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The anxiolytic-like profile of the nociceptin receptor agonist, endo-8-[bis(2-chlorophenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1] octane-3-carboxamide (SCH 655842): Comparison of efficacy and side effects across rodent species

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ABSTRACT

The endogenous opioid-like peptide, nociceptin, produces anxiolytic-like effects that are mediated via the nociceptin (NOP) receptor. Similarly, synthetic, non-peptide NOP agonists produce robust anxiolytic-like effects although these effects are limited by marked side effects. In the present studies, the effects of a novel NOP receptor agonist, SCH 655842, were examined in rodent models sensitive to anxiolytic drugs and tests measuring potential adverse affects. Oral administration of SCH 655842 produced robust, anxiolytic-like effects in three species, i.e., rat, guinea pig, and mouse. Specifically, SCH 655842 was effective in rat conditioned lick suppression (3–10 mg/kg) and fear-potentiated startle (3–10 mg/kg) tests, a guinea pig pup vocalization test (1–3 mg/kg), as well as in mouse Geller–Seifter (30 mg/kg) and marble burying (30 mg/kg) tests. The anxiolytic-like effect of SCH 655842 in the conditioned lick suppression test was attenuated by the NOP antagonist, J-113397. In mice, SCH 655842 reduced locomotor activity and body temperature at doses similar to the anxiolytic-like dose and these effects were absent in NOP receptor knockout mice. In rats, SCH 655842 did not produce adverse behavioral effects up to doses of 70–100 mg/kg. Pharmacokinetic studies in the rat confirmed dose-related increases in plasma and brain levels of SCH 655842 across a wide oral dose range. Taken together, SCH 655842 may represent a NOP receptor agonist with improved tolerability compared to other members of this class although further studies are necessary to establish whether this extends to higher species.

1. Introduction

Over the past decade, there has been considerable interest in the nociceptin opioid receptor (NOP), the most recently discovered member of the opioid receptor family. The NOP receptor shares approximately 60% homology with other opioid receptors (μ or MOP, κ or KOP, δ or DOP), but does not bind classic opioid ligands. Rather, the NOP receptor binds a high affinity endogenous ligand subsequently identified as OFQ/N or nociceptin (Meunier et al., 1995; Reinscheid et al., 1995).

The NOP receptor is expressed in brain regions involved in the integration of emotional stimuli such as fear and stress, including amygdaloid complex, hippocampus, septum, bed nucleus of the striata terminalis, and hypothalamic regions (Meunier, 1997; Mollereau et al., 1994; Mollereau and Mouledous, 2000). Accordingly, there has been considerable interest in the NOP receptor as a target for the treatment of anxiety-related disorders (see Civelli, 2008) which is supported by genetic and pharmacological evidence. Thus mice lacking either nociceptin or the NOP receptor exhibit increased anxiety-like behavior in models such as open-field, elevated plus-maze and light dark-aversion (Gavioli et al., 2007; Koster et al., 1999), and studies using intracerebroventricular (i.c.v.) injections of nociceptin demonstrate robust anxiolytic-like behavioral effects in both mice and rats (Griebel et al., 1999; Jenck et al., 1997).

Given such therapeutic potential of NOP agonists, a number of synthetic, brain-penetrant agonists have been identified. The prototype compound, Ro 64-6198, has confirmed and extended the anxiolytic potential of this drug class in multiple rodent species (Jenck et al., 2000;

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Nicolas et al., 2006; Varty et al., 2005). Newer, orally active NOP agonists have also been reported including SCH 221510 (Varty et al., 2008), PCPB and MCOPPB (Hirao et al., 2008a,b) with similar profiles.

However, these studies have also highlighted mechanism-based liabilities that could limit therapeutic potential. Specifically, Ro 64-6198 produces marked sedation, motor impairments and hypothermia; effects which are absent in NOP receptor knockout mice, confirming a target mechanism-based origin (Higgins et al., 2001; Varty et al., 2005). Such effects emerge at doses very similar to that required for anxiolysis (Jenck et al., 2000; Hirao et al. (2008a,b). However, the chemically distinct NOP agonist SCH 221510, exhibited approximately a 10-fold separation between the anxiolytic doses (3 mg/kg) and potential liability doses (>30 mg/kg) (Varty et al., 2008), suggesting that it may be possible to design NOP receptor agonists with a reduced propensity to produce unwanted side effects.

Recently, we described the synthesis and preliminary profile of a novel NOP receptor agonist, SCH 655842 (endo-8-[bis(2-chlorophenyl) methyl]-3-phenyl-8-azabicyclo [3.2.1]octane-3-carboxamide; see Fig. 1 and compound 24 in Ho et al., 2009). The present report describes a broad characterization of this compound across a variety of models sensitive to anxiolytic drugs and also tests measuring potential adverse effects. Furthermore, we determined plasma and brain levels of SCH 655842 at both an anxiolytic dose and a dose causing potential adverse effects to establish exposure multiple. The present studies suggest SCH 655842 has an improved tolerability profile compared to other NOP agonists currently reported in the literature.

2. Materials and methods

2.1. Drugs

SCH 655842 and J-113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one) were synthesized by the Chemical Research department of the Merck Research Labs, Kenilworth, NJ. Chlordiazepoxide hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). SCH 655842 was administered via oral gavage (p.o.) in 0.4% methylcellulose at dosing volumes of 5, 2, and 10 ml/kg for rat, guinea pig and mouse, respectively. J-113397 was administered intraperitoneally (i.p.) in saline at a dosing volume of 1 ml/kg. Chlordiazepoxide was dissolved in saline and administered i.p. at a dosing volume of 1 ml/kg. All drug doses were expressed as free base. SCH 655842 was administered 2 h prior to testing (except where indicated otherwise and for the rat and mouse locomotor activity studies when SCH 655842 was administered 1 h before testing), and J-113397 and chlordiazepoxide were administered 30 min prior to any test.

2.2. Subjects

Male CD and Wistar (only for fear-potentiated startle studies) rats (200–300 g), male C57BL/6 mice (20–25 g) and male and female Dunkin–Hartley guinea pig pups were purchased from Charles River Laboratories (Kingston, NY). As described by Varty et al. (2005), wild-type (WT) and NOP receptor knockout (KO) male and female mice were derived from animals originally obtained from Nishi et al. (1997) that were produced on a C57BL/6 and 129X1Sv mixed genetic background. Mice were derived from same genotype breeding. Rats were housed 3 per cage (housed individually for the conditioned lick suppression study), and mice were housed 5 per cage except for the NOP KO and WT mice which were housed individually for at least one week of acclimation prior to experiments. Guinea pig pups were 2–3 days of age upon arrival (transported and housed 3 per cage with the dam) and were used up to 21 days of age.

