Research Report

Olivary climbing fiber alterations in PN40 rat cerebellum following postnatal ethanol exposure

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ABSTRACT

Developmental ethanol exposure in rats during postnatal days (PN) 4–6 is known to cause significant loss of the cerebellar Purkinje cells. It is not known what happens to the surviving neurons as they continue to develop. This study was designed to quantify the interactions between the olivary climbing fibers and the Purkinje cells when the cerebellar circuits have matured. Rat pups were treated with a daily dose of ethanol (4.5 g/kg body weight) delivered by intragastric intubation on PN4, PN4–6, or PN7–9. The interactions between the climbing fibers and the Purkinje cells were examined on PN40 using confocal microscopy. Mid-vermal cerebellar sections were stained with antibodies to calbindin-D28k (to visualize Purkinje cells) and vesicular glutamate transporter 2 (VGluT2, to visualize climbing fibers). Confocal z-stack images were obtained from Lobule 1 and analyzed with Imaris software to quantify the staining of the two antibodies. The VGluT2 immunostaining was significantly reduced and this was associated with alterations in the synaptic integrity, and synaptic number per Purkinje cell with only a single exposure on PN4 enough to cause the alterations. Previously, we demonstrated similar deficits in climbing fiber innervation when analyzed on PN14 (Pierce, Hayar, Williams, and Light, 2010). The present study confirms that these alterations are sustained and further identifies the decreased synaptic density as well as alterations to the general morphology of the molecular layer of the cerebellar cortex that are the result of the binge ethanol exposure.

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Abbreviations: PN, postnatal days; FASD, Fetal Alcohol Spectrum Disorder; MolLay, molecular layer of cerebellar cortex; Soma, soma layer of cerebellar cortex; VGluT2, vesicular glutamate transporter 2; CalB, calbindin-D28k; BEC, blood ethanol concentration; PC, Purkinje cells

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1. Introduction

The cerebellum is vulnerable to developmental ethanol exposure, and in the rat, exposure on postnatal days (PN) 4–6 targets survival of the cerebellar Purkinje cells ( PCs) as well as the early distribution of olivary climbing fibers (Goodlett et al., 1990; Goodlett and Lundahl, 1996; Pierce et al., 1999; Dikranian et al., 2005; Pierce et al., 2010). The magnitude of the Purkinje neuron loss is directly proportional to the blood ethanol concentration achieved when the exposure lies within this vulnerable period (Bonthius and West, 1990; Goodlett et al., 1990, 1997, Pierce et al., 1999). The inferior olivary neurons are also vulnerable to ethanol and this period and reductions of as much as 25% are reported after ethanol exposure during this time frame (Napper and West, 1995; Dikranian et al., 2005). The rat brain development during the first week of postnatal life is a period of rapid growth similar to that found for human brain development during the early weeks of the third trimester (Dobbing and Sands, 1979; Dobbing, 1981; Cudd, 2005). Administering a high dose of ethanol to rat pups each day for the three day vulnerable period (PN4–6) is a well established model that mimics binge drinking during the human third trimester equivalent period (Hamre and West, 1993; Goodlett and Lundahl, 1996; Pierce et al., 1999; Dikranian et al., 2005; Pierce et al., 2010).

Binge drinking (five or more drinks at the same time or within a couple of hours) occurs in 32.6% of non-pregnant women (NSDUH, 2009). Studies of pregnant women report that within a couple of hours) occurs in 32.6% of non-pregnant their third trimester of pregnancy (NSDUH, 2009). Nevertheless, only 1% of pregnant women engage in binge drinking during the third trimester of pregnancy (NSDUH, 2009). Nevertheless, this 1% accounts for 40,000 to 60,000 children per year in the USA (NSDUH, 2009; Ventura et al., 2008). Interestingly, this rate for binge drinking during the third trimester is coincident with the estimated incidence rate for Fetal Alcohol Spectrum Disorder (FASD) of 1 in every 100 live births (Riley and McGee, 2005).

Previously, we reported that the cerebellar cortex of PN14 rat pups exposed to binge ethanol during PN4–6 showed significant alterations in the inferior olivary input. The number of Purkinje cells was decreased as previously reported, but additional alterations were identified that involve the development of the surviving Purkinje neurons and their receipt of significantly fewer climbing fiber inputs beyond what would be expected by the reduction in cell numbers (Pierce et al., 2010). This present study follows the same approach to identify the answer to several questions. Are the alterations in the climbing fiber input identified at PN14 sustained? Does the analysis of the co-localization of the climbing fiber and the Purkinje elements provide evidence of decreased synaptic contacts? Finally, is there evidence of delayed developmental damage in the group exposed to ethanol during PN7–9, when a reduction of PCs will not be produced?

