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Ethanol Exposure of Neonatal Rats Does Not Increase Biomarkers of Oxidative Stress in Isolated Cerebellar Granule Neurons

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Abstract

Oxidative stress is a candidate mechanism for ethanol neuropathology in Fetal Alcohol Spectrum Disorders. Oxidative stress often involves production of reactive oxygen species (ROS), deterioration of the mitochondrial membrane potential (MMP), and cell death. Previous studies have produced conflicting results regarding the role of oxidative stress and the benefit of antioxidants in ethanol neuropathology in the developing brain. This study investigated the hypothesis that ethanol neurotoxicity involves production of ROS with negative downstream consequences for MMP and neuron survival. This was modeled in neonatal rats at postnatal day 4 (P4) and P14. It is well established that granule neurons in the rat cerebellar cortex are more vulnerable to ethanol neurotoxicity on P4 than at later ages. Thus, it was hypothesized that ethanol produces more oxidative stress and its negative consequences on P4 than on P14. A novel experimental approach was employed in which ethanol was administered to animals *in vivo* (gavage 6g/kg), granule neurons were isolated 2-24 hr post-treatment, and ROS production and relative MMP were immediately assessed in the viable cells. Cells were also placed in culture and survival was measured 24 hr later. The results revealed that ethanol did not induce granule cells to produce ROS, cause deterioration of neuronal MMP, or cause neuron death when compared to vehicle controls. Further, granule neurons from neither P4 nor P14 animals mounted an oxidative response to ethanol. These findings do not support the hypothesis that oxidative stress is obligate to granule neuron death following ethanol exposure in the neonatal rat brain. Other investigators have reached a similar conclusion using either brain homogenates or cell cultures. In this context, it is likely that oxidative stress is not the sole and perhaps not the principal mechanism of ethanol neurotoxicity for cerebellar granule neurons during this stage of brain development.

Keywords

alcohol; neurotoxicity; reactive oxygen species; cerebellum; brain development

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Introduction

Ethanol exposure during brain development causes the neuropathology underlying Fetal Alcohol Syndrome and Alcohol-Related Neurodevelopmental Disorder (Stratton et al., 1996). The pathology of Fetal Alcohol Syndrome includes significant malformation and dysfunction in the brain including learning, behavioral, social, and psychiatric disorders that persist into adulthood (Sowell et al., 2002; Warren and Bast, 1988). Animal models (Bonthius and West, 1991; Driscoll et al., 1990; Green et al., 2002; Kane et al., 1997; Klintsova et al., 2002; Light et al., 2002b; Light et al., 1998) demonstrate that ethanol exposure during brain development causes significant neuronal loss (Hamre and West, 1993; Maier et al., 1999; Pierce et al., 1989; Pierce et al., 1997; Pierce et al., 1993; Pierce and West, 1987; Pierce et al., 1999). The neonatal rodent model of maternal drinking in the third trimester demonstrates striking Purkinje cell and granule neuron loss in the cerebellum (Goodlett et al., 1990; Light et al., 2002a; Pierce et al., 1999). A particular feature of this neurotoxicity is the differential temporal vulnerability of neuronal loss. When ethanol exposure occurs during the period postnatal day (P) 4-6, significant loss of neurons occurs reproducibly in the cerebellum (Goodlett and Eilers, 1997; Hamre and West, 1993; Miki et al., 1999; Pierce et al., 1999). In contrast, the periods prior to P4 and after P6 are less sensitive to ethanol neurotoxicity. The basis for this differential vulnerability has not been clearly established.

