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Neonatal Ethanol Disturbs the Normal Maturation of Parvalbumin Interneurons Surrounded by Subsets of Perineuronal Nets in the Cerebral Cortex: Partial Reversal by Lithium

Mariko Saito^{1,2}, John F. Smiley^{1,2}, Maria Hui¹, Kurt Masiello¹, Judith Betz¹, Maria Ilna³, Mitsuo Saito^{2,3} and Donald A. Wilson^{4,5}

¹Division of Neurochemistry, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA,

²Department of Psychiatry, NYU School of Medicine, New York, NY 10016, USA, ³Emotional Brain Institute, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA, ⁴Division of Analytical Psychopharmacology, Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA and

⁵Department of Child and Adolescent Psychiatry, NYU School of Medicine, New York, NY 10016, USA

Address for correspondence to Mariko Saito, Division of Neurochemistry, Nathan Kline Institute for Psychiatric Research, 140 Old Orangeburg Road, Orangeburg, NY 10962, USA. Email: Mariko.Saito@nki.rfmh.org

Abstract

Reduction in parvalbumin-positive (PV⁺) interneurons is observed in adult mice exposed to ethanol at postnatal day 7 (P7), a late gestation fetal alcohol spectrum disorder model. To evaluate whether PV⁺ cells are lost, or PV expression is reduced, we quantified PV⁺ and associated perineuronal net (PNN)⁺ cell densities in barrel cortex. While PNN⁺ cell density was not reduced by P7 ethanol, PV cell density decreased by 25% at P90 with no decrease at P14. PNN⁺ cells in controls were virtually all PV⁺, whereas more than 20% lacked PV in ethanol-treated adult animals. P7 ethanol caused immediate apoptosis in 10% of GFP⁺ cells in G42 mice, which express GFP in a subset of PV⁺ cells, and GFP⁺ cell density decreased by 60% at P90 without reduction at P14. The ethanol effect on PV⁺ cell density was attenuated by lithium treatment at P7 or at P14–28. Thus, reduced PV⁺ cell density may be caused by disrupted cell maturation, in addition to acute apoptosis. This effect may be regionally specific: in the dentate gyrus, P7 ethanol reduced PV⁺ cell density by 70% at P14 and both PV⁺ and PNN⁺ cell densities by 50% at P90, and delayed lithium did not alleviate ethanol's effect.

Key words: barrel cortex, Cat-315, dentate gyrus, fetal alcohol spectrum disorders, G42 mice

Introduction

Prenatal ethanol exposure can cause developmental impairment, leading to fetal alcohol spectrum disorders (FASD) with mild to severe symptoms including intellectual disabilities in offspring (Riley and McGee 2005). Binge-like ethanol exposure in neonatal rodents, equivalent to the third trimester of human fetuses, induces robust apoptotic neurodegeneration (Bonthius and West

1990; Ikonomidou et al. 2000; Olney et al. 2002; Guerri et al. 2009) and glial activation (Goodlett et al. 1993; Hashimoto et al. 2003; Lodge et al. 2009; Saito et al. 2010, 2012, 2015; Kane et al. 2011, 2012; Ahlers et al. 2015), as well as long-lasting anatomical, physiological, and behavioral deficits (Bonthius and West 1991; Wozniak et al. 2004; Ieraci and Herrera 2006; Wilson et al. 2011, 2016; Coleman et al. 2012; Sadrian et al. 2012, 2014; Smiley et al.

2015), providing an animal model for FASD. While there are various animal models for FASD, we have chosen to use this third trimester binge model, specifically a postnatal day 7 (P7) ethanol exposure model, which provides precise temporal control of exposure to help isolate the specific and synchronized effects of ethanol on the developing brain and the resulting changes in the adult brain. Using this model, we and others have found that P7 ethanol decreases PV neuron densities in various brain regions, such as the cortex and hippocampus of adult mice (Coleman et al. 2012; Sadrian et al. 2014; Smiley et al. 2015), and our studies have shown that decrease in GABA subtypes in the cortex is selectively severe compared with the decrease in total neuron number, which roughly corresponds to a reduction in cortex volume (Smiley et al. 2015). In the prefrontal cortex, PV⁺ cell densities are reduced by 20–30%, while NeuN⁺ cell densities remain the same (Smiley et al. 2015). Such PV neuron reduction may be related to the abnormal electrophysiology, deficits in memory function, and sleep problem in adult mice exposed to P7 ethanol (Sadrian et al. 2013, 2014; Wilson et al. 2016), because GABAergic interneurons are involved in many functions, such as cortical wiring, critical period plasticity, maintaining the balance of excitation and inhibition, and entraining network activity oscillations during and after brain development (Hashimoto et al. 2003; Lodge et al. 2009; Marin 2012; Le and Monyer 2013; Sadrian et al. 2013).

Decrease in PV neurons has been observed not only in FASD models but in various developmental disorders/injuries and in their animal models, including schizophrenia (Hashimoto et al. 2003; Lodge et al. 2009; Jodi et al. 2016), autism (Gogolla et al. 2009; Zikopoulos and Barbas 2013; Filice et al. 2016), and hypoxia (Dell'Anna et al. 1996; Faylor et al. 2010; Wang et al. 2011; Komitova et al. 2013). There may be common features among these disorders with dysregulated PV cell development, and the P7 ethanol model may provide insights into how PV cell densities are affected and how the reduction in PV cells is related to functional disturbances in these neurodevelopmental disorders.

However, mechanisms behind the long-lasting decrease of PV neurons have not been well explored in the P7 ethanol model. The reduction in PV neurons in the adult brain may be due to robust apoptotic neurodegeneration, which occurs within 24 h after P7 ethanol injection (Ikonomidou et al. 2000; Olney et al. 2002). Although PV expression is very low at P7 (del Rio et al. 1994; Itami et al. 2007; Lema Tome et al. 2008), precursors of PV basket cells are generated in medial ganglionic eminence during a protracted period of neurogenesis from embryonic day (E) 9.5–15.5 (Fishell and Rudy 2011), and post-mitotic immature PV cells are migrated into the cortex by around E15 (Behrens and Sejnowski 2009). P7 ethanol may induce apoptosis in these immature PV cells. Alternatively or additionally, P7 ethanol may affect maturation of PV cells and interfere with PV expression, because PV interneurons take a prolonged maturation step over the first 4 postnatal weeks during which PV expression increases (del Rio et al. 1994; Okaty et al. 2009). It is possible that the expression of PV protein/mRNA is reduced without cell loss as indicated in some models of schizophrenia, autism, and hypoxia (Powell et al. 2012; Komitova et al. 2013; Filice et al. 2016).

Thus, we have hypothesized that P7 ethanol not only causes acute apoptotic neurodegeneration but also disturbs PV interneuron maturation that continues long after ethanol injection at P7. To evaluate the possibility, the effects of P7 ethanol on PV cells were examined at P14 (juvenile/adolescent) and P90 (young adult) by measuring PV⁺ and/or perineuronal net (PNN)⁺ cell densities in the barrel cortex and dentate gyrus. PNNs are

