British Journal of Pharmacology (2005) 146, 33–40
www.nature.com/bjp

Nonphotic entrainment of the circadian body temperature rhythm by the selective ORL1 receptor agonist W-212393 in rats

*,[1]Koji Teshima, [1]Masanori Minoguchi, [1]Sayuri Tounai, [1]Atsuyuki Ashimori, [1]Junichi Eguchi, [2]Charles N. Allen & [3]Shigenobu Shibata

[1]Research Laboratory I (CNS), Pharmaceutical Research Unit, R&D Division, Mitsubishi Pharma Corporation, 3-7-25, Koyata, Iruma, Saitama 358-0026, Japan; [2]Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239-3098, U.S.A. and [3]Department of Pharmacology, School of Science and Engineering, Waseda University, 2-7-5, Higashifushimi, Nishitokyo, Tokyo 202-0021, Japan

1 We synthesized a small-molecule opioid receptor-like 1 (ORL1) receptor agonist, 2-{3-[1-((1*R*)-acenaphthen-1-yl)piperidin-4-yl]-2,3-dihydro-2-oxo-benzimidazol-1-yl}-*N*-methylacetamide (W-212393), and investigated its effect on the circadian body temperature rhythm of rats.

2 W-212393 has high affinity for ORL1 receptors in the rat cerebral cortex and human ORL1 receptors expressed in HEK293 cells with K_i values of 0.76 and 0.50 nM, respectively.

3 W-212393 concentration-dependently stimulated GTPγ^{35}S binding and its efficacy was similar to nociceptin/orphanin FQ (N/OFQ), suggesting that W-212393 is a full agonist at ORL1 receptors.

4 W-212393 dose-dependently occupied ORL1 receptors following intraventricular or intraperitoneal administration, suggesting that W-212393 is a brain-penetrating compound.

5 W-212393 (100 nM) and N/OFQ (100 nM) significantly suppressed the activity of spontaneously firing rat suprachiasmatic nucleus neurons. These suppressive effects were blocked by an ORL1 receptor antagonist, J-113397 (1 μM).

6 W-212393 (3 mg kg^{-1}, i.p.) induced a significant phase advance at circadian time 6 (CT6) and CT9, but not at other CTs. The magnitude of the W-212393 (0.3–3 mg kg^{-1}, i.p.)-induced phase advance was dose-dependent and greater than those produced by 8-hydroxy-2-(di-*n*-propylamino)tetralin (0.3–3 mg kg^{-1}, i.p.) or melatonin (0.3–3 mg kg^{-1}, i.p.). The W-212393 (3 mg kg^{-1}, i.p.)-induced phase advance was antagonized by J-113397 (10 mg kg^{-1}, i.p.).

7 W-212393 (3 mg kg^{-1}, i.p.) significantly accelerated the re-entrainment of the body temperature rhythm to a 6 h advanced light–dark cycle.

8 These results indicate that activation of ORL1 receptors contributes to the circadian entrainment and W-212393 may represent an interesting agent for the study of circadian rhythms.
British Journal of Pharmacology (2005) **146,** 33–40. doi:10.1038/sj.bjp.0706311;
published online 27 June 2005

Keywords: ORL1; nociceptin; orphanin FQ; circadian rhythm; nonphotic entrainment; SCN; W-212393

Abbreviations: CT, circadian time; N/OFQ, nociceptin/orphanin FQ; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; ORL1, opioid receptor-like 1; SCN, suprachiasmatic nucleus; ZT, zeitgeber time

Introduction

The opioid receptor-like 1 (ORL1) receptor, also referred to as NOP receptor, was cloned as the fourth member of the opioid receptor family (Bunzow *et al*., 1994; Mollereau *et al*., 1994). Various physiological roles have been ascribed to the nociceptin/orphanin FQ (N/OFQ)-ORL1 receptor system since the endogenous agonist, a heptadecapeptide named nociceptin (Meunier *et al*., 1995) or orphanin FQ (Reinscheid *et al*., 1995) (N/OFQ), was identified. The primary focus of N/OFQ research has been in the traditional opiate field of pain and analgesia (Mogil & Pasternak, 2001; Ko *et al*., 2002; Courteix *et al*., 2004). N/OFQ and ORL1 receptors mediate anxiolytic and antistress effects (Jenck *et al*., 1997; 2000), impair spatial learning (Sandin *et al*., 1997), block the rewarding potential of abused drugs (Sakoori & Murphy, 2004) or alcohol (Ciccocioppo *et al*., 2003; 2004; Kuzmin *et al*., 2003), and facilitate feeding (Pomonis *et al*., 1996).

The ORL1 receptor is highly expressed in the rat suprachiasmatic nucleus (SCN) and N/OFQ suppresses the activity of 88% of SCN neurons (Allen *et al*., 1999). Although it is well established that melatonin (Redman *et al*., 1983; Sack *et al*., 1991; Benloucif & Dubocovich, 1996) and 5-HT$_{1A}$ receptor agonists (Tominaga *et al*., 1992; Horikawa & Shibata, 2004) phase-shift circadian rhythms, merely 39 and 28% of SCN neurons respond to melatonin (Jiang *et al*., 1995) and 5-HT$_{1A}$ receptor agonists (Jiang *et al*., 2000), respectively. The observation that most SCN neurons respond to N/OFQ led to a hypothesis that N/OFQ would induce circadian phase changes *in vivo*. However, only moderate inhibition of light-induced phase advances and no direct phase shifts were observed following an unilateral injection of N/OFQ into the hamster SCN (Allen *et al*., 1999). To further investigate the

*Address for correspondence at: Research Laboratory I (CNS), Pharmaceutical Research Unit, R&D Division, Mitsubishi Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan; E-mail: Teshima.Koji@mk.m-pharma.co.jp

role of N/OFQ in the SCN, we synthesized a brain-penetrating small-molecule ORL1 receptor agonist, 2-{3-[1-((1*R*)-acenaphthen-1-yl)piperidin-4-yl]-2,3-dihydro-2-oxo-benzimidazol-1-yl}-*N*-methylacetamide (W-212393), and investigated its effect on the circadian body temperature rhythm of rats.

