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Validation of a Preclinical Spinal Safety Model

Effects of Intrathecal Morphine in the Neonatal Rat

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ABSTRACT

Background: Preclinical studies demonstrate increased neuroapoptosis after general anesthesia in early life. Neuraxial techniques may minimize potential risks, but there has been no systematic evaluation of spinal analgesic safety in developmental models. We aimed to validate a preclinical model for evaluating dose-dependent efficacy, spinal cord toxicity, and long-term function after intrathecal morphine in the neonatal rat.

Methods: Lumbar intrathecal injections were performed in anesthetized rats aged postnatal day (P) 3, 10, and 21. The relationship between injectate volume and segmental spread was assessed postmortem and by *in vivo* imaging. To determine the antinociceptive dose, mechanical withdrawal thresholds were measured at baseline and 30 min after intrathecal morphine. To evaluate toxicity, doses up to the maximum tolerated were administered, and spinal cord histopathology, apoptosis, and glial response were evaluated 1 and 7 days after P3 or P21 injection. Sensory thresholds and gait analysis were evaluated at P35.

Results: Intrathecal injection can be reliably performed at all postnatal ages and injectate volume influences segmental spread. Intrathecal morphine produced spinally mediated analgesia at all ages with lower dose requirements in younger pups. High-dose intrathecal morphine did not produce signs of spinal cord toxicity or alter long-term function.

Conclusions: The therapeutic ratio for intrathecal morphine (toxic dose/antinociceptive dose) was at least 300 at P3 and at least 20 at P21 (latter doses limited by side effects). These data provide relative efficacy and safety for comparison with other analgesic preparations and contribute supporting evidence for the validity of this preclinical neonatal safety model.

What We Already Know about This Topic

- ❖ There is a concern regarding potential toxicity of general anesthetics in newborns and infants
- ❖ Whether spinally administered drugs carry increased risk at early ages has not been studied

What This Article Tells Us That Is New

- ❖ In rats, the therapeutic to toxic ratio of spinal morphine was 300 when given 3 days after birth, and at least 20 when given 3 weeks after birth
- ❖ Assessing safety of spinal drugs in rat pups is possible, and morphine is not more toxic in newborn than in adolescent rats

◆ This article is accompanied by an Editorial View. Please see: Drasner K: Anesthetic effects on the developing nervous system: If you're not concerned, you haven't been paying attention. ANESTHESIOLOGY 2010; 113:10–2.

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BECAUSE of the plasticity of the developing nervous system, the efficacy and toxicity of analgesics and anesthetics may differ in early life. This has been emphasized by recent laboratory reports of developmental neuroapoptosis and long-term functional deficits after general anesthesia in the neonatal period (see recent reviews[1,2]). Increased utilization of neuraxial techniques has been suggested as a means to reduce these potential risks.[3] Selective spinally mediated analgesia has been demonstrated in developmental models of intrathecal and epidural administration, but there are significant age-related alterations in dose requirements and susceptibility to side effects.[4–9] Indeed, prolonged general anesthesia in postnatal day 7 (P7) rats has been reported to increase apoptosis in the spinal cord,[10] and the Food and Drug Administration Anesthetic and Life Support Drugs Advisory Committee has stated that "the potential for anesthetic agent-induced neurodegeneration at the level of the spinal cord should be evaluated, particularly with respect to the local anesthetics and opioids administered neuraxially."# Importantly, there has been no systematic evaluation of spinal drug toxicity and safety in early life.

Spinal opioids are administered for perioperative pain management in children *via* intrathecal or epidural routes, by bolus or infusion.[11] In surveys of pediatric anesthetists in the United Kingdom, 34% added opioid to caudal anesthetic blocks[12] and 85% to epidural analgesia, but the agent used and minimum age for using epidural opioids varied in different centers.[13] Neonatal spinal anesthesia is increasing in many centers,[14] but clinical utility is limited by the duration of action of spinal local anesthetics.[15,16] Addition of spinal analgesics such as opioids,[17–19] clonidine,[20,21] and neostigmine[22] has been used in infants to extend the duration of anesthesia or to enhance postoperative analgesia. Although the virtues of spinally administered analgesics and local anesthetics to control pain during and after surgery are evident, performance of regional anesthesia in healthy children must require demonstration of a high therapeutic ratio.[23] Concerns regarding potential toxicity of spinal analgesics continue to be raised in reviews and editorials,[24–28] and although major neurologic complications are apparently rare,[29,30] it has been suggested that a single case may be sufficient to change clinical practice, bring the technique into disrepute, and thus deny many children the benefits of regional analgesia.[31] Further, the "off label" use of agents for spinal delivery that occurs in the context of a clinical trial must require an informed consent that expresses the potential risks and safety of the neuraxial technique. In the absence of preclinical data, what can be said about the safety of any agent in the neonate? Such concerns have indeed resulted in changes in the policy that several journals**[32] have for accepting trials based on off-label neuraxial use. Further specific data comparing the efficacy and relative safety of spinal analgesics in preclinical trials are essential to inform the clinical choice between currently available and potential future spinal analgesics. Despite this imperative, there are no reports of models validated for the assessment of neuraxial drug safety in early development.

To develop such a model, we first confirmed the reliability and distribution characteristics of an intrathecal delivery protocol in rat pups at three developmental ages (P3, P10, and P21). Commencing with an investigation of intrathecal morphine, we evaluated analgesic efficacy in these age groups and then examined drug-exposed spinal tissue for histopathologic signs of neuronal injury. We specifically investigated the effect of intrathecal morphine at P3 or P21 on acute neuronal apoptosis in the spinal cord and evaluated long-term functional outcome by sensory hindlimb thresholds and gait analysis in adulthood. Our overall aim was to calculate a therapeutic ratio (toxic dose/analgesic dose) for intrathecal morphine at different postnatal ages, thus providing a basis for comparison with future analgesic studies.

Materials and Methods

All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of University of California, San Diego, La Jolla, California. Pregnant Holtzman Sprague–Dawley rats (Harlan, Indianapolis, IN) were housed in accordance with the National Institutes of Health guidelines on a 12-h light–dark cycle with free access to food and water. Male and female rat pups at P3, 10, and 21, with approximate mean body weights of 10, 30, and 65 g, respectively, were randomly assigned to treatment groups. Handling and separation from the litter were kept to a minimum. For prolonged experiments, pups were weaned into same-sex cages at P22.

Intrathecal Injection and Spread of Injectate

Pups were anesthetized with isoflurane (3–5%) in oxygen and air. Percutaneous intrathecal injections were made at the low lumbar level (intervertebral space L4–L5 or L5–L6) with a 30-gauge needle perpendicular to the skin. Injectate volumes of 0.5 or 1.0 μl/g body weight were delivered using a hand-driven microinjector (P3 and P10) or a 50-μl Hamilton syringe (P21). As previously described in adult rats, intrathecal placement was suggested by a lateral tail flick as the needle entered the subarachnoid space[33] and confirmed by the distribution of 5% methylene blue in the injectate (Fisher Scientific, Fair Lawn, NJ). Within 2 h of injection, animals were given 100 mg/kg pentobarbital intraperitoneally, exsanguinated by cardiac puncture, and the spinal cord was dissected. Intrathecal injections were defined by staining that was limited to the spinal cord and cerebrospinal fluid (CSF) without pooling of dye in the epidural space or within paravertebral tissues. In most cases, the injection site through the dura could be visualized, but there was no obvious damage to underlying structures. The spread of dye was assessed by

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# Food and Drug Administration Center for Drug Evaluation and Research: Anesthetic and Life Support Drugs Advisory Committee Meeting Minutes March 29, 2007. Available at: www.fda.gov/ohrms/dockets/ac/07/minutes/2007–4285m1-Final.pdf. Accessed October 10, 2009.

** ANESTHESIOLOGY Complete Instructions for Authors. Available at: http://journals.lww.com/anesthesiology/Pages/completeinstructionsforauthors.aspx. Accessed December 8, 2009.

Table 1. Postnatal Age and Dose of Intrathecal Morphine

Intrathecal Injectate		Average Total Dose per Animal		
Dose (per kg Body Weight)	Injectate Concentration	P3 (Mean Body Weight 10 g)	P10 (Mean Body Weight 30 g)	P21 (Mean Body Weight 65 g)
1 μg/kg	2 μg/ml	0.01 μg	0.03 μg	0.065 μg
3 μg/kg	6 μg/ml	0.03 μg	0.09 μg	0.195 μg
10 μg/kg	20 μg/ml	0.1 μg	0.3 μg	0.65 μg
30 μg/kg	60 μg/ml	0.3 μg	0.9 μg	1.95 μg
150 μg/kg	300 μg/ml			9.75 μg
0.3 mg/kg	0.6 mg/ml	3 μg		19.5 μg
3 mg/kg	6 mg/ml	30 μg		195 μg
10 mg/kg	20 mg/ml	100 μg		650 μg
30 mg/kg	60 mg/ml	300 μg		1.95 mg

Summary of intrathecal morphine administration by dose/kg based on mean body weight, injectate concentration, and average total dose per animal for three age groups.

P = postnatal age.

microscopic visualization and expressed as the number of vertebral segments above the injection level.

To assess intrathecal injectate distribution *in vivo*, P3 and P10 rats received intrathecal injections of 50% SAIVI™ Alexa Fluor® 680 in bovine serum albumin (Invitrogen, Eugene, OR) diluted in sterile saline. Injected volumes were 0.5, 1.0, or 1.5 μl/g body weight. Rats were anesthetized with isoflurane (1–2% in air/oxygen), and body temperature was maintained with a thermostatically controlled heat pad. Images were obtained at 30-min intervals for 2 h by the Xenogen®IVIS 100, *in vivo* imaging system (Caliper Life Sciences, Hopkinton, MA).

