1. Title page

A selective orexin-1 receptor antagonist attenuates stress induced hyperarousal without hypnotic effects

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2. Running title page

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3. Abstract

Orexins (OX) are peptides produced by perifornical and lateral hypothalamic (PeF/LH) neurons that exert a prominent role in arousal-related processes including stress. A critical role for the orexin-1 receptor (OX1R) in complex emotional behavior is emerging such as overactivation of the OX1R pathway being associated with panic or anxiety states. Here we characterize a brain penetrant, selective and high affinity OX1R antagonist compound 56 (N-([3-[(3-Ethoxy-6-methylpyridin-2-yl)carbonyl]-3-azabicyclo[4.1.0]hept-4-yl]methyl)-5-(trifluoromethyl)pyrimidin-2-amine). Ex vivo receptor binding studies demonstrated that after subcutaneous administration compound 56 crossed the blood brain barrier and occupied OX1Rs in the rat brain at lower doses than standard OX1R antagonists GSK-1059865, SB-334867 and SB-408124. While compound 56 did not alter spontaneous sleep in rats and in wild-type mice, its administration in OX2R knockout mice, selectively promoted REM sleep, demonstrating target engagement and specific OX1R blockade. In a rat model of psychological stress induced by cage exchange, the OX1R antagonist prevented the prolongation of sleep onset without affecting the sleep duration. In a rat model of panic vulnerability (involving disinhibition of the PeF OX region) to threatening internal state changes (i.e. intravenous sodium lactate infusion), compound 56 attenuated sodium lactate induced panic-like behaviors and cardiovascular responses without altering baseline locomotor or autonomic activity. In conclusion, OX1R antagonism represents a novel therapeutic strategy for the
treatment of various psychiatric disorders associated with stress or hyperarousal states.
4. Introduction

Orexins (OX), also known as hypocretins, are the common names given to a pair of excitatory neuropeptides called OX-A and OX-B derived from the common precursor prepro-OX that is exclusively produced by perifornical and lateral hypothalamic (PeF/LH) neurons (de Lecea et al., 1998; Sakurai et al., 1998). OX-producing neurons project widely to key areas of the brain (Peyron et al., 1998) and are predominantly involved in the control of wakefulness and also in the regulation of food intake, reward, addictive behaviors and stress (Li et al., 2013). The OX neuropeptides mediate their effect by stimulating two distinct G-protein coupled receptors, orexin-1 (OX1R) and orexin-2 (OX2R) that are co-located or selectively located in specific brain areas suggesting differentiated roles (Trivedi et al., 1998; Marcus et al., 2001). OX1Rs are more selectively expressed in the bed nucleus of the stria terminalis, amygdala, cingulate cortex and exclusive to noradrenergic neurons in the locus coeruleus which play a role in panic and anxiety. Conversely, OX2R’s are exclusive to histaminergic neurons in the tuberomammillary nucleus which play a critical role in wake promotion and predominantly expressed in the paraventricular hypothalamic nucleus (PVN) which is involved in the hypothalamo-pituitary-adrenal (HPA) axis regulation. In other brain regions like the dorsal raphe, the ventral tegmental area (VTA) or the prefrontal cortex (PFC), OX1R and OX2R are co-expressed.

While dual OX1/2R antagonists have been shown to promote sleep in various species by inhibiting the output of wake active neurons in the hypothalamus and brainstem regions (Hoyer and Jacobson, 2013), antagonism of the OX2R alone is sufficient to induce and prolong sleep in rodents (Dugovic et al., 2009; Mang et al.,
2012). In parallel, evidence has accumulated that demonstrates an involvement of the OX signaling via OX1R in reward pathways associated with drug dependence (Sharf et al., 2009). The role of the OX1R in emotional behavior is emerging (Johnson et al., 2012). Human subjects with panic anxiety symptoms have elevated levels of OX-A in the cerebrospinal fluid compared to subjects without panic anxiety symptoms (Johnson et al., 2010). Sodium lactate (NaLac) infusions or acute hypercapnia induction, which signal internal body state (interoceptive) threats, reliably cause panic attacks in humans activating OX neurons in the PeF of rats (Johnson et al., 2010; Johnson et al., 2012). This activation correlates with anxiety in the social interaction test. Blocking OX signaling with either siRNA or selective OX1R antagonists attenuates panic-like responses to NaLac. Overall, there is compelling evidence for the overactivation of the OX1R pathway in hyper-active states, thus conceptually a selective OX1R antagonist might normalize overexcited networks without inducing sedation. Noteworthy, OX1R antagonism fails to reduce anxiety-like behavior under baseline conditions in both elevated plus maze or social interaction test (Yeoh et al., 2014). Efficacy of an OX1R antagonist has been observed in fear-conditioned startle and in a resident-intruder paradigm involving social interaction (Steiner et al., 2012; Steiner et al., 2013a). OX neurons are activated during arousal or psychological stress but not by passive restraint stress or cold exposure (Furlong et al., 2009). In rats exposed to cage exchange, a model of psychological stress, cFos expression was elevated in hypothalamic OX neurons and in the PVN (involved in hypothalamic pituitary adrenal axis and autonomic control) relative to control (Cano et al., 2008).
Here we report the characterization of a novel selective OX1R antagonist recently described in the patent literature (compound 56) (Alvaro et al., 2010a) (Figure 1). We compared its properties to the literature standards GSK-1059865, SB-334867 and SB-408124 (Figure 1). In vitro affinity and potency for the human and rat OX1R was determined by radioligand binding and in vitro functional assays. Compound 56 was tritiated, tested as a tracer for autoradiography on rat tissue sections and in vivo target engagement was measured after subcutaneous injection of compound 56 in rat brain using ex vivo receptor occupancy. We have recently shown that while selective OX1R antagonism did not affect sleep wake states, additional pharmacological blockade of OX1R to OX2R antagonism elicited a disinhibition of rapid eye movement (REM) sleep at the expense of non-REM (NREM) sleep in rats (Dugovic et al., 2014). Therefore the effect of the selective OX1R antagonist on sleep was tested in OX2R knockout (KO) mice to demonstrate in vivo functional target engagement. Finally, the activity of compound 56 was evaluated in two models of stress-induced hyperarousal, a psychological exteroceptive stress (i.e. external threat) elicited by cage exchange and a previously described interoceptive stress (i.e. internal body state threat) elicited by intravenous NaLac provocation in a panic vulnerability model (Johnson et al., 2010) in rats.
5. Materials and Methods

All animal procedures performed in this study were in accordance with the Guide Care for and Use of Laboratory Animals adopted by the US National Institutes of Health (NIH Publication no. 80-23 revised 1996) and the guidelines of the Institutional Animal Care and Use Committee. Animals were housed individually under controlled conditions with a 12/12 h light/dark schedule and temperature of 22 ± 2°C. Food and water were provided ad libitum. Experiments were performed after animals had acclimated for at least one week unless stated otherwise.

