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Electrophysiological and Immunohistochemical Evidence for an Increase in GABAergic Inputs and HCN Channels in Purkinje Cells that Survive Developmental Ethanol Exposure

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Abstract

Ethanol exposures during the early postnatal period of the rat result in significant death of Purkinje cells (PCs). The magnitude, time-course, and lobular specificity of PC death have been well characterized in several studies. Additionally, significant reduction of climbing fiber inputs to the surviving PCs has been characterized. This study investigates whether further alterations to the cerebellar cortical circuits might occur as a result of developmental ethanol exposures. We first examined the firing pattern of PCs in acute slice preparations on postnatal days 13–15. While the basic firing frequency was not significantly altered, PCs from rat pups treated with ethanol on postnatal days 4–6 showed a significantly increased number of inhibitory postsynaptic potentials (IPSCs) and a larger I_h current. We conducted immunofluorescent studies to identify the probable cause of the increased IPSCs. We found a significant 21 % increase in the number of basket cells per PC and a near doubling of the volume of colocalized basket cell axonal membrane with PC. In addition, we identified a significant (~147 %) increase in HCN1 channel volume co-localized to PC volume. Therefore, the cerebellar cortex that survives targeted postnatal ethanol exposure is dramatically altered in development subsequent to PC death. The cerebellar cortical circuit that results is one that operates under a significant degree of increased resting inhibition. The alterations in the development of cerebellar circuitry following ethanol exposure, and the significant loss of PCs, could result in modifications of the structure and function of other brain regions that receive cerebellar inputs.

Keywords

Purkinje cells; Ethanol; Postnatal; Rat; Cerebellum; Basket cells; HCN1 channels

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Conflicts of Interest The authors have no conflicts to declare.

Introduction

Binge drinking by nonpregnant women is fairly common. The National Survey on Drug Use and Health reports that 32.6 % of nonpregnant women engage in binge alcohol use, defined as drinking five or more drinks at the same time or within a couple of hours [1]. However, these numbers drop dramatically when women are pregnant as only 1 % of pregnant women engage in binge drinking activities during the third trimester [2]. In the USA alone, this percentage translates into 40,000 to 60,000 children per year who were exposed to high levels of ethanol during the brain growth spurt.

An obvious solution to this dilemma is to abstain from ethanol exposure during fetal development. But this has not proven to be an achievable task. In fact, it was not until 2013 that Fetal Alcohol Spectrum Disorder (FASD) was officially recognized as a medical diagnosis and included as an appendix to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders [3]. In addition, although a wide range of estimates exist, the prevalence or frequency of FASD has not changed substantially over time and evidence suggests it may actually be increasing despite campaigns advising women to refrain from alcohol consumption during pregnancy [4].

Ethanol exposure during the development of the brain can be extremely damaging to neurons of the central nervous system. Heavy ethanol exposure disrupts the normal process of brain development, and as a result, various behaviors are affected ranging from motor control to cognitive deficits such as spatial working memory and executive functioning [4–8]. In particular, the human cerebellum is a region that exhibits relatively high vulnerability to developmental ethanol insults. Deficits in motor skills, including impaired performance of fine motor control involving speed, coordination, and balance can result from gestational ethanol exposure [9–11]. Various studies have reported disproportionate size reductions in the cerebellum, and alterations in white matter tracts and deep cerebellar nuclei [12–18]. Eyeblick classical conditioning, a simple form of motor learning that involves specific cerebellar structures [19], is also adversely affected by developmental ethanol exposure [20, 21].

The development of the rat brain involves stages similar to those of the human brain even though the rat brain at birth is immature compared to the human brain at birth [22–24]. Consequently, the first 10 postnatal days in the rat is a period of rapid brain growth similar to the human brain development during the third trimester [22–24]. Thus, ethanol exposure in the rat during postnatal days (PN) 4–6 is equivalent to ethanol exposure during the early third trimester in humans. The brain growth spurt during the third trimester of development is a critical period of brain development. One of the common patterns of ethanol abuse in pregnant women is abstinence during the first two trimesters followed by ethanol consumption during the third trimester [25].

Purkinje Cells and Ethanol Exposure

It is well documented that Purkinje cells (PCs) of the cerebellum can be killed by targeted developmental ethanol exposure. Studies in the rat have demonstrated significant loss of

these neurons caused by ethanol exposure on PN 4–6, with the amount of neuronal loss directly proportional to the blood ethanol concentration [26–30]. PCs are formed in the rat during gestational days 13–16 and are not mitotic after this neurogenesis period [31, 32]. Thus, when PCs die during the postnatal period, the loss is permanent. Interestingly, the window of vulnerability for PCs has a precise temporal boundary in that there is very little PC loss from ethanol exposures on PN 7 or later [30, 33, 34]. Additionally, although a recent study reported significant ethanol-induced loss of PCs following exposure on PN 0 or PN 2 [35], the general findings have not identified ethanol-specific PC loss before PN 4 [30, 36]. A single dose of ethanol was sufficient to produce the developmental loss of PCs which die by apoptosis triggered by the caspase cascade [27, 37, 38].

In addition to causing PC loss, developmental ethanol exposure causes depletion of a relatively larger proportion of cerebellar granule cells which may have greater sensitivity to ethanol than PCs (reviewed by [39]). However, our study focuses on PCs rather than the more abundant and frequently studied cerebellar granule cells because of the fact that the PCs are the only output neuron of the cerebellar cortex and because of the fact that granule cells remain mitotically active throughout most of the third trimester equivalent period [31]. As a result, although ethanol exposure during the early postnatal period causes a decrease in granule cell numbers compared to controls, the proportional balance between granule cells and PCs is maintained [40–44]. As stated above, PCs are not mitotically active during the postnatal period so their loss will not result in replacement.

The Cerebellum Afterward

Since the specific vulnerable window for the ethanol-induced loss of PCs has been determined and the time-course and manner of cell death have been characterized, our interest shifted to identifying the impact of this substantial neuronal loss on the subsequent development of the cerebellar cortex. An important goal for our laboratory has been to understand what happens to the maturation of the PCs and related neurons that have survived targeted postnatal (third trimester equivalent of human pregnancy) ethanol exposure. Our goal is to take advantage of the knowledge gained from this approach to develop new strategies for ameliorative treatments.

