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Neurochemical and Behavioral Outcomes of Intranasal Orexin Administration in Young and Aged Animals

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NEUROCHEMICAL AND BEHAVIORAL OUTCOMES OF INTRANASAL OREXIN ADMINISTRATION IN YOUNG AND AGED ANIMALS

by

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DEDICATION

"We cannot teach people anything; we can only help them discover it within themselves." –Galileo Galilei

I would like to dedicate these works to my wife, Meredith. Without your love and support, I fear none of this would have been possible. And to my brother, Blake, who has taught me the power of perseverance. I am forever grateful. Thank you both for taking this journey by my side to help me discover myself.

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ABSTRACT

Cognitive function represents the most important determinative factor for independent functioning in the elderly. As the existing pharmacotherapeutic tactics for treating Alzheimer's disease (AD) provide only modest benefits, novel treatment options are urgently needed. The hypothalamic orexin (hypocretin) system, a central integrator of physiological function, is anatomically and functionally positioned to modulate cognition. Indeed, mounting evidence suggests that a loss of orexin neurons and/or their peptides and receptors may underlie the cognitive decline observed during aging and AD. The current paucity of orexin receptor agonists has hindered the ability to study their potential as cognitive enhancers. Intranasal administration of orexin peptides circumvents these issues and others involving peptide delivery into the CNS. To investigate the overarching hypothesis that intranasal orexin administration improves the anatomical, neurochemical, and behavioral substrates of age-related cognitive dysfunction, the proceeding studies utilized a rodent model of aging in combination with acute intranasal administration of saline, orexin-A or the modified orexin-B analog [Ala^{11}, D-Leu^{15}]-orexin-B. These results highlight that intranasal OxA (5nmol) increases c-Fos expression, a marker for neuronal activation, in various telencephalic brain regions, including various subdivisions of the frontal cortex and the basal forebrain cholinergic system. Importantly, these brain regions are known to modulate attention and learning and memory in both young and aged subjects. Conversely, administration of intranasal [Ala^{11}, D-Leu^{15}]-orexin-B (5nmol), an orexin-2 receptor (Ox2R) selective peptide, elicited increases in c-

Fos expression that were far less reaching, suggesting that the orexin-1 receptor may provide a more important mechanistic role in this context. To ensure that intranasal orexin administration was affecting neurotransmission *in-vivo*, a separate cohort of animals were utilized for a prefrontal cortex (PFC) *in-vivo* microdialysis study, where counterbalanced animals received both intranasal saline and intranasal orexin-A over two separate sessions. Acetylcholine and glutamate in PFC dialysates were measured by high-performance liquid chromatography. These results show that intranasal orexin-A increases PFC acetylcholine and glutamate efflux, two putative neurochemical correlates of attentional function, in young and aged rats. Importantly, deficits in *in-vivo* cholinergic and glutamatergic neurotransmission play an essential role in the attentional dysfunction observed during aging. Therefore, to test whether intranasal orexin-A administration improves attentional capacity, a separate cohort of animals were trained and tested in an attentional set-shifting (ASST) behavioral paradigm. These results show that vehicle-treated aged animals are impaired in the extradimensional shift (EDS) stage compared to vehicle-treated young animals, highlighting that aging impairs PFC-mediated attentional function. Importantly, intranasal orexin-A in aged animals abolished the age-related EDS impairment relative to young control animals treated with saline. These results indicate that intranasal orexin-A may restore age-related PFC dysfunction to levels on par with their cognitively intact younger counterparts. Together, these studies highlight the putative mechanisms that underlie cognitive dysfunction during aging and provide insights into the possible therapeutic benefit of intranasal orexin-A in treating age-related cognitive dysfunction.

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CHAPTER 1

GENERAL INTRODUCTION

1.1: Significance

Cognitive impairment is a core feature of several neuropsychiatric and neurological disorders, including narcolepsy and age-related dementias. Demographic trends indicate an aging American populace; therefore, rates of age-related neurodegenerative diseases, including Alzheimer's disease (AD), are projected to increase significantly over the next several decades. While a plethora of approaches are currently being investigated to solve these disorders, it is of paramount importance that novel diagnostics, strategies, and therapies are developed to combat these devastating diseases. Current pharmacotherapeutic options for cognitive enhancement are scarce and limited in efficacy. Thus, novel treatment strategies are needed. The hypothalamic orexin (hypocretin) system, a central integrator of physiological function, plays an important role in modulating cognition. Several single- and dual-orexin receptor antagonists are available for various clinical and preclinical applications, but the paucity of orexin agonists has limited the ability to research their therapeutic potential. To circumvent this hurdle, direct intranasal administration of orexin peptides is being investigated as a prospective treatment for cognitive dysfunction, narcolepsy, or other disorders in which deficient orexin signaling has been implicated. The studies were designed to elucidate the possible mechanisms and therapeutic potential of intranasal orexin delivery. The results from these neurochemical and behavioral studies in young and aged animals

highlight the capacity for intranasal orexin administration to improve age-related deficits in neurotransmission and provide a potential framework for the use of intranasal orexin peptides in treating age-related cognitive dysfunction.

1.2: Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease that affects approximately 6 million Americans. The disease is currently the 6th leading cause of death in the United States with an estimated cost burden of 290 billion dollars in 2019 alone. Because of advances in other areas of medicine, life expectancy across the globe has risen and, therefore, cases of late-onset AD are expected to rise to 14 million by the year 2050 (Alzheimer's Association, 2019). Indeed, by definition, the largest risk factor for developing late-onset AD is age (Hebert *et al.* 1995).

The origins of AD date back to the early 20th century when German psychiatrist and neuropathologist Alois Alzheimer identified plaques and neurofibrillary tangles in a recently deceased patient that had been suffering from memory loss and hallucinations (Cipriani *et al.* 2011). While AD exists in both the early-onset and late-onset forms, the latter is much more common and its etiology far less understood. The primary pathological hallmarks of AD, amyloid-β plaques and hyperphosphorylated-tau in neurofibrillary tangles, gradually accumulate over the course of the disease with devastating effects to synapses and neurons. Thus, one of the main avenues of research in recent years has been in developing therapies for disrupting or preventing amyloid-β and hyperphosphorylated-tau pathologies. Unfortunately, none of the amyloid-β targeting therapies have passed phase 3 clinical trials due to lack of statistical significance on pre-

clinically defined endpoints. Even more concerning for this therapeutic route is that while these amyloid targeting therapies are efficacious in removing plaques, they are not efficacious at slowing disease progression (Selkoe and Hardy 2016; Scheltens *et al.* 2016). These concerns point to the complexity of AD and, unfortunately, suggest a probable need for individualized therapies and/or a combination of treatments.

1.3: Cholinergic receptors and neurotransmission

Acetylcholine is one of the major neurotransmitter in the brain with wide reaching activity and effects on cognition (Francis *et al.* 1999; Everitt and Robbins 1997; Mesulam 2013; Schliebs and Arendt 2011). Acetylcholine is synthesized within the presynaptic nerve terminals of cholinergic neurons by the enzyme choline acetyltransferase (ChAT). This enzyme uses acetyl CoA, a byproduct of glucose breakdown in the Krebs cycle, along with the main substrate choline to synthesize the neurotransmitter acetylcholine. Acetylcholine is then packaged into vesicles by the vesicular acetylcholine transporter (vAChT) where it is stored until its release during neurotransmission. After release into the synapse, acetylcholine will bind to specific post-synaptic receptors (M1 muscarinic receptors) in order to propagate a cholinergic signal from neuron to neuron. Within the synaptic cleft, acetylcholine is degraded by the enzyme acetylcholinesterase (AChE) into choline and acetate, which are ultimately taken back into nerve terminals and recycled for further production of acetylcholine (Tucek 1990). Once acetylcholine is released from the presynaptic terminal, it binds to two classes of receptors: nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). Nicotinic acetylcholine receptors are ligand-gated ion channels composed of five different subunits: two alpha subunits, one beta subunit, one gamma subunit, and one delta subunit. The nicotinic

receptors are primarily classified into two separate groups: heterometric receptors and homomeric receptors. The heteromeric receptors are primarily composed of two different types of subunits (Karlin and Akabas 1995; Taly *et al.* 2009). Among the heteromeric receptors, the α4β2 subtype is the most predominant receptor in the CNS. The homomeric receptors are composed of five identical subunits and is mainly represented by the α7 subtype within the CNS. Nicotinic acetylcholine receptors within the CNS can be located on somata, dendrites, axons, or axon terminals. Presynaptic and preterminal nAChRs enhance neurotransmitter release while postsynaptic nAChRs activate intracellular signaling mechanisms and gene transcription (McKay *et al.* 2007; Dani and Bertrand 2006). Acetylcholine also signals through metabotropic muscarinic receptors (mAChRs), which are G-coupled protein receptors coupled to either G_i or G_q. The M1, M3, and M5 subtypes are excitatory and are coupled to G_q and the M2 and M4 subtypes are inhibitory and are coupled to G_i (Felder 2018). The M1 receptor subtype is expressed postsynaptically and increases neuronal excitability. The M2 and M4 receptor subtypes represent the major presynaptic mAChRs in the brain and function to suppress cholinergic and glutamatergic neurotransmission. M2 can also be expressed presynaptically at GABAergic terminals. The M3 and M5 subtypes are expressed at lower levels in the brain, but function in a manner similar to M1 mAChRs (Lebois *et al.* 2018).

Cholinergic neurons function as interneurons or as projections neurons. Cholinergic interneurons of the striatum provide dense local innervation and function in sensorimotor planning and learning (Zhou *et al.* 2002). Cholinergic neurons of the pedunculopontine nucleus (PPT) and laterodorsal tegmentum (LDT) function as

regulators of REM sleep during the natural sleep/wake cycle (Dort *et al.* 2015). The primary source of cholinergic innervation to the neocortex is provided by cholinergic neurons within the basal forebrain. The basal forebrain cholinergic system (BFCS) is divided into four subdivisions (Ch.1-4). The medial septum (Ch.1) provides dense innervation to the hippocampus. The vertical limb of the diagonal band (Ch.2) provides a major source of innervation to the hippocampus and hypothalamus. The horizontal limb of the diagonal band (Ch.3) provides dense innervation of the olfactory bulb. Finally, the contiguous nucleus basalis/ventral pallidum/substantia innominata (Ch.4), sends widespread projections to the amygdala and neocortical areas like the prefrontal cortex, insular cortex, and posterior parietal cortex (Mesulam *et al.* 1983a).

1.3.1: The cholinergic hypothesis of Alzheimer's disease

The original hypothesis of AD was driven from studies noting cholinergic dysfunction during the disease: The first observation was a reduction of presynaptic cholinergic markers in the cortex of AD patients (Bowen *et al.* 1976; Davies and Maloney 1976). It was also discovered that the nucleus basalis degenerates during the disease (Mesulam 1976; Whitehouse *et al.* 1981). Finally, research into the effects of cholinergic agents on memory showed that cholinergic antagonists impair memory and cholinergic agonists improve memory (Drachman and Leavitt 1974). This hypothesis, although flawed in some ways, has validity as the primary therapeutics used to treat AD are cholinesterase inhibitors. Of the current FDA approved treatments for AD, 4 are acetylcholinesterase inhibitors that act by inhibiting the enzymatic breakdown of acetylcholine in the synaptic cleft. These treatments are approved for the treatment of mild to moderate AD. The only other FDA approved treatment for AD is memantine, an

NMDA receptor antagonist, which is approved alone or in combination with cholinesterase inhibitors for the treatment of moderate to severe AD. Unfortunately, neither class of drug is efficacious at halting progress of the disease, with most of the cognitive benefits occurring during the early phases of treatment (Yiannopoulou and Papageorgiou 2013; Lleó *et al.* 2005). Nonetheless, the progressive loss of basal forebrain cholinergic neurons and the ensuing degradation of afferents to the cortex, hippocampus, and amygdala represent a critical component of AD etiology (Cavedo *et al.* 2018; Sassin *et al.* 2000).

1.4: The hypothalamus

The hypothalamus, a diencephalic brain region, is the primary central nervous system locus for coordinating endocrine, autonomic, and behavioral responses to peripheral cues indicative of homeostatic status. The lateral hypothalamus (LH), originally defined as the 'feeding center' (Willie *et al.* 2002; Shimada *et al.* 1998; Sakurai *et al.* 1998), has abundant efferent and afferent connections to various brain regions, suggesting that the LH is highly involved in functions outside of feeding behavior. Indeed, lesioning studies indicate that the LH plays a crucial role in facilitating arousal (Lin *et al.* 1989; Swett and Hobson 1968; Robinson and Wishaw 1974; Jones 2003). Proper homeostatic and cognitive functioning in the elderly are extremely important factors for determining independent functioning in the elderly. In the elderly human population, physiological and homeostatic dysfunction, especially unexplained weight loss, may be key determinants for predicting cognitive decline into AD (Grundman 2005; Johnson *et al.* 2006; Cova *et al.* 2016; Buchman *et al.* 2005). This rapid, unexplained weight loss may represent an important clinical correlate of hypothalamic dysfunction in

the elderly. Although the mammalian hypothalamus contains a divergent array of nuclei and neural signaling molecules, the orexin (hypocretin) system plays a particularly prominent role in homeostatic regulation.

1.5: The orexin/hypocretin system

Two decades have passed since the discovery of the orexin/hypocretin (hereafter referred to as orexin) system by two separate laboratories in 1998. One group named the newly discovered pair of peptides hypocretin-1/hypocretin-2 due to their exclusive expression in the postero-lateral hypothalamus/perifornical area (de Lecea *et al.* 1998), while the other group named the pair of peptides orexin-A/orexin-B (OxA/OxB) after the Latin word 'orexis' meaning appetite (Sakurai *et al.* 1998). The gene that codes for orexin is structurally conserved among vertebrates and is comprised of two exons and one intron. Human orexin mRNA is composed of 616 bases which encodes the 131 amino acid prepro-orexin peptide. The prepro-orexin peptide ultimately serves as the precursor for the OxA and OxB peptide isoforms (Bai *et al.* 2018). Orexin-A contains 33 amino acid residues and orexin-B contains 28 amino acid residues, with the two peptides sharing a sequence homology of 46% (Sakurai *et al.* 1998; Alvarez and Sutcliffe 2002). During their original discovery, the orexin peptides were found to be endogenous ligands for two G-protein coupled receptors, the orexin-1/hypocretin-1 receptor and the orexin-2/hypocretin-2 receptor. The OxA peptide binds to the orexin-1 and orexin-2 receptors with approximately equal affinity, while the orexin-B peptide possesses a higher affinity for the OX_2 receptor (Smart and Jerman 2002; Ammoun *et al.* 2003; Gotter *et al.* 2012a; Leonard and Kukkonen 2014). Orexin neurons are glutamatergic (Rosin *et al.* 2003;

Torrealba *et al.* 2003) but also colocalize several other substances including dynorphin and neurotensin (Chou *et al.* 2001; Furutani *et al.* 2013).

1.5.1: Functional modulation of orexins

The orexin system originates with neurons largely restricted to a posterior hypothalamic level that includes the lateral hypothalamus, perifornical area, and parts of the dorsomedial nucleus, but gives rise to widespread projections that regulate limbic, cortical, and brain stem circuits (Peyron *et al.* 1998). Furthermore, orexin neurons receive reciprocal afferents, largely from limbic regions (Sakurai 2005; Yoshida *et al.* 2006). This diverse array of connections is consistent with the description of orexins as 'physiological integrators'(Li *et al.* 2014), but also suggests that orexins have the potential to modulate cognition. Furthermore, expression of orexin receptor mRNA is observed widely across the brain, with each receptor showing varied expression across the central nervous system (Marcus *et al.* 2001; Trivedi *et al.* 1998; Hervieu *et al.* 2001). Together, this heterogeneous collection of neural circuits suggests that orexin neurons have a distinct capacity to regulate endocrine, autonomic, and behavioral responses in order to maintain homeostasis (Li *et al.* 2014).

The extensive connectome of the hypothalamic orexin system puts it in a prime position to activate a multitude of phenotypic neurons throughout the brain. Indeed, orexin neuropeptides have been shown to excite neurons in a plethora of brain regions including GABAergic neurons in the medial septum, pyramidal neurons in the prefrontal cortex, and cholinergic neurons of the basal forebrain and laterodorsal tegmentum (Wu *et al.* 2002; Yan *et al.* 2012; Eggermann *et al.* 2001; Arrigoni *et al.* 2010; Burlet *et al.*

2018). Interestingly, recent work has demonstrated that orexin neurons are excited directly through the orexin-2 receptor and indirectly from orexin peptide excitation of glutamatergic neurons that synapse onto orexin neurons (Li *et al.* 2002; Yamanaka *et al.* 2010). Electrophysiological studies have also demonstrated that various neurotransmitters and neuropeptides can influence the activity of orexin neurons through direct and indirect pathways. For example, orexin neurons are depolarized in the presence of glutamatergic agonists for either the AMPA or NMDA receptor while antagonists for these receptors inhibit orexin neuron activity (Li *et al.* 2002). Furthermore, orexin neurons are hyperpolarized in the presence of agonists for the $GABA_A$ or the $GABA_B$ receptor (Xie *et al.* 2006; Li *et al.* 2002). Interestingly, application of antagonists for the AMPA, NMDA, and $GABA_A$ receptors blocked all synaptic events from orexin neurons, suggesting that glutamate and GABA are the primary sources of input to orexin neurons (Li *et al.* 2002). Furthermore, catecholamines appear to inhibit the activity of orexin neurons both directly and indirectly via modulation of GABAergic and glutamatergic input onto these neurons (Li *et al.* 2002; Yamanaka *et al.* 2003; Li 2005).

1.5.2: Orexin regulation of sleep/wakefulness

The importance of the orexin system in regulating sleep and wakefulness becomes evident in the neurological disorder, narcolepsy. Of the many responsibilities that the orexin system carries, its involvement in promoting wakefulness is arguably the clearest observable phenomenon. Now recognized as a specific loss of orexin neurons and a subsequent deficiency in orexin peptides (Burgess and Scammell 2012; Thannickal *et al.* 2000; Nishino *et al.* 2000), human narcolepsy is diagnosed in patients that present with diminished alertness, chronic daytime sleepiness, and, depending upon the narcoleptic

subtype, possible cataplexy, sleep paralysis, and hypnagogic hallucinations. The shortage of orexin transmission in narcoleptic symptomatology highlights the important role of orexin neurons in regulating sleep/wakefulness and their ability to inhibit spontaneous transitions into REM sleep. Indeed, during non-pathological conditions, orexin neurons are at peak activity during periods of wakefulness and show little to no activity during both REM and non-REM sleep (Lee 2005; Mileykovskiy *et al.* 2005; Ferrari *et al.* 2018). Furthermore, orexin overexpression in zebrafish induces a hyperlocomotive state and an phenotype that parallels states of insomnia (Sung *et al.* 2006). Recent mechanistic investigations have also revealed that orexin deficiencies result in deficits in theta-dominated wakefulness, a neural oscillation pattern that associated with goal-driven behaviors (Tyree *et al.* 2018; Vassalli and Franken 2017).

1.5.3: Orexins modulate cognitive function

While the orexin system is chiefly labeled as a 'physiological integrator', mounting evidence suggests that orexins may also modulate cognitive functions including attention, wakefulness/arousal, and learning and memory. Orexin modulation of cognitive function arises from multifarious interactions within telencephalic and hindbrain regions and their neurotransmitter systems. For example, orexins can modulate attentional function through connections with dopaminergic and noradrenergic systems of the ventral midbrain and locus coeruleus, respectively (Fadel and Deutch 2002; Horvath *et al.* 1999; Baldo *et al.* 2003; España *et al.* 2005; Vittoz and Berridge 2006). Furthermore, orexins can also facilitate attentional function through excitation of basal forebrain cholinergic neurons (Fadel and Burk 2010; Zajo *et al.* 2016) and modulation of glutamatergic thalamocortical synapses (Huang *et al.* 2006; Lambe *et al.* 2005; Song *et al.* 2006) that

ultimately alter prefrontal cortical release of acetylcholine and glutamate. Importantly, orexin-mediated effects on cognition are not limited to attention as OxA has also been shown to alter long-term synaptic plasticity, a presumed correlate of learning and memory, through coordinated alterations of cholinergic, glutamatergic, GABAergic, and noradrenergic transmission within the hippocampus (Selbach *et al.* 2004; Selbach *et al.* 2010; Yang *et al.* 2014). Indeed, the powerful effect of orexins on synaptic plasticity may underlie their role in persisting behavioral adaptations in a variety of contexts (Baimel and Borgland 2017; Borgland *et al.* 2006).

1.5.4: Orexin modulation of the basal forebrain

The basal forebrain is a highly complex subcortical brain region composed of series of heterogeneous subdivisions which include the medial septum (Ch.1), the vertical limb of the diagonal band of Broca (Ch.2), the horizontal limb of the diagonal band of Broca (Ch.3), and the ventral pallidum/ substantia innominata/ nucleus basalis (Ch.4) (Mesulam *et al.* 1983b). The subdivisions of the basal forebrain contain cholinergic, GABAergic, and glutamatergic neurons, with cholinergic and GABAergic neurons constituting the majority of the neurons present (Hur and Zaborszky 2005; Zaborszky *et al.* 1999; Gritti *et al.* 2003). Prior to the discovery of the orexin system, anterograde tracing studies demonstrated that projections originating from the lateral hypothalamic area, the putative location of orexin neurons, were observed to innervate basal forebrain cholinergic neurons (Cullinan and Záborszky 1991). Future tracing studies later confirmed that orexin immunoreactive fibers originating from the lateral hypothalamic area do indeed provide dense innervation to the basal forebrain (Peyron *et al.* 1998; Date *et al.* 1999). Moreover, orexin inputs to putative postsynaptic targets in the basal

forebrain have also been characterized. Specifically, appositions from orexin immunoreactive fibers have been observed on cholinergic-positive and parvalbumin-positive GABAergic cells in the ventral pallidum/substantia innominata, suggesting that these neurons of the basal forebrain are post-synaptic targets of orexin neurons (Fadel *et al.* 2005; Fadel and Burk 2010). Indeed, orexins directly modulate basal forebrain activity. For one, behavioral and electrophysiological evidence indicates that activation of basal forebrain PV+ GABAergic neurons is facilitated through the orexin-2 receptor (Mieda *et al.* 2011; Wu *et al.* 2002). Furthermore, *in-vitro* evidence suggests that OxA and orexin-B are approximately equipotent in exciting basal forebrain cholinergic neurons, suggesting that cholinergic activation is mediated primarily via the orexin-2 receptor (Eggermann *et al.* 2001). Other studies also point to the orexin-1 receptor having a crucial role in modulating the basal forebrain function, particularly functions involved in wakefulness. For instance, intrabasalis administration of OxA elicits a greater response on somatosensory cortical acetylcholine release than does OxB (Dong *et al.* 2006). Furthermore, the orexin-1 receptor antagonist SB-334867 abolishes stimulated acetylcholine release during feeding (Frederick-Duus *et al.* 2007). Ultimately, the orexin-1 and orexin-2 receptors may indeed play distinct and/or synergistic functional roles in modulating the basal forebrain cholinergic system in addition to other phenotypic neurons of the basal forebrain.

