Opioid Regulation of Pallidal Enkephalin Release: Bimodal Effects of Locally Administered Mu and Delta Opioid Agonists in Freely Moving Rats

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ABSTRACT
The globus pallidus and ventral pallidum receive dense enkephalinergic innervation from the dorsal and ventral striatum, respectively. A previous study demonstrated peripheral morphine administration to increase pallidal enkephalin release. To determine whether such opioid stimulatory effects may be mediated directly in the pallidum, in vivo microdialysis was used to study the effects of local administration of several concentrations of the mu receptor agonists morphine and morphine-6-glucuronide (M6G) as well as the the delta receptor agonist SNC80 on pallidal enkephalin release in freely moving rats. Low concentrations of morphine or M6G (1–10 nM) enhanced the release of enkephalins, an effect that was reversed by coadministration of the mu receptor antagonist β-funaltrexamine (β-FNA). A similar stimulatory effect was observed with a low concentration of SNC80 (50 nM), an effect that was blocked by the delta antagonist naltrindole (NTD). High concentrations of morphine (100 nM to 100 μM) had little or no effect, whereas M6G (10 μM) suppressed enkephalin release, an effect that was reversed by β-FNA. Similarly, a high concentration (5 μM) of SNC80 suppressed enkephalin release. However, this effect was not blocked by NTD but was attenuated by β-FNA, suggesting a mu receptor-mediated action. These results offer in vivo evidence of bimodal (i.e., stimulatory and inhibitory) effects of mu and delta opioid agonists on enkephalin release in the pallidum.

The GP and VP (referred to collectively as the pallidum) are key brain regions involved in the regulation of complex motor behavior (for reviews, see Mogenson and Yang, 1991; Napier, 1993; Swedlow and Koob, 1987). The VP (but not the GP) has also been implicated in reward circuitry and may represent a point of convergence for the rewarding effects of psychostimulant and opiate drugs (Johnson and Stellar, 1994; Johnson et al., 1993; Koob, 1992; Panagis et al., 1995; Robledo and Koob, 1993). The GP and VP receive dense enkephalinergic innervation from the caudate nucleus and nucleus accumbens, respectively (Cuello and Paxinos, 1978; Del Fiacco et al., 1982; Staines et al., 1980; Zahm et al., 1985), and the presence of mu and delta opioid receptors has been demonstrated in both regions (Mansour et al., 1988, 1993, 1995ab; Delfs et al., 1994; Bausch et al., 1995; Ding et al., 1996).

Activation of mu or delta receptors in either the GP or VP produces an increase in locomotor activity (Austin and Kalivas, 1990; Dewar et al., 1985; Hoffman et al., 1991; Joyce et al., 1981; Napier, 1992), an effect that, at least in the VP, may be mediated by inhibition of GABA release from the terminals of nucleus accumbens projection neurons (Austin and Kalivas, 1990), many of which are known to colocalize GABA and enkephalin (Zahm et al., 1985). The postsynaptic effects of opiates within the pallidum at the cellular level have been extensively studied with electrophysiological techniques and have provided evidence for both inhibitory and excitatory actions of opiate drugs on pallidal neurons (Frey and Huffman, 1985; Huffman and Felpel, 1981; Huffman and Frey, 1989; Mitrovic and Napier, 1995; Napier et al., 1983, 1992).

In view of the implied importance of pallidal opioid peptides in locomotor and, with respect to the VP, reward-related behaviors, it is important to investigate the factors regulating the release of these peptides in these brain regions. By combining microdialysis with a sensitive radioimmun...
munoassay (Maidment et al., 1989; Maidment and Evans, 1991), we recently showed that systemically administered morphine induces a dose-dependent increase in the release of pallidal opioid peptides, primarily Met- and Leu-enkephalin, in freely moving rats (Olive et al., 1995). As a first step in elucidating the mechanism(s) underlying this response, we sought to determine if opiate drugs could produce opioid peptide-releasing effects via a direct action in the pallidum.

