British Journal of Pharmacology (2003) 139, 1462–1468
www.nature.com/bjp

In vitro and *ex vivo* effects of a selective nociceptin/orphanin FQ (N/OFQ) peptide receptor antagonist, CompB, on specific binding of [^{3}H]N/OFQ and [^{35}S]GTPγS in rat brain and spinal cord

*,[1]Shizuo Yamada, [1]Toyofumi Kusaka, [1,3]Akihiko Urayama, [1]Ryohei Kimura & [2]Yasuo Watanabe

[1]Department of Biopharmacy, School of Pharmaceutical Sciences & COE21, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan and [2]Department of Pharmacology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan

1 A novel selective nociceptin/orphanin FQ (N/OFQ) peptide receptor antagonist, 1-[(3*R*,4*R*)-1-cyclooctylmethyl] - 3 - hydroxymethyl - 4 - piperidyl) - 3-ethyl-1,3 - dihydro - 2*H* - benzimidazol - 2 - one (CompB), inhibited specific binding of [^{3}H]N/OFQ to crude membranes from the rat brain and spinal cord in a concentration-dependent manner and their K_i values were 7.11 and 4.02 nM, respectively. Rosenthal analysis indicated that there was a significant increase in the K_d value for [^{3}H]N/OFQ binding in the brain and spinal cord in the presence of CompB (10 nM).

2 There was a dose-dependent increase in K_d values for [^{3}H]N/OFQ binding in the brain and spinal cord following i.v. injection of CompB at relatively low doses (0.69–6.88 μmol kg^{-1}), compared with the control values. In the spinal cord, enhancement with each dose was constantly greater and the duration of enhancement (6.88 μmol kg^{-1}) was significantly longer.

3 The degree of increase in K_d values for [^{3}H]N/OFQ binding after i.v. injection of CompB (6.88 μmol kg^{-1}) was significantly larger in the lumbar region of the spinal cord compared to other regions.

4 CompB (0.1, 0.3 μM) shifted the concentration–effect curves of N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord to the right.

5 The i.v. injection of CompB (6.88 μmol kg^{-1}) significantly suppressed the N/OFQ-stimulated [^{35}S]GTPγS binding in the rat spinal cord and shifted the concentration–effect curve to the right, while it produced little inhibitory effect in the brain. The present study has shown that CompB may exhibit pharmacological effects through a predominant blockade of N/OFQ peptide receptors in the spinal cord under *in vivo* conditions.

British Journal of Pharmacology (2003) **139,** 1462–1468. doi:10.1038/sj.bjp.0705371

Keywords: N/OFQ peptide receptor; [^{3}H]N/OFQ binding; [^{35}S]GTPγS binding; spinal cord; CompB; *ex vivo* effect

Abbreviations: CompB, 1-[(3*R*,4*R*)-1-cyclooctylmethyl]-3-hydroxymethyl-4-piperidyl)-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one; GTPγS, guanosine-5′-*o*-(3-thio)triphosphate; N/OFQ, nociceptin/orphanin FQ

Introduction

Nociceptin/orphanin FQ (N/OFQ), a heptadecapeptide isolated from rat and porcine brain, has been identified as an endogenous ligand of N/OFQ peptide receptor, an orphan G protein-coupled receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Cox *et al*., 2000). The sequences of human, mouse and rat complementary DNA for the N/OFQ peptide receptor have indicated a significant degree of homology with the 'classical opioid' receptors (μ, δ and κ) (Bunzow *et al*., 1994; Mollereau *et al*., 1994; Wang *et al*., 1994; Wick *et al*., 1994). Despite this homology with opioid receptors, potent opioid ligands failed to bind to N/OFQ peptide receptors. N/OFQ itself has high homology with opioid peptides, particularly dynorphin A, the endogenous ligand of the κ-opioid receptor, but this peptide exhibits very low binding affinity for opioid receptors (Henderson & McKnight, 1997).

Pharmacological studies have shown that N/OFQ may be involved in a wide variety of physiological functions: pain and analgesia (Mogil *et al*., 1996), learning and memory (Manabe *et al*., 1998), locomotion (Florin *et al*., 1996), feeding (Pomonis *et al*., 1996), stress and anxiety (Jenck *et al*., 1997) and neuronal differentiation (Saito *et al*., 1997) in the central nervous system. It is of interest to note that local microinjection of N/OFQ into the brain and spinal cord of rats and mice produced opposite effects on the nociceptive response (Calo' *et al*., 2000). Also, the intrathecal (i.t.) injection of low doses of N/OFQ causes allodynia and hyperalgesia (Okuda-Ashitaka *et al*., 1996; Hara *et al*., 1997; Minami *et al*., 1997; Sakurada *et al*., 1999), while higher doses produce analgesia (Xu *et al*., 1996; Erb *et al*., 1997). In relation to these effects, the N/OFQ peptide receptor has been suggested to be present in the brain and spinal cord following *in situ* hybridization analysis (Bunzow *et al*., 1994; Wick *et al*., 1994), and in peripheral tissues such as the intestine, vas deferens, liver and spleen (Wang *et al*., 1994).

