subtypes of the GABAAR within the somatosensory cortex. We tested several receptor subtypes based on the likelihood of their expression in neocortex at this stage of development in ferrets. Although we

### Table 2. Sample sizes for all experiments

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<tr>
<td>Normal</td>
<td>40</td>
<td>40</td>
<td>7</td>
<td>4</td>
<td>4,066</td>
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<tr>
<td>MAM-Tx</td>
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<td>6</td>
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<tr>
<td>Pyramidal cells: sIPSCs</td>
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<td>6</td>
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<td>GE cells: sIPSCs</td>
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<td>8</td>
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<tr>
<td>MAM-Tx</td>
<td>21</td>
<td>21</td>
<td>8</td>
<td>4</td>
<td>1,474</td>
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</table>

sIPSC, spontaneous inhibitory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current.
expected that GABA_A_R would be present at this age in ferret cortex, we were not able to visualize them. There were no obvious differences in the GABA_A_R between normal and MAM-Tx pyramidal cells after MAM treatment (Fig. 5, top). The GABA_A2aR and the GABA_A2β2R subunits, however, were increased in the parietal cortex after MAM treatment (Fig. 5, middle and bottom). These results indicate that GABA_A_R expression is increased, underlying the enhanced GABA_A_R-mediated synaptic transmission in MAM-Tx animals.

DISCUSSION

The present study uses a ferret model of CD to investigate the alterations of GABAergic synaptic transmission recorded in layer 2–3 pyramidal cells of the somatosensory cortex and on migrating GE-derived cells. We show that the basal activity mediated by GABA_A_R is enhanced within layer 2/3 and in cells leaving the GE in our model animals. An increased expression of GABA_A_R after MAM treatment is likely to underlie the changes in synaptic transmission. In our model, layer 4 formation is selectively disrupted by in utero administration of MAM on E33, leading to its profound reduction in size, a redistribution of interneurons, thalamic afferents, and GABA_A_R (Jablonska et al. 2004; Noctor et al. 1999; Palmer et al. 2008). We also observed that cells leaving the GE and traveling to the neocortex after MAM treatment display abnormal dynamic properties while en route and are abnormally positioned upon reaching their neocortical target (Abbah and Juliano 2013; Poluch et al. 2008). The present study evaluated the functional implications of these findings in two populations of cells: GABAergic neurons leaving the GE, and pyramidal cells in layer 2–3 of neocortex, presumably influenced by inhibitory synapses.
Treatment with MAM increases the amplitude of sIPSCs in layer 2/3 pyramidal cells. The increased amplitude of sIPSCs in pyramidal cells of layer 2/3 occurred without any significant change in frequency. This indicates that synaptic transmission through GABA_A receptor is upregulated in MAM-Tx animals, which is likely due to the increased GABA_A receptor density on the postsynaptic membrane previously observed in MAM-Tx animals (Jablonska et al. 2004). To further investigate the mechanism underlying the increase in sIPSC amplitude, we evaluated the mIPSCs in the same layer 2/3 pyramidal cells. mIPSCs are caused by the quantal release of GABA from presynaptic terminals of GABAergic interneurons in the absence of action potentials (Edwards et al. 1990). The number of presynaptic terminals of interneurons and/or the postsynaptic GABA_A receptor density on pyramidal cells determines the frequency of mIPSCs (Shao and Dudek 2005). In our study, both amplitude and frequency of mIPSCs were significantly increased in MAM-Tx animals relative to control. The increase in mIPSC frequency and amplitude signifies that both pre- and postsynaptic mechanisms are involved in altering GABAergic neurotransmission in our model animals. Presynaptic factors, such as alterations in the number of GABAergic interneurons and the quantal size of release, determine the frequency of response. It is possible that the GABAergic neurons in our model are altered in some way to induce a greater number of synapses or greater quantal release. We also know that after MAM treatment, subpopulations of interneurons show altered distributions (Poluch et al. 2008). Such redistributions may result in ectopic projections or altered synapses with upper cortical layer pyramidal cells. Region-specific alterations in local circuit connections occur in other models of CD (Brill and Huguenard 2010).

Postsynaptic mechanisms, such as the density and internalization of postsynaptic protein, modulate the size of GABAergic neurotransmission contributing to the change in mIPSC amplitude (DeFazio and Hablitz 1999; Goodkin et al. 2007; Hartmann et al. 2008; Kittler et al. 2000; Loup et al. 2006; Michels and Moss 2007). The frequency of sIPSCs, which is the summation of both action potential-dependent and -independent events, was altered to a lesser degree than the frequency of mIPSC in MAM-Tx animals. A number of factors may be responsible for this, such as the nature of the network of interneurons within layer 2/3 (Bacci et al. 2003; Tamas et al. 1998). Bacci and colleagues (2005) suggest that intrinsic modulation of selective populations of interneurons may have a strong effect on the excitatory activity of pyramidal cells. A number of extrinsic and intrinsic modulatory mechanisms are available, including autaptic transmission impacting on GABAergic cells, which subsequently influence the global signaling of excitatory pyramidal cells. Since we know that interneurons are abnormally placed in juvenile MAM-Tx ferrets, this may cause an abnormal influence on pyramidal cells.

