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N-Desmethylclozapine, a Major Clozapine Metabolite, Acts as a Selective and Efficacious δ-Opioid Agonist at Recombinant and Native Receptors

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The present study examined the effects of *N*-desmethylclozapine (NDMC), a biologically active metabolite of the atypical antipsychotic clozapine, at cloned human opioid receptors stably expressed in Chinese hamster ovary (CHO) cells and at native opioid receptors present in NG108-15 cells and rat brain. In CHO cells expressing the δ-opioid receptor (CHO/DOR), NDMC behaved as a full agonist both in stimulating [^{35}S]GTPγS binding ($pEC_{50} = 7.24$) and in inhibiting cyclic AMP formation ($pEC_{50} = 6.40$). NDMC inhibited [^{3}H]naltrindole binding to CHO/DOR membranes with competition curves that were modulated by guanine nucleotides in an agonist-like manner. Determination of intrinsic efficacies by taking into consideration both the maximal [^{35}S]GTPγS binding stimulation and the extent of receptor occupancy at which half-maximal effect occurred indicated that NDMC had an efficacy value equal to 82% of that of the full δ-opioid receptor agonist DPDPE, whereas clozapine and the other clozapine metabolite clozapine *N*-oxide displayed much lower levels of agonist efficacy. NDMC exhibited poor agonist activity and lower affinity at the κ-opioid receptor and was inactive at μ-opioid and NOP receptors. In NG108-15 cells, NDMC inhibited cyclic AMP formation and stimulated the phosphorylation of extracellular signal-regulated kinase 1/2 by activating the endogenously expressed δ-opioid receptor. Moreover, in membranes of different brain regions, NDMC stimulated [^{35}S]GTPγS binding and regulated adenylyl cyclase activity and the effects were potently antagonized by naltrindole. These data demonstrate for the first time that NDMC acts as a selective and efficacious δ-opioid receptor agonist and suggest that this unique property may contribute, at least in part, to the clinical actions of the atypical antipsychotic clozapine. *Neuropsychopharmacology* (2007) **32,** 773–785. doi:10.1038/sj.npp.1301152; published online 12 July 2006

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INTRODUCTION

N-desmethylclozapine (NDMC) and clozapine *N*-oxide (CNOX) are two major metabolites of the atypical antipsychotic clozapine, which lacks extrapyramidal side effects, displays high efficacy in refractory schizophrenia, improves negative symptoms, and reduces the risk of suicide in schizophrenia (Meltzer, 2002; Meltzer *et al*, 2003). Whereas CNOX can be rapidly re-converted to clozapine, NDMC reaches considerable plasma concentrations ranging from 49 to 140% of those of the parent compound in schizophrenic patients (Centorrino *et al*, 1994; Olesen *et al*, 1995; Aravagiri and Marder, 2001; Frazier *et al*, 2003) and from 50 to 220% in the rat (Baldessarini *et al*, 1993; Weigmann *et al*, 1999). In the rat brain, concentrations of NDMC higher than those in serum have been detected following clozapine administration, indicating that the metabolite can cross the blood–brain barrier (Weigmann *et al*, 1999). Moreover, a higher ratio of NDMC to clozapine in plasma was found to correlate positively with clinical improvements, indicating that NDMC may contribute to the clinical efficacy of clozapine (Weiner *et al*, 2004).

Pharmacological studies have shown that NDMC shares the clozapine ability to bind to a large array of neurotransmitter receptor systems. Thus, NDMC was found to display high affinity for serotonin $5HT_{2C}$ and $5HT_{2A}$ receptors, to potently block 5-HT_{2C} receptor-stimulated phosphoinositide hydrolysis in rat choroid plexus, and to bind to striatal dopamine (DA) D_1 and D_2 receptors (Kuoppamaki *et al*, 1993). Following *in vivo* administration, NDMC increased the expression of the immediate-early gene c-fos in rat brain with a regional specificity similar to that displayed by clozapine (Young *et al*, 1998).

An important feature of clozapine is its ability to bind with high affinity to acetylcholine (ACh) muscarinic receptors and to act as a mixed agonist/antagonist (Zorn *et al*, 1994; Zeng *et al*, 1997; Olianas *et al*, 1997, 1999; Michal

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et al, 1999). A similar property has recently been demonstrated for NDMC. Thus, in cell lines expressing the cloned human muscarinic receptor subtypes NDMC has been found to act as a partial agonist with potencies and efficacies higher than those of the parent drug (Sur *et al*, 2003; Weiner *et al*, 2004; Davies *et al*, 2005). In CA1 hippocampal neurons, NDMC was found to potentiate NMDA-mediated currents (Sur *et al*, 2003) and to induce phosphorylation of extracellular signal-regulated kinases (ERK) by activating muscarinic M_1 receptors (Weiner *et al*, 2004). This receptor has also been shown to mediate the increases in cortical ACh and DA release observed following systemic administration of NDMC in freely moving rats (Li *et al*, 2005). Besides stimulating muscarinic receptors, NDMC has been recently shown to act as a partial agonist in transfected cell lines overexpressing the human DA D_2 or D_3 receptor and the G protein Go, whereas clozapine behaved as an inverse agonist (Burstein *et al*, 2005).

Although the precise molecular mechanisms mediating the atypical antipsychotic profile of clozapine are still unknown, it is likely that both antagonist and agonist actions of the drug and its metabolite(s) at multiple neurotransmitter receptor systems may be involved (Roth *et al*, 2004). As NDMC is less characterized than its parent compound, the understanding of the role of NDMC in the therapeutic activity of clozapine requires the definition of its whole spectrum of pharmacological activities and molecular targets.

Previous studies have investigated the opioid system as one of the sites of action of clozapine in the brain. Chronic administration of clozapine to rats and mice increased proenkephalin mRNA expression in striatum and nucleus accumbens, dynorphin levels in *substantia nigra* (Angulo *et al*, 1990), and the density of brain μ- and δ-opioid receptors (Zang *et al*, 1995). In mice, the acute injection of clozapine was found to produce analgesia using the tail-flick test and this effect was antagonized by naloxone and selective μ- and κ-opioid receptor antagonists (Schreiber *et al*, 1999). Studies *in vitro* have shown that clozapine inhibited [^{3}H]Met-enkephalin binding to synaptosome-enriched fractions of rat whole brain and hippocampus with a micromolar potency (Somoza *et al*, 1981). In *Xenopus* oocytes co-injected with either the δ- or κ-opioid receptor mRNA and the G protein-activated inwardly rectifying potassium channel, Kobayashi *et al* (1998) reported that clozapine displayed both δ- and κ- receptor agonist activity and suggested that this effect may contribute to the antinociceptive and proconvulsant effect of the drug. Although early studies suggested possible links between opioids and schizophrenia (Pickar *et al*, 1982; Schmauss and Emrich, 1985; Brizer *et al*, 1985; Schmauss *et al*, 1987; Gulya, 1990), the involvement of the opioid system in the therapeutic actions of clozapine has been generally overlooked, mostly because of the low affinity of clozapine for the opioid receptors.

In the present study we have examined the interaction of NDMC with each of the cloned human opioid receptors individually expressed in Chinese hamster ovary (CHO) cells and evaluated its receptor activity by using different functional assays. Moreover, we investigated the actions of NDMC at opioid receptors naturally expressed in NG 108-15 cells and rat brain. In each receptor system, the effects of clozapine and CNOX were also analyzed.

Part of this study has previously been presented in an abstract form (Onali and Olianas, 2004a).

MATERIALS AND METHODS

Materials

[α-^{32}P]ATP (30–40 Ci/mmol), [2,8-^{3}H]cyclic AMP (25 Ci/mmol), [8-^{14}C]cyclic AMP (45.1 mCi/mmol), [2,8-^{3}H]adenine (28.8 Ci/mmol), [^{35}S]GTPγS (1306 Ci/mmol), [5′,7′-^{3}H]naltrindole ([^{3}H]NTI) (20 Ci/mmol) and [15,16-^{3}H]diprenorphine (53 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA, USA). FSK and GTPγS were from Calbiochem (San Diego, CA, USA) and Boehringer (Mannheim, Germany), respectively. Nociceptin/orphanin FQ (N/OFQ) was purchased from Neosystem (Strasbourg, France). TIPP was obtained from Bachem AG (Bubendorf, Switzerland). NDMC, (−)-U-50,488 hydrochloride (trans-(−)3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide hydrochloride), nor-binaltorphimine dihydrochloride (nor-BNI) and CTAP were from Tocris Cookson Ltd (Avonmouth, UK). DPDPE ((2-D-penicillamine, 5-D-penicillamine)-enkephalin) was purchased from Bachem AG (Bubendorf, Switzerland). Naloxone hydrochloride was from Salars (Como, Italy). Clozapine, CNOX, NTI, DAMGO ((D-Ala^2-*N*-methyl-Phe-Gly-ol^5)-enkephalin), and the other reagents were from Sigma RBI (St Louis, MO, USA).

Cell Culture

CHO-K1 cells (American Type Culture Collection) stably expressing the human δ-opioid receptor (CHO/DOR) (2250 fmol/mg protein), μ-opioid receptor-1 (CHO/MOR-1) (350 fmol/mg protein), κ-opioid receptor (CHO/KOR) (2800 fmol/mg protein), and nociceptin/orphanin FQ receptor (CHO/NOP) (400 fmol/mg protein) were generated by PolyFect (Qiagen) transfection with cDNAs cloned into pcDNA3.1 + vector (Invitrogen). The cDNAs were obtained from either UMR cDNA Resource Center (Rolla, MO, USA) or Top Gene Tec. (Montreal, Canada). Cells were grown as a monolayer culture in tissue culture flasks (Falcon) that were incubated at 37°C in a humidified atmosphere (5% CO_2) in Ham's F12 medium (GIBCO BRL) containing L-glutamine and sodium bicarbonate and supplemented with 10% heat inactivated foetal calf serum (GIBCO BRL), 0.5% penicillin/streptomycin (GIBCO BRL) and either 350 μg/ml hygromycin (GIBCO BRL) for CHO/DOR or 400 μg/ml geneticin (GIBCO BRL) for the other recombinant cell lines.

NG108-15 neuroblastoma × glioma hybrid cells (European Collection of Cell Cultures) were grown in DMEM supplemented with 2 mM L-glutamine, 0.1 mM hypoxanthine, 16 μM thymidine, 0.4 μM aminopterin, 0.5% penicillin/streptomycin, and 10% heat-inactivated foetal calf serum in a humidified 95% air and 5% CO_2 at 37°C.

Cell Membrane Preparation

Cells were grown in 100 mm plastic Petri dishes (Falcon), the culture medium was removed and the cells were washed

with ice-cold phosphate-buffered saline (PBS) (pH 7.4). Thereafter, the cells were 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 1000*g* for 2 min at 4°C. The supernatant was collected and centrifuged at 32 000*g* for 20 min at 4°C. The pellet was resuspended in the homogenization buffer at a protein concentration of 1.0–1.5 mg/ml and stored in aliquots at −80°C.

Dissection of Rat 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 according to the principles of laboratory animal care (Law on animal experiments in Italy, DL 116/92). Rats were killed by decapitation and the brain areas of interest were rapidly dissected as previously described (Onali and Olianas, 2002, 2004b). The frontal lobes and the tissue samples from individual slices were homogenized in an ice-cold buffer containing 10 mM HEPES-NaOH, 1 mM EGTA, 1 mM $MgCl_2$ (pH 7.40) using a Teflon-glass tissue grinder. The homogenate was centrifuged at 27 000*g* for 20 min at 4°C. The pellet was resuspended in the same buffer at a protein concentration of 0.8–1.0 mg/ml and used immediately for adenylyl cyclase assays or stored at −80°C for binding assays. For each experiment, a fresh tissue preparation was used.

Assay of [^{35}S]GTPγS Binding

Cell and tissue membranes were diluted 10-fold in an ice-cold buffer containing 10 mM HEPES/NaOH and 1 mM EDTA (pH 7.4), centrifuged at 32 500*g* for 30 min at 4°C and resuspended in the same buffer containing 0.1% bovine serum albumin (BSA). The binding of [^{35}S]GTPγS was assayed in a reaction mixture (final volume 100 μl) containing 25 mM HEPES/NaOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 10 kallikrein inhibitor units (KIU) of aprotinin, 10 μM leupeptin, 10 μM bestatin, 1.0 nM [^{35}S]GTPγS. GDP was added at the concentration of 5 μM for CHO/NOP, 10 μM for CHO/MOR-1, 30 μM for CHO/KOR and CHO/DOR, and 50 μM for rat 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 to determine the basal values. The reaction was started by the addition of [^{35}S]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 $MgCl_2$, immediately followed by rapid filtration on glass fiber filters (Whatman GF/C) presoaked in the same buffer. 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.

Assay of [^{3}H]Cyclic AMP Accumulation

CHO and NG108-15 cells grown in 36-mm plastic dishes were incubated in Ham's F-12 and DMEM, respectively, containing 10 μCi/ml of [^{3}H]adenine for 1 h at 37°C in an incubator. Thereafter, the medium was removed, and the cells were incubated in an oxygenated Krebs–HEPES uffer containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min at 37°C. Forskolin (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 [^{14}C]cyclic AMP (∼4000 c.p.m.). 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 additional 30 min. Following centrifugation at 20 000*g* for 5 min, the supernatant was collected and [^{3}H]cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns. The recovery of [^{3}H]cyclic AMP from each sample was corrected on the basis of the recovery of [^{14}C]cyclic AMP.

Assay of Adenylyl Cyclase

The adenylyl cyclase activities of dorsal striatum, nucleus accumbens, and frontal cortex were assayed in a reaction mixture (final volume 100 μl) containing 50 mM HEPES/NaOH (pH 7.4), 2.3 mM $MgCl_2$, 0.3 mM EGTA, 0.05 mM [α-^{32}P]ATP (150 c.p.m./pmol), 0.5 mM [^{3}H]cyclic AMP (80 c.p.m./nmol), 1 mM IBMX, 5 mM phosphocreatine, 50 U/ml of creatine phosphokinase, 100 μM GTP, 50 μg of BSA, 10 μg of bacitracin, and 10 KIU of aprotinin. FSK was present at the final concentration of 10 μM. The reaction was started by the addition of the tissue preparation (30–35 μg of protein) and was carried out at 25°C for 20 min. When the basal enzyme activity of the granule cell layer of rat olfactory bulb was measured, the [α-^{32}P]ATP concentration was 0.2 mM, FSK was omitted and the incubation was carried out at 30°C for 10 min. The reaction was stopped by the addition of 200 μl of a solution containing 2% (w/v) sodium dodecyl sulfate (SDS), 45 mM ATP, 1.3 mM cyclic AMP (pH 7.5). Cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns. The recovery of [^{32}P]cyclic AMP from each sample was calculated on the basis of the recovery of [^{3}H]cyclic AMP. Assays were carried out in duplicate.

Receptor Binding Assays

Radioligands used for receptor binding assays were [^{3}H]NTI in the experiments using CHO/DOR cell membranes and [^{3}H]diprenorphine in the experiments using CHO/MOR-1 and CHO/KOR cell membranes. For [^{3}H]NTI competition binding assays, CHO/DOR cell membranes (40–50 μg of protein) were incubated at 30°C for 60 min in a medium containing 25 mM HEPES/NaOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 150 mM KCl, 10 KIU of aprotinin, 10 μM leupeptin, 10 μM bestatin, and the indicated concentrations of the test compounds. When the effects of guanine nucleotides were examined, competition binding assays

were performed in the presence of 1.0 nM GTPγS and 30 μM GDP to keep the conditions similar to those used in the [^{35}S]GTPγS binding assays. The concentration of [^{3}H]NTI was 0.16 nM and the assay volume was 2.0 ml. Competition binding experiments in CHO/MOR-1 (70–80 μg of protein) or CHO/KOR (15–20 μg of protein) cell membranes were performed using 0.2 nM [^{3}H]diprenorphine under the experimental conditions indicated above with the exception that the assay volume was 1.0 ml. For saturation binding assays, membrane preparations of CHO/DOR (30 μg of protein), CHO/MOR-1 (60 μg of protein) and CHO/KOR (15 μg of protein) were incubated at 30°C for 120 min in the presence of either [^{3}H]NTI (from 8 pM to 1.5 nM) or [^{3}H]diprenorphine (from 20 pM to 3.0 nM). Nonspecific binding was determined in the presence of 10 μM naloxone. For each compound, control samples received an equal volume of vehicle. Reactions were terminated by the addition of 5 ml of ice-cold buffer containing 10 mM HEPES/NaOH (pH 7.4) and 1 mM $MgCl_2$ immediately followed by rapid filtration through glass fiber filters (Whatman GF/C) presoaked with 0.1% polyethylenimine. The filters were washed twice with 5 ml of buffer and the radioactivity trapped was determined by liquid scintillation spectrometry. Assays were performed in triplicate.

Indirect Immunofluorescence Analysis

Cells were grown in tissue culture chamber slides (Nunc Lab-Tek II treated with CC2) and serum starved for 12 h. Following the addition of fresh serum-free medium, the cells were treated with the test compounds and placed in the incubator for 20 min at 37°C. Thereafter, the medium was removed and the cells were rapidly washed with PBS. Following fixation with 4% paraformaldehyde in PBS for 10 min, the cells were treated with 0.5% Triton X-100 in PBS for 7 min, and blocked with 10% normal goat serum (NGS) in PBS for 20 min. The cells were then washed and incubated with a rabbit anti phospho-ERK1/2 affinity purified antibody (1 : 1000 dilution in PBS containing 0.25% Triton X-100 and 1.5% NGS; Neuromics, Northfield, MN) overnight at 4°C. After washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1 μg/ml in PBS containing 1.5% NGS; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 45 min at room temperature. After a final washing step, the glass slides were covered with coverslips in Gel Mount aqueous mounting medium (Sigma). The cells were analyzed with a Olympus IX51 microscope and images were captured with a Olympus digital camera. Negative controls were incubated with FITC-conjugated secondary antibody only. The percent of immunolabeled cells was determined by examining at least four fields in each sample and calculated by counting the total number of cells present in each optic field, which ranged from 80 to 100. Cell counting was performed by an investigator unaware of the drug treatment protocol.

Western Blot Analysis

For the assay of ERK1/2 phosphorylation in cell extracts, serum-starved NG-108-15 cells grown in 36 mm Petri dishes were treated with the test compounds as indicated above and cell extracts were prepared by scraping the cells in ice-cold PBS containing 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 0.1% phosphatase inhibitor cocktail 1 (Sigma), and a protease inhibitor cocktail (100 KIU/ml of aprotinin, 10 μg/ml of leupeptin, 10 μg/ml of soybean trypsin inhibitor, 3 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride). The samples were sonicated for 5 s in ice-bath and aliquots of the cell extracts containing equal amount of cell proteins were mixed with a 5 × solution of sample buffer (300 mM Tris-HCl, 10% SDS, 40% glycerol, 10% β-mercaptoethanol, and 0.008% bromophenol blue, pH 6.8), heated at 100°C for 5 min, and subjected to SDS-polyacrilamide gel electrophoresis. The proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp.), according to the method of Towbin *et al* (1979). The efficiency of the transfer was controlled by staining the gel with Coomassie Brilliant Blue and by following the transfer of prestained protein standards (Santa Cruz Biotechnology Inc.). Nonspecific binding sites were blocked by incubation in 20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween-20 (pH 7.6) (TBS-T buffer) containing 5% BSA for 1 h at room temperature. After washing with TBS-T buffer, the membranes were incubated overnight at 4°C with the anti-phospho-ERK1/2 antibody (1 : 1000 in TBS-T containing 0.1% BSA). For assurance that equivalent amount of total ERK was loaded in each lane, the blots were incubated with an anti-ERK1 affinity purified antibody (1 : 1000) (Neuromics). The membranes were then washed with TBS-T and incubated with a horseradish peroxidase-conjugated second antibody (1 : 10 000 Santa Cruz Biotechnology Inc.) for 45 min at room temperature. Immunoreactive bands were detected by using an enhanced chemiluminescence system (ECL Plus) and ECL Hyperfilm (Amersham). In each experiment, 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.). Band densities were determined by densitometric analysis using the NIH ImageJ software. In each experiment, the optical density of the phospho-ERK1/2 bands was normalized to the density of the corresponding ERK1 internal control.

Protein content was determined by the method of Bradford (1976) using BSA as a standard.

Statistical Analysis

Results are reported as mean ± standard error of the mean (SEM). Data from concentration–response curves were analyzed by the program Graph Pad Prism (San Diego, CA, USA.), which yielded agonist concentration producing half-maximal effect (EC_{50} values) and maximal effects (E_{max}). For statistical analysis, the EC_{50} values were converted to the logarithmic form (pEC_{50} = negative logarithm of EC_{50}) as they are log-normally distributed (Fleming *et al*, 1972). The percent of maximal effect (% E_{max}) by the drugs at the δ-opiod receptor was calculated as net maximal effect of the agonist/net maximal effect elicited by DPDPE × 100. Saturation binding data were analyzed by the LIGAND program (Munson and Rodbard, 1980).

Antagonist potencies in functional assays and radioligand competition curves were analyzed by nonlinear regression analysis and the antagonist inhibitory constant (K_i) was calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973). K_i values were converted to the logarithmic form (pK_i). The K_i/EC_{50} ratio was determined to be significantly different from one when the corresponding pK_i and pEC_{50} values were significantly different. When the difference was not significant, the ratio was considered equal to 1. The efficacy of the opioid receptor ligands in stimulating [^{35}S]GTPγS binding was calculated according to the method described by Ehlert (1985) and Quock *et al* (1999), in which efficacy $= (E_{max\text{-}A}/E_{max\text{-}B}) \times (K_i/EC_{50} + 1) \times 0.5$, where $E_{max\text{-}A}$ is the maximal effect elicited by the test compound, $E_{max\text{-}B}$ is the maximal effect elicited by DPDPE, respectively, K_i is the inhibition constant of the compound obtained from competition binding experiments in the presence of guanine nucleotides, and EC_{50} is the concentration of the compound producing half-maximal effect. Statistical analysis was performed by either Student's *t*-test when comparing two groups and one-way ANOVA followed by Dunnett *post hoc* test when comparing more than one group.

RESULTS

Effects in CHO Cells Expressing the Cloned Human Opioid Receptors

As shown in Figure 1a, in CHO/DOR cell membranes NDMC elicited a concentration-dependent stimulation of [^{35}S]GTPγS binding with a pEC_{50} of 7.24 ± 0.01 and a maximal effect corresponding to $124 \pm 5\%$ increase of basal activity. This stimulation was about 95% of that displayed by the full δ-opioid receptor agonist DPDPE (Table 1). Clozapine was less effective than NDMC and displayed a much lower potency ($pEC_{50} = 5.90 \pm 0.04$). On the other hand, CNOX was a weak agonist, eliciting a small stimulatory effect ($30 \pm 6\%$ increase) only at concentrations above 1 μM ($pEC_{50} = 5.41 \pm 0.02$). The δ-opioid receptor antagonist TIPP completely antagonized the stimulatory effects elicited by NDMC and clozapine with pK_i values of 9.43 ± 0.05 and 9.37 ± 0.06, respectively, which were similar to that displayed in blocking the DPDPE response ($pK_i = 9.63 \pm 0.03$) (Figure 2).

NDMC, clozapine, and CNOX failed to show agonist-like activity in CHO/MOR-1 cell membranes under conditions where the full μ-opioid receptor agonist DAMGO stimulated [^{35}S]GTPγS binding by 200% (Figure 1b). Similarly, in CHO/KOR cell membranes, NDMC and clozapine did not change [^{35}S]GTPγS binding at concentrations up to 1 μM and CNOX up to 10 μM (Figure 1c). At higher concentrations, NDMC and clozapine caused a stimulatory effect, which did not reach saturation at 100 μM, the highest concentration tested. For comparison, in these cells the κ-opioid receptor agonist (−)-U-50,488 stimulated [^{35}S]GTPγS binding by four-fold with a pEC_{50} of 9.24. Finally, in CHO/NOP cell membranes NDMC and the other two compounds were without effect up to 30 μM, whereas the NOP receptor agonist N/OFQ maximally stimulated [^{35}S]GTPγS binding by 130% (Figure 1d).

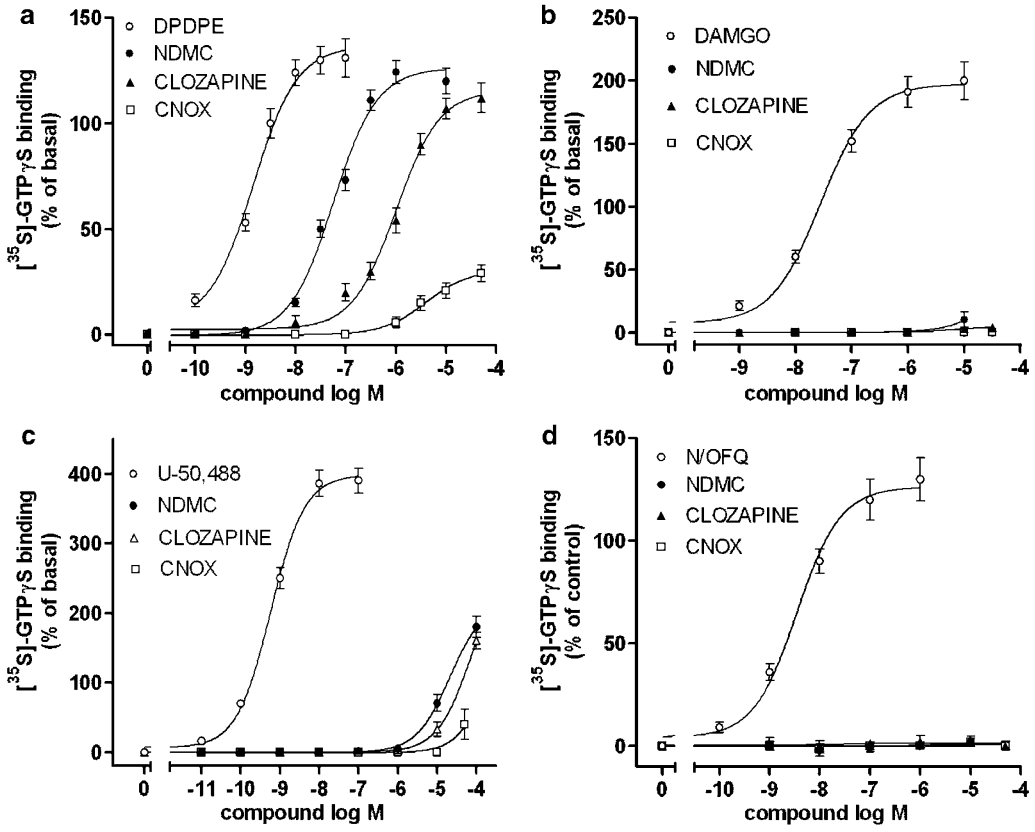

Figure 1 Effects of NDMC, clozapine, CNOX, and reference opioid agonists on [^{35}S]GTPγS binding in membranes of CHO/DOR (a), CHO/MOR-1 (b), CHO/KOR (c), and CHO/NOP (d) cells. Values are reported as percent of basal activity and are the mean ± SEM of three to six experiments.

Table 1 Potencies and Relative Efficacies of *N*-Desmethylclozapine, Clozapine, and Clozapine *N*-Oxide in Stimulating [^{35}S]GTPγS Binding at the Cloned Human δ-Opioid Receptor

Agonist	% E_{max}[a]	EC_{50} (nM) (*n*)	K_i[b] (nM) (*n*)	K_i/EC_{50}	Efficacy[c]
DPDPE	100	1.5±0.2 (5)	84.4±7.2 (4)	56.3	28.6
N-desmethylclozapine	95±3	57.5±1.5 (6)	2 636±140 (4)	45.8	23.4
Clozapine	82±4	1 250±110 (4)	27 900±2 400 (3)	22.3	9.50
Clozapine *N*-oxide	23±5	3 840±225 (3)	3 080±160 (3)	0.80[d]	0.23

[a]Percent of maximal stimulation of [^{35}S]GTPγS binding with respect to that obtained with DPDPE, which was set at 100%.
[b]Determined in competition binding experiments in the presence of 30 μM GDP and 1 nM GTPγS.
[c]Determined according to the equation described in Statistical analysis section.
[d]Not significantly different from 1; *n* = number of experiments.

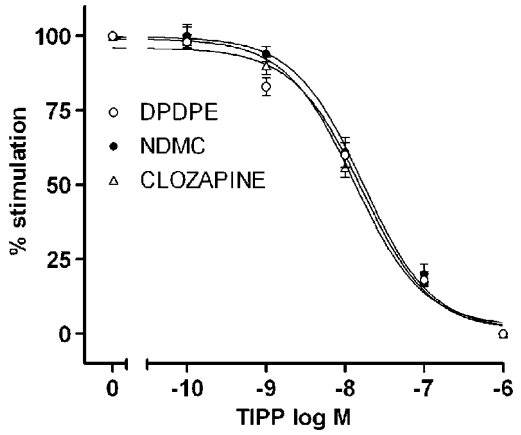

Figure 2 Antagonism of DPDPE-, NDMC- and clozapine-stimulated [^{35}S]GTPγS binding by TIPP in CHO/DOR cell membranes. Values indicate the percent of the stimulatory effect of either 0.1 μM DPDPE, 3 μM NDMC, or 30 μM clozapine at each concentration of TIPP and are the mean±SEM of three experiments.

In intact CHO/DOR cells, NDMC inhibited FSK-stimulated cyclic AMP accumulation with a pEC_{50} value of 6.40±0.04 and a maximal effect corresponding to 78±5% decrease of control activity ($n=3$) (results not shown). This effect was equal to 96±5% of the maximal inhibitory response elicited by DPDPE. Clozapine was significantly less potent ($pEC_{50}=5.05\pm0.06$, $p<0.01$) and less efficacious (67±3% inhibition), whereas CNOX showed little agonist activity, maximally inhibiting cyclic AMP accumulation by 16±4% with a pEC_{50} of 5.50±0.10 ($n=3$) (results not shown). In CHO/MOR-1 and CHO/KOR cells, NDMC, clozapine, and CNOX failed to significantly affect FSK-stimulated cyclic AMP accumulation at concentrations up to 30 μM ($n=3$) (results not shown).

Determination of Ligand-Binding Affinities

Increasing concentrations of NDMC inhibited [^{3}H]NTI binding to CHO/DOR membranes with a competition curve that was best fit by a two-site model, indicating the occurrence of a high- (52.8±4%, $pK_i=7.42\pm0.03$) and a low-affinity component ($pK_i=5.47\pm0.05$) (Figure 3a). In the presence of guanine nucleotides (30 μM GDP and 1 nM GTPγS), the NDMC competition curve was monophasic and shifted to the right, with a pK_i of 5.57±0.02. Clozapine inhibited [^{3}H]NTI binding with a pattern similar to that of NDMC (Figure 3b), but with lower pK_i values both in the absence (high-affinity component 50±3%, $pK_i=5.84\pm0.03$; low-affinity component $pK_i=4.46\pm0.05$) and in the presence of guanine nucleotides (single component with $pK_i=4.55\pm0.04$). Conversely, CNOX generated monophasic inhibition curves with similar pK_i values in the absence (5.59±0.05) and in the presence of guanine nucleotides (5.51±0.03) (Figure 3c).

In competition binding experiments performed using CHO/KOR cell membranes and in the absence of added guanine nucleotides, NDMC inhibited the binding of [^{3}H]diprenorphine (0.2 nM) with a steep monophasic competition curve and a pK_i of 4.76±0.04, whereas clozapine caused less than 50% inhibition at 100 μM, the highest concentration tested ($n=3$) (results not shown). Finally, in CHO/MOR-1 cell membranes, NDMC, clozapine and CNOX failed to affect the binding of 0.2 nM [^{3}H]diprenorphine at concentrations up to 50 μM ($n=3$) (results not shown).

Determination of δ-Opioid Receptor Agonist Efficacy

The efficacies of NDMC and the other two related compounds in stimulating [^{35}S]GTPγS binding to CHO/DOR membranes were determined according to the method of Ehlert (1985), which takes into consideration both the magnitude of the agonist effect and the extent of receptor occupancy at which half-maximal effect occurred (K_i/EC_{50} ratio). As shown in Table 1, NDMC exhibited a high K_i/EC_{50} ratio and an efficacy value equal to 82% of that displayed by DPDPE, whereas clozapine and CNOX had lower receptor reserve and efficacy values corresponding to 33 and 0.8% of that of the full agonist, respectively.

Effects in NG 108-15 Cells

In NG108-15 cells naturally expressing the δ-opioid receptors, NDMC inhibited FSK-stimulated cyclic AMP accumulation almost as effectively as DPDPE (65±5% reduction of control activity) and with a pEC_{50} value of 5.80±0.05 (Figure 4a). Clozapine was less potent ($pEC_{50}=5.01\pm0.06$) and less efficacious (43±4% inhibition of control activity) than the *N*-demethylated metabolite, whereas CNOX was inactive. NTI antagonized the

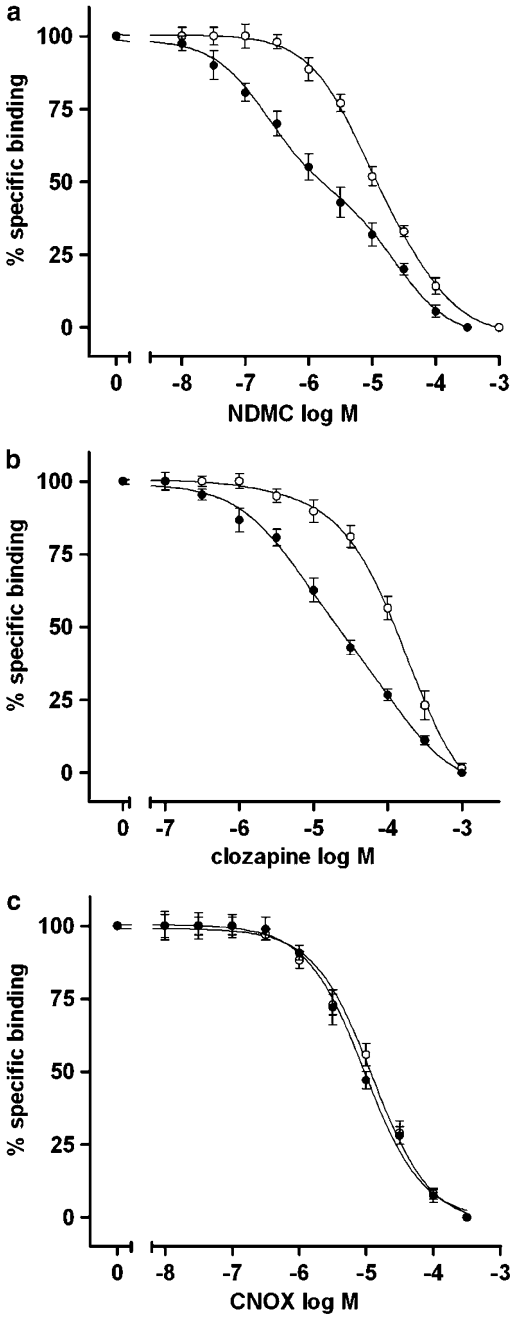

Figure 3 Competition for [^{3}H]NTI binding to CHO/DOR cell membranes by NDMC, clozapine, and CNOX in the absence (closed symbols) and in the presence (open symbols) of 30 μM GDP plus 1 nM GTPγS. Values indicate the percent of specific binding at each competitor concentration and are the mean ± SEM of three to four determinations.

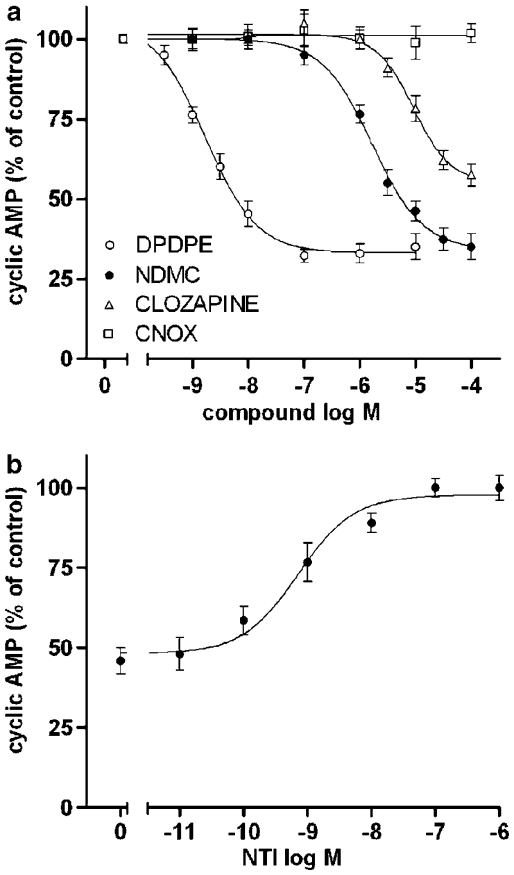

Figure 4 (a) Effects of DPDPE, NDMC, clozapine, and CNOX on FSK-stimulated cyclic AMP accumulation in NG108-15 cells. Values are reported as percent of control activity (vehicle) and are the mean ± SEM of four determinations. (b) Antagonism of NDMC (10 μM)-induced inhibition of cyclic AMP accumulation by NTI in NG108-15 cells. Values are reported as percent of control activity and are the mean ± SEM of three determinations.

NDMC inhibitory effect with a pK_i value of 10.14 ± 0.01, which was consistent with its δ-opioid receptor affinity (Figure 4b).

Immunocytochemical analysis showed that exposure of NG108-15 cells to NDMC (10 μM) for 20 min caused a marked stimulation of ERK1/2 phosphorylation, and this effect was antagonized by the concomitant addition of 1 μM NTI (Figure 5a). The percent values of immunolabeled cells were: control 24 ± 3, NDMC 60 ± 5 ($p < 0.05$ *vs* control), NTI 28 ± 5 ($p > 0.05$ *vs* control), and NTI + NDMC 32 ± 7 ($p > 0.05$ *vs* control) (one-way ANOVA, $n = 3$). In Western blot analysis using the same primary antibody, NDMC significantly stimulated the labeling of phosho-ERK1/2 immunoreactive bands by 55% and this effect was completely blocked by NTI, which *per se* caused no change (Figure 5b and c).

Effects in Rat Brain Membranes

In membranes of dorsal striatum, nucleus accumbens, and frontal cortex NDMC stimulated [^{35}S]GTPγS binding by 63.0 ± 4.0, 60 ± 3.0, and 72.0 ± 6.0% ($p < 0.001$), respectively, with pEC_{50} values of 6.18 ± 0.07, 6.15 ± 0.08, and 6.07 ± 0.05, respectively (Figure 6a–c). In the same brain regions, NDMC inhibited FSK-stimulated adenylyl cyclase activity by 31 ± 4, 24 ± 4, and 21 ± 3% ($p < 0.01$) with pEC_{50} values of 5.85 ± 0.06, 5.73 ± 0.03, and 5.76 ± 0.02, respectively. Conversely, in membranes of rat thalamus, which expresses a low level of δ-opioid receptors and a high density of μ-opioid receptors (Mansour *et al*, 1995), [^{35}S]GTPγS binding was weakly affected by NDMC (< 10 ± 3% increase at 10 μM, $n = 3$), but it was markedly stimulated by DAMGO (160 ± 8% increase at 1 μM, $n = 3$) (results not shown). In membranes of the granule cell layer of the main olfactory

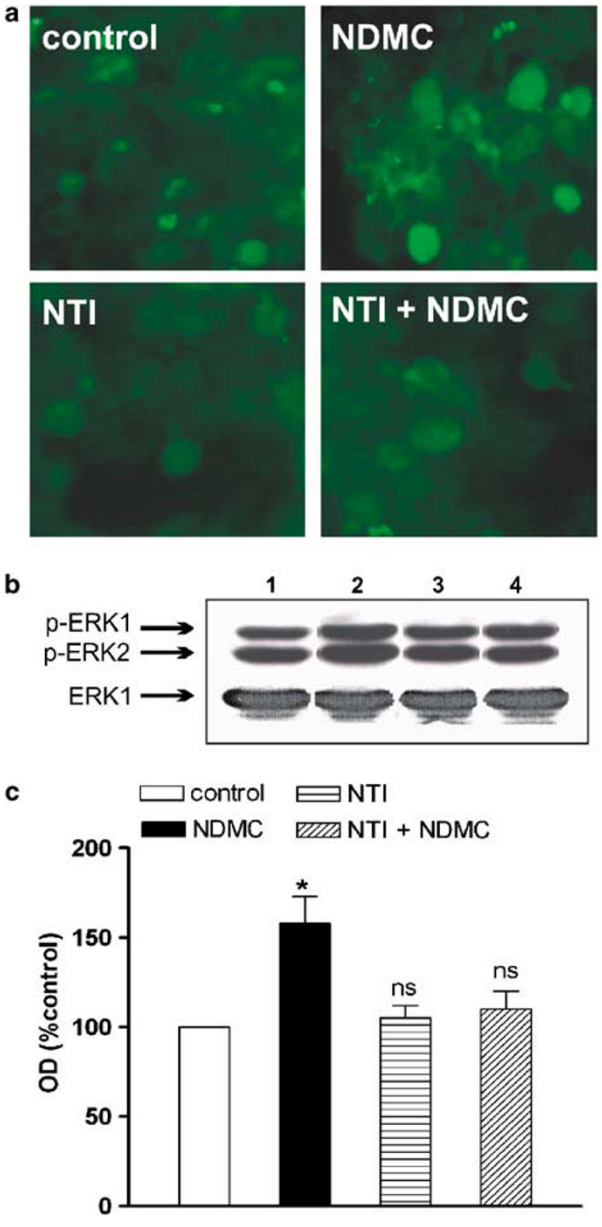

Figure 5 Stimulation of ERK1/2 phosphorylation by NDMC in NG108-15 cells. (a) Immunofluorescence analysis of phospho-ERK1/2 immunoreactivity. Cells were serum-starved for 12 h and then treated with either vehicle (control), 10 μM NDMC, 1 μM NTI, and NTI + NDMC for 20 min, fixed and immunostained with anti-phospho-ERK1/2 antibody followed by FITC-conjugated secondary antibody. Results are representative of three similar experiments. (b) Western blot analysis of phospho-ERK1/2 immunoreactivity. Serum-starved cells were treated for 20 min with vehicle (lane 1), 10 μM NDMC (lane 2), 1 μM NTI (lane 3), and NTI + NDMC (lane 4). Thereafter, cell extracts were prepared and equal amounts of proteins (30 μg) were loaded in each lane. Samples were subjected to immunoblotting with either anti-phospho-ERK1/2 antibody (top) or anti-ERK1 antibody (bottom). Results are representative of three similar experiments. (c) Densitometric analysis of immunoreactive phospho-ERK1/2 bands. The optical density of the phospho-ERK1/2 bands for each drug treatment was normalized to the density of the corresponding ERK1 band and is reported as percent of control. Values are the mean ± SEM of three experiments. *$p<0.05$ *vs* control, NS $p>0.05$ *vs* control by one-way ANOVA followed by Dunnett's test.

bulb, where previous studies have demonstrated the occurrence of a positive coupling of δ-opioid receptors to adenylyl cyclase (Onali and Olianas, 2004b), NDMC stimulated both [^{35}S]GTPγS binding and cyclic AMP formation by 170 ± 8 and 75 ± 4% ($p<0.001$), respectively, with pEC_{50} values of 5.88 ± 0.04 and 5.87 ± 0.05, respectively (Figure 6d). For comparison, DPDPE maximally stimulated [^{35}S]GTPγS binding by 74 ± 4, 80 ± 5, 83 ± 3, and 182 ± 9% in membranes of dorsal striatum, nucleus accumbens, frontal cortex, and olfactory bulb granule cell layer, respectively ($n=3$) (results not shown). Unlike NDMC, clozapine caused little changes in both functional assays, whereas CNOX was completely inactive. As shown in Figure 7, in each brain area NTI antagonized the NDMC stimulations of [^{35}S]GTPγS binding by 80–90% with pK_i values ranging from 10.53 to 10.15. Conversely, the κ-opioid receptor antagonist nor-binaltorphimine (nor-BNI) showed pK_i values of 8.19–8.50, which were about 100-fold lower than the affinity for the κ-opioid receptor ($pK_i = 10.20$). The μ-opioid receptor antagonist CTAP failed to counteract the agonist effects of NDMC in each brain region.

DISCUSSION

In the present study, we show that the clozapine major metabolite NDMC behaves as selective and efficacious agonist at the δ-opioid receptor. In CHO/DOR cells, the drug exhibited full agonist activity in both [^{35}S]GTPγS binding and cyclic AMP accumulation assays, and the activity was blocked by TIPP with a potency similar to that displayed in antagonizing DPDPE, demonstrating the mediation by a common recognition site. The functional selectivity for the δ-opioid receptor is indicated by the lack of agonist effects in both CHO/MOR-1 and CHO/NOP cells, and by the about 300- to 1000-fold higher threshold concentration for the activation of the κ-opioid receptor in CHO/KOR cells. Moreover, in radioligand binding assays, NDMC showed an affinity for the δ-opioid receptor, which was about 60-fold higher than that for the κ-opioid receptor and a very low affinity for the μ-opioid receptor. These results are in agreement with the K_i Database of the NIMH psychoactive drug screening program (http://pdsp.cwru.edu/pdsp.php), which reports an NDMC K_i value of 127.9 nM for the δ-opioid receptors and K_i values higher than 10 μM for both μ- and κ-opioid receptors. As expected for an agonist acting at a G protein-coupled receptor, in CHO/DOR cell membranes NDMC inhibited [^{3}H]NTI binding with shallow competition curves, indicating the presence of a high- and a low-affinity binding site. The addition of guanine nucleotides markedly reduced the fraction of the high-affinity component and shifted the competition curve to describe a single low-affinity binding site. This allosteric regulation by guanine nucleotides has been documented for agonists, but not for antagonists, at the δ-opioid receptor (Werling *et al*, 1988). When the efficacy was determined, we found that NDMC exhibited a relatively high K_i/EC_{50} ratio and an efficacy value slightly lower than that of the full agonist DPDPE, indicating that the drug activates δ-opioid receptor-mediated G protein signalling with high efficiency.

Previous studies have described partial agonist effects of NDMC at the cloned M_1–M_5 muscarinic receptors (Sur *et al*, 2003; Weiner *et al*, 2004; Davies *et al*, 2005) and D_2 and D_3 DA receptors (Burstein *et al*, 2005) with potencies generally comparable to those observed in CHO/DOR cells (pEC_{50}

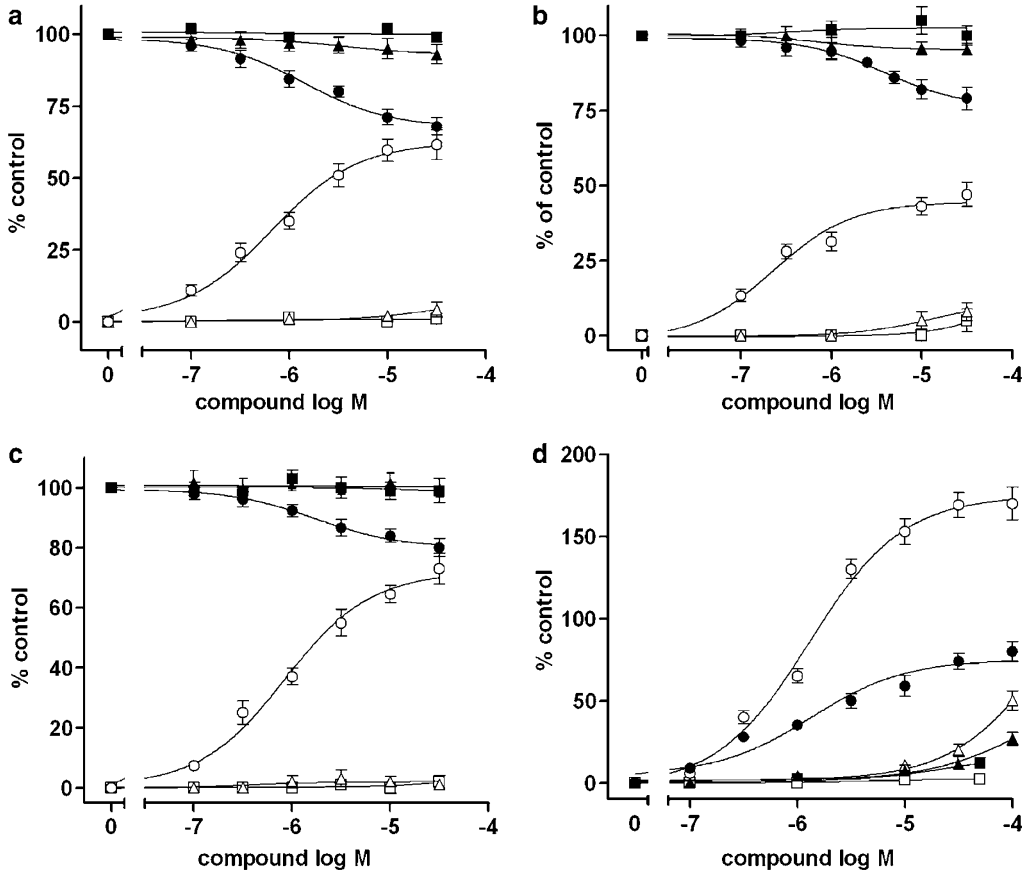

Figure 6 Concentration-dependent effects of NDMC (circles), clozapine (triangles), and CNOX (squares) on either [^{35}S]GTPγS binding (open symbols) or adenylyl cyclase activity (closed symbols) in membranes of dorsal striatum (a), nucleus accumbens (b), frontal cortex (c), and granule cell layer of main olfactory bulb (d). For [^{35}S]GTPγS binding assays, tissue membranes (3–4 μg of protein) were preincubated in the presence of the indicated concentrations of each drug for 20 min at 30°C. Thereafter, the reaction was started by the addition of 1 nM [^{35}S]GTPγS and continued for 40 min. Adenylyl cyclase activity was assayed in the presence of 10 μM FSK in dorsal striatum, nucleus accumbens and frontal cortex, and under basal condition in olfactory bulb granule cell layer. Values are reported as percent of control and are the mean ± SEM of three to four experiments.

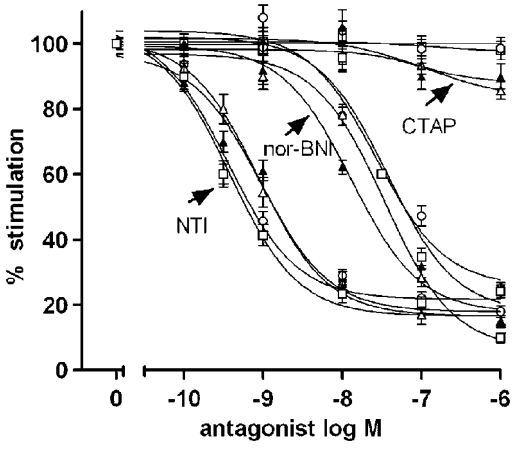

Figure 7 Antagonism of NDMC-induced stimulation of [^{35}S]GTPγS binding in different rat brain regions by NTI, nor-binaltorphimine (nor-BNI), and CTAP. Membranes of dorsal striatum (open triangles), nucleus accumbens (closed triangles), frontal cortex (circles), and olfactory bulb granule cell layer (squares) were preincubated in the presence of 10 μM NDMC and the indicated concentrations of the opioid receptor antagonists for 20 min at 30°C. Thereafter, the reaction was started by the addition of 1 nM [^{35}S]GTPγS and continued for 40 min. Values are reported as percent of NDMC stimulation at each antagonist concentration and are the mean ± SEM of three determinations. At the indicated concentrations, each antagonist *per se* failed to affect basal [^{35}S]GTPγS binding.

values = 7.24–6.40). For instance, in cells expressing M_1 muscarinic receptors NDMC stimulated intracellular Ca^{2+} levels with pEC_{50} values of 7.65 (Davies *et al*, 2005) and of 6.94 (Sur *et al*, 2003) and phosphoinositide hydrolysis with a pEC_{50} of 6.7 (Weiner *et al*, 2004). At D_2 and D_3 DA receptors, NDMC displayed agonist activity equal to 35 and 50% of that of pergolide at the concentration of 20 and 30 nM, respectively (Burstein *et al*, 2005). NDMC displayed higher potency ($K_i = 2.9$ nM) in antagonizing 5-HT_{2C} receptor-mediated phosphoinositide hydrolysis in rat choroid plexus and [^{3}H]ketanserin binding to 5-HT_{2A} in rat frontal cortex ($K_i = 7.4$ nM) (Kuoppamaki *et al*, 1993).

Clozapine displayed an agonist activity similar to that of its demethylated metabolite, being active in CHO/DOR cells but having little effects in CHO/KOR, MOR-1, and NOP cells. However, as δ-opioid receptor agonist, clozapine was much weaker than NDMC. Thus, clozapine affected [^{35}S]GTPγS binding and cyclic AMP accumulation with pEC_{50} values that were 18-fold lower than those of the demethylated metabolite. A similar difference was observed between the pK_i values of the two drugs for the high-affinity binding site at the δ-opioid receptor. Moreover, the calculated K_i/EC_{50} ratio of clozapine in stimulating [^{35}S]GTPγS binding was half of that of NDMC and the estimated efficacy value corresponded to only one-third of

that displayed by DPDPE, suggesting that the drug activates the δ-opioid receptor with a relatively low efficiency. The finding that clozapine possesses agonist activity at the δ-opioid receptor is in agreement with a previous study by Kobayashi *et al* (1998), who reported that in *Xenopus* oocytes co-injected with the G-protein-activated inwardly rectifying potassium channel and either the δ- or κ-opioid receptor mRNA, clozapine stimulated the δ-opioid receptor more potently and efficaciously ($EC_{50} = 4.56\,\mu M$; 60% of the DPDPE response) than the κ-opioid receptor ($EC_{50} = 30.2\,\mu M$; 20% of the U-50,488H response).

Differently from NDMC and clozapine, CNOX was a poor agonist at the δ-opioid receptor and failed to affect the other opioid receptor subtypes. Moreover, in competition binding assays, the compound inhibited the [^{3}H]NTI binding to CHO/DOR cell membranes with low affinity and in a guanine nucleotide-insensitive manner, a property typical of receptor antagonist. Indeed, CNOX showed a K_i/EC_{50} ratio close to unity and an estimated efficacy value less than 1% of that shown by DPDPE, indicating that the compound possesses a very low intrinsic activity.

As heterologous cell lines overexpressing the cloned receptors provide a system with a high stimulus-response efficiency that may greatly affect agonist intrinsic activity, it was important to investigate whether NDMC was able to activate the δ-opioid receptor in cell systems expressing receptors, G proteins, and effectors under native conditions. We found that in NG108-15 cells, which endogenously express δ-opioid receptors (Evans *et al*, 1992), NDMC inhibited FSK-stimulated cyclic AMP accumulation as effectively as DPDPE, clozapine was 25-fold less potent and significantly less efficacious, and CNOX was completely inactive. The involvement of the δ-opioid receptor in the NDMC response was demonstrated by the complete and potent blockade by NTI. Moreover, in the same cell system, NDMC induced ERK1/2 phosphorylation by acting at the δ-opioid receptor, as previously observed for other opioid agonists (Zhang *et al*, 1999). In neuronal cells, ERK1/2 is known to participate in molecular pathways regulating learning, memory, and synaptic plasticity (Thomas and Hunganir, 2004). Previously, it has been reported that in mice the systemic administration of NDMC increased phospho-ERK1/2 immunoreactivity in the hippocampus and this response was blocked by pretreatment with scopolamine, indicating the participation of muscarinic receptors (Weiner *et al*, 2004). Studies in rats have shown that clozapine induced ERK1/2 phosphorylation in prefrontal cortex by blocking serotonin $5HT_{2A}$ receptors and that ERK1/2 inhibitors prevented clozapine suppression of the conditioned avoidance response, a behavioral index of antipsychotic activity (Browning *et al*, 2005). It will be of interest to investigate whether in the brain NDMC can regulate ERK1/2 activity via δ-opioid receptors as found in NG108-15 cells.

The ability of NDMC to activate native δ-opioid receptor was also examined in distinct rat brain areas, such as dorsal striatum, nucleus accumbens, frontal cortex, and the granule cell layer of main olfactory bulb, which express high to intermediate concentrations of δ receptors together with other opioid receptors (Mansour *et al*, 1995). In each brain area, NDMC stimulated [^{35}S]GTPγS binding and regulated cyclic AMP formation in a manner qualitatively similar to δ-opioid receptor agonists. The maximal stimulations of [^{35}S]GTPγS binding by NDMC ranged from 75 to 100% of that of DPDPE and were potently blocked by NTI, but not by nor-BNI or CTAP. Moreover, NDMC had little effects in membranes of rat thalamus, which expresses a high concentration of μ-opioid receptors and a low level of δ receptors (Mansour *et al*, 1995). These data indicate that NDMC retains the ability to activate δ-opioid receptors endogenously expressed in brain. It is noteworthy that in the brain membrane preparations clozapine displayed negligible agonist activity, in agreement with the low efficacy value showed in CHO/DOR cells.

While in CHO/DOR cells NDMC stimulated [^{35}S]GTPγS binding with a nanomolar potency, the compound was significantly less potent in activating the native δ-opioid receptor with pEC_{50} values ranging from 5.73 to 6.18. Nonetheless, these values are within the range of NDMC plasma concentrations (175–435 ng/ml; 0.6–1.4 μM) measured in schizophrenics following chronic treatment with clozapine (Centorrino *et al*, 1994; Olesen *et al*, 1995; Aravagiri and Marder, 2001; Frazier *et al*, 2003). The concentrations reached by NDMC in the human brain under these conditions are unknown. Studies in rats have reported that brain levels of NDMC were relatively low as compared to those of clozapine (Baldessarini *et al*, 1993). However, in the study of Weigmann *et al* (1999), who analyzed the blood/brain distribution of clozapine and NDMC under steady-state conditions, the brain levels of NDMC reached a value of 2000 ng/g and were about threefold lower that those of clozapine. Although these values do not allow the determination of the drug concentrations in the biophase, they are comparable with the potencies of NDMC in activating the δ-opioid receptor in rat brain membranes. Moreover, whereas clozapine has higher affinity than NDMC at muscarinic M_1 and DA D_2/D_3 receptors and potently antagonizes NDMC agonist activity at these receptors (Sur *et al*, 2003; Weiner *et al*, 2004; Burstein *et al*, 2005; Li *et al*, 2005), the affinity of clozapine for the δ-opioid receptor is more than 10-fold lower than that of NDMC, suggesting that in the brain the demethylated metabolite could activate the receptors even in the presence of higher concentrations of the parent drug.

Clinical studies on the role of opioids in schizophrenia have yielded conflicting results, as some investigations reported beneficial effects with either opioid agonists (Brizer *et al*, 1985; Schmauss *et al*, 1987) or antagonists (Berger *et al*, 1981; Marchesi *et al*, 1995), whereas others observed either impairment of schizophrenic symptoms (Gerner *et al*, 1980; Judd and Janowsky, 1981) or no effects (Naber, 1988; Wonodi *et al*, 2004) with similar treatments. Moreover, most of the studies employed nonselective agents, making the understanding of the role of each opioid receptor difficult. Thus, at present the relevance of the selective δ-opioid agonist activity of NDMC for the antipsychotic action of clozapine remains uncertain. Nonetheless, it is possible that this property may have implications for clozapine activity in mood disorders. A number of evidence from animal studies indicate that δ-opioid receptors are highly expressed in cortical and limbic regions and are involved not only in analgesia, but also in the control of cognitive and emotional functions and motivation-related behaviors. Mice deficient in pre-proen-

kephalin, which generates the δ-preferring agonists enkephalins, showed increased levels of anxiety and males displayed offensive aggressiveness (Konig *et al*, 1996). Moreover, mice, in which the δ-opioid receptor gene has been disrupted by targeted deletion of exon 1, exhibited anxiogenic effects in the elevated-plus-maze and the light–dark box behavioral tests, whereas μ-opioid receptor deficient mice showed anxiolytic effects (Filliol *et al*, 2000). Importantly, mice lacking the δ-opioid receptors exhibited a depressive-like behavior in tests that reliably predict antidepressant effects in humans (Filliol *et al*, 2000). Thus, it can be proposed that an increased δ-opioid receptor activity by NDMC may contribute, at least in part, to the reported clinical efficacy of clozapine in ameliorating psychotic mood disorders (McElroy *et al*, 1991), in reducing the risk of suicide in schizophrenia and schizoaffective disorder (Meltzer *et al*, 2003; Kerwin and Bolonna, 2004) and in suppressing aggressive behaviors in psychotic patients (Kraus and Sheitman, 2005). A relevant role of δ-opioid receptors in depression is further supported by pharmacological data showing that the nonpeptidic δ-opioid receptor agonists (+)BW373U86 and SNC80 exerted antidepressive effects in animal models (Broom *et al*, 2002) and potentiated D_1 and D_2 DA receptor stimulation (Spina *et al*, 1998), which may improve cognition and depression, and that, conversely, δ-opioid receptor antagonists caused anxiety-related behaviors (Saito *et al*, 2005). Like antidepressant drugs (Nibuya *et al*, 1995), δ-opioid receptor agonists have been found to increase the hippocampal and cortical synthesis of brain-derived neurotrophic factor (BDNF) (Torregrossa *et al*, 2004), which is known to regulate neuronal development and plasticity and has been shown to possess antidepressant activity (Shirayama *et al*, 2002). Finally, selective activation of δ-opioid receptor has been shown to reverse the catalepsy induced by DA receptor antagonists and the parkinsonian symptoms in the MPTP-treated marmoset (Hille *et al*, 2001) and to cause convulsions (Comer *et al*, 1993), a relatively frequent side effect of clozapine therapy.

In conclusion, the present study demonstrates that NDMC behaves as a selective and efficacious δ-opioid agonist in recombinant and native receptor systems. This unique property provides the compound with the ability to affect those molecular pathways and neuronal circuits of the central opioid system specifically involved in the control of emotion and motivation and therefore may contribute to important features of the clozapine therapeutic action, particularly in improving mood disorders.

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