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Opioid effects on spinal [^{3}H]5-hydroxytryptamine release are not related to their antinociceptive action

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Abstract

Several opioid compounds were evaluated for an ability to modulate the K^+-stimulated release of [^{3}H]serotonin ([^{3}H]5-hydroxytryptamine, [^{3}H]5-HT) from rat spinal cord synaptosomal and tissue slice preparations. Selective κ-opioid receptor agonists depressed K^+-stimulated release of the radiolabelled transmitter from both tissue preparations, an effect which was reversed by norbinaltorphimine. Conversely, the selective μ- and δ-opioid receptor agonists [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE), respectively, enhanced the K^+-stimulated release of [^{3}H]5-HT. This effect was only seen using the tissue slice preparation. When used at concentrations near its reported K_d for μ-opioid receptors, the selective μ-opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) blocked the action of DAMGO, but had no effect on the action of DPDPE. However, higher concentrations of CTOP, as well as all effective concentrations of selective δ-opioid receptor antagonists, blocked the action of both DAMGO and DPDPE. All agonist effects on spinal 5-HT release, regardless of the tissue preparation, were only seen at high (μM) concentrations. Moreover, effects of the opioid agonists were not consistent with the reported involvement of spinal 5-HT neurotransmission in the mediation of their antinociceptive action. Thus, the ability of opioids to modulate spinal 5-HT release appears to be of minimal physiological significance.

Keywords: DAMGO ([D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin); DPDPE ([D-Pen^2,D-Pen^5]enkephalin); Naltrindole; ICI174864; 5-HT (5-hydroxytryptamine, serotonin); Spinal cord

1. Introduction

Spinopetal serotonergic neuronal systems have previously been shown to be involved in the mediation of antinociception. An enhancement of spinal serotonergic neurotransmission can be demonstrated following pharmacological or electrical activation of midbrain sites known to mediate analgesia (Lewis and Gebhart, 1977; Yaksh, 1979; Yaksh and Tyce, 1979; Yaksh and Wilson, 1979; Rivot et al., 1982). In addition, serotonergic receptor antagonists have been shown to reduce the effect of supraspinally (Yaksh, 1979) and systemically (Pekoe and Smith, 1982) administered opioids. Furthermore, the intrathecal administration of serotonin (5-HT) has been shown to produce analgesia which can be attenuated by 5-HT receptor antagonists (Schmauss et al., 1983; Kellstein et al., 1988; Solomon and Gebhart, 1988; Crisp and Smith, 1989).

The intrathecal administration of certain opioid drugs results in an antinociception which appears to be mediated in part by spinal serotonergic processes. Intrathecally applied β-endorphin (Crisp et al., 1989) or morphine (Kellstein et al., 1988; Crisp and Smith, 1989) produces antinociception which is blocked by 5-HT receptor antagonists. One possible mechanism of action of these opioids may be the regulation of spinal 5-HT release. The ability of opioids to modulate the release of [^{3}H]5-HT from a rat spinal cord synaptosomal preparation has previously been examined in this laboratory (Monroe et al., 1986), however the current study expands this work to include additional antinoci-

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ceptive agents, and a spinal cord slice preparation in which neuronal connectivity is less compromised. Compounds which were evaluated included morphine and β-endorphin, the κ-opioid receptor agonists ethylketocyclazocine and U50488H [*trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulfonate], the selective δ-opioid receptor agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE), and the selective μ-opioid receptor agonist [D-Ala^2,Gly-NMePhe^4,Gly-ol^5]enkephalin (DAMGO).