Although reduction in amplitude and frequency of IPSCs frequently associate with epilepsy and CD (Buckmaster and Dudek 1997; Hablitz and DeFazio 1998; Henry et al. 1993; Jacobs et al. 1999; McDonald et al. 1991; Rocha et al. 2007), the converse occurs in other models of these diseases. Our finding of increased GABAergic neurotransmission in layer 2/3 pyramidal cells corresponds with a recent finding of enhanced...
GABAergic activity in pediatric CD (Cepeda et al. 2012). Other studies demonstrate a similar increase in GABAergic activity. A freeze lesion model of CD reports increased IPSCs (Brill and Huguenard 2010; DeFazio and Hablitz 1999; Prince et al. 1997). In other models of CD, however, a reduction in inhibitory tone occurs, such as in utero exposure to carmustine, MAM, or radiation in rats (Karlsson et al. 2011; Xiang et al. 2006; Zhou and Roper 2010; Zhou et al. 2009). In these
models, the development of seizures is believed to be a consequence of reduced inhibitory drive similar to epileptogenic foci reported in animal models of epilepsy (Cossart et al. 2001; Hirsch et al. 1999; Kobayashi and Buckmaster 2003; Rice et al. 1996; Shao and Dudek 2005; Sun et al. 2007). This analysis is complicated further by evidence of elevated GABAergic signaling in other seizure models (Cossart et al. 2001; Gibbs et al. 1997; Nusser et al. 1998; Shao and Dudek 2005; Zhan and Nadler 2009). Overall this suggests that abnormal GABAergic signaling is a feature of abnormal cortical development, and that both increased and decreased GABA receptor neurotransmission contribute to impaired neural function.

**GABA<sub>A</sub>R-mediated neurotransmission enhances in migrating GE-derived neurons after MAM treatment.** The orientation and distribution of interneurons is altered in E33 MAM-Tx animals, in part due to elevated GABA<sub>A</sub>R-mediated activity (Poluch et al. 2008). Recently, we found that high levels of GABA<sub>A</sub>R alter the dynamic behavior of migrating cells leaving the GE (Abbah and Juliano 2013). To determine whether GE-derived cells receive abnormally increased GABA<sub>A</sub>R signaling after MAM treatment, we measured the sIPSCs. Both amplitude and frequency of sIPSCs were increased in MAM-Tx animals, suggesting that GABA<sub>A</sub>R synaptic transmission enhances in MAM-Tx animals. GABA signaling plays an important role in promoting migration of GE cells, and alterations in the basal level of GABA<sub>A</sub>R activity are likely to be deleterious in the overall migration of GE-derived interneurons. This was also demonstrated in a study by Heck and colleagues (2007), who used GABA<sub>A</sub> agonists and antagonists to show altered migration after disrupting these receptors. Elevated GABA<sub>A</sub>R-mediated activity, while initially promoting migration of cells derived from the GE, triggers early maturation of these cells by inducing precocious expression of the potassium chloride cotransporter (Abbah and Juliano 2013; Ganguly et al. 2001). During development, potassium chloride cotransporter maintains a high extracellular Cl<sup>-</sup> gradient and induces hyperpolarization of cells in response to GABA, playing a role in the developmental switch from the GABA-induced depolarization to hyperpolarization, which may also terminate cell migration (Bortone and Polleux 2009; Rivera et al. 1999). Thus the elevated functional responses to GABA<sub>A</sub>R synaptic transmission in cells migrating away from the GE supports the idea that an aberrant GABAergic environment contributes to both abnormal migration and disrupted processing of information.

The GE-derived cells that we studied were still en route to their target destination in the neocortex. Consequently, synaptic contacts with these cells are transient, and different regions of the neocortical environment during transit influence the level of GABA signaling. In our model, loss of layer 4 leads to redistribution of thalamic afferents and associated GABA<sub>A</sub>R; this may also alter the neocortical environment with respect to GABA signaling to influence the migration and positioning of...
migrating GE cells. The increase in both amplitude and frequency of sIPSCs suggests that both presynaptic mechanisms and postsynaptic GABA_A expression contribute to the elevated GABA_A-mediated increase in inhibitory synaptic neurotransmission.

