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Prenatal stress delays inhibitory neuron progenitor migration in the developing neocortex

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Summary

Prenatal stress has been widely demonstrated to have links with behavioral problems in clinical populations and animal models, however, few investigations have examined the immediate developmental events that are affected by prenatal stress. Here, we utilize GAD67GFP transgenic mice in which GABAergic progenitors express green fluorescent protein (GFP) to examine the impact of prenatal stress on the development of these precursors to inhibitory neurons. Pregnant female mice were exposed to restraint stress three times daily from embryonic day 12 (E12) onwards. Their offspring demonstrated changes in the distribution of GFP-positive (GFP+) GABAergic progenitors in the telencephalon as early as E13 and persisting until postnatal day 0. Changes in distribution reflected alterations in tangential migration and radial integration of GFP+ cells into the developing cortical plate. Fate mapping of GAD67GFP+ progenitors with bromodeoxyuridine injected at E13 demonstrated a significant increase of these cells at P0 in anterior white matter. An overall decrease in GAD67GFP+ progenitors at P0 in medial frontal cortex could not be attributed to a reduction in cell proliferation. Significant changes in *dlx2*, *nkx2.1* and their downstream target *erbb4*, transcription factors which regulate interneuron migration, were found within the prenatally-stressed developing forebrain, while no differences were seen in *mash1*, a determinant of interneuron fate, *bdnf*, a maturation factor for GABAergic cells or *fgf2*, an early growth/differentiation factor. These results demonstrate that early disruption in GABAergic progenitor migration caused by prenatal stress may be responsible for neuronal defects in disorders with GABAergic abnormalities like schizophrenia.

Keywords

Prenatal stress; GAD67; migration; inhibitory neurons; transcription factors; *erbb4*

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1. Introduction

Research on the effects of prenatal stress increasingly demonstrates that this adverse life event can be a significant risk factor for neuropsychiatric disorders. Stress in pregnant women has significant effects on fetal physiology (Monk et al., 2004; Wadhwa, 2005). Prenatal stress has also been linked to childhood behavioral, physiological, and emotional problems (Huot et al., 2004; Martini et al., 2010; O'Connor et al., 2003). Perinatal stressors have an association with symptom severity in Tourette syndrome (Leckman et al., 1990) and with autistic-like symptoms (Ronald et al., 2010). Psychiatric problems that develop in adolescence and young adulthood may also be related to maternal stress during pregnancy. Obstetric complications are more frequent in the maternal history of patients with bipolar disorder (Kinney et al., 1998). Furthermore, the development of schizophrenia has been shown to be correlated with stress experienced during pregnancy (Review: King et al., 2005). Changes in the brain occurring during prenatal stress must have long lasting consequences in order to explain the emergence of psychiatric illness years later. The mechanisms by which persistent cellular changes may be induced in the developing fetus by maternal stress are not understood.

Many of the neuropsychiatric disorders correlated with prenatal stress implicate the GABAergic system. Schizophrenia has been shown to be associated with a loss of GABAergic neurons in the hippocampus and neocortex and a change in their functioning, particularly those expressing parvalbumin and somatostatin (Hashimoto et al., 2008; Reynolds et al., 2004; Benes, 1999; Benes and Berretta, 2001). GABAergic abnormalities have also been implicated in the pathogenesis of autism and Tourette syndrome (Kataoka et al., 2010; Yip et al., 2008). The mechanisms by which these potentially critical changes in adult GABA neurons occur are not understood.

There are some data to suggest that prenatal stress and associated high levels of corticosteroids during embryonic development lead to changes in GABAergic populations in the adult brain which may reveal general pathological mechanisms involved in psychiatric disorders. Rat offspring of “stressed” mothers have fewer benzodiazepine binding sites in the amygdala and hippocampus, less GABA/benzodiazepine inhibitory activity and more sensitivity of GAD65 expression in these same regions in response to later corticosterone exposure (Barros et al., 2006; Stone et al., 2001; Weinstock, 1997). GABAergic synapses in the hypothalamus, as measured by vGAT immunocytochemistry, have also been shown to be increased (Viltart et al., 2006). Prenatal stress lowers the seizure threshold in the dorsal hippocampus (Edwards et al., 2002), suggesting a loss of inhibition of this region.

These results reflect changes in the adult population of GABAergic neurons following exposure to prenatal stress during critical periods of inhibitory neuron development. In rodents, GABAergic neurogenesis begins in the ganglionic eminence of the ventral telencephalon midway through embryonic development (~embryonic day 10–11) and continues for approximately a week (Fig 1A; for review see Corbin and Butt, 2011). GABAergic interneuron progenitors destined for the hippocampus and neocortex migrate away from the ventricular zone of the ventral telencephalon and tangentially move into the developing cortical plate in two migratory streams within the marginal zone and intermediate zone. After moving tangentially, GABAergic progenitors radially migrate and integrate into different cortical layers, a process that begins in lateral regions within 3 days of inhibitory neuron birth. Most migration is completed by birth. It is unknown what impact prenatal stress has on GABAergic progenitors that may result in abnormalities in the resulting mature neurons within adult brain networks. Some evidence for the impact of stress on GABAergic systems comes from studies in early postnatal life and adulthood in which acute and chronic stress leads to reductions in GABA signaling (review: Luscher et

al., 2011). However, GABAergic developmental events on which stress could impinge are significantly different in prenatal vs postnatal periods.

