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Chronic morphine increases Fos-positive neurons after concurrent cornea and tail stimulation

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Abstract

Objective—The aim of the present study was to examine the effect of chronic morphine exposure on diffuse noxious inhibitory controls in a large population of neurons throughout the medullary dorsal horn, as assessed using immunocytochemistry for c-Fos protein.

Background—Overuse of medications, including the opioids, to treat migraine headache can lead to progressively more frequent headaches. In addition, chronic daily headache sufferers and chronic opioid users both lack the inhibition of pain produced by noxious stimulation of a distal body region, often referred to as diffuse noxious inhibitory controls.

Methods—In urethane anesthetized rats, Fos-positive neurons were quantified in chronic morphine and vehicle treated animals following 52°C noxious thermal stimulation of the cornea with and without the application of a spatially remote noxious stimulus (placement of the tail in 55°C water).

Results—When compared to chronic morphine treated animals that did not receive the spatially remote noxious stimulus, chronic morphine treated animals given corneal stimulation along with the spatially remote noxious stimulus demonstrated a 163% increase ($p < 0.05$) in the number of Fos-positive neurons in the superficial laminae of the medullary dorsal horn and a 682% increase ($p < 0.01$) in deep laminae that was restricted to the side ipsilateral to the applied stimulus. In contrast, no significant difference was found in Fos-like immunoreactivity in vehicle treated animals given concurrent cornea and tail stimulation or only cornea stimulation in either superficial or deep laminae.

Conclusions—It is proposed that an increase in descending facilitation and subsequent loss of diffuse noxious inhibitory controls contributes to the development of medication overuse headache.

Keywords

Medication overuse headache; trigeminal nucleus; chronic morphine; pain; rat

Introduction

Opioids remain one of the most effective treatments for moderate to severe pain. Its long-term use, however, can lead to tolerance and in some cases increased pain, which is well documented in migraine sufferers^{1–4}. The use of symptomatic medications, including

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opioids, for the treatment of migraines can induce chronic daily headaches, a condition referred to as medication overuse headache (MOH) ⁵⁻⁷.

Studies in both humans and animals have implicated descending pain modulatory systems in the pathophysiology of MOH and other opioid-induced abnormal pain ⁸⁻¹². One such pain modulatory brain region affected by chronic morphine administration is the rostral ventromedial medulla (RVM) ^{2,10}. The RVM, via projections through the dorsal lateral funiculus (DLF), can both inhibit and facilitate nociceptive signals at the level of the spinal and medullary dorsal horn (MDH) ¹³. Following prolonged systemic exposure to morphine, an increase in the proportion of pro-nociceptive on-cells has been reported in the RVM ¹⁰. This relative increase in on-cells may contribute to opioid-induced abnormal pain. As evidence, inactivation of the RVM or lesions of the DLF attenuated thermal and tactile hypersensitivity produced by chronic morphine administration ¹².

Chronic morphine may affect descending modulation from other brain regions in addition to the RVM. A recent study found that, in morphine treated animals, MDH dura-sensitive neurons showed less inhibition to the application of a spatially remote noxious stimulus (diffuse noxious inhibitory controls, DNIC) ⁹. Impairment of DNIC also has been noted in chronic daily headache sufferers as well as in chronic opioid users ^{8,14-16}. The DNIC pathway includes connections from second-order dorsal horn neurons to the subnucleus reticularis dorsalis (SRD), which, in turn, projects to the spinal and MDH ¹⁷. In animals, DNIC is often examined using single unit recordings from MDH neurons, however its influence has also been examined using immunocytochemistry for Fos, the protein product of the proto-oncogene *c-fos* ¹⁸⁻²².

The quantification of Fos-like immunoreactivity (Fos-LI) as a marker for neuronal activity has been used extensively to study the activation of spinal and MDH neurons ²³. Following noxious stimulation, Fos-LI is produced in a somatotopically organized and intensity-dependent manner ²⁴⁻²⁸. Furthermore, treatments that inhibit nociceptive neurons, such as acute morphine administration or RVM stimulation, reduce the number of Fos positive neurons produced by noxious stimulation ²⁹⁻³¹. In the present study, the effect of prolonged systemic morphine exposure on DNIC was assessed by quantifying the number of Fos-positive neurons produced by noxious thermal stimulation of the cornea with and without noxious stimulation to the tail. This allowed for the examination of a large population of neurons throughout the MDH, which complements the previous single unit electrophysiology study performed in similarly treated rats ⁹.

