

Maturation of NK1 Receptor Involvement in the Nociceptive Response to Formalin

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ABSTRACT Administration of NK1 antagonists in adult animals attenuates the nociceptive response in the formalin test, indicating that the neurokinins and the NK1 receptor play a role in mediating this pain response. The number and distribution of NK1 receptors change dramatically during development, and the age at which they become involved in pain processing is not known. We examined the role of NK1 receptors in the formalin model in rats ranging in age between 3- and 21-days old. An NK1 antagonist, CP99,994, and its less active enantiomer CP100,263 were administered to the spinal cord (intrathecal), systemically (subcutaneous), or locally (intraplantar). Intrathecal administration of CP99,994, but not CP100,263, attenuated pain behaviors in the second phase of the formalin response in 14-day and 21-day old rats, but did not alter the pain response in 3-day or 10-day old rats. CP99,994 also reduced the expression of the *c-fos* protein in the superficial dorsal horn of 21-day old rats. Systemic and intraplantar injection of either CP99,994 or CP100,263 reduced the pain response to formalin in 3-day and 21-day old rats, suggesting a non-NK1 mediated mechanism of action. These results indicate that, within the spinal cord, NK1 receptors start to play a role in the pain response to formalin between 10 and 21 days. Moreover, analgesia induced by systemic or local injection of NK1 antagonists involves mechanisms other than, or in addition to, the NK1 receptor. **Synapse 36:254–266, 2000.**

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INTRODUCTION

Behavioral and physiological studies indicate that neonates are capable of responding to noxious stimulation at or before birth, and that nociceptive systems continue to develop well into postnatal life (Fitzgerald 1991; Yi and Barr 1995; Yi and Barr 1997). Behavioral responses to nociceptive stimuli start as general body movements, and develop into responses that are directed more specifically to the site of injury (Guy and Abbott, 1992; Teng and Abbott, 1998; Yi and Barr, 1995). Both the behavioral response and the expression of *c-fos*, a physiological marker of neuronal activation, induced by a formalin injection undergo changes through the first two weeks after birth until an adult-like pattern is observed around 15 days (Barr, 1998; Guy and Abbott, 1992; Teng and Abbott, 1998; Yi and Barr, 1995). The maturation of the physiological mechanisms involved in pain processing in adults follows a similar developmental course. It has been demonstrated that the neural pathways for the transduction of painful stimuli are present prior to birth, but are not found in adult patterns. In the rat, primary afferent fibers enter the dorsal horn lumbar segments late on

fetal day 19, reach lamina II at fetal day 19.5, and show adult-like distribution through the superficial dorsal horn at birth (Fitzgerald, 1991). Substance P (SP), a neurotransmitter associated with pain signaling, is found in small primary afferents prior to birth (Pickel et al., 1982). However, adult concentrations of SP are not reached until the second week of postnatal life (Coimbra et al., 1986; Fitzgerald and Gibson, 1984), and the electrophysiological and neurochemical properties of fine diameter fibers undergo significant maturation throughout the first two weeks of life (Fitzgerald et al., 1988). It is unknown whether these immature systems mediate the pain responses seen in infants. This study examined the involvement of the substance P receptor, NK1, in the response of infant rats to formalin.

In the adult, evidence indicates that the NK1 receptor plays a role in mediating responses to nociceptive

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stimuli. NK1 receptors are found in regions of the spinal cord associated with nociceptive pathways, including the superficial layers of the dorsal horn (Liu et al., 1997; Mantyh et al., 1995; Sakurada et al., 1995; Velazquez et al., 1997; Yashpal et al., 1995). Further, SP, a neurotransmitter that binds primarily to NK1 receptors (Chapman and Dickenson, 1993), is found in primary afferent fibers and in dorsal horn and spinally projecting brainstem neurons (De Biasi and Rustioni, 1988; Ruda et al., 1986; Traub, 1996). Noxious stimulation elicits the release of SP into the spinal fluid (Velazquez et al., 1997), and results in internalization of NK1 receptors (Mantyh et al., 1995; Marvizon et al., 1997), a process that appears to mediate receptor desensitization (Garland et al., 1996). Intrathecal application of SP excites nociceptive neurons in the dorsal horn (Cridland and Henry, 1986; Piercey et al., 1981), and elicits pain-like responses such as caudally directed biting (Mjelle-Joly et al., 1991; Mochhala and Sawynok, 1984; Piercey et al., 1981; Radhakrishnan and Henry 1995; Velazquez et al., 1997).

The results described above indicate that SP and its receptor, NK1, play a prominent role in the processing of nociceptive stimuli; however, attempts to block noxious stimulus evoked pain behaviors with NK1 antagonists have yielded mixed results. Systemic and intraplantar application of NK1 antagonists attenuate the pain response to noxious chemical stimulation, such as injection of formalin or capsaicin (Birch et al., 1992; Nagahisa et al., 1992; Rupniak et al., 1995; Santos and Calixto, 1997). However, this effect is not specific to the antagonists action on the NK1 receptor since similar levels of attenuation occur with the less active enantiomers (Nagahisa et al., 1992; Rupniak et al., 1995; Smith et al., 1994). These observations suggest that the antinociception produced by local or systemic application of NK1 antagonists is produced by mechanisms other than or in addition to those mediated by the NK1 receptor. In contrast, intrathecal application of NK1 antagonists produces a dose dependent attenuation of the second phase of the pain responses to formalin injection that is stereospecific (Chapman and Dickenson, 1993; Traub, 1996; Yamamoto and Yaksh, 1991). This indicates that spinal NK1 receptors are involved in chemonociception.

Although the role of spinal NK1 receptors in the mediation of nociception has been demonstrated in adult rats, little is known about the role of these receptors in infant rats. NK1 receptors are overexpressed at birth in brain and spinal regions where few receptors are seen in the adult, and adult patterns of NK1 receptor distribution do not appear until approximately 14.5–15 days after birth (Charlton and Helke, 1986). It is unknown whether the NK1 receptors found in neonatal rats play a functional role in the pain response to formalin. This study was designed to determine the age at which NK1 receptors are involved in this pain re-

sponse by assessing both *c-fos* expression and behavioral responses to formalin after administration of an NK1 antagonist, CP 99,994, or its inactive enantiomer, CP 100,263, across development. This study also assessed whether there are differences between systemic, intraplantar, and intrathecal administration of these antagonists, as in adult animals.

MATERIALS AND METHODS

All experiments were approved by both Hunter College and New York State Psychiatric Institute IACUCs and followed Ethical Guidelines of the Society for Neuroscience and the International Society for Developmental Psychobiology.

Subjects

Pups were the offspring of Long-Evans hooded rats that were mated in our laboratory. The parental animals were housed in standard laboratory cages in a colony room maintained at 22–24°C with a 12-hour light-dark photocycle with light onset at 0800 hours. Food and water were available ad libitum. Cages were checked twice daily, at approximately 0900 and 1800 hours. Pups found at either time were termed 0 days of age. In all experiments, no more than one pup per litter was in any given treatment condition; therefore the litter was the unit of analysis.

Surgeries

Subjects in the intrathecal condition had catheters placed into their spinal cords as described previously (Barr et al., 1992; Paredes et al., 1990). Catheters consisted of 1.5 cm of dialysis tubing (Spectra/Port, Houston, TX), with an internal diameter of 200 μm and a molecular weight cut-off (MWCO) of 13,000. This tubing was inserted 0.5 cm into a 4 cm length of Silastic brand tubing (Dow Corning, Midland, Michigan) with an internal diameter of 312 μm . The dialysis tubing was attached to the Silastic tubing with cyanoacrylate (Krazy Glue, Columbus, OH), and allowed to dry for a minimum of 24 hours. The catheter was tested by filling the tubing with saline prior to implanting it, and those with leaks were not used.