The climbing fibers, arising from the contralateral inferior olivary nucleus in the brain stem, represent one of two major extrinsic inputs to the Purkinje cells. The other major input is from the mossy fibers via granule cell axons known as parallel fibers (Altman and Bayer, 1997; Watanabe, 2008). Although the climbing fibers comprise only 1–2% of the synapses on the Purkinje cells, they have a critically important role in the proper functioning of the cerebellar circuitry (Strata and Rossi, 1998; Ito, 2001; Watanabe, 2008). The alterations of the climbing fiber development (e.g. glutamate receptor gene deletion) produces significant ataxia, motor discoordination, and disturbances of eye blink conditioning (Ichise et al., 2000; Hashimoto et al., 2001; Kishimoto et al., 2002; Kato et al., 2005). These behavioral deficits are very similar to those documented in rats postnatally exposed to ethanol (Meyer et al., 1990; Thomas et al., 1996, 1998; Klintsova et al., 2002; Green, 2004) and in human FASD (Streissguth et al., 1980; Roebuck et al., 1998; Coffin et al., 2005; Connor et al., 2006).

We hypothesized that ethanol exposure to the rat on PN4–6 would continue to induce, on PN40, significant alterations in the climbing fiber input to the Purkinje cells in support of a sustained negative impact on cerebellar functioning. In addition, these differences will be consolidated to the cerebellar molecular layer and not the soma, as this region contains much less innervation from the climbing fibers when mature. In addition, we hypothesize that the analysis of co-localization will show evidence of decreased synaptic contacts from the climbing fibers per PC. While we also suspect that the later ethanol exposure period (E7–9) will demonstrate detrimental impact on climbing fiber innervation, we do not expect this to be of great magnitude. To test these hypotheses, the climbing fiber synapses were visualized in Lobule I of the cerebellar sections immunostained for vesicular glutamate transporter 2 (VGLuT2) (Hioki et al., 2003; Miyazaki et al., 2003; Takagishi et al., 2007) with the Purkinje cell visualization using immunostaining for calbindin-D28k (CalB) (Light et al., 2002). Confocal z-stack images were 3D-reconstructed and quantified as the total expression intensity and the volume of expression in the soma and molecular layers, utilizing Imaris software (Bitplane AG, Zurich, Switzerland) (Hein et al., 2006; Marvizon et al., 2007; Nassimi et al., 2009).

2. Results

2.1. Blood ethanol concentration (BEC)

The mean BEC for the ethanol group (4.5 g/kg) was 365.4±38.6 mg/dl (±SEM) (n=7) as previously reported (Pierce et al., 2010).

2.2. Body weight data

The body weights of the rats at PN40 were analyzed for each group (Fig. 1). There was a significant interaction (F(6,39)=4.3; p<0.05); however, custom contrasts (p<0.05) did not verify any significant differences. This indicates that the general growth of the rats was not significantly affected by ethanol exposure.

2.3. Cell count data

Fig. 2 presents the results from the Purkinje cell counting within Lobule I. No significant interaction (F(6,30)=1.5; p>0.05) was identified; however, a significant main effect of the treatment (F(1,30)=16.3; p<0.01) and no significant main effect of timing (F(6,30)=1.5; p>0.05) were observed. Thus, postnatal ethanol exposure produced a significant decrease in the number of Purkinje neurons in Lobule I of the 40-day old rat.
as expected. In addition, we observed from the custom contrasts that significant differences were identified for the E4 vs C4 and the E4–6 vs C4–6 comparisons.

These data were obtained from 10 µm sections of the cerebellar midline and the number of animals within each group is slightly different from the numbers involved in the confocal analyses of 60 µm sections (see Table 1 and Experimental procedures).

2.4. Molecular layer analyses

Calbindin D28k positive immunofluorescent labeling was used to identify the Purkinje cells and concurrent VGluT2 positive immunofluorescent labeling was used to identify climbing fiber innervation of the Purkinje cells in Lobule I. VGluT2 immunoreactivity represents the nerve terminals of the climbing fibers from the inferior olivary nucleus. There are two main glutamate transporters in the cerebellar cortex, VGluT1 and VGluT2 (Hioki et al., 2003). Parallel fibers from granule cells in adult animals express VGluT1 (Hioki et al., 2003; Miyazaki et al., 2003). Climbing fiber terminals consistently exhibit VGluT2 immunoreactivity in the molecular layer (MolLay) and in the Purkinje cell soma layer (Hioki et al., 2003; Miyazaki et al., 2003; Takagishi et al., 2007). The immunostaining of VGluT1 and VGluT2 is localized only to nerve terminals and not to cell bodies or dendrites (Fremeau et al., 2004).

