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L-Type Calcium Channels Contribute to Ethanol-Induced Aberrant Tangential Migration of Primordial Cortical GABAergic Interneurons in the Embryonic Medial Prefrontal Cortex

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Abstract

Exposure of the fetus to alcohol (ethanol) via maternal consumption during pregnancy can result in fetal alcohol spectrum disorders (FASD), hallmarked by long-term physical, behavioral, and intellectual abnormalities. In our preclinical mouse model of FASD, prenatal ethanol exposure disrupts tangential migration of corticopetal GABAergic interneurons in the embryonic medial prefrontal cortex (mPFC). We postulated that ethanol perturbed the normal pattern of tangential migration via enhancing $GABA_A$ receptor-mediated membrane depolarization that prevails during embryonic development in GABAergic cortical interneurons. However, beyond this, our understanding of the underlying mechanisms is incomplete. Here, we tested the hypothesis that the ethanol-enhanced depolarization triggers downstream an increase in high-voltage activated nifedipine-sensitive L-type calcium channel (LTCC) activity, and provide evidence implicating calcium dynamics in the signaling scheme underlying the migration of embryonic GABAergic interneurons and its aberrance. Tangentially migrating Nkx2.1+ GABAergic interneurons expressed immunoreactivity to Cav1.2, the canonical neuronal isoform of the L-type calcium channel. Prenatal ethanol exposure did not alter its protein expression profile in the embryonic mPFC. However, exposing ethanol concomitantly with the LTCC blocker nifedipine prevented the ethanol-induced aberrant migration both *in vitro* and *in vivo*. In addition, whole-cell patch clamp recording of LTCCs in GABAergic interneurons migrating in embryonic medial prefrontal cortical slices revealed that acutely applied ethanol potentiated LTCC activity in migrating GABAergic interneurons. Based on evidence reported in the present study, we conclude that calcium is an important intracellular intermediary downstream of $GABA_A$ receptor-mediated depolarization in the mechanistic scheme of an ethanol-induced aberrant tangential migration of embryonic GABAergic cortical interneurons.

Significance Statement

The etiology of fetal alcohol spectrum disorders (FASD) takes place *in utero* when the fetus is exposed to alcohol. While the outcome of FASD has been well characterized, the mechanism underlying its embryonic etiology is incompletely understood. Here, we investigated the role of L-type voltage-gated calcium channels (LTCCs) in the ethanol-induced aberrant tangential migration of cortical GABAergic interneurons. The findings from our study highlight LTCCs as important regulators underlying the aberrant tangential migration resulting from prenatal ethanol exposure and suggest that they bear therapeutic potential in managing and treating FASD. The results also propose an interplay between chloride and calcium in the migrating embryonic interneurons, and exposure to ethanol may enhance this interaction, contributing to the etiology of FASD.

Introduction

Fetal alcohol spectrum disorders (FASD), hallmarked by lifelong physical, behavioral, and intellectual abnormalities, is the leading cause of preventable neurodevelopmental disorders (American Academy of Pediatrics, 2000; Carr et al., 2010; Mattson et al., 2011). Alcohol (ethanol) readily crosses the placenta, and can be detected in the fetus as well as in the amniotic fluid (Idänpään-Heikkilä et al., 1972; Cuzon et al., 2008). The National Survey on Drug Use and Health reported that, from 2015-2018, drinking prevalence for pregnant women in the past 12 months was 64.7%, current drinking was 9.8%, and current binge drinking was 4.5% (England et al., 2020). Of those respondents in their first trimester of pregnancy, 19.6% reported current alcohol use and 10.5% reported binge drinking. Many women are not aware that they are pregnant until after the 4th or 6th week of pregnancy, increasing the risk of ethanol consumption early in gestation (Floyd et al., 1999). The prevalence of FASD is estimated at 33.5 per 1000 live births in the USA, 22.7 globally, and as high as 113.22 in some populations

(Roozen et al., 2016). These statistics underscore that FASD is a significant public health concern. Understanding its embryonic etiology is thus critical for managing and treating FASD.

We have employed a mouse model of FASD in which pregnant mice consumed ethanol either chronically throughout gestation or in a binge-type pattern to investigate the effects of prenatal ethanol exposure on the development of the cerebral cortex (Cuzon et al., 2008; Skorput et al., 2015; Delatour et al., 2019, 2020). Ethanol exposure *in utero* throughout gestation altered tangential migration of corticopetal GABAergic interneurons, regulated by an ambient level of GABA in the embryonic telencephalon (Cuzon et al., 2006; 2008). Binge-type ethanol exposure also disrupted tangential migration, which was associated with a persistent interneuronopathy that manifested as an excitatory/inhibitory (E/I) imbalance, hyperactivity, and deficits in executive function in young adult mice (Skorput et al., 2015). In a more recent study (Skorput et al., 2019), acute exposure to ethanol enhanced the membrane depolarization mediated through $GABA_A$ receptors expressed in GABAergic interneurons (GINs) migrating in the embryonic telencephalon. This ethanol-induced enhancement was normalized by bumetanide, a blocker of the NKCC1 co-transporter. Co-treatment of pregnant dams with bumetanide and ethanol also mitigated aberrant tangential migration in the short term, as well as interneuronopathy and behavioral deficits in the long term. However, it remained unclear how the potentiation of GABA-mediated depolarization following ethanol exposure disrupted tangential migration. Here, we hypothesized that this ethanol-potentiated depolarization triggers downstream signaling molecules that enhance migration, notably an increase in the activity of voltage-gated calcium channels which, in turn, leads to an abnormally augmented level of tangential migration.

Indeed, calcium signaling is a key mechanism underlying neuronal migration, including tangential migration of cortical GINs (Komuro and Rakic, 1996, 1998; Soria and Valdeolmillos, 2002; Moya and Valdeolmillos, 2004; Inada et al., 2011). Migrating neurons undergo a wide range of cellular processes, including neurite extension, nucleokinesis, and trailing tail retraction

through cytoskeletal dynamics, all of which are regulated by calcium-dependent signaling (Gomez and Spitzer, 1999; Lautermilch and Spitzer, 2000; Gomez et al., 2001; Wen et al., 2004; Kerstein et al., 2017). Calcium influx through voltage-gated L-type calcium channels (LTCCs) has been implicated in various processes of neuronal and non-neuronal migration, including neurite extension and trailing tail retraction (Yang and Huang, 2005; Darcy and Isaacson, 2009; Bortone and Polleux, 2009; Danesi et al., 2018; Kamijo et al., 2018). Neurotransmitters such as glutamate and GABA have been reported to invoke calcium influx through LTCCs (Bortone and Polleux, 2009; Horigane et al., 2019). Furthermore, LTCCs have been linked to neuropsychiatric disorders, including autism spectrum disorders, timothy syndrome and schizophrenia (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Kabir et al., 2017; Danesi et al., 2018).

