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VISUALIZING ACUTE PAIN - MORPHINE INTERACTION IN DESCENDING MONOAMINE NUCLEI WITH FOS

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Abstract

The effect of morphine is often studied in the absence of pain, and it remains poorly understood if and how noxious stimulation may change the activity state of descending pain-modulatory pathways and their response to morphine. Immunohistochemical double-labeling technique with Fos and markers for noradrenergic and serotonergic neurons was used to examine if an intraplantar formalin injection (an acute noxious input) changed the effect of morphine on noradrenergic neurons of the A7 and A5 cell groups, and serotonergic neurons of the nucleus raphe magnus (NRM). Four groups of rats were analyzed: (1) CONTROL injected with normal saline subcutaneously, (2) rats treated with FORMALIN into the hind paw 30 minutes after subcutaneous normal saline injection, (3) rats injected with MORPHINE sulfate subcutaneously, and (4) rats treated with formalin into the hind paw 30 minutes after subcutaneous morphine injection (MORPHINE/FORMALIN). The average number of total Fos-labeled cells per section was unchanged in all areas of analysis in all treatment groups. However, the percentage of noradrenergic neurons in the A7 and A5 cell groups that contained Fos was significantly increased in the morphine/formalin group compared to all other groups, while no differences were found in serotonin cells in the NRM. In contrast with the view that morphine simply blocks access of nociceptive information to supraspinal brain areas, these data suggest that noxious stimulation has the capacity to modify the actions of morphine on brainstem noradrenergic nuclei, which may participate in descending pain-modulation as well as other behavioral responses to pain.

Keywords

brainstem; formalin; Fos; morphine; pain; rat

1. INTRODUCTION

Morphine activates descending pain-modulatory pathways originating from the brainstem that are thought to 'gate' access of nociceptive information to supraspinal areas at the level of the spinal cord dorsal horn (Basbaum and Fields, 1984; Fields and Basbaum, 1999; Jones, 1992;

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Mason, 1999). The antinociception produced by morphine is mediated in part by monoaminergic neurons including (1) spinally projecting serotonergic neurons of the nucleus raphe magnus (NRM) in the ventral medulla, as well as (2) spinally projecting noradrenergic neurons of the A7 and possibly those of the A5 cell groups in the brainstem (Jones, 1992).

Activation of neurons in NRM produces potent antinociception as a result of inhibition of spinal cord pain transmission pathways (Fields et al., 1991; Jones, 1992). Furthermore, NRM plays a major role in opiate-induced analgesia as it has been demonstrated that electrical or chemical lesion of the NRM prevents the antinociceptive effects of morphine (Chance et al., 1978; Proudfit and Anderson, 1975; Proudfit, 1980a; Proudfit, 1980b; Yaksh et al., 1977). Naloxone administered into the NRM attenuates the antinociception produced by systemic administration of morphine or electrical stimulation of this nucleus (Rivot et al., 1979). In addition, injection of morphine into the NRM attenuates the responses of dorsal horn neurons to noxious stimuli (Du et al., 1984).

Similar to serotonergic neurons of the NRM, noradrenergic neurons of the A7 cell group originating from the dorsolateral pontine tegmentum project to the superficial laminae of the rat spinal cord dorsal horn (Clark and Proudfit, 1991), where many second-order nociceptive neurons are located (Light, 1992). Both electrical (Yeomans et al., 1992) or chemical (Holden et al., 1999; Yeomans and Proudfit, 1992) stimulation of the A7 neurons produces antinociception that can be reduced by intrathecal injection of α_2 -adrenoreceptor antagonists. Finally, noradrenergic neurons of the A5 cell group innervate the deep dorsal horn and the intermediolateral cell column of the spinal cord (Byrum and Guyenet, 1987; Clark and Proudfit, 1993; Loewy et al., 1979b). These neurons appear to modulate nociception (Burnett and Gebhart, 1991; Miller and Proudfit, 1990) and cardiovascular reflexes in the rat (Drye et al., 1990; Loewy et al., 1979a; Loewy et al., 1979b; Loewy et al., 1986).

