0026-895X/92/040575-09$03.00/0

MOLECULAR PHARMACOLOGY, 42:575-583

κ-Opioid Inhibition of [^{3}H]Dopamine Release from Rat Ventral Mesencephalic Dissociated Cell Cultures

JACQUELINE A. M. SMITH, SANDRA E. LOUGHLIN, and FRANCES M. LESLIE

Departments of Pharmacology (J.A.M.S., S.E.L., F.M.L.) and Anatomy and Neurobiology (S.E.L.), California College of Medicine, University of California, Irvine, California 92717

Received May 12, 1992; Accepted July 23, 1992

SUMMARY

The present study investigated the effect of opioids on [^{3}H] dopamine release from mixed neuronal-glial cell cultures of embryonic rat ventral mesencephalon. Each of the major morphological types of dopaminergic cell was represented in these cultures. These cells exhibited specific uptake of [^{3}H]dopamine, which was subsequently released, in a calcium-dependent manner, in response to a double pulse of elevated extracellular potassium. Spontaneous and potassium-evoked [^{3}H]dopamine release was inhibited by κ- but not μ- or δ-opioid agonists. The selective κ_1 agonist (5α,7α,8β)-(−)-*N*-methyl-*N*-[7-(1-pyrollidinyl)-1-oxaspiro(4,5)-dec-8-yl]benzeneacetamide (U69593) produced a dose-dependent inhibition of dopamine release. The effect of U69593 was blocked by the nonselective opiate antagonist naloxone and the selective κ opioid antagonist norbinaltorphimine. κ-Opioid inhibition of potassium-evoked [^{3}H]dopamine release was maintained in the presence of tetrodotoxin. These results suggest that functional κ receptors, modulating dopamine release, are localized on the terminals of dopaminergic neurons.

Ventral mesencephalic dopamine neurons provide dense innervation of the caudate-putamen and nucleus accumbens (for review see Ref. 1). Proenkephalin and prodynorphin mRNA and enkephalin and dynorphin immunoreactivity are also abundant in these forebrain regions (2). In addition to the opioid peptides, μ-, δ-, and κ-opioid binding sites are present at moderate to high densities in rat caudate-putamen and nucleus accumbens (3, 4).

There is now substantial evidence to support the hypothesis that endogenous opioids play an important role in the regulation of ventral mesencephalic dopaminergic neurons. However, the precise mechanism underlying opioid modulation of dopamine function has yet to be established. *In vivo*, systemic administration of morphine increases striatal dopamine release and turnover (5, 6) and the firing rate of mesencephalic dopamine neurons (7). Studies using receptor-selective agonists have demonstrated that *in vivo* administration of both μ and δ agonists stimulates dopamine release (5, 8–11) and dopamine cell firing (12). μ agonists modulate dopamine release indirectly, whereas δ agonists may activate receptors that are localized presynaptically on dopamine terminals (9, 11, 13). In contrast, κ agonists inhibit the activity of mesencephalic dopaminergic neurons *in vivo* (8, 9, 14). However, direct administration of κ agonists onto the substantia nigra pars compacta does not influence cell firing (15, 16), and it has been suggested that κ-opioids indirectly control the activity of nigral dopamine cells (17).

The effects of opioids on [^{3}H]dopamine release from *in vitro* brain slice preparations of caudate-putamen or nucleus accumbens are not in complete agreement with the results of *in vivo* studies. μ agonists are without effect (18, 19), whereas δ agonists either have no effect (18, 19) or stimulate (5, 20) [^{3}H]dopamine release. κ-Opioids inhibit K^+- (18, 19, 21), *N*-methyl-D-aspartate- (22), and acetylcholine-stimulated (23) [^{3}H]dopamine release *in vitro*. Recent data suggest that, in contrast to the findings *in vivo*, this is through direct activation of receptors localized on the terminals of dopaminergic afferents (19, 23).

To determine the precise synaptic localization of striatal opioid binding sites, a number of groups have utilized autoradiographic techniques in combination with lesion of mesencephalic dopaminergic afferents. After 6-hydroxydopamine lesion of rat substantia nigra, the density of both μ and δ sites in the caudate-putamen is decreased (24, 25), but after lesion of the VTA and μ binding site density alone is reduced in the nucleus accumbens (26). In contrast, it has been reported that the density of κ-opioid binding sites in the caudate-putamen and

This work was supported by United States Public Health Service Grant NS19319 to F.M.L., in addition to Grant NS26761 and grants from the American Parkinson's Disease Association (Southern California Chapter) to S.E.L.

ABBREVIATIONS: VTA, ventral tegmental area; DMEM, Dulbecco's modified Eagle's medium; DAGOL, [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; TH, tyrosine hydroxylase; TTX, tetrodotoxin; U69593, (5α,7α,8β)-(+)-*N*-methyl-*N*-[7-(1-pyrollidinyl)-1-oxaspiro(4,5)-dec-8-yl]benzeneacetamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PBS, phosphate-buffered saline; TH-ir, tyrosine hydroxylase-immunoreactive; *df*, degrees of freedom; E15, gestational day 15; E17, gestational day 17; DIV, days *in vitro*; ANOVA, analysis of variance; GABA, γ-aminobutyric acid.

nucleus accumbens is unchanged (24, 26). Recently, it has been proposed that the lesion-induced changes in μ (27, 28) and δ (28, 29) sites are the result of transsynaptic effects. Together, these studies suggest that all three types of opioid binding site are present on nondopaminergic elements in the caudate-putamen and nucleus accumbens.

The discrepancies between *in vivo* and *in vitro* observations may be a function of the complexity of the neuronal circuitry in the striatum, which precludes precise determination of the location of functional opioid receptors. In the present study, a dissociated cell culture model of embryonic ventral mesencephalon was established in order to define the actions of selective opioids on dopamine release at the cellular level. The principal advantage of a cell culture model is that it lacks circuitry analogous to that present *in vivo* or in an *in vitro* brain slice. An additional advantage of this system over the majority of *in vitro* brain slice or synaptosome preparations is that it contains both intact cell bodies and terminal fields.

The effects of μ, δ, and κ_1 agonists on spontaneous and K^+-evoked [^{3}H]dopamine release from ventral mesencephalic cultures were investigated. Using an experimental design adapted from slice release studies, cells were exposed to a double pulse of the depolarizing stimulus such that dopamine release in the absence and presence of a test drug could be compared directly.

Experimental Procedures

Materials. Timed-mated pregnant Sprague-Dawley rats were obtained from Bantin and Kingman (Fremont, CA). The following materials were obtained from the sources indicated: DMEM/Ham's F12 (powder), fetal bovine serum, Hanks' balanced salt solution (without calcium and magnesium), and 24-well Nunc flat-bottomed tissue culture plates, Irvine Scientific (Irvine, CA); papain and DNase I, Worthington Biochemicals (Freehold, NJ); dispase, Boehringer Mannheim Biochemicals (Indianapolis, IN); rabbit anti-TH, Eugene Tech (Ramsey, NJ); biotinylated goat anti-rabbit immunoglobulin (Vectastain Elite ABC kit), Vector Labs (Burlingame, CA); [^{3}H]dopamine (24.1–30 Ci/mmol), New England Nuclear (Boston, MA); HEPES, glucose, poly-D-lysine (M_r 30,000–70,000), penicillin-streptomycin, benztropine, desmethylimipramine, U69593, naloxone, and pargyline, Sigma (St. Louis, MO); DAGOL and DPDPE, Peninsula Laboratories (Belmont, CA); norbinaltorphimine, Research Biochemicals Inc. (Natick, MD); and fluoxetine, Eli Lilly and Co. (Indianapolis, IN). All other reagents were of analytical grade.

