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Full Length Article

Evaluation of neurotoxicity and long-term function and behavior following intrathecal 1 % 2-chloroprocaine in juvenile rats

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ABSTRACT

Spinally-administered local anesthetics provide effective perioperative anesthesia and/or analgesia for children of all ages. New preparations and drugs require preclinical safety testing in developmental models. We evaluated age-dependent efficacy and safety following 1 % preservative-free 2-chloroprocaine (2-CP) in juvenile Sprague-Dawley rats. Percutaneous lumbar intrathecal 2-CP was administered at postnatal day (P)7, 14 or 21. Mechanical withdrawal threshold pre- and post-injection evaluated the degree and duration of sensory block, compared to intrathecal saline and naive controls. Tissue analyses one- or seven-days following injection included histopathology of spinal cord, cauda equina and brain sections, and quantification of neuronal apoptosis and glial reactivity in lumbar spinal cord. Following intrathecal 2-CP or saline at P7, outcomes assessed between P30 and P72 included: spinal reflex sensitivity (hindlimb thermal latency, mechanical threshold); social approach (novel rat versus object); locomotor activity and anxiety (open field with brightly-lit center); exploratory behavior (rearings, holepoking); sensorimotor gating (acoustic startle, prepulse inhibition); and learning (Morris Water Maze). Maximum tolerated doses of intrathecal 2-CP varied with age (1.0 μL/g at P7, 0.75 μL/g at P14, 0.5 μL/g at P21) and produced motor and sensory block for 10–15 min. Tissue analyses found no significant differences across intrathecal 2-CP, saline or naïve groups. Adult behavioral measures showed expected sex-dependent differences, that did not differ between 2-CP and saline groups. Single maximum tolerated in vivo doses of intrathecal 2-CP produced reversible spinal anesthesia in juvenile rodents without detectable evidence of developmental neurotoxicity. Current results cannot be extrapolated to repeated dosing or prolonged infusion.

1. Introduction

Spinal anesthesia with intrathecal or epidural local anesthetic is a feasible alternative to general anesthesia for selected surgical procedures in children of all ages (McCann et al., 2019; Whitaker and Williams, 2019). Neurological complications are rare, but transient neurological symptoms, more persistent deficits, and cauda equina syndrome have been reported. The risk of adverse events can be higher in neonates due to added technical difficulties and trauma during spinal injections, catheter-related problems, drug-related errors, or vascular puncture resulting in high plasma levels and systemic toxicity of local anesthetics (e.g. seizures, arrhythmias) (Hampl et al., 2014; Llewellyn and Moriarty, 2007; Long et al., 2016; Walker et al., 2018).

Neurotoxic effects of local anesthetics vary with the type of drug, formulation, concentration and duration of exposure (van Zuylen et al., 2019; Verlinde et al., 2016). All local anesthetics induce neurotoxicity and cell death following prolonged or high dose exposures in vitro, and cytotoxic mechanisms and potential preventive interventions have been

Abbreviations: 2-CP, 2-chloroprocaine; IT, intrathecal; P7, postnatal day 7.

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identified (Koo et al., 2021; Wang et al., 2021, 2010; Xu et al., 2018). In vivo studies of local anesthetic preparations with or without preservatives have evaluated acute dose-response, subsequent histopathological signs of nerve injury or inflammation, and long-term functional deficits across a range of species, but have focused on adult animals (Barsa et al., 1982; Drasner, 2005; Hampl et al., 2014; Takenami et al., 2012; Taniguchi et al., 2004).

Vulnerability to neurotoxicity differs in the immature nervous system, and new drugs or formulations require a pediatric study plan that includes preclinical safety data evaluating target organ systems and key developmental endpoints relevant to the route of administration (US Department of Health, 2020). Intrathecal and epidural techniques deliver drugs in close proximity to nerve roots and the spinal cord. As there are significant postnatal changes in spinal cord synaptic function, network organization and neuronal apoptosis across the first 3 postnatal weeks in juvenile rodents (Brewer and Baccei, 2020; Fitzgerald, 2005; Koch and Fitzgerald, 2013; Lawson et al., 1997; Walker et al., 2016), safety evaluations for spinal local anesthetics need to include in vivo animal models that ensure accurate intrathecal delivery and evaluate potential neurotoxicity across a range of postnatal ages (Suresh et al., 2018; Walker and Yaksh, 2012). In infant rats, no neurotoxicity was demonstrated following maximal tolerated intrathecal doses of the local anesthetics bupivacaine (Yahalom et al., 2011) or levobupivacaine (Hamurtekin et al., 2013). The amino-ester local anesthetic 2-chloroprocaine may have advantages in infants, as rapid metabolism by plasma esterases reduces the risk of systemic toxicity (e.g. seizures, cardiac arrhythmias) following inadvertent vascular injection (Boretsky, 2019; Walker et al., 2018), but neurotoxicity following intrathecal injection has not been assessed in developmental models.

In infants and young children, the need for general anesthesia in early life has been associated with subsequent cognitive and behavioral changes (Ing et al., 2021). Age- and dose-dependent neurotoxicity following general anesthetics and sedative drugs in early development have been replicated in multiple studies and species (Chinn et al., 2020; Jevtovic-Todorovic, 2018; Lee et al., 2021; Perez-Zoghbi et al., 2020; Talpos et al., 2019). While long-term functional impairments in cognitive and behavioral tasks have been identified in rodents (Jevtovic-Todorovic et al., 2003) and non-human primates (Talpos et al., 2019), standardized assessments of learning and memory, motor/sensorimotor function, emotionality and social behavior have not been consistently reported in anesthetic toxicity studies (Maloney et al., 2019a). Alongside assessments of spinal cord sensory and motor function following intrathecal local anesthetics (Hamurtekin et al., 2013; Yahalom et al., 2011) or analgesics (Walker and Yaksh, 2012), evaluation of higher order behavioral outcomes in adulthood will enhance the translational significance (Maloney et al., 2019a) of studies evaluating existing or new local anesthetics.

This study evaluates efficacy and safety following intrathecal injection of a new preparation of 1 % 2-chloroporocaine (2-CP) at postnatal day (P)7, 14 and 21. Outcomes following the maximum tolerated dose included tissue analyses in the spinal cord and brain one- and seven-days following injection, with treatment groups compared to IT saline and naïve controls. Importantly, long-term functional outcomes were assessed in adult males and females following intrathecal 2-CP or saline at P7, and included spinal cord reflex thresholds, locomotor activity, social approach, investigatory and anxiety-like behavior, sensorimotor gating, and spatial learning and memory.

2. Methods

2.1. Ethical approval

All reported experiments had appropriate Institutional Animal Care and Use Committee (IACUC) approval and were performed in AAALAC-accredited (Association for Assessment and Accreditation of Laboratory Animal Care) facilities at University of California, San Diego (UCSD).

2.2. Experimental animals

Pregnant Holtzman Sprague-Dawley rats (Harlan, Indianapolis, IN) and subsequent litters had free access to food (Teklad Rodent Diet 8604, Envigo, Madison, Wisconsin) and municipal tap water, in a 12 h light-dark cycle with room temperature maintained within the range 65–82 °F and relative humidity within the range 30–70 %. Animals were checked at least once every day, and the day of birth of pups assigned as postnatal day 0 (P0). Animals aged P21 (3 litters), P14 (3 litters), or P7 (4 litters plus 2 replacement litters) were assigned to the following experiments: dose finding, acute behavior, and tissue analysis (2 time points). Four additional P7 litters had injection of 2-CP or saline and were maintained for adult behavioral assessments. One litter at P3 received intrathecal ketamine as previously described (Walker et al., 2010) to act as a positive control for histology analyses (n = 8). Experimental groups included a total of 140 animals: dose finding, n = 21; acute behavior and histology, n = 68; replacement animals due to inadequate block (n = 6), mortality (n = 2), or difficulties with tissue processing in P7 animals (n = 11; 2CP n = 7, saline n = 3, naïve n = 1); and P7 injection for long-term behavior (n = 32).

Individual litters were restricted to a maximum of 12 for acute behavioral testing and tissue collection at 24 h or 7 days. Litters for long-term maintenance and adult behavior were restricted to 8 pups that were weaned into same-sex cages at P21, and hindlimb withdrawal reflex testing was performed at P28-35. Animals were then transferred to Dr Powell's protocol for higher order behavioral testing, performed in accordance with the Guiding Principles in the Care and Use of Animals (provided by the American Physiological Society) and the guidelines of the National Institutes of Health. Animals continued to be housed at UCSD Hillcrest Campus until approximately day 50 when they were transferred to The Scripps Research Institute San Diego for water maze testing. At the completion of experiments, animals were euthanized according to approved protocols. Reporting is in accordance with ARRIVE (Animals in Research: Reporting *in vivo* Experiments) Guidelines (Kilkenny et al., 2010) and relevant standards for preclinical developmental anesthetic neurotoxicity studies (Chinn et al., 2020).

2.3. Intrathecal injections and solutions

Percutaneous intrathecal injections were performed as previously described (Westin et al., 2010). Under brief isoflurane anesthesia (3%) with oxygen and room air via a nose cone, 1 % 2-CP (Chloroprocaine HCl 10 mg/mL, EU-DCP; LOT 17319; W.I.P. Code 59787) or the equivalent volume of sterile saline (NaCl 0.9 % solution injectable, Sintetica S.A. CH-6850; Batch No. 17403) was injected intrathecally at the L4-L5 or L5-L6 intervertebral space using a 30-gauge needle connected to a micro-injector and Hamilton syringe. Using this technique in rat pups aged from P3 to P21, we previously confirmed reliable spread across lumbar or up to mid-thoracic spinal segments with percutaneous intrathecal injectates of 0.5−1 μL /g of methylene blue dye followed by post-mortem dissection, and of fluorescent dye followed by in vivo imaging (Westin et al., 2010). All initial injections were performed by a single investigator (S.M.W), and subsequent P7 injections for replacement tissue analysis (K.E; n = 7 2-CP, n = 3 saline, n = 1 naive) used the same technique and resulted in the same degree of acute motor and sensory block following 2-CP.