*Author for correspondence;
E-mail: yamada@ys7.u-shizuoka-ken.ac.jp
[3]Current address: Division of Geriatrics, Department of Internal Medicine, Saint Louis University School of Medicine, St Louis, MO 63104, U.S.A.
Advance online publication: 7 July 2003

Radioligand binding studies of the N/OFQ peptide receptor have been performed in brain tissues of rats, humans, mice, frogs and Chinese hamster ovary (CHO) cells expressing N/OFQ peptide receptors (Adapa & Toll, 1997; Makman *et al*., 1997; Benyhe *et al*., 1999). However, to our knowledge, little information has been published on N/OFQ peptide receptor characteristics in the spinal cord. It is considered that this tissue may play a significant role in the appearance of allodynia and antinociception by N/OFQ (Hara *et al*., 1997; Minami *et al*., 1997; Sakurada *et al*., 1999; Meunier, 2000; Lu *et al*., 2001; Yamamoto *et al*., 2001). Thus, N/OFQ peptide receptors in the spinal cord could be a pharmacological target tissue for agonists and antagonists of these receptors. Recently, selective antagonists of N/OFQ peptide receptors have been successfully synthesized and their pharmacological effects have been investigated (Kawamoto *et al*., 1999; Meunier, 2000; Shinkai *et al*., 2000; Halab *et al*., 2002; Yamada *et al*., 2002; McDonald *et al*., 2003). CompB (1-[(3*R*,4*R*)-1-cyclooctylmethyl]-3-hydroxymethyl-4-piperidyl)-3-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one) has been found as a potent and selective nonpeptidyl N/OFQ peptide receptor antagonist without agonistic effects on other opioid receptors (Kawamoto *et al*., 1999; Ozaki *et al*., 2000). Most previous studies on novel agonists and antagonists of N/OFQ peptide receptors have involved in *in vitro* radioreceptor binding and behavioral pharmacological effects. However, these studies have substantiated little to the direct *in vivo* interaction of these agents with N/OFQ peptide receptors after systemic administration. In particular, N/OFQ peptide receptor binding characteristics of drugs in the spinal cord have been examined very little so far. Thus, it could be of general importance to verify whether ligands bind to receptors under the influence of various pharmacokinetic and pharmacodynamic factors (Beauchamp *et al*., 1995; Yamada *et al*., 1998). Therefore, the present study was undertaken to characterize the *in vitro* and *ex vivo* effects of CompB on specific [^{3}H]N/OFQ binding and on N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord of rats.

Methods

Drugs and chemicals

[Leucyl-^{3}H]N/OFQ (5.55 TBq mmol^{-1}) was purchased from Amersham Int. plc (Buckinghamshire, U.K.). [^{35}S]GTPγS (46.3 TBq mmol^{-1}) was purchased from DuPont-NEN Co. Inc. (Boston, MA, U.S.A.). CompB was donated by Banyu Co. Ltd (Tsukuba, Japan). The following agents were purchased from the companies indicated: N/OFQ, Peptide Inst. Inc. (Osaka, Japan); [Phe$^1\Psi$(CH_2-NH)-Gly_2]N/OFQ(1–13)NH_2 and naltrindole, Research Biochemical International (Natick, MA, U.S.A.); nor-binaltorphimine, CTOP (d-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) and GDP, Sigma Chemical Co., Inc. (St Louis, MO, U.S.A.); GTPγS, Wako Pure Chemical Ind. Ltd (Osaka, Japan). All solutions were prepared freshly each day. All other chemicals were obtained from commercial sources.

Drug administration

Male Sprague–Dawley rats at 7–9 weeks of age (Japan SLC Inc., Shizuoka, Japan) were housed three or four per cage in the laboratory with free access to food and water, and maintained on a 12 h dark/light cycle in a room with controlled temperature (24±1°C) and humidity (55±5%). Rats were fasted for 16 h before i.v. injection of CompB, and administered i.v. with CompB (0.69, 2.29, 6.88 μmol kg^{-1}) dissolved in 0.9% NaCl. The control rats were similarly treated with the vehicle. At 0.5 or 2 h after the drug injection, rats were killed by drawing blood from the descending aorta under light anesthesia with diethyl ether, and the brain and spinal cord were perfused with 0.9% NaCl from the aorta. Then, the brain (minus cerebellum) and spinal cord were removed. In the experiment examining the regional distribution of [^{3}H]N/OFQ binding sites, the spinal cord was dissected into cervical, thoracic and lumbar regions. All the procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

[^{3}H]N/OFQ binding assay

The tissues (brain and spinal cord) were homogenized in 19 volumes of ice-cold 50 mM Tris-HCl buffer (pH: 7.4) containing 2 mM EDTA, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride (*p*-APMSF) (buffer A) with a Polytron homogenizer, and the homogenate was centrifuged at 500 × *g* for 10 min. The supernatant was centrifuged at 40,000 × *g* for 15 min. The pellet was finally suspended in ice-cold buffer A containing 2 mg ml^{-1} bovine serum albumin (buffer B) and used in [^{3}H]N/OFQ binding assays. All steps for the tissue preparation were performed at 4°C. Yamada *et al*. (1980) have previously shown that the dissociation of agents from receptor sites at 4°C was much slower than at 25°C or 37°C. Thus, in the *ex vivo* experiment with CompB, the dissociation of these agents from receptor sites during tissue preparation (homogenization and suspension) appears to be minimized. In fact, there was no large difference in [^{3}H]N/OFQ binding parameters (K_d and B_{max}) between one washout and two washouts of the rat brain membranes at 0.5 h after i.v. injection of CompB (6.88 μmol kg^{-1}). Protein concentrations were measured by the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Binding assay of [^{3}H]N/OFQ was performed similarly as reported previously by Yamada *et al*. (2002). Briefly, membrane fractions (400–600 μg protein) prepared from rat tissues were incubated with different concentrations (0.01–0.6 nM) of [^{3}H]N/OFQ in a total volume of 500 μl of buffer B. Incubation was carried out for 60 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fiber filters presoaked in 0.1% polyethyleneimine solution for 60 min, and the filters were rinsed three times with 2 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid (2 l toluene, 1 l Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene), and then determined using a liquid scintillation counter. The specific binding of [^{3}H]N/OFQ was determined experimentally from the difference between counts in the absence and presence of 1 μM N/OFQ. All assays were conducted in duplicate.