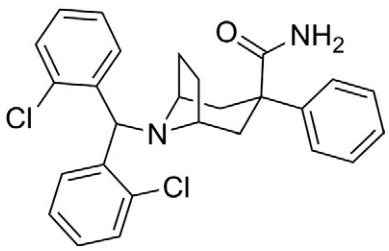

Fig. 1. Structure of SCH 655842 (endo-8-[bis(2-chlorophenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octane-3-carboxamide).

All animals were housed under a 12-h light-dark cycle (lights on 07:00 h) and allowed food and water ad libitum (except for rats for the Conditioned Lick Suppression assay which were water-restricted to 60 min of water access per day). All tests were performed during the light cycle between 09:00–17:00 h. All studies were conducted at an AAALAC-accredited facility in accordance with the NIH 'Guide to the Care and Use of Laboratory Animals' and the Animal Welfare Act, and guidelines established by the Institutional Animal Care and Use Committee (IACUC).

2.3. Behavioral studies: efficacy models

2.3.1. Rat conditioned lick suppression

Eight standard rat operant boxes (32×25×25 cm; L×W×H) (Med Associates, Georgia, VT) were used for the conditioned lick suppression test. Each chamber contained a stainless steel drinking spout that was connected to an external 200 ml bottle containing 0.2% saccharin solution. The bottom of each chamber was equipped with a metal grid that delivered foot shocks to the animal. Licks were recorded automatically by a lickometer connected to the bottle and a computer.

Forty rats were water deprived for 18–20 h and trained under a schedule of conditioned lick suppression. Under this schedule, each rat was presented with 20 trials each consisting of 23 s of unpunished drinking, followed by 7 s during which a tone was delivered to the apparatus. Licking during the first 2 s of the tone presentation went unpunished, whereas every lick during the final 5 s was punished by delivery of a foot-shock (0.7 mA, 500 ms duration). Rats were trained under this schedule 5 days per week until they made fewer than 5% of total licks during the unpunished phase. When rats had reached this criterion, they were tested during a session in which tone was presented but shock delivery was turned off. If rats made less than 5% of total licks in the tone periods under these conditions they were deemed to be conditioned and suitable for drug testing. On the day of testing, rats were brought to the test room and allowed to acclimate for at least 30 min. After administration of vehicle or a dose of drug, rats were placed into the test chambers and the number of licks made during both the unpunished (no tone) and punished (tone, no shock) components of the schedule were recorded for 20 trial presentations. Drug was administered using a within-subjects crossover design such that the effect of vehicle administration and up to 3 doses of drug were assessed in the same animal over several different test sessions. Drug testing occurred no more than twice a week (with at least 2 days between tests) and only following sessions in which rats made less than 5% of total licks during the tone periods with the shock turned off. On intervening days, rats continued to be trained in conditioning sessions with both tone and concomitant shock.

2.3.2. Rat fear-potentiated startle

All testing took place in the SR-LAB startle system (San Diego Instruments, San Diego, CA) equipped with a potentiated startle kit. Testing was conducted as previously described (Varty et al., 2008). Briefly, male Wistar rats were conditioned to associate the presentation of a light cue (3500 ms duration) with the imminent presentation of mild foot-shock (0.4 mA, 500 ms duration). Conditioning consisted of 20 presentations of the light/shock pairings [with an average inter-trial interval of 90 s] on two consecutive days. Following conditioning,

rats were exposed to a fear-potentiated startle session that comprised of 30 acoustic startle (105 dB, average inter-trial interval of 25 s, range 20–30 s) trials. The first 5 and last 5 trials were startle alone, and of the remaining 20 startle trials (presented in a pseudorandom order), 10 trials occurred in the presence of a light pair with the startle and 10 trials were startle alone. Any fear associated with the light would present itself as an elevated response to the startle stimuli. Rats were then assigned to balanced vehicle and drug groups based on both the startle response and the level of fear-potentiated startle. The following day, rats were tested in a fear-potentiated startle session again following the administration of either vehicle or drug.

2.3.3. Guinea pig pup vocalization assay

Dunkin–Hartley guinea pig pups (Charles River Laboratories, Kingston, NY) were used in a vocalization procedure described previously by Varty et al. (2005). Briefly, a guinea pig pup was removed from the dam and littermates and placed into a Plexiglas container. A blinded observer manually recorded the total number of separation-induced vocalizations during a 5 min period in a separate testing room. At least 24 h prior the first drug test, the pups were tested for their total number of baseline vocalizations and pups which made less than 200 calls within 5 min were excluded from the study. On the test day, all the animals were transferred from the colony room to a testing room. The animals were acclimatized in the room for at least 30 min and administered either vehicle or drug. Following a pretreatment time (during which the pups were returned to the dam), the total number of separation-induced vocalizations during a 5 min test was recorded.

2.3.4. Mouse Geller–Seifter

The mouse Geller–Seifter procedure has been described previously (see Varty et al., 2005). Briefly, food restricted C57BL/6 mice were trained to lever press for a pellet using a fixed-ratio-1 (FR-1) schedule of reinforcement, before being progressively increased to FR-10 (10 lever presses per pellet). After demonstrating stable FR-10 responding for one week, mice were trained on a Geller–Seifter conflict schedule. The 40 min Geller–Seifter testing schedule consisted of eight alternating 5 min phases of unpunished and punished responding. During the unpunished phase (house light on), the mice received a food pellet and during the punished phase (house light off and signaled by a tone), the mice received a food pellet paired to foot shock delivery (0.3 mA, 0.25 s). The animals were trained to meet or surpass a criterion (over 400 lever presses per session with a 70%:30% distribution of presses in the unpunished/punished phases) for eight consecutive testing days before any drug testing. On test days, drug or vehicle was administered and the number of responses was recorded. All studies used a within-subjects crossover design with 3–4 days between tests.

2.3.5. Mouse marble burying

Mice were transferred to the test room and allowed to acclimate for 30 min. After administration of vehicle or drug, mice were individually placed into a clear plastic cage ($46\times25\times15$ cm) containing 15 glass marbles of 1.5 cm diameter, evenly spaced on sawdust bedding (5 cm deep). Mice were left undisturbed in these cages for 60 min. After 60 min, an observer who was blind to the treatment removed the mice from the test cage and counted the number of buried marbles (more than 2/3 of the marble surface buried).