1.5.5: Orexin-neurotransmitter interactions: focus on attentional modulation

The studies mentioned herein primarily focus on the interactions between the orexin system and the neurotransmitters acetylcholine, glutamate, and GABA as they relate to cognitive function, especially attention. A substantial volume of evidence has

demonstrated clear orexin-cholinergic interactions and their potential role in modulating attentional function. A small and unstable neurotransmitter, acetylcholine is synthesized in presynaptic terminals of cholinergic neurons by the enzyme choline acetyltransferase (ChAT), a selective marker for cholinergic neurons in the brain. In addition to the basal forebrain, the primary source of acetylcholine for the neocortex, cholinergic neurons located in the laterodorsal tegmentum and pedunculopontine tegmentum are also important for orexin-cholinergic interactions. Indeed, orexins infused into the laterodorsal tegmentum, much like the basal forebrain, induce arousal and increase wakefulness (Thakkar *et al.* 2001; Xi *et al.* 2001; Espaa *et al.* 2001), which ultimately suggests that cholinergic innervation to the basal forebrain is vital for maintaining arousal (Ohno and Sakurai 2008; Yamanaka *et al.* 2003; Zhou *et al.* 2015). Both empirical and heuristic observations indicate that attentional function is dependent upon a state of arousal. This phenomenon is observed in narcoleptic patients where arousal and, often, attentional performance is impaired significantly. Together, this suggests that orexins are not only important, but necessary for proper attentional function. Functionally, orexins can modulate attention through several putative mechanisms. As previously mentioned, orexin neurons send robust projections to the basal forebrain, but also directly to the neocortex. Administration of OxA directly into the basal forebrain not only increases cortical acetylcholine release, but also enhances attentional capacity (Fadel *et al.* 2005; Zajo *et al.* 2016). Moreover, direct OxA administration into the basal forebrain also increases local glutamate release, in turn modulating the activity of basal forebrain cholinergic neurons (Frederick-Duus *et al.* 2007). Basal forebrain cholinergic neurons also receive help from GABAergic neurons facilitating arousal as orexins have been

found to directly excite wake promoting non-cholinergic neurons of the basal forebrain (Arrigoni *et al.* 2010). Lastly, direct infusion of orexin-B into the prefrontal cortex enhances accuracy during a highly demanding attentional task. Mechanistically, it was determined that the attentional enhancement occurred through excitation of the thalamo-cortical synapses activated by basal forebrain cholinergic neurons (Lambe *et al.* 2005).

1.5.6: Orexin function degenerates during aging

Because orexins play such a vital role in maintaining physiological homeostasis, dysregulation of the orexin system can result in a multitude of cognitive and behavioral deficits. This phenomenon is most clearly exemplified in narcolepsy, which is hallmarked by a selective loss of orexin neurons (Siegel 1999; Thannickal *et al.* 2009). While narcoleptic symptomatology is classically defined by disruption in the sleep/wake cycle, heuristic observations of narcoleptic patients have shown that narcoleptics display additional cognitive dysfunction, including deficits in sustained attention and olfactory discrimination (Rieger *et al.* 2003; Bayard *et al.* 2010; Naumann *et al.* 2006). Interestingly these deficits in attention and olfactory discrimination also arise during aging and early AD (Sarter and Turchi 2002; Wesson *et al.* 2010; Perry and Hodges 1999a; Hüttenbrink *et al.* 2013). The observed cognitive deficits in narcoleptics that arise from the loss of orexin neurons may also correlate to age-related cognitive dysfunction. Prior work from our lab and others has shown a selective age-related loss of orexin neurons and/or their peptides and receptors (Kessler *et al.* 2011; Downs *et al.* 2007; Porkka-Heiskanen *et al.* 2004; Sawai *et al.* 2010; Terao *et al.* 2002; Zhang *et al.* 2002). Moreover, recent post-mortem analysis of brains from patients with AD suggests a selective loss of orexin neurons (Davies *et al.* 2015; Fronczek *et al.* 2012). Collectively,

these findings provide convincing evidence that orexins modulate the underlying neural substrates of cognition and suggest that orexin-based therapeutics may be useful in the treatment of age-related cognitive disorders.

1.5.7: Orexins and Alzheimer's disease

Current research indicates that late-onset AD is an enormously complicated disorder composed of abnormal interactions among a multitude of biological systems. Although age is the strongest risk factor for AD (Hebert *et al.* 1995), the underlying cause, or more likely, causes, are still being investigated. The main pathological hallmarks of AD classically include amyloid-β plaques and neurofibrillary tangles. More recently, AD pathology has been consistently associated with neuroinflammation, altered insulin resistance, oxidative stress, and cerebrovascular dysfunction (Cavedo *et al.* 2018). These pathologies have been classically intertwined with the cholinergic lesion observed in AD (Mesulam 2013; Geula *et al.* 1998; Bergmann *et al.* 1978). More recently, however, these pathologies may be interconnected to the orexin system. In a recent study, the number of orexin neurons in AD patients was found to be reduced by 40% along with a 14% reduction in OxA within patient cerebrospinal fluid (Fronczek *et al.* 2012). However, more recent studies have found conflicting evidence, indicating that orexin levels are elevated in the cerebrospinal fluid (CSF) of AD patients (Liguori *et al.* 2016; Liguori 2017) and that increased OxA in the CSF correlates with amyloid-β_{42} levels (Gabelle *et al.* 2017). Furthermore, orexins have been recently found to inhibit uptake and degradation of amyloid-β in microglia (An *et al.* 2017). Despite these recent negative associations with AD, orexins have also been shown to have neuroprotective and cognitive enhancing effects. For example, in the SAMP8 mouse model of AD, ICV

microinjection of OxA improves memory (Jaeger *et al.* 2002). Furthermore, orexin receptor blockade in the CA1 region of the hippocampus impairs spatial memory retrieval (Akbari *et al.* 2008; Akbari *et al.* 2006), while administration of OxA into the hippocampus recovers impairments in learning and memory due to a painful stimulus (Raoof *et al.* 2014). Lastly, recent work characterizing orexin receptors has demonstrated that cells forming heterodimers between orexin receptors and the orphan receptor, GPR103, exert a neuroprotective effect against AD via upregulation $ERK_{1/2}$ phosphorylation (Davies *et al.* 2015; Chen *et al.* 2019).

1.5.8: Intranasal administration of orexins

The orexin system exerts a powerful influence over physiological and behavioral states by interacting with systems involved in sleep/wakefulness, energy homeostasis, addiction, stress responses, and cognition. These observations have given way to a substantial interest in developing therapeutic agents that target orexin receptors (Chieffi *et al.* 2017). To date, there are numerous selective and non-selective orexin receptor antagonists that have been developed (Skudlarek *et al.* 2017; Smart *et al.* 2001; Steiner *et al.* 2013; Roecker *et al.* 2016; Gotter *et al.* 2012a). Conversely, selective orexin receptor agonists with preclinical or clinical efficacy are scarce (Mieda and Sakurai 2013; Nagahara *et al.* 2015; Turku *et al.* 2017), ultimately leading to the use of orexin peptides as the agonists of choice. While early work in canine narcolepsy models suggested that systemic delivery of orexins could have therapeutic efficacy (Fujiki *et al.* 2003; John *et al.* 2000), concerns surrounding this route of administration include peripheral degradation, poor delivery across the blood-brain-barrier, and significant peripheral side effects (Dhuria *et al.* 2009; Hallschmid and Born 2008; Kastin and Akerstrom 1999). To

circumvent these issues, intranasal administration has been proposed as a feasible treatment route to target orexins and various other neuropeptides to the CNS (Dhuria *et al.* 2010; Hanson and Frey 2008; Lochhead and Thorne 2012). Intranasal administration of neuropeptides provides several benefits over systemic administration including targeted delivery to the CNS, reduced peripheral complications, and complete bypass of the blood-brain-barrier (Hanson and Frey 2008; Meredith *et al.* 2015; Lochhead and Thorne 2012; Spetter and Hallschmid 2015). The extent and time course of peptide delivery to the CNS depends on several factors such as peptide size, lipophilicity, and transportation methods from the olfactory mucosa into the brain (Lochhead and Thorne 2012; Dhuria *et al.* 2010; Meredith *et al.* 2015; Spetter and Hallschmid 2015). The mechanisms of intranasal orexin delivery to the CNS are not completely understood but available data suggest that proteins transported to the CNS start at olfactory and trigeminal nerve constituents of the nasal epithelium and proceed to the olfactory bulb and sensory/spinal trigeminal regions of the pons, the CNS origins of chemosensory and somatosensory innervation, respectively, of the nasal mucosa. Once in the CNS, peptides can then diffuse to various rostral and caudal brain regions (Thorne *et al.* 2004; Lochhead and Thorne 2012; Thorne *et al.* 2008). Brain penetration and distribution of peptides and proteins may be affected by multiple factors including molecular weight, tertiary structure, lipophilicity, and receptor localization. However, there are no broadly-applicable predictive models, emphasizing the importance of peptide-specific descriptions of distribution patterns in understanding behavioral and physiological effects of intranasal administration.

1.6: Summary

While cognitive deficits progress significantly from normal aging to mild cognitive impairment and finally to AD, an early feature of these age-related cognitive changes is a deterioration of attentional function (Sarter and Turchi 2002; Oken *et al.* 2012; Rizzo *et al.* 2000; Perry and Hodges 1999b; Scinto *et al.* 1994). The attentional deficits that occur during aging almost certainly involve the dysfunction of several neurotransmitter networks; however, these deficits prominently include dysfunction of the basal forebrain cholinergic system (BFCS) (Muir *et al.* 1992; Sarter and Bruno 1997; Robbins *et al.* 1997; Whitehouse 2004; Hasselmo and McGaughy 2004). These early discoveries made the BFCS a prominent target for treating age-related cognitive disorders (Robbins *et al.* 1997; Winkler *et al.* 1998). Nevertheless, the cholinesterase inhibitors used to treat these disorders provide only modest benefits on cognition and eventually lose their efficacy (Robbins *et al.* 1997; Winkler *et al.* 1998; Francis *et al.* 1999; Siegfried 1993; Ringman and Cummings 2006). Thus, age-related cognitive dysfunction is likely the consequence of a progressive degeneration of various interacting neurotransmitter systems. Taken together, the age-related cognitive deficits originally tied to BFCS dysfunction may ultimately arise from disruptions to the afferent regulation of the BFCS. Indeed, research over the previous two decades has keyed the hypothalamic orexin/hypocretin system as a prime contributor to the cognitive decline observed during aging.

Orexin projections to the basal forebrain have been shown to modulate cognition through connections to various telencephalic brain regions including the PFC, the agranular insular cortex—the putative 'interoceptive cortex'—, and the BFCS (Fadel and Burk 2010; Arrigoni *et al.* 2010; Huang *et al.* 2006; Lambe *et al.* 2005). Additionally, administration of

OxA has been shown to facilitate attentional processing through activation of basal forebrain cholinergic neurons, which in turn alter cortical acetylcholine release (Fadel *et al.* 2005; Fadel and Burk 2010; Zajo *et al.* 2016). OxA administration also modulates glutamatergic thalamocortical synapses and facilitates attention (Lambe *et al.* 2005; Huang *et al.* 2006; Song *et al.* 2006). Because orexins play such a vital role in maintaining physiological homeostasis, dysregulation of the orexin system can result in a multitude of cognitive and behavioral deficits. This phenomenon is most clearly exemplified in narcolepsy, which is hallmarked by a selective loss of orexin neurons (Siegel 1999; Thannickal *et al.* 2009). While narcoleptic symptomatology is classically defined by disruption in the sleep/wake cycle, heuristic observations of narcoleptic patients have shown that narcoleptics display additional cognitive dysfunction including deficits in sustained attention and olfactory discrimination (Rieger *et al.* 2003; Bayard *et al.* 2010; Naumann *et al.* 2006). Interestingly, narcoleptic patients also show subtle similarities in the cognitive deficits, namely deficits in attention and olfactory discrimination, that arise during aging and early AD (Sarter and Turchi 2002; Wesson *et al.* 2010; Perry and Hodges 1999a; Hüttenbrink *et al.* 2013). The observed cognitive deficits in narcoleptics that arise from the loss of orexin neurons may also correlate to age-related cognitive dysfunction. Prior work from our lab and others has shown a selective age-related loss of orexin neurons and/or their peptides and receptors (Kessler *et al.* 2011; Downs *et al.* 2007; Porkka-Heiskanen *et al.* 2004; Sawai *et al.* 2010; Terao *et al.* 2002; Zhang *et al.* 2002). Moreover, recent post-mortem analysis of brains from patients with AD suggests a selective loss of orexin neurons (Davies *et al.* 2015; Fronczek *et al.* 2012). Collectively, these findings provide convincing evidence that orexins modulate the underlying neural substrates of cognition

and suggest that orexin-based therapeutics may be useful in the treatment of age-related cognitive disorders.

Even though these observations provide a powerful framework for the therapeutic potential of intranasal OxA, studies investigating the mechanisms responsible for these behavioral observations remain limited. The available literature on intranasal orexin administration has primarily focused on the non-selective OxA neuropeptide; therefore, the contributions of each receptor to the behavioral and neurochemical observations cannot be determined. The scarcity of *in-vivo* studies using OxB administration stem from a multitude of reasons. These concerns are substantiated through work that shows limited diffusion of OxB across the blood-brain-barrier due to its low-lipophilic properties and rapid metabolic degradation by inactivating peptidases (Kastin and Akerstrom 1999). The affinity of OxB for the OX_2 receptor is roughly 10-fold higher than its affinity for the OX_1 receptor (Sakurai *et al.* 1998), making it difficult to draw receptor-specific mechanistic conclusions about physiological or behavioral responses to OxB. The development of a modified OxB peptide, [Ala^{11}, D-Leu^{15}]-OxB, with a reported 400-fold higher affinity for the OX_2 receptor vs. the OX_1 receptor, (Asahi *et al.* 2003) may offer a more selective tool for dissecting relative contributions of the orexin receptors to orexin peptide effects on physiological function (see (Putula *et al.* 2011) for potential caveats surrounding the relative selectivity and potency of this compound for the different orexin receptors *in vitro*). Ultimately, these studies were designed to assess the mechanisms and therapeutic potential underlying intranasal orexin administration, particularly as it relates to aging and age-related cognitive disorders.

1.7: Overarching hypothesis

I hypothesize that intranasal orexin administration improves age-related cognitive deficits by altering neurotransmission within brain regions that modulate attentional function (Figure 1.1). The pharmacological mechanisms through which intranasally administered orexin peptides improve cognition have yet to be fully determined. Accordingly, the studies planned here will expand upon the neurochemical and behavioral correlates of cognition within cholinergic, glutamatergic and GABAergic systems after intranasal orexin administration. Here, I will determine the mechanisms and therapeutic potential of intranasal OxA and intranasal [Ala^{11}, D-Leu^{15}]-OxB using immunohistochemistry, *in-vivo* microdialysis, and attentional set-shifting. I expect to find the following after intranasal orexin administration:

- Increased neuronal activation (i.e., c-Fos expression) within the PFC, BFCS, the insular cortex, and the hippocampus in young and aged animals treated with intranasal OxA
- Intranasal OxA will increase ACh and glutamate efflux in the PFC of young and aged animals
- Intranasal [Ala^{11}, D-Leu^{15}]-OxB administration will increase neuronal activation (i.e., c-Fos expression) of cholinergic neurons in the BFCS
- Intranasal OxA will decrease the number of trials needed to complete the extradimensional (EDS) stage of the attentional set-shifting task in young and aged animals

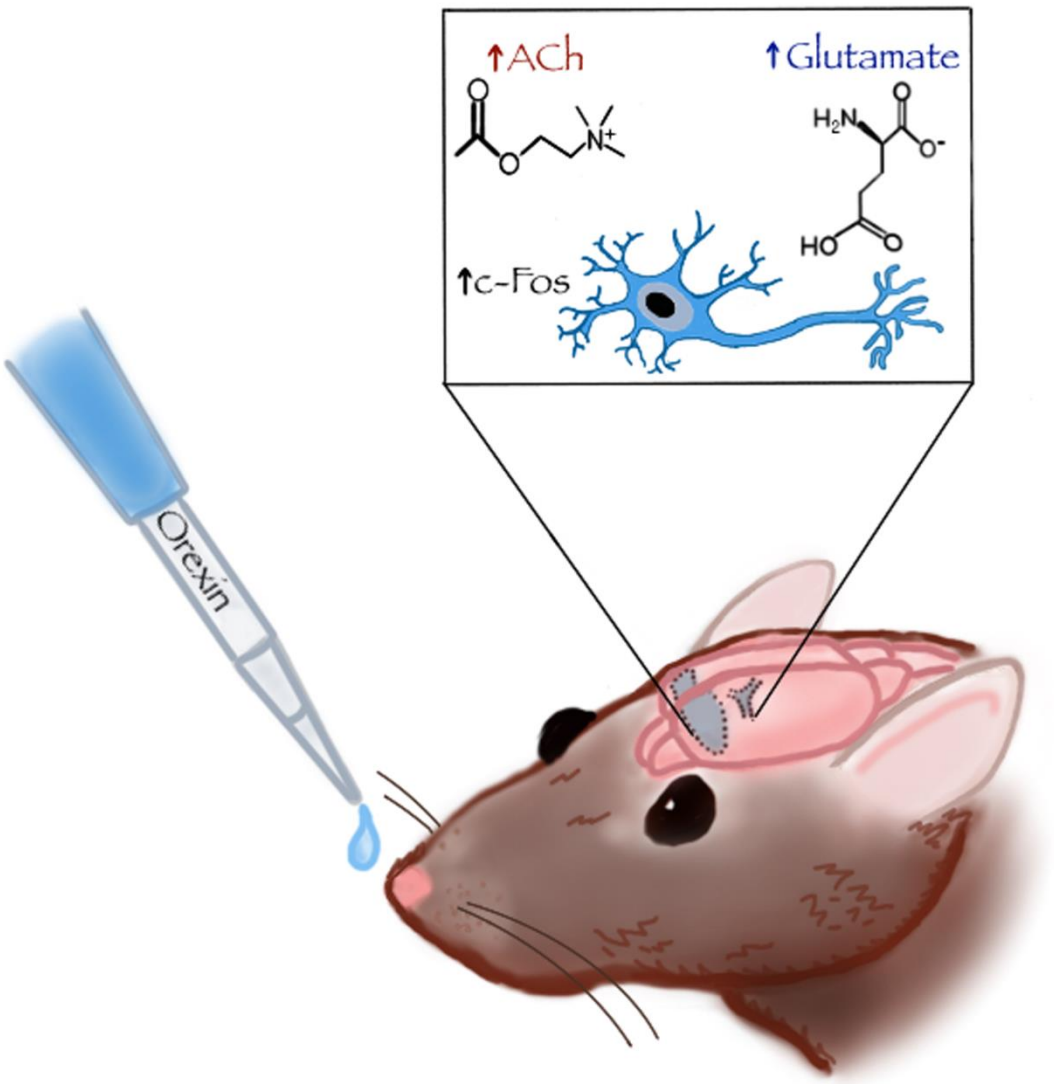

Figure 1.1 Summary figure for the overarching hypothesis. I hypothesize that intranasal orexin administration improves age-related cognitive deficits by altering neurotransmission within brain regions that modulate attentional function. Specifically, I predict that intranasal orexin administration will increase neuronal activation and neurotransmitter release in brain regions that support proper cognitive functioning during aging.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1: Rationale for animal care and use

Experimental methods, materials, and procedures for immunohistochemistry and *in-vivo* microdialysis were generally as described in our published works examining intranasal OxA administration in young and aged animals (Calva *et al.* 2018; Calva and Fadel 2018). Animal care and use practices, including rationale for use and number of animal subjects, were performed in accordance with protocols written under guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina (Animal Use Protocols #2248, #2409). Male Fischer 344/Brown Norway F1 hybrid rats (FBN/F1; Harlan/NIA) were utilized for all experiments. Young and aged animals were approximately 3-4 months and 26-28 months, respectively, upon arrival to the animal facility. The FBN/F1 strain has been extensively used by our laboratory and others for neurobiology of aging studies, because they have reduced susceptibility to several non-neurological age-related complications (e.g., intraperitoneal tumors) observed in other strains, even well into the third year of life (Lipman *et al.* 1996; Turturro *et al.* 1999). Therefore, we utilized the FBN/F1 hybrid strain in order to compare the effects of intranasal orexin administration on the neurobiological systems that are dysregulated during aging. As further proof of concept,

this strain of rat has been extensively used in our lab to study orexin-aging connections (Hagar *et al.* 2017; Kessler *et al.* 2011; Stanley and Fadel 2011; Stanley *et al.* 2012). All animals were housed in an environmentally controlled animal facility and kept on a 12:12 light: dark cycle with lights on at 07:00 hours. Animals were allowed access to standard rat chow and water *ad libitum* and all experiments were performed during the light phase of the light: dark cycle. Several measures were taken to minimize animal suffering during experimentation, including careful monitoring of anesthetic depth during surgery, administration of analgesics to ease post-operative pain, and using a within-subjects multi-session design for *in-vivo* microdialysis, thus reducing the number of animals needed to achieve equivalent statistical power. In lieu of performing power analyses to determine group sizes, prior studies using comparable treatment group sizes were consulted in order to obtain appropriate sample sizes for each experiment. The experimenter performing intranasal administration was not blinded to treatment conditions (saline vs. OxA/ [Ala^{11}, D-Leu^{15}]-orexin-B) during experimentation; however, the experimenter was unaware of these treatment conditions while conducting cell counts during histological imaging.

