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Inflammatory Pain and Hypersensitivity Are Selectively Reversed by Epidural Bupivacaine and Are Developmentally Regulated

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Background: **Low doses of local anesthetics applied to the young rat spinal cord *in vitro* have been shown to inhibit C-fiber–evoked responses. The aim of this work was to investigate whether such low doses applied epidurally selectively reduce nociceptive responses *in vivo* and to investigate the influence of postnatal development on such local anesthetic actions.**

Methods: **Three groups of rat pups aged 3, 10, and 21 days were studied. The threshold of the flexion withdrawal reflex to mechanical stimulation was determined in the hind limb at each age. Inflammatory pain was induced in the right hind limb with 2% carrageenan, causing a reduction in the sensory threshold on that side. The difference in threshold between the two sides represents inflammatory hypersensitivity. The effect of low-dose epidural bupivacaine on sensory thresholds and thus the induced hypersensitivity was also determined for each age group.**

Results: **Inflammatory hypersensitivity was selectively attenuated by very low doses of bupivacaine (concentration range. 0.004–0.0625%), which did not affect the sensory threshold in the contralateral uninflamed limb. This effect was also age-related, with younger rats being more sensitive than older rats.**

Conclusions: **The effects of epidural bupivacaine in the infant rat are developmentally regulated. Lower doses have a selective analgesic effect that decreases with increasing postnatal age.**

EPIDURAL analgesia is widely used in children and young infants for a variety of circumstances, including postoperative and neuropathic pain.[1–5] The mechanisms of epidural local anesthesia are complex and may be subject to developmental regulation, which would have important implications for pediatric pain management. Classically, epidurally administered local anesthetic drugs block sensory and motor nerve function in a concentration-dependent manner, such that it is possible to achieve selective differential sensory blockade without motor block by limiting the concentration of the drug, although the mechanism may be more complex than originally proposed.[6,7] Local anesthetics have recently been shown to selectively inhibit nociceptive C-fiber–induced activity in rat spinal cord *in vitro* at very low concentrations that would not normally be expected to affect nerve conduction.[8,9] Low doses of epidural local anesthetics *in vivo* might therefore have a "selective analgesic" effect while leaving background low-threshold tactile sensory thresholds intact. It has also been suggested that lower doses of local anesthetics are more effective in the very young.[10–12] Young nerves *in vitro* are more susceptible to the conduction blocking effects of local anesthetics,[13] but this has not been systematically demonstrated *in vivo*. The aim of the current experiments was to examine the influence of postnatal age and the presence of inflammation on the efficacy of epidural bupivacaine at low doses.

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Materials and Methods

Experiments were performed on male and female Sprague-Dawley rat pups from University College London Biological Services (London, United Kingdom) aged 3, 10, and 21 days and weighing approximately 10, 20, and 40 g, respectively. Experiments were performed under license in accordance with Home Office regulations (University College London Biological Services, London, United Kingdom).

Flexion Withdrawal Thresholds

Hind-limb flexion withdrawal thresholds to mechanical stimulation were determined using calibrated von Frey hairs (vFh) as described by Fitzgerald *et al.*[14] VFhs are calibrated nylon monofilaments that exert a reproducible stimulus strength in grams, logarithmically increasing in intensity, and expressed linearly on a scale of 1–18 as the vFh number (table 1). An increase of 1 in the vFh number corresponds to a 66% increase in the applied force. The stimulus was applied to the dorsal surface of the hind paw three times at 1-s intervals for each strength, starting at the lowest, until a reflex was elicited, and the threshold was recorded as the vFh number.