Pups were anesthetized with methoxyflurane (Metofane, Mallinckrodt Veterinary Inc, Mundelein, IL), and a caudal to rostral incision was made. Muscle and fascia were removed by blunt dissection, to expose vertebrae T8–T11. A laminectomy was performed, removing the dorsal surface of 2–3 vertebrae without damaging the dura. The dura lateral to the midline was punctured with a caudal movement of a curved 30-gauge hypodermic needle. The catheter was then inserted, so that the entire length of the dialysis tubing (1 cm) was in the subdural space. To better visualize the progress of the tubing, it was dipped into cresyl-violet dye. The dialysis portion of the catheter rested under

the dura and above the dorsal-lateral aspects of the spinal cord. Prior studies showed no detectable spinal cord damage, although dorsal roots on the side of the implant were sometimes damaged (Paredes et al., 1990; Barr, 1992). The silastic portion of the catheter was then anchored to the first intact vertebra rostral to the laminectomy with a single drop of cyanoacrylate. Both the incision and the open end of the tubing were closed with cyanoacrylate, and the animal was placed back with its littermates, into a cage that was kept warm by a heating pad. Pups were not placed back into the homecage with their mother to prevent the mother from damaging the catheters. Catheters were never damaged by littermates. Animals were allowed to recover for four hours before testing. Immediately prior to testing, subjects were observed for motor deficits and those that showed paralysis of their hindlimbs were not used. Placement of the catheter was checked by visual examination after the experiment.

Drug preparation and administration

The NK1 antagonist, CP99,994, its enantiomer CP100,263 (Pfizer, Inc., Groton, CN), and the NK1 antagonist LY703,606 (Research Biochemicals International, MA) were dissolved in 0.9% saline solution. Drugs were administered systemically through subcutaneous injection, locally through intraplantar (intradermal) injection, or directly to the spinal cord through intrathecal injection.

Intrathecal injections of the saline vehicle or 0.01 μg (0.03 nmol), 0.05 μg (0.15 nmol), 0.25 μg (0.75 nmol) of CP99,994 or CP100,263 were applied in 2 μl of saline to 3-day old (32 pups from 8 litters for CP99,994; 24 pups from 6 litters for CP100,263); 10-day old (32 pups from 8 litters for CP99,994; 24 pups from 6 litters for CP100,263); 14-day old (32 pups from 8 litters for CP99,994; 24 pups from 6 litters for CP100,263); and 21-day old (40 pups from 10 litters for CP99,994; 32 pups from 8 litters for CP100,263) subjects 10 minutes prior to behavioral testing. Since the catheter, including both the silastic and the dialysis tubing, held 3 μl of fluid, the syringe was filled with a total of 5 μl of the appropriate solution. The drug was applied by steadily injecting the solution at the rate of 1 μl every 15 seconds for a total of 75 seconds. The end of the tubing was again sealed with cyanoacrylate to prevent more of the solution from seeping into the spinal cord.

Three-day old (40 pups from 8 litters per drug) and 21-day old (40 pups from 8 litters per drug) subjects were given subcutaneous injections of saline or 1.0, 3.0, 10, or 30 mg/kg of CP99,994, CP100,263, or LY703,606 in the neck region 15 minutes prior to behavioral testing. Another group of 21-day old subjects received intraplantar injections of saline or 10, 30, or 100 μg or 100 μg subcutaneously of either CP99,994 (20 pups from 4 litters) or CP100,263 (25 pups from 5 litters) into the paw 5 minutes prior to testing.

Formalin

Pups were given a subcutaneous injection of 10 μl 2.0% formalin into the plantar surface of the hindpaw. Subjects that received intraplantar injections of the NK1 antagonists received the formalin injection into the same paw. The subjects that had intrathecal catheters placed in the spinal cord were injected in the paw contralateral to where the catheter was placed (the left paw). Immediately after the formalin injection, the subject's behavior was rated and recorded at 1 minute intervals for 1 hour after the formalin injection. Pain behavior was rated according to the 5-point scale previously described (Barr, 1998).

Histochemistry

Fos expression was analyzed in 21-day old subjects (12 pups from 4 litters) that received intrathecal injections of the vehicle, 0.01 μg or 0.25 μg CP99,994 and 14-day old subjects (8 pups from 4 litters) that had received intrathecal injections of the vehicle or 0.25 μg CP99,994. The expression of the Fos protein peaks approximately 2 hours after an injection of formalin in adult and infant rats (Yi and Barr 1995). Therefore, 2 hours after the formalin injection, subjects were injected intraperitoneally with an overdose of sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL). When deeply anesthetized, the animals were perfused intracardially with 0.9% phosphate buffered saline followed by 4% paraformaldehyde. After fixation, the spinal cords were removed, and the catheter placement visually checked in intrathecal subjects. Spinal cords were placed in 4% paraformaldehyde for a minimum of 24 hours. The tissue was moved into 30% sucrose for at least 24 hours prior to sectioning. The lumbar enlargement was sliced into thirty micron transverse sections on a cryostat and every third section was placed into 0.1 M phosphate buffer (PBS), pH 7.2. Sections were processed for the immunohistochemical localization of the Fos protein (Ab5, Cal Biochem, San Diego, CA) by the avidin-biotin-peroxidase (ABC) method, mounted on slides, and counted as described previously (Yi and Barr, 1995). Lumbar sections (L1–L6) were examined under a light microscope and labeled nuclei were counted using a drawing tube attachment. The spinal cord sections were subdivided into 5 regions (Fig. 1), and all visible labeled nuclei were counted within each region, irrespective of the density of the stain. The mean number of cell counts per region was calculated for each animal by averaging the number of stained nuclei from 5 maximally stained sections.

Statistics

For the behavioral analysis, the minute by minute data were averaged into 3-minute bins to decrease variability. A two-way analysis of variance (ANOVA)

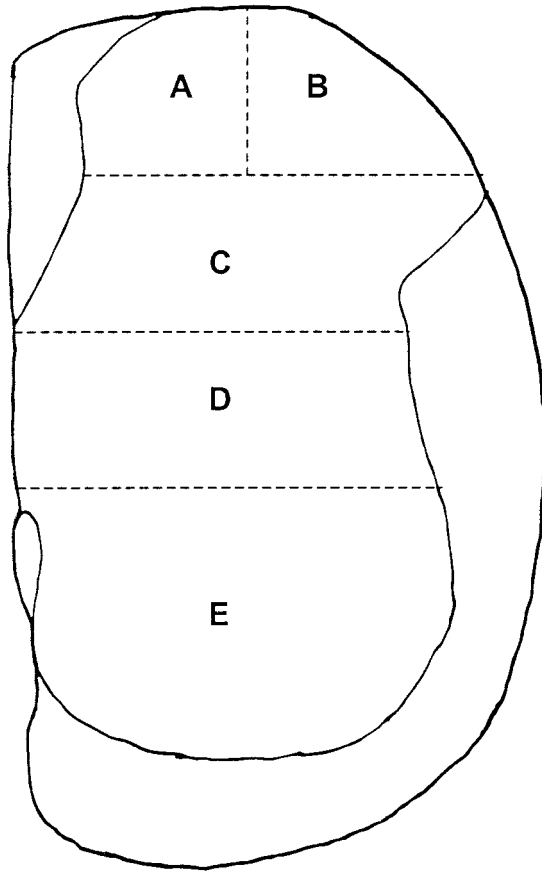


Fig. 1. Regions of the lumbar enlargement segment (L1–L6) of the spinal cord that were counted for *c-fos*. Areas **A** and **B** correspond to the medial and lateral superficial lamina respectively. Area **C** corresponds to laminae 3 and 4, area **D** to lamina 5, and area **E** to the ventral horn.

was performed, with the 20 3-minute bins and the drug dose as within-subject variables. For the *c-fos* analysis, the mean number of cell counts per region was calculated for each animal by averaging the number of stained nuclei from five maximally stained sections. Averaging multiple sections per animal increases the power of the ANOVA, therefore fewer subjects were required for each age and drug examined (Holson and Pearce, 1992). A two-way ANOVA was performed, with the region counted and the drug dose as within-subject variables. Statistical tests were considered significant if $p < .05$.

