Novel Orexin Receptor-2 Agonists Developed Using Structure-based Drug Design: Prototype Compounds Promote Wakefulness and Reduce Cataplexy in Orexin/Ataxin-3 and WT Mice

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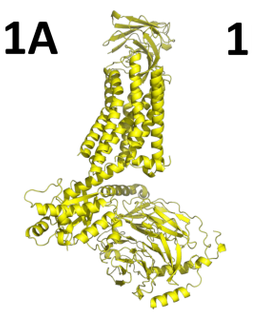
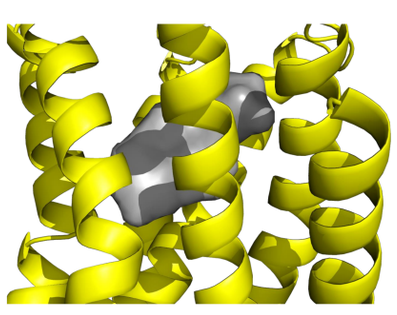
Introduction

Narcolepsy Type 1 (NT1) is caused by the profound loss of orexin (also known as hypocretin)-producing neurons, resulting in dysregulation of sleep/wake and energy homeostasis. The orexin/ataxin-3 (Atax) mouse recapitulates the orexin neurodegeneration of NT1, exhibiting cataplexy and decreased wakefulness. Reactivation of orexin receptor 2 (OX2R) has the potential to treat these and other symptoms of NT1. We have used the OX2R agonist-stabilized receptor (StaR®) protein to discover a pipeline of novel, selective OX2R agonists using high-resolution protein crystallography, cryo-EM, and structure-based drug design. Here, we present compounds from two lead series of orally available, selective, small molecule OX2R agonists with *in vivo* activity.

1A



1B



Full cryo-EM structure of OX2R-StaR® bound to a G protein, confirming stabilization of the agonist conformation (1A). Magnified view of orthosteric binding site with a novel, selective OX2R agonist (grey) bound (1B).

Materials & Methods

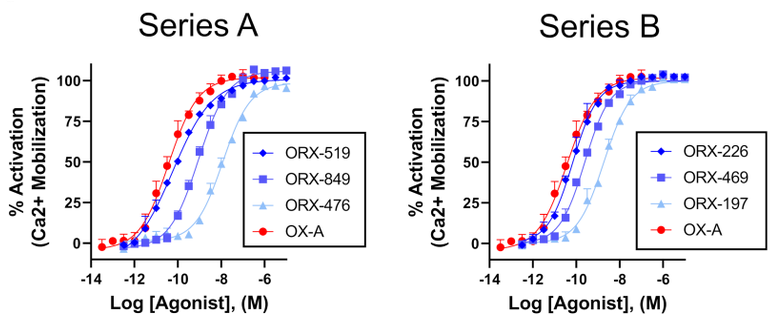
***In Vitro* Pharmacology. i)** Calcium mobilization (FLIPR) assays were performed in Chinese hamster ovary (CHO) cells stably expressing human recombinant OX2R or orexin receptor 1 (OX1R). Cells were loaded with a calcium-sensitive fluorescent dye and then monitored for changes in fluorescence that was agonist concentration-dependent. **ii)** Pathhunter β-arrestin recruitment assays were performed in CHO cells co-expressing the ProLink™ (PK)-tagged OX2R and Enzyme Acceptor (EA)-tagged β-Arrestin (Eurofins). Agonist-stimulated activation of the tagged OX2R resulted in β-arrestin recruitment and complementation of β-galactosidase enzyme fragments, PK and EA. The resulting functional enzyme hydrolyzed a substrate generating a chemiluminescent signal that was agonist concentration-dependent. **iii)** Whole cell current-clamp recordings were performed on histaminergic neurons of the ventral tuberomammillary nuclei (TMN) in brain slices from 3-4 week old male C57BL/6J mice (Evotec, in compliance with Italian Health Authorities). Membrane depolarization studies were performed in the presence of 1 µM tetrodotoxin. Firing rate studies were performed in the absence of tetrodotoxin.