lattice-like extracellular matrix, which are selectively formed around a set of PV neurons (Brauer et al. 1993; Hartig et al. 1994) and regulate the developmental plasticity of PV neurons (Celio et al. 1998; Ye and Miao 2013; Balmer 2016; Lensjo et al. 2017b). PNNs were detected by Wisteria Floribunda Agglutinin (WFA), a broad marker for PNNs, which binds to N-acetylgalactosamine of chondroitin sulfate, a main component of PNNs (Brauer et al. 1993; Hartig et al. 1994) and by anti-Cat-315 antibody, which recognizes a specific glycoform of aggrecan (chondroitin sulfate proteoglycan 1, CSPG 1) on a subset of PNNs in the cortex (Karetko-Sysa et al. 2014). If P7 ethanol decreases PV expression rather than cell loss, the expression of cellular components other than PV, such as PNNs, may not be down-regulated as shown in the case of animal models of autism (Filice et al., 2016). The possible immediate PV cell loss by P7 ethanol-induced apoptosis was assessed using GAD67-green fluorescent protein (GFP) BAC transgenic G42 mice that selectively express GFP in a subclass of PV-expressing basket interneurons (Chattopadhyaya et al. 2004). While PV is barely detectable at P7, GFP in G42 mice is already expressed (Chattopadhyaya et al. 2004). Therefore, P7 ethanol-induced acute apoptotic cell death can be observed in GFP⁺ cells. Finally, the effect of lithium co-treatment with ethanol at P7 or the effect of lithium treated after P14 (when the wave of acute cell death is over) on PV cell reduction were examined. We and others have shown that co-treatment with lithium prevents P7 ethanol-induced immediate apoptotic neurodegeneration, glial activation as well as electrophysiological abnormality and reduction in memory functions in adult animals (Zhong et al. 2006; Chakraborty et al. 2008; Young et al. 2008; Saito et al. 2010; Sadrian et al. 2012). If P7 ethanol affects prolonged PV cell maturation or causes delayed cell death, post-ethanol lithium treatment may still attenuate PV cell reduction, because delayed lithium treatment after the acute injury phase has been shown to exert therapeutic benefits through reduction in neuroinflammation, enhanced neurogenesis, and up-regulation of brain-derived neurotrophic factor (BDNF) (Xie et al. 2014; Taliyan and Ramagiri 2016).

Cumulative results of our present study suggest that P7 ethanol not only induces acute immature PV cell apoptosis but also disturbs prolonged PV cell maturation in the barrel cortex, while the process of PV cell reduction in the dentate gyrus is likely to be different from that of the barrel cortex.

Materials and Methods

Subjects

C57BL/6By mice, bred at the Nathan Kline Institute animal facility, were maintained on ad lib food and water at all times. All procedures were approved by the Nathan Kline Institute IACUC and were in accordance with NIH guidelines for the proper treatment of animals. P7 pups (both males and females) were injected subcutaneously with saline or ethanol as described (Olney et al. 2002; Saito et al. 2007). Each mouse in a litter was assigned to the saline or ethanol group. Ethanol treatment (2.5 g/kg) was delivered twice at a 2 h interval as originally described for C57BL/6 mice (Olney et al. 2002). Pups were returned to their home cage immediately following injections. Our previous studies showed that this P7 ethanol treatment induced a peak blood alcohol level (BAL) of 0.5 g/dL when truncal blood was collected at 0.5, 1, 3, and 6 h following the second ethanol injection and analyzed with an Alcohol Reagent Set (Pointe Scientific, Canton, MI, USA) (Saito et al. 2007). Under the

same P7 ethanol treatment conditions, it has been reported that initial BAL peaks are attained approximately 1 h after each injection, with BAL falling below half of this level 8 h after first ethanol exposure (Wozniak et al. 2004; Young and Olney 2006). Pups were weaned at P28 into group cages of littermates. Same-sex mice were housed together in cages in numbers between 2 and 4 per cage. For acute lithium treatment, saline or 0.6 M lithium chloride in saline was injected (10 μ L/g body weight, 6 mEq/kg body weight) intraperitoneally 15 min after the first ethanol injection at P7 as described previously (Zhong et al. 2006; Chakraborty et al. 2008). For the delayed chronic lithium treatment, 0.1 M or 0.2 M lithium chloride in saline was injected (10 μ L/g, 1 mEq/kg or 2 mEq/kg) once a day for 15 days from P14 to P28. Saline was injected in the same way as the control. GAD67-EGFP BAC transgenic mice (G42 mice) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the NKI animal facility. These mice selectively express green fluorescent protein (GFP) in a subclass of PV basket interneurons (Chattopadhyaya et al. 2004). G42 mice (hemizygous) were treated with saline/ethanol as described above for C57BL/6By mice. Animals used in this study are summarized in Table 1. Results of PV⁺, WFA⁺, Cat-315⁺, and GFP⁺ cell densities in the barrel cortex obtained as described in the result section are also listed in Table 1.

Immunohistochemistry and Cell Counting

In each experiment, 5–10 mice per each group derived from at least 4 different litters were used. Mice treated with ethanol, lithium, lithium+ethanol, or their controls were perfused with a solution containing 4% paraformaldehyde and 4% sucrose in cacodylate buffer (pH 7.2) at P7, P14, or P90, and the heads were removed and further fixed in the perfusion solution overnight. Then brains were removed, transferred to phosphate buffered saline (PBS) solution, and kept at 4 °C for 2–5 days until cut with a vibratome into 50 μ m thick coronal sections. The free-floating sections were rinsed in PBS, permeabilized in methanol for

10 min, and incubated for 30 min in blocking solution (PBS containing 5% BSA and 0.1% Triton X-100), followed by incubation overnight with antibodies against parvalbumin (PV25, Swant, Marly, Switzerland), GFP (ab290, Abcam, Cambridge, MA), Cat-315 (MAB1581, Millipore, Billerica, MA), cleaved caspase-3 (CC3) (#9664, Cell Signaling Technology, Danvers, MA), GAD67 (MAB5406, Millipore), glutamine synthetase (MAB302, Millipore) in PBS containing 3% BSA, and 0.1% Triton X-100. Validation/characterization of these antibodies was described in Supplementary Materials and Methods. Brain sections incubated with one of these primary antibodies were then rinsed in 0.1% Triton X-100 in PBS 3 times and incubated with another primary antibody for 2 h at r.t., followed by incubation with Alexa Fluoro594 (or 488) goat anti-rabbit (or mouse) IgG (or IgM for Cat-315) (Life Technologies, Grand Island, NY) in 0.1% Triton X-100 in PBS containing 1% BSA for 1 h at r.t. Sections were finally rinsed in PBS 3 times, mounted, and coverslipped using ProLong Gold Antifade Reagent (Life Technologies). For labeling with WFA, brain sections were incubated with biotin-conjugated WFA (Sigma, St. Louis, MO) for 2 h at r.t., followed by incubation with streptavidin-conjugated Alexa Fluor 594 (or 488) (Fisher Scientific, Pittsburgh, PA) for 2 h. For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, In Situ Cell Death Detection Kit, TMR red (Roche, Indianapolis, IN) was used. For dual labeling with CC3 immunostaining, free-floating brain sections were immunolabeled with CC3 antibody as described above, mounted on slides, dried, incubated in 0.3% Triton X-100 in PBS for 20 min at 85 °C, and then processed following the manufacturer's protocol. All photomicrographs were taken through a 4 $\times$, 10 $\times$, or 20 $\times$ objective with a Nikon Eclipse TE2000 inverted microscope attached to a digital camera DXM1200F. Because our previous studies (Smiley et al. 2015) showed that both 2-dimensional and stereological 3-dimensional counting methods gave similar significant reduction in PV cell densities in the cortex by P7 ethanol treatment, the 2-dimensional counting method was used in the present studies. The PV, WFA, Cat-315, GAD67, or GFP-positive cell number of each area of interest (AOI) and total