In vitro radioligand binding assays:

Human or rat OX1R binding was measured in competitive radioligand binding assays using [³H]SB-674042 (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((S)-2-(5-phenyl-(1,3,4)oxadiazol-2-ylmethyl)-pyrrolidin-1-yl)-methanone) as a tracer (4 nM, specific activity 35 Ci/mmol) (Langmead et al., 2004). Cell membranes were prepared from clonal CHO K1 cells transfected with the human OX1R or clonal HEK-293 cells transfected with the rat OX1R. Dilutions of test compounds were made in Dulbecco’s PBS from 10 mM stocks dissolved in DMSO. After a 60 minute incubation at room temperature, binding reactions were filtered. The membranes were counted in a scintillation counter. Non-specific binding was determined in the presence of 10 μM almorexant. Affinities of compounds for the human OX2R were measured by competitive radioligand binding using tritiated N-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-N-pyridin-3-ylmethyl-acetamide (EMPA) (2 nM, specific activity 27 Ci/mmol) (Malherbe et al., 2009). Cell membranes were prepared from a stable pool of
HEK-293 cells transfected with the human OX2R. Nonspecific binding was determined with 10 μM almorexant.

The Ki of the test compounds was calculated based on nonlinear regression (one site competition) using Graphpad Prism.

Compound 56 was tritiated via Pd-mediated hydrogenolysis (27.5 Ci/mmol, Moravek, CA) of the corresponding bromo precursor (The bromo precursor is available from HATU mediated amide-coupling of the corresponding amine and carboxylic acid fragments (Alvaro et al., 2010b; Maton et al., 2010; Verdelet et al., 2011) and its binding parameters (Kd and Bmax) for the human and rat OX1R were determined using identical conditions as those described for [3H]SB-674042.

The selectivity of compound 56 was evaluated in a large variety of ion-channels, transporters and receptor-binding assays. These assays were performed by Eurofins (Celles L’Evescault, France).

[^3H]Compound 56 in vitro receptor autoradiography on tissue section:

Adult male Sprague-Dawley rats (300-350 g, Charles River Laboratories, San Diego, CA) were euthanized using carbon dioxide and brains were immediately removed and rapidly frozen in dry-ice. Twenty-micron-thick coronal sections were cut using a Cryostat-microtome (Leica CM 1950) and thaw-mounted on adhesive microscope slides (Superfrost+ Plus, VWR). The sections were kept at –80°C until use. Brain sections were preincubated for 15 minutes at room temperature in 50 mM Tris, 10 mM MgCl2, 5 mM EDTA buffer at pH 7.4 and then incubated for 60 minutes in a fresh preparation of the
same buffer supplemented with 5 nM $[^3]$H)compound 56. Nonspecific binding was determined using adjacent sections incubated in the presence of 10 μM GSK-1059865. At the end of the incubation, sections were washed 3 times (10 minutes each) in ice cold buffer, dipped in deionized water and rapidly dried under a stream of cold air. Image acquisition was performed using a β-Imager TRacer (BioSpace, Paris, France).

In vitro functional assays (calcium mobilization assays):

Stably transfected CHO-K1 cells for the human OX1R or HEK-293 cells for the rat OX1R were used for the in vitro functional assays. The human OX2R functional assay used PFSK-1 cells which are a human neuroectodermal cell line that innately expresses the OX2R. Since the intracellular calcium response is transient and not consistent with equilibrium assumptions, the assays were performed by giving a standard, EC$_{80}$ dose of the OX agonist and calculating a pK$_B$ from inhibition of the agonist response by a dose range of the antagonists. The cells were plated in black 96 well tissue culture plates with clear bottoms at 50,000 cells/well and grown overnight at 37°C in 5% carbon dioxide (CO$_2$). Dilutions of the antagonist were prepared in Hanks Balanced Salt Solution (HBSS) from 10 mM DMSO stocks, while dilutions of OX peptides (OX-A for OX1R assays, OX-B for OX2R assays) were prepared in HBSS + 0.1% bovine serum albumin. On the day of the assay, a 2X dye-loading solution (BD Calcium Assay Kit) was added to the cells and incubated for 45 minutes at 37°C in 5% CO$_2$. Dilutions of the test compounds were added and the cells were incubated at room temperature for 15 minutes. The cell plate was then transferred to the Molecular Devices Fluorometric
Imaging Plate Reader (FLIPR) Tetra instrument, which adds the OX agonist and monitors changes in fluorescence which reflect intracellular calcium levels.

Results were calculated using GraphPad Prism (San Diego, CA) software. Raw data from the FLIPR Tetra was exported as the difference between maximum and minimum fluorescence observed for each well. A non-linear regression was used to determine the agonist EC$_{50}$ and antagonist IC$_{50}$ for each plate, then the antagonist K$_B$ was calculated according to Cheng and Prusoff (Cheng and Prusoff, 1973).

*Ex vivo receptor occupancy assays:*

Experiments were performed as previously described (Dugovic et al., 2009) in male Sprague-Dawley rats (300-400 g, Charles River Laboratories, San Diego, CA). The animals were euthanized using carbon dioxide and decapitated at different time points after drug administration (n = 3 per time point or dose regimen). Brains were rapidly frozen on powdered dry ice and stored at −80°C before sectioning. Plasma samples were also collected for bioanalysis (LC-MS/MS). Twenty micron thick tissue sections at the level of the tenia tecta were prepared for autoradiography. OX1R radioligand binding autoradiography was determined at room temperature with 5 nM $[^3]$H]SB-674042. Sections were incubated for 10 min to minimize dissociation. Nonspecific binding was determined in the presence of 10 $\mu$M GSK-1059865. Sections were allowed to dry before acquisition with $\beta$-Imager (BioSpace, Paris, France) for 6 h. Quantitative analysis was performed using the $\beta$-Imager TRacer. *Ex vivo* receptor labelling was expressed as the percentage of receptor labelling in corresponding brain areas (i.e. tenia tecta) of vehicle-treated animals. The percentage of receptor occupancy was plotted against time.
or dosage using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Percentage of receptor occupancy was also plotted against drug plasma or brain concentration. Pharmacokinetic parameters were analyzed using a non-compartmental model using the software package WinNonlin Version 4.0.1. (Pharsight, Palo Alto, CA).

Sleep recording and analysis in rats and mice:

Sleep experiments were conducted in male Sprague-Dawley rats (350-450 g, Harlan Laboratories, Livermore, CA) and in male C57Bl6 OX2R KO and corresponding wild-type mice (30-35 g, Charles River Laboratories, San Diego, CA) as described previously (Dugovic et al., 2009). Animals were chronically implanted with telemetric devices (Data Sciences International, St. Paul, MN) for the recording of electroencephalogram (EEG) and electromyogram (EMG) signals. Polysomnographic waveforms were analyzed per 10-s epoch and classified as wake, non-rapid eye movement (NREM) or rapid eye movement (REM) sleep by using the computer software program SleepSign (Kissei Comtec, Nagano, Japan). For each experiment, EEG and EMG signals were recorded for up to 6 h after administration of the tested compounds. Analysis of sleep parameters included latency to NREM sleep (defined as the time interval to the first six consecutive NREM epochs) and REM sleep (the first two consecutive REM epochs post-treatment), and the duration of NREM, REM and total sleep.

