Direct Agonist Activity of Tricyclic Antidepressants at Distinct Opioid Receptor Subtypes

Pierluigi Onali, Simona Dedoni, and Maria C. Olianas

Department of Neurosciences, Section of Biochemical Pharmacology, University of Cagliari, Cagliari, Italy

Received August 4, 2009; accepted October 13, 2009

ABSTRACT

Tricyclic antidepressants (TCAs) have been reported to interact with the opioid system, but their pharmacological activity at opioid receptors has not yet been elucidated. In the present study, we investigated the actions of amoxapine, amitriptyline, nortriptiline, desipramine, and imipramine at distinct cloned and native opioid receptors. In Chinese hamster ovary (CHO) cells expressing δ-opioid receptors (CHO/DOR), TCAs displaced [3H]naltrindole binding and stimulated guanosine 5’-O-[(3-[35S]thio)triphosphate ([35S]GTPγS) binding at micromolar concentrations with amoxapine displaying the highest potency and efficacy. Amoxapine and amitriptyline inhibited cyclic AMP formation and induced the phosphorylation of signaling molecules along the extracellular signal-regulated kinase 1/2 pathway and native opioid receptors. In Chinese hamster ovary (CHO) cells expressing κ-opioid receptors (CHO/KOR), TCAs, but not amoxapine, exhibited higher receptor affinity and more potent stimulation of [35S]GTPγS binding than in CHO/DOR and effectively inhibited cyclic AMP accumulation. Amitriptyline regulated ERK1/2 phosphorylation and activity in CHO/KOR and C6 glioma cells endogenously expressing κ-opioid receptors, and this effect was attenuated by the κ-opioid antagonist nor-binaltorphimine. In rat nucleus accumbens, amitriptyline slightly inhibited adenylyl cyclase activity and counteracted the inhibitory effect of the full κ agonist trans-3,4-dichloro-N-methyl-N-[2-[1-pyrrolidinyl]cyclohexyl]benzeneacetamide (U50,488). At the cloned μ-opioid receptor, TCAs showed low affinity and no significant agonist activity. These results show that TCAs differentially regulate opioid receptors with a preferential agonist activity on either δ or κ subtypes and suggest that this property may contribute to their therapeutic and/or side effects.

Although the inhibition of presynaptic reuptake of monoamines is considered to be the primary mechanism of action of tricyclic antidepressants (TCAs), it is well established that these drugs can act on multiple molecular targets by affecting the activity of distinct neurotransmitter receptor systems and ion channels (Baldessarini, 2006). These secondary actions have been generally related to TCAs’ adverse side effects, although some of them have been proposed to contribute to the therapeutic activity.

An interaction with the opioid system has long been shown to be involved in the analgesic and mood-elevating effects of TCAs. A number of studies have reported that the antinociceptive effects of TCAs are reversed by opioid receptor antagonists (Biegon and Samuel, 1980; Gray et al., 1998; Marchand et al., 2003; Benbouzid et al., 2008a,b) and that TCAs potentiate morphine-induced analgesia both in animals (Hamon et al., 1987) and in humans (Micó et al., 2006). In animal behavioral tests predictive of antidepressant effects in humans, such as the forced swimming and learned helplessness tests, the effects of TCAs have been found to be antagonized by blockade of opioid receptors, indicating the possible participation of opioid neurotransmission in the antidepressant activity of these drugs (Devoise et al., 1982; Tejedor-Real et al., 1995; Besson et al., 1999).

Although these studies implicate opioid mechanisms in the pharmacological activity of TCAs, the mode of action of these drugs on the opioid system has not been fully clarified. Some studies have reported that long-term antidepressant use increases enkephalin levels in different brain areas (De Felipe et al., 2003; Benbouzid et al., 2008a,b) and that TCAs differentially regulate opioid receptors with a preferential agonist activity on either δ or κ subtypes and suggest that this property may contribute to their therapeutic and/or side effects.

This work was supported by a grant from the Ministry of Education, University, and Research of Italy.

ABBREVIATIONS: TCA, tricyclic antidepressant; CHO, Chinese hamster ovary; [35S]GTPγS, guanosine 5’-O-[(3-[35S]thio)triphosphate; NTL, naltrexone; FSK, forskolin; (−)-U50,488, trans-(−)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide; nor-BNI, nor-binaltorphimine dihydrochloride; CTAP, d-Phe-Cys-Tyr-o-Trp-Arg-Thr-Pen-Thr-NH2; DPDPE, (2-D- penicillamine, 5-D-penicillamine)-enkephalin; DAMGO, d-Ala2-N-methyl-Phe-Gly-ol5)-enkephalin; pGSK, phospho-Ser9-glycogen synthase kinase-3β; pS6rp, phospho-Ser235/236 ribosomal protein; ERK, extracellular signal-regulated kinase; pERK1/2, phosphorylation of extracellular signal-regulated kinase 1/2; GSK, glycogen synthase; pAkt, phospho-Thr308-protein kinase B/Akt; CHO/DOR, CHO/KOR, CHO/MOR, CHO cells expressing the human δ-, κ-, and μ-opioid receptor, respectively; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ECL, enhanced chemiluminescence; ANOVA, analysis of variance.
et al., 1985; Hamon et al., 1987), whereas others observed no change or a decrease (Herman et al., 1986; Kurumaji et al., 1988). Radioligand binding studies examining the interactions of TCAs with opioid receptors also yielded contradictory results. For example, in vivo administration of TCAs has been found to cause a decrease, an increase, and no change in opioid receptor density in different brain areas (Stengaard-Pedersen and Schou, 1986; Hamon et al., 1987; Chen and Lawrence, 2004). Moreover, TCAs have been reported to be either devoid of activity at brain opioid receptors (Hall and Ogren, 1981) or capable of inhibiting opioid receptor binding at pharmacologically relevant concentrations (Bieggen and Samuel, 1980; Isenberg and Cicero, 1984; Wahlström et al., 1994). Thus, whether these drugs can directly act on opioid receptors and whether the distinct opioid receptor subtypes are differentially affected by TCAs still remain unresolved issues.

In the present study, we investigated the action of the TCAs amoxapine, amitriptyline, nortriptyline, desipramine, and imipramine at cloned human δ-, κ-, and μ-opioid receptors individually expressed in Chinese hamster ovary (CHO) cells. Moreover, the effects of some of these drugs at opioid receptors endogenously expressed in C6 glioma cells, rat dorsal striatum and nucleus accumbens, and postmortem human frontal cortex were also examined.

Materials and Methods

Materials. [α-32P]ATP (30–40 Ci/mmol), [2,8-3H]cyclosporin AMP (25 Ci/mmol), [8,14C]cyclic AMP (45.1 mCi/mmol), [2,8-3H]adenine (28.8 Ci/mmol), guanosine 5′-O-[(3′S)-thio]triphosphate ([35S]GTPγS) (1306 Ci/mmol), [15,16-3H]diprenorphine (53 Ci/mmol), and [5′,7′-3H]naltrindole ([3H]NTI) (20 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Forskolin (FSK) and GTPγS were from Calbiochem (San Diego, CA) and Boehringer Ingelheim GmbH (Ingelheim, Germany), respectively. (−)-U50,488 hydrochloride, nor-binaltorphimine dihydrochloride (nor-BNI), d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP), and NTI hydrochloride were from Tocris Bioscience (Bristol, UK). (2-D-Penicillamine, 5-D-penicillamine)-Enkephalin (DPDPE), and NTI hydrochloride were from Tocris Bioscience (Bristol, UK). Amoxapine, amitriptyline, nortriptyline, desipramine, and imipramine, and (α-Ala-2-N-methyl-Phe-Gly-ol 5)-enkephalin (DAMGO) were from Sigma-Aldrich (St. Louis, MO). Primary antibodies were as follows: rabbit polyclonal to phospho-Ser/Thr-glycogen synthase kinase-3β (pGSK), rabbit monoclonal to phospho-Ser235/236-S6 ribosomal protein (pS6rp), and rabbit polyclonal anti-extracellular signal-regulated kinase 1/2 (ERK1/2) from Cell Signaling Technology Inc. (Danvers, MA); rabbit polyclonal anti-phospho-ERK1/2 (pERK1/2) from Neurons (Northfield, MN); rabbit polyclonal anti-GSK-3β (GSK) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rabbit polyclonal antibody to phospho-Thr308-protein kinase B/Akt (pAkt) from GenWay (San Diego, CA); and rabbit polyclonal anti-actin from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit IgG and prestained protein standards were from Cell Signaling Technology, Inc. Unless otherwise specified, the other reagents were from Sigma-Aldrich.

Cell Culture. CHO-K1 cells (American Type Culture Collection, Manassas, VA) were grown as a monolayer culture in tissue culture flasks that were incubated at 37°C in a humidified atmosphere (5% CO2) in Ham's F-12 medium (Invitrogen, Carlsbad, CA) containing l-glutamine and sodium bicarbonate and supplemented with 10% fetal calf serum (Invitrogen) and 0.5% penicillin/streptomycin. CHO-K1 cells stably expressing the human δ-opioid receptor (CHO/DOR), κ-opioid (CHO/KOR), and μ-opioid receptor-1 (CHO/MOR-1) were generated as described previously (Olianas et al., 2006). CHO cells stably expressing the human muscarinic M1 (CHO/M1) and M4 (CHO/M4) were grown as previously reported (Olianas et al., 1999). CHO/DOR cells were maintained in the presence of 350 μg/ml hygromycin (Invitrogen), whereas for the other recombinant cell lines the growth medium contained 400 μg/ml geneticin (Invitrogen). C6 rat glioma cells (European Collection of Cell Cultures, Porton Down, Wiltshire, UK) were grown in Ham's F-12 medium supplemented with 2 mM l-glutamine, 0.5% penicillin/streptomycin, and 10% fetal calf serum in humidified 95% air and 5% CO2 at 37°C.

Cell Membrane Preparation. Cells were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4; scraped into an ice-cold buffer containing 10 mM HEPES/NaOH, pH 7.4, and 1 mM EDTA; and lysed with a Dounce tissue grinder. The cell lysate was centrifuged at 1000g for 2 min at 4°C. The supernatant was collected and centrifuged at 32,000g for 20 min at 4°C. The pellet was resuspended in homogenization buffer at a protein concentration of 1.0 to 1.5 mg/ml and stored in aliquots at −80°C.

Cell Treatments and Cell Extract Preparation. Cells were serum-starved for 24 h (CHO cells) or 48 h (C6 glioma cells) and then exposed to the test agents for the indicated time periods, washed with ice-cold PBS, and lysed by scraping into PBS containing 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate, 2 mM EDTA, 4 mM EGTA, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 20 mM okadaic acid, 0.1% phosphatase inhibitor mixture 1, and 1% protease inhibitor mixture. The samples were sonicated for 5 s in an ice bath and stored at −80°C. Aliquots of cell extracts were taken for protein determination. Protein content was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Dissection of Brain Regions and Membrane Preparation. Male Sprague-Dawley rats (200–300 g) were used. Animals were maintained in a 12-h light/dark cycle with food and water ad libitum. Experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and with the principles of Laboratory Animal Care in Italy (D.L. 116/92). Rats were killed by decapitation, and the dorsal striatum and nucleus accumbens were rapidly microdissected from 300-μm brain coronal slices as described previously (Olianas et al., 2006). Landmarks used for the dissection of the nucleus accumbens were the anterior commissure, the olfactory and lateral ventricles, the corpus striatum, the septal nuclei, and the olfactory tubercle.

Human frontal cortex tissue was obtained at autopsy from three male subjects (age 35–68 years, mean 56 years) within 26 to 36 h after death. The subjects had no history of neurological or psychiatric disease. The tissue was immediately frozen at −80°C and stored in liquid nitrogen. The specimens were provided by Professor R. Ambu (Department of Cytomorphology, University of Cagliari, Italy). The study was approved by the local ethics committee.

Freshly dissected or frozen tissue samples were homogenized in an ice-cold buffer containing 10 mM HEPES/NaOH, 1 mM EDTA, and 1 mM MgCl2, pH 7.40, using a Teflon-glass tissue grinder (Kontes, Vineland, NJ). The homogenate was centrifuged at 27,000g for 20 min at 4°C. The pellet was resuspended in the same buffer at a protein concentration of 0.8 to 1.0 mg/ml and used immediately for adenyl cyclase assays or stored at −80°C for binding assays.

Assay of [35S]GTPγS Binding. The binding of [35S]GTPγS was assayed in a reaction mixture (final volume 100 μl) containing 25 mM HEPES/NaOH, pH 7.4, 10 mM MgCl2, 1 mM EDTA, 150 mM KCl, 10 kalikrein inhibitor units of aprotinin, and 1.0 mM [35S]GTPγS. The GDP concentration was optimized for each receptor system and was 30 μM for CHO/KOR and CHO/DOR, 10 μM for CHO/MOR-1, and 50 μM for rat and human brain membranes. Membranes (2–4 μg of protein) were preincubated for 20 min at 30°C with the test compounds. For each compound, control samples received an equal volume (10 μl) of vehicle. The reaction was started by the addition of [35S]GTPγS and continued for 40 min at 30°C. The incubation was terminated by the addition of 5 ml of ice-cold buffer
containing 10 mM HEPES/NaOH, pH 7.4, and 1.0 mM MgCl2 immediately followed by rapid filtration on glass fiber filters (GF/C; Whatman International Ltd, Maidstone, UK). The filters were washed twice with 5 ml of buffer, and the radioactivity trapped was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 50 μM GTPγS. Assays were performed in duplicate.

Receptor Binding Assays. Receptor binding assays were carried out by using [3H]diprenorphine to label σ- and μ-opioid receptors and [3H]NTI to label δ-opioid receptors and by incubating the membrane preparations at 30°C for 120 min in a buffer containing 25 mM HEPES/NaOH, pH 7.4, 10 mM MgCl2, 1 mM EDTA, and 150 mM KCl. For saturation binding assays, the concentrations of [3H]diprenorphine and [3H]NTI ranged from 40 pM to 3 nM and from 20 pM to 2 nM, respectively. For competition binding assays, the concentration of either [3H]diprenorphine or [3H]NTI was 0.20 nM. Nonspecific binding was determined in the presence of 10 μM naloxone and corresponded to 4 to 12% and 12 to 30% of total [3H]diprenorphine and [3H]NTI binding, respectively. Triplicate determinations were made for each experiment. Reactions were terminated by filtration through Whatman GF/C filters presoaked with 0.1% polyethylenimine, which were washed three times with 5 ml of ice-cold buffer containing 10 mM HEPES/NaOH, pH 7.4, and 1 mM MgCl2. The radioactivity trapped was determined by liquid scintillation spectrometry.

Assay of [3H]Cyclic AMP Accumulation. CHO cells grown in 36-mm plastic dishes were incubated in Ham’s F-12 medium containing 10 μg/ml [3H]adenine for 1 h at 37°C in an CO2 incubator. Thereafter, the medium was removed, and the cells were incubated in an oxygenated Krebs-Hense buffer containing 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. FSK (10 μM) and the various test compounds were then added, and the incubation was continued for 10 min. Control samples were incubated in the presence of an equal volume of vehicle. The incubation was stopped by the aspiration of the medium and the addition of an ice-cold solution containing 6% (w/v) perchloric acid and 0.1 mM [14C]cyclic AMP (~4000 cpm). After 30 min at ice-bath temperature, the solution was neutralized by the addition of ice-cold 0.6 M KOH and left on ice for an additional 30 min. Centrifugation at 20,000g for 5 min, the supernatant was collected, and [3H]cyclic AMP was isolated by sequential chromatography on Dowex (Bio-Rad Laboratories, Hercules, CA) and alumina columns. The recovery of [3H]cyclic AMP from each sample was corrected based on the recovery of [14C]cyclic AMP.

Assay of Adenyl Cyclase Activity. The adenylyl cyclase activity was assayed in a reaction mixture (final volume 100 μl) containing 50 mM HEPES/NaOH, pH 7.4, 2.3 mM MgCl2, 0.3 mM EGTA, 0.05 mM [α-32P]ATP (150 cpm/μmol), 0.5 mM [3H]cyclic AMP (80 cpm/μmol), 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 100 μM GTP, 50 μg of BSA, 10 μg of bacitracin, and 10 kallikrein inhibitor units of aprotinin. FSK was present at the final concentration of 10 μM. The reaction was started by the addition of the tissue preparation (20–25 μg of protein) and was carried out at 25°C for 20 min. The reaction was stopped by the addition of 200 μl of a solution containing 2% (w/v) SDS, 45 mM ATP, and 1.3 mM cyclic AMP, pH 7.5. Cyclic AMP was isolated by sequential chromatography on Dowex (Bio-Rad Laboratories) and alumina columns. The recovery of [3H]cyclic AMP from each sample was calculated based on the recovery of [3H]cyclic AMP. Assays were carried out in duplicate.

Western Blot Analysis. Aliquots of cell extracts containing equal amount of protein were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Piscataway, NJ). The efficiency of the transfer was controlled by gel staining and by following the transfer of prestained protein standards. Nonspecific binding sites were blocked by incubation in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 7.6, containing 5% BSA for 1 h. After washing with Tris-buffered saline containing Tween 20, the membranes were incubated overnight at 4°C with one of the following primary antibodies: pERK1/2 (1:10,000; ERK1/2 (1:10,000), pGSK-3β (1:1000), GSK (1:1000), pAkt (1:1000), or actin (1:1000). The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000), and immunoreactive bands were detected by using enhanced chemiluminescence (ECL) Plus and ECL Hyperfilm (GE Healthcare). The size of the immunoreactive bands was determined by using molecular weight standards detected with a specific antibody suitable for the ECL system (Santa Cruz Biotechnology, Inc.). Bands densities were determined by densitometric analysis using Image Scanner III (GE Healthcare) and National Institutes of Health ImageJ software (Bethesda, MD). The optical density of phosphoprotein bands was normalized to the density of the corresponding total protein or actin band to yield the relative optical density value.

Assay of ERK1/2 Activity. ERK1/2 activity was measured by a nonradioactive kinase assay kit according to the manufacturer’s instructions (Cell Signaling Technology Inc.). In brief, serum-starved C6 glioma cells were treated with the test drugs for the indicated time periods, washed with ice-cold PBS, and incubated for 5 min with ice-cold cell lysis buffer (25 mM Tris-HCl, pH 7.50, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin). An aliquot of each cell extract was incubated overnight with immobilized antibody against phospho-ERK1/2 with gentle rocking at 4°C. After immunoprecipitation, the pellets were suspended in kinase buffer (25 mM Tris-HCl, pH 7.50, 5 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, and 10 mM MgCl2) supplemented with 200 μM ATP and 40 μg/ml Elk-1 fusion protein as a substrate and incubated for 30 min at 30°C. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using a rabbit polyclonal anti-phospho-Elk-1 (Ser383) (pElk-1) antibody (1:1000). For each sample, an aliquot of the corresponding cell extract was analyzed for total ERK1/2 protein, and the optical density of the pElk-1 band was normalized to the density of the ERK1/2 bands.

Statistical Analysis. Results are reported as mean ± S.E.M. Data from concentration-response curves were analyzed using GraphPad Software Inc. Prism (San Diego, CA), which yielded agonist concentration producing half-maximal effect (EC50 values) and maximal effects (E_max). The percentage of maximal effect (% E_max) by the drugs at 6- and κ-opioid receptors was calculated as E_max of the agonist/EC50 of the reference full agonist (DPDPE or U50,488, respectively) × 100. Saturation binding data were analyzed by the nonlinear curve-fitting program LIGAND, which provided ligand dissociation constant (K_d) and maximal binding capacity (B_max).

Antagonist potencies were analyzed by nonlinear regression analysis. When increasing concentrations of antagonists in the presence of a fixed concentration of agonist were examined, the antagonist inhibitory constant (K_i) was calculated according to the equation: K_i = IC50/1 + (A/EC50), where IC50 is the antagonist concentration producing half-maximal inhibition, A is the agonist concentration, and EC50 is the agonist EC50 value. When the effect of a fixed drug concentration in the presence of increasing agonist concentrations was examined, the K_i value was calculated according to the equation: EC50b = EC50a (1 + I/K_i), where EC50a and EC50b are agonist EC50 values in the absence and in the presence of the antagonist, respectively, and I is the concentration of the antagonist. Statistical analysis was performed by either Student’s t test when comparing two groups or one-way analysis of variance (ANOVA) followed by either Dunnett’s or Newman-Keuls post hoc tests when comparing more than two groups.

Results

Effects of TCAs in CHO/DOR. In CHO/DOR membranes, TCAs induced a complete displacement of [3H]NTI-specific binding (Fig. 1A). The dibenzoazepine amoxapine
the presence of either 10 μM amoxapine (10 μM) increased the $K_i$ of [3H]NTI from 55 ± 5 to 103 ± 9 μM (p < 0.05) without affecting the $B_{max}$ value (1470 ± 50 versus 1430 ± 45 fmol/mg protein) (Fig. 1B). On the other hand, at 10 μM amitriptyline did not significantly affect [3H]NTI saturation binding, whereas at 40 μM it increased the $K_i$ to 94 ± 5 μM (p < 0.05) without affecting the $B_{max}$ (1504 ± 55 fmol/mg protein). In functional assays, TCAs induced a concentration-dependent stimulation of [35S]GTPγS binding (Fig. 1C) with different potencies and efficacies. DPDPE, used as a reference agonist, stimulated [35S]GTPγS binding by 97 ± 4% with an EC50 value of 1.3 ± 0.3 nM. Amoxapine behaved as a full agonist displaying a maximal effect equal to that of the selective δ-opioid agonist DPDPE and a potency in the low micromolar range (Table 1). The other TCAs tested were 23- to 47-fold less potent than amoxapine but, with the exception of desipramine, showed only slightly lower efficacies. Addition of the δ-opioid receptor antagonist NTI (10 nM) completely antagonized the stimulatory effects elicited by the TCAs (Fig. 1D). Each TCA, tested at the same concentration range, failed to significantly stimulate [35S]GTPγS binding in membranes prepared from nontransfected CHO/K1 cells (result not shown). Moreover, each TCA (1–100 μM) failed to affect [35S]GTPγS binding in membranes prepared from either CHO/M4 ($B_{max}$ = 3200 ± 500 fmol/mg protein) or CHO/M4 ($B_{max}$ = 3500 ± 100 fmol/mg protein) cells, which were used as control for G

In intact CHO/DOR cells, amoxapine and amitriptyline inhibited FSK-stimulated cyclic AMP accumulation with EC50 values of 3.4 ± 0.5 and 16.2 ± 0.8 μM, respectively, and maximal effects corresponding to 41.1 ± 3.2 and 43.5 ± 4.5% decrease of control activity (p < 0.001), respectively (Fig. 2A). These effects were equal to approximately 72% of the maximal inhibitory response elicited by DPDPE (EC50 = 0.7 ± 0.2 nM) and were completely antagonized by the addition of 10 nM NTI (Fig. 2B).

Besides regulating cyclic AMP formation, δ-opioid receptors have been shown to modulate other intracellular signaling pathways, such as ERK1/2 phosphorylation cascade and the phosphatidylinositol 3-kinase pathway (Tegeder and Geisslinger, 2004). These pathways are known to regulate cell proliferation, differentiation, and neuronal survival and have been proposed to be molecular targets of antidepressants (Jope and Roh, 2006; Duman et al., 2007). Treatment of CHO/DOR with amoxapine (10 μM) caused a rapid increase of the phosphorylation of ERK1/2, Akt, and GSK-3β, which peaked at 5 min and remained elevated for at least 30 min (Fig. 3, A and B). Consistent with the stimulation of ERK1/2 activity, the drug induced a marked phosphorylation of S6 ribosomal protein (pS6rp), a downstream target of ERK1/2, which reached a plateau at approximately 15 min and lasted for at least 30 min. The stimulatory effects on ERK1/2 and GSK-3β phosphorylations were concentration-dependent with EC50 values of 5.1 ± 0.4 and 4.4 ± 0.6 μM, respectively, [35S]GTPγS. Values are expressed as percentage of control (vehicle + vehicle) and are the mean ± S.E.M. of three experiments. ***, p < 0.001; ns, not significantly different versus control by ANOVA followed by Dunnett’s test.
TABLE 1
Potencies and efficacies of TCAs in stimulating [35S]GTPγS binding in CHO/DOR cells

<table>
<thead>
<tr>
<th></th>
<th>[35S]GTPγS Assay</th>
<th>Cyclic AMP Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵦ µM</td>
<td>EC₅₀ µM</td>
</tr>
<tr>
<td>Amoxapine</td>
<td>2.5 ± 0.5</td>
<td>0.98 ± 0.08</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>28.1 ± 1.2</td>
<td>23.5 ± 1.2</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>30.0 ± 1.0</td>
<td>22.6 ± 2.0</td>
</tr>
<tr>
<td>Desipramine</td>
<td>40.1 ± 2.5</td>
<td>35.5 ± 3.5</td>
</tr>
<tr>
<td>Imipramine</td>
<td>49.8 ± 3.5</td>
<td>46.8 ± 4.0</td>
</tr>
</tbody>
</table>

a Determined by radioligand binding displacement.
b Percentage of maximal stimulation with respect to that elicited by DPDPE. Values are the mean ± S.E.M. of at least three experiments.

Effects of Amoxapine in Rat and Human Brain. In membranes of rat dorsal striatum and nucleus accumbens, amoxapine maximally stimulated [35S]GTPγS binding by 35 ± 3% (p < 0.01) and 28 ± 5% (p < 0.05) increase of control, respectively (Fig. 5A). The use of receptor subtype-selective antagonists indicated that the stimulatory effect of amoxapine (30 μM) was mediated by δ-opioid receptors. In fact, it was antagonized by NTI with a Kᵦ value of 25 ± 5 μM, which is similar to the affinity of the antagonist for δ-opioid receptors. In contrast, the selective κ-opioid receptor antagonist nor-BNI was severalfold less potent than NTI, displaying a Kᵦ of 3.2 ± 0.8 nM (Fig. 5B). This value is approximately 100-fold higher than nor-BNI affinity for the κ-opioid receptor and close to its affinity for δ-opioid receptors (Metcalfe and Coop, 2005). The selective μ-opioid receptor antagonist CTAP failed to cause a significant inhibition up to 1 μM (Fig. 5B), a concentration that was sufficient to completely block the stimulatory effect elicited by the μ-opioid receptor agonist DAMGO (100 nM) (result not shown). None of the antagonists significantly affected [35S]GTPγS binding per se. In rat nucleus accumbens, amoxapine inhibited FSK-stimulated adenylyl cyclase activity (18 ± 0.9% reduction of control activity, p < 0.05, equal to 68% of the DPDPE Eₘₐₓ) with an EC₅₀ of 4.5 ± 0.7 μM (Fig. 5C). This effect was completely blocked by the addition of 3 nM NTI. Finally, in membranes of postmortem human frontal cortex amoxapine stimulated [35S]GTPγS binding by 32 ± 6% (p < 0.05; equal to 48% of DPDPE Eₘₐₓ) with an EC₅₀ value of 3.6 ± 0.8 μM (Fig. 5D). This response also was blocked by the addition of 3 nM NTI (Fig. 5E).

Effects of TCAs in CHO/KOR. In receptor binding assays, TCAs completely displaced specific [3H]diprenorphine binding with a rank order of potencies opposite to that observed at CHO/DOR. In fact, amitriptyline, nortriptyline, desipramine, and imipramine showed Kᵦ values in the low micromolar range (1.8–3.3 μM), whereas amoxapine was much less potent (Fig. 6A; Table 2). Scatchard analysis of [3H]diprenorphine saturation binding data indicated that amitriptyline (10 μM) markedly decreased the affinity of the radioligand (Kᵦ value increased from 101 ± 9 to 340 ± 20 pM; p < 0.05), whereas amoxapine (10 μM) caused only a slight reduction of this parameter (Kᵦ 128 ± 16 pM) (Fig. 6B) and completely antagonized by 10 nM NTI (Fig. 3, C–J). Moreover, amoxapine (10 μM) failed to affect ERK1/2 and GSK-3β phosphorylations in nontransfected CHO-K1 cells (result not shown). The maximal stimulations of ERK1/2 and GSK-3β phosphorylations corresponded to 8.0- and 2.4-fold increase of control values (p < 0.001), respectively, and were equal to 66 and 70% of the maximal stimulations elicited by DPDPE, respectively. At the concentrations tested, amoxapine failed to affect the levels of total ERK1/2 and GSK-3β proteins (Fig. 3, E, G, and I).

Like amoxapine, amitriptyline caused a concentration-dependent stimulation of ERK1/2 and GSK-3β phosphorylation with EC₅₀ values of 10.7 ± 0.9 and 9.0 ± 0.5 μM, respectively, and Eₘₐₓ values corresponding to 550 ± 70 and 90 ± 10% increase of control values (p < 0.001), respectively (Fig. 4). The addition of NTI (10 nM) significantly attenuated the stimulatory effect of amitriptyline on ERK1/2 phosphorylation and completely blocked the induction of GSK-3β phosphorylation elicited by the antidepressant (Fig. 4).

![Figure 2](https://example.com/figure2.png) Amoxapine and amitriptyline inhibit cyclic AMP accumulation in CHO/DOR. A, cells prelabeled with [3H]adenine were incubated with FSK (10 μM) with and without NTI (10 nM) and then exposed to FSK (10 μM) with vehicle, amoxapine (30 μM), or amitriptyline (50 μM) for 15 min. Values are expressed as percentage of control. B, cells were preincubated for 5 min with and without NTI (10 nM) and then exposed to FSK (10 μM) with vehicle, amoxapine (30 μM), or amitriptyline (50 μM) for 15 min. Values are expressed as percentage of control and are the mean ± S.E.M. of three experiments. ***p < 0.001; ns, not significantly different versus control by ANOVA followed by Dunnett’s test.
Amoxapine stimulates ERK1/2 and phosphatidylinositol 3-kinase signaling pathways in CHO/DOR cells. A, time course of amoxapine-induced phosphorylation of ERK1/2 (pERK1/2), pS6 ribosomal protein (pS6rp), Akt (pAkt), and GSK-3β (pGSK). Cells were incubated with amoxapine (10 μM) for the indicated time periods, and phosphorylated proteins were measured in cell extracts by Western blot. Values are reported as percentage of control and are the mean ± S.E.M. of three experiments.

B, concentration-dependent stimulation of ERK1/2 phosphorylation with an EC50 value of 4.5 μM and an E(max) corresponding to 570% increase of control value (Fig. 6C). The TCA E(max) values ranged from 76 to 85% of that displayed by (-)-U50,488 (E(max) = 183 ± 8% increase of control value; EC50 = 0.60 ± 0.08 nM) (Fig. 6C; Table 2). In CHO/DOR cells, amitriptyline, nortriptyline, desipramine, and imipramine inhibited FSK-stimulated cyclic AMP accumulation by 30% at 100 μM (Fig. 6E). Both functional responses elicited by TCAs were completely blocked by nor-BNI (100 nM), which had no effect per se (Fig. 6, D and F).

In CHO/KOR cells, amitriptyline (1–50 μM) induced a concentration-dependent stimulation of ERK1/2 phosphorylation with an EC50 value of 4.5 ± 0.8 μM and an E(max) corresponding to 570 ± 95% increase of control value (p < 0.001) (Fig. 7, A and B). The addition of nor-BNI (100 nM) either vehicle or 10 nM NTI and then exposed to either vehicle or 10 μM amoxapine for additional 5 min. J, densitometric analysis of pGSK normalized to the density of total GSK. Values are reported as percentage of control and are the mean ± S.E.M. of three experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significantly different versus control by ANOVA followed by Dunnett’s test.
effectively antagonized the stimulatory effect elicited by amitriptyline.

**Effects of TCAs in C6 Glioma Cells.** C6 glioma cells are known to endogenously express /H9260-opioid receptors coupled to stimulation of ERK1/2 phosphorylation (Bohn et al., 2000). Moreover, it has been recently reported that in this cell line TCAs induced ERK1/2 activation through a monoamine-in-
dependent mechanism (Hisaoka et al., 2007). Therefore, it was of interest to investigate whether κ-opioid receptors could mediate the actions of TCAs on ERK1/2 signaling in this cell system. As shown in Fig. 7, C and D, a brief exposure of C6 cells to amitriptyline (15 μM) caused a stimulation of ERK1/2 phosphorylation, which corresponded to a 5.7-fold increase of control value \( (p < 0.001) \). The addition of nor-BNI (100 nM) reduced the amitriptyline stimulatory effect by 70% \( (p < 0.001) \). When ERK1/2 activity was measured after immunoprecipitation and determination of Elk-1 phosphorylation, it was found that amitriptyline (15 μM) stimulated the kinase activity by 4.8-fold \( (p < 0.001) \) (Fig. 7E and F) and that this response was reduced by approximately 50% after pretreatment with 100 nM nor-BNI \( (p < 0.01) \).

**Effects of Amitriptyline in Rat Nucleus Accumbens.** In membranes of rat nucleus accumbens, amitriptyline caused a slight but significant inhibition of PKA-stimulated cyclic AMP formation with an EC\(_{50}\) value of 6.2 ± 0.8 μM and an \( E_{\text{max}} \) corresponding to 12 ± 3% decrease of control activity \( (p < 0.05) \) (Fig. 8A). The inhibitory effect was completely antagonized by 10 nM nor-BNI (result not shown). When combined with \( \langle - \rangle \)U50,488 (300 nM), amitriptyline caused a concentration-dependent reduction of the inhibitory effect elicited by the full κ-opioid agonist, bringing the enzyme activity to the level determined by its maximal effect (Fig. 8A). In the same membrane preparations, \( \langle - \rangle \)U50,488 maximally inhibited FSK-stimulated cyclic AMP formation by 25 ± 4% with an EC\(_{50}\) of 57 ± 3 nM. In the presence of 30 μM amitriptyline, the \( \langle - \rangle \)U50,488 concentration-response curve was shifted to the right by 7.9-fold, yielding a \( K_i \) value of 4.3 ± 0.6 μM (Fig. 8B).

**Effects of TCAs in CHO/MOR.** All the TCAs tested completely displaced specific \( ^{3} \text{H} \)diprenorphine bound to μ-opioid receptors with relatively low potencies (Fig. 9A). The estimated \( K_i \) values were amitriptyline, 30 ± 2 μM; nortriptyline, 22 ± 3 μM; desipramine, 23 ± 4 μM; imipramine, 25 ± 3 μM; and amoxapine, 44 ± 5 μM. Scatchard analysis of \( ^{3} \text{H} \)diprenorphine saturation binding data indicated that, at the concentration of 30 μM, both amitriptyline and amoxapine decreased the affinity of the radioligand \( (K_a \) values were control, 118 ± 10 μM; amitriptyline, 370 ± 18 μM, \( p < 0.05 \); amoxapine, 275 ± 15 μM, \( p < 0.05 \)) without significantly changing the \( B_{\text{max}} \) (control, 600 ± 30 fmol/mg protein; amitriptyline, 620 ± 35 fmol/mg protein; amoxapine, 585 ± 29 fmol/mg protein) (Fig. 9B). In \( ^{35} \text{S} \)GTP\(_{\text{S}}\) binding assays, the TCAs caused a small and not significant stimulatory effect, which occurred at concentrations greater than 10 μM (Fig. 9C). Under the same conditions, the selective μ-opioid receptor agonist DAMGO increased \( ^{35} \text{S} \)GTP\(_{\text{S}}\) binding by 180 ± 12% \( (p < 0.001) \) with an EC\(_{50}\) value of 29 ± 5 nM. When examined in the presence of 30 nM DAMGO, the TCAs displayed a weak antagonistic activity with the following \( K_i \) values: amitriptyline, 36 ± 4 μM; nortriptyline, 25 ± 3 μM;
Discussion

In the present study, we show for the first time that widely used TCAs, such as amoxapine, amitriptyline, nortriptyline, desipramine, and imipramine, exert direct agonist activity at distinct opioid receptor subtypes. In CHO cells individually expressing the human receptors these drugs display differential affinity for $\delta$-, $\kappa$-, and $\mu$-opioid receptors. Thus, amoxapine, an antidepressant with rapid onset of therapeutic efficacy and atypical antipsychotic properties, preferentially bound to the $\delta$-opioid receptor, whereas amitriptyline, nortriptyline, imipramine, and desipramine showed higher selectivity for the $\kappa$-opioid receptor. All the TCAs examined had relatively low affinity for the $\mu$-opioid receptor. At each opioid receptor subtype, the TCAs completely displaced the specific binding of the radioligands and decreased the affinities of the radioligands without significantly affecting the $B_{\text{max}}$ values. These data indicated that TCAs interacted with the opioid receptors as competitive ligands.

In agreement with the radioligand binding data, TCAs showed differential agonist activity at the distinct opioid receptor subtypes. In fact, the drugs stimulated $\delta$- and $\kappa$-opioid receptors without significant agonist effects at $\mu$-opioid receptor. Moreover, important differences were observed in desipramine, $27 \pm 4 \, \mu M$; imipramine, $26 \pm 5 \, \mu M$; and amoxapine, $74 \pm 7 \, \mu M$ (Fig. 9D).

Fig. 8. Amitriptyline inhibits FSK-stimulated adenyl cyclase activity and antagonizes $(-)$-U50,488 inhibitory effect in rat nucleus accumbens. A, the enzyme activity stimulated by $10 \, \mu M$ FSK was assayed at the indicated concentrations of amitriptyline in the presence of either vehicle or $300 \, nM \, (-)$-U50,488. Values are expressed as percentage of control value (vehicle + vehicle) and are the mean $\pm$ S.E.M. of four experiments. B, antagonism of $(-)$-U50,488 induced inhibition of FSK-stimulated adenyl cyclase activity by amitriptyline. The enzyme activity was assayed at the indicated concentrations of $(-)$-U50,488 in the presence of either vehicle or $30 \, \mu M$ amitriptyline. Values are expressed as percentage of control activity (vehicle + vehicle) and are the mean $\pm$ S.E.M. of three experiments.

Fig. 9. Interaction of TCAs with $\mu$-opioid receptors. A, displacement of specific $[^{3}H]$diprenorphine binding in CHO/MOR-1 cell membranes by TCAs. The concentration of the radioligand was $0.2 \, nM$. Values are the mean $\pm$ S.E.M. of three experiments for each drug. B, Scatchard plot of $[^{3}H]$diprenorphine saturation binding carried out in the absence (control) and in the presence of either $30 \, \mu M$ amitriptyline or $30 \, \mu M$ amoxapine. Values are the mean of three experiments. C, effects of TCAs on $[^{35}S]$GTP$\gamma$S binding in CHO/MOR-1 cell membranes. The stimulatory effect of the full $\mu$-opioid agonist DAMGO is also reported. Values are the mean $\pm$ S.E.M. of three experiments for each drug. D, effects of TCAs on DAMGO-stimulated $[^{35}S]$GTP$\gamma$S binding in CHO/MOR-1 cell membranes. Membranes were preincubated with the indicated concentrations of the TCAs in the absence and in the presence of $30 \, nM$ DAMGO and then assayed for $[^{35}S]$GTP$\gamma$S binding. Values are expressed as percentage of the net DAMGO stimulatory effect at each drug concentration and are the mean $\pm$ S.E.M. of three experiments.
the drug potencies and efficacies at δ- and κ-opioid receptors. Thus, amoxapine was the most potent and efficacious δ-opioid receptor agonist but a weak stimulant of κ-opioid receptors. In contrast, classic TCAs displayed higher potencies at κ- than δ-opioid receptors. In line with the present study, Isenberg and Cicero (1984) reported that in rat brain membranes amoxapine inhibited the binding of the δ-opioid receptor agonist [3H][D-Ala²,D-Leu⁵]enkephalin 5-fold more potently than that of the nonselective opioid receptor ligand [3H]naltrexone. The preferential activity of classic TCAs at κ-opioid receptors correlates with previous radioligand binding studies reporting a higher selectivity of these drugs for κ- over µ- and δ-opioid binding sites in the human thalamic area (Wahlström et al., 1994).

The direct agonist activity of amoxapine was characterized at both cloned and native δ-opioid receptors. In CHO/DOR cells, the drug mimicked the responses to DPDPE in different functional assays, including stimulation of [35S]GTPγS binding, inhibition of cyclic AMP accumulation, activation of the ERK1/2 pathway, and induction of Akt and GSK-3β phosphorylations. In addition, amoxapine was capable of activating δ-opioid receptors endogenously present in rat dorsal striatum and nucleus accumbens and in human frontal cortex, indicating that the agonist activity was not limited to heterologous cell expression systems. Although in CHO/DOR cells amoxapine stimulated [35S]GTPγS binding almost as efficaciously as DPDPE, in all the other functional assays it consistently showed Emax values lower than those of the full agonist, indicating a partial agonist activity at the δ-opioid receptor. Likewise, the maximal responses elicited by classic TCAs at either κ- or δ-opioid receptors were significantly lower than those of the corresponding full agonist, suggesting that these drugs also act as partial agonists.

Because TCAs are known to interact with different neurotransmitter receptor systems and to affect several cellular events, it was important to assess the specificity of their agonist activity. In CHO/DOR cells and rat and human brain, the amoxapine responses were completely blocked by low nanomolar concentrations of NTI, indicating the involvement of δ-opioid receptors. With regard to the classic TCAs, in CHO/DOR cells the stimulation of [35S]GTPγS binding was totally blocked by NTI, and in CHO/KOR cells both stimulation of [35S]GTPγS binding and inhibition of cyclic AMP accumulation were completely antagonized by nor-BNI, indicating that the effects were specifically mediated by either δ- or κ-opioid receptors, respectively. The stimulations of ERK1/2 phosphorylation by amitriptyline observed in CHO/DOR, CHO/KOR, and C6 glioma cells were largely reduced by the opioid receptor antagonists but not completely antagonized, particularly at concentrations of the antidepressant higher than 10 to 15 μM. This indicates that amitriptyline could also induce ERK1/2 phosphorylation through an opioid receptor-independent mechanism. The molecular events mediating the latter effect are still unknown and require further investigation.

TCAs are known to accumulate in the brain where they reach concentrations severalfold higher than those in the blood. The plasma concentrations of the TCAs tested range from 0.2 to 1.6 μM (Baldessarini, 2006), whereas their brain-to-plasma ratios have been reported to be 10 to 35:1 (Glotzbach and Preskorn, 1982). In rats, the administration of 10 to 15 mg/kg desipramine or 20 mg/kg amoxapine produced brain concentrations equal to 20 to 40 μM and 13 μM, respectively (Biegon and Samuel, 1980; Kobayashi et al., 1992). Thus, the observed potencies of TCAs as δ- and κ-opioid receptor agonists are consistent with the brain concentrations reached by these drugs at clinically relevant doses.

The present demonstration that TCAs directly stimulate δ- and κ-opioid receptors raises the possibility that this property may contribute to their analgesic activity. Acute and chronic administrations of TCAs have been shown to display antinoceptive properties in various acute experimental pain models both in animals and humans, and in several of these tests a sensitivity to blockade by naltrexone has been observed (Micó et al., 2006). Recent studies have highlighted the participation of distinct opioid receptor subtypes. Thus, in the acetic acid-induced abdominal constriction test, the antinociceptive properties of chronic nortriptyline treatment was absent in mice deficient of δ-opioid receptors (Benbouzid et al., 2008b), and both δ- and κ-opioid receptor antagonists blocked the attenuation of neuropathic allodynia elicited by chronic TCA treatment (Benbouzid et al., 2008a). Moreover, supraspinal δ- and spinal μ-opioid receptors have been proposed to be involved in the antihyperalgesic effect of chronically administered clomipramine in a mononeuropathic pain model in rats (Marchand et al., 2003). Although it is possible that these effects may result from indirect actions of TCAs on the opioidergic system (i.e., enhanced release of endogenous opioid peptides), the present data support the idea that at least a portion of the TCA analgesic effect may involve a direct agonist activity at δ- and κ-opioid receptors. This direct mechanism may operate in conjunction with the primary action of these drugs on noradrenergic and serotoninergic systems (Micó et al., 2006).

It is also possible that the opioid agonist activity of TCAs, particularly at δ-opioid receptors, may contribute to their mood-elevating effects. Genetic and pharmacological studies have shown that δ-opioid receptors exert antidepressant activity (Filliol et al., 2000; Jutkiewicz, 2006). Cellular studies have reported that activation of δ-opioid receptors induces mitogenic responses (Wilson et al., 1997) and promotes neuroprotection and neurogenesis (Narita et al., 2006), which seem to be critical for the action of antidepressants (Duman et al., 2000). At the molecular level, these neurotrophic effects occur through recruitment of downstream intracellular molecules and phosphorylation events, particularly those associated with ERK1/2 and Akt/GSK-3β signaling pathways (Tegeder and Geisslinger, 2004), which, as shown in the present study, can be regulated by TCAs via activation of δ- and κ-opioid receptors. Moreover, both ERK1/2 and GSK-3β have been linked to the action of antidepressants (Jope and Roh, 2006; Duman et al., 2007). In C6 glioma cells, amitriptyline and other TCAs have been recently reported to regulate the production of glial cell line-derived neurotrophic factor, a stimulant of adult neurogenesis, by activating ERK1/2 (Hisaoka et al., 2007). Because the present study shows that in these cells amitriptyline-induced ERK1/2 stimulation is largely mediated by stimulation of κ-opioid receptors, it is possible that this receptor system also participates in TCA regulation of the synthesis of glial cell line-derived neurotrophic factor and other neurotrophic factors.
The agonist activity at δ- and κ-opioid receptors may also be involved in some TCA adverse effects. For example, the administration of nonpeptidic δ-opioid agonists is known to cause seizure in laboratory animals (Jutkiewicz, 2006), whereas activation of κ-opioid receptors may exert anticonvulsant activity (Loacker et al., 2007). In this context, the higher potency and efficacy of amoxapine at δ- versus κ-opioid receptors may correlate with the observed greater risk of seizure associated with amoxapine overdose, compared with other antidepressants (Litovitz and Troutman, 1983). TCAs are also known to induce a dysphoric-agitated state in some patients (Balldessarini, 2006). In animal models, the administration of κ-opioid receptor agonists has been reported to lower mood and exert prodepressive effects, whereas antagonists produce anxiolytic and antidepressant actions (Magee et al., 2003). Moreover, distinct stress-induced behavioral responses have been shown to be antagonized by κ-opioid receptor blockers or deletion of the prodynorphin gene, suggesting that dynorphin stimulation of κ-opioid receptors may mediate stress-induced dysphoria (McLaughlin et al., 2003).

Thus, the κ-opioid agonist activity may be related to TCA-induced mood worsening and anxiogenic reactions. However, there is also evidence that activation of κ-opioid receptors produces anxiolytic-like behavior (Wall and Mesnier, 2000). Moreover, the present study shows that in rat nucleus accumbens amitriptyline behaves as partial agonist at κ-opioid receptors and antagonizes the action of the full agonist (−)-U50,488. Thus, it is possible that in stress-related conditions, where an intense release of dynorphin can overstimulate κ-opioid receptors, the partial agonist activity of TCAs may reduce the level of receptor activation, thus attenuating, rather than enhancing, the aversive emotional consequences of increased κ-opioid transmission.

References
Wahlstrom A, Lenhammar L, Ask B, and Rane A (1994) Tricyclic antidepressants inhibit opioid receptor binding in human brain and hepatic morphine glucuronida-

Address correspondence to: Pierluigi Onali, Department of Neuroscience, Section of Biochemical Pharmacology, University of Cagliari, Cittadella Universitaria di Monserrato, 09042 Monserrato (CA), Italy. E-mail: onali@unic.it