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Effects of Orexin A on Attention in Rats

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Effects of Orexin A on Attention in Rats

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ABSTRACT

Orexins are neuropeptides released from neurons that are primarily localized in the hypothalamus but which project to several brain regions including to the basal forebrain, a region known to be crucial for normal attentional performance in rats. Our previous research demonstrated that orexin receptor blockade impairs attention, while infusions of orexin A into the lateral ventricle enhance attentional performance. The goal of the present experiments was to test whether the attention-enhancing effects of orexin A could be reproduced by infusions directly into the basal forebrain and whether the basal forebrain cholinergic inputs to the medial prefrontal cortex are crucial to the performance-enhancing effects of orexin A. Male FBNF1 hybrid rats were trained in a sustained attention task that required discrimination of visual signals (500, 100 or 25-ms illumination of a central panel light) from trials when no signal was presented. In experiment 1, after stable performance levels were established, rats received guide cannulae implanted bilaterally into the basal forebrain. In experiment 2, rats received both intraventricular guide cannula implantation and infusions of either the immunotoxin 192-IgG-saporin or vehicle into the medial prefrontal cortex. Postsurgically, rats were trained in a version of the task which increased attentional demands by presenting a visual distracter during the middle block of trials within a testing session. In experiment 1, rats then received 0 (vehicle), 0.1, 1.0 or 10.0pM orexin A in a counterbalanced order prior to task performance. Following vehicle administration, attentional performance decreased from block 1 to block 2, when the distracter was presented. This distracter-induced impairment in accuracy was attenuated following administration of the highest orexin A dose. In experiment 2, rats received infusions of 0 (vehicle), 10, 100 or 1000pM orexin A in a counterbalanced order prior to task performance. Compared with sham-lesioned animals, animals with a loss of cholinergic projections to the medial prefrontal cortex showed decreased accuracy at the shortest signal duration when the distracter was presented. Infusions of 100pM orexin A attenuated these impairments following the distracter in lesioned rats. The present results provide additional support that orexin A can enhance attentional performance under certain conditions and suggest that the basal forebrain is one structure that can mediate the effects of orexin A on attention.
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Defining Attention

The construct of attention has been the subject of rigorous and multipronged investigation in psychological research over the past several decades. Although colloquial concepts of the meaning of attention abound, converging on a single definition has proved more difficult in empirical research. Broadly, “attention” refers to the ability to detect and organize salient stimuli while disregarding extraneous information (McGaughy & Sarter, 1995; Burk, 2004). A fundamental relation between attention and working memory has been described, such that the capacity and efficiency of working memory is significantly impacted by increases in attentional load (Awh & Jonides, 2001; Kane et al., 2001; Jha, 2002; Burk, 2004). Thus, attention may be conceptualized as a filter which regulates the selection of the most relevant information for a given situation to be evaluated within the limited capacity of working memory (Cowan, 1995; Burk, 2004). The development of adaptive attentional processing is complex, involving both automatic bottom-up processing of stimuli and more voluntary top-down control of the allocation of attentional resources (Knudsen, 2007). Bottom-up processing is the signal driven level of attention by which exposure to the stimulus enhances the ability to process the signals. In contrast, top-down modulation refers to the more cognitive aspects of attentional processing, reflected in the ability to respond appropriately to task rules and filter irrelevant stimuli based on knowledge or practice. Additionally, attention has been described as a set of several integrated processes, including
selective focus on particular stimuli, attentional shift, and sustained attention over time (Bushnell & Strupp, 2009). These various aspects of attention may be individually taxed to study their contributions to normal attentional processing, which may help to elucidate the mechanisms by which a host of neurological conditions exert domain-specific effects on these processes (Gitelman, 2003).

Measuring Attention in Rats

Several behavioral measures have been developed with explicit attention demands that can be varied to study the components of attentional processing. The most extensively tested measure of attention in rats is the 5-choice serial reaction time test, in which rats are placed in a chamber and cued by a brief visual signal to enter one of five ports in order to receive a reward of food or water access (Carli et al., 1983; Bushnell & Strupp, 2009). This task can measure both sustained attention—by varying the duration of the visual signal and delay before the cue is presented—and selective attention, by introducing a distracting stimulus during task performance.

An alternative paradigm, the sustained attention task (SAT), has been developed based on a taxonomy of sustained attention to specifically tap the parameters of sustained attention in rats (Parasuraman et al., 1987; Bushnell, Kelly, & Crofton, 1994; McGaughy & Sarter, 1995). In this two-choice visual discrimination task, animals are required to discriminate between signal trials, in which lever presentation is preceded by the illumination of a panel light for a variable duration
(500, 100, or 25 ms in our lab) from nonsignal presentations in which animals must respond to the levers in the absence of the illumination of the panel light. Unlike vigilance tasks with simultaneous presentation of stimuli, the sustained attention task utilizes targets which are changes in a repetitive event from a single location, a manipulation which has been shown to impose greater memory load (Parasuraman, 1987). Rats are trained to respond by pressing one lever in response to signal trials and the other lever in response to non-signal trials in order to receive a reward—typically delivery of a food pellet or controlled volume of water. Several dimensions of this task make it ideal for taxing sustained attention. The presentation of signal and nonsignal trials occurs in an unpredictable order and the time between trials can be varied. This combined with the ability to employ a dynamic range of stimuli rather than just one signal type increases vigilance demands in order to remain successful at the task (McGaughy & Sarter, 1995; Burk, 2004). Further, previous research has focused specifically on the impact of acute distraction on sustained attentional performance. Typically, this involves a manipulation in which the houselight in the testing chamber is flashed during some trials, which has been found to disrupt performance (Newman & McGaughy, 2008; Hirsh & Burk, 2013). Similar patterns of impairment have been observed during the distracter condition in a version of the sustained attention task adapted for use in measuring human attentional performance, suggesting that in both rats and humans, introducing a visual distracter requires the recruitment of additional attentional resources in order
to filter out the irrelevant stimuli and continue to attend to the target (Bushnell et al., 2003; Demeter et al., 2008).