Several direct, as well as indirect, cellular mechanisms may contribute to ethanol-induced neuronal apoptosis during brain development. These include: altered neuronal metabolism (de la Monte et al., 2005; Li et al., 2002), glutamate excitotoxicity (Ikonomidou et al., 2000), disruption of apoptotic regulators (Heaton et al., 2006; Siler-Marsiglio et al., 2005a), limited neurotrophin support (Heaton et al., 2000b; Light et al., 2002b; Light et al., 2001), restricted synaptic development (Klintsova et al., 2002; West et al., 1994), and oxidative stress (Heaton et al., 2003; Heaton et al., 2002; Henderson et al., 1995; Ramachandran et al., 2003; Reyes et al., 1993; Siler-Marsiglio et al., 2005b; Smith et al., 2005). Oxidative stress can cause mitochondrial membrane depolarization, which is followed by cytochrome c release, caspase activation, and apoptosis (Bernardi et al., 2006; O'Rourke et al., 2005). In fact, ethanol treatment in cell culture as well as in the developing brain *in vivo* increases levels of ROS and free radicals, decreases endogenous antioxidant enzyme and metabolite concentrations, and produces lipid peroxidation (Heaton et al., 2003; Heaton et al., 2002; Henderson et al., 1995; Ramachandran et al., 2003; Siler-Marsiglio et al., 2005a; Siler-Marsiglio et al., 2004; Siler-Marsiglio et al., 2005b; Smith et al., 2005).

Although the involvement of ROS in ethanol-induced neurotoxicity has attracted much attention (Sun et al., 2001), a consensus of understanding has not been achieved. This important issue merits resolution because the existing observations provide potential for a pivotal relationship between ethanol-induced oxidative stress and ethanol-induced neurotoxicity.

The present study investigated the hypothesis that the developmental pattern of neuronal vulnerability to ethanol in the cerebellum is a function of the oxidative stress response in the tissue. It may be expected that ethanol exposure in neonatal animals would stimulate ROS production and that the level of ROS produced would be greater with exposure on P4 than on P14. This study contrasted, at P4 and P14, the oxidative response of granule neurons following ethanol exposure *in vivo*. To quantify changes in oxidative stress and determine the timing of cellular response after treatment, granule neurons were isolated 2, 12, and 24 hr after *in vivo* ethanol exposure and the viable neurons were immediately assayed for ROS generation. Mitochondrial membrane potential and cell survival were also measured because detrimental affects of ROS on these cellular mechanisms represent downstream consequences of oxidative stress.

Materials and Methods

The potential for ethanol to induce oxidative stress when administered *in vivo* was evaluated in P4 or P14 rats, an age of either relatively high or low ethanol sensitivity, respectively, in the cerebellum. Animals of each age were divided into three treatment groups: handled control (H); vehicle treated control (V); or ethanol treated (E). The treatment paradigm was designed to optimize detection of an acute alteration in the level of oxidative stress as well as its post-treatment time course. To this end, treatment with ethanol or vehicle was performed once per animal. Analyses were performed 2, 12, or 24 hr after the treatment in order to specifically encompass the period of time (1) a few hours prior to neuronal apoptosis, (2) at initiation of significant increase in neuronal apoptosis, and (3) at mid-peak level of neuronal apoptosis, in the cerebellum (Light et al., 2002a). Loss of granule neurons is essentially complete by 24 hr. Because quantitative measurement of ROS production can be more accurately performed *in vitro* than in the intact animal, granule neurons were isolated from fresh cerebella. Oxidative stress was determined in the freshly dissociated granule neurons immediately upon isolation from the tissue and measured as change in the level of ROS production or mitochondrial depolarization.

In the present report, *in vivo* refers to treatment of live animals. *In vitro* refers to assay of intact viable cells that were freshly isolated from treated animals; it does not refer to assay of cultured cells.

Ethanol Administration

Sprague Dawley CD rats were purchased from Charles River Laboratories and housed in the AAALAC-approved campus facility in the Division of Laboratory Animal Medicine. Animal protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult rats were bred to provide postnatal day 4 and day 14 (designated P4 and P14 respectively) pups for treatment.

Ethanol treated animals were administered a 15% w/v solution of ethanol (6 g/kg) in Intralipid-20%® (Fresenius Kabi Clayton, L.P.) vehicle by intragastric gavage (Kane et al., 1997; Light et al., 2002b; Light et al., 1998; Pierce et al., 1993; Pierce et al., 1999). The blood ethanol concentration was 355-385 mg/dl (n=6; Diagnostic Chemicals Limited kit) 1 hr post-treatment. Vehicle control animals were administered the same volume and concentration of vehicle by gavage. Handled control animals were weighed. In all treatment groups, the dam and pups were separated 5-10 min for weighing and gavage. Animals were sacrificed 2, 12, or 24 hr after treatment. On a given day, 6 pups from 1-2 litters were processed with 3 animals in each of 2 treatment groups, e.g., 3 E and 3 V. Each treatment group at each postnatal age and post-treatment interval contained 11-12 animals from 4 or more litters.