Materials and Methods

General methods

Male Sprague-Dawley rats were group housed (2-3/cage) in a climate-controlled environment with a 12 h light/dark cycle. Animals weighing 175-225 g were implanted subcutaneously with two 75 mg morphine sulfate or placebo pellets (generous gift of NIDA) under isoflurane anesthesia. Seven days post-implantation, morphine and vehicle treated animals were anesthetized with urethane (2.0 g/kg) and assigned to one of four treatment groups: 52°C corneal stimulation, 52°C corneal stimulation plus 55°C tail stimulation (as the DNIC stimulus), 55°C tail stimulation, or no stimulation.

Corneal stimulation, delivered to the left cornea, was applied for 20 seconds, repeated every minute, for 20 minutes using a 5 mm² contact thermode (Medoc, Minneapolis, MN). The cornea plus tail stimulation group had the distal third of the tail placed into a 55°C water bath ten seconds prior to and during the corneal stimulation. The tail was removed from the water bath at the same time that the thermal stimulus was discontinued. Animals that just

received tail stimulation had the distal third of the tail placed in the 55°C water bath for 30 s out of every minute for a total of 20 min. Control animals received neither corneal nor tail stimulation (No Stimulation). Animals were perfused two hours after the presentation of the first stimulus with heparinized 0.1 M phosphate buffer with saline (PBS) followed by 10% formalin. The brainstem was removed and post-fixed overnight in 10% formalin. All protocols were approved by the Committee on Animal Research at the University of New England, and animals were treated according to the policies and recommendations of the NIH guidelines for the handling and use of laboratory animals.

Immunocytochemistry

Alternate 50 µm Vibratome sections were collected into wells containing 0.1M PBS. Free floating sections were incubated for an hour in 3% normal goat serum before being agitated overnight at 4°C in a 1:1000 dilution of rabbit polyclonal anti-Fos antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1M PBS with 1% normal goat serum and 0.3% Triton X. The next morning, sections were washed in 0.1M PBS and incubated in a 1:200 dilution of biotinylated goat anti-rabbit secondary (Vector, Burlingame, CA) containing 1% normal goat serum for 60 min. After washing (0.1M PBS), sections were incubated in an avidin-biotin peroxidase (ABC, Vector) complex solution for 60 min. Sections were then reacted for 3-6 min with diaminobenzidine (0.7 mg/kg, Sigma, St Louis, MO) and hydrogen peroxide (0.17 mg/ml), intensified with nickel ammonium sulfate (1.25%) and cobalt chloride (1.0%). After a final wash, sections were mounted onto slides and coverslipped. Specific staining was lost either by omission of primary antibody or by pre-incubation of the primary antibody overnight with the original immunogen (Santa Cruz).

Quantification of Fos-positive neurons

Neurons located in the spinal trigeminal nucleus with Fos- LI were counted in all sections from 2 mm rostral to obex to 6 mm caudal to obex ²⁷. Under bright field illumination, Fos-positive neurons were clearly distinguished from background by the appearance of homogeneous brown-black precipitate. The number of Fos-positive neurons was quantified at 100× magnification by two individuals, both blinded to the experimental condition. After training sessions, variation in counts between the two investigators was less than ten percent. Separate counts were made for superficial (I-II) and deep (III-V) laminae of trigeminal subnucleus caudalis (Vc) and dorsal horn of the first and second cervical vertebrae (C1 and C2), as well as of Fos-positive neurons at the border of Vc and trigeminal nucleus interpolaris (Vi). In cases in which the cornea was not stimulated, the left side was considered the ipsilateral side.

Sections were organized in accordance with their AP levels with respect to obex in 0.5 mm increments and the mean number of Fos-positive neurons was averaged for each level. In addition, to assess the total number of Fos-positive neurons within the Vi/Vc transition region and the more caudal Vc/C1 transition regions, the average number of cells for each level within the two regions (Vi/Vc, 0.0 and -0.5 mm relative to obex, and Vc/C1, -3.0 to -5.0 mm relative to obex) were summed and the sums averaged over each treatment group.