Carrageenan Inflammation

An inflammatory reaction was induced in the right hind paw by local injection of a 2% solution of carrageenan. Animals were briefly anesthetized with halo-

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Table 1. Relation between von Frey Hair Number and Stimulus Intensity

von Frey Hair Number	Grams	Millinewtons	% Increase
4	0.0794	0.778	—
5	0.132	1.294	66
6	0.219	2.146	66
7	0.363	3.557	66
8	0.603	5.909	66
9	1.00	9.8	66
10	1.66	16.27	66
11	2.75	26.95	66
12	4.57	44.79	66
13	7.58	74.28	66
14	12.6	123.48	66
15	20.9	204.8	66
16	34.7	340.1	66
17	57.5	563.5	66
18	95.5	935.9	66

thane in oxygen, and the solution was injected using a 29-gauge needle into the plantar aspect of the paw, a volume of 5, 10, and 20 μl, respectively, at ages 3, 10, and 21 days, giving a uniform dosage of 10 mg/kg.

Epidural Injection Technique

At least 3 h after carrageenan injection, animals were briefly anesthetized with halothane in oxygen. Using a 1-ml syringe and 25-gauge needle, injections of bupivacaine or saline were made between the lower lumbar vertebrae into the epidural space using a loss of resistance to injectate technique.[15] Bupivacaine 0.004, 0.0075, 0.015, 0.03, 0.0625, and 0.125 or saline was injected in a volume of 0.025 ml at 3 days, 0.05 ml at 10 days, or 0.1 ml at 21 days. The dose range (0.1–3 mg/kg; table 2) and volume (2.5 ml/kg) of local anesthetic was proportionately similar at all ages for each concentration tested. All solutions contained 10% Evans Blue dye as a marker. At the end of each experiment after the animals were killed, a laminectomy was performed, and the spinal cord was observed to be free from staining after the intact dura was removed.

Dose and Age Groups

The withdrawal reflex could be reliably elicited at all ages within the range 0.004–0.125% bupivacaine. Higher concentrations ($>$ 0.125%) produced a mixed pattern of sensory and motor block of the hind limbs such that withdrawal was not reliably elicited, but this was not seen with solutions of 0.125% or lower. In practice, the effects of 0.015% bupivacaine at 10 and 21 days were so small that 0.0075 and 0.004% were not used. Therefore, data were collected in seven dose groups at 3 days and five dose groups at 10 and 21 days. The sample size was four for each dose and age group, a total 68 animals (n = 28, 20, and 20, respectively). A further four animals aged 3 days constituted a systemic control group, injected subcutaneously with 0.125% bupivacaine.

Sensory Testing

At time −180 (*i.e.*, 3 h before bupivacaine injection), baseline thresholds were determined in the right hind limb of a convenience sample of the animals (n = 11 per age group). The right hind paws of all the animals were then injected with carrageenan as previously described. Three hours later, at time 0, thresholds were determined in both hind limbs of all animals. The animals were then briefly anesthetized, and the bupivacaine–saline injection administered as described previously. After full recovery from anesthesia, 15 min after injection (time 15), thresholds were determined again in both hind limbs and at 15-min intervals for 90 min.

Statistical Analysis

The threshold data were analyzed in vFh number units corresponding to a logarithmic transformation.[15] This was necessary to overcome the marked heteroscedasticity evident on the original scale. Each increase in vFh number corresponds to a 66% increase in force (table 1), while each unit decrease corresponds to a 40% decrease in force. The average percent change in force is given by $\pm 100 \log_e(1.66) = \pm 50.7\%$; therefore, a unit change in vFh number corresponds approximately to a 50% change in force. Fractions of a unit correspond to fractions of 50%, *i.e.*, 0.1 vFh units = 5% change in force.[16]

Time trends in threshold in the control groups were assessed by analysis of covariance, including time (minutes), age (treated categorically), and animal (as a random effect).

Subsequent analyses were performed for thresholds at times 0 and 15, when the bupivacaine effect was consistently most marked. The results at later times were highly correlated with those at 15 min, and the extra complexity of using them in the analysis was not thought worthwhile given the limited extra information they provided. Analysis of covariance was used to adjust simultaneously for differences in threshold caused by age (treated categorically), dose (treated linearly on the log scale omitting the systemic control group), and the interaction of age and dose. A test was also performed for quadratic term in log dose. Nonsignificant terms were omitted from the analysis. Saline cannot be included on the log scale as it corresponds to zero dose; therefore, instead it was treated as 0.002% bupivacaine, *i.e.*, half the lowest true dose.