2. Materials and methods

All animals were used in accordance with guidelines established by the US Public Health Service 'Policy on the Humane Care and Use of Laboratory Animals'. Experimental protocols were approved by the WVU Animal Care and Use Committee. Male Sprague-Dawley rats (295–325 g, Hilltop Laboratory Animals, Scottdale, PA, USA.) were housed for at least 2 days prior to use in the animal facility at the WVU Health Sciences Center, and were given food and water ad libitum. The rats were killed by decapitation and the spinal cord was rapidly removed under hydrostatic pressure. Using a 12 ml syringe with an 18 gauge needle, ice-cold 0.32 M sucrose was injected into the caudal end of the vertebral canal, shearing the spinal cord free from its dorsal and ventral nerve roots and expelling it intact from the rostral end of the canal. For preparation of synaptosomes, the tissue was homogenized in 5 ml of 0.32 M sucrose using a Teflon/glass homogenizer, then centrifuged at $1000 \times g$ for 10 min in a Sorvall RC2B centrifuge. The resulting supernatant was decanted and centrifuged at $10\,000 \times g$ for 20 min, yielding a pellet enriched in synaptosomes (Gray and Whittaker, 1962). Slices of spinal cord were prepared using a McIlwain tissue chopper. Desheathed spinal cord was chopped twice at 0.3 mm intervals, rotated 90°, and chopped twice more. The resulting mince was suspended in 10 volumes of Beeson's solution having the following composition (mM): NaCl 126.5, KCl 1.4, $CaCl_2$ 1.1, $NaSO_4$ 0.5, KH_2PO_4 0.5, glucose 5.9, $MgCl_2$ 0.83, $NaHCO_3$ 27.5 (pH adjusted to 7.2; continuously gassed with a 95–5% mixture of O_2/CO_2), then centrifuged at $1000 \times g$ for 5 min.

The synaptosomal or slice preparations were resuspended in 10 volumes of gassed Beeson's solution, the container capped, and the tissue incubated for 10 min at 37°C. [^{3}H]5-HT was then added to produce a final incubation concentration of 100 nM, after which the tissue was incubated an additional 10 min at 37°C. Aliquots (0.2 ml) of the labeled tissue suspension were then pipetted into superfusion chambers (Williams et al., 1992) and superfused (0.5 ml/min) with gassed Beeson's solution for 60 min to allow [^{3}H]5-HT and [^{3}H]5-hydroxyindole-acetic acid ([^{3}H]5-HIAA) efflux to reach a constant level.

Five minute samples were collected just prior to, and immediately following the addition of opioid. A third, 10 min sample was collected, after which the tissues were lysed with 1 N HCl to determine the remaining tritium content of the tissue. [^{3}H]5-HT and [^{3}H]5-HIAA in superfusate fractions were separated by ion exchange chromatography (Williams et al., 1992) and quantitated using liquid scintillation spectrophotometry.

All drugs were initially screened for their ability to alter basal [^{3}H]-efflux in preliminary experiments which did not include a depolarizing (i.e. 15 mM K^+) stimulus. Effects on basal efflux were assessed by comparing the rate of ^{3}H-efflux/min in the third sample to that of the first. When opioid effects on K^+-stimulated release (i.e. release of [^{3}H]5-HT in excess of basal levels which was seen in response to an elevated concentration of K^+) were evaluated, the tissues were superfused with buffer containing 15 mM K^+ for the first 90 s (synaptosomes) or 5 min (slices) of the third fraction. To maintain isotonicity in these experiments, NaCl was reduced on an equimolar basis to the increase in KCl. K^+-stimulated [^{3}H]5-HT release was calculated as a percentage of available tissue stores (e.g. Monroe et al., 1986). In all experiments, when present, opioids were included in the superfusion buffer throughout the entire 15 min period prior to HCl lysis of the tissue.

Basal efflux levels averaged 3.5% of tissue stores. The [^{3}H]5-HIAA to [^{3}H]5-HT content ranged from 2.5:1 to 4:1 for the synaptosomal and slice preparations, respectively. [^{3}H]5-HIAA levels did not increase during periods of K^+-stimulation (see Monroe and Smith, 1985), whereas [^{3}H]5-HT levels increased 3–5-fold compared to basal efflux. Within each assay, all experimental conditions (including controls) were performed in triplicate. Each assay was performed at least three times. To control for potential day to day variability, each experimental condition was expressed as a percentage of a day matched control value. One way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) post hoc comparison, or Student's *t*-test (where appropriate), were used to determine statistical significance ($P < 0.05$).