Prenatal stress has also been discussed as a potential major influence on neuronal migration (Schneider et al., 2002) and gestational dexamethasone treatment has been demonstrated to have an impact on migration via an actin regulatory protein, caldesmon (Fukumoto et al., 2009). Given this framework, we examined the development of precursors to inhibitory neurons in the prenatally-stressed telencephalon using GAD67GFP knock-in mice (Tamamaki et al., 2003) which consistently label GABAergic progenitors in the embryonic brain. GFP-labeled interneuron progenitor populations within the ganglionic eminence and dorsal telencephalon were evaluated at multiple embryonic time points following prenatal stress. Beginning one day after the start of prenatal stress, both the proliferation and different phases of migration of these GABAergic neuron progenitors were evaluated to assess the impact of prenatal stress.

2. Materials and Methods

2.1. Mice

GAD67-GFP(Δ neo) mice (Tamamaki et al., 2003) were bred on a CD1 background. GAD67GFP^{+/-} male mice were used for mating with CD1 females lacking the GAD67GFP knock-in gene. Timed pregnancies were monitored following detection of vaginal plug on embryonic day 0 (E0) and thirty pregnant females were singly housed from E12. All experimental procedures involving animals were performed in accordance with the Yale Animal Resources Center and Institutional Animal Care and Use Committee (IACUC) policies.

2.2. Prenatal stress

Beginning on E12, half of the pregnant female mice were subjected to acute stress within a plexiglass restraint for 45 minutes under bright lights, three times daily during the daytime light cycle (at approximately 9 am, 12:30 pm, and 4 pm). The plexiglass restraints allowed pregnant females to change positions throughout each restraint period. .

2.3. Birth-dating, tissue collection and immunocytochemistry

Offspring brain tissue for immunocytochemistry and in situ hybridization was collected on E13, E14, E15 and P0 at approximately 10 am from 3–5 litters for each time point in each condition. This permitted analysis of GABAergic cells one, two, and three days after the start, as well as during, prenatal stress, to allow an investigation of the embryonic mechanisms of this process. We also examined the day of birth to understand cumulative effects at the termination of prenatal stress. In order to birth-date GABAergic progenitors, one-time bromodeoxyuridine (BrdU) injections (100 mg/kg in a 5 mg/ml solution of 0.07N NaOH sterile filtered) were given i.p. for each pregnant female prior to the first restraint period on either E12 or E13. For offspring tissue collected at E13, BrdU was injected at E12 (25 hours earlier) and for all other experiments was injected at E13. SRY genotyping was done to ensure use of only male offspring in all analyses.

The whole head, from E13 and E14 offspring, or the brain, from E15 and P0 offspring, was first rinsed in cold RNase-free PBS and then immersion fixed and post-fixed at least 12 hours in 4% paraformaldehyde/phosphate buffered saline. Following transfer to RNase-free 20% sucrose, tissue was embedded and cryo-sectioned (Leica, CM1900, Bannockburn, Illinois) at 25 μ m. Slide mounted sections were processed for staining first by blocking for 1 hour with 10% goat serum in PBS with 0.025% TritonX-100, 0.0125% Tween20 (PBS++) and then incubating for 24–48 hours at 4°C with 5% goat serum/PBS++ containing primary

antibodies as follows: 2-bromodeoxyuridine (BrdU) (rat 1:500; Accurate Chemicals, OBT0030, #H8913), Ki67 (mouse monoclonal 1:200, Novacastra, NCL-L-Ki67-MM1), caspase-3 (1:500; Cell Signaling, #9661), and green fluorescent protein (GFP) (1:1000; Abcam, AB13970, #660556). Brain sections were then washed three times in PBS followed by an incubation in 5% goat serum/PBS++ containing Alexa dye-conjugated secondary antibodies (1:500-1000; Molecular Probes). Fluorescently-labeled sections were coverslipped using mounting medium with DAPI (Vector Laboratories, #H-1200).

2.4. Cell counting

Three approaches were used to evaluate the distribution of cells within the developing cortex—stereological counts, measurement of cells distributed around the external circumference of the dorsal telencephalon, and bin counting from deep to superficial layers of the cortical plate. Stereological, unbiased estimates of GAD67GFP+, GAD67GFP+/BrdU+ (E13) double-labeled cells, and Ki67+ cells within the embryonic ventral and dorsal telencephalon and the neonatal frontal cortex, hippocampal CA and hippocampal dentate gyrus were obtained with a computer running the StereoInvestigator software (Microbrightfield, Colchester, Vermont) and coupled to a Zeiss Axioskope 2 Mot Plus (Carl Zeiss, Oberkochen, Germany) equipped with a digital camera and calibrated motorized stage controller that allows precise control of y-, x and z axes. Using the optical fractionator, nuclear profiles were counted in 3-dimensional counting boxes. Every 20th coronal section was used that contained any portion of the telencephalon. Variations between counts were assessed by the Gundersen coefficients of error and data were only used when cell count error values were below 0.15.

