Summer 2021

Anatomical Correlates of Age-Related Basal Forebrain Dysfunction

Brandy Lynn Somera

Anatomical correlates of age-related basal forebrain dysfunction

by

Brandy Lynn Somera

Bachelor of Science
Christopher Newport University, 2015

Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor in Philosophy in

Biomedical Science

Department Pharmacology, Physiology, & Neuroscience

School of Medicine

University of South Carolina

2021

Accepted by:

Jim Fadel, Major Professor

Ana Pocivavsek, Committee Member

Lawrence Reagan, Committee Member

Rekha Patel, Committee Member

Ray Swapan, Committee Member

Tracey L. Weldon, Interim Vice Provost and Dean of the Graduate School

DEDICATION

This dissertation is dedicated to my parents, Yira and Franklin Somera, for their love, support, and encouragement of my dreams. I could not have gotten this far without you.

ACKNOWLEDGEMENTS

Throughout this journey, I have grown and learned through the support and knowledge of all of those who surround me, and this work could not be possible without the help, advice, and support of several individuals. First, I would like to thank my mentor – Dr. Jim Fadel for encouraging me, and for teaching me that research is a journey of twists and turns where flexibility is key. I am especially grateful for you're calming demeanor and enthusiasm toward science which has spark a light in me. Thank you for supporting me and guiding me. To Dr. Larry Reagan– thank you for your listening ear and your honest advice. Your support, especially in the beginning of my journey, is what kept me going, and I cannot thank you enough for your empathy and understanding. And thank you Dr. Grillo, for your kindness and for providing me a role model. Your dedication to science and to your students is inspiring, and I can only ever hope to have the same dedication and love of science. Thank you for forgiving my stumbles, and providing me with help and patience when I needed it. To Jennifer Erichsen, I could not imagine my lab experience without you, thank you for being a listening ear during every crisis and for cheering on my small victories. To Jennifer Woodruff, I could not have done this work without your help and guidance, your patience in teaching me new techniques was vital to my dissertation. Thank you for always being available to talk through problems and help me begin new endeavors. To Marla Frick, thank you for always being willing to learn new things, and helping me in my projects, especially on the vibratome. Your positive and curious attitude always made my day! To

Jessica, you are a gem in the rough, you're bright and welcoming personality was always a day maker. Thank you for the much need encouragement through my interview process and for reminding me to stay confident! To Dr. Mott, thank you for always taking the time to talk me through the ins and outs of my graduate journey, your advice was pivotal to my success. To Nick, thank you for teaching me confocal and for allowing me to vent my excitement or fears at random! To Hannah Burzynski, for the occasional solace I need over unexpected ELISA data and for providing me critical notes during the learning process. To my committee, thank you all for challenging me to think deeper and for providing me with their time and such flexibility in unprecedented times. I'd especially like to thank Dr. Pocivavsek for coming in as the head of my committee when I was in need of her, and for taking the time to communicate to me all that I needed to know. You have been a blessing! To my family, I would like to thank my mother, Yira Somera, my father Franklin Somera, my sister Lorena Somera, and my brother, Eric Henson for all their love and support over the many years which have led to this event. I would also especially like to thank my husband, Brent Strickland, for being the most supportive, understanding, and, when I needed it, goofy husband I could have ever asked for – especially through the late nights, and the occasional quarter life crisis' I would experience. Lastly, I would like to thank everyone in the department for the most inspirational and encouraging family anyone could ask for!

ABSTRACT

The basal forebrain (BF) is comprised of several nuclei including the substantia innominata, medial septum, nucleus basalis and diagonal band of broca which are involved in cognitive functions including attention, motivation, and arousal. BF neurons are particularly vulnerable to dysfunction and degeneration in aged humans and, more dramatically, in diseases such as Alzheimer's disease (AD). Age-related BF dysfunction may reflect diminished afferent regulation as well as an altered local glial environment. Our lab has previously shown reduced orexin/hypocretin innervation of BF in aging, a phenomenon that may link afferent dysfunction with altered microglial homeostasis. There is little research examining these relationships involving afferent neuronal and glial cell populations in the BF as it relates to aging. The purpose of this study is to compare specific neuronal and glial populations to identify anatomical factors susceptible to age-related homeostatic dysfunction in the BF.

Several lines of evidence demonstrate the responsiveness of the BF to homeostatic stimuli including food related stimuli. Using a food-paired stimulus to examine effects of aging on physiologically-relevant afferent stimulation of this area, we deposited the retrograde neuronal tracer, cholera toxin B (CTb) in BF of aged (26-28 months) and young (2-3 months) F344/Brown Norway F1 hybrid rats and trained them for 7 days. We then combined neural tract-tracing with functional and phenotypic markers of activation to elucidate neural circuits that may underlie age-related loss of activation of BF neurons using immunohistochemistry.

Previous research in our lab has shown that a homeostatically- relevant circuits involving the orexin neuropeptide, show age-related degredation which may be linked to BF dysregulation. Additionally, orexin loss is linked to conditions such as narcolepsy, anorexia nervosa, age-related cognitive decline, and age-related neurodegenerative diseases, such as AD. To show that loss of orexin afferents affects inflammation in the BF via microglial dysregulation, we administered a miRNA-expressing lentivirus designed to knock down orexin expression (LV-PPOX) in the BF in young rats. We then analyzed phenotypic changes in microglia using immunohistochemistry and ELISA against a panel of pro- and anti-inflammatory cytokines. Changes in morphological and cytokine correlates of microglial activation following orexin loss can be seen in animals administered LV-PPOX. Together, these studies compare specific neuronal and glial populations of young and aged rats to identify anatomical factors susceptible to age-related dysfunction.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Aβ..... Amyloid β

AD..... Alzheimer ’s disease

ACh..... Acetylcholine

AChE.....Acetylcholine Esterase

APOE ε4 ..... ε4 allele of apolipoprotein E

APP ..... Amyloid Precursor Protein

BF..... Basal Forebrain

CCL.....Chemokine C-C motif ligand

CeA ..... Central Amygdala

ChAT.....Cholinesterase Acetyl Transferase

CtB ..... Cholera Toxin B Subunit

CSF ..... Cerebral Spinal Fluid

DA.....Dopamine

EEG..... electroencephalography

ELISA ..... enzyme-linked immunosorbent assay

fMRI.....functional magnetic resonance imaging

GABA .....Gamma-aminobutyric acid

Glu.....Glutamate

IC.....Insular Cortex

IHC..... Immunohistochemistry

IL..... Interleukin

ILC ..... Infralimbic Cortex

LH/PFA ..... Lateral Hypothalamus /Perifornical Area

LPS ..... Lipopolysaccharide

MCI ..... Mild Cognitive Impairment

NAc ..... Nucleus Accumbens

NBM ..... Nucleus Basalis of Meynert

NMDA ..... N-methyl D-aspartic acid

OXA ..... Orexin A

OXB ..... Orexin B

OXR1 ..... Orexin 1 Receptor

OXR2 ..... Orexin 2 Receptor

PAG ..... phate-activated glutaminase

PEN1 ..... Presenilin 1

PEN 2 ..... Presenilin 2

PFC/mPFC ..... Medial Prefrontal Cortex

PLC ..... prelimbic cortex

p-tau ..... tau protein

PV ..... Parvalbumin

SI ..... Substantia Innominata

TNFα ..... Tumor Necrosis Factor α

vGLUT ..... vesicular glutamate transporter

VTA ..... Ventral Tegmental Area

CHAPTER 1. GENERAL INTRODUCTION

1.1 Significance

Alzheimer's disease (AD) is distinguished by progressive brain atrophy which leads to memory loss, attentional deficit, homeostatic imbalance and sleep disturbance. Approximately 15-20% of Americans over the age of 65 will develop mild cognitive impairment (MCI) and 32% of those individuals go on to develop AD (Roberts & Knopman, 2013; Ward, Tardiff, Dye, & Arrighi, 2013). MCI is an age-associated memory impairment of which symptoms fall between normal age-related cognitive decline and severe dementia, but the neuronal anatomical correlates which cause this stepwise cognitive decline in aged individuals have yet to be fully understood. Moreover, the pathology which drives more severe forms of cognitive impairment which resembles a more severe form of MCI, such as AD, is still unknown.

Longitudinal studies comparing basal forebrain (BF) atrophy with Aβ deposition reveal abnormal degeneration in BF has been shown to precede the onset of AD (Schmitz & Nathan Spreng, 2016). In particular, the degeneration of the substantia innominata (SI)/ nucleus basalis of Meynert (NBM) nuclei of the BF is a hallmark of AD, and is associated with normal cognitive aging (Barragán Martínez, García Soldevilla, Parra Santiago, & Tejeiro Martínez, 2019; Kawas, Gray, Brookmeyer, Fozard, & Zonderman, 2000; Schmitz, Mur, Aghourian, Bedard, & Spreng, 2018). Evidence has shown the BF is

particularly vulnerable to neuronal dysfunction and moderate degeneration in aged humans which occurs more often in MCI and diseases such as AD (Fernández-Cabello et al., 2019; M. Grothe, Heinsen, & Teipel, 2013; Rossini, Rossi, Babiloni, & Polich, 2007; Wolf et al., 2014). This suggests that dysfunctional anatomical correlates of the BF, specifically in the SI/NBM, may be a key component in the spread and development of mild cognitive impairments and, in more severe cases, AD. This study investigates age-related changes in basal forebrain afferent and neuronal cell populations to identify vulnerable neuronal populations involved in age-associated cognitive decline and dysfunction. Results from this study will contribute toward the efforts to identify targets of age-related dysfunction which may play a role in age associated MCI and homeostatic dysfunction exacerbated during age-related neurodegenerative diseases, such as AD.

1.2 Alzheimer's Disease

Discovered in 1906 by Alois Alzheimer, AD was defined as a combination of cognitive deficits, psychiatric symptoms, and microscopic brain abnormalities (Roberson & Mucke, 2006). It was later understood that AD was a neurodegenerative disease, characterized by the loss of vulnerable cell populations, that results in widespread brain atrophy beginning in the BF (Kerbler et al., 2015). Other hallmarks of the disease include the extracellular deposition of amyloid-β (Aβ) and intracellular accumulation of tau. Today, AD affects more than 5.4 million people over the age of 65 in the U.S., and is estimated to affect 50 million individuals worldwide, a number that will escalate in the coming decade (Hebert, Weuve, Scherr, & Evans, 2013; Massoud & Gauthier, 2010). Moreover, the burden of these behavioral and psychologic changes falls greatly on both

care takers and the economic system, especially as the elderly population continues to grow. The behavioral changes associated with AD are distinguished by a progressive deterioration of memory, homeostatic balance, cognition, olfactory discrimination, learning and judgement (Auld, Kornecook, Bastianetto, & Quirion, 2002; Cacquevel, Lebeurrier, Cheenne, & Vivien, 2005; Gutiérrez et al., 1999; Mandrekar-Colucci & Landreth, 2012). However, our understanding of the development of the disease remains elusive and how the disease develops and progresses it is not yet fully understood.

AD has both genetic and environmental components which both can contribute to the development and progression of the disease. Genetically, AD has two distinct forms, familial and sporadic. Whereas familial AD is known to run in families, a majority of cases arise as late-onset sporadic which has no familial link. The familial form is due to mutations in three major genes including the amyloid precursor protein (APP) gene, presenilin1 (PSEN1) gene and presenilin 2 (PSEN2) gene(Armstrong, 2013; S. E. Hickman, Allison, & El Khoury, 2008; Mawuenyega et al., 2010). Interestingly, only 1% of cases are associated with genetic mutations such as APOE ε4. Most cases of AD (~99%) are considered sporadic with the major risk factor being age (Barragán Martínez et al., 2019; Hebert et al., 1995; Kawas et al., 2000). The development of sporadic AD usually occurs between the ages of 60-65, but the role of aging pathology is unclear (Dorszewska, Prendecki, Oczkowska, Dezor, & Kozubski, 2016; Lista et al., 2015). Many other factors may contribute to determining the sporadic AD form including anatomical changes, neuroinflammation, and aging. However, it is not yet known what specific underlying mechanisms and anatomical changes separate healthy normal aging

individuals from those who go on to develop AD. Moreover, the initial stages of AD, important in identification and prevention of AD, are not well understood.

1.2.1 Anatomical Correlates

Brain regions that are associated with higher cognitive functions, such as the BF and regions of BF innervation including the neocortex and hippocampus, are most vulnerable to the neurodegenerative characteristic pathology of Alzheimer's disease (McKinney, Coyle, & Hedreen, 1983; Whitehouse et al., 1982). Post-mortem studies reveal enlarged ventricles, cortical atrophy, and temporal atrophy including regions of the BF and hippocampus in patients diagnosed with AD (Bobinski et al., 1999; Hampel et al., 2018; Kerbler et al., 2015; M. M. Mesulam et al., 2002; M. Marsel Mesulam, 2004; Wood et al., 1983). Evidence has shown the BF is particularly vulnerable to neuronal dysfunction and moderate cell loss in aged humans, which occurs more dramatically in diseases such as AD (Fernández-Cabello et al., 2019; M. Grothe et al., 2013; Wolf et al., 2014). Recent research suggests that the BF degeneration may predict the onset of AD (Fernández-Cabello et al., 2019; Schmitz & Nathan Spreng, 2016). Moreover, changes in the BF volume predict the mild cognitive decline of normal aging patients and the cortical neuronal degeneration and cognitive decline seen in AD patients (Fernández-Cabello et al., 2019; M. Grothe et al., 2013; Schmitz & Nathan Spreng, 2016; Teipel et al., 2018; Wolf et al., 2014). This suggests that dysfunctional anatomical correlates of the BF may be a key component in the spread and development of AD.

1.2.2 Cholinergic Hypothesis

The discovery of the first neurotransmitter, Acetylcholine (ACh), in the early 20th century eventually led to a revolutionary change in the understanding of AD (H. Ferreira-Vieira, M. Guimaraes, R. Silva, & M. Ribeiro, 2016; Loewi, 1921). Following the histological analysis of several brains of patients with AD, researchers found that there was a severe deficit in cholinergic projections (P. Davies & Maloney, 1976). Specifically, the discovery of cholinergic cell loss in cortical neurons responsible for the innervation of the SI/NBM of the BF, in patients with AD, established cholinergic neuronal degeneration as a major symptom of AD (P. Davies & Maloney, 1976; M. M. Mesulam, 1976; Whitehouse, Price, Clark, Coyle, & DeLong, 1981). Moreover, it was found that, in patients with AD, there was a significant decrease in the enzyme responsible for the synthesis of ACh, choline acetyltransferase (ChAT), and a notable decrease in choline reuptake in the BF and hippocampus (Contestabile, 2011; Elaine K. Perry, Gibson, Blessed, Perry, & Tomlinson, 1977). Electrophysiological studies showing that there was a significant decrease in ACh release in cortical brain slices from AD patients which further supported the idea that cholinergic dysfunction plays a key role in AD (Nilsson, Nordberg, Hardy, Wester, & Winblad, 1986).

Cumulatively, these discoveries have led to the "cholinergic hypothesis" which states that the dysfunction of cholinergic neurons contributes to the cognitive decline in patients with AD (Raymond T. Bartus, 2000). We now know that a decline in ACh release by cholinergic neurons, specifically in the BF, plays an important role in cognitive impairment and attentional processing. The disruption of these cholinergic inputs leads to behavioral and attentional deficits in rats similar to symptoms of AD in

humans (Everitt & Robbins, 1997; Muir, Dunnett, Robbins, & Everitt, 1992). Moreover, the decline of BF cholinergic neurons and associated cognitive impairments can be seen in both normal aging and to a greater extent in aged individuals with AD (Raymond T. Bartus, Dean, Beer, & Lippa, 1982). Therefore, the loss of the cholinergic neurons in the BF during aging has been accepted as a major factor in cognitive dysfunction during aging and age-related degenerative diseases, such as AD. But, it is also evident that the cholinergic hypothesis can not account for the entirety of the pathophysiology of AD.

Research following has been unable to explain the cause for the loss of these cholinergic neurons. Moreover, acetylcholine esterase inhibitors used in the treatment of AD have been mildly effective. Some argue that the efficacy of the drug may be due to the inability to treat AD early, before significant neuronal degeneration. However, it is also evident that the loss of cholinergic neurons is not the only pathology present in AD. There are many important components associated with aging that present severe loss or dysfunction in age-related diseases (Ball et al., 1997; M. F. Sarter & Bruno, 1994; Schliebs & Arendt, 2011).

1.2.3 Amyloid Hypothesis

AD is a complex polygenic disease which involves a complexity of anatomical dysfunctions and molecular pathway disruptions. Three pathological changes are consistent with AD patients: abnormal Aβ distribution, neurofibrillary tangles due to tau protein (p-tau) and brain atrophy. The amyloid hypothesis states that AD is, at least in part, affected by an imbalance in Aβ metabolism, specifically Aβ production and clearance (Hardy & Selkoe, 2002). Amyloid, in the form of amyloid plaques outside

cells, and tau tangles, mostly composed of hyper-phosphorylated tau, are one of the first known anatomical features of late stages of AD. Many patients diagnosed with AD have extra-neuronal plaques of Aβ due to an underlying disruption of both the amyloid pathway and non-amyloid pathways which causes cleavage of APP into Aβ, particularly Aβ42. However, the cause of this disruption in the APP pathway is not yet fully understood. Moreover, researchers are shifting away from the over simplified assumption of linear causality introduced by the amyloid hypothesis.

1.2.4 The Inflammatory Hypothesis

Neuroinflammation, caused by the dysregulation of inflammatory cascades in the brain, is also considered a core pathological component of AD (Streit, Mrak, & Griffin, 2004). Neuroinflammation has been shown in both normal aged patients, and more severely in age individuals with neurodegenerative changes (Bettcher et al., 2017; Mosher & Wyss-Coray, 2014; Ownby, 2010; Ransohoff, 2016). It was first described in the 1980s as a congregation of high levels of astrocytic glial cells in both AD patients and some aging patients (Beach, Walker, & McGeer, 1989). Later studies indicated a direct correlation between immune-reactive microglia, using biomarker CD 68, and amyloid or tau pathology (Arends, Duyckaerts, Rozemuller, Eikelenboom, & Hauw, 2000). Moreover, post mortem studies indicate that increased markers of microglia were a consistent feature of AD (Hopperton, Mohammad, Trépanier, Giuliano, & Bazinet, 2018; Paasila, Davies, Kril, Goldsbury, & Sutherland, 2019). Some researchers hypothesize that neuroinflammation may perpetuate the formation of pathological markers, such as amyloid-β plaques and tau tangles, which leads to the neuronal death and dysfunction

seen in AD, but it is not yet fully understood (Solito & Sastre, 2012; Tuppo & Arias, 2005).

Recent evidence has shown that Aβ plaques are capable of interacting with microglial and astrocytic receptors that are involved in innate immune response (Minter, Taylor, & Crack, 2016) . This process involves the secretion of pro-inflammatory cytokines and has been shown to generate reactive oxygen species (ROS) that drive a dysregulated immune response which may contributes to neurodegeneration (Cagnin et al., 2001). In early rodent studies it has been shown that, following acute exposure of Lipopolysaccharide (LPS), a known proinflammatory virulence factor, there is a significant decrease in ChAT-positive cells in the BF and an increase in the production of Aβ and neuroinflammatory markers, including IL-1β, IL-6, and TNF-α (Brugg et al., 1995; Quan, Sundar, & Weiss, 1994; Willard, Hauss-Wegrzyniak, & Wenk, 1999). Thus, it has been hypothesized that glial function and cytokine activity play a key role in the brains immune response to neuronal degeneration.

Microglia are considered the main affector cell of the brain's innate immune system, and has been implicated in AD and other neurodegenerative diseases. Early histological studies showed an association between microglial populations and Aβ in AD patients (Perlmutter, Barron, & Chui, 1990; D. Walker & Lue, 2007). Moreover, increases in the expression of markers of microglia are widely reported in brains from patients with AD. Using positron emission tomography (PET) with benzodiazepine receptor ligand PK-11195, as a marker for microglia, the activation of microglia has been shown to precede cerebral atrophy in AD patients (Cagnin et al., 2001). This suggests that microglia activity exacerbates, or possibly triggers, symptoms of AD. Alternatively,

studies have suggested that microglia may impair Aβ clearance in the brain (Tejera et al., 2019). Activated microglia secrete proinflammatory cytokines and chemokines, including tumor necrosis factor α (TNFα), IL-1β, IL-6, and chemokine C-C motif ligand (CCL), which recruit more microglia and glial cells to the effected brain region.

Studies with measuring cytokine activity have also been associated with the onset of AD. Cerebral spinal fluid (CSF) and blood plasma levels of cytokines reveal a direct correlation between inflammatory markers, such as TNFα and CCL2 (Diniz et al., 2010; Tarkowski, Andreasen, Tarkowski, & Blennow, 2003; Westin et al., 2012). This supports the hypothesis that Aβ production and hyper-phosphorylated tau dysregulate the immune response system by recruiting microglia and astrocytes which fail to effectively clear the site; therefore, driving the production of more pro-inflammatory cytokine and chemokine production creating a cycle of chronic glial recruitment which may lead to cellular disturbance and neuronal degeneration seen in AD. Moreover, post-mortem histopathological and positron emission tomography reveals a co-localization of Aβ and tau pathology in AD (Fein et al., 2008; Ikonomovic et al., 2008; Teipel et al., 2005). Tau is a protein involved in the stabilization of microtubules, promotion of neurite outgrowth, and facilitation of enzyme anchoring and axonal transport of organelles. Interestingly, studies have demonstrated that both human and mouse microglia can uptake and release tau seeds, a form of tau capable of inducing tau accumulations, which supports the hypothesis that microglia may play a direct role in propagation AD by, in part, affecting the life cycle of seed-competent tau spreading in the brain (Hopp et al., 2018; Schlachetzki & Hull, 2009; Takeda et al., 2016, 2015).

Studies which use chronic LPS administration to induce a long-term inflammatory response in rats can reproduce cellular and behavioral responses similar to patients with AD, such as increased activation of microglia and astrogliosis, increased tissue levels of IL-1 and TNF-α, elevated expression of APP , and deficits in memory (Hauss-Wegrzyniak, Dobrzanski, Stoehr, & Wenk, 1998). This supported the hypothesis that, instead, a potentiated inflammatory response may contribute to the increase in Aβ production (Raskin, Cummings, Hardy, Schuh, & Dean, 2015). Furthermore, these studies collectively highlight the key role that inflammatory processes may play in early AD pathogenesis.