Animals and recordings. Atax mice and wild type (WT) colony mates (age ≥14 wk, Jackson Laboratory, Bar Harbor, ME, USA) were evaluated for sleep/wake using PiezoSleep (ver. 3.08, Adapt-a-base sensors, Signal Solutions LLC, Lexington, KY, USA), a rapid, non-invasive method for classifying sleep and wakefulness in 2 sec epochs by unsupervised machine learning. Atax and WT mice were surgically prepared with telemeters (HD-X02) for electroencephalogram (EEG), electromyogram (EMG) monitoring with concurrent video recording; arousal states were manually scored in 10 sec epochs using NeuroScore (Data Sciences Inc., St. Paul, MN, USA) by established methods[1]. Cataplexy was determined in Atax mice using consensus criteria[2]. In all studies, mice were recorded from home cages in LD12:12, with *ad libitum* food, water, and running-wheels. Separate groups of mice were used in the PiezoSleep validation (by genotype or treatment), PiezoSleep pharmacology, and EEG pharmacology studies. All experimental procedures were approved by the Institutional Animal Care and Use Committee at PsychoGenics.

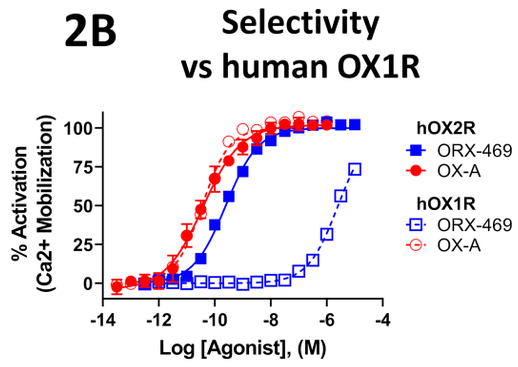
Formulation, dosing, and efficacy study design. Compounds were solubilized for oral dosing in 20% propylene glycol (PG) / 25% polyethylene glycol 400 (PEG) / water (ORX-849, 10 mL/kg) or 5% dimethyl sulfoxide (DMSO) / 20% solutol / 75% water (ORX-469, 5 mL/kg), serial diluted, and administered in a counterbalanced design. Data presented were the mean ± S.E.M. Significant differences from vehicle were determined by Holm-Sidak contrasts following repeated-measures analysis of variance.

Results

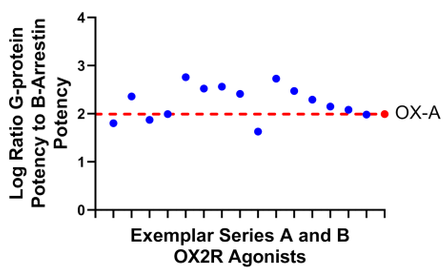
2A Human OX2R potency



2B Selectivity vs human OX1R



2C Unbiased agonism at human OX2R



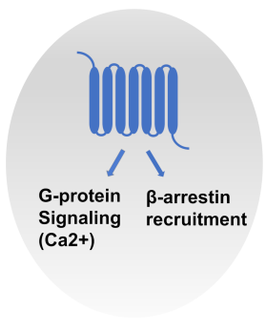


Table 1: Potency & Selectivity		hOX2R pEC50	Selectivity vs hOX1R
Series A	ORX-519	10.2	7100-fold
	ORX-849	8.8	480-fold
	ORX-476	7.9	850-fold
Series B	ORX-226	10.0	28000-fold
	ORX-469	9.5	8900-fold
	ORX-197	8.8	5900-fold

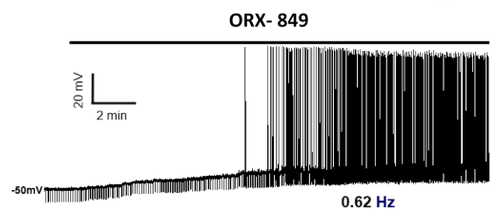
OX2R small molecule agonists showed high potency and selectivity vs OX1R

Agonist potencies were measured by calcium mobilization assay. OX2R agonist compounds from two distinct chemical series were shown to be potent, full OX2R agonists **(2A)** and selective versus OX1R **(2B, Table 1).**

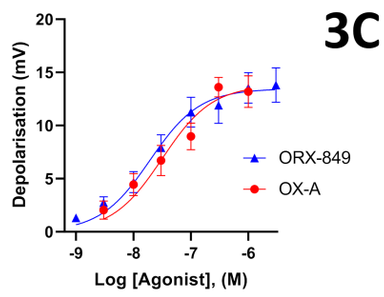
Compounds from each series demonstrated potencies at OX2R equivalent to the native ligand orexin A (OX-A, in red).

Compounds from both lead series stimulated multiple signaling pathways. Unbiased activation of the Gq G-protein pathway (Ca2+ mobilization) and β-arrestin pathway (recruitment) **(2C)** was evident from the activation profiles that were indistinguishable compared to the endogenous ligand OX-A.

