Delays in GABAergic Interneuron Development and Behavioral Inhibition after Prenatal Stress

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ABSTRACT: Prenatal stress is associated with altered behavioral, cognitive, and psychiatric outcomes in offspring. Due to the importance of GABAergic systems in normal development and in psychiatric disorders, prenatal stress effects on these neurons have been investigated in animal models. Prenatal stress delays GABAergic progenitor migration, but the significance of these early developmental disruptions for the continued development of GABAergic cells in the juvenile brain is unclear. Here, we examined effects of prenatal stress on populations of GABAergic neurons in juvenile and adult medial frontal cortex (mFC) and hippocampus through stereological counting, gene expression, and relevant anxiety-like and social behaviors. Postnatally, the total GABAergic cell number that peaks in adolescence showed altered trajectories in mFC and hippocampus. Parvalbumin neuron proportion in juvenile brain was altered by prenatal stress, but parvalbumin gene expression showed no differences. In adult brain, parvalbumin neuron proportions were altered by prenatal stress with opposite gene expression changes. Adult prenatally stressed offspring showed a lack of social preference on a three-chambered task, increased anxiety-like behavior on the elevated plus maze, and reduced center time in an open field. Despite a lack of significant group differences in adult total GABAergic cell populations, performance of these tasks was correlated with GABAergic populations in mFC and hippocampus. In conclusion, prenatal stress resulted in a delay in GABAergic cell number and maturation of the parvalbumin subtype. Influences of prenatal stress on GABAergic populations during developmentally dynamic periods and during adulthood may be relevant to the anxiety-like behaviors that occur after prenatal stress. © 2015 Wiley Periodicals, Inc. Develop Neurobiol 00: 000–000, 2015

Keywords: **prenatal stress; GABA; anxiety; medial frontal cortex; parvalbumin**

INTRODUCTION

Prenatal stress and maternal anxiety and depression are risk factors for some child behavioral disruptions and psychiatric disorders. Maternal depression during pregnancy has been linked to negative affect, ADHD, and conduct disorder in offspring (Huot et al., 2004; Martini et al., 2010). Maternal anxiety during the prenatal period has been correlated with an increased risk for behavioral and emotional deficits during childhood (O'Connor et al., 2003). In addition, prenatal stress has been associated with psychiatric diseases such as schizophrenia, autism, childhood anxiety, and Tourette syndrome (Leckman et al., 1990; King et al., 2005; Bergman et al., 2007; Ronald et al., 2010). To better understand the cellular and molecular mechanisms of outcomes after prenatal maternal stress, animal models have been used to study offspring brain and behavior (Stevens and Vaccarino, 2015).

Animal models have implicated multiple neural systems as mechanisms for prenatal stress effects.

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Among these, inhibitory neurons may be of particular interest due to their importance in processes of brain development and associations with the pathology of schizophrenia, autism, and anxiety (Fine et al., 2014). In adult rats, prenatal stress decreased levels of benzodiazepine receptors in hippocampus and increased GABAergic synapses in the hypothalamus (Barros et al., 2006; Viltart et al., 2006). Although mature neural systems after prenatal stress have been studied, developmental trajectories are less understood. The protracted nature of GABAergic system development (Le Magueresse and Monyer, 2013) and the lengthy process by which subtypes such as parvalbumin cells mature makes these particularly important processes to evaluate after early stress. Interestingly, early developmental processes including methylation in cortical interneurons (Matrisciano et al., 2013), the migration of GABAergic progenitors to the cerebral cortex (Stevens et al., 2013), and embryonic neurogenesis of GABAergic progenitors (Uchida et al., 2014) are altered by prenatal stress. How GABAergic cells then develop through vulnerable juvenile stages is a question of paramount interest to understanding mechanisms of disease, treatments, and preventive measures.

Long-term changes in brain function after prenatal stress have been in part evaluated by behavioral changes in animal models. Prenatal stress causes most consistently an array of inhibited behaviors in offspring with reduced activity in an open field, reduced social preference, and increased anxiety-like behavior in the elevated plus maze and light–dark box (Harmon et al., 2009; Laloux et al., 2012; Marrocco et al., 2012; Grigoryan and Segal, 2013). How altered neural development after early stress relates to these behavioral changes is not well understood.

In our previous work on prenatal stress, we have demonstrated changes in GABAergic cell migration into the cerebral cortex and hippocampus as well as changes in molecular aspects of these processes (Stevens et al., 2013). Other models in which GABAergic cell migration is affected during embryonic brain development show long-lasting alterations in cortical inhibitory neuron populations, particularly those of the parvalbumin subtype (Flames et al., 2004; Meechan et al., 2012; Lee et al., 2013; McCarthy et al., 2014; Nakai et al., 2014). Any changes due to prenatal stress in postnatal populations of cortical or hippocampal GABAergic neurons following altered progenitor migration have not been described. Here, we tracked how early GABAergic population changes progressed through juvenile and adult development. We also evaluated animal behavior for profiles of behavioral inhibition and whether this correlated with altered GABAergic populations.

METHODS

Mice

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

Prenatal Stress

Beginning on E12, half of the pregnant female mice were subjected to acute stress within a plexiglass restraint for 45 min 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. Litters were reduced to 7–9 pups at postnatal day 0 (P0), left with their mother until P24, and then weaned to single-sex group-housing. All evaluations here were performed on male offspring.

Brain Collection

Offspring brains were collected at four different time points: P0, P24, P48, and P150. At P0, animals were anesthetized with ice, rapidly decapitated, and the brain was immersion postfixed in 4% paraformadelhyde (PFA) for at least 4 h. At all other time points, deeply anesthetized animals were intracardiacally perfused with phosphate-buffered saline (PBS) followed by 4% PFA perfusion and immersion postfixation for at least 12 h.