2.4. Behavioral studies: liability models

2.4.1. Fixed-ratio responding

Standard operant boxes enclosed in sound-attenuating chambers (Med Associates, Georgia, VT) were used for mouse and rat fixed-ratio (FR) studies. All animals were food restricted and kept at 80% of their normal free-feeding body weight. Animals were trained to lever press for a pellet (food reward) on a FR-1 (one lever press per reward) schedule of reinforcement which was gradually increased to FR-10. On the test day, animals were administrated either vehicle or drug and placed into the box after an appropriate treatment time. Response rate was calculated by dividing the total number of presses by the total time the animals took to receive 100 pellets or 60 min, whichever occurred first. All the doses of drug were crossed-over in a within-subjects designed study, with 3–4 days between tests.

2.4.2. Rat and mouse locomotor activity

A VersaMax Animal Activity Monitor system (AccuScan Instruments Inc., Columbus, OH) was used for the rat locomotor activity studies, and a Tru Scan Photo Beam Activity Monitoring system (Coulbourn Instruments, Whitehall, PA) was used to measure mouse locomotor activity. Each apparatus consisted of a clear Plexiglas box (rat: $40\times40\times30$ cm; mouse $25\times25\times40$ cm) placed within the activity monitor. Interruption of infra-red photo beams within the locomotors chamber was used to monitor movement. Total locomotor distance, an indicator of ambulatory activity, was the dependent measure. After pretreatment, animals were placed individually into an activity chamber and behavior was assessed for 60 min.

2.4.3. Rat rotarod

The rat rotarod (Ugo Basile, Comerio, Italy) consisted of a 7 cm diameter drum divided by five flanges. The rotarod rotated at a speed of 16 revolutions per minute (RPM). Prior to the test day, rats were trained to remain on the rotarod for 2 min during two successive trials. Rats failing to meet this criterion were not used in subsequent drug studies. On the test day, drug or vehicle was administered and rats were tested on the rotarod for 2 min. Total time that rats remained on the rotarod was recorded (maximum time of 2 min).

2.4.4. Mouse rotarod

An EzRod Accelerating Rotarod system (AccuScan Instruments Inc., Columbus, OH) was used for mouse rotarod studies. A day before any drug test, mice were trained on the rotarod (rotating at a constant speed of 12 RPM) using ten 2 min trials with a 60 s inter-trial interval. Only mice that remained on the rotarod for the 2 min test were used in subsequent drug studies. On the day of testing, mice were treated with either drug or vehicle and then tested on the rotarod for 3 trials with a 60 s inter-trial interval. The time that mice maintained themselves on the rotarod from each trial (maximum time of 2 min) was recorded and the average time of 3 trials was used for data analysis.

2.4.5. Beam walking

Rats were pre-trained to traverse a beam (90 cm length, 2 cm width, 40 cm elevation) and enter a dark box until they reached a criterion of successfully traversing the beam within 60 s without more than three foot slips for two separate trials during the morning and afternoon. On the test day, animals reaching criterion were tested on the beam for two trials and total distance traversed along the beam was recorded. The best performance out of the two trials was used for data analysis.

2.4.6. Body temperature

A rectal probe and digital thermometer (Physitemp, Clifton, NJ) were used to measure core body temperature in rat and mouse. The probe was lubricated with mineral oil and inserted rectally for 3–5 s until the temperature reading stabilized. For rat and mouse, the core body temperature was recorded prior to drug administration and 2 h post-administration.

2.4.7. General observation study

Rats were treated with test compound and formally assessed for signs of sedation and postural changes associated with other NOP agonists, such as splayed hind limbs and flattened body posture (see

Varty et al., 2005). Behaviors were scored as either present or absent (0 = absent, 1 = present) at 30 min intervals for a total of 5 h (maximum score = 10) by 3 independent blinded observers. Chlordiazepoxide (30 mg/kg) was included for comparison. A total of six animals per group were used.

2.5. Rat pharmacokinetic study

Two separate pharmacokinetic studies were conducted in male CD rats with indwelling jugular vein cannula. In the first study, rats were dosed via oral gavage with SCH 655842 (3–70 mg/kg, $n=3$ per dose) and serial blood samples were collected at a series of time points (0.5, 1, 2, 4, 6 and 24 h) post-administration. In the second study, rats were dosed with SCH 655842 (10–70 mg/kg, $n=3$ per dose) and serial blood samples were collected at 0.5, 1, 2, 4, and 6 h time points and additionally, brains were collected at the final 6 h time point. Blood samples were spun in a centrifuge at 980 g for 10 min and plasma samples were extracted and stored at −20 °C until analyzed. Plasma samples were assayed for SCH 655842 using HPLC-MS/MS. Typically, a 25 µl plasma sample was placed in a 1 ml 96-well plate and subjected to protein precipitation by adding 75 µl of acetonitrile which contained the internal standard (SCH 75431, MS/MS transition was m/z 544 to m/z 306 at 24 eV). After vortexing for 60 s, samples were centrifuged at 980 g for 10 min and then 70 µl of the supernatant was transferred to a 350 µl 96-well plate. The samples and standard were then injected onto the HPLC-MS/MS system (typical injection volumes were 10 µl). The HPLC system consisted of a Shimadzu LC-10 AD pump and a LEAP CTC HTS PAL autosampler. Chromatographic separation of SCH 655842 and the internal standard was obtained on a Sprite Armor C18 (5 micron particle size) 40 × 2.1 mm (diameter) HPLC column using a gradient mobile phase system. The mobile phase A was 20/80 methanol/water + 10 mM ammonium acetate; the mobile phase B was methanol + 10 mM ammonium acetate + 0.6 ml/l of 10% acetic acid. The gradient was 99% A at the start, then 75% A at 0.1 min, 5% A at 1.2 min and held until 1.5 min when the gradient was increased back to 99% A at 1.7 min and held for a further 0.3 min. The mobile phase flow rate was held at 0.8 ml/min throughout the assay. The HPLC effluent was sent directly to the electrospray (ESI) source. The MS/MS system was a Thermo Finnigan TSQ Quantum operated in the positive ion mode. The selected reaction monitoring (SRM) transition for SCH 655842 was m/z 465 to m/z 235 with a collision energy setting of 26 eV. Calibration standard samples were prepared to match the expected levels of the samples (typically 1–5000 ng/ml) by spiking known concentrations of SCH 655842 into drug-free rat plasma. The calibration curve was processed and assayed along with the samples and the resulting standard curve was obtained after using least-squares linear regression with suitable weighting (typically $1/x2$) and used peak area ratios of the analyte and internal standard compared to the nominal concentrations. Brain samples were collected at a single time point at the end of a plasma collection period and were homogenized in a volume of deionized water (in ml) equal to four times the weight (in g) of the tissue. The spiked brain homogenate calibration standards and unknown samples were subjected to the same protein precipitation as the plasma samples. The supernatants were analyzed using the same LC-MS/MS method.