RESULTS

Intrathecal injection of CP99,994 and CP100,263

The effect of CP99,994 on formalin-induced nociceptive responding is depicted in Figure 2. CP99,994 decreased nociceptive responding in 21-day old and in 14-day old subjects, $p < .05$, and did not alter responses in the 10-day or the 3-day old rats, $p > .05$. To determine whether the antinociception observed in the 21 and 14 day old subjects was more pronounced in the first or the second phase, a separate ANOVA was

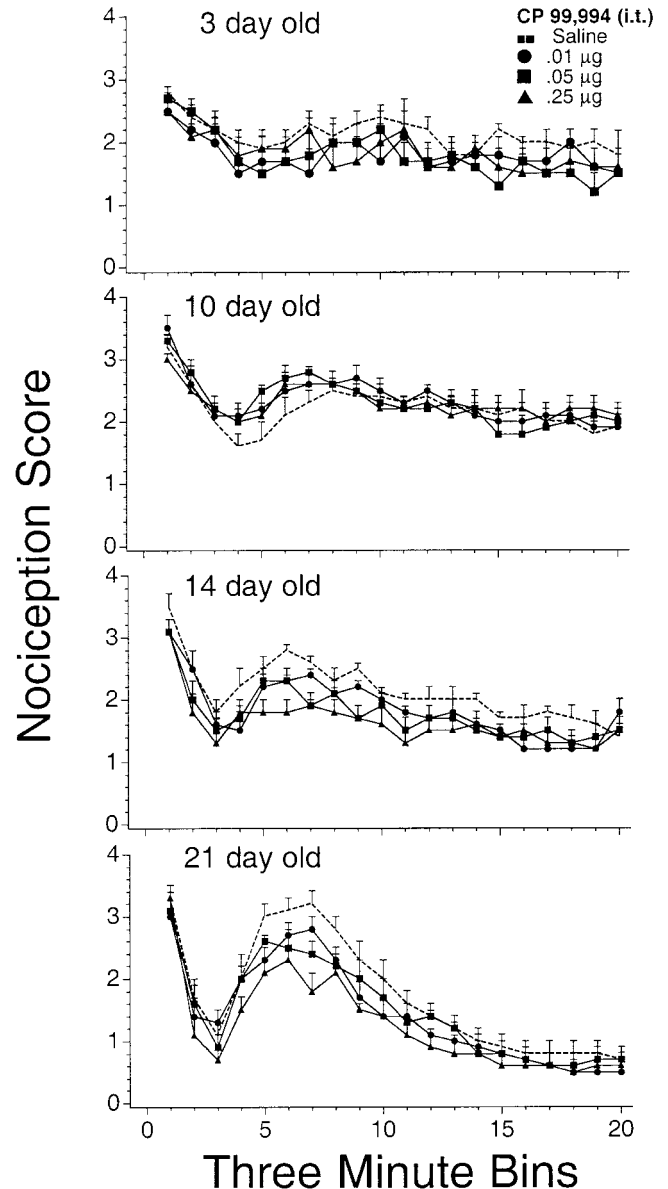


Fig. 2. Mean (\pm one SEM) nociceptive scores across the 20 3-minute bins in 3-day old rat pups, 10-day old, 14-day old, and 21-day old, that received intrathecal injections of the saline vehicle, 0.01 μg , 0.05 μg , or 0.25 μg of the NK1 antagonist, CP99,994. Application of CP99,994 10 min prior to a formalin injection (10 μl , 2%) reduced nociceptive responding in the 14-day and 21-day old subjects, but not the 10-day or 3-day old pups. The major effect was in the second phase.

performed that compared bin 1 with bins 4–8 across drug doses. The CP99,994 decreased responding equally during both phases of the formalin response in the 14 day old subjects (dose by phase interaction, $p > .05$). In contrast, the CP99,994 did decrease responding more in the second phase than in the first phase in the 21-day old subjects (dose by phase interaction, $p < .01$).

The formalin-induced nociceptive responding observed after intrathecal administration of CP100,263 is

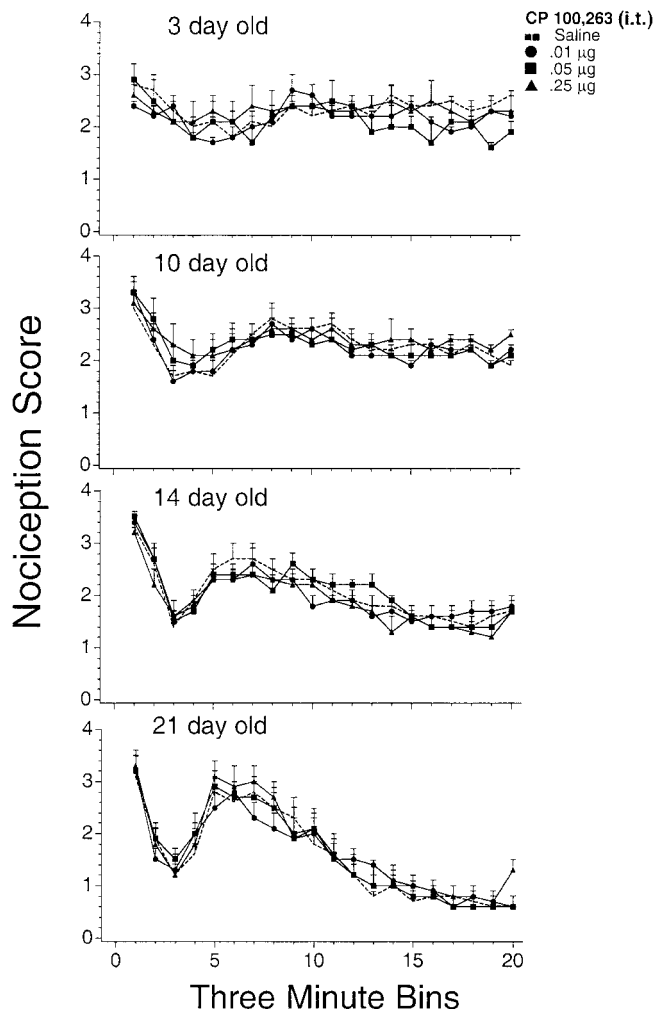


Fig. 3. Mean nociceptive scores across the 20 3-minute bins in 3-day old rat pups, 10-day old, 14-day old, and 21-day old, that received intrathecal injections of the saline vehicle, 0.01 μg , 0.05 μg , or 0.25 μg of the inactive enantiomer CP100,263. This drug did not alter nociceptive responding at any of the ages tested. Details are as in Figure 2.

depicted in Figure 3. This drug did not alter response at any of the ages tested. Neither the main effect of drug nor the drug by bin interaction was found to be significant, $p > .05$.

The effect of CP99,994 on Fos expression in 21-day olds is depicted in the top panel of Figure 4. Intrathecal administration of CP99,994 dose dependently decreased Fos expression in a region specific manner. There was no main effect of drug dose, $p > .05$; however both the main effect of region counted and the region by drug dose interaction were significant, $p < .05$. The expression of Fos was reduced in regions A and D, but not altered in the other regions. The CP100,263 did not alter Fos expression in 21-day old subjects. Although there was a main effect of region counted, $p < .05$, neither the main effect of drug, nor the drug by location interaction was significant, $p > .05$. The Fos expression in 14-day old subjects after

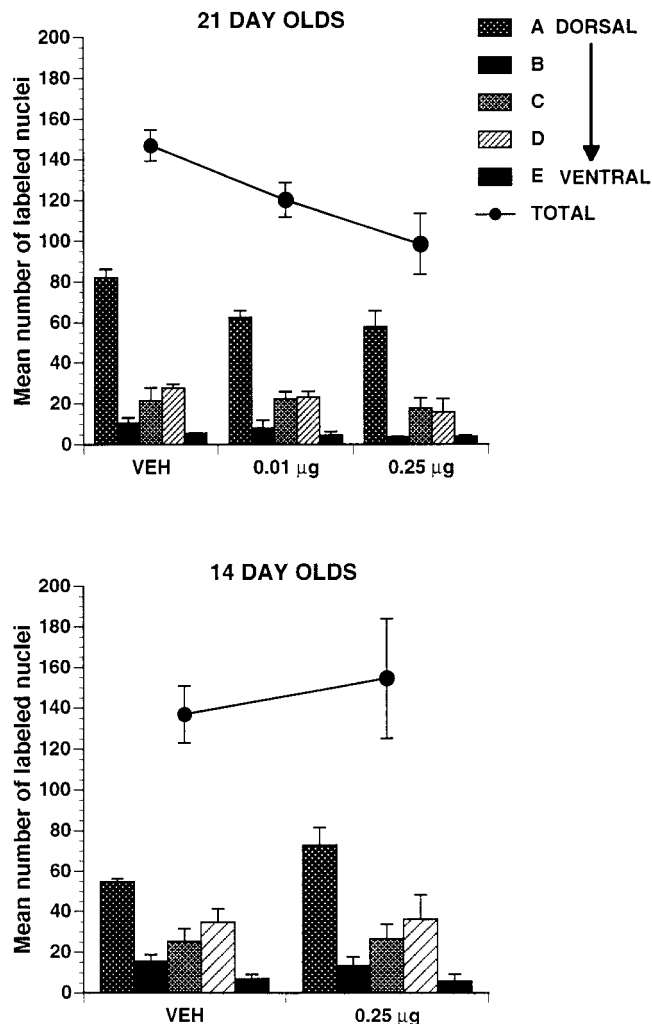


Fig. 4. The total number (filled circles) and the regional number (bar graphs) of nuclei labeled for *c-fos* in 21-day and 14-day old rats. Intrathecal injection of the CP99,994 decreased *c-fos* expression in the 21-day old subjects, particularly in the superficial lamina. The expression of *c-fos* was not altered in the 14-day old subjects. Error bars indicate one SEM.