Climbing Fiber Alterations

An initial area of interest was the climbing fiber innervation to the PC from the contralateral inferior olive. The climbing fibers have a critical role in the proper functioning of the cerebellar circuitry, yet they comprise only 1–2 % of the synapses on the PC [45–47]. We hypothesized that developmental ethanol exposure would negatively impact the establishment of synaptic contacts by climbing fibers onto PCs [34, 48]. We tested this hypothesis by using immunostaining for vesicular glutamate transporter 2 (VGluT2) to identify climbing fiber synapses in Lobule I [45–47] and calbindin-D28k to identify PCs [37]. Analyses were conducted at PN 14 to monitor the development of the climbing fiber synapses when they first contact the PC dendritic tree, then also at PN 40, a more mature stage of development.

The VGluT2 immunostaining was significantly reduced by the PN 4 and PN 4–6 but not PN7–9 exposures when the peak blood ethanol concentration was 365 ± 39 mg/dL [34, 48]. Additionally, we identified that the surviving neurons from these two exposure groups also had significantly fewer VGluT2-labeled synapses per PC when compared to controls [34, 48]. These effects were both dose- and temporally dependent, and a single ethanol exposure proved sufficient to cause alterations in the synaptic integrity. The main effect of developmental ethanol exposure, as noted, was that the ratio of climbing fiber synapses per PC was significantly reduced, both at PN 14 and PN 40 [34, 48].

A logical question that arises is whether the basic electrophysiology of the surviving PC has been also affected by the treatment. There have been few studies, especially from live tissue, evaluating the electrophysiological characteristics of PCs subsequent to postnatal ethanol exposure. One study reported data from extracellular recordings from PCs of adult animals that had been exposed to ethanol on PN 4–10 [49]. These animals received a total dose of 4.5 g/kg/day ethanol distributed in two consecutive feedings 2 h apart each day. The recordings were done in vivo with the animals under urethane anesthesia. In contrast to our findings regarding climbing fiber synapses, these authors identified no differences in the spontaneous activity or firing pattern of the PCs. However, although the authors reported difficulties identifying PCs by their complex spiking feature, they identified a significant decrease within the ethanol-exposed animals in the proportion of PCs generating complex spike bursts, indicating alterations in the climbing fiber input to the PCs [49]. We believe that the lack of complex spikes in PCs of ethanol-treated rats as reported by this study could be explained by the ongoing presence of urethane anesthesia as well as a decrease in the climbing fiber innervation of PCs resulting from the ethanol exposure [34, 48].

Another study utilized patch-clamp electrophysiology in vermal slices from the rat cerebellum following ethanol exposure from PN 2–12 [50]. A vapor inhalation technique was used to produce pup serum ethanol concentrations of approximately 160 mg/dL. PC recordings did not indicate any changes in the characteristics of the climbing fiber-evoked complex spike or the climbing fiber-induced long-term depression, when the animals were tested at PN 15–17 or PN 21–34. These results are really not surprising since the dose equivalent of ethanol exposure, as reflected in the serum ethanol level, was comparatively modest (160 mg/dL). Our studies of climbing fiber innervation to PCs on PN 14 also did not indicate a significant difference at a dose of 3.6 g/kg on PN 4–6 resulting in blood ethanol concentrations of 242 mg/dL [34].

Although we have accumulated a wealth of knowledge about the cellular and molecular mechanisms of acute ethanol toxicity (as reviewed by [51]), there are relatively few studies that have addressed the cellular mechanisms of the long-lasting effects of developmental ethanol exposure. In this study, our laboratory has investigated how GABAergic signaling in the cerebellum was affected by developmental ethanol exposure. We found that the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) was significantly higher in ethanol-treated rats compared to control rats. This result suggests that PCs in ethanol-treated rats may receive more inhibitory synapses. Additional experiments using immunofluorescence supported this interpretation and showed that the volume of basket cell (BC) axons that were in close proximity to PCs, such that their voxels of expression co-

localized, were greater on a per PC basis in ethanol-treated rats. This is in agreement with our electrophysiological findings and supports the hypothesis that PCs surviving ethanol exposure are under a greater inhibitory influence than controls. Furthermore, we also report that PCs from ethanol-treated rats show significantly greater amplitude of the I_h current compared to controls and immunofluorescence identification of the HCN1 (hyperpolarization-activated cyclic nucleotide-gated cation) protein support this finding. A schematic diagram summarizing the major changes in the cerebellar circuits that occur after developmental ethanol exposure is shown in Fig. 1.

Materials and Methods

Animal Model

The third trimester equivalent period in rats is the first 10 postnatal days [22, 24]. In our model, we expose the rat pups to ethanol on PN 4–6. Timed-pregnant Sprague-Dawley rats (Harlan, Inc.) delivered pups (designated PN 0) in the animal facilities at the University of Arkansas for Medical Sciences (UAMS) Division of Laboratory Animal Medicine. All experiments described below were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of UAMS prior to initiation of experimentation.

Ethanol (4.5 g/kg body weight) was administered as a single daily dose via intragastric intubation of a 15 % (w/v) ethanol solution in Intralipid-II® [37, 52]. Sham-intubated (SI) pups that receive the intragastric intubation without delivery of any solution serve as controls in this study.

Experiment 1: Electrophysiology of Surviving PCs

Procedure—This experiment was designed to characterize the basic electrophysiological properties of rat PCs from Lobule I of cerebellar vermal slices. Rat pups of either sex were randomly assigned to either controls or ethanol-treated groups. The ethanol-treated animals received 4.5 g/kg ethanol dose once daily for PN 4–6, utilizing intragastric intubations of 15 % (w/v) ethanol solution in Intralipid-II®. Sham-intubated rat pups served as controls.

