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Androgenic modulation of the chloride transporter NKCC1 contributes to age-dependent isoflurane neurotoxicity in male rats

Gregory A. Chinn, MD PhD[*], **Jennifer M. Sasaki Russell, PhD**, **Nicole A. Yabut, BS**, **Deenu Maharjan, MD**, **Jeffrey W. Sall, PhD MD**

University of California, San Francisco, Department of Anesthesia and Perioperative Care, San Francisco, CA

Abstract

Background—Cognitive deficits following perinatal anesthetic exposure are well established outcomes in animal models. This vulnerability is sex dependent and associated with expression levels of the Chloride transporters NKCC1 and KCC2. We hypothesized androgen signaling, NKCC1 function and the age of isoflurane exposure are critical for the manifestation of anesthetic neurotoxicity in male rats.

Methods—Flutamide, an androgen receptor antagonist, was administered to male rats on postnatal days 2, 4, 6 before 6 hours of isoflurane on postnatal day 7 (n_{total}=26). Spatial and recognition memory were subsequently tested in adulthood. NKCC1 and KCC2 protein levels were measured from cortical lysates by western blot on postnatal day 7 (n_{total}=20). Bumetanide, an NKCC1 antagonist, was injected immediately before isoflurane exposure(postnatal day 7) to study the effect of NKCC1 inhibition(n_{total}=48). To determine whether male rats remain vulnerable to anesthetic neurotoxicity as juveniles, postnatal day 14 animals were exposed to isoflurane and assessed as adults (n_{total}=30).

Results—Flutamide-treated male rats, exposed to isoflurane, successfully navigated the spatial (Barnes Maze probe trial F(1,151)=78, p<0.001; mean goal exploration (SD) 6.4(3.9) seconds) and recognition memory tasks (mean discrimination index (SD) 0.09(0.14), p=0.003), unlike isoflurane-exposed controls. Flutamide changed expression patterns of NKCC1 (Mean density (SD):Control 1.49(0.69), Flutamide 0.47(0.11), p<0.001) and KCC2 (median density (25^{th}percentile, 75^{th}percentile): Control 0.23(0.13, 0.49), Flutamide 1.47(1.18,1.62), p<0.001). Inhibiting NKCC1 with bumetanide was protective for spatial memory (probe trial F(1,162)=6.6, p=0.011; mean goal time 4.6(7.4) seconds). Delaying isoflurane exposure until postnatal day 14 in males preserved spatial memory(probe trial F(1,140)=28, p<0.001; mean goal time 6.1(7.0) seconds).

[*]**corresponding author**: Gregory.Chinn@ucsf.edu, 415) 514-3781, 1001 Potrero Ave, 3C39, San Francisco, CA 94110.

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Conclusions—Vulnerability to isoflurane neurotoxicity is abolished by blocking the androgen receptor, disrupting the function of NKCC1, or delaying the time of exposure to at least 2 weeks of age in male rats. These results support a dynamic role for androgens and chloride transporter proteins in perinatal anesthetic neurotoxicity.

Introduction

Animal models of early life anesthetic neurotoxicity have consistently found behavioral deficits after long perinatal exposures in species ranging from mice to rhesus macaques.[1,2] The extent to which perinatal anesthetic neurotoxicity occurs in humans remains undefined, though the results of several well conducted studies suggest that short exposures are benign[3–6] These results are consistent with preclinical studies which show that short anesthetic exposures do not cause significant behavioral deficits for a variety of species.[7,8] Regardless of the clinical effect, neonatal anesthetic exposure in animals is an important modulator of developmental plasticity with a mechanism that remains undefined.

Two important variables, sex and age at exposure, impact neurocognitive changes after anesthesia. Experimental animal models often exhibit worsened outcomes in males relative to females.[9,10] Similarly, age plays a critical role in susceptibility as a multitude of studies demonstrate deficits following exposures in utero through the perinatal period, while adult and even some juvenile animals do not develop neurocognitive changes after exposure.[1,2]

While the underlying mechanism of age- and sex-dependent susceptibility remains unknown, the developing GABAergic system is a prime candidate. There are at least three notable characteristics of this system which could impart vulnerability: volatile anesthetics act on the GABAa receptor, the inhibitory properties of the receptor mature in the early postnatal period, and the developmental timeframe is sexually distinct.

The flow of chloride into or out of the cell through the GABAa receptor determines the functional response to activation and is thought to be dependent on the relative expression of two solute carrier family proteins, NKCC1 and KCC2, which traffic chloride into (NKCC1) or out (KCC2) of the cell.[11] In the mature state, chloride flows into the cell following GABA activation. This direction is down the gradient established by KCC2 and hyperpolarizes the membrane. In the immature state, NKCC1 predominates, and the chloride gradient is reversed. This results in depolarization as chloride flows out of the cell through the GABA receptor. The transition of GABA from excitatory to inhibitory happens shortly after birth and is determined by the changing expression of NKCC1 and KCC2.[12] This developmental transition occurs at different rates and varies by cortical location in males and females with the transition occurring at a younger age in females in specific brain regions.[13–15]

We previously identified differences in susceptibility of male and female rats exposed to anesthesia at the same postnatal age,[9] and recently reported that in female rats both age of exposure and relative expression levels of NKCC1 and KCC2 were predictive of developing a behavior deficit.[15] These expression patterns were different for males and females across age with the ratio changing earlier in females. In a separate study, we found that gonadectomized male rats were protected, similar to females. Building on these data, the

current study was designed to 1) define the role of the androgen receptor in anesthetic neurotoxicity and developmental regulation of NKCC1 and KCC2 in cortex; 2) test NKCC1's functional role in developing cognitive deficits; and 3) explore how age and changes in cortex NKCC1 and KCC2 expression relate to vulnerability to cognitive deficits after anesthesia in male rats.

Methods

This manuscript adheres to the relevant sections of the ARRIVE guidelines.

Animals and Husbandry

Animal experiments were conducted according to the standards and protocols approved by the UCSF Institutional Animal Care and Use Committee. Neonatal all-male or all-female Sprague Dawley rat litters were purchased from Charles River (South San Francisco, CA) and delivered with a dam. Animals were housed as single sex litters in a single vivarium room and exposed to a reverse light-dark cycle (12h light-dark cycle, 18 to 25° C, 45 to 65% humidity). Food and water were available *ad libitum*. Animals were weaned on postnatal day 21 or 22 after which they were co-housed in groups of 4–6 from the same gender and litter. Animals were further segregated into groups of 2–3 on postnatal day 36, one week before behavioral testing began. Given the potential for environmental enrichment to influence behavior,[16] additional enrichment was limited to social housing and a single plastic tube (8cm diameter × 15cm long).

Isoflurane exposure

Isoflurane (ISO) was administered as previously described.[15,17] Animals were randomized to isoflurane exposure or control exposure while maintaining equal representations of each group across litters. For postnatal day 7 exposures, animals weighed 15 +/−5g. Briefly, animals were placed on a heated pad (Thermo Haake, Waltham MA) in a custom built exposure chamber. A humidified mix of air and oxygen (O_2) (40% O_2) was delivered to a Datex-Ohmeda isoflurane vaporizer (West Bloomfield, MI). Carbon dioxide (CO_2) absorbent pellets (Litholyme, Allied Healthcare, St. Louis, MO) were placed in the chamber. Isoflurane, CO_2, O_2 were monitored with a Datex-Ohmeda gas analyzer (West Bloomfield, MI). Isoflurane was down titrated from 2% to 1.4% , 0.8%, and 0.0% at two, four, and six hours respectively. Gas variables and pup temperatures were recorded continuously (Supplemental Fig. 3). Towards the end of exposure, non-exposed control animals (CON) were separated from dams for thirty minutes and placed on a warming pad in an anesthesia exposure chamber with room air. Limiting the control animal maternal separation was done to prevent confounding effects which can influence animal behavior in adulthood.[18,19] We conjecture that the isoflurane-exposed animals do not have awareness of maternal separation except for roughly 30 min following anesthesia emergence. In this and previous studies[15,20] our isoflurane exposure and control treatment did not translate into measurable nutritional deficiencies as weight curves for both groups are overlapping (Supplemental Fig. 2).