Measurement of GAD67GFP+ cells across the external circumference of the dorsal telencephalon was done by measuring with Neurolucida (Microbrightfield) both the surface perimeter with GAD67GFP+ cells immediately underlying the pia of the cortical plate in each section and the total external circumference from most lateral to most medial extent in every coronal section. The same point in the migratory path of GAD67GFP+ cells, as they enter an organized migratory stream, was used as the most lateral extent of the cortical plate. Following the dorsal extent of the pial surface of the telencephalon, the total circumference distance was measured. The distribution of GAD67GFP+ cells across surface area was then calculated as a percent of the total circumference and this was averaged across multiple coronal sections in the brain of each offspring.

In order to evaluate distribution of GAD67GFP+ cells within the radial thickness of the embryonic cortical plate, a blind method of counting cells within standardized bins across the cortical plate was adopted. Bin counts were made using 20× Apotome Z-stack micrographs obtained on an ApoTome equipped Axiovert 200M with Axiovision 4.5 software (Carl Zeiss, Thornwood, NY, USA) for right and left sides of four to six coronal sections by an experimenter blind to the animal condition. After counts were made within equally sized bin arrays placed across the cortical plate in a lateral and dorsal sector, cell counts were grouped into a single bin at the pial surface and three other equally sized larger bins that encompassed approximately a third of the layers of the cortical plate.

To evaluate differences, results from individual offspring within a single litter were averaged and then each litter was treated as a unit for each analysis. Cell densities within embryonic ventral and dorsal telencephalon and percent of circumference containing cells were compared using a student's t-test. Distribution of cells across the cortical plate was compared using repeated-measures ANOVA. Cell densities within neonatal cortex and hippocampus were compared using an ANOVA across gray matter regions and cell densities in the white matter were compared with a student's t-test.

2.5. In situ hybridization and qPCR

We included tissue samples from multiple NS and PS litters for *in situ* hybridization and qPCR analyses, to control for litter effects of PS and the precise timing of embryonic development at tissue collection. We also included samples from embryos that both did and did not carry the GAD67GFP+ transgene. Gene expression changes were identified in non-stressed (NS) and prenatally-stressed (PS) GAD67GFP+ offspring and then substantiated in NS and PS GAD67GFP-negative offspring. We saw similar effects in both groups of animals and show here the data from GAD67GFP- embryos. Quantification of the expression of *dlx1*, *dlx2*, *nkx2.1*, and *erbb4* was made using ventral telencephalic tissue while *bdnf* and *fgf2* was assessed in dorsal telencephalic tissue.

Collection and sectioning of brain tissue was performed as described above for immunocytochemistry using RNase free solutions. *In situ* hybridization was performed in frozen sections using the digoxigenin system as described previously (Ohkubo et al., 2004). Briefly, a riboprobe incorporating digoxigenin-labeled nucleotides was synthesized from linearized plasmids derived from the subcloned PCR fragments. Sections were fixed in 4% paraformaldehyde, washed in PBS, and dehydrated through grades of ethanol. Sections were then air dried, treated with proteinase K in buffer, fixed again, rinsed, acetylated, rinsed again in water, and air dried. Prehybridization was done with incubation in hybridization buffer followed by washes in 2X SSC and RNAase treatment. Tissue then underwent washes in 2X SSC, 50% heat-inactivated sheep serum in PBS. Sections were incubated overnight in a solution containing anti-digoxigenin Fab fragments, conjugated to alkaline phosphatase, preabsorbed with chick embryo powder to reduce background labeling. Following antibody incubation, sections were washed and incubated in developing buffer. The color reaction was carried out by incubation in developing buffer with NBT/BCIP. Finally, sections were washed in PBS and mounted in DAPI-containing mounting media.

To quantify the expression of genes involved in inhibitory neuron development, embryos were collected on E13, E14, and E15. The dorsal and ventral regions of the telencephalon were dissected out from the embryonic brains. The cerebral cortex and hippocampus were pooled in one sample from each embryo and the ganglionic eminences for a second sample, homogenized in Trizol reagent (Sigma) and frozen. After thawing, RNA was isolated using chloroform phase separation, precipitated with 75% alcohol, and re-suspended in water. RNA concentrations were determined using a Nanodrop Spectrophotometer (Thermo Scientific). cDNA was synthesized using Superscript III First Strand Synthesis Kit (Invitrogen). Quantitative PCR was carried out using Taqman Gene Assays (Applied Biosystems) for *dlx1* (ID Mm00438424_m1), *dlx2* (ID Mm00438427_m1), *nkx2.1* (ID Mm00447558_m1), and *erbb4* (ID Mm01256793_m1) using ventral telencephalic tissue and *bdnf* (ID Mm04230607_s1) and *fgf2* (ID Mm 00433287_m1) using dorsal telencephalic tissue. Beta-actin (predeveloped) was used as an endogenous control in all cases. qPCR was run using GeneAmp PCR Mastermix (Applied Biosystems) in a StepOne™ Instrument (Applied Biosystems). The cycle number threshold for signal detection for each gene of interest for each sample were normalized to that sample's cycle number threshold for beta-actin and the difference values in cycle number were converted to gene expression values using the formula:

$$[\text{expression} = 2^{-(\text{cycle number difference})}].$$

Gene expression values were averaged within each litter and then averages of litters in each condition were compared. Graphs depict gene expression values for PS brain normalized to gene expression values for NS brain. Genes which have similar developmental patterns of expression were grouped and NS and PS levels were compared across embryonic ages and specific gene by ANOVA.

2. Results

2.1. Distribution of GABAergic cells

In order to assess GABAergic progenitor populations during their proliferation and migration, we examined the GAD67GFP+ cells in three ways: 1) density within the ventral telencephalon (their birthplace and initial migrating zone), , and the dorsal telencephalon (their target zone) (Fig. 1A), 2) their circumferential distribution in their migratory paths tangentially around the dorsal telencephalon (Fig. 1A), and 3) their radial distribution as they integrate into the cortical plate after the change in direction to radial migration, across a grid overlaying the thickness of the dorsal telencephalon (Fig. 2G, 3A).

At embryonic day 13 (E13), the distribution of GAD67GFP+ cells in the early stages of migration around the neocortex was significantly different in offspring exposed to one day of stress compared to control offspring (Fig. 1B–E). The stereological count of GAD67GFP+ cells at E13 that had entered the dorsal telencephalon of PS offspring was 48% lower (Fig. 1F; $p < 0.05$, $n = 4, 3$). The density of GAD67GFP+ cells was unchanged in ventral telencephalon (Fig. 1G). The distribution of GABAergic progenitors within the superficial tangential migratory pathway was also altered after prenatal stress, with only 37.7% of the most superficial pathway within the cortical plate containing GAD67GFP+ cells in PS offspring compared to 56.6% in NS offspring (Fig. 1H; $p < 0.05$, $n = 3, 3$). This distribution change was present across all levels but more prominent in more caudal regions (Fig. 1I). As a control, we found that total circumference (size of the cortex) was not significantly different between PS and NS offspring (7.6% difference, $p = 0.22$, $n = 3, 3$).

The leading edge of GAD67GFP+ cells in the tangential migratory pathway of PS animals was also consistently less advanced at E14 (Fig. 2A–D). In the E14 telencephalon of control embryos, GABAergic precursors extended along the tangential migratory pathway 83.4% of the total circumference of the dorsal telencephalon that they will eventually occupy. However, in PS embryos, GABAergic cell distribution was only 69.1% of the dorsal telencephalic circumference (Fig. 2E, $p < 0.05$, $n = 4, 3$). Like at E13, differences in the circumferential distribution of GABAergic precursors between controls and PS animals at E14 were greater in caudal than in rostral regions (Fig. 2F).

The radial integration of GABAergic progenitors at E14 into the cortical plate was examined in the lateral region of cortex, where integration had begun at this stage of development. By superimposing a frame divided into horizontal bins over the thickness of cortex, the distribution of cells in layers of neocortex was quantified and averaged across all rostral to caudal sections (Fig. 2G). As expected, cells were most numerous in the two streams within the cortical plate, reflecting the two migratory pathways for cortical GABAergic progenitors: the most superficial region in the marginal zone (the first bin) and a deep region above the subventricular zone (the third bin) (Fig. 2G). There was no generalized effect of PS on this distribution at E14.

At E15, PS did affect radial migration of GABAergic progenitors as these cells reached the cortical plate and became incorporated into different layers (Fig 3A). In the dorsal region, GAD67GFP+ cells were redistributed in significantly greater proportions to the superficial layer after PS, with fewer cells in deeper cortical layers (all other bins) (Fig. 3B; $p < 0.05$, $n = 4, 4$). In lateral regions, similar to E14, there was no difference in the distribution of cells in the cortical plate of PS offspring (Fig. 3C). The effect on cortical integration in the dorsal region at E15 was also seen for absolute cell number across bins reflecting true increases and decreases in cell number (data not shown). At E15, analysis also showed qualitatively that tangential GAD67GFP+ cell distribution was again delayed by PS in the most medial region of cortex (Fig. 3D–G). This delay was reflected by GAD67GFP+ cells in the deeper

migratory path being clearly less advanced in PS animals (Fig. 3E,G brackets and arrows) than in NS animals (Fig. 3D,F).