However, noxious stimulation itself is known to cause activation of some of the same areas in the medulla that are activated by morphine (Bullitt, 1990; Chang et al., 1988). Concentrations of both serotonin and noradrenaline in the spinal cord dorsal horn are increased following noxious stimulation (in the form of subcutaneous formalin injection) (Omote et al., 1998). Furthermore, noxious stimulation in the form of formalin injection has been reported to increase Fos expression in serotonergic neurons of the NRM (Chen et al., 2003; Lang and Li, 1998), as well as tyrosine-hydroxylase containing neurons of the A7 and A5 cell groups in the rat (Han et al., 2003). However, no studies have investigated how morphine activation of monoamine neurons located in regions that contribute to pain sensitivity and the behavioral response to pain may be modified by the presence of noxious input. This interaction is important for understanding how the effects of morphine may be different in pain versus non-pain states. Therefore, the goal of the present study was to examine anatomical markers of neuronal activation within serotonergic neurons of the NRM and noradrenergic neurons of the A7 and A5 cell groups following morphine administration in the presence and absence of noxious stimulation. We examined if an intraplantar formalin injection (an acute inflammatory noxious input) changes the effect of morphine on those monoamine neurons of the brainstem using the expression of Fos, an indirect marker of neuronal activity. Detection of Fos has been widely employed for functional mapping of the brain areas activated in processes affecting the brain (Sagar et al., 1988; Sharp et al., 1993) and has been well established for noxious stimulation (Bullitt, 1990) as well as for morphine effects in rat (Chang et al., 1988).

2. RESULTS

A total of 4 different treatment groups were used in this study: (1) control, (2) formalin, (3) morphine, or (4) morphine/formalin group. Anatomical areas of interest included noradrenergic A7 and A5 cell groups, as well as serotonergic neurons of NRM in ventromedial

medulla (Fig. 1). Immunoreactive profiles that were counted included Fos immunoreactive nuclei, tyrosine-hydroxylase immunoreactive noradrenergic neurons of the A7 and A5 cell groups, tryptophan-hydroxylase (TPOH) immunoreactive serotonergic neurons of the NRM, as well as double-labeled profiles (noradrenergic or serotonergic neurons labeled with Fos).

2.1. Fos expression

To confirm that Fos was detectable using immunofluorescence, the sections analyzed were first examined for Fos immunolabeling in other brainstem areas besides those of our interest for all treatment groups. The average number of Fos cells per section was unchanged in all the areas of analysis following any of the treatment groups. Graphic representation of Fos expression density in different anatomical regions studied is illustrated in Fig. 2. Note that the highest density of Fos immunoreactive labeling was found in NRM region.

2.2. Fluorescent microscopic analysis of Fos expression in the A7 cell group

Anatomical location of the A7 cell group in the dorsolateral pontine tegmentum has been schematically illustrated in Figure 1A. The total number of 1610 noradrenergic neurons of the A7 cell group were counted in all 24 brains with an average number ($\pm$ SD) of 67 ± 18.5 tyrosine hydroxylase-containing neurons per rat. Percentage of noradrenergic neurons that were double-labeled with Fos showed statistically significant difference between the four treatment groups ($F(3,20)=8.23$, $p=0.001$). Pairwise comparisons with Bonferroni post-hoc test of all groups showed statistically significant increase in the percentage of double-labeled neurons in the morphine/formalin group compared to control ($p=0.002$), formalin ($p=0.004$), and morphine ($p=0.011$) groups in the area of the A7 cell group (Fig. 3). More specifically, the percent of double-labeled immunoreactive noradrenergic neurons of the A7 cell group that contained Fos labeling ($\% \pm$ SD) were $0.8\% \pm 0.9$ for control, $1.6\% \pm 2.5$ for formalin, $2.9\% \pm 6.0$ for morphine, and $13.1\% \pm 7.2$ for morphine/formalin treatment group (Fig. 3). As illustrated in Fig. 4A, double-labeled noradrenergic neurons of the A7 cell group were distinguished by the presence of both tyrosine hydroxylase immunoreactivity of the neuronal body (green) and Fos labeling of the nucleus (red) in individual neurons.