**Kinetics of GABA_A signaling are altered in MAM-Tx animals.** The measure of 10–90% rise time gives relative spatial information with respect to postsynaptic sites; changes in rise time deduce the locus of change in presynaptic terminal number. It is widely accepted that receptors located in the soma of postsynaptic membrane give a faster rise time compared with those located on the dendrites (Shao and Dudek 2005). In our study, there is no significant change in the rise time in either the sIPSCs or the mIPSCs of pyramidal cells in layer 2/3, indicating that there is no significant positional change in the active zones and receptor clusters between normal and MAM-Tx animals (Alberto and Hirasawa 2010; Xie et al. 1997). In the cells migrating from the GE, however, we observed a significant reduction in rise time after MAM treatment compared with normal. Although the reason for this change is unclear, one factor that may be responsible is the relative position of the migrating cells in the developing neocortex, which may affect the nature of synaptic contact they receive. The decay time constant is influenced by the density of postsynaptic receptors and receptor subtype composition (Shao and Dudek 2005; Xie et al. 1997); prolongation of decay time constant in both the sIPSCs and mIPSCs in MAM-Tx animals corresponds with increased expression of GABA_A. In this study and others by our group, we demonstrated increased GABA_A in this model of MAM treatment (Abbah and Juliano 2013; Jablonska et al. 2004). We also show an altered distribution in the composition of receptor subtypes, which vary with maturity, in this study and in previous work (Abbah and Juliano 2013; Jablonska et al. 2004). The amplitude of the IPSCs and decay time constant determines the charge transfer. In our study, the charge transfer was significantly increased for both sIPSC and mIPSC in cells leaving the GE and layer 2/3 pyramidal cells following treatment with MAM. This agrees with the increased amplitude and decay time reported here.

**Expression of GABA_A subunits is altered in MAM-Tx animals.** Our results show that GABAergic neurotransmission is enhanced in the neocortex of MAM-Tx animals, probably as a result of increased expression of GABA_A. We also demonstrated that GABA_A increase after E33 MAM treatment, primarily in the upper cortical layers using receptor binding and immunohistochemistry (Jablonska et al. 2004). Here we find an increase in the amplitude of sIPSCs and mIPSCs in pyramidal cells of layer 2/3 and a prolonged decay time. To further confirm that increased GABA_A expression is responsible for the enhanced GABAergic neurotransmission, we used Western blotting to measure the level of various subunits of GABA_A; GABA_A expression is increased in dysplastic cortex compared with control. The mammalian GABA_A is made of five subunits generated from 19 possible subunits (β1–3, γ1–3, δ, ε, θ, ρ, and ρ1–3) (Olsen and Sieghart 2008). Compelling evidence indicates that these subunits have differences in temporal expression and regional distribution during development (Araki et al. 1992; Carlson et al. 2010; Fritschy et al. 1994; Garrett et al. 1990; Hashimoto et al. 2009; Laurie et al. 1992; McKernan et al. 1991; Pirker et al. 2000; Yu et al. 2006). The GABA_A subunit is expressed early in development and mediates a number of developmental activities (Carlson et al. 2010; Fritschy et al. 1994; Laurie et al. 1992; Poulter et al. 1992). The increased expression of GABA_A and GABA_A appears to underlie the enhanced GABAergic neurotransmission reported here. Our laboratory also observed increased expression of GABA_A and GABA_A subunits in the neocortex of MAM-Tx animals at P0 (Abbah and Juliano 2013). A possible explanation for increased GABA_A expression in layer 2/3 of parietal cortex in MAM-Tx animals is the redistribution of thalamic afferents, which associate closely with GABA_A during development (Jablonska et al. 2004; Noctor et al. 2001; Palmer et al. 2001). In normal animals, thalamic afferents make synaptic contacts primarily in layer 4 (Jones and Burton 1976). This ensures that afferent information to the neocortex is first received in layer 4 before being transferred to the upper layers and lower layers 5 and 6 (Johnson and Alloway 1996; Senft and Woolsey 1991; White 1989). When layer 4 diminishes, the thalamic afferents and associated GABA_A become redistributed to upper cortical layers; this thalamic redistribution may account for the increase in GABAergic signaling in this region. Others report a close correlation between the expression of GABA_A and projection of thalamic afferents in the somatosensory and visual cortices of rodents (Brodie et al. 1996; Schlaggar and O’Leary 1994). Thalamic afferents play a significant role in the expression and regional distribution of GABA_A subunits within the visual and somatosensory cortical areas of rodents (Paysan et al. 1997). In rats, the expression of the α1-subunit, the predominant subunit of GABA_A in adult animals, is highest within the primary somatosensory (S1) and visual (V1) cortical areas; these regions also receive substantial terminations of thalamic afferents (Fritschy et al. 1994; Paysan et al. 1994). Huntsman and Jones (1998) reported that the mRNA of various GABA_A subtypes diminish in visual cortex after enucleation, also implicating thalamic influence on GABA_A expression.

GABA_A and GABA_A expression significantly increase in our model animals. The increase in these two receptor subunits represents an alteration in the stoichiometry of the GABA_A and may underscore the elevated GABA transmission in pyramidal cells of MAM-Tx juvenile ferrets. A change in the composition of the channels could easily lead to a change in the kinetics of response. The GABA_A subunit is presumed to be important for synaptic transmission and for the postsynaptic clustering of GABA_A during the development of synapses (Heck et al. 2003). In this regard, it could clearly play a role in the abnormal synaptic transmission observed here. It is not clear why we did not observe an increase or change in the α1-receptors in either the P0–P1 or P28–P38 animals, as we would expect these to increase as the animal matures. It may be that the ferret neocortex is still relatively immature, even at P28–P38, and does not express high levels of this receptor until a later date. In any case, the obvious increase and probable change in the composition of at least two GABA_A subtypes can clearly underlie the change in kinetics that we observed in our recordings.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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