Neural Mechanisms Underlying Attention

Some of the earliest work investigating the neural mechanisms responsible for attentional processing used electrode recordings to measure activity in the superior colliculus during shifts in visual attention in macaque monkeys (Wurtz & Goldberg, 1972). Subsequent innovations in imaging technology provided the opportunity for further exploration of brain regions active during attention demanding tasks, finding evidence of a role for both the posterior parietal lobe and the anterior cingulate in the neuroanatomy of attention (Posner & Petersen, 1990, Casey et al., 1997). The neural mechanisms required for normal attention performance are widely distributed throughout the brain. The basal forebrain, which has diffuse projections of both cholinergic and noncholinergic neurons throughout the brain, has been the subject of considerable focus for its role in attentional processing.

Although GABAergic and glutamatergic neurons greatly outnumber cholinergic neurons in the basal forebrain, this region serves as the primary extrinsic source of acetylcholine (ACh) throughout the cortex (Mesulam et al., 1983; Robbins et al., 1989; Sarter et al., 2001). The basal forebrain cholinergic system (BFCS) is composed of six distinct regions, termed regions Ch1-Ch6 (Mesulam et al., 1983). The Ch4 region of the BFCS contains the nucleus basalis magnocellularis (nBM) and
substantia innominata (SI) which are made up of a loosely clustered group of cholinergic neurons with diffuse projections to the prefrontal cortex (PFC) and other cortical regions (Mesulam et al., 1983). Using microdialysis, studies have confirmed that ACh levels in the rat cortex are elevated when animals are exposed to tasks which place explicit demands on attention compared with those that do not require sustained attention (Arnold et al., 2002; Sarter et al., 2001, 2005). In contrast, animals do not show an increase in cortical ACh release during performance on well-learned basic operant tasks, even when reinforcement rates were varied, presumably because the demands on attentional processing are lower in these tasks (Himmelhelber, Sarter, & Bruno, 1997).

Several studies have found these corticopetal cholinergic neurons in the basal forebrain to be necessary for normal attentional performance in rats. Excitotoxic lesions to the basal forebrain, for example, produce impairments in choice accuracy on the 5-choice serial reaction time test (Robbins et al., 1989). In a follow up study, Muir, Everitt, and Robbins (1995) found that treatments to restore cholinergic function with nicotine or the anticholinesterase physostigmine reversed these lesion-induced attentional deficits. Although this early work provided support for the growing body of literature implicating the basal forebrain in attention, the nonspecific nature of these lesions does not allow these attention mediating effects to be attributed exclusively to the loss of the cholinergic projections from the basal forebrain (Everitt & Robbins, 1997).
The development of immunotoxins provided the opportunity to investigate the contributions of individual neuron systems to attentional processing more discretely. The cholinotoxin 192-lgG-saporin has been particularly useful in isolating the role of the basal forebrain cholinergic system in attention. The action of 192-lgG-saporin is facilitated by the antibody 192-lgG, which targets the p75 nerve growth factor receptors expressed exclusively by the cholinergic inputs to the cortex from the basal forebrain, allowing saporin to be internalized and transported to the cell nucleus where it blocks protein synthesis within the ribosomes resulting in cell death (Wiley, Oeltemann, & Lappi, 1991). Injections of this cholinotoxin into the prefrontal cortex result in the selective destruction of these p75 expressing cholinergic neurons within the basal forebrain while leaving other neuronal systems intact (Holley, Wiley, Lappi, & Sarter, 1994). Experiments which have employed 192-lgG-saporin have found that when it is infused into the basal forebrain in varying doses, it produces a corresponding array of damage in which the severity of the damage predicted the degree of performance deficit on a 5-choice serial reaction time task (McGaughy, Dalley, Morrison, Everitt, & Robbins, 2002). Intrabasalis 192-lgG-saporin has also been found to decrease performance on the sustained attention task, specifically by decreasing accuracy in signal detection (McGaughy et al., 1996). Moreover, Holley, Turchi, Apple, and Sarter (1995) reproduced these decrements in signal detection accuracy utilizing intrabasalis infusions of benzodiazepine receptor agonists, which reduce the activity of basal forebrain cholinergic neurons resulting in diminished cortical ACh release (Sarter et al., 1990).
More intensive investigation of the cortical cholinergic projections from the basal forebrain has indicated that cortical acetylcholine release varies with attentional demand. Himmelheber and colleagues (2000) studied ACh efflux in the rat frontoparietal cortex using in vivo microdialysis as animals performed a sustained attention task. When a visual distracter was introduced during task performance, the rats showed an increase in cortical ACh efflux, presumably in response to the increased attentional demands created by the additional background noise. Rats trained on a version of the sustained attention task with minimal attentional demands do not show deficits in accuracy following loss of cortical cholinergic inputs. However, when multiple aspects of the task were manipulated to be more attention-demanding, rats with loss of basal forebrain corticopetal cholinergic neurons showed a significant decrease in signal detection (Burk, Lowder, and Altemose, 2008). Notably, only the manipulation of multiple task parameters to increase attentional demands—but not any one parameter alone—produced the observed disruptions in signal detection. Thus, no one parameter of the sustained attention task is independently crucial to observe deficits in attentional performance. Rather, multiple aspects of the task must be manipulated to be sufficiently attentionally taxing as to measure impairments in accuracy following lesions to the basal forebrain corticopetal cholinergic neurons. These findings provided support for previous reports that the efflux of cortical ACh facilitates the recruitment of additional attentional resources or increased attentional effort under more challenging conditions (Sarter, Gehring, & Kozak, 2006).
Regulation of basal forebrain neurons