The following drugs were purchased from the indicated sources: [^{3}H]5-HT (DuPont/NEN, Boston, MA); DPDPE and β-endorphin (Sigma, St. Louis, MO); DAMGO and ICI174864 (*N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; Aib = α-aminoisobutyric acid) (Cambridge Research Biochemicals, Wilmington, DE); morphine sulfate (Mallinkrodt, St. Louis, MO); naltrindole (Research Biochemicals, Natick, MA); D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (Bachem, Torrance, CA). Ethylketocyclazocine, U50488H, and norbinaltorphimine were graciously supplied by Ster-

ling-Winthrop (Rochester, NY), Upjohn (Kalamazoo, MI), and Dr. Philip Portoghese (Univ. of Minnesota), respectively.

3. Results

3.1. Synaptosomal tissue preparation

All of the compounds tested were initially evaluated at a 1 μM concentration for an ability to alter basal efflux. Only morphine and β-endorphin were found to cause a significant effect, increasing basal [^{3}H]5-HIAA efflux to 140 ± 4 and 127 ± 12 (mean $\pm$ S.E.M.) percent of control, respectively. Basal [^{3}H]5-HT efflux was not altered by 1 μM concentrations of any of the opioids tested.

Of all the opioids tested, only ethylketocyclazocine and U50488H altered K^+-stimulated [^{3}H]5-HT release (Fig. 1). Superfusion of the synaptosomes with 1 μM concentrations of the κ-opioid receptor agonists depressed release to 67 and 71 percent of control, respectively. Lower concentrations of ethylketocyclazocine did not significantly alter K^+-stimulated [^{3}H]5-HT release. The inhibitory effect of 1 μM concentrations of ethylketocyclazocine or U50488H was blocked by the co-superfusion of the synaptosomes with 10 nM norbinaltorphimine (Fig. 2).

3.2. Tissue slice preparation

None of the opioids tested altered basal [^{3}H]5-HIAA or [^{3}H]5-HT efflux when superfused at a 1 μM concentration. Superfusion of the tissue slices with 1 μM concentrations of the opioids resulted in mixed effects on K^+-stimulated [^{3}H]5-HT release (Fig. 1). Consistent with their action in the synaptosomal preparation, the addition of 1 μM concentrations of ethylketocyclazocine or U50488H to the superfusion buffer depressed K^+-stimulated [^{3}H]5-HT release to 79 and 81 percent of control, respectively. Conversely, DAMGO

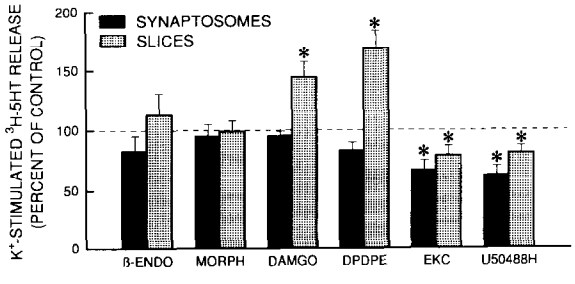

Fig. 1. The effect of a 1 μM concentration of opioid on the K^+-stimulated release of [^{3}H]5-HT from rat spinal cord synaptosomal and slice preparations. Release is expressed as percent of paired controls which were stimulated in the absence of opioid. Values are means $\pm$ S.E.M. from at least four experiments. For the synaptosomal data presented in this figure, control K^+-stimulated [^{3}H]5-HT release averaged $3.65 \pm 0.37\%$ ($n = 25$) of available tissue stores, which averaged 122436 ± 3665 DPM per superfusion chamber ($n = 107$). Using the slice preparation, control K^+-stimulated [^{3}H]5-HT release averaged $2.51 \pm 0.16\%$ ($n = 51$) of available tissue stores, which averaged 222894 ± 4522 dpm per superfusion chamber ($n = 188$). β-ENDO = β-endorphin; MORPH = morphine; EKC = ethylketocyclazocine. Significantly different from control * $P < 0.05$.