As the maximum tolerated doses of intrathecal local anesthetic vary with postnatal age (Hamurtekin et al., 2013), up-down dosing of different intrathecal injectate volumes (0.5, 0.75, 1, 1.5 and 1.75 μL /g) of 1 % 2-CP was performed at P7, P14 and P21. Doses resulting in significant respiratory depression or death were considered above the maximum tolerated and a lower dose was administered in the next animal. Inadequate block (no visible motor weakness at 2−3 min and minimal change in mechanical withdrawal threshold at 5 min) resulted in a higher dose being administered in the next animal. Animals used in dose finding experiments were terminally anesthetized following acute

behavioral assessments and were not used for further analyses.

For subsequent experimental groups, accurate intrathecal placement of an effective dose of 2-CP was defined as:

i) maintenance of adequate cardiorespiratory function (visible inspection of respiratory movement and general perfusion) to exclude an excessively high block or inadvertent intravascular injection/absorption;
ii) visible motor block restricted to both hind limbs (failure of hip flexion, dragging hindlimb), but with spontaneous return of forepaw movement indicating recovery from anesthesia at 2−3 min;
iii) bilateral loss of reflex withdrawal response to a supra-threshold mechanical stimulus at 3−5 min;
iv) sensory block (raised mechanical withdrawal threshold at 10 min).

Animals with inadequate block were removed from further evaluation and substituted with an additional animal in the same or subsequent litter to achieve the pre-determined number of age- and sex-matched animals with adequate block.

2.4. Experimental treatment groups

Rat pups were randomly assigned to receive intrathecal 2-CP or saline, with equal numbers of male and females in each treatment group, distributed across at least 3 litters to control for potential litter variability. Naïve littermates had the same degree of initial handling for recording of sex, weight, and mechanical withdrawal thresholds, and were then returned to the dam. For all interventions, rat pups were kept on a heating pad to maintain body temperature. The duration of general anesthesia (5−7 min), maternal separation and handling of pups was the same for both saline and 2-CP groups. Following behavioral assessment 30 min post-injection, animals were returned to the dam in the same bedding and home cage. The experimenter performing intrathecal injections was aware of treatment allocation during acute testing to confirm correct intrathecal placement and adequacy of local anesthetic block with 2-CP. Animals were then coded for tissue histology and longer-term behavioral evaluation, and subsequent investigators were unaware of treatment allocation. Following collection of all data, identification codes were provided to allow group-based statistical analyses.

At each age (P7, P14, P21), tissue analysis was performed at either 24 h or 7 days in 12 animals: maximum tolerated dose intrathecal 2-CP (n = 6); sterile saline (n = 4); and naive control (n = 2). Additional studies assessed potential long-term effects of intrathecal treatment on spinal reflex thresholds and higher order behaviors in adulthood following P7 intrathecal injection of 2-CP (n = 16; 8 male, 8 female) or saline (n = 16; male, 8 female).

2.5. Behavioral testing and assessment of spinal reflex function

Rat pups were divided into males and females, weighed, and baseline mechanical withdrawal thresholds for both hindpaws determined using calibrated von Frey filaments (0.4–15 g). Each von Frey filament was applied five times at 1 s intervals to the dorsal hindpaw.(Walker et al., 2012, 2010) The number of evoked withdrawal responses to each stimulus of increasing intensity was recorded until a given stimulus evoked five responses or a supra-threshold cut-off pressure was reached during local anesthetic motor/sensory block (limited to 8 g at P7 and 60 g at P14 and P21 to avoid tissue damage). Mechanical withdrawal threshold (MWT) was determined by plotting the number of withdrawal responses against the mechanical stimulus (force expressed as grams on $\log_{10}$ scale), constructing a sigmoidal stimulus-response curve with nonvariable slope using non-linear regression curve fit, and identifying the mid-point of the curve (50 % effective force; EF_{50}) (Hamurtekin et al., 2013; Walker et al., 2012, 2010; Westin et al., 2010). Following injection and recovery from anesthesia, pups were assessed for visible motor block (failure of hip flexion, dragging of hindlimbs, and no response to a suprathreshold mechanical stimulus). Animals were retained if dense motor block was apparent following intrathecal 2-CP, and motor function was normal following saline. Mechanical withdrawal thresholds were measured in both hindlimbs at 5, 10, 15, and 30 min following injection

Following IT 2-CP or saline at P7, hindlimb withdrawal responses were assessed at P28-30. Thermal withdrawal latency was determined using a modified Hargreaves Box (University Anesthesia Research and Development Group, University of California San Diego, La Jolla, California) with a glass surface (maintained at 30 °C) on which the rats were placed in individual Plexiglas cubicles and allowed to acclimatize for 30 min prior to testing. The thermal nociceptive stimulus from a focused projection bulb positioned below the glass surface was directed to the mid-plantar hindpaw. Latency was defined as the time required for the paw to show a brisk withdrawal as detected by photodiode motion sensors that stopped the timer and terminated the stimulus. In the absence of a response within 20 s, the stimulus was terminated (cut-off time). Thermal latency was the average of three measures from each hindpaw.

For tactile mechanical withdrawal thresholds, rats were acclimatized for 30 min in individual clear plastic compartments on a wire mesh-bottomed cage. To determine the 50 % mechanical threshold for paw withdrawal, von Frey hairs with bending force ranging from 0.4–15.1 g were sequentially applied perpendicularly to the plantar mid-hindpaw using a modified up-down method (increasing intensity if application of the filament for 5 s did not evoke withdrawal, decreasing intensity until lack of response) until six data points or the maximum or minimum stimulus was reached as previously described (Chaplan et al., 1994).

2.6. Euthanasia and tissue collection

Tissue was harvested 24 h following injection to assess acute tissue histopathology (including neuronal apoptosis), and at 7-days to assess subsequent signs of tissue injury (including histopathology, nerve injury, inflammation, and glial reactivity). Previous studies have identified increased neuronal apoptosis at 24 h, and increased glial reactivity 7 days following intrathecal ketamine in neonatal rodents (Walker et al., 2010), and impaired sensory function and neurotoxicity (histologic examination of cross sections of cauda equina) 7 days following intrathecal 2-CP in adult rodents (Taniguchi et al., 2004). Treatment group and age-matched naïve animals were deeply anesthetized with isoflurane and then received intraperitoneal pentobarbital 100 mg/kg (Beuthanasia®-D, Mercke Sharpe & Dohme, Madison. NJ). Animals were transcardially perfused with heparinized NaCl 0.9 % at 1 ml/g body weight followed by 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PFA, 1 mL/g). Spinal cord, cauda equina and brain tissue were harvested and placed in individual coded tubes.

Thoracolumbar spinal cord was post-fixed in 4 % PFA at 4 °C for 24−48 h then transferred to 30 % sucrose in phosphate buffered saline. Spinal cord was placed in OCT freezing medium (Tissue-Tek #4583), quickly frozen in dry ice and sectioned on a cryostat (Leica #CM1800). Sections were cut onto slides at 8−10 μm for H&E, and at 14 μm for immunostaining. Cauda equina was post-fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 24−48 h, then rinsed and stored in 0.1 M PB. Brains were post-fixed in 4 % PFA at 4 °C for 24−48 h, and then divided coronally and placed into tissue cassettes in 4 % PFA. Tissue was embedded in paraffin, then sectioned at 8−10 μm using a rotary microtome (Leica #RM2135 Bannockburn, IL) onto glass slides (Fisher SuperFrost Plus, Waltham, MA, USA).

2.7. Tissue staining and analysis

For all immunohistochemistry, primary antibody specificity was confirmed by including an isotype control (non-immune rabbit and/or

mouse IgG; Vector Laboratories #I-1000 and I-2000) diluted to the same concentrations as the primary antibodies (Cleaved Caspase 3, Iba1, GFAP). Multiple sections per animal provided averaged data, and statistical comparisons are based on n = number of animals.

2.7.1. Hematoxylin and eosin (H&E) histology

For spinal sections, H&E staining comprised: Hematoxylin, Gills #2 (Sigma #GHS232) for 30 s, dip in distilled H_2O with glacial acetic acid for 5 s, rinse, Eosin Y Alcoholic (Sigma #SLBZ2431) for 2 min., gradual dehydration in increasing concentrations of ethanol, clearing in CitriSolve (3 x 5 min) and then cover slipping using a non-aqueous mounting media (DPX). Brain sections were deparaffinized, rehydrated through graded concentration of ethanol, and H&E staining was performed as above.

Sections were viewed under light microscopy by a neuropathologist (M.R.G.) with histological assessments including signs of inflammation of spinal cord, dura, arachnoid, and nerve roots. A minimum of 7 sections throughout the brain were also processed with H&E staining and examined by a neuropathologist (M.R.G.).

2.7.2. Nerve root histology

Cauda equina tissue was rinsed in 0.1 M PB, post fixed in 1% osmium tetroxide, dehydrated in serial dilutions of alcohol, and embedded in araldite resin, as previously described.(Hamurtekin et al., 2013) Transverse, 1-μm-thick sections were cut on an automated Leica RM2065 microtome, stained with methylene blue, azure II and evaluated by light microscopy. Imaging was done using Openlab 4.04 imaging software (Improvision).

Cauda equina sections were closely examined by an experienced investigator (V.I.S.) for indices of Wallerian degeneration, including focal demyelination, disruption of myelin sheath, macrophage infiltration, axonal degeneration and periaxonal edema.

2.7.3. Caspase-3 immunohistochemistry

Immunohistochemistry with an antibody to activated caspase-3, evaluated apoptosis in spinal cord sections as per our previous protocols.(Hamurtekin et al., 2013; Walker et al., 2012) Following rinses in phosphate buffered saline (PBS), which was also used as the wash buffer between subsequent steps, nonspecific binding was blocked with 5 % normal goat serum (Vector Laboratories, Burlingame, CA) diluted in tris-buffered saline for 1 h at room temperature, and tissue was incubated overnight at room temperature with 1:200 rabbit monoclonal anti-Cleaved Caspase 3 (Cell Signaling, Beverly, MA #9664). Biotinylated goat anti-rabbit secondary IgG (Vector Laboratories #BA-1000) was followed by avidin-biotin-peroxidase (ABC-HRP; Vector #PK-6100) for one hour at room temperature. Staining was developed with 3,3′-diamniobenzidine (DAB; Vector Laboratories #SK-4100). Slides were dehydrated through graded ethanol, cleared in CitriSolve, cover-slipped with a non-aqueous mounting media (DPX), and coded.