2.2: Timeline for immunohistochemistry and *in-vivo* microdialysis experiments

Upon arrival, each batch of animals used for the immunohistochemistry or *in-vivo* microdialysis experiments followed the timeline outlined in Figure 2.1. Day 0 represents the day of arrival for each batch of animals used at the University of South Carolina School of Medicine animal facility. Beginning on Day 1, animals were handled and habituated for 7 days total (Day 0-7). After 1 week, animals were assigned to either the immunohistochemistry (c-Fos) or *in-vivo* microdialysis experimental groups. For the c-

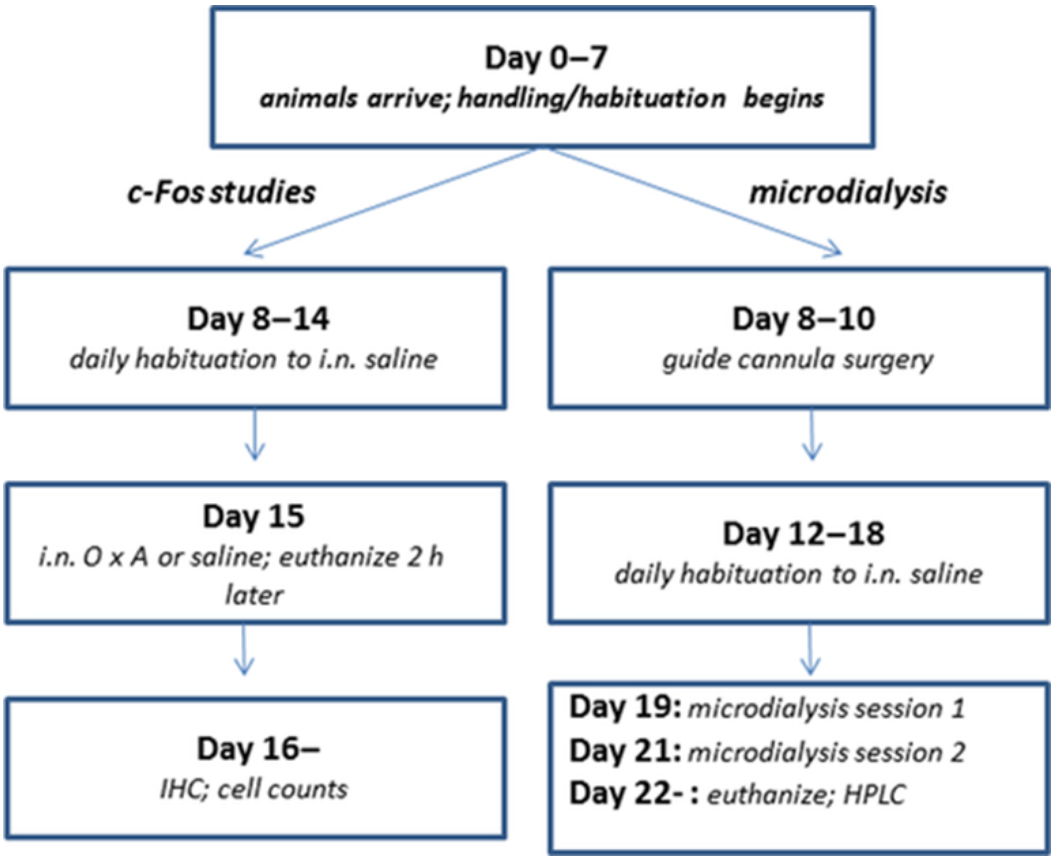

Figure 2.1 Experimental timeline for immunohistochemistry and *in-vivo* microdialysis

Fos experiments, animals received a daily habituation to intranasal saline for 7 days (Day 8-14). On testing day (Day 15), each rat received intranasal saline (50 µL of 0.9% saline), intranasal OxA (50 µL of a 100 µM solution; Enzo Life Sciences; Farmingdale, NY, USA), or intranasal [Ala^{11}, D-Leu^{15}]-orexin-B (50 µL of a 100 µM solution; Tocris Bioscience; Minneapolis, MN, USA) and was euthanized 2 hours post-administration. Processing of brain tissue, immunohistochemistry, cell counts, and data analysis started on Day 16 and continued until complete for each batch of rats used for c-Fos analysis. For the *in-vivo* microdialysis experiments, guide cannula surgery was performed during Days 8-10. Animals were allowed at least two full days of recovery before habituation in the microdialysis bowls. Following recovery, animals received a week of daily habituation to intranasal saline (Day 12-18). The first microdialysis session was performed on Day 19 and the second session on Day 21. Microdialysis experiments were counterbalanced so that half of the animals received OxA in the first session and saline in the second session; the order was reversed for the other half of the animals. On the day following the second microdialysis session (Day 22), animals were euthanized, their brains processed for histochemical verification of probe placement, and high-performance liquid chromatography (HPLC) analysis of dialysate samples began.

2.3: Stereotaxic surgery for implantation of guide cannulae

The stereotaxic surgical device allows for researchers to implant probes, fibers, and other devices or to inject substances into brain regions with extreme precision. Animals are first anesthetized, and their heads shaved to allow for access to the skin just above the skull. Once mounted in the stereotaxic device, the animal is sterilized and given an incision upon the midline of their head to access the skull surface. Once cleaned,

the bone sutures on the animal's skull are used to locate lambda and bregma in order to determine proper placement for the microdialysis probe. While under ketamine (90 mg/kg)/xylazine (2-10 mg/kg) anesthesia, animals used for *in-vivo* microdialysis received a single guide cannula (BASi, West Lafayette, IN, USA) inserted into the prefrontal cortex (PFC) at AP +2.8 mm, L ±0.5 mm, and DV -2.8 mm relative to bregma (Figure 2.2). Coordinates for guide cannula implantation were obtained from the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998). The intracerebral guide cannula was anchored in place using two to three skull screws and dental cement. Guide cannula placement was counterbalanced so that left and right hemispheres were represented equally. During the procedure, all animals were given a single dose of buprenorphine (0.01 mg/kg, s.c) to ease post-operative pain and were monitored until complete recovery.

2.4: Immunohistochemistry and immunofluorescence

Upon arrival, both young and aged animals were assigned to receive intranasal administration of either vehicle (50µL of 0.9% saline) or OxA (50µL of a 100µM solution; Enzo Life Sciences, Farmingdale, NY, USA). Animals used for immunohistochemistry were received in separate batches. For each batch, both treatment conditions were equally represented; however, treatment order within each batch was pseudo-randomly determined before each test day. Each animal received several days of gentle handling and habituation to intranasal saline administration prior to the test day. Briefly, each animal was loosely blanketed with a small cloth and held in a supine position so that only the animal's snout was protruding from a small opening. No restraint or anesthesia was used during intranasal habituation or treatment. On the test day, each rat was administered 50µL of saline or OxA. Intranasal administration of the

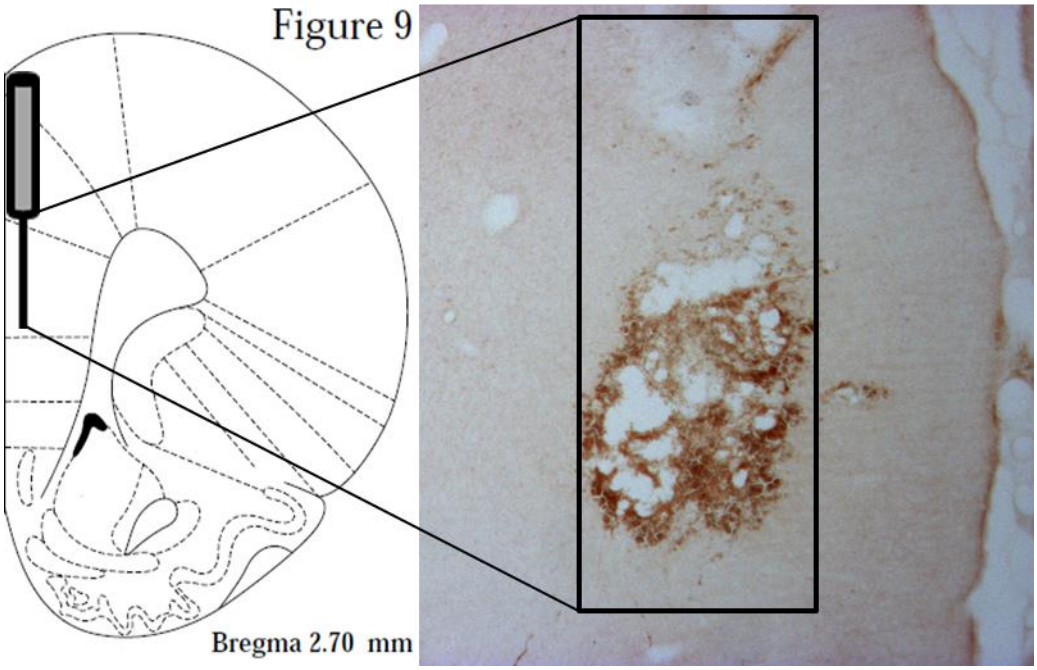

Figure 2.2 Probe placement verification with an acetylcholinesterase background stain. Intracerebral guide cannula was placed into each animals' prefrontal cortex at the coordinates: AP +2.8 mm, L ±0.5 mm, and DV -2.8 mm relative to bregma. An acetylcholinesterase background stain yielding a red-brown hue is indicative of the location of the microdialysis probe.

entire 50µL volume was delivered in four 12.5 µL aliquots (a total of 25 µL in each naris) over a 2-3-minute period. A separate set of young animals were assigned to receive either vehicle or a modified orexin-B peptide (50µL of a 100µM solution) serving as a potent and selective OX_2 receptor agonist ([Ala^{11}, D-Leu^{15}]-orexin-B; Tocris Bioscience; Minneapolis, MN, USA). While rat-to-human conversion of doses holds many caveats, based on conversion factors suggested by Nair and Jacob (Nair and Jacob 2016), our 5 nmol dose in rats would approximately equate to a 200 nmol dose in humans. While no studies to date have described intranasal [Ala^{11}, D-Leu^{15}]-OxB administration in humans, previous work in human narcoleptic patients suggests that a 435 nmol dose of intranasal OxA enhances wakefulness and attention. Given the lack of available comparative evidence, our doses of orexin appear practical.

Treatment group assignment for all immunohistochemistry experiments was pseudorandomized for each batch of animals such that treatment order (i.e. intranasal saline or intranasal orexin) was counterbalanced and equally represented for each batch. All animals were handled and habituated to intranasal saline administration prior to the treatment day. On treatment day, animals received their designated treatment and were subsequently sacrificed under heavy isoflurane anesthesia and perfused with phosphate buffered saline and 4% paraformaldehyde 2 hours post-administration to observe optimal c-Fos expression (Kaczmarek 1992). After an overnight post-fixation, each brain was coronally sectioned at a 50 µm thickness using a vibratome. A 1:4 serial sectioning method was utilized, thus allotting 200 µm between each representative section. Sections not immediately used for immunohistochemistry were stored in 30% sucrose/30% ethylene glycol anti-freezing solution at -20°C until use. Single and dual-label

immunohistochemistry followed similar protocols, where free-floating sections were incubated with a rabbit anti-c-Fos primary antibody (1:5000; Millipore, Billerica, MA, USA; catalog No. ABE457; RRID AB_2631318) for 48 hours at 4°C, followed by a biotinylated donkey anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch Laboratories Inc.; West Grove, PA, USA; code No. 711-065-152; RRID AB_2340593) for 1.5 hours at 23°C (RT), and a horseradish peroxidase conjugated streptavidin tertiary antibody (1:1600; Jackson ImmunoResearch Laboratories Inc.; code No. 016-030-084; RRID AB_2337238) for 1 hour at 23°C (RT). Staining for c-Fos was developed with 0.3% hydrogen peroxide and nickel-cobalt enhanced diaminobenzidine (DAB) to yield blue-black immunopositive nuclei. Dual-label staining for either choline acetyltransferase (ChAT) or parvalbumin (PV) used c-Fos stained sections that were subsequently incubated in either a goat anti-ChAT (1:3000; Millipore, Temecula, CA, USA; catalog No. AB144; RRID AB_90650) or a mouse anti-PV (1:4000; Sigma, St. Louis, MO, USA; catalog No. P3088; RRID AB_477329) primary antibody for 48 hours at 4°C. Secondary and tertiary steps followed with incubations in either an unlabeled donkey anti-goat (1:200; Jackson ImmunoResearch Laboratories Inc.; code No. 705-005-003; RRID AB_2340384) or an unlabeled donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories Inc.; code No. 715-005-150; RRID AB_2340759) secondary antibody for 2 hours at 23°C (RT), followed by incubations in either a goat peroxidase anti-peroxidase (1:500; Jackson ImmunoResearch Laboratories Inc.; code No. 123-005-024; RRID AB_2338953) or a mouse peroxidase anti-peroxidase (1:500; Jackson ImmunoResearch Laboratories Inc.; code No. 223-005-024; RRID AB_2339261) tertiary antibody for 1.5 hours at 23°C (RT). Immunostaining for ChAT or PV were developed with 3% hydrogen

peroxide and DAB to yield brown immunopositive cell bodies. Using a 0.15% gelatin solution, sections were mounted onto slides and allowed to dry overnight before dehydration, delipidation, and cover-slipping with DEPEX mounting medium.

In order to perform a qualitative examination of where intranasal OxA distributed within the brain, a subset of animals (n=4, young; n=4, aged) received intranasal administration of either saline or a modified OxA peptide labeled with a green-fluorescent fluorophore (5(6)-FAM-(Glu^{1})-OxA trifluoroacetate salt; BACHEM; Bubendorf, Switzerland). Animals receiving the fluorescein-tagged OxA peptide received 50 µL of a 500 µM dose split into four 12.5 µL increments over a two-minute period and were sacrificed thirty minutes post-treatment. This timepoint was chosen based upon a previous study showing peak appearance of ^{125}I–OxA in the CNS thirty minutes after intranasal delivery (Dhuria *et al.* 2009). Perfusion and brain sectioning were as described above. Sections were mounted onto slides with 0.15% gelatin, dried overnight, dehydrated only and cover-slipped using Permount mounting medium.

2.5: Microscopy and imaging

Histological experiments for single-labeled (c-Fos) cells, double-labeled (c-Fos + ChAT or PARV) cells, and AChE background staining were visualized using a Nikon E600 microscope fitted with a CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA). Fluorescence images were visualized using a Nikon E600 microscope or a Leica SP8 multiphoton confocal microscope (Leica Microsystems; Wetzlar, Germany) equipped with LAS AF 3 analysis software (Leica Microsystems; Wetzlar, Germany). Immunoperoxidase photomicrographs were captured using IP Lab Software (Scanalytics;

Trenton, NJ, USA). Images were imported into Adobe Photoshop 6.0 (Adobe Systems; San Jose, CA, USA) to adjust the image size and to make minor alterations to contrast and brightness. Brain regions where photomicrographs were captured are highlighted in the results via modified illustrations from the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998).

2.6: Comparative heatmap of c-Fos expression

A comparative heat map was generated utilizing single-label c-Fos data to better visualize differences in region-specific neuronal activation between intranasal treatment groups (i.e. saline, OxA, or [Ala^{11}, D-Leu^{15}]-OxB) across young and aged animals. The treatment groups used for producing the heat map were as follows: 1) Young saline (YS), 2) Young-OxA (YOA), 3) Young [Ala^{11}, D-Leu^{15}]-OxB (YOB), 4) Aged saline (AS), and 5) Aged OxA (AOA). For each brain region mapped, the data were computed across the total number of animals for each group with a minimum of n=7 animals per treatment group. The scaled colorimetric data within the heat map is represented as the ratio of average c-Fos densities for each brain region between the different treatment groups. All treatment groups were normalized to saline groups to yield the resulting heat map conditions: 1) YOA/YS, 2) YOB/YS, 3) AOA/AS, 4) AS/YS. The YOA and YOB experiments were performed at different time points; therefore, these groups were normalized using the corresponding YS group. In contrast, the AS/YS ratio was calculated using the average c-Fos densities pooled across both YS groups. Data calculations and analyses for the heat map were performed using Microsoft Excel 2016 for Macintosh (Microsoft Corporation, Redmond, WA, USA). The data was then

imported into MATLAB R2018a (MathWorks Inc., Natick, MA, USA) for generation of the colorimetric heat map.

2.7: *In-vivo* microdialysis

The *in-vivo* microdialysis technique is a commonly used tool in neuroscience research because of its ability to assess extracellular efflux of neurotransmitters real-time in freely behaving animals. The microdialysis probe itself is a semi-permeable membrane which, when in the brain, can collect neurotransmitters and neuropeptides. In concordance with the immunohistochemistry experiments, animals used for the *in-vivo* microdialysis experiments arrived in separate batches. Beginning 3 days after cannula implantation, animals were habituated in microdialysis bowls for 3-4 days and habituated with intranasal saline for 7 days. On microdialysis days, guide cannula stylets were removed and substituted with a microdialysis probe (BASi, West Lafayette, IN, USA) that extended 2 mm past the guide cannula. Probes were perfused at a 2 µl/min flow rate with artificial cerebrospinal fluid (aCSF, pH 7.4) containing: 150 mM NaCl, 3mM KCl, 1.7mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4.9 mM $_D$-glucose. Neostigmine bromide (50 nM; Sigma) was added to the aCSF to increase recovery of acetylcholine in collected dialysates. Dialysate collection started after a 3-hour discard period. Microdialysis sessions consisted of one hour (4 x 15-minute collections) of baseline collections followed by intranasal vehicle (0.9% saline) or OxA (100 µM; Enzo Life Sciences), administered in a total volume of 50 µl in 12.5 µl increments over a 2-minute period. The individual performing intranasal administration was not blinded to treatment conditions. Dialysate collection then continued for two hours (8 x 15-minute collections) post-treatment. Upon collection, dialysates were stored at -80ºC until analysis using

HPLC. All animals underwent two separate microdialysis sessions with an off day in-between and experiments were counterbalanced so that half of the animals received vehicle during session one, while the other half received OxA in session one. The day following the last microdialysis session, rats were euthanized and their brains processed for future probe placement verification using an acetylcholinesterase (AChE) background stain. Probe placement verification was performed after all HPLC samples were run for each respective animal. Any probe placement visualized outside the medial PFC excluded the animal from the study.

2.8: High-performance liquid chromatography and chromatogram analysis

Each 30 µl dialysate was split prior to analysis by high performance liquid chromatography with electrochemical detection (HPLC-ECD), with 20 µl analyzed for ACh and 10 µl analyzed for glutamate. ACh was analyzed using an HTEC-510 HPLC-ECD (EicomUSA; San Diego, CA, USA). Briefly, 20 µl of each dialysate was loaded into the AC-GEL separation column (2.0 ID x 150mm; EicomUSA) maintained at a constant 33ºC in combination with mobile phase (pH 8.5) containing 49.4 mM potassium bicarbonate ($KHCO_3$), 134.3 µM ethylenediaminetetraacetic acid disodium (EDTA-2Na), and 1.23 mM sodium 1- decanesulfonate. After analyte separation, post-column derivatization of ACh was attained through use of an AC-ENYM II enzyme reactor (1.0 ID x 4 mm; EicomUSA) containing acetylcholinesterase and choline oxidase, which generates hydrogen peroxide (H_2O_2) proportional to the amount of ACh present. The H_2O_2 is further broken down and detected on a platinum working electrode with an applied potential of +450 mV. The amount of ACh in each sample was measured by comparison with a three-point external standard curve with values predicted to be in

range of the collected dialysates. The limit of detection for this analysis was approximately 5 fmol/injection.

Glutamate levels in brain dialysates were analyzed using a CC-32 HPLC-ECD (BASi; West Lafayette, IN, USA) with modifications. First, 10 µl of each dialysate was loaded into the GU-GEL separation column (4.6 ID x 150mm; EicomUSA) in conjunction with a mobile phase (pH 7.2) containing 60 mM ammonium chloride-ammonium hydroxide, 134.3 nM EDTA-2Na, and 686 µM hexadecyltrimethylammonium bromide. After separation, post-column derivatization of glutamate was attained using an E-ENZ enzyme reactor (3.0 ID x 40 mm; EicomUSA) containing glutamate oxidase, which generates H_2O_2 proportional to the amount of glutamate present. The H_2O_2 is further broken down and detected on a 3.0 mm glassy carbon electrode (BASi) coated with a horseradish peroxidase osmium polyvinylpyridine solution (0 mV applied potential). The amount of glutamate in each dialysate was measured by comparison with a three-point standard curve using external standards expected to be in range of the collected dialysates. The limit of detection for this method was approximately 3 fmol/injection.

2.9: Attentional set-shifting paradigm

Cognitive impairment, particularly attentional dysfunction, is a principal feature of several neuropsychiatric and neurological disorders including narcolepsy (Rieger *et al.* 2003) and AD (Sarter and Turchi 2002; Perry and Hodges 1999b). These observed behavioral deficits stem, at least in part, from dysfunction within the prefrontal cortex (PFC) (Dannhauser *et al.* 2005; Hao *et al.* 2005; Sampath *et al.* 2017). Indeed, although

the ability to achieve simple cognitive tasks often remains intact, patients with neurological disorders often present with deficits in the ability to modify responses to complex behavioral tasks. In order to establish the homologous brain structure to the PFC in rodents, Birrell and Brown developed an attentional set-shifting task modeled after the Wisconsin Card Sorting Test (WCST) (Birrell and Brown 2000). In humans, the WCST is used to evaluate strategy-switching deficits in patients that have frontal lobe dysfunction (Lie *et al.* 2006; Nyhus and Barceló 2009). In primates, the medial prefrontal cortex facilitates the capacity to shift attention to different features of complex stimuli (Owen *et al.* 1991; Dias *et al.* 1996; Dias *et al.* 1997).

