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Lithium prevents long-term neural and behavioral pathology induced by early alcohol exposure

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Abstract

Fetal alcohol exposure can cause developmental defects in offspring known as fetal alcohol spectrum disorder (FASD). FASD symptoms range from obvious facial deformities to changes in neuroanatomy and neurophysiology that disrupt normal brain function and behavior. Ethanol exposure at postnatal day 7 in C57BL/6 mice induces neuronal cell death and long-lasting neurobehavioral dysfunction. Previous work has demonstrated that early ethanol exposure impairs spatial memory task performance into adulthood, and perturbs local and interregional brain circuit integrity in the olfacto-hippocampal pathway. Here we pursue these findings to examine whether lithium prevents anatomical, neurophysiological and behavioral pathologies that result from early ethanol exposure. Lithium has neuroprotective properties that have been shown to prevent ethanol-induced apoptosis. Here we show that mice co-treated with lithium on the same day as ethanol exposure, exhibit dramatically reduced acute neurodegeneration in the hippocampus and retain hippocampal-dependent spatial memory as adults. Lithium co-treatment also blocked ethanol-induced disruption in synaptic plasticity in slice recordings of hippocampal CA1 in the adult mouse brain. Moreover, long-lasting dysfunctions caused by ethanol in olfacto-hippocampal networks, including sensory-evoked oscillations and resting state coherence, were prevented in mice co-treated with lithium. Together, these results provide behavioral and physiological evidence that lithium is capable of preventing or reducing immediate and long-term deleterious consequences of early ethanol exposure on brain function.

Keywords

FASD; behavior; olfactory; lithium; neuroprotection

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Introduction

Alcohol consumption during pregnancy can lead to fetal alcohol spectrum disorder (FASD) in offspring, which describes a breadth of developmental impairments (Riley and McGee, 2005). FASD is one of the main causes of intellectual disability in western nations (Abel and Sokol, 1986), with neurobehavioral hallmarks such as deficits in learning, memory and mood. As many as one in 100 children born in the United States and Canada are diagnosed with FASD (May and Gossage, 2001, Chudley et al., 2005), ten percent of which fall under the more extreme designation of fetal alcohol syndrome (FAS) (Fox and Druschel, 2003). Ethanol exposure in neonatal rodents results in widespread and long-lasting neuropathologies comparable to human FASD (Hannigan, 1996). Animal binge models have therefore been established to examine the effects of alcohol consumption during the human third trimester equivalent developmental period (reviewed in Gil-Mohapel et al., 2010). This period is marked by a brain growth spurt with rapid synaptogenesis that makes the brain especially vulnerable to cytotoxic insult.

One of the more devastating consequences of neonatal ethanol exposure is an immediate and robust increase in apoptotic neurodegeneration (Ikonomidou et al., 2000, Olney et al., 2002a, Wozniak et al., 2004, Saito et al., 2007). This immediate wave of neuronal cell death induced by ethanol exposure (Obernier et al., 2002) is followed by longer lasting decreases in neurogenesis (Klintsova et al., 2007) and synaptogenesis (Sutherland et al., 1997, Medina et al., 2005). These developmental setbacks are believed to contribute to long-lasting neural behavioral deficits observed in animal models of FASD (Toso et al., 2005). A single day of ethanol exposure at P7 results in an immediate wave of neurodegeneration in select brain regions in mice, followed by long-lasting neurobehavioral deficits in spatial learning and memory (Wozniak et al., 2004, Wilson et al., 2011). Glycogen synthase kinase-3 β (GSK-3 β) is activated by ethanol exposure, which can lead to the initiation of mitochondria-mediated apoptotic caspase-3 signaling (Chakraborty et al., 2008, Saito et al., 2010b). Several pro-apoptotic pathways, including GSK-3 β , are inhibited by the long-standing psychoactive chemical lithium (Klein and Melton, 1996). Lithium has therefore been pursued in its capacity to limit neuronal cell death in models of neurodegenerative disease and alcohol-induced neuropathology (Chuang, 2005, Luo, 2010). For example, it has been shown that lithium can prevent ethanol-induced apoptosis in cultured rodent neurons (Takadera and Ohyashiki, 2004, Zhong et al., 2006), in *Drosophila* olfactory neurons (French and Heberlein, 2009), and in the P7 mouse brain (Zhong et al., 2006, Chakraborty et al., 2008, Young et al., 2008, Saito et al., 2010b). The protective effects of lithium against early ethanol exposure have thus far focused on the acute stage immediately following treatment. The effects of lithium against long-term ethanol-induced neurophysiological and behavioral dysfunction however, have not been explored.

The olfactory system is increasingly being utilized to assess neurobehavioral function, because deficits in olfactory sensory processing can often be the earliest manifestation of neurodegenerative diseases (Doty et al., 1988, Kesslak et al., 1988, Wesson et al., 2010a, Lutterotti et al., 2011). In both humans and rodents early alcohol exposure damages olfactory neuroanatomy and physiology (Loffe et al., 1984, Kaneko et al., 1993, Cortese et al., 1997, Slawecki et al., 2004, Parnell et al., 2009, Godin et al., 2010, Wilson et al., 2011). Furthermore, the olfactory system provides a major input to the hippocampal formation (Wilson and Sullivan, 2011), thus allowing use of natural sensory inputs (odors) to assay function of this critical area. The olfacto-hippocampal pathway is therefore a valuable tool to measure the neuropathological consequences of early ethanol exposure, and can be applied toward evaluating interventions. Here, we have examined the neuroprotective properties of lithium against long-term developmental deficits induced by ethanol in olfacto-

hippocampal circuit physiology, synaptic physiology and their associated behaviors. Our results suggest that co-treating mice with lithium on the same day as ethanol can prevent both immediate cell death and subsequent long-term disturbances caused by ethanol in hippocampal synaptic plasticity, local and regional olfacto-hippocampal circuit integrity, and associated behaviors known to be dependent on the integrity of said brain regions.