In the present study, we tested the hypothesis that an ethanol-induced potentiation of LTCCs contributes to the aberrant tangential migration of GABAergic interneurons. We report here that co-treatment with ethanol and nifedipine, an LTCC blocker, prevented the ethanol-induced aberrant tangential migration both *in vitro* and *in vivo*. In addition, we report that ethanol potentiates LTCC activity in migrating GABAergic interneurons in the embryonic cortex. We conclude that LTCCs play an important role in manifesting the aberrant migration of embryonic GABAergic interneurons induced by prenatal ethanol exposure and propose that they may be potential therapeutic targets for mitigating the teratogenic effects of ethanol on the pathoetiology of FASD.

Materials and Methods

Animals

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Dartmouth College Institutional Animal Care and Use Committee. The Nkx2.1-Cre transgenic mouse line (originally

obtained from Dr. Stewart Anderson; Xu et al., 2008) was crossed with the Ai14 Cre-reporter mouse line (Jackson laboratories, Bar Harbor, ME) to yield Nkx2.1Cre/Ai14 mice harboring tdTomato-fluorescent MGE-derived GABAergic interneurons (referred to as Nkx2.1+ GINs; Skorput et al., 2015). For time-pregnant mating, pairs of male and female mice were housed overnight, with the following day designated as E0.5. Embryonic day 13.5-16.5 embryos were included in this study as available. Based on results of Y-chromosome-specific genotyping (Stratman et al., 2003), no difference in the ratio of male to female offspring was noted between treatment groups. Embryonic days 13.5 - 16.5 were operationally defined to be within the age range roughly equivalent to mid-first trimester in humans (Clancy et al., 2001; Parnell et al., 2014).

Ethanol exposure paradigm

This study used the binge-type ethanol exposure paradigm reported in previous studies (Skorput et al., 2015, 2019; Delatour et al., 2019, 2020). Timed-pregnant mice were exposed to ethanol from E13.5 to E16.5, when tangential migration of MGE-derived cortical interneuron is at its peak in mice (Anderson et al., 2001; Batista-Brito and Fishell, 2009; Gelman et al., 2009; Hladnik et al., 2014; Jiménez et al., 2002; Marín and Rubenstein, 2001; Parnavelas, 2000). Pregnant dams were individually housed and fed a liquid diet (Research Diets, New Brunswick, NJ) containing alcohol (5% w/w) or isocaloric control diet containing maltose. Mice were maintained under normal 12/12 hr light/dark cycle and water was available ad libitum. The liquid food was replenished daily between 9:00 and 11:00 AM, when the amount consumed and the weight of the dams were measured. Following termination of the liquid diet on E16.5, the mice were returned to standard chow. Mean dam blood alcohol level (BAL), measured at 11:30 PM on E15.5, was 0.08%, consistent with previous binge ethanol measurements (Skorput et al., 2015, 2019). Blood was collected via tail-vein and assessed using an Analox Instruments GM7 series analyzer (Lunenburg, MA). Following the binge-type ethanol consumption paradigm,

pregnant dams carried their offspring to full term with no apparent effect on litter size. For nifedipine treatment, nifedipine stock was dissolved in DMSO and added to the control or 5% ethanol-containing liquid food to achieve 0.15 mg of nifedipine per kg body weight of the mice being fed. The final dilution of DMSO in the liquid food was 1:120,000.

Organotypic embryonic slice culture

Organotypic culture of embryonic slices was performed as described previously (Cuzon et al., 2006; Skorput et al., 2019). At embryonic day E14.5, time-pregnant dams were asphyxiated using CO_2 and embryos were removed by cesarean section. The tdTomato-expressing embryos were identified using fluorescence UV (Biological Laboratory Equipment Maintenance and Service, Budapest, Hungary). The brains were quickly harvested under a dissecting microscope and immersed in ice-cold slicing media (1:1 F12:DMEM) oxygenated by continuous bubbling with 95% O_2/5% CO_2. The dissected brains were embedded in 3.5% low melting point agarose in 1:1 F12:DMEM, and 250 µm-thick coronal slices were collected using a vibrating microtome (Electron Microscopy Sciences, Hatfield, Pennsylvania) in ice-cold slicing media with 95% O_2/5% CO_2.

The organotypic slices were placed on a fine nylon mesh supported on a U-shaped platinum wire in a 35-mm round Petri dish. To achieve air-liquid interface, 0.8 ml sterile filtered culture media (1:1 F12/DMEM, 1% penicillin/streptomycin, 1.2% 6 mg/ml glucose in DMEM, 10% fetal bovine serum, and 1% L-glutamine) was added. After the slice cultures were incubated in a humidified incubator (37° C, 5% CO2) for 1hr, sister cultures were randomly replenished with culture medium containing: (1) 6.5 mM EtOH, (2) 20 µM nifedipine, (3) 6.5 mM EtOH + 20 µM nifedipine, (4) 0.02% DMSO. We used this low concentration of ethanol for the organotypic slice culture experiment as it is equivalent to the blood alcohol concentration (30 mg/dL) observed in a mouse model of a low level of chronic ethanol consumption used in previous studies (Cuzon et al., 2008; Skorput and Yeh, 2016).

24 hours post-incubation, the slice cultures were washed in PBS and immerse-fixed in 4% paraformaldehyde/0.1M phosphate-buffered saline (PBS) overnight at 4°C. Following cryoprotection in 30% sucrose/0.1M PBS, the slice cultures were mounted on charged slides and coverslipped with FluorSave Reagent (Calbiochem, La Jolla, CA).

Electrophysiology

On E16.5, pregnant dams were euthanized with CO_2 aspyxiation and fetuses were removed by caesarian section. UV goggles were used to visualize Nkx2.1Cre/Ai14 embryos, which express tdTomato fluorescence in the cortical regions. The brains expressing tdTomato fluorescence were dissected in ice-cold oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.25 NaH_2PO_4, 2 $CaCl_2 \cdot 2H_2O$, 25 $NaHCO_3$, 25 D-glucose, pH 7.4 (adjusted with NaOH). The brains were embedded in 3.8% low-melting point agarose (Invitrogen, Carlsbad, CA). Coronal telencephalic slices (250-μm) containing the embryonic mPFC were obtained using a vibratome (Electron Microscopy Services, Hatfield, PA) and immersed in cutting solution containing (in mM): 3 KCl, 7 $MgCl_2$, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 28 $NaHCO_3$, 5 D-glucose, 110 sucrose, pH 7.4 (adjusted with 1 N NaOH). The slices were then stored in a reservoir of aCSF at room temperature. Only slices from the rostral telencephalon with the embryonic mPFC clearly discernable were used for recording.