We do know that genetic and molecular changes associated with AD point to the involvement of immune and inflammatory processes, a complete understanding of the mechanisms that underlie cell death in Alzheimer's disease, particularly in primary targets such as the BF, is not yet known. Recent data clearly show that changes in immune function can facilitate and trigger the pathophysiology of AD which has provided a new dimension to the pathogenesis of AD that may lead to novel ways of diagnosing and treating the disease. Studies have shown that aged individuals are vulnerable to dysregulation of the immune response leading to chronic inflammation, considered Inflammaging, but there is still debate as to whether neuroinflammatory features cause or contribute to age and age-related disease. The two hypothesis remain possible that inflammation could precede and predict AD or that neuroinflammation is a component of aging which is exacerbated in age-related diseases.

1.2.5 Current and Future Pharmaceutical approaches

The population of individuals who develop AD is expected to triple by 2020 generating increased burden on social and health services. This potential economic impact demonstrates the need to develop more effective therapies capable of identifying and preventing the progression of AD (Pais et al., 2020). Current pharmaceutical approaches involve targeting symptoms of AD rather than curing AD. These treatments involve acetylcholinesterase inhibitors which prevent breakdown of the neurotransmitter ACh to improve cognition, and a N-methyl D-aspartic acid (NMDA) receptor antagonist, which have been shown to prevent Aβ toxicity and inflammation associated with AD (Lahiri, Farlow, Greig, & Sambamurti, 2002; Mangialasche, Solomon, Winblad, Mecocci, & Kivipelto, 2010; Misra & Medhi, 2013). In AD patients, evidence of only modest improvements of cognitive symptoms after the application of acetylcholine esterase (AChE) inhibitors, as well as evidence of the existence of nonresponders, cast doubts on the cholinergic hypothesis. Moreover, the use of NMDA antagonists only provides some inflammatory relief to patients suffering AD. To date, there are no biomarkers or therapeutic mechanisms for identifying or eliminating the progression of Alzheimer's disease. Understanding and identifying the earliest pathological signs of AD is vital for effective early treatment of the disease.

The prevention of the onset or progression of AD at an early stage is critical to combating this disease. However, it is difficult to determine what constitutes normal age-related changes from pathological changes which may lead to AD. A central issue in understanding healthy versus pathological aging leading to neurodegenerative diseases is clarifying the structural identity of corresponding regions and cell populations

compromised during the aging process. The assumption that cholinergic denervation is the initial step to the development of AD is still in debate. Several studies suggest a myriad of potential targets disproportionately affected during the aging process including, immune cells and specific BF neuronal populations, thus rendering them susceptible to AD related pathologies(Allen, Abogadie, & Brown, 2006; Ball et al., 1997; Jim Fadel & Frederick-Duus, 2008; Ivana Gritti, Manns, Mainville, & Jones, 2003; Unal, Pare, & Zaborszky, 2015). Some have suggested that effective pharmacotherapies, administered in early stages that prevent initial neuronal dysfunction may involve chronic anti-inflammatory therapy(S. Hickman, Izzy, Sen, Morsett, & El Khoury, 2018; D. Walker & Lue, 2007; Wenk et al., 2000). However, identifying whether this is possible and if there are other key anatomical features which precede and predict the onset of AD are still not known.

1.3 Aging

Aging remains a central component and the leading risk in the development of AD. In the early 1970s, scientists noticed a drastic change in brain volume and weight in aged individuals, starting at around 50 years old, which is exacerbated individuals previously diagnosed with AD (Chrzanowska & Beben, 1973; Dekaban & Sadowsky, 1978). The loss of volume has been attributed to cortical thinning and neuronal loss in the hippocampus and BF (Brueggen et al., 2015; Colomb et al., 1996; Fleischman et al., 2014; Kilimann et al., 2014; Schliebs & Arendt, 2011). Imaging approaches, including functional magnetic resonance (fMRI) and electroencephalography (EEG), have shown an age-related decline in neuronal activation during a cognitive related tasks (Rossini et al., 2007). Cholinergic dysfunction is the most well-known aspect of age-related

cognitive decline which is seen more drastically in AD patients. It is thought that neuronal loss in the Basal Forebrain cholinergic system (BFCS) impedes synaptic processes located in cognitively relevant brain regions such as the cortex and hippocampus thus leading to cognitive deficits (Teipel et al., 2011). Age-dependent changes to BF input populations involved in cognition, attention and arousal, such as orexinergic and dopaminergic populations may also be contributing to age-dependent dysfunction in the BF. Additionally, changes in inflammation and pro-inflammatory cytokine release have been linked to aging and AD (Solito & Sastre, 2012; J. Zhang et al., 2016). Thus, aging has been associated with alterations in cognitive decline, inflammation, arousal, and homeostatic balance (Kessler, Stanley, Frederick-Duus, & Fadel, 2011).

1.3.1 Anatomical Components of Aging

The presentation of cognitive decline in aged individuals can range from mild memory loss to severe dementia, as seen in patients with AD. One component associated with the development of cognitive deficits seen in aged individuals is the neurotransmitter ACh. Early studies revealed a loss of biochemical markers for ACh, including a decrease in AChE and ChAT-positive neurons in aged patients which showed signs of age-related dementia (Bowen, Smith, White, & Davison, 1976; Elaine K. Perry et al., 1977). Later studies using cognitive enhancers demonstrated a clear link between cholinergic transmission and cognition in aged individuals (Riedel & Jolles, 1996). Conversely, antagonism of cholinergic receptors using the cholinergic antagonist, scopolamine, produces cognitive impairments (Budzynska et al., 2015; Ebert & Kirch, 1998). Because ACh has an important role in cognitive processes the loss of cholinergic

neurotransmission has been implicated in cognitive deficits associated with aging, and severe dementia (H. Ferreira-Vieira et al., 2016; Wilcock, Esiri, Bowen, & Smith, 1982). Specifically, the cholinergic projections of the BF, have been the most studied and considered an important component of age-related cognitive decline.

The BF has long been implicated in aging and age-related cognitive decline (Gu, Wortwein, Yu, & Perez-Polo, 2000). Moreover, the severity of dementia in AD patients can be attributed to the loss of cholinergic neurons in the BF (Raymond T. Bartus, 2000; Pappas, Bayley, Bui, Hansen, & Thal, 2000; E. K. Perry et al., 1985). Previous studies have shown that the BFCS experiences neuronal loss during aging and severe atrophy in patients diagnosed with AD (Wolf et al., 2014). Thus, aging has been associated with the loss of function of cholinergic neurons in the basal forebrain due to neuronal loss and dysregulation of cholinergic neurons left behind. This hypothesis is supported by studies which show that, in elderly patients, the loss of BF neurons strongly correlates to cognitive dysfunction in attentional and memory related tasks (M. J. Grothe, Heinsen, Amaro, Grinberg, & Teipel, 2016; Lammers et al., 2018; Wolf et al., 2014). Thus, studies today use the ablation of cholinergic neurons using lentiviral vectors, and cholinergic antagonists in the BF to mimic brain aging and the neurobehavioral deficiencies in AD (R. T. Bartus, Fleming, & Johnson, 1978; Gu et al., 2000).

Recently, new data has revealed that GABAergic neurons of the BF produce parallel inputs with cholinergic neurons to affecting regions. This suggests that other components of the BF and BFCS may play a role in age-related cognitive decline (Ivana Gritti, Mainville, Mancia, & Jones, 1997a). The input to the BF, or BF afferents, have also been implicated in age-related changes. Specifically, the cortical system,

dopaminergic ventral tegmental area (VTA) neurons and orexinergic projections stemming from the hypothalamus have shown age-related changes (Düzel et al., 2010; Jim Fadel & Frederick-Duus, 2008; Gielow & Zaborszky, 2017; Laszlo Zaborszky, Pang, Somogyi, Nadasdy, & Kallo, 1999). However, the exact relationship between these neuronal populations and age-related cognitive decline has yet to be defined. Moreover, whether these regions play a primary role in age-related dysfunction is not yet known.

1.3.2 Inflammaging

Inflammatory processes play an important role in the pathogenesis of aging by maintaining brain homeostasis and immunity. In particular, microglia are involved in these essential housekeeping functions by surveying the brain for unwanted pathogens and neuronal debris which can then be ingested and eliminated (Afridi, Lee, & Suk, 2020). Thus, as previously mentioned, microglia are considered a prominent immune cell of the brain and play a pivotal role in the maintenance of brain homeostasis (Aguzzi, Barres, & Bennett, 2013). Particularly microglia and astrocytes, are involved in homeostasis and defense against pathogens during the acute inflammatory phase (S. U. Kim & De Vellis, 2005). Some microglia survey and clear debris with little to no inflammation (M2 phenotype), whereas others are considered proinflammatory (M1 phenotype) and can cause high levels of inflammation and cytokine release. The M1 phenotype have been shown to contribute to neurodegeneration (Heppner, Ransohoff, & Becher, 2015; Martinez & Gordon, 2014; Schmitz, Soreq, Judes Poirier, & Nathan Spreng, 2020). Moreover, aging has been associated with an increase in the dysfunction microglial activation and mild loss of neuronal cells involved in these detrimental functions, such as cognition and homeostasis, in the BF (Schmitz & Nathan Spreng,

2016). Evidence also reveals a strong correlation between chronic neuroinflammation, homeostasis and impaired memory, especially in patients with AD(C. M. Duffy, Butterick, & Nixon, 2019). Specifically, microglial activation and cytokine release are important factors effected by chronic inflammation which is seen in aged individuals, and these factors are intensified in age related diseases, such as AD. It is thought that in cases of chronic inflammation these immune cells can cause neuronal damage if left unchecked (S. Hickman et al., 2018). Therefore, it has been hypothesized that loss of inputs originating from the basal forebrain might remove a key check on microglial inflammation in aged individuals (Schmitz et al., 2020; Schmitz et al., 2018).

Studies measuring cytokine release are uniquely capable of distinguishing between anti-inflammatory and pro-inflammatory actions of microglia. A study using aged individuals revealed that microglia become increasingly dysfunctional and release higher levels of pro-inflammatory cytokines compared to young individuls (Marschallinger et al., 2020; Solito & Sastre, 2012). Thus, the aging brain is characterized as having higher levels of pro-inflammatory cytokines, such as TNFα and IL-1β, and lower levels of anti-inflammatory cytokines indicating a low to mid-grade level of inflammation (Sparkman & Johnson, 2008). Moreover, microglia in an aged brains show increased levels of ROS and decreases in phagocytosis which indicates age-related dysfunction (Koellhoffer, McCullough, & Ritzel, 2017). As previously mentioned, brains diagnosed with AD have been associated with higher levels of activation and markers such as Amyloid-β (Hopperton et al., 2018). Thus, microglia, immune cells which typically play a prominent role in brain health and homeostasis, have

the potential to have a negative impact on cells during aging, and neurodegenerative disease.

1.3.3 Homeostatic Imbalance

Aging is linked to changes in physiological homeostatic functions such as dysregulation of food intake, weight, sleep cycles and metabolism (Chieffi et al., 2017; Espiritu, 2008; D. Frederick-Duus, Guyton, & Fadel, 2007; Hagar, Macht, Wilson, & Fadel, 2017). Aged individuals often experience a decrease in food intake followed by gradual weight loss, a phenomenon known as the "anorexia of aging" (Visvanathan, 2015). Previous studies have shown that age induced homeostatic disturbances leading to a decrease in food intake and weight loss precede cognitive decline and MCI (Cova et al., 2016; Danielle Frederick-Duus, 2008; Johnson, Wilkins, & Morris, 2006). Furthermore, studies have shown that a decrease in body mass may predict the development of MCI and dementia (Buchman et al., 2005; Johnson et al., 2006). Combined, these studies indicate a potential age-related mechanism which may precede and predict cognitive decline.

Alterations in homeostatic processes point towards mechanisms associated with hypothalamic dysfunction as a potential trigger initiating declining body mass, food intake, and weight loss associated with aging. The hypothalamus is the regulatory center for autonomic and endocrine homeostasis, and houses several neuropeptides and neuronal pathways associated with homeostatic regulation. It is possible that dysregulation of the neural pathways associated with homeostasis and cognitive function are disproportionately affected during the aging process making them vulnerable during

aging and in age-related diseases. Understanding the behavioral and neuroanatomical changes that occur in age healthy individuals vs. aged individuals susceptible to MCI and AD may reveal potential biomarkers for age-related diseases, such as AD.

Previous studies in our lab have linked neuroanatomical changes in orexin neurons produced by the hypothalamus, with food intake and weight gain (Jim Fadel, Bubser, & Deutch, 2002; Hagar et al., 2017). Moreover, orexin neurons regulate physiological and behavioral processes that have major impacts on energy balance and metabolic state (Inutsuka et al., 2014). Thus, age-related deficits in orexin innervation may play a key role in the physiological symptoms seen in aging and provide a link between homeostatic dysfunction and the onset of AD. Thus, age-related decline of the orexin system has been a key factor in the aging process (Danielle Frederick-Duus, 2008; Kessler et al., 2011). Research has shown an age-related decline in orexin concentration and neuropeptides which parallels physiological changes such as food intake and sleep (Desarnaud et al., 2004; Martone et al., 2013; Matsumura et al., 2002; Porkka-Heiskanen, Alanko, Kalinchuk, Heiskanen, & Stenberg, 2004). Moreover, age-related morphological changes of orexin neuron terminals points to an underlying dysfunction in orexin signaling during aging (J. H. Zhang, Sampogna, Morales, & Chase, 2005). Further studies analyzing receptor dysfunction and overall loss of orexin signaling during aging demonstrate a link to cognitive decline (Terao, Apte-Deshpande, Morairty, Freund, & Kilduff, 2002; J. H. Zhang, Sampogna, Morales, & Chase, 2002). In total, age-related dysfunctional orexin signaling in orexin may underlie age-related change in homeostatic and cognitive functions. Further studies analyzing the relationship

between homeostatic maintenance and orexin are still necessary in understanding the role of homeostatic dysfunction in aging and age-related neurodegenerative diseases.

1.3.5 Summary

Aging is associated with changes in neuroanatomical pathways associated with cognitive decline, inflammation and homeostatic imbalance. It is important to note that the reason some individuals may experience normal mild cognitive decline without developing severe cognitive impairments is not yet understood. Although the BF and cholinergic neurons of the BF have been expansively studied and implicated in cognitive aging, the BF is a complex structure and it is still unclear as to why some individuals experience more BFCS neuronal loss and to what extend age-associated cognitive decline can be related to BFCS in normal aging. (Ivana Gritti et al., 1997a, 2003). Moreover, treatments associated with increasing cholinergic transmission, such as AChE inhibitors, have not been successful in reversing cognitive deficits related to normal aging or AD. This suggests that there may be other components in the BF that are vulnerable to dysfunction in aging, or perhaps that neuronal populations communicating with the BF could be effecting the cognitive processes which are lost during aging and more severally in age-related diseases such as AD.

The role of inflammatory processes in aging is evident; however, there is still ongoing debate as to whether inflammation precedes or follows age-related dysregulation in the brain. The role of glial cell activation in the BF during the aging process has been sparsely researched and further insight into the potential relationship between aging, homeostatic neuronal pathways and microglial dysfunction is necessary. Microglia

contribute to overall brain homeostasis, and thus there could be pivotal to distinguishing unhealthy aging from healthy aging. Furthermore, our lab has shown that orexin plays an important role in homeostatic balance in aged animals and is capable of rescuing cognitive function by activating neurons in key cognitive areas such as the basal forebrain and prefrontal cortex (Calva & Fadel, 2020; Calva, Fayyaz, & Fadel, 2018, 2020). This suggests age-related orexin loss may contribute to key symptoms associated with aging including cognitive decline, inflammation, and homeostatic imbalance (J. R. Fadel, Jolivalt, & Reagan, 2013; Danielle Frederick-Duus, 2008; Porkka-Heiskanen et al., 2004). In this study, we hypothesize that age-related dysfunctional changes in neuronal groups associated with homeostatic regulation in BF may contribute to the vulnerability of these neurons during aging.

1.4 Basal Forebrain

Our understanding of the BF has shown significant advances in regards to the implications of its anatomical pathways. The BF is a heterogeneous set of subcortical nuclei which include the substantia innominata, medial septum, ventral pallidum, nucleus basalis and diagonal/vertical bands of broca which are located below the striatum and behind the frontal lobe (Laszlo Zaborszky, van den Pol, & Gyengesi, 2012). The BF nuclei have a heterogeneous set of neurons which have variable physiological properties and functions which represent their specific repertoire of afferent inputs (Hangya, Ranade, Lorenc, & Kepecs, 2015). The BF has been implicated in cognitive functions including attention, motivation, and arousal. Additionally, the BF integrates the signals from these areas by converging several signals which contribute to the output, or efferent, neurotransmitter release to areas of the brain including the hippocampus, amygdala,

Prefrontal Cortex (PFC), and Ventral Tegmental area (VTA). Some of these projections are considered bilateral to maintain bidirectional communication necessary for proper regulation. Thus, the BF has been described as a weigh station for several signaling pathways.

The BF regulates these functions through several neuronal populations including cholinergic, glutamatergic, GABAergic efferent neurons. The local cell populations, including the microglial cells and GABAergic interneurons, also play a pivotal role in regulating important signals and maintaining homeostatic balance. The anatomical complexity of the basal forebrain creates a challenge in understanding the functional of the BF, as well as the information processing characteristics which contribute to disease states. For this reason, research has only mildly elucidated the intricacies and complexities of the BF.

1.4.1 Acetylcholine and the Basal Forebrain Cholinergic System

The BFCS, a set of BF outputs which release ACh, plays an important role in cognitive functions including critical physiological processes, such as attention, memory, wakefulness and sleep, and decision-making (H. Ferreira-Vieira et al., 2016). The BFCS is located in the nucleus basalis magnocellularis (nBM), ventral pallidum/substantia innominata (VP/SI), diagnol bands of broca, and medial septum, first defined by as the Ch4 subgroup using Mesulam's nomenclature (M. -Marsel Mesulam, Mufson, Levey, & Wainer, 1983; M. Marsel Mesulam & Mufson, 1984). As mentioned previously, the BFCS has been the most widely studied set of BF efferent neurons and has been shown to be particularly vulnerable to dysfunction and degeneration in aged humans and, more

dramatically, in diseases such as Alzheimer's disease. Experiments performed using tract tracers and immunohistochemistry (IHC), have shown that the BFCS mainly sends outputs to the hippocampal formation, , the cortex, and the limbic system (Clark & Editors, n.d.; Gielow & Zaborszky, 2017). Although it was first hypothesized that these outputs were diffuse, it is now accepted that the BFCS provides topographically and functionally organized efferent to these limbic system and cortical areas of the brain, and that its wide spread projections provide a central modulating neurotransmitter system which is implicated in diverse behavioral processes (Bloem et al., 2014; M. M. Mesulam, 1996; Laszlo Zaborszky et al., 2015).

To study the functional significance of these outputs, injuries were introduced to BFCS using immunotoxins in rodent and non-primate models which led to attentional deficits in several animal models including monkeys and rats (Bucci, Holland, & Gallagher, 1998; Irle & Markowitsch, 1987; Voytko, 1996; Voytko et al., 1994). Early lesioning studies confirming cholinergic loss using ChAT activity demonstrated behavioral deficits following the loss of cholinergic neurons from the BF (Dekker, Connor, & Thal, 1991). Additionally, a more specific infusion of IgG-saporin, an immunotoxin which specifically destroys cholinergic neurons, into the BF produced cognitive deficits in several attention/cognitive tasks (Russchen, Amaral, & Price, 1985; Torres et al., 1994). The BFCS projects diffusely across the cortical mantle, where the functional topography is yet to be fully understood. Evidence has indicated that BF cholinergic neurons synapsing on glutamatergic neurons in the cortex and thalamus are involved in modulating the response of these neurons (McCormick, 1993). When cholinergic immunotoxins, target the low affinity form of the NGF receptor preferentially

expressed on cholinergic neurons, are applied to hippocampal regions which receive many cholinergic projections, memory deficits were shown, whereas cholinergic stimulation increased hippocampal memory formation. Thus, the BFCS expresses distinct functional connections, where outputs synapsing at the cortex modulate attentional and awareness, but ACh neurons synapsing at the hippocampus affect learning and memory (Ballinger, Ananth, Talmage, & Role, 2016; Bloem et al., 2014).

Post mortem studies of patients diagnosed with AD illustrate a significant decrease in cholinergic neurons, specifically in the nucleus basalis (Arendt, Bigl, Tennstedt, & Arendt, 1985; Hedreen, Struble, Whitehouse, & Price, 1984; Whitehouse et al., 1982). Early studies using scopolamine, a muscarinic receptor antagonist, showed a similar pattern of cognitive deficits as to what was observed in elderly individuals (Drachman & Leavitt, 1974; Ebert & Kirch, 1998). Thus, blocking a receptor whose modulator is acetylcholine causes similar attributes in young individuals as seen in elderly, such as attentional and memory deficits. This, and many following studies, led to the understanding that age-related cognitive deficits seen in elderly and AD patients could be caused by a loss in cholinergic function. Thus, the deficits could be reduced using the anticholinesterase, physostigimine, which was first shown in aged monkeys (R. T. Bartus et al., 1978; Raymond T. Bartus, 1979). Today, most treatments used to alleviate cognitive symptoms of AD are acetylcholine esterase inhibitors, which acts to prevent the enzyme, which normally breaks down Ach at the synapse, from degrading Ach (Pepeu, Giovannini, & Bracco, 2013). However, these treatments are only mildly effective because there are relatively few cholinergic neurons for the drug to act on, and

there is still very little understood about the pathways which contribute to cholinergic output.

1.4.2 GABA

While extensive research surrounding hypotheses about the cognitive and attentional functions mediated by the BFCS have been substantiated in recent years, knowledge about the behavioral and functional contributions of the large population of GABAergic neurons has remained scarce. Of particular interest are the GABAergic efferent neurons which have been shown to colocalize with the BF cholinergic neurons. Immunohistochemical studies, using ChAT and calcium-binding protein Parvalbumin (PV), have shown that GABAergic neurons outnumber cholinergic neurons 2:1, it is predicted that a similar number of GABAergic neurons and cholinergic neurons (1:1) form parallel projections while other GABAergic neurons are considered part of the local GABAergic interneuron population (Ivana Gritti, Mainville, & Jones, 1993; Kiss, Patel, Baimbridge, & Freund, 1990; M. Sarter & Bruno, 2002). It is important to note that in the BF, 90% of GABAergic (immunostained via GAD+) neurons are also PV+, and that these studies reflect staining with PV as a GABAergic marker (Ivana Gritti et al., 2003). Some studies suggest that BF interneurons (PV+) play a significant role in cortical response that perhaps contribute to attentional processes and executive functions by interacting with projecting GABAergic and cholinergic neurons (Freund & Meskenaite, 1992a; T. Kim et al., 2015). For example, the BF afferents from the PFC have been shown to target GABAergic neurons, and it is predicted that these neurons contribute to regulation of efferent neurons (Ronald P.A. Gaykema, Van Weeghel, Hersh, & Luiten, 1991; Ronald P.A. Gaykema & Zaborszky, 1997).