Methods

Animals

All experiments were performed in accordance with the guidelines for animal experimentation set by the Ethics Committee for Animal Use at Mitsubishi Pharma Corporation. Male Wistar rats were purchased from Charles River Japan (Yokohama, Japan). Animals were housed under standard laboratory conditions (temperature $23 \pm 1^{\circ}C$; relative humidity $55 \pm 5\%$; 12-h light/12-h dark cycle), with free access to food and water. After 1 week acclimation to the housing environment, rats were used for telemeter implant surgery at 7–8 weeks old, *ex vivo* binding at 6 weeks old, and electrophysiological recording at 3–4 weeks old.

Drugs and chemicals

W-212393 (Figure 1) and an ORL1 antagonist J-113397 (Ozaki *et al*., 2000) were synthesized at Mitsubishi Pharma Corporation (Japan). The endogenous agonist N/OFQ, the 5-HT_{1A} agonist (±)-8-OH-DPAT HBr, and melatonin were purchased from Sigma (St Louis, MO, U.S.A.). [Leucyl-^{3}H]nociceptin ([^{3}H]N/OFQ) and guanosine 5′-[γ-^{35}S]thiotriphosphate (GTPγ^{35}S) were purchased from Amersham Biosciences (Tokyo, Japan).

ORL1 receptor binding

In vitro competitive binding displacement analyses were performed with membranes prepared from rat cerebral cortex. The membranes were incubated for 20 min at 25°C with [^{3}H]N/OFQ (0.1 nM) in a reaction buffer (total volume 1 ml) containing 50 mM Tris, 2 mM EDTA, and 0.1% BSA, with a protease inhibitor Cocktail (Sigma), pH 7.4. The reaction was terminated by filtration through Whatman GF/B filters and rinsing three times with 3 ml of ice-cold Tris buffer. Nonspecific binding was determined in the presence of J-113397 (10 μM). *Ex vivo* ORL1 receptor binding was performed using the same methods except that membranes were prepared from the cerebral cortex of rats administered W-212393. Brains were obtained 15, 30, and 60 min after W-212393 was administered i.v., i.p., or p.o., respectively. Additional receptor binding and enzyme inhibition experiments were performed by MDS Pharma Services (Taipei, Taiwan).

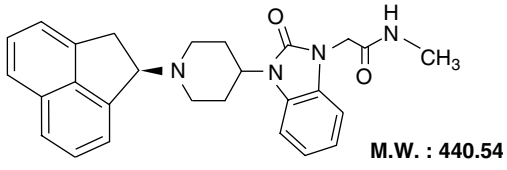


Figure 1 Chemical structure of W-212393.

GTPγ^{35}S binding

Agonist-mediated GTPγ^{35}S-binding analyses were performed with membranes prepared from HEK293 cells stably expressing hORL1 receptors. The membranes were incubated for 60 min at 30°C in GTPγ^{35}S (0.1 nM) and a reaction buffer (total volume 1 ml) containing 50 mM Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 0.1% BSA, with a protease inhibitor cocktail (Sigma), pH 7.4. The reaction was terminated by filtration through Whatman GF/B filters and rinsing three times with 3 ml of ice-cold Tris buffer. Nonspecific binding was determined in the presence of 10 μM unlabelled GTPγS.

Electrophysiology

Spontaneous firing of SCN neurons was recorded using a multielectrode dish (MED) system (Alpha MED Sciences Co., Ltd, Japan). Male Wistar rats were deeply anesthetized between zeitgeber time 3 (ZT3) and ZT4 with halothane, their brains removed and placed in ice-cold Krebs solution consisting of (in mM): NaCl 126; KCl 2.5; $NaHCO_3$ 26; NaH_2PO_4 1.24; $CaCl_2$ 0.5; $MgCl_2$ 4.0; glucose 10; saturated with 95% O_2 and 5% CO_2, pH 7.3–7.4. Coronal (200 μm thick) hypothalamic slices were cut with a microslicer (ZERO 1; Dosaka EM Co., Ltd, Japan) and placed on a MED probe (MED-P515A) containing 64 electrodes, each 50×50 μm, arranged in an 8×8 pattern with 150 μm gaps. The recording chamber was perfused (2 ml min^{-1}) with a bath solution identical to the slicing solution, except that the $CaCl_2$ was increased to 2.4 mM and $MgCl_2$ was reduced to 1.3 mM. N/OFQ or W-212393 was applied by bath perfusion. Recordings were performed 1–6 h after slice preparation. Data collection and analysis were performed using a computer with Performer 2.0 software (Alpha MED Sciences Co., Ltd, Japan). Action potentials with different amplitude were counted when the amplitude exceeded a threshold set as twice the baseline noise level at each electrode, consistent with the recording of several neurons.

Surgery to implant telemeters

Rats were anesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.), and a radio transmitter (TA10TA-F20, Data Sciences International (DSI), U.S.A.) was implanted in the abdominal cavity for monitoring the body temperature rhythm. Ampicillin sodium (Meiji Seika, Ltd, Japan) was injected into the femoral muscle after surgery.

Recording of body temperature rhythms

After recovery from telemeter implant surgery, the rats were individually housed in a soundproof box equipped with a signal receiver board. The body temperature of the rat was measured automatically every 5 min, and the results were saved to a computer. Temperatures higher than the mean body temperature calculated by the least-squares method were represented with a black line. The body temperature rhythm

was determined by plotting time on the horizontal axis, and days on the vertical axis.

Phase advance under constant dark conditions

After confirming a stable body temperature rhythm under constant dark conditions for at least 10 days, W-212393 or vehicle was administered i.p. at circadian time (CT) 0, 3, 6, 9, 12, 15, 18, or 21 (onset of body temperature elevation under constant dark conditions was set to be CT12) to obtain phase–response curves. W-212393, 8-OH-DPAT, and melatonin were administered i.p. at CT6 or 9 to examine the dose-dependency of phase-advancing effects, and J-113397 and W-212393 were simultaneously administered i.p. at CT6 to examine the antagonistic effect of J-113397 on W-212393-induced phase advances.

Effect on re-entrainment to a light–dark cycle

After confirming a stable body temperature rhythm under a 12 h light–dark cycle for longer than 10 days, the light cycle was advanced 6 h by advancing the initiation time of the light period. W-212393 was administered i.p. at ZT6 (the initiation time of light period was set to be ZT0).