Lastly, examination of the telencephalon on the day of birth (P0) demonstrated that GAD67GFP+ density was reduced by PS in total cortical gray matter by 23% ($p < 0.05$; $n = 4,4$), which did not differ across rostral to caudal levels. Analysis of medial regions of cortex, hypothesized to be most affected by migration disruptions, did not show consistent, significant decreases across regions—ANOVA analysis across regions and conditions showed a significant interaction ($p < 0.05$; $n = 5,4$). Post-hoc analysis showed that this interaction was mainly due to a 37% reduction in medial frontal cortex GAD67GFP+ cell density by PS (Fig. 4A,B; $p < 0.05$; $n = 5,4$) without significant changes in hippocampal regions. Early-born cells labeled with BrdU at E13 were not decreased significantly by PS in any region, although reductions were of similar magnitude to those for total GAD67GFP+ cells (Fig. 4D). Interestingly, early born GAD67GFP+ cells appeared to be redistributed at least in part from gray matter regions to the anterior subcortical white matter, a region that serves as a migratory pathway for progenitors targeted to frontal cortical regions (Fig. 4E,F). Within this region, there was a 59% increase in GAD67-GFP+/E13 BrdU+ double-labeled cells (Fig. 4G; $p < 0.05$, $n = 3,3$). There was no deficit in early born cells across the total cortical volume. These findings implicate a persistent change in migration to the most medial regions of the dorsal telencephalon due to prenatal stress that is more severe for early-born GABAergic cells.

2.2. GABAergic cell survival and proliferation

We examined whether changes in GABAergic progenitor cell numbers from E13 onwards following prenatal stress were due to changes in the survival or proliferation of these cells. Generally, these early processes were not altered by prenatal stress. Measures of BrdU labeled GAD67GFP+ cells at E13 after BrdU incorporation at E12 show that 24 hour survival of these early born cells was not deficient in PS embryos in either ventral or dorsal telencephalon (Fig. 5A,B). The overall population of 24 hour BrdU labeled cells within the ventral telencephalon at E13 was also not significantly decreased demonstrating that general cell survival in this region, regardless of expression of GAD67, was not affected by PS (data not shown). Survival was also qualitatively assessed with caspase-3 staining which showed no significant increase at E13 or E14 (Fig. 5C–F). Some increase in dorsal caspase-3 staining at E15 was found which was localized to non-GABAergic progenitors as demonstrated by a lack of co-labeling with GAD67GFP (Fig. 5G–J).

To examine whether proliferation of GABAergic progenitors was affected by PS, we assessed the cell cycle marker, Ki67, within the ganglionic eminences of E14 and E15 mice. The density of Ki67+ cells at E14 was not decreased in the ventral telencephalon of prenatally stressed offspring (Fig. 5K). At E15, there was a significant reduction in density of Ki67+ cells (Fig. 5L, $p < 0.05$, $n = 3,3$) suggesting a possible late-onset effect of prenatal stress on cell proliferation in the ganglionic eminence.

2.3. Mechanisms of GABAergic cell development

In order to understand the molecular mechanism by which prenatal stress altered inhibitory neuron migration and integration in the cortex, we examined by *in situ* hybridization a selected group of transcription factors known to play a role in neuronal differentiation and GABAergic cell migration, namely, *ngn2*, *mash1*, *dlx1*, *dlx2*, and *nkx2.1*.

PS exposure caused no apparent difference in expression at E13–E16 of *mash1* and *ngn2*, two transcription factors that are critical for neural determination and specification of ventral and dorsal telencephalic phenotypes, respectively, by *in situ* hybridization (Fig. 6A,B show

representative findings from E13). These data suggest that neither the differentiation of inhibitory nor excitatory progenitors were affected by prenatal stress. In contrast, *In situ* hybridization for *dlx1* and *dlx2*, two factors that promote cortical GABAergic phenotype and interneuron migration, demonstrated that both had altered expression in the ventral telencephalon of PS embryos (Fig. 6C,D). The changes in *dlx2* were more apparent than in *dlx1* by qualitative assessment. Quantification and comparison of gene expression levels to normalized controls by qPCR showed no significant alteration in *dlx1* expression by prenatal stress, but consistently decreased *dlx2* at all ages (Fig. 6F; $p < 0.05$; $n = 4,4$ at E13; 4,3 at E14; 4,3 at E15).

Expression of *nkx2.1* was decreased qualitatively at E13 (Fig. 6E). Downstream of *nkx2.1* via other transcription factors is *erbb4*, a receptor for the chemoattractant neuregulin-1. These two factors were consistently downregulated following prenatal stress (Fig. 6G; $p < 0.05$; $n = 3,4$ at E13; 3,3 at E14; 3,3 at E15 for *nkx2.1* and $n = 4,4$ at E13; 3,3 at E14; 3,3 at E15 for *erbb4*) demonstrating possible mechanisms by which GABAergic progenitor migration may be affected. In contrast, we found no significant systematic change in either brain derived neurotrophic factor (*bdnf*), which affects the migration of GABAergic progenitors by stimulation of cell motility, or fibroblast growth factor (*fgf2*) which has a role in the developing brain not known to be specific to inhibitory progenitors (Fig. 6H). However, the variability in the data from the use of embryonic brain across multiple litters may conceal more subtle alterations.

