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The NOP (ORL1) receptor antagonist Compound B stimulates mesolimbic dopamine release and is rewarding in mice by a non-NOP-receptor-mediated mechanism

[1]Miwako Koizumi, [1]Kazuto Sakoori, [1]Naoko Midorikawa & *,[1]Niall P. Murphy

[1]Neuronal Circuit Mechanisms Research Group, RIKEN Brain Science Institute, 2-1 Hirosawa, Wakoshi, Saitama 351-0198, Japan

1 Compound B (1-[(3*R*, 4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, CompB) is a nociceptin/orphanin FQ (N/OFQ) antagonist showing high selectivity for the NOP (ORL1) receptor over classical opioid receptors. We studied the effect of subcutaneous CompB administration on the release of mesolimbic dopamine (DA) and the expression of hedonia in mice.

2 CompB (0.3–30 mg kg^{-1}) dose dependently stimulated mesolimbic DA release as measured by *in vivo* freely moving microdialysis, without any change in locomotor activity. However, intracerebroventricular administered N/OFQ (endogenous agonist of the NOP receptor, 6 nmol) did not influence CompB- (10 mg kg^{-1}) induced DA release, despite clearly suppressing release when administered alone.

3 Studies using NOP receptor knockout mice and no-net-flux microdialysis revealed mildly, but not statistically significantly higher endogenous DA levels in mice lacking the NOP receptor compared to wild-type mice. Administration of CompB (10 mg kg^{-1}) induced identical increases in mesolimbic DA release in wild-type and NOP receptor knockout mice.

4 CompB was rewarding in approximately the same dose range in which CompB induced major increases in mesolimbic DA release when assayed using a conditioned place preference paradigm. The rewarding effect of CompB (30 mg kg^{-1}) was maintained in NOP receptor knockout mice.

5 These results show that CompB stimulates mesolimbic DA release and is rewarding by an action independent of the NOP receptor, the precise site of which is unclear. Consequently, caution should be exercised when interpreting the results of studies using this drug, particularly when administered by a peripheral route.
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Abbreviations: CompB, Compound B (1-[(3*R*, 4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one); DA, dopamine; NA, noradrenaline; Nac, nucleus accumbens; N/OFQ, nociceptin/orphanin FQ; VEH, vehicle

Introduction

Currently, four major classes of opioid peptides are recognized: endorphins, enkephalins, dynorphins, and the more recently discovered nociceptin (known also as orphanin FQ, abbreviated here to N/OFQ). Research in this and other laboratories suggests that these four endogenous opioid systems interact to buffer the expression of hedonia within the brain, and thus determine ongoing hedonic state. For example, when assayed in place conditioning paradigms, agonists of mu and delta opioid receptors (MOP and DOP, respectively) elicit rewarding effects, agonists of the kappa opioid receptor (KOP) elicit aversive effects (see Tzschentke, 1998) and agonists of the N/OFQ receptor (NOP, known also as ORL1 or OP_4) are either neutral (Devine *et al*., 1996; Ciccocioppo *et al*., 2000; Le Pen *et al*., 2002; Kuzmin *et al*., 2003) or mildly aversive (Sakoori & Murphy, 2004), depending on the species under study.

Several studies show that central administration of N/OFQ blocks the expression of some of the behavioural effects of abused drugs (Ciccocioppo *et al*., 1999; 2000; 2002; 2004; Murphy *et al*., 1999a; Kotlinska *et al*., 2002; Lutfy *et al*., 2002; Kuzmin *et al*., 2003; Zhao *et al*., 2003; Sakoori & Murphy, 2004), giving rise to the suggestion that drugs acting on this novel peptide system may be useful in the management of addictive disorders. Although a neurobiological explanation of these behavioural effects is far from complete, an important finding has been that N/OFQ suppresses basal (Murphy *et al*., 1996; Murphy & Maidment, 1999b; Koizumi *et al*., 2004) and drug-stimulated activity in the mesolimbic dopamine (DA) system (Di Giannuario *et al*., 1999; Di Giannuario & Pieretti, 2000; Lutfy *et al*., 2001), a neural pathway widely believed to be involved in the motivating, and perhaps rewarding properties of drugs.

*Author for correspondence; E-mail: nmurphy@riken.jp
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In light of these previous studies, recent studies in this laboratory have sought evidence that endogenous N/OFQ controls the expression of hedonia within the brain and plays some part in determining the activity of the mesolimbic DA system. In order to achieve this, two different experimental strategies have been employed: study of mice with null mutations of the NOP receptor gene (i.e. NOP receptor knockout mice) and administration of NOP receptor antagonists. We have found that heroin-stimulated mesolimbic DA release (and locomotion) is unchanged in NOP receptor knockout mice (Murphy *et al*., 2002), suggesting that in the stimulated state at least, endogenous N/OFQ plays no role in determining the activity of the mesolimbic DA system. However, the possibility of compensations for the null mutation of the NOP receptor during development restricts the interpretation of such studies. Consequently, a pharmacological approach has also been important.

NOP receptor antagonists may be classed roughly according to their structures, those being either peptide (similar to N/OFQ itself) or nonpeptide in nature. We have recently shown that the peptide NOP receptor antagonist UFP-101 (Calo *et al*., 2002) effectively blocks the suppressive effect of N/OFQ on mesolimbic DA release (Koizumi *et al*., 2004). However, when administered alone, UFP-101 does not influence mesolimbic DA release, suggesting that basal activity in this system is not determined by endogenous N/OFQ. Although such peptide antagonists are clearly able to reveal a great deal about the N/OFQ-NOP system, the necessity to administer them centrally precludes them from most clinical applications, in addition to making them less desirable for preclinical study. Consequently, a number of nonpeptide NOP receptor antagonists have been developed including JTC-801 (Yamada *et al*., 2002), SB-612111 (Zaratin *et al*., 2004), and the widely employed Compound B (1-[(3*R*, 4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, CompB; Ozaki *et al*., 2000a, b).

The objective of the current study was to seek support for our previous findings and establish the effectiveness of the commonly used nonpeptide NOP receptor antagonist CompB for future investigations. Thus, we studied the effect of peripheral CompB administration on endogenous DA levels in the nucleus accumbens (NAc, the terminal region of the mesolimbic DA system) and the expression of hedonia.