Given that the cortical cholinergic transmission facilitated by basal forebrain activation is critical for normal attentional performance, understanding the regulation of firing rates of basal forebrain corticopetal cholinergic neurons is important. Basal forebrain neurons are innervated by numerous neurotransmitter systems, projecting from several regions throughout the brain. The relative contributions of some of these inputs, which include GABAergic and glutamatergic systems, have been studied (Sarter et al., 1999). However, one more poorly characterized input is the hypothalamic orexinergic projections to the basal forebrain corticopetal cholinergic neurons. As the primary neurological structure responsible for coordinating the brain’s ability to detect and respond to external cues, the hypothalamus may be of particular significance in mediating attentional performance. A group of hypothalamic neuropeptides, the orexins, (orexin A and orexin B, also known as hypocretin 1 and hypocretin 2) have become the focus of a growing body of research since their simultaneous discovery by two independent lab groups in 1998 (de Lecea et al., 1998; Sakurai et al., 1998). Although orexin neuron cell bodies are localized to the hypothalamus and contiguous perifornical area, they have projections throughout the brain, suggesting a role for orexin neuron activity in numerous physiological processes ranging from feeding to coordination of sleep/wake cycles (De Lecea et al., 1998; Sakurai et al., 1998). Orexinergic neurons have robust projections into the basal forebrain, forming synapses on the cholinergic as well as noncholinergic neurons in this region (Cullinan & Zaborszky, 1991). Using
immunohistochemistry, Fadel, Pasumarthi, and Reznikov (2005) provided evidence for the direct relationship between orexin fibers and cholinergic neurons, reporting that orexin-immunoreactive fibers were widely distributed throughout cholinergic regions of the basal forebrain and positioned closely to these cholinergic neurons. Two types of orexin receptors are expressed in the neurons of the basal forebrain, the orexin 1 receptor (Ox1R) which binds with stronger affinity to orexin A, and the orexin 2 receptor (Ox2R) which binds both orexin A and orexin B with relatively equal affinity (Sakurai et al., 1998). It is hypothesized that these receptor subtypes play unique, complementary roles in modulating the activity of the basal forebrain (Fadel & Burk, 2010). Administration of orexin A to the basal forebrain in rats produces a substantial efflux of ACh in the cortex that is not observed when orexin is applied to the prefrontal cortex directly (Fadel et al., 2005). Moreover, intrabasalis infusion of an OxR1 antagonist blocks stimulated ACh release (Frederick-Duus et al., 2007). Although the lack of a commercially available antagonist for OxR2 places limitations on the ability to disambiguate which receptor subtypes are most influential in the excitatory effects of orexin in the basal forebrain, the available research clearly indicates a link between orexinergic neurons and the stimulated release of ACh to the cortex by the basal forebrain.

Orexins and attention

Initial physiological and behavioral studies related to orexin function focused largely on the role of orexin activity in regulating feeding behavior (Sakuri et al.,
Mouse models which have been genetically engineered to be deficient in leptin, a peptide hormone which acts within the hypothalamus to suppress appetite (ob/ob knockout mice), demonstrate diminished levels of the orexin precursor preprohypocretin mRNA (Yamamoto et al., 1999). Recent research has become increasingly focused on the influence of orexin on wakefulness, arousal, and attention. Human narcolepsy is marked by a profound loss of orexin peptides (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000), and individuals with this condition have been found to demonstrate impairments in attentional processing even during periods of normal wakefulness (Nauman et al., 2006). Further, mice which have been genetically modified to lack orexin producing neurons or orexin receptors exhibit symptoms analogous to those of human narcolepsy (Yamanaka & Tsunematsu, 2010). The recent development of techniques in optogenetics has allowed researchers to modulate the activity of orexinergic neurons in freely moving animals. In these experiments, orexin neurons which have been injected with light-sensitive proteins can be instantaneously activated or silenced using flashes of light, thus providing a model for isolating the effects of orexin neurons without allowing for other compensatory neuronal mechanisms to develop (Inutsuka & Yamanaka, 2013). The selective photostimulation of orexin neurons promotes the transition from non REM or REM sleep to wakefulness and increases the amount of wakefulness time (Adamantidis, et al., 2007; Carter et al., 2009). Aged rats show a specific and sizable decrease in orexin expression and number of receptors, suggesting that a reduction in orexin levels may be also an
important contributor to the attentional deficits resulting from age-related cognitive decline (Porkka-Heiskanen et al., 2004; Kessler, Stanley, Frederick-Duus, & Fadel, 2011).

Several lines of research suggest that both orexin A and orexin B may be effective in enhancing attention. Transnasal administration of orexin A in rhesus monkeys reduced cognitive deficits related to sleep deprivation (Deadwyler et al., 2007). In rats, administration of orexin B to the prefrontal cortex enhanced performance on a task that places high demands on attentional processing (Lambe et al., 2005). In contrast, both central and intrabasalis infusions of the orexin-1 receptor antagonist SB-334867 have been found to disrupt different aspects of attentional performance (Boschen, Fadel, & Burk, 2009). Specifically, we have previously observed impaired detection of the longest signal duration following systemically administered SB-334867, whereas intrabasalis SB-334867 decreased overall accuracy on trials with longer signal durations (Boschen, Fadel, & Burk, 2009). Finally, orexin A administered centrally via the lateral ventricle (LV) leads to improvements in sustained attention task accuracy on trials that required animals to respond in the absence of a signal presentation, without producing enhancements on other measures of task accuracy (Hirsh & Burk, 2011).

Rationale for Present Experiments

Collectively, the available literature suggests that the integrity of both the BFCS and orexin producing neurons are essential to normal attentional processing.
Given the accumulating neuroanatomical and behavioral data implicating these systems in attentional performance, the goal of the present studies was to localize the attention enhancing effects of orexin A and to clarify the roles of the basal forebrain corticopetal cholinergic neurons in this process. Thus, the primary objective of experiment 1 was to investigate whether orexin A infused directly into the basal forebrain would produce similar patterns of attentional enhancement to those observed previously in our lab following central administration of orexin A (Hirsch & Burk, 2011). In Experiment 2, we further investigated whether basal forebrain cholinergic neurons are necessary to mediate the relationship between orexin A and attention by measuring attentional performance following central infusions of orexin A in rats with and without lesions to medial prefrontal cortical cholinergic inputs from the basal forebrain.