Results were averaged and expressed as mean ± S.E.M. in defined time intervals. To determine whether differences were significant at a given interval, either paired...
Student’s t test or one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison post-hoc analysis was performed.

*Cage exchange stress model in rats and mice:*

The cage exchange stress procedure was performed in male Sprague-Dawley rats (350-450 g, Harlan, Livermorre, CA) and in male C57Bl6 mice (30-35 g, Jackson Laboratory, Sacramento, CA) at 4 h into the light phase, a period when the animals normally exhibit the maximal amount of physiological sleep. The animal was removed from its home cage and placed into a dirty cage previously occupied by another animal for at least one week during a 8h period (until dark onset). As a control procedure, the animal was removed from its home cage and returned to the same cage (brief handling).

*ACTH measurement in mice:*

Male C57Bl6 mice (30-35 g, Jackson Laboratory, Sacramento, CA) were used to assess ACTH serum levels. On the day of the experiment, a blood sample was collected in a serum separator tube (Becton, Dickinson and Company, New Jersey) via the submandibular punch of each mouse. Each tube was then allowed to sit on ice for one hour and then spun at 10000 rpm (Eppendorf Microfuge) at 4°C for ten minutes to allow for separation of the serum from the remaining whole blood constituents. Serum was then collected into a sterile Eppendorf tube and frozen at -80°C until the assay was performed. The Mouse Bone Magnetic Bead Luminex Panel (EMD Millipore, Billerica, MA) was used to measure serum levels of ACTH. Results were averaged and expressed
as mean ± S.E.M. A one-way ANOVA followed by a Neuman-Keuls post hoc analysis was used to determine if any of the treatment groups were significant from each other.

**Sodium lactate panic provocation model:**

Experiments were performed in male Sprague-Dawley rats (300-350 g, Harlan laboratory, Indianapolis IN). Prior to and during surgeries, rats were anesthetized with a nose cone connected to an isoflurane system (MGX Research Machine; Vetamic, Rossville IN). Radiotelemetry probes [Cat. no. C50-PXT, Data Science International (DSI) St. Paul MN] were surgically implanted into the peritoneal cavity and sutured to the muscle wall in order to assess general motor activity and core body temperature (CBT). A pressure transducer was implanted into the femoral artery to assess cardiovascular responses [i.e., mean arterial blood pressure (MAP) and heart rate (HR)]. Rats were also fitted with femoral venous catheters for 0.5M i.v. sodium lactate (NaLac) infusions, as previously described (Shekhar et al., 1996). After 3-5 days of recovery, rats were anesthetized and 26 gauge T-shaped cannulae (Cat. no. 3260PG, Plastics One Inc., Roanoake VA) were directed at cardio-excitatory PeF regions [(Shekhar and Keim, 1997) bregma: 1.2 mm posterior, +2.1 mm lateral, +9.1 mm ventral and adjusted for approaching at a 10 degree angle toward the midline with the stereotaxic incisor bar elevated 5 mm above the interaural line)] and cemented into place. The 22 gauge side arm was then attached, via PE-60 tubing, to an osmotic minipump (prefilled with l-AG solution chronically infused at 3.5 nmol/0.5μl per hour) and sutured into place subcutaneously at the nape of the neck (DURECT Corporation, Model no. 2002). Previous studies have determined that the dose of l-AG utilized here reduces local GABA...
concentrations by approximately 60% following unilateral infusions (Shekhar and DiMicco, 1987; Abshire et al., 1988; Shekhar et al., 1996; Shekhar and Keim, 1997; Shekhar et al., 2006).

Five days following L-AG infusion onset, in a counterbalanced design and with 48 hours between crossover, rats received a subcutaneous (s.c.) injection of either compound 56 at 3, 10, or 30 mg/kg, or vehicle as a control group 60 min prior to the 15 min 0.5 M NaLac challenge. DSI DATAQUEST software was used to monitor and record MAP, HR, CBT and motor activity which were recorded continuously in freely moving conscious rats and are expressed as a 20 min time course. The data reported are changes in MAP, HR, CBT and activity from the average of the baseline (t -5 to t -1) from each rat.

Baseline social interaction (SI) testing was done seven to eight days following radiotelemetry surgery recovery, and repeated again 2-3 days later during drug treatment crossover. On experimental drug testing days, the SI test was performed five min after the offset of the NaLac challenge with different partners each time. The SI box dimension were 0.9 m long x 0.9 m wide x 0.3 m height. The SI test is a validated test of experimental anxiety-like behavior in rats that is sensitive to FDA approved treatments for anxiety disorder symptom management that includes benzodiazepines and selective serotonin reuptake inhibitors (Sanders and Shekhar, 1995; Shekhar and Katner, 1995). All behavioral tests are digitally videorecorded with a camera above the box. The "experimental" rat and an unfamiliar "partner" rat are both allowed to individually habituate to the box for a 5 min period 24 h prior to each SI test. During the SI test, the two rats are placed together in the center of the box, and the total duration (sec) of non-aggressive physical contact (grooming, sniffing, crawling over and under, etc.) initiated
by the “experimental” rat is quantified over a 5 min duration. A baseline SI test was performed 72+ h after i.v. catheterization, but prior to osmotic minipump implantation. Another SI test was performed 5 days following minipump infusions and immediately following saline or NaLac infusions. Video recorded sessions were scored at a later time (by SDF), whom was blind to any drug treatment.

Following experiments, all rats were anesthetized and decapitated, their brains were removed, frozen, sectioned (30μm) and stained with Neutral Red for determination of injection cannulae placements.

Each dependent variable for assessment of behavior and radiotelemetry data was respectfully analyzed using a one way ANOVA, or a one way ANOVA with repeated measures with drug treatment as the main factor and time as repeated measures. In the presence of significant main effects post-hoc tests were conducted using a parametric Fisher’s LSD test. Within subjects time effects were also assessed using a Dunnett’s one way analysis with the min prior to the i.v infusion used as the control. Statistical significance was accepted with P < 0.05. All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., IL, USA) and all graphs were generated using SigmaPlot 2001 for Windows (SPSS Inc., IL, USA).