2.3. Fluorescent microscopic analysis of Fos expression in the A5 cell group

Figure 1A–C schematically illustrates the anatomical location of the A5 cell group in the ventrolateral region of the caudal pons and the rostral medulla. For the A5 cell group, total of 3224 noradrenergic neurons were counted in all 24 brains with an average number ($\pm$ SD) of 134 ± 30.1 tyrosine hydroxylase-containing neurons per rat. Similar to the noradrenergic A7 cell group, the percentage of noradrenergic A5 neurons that were double-labeled with Fos showed statistically significant difference between the four treatment groups ($F(3,20)=12.76$, $p<0.001$). Pairwise comparisons with Bonferroni post-hoc test of all groups showed statistically significant increase in the percentage of double-labeled neurons in the morphine/formalin group compared to control ($p<0.001$), formalin ($p<0.001$), and morphine ($p=0.011$) groups in the area of the A5 cell group (Fig. 3). More specifically, the percentage of double-labeled noradrenergic neurons ($\% \pm$ SD) that contained Fos immunoreactivity in the A5 cell group were $3.6\% \pm 3.2$ for control, $6.3\% \pm 5.1$ for formalin, $12.9\% \pm 10.5$ for morphine, and $29.3\% \pm 10.2$ for morphine/formalin treatment group (Table 1). Figure 4B illustrates representative example of double-labeled noradrenergic neurons of the A5 cell group that were characterized by the presence of both tyrosine hydroxylase immunoreactivity of the neuronal body (green) and Fos labeling of the nucleus (red) in individual neurons.

2.4. Fluorescent microscopic analysis of Fos expression in the Nucleus Raphe Magnus

The anatomical location of the NRM in the ventromedial medulla is schematically illustrated in Fig. 1B and C. The highest estimated density of Fos immunoreactive labeling was found in

the NRM region (Fig. 2). The total number of 15,818 serotonergic neurons of the NRM were counted in all 24 brains with an average number ($\pm$ SD) of 659 ± 204 TPOH-containing neurons per rat. In contrast to the noradrenergic A7 and A5 cell groups, the percentage of serotonergic neurons that were double-labeled with Fos showed no statistically significant difference between the four treatment groups ($F(3,20)=2.02$, $p=0.144$) in the NRM. More specifically, the percentage of double-labeled serotonergic neurons ($\% \pm$ SD) that contained Fos immunoreactivity in the NRM were $3.3\% \pm 2.3$ for control, $5.4\% \pm 3.2$ for formalin, $2.4\% \pm 0.9$ for morphine, and $4.6\% \pm 2.1$ for morphine/formalin treatment group (Fig. 3).

Representative photomicrograph of labeling that included serotonergic neurons of the NRM (green) and Fos labeling of nuclei (red) is illustrated in Fig. 4C. Double-labeled profiles were distinguished by the presence of both neuronal (green) and nuclear stain (red) in the individual neuron. For the purpose of identifying adrenergic neurons that might be present in the most caudal regions of the NRM, triple labeling using Fos, tyrosine hydroxylase, and TPOH was used to determine the localization of those neurons and confirm the lack of double-labeling with Fos (data not shown). Tyrosine-hydroxylase neurons were clearly detectable, and were found lateral to the NRM corresponding to the adrenergic neurons of the C1 cell group. Although those neurons contained light immunolabeling for TPOH, none were dually labeled with Fos immunoreactivity.

3. DISCUSSION

The present study demonstrates that acute noxious stimulation produced by intraplantar formalin injection, increased morphine induced Fos expression in distinct populations of monoamine neurons of the brainstem. These included noradrenergic neurons of the A7 and A5 cell groups, but not serotonergic neurons of the NRM (Fig. 5). Immunofluorescence techniques were used to double-label tyrosine hydroxylase immunoreactive noradrenergic neurons of the A7 and A5 cell groups, as well as TPOH immunoreactive serotonergic neurons of the NRM, with Fos-immunocytochemistry. Although morphine is thought to activate descending pain-modulatory pathways that gate the transmission of nociceptive information from the spinal cord to higher levels, our results suggest that the presence of a noxious stimulus modifies morphine's activation of supraspinal circuits associated with descending pain modulation.