intrathecal administration of CP99,994 is depicted in the bottom graph of Figure 4. CP99,994 did not alter Fos expression at this age. Although there was a main effect of region counted, $p < .05$, neither the main effect of drug nor the drug by region interaction were significant, $p > .05$.

The effects of surgery alone on Fos expression was assessed in 21-day old control subjects that received a saline, rather than a formalin, injection into the hindpaw. No Fos was observed in these animals (data not shown). Photomontages of Fos expression after intrathecal administration of saline or CP99,994 in 14-day old and 21-day old formalin injected subjects are depicted in Figures 5 and 6. The Fos expression in 10-day and 3-day old subjects was not analyzed because CP99,994 did not alter their behavioral responses to formalin.

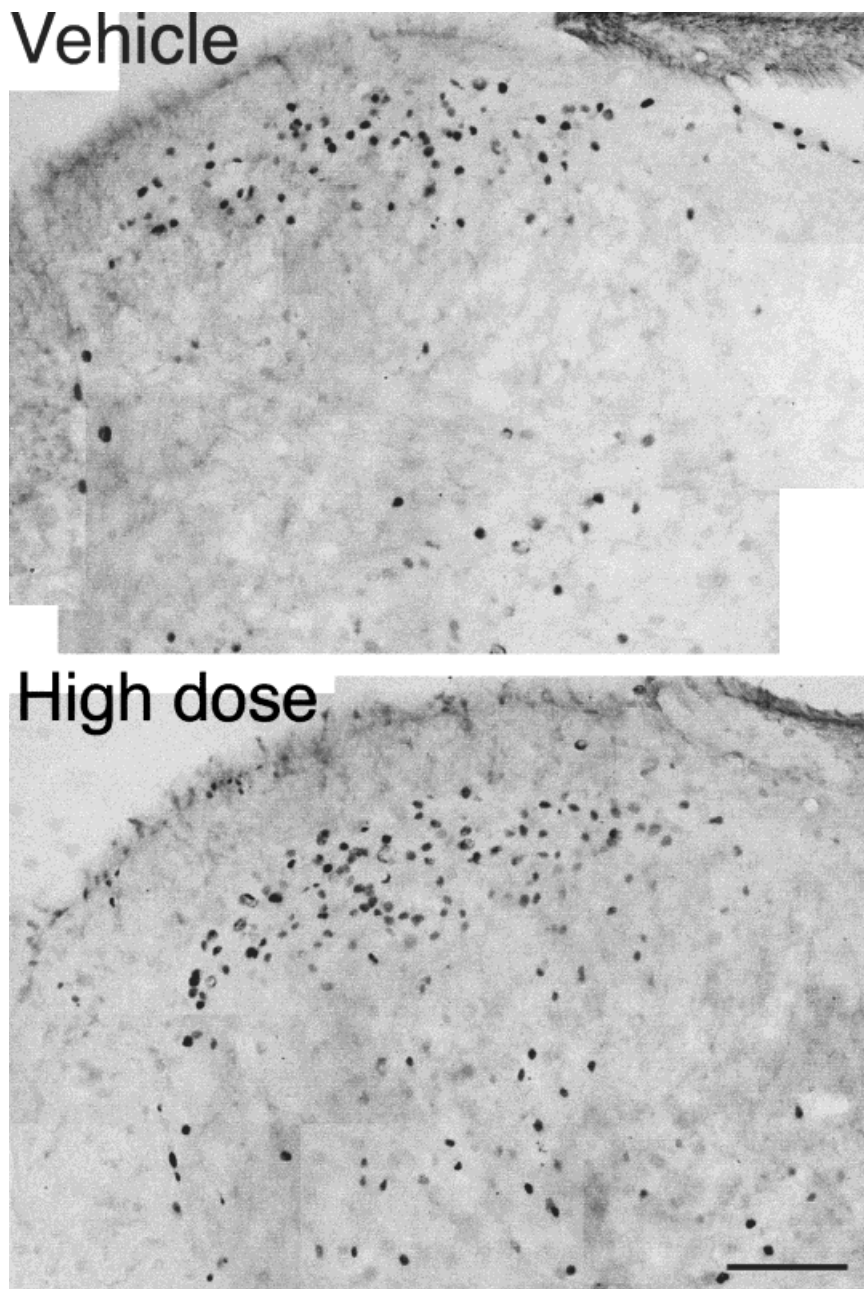


Fig. 5. Photomontage of Fos expression in the superficial lamina of the lumbar enlargement section (L1–L6) of one half of the spinal cord of 14-day old rats that received intrathecal injections of the saline vehicle (top panel) or the high dose of the NK1 antagonist CP99,994 (0.25 μ g, top panel) 10 min prior to the formalin injection. Medial is to the left and dorsal to the top. Intrathecal application of CP99,994 did not alter *c-fos* expression at this age. The bar indicates 10 μ m.

Systemic and local injections

Systemic. Subcutaneous injection of CP-99,994 (Figure 7), CP-100,263 (Figure 8), and LY-703,606 (data not shown) produced a significant reduction in nociception, but not likely through the NK1 receptor since CP-100,263 was as effective as CP99,994, $p < .01$. Both drugs were more effective in older animals, and at both ages the highest dose reduced pain behavior in the first phase as well as later during the test. For the 21-day old pups, this reduction included, but was not limited to, the second phase.

Local. Intraplantar injections of CP99,994 and CP100,263 were done only in 21-day old pups (Figure 9). Both drugs reduced nociception in a dose dependent

manner, $p < .001$. There were no differences between the two drugs, and both were effective in the initial phase of responding and during the second phase. When the highest dose (100 μ g) was given subcutaneously, there was no effect, demonstrating that the likely site of action is the paw. Note that the high intraplantar dose was lower than the doses given subcutaneously, on average 2.0 mg/kg for a 50 g rat pup. This replicates the failure of the low subcutaneous doses of the CP99,994 and CP100,263 to reduce nociception.