Chemicals:

SB-334867 and SB-408124 were purchased from Tocris Cookson (Ellisville, MO). Almorexant, GSK-1059865, compound 56, EMPA and SB-674042 were synthetized at Janssen Research & Development LLC. Peptides were obtained from Bachem (Torrance, CA). For in vitro assays, compounds were dissolved in dimethyl
sulfoxide (stock solution at 10 mM) and further diluted in assay buffer. For *in vivo*

studies, SB-334867, SB-408124 and compound 56 were formulated in 30% SBE-β-

Cyclodextrin. GSK-1059865 was formulated in 5% NMP, 20% Solutol, 75% HP-β-

Cyclodextrin (20%). All compounds were administered orally or subcutaneously in a

volume of 5 mL/kg (receptor occupancy studies) or 1 mL/kg (sleep and NaLac

experiments) in rats and in a volume of 10 mL/kg in mice.
Results

Compound 56 is a selective high affinity OX1R antagonist:

The affinity of compound 56 for the human and rat OX1R was determined by competitive radioligand binding using \(^{[3]H}\)-SB674042. GSK-1059865, SB-334867 and SB-408124, OX1R antagonist literature standards, were also included for comparison purpose. To determine the selectivity ratio versus the OX2R, the affinity of the compounds for the human OX2R was determined by competitive radioligand binding using \(^{[3]H}\)-EMPA as the radioligand.

Compound 56 showed high affinity binding to the human and rat OX1R, with \(K_i\) values of 3.6 and 3.2 nM, respectively (Table 1). GSK-1059865 also displayed high affinity for human and rat OX1R. Compound 56 showed greater affinity for the human OX1R compared to SB-334867 and SB-408124 (13 and 31 fold respectively). The binding selectivity of compound 56 at the human OX1R compared to the human OX2R is substantial (44 fold).

The four compounds were assayed by binding in a panel of 50 receptors, ion channels and transporters assays including adenosine (A1, A2A, A3), adrenergic (\(\alpha_1\), \(\alpha_2\), \(\alpha_1\)), angiotensin (AT1), dopamine (D1, D2), bradykinin (B2), cholecystokinin (CCKA), galanin (GAL2), melatonin ML1), muscarinic (M1, M2, M3), neurotensin (NT1), neurokinin (NK2, NK3), opiate (\(\mu\), \(\kappa\), \(\delta\)), serotonin (5-HT1A, 5-HT1B, 5-HT2A, 5-HT3, 5-HT5A, 5-HT6, 5-HT7), somatostatin, vasopressin (V1a), norepinephrine transporter, dopamine transporter and ion channels (sodium, calcium, potassium and chloride). Compound 56 at concentrations up to 1 \(\mu\)M had no significant affinity for any
receptor/transporter/ion channel (< 50 % inhibition at 1 μM) other than the OX1R. GSK-1059865 had some weak affinity for hNK₂ (Kᵢ = 920 nM) and hκ (Kᵢ = 620 nM). SB-334867 had weak affinity for the h5-HT₂B receptor (750 nM).

Compound 56 was radiolabeled and tested as a radioligand for the human and rat OX1R. Characterization of the binding of [³H]compound 56 to the human and rat OX1R was performed on membranes. Specific binding of [³H]compound 56 was saturable with a Kᵰ = 0.8 ± 0.1 nM and a Bₘₐₓ = 879 ± 26 fmol.mg for the human OX1R (Figure 2A) and a Kᵰ = 0.3 ± 0.1 nM and a Bₘₐₓ = 280 ± 8 fmol.mg for the rat OX1R (Figure 2B).

[³H]Compound 56 was then tested on rat brain tissue sections using autoradiography (Figure 2C). Specific labeling was observed in brain regions known to contain the OX1R with the highest binding densities observed in the locus coeruleus and tenia tecta. Specific binding densities were also observed in the cortex, hippocampus, hypothalamus and amygdala. Very low nonspecific binding was observed.

The functional antagonism of compound 56 for the human or rat OX1R was determined by measuring changes in intracellular calcium in cell culture assays in response to an EC₈₀ dose of OX-A. Functional antagonism data, summarized in Table 2, show that the high affinity OX1R binding of compound 56 is reflected in potent functional activity. The Kᵦ values correlated well with the Kᵢ values for the human and rat OX1R. The binding selectivity of compound 56 at the OX1R compared to the OX2R was confirmed at the functional level (Table 2).
Compound 56 crosses the blood brain barrier and occupies the OX1R in rat brain after systemic administration:

*In vivo* occupancy of the OX1R was assessed by *ex vivo* receptor binding autoradiography of $[^3H]$SB-674042 in rat brain tissue sections at the level of the tenia tecta. Time-dependency and dose-dependency were assessed after subcutaneous dosing (Figure 3).

After subcutaneous administration of a 10 mg/kg dose, compound 56 quickly occupied the OX1R in the rat brain (Figure 3A). The plateau for maximal receptor occupancy (above 90%) was achieved within 15 min and lasted for 4 hs. At the 6 h time point, occupancy levels dropped to 60-70%. GSK-1059865 had a very similar profile. SB-334867 achieved slightly lower levels of OX1R occupancy (80-90%) and started to significantly decrease (~ 50%) at the 4 h time point. Lower levels of OX1R occupancy (~ 70%) was observed for SB-408124 with a fast clearance (below 50% after 1 h).

Dose response studies were done at the 0.5 h time point to compare the four compounds (Figure 3B). The lowest ED$_{50}$ value was obtained for compound 56 (0.01 mg/kg) followed by GSK-1059865 (0.9 mg/kg). Much higher ED$_{50}$ values were measured for SB-334867 (7.0 mg/kg) and SB-408124 (7.6 mg/kg).

Blood brain barrier and pharmacokinetic parameters are listed in Table 3. Overall, there was a good correlation between receptor occupancy levels and plasma/brain concentrations, i.e. receptor occupancy levels dropped when plasma/brain levels dropped. Compound 56 had the highest brain to plasma ratio. Although, the highest plasma exposure values were reached after administration of SB-408124, this compound poorly crossed the blood brain barrier (brain to plasma ratio = 0.02).
Compound 56 was also found to cross the blood brain barrier after systemic administration in mice (10 mg/kg SC, $C_{\text{max}}$ in plasma: 1970 ng/mL, in brain: 929 ng/mL).

The OX1R antagonist compound 56 does not affect spontaneous sleep in rats:

Rats were orally dosed with the OX1R antagonist compound 56 (10 mg/kg) at the onset of the dark/active phase, the optimal phase to detect any hypnotic activity for OX1/2R antagonists. There were no significant differences between compound 56 and vehicle conditions (paired Student’s t test) in any of the sleep measures examined over the 6 h period following the treatment, specifically NREM sleep latency or duration (Figure 4A and B) and REM sleep latency or duration (Figure 4C and D).