Table 1 Animals used in this study

Mouse strain	Treatment	Age	N	Cell number/mm ²		
				PV ⁺ (GFP ⁺)	WFA ⁺	Cat-315 ⁺
C57BL/6By	Saline (P7)	P7	7	6.9 $\pm$ 1.8	n.d.	n.d.
C57BL/6By	EtOH (P7)	P7	7	4.7 $\pm$ 1.8	n.d.	n.d.
C57BL/6By	Saline (P7)	P14	8	186 $\pm$ 8.3	89.5 $\pm$ 2.6	n.d.
C57BL/6By	EtOH (P7)	P14	8	188 $\pm$ 5.8	106 $\pm$ 5.1	n.d.
C57BL/6By	Saline (P7)	P90	10	299 $\pm$ 13	209 $\pm$ 12	110 $\pm$ 4.4
C57BL/6By	EtOH (P7)	P90	10	228 $\pm$ 9.3	198 $\pm$ 9.6	101 $\pm$ 8.5
C57BL/6By	Saline + Saline (P7)	P90	7	278 $\pm$ 6.4	227 $\pm$ 11.9	117 $\pm$ 8.3
C57BL/6By	Saline + Lithium (P7)	P90	7	291 $\pm$ 13	225 $\pm$ 14.1	114 $\pm$ 7.0
C57BL/6By	EtOH + Saline (P7)	P90	7	219 $\pm$ 6.0	228 $\pm$ 9.6	108 $\pm$ 8.9
C57BL/6By	EtOH + Lithium (P7)	P90	7	274 $\pm$ 7.6	221 $\pm$ 9.6	109 $\pm$ 7.0
C57BL/6By	Saline (P7) + Saline (P14–28)	P90	7	278 $\pm$ 5.3	213 $\pm$ 9.3	110 $\pm$ 7.6
C57BL/6By	Saline (P7) + Lithium (P14–28)	P90	7	253 $\pm$ 12	208 $\pm$ 13.4	92.5 $\pm$ 8.3
C57BL/6By	EtOH (P7) + Saline (P14–28)	P90	7	182 $\pm$ 11	226 $\pm$ 8.6	116 $\pm$ 7.7
C57BL/6By	EtOH (P7) + Lithium (P14–28)	P90	7	234 $\pm$ 13	198 $\pm$ 11	116 $\pm$ 11
G42	Saline (P7)	P7	5	1.4 $\pm$ 0.9 (25.9 $\pm$ 3.4)	n.d.	n.d.
G42	EtOH (P7)	P7	5	0.8 $\pm$ 0.3 (37.9 $\pm$ 3.9)	n.d.	n.d.
G42	Saline (P7)	P14	6	198 $\pm$ 15 (22.5 $\pm$ 2.7)	153 $\pm$ 6.9	n.d.
G42	EtOH (P7)	P14	5	182 $\pm$ 25 (37.7 $\pm$ 2.6)	152 $\pm$ 7.8	n.d.
G42	Saline (P7)	P90	8	223 $\pm$ 13 (63.6 $\pm$ 8.3)	257 $\pm$ 4.9	112 $\pm$ 7.1
G42	EtOH (P7)	P90	7	176 $\pm$ 9.3 (25.3 $\pm$ 6.7)	242 $\pm$ 4.4	107 $\pm$ 7.1

Note: Cell densities (cell number/mm²) in the barrel cortex after various treatments are presented here as mean $\pm$ SEM. n.d., not detected.

dimensions of each AOI were measured using the Image-Pro software version 6.0 (Media Cybernetics, Silver Spring, MD). AOIs for cell counting were the barrel cortex including all layers and dentate gyrus. These AOIs were defined according to the Atlas of mouse brain (Paxinos and Franklin 2004). Dimensions of AOI analyzed were 0.7–2 mm² for barrel cortex and 0.4–0.7 mm² for dentate gyrus. The cell density of each AOI was calculated as the mean cell number per square millimeter from 5 to 9 mice (derived from 4 to 6 different litters) using 4–6 sections around bregma –0.22 to –1.70 mm for the barrel cortex and 3–6 sections around bregma –1.46 to –2.18 mm for the dentate gyrus per each mouse brain.

Statistics

Data were analyzed by Student's *t*-test for comparisons of 2 groups and one- or two-way ANOVA for comparisons of more than 2 groups/factors using SPSS statistics software (version 22). For post hoc analyses, Bonferroni or Fisher's LSD post hoc tests were used. For all analyses, *P* < 0.05 was considered statistically significant. Values are expressed as mean ± SEM obtained from 5 to 9 animals. Because no significant sex differences were observed either in the effects of ethanol on PV⁺, WFA⁺, and Cat-315⁺ cell densities in C57BL/6By mice or in the effects of GFP⁺ cell densities in G42 mice as shown in Results, both sexes were combined for statistical analyses. In the chronic lithium experiments, results of 0.1 M and 0.2 M (1 mEq/kg and 2 mEq/kg) lithium chloride were combined because there is no main effect of lithium doses (0.1 M and 0.2 M) as described in Results.

Results

Developmental Profiles of PV and PNN Expression in the Barrel Cortex

In order to clarify the effects of P7 ethanol on PV neuron development, we first examined developmental profiles of the expression of PV and the associated PNNs in the barrel cortex, where densities of PV⁺ and PNN⁺ neurons are high compared with other cortex regions at P14 (Fig. 1A). As reported earlier (Itami et al. 2007; Okaty et al. 2009; Ye and Miao 2013), both PV and PNN expression increased during brain development. While the number of PV⁺ cells and PNN⁺ cells detected by WFA lectin binding (WFA⁺ cells) was very low at P7, they increased at P14 (Fig. 1B). In the barrel cortex, PV⁺ cell density at P7 was $6.9 \pm 1.8/\text{mm}^2$ (*n* = 7) (Table 1), while, as shown in Figure 2A, PV⁺ cell density was $186.1 \pm 8.3/\text{mm}^2$ (*n* = 7) at P14. Weak WFA⁺ staining at P7 (Fig. 1B) appeared to be mostly associated with glutamine synthetase (GS)⁺ astrocytes (Fig. S1). In contrast, dual labeling with anti-PV antibody and WFA revealed that $95.8 \pm 1.1\%$ (*n* = 7) and $99.0 \pm 0.3\%$ (*n* = 5) of WFA⁺ cells were PV⁺ at P14 and P90, respectively, while PV⁺ cells without PNN expression were also present especially in layers other than 4 and 5 (Fig. 1B). PV⁺ cell density significantly (*P* < 0.001) increased between P14 ($186.1 \pm 8.3/\text{mm}^2$, *n* = 7) and P90 (298.7 ± 12.9 , *n* = 9), and WFA⁺ cell densities also significantly (*P* < 0.001) increased between P14 (89.5 ± 2.6 , *n* = 7) and P90 (208.6 ± 11.7 , *n* = 9) (Fig. 1B, Fig. 2A). Dual labeling using anti-PV and anti-Cat-315 antibody (another probe for PNN) showed that Cat-315⁺ cells were absent at P14 (Fig. 1C). Also, while $99.7 \pm 0.2\%$ (*n* = 5) and $97.2 \pm 0.1\%$ (*n* = 4) of Cat-315⁺ cells at P90 were PV positive (Fig. 1C) and WFA positive (Fig. 1D), respectively, Cat-315⁺ cell density was about 50% of WFA⁺ cell density (Figs 1D and 2A),

indicating developmentally delayed appearance of Cat-315 antigenicity in a subset of WFA⁺PV⁺ cells.

Effects of P7 Ethanol on PV Neurons in the Barrel Cortex of P14 and P90 Mice

Figure 2A shows PV⁺, WFA⁺, and Cat-315⁺ cell densities in the barrel cortex area (including all layers) measured at P14 and P90 after saline (Ctr) or ethanol (EtOH) treatment at P7. ANOVA using 2 factors (sex and treatment) indicated that there were no significant main effects of sex or interaction (sex × treatment) for PV⁺ (P14: *P* = 0.23 for main effect and *P* = 0.352 for interaction; P90: *P* = 0.16 and *P* = 0.202), WFA⁺ (P14: *P* = 0.257 and *P* = 0.492; P90: *P* = 0.541 and *P* = 0.802), or Cat-315⁺ cell density (P90: *P* = 0.411 and *P* = 0.915), while the significant main effect of treatment was observed in PV⁺ cell density at P90 (*P* < 0.001), but not in others (PV⁺ at P14, *P* = 0.569, WFA⁺ at P14, *P* = 0.088; WFA⁺ at P90, *P* = 0.832, or Cat-315⁺ cell density at P90, *P* = 0.843). In the present study, males and females were combined for statistical analyses, because no significant sex differences were observed in the effects of ethanol on PV⁺, WFA⁺, and Cat-315⁺ cell densities either at P90 or P14 as described above, although the sample size in each group was relatively low. Each experimental group had a similar distribution of males and females.

