Published in final edited form as:

Neuropharmacology. 2011 September ; 61(4): 614–621. doi:10.1016/j.neuropharm.2011.05.002.

Opioid-mediated regulation of A11 diencephalospinal dopamine neurons: pharmacological evidence of activation by morphine

Samuel S. Pappas¹, Tom Kennedy², John L. Goudreau^{2,3}, and Keith J. Lookingland^{1,2}

¹ Michigan State University, Department of Physiology, East Lansing, MI 48824

² Michigan State University, Department of Pharmacology and Toxicology, East Lansing, MI 48824

³ Michigan State University, Department of Neurology and Ophthalmology, East Lansing, MI 48824

Abstract

Dopamine (DA) neurons of the A11 diencephalospinal system represent the sole source of DA innervation to the spinal cord in mice, serving neuromodulatory roles in the processing of nociceptive input and movement. These neurons originate in the dorsocaudal diencephalon and project axons unilaterally throughout the rostrocaudal extent of the spinal cord, terminating predominantly in the dorsal horn. The density of A11 DA axon terminals in the lumbar region is greater in males compared to females, while in both sexes the activity of neurons terminating in the thoracic spinal cord is greater than those terminating in the lumbar region. The present study was designed to test the hypothesis that A11 DA neurons are activated by opioids. To test this hypothesis, male and female mice were systemically treated with agonists or antagonists acting at the μ -opioid receptor, and spinal cord concentrations of DA and its metabolite DOPAC were determined in the thoracic and lumbar spinal cord using high performance liquid chromatography coupled with electrochemical detection. Systemic administration of the μ -opioid agonist morphine led to a dose- and time-dependent increase in spinal cord DOPAC/DA ratio (an estimate of DA neuronal activity) in both male and female mice, with greater changes occurring in the lumbar segment. Blockade of opioid receptors with the opioid antagonist naloxone reversed the stimulatory effects of morphine on A11 DA neurons in both male and female mice, but had little to no effect on the activity of these neurons when administered alone. Present findings are consistent with the conclusion that spinal cord- projecting axon terminals of A11 DA neurons are activated by opioids in both male and female mice, most likely through a disinhibitory mechanism.

Keywords

Dopamine; Spinal Cord; Diencephalospinal; A11; mu-opioids; morphine

© 2011 Elsevier Ltd. All rights reserved.

Corresponding Author: Keith J. Lookingland, Ph.D., Associate Professor, Department of Pharmacology and Toxicology, B432 Life Sciences Building, Michigan State University, East Lansing, MI 48824, lookingl@msu.edu, 517-353-8971 (phone), 517-353-8915 (fax).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Of the diencephalic dopamine (DA) neuronal systems, A11 diencephalospinal DA neurons are unique in providing the sole source of spinal cord DA in mice (Pappas et al, 2010). A11 DA neuronal cell bodies originate in the caudal zona incerta (medial to the mamillothalamic tracts), dorso-caudal periventricular hypothalamus, and periventricular gray of the caudal thalamus and rostral midbrain (medial to the fasciculus retroflexus and ventral to the posterior commissure). These diencephalic neurons project axons through the dorsal longitudinal fasciculus of Schutz in the periventricular and periaqueductal gray (Bjorklund and Skagerberg, 1979), and descend unilaterally in the superficial dorsal horn and nearby dorsolateral funiculus of the spinal cord white matter. A small number of A11 DA axons descend in the area surrounding the central canal of the spinal cord.

A11 DA axons terminate at every spinal cord segment, predominantly in the dorsal horn, with additional projections to the zona intermedia and lamina X surrounding the central canal (Skagerberg et al., 1982; Ridet et al., 1992; Holstege et al., 1996). A minor DA axon terminal network is also present surrounding the ventral horn motor neurons of lamina IX (Yoshida et al., 1988). Neurochemical studies demonstrate measureable quantities of DA in the ventral horn, but highest concentrations in the dorsal horn (Basbaum et al., 1987; Fleetwood-Walker and Coote, 1981). In addition to spinal cord, the A11 neuronal system has rich intradiencephalic projections to the medial and midline thalamus and hypothalamus, and projections to other brain regions such as the dorsal raphe nucleus (Peyron et al., 1995) and frontal cortex. There may also be minor A11 projections to the central amygdaloid nucleus and lateral bed nucleus of the stria terminalis, although this is debated (Moriizumi and Leduc-Cross, 1992; Hasue and Shammah-Lagnado, 2002).

As A11 DA neurons project axons to several brain regions and terminate within different functionally distinct spinal cord laminae, it is understandable that many physiologically roles have been ascribed to this DA neuronal system. These include neuromodulatory effects on nociceptive input (Jensen and Yaksh, 1984; Barasi and Duggal, 1985; Fleetwood-Walker et al., 1988; Gao et al, 2001; Wei et al., 2009), locomotion (Barbeau and Rossignol, 1991; Barriere et al., 2004), movement (Zhu et al., 2007), cardiovascular function (Lahlou et al., 1990; Maisky and Doroshenko, 1991), and sex-specific roles (Pappas et al., 2010) including male sexual function (Giuliano et al., 2001, 2002). Dysfunction of this system is hypothesized to be one factor leading to the development of Restless Legs Syndrome (RLS) in humans (Trenkwalder and Paulus, 2005; Barriere et al., 2005), a sensorimotor disorder characterized by uncomfortable sensations and an urge to move the legs. The most common treatment for RLS is DA agonist administration, but the use of opioids such as morphine has also been reported to alleviate symptoms of RLS.

Morphine is an opiate alkaloid that is used as a potent analgesic acting as an agonist at the μ -opioid receptor, one of three main classes of G-protein coupled, inhibitory (Attali et al, 1989; Konkoy and Childers, 1989) opioid receptor (μ , κ , and δ receptors; Martin et al., 1976; Lord et al., 1977). μ -opioid receptors are widely located throughout the body, and are highly concentrated in regions involved with the processing of nociceptive stimuli, such as the periaqueductal gray matter, ventral medulla, and superficial dorsal horn of the spinal cord. μ -opioid receptors are also distributed in other regions of the nervous system, including midbrain and diencephalic regions containing DA neuronal systems.