[^{35}S]GTPγS binding assay

The [^{35}S]GTPγS binding assay was performed by a modification of the procedure described by Narita *et al*. (1999). Briefly, whole brain and spinal cord were homogenized in 19 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl using a Polytron homogenizer, and the homogenate was centrifuged at $500 \times g$ for 10 min. The supernatant was centrifuged at $40{,}000 \times g$ for 15 min. The pellet was finally suspended in ice-cold buffer and used in [^{35}S]GTPγS membrane assays. All steps were performed at 4°C. In the [^{35}S]GTPγS binding assay, membrane fractions (40–60 μg protein) prepared from rat tissues were incubated with various concentrations (0.03–3 μM) of N/OFQ, 100 μM GDP and 100 pM [^{35}S]GTPγS in a total volume of 1 ml of buffer. Incubation was carried out for 120 min at 25°C. The reaction was terminated by rapid filtration (Cell Harvester, Brandel Co., Gaithersburg, MD, U.S.A.) through Whatman GF/B glass fiber filters, and the filters were rinsed three times with 2 ml of ice-cold buffer. Tissue-bound radioactivity was extracted from the filters overnight in scintillation fluid (21 toluene, 1 l Triton X-100, 15 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene), and then determined using a liquid scintillation counter. The specific binding of [^{35}S]GTPγS was determined experimentally from the difference between counts in the absence and presence of 10 μM unlabelled GTPγS. All assays were conducted in duplicate.

Data analysis

The analysis of binding data was performed as described previously (Yamada *et al*., 1980). The apparent dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]N/OFQ (0.01–0.6 nM) were estimated by Rosenthal analysis of the saturation data (Rosenthal, 1967). The ability of N/OFQ, its analogs and opioid ligands to inhibit the specific binding of [^{3}H]N/OFQ (0.06 nM) was estimated from the IC_{50} values, which are the molar concentrations of unlabeled drug necessary to displace 50% of the specific binding of [^{3}H]N/OFQ (determined by log probit analysis). The inhibition constant, K_i was calculated from the equation, $K_i = IC_{50}/(1 + L/K_d)$, where L equals the concentration of [^{3}H]N/OFQ. Hill coefficients for the inhibition by N/OFQ, its analog and opioid ligands were obtained by Hill plot analysis. The pK_B values were calculated using the equation of Van Rossum *et al*. (1963): $pK_B = \log(r-1) - \log[\text{antagonist}]$, where r is the ratio of EC_{50} values of the agonist with and without antagonist.

The data are presented as the mean ± s.e.m. of three to six rats. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, and a value of $P < 0.05$ was considered significant.

Results

In vitro *inhibitory effects of CompB and other agents on specific [^{3}H]N/OFQ binding*

Specific binding of [^{3}H]N/OFQ at concentrations of 0.01–0.6 nM in rat brain and spinal cord was saturable and Rosenthal analysis showed a linear plot, suggesting a single population of binding sites with K_d values in the brain and spinal cord of 46.9 (brain) and 37.2 (spinal cord) pM. These values agreed well with the K_d value (50 pM) in brain membranes previously reported (Adapa & Toll, 1997). CompB (1–100 nM), N/OFQ (0.03–3 nM), [Phe$^1\Psi$(CH$_2$-NH)-Gly^2]N/OFQ(1–13)NH$_2$ (0.1–10 nM), naltrindole (1–100 μM) and nor-binaltorphimine (1–300 μM) inhibited specific [^{3}H]N/OFQ (0.06 nM) binding to rat brain and spinal cord in a concentration-dependent manner, in the order: N/OFQ > [Phe$^1\Psi$(CH$_2$-NH)-Gly^2]N/OFQ (1–13)NH$_2$ > CompB ≫ naltrindole > nor-binaltorphimine (Table 1). CTOP (0.1–10 μM) produced little inhibition of specific [^{3}H]N/OFQ binding in either tissue. The K_i value for CompB in the spinal cord was significantly lower than that in the brain. The Hill slope for each agent was close to unity.

Rosenthal analysis indicated that there was significant (152%) increase in the K_d value for brain [^{3}H]N/OFQ binding in the presence of CompB (10 nM) with little change in the B_{max} value, compared with the control values in the absence of this agent (Figure 1, Table 2). Furthermore, CompB (10 nM) caused a significant (159%) increase in the K_d value for spinal cord [^{3}H]N/OFQ binding with a slight but significant (17 %) reduction in the B_{max} value.

Table 1 *In vitro* inhibition by CompB, N/OFQ, its analog and opioid ligands of specific [^{3}H]N/OFQ binding in the brain and spinal cord of rats

Drugs	*Hill coefficients*	K_i *values* (*n*M)
Brain		
CompB	0.98 ± 0.06	7.11 ± 0.43
N/OFQ	1.12 ± 0.04	0.27 ± 0.01
[Phe$^1\Psi$(CH$_2$-NH)-Gly^2]-N/OFQ(1–13)NH$_2$	1.19 ± 0.03	1.03 ± 0.09
Naltrindole	0.91 ± 0.02	8435 ± 581
nor-Binaltorphimine	0.85 ± 0.07	34958 ± 2681
Spinal cord		
CompB	0.95 ± 0.05	4.02 ± 0.52
N/OFQ	1.09 ± 0.06	0.12 ± 0.01
[Phe$^1\Psi$(CH$_2$-NH)-Gly^2]-N/OFQ(1–13)NH$_2$	1.08 ± 0.01	0.58 ± 0.03
Naltrindole	0.96 ± 0.06	4310 ± 202
nor-Binaltorphimine	0.82 ± 0.03	10737 ± 1140

Drug-inhibition studies were performed with [^{3}H]N/OFQ (0.06 nM) in the brain and spinal cord of rats. Values are mean ± s.e.m. of three to four rats.