Transient OX1R blockade selectively promotes REM sleep in OX2R knockout mice:

Since the functional activity of an OX1R antagonist is not evident to reveal under baseline conditions, the challenge was to identify an in vivo pharmacodynamic model of efficacy. Previous studies have demonstrated that OX1R antagonists minimally affect spontaneous sleep-wake states in rodents, but produce a disinhibition of REM sleep in the presence of OX2R antagonism (Dugovic et al., 2014). In the present study, the in vivo functional target engagement was investigated in a model of permanent inhibition of OX2R, and the effects of compound 56 on sleep-wake states were examined in mice lacking the OX2R relative to their corresponding wild-type (WT) mice. As compared to vehicle, oral dosing of compound 56 (30 mg/kg) at 2 h into the light phase to OX2R KO mice significantly reduced the latency for REM sleep and prolonged the time spent in REM sleep during the first 6 h post treatment (Figure 5A), but did not alter REM sleep in...
WT mice (Figure 5B). Moreover, OX2R KO mice dosed with the OX1R antagonist exhibited a dysregulation in REM sleep onset as evidenced by the average of REM sleep latency occurring less than 10 min before the average of NREM sleep latency, indicative of sleep onset REM sleep (SOREM) episodes that were observed in 5 out of 7 mice. As opposed to REM sleep parameters, NREM sleep latency and NREM sleep duration were not significantly affected by the OX1R antagonist in both genotypes (Figure 5C and D).

Selective OX1R antagonism attenuates cage exchange stress-induced hyperarousal without affecting ACTH release:

The effect of the OX1R antagonist compound 56 was investigated in a rat model of acute psychological stress-induced hyperarousal elicited by cage exchange during the light/rest phase (Cano et al., 2008). Rats were dosed with compound 56 (10 mg/kg SC) or vehicle, and 30 min later (4 h into the light phase) were submitted to the cage exchange or control (brief handling) procedure. For each condition (cage exchange and control), animals were injected with the opposite treatment (vehicle or compound 56) in a counter-balanced manner and a cross-over design. Analysis of EEG sleep parameters revealed a significant main effect (four treatment conditions, cross-over design) for NREM sleep latency \( [F_{3,27} = 8.67, P<0.001] \), REM sleep latency \( [F_{3,27} = 6.47, P<0.01] \) and total sleep in the first 2 h \( [F_{3,27} = 5.18, P<0.01] \). In vehicle-treated rats, cage exchange stress induced a significant delay in both latencies for NREM sleep (\( P<0.05 \), Figure 6A) and REM sleep (\( P<0.05 \), Figure 6B) associated with a decrease in total sleep duration (\( P<0.05 \), Figure 6C) as compared to the control condition. Administration of compound 56 did not affect any EEG sleep parameters relative to vehicle in control
conditions, but significantly attenuated the increase in both NREM sleep latency (P<0.05, Figure 6A) and REM sleep latency (P<0.05, Figure 6B) induced by cage exchange. In contrast, the OX1R antagonist did not prevent the decrease in total sleep duration (Figure 6C). Thus, compound 56 could attenuate the sleep onset insomnia (i.e. prolongation of NREM and REM sleep latencies) elicited by cage exchange without impacting sleep duration.

To assess whether the cage exchange procedure would activate the HPA axis, ACTH levels were evaluated as a biomarker for the stress response. We examined the effects of the selective OX1R antagonist to address the involvement of OX1R in modulating the potential ACTH response to this psychological stress. ACTH serum levels were measured 20 min after the cage exchange or control conditions in mice treated at 4 h into the light phase with compound 56 (30 mg/kg SC) or vehicle 30 min before the procedure. The analysis of ACTH levels revealed a significant main effect (four treatment conditions in separate groups) [F3,44= 6.77, P<0.001]. Following cage exchange stress, vehicle-treated mice exhibited a 3.4 fold increase of ACTH levels as compared to the control conditions (P<0.05, Figure 6D), consistent with an activation of the HPA axis. Administration of compound 56, which had no effect by itself in control conditions, did not alter the cage exchange-induced ACTH release.

*Compound 56 attenuated NaLac-induced panic/anxiety-associated behavioral and physiological responses in panic-prone rats:*
In a final experiment, the effect of the OX1R antagonist compound 56 was investigated in a panic vulnerability to a well-known interoceptive panicogenic challenge (i.e. NaLac) following chronic disinhibition of the PeF OX region.

Analysis of autonomic and locomotor-associated behavioral responses: An overall ANOVA with time as a repeated measure detected a drug treatment x time interaction for NaLac-induced changes in MAP [F(57,608) = 1.8, P<0.001, Figure 7A], HR [F(57,608) = 1.6, P=0.005, Figure 7B], CBT [F(57,608) = 1.8, P<0.001, Figure 7D], and general motor activity [F(57,608) = 2.1, P=0.024, Figure 7E]. A within subjects analysis using a repeated measures ANOVA + Dunnett’s posthoc test revealed that compared to baseline NaLac: 1) increased MAP in the vehicle group [F(8,179) = 7.1, P<0.001], 3 mg/kg group [F(8,179) = 5.3, P<0.001], 10 mg/kg group [F(8,179) = 4.7, P<0.001], but not the 30 mg/kg group [F(8,179) = 1.6, P=0.067]; 2) increased HR in the vehicle group [F(8,179) = 9.5, P<0.001], 3 mg/kg group [F(8,179) = 4.6, P<0.001], 10 mg/kg group [F(8,179) = 5.6, P<0.001], but not the 30 mg/kg group [F(8,179) = 1.6, P=0.072]; 3) increased CBT in vehicle group [F(8,179) = 2.2, P=0.004], 10 mg/kg group [F(8,179) = 8.7, p<0.001], and 30 mg/kg group [F(8,179) = 2.1, P=0.008, Dunnett’s failed to reveal posthoc significance] but not the 3 mg/kg group [F(8,179) = 0.5, P=0.992]; 4) increased general motor activity in vehicle group [F(3,35) = 12.0, P<0.001], 3 mg/kg group [F(3,35) = 3.7, P=0.026], 10 mg/kg group [F(3,35) = 4.5, P=0.012], but not the 30 mg/kg group [F(3,35) = 2.2, P=0.114]. Final n’s/ group were 9/group.

Analysis of anxiety-associated behavioral responses following the NaLac infusion revealed the following:
An ANOVA with a Fisher’s LSD post hoc test revealed that rats treated with vehicle had a decreased social interaction duration following the NaLac challenge \( F(4,39) = 6.0, \ P=0.047, \text{ Figure 7C} \), which was attenuated with the 30 mg/kg dose of the compound 56, and approached significance with the 3 mg/kg dose \( \ P=0.076 \). Final n’s/group were respectively 9,9,8,9,9 with 1 video capture malfunction.

Histology revealed that all minipump cannulae placements resided in the PeF or just medial to the PeF in the dorsomedial hypothalamic nuclei (DMN) or dorsal hypothalamic area (DA) where acute disinhibition or chronic disinhibition + NaLac produces anxiety-associated behaviors and robust cardioexcitatory responses (Shekhar and DiMicco, 1987; Samuels et al., 2004; Johnson et al., 2008) (Figure 7F).
6. Discussion

In the present study, we characterized a novel selective OX1R antagonist that showed efficacy in two models of stress, a psychological stress elicited by cage exchange and a NaLac provocation panic model in rats. We also demonstrated that the compound did not alter sleep wake states in baseline conditions and therefore was devoid of any hypnotic effect.