In rodents, the attentional set-shifting task takes each animal through a series of increasingly difficult tasks in which they must dig in small containers to locate a food reward (Birrell and Brown 2000). The specific procedures for our attentional set-shifting task were adapted and modified from previous studies using similar methods (Birrell and Brown 2000; Lapiz-Bluhm *et al.* 2008; Snyder *et al.* 2012; Barense 2002; Liston *et al.* 2006; Bissonette *et al.* 2013). Young and aged FBN/F1 rats were approximately 3-4 months and 26-28 months, respectively, upon arrival to the animal facility. Upon arrival, both young and aged animals were assigned to receive intranasal administration of either vehicle (50μL of 0.9% saline) or OxA (50μL of a 100μM solution; Enzo Life Sciences, Farmingdale, NY, USA). All animals were acclimatized to individual housing for at least 4 days prior to the start of any behavioral procedures. For approximately one week prior to testing, each rat was maintained on a food restricted diet of approximately 14g per day, such that by testing day, each rat weighed approximately 80-90% of their free-feeding body weight. Each animal received several days of gentle handling and habituation to

intranasal saline administration prior to the testing day. All behavioral experiments were conducted during the light portion of the cycle, between 07:00 and 19:00 h. Our testing apparatus was a custom-built black rectangular Plexiglas arena with inner dimensions of 75L x 40W x 30H (Snyder *et al.* 2012). The testing arena contained a black Plexiglas removable divider to separate one-third of the arena from the remaining two-thirds of the arena. This smaller portion of the arena served as the starting box for each rat and as a holding area between each trial. Each trial began after lifting the removable divider, allowing the rats to access the remainder of the arena. The larger portion of the divided arena contained an irremovable and opaque Plexiglas panel to split the arena into two sections. These two sections served as the holding area for the digging bowls. A visual representation of the testing arena is shown in Figure 2.3. The digging pots used for these experiments were small plastic containers (internal rim diameter 7 cm; depth 3.5 cm). Each digging pot was distinguished by a pair of cues along two different stimulus dimensions: (1) the digging medium held in each pot and (2) an odor applied to the rim of each pot. The relevant/irrelevant dimensions and the positive/negative cue pairs for each stage of the task are shown in Table 2.1. To mark each digging pot with an odor, approximately 50 µl of extract was initially applied to the inner rim of the pot. Each digging pot receives only one odor and a different pot is used for each combination. No specific odors or media are necessary for this experiment; however, odor and media pairings should be different enough for each animal to discriminate between (i.e. do not pair lemon & lime or wood beads & plastic beads). The reward used for these experiments, buried approximately 2 cm beneath the surface of each medium, was a 'bacon softie' broken into small pieces. For each trial, a small amount of reward was

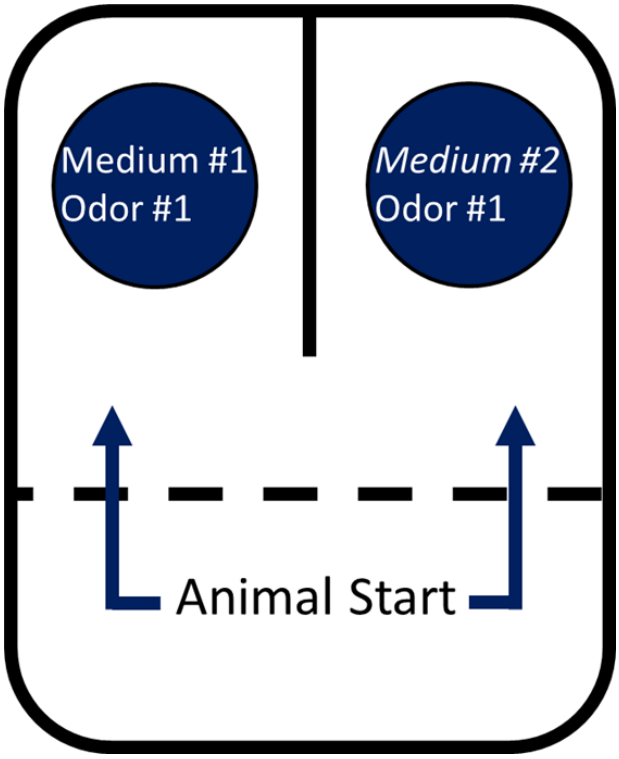

Figure 2.3 Representation of the testing arena used for the attentional set-shifting experiments. The dotted line represents the removable divider while the navy-blue circles represent the digging pots.

Table 2.1 Attentional set-shifting task stages progressing from simple discrimination to the extradimensional shift.

Task	Relevant Dimension	Irrelevant Dimension	(+)Cue Pairs (Rewarded)	(-)Cue Pairs (Not-Rewarded)
Simple Discrimination	Odor	None	Odor #1/Medium #1	Odor #2/Medium #1
Compound Discrimination	Odor	Medium	Odor #1/Medium #1 Odor#1/Medium #2	Odor #2/Medium #2 Odor#2/Medium #1
Intradimensional Shift	Odor	Medium	Odor #3/Medium #3 Odor#3/Medium #4	Odor #4/Medium #4 Odor#4/Medium #3
Reversal Learning	Odor	Medium	Odor #4/Medium #4 Odor#4/Medium #3	Odor #3/Medium #3 Odor#3/Medium #4
Extradimensional Shift	Medium	Odor	Medium #5/Odor #5 Medium #5/Odor #6	Medium #6/Odor #6 Medium #6/Odor #5

ground and applied to the surface of both pots to ensure that each animal was digging correctly based upon the positive cues rather than by smelling the food reward. After the animals were food restricted for at least 5 days, the 3-day behavioral procedure was conducted in the following manner:

Habituation Day (Day 1): before receiving their daily ration of standard rat chow, rats were trained to dig reliably in each pot for a food reward (bacon softie). First, two unscented plastic digging pots are placed into the home cage and baited. Each animal is first allowed to retrieve the reward from both pots without being covered. If the animal has not retrieved both food rewards after 5 minutes, the animal is given a 'time-out' period of 30 minutes and subsequently retried. Ultimately, each animal is required to dig for the food reward in each pot in 3 trials of 5 minutes each, with the food reward covered with an increasing amount of sawdust during each exposure. After the animal is digging reliably, it is placed in the testing arena and habituated to digging in the same manner described above.

Training Day (Day 2): before receiving their daily ration of standard rat chow, rats are trained on a simple discrimination (SD) task to a criterion of six consecutive correct trials. Each trial during the discrimination tasks was timed for up to 10 minutes. If the animal did not retrieve the reward after 10 minutes, a non-digging response, or 'error', was recorded. Any animal with six consecutive errors was subsequently excluded from further experimentation. All rats start by learning to associate the food reward with the positive odor cue (the starting media is sawdust in both digging pots). In these experiments, rosemary and orange were used as the odors. After six consecutive correct trials, the animals were then trained to discriminate between two different media to

achieve a reward. For these experiments, wood chips and gauze were used as the media. All animals were trained with these odors and media with the positive and negative cues randomly chosen for each animal. Importantly, the training stimuli were never used on testing days to ensure that animals were not remembering odor or media pairings.

Testing Day (Day 3): before receiving their daily ration of standard rat chow, each animal was tested on a series of five increasingly difficult discrimination tasks (Table 2.1). Again, the criterion used to proceed to the next stage of the experiment was six consecutive correct digging responses. The first stage, like training day, was a simple discrimination (SD) task. For the SD stage, half of the animals were required to discriminate between two odors (i.e. strawberry vs. cinnamon) to retrieve a reward while the other half were required to discriminate between two media (i.e. paper vs. felt) to retrieve a reward. Only one of the digging pots (i.e. the positive cue pot) contained the food reward. The second stage was a compound discrimination task (CD), where the irrelevant stimuli from the other dimension were introduced, but the animal was still required to discriminate between the two original stimuli from the SD stage. The third stage was an intradimensional shift (IDS), where all new stimuli were used, however, the task-relevant dimension was unchanged. For example, if the starting relevant dimension was odor, the IDS stage would introduce new odors and media, however, the relevant discriminatory dimension would still be odor. At this point in the task, the animal was given its predetermined treatment (i.e. intranasal saline or intranasal OxA). This timepoint for treatment was based upon the *in-vivo* microdialysis studies where the effect of intranasal OxA on PFC acetylcholine efflux lasted approximately 30-60 minutes. Because the ASST experiment lasts 2-3 hours for each animal, intranasal OxA was given

just before the reversal learning stage (REV) of the task to allow for optimal assessment of each treatment on prefrontal cortical function. The fourth stage of the task was a reversal learning (REV) stage, where the relevant dimension remains the same, however, the previously negative cue is now the positive cue. The fifth and final stage was an extradimensional attentional shift (EDS). In this stage, all new stimuli are introduced, but now the relevant dimension is changed. For example, if the starting relevant dimension was odor, the EDS stage would introduce new odors and media, and the relevant discriminatory dimension would change to media. For each animal, assignment to a positive or negative cue, relevant starting dimension, and left-right positioning of the pots within the arena were determined randomly in advance.

The main dependent measure in the attentional set-shifting task is the number of trials required to reach the established criterion (6 consecutive correct responses). A higher number of trials to completion indicates that the animal took more trials to effectively learn a new rule. Due to the increasing difficulty of each stage, the latter stages of the ASST experiment are expected to require more trials. The validity of the attentional set-shifting task (ASST) as a model of prefrontal cortical dysfunction has been demonstrated through lesioning studies to subsections of the rat prefrontal cortex. Lesioning the rat medial prefrontal cortex (mPFC) results in selective deficits on the extradimensional shift (EDS) stage of the ASST task, while lesions to the orbitofrontal cortex (OFC) selectively disrupted performance on the reversal learning (REV) stage of the task (Birrell and Brown 2000; McAlonan and Brown 2003). Furthermore, the validity of this task in measuring deficits in age-related cognitive performance is demonstrated in a study showing aged rats are impaired on the EDS stage of the task (Barense 2002).

While this task is at times complex, it is a powerful experimental tool that can be used to test hypotheses related to the mechanisms involved in treating age-related cognitive dysfunction.

2.10: Statistics and data analysis

For all immunohistochemistry experiments, single-labeled (c-Fos) and double-labeled (c-Fos + ChAT/PV) positive cells were counted within the confines of a reticle fixed into the eyepiece of the microscope. Counts for each brain region were determined by the total number of immunopositive nuclei/cells from two representative sections at different levels of the rostro-caudal gradient. Single-label c-Fos data were expressed as the density of immunopositive nuclei counted within the reticle area (c-Fos nuclei/mm^2). Statistical analyses of these data were analyzed by two-tailed unpaired *t*-tests (GraphPad Prism 8; GraphPad Software for Macintosh, La Jolla, CA, USA). Double-labeled neurons were expressed as the percentage of the total number of ChAT/PV neurons positive for c-Fos within the reticle area (i.e. % Double Labeled Neurons). Dual-label immunoperoxidase data were analyzed by two-tailed unpaired *t*-tests. Significant effects of treatment condition (i.e., OxA or saline) across age were determined by one-way ANOVA followed by Tukey's multiple comparisons test. A significance cutoff level of $p<0.05$ was used for all analyses.

For the *in-vivo* microdialysis data, baseline neurotransmitter efflux was obtained during the first four sample collections (i.e. timepoints 1-4) and averaged to yield mean basal efflux. For each sample analyzed, a raw value was obtained and expressed as pmol/20μL for ACh and μmol/l0μL for glutamate. The graphed *in-vivo* microdialysis

data are expressed as a percentage of the average baseline to account for individual variation in basal neurotransmitter efflux. Data were analyzed using two-way repeated-measure ANOVAs (GraphPad Prism 8; GraphPad Software; La Jolla, CA, USA) with treatment as a within-subjects variable, age as a between-subjects variable, and time as a repeated measure. Significant interactions and main effects of treatment (i.e., OxA or saline) or age were probed with Tukey multiple comparisons tests. A significance cutoff level of $p<0.05$ was used for all analyses.

For the attentional set-shifting experiments, trials to reach criterion (6 consecutive correct trials) were recorded for each rat. Because intranasal saline or intranasal OxA was given just before the REV stage of the ASST experiment, the first three stages and the final two stages were assessed differently. For the first 3 stages of the task (i.e. SD, CD, IDS; before treatment), the groups were collapsed to assess differences in task performance between young and aged animals. These results were analyzed using two-tailed unpaired t-tests. For the final 2 stages of the task (i.e. REV, EDS; after treatment), each treatment group was analyzed on its own using ordinary one-way ANOVAs followed by Holm-Sidak multiple comparisons to determine significant differences between the treatment groups. Data were represented as total number of trials to reach criterion (y-axis) for each stage (x-axis). A significance cutoff level of $p<0.05$ was used for all analyses.

CHAPTER 3

EFFECTS OF INTRANASAL OREXIN-A OR [ALA^{11}, D-LEU^{15}]-OREXIN-B ON NEURONAL ACTIVATION

3.1: Introduction

Because of orexin's role in important physiological and behavioral phenomena such as stabilization of sleep/wake states, food intake, addiction, and cognition there is much interest in orexin receptors as potential therapeutic targets. The two orexin peptides, OxA and orexin-B, act on two G protein-coupled receptors, with the orexin-1 receptor (Ox1R) binding OxA with high affinity and the orexin-2 receptor (Ox2R) binding both OxA and orexin-B with relatively high affinity (Smart and Jerman 2002; Ammoun et al. 2003; Gotter et al. 2012b; Leonard and Kukkonen 2014). Several single- and dual-orexin receptor antagonists have been developed (Smart et al. 2001; Gotter et al. 2012a; Steiner et al. 2013; Roecker et al. 2016; Skudlarek et al. 2017) but there is a paucity of selective orexin agonists that might have experimental or clinical utility (Mieda and Sakurai 2013; Turku et al. 2017). Administration of the native peptides, meanwhile, is limited by potential peripheral side effects and limited ability to target the brain due to peripheral degradation and the blood-brain barrier. One route that has been suggested for brain delivery of orexins and other neuropeptides is intranasal administration (Hallschmid and Born 2008; Hanson and Frey 2008; Dhuria et al. 2009; Lammers 2011; Spetter and Hallschmid 2015). Intranasal OxA, for example, has been shown to ameliorate attentional deficits produced by sleep deprivation in non-human

primates (Deadwyler et al. 2007). Rodent studies have further shown that intranasal OxA increases food intake and locomotor activity (Dhuria et al. 2016). Finally, limited human trials have suggested that intranasal OxA can ameliorate wakefulness, olfactory, and cognitive correlates of narcolepsy (Hallschmid and Born 2008; Baier *et al.* 2011; Weinhold *et al.* 2014a). Despite these intriguing findings, however, our understanding of the anatomical and neurochemical mechanisms that may underlie the effect of intranasal orexin on cognition and behavior remains extremely limited. Accordingly, the goal of this first experiment was to use immunohistochemistry to directly examine the effects of intranasal OxA and intranasal [Ala^{11}, D-Leu^{15}]-orexin-B on c-Fos expression, a marker for neuronal activation (Kaczmarek 1992), in multiple phenotypic neurons of young and aged rats.

3.2: Methods

The methods described herein are an abbreviated version of those described in 'Chapter 2: General Materials and Materials'. Young (3-4 months, 250-300g) and aged (26-28 months, 450-550g) male Fisher 344/Brown Norway F1 hybrid rats (Harlan/NIA) were used for all experiments. Upon arrival, animals were randomly assigned to receive intranasal administration of either vehicle (50 µl of 0.9% saline), OxA (50 µl of a 100 µM solution; Enzo Life Sciences), or [Ala^{11}, D-Leu^{15}]-orexin-B (50 µl of a 100 µM solution; Tocris Bioscience). Separate batches of animals were used for the immunohistochemistry experiments. On testing day, each batch of animals were dosed so that each treatment on a given day was represented equally, with the order of treatments for each animal being chosen pseudo-randomly. All solutions were administered in four 12.5 µl increments over a two-minute period. Each animal was habituated with intranasal

saline treatment for 7 days before experimentation. Beginning on day 8, animals received their assigned treatment and were sacrificed two hours later, the time at which c-Fos expression peaks (Kaczmarek 1992). Rats were anesthetized with isoflurane and perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA).

3.2.1: Immunohistochemistry

For single-label immunohistochemistry, free floating sections were incubated with a rabbit-anti c-Fos antibody for 48 h at 4ºC followed by a biotinylated donkey anti-rabbit secondary antibody for 1.5 h at room temperature (RT), and horseradish peroxidase conjugated streptavidin for 1 hour at RT. c-Fos staining was developed with 0.3% hydrogen peroxide and nickel-cobalt enhanced diaminobenzidine to yield blue-black immunopositive nuclei. For dual-label immunohistochemistry, the above steps were followed with incubation in either a goat anti-choline acetyltransferase or mouse anti-parvalbumin primary antibody for 48 h at 4ºC. Secondary and tertiary antibody incubation followed using either unlabeled donkey anti-goat or unlabeled donkey anti-mouse for 2 hours at RT, and either goat peroxidase anti-peroxidase or mouse peroxidase anti-peroxidase for 1.5 hour at RT. ChAT and PV immunoreactivity were developed with 3% hydrogen peroxide and diaminobenzidine to yield brown immunopositive cell bodies. Sections were mounted onto slides with 0.15% gelatin, dried overnight, dehydrated, delipidated, and cover-slipped using DEPEX mounting medium.

3.2.2: Immunofluorescence of peptide localization

In order to perform a qualitative examination of where intranasal OxA distributed within the brain, a subset of animals (n=4, young; n=4, aged) received intranasal

administration of either saline or a modified OxA peptide labeled with a green-fluorescent fluorophore 5(6)-FAM-(Glu[1])-OxA trifluoroacetate salt. Animals receiving the fluorescein tagged OxA peptide received 50 μL of a 500 μM dose split into four 12.5 μL increments over a two-minute period and were sacrificed thirty minutes post-treatment. This timepoint was chosen based upon a previous study showing peak appearance of ^{125}I–OxA in the CNS thirty minutes after intranasal delivery (Dhuria et al. 2009). Perfusion and brain sectioning were as described in the methods chapter above. Sections were mounted onto slides with 0.15% gelatin, dried overnight, dehydrated only and cover-slipped using Permount mounting medium.

3.2.3: Imaging

Histological experiments for single-labeled (c-Fos) cells, double-labeled (c-Fos + ChAT or PV) cells, and AChE background staining were visualized using a Nikon E600 microscope fitted with a CoolSNAP digital camera. Fluorescence images were visualized using a Nikon E600 microscope or a Leica SP8 multiphoton confocal microscope equipped with LAS AF 3 analysis software. Immunoperoxidase photomicrographs were captured using IP Lab Software. Images were imported into Adobe Photoshop 6.0 to adjust the image size and to make minor alterations to contrast and brightness. To indicate the approximate brain region where photomicrographs were obtained, diagrams and schematics of brain regions in the results section were adapted from The Rat Brain by Paxinos and Watson (Paxinos and Watson 1998).

3.2.4: Statistics and data analysis

For all immunohistochemistry experiments, single-labeled (c-Fos) and double-labeled (c-Fos + ChAT/PV) positive cells were counted within the confines of a reticle fixed into the eyepiece of the microscope. Counts for each brain region were determined by the total number of immunopositive nuclei/cells from two representative sections at different levels of the rostro-caudal gradient. Single-label c-Fos data were expressed as the density of immunopositive nuclei counted within the reticle area (c-Fos nuclei/mm^2). Statistical analyses of these data were analyzed by two-tailed unpaired *t*-tests (GraphPad Prism 8; GraphPad Software for Macintosh, La Jolla, CA, USA). Double-labeled neurons were expressed as the percentage of the total number of ChAT/PV neurons positive for c-Fos within the reticle area (i.e. % Double Labeled Neurons). Dual-label immunoperoxidase data were analyzed by two-tailed unpaired *t*-tests. Significant effects of treatment condition (i.e., OxA or saline) across age were determined by one-way ANOVA followed by Tukey's multiple comparisons test. A significance cutoff level of $p<0.05$ was used for all analyses.

3.3: Results

3.3.1: Intranasal 5(6)-FAM-(Glu^1)-orexin-A administration

To visually assess the possible modes by which intranasal OxA enters the brain and to provide qualitative supporting evidence for the above results, we intranasally administered a fluorescein-tagged OxA peptide 5(6)-FAM-(Glu¹)-OxA. This ultimately revealed a distribution of the peptide to both rostral and caudal brain regions. Prior work has suggested that intranasally delivered peptides reach the brain via diffusion along sensory trigeminal pathways (Hanson and Frey 2008; Chapman et al. 2013; Spetter and

Hallschmid 2015; Meredith et al. 2015). Therefore, we visualized delivery of the fluorescein-tagged peptide in the principal sensory and spinal subdivisions of the trigeminal nucleus (Figure 3.1; c, d), the sources of somatosensory innervation of the olfactory mucosa. The fluorescein-tagged OxA peptide was also detected in cortical regions, including the medial PFC (Figure 3.1; a, b). Due to the qualitative nature of these experiments, a relatively modest treatment group sizes were used (n=4, young; n=4, aged). This pattern of fluorescence was not observed following intranasal vehicle (saline) administration (n=4, young; n=4, aged).

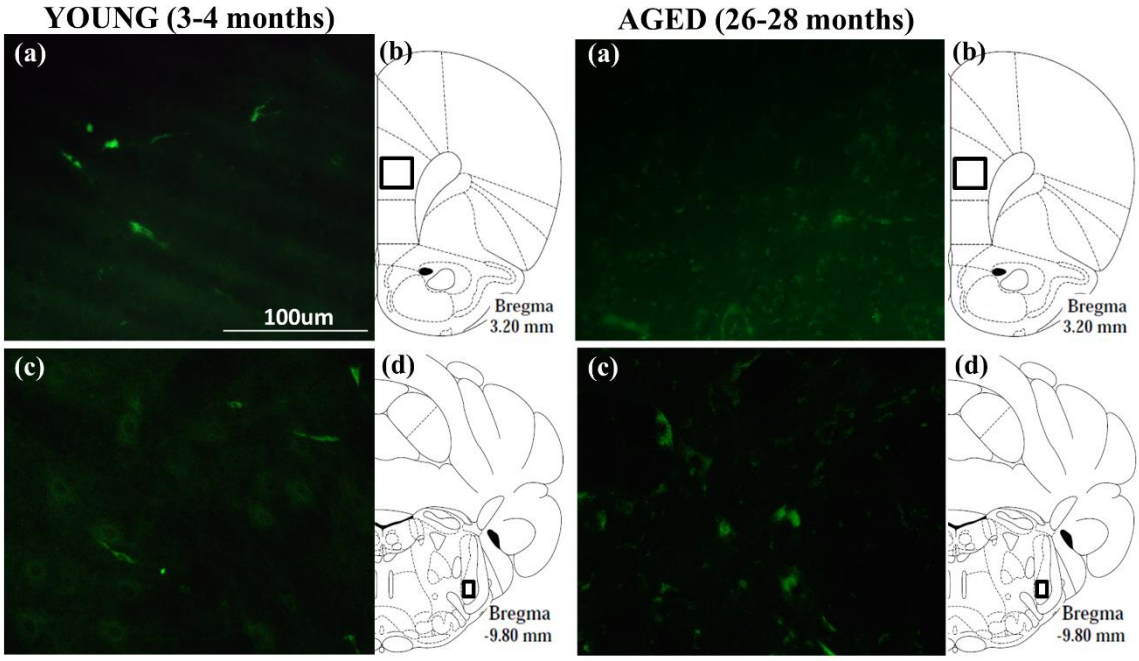

Figure 3.1 Localization of a green-fluorescent tagged OxA peptide in the brain after intranasal administration. (a) Typical distribution of the fluorescent-tagged OxA peptide (50 µl, 500 µM) in the PrLC after intranasal administration. Green fluorescence indicates the appearance of the labeled peptide in the PrLC. (b) Schematic indicating the approximate location (black-outlined square) within the PrLC where fluorescence photomicrographs were obtained. (c) Typical distribution of the fluorescent-tagged OxA peptide in the SpTrN. (d) Schematic indicating the approximate location (black-outlined square) within the SpTrN where fluorescence photomicrographs were obtained. Abbreviations: OxA, orexin-A; PrLC, prelimbic prefrontal cortex; SpTrN, spinal trigeminal nucleus. Scale bar approximately 100 µm.

3.3.2: Effects of intranasal orexin-A/ [Ala^{11}, D-Leu^{15}]-orexin-B on c-Fos expression

Intranasal administration of a fluorescein-tagged OxA peptide resulted in delivery to both rostral (prefrontal cortex) and caudal (spinal trigeminal nucleus) brain regions in young and aged animals indicating that intranasal orexins have the potential to quickly and efficiently enter the brain. Accordingly, the goal of the next set of experiments was to utilize immunohistochemistry to examine the effects of intranasal orexin delivery on c-Fos expression, a marker for neuronal activation.