Electrophysiology—At PN 13–15, the pups were decapitated and the cerebellum was removed and immersed in sucrose-artificial cerebrospinal fluid (sucrose-aCSF) equilibrated with 95 % O₂–5 % CO₂ (pH=7.38; composition, in mM: 26 NaHCO₃, 1 NaH₂PO₄, 2 KCl, 7 MgCl₂, 0.5 CaCl₂, 20 glucose, 0.4 ascorbic acid, 2 sodium pyruvate, and 234 sucrose). Parasagittal cerebellar slices (400 μm thick) were cut with a Vibratome 3000 (Vibratome, St. Louis, MO). The slices were allowed a 15-min period of recovery at 30 °C and then incubated at room temperature (22 °C) in aCSF equilibrated with 95 % O₂–5 % CO₂ (composition in mM: 124 NaCl, 26 NaHCO₃, 3 KCl, 2 MgCl₂, 2 CaCl₂, 0.4 ascorbic acid, 2 sodium pyruvate, and 20 glucose). For recording, a single slice was placed in a recording chamber that was continuously perfused at the rate of 1.5 mL/min with aCSF equilibrated with 95 % O₂–5 % CO₂. All recordings were performed at 30 °C. Neurons were visualized

using an upright microscope (Olympus BX50WI, Tokyo, Japan) equipped for epifluorescence.

Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm o.d., World Precision Instruments, Sarasota, FL) on a pipette puller (P-97, Sutter Instrument Company, Novato, CA). Extracellular recordings were obtained in the loose-patch configuration using patch pipettes (6–8 M Ω) filled with aCSF. To avoid mechanical stress effects on firing rate, we used pipettes that were preexposed to brain tissue to avoid direct apposition between the membrane and the pipette glass [53]. Extracellular spikes were recorded as action currents in voltage clamp mode at a holding potential of 0 mV and using a high-pass filter of 0.3–1 Hz. For intracellular recordings, patch pipettes (6–8 M Ω) were filled with a solution of the following composition (in mM): 125 potassium gluconate, 1 NaCl, 10 phosphocreatine ditris salt, 4 MgATP, 0.3 GTP, 0.5 EGTA, and 10 HEPES (pH 7.3 with NaOH, osmolarity 290 mOsm). The junction potential was 9–10 mV, and all reported voltage measurements were uncorrected for these potentials. Only neurons with access resistance <30 M Ω were included in this study. No series resistance compensation was performed. In all experiments, 0.02 % Lucifer Yellow was added to the intracellular solution for in situ labeling. The identity of PCs was confirmed by taking photos of Lucifer Yellow-labeled cells using an 8 megapixel digital camera (Nikon, Coolpix 8700) connected to the microscope eyepiece via an adapter (MM99–5700, Martin Microscope Company, Easley, SC).

Data Analysis—Recordings were made using a Multiclamp-700B amplifier and the analog signals were low-pass filtered at 2 kHz, and digitized at 5 kHz using a Digidata-1440A interface and pClamp10 software (Molecular Devices, Sunnyvale, CA). The detection of events was performed offline using Mini analysis program (Synaptosoft Inc., Decatur, GA; for details, see [54]). The times of occurrence of events were imported into Origin 7.0 (Microcal Software Inc., Northampton, MA) for further analysis using algorithms written in LabTalk. Data, expressed as mean $\pm$ SEM, were analyzed for significant differences using paired *t* test (Origin 7.0 software) unless otherwise stated.

We first sought to characterize the pattern of discharge of spontaneously active PCs. For this purpose, we used loose-patch extracellular recordings because in this noninvasive configuration, the spontaneous firing activity is stable and can be monitored for prolonged periods of time without rundown as in the whole-cell configuration [55, 56]. We then examined, using whole-cell patch clamping, the intrinsic and synaptic currents that modulate their firing activity. For firing pattern analysis, extracellular recordings were collected from five control animals and two ethanol-treated animals. For I_h and IPSC analysis, patch-clamp recordings were collected from three control animals and four ethanol-treated animals.

Experiment 2: Basket Cell Innervation of Surviving PCs

Procedure—This experiment was designed to evaluate the relative population numbers of rat PCs and BCs from Lobule I of cerebellar vermal slices as well as the ratio of BC axonal structures to PCs. Rat pups of either sex were randomly assigned to either controls or ethanol-treated groups. The ethanol-treated animals were fed 4.5 g/kg of ethanol once daily

for PN 4–6, utilizing intragastric intubations of 15 % (w/v) ethanol solution in Intralipid-II®. Sham-intubated rat pups served as controls.

Histology—Control ($n=12$) and E 4–6 ($n=12$) rat pups were anesthetized with pentobarbital on PN 23–25 and fixed by perfusion with 2 % paraformaldehyde-lysine phosphate. Sagittal slices of the cerebellar vermis were sectioned at 60- μ m thickness and processed for dual immunostaining in a free-floating configuration. Antibody to calbindin-D28k was used to identify PCs, and antibody to parvalbumin (MAB1572; Millipore, Inc.) was used since it is reported to label BCs, Stellate cells (SCs), as well as PCs [57] within the cerebellum. Since the PCs will be labeled with both red and green fluorescence, the selective identification of BCs and SCs relies on their reaction only to the anti-parvalbumin. Positive anti-parvalbumin neurons were identified as BCs or SCs if their position was within the proximal one-third or the distal two-thirds of the molecular layer, respectively [58]. For these studies, one 60- μ m slice from each animal was analyzed.

In order to obtain a more robust measure of the number of inhibitory synapses on the soma of PCs surviving E 4–6 exposure, we used an antibody (ab17126, Abcam Inc.) to the 200 kD Neurofilament Heavy protein (NF) that is reported to selectively label axonal structures of BCs within the cerebellum [59]. One sagittal slice (60 μ m) of the cerebellar vermis from each pup cerebellum (control $n=13$; E 4–6 $n=13$) was processed for dual immunostaining in a free-floating configuration along with the calbindin-D28k. Confocal image stacks (20 $\times$) having a size of 450 μ m $\times$ 450 μ m $\times$ ~40 μ m (z-axis), were obtained from a single layer of PCs along the bank region of Lobule I adjacent to Lobule II. These image stacks were reconstructed for quantitative analysis using the Imaris software package (Bitplane AG, Zurich).