A total of 115 animals were exposed to isoflurane with 2 mortalities. Both mortalities were in the postnatal day 7 exposure as there were 0 mortalities in the postnatal day 14 exposure

group. These rates are consistent with our previous studies.[15,17] 122 non-exposed control animals were utilized. All studies were designed with the ideals of replacement, refinement and reduction of animal usage. This will allow others using similar methods to make accurate power calculations and potentially reduce the number of animals used overall.

Flutamide Exposure

On postnatal day 2 all pups were randomly assigned to receive treatment with either flutamide, a selective antagonist of the androgen receptor (Sigma Aldrich, St. Louis, MO) or vehicle (Veh), sesame oil (Fisher Scientific, Waltham, MA). On postnatal days 2, 4, and 6, pups were given 250mg subcutaneous injections (volume 0.1mL) on their dorsum. Injection sites were sealed using Vetbond Surgical Adhesive (3M, Maplewood, MN).

Testosterone ELISA

Blood samples were taken from adult animals that were treated with flutamide or vehicle as neonates. Prior to cardiac perfusion at the time of sacrifice, animals were briefly anesthetized with isoflurane and ~ 1 mL blood was aspirated from the left ventricle into a heparinized syringe. The blood was then centrifuged for thirty minutes 14x*g* at 4°C, after which plasma was separated and stored at −20°C. An ELISA to quantify testosterone levels, EIA-1559 (DRG International, Inc, Springfield, NJ), was performed according to manufacturer's protocol.

Bumetanide Exposure

Bumetanide (Hospira, Lake Forest, IL) (0.25mg/mL) was diluted in PBS. Animals were randomized to be injected with bumetanide (dose of 1.8mg/kg (0.2mL volume)) or vehicle intraperitoneally fifteen minutes prior to isoflurane exposure. This dose has previously been reported to show an effect in neonatal rats.[21] Injection site was sealed with Vetbond.

Behavior Studies

All behavior testing was performed during the dark cycle between 0800 and 1700h. 70% ethanol was used to clean all testing materials between all procedures and rats. Visual cues were placed on the walls for the Barnes Maze task and within the testing boxes for all object recognition tasks. Experimenters were blinded to animal group at the time of the testing. For each cohort, order was initially randomized and animals were tested sequentially each time.

Barnes Maze

Barnes maze testing was performed similarly to our previous description.[15,17] In the training phase, testing began on postnatal day 43. Rats were first habituated to an escape box for two minutes then placed into a circular open field arena with twenty holes cut out along the perimeter. The position of the escape box was randomized for each animal (maintaining equal distribution of "goal" among groups) and the box was placed under the assigned goal hole. Movement and latency to goal was recorded with a camera (Basler aca1280, Basler Inc, Exton, PA) and tracking software (Ethovision XT 11.5, Noldus Information Technology, Inc, Leesburg, VA). Barnes maze training took place over the course of four days with one trial per day. A probe trial was performed one week after memory acquisition during which

the escape box was removed and animal movements and time spent investigating holes were recorded over ninety seconds. Our primary outcome was the time spent at the goal hole in the probe trial. In the bumetanide cohort, a second probe trial was conducted five weeks after the last day of the acquisition phase. Animals that did not find the position of the escape box by day four of the learning phase were not included in the probe trial analysis (Excluded 5 total: 1 Flutamide Control Cohort; 1 Bumetanide Cohort; 1 CON/Veh, 1 ISO/Veh; postnatal day 14 Male Cohort: 2 ISO).

Recognition memory tasks

Novel object recognition was performed in a standard way as previously described.[22] Movements were tracked with the aforementioned camera and software. In the exposure, rats were placed in a boxed arena and exposed to two identical objects for four minutes. After a two-minute delay, rats were placed back into the arena for the testing phase, where one of the identical objects had been replaced with a different object. Time spent investigating the novel object versus the familiar object was recorded for another four minutes. The discrimination index was calculated from these values (time exploring novel object minus the time exploring the familiar object, divided by the total time investigating both objects).

Object place recognition was performed in the same way as the novel object paradigm with the exception that during the exposure the rats were exposed to two different objects and in the test, one of the objects was switched with an object that is identical to the remaining object.[22] This object, while identical, should be identified as the novel target because of its location. For both recognition memory tasks, our primary outcome was the discrimination index.

Fluoro-Jade C Staining

Animals undergoing Fluoro-Jade C staining were cardiac perfused with ice cold 4% paraformaldehyde.[15] Brains were dissected and fixed overnight at 4°C, then sucrose sunk (30% sucrose), frozen with isopentane/dry ice and stored at −20°C. They were sectioned at 60 microns thickness on a sliding microtome. Sections were mounted on Superfrost slides (Fisherbrand, Waltham, MA) and stained with 0.0001% Fluoro-Jade C (Millipore, Billerica, MA, USA) according to manufacturer's protocol.

Stereology

Stereologic analysis was completed with software(Stereo Investigator 10, MBF Bioscience; Williston VT) utilizing the optical fractionator method by a blinded experimenter. 3–4 sections of each brain containing representative sections of hippocampus, laterodorsal thalamus and mediodorsal thalamus regions were traced bilaterally with 4x objective on an upright fluorescence microscope (Nikon Eclipse 80i, Nikon; Melville NY) equipped with a fluorescent lamp and a C11440 Hamamatsu camera (Bridgewater, NJ). Positive cells were counted according to standard stereology methods using a 40x objective lens. The counting frame was set to 300 × 300 and the systematic random sampling grid was set to 50% and 75% of the region of interest for isoflurane and control sections respectively.

Western blot

Western blots were conducted as previously described.[15] Briefly, animals were killed and their brains were immediately removed and placed on ice. Samples of frontal cortex were dissected from both hemispheres and placed in radioimmunopreciptation buffer (Boston Bioproducts, Ashland MA) with protease inhibitor cocktail (Fisher Scientific, South San Francisco, CA). Tissue was immediately homogenized and concentrations were determined by ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE). Standard protein electrophoresis was performed with Tris-glycine polyacrylamide 7.5% gels (Criterion TGX, Biorad, Hercules CA) loading buffer and running (Boston Bioproducts) Precision Plus Protein Standards Dual Color (Biorad). Semi-dry Transfer was to polyvinylidene fluoride membrane (Biorad) was performed with Trans-Blot SD device (Biorad). Blots were blocked with 5% non-fat dry milk (Biorad), then incubated with primary antibodies overnight: rabbit anti-NKCC1 1:1000 (#14581 Cell signaling Technologies; Danvers MA), rabbit anti-KCC2 1:1000 (#07–432 Millipore; Hayward CA), rabbit anti-GAPDH 1:2000 (#1440 Cell Signaling Technology). Secondary antibody goat anti-rabbit HRP 1:1000 (#A16104 Life Technologies, Carlsbad CA) was incubated for 1hr at room temperature then the blot was cut and processed individually with chemiluminescent substrate Super Signal West Pico Plus or Super Signal West Femto (NKCC1 only) and immediately imaged on ChemiDoc Touch imager (Biorad). Band densities were determined with Image Lab 6.0 (Biorad) and NKCC1 and KCC2 densities were normalized to GAPDH loading controls.