Statistical analysis

Average counts of Fos-positive neurons for each anterior-posterior level were calculated for each treatment group (e.g. corneal stimulation with morphine, corneal stimulation with vehicle). Individual comparisons across treatments for each rostrocaudal level were analysed with a two-way ANOVA where Fos-LI at each AP level for individual rats were treated as repeated measures. In addition, group averages for the total number of Fos-positive neurons located at the Vi/Vc transition region (total number of Fos positive neurons at AP levels 0.0 and -0.05) and superficial and deep regions at the Vc/C1 transition region (total number of

Fos positive neurons at AP levels -3.0 to -5.0) were compared with two-way ANOVAs where the stimulus was treated as one factor, and drug treatment (morphine or vehicle) was the other factor in the analyses. All data are based on averages from 4 rats per treatment condition except for 5 rats treated with morphine that were given corneal stimulation. Analysis was performed using SYSTAT® (v. 11). Significant differences between treatment group means was determined with Tukey-Kramer's post hoc test, with significance defined as $p < 0.05$. Hypotheses testing was two-tailed. Data are presented as mean $\pm$ SEM.

Results

Application of 52°C heat to the cornea produced two distinct regions of elevated levels of Fos-positive neurons in both vehicle and morphine treated animals: one located in the ventrolateral region at the transition between trigeminal subnucleus interpolaris and caudalis (Vi/Vc transition region, Fig. 1A), with peak Fos-LI observed at AP levels 0.0 mm and -0.5 mm posterior to obex (Fig. 1C), and another located more posterior at the transition between Vc and the first cervical vertebra (Vc/C1 transition region, Fig. 1B), with peak Fos-LI observed from -3.0 mm to -5.0 mm posterior to obex (Fig. 1C). In each of these regions, corneal stimulation evoked Fos-LI that was restricted to the side ipsilateral to the stimulus (Fig. 1C). Rats that did not receive noxious thermal stimulation of the cornea did not show significant levels of Fos-LI throughout the rostro-caudal length of the spinal trigeminal nucleus regardless of whether they also had been pretreated with morphine or vehicle or whether they received noxious tail stimulation or not (data not shown). In all treatment groups, additional Fos-LI was present in the medial solitary tract and the lateral reticular nucleus (Fig. 1A).

A two-way ANOVA with repeated measures performed on the Fos-positive neurons counted in the trigeminal nucleus on the side ipsilateral to the corneal stimulus showed a significant treatment effect ($F(7, 25)=20.50$, $p<0.001$). In addition, Fos-LI located on the ipsilateral side showed a significant anterior-posterior level effect ($F(16, 400)=53.341$, $p<0.001$) and interaction effect ($F(112, 400)=9.781$, $p<0.001$). Post hoc testing revealed that the number of Fos-positive neurons in chronic morphine treated rats that received cornea and tail stimulation were significantly different from all other treatment groups at 3.5 mm and 4.0 mm posterior to obex (Fig. 1C, $p<0.01$). At these two anterior-posterior levels, Fos-LI in chronic morphine treated animals receiving both cornea and tail stimulation was 201% (3.5 mm level) and 222% (4.0 mm level) greater than chronic morphine treated animals that received only cornea stimulation. When compared to vehicle treated animals that received both cornea and tail stimulation, Fos-LI was 233% (3.5 mm level) and 249% (4.0 mm level) greater in morphine treated animals given the same stimulus.

A two-way ANOVA with repeated measures comparing Fos-LI on the side contralateral to the corneal stimulus showed a significant treatment effect ($F(7, 25)=2.467$, $p=0.045$) and anterior-posterior level effect ($F(16, 400)=9.177$, $p<0.001$) but no interaction effect ($F(112, 400)=0.99$, $p=0.526$). However, post hoc analysis of means between treatment groups did not reveal any statistically significant differences.

In vehicle treated animals, corneal stimulation-evoked Fos-LI was observed mainly in superficial laminae at the Vc/C1 transition region, with far fewer Fos-positive neurons detected in deeper laminae (Fig. 2A). This pattern of Fos expression was not the case for morphine treated animals given both cornea and tail stimulation (Fig. 2B), in which significantly greater levels of Fos-LI were observed in deeper laminae.