Data analysis and statistics

Statistical analyses were performed with the SAS system, ver. 5.0. All values were presented as mean ± s.e.m. For competition-binding curves, IC_{50} values were calculated by fitting the logistic equation to the data by means of nonlinear regression. The inhibition constant, K_i, was calculated from the equation $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d is the equilibrium dissociation constant. Comparison of differences between two groups was performed using paired or unpaired Student's t-test. Statistical significances between more than two groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Receptor-binding profile

N/OFQ, W-212393, and J-113397 inhibited [^{3}H]N/OFQ binding to membranes prepared from rat cerebral cortex, with K_i values of 0.03 ± 0.002, 0.76 ± 0.14 and 2.26 ± 0.42 nM, respectively (Figure 2). W-212393 also showed high affinity for human ORL1 receptors expressed in HEK293 cells (B_{max}; 4.6 pmol mg^{-1} protein) and comparatively low affinities for the human μ receptor and human serotonin transporter (Table 1). W-212393 had no significant affinity (IC_{50} value $> 1\,\mu$M) for 40 other receptors or channel-binding sites, including adrenergic, dopamine, serotonin, acetylcholine, histamine, GABA, glutamate, Ca^{2+}, Na^+, and K^+ channels (see Appendix A). W-212393 also had no effect (IC_{50} value $> 10\,\mu$M) on the activity of 21 enzymes (see Appendix B).

Agonistic activity

N/OFQ and W-212393, but not J-113397, concentration-dependently stimulated GTPγ^{35}S binding in hORL1-expressing HEK cells (Figure 3). The EC_{50} values for N/OFQ and W-212393 were estimated to be 7.6 and 13.9 nM, with Hill coefficients of 0.63 and 0.70, respectively, by least-square fitting to a plot of the mean GTPγ^{35}S binding stimulated by N/OFQ or W-212393. W-212393 concentration-dependently stimulated GTPγ^{35}S binding and its efficacy was similar to N/OFQ, suggesting that W-212393 is a full agonist at ORL1 receptors.

Ex vivo *receptor occupancy*

W-212393 dose-dependently occupied ORL1 receptors after i.v. or i.p. administration (Figure 4). Following p.o. administration, ORL1 receptor occupancy by W-212393 was low, due to its poor oral bioavailability (data not shown). A dose of

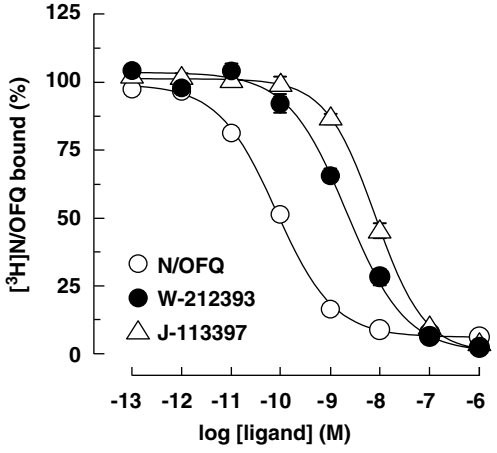


Figure 2 Inhibition curves of [^{3}H]N/OFQ binding to membranes prepared from rat cerebral cortex by N/OFQ, W-212393, and J-113397. Data indicate the means ± s.e.m. of four or five experiments.

Table 1 Binding affinity of W-212393 for ORL1, μ, δ, κ opioid receptors and serotonin transporter (SERT)

Receptor	*Hot ligand*	*Species*	*Receptor source*	K_i *value* (nM)
ORL1	[^{3}H]N/OFQ	Rat	Cerebral cortex	0.76 ± 0.14
ORL1	[^{3}H]N/OFQ	Human	Recombinant (HEK293)	0.50 ± 0.05
δ	[^{3}H]Naltrindole	Human	Recombinant (CHO)	> 1000[a]
κ	[^{3}H]Diprenorphine	Human	Recombinant (HEK293)	> 1000[a]
μ	[^{3}H]Diprenorphine	Human	Recombinant (CHO)	76.0 ± 27.8
SERT	[^{125}I]RTI-55	Human	Recombinant (HEK293)	11.2 ± 1.3

[a]IC_{50} (nM).

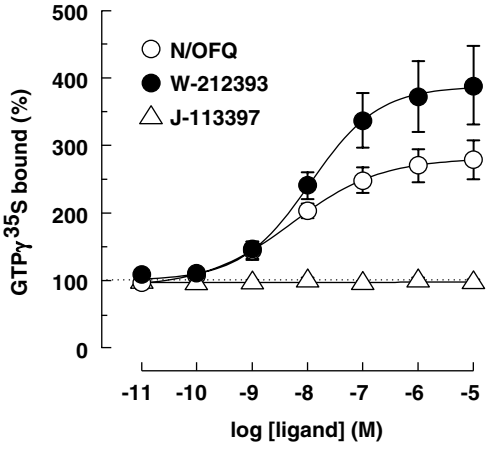


Figure 3 Effects of N/OFQ, W-212393, and J-113397 on stimulation of GTPγ^{35}S binding *in vitro*. Data indicate the means ± s.e.m. of three or four experiments.

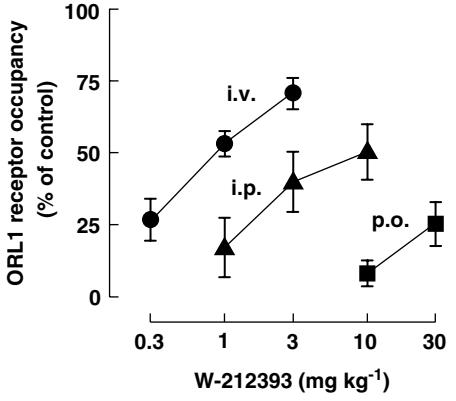


Figure 4 *Ex vivo* receptor occupancy of W-212393. *Ex vivo* receptor occupancy was assessed by [^{3}H]N/OFQ binding to cerebral cortex membranes prepared from rats administered W-212393. Each data point represents the mean ± s.e.m. of 4–10 rats.

3 mg kg^{-1}, i.p., resulted in 40.0 ± 10.5% receptor occupancy. These results indicate that W-21293 is a brain-penetrating compound.