Evaluation of Antinociceptive Effect of Intrathecal Morphine

To determine the antinociceptive dose of morphine, rat pups at P3, P10, or P21 were randomly assigned to treatment groups, and mechanical withdrawal thresholds were measured before and 30 min after intrathecal injections of saline or a range of doses of morphine (1–30 μg/kg at P3 and P10; 1–150 μg/kg at P21; see tables 1 and 2). For behavioral testing, pups were lightly restrained on a flat bench surface and calibrated von Frey hairs that deliver increasing mechanical stimuli (0.4–60 g) were applied to the dorsal surface of the hind paw five times at 1-s intervals. The number of evoked flexion withdrawals was recorded, and the maximum force applied was that which evoked five withdrawal responses. Pups (n = 5–12 per group) were then anesthetized and intrathecal injections of 0.5 μl/g body weight of saline or morphine sulfate (Merck, Rahway, NJ; powder reconstituted in sterile saline immediately before use; 2–300 μg/ml; see tables 1 and 2) were performed as described earlier.

In separate experiments, pups were given intrathecal morphine (30 μg/kg for P3 and P10; 150 μg/kg for P21; n = 5 each group), and 30 min later withdrawal thresholds were determined before and after intraperitoneal administration of 1 mg/kg naloxone. To compare the effect of systemic and

Table 2. Postnatal Age and Minimum Antinociceptive Effect of Intrathecal Morphine

Intrathecal Drug	P3			P10			P21		
	Baseline	30 min	n	Baseline	30 min	n	Baseline	30 min	n
Saline	1.9 ± 0.1	1.6 ± 0.2	9	3.8 ± 0.3	3.4 ± 0.4	9	8.3 ± 0.4	7.9 ± 0.5	11
Morphine 1 μg/kg	1.5 ± 0.1	1.6 ± 0.2	5	3.0 ± 2.9	2.9 ± 0.5	7	9.4 ± 0.3	9.6 ± 0.4	5
Morphine 3 μg/kg	1.7 ± 0.2	2.5 ± 0.6	4	4.3 ± 0.8	6.5 ± 0.7*	5	10.6 ± 1.0	12.1 ± 1.3	6
Morphine 10 μg/kg	1.5 ± 0.2	3.1 ± 0.4†	7	3.6 ± 0.7	7.1 ± 1.0‡	6	9.3 ± 0.6	10.7 ± 0.9	9
Morphine 30 μg/kg	1.4 ± 0.1	3.9 ± 1.9‡	5	3.2 ± 0.4	9.3 ± 0.8‡	10	9.3 ± 1.2	12.6 ± 1.5*	5
Morphine 150 μg/kg							8.4 ± 0.4	17.3 ± 2.1‡	12

Mechanical withdrawal threshold in grams (mean ± SEM) in groups of P3, P10, or P21 rat pups at baseline and 30 min after intrathecal injection of saline or morphine.

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ in comparison with saline, two-way repeated measures analysis of variance with Bonferroni *post hoc* comparison with saline.

P = postnatal day.

Table 3. Effects of High-dose Intrathecal Morphine in P3 and P21 Rat Pups

Intrathecal Injectate	P3 Intrathecal Injection							P21 Intrathecal Injection						
	Baseline	+30 min	n	+24 h	n	+7 d	n	Baseline	+30 min	n	+24 h	n	+7 d	n
Saline	1.1 ± 0.2 g	1.1 ± 0.1 g	7	1.2 ± 0.1	5	2.1 ± 0.6	4	7.4 ± 0.3 g	6.5 ± 0.4 g	5	7.0 ± 1.0	2	10.5 ± 2.1	3
Morphine 0.3 mg/kg	1.2 ± 0.1 g	7.4 ± 0.9 g*	7	1.1 ± 0.2	5	2.2 ± 0.4	4	7.0 ± 0.8 g	33.2 ± 10.9 g*	5	6.2 ± 1.3	2	9.3 ± 1.4	3
Morphine 3 mg/kg	1.2 ± 0.1 g	19.7 ± 2.0 g*	7	0.9 ± 0.1	5	2.3 ± 0.4	3	6.9 ± 0.6 g	47.6 ± 7.9 g†	6	4.7 ± 0.9	2	6.7 ± 0.9	4
Morphine 10 mg/kg	1.2 ± 0.1 g	23.1 ± 2.9 g†	5	0.9 ± 0.1	5				excitation	4				
Morphine 30 mg/kg		lethal							lethal	4				

Mechanical withdrawal threshold in grams (mean ±SEM) in groups of P3, P10, or P21 rat pups at baseline, 30 min, 24 h, or 7 days after intrathecal injection of saline or morphine.

* $P < 0.01$, † $P < 0.001$ two-way repeated measures analysis of variance with time and treatment as variables and Bonferroni *post hoc* comparison with saline.

P = postnatal day.

spinal administration, changes in mechanical threshold after interscapular subcutaneous injection of morphine (30 μg/kg for P3 and P10; 150 μg/kg for P21; n = 4–5) were also determined. All intrathecal solutions contained 5% methylene blue, and data were only included from animals in which intrathecal placement was confirmed postmortem. Control experiments indicated that methylene blue had no effect on baseline thresholds or behavior.

Evaluation of Toxicity of High-dose Morphine

In separate groups of animals, we evaluated spinal cord toxicity after incremental doses up to the maximum tolerable dose. In initial experiments, escalating log doses were administered intrathecally (0.3, 3, and 10 mg/kg) until the lethal dose was determined (30 mg/kg at both P3 and P21; see table 3). Dose-limiting side effects occurred at 10 mg/kg in P3 pups (sedation and respiratory depression) and more than 3 mg/kg in P21 pups (excitatory effects). As a result, P3 and P21 animals received intrathecal injections of saline, 0.3 or 3 mg/kg morphine, and an additional group of P3 animals received 10 mg/kg (n = 8–10 all groups). Animals received supplementary oxygen (100%; 2 l/min in recovery box) and were observed for side effects, and mechanical withdrawal thresholds were measured at baseline and 30 min after injection. Dye was not included in these injections and intrathecal placement was indicated by a tail flick on insertion and confirmed by a significant increase in mechanical withdrawal threshold 30 min after injection. Pups were returned to the litter for subsequent daily inspection without handling. On postinjection day 1 or 7, mechanical thresholds were determined and then animals were terminally anesthetized (100 mg/kg intraperitoneal pentobarbital) for histologic evaluation of the spinal cords.

Spinal Cord Preparation and Staining

After terminal anesthesia with intraperitoneal 100 mg/kg pentobarbital, animals were transcardially perfused with saline followed by 1 ml/g body weight of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). After laminectomy, spinal cords were carefully dissected and removed with the dura, dorsal root ganglions, and proximal nerve roots intact to minimize any physical trauma to the cord. The distance from the injection site caudally to the end of the dissected cord, and proximally to the lumbar enlargement, was noted. The tissue was postfixed in 4% paraformaldehyde for 6 h, transferred to 20% sucrose in 0.1 M PBS for 18–36 h, and then stored at 4°C in 30% sucrose in 0.1 M PBS. Three-millimeter-long transverse sections of the spinal cords, caudal to the lumbar enlargement and just rostral to the level of injections were cut, placed in optimum cutting temperature (OCT) Compound (Sakura Fintek, Torrance, CA) and frozen on dry ice. Using a cryostat (Leica CM 1800, San Marcos, CA), 7 and 14-μm sections were cut and mounted on Fisher Superfrost Plus (Fisher Scientific, Houston, TX) slides and stored at −70°C. Nerve root histology was not specifically evaluated in these studies that focused on the site of action of morphine in the spinal cord, but we consider them an essential addition to the protocol when evaluating local anesthetic toxicity.

Hematoxylin and Eosin. Seven-micron sections from all experimental groups at 1 and 7 days postinjection were stained with hematoxylin (Gill's II) for 30 s followed by 2 min in eosin Y (American MasterTech, Lodi, CA). Slides were then gradually dehydrated in increasing concentrations of ethanol followed by Citrisolve and coversliped (Permount; Fisher SP15, Fair Lawn, NJ). Coded sections were evaluated for histopathologic changes (in particular morphologic signs of apoptosis as well as neuronophagia, microglial nodules, demyelination, or gliosis) by a neuropathologist (M.G.) who was unaware of treatment group. The number of degenerating neurons per section was counted at 400 times magnification. Counts from at least four nonconsecutive sections of lumbosacral cord from each animal were averaged for statistical analysis.

Activated Caspase-3. Spinal cord sections from P3 animals were stained for activated caspase-3, which is the final member of an intracellular cascade activated during programmed cell death. After rinsing in distilled water and then in Tris-buffered saline (which was used as the wash buffer between steps throughout), slides were incubated in 3% peroxidase in methanol for 10 min. Slides were placed in upright staining holders (Thermo Shandon System, Waltham, MA),blocked with 0.3% Triton X-100 and 5% normal goat serum in Tris-buffered saline for 1 h at room temperature, then incubated overnight at 4°C with rabbit monoclonal antiactivated caspase, 3 (Cell Signaling, Beverly, MA)1:100 in blocking solution. Biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA) was applied at 1:250 for 30 min at room temperature, followed by avidin-biotin-peroxidase complex (ABC reagent; Vector Laboratories) for 30 min. Staining was developed with 3,3′-diaminobenzidine (DAB) (Vector Laboratories) for 8 min, and then slides were counterstained with hematoxylin, dehydrated, and cover-slipped (Permount; Fisher SP15). 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 by two investigators unaware of treatment group. Counts from at least four nonconsecutive sections of lumbosacral cord from each animal were averaged for statistical analysis.