*In vivo* microdialysis was used to locally administer morphine, its active metabolite M6G and the delta receptor agonist SNC80 directly into the pallidum of freely moving rats while simultaneously measuring extracellular enkephalin in this structure. Parts of this study have been reported previously in abstract form (Olive and Maidment, 1996).

**Methods**

**Animals and housing.** Adult male Sprague-Dawley rats (250–350 g; Harlan, Madison, WI) were housed individually in cylindrical cages (8 × 15 inches; Instech, Plymouth Meeting, MA) before and during dialysis procedures under a normal 12:12 hr light/dark cycle (lights on 7:00 a.m.) with food and water *ad libitum*. All pharmacological experiments were performed during the lights-on phase. All experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

**Surgical preparation.** Animals were anesthetized with halothane in a 1:1 mixture of O<sub>2</sub> and N<sub>2</sub>O. Guide cannulae, constructed from 22-gauge needles (Becton-Dickinson, Franklin Lakes, NJ) 13 mm in length, were fitted with stylettes and lowered into the brain to a depth of 2 mm above the GP/VP stereotaxic coordinates: AP, −0.8 mm; ML, ±2.9 mm; DV, −3.0 mm from bregma and the skull surface; Paxinos and Watson, 1986). Three 3/16-inch skull screws (Small Parts, Miami Lakes, FL) were also implanted into the skull for headstage stability. Cannulae were secured to the skull with methyl metacrylate dental cement (Hygenic, Akron, OH), and animals were allowed to recover for 3 to 6 days before dialysis probe implantation.

**Drugs.** Morphine sulfate and M6G were obtained from Sigma Chemical (St. Louis, MO); SNC80 was from Tocris Cookson (St. Louis, MO), NTD and β-FNA were from Research Biochemicals International (Natick, MA). Because of its poor solubility in water, SNC80 was dissolved in 45% (w/v) 2-hydroxypropyl-beta-cyclodextrin (Research Biochemicals, Natick, MA) before dilution in aCSF.

**Microdialysis procedures.** After recovery from surgery, animals were lightly restrained as described above and stylettes removed from the cannulae. CMA/12 microdialysis probes with 4 mm polycarbonate membranes (10,000 molecular weight cutoff; CMA, Acton, MA), continuously perfused with an aCSF [containing 125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub> (H<sub>2</sub>O), 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1.2 mM CaCl<sub>2</sub>, 5 mM d-glucose, 0.2 mM L-ascorbic acid and 0.025% (w/v) bovine serum albumin, pH 7.3–7.5] at a rate of 2.0 µl/min were slowly lowered stereotaxically into the GP/VP over a 10-min period to a final depth of −9.2 mm from the skull surface (fig. 1). Probes were secured with dental cement and attached to dual-channel liquid swivels (Instech, Plymouth Meeting, PA) for freely moving microdialysis procedures. Animals were allowed to recover from probe implantation for ≥12 hr before pharmacological experimentation.

On the following day, drugs were incorporated into the perfusion medium for two 2-hr periods, each followed by 3 hr of washout, via 240-µl sample injection loops on Rhodyne 7010 HPLC valves (Alltech, Deerfield, IL). For pharmacological receptor blockade studies, antagonists were reverse dialyzed 1 hr before and throughout the duration of the second 2-hr agonist infusion period.

Dialysis samples were collected at a flow rate of 2.0 µl/min every 30 min at room temperature into polypropylene microcentrifuge tubes (Fisher Scientific, Tustin, CA) via an Isco automated fraction collector (Isco, Lincoln, NE). Samples were then stored at −70°C before radioimmunoassay procedures.

**Euthanasia and verification of probe placement.** At the end of each experiment, animals were deeply anesthetized with Nembutal (150 mg/kg i.p.) and transcardially perfused with 100 ml of 0.1 M phosphate-buffered saline containing 0.1% heparin, pH 7.4, followed by 500 ml of 10% buffered formalin phosphate (pH 7.0, Fisher Scientific, Tustin, CA). Brains were removed, postfixed in the same fixative overnight and then cryoprotected in a solution containing 30% sucrose in 0.1 M phosphate buffer, pH 7.4, for 48 hr. Brains were stored at −70°C and then cut into 30-µm coronal sections on a cryostat, mounted onto gelatin-coated slides and stained with cresyl violet for verification of dialysis probe placement.