2.6. Statistical analysis

Unless otherwise noted, data were analyzed by one-way analyses of variance (ANOVA) using GraphPad Instat version 3.06 (GraphPad Software, San Diego, CA). In all studies, *post-hoc* comparisons were made using Dunnett's *t*-test and the significance level was $P<0.05$.

3. Results

3.1. Behavioral studies: efficacy models

3.1.1. Rat conditioned lick suppression

SCH 655842 dose-dependently increased the number of licks during the punished phase of the conditioned lick suppression test, with significant increases at doses of 3 and 10 mg/kg compared to the vehicle control group [$F(3,48)=4.8$, $P<0.01$] (Fig. 2A). The number of licks during the unpunished phase was not affected by SCH 655842 (Vehicle = 390 ± 91; 1 mg/kg = 300 ± 49; 3 mg/kg = 546 ± 127,

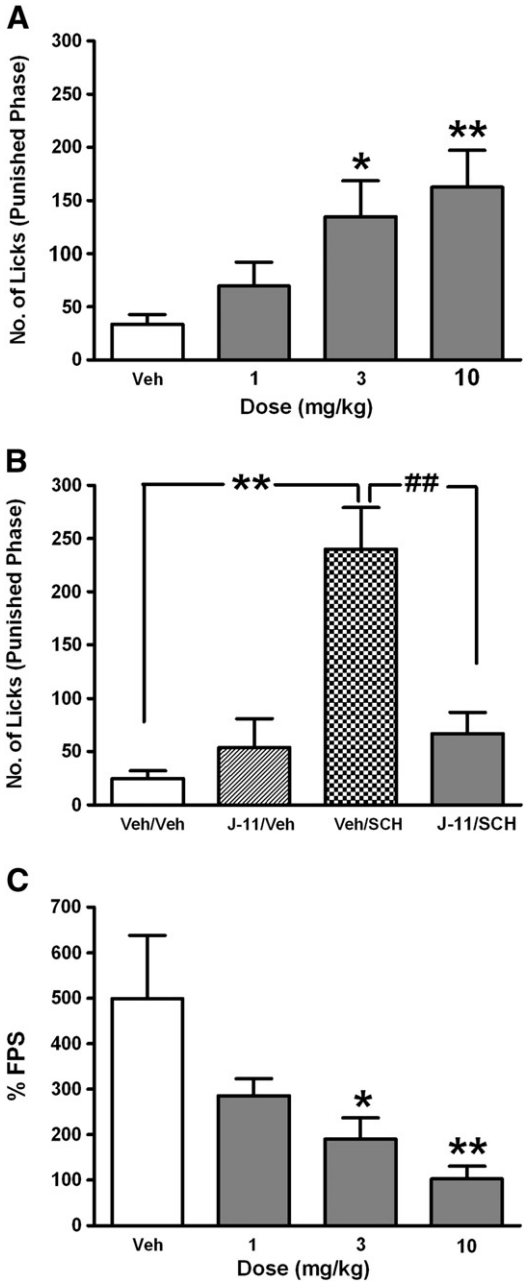

Fig. 2. The anxiolytic-like effects of SCH 655842 (1–10 mg/kg) in the rat and reversal by the NOP antagonist, J-113397. A) Effect of SCH 655842 in the rat conditioned lick suppression test and B) reversal of the anxiolytic-like effect with J-113397. C) Effect of SCH 655842 in the rat fear-potentiated startle (FPS) test. Each bar indicates mean ± standard error of the mean (S.E.M.) of 12–15 rats at each dose. Veh = Vehicle, J-11 = J-113397, SCH = SCH 655842. Significant differences from the vehicle group (Veh or Veh/Veh) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Significant differences from the SCH 655842 group (Veh/SCH) are indicated by ## $P<0.01$.

10 mg/kg = 304 ± 63). This result indicated that SCH 655842 had a selective anxiolytic-like effect in the conditioned lick suppression model.

To confirm SCH 655842 mediates its anxiolytic-like effects via the NOP receptor, rats were dosed with SCH 655842 (6 mg/kg) and the NOP antagonist, J-113397 (10 mg/kg) and tested in the conditioned lick suppression assay. There was a significant effect of treatment [$F(3,44) = 12.0$, $P<0.01$]. Rats treated with SCH 655842 alone exhibited significantly increased punished phase licks compared to the vehicle group (Fig. 2B). Administration of J-113397 blocked the anxiolytic-like effect of SCH 655842 such that the number of punished phase licks for this group (J-113397/SCH 655842) did not differ from the vehicle group (Vehicle/Vehicle). J-113397 alone had no effect on punished phase licking. Furthermore, there were no significant group differences in unpunished phase licking (Number of licks: Vehicle = 405 ± 102; J-113397 = 400 ± 96; SCH 655842 = 400 ± 72; J-113397 + SCH 655842 = 283 ± 57).

3.1.2. Rat fear-potentiated startle

SCH 655842 (1–10 mg/kg) significantly decreased the percentage of fear-potentiated startle in Wistar rats, compared to the vehicle group [$F(3,50) = 4.7$, $P<0.01$] (Fig. 2C). Specifically, rats treated with doses of 3 mg/kg ($P<0.05$) and 10 mg/kg ($P<0.01$) exhibited significantly lower percent fear-potentiated startle and these doses did not affect unconditioned startle response (Mean startle amplitude: Vehicle = 100 ± 13; 1 mg/kg = 79 ± 15; 3 mg/kg = 90 ± 17, 10 mg/kg = 88 ± 19).

3.1.3. Guinea pig pup vocalization

SCH 655842 significantly reduced the number of isolation-induced vocalizations in guinea pig pups [$F(3,34) = 32.6$, $P<0.001$] with >95% inhibition at doses of 1 and 3 mg/kg (Fig. 3A).