An acute 250-μm telencephalic slice was transferred to a recording chamber and stabilized by a platinum ring strung with thin plastic threads. The slice was maintained at 34 º C on a heated stage fit onto a fixed-stage upright microscope (BX51WI, Olympus, Melville, NY) and was perfused at a rate of 0.5-1.0 ml/min with oxygenated aCSF. Nkx2.1+ GINs were identified using a 40X water immersion objective (Olympus, Melville, NY) under fluorescence illumination and Hoffman Modulation Optics (Modulation Optics, Greenvale, NY). Images were displayed on a computer monitor through a video camera (Integral Technologies, Indianapolis, IN), which aided the navigation and placement of the recording and drug pipettes.

211 Recording pipettes for whole-cell patch clamp recording were pulled from borosilicate
212 glass capillaries (1.5 mm outer diameter, 0.86 mm inner diameter; Sutter Instrument Co.,
213 Novato, CA). The pipettes were back-filled with KCl internal-solution containing (in mM): 140
214 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, pH 7.4. The patch pipettes had resistances of 8-
215 10 MΩ. Recordings were made using an AxoPatch 700B amplifier (Molecular Devices Inc.,
216 Sunnyvale, CA). Membrane currents were digitized at 20 kHz (Digidata 1320A; Molecular
217 Devices), recorded with low-pass filtering at 10 kHz. Recordings were analyzed offline using
218 Mini Analysis (Synaptosoft) and Clampfit 10.3 (Skorput et al., 2019).
219 Nifedipine, TEA, and TTX were dissolved in aCSF immediately prior to recording to a
220 working concentration of 20 μM, 20 mM, and 1 μM, respectively. Ethanol was prepared fresh by
221 diluting 95% ethanol with aCSF to 18 mM for the electrophysiological experiments, which is
222 equivalent to the blood alcohol concentration of 80 mg/dl blood alcohol level attained in the
223 binge-ethanol exposure paradigm used in this and previous studies (Skorput et al., 2015,
224 Skorput et al., 2019).
225 Drug solutions were loaded into separate barrels of a 8-barrel drug pipette assembly that
226 was placed within 10 μm of the soma of the cell under study and applied using regulated pulses
227 of pressure (≤ 3 p.s.i.; Picospritzer, General Valve Corporation, Fairfield, NJ). The timing and
228 duration of the pressure pulses were controlled by a multi-channel timing unit and pulse
229 generator (Pulsemaster A300, WPI, Sarasota, FL). One of the barrels of the multi-barrel drug
230 pipette was filled with aCSF, which was applied between drug applications to clear drugs from
231 the vicinity of the cell and to control for mechanical artifacts that can occur due to bulk flow.
232
233 *Processing of embryonic tissues*

234 Time-pregnant dams were euthanized by CO_2 asphyxiation on E16.5, following control,
235 ethanol (5% w/w), nifedipine (0.15 mg/kg body weight) + ethanol (5% w/w), or nifedipine (0.15

mg/kg body weight) treatment. The embryos were removed, their brains dissected, and immerse-fixed in 4% PFA/0.1M PBS overnight at 4°C. Following cryoprotection in 30% sucrose/0.1M PBS overnight, 30-µm cryosections were cut with a sliding microtome. The slices were mounted on charged glass slides, DAPI counterstained, and coverslipped with FluorSave Reagent (Calbiochem, La Jolla, CA).

For immunohistochemistry, 30-µm coronal sections were collected into PBS and washed overnight at 4° C. The sections were then blocked for 30 minutes at room temperature in PBS containing 10% NGS and 0.025% Triton X-100. The sections were then incubated overnight at 4° C with rabbit anti-Cav1.2 primary antibody (Alomone Labs) at a dilution of 1:400 in PBS. The sections were washed in PBS 2X and incubated overnight with a 1:1000 dilution of Alexa Fluor 488 conjugated goat-anti-rabbit secondary antibody (Invitrogen, Grand Island, NY) in PBS. Negative control without primary antibody was routinely processed in parallel.

Immunofluorescence imaging and analysis

Fluorescent images were acquired using a CCD camera (Hamamatsu, Hamamatsu city, Japan) fit onto a spinning disk confocal microscope (BX61WI; Olympus, Melville, NY) and CellSens software (Olympus). Images were montaged using Photoshop to yield a full view of the region of interest.

For each embryonic brain, 10 consecutive sections of the embryonic mPFC beginning at equivalent rostral-caudal levels were imaged and analyzed for counts of Nkx2.1+ GINs. For each litter, sections from a minimum of 3 brains were imaged. The embryonic mPFC was delineated as part of the dorsomedial telencephalon based on DAPI counterstaining of the sections used for analyzing cells. Nkx2.1+ GINs were manually counted using Fiji's cell counting tool by trained experimenters blinded to the experimental condition.

For organotypic slice cultures, images were montaged with Photoshop to allow visualization of the cortex throughout its thickness, from the corticostriate juncture (CSJ) to the

dorsal apex. One 200-μm bin immediately proximal to the CSJ, was delineated as one of the regions of interest. Distal to the CSJ, five consecutive 100-μm bins spanning the thickness of the cortex were organized (Cuzon et al., 2006). The Nkx2.1+ GINs were manually quantified by trained experimenters blinded to the experimental condition using Fiji's cell counting tool. Cell counts in each 100-μm bin were normalized by counts from the 200-μm bin proximal to the CSJ from each tissue to calculate the crossing index (Cuzon et al., 2006).

Statistics

For histological analyses, n represents the number of litters to minimize litter effects. All groups of histological data were acquired from ten 30-μm tissue sections per animal from a minimum of three individual animals from multiple litters. For electrophysiological experiments, n also refers to the number of litters used. All statistical analyses were conducted using GraphPad Prism (Version 5.0). Power calculations were performed using the G*Power 3.1 software depending on whether the data were analyzed using t-test or ANOVA. Variance and expected differences were estimated by using group means and standard deviations from preliminary data or past experience in similar studies and reviews of related use in the literature. Group means were compared by paired t-test, one-way ANOVA or two-way ANOVA with appropriate post-hoc test as indicated, and reported in Results as mean (x) ± standard error.

Results

Binge exposure to ethanol does not affect Cav1.2 expression levels in the embryonic prefrontal cortex

We first asked whether LTCCs are expressed in Nkx2.1+ GINs as they migrate tangentially and become positioned in the cortical plate of the embryonic mPFC. Immunohistochemical staining of Cav1.2 in sections from Nkx2.1Cre-Ai14 mouse at E16.5 showed that virtually all cells express Cav1.2, including the Nkx2.1+ GINs (Fig. 1). We then

assessed if prenatal ethanol exposure alters the expression levels of Cav1.2 in the embryonic medial prefrontal cortex. To this end, pregnant dams were fed liquid diet containing 5% ethanol or isocaloric control from E13.5-E16.5. Embryonic brains dissected at E16.5 were stained for Cav1.2, and immunofluorescence intensity of Cav1.2 and tdTomato was measured using a spinning disk confocal microscope. Fluorescence intensity of Cav1.2 was normalized to that of tdTomato, which is expressed uniformly in the Nkx2.1Cre-Ai14 mice. There was no difference between the fluorescence intensity ratio between control and ethanol-treated embryos at E16.5, indicating that the binge exposure paradigm does not alter the expression levels of Cav1.2 (Fig. 2; Control; 1.07 ± 0.08, n = 4 litters; Ethanol; 1.00 ± 0.027, n = 5 litters; unpaired t-test, P>0.999).