**Materials and Methods—Experiment 1**

**Subjects**

Male FBNF rats (N=6) approximately three months of age at the beginning of the experiment were used. The rats were individually housed in hanging wire cages in a vivarium which was temperature and humidity controlled and operated on a 14:10 hour light/dark cycle. All rats were water restricted throughout the experiment, receiving water during behavioral testing and for 30 minutes after each testing session. Rats were trained five to seven days a week, and received at least one hour of water access on days when no behavioral testing occurred. Food was
provided *ad libitum* throughout the experiment. Animals were treated in accordance with the guidelines of the Animal Care and Use Committee at the College of William and Mary.

**Apparatus**

Animals were trained in the same box, located within a sound attenuating chamber, throughout the experiment. One side of each box contained two retractable levers, a water port positioned between the levers with a delivery dipper for providing reinforcement (0.01 ml tap water) for correct responses, and three panel lights. One panel light was located above each lever, and the third was located above the water port. The center light above the water port was used for the purposes of this study. A house light was positioned on the opposite side of each chamber for providing increased background noise during some trial blocks and dim illumination throughout testing sessions. Behavioral testing programs were operated by a personal computer using the Med-PC version IV software.

**Behavioral training prior to orexin administration**

Training occurred between 9:00 a.m. and 12:00 p.m. daily. Training sessions lasted for 40 min or until rats had completed 162 trials. In the initial shaping procedure, rats were trained to press an extended lever using an FR-1 schedule of reinforcement. Once animals maintained 120 lever presses for a period of three days they were allowed to continue shaping. In the next shaping task, rats were
required to learn to discern signal (1-s illumination of the central panel light) from
nonsignal trials in which the panel light remained unlit. Presentation of signals and
nonsignals was pseudo-randomized across all 162 trials in a session. After a signal or
no signal, the rats were cued to respond by extension of the levers into the
chamber. Rules for training were counterbalanced, such that half of the rats were
reinforced (3-s access to 0.01ml tap water) for pressing the left lever in response to
a signal, which was recorded as a hit. A miss was recorded for right lever presses
after a signal trial. For nonsignal trials, a press of the right lever was considered a
correct rejection and water access was provided, while a press of the left lever in
response to no signal presentation was recorded as a false alarm. The inter-trial
interval (ITI) was 12-s during this stage of training. An incorrect response during this
training phase would be followed by a correction trial which was the same trial type
as that in which the error occurred. If the rat responded incorrectly for three
consecutive trials, a forced trial occurred in which only the correct lever was
extended into the chamber for 60-s or until the rat responded. The rules of the task
were reversed for the other half of the rats such that a right lever press was
considered a hit following a signal presentation whereas the left lever was
considered a correct response to a nonsignal trial.

During the final phase of behavioral training animals were required to
discriminate between shorter and variable signal durations (500ms, 100ms, 25ms)
and nonsignals presented in random order across signal trials, and the ITI was varied
(9±3 s). These changes to the signal duration and event rate were designed to
increase the attentional demand (Parasuraman et al., 1987; Koelega et al. 1990; Burk, 2004). Each training session was comprised of 162 total trials (81 signal, 81 non-signal). For the signal trials, each of the three signal durations was presented for 27 trials within a session. Trials were presented in blocks of 18 (9 non-signal, 9 signal, with 3 of each signal duration) and trial types were selected randomly without replacement.

**Surgical procedures**

For rats receiving intrabasalis orexin administration, after stable performance levels were established (>70% hits on 500ms signal trials and 70% correct rejections on non-signal trials), animals received a surgical procedure in which guide cannulae were implanted bilaterally in the basal forebrain. On the night prior to surgery, rats were provided with 2.7 mg/ml acetaminophen via their drinking water. Rats were anesthetized via ip injections of 90.0 mg/kg ketamine combined with 9.0 mg/kg xylazine. Once animals were sedated, the surgical area was shaved with an electric razor and rats were positioned in a stereotaxic device with the incisor bar set at 3.3 mm below the interaural line. All surgical procedures were conducted under aseptic conditions. Target coordinates for guide cannulae implantation were established using the stereotaxic atlas (-1.3mm anterior-posterior (AP) and ± 2.7mm medial-lateral (ML) from bregma, -4.2mm from dura). An incision was made down the midline, exposing the skull, and holes were drilled over the target sites for cannulae implantation. An eight millimeter guide cannula was inserted in each side of the
basal forebrain, with internal cannula extending 3mm beyond the guide cannulae during infusion. Three stainless steel screws and dental cement were also used to secure the cannulae. Dummy cannulae were inserted to prevent blockage within the guide cannulae. Following surgery, animals were given a one week recovery period in which food and water were available ad libitum. Rats were then returned to water restriction and began to retrain on the behavioral task. All animals were housed in plastic tubs with wire tops following surgery for the duration of the experiment.

Drug Administration Procedures

Rats were considered candidates to begin infusion procedures when presurgical performance levels were matched. Prior to drug infusions, animals received at least two sham infusions, after which they were exposed to an augmented version of the behavioral task in which the houselight at the back of the chamber was flashed during the second block of trials (1.0 s on / 1.0s off) to increase attentional demands. These sham sessions were designed to allow rats to become accustomed to the infusion process before drug administration. Each animal received four orexin doses: vehicle solution, 0.1pM, 1.0pM, 10pM, infused in random order bilaterally into the basal forebrain. These doses were chosen because they fall within the range known to be sufficient to produce stimulation of cortical acetylcholine release when infused into the brain, and the ten-fold increase in dose was expected to generate measurable differences in the magnitude of effects (Fadel, Pasumarthi, & Reznikov, 2005). Infusions were made through the insertion of an
internal cannula attached to a Hamilton syringe by polyethylene tubing. A total volume of 0.5 μl solution was infused into each cannula at a rate of 1.0 μl/min (Hirsh & Burk, 2011). The internal cannula was left in place for one minute following the completion of each infusion to allow for drug diffusion. Animals were then immediately loaded into the chambers to begin behavioral testing. At least one day of training was allowed between each testing session to re-establish baseline performance. Figure 1 illustrates approximate sites of basal forebrain infusions.