Activated Caspase-3 positive cells were manually counted under high power light microscopy, and the average across up to 10 sections per animal was determined.

2.7.4. Fluoro-Jade C staining

The standard staining protocol was followed with distilled water washes between steps. Mounted spinal cord sections were immersed in 1 % sodium hydroxide in 80 % alcohol for 5 min; rinsed with 70 % alcohol; incubated with 0.06 % potassium permanganate for 10 min; stained with 0.00015 % Fluoro-Jade C (Chemicon, U.S.A. #AG325) with 1:5000 4′, 6-diamidino-2-phenylindole (DAPI, Molecular Probes) in 0.1 % acetic acid; cleared in CitriSolve (Decon Labs, Inc. #1601); and cover slipped with non-aqueous mounting media (DPX, Electron Microscopy Sciences #13512).

Spinal cord sections were examined using a Leica DM2000 fluorescence microscope with a Sola light engine light source and FITC filter at 100x total magnification, and positive cells were counted manually.

2.7.5. Glial reactivity

Astrocyte (glial fibrillary acidic protein, GFAP) and microglial (ionized calcium binding adapter molecule 1, Iba1) markers assessed glial reactivity in spinal cord sections. Following PBS rinses, slides were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) diluted in PBS with 0.3 % Triton X-100 (PBS-TX) for 1 h. Slides were incubated with rabbit anti-Iba1 (Wako #019-19741) and mouse anti-GFAP (Chemicon #MAB360) primary antibodies at 1:1000 in 1% Bovine Serum Albumin (Sigma #A7096) for 3 h at room temperature (RT), followed by secondary goat anti-rabbit or anti-mouse antibodies conjugated to a fluorescent marker (Alexa 488 or 594; 1:1000; Molecular Probes, Eugene, OR) for 1 h. Slides were rinsed and cover-slipped using ProLong Gold Antifade Reagent with 4′6-diamidino-2-phenylindole (Molecular Probes).

Fluorescent microscopy was performed using an Olympus BX-51 (Olympus America, Center Valley, PA) epifluorescent microscope and images were captured using a UPlanSApo 20x objective. Iba1 and GFAP fluorescent intensity measurements were quantified (Image J, GNU General Public License) at 4 regions of interest in the dorsal horn for at least 4 lumbar spinal sections per animal.

2.8. Higher-order behavior in young adult rodents: apparatus, methods and analysis

To minimize animal use, long-term behavioral assessments were restricted to the maximum tolerated dose in the youngest and most vulnerable age group (P7 IT injection of 1μL/g 2-CP or saline), and one saline group animal in which the eyes did not fully open was humanely euthanized and was not replaced. Behavioral tests were performed at intervals from P35 until P71, and sex was included as a biological variable (8 males and 8 females per group).

2.8.1. Social behavior

As failure to engage in positive social interactions throughout development can have persistent effects on brain function and on emotional, motivational and cognitive aspects of behavior (Trezza et al., 2011), social approach was measured in a modified 3-chamber social approach arena (Berg et al., 2018; Mouri et al., 2007). Rats were first habituated to the testing arena (48″ x 17″ x 11.5″ high acrylic chamber) without any objects/partner rats for 10 min. During the next 10 min period two plastic rectangular containers (11.5″ x 7−1/2″ x 5″ high) with vertical open spaces ~1−2 cm apart are inverted and placed on the left and right sides of the arena. A partner rat was placed under one of the containers while the other container was left empty, serving as a novel object. Stranger rats were age and sex-matched. The position of the partner rat and the empty cup was alternated and controlled for across experimental condition. An overhead camera was used to record the movement of the rat in the arena. A human observer scored time spent sniffing each plastic cage, using two stopwatches.

Dependent measures included the manually coded time spent with a stranger rat compared to time spent with an inanimate object.

2.8.2. Locomotor activity, investigatory behavior, anxiety-like behavior

To evaluate exploration and unconditioned anxiety (Mouri et al., 2007), locomotor activity and behavior was measured in the Behavioral Pattern Monitor (BPM; 30.5 × 61.0 × 28.0 cm black Plexiglas chamber with seven wall holes (three per side wall, one on the back wall) and three floor holes, each 2.5 cm in diameter (Geyer et al., 1986). Photocells in each hole detect investigatory nosepokes (holepokes). A 4 × 8 grid of infrared photobeams detected the animal's position in X–Y coordinates, and a second set of photobeams at a higher level detected rearing behavior. Every 55 ms, a microprocessor system recorded the status of all beams and stored this information for subsequent off-line analysis.

Rats were acclimated for 60 min in the testing room. Lights in the chambers were on to create a more anxiogenic environment. Raw data

was reduced to the X and Y coordinates of the rat in the chamber; further analyses produce specific measures of behavior.(Geyer et al., 1986) Locomotor activity was quantified by the number of crossings between any of eight equal square sectors within the BPM, and the number of holepokes and rearings were calculated. Data were examined in 10-min time resolutions (locomotor activity, time spent in center) or 30-min time resolutions (rearings, holepokes). Time spent in the center during the first 10-min block was used as a measure of anxiety-like behavior. Data were analyzed using two-way ANOVA with treatment as between-factor and time as a repeated measure with specific post hoc comparisons between drug and saline groups using Tukey's studentized range method where appropriate.

2.8.3. Acoustic startle and prepulse inhibition

Prepulse inhibition (PPI) of the acoustic startle response reflects the ability to adapt to a strong sensory stimulus (sensorimotor gating) and prevent attentional overload, and provides a model for impaired sensorimotor gating deficits associated with neuropsychiatric disorders (Geyer et al., 2001; Quednow et al., 2018) and anesthesia toxicity (Cabrera et al., 2020). Eight startle test chambers (SR-LAB system, San Diego Instruments, San Diego, CA, USA)(Halberstadt et al., 2016b; Ku et al., 2016) are sound-attenuated, lighted, and ventilated enclosures contain a clear nonrestrictive cylindrical Plexiglas stabilimeter, 8.2 cm in diameter. A high-frequency loudspeaker mounted 24 cm above the Plexiglas cylinder produces all acoustic stimuli, with peak and average amplitudes of the startle response detected by a piezoelectric accelerometer. At the onset of the startling stimulus, 100 1-ms readings are recorded, and the average amplitude is used to determine the magnitude of the startle response (measured in arbitrary units). A dynamic calibration system is used to ensure comparable stabilimeter sensitivity across test chambers, and sound levels are measured using the dB(A) scale.

Acoustic startle test sessions consist of startle trials (pulse-alone; 40-ms 120-dB pulse of broadband white noise) and prepulse trials (prepulse + pulse; 20-ms acoustic prepulse, 80-ms delay, then 40-ms 120-dB startle pulse; 100 ms onset–onset). There was an average of 15 s (range = 6–22 s) between trials. During each inter-trial interval, the movements of the animals were recorded to measure responding when no stimulus was present. Each startle session began with a 5-min acclimation period to a 65-dB broadband noise that was present continuously throughout the session. The startle test session contained 12 pulse-alone trials and 36 prepulse + pulse trials (12 prepulses each of 68, 71, and 77 dB; equivalent to 3, 6, and 12 dB above background) presented in a pseudo-randomized order. Six pulse-alone trials were presented at the beginning and the end of the test session but were not used in the calculation of PPI values.

The amount of PPI was calculated as a percentage score for each prepulse + pulse trial type:

$$\%PPI = 100 - [(\text{startle response for prepulse + pulse trial})/(\text{startle response for pulse-alone trial})] \times 100.$$

Startle magnitude was calculated as the average response to all of the pulse-alone trials. PPI data were analyzed with three-factor analysis of variance (ANOVA) with sex and drug treatment as the between-subjects factors and trial type (prepulse intensity) as a repeated measure. Because there was no significant interaction between drug and prepulse intensity, PPI data were collapsed across prepulse intensity and average PPI was analyzed with a two-factor ANOVA with sex and drug treatment group as between subject factors. Startle magnitude data were analyzed with two-factor ANOVA with sex and drug treatment as between subject factors. Post-hoc analyses were performed using Tukey's studentized range method.

2.8.4. Spatial learning and memory

The Morris water maze (MWM) comprises a large pool (180 cm inner diameter) filled with opaque water (temperature 21° C + 1°), with a platform (10 cm × 10 cm) submerged 3 cm below the surface, and a recording camera mounted above. On Day 0 rats were placed in water and allowed to locate the platform for 60 s. If unable to locate the platform, they were guided to the platform and allowed to sit for 10 s. On Days 1–5, rats were tested in two trials each day. Rats were placed in the maze in one of 4 randomly selected starting positions, and allowed to explore for a maximum of 60 s. Escape latency (i.e. time taken to find the hidden platform) and distance traveled was recorded. On Day 6, the platform was removed and the rat was placed in the maze and its search patterns for the missing platform measured to assess reference memory over a 60 s period. Data includes: time spent in target platform quadrant, number of crossings of the former platform location, number of crossings of former "zone P" (a 20 cm diameter circular area surrounding the former platform location), and time spent in "zone P".

For acquisition (Days 1–5), data were analyzed via 2-way ANOVA with IT injectate as a between subject variable and day as a repeated measure. For the probe trial, data were analyzed via 1-way ANOVA with IT injectate as a between subject variable.

