The Role of Basal Forebrain Orexin-2-Receptors in Attentional Performance in Rattus norvegicus

Abhya P. Vij

College of William and Mary

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The Role of Basal Forebrain Orexin-2-Receptors in Attentional Performance in *Rattus norvegicus*

Abhya Panya Vij

The College of William & Mary
Changes in homeostatic function, including sleep patterns and energy balance, are often predictive of age-related neurocognitive disorders such as Alzheimer’s and Parkinson’s disease. The basal forebrain has been implicated in both attentional processing and responses to homeostatic cues, suggesting a possible link between the significantly impaired attention task performance of Alzheimer’s and Parkinson’s patients and shifts in their homeostatic regulation. Recent research suggests a possible mechanism: the basal forebrain receives input from hypothalamic neurons that release the excitatory neuropeptides, orexin-A and orexin-B (orexins). Orexins bind with varying affinities to orexin-1- and orexin-2-receptors on cholinergic neurons in the basal forebrain and increase the rate at which they fire action potentials in response to attentional demands and regulation of homeostasis. Degeneration of these cholinergic projections to the cortex is a hallmark of Alzheimer’s and Parkinson’s, but it is currently unknown if impairment is due solely to loss of these projections, or if the decline can be attributed to loss of particular receptors on these neurons. In order to evaluate the necessity of orexin-2-receptors for attentional performance, TCS-OX2-29, an orexin-2-receptor selective, non-peptide antagonist was administered via bilateral infusion into the basal forebrain of Rattus norvegicus prior to completion of an attention task with a distracter present. The findings suggest a dose- and block-dependent decline in correct rejections at the 40 nM TCS-OX2-29 infusion into the basal forebrain on the sustained attention task with distracter compared to saline administration. An exploratory analysis technique revealed possible improvements in relative hits at 2 nM and 20 nM TCS-OX2-29 infusions into the basal forebrain. Combined, these findings suggest that the orexin-2-receptor is involved in attentional performance, and that blocking the orexin-2-receptor with TCS-OX2-29 may, at high doses, result in a decline in correct rejections, while lower doses of 2 nM and 20 nM may be involved in sensitization of the orexin-2-receptor, making it more likely to respond to binding of orexin A and orexin B.
OREXIN-2-RECEPTORS IN THE BASAL FOREBRAIN

The Role of Basal Forebrain Orexin-2-Receptors in Attentional Performance in *Rattus norvegicus*

Contents

Abstract

The Role of Basal Forebrain Orexin-2-Receptors in Attentional Performance in *Rattus norvegicus* 3

Introduction 5

Purpose ........................................ 6
Basal Forebrain Cholinergic System (BFCS) .................. 6
Orexinergic System .............................. 8
Peptides ..................................... 8
Receptors .................................... 9
Orexin-2-receptor antagonist: TCS-OX2-29 ............... 11

Materials and Methods 11

Subjects ........................................ 11
Testing Chambers .............................. 12
Attentional Performance Tasks ..................... 13
  Training Task ................................ 13
  Sustained Attention Task ..................... 14
  Sustained Attention Task with Distracter ......... 14
Surgical Procedure ............................ 15
Infusion Procedure ............................ 15
Performance Measures ......................... 17

Results 18

Baseline Performance Analysis (Block 1 vs. Block 2) ........ 20
Distracter Adaptation and Infusion-Induced Brain Damage .... 21
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitting a Sigmoidal × Exponential Surface</td>
<td>22</td>
</tr>
<tr>
<td>Fitting an Interpolated Cubic Spline Surface</td>
<td>26</td>
</tr>
<tr>
<td><strong>Discussion</strong></td>
<td></td>
</tr>
<tr>
<td>Traditional Analysis</td>
<td>37</td>
</tr>
<tr>
<td>Exploratory Analysis</td>
<td>38</td>
</tr>
<tr>
<td>Limitations</td>
<td>40</td>
</tr>
<tr>
<td>Further Study</td>
<td>41</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>43</td>
</tr>
<tr>
<td><strong>Appendix</strong></td>
<td>47</td>
</tr>
</tbody>
</table>
Introduction

Changes in homeostatic function, including sleep patterns and energy balance, may be predictive of age-related neurocognitive disorders such as Alzheimer’s and Parkinson’s disease (Fadel & Burk, 2010). The basal forebrain has been implicated in both attentional processing and responses to homeostatic cues, suggesting a possible link between the significantly impaired attention task performance of Alzheimer’s and Parkinson’s patients and shifts in their homeostatic regulation (Fadel & Burk, 2010).

Recent research suggests a possible mechanism: the basal forebrain receives input from hypothalamic neurons that release the excitatory neuropeptides (orexins), which bind with varying affinities to orexin-1- and orexin-2-receptors on cholinergic neurons in the basal forebrain. The binding of orexins to orexin-1- and orexin-2-receptors results in an increase the rate at which these cholinergic neurons fire action potentials in response to attentional demands and regulation of homeostasis (Fadel, Jolivalt, & Reagan, 2013). Degeneration of these cholinergic projections into the basal forebrain is a hallmark of both Alzheimer’s disease (Auld, Kornecook, Bastianetto, & Quirion, 2002) and Parkinson’s disease (Bohnen & Albin, 2011).

As humans age, neurons with receptors for orexins become sparser, limiting behavioral responses to homeostatic cues because there are fewer neurons that respond to orexins. The overall sensitivity of neurons to orexins is thus weakened. The link between shifts in homeostatic functions and the onset of aging related neurodegenerative disorders gives rise to the hypothesis that orexins and the cholinergic neurons they bind to in the basal forebrain cholinergic system (BFCS) may be involved in the onset of the characteristic attentional impairment observed in Alzheimer’s disease (Fadel & Burk, 2010). However, it is currently unknown if this impairment is due solely to loss of these hypothalamic projections to the basal forebrain, or if the decline can be attributed to loss of particular excitatory receptors on these neurons.
Purpose

The purpose of this study was to examine the role of basal forebrain orexin-2-receptors in attentional performance. This role was examined by administering TCS-OX2-29, an orexin-2-receptor selective antagonist, bilaterally into the basal forebrain, and measuring subject performance on a sustained attention task with distracter (Zajo, Fadel, & Burk, 2015), used to measure attentional performance in rats. The experimental hypothesis stated that if the dose of TCS-OX2-29 infused directly into the basal forebrain was increased, then attentional performance on the sustained attention task with distracter would decrease. Analysis of data was conducted using traditional ANOVA testing of each dose × trial block and dose × signal duration on performance measure. Additionally, models are described in the Results that measure the effects of repeated distracter exposure and repeated infusion into the basal forebrain, using sham and distracter-only trials. An exploratory analysis was conducted to determine if there was a difference between performance measures on experimental trials compared to performance at corresponding \((\text{distracter, infusion})\) points. These points represent expected performance, had the subject received a saline infusion or distracter-only trial, rather than a TCS-OX2-29 dose trial, at the given \((\text{distracter, infusion})\) point. Paired t-tests were then used to compare predicted (curve-generated) values with experimental values. This additional analysis allowed for some control over the possible learning curve that may accompany distracter exposure, and the possible exponential decay in performance that may accompany the physical damage caused by repeated infusions into the basal forebrain.