Table 2. Actual Dose for Each Concentration of Bupivacaine

Concentration (%)	Dose (mg/kg)
0.004	0.1
0.0075	0.2
0.015	0.4
0.03	0.8
0.0625	1.6
0.125	3.2

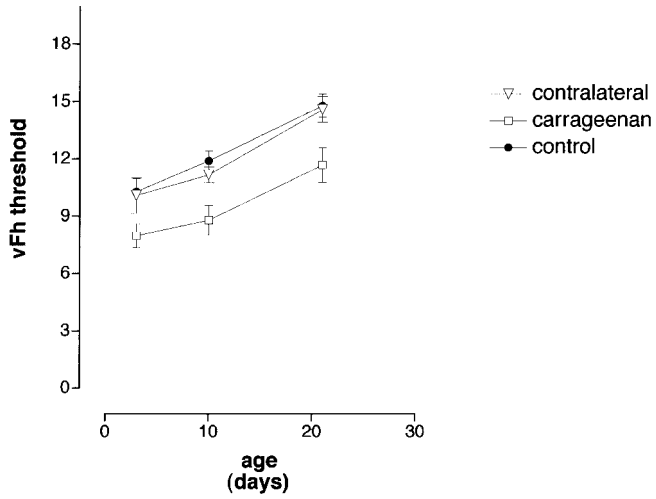

Fig. 1. Postnatal development of mechanical sensory thresholds. Thresholds in carrageenan-inflamed (*right*) and contralateral (*left*) hind limbs 3 h after carrageenan injection (n = 20 or 28 per age group) and in preinjection control (*right*) hind limbs (n = 11 per age group). vFh = von Frey hair.

Separate analyses were performed for the threshold in the inflamed right (carrageenan-treated) hind limb, the threshold in the left (contralateral) hind limb, and the difference in thresholds between right and left limbs. The latter analysis provides a paired comparison and represents the difference in threshold caused by the carrageenan inflammation, *i.e.*, inflammation hypersensitivity. Thresholds at time 15 were adjusted for thresholds at time 0 by including the time 0 threshold as a continuous covariate in the analysis of covariance. For the differential right–left threshold at time 15, both the mean and the difference of the two thresholds at time 0 were tested as covariates in the analysis, but neither was significant. Inspection of regression diagnostics for each analysis confirmed adequate normality and homoscedasticity of the residuals in each case.

Results

Postnatal Development of Mechanical Sensory Thresholds

Figure 1 shows that baseline sensory thresholds to mechanical stimulation increase with postnatal age, as has been reported previously.[14,15] Consistent age trends were observed in all three hind-limb groups: left (contralateral) at time 0, right (carrageenan) at time 0, and right (precarrageenan or control) at time −180. The contralateral and control groups were not significantly different ($P < 0.3$).

Effect of Carrageenan

Figure 1 also shows the effect of carrageenan inflammation on right hind-limb thresholds. Carrageenan causes a hypersensitivity or reduction in sensory threshold when compared with the contralateral limb at all ages, but more so in older animals. The mean ± SD of the difference in threshold between left and right hind limbs at time 0 was 2.1 ± 0.6, 2.3 ± 0.8, and 2.9 ± 0.9 vFh units, respectively, at 3, 10, and 21 days of age (age effect, $P = 0.002$).

Effect of Epidural Bupivacaine on Sensory Thresholds

Figures 2A–F summarize the effects of bupivacaine or saline on the thresholds of inflamed and contralateral limbs over time in each age group. The thresholds are plotted relative to the mean thresholds at time −180, showing the marked reduction in threshold caused by carrageenan inflammation. Animals injected with epidural saline or systemic bupivacaine showed only small changes in threshold over time, with no evidence of a peak at time 15 (time trend, $P = 0.2$).