Methods

Animals

Experimental protocols used throughout the study were approved by the RIKEN Brain Science Institute review committee and were in accord with NIH ethics guidelines. For the majority of studies (see details below), male C57BL6J mice (age: 68–74 days, Nihon Clea, Tokyo, Japan) were used. These mice were introduced into a temperature- and humidity-controlled colony room at least a week before the commencement of experiments. The remainder of experiments used both male and female, wild-type, heterozygous or NOP receptor knockout mice (age: 67–90 days) generated as described previously (Nishi *et al*., 1997) by mating of heterozygous pairs in a colony maintained in the RIKEN Brain Science Institute animal facility. The NOP receptor knockout mouse colony had been backcrossed 10 times onto a C57BL6 background. Mice from this colony were housed in cages separated by sex, but among same generation siblings of mixed genotypes. All animals were housed between two and five animals per cage under a 12 h : 12 h light/dark cycle (lights on 08:00) where they received standard lab chow and water *ad libitum*.

Procedure for microdialysis from the NAc

Animals were anaesthetised by intraperitoneal injection of ketamine (100 mg kg^{-1}, Wako, Osaka, Japan) and xylazine (10 mg kg^{-1}, Sigma, Tokyo, Japan) and placed into a stereotaxic frame adapted for mouse surgery. The skull surface was exposed prior to implantation of a concentric microdialysis probe, constructed as described previously (Maidment *et al*., 1989) using a 1 mm long, 300 μm diameter polyacrylonitrile membrane (AN69, Hospal, Tokyo, Japan), into the left NAc (coordinates relative to bregma in millimeters, A: +1.7; L: +0.8; V: −4.9) according to the Atlas of Franklin & Paxinos (1997). Microdialysis probes were continually perfused with an artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 0.9 mM NaH2PO4, 5 mM Na2HPO4, 1.2 mM CaCl2, 1 mM MgCl2 (pH 7.4) using syringe pumps (KDScientific, New Hope, PA, U.S.A.) at a rate of 2 μl min^{-1}. Three stainless-steel screws (1.52 mm OD, 3.18 mm length, Small Parts, Miami Lakes, FL, U.S.A.) were placed in the surface of the skull to serve as an anchor. Where necessary (see below), an intracerebroventricular (i.c.v.) guide cannula constructed from 23 G hypodermic steel tubing was implanted in the left lateral cerebroventricle (coordinates A: −0.3; L: +1.0; V: −2.0). The patency of the cannula was maintained by insertion of a dummy cannula of equal length constructed from 33 G tubing. The whole assembly was fixed to the skull with dental cement (Yamahachi Dental, Gamagoorishi, Aichi, Japan) prior to attaching the animal to a tether leading to a low-torque (model 375/D/22QM) dual channel liquid swivel (Instech Laboratories, Plymouth Meeting, PA, U.S.A.). Each animal was placed into a translucent microdialysis cage (18 cm diameter × 21 cm height) situated within a 25 cm × 25 cm locomotor activity monitoring arena (Truscan, Coulbourn Instruments, Allentown, PA, U.S.A.) consisting of an array of 16 × 16 evenly spaced photosensors that automatically recorded horizontal locomotor activity on a personal computer. Animals received food and water *ad libitum* in the microdialysis cages for the remainder of the experiment.

Unless otherwise stated, collection of dialysates commenced between 12:00 and 13:00 on the day following probe-implantation surgery. Dialysates were collected at 4°C automatically in 15 min intervals into plastic microvials preloaded with 3 μl of 12.5 mM perchloric acid/250 μM EDTA using refrigerated fraction collectors (Univentor, Malta). On completion of the experiment, dialysate samples were immediately capped and stored at −80°C until HPLC analysis. Throughout microdialysis experiments simultaneous locomotor activity was automatically recorded in 15 min sessions in synchrony with dialysate sample collection. Prior to all drug administrations, 1 h worth of samples (i.e. four dialysates) were collected to determine basal mesolimbic DA release. Unless otherwise stated, drugs were administered subcutaneously (s.c.) in 0.9% NaCl vehicle (VEH) at a volume of 10 ml kg^{-1}.

Overview of microdialysis experiments

Three individual sets of microdialysis experiments were performed to determine absolute basal neurotransmitter concentrations in the NAc of wild-type and NOP receptor knockout mice, and the effect, specificity, and reversibility of CompB actions on the mesolimbic DA system. A total of 155 mice were used for microdialysis studies, of which 38 were ultimately removed from the study due either to misplaced microdialysis probes, technical problems, or drug toxicity. In the first experiment, the responsiveness of the mesolimbic DA system to CompB was assessed over a wide dose range of CompB with simultaneous locomotor monitoring. In the second experiment, the specificity of CompB effects on mesolimbic DA release were assessed in mice lacking the NOP receptor following an assessment of the effect of NOP receptor gene knockout on basal extracellular DA concentrations by no-net-flux analysis. In the final experiment, the reversibility of CompB effects was tested by i.c.v. administration of N/OFQ.

Microdialysis experiment 1: accumbal dialysate DA concentrations and locomotion following CompB or morphine administration

Dialysate samples were collected from the NAc of C57BL6 during s.c. administration of VEH or CompB (dose range 0.3–30 mg kg^{-1}). Simultaneous locomotor activity was measured throughout the period of sample collection. As a positive control, an additional group of mice were administered 3 mg kg^{-1} of morphine.