2.9. Statistical analysis

Sample sizes were based on previous studies by the current investigators using the same methodology. For tissue analysis, multiple sections were evaluated to provide an average count for each animal, and analysis is based on number (n = 6 for 2-CP treatment group) of animals. Using the same protocols, we have previously identified statistically significant dose and/or age dependent differences with a minimum sample size of 4 animals per group.(Walker et al., 2012, 2010) At each postnatal age, changes in MWT pre- and post-injection of 2-CP or saline were analyzed by two-way ANOVA with repeated measures with time (baseline, 5, 10, 15 and 30 min following injection) and treatment group (2-CP vs saline) as variables, with Tukey *post-hoc* comparisons and reported *P* values adjusted for multiple comparisons.

Larger sample sizes were used for long-term functional outcomes to include sex as a biological variable,(Chinn et al., 2020) and litter as a random effect (Golub and Sobin, 2020). Mixed design general linear models assessed differences in dependent variables (left hindpaw reflex withdrawal thresholds, body weight) with sex and treatment group as between-subject factors and litter as a random effect. The degree and duration of acute sensory block at P7 was analyzed using mixed design GLM with repeated measures of MWT as within-subject effects, treatment group and litter as between-subject variables and degrees of freedom for within-subject effects corrected using Greenhouse-Geisser estimates of sphericity. Data were analyzed with SPSS V27 (IBM), and Prism® Version 9 (GraphPad, San Diego) was used for graphing.

Our primary hypothesis was that neonatal intrathecal 2-CP would not affect higher-order behavior in adult rats. Sample size was based on the minimum necessary to provide reliable estimates of treatment effects, based on our experience with these measures (Halberstadt et al., 2016a, b; Powell et al., 2015), and the learning and memory effects observed in the Morris Water Maze (Jevtovic-Todorovic et al., 2003) following general anesthetic exposure in juvenile rats. A sample comprising 8 males and 8 females in both 2-CP or saline groups is estimated to achieve a power of 0.80 to detect a moderate effect between 2-CP and saline and 0.99 for a large effect size. Details of analyses for each test paradigm are included with the Methods.

Higher order behavioral data were analyzed with SPSS V27 (IBM) for mixed effects models with sex and drug treatment as fixed factors and litter as a random factor. Where appropriate time, trial, or intensity were considered repeated measures. P values are reported to a minimum of $P < 0.001$, and differences reaching a rejection level of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Intrathecal 1 % 2-CP produces short-term reversible motor and sensory block in juvenile rodents

Dose-finding with an up-down method identified the maximum tolerated dose of 2-CP reliably associated with successful local anesthetic block at each postnatal age: 1μL/g 2-CP at P7, 0.75μL/g at P14 and 0.5μL/g at P21. These volumes of 2-CP or saline were used for all subsequent intrathecal injections. Following IT 2-CP, a significant increase in MWT (right shift and higher EF_{50}) was observed 10 min following 2-CP *vs* saline groups at all ages: 3.1 ± 0.2 *vs* 1.6 ± 0.04 g at P7 (Fig. 1A); 18.7 ± 1.5 *vs* 10.5 ± 0.2 g at P14 (Fig. 1B); 16.4 ± 1.1 *vs* 10.3 ± 4.0 g at P21 (EF_{50} ± SE g) (Fig. 1C), with a return to baseline by 15−30 min (Fig. 1D–F). At all postnatal ages, baseline MWT did not differ between treatment groups (2-CP, saline, naïve) or between right and left hind paws (Fig. 1D-F). For P7 experimental animals (n = 56 from 7 litters), baseline MWT showed no main effect of sex ($F_{1,24} = 0.7$, P = 0.44), treatment group (saline, 2-CP or naive; $F_{2,24} = 2.1$, P = 0.17) or litter ($F_{6,24} = 2.7$, P = 0.39). Intrathecal 2-CP produced motor block at 5 min (lack of response to suprathreshold mechanical stimulus), ongoing sensory block at 10 min, and MWT returned towards baseline by 15 min (Fig. 1D-F).

Additional groups of P7 males and females for long-term behavioral

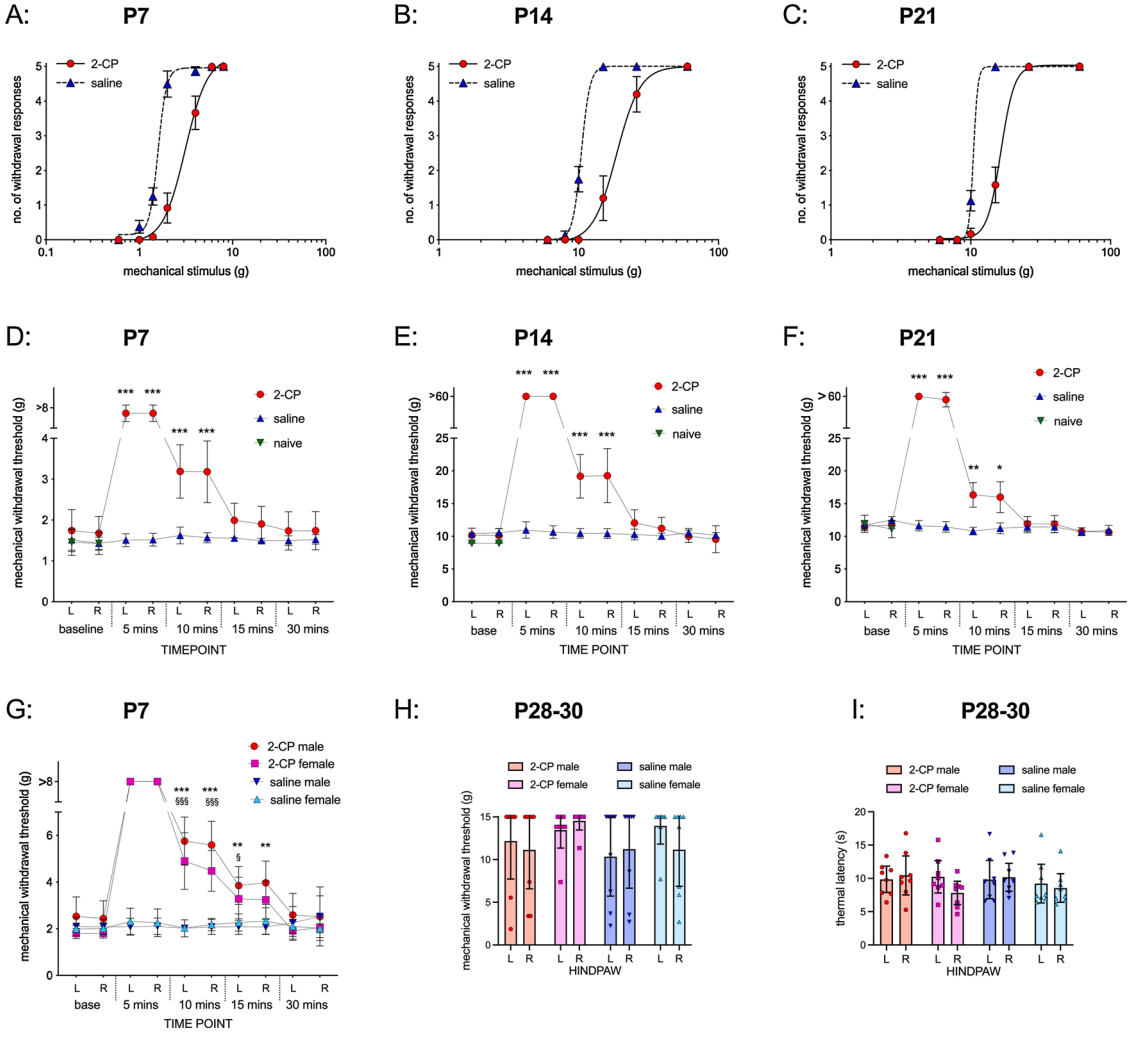

Fig. 1. Intrathecal 2-chloroprocaine (2-CP) produces short-term mechanical and sensory block at all postnatal ages.
(A–C): Mechanical stimulus (g force vFh applied to left hindpaw) vs response (number of withdrawal responses to 5 applications of vFh) relationship for left hindpaws 10 min following intrathecal injection of 2-CP or sterile saline. At each postnatal age P7 (A), P14 (B) or P21 (C) 2-CP injection results in a left shift (i.e. higher mechanical force to evoke reflex withdrawal due to sensory block). Data points = mean ± SEM.
(D–F): Mechanical withdrawal threshold (MWT; EF_{50} from individual stimulus-response relationships) in 2-CP, saline and naïve groups at postnatal day P7, 14 or 21 for the left (L) and right (R) hindpaw. Five minutes following 2-CP injection, MWT values are represented as the maximum applied force (10 g at P7, 60 g at P14 and P21). (D): At P7, baseline MWT did not differ (no main effect of group, $F_{(2,\ 21)} = 0.63$, $P = 0.54$; or paw $F_{(1,21)} = 1.24$, $P = 0.28$) but showed subsequent main effects of time ($F_{9,162} = 54.8$; P < 0.001) and treatment group ($F_{1,18} = 136$; P < 0.001). (E) At P14, baseline MWT did not differ (no main effect of group, $F_{(2,\ 21)} = 2.1$, P = 0.15 or paw, $F_{(1,\ 21)} = 0.29$, P = 0.59) but showed subsequent main effects of time ($F_{9,162} = 387$; P < 0.001) and group ($F_{1,\ 18} = 322$; P < 0.001). (F) At P21, baseline MWT did not differ (no main effect of group, $F_{(2,\ 21)} = 1.3$, P = 0.29; or paw $F_{(1,\ 21)} = 0.07$, P = 0.79) but showed subsequent main effects of time ($F_{9,162} = 157$; P < 0.001) and group ($F_{1,\ 18} = 321$; P < 0.001). ***P < 0.001, **P < 0.01, *P < 0.05 two-way repeated measures ANOVA with Tukey *post-hoc* comparisons. Data points = mean [95 %CI]; at each age, $n = 12$ 2-CP, $n = 6$ saline, $n = 4$ naïve. (G): Additional male and female P7 rats received intrathecal 2-CP or saline. Data points = mean [95 %CI], $n = 8$ animals per group. ***P < 0.001, **P < 0.01 2-CP male vs saline male; §§§P < 0.001, §P < 0.05 2-CP female vs saline female (2-way repeated measures with Tukey *post-hoc* comparisons). (H–I): Intrathecal 2-CP at P7 had no significant long-term effect on hindlimb reflex response. Values for mechanical withdrawal threshold (H) and thermal escape latency (I) did not differ between left and right paws, males and females or 2-CP versus saline groups. Data points = individual values, bars = mean [95 %CI], $n = 8$ per group.

assessments received 1μL/g intrathecal 2-CP or saline. For baseline MWT, there was no main effect of sex ($F_{1,16} = 1.9$, $P = 0.26$), treatment group ($F_{1,16} = 4.4$, $P = 0.12$) or litter ($F_{3,16} = 1.4$, $P = 0.72$), and no treatment group by litter interaction ($F_{3,16} = 0.27$, $P = 0.84$). Within-subject changes in left MWT following intrathecal injection demonstrated a main effect of time ($F_{1.1,25.4} = 19.2$, $P < 0.001$) and time by drug interaction ($F_{1.1,25.4} = 18.6$, $P < 0.001$) but no drug by litter interaction ($F_{3,24} = 0.63$, $P = 0.60$). Within saline or 2-CP groups, MWT did not differ between left and right hindpaws or between male and females (Fig. 1G).

