Parallel studies have demonstrated that enkephalin release from nerve terminals in the pallidum (globus pallidus and ventral pallidum) can be modulated by locally applied opioid drugs. To investigate further the mechanisms underlying these opioid effects, the present study examined the presynaptic and postsynaptic localization of δ (DOR1) and μ (MOR1) opioid receptors in the dorsal and ventral striatopallidal enkephalinergic system using fluorescence immunohistochemistry combined with anterograde and retrograde neuronal tracing techniques. DOR1 immunostaining patterns revealed primarily a postsynaptic localization of the receptor in pallidal cell bodies adjacent to enkephalin- or synaptophysin-positive fiber terminals. MOR1 immunostaining in the pallidum revealed both a presynaptic localization, as evidenced by punctate staining that co-localized with enkephalin and synaptophysin, and a postsynaptic localization, as evidenced by cytoplasmic staining of cells that were adjacent to enkephalin and synaptophysin immunoreactivities. Injections of the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHA-L) or the retrograde tracer Texas Red-conjugated dextran amine (TRD) into the dorsal and ventral striatum resulted in labeling of striatopallidal fibers and pallidostratial cell bodies, respectively. DOR1 immunostaining in the pallidum co-localized only with TRD and not PHA-L, whereas pallidal MOR1 immunostaining co-localized with PHA-L and not TRD. These results suggest that pallidal enkephalin release may be modulated by μ opioid receptors located presynaptically on striatopallidal enkephalinergic neurons and by δ opioid receptors located postsynaptically on pallidostratial feedback neurons.

Key words: opioid; striatum; nucleus accumbens; enkephalin; delta receptor; mu receptor; globus pallidus; ventral pallidum

Neurons of the dorsal striatum (caudate putamen) and ventral striatum (nucleus accumbens) exhibit some of the highest concentrations of preproenkephalin mRNA expression in the brain (Yoshikawa et al., 1984; Khachaturian et al., 1985, 1993; Shivers et al., 1986; Harlan et al., 1987; Hurd, 1996). A large proportion of these dorsal and ventral striatal enkephalinergic neurons are medium-sized spiny neurons that project to the globus pallidus (GP) and ventral pallidum (VP), respectively (referred to herein collectively as the pallidum) (Cuello and Paxinos, 1978; Staines et al., 1980; Correa et al., 1981; Del Fiacco et al., 1982; Khachatyan et al., 1983; Groenewegen and Russchen, 1984; Gerfen and Young, 1988). Indeed, very few pallidal neurons synthesize enkephalins (Hökfelt et al., 1977; Johansson et al., 1978; Sar et al., 1978; Finley et al., 1981; Khatchaturian et al., 1983; Williams and Dockray, 1983; Fallon and Leslie, 1986; Harlan et al., 1987; Mansour et al., 1993; Hurd, 1996), indicating that the vast majority of enkephalin in this region is contained in striatopallidal efferent fibers.

Previous studies have implicated the striatopallidal pathways in the rewarding effects of both opiate and psychostimulant drugs (Zito et al., 1985; Hubner and Koob, 1990; Robledo and Koob, 1993; Gong et al., 1996, 1997). However, the mechanisms governing the release of enkephalins in this system have not been thoroughly investigated. We have previously demonstrated that peripherally administered morphine induced a dose-dependent increase in pallidal enkephalin release (Olive et al., 1995). More recently we showed that μ and δ opioid agonists applied locally into the pallidum have bimodal effects on Met- and Leu-enkephalin release in this structure, such that low concentrations of these compounds enhance enkephalin release, whereas high concentrations inhibit the release of these peptides (Olive and Maidment, 1996).