Experimental Procedures

Subjects

Male and female C57BL/6By mice were used as subjects. Mice were bred and born in the Nathan Kline Institute animal facility, and dams were maintained on *ad lib* food and water at all times. All procedures were approved by the Nathan Kline Institute IACUC and were in accordance with NIH guidelines for the proper treatment of animals. Dams and their litters were housed individually in standard mouse cages. Direct injection of ethanol into postnatal day 7 (P7) mice is a well-established method used to model early ethanol neuropathology (Olney et al., 2002b, Wozniak et al., 2004, Izumi et al., 2005, reviewed in Gil-Mohapel et al., 2010). This is done primarily to focus insult during the brain growth spurt period that takes place in rodents, developmentally equivalent to third trimester gestation in humans (Schlessinger et al., 1975). P7 pups were injected subcutaneously with ethanol (2.5 g/kg) twice at 0 hr and 2 hrs as originally described for C57BL/6 mice (Olney et al., 2002a, 2002b). This treatment induces a peak blood alcohol level of ~0.5g/dL when the truncal blood is collected at 0.5, 1, 3, and 6 hrs following the second ethanol injection, as analyzed using Alcohol Reagent Set (Pointe Scientific, Canton, MI). This alcohol level is similar to those reported by others (Wozniak et al., 2004, Young and Olney, 2006, Saito et al., 2007). Lithium chloride (0.6 M LiCl in saline, 10 μ l/g, 6 m Eq/kg body weight) or saline was injected intraperitoneally 15 min after the first ethanol injection as described in (Zhong et al., 2006; Chakraborty et al., 2008). After injections, pups were returned to the litter. Weaning occurred at P25-30. Animals were tested when 3 months old.

Fluoro-Jade C staining

Nineteen hours after the first ethanol/saline injection, both male and female mice were transcardially perfused with 4% paraformaldehyde and 4% sucrose in 67 mM cacodylate buffer (pH 7.2). The mouse heads were then removed and post-fixed overnight in perfusion solution. Mouse brains were then removed and transferred to phosphate buffered saline (PBS) solution, and kept at 4°C for 2–5 days until sectioned with a vibratome at 50 μ m thickness. Sections were stained with Fluoro-Jade C solution (Millipore) following the manufacturer's instructions, slightly modified as follows: sections were mounted onto slides, dried and immersed in an 80% ethanol solution containing 1% sodium hydroxide for 5 min. They were then rinsed for 2 min in 70% ethanol and incubated in 0.06% potassium permanganate solution for 3 min, followed by a 1–2 min rinse in water. Slides were then incubated for 10 min in a 0.0001% solution of Fluoro-Jade C dissolved in 0.1% acetic acid, rinsed three times with distilled water, allowed to dry, cleared in xylene, and coverslipped with DPX (Sigma). The extent of neurodegeneration detected by Fluoro-Jade C positive staining was quantified in the retrosplenial (RS) cortex and the cornu ammonis (CA) brain regions. The number of stained cells in each area of interest (AOI), and total dimensions of the AOI itself were measured using Image-Pro software (MediaCybernetics – version 4.5, Silver Spring, MD, USA). The boundary of each region was defined using the Atlas of the Developing Mouse Brain (Paxinos et al., 2007). Fluoro-Jade C stained not only irregular-shaped degenerating cell bodies, but also other structures such as fragmented axons and dendrites. For quantification, only the degenerating cell bodies were counted. Automatic cell counting after correction (splitting adjacent cells and excluding artifacts) gave similar results to those of manual counting. The extent of neurodegeneration was expressed as the number

of Fluoro-Jade C-stained cells per square millimeter. For each treatment group four to five brains were analyzed, and five sections per brain were counted to yield the average number of positively stained cells per brain. RS and CA sections analyzed were located between 4.35 and 5.19 mm from the most rostral part of the P6 brain according to the mouse brain atlas (Paxinos et al., 2007).

***In vitro* hippocampal physiology**

Three month old male and female mice, treated at P7 with saline (n=5), ethanol (n=5), lithium (n=5) or EtOH+Li n=5), were sacrificed by cervical dislocation followed by decapitation. Hippocampi were quickly removed. Transverse hippocampal slices (400 μ m) were cut and recorded according to standard procedures (Vitolo et al., 2002). Following cutting, hippocampal slices were transferred to a recording chamber where they were maintained at 29° C and perfused with artificial cerebrospinal fluid (ACSF) continuously bubbled with 95% O₂ and 5% CO₂. The ACSF composition in mM was: 124.0 NaCl, 4.4 KCl, 1.0 Na₂HPO₄, 25.0 NaHCO₃, 2.0 CaCl₂, 2.0 MgSO₄, 10.0 glucose, osmolarity at 290–300 mOsmol/L. CA1 field-excitatory-post-synaptic potentials (fEPSPs) were recorded by placing both the stimulating and the recording electrodes in CA1 *stratum radiatum*. Basal synaptic transmission (BST) was assayed by plotting the stimulus voltages against slopes of fEPSP. For long-term potentiation (LTP) experiments, a 10 min baseline was recorded every min at an intensity that evokes a response ~35% of the maximum evoked response. LTP was induced using theta-burst stimulation (4 pulses at 100 Hz, with the bursts repeated at 5 Hz, and each tetanus including 3× 10-burst trains separated by 15 seconds). Responses were recorded for 2 hrs after and measured as fEPSP slope expressed as percentage of baseline. The results were expressed as mean $\pm$ Standard Error Mean (SEM).

***In vivo* olfacto-hippocampal physiology**

Mice tested in the behavioral assays described below were subsequently used for electrophysiological analyses (n = 10 saline, 10 ethanol only, 6 lithium only and 11 EtOH +Li). Mice were anesthetized with urethane (1.5 g/kg) and placed in a stereotaxic apparatus. Electrodes were directed by stereotaxic coordinates according to (Paxinos et al., 2007) to the OB at Bregma 4.5mm, midline offset 1.0 mm, depth 1.5 mm; anterior piriform cortex at Bregma 0.2 mm, midline offset 2.8 mm, depth 4.0 mm; and dorsal hippocampus at Bregma –2.0 mm, midline offset 1.0 mm, depth 2.0 mm for all *in vivo* recordings. Respiration was monitored with a piezoelectric plethysmograph strapped to the animal's chest. Monopolar tungsten electrodes were placed in the olfactory bulb (granule cell layer), anterior piriform cortex (aPCX) (Layer III), and dorsal hippocampus (primarily CA1), and recordings made relative to a scalp reference electrode. Signals were amplified and filtered (0.5 to 300 Hz), digitized at 10 kHz, and stored and analyzed with Spike2 software (Cambridge Electronic Design Inc., Cambridge, England). The odor stimulus (2 seconds of isoamyl acetate diluted 1:100 in mineral oil) was repeated 4 times, and the mean baseline (2 seconds prior to odor onset) and odor-evoked activity (2 seconds from odor onset) were used in the analyses. Spontaneous and odor-evoked local field potentials (LFP) were analyzed (Wesson et al., 2010a) in all 3 regions with fast-fourier transforms (FFT; 2.49 Hz bins). Coherence of spontaneous LFPs between the piriform cortex and dorsal hippocampus were determined using a “cohere” script function within Spike2 at 5-Hz resolution. For paired-pulse analysis, the olfactory bulb electrode was additionally used as a stimulating electrode to evoke monosynaptic potentials in the piriform cortex for assessment of local circuit function and feedback inhibition (Ketchum and Haberly, 1993, Neville and Haberly, 2004). Two, equal intensity constant current pulses (0.1 mA) were delivered at varying interstimulus intervals (20, 50, 100, 300 milliseconds) with a 10-second interpair interval. The slope of the initial positive-evoked potential was used as a measure of response amplitude and the slope of the response to the second (test pulse) was expressed as a percentage of the response to the first

(conditioning) pulse. Comparisons were made between treatment groups using analyses of variance (ANOVAs) and post-hoc Fisher tests.

