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Further characterization of the effect of ethanol on voltage-gated Ca^{2+} channel function in developing CA3 hippocampal pyramidal neurons

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Abstract

Developmental ethanol exposure damages the hippocampus, a brain region involved in learning and memory. Alterations in synaptic transmission and plasticity may play a role in this effect of ethanol. We previously reported that acute and repeated exposure to ethanol during the 3rd trimester-equivalent inhibits long-term potentiation of GABA_A receptor-dependent synaptic currents in CA3 pyramidal neurons through a mechanism that depends on retrograde release of brain-derived neurotrophic factor driven by activation of voltage-gated Ca^{2+} channels (Zucca and Valenzuela, 2010). We found evidence indicating that voltage-gated Ca^{2+} channels are inhibited in the presence of ethanol, an effect that may play a role in its mechanism of action. Here, we further investigated the acute effect of ethanol on the function of voltage-gated Ca^{2+} channels in CA3 pyramidal neurons using Ca^{2+} imaging techniques. These experiments revealed that acute ethanol exposure inhibits voltage-gated Ca^{2+} channels both in somatic and proximal dendritic compartments. To investigate the long-term consequences of ethanol on voltage-gated Ca^{2+} channels, we used patch-clamp electrophysiological techniques to assess the function of L-type voltage-gated Ca^{2+} channels during and following ten days of vapor ethanol exposure. During ethanol withdrawal periods, the function of these channels was not significantly affected by vapor chamber exposure. Taken together with our previous findings, our results suggest that 3rd trimester-equivalent ethanol exposure transiently inhibits L-type voltage-gated Ca^{2+} channel function in CA3 pyramidal neurons and that compensatory mechanisms restore their function during ethanol withdrawal. Transient inhibition of these channels by ethanol may be, in part, responsible for the hippocampal abnormalities associated with developmental exposure to this agent.

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Conflict of interest statement

The authors declare that they have no competing interests.

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Keywords

vapor chamber; calcium; voltage-gated channel; hippocampus; neonatal; third trimester

1. Introduction

Fetal alcohol spectrum disorder (FASD) is characterized by increased incidence of neurological and psychiatric conditions, including learning and memory alterations (Davis et al., 2013). The mechanisms responsible for these effects of ethanol are not fully understood, although studies suggest that alterations in synapse formation, refinement, and/or maintenance contribute to the pathophysiology of FASD (Valenzuela et al., 2011). A critical period for normal synapse development is the third trimester (first 1–2 weeks of postnatal life in rats), a period of gestation where a significant number of pregnant women drink alcohol (Ethen et al., 2009). One of the mechanisms of synapse formation is activity-dependent plasticity, which is essential for stabilization of functional synapses and pruning of unneeded synapses (Stoneham et al., 2010). Ethanol exposure during the third trimester-equivalent impairs activity-dependent plasticity mechanisms at both glutamatergic synapses (Puglia and Valenzuela, 2010a; Puglia and Valenzuela, 2010b) and at GABAergic synapses. Specifically, we found that acute and chronic ethanol exposure blocks long-term potentiation of GABA_A receptor-mediated spontaneous postsynaptic currents (LTP-GABA_AR) in developing CA3 pyramidal neurons, which depends on local, retrograde release of brain-derived neurotrophic factor (BDNF) from these neurons (Zucca and Valenzuela, 2010). This plasticity mechanism is thought to participate in the strengthening and stabilization of synaptic connections between GABAergic interneurons and CA3 pyramidal neurons (Caillard et al., 1999; Gubellini et al., 2005).

Although the mechanism underlying the effect of third trimester-equivalent ethanol exposure on LTP-GABA_AR remains to be elucidated, there is evidence that inhibition of L-type voltage-gated Ca²⁺ channels may play a role in this process. The retrograde release of BDNF that is required for the induction of LTP-GABA_AR depends on Ca²⁺ influx via L-type voltage-gated Ca²⁺ channels (Gubellini et al., 2005; Zucca and Valenzuela, 2010). Slice electrophysiological studies showed that acute exposure to ethanol inhibits L-type voltage-gated Ca²⁺ channel currents in CA3 pyramidal neurons from neonatal rats (Zucca and Valenzuela, 2010). Repeated ethanol vapor chamber exposure during the third trimester equivalent also decreased the amplitude of the voltage-gated currents evoked in CA3 pyramidal neurons by the depolarizing pulses used to induce LTP-GABA_AR (Zucca and Valenzuela, 2010).