Microdialysis experiment 2: assessment of absolute extracellular basal neurotransmitter concentrations and determination of the effect of CompB on accumbal dialysate DA concentrations in NOP receptor knockout mice

In order to determine if deletion of the NOP receptor gene results in altered extracellular neurotransmitter turnover in the NAc *per se*, a no-net-flux analysis of absolute extracellular noradrenaline (NA), DA and serotonin (5-HT) concentrations was performed (Justice, 1993). In these studies, both male and female, wild-type and NOP receptor knockout animals underwent stereotaxic surgery for implantation of a microdialysis probe into the NAc as described above. The morning following surgery, animals were switched to an aCSF (comprised of 125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH2PO4, 5 mM Na_2HPO4, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) containing 0.2 mM ascorbic acid (in order to stabilise DA in the perfused aCSF and that collected from the probe) perfused at a flow rate of 0.5 μl min^{-1} commencing at 09:00. Four different concentrations (0, 5, 10 and 20 nM, defined as [Cin], as this was the concentration of each neurotransmitter perfused into the input of the probe) of NA, DA and 5-HT were perfused in random order for periods of 2.5 h each for a total of 10 h. Changes between concentrations were made using a liquid switch (Univentor). Dialysates were collected at 30 min intervals (without the addition of 12.5 mM perchloric acid/250 μM EDTA) throughout the entire collection period. Analysis of the profile of each monoamine concentrations in serial dialysates during this procedure showed that the output NA, DA and 5-HT concentrations typically achieved equilibrium by the final 30 min period of each 2.5 h perfusion period. The final dialysate (defined as [Cout], as this was the concentration of each neurotransmitter recovered from the output of the probe) of each 2.5 h period was used for calculation of the point of no-net-flux. The net change in NA, DA and 5-HT ([Cin]−[Cout]) was plotted against [Cin] and a linear regression made to determine the [Cin] concentration at which [Cin]−[Cout] is equal to zero (i.e. the point of 'no-net-flux'). As animals of different genotypes and sexes were used in this part of the study, individual calculations of the average absolute extracellular NA, DA and 5-HT concentrations were made.

Following no-net-flux experiments, probes were immediately switched back to the original aCSF. The following day, mice were administered either a single dose of VEH or CompB (10 mg kg^{-1}) to determine the effect on mesolimbic DA release in the absence of the NOP receptor.

Microdialysis experiment 3: effect of i.c.v. N/OFQ administration on CompB-induced changes in accumbal dialysate DA concentrations

In order to determine the reversibility of the CompB-induced effects on mesolimbic DA release, N/OFQ (6 nmol) was administered i.c.v. to male C57BL6 mice during peripheral administration of CompB (10 mg kg^{-1} s.c.). Central N/OFQ administration was achieved by insertion of 30.5 mm injector constructed of 30 G steel tubing through an i.c.v. guide cannula implanted at the time of surgery for implantation of the microdialysis probe. The injector was attached to a 10 μl Hamilton syringe *via* PFA tubing ($\varnothing$ 0.40 mm ID, $\varnothing$ 0.60 mm OD). Injections were made in 3 μl of 0.9% NaCl VEH over a 10 s period immediately prior to CompB administration.

Place conditioning

Place conditioning was conducted in 25 cm × 25 cm × 40 cm (W × D × H) locomotor activity monitoring boxes (Truscan, Coulbourn Instruments) divided into two equal sized compartments (25 cm × 12.5 cm × 40 cm) containing visual, tactile, and olfactory cues as described previously (Sakoori & Murphy, 2004). Two sets of place conditioning experiments were performed to determine the dose responsiveness of CompB effects and the specificity of CompB action. Male C57BL6 mice were used in the first set of experiments, whereas male and female, wild-type, heterozygous and NOP receptor knockout mice were used in the second set of experiments. In the latter case, mice were tested in sets separated by sex. A total of 144 mice were used for place conditioning studies, of which six were removed due to a technical problem.

Conditioning was performed using an unbiased experimental design (see Sakoori & Murphy, 2004) consisting of a 20 min pretest of initial preference (in the drug-free state, day 1) and eight 40 min conditioning sessions (i.e. four VEH and four drug session given on alternate days, days 2–9). The expression of place preference to the drug-paired compartment was serially tested (20 min test in the drug-free state) on three occasions subsequent to drug conditioning: the day following conditioning (day 10), 29 days later (day 39), and 39 days after the second test (day 78). During drug conditioning sessions,

animals received a s.c. injection of VEH, morphine (3 mg kg^{-1}), or CompB (0.3–30 mg kg^{-1}). Locomotor activity was automatically recorded throughout all conditioning sessions. Place preference was calculated by subtraction of time spent in the drug-paired compartment during the pretest from the time spent in the same compartment during the tests for each animal. Following each test, animals remained in their home cages in their original groupings.

Neurochemical analysis and verification of probe and cannulae placements

Dialysate NA, DA and 5-HT contents were determined by HPLC with electrochemical detection as described previously (Koizumi *et al*., 2004). Data are presented uncorrected for the addition of 12.5 mM perchloric acid/250 μM EDTA (where applicable).

Neuroanatomical positions of microdialysis probes and injection cannulae were determined by histological means as described previously (Koizumi *et al*., 2004). Only animals with microdialysis probes located within the NAc between the anterior–posterior coordinates of +0.62 and +1.78 mm relative to bregma (according to the Atlas of Franklin & Paxinos, 1997) were included in subsequent analyses, and (where applicable) i.c.v. cannulae clearly targeted at the lateral ventricle.

Drugs and reagents

N/OFQ was synthesised and purified by HPLC by the RIKEN Brain Science Institute core facility. Structure and purity were confirmed by HPLC, mass spectrometry, and amino-acid analysis. Actual peptide content was determined in comparison to commercially available N/OFQ peptide (Peptide Institute Inc., Osaka, Japan). CompB (a gift from Banyu Pharmaceutical Company, Japan) and morphine hydrochloride (Sankyo Co, Tokyo, Japan) were dissolved to give final concentrations corrected for both molecular water and hydrochloride content. Unless otherwise stated, reagents were purchased from Wako (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma (Tokyo, Japan)

Data analysis and statistics

Points of no-net-flux, probe recoveries, and absolute dialysate DA concentrations were analysed by one-way analysis of variance (ANOVA). Basal levels of mean dialysate DA concentrations (i.e. mean of four predrug dialysate concentrations) and total locomotion were analysed by either one- or three-way ANOVA as appropriate. For comparison of changes in DA after drug administration, dialysate DA concentrations were normalised to the respective mean basal concentration. Differences in DA release following drug treatment were identified by univariate repeated measures ANOVA of the 3 h postdrug administration period using treatment, genotype, and sex as main factors as appropriate. Locomotion data were analysed in the absolute form (i.e. in the raw form, not normalised) in the same way. Conditioned place preference scores were analysed by univariate repeated measures ANOVA taking treatment, genotype and sex as main factors where appropriate. All results are expressed as the mean ± standard error of the mean (s.e.m.). A Student–Newman–Keuls *post hoc* analysis was used to determine group differences in main effects whenever the ANOVA yielded a globally significant F score. Contrast *post hoc* analysis was used to identify specific group differences when significant interactions of main effects together, or with time, were identified. ANOVA testing was made using SuperANOVA software (Abacus Concepts, Berkeley, CA, U.S.A.). Statistical significance was taken at *P*-values less than 0.05.