Cresyl violet histology

Following electrophysiological recordings, brains were removed, fixed in 10% formalin, and subsequently sectioned coronally at a 40 μ m thickness, and stained with cresyl violet to confirm electrode recording sites in the *in vivo* experiments.

Behavior

A hippocampal-dependent object placement task was adapted for mice from (Luine et al., 2003, Macbeth et al., 2008). Mice treated at P7 were tested at 3 months of age (P90-P100). Mice were acclimated to an open Plexiglas arena (20 cm $\times$ 40 cm $\times$ 20 cm) lined with fresh corncob bedding twice a day with inter-trial intervals (ITI) of 10 min on day 1, and 4 hours on day 2. On the third day mice were placed in the same arena with two identical objects (amber glass vials, 2 cm diameter $\times$ 5 cm tall) placed equidistant to adjacent corners of the arena. Investigation duration was quantified over a 5 min time period (Trial 1) then mice were returned to their litter cage. Investigation was scored as time of nose pointing toward the object within 2 cm. Objects and arena were wiped down with 70% ethanol between each session. All alcohol odor was evaporated before each trial. Four hours after training, one object was moved longitudinally to a position diagonal from the object retaining its original position. Investigation time for each object was quantified as a percentage of total investigation time. Mice that did not investigate both objects within the training or test duration were removed from analysis, and the number of animals excluded did not vary between groups. Behavior on the training and testing days were videotaped for offline analyses. Investigation times were compared within each condition between training and test trials using a student's t-test to confirm the behavioral task effectiveness in controls. One-way ANOVA comparison of moved object investigation times during the test trial (trial 2) were made across conditions to determine statistical significance of variance between treatments.

For olfactory behavioral analyses we chose two behaviors known to be dependent on the olfactory bulb and piriform cortex – short-term odor habituation and cross-habituation (Wilson and Linster, 2008). Short-term habituation involves a necessary synaptic depression within the piriform cortex (Best et al., 2005), and the specificity of this habituation involves circuitry within the olfactory bulb and cortex (Linster et al., 2009). For these tests, the Wesson olfactory behavioral assay (Wesson et al., 2010b) was performed. A cotton applicator with 200 μ L of each odor (a 10^{-3} dilution in mineral oil) was presented to individually housed mice. Four molecularly and perceptually diverse monomolecular odorants were used (acetic acid, eugenol, heptanal, nonane; Sigma, St. Louis, MO). The applicator end was inserted into a plastic pipette tip to prevent physical contact with the odorant and presented through a port hole at one side of the cage for 20 seconds. Investigation times were recorded as the duration of time the mouse spent sniffing within 1 cm of the port hole. Each odorant was presented four times (four trials/odor, four odors total) with a 30 second intertrial interval (ITI). After the fourth trial presentation of an odor, the first trial of the next odor was presented 30 seconds later with a repeating ITI sequence.

Results

Ethanol-induced neuronal cell death is prevented by co-treatment with lithium

Ethanol induces caspase-3 activation in P7 mice, which is followed by rapid neurodegeneration (Olney et al., 2002a, Saito et al., 2007). We and others have demonstrated that co-treatment with lithium potentially blocks such ethanol-induced caspase-3

activation (Zhong et al., 2006; Chakraborty et al., 2008; Young et al., 2008; Saito et al., 2010). However, it has not been shown whether lithium actually blocks ethanol-induced neurodegeneration, which follows caspase-3 activation. Here, we examined the effects of lithium on ethanol-induced neurodegeneration as detected by Fluoro-Jade C staining (Fig. 1). Fluoro-Jade C solution has been shown to label degenerating neurons (Schmued et al., 2005). Quantification of Fluoro-Jade-stained cells was performed in the retrosplenial cortex and hippocampal (CA) regions. The retrosplenial cortex was chosen because ethanol induces robust caspase-3 activation and neurodegeneration in this region (Olney et al., 2002b; Saito et al., 2007), and because the region has been implicated in tasks involving spatial navigation (Maguire, 2001) and memory (Svoboda et al., 2006). We also selected the hippocampal region, where substantial cell death was previously observed (Wilson et al., 2011), and which was related to LTP and neurobehavioral studies described below. Two-way ANOVA showed that there were significant effects of ethanol (versus saline) [$F(1,12)=387.6$, $p < 0.001$] and lithium (versus saline) [$F(1,12)=339.7$, $p < 0.001$], and a significant interaction between ethanol and lithium treatments [$F(1,12)=336.8$, $p < 0.001$]. Comparison using one-way ANOVA with Bonferroni's post hoc tests indicated that saline and lithium groups were not statistically significantly different from one another, while ethanol mice had significantly greater proportions of neuronal death in both the retrosplenial cortex and hippocampus (treatment $\times$ FJ positive cells/mm², *Retrosplenial Cortex*: $F(3,12)=295.8$, $p < 0.001$; **Saline** = 5 ± 1 , $n = 4$, $p < 0.001$ vs. Ethanol; **Lithium** = 3 ± 0.9 , $n=4$, $p < 0.001$ vs. ethanol; **Ethanol** = 895 ± 62 , $n = 4$, $p < 0.001$ vs. all other groups; *Hippocampus*: $F(3,12)=65.9$, $p < 0.001$; **Saline** = 3 ± 0.3 , $n = 4$, post-hoc test $p < 0.001$ vs. ethanol; **Lithium** = 4 ± 0.8 , $n=4$, $p < 0.001$ vs. ethanol; **Ethanol** = 134 ± 17 , $n = 4$, $p < 0.001$ compared to all other groups). Lithium co-treatment with ethanol showed dramatically reduced neurodegeneration compared to P7 ethanol-treated mice, and although not completely prevented, was comparable to the baseline detected in controls (*Retrosplenial Cortex*: **EtOH+Li** = 35 ± 10 , $n = 4$, $p < 0.001$ vs. ethanol; *Hippocampus*: **EtOH+Li** = 10 ± 1 , $n = 4$, $p < 0.001$ vs. ethanol). These results concur with our previous findings that ethanol-induced caspase-3 activation is almost completely blocked by lithium treatment in the P7 mouse forebrain (Chakraborty et al., 2008, Saito et al., 2010a), and thus establishes a model of neuroprotection with which we further examined physiology and behavior.