ANOVA using 2 factors (time and treatment) indicated that there were a statistically significant interaction between time (P14 and P90) and treatment (control and ethanol) [*F* (1,28) = 13.1, *P* = 0.001, *n* = 7 for P14 groups, *n* = 9 for P90 groups] and significant main effects of time [*F* (1,28) = 56.8, *P* < 0.001] and treatment [*F* (1,28) = 11.7, *P* = 0.002]. Fisher's LSD post hoc test showed that ethanol decreased PV cell densities at P90 (*P* < 0.001), but not at P14 (*P* > 0.20) (Fig. 2A). Also, significant increases in PV cell densities were observed between P14 and P90 in both control (*P* < 0.001) and ethanol (*P* < 0.0025) groups (Fig. 2A). PV cell densities in the P90 barrel cortex of the control group (299 cells/mm²) and the ethanol group (228 cells/mm²) were slightly higher than PV cell densities in the whole cortex of the control group (248 cells/mm²) and the ethanol group (202 cells/mm²) measured by us previously (Smiley et al. 2015). In contrast, there were no significant main effect of treatment (*P* = 0.765) or interaction between time and treatment on WFA⁺ cell densities (*P* = 0.078, *n* = 7 for P14 groups, *n* = 9 for P90 groups), while there was a significant main effect of time [*F* (1,28) = 138.1, *P* < 0.0005]. The LSD post hoc test indicated that WFA⁺ cell densities increased between P14 and P90 in both control (*P* < 0.001) and ethanol (*P* < 0.001) groups (Fig. 2A). Although ANOVA indicated that ethanol treatment did not change WFA⁺ cell densities, *P*-value of interaction was close to 0.05 and there was a tendency of slight increase in the WFA⁺ cell densities at P14. As shown in Figure 2B, when PV⁺ and WFA⁺ cell densities were measured only in layers 4 and 5, ANOVA showed that there was a significant interaction [*F* (1,24) = 4.41, *P* = 0.046, *n* = 7 per group] between 2 factors, and the LSD test indicated that WFA⁺ cell density was significantly (*P* < 0.01) higher in ethanol-treated samples. Cat-315⁺ cells were absent at P14 (Fig. 1C), and the number was not significantly different between the control and ethanol groups (*P* = 0.316, *n* = 7 per group) at P90 (Fig. 2A). Thus, P7 ethanol significantly reduced the PV cell density at P90, but the reduction was not apparent at P14. In contrast, P7 ethanol did not reduce either the number of WFA⁺ or Cat-315⁺ cells at P90, and the density of WFA⁺ cells was significantly higher in ethanol-treated brain at P14 in the layers 4 and 5. These results suggest that a part of the PV⁺ cell reduction by

Figure 1. Developmental changes in PV and PNN expression in the cortex. (A) A representative image of P14 mouse brain sections dual-labeled with anti-PV antibody (red) and biotin-conjugated WFA (green). The expression of PV and PNN was especially strong in layer 4 of the barrel cortex. S1BF, barrel field primary somatosensory cortex; CA3, hippocampus CA3 region; RT, reticular thalamus. Scale bar = 500 μ m. (B) The expression of PV and PNN in the barrel cortex is compared between P7, P14, and P90 brains. These brain sections were dual-labeled with anti-PV antibody (red) and biotin-conjugated WFA (green). Scale bar = 100 μ m. (C) The brain sections of P14 and P90 mice were dual-labeled with anti-PV antibody (red) and anti-Cat-315 antibody (green). The overlaid images labeled with both antibodies in the barrel cortex indicate no Cat-315 expression at P14. Scale bar = 50 μ m. (D) P90 brain sections were dual-labeled with biotin-conjugated WFA (red) and anti-Cat-315 antibody (green). Scale bar = 50 μ m.

ethanol was due to the decrease in expression levels of PV in WFA⁺ cells rather than the cell loss/death, or alternatively, PV⁺ cell reduction was more prevalent in cells that lacked PNNs. In Figure 2C, P90 brain sections from control and ethanol groups were dual-labeled with anti-PV antibody and WFA. The overlaid images showed that while virtually all WFA⁺ cells were PV⁺ in the control groups, P7 ethanol increased WFA⁺ cells with faint or no PV expression. For quantitative analyses, densities of PV⁺WFA⁻ (PV only), PV⁻WFA⁺ (WFA only), and PV⁺WFA⁺ (PV+WFA) cells were measured (Fig. 2D). ANOVA showed that there were significant main effects of treatment (saline/ethanol) [$F(1,36) = 5.48$, $P = 0.025$, $n = 7$ per group] and cell types

[$F(2,36) = 369.6$, $P = 0.001$] as well as significant interaction [$F(2,36) = 25.0$, $P = 0.001$]. LSD post hoc test indicated that the densities of all 3 cell types were significantly ($P < 0.0005$) different between the saline and ethanol group. It showed that although $99.2 \pm 0.4\%$ ($n = 7$) of WFA⁺ cells were PV⁺ in the control groups, there was a significant elevation of WFA⁺ cells without PV expression along with a significant decrease in PV⁺WFA⁺ cells. In Figure 2E, P90 brain sections were dual-labeled with anti-PV and anti-Cat-315 antibodies. It showed that there were a substantial number of PV cells without Cat-315 expression in the control group, but the cell type decreased in the ethanol group. For quantitative analyses, densities of PV⁺Cat-315⁻ (PV only),

Figure 2. Effects of P7 ethanol on PV⁺ and PNN⁺ cell density at P14 and P90. (A) Mice were exposed to saline (Ctr) or ethanol (EtOH) at P7, perfusion-fixed at P14 or P90, and brain sections were labeled with the anti-PV antibody, biotin-conjugated WFA, and anti-Cat-315 antibody, and the densities of labeled PV⁺ cells, WFA⁺ cells, and Cat-315⁺ cells were measured in the barrel cortex including all layers as described in Materials and Methods. Values (cell number/mm²) are expressed as mean $\pm$ SEM from 7 to 9 animals. There were significant differences *between Ctr and EtOH groups in the densities of PV⁺ cells at P90 ($P < 0.001$), #between P14 and P90 in PV⁺ and WFA⁺ cell densities in Ctr group ($P < 0.001$), and \$between P14 and P90 in PV⁺ ($P < 0.0025$) and WFA⁺ cell densities ($P < 0.001$) in EtOH group. (B) After saline or ethanol treatment at P7, mice were perfusion-fixed at P14, and brain sections were labeled with anti-PV antibody and biotin-conjugated WFA. The densities of labeled PV⁺ cells and WFA⁺ cells were measured in the layer 4 and 5 of the barrel cortex. Values (cell number/mm²) are expressed as mean $\pm$ SEM from 7 animals. *indicates that there was a significant difference between saline and ethanol groups ($P < 0.01$). (C) Brain sections from P90 mice exposed to saline (Ctr) or ethanol (EtOH) at P7 were dual-labeled with anti-PV antibody (green) and biotin-conjugated WFA (red). Merged images are shown here. Ethanol treatment increased WFA⁺PV⁺ cells. Scale bar = 50 μ m. (D) Brain sections were processed as described in C, and densities of PV⁺WFA⁺ (PV only), PV⁺WFA⁺ (WFA only), and PV⁺WFA⁺ (PV+WFA) cells were measured. Values (cell number/mm²) are expressed as mean $\pm$ SEM from 7 animals. *The LSD post hoc test after ANOVA showed that cell densities were significantly ($P < 0.0005$) different between the saline and ethanol group in PV⁺WFA⁺, PV⁺WFA⁺, and PV⁺WFA⁺ groups. (E) Brain sections from P90 mice exposed to saline (Ctr) or ethanol (EtOH) at P7 were dual-labeled with anti-PV antibody (green) and anti-Cat-315 antibody (red). Merged images are shown here. Scale bar = 50 μ m. (F) Brain sections were processed as described in E, and densities of PV⁺Cat-315⁺ (PV only), PV⁺Cat-315⁺ (Cat-315 only), and PV⁺Cat-315⁺ (PV+Cat-315) cells were measured. Values (cell number/mm²) are expressed as mean $\pm$ SEM from 6 animals. The LSD post hoc test after ANOVA showed that densities of PV⁺Cat-315⁺ cells were significantly ($P < 0.0005$) different between the saline and ethanol group, while densities of PV⁺Cat-315⁺ (Cat-315 only) and PV⁺Cat-315⁺ (PV+Cat-315) were not significantly different.