Drugs acting as agonists or antagonists at μ -opioid receptors produce a characteristic, differential response in central DA neuronal populations. Morphine administration and the resulting activation of μ -opioid receptors leads to the activation of midbrain A8/9 and A10 DA neurons that project to the striatum, nucleus accumbens, and prefrontal cortex (Di

Chiara and Imperato, 1988; Johnson and North, 1992). The regulation of diencephalic DA systems by opioid-mediated mechanisms is more complex and multifaceted. The endogenous opioid peptide β -endorphin has been implicated in the regulation of diencephalic DA neurons through the action of μ -opioid receptors, and enkephalin may exert an effect on both μ - and δ -opioid receptors in this region (Lookingland and Moore, 2005). A12 tuberoinfundibular neurons are inhibited by β -endorphin, but not under normal physiological conditions. In contrast, A13 incertohypothalamic and A14 periventricular DA neurons terminating in the preoptic area are stimulated following activation μ -opioid receptors. Activity of periventricular-hypophysial DA neurons remains unaltered following activation or inhibition of μ -opioid receptors. The effects of opioids on the A11 DA system are unknown.

Several lines of evidence suggest that DA released from descending A11 fibers in the superficial dorsal horn plays an inhibitory role in the processing of nociceptive stimuli (Jensen and Yaksh, 1984; Barasi and Duggal, 1985; Fleetwood-Walker et al., 1988; Gao et al., 2001; Wei et al., 2009; Charbit et al., 2009), and the presence of inhibitory D2/3 receptors in the dorsal horn of the cervical and lumbar spinal cord (Yokoyama et al., 1994; Levant and McCarron, 2001; Zhu et al., 2007) and trigeminocervical complex (Bergerot et al., 2007; Charbit et al., 2009) further supports an antinociceptive role for descending A11 diencephalospinal DA tracts.

As opioid receptors are concentrated in regions containing A11 DA neuronal cell bodies and axon terminals, μ -opioid activation increases the activity of nearby diencephalic DA neurons, and administration of opioids relieves the symptoms of RLS, it was hypothesized that administration of morphine would lead to a significant increase in A11 DA activity within the spinal cord of male and female mice through a 'dis-inhibitory' role of opioid receptors located on an inhibitory neuron antecedent to the A11 system.

2. Materials and Methods

Animals

Age-matched 8–10 week old male and female mice obtained from Jackson Laboratories (Bar Harbor, Maine) were used for this study. Animals were housed four per cage, maintained in a temperature-controlled ($22 \pm 1^\circ\text{C}$) and light-controlled (12 hr light-dark cycle) room, and provided with food and tap water *ad libitum*. Tissue harvesting was performed between 0800–1000 h to control for possible circadian variations in DA neuronal activity. The Michigan State University Institutional Animal Care and Use Committee approved all experiments performed. These experiments were conducted following the U.S. National Institutes of Health guidelines for the care and use of laboratory animals and were compliant with the provisions of the Animal Welfare Act. The minimal number of animals required to achieve sufficient statistical power was used.

Drugs

Morphine sulfate pentahydrate (Baxter Scientific) was diluted as a stock solution of 7.52mg/mL morphine (accounting for the $\text{H}_2\text{SO}_4 \cdot 5\text{H}_2\text{O}$; all doses will be reported as mg/kg of the free base). On the day of injection, the stock solution was diluted with sterile saline into working solutions of 0.5, 1, or 2 mg/mL, such that final doses would equal 5, 10 or 20 mg/kg morphine with an injection of 10mL/kg. Naloxone-HCl (USP grade, Fisher Scientific) was dissolved in sterile saline at a concentration of 10 mg/mL, free-base. On the day of injection, the stock solution was diluted with sterile saline into a working solution of 1 mg/mL, for a final dose of 10 mg/kg. Mice received injections of morphine, naloxone, or saline

vehicle (10 mL/kg) and were sacrificed by decapitation at varying times afterward, as indicated in figure legends.

Tissue Dissection and Neurochemical Analysis

Mice were sacrificed by decapitation, and brains and spinal cords were removed, and fresh tissue dissection of the median eminence was performed. Brains and spinal columns were then quickly frozen over dry ice. Coronal brain sections (500 μ m) were prepared on a cryostat (-10°C) and the striatum and nucleus accumbens were microdissected using a modified 18- or 21-gauge needle, respectively. Samples containing approximately 50 μ g total protein were placed into ice-cold 0.1M phosphate-citrate buffer with 15% methanol. Samples were assayed for neurochemical content using high performance liquid chromatography coupled to electrochemical detection (HPLC-ED; Lindley et al., 1990). Consecutive spinal cord segments delimited by their vertebral widths were dissected with a scalpel, mounted on a glass slide, and refrozen over dry ice as previously described (Pappas et al., 2008, 2010). Spinal column segments within thoracic T6-T9 or lumbar L1-L3 vertebrae were viewed with a dissecting microscope, and bilateral micropunches of gray matter were taken from each segment using a modified 24-gauge needle. Samples were pooled into tubes corresponding to thoracic or lumbar regions, placed into 0.1 M phosphate-citrate buffer containing 15% methanol, and assayed using HPLC-ED. Brain and spinal cord concentrations of DA and DOPAC were calculated by normalizing samples to protein content determined by Lowry protein assay (Lowry et al., 1951).

Statistical Analysis

To compare among groups, one-way analysis of variance was performed using Sigmaplot Software version 2.03 (SysStat Software, Point Richmond, CA). If a significant interaction was detected by ANOVA, Tukey's post hoc test was used for multiple comparisons and differences with a probability of error of less than 5% were considered statistically significant ($P \leq 0.05$).

3. Results

As demonstrated in Figure 1 for male mice, under basal conditions DA concentrations were higher in the lumbar spinal cord, while the DOPAC/DA ratio was higher in the thoracic spinal cord. A single injection of morphine led to a significant increase in the DOPAC/DA ratio in both regions. The lumbar DOPAC/DA ratios were increased at every morphine dose, while thoracic ratios were only affected at the highest morphine dose (Figure 1A). This effect was associated with an increase in DA concentrations within the thoracic but not lumbar spinal cord. Elevated thoracic DA concentrations in morphine-treated mice were at similar levels to those of saline-treated control lumbar values (Figure 1B). These neurochemical changes were time-dependent, increasing to highest levels at 60 min, and returning to control levels by 120 min (Figures 1C and 1D).