The maximal effect of epidural bupivacaine was consistently at time 15. Figures 2B, D, and F show that peak thresholds in the contralateral, uninflamed limb increased with concentration ($P < 0.0001$) as expected from local anesthetic conduction block. The minimum visibly effective concentration was 0.0625% at 3 days and 0.125% at 10 and 21 days, a highly significant trend ($P < 0.001$). This disappeared when adjusted for the threshold at time 0 ($P = 0.3$), indicating that the same age trend was present in the threshold at time 0. In addition, the threshold–concentration relation was steeper in the younger animals (concentration by age interaction, $P = 0.02$).

Effect of Epidural Bupivacaine on Carrageenan-induced Hypersensitivity

Figures 2A, C, and E show that thresholds in the carrageenan-inflamed limbs of the saline and systemic bupivacaine control animals decreased slightly with time, so that the hypersensitivity increased slightly throughout the experiment. The mean decrease in threshold during the 90 min amounted to 0.50 ± 0.14 vFh units (time trend, $P = 0.0005$).

The hypersensitivity was reduced by epidural bupivacaine at all ages, again with a peak effect at time 15. The minimum visibly effective concentrations on the time 15 threshold at 3, 10, and 21 days were 0.004, 0.03, and 0.0625%, respectively. The effects of both concentration ($P < 0.0001$) and age ($P < 0.02$) were significant, but there was no interaction between them (dose by age interaction, $P = 0.2$).

Focusing on the paired differences in threshold between inflamed and normal limbs, the response to bupivacaine at different concentrations in the three age groups was complex, as shown in figure 3. Bupivacaine at the highest concentration of 0.125% raised thresholds to a similar extent in both limbs irrespective of age, as expected from local anesthetic block, giving a difference close to zero at all ages. Similarly, at the lowest concen-