Over the last 15 years, most of the drug discovery efforts in the OX field have been directed towards developing nonselective OX receptor antagonists. At least four dual OX receptor antagonists have entered human trials for the treatment of sleep related disorders (Hoyer and Jacobson, 2013). Recently, the U.S. Food and Drug Administration approved Belsomra (Suvorexant) for the treatment of insomnia (http://www.fda.gov/). It is now well established that selective OX2R antagonists have robust hypnotic activity in preclinical species and transient pharmacological inhibition of the two receptors, either by a dual OX1/2R antagonist or by simultaneous blockade of OX1R to OX2R antagonism, disrupts sleep architecture by shifting the balance in favor of REM sleep at the expense of NREM sleep (Dugovic et al., 2014). The role of the OX1R in emotional behavior is starting to emerge (Johnson et al., 2012). Most of these preclinical studies used the OX1R antagonist SB-334867 (Smart et al., 2001) which has been reported to be hydrolytically unstable, and therefore possible confounding effects on in vivo and in vitro studies cannot be ruled out (McElhinny Jr et al., 2012). We confirmed that SB-334867 has moderate in vitro affinity for the OX1R and a decent selectivity profile. Our data showed that SB-334867 occupied the OX1R for several hours after systemic
administration indicating that some of the effects of SB-334867 can be attributed to OX1R blockade. The related compound, SB-408124 is more stable but has lower in vitro affinity/potency for the OX1R (Langmead et al., 2004). Despite poor brain penetration sufficient exposure was achieved to sustain significant level of OX1R occupancy. We also confirmed and extended the excellent selectivity profile of GSK-1059865 (Gozzi et al., 2011). Compound 56 had a similar in vitro profile to GSK-1059865 but slightly better brain penetration. Compound 56 was therefore selected for thorough in vivo characterization.

Previous studies have demonstrated that OX1R antagonists minimally affect sleep-wake states in basal conditions (Smith et al., 2003; Dugovic et al., 2009; Gozzi et al., 2011; Steiner et al., 2013a; Dugovic et al., 2014), but produce a disinhibition of REM sleep in the presence of OX2R antagonism (Dugovic et al., 2014). In rats, the co-administration of the OX2R antagonist JNJ-10397049 and the OX1R antagonist SB-408124 or GSK-1059865 promoted REM sleep whereas REM sleep was not affected either by OX2R or OX1R blockade alone (Dugovic et al., 2009; Dugovic et al., 2014). Consistent with these data, we found that compound 56 was devoid of sleep promoting effects under basal conditions in rats and WT mice. In line with our previous results on simultaneous pharmacological OX1R and OX2R blockade, compound 56 did promote REM sleep in OX2R KO but not WT mice. These experiments showed that compound 56 fully engaged the OX1R and elicited specific OX1R blockade.

There is literature supporting the view that OX receptor antagonists do not alter basal anxiety levels in rodents but show efficacy when anxiety levels have been exacerbated (Steiner et al., 2012; Steiner et al., 2013a). Negative data with OX receptor
antagonists in classical anxiety models like elevated plus-maze or social interaction (under baseline conditions) have been reported (Yeoh et al., 2014). Therefore we tested compound 56 in two models of psychological exteroceptive (i.e. external threat) and interoceptive (i.e. internal body state threat) stress induced hyperarousal. In future experiments, it would be interesting to test the fear potentiated startle paradigm as another selective OX1R antagonist showed anxiolytic effects in this model (Steiner et al., 2013a). OX’s exert a prominent role in arousal-related process including stress by activating OX1R and OX2R. Stress and corticotrophin-releasing factor (CRF) activate OX neurons, and OX administration stimulates hyperarousal, ACTH and corticosterone release [for review see (Berridge et al., 2010)]. We first examined whether a selective OX1R antagonist could attenuate the arousal effects of a psychological stress elicited by cage exchange on EEG sleep in rats and on potential HPA axis activation in mice. Previous studies have shown that psychological stress induced by cage exchange produced an increase in body temperature, activity, arterial pressure and heart rate in mice (Oka et al., 2003; Lee et al., 2004) and acute insomnia in rats (Cano et al., 2008). In accordance with these results, we found that rats exposed to this psychological stressor exhibited sleep disturbances including increased NREM and REM sleep latencies and decreased total sleep time for at least 2 h. When the animals were pretreated with an OX1R antagonist before the cage exchange stress the effect on sleep latency, an index of the level of arousal, was attenuated whereas the compound had little impact on sleep duration. The normalization of both NREM and REM sleep latencies by compound 56 administration was not the result of a soporific effect since the associated decrease in total sleep time was not altered between vehicle and compound treated animals submitted
to cage exchange. Together with our finding that compound 56 was inactive on spontaneous sleep, these data suggest that OX1R antagonism may inhibit the cage exchange stress-induced phasic orexin activation without affecting the physiological tonic excitability of orexin neurons regulating the basal sleep-wake cycle. In a fear-potentiated startle paradigm, another selective OX1R antagonist ACT-335827 has been shown to elicit anxiolytic-like effects without promoting sleep in rats (Steiner et al., 2013a). In addition to a number of alterations in the autonomic system previously reported following cage exchange in mice (Oka et al., 2003; Lee et al., 2004), our present data showed an increase in ACTH levels revealing an activation of the HPA axis. In contrast to the hyperarousal response, OX1R blockade did not affect the cage exchange stress-induced ACTH release in mice, suggesting that OX1R are not directly involved in HPA axis activation elicited by this psychological stress model. A putative OX2R mediated effect might be rather expected since increase in ACTH levels by swimming stress was attenuated with prior central infusion of an OX2R antagonist (Chang et al., 2007), a result consistent with the predominance of OX2R in the PVN (Marcus et al., 2001). However, in a later study administration of the dual OX1/2R antagonist almorexant in rats failed to alter the release of corticosterone in basal and various stress conditions (novelty, social stress and restraint stress) or the release of ACTH upon pharmacological challenge with CRF (Steiner et al., 2013b). It remains uncertain whether testing a selective OX2R antagonist in this particular model is relevant since the intrinsic sleep-promoting properties of the antagonist would be a confounding factor.

As a second model we assessed the effect of the OX1R antagonist compound 56 in a panic vulnerability model involving chronic disinhibition of the PeF OX regions that
has been utilized for over 15 years [see review (Johnson and Shekhar, 2012)]. Previously, Johnson and colleagues determined that chronically disinhibiting the PeFOX region was critical for stress (NaLac)-induced panic responses in a rat model of panic vulnerability that has robust postdictive, face, predictive, and construct validity. Here we showed that the highest dose of compound 56 (30 mg/kg) attenuated the anxiety-like behavior (reduced social interaction), locomotor, and cardioexcitatory responses induced by the NaLac challenge. The lowest dose tested (3 mg/kg) was also efficacious on locomotor and cardiovascular parameters but not on the behavioral readout. Surprisingly, the intermediate dose was not efficacious on any parameters excepted on the suppression of NaLac induced locomotor activity. There were no significant side effects of compound 56 at any of the doses tested on sedation as assessed by monitoring baseline locomotion or autonomic activity. Compound 56 was also tested in a rat model of CO₂-induced panic (Johnson et al., in preparation). As observed in the NaLac model, compound 56 had no effect on baseline cardiovascular or locomotor activity, and a consistent attenuation/blockade of CO₂-induced anxiety behavior in the social interaction test was observed at 30 mg/kg. An attenuation of the pressor and bradycardia response was also observed at 10 and 30 mg/kg with a more clear dose response relationship. Thus, overall the efficacy of compound 56 in these two models of panic was consistent. In the cage exchange paradigm and the NaLac provoked panic model, efficacy was observed at doses that achieved maximal levels of OX1R occupancy. However, the lowest doses of compound 56 which were ineffective on the anxiety response in the NaLac model were also found to be near maximal level of receptor occupancy. These data indicate that the receptor occupancy assay is not predicting the level of OX1R needed for efficacy in this
behavioral model. The reason for this discrepancy is not entirely clear. OX1R occupancy levels were measured in the tenia tecta, a small region where high densities were detected. For practical reasons we were not able to measure OX1R occupancy in the hypothalamus, BNST, or amygdala which are more closely related to the site of action for the anti-panic response. Binding densities in these regions are relatively low and therefore only weak signal can be detected in these regions.

Although OX appears to play an important role in the aforementioned stress paradigms (e.g., exteroceptive cage exchange and interoceptive NaLac or CO₂), not all stressors engage the OX system. Restraint and cold stress for example are not effective stimuli for OX activation and OX1R and ORX2 blockade does not alter the pressor and tachycardia responses to these stressors (Furlong et al., 2009). As active wake/hyperactivity is a prerequisite component to activate OX neurons, non-standard preclinical stress models could reveal the potential anxiolytic effects of selective OX1R antagonists. Clinical translations of these findings in the context of complex nervous system disorders remain to be established.

In conclusion, our data indicate that under aversive conditions, hyperarousal and anxiety-associated behavioral and physiological responses can be reduced by a selective OX1R antagonist that could represent a new therapeutic strategy without affecting sleep per se.
7. Acknowledgment

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8. Authorship Contributions

Participated in research design: Pascal Bonaventure, Philip L. Johnson, Anantha Shekhar, Timothy Lovenberg, Christine Dugovic

Conducted experiments: Sujin Yun, Stephanie D. Fitz, Diane Nepomuceno, Brian Lord, Michelle Wennerholm, Jonathan Shelton

Contributed new reagents or analytic tools: Brock Shireman, Terry Lebold, Nicholas Carruthers

Performed data analysis: Pascal Bonaventure, Philip L. Johnson, Diane Nepomuceno, Brian Lord, Jonathan Shelton, Christine Dugovic

Wrote or contributed to the writing of the manuscript: Pascal Bonaventure, Phil Johnson, Christine Dugovic
9. References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.


hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:1 page following 696.


10. Footnotes

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11. Figure Legends

**Figure 1:** Chemical structure of OX1R antagonists. Compound 56: (N-([3-[(3-Ethoxy-6-methylpyridin-2-yl)carbonyl]-3-azabicyclo[4.1.0]hept-4-yl]methyl)-5-(trifluoromethyl)pyrimidin-2-amine); GSK-1059865: (5-Bromo-N-([1-[(3-fluoro-2-methoxyphenyl)carbonyl]-5-methylpiperidin-2-yl]methyl)pyridin-2-amine; SB-408124: 1-(6,8-Difluoro-2-methylquinolin-4-yl)-3-[4-(dimethylamino)phenyl]urea; SB-334867: 1-(2-Methyl-1,3-benzoxazol-6-yl)-3-(1,5-naphthyridin-4-yl)urea.

**Figure 2:** Characterization of [³H]compound 56. A and B) Saturation study: total, specific and nonspecific binding of [³H]compound 56 to CHO or HEK-293 cells stably expressing the human (A) or rat (B) OX1R receptor with increasing radioligand concentration. Nonspecific binding was defined as that remaining in the presence of 10 μM almorexant. Vertical lines show S.E.M. Data are the mean of 3 (rOX1R) or 4 (hOX1R) experiments. C) Digitized computer images of the distribution of [³H]compound 56 binding sites in coronal rat brain sections at 3 different levels, nonspecific binding was determined in the presence of 10 μM GSK-1059865. Color represents relative levels of optical density ranging from red >yellow>green>blue>black. Scale bar, 0.25 cm. Cx, cortex; TT, tenia tecta; Hip, hippocampus; Amg, amygdala; LC, locus coeruleus.

**Figure 3:** Ex-vivo brain OX1R binding autoradiography by compound 56, GSK-1059865, SB-334867 and SB-408124. (A) Duration of occupancy for OX1R antagonists post subcutaneous administration in rats (10 mg/kg). (B) Occupancy versus dose measured at
0.5 h. Results are expressed as average percentage receptor occupancy vs. vehicle treated rats ± S.E.M. (n = 3). OX1R occupancy was measured in the tenia tecta.

**Figure 4:** Effects of the OX1R antagonist compound 56 on sleep parameters in rats. NREM latency (A) and NREM duration (B), and REM latency (C) and REM duration (D) are determined for the 6h period after oral dosing (10 mg/kg) at the onset of the dark phase. Results are expressed in minutes and are represented as means ± S.E.M. of 8 animals.

**Figure 5:** REM sleep-promoting effects of the OX1R antagonist compound 56 in OX2R KO. REM sleep latency and duration in OX2R KO (A) and OX2R WT (B), and NREM sleep latency and duration in OX2R KO (C) and OX2R WT (D) for the 6h period after oral dosing (30 mg/kg) during the light phase are expressed in minutes. Values are means ± S.E.M. of 7 OX2R KO and 5 OX2R WT mice. *P<0.05 and **P<0.01 versus vehicle as determined by paired Student’s t test for each genotype.

**Figure 6:** Effects of the OX1R antagonist compound 56 on cage exchange stress-induced hyperarousal in rats and ACTH release in mice. Vehicle or compound 56 was administered subcutaneously (10 mg/kg in rats and 30 mg/kg in mice) 30 minutes prior to cage exchange (CE) or control (Cont) condition. NREM latency (A), REM latency (B), and total sleep (C) in rats for the first 2 h are expressed in minutes and are represented as means ± S.E.M. of 7 animals (four treatment conditions, cross-over design). ACTH release (D) in mice is expressed in pg/mL of serum and are represented as means ±
S.E.M. of 11-13 animals per condition (4 treatment conditions). Statistical significance (*P<0.05, **P<0.01 and ***P<0.00) was based on one-way ANOVA followed by Newman-Keuls Multiple Comparison test.