Results

Histological analysis of microdialysis probe positions

Microdialysis probes were distributed between the anterior–posterior coordinates of 1.78 and 0.62 mm relative to bregma. Probes sampled primarily from the interface of the shell and core regions of the NAc (Figure 1). No systematic differences between the locations of probes between groups were evident.

Microdialysis experiment 1: accumbal dialysate DA concentrations and locomotion following CompB or morphine administration

No significant differences in absolute accumbal dialysate DA concentrations were found between treatment groups prior to drug administration. The mean basal accumbal dialysate DA concentration was 0.444 ± 0.019 nM. DA levels showed a slight tendency to decrease progressively in the hour sampling period prior to drug administration. CompB induced a dose-dependent increase in accumbal dialysate DA concentrations in the NAc (Figure 2a). A significant main effect of treatment

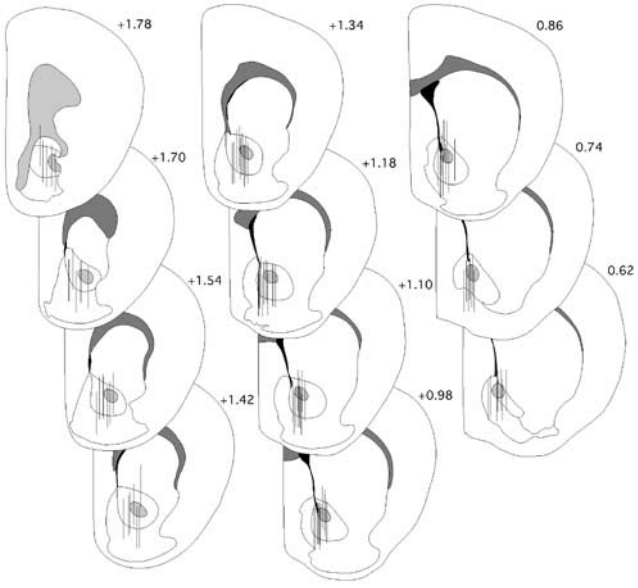

Figure 1 Schematic representation of coronal sections illustrating the locations of microdialysis probes in the NAc. Vertical lines represent axis of the area of active membrane. Samples were collected from a total of 117 microdialysis probes, of which the locations of 87 are shown in the figure. The locations of the remaining 30 probes existed in places already marked by the 87 locations shown. Figures adapted from Franklin & Paxinos (1997). Numbers represent anterior–posterior coordinate (in mm) relative to bregma.

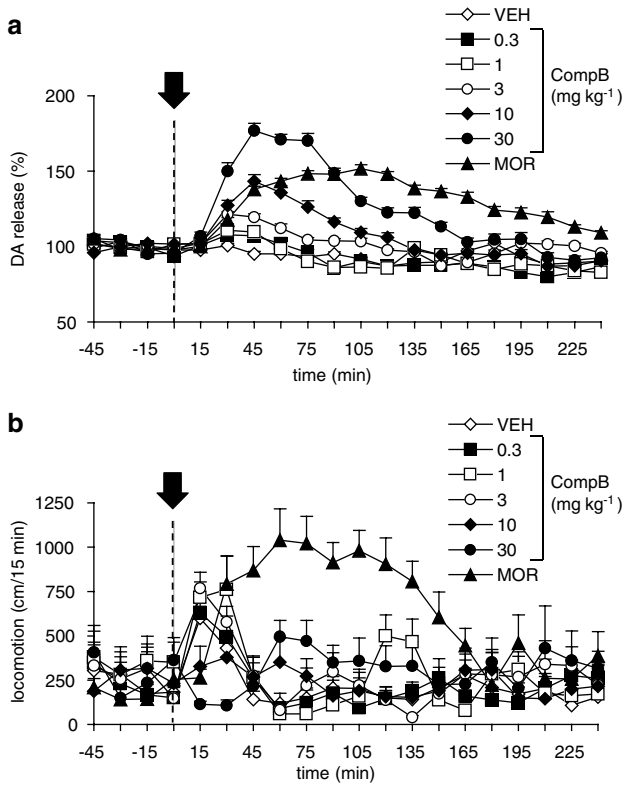

Figure 2 Effect of s.c. CompB administration or morphine (MOR, 3 mg kg^{-1}, arrow) on accumbal dialysate DA concentrations and locomotion in mice. (a) Both CompB and MOR administration stimulated increases in accumbal DA concentrations. Data are expressed as a percentage (mean ± s.e.m.) of the mean DA concentration of four predrug dialysate samples. *n* per group: 6–11. (b) CompB had no statistically significant effect on locomotor activity, although the highest dose tested tended to suppress locomotion in the immediate postinjection 30 min period. Data are expressed in the absolute form (mean ± s.e.m.). *n* per group: 8–13.

($F6,50 = 15.198$, $P < 0.0001$), time ($F11,547 = 41.572$, $P < 0.0001$), and a significant interaction ($F66,547 = 8.851$, $P < 0.0001$) between the two was evident. *Post hoc* analysis revealed a significant increase in accumbal dialysate DA concentrations was evident in mice administered 10 or 30 mg kg^{-1} CompB or 3 mg kg^{-1} morphine when compared to mice administered VEH. Comparison of the profiles of accumbal dialysate DA concentrations showed that CompB induced more rapid but shorter-lived increases than morphine.

No significant differences in total predrug locomotion between treatment groups were evident ($F6,61 = 0.657$, NS). A significant main effect of treatment ($F6,61 = 25.440$, $P < 0.0001$), time ($F11,671 = 6.358$, $P < 0.0001$), and a significant interaction ($F66,671 = 4.466$, $P < 0.0001$) between the two was evident on locomotion following drug administration. Compared to VEH-treated mice, significant increases in locomotion were observed in mice administered morphine only, with the peak effect occurring approximately 1 h after injection (Figure 2b). In contrast, locomotor activity was not changed following CompB administration at any of the doses tested, although short-term depressions in locomotor activity were seen immediately after injection of the highest dose tested. It is notable that two of the 13 mice administered the highest dose (30 mg kg^{-1}) of CompB experienced fatal seizures, although no such effect was observed after administration of the same dose during conditioned place preference experiments (see below).