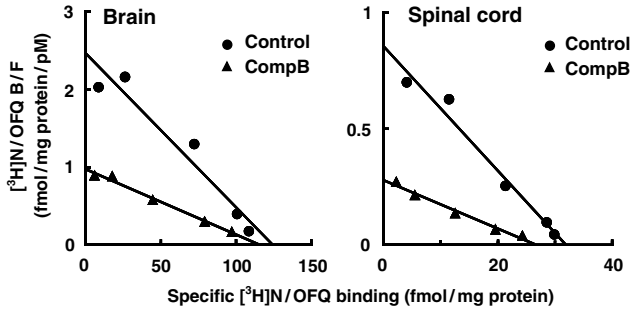

Figure 1 Rosenthal plots of specific [^{3}H]N/OFQ binding in the brain and spinal cord of rats in the absence (control) and presence of CompB. Specific binding of [^{3}H]N/OFQ (0.01–0.6 nM) in rat tissues was measured in the absence and presence of CompB (10 nM). Values are means of four rats.

Table 2 K_d and B_{max} values for specific [^{3}H]N/OFQ binding in the brain and spinal cord of rats in the absence and presence of CompB

	K_d values (pM)	B_{max} values (fmol mg protein^{-1})
Brain		
Control	46.9±1.0	123±5
CompB (10 nM)	118±7***	114±6
Spinal cord		
Control	37.2±3.2	31.8±0.5
CompB (10 nM)	96.3±3.5***	26.3±0.9**

Rosenthal analysis was performed with [^{3}H]N/OFQ (0.01–0.6 nM) binding in the brain and spinal cord of rats, in the absence and presence of CompB (10 nM). Values are mean±s.e.m. of four rats. Asterisks show a significant difference from the control values, $^{**}P<0.01$, $^{***}P<0.001$.

Effect of i.v. injection of CompB on specific [^{3}H]N/OFQ binding

The effects of i.v. injection of CompB on specific [^{3}H]N/OFQ binding in the rat brain and spinal cord were investigated. As shown in Figure 2 and Table 3, there was a dose-dependent increase in K_d values for specific [^{3}H]N/OFQ binding in both tissues, 0.5 h after i.v. injection of CompB at doses of 0.69, 2.29 and 6.88 μmol kg^{-1}, compared with the control values. The increased rates (59.1, 139 and 417%, respectively) in the spinal cord at these doses were constantly greater than those (31.3, 91.8 and 211%, respectively) in the brain. The increased rate at the i.v. dose of 6.88 μmol kg^{-1} was significantly ($P<0.05$) larger in the spinal cord than in the brain. Further, even at 2 h after i.v. injection of this dose of CompB, there was still a significant increase (42.3%) in the K_d for specific [^{3}H]N/OFQ binding only in the spinal cord (data not shown). On the other hand, B_{max} values for [^{3}H]N/OFQ binding in the brain and spinal cord of these CompB-injected rats were comparable to the control values (Table 3). Further, the effects of CompB on specific [^{3}H]N/OFQ binding were examined in three regions of rat spinal cord. As shown in Table 4, an i.v. injection of CompB (6.88 μmol kg^{-1}) produced a significant increase in K_d values for specific [^{3}H]N/OFQ binding in each region of rat spinal cord with no effect on the B_{max} values, and enhancement in the lumbar region (543%) was significantly larger than that (each 289%) in the cervical or thoracic regions.

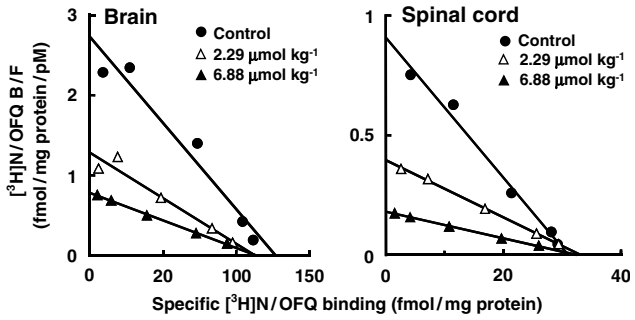

Figure 2 Rosenthal plots of specific [^{3}H]N/OFQ binding in the brain and spinal cord of vehicle-(control) and CompB-administered rats. Rats received CompB (2.29 and 6.88 μmol kg^{-1} i.v.), and were killed at 0.5 h after the administration. Each point represents the mean of six control- and four or five CompB-treated rats.

Effects on N/OFQ-stimulated [^{35}S]GTPγS binding

N/OFQ (0.03–3 μM) stimulated specific [^{35}S]GTPγS binding in the brain and spinal cord of rats in a concentration-dependent manner, and CompB at concentrations of 0.1 and 0.3 μM shifted the concentration–effect curves of N/OFQ-stimulated [^{35}S]GTPγS binding to the right (Figure 3). The pK_B values of CompB for the competitive inhibition of N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord were 7.79±0.16 and 7.69±0.17, respectively. There was little difference between these tissues in the inhibitory potency of CompB.

The i.v. injection of CompB (6.88 μmol kg^{-1}, 0.5 h later) significantly suppressed the N/OFQ-stimulated [^{35}S]GTPγS binding in the rat spinal cord and it shifted the concentration–effect curve to the right (Figure 4b). On the other hand, in the brains of these CompB-injected rats, there was little change in N/OFQ-stimulated [^{35}S]GTPγS binding (Figure 4a).

Discussion

Binding characteristics of CompB to N/OFQ peptide receptors and its effect on N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord of rats were examined *in vitro* and *ex vivo*. N/OFQ and [Phe$^1\Psi$(CH$_2$-NH)-Gly^2]N/OFQ(1–13)NH$_2$ were the most potent inhibitors of specific [^{3}H]N/OFQ binding *in vitro*, whereas the binding was inhibited or unaffected by extremely high concentrations of opioid ligands. Thus, specific [^{3}H]N/OFQ binding to crude membranes of the brain and spinal cord exhibited pharmacological specificity that characterized N/OFQ peptide receptors, in accordance with previous observations (Adapa & Toll, 1997). In agreement with previous observations using recombinant CHO cell membranes (Ozaki *et al*., 2000), it was shown that CompB bound to N/OFQ peptide receptors with very high (nanomolar) affinity in the brain and spinal cord of rats, with much greater affinity than opioid ligands such as naltrindole, and thus displayed a high degree of selectivity. This agent appeared to be the most potent inhibitor of N/OFQ peptide receptors, when compared with other antagonists of N/OFQ peptide receptors currently reported (Meunier, 2000; Ozaki *et al*., 2000; Halab *et al*., 2002; Yamada *et al*., 2002). Rosenthal analysis revealed that CompB increased K_d values for [^{3}H]N/OFQ binding in the brain and spinal cord, and that it caused a