3.3.2.a: Effects of intranasal orexin-A on c-Fos in young animals

Intranasal administration of OxA significantly increased c-Fos expression in multiple brain regions of young animals (Table 3.1). In the cortex, intranasal OxA increased c-Fos expression in the prelimbic (PrLC; t_{14}=3.163, p=0.0069), agranular insular (AIC; t_{14}=3.291, p=0.0054), ventral orbital (VOC; t_{13}=2.357, p=0.0348), and piriform areas (PirC; t_{14}=2.259, p=0.0404). Importantly, this effect was not a global phenomenon as intranasal OxA failed to significantly alter c-Fos expression within the claustrum, medial orbital cortex (MOC), and all regions of the hippocampus. In fact, there was a strong trend for decreased c-Fos expression in the infralimbic cortex (t_{14}=2.092, p=0.0551) after intranasal OxA administration. We also considered the possibility that intranasal OxA delivery may alter c-Fos expression within brainstem regions involved in arousal. Indeed, intranasal OxA administration significantly increased c-Fos expression within the pedunculopontine nucleus (PPTg; t_{13}=2.692, p=0.0185) (Table 3.1). As an example, density measurements for c-Fos were made using a 0.179 mm x 0.179 mm area in the center of the prelimbic cortex. For c-Fos counts within

brainstem regions, measurements represent the total number of c-Fos positive nuclei within the entire brain region.

Table 3.1 c-Fos densities (nuclei/mm^2) in each brain region after intranasal vehicle (saline) or intranasal OxA (50 µl, 100 µM) administration in young animals.

c-Fos density (nuclei/mm^2)		
Brain Region	**Vehicle**	**100 uM OxA**
Piriform Cortex*	582 ± 38.24, n=8	777.3 ± 77.55, n=8
Agranular Insular Cortex**	125 ± 10.23, n=8	277.3 ± 45.15, n=8
Prelimbic Cortex**	859.4 ± 86.19, n=8	1234 ± 81.4, n=8
Ventral Orbital Cortex*	703.1 ± 80.97, n=8	959.8 ± 70.45, n=7
Infralimibc Cortex **(p=0.0551)**	890.6 ± 38.27, n=8	714.8 ± 74.8, n=8
Medial Orbital Cortex	609.4 ± 81.62, n=8	660.7 ± 56, n=7
Perirhinal Cortex	334.7 ± 78.12, n=8	394.9 ± 47.22, n=8
Claustrum	605.5 ± 45.34, n=8	722.7 ± 69.23, n=8
Hippocampus (CA3)	17.35 ± 4.1, n=8	23.47 ± 3.085, n=8
Hippocampus (CA1)	14.54 ± 3.437, n=8	19.39 ± 3.876, n=8
Hippocampus (Dentate Gyrus)	73.98 ± 3.734, n=8	85.71 ± 10.09, n=8
Pedunculopontine Nucleus*	4.571 ± 1.043 n=7	8.125 ± 0.8332 n=8
***= p<0.05; **=p<0.01**		

3.3.2.b: Effects of intranasal [Ala^{11}, D-Leu^{15}]-orexin-B on c-Fos in young animals

Intranasal [Ala^{11}, D-Leu^{15}]-OxB administration increased neuronal activation (c-Fos expression) in cortical and basal forebrain regions (Figure 3.2). In the cortex, intranasal [Ala^{11}, D-Leu^{15}]-OxB administration significantly increased activation in the

piriform cortex (t_{12} = 3.224, p = 0.0073) and the agranular insular cortex (t_{12} = 2.519, p = 0.0269) when compared to intranasal saline treated animals. In addition, there a was a strong trend for increased activation in the prelimbic cortex (t_{12} = 2.033, p = 0.0647). In

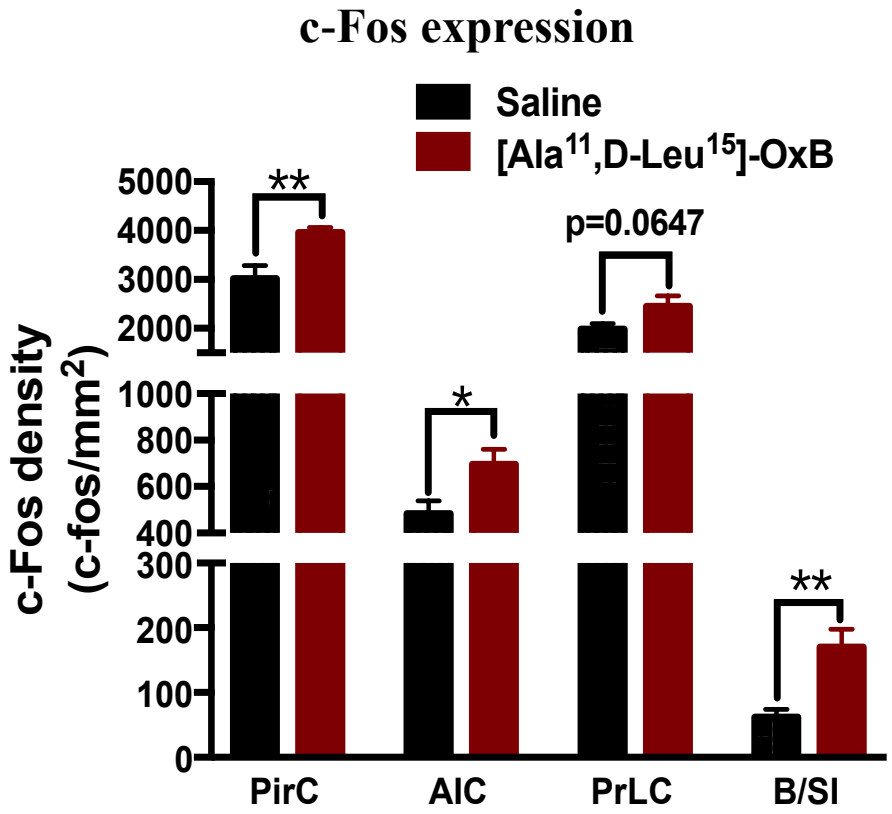

Figure 3.2 Neuronal activation (c-Fos expression density) in cortical and basal forebrain regions following intranasal [Ala^{11}, D-Leu^{15}]-OxB administration in young rats. Single-labeled c-Fos densities in brain regions of animals treated with intranasal vehicle (saline; PirC, AIC, PrLC, B/SI, *n=7* rats) or intranasal [Ala^{11},D-Leu^{15}]-OxB (50 µL, 100 µM; PirC, AIC, PrLC, B/SI, *n=7* rats). Intranasal [Ala^{11}, D-Leu^{15}]-OxB administration significantly increased c-Fos expression within the PirC, AIC, and B/SI regions compared to vehicle treated controls. There was a strong trend for increased c-Fos expression within the PrLC after intranasal [Ala^{11}, D-Leu^{15}]-OxB administration. Abbreviations: Ala, Alanine; D-Leu, D-Leucine; OxB, orexin-B; PirC, piriform cortex; AIC, agranular insular cortex; B/SI, nucleus basalis/substantia innominata; PrLC, prelimbic prefrontal cortex. Error bars represent SEM. $^{**}p < 0.01$, $^{*}p < 0.05$

the basal forebrain, intranasal [Ala^{11}, D-Leu^{15}]-OxB administration significantly increased c-Fos expression within the nucleus basalis/substantia innominata (t_{12} = 3.663, p = 0.0032) compared to intranasal saline treatment. Density measurements for c-Fos were obtained using a 0.032 mm^2 area within cortical regions and a 0.1225 mm^2 area within basal forebrain regions. While intranasal [Ala^{11}, D-Leu^{15}]-OxB did elicit increases in c-Fos expression within some brain regions, intranasal OxA was able to increase c-Fos expression across a broader range of the neuraxis, including the prefrontal cortex. Therefore, the studies discussed henceforth focus on examining the effects of intranasal OxA on neurotransmission and cognition.

3.3.2.c: Effects of intranasal orexin-A on c-Fos in aged animals

Intranasal administration of OxA significantly increased c-Fos expression in multiple brain regions of aged animals (Table 3.2). In the cortex, intranasal OxA increased c-Fos expression in the prelimbic cortex (PrLC; t_{14}=4.276, p=0.0008) and agranular insular cortex (AIC; t_{14}=3.222, p=0.0061). Importantly, intranasal OxA did not globally excite all brain regions as there were no significant changes in c-Fos expression within the piriform cortex, ventral orbital cortex, nucleus accumbens shell, retrosplenial cortex, and regions CA3 and CA1 of the hippocampus. Interestingly, there was a strong trend for decreased c-Fos expression in the infralimbic cortex (t_{14}=1.91, p=0.0769) after intranasal OxA administration. We also considered the possibility that intranasal OxA delivery may alter c-Fos expression in brain regions outside of the cortex. Indeed, intranasal OxA also significantly increased c-Fos expression in the claustrum (Cl; t_{14}=3.055, p=0.0086) and in the dentate gyrus of the hippocampus (DG; t_{14}=2.497, p=0.0256) (Table 3.2). As an example, density measurements for c-Fos were made using

a 0.179 mm x 0.179 mm area in the center of the prelimbic cortex. For brainstem c-Fos counts, measurements represent the total number of c-Fos positive nuclei within the entire brain region.

Table 3.2 c-Fos densities (nuclei/mm^2) in each brain region after intranasal vehicle (saline) or intranasal OxA (50 µl, 100 µM) administration in aged animals.

c-Fos density (nuclei/mm^2)		
Brain Region	**Vehicle**	**100 uM OxA**
Piriform Cortex	3770 ± 407.2, n=8	4469 ± 422.4, n=8
Agranular Insular Cortex**	285.2 ± 51.29, n=8	699.2 ± 117.8, n=8
Prelimbic Cortex***	2023 ± 120.3, n=8	3141 ± 231.9, n=8
Ventral Orbital Cortex	2754 ± 277.6, n=8	3574 ± 379.6, n=8
Infralimibc Cortex **(p=0.0769)**	2102 ± 110.9, n=8	1816 ± 99.95, n=8
Claustrum**	1805 ± 96.45, n=8	2238 ± 104.1, n=8
Nucleus Accumbens Shell	620.4 ± 47.92 n=8	754.1 ± 65.74 n=8
Retrosplenial Cortex **(p=0.0988)**	1043 ± 102.1 n=8	1376 ± 158.0 n=8
Hippocampus (CA3) **(p=0.0813)**	33.16 ± 5.142 n=8	47.70 ± 5.784 n=8
Hippocampus (CA1)	54.59 ± 9.899 n=8	72.70 ± 13.77 n=8
Hippocampus (Dentate Gyrus)*	138.3 ± 11.84 n=8	185.2 ± 14.60 n=8
***= p<0.05; **=p<0.01; ***=p<0.001**		

3.3.2.d: Effects of intranasal orexin administration across treatment and age

In addition to using intranasal [Ala^{11}, D-Leu^{15}]-OxB administration in young animals, we have combined intranasal OxA administration and immunoperoxidase staining for c-Fos in aged animals. To compare the effects aging and intranasal orexin administration (i.e. OxA or [Ala^{11}, D-Leu^{15}]-OxB) in multiple brain regions, we

constructed a heatmap using c-Fos expression ratios that were normalized to intranasal saline treated controls (Figure 3.3). The range of ratios that we observed fell between 0.4 and 3.8, with lower scores lighter in color and higher scores darker in color. Scores above 1 indicate higher c-Fos expression when compared to saline treated controls and vice-versa for scores below 1. On average, the effects of intranasal OxA treatment in aged animals were more robust than the intranasal OxA treatment in young animals. Aged animals also exhibited higher 'basal' c-Fos expression in most brain regions compared to young animals, as indicated by the AS/YS treatment comparison. When comparing the effects between the OxA and [Ala^{11}, D-Leu^{15}]-OxB treatments (i.e. YOA/YS vs. YOB/YS), the YOA/YS group shows higher c-Fos expression ratios in most brain regions. Accordingly, YOA/YS and YOB/YS groups qualitatively suggest that intranasal OxA and intranasal [Ala^{11}, D-Leu^{15}]-OxB administration increases c-Fos expression in young animals. Further statistical analysis on these differences from prior intranasal OxA studies and from our data presented above (Figure 3.3) show that significant differences in c-Fos expression are localized to specific brain regions. Specifically, in the cortex of young animals, intranasal OxA significantly increased c-Fos expression in the piriform, agranular insular, prelimbic, and ventral orbital cortices (Calva *et al.* 2018). Significant increases in cortical c-Fos expression after intranasal [Ala^{11}, D-Leu^{15}]-OxB were limited to the piriform and agranular insular cortices. In addition, the higher ratios present in the AS/YS group suggests that aged animals exhibit higher basal levels of activation.

3.3.3: Effects of intranasal orexin-A on c-Fos expression in cholinergic neurons

Given the bounty of evidence showing that OxA activates the BFCS (Eggermann et al. 2001; Fadel et al. 2005; Fadel and Frederick-Duus 2008; Arrigoni et al. 2010; Fadel

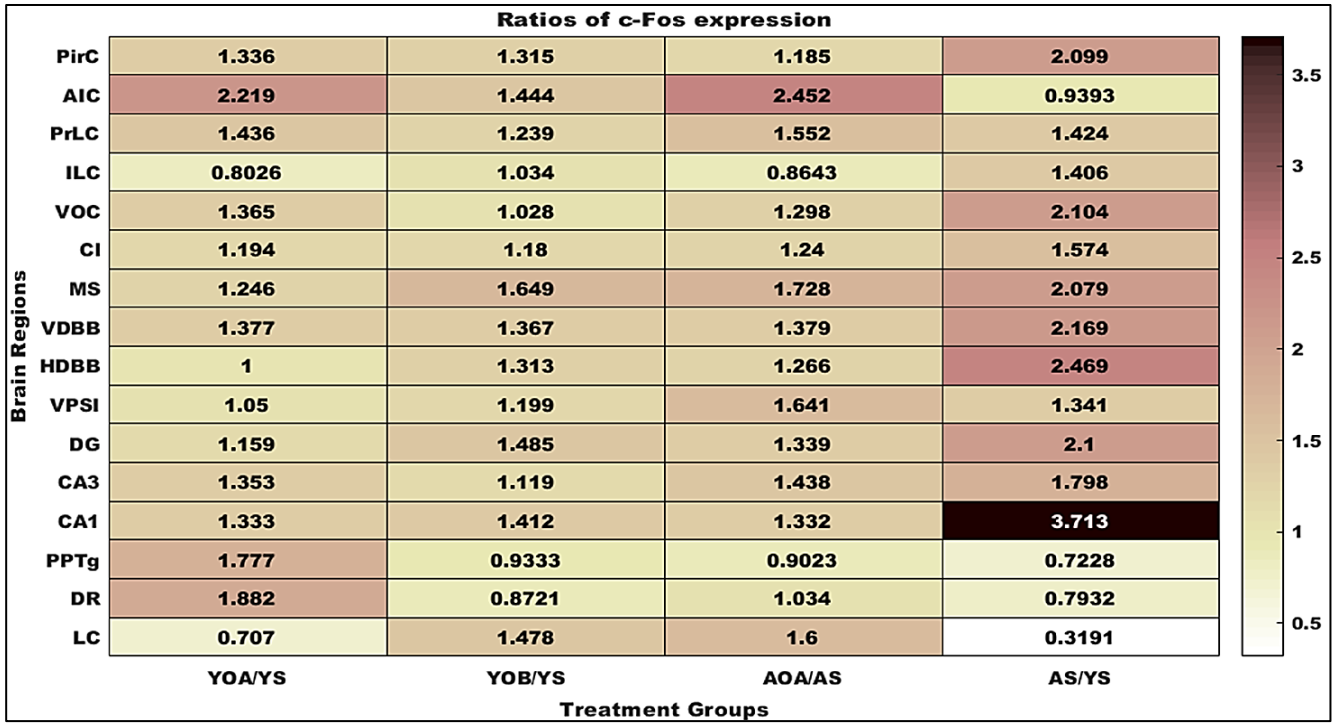

Figure 3.3 Heatmap of c-Fos expression ratios after treatment with intranasal orexin. Treatment groups: 1) YOA/YS (YOA and YS, *n=8* rats), 2) YOB/YS (YOB and YS, *n=7* rats), 3) AOA/AS (AOA and AS, *n=8* rats), and 4) AS/YS (AS, *n=8* rats; YS, *n=15* rats). Abbreviations: YOA/YS, young-orexin-A/young saline; YOB/YS, young-[Ala^{11},D-Leu^{15}]-orexin-B/young saline; AOA/AS, aged-orexin-A/aged saline; AS/YS, aged saline/young saline; OxA, orexin-A; [Ala^{11},D-Leu^{15}]-OxB, [Ala^{11},D-Leu^{15}]-orexin-B; PirC, piriform cortex; AIC, agranular insular cortex; PrLC, prelimbic cortex; ILC, infralimbic cortex; VOC, ventral orbital cortex; Cl, claustrum; MS, medial septum; VDBB, vertical limb of the diagonal band; HDBB, horizontal limb of the diagonal band; VP/SI, ventral pallidum/substantia innominata; DG, dentate gyrus; CA3, cornu ammonis 3; CA1, cornu ammonis 1; PPTg, pedunculopontine tegmentum; DR, dorsal raphe; LC, locus coeruleus.

and Burk 2010), we investigated if intranasal administration of OxA would elicit a similar response in young and aged animals. Indeed, analysis by one-way ANOVA indicated a significant effect of treatment for the medial septum (MS; $F_{3,28}$ = 9.516, p=0.0002), the vertical limb of the diagonal band (VDBB; $F_{3,28}$=13.16, p<0.0001), the horizontal limb of the diagonal band (HDBB; $F_{3,28}$=12.56, p<0.0001), and the ventral pallidum/substantia innominata (VP/SI; $F_{3,28}$=12.34, p<0.0001). Further analysis with Tukey's multiple comparisons test revealed that intranasal OxA increased c-Fos expression in cholinergic (ChAT+) neurons of the MS (p=0.0168), VDBB (p=0.0287), and the VP/SI (p=0.0007) of aged animals compared to treatment with intranasal saline. In young animals, Tukey's multiple comparisons test revealed that intranasal OxA increased c-Fos expression in ChAT+ neurons the VDBB (p=0.0002) and VP/SI (p=0.0040). Interestingly, both portions of the diagonal band exhibited higher 'basal' levels of cholinergic neuron activation in aged animals compared to young animals (Figure 3.4). Furthermore, intranasal OxA activated a higher percentage of ChAT+ neurons in the MS and HDBB in aged animals, although activation in the HDBB is quite high under control conditions (Figure 3.4). Overall, data are expressed as the percentage of ChAT+ neurons that also express c-Fos (Figure 3.4). Single and double labeled ChAT neurons were counted within the same 0.45mm x 0.45 mm area for each basal forebrain region so that direct comparisons could be established. As an example of the area where c-Fos counts were obtained, the reticle area in the VDBB is depicted in the adapted diagram (Figure 3.5). Double–labeled ChAT neurons of the VDBB are represented by arrows pointing to the blue-black immunoreactivity for c-Fos and brown immunoreactivity for ChAT (Figure 3.5)

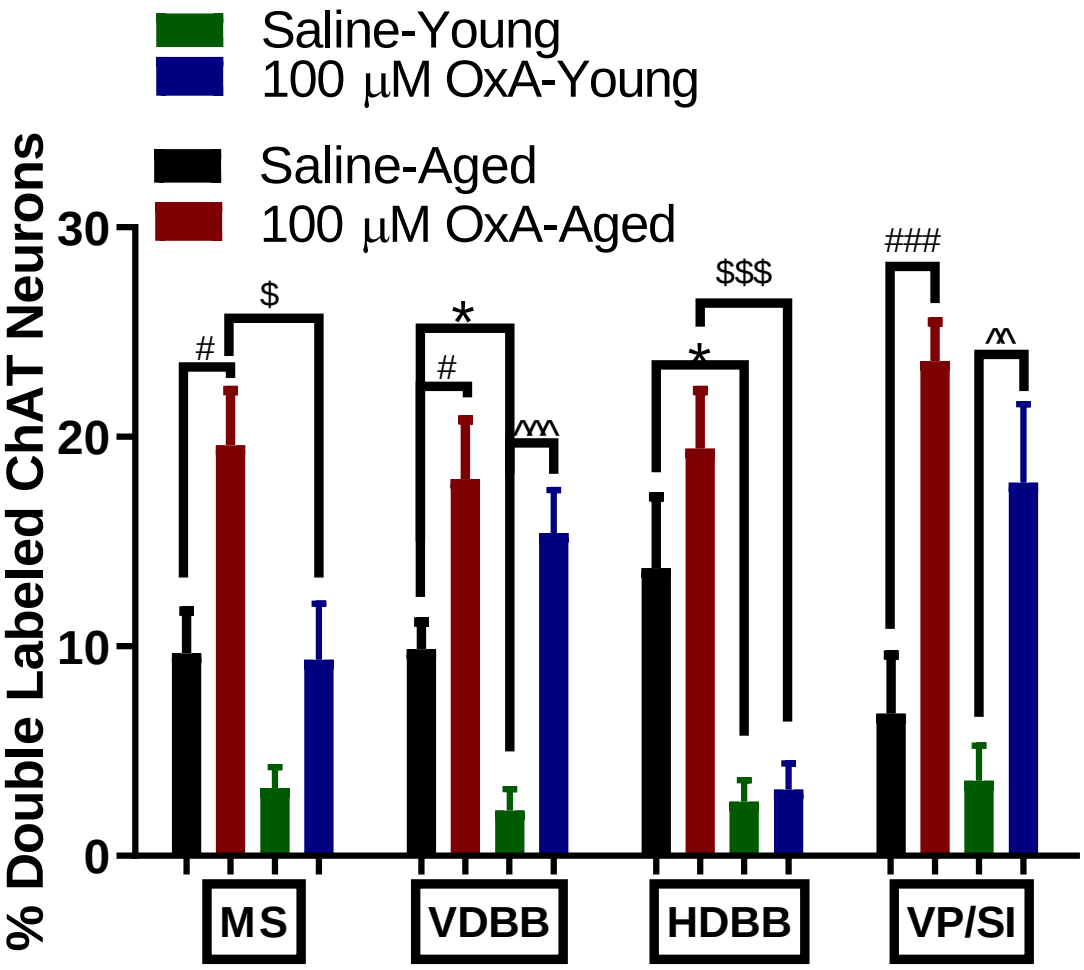

Figure 3.4 Activation of cholinergic neurons in the basal forebrain by intranasal OxA administration. Intranasal OxA significantly increased the percentage of dual-labeled ChAT neurons in the MS, VDBB, and VP/SI of aged animals (n=8, saline; n=8, OxA). In young animals, intranasal OxA significantly increased c-Fos expression in ChAT+ neurons within the VDBB and VP/SI. Data are represented as the percentage of double-labeled (c-Fos/ChAT) neurons relative to the total number of ChAT-positive neurons within the basal forebrain. #/$/*=p<.05, ^^=p<.01, $$$/###/^^^=p<.001