Basal Forebrain Cholinergic System (BFCS)

The basal forebrain encompasses the septum, diagonal band nuclei, and substantia innominata (Ramachandran, Gupta, & Tranel, 2012). Notably, the basal forebrain has been implicated in attention, motivation, memory, and aging-related neurodegenerative diseases including Alzheimer’s and Parkinson’s (Blanco-Centurion, Gerashchenko, & Shiromani, 2007).
The cholinergic system refers to projections leaving the basal forebrain that release the neurotransmitter, acetylcholine, in the hippocampus and cerebral cortex (Ramachandran et al., 2012). Unlike the acetylcholine mechanism at the neuromuscular junction, which initiates muscle contraction by opening ligand-gated sodium channels in the central nervous system, here acetylcholine acts primarily through metabotropic means at G protein-coupled receptors (Picciotto, Higley, & Mineur, 2012).

A study conducted by Henny and Jones (2008) of the rat basal forebrain showed that of the neuronal terminals projecting from the basal forebrain into the prefrontal cortex, about 19 percent are cholinergic. Miettinen, Kalesnykas, and Koivisto (2002) estimated that there were $26390 \pm 1097$ cholinergic neurons in the rat basal forebrain. Within the diagonal band of Broca (a part of the basal forebrain), Miettinen et al. (2002) reported $9647 \pm 504$ cholinergic neurons. Likewise, the horizontal band of Broca housed $9403 \pm 484$ cholinergic neurons, and the nucleus basalis encompassed $7312 \pm 281$ cholinergic neurons (Miettinen et al., 2002). Communication between the basal forebrain and lateral hypothalamus is bidirectional; many orexin neurons within the hypothalamus are known to synapse onto cholinergic and non-cholinergic neurons in the basal forebrain (Zaborszky, Cullinan, & Braun, 1991).

Cholinergic neurons do not maintain distinct connections when compared to nearby non-cholinergic neurons (Grove, 1988). Because these clusters of cholinergic neurons are widespread throughout the basal forebrain, the BFCS may play an important role in coordinating interactions between specialized cortical and subcortical networks. This could account for why the BFCS is implicated in the development of both cortical and subcortical attentional networks (Parikh & Sarter, 2008). In other words, the basal forebrain cholinergic system could be involved in the development of both cortical (i.e., Alzheimer’s) and subcortical dementias (i.e., motor symptoms of Parkinson’s).
Orexinergic System

The orexinergic system is comprised of two orexin neuropeptides, orexin A (OxA) and orexin B (OxB), and two receptors, known as orexin-1-receptor (OX1R) and orexin-2-receptor (OX2R) (Sakurai et al., 1998).

Originally, orexins were considered modulators of hunger cues and sleep/wake cycles, but since the initial discovery of the orexin system by Sakurai et al. in 1998, components of the system have been implicated in several homeostatic processes. Scientists noted dramatic reductions in functional orexin-1- and orexin-2-receptors in canine narcolepsy, suggesting orexin system involvement in sleep patterns (Lin et al., 1999). As previously discussed, changes in sleep patterns may be an early sign of neurodegenerative disorders. Further, orexin-producing cells are responsive to leptin and ghrelin (Ramachandran et al., 2012), which help modulate food intake through activating satiety and hunger responses, respectively. Hunger, too, is a homeostatic cue that also may be affected by early degeneration in neurodegenerative disorders (Fadel & Burk, 2010). Additionally, orexins may be involved in maintenance of attentional processes. Wheeler et al. (2014) showed that loss of orexin neurons disrupts acquisition of conditioned orienting responses. Boschen, Fadel, and Burk (2009) showed that both systemic injection and direct infusion into the basal forebrain of SB-33467, an orexin-1-antagonist, impairs attentional performance. These relationships suggest a strong link between homeostatic changes that precede the impaired attentional performance notable in Alzheimer’s and Parkinson’s patients, and implicates the degeneration of the orexinergic system as a possible mechanism for the aging-related decline.

Peptides. Both OxA and OxB are produced by a small population of neurons in the hypothalamus, and derived from prepro-orexin via proteolytic cleavage (Sakurai et al., 1998). These two neuropeptides share about 64 percent sequence homology, but differ significantly in binding affinity for OX1R and OX2R (Sakurai et al., 1998). OxA binds with approximately equal affinities to both receptors, however OxB binds with 10-fold higher affinity to OX2R than to OX1R (Sakurai et al., 1998).
Receptors. Both OX1R and OX2R are G-protein coupled receptors (Sakurai et al., 1998). Their implication in learning, memory and attention is based on the widely accepted premise that highly selective changes in the strength of synaptic connections between neurons in the brain contribute to learning and memory. There are two main mechanisms by which both OX1R and OX2R may be involved in long term changes at the synaptic terminal and post-synaptic membrane, and a third mechanism by which only OX2R may be involved. The first two mechanisms involve the coupling of G_{q/11} to OX1R and OX2R upon binding of OxA or OxB to either receptor. The G_{q/11} effector, phospholipase C, goes on to hydrolyze PIP2, a phospholipid found in the plasma membrane, forming second messengers IP3 and diacylglycerol. Diaglycerol attaches protein kinase C to the plasma membrane, while IP3 binds to the IP3 receptor on the cytoplasmic side of the endoplasmic reticulum. The IP3 receptor opens as a calcium-selective ion channel, and calcium rushes out of the endoplasmic reticulum. This calcium binds to and activates protein kinase C at the neuronal membrane (Newton, 1995).

The first mechanism is dependent upon this activation of protein kinase C. Protein kinase C is known to be involved in the maintenance and persistence of long term potentiation, which is integral to learning plasticity (Routtenberg, 1986). Activation of protein kinase C has been implicated in the persistence, or delayed decay, of a response to a stimulus (Routtenberg, 1986).

However, the impacts of intracellular calcium are not limited to the protein kinase C pathway. The second mechanism involves the increased intracellular calcium that results from the binding of IP3 to the IP3 receptor during the aforementioned cascade. Calcium has been heavily implicated in learning plasticity. In particular, there is strong evidence for calcium involvement in generation of long term potentiation, or the selective strengthening of synapses after they have been strongly activated before. When intracellular calcium concentration increases, calmodulin-dependent kinase II in the dendritic spines can be activated (Herring & Nicoll, 2016). Calmodulin-dependent kinase II can then go on to phosphorylate AMPA receptors, which increases
conductance of AMPA channels (Herring & Nicoll, 2016). Ehlers (2000) also showed that calmodulin-dependent kinase II can induce the incorporation of additional AMPA receptors into the post-synaptic membrane. Both of these changes serve to strengthen a synapse following strong activation (Herring & Nicoll, 2016).

The third mechanism is specific to OX2R, which can couple to G\textsubscript{s} or G\textsubscript{i} (Tang et al., 2008) and then triggers a cyclic AMP (cAMP) signalling cascade. In the case of G\textsubscript{s} the alpha subunit binds to adenylyl cyclase, activating it. Activated adenylyl cyclase goes on to catalyze the conversion of ATP to cAMP. Increases in intracellular concentration of cAMP can result in activation of protein kinase A, which phosphorylates AMPA and transcription factors that can regulate gene expression. Changes in AMPA receptor expression and gene expression of other proteins can influence long-term potentiation (Herring & Nicoll, 2016).