3.1. Methodological Considerations

Fos expression is thought to correlate with biochemical activity of cells (Hunt et al., 1987; Morgan et al., 1987) and is used as a marker of neuronal activity (Sagar et al., 1988; Sharp et al., 1993). However, Fos is an indirect marker of activity and as such is vulnerable to both false-negative and false positive error. For example, some neurons when activated may activate other immediate-early genes but not c-fos. Likewise, the amount of activation that is sufficient to initiate expression of c-fos may be different for different types of neurons. Taken together, this might lead to under-detection of activation (Dragunow and Faull, 1989). However, the major advantage of Fos is that it is robustly expressed and can be easily detected and quantified. More specifically, Fos protein expression has been well established for sensory (Hunt et al., 1987) and noxious stimulation (Bullitt, 1990; Hunt et al., 1987; Leah et al., 1989). In addition, it has been well established for morphine (Chang et al., 1988; Liu et al., 1994) and several drugs of abuse (Heilig et al., 1993; Persico et al., 1993; Umino et al., 1995) and has provided insight into the neuroanatomical regions activated by these drugs.

3.2. Noradrenergic Neurons

Noxious Stimulation—The results of the present study did not show any increase in Fos expression in either noradrenergic or non-noradrenergic neurons of the A7 and A5 cell groups following noxious stimulation alone in the form of intraplantar formalin injection. In contrast, it has been reported that the concentration of noradrenaline in the spinal cord dorsal horn

increases following subcutaneous 2% formalin injections (Omote et al., 1998), implicating the activation of bulbo-spinal noradrenergic descending inhibitory systems in the presence of formalin. In addition, Han et al. (2003) reported an increase in Fos expression in noradrenergic neurons of the rat brainstem including A7 and A5 cell groups after somatic as well as visceral noxious stimulation induced by 10% formalin. The discrepancy between the latter study and the current study might be due to the difference in formalin concentration used for noxious stimulation. Since we used a lower concentration of formalin (2.5%) it is possible that this concentration is below the threshold for affecting Fos expression in noradrenergic neurons of the A7 and A5 cell groups.

Morphine Effects—Current results did not show any changes in Fos expression in either noradrenergic or non-noradrenergic neurons of the A7 and A5 cell groups following systemic administration of morphine. In contrast, μ -opioid receptors are found in the area of the dorsolateral pontine tegmentum where noradrenergic A7 neurons are located, as well as in the ventrolateral medulla that includes noradrenergic A5 cell group (Herkenham and Pert, 1982; Mansour et al., 1987). Also, microinjection of morphine into the A7 cell group produces antinociception that can be reversed by intrathecal injection of α_2 -adrenoceptor antagonists (Holden et al., 1999). These findings and others (Fang and Proudfit, 1996; Fang and Proudfit, 1998; Yaksh, 1979) have lead to the hypothesis that morphine administration leads to disinhibition of spinally projecting noradrenergic neurons. The lack of Fos expression in these neurons further raises the possibility of sub-threshold activation of these areas by morphine administration.

Formalin Enhances Morphine Effect—In contrast to each treatment alone, acute noxious stimulation in the form of intraplantar 2.5% formalin injection in combination with morphine administration activated Fos expression in noradrenergic neurons of the A7 and A5 cell groups. These results implicate an interaction between morphine and noxious input (in the form of formalin injection) in supraspinal brain areas that involve A7 and A5 cell group. Since the total number of Fos density was unchanged among different treatment groups, our results implicate that in the morphine/formalin group there is an inactivation of the non-noradrenergic and activation of noradrenergic neurons of the A7 and A5 cell groups. Anatomical studies have demonstrated that the descending axons arising from the noradrenergic neurons of the A7 cell group travel in the ipsilateral-dorsolateral funiculus and terminate in the ipsilateral dorsal horn (laminae I–IV) at all spinal levels (Clark and Proudfit, 1991; Clark and Proudfit, 1992), where many second order nociceptive neurons are located (Light, 1992). These anatomical observations underlie pharmacological studies which have demonstrated that electrical (Yeomans et al., 1992) and chemical stimulation of the A7 cell group (Holden et al., 1999; Yeomans and Proudfit, 1992) leads to antinociception that can be reversed by intrathecal α_2 -adrenergic antagonists suggesting that the A7 cell group is a major source of the descending noradrenergic neurons that comprise an important part of descending pain-modulatory pathways. In contrast, although noradrenergic neurons of the A5 cell group could play a role in regulating pain processing, they are better characterized to function in autonomic control at the level of the spinal cord (Burnett and Gebhart, 1991; Loewy et al., 1979a; Stanek et al., 1984). Indeed, noradrenergic neurons of the A5 cell group have been demonstrated to innervate the intermediolateral cell column in thoracic spinal cord segments (Byrum and Guyenet, 1987; Clark and Proudfit, 1993; Loewy et al., 1979b; Romagnano et al., 1991) and produce a depressor response (Burnett and Gebhart, 1991; Stanek et al., 1984). Taken together, these observations suggest the model that noxious stimulation could modify the effects of morphine in the A7 and A5 noradrenergic cell groups to influence pain sensitivity and concomitant autonomic functions, respectively (Fig. 5).