3.1.4. Mouse Geller–Seifter

SCH 655842 (3–30 mg/kg) produced a dose-dependent increase in the percentage of punished lever presses [$F(3,40) = 19.7$, $P<0.01$] with a significantly increase at a dose of 30 mg/kg (Fig. 3B). Furthermore, SCH 655842 had no effect on the number of unpunished presses, compared to the vehicle group (Number of lever presses: Vehicle = 662 ± 43; 3 mg/kg = 661 ± 57; 10 mg/kg = 669 ± 48, 30 mg/kg = 598 ± 61).

3.1.5. Mouse marble burying

SCH 655842 significantly decreased the number of buried marbles [$F(3,33) = 6.2$, $P<0.01$] at a dose of 30 mg/kg (Fig. 3C), compared to the vehicle group.

3.2. Behavioral studies: liability models

3.2.1. Fixed-ratio responding

Rats dosed with up to 100 mg/kg SCH 655842 did not exhibit any difference compared to vehicle group in terms of their lever response rate (Lever presses per sec: Vehicle = 0.89 ± 0.08, 30 mg/kg = 0.87 ± 0.08, 70 mg/kg = 0.88 ± 0.08, 100 mg/kg = 0.81 ± 0.12). Furthermore, SCH 655842 (3–30 mg/kg) was also tested in a mouse Fixed-Ratio assay and likewise did not produce any change in response rate, compared to the vehicle group (Lever presses per sec: Vehicle = 0.36 ± 0.04, 3 mg/kg = 0.36 ± 0.04, 10 mg/kg = 0.35 ± 0.05, 30 mg/kg = 0.38 ± 0.04).

3.2.2. Locomotor activity

In rats, there was a significant effect of treatment on total distance traveled [$F(4,35) = 7.4$, $P<0.001$], but only chlordiazepoxide (30 mg/kg) significantly decreased locomotor activity compared to vehicle group (Fig. 4A). SCH 655842 produced a moderate increase in locomotor activity at a dose of 30 mg/kg, but it was not significant. However, SCH 655842 produced a significant effect on total distance traveled [$F(4,33) = 29.8$, $P<0.001$] in the mouse. Specifically, the dose of 3 mg/kg significantly increased locomotor activity, while doses of 30 and 100 mg/kg significantly reduced locomotor activity (Fig. 4B).

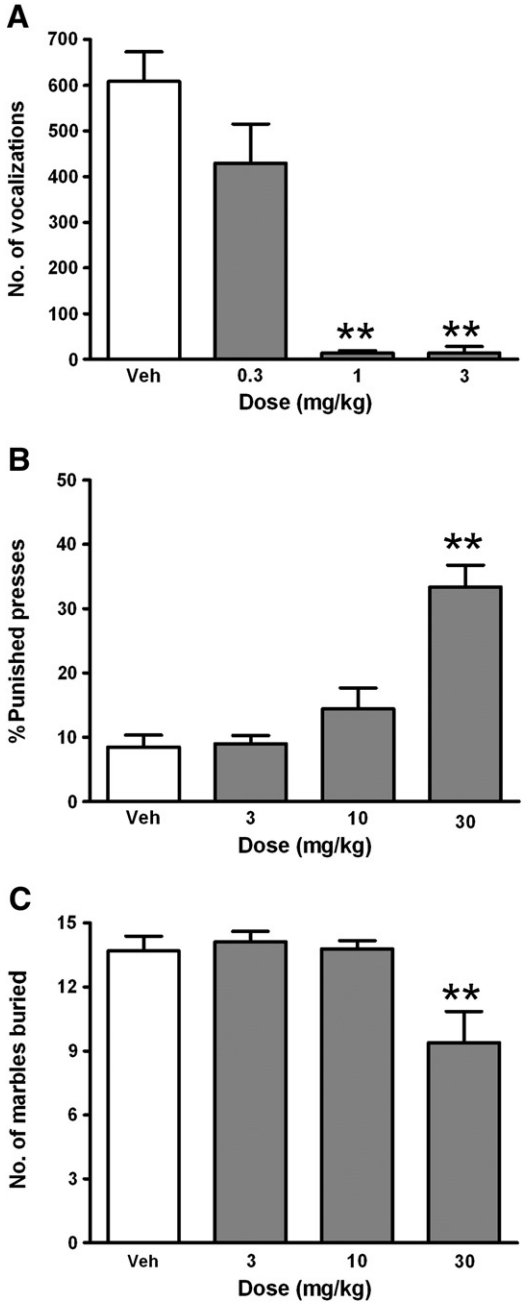

Fig. 3. The anxiolytic-like effects of SCH 655842 (0.3–30 mg/kg) in A) guinea pig pup vocalization, B) mouse Geller–Seifter and C) mouse marble burying assays. Each bar indicates mean ± S.E.M. of 10 guinea pig pups or 8–11 mice at each dose. Significant differences from vehicle (Veh) are indicated by $^{**}P<0.01$.

3.2.3. Rotarod

In the rat rotarod study, there was a significant effect of treatment on the time spent on the rotarod [$F(4,33) = 18.8$, $P<0.001$]. SCH 655842, at dose of 100 mg/kg, produced a moderate decrease of the time spent on the rotarod while animals treated with chlordiazepoxide (30 mg/kg) were unable to maintain themselves on the rotarod (Fig. 4C). Furthermore, in the mouse, SCH 655842 produced a significant decrease in rotarod performance at doses of 30 and 100 mg/kg [$F(4,38) = 19.9$, $P<0.001$] (Fig. 4D).