Immunohistochemistry

Brains from offspring were rinsed with PBS and transferred to 20% sucrose for at least 15 h. Tissue was embedded and cryosectioned (Leica, CM1900, Bannockburn, Illinois) at 25 μm (P0) or 50 μm (P24, P48, and P150). Slide-mounted (P0) or free-floating (all other ages) sections were processed for staining first by blocking for 1 h with 10% goat serum in PBS with 0.025% TritonX-100, 0.0125% Tween20 (PBS++) and then incubating for 24–48 h at 4°C with 5% goat serum/PBS++ containing primary antibodies as follows: parvalbumin (PV) (1:4000; Sigma, SAB4200545) 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).

Cell Counting

Stereological counting was utilized to obtain reliable estimates of cell number as done previously (Stevens et al., 2010). Stereological, unbiased estimates of GAD67GFP+, and Parvalbumin+ cell number and density within the neonatal, adolescent, and adult medial frontal cortex (mFC) and hippocampal cornus ammonis (hippocampal CA) were obtained with a computer running the StereoInvestigator software (Microbrightfield, Colchester, VT) 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 three-dimensional counting boxes. Every 10th coronal section was used that contained any portion of mFC or hippocampal CA. Variations of counts were assessed by the Gunderson coefficients of error and data were only used when cell count error values were below 0.15.

Quantitative PCR

To quantify the expression of inhibitory neuron genes, brain tissue was collected at P27 and P150. Deeply anesthetized animals were rapidly decapitated and brain tissue was dissected out, sectioned on ice at 100 micrometers, and flash frozen on dry ice. Samples of mFC and hippocampal CA were collected using a micro-punch from 2 sections bilaterally and processed for total mRNA using an RNeasy Mini Kit (Qiagen). RNA concentrations were determined using a Nanodrop Spectrophotometer (Thermo Scientific). cDNA was synthesized using the Transcriptor First-Strand cDNA Synthesis kit (Roche). Quantitative PCR was carried out using Taqman Gene Assays (Applied Biosystems) for GAD1 (ID Mm00725661_s1) and parvalbumin (ID Mm00443100_m1). Beta-actin (predeveloped) was used as an endogenous control in all cases. qPCR was run using GeneAmp PCR Mastermix (Applied Biosystems) in a 7900HT or StepOne™ Instrument (Applied Biosystems). The cycle number threshold for signal detection for each gene of interest for each sample was normalized to that sample's cycle number threshold for beta-actin and the difference in cycle number (ΔC_T) was converted to gene expression values using the formula: [expression$=2^{-\Delta C_T}$].

Graphs depict the gene expression values for all averaged prenatal stress individual samples normalized to average gene expression values for control samples in the same region. Gene expression levels were grouped for analysis at each age across brain regions.

Behavioral Tests

All behavior assessments were performed on 2–3-month-old male mice during the light cycle in a dedicated testing room with only one behavior assessment performed per day, allowing mice to habituate to the testing room for 60 min prior to testing. Unless otherwise noted, mice remained in their home cage with cage-mates immediately before and after assessments. At least 3 weeks elapsed after testing before brain tissue was collected.

Social Approach. In a three-chamber social approach apparatus (Nadler et al., 2004), mice were tested for social preference and recognition in a well-lit room on a single day. Two male, nonexperimental "stranger" mice of the same strain and age were habituated for 5 min to small cylinders in one end of each side chamber. Individual experimental mice were habituated to the center chamber for 5 min. Subsequently, the experimental mouse underwent a 10 min trial moving through all 3 chambers with only one stranger present and then a second, 10 min trial in which the first stranger mouse and the second stranger mouse were present. Movement was recorded using an overhead camera and Anymaze software (Stoelting, Wood Dale, Illinois) and evaluated for the amount of time the experimental mouse spent around the cylinder on each side. Social discrimination index was calculated from behavior during the first trial taking the quotient of the time spent with the first stranger and the time spent with both cylinders overall. Social learning coefficient was calculated from the time spent with the second stranger divided by the time spent with both cylinders overall.

Elevated Plus Maze. In a Stoelting (Wood Dale, Illinois) Elevated Plus Maze, mice were tested for anxiety-like behavior in a well-lit room for 5 min on a single day. Mouse movement throughout the maze was recorded using an overhead camera. Anymaze software coded and assessed the amount of time spent in the closed arms, open arms, and the center of the maze.

Open Field. In a rectangular plastic arena, approximately 1800 cm^2 in area, mice were tested for locomotor activity in a well-lit room for 30 min on two consecutive days. Test mice were placed in the corner of the arena and their movements recorded using an overhead camera and Anymaze software. The amount of time spent in the center 70% of the arena was measured for each day to assess behavioral inhibition.

Statistical Analysis

Analysis of variance (ANOVA) approaches (SPSS; IL, USA) were used to compare the number of GAD67GFP+ cells across different ages within each region in each condition and the proportion of GAD67GFP+ cells that were parvalbumin+ across each region within each age. Post-hoc two-tailed Student's *t*-tests were used to compare the number of GAD67GFP+ cells at P0 and P24. GAD67GFP+ cell densities, qPCR gene expression values