Statistics

Statistical analysis was performed using Prism 8.3 software (GraphPad Software, San Diego, CA). Group size for behavior experiments was determined from previous studies.[15,17] For an alpha of 0.05 and an effect size ranging from 0.98 to 1.5 depending on the specific task, a group size of 8 to 13 animals is predicted to give a power of 0.8. For the cell death assay, we previously found a very large effect size of 6.5 with an alpha 0.05, which gave a power of 0.99 with groups of 3.[15] Animal numbers are reported as “n”. Means and standard deviations are reported as the mean value followed by the standard deviation in parentheses for all parametric analyses. For non-parametric data, Median values followed by 25^{th} and 75^{th} quartiles in parentheses are given. For all experiments, data were first subjected to Shapiro-Wilk normality testing which informed the choice of subsequent parametric or non-parametric statistical tests. For all analyses, alpha was set to 0.05. No outliers were detected or eliminated from any of the analyses. With the exception of 5 animals (identified above) that did not meet criteria for learning the position of the goal in the Barnes Maze, no other data were excluded from analyses.

In the Barnes maze training analysis, a two-way, repeated measures ANOVA was utilized with individual subjects as a blocking factor. This allowed for testing the effect of treatment, day of training, interaction of treatment and training, as well as the individual subjects effect on maze performance (time to escape). The choice of repeated measures (subjects blocking) design was supported by subject F-tests which suggest rejecting the null hypothesis (that all subjects are the same) for all of the trials. In the probe trial, the time exploring equidistant positions (e.g. +/−1 hole, +/−2 hole etc.) was averaged and compared to the time spent

exploring the goal hole with Dunnett's multiple comparison test.[15,17] To compare these patterns across groups, a curve comparison was made using the extra sum of squares F-test to ask whether the pattern fit a one-phase decay function or a straight line. We interpreted a p value <0.05 to favor the one-phase decay function over a straight line, and concluded that patterns of exponential decay were different from linear functions which formed the basis of comparisons across groups.

In the recognition memory experiments, unpaired, two-tailed t-Tests comparing the time investigating novel and familiar objects were used and a two-tailed one sample t-Test to compare the difference of the discrimination index from zero. In the western blot studies, differences between groups were tested with an unpaired, two-tailed t-Test or two-tailed Mann-Whitney U test for non-normal datasets. In the cell death experiments, one-way ANOVA was used to test for effect of group and post-hoc Sidak's multiple comparison was used to compare the two isoflurane exposed groups.

Results

Androgen receptor inhibition prior to isoflurane exposure protects males from cognitive deficit

Male rats, exposed to six hours of isoflurane at postnatal day 7 (n=13), learned the position of the escape box in the Barnes maze similar to controls (n=17) with a decrease in latency over the training period by repeated measures ANOVA $F_{day}(3, 84)=40$, $p<0.001$; there was no effect of group or interaction. (Fig. 1 B). In the probe trial, delayed 1 week after learning, non-exposed (Control) animals spent on average 6.0(4.7) seconds exploring the goal which was statistically more time than any other position, displaying a memory of the escape box location (Dunnett's test goal vs positions +/− 1,2,3,4,5,6,7,8,9,opposite). Additional analysis showed these data fit a one-phase decay model ($F(1, 140)=6.5$, $p=0.012$) compared to a linear function. In contrast, isoflurane exposed males were unable to differentiate the goal from any other position (mean time at goal 2.3(2.5) seconds) and the corresponding curve is best described by a linear function ($F(1,184)=0.18$, $p=0.675$) (Fig. 1 C)).

We previously found that unlike males, female rats exposed to six hours of isoflurane on postnatal day 7 do not develop a spatial memory deficit, however those exposed on postnatal day 4 are susceptible.[15] We hypothesized that androgen signaling plays a role in the prolonged susceptibility of males relative to females. We took a pharmacologic approach by injecting the androgen receptor blocker flutamide on postnatal days two, four, and six. We first completed a control cohort comparing flutamide (n=16) to vehicle (n=14) and found no effect on spatial memory or the object recognition tasks (Supplemental Fig. 1). There were no differences in weights between the groups nor were there any differences in serum testosterone levels in adulthood as measured by ELISA (Flutamide n=14; Control n=12) (means(SD): flutamide 1.90 (0.53)ng/mL, vehicle 1.97 (0.45)ng/mL; unpaired, t-Test $p=0.735$ (Supplemental Fig. 2)). A second cohort of flutamide (n=14) and control (n=14) animals was studied with the addition of a six hour isoflurane exposure at postnatal day 7. In this cohort, both groups learned the position of the goal in the Barnes maze and were not different during this acquisition phase (two-way repeated measures ANOVA $F_{day}(3, 78)=24$, $p<0.001$; $F_{group}(1, 26)=0.34$, $p=0.567$; $F_{interaction}(3,78)=2.1$, $p=0.112$)(Fig. 1E). Animals

treated with isoflurane and vehicle (ISO/Veh) performed poorly in the probe trial, spending a mean 2.4(3.5) seconds at the goal with no differences between the time at the goal and any other position. A curve analysis of these data favored a linear function ($F(1,151)=1.5$, $p=0.229$). In contrast, the flutamide treated males that were exposed to isoflurane at postnatal day 7 (ISO/Flutamide) were able to successfully differentiate all positions from the goal hole spending on average 6.4(3.9) seconds at the goal (Fig. 1 F). This pattern was different from the ISO/Veh group as it fit a one phase decay ($F(1,151)=79$, $p<0.001$). This protective effect of flutamide was further demonstrated in recognition memory domains as the ISO/Veh group showed no difference between discrimination of novel and familiar objects in the Object Place Test(mean discrimination index(SD): 0.07 (0.20) one sample t-Test $p=0.181$) but the ISO/Flutamide group was able to successfully discriminate the target (mean discrimination index(SD): 0.09 (0.14); one sample t-Test $p=0.032$) (Fig. 1J). Novel object recognition testing revealed successful discrimination by both groups (mean discrimination index(SD): ISO/Veh 0.30 (0.24), ISO/Flutamide 0.41 (0.28); one sample t-Test $p<0.001$ for both groups) (Fig. 1I) as is consistent with previous reports.[15,17,22]

We previously reported a change in the protein levels of NKCC1 and KCC2 in females which occurs between postnatal day 4 and 7; corresponding to the animal's loss of susceptibility to the isoflurane induced memory deficit over this time frame. We hypothesized that in males, flutamide would change the protein levels of these chloride transporters to reflect a mature pattern as in females. To test this hypothesis a third cohort of animals was administered flutamide in the same way as the previous experiments and was sacrificed on postnatal day 7 (with no isoflurane exposure)(Fig. 2). Westerns blots were conducted on lysates from bilateral frontal cortex and demonstrated robust changes in the protein levels of NKCC1 and KCC2 with flutamide treated animals (n=10) displaying a more mature pattern (Low NKCC1 and high KCC2) compared to vehicle treated animals (n=10) (Mean density(SD) NKCC1: Control 1.49(0.69), Flutamide 0.47(0.11), t-Test $p<0.001$; Median density(25^{th} percentile, 75^{th} percentile) KCC2: Control 0.23(0.13, 0.49), Flutamide 1.47(1.18,1.62), Manny-Whitney U test $p<0.001$).