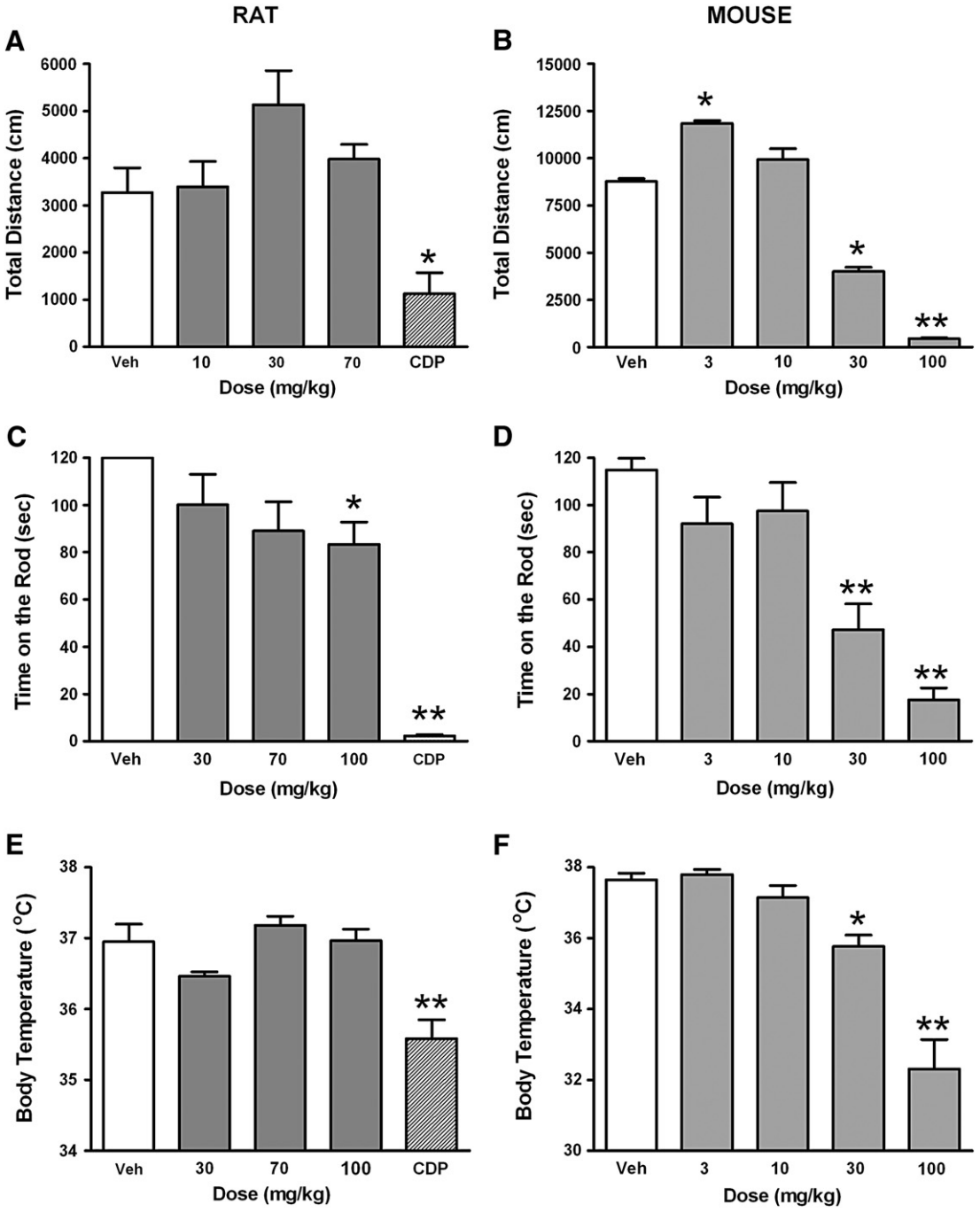

Fig. 4. Effect of SCH 655842 (3–100 mg/kg) and chlordiazepoxide (CDP: 30 mg/kg) on rat (left figures) and mouse (right figures) locomotor activity (panels A and B), rotarod performance (panels C and D) and core body temperature (panels E and F). Each bar indicates mean ± S.E.M. of 6–8 animals. Significant differences from vehicle (Veh) are indicated by $^{*}P<0.05$ and $^{**}P<0.01$.

3.2.4. Body temperature

In rats there was a significant effect of treatment on core body temperature [$F(4,35)=11.0$, $P<0.001$]. Only chlordiazepoxide-dosed animals exhibited a significant decrease in core temperature compared to the vehicle-dosed animals (Fig. 4E). In mice, SCH 655842 significantly reduced body temperature [$F(4,33)=29.3$, $P<0.001$] at doses of 30 and 100 mg/kg (Fig. 4F).

3.2.5. Beam walking

There was a significant effect of treatment on distance traversed on the beam [$F(5,40)=586.7$, $P<0.001$] but similar to body temperature this was due to a significant effect of chlordiazepoxide. Rats treated with SCH 655842 (10–100 mg/kg) successfully completed the 90 cm beam ($n=8$ per group) while rats treated with chlordiazepoxide ($n=6$) only traversed an average of 14 ± 9 cm (data not shown). In a separate experiment, a 4 h pretreatment with SCH 655842 (30–70 mg/kg) also failed to affect beam walking (data not shown).

3.2.6. General observation study

In addition to the aforementioned liability studies which focused on a 2 h pretreatment for consistency with the efficacy studies, a further experiment was conducted where animals were continuously monitored for up to 5 h post-dosing, with formal assessments of sedation, splayed hind limbs and flattened body posture made at 30 min intervals

by 3 independent observers. With the exception of a modest increase in flattened body posture at the 70 mg/kg dose (Total score: 0–150 min: Vehicle = 0, SCH 655842 70 mg/kg = 2.5 ± 0.5; 150–300 min: Vehicle = 0, SCH 655842 70 mg/kg = 1.5 ± 0.5) there were no group differences on the other measures.

3.2.7. Behavioral studies with NOP receptor KO mice

A two-way ANOVA (treatment × genotype) was used to analyze the effect of SCH 655842 (100 mg/kg; dose chosen based on dose-response data in C57BL/6 mice, see Fig. 4) of SCH 655842 in wild-type and NOP receptor KO mice. There was a significant interaction between treatment and genotype on both the locomotor activity [$F(1,36) = 15.5$, $P<0.001$] and core body temperature [$F(1,36) = 21.6$, $P<0.0001$] measures. Wild-type mice treated with SCH 655842 displayed significantly decreased locomotor activity and body temperature (Fig. 5). In contrast, NOP receptor KO mice treated with SCH 655842 did not exhibit any significant difference in locomotor activity, compared to KO vehicle controls, and exhibited a slight increase in body temperature.

3.3. Rat pharmacokinetic study

In the first study, SCH 655842, at doses of 3–70 mg/kg, produced a dose-dependent increase in plasma exposure measured as the total exposure during the 24 h sampling period (AUC, see Table 1). Additionally, the plasma concentration at 2 h, the time point when most of the behavioral tests were conducted in the rat, also increased in a dose-dependent manner. Comparing the concentrations of SCH 655842 at doses of 3 mg/kg (efficacious dose in rat anxiety tests) and 70 mg/kg (dose at which mild behavioral effects are observed) there are 34-fold (based on the AUC measure) and 23-fold (based on the C_{2h} measure) exposure differences between the doses, suggesting that there is a large efficacy-safety window for SCH 655842 in the rat.

In the second study, both the plasma and brain concentrations of SCH 655842 increased in a dose-dependent manner when measured 6 h after administration. Over the 10–70 mg/kg dose range of SCH 655842, the brain:plasma ratio remained in the range of 0.4–0.6 (see Table 1).

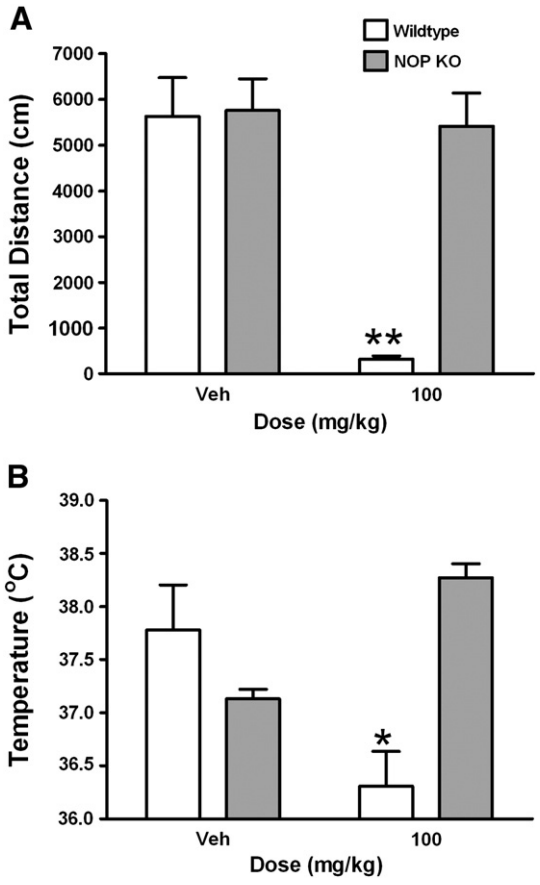

Fig. 5. Effect of SCH 655842 (100 mg/kg) on A) locomotor activity and B) body temperature in wild-type and NOP receptor knockout (KO) mice. Each bar indicates mean ± S.E.M. of 10 mice. Significant differences from vehicle (Veh) are indicated by *$P<0.05$ and **$P<0.01$.

Table 1
Levels of SCH 655842 (3–70 mg/kg) in rat plasma and brain.

Dose (mg/kg)	Study 1		Study 2		
	$AUC_{0-24\ h}$ (ng h/ml)	$C_{2\ h}$ (ng/ml)	$C_{6\ h}$ (ng/ml) plasma	$C_{6\ h}$ (ng/g) brain	Brain:plasma ratio
3	253 ± 23	37 ± 4	NT	NT	NT
10	1740 ± 147	360 ± 81	126 ± 31	46 ± 8	0.43 ± 0.13
30	3190 ± 785	310 ± 25	223 ± 67	101 ± 27	0.46 ± 0.06
70	8470 ± 960	850 ± 14	335 ± 34	198 ± 29	0.59 ± 0.06

The data presented are from 2 separate studies (see Materials and methods for detail).
$AUC_{0-24\ h}$: Area under the curve–total plasma exposure during the 24 h sampling period.
$C_{2\ h}$ and $C_{6\ h}$: SCH 655842 concentration 2 h and 6 h after administration, respectively.
NT = not tested.

4. Discussion

Synthetic agonists of the NOP receptor have been shown to produce robust anxiolytic-like effects in rodents (see Hirao et al., 2008a,b; Jenck et al., 2000; Varty et al., 2005, 2008) but they have also been shown to produce sedative-like side effects that may limit their therapeutic window (see Higgins et al., 2001; Jenck et al. 2000; Varty et al. 2005). However, there was some evidence of separation between the efficacious, anxiolytic doses and doses that produce adverse effects, suggesting that it may be possible to develop a synthetic, orally active NOP agonist that could be progressed into the clinical setting. We have recently described a novel, orally active NOP agonist, SCH 655842, a functionally selective NOP receptor agonist (NOP $EC_{50} = 6$ nM; MOP $EC_{50} = 376$ nM) with a binding affinity of 1.7 nM at the human NOP receptor (compound 24, Ho et al., 2009; see Fig. 1). The main aim of the present study was to characterize SCH 655842 in terms of the compound's in vivo profile in anxiety and side effect tests in order to determine the separation between efficacious and liability doses, and then, using pharmacokinetic studies, translate this dose separation into an exposure multiple that could be used to determine the potential therapeutic window.

In the first part of our studies, SCH 655842 was tested in a variety of standard animal models that are sensitive to clinically used anxiolytic drugs. The set of models used included tests of acute stress (vocalization and marble burying), conditioned fear (fear-potentiated startle) and conflict fear (conditioned lick suppression and Geller–Seifter) across three rodent species. Importantly, the tests chosen required either a reduction in a behavior (vocalizations, burying marbles) or an increase in a behavior (punished licks) allowing for the separation of anxiolytic effects from potential secondary behavioral effects such as sedation or stimulation. In all of the tests across the three species, SCH 655842 produced effects consistent with anxiolysis and the magnitude of these effects were similar to benzodiazepines evaluated in these models (see Table 2 and Varty et al., 2005, 2008). Importantly, to confirm the mechanism of action, the anxiolytic-like effect of SCH 655842 in the rat conditioned lick suppression model was completely attenuated by the NOP receptor antagonist, J-113397 (Ozaki et al., 2000). This finding is consistent with our previous work demonstrating that J-113397 blocked the anxiolytic-like effects of the NOP agonists, Ro 64-6198 and SCH 221510, in the same rat conditioned lick suppression test (Varty et al., 2005, 2008). Although

Table 2
Comparison of the minimal effective doses of the NOP receptor agonists, SCH 655842 and Ro64-6198 and benzodiazepine anxiolytics (either chlordiazepoxide or diazepam) in models of anxiolytic-like activity.

	SCH 655842	Ro64-6198[a]	Benzodiazepine[b]
Rat CLS	3 mg/kg	3 mg/kg	6 mg/kg
Rat FPS	3 mg/kg	NT	10 mg/kg
GPPV	1 mg/kg	0.3 mg/kg	10 mg/kg
Mouse Geller–Seifter	30 mg/kg	3 mg/kg	3 mg/kg

NT: Not tested.
[a] Varty et al., 2005
[b] Varty et al., 2008.

not tested here, we have also shown previously that the anxiolytic-like effects of NOP agonists are insensitive to the mu opioid antagonist, naltrexone (Varty et al., 2005, 2008).