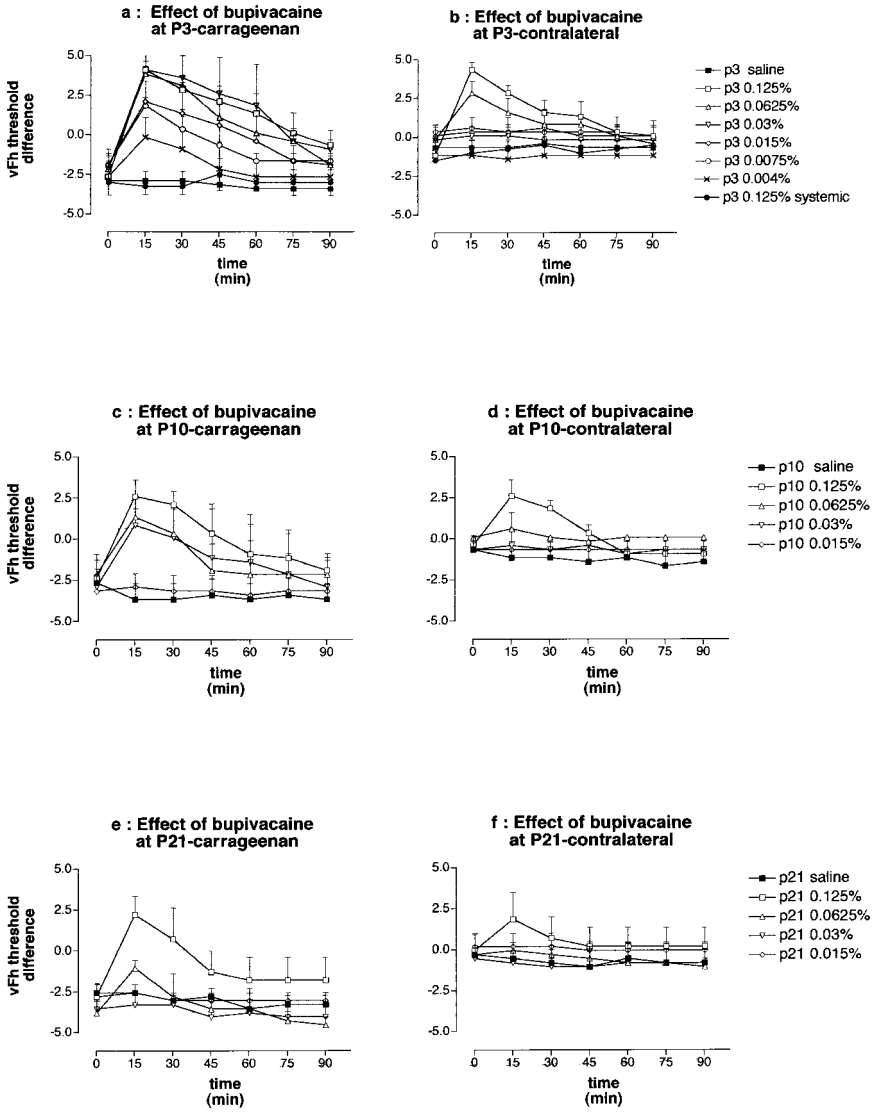

Fig. 2. (*A–F*) Effects of 0.004–0.125% epidural bupivacaine, 0.125% systemic bupivacaine, and saline on mechanical sensory thresholds in carrageenan inflamed (*A*, *C*, and *E*) and contralateral (*B*, *D*, and *F*) limbs at postnatal (P) ages 3, 10, and 21 days. Thresholds at time 0 are reduced in the inflamed limbs at all ages. Epidural bupivacaine reverses this decrease in threshold, with the minimum effective concentration increasing with postnatal age. vFh = von Frey hair.

tration (saline, treated as 0.002%), the threshold difference was −2 to −3 at all ages, reflecting the relative hypersensitivity of the inflamed limb as seen at time 0. Intermediate concentrations (0.004–0.0625%) affected thresholds differentially by age; at 21 days, the inflammatory hypersensitivity was only reversed by 0.0625% bupivacaine, whereas at 3 and 10 days, the required concentrations were lower. In addition, at 3 days, 0.004% and higher concentrations of bupivacaine not only reversed the hypersensitivity but actually raised the threshold in the inflamed limb above that for the control limb. This pattern, summarized by quadratics in log concentration for each age group (fig. 3), was highly significantly different between groups (interaction of age and $\log^2$ concentration, $P < 0.0001$).

Discussion

We have found that very low doses of epidural bupivacaine are effective at reversing inflammation-induced hypersensitivity in young rats. These doses are too low to affect mechanical sensory thresholds in the untreated limb. This "analgesic" effect of low-dose epidural bupivacaine is greatest in younger rat pups. This is the first time these low-dose effects have been systematically demonstrated *in vivo*, and they highlight the importance of development when considering the appropriate use of epidural local anesthetics in early life.

We used sensory thresholds of the flexion withdrawal reflex to mechanical stimulation to investigate the effect of bupivacaine on untreated and carrageenan-treated hind limbs. This reflex threshold has been extensively used in the study of human and animal models of pain behavior.[14,15,17–20] During normal circumstances, thresholds for the withdrawal reflex increase with postnatal age, and this is thought to reflect maturation of the central nervous system.[21] In the adult, the reflex can only be evoked by Aδ- and C-fiber–mediated noxious stimulation.[22,23] In the neonate, the flexion withdrawal reflex may also be evoked by nonnoxious mechanical