Neurotransmitter release can be autoregulated by at least two receptor-mediated mechanisms: receptors located presynaptically on the terminal bouton (i.e., autoreceptors) and receptors located postsynaptically on neurons that have recurrent projections onto the neurotransmitter-releasing neuron (Chesselet, 1984). As evidenced by ligand-binding, immunohistochemical, and mRNA expression data (Mansour et al., 1988, 1993, 1995a,b; Delfs et al., 1994; Bausch et al., 1995; Ding et al., 1996), the pallidum contains low to moderate levels of μ and δ receptors, which are thought to be the endogenous receptors for enkephalins (Raynor et al., 1994). The present study used fluorescent neuronal tracing and immunohistochemistry combined with confocal microscopy to determine the presynaptic versus postsynaptic localization of μ and δ opioid receptors within the striatopallidal system.

MATERIALS AND METHODS

Neuronal tracing. All experiments used adult male Sprague Dawley rats (250–350 gm; Harlan, Madison, WI). Tracing of striatopallidal and pallidostratial pathways was achieved by injecting animals with one of two neuronal tracers under halothane anesthesia in a 1:1 mixture of O2.
Tokyo, Japan) to a tip diameter of 10–15 μm were back-filled with a solution containing 2.5% (w/v) PHA-L in 0.1 M PBS, pH 7.4. Positive current (5 μA) was pulsed into the solution via a silver chloride wire at a rate of 7 sec on and 7 sec off for 15–20 min per injection site using a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel) and a World Precision Instruments (New Haven, CT) 160 microiontophoresis controller. After iontophoresis of the tracer solution, the micropipette was left in place for a further 10 min before slow withdrawal over a 5 min period. Holes in the skull were covered with bone wax, and animals were killed 4–7 d later.

Retrograde labeling of pallidostriatal neurons was achieved by injecting 200–300 nl of a 0.5% (w/v) solution of Texas Red-conjugated dextran amine (10,000 molecular weight; Molecular Probes, Eugene, OR) (Fritzsch, 1993) in 0.1 M PBS, pH 7.4, unilaterally into the dorsal striatum (n = 6) or nucleus accumbens (n = 6) by iontophoresis according to the method of Gerfen and Sawchenko (1984). Five different locations within each of these two structures were targeted using the following stereotaxic coordinates according to the atlas of Paxinos and Watson (1986): dorsal striatum, anteroposterior (AP), 1.6 to 7.4 mm from bregma and skull surface; and nucleus accumbens, AP, 1.0 to 1.7 mm; ML, 0.7 to 1.9 mm; and DV, 6.6 to 7.4 mm from bregma and skull surface (Fig. 1). Brieﬂy, glass micropipettes (A-M Systems, Everett, WA) pulled with a vertical microelectrode puller (PE-2; Narishigi Instruments, Deerfield, IL) and placed into culture plate wells containing 0.1 M PBS, pH 7.4.

Immunochemistry. For all immunohistochemical procedures, animals were deeply anesthetized with Nembutal (150 mg/kg, i.p.) and perfused with 200 ml of 0.1 M PBS containing 0.1% heparin, pH 7.4, followed by 800 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Perfusion was performed with ice-cold fixatives in an ambient temperature of 4°C. Brains were then removed and post-fixed in the same fixative solution at 4°C overnight and then transferred to 0.1 M PBS containing 30% (w/v) sucrose, pH 7.4, for 48 hr. Sagittal sections (40–50 μm) were cut on a Jung Frigocut 2800E cryostat (Leica, Deerfield, IL) and placed into culture plate wells containing 0.1 M PBS, pH 7.4.

All immunohistochemistry was performed on free-floating sagittal sections. Sections were incubated with primary antisera for 24 hr at 4°C under gentle agitation in PBS solution containing 0.3% (v/v) Tween 20, 1% (w/v) bovine serum albumin (fraction V, protease free) and 10% (v/v) normal goat or donkey serum, pH 7.4 (all chemicals from Sigma, St. Louis, MO). Antisera to the δ opioid receptor (DOR1), μ opioid receptor (MOR1), Met- and Leu-enkephalin, PHA-L, and synaptophysin were used in various combinations (see Antisera and Controls for sources and titers).