Fig. 3 shows the rendered Imaris image with each channel shown independently, (A. CalB, B. VGluT2) and merged (C). The blue line outlines the Soma region to indicate the division of these images into the Soma and the Molecular Layer (MolLay) regions for analyses.

Within these two specific regions (Soma and MolLay), the analysis of the climbing fiber development as measured by VGluT2 immunostaining was determined as the summed intensity or volume of the voxels within the VGluT2 channel in the region. The volume represents the size of the tissue compartment expressing the protein and the intensity was interpreted as a measure of the total expression level of that protein. Since it is known that ethanol exposure results in a decreased number of Purkinje cells, the summed VGluT2 volume or summed VGluT2 intensity of each sample was divided by the total volume of the calbindin-D28k channel (Purkinje cell specific) of that same sample and region in order to normalize the data and obtain the VGluT2 volume or intensity per micron³ of the Purkinje cell tissue. Thus any group differences in these values are not the result of the ethanol-induced loss of the Purkinje cells. These two endpoints were termed VGluT2 Intensity Ratio, and VGluT2 Volume Ratio (Table 2). The Imaris software also allowed the construction of a channel that includes only those voxels that

<table>
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<th>Group</th>
<th>Description</th>
<th>Confocal Imaging</th>
<th>Cell Counting</th>
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<tr>
<td>E4</td>
<td>Ethanol exposed PN4 only</td>
<td>7</td>
<td>8</td>
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<tr>
<td>E4–6</td>
<td>Ethanol exposed PN4–6</td>
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<td>5</td>
</tr>
<tr>
<td>C4</td>
<td>Isocaloric control PN4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>C4–6</td>
<td>Isocaloric control PN4–6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>C7–9</td>
<td>Isocaloric control PN7–9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MRC</td>
<td>Mother reared control</td>
<td>8</td>
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contained both red and green fluorescence and was termed the co-localized channel. As described above the summed volume or intensity of the co-localized voxels in each region of interest was divided by the total volume of the calbindin-D28k channel in order to normalize these measures to the volume of the Purkinje cell tissue. These two end-points were termed Co-localized Intensity Ratio and Co-localized Volume Ratio (Table 2).

We have interpreted the co-localized end-points as representing the portion of the climbing fiber tissue that had made putative functional contacts with the Purkinje cells. Each of these four endpoints was determined in the Soma and Molecular Layer regions of each tissue sample independently and in a blind-to-treatment manner.

By PN40, the VGluT2 positive immunofluorescent labeling was represented as distinct puncta in the Molecular Layer region of Lobule I. The number of these puncta was determined using the Imaris program, and the data were normalized by dividing the number of puncta by the number of Purkinje cells in the specific confocal slice (VGluT2 Puncta/PC) (Table 2). In addition, the co-localized puncta were also counted and normalized to the number of Purkinje cells in the specific confocal section (CoL-Puncta/PC) (Table 2).

Finally, the width (or radial distance) of the molecular layer was measured using the reconstructed images from the confocal image z-stacks obtained from the mid-sagittal slices of the Lobule I region. The measurements were made of the molecular layer by recording the distance from the edge demarcated between the soma layer and the molecular layer to the pial surface of the molecular layer using only the CalB channel. For each tissue image analyzed, we calculated an average of four of these measurements that were made across the extent of the