PV⁺Cat-315⁺ (Cat-315 only), and PV⁺Cat-315⁺ (PV+Cat-315) cells were measured (Fig. 2F). ANOVA showed that there were significant main effects of treatment [$F(1,33) = 17.39$, $P < 0.0001$, $n = 6$ per group] and cell types [$F(2,33) = 156.5$, $P < 0.001$] as well as

significant interaction [$F(2,33) = 22.6$, $P < 0.001$]. The LSD test indicated that densities of PV⁺Cat-315⁺ cells, but not the other cell types, were significantly ($P < 0.0005$) different between the control and ethanol groups. $99.4 \pm 0.3\%$ ($n = 6$) of Cat-315⁺ cells

were PV⁺, and ethanol did not reduce PV⁺Cat-315⁺ cell density and did not increase PV[−]Cat-315⁺ cell density (Fig. 2E,F), suggesting that at least a part of the ethanol-induced PV cell reduction is due to decrease in PV expression in WFA⁺Cat-315[−] cells, although further characterization of WFA⁺ PV[−] cells in ethanol group is necessary to confirm the origin of these cells.

GFP⁺ Cell Densities in P90 G42 Mice Were Reduced by P7 Ethanol

To examine further the possible interference of PV maturation by P7 ethanol, GAD67-GFP transgenic G42 mice were used. In the cortex of G42 mice, it has been shown that GFP was expressed in a certain subset of PV neurons and their immature forms under the control of GAD67 gene promotor (Chattopadhyaya et al. 2004).

As shown in representative images in Figure 3A and quantitative results in Figure 3B, P7 ethanol reduced the density of GFP⁺ cells in the barrel cortex at P90. In these and the following analyses, both males and females were combined because ANOVA using 2 factors (sex and treatment) indicated that there were no significant main effects of sex or interaction (sex × treatment) for GFP⁺ cell densities ($P = 0.225$ for main effect and $P = 0.777$ for interaction) in the barrel cortex at P90. In Figure 3B, ANOVA using 2 factors (time and treatment) indicated that there was a statistically significant interaction between time (P14 and P90) and treatment (control and ethanol) on GFP⁺ cell densities [$F(1, 21) = 20.8$, $P < 0.0005$, $n = 6$ and 5 for P14 groups, $n = 7$ for P90 groups], and the LSD post hoc test showed that ethanol decreased GFP⁺ cell densities at P90 ($P < 0.001$), but not at P14 ($P > 0.1$). Also, the significant increase in GFP⁺ cell densities was observed between P14 and P90 in control ($P < 0.001$) but not in ethanol group ($P > 0.1$). When P90 G42 brain sections from control and ethanol groups were dual-labeled with anti-GFP and anti-PV antibody (Fig. 3C), $92.9 \pm 2.2\%$ ($n = 6$) of GFP⁺ cells were PV⁺ in the barrel cortex of the control, and ethanol reduced the proportion of GFP⁺PV⁺ cells ($P = 0.02$ by Student's *t*-test) (Fig. 3D). When brain sections were dual-labeled with anti-GFP antibody and WFA (Fig. 3E), $91.3 \pm 0.9\%$ ($n = 5$) of GFP⁺ cells were WFA⁺ in the control, and ethanol reduced the proportion of GFP⁺WFA⁺ cells ($P = 0.01$) (Fig. 3F). However, when brain sections were dual-labeled with anti-GFP and anti-Cat-315 antibody (Fig. 3G), only $20.4 \pm 1.3\%$ of GFP⁺ cells in the control group were associated with Cat-315⁺ PNN, and ethanol did not decrease the percentage of GFP⁺Cat-315⁺ cells ($P = 0.340$) (Fig. 3H). In agreement with the reduction of GFP⁺ cell density in the ethanol group at P90, P7 ethanol reduced GAD67⁺ cell densities in the barrel cortex of P90 C57BL/6By mice ($P = 0.0028$ by Student's *t*-test, $n = 6$) (Fig. 3I). As shown in Table 1, the effects of ethanol on densities of PV⁺, WFA⁺, and Cat-315⁺ cells in G42 mice were similar to those of C57BL/6By mice, although PV cell densities in both control and ethanol groups were significantly lower than those of C57BL/6By mice. Because GFP expression, unlike PV, was expressed early in the first postnatal week (Chattopadhyaya et al. 2004), we evaluated ethanol-induced apoptosis in GFP⁺ cells in the barrel cortex at P7. P7 ethanol exposure in G42 mice induced caspase-3 activation (detected by anti-CC3 antibody) in $10.3 \pm 0.4\%$ ($n = 4$) of GFP⁺ cells 8 h after the ethanol exposure, indicating that some immature PV cells were acutely killed by P7 ethanol. As shown in Table 1, the total GFP⁺ cell densities in the control and ethanol groups at P7 (8 h after saline/ethanol exposure) were 25.9 ± 3.4 and 37.9 ± 3.9 ($n = 5$) cells/mm², respectively, although CC3⁺ cells were barely detected in control cells. TUNEL staining

which was detected in $88.2 \pm 2.7\%$ ($n = 3$) of CC3⁺ neurons 8 h after P7 ethanol treatment (Fig. S2) can be used as another apoptotic marker. We found that $7.9 \pm 3.3\%$ ($n = 4$) of GFP⁺ cells were TUNEL positive 8 h after P7 ethanol treatment. However, severe (about 60%) reduction of GFP⁺ cell density at P90, which was not observed at P14 (Fig. 3A,B), and the unchanged WFA⁺ cell density in ethanol-treated G42 mice at P90 (Table 1) suggest that in addition to the initial cell loss by apoptosis, there was a disturbance in development of GAD67 expression along with PV expression (Fig. 2) in subsets of PV neurons, which are likely to be surrounded by WFA⁺Cat-315[−] PNN.

PV Cell Reduction by P7 Ethanol in the Dentate Gyrus

P7 ethanol induces PV cell reduction in various regions of adult brain (Coleman et al. 2012; Sadrian et al. 2014; Smiley et al. 2015). We asked whether the effects of ethanol on PV neurons in the dentate gyrus are similar to those in the barrel cortex. In these analyses, both males and females were combined because ANOVA using 2 factors (sex and treatment) indicated that there were no significant main effects of sex or interaction (sex × treatment) for PV⁺ ($P = 0.218$ for main effect and $P = 0.287$ for interaction), for WFA⁺ ($P = 0.529$ for main effect and $P = 0.805$ for interaction) and for Cat-315⁺ ($P = 0.782$ for main effect and $P = 0.997$ for interaction) in the dentate gyrus at P90. Figure 4A shows representative images of the effects of ethanol on PV⁺ and PNN⁺ cells at P14 and P90, and Figure 4B shows the quantitative results. ANOVA using 2 factors (time and treatment) indicated that there was a significant main effect of treatment on PV⁺ cell densities [$F(1,28) = 115.1$, $P < 0.0005$, $n = 7$ for P14, and $n = 9$ for P90 groups], but no significant main effect of time or interaction. The LSD post hoc test indicated that P7 ethanol significantly reduced PV cell densities in both P14 and P90 ($P < 0.001$) (Fig. 4B). As for WFA cell density, ANOVA using 2 factors (time and treatment) indicated that there was a significant interaction [$F(1,28) = 12.6$, $P = 0.001$, $n = 7$ for P14, and $n = 9$ for P90 groups], and the LSD test showed that P7 ethanol-induced significant reduction in WFA⁺ cells detected at P90 ($P < 0.001$), but not at P14 ($P > 0.1$). Cat-315⁺ cells at P90 showed no significant differences between control and ethanol groups ($P = 0.09$, $n = 5$) (Fig. 4B). Dual labeling with PV and WFA indicated that virtually all WFA⁺ cells were PV positive both in control and ethanol-treated samples (data not shown). However, there are some PV negative cells on superficial granule cell layers, which were faintly stained with WFA in both control and ethanol-treated cells. Those cells were excluded when counting WFA⁺ cell densities. There were no GFP⁺PV⁺ cells in the dentate gyrus of G42 mice either at P14 or P90 (data not shown). While some GFP⁺ cells were found in the dentate gyrus at P14, they were PV negative and appeared to be differentiating granule cells as described previously (Cabezas et al. 2013). Thus, PV cells in the barrel cortex and the dentate gyrus were differentially affected by P7 ethanol.