Fluoro-Jade C. Fluoro-Jade C staining, a sensitive marker of neuronal degeneration, was performed as described previously[34] on tissue collected 24 h after injection. Briefly, 14-μm spinal cord sections were rinsed in distilled water and then immersed in 1% sodium hydroxide in 80% ethanol for 5 min. After 2-min rinses in 70% ethanol and then in water, slides were incubated for 10 min in 0.06% potassium permanganate solution followed by 10 min in 0.0002% solution of Fluoro-Jade C (Chemicon, Temecula, CA) and 0.01% of 4′6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) dissolved in 0.1% acetic acid vehicle. Slides were rinsed in distilled water and air dried in a dark incubator for 30 min at 37°C before cover-slipping with dibutyl phthalate polystyrene exylene nonfluorescent mounting medium. Slides were coded, examined under the appropriate wavelength fluorescent microscopy, and two investigators unaware of treatment group counted the number and location of immunofluorescent cells. Counts from at least four nonconsecutive sections of lumbosacral cord from each animal were averaged for statistical analysis.

Glial Fibrillary Acidic Protein and Ionized Calcium-binding Adapter Molecule-1. Tissue obtained 7 days after intrathecal injection was examined with antibodies against astrocyte and microglial markers (glial fibrillary acidic protein and ionized calcium-binding adapter molecule-1 [Iba-1], respectively). After rehydration in 0.1 M PBS, slides were rinsed in 0.1% Triton X-100 in PBS (which was used for rinses between steps throughout) and were then incubated in 5% goat blocking serum at room temperature for 1 h. Slides were incubated overnight at 4°C with primary antibodies, 1:500 mouse anti-glial fibrillary acidic protein (Chemicon) and 1:1000 rabbit anti-Iba-1 (WAKO, Richmond, VA), diluted in 1% bovine serum albumin and 0.1% Triton X-100 in PBS. In negative control slides, primary antibodies were replaced with control immunoglobulin G in a similar dilution, and lack of staining was confirmed. After 1 h at room temperature with secondary antibodies, 1:250 Alexa Fluor 488 goat anti-mouse and 1:250 Alexa Fluor 594 goat anti-rabbit (Molecular Probes), slides were rinsed and cover slipped with Prolong Gold antifade mounting medium with 4′6-diamidino-2-phenylindole. From Iba-1 immunolabeled spinal cord sections, the central region, left and right dorsal horn, and left and right ventral horns were imaged using the same 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 (Image Pro Plus software; Media Cybernatics Inc, Silver Spring, MD). Using Image-J,†† image-coded sections were analyzed after colorsplit using the green channel only, then were manually given an individual threshold for background subtraction and analyzed for area fraction (area of positively stained cells as a percentage of total area = 1,280 × 1,024 pixels). Photographs from four nonconsecutive sections of lumbosacral cord from each animal were used and measurements from central, dorsal, and ventral regions were averaged for statistical analysis.

Control Groups

To provide a positive control for glial activation and neuronal injury, a single-level low-thoracic laminectomy was performed in anesthetized P3 pups, and 0.4 μl of 20 nM *N*-methyl-D-aspartate (NMDA) was injected intraspinally using a Hamilton syringe and 30-gauge needle.[35] Spinal cords were harvested on postinjection day 1 (for activated caspase-3 and Fluoro-Jade C counts) or 3 (for glial fibrillary acidic protein and Iba-1 immunohistochemistry). Paraffin-embedded neonatal rat brain tissues, which were exposed to a hypoxic–ischemic insult,[36] were also used as a positive control to confirm activated caspase-3 staining. Animals injected with intrathecal saline and naïve age-matched animals were used as negative controls.

Long-term Functional Outcome

Long-term functional effects of intrathecal morphine were examined by measuring sensory thresholds and by analyzing gait at P35 in separate groups of animals that received intrathecal injections on P3 or P21. Male and female P3 rat pups were equally divided into treatment groups: intrathecal saline (n = 11); morphine 0.3 mg/kg (n = 8); or morphine 3 mg/kg (30-fold antinociceptive dose; n = 10). Pups were returned to the dam and then weaned into same-sex cages at P21. Separate P21 pups received intrathecal saline (n = 13) or morphine 4.5 mg/kg (30-fold antinociceptive dose; n = 12) and were maintained in same-sex cages. Correct intrathecal placement was confirmed by a significant increase in mechanical withdrawal threshold 30-min postinjection. An additional age-matched naïve control group had no prior anesthesia or intrathecal injection (n = 4).

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†† Image Processing and Analysis in Java. Available at: http://rsbweb.nih.gov/ij/. Accessed August 31, 2009.

Mechanical withdrawal thresholds were determined by applying calibrated von Frey hairs to the plantar surface of the hind paw and using a modified version of the up–down method as described previously.[37,38] Rats were allowed to acclimatize for at least 30 min in a clear plastic cage with a wire mesh bottom. The 50% paw withdrawal threshold was determined with a series of von Frey filaments (Stoelting, Wood Dale, IL) beginning with a buckling weight of 2.0 g up to a maximum of 15 g. If paw lifting occurred, the next weaker filament was applied, but if application of the filament for 5 s did not elicit a withdrawal response the next stronger filament was used.

Thermal withdrawal latency was determined using a modified Hargreaves Box (University Anesthesia Research and Development Group, University of California, San Diego, La Jolla, CA), consisting of a glass surface (maintained at 30°C) on which the rats were placed in individual clear plastic cubicles.[39] The thermal nociceptive stimulus originates from a focused projection bulb positioned below the glass surface. A timer was activated by the light source, and 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 (cutoff time). Three measures were obtained from each hind paw and latency expressed as mean ± SEM.

Gait analysis was performed as the animal crossed the glass runway of the CatWalk® system (Noldus Information Technology, Wageningen, The Netherlands). At P22–25, rats were placed on one end of the runway and allowed to explore the environment for about 5 min for 3 consecutive days. Animals then commenced a training paradigm, as described previously.[40,41] Briefly, animals were deprived of food for at least 3 h before testing, and then were allowed to spontaneously cross the runway toward food rewards positioned at the farther end. Training continued for 2 weeks, and when the animals reached the age of 5 weeks runway crossings were recorded if they met the following criteria: (1) a maximal time of 2 s for crossing the 60-cm-long part of the runway used for gait recording, and (2) there were no intermediate stops during the crossing. Three crossings per animal were analyzed using the CatWalk® 7.1.6 software.

Statistical Analysis

The effect of injectate volume on segmental spread of intrathecal dye at P3 or P10 was compared with a Mann–Whitney two-tailed test. For each test condition in the neonatal behavioral studies, 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 nonvariable slope was constructed using nonlinear regression curve fit. The mid point of the curve (50% effective force) was determined and designated the threshold as previously described.[6] Mechanical thresholds before and 30 min after intrathecal saline or morphine were analyzed by repeated measures two-way analysis of variance (ANOVA) with time and treatment as variables and Bonferroni *post hoc* tests for comparison with saline. At P35, mechanical withdrawal thresholds were evaluated using the up–down method, and values were calculated as described[38] and represented as mean ± SEM. Thermal withdrawal latency was designated as the mean of three values for each hind paw. Data were normally distributed (D'Agostino and Pearson normality test) and treatment groups were compared with one-way ANOVA followed by Bonferroni *post hoc* tests for multiple comparisons. Prism version 5.0 (GraphPad, San Diego, CA) was used for analysis and $P < 0.05$ was reported as statistically significant.

Results

Intrathecal Injection and Spread of Injectate

After initial experiments to refine the technique, prospective injections of a large number of animals were undertaken (P3, n = 60; P10, n = 64; P10, n = 52), and the success rate of intrathecal injection was 83–85% at all ages. In animals with confirmed intrathecal placement, segmental distribution related to age and injectate volume is plotted in figure 1A. At P3, 1.0 and 0.5 μl/g intrathecal injectate resulted in spread across 14.9 (95% CI 12.7–17.1; n = 24) *versus* 8.3 (95% CI 6.9–9.6; n = 26) segments, respectively ($P < 0.01$; Mann–Whitney); and at P10 across 12.1 (95% CI 9.5–14.7; n = 22) *versus* 7.7 (95% CI 6.3–9.1; n = 31) segments, respectively ($P < 0.01$; Mann–Whitney). In P21 animals, the spread after 0.5 μl/g injectate was less than that in younger animals (mean 5.2; 95% CI 4.2–6.2; n = 44). Because 0.5 μl/g reliably produced spread across lumbar and low-thoracic segments at all ages, this injectate volume was used for subsequent experiments. Greater postmortem segmental spread after 1.0 *versus* 0.5 μl/g of dye in P10 pups is illustrated in figures 1B and C, respectively. The relationship between injectate volume and intrathecal spread was also confirmed *in vivo*. Fluorescent dye 0.5 μl/g produced spread over lumbar and low-thoracic segments in a P10 (fig. 1D) and P3 rat (fig. 1E). A higher injectate volume of 1.5 μl/g produced greater spread (fig. 1F), and fluorescence extended into the cisterna magna and occasionally into the cerebral ventricles in some animals.