**Histological procedures**

After being deeply anesthetized via an ip injection of 90 mg/kg ketamine and 9.0 mg/kg xylazine, rats were transcardially perfused with 10% sucrose followed by 10% formalin at a pressure of 300mmHg using a Perfusion One apparatus. The brains were then harvested and placed in formalin for 48 hours before being put into a 30% sucrose solution in phosphate buffered saline for at least three days. The tissue was then sectioned in 50μl slices using a freezing microtome. Sections nearest the cannula sites were stained using cresyl violet and viewed under a microscope to assess cannula placement.

**Materials and Methods—Experiment 2**

**Subjects**

Male FBNF rats approximately three months in age at the beginning of the study were used. The rats were housed under similar conditions as in experiment 1.

**Surgical Procedures**
Presurgical training, determination of surgical candidacy and surgical preparations were identical to those in experiment 1. Target coordinates for implantation of a single guide cannula into the lateral ventricle were established using the stereotaxic atlas (-0.8mm AP, 1.6mm ML, -2.5mm DV from bregma). The hemisphere in which the cannula was implanted was counterbalanced, such that half of the animals received a cannula in the left hemisphere and half received a cannula in the right hemisphere. An eight millimeter guide cannula was inserted into the lateral ventricle, with internal cannula extending 1mm beyond the guide cannula during infusion (Figure 2). Three stainless steel screws and dental cement were again used to secure the cannula. During the same surgery, rats received infusions of either the immunotoxin 192-IgG-saporin (0.2 μg/μl; 0.5μl per site) or saline into the medial prefrontal cortex (3.7mm AP, 0.7mm ML, -3.5 mm from dura; 2.6 mm AP, 0.7mm ML, -3.5 mm from dura). Postsurgically, animals were again given a one week recovery period during which food and water were available ad libitum, and then returned to water restriction and retrained on the behavioral task.

Postsurgical Behavioral Testing Procedures

Following the recovery period, all animals completed 15 sessions on the same version of the sustained attention task on which they were trained prior to surgery in order to assess any effects of lesion on performance before exposure to drug infusions. Performance was averaged across blocks of 3 sessions, thus a total of five 3-session blocks were used for subsequent analysis.

Drug Administration Procedures
Drug infusion procedures were performed similarly to those in experiment 1, except that the drug doses were increased (0, 10pM, 100pM, 1000pM) to account for infusion into the LV instead of directly into the basal forebrain. On sham and drug infusion sessions, rats were tested in a version of the sustained attention task with the flashing houselight distracter presented during the second block of trials (trials 55-108) within the session.

**Histological Procedures**

After completion of all behavioral testing, rats were deeply anesthetized and transcardially perfused using 10% sucrose followed by 4% paraformaldehyde. The harvested brains were placed in the same paraformaldehyde fixative for at least 24 hours before being placed in a 30% sucrose phosphate buffer for cryoprotection and refrigerated. Sections (50μM) from the prefrontal cortex region as well as those near the cannula site were preserved in an antifreeze solution until they underwent acetylcholinesterase (AChE) fiber staining. AChE histochemistry was performed on frozen tissue using previously described procedures (Tago et al., 1986; McGaughy et al., 1996). Brain tissue sections were first rinsed in a 0.1 M phosphate buffer solution and then incubated in hydrogen peroxide for 20 minutes. Sections were then rinsed in maleate buffer before being immersed in a solution composed of 0.5 ml of 0.1 M sodium citrate, 1.0 ml of 5 mM potassium ferricyanide, 1.0 ml of 30 mM cupric sulfate and 10.0 mg of acetylthiocholine into 197.5 ml of 0.1 M maleate buffer for 30 minutes. After rinsing in 50.0 mM Tris buffer (pH 7.6), sections were incubated for 10 minutes in the solution prepared using the provided instructions in
a diaminobenzidine (DAB) kit. At the end of the incubation period, drops of 0.1% hydrogen peroxide were added to the sections until tissue staining was complete. Brain tissue sections were mounted on gelatin coated slides and allowed to dry. Slides were then dehydrated and coverslipped.

**Behavioral Measures and Statistical Analyses**

Performance accuracy in the sustained attention task is determined through the assessment of hits, misses, correct rejections, and false alarms. The number of hits (H), misses (M), correct rejections (CR), false alarms (FA) and omissions were recorded for each animal during each testing session. The relative number of hits and correct rejections per block and for the overall testing session were calculated as \( \frac{H}{H+M} \) and \( \frac{CR}{CR+FA} \) respectively. The range of scores for relative hits is from 0 (the animal pressed the correct rejection/miss lever every time a signal was presented) to 1 (the animal pressed hit/false alarm lever following each signal presentation). The range of scores is the same for relative correct rejections, with the opposite lever being pressed in response to no signal presentation for values of 0 and 1. For the distracter task, all measures were calculated for the entire session, as well as for each of three 54-trial blocks within a session. To determine the effect of the distracter for each rat, a distracter score was calculated, reflecting the difference between all behavioral measures between block 1 (standard task with no distracter) and block 2 (distracter presentation). Thus, positive values indicate greater accuracy during block 1, whereas negative values indicate greater accuracy.
during block 2 and a greater difference in accuracy between block 1 and block 2 is indicated as values become increasingly positive or negative.

The overall SAT accuracy, which takes into account performance on both signal and non-signal trials, was also calculated for each animal using the formula SAT=((H-FA)/(2x(H+FA)-(H+FA)^2)) (McGaughy et al., 1996; Boschen et al., 2009). The range of scores for the SAT measure is from -1 to 1, where a score of 1 indicates 100% correct responses on signal and non-signal trials and a value of 0 indicates an inability to discriminate between signal and non-signal trials. Analyses of omissions were conducted separately from those of hits and correct rejections. Data were analyzed with SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). A level of α=0.05 was used as the criterion for statistical significance.