3.3. Serotonergic Neurons

In the present study, there was no increase in Fos expression in either serotonergic or non-serotonergic neurons of the NRM following noxious stimulation alone in the form of intraplantar 2.5% formalin injection, morphine injection, or both combined. These observations were somewhat unexpected as noxious stimulation by itself is thought to increase serotonin levels in the spinal cord (Omote et al., 1998). Furthermore, morphine microinjection into the NRM inhibits nociceptive neuronal responses in the spinal cord dorsal horn (Du et al., 1984) and naloxone administered into the NRM attenuates the analgesia produced by systemic administration of morphine (Rivot et al., 1979). However, some evidence suggests that neither stimulus alone may be robust enough to activate Fos. For example, Chen et al. (2003) reported the highest proportion of double-labeled serotonergic neurons with Fos in the NRM and the surrounding nuclei of the reticular formation after somatic and visceral noxious stimulation in the form of 10% formalin solution but not 1 or 5% solutions. Also, *in vivo* single unit recordings have demonstrated that the serotonergic cell population of the NRM is not excited by analgesic doses of morphine (Gao et al., 1998). In addition, current understanding of the role of NRM in nociception and antinociception suggests the likelihood that under each condition, different non-serotonergic “ON” or “OFF” cells may change activity state (see Review by Mason (1999)). This potential change in populations of cells, without a change in total number, would be difficult to detect using the current methods.

3.4. Conclusions

The results provide evidence that noxious stimulation (in the form of 2.5% formalin injection) increases morphine effects in the noradrenergic neurons of the A7 and A5 cell group that are known to project to the spinal cord dorsal horn and intermediolateral column, respectively. The interaction between morphine and noxious stimulation was specific to these groups such that no effect was seen in serotonergic neurons of the NRM. Contrary to the common perception that morphine simply inhibits the access of nociceptive information to supraspinal areas by preventing activation of spinothalamic neurons at the level of the spinal cord dorsal horn, these data indicate that noxious stimulation has the capacity to modify the actions of morphine on brain areas that may contribute to the descending pain-modulatory pathways as well as other physiological changes that occur under these conditions. The interaction between these stimuli is important for understanding the neurobiological consequences of opioid administration on patients with and without pain.

4. EXPERIMENTAL PROCEDURE

4.1. Animal Care and Use

Adult male Sprague-Dawley rats (250–300 g; derived from Sasco; Charles River Laboratories International, Inc. Wilmington, MA) were housed in groups of two, and were maintained on a 12-h light/dark cycle. Food and water were given *ad libitum*. This type of rat is selected because it represents typical experimental subject used in the literature. Rats were handled for at least 3 consecutive days before experimental procedures to minimize stress induced by experimental handling. A total of 24 unanesthetized animals in 4 experimental groups (n=6/group) were used in this study. They include: **(1) control group** that received normal saline subcutaneously in a suprascapular location, **(2) formalin group** that was treated with formalin into the hind paw 30 minutes after subcutaneous normal saline injection, **(3) morphine group** that received only morphine injection subcutaneously, and **(4) morphine-formalin group** that was treated with formalin into the hind paw 30 minutes after subcutaneous morphine injection. The delay after morphine administration was to ensure that morphine had reached peak antinociceptive effects. Care and use of animals were approved by the Institutional Care and Use Committee at Children’s Hospital Boston, and were consistent with the National Institutes of Health.

4.2. Drugs

Morphine sulfate (Baxter Healthcare Corp., Deerfield, IL) or equal volume of normal saline control was used subcutaneously in one dose 30 minutes before formalin injection. Morphine was given at 5 mg/kg dose. Behavioral studies in rat demonstrated that morphine potency varies with the nociceptive test and has been the lowest for chemical stimuli (10mg/kg) and the highest with mechanical stimuli (3 mg/kg) (Morgan et al., 2006). In addition, this dose was selected because it produces place preference (Bardo et al., 1995; Cicero et al., 2000) and antinociception but no sedation in rats (Abbott et al., 1995). Subcutaneous injection of either morphine or normal saline was done in the suprascapular region.