In this study, we investigated the acute and chronic effects of ethanol exposure on the function of L-type voltage-gated Ca²⁺ channels during the third trimester equivalent. Using Ca²⁺ imaging techniques, we determined that acute ethanol reduces Ca²⁺ transients mediated by voltage-gated Ca²⁺ channels equally in both somatic and dendritic compartments of CA3 pyramidal neurons. However, patch-clamp electrophysiological recordings revealed that ethanol inhibition of L-type voltage-gated Ca²⁺ channels does not persist during ethanol withdrawal periods.

2. Results

2.1. Acute ethanol exposure decreases action potential-induced Ca^{2+} transients in CA3 pyramidal neurons

Our previous study (Zucca and Valenzuela, 2010) showed electrophysiological evidence consistent with acute ethanol-induced inhibition of voltage-gated Ca^{2+} channels in CA3 pyramidal neurons. However, our previous studies did not assess whether ethanol has differential effects on the function of dendritic or somatic voltage gated Ca^{2+} channels. Here, we explored this issue using fluorescence Ca^{2+} imaging. Brain slices were obtained from rats between postnatal day (P) 7 and P9. Whole-cell current-clamp electrophysiology was used to induce action potentials (APs) in CA3 pyramidal neurons. CA3 neurons were allowed to equilibrate with the ratiometric fluorescent Ca^{2+} indicator Fura-2 for 20 minutes. During the 20 minute equilibration, slices were continuously bathed with artificial cerebrospinal fluid (ACSF) or ACSF containing 80 mM ethanol. Three Ca^{2+} transients at 30 second intervals were induced with bursts of 10 action potentials at 20 Hz. F/F_i was measured ratiometrically at ~4 Hz with 350 and 380 nm excitation and ~520 nm emission. Representative ratiometric images of F/F_i are shown for control and ethanol exposed slices in Figure 1A and B, respectively. Ca^{2+} transients were measured at the soma and proximal dendrite (shown in Figure 1A and B), and representative traces are shown in Figure 1C and D. Slices that were exposed to 80 mM ethanol for 20 min exhibited significantly smaller Ca^{2+} transients in both the soma and proximal dendrites compared to slices that were only exposed to ACSF (Figure 1E and F). The peak amplitudes of those responses were significantly reduced by ethanol in both the soma ($t(10)=2.899$, $P=0.0159$; 6 cells from 5 animals) and proximal dendrite ($t(10)=2.801$, $P=0.0188$; 6 cells from 5 animals)(Figure 1G and H). Overall these studies suggest that both dendritic and somatic voltage gated Ca^{2+} channels have similar sensitivity to ethanol. Furthermore, these studies support our hypothesis that ethanol inhibition of dendritic voltage-gated Ca^{2+} channels could reduce the release of BDNF from proximal dendrites.

2.2. Withdrawal from third trimester-equivalent alcohol exposure does not alter passive membrane characteristics

To model third trimester ethanol exposure, we used a vapor inhalation system to expose neonatal pups with their mother to ethanol (Morton et al., 2014). Timed-pregnant rats (gestational days 12–15) were delivered from Harlan Laboratories (Indianapolis, IN) and animals were exposed to either air or ethanol from P2 to P12 for 4 hours every day (Figure 2A). Specifically, we investigated whether voltage-gated Ca^{2+} channel function was affected at P6-P8 and P13-P15 during ethanol withdrawal periods (i.e. 20–40 hours after the last ethanol exposure). Average pup serum ethanol concentration (SEC) was near 60 mM (or 0.28 g/dL; legal intoxication limit = 0.08 g/dl) immediately following the four hr long-exposure (10 animals from 4 litters) (Figure 2B). The average vaporized ethanol concentration in the chamber was 3.359 ± 0.3955 g/dL. The ethanol exposed pup weights ($21.67 \text{ g} \pm 1.176$, $n=15$) were not significantly different from those of the air exposed ($23.8 \text{ g} \pm 1.265$, $n=16$) pups at P12 ($t(29)=1.234$, $P=0.2271$). Third trimester-equivalent ethanol exposure did not persistently affect cell capacitance (P6-P8, $t(12)=0.4741$, $P=0.6440$); P13-P15, Mann-Whitney, $U=48.50$, $P=0.9278$; 8 animals from 4 litters). The membrane

resistance was lower in both the air and ethanol exposed animals at P13-P15 with respect to P6-P8, but was not altered by ethanol exposure at either age (P6-P8, $t(11)=1.197$, $P=0.2563$; 7 animals from 4 litters; P13-P15, $t(18)=1.419$, $P=0.1730$; 10 animals from 4 litters).