Additional analysis was performed on total Fos-LI located at the Vc/C1 transition region (from 3.0-5.0 mm posterior to obex) in both superficial (I-II) and deep (III-V) laminae. A

two-way ANOVA of superficial Fos-LI showed a main effect of stimulus ($F(3,25) = 57.21$, $p < 0.001$), a main effect of drug treatment ($F(1,25)$, $p=0.04$), and an interaction effect ($F(3,25)=7$, $p<0.001$). Post hoc analysis revealed that, as expected, in superficial laminae of Vc/C1 stimulation of the cornea in both vehicle and morphine treated rats induced an increase in Fos-LI when compared to their respective unstimulated vehicle and morphine control groups (Fig. 3A, $p<0.01$). In vehicle treated rats, the inclusion of tail stimulation with cornea stimulation (DNIC treatment) produced a non-significant 30% decrease in Fos-LI when compared to Fos-LI induced by cornea stimulation alone (Fig. 3A, $p>0.05$). A comparison of vehicle and morphine treated animals that received only cornea stimulation found no significant differences in the amount of Fos-LI (Fig. 3A, $p>0.05$). However, morphine treated rats that received cornea plus tail stimulation (DNIC treatment) demonstrated a 210% increase in Fos-LI when compared to vehicle treated DNIC animals (Fig. 3A, $p<0.01$), and this same group of rats showed a 163% increase relative to morphine treated animals that received only corneal stimulation (Fig. 3A, $p<0.05$). In rats that did not receive corneal stimulation, tail stimulation alone had no effect of Fos-LI when compared to animals unstimulated animals (Fig. 3A, $p<0.05$). Furthermore, similar levels of Fos-LI were observed in morphine and vehicle treated animals that received no stimulation (Fig. 3A, $p<0.05$).

Similar trends were observed when Fos-LI in deep laminae of Vc/C1 was analyzed. A two-way ANOVA showed a main effect of stimulus ($F(3,25)=6.04$, $p=0.002$) and an interaction effect ($F(3,25)=3.79$, $p=0.023$) but no main effect of drug treatment ($F(1,25)=1.684$, $p=0.206$). Post hoc analysis indicated that morphine treated animals given both cornea and tail stimulation (DNIC) showed a significant, 420% increase in Fos-LI when compared to vehicle treated animals that received cornea and tail stimulation, and a 682% increase in Fos-LI when compared to morphine treated rats that received only corneal stimulation (Fig. 3B, $p<0.05$). In vehicle treated rats, the inclusion of tail stimulation produced a non-significant 33% decrease in Fos-LI induced by corneal stimulation (Fig. 3B, $p>0.05$). Similar to Fos-LI expression in superficial laminae, tail stimulation and morphine treatment did not affect Fos-LI in deep lamina in rats that did not receive corneal stimulation. Finally, when compared to treatment groups that did not receive corneal stimulation, only corneal stimulated rats that received morphine treatment and concurrent tail stimulation demonstrated a statistically significant increase in Fos-LI (Fig. 3B, $p<0.01$).

At the Vi/Vc transition region, a two-way ANOVA revealed a main effect of stimulus ($F(3,25)=10.025$, $p<0.001$) but not drug treatment ($F(1,25)=2.29$, $p=0.143$) or interaction ($F(3,25)=0.474$, $p=0.703$). Post hoc testing revealed that the mean Fos-LI in morphine treated animals that received both corneal and tail stimulation was significantly greater than all groups that did not receive corneal stimulation (Fig. 3C, $p<0.05$). The increase in Fos-LI in the other groups that received corneal stimulation did not reach statistical significance when compared to groups that did not receive corneal stimulation (Fig. 3C).

Discussion

The present study examined the effect of 7-day sustained morphine exposure on the expression of Fos-LI following noxious thermal stimulation of the cornea in the presence and absence of a DNIC stimulus (placement of the tail in 55°C water). The cornea was chosen as the site for stimulation because it is densely innervated exclusively by small diameter primary afferents, is easily accessible, and a previous study found a lack of DNIC in chronic migraine patients using the eye blink reflex^{15,32-35}. Furthermore, the receptive fields of dura-sensitive trigeminal nucleus caudalis neurons often include the cornea, indicating significant convergence in the primary afferent neurons that innervate these two structures³⁶⁻³⁹. Thus, the cornea is an ideal site for stimulation that can provide insights into

the central processing of headache-related information. The results from this study may also be relevant to other chronic pain syndromes in which DNIC is attenuated, such as fibromyalgia⁴⁰.

As expected, noxious stimulation of the cornea produced Fos-LI in two main regions of the spinal trigeminal nucleus, the Vi/Vc transition region and the Vc/C1 transition region^{27,41,42}. Corneal stimulation evoked Fos-LI was not significantly affected by the DNIC stimulus in vehicle treated animals. In contrast, corneal stimulation induced Fos-LI was increased at the Vc/C1 transition region in morphine treated animals that also received concomitant tail stimulation (DNIC). The phenomenon of DNIC, sometimes referred to as counter-irritation, was initially described as inhibition of dorsal horn nociceptive activity by a distal noxious stimulus⁴³⁻⁴⁵. Analogous results have been obtained in human studies, using either psychophysical measurements or nociceptive reflexes as endpoints⁴⁵⁻⁴⁷. Neurons responsible for producing the inhibition appear to be located in the SRD and project to the dorsal horn via the DLF^{19,48-52}.