NOP receptors are widely distributed in the brain (Mollereau and Mouledous, 2000) including forebrain and brain stem. As such, the second part of the present studies focused on establishing any behavioral liabilities associated with SCH 655842. To achieve this, SCH 655842 was dosed up to 100 mg/kg (thereby achieving 30-fold dose multiples over the efficacious dose of 3 mg/kg in the rat) and tested in a variety of assays measuring potential liabilities for NOP agonists such as sedation, motor impairment and hypothermia. Indeed, similar to other NOP agonists such as nociceptin and Ro 64-6198, SCH 655842 produced hypoactivity, motoric impairment and hypothermia, particularly in the mouse. In the mouse, there is no apparent safety window. Moreover, the reduction in activity at 30 mg/kg makes interpretation of the marble burying result (MED = 30 mg/kg) challenging. However, an interesting observation from these studies was that SCH 655842 may have an improved safety window compared to Ro 64-6198, at least in the rat (see Tables 2 and 3 for comparison of minimal effective doses in efficacy and liability models). For example, a comparison of the minimal effective doses for efficacy in the rat conditioned lick suppression model, compared to effects on motor deficits, suggests at least a 23-fold difference in dose for SCH 655842 (3 vs. 70 mg/kg), compared to 3-fold for Ro 64-6198 (10 vs. 30 mg/kg, see Varty et al., 2005). Furthermore, when comparing the plasma levels of SCH 655842 at the same two doses, the same 23-fold difference is evident.

In terms of an explanation for this apparent difference in safety window between Ro 64-6198 and SCH 655842, both drugs have excellent functional selectivity for human NOP vs. human MOP receptors, i.e., SCH 655842: 63x; Ro 64-6198: 900x (Ho et al., 2009; McLeod et al., 2004). Therefore, it seems unlikely that the difference is due to mu opioid receptor activity. Also, antagonist studies and experiments in NOP receptor knockout mice with SCH 655842 and Ro 64-6198 suggest that both efficacy and neurological side effects are NOP receptor mediated (Higgins et al., 2001; Varty et al., 2005). Extensive pharmacokinetic studies with SCH 655842 suggest the drug has good oral bioavailability, with reasonable CNS penetration and a linear plasma exposure over the 3–70 mg/kg dose range, which suggest the likelihood for increasing NOP receptor occupancy that is proportional to increasing drug dose. At the present time, the *in vitro* characterization of SCH 655842 has been confined to cloned cell lines expressing human NOP receptors, where a full agonist profile has been established using a GTPγS shift assay (Ho et al., 2009). Extension of these studies into native tissue preparations may provide a means to explore possible functional differences between SCH 655842 and Ro 64-6198. As agonist efficacy is dependent on receptor density and coupling efficiency, both of which may be tissue and model dependent, this may be overestimated in systems expressing high levels of receptor (e.g., see McDonald et al., 2003). Alternative differences may relate to agonist features such as rates of receptor desensitization and/or internalization, which have been investigated with Ro 64-6198 (Dautzenberg et al., 2001), but not for SCH 655842. Finally, the possibility for a functional heterogeneity of NOP receptors should be considered. On the basis that Ro 64-6198 only produced hypoactivity in mice, yet nociceptin produced a biphasic hyper- and hypoactivity, Kuzmin et al. (2004) have proposed the existence of functional subtypes of NOP receptors, with Ro 64-6198 only affecting one subpopulation. The fact that SCH 655842 also elicits a biphasic motor response in this species may suggest an interaction across a broader population of NOP receptors.

Table 3
Comparison of the minimal effective doses of the NOP receptor agonists, SCH 655842 and Ro64-6198, and benzodiazepine anxiolytics (either chlordiazepoxide or diazepam) in liability models.

	SCH 655842	Ro64-6198[a]	Benzodiazepine[a]
Rat LMA	>70 mg/kg	10 mg/kg	10–30 mg/kg
Rat rotarod	100 mg/kg	30 mg/kg	10–30 mg/kg
Rat beam walk	>100 mg/kg	30 mg/kg	10–30 mg/kg
Rat fixed-ratio	>100 mg/kg	10 mg/kg	30 mg/kg
Mouse LMA	100 mg/kg	3 mg/kg	NT
Mouse body temp	30 mg/kg	10 mg/kg	NT
Mouse rotarod	30 mg/kg	30 mg/kg	NT
Mouse fixed-ratio	>30 mg/kg	10 mg/kg	NT

NT: Not tested.
[a] From Varty et al., 2005.

In order to establish the therapeutic potential of NOP agonists it will be important to examine their tolerability profile in higher species. Early studies conducted in the primate seem encouraging as the intrathecal administration of OFQ/N and UFP-112 appears to result in antinociceptive efficacy at concentrations without obvious side effects (Hu et al., 2009; Ko and Naughton, 2009). Furthermore, Ko et al. (2009) have reported that systemically administered Ro 64-6198 (0.0001–0.06 mg/kg) to rhesus monkeys reduced the pain responses to an acute noxious stimulus (50 °C water), and capsaicin-induced allodynia, and did not elicit any observable signs of sedation, respiratory depression or abuse liability. These findings suggest that NOP agonists may have a preferred profile to MOP receptor agonists as treatments for pain (Ko et al., 2009). However, as these authors discuss, higher dose studies are necessary to establish the identity of any dose-limiting side effects and where they emerge in relation to therapeutic effects. Clearly, evaluation of SCH 655842 in a primate anxiety model would be of significant interest as well as establishing any side effect liability in this species. Podlesnik et al. (2011) recently demonstrated that Ro 64-6198 has antinociceptic properties in rhesus monkeys, which suggests that this compound is well tolerated in non-human primates.

In summary, we have reported on the in vivo behavioral profile of the novel NOP agonist SCH 655842. An interesting feature of this drug was that it appears to retain the efficacy characteristic of NOP agonists in rodent tests of anxiety, yet may show a better margin between efficacy and neurological side effects also mediated via the NOP receptor. Importantly, this margin is evident across a treatment range in which plasma exposure is proportional to dose. Whether this margin extends to other species, such as primate, is for further investigation. If so, this may open avenues for the identification of a class of NOP agonists with therapeutic potential in multiple indications, including anxiety.

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