After incubaon with primary antisera, sections were rinsed in PBS and incubated with ﬂuorescein-conjugated secondary antibody in PBS containing 2% (v/v) normal goat or donkey serum for 2–3 hr at room temperature under gentle agitation. Sections were then rinsed in PBS containing 0.1% (v/v) Tween 20, mounted onto slides, and coverslipped with a glycerol solution containing ProLong AntiFade reagent (Molecular Probes). Sections were then examined by confocal laser scanning microscopy.

Antisera and controls. A rabbit polyclonal antisera to residues 3–17 of DOR1 was used at a 1:100 dilution (Incstar, Stillwater, MN, antibody 442E) (Dado et al., 1993, Arvidsson et al., 1995a; Elde et al., 1995; Lai et al., 1996). Preabsorption of this primary antiserum with the synthetic epitope sequence DOR1 3–17 (LVPSARAEQLSSPLV; a generous gift of Dr. Robert Elde, University of Minnesota) at 10−4 M overnight at 4°C was used as a control. A rabbit polyclonal antiserum raised in our laboratory (Sternini et al., 1996) to residues 387–398 of MOR1 was used.

Figure 1. Diagrammatic representation of rat brain sagittal sections showing locations of iontophoretic deposits of PHA-L (∗) and microinjections of TRD (vertical lines). Also shown are regions of the globus pallidus (light gray shading) and ventral pallidum (dark gray hatched shading) examined in the present study. Planes of section are adapted from experimental tissue and the atlas of Paxinos and Watson (1986) and delineated by number of millimeters lateral (L) to the midline, CPu, Caudate-putamen; NAcSh, shell region of nucleus accumbens; NAcC, core region of nucleus accumbens.
RESULTS

Cellular distribution of DOR1 immunoreactivity in the pallidum and co-localization with presynaptic markers

The GP and VP were anatomically defined according to the criteria of Paxinos and Watson (1986) as shown in Figure 1. Low to moderate levels of DOR1 staining were evenly distributed throughout the GP (Fig. 2A) and VP (Fig. 2C). Preincubation of the DOR1 antiserum with the peptide sequence to which it was raised (LENLEAETAPLP) at $10^{-4}$ M for 12–24 hr substantially reduced DOR1 immunostaining (Fig. 3B). Higher magnification revealed two different cellular distributions of DOR1 staining. In the more concentrated patches of the GP and VP, fine and diffuse puncta surrounded and formed the outline of what were considered to be small cell bodies (8–15 μm in diameter), which themselves were devoid of MOR1 immunoreactivity (Fig. 3D,E). The other type of staining pattern was an amorphous distribution within the cytoplasm of similar sized cells in the VP (Fig. 3F) and GP (data not shown). As with DOR1, no clear enhancement of cell perimeter labeling indicative of plasmalemmal staining was apparent. Also similar to DOR1, MOR1 staining of fibrous arborizations could not be observed, although occasional single long fiber processes were seen (data not shown). Double-labeling experiments showed that the fine and diffuse punctate MOR1 immunoreactivity was highly co-localized with synaptophysin in the VP (Fig. 3G) and GP (data not shown). This MOR1 immunoreactivity could also be seen to co-localize with diffuse enkephalin-immunoreactive puncta surrounding small unlabeled cells in the GP (Fig. 3H) and VP (data not shown). Conversely, cytoplasmic MOR1 staining in the pallidum did not co-localize with synaptophysin (data not shown) or enkephalin (Fig. 3I). No major differences between the GP and VP were observed with regard to MOR1 morphology and co-localization with presynaptic markers.

Neuronal tracing

To verify that the potential presynaptic or postsynaptic localization of MOR1 or DOR1 immunoreactivity was related to striatopallidal inputs, anterograde labeling of dorsal and ventral striatopallidal neurons was achieved by injection of the specific anterograde tracer PHA-L into the dorsal caudate nucleus or nucleus accumbens (core and shell) (Fig. 1). Labeling of long fiber processes and punctate terminals was seen at low to moderate intensity throughout the GP and VP, with very little or no retrograde labeling of pallidal cell bodies. A limited amount of co-localization of MOR1 immunoreactivity with PHA-L-labeled fiber processes was observed within the GP (Fig. 4A) and VP (not shown). No such co-localization of DOR1 and PHA-L was found in the GP or VP (Fig. 4B).