Nifedipine co-treatment in vitro prevents the ethanol-induced aberrant tangential migration

To determine the involvement of LTCCs in ethanol-induced aberrant tangential migration, organotypic slice cultures containing the embryonic mPFC were prepared from E14.5 Nkx2.1Cre-Ai14 brains. The slices were incubated in either control or 6.5 mM ethanol-containing medium without or with 20 μM nifedipine. Following 27 hours of incubation, the slices were fixed in 4% paraformaldehyde/PBS and tangential migration of MGE-derived interneurons was subsequently assessed by counting the number of cells in consecutive 100-μm bins in the mPFC beginning at the CSJ. The number of cells in each bin was normalized to the number of cells in the 200-μm bin delineated immediately proximal to the CSJ to calculate the crossing index (Figure 3A). When the number of Nkx2.1+ GINs per 100-μm bin was compared among the different treatment groups, ethanol-treated organotypic slice cultures had significantly higher number of cells compared to those in the control group (Fig. 3B; Vehicle; 216.9 ± 26.69 cells n = 8 cultures, EtOH 319.6 ± 33.54 cells, n = 9 cultures, one-way ANOVA, Tukey's post-hoc test, $p<0.05$). On the other hand, the number of Nkx2.1+ GINs in the organotypic cultures co-treated with LTCC blocker nifedipine and ethanol were similar to that of control (Nifed + EtOH; 216.1 ±

14.71 cells, n = 10 cultures, p>0.999). At the concentration of nifedipine used (20 μM), treatment with the LTCC blocker alone did not alter the density of Nkx2.1+ GINs in the cortex compared to controls (Nifed; 207.2 ± 24.69, 6 cultures; P>0.999). When the crossing index was analyzed with two-way ANOVA, the number of Nkx2.1+ GINs was significantly higher in ethanol treated cultures compared to control in cortical regions more distal to the CSJ (Fig. 3C). Cultures treated with nifedipine alone and those co-treated with nifedipine and ethanol resulted in a similar crossing index as that of control in all bins. These results implicate calcium flux through the LTCCs being involved in the ethanol-induced aberrant tangential migration, and that blocking LTCCs with nifedipine can normalize the effect of ethanol.

Nifedipine co-treatment in vivo prevents the ethanol-induced aberrant tangential migration

To investigate if the preventive effect of nifedipine when co-treated with ethanol seen *in vitro* can be replicated *in vivo*, we fed pregnant dams harboring Nkx2.1Cre-Ai14 embryos with a liquid diet containing ethanol (5% EtOH w/w) as well as nifedipine (0.15 mg/kg body weight dissolved in DMSO) from E13.5 - E16.5 according to the timeline outlined in Figure 4A. The dose of nifedipine we used was based on the medical dose of 10 mg prescribed to pregnant women for emergency hypertension and that of the average body weight for first trimester pregnant women is 79 kg (ACOG Committee Opinion No. 767: “Emergent Therapy for Acute-Onset, Severe Hypertension During Pregnancy and the Postpartum Period”, 2019). On E16.5, we analyzed the number of Nkx2.1+ GINs in the embryonic mPFC of the progeny (Fig. 4B and C). The laminar localization of these neurons in the embryonic mPFC was not systematically analyzed. Ethanol treatment significantly increased the number of Nkx2.1+ GINs in the mPFC (Fig. 4C; Control; 190.9 ± 22.87 cells, n = 6 litters, EtOH; 369.3 ± 3.063 cells, one-way ANOVA, Tukey’s post-hoc test, p<0.0005, n = 3 litters). Nifedipine treatment during the binge-exposure to alcohol attenuated the number of Nkx2.1+ GINs to a level similar to that of control (Nifed +

EtOH; 173.0 ± 24.20 cells; p>0.99, n = 4 litters). Nifedipine alone, at the concentration used in this study, did not alter the tangential migration of GABAergic interneurons into the mPFC compared to controls (Nifed; 102.4 ± 27.88 cells; p>0.99, n = 4 litters). Thus, the effect of nifedipine in preventing the ethanol-induced enhancement of Nkx2.1+ GINs seen *in vitro* in organotypic slices is recapitulated *in vivo.*

Ethanol exposure increases L-type calcium channel activity

We next assessed whether ethanol exposure directly affects LTCC-activated currents in tangentially migrating GABAergic interneurons. We performed whole-cell patch clamp recordings from Nkx2.1+ GINs in acute slices from the embryonic (E16.5) Nkx2.1/Ai14 mouse brains. A multi-barrel drug pipette was used to focally apply 18 mM ethanol, 20 μM nifedipine, 1 μM TTX, 20 mM TEA, and aCSF to cells being recorded from (Fig. 5A). Calcium current mediated by LTCCs was isolated by applying a depolarizing +40 mV voltage step from a holding potential of -60 mV. Nifedipine application blocked 65.35% of the inward current (Fig. 5C, control; 37.49 ± 7.025, nifedipine; 12.99 ± 3.240, n = 7 litters, p<0.01). The inward current mediated through nifedipine-sensitive LTCCs was isolated by subtracting the current recorded in presence of nifedipine from that without nifedipine. This current was measured again before and during acute 18mM ethanol application (Fig. 5B). We found that acute ethanol exposure potentiated the LTCC-activated current amplitude by 33.16% (Fig. 5D, Control; 27.31 ± 7.158, Ethanol; 40.86 ± 11.81 , n = 7 litters, paired t-test, p<0.05). These results indicate that ethanol augments LTCC-mediated currents in embryonic Nkx2.1+ GINs and, thus, calcium influx consequent to an ethanol-induced potentiation of $GABA_A$ receptor-mediated depolarization (Skorput et al., 2019).