Table 3 K_d and B_{max} values for specific [^{3}H]N/OFQ binding in the brain and spinal cord of rats at 0.5 h after i.v. injection of CompB

	Doses (μmol kg^{-1})	K_d *values* (pM)	B_{max} *values* (fmol mg protein^{-1})
Brain			
Control		45.0±1.7	122±4
CompB	0.69	59.1±7.5	112±5
	2.29	86.3±10.3**	110±9
	6.88	140±12***	113±7
Spinal cord			
Control		35.0±2.6	31.5±1.0
CompB	0.69	55.7±4.3**	41.5±4.5
	2.29	83.8±4.6***	32.5±3.2
	6.88	181±6***,$^+$	32.3±2.6

Rats received 0.69, 2.29 and 6.88 μmol kg^{-1} (i.v.) of CompB, and were killed 0.5 h after the injection. Specific binding of [^{3}H]N/OFQ (0.01–0.6 nM) in rat tissues was measured. Values are mean±s.e.m. of six control rats and four to five CompB-treated rats. Asterisks show significant differences from the control values, $^{**}P<0.01$, $^{***}P<0.001$. The symbol ($^+$) indicates a significant difference from the increase rate (%) of the K_d value in the brain at the same dose, $^+P<0.05$.

Table 4 K_d and B_{max} values for specific [^{3}H]N/OFQ binding in each region of rat spinal cord at 0.5 h after i.v. injection of CompB

Spinal cord regions	K_d *values* (pM)	B_{max} *values* (fmol mg protein^{-1})
Cervical region		
Control	28.8±0.9	29.3±1.0
CompB	112±2***	28.8±2.9
Thoracic region		
Control	35.7±2.4	30.7±2.0
CompB	139±10***	28.1±1.8
Lumbar region		
Control	22.1±1.6	28.0±0.8
CompB	142±9***,†	29.4±0.7

Rats received 6.88 μmol kg^{-1} (i.v.) of CompB, and were killed at 0.5 h after the injection. Specific binding of [^{3}H]N/OFQ (0.01 – 0.6 nM) in rat spinal cord regions was measured. Values are mean±s.e.m. of five control rats and three to four CompB-treated rats. Asterisks show a significant difference from the control values, $^{***}P<0.001$. The symbol (†) indicates a significant difference from the increase rate (%) of the K_d value in the cervical or thoracic region, $^+P<0.05$.

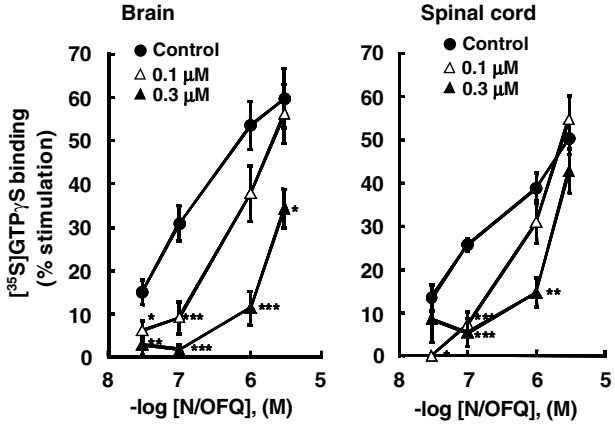

Figure 3 Concentration–effect curves of N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord of rats in the absence (control) and presence of CompB. The ordinate is per cent stimulation over basal [^{35}S]GTPγS binding in the absence of N/OFQ. The abscissa is −logarithmic molar concentrations of N/OFQ (0.03–3 μM). Each point represents the mean±s.e.m. of six control and four or five CompB-treated rats. Asterisks show a significant difference from the control values, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

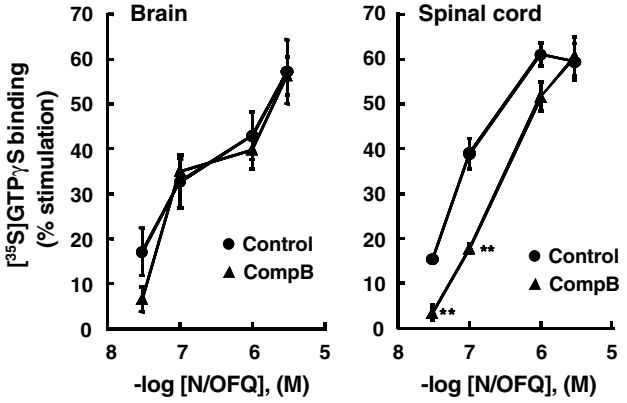

Figure 4 Concentration–effect curves of N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord of vehicle- (control) and CompB-administered rats. Rats received CompB (6.88 μmol kg^{-1}, i.v.), and were killed at 0.5 h after the administration. The ordinate is per cent stimulation over basal [^{35}S]GTPγS binding in the absence of N/OFQ. The abscissa is −logarithmic molar concentrations of N/OFQ (0.03–3 μM). Each point represents mean±s.e.m. of four rats. Asterisks show a significant difference from the control values, $^{**}P<0.01$.

concomitant reduction of the B_{max} value only in the spinal cord. This observation may indicate that CompB binds to brain N/OFQ peptide receptors in a competitive and reversible manner and that this agent binds, partially in a noncompetitive manner in addition to a competitive manner, to spinal cord receptors, suggesting higher affinity of CompB in spinal cord N/OFQ peptide receptors.