Microdialysis experiment 2:assessment of absolute extracellular neurotransmitter concentrations and determination of the effect of CompB on accumbal dialysate DA concentrations in NOP receptor knockout mice

No-net-flux analysis reliably determined accumbal absolute extracellular concentrations of DA only. Extreme low accumbal NA and 5-HT concentrations compounded with difficulties in their measurement by HPLC precluded a reliable no-net-flux determination of absolute extracellular NA and 5-HT concentrations. Examples of DA [Cout] concentration profiles are shown in Figure 3. Both male and female animals showed nonsignificant trends towards higher accumbal extracellular DA concentrations as a result of NOP receptor knockout (Figure 4 and Table 1).

On the second day of sampling, NOP receptor knockout animals showed significantly higher accumbal dialysate DA concentrations ($F1,17 = 6.474$, $P = 0.0209$). No significant effect of sex or interaction with any other factor was observed. Basal accumbal dialysate DA concentrations were 0.272 ± 0.046, 0.361 ± 0.042, 0.473 ± 0.109, and 0.547 ± 0.099 nM for wild-type male, NOP receptor knockout male, wild-type female, and NOP receptor knockout female animals, respectively. A significant main effect ($F1,17 = 32.036$, $P < 0.0001$) of CompB (10 mg kg^{-1}) administration was found in addition to a significant effect of time ($F11,184 = 14.181$, $P < 0.0001$) and interaction between treatment and time ($F11,184 = 4.939$, $P < 0.0001$), although no significant main effect of genotype or interaction between genotype and treatment was evident. Furthermore, no significant effect of sex or interaction with any of the other two factors was evident. Thus, CompB administration induced similar increases in accumbal dialysate DA concentrations in both wild-type and NOP receptor knockout animals with sex having no bearing on this effect (Figure 5).

Microdialysis experiment 3: effect of i.c.v. N/OFQ administration on CompB-induced changes in accumbal dialysate DA concentrations

No significant differences in basal accumbal dialysate DA concentrations were found prior to drug treatment. The mean basal DA concentration across all treatment groups was 0.374 ± 0.020 nM. Statistical analysis of the postdrug period showed a significant main effect of treatment ($F3,26 = 16.427$, $P < 0.0001$), time ($F11,285 = 8.042$, $P < 0.0001$), and interaction ($F33,285 = 6.260$, $P < 0.0001$) between treatment and time. I.c.v. administration of 6 nmol N/OFQ induced a significant and long-term suppression in accumbal dialysate DA concentrations with a nadir of approximately 50% basal levels occurring 45 min after injection (Figure 6). CompB (10 mg kg^{-1}) administration stimulated accumbal dialysate DA concentrations in a manner similar to Experiment 1. However, coadministration of 6 nmol N/OFQ did not influence the increase in accumbal dialysate DA concentrations induced by Comp B administration.

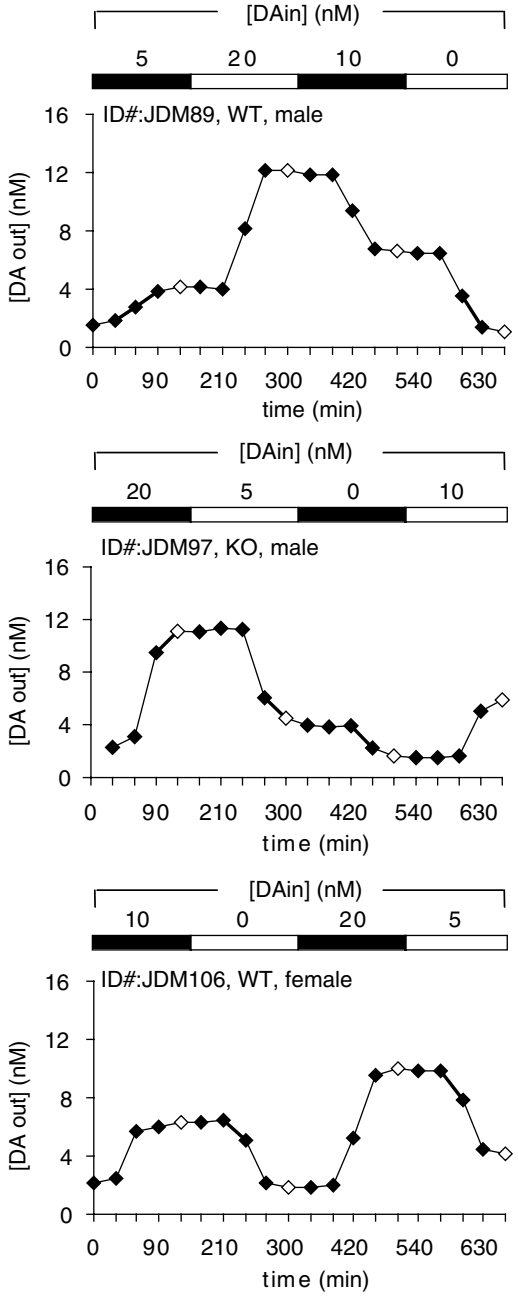

Figure 3 Representative plots from three individual experiments showing the time course of DA recovered from microdialysis probes placed in the NAc of mice of various genotypes and sexes during no-net-flux determinations. Black and white bars indicate perfused concentrations of neurotransmitters made in random order. Note the lag between changing perfused concentration and achievement of equilibrium in the recovered dialysates. This lag was due to the slow perfusion rate, dead space between the dialysis membrane and collection tube, and time required to reach dynamic equilibrium. Open symbols represent dialysate samples used for the determination of points of no-net-flux. WT, wild type; KO, NOP receptor knockout.

Hedonic effect of CompB in the place conditioning paradigm

Similar to microdialysis studies, CompB induced no changes in locomotion during conditioning sessions (data not shown). Mice conditioned with CompB showed a dose-dependent place preference for the drug-paired compartment with a significant main effect of treatment ($F6,59 = 7.043$, $P < 0.0001$), time ($F2,118 = 8.501$, $P = 0.0004$), and interaction between treatment and time ($F12,118 = 2.916$, $P = 0.0014$). Contrast analysis showed significant conditioned place preferences for morphine and the two highest doses of CompB at all time points after conditioning tested (Figure 7a). Furthermore, significant increases in the strength of the expressed conditioned place preference between the tests on days 10 and 39 were seen for those same treatments. Although contrast analysis showed no significant difference between the magnitude of the conditioned place preference for 10 and 30 mg kg^{-1} CompB during the test performed on day 10, the conditioned place preference expressed by animals conditioned with 30 mg kg^{-1} CompB was significantly greater than those conditioned with 10 mg kg^{-1} CompB on days 39 and 74. Thus, the dose responsive of the rewarding properties of CompB was most clearly expressed during repeated testing.

No significant main effect of genotype was found on the magnitude of conditioned place preferences to CompB (30 mg kg^{-1}), although a significant main effect of treatment ($F1,60 = 41.825$, $P < 0.0001$) was evident reflecting an equally sized place preference to CompB across all genotypes (Figure 7b). Similar to the dose–response study, CompB-induced conditioned place preferences were expressed most strongly when tested 30 days after the final conditioning session (i.e. day 39) as reflected by a significant main effect of time ($F2,119 = 20.228$, $P < 0.0001$) and interaction between time and treatment ($F2,119 = 6.521$, $P = 0.0021$). No significant effect of sex on the strength of place preference or interaction with any other factor was found during any of the tests for conditioned place preference.

Discussion

The aim of the current study was to test the hypothesis that endogenous N/OFQ determines basal activity in the mesolimbic DA system and the expression of hedonia within the brain by studying NOP receptor knockout mice and application of the nonpeptide NOP receptor antagonist CompB. This hypothesis was based on two main lines of previous study, these being (1) N/OFQ blocks the reinforcing or rewarding potential of several abused drugs (Ciccocioppo *et al*., 1999; 2000; 2002; 2004; Murphy *et al*., 1999a; Kotlinska *et al*., 2002; Lutfy *et al*., 2002; Kuzmin *et al*., 2003; Zhao *et al*., 2003; Sakoori & Murphy, 2004) and (2) N/OFQ suppresses basal and drug-stimulated activation of the mesolimbic DA system (Murphy *et al*., 1996; Di Giannuario *et al*., 1999; Murphy & Maidment, 1999c; Di Giannuario & Pieretti, 2000; Lutfy *et al*., 2001; Koizumi *et al*., 2004). The over-riding finding was that CompB induces mesolimbic DA release and was rewarding, in the absence of changes in locomotion, yet surprisingly, this action was *not* mediated through the NOP receptor.

CompB is a relatively recently developed NOP receptor antagonist showing high selectivity for the NOP receptor over classical opioid receptors (Ozaki *et al*., 2000a, b). Thus far, the majority of published studies using CompB have been performed *in vitro*. These studies may be approximately divided into those either characterising the efficacy and selectivity of CompB itself (e.g. Bigoni *et al*., 2000; Ozaki *et al*., 2000a, b; Ichikawa *et al*., 2001) or those employing

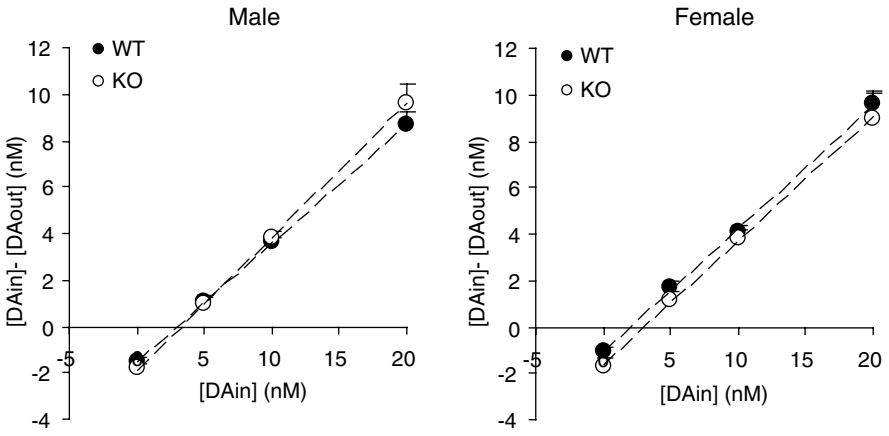

Figure 4 No-net-flux plots of DA concentrations in the NAc of mice of various genotypes and sexes. Points of no-net-flux (equivalent to the actual absolute extracellular neurotransmitter concentration) were calculated by linear regression of [Cin]−[Cout] against [Cin] followed by determination of [Cin] at the point where [Cin]−[Cout] was zero under steady-state conditions (see Methods for more details). Points represent the mean±s.e.m. See Table 1 for calculated points of no-net-flux, extraction fractions, and *n*'s per group. WT, wild type; KO, NOP receptor knockout.

Table 1 Basal extracellular DA concentrations and extraction fractions in the NAc of male and female, wild-type, and NOP receptor knockout mice determined by no-net-flux analysis

	Wild type			*NOP receptor knockout*		
	Male	*Female*	*Combined*	*Male*	*Female*	*Combined*
Extracellular concentration (nM)	2.915±0.356 (6)	1.975±0.441 (5)	2.487±0.295 (11)	3.168±0.485 (6)	2.945±0.659 (4)	3.079±0.350 (10)
Extraction fraction (%)	50.9±3.1 (6)	53.0±3.5 (5)	51.8±2.1 (11)	57.0±4.7 (6)	53.1±5.9 (4)	55.5±3.4 (10)

Data shown as mean±s.e.m. Numbers within parentheses represent *n*.

CompB for verifying that actions of N/OFQ are mediated through the NOP receptor (e.g. Chin *et al*., 2002; Mela *et al*., 2004). *In vivo* studies employing CompB are fewer, although an appreciable number have recently accumulated. The majority of these studies have used direct administrations of CompB into discrete regions of the central nervous system. These studies show that CompB blocks several actions of N/OFQ including its antitussive properties (Bolser *et al*., 2001) and the ability of N/OFQ to suppress behavioural sensitization to cocaine (Lutfy *et al*., 2002). Least of all are studies employing peripheral administrations of CompB. However, it is this final class of studies that are particularly relevant to the studies presented here. These show that peripheral CompB administration (at a dose of 20 mg kg^{-1}) induces an increase in NA concentrations in the amygdala, which is reversible by local application of N/OFQ (Kawahara *et al*., 2004). Of particular relevance, CompB (also at a dose of 20 mg kg^{-1}) possesses antidepressant-like properties (Redrobe *et al*., 2002), which concurs well with the current study as antidepressant drugs, by their nature, act to raise hedonic state.