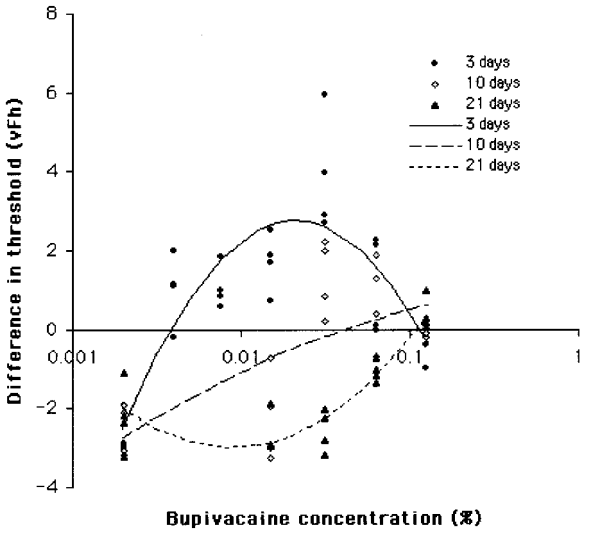

Fig. 3. Scatterplot by age of the differential threshold in carrageenan-inflamed *versus* normal limbs at time 15 against $\log_{10}$ dose of bupivacaine, with the data "dithered" in the y direction. Saline is treated as a concentration of 0.002%. The quadratic regression lines for each age are superimposed, showing a highly significant dose squared by age interaction.

stimulation mediated by Aβ fibers, reflecting differences in sensory processing in development.[24,25] Nevertheless, in common with the adult reflex, the neonatal flexion withdrawal reflex clearly demonstrates a hypersensitivity response to injury, manifested as a reduction in threshold to mechanical stimulation that is reversed by analgesia and topical local anesthetics.[15,26]

Carrageenan induces a local inflammatory response and hyperalgesia when injected subcutaneously and has been commonly used in pharmacologic tests of antiinflammatory and analgesic drugs.[27–30] Carrageenan-induced inflammatory hypersensitivity in rats has been measured as a decrease in vocalization threshold to paw pressure, reflex withdrawal latency to noxious heat, and withdrawal threshold to a mechanical stimulus.[15,31,32] In these studies, hypersensitivity was found to develop quickly, reaching a peak after 2–4 h and remaining stable for up to 8 h. Our results are in broad agreement with this, although the dose administered varies slightly between studies, perhaps accounting for the small differences observed.[15,31,32] Although the inflammatory process itself is not fully mature at birth and undergoes considerable development during the postnatal period,[33] inflammatory hypersensitivity has been clearly demonstrated in the neonate to both carrageenan and mustard oil.[15,34]

The mechanism of epidural local anesthesia is complex, and the changes in efficacy with development reported here may be related to one or more known aspects of local anesthetic activity. The principal site of action of epidural local anesthetics is on the spinal nerve roots; however, they may also have significant effects on the paravertebral nerves and in the spinal cord.[35] In a study of the sites of action of epidural local anesthetics *in vivo* using sensory evoked potentials, bupivacaine exerted its principal effects in the dorsal root entry zone and the long tracts of the spinal cord.[36] It is possible that developmental effects are caused by increased access to the site of action in younger animals, although this does not explain the selective analgesic effect. To our knowledge, developmental changes in dural penetration of local anesthetics or indeed other molecules have not been described. A further possibility could be related to the action of local anesthetics on ion channels in the dorsal root or spinal cord itself.

Local anesthetics block nerve impulse conduction by inhibition of voltage-gated Na^+ channels. Subtypes of sodium channels have been described and, in particular, tetrodotoxin-resistant channels are thought to be important for nociceptor sensitization and hyperalgesia in inflammatory states,[37,38] and their expression in dorsal root ganglion cells may be developmentally regulated. The Na^+ channel exists in a series of conformational changes known as "activated" or conducting and at least two other nonconducting states, "resting" and "inactivated." Local anesthetics inhibit the changes in conformation that underlie the activation process, and this inhibition increases with repetitive depolarizations, a process known as *state-dependent* or *phasic block*.[39] The existence of phasic block implies that a resting nerve is less sensitive to local anesthetic–induced conduction blockade than a nerve that is being repetitively stimulated. Both lidocaine and bupivacaine have been shown to block inactivated Na^+ channels at lower concentrations than that required to block channels in the resting state.[40] State dependence also favors the block of small sensory fibers that generate long action potentials at high frequency.[41] The rate of firing is one of the many factors affecting the C_m of a drug, *i.e.*, the minimum concentration of local anesthetic necessary to block impulse conduction along a given nerve fiber.[11,41] Other factors affecting C_m include nerve fiber size, degree of myelination, length of nerve exposed to drug, local pH, and calcium concentration. Degree of myelination and the distance between nodes of Ranvier in myelinated fibers are also thought to be important factors in differential nerve blockade.[42,43] Developmental changes in these factors are likely to be responsible for the observation that lidocaine block of myelinated and unmyelinated fibers is more effective *in vitro* in nerves from young compared with adult or aged animals.[13]

