IBRO Neuroscience Reports 14 (2023) 483–493

Contents lists available at ScienceDirect

IBRO Neuroscience Reports

journal homepage: www.sciencedirect.com/journal/IBRO-Neuroscience-Reports

Research Paper

Early-life propofol exposure does not affect later-life GABAergic inhibition, seizure induction, or social behavior

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ARTICLE INFO

Keywords:
Propofol
Early-life anesthesia
Brain development
GABAergic inhibition
Social behavior
Cognitive function

ABSTRACT

The early developing brain is especially vulnerable to anesthesia, which can result in long lasting functional changes. We examined the effects of early-life propofol on adult excitatory-inhibitory balance and behavior. Postnatal day 7 male mice were exposed to propofol (250 mg/kg i.p.) and anesthesia was maintained for 2 h; control mice were given the same volume of isotonic saline and treated identically. The behavior and electrophysiology experiments were conducted when the mice were adults. We found that a 2-h neonatal propofol exposure did not significantly reduce paired pulse inhibition, alter the effect of muscimol (3 μM) to inhibit field excitatory postsynaptic potentials or alter the effect of bicuculline (100 μM) to increase the population spike in the CA1 region of hippocampal slices from adult mice. Neonatal propofol did not alter the evoked seizure response to pentylenetetrazol in adult mice. Neonatal propofol did not affect anxiety, as measured in the open field apparatus, depression-like behavior, as measured by the forced swim test, or social interactions with novel mice, in either the three-chamber or reciprocal social tests. These results were different from those with neonatal sevoflurane which demonstrated reduced adult GABAergic inhibition, increased seizure susceptibility and reduced social interaction. Even though sevoflurane and propofol both prominently enhance GABA inhibition, they have unique properties that alter the long-term effects of early-life exposure. These results indicate that clinical studies grouping several general anesthetic agents in a single group should be interpreted with great caution when examining long-term effects.

1. Introduction

Propofol is a commonly used intravenous agent for anesthesia and procedural sedation in neonates, children and adults; its predominate mechanism of action is to enhance the effect of the inhibitory anesthetic GABA. Sevoflurane, a volatile anesthetic commonly used in neonates, children and adults, shares propofol's rapid onset and emergence and both potentiate $GABA_A$ receptor mediated GABAergic inhibitory neurotransmission (Garcia et al., 2010). We have previously studied sevoflurane neonatal anesthesia and will compare these results to the current results with propofol in the discussion section.

While periods of anesthesia less than 1 h in animals and children do not appear to lead to problems, more prolonged or repeated anesthesia may lead to neurodevelopmental changes and deficits in learning and behavior that extend into later years (Ing et al., 2021; Walkden et al., 2020). In humans the developing brain is vulnerable to anesthetics because neuronal genesis and maturation and the pruning of excess neurons by apoptosis begins mid-pregnancy and continues to be active through the perinatal period (Andropoulos, 2018). General anesthetics have been shown to trigger dose-dependent apoptosis in the developing brain and long-term effects on behavior, learning and memory (Ing et al., 2021; Jevtovic-Todorovic et al., 2003; Olsen, Brambrink, 2013). Previous studies of the long-term effects of early life propofol administration have yielded mixed results, with some studies showing long-term effects only after multiple exposures (Chen et al., 2016; Zhou et al., 2021). In humans it has been easier to show behavioral effects of early life anesthesia than deficits in learning and memory (Ing et al., 2021; Walkden et al., 2020). Social and anxiety-like behaviors are examined in

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https://doi.org/10.1016/j.ibneur.2023.05.007
Received 9 March 2023; Accepted 13 May 2023
Available online 15 May 2023

our study since these have been seen to be affected in some clinical studies of early-life anesthesia (Ing et al., 2021; Walkden et al., 2020).

Clinical anesthesia practice mandates continuous monitoring for hypoxia and hypotension, however animal studies have been less rigorous in this regard (Trapani et al., 2000). Many animal studies either measure blood oxygen levels at a single time point or take measurements from animals only treated similarly to the experimental ones (Gonzales et al., 2015; Karen et al., 2013; Wan et al., 2021; Zhou et al., 2021). These techniques would miss intermittent periods of hypoxia or hypoperfusion which could induce hypoxic/ischemic neuronal damage. The current study continuously monitors the mice with a pulse oximeter, which is a standard of care for clinical anesthesia. Neonatal animals are particularly susceptible to decreased respiration and blood pressure during which hypoxic or ischemic neuronal damage could lead to changes in behavior, learning and memory independent of direct anesthetic effects.

While all general anesthetics decrease consciousness, anesthetics have unique mechanisms of actions; sevoflurane and propofol primarily enhance GABA inhibition while ketamine acts primarily on glutamate receptors (Campagna et al., 2003; Forman and Chin, 2008; Garcia et al., 2010; Krasowski and Harrison, 1999; Rudolph and Antkowiak, 2004). However, even anesthetics with similar receptor actions have unique secondary effects. Volatile anesthetics release of calcium from intracellular stores, alter microRNA expression and activate second messengers; intravenous anesthetics may selectively activate different second messenger signaling cascades in addition to their effects on neurotransmitter receptors (Campagna et al., 2003; Forman and Chin, 2008; Garcia et al., 2010; Krasowski and Harrison, 1999; Lin et al., 2018; Rudolph and Antkowiak, 2004; Wang et al., 2012).

Previously we found that neonatal sevoflurane led to behavioral changes in mice, particularly with regard to social interactions (Lin et al., 2016). Neonatal sevoflurane reduced adult GABA inhibition in the adult hippocampus and the mice exhibited behavioral changes and were more susceptible to induced seizures (Lin et al., 2021). Since propofol, like sevoflurane, has its predominant anesthetic action by enhancing GABA activity, in the current study we examine neonatal propofol for long-term effects on GABAergic inhibition and behavior in adult mice.

2. Experimental procedures

A total of 102 male C57BL6/J mice were used throughout the study, which was approved by the State University of New York, Downstate Health Sciences University Institutional Animal Care and Use Committee. All experiments were performed in accordance with relevant guidelines and regulations. We obtained C57BL6/J female and male mice from Taconic Biosciences (Germantown, NY) and bred them in house. This was done to avoid the effects of shipping trauma on pregnant or neonatal mice. The methods used in this paper have been described in detail in previous publications (Lin et al., 2021, 2016; Liu et al., 2018).

The effects of early-life propofol exposure on adult electrophysiology and behavior were examined using C57BL6/J male mice (Fig. 1A). One group of mice received 250 mg/kg of the clinical formulation of propofol (i.p.) (propofol emulsion in intralipid; Hospira Inc., Lake Forest, IL) on postnatal day 7, while a control group was injected with the same volume of isotonic saline. We chose postnatal day 7 since it is a critical time period during neuronal development for anesthetic induced behavioral changes and it is the time we used for previous studies (Lin et al., 2021; Lin et al., 2016; Zhou et al., 2021). We did not control for the propofol vehicle, intralipid, since it is always present in the clinical formulation of propofol. We chose the lowest propofol dose that gave complete anesthesia for 2 h but did not affect heart rate or oxygenation; propofol emulsion is slowly absorbed when given i.p. Clinically propofol is administered i.v. but reliable i.v. access is difficult in neonatal mice so we administered propofol i.p. All male mice in a litter were subjected to either propofol or saline on the same day. Two male mice were removed from the Dam at a time, one was given propofol the other saline, they were both apart from the Dam for 2.5 h; if there were an odd number of male mice in a litter the last mouse was not paired when subjected to either saline or propofol. The pups were placed on a 37° C heating pad during anesthesia to maintain normal body temperature. A pulse oximeter sensor (MSTAT-4 mm, Kent Scientific Corporation, Torrington, CT, USA) was placed on one of the hind paws of all anesthetized pups; the peripheral capillary oxygen saturation and heart rate were measured continuously and recorded every 5 min. The heart rate and oxygen saturation were maintained within the normal rage throughout the experiment (Fig. 1B, C). Thirty minutes after the 2-h treatment (propofol or saline), the pups were returned to their home cage and reunited with their dams. The Dam accepted the returned neonates without any