OX2R and OX1R differ in their distribution within the brain. Trivedi, Yu, Macneil, Ploeg, and Guan (1998) analyzed mRNA expression of OX1R and OX2R in the rat brain. They noted that OX1R mRNA expression is highest in the ventromedial hypothalamic nucleus, tenia tecta, hippocampal formation, dorsal raphe, and locus coeruleus (Trivedi et al., 1998). In contrast, OX2R mRNA expression is highest in the paraventricular nucleus, cerebral cortex, nucleus accumbens, subthalamic and paraventricular thalamic nuclei, and the anterior pretectal nucleus (Trivedi et al., 1998). Staining for these receptors showed that both receptors populate the hypothalamus, and that OX1R is also localized to the locus coeruleus, while OX2R is present throughout the cortex (Mould, Brown, Marshall, & Langmead, 2013). These differences in distribution suggest that these receptors may have distinct roles in the modulation of homeostatic functions (Ebrahim, Howard, Kopelman, Sharief, & Williams, 2002).

OX1R and OX2R also have varying affinities for ligands OxA and OxB. OX1R binds with 100 to 1000-fold higher affinity to OxA than to OxB (Ebrahim et al., 2002). OX2R is less selective, and binds to both OxA and OxB with approximately equal affinity (Ebrahim et al., 2002).
Orexin-2-receptor antagonist: TCS-OX2-29

TCS-OX2-29 (Tocris) is an OX2R-selective non-peptide antagonist. Supplier data states that TCS-OX2-29 exhibits 250-fold selectivity for the OX2R compared to OX1R and other ion channels and transporters. TCS-OX2-29 has a molecular weight of 433.97 grams per mole, and chemical formula: \( C_{23}H_{31}N_3O_3 \cdot HCl \). This antagonist is soluble up to 25 mM in dimethyl sulfoxide (DMSO). All solutions used in this study were diluted in DMSO to concentrations lower than 25 mM.

Several OX1R and OX2R antagonists have been developed, although specificity, half lives, and IC50 values vary. A study conducted by Mould et al. (2013) involved competition kinetics analyses of several well-known OX2R antagonists including almorexant, suvorexant, SB-408124, SB-334867, EMPA, and TCS-OX2-29. This study showed that TCS-OX2-29 has both the highest dissociation rate \( (k_{off} = 0.22/\text{min}) \) and that TCS-OX2-29 has the shortest dissociation half-life of the compounds analyzed. Additionally, TCS-OX2-29 has the largest \( K_i \) of the compounds tested, and therefore the lowest binding affinity to OX2R compared to the other antagonists. The IC50 of TCS-OX2-20 in 40 nM. Taken together, these attributes suggest that TCS-OX2-29 is highly selective for OX2R, but compared to other antagonists, TCS-OX2-29 binds to OX2R only transiently.

Materials and Methods

Subjects

Seven male Fischer Brown Norway F1 hybrid rats were used (Charles River Laboratories, Wilmington, MA). All rats were housed in pairs during training, and switched to individual housing following surgery in order to prevent interference with the implanted guide cannulae by a cagemate. The vivarium where the rats were housed throughout the study was temperature and humidity controlled and operated on a 14:10 hour light/dark cycle (lights on 0600). In order to minimize omissions by subjects on training and sustained attention tasks (for which water was used as a reward for correct responses), all rats were water restricted throughout the experiment. Subjects received
water during daily training or testing sessions and for 10 minutes after each session. The latency of water access following a session ranged from 15-30 minutes after completion of the session. This varied delay in water access was incorporated to discourage omissions during sessions. Rats were trained five to seven days a week, and received at least 20 minutes of water access on days when no behavioral testing occurred. Food was provided *ad libitum* throughout the experiment. Training procedures, surgical implantation, and sustained attention task administration closely followed methods used by Zajo et al. (2015) and Kozikowski and Burk (2016).

Animals were treated and handled in accordance with the guidelines of the Animal Care and Use Committee at The College of William and Mary.

**Testing Chambers**

Rats were trained and tested in individual chambers, each of which was mounted within a sound attenuating box (Med Associates, Inc.). Further information regarding illumination levels within these chambers has been documented (Burk, 2004).

One wall of each chamber had a water port positioned with a dipper that could hold a volume of 10μL. This dipper was controlled externally through the MED-PC IV software, and programmed to provide water rewards of 10μL for each correct response during a session.

Subject responses were recorded in the form of lever presses. Each chamber had one retractable lever on either side of the water port (two total levers per chamber). Above the water port and each lever was a single panel light, for a total of three panel lights. These lights were used as stimuli; illumination was programmed within the MED-PC IV software. A house light was positioned on the opposite side of each chamber and was off during sessions unless otherwise specified in a particular task.

At the start of daily testing, all the boxes were tested to ensure proper panel light, house light, lever and dipper function.
Attentional Performance Tasks

**Training Task.** Subjects were initially trained on a shaping task to learn to press an extended lever for a water reward. The task employed an FR-1 schedule of reinforcement. In order to prevent subjects from favoring one of the two levers over the other, if one lever was pressed more than five consecutive times, the subject would not be rewarded until the other lever was pressed. Subjects continued testing on this task until they received 120 or more rewards of a possible 162 rewards for three consecutive sessions.

Following successful shaping, subjects began training to distinguish between two trial types: signal trials, which included a single second illumination of the middle panel light, and nonsignal trials, in which there was no illumination of the middle panel light. In order to respond to the signal stimulus, rats were trained to press one of the two levers. Half the subjects, randomly selected, were rewarded with three seconds of access to 10 µL for pressing the right side lever in response to a signal trial. The other half of the subjects were rewarded with three seconds of access to 10 µL for pressing the left side lever in response to a signal trial, and for pressing the right side lever in responses to a nonsignal trial. For the other half of the subjects, the rats were rewarded for pressing the right side lever in response to a signal trial, and for pressing the left side lever in response to a nonsignal trial. Animals were cued to respond by the extension of both response levers after each signal or nonsignal. There was a twelve second pause between trials.

During this training period, attempts were made to modify incorrect responses using correction trials (repeats of the incorrect trial). If, following an incorrect response, a subject responded incorrectly to three consecutive correction trials, a forced correction trial was initiated, in which only the correct lever was extended for 90 seconds for the rat to press in response to the signal or nonsignal.

After rats performed this task with greater than 70 percent relative hits in response to the 500-ms signal, and greater than 70% correction rejections for a minimum of three consecutive days, they were switched to the Sustained Attention Task.
Sustained Attention Task. The purpose of the Sustained Attention Task was to create a more attentionally demanding environment by requiring subjects to differentiate between 5-ms, 100-ms, and 500-ms signals (illumination of the central panel light). Additionally, the time between trials was varied between six and twelve seconds to further increase attentional demands. As during training sessions, for animals that were trained to press the right side lever in response to a 1-ms illumination, a right lever press was documented as a 'hit' for 5-ms, 100-ms, and 500-ms signal trials, and a left lever press was documented as a 'correct rejection' on nonsignal trials. If an animal in this group pressed the left lever in response to a signal trial, this response was documented as a 'miss.' If an animal in this group pressed the right side lever in response to a nonsignal trial, this response was documented as a 'false alarm.' Finally, if an animal in this group did not press either lever in response to a signal or nonsignal trial, this was recorded as an 'omission.' These rules were reversed for the rats originally trained to press the left side lever in response to a signal trial.