Intrathecal 2-CP had no significant long-term effect on hindlimb reflexes at P28-30. For MWT, there was no main effect of treatment group ($F_{1,16} = 0.68$, $P = 0.47$), sex ($F_{1,16} = 0.21$, $P = 0.68$) or litter ($F_{3,16} = 0.74$, $P = 0.60$) (Fig. 1H). Similarly, thermal escape latency showed no main effect of treatment ($F_{1,16} = 0.36$, $P = 0.59$), sex ($F_{1,16} = 0.19$, $P = 0.69$) or litter ($F_{3,16} = 0.95$, $P = 0.53$) (Fig. 1I).

We have previously performed gait analysis at P35 (CatWalk® system), and found no differences following maximum tolerated intrathecal doses of 0.5 % bupivacaine (Hamurtekin et al., 2013), but persistent alterations following IT ketamine at P3 (Walker et al., 2010). Additional experiments performed at that time found no difference from saline controls in static or dynamic gait parameters following intrathecal 2-CP at P3 or P21, but as a different preparation and dose was used (3 % 2-CP 1μL/g at P3 and 0.7 μL/g at P21), these results are not reported in detail.

Body weight increased with postnatal age (14.3 ± 0.23 g at P7; 26.0 ± 0.58 g at P14; 41.3 ± 2.61 at P21; n = 24 at all ages) but did not differ across 2-CP, saline or naïve groups (main effect of age $F_{2,63} = 383$, $P < 0.001$, but not group $F_{2,64} = 1.9$, $P = 0.15$). All groups gained weight in the 24 h post injection, including those receiving 2-CP at P7 (14.6 ± 0.6–17.0 ± 0.8 g), P14 (26.4 ± 1.4–29.2 ± 1.6 g) or P21 (42.5 ± 4.7–45.8 ± 3.9 g) suggesting that treatment did not impair feeding or maternal care. In P7 litters for long-term behavioral assessments, body weight at baseline showed no main effect of sex ($F_{1,16} = 0.56$, $P = 0.51$), treatment group ($F_{1,16} = 4.5$, $P = 0.13$) or litter ($F_{3,16} = 7.1$, $P = 0.27$). By P28-30, body weight was greater in males vs females (97.9 ± 8.9. vs 86.1 ± 6.9 g; main effect of sex ($F_{1,16} = 35$, $P = 0.01$) but did not differ based on treatment group ($F_{1,16} = 2.8$, $P = 0.20$) or litter ($F_{3,16} = 91$, $P = 0.9$).

3.2. *Single-dose intrathecal 2-CP did not produce acute tissue toxicity in juvenile rodents*

Histopathological evaluation of lumbar spinal cord sections stained with H&E identified occasional indices of focal dural thickening and inflammation (4 of 72 animals), and mild leptomeningeal inflammation (8 of 72 animals) that were not specific to group (naïve, saline or 2-CP), or to postnatal age at the time of injection (P7, P14, P21) or sacrifice (1 or 7 days). Spinal cord or nerve root inflammation was not seen in any animals. No pathologically significant abnormalities were found in H&E sections from the brain. Incidental findings (microglial nodules in 3 animals) were not specific to any treatment group or time point.

Nerve roots of the cauda equina (3–5 sections per animal) from P7, P14 and P21 pups were examined 1 and 7 days after intrathecal saline or 2-CP and in age-matched naïve animals. Largely intact axonal morphology was observed in the endoneurium of the cauda equina in 2-CP and saline treatment groups following injection at all postnatal ages.

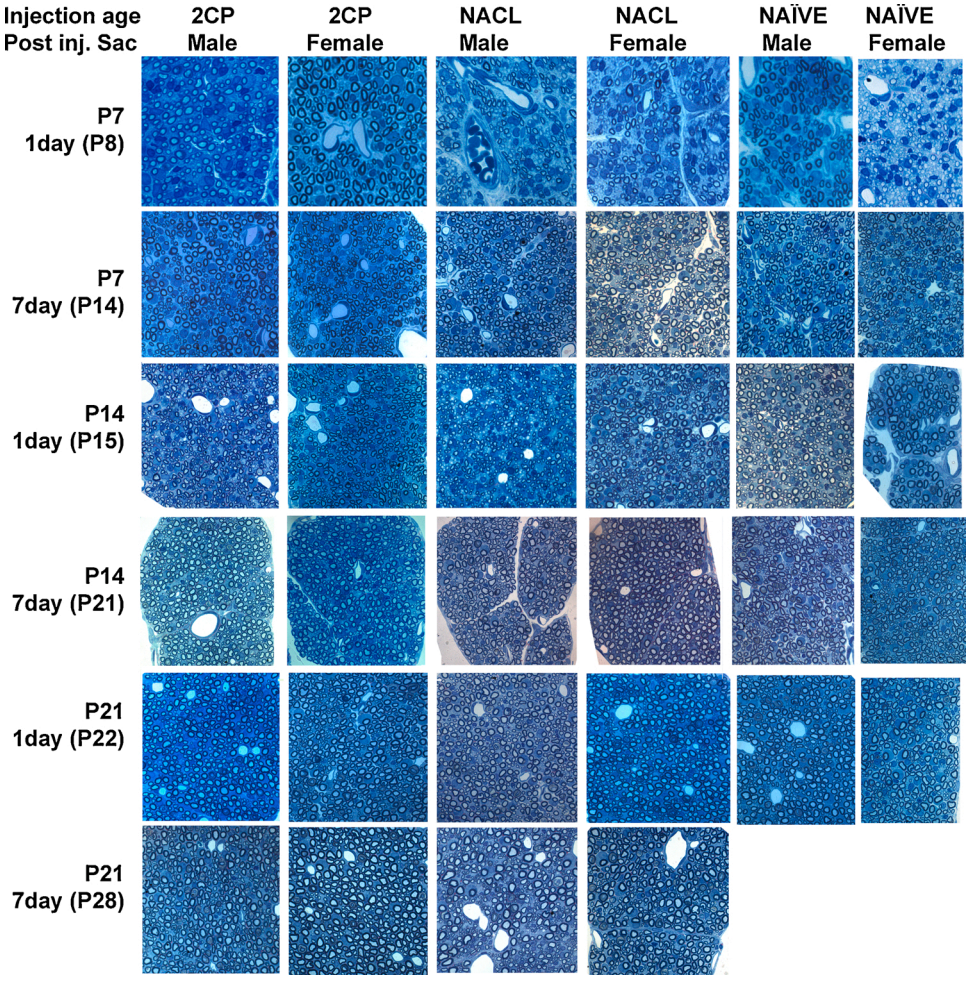

Fig. 2. Histology of the cauda equina is influenced by postnatal age but not by intrathecal saline or 2-CP. Representative sections of cauda equina from female and male P7, P14 and P21 rats at 1 day and 7 days after intrathecal saline or 2-CP and from naïve controls. The nerves show largely intact myelinated axons and normal Schwann cell morphology in the experimental and control groups. Transverse 1-μm-thick araldite sections stained with methylene blue azure II. Light microscopy magnification x 500. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The cauda equina histology was judged to be uniformly comparable to that noted in the corresponding naïve tissues. Enlarged Schwann cell cytoplasm was observed in P7 and P14 groups, and is characteristic of the proliferative Schwann cell phenotype during normal development (Webster, 1993). No apparent differences between treatment groups were observed, regardless of sex or age of the animals (Fig. 2).

There were no significant differences in activated caspase-3 positive cell counts 24 h (Fig. 3A) or 7 days following intrathecal 2-CP or saline (main effect of age, $F_{2,28} = 39$ $P < 0.001$; but not group, $F_{2,28} = 1.63$ $P = 0.21$). There were no apparent sex differences in the 2-CP groups, but the sample size was limited (3 males and 3 females). Increased numbers of large neurons positive for activated caspase-3 were only seen in the positive control group, 24 h following P3 ketamine. Staining with FluoroJade C did not identify any differences between 2-CP, saline and naïve animals at P7, P14 or P21.

There was no evidence of altered glial reactivity in the spinal cord at 24 h or 7 days following intrathecal 2-CP or saline (Fig. 3B–D). Iba-1 immunofluorescence (Fig. 3C) and GFAP immunofluorescence (Fig. 3D) in lumbar dorsal horn 7 days following injection did not differ from age-matched controls. Similarly, no differences were seen at 24 h for Iba1 (no main effect of group, $F_{2,27} = 1.01$ $P = 0.14$) or GFAP (no main effect of group, $F_{2,26} = 0.18$ $P = 0.83$).