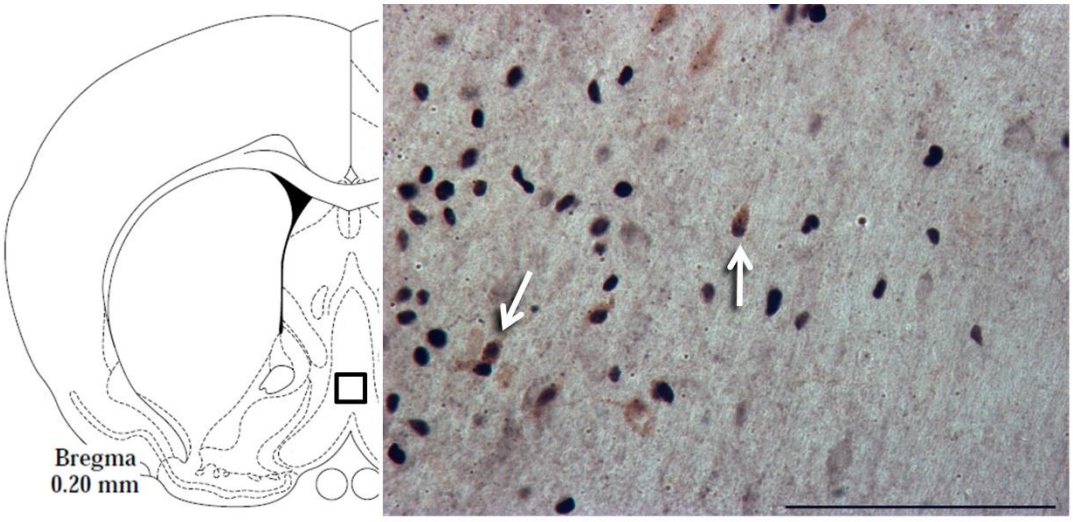

Figure 3.5 Representative photomicrograph depicting double-labeled cholinergic (ChAT+) neurons in the basal forebrain. Intranasal OxA significantly increased the percentage of dual-labeled ChAT neurons in the VDBB compared to vehicle treated controls in both young and aged animals. The bold black square on the schematic indicates the approximate location within the VDBB where c-Fos/ChAT counts and photomicrographs were obtained. The photomicrograph on the right shows typical dual-label immunohistochemistry for c-Fos (blue/black) and ChAT (brown) neurons within the VDBB (arrows). Abbreviations: OxA, orexin-A; ChAT, choline acetyltransferase; VDBB, vertical limb of the diagonal band. Scale bar represents approximately 100 µm

3.3.4: Effects of intranasal [Ala^{11}, D-Leu^{15}]-OxB on c-Fos expression in cholinergic neurons

Our work in young and aged animals utilized intranasal administration of the OxA peptide to study its effects on cholinergic neurons (Calva *et al.* 2018; Calva and Fadel 2018); therefore, the receptor mechanisms by which intranasal orexin administration may activate basal cholinergic neurons remained unresolved. Accordingly, we examined the effects of intranasal [Ala^{11}, D-Leu^{15}]-OxB administration, a selective OX_2 receptor agonist, on c-Fos expression within cholinergic (ChAT+) neurons of the basal forebrain. Ultimately, we discovered that intranasal [Ala^{11}, D-Leu^{15}]-OxB significantly increased c-Fos expression within cholinergic neurons of the medial septum compared to intranasal saline administration (t_{12} = 2.704, p = 0.0192). Intranasal [Ala^{11}, D-Leu^{15}]-OxB administration did not significantly alter activation of cholinergic neurons within any other subdivision of the basal forebrain (data not shown).

3.3.5: Effects of intranasal orexin administration on c-Fos in GABAergic neurons

To analyze the effects of intranasal OxA on neuronal subpopulations in the cortex, we stained cells for parvalbumin (PV), a marker for fast-spiking GABAergic interneurons in the cortex (Hu et al. 2014). Indeed, analysis by one-way ANOVA indicated a significant effect of treatment in the prelimbic cortex (PrLC; $F_{3,28}$ = 14.44, $p<0.0001$), the ventral orbital cortex (VOC; $F_{3,27}$=7.120, p=0.0011), and in the claustrum (Cl; $F_{3,28}$=5.561, p=0.0040). Further analysis with Tukey's multiple comparisons test revealed that intranasal OxA decreased c-Fos expression in parvalbumin (PV+) GABAergic interneurons of the prelimbic cortex (p=0.0118) of aged animals compared to

treatment with intranasal saline. In young animals, Tukey's multiple comparisons test revealed that intranasal OxA decreased c-Fos expression in parvalbumin (PV+) GABAergic interneurons of the prelimbic cortex (p=0.0038) and the ventral orbital cortex (p=0.0007). Interestingly, PV+ GABAergic interneurons of the prelimbic cortex show higher 'basal' activity under control treatment conditions in aged animals compared to young animals (Figure 3.6). Furthermore, while 'basal' activation levels of PV+ GABAergic interneurons in the claustrum are not significantly different, under OxA treatment conditions, aged animals exhibit significantly higher activity within this subset of neurons compared to young animals treated with intranasal OxA (Figure 3.6).

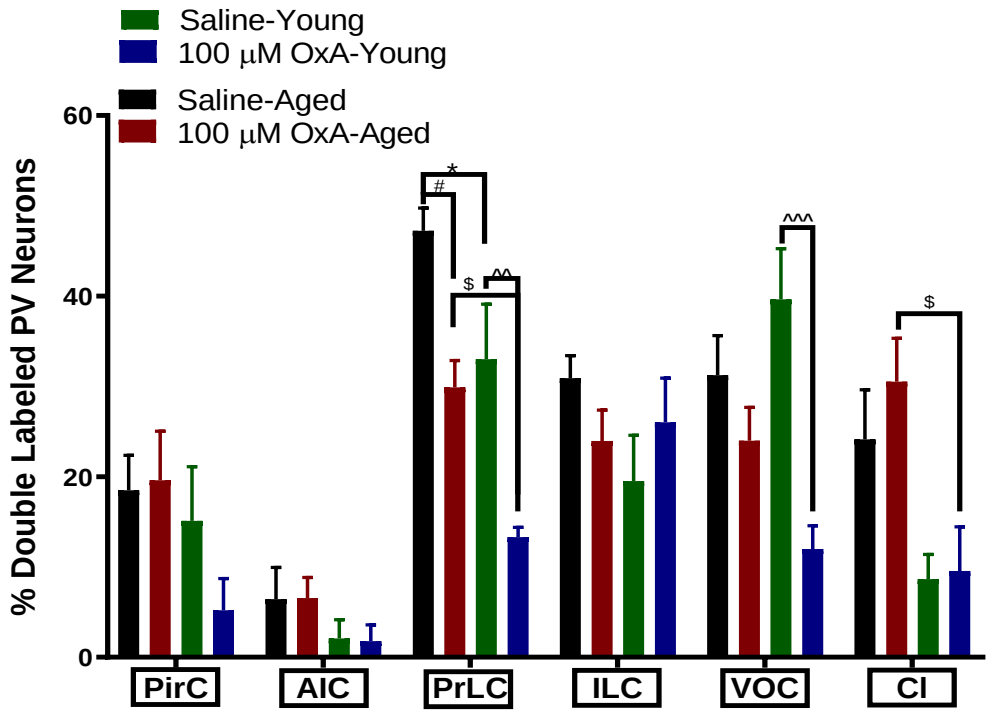

Figure 3.6 Activation of PV+ GABAergic interneurons neurons in the cortex by intranasal OxA administration. In aged animals, intranasal OxA significantly decreased the number of active PV+ GABAergic interneurons in the PrLC (n=8, saline; n=8, OxA). In young animals, intranasal OxA significantly decreased the number of active PV+ GABAergic interneurons in the PrLC, and VOC (n=8, saline; n=8, OxA). Data are represented as the percentage of double-labeled (c-Fos/PV+) neurons relative to the total number of PV+ neurons. #/$/*=p<.05, ^^=p<.01, ^^^=p<.001

Data are expressed as the percentage of PV positive neurons also expressing c-Fos (Figure 3.6). Single-labeled and double-labeled PV neurons were counted within the same 0.179mm x 0.179mm reticle area as c-Fos so that direct comparisons could be established. Double–labeled PV neurons of the prelimbic cortex are depicted by arrows showing blue-black immunoreactivity for c-Fos and brown immunoreactivity for PV (Figures 3.7).

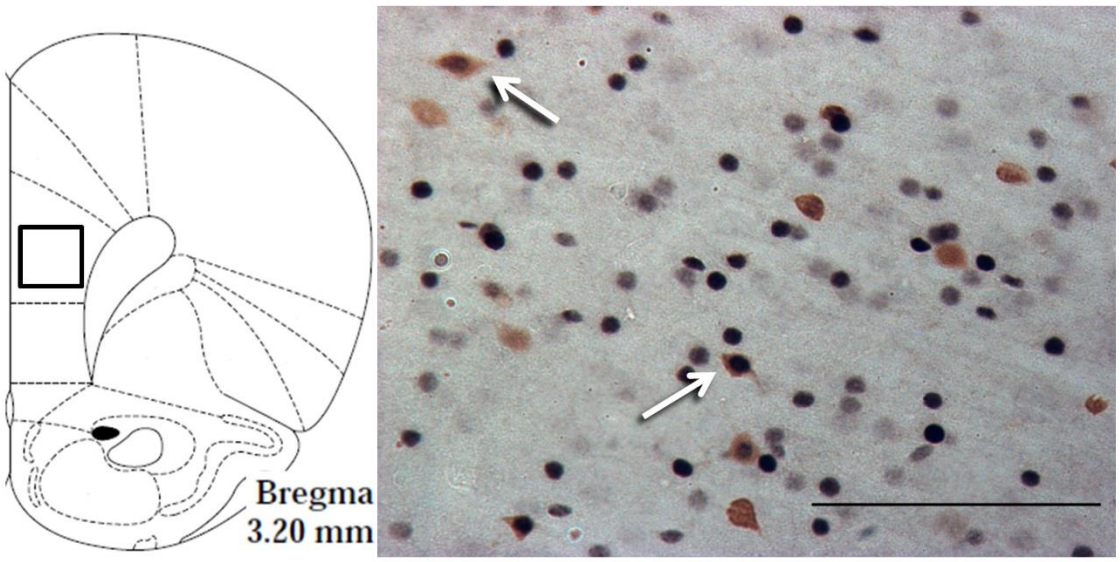

Figure 3.7 Representative photomicrograph depicting double-labeled parvalbumin positive (PV+) GABAergic interneurons in the cortex. Activation of PV+ GABAergic interneurons in cortical brain regions after intranasal OxA administration. Intranasal OxA significantly decreased the percentage of dual-labeled PV neurons in the PrLC of young and aged animals. The bold black square on the schematic indicates the approximate location within the PrLC where c-Fos/PV counts and photomicrographs were obtained. The photomicrograph on the right shows typical dual-label immunohistochemistry for c-Fos (blue/black) and PV (brown) neurons within the PrLC (arrows). Abbreviations: PV, parvalbumin; OxA, orexin-A; PrLC, prelimbic prefrontal cortex. Scale bar represents approximately 100 µm

Importantly, these observations indicate that intranasal OxA administration decreases activation of fast-spiking PV+ GABAergic interneurons in the PFC. These cells are the principal interneuron phenotype within the cortex and function to gate the firing

of cortical pyramidal neurons (Kawaguchi and Kubota 1997; Kelsom and Lu 2013; Xu *et al.* 2010; Hu *et al.* 2014; Sohal *et al.* 2009; Kim *et al.* 2015). While the mechanisms driving this inhibition remain unknown, one possibility is through inhibition from basal forebrain PV+ projection neurons that preferentially synapse onto PV+ cortical interneurons (Henny and Jones 2008; Freund and Meskenaite 1992). In support of this argument, we have data from aged animals that highlight the potential for this mechanism. When analyzing the effects of intranasal OxA administration on cholinergic neurons of the basal forebrain, we also investigated its effects in PV+ GABAergic neurons of the basal forebrain of aged animals (Figure 3.8). Unfortunately, due to staining issues during immunohistochemistry, we were unable to achieve proper staining

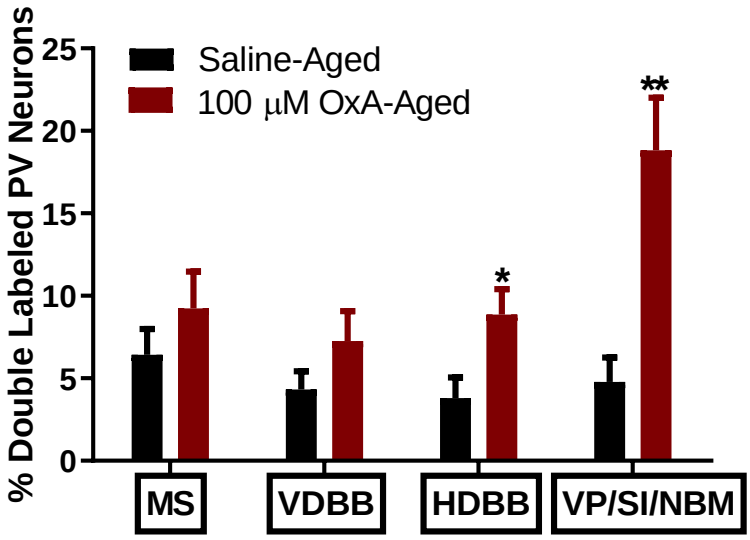

Figure 3.8 Activation of PV+ GABAergic neurons in the basal forebrain after intranasal OxA administration in aged animals. Intranasal OxA significantly increased the percentage of dual-labeled PV+ neurons in the horizontal limb of the diagonal band (HDBB) and in the ventral pallidum/substantia innominata/nucleus basalis (VP/SI/NBM) continuum of aged animals (n=8, saline; n=8, OxA). Data are represented as the percentage of double-labeled (c-Fos/PV+) neurons relative to the total number of PV+ neurons. *=p<.05, **p<.01

of PV+ neurons in the basal forebrain of young animals. Interestingly, intranasal OxA selectively increases c-Fos expression within PV+ GABAergic neurons of the horizontal limb of the diagonal band (HDBB; t_{13}=2.580, p=0.02290) and the contiguous ventral pallidum/substantia innominate/nucleus basalis region of the basal forebrain (VP/SI/NBM; t_{13}=4.175, p=0.0011). Activation of these neurons via orexins may ultimately function to regulate cortical gamma band oscillations (Kim *et al.* 2015), a putative electrophysiological correlate of attention and feature binding (Gray and Singer 1989; Tiitinen *et al.* 1997).

3.4: Discussion

These studies highlight that intranasal orexins selectively increase neuronal activation in distinct cortical, basal forebrain, and brainstem regions. While intranasal [Ala^{11}, D-Leu^{15}]-OxB selectively activates the piriform and agranular insular cortices, intranasal OxA activates a broader range of cortical regions including the prelimbic and ventral orbital cortices. These studies also demonstrate that intranasal OxA activates basal forebrain cholinergic neurons in young and aged animals, which suggest a capacity for intranasal OxA to modulate cholinergic neurotransmission across the lifespan.

3.4.1: Effects of intranasal orexin administration on cortical activation

Orexin neurons send widespread projections to the cortex that modulate various aspects of cognition, especially those related to attentional function. Correspondingly, intranasal [Ala^{11},D-Leu^{15}]-OxB and intranasal OxA (Calva *et al.* 2018; Calva and Fadel 2018) increased c-Fos expression in the agranular insular cortex, a brain region that facilitates interoceptive attention to an organism's physiological status (Craig and Craig 2002; Avery *et al.* 2017; Hassanpour *et al.* 2017). Orexin's actions in the insular cortex

may help promote appropriate behavioral responses to homeostatic challenges, consistent with orexin's role as a physiological integrator. Indeed, previous evidence highlights that modulation of orexin expression alters both behavioral and insular cortical cholinergic responses to food-paired stimuli in food-restricted animals (Hagar *et al.* 2017).

Increased c-Fos expression, after intranasal OxA or [Ala^{11}, D-Leu^{15}]-OxB, was also observed in the piriform cortex, suggesting that activation of this brain region is primarily mediated through the OX_2 receptor. This is consistent with OX receptor mRNA expression patterns in the rat piriform cortex that indicate the selective presence of the OX_2 receptor (Marcus *et al.* 2001). The piriform cortex plays an important role in olfactory discrimination (Stettler and Axel 2009; Bekkers and Suzuki 2013). Interestingly, olfactory dysfunction occurs during aging and age-related cognitive disorders (Enwere 2004; Mobley *et al.* 2014; Kovács 2004), and may serve as an early predictor for AD (Sohrabi *et al.* 2012; Djordjevic *et al.* 2008; Hüttenbrink *et al.* 2013). Orexin modulation of olfactory function has been demonstrated by studies that show i.c.v. administration of OxA enhances olfactory sensitivity to odors (Prud'homme *et al.* 2009; Julliard *et al.* 2007). Together, these findings suggest that OX_2 receptor-mediated activation of the piriform cortex by OxA or [Ala^{11}, D-Leu^{15}]-OxB may serve to enhance odor discrimination and olfactory function within the piriform cortex.

Orexin neurons also densely innervate the PFC where they modulate neurotransmission related to attentional processing (Zajo *et al.* 2016; Fadel *et al.* 2005; Huang *et al.* 2006; Vittoz and Berridge 2006). Though we observed a strong trend for increased c-Fos expression in the prelimbic PFC after intranasal [Ala^{11}, D-Leu^{15}]-OxB administration, our overall observations indicate that orexin-mediated enhancement of

attentional function may primarily occur via the OX_1 receptor. The extent to which intranasal orexin administration modulates neurotransmission and cognitive function is discussed in the upcoming chapters.

3.4.2: Effects of intranasal orexin administration on GABAergic neurons in the basal forebrain

These observations indicate that intranasal OxA administration decreases activation of fast-spiking PV+ GABAergic interneurons in the PFC of both young and aged animals (Calva *et al.* 2018; Calva and Fadel 2018). These cells are the principal interneuron phenotype within the cortex and function to gate the firing of cortical pyramidal neurons (Kawaguchi and Kubota 1997; Kelsom and Lu 2013; Xu *et al.* 2010; Hu *et al.* 2014; Sohal *et al.* 2009; Kim *et al.* 2015). While the mechanisms driving this inhibition remain unknown, one possibility is through inhibition from basal forebrain PV+ projection neurons that preferentially synapse onto PV+ cortical interneurons (Henny and Jones 2008; Freund and Meskenaite 1992). Anatomical evidence suggests that the large majority of these PV+ basal forebrain neurons are GABAergic (Gritti *et al.* 2003; Mckenna *et al.* 2013). Functionally, these PV+ basal forebrain projection neurons regulate cortical gamma band oscillations (Kim *et al.* 2015), a putative electrophysiological correlate of attention and feature binding (Gray and Singer 1989; Tiitinen *et al.* 1997). Behavioral and electrophysiological evidence suggests that activation of basal forebrain PV+ neurons is mediated primarily through the OX_2 receptor (Wu *et al.* 2002; Mieda *et al.* 2011). Given the prominent role of the orexin system in modulating arousal/wakefulness (Sakurai 2002; Jones 2008), intranasal orexins may ultimately regulate cortical activation through modulation of these PV+ neurons in the basal forebrain.

3.4.3: Effects of intranasal orexin administration on cholinergic neurons in the basal forebrain

Orexin neurons are anatomically and functionally positioned to modulate cholinergic neurotransmission. Specifically, orexin neurons modulate the basal forebrain cholinergic system (BFCS), the primary source of cholinergic neurotransmission to the cortex (Fadel and Burk 2010; Villano *et al.* 2017). Several studies illustrate interactions between OxA and the basal forebrain. In particular, infusion of OxA directly into the basal forebrain modulates cholinergic-dependent attentional processing and potently increases cortical ACh release (Fadel *et al.* 2005; Zajo *et al.* 2016). Furthermore, intranasal OxA activates cholinergic neurons of the ventral pallidum/substantia innominata and vertical limb of the diagonal band of young animals (Calva *et al.* 2018) and activates cholinergic neurons of the medial septum, vertical limb of the diagonal band, and ventral pallidum/substantia innominate of aged animals. In contrast, intranasal administration of [Ala^{11}, D-Leu^{15}]-OxB selectively activates cholinergic neurons of the medial septum (Calva and Fadel 2018) . This pattern of activation is consistent with *in-situ* evidence in rats that describes the selective presence of the OX_2 receptor in the medial septum (Marcus *et al.* 2001). As described above, intranasal OxA administration also enhances activation of cholinergic neurons in the basal forebrain of aged animals, indicating that intranasal OxA may be a viable therapeutic for treating age-related deficits in neurotransmission. Accordingly, the effects of intranasal OxA administration on cholinergic neurotransmission and cognitive function will be reviewed in the upcoming chapters.

CHAPTER 4

EFFECTS OF INTRANASAL OREXIN-A ADMINISTRATION ON PFC NEUROTRANSMISSION AND ATTENTIONAL PERFORMANCE

4.1: Introduction

Collectively, the experiments presented in Chapter 3 demonstrate that intranasal OxA administration increased c-Fos expression in many, but not all areas of cerebral cortex, basal forebrain, and brainstem. The selective nature of OxA-elicited neuronal activation was further demonstrated by a limited phenotypic analysis of these effects, which revealed increased activation of basal forebrain cholinergic neurons but decreases in Fos expression in GABAergic, PV+ interneurons in prelimbic and ventral orbital cortices. Additionally, while intranasal [Ala^{11}, D-Leu^{15}]-OxB selectively activates the piriform and agranular insular cortices, intranasal OxA activates a broader range of cortical and subcortical brain regions. These studies also demonstrate that intranasal OxA activates basal forebrain cholinergic neurons in young and aged animals, which suggest a capacity for intranasal OxA to modulate cholinergic neurotransmission across the lifespan. Our understanding of the effects of intranasal orexin administration on neurotransmission and cognition will continue to evolve in the analysis of the following studies using *in-vivo* microdialysis in the prefrontal cortex. Orexin neurons also densely innervate the PFC where they modulate neurotransmission related to attentional processing (Zajo *et al.* 2016; Fadel *et al.* 2005; Huang *et al.* 2006; Vittoz and Berridge 2006). Prior studies suggest that direct intrabasalis application of OxA increases cortical

acetylcholine and enhances attentional function (Fadel *et al.* 2005; Zajo *et al.* 2016). Combined with the evidence from our immunohistochemical studies, this suggests that intranasal OxA may enhances attentional processing in the PFC via stimulated acetylcholine release. Though we observed a strong trend for increased c-Fos expression in the prelimbic PFC after intranasal [Ala^{11}, D-Leu^{15}]-OxB administration, our overall observations indicate that orexin-mediated enhancement of attentional function may primarily occur via the orexin-1 receptor. The purpose of these studies is to investigate the extent to which intranasal orexin administration modulates neurotransmission and cognitive function in young aged animals.