4.3. Formalin Injection

A total of 12 rats received a formalin injection in the hindpaw (Abbott et al., 1995; Dubuisson and Dennis, 1977). Following acclimatization to the environment, animals were given a subcutaneous injection of 50 μ l of 2.5% formalin in normal saline into the distal plantar region of the single hind paw. The injection was done as quickly as possible using a 30-gauge needle. Formalin solution was prepared from commercially available stock formalin by further diluting it with isotonic saline to reach 2.5% concentration (Luccarini et al., 2006). Stock formalin solution is an aqueous solution of 37% formaldehyde (Sigma, St. Louis, MO). Formalin injection followed either saline or morphine subcutaneous injection after 30 minutes. Although we did not quantitatively measure behavior following formalin injection alone, rats displayed spontaneous pain behavior, characterized by increased paw flinching, licking, and keeping the paw elevated as originally described by Dubuisson and Dennis (1977). Also, although post-formalin swelling and redness were not measured, increase in edema and erythema of the formalin-injected paw has been noted. The behavioral antinociceptive effects after morphine injection are evident in rats for approximately 15–90 minutes, with a peak at around 60 minutes. Therefore, formalin was administered at the time when morphine has taken effect. In addition, our qualitative behavioral observations agree with report by Dubuisson and Dennis (1977) who described that despite swelling and reddening of the formalin-injected paw, it was difficult to distinguish behavior between morphine/formalin treated rats from the ones in the control group. After formalin injection, animals were put back into the separate cages for two hours allowing for Fos protein expression. Fos protein expression generally peaks at 1 hour and disappears by 3–4 hours (Herdegen and Leah, 1998) after either short or continuous stimulus. Therefore, time point selected for sampling perfusion was 2 hours after noxious stimulation and 2 hours and 30 minutes after systemic morphine administration. Experimental groups were matched and different groups were perfused on the same day. Tissue from animals in each group was processed in parallel.

4.4. Perfusion

Two hours after formalin injection and 2 ½ hours after saline or morphine subcutaneous injection, each animal was deeply anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneally). Perfusion was done through ascending aorta with 50 ml of normal saline, followed by 250 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4, room temperature). Brains were removed and stored in the same fixative solution overnight (4°C) before cryoprotection in 30% sucrose solution in 0.1M PB for at least 48 hours. Subsequently, brains were frozen and 40 μ m coronal sections were cut on a freezing microtome. The free-floating sections were subsequently processed for immunostaining.

4.5. Immunocytochemistry

We analyzed one third of collected coronal brain sections for Fos expression and noradrenergic neurons double-labeling, another third for Fos expression and the serotonergic neurons double-labeling, and the last third for triple-labeling of all profiles. Primary antisera were diluted in

0.1M PB with saline, 0.3% Triton X-100 (PBST), 0.04% bovine serum albumin (BSA), and 0.1% sodium azide and incubated with the tissue for 2–3 days at 4 °C. Fos immunoreactivity was detected by incubating sections in rabbit anti-Fos antisera (Oncogene, Cambridge, MA) diluted 1:10,000. Noradrenergic neurons were labeled using an antiserum raised in mouse against tyrosine hydroxylase, the synthesizing enzyme for catecholamine neurons (Chemicon International, Temecula, CA) diluted 1:2000. Similarly, serotonin neurons were detected using an antiserum raised against TPOH, the synthetic enzyme for serotonin, raised in sheep (Chemicon International, Temecula, CA) 1:1000. Although this antiserum has some cross-reactivity to tyrosine hydroxylase, which is present in some adrenergic neurons in the ventral medulla, it was selected for its very high sensitivity for TPOH. Free-floating sections were rinsed with 0.1 M PB in saline prior to further processing. Donkey anti-rabbit secondary antiserum for Fos was conjugated to CY3 (red fluorophore; Jackson ImmunoResearch, West Grove, PA). Also, secondary antisera raised in donkey (anti mouse or anti sheep) were conjugated to Alexa-488 (green fluorophore; Invitrogen, Carlsbad, CA). All secondary antisera were diluted 1:200. For triple-labeling experiments, secondary anti-TPOH antibody was detected with a donkey anti-sheep antisera conjugated to Alexa-647 (Invitrogen, Carlsbad, CA). Sections were rinsed in 0.1 M PB in saline solution prior to mounting on slides in 0.05M PB. After drying, mounted sections were coverslipped with 90% glycerol solution.