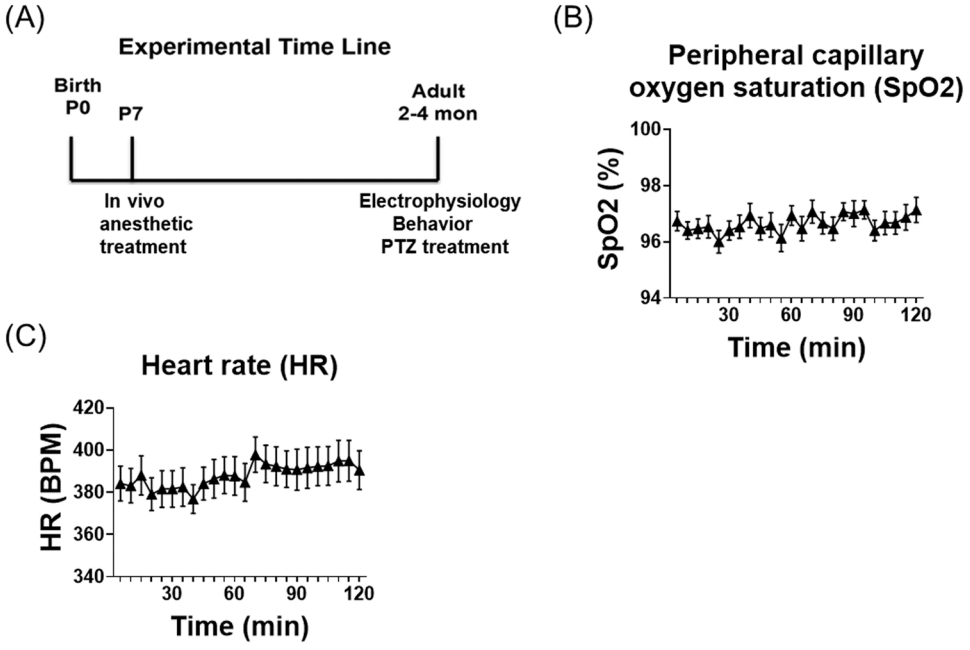

Fig. 1. Experimental timeline and physiological parameters during anesthesia A. All experiments were conducted following this established timeline. Mice were treated with propofol on postnatal day 7 (P7) and were examined for electrophysiology, seizure induction or behavior as adults at 2-4 months. The timing of the adult study depended on the parameter examined. B. and C. During the propofol anesthesia peripheral capillary oxygen saturation (SpO2) and heart rate (HR) were measured continuously with a pulse oximeter designed for small rodents (values are the mean ± the standard error of the mean; n = 15 mice).

observable problems. All pups were then reared and weaned following standard institution procedures and examined as adults.

2.1. Electrophysiological procedures

Adult mice (2–3 months of age) were sacrificed by cervical dislocation; their brains were rapidly removed, placed in ice cold artificial cerebrospinal fluid (aCSF) (2–4 °C), and the hippocampi were dissected from the rest of the brain and the dorsal hippocampus sectioned into 500 μm thick slices. The dorsal hippocampus was examined since it provides good slices for electrophysiological studies and it is the region we have used in our previous studies; the choice, vs the ventral hippocampus, was not based on behavioral or physiological differences. The hippocampal slices were incubated in artificial cerebrospinal fluid (aCSF) that was equilibrated with 95 % O_2 and 5 % CO_2, for 2 h at room temperature (approximately 25 °C). The composition of the aCSF was, in mmol/L NaCl, 126; KCl, 3; KH_2PO_4, 1.4; $NaHCO_3$, 26; $MgSO_4$, 1.3; $CaCl_2$, 1.4; glucose, 10; at pH, 7.4. Slices were transferred to a recording chamber and maintained at 28–30 °C in this chamber during the electrophysiological recordings (Liu et al., 2018). Recordings were carried out using glass-micropipettes (1 M NaCl) and analyzed with pClamp 9 software (Axon Instruments, Foster City, CA).

Hippocampal slices were perfused with aCSF at a rate of 3.0 ml•min^{-1} in a physiologic recording chamber (Fine Science Tools, Foster City, CA). A bipolar stimulating electrode was placed in the Schaffer collateral pathway, and paired stimulation pulses were applied with interpulse intervals of 30, 70, and 200 ms. The population spikes in response to the paired pulse stimulation were recorded extracellularly from the CA1 pyramidal cell layer (Shin et al., 2011) . The stimulation intensity was set to a value which yielded a response of 70–80 % of the maximal amplitude of the population spike. Subsequent analysis averaged 3 trials for each individual mouse and each interpulse interval. Inhibition was measured using the paired pulse paradigm and quantified as P2/P1 as a percentage. *P*2 is the amplitude of the population spike in response to the second stimulus, and P1 is the amplitude of the population spike in response to the first stimulus (Shin et al., 2011).

The GABA agonist muscimol (3 μM) (Sigma-Aldrich, St. Louis, MO) was perfused to directly examine inhibition (Yamamoto et al., 2011). Field excitatory postsynaptic potentials (fEPSP) from CA1 stratum radiatum were recorded after single stimuli (Liu et al., 2018). The stimulation intensity was set to 70–80 % of the maximal slope of fEPSP. The average slope of the 30 min baseline recordings was set to 100 %, and the slope as a percentage of the baseline level was calculated for analysis of the inhibitory effect of muscimol.

In separate experiments, we examined the effect of the GABA antagonist bicuculline. The stimulation intensity was set to 40–50 % of the maximum amplitude of the population spike (Liu et al., 2018). The average amplitude of the 30 min baseline recording was set as 100 %, and every stimulus was converted into a percentage of the baseline level. When the baseline response stabilized after 30 min, 100 μM bicuculline methiodide (Sigma-Aldrich, St. Louis, MO) was perfused through the recording chamber (Yamamoto et al., 2011).

2.2. Seizure induction

In order to test susceptibility to induced seizures, adult mice (2 months of age) that were treated neonatally with either propofol or saline received an injection of pentylenetetrazol (PTZ 45 mg kg^{-1} i.p.) (Sigma-Aldrich, St. Louis, MO) (Del Vecchio et al., 2004; Roberson et al., 2007). The mice were then placed in an open field apparatus for 30 min and their behavior recorded on a video camera. Seizure intensity was scored by investigators who were blind to the treatment status based on the Racine scale commonly used to assess the intensity of a seizure in rodent models of experimental epilepsy (Racine, 1972). This scale defined seizure intensity in five stages described as: (1) mouth and facial twitching; (2) heading nodding; (3) fore- limb clonus; (4) seizures characterized by rearing; (5) seizures characterized by rearing and falling. After a single injection of PTZ, we observed the seizure intensity was most robust within the first 10 min after the injection; therefore, only that portion of the data was used for comparison. For the latency to seizure intensity stage analysis, mice that did not reach that stage were assigned a time of 30 min (1800 s) for that stage since this was the total duration of observation for the experiments.

2.3. Behavioral tests

The behavioral tests were conducted for the propofol and the saline treated mice serially for the open field, novel object, 3 chamber social interaction and reciprocal social interaction tests; less stressful tests were done before more stressful tests. The animals were allowed to rest in their home cage for at least 5 days between each test. Mice were first given the open field test, the number of animals examined in the other tests was reduced to allow equal numbers of propofol and saline mice from the same litters. The forced swim test was done on a different cohort of animals not subjected to any other behavioral test. We decided to do this test after we had mostly completed the other behavioral studies because recent results found that neonatal ketamine altered forced swim behavior (Lin et al., 2021). For all tests, propofol and saline treated mice from the same litter were done on the same day. The tests were either scored automatically by computerized tracking or by blinded observers examining video tapes.

2.4. Open field test

To assess general locomotor activity and anxiety-like behavior of the mice, an open field test was performed on adult mice (2–3 months old) treated neonatally with either propofol or saline (Crawley, 1985). In a brightly lit room, each mouse was allowed to freely explore in a square open field arena (41 cm on each side) for 30 min. Locomotion patterns such as distance traveled and time spent in the center vs. the periphery of the arena were automatically measured using a computerized tracking apparatus (Versadat, Versamax, Groovy, CA, USA).