Lithium counteracts ethanol-induced hippocampal dysfunction *in vitro*

Ethanol exposure during synaptogenesis has been shown to cause changes in synaptic plasticity (Izumi et al., 2005, Wijayawardhane et al., 2007, Wijayawardhane et al., 2008) and behavior (Goodlett and Peterson, 1995, Goodlett and Johnson, 1997, Girard et al., 2000, Willoughby et al., 2008) that persist throughout development into adulthood. The molecular mechanisms responsible for these long-lasting deficits are largely unknown. We performed a series of adult hippocampal slice recordings *in vitro* to examine whether lithium, which protects neurons from P7 ethanol-induced neurodegeneration, prevents ethanol-induced impairments of synaptic plasticity. We first determined the electro-physiological parameters of synaptic plasticity, specifically the I/O responses of fEPSP and the LTP of fEPSP, in the Schaffer collateral pathway of hippocampal slices prepared from adult animals treated with saline, ethanol, lithium and EtOH+ Li at P7. Robust I/O responses of fEPSP were evoked by increasing the intensity of stimuli in all groups. The I/O curve of fEPSP was not altered by ethanol, lithium, or EtOH+Li treatments compared to saline controls ($p > 0.05$), suggesting that neither ethanol nor lithium significantly affects the magnitude of fEPSPs from pyramidal cells over the entire range of stimulation intensities (Figure 2b).

Prior to tetanic stimulation, baseline fEPSP were recorded with stimulation at an intensity set to evoke ~35% of the maximum evoked response. These test pulses were delivered at 60-second intervals. Tetanic stimulation evoked typical LTP of fEPSP in slices from adult mice

treated at P7 with saline, ethanol, lithium, and EtOH+Li. These responses were stable over 120 minutes (Figure 2c). However, tetanic stimulation evoked a significantly reduced magnitude of LTP of fEPSP in slices prepared from the P7 ethanol treated animals (fEPSP normalized as percentage of pre-tetanic baseline: **1 min** = 227% $\pm$ 11, **60 min** = 173% $\pm$ 14 and **110 min** = 163% $\pm$ 10, number of animals = 5, 10 slices) compared to saline treated animals (**1 min** = 344% $\pm$ 11, **60 min** = 315% $\pm$ 12 and **110 min** = 323% $\pm$ 13, number of animals = 5, 10 slices). Using a 2 $\times$ 2 ANOVA [ethanol treatment $\times$ lithium treatment], there was a significant group interaction ($F_{(1,44)} = 11.32$, $p = 0.0016$; post-hoc test: **saline vs. ethanol** significantly different at all post-tetanic stimulation time intervals [1 min, 60 min, 110 min], $p < 0.001$).

The magnitude of LTP of fEPSP in slices prepared from lithium only treated mice (**1 min** = 339% $\pm$ 11, **60 min** = 301% $\pm$ 14 and **110 min** = 298% $\pm$ 12, $n=5$, 10 slices) did not differ significantly from saline treated mice (post-hoc test: **saline vs. lithium** at each time interval, $p > 0.05$ NS). However, co-treatment of P7 ethanol-exposed mice with lithium significantly reduced long-term deficits in the magnitude of LTP of fEPSP, both in initial induction as well as maintenance, extending over a 120 minute period (**1 min** = 345% $\pm$ 12, **60 min** = 255% $\pm$ 13 and **110 min** = 244% $\pm$ 17, number of animals = 5, 10 slices; post-hoc test: **ethanol vs. EtOH+Li** significantly different at all post-tetanic stimulation time intervals [1 min, 60 min, 110 min] $p < 0.001$). These results demonstrate that lithium treatment significantly reduced ethanol-induced deficits in LTP. These results are congruent with recent findings that early ethanol exposure in postnatal rodents results in an immediate decrease in LTP (Puglia and Valenzuela, 2010) that can last several weeks (Izumi et al., 2005), and further establish that single-day exposure at P7 causes long-term LTP impairment into adulthood. The ethanol-induced LTP decrease was not completely rescued by lithium however, as LTP levels in **EtOH+Li** slices, while significantly different from ethanol alone, were also significantly different from saline and from lithium only (post-hoc test: **EtOH+Li vs. saline** and **EtOH+Li vs. Lithium only** at 60 min and 110 min post-tetanic stimulation time intervals, $p < 0.01$). The results demonstrate that lithium significantly reduces an early ethanol-induced perturbation of LTP plasticity in the mouse hippocampus.

Lithium treatment reduces ethanol-induced deficits in olfacto-hippocampal function *in vivo*

Fetal alcohol exposure has been shown to alter olfactory function and discrimination (Youngentob and Glendinning, 2009, Akers et al., 2011). Impairments in memory and executive function are associated with damage incurred by the hippocampus (Uecker and Nadel, 1996, Green et al., 2009, Furumiya and Hashimoto, 2011), while the olfacto-hippocampal network is implicated in olfactory learning and memory (Martin et al., 2007). We therefore utilized the olfacto-hippocampal network as a model system to study lithium-mediated prevention of ethanol-induced deficits *in vivo* by performing LFP recordings in the olfactory bulb (OB), anterior piriform cortex (aPCX) and CA1 dorsal hippocampus (dHipp). Saline only ($n = 8$) and lithium only ($n = 6$) control groups did not differ in any of the physiological parameters described below (non-significant post-hoc comparisons from all ANOVAs performed), and are therefore combined and described collectively in our *in vivo* analyses as the “control” group.

As a test of local olfactory circuit integrity in ethanol-exposed mice, we measured evoked monosynaptic potentials in the anterior piriform cortex in response to OB paired-pulse stimulation (first pulse = conditioning, second pulse = test, equal intensity; see Figure 3). Local synaptic inhibition is typically expressed as paired-pulse depression following stimulation (Ketchum and Haberly, 1993). This was indeed the case in our control mice (**20 ms** = 82.6 $\pm$ 3.6; **50 ms** = 84%.4 $\pm$ 2.2, $n = 14$; see Figure 3b). In contrast, ethanol treated

mice showed a statistically significant increase in test pulse response magnitude relative to the conditioning pulse response at the 50 ms interval (2×2 ANOVA significant interaction: saline, ethanol group × saline, lithium treatment, $F_{(1,24)} = 6.60$, **ethanol** factor $p < 0.001$). This shift from depression to facilitation implies local feedback inhibition dysfunction in the anterior piriform cortex of early ethanol treated mice. These results agree with our previously reported data (Wilson et al., 2011). We then pursued whether lithium co-treatment at P7 could prevent long-term ethanol-induced disruption of local anterior piriform cortex synaptic inhibition. Paired pulse response curves of littermates co-treated with both ethanol and lithium at P7 retained inhibition equivalent to controls (**50 ms** = $84.53\% \pm 9.6$, two-way ANOVA: $F_{(1,24)} = 6.60$, saline factor $p = 0.83$, NS). Thus, the neuroprotective properties of lithium were capable of preserving local inhibition in the anterior piriform cortex that is otherwise compromised into adulthood by P7 ethanol exposure.