**Radioimmunoassay for enkephalins.** A highly sensitive solid-phase “universal” opioid peptide radioimmunoassay was used to analyze pallidal dialysate opioid peptide content, as described elsewhere (Maidment et al., 1989; Maidment and Evans, 1991). Briefly, after acetylation, dialysis samples or standard concentrations of Met-enkephalin were incubated, together with the radioactive tracer peptide <sup>125</sup>I-N-Ac-γ-endorphin, in Immulon-4 microplate wells (Dynatech Laboratories, Chantilly, VA) to the surface of which was bound rabbit antiserum to the sequence N-Ac-Tyr,Gly,Gly,Phe,Met/Leu,X; a sequence common to all receptor-active endogenous enkephalins, endorphins and dynorphins. Removal of unbound tracer was accomplished by washing the wells which were then assayed for bound tracer peptide using a Micromedic Gamma Counter (Rohm and Haas, Huntsville, AL). The detection limit of this assay was 0.1 fmol, and the ED<sub>50</sub> was −1.5 fmol. Although this assay recognizes all three major classes of endogenous opioid peptides, previous HPLC analysis has shown the primary opioid peptides recovered from pallidal dialysates to be Met- and Leu-enkephalin (Maidment et al., 1989; Maidment and Evans, 1991).

**Statistical analysis.** Femtomole values for each 30-min sample were transformed to percentage of basal enkephalin release, assigning a value of 100% to the average enkephalin level in the six 30-min base-line samples collected before drug administration (fig. 2). All data are presented as mean ± S.E.M.. Throughout the text and figure legends, n refers to the number of animals. Because the time course of drug effects varied between animals, statistical dose-response analysis was performed on data averaged over 2-hr periods, comparing the 2 hr before the first administration with the two 2-hr drug infusion periods using one-way repeated measures analysis of variance (ANOVA) followed by a Dunnett’s posthoc test (Super-ANOVA; Abacus Concepts, Berkeley, CA). These data are presented as percent increase or decrease from base line (figs. 3–5). P ≤ 0.05 was considered to be statistically significant. Because the effects of
some opioid compounds has been reported to be prolonged by sus-
ception in 2-hydroxypropyl-b-cyclodextrin (Jang et al., 1992; Meert et al., 1992), data from the 30-min period following cessation of SNC80 administration was included in the analysis. Data from one or two animals from each group were discarded from analysis for one of three reasons: (1) the probe was placed outside of the GP/VP, (2) basal dialysate levels of enkephalins were not detectable and (3) dialysate levels of enkephalins were >2 S.D.s from mean values for a particular time point. In the latter case, such variability was attributed to inaccuracies in the pipetting of sample and/or tracer peptide in the radioimmunoassay.

Fig. 2. Time course of effects of local perfusion (denoted by bar) of A, artificial CSF (n = 9), B, 10 nM morphine (n = 8), C, 50 nM SNC80 (n = 6), and D, 5 µM SNC80 (n = 6) on enkephalin release from the pallidum. Data are expressed as percent of base-line enkephalin release (mean ± S.E.M.). Basal opioid peptide immunoreactivity levels were 0.36 ± 0.08, 0.57 ± 0.10, 0.37 ± 0.07, 0.87 ± 0.24 fmol/30 min for A through D, respectively. See figure 3 and Methods for statistical analysis of these data.