3.3. Adult behavioral outcomes did not differ following intrathecal 2-CP or saline at P7

Male and female rats treated at P7 with intrathecal saline or 2-CP were evaluated at intervals from P35 to P71 to assess long term behavioral outcomes (Fig. 4). Prior intrathecal 2-CP at P7 had no effect on social approach behavior in young adult rats. Rats showed the expected high preference for the social stimulus (i.e. novel rat) compared to the inanimate object (main effect $F_{1,26} = 37.26$, $P < 0.001$), indicating ample range to see a reduction related to prior treatment (Fig. 4A). Overall, there was a main effect of sex ($F_{1,26} = 5.51$, $P < 0.05$) as males spent an increased percentage of time sniffing the novel rat, and an object x sex interaction ($F_{1,26} = 9.7$, $P < 0.01$), but no differences between 2-CP and saline groups in males or females (Fig. 4A and B).

Locomotor activity, exploratory behavior and anxiety-like behavior in a brightly lit arena did not differ between 2-CP and saline groups (Fig. 4C–G). Female and male rats habituated to the arena, as demonstrated by decreased transitions over the 60-min session (main effect of time; $F_{5,70.9} = 70.96$, $P < 0.001$), but there were no differences between 2-CP and saline groups nor any interactions between treatment and time (Fig. 4C and D). During the first 10 min, female rats spent more time exploring the brightly-lit center of the field (main effect of sex; $F_{1,27} = 6.55$, $P = 0.016$), but this was not influenced by prior IT treatment (no main effect of group; $F_{1,27} = 0.016$ $P = 0.90$) (Fig. 4E). Investigatory behavior was reduced in males, with reduced rearing (main effect of sex $F_{1,27} = 8.4$, $P < 0.01$) (Fig. 4F) and holepoking behavior (main effect of sex; $F_{1,27} = 19.8$, $P < 0.001$)(Fig. 4G), but 2-CP and saline groups did not differ in male or female rats.

Prepulse inhibition (PPI) of the acoustic startle response did not differ between saline and 2-CP groups (Fig. 4H–J). As predicted, increasing prepulse intensity (68−77 dB) was associated with increased

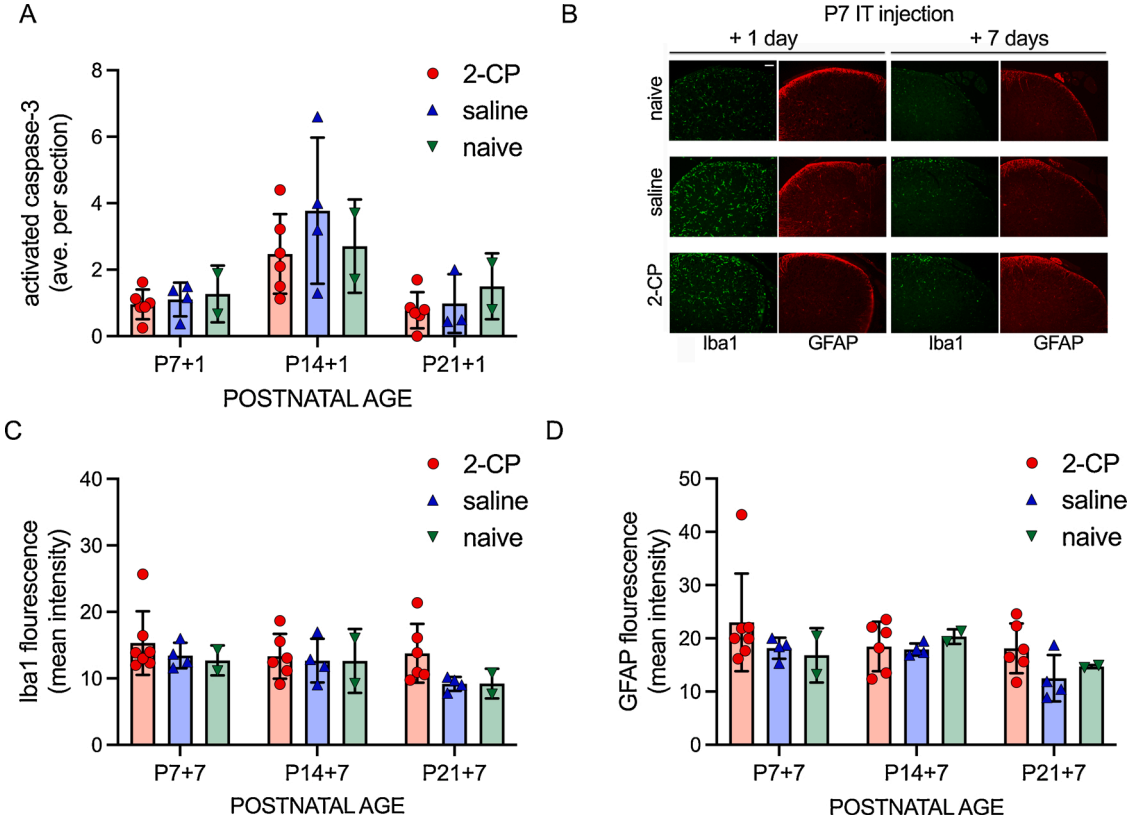

Fig. 3. Spinal cord tissue analyses did not differ following intrathecal 2-CP or saline.
(A): Twenty-four hours following intrathecal injection, positive activated caspase-3 (ac-3) cell counts following 2-CP did not differ from age-matched saline or naïve controls. Individual data points = average count from 10 sections per animal; bars = mean ± SD. (B): Representative dorsal horn sections with staining for Iba1 (green) and GFAP (red) immunoreactivity one and seven days following intrathecal injection of 2-CP or saline and age-matched naïve animals. (C): Iba1 immunoreactivity did not differ 7 days following intrathecal injection of 2-CP or saline at P7, P14 or P21, and was comparable with age-matched naïve data (no main effect of group $F_{2,28} = 2.11$ $P = 0.14$ or age $F_{2,28} = 1.89$ $P = 0.17$). (D): GFAP immunoreactivity did not differ 7 days following intrathecal injection of 2-CP or saline at P7, P14 or P21, and was comparable with age-matched naïve data ($F_{2,28} = 1.80$ $P = 0.18$ or age $F_{2,28} = 1.81$ $P = 0.18$). (E–F): Individual data points = average intensity for each animal (4 sections per animal; ROI intensity minus background at 4 dorsal horn locations); bars = mean ± SD.