Inhibition of NKCC1 with Bumetanide protects against isoflurane associated deficits

To specifically investigate the role of NKCC1 in the manifestation of the behavior phenotype, we blocked function of NKCC1 with bumetanide, an NKCC1 antagonist.[23] There were three groups of animals: those exposed to bumetanide (ISO/bumetanide n=15) or vehicle (ISO/Veh n=15) prior to isoflurane and those injected with vehicle and not exposed to isoflurane (CON/Veh n=18). In the Barnes Maze task, we found a strong learning effect for day of training during the acquisition phase (two-way repeated measures ANOVA $F(3, 135)=224$, $p<0.001$) (Fig. 3B). There was no effect of group or interaction. Performance in the probe trial after 1 week revealed CON/Veh and ISO/bumetanide treated animals fit a curve favoring one phase decay ($F_{CON/Veh}(1,184)=10$, $p=0.002$; $F_{ISO/Bumetanide}(1,162)=6.6$, $p=0.011$), compared to the ISO/Veh group whose extra sum of squares F-test fit favored a linear function ($F_{ISO/Veh}(1,151)=1.9$, $p=0.174$)(Fig. 3 C). A second probe trial was conducted five weeks after the initial learning phase. The curve remained linear for ISO/Veh, while the CON/Veh and ISO/bumetanide followed a one phase decay. Mean time exploring the goal was 1.3(2.6) seconds for ISO/Veh, 4.3(5.7) seconds for Con/Veh, and 4.6(7.4)

seconds for ISO/bumetanide. CON/Veh and ISO/bumetanide also showed differences from goal for positions +/−4,5,6,7,8,9, opposite and +/−5,6,7,8,9,opposite, compared to no differences for ISO/Veh in this long delay probe trial (Fig. 3D). Animal weight was recorded over the developmental window and no differences between any of the groups were observed (Supplemental Fig. 2).

Postnatal day 14 male rats have lost vulnerability to isoflurane associated deficits

Previously, we showed that female rats are susceptible on postnatal day 4 but have lost vulnerability by postnatal day 7.[15] Here we show that males are susceptible on postnatal day 7 but can be protected with flutamide administered prior to exposure. However, we do not know when this loss of vulnerability occurs in males. We aimed to define anesthetic susceptibility in males at the timepoint of postnatal day 14. On postnatal day 14, we exposed male rats to the same six hour isoflurane exposure(n=15) or control (n=15) as previously described. Barnes maze learning showed both groups learned the position of the escape box over 4 days (Fig. 4 B–C) (two-way repeated measures ANOVA $F_{group}(1,28)=0.23$, $p=0.632$; $F_{day}(3, 84)=19$, $p<0.001$; $F_{interaction}(3, 84)=0.67$, $p=0.571$). In the Probe trial after exposure at postnatal day 14, both groups successfully discriminated the goal from other positions (CON+/−4,5,6,7,8,9,opposite; ISO +/−1,2,3,4,5,6,7,8,9,opposite). Mean time spent exploring the goal was 4.3(5.0) seconds for controls and 6.1(7.0) seconds for isoflurane exposed animals. Curve comparison found one phase decay is preferred over straight line for both the control ($F(1,162)=7.5$, $p=0.007$) and isoflurane treated animals ($F(1,140)=28$, $p<0.001$). Thus, postnatal day 14 represents a defined point where the susceptibility to a memory deficit after isoflurane in male rats has ceased.

The expression of NKCC1 and KCC2 in the cortex was measured in male (n=10) and female (n=10) rats at postnatal day 14 by western blot analysis and found to be no different (Mean density(SD) NKCC1: Male 0.98(0.52), Female 1.06(0.15), t-Test $p=0.649$; Median density(25^{th}percentile,75^{th}percentile) KCC2: Male 1.06(0.59,1.28), Female 0.85(0.76,1.17), Mann-Whitney U test $p=0.912$)(Fig. 4 D–H). These results suggest that by postnatal day 14, the developmental sex-difference observed at postnatal day 7 in NKCC1 and KCC2 levels has abated.[15]

Cell death does not predict spatial memory performance

As treatment with both flutamide and bumetanide attenuates the deleterious effects of volatile anesthetics on memory, we next explored the effects of these interventions on cell death. We specifically studied the hippocampus (HC), laterodorsal thalamus (LDT) and mediodorsal thalamus (MDT) as we previously reported cell death in these regions with the same anesthetic exposure.[15] We found a group effect by one-way ANOVA for *most* groups (Fig. 5)(Flutamide: $F_{HC}(2, 6)=5.6$, $p=0.043$, $F_{LDT}(2, 6)=5.1$, $p=0.050$, $F_{MDT}(2, 6)=3.0$, $p=0.12$; Bumetanide: $F_{HC}(2, 6)=5.2$, $p=0.048$, $F_{LDT}(2, 6)=6.2$, $p=0.034$, $F_{MDT}(2, 6)=5.5$, $p=0.044$). Comparing between the animals that received treatment vs vehicle that were also exposed to isoflurane, post-hoc Sidak's multiple comparison testing revealed no differences between the vehicle control and treatment (Mean Cells/mm^3(SD) Flutamide Cohort: HC Iso/Veh 28(17), Iso/Flu 31(5), $p=0.454$; LDT Iso/Veh 2300(1460), Iso/Flu 1540(475), $p=0.335$; MDT Iso/Veh 654(558), Iso/Flu 532(192), $p=0.677$; Bumetanide Cohort: HC

Iso/Veh 37(16), Iso/Bum 17(9), p=0.067; LDT Iso/Veh 262(2030), Iso/Bum 1680(856), p=0.590; MDT Iso/Veh 591(262), Iso/Bum 426(228), p=0.590).

Discussion

We previously reported that Isoflurane exposure in female rats at postnatal day 4 but not 7 is followed by a memory deficit and that protein levels of NKCC1 and KKC2 in males and females are associated with a temporally-defined susceptibility to anesthesia induced memory deficits.[15] Here we show that blocking androgen receptors through flutamide administration prior to isoflurane exposure in postnatal male rats protects against this behavioral deficit. Flutamide also alters NKCC1 and KCC2 protein expression patterns in males to be similar to those we reported in adults and in female rats at postnatal day 7 when they are no longer susceptible to the memory deficit. Flutamide alone does not affect adult male rats in terms of weight gain, serum testosterone levels as young adults, or ability to perform spatial and recognition memory tasks. The observed effects of flutamide on NKCC1/KCC2 expression and behavioral outcomes suggest that regulation of these specific chloride transporters by androgens is a critical component for anesthetic susceptibility. We also show that this deficit is linked to chloride transporter function, as acute blockade of NKCC1 with bumetanide during isoflurane exposure prevents the cognitive deficit in adulthood. Finally, we narrow the range of days over which male rats are susceptible as postnatal day 7 exposure leads to a memory deficit while postnatal day 14 exposure does not.