Cerebellar Granule Neurons

Cerebella were dissociated and cerebellar granule neurons were isolated as described previously (Chang et al., 1996,1997; Chang and Liu, 1997,1998; Kane et al., 1996a,b). Animals were sacrificed 2 hr, 12 hr, or 24 hr after ethanol treatment by an overdose of CO_2 and cervical transection. The cerebellum of each animal was minced, digested with trypsin (0.3 mg/ml) for 5 min, and mixed with an equal volume of dissociation buffer (80% DMEM medium, 10% F12 medium, 10 % fetal bovine serum, and 0.1 mg/ml DNase). The tissue was centrifuged (110*x*g, 5 min) and the pellet was dissociated with 1 ml dissociation buffer in a 12×75 mm tissue culture tube. Dissociated cells were collected in the supernatant after the tissue settled by gravity in the tube for 10 min. The dissociation process was repeated on the tissue pellet. The dissociated cells were pooled, centrifuged, and resuspended in 1.5 ml Hank's Balanced

Salt Solution (HBSS). The neurons isolated by this method are >95% granule neurons based on staining of astrocytes and microglia, morphological differentiation of small granule neurons and larger Purkinje neurons, and cell counts (data not shown).

Viability of the cell isolates was assessed by trypan blue dye exclusion and cell counts. This was performed in order to standardize the number of viable cells for each sample and each assay. Thus, the results within each assay are based on an equivalent number of viable cells from each sample. Freshly isolated cerebellar granule neurons from handled control P4 (n=11) and P14 (n=12) animals exhibited similar viability of 97.9 ± 0.3% and 98.6 ± 0.2% respectively. There was no difference in the viability of freshly isolated cells due to treatment. Each cell isolate in the study contained greater than 95% viable cells.

Using parallel aliquots of each cell isolate, the production of reactive oxygen species (ROS) and the mitochondrial membrane potential (MMP) were analyzed immediately, within 1 hr. In addition, aliquots of each cell isolate were placed in culture and neuron survival was measured 24 hr later. All three experimental assays (ROS, MMP, and 24-hr survival) were performed on aliquots of the same sample; that is, every sample was subjected to all three biological assays.

Production of Reactive Oxygen Species (ROS)

Quantitative analysis of ROS production by granule neurons was performed with oxidative conversion of the fluorescent marker H_2DCF-DA (2′, 7′-dichlorodihydrofluorescein diacetate; Molecular Probes, Eugene, OR). Aliquots of the granule neuron suspensions described above were used in this assay. Viable cells (50,000 in 50 μl HBSS) were mixed with 50 μl of 20 μM H_2DCF-DA in 96-well plates and incubated at 37°C for 1 hr. H_2DCF-DA is converted to DCF-DA in the presence of ROS. Thus, relative DCF-DA fluorescence corresponds to the relative level of ROS production (Rosenkranz et al., 1992). Fluorescence of DCF-DA was quantified with a Spectra Max Gemini XS (Molecular Devices, Sunnyvale, CA) at an excitation of 485 nm, emission of 535 nm, and cutoff of 530 nm. The results were expressed as mean ± SE fluorescence units (FU) of DCF-DA at 535 nm.

Change in Mitochondrial Membrane Potential (MMP)

The change in MMP was quantified with the dye JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolocarbocyanine iodide, Molecular Probes, Eugene, OR) as described previously (Garg and Chang, 2004,2006). To prepare JC-1, a 5 mM stock solution was prepared in dimethyl sulfoxide, subsequently diluted 1:4 with 5% BSA, further diluted 1:199 in HBSS, and filtered through a 0.2 μm membrane. Binding of the dye to mitochondria was detected by fluorescence spectroscopy as two emission peaks. A green peak at 545 nm represents JC-1 monomers. A red peak at 595 nm represents JC-1 aggregates, which form at the mitochondrial membrane in the presence of a negative membrane potential in healthy cells. The intensity ratio of these two peaks is a relative measure of mitochondrial potential. An increased 545/595 ratio represents deterioration of the MMP, depolarization, and reduced mitochondrial integrity. The results were expressed as mean ± SE of the ratio.