Basal segmental differences in DOPAC/DA ratios were present in female mice, with thoracic spinal cord values found to be higher than those for lumbar spinal cord (Figure 2A). These differences were not as robust as in males, but segmental differences in DA concentration were comparable (Figure 2B). Systemic morphine administration caused a significant increase in DOPAC/DA ratios and DA concentrations in the spinal cord of female mice, with a significant effect present at all doses (Figures 2A and 2B). These morphine-induced neurochemical changes were time-dependent, although in a pattern different from males. DOPAC/DA ratios were increased by 60 min in both thoracic and lumbar regions, but remained high throughout the entire 120 min time course (Figure 2C).

DA concentrations were increased in both spinal segments following morphine administration, and remained higher for the entire time course (Figure 2D).

To confirm that morphine-induced changes in spinal cord DA neurochemical activity were due to an opioid receptor mediated mechanism and to determine if endogenous opioids tonically regulate A11 DA neurons, male and female mice were pretreated with the opioid receptor antagonist naloxone 30 min prior to morphine or saline treatment. As shown in Figure 3 for male mice, morphine alone caused a significant increase in DOPAC/DA ratios and DA concentrations in both the thoracic and lumbar spinal cord; pretreatment with naloxone blocked these morphine induced changes. DOPAC/DA ratios were significantly decreased in the thoracic segment following naloxone treatment, with no effect in the lumbar segment of the spinal cord (Figure 3A). No changes in spinal cord DA concentrations occurred following naloxone treatment alone (Figure 3B). In female mice, pre-treatment with naloxone blocked the morphine-induced changes in spinal cord DOPAC/DA ratios and DA concentrations (Figure 3C and 3D). No significant changes to these values occurred following naloxone treatment alone or in the presence of morphine.

Three brain regions including the striatum, nucleus accumbens, and median eminence served as controls. As positive control, concentrations of DOPAC and the DOPAC/DA ratio were increased in the striatum and nucleus accumbens following morphine administration (Table 1). These morphine induced changes were reversed by pretreatment with naloxone, but naloxone alone had no effect. Morphine significantly increased median eminence DOPAC concentrations and DOPAC/DA ratio in females (positive control), but had no effect in males (negative control). DA concentrations were not changed in any control region following treatment with morphine or naloxone.

4. Discussion

4.1. Spinal cord DA and DOPAC/DA and effects of morphine

Administration of the μ -opioid agonist morphine produced an increase in A11 diencephalospinal neuronal DA synthesis and metabolism, an effect reflected by changes in DA and DOPAC concentrations. In both the thoracic and lumbar segments of the spinal cord, morphine administration elicited a dose- and time-dependent increase in A11 DA neuronal activity (i.e. ratio of DOPAC/DA) in both male and female mice. While this stimulatory effect was present at all morphine doses for the lumbar region, A11 DA neuronal activity was increased in the thoracic region only at the highest dose in males. This latter observation may represent a segmental difference in the extent of μ -opioid regulation of A11 DA neurons terminating in these regions. It is notable that thoracic DOPAC/DA values are higher than those for the lumbar segment under normal physiological conditions (Pappas et al., 2008), and lumbar segment values only exceeded the saline control values of the thoracic segment following administration of the highest morphine dose. The segmental difference in DOPAC/DA ratio and response to morphine administration was also present in females, but was smaller in magnitude when compared to males

The basal segmental difference in DOPAC/DA ratios could be attributed, in part, to differences in μ -opioid mediated regulation of A11 DA neuronal activity within the spinal cord. Gray matter in the thoracic spinal cord may contain a greater number of μ -opioid receptors than lumbar gray, or may contain segmental differences in tonic endogenous μ -opioid mediated activation of presynaptic DA axon terminals. The decrease in DA neuronal activity seen following administration of the opioid antagonist naloxone (given alone or in combination with morphine) suggests a minor tonic μ -opioid mediated regulation in the thoracic spinal cord of male mice. In contrast, naloxone produced no effect in the lumbar spinal cord of males or in either region of females, suggesting there is no tonic μ -opioid

mediated regulation of A11 DA neuronal activity in these contexts. As such, these findings may represent a sex difference in the spinal cord response to opioid administration.

Differences in opioid mediated regulation of A11 diencephalospinal DA neurons may also explain why the segmental differences in DOPAC/DA ratios are more pronounced in males. Indeed, segmental differences in basal activity were not as robust in females, and no segmental differences in response to μ -opioid administration were observed in females. Male rodents are more sensitive to opioid analgesia than females; e.g., the effective dose of morphine is approximately half of the female level (Cicero et al., 1997; Barrett et al., 2002). Sex-specific differences in response to morphine administration are not due to differences in the pharmacokinetics of morphine, as the peak levels of morphine in brain and blood, elimination half-life from the serum, and disappearance from the brain are similar in males and females after subcutaneous injection (Cicero et al., 2007).

4.2 Opioid receptor localization

It is unknown whether the opioid receptors mediating the effects of morphine and naloxone are present in the A11 DA cell bodies or axon terminals (or both). It is possible that μ -opioid receptors located in the diencephalic cell body region are affecting the A11 DA neuronal system, as μ -receptors are robustly expressed in the periventricular and periaqueductal gray regions containing these neurons (Herkenham et al., 1980; Lewis et al., 1983). On the other hand, if variation in μ -opioid receptor densities underlie the observed segmental differences in basal and morphine-activated spinal cord DA activity, then variation at the spinal cord level is most plausible. Single A11 diencephalospinal DA neurons likely project axon terminal collaterals to many, if not all, spinal cord segments (Skagerberg et al., 1985; Qu et al., 2006; Pappas et al., 2010). The lack of any clear topographical organization of this spinal cord- projecting DA system favors a spinal cord site of action of opioids as a plausible explanation for the differential segmental responses to morphine and naloxone observed in A11 diencephalospinal DA neurons.

