Intrathecal clonidine in the neonatal rat: dose-dependent analgesia and evaluation of spinal apoptosis and toxicity

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Abstract

BACKGROUND: Neuraxial clonidine is utilized for peri-operative analgesia in children of all ages. Preclinical studies in the postnatal rat allow comparison of the relative toxicity and safety of spinal analgesics throughout postnatal development.

METHODS: Rat pups aged 3, 7 or 21 postnatal (P) days were briefly anesthetized for intrathecal injections of saline or clonidine. At each age, the maximum tolerated, anti-nociceptive (increased hindlimb mechanical withdrawal threshold) and anti-hyperalgesic (hindpaw carrageenan inflammation) doses were determined. Lumbar spinal cord sections were assessed for apoptosis and cell death (histology, activated caspase-3 immunohistochemistry, Fluoro-Jade C staining), histopathology (haematoxylin and eosin staining), and increased glial reactivity (microglial and astrocytic markers). P3 intrathecal ketamine sections served as positive controls. In additional groups, thermal latency and mechanical withdrawal threshold were measured at P35.

RESULTS: Intrathecal clonidine produces age- and dose-dependent analgesia in rat pups. Maximal doses of clonidine did not alter the degree or distribution of apoptosis or increase glial reactivity in the neonatal spinal cord. No spinal histopathology was seen 1 or 7 days following injection at any age. Intrathecal clonidine did not produce persistent changes in reflex sensitivity to mechanical or thermal stimuli at P35.

CONCLUSIONS: Intrathecal clonidine in the postnatal rat did not produce signs of spinal cord toxicity, even at doses much greater than required for analgesia. The therapeutic ratio (maximum tolerated dose/anti-hyperalgesic dose) was >300 at P3, >30 at P7, and >10 at P21. These data provide additional information to inform the clinical choice of spinal analgesic agent in early life.

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Introduction

Preclinical work demonstrated that spinal delivery of clonidine produced potent analgesia (1,2) through an effect upon spinal alpha$_2$-adrenergic receptors (3). Subsequent human studies with neuraxial clonidine demonstrated clinical efficacy in adults (4) for the management of acute post-operative (5,6), labor (7,8) and post-cesarean (9) pain; as well as for longer-term management of chronic neuropathic (10,11) and cancer pain (12). In pediatric practice, spinal clonidine has been used primarily for peri-operative pain management; either as an additive to prolong analgesia with single-shot caudal local anesthetic (13-15) or to improve analgesia and reduce local anaesthetic requirements in epidural infusions (16,17). Although the majority of controlled trials have been conducted in infants and children, neuraxial clonidine has also been used in neonates, via either the caudal (18-20) or intrathecal route (21,22).

Laboratory studies have confirmed that spinal alpha$_2$ agonist analgesic mechanisms are functional from early development, and intrathecal clonidine (23) and epidural dexmedetomidine (24,25) have both anti-hyperalgesic and anti-nociceptive effects in rat pups from the third postnatal (P3) day. Epidural dexmedetomidine produces spinally-mediated dose-dependent analgesic effects at all postnatal ages, but the sensitivity to sedative (24) and cardiovascular (25) side-effects is also increased in early life.

The benefits of spinal analgesia must always be balanced by the potential risk of neurotoxicity when drugs are administered in relatively high concentrations close to the spinal cord, and adequate preclinical testing before routine clinical use has been advocated (26-28). In adult animals, repeated injections or continuous infusions of spinally administered clonidine for 14 days or longer, have not produced neurotoxic effects in rats (29,30) or dogs (31,32), and bolus doses did not reduce spinal cord blood flow in sheep (33) or pigs (34).

Responses to analgesics and anesthetics may differ in the developing nervous system (35). In particular, anesthetics with γ–amino butyric acid (GABA) agonist or n-methyl-D-aspartate (NMDA) antagonist action increase neuronal apoptosis or cell death in the primate (36-39) and rodent brain (40-42). Accordingly, it is important when evaluating novel neuraxial therapeutics for neonates and infants, to define the potential effects of the agent with respect to the developing

spinal cord. We have recently developed a model for assessing spinal toxicity in the neonatal rat that includes evaluation of: i) dose-dependent analgesic effects; ii) histopathology, neuronal apoptosis and glial reactivity in the spinal cord at doses up to the maximum tolerated; and iii) functional outcomes (43,44). Calculation of a therapeutic ratio (toxic dose/analgesic dose) allows comparison of the effects of different drugs at different postnatal ages. In this model, morphine had a high therapeutic ratio, i.e. toxicity was not seen at 300 times the analgesic dose in neonatal rats (44). In contrast, intrathecal ketamine increased apoptosis and glial reactivity in the spinal cord and produced persistent changes in gait and mechanical threshold in the same dose range as analgesia, i.e. its therapeutic ratio was <1 (43). As clonidine is a potential alternative to ketamine for caudal analgesia in pediatric patients (15), we have now evaluated the analgesic effects and potential for spinal apoptosis and toxicity following single-dose intrathecal clonidine in the postnatal rat.

Methods

Experimental animals

The study protocol and experiments were performed with personal and project licenses approved by the UK Home Office, in a laboratory approved for regulated procedures, and in accordance with the requirements for the care of laboratory animals of the United Kingdom Animal (Scientific Procedures) Act 1986. Sprague-Dawley dams and litters were bred in-house in the Biological Services Unit University College London, and were maintained on a 12-h light/dark cycle at constant ambient temperature with free access to food and water. Postnatal (P) day 3, 7 or 21 male and female rat pups, with mean body weights of 10, 16 and 54 grams respectively, were assigned to treatment groups. Within each litter, pups were randomly selected, numbered, and assigned to treatment groups. Data from animals within the same litter were treated as independent values, and treatment groups and group comparisons comprised animals from more than one litter. Overall, 12 litters from each age group were utilized. An independent colleague coded drug solutions to ensure the experimenter was unaware of treatment allocation during testing. The degree of handling and duration of maternal separation was minimized, and was the same for saline control and clonidine treatment animals. Litters were restricted to a maximum of 12, and pups were weaned into same sex cages at P21.

Behavioral testing

In P3, P7 and P21 rats, mechanical withdrawal thresholds were measured with hand-held von Frey hairs (vFh) that apply a logarithmically increasing force (vFh 5 = 0.13g to vFh 13 = 7.8g)(45). Testing was performed on the dorsal hindpaw as it is difficult to maintain body temperature or reliably apply mechanical stimuli to the plantar surface through an elevated mesh platform in small pups. Rats were lightly restrained on a flat bench surface and von Frey hairs were applied 5 times at one-second intervals to the dorsal surface of the hindpaw, as previously described (24,43,45,46). The number of evoked flexion reflexes to increasing intensity stimuli was recorded, until a given stimulus evoked five withdrawal responses or a supra-threshold cut-off pressure was reached (7.8g at P3, 13 g at P7, or 60g at P21).