**Histological Analyses**

AChE-positive fibers were quantified in the prelimbic/infralimbic (PI/IL) region of the cortex as well as the primary motor (M1) regions in sections from both lesioned and sham lesioned animals. AChE-positive fiber density was quantified using a modified counting grid method described by Burk, Lowder, and Altemose (2008). Using an Olympus BX-51 light microscope with an objective lens magnification of 40X, three lines that bisected each other at the midpoint were placed over the image using the Grid Mask function in ImagePro Discovery. Grid parameters were set to a radius of 400 with segments of 10. Image size was set to 50%. Each time a fiber crossed the lines was counted and a percent loss was
determined. Figure 3 includes images representative of sections from lesion and sham-lesion groups.

Results – Experiment 1

Six rats maintained stable task performance throughout postsurgical testing and had cannula placement confirmed by identifying tissue loss and gliosis indicating the location of the guide cannulae. Intrabasalis orexin A did not affect performance on the standard task without the distracter (block 1). A dose (0, 0.1, 1, and 10pM orexin A) x signal duration (500, 100, and 25ms) ANOVA was conducted for the SAT measure which yielded no significant effect of orexin A dose. A similar set of analyses was conducted for the relative hits, as well as nonsignal trials and omission rates. These analyses also yielded no significant effect of drug dose on any of these measures during the first block of trials (Figure 4).

Distracter scores (b1-b2) were calculated for each rat for both relative hits and the SAT measure to assess the effects of the distracter. This measure indicates the impairment in performance in response to the visual distracter presented during block 2, with lower scores representing less of a decrease in attentional performance between the first and second block of trials. A dose (0, 0.1, 1.0, 10pM orexin A) x signal duration (3 levels) yielded a significant dose x signal duration interaction (F(6,30) = 2.866, p = 0.025) for the difference score SAT measure. Follow up analyses revealed a significant main effect of dose at the 500ms level (F(3,15)= 6.697, p = .004), but not at the 100ms or 25ms signal durations (Figure 5). For hits,
this effect was mirrored by analyses of accuracy on signal trials. For hits, there was a significant dose x signal duration interaction for the distracter score ($F(6, 30) = 2.689$, $p=0.033$). Follow up analyses again revealed a main effect of dose at the 500ms signal duration ($F(3, 15) = 6.697$, $p = .004$), but not at the 100ms or 25ms levels (figure 6). Further analyses via paired sample t-tests compared each orexin A dose with vehicle administration. Performance following 10 pM orexin A was significantly different compared to vehicle at the longest (500 ms) signal duration ($t(5)=2.766$, $p=0.040$). Thus, at the longest (500 ms) signal duration, the 10pM orexin A dose decreased the distracter score for relative hits (b1-b2) compared with vehicle administration, suggesting that orexin A infusions decreased distractibility at this dose. There were no significant effects of orexin A dose on correct rejections or omissions for the distracter score.

Finally, dose x signal duration ANOVAs were conducted for the SAT measure, relative hits, correct rejections, and omissions on the standard task following the distracter (block 3). These analyses also yielded no significant effect of orexin A dose during the third block of trials (Figures 7, 8).

Results – Experiment 2

Histological Analyses

One lesioned animal was dropped from analysis due to difficulties with histological assessment. All remaining lesioned animals and a subset of the sham-lesioned animals were included in subsequent histological analysis. On average,
lesioned animals showed a 60.8% loss of AChE-positive fibers compared to sham
lesioned animals. A t-test comparing the mean AChE fiber counts from the medial
PFC and M1 regions was conducted, and results from this analysis confirmed that
lesioned animals showed significantly fewer AChE fibers in the prelimbic portion of
the medial prefrontal cortex than sham-lesioned animals (t(14)=10.769, p < .001;
mean ± SEMs for AChE-positive fiber counts: sham-lesioned animals, 61.18 ±3.61;
lesioned animals, 23.98 ±1.62). These analyses yielded no significant differences in
AChE-positive fiber counts in the primary motor cortex (t(14)=.268, p=.792; mean ±
SEMs for AChE-positive fiber counts: sham-lesioned animals, 61.59 ± 2.79; lesioned
animals, 60.68 ± 2.07).

Performance prior to orexin A infusions

The data reported here are from the 19 rats (10 lesion, 9 sham-lesion) which
maintained stable performance levels across infusion sessions. No significant
differences were observed in performance between lesion and sham-lesion groups
on the three days prior to surgery. A repeated-measures block (5 levels) x signal
duration (3 levels) x lesion (2 conditions) ANOVA on the relative hits, correct
rejections, and omissions during the first 15 days of training on the standard task
following surgery found a main effect of signal duration on relative hits (F(2,34)=
288.35, p < 0.001). Rats exhibited signal duration dependent accuracy, with higher
hit rates following longer signal durations. These analyses yielded no significant
effect of lesion on any measure. To further confirm there were no differences
between sham-lesioned and lesioned rats prior to drug exposure, the first and last
three sessions of the 15 day training period were assessed, and revealed no significant group differences in accuracy on relative hits, correct rejections, or omissions.