The importance of steroid hormones in guiding sex-specific brain development has long been appreciated.[24] For many years it was presumed that sexual dimorphism resulted primarily from exogenous testosterone, aromatized to estrogen in the brain, which then exerts masculinizing effects.[25] However, it is now understood that this process is substantially more complex; mediated through local and systemic sex hormone signaling, including both androgens and estrogens, with specific regionalization in the brain.[26] In our study, we show a specific role for androgen receptors in the susceptibility of postnatal male rats to isoflurane. This period of susceptibility is associated with an immature expression pattern of NKCC1 and KCC2 in the cortex. Treatment with flutamide is similar to testicular-feminization, in which genetically male individuals are born with a non-functioning androgen receptor.[27] In rodent models, all sexually dimorphic nuclei examined with testicular feminized animals show loss of male characteristics,[27] which highlights the importance of androgen receptors in brain patterning. We previously found that neonatally-gonadectomized males were also protected from the isoflurane mediated deficit[20] similar to the current flutamide studies. However, in contrast to gonadectomy and testicular feminization models, our flutamide intervention targets a narrower time period encompassing the isoflurane exposure, and does not cause irreversible gonadal inhibition as testosterone levels are normal in adulthood after neonatal flutamide treatment. Additionally, flutamide alone was not sufficient to affect spatial memory performance (Supplemental Fig. 1), which is important given the role of early life sex hormones effect on spatial memory.[28]

Anesthetic toxicity is dependent on the developmental stage during exposure, providing critical insight into the underlying mechanism of injury. Our study builds on previous work

in female rats which have a susceptibility window across postnatal day 4 that is closed by postnatal day 7. In our current study, males exposed on postnatal day 7 have a phenotype similar to postnatal day 4 females in that they are susceptible to anesthetic deficit and express immature protein patterns of NKCC1 and KCC2. The administration of flutamide changes the protein levels of NKCC1 and KCC2 such that the males reflect the pattern of postnatal day 7 females; KCC2 increases and NKCC1 decreases, which corresponds with protection from the neurotoxic insult. The postnatal day 14 exposure in males did not result in cognitive deficits and there was no difference in the specific chloride transporter's expression between males and females. This suggests that vulnerability following isoflurane exposure is minimized by postnatal day 14 through a similar mechanism that protects the female brain at postnatal day 7.[15] These differing outcomes after anesthetic exposure support a dynamic model where susceptibility is regulated through the developmental effects of endogenous sex steroids, structural dimorphisms, and age.[26] Intriguingly, others have also shown that GABAergic anesthetics administered in the second week of life in rodents can result in lifelong neuroanatomic changes[29,30] which are often associated with improved cognition. Although not designed to evaluate this directly, our study also shows very high performance in isoflurane-exposed males on P14. In contrast, Zhu and colleagues, using different species and exposure paradigms, reported worsened cognitive performance after anesthetic exposure at this age[31].

As an NKCC1 inhibitor, bumetanide can suppress GABA excitability in the neonatal period.[32] It is presumably through this action that it has been used to rescue the effects of early life anesthetics in our study and others.[21,33] In this study we find that the single dose of bumetanide prior to isoflurane exposure changed behavior in the Barnes Maze Probe trial at one week and five weeks following initial training. Taken with the KCC2 expression data, these behavioral data suggest that conditions for anesthetic neurotoxicity are met when KCC2 levels are low and NKCC1 is high. However, blockade of NKCC1 leading to a functionally adult phenotype, is sufficient to protect against the deficit at a developmental stage it would normally occur.

Interestingly, unrelated models of neurological injury in neonates have also implicated NKCC1 function in their disease pathways. In a completely different neonatal insult model than anesthetic neurotoxicity, maternal deprivation, bumetanide was also found to be protective.[18] In another application, the action of bumetanide was posited to be useful in treating refractory neonatal seizures[23] although side effects far overshadowed any potential benefit in a small clinical trial.[34] In the field of anesthetic neurotoxicity, volatile anesthetics can produce seizure-like activity in neonatal animals that is dependent on GABA excitability.[33] Furthermore, sex differences may underlie susceptibility to neonatal seizures in animals[35] and humans.[36–38] Although these phenomena are studied by very different fields, the mechanistic commonality of NKCC1 function during a narrow developmental window suggests they are related, and may be manifestations of different perturbations of the same underlying developmental program.

How the proposed interaction of anesthetics and GABA development translates into a cognitive deficit remains largely unknown. This question is further complicated by the fact that anesthetic agents themselves can influence the expression patterns of these chloride

transporters,[39] possibly extending the critical period or irreparably altering normal development in this sensitive window. Volatile anesthetic exposure can also lead to DNA methylation of specific genes including chloride transporters which can alter expression of these molecules in the next generation.[40,41]

Cell death was initially thought to play an outsized role in the mechanism of anesthetic toxicity given the reproducible findings in animal models from worms to non-human primates.[42,43] However, this assumption has been challenged with a number of preclinical studies showing a dissociation between apoptosis and behavioral deficits. For example, apoptosis can be prevented by inhibiting the P75 neurotrophin receptor after neonatal isoflurane or propofol exposure,[44,45] but this does not protect against the lasting cognitive deficit.[46] Similarly, in our current study, we found elevated levels of cell death in both flutamide and bumetanide treatments when paired with isoflurane exposure. Yet despite having similar levels of apoptosis, there was better cognitive performance in the Barnes maze of the flutamide and bumetanide groups compared to vehicle treated animals. Instead of simply losing critical cells through apoptosis, the neurotoxic insult may exert its effect by influencing processes like synaptogenesis or cell maturation. In this way, normal development is altered during this critical window, and the effects are later unmasked with behavioral deficiencies in adulthood.

There are several limitations with the studies reported here. The link between GABA function and toxicity remains associative and theorized based on measured changes in the expression pattern of NKCC1 and KCC2. However, pharmacological blockade of NKCC1 does result in a change in cognitive behavior, strongly supporting our hypothesis. We have also taken a pharmacologic approach instead of a genetic one (in using rats as opposed to transgenic mice). While a genetic model could be very valuable in testing the specific genetic mechanism, we have prioritized *cognitive deficit* as the clinically relevant outcome. Because of this, using rats in well-defined cognitive tests has distinct advantages in reproducibility and sensitivity compared to mice. In addition, the pharmacologic manipulations allowed for temporal interventions that were not permanent, unlike genetic manipulations.

The expression of NKCC1 and KCC2 is regulated by many factors including age, sex, brain region and exposure to anesthetic agents. Interpretation of our studies should therefore be limited as our experiments represent small subset of conditions defined by these factors. This leaves open the possibility that NKCC1 and KCC2 might play significant roles in cognition after anesthesia exposure, which we have yet to explore. Finally, the exposure to volatile anesthetic in perinatal rats should be extrapolated with caution to humans for several reasons including technical limitations in our inability to mechanically ventilate multiple animals at a time.

In conclusion, these studies identify important factors for susceptibility to anesthetic neurotoxicity including a role for androgen receptors and the chloride transporters, NKCC1 and KCC2. These developmental insights also have practical significance for the design and reporting of results in the field of sex-difference and developmental neurotoxicity and may