Electrophysiological properties

If W-212393 is acting as an agonist at the ORL1 receptor, it should inhibit the activity of spontaneously firing SCN neurons, similar to N/OFQ (Allen *et al*., 1999). To test this hypothesis, the spontaneous firing of SCN neurons was recorded using a microelectrode array (Figure 5a and b). N/OFQ (100 nM) reduced the spontaneous firing rate 34.7 ± 5.0% from 9.24 ± 0.92 to 6.09 ± 0.89 Hz ($n = 4$ slices; $P < 0.01$; Figure 5c). W-212393 (100 nM) also reduced the spontaneous firing rate 68.3 ± 5.9% from 8.35 ± 0.44 to 2.68 ± 0.49 Hz ($n = 8$ slices; $P < 0.001$; Figure 5d). W-212393 produced a significantly greater reduction than N/OFQ in the spontaneous firing frequency ($P < 0.01$, Student's *t*-test). Suppressive effects of both N/OFQ and W-212393 did not recover after extensive washes for up to 40 min. The ORL1 receptor antagonist J-113397 (1 μM) prevented the reduction of the spontaneous firing rate by N/OFQ (100 nM) and W-212393 (100 nM). The mean firing frequency was 7.94 ± 0.63 Hz in control, 6.95 ± 0.76 Hz in N/OFQ plus J-113397, 6.47 ± 0.61 Hz in W-212393 plus J-113397 ($n = 4$ slices; Figure 5e).

Phase–response curve for W-212393

Representative records of effects of vehicle and W-212393 injections at CT6 on the body temperature rhythm and the phase–response curve produced by W-212393 are shown in Figure 6a and b, respectively. The administration of W-212393 (3 mg kg^{-1}, i.p.) induced a significant phase advance of 1.78 ± 0.52 h ($n = 7$, $P < 0.01$) at CT6 and 1.77 ± 0.23 h ($n = 8$, $P < 0.001$) at CT9, but not at other CTs. Vehicle produced little effect on the phase of body temperature rhythm (0.09 ± 0.07 h at CT6, $n = 7$; 0.11 ± 0.05 h at CT9, $n = 7$).

Dose dependency of W-212393 and comparison with 8-OH-DPAT and melatonin

Phase-advancing effects of W-212393, 8-OH-DPAT and melatonin were compared at doses of 0.3, 1, and 3 mg kg^{-1}, i.p. administered at CT6 or 9. The magnitude of the W-212393-induced phase advance at both CT6 and 9 was dose-dependent and much greater than those produced by 8-OH-DPAT or melatonin (Figure 7).

Antagonistic effect of J-113397 on the W-212393-induced phase advance

Representative records of the effects of vehicle, W-212393 (3 mg kg^{-1}, i.p.) alone and W-212393 (3 mg kg^{-1}, i.p.) with J-113397 (10 mg kg^{-1}, i.p.) are shown in Figure 8a. The W-212393-induced phase advance was antagonized by the ORL1 receptor antagonist, J-113397 (0.09 ± 0.06 h for vehicle, 2.48 ± 0.44 h for W-212393 alone; $P < 0.001$ vs vehicle, 0.01 ± 0.08 h for J-113397 alone and 0.77 ± 0.17 h for J-113397 with W-212393; $P < 0.001$ vs W-2123933 alone; Figure 8b).

Acceleration of re-entrainment to a light–dark cycle by W-212393

Representative records of effects of vehicle and W-212393 at ZT6 on the re-entrainment of the body temperature rhythm to a 6 h advanced light–dark cycle are shown in Figure 9a and the averages of phase shifts on each day were plotted in Figure 9b. When rats were injected with W-212393 (3 mg kg^{-1}, i.p.) at ZT6, it took only 3–4 days for re-entrainment. In contrast, when rats were injected with vehicle, it took 8–9 days for complete re-entrainment to a new light–dark cycle. W-212393 significantly accelerated the re-entrainment of the body temperature rhythm to a 6 h advanced light–dark cycle (5.82 ± 0.23 h for W-212393 and 2.06 ± 0.58 h for vehicle on the fourth day, $n = 7$, $P < 0.001$).

Discussion

The aim of the present study was to investigate the physiological roles of the N/OFQ-ORL1 receptor system in the circadian rhythm (Allen *et al*., 1999). To facilitate these studies, we synthesized a small-molecule ORL1 receptor