*Effects of Orexin A Infusions on attention task performance*

Following drug infusion, omissions increased substantially at the highest orexin dose (1000 pM), rendering the measures of accuracy difficult to interpret. Therefore, task performance was subsequently analyzed following 0 (vehicle), 10pM and 100pM orexin A infusions. The overall SAT measure, taking into account performance on both signal and non-signal trials was calculated for all animals and these values were analyzed using lesion (2 conditions) x dose (0, 10, 100pM orexin A) x signal duration (3 levels) ANOVA for the first block of trials. This analysis yielded a significant lesion x dose x block interaction (F(4.64) = 2.983, p = 0.025) for the SAT measure during block 1. The basis for this interaction appeared to be differences at the 25ms signal duration, however follow up analyses did not reveal any statistical differences between lesioned and sham-lesioned groups at this level. A similar analysis was conducted for relative hits, correct rejections, and omissions during block 1 of trials and yielded no significant effects (figures 9, 10). The distracter score (b1-b2) was calculated for each behavioral measure similarly to experiment 1. For SAT, a lesion (2 conditions) x dose (0, 10, 100pM orexin A) x signal duration (3 levels) ANOVA yielded a lesion x dose x signal duration interaction which was approaching significance (F(4,64) = 2.313, p = 0.067). For relative hits, these analyses revealed a significant lesion x dose x signal duration interaction (F(4,68) = 2.656, p = 0.040) for
the distracter score. This interaction was further assessed by conducting dose X lesion ANOVAs at each signal duration. For the distracter score for relative hits, there was a significant dose x lesion interaction \(F(2,34) = 4.965, p=0.013\) at the 25ms signal duration, but not on 500-ms or 100-ms signal trials (figure 11). This dose X lesion interaction was further assessed with separate one-way ANOVAs for dose for sham-lesioned and lesioned animals. No significant effects of dose were observed for the sham-lesioned animals \(F(2,16) = .676, p = .523\). There was, however, a significant main effect of dose on accuracy at the 25ms signal duration for the lesioned group \(F(2,18) = 6.922, p = 0.006\). To clarify the nature of this effect, follow up analysis via paired sample t-tests comparing each orexin A dose with vehicle administration for the lesioned animals were conducted. These analyses yielded a significant difference in the b1-b2 scores following vehicle versus 100 pM orexin A \(t(9) = 2.973, p = 0.016\). Thus, at the 25 ms signal duration, the 100pM orexin A dose decreased the distracter score for relative hits compared with vehicle administration, indicating that the lateral ventricle infusions of 100pM orexin A may reduce distractibility at the 25ms level. There were no significant effects of orexin A dose on correct rejections or omissions for the distracter score.

Finally, dose x signal duration ANOVAs were conducted for the SAT measure, relative hits, and correct rejections, and omissions on the standard task following the distracter (block 3). These analyses also yielded no significant effect of orexin A dose during the third block of trials (figures 12, 13).
Discussion

*Effects of intrabasalis orexin A infusions*

The present experiments tested whether the attentional enhancements produced by orexin A are mediated by the basal forebrain. Effects of the distracter were determined by distracter scores that compared differences in accuracy on block 1 to block 2 of sessions. Infusions of orexin A directly into the basal forebrain diminished the impairment in performance in response to the greater attentional demands presented during the distracter task, particularly at the highest dose and at the 500ms signal duration. Sarter et al. (2005) identified two distinct mechanisms which are involved in the activation of the cortical cholinergic system during attention demanding tasks. Signal driven processing reflects the bottom-up level of processing by which the exposure to the stimulus enhances the ability to process the signals, while cognitive modulation of signal detection reflects the ability to respond appropriately based on task rules. Boschen et al. (2009) found that intrabasalis administration of the selective orexin-1 receptor antagonist SB-334867 resulted in a pattern of impairments only at the longest signal duration. Boschen et al. (2009) interpreted this finding to reflect an impairment in activating the appropriate rules after signal presentation. Similarly, intrabasalis orexin A may have enhanced the ability to generate the correct signal guided response without significantly impacting signal driven processing. Enhancements were observed only on distracter scores in the present experiment, suggesting that these beneficial effects occur primarily under conditions that increase demands on attention.
**Effects of LV orexin A infusions in sham-lesioned animals**

Overall, no significant effects of LV orexin A infusions were observed in the sham-lesioned animals. Previous findings in our lab suggest that orexin A infused into the lateral ventricle could be expected to produce an increase in accuracy on correct rejections in intact animals (Hirsh & Burk, 2011). In the current study, rats reached higher levels of accuracy on nonsignal trials during the training period before beginning infusions (correct rejections following vehicle administration, 74.3% in the previous study vs. 82% in the present experiment). Thus, one possible explanation is that the higher levels of training and performance accuracy at baseline among animals in the present experiment may have limited our ability to observe noticeable changes in performance above and beyond their baseline performance. Alternatively, it may be that, in the previous experiment, the rats were still learning the task and that orexin A has more beneficial effects on learning. Previous research has demonstrated that LV infusions of orexin A can enhance learning in a passive avoidance task (Telegdy & Adamik, 2002), whereas administration of an orexin A antagonist to the ventral tegmental area (VTA) impairs learning of reward-associated cues (Harris et al., 2007). Additionally, future research could investigate whether orexin A enhances learning through overlapping neural circuits compared with any effects on attention.

**Effects of loss of mPFC cholinergic inputs**
Lesions of the mPFC cholinergic inputs did not significantly affect standard task performance. Under the distracter condition, sham-lesioned rats demonstrated an increase in accuracy at the 25ms signal duration from block 1 to block 2, presumably because the houselight flashes during the second block of trials instead of remaining consistently illuminated resulting in some of the 25ms signal presentations occurring when the chamber is dark. Thus, during the distracter task, some of the brief 25ms signals may be easier to discern than during the standard task. Lesioned animals demonstrated a decrease in accuracy on 25-ms signal trials when the distracter was presented, suggesting that loss of mPFC cholinergic inputs may impair the ability to fully take advantage of this benefit of the distracter at the shortest signal duration. However, following administration of orexin A, lesioned animals were able to recover a pattern of accuracy similar to that of animals with intact mPFC cholinergic systems. For lesioned rats, orexin A did not affect performance at the 500ms or 100ms signal durations. Taken together, these results suggest that intraventricular orexin A may enhance attentional performance at the shortest signal durations by altering the rats’ criterion for identifying events as signals, which would be expected to be most salient at the 25ms level when it is the most difficult to discern signal from nonsignal presentation.