Data Analysis—For the quantification of BC and SC numbers, the entire image was utilized and a demarcation line was drawn to separate the proximal one-third from the distal two-thirds of the molecular layer. Molecular layer neurons were counted as BCs if they resided within the proximal region and as SCs if they were located within the distal two-thirds region [58]. PCs were easily identified and counted based on calbindin-D28k positive labeling.

In order to analyze the immunostaining of NF which represents the axons of BCs, we designated a specific region of interest (ROI) that included the volume between the interior point just below the pinceau and the proximal one-third of the molecular layer. We constructed an isosurface of both individual channels as well as the co-localized channel (CoLoc) that represents the confluence of BC axons with PC elements. The numbers of PCs were counted in each slice and the BC axon and Co-Loc volumes were expressed on a per PC basis to account for the ethanol-induced loss of PCs.

Experiment 3: HCN1 Expression at Basket Cell Innervation of PCs

Procedure—This experiment was designed to look at the HCN1 expression at the BC innervation of PCs from Lobule I of cerebellar vermal slices. Rat pups of either sex were randomly assigned to either controls or ethanol-treated groups. Rat pups ($n=12$) were treated

with a daily dose (4.5 g/kg) of ethanol (E 4–6) delivered by intragastric intubation of a 15 % (w/v) ethanol solution in Intralipid-II on PN 4–6. Control animals ($n=9$) received SI.

Histology—For rats between PN 41 and 48, the animals were anesthetized with pentobarbital and perfused with 2 % paraformaldehyde-lysine-periodate (PLP). Mid-vermal sections (60 μ m) from the cerebellum were cut with a cryostat and concurrently stained using a free-floating configuration with an antibody to HCN1[60] (gift of Dane Chetkovich, MD/PhD, Northwestern University Institute of Neuroscience) and an antibody to calbindin-D28k (to visualize PCs). For these studies, one 60- μ m slice from each animal was analyzed.

Data Analysis—For the analysis of HCN1 channel expression, the ROI included the soma and the area just interior to the soma where the pinceau was identified. The isosurface for calbindin-D28k expression representing PCs soma and the initial axon segment as well as HCN1 channel expression were identified. The Imaris software allowed the construction of a co-localized channel (CoLoc) that includes only those voxels that contained both red and green fluorescence. We have interpreted the co-localized end points as representing the portion of the HCN1 channel expressing tissue that made putative functional contacts with PCs.

Three end points were evaluated; all of them were related to the total volume of expression: the calbindin-D28k soma (S) volume per PC (CalB-S Vol/PC); the HCN1 soma (S) volume per PC (HCN1-S Vol/PC), and the co-localized soma volume (CoLoc-S Vol/PC). All end points were expressed on a per PC basis to normalize to the reduced number of PCs that result from E 4–6 exposure.

Results

Experiment 1: Electrophysiology of Surviving PCs

PCs undergo continuous changes in their morphological and electrophysiological properties and synaptic connectivity throughout development [61, 62]. To reduce the variability in the electrophysiological properties due to developmental changes, we restricted our recordings (Fig. 2) to PN 13–15 and to Lobule I since this portion of the cerebellum demonstrated the greatest reduction of PCs following postnatal ethanol exposure [26, 63, 64]. We first sought to characterize the pattern of discharge of spontaneously active PCs and to examine how inhibitory and excitatory inputs modulate their firing activity. For this purpose, we used loose-patch extracellular recordings because in this noninvasive configuration, the spontaneous firing activity is stable and can be monitored for prolonged periods of time without running down as in whole-cell configuration [55, 65].

The mean firing frequency of PCs (calculated over 6–8min of recording) was not significantly different ($p=0.98$, unpaired t test) in ethanol-treated rats (34.2 ± 2.3 Hz, $n=18$, range 16– 55 Hz) compared to control rats (34.2 ± 4.6 Hz, $n=19$, range 7–81 Hz). The “ n ” for reporting this data is the actual number of PCs obtained from the cerebellar slices of the number of pups identified in the methods section. The mean, median, standard deviation, and coefficient of variation of the interspike interval (ISIs) were also not statistically different ($p=0.23$, 0.11, 0.22, and 0.44, respectively, unpaired t test). Additional analysis on

the regularity of firing reveals the mean mode (interspike interval at the peak of distribution) of the ISIs was nonsignificantly shorter ($p=0.06$) in ethanol-treated rats (23 ± 1.6 ms, $n=18$, range 12–37 ms) compared to control rats (29 ± 2.8 ms, $n=19$, range 9–57 ms). Even though it does not meet statistical significance, this result suggests that the preferred firing frequency of PCs may be slightly higher in ethanol-treated vs. control rats. In both control and ethanol-treated rats, the ISI histograms (Fig. 2) demonstrated a skewed distribution.

A small proportion of cells (6 of 15 PCs from control animals and 4 of 18 cells from ethanol-treated animals) exhibited spike bursts occurring at regular intervals (0.3–1.5 bursts/s) and separated by a pause (>200 ms). This bursting was identifiable by the presence of a bimodal distribution in the histogram of ISIs (although the second peak was quite small compared to the first). To further quantify this firing pattern, we defined a new parameter called “Asymmetry Index” as the ratio of the number of ISIs that were longer than the mode, divided by the number that were shorter than the mode. The asymmetry index was significantly larger ($p=0.0049$) in PCs from ethanol-treated (2.74 ± 0.19) compared to control (1.97 ± 0.17) pups. This indicates that the ISI distribution in ethanol-treated rats was more skewed than control rats because the PCs surviving ethanol treatment have a relatively higher proportion of longer ISIs than shorter ISIs. These relatively longer ISIs, or pauses, are most likely caused by inhibitory synaptic inputs as has been shown previously in vitro [66] and in vivo [67, 68]

Figure 3 shows the frequency of spontaneous IPSCs from PCs of the ethanol-treated and control rats (mean $\pm$ SEM). Ethanol exposure resulted in a significant ($p=0.0004$; unpaired t test) increase in the IPSCs frequency (20.6 ± 1.5 Hz; $n=14$) compared to control rats (10.8 ± 1.9 Hz; $n=13$). This suggests that PCs in ethanol-treated rats may receive more inhibitory synapses or that the inhibitory synapses have a higher probability of releasing GABA and this result is consistent with the skewed distribution evident in the frequency histograms of Fig. 2.