To investigate the ethanol-induced neural dysfunction and potential lithium-mediated preservation of olfactory information flow in the olfacto-hippocampal pathway, we performed *in vivo* LFP recordings on anesthetized adult mice exposed to ethanol at P7 (Figure 4). Two second odor puffs were administered four times and odor-evoked LFP power spectra were quantified by FFT. Odor-evoked activity in the hippocampus showed a significant overall group affect, with enhanced responses in ethanol treated mice (ANOVA: treatment group [4] × odor-evoked power at frequency, $F_{(3,24)} = 3.09$, $p = 0.046$; Fisher's post-hoc tests for **saline vs. ethanol** and **ethanol vs. EtOH+Li** revealed significant differences at beta band frequencies [15Hz–34Hz], $p < 0.05$). This supports our previously reported data (Wilson et al., 2011). Lithium co-treatment at P7 prevented this early ethanol-induced elevation of odor-evoked power in the dorsal hippocampus (Post-hoc test: ethanol vs. EtOH+Li, $p < 0.05$ for all three frequencies reported above, $p < 0.01$ vs. ethanol; see Figure 5c). In contrast to the hippocampus, odor-evoked activity in the adult OB and aPCX of P7 ethanol-exposed mice were not significantly different from controls (Figure 5a).

Considering the increased odor-evoked hippocampal activity and the changes in piriform cortex paired-pulse inhibition in ethanol-exposed mice, it is apparent that information flow along the olfacto-hippocampal pathway is distorted at multiple points. The hippocampus receives olfactory input from both the olfactory bulb and piriform cortex via the lateral entorhinal cortex for integration into olfactory-based memory and behavior (Staubli et al., 1986, Martin et al., 2007). As a measure of functional connectivity between brain regions, we measured spontaneous resting state coherence between the OB and anterior piriform cortex (OB:aPCX), the anterior piriform cortex and dorsal hippocampus (aPCX:dHipp), and regional olfacto-hippocampal network coherence between the OB and dorsal hippocampus (OB:dHipp) in anesthetized adult mice exposed to ethanol at P7 (Figure 6). Ethanol-exposed mice exhibited significantly elevated coherence between the OB and hippocampus compared to saline and lithium only controls, distinguished in the beta band region (ANOVA: treatment group [4] × coherence at frequency, $F_{(3,36)} = 3.74$, $p = 0.019$; Fisher's post-hoc test: **saline vs. ethanol** significantly different at delta [0–5Hz], theta [5–15Hz] and beta [15–35] frequency ranges, $p < 0.05$). The rise in spontaneous coherence in P7 ethanol-exposed mice supports our previously reported observations (Wilson et al., 2011). There was an slight increase in coherence between the other two region pairs (OB:aPCX and aPCX:dHipp); however, there was no significant overall group effect observed ($p = 0.21$ and 0.053 , respectively).

We next tested whether lithium could prevent ethanol-induced changes in functional connectivity along the olfacto-hippocampal pathway as demonstrated by spontaneous coherence between the OB and hippocampus. Lithium co-treatment of P7 ethanol-exposed mice resulted in a marked return of coherence between these regions to control levels, with significantly lower coherence than mice exposed to ethanol alone (Fisher's post-hoc test:

ethanol vs. EtOH+Li significantly different in the beta band frequency range, $p < 0.05$, see Figure 6c). Lithium therefore significantly prevented elevated coherence in the beta band, but not in the delta and theta band frequency range. As noted above, there was no significant difference of coherence between lithium and saline treated control groups (post-hoc test: **Saline only vs. Lithium only**, $p > 0.50$). These results, in combination with the lithium-mediated protection of piriform cortical inhibition and odor-evoked activity in the hippocampus, provide *in vivo* physiological evidence that lithium is sufficient to prevent long term ethanol-induced defects in olfacto-hippocampal processing.

Early ethanol exposure does not affect odor habituation

Defects in olfactory processing can arise as behaviorally measureable defects in odor discrimination. P7 ethanol-exposed mice show numerous alterations in olfactory physiology that are prevented by lithium co-treatment. In order to assess whether these differences manifest in olfactory behavior, we performed a standardized odor habituation task using a series of four pure and molecularly distinct odors (Wesson et al., 2010b, Wilson et al., 2011). Both short-term odor habituation and piriform cortical odor adaptation involve depression of olfactory bulb input synapses to piriform cortical pyramidal cells (Best and Wilson, 2004, Best et al., 2005). However, despite the significant defects we detected in olfactory information flow physiologically, no change in odor habituation behavior was observed between treatment groups. All groups habituated normally by the fourth trial of each odor (Figure 7a). In addition to repeated odor habituation, we examined cross-habituation between odors by quantifying the amount of time spent investigating a novel odor during the first trial, after already habituating to a previous odor. The difference in investigations between trial 1 of odor $n+1$ was compared with the investigation of trial four of odor n . Over the course of the four odors, both saline treated and lithium only treated mice spent less time on average investigating the first trial of a novel odor compared to the last trial of the odor presented before it (**Saline** = $-0.80s$ / odor presented ± 0.11 , $n=10$; **Lithium** = $-0.69s$ / odor presented ± 0.11 , $n=8$) compared to ethanol-treated mice (**Ethanol** = $-0.23s$ / odor presented ± 0.15 , $n=13$). This decrease in cross-habituation was counteracted by co-treatment of lithium **EtOH+Li** = $-0.88s$ / odor presented ± 0.10 , $n=8$. This decrease implies some cross-habituation, perhaps reflecting limited odor discrimination in all groups (Fletcher and Wilson, 2002). There was a trend for decreased cross-habituation (i.e., enhanced odor discrimination) in ethanol mice; however, the interaction effect in a two-way ANOVA (saline and ethanol groups $\times$ saline and lithium co-treatments) was not quite statistically significant ($F_{(1,36)} = 3.32$, $p = 0.07$). The non-significant tendency for decreased cross-habituation in ethanol mice was not an artifact of varying interest in the task based on treatments, as initial odor investigation times for the first trial of the first odor was consistent across all groups (**Saline** = $4.7s \pm 0.68$; **Lithium** = $4.8s \pm 0.78$; **Ethanol** = $5.1s \pm 0.71$; **EtOH+Li** = $4.8s \pm 1.13$; see Figure 7b), as was odor habituation, described above.