Trials were divided into three blocks of 54 trials each. There were 27 signal and 27 nonsignal trials per block, and each signal duration (500-ms, 100-ms, and 25-ms) was presented a total of 27 times throughout the entire testing session. Trials were selected randomly without replacement.

Once subjects had again reached greater than 70% relative hits in response to the 500-ms signal, and greater than 70% correct rejections for a minimum of three consecutive days, they were surgically implanted with guide cannulae bilaterally into the basal forebrain. Following a minimum seven-day recovery period after surgery, rats continued testing on the Sustained Attention Task until they reached pre-surgical accuracy in hits in response to 500-ms signals and correct rejections.

Sustained Attention Task with Distracter. This task was identical to the Sustained Attention Task, however during the second trial block (trials 55 through 109) the houselight flashed on and off (2 second period) repeatedly throughout the block. The flashing house light serves to produce a cognitively demanding condition that is thought to disrupt attentional performance in several neuropsychiatric conditions.
OREXIN-2-RECEPTORS IN THE BASAL FOREBRAIN

(Sarter, Hasselmo, Bruno, & Givens, 2005). The Sustained Attention Task with Distracter was used for all experimental trials during which data was recorded for analysis, but in between data collection days, which occurred at a maximum rate of one data collection day every other day, rats completed the Sustained Attention Task (without distracter).

**Surgical Procedure**

At least 12 hours prior to surgery, rats received 2.6 mg/mL acetaminophen in their drinking water. The following morning, rats received 90 mg/kg ketamine combined with 9.0 mg/kg xylazine for anesthesia. In order to ensure that subjects were sufficiently anesthetized, the withdrawal reflex of the back paws was checked. Once the response was negative, the surgical area was shaved, and subjects were positioned in a stereotaxic apparatus. All surgical procedures were conducted under aseptic conditions.

All subjects were received implants of 8-mm, 22 guage guide cannulae bilaterally into the basal forebrain (−1.3mm anterior-posterior (AP) and +2.7mm medial-lateral (ML) from bregma and −4.2mm from dura). Following implantation of the guide cannulae, three stainless steel screws were secured in the skull and the surgical area around the screws and cannulae was filled with dental cement. Subjects were given a minimum of one week to recover following surgical implantation of guide cannulae bilaterally into the basal forebrain. During the recovery period, animals had free access to water and food available *ad libitum*. Following this recovery period, subjects were returned to the water-restricted schedule and continued on the Sustained Attention Task training until their performance was comparable to pre-surgical performance (greater than 0.7 rH500 and 0.7 rCR) for at least three consecutive days.

**Infusion Procedure**

Infusions were made through the insertion of an internal cannula (28 gauge) that extended 3mm beyond the guide cannula, attached to a Hamilton syringe by polyethylene tubing. For each infusion, the right hemisphere was infused prior to the left hemisphere. The internal cannula was left in place for one minute following the
completion of each infusion to allow for drug diffusion (Zajo et al., 2015). Bilateral infusions of 0.5 µL saline (control) or 0.5 µL of 2 nM, 20 nM, or 40 nM TCS-OX2-29, diluted in DMSO, were administered to subjects at a rate of 0.5 µL/min immediately before beginning the Sustained Attention Task with Distracter. While not in use, TCS-OX2-29 solutions, which were aliquoted into single-use vials, were kept frozen at 4°C. Solutions were thawed immediately prior to use. Subjects were tested on Sustained Attention Task with Distracter sessions immediately following infusion.

These trials were conducted with at least one non-infusion, non-distracter day between trials to allow subjects to return to baseline performance between experimental trials. Dose order was randomized in order to minimize ordering effects. Each subject received a minimum of one sham infusion with distracter trial. Four subjects also completed distracter only (with no infusion) trials at the before the first infusion session and following the final infusion session. Sham and distracter only sessions were used to model potential effects of distracter adaptation and infusion-induced brain damage on attentional task performance. Subject 5502 was used as a surgical control. This rat received surgery to implant guide cannulae bilaterally into the basal forebrain, but did not receive any infusions, and therefore data from this subject is not included in statistical analysis of dose-dependent performance. The table below shows the in which each subject completed experimental trials. "Sham" denotes a saline infusion, and 'distr.' denotes that the subject performed the experimental task (Sustained Attention Task with Distracter) without any infusion.
Table 1. Trials for each subject in the order conducted.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Trial</th>
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<td>2</td>
<td>sham</td>
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<tr>
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<td>20</td>
<td>40</td>
<td>distr.</td>
<td>-</td>
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</table>

Performance Measures

Subject responses to each trial were recorded within the MED-PC IV software. Data were converted using MEDPC2XL into spreadsheet format for analysis. Once uploaded to Google Sheets, a macro (Appendix) was scripted and applied within the Google Sheets platform to calculate the relevant measures. These measures included: the number of hits (H500, H100, or H25, corresponding to number of hits on 500-ms, 100-ms, and 25-ms signal trials, respectively), misses (M500, M100, M25, corresponding to misses on 500-ms, 100-ms, and 25-ms signal trials, respectively), correct rejections (CR), false alarms (FA) and omissions. These measures were used to calculate fraction hits and ultimately, a Sustained Attention Test score (SAT) for each block of trials. All unscripted measures are described, with abbreviations, in Table 2.
Table 2. Performance measures, abbreviations, and formula for calculation for each measure examined by statistical analysis.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Abbreviation</th>
<th>Formula</th>
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<tr>
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<td>( \frac{H_{500}}{H_{500} + M_{500}} )</td>
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<tr>
<td>Fraction Hits at 100-ms</td>
<td>rH100</td>
<td>( \frac{H_{100}}{H_{100} + M_{100}} )</td>
</tr>
<tr>
<td>Fraction Hits at 25-ms</td>
<td>rH25</td>
<td>( \frac{H_{25}}{H_{25} + M_{25}} )</td>
</tr>
<tr>
<td>Fraction Hits Overall</td>
<td>rHO</td>
<td>( \frac{1}{3} \times (rH_{500} + rH_{100} + rH_{25}) )</td>
</tr>
<tr>
<td>Fraction Correct Rejections</td>
<td>rCR</td>
<td>( \frac{CR}{CR + FA} )</td>
</tr>
<tr>
<td>Fraction Correct Rejections for Block y</td>
<td>rCRY</td>
<td>( \frac{CR_y}{CR_y + FA_y} )</td>
</tr>
<tr>
<td>Fraction Hits for Block y</td>
<td>rHOy</td>
<td>Using only Block y trials: ( \frac{1}{3} \times (rH_{500} + rH_{100} + rH_{25}) )</td>
</tr>
<tr>
<td>Fraction Hits at x Signal</td>
<td>rHx</td>
<td>if ( x = 500 ), ( rH_x = rH_{500} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>if ( x = 100 ), ( rH_x = rH_{100} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>if ( x = 25 ), ( rH_x = rH_{25} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>if ( x = O ), ( rH_x = rHO )</td>
</tr>
<tr>
<td>Sustained Attention Test Overall</td>
<td>SAT</td>
<td>( SAT = \frac{(rHO - FA)}{(2 \times (rHO + FA) - (rHO + FA)^2)} )</td>
</tr>
<tr>
<td>Sustained Attention Test at y Block</td>
<td>SATBy</td>
<td>( SAT_{By} = \frac{(rHO_y - FA)}{(2 \times (rHO_y + FA) - (rHO_y + FA)^2)} )</td>
</tr>
</tbody>
</table>

Results

Analysis using repeated measures, three-factor (dose, signal duration and block number) repeated measures ANOVA showed a significant effect of block on \( SAT_{By} \) \( (\alpha = 0.05, \ df = 2, \ F = 5.057, \ p = 0.038) \), as well as a significant effect of signal duration on SAT score at each signal duration \( (df = 2, \ F = 5.057, \ p < 0.001) \).