2.5. Novel object recognition test

The novel object recognition test is carried out on two consecutive days and examines learning and memory-like behavior on adult male mice (2–3 months old) treated neonatally with either propofol or saline (Leger et al., 2013). The test was conducted in a room with dim lighting. Day 1 is considered the familiarization phase during which the mouse was habituated in a standard open field apparatus for 10 min and then taken out of the arena while two identical objects were placed in the center of the arena positioned 12 cm from each other such that the mouse could travel freely across the center of the arena without obstruction. The mouse was placed back in the arena and allowed 10 min to become familiar with the two identical objects. Day 2 is considered the test phase, one of the two identical, now familiar, objects was taken out of the arena and replaced with a novel object. The same mouse was then placed in the arena and allowed 10 min of exploration time. The times spent sniffing and interacting with (attempting to climb up or jump on or at) the familiar and the novel objects were scored for each mouse.

2.6. Three-chamber social behavior test

The three-chamber social test paradigm was used to study self-directed social behavior of adult mice (3–4 months old) treated neonatally with either propofol or saline (Nadler et al., 2004). The two side chambers each contained a small cage (wire pencil cup): a target novel mouse was confined in the cage on one of the two sides of the three-chamber apparatus, and the other side contained an empty cage. The small cage had a weighted cup on top of it which prevented the

subject mouse from climbing on the cage. The target mouse was of the same strain (C57BL6/J) and sex (male) but had never interacted or been housed with the subject mouse. Confining the target mouse prevented physical contact between the two unfamiliar mice while providing olfactory, visual, and auditory interactions. The neonatally treated adult subject mouse was placed in the center chamber and allowed to explore all chambers for 10 min. The time the subject mouse interacted with either an empty cage (object) or the cage with the novel target mouse was scored and indicated the degree of social interaction of the neonatally treated mouse. The time interacting with the novel mouse was scored only when directly interacting with the mouse, primarily sniffing, and did not include time standing against the cage when the target mouse was not adjacent to it.

2.7. Reciprocal social behavior test

The reciprocal social interaction paradigm is designed to provide detailed and direct insight into how two unfamiliar mice interact in a clean cage with fresh bedding that neither mouse had been in previously (Silverman et al., 2010). An adult mouse (3–4 months old) treated neonatally with either propofol or saline was placed in a large cage for 10 min and then a novel target mouse is added for an additional 10 min so that they could interact directly. The target mouse was of the same strain (C57BL6/J) and sex (male) but had never interacted or been housed with the subject mouse, the target mouse was a different mouse from the target mouse used in the three chamber experiments. There was no aggressive behavior between the mice, most probably because the cage was not home territory to either mouse. There was only very brief nose to nose sniffing which could not be meaningfully scored. The most predominant behaviors of the treated mouse's interaction with the target mouse were scored. These behaviors included push-crawl/following, arena exploration, and self-grooming, Anogenital sniffing is included in push-crawl and following behavior since some of the following behavior is an attempt at anogenital sniffing of the moving target mouse.

2.8. Forced swim test

The forced swim test was designed to examine depression-like behavior (Costa et al., 2013). A 2000 ml beaker was filled with 1400 ml tap water at 25° C to ensure the mice couldn't touch the bottom of the beaker. Neonatally treated adult mice (2–3months old) were placed in the water filled beaker, observed and videotaped for 6 min. After 6 min, mice were removed from the beaker to a dry cage. A heater was used to keep the temperature of the cage at 37° C. The 6 min session was divided into pretest (the first 2 min) and test (the last 4 min); data was only analyzed for the last 4 min (Roni and Rahman, 2015;Yankelevitch-Yahav et al., 2015) Their behaviors were recorded by an observer blind to the treatment grouped in minute-by-minute blocks. The amount of time that mice spent mobile (swimming/climbing behavior) and immobile (floating behavior) was scored for each mouse. Since this test is stressful to the mice, no other procedures were carried out on these mice after this test.

2.9. Statistical analysis

GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA) was used to carry out the statistical analyses using a fixed effect model. The data were normally distributed, all data with unequal variance used Welch's correction and the Bonferroni correction was used for multiple comparisons. We state in the results section for each experiment when these corrections were used. Data with one variable were analyzed using an unpaired t-tests with Welch's correction. Data with two variables were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparaisons tests. Values given in the text are the mean ± the standard error of the mean.

3. Results

During the 2-h P7 propofol treatment, peripheral capillary oxygen saturation (SpO2) and heart rate (HR) were monitored with a pulse oximeter sensor. An average SpO2 of 97 ± 0.1 % (mean ± S.E.M.) and HR of 388 ± 1.8 beats per min (BPM) (n = 15 propofol group) indicate the mice were in a physiological healthy state. There were no time points with significant decreases of SpO2 or changes in heart rate. (Fig. 1B, C).

4. The effect of neonatal exposure to propofol on excitatory/inhibitory neurotransmission later in life

The paired-pulse stimulation paradigm was used to measure recurrent synaptic inhibition. The percentage of the amplitude of the population spike2 compared to population spike 1 (PS2/PS1 × 100) was analyzed at inter-pulse intervals of 30 ms, 70 ms and 200 ms (Fig. 2A, B); there was no difference between saline and propofol groups (Two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1,\ 24} = 0.8448$, $P > 0.05$ for treatment; $F_{2,\ 24} = 3.122$, $P > 0.05$ for interval; $F_{2,\ 24} = 0.0364$, $P > 0.05$ for interaction between treatment and interval. Bonferroni multiple comparison test $P > 0.05$ for treatment at intervals 30 ($t_{24} = 0.3642$), 70 ($t_{24} = 0.4890$) and 200 ms ($t_{24} = 0.7387$); n = 5 saline, n = 5 propofol groups). The paired-pulse inhibition responses at the 30 ms interval were 56 ± 14 % for saline group and 63 ± 13 % for propofol group (Fig. 2B). At the 200 ms inter-pulse interval the paired-pulse responses were 87 ± 14 % and 101 ± 11 % for saline and propofol groups respectively.

5. The effect of neonatal exposure to propofol on GABA receptor function later in life

The effect of neonatal propofol treatment on GABA receptor function in adult animals was examined. We applied the $GABA_A$ receptor agonist muscimol (3 μM) to hippocampal slices from neonatally treated adult mice and recorded neurotransmission in the CA1 region. The slope of the field EPSP was expressed as the percent change from the baseline (averaged for 30 min before muscimol) (Fig. 2C). The mean field EPSP slopes after muscimol (3 μM) were 55 ± 3 % in saline group and 56 ± 3 % in propofol group (35–90 min). The decrease in the field EPSP after muscimol was significant in both saline and propofol groups, and no difference was found between the saline and propofol groups (Two-way repeated measures ANOVA from 35 min to 90 min: $F_{1,\ 8} = 0.0751$, $P > 0.05$ for treatment; $F_{2,\ 16} = 112.4$, $P < 0.001$ for time; $F_{11,\ 88} = 0.1722$, $P > 0.05$ for interaction between treatment and time; n = 5 saline, n = 5 propofol groups) (Fig. 2D).

Bicuculline (100 μM), a $GABA_A$ receptor antagonist, is expected to enhance excitatory output by blocking the $GABA_A$ receptor. We analyzed this increase of excitation by measuring the amplitude of the population spike. In both the saline and propofol groups, there was an increase in excitation when compared to the baseline before bicuculline (Fig. 2E). The mean population spike amplitudes after bicuculline (35–90 min) were 159 ± 3 % of baseline for the saline group and 147 ± 3 % for the propofol group. The increase in excitation was not different between these 2 groups (Two-way repeated measures ANOVA from 35 min to 90 min: $F_{1,\ 8} = 1.086$, $P > 0.05$ for treatment; $F_{2,\ 18} = 14.02$, $P < 0.001$ for time; $F_{11,\ 88} = 0.2958$, $P > 0.05$ for interaction between treatment and time; n = 5 saline, n = 5 propofol groups) (Fig. 2 F).

In conclusion, the experiments with muscimol and bicuculline demonstrated that $GABA_A$ receptor mediated inhibition was not affected by neonatal propofol treatment and the balance of excitatory/inhibitory output was not changed in the neonatally treated propofol group later in life.