It has been well established that the BF corticopetal nuclei contain BF cholinergic (ChAT+) and GABAergic (PV+) neurons which project cortically (Ferguson & Gao, 2018; Hooly Moore, Sarter, & Bruno, 1995; M. Sarter & Bruno, 2002; Laszlo Zaborszky et al., 1999). The stimulation of the BF GABA corticopetal projections inhibits the excitability of cortical interneurons, which demonstrates a potent cortical disinhibition (M. Sarter & Bruno, 2002; M. F. Sarter & Bruno, 1994). Furthermore, studies have shown that large PV+ neurons of the BF that project cortically synapse on pyramidal cells of the cortex (Ivana Gritti et al., 1993, 1997a, 2003). These PV neurons from the BF play a significant role in regulating the glutamatergic pyramidal and GABAergic PV interneuron activity in the cortex which contribute to the balance of excitatory/inhibitory activity (feedforward/feedback inhibition) that drives behavioral responses such as planning, decision-making, attention, and working memory (Ferguson & Gao, 2018; Freund & Meskenaite, 1992b; H. Hu, Gan, & Jonas, 2014; Packer & Yuste, 2011). Although the BF GABAergic neurons within the corticoseptal pathway have been studied, little is known about how the local GABAergic interneurons may regulate other pathways and behaviors. Moreover, although there is debate as to whether the GABAergic neurons of the BF are compromised during AD, their role in aging and these age-related diseases has not yet been elucidated (Hä et al., n.d.; M. Sarter & Bruno, 2002).

1.4.3 Glutamate

The BF glutamatergic neurons are the least understood of the three neuronal phenotypes in the BF. Immunohistochemical staining of glutamatergic neurons, using vesicular glutamate transporter marker (vGLUT) and phosphate-activated glutaminase

(PAG), revealed that glutamatergic neurons are a separate phenotype form GABAergic and, in some cases, cholinergic neurons (Fremeau, Voglmaier, Seal, & Edwards, 2004; Ivana Gritti et al., 1993; Herzog et al., 2001; Manns, Mainville, & Jones, 2001). Studies have revealed that glutamatergic afferents of the BF originate from the cortex, amygdala, and telencephalic areas including the VTA (Ronald P.A. Gaykema et al., 1991; Haring & Wang, 1986; L. Zaborszky, Gaykema, Swanson, & Cullinan, 1997). Microdialysis studies, which administered amphetamine, which acts primarily on NMDA and dopaminergic receptors, to the SI/NBM of the BF demonstrated that excitation of the glutamate receptors in the BF can excite cholinergic neurons to release Ach in the PFC (Lamour, Dutar, Rascol, & Jobert, 1986). Moreover, stimulation NMDA in the BF stimulated cortical Ach release in response to a food-paired behavioral stimulus (Arnold, Fadel, Sarter, & Bruno, 2001; Jim Fadel, Sarter, & Bruno, 2001). This indicates a potential role for BF glutamate in attentional and cognitive function. Later studies demonstrated a role for these cholinergic and glutamatergic transmission in top-down regulation, the regulation of attentional functions of environmental stimuli, in the PFC (Nelson, Sarter, & Bruno, 2005).

1.4.4 Microglia

Microglia are prominently known for their role in the innate immune system which guards against neuronal injury and infection. While it is well understood that microglia play a central role in detecting and clearing pathogens and unwanted debris in the brain, there is little understanding of different functions microglia may have in different regions of the brain. Recent studies have found that neuronal signaling exerts a significant impact on proinflammatory processes of glial cells. In particular, ACh release

has demonstrated anti-inflammatory actions on microglia, termed the cholinergic anti-inflammatory pathway (Pavlov & Tracey, 2005). It has also been hypothesized that cholinergic neurons of the BF can regulate microglial activity in the brain through cholinergic modulation (Gamage et al., 2020). This mechanism for neuronal-glial interaction has been demonstrated in hippocampal microglia in which nicotinic acetylcholine receptor α7 subunit (nAChRα7) agonists suppresses glial TNFα release (H. Wang et al., 2003). Thus, a role for neuronal-microglial communication is emerging, and it is clear is clear that ACh is necessary to maintain microglial homeostasis. However, the exact mechanism for neuronal-glial interactions are not known and whether other phenotypes of neurons may play similar or opposing roles has yet to be investigated.

Microglia have been known to impact the BF neuronal environment during aging and age-related diseases. A loss of BFCS neurons, which have been shown to occur in aging and AD, results in microglial reactivity to Aβ and Tau protein which may contributes to uncontrolled inflammation and unnecessary neuronal death (Schmitz et al., 2020). In aging humans, significant levels of microglial inflammation, measured via cytokine release, have been demonstrated (Chung et al., 2009; Rea et al., 2018; Schliebs & Arendt, 2011). are In a rat Aβ42 lesion model, BF cholinergic neurons in the SI/NBM showed significant degeneration and high levels of microglial activation (Nyakas, Granic, Halmy, Banerjee, & Luiten, 2010). Moreover, it is still not fully understood as to how the loss of BF cholinergic tone to the forebrain, which occurs in aging and age-related diseases such as AD, may affect microglial in the BF and major BF efferent targets, such as the hippocampus and cortex.

1.4.5 Summary

The BF several neuronal phenotypes including cholinergic, glutamatergic, and GABAergic neurons, as well as glial cells. The BF consists of efferent cholinergic and glutamatergic component that serves to amplify sensory input and promote attentional and cognitive processing, and a GABAergic component that projects cortically mediating executive functions which require a flexible modulation. Meanwhile, GABAergic interneurons of the BF are involved in the regulating bilateral feedback from several pathways. Collectively the BF integrates circuits involved in sensory, cognitive, attentional and decision making processes in order to provide a context-adequate behavioral response. Additionally, it has been shown to be susceptible to aging and is thought to play a major role in cognitive and attentional deficits seen in AD patients. It is clear that our understanding of the functions of the BF will remain inadequate in the absence of evidence concerning the role of its GABAergic and other noncholinergic components. Moreover, our understanding of the pathways in which the BF integrates may provide a clue as to what components of the BF are vulnerable to dysfunction in aging and age-related disease.

1.5 Inputs to the Basal Forebrain

The BF plays a significant role in the information processing and integrating several signals from several neuronal types in the brain. Importantly, the BF receives several inputs from various brain regions including the insular cortex (IC), medial prefrontal cortex (PFC), lateral hypothalamus (LH), VTA, nucleus accumbens (NAc) and amygdala. These projections make contact with several populations in the BF including

BFCS, glutamatergic neurons, GABAergic neurons and microglia mentioned previously. We know the loss of BF neurons can effect neural networks which rely on BF input to provide necessary communication attributed to the cognitive and homeostatic balance necessary for proper function. It is also possible that the dysfunctional signaling contacting the local BF neurons may contribute in some way to neuronal dysregulation and degeneration. Recent evidence has documented details of the afferent networks of the basal forebrain (R. P.A. Gaykema, Gaal, Trader, Hersh, & Luiten, 1991; Heimer & Alheid, 1991; L. Zaborszky, Cullinan, & Braun, 1991). These regions which send afferent inputs to the BF may be involved in the circuits which seem to manifest the neuronal dysfunction and degeneration in age-related diseases, such as AD (R. Hu, Jin, He, Xu, & Hu, 2016; M. Sarter, Bruno, & Turchi, n.d.). For example, studies using in vivo microdialysis to measure cholinergic release in several brain regions following operant tasks have revealed a role for cortical neurons associated with the BFCS to contribute to attentional processing associated with the cognitive symptoms of AD which show mild cognitive decline during the aging process (J. Fadel & Burk, 2010a; Hasselmo & Sarter, 2011; Kessler et al., 2011; Holly Moore, Sarter, & Bruno, 1992; M. Sarter, Givens, & Bruno, 2001). (Auerbach & Segal, 1996; Blitzer, Gil, & Landau, 1990; Hasselmo & Sarter, 2011). This suggests that cholinergic dysfunction strongly correlates to a dysfunction in neuronal inputs associated with cognitive function. However, it is still not known how these regions are effected during normal age relate changes and there is still much to learn about afferent neuronal populations which make contacts with the BFCS and local cell populations.

1.5.1 The Prefrontal Cortex

Several tract-tracing studies using anterograde tracers have suggested that the cortex sends a significant portion of projections to the basal forebrain (Haring & Wang, 1986; M.-M. Mesulam & Mufson, 1981). Tract tracing studies paired with cholinergic immunohistochemistry have revealed that PFC and insular cortex (IC) send highly organized bilateral projections between cortices and the BFCS (Chaves-Coira, Rodrigo-Angulo, & Nuñez, 2018; Ronald P.A. Gaykema et al., 1991). Later studies found that the boutons of the neurons from the PFC were both excitatory, glutamatergic neurons, and inhibitory, GABAergic neurons (L. Zaborszky et al., 1997). Recent findings have shown that the presence of specific neuronal networks between the BF and cortical regions, such as the PFC and IC, that may play different roles in the control of cortical activity (Chaves-Coira, Martín-Cortecero, Nuñez, & Rodrigo-Angulo, 2018).

The medial PFC sends important GABAergic and glutamatergic projections to the BF, and is thought to be central to processing and cognition, including decision-making and memory. The medial PFC has two sub regions, the prelimbic cortex (PLC) located in the most medial portion of the frontal cortex under the cingulate, and the infralimbic cortex (ILC) located directly below the PLC. The ILC and PLC have both been shown to send major connections to the BF (Vertes, 2003; L. Zaborszky et al., 1997; Laszlo Zaborszky et al., 2015). Studies using appetitive lever press rewards showed an increased activation of PFC and ILC excitatory neurons which indicated a role for both regions in homeostatic response (Burgos-robles, Bravo-rivera, & Quirk, 2013). The PLC and ILC bilateral projections are also thought to play an important role in fear and memory extinction by sending important projections to the BFCS which end in the amygdala and

hippocampus (Knox, 2016; Knox & Keller, 2016). Perhaps, the most interesting hypothesis involves the significance of the BF in mediating both fear/extinction and reward memories to be sent to the hippocampus and amygdala. Thus, findings have revealed specific areas of the BF project bilaterally to the PFC, however, their role in aging and age-related dysfunction has yet to elucidate.

1.5.2 Insular Cortex

The IC, also known as the insular lobe, is a portion of the cerebral cortex beneath the lateral sulcus and located in the center of both cerebral hemispheres of the brain. It is divided into three regions which are adjacent to each other, the agranular, dysgranular and granular layers (Augustine, 1996). The IC was first proposed to have a role in taste and feeding behaviors, including cue potentiated feeding (Augustine, 1985; S. Cole, Hobin, & Petrovich, 2015; Sindy Cole, Keefer, Anderson, & Petrovich, 2020). Early analysis of neuronal activity in monkeys concluded that only 10% of the neurons in the IC are related to taste, 20% are related to sensory or motor responses, but most neurons in the IC were considered a mystery. We now know that the IC sends projections to the cortex, amygdala, limbic system, thalamus, and basal ganglia, and receives projections from these same regions (Flynn, Benson, & Ardila, 1999; M. Nagai, Kishi, & Kato, 2007; Michiaki Nagai, Scheper, Lenarz, & Förster, 2021). Following research has shown that the IC is activated in response to several stimuli involved in maintaining the homeostatic balance of one's state of being, these stimuli include hunger, thirst, itch, touch, body temperature, heartbeat, gastric, and bladder signals (A. D. Craig, 2003, 2013; A. D. B. Craig, 2011; Teipel et al., 2018). Moreover, studies have shown that after chewing,

salivating, or swallowing, there are changes in insular cortex signaling, (Flynn et al., 1999).

There has been some evidence that illustrates several neurochemical changes are occurring in the IC during interoceptive awareness. Our lab has shown that following food-paired stimuli the IC increased efflux of several neurotransmitters including GABA, glutamate, and ACh and that these signals are reduced in aged models (Hagar et al., 2017). Moreover, the BFCS has been known to send cholinergic and GABAergic projections to the IC which, in response to homeostatic cues, are activated (Afif & Mertens, 2010; Evrard, 2019; Rodríguez-García, María, & Miranda, 2016; M. Sarter & Bruno, 2002). Given the bidirectional relationship between the IC and BF, in which the IC has been shown to send mainly glutamatergic projections to the BFCS and GABAergic efferent and local neurons, it has been hypothesized that the IC is responsible for processing several signals to sense of the physiological condition of the body, named interception (A. D. Craig, 2003, 2013; A. D. B. Craig, 2011). Thus, this circuitry may be important for biasing attentional resources toward external stimuli related to underlying physiological status which becomes deficient with age. However these age-related changes are still not well understood, and it is still not known how these changes may affect the BF during aging.

1.5.3 Nucleus Accumbens

The NAc is a heterogeneous region, located in the rostral and ventral forebrain, and is split into two primary segments: a medial shell and lateral core. The NAc is thought to receive a significant portion of the inputs from the basal ganglia, but also

receives inputs from glutamatergic neurons of the amygdala, hippocampus, thalamus, and PFC (H. J. Groenewegen, der Zee, te Kortschot, & Witter, 1987). As a whole, the NAc is a significant player in controlling the biological drives necessary for survival including feeding, reproduction, and reward/motivation (Boswell & Kober, 2016; Pratt, Will, Kelley, & Baldo, n.d.; Rebec, Grabner, Johnson, Pierce, & Bardo, 1996). However, the shell and core subregions receive different inputs which suggests that they may serve distinct behavioral functions (Salgado & Kaplitt, 2015; Zahm, 2000). This has been supported in tract tracing studies in which the afferents entering the shell, including the piriform and infralimbic cortex, differ from the afferents entering the core, including the insular and prelimbic cortex (Brog, Salyapongse, Deutch, & Zahm, 1993). Glutamatergic and dopaminergic inputs into the shell play a role in reinstatement drug seeking behavior, this has been implicated in cortical shell afferents and VTA afferents (Bossert et al., 2012; Henk J. Groenewegen, Wright, Beijer, & Voorn, 1999; Sesack, Deutch, Roth, & Bunney, 1989). However, inputs into the core are involved in the cognitive processing of functions related to reward and reinforcement via afferent regions including the lateral hypothalamus, temporal lobe, and brain stem (Brog et al., 1993).

The main efferent neurons from the NAc are medium spiny neurons (>90%) that send GABAergic projections to various areas of the mesencephalon and basal ganglia. Lesioning studies have shown that lesions of the nucleus accumbens result in a depletion of GABAergic activation of the BF (Záborsky & Cullinan, 1992; L. Záborszky, Carlsen, Brashear, & Heimer, 1986). More recent work has shown that the BFCS is innervated by GABAergic afferents originating largely in the NAc (M. Sarter, Bruno, & Turchi, 1999). Furthermore, other studies using dopaminergic receptor antagonist, resulted in

attenuation of GABAergic output to the BF and support the hypothesis that NAc GABAergic neurons can control the excitability of basal forebrain cholinergic neurons (M. Sarter et al., 1999). In response, the BF sends inhibitory outputs to the VTA which makes dopaminergic contacts on the NAc to form a bilateral disinhibitory circuit (J. Wang et al., 2021). Thus, the BF, VTA and NAc form an intimate pathway which is highly involved in motivational recruitment of attention by homeostasis-associated cues (Tashakori-Sabzevar & Ward, 2018). However, this relationship is not yet fully understood as it relates to aging and age-related diseases.

1.5.4 VTA

The VTA is located in the midline of the lower midbrain next to the substantia nigra, and contains glutamatergic, GABAergic, and dopaminergic neurons. Although the VTA contains many types of neurons, it is characterized by its large population of dopaminergic neurons (Morales & Margolis, 2017). The VTA plays a significant role in decision making, working memory, stimulus salience and positive/negative reinforcement (Adcock, Thangavel, Whitfield-Gabrieli, Knutson, & Gabrieli, 2006; Berridge, 2007; Bromberg-Martin, Matsumoto, & Hikosaka, 2010; Salamone & Correa, 2012). The lateral hypothalamus, and dorsal raphe are the largest sources of inputs to the VTA, but the VTA is also known to make microcircuits with nearly all brain regions it projects too (Beier et al., 2015; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012).

The dopaminergic neurons of the VTA project through two distinct pathways, the mesocortical pathway and the mesolimbic pathway. The mesocortical pathway projects to several cortices including the PFC, cingulate cortex, orbital cortex, sensory cortex, and

motor cortex, and contributes to motivation and executive function. Meanwhile, the mesolimbic cortex projects to the NAc, and amygdala and is associated with reward and reinforcement. Although most of these functions are attributed to the dopaminergic system, it is thought that certain populations of GABAergic and glutamatergic output neurons can also play a role in motivational and reward behavior (Qi et al., 2016; Stamatakis et al., 2013; Van Zessen, Phillips, Budygin, & Stuber, 2012). One such area that may receive projections from several neuronal cell types of the VTA is the BF. It is hypothesized that the BF plays a key role in mediating inputs from the brainstem, including the VTA and substantia nigra and that dopaminergic, glutamatergic and GABAergic of the VTA project to the BF, but it is not known whether all of these projections synapse on BF cholinergic neurons (L. Zaborszky et al., 1997). Recent studies have shown that the BF cholinergic neurons projecting to the PFC receive inputs from the VTA. (Gielow & Zaborszky, 2017). Additionally, there is some evidence indicating these connections may play a role in reward uncertainty, value, and punishment decision making, but further investigation into the behavioral role of these inputs to BF neurons is needed to uncover the specific behavioral components of these pathways (Monosov, Leopold, & Hikosaka, 2015).

1.5.5 The Lateral Hypothalamus and the Orexinergic System

As mentioned previously, the BF is regulated by a variety of inputs, including the LH, whose cell bodies are located within the postero-lateral hypothalamus (Villano et al., 2017). Early lesioning studies demonstrated that the ablation of LH neurons would inhibit feeding and drinking behaviors (Anand & Brobeck, 1951; Morrison & Mayer, 1957). Thus, the LH is considered to be the feeding center of the brain and plays an

important role in homeostatic physiology and behavior (Stuber & Wise, 2016). The LH contains a plethora of neurotransmitters and neuropeptides, including glutamate, GABA, melanin concentrating hormone, and orexin, cell populations that are important for regulating feeding and reward. It is worth noting that some of these cells groups regulate feeding within the same neurons. For example, studies using immunohistochemistry have shown that glutamate containing neurons also contain orexin neuropeptides (Rosin, Weston, Sevigny, Stornetta, & Guyenet, 2003).

In the late 1990's, two separate research groups, Lecea and Sakurai, simultaneously discovered the neuropeptide orexin, also termed hypocretin (L. De Lecea et al., 1998; Sakurai et al., 1998). They found that these orexin neurons exclusively originate from the LH and supported a role for orexin in promoting feeding behavior and energy homeostasis (Sakurai et al., 1998). Orexin neuropeptides, including orexin A (OXA) and orexin B (OXB), bind to two G protein-coupled receptors, the orexin 1 receptor (OXR1) binds OXA; whereas the orexin 2 receptor (OXR2) binds OxA and OxB with roughly equal affinity (Ammoun et al., 2003; Scammel & Winrow, 2011; Smart & Jerman, 2002; Thompson, Xhaard, Sakurai, Rainero, & Kukkonen, 2014). The orexin neurons project diffusely across the mammalian brain to areas including the PFC, VTA, BF, NAc, and other hypothalamic regions (Eggermann et al., 2001; Kukkonen, Holmqvist, Ammoun, & Åkerman, 2002; Peyron et al., 1998). Due to the extensive terminal fields of the orexin neurons, signaling is well positioned to integrate multiple physiological processes such as arousal, energy homeostasis, reward seeking, autonomic function, and cognition. This wide variety of connections of orexin projecting neurons demonstrates a wide range of cognitive, behavioral function which have collectively

earned their description as “physiological integrators” (Luis De Lecea, Sutcliffe, & Fabre, 2002; J. R. Fadel et al., 2013).

Orexins provide substantial innervation to nuclei that are the source of diffuse neuromodulatory transmitters, including dopamine neurons of the ventral tegmental area, cholinergic neurons of the basal forebrain, and cholinergic and glutamatergic neurons of the PFC (Calva et al., 2018; Espana, Baldo, Kelley, & Berridge, 2001; C. Fadel et al., 2005; Jim Fadel et al., 2002). Our lab has shown that orexin innervation of the VTA contributes to the reported changes in motivated behaviors related to food and drug reward (Morgan, Harrod, Lacy, Stanley, & Fadel, 2013). Recent research has also shown that orexin neurons promote arousal, cognition, and attention through their projections to the BFCS and PFC. Our lab has demonstrated that orexin administration results in an increase in acetylcholine and glutamate from the PFC (Calva & Fadel, 2020; Calva et al., 2018; J. Fadel, Pasumarthi, & Reznikov, 2005). We hypothesize that the release orexin in the PFC and BF activates cortical neuronal activity is vital to attentional and cognitive processing, as well as maintenance of energy homeostasis. We further devise that these afferent pathways are vulnerable during aging. Previous studies in our lab and others have shown that aged animals are unresponsive to orexin and observe a reduction in orexin neurons innervating cholinergic neurons of the LH (>40%) and BF (Danielle Frederick-Duus, 2008; Kessler et al., 2011). These alterations in orexin regulation may contribute to the age-related dysfunctions in arousal, attention, and cognition seen in aged individuals, and may represent a highly vulnerable set of neurons susceptible to age-related diseases, such as AD.