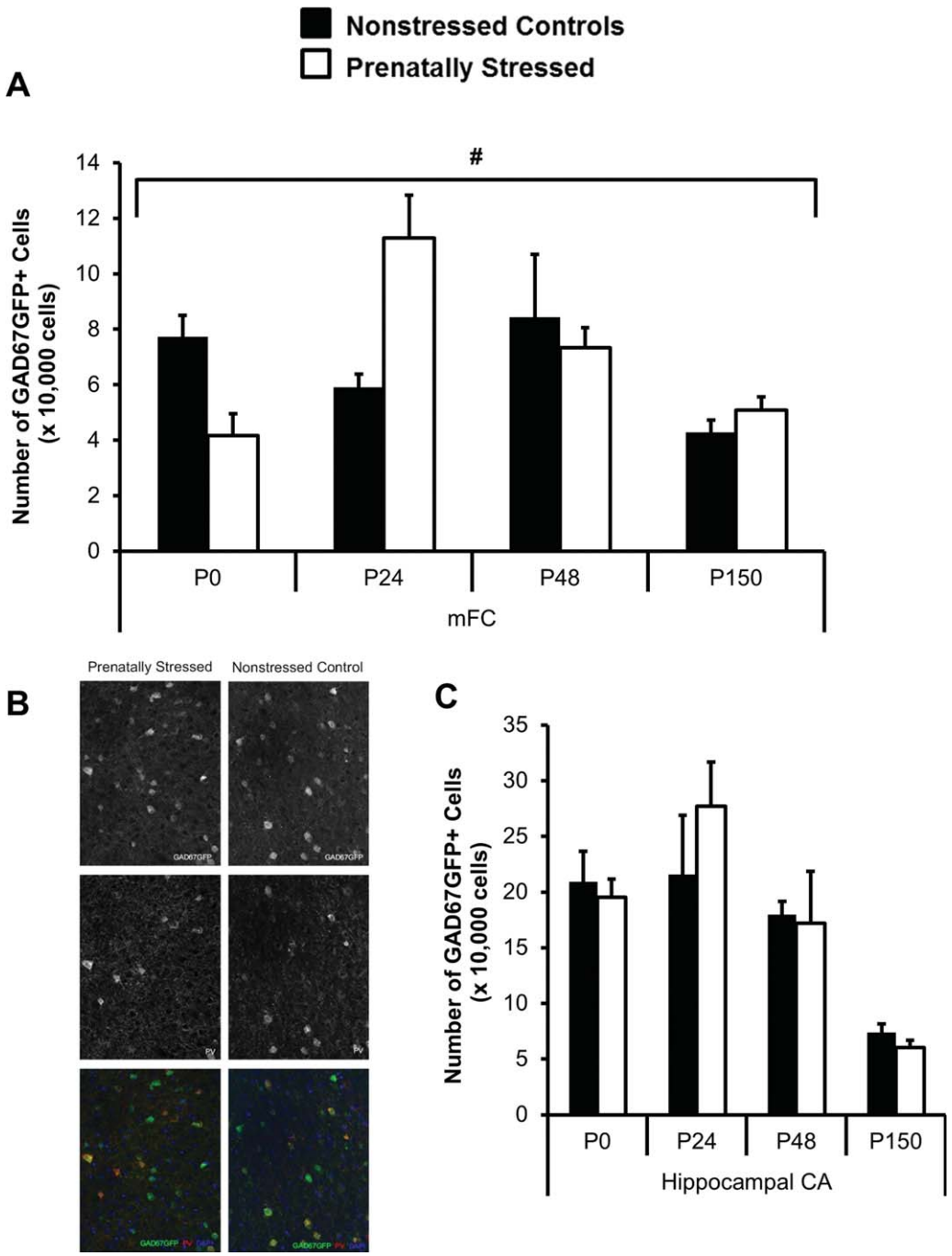

Figure 1 Changes in GABAergic cell number independent of regional volume or density in dorsal regions from birth through adulthood after prenatal stress. Stereological counts of GAD67GFP+ cells in mFC (examples of immunostaining for counting at P24 shown in B) were altered after prenatal stress (A) while cell number in hippocampal CA (C) was not significantly changed ($^{\#}p < 0.05$ for interaction of age by stress by ANOVA).

within each region in each condition, and differences on behavioral tasks were compared using two-tailed Student's *t*-tests. Pearson's coefficient was calculated across all individuals in both conditions to determine the correlation between inhibited behavior and GAD67GFP+ cells populations and two-tailed significance was tested.

Table 1 GAD67GFP+ Cell Density in Dorsal Forebrain Regions from Day of Birth, Adolescence, and Adulthood in Nonstressed (NS) and Prenatally Stressed (PS) Offspring and Volume of Regions Analyzed

	Age	NS GAD67GFP+ cell density (± SEM) (cells/μm^3)	PS GAD67GFP+ cell density (± SEM) (cells/μm^3)	% change	p-value	ANOVA	NS region volume (±SEM) (μm^3)	PS region volume (± SEM) (μm^3)	p-value	ANOVA
mFC	P0	1.23E-04 (±1.3E-05)	8.34E-05 (±8.0E-06)	−32.4	**0.03**	**Age effect** ***p* < 0.001**	6.55E+08 (±8.4+E07)	5E+08 (±1.47E+08)	0.36	**Age effect** ***p* < 0.001**
	P24	1.94E-05 (±1.5E-06)	2.47E-05 (±1.5E-06)	26.9	0.07	Stress effect $p = 0.060$	3.10E+09 (±4.7E+.08)	4.6E+09 (±4.81E+08)	0.09	**Stress effect** ***p* = 0.009**
	P48	2.55E-05 (±3.4E-06)	2.42E-05 (±1.9E-06)	−5.2	0.75	**Age × stress** ***p* = 0.007**	3.21E+09 (±4.3E+08)	3E+09 (±1.2E+08)	0.71	**Age × stress** ***p* = 0.002**
	P150	2.09E-05 (±2.4E-06)	1.68E-05 (±1.6E-06)	−19.8	0.18		2.10E+09 (±6.5E+07)	3.07E+09 (±2.1E+08)	**<0.01**	
Hippocampal CA	P0	1.24E-04 (±1.8E-05)	8.47E-05 (±9.4E-06)	−31.5	0.10	**Age effect** ***p* < 0.001**	1.46E+09 (±3.8E+08)	2.36E+09 (±1.6E+08)	0.07	**Age effect** ***p* < 0.001**
	P24	1.75E-05 (±2.3E-06)	2.32E-05 (±4.4E-06)	32.4	0.31	Stress effect NS Age × stress NS	1.20E+10 (±1.5E+09)	1.22E+10 (±5.7E+08)	0.94	Stress effect NS Age × stress NS
	P48	1.27E-05 (±3.7E-06)	1.30E-05 (±2.9E-06)	2.8	0.91		1.42E+10 (±1.2E+09)	1.30E+10 (±8.5E+08)	0.45	
	P150	8.22E-06 (±2.6E-06)	6.75E-06 (±4.5E-07)	−17.8	0.14		9.14E+09 (±2.4E+08)	8.87E+09 (±7.6E+08)	0.82	