Figure 4 shows that PCs from ethanol-treated rats have a significantly greater ($p<0.01$, unpaired t test) amplitude of the hyperpolarization-activated inward current (I_h) generated by -50 mV hyperpolarizing steps (control: 368 ± 19 pA $n=5$; ethanol-treated: 563 ± 45 pA, $n=7$; $p<0.01$; mean $\pm$ SEM) In contrast, the input resistance in control (42 ± 5 M Ω ; $n=5$) was not significantly different ($p=0.27$, unpaired t test) from ethanol-treated rats (36 ± 4 M Ω ; $n=7$). Because I_h has been shown to contribute to the pacemaker activity of many neurons, the presence of a relatively larger I_h in PCs of ethanol-treated animals may explain, at least in part, the different pattern of PC firing. The time constant of the I_h current in controls (70 ± 10 ms; $n=5$) was not significantly different ($p=0.97$, unpaired t test) from ethanol-treated rats (69 ± 16 ms; $n=7$), suggesting that I_h channels have the same kinetic properties (and maybe the subunit composition) in both groups of rats. The increase in I_h in PCs is consistent with a study showing that acute ethanol administration increased the firing of hippocampal interneurons by enhancing I_h and this effect was more pronounced in younger animals [69]. It has also been suggested that the acute ethanol-induced increase in firing of PCs, in the presence of glutamatergic and GABAergic receptor blockers [70], could be due in part to enhancement of I_h .

Experiment 2: Basket Cell Innervation of Surviving PCs

It seems clear from the above results that ethanol exposure (PN 4–6) has a far more reaching impact on the cerebellar cortex than simply the loss of PC numbers. The mounting evidence suggests that PCs surviving the apoptotic-induced death produced by PN 4–6 ethanol exposure develop as mature neurons that are under an increased magnitude of tonic inhibition. Since the above data suggest that BCs are likely to mediate this increased inhibitory tone, we decided to address this possibility further using immunofluorescence techniques.

Figure 5 presents the results (mean±SEM) of the numbers of PCs, BCs, and SCs within Lobule I of control (SI) and ethanol-exposed (E 4–6) rat cerebellum at PN 23–25. As seen in Fig. 5a, the total numbers of BCs and SCs were significantly ($p=0.02$, unpaired t test) decreased by the E 4–6 treatment (~21 %). When expressed on a per PCs basis (Fig. 5b), the difference was also significant ($p=0.01$; unpaired t test) with approximately 21 % more BCs and SCs per PC. When considering only the total number of BCs (Fig. 5c), E 4–6 treatment results were not significant ($p=0.07$; unpaired t test); however, when scaled to the total number of PCs in the section analyzed (Fig. 5d), E 4–6 treatment produced a significant ($p=0.05$, unpaired t test) increase in the number of BCs per PC that was roughly 21 %.

Admittedly, the numerical quantitation of neurons in this manner is likely to be less accurate from an absolute perspective. Nevertheless, it is encouraging that the findings are consistent with the electrophysiological findings reported above.

Figure 6 presents the results (mean±SEM) of the analyses of BC-axons and PC-soma. Figure 6a shows the total number of PCs in each slice from SI and E 4–6 groups. As discussed above, the E 4–6 treatment produced a significant ($p=0.03$, unpaired t test) decrease of approximately 30 % in PCs which is in agreement with previously published data [63, 64]. Consequently, we expressed the quantitative data for BC axons and co-localization on a per PC basis. Figure 6b presents the total volume of BC-axons per number of PCs per slice. Although a difference in the mean values was evident, it does not reach significance ($p=0.5$, unpaired t test) largely due to a substantial level of variation in the data. In Fig. 6c, the volume of co-localized voxels (representing both the presence of BC axon and PC-soma immunofluorescence) scaled to a per PC basis revealed a significant ($p=0.02$, unpaired t test) and roughly 100 % increase in the E 4–6 group. This means that the volume of BC-axons in virtual contact with PCs was greater, on a per PC basis, in PCs that survived E 4–6 exposure. This result is in agreement with our findings above and supports the hypothesis that PCs surviving ethanol exposure are under a greater inhibitory influence than controls.

As stated in the methods, the histological analyses were conducted in tissue from PN 23–25 rats while the electrophysiological analyses were conducted in tissue from PN 13–15 rats. Although the findings are in agreement, the impact of the developmental difference must be addressed. We conducted an analysis of BCs and PCs numbers in tissue of PN 14 rats and found that the ratio of BCs/PCs was increased in the E 4–6 group by ~20 % (data not shown). In contrast, immunofluorescence analysis of PN 14 tissue for BC axons, using the antibody (ab17126, Abcam Inc.) to the 200 kD NF, did not demonstrate sufficient quality of detection to allow for confocal analysis.

That this greater inhibitory influence is mediated by an increased BC innervation is consistent with the known developmental changes of these neurons. While PCs are vulnerable to apoptotic death during the PN 4–6 period, BCs are likely less affected by ethanol exposure because at this stage they are still completing their migratory processes through the molecular layer [71]. It is not until PN 9 that the BC axons reach the stage of synaptogenesis with PC soma and subsequently trigger the relocation of climbing fiber synapses from the soma to the proximal dendritic region [71–73].