2.3. Withdrawal from third trimester-equivalent ethanol exposure does not alter voltage-gated Ca^{2+} channel currents or L-type channel contribution to these currents

To assess if withdrawal from chronic alcohol exposure during the third trimester-equivalent altered voltage-gated Ca^{2+} channel currents, we used whole-cell voltage-clamp electrophysiology and pharmacologically isolated the currents mediated by these channels. In these experiments, we used Ba^{2+} as the charge carrier due the increased conductance of this ion through L-type channels and to reduce Ca^{2+} -dependent inactivation of these channels. Voltage-gated Ca^{2+} channels currents were induced with voltage steps every 30 seconds and L-type voltage-gated Ca^{2+} channels were blocked with 100 μM verapamil. Representative Ba^{2+} currents in the absence and presence of verapamil are shown in Figure 3A and B for P6-P8 and P13-P15 animals, respectively. Third trimester-equivalent ethanol exposure did not affect the peak amplitude of the Ba^{2+} currents at either age (P6-P8, air vs EtOH, $t(7)=1.156$, $P=0.2858$, 6 animals from 3 litters; P13-P15, air vs EtOH, $t(11)=0.6236$, $P=0.5456$, 7 animals from 4 litters). To calculate the % L-type contribution, we subtracted the Ba^{2+} currents following verapamil treatment from baseline currents. Third trimester-equivalent ethanol exposure did not alter the L-type channel contribution at either age relative to air exposed animals (Figure 3E and F)(P6-P8, air vs EtOH $t(7)=0.5062$, $P=0.6283$, 6 animals from 3 litters; P13-P15, air vs EtOH $t(11)=0.05479$, $P=0.9473$, 7 animals from 4 litters). Furthermore, ethanol exposure did not significantly affect the fast or the slow decay time constants of baseline currents from voltage-gated Ca^{2+} channels relative to air exposed animals ((P6-P8; Interaction, $F(1,8)=0.3718$, $P=0.5589$; tau, $F(1,8)=51.41$, $P<0.0001$; air vs EtOH, $F(1,8)=0.9307$, $P=0.3629$)(P13-P15; Interaction, $F(1,12)=1.032$, $P=0.3296$; tau, $F(1,12)=294.5$, $P<0.0001$; air vs EtOH, $F(1,12)=3.394$, $P=0.09$)). Furthermore, in the presence of verapamil the decay constants were not significantly different between air and ethanol exposed animals ((P6-P8, Interaction, $F(1,10)=0.0025$, $P=0.9578$; tau, $F(1,10)=101.5$, $P<0.0001$; air vs EtOH, $F(1,10)=1.360$, $P=0.2706$)(P13-P15, Interaction, $F(1,10)=0.4770$, $P=0.5055$; tau, $F(1,10)=108.7$, $P<0.0001$; air vs EtOH, $F(1,10)=0.4915$, $P=0.4993$)). These studies suggest that withdrawal from third trimester-equivalent ethanol exposure does not have persistent effects on voltage-gated Ca^{2+} channels in developing CA3 pyramidal neurons.

3. Discussion

We previously demonstrated that ethanol acutely inhibits L-type voltage-gated Ca^{2+} channel currents in CA3 pyramidal neurons using somatic whole-cell patch-clamp electrophysiological recordings (Zucca and Valenzuela, 2010). Under these conditions, control of the membrane potential is limited to somatic and perisomatic regions (Williams and Mitchell, 2008). However, dendritic Ca^{2+} influx via these channels is likely responsible for the retrograde release of BDNF that mediates LTP-GABA_AR (Dean et al., 2009; Kuczewski et al., 2008; Magby et al., 2006; Wong et al., 2015). Consequently, we further characterized the acute effect of ethanol using Ca^{2+} imaging techniques, which allowed