DISCUSSION

Administration of the NK1 antagonists produced antinociception in the formalin test that depended on

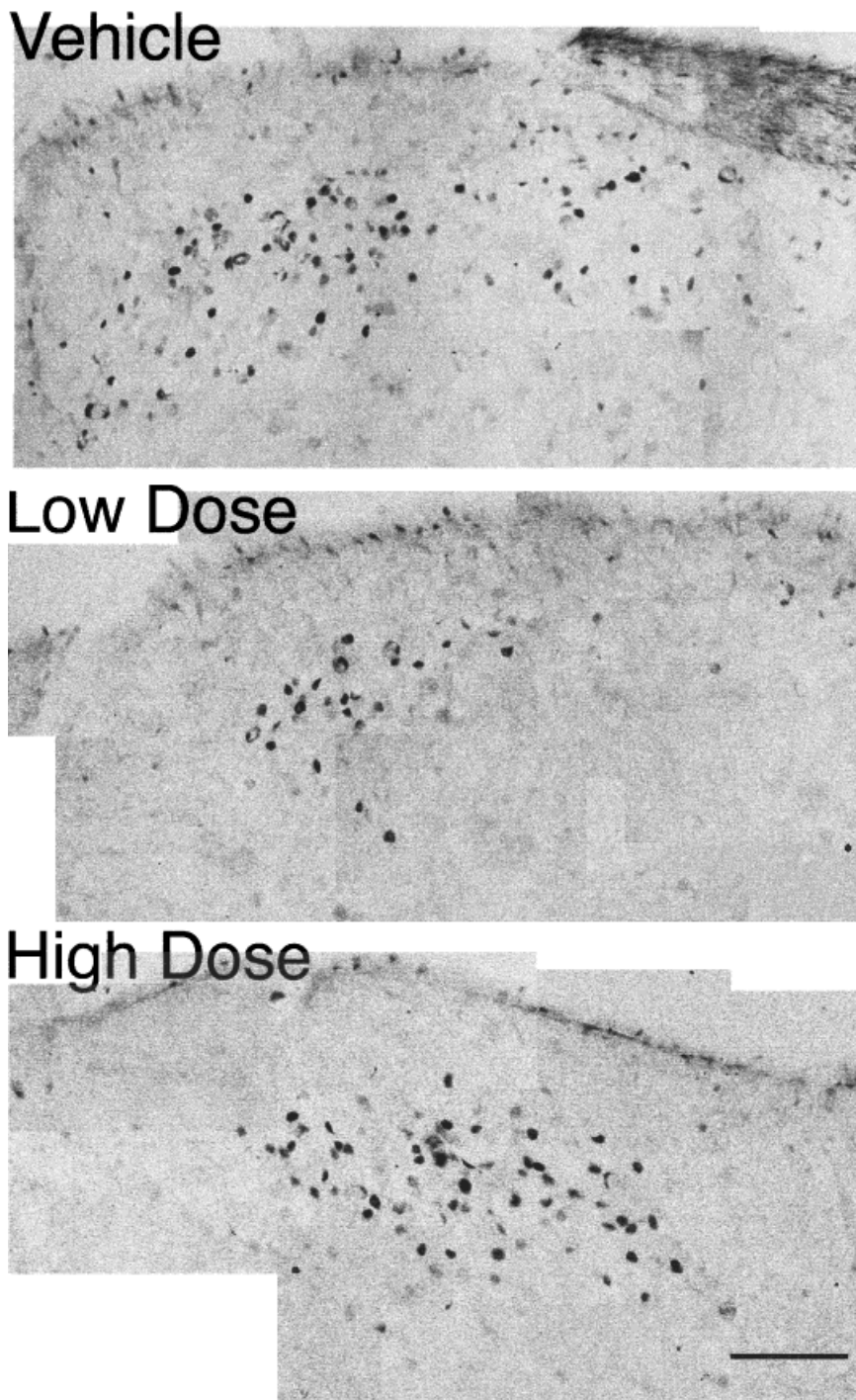


Fig. 6. Photomontage of Fos expression in the superficial lamina of the lumbar enlargement section (L1–L6) of the spinal cord of 21-day old rats treated with the saline vehicle (top panel), the low dose (0.01 μg , middle panel) or the high dose (0.25 μg , bottom panel) of the NK1 antagonist, CP99,994. Both doses reduced *c-fos* expression compared to the vehicle control. The bar indicates 10 μm .

both the route of administration and the age tested. Intrathecal administration of CP99,994, but not the less active enantiomer CP100,263, attenuated response on the formalin test in 14 and 21-day old subjects, demonstrating that this effect was dependent on NK1 receptors. In the 21-day old subjects, this antinociception was more pronounced during the second phase than the first phase of the response. Neither drug altered nociceptive response in the 3-day or 10-day old subjects. These findings indicate that the NK1

receptors are not involved in chemonociception in infant rats, and that they do not start to play a role in this response until approximately postnatal day 14.

Intrathecal administration of CP99,994 also diminished Fos staining in the 21-day old pups, and this reduction was seen primarily in the medial superficial lamina of the dorsal horn. A slight reduction was seen in the deeper lamina, particularly in lamina 5 (region D, Figs. 1 and 4). Both regions are involved in the processing of nociceptive stimuli. No changes were seen

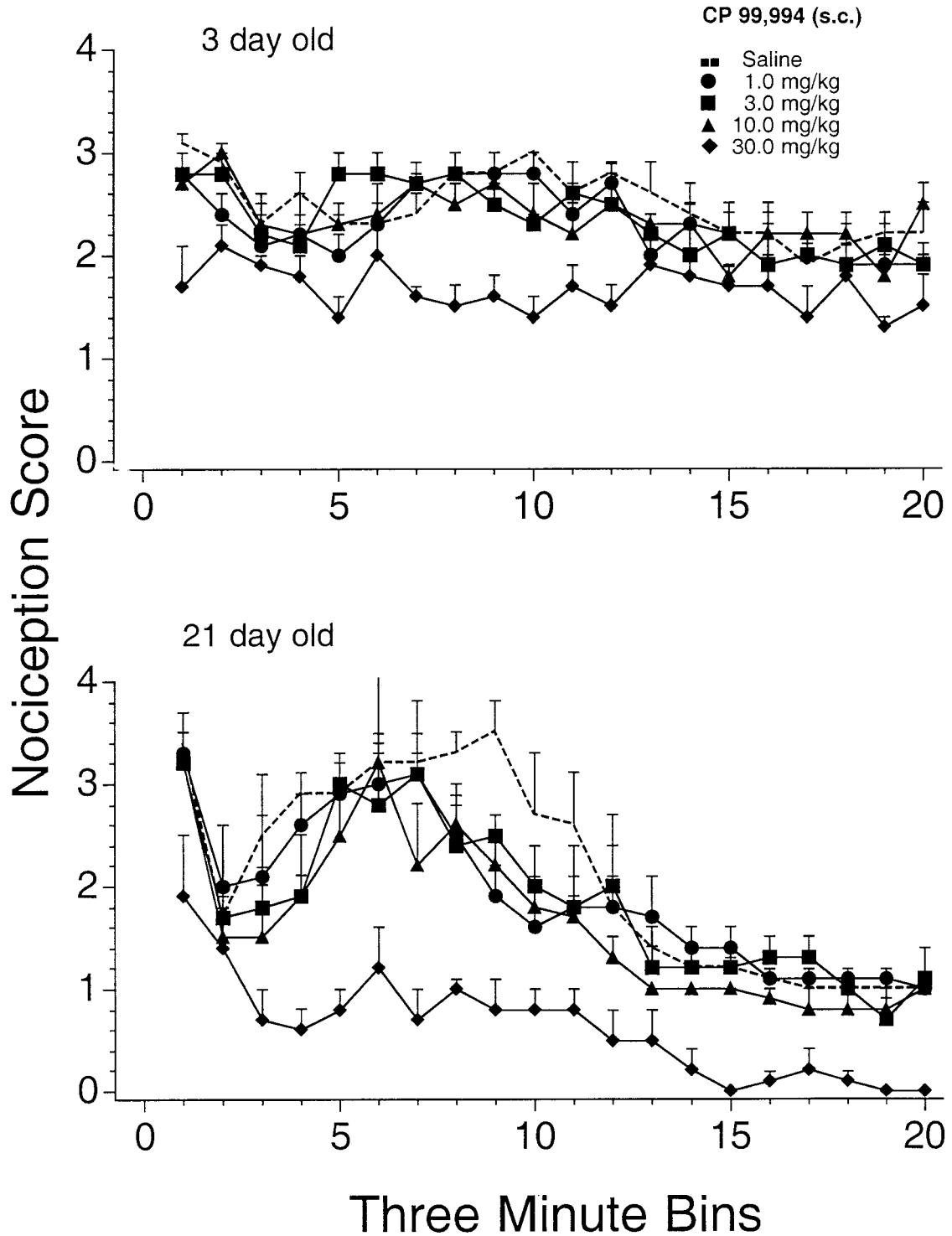


Fig. 7. Mean nociceptive scores across the 20 3-minute bins in rat pups 3-days old, or 21-days old, that received subcutaneous injections of the saline vehicle, 1.0 mg/kg, 3.0 mg/kg, 10.0 mg/kg, or 30.0 mg/kg of the NK1 antagonist CP99,994 prior to the formalin test. The high-

est dose attenuated the nociceptive response in both the 3-day and the 21-day old subjects. The other 3 doses slightly attenuated the responding during the second phase in the 21-day pups. Error bars indicate the SEM.