Experiment 3: HCN1 Expression at Basket Cell Innervation of Purkinje Cells

Based on the above electrophysiological data, especially in regards to the I_h current, we decided to conduct an immunofluorescence analysis of inhibitory inputs to the PCs. BC axon terminals are known to synapse on the soma and initial axonal segment of PCs forming the pericellular basket and pinceau structures, respectively. This innervation is believed to provide coincident inhibitory inputs to the PCs and to synchronize the output of the cerebellar cortex. We examined the immunofluorescence distribution of HCN1 (hyperpolarization-activated cyclic nucleotide-gated cation) channels that mediate the I_h current (hyperpolarization-activated mixed cation current). HCN1 subunits were found to be densely clustered in the presynaptic inhibitory terminals that surround PCs and originate from BCs [74]. The I_h current facilitates neuronal synchronization and can contribute to the pacemaker activity of PCs. Abnormally synchronized activity of PCs can lead to impaired movement.

Figure 7a presents four images showing the PC/HCN1 immunofluorescence from an SI and E 4–6 Lobule I cerebellar slice. The top two images show the PC soma revealed by calbindin-D28k (red) and HCN1 of the soma region revealed by anti-HCN1 (green). Analysis with the Imaris software allows the creation of a channel (yellow) composed exclusively of co-localized voxels (those having both red and green fluorescence) and this represents areas of close association between HCN1 channels and PC soma.

Figure 7b presents the quantitative analysis (mean $\pm$ SEM) of the total volumes of the different channels relative to the number of PCs contained within the slice. It is critical to remember that a primary effect of E 4–6 exposure is to kill PCs so the number of these cells per slice is expected to be different. Thus, expression of total volume data of immunofluorescence on a per PC basis is necessary. In this study, the number of PCs per slice was significantly ($p<0.0001$, unpaired t test) lower in the E 4–6 rats (SI=31.6 $\pm$ 1.5 $n=9$; E 4–6 20.0 $\pm$ 1.5 $n=12$) compared to SI rats.

The total PC soma volume/PC was not different between SI and E 4–6 rat pups; in contrast, significant increases in the total HCN1 volume/PC and co-localized volume/PC were identified. This is consistent with the electrophysiological evidence showing increased inhibitory input to PCs and increased amplitude of the I_h current (Fig. 3, above). These results demonstrate an increased expression of HCN1 channels primarily on the pericellular basket and pinceau structures of the BC axons as they innervate PCs.

As stated in the methods, the histological analyses for HCN1 protein were conducted in tissue from PN 41–48 rats. We were unable to obtain detectable immunofluorescence

staining sufficient for confocal analysis in tissues from PN 14 rats; however, we were able to conduct similar analyses in tissues from PN 21 rats. Quantitative analysis of these data show relatively high variability of immunofluorescence intensity, nevertheless, a significant increase ($p<0.05$) in colocalized volume/PC was identified when comparing E 4–6 to SI rats (data not shown).

Discussion

Extensive work has been conducted to examine the nature, timing, dose-effect relationships, and even prevention of the loss of PCs produced by postnatal (PN 1–9) ethanol exposures; however, a near paucity of data exists that would provide insights into how the cerebellum matures and functions after a significant loss of PCs. In this light, the findings we report here are quite exciting. The effects of developmental ethanol exposure go far beyond the loss of PCs. For example, we previously showed that there are major alterations in the input to PCs derived from the climbing fibers from the inferior olive [34, 48].

The working hypothesis that emerges is that PCs surviving PN 4–6 ethanol exposure continue their developmental maturation within a cerebellar cortex whose interneurons and glia have not been adversely impacted by the targeted ethanol exposure, yet the synaptic input from other regions is greatly reduced. As a result, the PCs at PN 13–15 are under nearly twice the magnitude of inhibitory impulses in ethanol-treated rats compared to controls (Fig. 3). The finding of an increased magnitude of the I_h current provides support for this hypothesis since this is one mechanism for the cell to respond to increased inhibition by a rebound excitation, which could lead to enhanced membrane potential oscillations and possible bursting (Figs. 4 and 7). This result is supported by a previous study showing that activation of molecular layer interneurons produced a hyperpolarizing inhibitory postsynaptic potentials (IPSP) in PCs leading to activation of I_h which caused rebound depolarization [75].

Our studies identifying increased BC number relative to PC number and/or increased BC axonal contacts with PC soma are consistent with the above increased inhibitory input (Figs. 5 and 6). In fact, although our immunofluorescent studies were conducted at a later developmental age than the electrophysiological studies, the agreement between the morphological and the electrophysiological findings suggests that these changes are sustained rather than transient.

The age difference between the electrophysiological and morphological studies was driven by technical difficulties and practical reasons. We chose younger rats for electrophysiological recordings in order to maximize slice viability because slices obtained from younger animals are known to show less signs of traumatic injury caused by sectioning [76, 77]; and even despite our use of the favored sucrose-aCSF solution during slice preparation [78]. Although it has been possible to obtain electrophysiological recordings from PCs up till age PN 90 [61], the purpose of our recordings was to investigate the intrinsic and synaptic electrical properties of the PCs under optimal conditions. During our preliminary experiments, we could not record spontaneous IPSPs at relatively old ages (PN 40, data not shown). As a consequence, we opted to use cerebellar slices from rats at PN 13–

15 in order to examine the inhibitory synaptic properties as well as the amplitude of I_h at an age when the spontaneous IPSPs are mostly active and not compromised by slice viability. Previous studies have shown that at a similar younger age, PCs in brain slices exhibited relatively high rate of spontaneous IPSPs and infrequent spontaneous EPSPs [66, 79]. This lack of spontaneous EPSPs in vitro could be explained by the findings that granule cells, which provide excitatory inputs to PCs, are mostly silent [80] under these conditions. In contrast, molecular layer interneurons, which provide inhibitory input to PCs, are spontaneously active [66] and coupled by electrical and chemical synapses [81]. Therefore, there could be many reasons why PCs lose their spontaneous IPSPs at older age such as a decrease in the probability of spontaneous release of GABA, a decrease in the spontaneous firing activity of molecular layer interneurons, in addition to a decrease in slice viability affecting especially synaptic transmission.