Discussion

We sought to contribute to elucidating the mechanisms underlying the embryonic etiology of FASD in this study. This was motivated by our previous studies that showed ethanol altering tangential migration of GABAergic interneurons (Cuzon et al., 2008; Skorput et al. 2015; Skorput et al. 2019). The major findings of this study are three-fold. First, we showed that Cav1.2 (or $\alpha 1_C$) is ubiquitously expressed in the embryonic mouse cortex. Second, nifedipine, presented either directly to organotypic slices *in vitro* or through *in vivo* treatment of ethanol-consuming pregnant dams, prevents the ethanol-induced aberrant tangential migration in the fetal cortex. Third, we provide evidence that ethanol enhances calcium influx through LTCCs in migrating embryonic GABAergic interneurons. Collectively, these findings provide evidence for calcium and LTCCs being important regulators of the ethanol-induced aberrant tangential migration of GINs in the embryonic mPFC.

Figure 6 integrates the results of the present study and those of a recent one (Skorput et al., 2019) that, together, led to formulating an LTCC-based subcellular mechanistic scheme by which ethanol may mobilize tangential migration in embryonic GINs. Under normal control conditions (Fig. 6A), the preponderance of the NKCC1 chloride importer vis-à-vis the KCC2 chloride exporter generates a net high intracellular level of chloride that maintains a depolarized membrane potential in embryonic GINs. In addition, tonic activation of $GABA_A$ receptors by an ambient presence of GABA in the extracellular milieu (Cuzon et al., 2006) leads to a net chloride efflux and depolarization of the membrane. Ethanol exposure (Fig. 6B) does not directly affect the action of the NKCC1 cotransporter but potentiates chloride efflux through $GABA_A$ receptors, resulting in enhanced depolarization (Skorput et al., 2019) which goes on to promote downstream processes that either enhance LTCC activity (Fig. 6B) or trigger other mechanisms that increase intracellular calcium dynamics. One such mechanism, as postulated in Figure 6, may be a surrogate activation of the calcium-permeable NMDA receptors following the depolarization-dependent release of the magnesium block. Whether and how such surrogate

389 interacts and comes into play in conferring ethanol's influence on the tangential migration of
390 cortical GINs await experimental elucidation.

391 Our results indicate that, although the embryonic neocortex expresses Cav1.2, or $\alpha 1_C$, at
392 E16.5, prenatal exposure to ethanol did not affect Cav1.2 expression levels in the embryonic
393 prefrontal cortex. It should be noted that this finding deviates from earlier studies in which
394 chronic ethanol exposure was shown to increase calcium uptake through LTCCs, presumably
395 through the increase in expression of α1 and α2/δ1 subunits of LTCCs in cortical neurons and
396 $\alpha 1_C$, α2, and $\beta 1_b$ in neural crest-derived cell line PC12 (Katsura et al., 2006; Walter et al., 2000).
397 A parsimonious explanation to account for the apparent discrepant results regarding LTCC
398 expression might be due to the use of high doses of ethanol in the previous studies (50-150
399 mM) vis-à-vis the more moderate concentrations of ethanol (6.5 mM and 18 mM) used here. In
400 addition, the earlier studies employed a chronic ethanol exposure paradigm (Katsura et al.,
401 2006; Walter et al., 2000), and this differs from the binge-type ethanol exposure early in
402 gestation employed in our study.

403 Nifedipine treatment in ethanol-exposed organotypic slice cultures prevented the
404 aberrance in tangential migration. Maternal nifedipine treatment along with binge exposure to
405 ethanol also normalized migration disrupted by ethanol *in vivo*. Nifedipine, as a dihydropyridine
406 calcium channel blocker, can have wide-ranging systemic effects, including activation of the
407 reflex sympathetic nervous system, increase in myocardial oxygen supply, and decrease in
408 blood pressure (Gibbons et al., 2003; Abrams et al., 2007). Pregnant mice treated with
409 nifedipine may be subject to such physiological changes, independent of or in addition to its
410 effects on the embryonic central nervous system. However, we favor the prospect that
411 nifedipine's effect on the tangential migration of Nkx2.1+ GINs most likely involves a direct
412 effect, vis-à-vis, secondary effects on physiological alterations, as similar outcomes were
413 recapitulated in our organotypic slice culture experiments that would have arguably
414 circumvented any direct systemic effects.

In the present study, acute exposure to ethanol enhanced the activity of LTCCs. We note that acute ethanol exposure has been shown to inhibit the function of LTCCs in several neuronal preparations (Wang et al., 1991; Wang et al., 1994; Mullikin-Kilpatrick and Treistman, 1995; Zucca et al., 2010; Mah et al., 2011; Morton et al., 2016). Such differences may arise from the fact that embryonic neurons have relatively depolarized resting membrane potentials (approximately -40 mV; Skorput et al., 2019), such that membrane depolarization exerted by traditionally hyperpolarizing neurotransmitters such as GABA might be more conducive to reaching the activation potential for LTCCs (Nakanishi and Okazawa, 2006; Horigane et al., 2019). We hypothesized that ethanol, by potentiating depolarization through $GABA_A$ receptors, may further activate LTCCs, increasing calcium influx into the migrating embryonic GABAergic interneurons (Fig. 6B). This is in line with reduced calcium signaling observed in tangentially migrating interneurons with KCC2 upregulation, which decreases GABA-induced depolarization (Bortone and Polleux, 2009). In this study, we did not confirm the role of GABA as a mediator of the observed increase in calcium influx through LTCCs. Nonetheless, our findings provide the basis for investigating further the interplay between $GABA_A$ receptor and LTCCs activation in embryonic Nkx2.1+ GINs upon ethanol exposure.

L-type calcium channels are predictably not the only voltage-gated calcium channels that immature GABAergic cortical interneurons express. Thus, the involvement of L-type calcium channels does not preclude the role of other voltage-activated calcium channels in regulating the tangential migration of Nkx2.1+ GINs. Ligand-gated ion channels other than $GABA_A$ receptors may also play a role. For example, NMDA receptors are expressed in immature GABAergic cortical interneurons (Soria and Valdeolmillos, 2002). Since NMDA receptor activation relies on membrane depolarization-induced release of magnesium block, a GABA-mediated depolarization may facilitate NMDA receptor activation and, thereby, promote calcium influx in immature GABAergic interneurons. This surrogate activation of NMDA receptors may be augmented with ethanol exposure (Fig. 6). Future investigative work will need to confirm the

potential involvement of NMDA and interplay with $GABA_A$ receptor activation in this mechanistic signaling scheme.

The present study demonstrated that ethanol increases LTCC-mediated channel activity (Fig. 6B). However, how this would enhance tangential migration of cortical GABAergic interneurons still remains unresolved. Calcium signaling is critical in activating the downstream cytoskeletal dynamics in mechanisms underlying neuronal migration (Gomez and Spitzer, 1999; Lautermilch and Spitzer, 2000; Gomez et al., 2001; Wen et al., 2004; Kerstein, Patel, and Gomez, 2017). Live imaging studies addressing changes in filopodia motility, somal translocation, or trailing tail retraction, as downstream mechanisms of ethanol-induced potentiation of LTCC-mediated calcium current will further elucidate how ethanol affects normal tangential migration of primordial GABAergic cortical interneurons and, thus, contribute to our understanding of the pathoetiology of FASD.