1.6 Hypothesis

Age-related BF dysfunction may reflect diminished afferent regulation as well as an altered local environment. We have previously shown reduced orexin/hypocretin innervation of BF in aging, a phenomenon that may link afferent dysfunction with altered microglial homeostasis. There is little research examining these relationships involving afferent neuronal and glial cell populations in the BF as it relates to aging. The purpose of this study is to compare specific neuronal and glial populations to identify anatomical factors susceptible to age-related homeostatic dysfunction in the BF using behavioral tasks, IHC, and enzyme-linked immunosorbent assay (ELISA). I hypothesize that aging contributes to dysfunctional changes in neuronal and microglial activation in the BF. I aim to identify age-related changes in afferent regulation, neuronal activation, and microglial morphology following changes in homeostatically relevant stimuli.

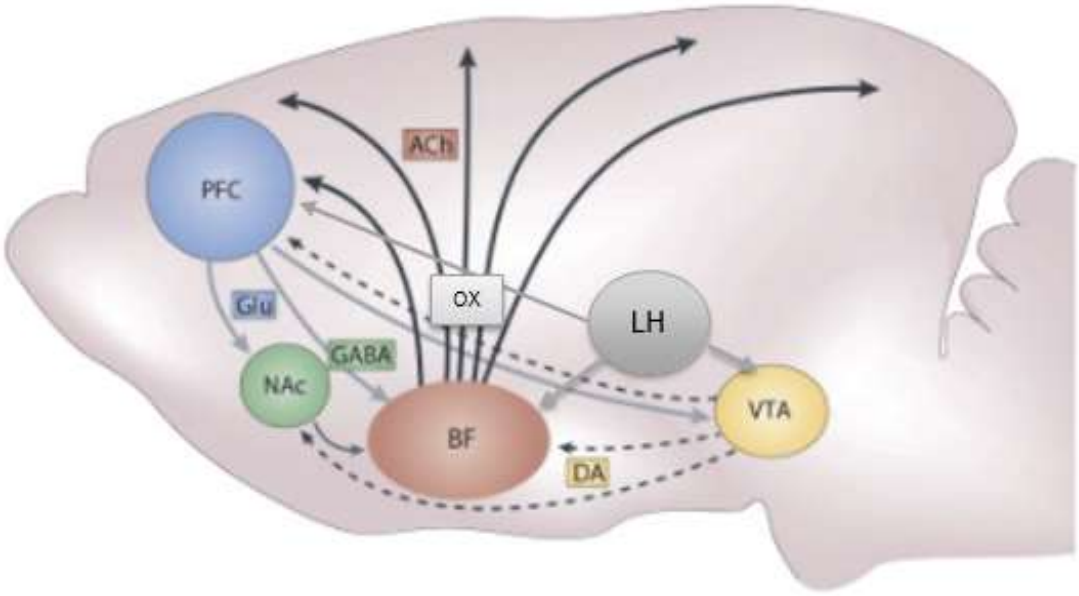

Figure 1.1 Overall BF afferent circuitry investigated. A schematic summary of inputs to the BF involved in age-related processing of salient stimuli.

1.6.1 Specific Aims

Aim 1: To identify age-related changes in neuronal activation of basal forebrain afferent population.

Aim 2: To evaluate age-related changes in activation of specific neuronal populations in the BF.

Aim 3: Investigate the role of orexin-microglial interactions as it relates to age-related BF homeostatic function.

Together, these studies will reveal targets of age-related dysfunction which may play a role in age associated MCI that may be exacerbated during age-related diseases such as Alzheimer's disease.

CHAPTER 2. GENERAL METHODS

2.1 Rationale for animal care and use

All Animal care and use procedures were carried out in accordance with protocols written under the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals approved by Institutional Animal Care and Use Committee at the University of South Carolina School of Medicine (Animal Use Protocols #). Every effort was made to minimize the number of animals used and their suffering.

2.2 Subjects, Handling, and Habituation

Male, F344/Brown Norway F1 hybrid rats (250-300g; National Institute of Aging Colony, Baltimore, MD, USA) were initially pair-housed on a 12-h light/dark cycle (lights on at 07:00 h) and fed with standard laboratory chow and water freely available. Young rats were approximately 3-4 months and aged rats were approximately 26-28 months upon arrival to the animal facility. The FBN/F1 hybrid rat strain has been continuously used by our laboratory and other labs conducting aging studies due to their reduced susceptibility to several non-neurological age-related complications, including intraperitoneal tumors which are frequently observed in other strains (Lipman, Chrisp, Hazzard, & Bronson, 1996; Van Der Staay & Blokland, 1996). Therefore, the FBN/F1 hybrid strain was used to compare the effects of cognition in a food-paired stimulus test to determine the neuronal activation dysregulation during aging. Furthermore, previous

lab work has used this strain of rat to study orexin-aging connections (Calva & Fadel, 2020; Calva et al., 2018; D. Frederick-Duus et al., 2007; Hagar et al., 2017; Kessler et al., 2011). All animals were handled daily during the first week while being housed in an environmentally controlled animal facility and kept on a 12:12 light: dark cycle with lights on at 07:00 h. Animals were given standard rat chow, ad libitum. During the second week, animals were separated and food intake of freely available standard chow was measured. On the third week, animals were given a food restricted diet that consisted of 80% of their standard diet. Several measures were taken to minimize animal suffering during experimentation, including careful monitoring of animal health during habituation, monitoring anesthetic state during surgery, administration of analgesics to ease post-operative pain.

2.3 Stereotaxic Surgery

Stereotaxic surgery is used for site-specific targeting of specific brain regions using 3 sets of coordinates generated in relation to bregma, the intersection of the coronal and sagittal sutures of the skull. To begin, all animals were anesthetized using isoflurane (Piramal Critical Care Inc., Bethlehem, PA, USA) using E-Z anesthesia system (Euthanex Corps, Palmer, PA, USA). Animals were mounted on stereotaxic surgical device (Stoelting, Dale, IL, USA) which allows for injection of a substance into brain regions with extreme precision, and monitored throughout the surgery in order to maintain a sufficient level of anesthesia by a lack of response to a tail pinch. Following anesthesia, animals were shaved to allow for access to the skin just above the skull and wiped with hydrogen peroxide pads (Medline, Lo# QH17713L, Cat # MDS093917, Mundelein, IL, USA). Following sterilization, an incision is made, approximately one

inch, along the midline of the skull, and bregma is identified. Using the stereotaxic device, and bregma as the reference point, the coordinates for the anterior/posterior (AP), dorsal ventral (DV), and medial lateral (L) were calculated and the target sites were marked. Using a Dremel tool, holes were drilled and an injection needle (Hamilton Company, Reno, NV, USA) with the desired substance is inserted in to the BF (Young: AP -.8 mm, L ±2.5 mm, and DV -8.0 mm; Aged: AP -1.1 mm, L ±2.8 mm, and DV -8.3 mm) or LH (Young: AP -2.5 mm, L ±1.2 mm, and DV -9.0 mm; Aged: AP −2.9 mm, L +1.6 mm, DV −9.4 mm relative). Coordinates for guide cannula implantation were obtained from the Paxinos and Watson rat brain atlas (Paxinos & Watson, 1998). Lastly, all animals were given a single dose of buprenorphine (0.01 mg/kg) to ease post-operative pain, given a triple antibiotic ointment (Acme United Corp., Fairfield, CT, USA) and were monitored until complete recovery.

2.4 Stereotaxic Injections

Injections were implemented using a stereotaxic surgical device (Stoelting; Dale, Illinois, USA). Each animal was injected individually and injections were performed in a separate sterile environment.

2.4.1 Cholera Toxin B Subunit

To analyze basal forebrain afferent projections from the basal forebrain CtB was injected in both aged (26-28 months) and young rats (2-3 months). Under isoflurane anesthesia, 1ul of a retrograde neural tract tracer CtB (cholera toxin B subunit; Lot #10331A1; stores at 2-8º C; List Biological laboratories, Inc.) was injected unilaterally into the basal forebrain (AP -.8 mm, L ±2.5 mm, and DV -8.0 mm; Aged: AP -1.1 mm, L ±2.8 mm, and DV -8.3 mm) of animals (n=6-8/group). Animals were allowed one week

(7 days) recovery. During this time handling, habituation to chambers, and food intake was measured. Following immunohistochemistry, CtB infusion produced bilateral expression within and around the substantia innominata. In some cases, mild expression was seen in the ventral pallidum (VP) and medial septum (MS). CtB expression was observed using Peroxidase (DAB) immunohistochemistry. Additionally, aged and young rats showed similar spread of CtB expression following injections.

2.4.2 Lentiviral Injections

Lentiviral vectors are well recognized as good vehicles for gene delivery. Lentiviral vectors are capable of efficiently transducing post-mitotic cells, and stably integrating into the host genome allowing for long-term expression. A lentiviral vector containing a DNA antisense strain, prepro-orexin, was injected into the LH to transduce non-dividing cells long-term. This provides a unique ability to downregulate orexin expression by gene mediated transfer. The transgene expression cassette SPWG-ORX (Figure 2.1) was constructed at the University of South Carolina School of Medicine Pharmacology, Physiology, and Neuroscience Virus Core Facility.

This lentivirus vector was produced by transfecting human embryonic kidney (HEK) 293T cells with a transgene expression cassette, an envelope cassette, a packaging cassette, and a vector cassette. The transgene expression cassette contains a prepro-orexin DNA antisense, phosphoglyceatekinase-1 promoter for ubiquitous expression (PGK-1p), an internal ribosome entry site (IRES), and enhanced green fluorescent protein (GFP) expression marker, and a post-translational regulatory element, woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which increases gene expression by greater than fivefold. The envelope cassette encodes the vesicular stomatitis virus G

glycoprotein gene which allows the vector to gain entry into the cells, and permits transduction of several cell types, stabilizes the vector, and increases infection rate. The packaging cassette, pCMVpR8.92, encodes lentiviral vector trans-regulatory elements which encode for transcription factors necessary for effective transduction and replication of the lentivirus. The vector cassette contains full-length vector RNA (HIV-1 Tat-dependent vector) with cis-acting elements necessary for packaging, reverse transcription, and stable expression by insertion into the host DNA (Cockrell & Kafri, 2007; Wong et al., 2006). Co-transfection produced virus stocks with 5x106 tu/µL.

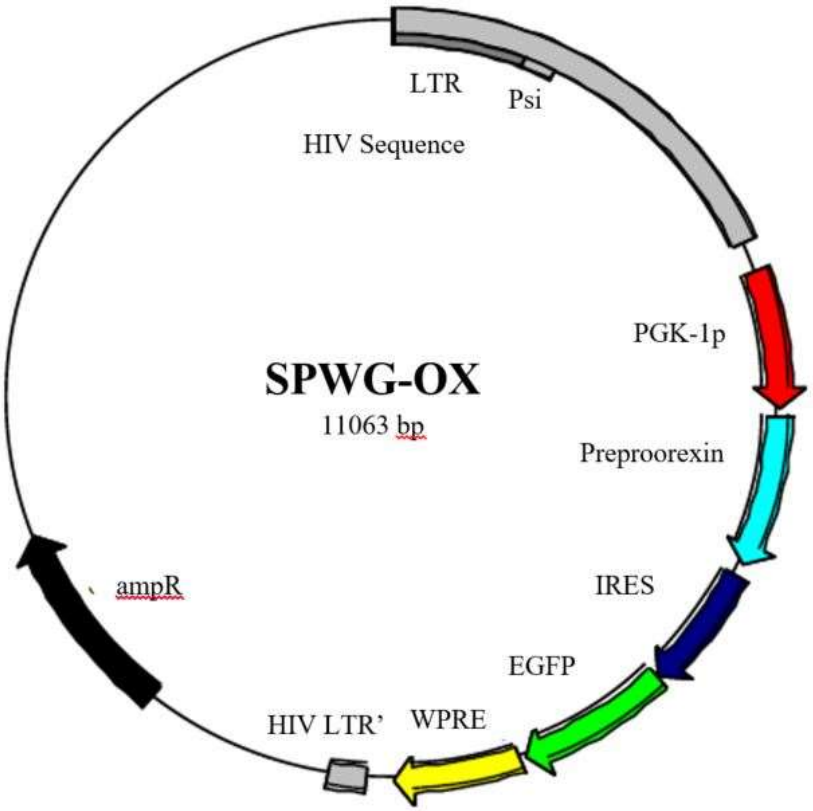

Figure 2.1 SPWG-OX. Plasmid vector containing prepro-orexin antisense gene construct.

2.5 Entrainment

Prior to entrainment, all animals were handled daily for 7 days. The following week, animals were handled and food intake was recorded for 7 days while animals were habituated in microdialysis bowls (parabolic clear plastic bowls; Bioanalytical Systems, Inc., West Lafayette, IN, USA; BAS). On the third week, the animals were food restricted to 80% of their regular intake and underwent entrainment. During this time, animals were monitored daily for food intake, water intake, and weight gain/loss. For the entrainment, animals were either trained to associate a single palatable treat (bacon softie) with sudden darkness or habituated in microdialysis bowls as controls. The food paired stimulus (bacon/darkness) was chosen because this entrainment has been shown to increase in prefrontal cortical acetylcholine release (J. Fadel, Sarter, & Bruno, 1999; Jim Fadel, Moore, Sarter, & Bruno, 1996; D. Frederick-Duus et al., 2007; Inglis, Day, & Fibiger, 1994). This homeostatically relevant stimulus was given at the same time each day every day at 10:00. The amount of time that lapsed prior to consuming the bacon softie was recorded (latency to feed), and animals were taken back to their initial housing premises 30 minutes after consumption. Animals continued to be closely monitored for food intake, water intake, and weight gain/loss. On the 7th day of training, the animals were either given one last entrainment stimulus (E1) or not given the stimulus (C1) before being sacrificed. Animals that were not trained at all (C2) were also sacrificed on this day. Trunk blood was collected in BD vacutainers, centrifuged, and supernatant (plasma) was stored at -20°C for ELISA.

2.6 Trans-Cardial Perfusion and Tissue Collection

All experiments were completed with animals deeply anesthetized with isoflurane and sacrificed. In the first two experiments (Chapter 3 and 4) all animals were transcardial perfused using phosphate buffer saline (PBS) and 4% paraformaldehyde (4% PFA). The pre-fixed brains were then removed from the skull placed in 4% PFA for an additional 24-48 h. The following day the brains were blocked into 3 sections and sliced using a vibratome (Electron Microscopy Sciences, OT-4000) into 50 micron thick slices. The slices of brain tissue were collected and free floated in six wells of the 1X Tris-buffered saline (TBS; prepared with 0.08M Tris base (tris hydroxymethyl aminomethane), 0.42M Tris Hydrochloride, 1.54M Sodium Chloride and diluted to 1%), then transferred to 30% sucrose PBS, anti-freezing solution (500 ml 0.1 PB, 300 ml ethylene glycol and 300 gm sucrose), for long term storage.

In experiments were animal brain homogenates were used for ELISA and immunohistochemistry, brains were extracted and half the brain was placed directly on dry ice and half post-fixed in a 4% paraformaldehyde solution. The flash frozen half of the brain was then stored at -80^{o} C to await further processing, and the post-fixed half of the brain was blocked into 3 sections and sliced, similar to experiments 1 and 2, and placed in antifreeze solution for long term storage.

2.7 Immunohistochemistry

The immunohistochemistry technique uses antibodies which are conjugated to enzymes that catalyze reactions in order to form detectable compounds, either via fluorescence or DAB, to visualize and localize specific antigens in a tissue samples in the brain.

2.7.1 Timeline

For immunohistochemistry studies, all animals maintained a strict timeline (Figure2.2). Upon arrival, animals were habituated for 7 days. Animals were then handled while food intake and body weight was measured daily for 7 days. Following bowl habituation in microdialysis bowls in a separate room dedicated to animal testing, animals were either given paired presentation of a sudden darkness in the testing room with palatable food (E1; young n=8; aged n=8), no food paired stimulus training on the last day (young C1 n =8; aged C2 n= 8) , or no food paired stimulus training on any day (C2) while on an 80% diet restriction (80% of normal chow intake) (single bacon flavored chow; a single paired presentation per day; always given at 11:00 h+/- 15min). After seven consecutive days of training, the latency of the animals to consume the bacon flavored chow (Bacon softies; Bioserve, Fleming, NJ) was generally less than 5 seconds and rats were either presented the paired stimulus 1 h prior to perfusion with 4% paraformaldehyde and .1M PBS (young E1 n=8; aged E1 n=8) or left in the parabolic plastic bowls with no presentation of the food paired stimulus (young C1/C2 n=8; aged n=8). The light were kept off 1 h post food presentation on each day (red light present for trainee visualization). During the first two days animals which did not consume food (bacon softie) after 5:00 minutes were left with food in chamber and time to latency was considered 5:00 minutes. During this training no animals failed to exhibit latency improvement during the 7 (E1) or 6 day trainings (C1). Animals categorized in group C2 were neither exposed to bacon treats nor darkness during training times (11:00).

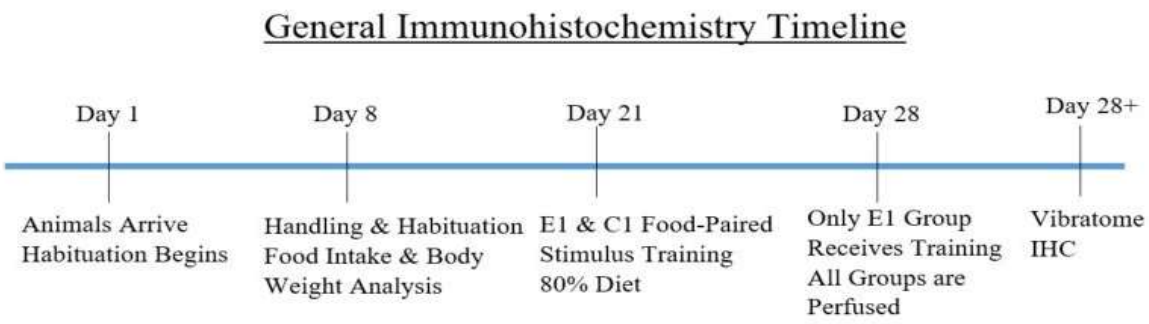

Figure 2.2 General Timeline of Immunohistochemistry experiment.

2.7.2 Injection Placement Verification

CtB staining using immunohistochemistry verified the CtB injection site placement in each rat. The verification was conducted on 50 µm coronal sections of the BF that were sectioned on the vibratome in 1x TBS and stored in anti-freezing solution (500ml .1M PB, 300 ml ethylene glycol, 300 g sucrose). Free-floating sections were placed in a petri dish and BF brain tissue sections were taken for staining. Sections were washed thoroughly (5 rinses at 10 min each) in 1x TBS prior to immunohistochemistry of αCtB (goat) (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA cat# 705-005-003) overnight at room temperature followed by secondary antibody staining of unlabeled donkey αgoat (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 132096) at 2 h. Tissue was then washed and incubated in Goat Pap (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #11945) for 1.5 hs and washed again. Tissue sections were developed using DAB (Sigma-Aldrich co., St. Louis, MO, USA, Lot#MKCK2487) and 30% hydrogen peroxidase (VWR Life Sciences, Solon, Ohio, USA, Lot # 18K1256212) to produce a brown staining of the CtB injection site in the BF. Sections containing the CtB verification were mounted onto

slides using 0.15% gelatin solution (.15g gelatin to 100 ml diH20). After drying overnight, the tissue sections were further dehydrated via ascending ethanol concentrations (50 – 100%) followed by Citrosol™ for 10 min in each. The slides were cover slipped using DPX mounting medium (VWR International, West Chester, PA). CtB injection site was verified (Figure 2.3) using a rat brain atlas (Paxinos & Watson 1998).Animals with inaccurate probe placement were excluded from further analysis of any type.

2.7.3 Immunoperoxidase following CtB

For the immunoperoxidase staining the Fadel lab protocol, previously described by Fadel and colleagues in 2002, was used (Fadel, Bubser et al. 2002). Perfused brains stored in 4% paraformaldehyde were coronally sectioned (50 microns) using a vibratome. All tissue for Immunohistochemical analysis was processed according to previously described protocols with minor modifications (Frederick-Duus et al., 2007, Hagar, calva

Rat brains treated with CtB were double labeled for rabbit α-cFos (1:500; Millipore Corp.,Temecula, CA ; Cat.# ABE457; Lot#3041795) and either goat α-CTb(1:5000; List Biological laboratories Inc., Campbell, CA; Lot #7032A6), goat α-ChAT (1:3000; Millipore, Temecula, CA, USA;, Lot# 2762374, Cat#AB144), mouse α-Parvalbumin (PV; 1:3000; Sigma, Saint Louis, Missouri, USA; Cat#P3088, Batch# 016MA847V), vGluT (Millipore; Temecula, CA, USA; Cat#AB5905; Lot#2733748) , or mouse α-Tyrosine Hydroxylase (TH; 1:3000; Immunostar, Hudson, WI; Lot# 907001; Cat.#22941) to identify activated basal forebrain afferents, cholinergic neurons, parvalbumin positive neurons, or dopaminergic neurons, respectively. Tissue was cleaned in 1x TBS washes five times for 10 minutes, transferred into methanolic peroxide (90 ml

1x TBS, 10 ml, 100 % methanol and 2ml 30% hydrogen peroxide) for 15 minutes to terminate any endogenous peroxidase activity, and to eliminate background staining, the tissue was blocked with TBS+ (100 ml 10X TBS, 40ml normal horse serum, 2ml Triton X-100, q.s. to 1L with pure diH20). Following blocking, sections were incubated in rabbit α-cFos antibody for 48 h. Tissue was then incubated with biotinylated donkey anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 116529) for 1.5 h followed by horseradish peroxidase-conjugated streptavidin (SHRP; 1:1,600; Jackson; cat# 016–030–084) for 1 h. cFos labeling was visualized by adding 30% hydrogen peroxide (H202; 10 µl 30% H202 in 1ml TBS) to the tissue sections in the presence of diaminobenzidine and nickel-cobalt solution (3.0 gm nickel ammonium sulfate, 0.3 gm cobalt chloride q.s.100 ml with distill water) filtered through a .2 µm syringe, generating a blue/black stain in cFos-immunoreactive areas . After an appropriate development time, all sections were rinsed with 1X TBS (4 X 10 min).