Effect of Postnatal Age on Withdrawal Thresholds and Response to Intrathecal Morphine

Baseline mechanical thresholds increased with postnatal age, consistent with the previously reported normal developmental profile.[6,7,42] Before injection, the baseline threshold (mean ± SEM) calculated from the midpoint of the stimulus–response curve (fig. 2) was 1.60 ± 0.04 g at P3 (n = 58; 1.51–1.69; 95% CI), 3.23 ± 0.14 g at P10 (n = 70; 2.96–3.59; 95% CI), and 9.24 ± 0.23 g at P21 (n = 59; 8.79–9.69; 95% CI). To confirm intrathecal injection, methylene blue was included in all injectates assessing the antinociceptive effect of morphine. The dye does not influence threshold values because there were no significant differences between mechanical thresholds at baseline and after injection of saline with methylene blue at any age.

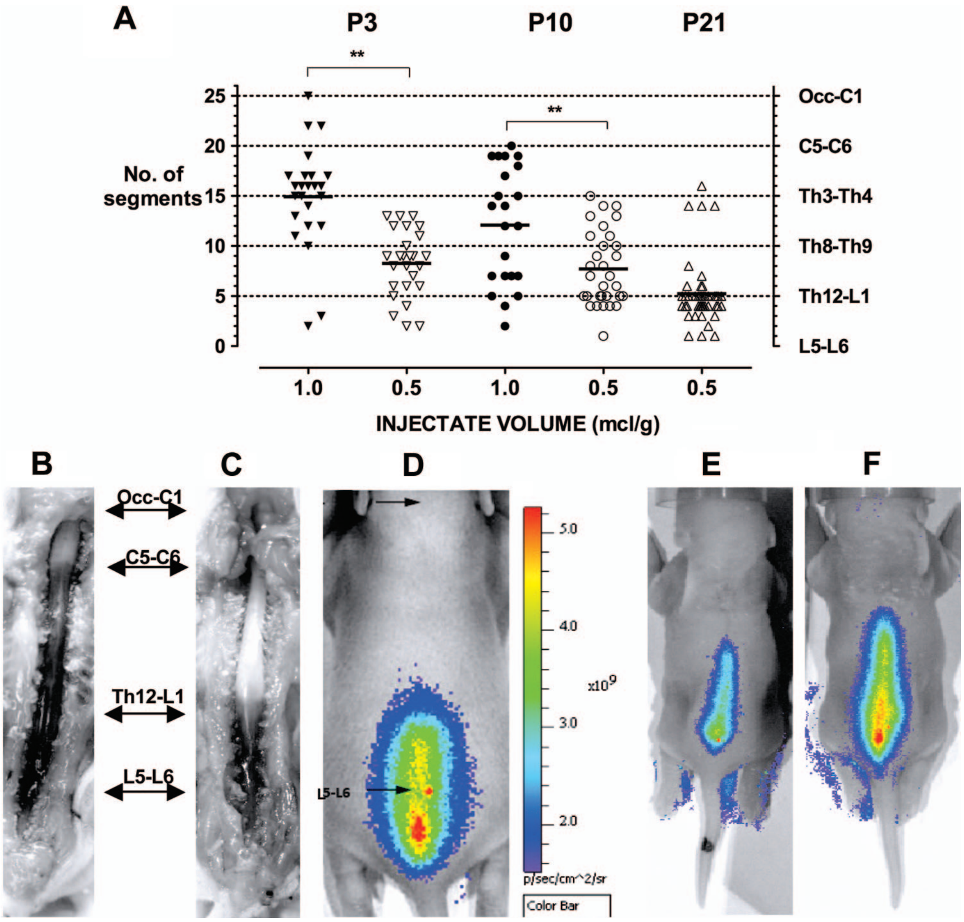

Fig. 1. Intrathecal drug distribution in postnatal day (P)3, 10, and 21 rats is dependent on injectate volume. The scattergram (*A*) shows the number of segments of intrathecal spread above the injection site (L5–L6) for different volumes in P3 (mean body weight ± SEM = 10 ± 1 g; 1.0 μl/g, n = 24; 0.5 μl/g, n = 26), P10 (body weight 25 ± 6 g; 1.0 μl/g, n = 22; 0.5 μl/g, n = 31), and P21 (66 ± 12 g; 0.5 μl/g, n = 44) rats. Each point indicates the result from a single rat. Segmental spread is significantly increased by increasing injectate volume in P3 and P10 rats (** $P < 0.01$ Mann–Whitney two-tailed test 0.5 *vs.* 1.0 μl/g). Representative postmortem images in P10 rats showing spread after injection of 1.0 μl/g (*B*) and 0.5 μl/g (*C*) methylene blue. Using an *in vivo* imaging system (Xenogen®IVIS 100; Caliper Life Sciences, Hopkinton, MA), similar spread is seen 60 min after injection of 0.5 μl/g fluorescent dye (*D*). *In vivo* images in P3 pups 120 min after injection of 0.5 μl/g (*E*) or 1.5 μl/g (*F*) fluorescent dye also demonstrate increased spread with the higher volume. C = cervical; L = lumbar level; Occ = occipital; Th = thoracic.

Intrathecal morphine produces dose-dependent increases in mechanical withdrawal threshold at all postnatal ages. The effect of increasing doses of intrathecal morphine (micrograms per kilogram body weight) on withdrawal thresholds 30 min after injection is shown in table 2. The mechanical withdrawal threshold was significantly increased in comparison with the saline control group by 10–30 μg/kg morphine at P3, 3–30 μg/kg at P10, and by 30–150 μg/kg at P21.

As baseline values for withdrawal threshold increase during postnatal development (fig. 2), it is difficult to compare raw data from different age groups. Therefore, data are also expressed as the percentage change from baseline for each age and treatment group (fig. 3). This allows each animal to act as its own control thus reducing variability and also allows comparison of the relative dose–response across age groups. Sensitivity to morphine is greater in younger pups because 10 μg/kg morphine at P3 and P10 significantly increases the mechanical withdrawal threshold, but a higher dose (150 μg/kg) is required to produce the same effect at P21. Importantly, these data define the minimum intrathecal morphine dose that produces a statistically significant analgesic effect at different ages.

At all ages, effects of intrathecal morphine were naloxone reversible because thresholds returned to baseline when naloxone was administered 30 min after an antinociceptive dose of morphine (30 μg/kg at P3 and P10, 150 μg/kg at P21; not significant *vs.* saline; $P < 0.001$ *vs.* intrathecal morphine alone, one-way ANOVA with Bonferroni *post hoc* comparison; fig. 3). Doses of morphine that significantly increased withdrawal threshold after intrathecal administration had no effect on mechanical thresholds when given systemically (subcutaneous 30 μg/kg at P3 and P10 or 150 μg/kg at P21), suggesting that intrathecal administration is producing spi-

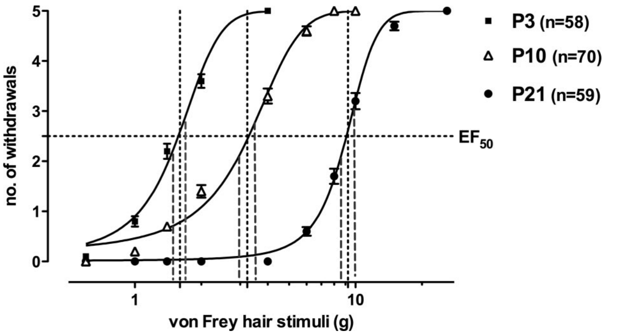

Fig. 2. Mechanical withdrawal thresholds of the hind limb increase with postnatal age. Number of withdrawals (out of a total of 5 stimulus applications) evoked by increasing force (in grams) of mechanical stimuli (von Frey hairs) in postnatal (P) day 3, 10, and 21 animals. Threshold (in grams) is defined as the midpoint (*i.e.*, EF_{50} = effective force 50%) of the nonlinear regression curve and is 1.60 (95% confidence interval [CI], 1.51–1.69) g at P3, 3.23 (95% CI, 2.96–3.59) g at P10, and 9.24 (95% CI, 8.79–9.69) g at P21. Data points = mean ± SEM (n = 58–70); *dashed lines* = 95% CI for EF_{50} values.

nally mediated effects (not significant subcutaneous *vs.* saline, $P < 0.01$ subcutaneous *vs.* same dose intrathecal, one-way ANOVA with Bonferroni *post hoc* comparison; fig. 3).

Response to High-dose Intrathecal Morphine

Separate groups of animals received high doses of intrathecal morphine (milligram per kilogram body weight) at P3 and P21 to evaluate spinal toxicity. Methylene blue was not added to injections for toxicity evaluation, but correct intrathecal placement was confirmed by the marked increases in withdrawal threshold produced by these doses (table 3). In addition, age- and dose-dependent changes in general behavior were seen. Systemic administration of high-dose morphine (up to 50 mg/kg subcutaneously) has been reported to produce a rigid Straub tail only in pups older than P12,[43] but high doses of intrathecal morphine produced this effect in both P3 and P21 rats. Dose escalation was limited by respiratory depression in P3 pups, with 10 mg/kg intrathecal morphine producing visible slowing of the respiratory rate, marked sedation, and cyanosis in the absence of supplemental oxygen. In P21 pups, excitatory effects and convulsions occurred at 10 mg/kg. Intrathecal morphine 30 mg/kg rapidly produced lethal respiratory depression in both age groups. Intrathecal morphine at P3 or P21 had no residual effect on mechanical withdrawal thresholds 24 h after injection (table 3), as values did not differ from age-matched saline control groups ($P = 0.8$, saline *vs.* morphine 0.3, 3, and 10 mg/kg at P3; $P = 0.6$, saline *vs.* morphine 0.3 and 3 mg/kg at P21; one-way ANOVA with Bonferroni *post hoc* comparison). Seven days after P3 injection, mechanical thresholds had increased in all groups to values consistent with the age of P10, but did not differ across saline or intrathecal morphine groups ($P = 0.9$, saline *vs.* morphine 0.3, 3, and 10 mg/kg at P3; $P = 0.56$, saline *vs.* morphine 0.3 and 3 mg/kg at P21; one-way ANOVA with Bonferroni *post hoc* comparison).