hold clinical relevance for future study given the disproportionate distribution of surgical problems in very young boys and girls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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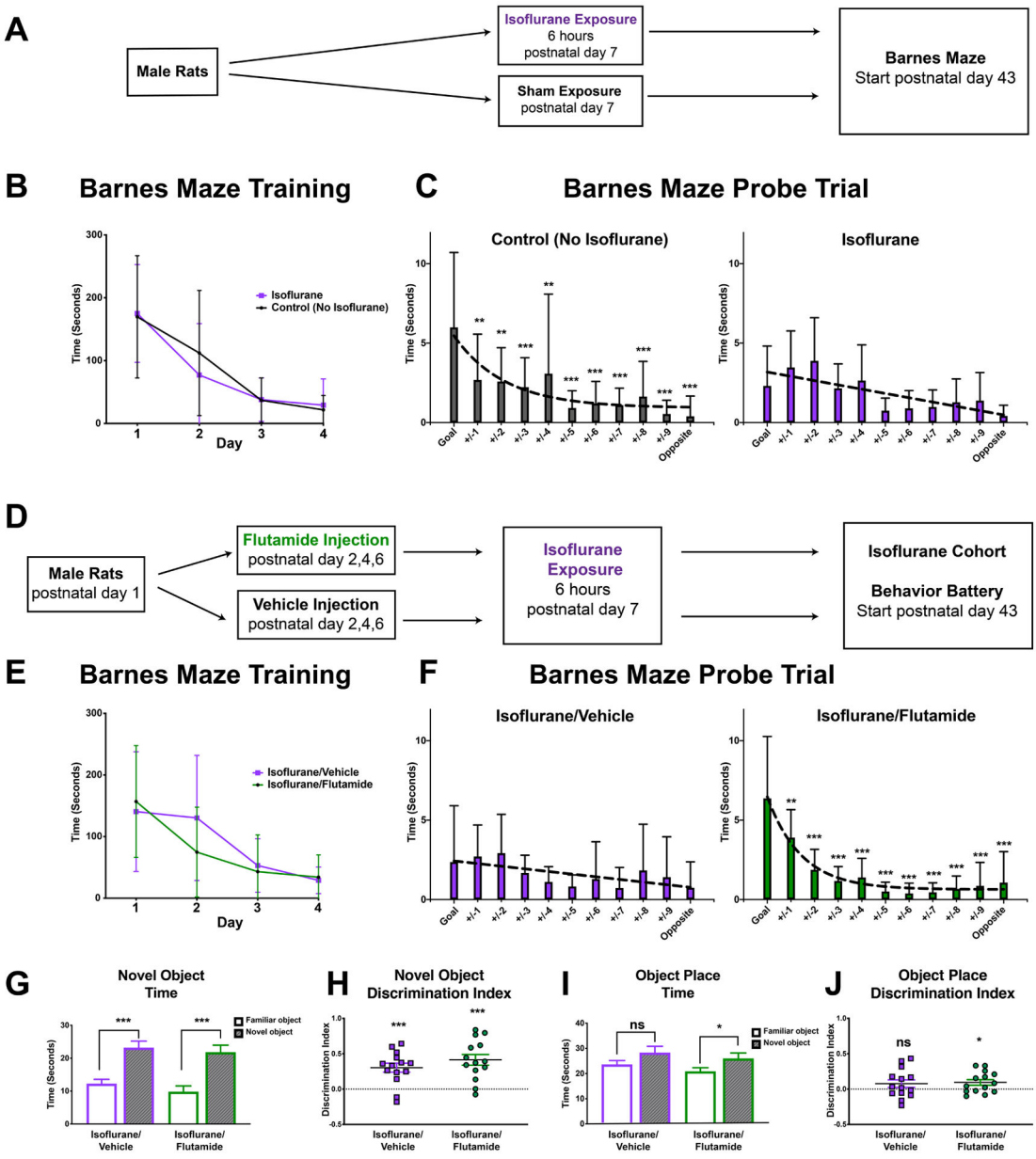

Figure 1.

Flutamide protects against Spatial and Recognition Memory Deficits

A. Experimental overview: male rats exposed to sham (n=17) or isoflurane (n=13) at postnatal day 7. Animals underwent Barnes Maze testing starting at postnatal day 43. B. Behavior Training. All animals acquired the goal over the course of four training days C. Probe trial. Control animals differentiated the goal from every other averaged position by Dunnett's multiple comparison analysis. This pattern fit a curve with one-phase decay by extra sum of squares F-test ($F(1,140)=6.5$, $p=0.012$). Isoflurane exposed animals were unable to differentiate any position from goal and showed a linear relationship among all positions ($F(1,184)=0.18$, $p=0.675$). D. Experimental overview: male rats were injected with flutamide (n=14) or vehicle (n=14) then exposed to isoflurane and subjected to behavior

battery. E. Barnes Maze Training. No difference among acquisition. F. Probe trial. ISO/Veh animals did not differentiate the goal from other positions while ISO/flutamide treated animals successfully discriminated every position by Dunnett's multiple comparison. Curve fit for ISO/flutamide favored a one-phase decay ($F(1,151)=79$, $p<0.001$). G-H. Novel object recognition testing showed differences for both groups the discrimination index for the novel object (one sample t-Test $p<0.001$ for both groups). I-J. Object Place Recognition- ISO/Veh animals were unable to discriminate the novel from the familiar object ($p=0.181$) while ISO/flutamide animals were able to discriminate ($p=0.032$). Error bars represent standard deviations. ns=not significant, *$p<0.05$, ** $p<0.01$, ***$p<0.001$

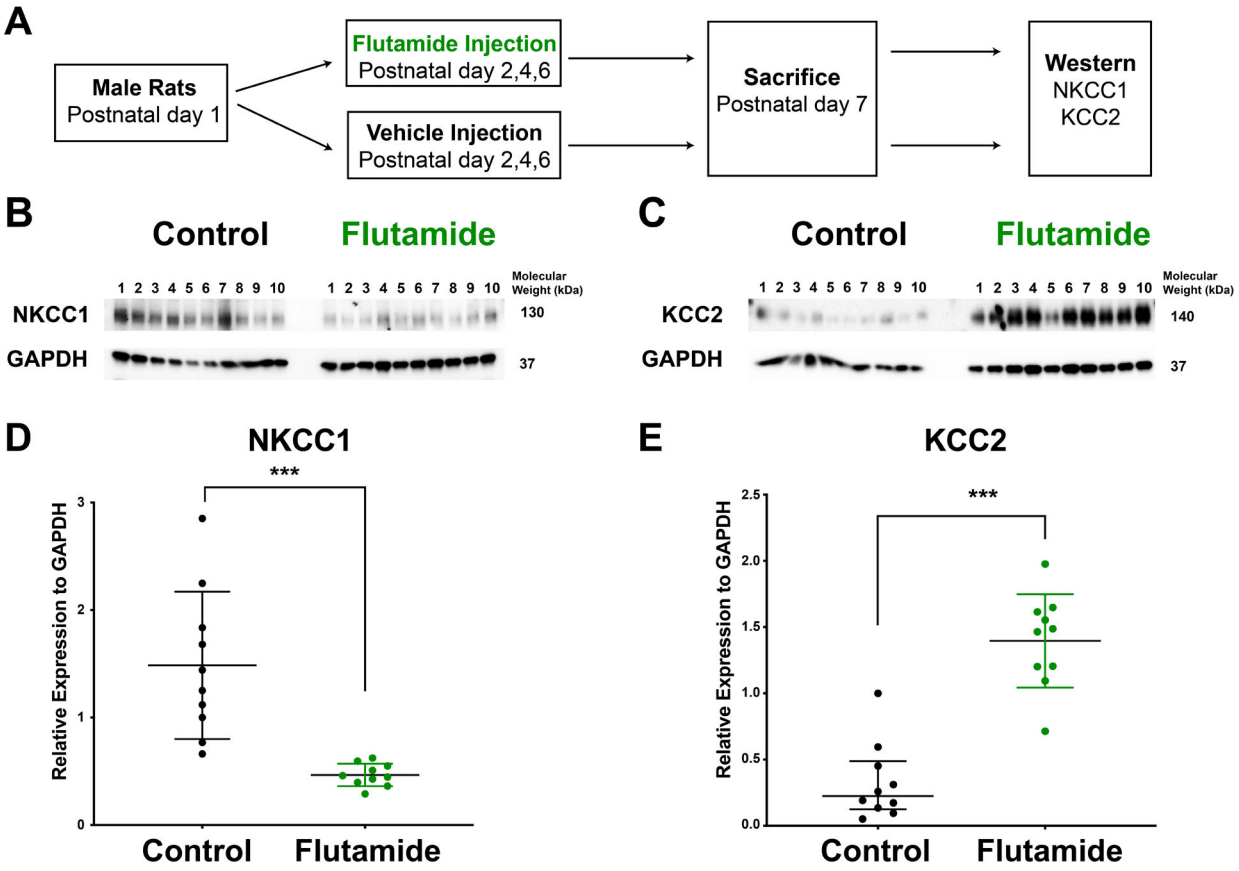

Figure 2.