For the second development, tissue was incubated in goat α-CTb antibody, α-ChAT, α-Parvalbumin, or α-TH for 48 h at room temperature on a nutator. Tissue was washed (.1M PB 1x TBS) three times for 10 minutes and placed in unlabeled donkey anti-goat antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 132096) or unlabeled donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot#87269) for 2 hr followed by followed by an incubation in Goat PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #11945) or Mouse PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #131380). Tissue was developed using diaminobenzidine (DAB) (Sigma-Aldrich co., St. Louis, MO, USA, Lot#MKCK2487), generating a brown

immunoprecipitate to produce cFos nuclear labeling in either CTb, ChAT, PV, or TH-positive fibers. Tissue was then mounted of microscope slides (VWR Life Sciences, Radnor, PA, USA, and Cat # 16005-106) and dehydrated. After dehydration using ethanol (50%-100%) and two 10 min soaks in Citrosol, the slides were cover slipped using DPEX Mounting Medium (Electron Microscopy Sciences, Hatfield, PA, USA, Cat# 13515) and microcover glass (VWR Life Sciences, Solon, Ohio, USA, Cat # 48393) and left overnight to dry.

2.7.4 Immunoperoxidase following Lentiviral Injection

Post-fixed brains stored in 4% paraformaldehyde for 24 hr were coronally sectioned (50 microns) using a vibratome. It should be noted that one hemisphere of the rat brain was allocated for ELISA and one hemisphere was allocated to immunohistochemistry. Rat brains injected with the lentivirus containing preproorexin antisense (LV-PPOX) or lentivirus containing no antisense and only the GFP gene (LV-GFP) were double labeled for rabbit αIb1A (1:5000; Wako Pure Chemical Industries; Osaka, Japan; Cat# 019-19741; Lot# PTR2404), and either goat α-Orexin-A (OXA; 1:500; Santa Cruz Biotechnology Inc.; Dallas, TX, USA; Cat.# sc80263), goat α-ChAT (1:3000; Millipore, Temecula, CA, USA;, Lot# 2762374, Cat#AB144), or mouse α-Parvalbumin (PV; 1:3000; Sigma, Saint Louis, Missouri, USA; Cat#P3088, Batch# 016MA847V) to identify microglia, and orexin neurons, cholinergic neurons, or parvalbumin positive neurons, respectively. Tissue was cleaned in 1x TBS washes five times for 10 minutes, transferred into methanolic peroxide (90 ml 1x TBS, 10 ml, 100 % methanol and 2ml 30% hydrogen peroxide) for 15 minutes to terminate any endogenous peroxidase activity, and to eliminate background staining, the tissue was blocked with

TBS+ (100 ml 10X TBS, 40ml normal horse serum, 2ml Triton X-100, q.s. to 1L with pure diH20). Following blocking, sections were incubated in rabbit α-Ib1A antibody for 48 h. Tissue was then incubated with biotinylated donkey anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 116529) for 1.5 h followed by horseradish peroxidase-conjugated streptavidin (SHRP; 1:1,600; Jackson; cat# 016–030–084) for 1 h. cFos labeling was visualized by adding 30% hydrogen peroxide (H202; 10 µl 30% H202 in 1ml TBS) to the tissue sections in the presence of diaminobenzidine and nickel-cobalt solution (3.0 gm nickel ammonium sulfate, 0.3 gm cobalt chloride q.s.100 ml with distill water) filtered through a .2 µm syringe, generating a blue/black stain in cFos-immunoreactive areas . After an appropriate development time, all sections were rinsed with 1X TBS (4 X 10 min).

For the second development, tissue was incubated in goat α-OXA antibody, goat α-ChAT, or mouse α-Parvalbumin for 48 h. Tissue was washed (.1M PB 1x TBS) three times for 10 minutes and placed in unlabeled donkey anti-goat antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 132096) or unlabeled donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot#87269) for 2 h followed by followed by an incubation in Goat PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #11945) or Mouse PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #131380). Tissue was developed using diaminobenzidine (Sigma-Aldrich co., St. Louis, MO, USA, Lot#MKCK2487) to produce microglial labeling alongside either OXA, ChAT, or PV neurons. After dehydration using ethanol (50%-100% 2 minutes each) and Citrosol, the slides were cover slipped using DPEX Mounting Medium (Electron

Microscopy Sciences, Hatfield, PA, USA, Cat# 13515) and micro-cover glass (VWR Life Sciences, Solon, Ohio, USA, Cat # 48393) in a dark room. The slides were then dried for 48 hs prior to cleaning and imaging.

2.7.5 Immunofluorescence

CtB injected tissue was cut on the vibratome (50 microns) and stored in antifreeze solution. For immunofluorescence, the tissue was washed (5 times for 10 minutes each), placed in methanolic peroxide (90 ml 1x TBS, 10 ml, 100 % methanol and 2ml 30% hydrogen peroxide) for 15 minutes, and blocked with TBS+ (100 ml 10X TBS, 40ml normal horse serum, 2ml Triton X-100, q.s. to 1L with pure diH20) for 20 min. The tissue was then triple labeled for rabbit α-cFos (1:500; Millipore Corp., Temecula, CA ; Cat.# ABE457; Lot#3041795), goat α-CTb(1:5000; List Biological laboratories Inc., Campbell, CA; Lot #7032A6), goat α-ChAT (1:3000; Millipore, Temecula, CA, USA;, Lot# 2762374, Cat#AB144), and mouse α-Tyrosine Hydroxylase (TH; 1:3000; Immunostar, Hudson, WI; Lot# 907001; Cat.#22941) followed by incubation with fluorescent secondary antibodies for rabbit (1:250), goat(1:100), and mouse (1:100) while covered in the dark. Sections were then washed (5 rinses 10 minutes each), placed on microscope slides using .15% gelatin solution, and covered in a box to dry overnight. The slides were then dehydrated using ethanol (50% -100% 2 minutes each), cover slipped using Permount mounting medium (Electron microscopy Sciences; Hatfield, PA, USA; Cat #17986-05; Lot #162767) and sealed with fingernail polish (Sally Hansen). The slides were then dried for 48 hs in a box to avoid light exposure prior to cleaning and imaging.

2.8 Microscopy

After dehydration and cover slipping, tissue sections were examined by light microscopy (Nikon Eclipse E600). Two serial sections per animal at approximately 1.6 mm rostral to bregma were analyzed in a 400,000 mm2 area within each area. An average was calculated for each animal based on those numbers.

2.8.1 Immunoperoxidase Photography

For all immunoperoxidase slides, images were visualized using the Nikon Eclipse was fitted with CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA). In partnership with the IP Labs Software (Scanalytics; Trenton, NJ, USA) and images were imported to Adobe Photoshop 7.0 (Adobe Systems; San Jose, CA, USA) to adjust for image size, contrast and brightness. Two images were taken of each animal and peroxidase staining was quantified manually in each brain region (Figure 2.3). All images were formatted into the same pixel size before quantification.

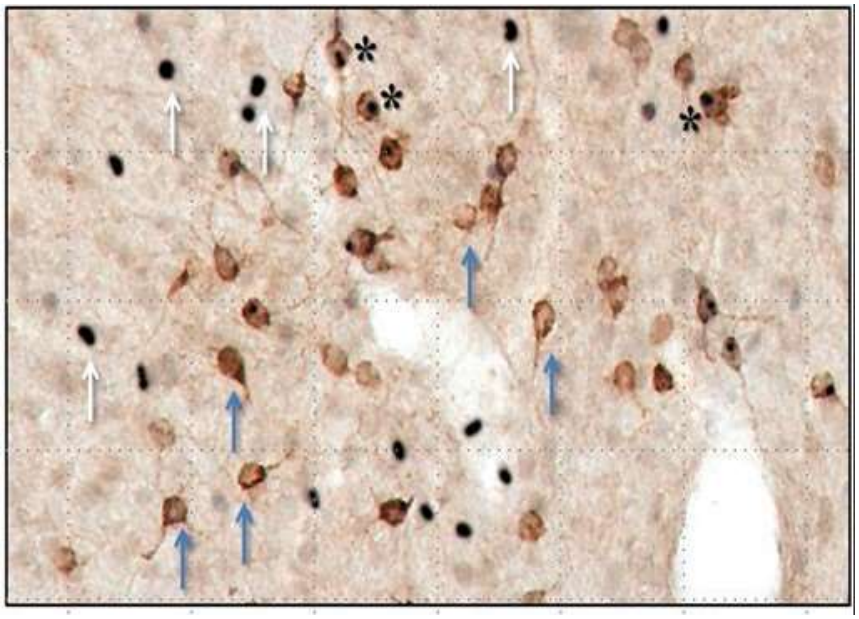

Figure 2.3 A representative pictograph of a brain tissue slice (Cl) with cFos (black; white arrow), CtB (brown; blue arrow), and cFos/CtB (astrick*) labeled neurons.

2.8.2 Immunofluorescence Photography

Fluorescent images of brain tissue sections were acquired at 10x magnification and identified based on corresponding rat atlas reference figures in the Rat Brain Atlas. Fluorescence images were visualized using a Nikon E600 microscope or a Zeiss LSM700 (Zeiss; Oberkochen, Germany) equipped with a scan head with two photomultiplier tube (PMT) detectors on an inverted Axio Observer Z1 stand. The Zeiss confocal microscope contains four lasers which produce lines at 405nm, 635nm, 555nm and 488nm that allow for visualization of triple labeled immunofluorescence.

2.9 Assays

To assess the cytokine activity, an ELISA was performed using both blood plasma and brain homogenates from brain punches of the BF. Prior to analysis of brain homogenates, protein levels were analyzed using a BCA protein Assay to

2.9.1 Timeline

For the microglia studies, animals were habituated for 7 days prior to stereotaxic injections of the SPWG-OX virus (Figure 2.1). Following surgical injections, animals were given food and water ad libitum for 3 weeks to allow the lentivirus to achieve maximum efficacy while food intake and body weight measurements were taken daily. The animals were then tested for cognitive/attentional deficits using food-paired stimulus (bacon/darkness) and sacrificed with fresh brains removed and cut in half sagittaly. One half of the brain was placed directly on dry ice in aluminum foil for preservation of fresh tissue, and the other hemisphere was post-fixed in 4% paraformaldehyde in a glass 20ml vial. The half of the brain designated to the ELISA study was processed for BF brain

punches, and the post-fixed portion was sliced on the vibratome and tissues slices placed in antifreeze solution in -20°C freezer to await immunohistochemistry.

2.9.2 Plasmid Collection

To perform ELISA on blood plasma, blood was collected on ice in EDTA- treated test tube (BD; Cat # 367835; Franklin Lakes, NJ, USA) prior to perfusion. All blood was transferred on ice and then spun down for 15 minutes at 30,000 x g in a centrifuge machine at 4°C. The supernatant from the plasma was then transferred to an Eppendorf tube and placed in a freezer at -80°C.

2.9.3 Brain Homogenate Punches

For brain homogenates, punches were taken using a freezing microtome and the rat brain atlas. Inactive lysis buffer cocktail was prep before taking brain punches (.8g NaCl, 1ml 1% Tergitol, 10ml 10% glycerol, .315g Tris HCl; q.s. 100ml with HPLC grade water). After taking brain punches, tissue was immediately weighed and placed in active lysis buffer solution (100 µl phosphatase inhibitor cocktail, 100 µl protease inhibitor cocktail, 10ml inactive lysis buffer) and lysis buffer was added at 1mg brain tissue: 5 µl buffer solution in safe lock Eppendorf tubes (Eppendorf North America Inc., USA; Lot J189122H; Cat #022363204) on ice. Before tissue is homogenized, 0.1g of .5mm Zirconium Oxide beads (Next Advance; Troy, NY, USA; Cat# ZROB05) is added to each safe-lock Eppendorf tube, and tubes are placed in a bullet blender (Next Advance; Troy, NY, USA; Cat# G14G15) for 5 minutes at speed 10 in 4°C. Tubes are aliquoted into Eppendorf tubes (50ul each) and immediately placed in -80°C freezer to await ELISA or BCA Protein Assay.

2.9.4 BCA Protein Assay

Brain Tissue homogenates are removed form -80^{o}C freezer and immediately placed on ice. BCA working reagent is determined and working reagent is prepared (400 µl Reagent A, 20ml reagent B) and vortexed. Standards are prepared in accordance to the BCA protein Assay Kit (Thermo Scientific; Rockford, IL, USA; Cat#23225) using 2mg/ml albumin standard ampules. Tissue samples are diluted at 1:12.5 in HPLC grade water (4µl sample: 46 µl HPLC grade Water) in Eppendorf tubes. To a sample plate, 25 µl of tissue sample or standard is added to each well. To the same plate, 200 µl of working reagent is added to each well using multichannel pipette. Plate is then placed in oven at 37^{o}C for 30 minutes, and then cooled for 15 minutes outside the oven before being read using a Biotek Synergy 2 Plate Reader (Biotek instruments Inc.; Winooski, VT, USA; Cat #7131000).

2.9.5 ELISA

Blood plasma of ages and young animals were analyzed using a 12-Plex Th1/Th2 Bio-Plex kit (Bio-Rad Laboratories Inc.; Cat # 171K1002M; USA) immediately following perfusion. To analyze cytokines, the Bio-Plex assay uses premixed coupled magnetic beads and detection antibodies to detect a total of 12 different rat cytokines including: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, IFN-γ, and TNF-α. Solutions for the assay were brought to room temperature for 45 minutes and capture beads, lyophilized standards vial, secondary antibody, and tertiary antibody (SAPE) were placed on ice. The dilution standards were prepared using the provided diluent and reconstituted standard. Blood plasma or brain homogenates, previously stored in Eppendorf tubes, were thawed and diluted to 1:4 (for plasma) or 1:3 (brain

homogenates) with diluent. Tube of magnetic beads (575 µL) was then vortexed and diluted into 15 ml conical tube (Corning; Cat# 430790; Lot # 19418049; Mexico) with assay buffer (5175 µL). Diluted beads were then added to be bottom of a 96-well plate, followed by 50 µL of standards and samples using a P-1000 and P-200 multichannel pipette. The assay plate was incubated on an orbital shaker plate for 1 h at room temperature. After 3 washes with wash buffer using a wash station (MAGx2 protocol), the detection antibody (300 µL) was added to each well along with the detection antibody diluent (2700 µL), followed by a 30 minute incubation at room temperature on an shaker plate. The plate was then run through the wash station (MAG 3x protocol) to remove left over detection antibody. Finally the tertiary antibody, SAPE (60 µL) and assay buffer (5940 µL), was added to each well using a multichannel pipette and the plate incubated for ten minutes on a plate shaker. The plate was runt through a wash station (MAG3x) and beads were resuspended in assay buffer (125 µL). The plate was read using a Luminex Bio-Plex Multiplex plate reader that uses high photomultiplier voltage to detect cytokines. Results were interpreted from a logarithmic standard curve.

2.10 Statistic analysis

Differences in latency to feed were tested by a one-way analysis of variance (ANOVA) post hoc test with significance $p < .05$. Percent double labeled to either CtB, ChAT, PV, or TH were tested by a two-way ANOVA. The between groups factors were administration of food-paired stimulus (i.e. C1, C2, E1) and age (i.e. young and old), where 2-3 months is considered young and 26-28 months is considered old. Young and old groups were analyzed separately as determined as well. Sample size was determined by power analysis for the food-paired stimulus study and suggested that 8 animals per

group were sufficient to achieve statistical significance given an effect size of $f = 0.6$ and $\alpha = 0.05$. N-sizes for plasma measures were 8 per group. All values are expressed as mean ± SEM. All statistical tests were done using GraphPad Prism version 5.02 (GraphPad Software Inc.; San Diego, CA). Immunohistochemical data were expressed as percent of cFos/CTb, cFos/TH, cFos/ChAT, cFos/Parvalbumin relative to CTb, TH, ChAT, or Parvalbumin labeled cells, respectively.

BCA protein assays were analyzed using Excel and protein contents (ug/ml) were then interpolated in GraphPad Prism 9 using a standard curve. ELISA data (pg/ml) was normalized to protein content (ug/ml) and graphed using an analysis of variance (ANOVA) with significance set at $p < 0.05$. Cytokines were analyzed as one-way ANOVA or paired/unpaired t-test.

CHAPTER 3. AGE RELATED CHNAGES IN BF AFFERENT ACTIVATION

Aim 1: To identify age-related changes in neuronal activation of basal forebrain afferent population following a food-paired stimuli in aged and young rats.

3.1 Rationale

The ability to detect and interpret internal and external stimuli to make appropriate decisions requires focusing on relevant stimuli. Many brain regions are implicated in maintaining an adequate level of signal/noise ratio for stimulus discrimination, and stimulus valence evaluation (Aitta-Aho et al., n.d.; Ronald P.A. Gaykema et al., 1991; Morales & Margolis, 2017; Villano et al., 2017). Recent evidence suggests that sensory and decision-making circuits must interact via a common neuronal pathway designated to integrate signals in order to elicit an appropriate behavioral response. The BF has been implicated in the integration of signals and plays a vital role in top-down processes in which the BF moderates how attentional resources are allocated (J. Fadel & Burk, 2010b; Viggiano et al., 2014). The heterogeneous afferent and efferent neurons of the BF are capable of interpreting and communicating these diverse signals, and it is important to note that the BF forms many bidirectional signaling pathways which includes most of the cortical mantle and sensory processing circuits modulating different aspects of the sensory decision-making process (I. Gritti et al., 2006; Hasselmo & Sarter, 2011; Laszlo Záborszky et al., 2018).

The functional role of the BF efferents, particularly the BFCS, has been extensively studied. Previous studies have established a role for cortically projecting basal forebrain cholinergic neurons (BFCN) in the modulation of attention (Everitt & Robbins, 1997; Lammers et al., 2018; McGaughy & Sarter, 1998). These anatomical studies demonstrated that these cholinergic neurons are interspersed with GABAergic projecting neurons and may form highly organized inputs onto specific cortical groups which receive specific combinations of inputs to influence attention (Gielow & Zaborszky, 2017; Ivana Gritti et al., 1993; Kondo & Zaborszky, 2016; Laszlo Zaborszky et al., 2015). Hippocampal BF efferents have been shown to influence memory and learning aspects of behavioral tasks. The control of these efferent signals is achieved through a variety of inputs stimulating different cholinergic, and potentially GABAergic, neurons. Several studies have defined BF afferent populations which contribute to this input-output relationship. However, the role of these BF afferents during aging is yet to be fully understood.

Evidence indicates that age-related cognitive deficits, particularly in memory, learning, decision making and attention, are in part due to a dysregulation in the signaling mechanisms of the BF; however, it is not yet known what particular afferent and local neuronal pathways or populations may be implicated in this dysfunction. Behavioral cognitive and attentional deficits have been demonstrated in both normal aging and pathological age-related disorders have been associated with basal forebrain dysfunction (Baxter & Chiba, 1999). Moreover, homeostatic dysfunction has been shown to disrupt cognitive function in normal aged patients, and in rodent models suggesting a relationship between aging and homeostatic dysfunction (J. R. Fadel et al., 2013; Herman

et al., 2016; Teipel et al., 2018). Evidence suggests that the BFCS undergoes moderate degenerative changes during aging which are related to mild attentional and cognitive decline (Schliebs & Arendt, 2011). Thus, we aim to identify age-related changes in specific BF afferent populations.

3.2 Hypothesis

Because there is growing evidence suggesting that BF neurons receive a specific combination of inputs related to differing functions, first we predict that there are specific BF afferent circuits involved in age-relate homeostatic dysfunction. I hypothesize that age-related attentional deficits related to the attentional processing of homeostatically relevant stimuli is associated with dysfunctional neuronal activation of specific BF afferents. In my studies, I have reproduced a previously demonstrated age dependent relationship between a dark/food-paired stimulus and age. Using this behavioral technique in combination with neuronal tract tracing and histochemical analysis, I examine the effects of aging on afferent input stimulation in the BF. I predict that neuronal activation of specific BF afferents will be uncovered. This approach will reveal several circuit motifs vulnerable to age-related dysfunction which will enable future anatomical studies to define specific cell signaling mechanisms that are effected by aging and age-related diseases.

3.3 Approach

All experiments were carried out in accordance with NIH Protocols and IACUC approval. The methods described herein are an abbreviated version of those in Chapter 2: General Methods. Experiments were carried out in six separate batches. All methods are depicted in a timeline (Figure 3.1).

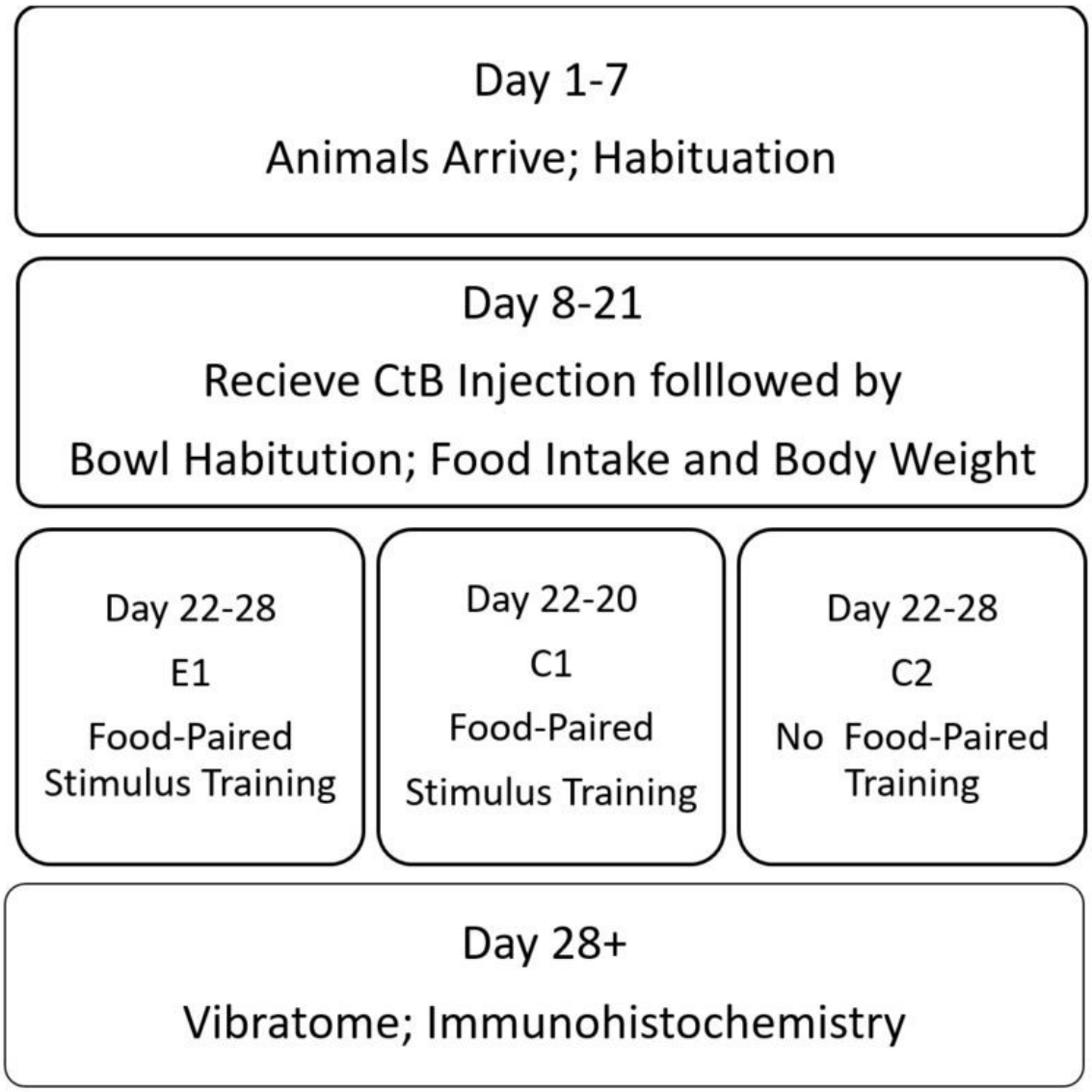

Figure 3.1 Timeline of CtB Injection experiment on young (n=24) and age rats (n=24). Rats were further divided into 3 subcategories based on food paired stimulus training (E1, C1, and C2).