Results

Concentration-response analysis of morphine, M6G and SNC80. The mean ± S.E.M. basal level of pallidal dialysate opioid peptide immunoreactivity was 0.55 ± 0.03 fmol/30 min (n = 126). Reverse dialysis of aCSF had no significant effect on pallidal dialysate enkephalin levels (fig. 2A). Morphine, however, produced concentration-dependent changes in recovered enkephalin (Figs. 2B and 3A). A bell-shaped concentration-response curve was obtained with significant increases in release occurring in the range of 0.1 to 10 nM, with maximum increases of 77 ± 24% (infusion 1) and 82 ± 36% (infusion 2) at the 1 nM dose (F(2,15) = 4.0). No
significant increase was observed in the range of 100 nM to 100 µM. Indeed, at the highest dose tested (100 µM) a significant decrease (−28 ± 12%, F(2,8) = 4.9) was observed, although only during the second of the two infusions. Similarly, at the lowest dose tested (0.1 nM), only the second infusion produced a significant increase in release (48 ± 15%, F(2,12) = 7.0).

A distinct bimodal concentration-response effect was seen after local infusion of the morphine metabolite M6G into the pallidum (fig. 3B). The lowest concentration tested (10 nM) significantly increased enkephalin release during both infusions (69 ± 16% and 48 ± 21%, F(2,14) = 5.5). A concentration of 500 nM M6G induced a smaller, statistically insignificant increase during both infusions. A high concentration (100 µM) of M6G significantly reduced basal enkephalin release during both infusions (−31 ± 12% and −29 ± 10%, F(2,10) = 5.9).

A similar bimodal concentration-response effect was seen with local administration of the delta opioid agonist SNC80 into the pallidum (fig. 3C). The lowest concentration (5 nM) had no significant effect on basal enkephalin release, whereas a 50 nM concentration produced a significant but delayed (fig. 2C) increase in enkephalin release during both infusions (61 ± 15% and 73 ± 25%, F(2,10) = 5.4). A higher concentration of SNC80 (500 nM) had a small but statistically insignificant effect on enkephalin release during both infusions, and the highest concentration tested (5 µM) produced a significant reduction in enkephalin release during both infusions (−43 ± 10% and −38 ± 12%, F(2,10) = 11.3, P < 0.05) (fig. 2D).

Blockade of morphine, M6G and SNC80 effects with selective antagonists. The opioid receptor subtypes involved in the stimulatory (i.e., enkephalin release-enhancing) effects of morphine, M6G and SNC80 were examined by coapplication of the mu receptor antagonist β-FNA or the delta receptor antagonist NTD during the second infusion period. β-FNA (100 nM) abolished the stimulatory effect of morphine (10 nM) (fig. 4A) and M6G (10 nM) (fig. 4B). Similarly, the enkephalin release-enhancing effect of SNC80 (50 nM) was blocked by coadministration of NTD (100 nM) (fig. 4C).

The inhibitory (i.e., enkephalin release-suppressing) effects of the higher concentrations of M6G and SNC80 were investigated in a similar manner. The ability of M6G (10 µM) to inhibit the release of enkephalins was blocked by incorporation of β-FNA (100 nM) during the second infusion (fig. 5A). NTD (100 nM) did not block the inhibitory effect of SNC80 on enkephalin release (fig. 5B). However, β-FNA (100 nM) was effective in attenuating this effect (fig. 5B).

Discussion

Several behavioral and electrophysiological studies have demonstrated the importance of opioid receptors in modulating the output of both the GP and VP (Austin and Kalivas, 1990; Dewar et al., 1985; Frey and Huffman, 1985; Hoffman et al., 1991; Huffman and Felpel, 1981; Huffman and Frey, 1989; Johnson and Napier, 1997; Joyce et al., 1981; Mitrovic and Napier, 1995; Napier, 1992; Napier et al., 1983, 1992). It was therefore of interest to examine the factors regulating the release of these peptides in the pallidum. We have previously reported that peripheral administration of the pre-dominantly mu opioid receptor agonist morphine (10 mg/kg i.p.) induces a dose-dependent increase in the release of endogenous pallidal opioid peptides, primarily Met- and Leu- enkephalin (Olive et al., 1995). In an effort to determine the locus of this action, we investigated the effect of morphine and other opiate drugs on opioid peptide release after direct application to the pallidum. This was particularly pertinent
in view of a previous report demonstrating differential effects of systemic vs. local administration of morphine on pallidal neuron activity (Napier et al., 1992).