Flutamide Alters Chloride transporters at postnatal day 7

A. Experimental overview: male rats were injected with either flutamide (n=10) or vehicle (n=10) and sacrificed on postnatal day 7. B-C. Cortical lysates were made and subjected to western blot analysis for NKCC1 and KCC2. D-E. quantification of blot after normalization to GAPDH showed decrease in NKCC1 with flutamide treatment by unpaired t-Test ($p<0.001$) and an increase in KCC2 by Mann-Whitney U test ($p<0.001$). Error bars represent standard deviations or interquartile range (E). ***$p<0.001$

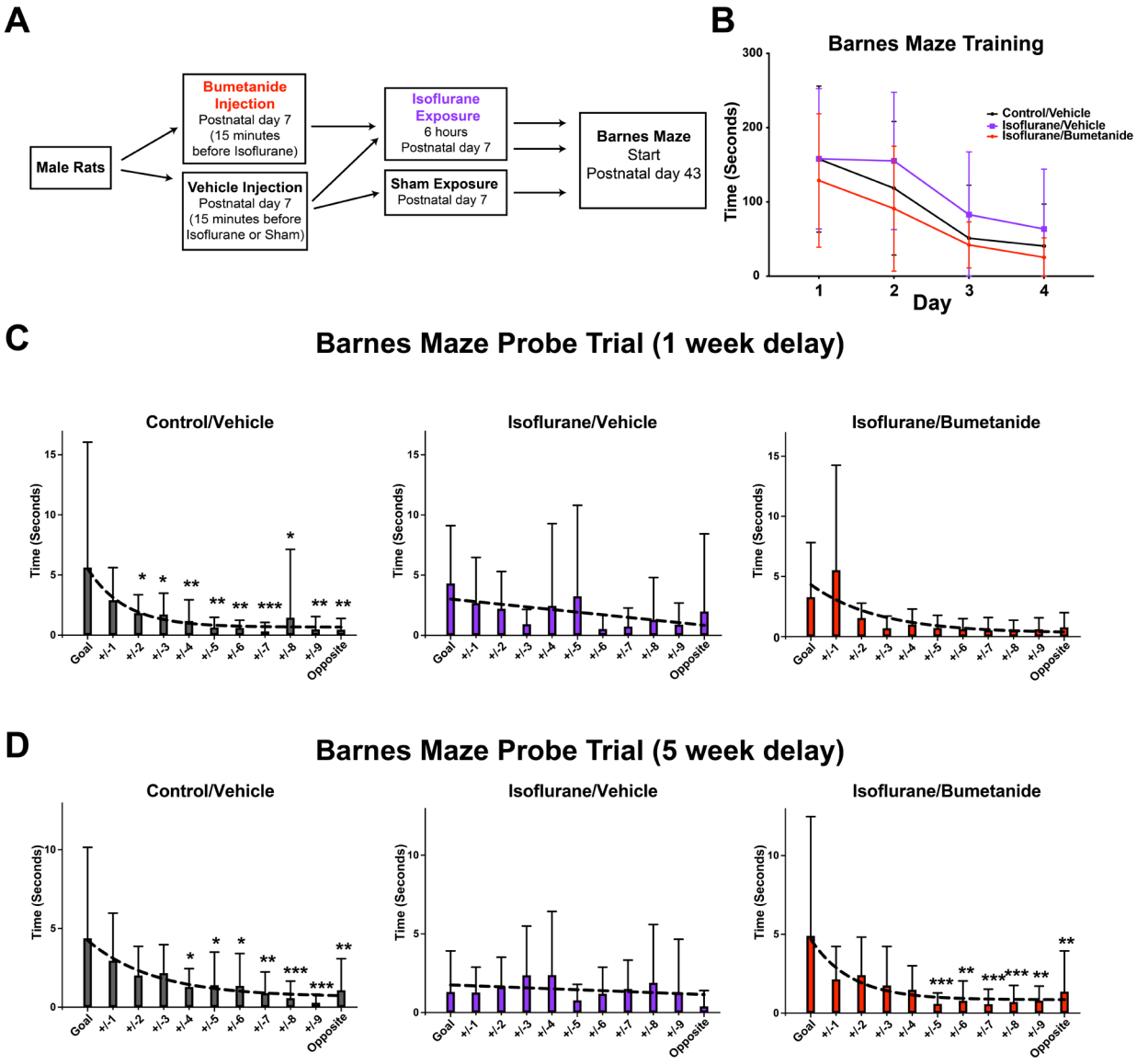

Figure 3.

Bumetanide protects against recognition memory deficit

A. Experimental overview: Bumetanide (n=15) or vehicle (n=15) was injected prior to isoflurane exposure. Non-exposed, vehicle injected controls were also used (n=18). Barnes maze testing followed at postnatal day 43. B. All groups acquired the goal position over 4 days. C. Probe trial after 1 week showed no difference in goal vs other positions for either ISO/Veh or ISO/bumetanide by multiple comparison, but curve fit showed preference for linear fit by sum of squares F-test for ISO/Veh ($F(1,151)=1.9$, $p=0.174$) and a one-phase decay for ISO/bumetanide ($F(1,162)=6.6$, $p=0.011$). D. Repeat probe trial 5 weeks after initial acquisition showed differences in positions +/−5,6,7,8,9,opposite in the ISO/bumetanide, but no differences in the ISO/Veh. Error bars represent standard deviations. *$p<0.05$, ** $p<0.01$, ***$p<0.001$