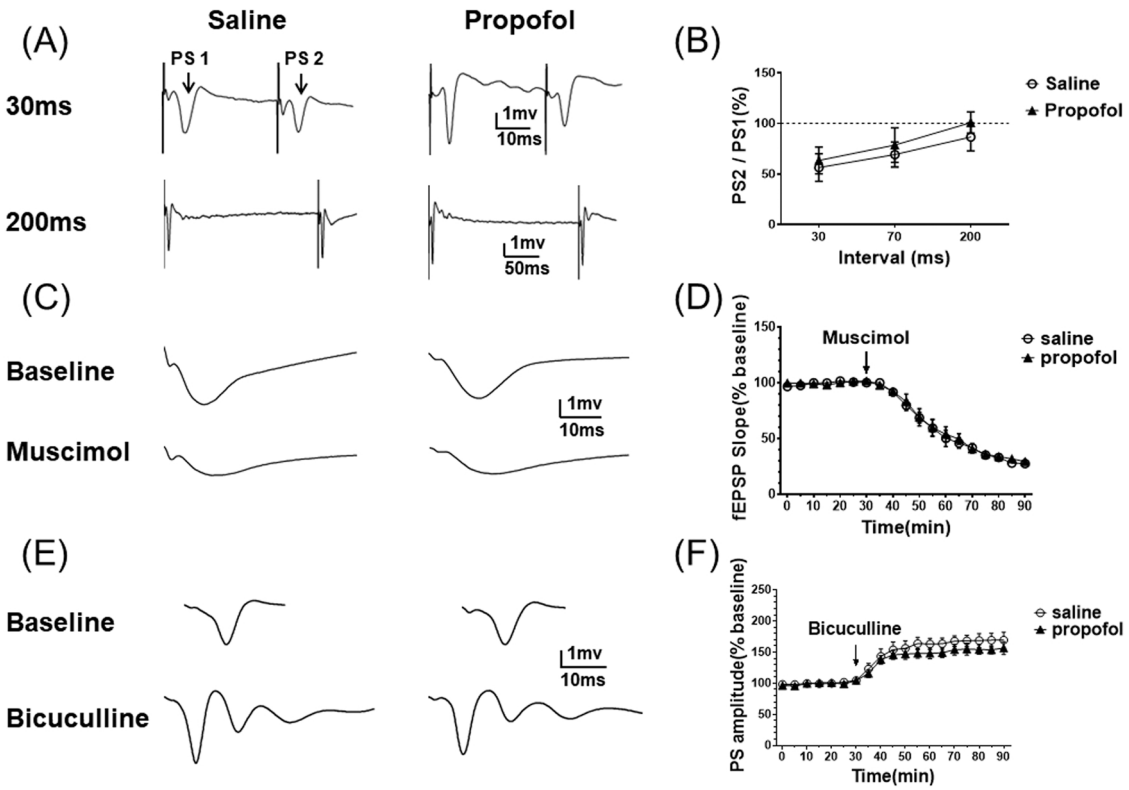

Fig. 2. Early-life propofol treatment did not alter excitatory/inhibitory neurotransmission later in life. A and B. The population spikes (PS) in the CA1 pyramidal cell layer of adult hippocampal slices are shown after paired pulse stimulation to the Schaffer collaterals. Adult mice treated with propofol on P7 did not demonstrate a reduction of paired pulse inhibition (PS2/PS1) compared to saline treated controls. Increasing the time between the paired pulses reduced the inhibition in propofol treated and controls equally (values are the mean ± the standard error of the mean, saline n = 5, propofol n = 5). C and D. The field excitatory post synaptic potentials (fEPSP) in the CA1 dendritic layer of adult hippocampal slices are shown after stimulation to the Schaffer collaterals. Muscimol, a GABA agonist, reduced the fEPSPs equally in both the neonatally propofol and saline treated mice (values are the mean ± the standard error of the mean, saline n = 5, propofol n = 5). E and F. The population spikes (PS) in the CA1 pyramidal cell layer of adult hippocampal slices are shown after submaximal stimulation to the Schaffer collaterals. Bicuculline, a GABA antagonist, increased the size of the population spike equally in neonatally propofol and saline treated mice (values are the mean ± the standard error of the mean, saline n = 5, propofol n = 5).

6. The effect of neonatal exposure to propofol on seizure intensity later in life

An imbalance in excitatory/inhibitory neurotransmission may cause seizures and epilepsy (Ben-Ari, 2006). Sevoflurane increased susceptibility to induced seizures (Lin et al., 2021), therefore neonatal propofol treatment was examined to determine whether it would also increase seizure susceptibility. There were similar changes in seizure intensity stages when pentylenetetrazole (PTZ) was injected into adult mice treated neonatally with either saline or propofol (Two-way repeated measures ANOVA from 1 min to 10 min: $F_{1,\ 18} = 0.1050$, $P > 0.05$ for treatment; $F_{3,\ 63} = 13.30$, $P < 0.001$ for time; $F_{9,\ 162} = 1.034$, $P > 0.05$ for interaction between treatment and time; n = 11 saline, n = 9 propofol groups) (Fig. 3 A). The average seizure intensity stage during the first 10 min after PTZ injection was 1.87 ± 0.10 in saline group and 1.94 ± 0.12 in propofol group. In addition, there were no differences in the latency to start any seizure intensity stage above 3 between the saline and propofol groups (Two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1,\ 54} = 1.634$, $P > 0.05$ for treatment; $F_{2,\ 54} = 2.720$, $P > 0.05$ for seizure intensity stage; $F_{2,\ 54} = 0.02668$, $P > 0.05$ for interaction between treatment and seizure intensity stage; Bonferroni multiple comparison test: $P > 0.05$ for treatment at seizure intensity stage 3 ($t_{54} = 0.9266$), 4 ($t_{54} = 0.6449$) and 5 ($t_{54} = 0.6424$); n = 11 saline, n = 9 propofol groups)(Fig. 3B). The average latencies to reach stage 5 were 1101 ± 209 s and 913 ± 239 s for the saline and propofol groups. The seizure data provide further evidence that neonatal propofol treatment does not affect the balance of excitatory/inhibitory neurotransmission in adult mice.

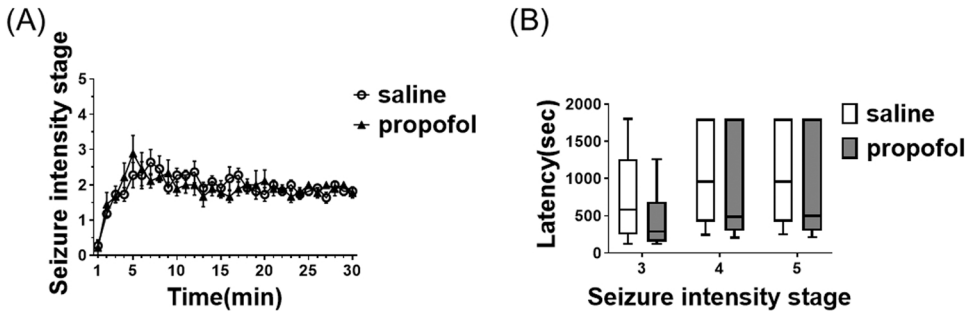

Fig. 3. Early-life propofol was not associated with increased induced seizures later in life. There was no significant difference between neonatally treated propofol and saline mice in Pentylenetetrazole (PTZ) induced in seizure intensity or the latency until a certain seizure stages. Seizure stages graded on the Racine scale. (values are A. the mean ± the standard error of the mean, saline n = 11, propofol n = 9 and B are the median, box 25th and 75th percentile and whisker 5th and 95th percentile, saline n = 11, propofol n = 9).

7. The effect of neonatal exposure to propofol on locomotor, anxiety-like, cognition, social ability and depression-like behavior later in life

7.1. Open field behavior

The open-field apparatus was used to examine the locomotion and spontaneous behavior as well as provide a general physical assessment (Fig. 4 A–E) (Crawley, 1985; Crawley et al., 2007). We observed no difference between propofol and saline treated groups on locomotion. No differences were found for total distance travelled in the saline and propofol treated groups (Fig. 4A), 3427 ± 378 cm vs 4941 ± 715 cm respectively; (unpaired t-test with Welch correction, $P > 0.05$; $n = 11$ saline, $n = 12$ propofol groups) and time spent moving 372 ± 30 s vs 488 ± 48 s respectively (Fig. 4E); (unpaired t-test with Welch correction, $P > 0.05$; $n = 11$ saline, $n = 12$ propofol groups) between two groups in the open field arena.