4.2: Methods

4.2.1: Stereotaxic surgery for implantation of guide cannulae

Under ketamine (90 mg/kg)/xylazine (2-10 mg/kg) anesthesia, animals used for *in-vivo* microdialysis received a single guide cannula inserted into the prefrontal cortex (PFC) at AP +2.8 mm, L ±0.5 mm, and DV -2.8 mm relative to bregma. Coordinates for guide cannula implantation were obtained from the Paxinos and Watson rat brain atlas (Paxinos and Watson 1998). The intracerebral guide cannula was anchored in place using two to three skull screws and dental cement. Guide cannula placement was counterbalanced so that left and right hemispheres were represented equally. During the procedure, all animals were given a single dose of buprenorphine (0.01 mg/kg, s.c.) to ease post-operative pain and were monitored until complete recovery.

4.2.2: *In-vivo* microdialysis

In concordance with the immunohistochemistry experiments, animals used for the *in-vivo* microdialysis experiments arrived in separate batches. Beginning 3 days after cannula implantation, animals were habituated in microdialysis bowls for 3-4 days and habituated with intranasal saline for 7 days. On microdialysis days, guide cannula stylets were removed and substituted with a microdialysis probe that extended 2 mm past the guide cannula. Probes were perfused at a 2 µl/min flow rate with artificial cerebrospinal fluid (aCSF, pH 7.4) containing: 150 mM NaCl, 3mM KCl, 1.7mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4.9 mM $_D$-glucose. Neostigmine bromide (50 nM) was added to the aCSF to increase recovery of acetylcholine in collected dialysates. Dialysate collection started after a 3-hour discard period. Microdialysis sessions consisted of one hour (4 x 15-minute collections) of baseline collections followed by intranasal vehicle (0.9% saline) or OxA (100 µM), administered in a total volume of 50 µl in 12.5 µl increments over a 2-minute period. The individual performing intranasal administration was not blinded to treatment conditions. Dialysate collection then continued for two hours (8 x 15-minute collections) post-treatment. Upon collection, dialysates were stored at -80ºC until analysis using HPLC. All animals underwent two separate microdialysis sessions with an off day in-between and experiments were counterbalanced so that half of the animals received vehicle during session one, while the other half received OxA in session one. The day following the last microdialysis session, rats were euthanized and their brains processed for future probe placement verification using an acetylcholinesterase (AChE) background stain. Probe placement verification was performed after all HPLC samples were run for

each respective animal. Any probe placement visualized outside the medial PFC excluded the animal from the study.

4.2.3: High-performance liquid chromatography and chromatogram analysis

Each 30 µl dialysate was split prior to analysis by high performance liquid chromatography with electrochemical detection (HPLC-ECD), with 20 µl analyzed for ACh and 10 µl analyzed for glutamate. ACh was analyzed using an HTEC-510 HPLC-ECD (EicomUSA; San Diego, CA, USA). Briefly, 20 µl of each dialysate was loaded into the AC-GEL separation column (2.0 ID x 150mm; EicomUSA) maintained at a constant 33ºC in combination with mobile phase (pH 8.5) containing 49.4 mM potassium bicarbonate ($KHCO_3$), 134.3 µM ethylenediaminetetraacetic acid disodium (EDTA-2Na), and 1.23 mM sodium 1- decanesulfonate. After analyte separation, post-column derivatization of ACh was attained through use of an AC-ENYM II enzyme reactor (1.0 ID x 4 mm; EicomUSA) containing acetylcholinesterase and choline oxidase, which generates hydrogen peroxide (H_2O_2) proportional to the amount of ACh present. The H_2O_2 is further broken down and detected on a platinum working electrode with an applied potential of +450 mV. The amount of ACh in each sample was measured by comparison with a three-point external standard curve with values predicted to be in range of the collected dialysates. The limit of detection for this analysis was approximately 5 fmol/injection.

Glutamate levels in brain dialysates were analyzed using a CC-32 HPLC-ECD (BASi; West Lafayette, IN, USA) with modifications. First, 10 µl of each dialysate was loaded into the GU-GEL separation column (4.6 ID x 150mm; EicomUSA) in

conjunction with a mobile phase (pH 7.2) containing 60 mM ammonium chloride-ammonium hydroxide, 134.3 nM EDTA-2Na, and 686 μM hexadecyltrimethylammonium bromide. After separation, post-column derivatization of glutamate was attained using an E-ENZ enzyme reactor (3.0 ID x 40 mm; EicomUSA) containing glutamate oxidase, which generates H_2O_2 proportional to the amount of glutamate present. The H_2O_2 is further broken down and detected on a 3.0 mm glassy carbon electrode (BASi) coated with a horseradish peroxidase osmium polyvinylpyridine solution (0 mV applied potential). The amount of glutamate in each dialysate was measured by comparison with a three-point standard curve using external standards expected to be in range of the collected dialysates. The limit of detection for this method was approximately 3 fmol/injection.

4.2.4: Attentional set-shifting paradigm

In rodents, the attentional set-shifting task takes each animal through a series of increasingly difficult tasks in which they must dig in small containers to locate a food reward (Birrell and Brown 2000). The specific procedures for our attentional set-shifting task were adapted and modified from previous studies using similar methods (Birrell and Brown 2000; Lapiz-Bluhm *et al.* 2008; Snyder *et al.* 2012; Barense 2002; Liston *et al.* 2006; Bissonette *et al.* 2013). Young and aged FBN/F1 rats were approximately 3-4 months and 26-28 months, respectively, upon arrival to the animal facility. Upon arrival, both young and aged animals were assigned to receive intranasal administration of either vehicle (50μL of 0.9% saline) or OxA (50μL of a 100μM solution; Enzo Life Sciences, Farmingdale, NY, USA). All animals were acclimatized to individual housing for at least 4 days prior to the start of any behavioral procedures. For approximately one week prior

to testing, each rat was maintained on a food restricted diet of approximately 14g per day, such that by testing day, each rat weighed approximately 80-90% of their free-feeding body weight. Each animal received several days of gentle handling and habituation to intranasal saline administration prior to the testing day. All behavioral experiments were conducted during the light portion of the cycle, between 07:00 and 19:00 h. Our testing apparatus was a custom-built black rectangular Plexiglas arena with inner dimensions of 75L x 40W x 30H (Snyder *et al.* 2012). The testing arena contained a black Plexiglas removable divider to separate one-third of the arena from the remaining two-thirds of the arena. This smaller portion of the arena served as the starting box for each rat and as a holding area between each trial. Each trial began after lifting the removable divider, allowing the rats to access the remainder of the arena. The larger portion of the divided arena contained an irremovable and opaque Plexiglas panel to split the arena into two sections. These two sections served as the holding area for the digging bowls. A visual representation of the testing arena is shown in Figure 2.3. The digging pots used for these experiments were small plastic containers (internal rim diameter 7 cm; depth 3.5 cm). Each digging pot was distinguished by a pair of cues along two different stimulus dimensions: (1) the digging medium held in each pot and (2) an odor applied to the rim of each pot. The relevant/irrelevant dimensions and the positive/negative cue pairs for each stage of the task are shown in Table 2.1. To mark each digging pot with an odor, approximately 50 µl of extract was initially applied to the inner rim of the pot. Each digging pot receives only one odor and a different pot is used for each combination. No specific odors or media are necessary for this experiment; however, odor and media pairings should be different enough for each animal to discriminate between (i.e. do not

pair lemon & lime or wood beads & plastic beads). The reward used for these experiments, buried approximately 2 cm beneath the surface of each medium, was a 'bacon softie' broken into small pieces. For each trial, a small amount of reward was ground and applied to the surface of both pots to ensure that each animal was digging correctly based upon the positive cues rather than by smelling the food reward. After the animals were food restricted for at least 5 days, the 3-day behavioral procedure was conducted in the following manner:

Habituation Day (Day 1): before receiving their daily ration of standard rat chow, rats were trained to dig reliably in each pot for a food reward (bacon softie). First, two unscented plastic digging pots are placed into the home cage and baited. Each animal is first allowed to retrieve the reward from both pots without being covered. If the animal has not retrieved both food rewards after 5 minutes, the animal is given a 'time-out' period of 30 minutes and subsequently retried. Ultimately, each animal is required to dig for the food reward in each pot in 3 trials of 5 minutes each, with the food reward covered with an increasing amount of sawdust during each exposure. After the animal is digging reliably, it is placed in the testing arena and habituated to digging in the same manner described above.

Training Day (Day 2): before receiving their daily ration of standard rat chow, rats are trained on a simple discrimination (SD) task to a criterion of six consecutive correct trials. Each trial during the discrimination tasks was timed for up to 10 minutes. If the animal did not retrieve the reward after 10 minutes, a non-digging response, or 'error', was recorded. Any animal with six- consecutive errors was subsequently excluded from further experimentation. All rats start by learning to associate the food

reward with the positive odor cue (the starting media is sawdust in both digging pots). In these experiments, rosemary and orange were used as the odors. After six consecutive correct trials, the animals were then trained to discriminate between two different media to achieve a reward. For these experiments, wood chips and gauze were used as the media. All animals were trained with these odors and media with the positive and negative cues randomly chosen for each animal. Importantly, the training stimuli were never used on testing days to ensure that animals were not remembering odor or media pairings.

Testing Day (Day 3): before receiving their daily ration of standard rat chow, each animal was tested on a series of five increasingly difficult discrimination tasks (Table 2.1). Again, the criterion used to proceed to the next stage of the experiment was six consecutive correct digging responses. The first stage, like training day, was a simple discrimination (SD) task. For the SD stage, half of the animals were required to discriminate between two odors (i.e. strawberry vs. cinnamon) to retrieve a reward while the other half were required to discriminate between two media (i.e. paper vs. felt) to retrieve a reward. Only one of the digging pots (i.e. the positive cue pot) contained the food reward. The second stage was a compound discrimination task (CD), where the irrelevant stimuli from the other dimension were introduced, but the animal was still required to discriminate between the two original stimuli from the SD stage. The third stage was an intradimensional shift (IDS), where all new stimuli were used, however, the task-relevant dimension was unchanged. For example, if the starting relevant dimension was odor, the IDS stage would introduce new odors and media, however, the relevant discriminatory dimension would still be odor. At this point in the task, the animal was

given its predetermined treatment (i.e. intranasal saline or intranasal OxA). This timepoint for treatment was based upon the *in-vivo* microdialysis studies where the effect of intranasal OxA on PFC acetylcholine efflux lasted approximately 30-60 minutes. Because the ASST experiment lasts 2-3 hours for each animal, intranasal OxA was given just before the reversal learning stage (REV) of the task to allow for optimal assessment of each treatment on prefrontal cortical function. The fourth stage of the task was a reversal learning (REV) stage, where the relevant dimension remains the same, however, the previously negative cue is now the positive cue. The fifth and final stage was an extradimensional attentional shift (EDS). In this stage, all new stimuli are introduced, but now the relevant dimension is changed. For example, if the starting relevant dimension was odor, the EDS stage would introduce new odors and media, and the relevant discriminatory dimension would change to media. For each animal, assignment to a positive or negative cue, relevant starting dimension, and left-right positioning of the pots within the arena were determined randomly in advance.

4.2.5: Statistics and data analysis

For the *in-vivo* microdialysis data, baseline neurotransmitter efflux was obtained during the first four sample collections (i.e. timepoints 1-4) and averaged to yield mean basal efflux. For each sample analyzed, a raw value was obtained and expressed as pmol/20μL for ACh and μmol/l0μL for glutamate. The graphed *in-vivo* microdialysis data are expressed as a percentage of the average baseline to account for individual variation in basal neurotransmitter efflux. Data were analyzed using two-way repeated-measure ANOVAs (GraphPad Prism 8) with treatment as a within-subjects variable, age as a between-subjects variable, and time as a repeated measure. Significant interactions

and main effects of treatment (i.e., OxA or saline) or age were probed with Tukey multiple comparisons tests. A significance cutoff level of $p<0.05$ was used for all analyses.

For the attentional set-shifting experiments, trials to reach criterion (6 consecutive correct trials) was recorded for each rat. Because intranasal saline or intranasal OxA was given just before the REV stage of the ASST experiment, the first three stages and the final two stages were assessed differently. For the first 3 stages of the task (i.e. SD, CD, IDS; before treatment), the groups were collapsed to assess differences in task performance between young and aged animals. These results were analyzed using two-tailed unpaired t-tests. For the final 2 stages of the task (i.e. REV, EDS; after treatment), each treatment group was analyzed on its own using ordinary one-way ANOVAs followed by Holm-Sidak multiple comparisons to determine significant differences between the treatment groups. Data were represented as total number of trials to reach criterion (y-axis) for each stage (x-axis). A significance cutoff level of $p<0.05$ was used for all analyses.

4.3: Results

4.3.1: Intranasal orexin-A: PFC glutamate efflux in young and aged rats

After observing increased c-Fos expression in the prelimbic prefrontal cortex (PFC) of both young and aged rats, we then investigated the effects of intranasal OxA on PFC neurotransmission using microdialysis. When analyzing the results of intranasal OxA administration on glutamate efflux in the PFC, intranasal OxA resulted in a significant TIME x TREATMENT interaction ($F_{33,330}$=1.560, p=0.0289). There was no

significant main effect of TIME ($F_{2.029,60.88}$=0.2592, p=0.7757) or TREATMENT ($F_{3,30}$=1.771, p=30.1740). Multiple comparisons indicated that the young saline and aged saline treated groups had significantly different glutamate efflux at timepoints 11 and 12 with the young saline group exhibiting higher glutamate efflux. Furthermore, young intranasal OxA treated animals also exhibited significantly higher glutamate efflux at timepoints 11 and 12 compared to aged saline treated animals. Interestingly, glutamate efflux in intranasal OxA treated aged animals was not significantly different from young OxA or young saline treatment groups which may indicate that aged animals, under control conditions, have deficits in glutamate neurotransmission or gliotransmission. Multiple comparisons yielded no other significant effects (Figure 4.1).

4.3.2: Intranasal orexin-A: PFC acetylcholine efflux in young and aged rats

When analyzing the results of intranasal OxA administration on acetylcholine efflux in the PFC, intranasal OxA significantly increased ACh efflux in the PFC as indicated by a significant main effect of TIME ($F_{11,352}$=17.06, p<0.001), a significant main effect of TREATMENT ($F_{3,32}$=10.21, p<0.0001), and a significant TIME x TREATMENT interaction ($F_{33,352}$=5.025, p<0.0001). Further analysis with Tukey multiple comparisons indicated that young animals treated with intranasal OxA had significantly increased ACh efflux compared to young animals treated with intranasal saline. The significant increase in ACh persisted from collections five through nine (approximately 1 hour and 15 minutes). Aged animals treated with intranasal OxA also exhibited an increase in PFC ACh release that was significantly different from aged matched controls. In the aged animals that received intranasal OxA, the significant increase in PFC ACh efflux lasted from collections six through seven (approximately 30

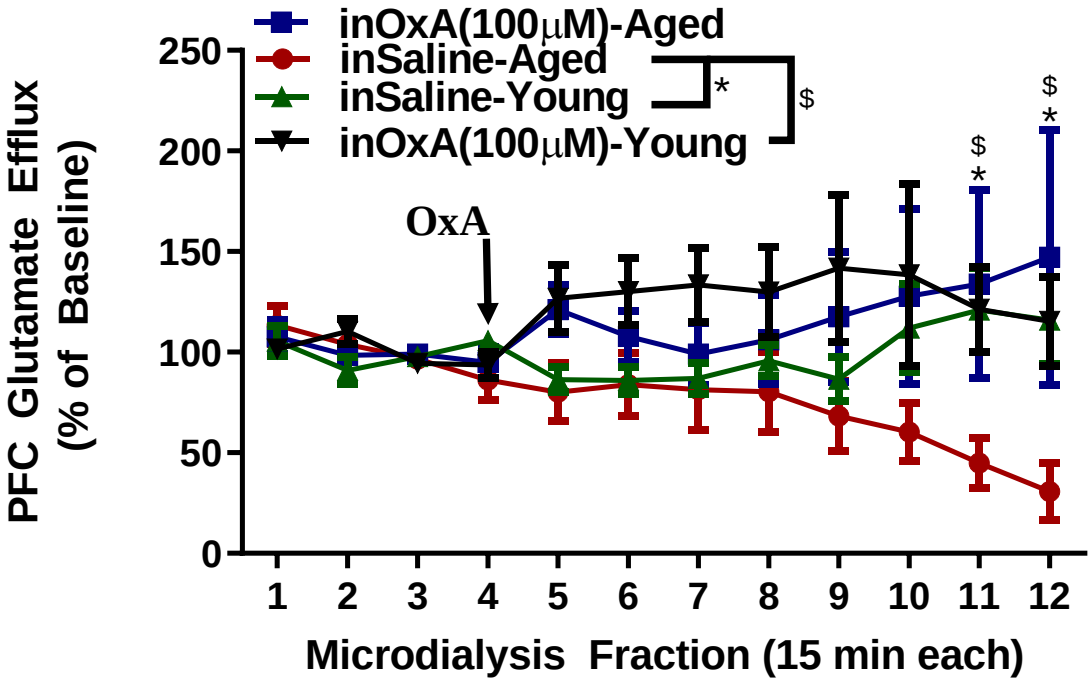

Figure 4.1 PFC glutamate efflux after intranasal OxA administration in young and aged animals. Statistical analyses for PFC glutamate efflux revealed a significant TIME x TREATMENT interaction (**p=0.0289**). Tukey multiple comparisons: Aged animals (n=7, saline; n=7 OxA) and Young animals (n=10, saline; n=10, OxA). Young saline and aged saline treated groups had significantly different glutamate efflux at timepoints 11 and 12 with the young saline group exhibiting higher glutamate efflux. The young intranasal orexin-A treated animals also exhibited significantly higher glutamate efflux at timepoints 11 and 12 compared to aged saline treated animals. */$=$p<0.05$

minutes). In addition to having a shorter time course of intranasal OxA elicited ACh efflux, peak efflux in aged animals was also significantly lower compared to young animals treated with intranasal OxA. Young animals treated with intranasal OxA had a peak ACh efflux that was approximately 233% of baseline while aged animals treated with intranasal OxA had a peak ACh efflux at around 186% of baseline (Figure 4.2). Probe placement verification via acetylcholinesterase background staining stain revealed that the 2-mm active membrane portion of the dialysis probe was centered in the prelimbic portion of the PFC with a small degree of overlap dorsally into the anterior cingulate cortex or ventrally into infralimbic portion of the prefrontal cortex (Figure 2.2, Chapter 2)

4.3.3: Attentional set-shifting: effects of intranasal orexin-A on attentional function

Proper cholinergic neurotransmission in the PFC is crucial for sustained attention (Himmelheber *et al.* 1997; Dalley *et al.* 2001; Sarter *et al.* 2005) and decreased ACh within the PFC represents a neurochemical correlate of age-related attentional dysfunction (Herzog *et al.* 2003; Burk *et al.* 2002; Muir 1997). Attentional function is modulated by the orexin system through its action on converging glutamatergic and cholinergic inputs that ultimately arrive at the PFC (Huang *et al.* 2006; Lambe *et al.* 2005; Fadel *et al.* 2005). Prior work has previously demonstrated a capacity for OxA to increase cortical ACh levels and attentional processing, even among rats with lesions to the BFCS inputs to the PFC (Fadel and Burk 2010; Zajo *et al.* 2016). These studies, combined with our studies highlighting increased PFC efflux of ACh after intranasal OxA administration, suggest that intranasal OxA may ameliorate age-related deficits in attention. To test the hypothesis that intranasal OxA

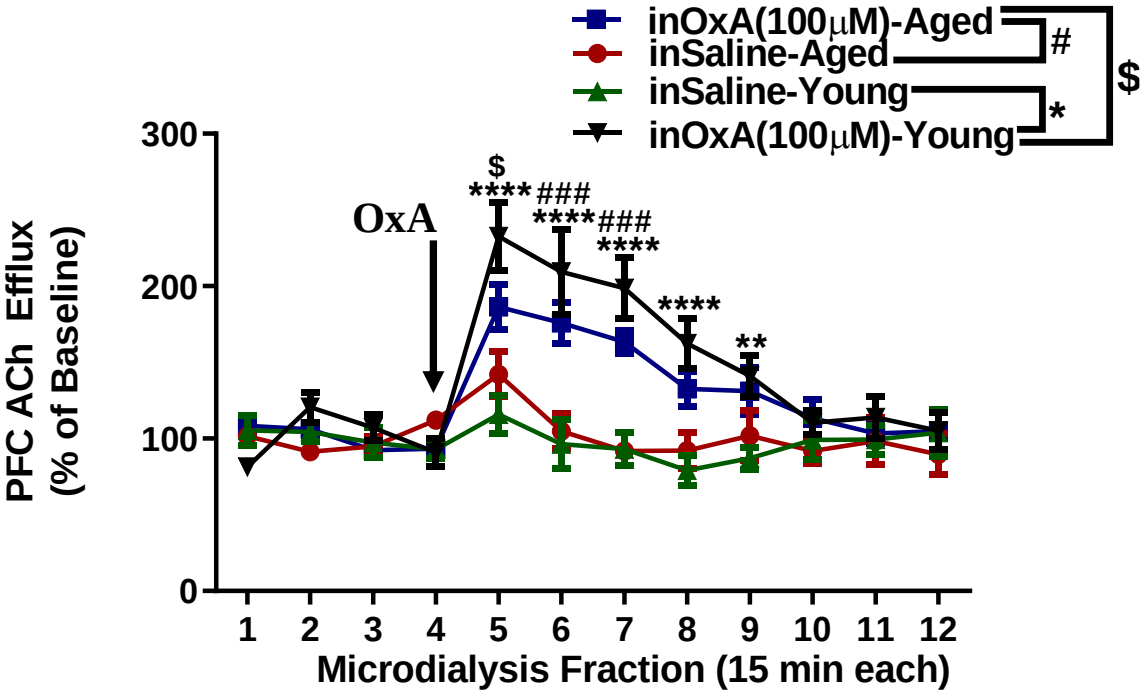

Figure 4.2 PFC acetylcholine efflux after intranasal OxA administration in young and aged animals. Intranasal OxA significantly increased acetylcholine (ACh) efflux within the prefrontal cortex (PFC) relative to treatment with intranasal saline. Statistical analyses for PFC ACh efflux revealed a significant effect of treatment (**p<0.0001**) and a TIME x TREATMENT interaction (**p<.0001**). Tukey multiple comparisons: In young animals (n=10, saline; n=10 OxA), intranasal OxA significantly increased PFC ACh efflux from timepoints 5-9. In aged animals (n=8, saline; n=8, OxA), intranasal OxA significantly increased PFC ACh efflux from timepoints 6-7. Young intranasal OxA treated animals had a significantly higher peak efflux of ACh in the PFC compared to aged animals treated with intranasal OxA ****= p<0.0001, ###=p<0.001, **=p<0.01, $=p<0.05

improves aged related impairments in attentional performance, I used the attentional set shifting paradigm along with intranasal administration of saline or OxA to determine the therapeutic potential of OxA (Figure 4.3). I hypothesized that I would see age-related impairments on the EDS stage of the task as aged animals have previously been reported to be impaired on this stage (Barense 2002). I also hypothesized that intranasal OxA would improve age-related attentional deficits on the EDS stage of the task through increased acetylcholine release in the PFC (Calva *et al.* 2018; Calva and Fadel 2018). Two-tailed unpaired t-tests on the first three stages of the task (before treatment) indicated that there were no significant differences in performance between young and aged animals. One-way ANOVAs on the final two stages of the task revealed a significant effect between treatment groups on the EDS stage ($F_{3,24}$=3.444, p=0.0326). Holm-Sidak multiple comparisons revealed that aged animals treated with saline were significantly impaired on the EDS stage of the task compared to young animals treated with saline (p=0.0227). While intranasal OxA did not significantly improve performance in aged animals compared to aged animals treated with saline, performance of the aged-OxA group on the EDS stage was not significantly different from young saline or young OxA treated animals. A one-way ANOVA also revealed a significant effect between treatment groups on the REV stage of the ASST task ($F_{3,24}$=3.120, p=0.0448). Holm-Sidak multiple comparisons revealed that young animals treated with OxA performed significantly worse on the REV stage compared to young animals treated with saline (p=0.0409).