μ -, δ -, and κ -opioid receptors are present throughout the spinal cord, but μ - and δ -binding sites are more concentrated in the rostral portions of the spinal cord (Gouarderes and Cros, 1984, 1985). As such, thoracic μ -opioid regulation of DA neuronal activity is logical. On the other hand, other studies reported a preponderance of μ -opioid receptors at all spinal segments (Besse et al., 1990, 1991) and a similar rostro-caudal distribution of all opioid receptor types along the spinal cord (Traynor and Wood, 1987).

4.3. Neurochemical estimates of DA neuronal activity following morphine administration

Increased DOPAC/DA ratios provide a surrogate marker of increased DA neuronal activity, as these values reflect the release, reuptake, and metabolism of DA (Lookingland and Moore, 2005). Morphine, acting via post-synaptic μ -opioid receptors, would be expected to inhibit a downstream neuron and could not activate A11 diencephalospinal DA neurons directly. The most logical scenario, therefore, is that morphine inhibits tonically active afferent inhibitory inputs to A11 diencephalospinal DA neurons.

An increase in neuronal activity is often reflected by decreased (rather than increased) DA concentrations, as synthesis is unable to achieve the level of activity required to maintain normal levels of transmitter in the face of increased release. One example of an increase in DA neuronal activity leading to a decrease in DA concentration is the nigrostriatal DA neuronal system response to D2 antagonist administration (Pappas et al., 2008). In contrast increases in DA concentrations following pharmacological manipulations are often indicative of decreased neuronal activity, with synthesis maintained, but release halted. It is therefore possible that morphine administration and subsequent activation of μ -opioid

receptors is decreasing the activity of spinal cord- projecting A11 DA neurons. On the other hand, morphine induced a substantial increase in DOPAC concentrations and in the DOPAC/DA ratio, while naloxone decreased the DOPAC/DA ratios. It is therefore more likely that the present results reflect an increase in neuronal activity with an accompanying increase in synthesis of DA.

Increases in spinal cord DA concentrations may arise from descending norepinephrine (NE) neurons, but this is not likely. Although there is approximately 10 times more NE than DA in the spinal cord in non-treated animals, the vast majority of spinal cord DA is present in DA-containing axon terminals, and not simply as NE precursor (Commissiong et al., 1978; Mouchet et al., 1982; Skagerberg et al., 1982; Holstege et al., 1996; Pappas et al., 2010). However, if morphine is activating NE neurons terminating in the spinal cord, DA-beta-hydroxylase may become rate limiting, leading to an accumulation of DA within descending NE terminals. This scenario could account for an increase in DA concentrations, but would not rule out the possibility that morphine also activated descending A11 DA neurons. In addition, studies in rats reported no changes in NE turnover or NE metabolite content (3-methoxy-4-hydroxyphenylglycol) after either low (2.5 mg/kg; Weil-Fugazza and Godefroy, 1991) or high morphine doses (40 mg/kg; Karoum et al., 1981), suggesting that changes in spinal cord NE activity do not account for the morphine-induced DA concentration changes seen in the present study. NE metabolites were not measured in this study, so a neurochemical estimate of NE turnover was not possible.

The finding of increased A11 diencephalospinal DA neuronal activity following morphine is in agreement with early studies by Karoum et al. (1981), who showed that lumbar spinal cord steady state DA concentrations and lumbar ventral horn DA turnover rates increased after 40 mg/kg morphine-sulfate in rats. Later studies also showed increased lumbar DA metabolism following 2.5 mg/kg morphine (Weil-Fugazza and Godefroy, 1991). In contrast, another early study reported no changes in DA or DOPAC levels in the spinal cord of rats after 10 mg/kg morphine-HCl (Commissiong et al., 1983). These seemingly contradictory findings may be due to differences in the dose of morphine administered, particularly since the early studies used different drug preparations. Changes to spinal cord DA neurochemistry were dose-dependent in the present study, so dose differences between laboratories is a reasonable explanation for this discrepancy.

4.4. Mechanisms of mu-opioid regulation of diencephalospinal DA neurons

While μ -opioid receptors are inhibitory, morphine administration led to an increase in DA neuronal synthesis and metabolism in the present study. It is plausible that morphine activated μ -opioid receptors present on an inhibitory neuron preceding the A11 DA axon terminal; e.g. an inhibitory GABAergic neuron with axons terminating on A11 axon terminals at the spinal cord level. In this scenario, the morphine-induced activation of inhibitory μ -opioid receptors on the neuron upstream of the A11 diencephalospinal DA neuron would remove tonic inhibitory input to the A11 system, thereby increasing the levels of DA synthesis, release, and metabolism via dis-inhibition.

Second messenger systems common to both μ -opioid and DA receptors represent another possible mechanism for cross-talk of ligands acting μ and DA receptors of the same neurons. For example, morphine-induced inhibition of μ -opioid receptor-linked adenylate cyclase could be opposed by activation of D1 receptors or facilitated by activation D2 receptors. The μ -opioid receptor is primarily coupled to heterotrimeric Gi/Go proteins. Activation of the μ -opioid receptor leads to several downstream second messenger effects (Connor and Christie, 1999), including inhibition of adenylate cyclase, inhibition of Ca^{2+} mobilization, and stimulation of K^{+} channels (Loh and Smith, 1990; Connor and Christie, 1999; Ikeda et al., 2002; Waldhoer, 2004). DA receptors are also G-protein coupled

receptors and are classified into two main categories, D1 and D2, based on the characteristic synaptic response following activation (Cools and Van Rossum, 1976; Garau et al., 1978; Keababian and Calne, 1979; Missale et al., 1998). Through a coupling to G α s proteins, activation of D1 family receptors is stimulatory to adenylate cyclase, leading to increased intracellular cAMP concentrations in the post-synaptic neuron (Keababian et al., 1971; Brown et al., 1977; Monsma et al., 1990). D1 receptors may also be linked to G-q proteins, which increase intracellular Ca²⁺ concentrations and activate PLC (Felder et al., 1989; Undie and Friedman, 1990, 1992; Undie et al., 1994; Wang et al., 1995). In contrast, D2 family receptors are coupled to various inhibitory G proteins, and activation of these receptors leads to inhibition of adenylate cyclase (De Camilli et al., 1979; Meunier and Labrie, 1982; Onali et al., 1985), activation of K⁺ channels (Lacey et al., 1987), inhibition of Ca²⁺ mobilization, and stimulation of mitogenesis (Lajiness et al., 1993).