While the diagnosis of FASD occurs postnatally, its embryonic etiology is incompletely understood. The current study focused on investigating how ethanol as a teratogen disrupts the process of migration in the embryonic neocortex. We demonstrated that ethanol increases chloride efflux to enhance GABA-induced depolarization, and increases LTCC activity in migrating interneurons. Ethanol may also activate NMDA receptors, which are calcium permeable, and enhanced activation of voltage-gated calcium channels may increase calcium influx into the cell, which may act on downstream targets to alter cytoskeletal dynamics. Overall, data presented here point to LTCCs playing an important role in the ethanol-induced aberrant tangential migration of cortical GABAergic interneurons. We propose that the inhibition of LTCCs by nifedipine, as an FDA approved drug, may bear therapeutic potential in treating and managing FASD.

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589

590 **Figure Legends**
591
592 **Figure 1. Cav1.2 is expressed in the embryonic medial prefrontal cortex.**
593 (A) Representative images of histological sections from Nkx2.1/Ai14 E16.5 mouse brain (A1)
594 immunostained for Cav1.2 (A2) and overlayed with images of DAPI counterstaining and
595 Nkx2.1/tdTomato-fluorescent GABAergic interneurons (A3). Images were captured at 10X
596 magnification on a spinning disk confocal microscope. (B) Representative images at 40X
597 magnification. These images are magnified images of the area demarcated by the white box in
598 A1-A3. (C) Representative images of no primary antibody negative control of Cav1.2 staining.
599
600 **Figure 2. Prenatal ethanol exposure does not alter Cav1.2 expression.**
601 (A) Representative images of Cav1.2 staining overlayed with DAPI and Nkx2.1 in the medial
602 prefrontal cortex (mPFC) of control (A1) and ethanol-fed (A2) E16.5 mouse brain. (B)
603 Quantification of fluorescence intensity ratio of Cav1.2 to Nkx2.1 in the mPFC of control and
604 ethanol-treated mice. Unpaired t-test. See Results for statistical details.
605
606 **Figure 3. Nifedipine prevents ethanol-induced aberrant migration in organotypic slice**
607 **cultures**
608 (A) Representative image of Nkx2.1/Ai14 embryonic mouse brain with 5 bins (100-μm wide
609 each) above corticostriate juncture (CSJ) and one 200-μm bin below CSJ. (B) Quantification of
610 mean Nkx2.1+ cells per individual bins in control (Vehicle; 3 litters, 3 females, 5 males), ethanol
611 (EtOH; 3 litters, 6 females, 3 males), ethanol + nifedipine (EtOH + Nifed; 3 litters, 7 females, 3
612 males), and nifedipine (Nifed; 3 litters, 2 females, 4 males) treated organotypic slice cultures.
613 (C) Quantification of crossing index for control (Vehicle), ethanol (EtOH), ethanol + nifedipine
614 (EtOH + Nifed), and nifedipine (Nifed) treated organotypic slice cultures. * compared to control,

615 # compared to EtOH + Nifed. *, # = p<0.05, **, ## = p<0.01, ***, ### = p<0.001, two-way
616 ANOVA with Tukey's post hoc test
617
618 **Figure 4. Maternal nifedipine treatment normalizes ethanol-induced enhancement of**
619 **tangential migration in vivo.**
620 (A) Experimental timeline. Graded blue box highlights the period of tangential migration,
621 beginning at ~E01.5 and tapering out by ~P0. Binge-type ethanol exposure and nifedipine co-
622 treatment begins on E13.5 and ends on E16.5 (red dashed lines). (B) Fluorescent images of
623 mPFC counterstained with DAPI in mice that received control, binge-type maternal ethanol
624 consumption from E13.3 - E16.5 (EtOH), ethanol exposure in combination with maternal
625 nifedipine treatment in liquid diet (0.15 mg/kg body weight) (EtOH+Nifed), or nifedipine only
626 treatment (Nfed). Scale bars = 100 μm. (C) Quantification of $Nkx2.1^{+}$ cells in all treatment
627 groups. ** = p < 0.01 compared to control. one-way ANOVA with Tuckey's post hoc test.
628
629 **Figure 5. Ethanol acutely potentiates calcium current mediated by L-type calcium**
630 **channels.**
631 (A) Representative images of the recording setup. Hoffman modulation contrast image of an
632 acute slice from E16.5 mouse brain with the patch clamp recording pipette (A1, right) pointed at
633 a tdTomato-fluorescent Nkx2.1+ GABAergic interneuron to be recorded in the whole-cell voltage
634 clamp configuration. A multi-barrel drug pipette is navigated to the vicinity of the cell being
635 recorded (A1, left). (B) Calcium current mediated by L-type voltage-gated calcium channels was
636 isolated by performing 40-mV depolarizing voltage steps in the presence of TTX, TEA (black
637 trace). This current was subtracted with current obtained from 40-mV depolarizing steps in the
638 presence of TTX, TEA, and nifedipine (Nifedipine, gray trace). L-type calcium current was
639 measured again in the presence of 18 mM ethanol (EtOH, red trace). (C) Quantification of peak
640 amplitude of calcium current mediated by LTCC in the presence of TTX and TEA (Control) and

nifedipine (Nifedipine). ** = p<0.01 paired t-test. (D) Peak amplitude of calcium current mediated by LTCC in the before (Control) and during acute 18mM ethanol exposure (EtOH). * = p<0.05 unpaired t-test

Figure 6. Schematic diagram summarizing the effect of ethanol on migrating embryonic cortical GABAergic interneurons. (A) The depolarizing action of GABA in embryonic cortical GINs is established by the predominant expression of NKCC1 chloride importer relative to that of the KCC2 chloride exporter, resulting in heightened intracellular chloride. GABA binding to its cognate $GABA_A$ receptor thus causes chloride efflux and membrane depolarization. (B) Following ethanol exposure, chloride efflux from $GABA_A$ receptors is enhanced, shifting the membrane potential of embryonic GABAergic interneurons to more depolarized levels. This enhanced depolarization of the membrane potential promotes the activation of L-type voltage-gated calcium channels (LTCC). It is postulated that the increased depolarization may also activate NMDA receptors by releasing the voltage-dependent Mg^{++} block. The increase in the activity of calcium-permeable channels may, in turn, increase calcium influx, raising intracellular calcium, and further activate downstream calcium signaling mechanisms. The net result of this chloride-to-calcium interplay mobilizes changes in the actin-microtubule dynamics and promote migration of cortical GABAergic interneurons.