3. Discussion

Here we demonstrate that a well-validated model of prenatal stress in mice resulted in changes in the distribution of GAD67GFP+ GABAergic cells within the dorsal telencephalon of the offspring that support a model in which stress impairs the migration of GABAergic progenitors and their subsequent integration in the cerebral cortex. This change in the migration of GABAergic progenitors began after only one day of stress and was still observable after the last day of stress. In addition, there were no changes in proliferation or survival of these early born cells during the first three days of prenatal stress. Lastly, we demonstrated that concurrent with this change in GABAergic progenitor distribution, the expression of the transcription factors *dlx2* and *nkx2.1* and one of their downstream targets, *erbb4*, was significantly and consistently decreased during prenatal stress suggesting a mechanism by which prenatal stress impacts GABAergic neuron development.

In the developing cortical plate of prenatally stressed animals, the density of GABAergic progenitors was reduced and the leading edge of migration in the most superficial migratory pathway was less advanced. Both of these findings suggest that tangential movement of GABAergic progenitors was delayed by prenatal stress. We concluded that a delay in migration was the most likely process to account for these changes, by ruling out any decrease in the survival of GABAergic progenitors and by demonstrating no alteration in proliferation within the ganglionic eminences after prenatal stress, both of which could have contributed to the finding that fewer GAD67GFP+ cells were entering the dorsal telencephalon. Previous work has demonstrated that prenatal corticosteroids do alter the migration of cortical excitatory progenitors (Fukumoto et al., 2009), providing converging evidence that migration of embryonic progenitors is susceptible to this type of environmental impact. The timing of neuronal migration coincides with periods during which rodents and nonhuman and human primates have demonstrated susceptibility to prenatal stress—the last gestational week for rodents and the late first and early second trimester for humans (Davis and Sandman, 2010; Schneider et al., 2002; Stone et al., 2001). Because of consistent effects seen on migration from E13–E15, these findings also suggest

that GABAergic progenitor migration was influenced by mechanisms occurring at the earliest time point of prenatal stress (on E12).

The alterations seen in GABAergic progenitors from E13 to E15 were more significant in more caudal regions of the embryonic telencephalon. This could reflect an amplification of the effects on migration of early GABAergic progenitors which must travel greater distances from medial ganglionic eminence to caudal regions (Miyoshi et al., 2010). However, there may also be region-specific components of GABAergic progenitor migration that have not been identified here. Cell-autonomous components of MGE- and CGE-derived progenitors may be differentially affected by prenatal stress and/or signaling molecules such as TBR2, NRG1, or CXCL12 (Neddens and Buonanno, 2010; Sessa et al., 2010; Tiveron et al., 2006) expressed in rostral and caudal regions of the dorsal telencephalon that are targets for GABAergic progenitors may be affected by prenatal stress in a regionally-specific manner.

We found that in the most dorsal regions of the cortical plate, prenatal stress resulted in significantly less integration of GAD67GFP+ cells radially into all layers. This suggests that the processes induced by PS influencing GABAergic progenitors in the tangential migratory processes were generally continued in the next stage of these cells' development. The greater effect seen in dorsal regions at E15 than in lateral regions at either E14 or E15 also suggests that the effects of prenatal stress are not more prominent in regions that have earlier arriving precursors; if this was the most critical aspect of the effects of stress, deficits in the E14 lateral cortical plate would have been comparable to those seen in the E15 dorsal cortical plate, as both evaluated early arriving precursors. Instead, cell migration over longer distances and integration into more dorsal regions may be processes more affected by PS.

GABAergic cells were persistently deficient in neonatal (P0) dorsal telencephalon, a result which was disproportionately greater in medial frontal regions. Medial frontal cortex showed a greater deficiency of GAD67GFP+ cells than in overall cortex and more posterior hippocampal regions. Thus, despite findings at E13–E14 that posterior regions were more affected by PS, by P0 the more anterior regions were more significantly affected. An increase in early-born progenitors in the deep migratory white matter pathway after PS converges with other evidence from our study suggesting a delayed migration into medial cortical regions. The sensitivity of these early-born cells to the effects of prenatal stress most likely reflects the importance of timing of when these cells were born and the distance over which they traveled to reach their targets.

Altered migration of GABAergic progenitors could be a source of significant changes in inhibitory functioning in the mature telencephalon following prenatal stress (Barros et al., 2006; Edwards et al., 2002; Stone et al., 2001; Viltart et al., 2006; Weinstock, 1997). Alterations in the functioning or location of inhibitory cells during development even temporarily, however, could independently influence the development of other neural circuits, with persistent consequences into adulthood.