For DA neurons with presynaptic D2 autoreceptors, cross-talk at the second messenger level could provide an explanation for observed effects of morphine on DA neuronal activity and nerve terminal DA homeostasis. A11 diencephalospinal neurons, however, do not appear to have functional D2 autoreceptors (Pappas et al. 2008) and the D1 receptor is expressed in post-synaptic (non-DA) neurons. As such, there is no clear substrate for a second messenger interaction-based mechanism for the effects of mu-opioid receptor activation on diencephalospinal neurons at the spinal cord level. While mu opioid receptors are expressed in the spinal cord, the precise localization of mu opioid receptors on specific neuronal phenotypes is not known. If mu opioid receptors were located on the A11 diencephalospinal nerve terminal, then second messenger-mediated effects on DA neuronal activity and homeostasis would merit further investigation.

4.5. Experimental control regions

As experimental controls for comparison with spinal cord findings, microdissections of the DA axon terminal regions of the dorsal striatum, nucleus accumbens, and median eminence were also examined in male and female mice. Median eminence concentrations of DA and DOPAC, and the DOPAC/DA ratio remained unchanged following administration of morphine or naloxone in males, but morphine increased DOPAC concentrations and the DOPAC/DA ratio in females. As there are well-documented sex differences in the basal activity of DA neurons terminating in the median eminence (Lookingland and Moore, 2005), a sex-specific effect of morphine is to be expected. Endogenous β -endorphins are *inhibitory* to A12 tuberoinfundibular DA neurons terminating in the median eminence (Haskins et al., 1981; Lookingland and Moore, 1985; Loose and Kelly, 1990; Callahan et al., 1996; Andrews and Grattan, 2003; Tavakoli-Nezhad and Arbogast, 2010), but not under normal, basal conditions (Deyo et al., 1979). A delayed *activation* of A12 DA neurons 4 hours after morphine has been observed, as evidenced by increased DA turnover in the median eminence and increased DA concentrations in the pituitary portal blood (Gudelsky et al., 1986). The later onset effect of morphine was hypothesized to be due to a secondary effect of prolactin or α -MSH feedback on tuberoinfundibular neurons, as opposed to acute direct effect of opioids on these neurons. It is possible that there is a different time-course for this secondary effect in mice as compared to the findings in rats (Gudelsky et al., 1986). A potential species difference in the acute response of A12 tuberoinfundibular DA neurons to morphine is also possible, as previous studies were performed in rats (Deyo et al., 1979).

In the striatum, the major axon terminal region of the A8/9 nigrostriatal DA neuronal system, DA concentrations remained unchanged, but DOPAC concentrations were significantly increased following morphine. The morphine induced increase in striatal DOPAC is consistent with a stimulatory role of μ -opioid receptors on midbrain DA neuronal systems (Iwatsubo and Clouet, 1977; DiChiara and Imperato, 1988) through the inhibition of local inhibitory interneurons (Johnson and North, 1992). No changes occurred with

naloxone treatment alone; indicating a lack of μ -opioid receptor mediated tonic regulation within the nigrostriatal DA neuronal system.

Nucleus accumbens DOPAC concentrations and the DOPAC/DA ratio were also significantly increased following morphine treatment and these effects were blocked by naloxone pre-treatment. The observed effects of opioid agonists and antagonists on mesolimbic DA neurons are in agreement with previous studies (DiChiara and Imperato, 1988; Johnson and North, 1992), and reflect an activation of the midbrain A10 mesolimbic DA neuronal system through inhibition of local inhibitory interneurons (Johnson and North, 1992).

4.6. Summary

In summary, axon terminals of spinal cord-projecting A11 diencephalospinal DA neurons demonstrate several regional segmental differences in both male and female mice. Thoracic spinal cord DA concentrations are lower than lumbar concentrations, but contain higher DOPAC/DA ratios, reflecting a lower density of axon terminals, but higher activity. A single injection of morphine causes an increase in spinal cord DA neuronal activity in a dose- and time-dependent, naloxone-reversible manner. The observations reported herein are consistent with the conclusion that μ -opioid receptors regulate the activity of A11 spinal cord-projecting DA neurons through a dis-inhibitory mechanism, possibly in a segment specific fashion. Opioid-mediated segmental regulation of diencephalic A11 DA neurons may be one factor underlying the beneficial effect of morphine treatment for the symptoms of RLS, and may represent a parallel opioid pathway for the inhibition of nociceptive input to the dorsal horn.

Abbreviations

DA	Dopamine
DOPAC	3,4-Dihydroxyphenylacetic acid
NE	Norepinephrine

References

- Andrews ZB, Grattan DR. Opioid receptor subtypes involved in the regulation of prolactin secretion during pregnancy and lactation. *J Neuroendocrinol.* 2003; 15:227–236. [PubMed: 12588510]
- Attali B, Saya D, Vogel Z. Kappa-opiate agonists inhibit adenylate cyclase and produce heterologous desensitization in rat spinal cord. *J Neurochem.* 1989; 52:360–369. [PubMed: 2536070]
- Barasi S, Duggal N. The effect of local and systemic application of dopaminergic agents on tail flick latency in the rat. *Eur J Pharmacol.* 1985; 117(3):287–294. [PubMed: 3841070]
- Barbeau H, Rossignol S. Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic, and dopaminergic drugs. *Brain Res.* 1991; 546:250–260. [PubMed: 2070262]
- Barriere G, Mellen N, Cazalets JR. Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. *Eur J Neurosci.* 2004; 19:1325–1335. [PubMed: 15016090]
- Barriere G, Cazalets JR, Bioulac B, Tison F, Ghorayeb I. The restless legs syndrome. *Prog Neurobiol.* 2005; 77:139–165. [PubMed: 16300874]
- Basbaum AI, Godefroy F, Weil-Fugazza J. A new microdissection technique for regional biochemical analysis of the rat spinal cord: serotonin, norepinephrine, dopamine, and uric acid. *Brain Res.* 1987; 419:229–238. [PubMed: 2445422]
- Basbaum, AI.; Jessell, TM. The Perception of Pain. In: Kandel, ER.; Schwartz, JH.; Jessell, TM., editors. *Principles of Neural Science.* 4. McGraw-Hill; 2000. p. 472-492.