The time spent in the center verses the edges of the chamber is an indication of less anxiety, with anxious mice spending less time exploring the center of the field. As a measurement of anxiety-like behavior, we examined the following parameters: center distance travelled for the saline and propofol treated mice (Fig. 4B), 1109.9 ± 194.1 cm vs 1707.3 ± 274.8 cm respectively (unpaired t-test with Welch correction, $P > 0.05$; $n = 11$ saline, $n = 12$ propofol groups); center versus total distance travelled percentage 31 ± 3 % vs 34 ± 3 % (unpaired t-test with Welch correction, $P > 0.05$; $n = 11$ saline, $n = 12$ propofol groups) (Fig. 4C); and center versus total time percentage, 22 ± 3 % vs 26 ± 4 % (unpaired t-test with Welch correction, $P > 0.05$; $n = 11$ saline, $n = 12$ propofol groups)(Fig. 4D). We found no significant difference between the two groups for any these three related parameters.

7.2. Novel object recognition behavior

The novel object recognition test offers no external stimuli or reinforcement and we used it to examine cognitive function of adult mice (Leger et al., 2013). During day 1, both saline and propofol groups showed no differential preference for either of the two identical objects (Fig. 4F); this was analyzed by the time spent exploring each object (two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1, 36} = 2.696$, $P > 0.05$ for treatment; $F_{1, 36} = 0.2218$, $P > 0.05$ for object; $F_{1, 36} = 0.07146$, $P > 0.05$ for interaction between treatment and object; Bonferroni multiple comparison test: $P > 0.05$ for exploration time between object1 and object2 in saline group ($t_{36} = 0.5220$); $P > 0.05$ for exploration time between object1 and object2 in propofol group ($t_{36} = 0.1440$); $n = 10$ saline, $n = 10$ propofol groups). The mean exploration time for the two identical objects (object1 and object2) of saline group were 21 ± 6 s and 26 ± 9 s. The mean exploration time for the two identical objects (object1 and object2) of propofol group were 12 ± 3 s and 14 ± 3 s (Fig. 4F). The propofol mice spent less time interacting with the objects on day 1, the familiarization period with these objects.

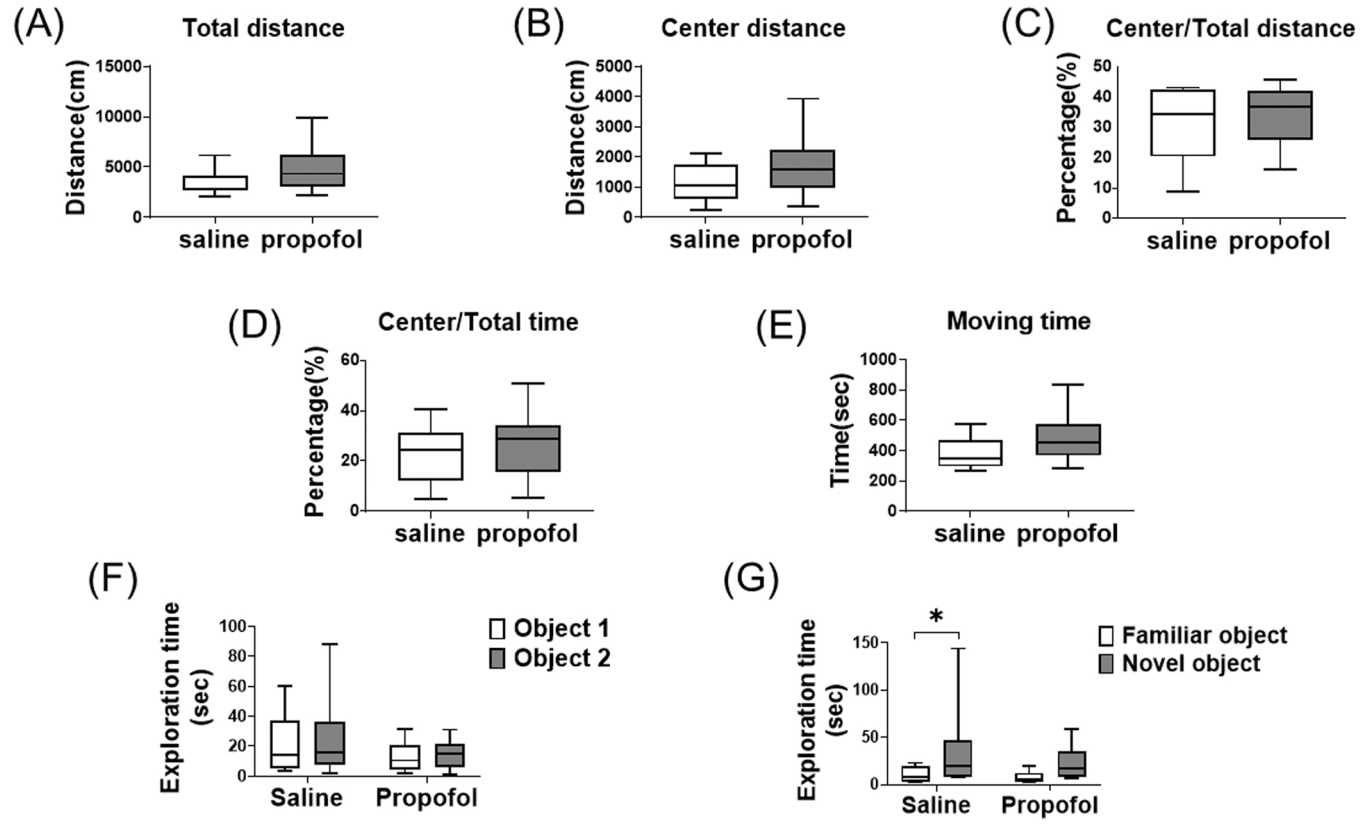

Fig. 4. Early-life propofol did not alter open field behavior later in life. A, B, and C. Total distance, center distance and the percentage of center to total distance were not different between the neonatally treated propofol and saline mice in the open field apparatus. D and E. The percentage of center to total time and the moving time were also not different between the propofol and saline treated mice. We detected no differences between the neonatally treated propofol and saline mice on the open field apparatus. (values are the median and first and third quartiles, saline $n = 11$, propofol $n = 12$). F and G. The novel object recognition test led to ambiguous results. The object exploration time was not different between the propofol and saline groups on the first day for the 2 objects. One object was switched to a novel object on the second day and the time interacting with the novel object was compared for the propofol and saline groups. A multiple comparison test indicated significantly more interaction with the novel object in the saline, but not the propofol neonatally treated mice. However, a simple t-test indicated significantly more interaction with the novel object in both the saline and propofol treated mice. (values are the median and first and third quartiles, saline $n = 10$, propofol $n = 10$) Thus, experiments examining propofols effect on novel object recognition are not conclusive.

On day 2, one of the objects was changed to a novel object, an object the mouse had not been previously exposed to (Fig. 4G). The mean exploration time for the familiar and novel object of saline group were 10 ± 3 s and 38 ± 15 s. The mean exploration time for the familiar and novel object of propofol group are 8 ± 2 s and 24 ± 6 s. The saline group, but not propofol group, spent significantly more time exploring the novel object (Two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1,\ 36} = 1.091$, $P > 0.05$ for treatment; $F_{1,\ 36} = 7.450$, $P < 0.01$ for object; $F_{1,\ 36} = 0.5646$, $P > 0.05$ for interaction between treatment and object; Bonferroni multiple comparison test: $P < 0.05$ for exploration time between familiar object and novel object in the saline group ($t_{36} = 2.461$); $P > 0.05$ for exploration time between familiar object and novel object in the propofol group ($t_{36} = 1.399$); n = 10 saline, n = 10 propofol groups) (Fig. 4G). This lack of significant increased interest in the novel object after neonatal propofol treatment with this multiple comparison test may indicate that propofol treatment impairs learning and memory in adult mice. However, Student's t-test indicated significantly more interaction with the novel object than the familiar object in the propofol group ($t_{10} = 2.530$; $P < 0.03$). This difference between multiple and single comparison tests, coupled with the difference in interaction with objects between saline and propofol treated mice during the familiarization period on day 1 make any conclusion from this test ambiguous.