Additionally, there was a significant effect of block \( \times \) dose on SAT score \( (df = 6, \ F = 6.242, \ p < 0.001) \). In order to isolate effects of block \( \times \) dose on \( rHO \) and \( rCR \), both components of the SAT score were analyzed. A repeated measures one-factor ANOVA showed that the effect of dose on \( rHO_y \) was not significant during
Block 1 ($F = 1.548, \ p = 0.253$), Block 2 ($F = 0.533, \ p = 0.668$), or Block 3 ($F = 2.279, \ p = 0.132$). Similarly, a repeated measures one-factor ANOVA showed that the effect of dose on \( rCRy \) was not significant during Block 1 ($df = 3, \ F = 1.328, \ p = 0.311$) or Block 2 ($df = 3, \ F = 1.026, \ p = 0.416$). The effect of dose on \( rCR3 \) was significant for Block 3 ($df = 3, \ F = 4.010, \ p = 0.034$). Taken together, these data suggest that for Blocks 1 and 2, both changes in \( rHOy \) and \( rCRy \) contribute to the significance of the block \( \times \) dose effect on SAT. In Block 3, the significance of the effect of dose on \( rCR3 \) suggests that changes in block \( \times \) dose dependent changes in correct rejections contribute more to the change in SAT than does \( rHO3 \).

Figure 1. The effect of TCS-OX2-29 dose on overall SAT score.

Although the SAT score is a useful, well characterized (McGaughy & Sarter, 1998) statistic for analyzing behavioral data on a sustained attention task, the formula for the score does not include an omissions factor. Thus, a subject can correctly respond to as little as a single cue, and then omit all additional trials, and have the same SAT score as an animal that responds correctly to each of the 162 trials. The dataset of experimental sessions did include trials with non-eligible omissions at all dose levels. A repeated measures ANOVA analyzing dose \( \times \) block showed that there was not a significant block-dependent increase in omissions ($df = 18, \ F = 3.715, \ p = 0.072$) nor
was there a significant effect of dose \times block on number of omissions \\
(df = 18, F = 2.359, p = 0.062). Both effects are approaching significance at \alpha = 0.05. 
An increase in omissions in later blocks is an expected effect observed in sustained 
attention task data in rats (McGaughy & Sarter, 1998), possibly due to decreases in 
motivation following each additional reward.

Omissions data is particularly relevant in the context of the orexinergic system, 
because orexins are involved in regulating sleep/wake cycles as well as feeding behavior 
(Ramachandran et al., 2012). In this study, sleep cycles and feeding behavior were not 
monitored. However, in the sustained attention tasks, shifts in the sleep/wake resulting 
in fatigued subjects, and a decreased thirst drive resulting from changes in feeding 
behavior could contribute to increased omissions in experimental trials. Because there 
was no significant difference in number of omissions between saline trials and 
TCS-OX2-29 trials, the omissions data suggest that there was not a significant increase 
in fatigue or significant decrease in thirst contributing to behavioral performance on the 
sustained attention task with distracter.

**Baseline Performance Analysis (Block 1 vs. Block 2)**

Baseline performance on the Sustained Attention Task was established for each 
experimental trial, using the values from Block 1. Comparing Block 2 values on 
rH$500$, $rH100$, $rH25$, $rHO$, $rHy$, and $SAT$ to those for Block 1 allowed control over 
day-to-day variation in subject performance on the task. Because ANOVA showed no 
significant difference in hits from a dose \times signal duration \\
(df = 2, F = 1.702, p = 0.164), all additional analyses used $rHO$ and $rHOy$.

Because Block 2 included a distracter, while Block 1 did not, analysis was 
conducted to examine dose-dependent differences between these two blocks. 
Accordingly, differences were computed for $(rHO1 - rHO2)$ and $(rCR1 - rCR2)$. A 
positive value from either of these difference computations would indicate that the 
subject performed better in Block 2 with distracter present than in Block 1 without 
distracter present. A repeated measures ANOVA showed no significant dose effect on
(rHO1 - rHO2) values $\alpha = 0.05$ ($df = 3, F = 3.049, p = 0.061$).

Figure 2. The effect of TCS-OX2-29 dose on rHO1 - rHO2. A positive value indicates that relative hits decreased from Block 1 to Block 2, while a negative value indicates that relative hits increased from Block 1 to Block 2.

**Distracter Adaptation and Infusion-Induced Brain Damage**

The flashing house light serves to produce a cognitively demanding condition that is thought to disrupt attentional performance in several neuropsychiatric conditions (Sarter et al., 2005). The improvement in performance as subjects are repeatedly exposed to the distracter during the sustained attention task has been examined by Kozikowski and Burk (2016). Results showed that subjects performed significantly worse than baseline during the first exposure to the distracter, and that this effect was not statistically significant after the first exposure to the distracter (Kozikowski & Burk, 2016). However, in the study by Kozikowski and Burk (2016), the flashing houselight distracter was present throughout the testing session, rather than during a single block. We intentionally limited distracter exposure to a single block in order to minimize distracter-related learning. Still, controlling for these repeated exposures to distracter should serve to normalize the data. Additionally, the physical damage that results from repeatedly inserting the internal cannulae to deliver TCS-OX2-29 could
cause damage to neurons in the infusion region of the basal forebrain, which may
decrease scores on performance measures including $rHxy$, $rCRy$, and $SAT$.

Combined, these effects may pose confounds to performance data from infusion
trials. A subject may improve in performance with a distracter present from the first
trial to the second trial according to a sigmoidal learning curve. Simultaneously,
repeated infusions may further impair the performance of a subject on the distracter
task according to an exponential decay function, independently of the effects of
TCS-OX2-29.

In order to evaluate the significance of these effects and isolate this influence on
the dataset, data from subjects receiving saline infusions on their second, third, fourth,
fifth, sixth and seventh exposures to distracter were compiled. After performing T-tests
to compare the rHx, rCR, and omissions values for subjects performing the distracter
task but receiving no infusion compared to subjects performing the distracter task after
receiving a saline infusion, and finding no significant difference between saline trials and
distracter-only trials, data from distracter-only trials were also incorporated into the
model.

**Fitting a Sigmoidal × Exponential Surface.** The learning curve is
predicted to be sigmoidal, while the effects of repeated infusions are predicted to follow
an exponential decay curve. Therefore, a sigmoidal × exponential surface fit is
appropriate. The general model, $(A \times exp(B \times i)) \times (C/(1 + exp(-D \times d)))$ was fit to the
data. The variables 'd' and 'i' correspond to the number of distracter exposures and the
number of infusions, respectively. The vertical axis lists the variable being plotted.
Figure 3. The effect of distracter exposure and repeated infusions on \( rHO1 \).