visualization of voltage-gated Ca^{2+} channel function at dendritic sites. In agreement with our previous findings (Zucca and Valenzuela, 2010), ethanol acutely inhibited Ca^{2+} transients in developing CA3 pyramidal neurons and the magnitude of the effect was similar at both somatic and proximal dendritic sites. However, this effect was smaller than what we had previously detected using electrophysiological techniques (Zucca and Valenzuela, 2010), despite using a higher concentration of ethanol for the Ca^{2+} imaging studies. Differences in experimental conditions (i.e. AP induced Ca^{2+} transients vs membrane voltage steps) may explain the discrepancies in the results between the two studies. Furthermore, we used slightly younger rats for the slice electrophysiology studies. It should also be kept in mind that L-type voltage-gated Ca^{2+} channels contribute to only 20–30% of the total Ca^{2+} influx in developing CA3 pyramidal neurons (Morton et al., 2013). Considering that acute ethanol appears to selectively target L-type voltage-gated Ca^{2+} channels in these neurons, we expected that the ethanol effect on total Ca^{2+} transients mediated by multiple Ca^{2+} channel subtypes would only result in a maximum of a 30% reduction. Additional studies should further characterize the acute actions of ethanol on Ca^{2+} transients mediated by L-type voltage-gated Ca^{2+} channels only, including those that take place at more distal dendritic sites. To test for the persistence of the ethanol-induced inhibition of voltage-gated Ca^{2+} channel function in developing CA3 pyramidal neurons, we assessed voltage-gated Ca^{2+} channel function during withdrawal periods. We previously found that in-vivo exposure to ethanol during the third trimester equivalent inhibited currents evoked by the depolarizing pulses used to induce LTP-GABA_AR (Zucca and Valenzuela, 2010). We show here that third trimester-equivalent ethanol exposure did not affect total voltage-gated Ca^{2+} channel currents nor L-type channel contribution during ethanol withdrawal. In our previous study (Zucca and Valenzuela, 2010), we prepared the brain slices within an hour of the end of the 4 hr ethanol exposure, whereas in this study, we collected the tissue approximately 20 – 40 hours after the last exposure. Therefore, it is possible that voltage-gated Ca^{2+} channel function is inhibited while ethanol is present and fully recovers during withdrawal. Indeed, rapid development of tolerance of L-type voltage-gated Ca^{2+} channels to ethanol has been documented in neurohypophysial terminals (Pietrzykowski et al., 2013). Moreover, the pups were older at the time of slice collection in the present study (P6-8 and 13-15) vs. our previous study (P4-6), suggesting that the effect of ethanol on voltage-gated Ca^{2+} channels is age-dependent. In addition, for the present studies, we used the L-type voltage-gated Ca^{2+} channel blocker verapamil instead of nifedipine because the latter has been shown to have off target effects, including agonism of transient receptor potential cation channels; specifically, member 3 of subfamily M (i.e., TRPM3 channels) (Hirasawa and Pittman, 2003; Wagner et al., 2008; Zamudio-Bulcock et al., 2011). Taken together, the findings of our study suggest that third trimester-equivalent ethanol causes a transient inhibition of L-type voltage-gated Ca^{2+} channel function in CA3 pyramidal neurons that recovers during ethanol withdrawal. Prolonged exposure (24 hours) to ethanol in cultured hippocampal neurons has been shown to increase the surface expression of L-type voltage-gated Ca^{2+} in the soma and growth cones (Mah et al., 2011). However, under our experimental conditions of repeated ethanol exposure/withdrawal cycles, we did not detect this compensatory effect. Other studies have demonstrated longer-lasting reductions in voltage-gated Ca^{2+} channel function in neurons from prenatally ethanol-exposed rodents (Lee et al., 1996; Servais et al., 2007). Ethanol exposure during the

third trimester equivalent has also been shown to induce long-lasting alterations in voltage-gated Ca^{2+} channel function in distal dendrites of layer 5 cortical neurons (Granato et al., 2012). Therefore, it is important to perform a more detailed characterization of the effect of ethanol on hippocampal neurons and other neuronal population during development.