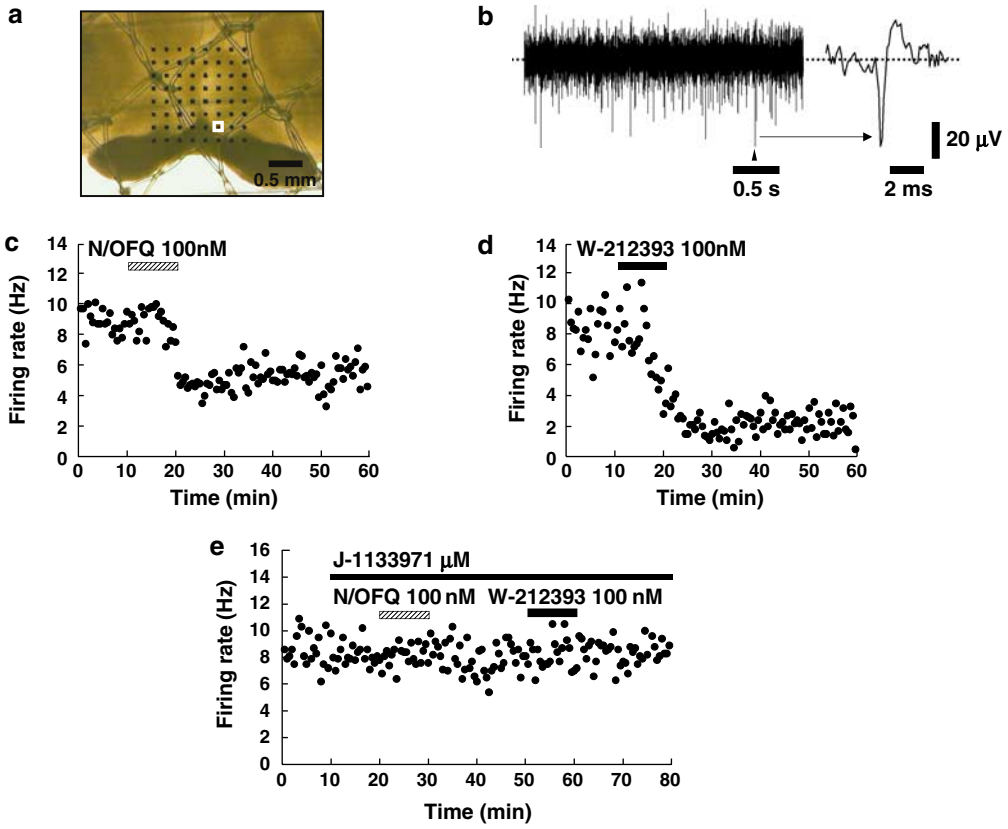


Figure 5 Effects of N/OFQ and W-212393 on the spontaneous action potential firing of SCN neurons. (a) SCN slice placed on MED. (b) Representative trace of spontaneous firing activity recorded by the marked electrode (white square) in (a). Effect of N/OFQ (c) and W-212393 (d). N/OFQ and W-212393 were applied by bath perfusion for 10 min. Both N/OFQ and W-212393 significantly suppress the activity of spontaneously firing rat SCN neurons. The suppressive effects of N/OFQ and W-212393 did not recover after extensive washes for up to 40 min. (e) ORL1 receptor antagonist J-113397 (1 μM) prevented the reduction of the spontaneous firing rate by N/OFQ (100 nM) and W-212393 (100 nM). J-113397 was applied by bath perfusion 10 min before N/OFQ application. N/OFQ and W-212393 were applied by bath perfusion for 10 min with 20 min interval.

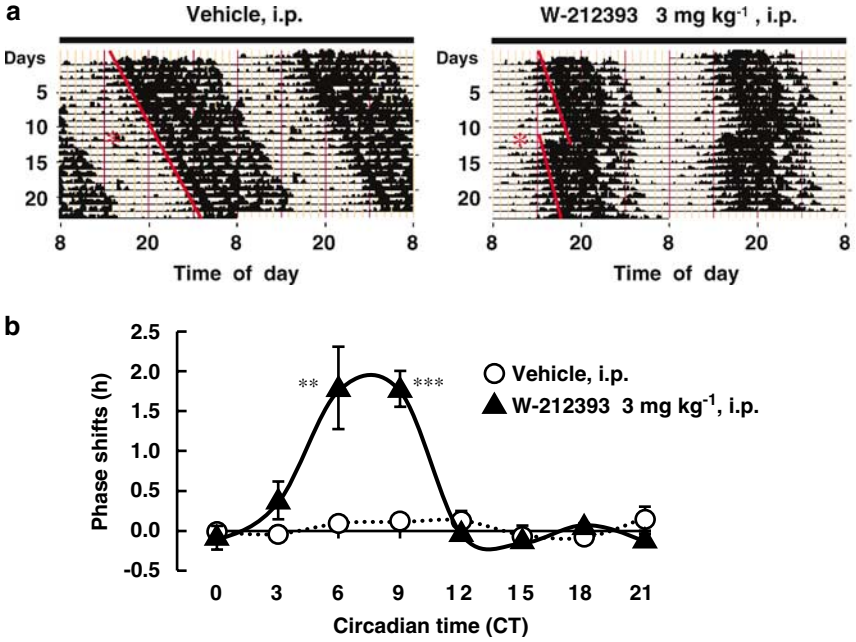


Figure 6 Phase–response curve for W-212393 in rats. (a) Representative double-plotted body temperature rhythm of vehicle (left) and W-212393 (right) injected at CT6. The time of day is indicated horizontally and consecutive days vertically. Rats were maintained in constant darkness until a stable free-running rhythm was observed for longer than 10 days. The asterisk in the figure indicates the injection time. (b) Mean phase shifts induced by W-212393 (3 mg kg^{-1}, i.p.) or vehicle administration at CT0, 3, 6, 9, 12, 15, 18, and 21. Data indicate the mean $\pm$ s.e.m. of 6–8 rats. $^{**}P<0.01$, $^{***}P<0.001$ in comparison with vehicle by Student's *t*-test.

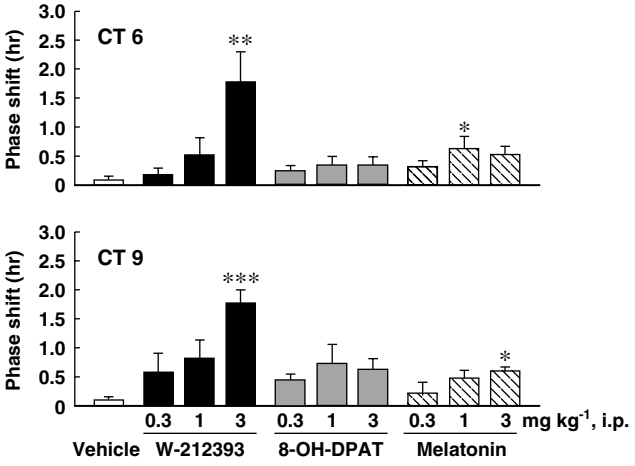
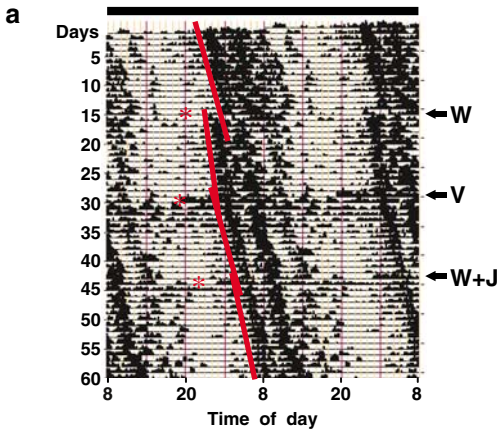


Figure 7 Effects of W-212393, 8-OH-DPAT, and melatonin on the circadian rhythm of body temperature in rats. Drugs were injected at CT6 (upper) or CT9 (lower). Data indicate the mean ± s.e.m. of 5–8 rats. $*P<0.05$, $**P<0.01$, $***P<0.001$ in comparison with vehicle by Dunnett's multiple comparison test.