Aliquots of the granule neuron suspensions described above were used in this assay. Viable cells (250,000 in 250 μl HBSS) were collected by centrifugation (110*x*g, 5 min), resuspended in 250 μl of 5 μM JC-1, and incubated at 37°C for 15 min. Cells were centrifuged, resuspended and centrifuged again. Then the cell pellet was resuspended in 200 μl HBSS and transferred to a 96-well plate. Fluorescence was quantified with a Spectra Max Gemini XS with an excitation of 485 nm, emission of 545 nm and 595 nm, and cutoff of 530 nm. The relative intensity of the two peaks was expressed as the ratio of FU at 545nm/595nm.

Granule Neuron Survival

Survival of isolated cells following culture for 24 hr was assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (Chang and Liu, 2001; Garg and Chang, 2003; Kane et al., 1996a, b; Liu et al., 1997). Freshly isolated cell aliquots were plated in poly-L-lysine (25 mg/l) coated 96-well plates (150,000 viable cells/well) in medium containing 80% DMEM medium, 10% F12 medium, 10% fetal bovine serum, 1.4 mM glutamine, and 25 mM KCl. After overnight culture, the cells were incubated in DMEM containing 100 μg/ml of MTT for 1 hr. The dye was solubilized with dimethyl sulfoxide. The optical density at 570 nm was quantified with a Spectra Max 190 (Molecular Devices, Sunnyvale, CA).

Data Analysis

This set of experiments was performed in 161 animals. Each experimental group contained 11-12 animals, providing sufficient power for analyses. The impact of age (P4 vs. P14), treatment (H vs. V vs. E), and post-treatment time interval (2 hr vs. 12 hr vs. 24 hr) on each of the three outcomes was analyzed using three-way analysis of variance (ANOVA) including all possible interaction terms. The design did not include repeated measures. Each outcome measure (relative ROS production, relative MMP, and 24-hr survival) was analyzed separately.

Three-factor interactions terms were not statistically significant (DF 4) for any of the three outcome measures. Because the design had an unequal n, and the ANOVA models for ROS production and MMP contained statistically significant two-factor interaction terms, these variables were compared as tests of simple effects of least squares means. The least squares means are a model adjustment for the imbalance in the design, and the simple effects compare the effect of one factor while controlling for the other two. Nominal p-values were determined, and Bonferroni adjusted significance determinations were made, maintaining hypothesis-wide alpha levels at 0.05. The adjusted significance levels are noted in footnotes accompanying the tables. Because the only significant effect in the ANOVA model for 24-hr survival was a main effect for age, this variable was reported according to the observed means ± SE and compared between age groups using a *t*-test. All statistical evaluations were performed with SAS® 9.1 (SAS Institute, Cary, NC).

Results

The experiments and analyses tested the hypotheses that cerebellar granule neurons from P4 and P14 animals mount distinct oxidative responses to ethanol exposure. To this end, neurons were freshly isolated from rats that were handled controls, vehicle controls, or were given ethanol *in vivo*. The cells were isolated 2, 12 or 24 hr post-treatment. ROS production, change in MMP, and 24-hr survival were measured to evaluate oxidative stress and its downstream consequences. Cellular levels of each of these biomarkers varied between animals that were treated at 4 versus 14 days of age ($p<0.0001$ ROS and MMP; $p=0.0033$ survival) — because of a singular difference found in P14 animals 12-24 hr after treatment as detailed below.

The level of ROS production by the neurons, as assessed by H_2DCF-DA conversion, was significantly different between the P4 and P14 animals. ROS production was also significantly different between the three treatment groups. This was revealed as a main effect of age ($p<0.0001$, F-factor 19.92, DF 1) and treatment ($p=0.0016$, F-factor 6.75, DF 2) in the ANOVA. The statistical analysis also revealed a significant interaction between age and treatment ($p=0.0216$). The post-treatment time interval did not affect production of ROS and this was reflected in the lack of a main effect or interaction of this factor.