Lithium Attenuated Ethanol-Induced Reduction in PV⁺ Cell Densities

We have shown previously that co-treatment with lithium attenuates ethanol-induced apoptotic neurodegeneration, glial activation, and long-term behavioral and electrophysiological deficits (Chakraborty et al. 2008; Saito et al. 2010; Sadrian et al. 2012). In the present study, the effects of co-treatment as well as delayed treatment with lithium on PV⁺ and WFA⁺ cell densities were examined (Fig. 5). Bar graphs A–H show PV or WFA

Figure 3. Densities of GFP⁺ cells in G42 mice decreased by P7 ethanol. (A) Brain sections from P90 G42 mice exposed to saline or ethanol at P7 were labeled with anti-GFP antibody. Scale bar = 200 μ m. (B) Brain sections from P14 and P90 G42 mice exposed to saline or ethanol at P7 were labeled with anti-GFP antibody, and the GFP⁺ cell densities in the barrel cortex were measured. Values (cell number/mm²) are expressed as mean $\pm$ SEM from 5–7 animals. *indicates that there was a significant ($P < 0.001$) difference between saline and ethanol groups at P90 and #indicates that there was a significant ($P < 0.001$) difference in controls between P14 and P90. (C) Brain sections from P90 G42 mice exposed to saline or ethanol at P7 were labeled with anti-GFP (green) and anti-PV antibody (red). Merged Images are shown here. Scale bar = 50 μ m. (D) Brain sections were processed as described in C, and the percent distribution of GFP⁺PV⁺ (GFP only), GFP⁺PV⁺ (PV only), and GFP⁺ + PV⁺ (GFP + PV) cells was expressed as mean $\pm$ SEM from 6 animals. *indicates that there was a significant ($P < 0.02$) difference between saline and ethanol groups in the percentage of GFP⁺ PV⁺ (GFP + PV) cells at P90. (E) Brain sections from P90 G42 mice exposed to saline or ethanol at P7 were labeled with anti-GFP (green) and WFA (red). Merged Images are shown here. (F) Brain sections were processed as described in E, and the percent distribution of GFP⁺WFA⁺ (GFP only), GFP⁺WFA⁺ (WFA only), and GFP⁺WFA⁺ (GFP + WFA) cells was expressed as mean $\pm$ SEM from 6 animals. *indicates that there was a significant ($P < 0.01$) difference between saline and ethanol groups in the percentage of GFP⁺WFA⁺ (GFP + WFA) cell densities at P90. (G) Brain sections from P90 G42 mice exposed to saline or ethanol at P7 were labeled with anti-GFP

A EtOH effects on PV⁺ and PNN⁺ cells in the dentate gyrus B EtOH effects on PV⁺ and PNN⁺ cell densities at P14 and P90 in the dentate gyrus

Figure 4. P7 ethanol reduced PV⁺ cell densities in the dentate gyrus both at P14 and P90. (A) Brain sections from P14 and P90 mice exposed to saline or ethanol at P7 were labeled with anti-PV antibody, WFA, or anti-Cat-315 antibody. The representative images show the dentate gyrus area. Scale bar = 200 μm. (B) Brain sections were prepared as described in A, and the densities of labeled PV⁺ cells, WFA⁺ cells, and Cat-315⁺ cells were measured in the dentate gyrus. Values (cell number/mm²) are expressed as mean ± SEM from 7 to 9 animals. *indicates that there were significant differences between Ctr and EtOH groups in the densities of PV⁺ at P14, PV⁺ at P90, and WFA⁺ at P90 ($P < 0.001$).

cell densities with representative images under the graphs. In Figure 5A, saline or lithium was injected 15 min after the first saline/ethanol injection as described previously (Zhong et al. 2006; Chakraborty et al. 2008; Saito et al. 2010), and PV⁺ cell densities in the barrel cortex were measured at P90. There was a statistically significant difference between groups, saline followed by saline (S/S), ethanol followed by saline (E/S), saline followed by lithium (S/Li), and ethanol followed by lithium

(E/Li) groups, as determined by one-way ANOVA [$F(3,24) = 13.40$, $P < 0.001$]. A Bonferroni post hoc test showed that E/S group cell density was significantly lower than all other groups ($P < 0.001$), and there were no significant differences between the other 3 groups, indicating that lithium reversed PV⁺ cell reduction by ethanol. Figure 5B shows the effects of the same lithium treatment on WFA⁺ cell densities in the barrel cortex at P90. One-way ANOVA indicated that there were no statistically significant differences between groups [$F(3,24) = 0.075$, $P = 0.973$]. Figure 5C shows the effects of chronic lithium (0.1 M and 0.2 M, 10 μL/g body weight) treatment which started a week after ethanol treatment and lasted for 15 days on PV⁺ cell densities in the barrel cortex at P90. For statistical analyses, data from 0.1 M and 0.2 M lithium were combined because ANOVA [treatment (saline+lithium, EtOH+lithium) × dose of lithium (0.1 M and 0.2 M)] indicated that there was no significant main effect of dose or no significant interaction between treatment and dose for either PV cell densities in the barrel cortex (main effect, $P = 0.545$; interaction $P = 0.617$) or in the dentate gyrus (main effect, $P = 0.419$; interaction, $P = 0.998$). In the barrel cortex (Fig. 5C), one-way ANOVA indicated significant differences between groups, $F(3,24) = 13.99$, $P < 0.001$, and a Bonferroni's post hoc test indicated that the E/S group was significantly different from all other groups ($P = 0.015$). The difference between the S/S group and E/Li group was almost significant ($P = 0.051$), suggesting that delayed lithium partially attenuated reduction of PV cell density by ethanol. Figure 5D shows the effects of delayed lithium on WFA⁺ cell densities in the barrel cortex at P90. ANOVA showed that there were no significant differences between groups [$F(3,24) = 1.21$, $P = 0.329$]. Figure 5E shows the effects of acute co-treatment with lithium on PV cell densities in the dentate gyrus. ANOVA indicated that there were significant differences between groups [$F(3,22) = 38.78$, $P < 0.001$] and a Bonferroni's post hoc test showed that PV cell density in the E/S group was significantly different from all other groups ($P < 0.02$). Also, E/Li group was significantly ($P < 0.02$) different from all other groups, indicating that lithium co-treatment partially attenuated ethanol-induced reduction of PV cell density. In the dentate gyrus, the reduction of the WFA⁺ cell density by P7 ethanol at P90 was also restored by P7 lithium (Fig. 5F): One-way ANOVA indicated that there were statistically significant differences between groups [$F(3,23) = 16.3$, $P < 0.001$], and E/S group was significantly ($P < 0.001$) different from all other groups by Bonferroni post hoc test, while there were no significant differences between the other 3 groups. Figure 5G shows the effects of delayed lithium treatment on PV⁺ cell densities in the dentate gyrus at P90. Although one-way ANOVA indicated significant difference between groups [$F(3,24) = 43.9$, $P < 0.001$], and E/S and E/Li groups were significantly ($P < 0.001$) different from other groups, there was no significant ($P = 1$) difference between the E/S and E/Li groups. In Figure 5H, the effects of delayed lithium on WFA cell densities in the dentate gyrus were shown. Similar to the effects on PV⁺ cell densities, ANOVA indicated significant differences between groups [$F(3,24) = 9.4$, $P < 0.001$], and E/S and E/Li groups were significantly ($P = 0.03$) different from other groups. However, there was no significant difference between the E/S and E/Li groups ($P = 1$).