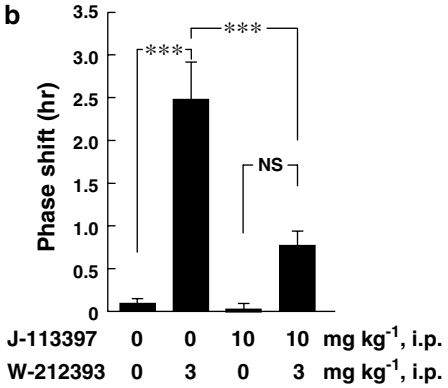
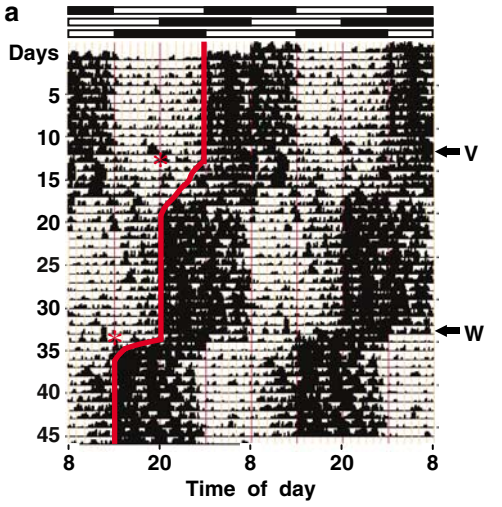


Figure 8 Inhibition of W-212393-induced phase advance by J-113397. (a) Representative records of the effects of vehicle (V), W-212393 alone (W), and W-212393 with J-113397 (W + J) on the body temperature rhythm at CT6. The time of day is indicated horizontally and consecutive days vertically. Rats were maintained in constant darkness until a stable free-running rhythm was observed for longer than 10 days. Asterisk in the figure indicates the time of injections. (b) The mean phase shifts induced by vehicle, W-212393 alone, J-113397 alone, and W-212393 with J-113397. W-212393 and J-113397 were simultaneously administered i.p. at CT6. Data indicate the mean ± s.e.m. of six or seven rats. $***P<0.001$ in comparison by Tukey's multiple comparison test.



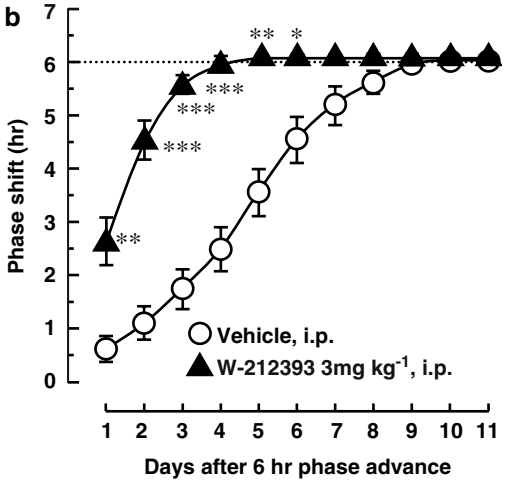


Figure 9 Effect of W-212393 on re-entrainment of the body temperature rhythm to a 6 h advanced light–dark cycle. (a) Representative records of the effects of vehicle (V) and W-212393 (W) injected at ZT6. The time of day is indicated horizontally and consecutive days vertically. Upper, middle, and lower bars on top of the figure show the light (open bar)–dark (solid bar) cycle during days 1–13, 14–34, and 35–46, respectively. Asterisk in the figure indicates the time of injections. (b) The mean phase shifts plotted each day after a 6 h advanced light–dark cycle. Data indicate the mean ± s.e.m. of seven rats (crossover administration). $*P<0.05$, $**P<0.01$, $***P<0.001$ in comparison with vehicle by paired Student's *t*-test.

agonist capable of crossing the blood–brain barrier. W-212393 has a high affinity for both rat and human ORL1 receptors, with K_i values of 0.76 and 0.50 nM, respectively, and a relative selectivity over other receptors. The affinity of W-212393 for the ORL1 receptor is lower than that of N/OFQ (0.76 vs 0.03 nM). The first reported synthetic ORL1 receptor agonist, Ro 64-6198, also has high affinity for recombinant human ORL1 receptor, with a K_i value of 0.39 nM (Jenck *et al*., 2000). Although these two compounds have almost equal affinity for ORL1 receptors, their binding profiles differ, namely W-212393 has lower affinities for κ, D_2 and σ receptors and higher affinity for the serotonin transporter than Ro 64-6198. Two subsets of ORL1 receptors may exist in the ventrolateral periaqueductal gray, one of which is sensitive to both N/OFQ

and Ro 64-6198, and the other is sensitive only to N/OFQ but not to Ro 64-6198 (Chiou *et al*., 2004). Functional heterogeneity of ORL1 receptors was also suggested by an *in vivo* study showing that Ro 64-6198 produced some but not all of the physiological responses generated by N/OFQ (Kuzmin *et al*., 2004). W-212393 will be expected to be a useful tool to investigate the heterogeneity of ORL1 receptors.

W-212393 induced a significant phase advance at CT6 and 9, but not at other CTs. The phase–response curve was similar to other nonphotic entrainment patterns. Our results indicate that bilateral N/OFQ activation of ORL1 receptors is required to produce a phase shift since no direct phase shifts were observed following a unilateral injection of N/OFQ into the hamster SCN (Allen *et al*., 1999). Administration of W-212393 to hamsters may bring additional insights. Nonphotic entrainment is produced not only by drugs but also by behavioral cues, such as forced running (Maywood *et al*., 1999). W-212393 induced a significant phase advance and ataxia, but not hyperactivity, at an i.p. dose of $3\,mg\,kg^{-1}$. The W-212393-induced phase advance was antagonized by an ORL1 receptor antagonist, J-113397. These results indicate that W-212393-induced phase advance is not induced by behavioral cues and is mediated by the ORL1 receptor.