To determine whether MOR1 or DOR1 immunoreactivity is localized on pallidosstriatal feedback neurons, a retrograde dextran amine tracer (TRD) was injected into the dorsal caudate nucleus and nucleus accumbens (core and shell) of a separate set of animals (Fig. 1). This tracer was found to retrogradely label a large number of cell bodies throughout the pallidum. Punctate MOR1 immunoreactivity could be seen to surround such retrogradely labeled neurons, but no co-localization of MOR1 and TRD was seen within the cytoplasm of these cell bodies in the VP (Fig. 4C) or GP (data not shown). In contrast, DOR1 immunoreactivity could be found in cell bodies that had retrogradely transported the dextran amine to the VP (Fig. 4D) and GP (data not shown), indicating that DOR1 receptors are expressed by pallidosstriatal neurons.

DISCUSSION

We used specific polyclonal antibodies to examine the cellular distribution of MOR1 and DOR1 immunoreactivities in both the striatopallidal and pallidosstriatal pathways. Both the GP and VP had low to moderate levels of MOR1 and DOR1 staining, in agreement with other immunohistochemical and in situ hybridization studies (Mansour et al., 1988, 1993, 1995a,b; Churchill et
The regional distribution of MOR1 and DOR1 immunoreactivities within the pallidum was also consistent with the findings of other investigators. Thus, a diffuse and relatively homogeneous distribution of DOR1 was observed throughout the GP and VP (Mansour et al., 1994a, 1995b). MOR1 immunoreactivity was heterogeneously distributed in these structures, with a greater clustering of MOR1-stained cells in the caudal regions of the GP (Mansour et al., 1994a,b, 1995b; Ding et al., 1996) and rostro-dorsal parts of the VP (Churchill et al., 1990; Delfs et al., 1994; Mansour et al., 1994b).

Many studies have localized $\mu$ and $\delta$ opioid receptors presynaptically and postsynaptically with respect to enkephalinergic terminals in other regions of the CNS at the light microscopic (Arvidsson et al., 1995a,b; Guttenberg et al., 1996) and electron microscopic levels (Cheng et al., 1995, 1996a,b; Svingos et al., 1995, 1996; van Bockstaele et al., 1996; Wang et al., 1996). Studies examining presynaptic versus postsynaptic localization of opioid receptors in the striatopallidal system have used autoradiographic binding techniques after striatal lesions. One group found no change in ventral pallidal $\mu$ receptor binding after lesions of the ventral striatum (Churchill et al., 1990), whereas others found significant decreases in both pallidal $\mu$ and $\delta$ receptor binding after lesions of the dorsal striatum (Abou-Khalil et al., 1984; Waksman et al., 1987). However, changes in radiolabeled ligand binding after lesions are difficult to interpret with respect to precise presynaptic or postsynaptic localization of the receptor of interest. Lesion-induced increases in the number of postsynaptic

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**Figure 2.** Confocal microscopic images of rat brain sagittal sections showing distribution and morphology of DOR1 immunoreactivity (green) within the pallidum. A, Low magnification of DOR1 immunoreactivity in the GP (also see Fig. 1). B, Reduction of staining in the GP for DOR1 after preabsorption of the primary antibody with the cognate peptide. C, Low magnification of DOR1 immunoreactivity in the VP just beneath the posterior limb of the anterior commissure (ACP) (also see Fig. 1). D–F, Morphology of a typical cell body in the GP labeled for DOR1 at a higher magnification, viewed at step sizes of 1.5 $\mu$m. Arrows indicate approximate location of nucleus, and arrowheads demarcate cytoplasmic staining. G, Representative image of a DOR1-immunoreactive cell body in the VP with diffuse cytoplasmic staining and a small population of more intense vesicular compartment-like structures. Double labeling revealed punctate staining of synaptophysin (H, red) and enkephalin (I, red) surrounding DOR1-labeled cell bodies (in GP and VP, respectively) with no apparent co-localization. Scale bars: A, B, 250 $\mu$m; C, 100 $\mu$m; D–I, 5 $\mu$m.
receptors attributable to receptor upregulation or, conversely, decreases attributable to trans-synaptic degeneration could also account for the observed changes (or lack thereof) in radioligand binding. The increased resolution offered by immunohistochemical localization of receptors combined with co-localization of presynaptic and postsynaptic markers circumvents these limitations.