Density of GAD67GFP+ cells was significantly altered in mFC ($p < 0.001$ for interaction of age by stress by ANOVA), but not hippocampal CA. The total volume of mFC was also altered ($p < 0.001$ for interaction of age by stress by ANOVA), while the volume of hippocampal CA was not affected by stress or an interaction of stress and age.

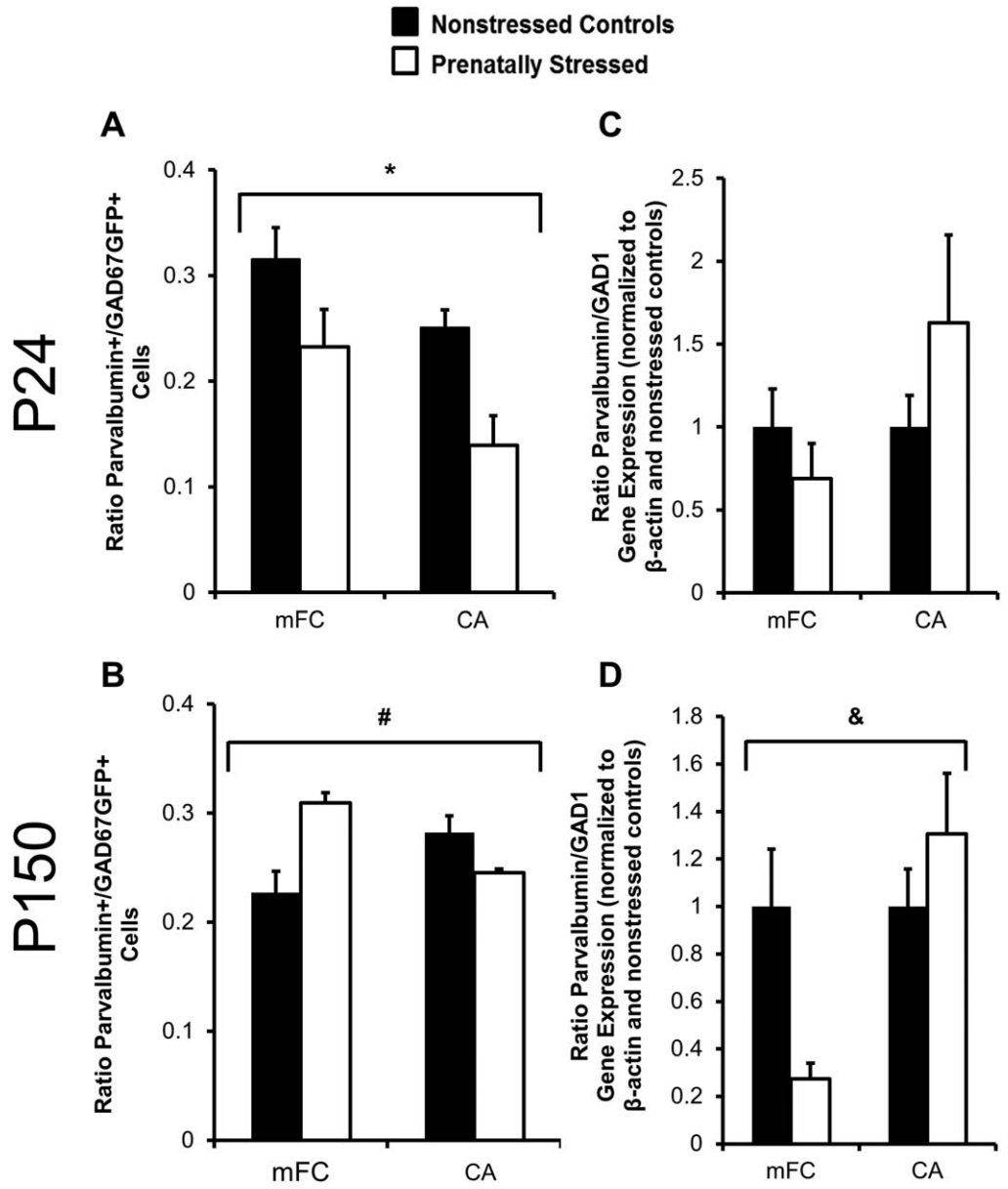

Figure 2 Ratios of Paravalbumin+ (PV)-to-GAD67+ cell number and gene expression after prenatal stress in adolescence and adulthood. Prenatal stress decreased the ratio of PV+-to-GAD67+ cells in mFC and hippocampal CA at P24 (A) and in hippocampal CA at P150 (B). The ratio was increased after prenatal stress at P150 in mFC (B). No significant changes were found in the gene expression at P24 (C), but PS did affect PV/GAD1 expression at P150 (D) ($^{*}p < 0.01$ for stress by ANOVA; $^{\#}p = 0.001$ for interaction of condition by region by ANOVA; $^{\&}p < 0.05$ for interaction of condition by region by ANOVA).