Long-term ethanol-induced spatial memory impairment is prevented by lithium

Fetal alcohol exposure can generate neurobehavioral deficits, particularly in learning and memory in both mice and humans (Uecker and Nadel, 1996, Wozniak et al., 2004, Willoughby et al., 2008). This has been attributed, at least in part to increases in neurodegeneration (Ikonomidou et al., 2000, Saito et al., 2010b) and decreases in neurogenesis (Klintsova et al., 2007) in the hippocampus, although other brain regions affected by ethanol may contribute to this neurobehavioral deficiency (Wozniak et al., 2004). A consensus is that spatial memory at the very least relies on the functional integrity of hippocampal circuits (Matthews and Silvers, 2004). It has been shown that ethanol exposure at P7 mice causes neurodegeneration in select regions of the brain, including the hippocampus, and is correlated with a long-term impairment in spatial memory (Wozniak et al., 2004). Since we found lithium co-treatment in P7 ethanol mice is capable of

dramatically reducing neurodegeneration in the hippocampus and preventing most neurophysiological consequences in the olfacto-hippocampal circuit *in vitro* and *in vivo*, we tested whether lithium could prevent the ethanol-induced behavioral impairment of spatial memory in a standardized object placement task (see Materials and Methods). Starting locations of the identical objects in the observation arena were balanced, and none of the treatment groups showed an overall preference for either object location during training, as each was investigated equally and equivalent to chance (Figure 7c). One of the two identical objects was then moved to a novel location with a four hour ITI between the training (trial 1) and test trial (trial 2). The investigation times for the moved object were normalized in proportion to total investigation times for both (unmoved + moved) objects as a measure of spatial memory. Both saline only and lithium only mice showed a significant increase in the percentage of investigations for the moved object during the test trial (**Saline**: Trial 2 = 64.0% $\pm$ 2.9 vs. Trial 1 = 50.1%, $p < 0.05$, $n = 6$; **Lithium**: Trial 2 = 61.9% $\pm$ 2.1 vs. Trial 1 = 47.8% $\pm$ 3.0, t -test (9) = 26.8, $p = 0.0019$), thus displaying a functional spatial memory (Luine et al., 2003). These two equivalent data sets were therefore combined and expressed generally as the control group. P7 ethanol-exposed mice did not show a significant increase in investigation time (**Ethanol**: Trial 2 = 51.9% $\pm$ 4.65 versus Trial 1 = 47.6% $\pm$ 4.65, t -test (5) = 11.15, $p = 0.47$), indicating impaired spatial memory. These results concur with our previous findings in P7 ethanol-exposed mice (Wilson et al., 2011). Interestingly, spatial memory task performance was retained in ethanol-exposed mice co-treated with lithium (**EtOH+Li**: Trial 2 = 65.6% $\pm$ 2.64 versus Trial 1 = 50.99% $\pm$ 4.58, t -test (6) = 15.36, $p < 0.030$). There was no significant difference between raw investigation times during the test trial between conditions ($p = 0.42$). Additionally, moved object test trial investigations were compared across conditions, using a one-way ANOVA and Fisher's post-hoc test. Control mice and ethanol mice co-treated with lithium had significantly increased moved object investigation during the test trial compared to ethanol-exposed mice ($p < 0.05$). Therefore, lithium co-treatment given on the day of binge ethanol exposure (P7) was sufficient to maintain spatial memory task performance into adulthood. This behavioral result corresponds well with our neuroanatomical and neurophysiological data above.

Discussion

We have demonstrated that the neuroanatomy, *in vitro* synaptic plasticity, and *in vivo* olfacto-hippocampal information flow are impacted into adulthood in mice exposed to binge ethanol at P7. These neural defects correlate with behavioral impairments in hippocampal-dependent spatial memory observed in the same animals, and as reported elsewhere (Wozniak et al., 2004, Wilson et al., 2011). Although short-term odor memory in the form of habituation was preserved in ethanol mice, odor discrimination as measured by cross-habituation was slightly but not significantly enhanced ($p = 0.07$). Most importantly, we have shown that lithium co-treatment on the day of ethanol exposure was sufficient to prevent ethanol-induced neurobehavioral pathologies at every level examined, ranging from neural survival to synaptic physiology to neural circuit function to behavior.

The present results replicate our previous findings (Wilson et al., 2011) that while damage does occur in the olfactory system, the adult hippocampus is more robustly affected by P7 ethanol at the neuroanatomical, neurophysiological and behavioral levels. Furthermore, functional connectivity between components of the olfacto-hippocampal pathway is disrupted. The difference in sensitivity between the hippocampus and olfactory system most likely reflects respective differences in developmental maturity at P7 (Farbman, 1992, Zhao et al., 2006). For example, although extensive postnatal neurogenesis and synaptogenesis occur in the rodent olfactory system (Bayer, 1983, Carleton et al., 2003), olfactory sensory neurons, olfactory bulb mitral cells, and piriform cortical pyramidal cells all develop very early during ontogeny (Schwob and Price, 1978, Bayer, 1983, 1986, Brunjes and Frazier,

1986), allowing simple olfactory-guided behavior even in utero (Smotherman et al., 1990). In contrast, hippocampal dependent behaviors emerge several weeks later (Chun and Johnson, 2007). Additionally, ethanol exposure as late as adolescence can induce long-lasting modifications in EEG and auditory event related potentials (Slawecki et al., 2001, Ehlers and Criado, 2010). Work ongoing is examining the age-sensitivity of the effects reported here. Finally, while degenerating neurons appear to be removed by activated microglia within 72 h (Olney et al., 2002b, Saito et al., 2010b), persisting cell loss has been reported in the anterior cingulate cortex, CA1, and thalamus at P14 (Ieraci and Herrera, 2006) and in anterodorsal nucleus of the thalamus at P14, P30 and P90 (Wozniak et al., 2004) in P7 mice treated under the same ethanol conditions as the present study. In addition to permanent loss of neurons present at the time of ethanol exposure, P7 ethanol exposure also decreases neurogenesis in the adult hippocampus (Ieraci and Herrera, 2007).