4.4: Discussion

The collective microdialysis results demonstrate that intranasal OxA administration rapidly and significantly increases ACh and glutamate within the PFC, an

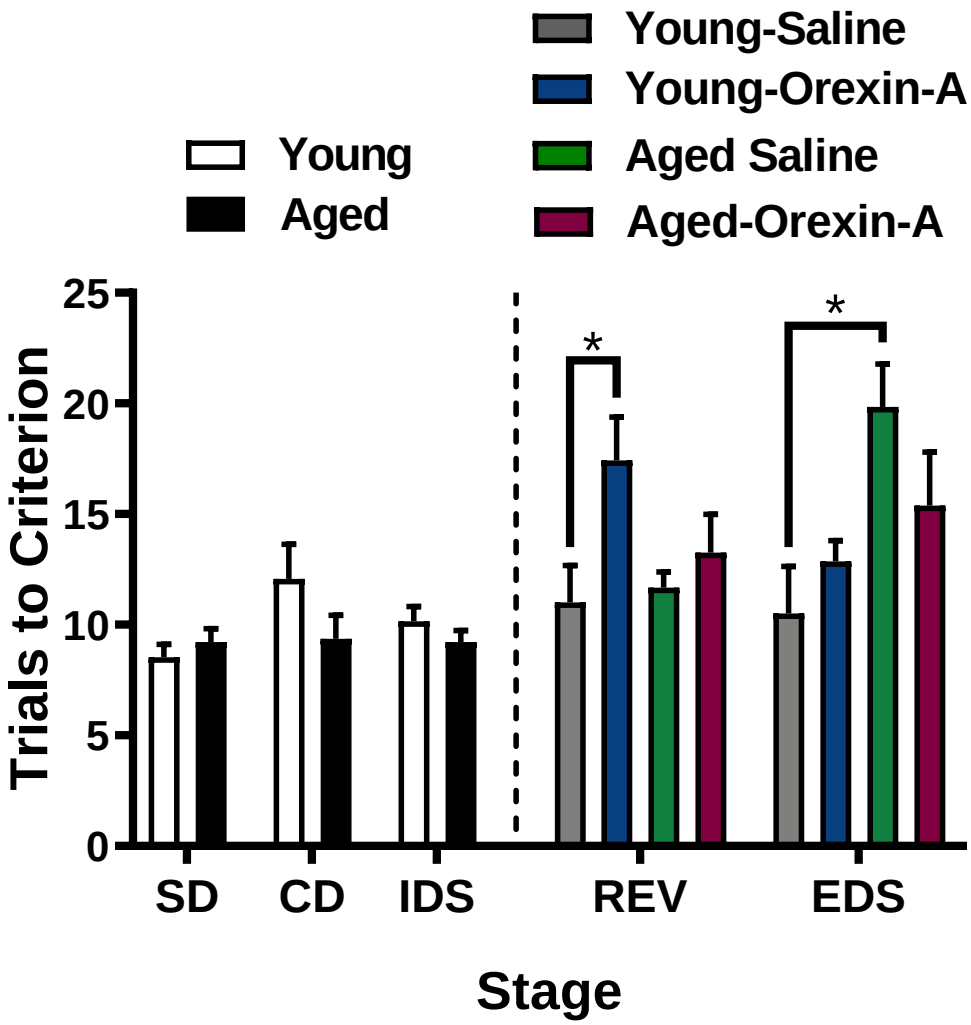

Figure 4.3 Performance of young and aged animals treated with intranasal saline or intranasal OxA in the attentional set-shifting task. Young animals treated with intranasal OxA (n=7) were significantly impaired on the REV stage of the task compared with young animals treated with intranasal saline (n=7). Aged animals treated with saline (n=6) were significantly impaired on the EDS stage of the task compared with young animals treated with intranasal saline. Intranasal OxA treatment in aged animals (n=8) did not significantly improve performance on the EDS stage or REV stage of the task.

area important for multiple aspects of executive and cognitive function. Thus, in this rodent model, intranasal OxA proved effective at reaching the brain and modulating the activity of prefrontal cortical neurotransmitter systems that modulate attentional function.

4.4.1: Mechanistic considerations for neuronal activation and neurotransmission

The effects of intranasal OxA administration on c-Fos are corroborated by our effects seen through *in-vivo* microdialysis. In summary, intranasal OxA significantly increased both acetylcholine efflux within the PFC. The Ox1R and Ox2R are both expressed in the basal forebrain (Marcus et al. 2001). While direct receptor mechanisms involving intranasal OxA administration cannot be determined from these studies alone, prior studies indicate that our effects may primarily be an Ox1R-mediated phenomenon. For example, intrabasalis administration of OxA elicits a greater response on somatosensory cortical ACh release than OxB (Dong et al. 2006). Studies from our lab corroborate this finding as the specific Ox1R antagonist SB-334867 block stimulated ACh release during feeding (Frederick-Duus et al. 2007). Furthermore, the results from the intranasal [Ala^{11}, D-Leu^{15}]-OxB studies suggested that activation of cholinergic neurons in the basal forebrain was limited to the medial septum, which is not a major source of cholinergic input to the prefrontal cortex (Mesulam *et al.* 1983a). Still, it is possible that the Ox2R plays a large role in the effects mediated by intranasal OxA administration as previous work using *in vitro* electrophysiology has reported that OxB is as effective as OxA at exciting basal forebrain cholinergic neurons (Eggermann et al. 2001). Importantly, the large increases in ACh efflux in young rats (233% of baseline) and aged rats (186% of baseline) that we observed translate to previous work showing

that high levels of ACh in the hippocampus and neocortex set the dynamics for increased attention (Hasselmo and McGaughy 2004).

Changes to glutamatergic transmission in the PFC after intranasal OxA administration in young and aged animals were less clear. Statistical analyses indicated that young saline and aged saline treated groups had significantly different glutamate efflux at timepoints 11 and 12 with the young saline group exhibiting higher glutamate efflux. Interestingly, PFC glutamate efflux in aged animals treated with intranasal OxA did not differ significantly from the young OxA or young saline treatment groups which may indicate that aged animals, under control (i.e. saline treatment) conditions, have dysfunction in glutamatergic neurotransmission or gliotransmission. Mechanistically, these effects can arise from a variety of sources. In brain microdialysis, a large majority of the amino acid neurotransmitters sampled under basal conditions are derived from reverse transporter activity or from glial cells (Timmerman and Westerink 1997; Westerink 1995). Intranasal OxA could also be acting locally within the PFC as orexin receptors are expressed in this brain region (Hervieu et al. 2001; Marcus et al. 2001) and OxA has been shown stimulate presynaptic glutamate release in the PFC (Lambe et al. 2007). Visualization of (5(6)-FAM-(Glu¹)-Hypocretin-1) in the PFC via fluorescence supports a direct effect of intranasal OxA on PFC pyramidal neurons. Indirectly, increases in PFC glutamate efflux could also stem from excitation of glutamatergic thalamocortical synapses originating from the paraventricular nucleus (Lambe et al. 2005; Huang et al. 2006) or from orexin neurons that co-express glutamate (Rosin *et al.* 2003; Blanco-Centurion *et al.* 2018; Schöne and Burdakov 2012). The latter possibility seems unlikely, however, as there were no differences in c-Fos expression in orexin

labeled neurons between intranasal vehicle and intranasal OxA treated animals (data not shown).

4.4.2: Therapeutic potential of intranasal OxA for attentional dysfunction

Intranasal OxA in young and aged animals elicited an interesting set of behavioral responses in the attentional set-shifting task. Aged Long-Evans rats have previously been shown to exhibit impaired performance on the EDS stage of the ASST experiment (Barense 2002), a behavioral correlate of mPFC dysfunction (Birrell and Brown 2000). Indeed, in our ASST paradigm, aged animals treated with intranasal saline were impaired on the EDS stage compared with young animals treated with saline. Altogether, this suggests that aged animals display impaired PFC mediated attentional performance compared to young animals and that these deficits are extended across multiple strains (Barense 2002). Furthermore, while intranasal OxA did not significantly improve performance in aged animals compared to aged saline treated animals, EDS performance of the aged-OxA group was not significantly different from young-saline or young OxA treated animals. Together, this would suggest that intranasal OxA returns PFC mediated attentional function in aged animals to levels on par with their younger counterparts. Indeed, this putative effect is supported by the following evidence: (1) intranasal OxA increases PFC ACh release (Calva *et al.* 2018) and (2) direct OxA application into the basal forebrain enhances cholinergic dependent attentional processing (Fadel *et al.* 2005; Zajo *et al.* 2016). Finally, intranasal OxA administration impaired the performance of young animals on the REV stage of the ASST task compared to young animals treated with intranasal saline. Ultimately, this suggests that acute administration of OxA to cognitively intact younger subjects does not enhance attentional performance and may significantly impair cognitive performance overall.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1: Conclusions

The OxA neuropeptide exerts equal affinity for both orexin receptor subtypes; therefore, the neurochemical and behavioral observations surrounding this neuropeptide cannot be attributed to one specific receptor. In addition, because of caveats surrounding the use of G-protein-coupled receptor agonists (e.g., differences in penetrance, unknown brain concentrations with systemic administration, etc.) it is difficult to say definitively what the receptor mechanisms are that mediate single-dose *in-vivo* responses. However, the available evidence suggests that our effects following intranasal orexin are primarily mediated via the OX_1 receptor, particularly those involving the basal forebrain cholinergic system. For example, systemic or intrabasalis administration of the specific OX_1 receptor antagonist SB-334867 attenuates ACh release that is induced during feeding (Frederick-Duus *et al.* 2007). Additionally, OxA administration into the basal forebrain results in greater ACh release in the somatosensory cortex compared to OxB (Dong *et al.* 2006). Furthermore, the observations described above indicate that intranasal administration of the OX_2 receptor agonist [Ala^{11}, D-Leu^{15}]-OxB results in the activation of fewer brain regions than intranasal OxA. Nevertheless, the OX_2 receptor likely plays an important role in various aspects of orexin mediated neurotransmission. For example, we observed that medial septal cholinergic neurons were selectively activated by [Ala^{11},

D-Leu^{15}]-OxB. Furthermore, previous evidence indicates that orexins likely mediate activation of BF PV+ neurons through the OX_2 receptor (Wu *et al.* 2002; Mieda *et al.* 2011). Intriguingly, *in-vitro* evidence indicates that BF cholinergic neurons potently excite BF PV+ and other GABAergic neurons (Yang *et al.* 2014), suggesting that cholinergic and GABAergic systems in the BF work in tandem to modulate cortical activity. Because intranasal OxA and [Ala^{11}, D-Leu^{15}]-OxB affect distinct cholinergic and GABAergic systems, this evidence indicates that intranasal orexins facilitate cognition, in part, through coordinated activation of cholinergic and GABAergic neurotransmission in the basal forebrain. These putative mechanisms that underlie the behavioral and neurochemical correlates of intranasal orexin administration are outlined in a summary figure (Figure 5.1).

5.1.1: Therapeutic implications of intranasal orexin administration

Accumulating behavioral evidence from both animals and humans suggests that intranasal OxA administration may be useful in treating a variety of cognitive disorders. Recent rodent studies have demonstrated that intranasal OxA administration increases locomotion and food intake (Dhuria *et al.* 2016). Additionally, intranasal OxA administration in sleep deprived rhesus macaque monkeys improves performance in a short-term memory task and alters local cerebral glucose metabolism (Deadwyler *et al.* 2007). Of clinical significance, intranasal OxA administration in patients with narcolepsy has been shown to decrease the number of spontaneous wake-REM sleep transitions, improve deficits in olfactory acuity, and enhance divided attention (Baier et al., 2011, 2008; S L Weinhold et al., 2014). The subtle similarities in the cognitive deficits between narcolepsy and some forms of age-related cognitive decline, especially deficits in

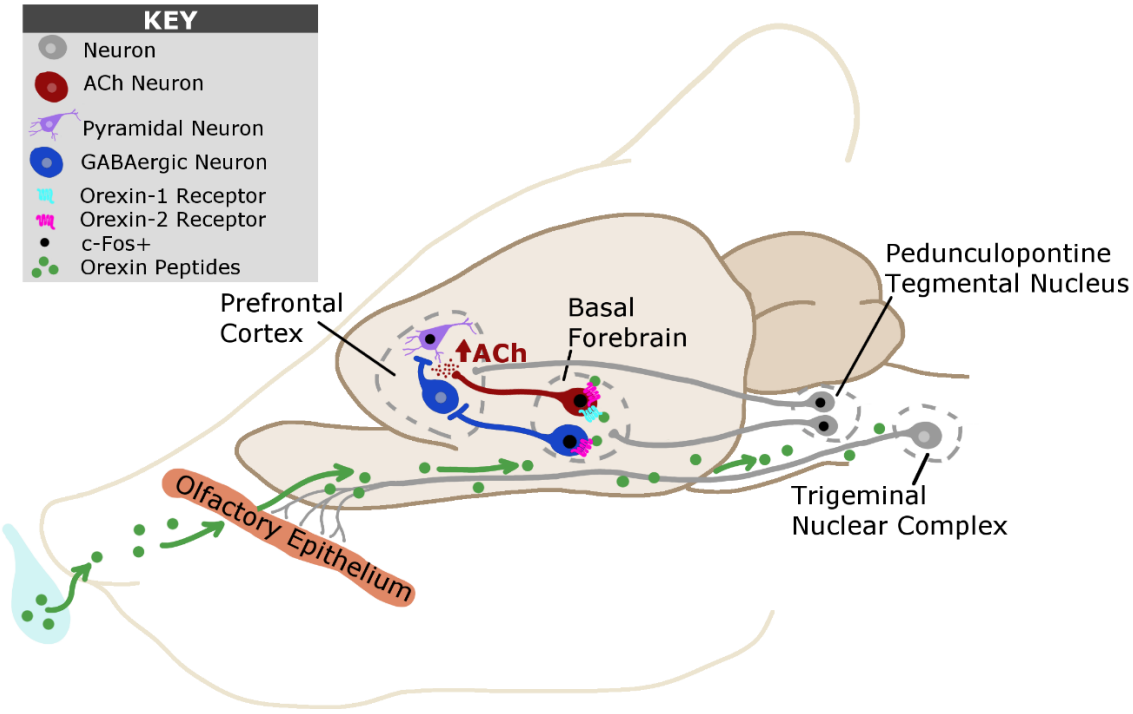

Figure 5.1 Putative mechanisms underlying intranasal orexin entry and action within the brain. Orexins administered via the intranasal route are hypothesized to enter the brain via two main mechanisms: 1) diffusion across the olfactory epithelium into olfactory and rostral brain areas and 2) extra-axonal diffusion along trigeminal sensory pathways into brainstem regions. After accessing the CNS, our data suggests that orexins activate basal forebrain cholinergic neurons via the orexin-1 or orexin-2 receptor. Excitation of these neurons by orexins ultimately increases acetylcholine efflux within the prefrontal cortex, a putative neurochemical correlate of attention. We also observed that intranasal orexin-A administration increases neuronal activation of excitatory pyramidal neurons and decreases neuronal activation of inhibitory parvalbumin-positive GABAergic interneurons within the prefrontal cortex. This dichotomy may ultimately arise from orexin-2 receptor-mediated excitation of parvalbumin-positive GABAergic neurons within the basal forebrain. These inhibitory projections neurons of the basal forebrain preferentially synapse onto cortical GABAergic interneurons. Finally, we observed that intranasal orexin-A administration can activate brainstem neurons of the pedunculopontine tegmental nucleus, which may also modulate activity within the basal forebrain and/or cortex.

attention, hint at the involvement of the orexin system (Hüttenbrink *et al.* 2013; Perry and Hodges 1999a; Sarter and Turchi 2002; Wesson *et al.* 2010). Indeed, aged animal models are associated with a reduction in orexin neurons and/or neuropeptide expression (Terao *et al.* 2002; Porkka-Heiskanen *et al.* 2004; Kessler *et al.* 2011). Furthermore, a reduced number of orexin neurons has also been observed, post-mortem, in patients with AD and dementia with Lewy bodies (Fronczek *et al.* 2012; Kasanuki *et al.* 2014).

The neuronal and pharmacological mechanisms underlying the effect of orexins on cognitive function remain to be fully elucidated. Advancements may include peptide or vehicle modifications that enhance brain penetrance, such as inclusion of cyclodextrin compounds to promote bioavailability of intranasally-administered proteins and peptides (Meredith *et al.* 2015). Development of novel ligands such as the non-peptide OX_2 receptor agonist YNT-185, which has recently shown promise in murine narcolepsy models (Irukayama-Tomobe *et al.* 2017; Takenoshita *et al.* 2018), will also facilitate advancements in this field. Furthermore, it will be important to more fully examine and clarify potential negative effects of intranasal orexin on disease processes such as amyloid plaque formation (Kang *et al.* 2009; Liguori 2017) prior to clinical implementation. Nonetheless, as described above, intranasal orexin administration rapidly enters the brain and targets brain regions and neurotransmitter systems that mediate proper cognitive functioning. Ultimately these studies provide mechanistic evidence for the therapeutic potential of intranasal orexin administration in treating age-related cognitive dysfunction.

5.2: Future Directions

Several future avenues of research can be taken to further enhance the understanding of the results presented here. While these experiments provide a therapeutic context for acute intranasal orexin administration in treating age-related cognitive dysfunction, further work utilizing chronic administration will be needed to determine if long-term dosing enhances or is detrimental to cognitive function. Furthermore, dose-response studies using intranasal orexin would help determine the doses needed for optimal cognitive enhancement in aged animals. Dose-response studies would also help clarify the possible detrimental effects of intranasal OxA in cognitively intact younger animals. Importantly, both the young and aged animals demonstrate an ability to perform the attentional set-shifting task. This is highlighted by the fact that neither the young or aged animal groups exhibit behavioral deficits on the first three stages of the behavioral task which, classically, are less cognitively demanding compared with the reversal learning and extradimensional shift. The fact that aged animals take more trials to complete difficult stages of this behavioral task indicate that this paradigm may be useful in testing other investigative treatments for age-related cognitive dysfunction. Ultimately, the gold-standard for determining therapeutic efficacy, or lack thereof, lies within dose-response studies with acute and chronic dosing regiments. Unfortunately, limited studies have assessed multiple doses of orexin-A, particularly intranasal OxA, in modulating behavioral responses. Nonetheless, preliminary studies using a radiolabeled OxA peptide indicate that intranasal administration of 10 nmol and 100 nmol OxA reach various regions of the brain in a dose-dependent manner (Dhuria *et al.* 2016). Interestingly, recent work has suggested that higher i.p doses of OxA in rats

(40 µg/kg) attenuates prepulse inhibition (PPI) in non-sleep deprived rats, while lower i.p doses of OxA (10 µg/kg) ameliorates impairments in PPI in sleep-deprived rats (Öz *et al.* 2018). These data highlight the importance of optimizing the appropriate dose in behavioral experiments and to the critical need for testing during all phases of the sleep/wake cycle. Apart from OxA, the newly developed YNT-185 compound, an orexin-2 receptor selective agonist, has also shown promise in dose-response studies. Administration of YNT-185 to wild-type mice via three separate modes of delivery (i.c.v., i.p., i.v) all resulted in dose-dependent enhancement of wakefulness during the light phase. Furthermore, YNT-185, in a dose-dependent manner, was also able to alleviate spontaneous transitions from wakefulness to REM sleep in multiple models of mouse narcolepsy. Of additional importance, no desensitization to the YNT-185 compound was observed in the narcoleptic mouse models (Irukayama-Tomobe *et al.* 2017). Together, these collective studies highlight the importance for utilizing both chronic administration paradigms as well as experimental designs that account for dose optimization for intranasal OxA administration.

While the interactions between orexins and the systems that support proper cognition are multifarious, these inextricable associations and results may ultimately be explained via an inverted U-shaped curve that shifts across the lifespan. For example, if orexin levels are deficient, as may be the case in aged animals (Kessler *et al.* 2011), a lower dose of intranasal OxA may be sufficient to ameliorate cognitive deficits, conceivably from a compensatory upregulation of orexin receptors. Conversely, in younger animals with an intact orexin system, intranasal orexin-A may ultimately perturb normal cognitive function, perhaps through an overabundance of excitatory

neurotransmission within the PFC. Ultimately, dose-response studies using intranasal OxA and other orexin receptor targets will elucidate the nature of these inverted U-shaped curves and how they might shift during aging.

Finally, these experiments should, of course, be repeated in female rats to determine if there are sex differences in the ability of intranasal OxA to treat age-related cognitive dysfunction. Assessing these sex differences will be particularly important due to the new body of evidence highlighting sex differences in orexin mediated responses to stress and cognitive flexibility (Grafe *et al.* 2017) .

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