Our attempts at HCN1 immunostaining in PN 14 rats were not successful. However, we were able to obtain quantitative data for HCN1 immunostaining from PN 21 rats but the intensity of immunofluorescence exhibited large variability. As a result, we elected to use tissue from PN 41–48 rats for the quantitative immunofluorescent analysis of HCN1. That our findings, morphologically are in concert with the PN 13–15 electrophysiology are encouraging. Indeed, although our data from the PN 21 rats involved high variability, they were consistent with the significant increase in co-localization of HCN1 immunostaining with PC structures when characterized on a per PC basis (data not shown). Nevertheless, additional studies at PN 41–48 involving electrophysiological or functional (behavioral) outcomes are warranted to provide further interpretative guidance in regards to the present findings. We have been working on a functional analysis of rat licking [82] that may be useful in this regard. Finally, it is also possible that different HCN subunit proteins might coexist in PCs of younger animals. A recent study producing a PC selective knockout of the HCN1 protein still required several weeks of life before the HCN1 protein completely disappeared [83].

A further caveat that must be addressed in regards to the electrophysiological data reported herein concerns the relatively small number of treated and control rat pups from which PCs were recorded. Although the statistical analyses conducted on the total number of recorded cells were sufficiently powered, conclusions from these data alone would need to be considered preliminary because they were derived from a relatively small number of pups. Nevertheless, substantiation of the electrophysiological findings is provided by the immunofluorescence data which was conducted using a substantial number of control and treated rat pups sufficient to provide appropriate statistical power. The finding that the immunofluorescence data are in concert with the electrophysiological data allows us to feel confident in reporting them altogether.

All the present work was conducted on PCs located in Lobule I of the midline vermal cerebellar region. While the consistency of the cerebellar cortex in cellular make-up and electrophysiological function is well known with few exceptions [84, 85], it is also clear that certain regions of the cerebellum show differential sensitivity to the effects of developmental ethanol [30, 37, 63, 64]. Lobule I of the cerebellum demonstrates the greatest reduction of PCs (45 %) following postnatal ethanol exposure [26, 63, 64]. As a result, the findings we

report are focused on that specific region of the cerebellum. Further studies in other cerebellar regions are warranted and currently underway.

Our study shows that PCs that survive PN 4–6 ethanol exposure receive relatively greater inhibitory inputs. The developmental exposure to ethanol seems to mirror its acute effects. For example, acute application of alcohol was also found to potentiate presynaptic release of GABA on PCs via cAMP-PKA mechanism [86]. Our electrophysiological findings are in agreement with our immunofluorescence data which showed that, while the excitatory climbing fiber inputs were reduced significantly, the inhibitory BC inputs were increased significantly. So, what is the likely impact of this increased inhibition on the circuit-level functions of the cerebellum? We hypothesize that the synchronized activity of PCs could become impaired after developmental ethanol exposure. As a consequence, PCs which survive the ethanol treatment may exhibit an altered synchronized activity caused by a change in GABAergic synaptic transmission.

Synchronous IPSCs in paired recordings of PCs were first reported in cerebellar slices from 9- to 15-day-old rats [79, 87]. The mechanism of these synchronous inhibitory inputs, however, have not been clearly resolved because they have been studied under different experimental conditions. For example, it was shown that in juvenile mice (4–14 days old), PCs make monosynaptic GABAergic connections with neighboring PCs via their axon collaterals. These connections change from excitatory to inhibitory throughout this developmental period depending on the chloride equilibrium potential, and they are pruned in older mice [62]. Studies in adult rats in vivo suggested that PC recurrent inhibitory synapses [67], near-simultaneous climbing fiber excitatory input [88], and parallel-fiber-mediated common excitatory input [56, 89] all contribute to PC synchronicity. In adult mice in vitro, PCs exhibit rhythmic IPSPs, spikelets, and dye couplings only during nicotine-induced network oscillations but not in resting conditions. Moreover, phasic inhibitory inputs were found to be necessary for the generation of gamma rhythm whereas gap junctions contribute to the generation of very fast oscillations [90]. Experimental data and computational modeling indicated that the spikelets might be due to the electrical coupling between the proximal axons of PCs [91]. Thus, the inhibitory inputs to PCs seem to be involved in controlling the timing of firing and the generation of cerebellar network rhythmic oscillations. Our previous studies identified deficits of climbing fiber excitatory input to PCs surviving ethanol exposure [34, 48]. The present study identifies an increased presence of inhibitory synapses to PCs surviving the ethanol exposure. Clearly the combined effects of these two alterations would be a substantial inhibition of PCs in these rats. This inhibition is likely mitigated to some degree by the increased I_h amplitude. Several studies have shown that PCs exhibit a hyperpolarization-activated current (I_h) that modulates their firing pattern and interacts with their inhibitory inputs [75, 83, 92–96]. Thus, our findings of increased I_h amplitude in the PCs surviving ethanol exposure may serve to modulate this increased inhibitory status. Our analysis of the spontaneous firing frequency of PCs (Fig. 2) suggests an alternative hypothesis. Although somewhat preliminary, we found that the ISI distribution was more skewed in ethanol-treated versus control rats resulting in longer ISIs, or pauses, which are most likely caused by inhibitory synaptic inputs as has been shown previously in vitro [66] and in vivo [67, 68]. These increased pauses may allow for greater synchrony across PCs [79, 87] thus mitigating, to some degree, the decrease in climbing

fiber excitatory inputs [34, 48] which additionally contribute to PC synchronization [97]. Further studies are warranted to explore this possibility.

A final and perhaps more intriguing possibility for understanding the functional impact of altered development of the cerebellum subsequent to PN 4–6 ethanol exposure involves the evidence that the cerebellum has an important role in cognitive functions [98, 99]. It is possible that the development of forebrain regions could be adversely affected subsequent to ethanol-induced alterations in cerebellar development [100]. This would imply that we might need to add developmental ethanol exposure to the list of potential causative agents of Autism Spectrum Disorders (ASD). This is not a novel suggestion. Previously, a magnetic resonance imaging study identified hypoplasia of the cerebellar Lobules VI and VII in the vermis of ASD patients [101]. This study also indicated that Lobules I–V of the vermis in these patients were not significantly altered. Another study reported a significant reduction in gray matter content of the vermis and Crus-II regions of the cerebellum in low-functioning ASD children [102]. Other reports also substantiate these findings. For example, eyeblink conditioning has been demonstrated to be defective in many types of neurodevelopmental disorders, involving children and animal models [103, 104].