(green) and anti-Cat-315 (red) antibody. Merged images are shown here. (H) Brain sections were processed as described in G, and the percent distribution of GFP⁺Cat-315⁺ (GFP only), GFP⁺Cat-315⁺ (Cat-315 only), and GFP⁺Cat-315⁺ (GFP+Cat-315) cells was expressed as mean ± SEM from 5 animals. There was no significant difference between saline and ethanol groups for the percentage of GFP⁺Cat-315⁺ (GFP+Cat-315) cells at P90 ($P = 0.340$). (I) Brain sections from P90 C57BL/6By mice exposed to saline or ethanol at P7 were labeled with anti-GAD67 antibody and cell densities were measured. Scale bar = 50 μm. Values (cell number/mm²) are expressed as mean ± SEM from 6 animals. *Significant difference between saline and ethanol groups, $P = 0.0028$.

Figure 5. The effects of lithium on P7 ethanol-induced reduction in PV⁺ cells. (A) Brain sections were obtained from P90 mice injected acutely with saline followed by saline (S/S), ethanol followed by saline (E/S), saline followed by lithium (S/Li), or ethanol followed by lithium (E/Li) at P7 as described in Materials and Methods. Sections were labeled using anti-PV antibody, and PV⁺ cell densities in the barrel cortex were compared between groups ($n = 7$). *indicates that the E/S group was significantly different from all other groups by Bonferroni's post hoc test after ANOVA ($P < 0.001$). There were no significant differences between the other 3 groups. The lower panel shows representative images of P90 mice in S/S, E/S, S/Li, and E/L groups. Scale bar = 200 μ m. The direction of each image is dorsal (top) to ventral (bottom). (B) Brain sections were obtained as described in A, and WFA⁺ cell densities in the barrel cortex were compared between groups ($n = 7$). There were no significant differences among the treatment groups by ANOVA. The lower panel shows representative images. (C) Mice injected with saline or ethanol at P7 were injected chronically with saline or lithium between P14 and P28. At P90, brain sections were labeled using anti-PV antibody, and PV cell densities in the barrel cortex were compared between groups ($n = 7$). *indicates that the E/S group was significantly different from all other groups by Bonferroni's post hoc test after ANOVA ($P = 0.015$). There were no significant differences among the other 3 groups. The lower panel shows representative images. (D) Brain sections were obtained as described in C, and WFA⁺ cell densities in the barrel cortex were compared between groups ($n = 7$). No significant differences were found among the treatment groups by ANOVA. The lower panel shows representative images. (E) Brain sections were obtained as described in A, and PV⁺ cell densities in the dentate gyrus were compared between groups ($n = 7$). *E/S and *E/Li groups were significantly ($P < 0.02$) different from all other groups by Bonferroni's post hoc test after ANOVA. The lower panel shows representative images. (F) Brain sections were obtained as described in A, and WFA⁺ cell densities in the dentate gyrus were compared between groups ($n = 7$). *indicates that the E/S group was significantly different from all other groups by Bonferroni's post hoc test after ANOVA ($P < 0.003$). The lower panel shows representative

Thus, our data indicated that co-treatment with lithium attenuated ethanol-induced decrease in the PV⁺ cell density in both barrel cortex and dentate gyrus, while the delayed chronic lithium treatment partially restored PV⁺ cell density only in the barrel cortex. Neither co-treatment nor delayed lithium treatment affected WFA⁺ cell densities significantly in the barrel cortex, while co-treatment, but not delayed lithium treatment, attenuated ethanol-induced reduction of WFA⁺ cell densities in the dentate gyrus.

Discussion

The present study indicates that ethanol exposure in neonatal mice, used as a model for FASD, affects development of PV neurons in the barrel cortex and the dentate gyrus, but the mechanisms may be different in these brain regions.

In agreement with our previous studies showing that ethanol exposure in P7 mice preferentially reduces the cell density of GABAergic neurons in the P90 mouse cortex (Smiley et al. 2015), P7 ethanol reduced the PV⁺ cell density by 25% in the P90 barrel cortex (Fig. 2A). Such decrease in PV neurons in the adult brain may partially be due to acute ethanol-induced apoptotic cell death, because we found that caspase-3 was activated and TUNEL staining was positive 8 h after P7 ethanol exposure in 8–10% of GFP⁺ cells in G42 mice that selectively express GFP in a subclass of PV interneurons and their immature forms (Chattopadhyaya et al. 2004). However, GFP⁺ cell number was drastically (~60%) reduced at P90 despite the lack of reduction at P14 (Fig. 3B). Also, the density of PNN⁺ cells, which were detected by WFA and/or anti-Cat-315 antibody, and more than 99% of which were co-localized with PV⁺ cells in the control group (Fig. 2D,F), did not significantly decrease at P90 by P7 ethanol treatment (Fig. 2A). Instead, P7 ethanol increased WFA⁺ cells with faint or no PV expression at P90 (Fig. 2C,D). These results and the data indicating no significant difference in the PV⁺ cell density between control and ethanol groups at P14 (Fig. 2A) suggest that PV⁺ cell reduction is partially due to the delayed down-regulation of PV expression during the maturation of PV neurons, in addition to acute cell death, although more precise characterization of WFA⁺PV[−] cells may be necessary. Results showing that lithium treatment 7 days after P7-induced apoptosis partially restored the PV cell density supports the notion that delayed lithium may enhance normal PV cell maturation in addition to the acute anti-apoptotic effects. It has been reported that lithium exerts neuroprotection in animal models of neurodegenerative diseases, neurodevelopmental disorders, and acute brain injuries by various mechanisms, such as preventing apoptosis, increasing neurotrophins and cell-survival molecules, modulating inflammatory molecules, and enhancing neurogenesis (Forlenza et al. 2014; Leeds et al. 2014; Lazzara and Kim 2015; Dell'Osso et al. 2016). While, it is believed that anti-apoptotic effects of lithium are mainly due to its inhibitory action on glycogen synthase kinase-3 (GSK-3) (Leeds et al., 2014; Dell'Osso et al. 2016), mechanisms behind other effects of lithium have not been well-elucidated. The beneficial effects of delayed lithium treatment may also be exerted by inhibition of secondary (delayed) apoptosis as suggested in the case of neonatal rat cerebral hypoxia-ischemia model

(Xie et al. 2014) or adult rat ischemic reperfusion injury model (Taliyan and Ramagiri 2016). Lithium may also support normal brain development through enhanced production of neurotrophic factors, such as BDNF that is known to play a critical role in cortical development, synaptic plasticity, neuronal differentiation, and survival (Dell'Osso et al. 2016). BDNF may control differentiation/maturation of PV neurons as demonstrated in fast-spiking cell culture (Berghuis et al. 2004) and in the developing mouse barrel cortex (Itami et al. 2007).

It has been demonstrated that PV cell loss and reduction in PV expression have opposing effects on neural activity in the cortex. While PV cell loss enhances excitatory activity, the decrease in PV expression enhances inhibitory activity (Filice et al. 2016). In our experiments, however, GFP⁺ cell density in P90 G42 mice and GAD67⁺ cell density in P90 C57BL/6By mice were reduced by 60% and 35%, respectively, by P7 ethanol (Fig. 3B,I), indicating that not only PV but also GAD67 expression was disturbed. Since GAD67 expression appears to be directly correlated with inhibitory activity (Lazarus et al. 2015), P7 ethanol-induced decrease in PV and GAD67 expression is likely to result in enhanced excitatory activity, which is consistent with our previous electrophysiological findings (Wilson et al. 2011; Sadrian et al. 2014).