To our knowledge, the current study is the first to assess directly an action of CompB administered alone in mice lacking the NOP receptor. In many respects, assaying pharmacological effects in animals lacking the gene of the hypothesised cognate receptor may be considered the acid test of drug specificity. Consequently, the current study clearly identified CompB as capable of neurochemical and behavioural actions *independent* of the NOP receptor. This conclusion was achieved as central N/OFQ administration did not influence the neurochemical actions of CompB. Moreover, and perhaps more decisively, the neurochemical and behavioural effects of CompB were maintained in mice deficient of NOP receptors. It is important to note that the effects of CompB on mesolimbic DA release and hedonic state were practically identical in wild-type and NOP receptor knockout mice, that is, they were neither stronger nor weaker, suggesting that the NOP receptor plays no role at all in mediating these effects.

Identifying the site of these non-NOP-receptor-mediated actions is likely to be challenging. Previous attempts at developing CompB as an *in vivo* imaging agent have been unsuccessful due to widespread and nonspecific binding of CompB at receptor and nonreceptor sites (Ogawa *et al*., 2003). Although these studies were unable to determine what these additional binding sites were, the inability of the general opiate antagonist naloxone to affect CompB binding suggests they are not classical opioid receptors. Aside from this study, very few reports are available to indicate what the site of CompB action observed in the current study might be, making it difficult to speculate on how CompB may be acting. Agonistic properties at other G-protein-coupled receptor may be eliminated as a possible mechanism, since [^{35}S]GTPγS binding is absent following treatment with CompB in tissue prepared from NOP receptor knockout mice (Ichikawa *et al*., 2001). However, this observation goes only a short way to revealing the full pharmacological profile of CompB. Numerous alternative sites exist within the peripheral and central nervous system at which drug action may occur, including ion channels, transporters, and intracellular receptors. It is essential to note that a peripheral route of CompB was

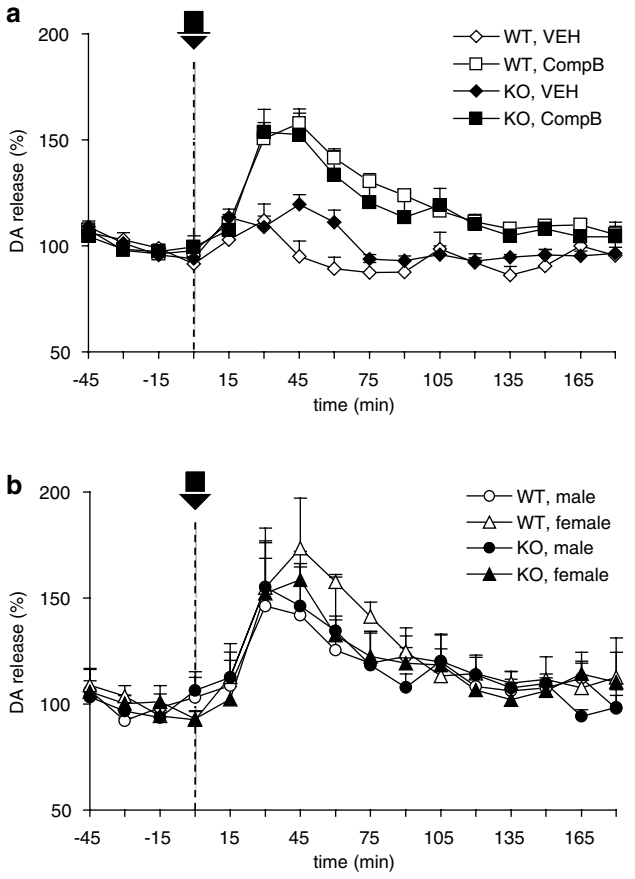

Figure 5 Effect of s.c. VEH or CompB administration (10 mg kg^{-1}, arrow) on accumbal dialysate DA concentrations of male and female, wild-type, and NOP receptor knockout mice. Data are expressed as a percentage (mean ± s.e.m.) of the mean accumbal DA dialysate concentration of four predrug dialysate samples. (a) Mean data averaged across sexes, separated by treatment and genotype. The stimulatory effect of CompB on accumbal dialysate DA concentrations was unaltered by deletion of the NOP receptor gene. *n* per group: 6–7. (b) Mean data separated by sexes for all CompB-treated animals. The response to CompB was the same in male and female, knockout, and wild-type mice. VEH-treated animals not shown for clarity. *n* per group: 3. WT, wild type; KO, NOP receptor knockout.

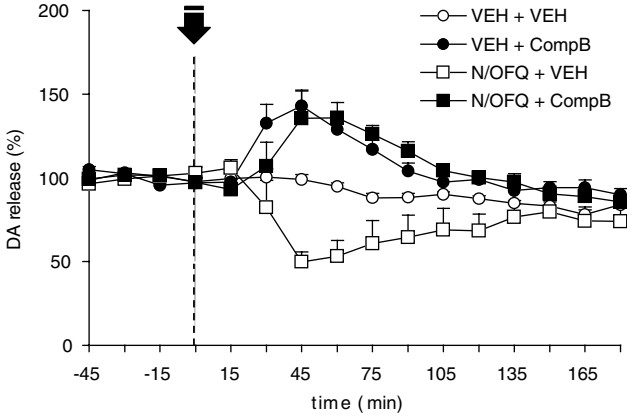

Figure 6 Effect of i.c.v. N/OFQ administration (6 nmol, arrow) on s.c. CompB- (10 mg kg^{-1}, arrow) stimulated accumbal dialysate DA concentrations. Administration of N/OFQ alone induced a long-lasting depression in basal accumbal dialysate DA concentrations. However, the same dose of N/OFQ had no effect on CompB-stimulated accumbal dialysate DA concentrations. Data are expressed as a percentage (mean ± s.e.m.) of the mean accumbal dialysate DA concentration of four predrug dialysate samples. *n* per group: 7–8.