- Besse D, Lombard MC, Zajac JM, Roques BP, Besson JM. Pre- and postsynaptic distribution of mu, delta, and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Res.* 1990; 521(1–2):15–22. [PubMed: 2169958]
- Besse D, Lombard MC, Besson JM. Autoradiographic distribution of mu, delta and kappa opioid binding sites in the superficial dorsal horn, over the rostrocaudal axis of the rat spinal cord. *Brain Res.* 1991; 548(1–2):287–291. [PubMed: 1651143]
- Bergerot A, Storer RJ, Goadsby PJ. Dopamine inhibits trigeminovascular transmission in the rat. *Ann Neurol.* 2007; 61:251–262. [PubMed: 17387726]
- Bjorklund A, Skagerberg G. Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Res.* 1979; 177:170–175. [PubMed: 497819]
- Callahan P, Baumann MH, Rabii J. Inhibition of tuberoinfundibular dopaminergic neural activity during suckling: involvement of mu and kappa opiate receptor subtypes. *J Neuroendocrinol.* 1996; 8:771–776. [PubMed: 8910807]
- Charbit AR, Akerman S, Holland PR, Goadsby PJ. Neurons of the dopaminergic/calcitonin gene-related peptide A11 cell group modulate neuronal firing in the trigeminocervical complex: an electrophysiological and immunohistochemical study. *J Neurosci.* 2009; 29(40):12532–41. [PubMed: 19812328]
- Commissiong JW, Galli CL, Neff NH. Differentiation of dopaminergic and noradrenergic neurons in rat spinal cord. *J Neurochem.* 1978; 30:1095–1099. [PubMed: 660186]
- Commissiong JW. Mass fragmentographic analysis of monoamine metabolites in the spinal cord of rat after the administration of morphine. *J Neurochem.* 1983; 41:1313–1318. [PubMed: 6194263]
- Connor M, Christie MJ. Opioid receptor signaling mechanisms. *Clin Exp Pharmacol Physiol.* 1999; 26:493–499. [PubMed: 10405772]
- Deyo SN, Swift RM, Miller RJ. Morphine and endorphins modulate dopamine turnover in rat median eminence. *Proc Natl Acad Sci USA.* 1979; 76(6):3006–3009. [PubMed: 288082]
- Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentration in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci USA.* 1988; 85:5274–5278. [PubMed: 2899326]
- Fleetwood-Walker SM, Coote JH. Contribution of noradrenaline-, dopamine-, and adrenaline-containing axons to the innervation of different regions of the spinal cord of the cat. *Brain Res.* 1981; 206:95–106. [PubMed: 7470896]
- Fleetwood-Walker SM, Hope PJ, Mitchell R. Antinociceptive actions of descending dopaminergic tracts on cat and rat dorsal horn somatosensory neurons. *J Physiol.* 1988; 399:335–348. [PubMed: 2841456]
- Gao X, Zhang Y, Wu G. Effects of dopaminergic agents on carrageenan hyperalgesia after intrathecal administration to rats. *Eur J Pharmacol.* 2001; 418(1–2):73–77. [PubMed: 11334867]
- Giuliano F, Allard J, Rampin O, Droupy S, Benoit G, Alexandre L, Bernabe J. Spinal proerectile effect of apomorphine in the anesthetized rat. *Int J Imp Res.* 2001; 13:11–115.
- Giuliano F, Allard J, Rampin O, Droupy S, Benoit G, Alexandre L, Bernabe J. Pro-erectile effect of systemic apomorphine: existence of a spinal site of action. *J Urol.* 2002; 167:402–406. [PubMed: 11743364]
- Gouarderes C, Cros J. Opioid binding sites in different levels of rat spinal cord. *Neuropeptides.* 1984; 5(1–3):113–116. [PubMed: 6099476]
- Gouarderes C, Cros J, Quirion R. Autoradiographic localization of mu, delta and kappa opioid receptor binding sites in rat and guinea pig spinal cord. *Neuropeptides.* 1985; 6(4):331–342. [PubMed: 2995864]
- Haskins JT, Gudelsky GA, Moss RL, Porter JC. Iontophoresis of morphine into the arcuate nucleus: effects on dopamine concentrations in hypophysial portal plasma and serum prolactin concentrations. *Endocrinology.* 1981; 108(3):767–771. [PubMed: 7460840]
- Hasue RH, Shammah-Lagnado SJ. Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. *J Comp Neurol.* 2002; 454(1):15–33. [PubMed: 12410615]