A number of studies have demonstrated that ethanol exposure can induce neuronal circuit abnormalities in the CA3 hippocampal subfield, a region that has been implicated in the pathophysiology of learning and memory alterations associated with FASD (Berman and Hannigan, 2000; Farr et al., 1988; Livy et al., 2003; Maier and West, 2001; Sakata-Haga et al., 2003). It is possible that the inhibitory effects of ethanol exposure on L-type voltage-gated Ca^{2+} channel function could contribute to these abnormalities, even if these are only transient, via alterations in Ca^{2+} -dependent processes such as gene transcription, protein synthesis, and receptor trafficking, all of which can impact development and refinement of neuronal circuits (Greer and Greenberg, 2008). A better understanding of the role of L-type voltage-gated Ca^{2+} channels in the mechanism by which ethanol affects neuronal development may result in the identification of better interventions against FASD-related learning and memory deficits.

4. Experimental Procedures

4.1. Animals and Slice preparation

The University of New Mexico Health Sciences Center Institutional Care and Use Committee approved all animal procedures. Pregnant Sprague Dawley dams between gestational day 12 and 15 were received from Harlan Laboratories (Indianapolis, IN). Both male and female pups were used for all experiments. Animals were heavily anesthetized with 1 g/kg ketamine followed by decapitation. Brain tissue was removed and incubated for 2–4 minutes in oxygenated ice cold cutting solution (in mM): sucrose, 220; KCl, 2; NaH_2PO_4 , 1.3; NaHCO_3 , 26; MgSO_4 , 12; CaCl_2 , 0.2; glucose, 10; ketamine, 1 mg/ml. Coronal slices were generated using a vibrating slicer (1000 Plus Vibratome, Leica, Bannockburn, Illinois) at a thickness of 300 μm . Slices were incubated in oxygenated artificial cerebrospinal fluid (ACSF) (in mM): NaCl, 125; KCl, 2; NaH_2PO_4 , 1.3; NaCO_3 , 26; glucose, 10; CaCl_2 , 2; MgSO_4 , 1, at 35°C for 40 minutes and allowed to recover at room temperature (21–22°C) for at least 30 minutes prior to recording. All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless specified.

4.2. Electrophysiological Recordings

Slices were maintained in ACSF during recording at approximately 32°C. CA3 pyramidal neurons were identified by morphology under video monitoring of infra-red Differential Interference Contrast Microscopy using a BX51WI upright microscope (Olympus, Center Valley, PA) and a LUMPlan FI/IR 40 $\times$ water immersion lens with 0.8 N.A. Recording electrodes were pulled using a DMZ-Universal Puller (Zeitz Instruments, Martinsreid, Germany) resulting in resistances between 2 and 5 M Ω . Electrodes were filled with internal solution containing the following (in mM): K-gluconate, 135; NaCl, 8; MgCl_2 , 1; HEPES, 10 (pH 7.2 with KOH); ATP Mg^{2+} salt, 2; GTP Na^+ salt, 0.3; EGTA, 0.05. Recordings were obtained with a Multiclamp 700B amplifier connected to a Digidata 1440A and data were

acquired with pClamp 10 software (Molecular Devices Sunnyvale, CA). In the whole-cell configuration, we compensated for junction potential, membrane capacitance, and 70% of series resistance. Voltage-gated Ca^{2+} channel currents were recorded in presence with 1 μM tetrodotoxin (Tocris, Bristol, United Kingdom), 10 μM GABAzine (Tocris), 50 μM DL-(2R)-amino-5-phosphonovaleric acid (Tocris), 10 mM tetraethylammonium, and 1 mM kynurenic acid.

4.3. Calcium Imaging

In the whole-cell patch-clamp configuration, CA3 pyramidal neurons in slices from P7 to P9 rats were filled with the K-Gluconate internal solution (described above) supplemented with Bis-Fura-2 hexapotassium salt (150 μM) (Invitrogen, Carlsbad, CA). Neurons were allowed to dialyze for 20 minutes prior to Ca^{2+} imaging. Ca^{2+} transients were acquired using a Polychrom V imaging system from TILL photonics (Grafelfig, Germany) equipped with a monochromator and an optical fiber to deliver light to the microscope. Within the imaging path a 500 nm dichroic mirror and emission filter 525/50 nm were used to image the fluorescence of Fura-2 at both 350 nm and 380 nm. Data acquisition and analysis were performed with TILLvisION imaging software version 4.5.41 (TILL Photonics). Images were acquired at 350 nm and 380 nm at approximately 4 Hz. Ca^{2+} transients were measured at the soma and dendrites (approximately 20–40 μm from the soma). Ratiometric F/F_i images were produced from background-subtracted 350 nm and 380 nm images. Three Ca^{2+} transients were induced 30 seconds apart by 10 action potentials induced by 5 ms, 1.5 nA current injection, at 20Hz. Ca^{2+} transients were normalized to the pre-action potential baseline fluorescence and averaged for the three Ca^{2+} transients and presented as % baseline (F/F_i).