Our findings that proliferation within the ganglionic eminence was not significantly affected until prenatal stress had been continued more chronically (i.e. a significant change at E15 after 3 days) broadens our understanding of how stress or exogenous corticosteroids affect dividing cells. While prenatal stress has been shown to influence later postnatal proliferation and neurogenesis (Lemaire et al., 2006; Ulupinar et al., 2006), no previous studies have addressed how early embryonic neurogenesis is affected. The initial resistance of progenitor proliferation to stress may be due to compensatory mechanisms within the ventral telencephalon or the lack of the specific glucocorticoid responsive elements expressed at early time points that influence proliferation. Similarly, our findings that apoptosis of GABAergic progenitors was not a significant effect of prenatal stress suggest that these cells

are not sensitive to the pro-apoptotic effects of stress seen in other forebrain regions (Fujioka et al., 1999).

Transcription factors involved in GABAergic cell migration were altered by prenatal stress in expression level but not in spatial distribution. As early as 24 hours after stress was induced and persisting at least into the 4th day of prenatal stress, expression of *dlx2*, *nkx2.1* and its downstream target, *erbb4*, were reduced. *Dlx1* and *Dlx2* both promote migration of inhibitory precursors through transcriptional regulation of other transcription factors such as *lhx6* and *arx* (Anderson et al., 1997; Cobos et al., 2007; Colasante et al., 2008) but also through independent regulation of other genes involved in migration (Fulp et al., 2008). *Nkx2.1* also regulates inhibitory precursor migration (Butt et al., 2008; Nobrega-Pereira et al., 2008). The persistent downregulation of *dlx2* and *nkx2.1* may be the mechanism by which migration is delayed in prenatally stressed offspring, although the promoter elements in these genes that are directly responding to glucocorticoids or other components of prenatal stress response in the ventral telencephalon have not been identified. Possible increases in expression of *dlx1* after stress may represent a compensatory response in inhibitory progenitors. All of these transcription factors are also important for the differentiation and fate of inhibitory precursors suggesting that migration changes may occur simultaneously with other alterations of GABAergic progenitors, i.e., a change in cell phenotype. For example, the ability of these cells to make GABA may be reduced due to the reduction of *dlx2*, which could, in turn, alter activation of GABA_A receptors, significantly affecting the migration of GABAergic progenitors (Cuzon et al., 2006). The similarity of *mash1* expression in non-stressed and prenatally-stressed mice did suggest that the general ventral fate of these cells was not disrupted (Fode et al., 2000). While other prenatal environmental exposures with relevance for later behavioral disorders have been shown to alter transcription factors of the ganglionic eminence (Godin et al., 2011; Oskvig et al., 2012), this is the first data showing that prenatal stress affects early regulators of cortical inhibitory neurons.

Our finding does not rule out the possibility that prenatal stress may also influence the migration of GABAergic progenitors through other mechanisms, such as excess expression of the cell motility protein caldesmon involved in radial migration of cortical excitatory progenitors (Fukumoto et al., 2009). Growth factors are also a potential mediator of the effects of prenatal stress on GABAergic progenitors. We demonstrated no significant change in *bdnf*, a growth factor that acts as a permissive cue for interneuron migration (Polleux et al., 2002) and has been shown to be altered postnatally by prenatal stress (Van den Hove et al., 2006). However, further examinations of the contribution of growth factors to the developmental mechanisms occurring during prenatal stress are warranted.

Conclusions

The effects of prenatal stress on inhibitory neuron precursor migration may demonstrate one component of the developmental pathophysiology of stress-related behavioral problems. Alterations in the migration of embryonic neuronal precursors may arise from environmental influences, genetic variation or the interaction of these two. One gene implicated in trait anxiety, *MAMDC1* or *MDGA1*, is involved in neuronal migration (van den Oord et al., 2008; Takeuchi and O'Leary, 2006) suggesting that a subset of anxiety problems, which have links to prenatal stress (Bergman et al., 2007; O'Connor et al., 2002; Vallee et al., 1997) may result from altered neuronal migration within the brain. *ErbB4*, which we report decreased by prenatal stress, and its ligand *Nrg-1* affect parvalbumin+ cell migration (Flames et al., 2004; Martini et al., 2009; Neddens and Buonanno, 2010) and the genes for these proteins are also associated with schizophrenia (Flames et al., 2004). Mouse models of schizophrenia, including the 22q11 chromosomal deletion and the *DISC-1* mutation, have

been shown to have a significant impairment in neuron migration (Meechan et al., 2009; Meyer and Morris, 2009). Together with data from our prenatal stress model, these findings suggest that the etiology of psychiatric disorders may occur during early phases of development with specific implication for the migration of neurons that occurs during this period.

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Figure 1.