Coefficients (with 95% confidence bounds):

\[
\begin{align*}
A &= -0.826 \ (-5.531\times10^7, 5.531\times10^7) \\
B &= -0.1052 \ (-0.2635, 0.05316) \\
C &= -0.9769 \ (-6.542\times10^7, 6.542\times10^7) \\
D &= 0.2134 \ (-0.6467, 1.074)
\end{align*}
\]

Goodness of fit:

- SSE: 1.038
- R-square: 0.143
- Adjusted R-square: -0.01764
- RMSE: 0.2547
Figure 4. The effect of distracter exposure and repeated infusions on \( rHO2 \).

Coefficients (with 95% confidence bounds):

- \( A = 0.7785 \) \((-4.775e+07, 4.775e+07)\)
- \( B = -0.1941 \) \((-0.3974, 0.009121)\)
- \( C = 1.012 \) \((-6.21e+07, 6.21e+07)\)
- \( D = 0.2326 \) \((-0.7209, 1.186)\)

Goodness of fit:

- SSE: 1.02
- R-square: 0.2352
- Adjusted R-square: 0.09175
- RMSE: 0.2524
Figure 5. The effect of distracter exposure and repeated infusions on rHO3.

Coefficients (with 95% confidence bounds):

A = 0.4169 (-8.652e+07, 8.652e+07)
B = -0.2336 (-0.8265, 0.3594)
C = 0.414 (-8.593e+07, 8.593e+07)
D = 0.3882 (-6.055, 6.831)

Goodness of fit:

SSE: 0.43
R-square: 0.09938
Adjusted R-square: -0.06949
RMSE: 0.1639

**Fitting an Interpolated Cubic Spline Surface.** Given the R-square and adjusted R-square scores listed for Figures 3 through 5, the generated surfaces are poor fits for the data presented. It is general practice to fit according to the expected functions, rather than arbitrarily increasing polynomial degree to achieve the highest possible R-square or adjusted R-square, because doing so would increase the probability of Type I error. Theoretically, repeated exposures to distracter should result in a learning curve as the animals get used to the distracter, and repeated infusions should result in an exponential decay curve as the first infusion should cause more damage than the next, which should cause more damage than the next, and so on. However, the relationship may be more complex. It is possible that neuronal plasticity is allowing synapses to circumvent the infusion area, more quickly in some subjects than others. It is also possible that given the known homeostatic components influenced by the orexinergic system (for example sleep and feeding behavior), subject motivation and sleep/wake cycles may be fluctuating and affecting the \( rHOy \) data, in ways that cannot be predicted by a general sigmoidal \( \times \) exponential model.

The purpose of developing this distracter \( \times \) infusion surface model is to be able to predict what the performance of a subject would have been, had the animal received saline rather than TCS-OX2-29 during any given experimental trial. For this reason, a better fit than what was possible with the sigmoidal \( \times \) exponential model on the current dataset is necessary. Rather than arbitrarily assigning a higher order polynomial fit to the data, an interpolated fit has been selected to present a second model. Although the following figures present models with better goodness of fit values than the previously presented models, they do not attempt to assert any direct relationship between the variables. Rather, an interpolated surface was applied to establish a smooth gradation connecting each point.

According to Liu (n.d.), there are two relevant methods of interpolation: linear interpolation and cubic interpolation. A linear interpolation is useful for points related by a linear relationship, but for points close together, a linear spline interpolation cannot
capture the curvature of a function (Liu, n.d.). The first derivative of the function are discontinuous at the measured nodes (Liu, n.d.). This discontinuity at measured nodes may result in interpolated values near measured nodes that are more different (as a linear fit has been applied) than the predicted values would be in reality (Liu, n.d.).

The goal of cubic spline interpolation, on the other hand, is to obtain an interpolation formula that is continuous in both the first and second derivatives, both within the intervals and at the interpolating nodes, ultimately resulting in a smoother interpolating function (Liu, n.d.). For the context of distracter × infusion relative hits and omissions, a smooth function was preferred, therefore cubic spline interpolation was selected. Statistical analysis using both the sigmoidal × exponential fit and the cubic spline interpolation fit is included.

Figure 6. The effect of distracter exposure and repeated infusions on \( rH01 \), with a piecewise, interpolated fit.
Goodness of fit:

SSE: 0.659

R-square: 0.456

Figure 7. The effect of distracter exposure and repeated infusions on rHO2, with a piecewise, interpolated fit.

Goodness of fit:

SSE: 0.3928

R-square: 0.7054
Figure 8. The effect of distracter exposure and repeated infusions on \( rHO3 \), with a piecewise, interpolated fit.

Goodness of fit:

SSE: 0.05093

R-square: 0.8933

Both the sigmoidal × exponential fit and the interpolated fit were used to separately approximate ‘predicted’ values on \( rHOy \) for each (distracters, infusions) point. These predictors were intended to represent the values \( rHOy \) values that subjects would have received if, rather than an experimental trial, they had received a saline trial in its place. For example, subject 5701 received a 2 nM infusion of TCS-OX2-29 as the third exposure to distracter and second infusion. Based on the sigmoidal × exponential fit data, this suggests that had the subject received a saline trial as the third exposure to distracter and second infusion,
predicted \( rHO_1 = 0.4277861404 \), \( rHO_2 = 0.2619720761 \), \( rHO_3 = 0.02571390485 \).

Based on the interpolated fit, if the subject had received a saline trial as the third exposure to distracter and second infusion,

predicted \( rHO_1 = 0.425 \), \( rHO_2 = 0.2292 \), \( rHO_3 = 0.00129 \). The performance of subject 5702 with the 2 nM TCS-OX2-29 infusion was actually

\( rHO_1 = 0.74 \), \( rHO_2 = 0.45 \), \( rHO_3 = 0.22 \).

Figure 9. The effect of dose on \( rHO_1 \), with comparison of the sigmoidal × exponential prediction and the interpolated cubic spline prediction.

Figure 10. The effect of dose on \( rHO_2 \), with comparison of the sigmoidal × exponential prediction and the interpolated cubic spline prediction.
After completing predictions for all subjects corresponding to (distracter, infusion) values for each trial the subjects completed, a series of paired T-tests were completed comparing the experimental values to predicted values from both the sigmoidal × exponential and the interpolated fits. Notably, Figures 9 through 11 suggest that many of the experimental values of $rHO_y$ were significantly higher than the predicted values from both models. Analysis using paired T-tests, comparing actual values to predicted values with both models, showed that there was a significant difference in Block 2 between the interpolated $rHO_2$ and actual $rHO_2$ for the 20 nm dose ($p = 0.033$) and 40 nm dose ($p = 0.008$). These differences could not be computed using the sigmoidal × exponential fit for the 20 nm dose and the 40 nm dose because the standard error of the difference was 0. In Block 3, there was a significant difference between the interpolated $rHO_3$ and actual $rHO_3$ at the 0 nm dose ($p = 0.025$), at the 2 nm dose ($p = 0.018$), at the 20 nm dose ($p = 0.035$), but not at the 40 nm dose ($p = 0.080$). Using the sigmoidal × exponential fit, there was a significant difference between the predicted $rHO_3$ and
actual $rHO3$ at the 0 nM dose ($p = 0.025$), 2 nM dose ($p = 0.017$), and at the 20 nM dose ($p = 0.027$) as well. Again, there was no significant difference at the 40 nM dose ($p = 0.076$). In contrast to the traditional analysis using repeated measures ANOVA at the beginning of the Results section, which showed no significant dose dependent difference in relative hits at each block, all of the significant differences determined by these paired T-tests indicated dose dependent improvement with TCS-OX2-29 on the relative hits measure, compared to the predicted relative hits if saline had been infused. However, these contrasting results do not necessarily imply that either analysis technique is inaccurate. The relative hits measure, like the SAT score, can be artificially high in trials with significant omissions. If there is a dose-dependent effect of TCS-OX2-29 on the number of omissions, and if the number of omissions increases at higher doses of TCS-OX2-29, then it is possible that subjects are responding to fewer trials and therefore the relative hits values analyzed above are not truly representative of performance.