RESULTS

Developmental Alterations in GABAergic Cell Populations

GAD67GFP+ cell populations were examined at multiple time points from birth through adulthood to understand the trajectory of neural development. To compare populations across different ages, we first examined the trajectory of total GAD67GFP+ cell number, independent of volume or cell density. An altered developmental time course was observed in the medial frontal cortex (mFC); prenatally stressed mice had a significantly lower GAD67GFP+

Table 2 Parvalbumin+ Cell Density and Gene Expression Independent and As a Ratio of Total GAD67+ Cells or *gad1* Gene Expression in Dorsal Forebrain Regions from Adolescence to Adulthood in Nonstressed (NS) and Prenatally Stressed (PS) Offspring

	Region	NS PV+ cell number (±SEM) (cells)	PS PV+ cell number (±SEM) (cells)	*p*-value	NS PV+/ GAD67+ cell ratio (±SEM)	PS PV+/ GAD67+ cell ratio (±SEM)	*p*-value	NS pv gene expression (±SEM)	PS pv gene expression (±SEM)	*p*-value	NS pv/gad1 ratio gene expression (±SEM)	PS pv/gad1 ratio gene expression (±SEM)	*p*-value
P24	mFC	13936 (±2111)	21021 (±2058)	↑**0.03**	0.315 (±0.026)	0.233 (±0.035)	↓0.10	1.0 (±0.21)	0.94 (±0.28)	↓0.80	1.0 (±0.23)	0.69 (±0.21)	↓0.42
	CA	54123 (±13585)	36464 (±1685)	ANOVA stress × region, ***p* < 0.05** ↓0.27	0.251 (±0.017)	0.139 (±0.028)	ANOVA stress, ***p* = 0.01** ↓**0.03**	1.0 (±0.23)	1.62 (±0.33)	↑0.16	1.0 (±0.19)	1.63 (±0.53)	↑0.20
P150	mFC	11919 (±1057)	15745 (±1189)	↑**0.04**	0.245 (±0.009)	0.282 (±0.019)	↑0.16	1.0 (±0.20)	0.86 (±0.16)	↓0.62	1.0 (±0.27)	0.27 (±0.06)	↓**0.04**
	CA	21092 (±3149)	14483 (±2032)	ANOVA stress region, ***p* < 0.05** ↓0.10	0.309 (±0.003)	0.227 (±0.015)	ANOVA stress × region, ***p* = 0.001** ↓**<0.01**	1.0 (±0.21)	1.56 (±0.27)	↑0.16	1.0 (±0.16)	1.31 (±0.26)	ANOVA stress × region, ***p* < 0.05** ↑0.38

Pv and *pv*/gad1 gene expression is reported relative to expression of beta-actin and normalized to nonstressed controls.

neuronal number at postnatal day 0 (P0) and significantly more GAD67GFP+ neurons at P24 [Fig. 1(A)] (ANOVA for stress by age $p = 0.001$ and post-hoc *t*-tests $p < 0.01$ for P0 and $p < 0.05$ for P24). At P48 and P150, differences in mFC GABAergic cell number were no longer present after prenatal stress. In hippocampal CA, no significant differences in the trajectory of GAD67GFP+ cell number were observed after prenatal stress, but a similar tendency was seen for increased cell number at P24 and no differences at later ages [Fig. (1)B]. Although the trajectory of mFC volume was also changed after prenatal stress (Table 1, ANOVA for stress by age interaction $p = 0.009$), the trajectory of GAD67GFP+ cell density in mFC was still altered after prenatal stress. The normal drop in density as the brain grows postnatally was apparent in both groups, but on a different time course after prenatal stress, mainly driven by a significant decrease in density at P0 and a trend increase in density at P24 (Table 1, ANOVA for stress by age interaction $p < 0.001$ and post-hoc *t*-tests $p < 0.01$ for P0 and $p = 0.07$ for P24). In hippocampal CA, no significant differences in volume of GAD67GFP+ cell density were found due to prenatal stress.

Effects of Prenatal Stress on Parvalbumin Cell Maturation

Parvalbumin is a calcium-binding protein in one subclass of interneurons of the mFC and hippocampus that begins to be expressed 2–3 weeks after birth in mice. Because we found significant differences in total GABAergic population trajectories which could change parvalbumin cells accordingly, we examined changes in parvalbumin (PV)-expressing cells relative to total GAD67GFP+ cells and changes in the expression of the *parvalbumin* (*pv*) gene itself relative to *gad1* gene expression.

In general, we found that parvalbumin+ (PV) cell population ratios were significantly affected by prenatal stress, with a decrease in populations across regions in adolescent brain and divergent trajectories in mFC and hippocampal CA into adulthood [Fig. 2(A,B)] (ANOVAs for stress $p = 0.01$ at P24 and for stress by region interaction $p < 0.0001$ at P150). This was driven mainly by a decreased PV+ ratio in hippocampus (Table 2, *t*-test $p < 0.05$) although a non-significant decrease in the mFC PV+ ratio was also seen. PV+ cell numbers alone did not show a consistent change at P24 (Table 2, ANOVA for stress by region interaction $p < 0.05$), demonstrating that the main effect of stress on PV+/GAD67+ ratios may be attributable to a general delay in maturation, but with PV+ and GAD67GFP+ cell number not reaching the same developmental stage at P24 in mFC and hippocampus. At postnatal day 150, prenatally stressed mice continued to show a decreased ratio of PV+ to GAD67GFP+ cells in hippocampal CA, but the ratio was increased in mFC [Fig. 2(B)] (ANOVA for condition by region $p = 0.001$). Accordingly, the independent PV+ cell number was decreased in hippocampal CA and increased in mFC at P150. This demonstrated that after prenatal stress, PV+ cell changes eventually matched by adulthood those of total GABAergic population changes (Table 2, ANOVA stress by region interaction $p < 0.05$).This suggested an impairment in maturation of this cell type after prenatal stress that was overcorrected by adulthood only in mFC.