Ozaki *et al.* (2000) have shown that subcutaneous (s.c.) administration of CompB at doses of 6.88–68.8 μmol kg^{-1} dose-dependently suppresses hyperalgesia elicited by intracerebroventricular (i.c.v.) injection of N/OFQ in a tail-flick test with mice. In accord with this *in vivo* pharmacological finding, the i.v. injection of lower doses (0.69–6.88 μmol kg^{-1}) of CompB produced dose-dependent increases of K_d values for [^{3}H]N/OFQ binding in the brain and spinal cord of rats. The enhancement by CompB in both tissues was significant 0.5 h after the i.v. injection, except in the brain at the dose of 0.69 μmol kg^{-1}. Further, the extent and duration of enhancement by CompB (6.88 μmol kg^{-1}) were significantly greater in the spinal cord than in the brain. These *ex vivo* data seem to agree with the *in vitro* reduction by CompB in the number

(B_{max}) of [^{3}H]N/OFQ binding sites in the spinal cord. Therefore, it is conceivable that the N/OFQ peptide receptor-binding activity of CompB is significantly greater and of a longer duration in the spinal cord than in the brain under *in vivo* conditions.

It is well known that a stimulation by agonists such as N/OFQ, of G-protein-coupled receptors, results in increased amounts of specific [^{35}S]GTPγS binding in brain membranes (Narita *et al*., 1999). In the present study, CompB competitively antagonized N/OFQ-stimulated [^{35}S]GTPγS binding in the brain and spinal cord of rats with approximately equal potency *in vitro*. Moreover, i.v. injection of CompB at the dose of 6.88 μmol kg^{-1} shifted the concentration–response curve for N/OFQ-stimulated [^{35}S]GTPγS binding in the spinal cord to the right, suggesting effective antagonism of N/OFQ-mediated physiological responses. However, N/OFQ-stimulated [^{35}S]GTPγS binding in the brain was suppressed little by i.v. injection of CompB, in spite of a significant degree of *ex vivo* binding to N/OFQ peptide receptors and of *in vitro* competitive inhibition of the N/OFQ-stimulated [^{35}S]GTPγS binding. Although the apparent discrepancy for these results needs to be investigated further, it could be considered that the extent of N/OFQ peptide receptor occupancy of CompB in the brain was not sufficient enough to suppress the N/OFQ-mediated response. In other words, the extent of receptor binding might be below the threshold of N/OFQ peptide receptor occupancy by antagonists to inhibit N/OFQ-mediated physiological response significantly. Alternatively, the N/OFQ-stimulated [^{35}S]GTPγS binding in the brain is expected to be influenced by multiple endogenous factors under *in vivo* conditions, because physiological functions mediated through G-protein-coupled receptors are generally regulated in a more complicated manner in the brain than in the spinal cord. Also, it may be speculated that the observed difference between the brain and spinal cord in the *ex vivo* binding and antagonistic activities of CompB in N/OFQ peptide receptors may be attributable to a significant distinction between these sites in pharmacokinetic factors determining the actual concentration of this agent in the vicinity of N/OFQ peptide receptors. They include the biological stability and the amount of delivery to the receptor site depending on the extent and/or rate of CompB transported (influx, efflux) through the blood–brain barrier or through the blood–cerebrospinal barrier of rats. Until the concentration of CompB in the brain and spinal cord after the systemic injection is definitely known, therefore, we should continue to interpret relative greater potency of CompB in the spinal cord N/OFQ peptide receptors with caution. Interestingly, Narita *et al*. (1999) have found high levels of N/OFQ-stimulated [^{35}S]GTPγS binding in the mouse lumbar spinal cord, which corresponds to the areas in which N/OFQ-like immunoreactive fibers are concentrated. Inasmuch as i.v. injection of CompB bound more extensively to N/OFQ peptide receptors in the lumbar region rather than in other regions (Table 4), this agent may predominantly manipulate physiological responses mediated by the stimulation of N/OFQ peptide receptors in the lower region of the spinal cord.

The i.c.v. and i.t. injections of N/OFQ have been reported to elicit various physiological responses possibly through interactions with N/OFQ peptide receptors (Meunier, 2000). In fact, there seems to be a significant difference in pharmacological effects produced by i.c.v. and i.t. injections of N/OFQ in rodents. The spinal and supraspinal contribution of N/OFQ peptide receptor systems may differ quantitatively and qualitatively with pain modalities (Yamamoto *et al*., 2001). Recently, Yamamoto *et al*. (2001) have shown that the analgesic effect mediated by N/OFQ was antagonized by the i.t. injection of CompB with 10 times larger potency than by the i.c.v. injection. Such greater sensitivity of CompB in the spinal cord than in the supraspinal brain sites may be well explained by our data obtained in the present study. Calo' *et al*. (2000) have reported that the local microinjection of N/OFQ into the brain and spinal cord of rats has apparently opposing effects on the nociceptive response. Further, opposite spinal effects of N/OFQ, that is, pronociceptive *versus* antinociceptive, have been reported. The i.t. injection of low doses (picogram or nanogram order) of N/OFQ cause allodynia and hyperalgesia (Okuda-Ashitaka *et al*., 1996; Hara *et al*., 1997; Minami *et al*., 1997; Sakurada *et al*., 1999), while higher doses produce analgesia (Xu *et al*., 1996; Erb *et al*., 1997). Jia *et al*. (1998) have reported that [^{3}H]N/OFQ binding in the rat spinal cord is significantly increased during persistent peripheral inflammation. Taken together, the results obtained here suggest that CompB could be useful for exhibiting the pharmacological effects against peripheral inflammation possibly through the relatively selective blockade of N/OFQ peptide receptors in the spinal cord. In conclusion, the present study has provided the first *in vivo* evidence that the systemic injection of relatively low doses of CompB binds significantly to N/OFQ peptide receptors in the rat spinal cord.

We thank Drs S. Ozaki and H. Ohta of Banyu Co. Ltd (Tsukuba, Japan) for providing CompB and for helpful discussions.