Limitations and Future Directions

The present study utilized a well-defined test of attention to measure enhancements in attentional processing, however a number of important limitations
should be considered. Of course, infusion studies convey an inherent risk of affecting circuitry outside of the intended target. The mRNA for both Ox1 and Ox2 receptors is widely distributed throughout the brain, and orexins have been shown to produce excitatory actions on many neuronal systems in addition to the BFCS, including noradrenergic locus coeruleus neurons, histaminergic neurons, and cholinergic mesopontine neurons (Prashant et al., 1998; Bayer et al., 2004). Infusions into the lateral ventricle allow the spread of orexin A to diffuse brain regions, thus, the effects of the drug on attentional performance cannot be attributed exclusively to its interaction with the basal forebrain. Although intrabasalis infusions of orexin A allow for the more specific targeting of the region of interest, orexin receptors are found on both cholinergic and non-cholinergic neurons within the basal forebrain. Future studies should be directed at clarifying the neuroanatomical circuitry underlying the effects of orexins in the basal forebrain by examining orexin A induced changes in attentional performance in animals with lesions specific to the noncholinergic basal forebrain neurons.

Histological analyses of AChE-positive fibers indicated that there was a significant, but not total, loss of cholinergic projections to the prelimbic region of the cortex. Thus, it is likely that some cholinergic transmission is still occurring in this region via these intact neurons. It is unknown whether the attention enhancing effects of orexin A in experiment 2 can be attributed to greater activation of the remaining cholinergic neurons or the interactions of orexin with other neuronal systems. Moreover, the potential compensatory processes to provide some
functional recovery following loss of cholinergic projections were not characterized in the present experiment. Infusions of orexin A have been shown to increase ACh efflux within the PFC (Fadel et al., 2005). A microdialysis study would be useful in confirming the effects of the lesions produced by the selective cholinotoxin 192-IgG-saporin on ACh availability.

Collectively, the results of the present study contribute to the existing body of literature indicating the effectiveness of the distracter condition on the SAT task in impairing attentional performance. Further, although these results differ somewhat from previous findings in our lab in which the enhancements were observed exclusively on nonsignal trials, the present data are consistent with the idea that orexin A administration improves performance on a task specifically designed to tax sustained attention. Our results indicated that some of the attention-enhancing effects of orexin A may be mediated by the basal forebrain cholinergic system, however, it is likely that other noncholinergic neuronal systems also interact with orexin to produce its effects. Future studies should be aimed at gaining a better understanding the clinical utility of the effects of orexin A. Finally, it is important to note that although promising, the size of the attention-enhancing effects of orexin A is small. While our results contribute to the existing literature suggesting that orexins are involved in the modulation of normative attentional performance, further research is needed to determine the clinical utility of orexin A and investigate more viable mechanisms for administration in human populations.
Figure 1. Cannula placements within the basal forebrain at -1.3 mm to bregma. Circles indicate the location of the infusion cannula, which extended 3 mm beyond the guide cannula.
Figure 2. Cannula placement for lateral ventricle infusions (0.8mm AP, 1.6mm ML, -2.5mm DV from bregma), with internal cannula extending 1mm beyond guide cannula.
Figure 3. The figure depicts acetylcholinesterase (AChE)-positive fiber staining in sham-lesioned and lesioned tissue. The column on the left contains images from a sham-lesioned animal, and the right column (b., d.) are from a lesioned animal. Images in the top row (a., b.) are from sections in the primary motor cortex (M1), whereas images in the bottom row (c., d.) are from sections in the medial prefrontal cortex (mPFC). Lesioned animals exhibited a significant loss of AChE-positive fibers in the infralimbic region compared to sham-lesioned animals.
Intrabasalis orexin A
Hits Block 1

Figure 4. Attention task performance following intrabasalis administration of orexin A (0.0, 0.1, 1.0, 10.0 pM, n=6). There were no effects of drug dose on relative hits during block 1 of the attention task. Error bars represent standard errors of the mean.
Figure 5. SAT measure, which takes into account accuracy on trials both with and without signal presentation, for difference scores (Block 1-Block 2). Intrabasalis orexin A at the highest (10pM) dose significantly reduced the measure of overall distracter-induced impairment at the longest (500ms) signal duration. Error bars represent the standard error of the mean.
Intrabasalis Orexin A
Difference Scores Relative Hits

Figure 6. Attention task performance following intrabasalis administration of orexin A (0.0, 0.1, 1.0, 10.0 pM, n=6). Compared to vehicle, intrabasalis orexin A (10.0 pM) significantly reduced the decrease in accuracy on relative hits from block 1 to block 2 in which the distracter was presented at the longer signal duration (p<0.05). Error bars represent standard errors of the mean.
Figure 7. Attention task performance following intrabasalis administration of orexin A (0.0, 0.1, 1.0, 10.0 pM, n=6). There were no effects of drug dose on relative hits during block 3 of the attention task. Error bars represent standard errors of the mean.
Figure 8. Attention task performance following intrabasalis administration of orexin A (0.0, 0.1, 1.0, 10.0 pM, n=6). Correct rejections were unaffected by intrabasalis orexin A at any dose.
Figure 9. Attention task performance following lateral ventricle administration of orexin A (0.0, 10.0, 100pM). There were no significant effects of drug dose on accuracy at any signal duration for sham-lesioned animals (n=9).
Figure 10. There were no significant effects of drug dose on relative hits at any signal duration during block 1 for animals with 192-IgG-saporin induced lesions to the medial prefrontal cortex (n = 10).
Figure 11. Animals with 192-IgG-saporin induced lesions exhibited a shift in the pattern of accuracy changes from block 1 to block 2 for relative hits following infusions of the highest orexin A dose. The performance of sham-lesioned animals was not affected by orexin A at any dose. Error bars represent standard error of the mean.
Figure 12. There were no significant effects of drug dose on the accuracy of sham-lesioned animals during standard task performance on the third block of trials following the distracter task. Error bars represent the standard error of the means.
Figure 13. There were no significant effects of drug dose on the accuracy of sham-lesioned animals during standard task performance on the third block of trials following the distracter task. Error bars represent the stand error of the mean.
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