4.6. Mapping and Cell Counts

All counting of neurons were done under fluorescent microscope (Olympus IX81; Olympus America Inc. Melville, NY, USA) equipped with a camera and digital microscopy software (*Slidebook* v4.2, Olympus). The area defined by each subregion was based on the extent of the cellular groups comprising the region and specific landmarks (Fig.1). Since the monoamine nuclei are not tightly bound nuclei and have no clear anatomical boundaries, Fos immunolabeling was counted only in the vicinity of the monoamine neurons and dendrites comprising the specific nucleus. The rostrocaudal extent of the A7 cell group area of quantification corresponded to plates 54 and 55 of Paxinos' atlas (Paxinos and Watson, 1997) while A5 cell group corresponded to plates 54–64. Finally, area of NRM corresponded to plates 58–66 of Paxinos' Atlas. For each rat, an average number of brain sections photographed and subsequently analyzed were: 10 sections for the A7 cell group (total 250 sections/24 brains), 23 sections for the A5 cell group (total 551 sections/24 brains), and 33 sections for the NRM (total 790 sections/24 brains). For each area, a minimum of 6 rats contributed to the mean number of the total counted immunolabeled profiles in each group. Double-labeled neurons were manually enumerated from the photographs by visualizing the individual and merged images of each fluorophore. A nucleus was counted as Fos positive if it was entirely filled with reaction product. Double-labeled neurons were considered positive if the nucleus was entirely filled with labeling for Fos, and the surrounding cell body and proximal dendrites filled with labeling for tyrosine hydroxylase or TPOH, as visualized by two different fluorophores. Sections were selected, photographed, and counted by an observer blind to the treatment group.

4.7. Statistical Analysis

The total number of cells containing tyrosine hydroxylase and TPOH, with and without Fos, and the total number of cells containing Fos immunolabeling only was summed per animal. The number of sections analyzed from each animal varied, due to individual differences and technical issues. To account for this intrinsic variation, the total number of Fos cells was divided by the number of sections sampled to yield a density of Fos immunolabeling for each rat. There was a low density of monoamine cells per section, particularly in A5 and A7, which could inadvertently skew observations relating to the appearance of Fos specifically in these cells. To account for this, and for the variation in the number of sections sampled, we calculated the percent of monoamine cells that were dually labeled with Fos per brain. Individual densities

or percentages were averaged to yield a group mean $\pm$ SD for each pharmacological group. Differences among the four different treatment groups were determined by using analysis of variance (ANOVA) with a Bonferroni post-hoc test for multiple comparisons in order to protect against type 1 errors. Two-tailed p value less than 0.05 with Bonferroni post-hoc test was considered statistically significant. Statistical analysis was performed using the SPSS software package, v.16.0 (SPSS Inc., Chicago, IL).

ABBREVIATIONS

ANOVA	Analysis of variance
BSA	bovine serum albumin
NRM	nucleus raphe magnus
PB	phosphate buffer
PBST	phosphate buffer with saline and Triton
TPOH	tryptophan hydroxylase

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Figure 1. Anatomical regions of analysis

Schematic representation of rat brain representative transverse brainstem sections modified from Paxinos Atlas (Paxinos and Watson, 1997). **Panels A–C** correspond to Paxinos Atlas plates 54, 59, and 62, respectively. Anatomical areas of analysis are marked by square and monoamine neuronal cell groups analyzed are illustrated as black circles. These include: tyrosine hydroxylase-immunoreactive noradrenergic neurons of the A7 cell group in the pons (**Panel A**) and the A5 cell group throughout the distal brainstem (**Panels A–C**), as well as tryptophan hydroxylase-immunoreactive serotonergic neurons of the nucleus raphe magnus (NRM) in the ventromedial medulla (**Panel B and C**). **Abbreviations:** 4V, 4th ventricle; 6, 6th cranial nerve; 7, facial nucleus; 7n, 7th cranial nerve; g7, genu facial nerve; GiA,

gigantocellular reticular nucleus pars alpha; **LPGi**, lateral paragigantocellular nucleus; **LVPO**, lateroventral periolivary nucleus; **LSO**, lateral superior olive; **me5**, mesencephalic 5th tract; **ml**, medial lemniscus; **vlPAG**, ventrolateral periaqueductal gray; **py**, pyramidal tract; **scp**, superior cerebellar peduncle; **sp5**, spinal trigeminal tract.