in the lateral superficial lamina (region B) or in the ventral horn (region E). As with the behavioral response, no changes were observed after intrathecal ad-

ministration of CP100,263. Thus, blockade of spinal NK1 receptors in 21-day old rats suppresses the immunoreactive neurons of the dorsal horn, and, to a lesser

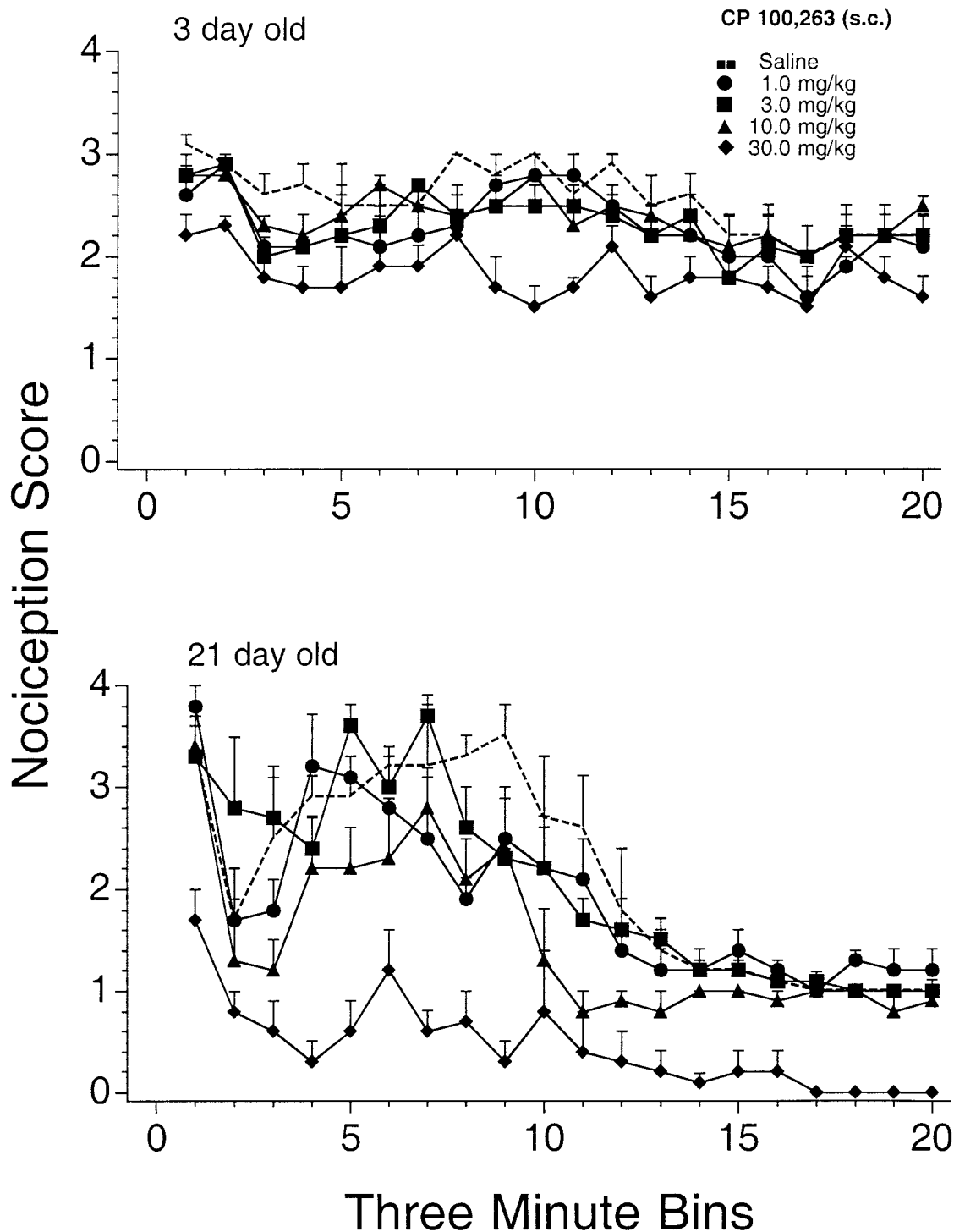


Fig. 8. Mean nociceptive scores across the 20 3-minute bins in rat pups 3-days old, or 21-days old, that received subcutaneous injections of the saline vehicle, 1.0 mg/kg, 3.0 mg/kg, 10.0 mg/kg, or 30.0 mg/kg

of the inactive enantiomer CP100,263. As with the CP99,994, the highest dose attenuated the nociceptive responding in both ages. Error bars indicate the SEM.

degree, more ventral regions (region E). No changes in Fos expression were observed after CP99,994 administration in 14-day old subjects. The reason for the discrepancy between the behavioral and *c-fos* expression

in the 14-day old subjects is unknown. It may be that the expression of *c-fos* is not as sensitive to the effects of CP99,994 as is the behavioral response. Alternatively, the *c-fos* expression may reflect processes in

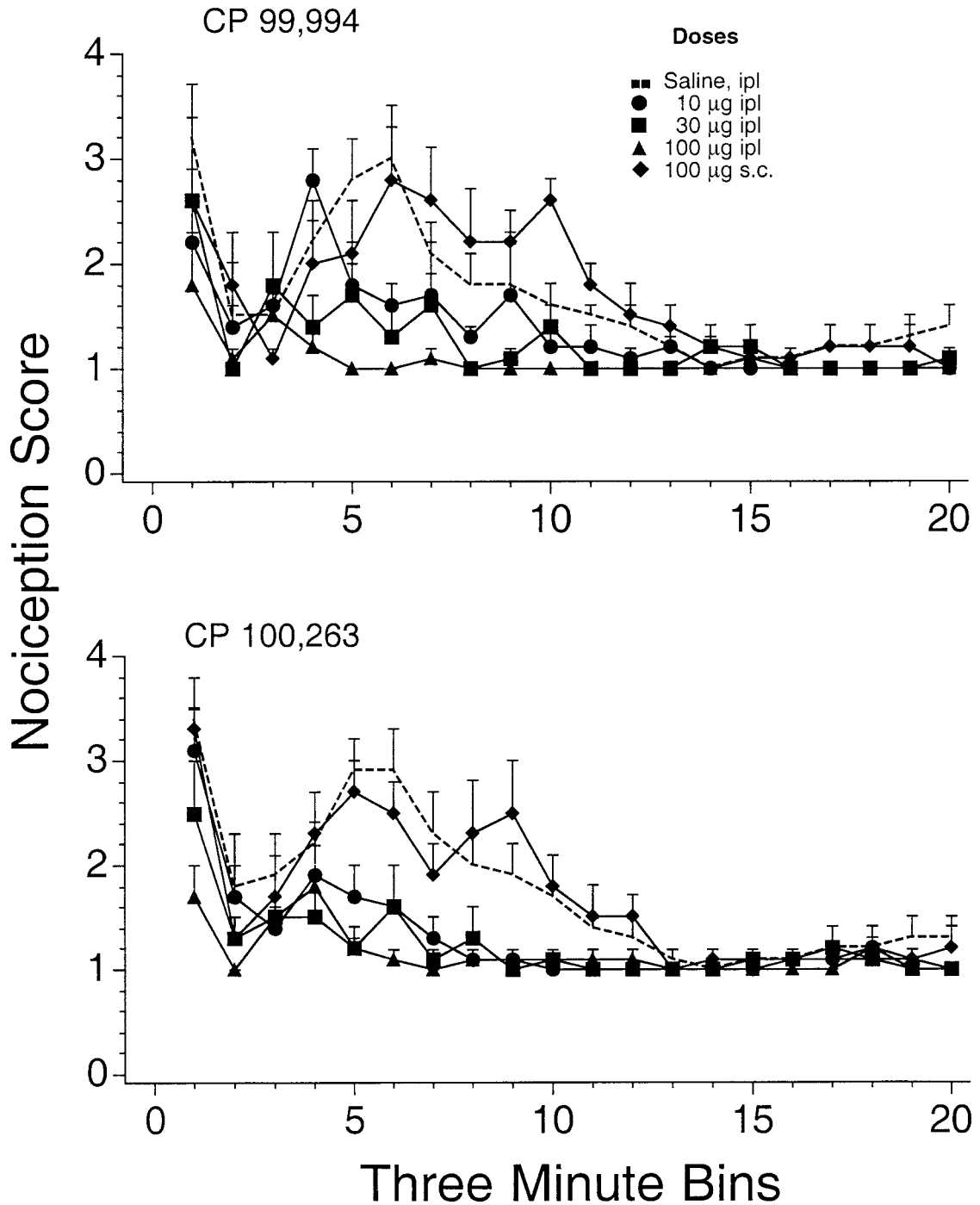


Fig. 9. Mean nociceptive scores for 21-day old pups that received intraplantar injections of the saline vehicle, 10 µg, 30 µg, or 100 µg, or 100 µg subcutaneously of either the NK1 antagonist CP99,994, or CP100,263. All intraplantar doses of both compounds reduced noci-

ceptive responding. In contrast, the subcutaneous injection of the highest dose (diamonds) did not alter nociceptive responding, confirming the data in Figures 7 and 8. Error bars indicate the SEM.