<table>
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<th>Table 2 – Definition of image analysis end-points.</th>
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<tr>
<td>VGluT2 Intensity Ratio</td>
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<td>VGluT2 Volume Ratio</td>
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<td>Co-localized Intensity Ratio</td>
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<td>Co-localized Volume Ratio</td>
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<tr>
<td>VGluT2 Puncta/PC</td>
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<tr>
<td>CoL-Puncta/PC</td>
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<td>MolLay-Width</td>
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Fig. 3 – Rendered images of Lobule I Purkinje cells with VGluT2/ climbing fiber innervation. Images were produced using Imaris software. A. Rendered image of the calbindin-D28k (CalB) immunostaining demonstrating cerebellar Purkinje cells. Note that the soma layer is outlined in blue. The scale bar in each image is 30 μm. B. Rendered image of the VGluT2 immunostaining. This represents the VGluT2 positive portion of the climbing fibers from the inferior olive. The VGluT2 immunostaining is represented as green puncta and the specific VGluT2 immunostaining that is co-localized with CalB is represented as yellow puncta. C. Combined red and green channels showing the interactions of the Purkinje cells with the climbing fibers.
The MolLay Width (Table 2) provided a comparative metric of the developmental extent of the cerebellar cortex. The results of the analyses of the climbing fiber innervation within the MolLay region are presented in Figs. 4–6. For the climbing fiber innervation to the MolLay region, there was no significant interaction (F(2,29) <1), and no significant main effect of the treatment (F(1,29) <1) or timing (F(2,29) <1). MRC40=275.81±35.14. B. The co-localized expression intensity relative to the total PC tissue volume (Co-localized Intensity-Ratio). There were no significant control group differences (F(3, 20)=0.46; p>0.05) that impact the conclusions. A significant interaction (F(2, 29)=5.1; p<0.02) was observed. Custom contrasts at the p<0.05 level identified significant differences for E4 vs C4 (*) and for E4–6 vs C4–6(†). MRC40=148.88±13.39. C. VGlut2 expression volume relative to the total PC tissue volume (VGlut2 Volume-Ratio). There were no significant control group differences (F(3, 20)=0.72; p>0.05) that impact the conclusions. A significant main effect of the treatment (F(1,29) =22.9; p<0.01) and no significant main effect of timing (F(2, 29)=2.8; p>0.05) was observed. Custom contrasts at the p<0.05 level identified significant differences only for E4 vs C4 (*), E4 vs E7–9 (†), E4–6 vs C4–6 (‡), and E4–6 vs E7–9 (††). MRC40=0.06±0.005. D. The co-localized expression volume relative to the total PC tissue volume (Co-localized Volume-Ratio). There were no significant control group differences (F(3, 20)=1.46; p>0.05) that impact the conclusions. A significant interaction (F(2, 29)=5.1; p<0.02) was observed. Custom contrasts at the p<0.05 level identified significant differences for E4 vs C4 (*), E4 vs E7–9 (†); E4–6 vs C4–6 (‡), E4–6 vs E7–9 (††); and E7–9 vs C7–9 (‡‡). MRC40=0.03±0.002.

Fig. 4 – Alterations in Climbing Fiber endings in the Molecular Layer of Lobule I on PN40 following ethanol exposure at 4.5 g/kg during the early postnatal period. MRC40= mother reared control group; C4, C4–6, C7–9= Isocaloric vehicle control groups; E4, E4–6, E7–9 = Ethanol exposure groups; (see Table 1). Error bars represent SEM. A. VGlut2 expression intensity relative to the total PC tissue volume (VGlut2 Intensity-Ratio). There were no significant control group differences (F(2,29)=0.05; p>0.05) that impact the conclusions. There was no significant interaction (F(2,29) <1) and no significant main effect of treatment (F(1,29) <1) or timing (F(2,29) <1). MRC40=275.81±35.14. B. The co-localized expression intensity relative to the total PC tissue volume (Co-localized Intensity-Ratio). There were no significant control group differences. A significant interaction (F(2,29) =5.1; p<0.02) was observed. Custom contrasts at the p<0.05 level identified significant differences for E4 vs C4 (*) and for E4–6 vs C4–6(†). MRC40=148.88±13.39. C. VGlut2 expression volume relative to the total PC tissue volume (VGlut2 Volume-Ratio). There were no significant control group differences (F(3, 20)=0.72; p>0.05) that impact the conclusions. A significant main effect of the treatment (F(1,29) =22.9; p<0.01) and no significant main effect of timing (F(2, 29)=2.8; p>0.05) was observed. Custom contrasts at the p<0.05 level identified significant differences only for E4 vs C4 (*), E4 vs E7–9 (†), E4–6 vs C4–6 (‡), and E4–6 vs E7–9 (††). MRC40=0.06±0.005. D. The co-localized expression volume relative to the total PC tissue volume (Co-localized Volume-Ratio). There were no significant control group differences (F(3, 20)=1.46; p>0.05) that impact the conclusions. A significant interaction (F(2, 29)=5.1; p<0.02) was observed. Custom contrasts at the p<0.05 level identified significant differences for E4 vs C4 (*), E4 vs E7–9 (†); E4–6 vs C4–6 (‡), E4–6 vs E7–9 (††); and E7–9 vs C7–9 (‡‡). MRC40=0.03±0.002.
The VGluT2 Volume Ratio within the MolLay region (Fig. 4C) did not show a significant interaction ($F(2, 29)=1.1; p>0.05$). However, a significant main effect of the treatment ($F(1,29)=22.9; p<0.01$) with no significant main effect of timing ($F(2, 29)=2.8; p>0.05$) was observed. Clearly, postnatal ethanol exposure results in a loss of volume for the climbing fiber tissue within the cerebellar cortex. The custom contrasts at the $p<0.05$ level provide further insights as significant differences were identified for the E4 vs C4, E4 vs E7–9, E4–6 vs C4–6, and E4–6 vs E7–9 comparisons.