We found that local administration of low concentrations (nanomolar range) of both morphine and the active morphine metabolite M6G induced an increase in pallidal enkephalin release similar to peripheral administration. However, as the concentration of morphine or M6G was increased into the micromolar range, this effect was lost and, at the highest concentrations tested (100 and 10 μM, respectively), was replaced by a decrease in enkephalin release. Given that both morphine and M6G are relatively selective for the μ opioid receptor at low nanomolar concentrations (Abbott and Palacios, 1988; Chen et al., 1991; Mignat et al., 1995; Raynor et al., 1994) and that the enkephalin-releasing effects of these locally perfused drug concentrations were completely reversed by coadministration of the μ opioid receptor antagonist β-FNA, it is likely that the stimulatory effects of low concentrations of morphine and its metabolite in the pallidum are mediated predominantly by the μ opioid receptor. (The cross-reactivity of the more μ-selective peptide agonists such as DAMGO with the radioimmunoassay at the high concentrations necessary to be incorporated in the dialysate perfusion medium prevented their use in this study.) However, a potential role for δ opioid receptors in mediating opioid-induced endogenous opioid peptide release is implicated by the observation that the δ opioid receptor agonist SNC80 also produced an increase in pallidal enkephalin release and that this effect was completely reversed by coadministration of the δ-selective antagonist NTD. (Again, δ-selective peptide agonists such as DPDPE cross-reacted at high concentrations.) Taken together, it is apparent from these data that stimulation of both μ and δ opioid receptors within the pallidum at low agonist concentrations can enhance the release of enkephalins from this structure.

Both μ and δ opioid receptors are generally considered to decrease transmitter release via coupling to inhibitory G proteins, leading to inhibition of voltage-gated calcium channels and/or activation of potassium channels (Huang, 1995; Mulder and Schoffelmeer, 1993; Sarne et al., 1996). However, an increasing number of reports have demonstrated stimulatory effects of μ and δ opioid receptor agonists on neurotransmitter release, including enkephalins, when applied at low concentrations (Barke and Hough, 1994; Gintzler and Xu, 1991; Mauborgne et al., 1987; Xu et al., 1989). Such effects are often explained by disinhibitory mechanisms that may, indeed, be responsible for our current results, perhaps involving local inhibition of GABA release, for instance (Cohen et al., 1992; Johnson and North, 1992; Zieglgansberger et al., 1979). An alternative explanation is offered by several reports, using simple cellular systems, that at low agonist concentrations, both μ and δ opioid receptors mediate a direct stimulatory action on transmitter release (Cahill et al., 1993; Fan et al., 1995; Hirai and Katayama, 1988; Tang et al., 1994). Such effects are proposed to be mediated by increased Na⁺ and Ca²⁺ conductances, decreased K⁺ conductance and/or increased adenylate cyclase and protein kinase C activity (Crain and Shen, 1996; Huang, 1995; Sarne et al., 1996; Smart and Lambert, 1996). We failed to observe a morphine-induced decrease in enkephalin release with all but the highest concentration tested (100 μM), and then only during the second infusion (perhaps reflecting a need for accumulation of the drug from the first and second infusions to attain a sufficiently high concentration). This result is in concordance with several reports studying enkephalin release in the striatum and spinal cord (Osborne and Herz, 1980; Richter et al., 1979; Tseng et al., 1985). However, many others have found that micromolar concentrations of morphine do inhibit the release of enkephalins from other regions of the central nervous system in vitro (Glass et al., 1986; Sawnyok et al., 1980) or in anesthetized in vivo preparations (Collin et al., 1994; Jhamandas et al., 1984; Ueda et al., 1986; Yaksh and Elde, 1981), most likely through presynaptic μ and δ receptor-mediated mechanisms (Bourgoin et al., 1991, 1994; Collin et al., 1992; Nikolarakis et al., 1989). However, none of these studies examined enkephalin release from the pallidum. We did observe a significant suppression of enkephalin release after local perfusion of a high concentration of M6G (10 μM), an effect that was blocked by coadministration of β-FNA, indicating a μ receptor-mediated mechanism of action. The δ agonist SNC80 also produced a suppression of pallidal enkephalin release when locally administered at a relatively nonselective concentration of 100 nM NTD (morphine and M6G are relatively selective for the μ receptor at low nanomolar concentrations (Abbott and Palacios, 1988; Chen et al., 1991; Mignat et al., 1995; Raynor et al., 1994).
tive micromolar concentration (Bilsky et al., 1995; Knapp et al., 1996). This effect appears to be mediated by μ receptors because it was attenuated by β-FNA but not NTD.