Preparation of ventral mesencephalic cultures. Ventral mesencephalic cultures were prepared using a modification of the procedure described by Raymon and Leslie (30). Briefly, timed-mated pregnant rats were sacrificed by decapitation on E15 (day of insemination = day 0) and the embryos (crown to rump length, 11.0 mm) were dissected by caesarean section and placed in a sterile solution containing 277 mM NaCl, 10 mM KCl, 3.2 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 44 mM dextrose (saline I), and 1% penicillin-streptomycin. The ventral mesencephali, containing the substantia nigra and VTA dopamine cell groups, were dissected under sterile conditions. Tissue pieces from 15–20 embryos were pooled, rinsed with saline I, and then incubated for 15 min at 37° with 0.01% (w/v) papain, 0.1% dispase, 0.01% DNase I, in Hanks' balanced salt solution without calcium and magnesium. Tissue pieces were triturated with a fire-polished pipette, incubated for 30 min at 37°, triturated again, and incubated for an additional 30 min. The resulting cell suspension was centrifuged for 5 min at 3000 × *g*, and the cell pellet was resuspended in 0.01% DNase I in DMEM/Ham's F12 (1:1) supplemented with 5 mM HEPES, 0.6% glucose, and 14 mM $NaHCO_3$ (DMEM/H12). The cell suspension was incubated for 30 min at 37°, triturated, and recentrifuged as described above. The resulting pellet was resuspended in DMEM/H12 and passed through a 133-μm Nitex mesh. An aliquot of the eluant was taken for determination of viable cell number by trypan blue exclusion. Cells were plated at a concentration of 1.1×10^6 cells/ml of DMEM/H12 containing 10% fetal bovine serum, on 24-well tissue culture plates that had been coated with poly-D-lysine (10 μg/ml). For immunocytochemical studies, cells were plated in eight-well LabTek chamber slides (Nunc). Cultures were maintained in a stable environment at 37°, 95% humidity, and 5% CO_2. Twenty-four hours after the day of plating was considered 1 DIV. A complete medium change was performed at the start of 3 and 6 DIV. TH immunocytochemistry and [^{3}H]dopamine uptake and release assays were performed on cells at 8 DIV.

TH immunocytochemistry. Cells were incubated at 22° for 30 min with 4% paraformaldehyde in 0.1 M PBS and for 5 min with 0.2% (v/v) Triton X-100 in PBS. They were washed for 10 min with four changes of PBS before incubation for 30 min with 10% normal goat serum in PBS containing 0.25% Triton X-100 (blocking serum). Cells were incubated overnight at 4° with a 1/1000 dilution of rabbit anti-TH. All antibody dilutions were made in blocking serum. Cells were incubated for an additional 60 min at 22°, washed for 30 min with four changes of PBS, and incubated with biotinylated goat anti-rabbit immunoglobulin (1/200) for 60 min. Cells were washed for 30 min as described previously, incubated for 60 min at 22° with the avidin-biotin complex, and washed again. Cells were then incubated for 8 min with 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride in 0.1 M Tris·HCl buffer, pH 7.6, containing 0.01% (v/v) H_2O_2, to allow formation of the reaction product. They were then washed for 30 min with PBS as described above, coverslipped with 10% glycerine in PBS, and viewed by light-field microscopy.

[^{3}H]Dopamine uptake. The [^{3}H]dopamine uptake procedure was a modification of that described by Prochiantz *et al.* (31). Briefly, cells were preincubated for 15 min at 37° in Krebs-HEPES buffer (3 mM KCl, 1.2 mM KH_2PO_4, 0.4 mM $MgSO_4$, 122 mM NaCl, 1.3 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, 100 mM ascorbic acid, 1 μM pargyline) in the presence or absence of catecholamine uptake inhibitors. [^{3}H]Dopamine was added at a final concentration of 50 nM and the cells were incubated for an additional 15 min. The cells were then rapidly washed three times with ice-cold buffer. To determine the intracellular accumulation of [^{3}H]dopamine, the cells were lysed with 95% ethanol/5% 0.1 M HCl for 30 min and an aliquot was taken for determination of radioactive content by liquid scintillation counting. All experiments were performed in triplicate.

[^{3}H]Dopamine release. The procedure for [^{3}H]dopamine release was a modification of that described by Mount *et al.* (32). Cells were rinsed once with Krebs-HEPES buffer and then incubated for 20 min at 37° in buffer containing 50 nM [^{3}H]dopamine. After loading, the cells were washed five times for 5 min each at 22° to remove unincorporated radioactivity. To stimulate neurotransmitter release, 25 and 50 min after loading with [^{3}H]dopamine the cells were exposed to a 5-min pulse (S1 and S2, respectively) of Krebs-HEPES buffer containing 20 mM KCl. To maintain the same osmolarity the KCl replaced an equimolar concentration of NaCl in the buffer. Cells were washed four times for 5 min each between the two pulses. Fractions were collected 20 (basal A), 25 (S1), 30 (basal B), 45 (basal C), and 50 min (S2) after loading with [^{3}H]dopamine, and aliquots were taken for determination of radioactivity content. To determine the residual intracellular concentration of radioactivity the cells were lysed with 95% ethanol/5% 0.1 M HCl, as described above for [^{3}H]dopamine uptake.

To determine the optimal concentration of KCl for the stimulation of [^{3}H]dopamine release, cells were exposed to a constant 20 mM K^+ S1 pulse and increasing concentrations of K^+ for the S2 pulse. The concentration of NaCl in the Krebs-HEPES buffer was reduced correspondingly, as described above. To investigate the Ca^{2+} dependency of [^{3}H]dopamine release, cells were exposed to a 20 mM K^+ S1 pulse and then washed with Ca^{2+}-free Krebs-HEPES buffer containing 100 μM EGTA. Cells were then incubated for 5 min with Ca^{2+}-free Krebs-

HEPES buffer containing 100 μM EGTA and 20 mM K^+, for the S2 pulse.

Characterization of drug effects on [^{3}H]dopamine release. In pharmacological studies, cells were incubated with drug during the 20-min washing period between the S1 and S2 pulses and for the duration of the S2 pulse. To examine drug effects on spontaneous [^{3}H]dopamine efflux, the 5-min fractions from the periods immediately after (basal B) and 15 min after (basal C) exposure to the drug were collected. To characterize drug effects on K^+-evoked [^{3}H]dopamine release alone, spontaneous efflux was subtracted from both the control and drug-treated S2 pulses. Spontaneous efflux of [^{3}H]dopamine during the 5-min S2 pulse was not significantly different from that in the preceding basal C fraction. Therefore, it was considered appropriate to use the basal C fraction as a measure of spontaneous efflux for the subsequent S2 pulse.

Data analysis. To compensate for the inherent variability in the extent of neurotransmitter uptake between different culture preparations, [^{3}H]dopamine release data were expressed as ratios with respect to the appropriate internal controls, determined before drug exposure or changes in buffer composition. Data are reported as the mean ± standard error of at least three experiments, each performed on independent culture preparations. All experiments were performed in triplicate.

For analysis of combined effects on spontaneous and evoked [^{3}H]dopamine release, data were expressed as the ratio of [^{3}H]dopamine release in the S2 pulse to that in the S1 pulse. S2/S1 ratios for 20 mM K^+-evoked [^{3}H]dopamine release in control and drug-treated cells were compared using one-way ANOVA. *Post hoc* comparisons of individual drug treatments were made using Newman-Keuls analysis. *Post hoc* comparisons of individual drug treatments with control were made using Dunnett's *t* test. To examine drug effects on spontaneous and evoked release independently, spontaneous (basal C) and evoked (S2 − basal C) [^{3}H]dopamine release values were expressed as ratios with respect to their appropriate internal controls, basal A and S1, respectively. Basal C/basal A and (S2 − basal C)/S1 ratios in control and drug-treated cells were compared using Student's paired *t* test.

To quantify the degree of opioid inhibition of spontaneous and K^+-evoked [^{3}H]dopamine release, respectively, data were expressed as a percentage with respect to the drug-free control, as follows: drug (basal B or C)/control (basal B or C) × 100 and drug (S2 − basal C)/control (S2 − basal C) × 100.