We also analyzed the gene expression of *pv* and *gad1* in mFC and hippocampal CA as a complementary measure to cell numbers and densities. This allowed us to understand whether the populations' expression of the *pv* gene changed in accordance or in compensation to cell numbers across development. The pattern of *pv* relative to *gad1* expression after prenatal stress did not follow the same pattern as PV+-to-GAD67+ cell ratios. At P24, specifically, when PV+/GAD67+ cell ratio was decreased, there were no significant differences in *pv*-to-*gad1* ratio of gene expression [Fig. 2(C) and Table 2]. No differences were found in *pv* gene expression independently at P24 (Table 2) demonstrating the importance of understanding *pv* expression as a function of the population's *gad1* expression. However in adulthood, the ratio of *pv*-to-*gad1* gene expression was opposite to that seen for cell number ratios at the same time point [Fig. 2(D) and Table 2] (ANOVA for stress by region interaction $p < 0.05$). This was alongside a finding that *pv* gene expression independently was not significantly changed just as in adolescence (Table 2). These findings show that the developmental trajectory of a specific GABAergic subtype is altered by prenatal stress; cell number changes after prenatal stress may be accompanied by gene expression compensation which occurred differently at P24 than at P150.

Prenatal Stress, Adult Behavior, and GAD67GFP+ Correlations

While there were no significant differences in GAD67GFP+ cell number after prenatal stress in adult mFC or hippocampal CA [Fig. 1(A,B)], changes in adult behavior were observed. To understand the long-term effects of prenatal stress and potential connections to neuropsychiatric disorders, mice underwent testing in the elevated plus maze, open field, and three-chamber social behavior task in adulthood. Nonstressed control mice showed a

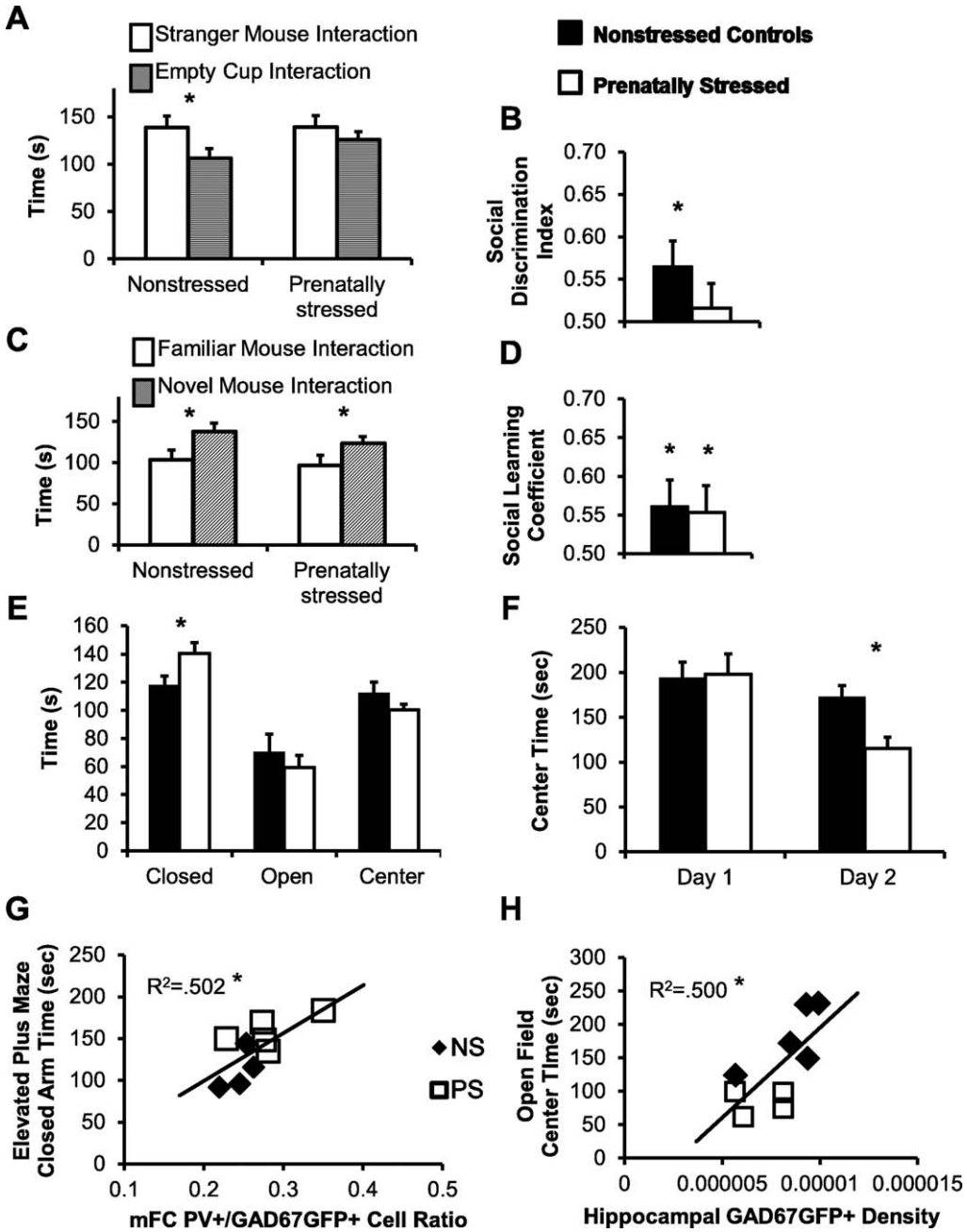

Figure 3 Effect of prenatal stress on behavioral outcomes and the relationship of behavior with neurobiological changes. Both nonstressed and prenatally stressed mice spent more time with a "stranger" mouse than an empty cup (A), but only nonstressed controls had a significantly higher social discrimination index than chance (B). Nonstressed and prenatally stressed mice spent more time with a novel than familiar mice (C) and had higher social learning coefficient than chance (D). Prenatally stressed mice spent more time in the closed arm of the elevated plus maze (E) and less time in the center of the open field on Day 2 of testing (F). A positive correlation was found between the mFC PV+/GAD67GFP+ cell ratio and the time spent in the closed arm of the elevated plus maze (G) and a positive correlation was found between hippocampal GAD67GFP+ cell density and the time spent in the center of the open field (H) (*$p < 0.05$ by two-tailed student's *t*-test or for two-tailed significance of Pearson's correlation).