Thus, it would seem that both μ and δ receptors within the pallidum mediate an opiate-induced increase in enkephalin release at low agonist concentrations (nanomolar), whereas at higher concentrations (micromolar) of exogenously applied agonists, a μ receptor-mediated decrease in release predominates. Whether these different responses result from activation of populations of receptors localized to separate neuronal components of the pallidum or from differential coupling of the same receptors to specific stimulatory vs. inhibitory G proteins at different agonist concentrations remains unclear.

Immunohistochemical data from our laboratory (Olive et al., 1997) demonstrated the presence of μ but not δ receptors on enkephalinergic terminals in the pallidum, whereas both μ and δ receptors were identified on postsynaptic structures in this region. Using retrograde tracers, cells expressing δ receptors were found to project directly from the pallidum to the striatum (Olive et al., 1997). Because previous studies have identified GABA as a neurotransmitter in such pallidostriatal projections (Churchill and Kalivas, 1994; Rajakumar et al., 1994), it can be postulated that inhibition of such inhibitory GABAergic feedback neurons via activation of δ-opioid receptors is responsible for the μ-mediated increase in pallidal extracellular enkephalin observed in the present study. Electrophysiological studies have indeed demonstrated a predominant inhibitory response of pallidal neurons to δ receptor agonists (Mitrovic and Napier, 1995). μ agonists, on the other hand, while producing inhibitory responses in most pallidal neurons, can induce excitatory responses in others, at least after ionotophoretic application (Huffman and Frey, 1989; Napier et al., 1992). If both sets of pallidal output neurons feed back to the dorsal and/or ventral striatum to regulate the activity of the enkephalinergic projection, this could perhaps explain the bimodal effect on enkephalin release. However, there is no evidence for differential sensitivity to μ agonists of the pallidal neurons responding with excitation vs. inhibition (Huffman and Frey, 1989; Napier et al., 1992). Similarly, individual pallidal neurons respond to opiate drugs with a monophasic rather than a biphasic concentration-response curve (Frey and Huffman, 1985; Huffman and Frey, 1989; Mitrovic and Napier, 1995; Napier et al., 1992). Thus, a purely postsynaptic feedback mechanism is unlikely. The localization of μ receptors both presynaptic and postsynaptic to striatopallidal fibers provides a more plausible explanation whereby, for instance, the presynaptic μ receptors mediate enhancement of release directly while the postsynaptic μ receptors mediate inhibition of enkephalin release. (Presumably through a polysynaptic feedback mechanism because we were unable to locate μ receptors on pallidostriatal neurons (Olive et al., 1997)). Differences in the presynaptically vs. postsynaptically mediated dose-response relationships due to factors such as the influence of other inputs to the postsynaptic neurons producing a “breakthrough” effect or the possible involvement of subtypes of the μ receptor could lead to an overall biphasic effect on release. It is also possible that enkephalin terminals in the GP and VP are differentially regulated by opioid receptor activation. The size of the probes and their placement used in this study did not allow differentiation of the two structures. In summary, local administration of μ and δ receptor agonists produces an increase in endogenous extracellular opioid peptide levels in the pallidum, an effect reversible with β-FNA and naltrindole, respectively. As the concentration is increased into the micromolar range, this effect is replaced by an inhibitory response that appears to be mediated by μ receptors. Therefore, when using opioid receptor subtype-selective agonists to study pallidal opioid receptor involvement in behavioral or electrophysiological models, the possible effects of endogenously released opioid peptides exhibiting a different spectrum of opioid receptor activity need to be considered.

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References