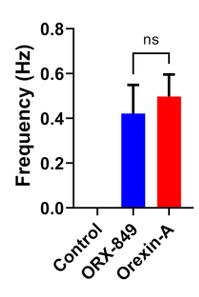
3A Whole cell recordings



3B

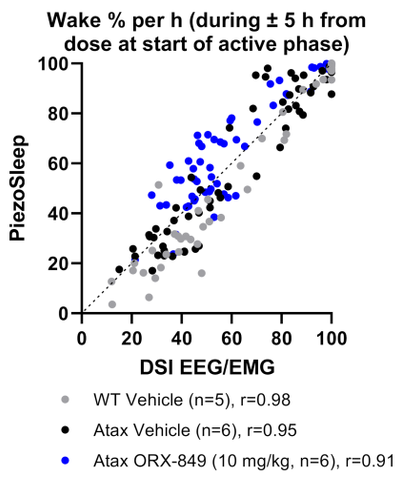


3C



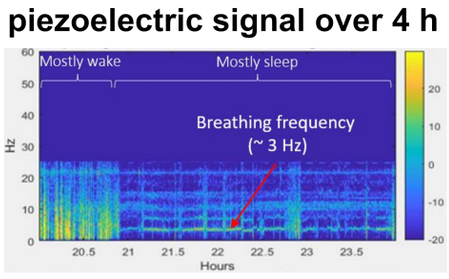
OX2R small molecule agonists activated endogenous mouse OX2Rs from histaminergic neurons in the TMN brain slice. Representative trace of firing rate from whole cell recordings in the presence of acute ORX-849 **(3A).** Membrane depolarization EC50s for ORX-849 and OX-A were 89 nM (max 12.5 mV) and 32 nM (max 13.8 mV), respectively **(3B).** ORX-849 and OX-A at 300 nM depolarized TMN neurons and increased the firing rate **(3C).**

4A PiezoSleep vs EEG

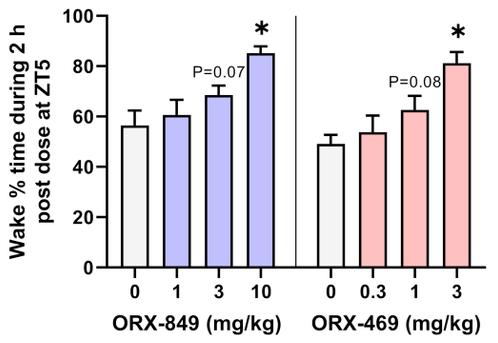


Wake time detected by PiezoSleep highly correlated with EEG/EMG-defined wakefulness (4A). Simultaneous piezoelectric and EEG/EMG/video recordings were collected from the same individual mice. PiezoSleep transforms breath rate and movement signals into a sleep/wake decision statistic **(4B).**To obtain a range of sleep/wake behaviors, Atax and WT mice were administered vehicle or ORX-849 at the start of the dark period, and wakefulness was quantified hourly for 5 h before and after dosing. Wake % per h classified by PiezoSleep correlated with time awake as determined by EEG/EMG/video. Pearson r = 0.91-0.98, P < 0.0001.

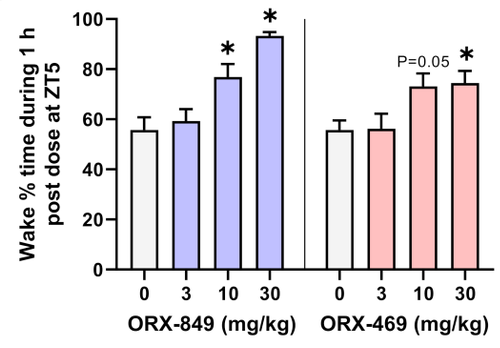
4B Spectrogram of piezoelectric signal over 4 h



4C Atax mice (2 h)



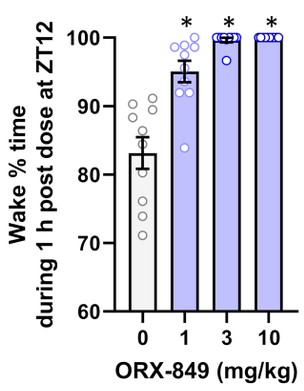
4D WT mice (1 h)