We have shown that epidural bupivacaine may also have an analgesic effect independent of sensory blockade, with very low doses reversing inflammatory hypersensitivity. This analgesic effect was most striking at younger ages. This is consistent with the earlier report that very low concentrations of lidocaine selectively modify nociceptive transmission within the spinal cord *in vitro* in rats. Using ventral root potential recordings as

a measure of spinal reflex activity after dorsal root stimulation, low concentrations of lidocaine have been found to selectively attenuate the C-fiber–evoked response while leaving both the component caused by low-threshold Aβ fibers and peripheral nerve impulse transmission intact.[8,9] Interestingly, it was noted in these studies that the effective concentration was 3.6–36 μM at age 1–5 days and 40–60 μM at age 10–12 days. Systemically administered low-dose local anesthetics have also been shown to reduce nociceptive activity in the adult rat spinal cord at doses that did not affect peripheral nerve conduction,[44] and to block secondary hyperalgesia in humans.[45]

The mechanism of action for this analgesic effect is unknown but could be conduction block of intraspinal presynaptic terminals or, more likely, a postsynaptic effect on membrane ion channels, antagonism at neurotransmitter receptors, or interruption of second messenger pathways.[8] Local anesthetics have also been shown to inhibit K^+ channels, Ca^{2+} channels, and a number of membrane-associated enzymes and second messenger systems, including the cyclic adenosine monophosphate–protein kinase A and calcium–calmodulin-dependent protein kinase–mediated pathways, although these actions have not been explicitly implicated in putative analgesic mechanisms.[46–50]

The fact that very low doses of epidural bupivacaine can reduce allodynia or inflammatory hypersensitivity in infant rats more effectively with increasing immaturity may have important implications for clinical practice. The dose–response effect of epidural bupivacaine on somatosensory and motor function has been studied in detail in adult humans,[51] where four concentrations of epidural bupivacaine (0.5, 0.25, 0.125, and 0.075%) were compared. Concentrations less than 0.25% produced a selective sensory block and differential sensory block in the order heat > mechanical > electrical, which was concentration-dependent, and the lowest concentration, 0.075%, induced hypoalgesia for heat only. It was concluded that bupivacaine 0.125%, the highest dose used in our study, is the most suitable concentration for the treatment of pain, and this corresponds with current clinical practice in both adults and children.[51–54]

Such studies have not been possible in children, particularly neonates and premature infants, because of the lack of reliable and sensitive pain measurement tools. As a consequence, there are few data on the influence of development on the efficacy of epidural local anesthetics available in humans. A small number of investigations have attempted to define the optimum concentrations at different ages in children, although there are none comparing efficacy between neonates and older children.[52,55,56] At present, in young patients, the doses of local anesthetics used clinically are generally based on weight rather than age or maturity,[2,57] although this practice has recently been questioned.[58] Reports of increased toxicity of local anesthetics in neonates and infants,[59,60] especially bupivacaine, which is at present the most widely used drug in pediatrics, has led to some practitioners using less than the recommended dose,[61] although systematic studies of the efficacy of these reduced doses are lacking. The findings of this investigation suggest that very young patients may be more sensitive to the therapeutic effects of epidural local anesthetics. This may have important implications for the present and future treatment of these patients, and further clinical investigation is needed.

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