Because the effect of treatment on ROS production differed by the age of the animal, as evidenced by the statistically significant age by treatment interaction, change within each age group was investigated. The constitutive level of ROS production was equivalent in H animals at P4 and P14 (Fig. 1). There was elevated ROS production in V and E animals at P14 compared to P4. The difference was significant at 12 and 24 hr post-treatment in the V group and at 24 hr in the E group (Table 1). These analyses found that treatment with either vehicle or ethanol produced a distinct ROS response at P14.

Similarly, change within individual treatment groups was evaluated, thus exploring interaction between age and treatment. The results were clear; changes in ROS production based on treatment were due primarily to effects within the P14 group and not the P4 group (Fig. 1). Potential differences between treatment groups for specific combinations of age and post-treatment interval were explored. This revealed that neurons from the P4 animals produced the same amount of ROS regardless of the treatment group and the post-treatment interval. In P14 animals, 12-24 hr after treatment, the three treatment groups produced different amounts of ROS ($p=0.0027$), with the highest values in the V and E groups ($p=0.0006$ and $p=0.0118$ respectively). There was no difference in ROS production between V and E animals at any post-treatment interval. Thus, the effect of treatment and/or age on ROS production by granule neurons was specific to P14 animals and was based on treatment with either vehicle or ethanol.

Change in the mitochondrial membrane potential was assayed in aliquots of the same cell isolates used to determine ROS levels. MMP, as assessed with the JC-1 545/595 nm emission intensity ratio, was significantly different between the 4- and 14-day old animals. This was revealed as a main effect in the ANOVA ($p<0.0001$, F-factor 39.38, DF 1). There was no difference in MMP between the three treatment groups. Likewise, MMP did not vary with the post-treatment time interval. There was no difference in MMP between treatment groups for particular combinations of age and post-treatment interval. Interactions between age and treatment ($p=0.0060$) and between age and post-treatment interval ($p=0.0151$) were found. Thus, age and the relationship of age to treatment or to post-treatment interval determined the MMP in isolated granule neurons.

Because the treatment differences in MMP varied based on the age of the animal, change within each age group was investigated. Control H animals in the P4 group and the P14 group exhibited equivalent MMP (Fig. 2). A difference was observed in the V group and in the E group when P4 and P14 animals were compared. P14 animals showed a decrease in the 545/595 ratio, indicating increased mitochondrial polarity and integrity, compared to P4 animals 12-24 hr after treatment with vehicle or ethanol (Table 2).

To assess the potential parallel between oxidative stress and neuron survival, aliquots of the same cell isolates used above were placed in culture and their survival was measured 24 hr later. Analysis established that the ability of the isolated cells to survive in culture depended solely on the age of the animal. Better survival was observed ($p=0.0033$) in 4-day-old animals (0.269 ± 0.085; $n=84$) compared to 14-day-old animals (0.225 ± 0.097; $n=84$). Neither the treatment group nor the post-treatment time interval had an influence on neuron survival, either as main effects or in interaction effects.

Discussion

Based on multiple reports, it has been hypothesized that ethanol induces production of ROS and, further, that antioxidants protect neurons from ethanol-induced death by blocking production of ROS (Heaton et al., 2000a; Heaton et al., 2003; Heaton et al., 2002; Henderson et al., 1995; Ramachandran et al., 2003; Siler-Marsiglio et al., 2004; Siler-Marsiglio et al., 2005b). However, other studies have failed to support the hypothesis (Edwards et al., 2002;

Grisel and Chen, 2005; Pierce et al., 2006; Smith et al., 2005; Tran et al., 2005). The present study employed a unique combination of features to address the hypothesis. In this study, exposure to ethanol occurred in living animals at either 4 or 14 days of age. Treatment was followed by isolation of fresh, viable neurons over a time course of 2-24 hr and immediate assay of oxidative biomarkers. Previous investigations have analyzed brain homogenates from treated animals or have analyzed cultured cells. Although homogenates provide analyses of mixtures of neurons and glia, and cultures provide analyses of rapid changes in a single cell type, it is important to extend our understanding to the level of individual cell types within the context of the living animal. The design of this study provided not only the complexity of ethanol effects that occur in the whole animal but also provided analysis of neuron-specific consequences.