Finally, the Co-localized Volume Ratios within the MolLay (Fig. 4D) showed a significant interaction ($F(2, 29)=5.1; p<0.02$). Custom contrasts at the $p<0.05$ level identified significant differences for E4 vs C4, E4 vs E7–9; E4–6 vs C4–6, E4–6 vs E7–9; and E7–9 vs C7–9. Thus, the impact of ethanol exposure on the climbing fiber innervation of the cerebellar cortex is a significant and somewhat profound decrease in both the climbing fiber tissue and that portion that is co-localized with the Purkinje cell targets.

Since the VGluT2 positive immunofluorescent labeling at PN40 consists of distinct puncta throughout the MolLay region of Lobule I (see Fig. 3B) we counted these puncta and then normalized them to the number of Purkinje cells (PC) within the analyzed slice (Fig. 5). The VGluT2 Puncta/PC (Fig. 5A) showed no significant interaction ($F(2, 29)=3.2; p>0.05$) and no significant main effect of the treatment ($F(1,29)=1.9; p>0.05$). However, a significant main effect of timing ($F(2, 29)=5.4; p<0.02$) was observed. Custom contrasts at the $p<0.05$ level identified significant differences only for E4 vs E7–9(*) and E4–6 vs E7–9 †. MRC40=112.05±12.0. B. Total co-localized puncta present in the Molecular Layer region expressed on a per Purkinje cell basis. (CoL-Puncta/PC). There were no significant control group differences ($F(3, 20)=0.17; p>0.05$) that impact the conclusions. A significant interaction ($F(2, 29)=3.2; p>0.05$) and no significant main effect of the treatment ($F(1,29)=1.9; p>0.05$) was identified. However a significant main effect of timing ($F(2, 29)=5.4; p<0.02$) was observed. Custom contrasts at the $p<0.05$ level identified significant differences for E4 vs C4 (*), E4–6 vs C4–6(†), and E4–6 vs E7–9 †. MRC40=56.27±9.48.

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present. This main effect of timing consisted of differences between the 4 and 4–6 groups compared to the 7–9 period. The effect is clarified when the custom contrasts are inspected and reveal significant differences only for E4 vs E7–9, and E4–6 vs E7–9.

The analysis of the Co-localized Puncta/PC (Fig. 5B) values reveals a significant interaction ($F_{(2, 29)}=3.7; p<0.05$). The custom contrasts identify significant differences for E4 vs C4, E4–6 vs C4–6 and E4–6 vs E7–9.

2.5. Molecular layer width

The apparent width or radial distance of the molecular layer (Fig. 6) did not show a significant interaction ($F_{(2, 29)}=2.2; p>0.05$). However, significant main effects of both treatment ($F_{(1, 29)}=6.9; p<0.02$) and timing ($F_{(2, 29)}=4.9; p<0.02$) were identified. We conducted the custom contrasts at the $p<0.05$ level in order to obtain further insights and observed significant differences for E4 vs E7–9, E4–6 vs C4–6, and E4–6 vs E7–9.
2.6. Soma region analyses

The analysis of these parameters in the Soma region of PN40 rats revealed only a main effect of ethanol to decrease the Co-localized Volume Ratios (Fig. 7D).

3. Discussion

This study confirms that the Purkinje neurons surviving the extensive neuronal death induced by binge-like ethanol exposure during the postnatal vulnerable period of the rat are also damaged in their interaction with the critical input of inferior olivary neurons. Previously we showed significant decreases of the climbing fiber innervation at PN14 of the rat brain development (Pierce et al., 2010), and the present study demonstrates that those decreases are sustained, and likely permanent. Our normalization of the climbing fiber measurements to the volume (or number) of the Purkinje neurons (that survived ethanol exposure) allows the interpretation that these alterations were not merely reflective of the Purkinje cell loss; rather they demonstrate an altered trajectory of development in the cerebellar cortex.

Two important aspects of the climbing fiber innervation of the Purkinje cells were decreased in the E4 or E4–6 groups. The VGluT2 Volume Ratio (Fig. 4) identifies the total volume of the tissue expressing VGluT2, and thus the climbing fiber innervation (normalized to the total volume of the PC tissue). Both E4 and E4–6 treatments resulted in significant decreases in this parameter; however, they were not significantly different from each other. This supports the conclusion that a single binge exposure to ethanol is sufficient to produce the damage identified providing it is delivered during the PN4–6 vulnerable period. We therefore conclude that either fewer climbing fibers are providing the innervation or there is a decreased branching of the climbing fibers present. The latter seems perhaps more likely in the light of the normal condition of one climbing fiber innervating a Purkinje cell. The analysis of the Co-localized Volume Ratio and the Co-localized Intensity Ratio (Fig. 4D) demonstrates that the actual magnitude of the climbing fiber innervation to the surviving Purkinje cell is decreased with E4 and E4–6 exposures. Assuming that the co-localized endpoints are surrogates of the functional contacts between the climbing fibers and the Purkinje cells then a decrease in the functional interactions between the climbing fibers and the Purkinje cells is suggested.