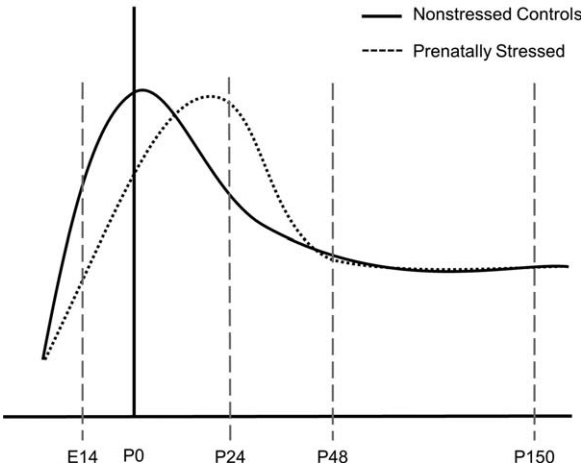

Figure 4 Proposed model of the altered trajectory of GABAergic cell populations after prenatal stress.

normal preference for spending more time with a stranger mouse than an empty cylinder while prenatally stressed mice did not [Fig. 3(A,B)]. Social preference index was significantly above chance for nonstressed mice, but not for their prenatally stressed counterparts [Fig. 3(B)] (nonstressed controls $p < 0.05$, prenatally stressed n.s.). However, both nonstressed and prenatally stressed mice showed normal social recognition, spending more time with a novel than a familiar mouse [Fig. 3(C,D), $p < 0.05$ for both].

After prenatal stress, mice spent more time in the closed arm of the elevated plus maze compared to nonstressed controls [Fig. 3(E), $p < 0.05$]. In the open-field task, prenatally stressed mice spent less time in the exposed center of the apparatus on the second day of testing [Fig. 3(F), $p < 0.05$].

Interestingly, correlations were found between cortical interneuron populations and behavior of individual animals. The amount of time spent in the closed arm of the elevated plus maze correlated significantly with the density of GAD67GFP+ cells in mFC ($R = -0.508$, $p < 0.05$), hippocampal GAD67GFP+ density ($R = -0.707$, $p < 0.05$), and the ratio of PV+/GAD67GFP+ cells in mFC and hippocampal CA [Fig. 3(G), $R = 0.709$, $R = -0.739$; $p < 0.05$]. There were also correlations between the amount of time spent in the center of the open field apparatus and the density of GAD67GFP+ cells in the hippocampal CA [Fig. 3(H), $R = 0.707$, $p < 0.05$] and the hippocampal ratio of PV+/GAD67GFP+ cells ($R = 0.636$, trend at $p = 0.06$). Social behavior only significantly correlated with hippocampal PV+/GAD67GFP+ ratio ($R = 0.688$, $p < 0.05$). These results show that prenatal stress resulted in alterations of behavior in adult mice that are related to the inhibitory neurons of mFC and hippocampus.

DISCUSSION

In this study, we have shown that prenatal stress altered GABAergic populations, GABAergic cell gene expression, and behavioral outcomes of mice through multiple postnatal stages of development. Our findings are consistent with previous work from our lab showing delayed migration of GABAergic progenitors (Stevens et al., 2013). The current findings support a continued delayed time course for GABAergic development after birth and in adolescence that eventually equilibrates in adulthood. Although we found here no significant differences in total GABAergic populations in adulthood, the proportion of parvalbumin+ cells was altered. Last, prenatally stressed adult mice displayed more anxious-like behaviors across multiple tasks, which were correlated with GABAergic cell densities and the proportion of PV+/GAD67GFP+ cells in medial frontal cortex (mFC) and hippocampus.

In the normal mouse dorsal forebrain, inhibitory neuron populations increase in early postnatal life until reaching a peak and undergoing pruning by apoptotic processes (Southwell et al., 2012). The changes we found suggest that the normal peak of inhibitory neuron populations in the cortex was delayed by prenatal stress from its normal time of occurrence during postnatal days 5–10 leading to a temporary and atypically high number of GABAergic cells at the time of weaning. Based on these findings, we propose a model of GABAergic neuronal population developmental trajectory in prenatally stressed versus nonstressed mice (Fig. 4). Our model suggests a delay after prenatal stress in the cellular processes that lead to populating, pruning, and molecular differentiation of GABAergic cells in mFC and other brain regions (Patz et al., 2003; Southwell et al., 2012) and a normalization of the total population by adulthood. While we found no significant effect of stress in hippocampal GABAergic cell numbers, a similar pattern of atypically high GABAergic cell number was present at weaning (Fig. 1B). Thereafter, at postnatal day 48 and 150, there were no significant differences between prenatally stressed and nonstressed animals in their mFC or hippocampal GABAergic populations suggesting that cellular pruning is delayed but still occurs to equalize populations by adulthood. The importance of these GAD67+ populations and the developmental changes they undergo after prenatal stress may be reflected by the correlations of behavior

with small range of individual variability in total GABAergic populations in adult brain. For these studies focused on the underpinnings of anxiety-like behavior, we chose to examine GAD67 (*gad1*) and not its partner, GAD65 (*gad2*), because of previous data demonstrating significant changes after prenatal stress only in *gad1*, not *gad2* (Dong et al., 2015). Interestingly, while *gad1* and *gad2* are both developmentally regulated in medial frontal cortical regions (Huang et al., 2007), *gad1* is more implicated than *gad2* in the genetics of anxiety disorders (Hettema et al., 2006). There may be important differences in susceptibility of *gad1* and *gad2* in developmentally regulated behavioral problems.