7.3. Reciprocal social interaction behavior

The reciprocal social interaction test is designed to examine the interaction of two unfamiliar mice in a neutral cage that neither has been in before. We scored three primary behaviors of the subject mouse's interaction with the target mouse, push-crawl and following behavior (Fig. 5A), arena exploration (Fig. 5B) and self-grooming (Fig. 5C); push-crawl and following behavior is an important social behavior for mice. We measured the times spent by adult mice carrying out each of these behaviors after neonatal treatment with either saline or propofol. The time for push-crawl and following behavior, 108 ± 19 s vs 103 ± 9 s (unpaired t-test with Welch correction, $P > 0.05$; n = 10 saline, n = 10 propofol groups); arena exploration, 458 ± 18 s vs 469 ± 7 s (unpaired t-test with Welch correction, $P > 0.05$; n = 10 saline, n = 10 propofol groups) and self-grooming, 34 ± 9 s vs 28 ± 6 s (unpaired t-test with Welch correction, $P > 0.05$; n = 10 saline, n = 10 propofol groups), were not significantly different between the saline and propofol groups.

7.4. Three-chamber social behavior

Three-chamber social test is widely used to assess general sociability and interest in rodents. The subject mouse is free to explore all three chambers (object chamber, mouse chamber and center chamber) during the test period. The interactions between the subject mouse and a novel object (empty pencil cup), the subject mouse and a target novel mouse (mouse confined under pencil cup) were timed and scored (Fig. 5D). The times spent in the object chamber (chamber with empty cage, novel object) and mouse chamber (chamber with novel mouse in a cage) were scored (Fig. 5E). Both the saline and propofol groups showed significant differences between the time interacting with a novel mouse and a novel object (Two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1,\ 36} = 1.620$, $P > 0.05$ for treatment; $F_{1,\ 36} = 48.19$, $P < 0.001$ for target object and mouse; $F_{1,\ 36} = 0.01537$, $P > 0.05$ for interaction between treatment and target; Bonferroni multiple comparison test: $P < 0.001$ for time interacting between object and mouse in saline group ($t_{36} = 4.821$); $P < 0.001$ for time interacting between object and mouse in propofol group ($t_{36} = 4.997$); n = 10 saline, n = 10 propofol groups). The average times interacting with the novel object and the novel mouse in the saline group were 55 ± 4 s and 138 ± 14 s, respectively. The average times interacting with object and mouse in the propofol group were 38 ± 6 s and 124 ± 19 s. There were significant

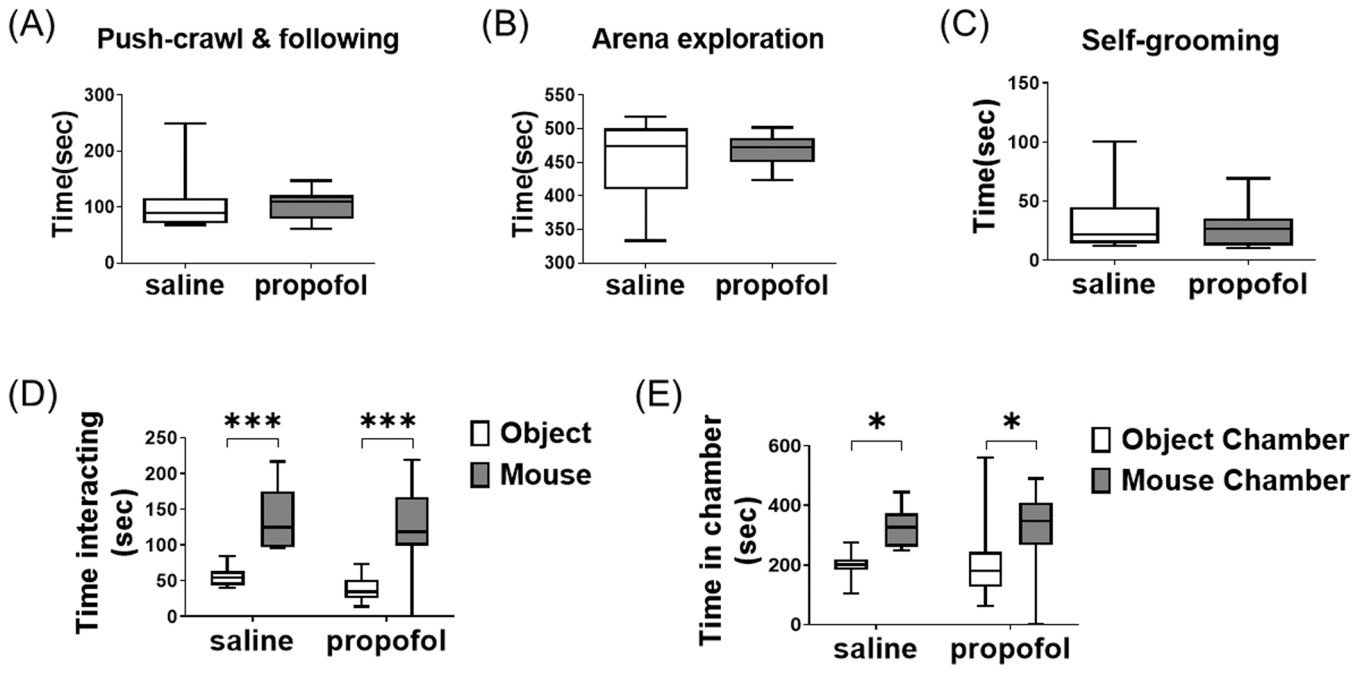

Fig. 5. Early -life propofol did not alter social interactions between experimental and novel target mice. A, B and C. The experimental mice were placed in the same cage as an untreated novel target mouse of the same strain that they had never interacted with before. There was no difference in later-life social interaction (push-crawl and following behavior), arena exploration or self-grooming between the neonatally treated propofol and saline mice. (values are the median and first and third quartiles, saline n = 10, propofol n = 10) D and E. The experimental mice were placed in a three-chamber apparatus, one chamber had a novel mouse confined in a small cage, one chamber had an identical empty cage and the middle chamber was empty. Both the neonatally treated propofol and saline treated mice spent more time interacting with the novel mouse and more time in the chamber with the novel mouse. (values are the median and first and third quartiles, saline n = 10, propofol n = 10) We detected no alteration in social interactions with neonatal propofol treatment.

differences between the time interacting with the novel object and the novel mouse in both the saline and propofol groups; this indicates that propofol did not lead to social disfunction.

There were differences between time spent in the chamber with the novel object and the novel mouse for both the saline and propofol groups (Two-way ANOVA followed by Bonferroni's multiple comparisons test: $F_{1, 36} = 0.002441$, $P > 0.05$ for treatment; $F_{1, 36} = 12.94$, $P < 0.001$ for chamber; $F_{1, 36} = 0.02663$, $P > 0.05$ for interaction between treatment and chamber; Bonferroni multiple comparison test: $P < 0.05$ for time spent between object chamber and mouse chamber in saline group ($t_{36} = 2.659$); $P < 0.05$ for time spent between object chamber and mouse chamber in propofol group ($t_{36} = 2.429$); n = 10 saline, n = 10 propofol groups). In the saline treated group, the mean time spent in the object and mouse chambers were 199 ± 14 s and 324 ± 20 s; the times for the neonatally treated propofol group were 206 ± 44 s and 321 ± 45 s respectively. This result agrees with the interaction time data and further supports that neonatal propofol treatment does not impact social interaction later in life.