To confirm that W-212393 is acting in the SCN, the direct effect of W-212393 on the activity of SCN neurons was examined by using a MED system. Circadian changes of spontaneous firing rate were recorded by means of MED system for several days in dispersed and slice cultures of the rat SCN (Honma *et al*., 1998; Ikeda *et al*., 2003). We used an acutely prepared SCN slice in this study. Both N/OFQ and W-212393 significantly suppressed the activity of spontaneously firing rat SCN neurons at a concentration of 100 nM, and these suppressive effects were completely blocked by an ORL1 receptor antagonist, J-113397. These results indicate that the suppressive effects of N/OFQ and W-212393 on the activity of SCN neurons are mediated by the ORL1 receptor. The suppressive effects of both N/OFQ and W-212393 were long lasting, and did not recover after extensive washes for up to 40 min. An outward current activated by N/OFQ (Allen *et al*., 1999) was also long lasting compared with those by melatonin (Jiang *et al*., 1995) or 5-HT_{1A} receptor agonists (Jiang *et al*., 2000). The long lasting inhibitory effect of W-212393 on neural activity in SCN may contribute to the significant phase advance compared with those of melatonin or 8-OH-DPAT, as well as higher percentage of SCN neurons that responded to N/OFQ. Our experiments showed that the MED system is also useful for recording of spontaneous neural activity in acutely prepared SCN slice.

Additional evidence that W-212393 can penetrate the blood–brain barrier to occupy ORL1 receptors was obtained from *ex vivo* receptor experiments. Despite a low oral bioavailability (3.7%) and a medium parenteral bioavailability (13.0%), W-212393 showed relatively high brain penetration after an i.v. injection. At an i.v. dose of $1\,mg\,kg^{-1}$, $53.3 \pm 4.5\%$ receptor occupancy was observed. Loss of righting reflexes were also observed at this W-212393 dose, suggesting that less than 50% receptor occupancy is sufficient for the pharmacological effects of W-212393. Indeed, W-212393 produced a significant phase advance at $3\,mg\,kg^{-1}$ i.p., with $40.0 \pm 10.5\%$ receptor occupancy.

Furthermore, W-212393 significantly accelerated the re-entrainment of the body temperature rhythm to a 6 h advanced light–dark cycle, suggesting that W-212393 may represent an interesting tool for treatment of circadian rhythm disorders such as jet lag or delayed sleep phase syndrome.

In conclusion, the present study demonstrates that daytime administration of an ORL1 agonist W-212393 induces a phase advance in the body temperature rhythm of rat. This is the first report that activation of ORL1 receptor can reset the circadian pacemaker of rat.

We thank Dr T. Horikawa, Mr J. Ando and Ms T. Ohbora for pharmacokinetics study of W-212393 in rats.

References

ALLEN, C.N., JIANG, Z.G., TESHIMA, K., DARLAND, T., IKEDA, M., NELSON, C.S., QUIGLEY, D.I., YOSHIOKA, T., ALLEN, R.G., REA, M.A. & GRANDY, D.K. (1999). Orphanin-FQ/nociceptin (N/OFQ) modulates the activity of suprachiasmatic nucleus neurons. *J. Neurosci.,* **19,** 2152–2160.

BENLOUCIF, S. & DUBOCOVICH, M.L. (1996). Melatonin and light induce phase shifts of circadian activity rhythms in the C3H/HeN mouse. *J. Biol. Rhythms,* **11,** 113–125.

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

CHIOU, L.-C., CHUANG, K.-C., WICHMANN, J. & ADAM, G. (2004). Ro 64-6198 [(1*S*,3*aS*)-8-(2,3,3*a*,4,5,6-hexahydro-1*H*-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one] acts differently from nociceptin/orphanin FQ in rat periaqueductal gray slices. *J. Pharmacol. Exp. Ther.,* **311,** 645–651.

CICCOCIOPPO, R., ECONOMIDOU, D., FEDELI, A., ANGELETTI, S., WEISS, F., HEILIG, M. & MASSI, M. (2004). Attenuation of ethanol self-administration and of conditioned reinstatement of alcohol-seeking behaviour by the antiopioid peptide nociceptin/orphanin FQ in alcohol-preferring rats. *Psychopharmacology (Berlin),* **172,** 170–178.

CICCOCIOPPO, R., ECONOMIDOU, D., FEDELI, A. & MASSI, M. (2003). The nociceptin/orphanin FQ/NOP receptor system as a target for treatment of alcohol abuse: a review of recent work in alcohol-preferring rats. *Physiol. Behav.,* **79,** 121–128.

COURTEIX, C., COUDORE-CIVIALE, M.A., PRIVAT, A.M., PELISSIER, T., ESCHALIER, A. & FIALIP, J. (2004). Evidence for an exclusive antinociceptive effect of nociceptin/orphanin FQ, an endogenous ligand for the ORL1 receptor, in two animal models of neuropathic pain. *Pain,* **110,** 236–245.

HONMA, S., SHIRAKAWA, T., KATSUNO, Y., NAMIHIRA, M. & HONMA, K. (1998). Circadian periods of single suprachiasmatic neurons in rats. *Neurosci. Lett.,* **250,** 157–160.

HORIKAWA, K. & SHIBATA, S. (2004). Phase-resetting response to (+)8-OH-DPAT, a serotonin 1A/7 receptor agonist, in the mouse *in vivo*. *Neurosci. Lett.,* **368,** 130–134.

IKEDA, M., SUGIYAMA, T., WALLACE, C.S., GOMPF, H.S., YOSHIOKA, T., MIYAWAKI, A. & ALLEN, C.N. (2003). Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons. *Neuron,* **38,** 253–263.

JENCK, F., MOREAU, J.L., MARTIN, J.R., KILPATRICK, G.J., REINSCHEID, R.K., MONSMA JR, 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.

JENCK, F., WICHMANN, J., DAUTZENBERG, F.M., MOREAU, J.L., OUAGAZZAL, A.M., MARTIN, J.R., LUNDSTROM, K., CESURA, A.M., POLI, S.M., ROEVER, S., KOLCZEWSKI, S., ADAM, G. & KILPATRICK, G. (2000). A synthetic agonist at the orphanin FQ/nociceptin receptor ORL1: anxiolytic profile in the rat. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 4938–4943.

JIANG, Z.-G., NELSON, C.S. & ALLEN, C.N. (1995). Melatonin activates an outward current and inhibits Ih in rat suprachiasmatic nucleus neurons. *Brain Res.,* **687,** 125–132.

JIANG, Z.-G., TESHIMA, K., YANG, Y., YOSHIOKA, T. & ALLEN, C.N. (2000). Pre- and postsynaptic actions of serotonin on rat suprachiasmatic nucleus neurons. *Brain Res.,* **866,** 247–256.