addition to nociception. It has been demonstrated that Fos staining also occurs in response to a combination of inflammation and pain (Buritova et al., 1996; Honore et al., 1996). In the formalin model, the second phase is associated with inflammation (Tjolsen et al., 1992) and is more pronounced in the 21-day old subjects than the

14-day olds. Therefore, the combination of inflammation and nociception may account for the differences in the effectiveness of CP99,994 in reducing *c-fos* expression at the different ages.

The behavioral data suggest that spinal NK1 receptors are not involved in the nociceptive response to

formalin in infant rats 10 days old and younger and start to play a role in this response between 10 and 14-days after birth. Several physiological changes in the pain system occur over the first two postnatal weeks that may account for these data. Substance P, is found prenatally in primary afferent fibers (Pickel et al., 1982) and NK1 receptors, activated by SP, are overexpressed at birth (Charlton and Helke, 1986). The adult-like concentrations of SP and distribution of NK1 receptors are not found until the third week of postnatal life (Charlton and Helke, 1986; Coimbra et al., 1986; Fitzgerald and Gibson, 1984). Moreover, the electrophysiological and neurochemical properties of fine diameter nociceptors undergo significant maturation throughout the first weeks of life (Fitzgerald et al., 1988), and the behavioral manipulations that specifically test C-fiber function, such as the reflexive withdrawal or *c-fos* expression in response to mustard oil or neurogenic edema, are not observed until 10–11 days postnatally (Fitzgerald and Gibson, 1984; Williams et al., 1990). The maturation of the NK1 receptors and the C-fiber and SP distribution into adult like patterns corresponds to the time that we observed the effects of the NK1 receptor antagonists in nociceptive processing. Whether the failure of the NK1 antagonist to attenuate pain responses in younger rats is due to immature NK1 receptors or because the endogenous agonist, SP, is not released to activate these receptors cannot be determined by administration of the antagonists alone. Future studies looking at the effects of administration of NK1 agonists are needed to resolve this issue.

Changes in the response to formalin over the first two postnatal weeks have been demonstrated previously (Barr, 1998; Guy and Abbott, 1992; Teng and Abbott, 1998; Yi and Barr, 1995). Very young animals respond to noxious stimulation with general body movements that become more localized and adult-like behavioral responses emerge between 14 and 21 days of age. The responses to formalin reported here are consistent with these observations. The response of the 3-day olds showed an overall decrease in nociceptive score over time, but the biphasic response typically seen in adult animals was not observed. The biphasic nature grew more pronounced in the 14-day olds, and was most pronounced in the 21-day old subjects. These data suggest that the NK1 receptors that are involved in mediating the second phase of the response. Thus the appearance of the maturation of functional spinal cord NK1 receptors, the second phase of formalin responding, and the late onset of NK1 antagonist antinociception, are all interrelated. Indeed, studies in the adult literature have shown that intrathecal administration of NK1 antagonists attenuate the second phase of the formalin response in the rat, but have no impact on the first phase (Traub, 1996). However, caution is warranted in this conclusion since the NK1 antagonists have been found to block both phases of the for-

malin response in other species, such as the mouse (Garret et al., 1991; Rupniak et al., 1993; Sakurada et al., 1995).

Systemic and local administration of the NK1 antagonists CP99,994 and LY703,606 were antinociceptive. However, both the NK1 antagonists, and the less active enantiomer CP100,263 attenuated nociceptive response in both 3-day and 21-day old subjects, suggesting that the antinociception is not specific to antagonism of the NK1 receptor, or that actions other than those at the NK1 receptor override any specific NK1 antagonism. In the adult literature, several researchers have reported that systemic administration of NK1 antagonists produces an antinociception that is not limited to the active enantiomers (Rupniak et al., 1995; Smith et al., 1994). Peripheral administration of high doses of these antagonists also attenuated both the early and the late phases of the formalin response in the adult (Seguin et al., 1995). We also found that both phases of the formalin response were reduced after intradermal administration of the highest dose (100 µg) of both CP99,994 and CP100,263 in the 21-day old subjects, and subcutaneous administration of the highest dose (30 mg/kg) of both drugs in 3-day and 21-day old subjects.

One possible explanation for these findings is that the systemic administration may produce its effect at multiple sites, including the periphery and the brain. It has been demonstrated that both CP99,994 and CP100,263 are capable of crossing the blood brain barrier (McLean et al., 1993), therefore it would be expected that subcutaneous administration of the CP99,994 would be effective in the same manner as the intrathecal administration. However, since the subcutaneous and the local administration of both the CP99,994 and its less active enantiomer produced analgesia, and since the inactive enantiomer did not produce antinociception when administered intrathecally, the nonneurokinin effects may mask, or enhance, the effects that antagonism of the spinal NK1 receptors would have produced.

It is possible that these compounds produce analgesia by blocking ion channels (Rupniak et al., 1995). Several of the NK1 antagonists have been shown to bind to L-type calcium channels, and it has been proposed that the antinociception induced by systemic administration of these compounds is due to the blockade of these channels (Rupniak et al., 1995). One potential problem for this explanation is that it has been demonstrated that CP99,994 and CP100,263 have lower affinity for these calcium channels at the dihydropyridine site than other compounds such as CP-96,345 (McLean et al., 1993). However, it has been shown that these compounds have equivalent affinity for another binding site on the calcium channel, the phenylalkylamine site (Lombet and Spedding, 1994). Moreover, blockade of other ion channels, such as the sodium

channels, might cause antinociception (Karlsson et al., 1994; Rupniak et al., 1995). Understanding the mechanisms by which the peripheral and systemic administration of these compounds produced antinociception might provide important advances in the understanding and development local analgesics.

The data presented here demonstrate that intrathecal injection of NK1 antagonists produced antinociception in a stereospecific manner in rats 14-days of age and older while peripheral administration of these compounds produced antinociception that was not stereospecific. This indicates that the antinociception produced by the intrathecal administration of the NK1 antagonist is produced through actions at the NK1 receptor while the systemic and local administration of these compounds produces antinociception through mechanisms other than the NK1 receptor. The results from the intrathecal injections also show that the spinal NK1 receptors start to play a role in nociceptive processing in the rat between the ages of 10 and 14-days after birth, in agreement with other data suggesting a late appearance of substance P and C-fiber mediated nociception.