Results

Morphological characterization. In ventral mesencephalic cultures, TH-ir and nonimmunoreactive cell types were easily differentiated. Three morphological types of TH-ir cells, fusiform, polygonal, and trigonal, with diameters of approximately 15–45 μm were present in the cultures (Fig. 1). Fusiform and trigonal cell types were particularly abundant. Medium-sized fusiform cells, with primary dendrites arising from opposite poles (Fig. 1B), made up a large proportion of the TH-ir cells. Many larger cells, with trigonal somas, had one large dendrite and one or more smaller dendrites (Fig. 1, A and C). In addition, smaller TH-ir cells with round cells bodies were present. A rich plexus of TH-ir processes, many of which appeared varicose, was present between cell bodies.

[^{3}H]Dopamine uptake. Ventral mesencephalic cultures at 8 DIV exhibited specific uptake of [^{3}H]dopamine in Krebs-HEPES buffer at 37°. After a 20-min incubation, [^{3}H]dopamine (50 nM) uptake was 2022 ± 283 fmol/well (three experiments). The dopamine uptake inhibitor benztropine (1 μM) inhibited uptake 75.5 ± 3.9% (four experiments) with respect to control, in contrast to the norepinephrine uptake inhibitor desmethylimipramine (1 μM) and the serotonin uptake inhibitor fluoxetine (1 μM), which inhibited uptake by 13.7 ± 3.3% (three

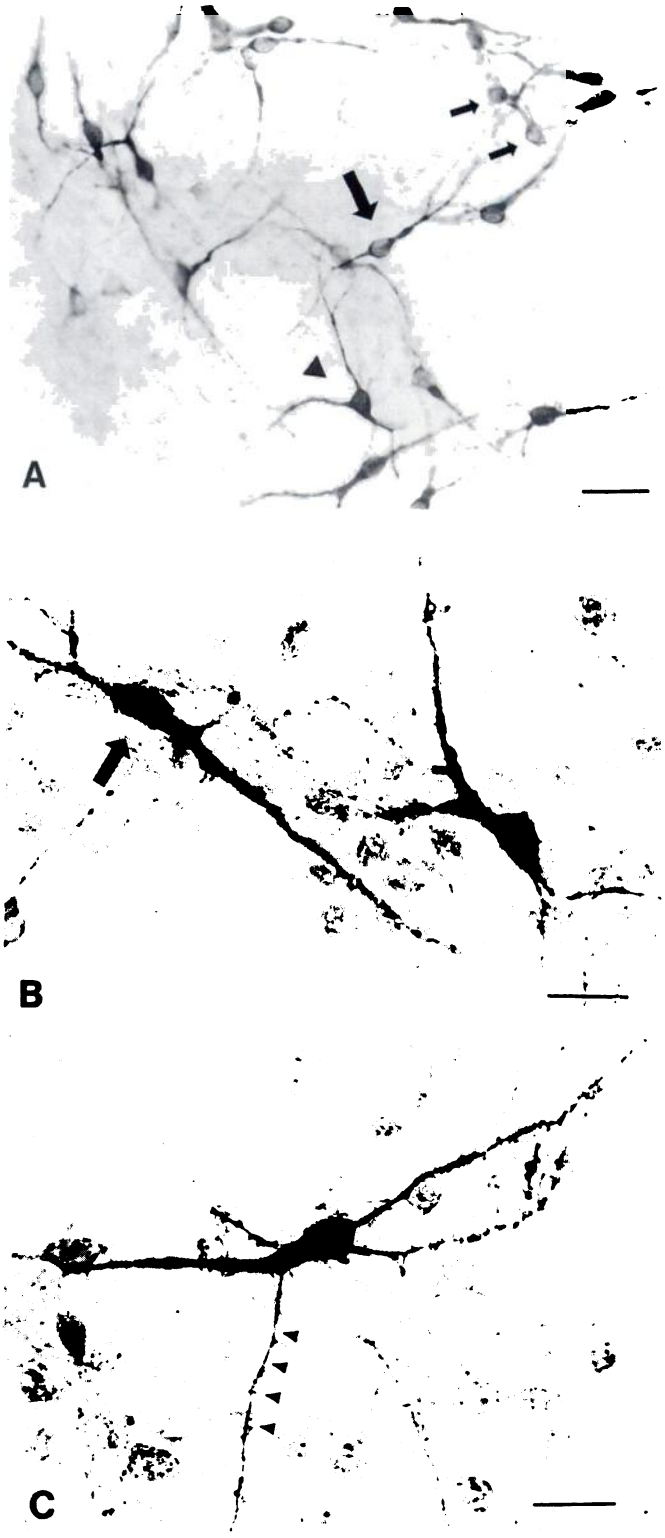

Fig. 1. Photomicrographs of TH-ir cells in ventral mesencephalic cultures. Cells of multiple morphologies are present (A), including fusiform (*large arrow*), trigonal (*arrowhead*), and small round cells (*small arrows*). At higher power (B and C), very fine varicose processes are seen between cells. A fusiform cell is indicated by the *arrow* in B. A process that appears to be an axon leaves the cell body in C (*small arrowheads*). *Scale bar*, 100 μm in A and 50 μm in B and C.

experiments) and 8.8 ± 5.0% (three experiments), respectively. Consistent with previous observations (32, 33), specific [^{3}H]dopamine uptake (defined with 1 μM benztropine) increased as a function of time in culture and was maximal at 6–8 DIV (data not shown). Furthermore, specific [^{3}H]dopamine uptake was completely inhibited in Na^+-free Krebs-HEPES buffer in which 122 mM choline chloride was substituted for NaCl (data not shown).

Characteristics of [^{3}H]dopamine release. Ventral mesencephalic cultures demonstrated consistent robust release of [^{3}H]dopamine at 22° in response to two pulses of a depolarizing concentration of K^+ applied 25 and 50 min after loading with [^{3}H]dopamine (Fig. 2). [^{3}H]Dopamine release during the S1 and S2 pulses was increased 1.6- and 2.1-fold, respectively, with respect to spontaneous [^{3}H]dopamine efflux determined in the fractions immediately before the 20 mM K^+ pulses. [^{3}H]Dopamine released during the S1 and S2 pulses was 179 ± 15 fmol/well (16 experiments) and 126 ± 14 fmol/well (16 experiments), which corresponded to 17.8 ± 1.1% (16 experiments) and 12.1 ± 0.7% (16 experiments), respectively, of the residual intracellular content of [^{3}H]dopamine at the end of the release period. The S2/S1 ratio was 0.70 ± 0.02 (16 experiments) for cells that received a double pulse of 20 mM K^+. This was significantly different from a value of 0.25 ± 0.01 (16 experiments) for cells that were exposed to a pulse of 20 mM K^+ for S1 and basal Krebs-HEPES buffer for S2 ($p < 0.0005$; paired Student's t test). Thus, 20 mM K^+-evoked [^{3}H]dopamine release was increased significantly with respect to spontaneous efflux.

K^+-stimulated [^{3}H]dopamine release was inhibited in the absence of extracellular calcium (Fig. 3). The S2/S1 ratio for a double 20 mM K^+ pulse in the absence of calcium was significantly different from that in the presence of calcium ($p < 0.01$; Newman-Keuls). However, there was no significant difference between the S2/S1 ratios for cells that received a single pulse of 20 mM K^+ for S1 and were then exposed to basal Krebs-HEPES buffer, in the absence or presence of calcium, for S2 ($p > 0.05$; Newman-Keuls).

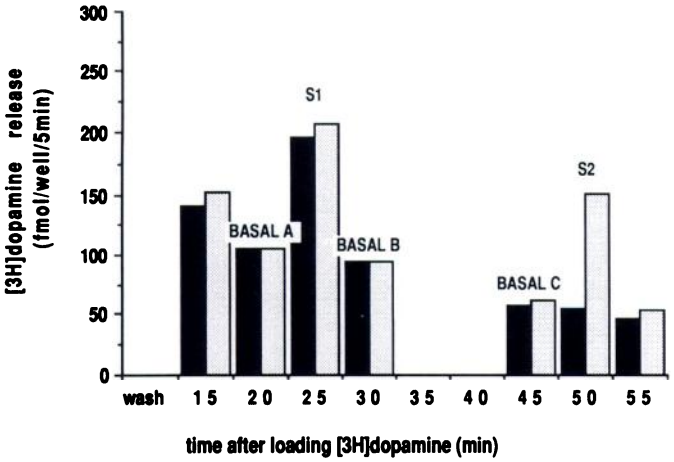

Fig. 2. Spontaneous and 20 mM K^+-evoked release of [^{3}H]dopamine from ventral mesencephalic cultures. Cultures were loaded for 20 min with [^{3}H]dopamine and subsequently washed with Krebs-HEPES buffer every 5 min, for 60 min. [^{3}H]Dopamine content of these 5-min fractions was determined by liquid scintillation counting. Cells were exposed to a single (■) or double (□) 5-min pulse of 20 mM K^+ Krebs-HEPES buffer at 25 min (S1) and 50 min (S2) after loading. Data shown are from a representative experiment. A similar profile of [^{3}H]dopamine release was obtained in all other cultures.

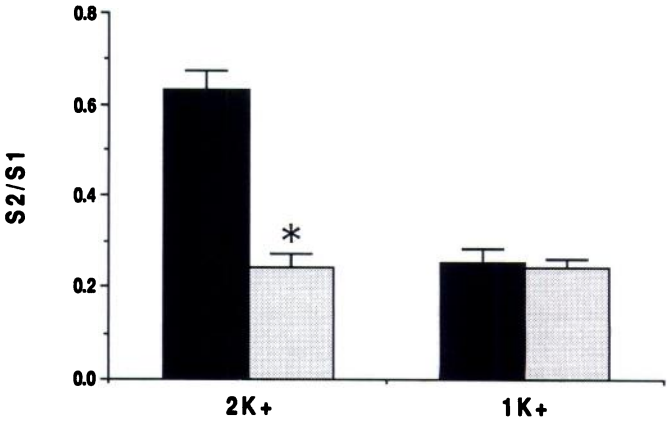

Fig. 3. Spontaneous and 20 mM K^+-evoked [^{3}H]dopamine release from ventral mesencephalic cultures in Krebs-HEPES buffer in the presence or absence of extracellular calcium. Cultures were exposed to a double pulse of 20 mM K^+ ($2K^+$) or a single pulse of 20 mM K^+ for S1 and basal Krebs-HEPES buffer for S2 ($1K^+$). After the S1 pulse, cells were incubated in Krebs-HEPES buffer (■) or Krebs-HEPES buffer without Ca^{2+}, containing 100 μM EGTA (□), for the duration of the washing period and the S2 pulse. Data are expressed as the ratio of [^{3}H]dopamine released in S2/S1 pulses. Data represent the mean ± standard error of three experiments. One-way ANOVA, $F = 38.3$; df 3,8; $p < 0.0001$. *, $p < 0.01$, significantly different from control S2/S1 in the presence of Ca^{2+} (Newman-Keuls). In the presence of calcium, [^{3}H]dopamine release during the S1 and S2 pulses was 164 ± 23 and 105 ± 19 fmol/well (three experiments), respectively.

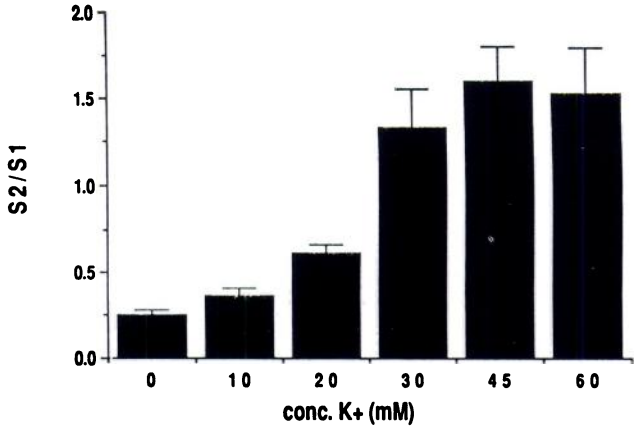

Fig. 4. [^{3}H]Dopamine release from E15 rat ventral mesencephalic cultures in response to increasing concentrations of K^+. Cells were exposed to a 20 mM K^+ S1 pulse and an S2 K^+ pulse at the concentration indicated, as described in Experimental Procedures. Basal Krebs-HEPES buffer is indicated as zero. Data are expressed as the ratio of [^{3}H]dopamine release in S2/S1 fractions. Data represent the mean ± standard error of three experiments. [^{3}H]Dopamine release for the 20 mM K^+ S1 pulse and a 20 mM K^+ S2 pulse was 142 ± 20 and 90 ± 19 fmol/well (four experiments), respectively.

There was a dose-dependent increase in [^{3}H]dopamine release with increasing K^+ concentrations (Fig. 4). K^+-evoked release could be detected at 10 mM K^+ and increased to reach maximal stimulation at 45–60 mM K^+. Twenty millimolar K^+ was chosen as the standard depolarizing concentration for both S1 and S2 pulses in all subsequent experiments, because it produced submaximal stimulation of [^{3}H]dopamine release.

Effect of opioids on [^{3}H]dopamine release. The μ-selective agonist DAGOL and the δ-selective agonist DPDPE at a concentration of 500 nM had no significant effect on the S2/S1 ratio for [^{3}H]dopamine release ($p > 0.05$; Dunnett's t test) (Fig. 5). In contrast, the κ_1-selective agonist U69593 (500 nM) and

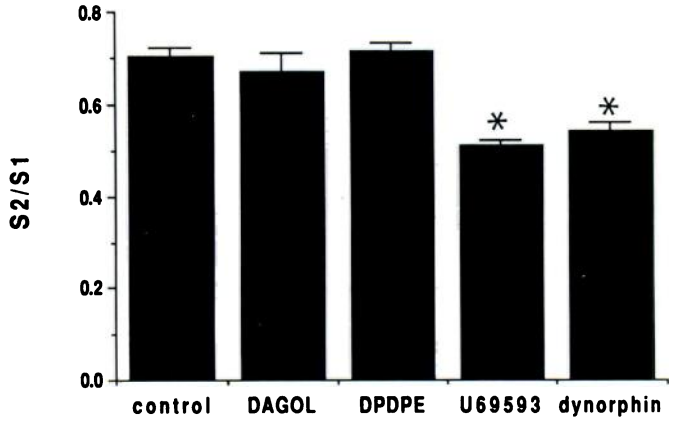

Fig. 5. Effect of μ-, δ-, and κ-opioid agonists on 20 mM K^+-evoked [^{3}H]dopamine release from ventral mesencephalic cultures. After the S1 pulse, cells were incubated with Krebs-HEPES buffer containing DAGOL (500 nM), DPDPE (500 nM), U69593 (500 nM), or dynorphin-1–17 (200 nM) for the duration of the washing period and the S2 pulse. Data represent the mean ± standard error of the ratio of [^{3}H]dopamine release in S2/S1 fractions in three or four experiments. One-way ANOVA, $F = 11.01$; df 4,11; $p < 0.005$. *, $p < 0.01$, significantly different from control S2/S1 ratio (Dunnett's t test). Control [^{3}H]dopamine release was 173 ± 19 and 121 ± 14 fmol/well (six experiments) for the S1 and S2 pulses, respectively.

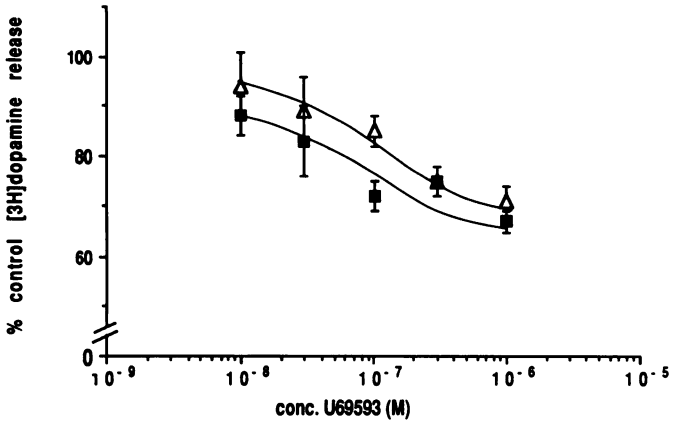

Fig. 6. Effect of U69593 on spontaneous (△) and 20 mM K^+-evoked (■) [^{3}H]dopamine release from ventral mesencephalic cultures. After the S1 pulse, cells were incubated with the appropriate concentration of U69593 for the duration of the washing period and the S2 pulse. Data are expressed as percentage of drug-free control values, as described in Experimental Procedures. Spontaneous [^{3}H]dopamine efflux was determined immediately after exposure to U69593 (basal B fraction). Data represent the mean ± standard error of three to nine experiments. Control [^{3}H]dopamine release was 83 ± 6 and 97 ± 18 fmol/well (nine experiments) for the basal B and (S2 − basal C) fractions, respectively.

the endogenous opioid peptide dynorphin-1–17 (200 nM) significantly inhibited the S2/S1 ratio ($p < 0.01$; Dunnett's t test) (Fig. 5).

Because the S2/S1 ratio represents a composite measure of both basal and K^+-evoked [^{3}H]dopamine release, the effects of the selective opioids on spontaneous and K^+-evoked efflux were characterized independently. U69593 exhibited dose-dependent inhibition of both spontaneous and K^+-evoked [^{3}H]dopamine release (Fig. 6). U69593 inhibition of spontaneous efflux was apparent within 5 min (basal B) and was sustained after 15 min (basal C) of exposure to the drug. Maximum inhibition of spontaneous and K^+-evoked release was observed in the presence of 1 μM U69593, at which concentration release represented 71 ± 3% and 67 ± 2% (three experiments), respectively, of control values. The EC_{50} values for U69593 for inhibition of spontaneous and K^+-evoked release were approximately 60 and 20 nM, respectively. In contrast, DAGOL and DPDPE had no effect on either spontaneous or evoked [^{3}H]dopamine release.

The inhibitory effects of U69593 (100 nM) on combined spontaneous and K^+-evoked [^{3}H]dopamine release were reversed by the nonselective opiate antagonist naloxone (1 μM) and the κ-selective antagonist norbinaltorphimine (10 nM) (Fig. 7). Naloxone (1 μM) alone produced a slight inhibition of [^{3}H]dopamine release, but this was not statistically significant ($p > 0.05$; Newman-Keuls).

TTX, which potently inhibits one class of voltage-sensitive Na^+ channels, reduced spontaneous [^{3}H]dopamine efflux but did not influence 20 mM K^+-evoked [^{3}H]dopamine release (Fig. 8). TTX (500 nM) and U69593 (100 nM) produced a similar degree of inhibition of spontaneous [^{3}H]dopamine efflux. However, the inhibitory effects of TTX and U69593 were nonadditive, suggesting that κ-opioid modulation of spontaneous efflux had been blocked by TTX (Fig. 8A). Spontaneous efflux was 65 ± 2% (four experiments) of control in the presence of TTX alone and 71 ± 7% (four experiments) in the presence of both drugs. In contrast, TTX did not inhibit K^+-evoked [^{3}H]dopamine release, and the effect of U69593 was maintained in the presence of TTX (Fig. 8B). Thus, in the presence of both drugs K^+-evoked release was 59 ± 5% (four experiments) of control, compared with 115 ± 14% (four experiments) in the presence of TTX alone.

Discussion

Characteristics of embryonic rat ventral mesencephalic culture. Ventral mesencephalic cultures contained both catecholaminergic and noncatecholaminergic neuronal cell types, as defined by immunohistochemical techniques using antibodies directed against TH, the rate-limiting enzyme in

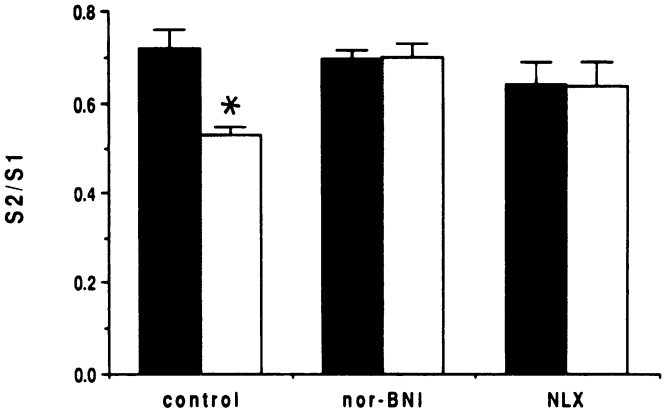

Fig. 7. Effect of U69593 on 20 mM K^+-evoked [^{3}H]dopamine release from ventral mesencephalic cultures in the presence or absence of naloxone and norbinaltorphimine. Cultures were exposed to an S1 pulse and then incubated with Krebs-HEPES buffer (*control*) or Krebs-HEPES buffer containing 1 μM naloxone (*NLX*) or 10 nM norbinaltorphimine (*nor-BNI*), in the absence (■) or presence (□) of 100 nM U69593, for the duration of the washing period and the S2 pulse. Data are expressed as the ratio of [^{3}H]dopamine release in S2/S1 fractions. Data represent the mean ± standard error of three or four experiments. One-way ANOVA, $F = 3.68$; df 5,20; $p < 0.025$. *, $p < 0.05$, significantly different from control S2/S1 ratio (Newman-Keuls). Control [^{3}H]dopamine release was 212 ± 37 and 156 ± 35 fmol/well (five experiments) for the S1 and S2 pulses, respectively.

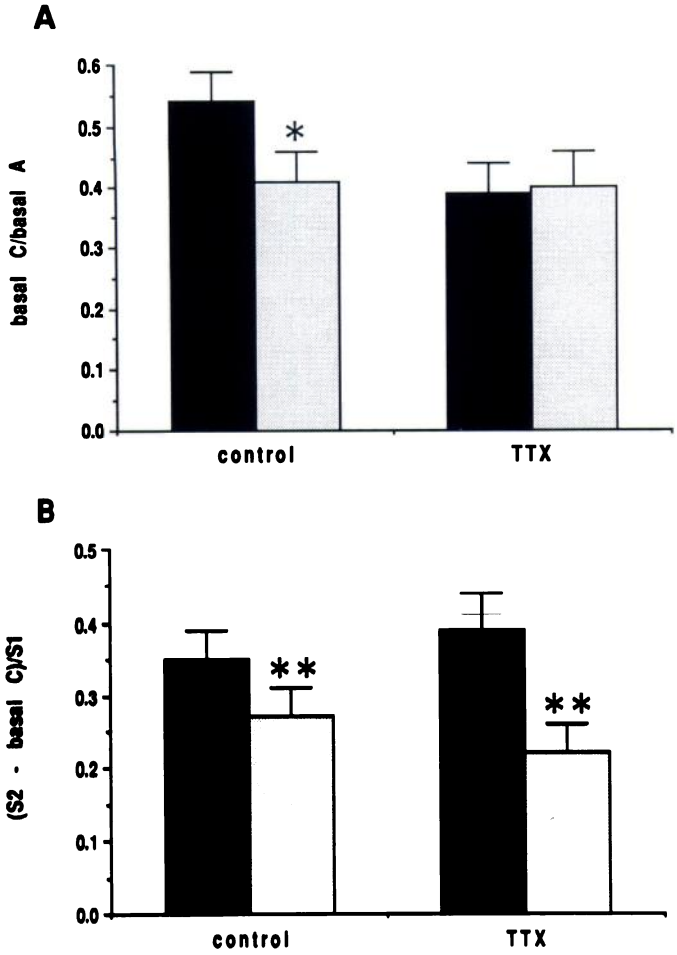

Fig. 8. Effect of U69593 and TTX on spontaneous (A) and 20 mM K^+-evoked (B) [^{3}H]dopamine release from ventral mesencephalic cultures. Cultures were exposed to an S1 pulse and then incubated with Krebs-HEPES buffer (*control*) or Krebs-HEPES buffer containing 500 nM TTX (*TTX*), in the absence (■) or presence (□) of 100 nM U69593, for the remainder of the experiment. Spontaneous [^{3}H]dopamine efflux in the presence of drug was determined immediately before the S2 pulse (basal C). Data are expressed as spontaneous and evoked release ratios, as described in Experimental Procedures. Data represent the mean ± standard error of four experiments. *, $p < 0.01$, significantly different from control ratio in the absence of U69593; **, $p < 0.005$, significantly different from corresponding control or TTX ratio in the absence of U69593. Control [^{3}H]dopamine release was 131 ± 9, 207 ± 18, 71 ± 5, and 73 ± 13 fmol/well (four experiments) for the basal A, S1, basal C, and (S2 − basal C) fractions, respectively.

catecholamine synthesis. The morphology of the TH-ir cells was very similar to that which has been observed in Golgi preparations of substantia nigra and VTA or in sections retrogradely labeled after tracer injections into terminal fields (see Ref. 1 for review). The medium-sized fusiform cells, with primary dendrites arising from opposite poles, were very similar to cells of the dorsal substantia nigra pars compacta and the VTA that contribute to the dopaminergic innervation of limbic structures. The larger trigonal cells with multiple dendrites were similar to those found in ventral substantia nigra pars compacta and VTA. *In vivo*, cells with similar morphology extend a large dendrite ventrally into the substantia nigra pars reticulata and project axons to multiple forebrain regions. In addition, small cells reminiscent of interfascicular nucleus cells were present. In the present study, ventral mesencephalon tissue pieces were dissociated using a combination of enzymatic and mechanical techniques, which produced a more homogeneous cell suspension than mechanical dissociation alone. Each of the major morphological types of dopaminergic cells was represented in these ventral mesencephalic cultures, confirming that this method is appropriate for the establishment of cultures that contain cell types equivalent to those *in vivo*.

The present study confirms earlier reports (31–34) that catecholamine neurons in cultures from embryonic rat ventral mesencephalon have dopaminergic properties. Indeed, [^{3}H]dopamine uptake into ventral mesencephalic cultures at 8 DIV was inhibited by benztropine, a relatively specific inhibitor of dopamine uptake into dopaminergic neurons (31), but not by inhibitors of norepinephrine or serotonin uptake. Although Na^+-dependent, high affinity uptake of [^{3}H]dopamine into astrocyte cultures has been reported, this has a low concentrative capacity (35) and, as such, would represent only a small fraction of the uptake into dopaminergic neurons. The observation that ventral mesencephalic cultures exhibited robust, calcium-dependent, [^{3}H]dopamine release is also inconsistent with neurotransmitter uptake into glial cells, which do not show K^+-stimulated release (36).

[^{3}H]Dopamine release in response to a number of different depolarizing stimuli, such as elevated extracellular K^+, veratridine, and glutamate, has been widely characterized in ventral mesencephalic cultures (32–34, 36). In each of these studies a single exposure to the depolarizing stimulus was used. In contrast, the present study adopted a double-K^+ pulse paradigm that was considered more appropriate for characterization of the effects of inhibitory agents. Test drugs were introduced after the initial S1 pulse, with an equilibration period before the S2 pulse in the presence of the test agent. The first pulse represents an internal control for the second (test) pulse, therefore compensating for variations in the extent of neurotransmitter uptake by dissociated cell culture preparations, which have been noted previously (34). The biochemical identity of the radiolabeled compounds released was not examined. However, others have demonstrated that radioactive efflux from ventral mesencephalic cultures is an appropriate measure of [^{3}H]dopamine release (32, 36). In accordance with the method used extensively by Mount *et al.* (32), dopamine uptake inhibitors were not included in [^{3}H]dopamine release assay buffers. The inclusion of such inhibitors would be expected to increase the signal to noise ratio. However, the assay sensitivity was adequate for the purposes of the present study.

[^{3}H]Dopamine release from ventral mesencephalic cultures increased as the extracellular concentration of K^+ was increased, and release was maximal at 45–60 mM K^+. Twenty millimolar K^+ was chosen as the standard concentration for characterization of drug effects because this concentration elicited submaximal stimulation. This is in contrast to the majority of previous studies, which have used 50–60 mM K^+, a concentration range that in this culture system produced supramaximal responses. The choice of K^+ concentration is critical to the characterization of opioid inhibition of neurotransmitter release, because the inhibitory effect of opioids is dependent upon stimulus intensity (37). μ-Opioid inhibition of K^+-evoked [^{3}H] norepinephrine release from rat cortical slices was attenuated when the K^+ concentration was increased from 20 mM to 50 mM (37).

Effect of κ-opioids on [^{3}H]dopamine release. Both spon-

taneous and 20 mM K^+-evoked [^{3}H]dopamine release from embryonic ventral mesencephalic cultures was inhibited by U69593 and dynorphin-1–17, both of which exhibit high affinity for the κ_1 receptor (4, 38). U69593 produced a dose-dependent inhibition of both spontaneous and K^+-evoked [^{3}H]dopamine release, which was reversed by naloxone and norbinaltorphimine. It is most unlikely that these observations represented stimulation of [^{3}H]dopamine uptake because, to date, only inhibition of [^{3}H]dopamine uptake by κ-opioid agonists has been reported, and this was at a concentration (40 μM) much higher than those used presently (39). Furthermore, the EC_{50} for U69593 inhibition of evoked release was 20 nM, which is in agreement with the literature value of 10 nM for inhibition of [^{3}H]dopamine release from rat striatal slices (40).

The maximum degree of inhibition by U69593 (approximately 30%) is comparable to that reported for inhibition of K^+-stimulated [^{3}H]dopamine release from rat striatal slices by both selective κ_1 and nonselective κ agonists (40). These observations are consistent with the presence of two populations of dopaminergic neurons in ventral mesencephalic cultures, one of which does not express κ receptors. These may correspond to the two major TH-ir cell types identified using immunohistochemical techniques. An alternative explanation is the existence of two mechanisms of cellular dopamine release, only one of which is modulated by κ receptors.

Spontaneous [^{3}H]dopamine efflux was attenuated by TTX. This confirms a previous report (32, 41) that neuronal cells in ventral mesencephalic cultures undergo spontaneous firing. The effects of TTX and U69593 on spontaneous efflux were nonadditive, suggesting that in this culture system κ opioids inhibit dopamine efflux from tonically active cells. κ-opioid inhibition of spontaneous dopamine efflux has also been demonstrated *in vitro*, in brain slice preparations of rat (40) and cat caudate nucleus (23).

U69593 inhibition of K^+-stimulated [^{3}H]dopamine release from ventral mesencephalic cultures was maintained in the presence of TTX. The mechanism of action of κ-opioids on evoked release, therefore, is independent of Na^+ action potentials, providing further evidence that functional κ receptors are localized on the terminals of dopaminergic neurons. This supports previous findings *in vitro*. Thus, κ-opioid inhibition of acetylcholine-evoked [^{3}H]dopamine release from slices of cat caudate-nucleus was maintained in the presence of TTX, consistent with a direct effect on the terminals of dopaminergic afferents and not local neuronal circuits (23). Indeed, U50488 inhibited [^{3}H]dopamine release from rat striatal synaptosomes (19).

The results of neurotransmitter release studies are not in agreement with earlier receptor binding studies, which have postulated an exclusively postsynaptic localization for κ receptors in the caudate-putamen and nucleus accumbens (24, 26). However, more recent studies in this laboratory have demonstrated that the density of [^{3}H]diprenorphine-labeled κ binding sites in rat caudate-putamen was decreased both 2 and 24 weeks after 6-hydroxydopamine lesion of the substantia nigra (28). Consistent with these observations, striatal κ binding site density was decreased in the mutant Weaver mouse, in which mesencephalic dopaminergic neurons have undergone spontaneous degeneration (42). Electron microscopic autoradiographic techniques also have provided evidence for the localization of striatal κ sites at axo-axonic interfaces that may correspond to dopaminergic afferents (43).

The substantia nigra pars compacta, the source of the majority of the striatal dopaminergic afferents, has a high density of μ sites but is devoid of κ sites (3). Moreover, *in vitro* electrophysiological studies have failed to demonstrate a direct effect of κ agonists on dopaminergic neurons in either the substantia nigra pars compacta (16) or the VTA (44). Together with the present findings, these observations suggest that functional κ receptors are localized selectively on the terminals of ventral mesencephalic dopamine neurons but are absent from, or present in very low density on, the cell body. The distribution of κ receptors may be analogous to that of dopamine D1 receptors (45). In the dentate gyrus and cerebellum, D1 receptors are synthesized in the cell body but are not expressed there, being transported along fiber projections to the dendritic or axonal fields, respectively. Localization of κ receptor mRNA, and hence the sites of receptor synthesis, awaits the cloning of the receptor.

Effect of μ- and δ-opioids on [^{3}H]dopamine release. DAGOL and DPDPE did not influence [^{3}H]dopamine release from ventral mesencephalic cultures, consistent with *in vitro* observations that μ and δ agonists do not modulate stimulus-evoked release of [^{3}H]dopamine from slices of rat caudate-putamen (18, 19, 22) or nucleus accumbens (21). *In vivo*, μ agonists stimulate striatal dopamine release indirectly through a receptor population localized on intrinsic neurons or nondopaminergic afferents (9, 11, 13). At the level of the mesencephalon, μ agonists indirectly excite VTA (44) and substantia nigra pars compacta (16) dopamine neurons by hyperpolarization of a secondary population of inhibitory GABAergic interneurons. Because ventral mesencephalic cultures do not have a circuitry equivalent to that *in vivo*, the lack of effect of μ agonists on [^{3}H]dopamine release is consistent with the hypothesis that μ effects are mediated indirectly.

The negative results obtained with DPDPE in the present study are inconsistent with the hypothesis that *in vivo* δ agonists stimulate dopamine release directly (9, 11, 13). The discrepancies between the *in vivo* and *in vitro* effects of δ agonists may be a consequence of a preferential effect of δ agonists on the release of newly synthesized dopamine (20). In the present study, opioid effects on the release of presynthesized dopamine were selectively characterized because ventral mesencephalic cells were loaded with [^{3}H]dopamine and not a precursor molecule. Another factor that must be taken into consideration, however, is whether these cultures express δ receptors. Cultures were established from embryonic neurons, which are at an immature stage of development and may not express their full complement of receptors.

Ontogenetic expression of opioid receptors. Few studies have characterized the prenatal development of opioid receptor binding sites and those that have done so have used nonselective opioid radioligands (see Ref. 46 for review). ^{125}I-β-Endorphin-labeled binding sites, which are thought to represent μ sites, are present in the striatal anlage and ventral mesencephalon by E15 (46). To our knowledge the first appearance of κ sites prenatally in rat brain has not been established. However, μ and κ binding sites and functional receptors have been demonstrated in E15-E17 rat striatal cultures (47, 48) and E17 brain slices (49), respectively. The present study has extended

these observations to demonstrate functional κ receptors in rat ventral mesencephalic cultures derived from E15 brain.

The lack of effect of DPDPE on [^{3}H]dopamine release in ventral mesencephalic cultures may be a function of the late ontogenetic expression of δ receptors, with respect to both μ and κ receptors. Significant densities of δ binding sites or functional receptors are not detectable in rat brain until at least 7 days postpartum (46, 49). Indeed, δ sites have not been detected in E17 rat striatal cultures (48), although this brain region expresses δ receptors at high density in the adult (3). Thus, the present culture system may not be an appropriate model for characterization of δ receptor-mediated modulation of dopamine release.

In conclusion, the present study has demonstrated κ- but not μ- or δ-opioid modulation of dopamine release from embryonic rat ventral mesencephalic cultures. κ agonists inhibited dopamine release directly, providing further evidence for the presynaptic localization of κ receptors in the rat striatum. Functional κ receptors are present early during development and may, therefore, represent a potential site for opioid modulation of dopamine release during ontogeny. Embryonic rat ventral mesencephalic cultures represent an excellent model to examine further the mechanisms by which κ-opioids modulate neurotransmitter release.

Acknowledgments

We would like to thank H. K. Raymon for all her advice with respect to the tissue culture.

References

1. Fallon, J. H., and S. E. Loughlin. The substantia nigra, in *The Rat Nervous System* (G. Paxinos, ed.). Academic Press, Sydney, Australia, 353–374 (1985).
2. Graybiel, A. M. Neurotransmitters and neuromodulators in the basal ganglia. *Trends Neurosci.* **13**:244–254 (1991).
3. Mansour, A., H. Khachaturian, M. E. Lewis, H. Akil, and S. J. Watson. Autoradiographic differentiation of *mu, delta* and *kappa* opioid receptors in the rat forebrain and midbrain. *J. Neurosci.* **7**:2445–2464 (1987).
4. Unterwald, E. M., C. Knapp, and R. S. Zukin. Neuroanatomical localisation of κ_1 and κ_2 opioid receptors in rat and guinea-pig brain. *Brain Res.* **562**:57–65 (1991).
5. Chesselet, M. F., A. Cheramy, T. D. Reisine, and J. Glowinski. Morphine and δ-opiate agonists locally stimulate *in vivo* dopamine release in cat caudate nucleus. *Nature (Lond.)* **291**:320–322 (1981).
6. Wood, P. L., H. S. Kim, C. Cosi, and S. Iyengar. The endogenous *kappa* agonist, dynorphin-(1–13), does not alter basal or morphine-stimulated dopamine metabolism in the nigrostriatal pathway of the rat. *Neuropharmacology* **26**:1585–1588 (1987).
7. Matthews, R. T., and D. C. German. Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. *Neuroscience* **11**:617–625 (1984).
8. Di Chiara, G., and A. Imperato. Opposite effects of *mu* and *kappa* opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. *J. Pharmacol. Exp. Ther.* **244**:1067–1080 (1988).
9. Spanagel, R., A. Herz, and T. S. Shippenberg. The effects of opioid peptides on dopamine release in the nucleus accumbens: an *in vivo* microdialysis study. *J. Neurochem.* **55**:1734–1740 (1990).
10. Pentney, R. J. W., and A. Gratton. Effects of local *delta* and *mu* opioid receptor activation on basal and stimulated dopamine release in striatum and nucleus accumbens of rat: an *in vivo* electrochemical study. *Neuroscience* **45**:95–102 (1991).
11. Dourmap, N., A. Michael-Titus, and J. Costentin. Differential effect of intrastriatal kainic acid on the modulation of dopamine release by μ- and δ-opioid peptides: a microdialysis study. *J. Neurochem.* **58**:709–713 (1992).
12. Latimer, L. G., P. Duffy, and P. W. Kalivas. *Mu* opioid receptor involvement in enkephalin activation of dopamine neurons in the ventral tegmental area. *J. Pharmacol. Exp. Ther.* **241**:328–337 (1987).
13. Arenas, E., J. Alberch, and J. Marsal. Dopaminergic system mediates only δ-opiate inhibition of endogenous acetylcholine release evoked by glutamate from rat striatal slices. *Neuroscience* **42**:707–714 (1991).
14. Manzanares, J., K. J. Lookingland, and K. E. Moore. *Kappa* opioid receptor-mediated regulation of dopaminergic neurons in the rat brain. *J. Pharmacol. Exp. Ther.* **256**:500–505 (1991).
15. Lavin, A., and M. Garcia-Munoz. Electrophysiological changes in substantia nigra after dynorphin administration. *Brain Res.* **369**:298–302 (1985).
16. Lacey, M. G., N. B. Mercuri, and R. A. North. Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *J. Neurosci.* **9**:1233–1241 (1989).
17. Walker, J. M., L. A. Thompson, J. Frascella, and M. W. Friederich. Opposite effects of μ and κ opiates on the firing-rate of dopamine cells in the substantia nigra of the rat. *Eur. J. Pharmacol.* **134**:53–59 (1987).
18. Schoffelmeer, A. N. M., K. C. Rice, A. E. Jacobson, J. G. Van Gelderen, F. Hogenboom, M. H. Heijina, and A. H. Mulder. μ-, δ- and κ-opioid receptor mediated inhibition of neurotransmitter release and adenylate cyclase activity in rat brain slices: studies with fentanyl isothiocyanate. *Eur. J. Pharmacol.* **154**:169–178 (1988).
19. Werling, L. L., A. Frattali, P. S. Portoghese, A. E. Takemori, and B. M. Cox. *Kappa* receptor regulation of dopamine release from striatum and cortex of rats and guinea-pigs. *J. Pharmacol. Exp. Ther.* **246**:282–286 (1988).
20. Petit, F., M. Hamon, M.-C. Fournie-Zaluski, B. P. Roques, and J. Glowinski. Further evidence for a role of δ-opiate receptors in the presynaptic regulation of newly synthesised dopamine release. *Eur. J. Pharmacol.* **126**:1–9 (1986).
21. Heijina, M. H., M. Padt, F. Hogenboom, P. S. Portoghese, A. H. Mulder, and A. N. M. Schoffelmeer. Opioid receptor-mediated inhibition of dopamine and acetylcholine release from slices of rat nucleus accumbens, olfactory tubercle and frontal cortex. *Eur. J. Pharmacol.* **18**:267–278 (1990).
22. Werling, L. L., H. M. Jacocks, and P. N. McMahon. Regulation of [^{3}H] dopamine release from guinea-pig striatum by NMDA receptor/channel activators and inhibitors. *J. Pharmacol. Exp. Ther.* **255**:40–45 (1990).
23. Gauchy, C., M. Desban, O. Krebs, J. Glowinski, and M. L. Kemel. Role of dynorphin-containing neurons in the presynaptic inhibitory control of the acetylcholine-evoked release of dopamine in the striosomes and the matrix of the cat caudate nucleus. *Neuroscience* **41**:449–458 (1991).
24. Eghbali, M., C. Santoro, W. Paredes, E. L. Gardner, and R. S. Zukin. Visualisation of multiple opioid-receptor types in rat striatum after specific mesencephalic lesions. *Proc. Natl. Acad. Sci. USA* **84**:6582–6586 (1987).
25. Waksman, G., E. Hamel, P. Delay-Goyet, and B. P. Roques. Neutral endopeptidase-24.11, μ and δ opioid receptors after selective brain lesions: an autoradiographic study. *Brain Res.* **436**:205–216 (1987).
26. Unterwald, E. M., A. Tempel, G. F. Koob, and R. S. Zukin. Characterisation of opioid receptors in rat nucleus accumbens following mesolimbic dopaminergic lesions. *Brain Res.* **505**:111–118 (1989).
27. Trovero, F., D. Herve, M. Desban, J. Glowinski, and J.-P. Tassin. Striatal opiate *mu*-receptors are not located on dopamine nerve endings in the rat. *Neuroscience* **39**:313–321 (1990).
28. Smith, J. A. M., S. E. Loughlin, and F. M. Leslie. Long-term changes in striatal opioid binding sites after 6-hydroxydopamine lesion of substantia nigra. *Soc. Neurosci. Abstr.* **17**:451 (1991).
29. Dilts, R. P., and P. W. Kalivas. Autoradiographic localisation of *delta* opioid receptors within the mesocorticolimbic dopamine system using radioiodinated [2-D-penicillamine,5-D-penicillamine]enkephalin (^{125}IDPDPE). *Synapse* **6**:121–132 (1990).
30. Raymon, H. K., and F. M. Leslie. Characterisation of specific [^{3}H]norepinephrine uptake in dissociated noradrenergic cell cultures from embryonic rat rhombencephalon. *Soc. Neurosci. Abstr.* **17**:363 (1991).
31. Prochiantz, A., U. Di Porzio, A. Kato, B. Berger, and J. Glowinski. *In vitro* maturation of mesencephalic dopaminergic neurons from mouse embryos is enhanced in presence of their striatal target cells. *Proc. Natl. Acad. Sci. USA* **76**:5387–5391 (1979).
32. Mount, H., S. Welner, R. Quirion, and P. Boksa. Glutamate stimulation of [^{3}H]dopamine release from dissociated cell cultures of rat ventral mesencephalon. *J. Neurochem.* **52**:1300–1310 (1989).
33. Ahnert-Hilger, G., J. Engele, I. Reisert, and C. Pilgrim. Different developmental schedules of dopaminergic and noradrenergic neurons in dissociation culture of fetal rat midbrain and hindbrain. *Neuroscience* **17**:157–165 (1986).
34. Engele, J., C. Pilgrim, M. Kirsch, and I. Reisert. Different developmental potentials of diencephalic and mesencephalic dopaminergic neurons *in vitro*. *Brain Res.* **483**:98–109 (1989).
35. Semenoff, D., and H. K. Kimelberg. Autoradiography of high affinity uptake of catecholamines by primary astrocyte cultures. *Brain Res.* **348**:125–136 (1985).
36. Barochovsky, O., and H. F. Bradford. Development of transmitter releasing capacity in neuron-enriched tissue cultures. *J. Neurochem.* **48**:787–797 (1987).
37. Werling, L. L., S. R. Brown, and B. M. Cox. Opioid receptor regulation of the release of norepinephrine in brain. *Neuropharmacology.* **26**:987–996 (1987).
38. Nock, B., A. L. Giordano, T. J. Cicero, and L. H. O'Connor. Affinity of drugs and peptides for U69593-sensitive and -insensitive *kappa* opiate binding sites: the U69593-insensitive site appears to be the β-endorphin-specific *epsilon* receptor. *J. Pharmacol. Exp. Ther.* **254**:412–419 (1990).
39. Clow, D. W., and K. Jhamandas. Effects of a κ-receptor agonist, U50488, on the release of endogenous brain dopamine. *Can. J. Physiol. Pharmacol.* **66**:128–133 (1988).
40. Mulder, A. H., D. M. Burger, G. Wardeh, F. Hogenboom, and L. A. Frankhuyzen. Pharmacological profile of various κ-agonists at κ-, μ- and δ-opioid receptors mediating presynaptic inhibition of neurotransmitter release in rat brain. *Br. J. Pharmacol.* **102**:518–522 (1991).
41. Mount, H., R. Quirion, J. Kohn-Alexander, and P. Boksa. Subtypes of

excitatory amino acid receptors involved in the stimulation of [^{3}H]dopamine release from cell cultures of rat ventral mesencephalon. *Synapse* **5**:271–280 (1990).
42. Loughlin, S. E., and F. M. Leslie. Opioid receptor changes in Weaver mouse striatum. *Brain Res.* **585**:149–155 (1992)
43. Jomary, C., J. E. Gairin, and A. Beaudet. Synaptic localisation of κ opioid receptors in guinea-pig neostriatum. *Proc. Natl. Acad. Sci. USA* **89**:564–568 (1992).
44. Johnson, S. W., and R. A. North. Opioids excite dopamine neurons by hyperpolarisation of local interneurons. *J. Neurosci.* **12**:483–488 (1992).
45. Mansour, A., J. H. Meador-Woodruff, Q. Zhou, O. Civelli, H. Akil, and S. J. Watson. A comparison of D1 receptor binding and mRNA in rat brain using receptor autoradiographic and *in situ* hybridisation techniques. *Neuroscience* **46**:959–971 (1992).
46. Loughlin, S. E., and F. M. Leslie. Opioid receptors and the developing nervous systems, in *Developing Nervous Systems* (I. S. Zagon and P. J. McLaughlin, eds). Chapman and Hall, London, in press.
47. Van Vliet, B. J., A. H. Mulder, and A. N. M. Schoffelmeer. *Mu*-opioid receptors mediate the inhibitory effect of opioids on dopamine-sensitive adenylate cyclase in primary cultures of rat neostriatal neurons. *J. Neurochem.* **55**:1274–1280 (1990).
48. Vaysse, P. J.-J., R. S. Zukin, K. L. Fields, and J. A. Kessler. Characterisation of opioid receptors in cultured neurons. *J. Neurochem.* **55**:624–631 (1990).
49. De Vries, T. J., F. Hogenboom, A. H. Mulder, and A. N. M. Schoffelmeer. Ontogeny of μ-, δ- and κ-opioid receptors mediating inhibition of neurotransmitter release and adenylate cyclase activity in rat brain. *Dev. Brain Res.* **54**:63–69 (1990).

Send reprint requests to: F. M. Leslie, Ph.D., Department of Pharmacology, College of Medicine, University of California, Irvine, CA 92717.