While DNIC has often been described in single-unit recordings of nociceptive neurons in the spinal and MDH, the application of heterotopic noxious stimulation has also been demonstrated to reduce noxious stimulation-evoked Fos-LI in the spinal cord lumbar dorsal horn^{18,20-22,53,54}, however to a much lesser extent. In studies that use single unit recordings of nociceptive neurons, 50-80% inhibition of noxious stimulation evoked activity is commonly observed with DNIC, however these studies tend to stimulate less frequently than the protocol used in the present study^{18,21,22,54,55}. In comparison, two studies using Fos-LI to measure the effect of DNIC found only a 15% and 33% reduction in animals that received concomitant heterotopic noxious stimulation^{20,53}. Although not statistically significant, we found a similar trend, with cornea plus tail stimulation producing a 30-33% reduction in Fos-LI when compared to those that received only corneal stimulation in vehicle treated animals. It is possible that the use of repetitive stimulation to induce Fos-LI reduced the inhibition normally seen with noxious stimulation of the tail in electrophysiological studies.

Recently, dysfunction of the DNIC system has been implicated in several chronic pain conditions. Compromised DNIC has been described in patients with fibromyalgia, irritable bowel syndrome, temporomandibular disorder and chronic daily headache, including chronic tension-type headache and chronic migraine^{14-16,56-59}. In one study, capsaicin application to the hand increased blink reflexes and subjective pain sensations induced by supraorbital nerve stimulation in chronic migraine, but not control, subjects¹⁵. Similarly, a study comparing the suppression of the nociceptive flexion RIII reflex by the cold pressor test in normal subjects with those who suffer from either chronic tension type headache or migraine headache found significant alterations in DNIC. While the cold pressor test suppressed the RIII nociceptive reflex in normal subjects, it enhanced the RIII nociceptive reflex in the headache sufferers⁶⁰. The enhancement of noxious stimulation-evoked responses in these studies suggests the presence of increased descending facilitation, as does our finding that concomitant heterotopic noxious stimulation produced an increase in Fos-LI in chronic morphine treated animals.

Another study has demonstrated an attenuation of DNIC in chronic opioid users⁸, which may be related to the development of medication overuse headache in migraineurs, as well as in sufferers of MOH prior to medication withdrawal⁶¹. Consistent with these findings, a recent study in rats found that chronic morphine exposure reduced DNIC in dura-sensitive neurons recorded in the MDH⁹. In the present study, the increase in Fos-LI observed at the Vc/C1 transition region in animals given concomitant noxious stimulation is consistent with this previous work. The dramatic increase in Fos-LI in these animals, however, was somewhat unexpected as the single unit recordings indicated that chronic morphine

produced only a loss of DNIC, and did not enhance activity above control levels. This discrepancy may be due to the aforementioned differences in protocols used to elicit neuronal activity versus Fos-LI. Alternatively, the addition of a DNIC stimulus in chronic morphine treated animals may activate an additional subset of normally quiescent neurons that were not previously sampled in the electrophysiology experiments, yet uncovered using Fos-LI. The dramatic increase in Fos-LI in morphine treated animals presented with concurrent corneal and tail stimulation seems to indicate an increase in descending facilitation under these conditions, rather than simply a decrease in descending inhibition.

Evidence indicates an involvement of RVM pain facilitating neurons in chronic morphine-induced changes in sensory processing at the level of the dorsal horn^{2,11,62,63}. Chronic morphine caused an increase in pronociceptive on-cells within the RVM¹⁰, and inactivation of the RVM reduced chronic morphine-induced behavioral hyperalgesia⁶³. Furthermore, DLF lesions attenuated the increase in excitatory transmission within the spinal cord dorsal horn observed after sustained morphine exposure⁶². Since RVM pronociceptive on-cells are activated by noxious stimulation, it is possible that greater activation of these cells, elicited by the DNIC stimulus in chronic morphine treated animals, is responsible for the increased Fos-LI and reduction in DNIC.