All experiments were conducted using young (3-4 months) and aged (26-28 months) male Fisher 344/Brown Norway F1 hybrid rats (National Institute of Aging Colony, Baltimore, Maryland, USA. Upon arrival, animals were randomly assigned to receive C1 (no training), C2 (training on 6 of 7 days), or E1 (training all 7 days). All animals received CtB Injections (Figure 3.2) following Habituation for 7 days.

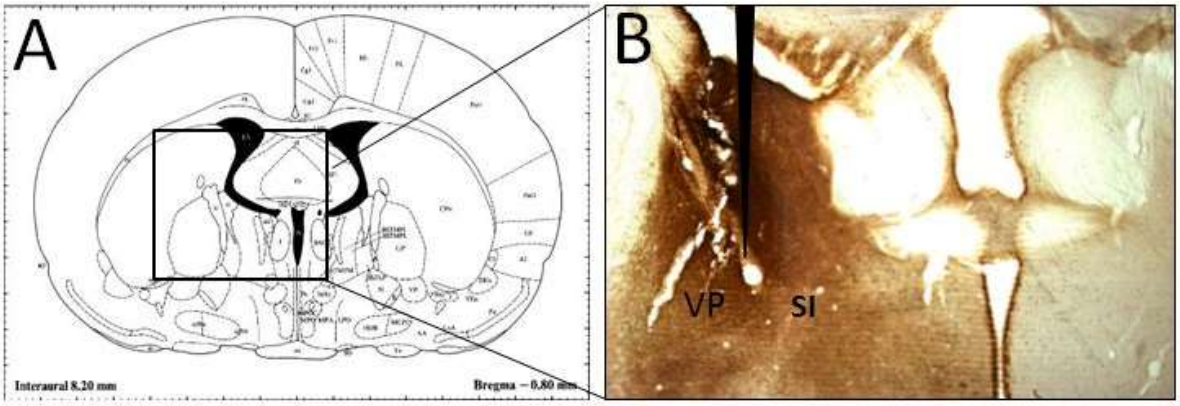

Figure 3.2. A typical CTb deposit site.
(A) Diagram of SI region of the BF. **(B)** CTb deposit site (indicated by black elongated arrow) following injection of CTb (brown) in the SI/VP region of a young rat.

3.3.1 CTb Retrograde Tracer Injection

A retrograde neuronal tracer CTb (1ul) was injected unilaterally in the BF of aged (26-28 months) (n=24) and young (2-3 months)(n=24) F344/Brown Norway F1 hybrid rats (250-300g; National Institute of Aging Colony, Baltimore, MD, USA) following one week of handling and habituation. Injections targeted the SI/NBM of the BF (Stereotaxic Coordinates: AP -.8 mm, L ±2.5 mm, and DV -8.0 mm; Aged: AP -1.1 mm, L ±2.8 mm, and DV -8.3 mm) Animals were allowed one week for recovery during which handling, habituation to chambers, and food intake was measured. CtB infusion was used to

produce bilateral expression. The injection site was later observed using immunohistochemistry (Figure 3.2). Starting at Day 8 prior to CtB injection and daily until day 28 when rats were perfused and brain tissue was processed for immunohistochemistry, all animals were monitored daily for any changes in body weight or food intake.

3.3.2 Food-paired training

During day 14-21 young or aged rats were categorized into 3 subcategories based on food/darkness training assignments. Control groups (C1; aged n=8; young n=8) were habituated in microdialysis bowls for 1 h until day 7 in which rats were perfused with 4% paraformaldehyde 1 h after the completion of daily bowl habituation. Control groups which received food/darkness training on day 1-6 (C2; aged n=8; young n=8) were only habituated in microdialysis bowls on day 7 prior to perfusion. Experimental groups (E1; aged n=8; young n=8) were given the food/darkness training daily for 7 days, and animals were then perfused on day 7 1 h after being given the food/darkness stimulus. The food/darkness training consisted of darkness (switching off the light) followed by the presentation of a palatable treat (Bacon softie). During day 14-21 rats were given an 80% diet restriction. Calculation for the daily amount each at would receive were taken from food intake data taken one week prior.

3.3.3 Histology

Following perfusion with 4% paraformaldehyde, brains were removed and cut on a vibratome in 1X TBS and placed in antifreeze solution for storage in -20^{o}C freezer. Prior to staining, tissue was washed in 1x TBS, transferred into methanolic peroxide, and blocked with TBS. Tissue sections were double labeled for rabbit α-cFos (1:500;

Millipore Corp., Temecula, CA) and goat α-CTb (1:5000; List Biological laboratories Inc.) to identify activated basal forebrain afferents. Following incubation in rabbit α-cFos, sections were incubated biotinylated donkey anti-rabbit secondary antibody for 1.5 hs followed by horseradish peroxidase-conjugated streptavidin for 1 h. cFos labeling was visualized by adding 30% hydrogen peroxide to the tissue sections in the presence of diaminobenzidine and nickel-cobalt solution. After an appropriate development time, all sections were rinsed with 1X TBS (4 X 10 min) and placed directly in goat α-CtB primary antibody for 24 hs at room temperature. Tissue was then washed and placed in unlabeled donkey α-goat secondary antibody for 2 hs at room temperature followed by goat PAP for 1.5 h at room temperature. Tissue was then washed and visualized by adding 30% hydrogen peroxide to the tissue sections in the presence of diaminobenzidine to produce a brown staining (Figure 3.2).

For single-label immunohistochemistry to identify either CtB Density or cFos density, free floating sections were incubated with a rabbit α cFos or goat αCtB antibody for 48 hr at 4ºC followed by a biotinylated donkey α-rabbit or biotinylated donkey α-goat secondary antibody for 1 ½ hs at room temperature, and horseradish peroxidase conjugated streptavidin for 1 h at room temperature. Staining was developed using 0.3% hydrogen peroxide and nickel-cobalt enhanced diaminobenzidine to yield blue-black nuclei of activated neurons or CtB afferent fibers. All sections were mounted onto slides with 0.15% gelatin, dried overnight, dehydrated (ethanol 50%-100%), delipidated (Citrosol), and cover-slipped using DEPEX mounting medium.

For immunofluorescence in the VTA, tissue was triple labeled for rabbit α-cFos (1:500; Millipore Corp.), mouse α-TH (1:500; Immunostar) and goat α-CTb (1:5000; List Biological laboratories Inc.) to confirm dopaminergic activation of BF afferents n=4, young; n=4, aged). Perfusion and brain sectioning were similar to the methods described in chapter 2. Tissue was then triple labeled for rabbit α-cFos, goat α-CTb, and mouse α-Tyrosine followed by incubation with fluorescent secondary antibodies for rabbit (1:250), goat (1:100), and mouse (1:100) while covered in the dark. Sections were then washed and placed on microscope slides using .15% gelatin solution, and covered in a box to dry overnight. Sections were mounted onto slides with 0.15% gelatin, dried overnight in a dark box, dehydrated only and cover-slipped using Permount mounting medium and finger nail polish.

3.3.4: Microscopy and Imaging

Histological experiments for single-labeled (cFos and CtB) neurons, and double-labeled (cFos/CtB) neurons were visualized at 10x-20x magnification using a Nikon E600 microscope fitted with a CoolSNAP digital camera. Immunoperoxidase photos were captured using IP Lab Software. Fluorescence images were visualized at 10x-20x magnification on a Zeiss LSM700 (Zeiss; Oberkochen, Germany) equipped with a scan head with two photomultiplier tube (PMT) detectors on an inverted Axio Observer Z1 stand.

3.3.5 Data Analysis

For all immunohistochemistry experiments, single-labeled (cFos) and double labeled (cFos/CtB) positive neurons 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/CtB labeled neurons and expressed as a percentage of cFos labeled in CtB cells. Two representative images of each animal in each region were averaged to obtain one data point. Single-label cFos and single-labeled CtB was expressed as the density of immunopositive nuclei counted within the reticle area (cFos or CtB nuclei/mm2) and qualitatively expressed in table format.

Statistical analyses of these data were analyzed by two-way ANOVA or unpaired two-tailed t-test using GraphPad Prism 8 Software. To analyze the relationship between age (young and old) and training effect (C1, C2, E1) a two-way ANOVA was performed. Significant effects of CtB, cFos density, body weight, feeding latency, and food intake between aged or young animals were tested using multiple unpaired t-tests or one-way ANOVA (significant effects of treatment condition (i.e. E1, C1, C2) across age were determined followed by Tukey's multiple comparisons test). The significance level was determined as $p<0.05$ for all analyses.

3.4 Results

3.4.1 Effects of Age on Latency to Feed

To confirm that aging can affect the cognitive ability to sense and attend to a homeostatically relevant stimuli, food-paired (dark/bacon softie) stimulus training was enacted (Figure 3.3). Latency to feed was used to test the ability to process the presentation of a bacon softie following a quick shut off of the lights. A greater latency to feed was expected for the aged E1; 26-28 months) group in comparison to their younger (E1; 2-3 months) counterparts over a 7 day period. Two trends are evident among the groups: both groups show a general decrease in response latency over time and the age group had a greater overall response latency than the young group. A two-way ANOVA indicated a

significant difference between the age and young animal's response latency ($p<.0001$; $F(1, 14) = 130.6$; column factor). The ability for aged animals to respond and attend to the conditioned stimulus was observably longer than young animals from day to day. A post ho Turkeys Multiple Comparisons test was performed which revealed a significant difference between Day 3-7 in aged and all days in young animals. Thus, the food-paired stimulus (darkness bacon softie) was a successful indicator of the gap between aged vs. young cognitive ability to attend to external homeostatically relevant stimuli. Previous work showed food restricted aged rats have increased latency to food paired stimuli compared to young control (D. Frederick-Duus et al., 2007; Danielle Frederick-Duus, 2008).

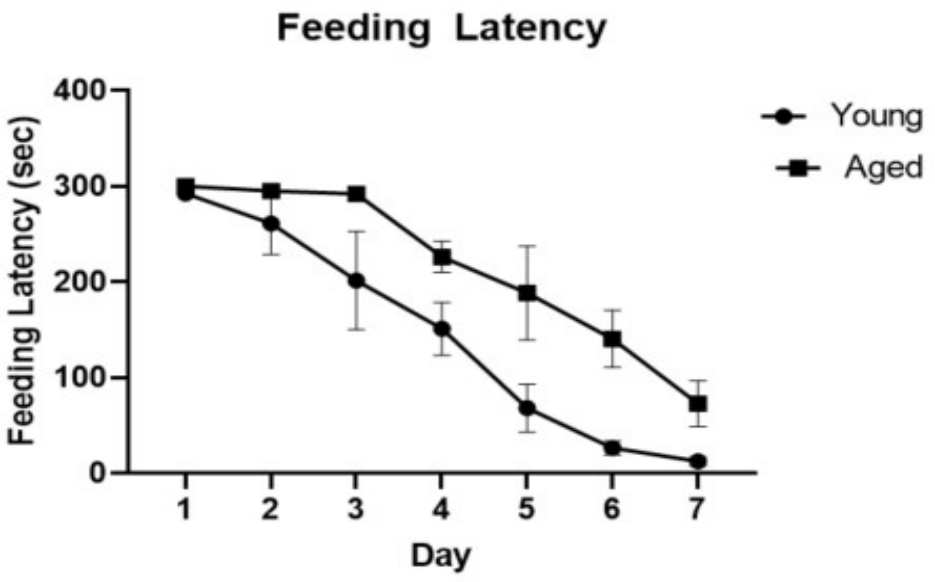

Figure 3.3 Food-Paired stimulus training over 7 days between aged and young rats. Data reveals a significant increase in feeding latency of age rats compared to younger counterparts ($p<.0001$; $F(1, 14) = 130.6$) and between days (3-7) using Tukey's post hoc analysis.

3.4.2 Effects of Age on cFos Density

To confirm that aging can produce a significant decrease in activation, immunohistochemistry was employed to measure the mean density of activation in several brain regions including the PLC, ILC, IC, LH, CL, NAc, CEA, and VTA (Table 3.1). CFos expression was measured (cFos nuclei per mm^2) in C1, C2, and E1 groups of young and aged animals. Density measurements for cFos were taken in a 0.49 mm x 0.49 mm area in the center of the associated regions. For cFos counts within a region, total number was represented as a cFos positive nuclei. A two-way ANOVA was used to indicate whether a significant differences between E1, C1, C2 groups of young and aged animals in each separate brain region. A post hoc Turkeys Multiple Comparisons test was performed to reveal a significant difference between groups of young and aged animals.

There was a significant increase in cFos density of young E1 animals vs. aged E1 groups in the PLC (P=.0437; F (1, 6) = 28.2), NAc (p=.0344; F (1, 6) =17.4), LH (p=.0356; F (1,6)= 39.2) and VTA (p=0.013; F (1, 6) = 26.78). A trend in the ILC (p=.059; F (1, 6) = 28.9) reveals a decrease in young cFos activation compared to aged groups. Following comparisons of age groups, a quantification of the cFos density in each group (C1, C2, E1) for both young and aged rats using a post hoc analysis, Tukeys Multiple Comparisons test, was performed which revealed a significant decrease in age E1 groups vs young E1 groups in the PLC (p=.0319), NAc (p=.0147), and VTA (p=.0011). A significant increase in aged E1 compared to young E1 groups in the ILC (p=.0411) was also revealed which indicated that the ILC and PLC have opposing cFos activation in response to a food paired stimulus.

Table 3.1 Density of cFos indicated via cFos labeled nuclei (per mm2). Table illustrates cFos density of labeled nuclei in PLC, ILC, IC, LH, Cl, NAcc, CeA, and VTA following food paired stimulus (C1, C2, E1). There is a significant increase between young E1 compared to old E1 groups (with *=P<.05) of PLC, NAcc, and VTA. The ILC demonstrates an opposing trend with aged showing higher levels of cFos density compared to young. This suggests a potentially significant role in the processing of homeostatically relevant stimuli which is lost during aging.

cFos Density (cFos nuclei per mm^2)						
Area	**Young**			**Old**		
	C1	C2	E1	C1	C2	E1
PLC	21.94	22.86	34.69*	22.45	23.45	22.96
ILC	22.53	22.04	17.69	20.41	22.45	15.82
IC	14.80	13.88	15.95	12.93	11.56	16.33
LH	16.42	14.92	18.31*	15.21	16.32	14.21
Cl	18.28	17.89	16.93	17.74	17.85	15.31
NAc	33.16	30.20	44.30*	27.40	34.69	37.31
CeA	26.02	20.41	19.73	20.41	18.37	19.23
VTA	20.41	17.96	29.25*	13.61	12.93	15.31

Effects of Age on CtB density

Using immunohistochemistry, CtB fibers where quantified for several BF afferent regions including the PLC, ILC, IC, LH, CL, NAc, CeA. There was no significant difference in CtB fiber density between young and age animals. This was confirmed using a t-test between young and aged groups in each individual BF afferent regions. However, CtB density was observably uniform across age (young, aged) and group (E1, C1, C2). Density of fiber was determined using a 0.49 mm x 0.49 mm area in the center of the associated regions and were quantified using Excel.

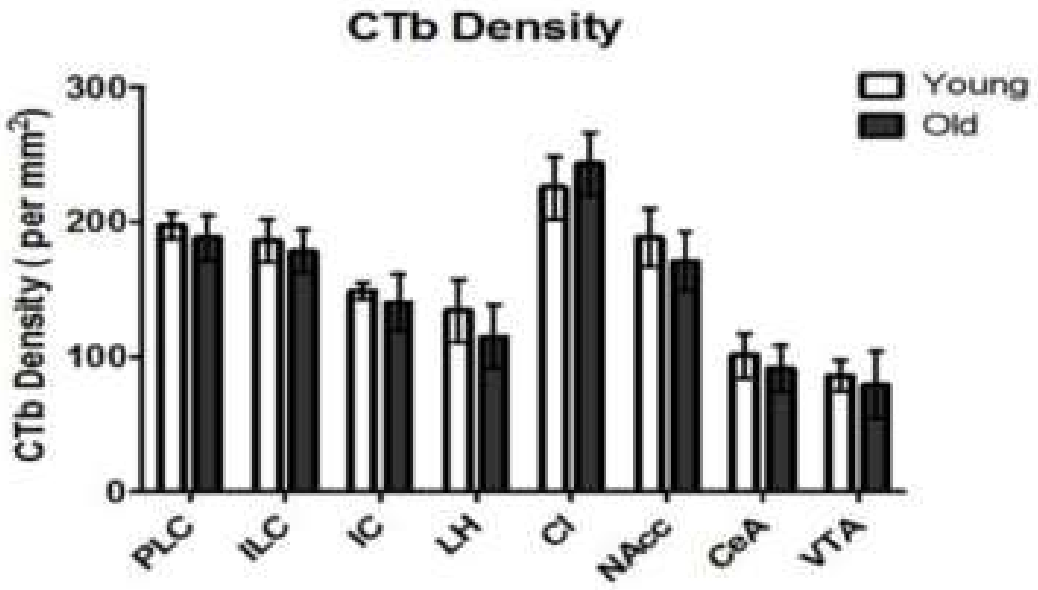

Figure 3.4 CtB density of several BF afferents including PLC, ILC, IC, LH, NAc, CeA, and VTA showed no significant changes indicating no BF Afferent loss between young and aged animals.

3.4.3 Effects of Age and Food-paired Stimulus in Cortical Regions

To determine the effects of aging on BF afferent activation, immunohistochemistry was employed to measure the number of cFos/CtB neurons over CtB+ neurons in the several brain regions including the PLC, ILC, and IC (Figure 3.5). Analysis using light microscopy and diaminobenzidine (DAB) reveal cFos labeled nuclei (black) and CTb labeled soma (Brown). Double labeling of CTb-cFos positive cells are expressed as a percentage of the total CTb labeling for PLC, ILC, and IC in young and aged rats. A two-way ANOVA revealed a significant variation between stimuli (C1, C2, E1; $p=0.003$; $F(2,33)=10.6$). Following a two-way ANOVA paired with a Tukey's Multiple Comparison test, the PLC shows a significant increase in the percentage of double labeled neurons when exposed to dark-food paired stimulus 1 h prior to perfusion (young E1 vs age E1; $p=0.0397$) and between young rats which received food-paired stimulus training (E1) and controls (C1 vs E1; $p=0.0131$) (C2 vs E1; $p=0.001$). In the ILC, a two-way ANOVA reveals a stimulus effect (Column factor; E1, C1, C2; $p<0.0001$). However, no changes in amount of basal forebrain afferent activation of younger rats (E1) compared to age counterparts (E1). Interestingly, the ILC shows an opposing effect between column factor (stimuli; $p=0.04111$; $F(2,33)=12.1$) which is exemplified in Tukey's Multiple Comparison test between the young E1 groups which have a significantly lower activation of basal forebrain afferents compared to controls (E1 vs. C1; $p=0.001$)(E2 Vs. C2; $p=0.0013$), and opposing effects in aged E1 and control groups (E1 vs. C1; $p=0.0158$)(E2 Vs. C2; $p=0.0003$). The IC has no significant age-related change in the basal forebrain afferent activation following a food paired stimulus (E1) in young rats compared to aged rats using a two-way ANOVA.