References

ADAPA, I.D. & TOLL, L. (1997). Relationship between binding affinity and functional activity of nociceptin/orphanin FQ. *Neuropeptides,* **31,** 403–408.

BEAUCHAMP, H.T., CHANG, R.S.L., SIGEL, P.K.S. & GIBSON, R.E. (1995). *In vivo* receptor occupancy of the angiotensin II receptor by nonpeptide antagonists: Relationship to *in vitro* affinities and *in vivo* pharmacologic potency. *J. Pharmacol. Exp. Ther.,* **272,** 612–618.

BENYHE, S., MONORY, K., FARKAS, J., TOTH, G., GUERRINI, R., SALVADORI, S., OROSZ, G., WOLLEMANN, M. & BORSODI, A. (1999). Nociceptin binding sites in frog (*Rana esculenta*) brain membranes. *Biochem. Biophys. Res. Commun.,* **260,** 592–596.

BUNZOW, J.R., SAEZ, C., MORTRUD, M., BOUVIER, C., WILLIAMS, J.T., LOW, M. & GRANDY, DK. (1994). Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. *FEBS Lett.,* **347,** 284–288.

CALO, G., GUERRINI, R., RIZZI, A., SALVADORI, S. & REGOLI, D. (2000). Pharmacology of nociceptin and its receptor: a novel therapeutic target. *Br. J. Pharmacol.,* **129,** 1261–1283.

COX, B.M., CHAVKIN, C., CHRISTIE, M.J., CIVELLI, O., EVANS, C., HAMON, M.D., HOELLT, V., KIEFFER, B., KITCHEN, I., MCKNIGHT, A.T., MEUNIER, J.C. & PORTOGHESE, P.S. (2000). Opioid receptors. In : *The IUPHAR Compendium of Receptor Characterizaion and Classification* ed. D. Girdlestone, pp. 321–333. London: IUPHAR Media Ltd.

ERB, K., LIEBEL, J.T., EGEDER, I., ZEILHOFER, H.U., BRUNE, K. & GEISSLINGER, G. (1997). Spinally delivered nociceptin/orhanin FQ reduces flinching behavior in the rat formalin test. *Neurol. Rep.,* **8,** 1967–1970.

FLORIN, S., SUAUDEAU, C., MEUNIER, J.-C. & COSTENTIN, J. (1996). Nociceptin stimulates locomotion and exploratory behaviour in mice. *Eur. J. Pharmacol.,* **317,** 9–13.

HALAB, L., BECKER, J.A., DARULA, Z., TOURWE, D., KIEFFER, B.L., SIMONIN, F. & LUBELL, W.D. (2002). Probing opioid receptor interactions with azacycloalkane amino acids. Synthesis of a potent and selective ORL1 antagonist. *J. Med. Chem.,* **45,** 5353–5357.

HARA, N., MINAMI, T., OKUDA-ASHIAKA, E., SUGIMOTO, T., SAKAI, M., ONAKA, M., MORI, H., IMANISHI, T., SHINGU, K. & ITO, S. (1997). Characterization of nociceptin hyperalgesia and allodynia in conscious mice. *Br. J. Pharmacol.,* **121,** 401–408.

HENDERSON, G. & MCKNIGHT, A.T. (1997). The orphan opioid receptor and its endogenous ligand-nociceptin/orphanin FQ. *Trends Pharmacol. Sci.,* **18,** 293–300.

JENCK, F., MOEAU, J.-L., MARTIN, J.R., KILPATRICK, G.J., REINSCHEID, R.K., MONSMA, F.J., NOTHACKER, H.-P. & CIVELLI, O. (1997). Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 14854–14858.

JIA, Y., LINDEN, D.R., SERIE, J.R. & SEYBOLD, V. (1998). Nociceptin/orphanin FQ binding increases in superficial laminae of the rat spinal cord during persistent peripheral inflammation. *Neurosci. Lett.,* **250,** 21–24.

KAWAMOTO, H., OZAKI, S., ITOH, Y., MIYAJI, M., ARAI, S., NAKASHIMA, H., KAO, T., OHTA, H. & IWASAWA, Y. (1999). Discovery of the first potent and selective small molecule opioid receptor-like (ORL1) antagonist: 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl - 1,3- dihydro-2*H*-benzimidazol-2-one (J-113397). *J. Med. Chem.,* **42,** 5061–5063.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

LU, J.T., HUANG, Y.H., PALMER, P.P., XIE, G.X., GABRIEL, A., GROND, S. & YU, L.C. (2001). Blockade effects of (Nphe1)nociceptin(1-13)-NH_2 on anti-nociceptin induced by intrathecal administration of nociceptin in rats. *Regul. Pept.,* **101,** 81–85.

MAKMAN, M.H., LYMAN, W.D. & DVOKIN, B. (1997). Presence and characterization of nociceptin (orphanin FQ) receptor binding in adult rat and human fetal hypothalamus. *Brain Res.,* **762,** 247–250.

MANABE, T., NODA, Y., MAMIYA, T., KATAGIRI, H., HOUTANI, T., NISHI, M., NODA, T., TAKAHASHI, T., SUGIMOTO, T., NABESHIMA, T. & TAKESHIMA, H. (1998). Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. *Nature,* **394,** 577–581.

MCDONALD, J., CALO, G., GUERRINI, R. & LAMBERT, D.G. (2003). UFP-101, a high affinity antagonist for the nociceptin/orphanin FQ receptor: radioligand and GTPγ^{35}S binding studies. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **367,** 183–187.

MEUNIER, J.C. (2000). The potential therapeutic value of nociceptin receptor agonists and antagonists. *Exp. Opin. Ther. Patents,* **10,** 371–388.

MEUNIER, J.C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.-L., GUILLEMOT, J.C., FERRARA, P., MONSARRAT, B., MAZARGUIL, H., VASSART, G., PARMENTIER, M. & COSTENTIN, J. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1 receptor. *Nature,* **377,** 532–535.