**Figure 7**: The effects of systemic subcutaneous injections of 3, 10 and 30 mg/kg doses of compound 56 on sodium lactate (NaLac)-induced panic-associated autonomic responses [(A) mean arterial blood pressure (MAP); (B) heart rate (HR); and (D) core body temperature (CBT)]; and behavioral responses [(C) social interaction duration and (E) general motor activity] in rats made panic-prone with chronic reductions of GABA synthesis in the perifornical hypothalamic (PeF) region [i.e., local l-allo-glycine (l-AG) infusions]. Line graphs are expressed as average percentage ± S.E.M. (n = 9/group). + symbol in Figures A,B,D,E indicate between subjects differences using a Fisher’s LSD posthoc test that was protected with an ANOVA at each time point and significance with a one way ANOVA with repeated measures P<0.05. * symbol in Figures A,B,D,E indicate within subjects differences using a Dunnett’s posthoc test against baseline that was protected with a repeated measures P<0.05. Bar graph in Figures C is expressed as average percentage ± S.E.M. (n=9,9,8,9,9), and + and * symbols indicate between subjects differences using a Fisher’s LSD posthoc test that was protected with an ANOVA P<0.05. There was no significant baseline HR, MAP, CBT or general motor activity between groups (see legends on figures). (F) is a coronal brain section from a standard stereotaxic atlas of the rat brain (Paxinos and Watson, 2005) illustrating cannula placements (black circles) in the PeF region and medial nuclei. Additional abbreviations:
3V, 3rd ventricle; DA, dorsal hypothalamic area; DMN, dorsomedial hypothalamic nucleus; f, fornix; LH, lateral hypothalamus; mt, mammillothalamic tracts.
12. Tables

**Table 1:** Comparative binding parameters ($K_i$ values) of compound 56, GSK-1059865, SB-334867 and SB-408124 at human OX1R, rat OX1R and human OX2R. The selectivity ratio versus the human OX2R is also listed. Data represent the mean ± S.E.M. of at least 6 determinations each performed in duplicate.

<table>
<thead>
<tr>
<th>Ki (nM)</th>
<th>hOX1R CHO [³H]SB-674042</th>
<th>rOX1R HEK-293 [³H]SB-674042</th>
<th>hOX2R PFSK-1 [³H]EMPA</th>
<th>hOX2R/hOX1R ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 56</td>
<td>3.6 ± 0.9</td>
<td>3.2 ± 0.4</td>
<td>160 ± 5</td>
<td>44</td>
</tr>
<tr>
<td>GSK-1059865</td>
<td>5.0 ± 0.3</td>
<td>6.4 ± 0.2</td>
<td>409 ± 10</td>
<td>82</td>
</tr>
<tr>
<td>SB-334867</td>
<td>47.6 ± 6.4</td>
<td>144 ± 7</td>
<td>3781 ± 280</td>
<td>79</td>
</tr>
<tr>
<td>SB-408124</td>
<td>112 ± 8</td>
<td>375 ± 56</td>
<td>&gt;10,000</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Table 2: Comparative *in vitro* functional potencies (Kn values) of compound 56, GSK-1059865, SB-334867 and SB-408124 at human OX1R, rat OX1R and human OX2R.

Data represent the mean ± S.E.M. of at least 3 determinations each performed in duplicate.

<table>
<thead>
<tr>
<th>Kn (nM)</th>
<th>hOX1R CHO</th>
<th>rOX1R HEK-293</th>
<th>hOX2R PFSK-1</th>
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</thead>
<tbody>
<tr>
<td>Compound 56</td>
<td>2.9 ± 0.5</td>
<td>5.2 ± 1.1</td>
<td>92 ± 22</td>
</tr>
<tr>
<td>GSK-1059865</td>
<td>13.3 ± 1.5</td>
<td>20.7 ± 3.8</td>
<td>304 ± 31</td>
</tr>
<tr>
<td>SB-334867</td>
<td>163 ± 29</td>
<td>232 ± 53</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>SB-408124</td>
<td>127 ± 16</td>
<td>210 ± 55</td>
<td>&lt;5.0</td>
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Table 3: Pharmacokinetic and blood brain barrier parameters of subcutaneous administration of a 10 mg/kg dose of compound 56, GSK-1059865, SB-334867 and SB-408124 in rats.

<table>
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<tr>
<th></th>
<th>Compound 56</th>
<th>GSK-1059865</th>
<th>SB-334867</th>
<th>SB-408124</th>
</tr>
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<td><strong>Plasma</strong></td>
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<tr>
<td>$T_{\text{max}}$ (h)</td>
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<td>2.00</td>
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<tr>
<td>$T_{1/2}$ (h)</td>
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<td>$C_{\text{max}}$ (ng/mL)</td>
<td>1871</td>
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<td>5407</td>
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<tr>
<td>$AUC_{\text{inf}}$ (h*ng/mL)</td>
<td>3350</td>
<td>9142</td>
<td>18068</td>
<td>153418</td>
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<tr>
<td><strong>Brain</strong></td>
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<tr>
<td>$T_{\text{max}}$ (h)</td>
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<td>$C_{\text{max}}$ (ng/mL)</td>
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<td>$AUC_{\text{inf}}$ (h*ng/mL)</td>
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<td>Brain/plasma ratio</td>
<td>0.48</td>
<td>0.34</td>
<td>0.29</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Figure 1

Compound 56

GSK-1059865

SB-408124

SB-334867
**Figure 2**

**A** Human OX1R (CHO)

![Graph showing binding affinity](image)

- Total binding
- Non-specific binding
- Specific binding

- $K_d = 0.8 \pm 0.1 \text{ nM}$
- $B_{max} = 879 \pm 26 \text{ fmol/mg}$

**B** Rat OX1R (HEK-293)

![Graph showing binding affinity](image)

- Total binding
- Non-specific binding
- Specific binding

- $K_d = 0.3 \pm 0.1 \text{ nM}$
- $B_{max} = 280 \pm 8 \text{ fmol/mg}$

**C**

- [3H] Compound 56
- Non-specific binding

- Cx
- Hip
- Hyp
- LC

- Scale bar

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Figure 3

A

% OX1R Occupancy vs. Time (hr)

B

% OX1R Occupancy vs. Dose (mg/kg)

Legend:
- Red: Compound 56
- Purple: SB-408124
- Green: GSK-1059865
- Blue: SB-334867

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 4

A. NREM Latency

B. NREM Duration

C. REM Latency

D. REM Duration

Vehicle Compound 56
Figure 5

A  OX2 KO

B  OX2 WT

C

D

Vehicle  Compound 56
Figure 6

A

NREM Latency

B

REM Latency

C

Total Sleep / 2h

D

ACTH

Vehicle + Control
Vehicle + Cage Exchange
Compound 56 + Control
Compound 56 + Cage Exchange

** |

*** |

* |

** |

* |

Minutes

Minutes

Minutes

Minutes

PG/mL

PG/mL

PG/mL

PG/mL
Figure 7

A. Arterial Blood Pressure

B. Heart Rate

C. Social Interaction

D. Core Body Temp

E. Locomotor Activity

F. I-AG minipump Cannula Placements

-3.24 to -3.48 mm from Bregma