Figure 1

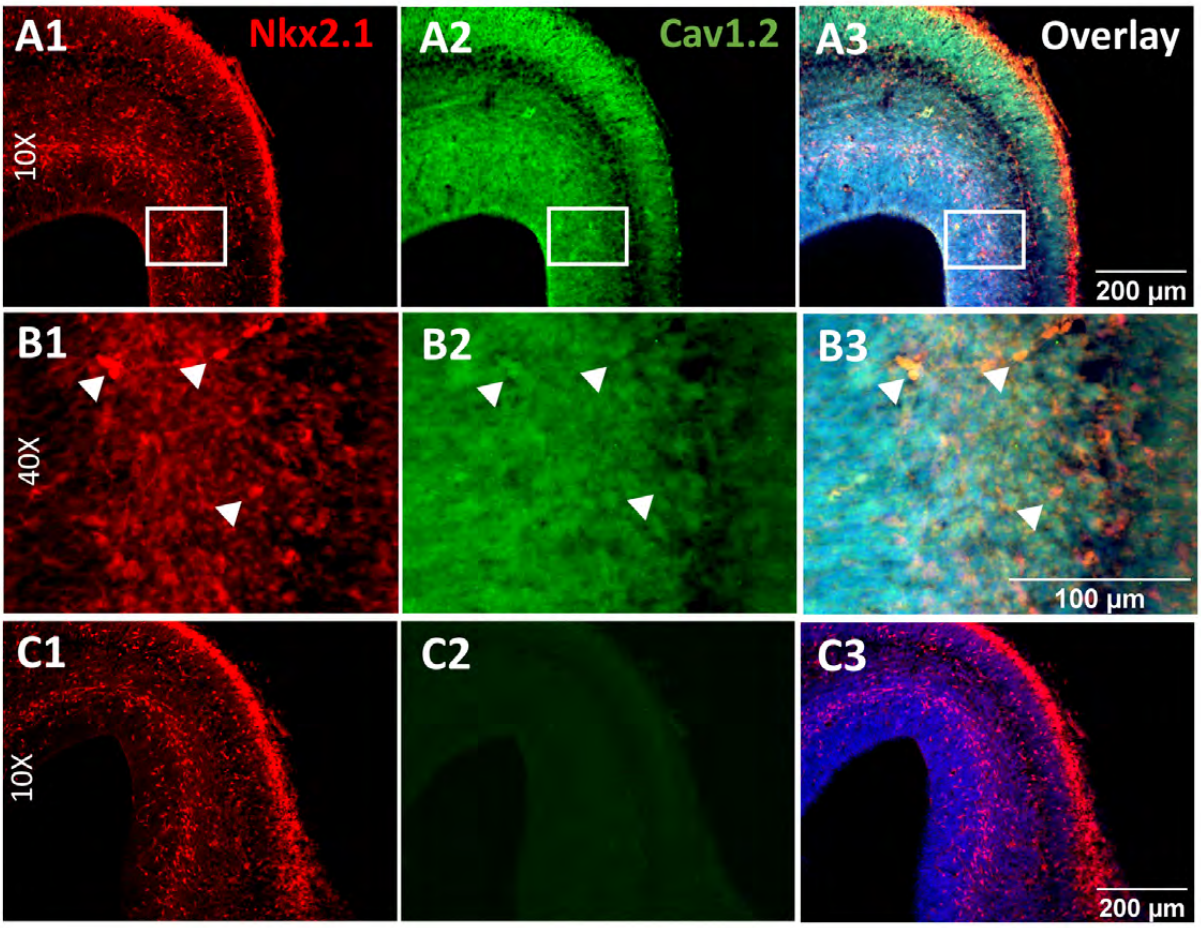

Figure 2

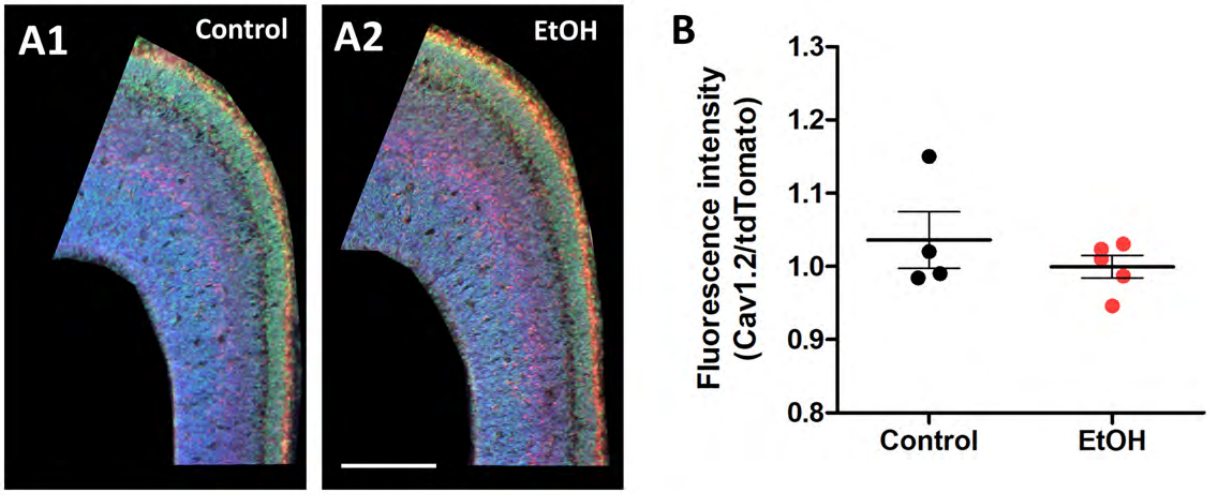

Figure 3