KO, M.C.H., NAUGHTON, N.N., TRAYNOR, J.R., SONG, M.S., WOODS, J.H., RICE, K.C. & MCKNIGHT, A.T. (2002). Orphanin FQ inhibits capsaicin-induced thermal nociception in monkeys by activation of peripheral ORL1 receptors. *Br. J. Pharmacol.,* **135,** 943–950.

KUZMIN, A., SANDIN, J., TERENIUS, L. & OGREN, S.O. (2003). Acquisition, expression, and reinstatement of ethanol-induced conditioned place preference in mice: effects of opioid receptor-like 1 receptor agonists and naloxone. *J. Pharmacol. Exp. Ther.,* **304,** 310–318.

KUZMIN, A., SANDIN, J., TERENIUS, L. & OGREN, S.O. (2004). Evidence in locomotion test for the functional heterogeneity of ORL-1 receptors. *Br. J. Pharmacol.,* **141,** 132–140.

MAYWOOD, E.S., MROSOVSKY, N., FIELD, M.D. & HASTING, M.H. (1999). Rapid down-regulation of mammalian *Period* genes during behavioral resetting of the circadian clock. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 15211–15216.

MEUNIER, J.-C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.L., GUILLEMOT, J.C., FERRARA, P., MONSERRAT, 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.

MOGIL, J.S. & PASTERNAK, G.W. (2001). The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol. Rev.,* **53,** 381–415.

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.

OZAKI, S., KAWAMOTO, H., ITOH, Y., MIYAJI, M., IWASAWA, Y. & OHTA, H. (2000). A potent and highly selective nonpeptidyl nociceptin/orphanin FQ receptor (ORL1) antagonist: J-113397. *Eur. J. Pharmacol.,* **387,** R17–R18.

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

REDMAN, J., ARMSTRONG, S. & NG, K.T. (1983). Free-running activity rhythms in the rat: entrainment by melatonin. *Science,* **219,** 1089–1091.

REINSCHEID, R.K., NOTHACKER, H.P., BOURSON, A., ARDATI, 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 opioidlike G protein-coupled receptor. *Science,* **270,** 792–794.

SACK, R.L., LEWY, A.J., BLOOD, M.L., STEVENSON, J. & KEITH, L.D. (1991). Melatonin administration to blind people: phase advances and entrainment. *J. Biol. Rhythms,* **6,** 249–261.

SAKOORI, K. & MURPHY, N.P. (2004). Central administration of nociceptin/orphanin FQ blocks the acquisition of conditioned place preference to morphine and cocaine, but not conditioned place aversion to naloxone in mice. *Psychopharmacology (Berlin),* **172,** 129–136.

SANDIN, J., GEORGIEVA, J., SCHOTT, P.A., OGREN, S.O. & TERENIUS, L. (1997). Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learning in rats. *Eur. J. Neurosci.,* **9,** 194–197.

TOMINAGA, K., SHIBATA, S., UEKI, S. & WATANABE, S. (1992). Effects of 5-HT1A receptor agonists on the circadian rhythm of wheel-running activity in hamsters. *Eur. J. Pharmacol.,* **214,** 79–84.

Appendix A

See Table A1.

Table A1 Receptors and ion channels with no binding affinity for W-212393 ($IC_{50} > 1000$ nM)

Adenosin A_1 (human)	Opioid κ (human)
Adrenergic α_1, nonselective (human)	Opioid δ (human)
Adrenergic α_2, nonselective (human)	Serotonin 5-HT_{1A} (rat)
Adrenergic β_1 (human)	Serotonin 5-HT_2 (human)
Adrenergic β_2 (human)	Serotonin 5-HT_3 (human)
Adrenergic β_3 (human)	Serotonin 5-HT_4 (guinea-pig)
Bradykinin B_2 (human)	Serotonin 5-HT_{5A} (human)
Dopamine D_1 (human)	Serotonin 5-HT_6 (human)
Dopamine D_2 (human)	Serotonin 5-HT_7 (human)
Dopamine D_3 (human)	Sigma, nonselective (guinea-pig)
Dopamine $D_{4.4}$ (human)	Somatostatin sst1 (human)
Dopamine D_5 (human)	Dopamine transporter (human)
$GABA_A$, agonist site (rat)	Norepinephrine transporter (human)
$GABA_A$, benzodiazepine, central (rat)	
$GABA_B$, nonselective (rat)	Calcium channel, L-type (rat)
Glucocorticoid (human)	Calcium channel, N-type (rat)
Glutamate, nonselective (rat)	Sodium channel, Site 1 (rat)
Histamine H_1 (human)	Potassium channel [K_{ATP}] (hamster)
Melatonin ML (MT), nonselective (chicken)	Potassium channel [SK_{Ca}] (rat)
Muscarinic, nonselective, Central (rat)	
Neuropeptide Y_1 (human)	
Nicotinic acetylcholine (human)	

Appendix B

See Table B1.

Table B1 W-212393 had no effect on enzyme activity ($IC_{50} > 10\ \mu$M)

ATPase, H^+/K^+ (rabbit)
Carbonic anhydrase (human)
Catechol-*O*-methyltransferase (COMT) (porcine)
Cyclooxygenase (COX-1) (human)
HMG-CoA reductase (rat)
Lipoxygenase (5-LO) (human)
Monoamine oxidase (MAO-A) (human)
Monoamine oxidase (MAO-B) (human)
Nitric oxide synthase, inducible (iNOS) (mouse)
Nitric oxide synthase, Neuronal (nNOS) (rat)
Phosphodiesterase (PDE1) (bovine)
Phosphodiesterase (PDE5) (human)
Phospholipase (PLA2-I) (porcine)
Phospholipase (PLC) (bacillus cereus)
Protein serine/threonine kinase (Ca^{2+}/calmodulin-dependent II) (rat)
Protein serine/threonine phosphatase (calcineurin, PP2B) (human)
Protein serine/threonine kinase (p38α) (human)
Protein serine/threonine kinase (PKA), nonselective (bovine)
Protein serine/threonine kinase (PKC), nonselective (rat)
Protein tyrosine phosphatase (PTP1B) (human)
Tyrosine hydroxylase (rat)

(Received March 11, 2005
Revised April 21, 2005
Accepted May 18, 2005
Published online 27 June 2005)