7.5. Forced swim behavior

The forced swim test is widely used as an assessment for depression-like behavior in rodents and is also used as a pharmaceutical screening tool for potential antidepressant treatments (Bogdanova et al., 2013). We scored the time spent floating (Fig. 6A), swimming (Fig. 6B) and climbing (Fig. 6C) during the test, and we found no difference in any of these behaviors between the saline and propofol groups: Floating (two-way repeated measures ANOVA from 3 min to 6 min, $F_{1, 27} = 0.6082$, $P > 0.05$ for treatment; $F_{2, 66} = 2.043$, $P > 0.05$ for time; $F_{3, 81} = 0.5213$, $P > 0.05$ for interaction between treatment and time; n = 13 saline, n = 16 propofol groups); Swimming (two-way ANOVA repeated measures from 3 min to 6 min, $F_{1, 27} = 1.037$, $P > 0.05$ for treatment; $F_{2, 58} = 1.627$, $P > 0.05$ for time; $F_{3, 81} = 0.6658$, $P > 0.05$ for interaction between treatment and time; n = 13 saline, n = 16 propofol groups); Climbing (two-way ANOVA repeated measures from 3 min to 6 min, $F_{1, 27} = 1.275$, $P > 0.05$ for treatment; $F_{2, 57} = 2.219$, $P > 0.05$ for time; $F_{3, 81} = 0.9938$, $P > 0.05$ for interaction between treatment and time; n = 13 saline, n = 16 propofol groups). We also measured the latency to immobility, and this analysis also showed no difference between neonatally treated saline and propofol groups (30.1 ± 5.4 s vs 26.1 ± 3.0 s; unpaired t-test with Welch correction, $P > 0.05$; n = 13 saline, n = 16 propofol groups). A commonly used indication of depression-like behavior is a reduction in the time swimming or climbing, but we did not observe this when the propofol group was compared to the saline group. This indicates neonatal propofol treatment did not affect depression-like behavior in adult mice.

8. Discussion

Numerous studies have examined the long-term effects of neonatal propofol, however the differences in anesthetic duration (1–5 h) and concentration (20–200 mg/kg) have yielded mixed results making their interpretation difficult (Chen et al., 2016; Creeley et al., 2013; Karen et al., 2013; Wan et al., 2021; Zhong et al., 2018; Zhou et al., 2021). A number of studies have demonstrated an increase in apoptosis, but many of them have not monitored continuously for hypoxia or hypoperfusion. Other studies have found that a single dose of propofol in the neonatal period did not lead to long-term effects and that multiple applications of propofol neonatally are required to show a deficit; however, these studies used lower doses and/or shorter anesthetic times than we used

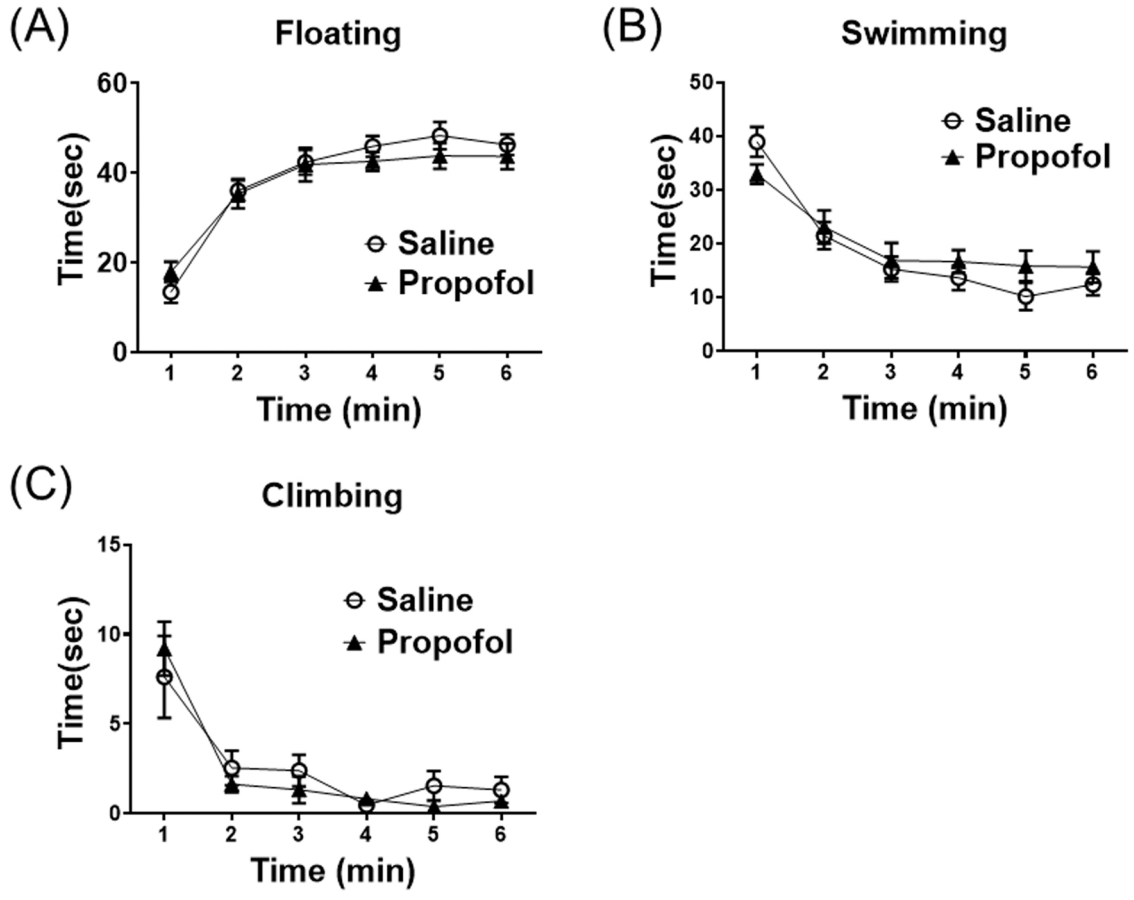

Fig. 6. Early-life propofol did not alter the forced swim behavior of mice. A, B, and C. The neonatally treated propofol and saline mice did not show a difference in floating, swimming or climbing behavior when placed in the water for 6 min. (values are the mean ± the standard error of the mean, saline n = 13, propofol n = 16).

(Chen et al., 2016; Gonzales et al., 2015; Wan et al., 2021; Zhou et al., 2021). Our study slots in between these single and multiple propofol applications in that it examines one longer (2-h) anesthetic period. There is little evidence either in animals or clinically that periods of anesthesia of 1 h or less, with any agent at an anesthetic concentration, leads to long-term alterations in learning, memory or behavior(Chen et al., 2016; Ing et al., 2021; Walkden et al., 2020; Zhou et al., 2021). One recent study found a long-term loss of inhibitory neurons after three, but not one, neonatal propofol applications; we did not find a long-term deficit in GABA inhibition after one 2-h propofol anesthetic period, which is a total anesthetic time intermediate between one and three 1-h anesthetic periods (Zhou et al., 2021). However, in a previous study, we found that neonatal sevoflurane (2-hrs) did reduce long-term GABAergic inhibition similar to what the other study found after multiple propofol applications (Lin et al., 2021). One interpretation of these results is that sevoflurane and propofol can have long-term effects on GABAergic inhibition but that the volatile anesthetic sevoflurane is more potent in generating this side effect. These studies point to the importance of length and repetition of anesthesia. A study in rhesus monkeys found that 5-hrs of neonatal propofol led to apoptosis and brain damage but caused less damage than the volatile anesthetic isoflurane, this indicated reduced long-term effects with propofol compared to volatile anesthetics (Creeley et al., 2013). This study and our previous studies examined the effects of GABA inhibition in the hippocampus, it is likely, and other studies support this (Zhou et al., 2021), that anesthetics affect GABAergic inhibition in other regions of the brain. The behavioral and seizure effects of anesthetics are likely due to GABA actions on regions of the brain in addition to the hippocampus, we just use the hippocampus as a model region to examine GABAergic inhibition.

We chose a dose of propofol that would provide approximately 2 h of anesthesia while maintaining normal heart rate and oxygenation. We chose 2 h because this is the time of anesthesia used in our previous studies examining sevoflurane and ketamine and is a reasonable time for a surgical procedure. We had approximate equivalence of anesthetic depth between sevoflurane (2.4 %) and propofol (250 mg/kg), although recovery from anesthesia was quicker in the sevoflurane treated mice. The ketamine treated mice were less deeply anesthetized, we used a dose of 10 mg/kg every 20 min (total dose 40 mg/kg) to achieve 2 h of anesthesia. However, ketamine, a dissociative anesthetic, is used differently in clinical situations, it does not provide deep anesthesia without movement. We approximated the state of anesthesia for ketamine's clinical use (Green et al., 2011) and discuss this in detail in our previous paper examining ketamine and sevoflurane (Lin et al., 2021). The following conditions apply to all of the studies we have carried out. Both anesthesia and control neonatal mice are removed from the dam for the same period of time and treated similarly. The concentration of propofol used fully anesthetized the animal (no response to tail pinch); throughout the anesthesia, the heart rate and oxygenation, as measured with a pulse oximeter, were within normal limits. Similar numbers of control and anesthesia treated mice come for the same litter to avoid maternal behavior related differences. Behavior tests on mice exposed to either saline or propofol from the same litter were done on the same day.