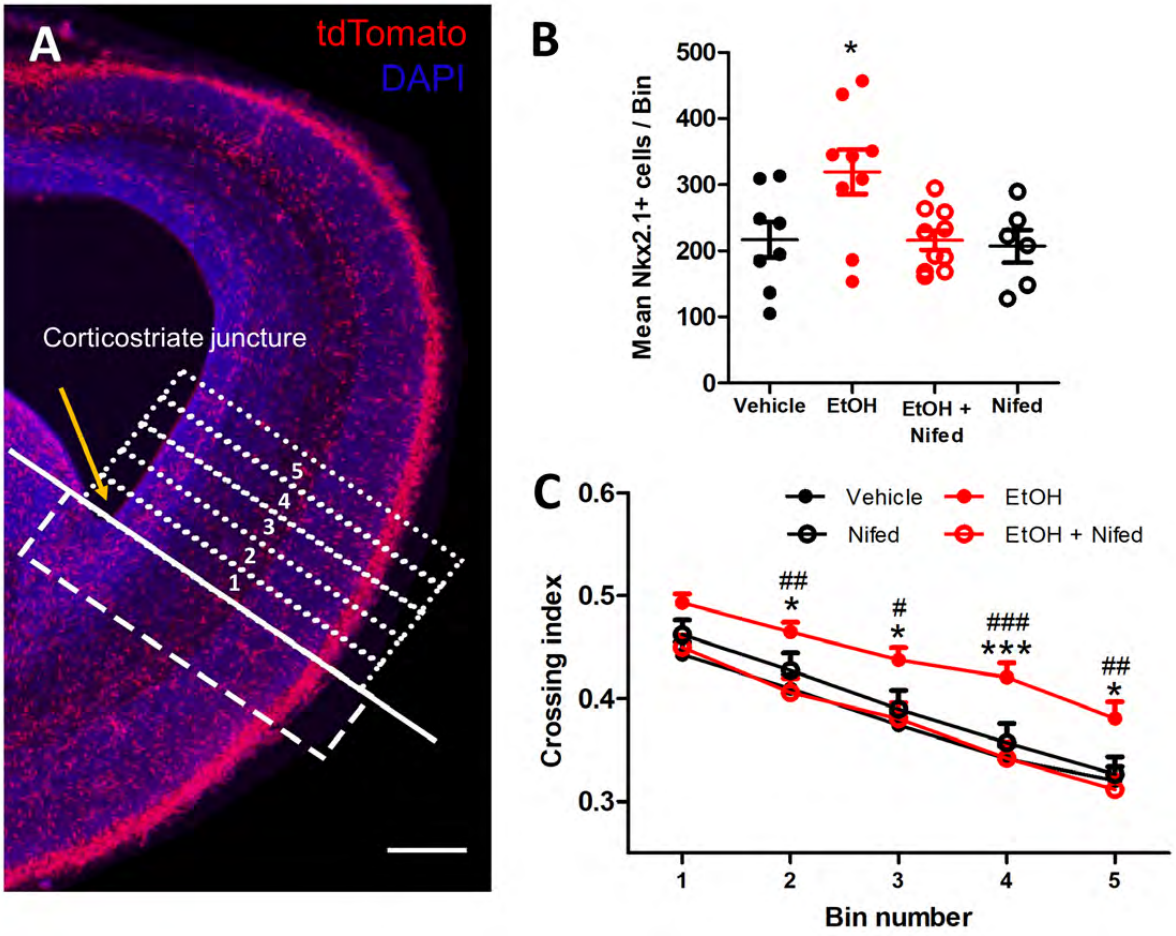

Figure 4

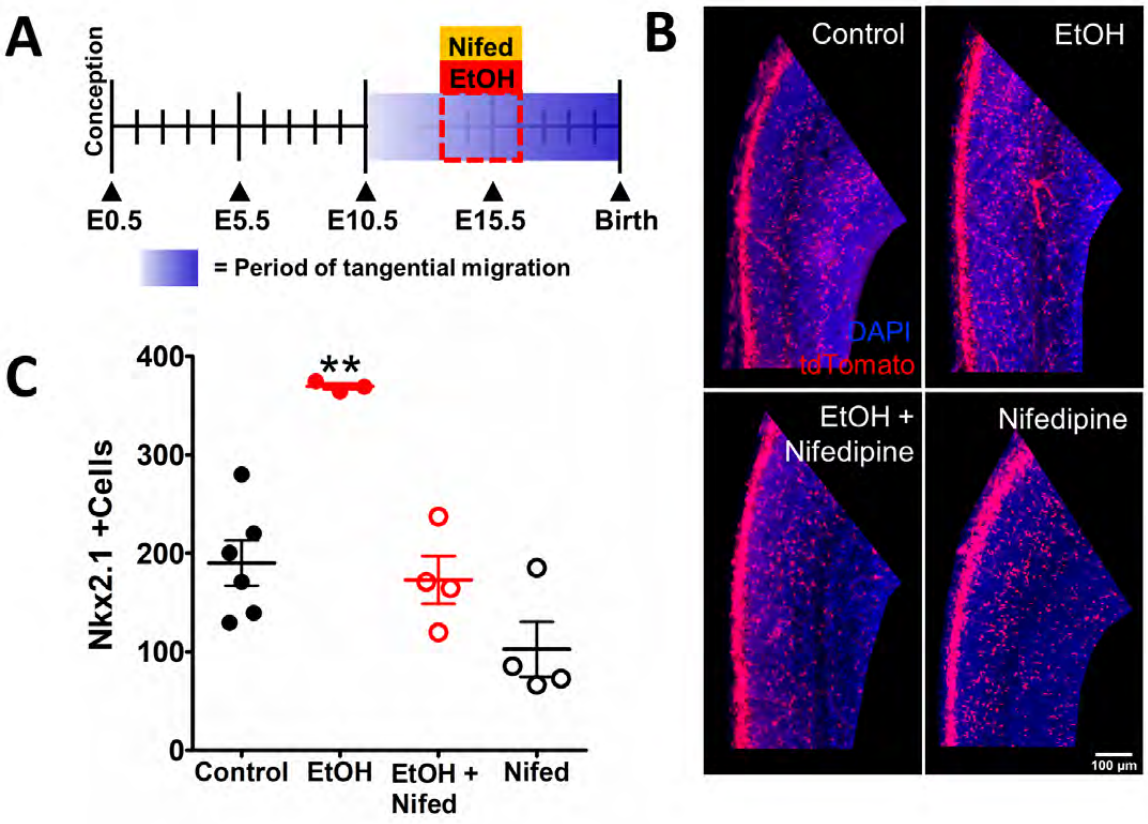

Figure 5

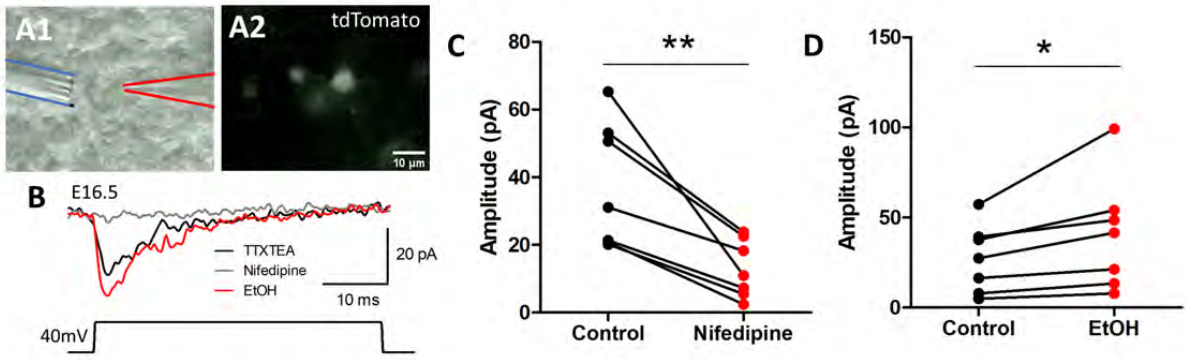

Figure 6