Migration delay due to PS after one day. Normal events in GABAergic progenitor development are demonstrated in this diagram (A). Nonstressed offspring (B,D) and PS offspring (C,E) showed significant changes in distribution of GAD67GFP+ cells in the pathway of tangential migration across rostral (B,C) to caudal (D,E) regions of the E13 forebrain. (F,G) Stereological counts indicating that PS significantly decreased the density of GAD67GFP+ cells in the E13 dorsal telencephalon (F) but not ventral telencephalon (G). PS offspring also showed a decrease at E13 in the circumferential distribution of GAD67GFP+ cells in dorsal telencephalon (H) that was greater in caudal regions (I) (* indicates $p < 0.05$ by two-tailed student's t-test; arrows indicate the leading edge of migration of GAD67GFP+ cells). Scale bar=200 μm .

Figure 2.

Migration delay due to PS after two days. Nonstressed offspring (A,C) and PS offspring (B,D) again showed significant changes in distribution of GAD67GFP+ cells in the pathway of tangential migration across rostral (A,B) to caudal (C,D) regions of the E14 forebrain. (E,F) Stereological counts indicating that PS offspring showed a decrease at E14 in the circumferential distribution of GAD67GFP+ cells in dorsal telencephalon (E) that was greater in caudal regions (F). (G,H) Bin-counting in lateral areas of the cortex, shown by schematic (G), demonstrated no significant difference with PS in the percentages of total GFP+ cells in different layers of cortex averaged across all coronal sections at E14 (H) (* indicates $p < 0.05$ by two-tailed student's t-test; arrows indicate the leading edge of migration of GAD67GFP+ cells) Scale bar=200 μm .

Figure 3.

Migration delay due to PS after 3 days. (A) Schematic showing overlay of bins to count percentages of total cells in different layers in two regions, dorsal and lateral, of cortex (B,C). At E15, bin counting demonstrated that PS induced significant alterations in cortical integration of GAD67GFP+ cells in E15 dorsal cortex (B) but not in lateral (C) cortex when averages across all coronal sections were compared. (D–G) At the leading edge of migration in medial cortical sectors, nonstressed (D,F) and PS offspring (E,G) showed significant changes in radial integration of GAD67GFP+ cells in both rostral (D,E) and caudal (F,G) regions of the forebrain (brackets indicate cells at the leading edge of the deep migratory path, arrows indicate the cortically-integrating GAD67GFP+ cells in the dorsal area; LV=

lateral ventricle, # indicates $p < 0.05$ for interaction of condition by bin by ANOVA). Scale bar=200 μm .

Figure 4.

Migration delay due to PS after 7 days. Nonstressed offspring (A) and PS offspring (B) showed significant changes in density of GAD67GFP+ cells and GAD67GFP+/BrdU+(E13) cells in medial frontal regions of the P0 cortex. PS significantly affected medial regions of the dorsal telencephalon at P0 with decreased density of GAD67GFP+ cells (C) but more substantial decreases GAD67GFP+/BrdU+(E13) cells (D). PS offspring (F) compared to nonstressed controls (E) also showed an increase in density of GAD67GFP+/BrdU+(E13) cells in anterior subcortical white matter, traced by dotted lines (G) (arrows indicate double-labeled GAD67GFP+/BrdU+ cells; LV=lateral ventricle, # indicates $p<0.05$ for interaction of condition by region by ANOVA, * indicates $p<0.05$ by two-tailed student's t-test). Scale bar=200 μ m.

Figure 5.

No changes in GABAergic survival or proliferation due to PS. At E13, NS and PS offspring showed no significant differences in survival of GAD67GFP+ cells born at E12 in the ventral (A) or dorsal (B) telencephalon. NS (C,E,G,I) and PS (D,F,H,J) offspring showed no difference in apoptotic cells at E13 (C,D) or E14 (E,F) but an increase in apoptosis at E15 (G,H) that was specific to non-GABAergic cells (I,J). Proliferative markers in the ganglionic eminence of NS and PS offspring were similar at E13 (K) and showed a trend difference at E14 (L) that was greater in caudal regions (H) (* indicates $p < 0.05$ by two-tailed student's *t*-test; arrows indicate caspase-3+ cells that show no labeling with GAD67GFP). Scale bar=200 μ m.

Figure 6.

Decreased transcription factors (TFs) for GABAergic migration due to PS. NS and PS offspring did not differ in TFs determining dorsal-ventral fate (A, B) at E13. Variable decreases in expression with PS without changes in localization was shown at E13 for TFs involved in GABAergic progenitor migration, *dlx1* (C), *dlx2* (D), and *nkx2.1* (E).

Quantification of gene expression changes with PS in the ventral telencephalon by qPCR in basal ganglia for these TFs demonstrated that *dlx1* showed no consistent alteration at E13 (F, left) but *dlx2* (F, right), *nkx2.1* (G, left), and their downstream target *erbb4* (G, right) were decreased at all embryonic time points. Quantification of gene expression in dorsal telencephalon for *bdnf* and *fgf2* showed no significant changes with PS (H) (* indicates

p<0.05 for main effect of condition by one-way ANOVA, *^ indicates p<0.05 for interaction of condition by age by two-way ANOVA). Scale bar=200 μ m.