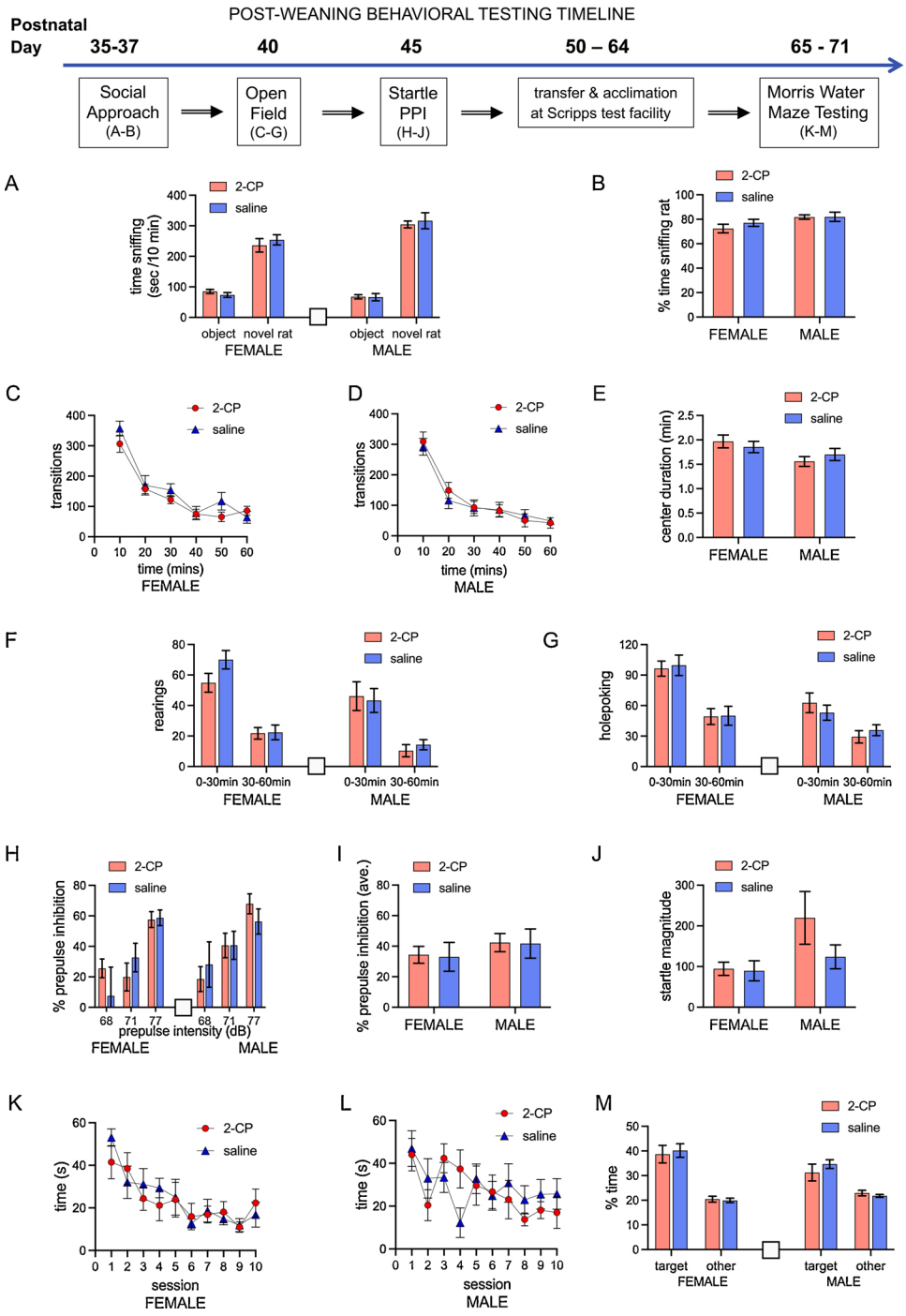

Fig. 4. Behavioral assessments sequentially performed at ages from postnatal day (P)35 to 71 did not differ following P7 intrathecal injection of 2-chlorprocaine (2-CP) or saline in males or females.
(A–B): At P35-37, behavior in a 3-chamber social approach arena was assessed. Time spent approaching and sniffing the novel rat (seconds during 10 min) was higher than time spent with an inanimate object but there was no main effect of treatment in females ($F_{1,26} = 0.04$, $P = 0.84$) or males ($F_{1,28} = 0.11$, $P = 0.74$)(A). Male rats spent an increased percentage of time sniffing the novel rat (main effect of sex $F_{1,27} = 5.1$, $P = 0.03$), but there was no main effect of treatment group ($F_{1,27} = 0.60$ $P = 0.44$)(B). (C–D): At P40, the number of transitions across a grid in the Behavioral Pattern Monitor reduced with time (10 min blocks from 0 to 60 min.) in both females (C) and males (D). (E) During the first 10 min, female rats spent more time exploring the brightly-lit center of the field, but 2-CP and saline groups did not differ in females or males. (F–G) Investigatory behavior measured by number of rearings (F) and holepoking behaviors (G) was plotted in 0–30 and 30−60 min blocks, and was less in males (main effect of sex, $F_{1,27} = 19.8$, $P < 0.001$) but did not differ between 2-CP and saline groups. (H–J): Prepulse inhibition of the acoustic startle response (ASR) was assessed at P45. (H) Percentage inhibition of ASR increased at higher prepulse intensities (68−77 dB) in females and males. (I) Average percentage PPI did not differ between groups. (J) Startle magnitude in response to the 120 dB pulse trials was not influenced by treatment group ($F_{1,27} = 1.63$, $P = 0.21$), and post-hoc comparisons found no significant differences between any groups. (K–M): In the Morris Water Maze, time taken in seconds to reach a hidden platform was measured across sessions 1–10 (2 sessions/day for 5 days) and reduced with time in both females (K) and males (L). (M) The percent time confirms increased time spent in the target quadrant (where the platform had been located previously) compared to all other quadrants, but this was not influenced by treatment group or sex. Data points = mean ± SEM; n = 7–8 per group.

inhibition (main effect of intensity; $F_{2,60.47} = 32.18$, $P < 0.001$), but the degree of prepulse inhibition did not differ between saline and 2-CP groups at any intensity (Fig. 4H). Similarly, average percentage PPI was not influenced by sex ($F_{1,24.74} = 1.89$, $P = 0.18$) or treatment ($F_{1,25.24} = 0.10$, $P = 0.75$) (Fig. 4I). Despite increased variability in the male 2-CP group, the magnitude of the acoustic startle response in the 120 dB pulse trials was not influenced by treatment group ($F_{1,25.98} = 1.75$, $P = 0.20$) and there were no interactions between sex and treatment ($F_{1,26.18} = 1.03$ $P = 0.32$) (Fig. 4J).

Intrathecal 2-CP did not produce any enduring effects on spatial learning and memory (Fig. 4K–M). Latency to reach a hidden platform in the Morris water maze decreased over the course of the 10 sessions (main effect of session; $F_{9,243} = 8.56$ $P < 0.001$) indicating that female (Fig. 4K) and male rats (Fig. 4L) learned the location of the platform. There were no significant effects of treatment group, sex, or sex by group interactions. Spatial memory for platform location was maintained in all groups, as rats spent more time in the quadrant where the platform had been located previously (main effect of quadrant; $F_{1,27} = 57.8$, $P < 0.001$) (Fig. 4M) Females spent slightly more time in the target quadrant compared to male rats (main effect of sex; $F_{1,27} = 4.9$, $P < 0.05$; and sex x quadrant interaction, $F_{1,27} = 4.9$, $P = 0.03$); however, there were no differences between 2-CP and saline groups (Fig. 4M). Results are not influenced by locomotor activity, as there were no differences in distance travelled between 2-CP versus saline groups in females (1464 ± 31 *vs* 1511 ± 42 cm; mean ± SEM) or males (1441 ± 88 *vs* 1380 ± 69) during the probe test, and overall no main effect of sex ($F_{1,27} = 1.5$ $P =$

0.24) or intrathecal treatment group ($F_{1,27} = 0.01$, $P = 0.92$).

4. Discussion

Age-adjusted maximum tolerated intrathecal doses of 1 % 2-CP (1.0 μL/g at P7, 0.75 μL/g at P14 and 0.5 μL/g at P21) resulted in effective and quantifiable sensory and motor block in juvenile rats. Following resolution of acute block, mechanical withdrawal threshold did not differ from age-matched intrathecal saline or naïve controls at 30 min or 24 h following injection at any age, and there were no long-term effects on spinal sensorimotor function in males or females receiving intrathecal 2-CP or saline at P7. Examination of spinal cord, cauda equina and brain tissue at one- or seven-days post intervention found no significant tissue pathology specific to intrathecal treatment (2-CP vs saline) or related to brief general anesthesia (saline vs naïve). In addition, spinal cord apoptosis and glial reactivity did not differ between 2-CP and age-matched saline groups. Finally, exposure to spinal anesthetic doses of 2-CP on postnatal day 7 did not produce any enduring effects on social behavior, spatial learning and memory, sensorimotor gating, or anxiety-like behavior in young adult male or female rats.

Intrathecal 2-CP produced reversible motor and sensory block in juvenile animals. The 10−15 min duration of block was shorter than the 30−40 min block previously demonstrated in vitro in P7 rodents following 0.5 μL/g 0.5 % levobupivacaine (Hamurtekin et al., 2013) and 0.5−1.0 μμL/g 0.75 % bupivacaine (Yahalom et al., 2011). Maximum tolerated volumes of 1 % 2-CP were highest at P7, and likely reflect the greater relative cerebrospinal fluid (CSF) volume in younger animals (Bass and Lundborg, 1973; Ghersi-Egea et al., 2015). As there are age-dependent changes in CSF regulation and ion composition (Xu et al., 2021), CSF efflux mechanisms (Saunders et al., 2018), and the vulnerability of the developing central nervous system to anesthetic and sedative/analgesic toxicity (Deng et al., 2014; Walker and Yaksh, 2012), there is a clear need for the efficacy and developmental toxicology of neuraxial local anesthetics to be assessed across a range of postnatal ages. In human infants, the reduced duration of action and higher dose requirements for spinal local anesthetics have also been attributed to age-related changes in CSF volumes (11−15 ml/kg in infants, 4 mL/kg in older children and 2 mL/kg in adults)(Boretsky, 2019). This is reflected by evidence-based recommended volumes for intrathecal tetracaine 0.5 %: 0.13 mL/kg (1 mg/kg) in infants weighing less than 4 kg compared to 0.07 mL/kg (0.5 mg/kg) in infants weighing more than 4 kg; and the reduced duration of action and potency of 2-CP compared to bupivacaine and levobupivacaine is also highlighted (Suresh et al., 2018). Spinal anesthesia is feasible as a solo technique for neonates and infants requiring lower abdominal and lower limb surgeries, and can minimize or avoid exposure to general anesthetic and sedative drugs (Disma et al., 2018; Whitaker and Williams, 2019). The incidence of intraoperative hypotension was reduced with awake regional versus general anesthesia (median duration 54 min) for neonatal inguinal hernia repair (McCann et al., 2017) and neurodevelopmental outcomes were equivalent at 5-years of age (McCann et al., 2019). The short duration of action of intrathecal 2-CP limits clinical utility for single-dose spinal anesthesia (Suresh et al., 2018), but 3 % 2-CP (increments of 0.5 mL/kg/dose up to 2 mL/kg followed by 1.5 mL/kg/hr) via a caudal epidural catheter has been used as the sole anesthetic (i.e. no additional sedative or general anesthetic) for neonates undergoing inguinal hernia (Mueller et al., 2017).

Dose-dependent neurotoxic effects of local anesthetics vary with the agent, its potency, and the dose or duration of exposure (van Zuylen et al., 2019; Verlinde et al., 2016). Proposed cellular mechanisms include non-specific cell death, increased apoptosis and alterations in inflammatory signaling pathways (Kan et al., 2018; Takenami et al., 2012, 2009; Taniguchi et al., 2004; Verlinde et al., 2016). Laboratory studies in adult animals reported different degrees of toxicity and morphological changes with earlier 2-CP formulations that were attributed to either the drug (Taniguchi et al., 2004), differences in relative dosing, the pH of the solution, or the preservative bisulfite (Hampl et al., 2014; Takenami et al., 2015). As also seen following levobupivacaine at P3 or P7 (Hamurtekin et al., 2013) and bupivacaine at P7, P14 or P21 (Yahalom et al., 2011), there was no increase in apoptosis in the spinal cord following 1% 2-CP at any postnatal age. Injection of lidocaine into the dorsal root ganglia of adult animals increased reactivity in satellite glial cells (increased GFAP immunoreactivity) (Puljak et al., 2009), and intrathecal ketamine at P3 increased reactivity of spinal microglia and astrocytes (increased Iba1 and GFAP immunoreactivity) (Walker et al., 2010), but no alteration in glial reactivity was seen following intrathecal levobupivacaine at P3 or P7 (Hamurtekin et al., 2013) or here following 2-CP at P7, P14 or P21. Although some local anesthetics dose-dependently reduce cell viability and increase apoptosis of developing motor neurons in cell culture (Koo et al., 2021), P7 intrathecal 2-CP did not alter long-term motor function, as withdrawal reflexes at P28-30, spontaneous locomotor activity and rearing in an open field at P40, and distance travelled in the Morris water maze probe test at P65 did not differ between 2-CP and saline groups.