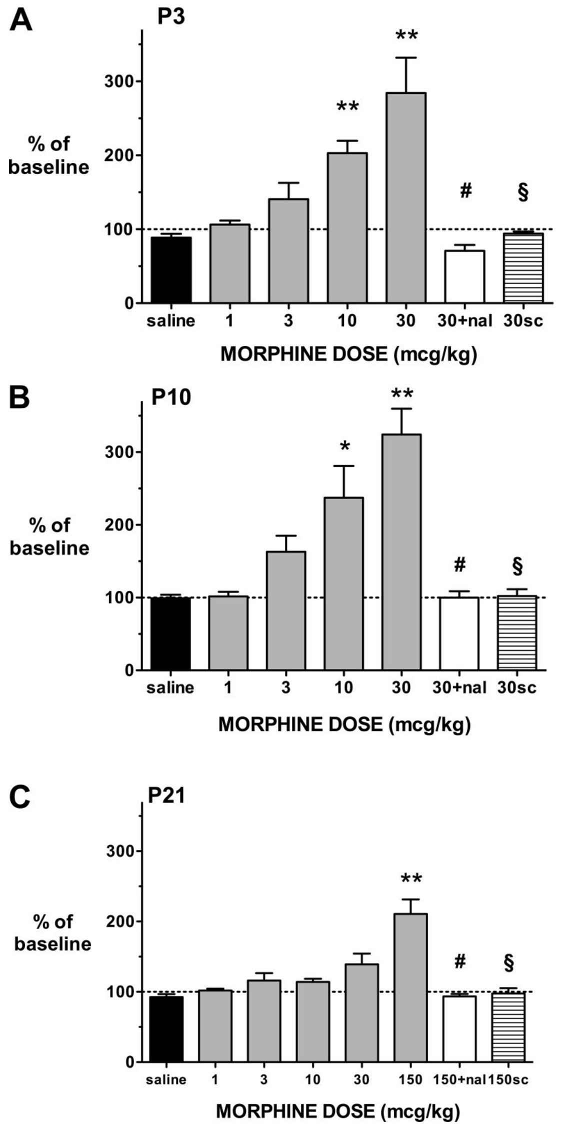

Fig. 3. Intrathecal morphine produces a dose-dependent naloxone-reversible antinociceptive effect at all postnatal ages at doses that have no effect after systemic delivery. Mechanical withdrawal thresholds 30 min after intrathecal injection are represented as a percent change from baseline threshold in postnatal day (P) 3 (*A*), P10 (*B*), and P21 (*C*) rats. Mechanical threshold is significantly increased after doses of 10 and 30 μg/kg intrathecal morphine in P3 and P10 rats, respectively, and 150 μg/kg in P21 rats. Effects of intrathecal morphine are antagonized by coadministration of naloxone (nal), and the maximal dose given subcutaneously (sc) has no effect on mechanical threshold. * $P < 0.05$, ** $P < 0.01$ morphine *vs.* saline; # $P < 0.01$ naloxone plus maximal dose intrathecal morphine *vs.* maximal dose intrathecal morphine; § $P < 0.01$ subcutaneous *vs.* intrathecal administration of maximal dose morphine. *Bars* = mean ± SEM; n = 5–12 per group; one-way analysis of variance with Bonferroni multiple *post hoc* comparison with saline.

Evaluation of Spinal Toxicity after High-dose Intrathecal Morphine

Systematic examination of coded hematoxylin and eosin sections by a neuropathologist (M.G.) found no major his-

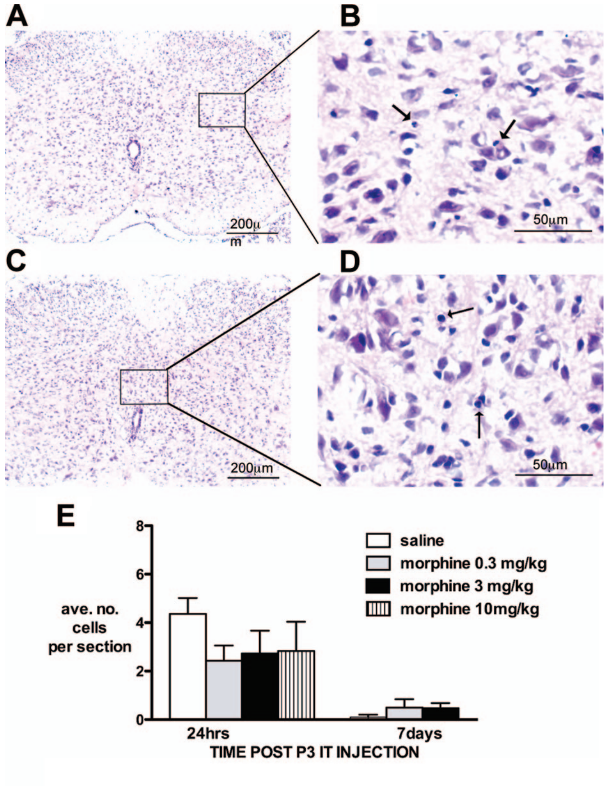

Fig. 4. Intrathecal morphine at postnatal day (P) 3 does not increase the number of degenerating neurons in the spinal cord 24 h or 7 days postinjection. (*A–D*) Representative transverse spinal cord sections stained with hematoxylin and eosin 24 h after morphine 0.3 mg/kg (*A* and *B*) or morphine 10 mg/kg (*C* and *D*). *Arrows* point to degenerating neurons that have a shrunken appearance and condensed nuclei in the high-power expanded images (*B* and *D*). (*E*) Histogram shows the number of degenerating neurons for each treatment group at 24 h and 7 days after intrathecal (IT) injection of saline or morphine in P3 rat pups. The number of cells for each animal was averaged from at least four lumbosacral transverse spinal cord sections. *Bars* = mean ± SEM for n = 4 animals per treatment group.

topathologic effects (gliosis, necrosis, or inflammation) in the spinal cords of saline- or morphine-treated animals either 1 or 7 days after intrathecal injection in P3 and P21 pups. Degenerating neurons, most of which had apoptotic morphology (figs. 4A–D), were evident in saline-treated groups 24 h after P3 injection (mean ± SEM: 4.3 ± 0.7 cells per section), but fewer than one cell per section was seen at P10 (7 days after P3 injection). The number of degenerating neurons was not influenced by any dose of morphine at P3 (fig. 4E). In older animals receiving intrathecal saline or morphine at P21, degenerating neurons were rarely seen (0–2 cells across four sections) and numbers were not influenced by morphine treatment.

Similar numbers of activated caspase-3-positive cells were present 24 h after P3 injection of saline (4.3 ± 0.9 cells per section), with the majority distributed throughout the dorsal horn (fig. 5A). Intrathecal morphine did not increase the number or alter the distribution of caspase-3-positive cells (fig. 5B). In addition, the number of caspase-3-positive cells was similar in age-matched naïve animals (4.3 ± 1.0 cells/section), suggesting that neither the brief anesthesia nor the intrathecal injection is responsible for the baseline level of apoptosis.

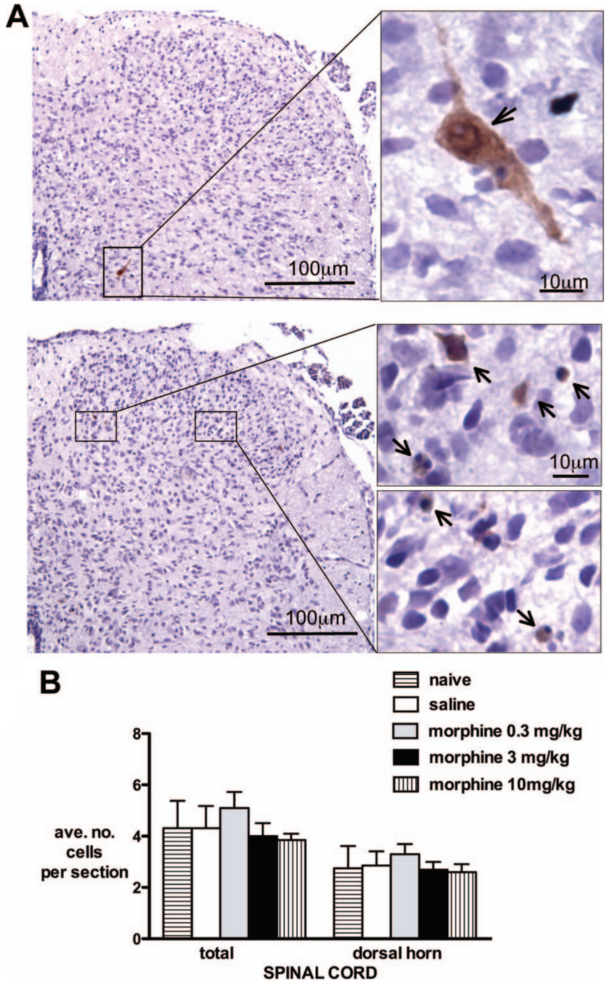

Fig. 5. Intrathecal morphine at postnatal day (P) 3 does not increase the number of apoptotic neurons in the spinal cord. (*A*) Representative transverse spinal cord sections immunostained with activated caspase-3 antibody 24 h after intrathecal injection of morphine 0.3 mg/kg. In high-power insets, caspase-positive cells are identified by *arrows*. (*B*) The number of activated caspase-3 positive cells in the whole spinal cord section (total) and the number located within the dorsal half of the cord (dorsal horn) are shown in naïve P4 pups, and 24 h after intrathecal injection on P3 of saline or morphine. At least four lumbosacral transverse spinal cord sections were analyzed from each animal and the number averaged. *Bars* = mean ± SEM for n = 4 animals per treatment group.