Figure 2. Estimated Density of Fos Immunoreactive Nuclei

Graph represents biased estimates of density ($\#Fos/section/brain \pm SD$) of Fos immunoreactive nuclei in different brain regions that include noradrenergic neurons of the A7 and A5 cell groups, as well as serotonergic neurons of the nucleus raphe magnus (NRM). Presented number of Fos included both single and double-labeled Fos cells. Analysis included four different experimental groups: (1) Control, (2) Formalin, (3) Morphine, and (4) Morphine/Formalin group (M/F). Please note that there is no statistically significant difference in Fos expression among any of the four treatment groups with respect to each anatomical area (A7, A5, NRM).

Figure 3. Estimated Percentages of Double Labeled Immunohistochemical Profiles/Brain

Anatomical regions of analysis include noradrenergic neurons of the A7 and A5 cell groups, as well as serotonergic neurons of the nucleus raphe magnus (NRM). Graph illustrates biased estimates of the average number of double-labeled profiles $\pm$ SD as a percentage of density of either noradrenergic (A7 and A5 cell groups) or serotonergic neurons (NRM). Analysis included four different pharmacological groups: (1) Control, (2) Formalin, (3) Morphine and (4) Morphine/Formalin group (M/F). Note statistically significant difference in percentage of double-labeled neurons in the group that received formalin injection following morphine administration (M/F) in the A7 ($F(3,20)=8.23$, $p=0.001$) and the A5 ($F(3,20)=12.76$, $p<0.001$) cell groups only, whereas there is no statistical difference in the area of the NRM ($F(3,20)=2.02$, $p=0.144$). Two-tailed p value less than 0.05 with Bonferroni post-hoc test were considered statistically significant. More specifically, pairwise comparisons with Bonferroni post-hoc tests of all groups showed statistically significant increase in the percentage of double-labeled neurons in the M/F group compared to control ($p=0.002$), formalin ($p=0.004$), and morphine ($p=0.011$) in the area of the A7 group. Similarly, statistically significant increase in the percentage of double-labeled neurons in the M/F group compared to control ($p<0.001$), formalin ($p<0.001$), and morphine ($p=0.011$) was also found in the area of the A5 cell group. Asterisks (*) denote statistically significant increase in values.

Figure 4. Immunohistochemical Labeling

Representative photomicrographs on the left illustrate examples of immunocytochemical labeling in each of the analyzed anatomical regions. Photomicrographs for A7 (**A**) and A5 (**B**) cell groups are from morphine/formalin treatment group, whereas the representative photomicrograph of the NRM (**C**) is from the control treatment group. Panels on the right represent enlarged areas that were marked with asterisk (*) on the corresponding left panels. Double-labeled profiles were distinguished by the presence of neuronal (green) and nuclear stain (red) in the individual neuron (arrows). Arrowheads indicate Fos-immunolabeling only (red). Both noradrenergic (**A and B**) and serotonergic neurons (**C**) are green. Scale bar 100 μm throughout.

Figure 5. Model

Schematic representation modified from Paxinos atlas (Paxinos and Watson, 1997) of descending pain modulatory pathways that in part include projections of noradrenergic neurons (NA) from the A7 cell group and serotonergic neurons (5-HT) from the nucleus raphe magnus (NRM) to the spinal cord dorsal horn (SCDH) that inhibit nociceptive transmission. Noradrenergic neurons of the A5 cell group are known to project to intermediolateral column (IMLC) of the spinal cord where they influence autonomic functions. Descending projection pathways that appear activated by acute pain (as intraplantar 2.5% formalin injection) in the presence of morphine are illustrated as straight line and include noradrenergic neurons of the A7 and A5 cell groups, but not serotonergic neurons of the NRM (dashed line).

Abbreviations: **IC**, inferior colliculus; **SCVH**, spinal cord ventral horn; **SMT**, spinomesencephalic tract; **SRT**, spinoreticular tract; **STT**, spinothalamic tract.