Based on the hypothesis that ethanol neurotoxicity is mediated by oxidative stress, and given differential granule neuron vulnerability to ethanol *in vivo* at P4 versus P14, it was predicted that ethanol would generate an increased oxidative response at P4 relative to P14. However, this was not observed. In fact, ethanol did not produce an oxidative response in granule neurons at either age. Ethanol treatment did not increase neuronal ROS production when compared to vehicle control in either P4 or P14 animals. Ethanol did not cause mitochondrial depolarization in neurons from either P4 or P14 animals. Furthermore, the ability of the neurons to survive was not impaired by ethanol at either age.

These results suggest that oxidative stress is not obligate to granule neuron death associated with neonatal ethanol exposure. This was notable based on previous work of other investigators and current hypotheses (Heaton et al., 2000a; Heaton et al., 2003; Heaton et al., 2002; Henderson et al., 1995; Ramachandran et al., 2003; Siler-Marsiglio et al., 2004; Siler-Marsiglio et al., 2005b). However, it is consistent with reports from several investigators suggesting that oxidative stress is not involved in ethanol neurotoxicity (Edwards et al., 2002; Grisel and Chen, 2005; Pierce et al., 2006; Smith et al., 2005; Tran et al., 2005). That being said, the present findings do not exclude the possibility that ethanol produces oxidative stress that contributes to neuronal death. Although the post-treatment sampling intervals were selected to precede and encompass the period of granule neuron apoptosis that occurs in this model (Light et al., 2002a), a different sampling strategy may provide different results. Perhaps the process of tissue isolation, although rapid, altered oxidative events that were occurring in the animal. Glial cells are a significant source of ROS *in situ*; their removal during the preparation of a purified population of granule neurons alters the neuronal environment relative to the intact brain. Alternatively, perhaps granule neurons are more resistant to pro-oxidative insult than other neuronal populations. It would be interesting to employ the model used in the present study to examine ROS production, MMP, and survival in other neuronal populations following ethanol exposure *in vivo*.

Although an ethanol-specific effect was not identified, neurons from P14 animals given either vehicle or ethanol produced more ROS 24 hr after treatment than handled controls. However, cellular integrity as assessed by MMP was stabilized 12 and 24 hr after treatment, and cell survival was not altered. No such effect was found in P4 animals. The P14-specific, 24 hr-specific increase in ROS may reflect differential cell vulnerability during tissue processing or reflect unique characteristics within the parenchyma. This model will be useful to further investigate the apparent dissociation of oxidative stress and mitochondrial depolarization in these neurons.

In conclusion, the basic question of cause and effect between oxidative stress and ethanol neurotoxicity has not yet been unraveled. The mechanisms underlying ethanol neuropathology in fetal development are likely multi-faceted and complex. Our current understanding suggests that oxidative stress is one of multiple mechanisms that can contribute to ethanol neurotoxicity.

Probing causative mechanisms and understanding their role in fetal alcohol exposure continues to provide new insights, and non-oxidative mechanisms are likely to be uncovered.

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Abbreviations

DF, degree of freedom
E, ethanol treated
FU, fluorescence units
H, handled control
HBSS, Hank's balanced salt solution
H_2DCF-DA, 2′, 7′-dichlorodihydrofluorescein diacetate
JC-1, 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolocarbocyanine iodide
MMP, mitochondrial membrane potential
MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OD, optical density
P, postnatal day
ROS, reactive oxygen species
V, vehicle control