Long-term alterations in higher order behaviors in rodents have been reported following early life exposure to inhaled or systemically administered general anesthetic and/or sedative drugs (Cabrera et al., 2020; Jevtovic-Todorovic, 2018; Jevtovic-Todorovic and Brambrick, 2018; Maloney et al., 2019a). Sex-dependent differences vary across paradigms (Cabrera et al., 2020), but have not been evaluated in all studies. For example, deficits in social recognition following isoflurane during the first postnatal week have been reported in either male (Lee et al., 2014) or female (n = 15, 5 litters C57BL/6 mice) (Maloney et al., 2019b) rodents. Despite no change following 6 h isoflurane 0.75 % combined with nitrous oxide and midazolam at P7 (n = 9–11 Sprague-Dawley rats) (Jevtovic-Todorovic et al., 2003), deficits in prepulse inhibition were reported in adult rodents following 6 h 1.2 % isoflurane (6 h at P4-5) (Seubert et al., 2013), and in males but not females following 2.1 % sevoflurane (6 h at P4-6) (Xu et al., 2015) and propofol (140 mg/kg over 6 h at P4-6) (Tan et al., 2014). Following isoflurane or fentanyl-ketamine at P14 outer crossings in an open field were increased at P30 (n = 15−16) but not at P60 (n = 11–14 male Wistar rats) (Medeiros et al., 2011), and following sevoflurane (2% for 2−4 h) at P7 distance moved in an open field was increased compared to controls at P35 (n = 12 Wistar male or female rats) (Zheng et al., 2013). Total ambulations/crossings were lower in male mice only (n = 20, 5 litters C57BL/6 mice) following repeated isoflurane at P3, 5 and 7 (Maloney et al., 2019b). The Morris Water Maze test identified impaired learning and spatial memory in adult rodents following early life exposure to general anesthetic combinations (isoflurane, nitrous oxide, midazolam at P7; n = 9–11 Sprague-Dawley rats) (Jevtovic-Todorovic et al., 2003) and induction agents (propofol, ketamine, and thiopentone) (Maloney et al., 2019a). Here, we also included a battery of recommended and standardized post-weaning tests to assess adult functional outcome including: learning and memory (i.e. Morris water maze testing); motor/sensorimotor function (i.e. spinal reflex thresholds, locomotor activity, PPI); emotionality (i.e. open field behavior, acoustic startle, exploratory behavior); and social function (i.e. social approach) (Maloney et al., 2019a). Despite the increased relative dose of IT 2-CP in the youngest animals, and the potential vulnerability of many brain regions to neurotoxicity at P7 (Maloney et al., 2019a), behavioral outcomes did not differ following prior intrathecal 2-CP or saline. While expected sex-dependent differences were noted in some behaviors (i.e. increased time sniffing novel rat in males, increased time exploring brightly-lit arena in females), outcomes following IT 2-CP or saline did not differ in males or females.

The formulation of 2-CP for clinical use has changed multiple times since its introduction in 1952, with concentrations ranging from 1 to 3 %, solutions with or without epinephrine, and earlier preparations including preservatives such as sodium bisulfite or methylparaben (Goldblum and Atchabahian, 2013; Moore et al., 1982; Pollock, 2012).

Larger epidural volumes of preservative containing 2–3 % 2-CP were associated with neurological sequelae following unintentional intrathecal injection, transient neurologic symptoms (TNS), and variable levels of back pain in adults (Heydinger et al., 2021; Moore et al., 1982; Pollock, 2012; Stevens et al., 1993). More recently, a meta-analysis including 2 clinical studies with 1% preservative-free 2-CP (Casati et al., 2007; Teunkens et al., 2016) reported no difference in the risk of TNS following 2-CP or lidocaine (Forget et al., 2019). In adults, the short duration of action of IT 1% 2-CP has potential advantages over other intrathecal local anesthetics, with earlier ambulation and discharge following selected day-case procedures (Bhaskara et al., 2019; Ghisi et al., 2021; Saporito et al., 2019). While spinal anesthesia in neonates and infants can minimize or avoid exposure to general anesthetics (Disma et al., 2018; Whitaker and Williams, 2019), clinical utility is limited by the shorter duration of action of spinal local anesthetics at this age (Suresh et al., 2018). Permanent neurological deficits are very rare following regional anesthesia in children (Walker et al., 2018), or neonatal neuraxial catheter techniques (Llewellyn and Moriarty, 2007; Long et al., 2016), and the risk of transient neurological deficit following neuraxial and peripheral blocks in pediatric series is 2.4:10,000 [95 %CI 1.6–3.6:10,000] (Walker et al., 2018). However, there is little comparative data evaluating potential differences between available local anesthetics and preparations.

In clinical practice, local anesthetic toxicity can refer to: neurotoxicity associated with relatively high concentrations of drug being delivered in close proximity to the spinal cord and nerve roots (as discussed above); extensive cephalad spread and cardiorespiratory compromise, particularly if higher doses required for epidural administration are delivered directly into the CSF following unrecognised dural puncture or catheter migration; or systemic toxicity due to high plasma concentrations following inadvertent vascular injection or accumulation of drug and metabolites with prolonged infusion (van Zuylen et al., 2019). As 2-CP is an amino-ester that is rapidly metabolized by plasma pseudocholinesterase and has a short plasma half-life (Boretsky, 2019), it may reduce the risk of local anesthetic accumulation and systemic toxicity (Walker et al., 2018), particularly when higher total doses of local anesthetic are used for prolonged epidural infusion or paravertebral blocks in infants (Boretsky, 2019; Greco and Boretsky, 2020; Heydinger et al., 2021). While self-limiting acute systemic toxicity has been reported in infants following large bolus doses or inadvertent intravascular injection of 2-CP,(Cladis and Litman, 2004; Hernandez and Boretsky, 2016; Walker et al., 2018) low plasma concentrations have been documented in some series (Heydinger et al., 2021; Suresh et al., 2018). Epidural infusion increases clinical utility of short-acting local anesthetics such as 2-CP, but reports in neonates and infants vary widely in the preparation and concentration (1 %–3 %) used, duration (1–11 days) of infusion, and hourly doses (0.25–1.6 ml/kg/hr; 3.75–25 mg/kg/hr) (Gibbs et al., 2020; Muhly et al., 2015; Relland et al., 2021; Ross et al., 2015; Veneziano et al., 2016). The current data do not evaluate safety of prolonged epidural or intrathecal administration.

This single-dose study in juvenile rodents has limitations. Percutaneous intrathecal injection was performed under brief general anesthesia (5−8 min) and rapid recovery facilitated assessment of early motor and sensory block. Rodents were closely observed but physiological parameters were not monitored or compared in 2-CP and saline groups. Comparison with naïve controls (albeit small numbers) showed no detectable differences in reflex thresholds, weight gain or tissue outcomes at 24 h or 7 days related to the anesthesia, handling and maternal separation in treatment groups. Dose-response relationships were evaluated for acute efficacy, but as in previous developmental evaluations of intrathecal toxicity with bupivacaine (Yahalom et al., 2011) and levobupivacaine (Hamurtekin et al., 2013), tissue and functional outcomes were assessed following single age-adjusted doses of 2-CP. As maximum tolerated doses were not associated with adverse tissue pathology, lower doses were not assessed. Drug was delivered as a single percutaneous injection, as tissue outcomes following infusion in juvenile rodents can be confounded by the large relative size of the catheter and the need for open dissection for accurate placement (Hughes and Barr, 1988). As a result, the current safety data cannot be extrapolated to repeated boluses or to continuous infusion. The sample sizes for tissue analyses were small (n = 6 for 2-CP), but the same protocols identified significant changes in apoptosis and glial reactivity following intrathecal ketamine in P3 rodents (n = 4), and associated long-term functional changes in sensory threshold and gait (n = 10; 5 male, 5 female). In adult rats, larger sample sizes (n = 27) identified alterations in tail flick latency and nerve injury score 7 days following similar volumes (1–1.2 ml/g infused over 2 h) but higher concentrations of 2-CP (preservative-free 3% chloroprocaine, Nesacaine-MPF)(Taniguchi et al., 2004). Importantly, no differences in long-term function with spinal reflex or higher order behavioral measures were identified in adult male or female rodents following the maximum tolerated single dose of 2-CP. However, the current data do not exclude the possibility of more subtle signs of neurotoxicity being detected in larger samples.

5. Conclusions

Intrathecal 1 % 2-CP produces reliable, brief and reversible motor and sensory block of the hindlimbs in juvenile rodents. No tissue pathology in the spinal cord, cauda equina and brain was specifically related to maximum tolerated doses of 2-CP at P7, P14 or P21. Spinal reflex function, locomotor activity and higher order behavioral measures did not differ in adult male or female rats following intrathecal saline or 2-CP at P7. While these laboratory data failed to identify any adverse effects following a single dose of intrathecal 2-CP in early development, inadvertent intrathecal administration or prolonged infusions may deliver higher doses of local anesthetic in close proximity to the developing nervous system and the effects of such exposure cannot be extrapolated from the results reported here. As spinal anesthesia can provide effective perioperative analgesia and reduce general anesthetic requirements in human infants, the potential or relative neurotoxicity of local anesthetics preparations and spinal analgesic agents require ongoing evaluation in preclinical developmental models.

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CRediT authorship contribution statement

Suellen M. Walker: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Shelle Malkmus:** Investigation, Data curation, Writing - review & editing, Visualization. **Kelly Eddinger:** Investigation, Formal analysis, Writing - review & editing, Visualization. **Joanne Steinauer:** Investigation, Formal analysis, Writing - review & editing, Visualization. **Amanda J. Roberts:** Investigation, Formal analysis, Writing - review & editing, Visualization. **Veronica I. Shubayev:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision. **Marjorie R. Grafe:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization, Supervision. **Susan B. Powell:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Tony L. Yaksh:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Suellen Walker reports financial support was provided by Sintetica S.A., Via Penate 5, 6850 Mendrisio, Switzerland. Tony Yaksh reports financial support was provided by Sintetica S.A., Via Penate 5, 6850 Mendrisio, Switzerland. Veronica Shubayev reports financial support was provided by Sintetica S.A., Via Penate 5, 6850 Mendrisio, Switzerland. Marjorie Grafe reports financial support was provided by Sintetica S.A., Via Penate 5, 6850 Mendrisio, Switzerland. Susan Powell reports financial support was provided by Sintetica S.A., Via Penate 5, 6850 Mendrisio, Switzerland.

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