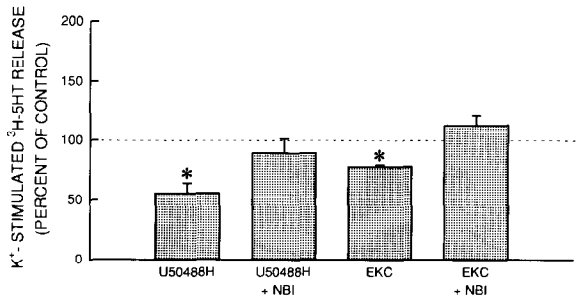

Fig. 2. The effect of 10 nM norbinaltorphimine (NBI) on the ability of 1 μM U50488H or ethylketocyclazocine (EKC) to decrease the K^+-stimulated release of [^{3}H]5-HT from rat spinal cord synaptosomes. Release is expressed as percent of paired controls which were stimulated in the absence of opioid or antagonist, which by itself did not affect release. Values are means $\pm$ S.E.M. from three experiments. Control K^+-stimulated [^{3}H]5-HT release for the data presented in this figure averaged $1.38 \pm 0.10\%$ ($n = 9$) of available tissue stores, which averaged 85073 ± 3844 dpm per superfusion chamber ($n = 41$). Significantly different from control * $P < 0.05$.

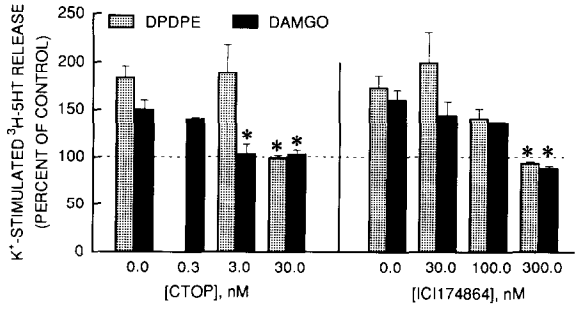

Fig. 3. The effect of CTOP (left) and ICI174864 (right) on the ability of a 1 μM concentration of DPDPE and DAMGO to enhance the K^+-stimulated release of [^{3}H]5-HT from rat spinal cord slices. Release is expressed as percent of paired controls which were stimulated in the absence of opioid or antagonists, which by themselves did not affect release. Values are means $\pm$ S.E.M. from four experiments. Both agonists significantly enhanced release compared to control ($P < 0.05$). For the data presented in the left panel, DAMGO: control K^+-stimulated [^{3}H]5-HT release averaged $3.40 \pm 0.16\%$ ($n = 30$) of available tissue stores, which averaged 185091 ± 5497 dpm per superfusion chamber ($n = 88$); DPDPE: control K^+-stimulated [^{3}H]5-HT release averaged $2.92 \pm 0.19\%$ ($n = 20$) of available tissue stores, which averaged 197524 ± 6151 DPM per superfusion chamber ($n = 54$). For the data presented in the right panel, DAMGO: control K^+-stimulated [^{3}H]5-HT release averaged $3.20 \pm 0.14\%$ ($n = 29$) of available tissue stores, which averaged 192580 ± 5033 dpm per superfusion chamber ($n = 86$); DPDPE: control K^+-stimulated [^{3}H]5-HT release averaged $3.09 \pm 0.15\%$ ($n = 28$) of available tissue stores, which averaged 185480 ± 5454 dpm per superfusion chamber ($n = 81$). Significantly different from agonist alone * $P < 0.05$.

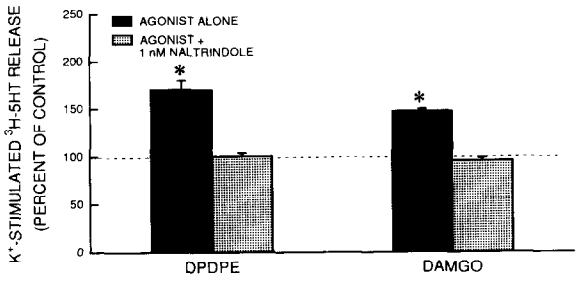

Fig. 4. The effect of 1 nM naltrindole on the ability of 1 μM DAMGO or DPDPE to increase the K^+-stimulated release of [^{3}H]5-HT from rat spinal cord slices. Release is expressed as percent of paired controls which were stimulated in the absence of opioid or antagonist, which by itself did not affect release. Values are means ± S.E.M. from three experiments. Control K^+-stimulated [^{3}H]5-HT release for the data presented in this figure averaged 3.21 ± 0.23% ($n = 17$) of available tissue stores, which averaged 184254 ± 7946 dpm per superfusion chamber ($n = 49$). Significantly different from control * $P < 0.05$.

and DPDPE were found to increase K^+-stimulated [^{3}H]5-HT release by approximately 50 percent. Concentrations of DPDPE or DAMGO lower than 1 μM had no effect on K^+-stimulated [^{3}H]5-HT release. The addition of β-endorphin (1 μM) or morphine (1 nM to 1 μM) to the superfusion buffer also had no effect on K^+-stimulated [^{3}H]5-HT release (Fig. 1).