Further support for the above findings is seen in the analyses of the number of the climbing fiber puncta per Purkinje cell (Fig. 5). Again, the VG2 Puncta/PC shows significant decreases for E4 and E4–6 only when compared to the E7–9 group. However, when analysis is focused on the co-localized puncta (CoL-Puncta/PC, Fig. 5B) the E4 treatment resulted in a significant decrease compared to the controls and the E4–6 resulted in a significant decrease compared to the control and E7–9 (but not E4). Again the conclusion that the surviving Purkinje cells receive fewer climbing fiber synaptic contacts is supported. These findings strongly point to a cerebellar circuit that is likely deficient since the climbing fiber innervation, while relatively minor compared to the total synaptic innervation of the Purkinje neurons, is critical in triggering long-term depression (Carta et al., 2006).

According to the literature, the number of inferior olivary neurons is decreased following E4 or E4–6 ethanol exposure (Napper and West, 1995; Dikranian et al., 2005). The loss of the inferior olivary neurons is not trivial, since the climbing fibers are the axons of these neurons. However, it is important to point out that the timing of ethanol exposure in these studies occurs during the creep stage (PN3–7) of the climbing fibers development when the inferior olivary axons have an overabundance of branches. During normal development, a single inferior olivary axon at PN3–7 will have more than 100 climbing fiber branches (Sotelo, 2004; Sugihara, 2006). The climbing fiber innervation to the Purkinje neurons during this period is concentrated around the soma and each Purkinje cell may receive input from 14 climbing fiber branches, since the ratio of the Purkinje neurons to the inferior olivary neurons is 7 to 1 (Mason et al., 1990; Altman and Bayer, 1997; Lopez-Bendito et al., 2001; Sotelo, 2004; Sugihara, 2006). Even if postnatal ethanol exposure reduced the number of the inferior olivary neurons in a greater proportion than the Purkinje cells, there would still be an overabundance of climbing fiber branches to potentially produce the typical development of the climbing fiber distribution.

We have also identified evidence of significant remodeling of the molecular layer when a substantial number of the Purkinje cells have been killed. This remodeling appears as a significant reduction of the radial width of the molecular layer (Fig. 6) following E4 and E4–6 treatments. This finding suggests a significant remodeling of the developing molecular layer following the loss of more than a third of the Purkinje neurons. The result is a molecular layer that contains the same total volume of the Purkinje cell tissue compacted within a shorter radial width molecular layer suggesting that the dendritic tree of these neurons developed within the space formerly occupied by the now dead Purkinje neurons. Thus, the density of the molecular layer is maintained, while the radial width is reduced.

During normal cerebellar development the climbing fibers at PN15 have usually reached the adult configuration with their extensive distribution around the proximal dendrites of the Purkinje cells and minimal soma based contacts (Lopez-Bendito et al., 2001; Sotelo, 2004; Sugihara, 2006; Hashimoto et al., 2009). In the present studies, none of the ethanol exposures produced any significant alteration of the climbing fiber innervation within the Soma region. Previously, we identified the alterations of the climbing fiber innervation in the Molecular Layer region at PN14 that were mirrored by similar alterations in the Soma region (Pierce et al., 2010). We conclude, that the differences in

| Table 3: Number of liters represented within the experimental groups (PC counting). |
|-----------------|-----|-----|-----|-----|-----|-----|-----|
| E4  | E4–6 | E7–9 | C4  | C4–6 | C7–9 | MRC |
| 4.5 g/kg | 7 (5) | 7 (6) | 5 (5) | 3 (5) | 6 (5) | 4 (4) | 8 (9) |
the climbing fiber development we have detailed, both at PN14 (Pierce et al., 2010) and in the present report at PN40, are not the result of a failure of the climbing fiber translocation from the soma to the proximal dendritic tree structures.

A significant decrease in the Colocalized Volume Ratio (Fig. 4D) was identified for E7–9 ethanol exposure compared to the controls (C7–9). The magnitude of this decrease was roughly 50% of the reduction produced by E4 or E4–6 (Fig. 4D). This suggests that the present findings are not dependent upon the Purkinje cell loss but rather are concordant with that effect. However, the majority of the Purkinje cell and the climbing fiber alterations from ethanol exposure occurred during the PN4–6 time period.

In summary, we have shown for the first time that binge-like ethanol exposure during the rat cerebellar vulnerable period (PN4–6) for the Purkinje cell development produces significant and permanent alterations of the climbing fiber innervation of those Purkinje neurons that survive the well characterized ethanol-induced death. Thus, the damage from the third-trimester equivalent ethanol exposure to the human fetus is likely to consist of more than just a reduced number of Purkinje neurons, but also includes altered functional connections of the cerebellar cortical circuit. Further studies to identify these alterations are underway.

4. Experimental procedures

4.1. Animal and tissue preparation

Timed-pregnant Sprague–Dawley rats (Harlan) delivered pups (designated PNO) in the University of Arkansas for Medical Sciences (UAMS) Division of Laboratory Animal Medicine. All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of UAMS prior to the initiation of experimentation. Efforts to minimize the number of animals as well as to reduce any animal discomfort were included in this review. In addition, all experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals.

The rat pups of either sex were randomly assigned on PN4 to one of the experimental groups (see Table 1). Eight dams were utilized to produce 12 l of rat pups. Each experimental group was comprised of pups from at least 3 (and in most cases 5–8) different litters (see Table 3). Ethanol exposure (E groups) was given at the dose of 4.5 g/kg body weight. Ethanol was administered one time a day, for one day on PN4 (E4 group), or for 3 days from PN4–6 (E4–6 group) or PN7–9 (E7–9 group). Intragastric intubation of a 15% (w/v) ethanol solution in Intralipid-10%, as previously described (Light et al., 2002), was utilized to deliver the daily ethanol dose. Isocaloric (vehicle) control pups (C groups) were treated in an identical manner, with the substitution of an isocaloric quantity of dextrose for the ethanol. Mother-reared controls (MRC) were not intubated. When performing the intubation process, all pups (representing all groups) were removed together from the mother, weighed and kept in a warmed chamber. Pups were returned to the mother within 30–40 min.

PN40 was chosen for our analyses since by this time the final stage of climbing fiber innervation and maturation will have been established and the adult configuration attained. Three periods of the Purkinje cell maturation have been identified (McKay and Turner, 2005). The early postnatal period (PN0–PN9) is characterized primarily by the establishment of a monolayer configuration and the development of the cell soma and the beginnings of the dendritic structures. The period of rapid maturation (PN12–PN18) is characterized by a significant expansion of the dendritic tree. The final stage (PN18–PN90) has minor refinements of the cell output and a plateau in the growth of the dendritic area after PN30 (McKay and Turner, 2005). By PN40, the total dendritic length and the area of the Purkinje neurons are nearly identical to that seen at PN90 (McKay and Turner, 2005).

On PN40, rats were weighed and then processed for the collection of the cerebellum to be analyzed by confocal microscopy. After an intraperitoneal injection of 50 mg/kg pentobarbital, intracardial perfusion with 0.05% heparin/0.9% NaCl was followed by 4% paraformaldehyde-lysine-periodate fixative (PLP), pH 7.4 (Pierce et al., 2006, 2010). The cerebella were removed and post-fixed in PLP at 4 °C overnight then cryoprotected with 30% sucrose in phosphate buffered saline (PBS), pH 7.4, and stored at 4 °C. Mid-vermal sections (60 μm thick) were prepared by cryostat sectioning and stored free floating in 0.1 M glycine/PBS with 0.02% Microcide. Additional mid-vermal sections of the same tissues were cut at 10 μm and collected on glass slides for cell counting.

Tissue was prepared for confocal imaging using immunofluorescence techniques. The Purkinje cells, including their somas and dendritic structures, were selectively identified using monoclonal antibody to calbindin-D28k (CalB; 1:5000, clone CB955, Sigma Chemical Co.) (Light et al., 2002; Pierce et al., 2010). The antibody to vesicular glutamate transporter 2 (VGluT2; 1:5000, AB5907, Chemicon, Millipore Corp.) was used to visualize glutamate transporters within the climbing fibers (Hioki et al., 2003; Miyazaki et al., 2003; Takagishi et al., 2007; Pierce et al., 2010). The tissue was double-labeled with antibodies to CalB and VGluT2. Appropriate secondary antibodies (Alexa Fluor 594, Molecular Probes; Fluorescein conjugated affinity purified secondary, Millipore Corp.) were utilized to label the Purkinje cells which were visible with fluorescence microscopy as red, and the climbing fibers as green.

4.2. Cell counting

Cell counts from Lobule 1 were accomplished using mid-vernral sections that were cut at 10 μm. The tissue sections were prepared for cell counting using immunohistochemical techniques to visualize the Purkinje cells. The monoclonal antibody to calbindin-D28k (CalB; 1:500, clone CB955, Sigma Chemical Co., St. Louis, MO) was used to selectively identify the Purkinje cells, coupled with 3,3′-diaminobenzidine (DAB, Vector Laboratories) (Light et al., 2002; Pierce et al., 2010). Cell counting was done using a Zeiss Axioskop (Carl Zeiss, Thornwood, NY). One mid-vernral section of the cerebellum from each rat in the various groups was analyzed and all the Purkinje cells in Lobule I were counted (Light et al., 2002). The purpose of the cell counts was to confirm the presence of the decreased Purkinje cells consistent with the literature reports.
In some cases we were not successful in obtaining useful sections of both 10 μm and 60 μm from the same cerebellum. Thus, there are different numbers of animals within the seven groups of the PC counting compared to the confocal studies. The numbers for both the PC counting and the confocal analyses are presented in Table 1. There is no doubt that the stereological technique for cell counting provides a greater precision in terms of the total number of cells within a defined region (Idrus et al., 2010). Nevertheless, we have previously demonstrated that counting one mid-vermal section provides identical data in terms of the relative magnitude of cell loss and is an appropriate approach in the present study for confirming the well characterized cell loss (Pierce et al., 1999).

4.3. Blood ethanol concentrations

Blood ethanol concentrations (BEC) were determined from the separate groups of pups. On PN4, pups were given a single ethanol dose, then analyzed 2.5 h later. The animals were quickly decapitated and trunk blood was collected for analysis using head-space gas chromatography with flame-ionization detection per our established procedures (Ge et al., 2004).

4.4. Confocal imaging

Confocal image z-stacks were obtained from the mid-sagittal slices of the Lobule I region, most adjacent to Lobule X. Lobule I was chosen because the Purkinje cell loss in that lobule is greater than in any of the other lobules (Pierce et al., 1989; Bonthius and West, 1990; Pierce et al., 1993). The sampling area was approximately 500 × 500 μm in each tissue. All tissues from each of the experimental and control groups were stained together as a cohort to ensure that the immunofluorescent staining was comparable across samples. In addition the confocal image acquisition was conducted so that all samples were imaged using consistent settings for laser power and detector gain.

The confocal images were collected from a Zeiss Pascal confocal microscope with a 20× objective using the following parameters. Resolution was set at 512 × 512 pixels with 12 bit color. Multitrack settings were used with a 543 nm HeNe laser and a 488 nm argon laser. The optical slice was set at 2.0 μm with the slice thickness at 0.75 μm. This oversampling in the z-axis improved the resolution in that axis. Four scans were made per line with the mean of the signal recorded. The pinhole was set at 1 Airy unit.

4.5. Image analysis

One confocal image stack represented the data from one animal within a specific group. The confocal z-stacks were reconstructed into 3D image projections using the Imaris software (Bitplane AG, Zurich, Switzerland). The reconstructed images were first cropped into 3D image projections using the Imaris software (Bitplane AG, Zurich, Switzerland). The reconstructed images were then analyzed as a test of data consistency and to ensure the absence of the control group differences.

4.6. Statistical analysis

Our hypothesis addresses specific questions that relate to the treatment-timing combinations. As a result our first task was to identify whether the four control groups necessitated by these experiments were homogeneous across the different variables. To address this question we conducted a preliminary analysis of variance (ANOVA) including only the four control groups (MRC, C4, C4 – B, and C7 – 9). This analysis served as a test of data consistency and to ensure the absence of the control group differences.

Since there were no significant control group differences, the analysis for the treatment-timing specific alterations involved a 2 × 3 factorial arrangement ANOVA. The six groups were: E4, E4 – E6, E7 – 9, C4, C4 – 6, and C7 – 9. Our approach consisted of first observing the interaction effect of E/C and timing. If this effect was significant at the 5% level, we directly observed the significance of the six custom contrasts (simple effects) of interest. The six custom contrasts of interest were: E4 vs C4, E4 vs E4 – E6, E4 vs E7 – 9, E4 – 6 vs C4 – 6, E4 – 6 vs E7 – 9; and E7 – 9 vs C7 – 9.

In the case of a non-significant interaction effect (p > 0.05) we observed the main effects, that is, the means of the E/C across timing, and the means of the timing, across E/C. In some situations, we included the significance of the six custom contrasts in order to provide additional insights.

We conducted the specific custom contrasts of interest using the mean-squared error values. Bonferroni multiple contrast methods were applied to control for type I error. All statistical analyses were conducted using SAS software (SAS Institute) and graphs were constructed using Prism (GraphPad, Inc.) software.

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