We have shown a discrete presynaptic localization of $\mu$ opioid receptors within the striatopallidal projection systems, as evidenced by MOR1 immunoreactivity in the GP and VP that surrounded cell body-shaped structures and showed co-localization with synaptophysin, enkephalin, and anterogradely labeled striatopallidal fibers. Other investigators have also found $\mu$ receptors to be presynaptic on enkephalinergic terminals in the striatum and nucleus accumbens (Svingos et al., 1995, 1996; Guttenberg et al., 1996). It is therefore likely that MOR1 serves as a presynaptic autoreceptor regulating the release of enkephalins from striatopallidal projection neurons. Given the predominant inhibitory effects of opiates on neurotransmitter release via $G_{i/o}$ or $G_{i}$-protein-coupled mechanisms (see Mulder and Schoffelmeer, 1993; Huang, 1995; Sarne et al., 1996), such receptors may mediate the inhibitory effects of high concentrations of $\mu$ agonists on enkephalin release observed in microdialysis studies conducted in our laboratory (Olive and Maidment, 1996). It should also be noted, however, that activation of presynaptic $\mu$ receptors has been demonstrated to mediate stimulatory effects on enkephalin release under certain circumstances, particularly at

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**Figure 3.** Confocal microscope images of rat brain sagittal sections showing distribution and morphology of MOR1 immunoreactivity (green) within the pallidum. A, Low magnification of the GP. Note the clusters of higher intensity of MOR1 staining along the dorsocaudal border of the globus pallidus (asterisk) and a patch of MOR1 immunoreactivity in the striatum (arrow). B, Reduction of staining for MOR1 after preabsorption of the primary antibody with the cognate peptide. C, Low magnification of the VP just beneath the posterior limb of the anterior commissure (ACP) (also see Fig. 1). Note the higher intensity of MOR1 staining along the dorsal rim of the VP. D, E, High magnification showing punctate MOR1 staining surrounding spherical entities in the subcommissural ventral pallidal region (D) and globus pallidus (E). F, Typical morphology of cell bodies in the VP with cytoplasmic (arrowhead) staining of MOR1. G, High magnitude showing high degree of co-localization (yellow) of MOR1 (green) with synaptophysin (red) immunoreactivity in the subcommissural VP. H, Co-localization (yellow, arrows) of MOR1 (green) with enkephalin (red) surrounding a putative cell body in the GP. I, Lack of co-localization of cytoplasmic MOR1 staining (green) in the GP with synaptophysin (red). Scale bars: A, B, 250 $\mu$m; C, 100 $\mu$m; D–I, 5 $\mu$m.
low agonist concentrations, possibly via coupling to Gs (Xu et al., 1989; Gintzler and Xu, 1991). The possibility that these presynaptic receptors mediate the stimulatory effects of low concentrations of morphine on pallidal enkephalin release cannot therefore be ruled out (Olive et al., 1995; Olive and Maidment, 1996).

It should also be noted that studies at the electron microscopic level are needed to confirm the precise presynaptic ultrastructural localization of μ receptors within the pallidum.