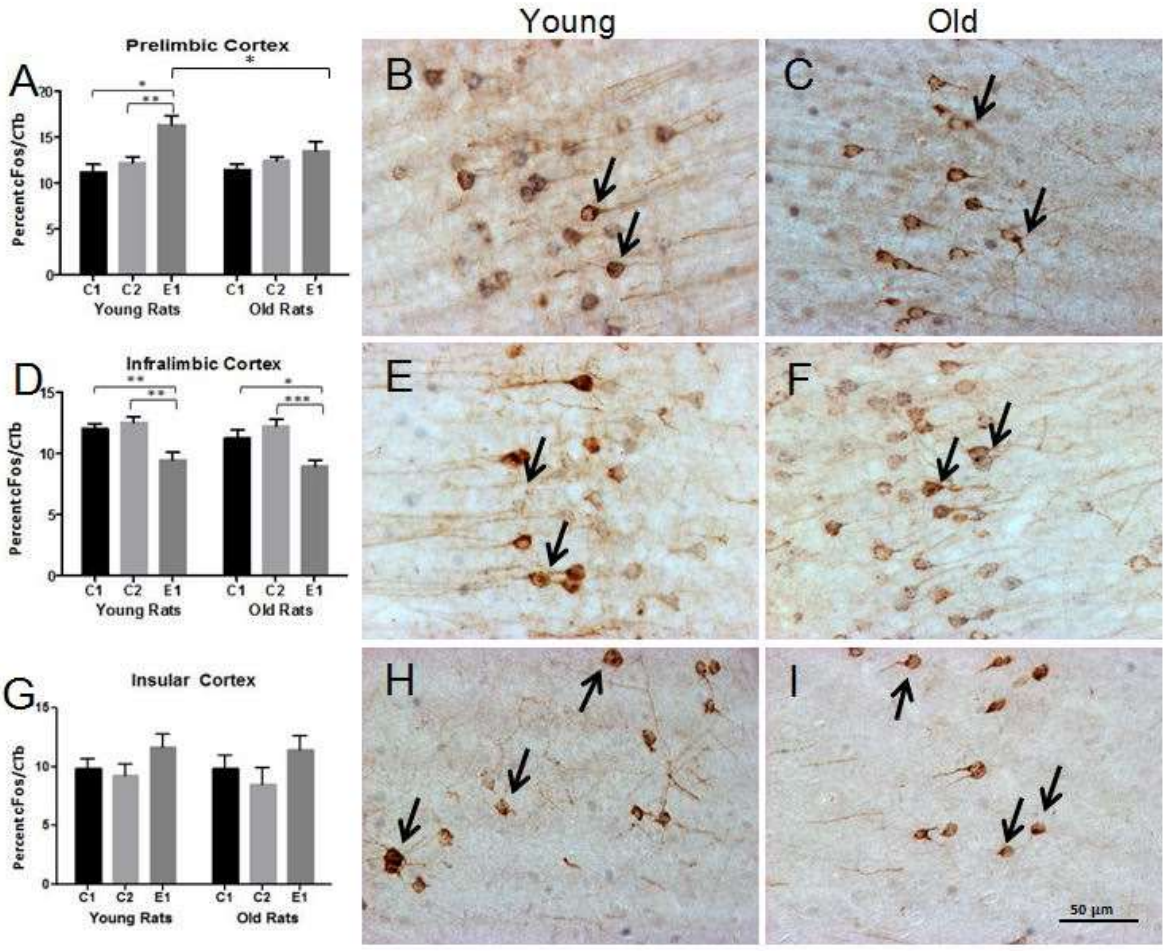

Figure 3.5 Double labeled immunohistochemistry of cortical regions. (A) A significant change between young and aged rats both between age groups and following food paired stimulus (E1) and controls (young C1 vs C2) in cFos labeled CTb neurons in the PLC. (B)(E)(H) Representative image (10X) of cFos/CTb+ double labeling (black arrow) in the PLC, ILC, and IC in young rat after the food paired stimulus (E1), respectively. (C)(F)(I) Representative image (10X) of an old rat after the food paired stimulus (E1) in the PLC, ILC, and IC, respectively. (D) Double labeled cFos/CTb in the ILC following food paired stimulus. No significant changes in double labeling between age groups. However stimulus groups (young C1 vs E1 and C2 vs E1) show changes in ILC in cFos/CTb labeled neurons. (G) No significant changes between age group or stimuli group double labeling (cFos/CTb).

3.4.5 Effects of Age and Food-paired Stimulus in Medial Regions

To investigate the effects of aging on BF afferent activation in medial brain regions, immunohistochemistry was used to measure the number of cFos/CtB neurons over CtB+ neurons in the medial brain regions including the NAc and Cl (Figure 3.6). The two-way ANOVA was used on data which was obtained using light microscopy and DAB reveal cFos labeled nuclei (black) and CTb labeled soma (Brown). Black arrows indicated cFos/CtB+ neurons in each brain region. Double labeling of CTb-cFos positive cells is expressed as a percentage of the total CTb labeling for young and aged rats. In the NAc, a two-way ANOVA revealed a significant variation between stimuli (C1, C2, E1; $p=0.0004$; $F(2, 33) = 10.16$). Following a two-way ANOVA paired with a Tukey's Multiple Comparison test, the PLC shows a significant increase in the percentage of double labeled neurons when exposed to dark-food paired stimulus 1 h prior to perfusion (young E1 vs age E1; $p=0.0086$). Tukey's Multiple Comparison test also revealed a significant difference between young rats which received food-paired stimulus training (E1) and controls (C1 vs E1; $p=0.0229$) (C2 vs E1; $p=0.0092$), and between aged E1 and C1 control groups ($p=0.0422$). In the Cl, following a two-way ANOVA, there was no significant age-related change (row factor) in BF afferent activation following a food paired stimulus) in young rats compared to aged rats. Additionally, Tukey's Multiple Comparison test did not determine any between group and age effects in the Cl.

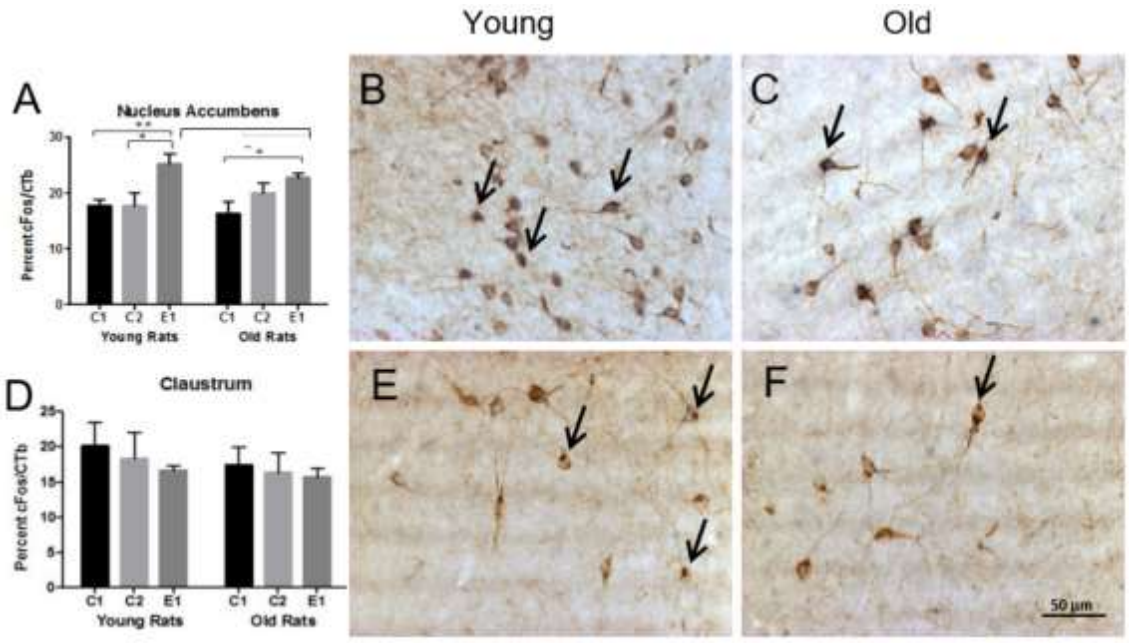

Figure 3.6 Double labeled immunohistochemistry of medial regions. (A) Double labeled cFos/CTb in the NAcc following food paired stimulus. A significant change in double labeling between age groups (young E1 vs Old E1) and between stimuli groups (young C1 vs E1 and C2 vs E1)(old C1 vs E1. (B)(E) Representative image (10X) of cFos/CTb double labeling(black arrow) in the Cl and Nac in young rat after the food paired stimulus (E1), respectively. (C)(F) Representative image (10X) of an old rat after the food paired stimulus (E1) in the Cl and Nac, respectively. (D) No significant change between young and old rats both between age groups and between stimuli groups (C1, C2, E1) in cFos labeled CTb neurons in the Cl.

3.4.6 Effects of Age and Food-paired Stimulus in Caudal Regions

To test the hypothesis that aging causes dysregulation of BF afferent activation, using immunohistochemistry, I measured the number of cFos/CtB neurons over CtB+ neurons in the caudal brain regions including the LH, CeA, and VTA following a food-paired stimulus (Figure 3.7). In the LH, a two-way ANOVA resulted in a no significant age or stimuli factor changes in basal forebrain afferent activation following a food paired stimulus. (E1) Tukey's Multiple Comparison test did not determine any across groups or age effects. The C2 and E1 young groups showed a slight trend ($p=.1768$). However, a greater trend between young E1 and aged E1 groups is present ($p=0.0565$).

Curiously, analysis of the CeA reveals a significant decrease in the food conditioned young rats who did not receive the bacon treat stimulus on the 7th day (C2) compared to habituated controls (C1) following a two-way ANOVA (column factor; stimuli; $p=0.0473$; $F(2,33) = 18.683$) paired with a Tukey's Multiple Comparison test (Young C1 vs. C2; $p = .0485$). Additionally a slight trend between young trained rats (E1) and aged trained rats (E1) is present ($p=0.0657$).

The VTA, following a two-way ANOVA, confirms a significant change in row factor (age; $p<0.0001$; $F (1, 33) = 25.77$) and column factor (stimuli; $p=0.0011$; $F (2, 33) = 8.487$). Moreover, there is a significant difference between young E1 and control groups (E1 vs C1; $p<0.001$) (E1 vs C2; $p<0.0001$). There is also a significant different between young and aged E1 groups ($p=0.001$). No other significant changes are present.

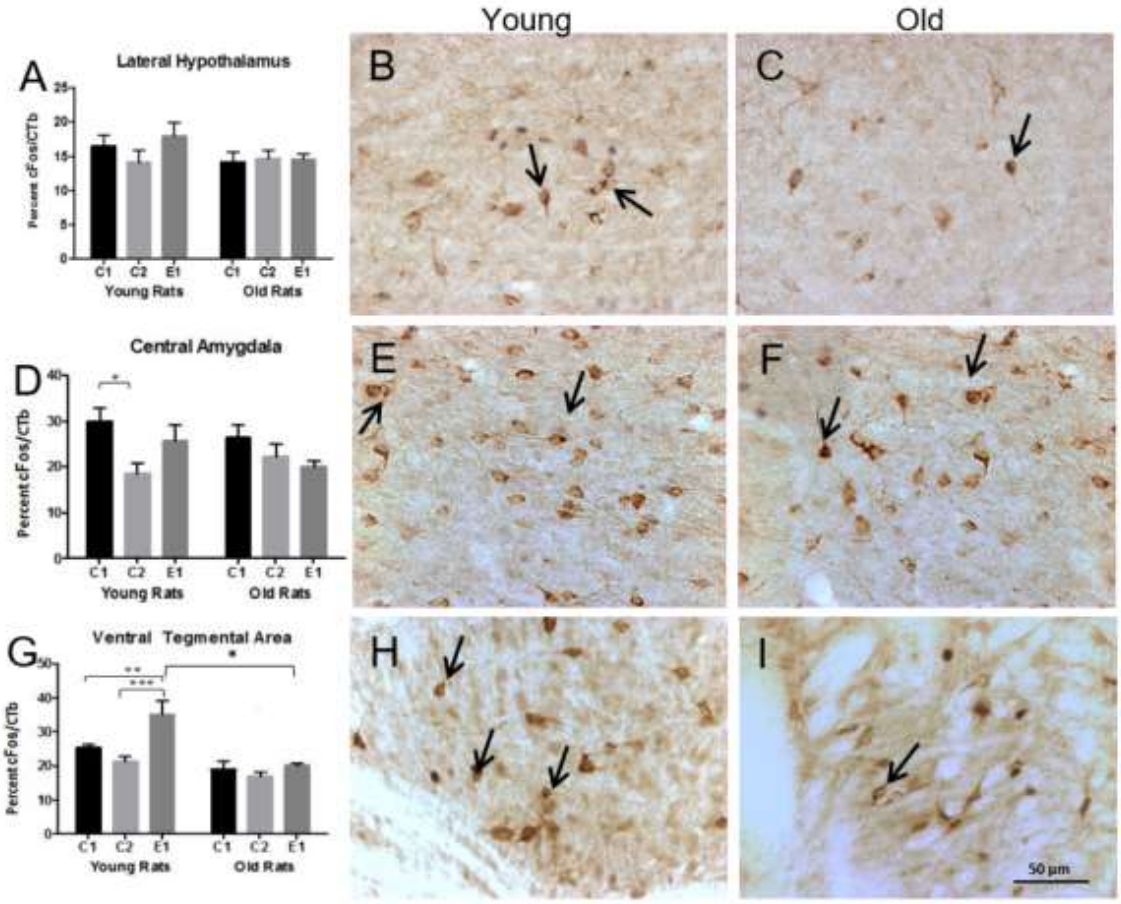

Figure 3.7 Double labeled immunohistochemistry of caudal regions. (A) No significant change between young and old rats both between age groups and stimuli groups (C1, C2, E1 in cFos labeled CTb neurons in the LH. (B)(E)(H) Representative image (10X) of cFos/CTb double labeling (black arrow) in the LH, CeA, and VTA in young rat after the food paired stimulus (E1), respectively. (C)(F)(I) Representative image (10X) of an old rat after the food paired stimulus (E1) in the LH, CeA, and VTA, respectively. (D) Double labeled cFos/CTb in the CeA following food paired stimulus. No significant changes in double labeling between age groups. However stimulus groups (young C1 vs C2 show no changes in cFos/CTb labeled neurons. (G) A significant change between age groups (young vs old E1) and stimuli groups (young C1 vs E1 and C2 vs E1) in double labeling (cFos/CTb).

3.4.7 Dopaminergic neurons of the VTA

To analyze activational changes in the dopaminergic population of neurons in the VTA, BF afferent activation of dopaminergic neurons was performed using double immunofluorescence (Figure 3.8). Pictographs of double labeled and triple labeled neurons were analyzed (Figure 3.9) and doubled labeled neurons in the VTA from each group were counted manually and a two-way ANOVA revealed no significant change in cFos/TH labeled neurons between groups. Triple labeled immunofluorescence revealed that the BF inputs (green; CtB) originating in the VTA were immunopositive for cFos and TH.

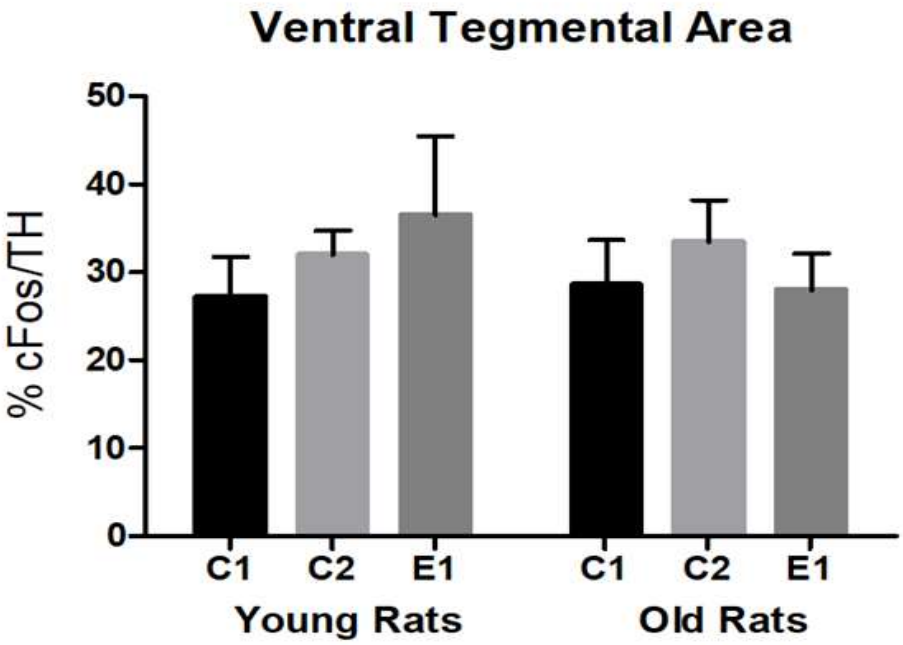

Figure 3.8. Double Labeled Immunohistochemistry. No significant difference between young and aged dopaminergic activation following food-paired stimulus.

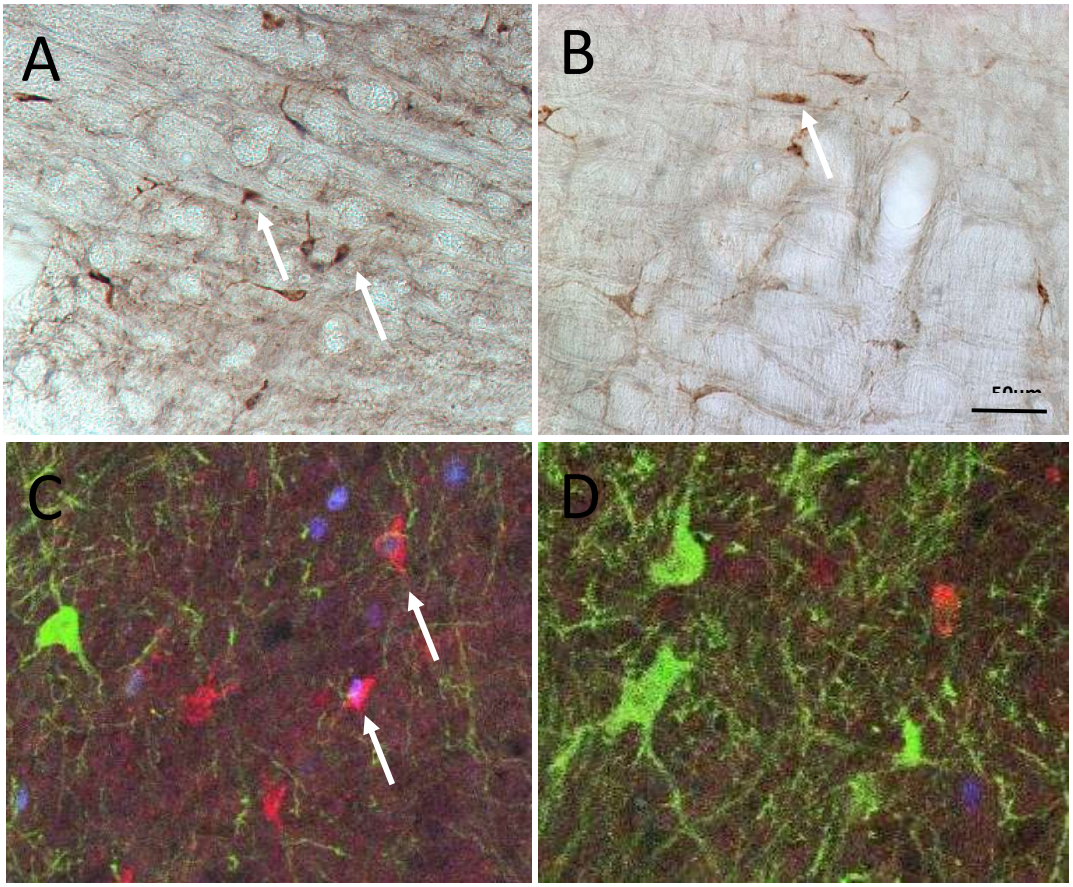

Figure 3.9. Immunohistochemistry and Immunofluorescence. **(A)(B)** Representative pictures of the VTA of young (left) & aged (right) showing cFos (black) and TH (brown) labeled neurons after DAB immunohistochemistry. **(C)(D)** Representative images of immunofluorescence using TH (Red), cFos (blue) and CtB (green) in the VTA.

3.5 Discussion

These data suggest that there are age-related changes in behavioral and neuronal activation associated with the BF in response to a food-paired stimulus. Interestingly, an age-related loss in neuronal activation, but not in BF input can be seen following the presentation of a food-paired stimulus. Moreover, age-related attentional deficits related to the attentional processing of homeostatically relevant stimuli are associated with age-related dysfunctional neuronal activation of specific BF afferents including the PLC, LH, NAc, and VTA.

3.5.1 Latency to Feed

The latency to feed behavioral paradigm demonstrates the animals' ability to recognize hunger and divert attentional resources to fulfilling that hunger. When rats are placed on an 80% food restricted diet and presented with a conditioned food-paired stimulus (darkness/bacon softie), the aged animals showed a greater latency to feed over the 7 day period than the young animals. This demonstrated an attententional imbalance to homeostatically relevant cues in aged animals. The significantly different behavioral response between age and young rats leads to the conclusion that aged rats have a cognitive impairment related to processing attentional stimuli associated with food intake. Interestingly, this phenomenon is seen in aged humans (J. E. Morley, 2001; John E. Morley & Thomas, 1999) which indicates a conserved behavioral deficit. Moreover, the feeding latency mirror the BF afferent neuronal dysfunction seen between full trained (E1) aged and young rats in the PLC, LH, NAc, and VTA which suggests that this behavioral deficit is associated with specific BF inputs.

Previously, studies in our lab have shown that feeding latency is mediated in part by orexin and attributed to a loss in BF cholinergic innervation of the PLC (Jim Fadel & Frederick-Duus, 2008; Kessler et al., 2011). Studies have also demonstrates a bilateral innervation between the PLC and BF which may be involved in recruitment of cognitive resources in order to respond to homeostatically relevant external cues (Tashakori-Sabzevar & Ward, 2018). Together these data suggest that age-related homeostatic attentional deficit, observed in rats and humans, and may be due to a cognitive impairment.

3.5.2 cFos and CtB Density

cFos is a proto-oncogene that is expressed in neurons following depolarization. Using immunohistochemistry, the protein product, cFos protein, can be used as a marker for neuronal activity. In this study, I characterized brain regions showing cFos activation in rats following entrainment using a food-paired stimulus for 7 days (E1), and compared these to levels of activation in untrained (C1) or 6 day trained (C2) control groups of both young and aged rats. Our results show that there is a significant decrease in neuronal activation of aged rats in the PLC, NAc, LH, and VTA which indicated overall neuronal dysfunction in aged rats in these brain region.

To test the hypothesis that the neuronal dysfunction occurring between aged and young groups is not due to a loss in BF afferent neurons, CtB density was measured using immunohistochemistry for the CtB retrograde tracer previously injected in the BF of young and aged animals. T-test reveal no significant difference between aged and young rats in any brain areas of interest including the PLC, ILC, IC, LH, NAc, CeA and VTA.

Thus, we concluded that the neuronal dysfunction is not due to BF afferent loss but perhaps the dysfunctional regulation of BF afferent regions.

3.5.3 Age-related effects of food paired stimulus

Previous studies have suggested that age induced homeostatic disturbances leading to a decrease in food intake and weight loss precede cognitive decline and MCI (Cova et al., 2016; Danielle Frederick-Duus, 2008; Johnson et al., 2006). To test the hypothesis that specific BF afferents are associated with this age-related dysfunction, I administered a homeostatically relevant stimulus, previously shown to be effected by the aged condition. Aged rats showed a significant decrease in afferent activation following entrainment in several brain regions suggesting a role in age-related input alterations that may correlate with homeostatic dysfunction in the BF. The BF plays an important role in the information processing and integrating several and receives several inputs from various brain regions. The dysfunctional signaling of inputs to the BF neurons may contribute in some way to neuronal dysregulation seen during aging. Thus, the BF afferents experiencing neuronal dysregulation are considered vulnerable to age related changes, and identifying these vulnerable populations may contribute to our understanding of aging, and age-related diseases.