The open field apparatus is used to test mobility and anxiety, anxious mice spend less time in the center of the apparatus (Crawley, 1985; Crawley, 2007). The percent time and distance in the center region was not different in the propofol treated mice indicating these mice did not have increased anxiety. Neonatal sevoflurane also did not alter the percent time and distance in the center; however, neonatal ketamine significantly reduced adult mouse center time and distance (Lin et al., 2021). These results indicate that neonatal ketamine, but not sevoflurane or propofol, increases anxiety-like behavior in mice.

The novel object recognition test is used to examine cognitive function in mice. The saline treated mice showed increased interaction time with the novel object while the neonatal propofol treated mice did not show significantly more interest, as adults, in the novel object when examined using a multiple comparison test, this might indicate decreased memory and/or cognition. However, the less stringent t-test did indicate increased interest in the novel object for the propofol treated mice which suggests no effect on learning or memory; thus, the results of these experiments are ambiguous and no firm conclusions can be made from them. Propofol affects GABAergic transmission in many brain regions and the interpretation of the behavioral effects we examined may be complicated by these multiple effects (Garcia et al., 2010; Zhou et al., 2021). Sevoflurane treated mice clearly demonstrated less interest in the novel object and reduced learning and memory which suggests that sevoflurane has a greater effect than propofol (Lin et al., 2016).

In the current study we examined social interactions using the three-chamber test, which restricts physical contact with a target mouse, and a reciprocal interaction test, which allows the treated and target mouse to freely interact. In both tests the saline and propofol treated mice exhibited the same interest and interaction with the target mouse. We did not detect an effect of neonatal propofol on adult social behavior. This result was different from our results with neonatal sevoflurane which demonstrated decreased social interaction with the target mouse in both tests (Lin et al., 2016) These normal social interaction results with 2-h neonatal propofol are similar to those found by others after one short neonatal propofol exposure but different from their three propofol exposure results which found social deficits similar to those we found with 2-h sevoflurane (Gonzales et al., 2015; Karen et al., 2013; Zhou et al., 2021). It is possible that neonatal propofol has similar long-term effects but is either less potent or requires longer or multiple anesthetic periods than sevoflurane to elicit its effects.

We examined depression-like behavior after neonatal propofol using the forced swim test (Costa et al., 2013). We found no difference between the neonatally treated propofol and the saline controls in the forced swim test, this indicates that propofol did not increase depression like behavior in mice. This was different from the results we found with neonatal ketamine which increased floating time and reduced swimming and climbing in adult mice which is an indication of depression like behavior after neonatal ketamine (Lin et al., 2021).

In a previous study we found that the adult mice, neonatally treated with sevoflurane, had reduced GABAergic inhibition and increased induced seizure response, we did not find this result in mice neonatally treated with ketamine (Lin et al., 2021). Since the predominant anesthetic effect of sevoflurane is to enhance GABA inhibition and ketamine blocks NMDA receptors (Campagna et al., 2003; Garcia et al., 2010; Goa et al., 1999), we postulated that neonatal sevoflurane enhanced GABAergic transmission during development leading to a reduction in GABA receptors and/or inhibitory interneurons and thereby reduced GABA based inhibition in the adult (Lin et al., 2021). Since propofol also primarily acts to enhance GABA inhibition we hypothesized that it too would lead to GABA down regulation and less inhibition in adult mice, our results did not support hypothesis. While neonatal sevoflurane reduced paired pulse inhibition, reduced muscimol inhibition of the field EPSP, increased population spike amplitude with bicuculline and increased the seizure intensity compared to controls, (Lin et al., 2021) neonatal propofol had none of these effects. Saline and propofol neonatally treated mice showed no differences in paired pulse inhibition, muscimol inhibition of the field EPSP, population spike amplitude with bicuculline or seizure intensity. Thus, even though sevoflurane and propofol share a primary anesthetic action of increasing GABA inhibition, 2 h of neonatal propofol did not lead to persistent long-term effects on GABAergic inhibition in adult animals. There may be some other action of sevoflurane, perhaps enhanced release of calcium from intracellular organelles, altered microRNA expression or a differential effect on second messenger pathways leading to the differences in their long-term effects (Campagna et al., 2003; Forman and Chin, 2008; Krasowski and Harrison, 1999; Lin et al., 2018; Rudolph, 2001; Wang et al., 2012). Alternatively, as previously discussed, it could be that propofol is less potent in eliciting long-term effects than sevoflurane and requires longer anesthetic periods or multiple anesthetic applications to

alter GABAergic inhibition. One study found that multiple, but not single, 1-h neonatal exposures to propofol induced long-term loss of GABAergic interneurons (Zhou et al., 2021).

Both propofol and sevoflurane have cellular effects in addition to their enhancement of GABAergic inhibitory activity. Even their GABA enhancement could be working through different GABA receptors and/or cell types leading to the differential long-term effects of these two agents. Recent studies have found that neonatal propofol can alter intracellular calcium, protein kinase activity, ERK1/2 signaling pathways and neurotrophic factors which can lead to long-term effects in the nervous system including apoptosis and reduced survival of neuronal stem cells (Karen et al., 2013; Wan et al., 2021; Zhong et al., 2018). However, whether these are direct effects of propofol or secondary to ischemia is not clear since most of the animal studies did not monitor adequately or continuously for hypoxia or hypoperfusion during anesthesia.

There are a number of limitations to our study. We only examined male mice to reduce the variability and the number of animals required for the study. Therefore, our results cannot be extrapolated to female mice since other studies have found a difference in some behavioral outcomes when male and female are examined after neonatal propofol (Gonzales et al., 2015). We used a high concentration of propofol (250 mg/kg) that fully anesthetized the mice for 2 h but have not examined other doses and durations of propofol anesthesia which may yield different results. We administered propofol i.p., but clinically it is administered i.v.; this affects propofol's pharmacokinetics. Anesthetics affect many regions of the brain, however, we only examined inhibition in the hippocampus where it can be examined in detail in brain slices. It is likely the behavioral and seizure effects we observe are due to GABAergic changes in other areas of the brain but we did not examine this directly. Most importantly, great caution must be used when trying to extrapolate results in rodents to those in humans. Mice undergo rapid neuronal development and are most susceptible to anesthetics on postnatal day 7, human neuronal development occurs over a longer time span so a short anesthetic exposure might have less of an effect in humans.

Our results indicate that propofol, when used for anesthesia in neonatal mice, has markedly different long-term effects compared to sevoflurane, an anesthetic that also enhances GABAergic inhibition as a mechanism of its anesthesia inducing property. We found that a 2-h neonatal propofol exposure, unlike sevoflurane, did not reduce GABAergic inhibition in adult mice and did not increase evoked seizure susceptibility. Neonatal propofol, unlike sevoflurane did not affect social interactions with novel mice. Unlike neonatal ketamine, an anesthetic that primarily blocks the excitatory NMDA receptors, propofol did not increase depression-like behavior as measured by the forced swim test or anxiety-like behavior as measured in the open field test. Thus, neonatal exposure to different anesthetics has unique long-term effects on adult behavior and GABAergic inhibition. These results indicate that clinical studies grouping several general anesthetic agents in a single group should be interpreted with great caution when examining long-term effects.

Declaration of Competing Interest

None.

Acknowledgements

We are thankful for the generous funds to carry out the experiments that were provided by "The Garry S. and Sarah Sklar Professorship in Anesthesiology" and the Anesthesiology Department through the Brooklyn Anesthesia Research Division of University Physicians of Brooklyn, Brooklyn, New York.

Author credits

J Liu, D Lin, JE Cottrell and IS Kass: Designed the study and edited the manuscript J Liu and A Yau: carried out the study and analyzed the data. J Liu and IS Kass wrote the manuscript.

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