μ opioid receptors were also localized to postsynaptic structures within the pallidum but, unlike DOR1, did not appear to be present on pallidostratial neurons, as evidenced by lack of co-localization with TRD. (It should be noted that our data do not entirely eliminate the possibility that MOR1 is expressed in pallidostratial neurons that were not labeled by the TRD injections.) Therefore, if postsynaptic μ receptors are mediating the observed stimulatory and/or inhibitory effects of locally administered morphine on enkephalin release in the pallidum (Olive and Maidment, 1996), it is necessary to invoke polysynaptic feedback mechanisms. In this regard, electrophysiological studies have reported both excitatory and inhibitory responses of VP neurons to microiontophoretic application of morphine (Napier et al., 1992; Chrobak and Napier, 1993; Mitrovic and Napier, 1995; Johnson and Napier, 1997) and primarily inhibitory responses of GP neurons (Huffman and Felpel, 1981; Stone, 1983; Napier et al., 1983, 1992).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Confocal microscopic images showing co-localization of neuronal tracers with MOR1 and DOR1. A, Co-localization (yellow, arrows) of MOR1 immunoreactivity (green) with PHA-L-labeled striatopallidal fibers (red) in the globus pallidus. B, Lack of co-localization of DOR1 (green) on PHA-L-labeled striatopallidal fibers (red) in the globus pallidus. C, Lack of co-localization of TRD (red) with punctate MOR1 immunoreactivity (green) in the ventral pallidum. D, Co-localization (yellow, arrows) of DOR1 immunoreactivity (green) in TRD-labeled (red) pallidostratial cell bodies. Scale bars: A, 20 μm; B–D, 10 μm.

We failed to find evidence of a presynaptic localization of DOR1 immunoreactivity in the GP and VP. No staining of fibers or varicosities was apparent, and similarly, no colocalization with synaptophysin or enkephalin immunoreactivity was observed. Previous immunohistochemical studies using the same (Dado et al., 1993; Arvidsson et al., 1995a) or different (Cheng et al., 1995; Svingos et al., 1995) antisera have demonstrated that δ receptors can exist presynaptically in other regions of the nervous system. Indeed, we did find evidence of a presynaptic localization of DOR1 in the median eminence and in the dorsal horn of the spinal cord (data not shown), in agreement with the initial characterization of this antiserum (Dado et al., 1993; Arvidsson et al., 1995a). This suggests that the δ receptor agonist-induced stimulation of pallidal enkephalin release revealed by microdialysis (Olive and Maidment, 1996) is mediated by activation of a positive feedback loop. The demonstration of a discrete postsynaptic localization of δ opioid receptors in the GP and VP within cell bodies of neurons retrogradely labeled by injection of TRD into the dorsal and ventral striatum is consistent with the hypothesis that such feedback may be direct. Considerable anatomical and electrophysiological evidence exists for a direct pathway from the
The release may be modulated by pharmacological experiments suggest that pallidal enkephalin pathways is warranted. Two similar but nevertheless disparate opioidergic striatal output between the GP and VP. More extensive comparison of these to colocalization of such markers with either MOR1 or DOR1 in the nucleus accumbens, but we found no differences with regard to the postsynaptic effects of opioids in the GP and VP cited above. Such differences could theoretically be attributable to differences in presynaptic versus postsynaptic localization of μ and δ opioid receptors between the GP and VP or to differences in opioid receptor density or signal transduction mechanisms between the GP and VP. However, we failed to find any obvious differences between the GP and VP in terms of the general morphology of MOR1 and DOR1 staining and co-labeling with presynaptic markers. Deposits of anterograde and retrograde tracer were made into the caudate nucleus and into both the core and shell of the nucleus accumbens, but we found no differences with regard to colocalization of such markers with either MOR1 or DOR1 between the GP and VP. More extensive comparison of these two similar but nevertheless disparate opioidergic striatal output pathways is warranted.

Taken together, the results of the current study and our parallel pharmacological experiments suggest that pallidal enkephalin release may be modulated by μ opioid receptors located presynaptically on striatopallidal enkephalineric fibers and by δ opioid receptors located postsynaptically to these enkephalineric terminals on feedback neurons that project to the striatum. The possibility also exists that μ receptors located postsynaptically on pallidal neurons contribute to a polysynaptic feedback loop regulating pallidal enkephalin release. Further studies at the electron microscopic level are warranted to examine the ultrastructural localization of μ and δ opioid receptors in striatopallidal pathways.

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