Fluoro-Jade C has higher resolution than its predecessors Fluoro-Jade and Fluoro-Jade B, and because it stains all degenerating neurons, higher numbers were found in comparison with caspase-3 staining. However, the same pattern of distribution was seen (*i.e.*, the majority were located in the dorsal horn), and there were significantly more positive cells in the saline group in the younger animals (10.1 ± 1.8 cells/section at P3 and 3.5 ± 0.58 cells/section at P21; $P < 0.05$

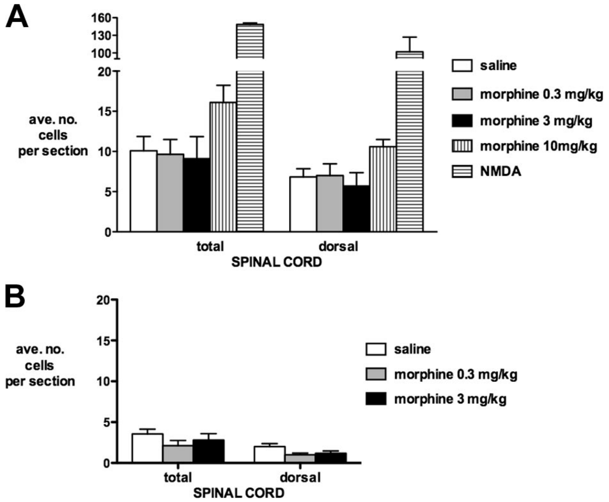

Fig. 6. Intrathecal morphine does not increase Fluoro-Jade C- positive neurons in the spinal cord. The number of degenerating neurons stained with Fluoro-Jade C in the whole spinal cord section (total) and the number located within the dorsal half of the cord (dorsal horn) 24 h after intrathecal injection of saline or morphine on postnatal day (P) 3 (*A*) and P21 (*B*) are shown. Numbers were markedly increased after intraspinal *N*-methyl-D-aspartate (NMDA), which served as a positive control. At least four lumbosacral transverse spinal cord sections were analyzed from each animal and the number averaged. *Bars* = mean ± SEM for n = 4–5 animals per treatment group.

Student unpaired two-tailed *t* test; fig. 6). Although not statistically significant, the number of Fluoro-Jade C cells was increased after the maximal dose of morphine 10 mg/kg at P3 (16.1 ± 2.1 cells/section), which may be secondary to the respiratory depression associated with this dose. Lower doses of morphine had no effect on the number of positive cells after P3 (fig. 6A) or P21 injection (fig. 6B). Intraspinal NMDA served as an effective positive control as it produced marked histologic changes at the site of injection and increased the number of activated caspase-3 and Fluoro-Jade C staining cells (fig. 6A).

Seven days after injection of intrathecal morphine in P3 or P21 rat pups, there was no discernible alteration in spinal cord staining with microglial or astrocytic markers (fig. 7). The area fraction of Iba-1 immunoreactivity did not differ between saline- and morphine-injected animals (fig. 7A), whereas a clear increase was seen after intraspinal NMDA, which acted as a positive control for both altered microglial (figs. 7A and E) and astrocytic staining (fig. 7I).

Functional Analysis at P35

At P35, mechanical withdrawal thresholds and thermal withdrawal latencies were used to evaluate spinal reflex sensitivity. No significant differences in mechanical withdrawal threshold (fig. 8A) or thermal withdrawal latency (fig. 8B) were found related to prior intrathecal treatment or age at the time of injection (not significant, one-way ANOVA with Bonferroni *post hoc* comparison of all groups). Animals were also tested for their locomotor performance and gait on the CatWalk® runway system. Both static and dynamic gait parameters were assessed (table 4). No significant difference in any analyzed parameter was observed when comparing naïve age-matched controls or animals injected at P3 or P21 with saline or morphine (not significant, one-way ANOVA with Bonferroni *post hoc* comparison).

Discussion

Our aim was to develop a model for preclinical safety evaluation of intrathecal analgesics in neonatal rats that encompasses dose-dependent analgesic efficacy, histologic indicators of spinal cord toxicity, and long-term functional outcome. Intrathecal morphine produces spinally mediated analgesia at all postnatal ages, and dose requirements are lower in younger pups. High doses of intrathecal morphine at P3 or P21 did not produce appreciable increases in neuronal injury, apoptosis, or glial response, and there were no long-term changes in hindlimb sensory thresholds or gait. The therapeutic ratio for intrathecal morphine (toxic dose relative to antinociceptive dose) was at least 300 at P3 and at least 20 at P21 (the latter being limited by side effects at higher morphine doses). This work provides a model for evaluating preclinical toxicity in neonatal animals and comparing the relative safety of currently used and future spinal analgesic and anesthetic preparations. These results stand in marked contrast to the effects observed in this model with intrathecal ketamine as reported in the companion article.[44] Issues pertinent to the validity of this model are outlined below.

Injection Technique

Percutaneous intrathecal injection can be performed reliably in rat pups, using methodology similar to that previously described in adult rats[33] and mice (20–25 g).[45] An injectate volume of 0.5 μl/g produced spread to the low- to mid-thoracic segments at P3 and is consistent with previous reports injecting 4 μl *via* a low-thoracic catheter in P3 pups.[8,9] These volumes are relatively larger than have been reported in adults and may relate to developmental changes in CSF volume and kinetics. CSF volume relative to body weight is larger (~9.4 μl/g at P5 *vs.* 4 μl/g at P30), and the rate of CSF formation is significantly slower due to immaturity of choroid plexus secretion in young animals.[46] In additional experiments (not detailed here), we also found that 0.5 μl/g local anesthetic (0.5% bupivacaine) was required to reliably produce motor block of the hindlimbs in P3 pups. This is consistent with the relatively higher volumes of local anesthetic that are clinically used to achieve spinal blockade in neonates and infants because the volume of CSF is twice that in adults (4 ml/kg *vs.* 2 ml/kg) and the proportion of spinal *versus* cerebral CSF is greater (50 *vs.* 25%).[47] By contrast, morphine dose requirements were lower in younger pups because the greater pharmacodynamic sensitivity to this drug is more dependent on total dose. Similarly, in the central