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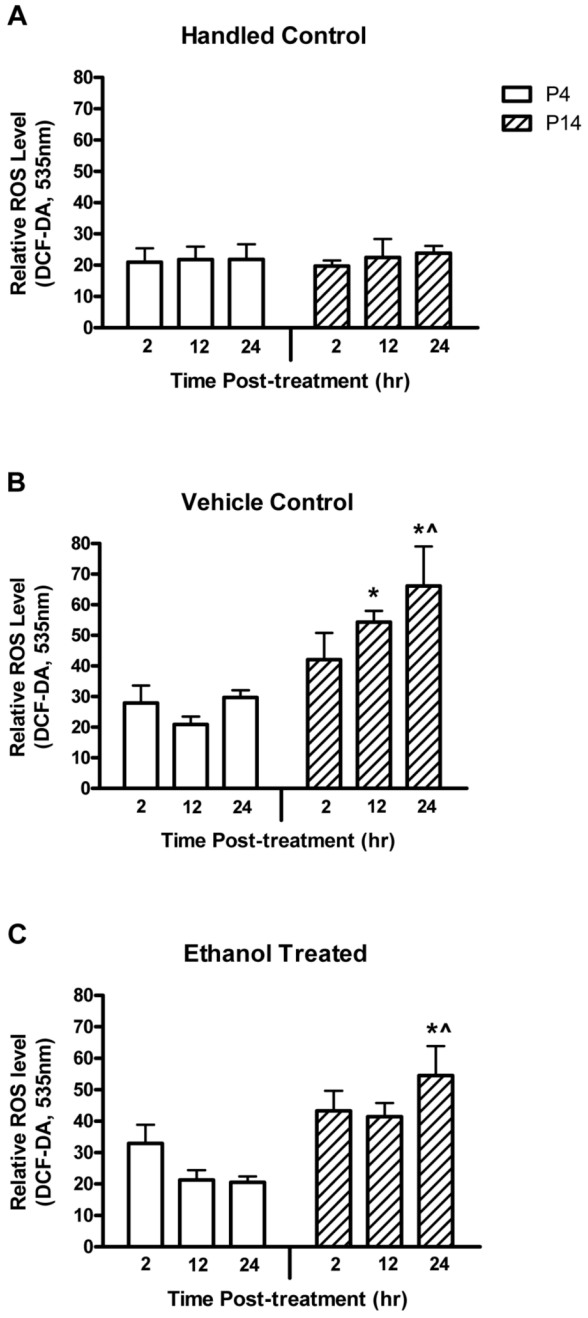

Figure 1.

Ethanol treatment did not increase ROS production in either P4 or P14 animals relative to vehicle controls. Neurons were isolated from handled control (A), vehicle control (B), or ethanol treated (C) animals 2-24 hr post-treatment. The level of ROS was determined by H_2DCF-DA conversion to DCF-DA. Increased DCF-DA fluorescence at 535nm reflects an increased ROS level. The experimental means ± SE are illustrated. The least squares means and the results of the statistical comparisons are detailed in Table 1. No effect specific to ethanol treatment was observed. Higher ROS production was found 24 hr after treatment in P14 animals given either vehicle or ethanol when compared to P4 animals or handled controls. Significant compared to *P4, ^H.