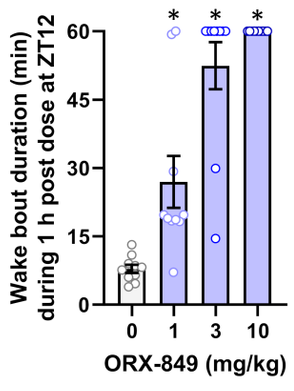
Representative Series A (blue) and Series B (red) OX2R agonists promoted wakefulness at increasingly low doses and for longer durations of action in PiezoSleep. Compounds were orally administered at 5 h after lights on (zeitgeber time, ZT5) in Atax **(4C)** and WT **(4D)** mice (each n=16). Dose-related increases in time awake were observed for both compounds. In Atax mice for two hours post dose, ORX-849 (10 mg/kg) and ORX-469 (3 mg/kg) increased time awake. In WT mice, ORX-849 and ORX-469 at 10 and/or 30 mg/kg increased time awake during the first hour post dose. *P < 0.05 vs. vehicle

Consolidation of wakefulness:

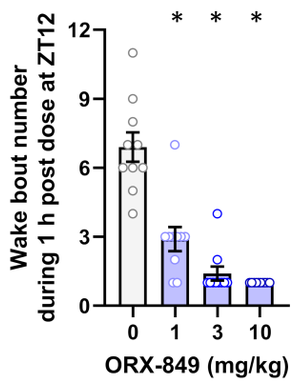
5A Wake time



5B Wake bout duration ↑

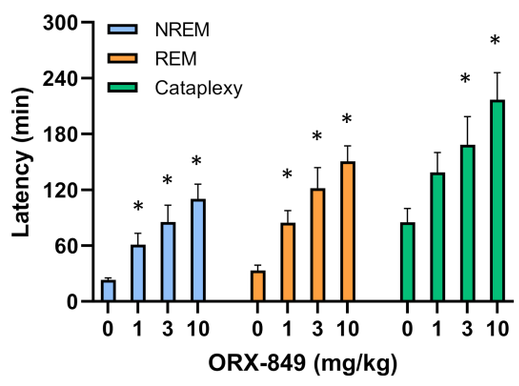


5C Wake bout frequency ↓

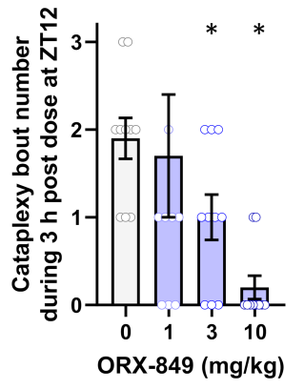


ORX-849 increased time awake (5A) and consolidated wakefulness (5B-C) during the active phase in Atax mice as determined by EEG/EMG/video. After oral administration at the start of the dark period, ORX-849 (≥ 1 mg/kg) increased time awake, increased the mean wake bout duration, and decreased the number of wake bouts during the first hour post dose. *P < 0.05 vs. vehicle

6A Latencies



6B Cataplexy



ORX-849 increased the latency to rapid-eye-movement (REM) sleep, non-REM (NREM) sleep, and to the first occurrence of cataplexy in Atax mice (6A); cataplexy bout numbers decreased after ORX-849 (6B). Dose-related increases in the time to ≥ 30 sec of NREM sleep, ≥ 20 sec of REM sleep, and ≥ 10 sec of cataplexy were observed after oral ORX-849 (1-10 mg/kg) administered at ZT12. ORX-849 decreased the number of cataplexy episodes during the 3 h post dose. Cataplexy bouts of 4 and 7 episodes included in the analysis in 6B are not shown (out of range on y-axis). P < 0.05 vs. vehicle

Conclusions

- Orexia has developed exceptionally potent and selective OX2R full agonists with demonstrated *in vitro* activity equivalent to the endogenous peptide, OX-A, in cells expressing recombinant OX2R and at endogenous OX2R expressed in TMN of acute mouse brain slices
- OX2R agonists from multiple series increase time awake, consolidate wakefulness, and suppress cataplexy in Atax mice and are also active in WT mice
- The newest compounds show substantially increased potency with pEC50s >10 and are progressing rapidly through the discovery pipeline
- Structure based drug design has enabled the discovery of novel, potent OX2R agonists which have the potential to treat narcolepsy and reduce excessive sleepiness in other disorders with high unmet need

References

1.Black, SW et al., SLEEP 2013;36(3):325
2.Scammell, TE et al., SLEEP 2009;32(1):111

Acknowledgements

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