The release enhancing action of 1 μM DAMGO was blocked by a 3 nM concentration of the selective μ-opioid receptor antagonist CTOP (Fig. 3). The same concentration of antagonist failed to alter the action of 1 μM DPDPE (Fig. 3). Both agonists were, on the other hand, blocked by a 30 nM concentration of CTOP (Fig. 3).

The addition of a 300 nM concentration of the selective δ-opioid receptor antagonist ICI174864 to the superfusion media resulted in an antagonism of the actions of both DAMGO and DPDPE (Fig. 3). Lesser concentrations failed to antagonize either agonist (Fig. 3). The release enhancing effects of DAMGO and DPDPE were also completely blocked by the addition of a 1 nM concentration of the selective δ-opioid receptor antagonist naltrindole to the superfusion media (Fig. 4).

4. Discussion

The effects of ethylketocyclazocine and U50488H on K^+-stimulated 5-HT release from spinal cord tissue are apparently mediated via κ-opioid receptors which are located directly on 5-HT nerve terminals. Both compounds which selectively interact with κ-opioid receptors depressed the K^+-stimulated release of [^{3}H]5-HT from spinal cord nerve terminal (synaptosomal) and slice preparations. Moreover, norbinaltorphimimine, the selective κ-opioid receptor antagonist, blocked the effect of both agonists. In contrast, the effects of DAMGO and DPDPE appear to be mediated via receptors which are located on neurons that converge on spinal serotonergic processes. The ability of these peptides to enhance release was only seen in the spinal cord slice preparation.

The receptors mediating the effects of DPDPE and DAMGO are more difficult to characterize. The ability of DPDPE to enhance K^+-stimulated [^{3}H]5-HT release is blocked by concentrations of the δ-opioid receptor antagonists ICI174864 (300 nM) and naltrindole (1 nM) which are selective for the δ-opioid receptor subtype (Cotton et al., 1984; Portoghese et al., 1988, respectively), suggesting an interaction with δ-opioid receptors. Similarly, the effect of DAMGO would appear to be mediated via μ-opioid receptors. A concentration of CTOP (3 nM) which is consistent with the affinity of μ-opioid receptors for the antagonist (Pelton et al., 1986) completely blocked the effect of DAMGO, while having no effect on the ability of DPDPE to enhance release. However, superfusion of the tissue slices with 30 nM CTOP blocked the actions of both DAMGO and DPDPE. In addition, when used at a concentration which approximates the reported affinity of the δ-opioid receptor for the antagonist (300 nM; Cotton et al., 1985), ICI174864 also effectively blocked the ability of DAMGO to enhance release.

These observations can not be explained by a non-selective interaction of the antagonists with other opioid receptor subtypes. Based on the reported affinity of the δ-opioid receptor for CTOP (5600 nM; Kazmierski et al., 1988), an effective interaction of the antagonist with this opioid receptor subtype is unlikely at a 30 nM concentration. Furthermore, concentrations of ICI174864 up to 5 μM have been demonstrated to be ineffective in blocking μ-opioid agonist-mediated effects (Cotton et al., 1984). Moreover, when used at a concentration selective for interactions with δ-opioid receptors, naltrindole also blocked the effect of DAMGO.

The ability of μ- and δ-opioid receptor antagonists to block the actions of both DAMGO and DPDPE does not result from interactions with the reported μ/δ-opioid receptor complex. The results of antinociceptive and biochemical studies have suggested the existence of physically associated μ- and δ-opioid receptors, based on the ability of δ-opioid receptor agonists and antagonists to modulate the actions of μ-opioid receptor agonists (Heyman et al., 1989a,b; Schoffelmeer et al., 1992). However, Schoffelmeer and co-workers (1992) have shown that antagonism of the regulation of adenylate cyclase in the striatum, a μ/δ-opioid receptor complex-mediated effect, requires concentrations of CTOP and naltrindole which are considerably higher than those needed to block μ- or δ-opioid receptor-mediated regulation of neurotransmitter re-

lease and, higher than those concentrations which were effective in the current study.