In order to evaluate this hypothesis, interpolant cubic spline fits were applied to omissions data from the model dataset, which was composed of only saline infusion and distracter only trials. A sigmoidal × exponential fit was not applied, because the number of omissions may not be related to a learning curve or damage to the brain region directly. Fatigue and changes in motivation due to administration of TCS-OX2-29, rather, may be responsible for the primary effect. The fits applied in Figures 12 through 14 all suggest a trend toward increased omissions with increased exposure to distracter and increased infusions.
Figure 12. The effect of repeated distracter exposure and repeated infusions on omissions in Block 1, using an interpolated cubic spline fit.

Goodness of fit:

SSE: 2421

R-square: 0.4487
Figure 13. The effect of repeated distracter exposure and repeated infusions on omissions in Block 2, using an interpolated cubic spline fit.

Goodness of fit:

SSE: 2611

R-square: 0.4696
Figure 14. The effect of repeated distracter exposure and repeated infusions on omissions in Block 3, using an interpolated cubic spline fit.

Goodness of fit:

SSE: 2605

R-square: 0.6436

These interpolations were used to predict the estimated number of omissions expected, if subjects had received a saline infusion in place of the corresponding TCS-OX2-29 experimental trial. The following figures show the predicted omissions compared to actual omissions.
Figure 15. Comparison of Interpolated Cubic Spline predicted omissions in Block 1, compared to measured omissions.

Figure 16. Comparison of Interpolated Cubic Spline predicted omissions in Block 2, compared to measured omissions.
Follow up analysis using paired T-tests showed that there was a significant difference in the predicted number of omissions and actual number of omissions in Blocks 2 and 3 at the 20 nM dose. At this dose, there were fewer than the predicted number of omissions. There was no significant difference in predicted number of omissions compared to actual omissions at the 0 nM (saline), 2 nM, and 40 nM doses Blocks 1, 2 or 3.

Discussion

Traditional Analysis

This study examined whether antagonism of OX2R bilaterally in the basal forebrain using TCS-OX2-29 could inhibit attentional performance via direct infusions into the basal forebrain. Analyses were conducted with traditional ANOVA testing for sustained attention task data, implemented previously by Kozikowski and Burk (2016) and Zajo et al. (2015). The results described suggest that there is a significant decline in the SAT score measure of performance dependent on TCS-OX2-29 dose and block number. Further analyses suggested trends that this effect was due both to the relative hits during each block and to the relative correct rejections at each block, however, the
dose × block effect was not significant on relative hits for any blocks, and was only significant during Block 3 for relative correct rejections. Baseline performance analysis showed that there was no significant effect of TCS-OX2-29 dose on subject performance differences between Block 1 and Block 2. Notably, several dose effects were approaching significance, including the block dependent increase in omissions ($F = 3.715, p = 0.072$), dose × block effect on number of omissions ($F = 2.359, p = 0.062$) and the dose effect on the baseline adjusted Block 2 relative hits ($F = 3.049, p = 0.061$).

The results of this well-documented method of analysis suggest that combined, block number and dose of TCS-OX2-29 may have an effect on SAT score performance by affecting both the fraction of hits on a task and the fraction of correct rejections. Additional trials to increase the power and sample size of the analysis are necessary to elucidate the effects of dose on number of omissions, dose × block on number of omissions, and dose on the relative hits in Block 2, adjusted for baseline performance from Block 1. The trend toward a dose × block dependent increase in omissions, while not significant, may suggest that the subjects experience a speed-accuracy tradeoff. Due to the antagonism of TCS-OX2-29 receptors in the basal forebrain, the neurons with orexin-2-receptors likely respond less frequently to local orexins A and B. Subjects may respond more slowly to stimuli and thus be scored with higher ’omissions,’ while maintaining similar relative hits values compared to their saline trials.

Exploratory Analysis

As described previously, an exploratory analysis was performed using models of the effect of repeated distracter exposures and repeated infusions on overall relative hits and omissions during each block. Two models, a sigmoidal × exponential fit and an interpolated cubic spline fit, were developed for the relative hits during each block, to allow for interpolation of relative hits during each block at combinations of distracter exposures and repeated infusions under sham infusion and distracter-only conditions. When analyzed in comparison to the predicted relative hits values, there was no significant improvement or decline in performance during Block 1, the well-trained
version of the task that corresponded to the sustained attention task without distracter that the subjects practiced daily. However, when compared with predicted performance using a sigmoidal $\times$ exponential and an interpolated model, subjects had higher relative hits values in Block 2, the more attention-demanding task, at 20 nM and 40 nM doses of TCS-OX2-29. Accordingly, TCS-OX2-29 does not appear to significantly impact relative hits in the first block, but may have an impact when attentional demands increase. An analogous conclusion was presented by Zajo et al. (2015), in a study that administered orexin A to improve attention performance. Improvement was significant only in Block 2, when attentional demands were increased using a flashing distracter.

In the final block, there was a dose dependent significant difference in correct rejections, and when both prediction models were used, there was also a significant difference in relative hits with the sham infusion, 2 nM, and 20 nM infusions. The number of omissions dependent on dose $\times$ block was approaching significance when analyzed using traditional ANOVA testing, and it is possible that increased omissions in Block 3 were artificially contributing to higher variation in relative hits scores, because a subject that responds to only a few trials can easily achieve a significantly higher relative hits value than a subject that responds to all the trials. Interpolated predictions of the expected number of omissions using saline and distracter-only trials suggested that at the 20 nM dose in both Blocks 2 and 3, subjects omitted significantly fewer trials than were predicted by the model. At other doses and in other blocks, there was no significant difference between the interpolated number of omissions compared to actual omissions.

Because there was no significant increase in omissions using this analysis method, the resultant findings using this exploratory analysis differ from the findings of the traditional analysis. According to this exploratory analysis, in both Blocks 2 and 3, subjects that received 20 nM TCS-OX2-29 infusions had decreased omissions compared to the interpolated number of omissions, and increased relative hits compared to the predicted fraction of relative hits using both the interpolated and sigmoidal $\times$ exponential models. At all other doses of TCS-OX2-29, there was no significant
difference in omissions. Thus, according to this exploratory analysis, subjects had increased values of relative hits during Block 2 with 20 nM and 40 nM infusions, with decreases and no changes in relative hits, respectively. In block 3, subjects had increased values of relative hits with 0 nM, 2 nM and 20 nM doses of TCS-OX2-29, with either no significant difference in, or decreases in, the number of omissions compared to the predicted omissions values.