The PFC and IC send highly organized bilateral projections between cortices and the BFCS (Chaves-Coira, Rodrigo-Angulo, et al., 2018; Ronald P.A. Gaykema et al., 1991). Following entrainment, neuronal activation following a food-paired stimulus revealed an age-related decrease in neuronal activation of aged rats compared to young rats in the PLC but an opposite effect was seen in the ILC. Moreover, the IC did not show significant age-related effects. The ILC and PLC have both been shown to send major

connections to the BF (Vertes, 2003; L. Zaborszky et al., 1997; Laszlo Zaborszky et al., 2015).. The opposing effects seen in the PLC and ILC may be due to their differing roles in mediating both fear/extinction and reward memories. The PLC is thought to exert top-down control over subcortical structures, including the BF, to regulate appropriate behavioral responses. Importantly, the PLC and ILC regulate the expression and suppression of fear and learning in rodents, respectively. Thus, one may conclude that the opposing effects of the BF afferents, PLC and IL, are due to their role in mediating such effects, and that these circuits are highly effected by age-related cognitive neuronal dysfunction. The IC, however, has been shown to activate in response to one's current interoceptive state which has been shown to have age-related dysregulation associated with the age-related loss of orexin (Hagar et al., 2017). Interestingly, the brain region responsible for the production of orexin, LH, had a trend which revealed an age-related loss in neuronal activation of BF intended circuits. Thus, orexinergic innervation of the BF and IC may contribute to the slight trends of neuronal dysfunction seen in aged animals.

Appetitive food stimuli have been shown to stimulated dopamine transmission in the core of the NAc (Bassareo & Di Chiara, 1999) and is a significant player in controlling the biological drives necessary for survival including feeding, reproduction, and reward/motivation (Boswell & Kober, 2016; Pratt et al., n.d.; Rebec et al., 1996). A significant increase in neuronal activation of BF afferents between E1 and control groups (C1,C2) of young animals located in the NAc was observed following a food-paired stimulus. Interestingly, the LH, IC, and PFC have been shown to innervate the NAc which are involved in cognitive processing of functions related to reward/reinforcement

(Brog et al., 1993). Furthermore, the NAc and VTA share an intimate pathway which is highly involved in motivational recruitment of attention by homeostasis-associated cues (Tashakori-Sabzevar & Ward, 2018). Thus, it was not surprising to see that the VTA also showed an increase in E1 neuronal activation in young animals compared to controls. The VTA and NAc also showed an age-dependent effect on entrained (E1) animals where aged rats showed a significant decrease in neuronal activation of BF afferent compared to young which suggests that this effect is blunted during aging. However, the exact cause for this neuronal dysfunction is unknown.

Like the BF, the VTA hosts a heterogeneous neuronal population. Previous, reports reveal a robust dopaminergic inputs in the basal forebrain from the ventral tegmental area (Zahm & Trimble, 2008). This data suggests that dopaminergic neurons from the ventral tegmental area may play a role in the processing of homeostatically relevant conditioned stimuli. However, there may be other subpopulations from the VTA that send projections in response to such a stimulus that the BF is mediating (Ronald P.A. Gaykema & Zaborszky, 1997; Hur & Zaborszky, 2005). Moreover, it has been suggested that PFC projecting cholinergic neurons in the BF receive input from the VTA (Gielow & Zaborszky, 2017). This may indicate that a circuit motif between the VTA>BF>PFC plays a role in mediating the attentional and cognitive decision making processes of homeostatically relevant cues, and that this circuit is greatly hampered during aging. This may indicate a circuit motif which is vulnerable to aging and age-related changes.

Together, this approach has revealed several BF afferents areas that are potentially vulnerable to age-related dysfunction, which will enable future anatomical studies to define specific cell signaling mechanisms that are effected by aging and age-

related diseases. Additionally, a circuit motif is revealed which is blunted during aging. Thus, these studies indicate a potential age-related mechanism which may precede and predict cognitive decline.

CHAPTER 4. AGE-RELATED CHANGES IN BF NEURONAL ACTIVATION

Aim 2: Identify age-related changes in neuronal activation of cell populations in the basal forebrain.

4.1 Rationale

Alzheimer's disease is associated with BFCS cell loss and degeneration, specifically in the SI (Wolf et al., 2014). Similarly, moderate degenerative and cognitive changes have been described in normal aging (M. J. Grothe et al., 2016; Wolf et al., 2014). Extensive evidence involving the manipulation of the BFCS has shown that lesions to the BFCS produce similar cognitive and attentional changes seen in normal aged and AD patients (J. R. Fadel et al., 2013; McGaughy, Kaiser, & Sarter, 1996). However, the role of GABAergic and glutamatergic neurons in the BF has not been as extensively studied.

Some evidence has shown that excitation of the glutamate receptors in the BF can excite cholinergic neurons to release ACh in the PFC. This paired with research stating a role for GABAergic neurons, through the use of GABAergic agonist (chlordiazepine), in decreasing BF cholinergic efflux of ACh suggests that GABAergic and glutamatergic neurons play an important role in mediating BF cholinergic transmission (Jim Fadel et al., 1996; Holly Moore et al., 1992; M. F. Sarter & Bruno, 1994).

In immunohistochemistry studies comparing calcium binding protein Parvalbumin (PV) and choline acetyl transferase (ChAT), a cholinergic marker, GABAergic neurons tend to outnumber cholinergic neurons 2:1 (M. Sarter & Bruno, 2002). Thus, it is widely accepted that, compared with the number of cholinergic neurons, an equal number of GABAergic neurons project to the cortex in congruence with the BFCS (Ivana Gritti, Mainville, Mancia, & Jones, 1997b; Laszlo Zaborszky et al., 1999). It is also believed that glutamatergic axons from the Prefrontal Cortex (PFC) terminate exclusively on the noncholinergic neurons on the BF and that glutamatergic containing neurons project to the PFC (Laszlo Zaborszky et al., 1999). This suggests a bidirectional relationship between the PFC and, several populations of neurons in the BF including GABAergic, glutamatergic, and cholinergic neurons. However, little is understood about the behavioral effects on GABAergic and glutamatergic neurons in the BF, it can reasonably hypothesized that GABAergic and glutamatergic neurons in the SI may play a role in mediating cognitive and attentional processes and that these processes may undergo age-dependent alterations similar to what is seen in cholinergic neurons of the BF .

4.2 Hypothesis

Because there is growing evidence suggesting that the heterogeneous population of neurons in the BF may be intimately involved in mediating the BFCS, I predict that there are specific BF neuronal cell populations that are involved in age-relate homeostatic dysfunction. I aim to identify a change in activation of GABAergic and glutamatergic neurons, following a homeostatically relevant stimulus previously described, between young and aged rats. Furthermore, I hypothesize that age-related attentional deficits

related to the attentional processing of homeostatically relevant stimuli is associated with dysfunctional neuronal activation of specific BF populations. In my studies, I have reproduced a previously demonstrated age dependent relationship between a dark/food-paired stimulus and age. Using this behavioral technique in combination with neuronal histochemical analysis, I examine the effects of aging on local BF stimulation in the SI. This approach will enable future anatomical studies to define specific cell signaling circuits between BF afferents and local BF neurons to reveal the mechanisms that are effected by aging and age-related diseases.

4.3 Approach

All experiments were carried out in accordance with NIH Protocols and IACUC approval. The methods described herein are an abbreviated version of those in Chapter 2: General Methods. Experiments were carried out in six separate batches. All methods are depicted in a timeline (Figure 3.1). All experiments were conducted using young (3-4 months, 250-300g) and aged (26-28 months, 450-550g) male Fisher 344/Brown Norway F1 hybrid rats (National Institutes of Aging Colony, Baltimore, Maryland, USA). Animals were ordered in four separate batches. Upon arrival, animals were randomly assigned to receive C1 (no training), C2 (training on 6 of 7 days), or E1 (training all 7 days). All Animals received CtB Injections following Habituation for 7 days and were restricted to an 80% diet of normal ad libitum food weights.

4.3.1 Food-paired training

During day 14-21 young or aged rats were categorized into 3 subcategories based on food/darkness training assignments. Control groups (C1; aged n=8; young n=8) were habituated in microdialysis bowls for 1 h until day 7 in which rats were perfused with 4%

paraformaldehyde 1 h after the completion of daily bowl habituation. Control groups which received food/darkness training on day 1-6 (C2; aged n=8; young n=8) were only habituated in microdialysis bowls on day 7 prior to perfusion. Experimental groups (E1; aged n=8; young n=8) were given the food/darkness training daily for 7 days, and animals were then perfused on day 7 1 h after being given the food/darkness stimulus. The food/darkness training consisted of darkness (switching off the light) followed by the presentation of a palatable treat (Bacon softie). During day 14-21 rats were given an 80% diet restriction. Calculation for the daily amount each at would receive were taken from food intake data taken one week prior.

4.3.2 Histology

Following perfusion with 4% paraformaldehyde, brains were removed and cut on a vibratome in 1X TBS and placed in antifreeze solution for storage in -20^{o}C freezer. Prior to staining, tissue was washed in 1x TBS, transferred into methanolic peroxide, and blocked with TBS. Tissue sections were double labeled for rabbit α-cFos (1:500; Millipore Corp., Temecula, CA ; Cat.# ABE457; Lot#3041795). Tissue was then incubated with biotinylated donkey anti-rabbit secondary antibody for 1.5 hs followed by horseradish peroxidase-conjugated streptavidin (SHRP; 1:1,600; Jackson; cat# 016–030–084) for 1 h. cFos labeling was visualized by adding 30% hydrogen peroxide (H202; 10 µl 30% H202 in 1ml TBS) to the tissue sections in the presence of diaminobenzidine and nickel-cobalt solution (3.0 gm nickel ammonium sulfate, 0.3 gm cobalt chloride q.s.100 ml with distill water) filtered through a .2 µm syringe, generating a blue/black stain in cFos-immunoreactive areas. After an appropriate development time, all sections were rinsed with 1X TBS (4 X 10 min).

For the second development, tissue was incubated in and either goat α-ChAT (1:3000; Millipore, Temecula, CA, USA;, Lot# 2762374, Cat#AB144), mouse α-Parvalbumin (PV; 1:3000; Sigma, Saint Louis, Missouri, USA; Cat#P3088, Batch# 016MA847V), or vGluT (Millipore; Temecula, CA, USA; Cat#AB5905; Lot#2733748) to identify activated BF cholinergic neurons, GABAergic, or glutamatergic neurons, respectively. After 48 h at room temperature on a nutator, tissue was washed (.1M PB 1x TBS) 3 times for 10 minutes and placed in unlabeled donkey anti-goat antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot# 132096) or unlabeled donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot#87269) for 2 h followed by followed by an incubation in Goat PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #11945) or Mouse PAP (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; Lot #131380). Tissue was developed using diaminobenzidine (DAB), generating a brown immunoprecipitate to produce cFos nuclear labeling in either CTb, ChAT, PV, or TH-positive fibers. Tissue was then mounted of microscope slides (VWR Life Sciences, Radnor, PA, USA, Cat # 16005-106) and dehydrated. After dehydration using ethanol (50%-100%) and two 10 min soaks in Citrosol, the slides were cover slipped using DPEX Mounting and microcover glass and left overnight to dry before being cleaned for imaging.

4.3.3: Microscopy and Imaging

Histological experiments for double-labeled (cFos and PV/ChAT/vGluT) neurons, were visualized at 10x-20x magnification using a Nikon E600 microscope fitted with a

CoolSNAP digital camera. Immunoperoxidase photos were captured using IP Lab Software.

4.3.4 Data Analysis

For all immunohistochemistry experiments, neurons 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 cFos and ChAT/PV/vGluT labeled neurons and expressed as a percentage of cFos labeled in each respective neuronal cell. Two representative images of each animal in each region were averaged to obtain one data point.

Statistical analyses of these data were analyzed by two-way ANOVA or unpaired two-tailed t-test using Graphpad Prism 8 Software. To analyze the relationship between age (young and old) and training effect (C1, C2, E1) a two-way ANOVA was performed. Significant effects of CtB, cFos density, body weight, feeding latency, and food intake between aged or young animals were tested using multiple unpaired t-tests or one-way ANOVA (significant effects of treatment condition (i.e. E1, C1, C2) across age were determined followed by Tukey's multiple comparisons test). The significance level was determined as $p<0.05$ for all analyses.

4.4 Results

The basal forebrain contains several nuclei and neuronal populations including cholinergic, glutamatergic and GABAergic neurons. To test the hypothesis that changes in neuronal activation during aging may be neuronal subtype specific, double label histochemistry using α-CFos and either α-ChAT or α-Parvalbumin or α-vGluT was performed. A two-way ANOVA followed by a Tukey's Multiple Comparison test

demonstrated a significant changes between column factors (stimuli) and a slight trend between row factor (age)($p=.09211$; $F(3,30)=2.8.11$) in activated PV cells. A significant change in PV neuronal activation between young and aged entrained rats (E1) ($p=.0478$) is seen following Tukey's Multiple Comparison test and a trend ($p=.0746$) can be seen in young rats between controls (C1) and entrained (E1) rats. However, no significant changes in cFos labeled ChAT or vGluT labeled cells following food-paired stimulus for young or old rats is seen following a two-way ANOVA or Tukey's Multiple Comparison test.

4.5 Discussion

Studies in which cholinergic neurons are lesioned in the SI produce age-related deficits in attentional tasks (M. Sarter et al., 2001). Because GABAergic and cholinergic neurons in the SI are largely overlapping, an age-related deficits in both cholinergic and glutamatergic neurons was expected. Interestingly, GABAergic PV+ neurons had the most prevalent deficit in awareness and entrainment to a palatable conditioned stimulus in aged rats compared to their younger counterparts, although slight attenuation of cholinergic activation is present. Cortically projecting PV+ neurons from the BF have been shown to play an important role in regulating cortical gamma band oscillations important in awareness and arousal (T. Kim et al., 2015). Studies using optogenetic approaches have highlighted a role for cholinergic neurons in reward/punishment schemes as well as reward timing while PV+ GABAergic neurons have been attributed to behavioral state control and arousal (S. C. Lin, Brown, Shuler, Petersen, & Kepecs, 2015). My data supports a role for PV+ GABAergic neurons in arousal and attention related to a homeostatically relevant stimulus, and presents a novel age-dependent deficit in PV activation of BF neurons upon the presentation of a food-paired conditioned stimulus. This data suggests that PV neurons

in the SI play an important role in recognizing homeostatically relevant cues, but this behavior is severally disrupted during the aging process.

A separate set of neurons in the BF (SI) have recently been identified which produce low tonic firing and cortical modulation consistent with projecting neurons (Avila & Lin, 2014; S. C. Lin, Gervasoni, & Nicolelis, 2006; S. C. Lin & Nicolelis, 2008). These neurons have been shown to play a key role in motivational salience and are considered salient encoding. The identity of these neurons has been highly disputed and often mistaken as cholinergic neurons; however, recent evidence suggests otherwise (S.-C. Lin, Brown, Hussain Shuler, Petersen, & Kepecs, 2015). These neurons are thought to be potentially GABAergic or glutamatergic neurons, although this is disputed (Henny & Jones, 2008; Hur & Zaborszky, 2005). Analysis of glutamatergic (vGluT) and GABAergic (PV) neurons using a salient conditioned stimulus supports the idea for PV projecting neurons as the mystery neuronal subset. Further studies are needed to confirm whether all or most of these activated BF neurons were cortically projecting.

The BF cell populations which have been analyzed have been implicated in arousal, learning, and attention, and the disruption of these neurons during aging has been linked to many neurological disorders, such as coma and Alzheimer's disease. Although, the BF is known for the role its cholinergic neurons play in cognition, recent studies targeting specific BF cell types have led to a new insights on cell-type-specific circuit mechanisms during behavior. In my study, a conditioned salient stimuli approach has enable me to determine changes in age-related behavior and neuronal dysfunction under which noncholinergic BF neurons are key players. Thus, I have identified a behavioral approach which influences GABAergic mechanisms (PV).

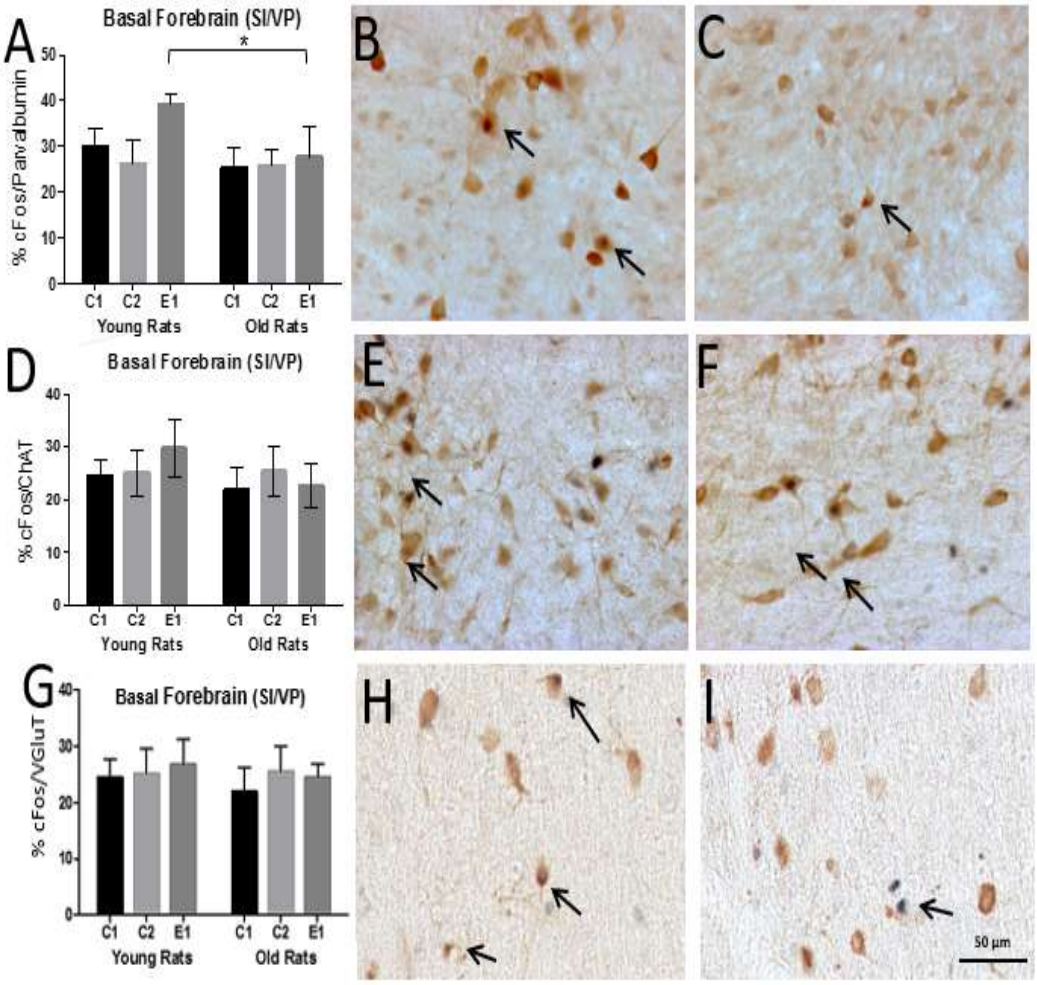

Figure 4.1 Immunohistochemistry in the Basal Forebrain.
(A) A significant change between young and aged rats following food-paired stimulus (E1) in PV+ activation within the basal forebrain. (B)(E)(H) Representative image (10X) of cFos/PV+ double labeling (black arrow) in the basal forebrain in young rat after the food paired stimulus (E1). (C)(F)(I) A representative image (10X) of an old rat after the food paired stimulus (E1). (D) Double labeled(black arrow) cFos/ChAT in the basal forebrain following habituation (C1), Foor-paired stimulus training with no CS+ on day 7 (C1) and Food-paired stimulus for 7 days (C2). No significant changes in basal forebrain ChAT activation between young and old rats. (G) Double labeled(black arrow) cFos/vGluT in the basal forebrain following habituation (C1), Foor-paired stimulus training with no CS+ on day 7 (C1) and food-paired stimulus for 7 days (C2). No significant changes in basal forebrain vGluT activation between young and old rats.

CHAPTER 5. AGE-RELATED CHANGES IN BF MICROGLIAL ACTIVATION

Specific Aim 3: Investigate the role of orexin-microglial interactions as it relates to age-related BF homeostatic function.

5.1 Rationale

The orexin system originates in the LH and projects to several regions in the brain including the BF. Orexins are an excitatory neuropeptide originally discovered in the late 1990s, which are involved in a broad range of homeostatic signaling including appetitive behavior, wakefulness, and arousal (D. Frederick-Duus et al., 2007; Sakurai & Mieda, 2011; Villano et al., 2017). It was later understood that two Orexins, OXA and OXB, are involved in integrating a variety of homeostatic signals. Both OXA and OXB are encoded by a single precursor polypeptide, named prepro-orexin (Kukkonen et al., 2002). The presence of mRNA for both OXR1 and OXR2 was first discovered in the BF using quantitative PCR and in situ hybridization (Marcus et al., 2001). Due to the BFCS role in attentional processing, it was thought that the orexin system may play an important role in biasing attentional resources towards external stimuli that are important for homeostatic balance (Hagar et al., 2017). Thus, orexin inputs to the BFCS are thought to provide anatomically important interactions that are particularly important in functional roles involving arousal and attention, specifically for allocating importance to

specific interoceptive cues related to external stimuli (J. Fadel & Burk, 2010b; Jim Fadel & Frederick-Duus, 2008).

The ability to integrate environmental cues that are related to interoceptive status combined with the broad range of functions influenced by the orexin system has earned them their description as "physiological integrators". This is supported by previous studies that reveal an age-related decline of orexin function both at a molecular and behavioral level leads to homeostatic dysfunction and a loss in cue-related homeostatic behaviors (Kessler et al., 2011). Furthermore, inhibition of orexin expression using a lentivirus preproorexin antisense, revealed that the loss of orexin expression in young rats mimicked similar neurochemical and feeding behaviors seen in rats with age-related loss of orexin suggesting that orexin loss is an important aspect in age-related homeostatic dysfunction (Hagar et al., 2017).

Previous studies using immunofluorescence revealed expression of OXR1 by glial cells and OX1R receptor is upregulated in microglia after murine controlled cortical impacts models (Mihara et al., 2011). Thus, Orexin receptors are known to be located on many cell populations as well as glial cells including microglia and may mediate inflammatory responses in the BF via OXR1 and OXR2 (C. M. Duffy et al., 2019; Cayla M. Duffy et al., 2015; