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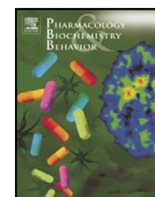

Ex vivo delta opioid receptor autoradiography: CNS receptor occupancy of two novel compounds over their antihyperalgesic dose range

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ABSTRACT

Discovered as part of an effort to identify delta opioid (DOPr or DOR) agonist analgesics, JNJ-20788560 and JNJ-39204880 exhibited high DOR affinity, with K_i values of 1.7 and 2.0 nM, respectively, and were selective for DOR over the mu opioid receptor (MOPr or MOR), with 596- and 122-fold selectivity, respectively. Both compounds stimulated DOR but not MOR induced GTPγS binding and were effective antihyperalgesic agents in the complete Freund's adjuvant model of thermal hyperalgesia in the rat, with oral ED_{50} values of 13.5 and 35 mg/kg, corresponding to plasma levels of 1 and 9 µM, respectively. Autoradiographic analysis of DOR and MOR occupancy in sections of brain (striatum) and lumbar spinal cord (L4–L6) was determined ex vivo, using radiolabeled naltrindole or DAMGO. Quantitative image analysis resulted in striatal DOR ED_{50} values of 6.9 and 10.7 mg/kg, for JNJ-20788560 and JNJ-39204880 respectively, and spinal cord values of 6.4 and 3.2 mg/kg, respectively. Neither compound dose-dependently occupied MOR within the dose range studied. Thus, this study confirmed the DOR selectively over MOR of both compounds following their oral administration, and further demonstrated dose-dependent DOR occupancy by each compound across its antihyperalgesic dose range. Importantly, these in vitro, in vivo, and ex vivo data revealed that the greater in vitro potency of JNJ-20788560 was paralleled by its greater in vivo potency, although JNJ-39204880 achieved higher plasma levels following its oral administration. The receptor occupancy levels observed at the pharmacologic ED_{50} doses of these compounds suggest the need for greater target engagement by JNJ-39204880 than by JNJ-20788560 to elicit a similar therapeutic response.

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1. Introduction

Opioid receptors mediate antinociceptive and antihyperalgesic effects through mu, delta and kappa subtypes located both peripherally and in the central nervous system. Often, in vitro binding and functional assays are used to characterize the interactions (binding affinity and functional potency) of compounds with these receptors. But the receptor selectivity observed in assays conducted in vitro is not always paralleled by the profile observed following the drug's in vivo administration, especially by the oral route, due to variables such as pharmacokinetic properties of the compounds as well as differences among tissues in receptor distribution and functional coupling. Furthermore, the in vivo profile of a drug can be altered by clinical pathology or treatments comprising the induction of an animal model of disease, such as pain, which may induce changes in receptor distribution or trafficking and/or in the signaling pathways engaged by the receptor.

Delta opioid receptor (DOR) agonists are more effective therapeutic agents in animal models of thermal inflammatory hyperalgesia than in nociceptive models (Cahill et al., 2003; Codd et al., 2009; Fraser et al., 2000; Hurley and Hammond, 2000). This enhanced antihyperalgesic effect is paralleled by DOR up regulation and trafficking to the plasma membrane in the dorsal horn in rats receiving an intraplantar (i.pl.) injection of complete Freund's adjuvant (CFA) (Cahill et al., 2003). But CFA treatment has been demonstrated to also alter the pharmacokinetics of some compounds, increasing blood brain barrier permeability (Brooks et al., 2005), increasing the blood concentration of α1 acid glycoprotein (Huber et al., 2001) (which may increase plasma protein binding of some compounds), and altering compound metabolism. For example, the clearance of the DOR agonist SNC80 is decreased in animals treated with CFA, but other changes consequent to CFA administration, such as hepatic and possibly intestinal losses, obviate this potentially advantageous change (Projean et al., 2007).

Short of positron emission tomography, information regarding in vivo drug–receptor interactions can be obtained indirectly by ex vivo autoradiographic studies, in which the drug of interest is administered by a specified route to animals treated similarly to those used in the pharmacodynamic study. Tissue obtained from these animals can be probed with radiolabeled ligands selective for the receptor of interest, lower levels of radiolabeling being observed if the receptor is already occupied by the compound administered in vivo.

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In the present study, two structurally diverse DOR agonists were studied in an ex vivo format using rats treated with CFA. Following oral administration, each compound was shown to dose-dependently occupy DOR in brain (striatum) and spinal cord (L4–L6) over the range of doses administered, which was pharmacologically active in models of inflammatory pain. In the same studies, the compounds did not dose-dependently occupy the mu opioid receptor (MOR), confirming the compound profiles defined in vitro and supporting the role of CNS DOR in the antihyperalgesic effect of the DOR agonists.

2. Materials and methods

2.1. Chemicals

JNJ-20788560, 9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9*H*-xanthene-3-carboxylic acid diethylamide (Codd et al., 2009), and JNJ-39204880, [4-(4-methoxy-phenoxy)-[2,5′]bipyrimidinyl-5-yl]-pyrrolidin-2-ylmethyl-amine, shown in Fig. 1, were synthesized as their hydrochloride salts in J&J PRD laboratories. Radiolabeled compounds were obtained from Perkin Elmer, (Boston, MA), $MgCl_2$ and Tris from Fisher (Pittsburgh, PA), sucrose from EM Science (Gibbstown, NJ), dithiothreitol from Fluka (Buchs, Switzerland) and NaCl, HEPES, EDTA, GDP and CFA from Sigma (St. Louis, MO).

2.2. Animals

All experimental procedures were approved by the Animal Care and Use Committee of J&J PRD and were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Research Council and The International Association for the Study of Pain. Rats (strains used are specified in each procedure below) were purchased from Charles River (Kingston, NY), acclimated for at least a week prior to use to the 12/12 hr light dark cycle, and given free access to food and water.

2.3. In vitro opioid receptor binding assays

Binding assays were performed essentially as previously described (Codd et al., 2006). Male Wistar rats were killed by cervical dislocation, and their brains were removed and placed immediately on dry ice. On the day of the experiment, brains were removed from their −80 °C storage and placed in ice cold 50 mM Tris–HCl buffer (pH 7.4). The forebrains were separated from the remainder of the brain by a coronal transection, beginning dorsally at the colliculi and passing ventrally through the midbrain–pontine junction. After dissection, the forebrains were homogenized in Tris buffer in a Teflon®-glass homogenizer. The homogenate was diluted to a concentration of 1 g forebrain/80 mL Tris buffer and centrifuged at 39,000 × *g* for 10 min. The pellet was resuspended in the same volume of Tris buffer, containing 5 mM $MgCl_2$, with several brief pulses from a Polytron homogenizer. This particulate preparation was used for the opioid binding assays. Following incubation with 2 to 3 nM of the delta selective ligand $[^3H]$-DPDPE or 0.8 nM of the mu selective ligand $[^3H]$-DAMGO at 25 °C for 2.5 h in a 96-well plate in a total volume of 1 mL, the plate contents were filtered through Wallac filtermat B sheets on a Tomtec 96-well harvester. The filters were rinsed three times with 2 mL of 10 mM HEPES (pH 7.4), and dried in a microwave oven. To each sample area, LKB BetaPlate Scint scintillation fluid was added, and the resulting radioactivity was quantified on a LKB 1205 BetaPlate liquid scintillation counter. K_d and K_i values were calculated using GraphPad Prism (San Diego, CA).

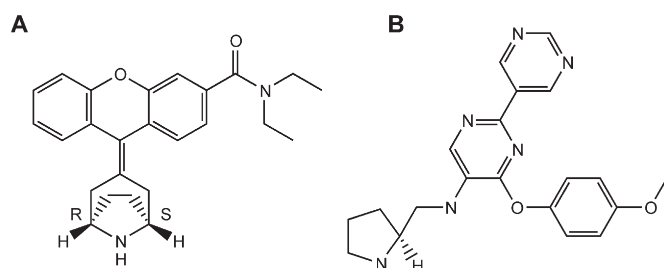

Fig. 1. Structure of (A) JNJ-20788560 and (B) JNJ-39204880.

2.3.1. Agonist-stimulated GTPγS functional assays

Functional assays were performed essentially as previously described (Codd et al., 2006). Membranes from endogenous DOR-expressing NG-108 cells or recombinant MOR-expressing Chinese hamster ovary cells (CHO-hMOR) were purchased from Receptor Biology, Inc. (Baltimore, MD). A vial containing 1 mL of membrane was added to 15 mL cold 50 mM HEPES buffer (pH = 7.6) buffer, containing 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 50 μM GDP and 1 mM EDTA, homogenized with a Polytron, and centrifuged at 3000 rpm for 10 min. The supernatant was then centrifuged at 18,000 rpm for 20 min, and the pellet was resuspended with a Polytron in 10 mL of assay buffer.

The receptor-containing membranes were preincubated in the assay buffer with wheat germ agglutinin-coated SPA beads (Amersham, Piscataway, NJ) at 25 °C for 45 min. The SPA bead coupled membranes were then incubated with 0.5 nM $[^{35}S]$-GTPγS; each assay well contained 1 mg of SPA beads and 3–5 μg of membrane protein. The basal binding was defined as that taking place in the absence of added test compound and taken to be 100%, with agonist-stimulated binding rising to levels significantly above this value. A range of concentrations of receptor agonists was used to stimulate $[^{35}S]$-GTPγS binding. Both basal and non-specific binding were measured in the absence of agonist; the non-specific binding determination included 10 μM unlabeled GTPγS. Radioactivity was quantified on a Packard TopCount (Perkin Elmer, Boston, MA), and the percent stimulation was calculated as:

$$\frac{(\text{test compound cpm–non} - \text{specific cpm})}{(\text{basal cpm–non} - \text{specific cpm})} \times 100$$

EC_{50} values were calculated using GraphPad Prism.

2.4. Ex vivo autoradiography

To determine the DOR and MOR occupancy in rat brain and lumbar spinal cord of orally administered compounds, ex vivo autoradiography was used as previously described (Langlois et al., 2001). Key features of this procedure, designed to minimize the dissociation of drug-bound receptor formed in vivo (Yu et al., 2004), include immediate freezing of the tissue, lack of tissue preincubation (Marcus et al., 2005; Schotte et al., 1996), short incubation times with the radiolabeled ligands (Marcus et al., 2005; Schotte et al., 1996), and brief washes with ice cold buffer and water at the end of the incubation (Schotte et al., 1996). Furthermore, incubations conducted in parallel with tissue from vehicle treated animals are essential to study design and interpretation (Marcus et al., 2005).

Sprague–Dawley rats were injected with CFA (100 μL of 1:1 CFA: saline, 100 μL) i.pl. 24 h prior to oral dosing and fasted overnight. Three rats per compound per dose were orally administered vehicle or the indicated doses of JNJ-20788560 or JNJ-39204880. At the time of maximal plasma exposure for each compound (previously determined as 1 h for JNJ-20788560 and 2 h for JNJ-39204880), blood samples were collected for determination of drug levels in plasma (see below). The animals were euthanized, and their brains and spinal cords (L4–L6) were immediately harvested and rapidly frozen in dry-ice cooled isopentane (−20 °C). Brain or spinal cord cross-sections (20 μm thick) were cut using a cryostat-microtome and thaw-mounted on microscope slides. Occupancy of DOR and MOR by the orally administered compound was measured bilaterally in brain

(striatum only) or spinal cord (the entire slice) of each individual rat by incubation of the thaw-mounted slices for 10 min at room temperature with, respectively, 0.2 nM [^{3}H]-naltrindole (NTI) or 2 nM [^{3}H]-DAMGO in 50 mM Tris–HCl buffer (pH = 7.4), containing 5 mM $MgCl_2$, 40 µg/mL bacitracin and 0.1% BSA. In each case, a 1 µM concentration of the respective non-radiolabeled ligand was used to determine non-specific binding. The slides were then quickly rinsed, first with ice cold buffer and then with ice cold water, to remove unbound radiolabel, and allowed to dry for two days at room temperature. Quantitative autoradiographic analysis was performed following image acquisition for 1 or 4 h for NTI and DAMGO, respectively, with a Beta-imager (Biospace Labs, Cambridge, MA). The percentage of receptor occupancy was plotted against the log of the orally administered dose, and ED_{50} values were calculated for each compound in striatum and spinal cord by non-linear regression analysis using GraphPad Prism (San Diego, CA).

2.5. Determination of plasma drug levels

Blood samples were collected into heparinized tubes and centrifuged to remove cells, and the resulting plasma was extracted with acetonitrile. Following centrifugation, the supernatant was removed. A 50 µL sample of plasma was added to 150 µL of acetonitrile containing an internal standard (1 µg/mL propranolol in acetonitrile), vortexed for 1 min and then centrifuged at 3400 rpm at 10 °C for 10 min. An aliquot of each supernatant was transferred to a 96 well plate for LC/MS/MS analysis on a system consisting of a Shimadzu LC-10AD pump with a Leap HTS PAL autosampler, and an AB/MDS Sciex API 4000 mass spectrometer with a turbo ion spray interface.

2.6. Rat CFA radiant heat test

Male Sprague–Dawley rats were injected with CFA (100 µL of 1:1 CFA:saline, 100 µL) subcutaneously into the sub-plantar tissue of the left hind paw to stimulate an inflammatory reaction. Twenty-four hours later and after fasting overnight, the response time of the animal to a thermal stimulus (Hargreaves et al., 1988) was reevaluated and compared to the animal's baseline response time (preceding CFA treatment). Only rats that exhibited at least a 25% reduction in response latency from baseline were included in further analysis. Following post-treatment latency assessment, rats were orally administered test compound or vehicle (hydroxypropylmethylcellulose). At the previously determined time of maximum anti-hyperalgesic effect, 1 h for JNJ-20788560 and 2 h for JNJ-39204880, the response latency of each animal was again assessed. The percent reversal of hyperalgesia was calculated for each animal as:

$$(\text{treatment response–CFA response}) / [(\text{pre CFA response–post CFA response})] \times 100$$

Therefore, a return to normal pre-treatment thresholds was equal to 100% reversal. Average % reversal of hyperalgesia was then calculated for each treatment group. ED_{50} values and associated statistics were calculated using PharmTools Plus software (The McCary Group, Schnecksville, PA).

3. Results

3.1. In vitro DOR pharmacology

Assayed in vitro, JNJ-20788560 and JNJ-39204880 exhibited high affinity for the rat brain DOR (2.0 and 1.7 nM, respectively, Table 1), and were selective for DOR over MOR. Both compounds exhibited agonist activity in a DOR GTPγS assay; however, due to the weak binding affinity of the compounds at MOR, neither EC_{50} values nor fold selectivity vs DOR could be determined in the MOR GTPγS assay (Table 1). The K_i and EC_{50} values shown were obtained from concentration response studies conducted in triplicate and are representative of determinations made independently on multiple occasions.

Table 1
In vitro binding affinity and GTPγS potency at delta and mu opioid receptors.

Compound	Binding affinity (K_i, nM)		Delta selectivity	GTPγS EC_{50} (nM)	
	DOR	MOR		DOR	MOR
JNJ-20788560	2.0	1191	596	5.6	>10,000
JNJ-39204880	1.7	208	122	38	>10,000

3.2. Ex vivo receptor occupancy

3.2.1. Dose dependence of plasma exposure in CFA pretreated rats

Administered orally to rats treated with CFA 24 h earlier, both JNJ-20788560 and JNJ-39204880 exhibited dose-dependent plasma exposure (Fig. 2). Across the dose range of 1.2 to 150 mg/kg, p.o., plasma levels of JNJ-20788560 rose from 120 nM to 8.6 µM. In the dose range of 3 to 300 mg/kg, plasma levels of JNJ-39204880 rose from 1.7 nM to 30 µM.

3.2.2. Receptor occupancy

Specific DOR and MOR binding was observed in brain and spinal cord slices from CFA treated rats (Fig. 3). Furthermore, as shown in Fig. 4, across their pharmacologic dose range, orally administered JNJ-20788560 and JNJ-39204880 dose-dependently occupied DOR in striatum and spinal cord (L4–L6), with ED_{50} values of 3–10 mg/kg.

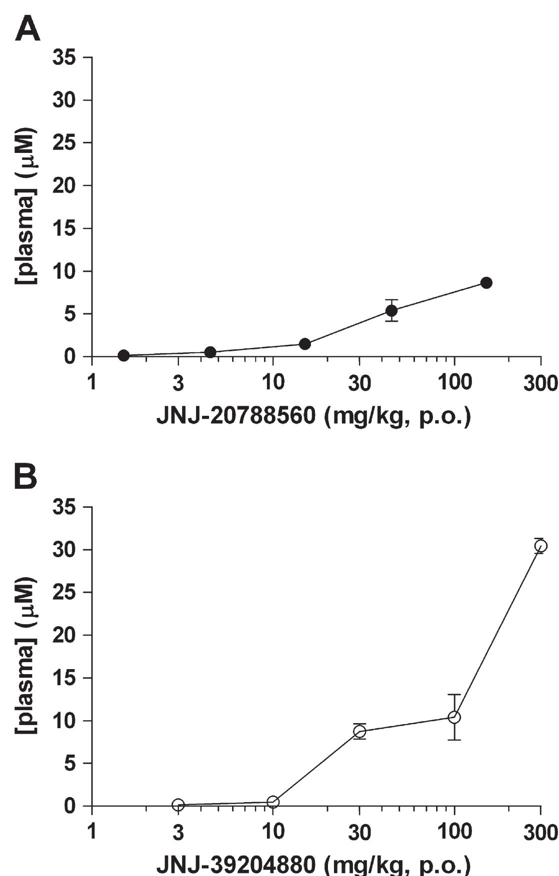

Fig. 2. Plasma drug concentrations of JNJ-20788560 (A) and JNJ-39204880 (B) following oral administration to CFA treated rats. Blood was collected 1 h after JNJ-20788560 and 2 h after JNJ-39204880 administration. Data were obtained from the same rats whose brain and spinal cord DOR occupancy are shown in Fig. 4 (N = 3/drug/dose).

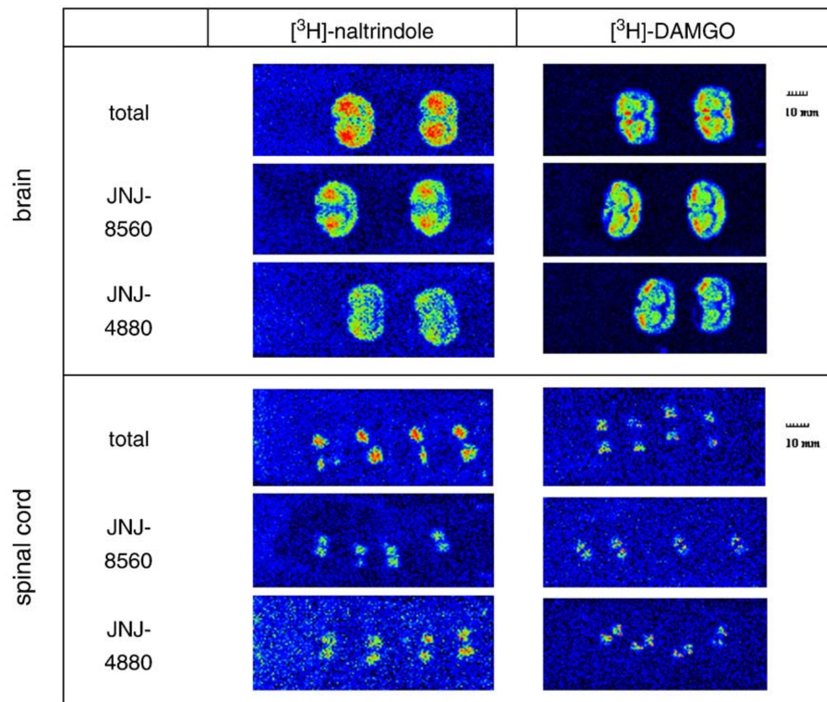

Fig. 3. Representative digital images of brain and spinal cord sections acquired with the Beta-Imager, illustrating total binding as well as binding at the approximate antihyperalgesic ED_{50} dose for each compound, 15 mg/kg for JNJ-20788560 (JNJ-8560) and 30 mg/kg for JNJ-39204880 (JNJ-4880) at DOR ([^{3}H]-naltrindole) and MOR ([^{3}H]-DAMGO).

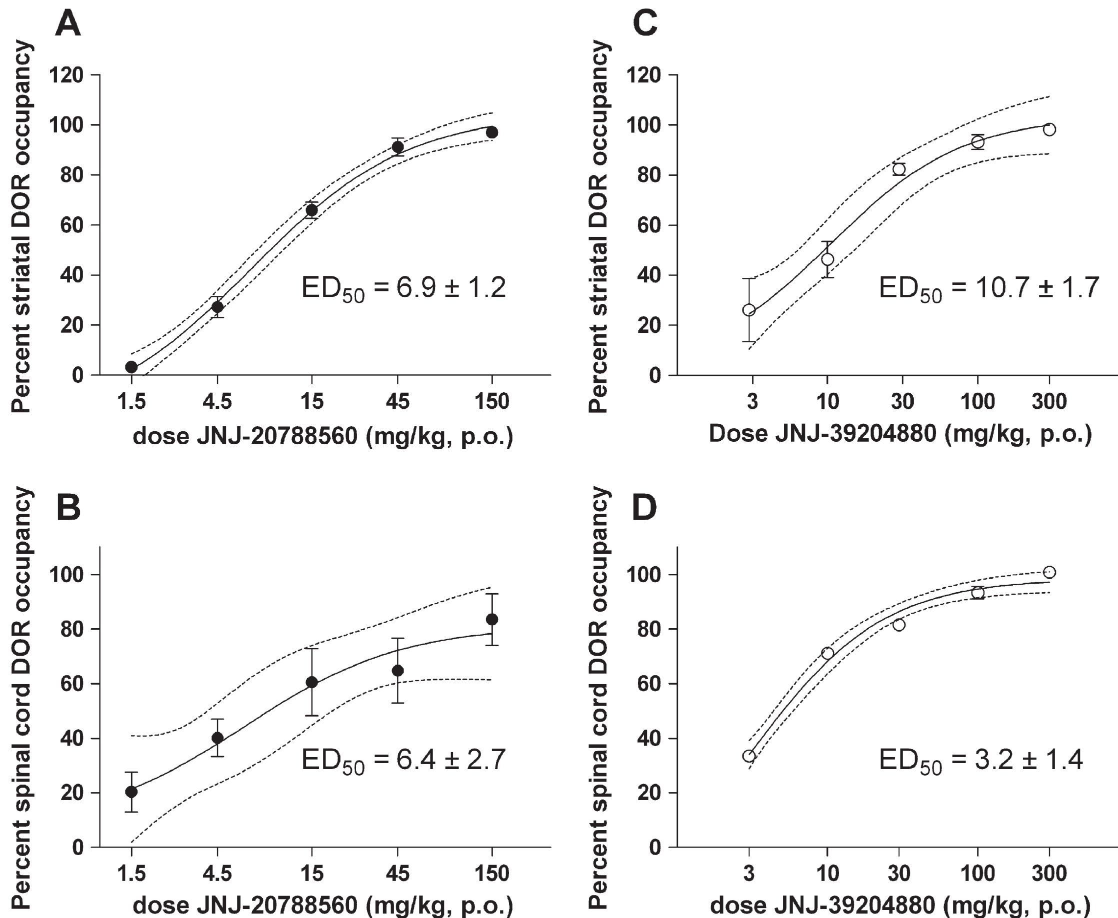

Fig. 4. Ex vivo receptor occupancy by JNJ-20788560 (A, B) and JNJ-39204880 (C,D) in striatum and spinal cord in CFA treated rats. Shown are the mean ± SEM as well as the 95% CL of the non-linear regression analysis of the data obtained from three rats/dose/drug; the striatal and spinal cord sections were obtained from the same rats.

Table 2
ED_{50} values for DOR and MOR ex vivo receptor occupancy by JNJ-20788560 and JNJ-39204880 in striatum and spinal cord in CFA treated rats. Shown are the mean ± SEM.

	DOR ED_{50} (mg/kg, p.o.)		MOR ED_{50} (mg/kg, p.o.)	
	JNJ-20788560	JNJ-39204880	JNJ-20788560	JNJ-39204880
Striatum	6.9 ± 1.2	10.7 ± 1.7	>150	>300
Spinal Cord	6.4 ± 2.7	3.2 ± 1.4	>150	>300

Corresponding plasma levels were about 1 µM for JNJ-20788560 and 0.2–0.5 µM for JNJ-39204880. In contrast, neither compound dose-dependently occupied MOR (Table 2), demonstrating the DOR selectivity of the compounds in vivo following their oral administration to CFA treated rats.

3.3. Rat complete Freund's adjuvant hyperalgesia

In keeping with their DOR agonist pharmacology, both compounds were effective antihyperalgesic agents, exhibiting dose-dependent reversal of CFA-induced thermal hyperalgesia in the rat. Oral ED_{50} values of 13.5 ± 5.7 and 35.4 ± 18 mg/kg were obtained for JNJ-20788560 and JNJ-39204880, respectively. At a dose of 100 mg/kg, each compound induced a reversal in hyperalgesia of approximately 85%.

3.4. Receptor occupancy at ED_{50} dose in CFA treated rats

Because the ex vivo receptor occupancy studies were conducted on CFA treated rats, the resulting data reflect the receptor occupancy in the striatum and the spinal cord under the conditions actually used in the pharmacologic model, thus enabling determination of the receptor occupancy at the pharmacologic ED_{50} dose. JNJ-20788560 occupied about 60% of DOR in the striatum and spinal cord at its CFA ED_{50} dose of 13.5 mg/kg, whereas JNJ-39204880 exhibited an average receptor occupancy of approximately 84% at its CFA ED_{50} dose of 35 mg/kg, p.o. (Table 3). The ED_{50} values obtained in the CFA radiant heat test correspond to plasma concentrations of approximately 1 and 9 µM of JNJ-20788560 and JNJ-39204880, respectively.

4. Discussion

JNJ-20788560 and JNJ-3904880 are structurally diverse compounds that exhibited high affinity for DOR and functional selectivity for DOR over MOR in vitro. Although their binding affinities at DOR were virtually identical, the affinities of the two compounds at MOR differed somewhat, with the result that JNJ-20788560 exhibited greater DOR selectivity than did JNJ-39204880.

Importantly, the high affinity of JNJ-20788560 and JNJ-39204880 for DOR measured in vitro was reflected in their CNS DOR occupancy following oral administration. In an ex vivo receptor occupancy study, the two compounds exhibited robust DOR occupancy in both striatum and spinal cord. In contrast, the compounds did not dose-dependently occupy MOR under the same conditions, maintaining their DOR selectivity (over MOR) across their full pharmacologic dose range.

JNJ-20788560 exhibited virtually identical ED_{50} values for occupancy of DOR in striatum and in spinal cord, whereas JNJ-39204880 occupied spinal DOR at a lower dose than that at which it occupied the receptor in striatum (three-fold preference for spinal DOR). It is not known whether this tissue preference is due to a greater access of JNJ-39204880 to the spinal cord than to the brain, or if it derives from the properties of DOR as constituted in brain vs spinal cord. Differences between brain and spinal cord DOR have previously been noted, the terms delta1 sometimes applied to spinal and delta2 to brain DOR sites (Traynor and Elliott, 1993). The functional consequences of these DOR subtypes have been explored (Hurley et al., 1999) and may play a role in the interaction of spinal and supraspinal opioid sites in mediating antinociception. Thus, whereas administration of the delta1 selective agonist DPDPE both spinally and supraspinally produced merely an additive antinociceptive effect, concurrent administration of the delta2 selective agonist deltorphin II at the two sites effected synergistic antinociception (Hurley et al., 1999).

Despite their virtually identical DOR binding affinities in vitro, the GTPγS functional potencies of the two compounds differed somewhat, with JNJ-20788560 having a DOR potency about seven times that of JNJ-39204880. Notably, this in vitro potency difference was reflected in the relative antihyperalgesic potencies of the compounds, with JNJ-20788560 being somewhat more potent in attenuating thermal hyperalgesia than was JNJ-39204880. Determination of the plasma levels corresponding to the CFA radiant heat ED_{50} values of the two compounds revealed that the plasma level of JNJ-39204880 was about six times that of JNJ-20788560, (i.e., 9 µM vs 1.6 µM), at the 50% level of effect. Thus, the in vivo potencies of the drugs, when expressed as plasma levels present at their ED_{50} doses, were directly proportional to their potencies as measured in the GTPγS assay in vitro.

Importantly for the relationship among the several parameters herein quantified, receptor occupancy, pharmacokinetic profiles, and the pharmacodynamic effect, all were measured in animals pretreated with CFA via intraplantar administration, assuring control of potential pharmacokinetic consequences of CFA administration, such as alterations in compound distribution (Brooks et al., 2005; Huber et al., 2001) or metabolism (Hung et al., 2006; Subramanian and Ramalingam, 2000), as well as CFA-induced changes in DOR expression, localization/distribution or functional competence (Cahill et al., 2007, 2003). The dose-dependent CNS DOR occupancy of these compounds across their pharmacologic dose range supports the notion that their dose-dependent antihyperalgesic effect is mediated by DOR. Conversely, the lack of dose-dependent CNS MOR occupancy by the compounds across their pharmacologic dose range suggests that their antihyperalgesic effect is not MOR mediated. Thus, taken together, these data support the notion that DOR agonists are capable of ameliorating inflammatory hyperalgesia via CNS (spinal and supraspinal) DOR.

The data suggest additionally that the level of receptor occupancy required for the induction of a therapeutic effect (e.g., antihyperalgesia) is not fixed but is compound dependent. It is tempting to speculate, given the potency differences between these two compounds, that higher receptor occupancy levels of a less potent compound are needed to provide a level of effect similar to that achieved at lower receptor occupancy levels by a more potent compound. Study with other compounds targeting the same receptor will be helpful in establishing the scope of applicability of this observation.

Table 3
Percent DOR occupancy, determined by ex vivo receptor autoradiography, at compound ED_{50} value in CFA radiant heat test. Shown are the means ± SEM calculated from the behavioral responses of rats over a dose range of 1–100 mg/kg (JNJ-20788560) or 10–300 mg/kg (JNJ-39204880).

	% DOR occupancy at pharmacologic ED_{50} dose	
	JNJ-20788560	JNJ-39204880
Striatum	63 ± 4.9	80 ± 9.1
Spinal cord	58 ± 15	88 ± 2.8

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