Our studies further indicated that there were different populations of PV⁺ cells in the adult barrel cortex; PV cells surrounded by PNN detected by WFA (PV⁺WFA⁺) and PV cells without PNN (PV⁺WFA[−]) (Fig. 1). PV⁺WFA⁺ cells were further divided to PV⁺WFA⁺Cat-315⁺ cells and PV⁺WFA⁺Cat-315[−] cells (Fig. 1). While we found that P7 ethanol generated WFA⁺ cells with undetectable or very low levels of PV at P90 (Fig. 2C,D), there were very few Cat-315⁺PV[−] cells (Fig. 2E,F), suggesting that Cat-315⁺ cells or the immature Cat-315⁺ cells are more resistant to P7 ethanol. This may be related to the observation that P7 ethanol decreased GFP⁺ cells, approximately 75% of which were Cat-315 negative (Fig. 3H), more strikingly than PV⁺ cells (Fig. 3A,B). It is interesting that Cat-315 was hardly expressed at P14, while WFA⁺ cells were already abundant at this age (Fig. 1B,C). This may suggest that PV⁺ WFA⁺Cat-315⁺ cells are more mature than PV⁺ WFA⁺Cat-315[−] cells, or early developed PV⁺WFA⁺Cat-315[−] cells and late developed PV⁺WFA⁺Cat-315⁺ were derived from genetically different precursors. It has been reported that PNN expression may have an important and specific role in activity-dependent plasticity in the rodent somatosensory cortex, and the completion of PNN and PV maturation corresponds to the end of critical period for plasticity in that circuit. Specifically, Cat-315⁺ cells seem to be more involved in the activity-dependent plasticity because the sensory deprivation of barrel cortex reduced Cat-315⁺ cells but not WFA⁺ cells (McRae et al. 2007). Clarification of subgroups of PV neurons, which are sensitive to early ethanol exposure, needs further investigation.

PV cell reduction has been reported in various developmental diseases, and down-regulation in PV expression rather than cell loss similar to our study has been reported in rodent models of hypoxia (Komitova et al. 2013) and schizophrenia (Tseng et al. 2009; Powell et al. 2012) and human schizophrenics (Enwright et al. 2016). Also, PV down-regulation with unchanged PNN expression has been reported in a model of

images. (G) Brain sections were obtained as described in C, and PV cell densities in the dentate gyrus were compared between groups. *The E/S and E/Li groups were significantly different from S/S and S/Li groups ($P < 0.001$), while there was no difference between E/S and E/Li groups ($P = 1$) by Bonferroni's post hoc test after ANOVA. The lower panel shows representative images. (H) Brain sections were obtained as described in C, and WFA cell densities in the dentate gyrus were compared between groups. *The E/S and E/Li groups were significantly different from S/S and S/Li groups ($P < 0.001$), while there was no difference between E/S and E/L group ($P = 1$) by Bonferroni's post hoc test after ANOVA. The lower panel shows representative images.

autism (Filice et al. 2016). Decreased PV and GAD67 expression is also reported in animal models of schizophrenia using ketamine (Behrens et al. 2007).

It is yet unknown how delayed reduction of PV and GAD67 expression occurs following developmental stressors including ethanol. In the present study, P7 ethanol did not induce reduction of PV or GFP cells at P14 despite some cell death at P7, and there is a significant increase in WFA⁺ cells in the layer 4 and 5 of the barrel cortex of C57BL/6By mice (Fig. 2B). While elevation in the density of GFP⁺ cells or WFA⁺ cells observed at P14 may not reflect increases in the total number of these cells in barrel cortex because of the possible reduction of cortex volume by P7 ethanol (Ikonomidou et al. 2000; Smiley et al. 2015), it is possible that ethanol causes precocious maturation of PNNs, resulting in no reduction or some increase in PV⁺/GFP⁺ cells at P14, but less increase in PV cells later between P14 and P90 because of the disruption of normal PV neuron development. CSPG, which is the major component of PNN, is produced partly by astrocytes (Faissner et al. 2010), as also suggested in the present study by dual labeling using WFA and glutamine synthetase antibody at P7 (Fig. S1), and it has been indicated that ethanol inhibits arylsulfatase B activity and increases CSPG levels in astrocytes, which may lead to altered brain connectivity and decrease in neuronal plasticity (Zhang et al. 2014). P7 ethanol-induced astrocyte activation observed in our previous study (Saito et al. 2015) may also affect CSPG levels. It is also possible that PV⁺ cell reduction is caused by disturbance in NMDA signaling known to be triggered by ethanol (Hoffman et al. 1989; Nagy 2004), because PV⁺ cell development is dependent upon excitatory neuronal input and activity-dependent neurotrophin signaling (Patz et al. 2004). In line with this, treatment of neonatal animals with the NMDA receptor antagonist ketamine results in decreased PV immunoreactivity with maintained interneuron numbers (Powell et al. 2012). It has been also reported that ethanol exposure in the developing brain induces epigenetic modification, specifically changes in DNA methylation (Basavarajappa and Subbanna 2016; Laufer et al. 2017; Mandal et al. 2017), which may lead to deficits in PV cell maturation. Finally, schizophrenia has been associated with a modification in expression of transcription factors and co-activators involved in the developmental regulators of cortical PV neurons (Volk et al. 2016), providing another target of ethanol-induced change in these cells.

In contrast to the barrel cortex, P7 ethanol induced strong reduction in PV⁺ cell densities in the dentate gyrus of both P14 and P90 mice (Fig. 4). Also, WFA⁺ cell density was decreased at P90 (Fig. 4). It is possible that these PV neurons or immature PV neurons were lost immediately after ethanol treatment by acute apoptotic neurodegeneration instead of prolonged cell loss or disturbance in PV neuron maturation. In agreement with the notion, while acute lithium treatment attenuated PV⁺ cell density reduction in the dentate gyrus at P90 (Fig. 5B), delayed chronic lithium did not show significant effects on PV⁺ cell densities (Fig. 5E). PNNs show regional variations in their components, amounts, and the developmental timing of the expression (Seeger et al. 1994; Lensjo et al. 2017a), and the differences in PNN expression in the cortex and hippocampus have been reported (Lensjo et al. 2017a; Lensjo et al. 2017b). Also, in our experiments, the expression pattern of PNN in the dentate gyrus was different from that in the barrel cortex. These regional developmental or molecular differences in PNNs may confer differences in the effects of P7 ethanol on immature PV cells in these regions, because PNNs show vast effects, including neuroprotective effects (Cabungcal et al.

2013), on PV neurons. Although some cleaved caspase-3⁺ cells are detected in the dentate gyrus 8 h after P7 ethanol exposure (data not shown), whether these were PV⁺ cells (immature PV cells) or not could not be determined in the present study because the PV expression was low at this age, and because GFP⁺ cells in the dentate gyrus of P7 G42 mice appeared to be granule cells at a certain stage of development as previously reported (Cabezas et al. 2013).

One of the limitations of our current study is the use of G42 mice. Because GFP⁺ cells in G42 mice represent only a subgroup of PV⁺ cells, findings obtained from GFP⁺ cells may not be applicable to other subgroups of PV⁺ cells, although this feature may be useful once the nature of GFP⁺PV⁺ cells is clarified. Also, as shown in Figure 3B, the density of GFP⁺ cells increased even after P14 in controls. To clarify the PV cell fate after P7 ethanol treatment, genetic lineage tracing studies using Cre-driver for genes expressed in PV cell precursors before P7 and diminished thereafter, such as Nkx2.1 (Xu et al. 2008; Taniguchi et al. 2011), may be important. The use of such mice may clarify whether WFA⁺PV⁺ cells observed in P7 ethanol-treated mice are immature/dysregulated PV cells or not, which is difficult to prove decisively in the current study.

Thus, while further investigation is necessary, our studies suggest that P7 ethanol disrupts PV cell maturation in the adult barrel cortex, which may be rescued by delayed chronic lithium treatment.

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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Notes

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