To evaluate any persistent changes in sensory function following intrathecal injection at P3, P7 or P21, sensory thresholds were measured at 5 weeks of age (P35). Following habituation on an elevated mesh platform, the mechanical stimulus (electronic von Frey device; Dynamic Plantar Aesthesiometer, Ugo Basile) was applied to the mid-plantar surface of the hindpaw. The mechanical threshold was averaged from 3 measures of the force (0 to 50 grams; ramp 20 grams/sec) required to produce hind-limb withdrawal. Thermal withdrawal latency was determined using a modified Hargreaves Box (University Anesthesia Research and Development Group, University of California San Diego, La Jolla, California)(47), consisting of a glass surface (maintained at 30 °C) on which the rats were placed in individual Plexiglas cubicles. 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 seconds, the stimulus was terminated (cut-off time). Thermal latency was the average of three measures from each hindpaw.

Intrathecal injection technique

Pups were anesthetized with halothane (3-5%) in oxygen and air. Percutaneous intrathecal injections were made at the low lumbar level (intervertebral space L4-5 or L5-L6) with a 30-gauge needle perpendicular to the skin, connected to a micro-injector and 50 μl Hamilton syringe. Based on our previous work (44), an injectate volume of 0.5 μl per gram bodyweight was used as this provides a uniform spread across lumbar and low thoracic segments in rat pups of all current age (and corresponding weight) groups. Following preparation of different concentrations of clonidine, an independent colleague coded samples of drug and saline, to ensure the experimenter (SMW) was unaware of treatment group during behavioral testing.

Intrathecal clonidine dose

Determination of maximum tolerated dose.

In pilot experiments, intrathecal clonidine hydrochloride (Sigma-Aldrich, St Louis, MO) dissolved in sterile saline were administered to P3, P7 and P21 pups in escalating doses of 0.1, 0.3, 1, 3, 10, and 30mg/kg. Dose escalation was limited by behavioral disturbance and weight loss, rather

than by acute cardiovascular collapse or sedation and respiratory depression. Doses resulting in marked behavioral disturbance (reduced activity and freezing, alternating with agitation or excitation) were also associated with failure of normal daily weight gain or actual weight loss. Behavioral and motor changes were more apparent in the older pups, and occurred at lower doses. Following doses of 3mg/kg clonidine and above in P21 pups, piloerection, urinary voiding and shivering with significant hypothermia (temperature <31C) were also observed. As a result, temperature was monitored at intervals in all pups by holding a surface electrode between the upper limb and thorax in the 'axilla' of P3 pups or inserting a rectal probe in P10 or P21 pups. A warming protocol (thermostatically controlled heating blanket within open topped Perspex container) was established to ensure that body temperature was maintained after injection of saline or clonidine in subsequent experimental groups. The maximum tolerated dose of intrathecal clonidine was defined as the dose that did not produce weight loss in individual animals (when comparing values pre- and 24 hrs post-injection of clonidine) or significant group differences from saline controls. This provided a quantifiable outcome that could be standardized across the postnatal ages. In subsequent experimental groups, the maximum tolerated dose at each age produced mild acute behavioral changes, but body temperature and body weight were maintained.

For clarity, intrathecal doses are expressed in terms of mg/kg. However, as the concentration of drug is important for the direct effect upon local tissues after intrathecal delivery, the range of concentrations and mean total dose for each treatment group are also presented in Table 1.

Evaluation of anti-nociceptive doses.

Intrathecal clonidine doses up to the maximum tolerated were administered to evaluate acute anti-nociceptive effects. Individual pups received single doses of saline or clonidine. Mechanical withdrawal threshold was determined at baseline, correct intrathecal placement was confirmed by a significant increase in threshold 30 minutes after injection, and thresholds were again measured prior to sacrifice and spinal cord harvesting at 24 hours or 7 days post injection. Single injections of the maximum tolerated dose were administered for tissue analysis (44), as our model aims to

deliver the highest possible concentration of drug to evaluate local tissue toxicity, and repeated administration is not feasible as intrathecal catheters can produce confounding injuries in small pups (23). To confirm that effects at these doses were not solely due to systemic absorption, mechanical thresholds 30 minutes following subcutaneous administration of clonidine in the midlumbar region (10mg/kg at P3 or 3mg/kg at P7 and P21 in 5 μl/g; n=6 per group) were also evaluated.

In separate groups of animals, the maximum tolerated dose of intrathecal clonidine was administered to P3 (10mg/kg), P7 (3mg/kg) or P21 (1mg/kg) pups (n=8 per group). Control animals received intrathecal saline (n=6-8 per group). Mechanical withdrawal thresholds were measured at baseline and 30 minutes after injection to confirm intrathecal placement. Animals were maintained and regularly reviewed in the UCL Biological Services Unit, weaned into same-sex cages at P21, and weight, mechanical withdrawal threshold and thermal withdrawal latency were measured at 5 weeks of age.

Evaluation of anti-hyperalgesic doses.

Hindpaw inflammation was induced in P3, P7 and P21 pups by injection of 1 μl/g of 2% lambda carrageenan (Sigma-Aldrich) into the mid-plantar surface of the left hindpaw under brief halothane (2-4%) in oxygen anesthesia (24). Mechanical withdrawal thresholds were measured at baseline and 3 hours following carrageenan to quantify the degree of hyperalgesia. Intrathecal saline or clonidine (0.03 – 0.3 mg/kg; n=5-6 all groups) was administered as described above. Mechanical withdrawal thresholds were measured 15 and 30 minutes following injection, with the investigator (SMW) unaware of the treatment allocation.

Spinal cord preparation, staining and analysis

Spinal cord tissue was analyzed 24 hours and 7 days after injection as previously described (43,44) (n=4 per treatment group). In brief, animals were terminally anesthetized with 100mg/kg intraperitoneal pentobarbitone, and then transcardially perfused with heparinised saline followed by 4% paraformaldehyde. Spinal cords were dissected, postfixed in 4% paraformaldehyde, and then cryoprotected in 30% sucrose. Using a cryostat, 7 and 14 micron

sections of lower lumbar spinal cord were taken and mounted on Fischer Superfrost Plus (Fischer Scientific, Houston, TX) slides and stored at -70^{0}C.

For each analysis, sections were coded and the investigator was unaware of the treatment group. For quantitative analyses, at least 4 non-consecutive sections of lumbosacral cord from each animal were examined and the mean determined. Different sections were utilized for caspase-3 immunohistochemistry, Fluoro-Jade C staining or haematoxylin and eosin (H&E) staining at 24 hours, and for H&E staining and glial immunoreactivity at 7 days. Sections from P3 pups that had received 10mg/kg intrathecal preservative-free ketamine hydrochloride (Sigma-Aldrich, St Louis, MO) as previously described (43), were stained in parallel to act as a positive control for the staining protocol and to ensure the sample size was sufficient to detect treatment effects previously associated with both structural and functional changes (43). All statistical analysis was calculated with *n* representing the number of animals within each treatment group.

Fluoro-Jade C staining.

Fluoro-Jade C (FJ-C; Millipore, Temecula, CA) staining was performed as previously described (48) on 14 micron spinal cord sections prepared from tissue collected 24 hours following intrathecal injection. In addition to 0.0001% FJ-C, slides were incubated with the DNA stain 4',6-diamidino-2-phenylindole (DAPI 0.0001%; MP Biomedicals, Solon, OH) to aid visualization and confirm tissue integrity of the sections. Slides were examined with the appropriate wavelength fluorescent microscopy, and the number of immunofluorescent cells and their distribution in the dorsal horn, ventral horn or adjacent to the central canal were recorded.

Activated caspase-3 immunohistochemistry.

To further assess apoptosis, additional spinal cord sections obtained from P3 and P7 animals 24 hours following intrathecal injection were incubated with monoclonal activated caspase-3 antibody (1:100 Cell Signaling, Beverly, MA) for 72 hours at 4C. Biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA) was applied at 1:250 for 45 min at room temperature, followed by avidin-biotin-peroxidase complex (ABC reagent; Vector, Burlingame, CA) for 30 min. Staining was developed with DAB (Vector, Burlingame, CA) for 12 min. Sections were lightly counterstained with haematoxylin, dehydrated and coverslipped (Permount, Fischer SP15, Fair Lawn, NJ). Slides were coded and the number and location (dorsal horn, ventral horn

or adjacent to central canal) of caspase-3 immunoreactive cells were counted under high power light microscopy.

Histology.

A neuropathologist (MG) evaluated seven-micron spinal cord sections from all experimental groups, sacrificed at 1 or 7 days post-injection. Haematoxylin and eosin stained sections were examined for histopathological changes (including tissue necrosis, gliosis, and inflammation) and apoptotic cell numbers were counted under high power.

Glial fibrillary acidic protein (GFAP) and ionized calcium binding adapter molecule 1 (Iba1) immunohistochemistry.

Tissue obtained 7 days following intrathecal injection was incubated with primary antibodies against astrocyte (1:1500 mouse anti-GFAP; Chemicon, Temecula, CA) and microglial (1:1000 rabbit anti-Iba-1; WAKO, Richmond, VA) markers for 48 hours at 4C. Flourescent secondary antibodies 1:250 Alexa 488 goat anti-mouse and 1:250 Alexa 594 goat anti-rabbit (Molecular Probes, Eugene, OR) were applied for 1 hour at room temperature. Slides were coverslipped with ProLong antifade reagent with DAPI (Molecular Probes, Eugene, OR). Spinal cord sections were imaged using standardized settings on a microscope (Olympus BX51 microscope with appropriate wavelength fluorescence illuminator; Olympus America Inc, Center Valley, PA) equipped with a digital camera and image-capture software. The mean intensity of immunofluorescence within a fixed area region of interest in the dorsal horn was quantified for each section (Image Pro Plus software, Media Cybernatics Inc, Silver Spring, MD).

Statistical Analysis

For evaluation of mechanical withdrawal threshold in pups, the number of withdrawal responses was plotted against the mechanical stimulus (force expressed as grams on $\log_{10}$ scale). A sigmoidal stimulus-response curve with non-variable slope was constructed using non-linear regression curve fit. The mid point of the curve (50% effective force; EF_{50}) was determined and designated the threshold (24,46). In adults, mechanical withdrawal thresholds and thermal latencies were the mean of 3 values for each hindpaw. The degree of reversal of inflammatory hyperalgesia for each animal was calculated from the mechanical withdrawal thresholds at different time points: [(30 minutes post-clonidine - inflamed) / (baseline – inflamed) x 100].

Treatment groups were compared with one way analysis of variance (ANOVA) followed by Bonferroni's *post*-hoc test for multiple comparisons or Dunnett's multiple comparison, or Student's t-test if comparison was limited to 2 groups. P35 sensory threshold values were tested for normality (D'Agostino and Pearson normality test) and also assessed by two-way ANOVA with treatment (saline or different clonidine doses) and sex (male or female) as variables. Data was analyzed using Prism Version 5.0 (GraphPad, San Diego, CA). $P<0.05$ was considered statistically significant.

Results

Intrathecal clonidine produces dose-dependent analgesic effects at all postnatal ages

Effects of clonidine on mechanical withdrawal threshold.

The maximum tolerated intrathecal clonidine dose was 10mg/kg at P3 and 3mg/kg at P7. These doses produced mild behavioral effects initially, that did not preclude sensory testing at 30 minutes, and body weight was maintained at 24 hours and 7 days. In P21 pups, 3mg/kg clonidine produced more marked behavioral effects (reduced movement but initial excitatory responses on handling), but minimal weight loss at 24 hours (56.7±1.1g at baseline vs 54.7±1.8g), and this dose was used for acute evaluations. However, as body weight was lower than saline controls by 3 days post-injection, the lower dose of 1mg/kg was designated the maximum tolerated dose in P21 pups for evaluation at 7 days post injection and at P35.

Mechanical withdrawal thresholds increase with postnatal age, from a mean of 1.3g at P3, 1.6g at P7 to 11.9g at P21 (Table 2). Intrathecal clonidine produces dose-dependent anti-nociceptive effects at all postnatal ages. Thirty minutes following intrathecal injection, mechanical withdrawal thresholds did not differ from baseline in the saline control groups, but were significantly increased by 1mg/kg clonidine at all postnatal ages ($P<0.05$ or $P<0.01$; one way ANOVA with Dunnett's comparison to baseline; Fig. 1). Systemic administration of the highest clonidine dose also increased mechanical withdrawal threshold compared with baseline, but effects were significantly less than when the same dose was administered intrathecally at P3 (10mg/kg s.c. vs IT = 1.3±0.08g vs 3.0±0.14g; $P<0.01$), at P7 (3mg/kg s.c. vs IT = 2.8±0.19g vs

3.8 ± 0.25g; P<0.05), or at P21 (3mg/kg s.c. vs IT = 30±3g vs 74±6g; P<0.01 one way ANOVA with Bonferroni post hoc comparisons).

Inflammatory hyperalgesia.

Three hours following hindpaw injection of carrageenan, mechanical withdrawal thresholds in the inflamed paw were significantly decreased at all postnatal ages (Fig. 2A; P<0.001 paired two-way Student's t-test). The minimum dose of intrathecal clonidine that produced a significant reversal of inflammatory hyperalgesia was 0.03mg/kg at P3 and 0.1mg/kg at P7 (P<0.01), and 0.3mg/kg at P21 (P<0.05, one way ANOVA with Dunnett's comparison to saline; Fig. 2B). Consistent with previous data following epidural dexmedetomidine (24), anti-hyperalgesic effects of intrathecal clonidine were seen at lower doses than anti-nociceptive effects at all ages. In addition, age-dependent analgesic effects were evident following inflammation, with lower dose requirements in the younger pups.

Intrathecal clonidine does not produce persistent changes in sensory thresholds

Following intrathecal injection of clonidine 10mg/kg at P3, 3mg/kg at P7 or 1mg/kg at P21, mechanical withdrawal thresholds (Table 2) and body weight (Table 3) did not differ from saline control groups 24 hours or 7 days following injection. In additional animals, more persistent effects on spinal reflex sensitivity were assessed at P35. There were no significant differences in mechanical withdrawal threshold or thermal withdrawal latency following intrathecal injection of clonidine or saline at P3, P7 or P21 (Table 2; n.s. one way ANOVA with Bonferroni multiple *post-hoc* comparisons). There was no main effect of treatment ($F_{3,36}$ = 0.43, P=0.73) or sex ($F_{1,36}$ = 0.71, P=0.55) on mechanical withdrawal threshold at P35. Similarly, there was no main effect of treatment ($F_{3,36}$ = 0.23, P=0.87) or sex ($F_{1,36}$ = 0.43, P=0.43) on thermal withdrawal latency at P35. Treatment accounted for 5.3% and 4%, and sex for <0.1% and 1.6% of total variance in mechanical threshold and thermal latency respectively (two-way ANOVA with treatment and gender as variables).

Maximal tolerated doses of intrathecal clonidine do not increase apoptosis or produce histopathological changes in rat pups

The number of Fluoro-Jade C positive neurons 24 hours following saline injection decreased with age (P4: 11.1±1.02; P8: 6.5±1.2; P22: 2.5±0.5; P<0.01 one way ANOVA linear trend) and the

majority were found in the dorsal horn (Fig. 3A,B). Intrathecal clonidine had no effect on the number or distribution of Fluoro-Jade C positive cells at any age. By contrast, intrathecal ketamine 10mg/kg at P3 significantly increased the number of Fluoro-Jade C positive cells in the lumbar spinal cord, with the greatest increase in the dorsal horn (Fig. 3A).

Similarly, maximum tolerated doses of intrathecal clonidine at P3 or P7 did not increase apoptotic cell counts as identified by activated caspase-3 immunohistochemistry (Fig. 4). Significant increases were seen in the P3 ketamine 10mg/kg positive control group.

Spinal cord sections stained with haematoxylin and eosin were evaluated for histopathological changes after intrathecal injection. The age related decrease in apoptosis is again demonstrated in data from animals 24 hours post injection (P4, P8, P22) and 7 days post injection (P10, P14, P28). Within each age group, the number of apoptotic profiles following any dose of intrathecal clonidine did not differ from the saline control group (Fig. 5). None of the spinal cords showed histopathological changes such as necrosis, inflammation, or microglial nodules.

Intrathecal clonidine does not increase glial reactivity in the neonatal spinal cord

Glial reactivity 7 days following intrathecal injection was assessed by immunohistochemistry for microglial (Iba1; Fig 6A,B) and astrocytic (GFAP; Fig.6C) markers. The mean density of Iba-1 or GFAP immunofluorescence in the dorsal horn was not altered by maximum doses of intrathecal clonidine at P3, P7 or P21. However, significant increases were seen in the P3 ketamine group (Fig. 6).

Postnatal age and therapeutic ratio of intrathecal clonidine

Based on the above results, the therapeutic ratio calculated as the quotient of the maximum tolerated dose and the minimum dose that significantly increased mechanical threshold in the uninjured paw was calculated as >10 at P3 (10/1) and >3 at P7 and P21 (3/1). Using the anti-hyperalgesic dose as the measure of analgesic activity, the therapeutic ratio at P3 was >300 (10/0.03), at P7 was >30 (3/0.1), and at P21 was >10 for acute apoptosis (3/0.3) and >3 for persistent sensory effects (1/0.3). As the maximum dose was limited by systemic side-effects and there were no signs of local spinal toxicity, the ratio for spinal effects is designated as greater than the given ratio rather than an absolute value.

Discussion

These are the first systematic studies evaluating the potential spinal toxicity of intrathecal clonidine in a neonatal model. Intrathecal clonidine dose-dependently reverses inflammatory hyperalgesia at all postnatal ages, and higher doses increase sensory thresholds in uninjured paws (i.e. have anti-nociceptive effects). Single maximum tolerated doses/concentrations of intrathecal clonidine did not produce signs of local tissue toxicity at any postnatal age as measured by several criteria. Firstly, intrathecal clonidine did not increase apoptotic cell counts identified by activated caspase-3 immunohistochemistry or histological examination, or alter the number or distribution of Fluoro-Jade C positive neurons 24 hours following injection. Intrathecal clonidine did not produce histopathological changes in the spinal cord or alter markers of microglial or astrocytic reactivity. Finally, intrathecal clonidine had no persistent effects on spinal cord reflex sensitivity to mechanical or thermal stimuli at P35. The absence of effects of clonidine were similar to those following morphine (44), but in contrast to the effects following intrathecal ketamine in P3 animals as reported previously (43) and confirmed in parallel sections in the current study.

Clonidine is an alpha-adrenergic agonist with an approximate 160-fold selectivity for the $alpha_2$ vs $alpha_1$ receptor (49). Early preclinical work pointed to the potent spinal action of $alpha_2$ agonists such as clonidine in regulating the processing of nociceptive information following both tissue and nerve injury in adult animals (1,2,50). Spinal $alpha_2$ agonists have age- and dose-dependent analgesic effects throughout postnatal development (23-25). The current study confirms reversal of hyperalgesia by intrathecal clonidine, with lower dose requirements in the youngest pups. Higher intrathecal doses are anti-nociceptive, and although maximum tolerated intrathecal doses produce additional systemic side-effects, analgesic effects are still more marked than when the same dose is administered systemically.

Safety evaluation of spinal clonidine has been undertaken in adult dogs after repeated bolus (32) or extended continuous epidural (31) and intrathecal (51) delivery. No evidence of spinal pathology was observed. Similarly, intrathecal administration for 14 days did not result in toxicity in the adult rat (29,30). These findings are based on histological evaluation performed by

a neuropathologist, and similar methodology here has also failed to demonstrate histopathology in the cord of neonatal and young rats following single dose intrathecal clonidine. Of particular interest, clonidine has been reported to a have anti-inflammatory actions (52-55), and co-administration reduces the local inflammatory effects evoked by chronic low dose intrathecal morphine in the canine model (51). Alpha$_2$ agonists may also have specific advantages in combination therapy in early development. Systemic dexmedetomidine reduced general anesthetic-related apoptosis in P7 rats, although efficacy varied in different brain regions (56,57), and effects in the spinal cord have not been evaluated. The effect of combinations of different intrathecal analgesics and local anesthetics throughout postnatal development warrant future evaluation.

Given the concern regarding general anesthesia in neonates and increased interest in neuraxial anesthesia (58), we have emphasized the need to develop a safety database to define the potential toxicity of agents for spinal delivery at different developmental ages (59). The present work with clonidine, and previous work with morphine (44), ketamine (43), and bupivacaine (60) represent the only systematic preclinical spinal safety data in postnatal rats. Several points should be emphasized. Assessment of developmental safety must take cognizance of the fact that there are age-dependent changes in connectivity in the developing spinal cord (35,61), and treatments that alter afferent traffic, such as NMDA antagonists (43) and general anesthetics (60,62) can increase apoptosis in the spinal cord, and persistently modify spinal cord structure and function (43,63,64). Accordingly, as in our previous studies (43,44), we specifically examined treatment effects at P3, P7 and P21; a developmental time span that extends from the critical neonatal period through to childhood in humans. Consistent with our current and previous reports, spontaneous apoptosis in the spinal cord of postnatal rats occurs largely in the dorsal horn from P2-P5, decreasing by P8-10 (65,66). The brief anesthesia used for our intrathecal injection protocol has no additional impact, as the number of apoptotic cells did not differ between naïve and intrathecal saline animals (44). Here, maximum tolerated doses of clonidine did not alter the number or distribution of apoptotic cell profiles as assessed by histological examination, activated-caspase 3 immunohistochemistry or Fluoro-Jade C staining. In

addition, there was no evidence of a delayed injury response as there was no histopathological change or increase in glial reactivity 7 days following injection. Following general anesthetic exposures that increase cortical and hippocampal apotosis in rodents, changes in higher cognitive functions such as learning and memory have been reported at intervals from 4 weeks to 8 months (67). We evaluated sensory thresholds at P35, when both sensory (61) and motor (68,69) components of reflex function are fully mature, and descending modulation from the rostroventral medulla has reached adult patterns by P25 (70). Whereas intrathecal ketamine at P3 increased apoptosis and altered mechanical withdrawal thresholds and gait (43), single maximal doses of intrathecal clonidine did not alter reflex sensitivity at P35.

Clonidine has an established role as a spinal adjuvant analgesic in pediatric practice, and clonidine via the intrathecal (71) or caudal/epidural (72) route has a greater effect than the same dose intravenously. Epidural clonidine 0.08-0.12 μg/kg/hr produces dose-dependent analgesia when added to local anesthetic infusions (17), and higher doses of clonidine alone (0.2μg/kg/hr preceded by bolus of 2μg/kg) provide analgesia at rest following abdominal surgery (73). Clonidine 1-2μg/kg prolongs analgesia when added to caudal local anesthetic, with cardiovascular and sedative side-effects at doses of 5μg/kg in children (15), but sensitivity to side-effects (apnoea, oxygen desaturation, and bradycardia) is greater in neonates (18-20). Similarly, when added to *intrathecal* local anesthetic, clonidine prolonged analgesia in neonates (21), but an increased proportion were sedated and developed self-limiting apnea postoperatively (22). Caudal dexmedetomidine has analgesic properties (74) similar to clonidine (75) in infants and children, but there has been limited preclinical evaluation of toxicity (59,76).

The merits of different spinal analgesics have been discussed in several clinical reviews, but concerns about the relative safety of different drugs and preparations have also been expressed (15,77-79). Current practice differs across centers and countries as the availability of preparations varies and there is limited clinical data relating to comparative efficacy or long-term outcomes (16,80). Within study comparisons of caudal clonidine and ketamine have variably reported more prolonged analgesia with ketamine (81,82) or no difference (83). Addition of clonidine to ketamine further prolonged analgesia in one study (84), but provided no benefit in

another (85). Between 2002 and 2009, the proportion of pediatric anesthetists adding clonidine to caudal local anesthetic in the UK increased, and use of caudal ketamine remained relatively stable (13,14). However, decreased use of ketamine in Europe (86) has been attributed to guidelines reporting laboratory toxicity with preservative-free ketamine in adult animals (87). Our previous study (43) and the direct comparisons utilized here, suggest that ketamine has specific developmental effects that reduce the safety margin for spinal administration in early development.

One of the conundrums facing the interpretation of preclinical safety data is the translation of dosing from the animal model to the human condition. We have suggested that one useful strategy is to compare the therapeutic ratio (i.e. the minimum spinal dose at which toxicity is observed divided by the maximally therapeutic dose), as this would provide a conservative estimate for comparison of different agents. The definition of the therapeutic dose clearly depends upon the end point selected. In previous work, we evaluated anti-nociceptive effects of morphine to a mechanical stimulus (44). As ketamine has minimal effects on baseline thresholds, we evaluated anti-hyperalgesic effects following hindpaw inflammation (43). Here we evaluated both anti-nociceptive and anti-hyperalgesic effects of clonidine, and as shown previously in rat pups following epidural morphine (88,89) or epidural dexmedetomidine (24), lower doses of intrathecal clonidine were required to reverse inflammatory hyperalgesia than to produce anti-nociceptive effects. Analgesic dose requirements for morphine, ketamine and clonidine were age-dependent and lowest at P3. As analgesic requirements vary with the type of drug and with age (e.g. carrageenan-induced hyperalgesia was reversed by clonidine 30mcg/kg and ketamine 3mg/kg at P3, and by clonidine 300mcg/kg and ketamine 15mg/kg at P21), equi-analgesic effects rather than absolute doses are used to calculate the relative therapeutic ratio. Using anti-hyperalgesic doses, therapeutic ratios for intrathecal clonidine were >300 at P3, >30 at P7, and >10 at P21. Similarly, maximal doses of morphine did not produce toxicity, and the therapeutic ratio was high (44). By contrast, intrathecal ketamine increased apoptosis in the same dose range as analgesia, and the calculated therapeutic ratio did not exceed 1 (43). This approach has potential weaknesses. From the perspective of the spinal agent, the principal variable defining

local tissue effects are the local concentration of the drug in the spinal canal and the duration of that exposure (26). As repeated dosing via intrathecal catheters is not practical in small pups, single doses were given and the maximum testable dose of clonidine, morphine and ketamine was limited by systemic side effects. While systemic side effects are of concern, our principal focus for toxicity is the local effect upon spinal tissues. In the event that the maximum tolerable dose after intrathecal delivery is not associated with toxicity, we can only conclude that the therapeutic ratio is at least, or greater than, a certain value. This value will be low if systemic side-effects preclude assessment of higher doses/concentrations. Conversely, if local spinal toxicity occurs at a dose lower than the therapeutic analgesic effect, one can assert that the therapeutic ratio is 1 or less. We would argue that despite these limitations on achieving absolute values, this methodology allows us to compare the relative safety margin of different drugs at given stages of development. A relatively wide safety margin has been demonstrated with morphine (44) and now with clonidine. The comparisons with ketamine presented here and previously (43), confirm that spinal clonidine has a wider safety margin than spinal ketamine in the neonatal rat. These data provide additional information to inform the clinical choice of spinal analgesic in early life.

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Figure Legends

Figure 1. Dose-dependent anti-nociceptive effects of intrathecal clonidine. Mechanical withdrawal thresholds at baseline and 30 minutes following intrathecal (I.T.) injection of saline or clonidine (CL) 1, 3 or 10mg/kg in postnatal day (P) 3, 7 or 21 rat pups are shown. Data points = mean±SEM, n=6-8 per treatment group, *P<0.05 ***P<0.001 one way ANOVA with Dunnett's comparison to baseline.

Figure 2. Anti-hyperalgesic effects of clonidine. A, Mechanical withdrawal thresholds are significantly reduced from baseline 3 hours following hindpaw carrageenan injection at postnatal day (P) 3, 7, and 21. Bars = mean±SEM, ***P<0.001 Student's two-tailed paired t-test. B, The degree of reversal of carrageenan-induced hindpaw inflammatory hyperalgesia [30mins post dose – inflamed/baseline – inflamed) x100] is shown following intrathecal (I.T.) saline (0) or increasing doses of intrathecal clonidine at postnatal (P) day 3, 7 and 21. Data=mean±SEM, n=5-7 per group, *P<0.05 **P<0.01 vs saline, one way ANOVA with Dunnett's comparison to saline.

Figure 3. Fluoro-Jade C positive cell counts 24 hours following intrathecal injection at postnatal day 3 (A), 7 or 21 (B). The number of cells in the whole section (total) and the proportion distributed within the dorsal horn (dorsal horn) are averaged from at least 4 non-consecutive sections per animal. I.T.=intrathecal; P=postnatal day; CL=clonidine 1, 3 or 10mg/kg; ket 10 = ketamine 10mg/kg. Bars = mean±SEM, n= 4 animals per group. **P<0.01 ket 10 vs all groups in total section; $^{\#\#}$P<0.01 ket 10 vs all groups in dorsal horn; one way ANOVA with Bonferroni post-hoc comparisons.

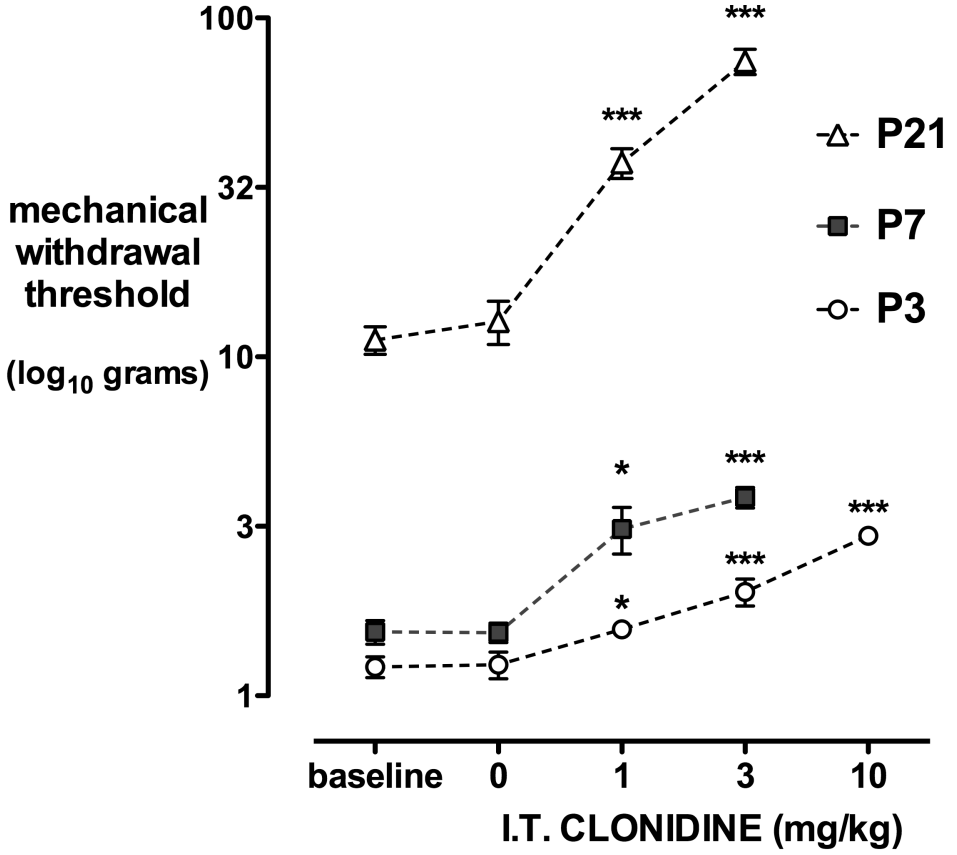
Figure 4. A, Example of lumbar spinal cord section following intrathecal ketamine at P3 with activated-caspase 3 immunopositive cells stained brown and highlighted with arrows. B, Activated-caspase 3 immunopositive cell counts 24 hours following intrathecal injection at postnatal day 3 or 7. Numbers are averaged from at least 4 non-consecutive sections per animal.

I.T=intrathecal; P=postnatal day; CL=clonidine 1, 3 or 10mg/kg; ket=ketamine 10mg/kg. Bars = mean±SEM, n= 4 animals per group. **P<0.01 ket 10 vs all other P3 groups; one way ANOVA with Bonferroni post-hoc comparisons.

Figure 5. Apoptotic cell counts from haematoxylin and eosin stained sections. Numbers are averaged from at least 5 sections per animal 24 hours or 7 days following intrathecal injection on postnatal day 3, 7 and 21. Counts following intrathecal saline or any dose of intrathecal clonidine did not differ at any time point (n.s. one way ANOVA).

Figure 6. A, Representative spinal cord sections demonstrating Iba1 immunofluorescent staining 7 days following injection of i) intrathecal clonidine 10mg/kg or ii) intrathecal ketamine 10mg/kg. B, Quantification of Iba1 immunofluoresence in the dorsal horn 7 days following intrathecal (I.T.) saline, clondine 1, 3 or 10mg/kg (CL 1, 3, 10) or ketamine 10mg/kg (ket 10) at postnatal day (P)3, 7 or 21. C, Quantification of GFAP immunofluoresence in the dorsal horn in the same experimental groups as above. Bars=mean±SEM, *n*=3-4 animals per group. *P<0.05 ketamine 10mg/kg vs saline or clonidine 10mg/kg at P3; one way ANOVA with Bonferroni post-hoc comparisons.

FIGURE 1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(log10 grams)
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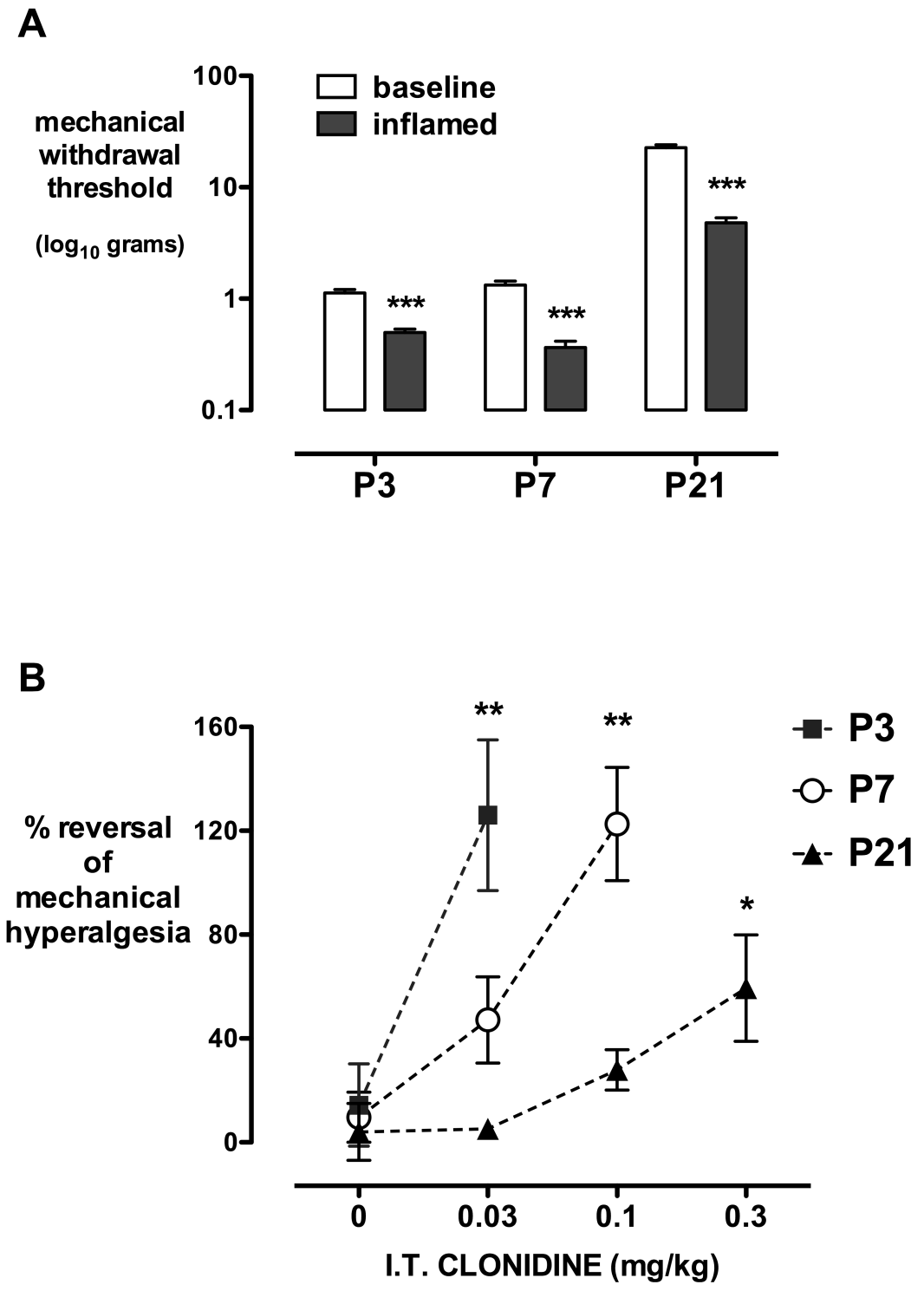
FIGURE 2


A
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80
40
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% reversal of mechanical hyperalgesia
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**
*
P3
P7
P21
0
0.03
0.1
0.3
I.T. CLONIDINE (mg/kg)

FIGURE 3

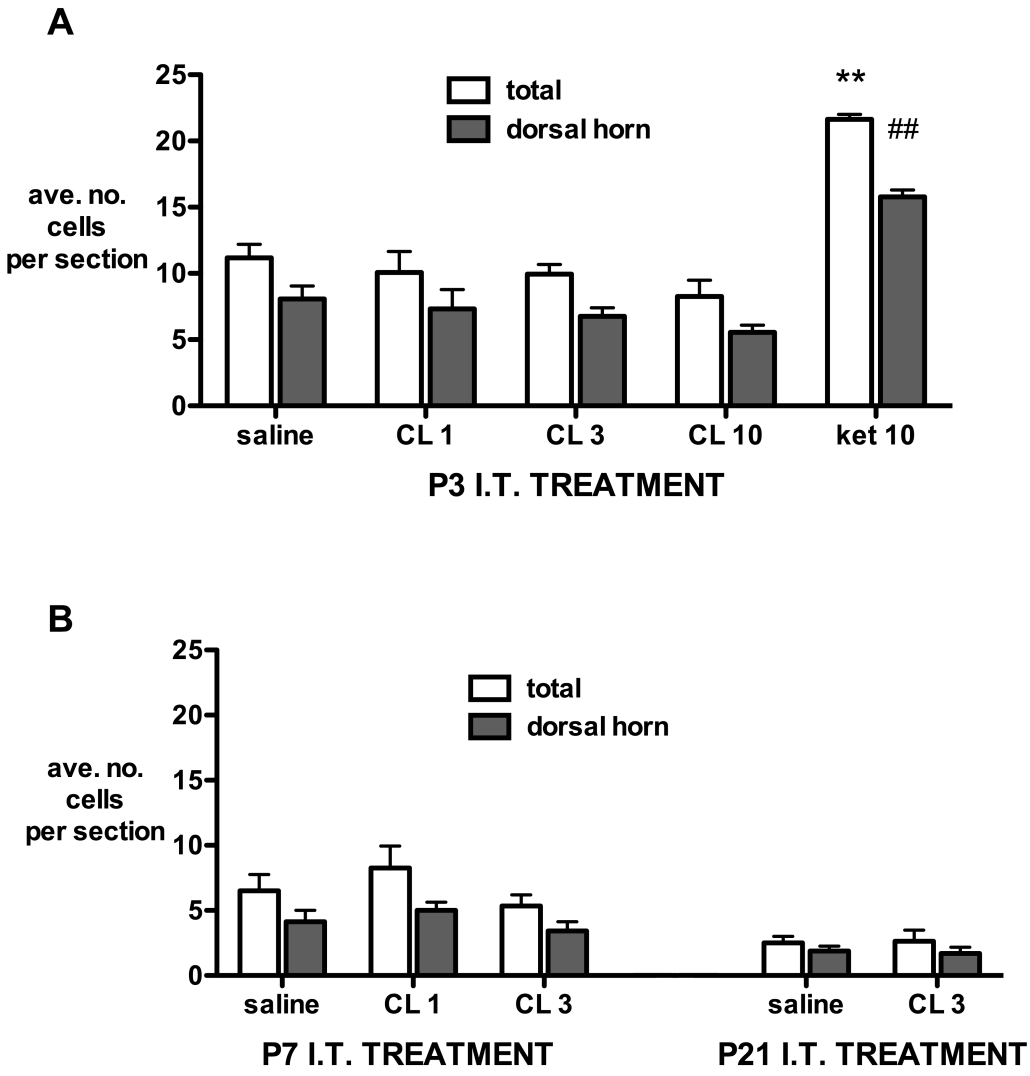

A
25
20
15
10
5
0
ave. no. cells per section
total
dorsal horn
**
##
saline
CL 1
CL 3
CL 10
ket 10
P3 I.T. TREATMENT
B
25
20
15
10
5
0
ave. no. cells per section
total
dorsal horn
saline
CL 1
CL 3
saline
CL 3
P7 I.T. TREATMENT
P21 I.T. TREATMENT

FIGURE 4

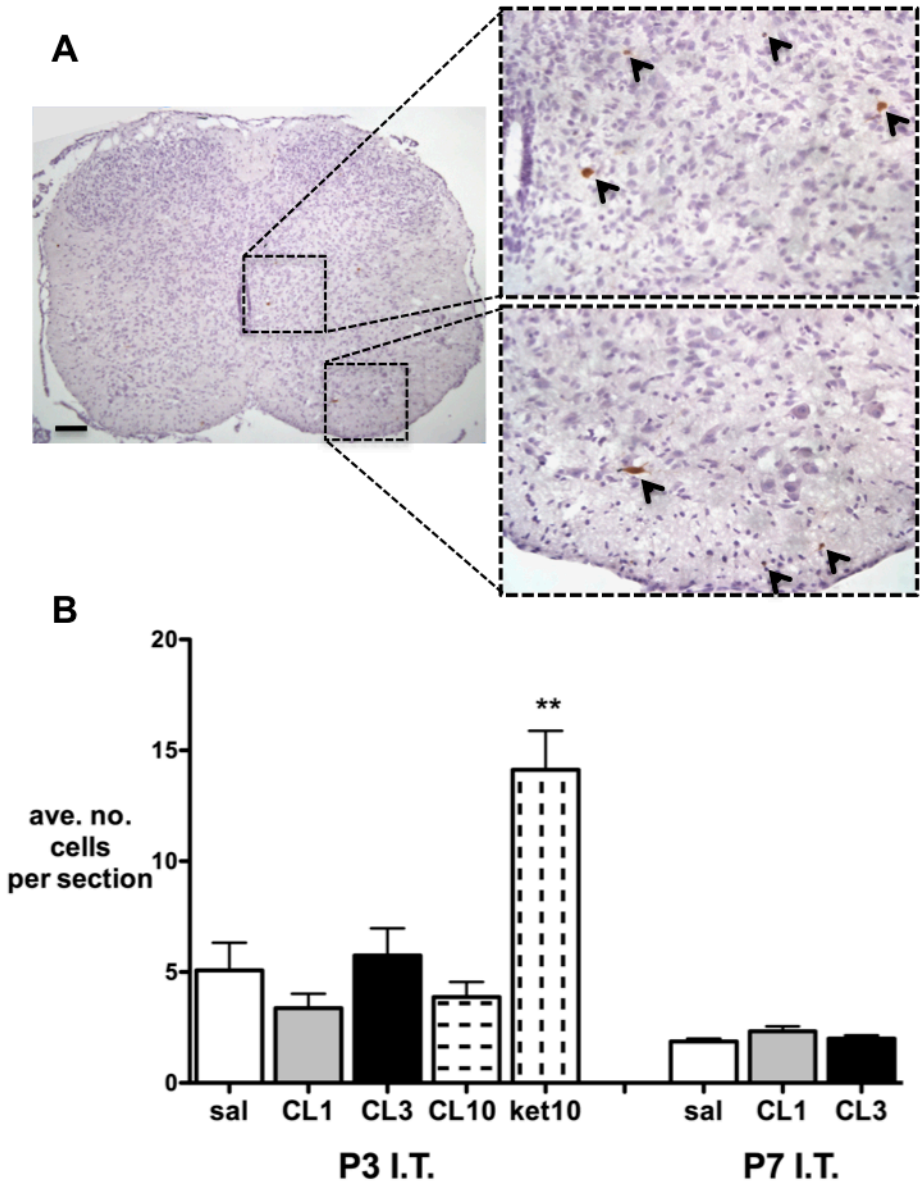

A
B
ave. no. cells per section
20
15
10
5
0
**
sal
CL1
CL3
CL10
ket10
sal
CL1
CL3
P3 I.T.
P7 I.T.

FIGURE 5

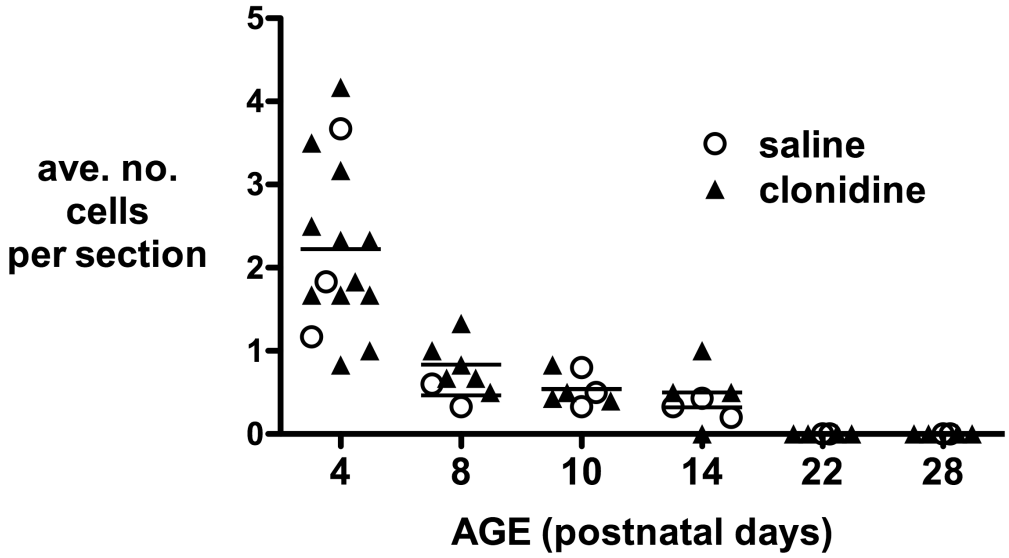
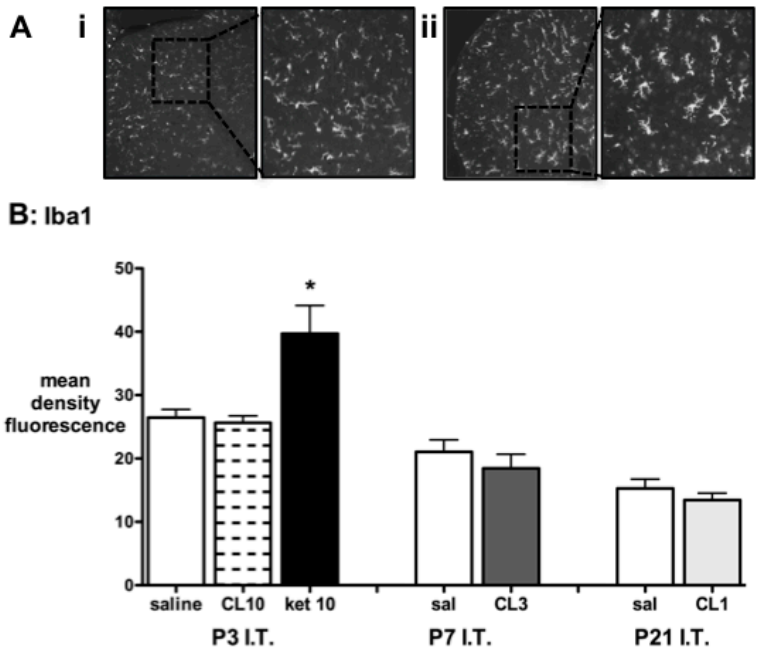

ave. no.
cells
per section
5
4
3
2
1
0
saline
clonidine
4
8
10
14
22
28
AGE (postnatal days)

FIGURE 6


A
i
ii
B: Iba1
mean density fluorescence
50
40
30
20
10
0
*
saline
CL10
ket 10
sal
CL3
sal
CL1
P3 I.T.
P7 I.T.
P21 I.T.
C: GFAP
mean density fluorescence
150
100
50
0
*
sal
CL10
ket 10
sal
CL3
sal
CL1
P3 I.T.
P7 I.T.
P21 I.T.