Ethanol exerts its toxic effects on central nervous system development through multiple pathways leading to increased apoptotic neurodegeneration (West et al., 1986, Ikonomidou et al., 2000), decreased cell motility (Carter et al., 2008), decreased neurogenesis (Nixon and Crews, 2002), and decreased pro-survival signaling (Young et al., 2008, Eade et al., 2010). To study the protective properties of lithium we have employed the P7 ethanol exposure binge model. P7 is a significant stage in rodent brain development at the peak of the “growth spurt” period (Olney et al., 2002b) - a period which occurs in human brain development during gestation in the third trimester (Dobbing and Sands, 1973). FASD pathology has therefore been associated with the well-documented consequence of neuronal cell death caused by alcohol during this critical stage of development. Early ethanol exposure promotes glycogen synthase kinase-3 β (GSK-3 β) activation, which is one of the more thoroughly studied mechanisms of neuronal cell death signaling (reviewed in Luo, 2010). Once active, GSK-3 β promotes multimodal neurodegeneration by eliciting pro-apoptotic pathways via caspase-3 (Takadera and Ohyashiki, 2004, Young et al., 2005, Zhong et al., 2006), tau cleavage (Saito et al., 2010b), and the subsequent aggregation of cleaved tau (Slawecki et al., 2001). Ethanol may enhance the apoptotic pathway by activating GSK-3 β , and by suppressing pro-survival kinases, such as ERK (Young et al., 2008), Akt and AMPK (Chakraborty et al., 2008). In addition to its traditional role as a psychoactive drug, lithium promotes the inhibition of GSK-3 β (Jope, 2003). In fact, *in vivo* studies have supported a model of lithium protection from ethanol induced neurodegeneration through GSK-3 β inhibition (Chakraborty et al. 2008., Saito et al., 2010b). However, *in vitro* work suggests that lithium's role as a neuroprotectant against ethanol toxicity is independent of GSK-3 β inhibition (Zhong et al., 2006). Lithium, thus, may work through a variety of mechanisms. For example, it is likely that the potential activation of Akt and AMPK by lithium (Chakraborty et al., 2008), which implicates cell survival (Blazquez et al., 2001, Culmsee et al., 2001, Kuramoto et al., 2007, Manning and Cantley, 2007), may also contribute to the neuroprotective action of lithium. There are additional modes through which lithium may be considered a neuroprotectant against fetal alcohol toxicity. For example, lithium also prevents neurodegeneration through the inhibition of Bax and p53 signaling, as well as by promoting the neurotrophic activity of BDNF, VEGF and CREB (Chuang, 2004). Further work is required to sort out the mechanisms of lithium protection against ethanol-induced neuropathology.

Our data support the hypothesis that a major cause of lasting early ethanol-induced neurologic defects is due to the wave of cell death of neurons, glia and stem cells that immediately follows ethanol exposure. Loss of these cell populations early in brain development may initiate a cascade of effects on subsequent neural circuit maturation. Specific effects are likely dependent on the age when toxic exposure occurs relative to the maturation of specific circuits. For example, ethanol induces the loss of GABAergic interneurons (Granato, 2006). GABA-mediated inhibition plays a major role in mature

circuit function; including, control of cell excitability, lateral and feedback inhibition, and imposing temporal structure on spike trains and circuit oscillations (Buzsaki, 2006). Given these effects, loss of GABAergic interneurons during early development would be expected to have major effects on circuit maturation by influencing such basic and critical processes as activity-dependent synapse maintenance and pruning, dendritic structure, cell survival and circuit connectivity. These effects may be limited to one circuit or another depending on the time, intensity and duration of cytotoxic insult. Similar scenarios can be envisioned for disruption of other cellular phenotypes. For example, loss or reduction in stem cell populations may limit the ability of adult circuits to support the neurogenesis required for memory (Nixon and Crews, 2002, Klintsova et al., 2007) or pattern separation (Sahay et al., 2011). Lithium treatment may block the pathological effects of ethanol exposure at P7 by preventing the initial wave of cell death that follows, resulting in near normal adult neurobehavioral expression.

Although short-term odor habituation was not affected by early ethanol exposure, olfactory information flow is misrepresented in mice exposed to ethanol at P7, indicated by relative increases in odor-evoked oscillations in the OB and hippocampus, reduced paired-pulse facilitation in the piriform cortex, and elevated LFP coherence throughout the olfacto-hippocampal pathway. Interestingly, early ethanol exposed mice also exhibited a tendency toward lower average cross-habituation to novel odors ($p = 0.07$), suggesting enhanced odor discrimination of molecularly dissimilar odorants. Together, these results suggest a long-lasting early ethanol induced hyper-excitability (reduced inhibition, elevated sensory responsiveness and increased functional connectivity) in the olfacto-hippocampal system. These effects were prevented with lithium co-treatment. The slight improvement in odor discrimination could result from the enhanced odor-evoked activity we observed, given that odor discrimination improves as stimulus intensity increases above detection threshold (Daly et al., 2008). Using a prolonged gestational exposure model in rats, Youngentob and colleagues have reported a long-lasting enhancement in both behavioral and olfactory epithelial response to ethanol odors, though they found their enhancement was largely limited to ethanol odor itself (Youngentob et al., 2007, Eade et al., 2010). Nonetheless, this suggests that peripheral changes could also contribute to the enhanced odor-evoked responses we observed here in central structures.

In contrast to the effects of the P7 binge model used here, prolonged ethanol exposure during prenatal development through maternal consumption can impair adult odor discrimination (Akers et al., 2011). Prolonged gestational ethanol exposure, or binge exposure during very early gestational ages (e.g., embryonic day 7) in mice can produce severe reductions in olfactory bulb development or even agenesis of the olfactory bulb (Godin et al., 2010, Akers et al., 2011). In contrast, as shown here, brief exposure in more developed mice has limited effects on olfactory bulb gross anatomy, though it significantly modifies olfactory system function.

In summary, lithium exhibits a profound capacity to prevent a broad range of anatomical, physiological and behavioral effects of ethanol-induced developmental neuropathology when administered at the same time as ethanol. Clinically, the lithium-mediated prevention of ethanol-induced neural and behavioral dysfunction presented here are particularly exciting if lithium's neuroprotective capacity is not limited to ethanol toxicity, but rather extends to other forms of neuropathology. For example, lithium's inhibition of GSK-3 β mediated apoptosis, tau cleavage and A β accumulation, support a potential therapeutic role in neurodegenerative diseases and acute ischemia (Chuang, 2004, Rowe and Chuang, 2004, Hunsberger et al., 2009). Recent studies have also shown neuroprotective effects of lithium treatment following traumatic brain injury (Zhu et al., 2010, Yu et al., 2011). Mice treated with lithium alone did not differ in any of our neurobehavioral measures from saline injected

mice. While the consequences of lithium treatment to human fetal development are currently not known, this study provides incentive to explore the clinical potential of lithium as a prophylactic against early alcohol-induced neurobehavioral pathology. More work is required to examine whether lithium itself would be a feasible approach of limiting ethanol-induced neuropathology, or whether downstream cellular effectors of lithium action could be targeted to minimize collateral damage.