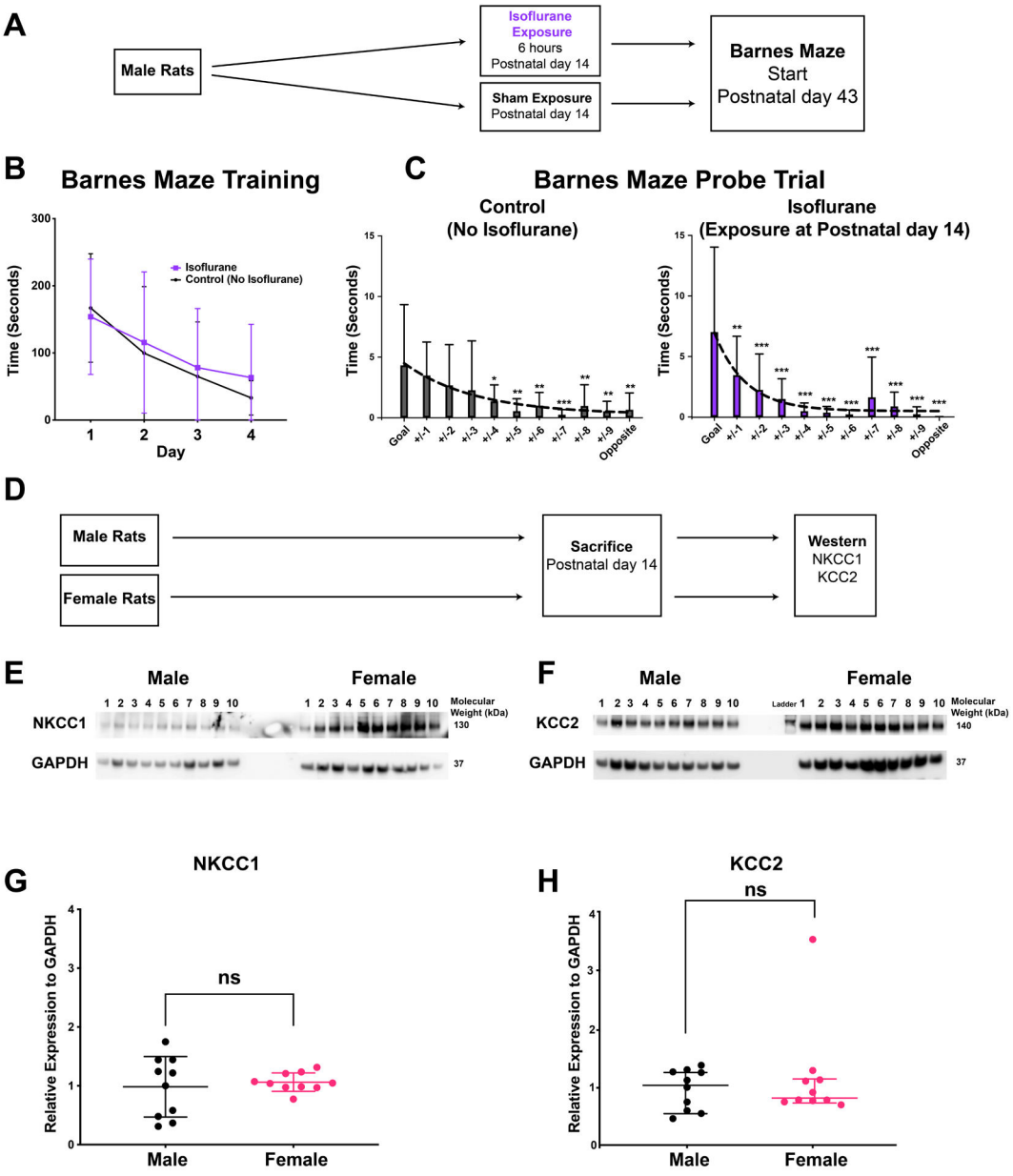

Figure 4.

Male susceptibility window has closed by postnatal day 14, Chloride transporter levels are not different in males and females

A. Experimental overview: Male rats were exposed to isoflurane (n=15) or sham at postnatal day 14 (n=15) and underwent Barnes Maze testing at postnatal day 43. B. Both groups acquired the position of the goal hole. C. Both groups differentiated the goal from at least part of the maze by Dunnett's multiple comparison, and curve fit analysis favored one phase decay functions over linear ($F_{CON}(1,162)=7.5$, $p=0.007$; $F_{ISO}(1,140)=28$, $p<0.001$). D. Experimental overview: male (n=10) and female (n=10) rats were sacrificed at postnatal day 14. E-F. Cortical lysates were subjected to western blot analysis for NKCC1 and KCC2. G-H. After normalization to GAPDH, neither protein showed a difference between male and

females. Error bars represent standard deviations, or interquartile range (H). ns=not significant, *p<0.05, ** p<0.01, ***p<0.001

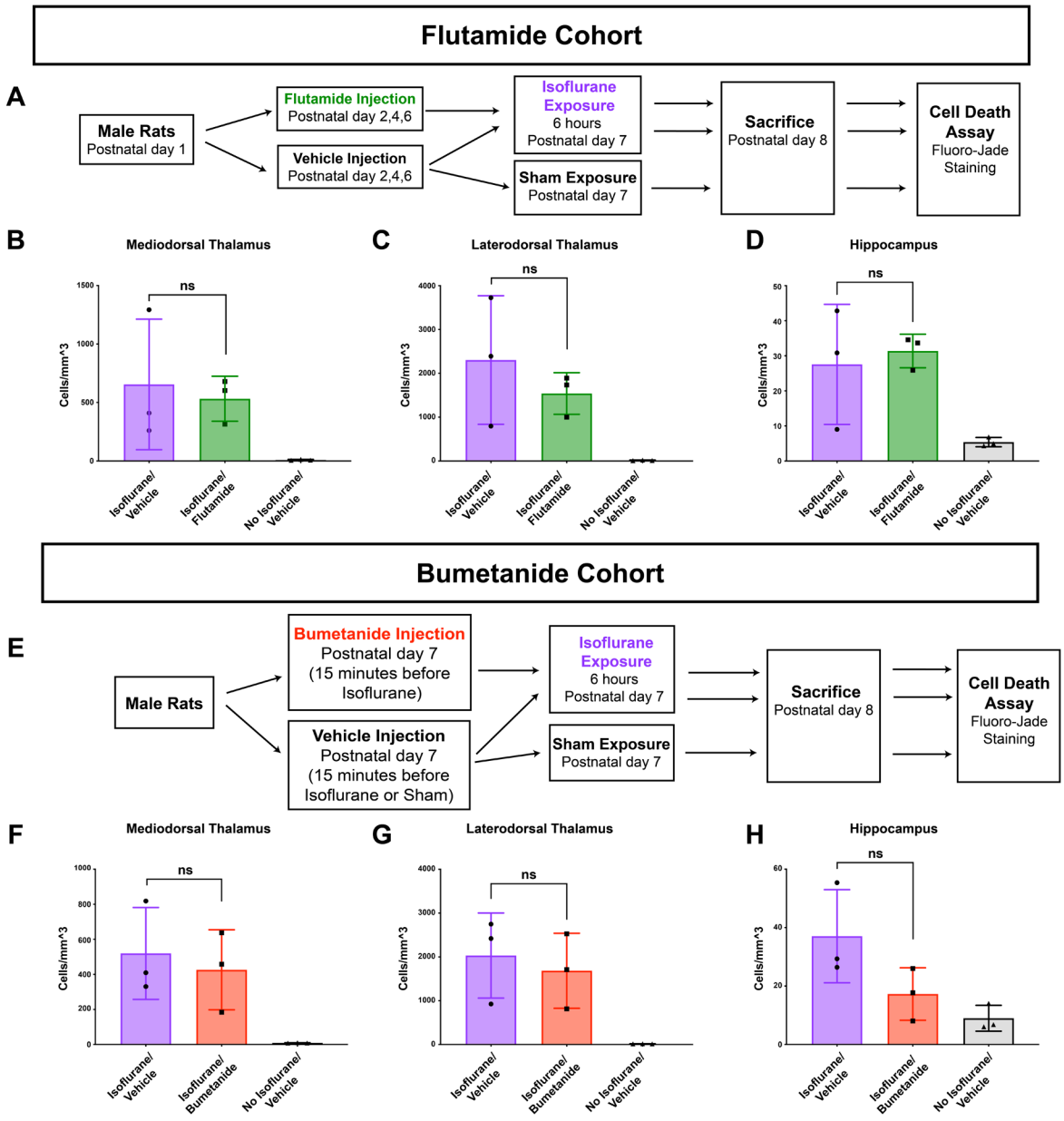

Figure 5.

Cell death occurs after ISO exposure with Flutamide or Bumetanide

A. Experimental overview: Male rats were injected with flutamide (n=3) or vehicle (n=3), then exposed to isoflurane or control conditions (n=3) on postnatal day 7. On postnatal day 8 animals were sacrificed, brains were used in a cell death assay. B-D. Stereologic quantification of Fluoro-Jade C positive cells in mediodorsal thalamus, laterodorsal thalamus and hippocampus. There was no difference between either ISO exposed group by Sidak's multiple comparison testing. E. Experimental overview: Bumetanide was injected prior to postnatal day 7 isoflurane exposure; postnatal day 8 animals were sacrificed and cell death assay completed. F-H. Stereologic quantification of Fluoro-Jade C positive cells showed no

difference between isoflurane exposed groups for any brain region. Error bars represent standard deviations. ns=not significant