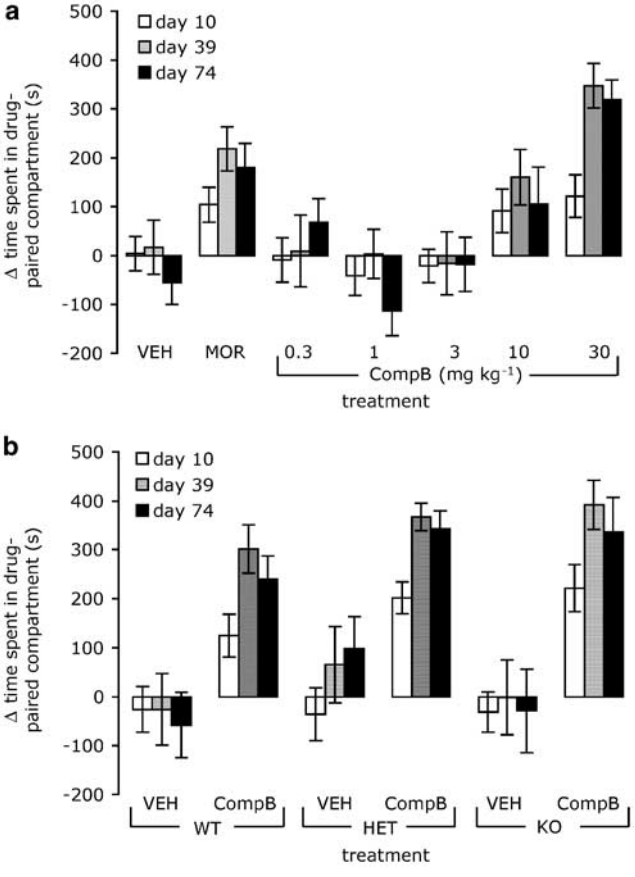

Figure 7 Hedonic properties of CompB as assessed by place conditioning. (a) Mice conditioned with CompB (0.3–30 mg kg^{-1} s.c.) developed a dose-dependent conditioned place preference that was expressed most strongly during repeated testing. MOR, 3 mg kg^{-1} morphine s.c. *n* per group: 9–11. (b). The rewarding effect of CompB (30 mg kg^{-1}) was unaltered by deletion of the NOP receptor gene. *n* per group: 11–13. WT, wild type; HET, heterozygote; KO, NOP receptor knockout.

employed in the current study. Without evidence to suggest otherwise, a peripheral site of action is just as likely as a central site of action. For example, CompB could be eliciting the neurochemical and behavioural responses observed in the current study by a direct or, possibly, indirect action on afferent peripheral nerves. Indeed, stimulation of the vagus nerve has been considered useful for the treatment of several psychiatric disorders including depression (see George *et al.*, 2000). Clearly, much further study is necessary to identify both the pharmacological and anatomical sites of CompB action.

In order to gain an understanding of the role of endogenous N/OFQ in the maintenance of basal mesolimbic activity, we performed a no-net-flux analysis of absolute basal neurotransmitter concentrations in the NAc of wild-type and NOP receptor knockout mice. We found that mice lacking the NOP receptor showed tendencies towards (but not statistically significant) higher basal levels of DA in the NAc when analysed by no-net-flux microdialysis. Furthermore, a statistically significant difference between NOP receptor knockout

and wild-type mice was found in basal dialysate DA contents on the day following sampling for no-net-flux analysis. In general, comparisons of absolute neurotransmitter concentrations in dialysates are made within 24 h after microdialysis probe insertion and, as such, do not reflect actual endogenous neurotransmitter concentrations as reliably as that determined by no-net-flux analysis. Taken together, these findings suggest that endogenous N/OFQ places only a weak suppressive tone, if any, on mesolimbic activity as suggested by our previous studies (Murphy *et al*., 2002; Koizumi *et al*., 2004), despite neuroanatomical studies showing widespread expression of NOP receptors in mesolimbic DA neurons (Maidment *et al*., 2002; Norton *et al*., 2002). However, it is important to bear in mind that compensatory changes may have occurred during development in NOP receptor knockout animals. Previous studies of the closely related kappa opioid system reveal that deletion of the KOP receptor gene leads to upregulated basal DA levels in the NAc (Chefer *et al*., 2000), as predicted by the suppressive effect of dynorphin and other kappa agonists on mesolimbic DA release. Although both KOP receptor agonists (see Murphy *et al*., 2002 for references) and NOP receptor agonists (Ciccocioppo *et al*., 1999; 2000; 2004; Murphy *et al*., 1999a; Kotlinska *et al*., 2002; Kuzmin *et al*., 2003; Zhao *et al*., 2003; Sakoori & Murphy, 2004) block the rewarding potential of several abused drugs, KOP receptor agonists are considerably more aversive than agonists of the NOP receptor (see Tzschentke, 1998 for references). Thus, it may not be entirely unexpected that endogenous N/OFQ tone plays less of a role in determining either basal or stimulated activity of mesolimbic DA neurons.

In addition to the unexpected finding that CompB acts at a site other than the NOP receptor, the current study yielded the interesting observation that CompB-induced mesolimbic DA release and reward is not accompanied by any change in locomotor activity. In fact, at the highest doses tested, where the strongest rewarding effect was found, locomotion was mildly suppressed by CompB. Although it is not a universal phenomenon, rewarding drugs tend to both activate the mesolimbic system and induce increases in locomotion, a realisation that has been hypothesised to be linked in the past (Wise & Bozarth, 1987). However, as the site at which CompB induced the neurochemical and behavioural actions in the current study is far from clear, it is currently difficult to speculate on whether CompB simply lacks inherent locomotor properties or that CompB acts concurrently at many sites with an overall outcome of no effect on locomotion. Nonetheless, the observation itself is interesting and may be worthy of further study.

In summary, the NOP receptor antagonist CompB stimulates mesolimbic DA release and induces reward by a non-NOP-receptor-mediated mechanism. Consequently, we strongly recommend that care be taken in the interpretation of experimental data obtained using this drug, particularly when administered *in vivo* by a peripheral route.

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