MINAMI, T., OKUDA-ASHITAKA, E., NISHIZAWA, M., MORI, H. & ITO, S. (1997). Inhibition of nociceptin-induced allodynia in conscious mice by prostaglandin D_2. *Br. J. Pharmacol.,* **122,** 605–610.

MOGIL, J.S., GRISEL, J.E., REINSCHEID, R.K., CIVELLI, O., BELKNAP, J.K. & GRANDY, D.K. (1996). Orphanin FQ is a functional anti-opioid peptide. *Neuroscience,* **75,** 333–337.

MOLLEREAU, C., PARMENTIER, M., MAILLEUX, P., BUTOUR, J.L., MOISAND, C., CHALON, P., CAPUT, D., VASSART, G. & MEUNIER, J.C. (1994). ORL1, a novel member of the opioid receptor family: cloning, functional expression and localization. *FEBS Lett.,* **341,** 33–38.

NARITA, M., MIZOGUCHI, H., OJI, D.E., DUN, N.J., HWANG, B.H., NAGASE, H. & TSENG, L.F. (1999). Identification of the G-protein-coupled ORL1 receptor in the mouse spinal cord by [^{35}S]-GTPγS binding and immunohistochemistry. *Br. J. Pharmacol.,* **128,** 1300–1306.

OKUDA-ASHITAKA, E., TACHIBANA, S., HOUTANI, T., MINAMI, T., MASU, Y., NISHI, M., TAKESHIMA, H., SUGIMOTO, T. & ITO, S. (1996). Identification and characterization of an endogenous ligand for opioid receptor homologue ROR-C: its involvement in allodynic response to innocuous stimulus. *Brain. Res. Mol. Brain Res.,* **43,** 96–104.

OZAKI, S., KAWAMOTO, H., ITOH, Y., MIYAJI, M., AZUMA, T., ICHIKAWA, D., NAMBU, H., IGUCHI, T., IWASAWA, Y. & OHTA, H. (2000). *In vitro* and *in vivo* pharmacological characterization of J-113397, a potent and selective non-peptidyl ORL1 receptor antagonist. *Eur. J. Pharmacol.,* **402,** 45–53.

POMONIS, J.D., BILLINGTON, C.J. & LEVINE, A.S. (1996). Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. *Neurol. Rep.,* **8,** 369–371.

REINSCHEID, R.K., NOTHACKER, H.-P., BOURSON, A., ARDTI, A., HENNINGSEN, R.A., BUNZOW, J.R., GRANDY, D.K., LANGEN, H., MONSMA, JR, F.J. & CIVELLI, O. (1995). Orphanin FQ: a neuropeptide that activates an opioid like G protein-coupled receptor. *Science,* **270,** 792–794.

ROSENTHAL, H.E. (1967). Graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.,* **20,** 525–532.

SAITO, Y., MARUYAMA, K., SAIDO, T.C. & KAWASHIMA, S. (1997). Overexpression of a neuropeptide nociceptin/orphanin FQ precursor gene, N23K/N27K, induces neurite outgrowth in mouse NS20Y cells. *J. Neurosci. Res.,* **48,** 397–406.

SAKURADA, C., SAKURADA, S., KATSUYAMA, S., SASAKI, J., TAN-NO, K. & SAKURADA, T. (1999). Involvement of tachykinin NK1 receptors in nociceptin-induced hyperalgesia in mice. *Brain Res.,* **841,** 85–92.

SHINKAI, H., ITO, T., IIDA, T., KITAO, Y., YAMADA, H. & UCHIDA, I. (2000). 4-Aminoquinolines: novel nociceptin antagonists with analgesic activity. *J. Med. Chem.,* **43,** 4667–4677.

VAN ROSSUM, J.M., HURKMANS, J.A.T.M. & WOLTERS, C.J.J. (1963). Cumulative dose–response curves II. Techniques for the making of dose–response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn.,* **143,** 299–330.

WANG, J.B., JOHNSON, P.S., IMAI, Y., PERSICO, A.M., OZENBERER, B.A., EPPLER, C.M. & UHL, G.R. (1994). cDNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS Lett.,* **348,** 75–79.

WICK, M.J., MINNERATH, S.R., LIN, X., ELDE, R., LAW, P.Y. & LOH, H.H. (1994). Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors. *Brain Res. Mol. Brain Res.,* **27,** 37–44.

XU, X.J., HAO, X. & WIESENFELD-HALLIN, Z. (1996). Nociceptin or antinociceptin: potent spinal antinocicepive effect of orphanin FQ/nociceptin in the rat. *Neurol. Rep.,* **7,** 2092–2094.

YAMADA, H., NAKAMOTO, H., SUZUKI, Y., ITO, T. & AISAKA, K. (2002). Pharmacological profiles of a novel opioid receptor-like1 (ORL_1) receptor antagonist, JTC-801. *Br. J. Pharmacol.,* **135,** 323–332.

YAMADA, S., UCHIDA, S. & KIMURA, R. (1998). Integration of pharmacokinetics and pharmacodynamics in drug development–*in vivo* receptor occupancy by calcium antagonist and angiotensin II receptor antagonist. *Curr. Top. Pharmacol. (Res. Trends),* **4,** 239–250.

YAMADA, S., YAMAMURA, H.I. & ROESKE, W.R. (1980). Characerization of *alpha*-1 adrenergic receptors in the heart using [^{3}H]WB4101: effect of 6-hydroxydopamine treatment. *J. Pharmacol. Exp. Ther.,* **215,** 176–185.

YAMAMOTO, T., SAKASHITA, Y. & NOZAKI-TAGUCHI, N. (2001). Antagonism of ORL1 receptor produces an algesic effect in the rat formalin test. *Neurol. Rep.,* **12,** 1323–1327.

(Received April 14, 2003
Accepted May 9, 2003)