Neurons located in the subnucleus reticularis dorsalis (SRD), which project directly to the spinal and medullary dorsal horn, appear to be responsible for producing DNIC^{18,50,64-66}. DNIC is unaffected by brainstem transections rostral to the SRD, whereas DNIC is abolished when transections are performed caudal to the SRD^{67,68}. Additionally, direct lesions of the SRD (and not the RVM) attenuate DNIC^{45,69,70}. Although RVM lesions do not normally affect DNIC, the descending inhibition produced by DNIC stimuli and descending facilitation produced by activation of RVM on-cells appear to interact under certain conditions. After chronic morphine, in which DNIC is attenuated, inactivation of the RVM was able to reinstate DNIC in dura-sensitive trigeminal neurons⁹. Thus, it appears that the loss of DNIC after chronic morphine may be due to an increase in descending facilitation, whereby an increase in facilitation from the RVM counteracts the inhibition normally produced by the SRD. The exact nature of the interaction between the RVM and SRD remains to be elucidated. While neurons in both the RVM and SRD project to the dorsal horn, the SRD also receives direct input from the RVM¹⁷.

The absence of morphine-induced changes in Fos-LI at the Vi/Vc transition region was not unexpected. Neurons at the Vi/Vc transition region control tearing and blinking responses to corneal stimulation⁷¹ and do not typically demonstrate inhibition with the presentation of a heterotopic noxious stimulus²². Furthermore, unlike corneal-responsive neurons located at the Vc/C1 transition region, Vi/Vc neurons are usually excited rather than inhibited by morphine administration⁷².

In summary, chronic administration of morphine produced an increase in Fos-LI at the Vc/C1 transition region when corneal stimulation was paired with noxious stimulation of a distal region of the body. These results strengthen previous findings demonstrating a lack of DNIC in dura-sensitive neurons following chronic morphine exposure⁹. Deficiencies in DNIC, caused either by a decrease in descending inhibition or an increase in descending facilitation, may be particularly relevant as a cause of medication overuse headache. Additional studies are necessary to determine whether other classes of migraine medications produce similar effects.

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Abbreviations

DNIC	Diffuse noxious inhibitory controls
MDH	medullary dorsal horn
MOH	medication overuse headache
RVM	rostral ventromedial medulla
DLF	dorsal lateral funiculus
PBS	phosphate buffer with saline
Fos-LI	Fos-like immunoreactivity
Vc	trigeminal subnucleus caudalis
Vi	trigeminal subnucleus interpolaris
SRD	subnucleus reticularis dorsalis
NTS	nucleus tractus solitarius
LRN	lateral reticular nucleus

Figure 1. Fos-like immunoreactivity in the spinal trigeminal nucleus following corneal stimulation. **A)** Camera lucida drawing of a representative section at the Vi/Vc transition region in a morphine treated animal that received cornea and tail stimulation. **B)** Drawing of a representative section at the Vc/C1 transition region in the same animal depicted in panel A. **C)** Stimulation of the cornea evoked Fos-LI at both the Vi/Vc and Vc/C1 regions, ipsilateral to the side of stimulation. Concomitant cornea and tail stimulation produced greater Fos-LI at the Vc/C1 region in chronic morphine treated animals when compared to all other groups. Open symbols, vehicle treatment; closed symbols, morphine treatment; squares, cornea plus tail stimulation; circles, cornea only stimulation. aa, $p < 0.01$ vs all other treatment groups at the same anterior-posterior (AP) level. NTS, nucleus tractus solitarius; LRN, lateral reticular nucleus.

Figure 2.

Fos-like immunoreactivity at the Vc/C1 transition region in morphine treated animals given (A) corneal stimulation and (B) concomitant corneal and tail stimulation. Without the DNIC stimulus, Fos-LI was localized mainly in the superficial laminae with relatively few deep cells, whereas Fos-LI in chronic morphine treated animals given cornea and tail stimulation was located in both the superficial and deep laminae.

Figure 3.

Total number of Fos-positive neurons located in **A)** superficial laminae at the Vc/C1 transition region, **B)** deep laminae at the Vc/C1 transition region, and **C)** the Vi/Vc transition region. All counts represent Fos-LI located on the side ipsilateral to the corneal stimulus in vehicle (open bars) and chronic morphine (solid bars) treated animals (see text for details). aa, $p < 0.01$ vs comparable groups that did not receive corneal stimulation; b, $p < 0.05$, bb, $p < 0.01$ vs vehicle treated group with corneal and tail stimulation; c, $p < 0.05$ vs morphine treated group with corneal stimulation but no tail stimulation.