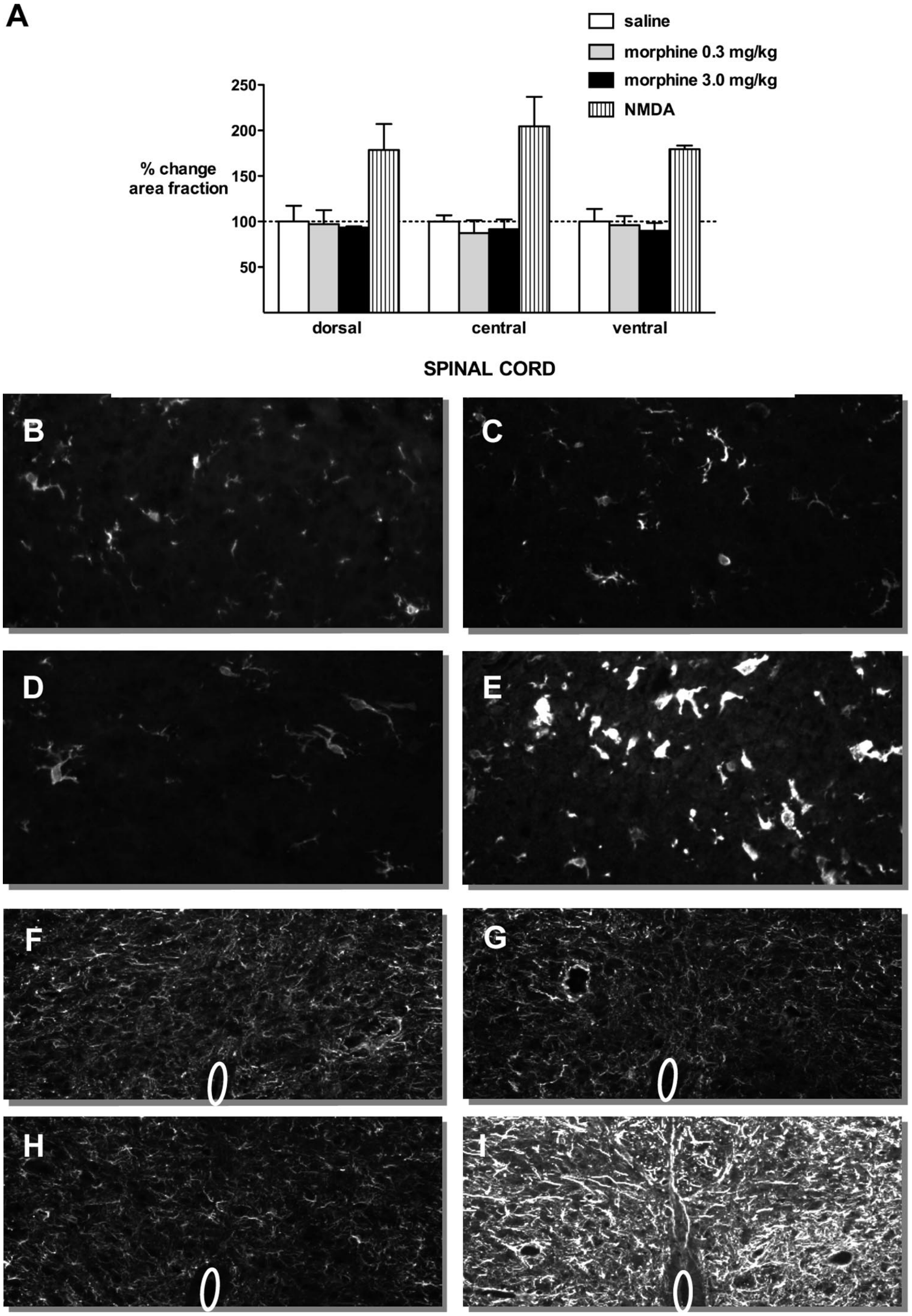

Fig. 7. Intrathecal morphine does not alter microglial or astrocyte marker staining in the spinal cord 7 days after injection. (*A*) Histogram represents area fraction immunoreactivity for ionized calcium binding adapter molecule 1 (Iba1) in transverse spinal cord sections from postnatal day (P) 3 rats. Results are expressed as percentage change from saline injection animals 7 days after injection of intrathecal saline or morphine (0.3 or 3 mg/kg) in images taken from the dorsal horn, adjacent to the central canal, and ventral horn of lumbosacral spinal cord. The area of Iba1 staining was markedly increased 3 days after intraspinal injection of *N*-methyl-D-aspartate (NMDA), which served as a positive control. Representative high-power images of spinal cord sections showing Iba1 immunoreactivity 7 days after intrathecal injection at P3 of morphine 0.3 mg/kg (*B*), morphine 3 mg/kg (*C*), or saline (*D*); and 3 days after intraspinal injection of NMDA (*E*). Representative images from the dorsal half of the spinal cord showing glial fibrillary acidic protein immunoreactivity 7 days after intrathecal injection at P3 of morphine 0.3 mg/kg (*F*), morphine 3 mg/kg (*G*), or saline (*H*); and 3 days after intraspinal injection of NMDA (*I*). The oval symbol overlies the central canal.

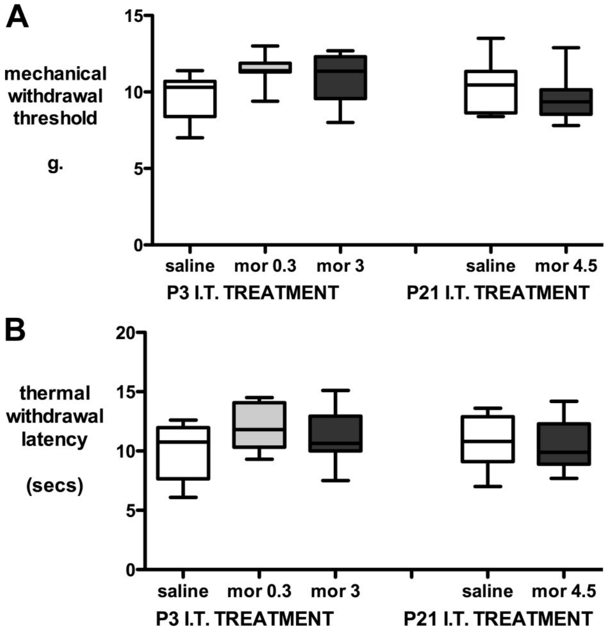

Fig. 8. Behavioral thresholds at P35 after intrathecal injection of saline or morphine on postnatal day (P) 3 or 21. Hindlimb mechanical withdrawal thresholds (*A*) and thermal withdrawal latencies (*B*) are shown in animals that received intrathecal (IT) saline, 0.3 mg/kg morphine (mor 0.3) or 3 mg/kg morphine (mor 3), at P3 (n = 10 all groups), or saline or 4.5 mg/kg morphine (mor 4.5) at P21 (n = 12 both groups). Box and whisker plot with 5–95% confidence intervals; n = 10 per group. Values did not differ significantly across groups; one-way analysis of variance with Bonferroni *post hoc* comparisons.

nervous system, antinociceptive responses occur at much lower brain morphine levels in rat pups.[48]

In pediatric practice, bolus or brief perioperative infusions of spinal analgesics are commonly used for perioperative pain management, and prolonged intrathecal or epidural analgesic infusions are usually limited to children with cancer pain uncontrolled by less invasive measures.[49] Therefore, we used a single percutaneous injection of increasing doses to maximize spinal cord drug exposure. This methodology also allowed us to avoid several confounding factors. Catheter insertion has been described in infant rats but a laminectomy is required,[8] and the catheter must be placed contralateral to the limb being tested, because it produces disruption of the dorsal root ganglion on the side of the catheter and deformation of the underlying cord.[9] In addition, use of a percutaneous technique minimized the duration of anesthesia and maternal separation and also avoided potential disruption of the catheter or surgical wound by the dam.

Actions of Intrathecal Morphine

Intrathecal morphine produced dose-dependent naloxone-reversible (*i.e.*, opiate receptor-mediated) antinociceptive effects at all ages. Effects were spinally mediated as the same dose administered systemically did not increase the mechanical withdrawal threshold. Dose requirements were lower in younger pups, and increases in paw withdrawal latency from a fixed mechanical stimulus have similarly been reported after 10 and 30 μg/kg intrathecal morphine at P4.[8] Variability in reported sensitivity to morphine in early life likely relates to differences in the dose range studied and the test stimulus (mechanical or thermal) used. As seen here, when assessing changes in mechanical withdrawal threshold dose requirements were lower in younger pups after epidural[7] and systemic[42] morphine, but were less apparent when a thermal stimulus was used to assess withdrawal latency. This may relate to postnatal changes in μ-opioid receptor distribution, because an increased proportion of large A-fiber dorsal root ganglion neurons express functional μ-opioid receptors in early life, but there is relatively constant expression on small thermoreceptive C-fibers.[42,50] In addition, the significant changes in the density and distribution of μ-opioid receptors in the spinal cord in the first 3 postnatal weeks[51] would influence intrathecal morphine dose requirements.

Table 4. Gait Parameters in Adult Animals after Intrathecal Treatment at P3 or P21

	Static Parameters		Dynamic Parameters			
Treatment	Print Area	Print Intensity	Regularity Index	Duty Cycle	Stride Length	Stability of Gait
Naïve (n = 4)	36.4 ± 1.8	157.5 ± 4.8	99.5 ± 0.5	56.4 ± 2.6	101.4 ± 4.4	29.6 ± 1.8
P3 saline (n = 11)	40.3 ± 3.5	151.4 ± 4.6	99.8 ± 0.2	54.1 ± 1.4	107.5 ± 2.1	27.6 ± 0.7
P3 morphine 0.3 mg/kg (n = 10)	48.2 ± 4.7	151.3 ± 1.9	99.8 ± 0.2	57.3 ± 1.3	106.2 ± 2.8	28.0 ± 0.8
P3 morphine 3 mg/kg (n = 8)	46.9 ± 3.8	147.9 ± 1.7	99.7 ± 0.3	58.4 ± 1.3	104.2 ± 3.9	30.5 ± 0.8
P21 saline (n = 13)	38.2 ± 3.6	142.0 ± 4.5	99.3 ± 0.4	54.0 ± 1.5	108.4 ± 3.2	28.6 ± 0.7
P21 morphine 4.5 mg/kg (n = 12)	38.6 ± 4.7	145.7 ± 4.3	99.8 ± 0.2	53.1 ± 1.6	110.3 ± 3.0	27.9 ± 1.0

At P35, gait parameters are shown for naïve age-matched control animals and after intrathecal treatment at postnatal day (P) 3 or 21. Duty cycle = ratio between stance duration and full stepcycle duration (stance phase duration/[stance + swing phase duration]); print area = surface area of floor contacted by hindpaw; print intensity = intensity of pixels forming area of paw contact; regularity index = index for degree of interlimb coordination during gait; stability of gait = distance between two hindpaws measured perpendicular to walking direction; stride length = distance between placement of hindpaw and subsequent placement of same paw.

As we wished to maximize spinal drug exposure, we escalated intrathecal morphine doses in sequential groups of P3 or P21 pups until a maximum tolerated dose was reached. Consistent with previous reports, excitatory effects and convulsions limited dose escalation in P21 but not P3 pups. Resistance to seizures and convulsions in P1-P5 pups, but not older rats, has similarly been reported after high-dose systemic morphine (up to 50 mg/kg)[43] and intracerebroventricular morphine.[52]

Intrathecal Dosing: Preclinical versus *Clinical*

In the current studies, the doses are presented as drug mass per kilogram body weight. This is in accord with many clinical studies. In this study, the minimal analgesic dose was 10 μg/kg in P3 rats, which is in the range of reported benefit (5–7 μg/kg) in human infants and children.[17,53–55] However, we would stress that the current thinking regarding intrathecal toxicity is strongly related to local concentration.[56] In these preclinical studies, the analgesic and maximum tolerable concentrations of morphine were 20 μg/ml and 6–20 mg/ml, respectively. In children, epidural infusion concentrations are in the range of 10 μg/ml morphine,[11] and 30 μg/ml morphine has been infused intrathecally after cardiac surgery,[18] but the concentration used for intrathecal and epidural or caudal boluses will vary and often depend on the volume of added local anesthetics.