The action of opioids on neurotransmitter release varies, depending on the given neurotransmitter, species, and central nervous system region evaluated. Using tissues from a number of species, several investigators have demonstrated the activation of κ-opioid receptors to depress [^{3}H]-neurotransmitter release ([^{3}H]5-HT, Passarelli and Costa, 1989; [^{3}H]-dopamine, Smith et al., 1992; Mulder et al., 1991; [^{3}H]-norepinephrine, Kinouchi et al, 1989; Werling et al., 1989). However, in contrast to these and the current results, U50488H has been reported to enhance the release of [^{3}H]5-HT from mouse brain slices and spinal cord synaptosomes (Ho and Takemori, 1990). Furthermore, a κ-opioid receptor mediated enhancement of neurotransmitter release has been reported for acetylcholine in mouse cerebral cortex (Ennis and Stephens, 1984) and substance P in rat trigeminal slices (Suarez-Roca and Maixner, 1993). Passarelli and Costa (1989) have reported an inhibitory action of DAMGO and DPDPE on the K^+-stimulated release of [^{3}H]5-HT from hippocampal slices, however, Spanagel et al. (1990) demonstrated a faciliatory action of the peptides on dopamine release in vivo.

In addition, conflicting reports exist regarding the role of spinal 5-HT neurotransmission in mediating the analgesic effects of opioids. Studies using intrathecally applied receptor antagonists suggest a serotonergic component to the antinociception resulting from spinally applied morphine and β-endorphin (Kellstein et al., 1988; Crisp and Smith, 1989; Crisp et al., 1989; Schaus et al., 1991). However in another study, spinally applied morphine was found to have no effect on spinal 5-HIAA levels (Vasko et al., 1984). Similarly, Bineau-Thurotte and co-workers (1984) have shown that the release of 5-HT from rat spinal cord slices is unaffected by the presence of morphine in the superfusion media. Finally, using in vivo microdialysis, Matos and coworkers (1992) failed to demonstrate a clear correlation between spinal 5-HT release and analgesia resulting from systemically applied morphine, although the drug did increase 5-HIAA levels in some of the animals.

In mice, antinociception resulting from the stimulation of κ-opioid receptors has also been reported to be dependent on spinal 5-HT neurotransmission (Von Voigtlander et al., 1984; Ho and Takemori, 1989). Given the contrasting effects of κ-opioid receptor selective compounds on spinal 5-HT release in the mouse (Ho and Takemori, 1990) and rat (current study), it is likely that selective κ-opioid receptor agonists produce analgesia via a different mechanism in the rat. Similar species differences may also exist in the mechanism by which DAMGO and DPDPE produce analgesia. In mice, the intrathecal administration of DAMGO has been reported to produce antinociception which involves a serotonergic component (Schaus et al., 1991). However, Spanos et al. (1989) have reported that the antinociception resulting from the intrathecal administration of either compound is not antagonized by intrathecally applied 5-HT receptor antagonists in rats. Thus, it would appear that the effect of these peptides detected in the current study is not related to their antinociceptive action in rats. On the other hand, since a whole spinal cord preparation was used in the studies described herein, it could be that a facilitatory action on 5-HT release from spinal neurons involved in the mediation of antinociception could be masked by an inhibitory action on a larger population of spinal 5-HT neurons which are not involved in analgesia.

In summary, the activation of κ-opioid receptors located directly on 5-HT nerve endings results in an inhibition of 5-HT release from rat spinal cord tissue. Conversely, spinal 5-HT release is enhanced by DAMGO and DPDPE. The receptors mediating the action of the peptides are located on neuronal processes which converge on 5-HT neurons, but can not be clearly characterized as μ- or δ-opioid in nature. The ability of the opioids to regulate spinal 5-HT release is not related to their antinociceptive action since the effect of a given opioid on spinal 5-HT release does not correlate with the reported involvement of spinal 5-HT neurotransmission in the mediation of its antinociceptive action. Moreover, the physiological significance of the opioid regulation of spinal 5-HT release can be questioned given that high concentrations of agonists were required to produce an effect.

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