Highlights

- Ethanol exposure during early development induces an immediate wave of cell death
- This exposure results in impaired hippocampal synaptic plasticity in adult mice
- There is an impairment in information flow through the olfacto-hippocampal circuit
- Finally, these long-lasting physiological changes are associated with impaired memory
- Co-treatment with lithium prevents cell death, and all neurobehavioral disruptions

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Figure 1.

Lithium blocks acute ethanol-induced neurodegeneration. P7 mice were treated with saline (control), ethanol, lithium, and ethanol+lithium, and the brains were perfusion-fixed 19 hrs after treatment. Brain sections were then stained with the Fluoro-Jade C reagent. **A)** Representative images show the retrosplenial (RS) cortex and the hippocampus CA1 regions. **B)** Quantification of Fluoro-Jade-stained cells in the retrosplenial cortex. **C)** Quantification of Fluoro-Jade-stained cells in the *cornu ammonis* (all CA regions). Ethanol groups were significantly different from all other groups as determined by a one-way ANOVA with the Bonferroni post-hoc test (* $p < 0.05$)

Figure 2.

Lithium rescues P7 ethanol-induced synaptic plasticity measured in adult mice hippocampus. **A)** Schematic diagram of stimulating and recording electrode positions in CA1 region of the hippocampus (DG = dentate gyrus). **B)** Summary graph to show field input/output relationships for P7 saline, ethanol, lithium and EtOH+Li. **C)** The superimposed fEPSPs illustrate respective recordings from representative experiments taken at 0–10 min (dotted line) prior to, and 80–90 min (solid line) post tetanic stimulation. Scale: 1 mV, 10 ms. **D)** Time course of the averages of fEPSP slopes from slices obtained from saline, ethanol, lithium and EtOH+Li treated mice. fEPSP slopes were normalized to the average value during the 10 min before stimulation in each experiment. Arrows show the time of tetanic stimulation (4 pulses at 100 Hz, with bursts repeated at 5 Hz, and each tetanus including three 10-burst trains separated by 15 s). **E)** Combined plots of the averages of fEPSP slopes at several time points. Each point is mean $\pm$ SEM ($n = 5$ mice/group; 10 slices/group). Statistical significance tested by two-way ANOVA *** $P < 0.001$.

Figure 3. Paired-pulse analysis of local synaptic function in the anterior piriform cortex. **A)** Representative responses recorded in the Layer III of the anterior piriform cortex to olfactory bulb stimulation (stimulus artifact at arrows). The onset slope (post artifact) of the response to the second (test) pulse was expressed as a ratio with the onset slope of the response to the first (conditioning pulse). **B)** Mice exposed to ethanol at P7 show impaired paired-pulse depression with a shift to paired-pulse facilitation. Lithium co-treatment of P7 ethanol-exposed mice prevents this shift. Statistical significance at each time interval tested by two-way ANOVA, * $P < 0.01$.

Figure 4.

A) A representative LFP trace with basal activity preceding a two second odor stimulation, producing evoked potentials recorded in the olfactory bulb (OB), anterior piriform cortex (aPCX) and dorsal hippocampal CA1 (dHipp) *in vivo*. The colorimetric representation of FFT analysis before, during and after odor stimulation, reveal comparative differences in baseline, odor-evoked and recovery power spectra respectively (AU: arbitrary units). **B)** Electrode recording sites labeled with red dots on coronal plane diagrams. Each diagram is derived from a line along the rostral-caudal axis of the upper sagittal view. Arrows indicate the magnified area of interest (aPCX and dHipp only). cg = cingulate, dhc = dorsal hippocampal commissure, DG = dentate gyrus, EPL = external plexiform layer, GCL = granule cell layer, GL = glomerular layer, MCL = mitral cell layer, ONL = olfactory nerve layer, lo = lateral olfactory tract. Roman numerals delineate cell layers within the piriform cortex. Scale bars = 0.25 mm

Figure 5.

Odor-evoked LFP power in the (A) olfactory bulb (OB), (B) anterior piriform cortex (aPCX) and (C) dorsal hippocampus (dHipp). (D) Total mean LFP increases relative to baseline from all frequency bands (0–80Hz), plotted for each recording location. OB and aPCX showed no significant difference between groups. Beta band frequency range for which significance was found between groups is highlighted by a white background, with other unaffected bands shaded in grey in (C). Statistical significance between ethanol and control groups, as well as ethanol and lithium co-treatment groups were attained by repeated measures ANOVA * $p < 0.05$.

Figure 6.

Regional olfacto-hippocampal network spontaneous coherence comparison, recorded between the (A) olfactory bulb and anterior piriform cortex (OB:aPCX), (B) anterior piriform cortex and dorsal Hippocampus (aPCX:dHipp) and the (C) olfactory bulb and dorsal hippocampus (OB:dHipp). Ethanol enhanced spontaneous coherence between the OB and dHipp. This elevated coherence was effectively prevented in the beta frequency band range in mice co-treated with lithium. (D) Total mean coherence from all frequency bands (0–80Hz) from all brain region pairs, providing an overall view of ethanol-induced effects, and lithium-mediated prevention of the ethanol effect. OB:aPCX and aPCX:dHipp showed no statistical significance between groups. Statistical analysis by repeated measures ANOVA revealed significance between the ethanol group and the control and lithium co-treatment groups (* $p < 0.05$). Significance between ethanol and control groups only (# $p < 0.05$) represented in delta (0–5Hz) and theta (5–15Hz) bands, as lithium co-treatment did not return coherence to control levels at all frequency bands affected by P7 ethanol. Unaffected frequency bands are shaded in grey in (C).

Figure 7.

Odor habituation and spatial memory behavior experiments. **A)** Standardized odor habituation task consisting of a single pure odor being presented to individually housed mice four times (trials 1–4) for 20 seconds each with a 30 second inter-trial interval (ITI). Investigation times are averaged for each trial with a group. Immediately after the fourth ITI a different molecularly distinct odor is presented for a total series of four odors with four trials each. All conditions displayed a typical habituation curve. **B)** Odor series habituation was observed in all conditions (lower panel), measured as the decline in first trial odor investigation times versus the first trial investigation time of the previously presented odor; however, ethanol-exposed mice showed a markedly higher retention of first trial investigation through the series compared to control and lithium co-treatment mice (though no significant difference between groups was found). This tendency was not due to varying interest in the task between treatment groups, as all groups showed similar first odor-first trial investigation times (upper panel). **C)** Spatial memory task performance was displayed by control mice, with a significant increase in sniff investigation of the moved object in proportion to the unmoved object (* $p < 0.05$). Ethanol mice showed impaired spatial memory with no significant difference ($p = 0.47$). Lithium co-treatment of P7 ethanol mice was effective at retaining spatial memory into adulthood, as investigation times were significantly increased for the moved object (* $p < 0.05$).