This improvement on both relative hits and omissions measures is in contrast to the original hypothesis, that higher doses of TCS-OX2-29 infusion into the basal forebrain bilaterally would disrupt attention by antagonizing orexin-2-receptors, and does not support the speed-accuracy tradeoff explanation provided. Notably, allosteric sensitization of nicotinic receptors at following interaction with a subgroup of cholinesterase inhibitors, known as allosterically potentiating ligands (Maelicke et al., 2001). Following interaction at the nicotinic receptor, receptor sensitization occurs, which means that interaction of the receptor with the ligand, acetylcholine, is more likely to induce channel opening (Maelicke et al., 2001). Similarly, the interaction at orexin-2-receptors of TCS-OX2-29, with a relatively high dissociation rate of $k_{off} = 0.22/\text{min}$ and relatively short dissociation half life, may actually result in sensitization of the orexin-2-receptor, making it more likely to respond to binding of orexins A and B. Because the dissociation rate is high and the dissociation half life is short, the brief binding of TCS-OX2-29 at the orexin-2-receptor may serve to improve performance, by increase activity of these neurons rather than inhibiting activity.

Limitations

Because infusions must be carried out by physically inserting a cannula into the basal forebrain, brain damage following surgery and brain damage resulting from repeated infusions is inevitable. We attempted to minimize the damage by placing the guide cannula 3-mm above the target site. Additionally, placement of the guide cannulae can only be established based on typical location of the basal forebrain in the average rat (using relative distances from bregma and dura). Thus, cannula placement
cannot be confirmed until after completing experimental sessions. Further, there exists subject-to-subject variation in the number of OX2Rs in the basal forebrain, as evidenced by the ranges of OX2R receptors in the rat basal forebrain documented by Miettinen et al. (2002). Performance on the sustained attention task with distracter may also be affected by the amount of previous experience with distracter. A study conducted by Kozikowski and Burk (2016), in which a distracter was presented throughout the entire testing session, the effect of distracter exposure is only significant for the first experience with distracter. In this experiment, distracter exposure occurred only during Block 2 of the testing session. This was done explicitly to minimize the effect of distracter-related learning on the SAT scores, relative hits, and correct rejections. However, the trends depicted in Figures 3 through 8 do suggest that relative hits trended toward higher values with additional distracter exposures and fewer infusions. Finally, established methods of analysis measuring SAT score, relative hits, and relative correct rejections do not account for omissions, and thus a score on any of these measures can be artificially high if a subject correctly responds to a few stimuli and omits all additional trials.

Further Study

Additional research should, most importantly, include additional test subjects in order to increase power and sample size and minimize the probability that any differences between dose are due to chance. Increased sample size will simultaneously provide additional data to construct more robust sigmoidal × exponential and interpolated models of the combined effects of distracter exposure and repeated infusions bilaterally into the basal forebrain. Additionally, latency analysis should be conducted to determine if subjects were responding more slowly to stimuli in an TCS-OX2-29 dose-dependent manner. Histological analysis should be completed and guide cannula placement should be confirmed for each subject, to ensure that infusions of TCS-OX2-29 were administered to corresponding locations in each subject.

Patch clamp analyses of the orexin-2-receptor in the presence of TCS-OX2-29,
conducted according to the methodology described by Maelicke et al. (2001) would be beneficial to establish stronger understanding of the effect of TCS-OX2-29 on the orexin-2-receptor. An experiment using patch clamp could be used to determine if TCS-OX2-29, at doses corresponding to the 2 nM and 20 nM doses described in this study may potentiate the orexin-2-receptor. Additionally, such an experiment could provide valuable information regarding dose-dependent effects of TCS-OX2-29 on the orexin-2-receptor.

Finally, analysis measures should be developed and documented to account for omission rates in an overall performance score. One approach would involve analyzing total hits and correct rejections rather than calculating relative hits and relative correct rejections. However, this method would require hits, misses, correct rejections, false alarms, and omissions to all be analyzed separately, as none of these measures include information about any other measures, unlike the relative values (for example, relative hits contains information about the number of hits and the number of misses). Thus, it would be more difficult to account for interplay between these factors.

Alternatively, a composite score similar to the SAT score could be developed with an omissions factor included. However, considerations in developing such a score would need to include appropriately weighting the omissions factor. That is, if the entire SAT score were multiplied by \((1 - \text{omissions})/\text{total stimuli}\), a subject that completed a single correct hit and omitted the rest of the trials during Block 1 would have the same SAT score as a subject that performed pressed the ‘no signal’ lever in response to 26 of the 27 nonsignal trials. Also, adding an omissions factor would introduce the omissions error into the measure. Therefore, the definition of a new measure would need to be carefully assigned prior to using the score for analysis.
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The code listed below is compatible with Google Scripts for bulk data analysis on Training and Sustained Attention Task (with and without distracter) sessions using MEDPC2XL output with the "attentionask.mtp" settings file implemented. The code can output overall values for rH500, rH100, rH25, rCR, FA, and Omissions. Slight modifications to the provided code also allow the user to generate block-wise data.

```javascript
function dayRpt() {
    var s, data, i, rw=[], j, sum=[], test, k, rpt;
    s=SpreadsheetApp.getActiveSpreadsheet().getSheetByName("Raw");
data=s.getDataRange().getValues();
    for (i in data) {
        if (data[i][0]=="Subject") rw.push(Number(i));
    }
rw.push(data.length);
    for (i=0;i<rw.length;i++) {
        try {
            data[rw[i]][9]="Record: ";
            data[rw[i]][10]=data[rw[i]+1][0];
            data[rw[i]][11]=data[rw[i]+1][1];
j=rw[i]+5;
            sum=data[j];
j++;
            while (typeof(data[j][0])="number" & j<rw[i+1]) {
                for (k=0;k<sum.length;k++) sum[k]+=data[j][k];
j++;
            }
rpt=buildrpt(sum);
            for (k in rpt) data[rw[i]][12+Number(k)]=rpt[k];
s.getDataRange(rw[i]+1,1,1,data[rw[i]].length).setValues([data[rw[i]]]);
        }
    }
}
```
function buildrpt(sum) {
  var out=[]
  // overall hit values
  out.push(sum[3]/(sum[3]+sum[6]));
  out.push(sum[4]/(sum[4]+sum[7]));
  out.push(sum[8]/(sum[8]+sum[9]));
  [8]+sum[9]));

  // overall lat values
  out.push(sum[10]/(sum[10]+sum[13])); // h500 lat
function create() {
    var sheet1 = SpreadsheetApp.getActiveSpreadsheet().
        getSheetByName('Raw');
    var sheet2 = SpreadsheetApp.getActiveSpreadsheet().
        getSheetByName('Report');
    var dest;
    var rowCount = 1;
    var sheet1Length = sheet1.getLastRow();

    var range1 = sheet1.getRange(1,10);
    var recLine = range1.getValue();
    for (var j = 1; j < sheet1Length; j++) {
        range1 = sheet1.getRange(j,10);
        recLine = range1.getValue();
        if (recLine == 'Record: ') {
            var copyrange = sheet1.getRange(j,11,j,18);
            copyrange.copyValuesToRange(sheet2, 1, 8, rowCount+1,
                                          rowCount+1);
            rowCount++;
        }
    }
}