REFERENCES

- Barr GA. 1998. Maturation of the biphasic behavioral and heart rate response in the formalin test. *Pharmacol Biochem Behav* 60:329–35.
- Barr GA. 1992. Neuropharmacology: concepts and methods of study. In: Shair HN, Barr GA, Hofer MA, editors. *Developmental psychology: new methods and changing concepts*. New York: Oxford University Press, p 321–341.
- Barr GA, Miya DY, Paredes W. 1992. Analgesic effects of intraventricular and intrathecal injection of morphine and ketocyclazine in the infant rat. *Brain Res* 584:83–91.
- Birch PJ, Harrison SM, Hayes AG, Rogers H, Tyers MB. 1992. The non-peptide NK1 receptor antagonist, (+/-)-CP-96,345, produces antinociceptive and anti-oedema effects in the rat. *Br J Pharmacol* 105:508–10.
- Buritova J, Honore P, Besson JM. 1996. Ketoprofen produces profound inhibition of spinal c-Fos protein expression resulting from an inflammatory stimulus but not from noxious heat. *Pain* 67:379–89.
- Chapman V, Dickenson AH. 1993. The effect of intrathecal administration of RP67580, a potent neurokinin 1 antagonist on nociceptive transmission in the rat spinal cord. *Neuroscience Letters* 157:149–52.
- Charlton CG, Helke CJ. 1986. Ontogeny of substance P receptors in rat spinal cord: quantitative changes in receptor number and differential expression in specific loci. *Brain Res* 394:81–91.
- Coimbra A, Ribeiro, Da Silva A, Pignatelli D. 1986. Rexed's laminae and the acid phosphatase (FRAP)-band in the superficial dorsal horn of the neonatal rat spinal cord. *Neurosci Lett* 71:131–6.
- Cridland RA, Henry JL. 1986. Comparison of the effects of substance P, neurokinin A, physalaemin and elodeisin in facilitating a nociceptive reflex in the rat. *Brain Res* 381:93–9.
- De Biasi S, Rustioni A. 1988. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci USA* 85:7820–4.
- Fitzgerald M. 1991. Development of pain mechanisms. *Br Med Bull* 47:667–75.
- Fitzgerald M, Gibson S. 1984. The postnatal physiological and neurochemical development of peripheral sensory C fibres. *Neuroscience* 13:933–44.
- Fitzgerald M, Zeller WP, Goto M, Anderson CL, Hurley RM. 1988. Concurrent clinical and metabolic derangements in the newborn rat: a late phase sepsis model. *Ann Clin Lab Sci* 18:229–34.
- Garland AM, Grady EF, Lovett M, Vigna SR, Frucht MM, Krause JE, Bunnnett NW. 1996. Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors. *Mol Pharmacol* 49:438–46.
- Garret C, Carruette A, Fardin V, Moussaoui S, Peyronel JF, Blanchard JC, Laduron PM. 1991. Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc Natl Acad Sci USA* 88:10208–12.
- Guy ER, Abbott FV. 1992. The behavioral response to formalin in preweanling rats. *Pain* 51:81–90.
- Holson RR, Pearce B. 1992. Principles and pitfalls in the analysis of prenatal treatment effects in multiparous species. *Neurotoxicol Teratol* 14:221–8.
- Honore P, Buritova J, Besson JM. 1996. Intraplantar morphine depresses spinal c-Fos expression induced by carrageenin inflammation but not by noxious heat. *Br J Pharmacol* 118:671–80.
- Karlsson U, Nasstrom J, Berge OG. 1994. (+/-)-CP-96,345, an NK1 receptor antagonist, has local anaesthetic-like effects in a mammalian sciatic nerve preparation. *Regul Pept* 52:39–46.
- Liu H, Mantyh PW, Basbaum AI. 1997. NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature* 386:721–4.
- Lombet A, Spedding M. 1994. Differential effects of non-peptidic tachykinin receptor antagonists on Ca²⁺ channels. *Eur J Pharmacol* 267:113–5.
- Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE. 1995. Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. *Proc Natl Acad Sci USA* 92:2622–6.
- Marvizon JC, Martinez V, Grady EF, Bunnnett NW, Mayer EA. 1997. Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J Neurosci* 17:8129–36.
- McLean S, Snider RM, Desai MC, Rosen T, Bryce DK, Longo KP, Schmidt AW, Heym J. 1993. CP-99,994, a nonpeptide antagonist of the tachykinin NK1 receptor. *Regul Pept* 46:329–31.
- Mjelle-Joly N, Lund A, Berge OG, Hole K. 1991. Potentiation of a behavioural response in mice by spinal coadministration of substance P and excitatory amino acid agonists. *Neurosci Lett* 133:121–4.
- Moochhala SM, Sawynok J. 1984. Hyperalgesia produced by intrathecal substance P and related peptides: desensitization and cross desensitization. *Br J Pharmacol* 82:381–8.
- Nagahisa A, Asai R, Kanai Y, Murase A, Tsuchiya NM, Nakagaki T, Shieh TC, Taniguchi K. 1992. Non-specific activity of (+/-)-CP-96,345 in models of pain and inflammation. *Br J Pharmacol* 107:273–5.
- Paredes W, Hughes HE, Giordano J, Barr GA. 1990. Methods of injecting drugs directly into the spinal cord of neonatal rats. *Lab Animal* 19:39–41.
- Pickel VM, Sumal KK, Miller RJ. 1982. Early prenatal development of substance P and enkephalin-containing neurons in the rat. *J Comp Neurol* 210:411–22.
- Piercey MF, Schroeder LA, Folkers K, Xu JC, Horig J. 1981. Sensory and motor functions of spinal cord substance P. *Science* 214:1361–3.
- Radhakrishnan V, Henry JL. 1995. Antagonism of nociceptive responses of cat spinal dorsal horn neurons in vivo by the NK-1 receptor antagonists CP-96,345 and CP-99,994, but not by CP-96,344. *Neuroscience* 64:943–58.
- Ruda MA, Bennett GJ, Dubner R. 1986. Neurochemistry and neural circuitry in the dorsal horn. *Prog Brain Res* 66:219–68.
- Rupniak NM, Webb JK, Williams AR, Carlson E, Boyce S, Hill RG. 1995. Antinociceptive activity of the tachykinin NK1 receptor antagonist, CP-99,994, in conscious gerbils. *Br J Pharmacol* 116:1937–43.
- Rupniak NMJ, Boyce S, Williams AR, Cook G, Longmore J, Seabrook GR, Caesar M, Iversen SD, Hill RG. 1993. Antinociceptive activity of NK1 receptor antagonists: non-specific effects of racemic RP67580. *British Journal of Pharmacology* 110:1607–13.
- Sakurada T, Katsumata K, Yogo H, Tan NK, Sakurada S, Ohba M, Kisara K. 1995. The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. *Pain* 60:175–80.
- Santos AR, Calixto JB. 1997. Further evidence for the involvement of tachykinin receptor subtypes in formalin and capsaicin models of pain in mice. *Neuropeptides* 31:381–9.
- Seguin L, Le Marouille, Girardon S, Millan MJ. 1995. Antinociceptive profiles of non-peptidergic neurokinin1 and neurokinin2 receptor antagonists: a comparison to other classes of antinociceptive agent. *Pain* 61:325–43.
- Smith G, Harrison S, Bowers J, Wiseman J, Birch P. 1994. Non-specific effects of the tachykinin NK1 receptor antagonist, CP-99,994, in antinociceptive tests in rat, mouse and gerbil. *Eur J Pharmacol* 271:481–7.

- Teng CJ, Abbott FV. 1998. The formalin test: a dose-response analysis at three developmental stages. *Pain* 76:337-47.
- Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. 1992. The formalin test: an evaluation of the method [see comments]. *Pain* 51:5-17.
- Traub RJ. 1996. The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. *Pain* 67:151-61.
- Velazquez RA, Kitto KF, Larson AA. 1997. CP-96,345, which inhibits [3H] substance P binding, selectively inhibits the behavioral response to intrathecally administered N-methyl-D-aspartate, but not substance P, in the mouse. *J Pharmacol Exp Ther* 281:1231-7.
- Williams S, Evan G, Hunt SP. 1990. Spinal c-fos induction by sensory stimulation in neonatal rats. *Neurosci Lett* 109:309-14.
- Yamamoto T, Yaksh TL. 1991. Stereospecific effects of a nonpeptidic NK1 selective antagonist, CP-96,345: antinociception in the absence of motor dysfunction. *Life Sci* 49:1955-63.
- Yashpal K, Pitcher GM, Henry JL. 1995. Noxious peripheral stimulation produces antinociception mediated via substance P and opioid mechanisms in the rat tail-flick test. *Brain Res* 674:97-103.
- Yi DK, Barr GA. 1995. The induction of Fos-like immunoreactivity by noxious thermal, mechanical and chemical stimuli in the lumbar spinal cord of infant rats. *Pain* 60:257-65.
- Yi DK, Barr GA. 1997. Formalin-induced c-fos expression in the spinal cord of fetal rats. *Pain* 73:347-54.