While the molecular and cellular mechanisms responsible for the temporary delay in GABAergic cell development are not clear, the early impact of maternal stress beginning at day E12 on these cells is likely important given the lack of change found in GABAergic migration and total populations when stress is begun later in embryonic development (Uchida et al., 2014). Our previous work showed that transcription factors responsible for migration and other processes of GABAergic cell development like *dlx2* and *nkx2.1* were affected as early as 1 day after prenatal stress began, embryonic day 13 (Stevens et al., 2013). Alterations in these cell-intrinsic regulators of GABAergic cell development and others such as NPAS1, KCC2, and Sox6 (Batista-Brito et al., 2009; Bortone and Polleux, 2009; Stanco et al., 2014) could arise in early development, persist, and be responsible for multiple developmental changes. Because of the importance of GABAergic network activity for GABA neuron development (Manent et al., 2005; Baho and Di Cristo, 2012), altered network characteristics of developing GABAergic cells may cause a more protracted maturation of the same cell population. Alternately, indirect mechanisms such as through alterations in glial cells that are important for cortical interneuron development postnatally (Barros et al., 2006; Smith et al., 2014) may also be involved.

To better understand the maturation of GABAergic systems, the parvalbumin subtype of cortical interneurons was evaluated as one assessment of how prenatal stress influences GABAergic cell differentiation during development. In both the mFC and CA at P24, prenatal stress decreased the ratio of parvalbumin+ among all GAD67GFP+ cells with no significant differences in gene expression. Because parvalbumin is a late onset protein, the decrease in ratio of cell number suggests that prenatally stressed inhibitory neurons in dorsal regions are less mature at P24. These findings are similar to effects of later embryonic stress (Uchida et al., 2014) and of early postnatal stress on parvalbumin interneurons in mFC and hippocampus (Brenhouse and Andersen, 2011; Giovanoli et al., 2014), the latter implicating altered maternal postnatal care (Smith et al., 2004) in the effects seen here after prenatal stress. Alterations in these populations in the juvenile mFC may implicate prenatal stress in the sensitive periods in cortical plasticity that rely on GABAergic activity and the mechanisms of parvalbumin neuron development (Hensch, 2005) and related factors including Otx2 and perineuronal nets that may be influenced by stress (Beurdeley et al., 2012; Morishita et al., 2015). One form of prenatal stress, prenatal exposure to maternal depression, has been shown to alter developmentally sensitive periods in a related way (Weikum et al., 2012).

Changes after prenatal stress in cell ratios were more similar to independent PV+ cell numbers in hippocampal CA than mFC, likely due to the larger changes in the GAD67GFP+ population as a whole in mFC. Hippocampal CA showed consistently decreased PV+ cell populations and ratios after prenatal stress while mFC had dynamic PV+ cell ratios at the same time that independent PV+ cell numbers were consistently increased. This finding that PV+ cell numbers did not change on the same time scale or to the same degree as the total GABAergic population in mFC may suggest that this region is more susceptible to a dysregulated total GABAergic population.

In adulthood, the decrease in PV+-to-GAD67GFP+ cell ratio was still present in hippocampal CA, but an "overcorrection" had occurred in mFC. We also examined PV and Gad1 gene expression which may parallel PV+ cell numbers, but may alternatively become up- or downregulated in response to a system with abnormal cell numbers (Saji et al., 1994). We found a decreased PV-to-Gad1 gene expression ratio after prenatal stress in mFC and an increased ratio in hippocampal CA. These changes were in the opposite direction of changes in PV+ cell number and may reflect gene expression levels compensating for the imbalance in differentiated PV+ cells in these regions or the opposite, with abnormal levels of gene expression becoming compensated for post-transcriptionally and resulting in altered protein labeling with PV. These results contribute to growing evidence that prenatal or early-life stress alters the parvalbumin subtype of cortical interneurons in multiple ways (Helmeke et al., 2008; Zohar et al., 2015). Differences in the timing of early life stress effects may result from different types and timing of stress. In general, these changes suggest that the cellular

composition of GABAergic cells is disrupted by prenatal stress and remains altered in adulthood, affecting both gene and protein expression.

At P150, when mice had reached adulthood, an impact of prenatal stress was no longer seen in the total number of GAD67+ cells in mFC or hippocampal CA. However, changes in behavior of P150 mice were observed. Prenatally stressed mice exhibit decreased sociability, shown through the first phase of the three-chambered social task, and increased anxiety, shown through elevated plus maze and open-field task. These behaviors correlated with mFC and hippocampal CA GAD67+ cell density and PV+/GAD67GFP+ cell ratio, which suggest the importance of inhibitory cell densities and the developmental processes that influence them in dorsal forebrain regions. Although significant cell count changes diminish by P150, correlations suggest that the GABAergic and PV+ cell density in dorsal regions may impact behavior. The importance of acute local GABAergic activity in these regions has been demonstrated for anxiety-like behaviors in rodents (Bi et al., 2013).

The behavioral findings here contribute to the relevance of the prenatal stress model for neuropsychiatric disorders (Fine et al., 2014). Inhibited sociability is a central symptom of autism and increased anxiety has been observed in generalized anxiety disorder and schizophrenia. The association of these behaviors with dorsal forebrain GABAergic populations after prenatal stress provides a possible mechanism for how exposure to prenatal stress becomes a risk factor for neuropsychiatric disorders, in which GABAergic systems have been shown to differ postmortem (Fatemi et al., 2002; Lewis, 2012). The links between neural mechanism and behavioral outcomes in this model suggest that one component of effective intervention may be to normalize either acute GABAergic neuron function or altered development in at-risk populations (Benham et al., 2014; Cellot and Cherubini, 2014).

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