When evaluating susceptibility to apoptosis in laboratory studies, a major difficulty is relating results to "clinically relevant" doses and outcomes. Studies of general anesthetic apoptosis have related doses producing toxicity in the laboratory to either similar clinical plasma levels[57] or similar functional effects (*e.g.*, ED_{50} for sedation).[58] Here, our aim was not to evaluate "clinical" doses but to maximize spinal drug exposure to assess the potential for toxicity in the minimum number of animals.[59] By expressing the dose associated with toxicity as a ratio of the functional antinociceptive dose, a therapeutic ratio can be estimated, the apparent margin of safety for a given drug can be assessed, and the relative propensity for toxicity of different drugs given by this route can be compared.[59,60] Because of differences in underlying mechanisms, analgesic effects may not run in parallel with all forms of toxicity in all species. However, the degree of neuroapoptosis after general anesthesia varies with dose and age in rodents[58,61,62] and primates,[63] thus emphasizing the need to evaluate a range of doses at different ages in a developmental model of spinal toxicity.

Assessment of Spinal Cord Pathology

In adults, chronic intrathecal infusion of morphine has been used for clinical management of both cancer[64] and noncancer pain.[65] Although pericatheter granulomas have been associated with high-concentration intrathecal morphine infusions in clinical[66] and laboratory studies,[56,67] such outcomes have not been noted with single or repeated bolus delivery of intrathecal or epidural morphine, and morphine did not produce histopathology in the spinal cord.[68–70] In cancer patients at postmortem, tumor-related pathology has been found in the spinal column, but no additional neurologic deficits or neuropathologic changes have been attributed to chronic morphine infusion.[71,72] In the current studies, we assessed histopathologic changes after intrathecal morphine at younger ages, and in accordance with adult studies, found no changes after maximum-tolerated single doses of morphine in P3 and P21 pups.

Of particular relevance to this neonatal model, we also evaluated the effect of morphine on developmentally regulated apoptosis in the spinal cord. In the rodent brain, vulnerability to the proapoptotic action of drugs such as NMDA antagonists is dependent on postnatal age, and both the magnitude and region of peak susceptibility change during the first 3 postnatal weeks, with increased apoptosis only occurring in regions with an appreciable rate of spontaneous or physiologic apoptosis.[73] Studies investigating general anesthetic toxicity in the brain have used rodents between P5 and P10, with the majority at P7 when apoptosis is prevalent in the cortex.[1] Although apoptosis in the spinal cord has been reported after general anesthesia at P7,[10] the period of peak susceptibility in the spinal cord may differ from the cortex. Spontaneous apoptosis in the spinal cord occurs predominantly in the ventral horn prenatally and dorsal horn postnatally,[74,75] with the number of apoptotic cells highest at P0–P2, lower at P4–P8, and negligible at P10[76]. This is consistent with the current findings in the saline control groups, as spontaneous apoptosis was detected at P4 but few degenerating neurons were visible at older ages (P10 or P22). In the accompanying article, we have also confirmed that apoptosis occurs predominantly in the dorsal horn at P3 and has decreased by P7.[44] Importantly, there were no differences in caspase-3 positive cell counts in naïve animals and those receiving intrathecal saline, confirming that effects are not due to the brief general anesthesia or to injection trauma.

Prolonged opioid exposure has been shown to produce apoptosis in adult rodents, and this form of neurotoxicity may contribute to the neurologic impairments associated with opioid abuse.[77] Dose-related increases in apoptotic cells were found in lamina I and II after 7 days of intrathecal morphine,[78] and chronic systemic morphine, but not a single dose, produced apoptosis in the brains of adult mice.[79] Both extrinsic and intrinsic apoptotic pathways were activated, and the precipitating factors and mechanisms relevant to tolerance and withdrawal in the adult are likely to differ from the enhancement of physiologic or spontaneous apoptosis after anesthetic and analgesic exposure in early development. Systemic opioids have not been associated with developmental apoptosis in the rodent brain. A single dose of 10 mg/kg subcutaneous morphine did not increase apoptosis in P3 rats, but when coadministered with caffeine the number of Fluoro-Jade B (but not caspase-3)-positive cells was greater than that after caffeine alone.[80] In pregnant guinea pigs (gestational age G50), 4 h of anesthesia with isoflurane and nitrous oxide produced apoptosis in the brains of offspring, but the same duration of fentanyl infusion did not.[81] In contrast, prolonged *in vitro* exposure to morphine produced apoptosis in hippocampal cell cultures from E16 mouse.[82] Morphine also dose dependently increased apoptosis of neurons and microglia, but not astrocytes, in cell cultures from human fetal brain (16–22 weeks' gestational age), and its effects were reduced by caspase-3 inhibitors and pretreatment with naloxone.[83] However, it is difficult to correlate the relative doses with *in vivo* and *in vitro* exposure, and cultures were treated with morphine for prolonged periods (5–7 days).[82,83] Our results suggest that single intrathecal doses of morphine, even in maximum tolerated doses, do not significantly increase apoptosis in the spinal cord. This was confirmed with multiple methodologies, and consistent results were obtained with histopathologic evaluation, activated caspase-3 immunohistochemistry, and Fluoro-Jade C staining.

Opioids activate glial cells in the central nervous system, releasing proinflammatory mediators, which in turn alter opioid-mediated efficacy and side effects.[84,85] Attenuation of microglial activation by minocycline potentiated morphine-induced analgesia and reduced respiratory depression.[86] The impact of glial activation on the pharmacodynamic response to morphine has not been evaluated in early development and is beyond the scope of the current study. Our primary aim was to determine whether a glial response to neuronal injury could be detected 7 days after intrathecal injection. Although the spinal cord microglial response to peripheral nerve injury varies with postnatal age,[35,87] microglia are capable of responding to direct insults such as intrathecal NMDA as shown here at P3 and previously at P10.[35] In human fetal cell culture, prolonged opioid exposure produced apoptosis in neurons and microglia, but had no effect on astrocytes.[83] A higher proportion of astrocytes express μ receptors in early development, and 20 mg/kg subcutaneous morphine reduced astrocyte prolife ratio in the subventricular zone of the P5 mouse.[88] In this study, we found no change in the expression of microglial or astrocytic markers (Iba-1 and Glial fibrillary acidic protein, respectively) in the spinal cord 7 days after single intrathecal doses of morphine at P3 or P21.

Functional Assessments

The need for appropriate end points when assessing neurodevelopmental outcome after early anesthesia exposure has been highlighted in a recent editorial.[89] As our aim was to evaluate spinal cord toxicity, we used sensory thresholds mediated by spinal systems to evaluate long-term functional outcome. Here, intrathecal morphine at P3 or P21 had no effect on hindpaw mechanical withdrawal threshold or thermal withdrawal latency at P35. In addition, sensorimotor coordination and motor function were assessed by a computerized gait analysis system that has been used previously to assess functional deficits and recovery after peripheral nerve lesions[40,90] and spinal cord injury.[41,91] Single doses of intrathecal morphine at P3 or P21 had no long-term effect on weight bearing (assessed by static CatWalk® parameters) or on coordination and gait stability (assessed by dynamic parameters). Brief general anesthesia at P3 or P21 had no effect on gait, because measures in saline control animals did not differ from age-matched naïve animals.

Clinical Implications

Neuraxial drugs can be delivered into the epidural/caudal or intrathecal space. In this study, we evaluated the effects of intrathecal drug administration to maximize exposure of the target tissue (*i.e.*, spinal cord) to the drug. Although it may be argued that administration *via* the more commonly used epidural/caudal route would diminish the exposure of the cord to high concentrations, we would note the following points. (1) Intrathecal morphine has been used in children after major surgery for many years, administered either as a single bolus[55,92,93] or *via* continuous infusion[94,95]; and intrathecal morphine has also been administered to neonates and infants after cardiac surgery[17,18,96,97] and major craniofacial surgery.[98] (2) The use of spinal anesthesia in neonates and infants is increasing,[14] and the limited duration of action of local anesthesia may be improved by coadministration with spinal opioid.[19] (3) Although there have been no direct comparisons of dose–response *via* the two routes, higher bolus doses (15–50 μg morphine) are administered epidurally or caudally in children,[11] whereas 4–5 μg/kg morphine is effective after intrathecal delivery,[53–55] suggesting that similar target concentrations in the cord are required to achieve analgesia and thus have potential for toxicity regardless of whether initial administration is epidural or intrathecal. (4) Inadvertent dural puncture is a rare but recognized complication of pediatric epidural[29] and caudal techniques[30] and may result in delivery of higher doses of drug into the CSF.

Here, we focused on toxicity at the site of opioid action in the spinal cord. Because local anesthetics have been shown to produce histopathology in nerve roots[99,100] and the spinal cord[101] in adult animals, evaluating the developmental toxicity of local anesthetic preparations will require additional histopathologic examination of the nerve roots. *In vitro*, local anesthetics induce apoptosis in cultured cell lines derived from rat dorsal root ganglia[102] and human neurons[103] but *in vivo* apoptosis at different postnatal ages requires further evaluation.

There is currently insufficient clinical evidence to fully inform the choice between different spinal analgesic agents in children,[13] and our aim is to begin to provide preclinical comparative data regarding the relative safety of different drugs at different postnatal ages. The current data suggest that the therapeutic ratio for morphine is high (at least 300 at P3 and 20 at P21), as single-dose intrathecal morphine in rat pups did not produce acute histopathology in the spinal cord or long-term changes in function. When choosing between two spinal analgesic drugs with similar benefits in terms of clinical analgesic efficacy, it would seem prudent to choose the drug with a higher therapeutic ratio to minimize risk. In the companion article,[44] a comparable analysis has been undertaken with ketamine and the outcome with this intrathecal drug is considerably different. Such data suggest that the model as described can distinguish agents based on their ability to yield neurotoxocity after intrathecal delivery of critical doses and concentrations.

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