These findings open up the possibility that the developmental alterations of the cerebellum not only impact its motor functions but it might also compromise the normal development of forebrain regions that are involved in cognitive functions and which receive input from the now dysfunctional cerebellum [100]. Thus, the varied patterns of social and behavioral alterations seen in children experiencing neurodevelopmental damage may be dependent on the timing of the cerebellar damage during fetal development.

Summary

Our study shows that in Lobule I of the vermis, PCs that survive PN 4–6 ethanol exposure receive relatively greater inhibitory inputs. These electrophysiological findings are in agreement with our immunofluorescence data which showed a substantial increase in the inhibitory inputs from BCs along with a previously reported reduction of the excitatory inputs from climbing fibers. Since inhibitory inputs to PCs seem to be involved in controlling the timing of firing and the generation of cerebellar network rhythmic oscillations, it is therefore possible that Purkinje cell firing would show significant and dramatic alterations in synchrony in rats exposed to ethanol on PN 4–6.

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Fig. 1.

Schematic diagram showing the major changes that occur in the cerebellar circuits after developmental ethanol exposure. Ethanol treatment of rats during PN 4–6 causes loss of PCs, shrinkage of the molecular layer, deterioration in the climbing fibers that innervate PCs, increase in GABAergic innervation to PCs from molecular layer interneurons, and increases in HCN1 channels on PC soma and inhibitory axons that innervate PCs

Fig. 2.

Interspike interval (ISI) distributions. Histograms of the ISIs were constructed from recordings of 6–8 min from each PC. The histograms of the ISIs were binned at 1 ms and smoothed using 3 point adjacent averaging. Then they were normalized to the total number of ISI intervals in each histogram

Fig. 3. Recordings from PCs of Lobule I in slices taken on PN 13–15 from Control (C) and Ethanol (E)-treated (PN 4–6) rat pups. Values are mean±SEM. **a** Voltage clamp recordings at holding potential of -40 mV and current traces of 1 s duration. **b** The frequency of spontaneous IPSCs was significantly ($*p<0.01$) higher (unpaired t test) in ethanol-treated rats compared to Controls; suggesting that PCs from the ethanol-treated rats may receive more inhibitory synapses

Fig. 4.

Recordings of PCs of Lobule I in slices from Control (C) and ethanol-treated (4.5 g/kg/day, PN 4–6; E) PN 13–15 rat pups. Values are mean $\pm$ SEM. **a** Current clamp—500 ms pulses with amplitudes from $-1,000$ pA to $+600$ pA (200 pA increments). Note that the sag in the membrane potential in response to hyperpolarizing current pulses and the rebound depolarization at the end of current pulses were greater in magnitude in Ethanol vs. Control. Voltage steps of 500 ms pulses (-60 mV below holding potential, 10 mV increments). Note that the amplitude of the time-dependent inward current was larger in ethanol-exposed vs. control rats. **b** Group data: Control (368 ± 19 pA; $n=5$); Ethanol (563 ± 45 pA; $n=7$); The ethanol-treated group showed a significant increase in amplitude ($*p<0.01$; unpaired t test) compared to Controls. The input resistance ($C=42\pm5$ MOhm, $n=5$; $E=36\pm4$ MOhm; $p=0.27$) between the two groups was not significantly different

Fig. 5.

Immunofluorescence analysis of Basket (*BCs*), Stellate (*SCs*), and Purkinje (*PCs*) cell numbers within Lobule I of control (SI) and ethanol-exposed (E 4–6) rat cerebellum at PN 23–25. Values are mean±SEM. **a** The total numbers of *BCs* and *SCs* are decreased by E 4–6 treatment by 21 % which is statistically significant ($p=0.04$, unpaired t test). **b** When the total number of *BCs* and *SCs* are expressed on a per *PCs* basis the significant difference shows a 21 % increased number per *PC* ($p=0.01$; unpaired t test). **c** Considering only total *BCs* numbers the decrease with E 4–6 is greater in percentage but not statistically significant ($p=0.01$, unpaired t test). **d** When the ratio of *BCs* to *PCs* is analyzed the E 4–6 treatment shows a significant ($p=0.05$, unpaired t test) increase of roughly 21 %

Fig. 6.

Sagittal slices of the cerebellar vermis at 60 μm thickness from PN 23–25 control (SI) and E 4–6 rat pups were processed for immunofluorescence techniques in a free-floating configuration using anti-200 kD Neurofilament Heavy protein to identify BC-axons and calbindin-D28k to identify PC-soma. Confocal image stacks (20×) having a size of 450 mm×450 mm×~40 mm (z-axis) were obtained from a single layer of PCs along the bank region of Lobule I adjacent to Lobule II. These image stacks were reconstructed for quantitative analysis using the Imaris (Bitplane AG, Zurich) software package. Values are mean ±SEM. **a** Shows the decrease in the number of PCs per slice due to the E 4–6 exposure. **b** Shows the total BC axon volume/per PC within the slice. No significant difference is noted due to large variation. **c** When the total BC axon volume co-localized with PC soma is calculated and expressed per PC within the slice a significant increase is evident

Fig. 7. Immunofluorescence analysis of HCN1 channels on the PC soma within Lobule I of control (SI; $n=9$) and ethanol-exposed (E 4–6; $n=12$) rat cerebellum At PN 41–48. Values are mean $\pm$ SEM. **a** Confocal images of an SI and E 4–6 section showing PC soma (red) revealed by calbindin-D28k and anti-HCN1 (green). The images below are the same sections with the HCN1 channel turned off and the col-localized voxel channel (yellow) turned on to demonstrate only the HCN1 protein associated with PC soma. Scale bars are 30 μ m. **b** Quantitative analysis of the total volume of voxels from each fluorescence channel representing PC soma, HCN1 protein, and co-localization of both. Total volume data are

scaled to the number of PCs analyzed within a slice to neutralize the impact of E 4–6-induced PC loss

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