- Herkeham M, Pert CB. In vitro autoradiography of opiate receptors in rat brain suggests loci of "opiateergic" pathways. *Proc Natl Acad Sci USA*. 1980; 77(9):5532–5536. [PubMed: 6254088]
- Holstege JC, VanDijken H, Buijs RM, Goedknecht H, Gosens T, Bongers CMH. Distribution of dopamine immunoreactivity in rat, cat, and monkey spinal cord. *J Comp Neurol*. 1996; 376:631–652. [PubMed: 8978475]
- Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I, Niki H. Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? *Neurosci Res*. 2002; 44:121–131. [PubMed: 12354627]
- Iwatsubo K, Clouet DH. Effects of morphine and haloperidol on the electrical activity of rat nigrostriatal neurons. *J Pharmacol Exp Ther*. 1977; 202(2):429–436. [PubMed: 886472]
- Jensen TS, Yaksh TL. Effects of an intrathecal dopamine agonist, apomorphine on thermal and chemical evoked noxious responses in rats. *Brain Res*. 1984; 296:285–293. [PubMed: 6322926]
- Johnson SW, North RA. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci*. 1992; 12:483–488. [PubMed: 1346804]
- Karoum F, Wyatt RJ, Costa E. A comparative study on the effects of morphine on central, peripheral, and ganglionic stores of catecholamines. *J Pharmacol Exp Ther*. 1981; 216(2):321–328. [PubMed: 6257887]
- Konkoy CS, Childers SR. Dynorphin-selective inhibition of adenylyl cyclase in guinea pig cerebellum membranes. *Mol Pharmacol*. 1989; 36:627–633. [PubMed: 2572962]
- Lahlou S, Peteijean P, Pellissier G, Mouchet P, Feuerstein C, Demenge P. Rostrocaudal localization of cardiovascular responses induced by intrathecal administration of apomorphine in conscious, freely moving rats. *J Cardiovasc Pharmacol*. 1990; 16:331–337. [PubMed: 1697391]
- Levant B, McCarson KE. D3 dopamine receptors in rat spinal cord: implications for sensory and motor function. *Neurosci Lett*. 2001; 303:9–12. [PubMed: 11297811]
- Lewis ME, Khachaturian H, Watson SJ. Comparative distribution of opiate receptors and three opioid peptide neuronal systems in rhesus monkey central nervous system. *Life Sci*. 1983; 33(Suppl 1): 239–242. [PubMed: 6141488]
- Lindley SE, Gunnet JW, Lookingland KJ, Moore KE. 3,4-Dihydroxyphenylacetic acid concentrations in the intermediate lobe and neural lobe of the posterior pituitary gland as an index of tuberohypophysial dopaminergic neuronal activity. *Brain Research*. 1990; 506(1):133–8. [PubMed: 2302550]
- Loh HH, Smith AP. *Ann Rev Pharmacol Toxicol*. 1990; 30:123–147. [PubMed: 2160790]
- Lookingland KJ, Moore KE. Differential effects of morphine on the rates of dopamine turnover in the neural and intermediate lobes of the rat pituitary gland. *Life Sci*. 1985; 37(13):1225–1229. [PubMed: 2995741]
- Lookingland, KJ.; Moore, KE. Functional neuroanatomy of hypothalamic dopaminergic neuroendocrine systems. In: Dunnet, SB., editor. *Handbook of Chemical Neuroanatomy*. Vol. 21. Elsevier; Amsterdam: 2005. p. 435-523.
- Loose MD, Kelly MJ. Opioids act at μ -receptors to hyperpolarize arcuate neurons via an inwardly rectifying potassium conductance. *Brain Res*. 1990; 513:15–23. [PubMed: 2161696]
- Lord JA, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. *Nature*. 1997; 267:495–499. [PubMed: 195217]
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265–275. [PubMed: 14907713]
- Maisky VA, Doroshenko NZ. Catecholamine projections to the spinal cord in the rat and their relationship to central cardiovascular neurons. *J Auton Nerv Syst*. 1991; 34(2–3):119–128. [PubMed: 1918805]
- Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther*. 1976; 197(3):517–532. [PubMed: 945347]
- Morizumi T, Leduc-Cross B. Is there a dopaminergic projection from the A11 catecholamine cell group to the amygdala? *Exp Brain Res*. 1992; 88:451–454. [PubMed: 1349535]
- Mouchet P, Guerin B, Feuerstein C. Dissociate destruction of noradrenaline and dopamine descending projections in the thoracic spinal cord of the rat. *Life Sci*. 1982; 30:373–381. [PubMed: 7070213]

- Pappas SS, Behrouz B, Janis KL, Goudreau JL, Lookingland KJ. Lack of D2 receptor mediated regulation of dopamine synthesis in A11 diencephalospinal neurons in male and female mice. *Brain Res.* 2008; 1214:1–10. [PubMed: 18462709]
- Pappas SS, Tiernan CT, Behrouz B, Jordan CL, Breedlove SM, Goudreau JL, Lookingland KJ. Neonatal androgen dependent sex differences in lumbar spinal cord DA concentrations and the number of A11 diencephalospinal dopamine neurons. *J Comp Neurol.* 2010; 518(13):2423–2436. [PubMed: 20503420]
- Peyron C, Luppi PH, Kitahama K, Fort P, Hermann DM, Jouvet M. Origin of the dopaminergic innervation of the rat dorsal raphe nucleus. *Neuroreport.* 1995; 6(18):2527–2531. [PubMed: 8741755]
- Qu S, Ondo WG, Zhang X, Xie WJ, Pan TH, Le WD. Projections of diencephalic dopamine neurons into the spinal cord in mice. *Exp Brain Res.* 2006; 168(1–2):152–156. [PubMed: 16044299]
- Ridet JL, Sandillon F, Rajaofetra N, Geffard M, Privat A. Spinal dopaminergic system of the rat: light and electron microscopic study using an antiserum against dopaminergic, with particular emphasis on synaptic incidence. *Brain Res.* 1992; 598:233–241. [PubMed: 1486484]
- Skagerberg G, Bjorklund A, Lindvall O, Schmidt RH. Origin and termination of the diencephalospinal dopamine system in the rat. *Brain Res Bull.* 1982; 9:237–244. [PubMed: 7172029]
- Skagerberg G, Lindvall O. Organization of diencephalic dopamine neurones projecting to the spinal cord in the rat. *Brain Res.* 1985; 342:340–351. [PubMed: 4041835]
- Tavakoli-Nezhad M, Arbogast LA. Mu and kappa opioid receptor expression in the mediobasal hypothalamus and effectiveness of selective antagonists on prolactin release during lactation. *Neuroscience.* 2010; 166(2):359–367. [PubMed: 20045447]
- Traynor JR, Wood MS. Distribution of opioid binding sites in spinal cord. *Neuropeptides.* 1987; 10(4):313–320. [PubMed: 2829051]
- Trenkwalder C, Paulus W. The restless legs syndrome. *Lancet Neurol.* 2005; 4:465–475. [PubMed: 16033689]
- Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. *Ann Rev Biochem.* 2004; 73:953–990. [PubMed: 15189164]
- Wei H, Viisanen H, Pertovaara A. Descending modulation of neuropathic hypersensitivity by dopamine D2 receptors in or adjacent to the hypothalamic A11 cell group. *Pharmacol Res.* 2009; 59(5):355–363. [PubMed: 19416636]
- Weil-Fugazza J, Godefroy F. Further evidence for the involvement of the diencephalo-dopaminergic system in pain modulation: a neurochemical study on the effect of morphine in the arthritic rat. *Int J Tiss Reac.* 1991; 13(6):305–310.
- Yokoyama C, Okamura H, Nakajima T, Taguchi J, Ibata Y. Autoradiographical distribution of [3H]YM-09151–2, a high affinity and selective antagonist ligand for the dopamine D2 receptor group in the rat brain and spinal cord. *J Comp Neurol.* 1994; 344:121–136. [PubMed: 7914895]
- Yoshida M, Tanaka M. Existence of a new dopaminergic terminal plexus in the rat spinal cord: assessment by immunohistochemistry using anti-dopamine serum. *Neurosci Lett.* 1988; 94:5–9. [PubMed: 3071747]
- Zhu H, Clemens S, Sawchuk M, Hochman S. Expression and distribution of all dopamine receptor subtypes (D1–D5) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic in situ hybridization study. *Neuroscience.* 2007; 149:885–897. [PubMed: 17936519]