4.4. Statistical Analysis

All graphs and statistical analyses were performed with Prism 5.0 (GraphPad, San Diego, CA). The level of significance was considered when $p < 0.05$. A statistical unit (n) was considered an individual slice for the acute alcohol experiments and an animal for third trimester-equivalent ethanol exposure experiments (data generated from multiple cells or tissue sections from a single animal were averaged to yield a unit of determination). All data sets were tested for a normal distribution with the D'Agostino and Pearson's test. If data sets followed a normal distribution, a two-tailed student's t-test or two-way ANOVA were used to analyze the data. If the data did not follow a normal distribution a Mann-Whitney test was used. Data are presented as mean $\pm$ S.E.M.

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Highlights

- Acute ethanol reduces somatic and dendritic Ca^{2+} transients in CA3 pyramidal neurons.
- Ca^{2+} channel function is not altered during withdrawal from repeated ethanol exposures.
- The contribution of L-type voltage-gated Ca^{2+} channels is not altered during withdrawal periods.

Figure 1. Acute ethanol inhibits action potential-induced Ca^{2+} transients

CA3 pyramidal neurons were filled with 150 μM Bis-Fura-2 hexapotassium salt for 20 min. Using whole-cell current-clamp electrophysiology, we induced 10 action potentials at 20 Hz with 1.5 nA current injections. The resulting Ca^{2+} transients were monitored by imaging with 350 nm and 380 nm light at ~ 4 Hz. Ratiometric images were generated to calculate F/F_i and representative images of a Ca^{2+} transient are shown for ACSF controls (CTR; A) and 80 mM ethanol (EtOH; B) (Scale bar = 5 μm). Arrows indicate when action potentials were induced. Representative traces (normalized to baseline) from somatic and dendritic regions outlined in A and B are shown for control (C) and ethanol (D) treated cells. Lines underneath traces indicate when the action potentials were induced. Mean Ca^{2+} transients from the soma (E) and dendritic (F) regions are shown from control and ethanol treated cells. Lines underneath traces indicate when the action potentials were induced. The Ca^{2+} transient peak amplitudes from control and ethanol treated cells are compared for somatic (G) and dendritic (H) regions. * $P < 0.05$.

Figure 2. Passive membrane properties of CA3 pyramidal neurons following withdrawal from third trimester-equivalent ethanol exposure

A diagram of the experimental paradigm is shown in A. Time pregnant rats were received between gestational (G) G12-G15. Date of birth was considered postnatal day (P) P0 and animals were exposed to air or vaporized ethanol for 4 hours per day between P2 and P12. Voltage-gated Ca^{2+} channel currents were assessed at P6-P8 and P13-P15. All animals that were used for experiments were withdrawn from alcohol for 20–40 hours. (B) Pups were randomly collected immediately following the ethanol exposure to monitor serum ethanol concentration (SEC). Using whole-cell voltage-clamp electrophysiology we measured the cell capacitance (C and D) and membrane resistance (E and F) in P6-P8 and P13-P15 animals, respectively, from air and ethanol exposed animals.

Figure 3. Voltage-gated Ca^{2+} channel currents and L-type channel contribution following withdrawal from third trimester-equivalent ethanol exposure

Using whole-cell voltage-clamp electrophysiology, we induced voltage-activated channel currents by stepping the membrane potential from -70 mV to 0 mV for 200ms. After baseline currents were established, L-type channels were blocked with $100 \mu\text{M}$ verapamil. Representative Ba^{2+} currents are shown before and after verapamil application for air and ethanol exposed animals at P6-P8 (A) and P13-P15 (B). Mean baseline peak amplitudes for air and ethanol treated animals are shown for P6-P8 (C) and P13-P15 (D). L-type channel contribution was calculated by subtracting the remaining Ba^{2+} current in presence of verapamil from the baseline current and presenting the data as a percent of the baseline peak amplitude for air and ethanol treated animals at P6-P8 (E) and P13-P15 (F). Baseline currents were fit with a second exponential to determine the decay time constants for both air and ethanol exposed animals at both P6-P8 (G) and P13-P15 (H).