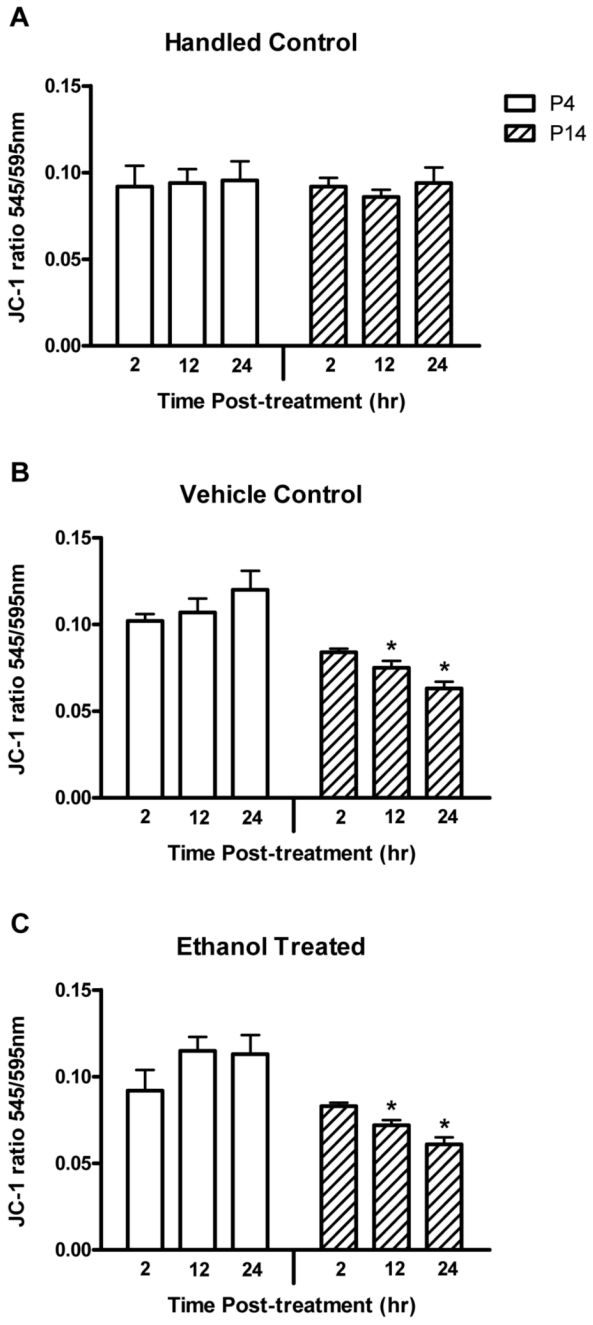

Figure 2.

Ethanol treatment did not cause depolarization of the mitochondrial membrane in either P4 or P14 animals relative to vehicle controls. Neurons were isolated from handled control (A), vehicle control (B), or ethanol treated (C) animals 2-24 hr post-treatment. Change in the MMP was quantified with the fluorescent dye JC-1. A JC-1 emission shift from 595 nm to 545 nm (increase in the 545/595 ratio) reflects depolarization and deterioration of mitochondrial integrity. The experimental means ± SE is illustrated. The least squares means and the results of the statistical comparisons are detailed in Table 2. No effect specific to ethanol treatment was observed. The MMP was more negative, more stable, in P14 animals compared to P4

animals 12 and 24 hr after they were given either vehicle or ethanol. Significant compared to *P4.

Table 1

Comparison of ROS Levels between Ages by Treatment and Time

Treatment Group	Post-Treatment Interval (hr)	Age		Nominal p-value
		P4	P14	
H	2	21.95 (±10.43)	19.80 (±10.43)	0.884
	12	21.75 (±10.43)	22.48 (±10.43)	0.961
	24	21.88 (±10.43)	23.83 (±10.43)	0.895
V	2	27.92 (±6.95)	42.10 (±6.02)	0.126
	12	20.89 (±6.02)	54.33 (±6.02)	0.0001*
	24	29.69 (±6.29)	66.04 (±6.02)	<0.0001*
E	2	32.89 (±6.95)	43.33 (±6.02)	0.258
	12	21.31 (±6.02)	41.43 (±6.02)	0.020
	24	20.50 (±6.02)	54.54 (±6.02)	0.0001*

Results are presented as least-squares mean (± standard error).

*Significant, compared to adjusted p-value = 0.0056.

Table 2

Comparison of Relative MMP between Ages by Treatment and Time

Treatment Group	Post-Treatment Interval (hr)	Age		Nominal p-value
		P4	P14	
H	2	0.092 (±0.011)	0.092 (±0.011)	0.987
	12	0.094 (±0.011)	0.086 (±0.011)	0.585
	24	0.096 (±0.011)	0.094 (±0.011)	0.923
V	2	0.102 (±0.007)	0.084 (±0.006)	0.072
	12	0.107 (±0.006)	0.075 (±0.006)	0.0005*
	24	0.120 (±0.007)	0.063 (±0.006)	<0.0001*
E	2	0.092 (±0.007)	0.083 (±0.006)	0.358
	12	0.115 (±0.006)	0.072 (±0.006)	<0.0001*
	24	0.113 (±0.006)	0.061 (±0.006)	<0.0001*

Results are presented as least-squares mean (± standard error).

*Significant, compared to adjusted p-value = 0.0056.