- Morphine administration increases dopamine synthesis and metabolism in A11 diencephalospinal dopamine neurons
- Opioid-mediated activation of dopamine neurons occurs in both male and female mice
- Basal thoracic spinal cord DOPAC/DA ratios are higher than Lumbar spinal cord ratios.
- Opioid receptors regulate the activity of A11 spinal cord- projecting DA neurons, likely through a dis-inhibitory mechanism

MALE

Figure 1.

Dose response (Panels A and B) and time course (Panels C and D) effects of systemic morphine administration on spinal cord DA concentrations and DOPAC/DA ratios in male mice. Male mice ($n = 8/\text{group}$) received subcutaneous injections of morphine (5, 10, or 20 mg/kg) or saline vehicle (10 mL/kg) and were sacrificed 1 hr later (Panels A and B). Male mice ($n = 8/\text{group}$) received subcutaneous injections of morphine (20 mg/kg) or saline vehicle (10 mL/kg) and were sacrificed 30, 60, or 120 min later (Panels C and D). Open symbols represent values not different from saline treated controls and error lines represent ± 1 SEM. * with closed symbols represent values significantly higher than saline control within each region. # indicates values control values significantly different between regions ($P \leq 0.05$, two-way ANOVA with post-hoc Tukey's test).

FEMALE

Figure 2.

Dose response (Panels A and B) and time course (Panels C and D) effects of systemic morphine injection on spinal cord DA concentrations and DOPAC/DA ratios in female mice. Female mice ($n=6-8$ /group) received subcutaneous injections of morphine (5, 10, or 20 mg/kg) or saline vehicle (10 mL/kg) and were sacrificed 1 hr later (Panels A and B). Female mice ($n=8$ /group) received subcutaneous injections of morphine (20 mg/kg) or saline vehicle (10 mL/kg) and were sacrificed 30, 60, or 120 min later (Panels C and D). Open symbols represent values not different from saline treated controls and error lines represent ± 1 SEM. * with closed symbols represent values significantly higher than saline control within each region. # indicates control values significantly different between regions ($P \leq 0.05$, two-way ANOVA with post-hoc Tukey's test).

Figure 3.

Effects of morphine and naloxone on spinal cord DA concentrations and DOPAC/DA ratio in male (panels A and B) and female (panels C and D) mice. Male or female mice ($n=7-8$ /group) received a single subcutaneous injection of saline vehicle (10 mL/kg), morphine (20 mg/kg), naloxone (10 mg/kg), or naloxone (10 mg/kg) followed by morphine 30 min later (20mg/kg), and were sacrificed 1 hr after the morphine injection. Columns represent the mean and vertical lines + 1 SEM. * indicates values significantly different from saline treated controls ($P \leq 0.05$, one-way ANOVA with post-hoc Tukey's test).

Striatum, nucleus accumbens, and median eminence concentrations of DA and DOPAC and the ratio of DOPAC/DA in male and female mice following administration of saline, morphine (20 mg/kg), naloxone (10 mg/kg) + morphine (20 mg/kg) or naloxone (10 mg/kg). Mean and (1 SEM), n=8 mice per group.

Table 1

	DA (ng/mg protein)				DOPAC (ng/mg protein)				DOPAC/DA			
	Saline	Morphine	Naloxone + Morphine	Naloxone	Saline	Morphine	Naloxone + Morphine	Naloxone	Saline	Morphine	Naloxone + Morphine	Naloxone
Striatum												
Male	145.5 (5.9)	143.8 (4.3)	138.0 (6.4)	142.9 (3.4)	6.6 (0.3)	12.5* (0.4)	7.5 (0.3)	6.8 (0.4)	0.045 (0.002)	0.087* (0.003)	0.054 (0.002)	0.048 (0.003)
Female	194.9 (5.1)	190.3 (4.2)	190.4 (9.2)	182.0 (8.8)	7.7 (0.6)	16.2* (0.6)	11.5 (0.9)	8.7 (0.6)	0.039 (0.002)	0.085* (0.002)	0.060 (0.003)	0.048 (0.004)
Nucleus Accumbens												
Male	149.0 (5.7)	135.7 (4.9)	122.6 (5.1)	134.7 (4.5)	10.6 (0.4)	24.1* (1.1)	11.6 (0.4)	11.7 (0.4)	0.071 (0.002)	0.178* (0.006)	0.095 (0.003)	0.087 (0.004)
Female	129.9 (11.6)	126.1 (6.0)	135.2 (5.5)	117.5 (9.0)	10.1 (0.4)	22.3* (1.9)	14.8 (1.2)	10.3 (0.7)	0.080 (0.004)	0.179* (0.016)	0.109 (0.006)	0.089 (0.004)
Median Eminence												
Male	182.0 (25.2)	192.7 (10.6)	195.7 (12.2)	182.93 (21.1)	5.1 (0.9)	7.3 (0.9)	5.6 (0.7)	4.5 (0.6)	0.028 (0.003)	0.037 (0.003)	0.028 (0.002)	0.024 (0.001)
Female	139.0 (8.9)	121.9 (8.8)	115.5 (7.7)	104.12 (7.1)	5.8 (0.4)	7.4 (0.5)	5.6 (0.5)	4.4 (0.3)	0.042 (0.002)	0.062 (0.003)	0.050 (0.005)	0.043 (0.002)

* Significantly different from saline treated control levels (P<0.05, one-way ANOVA).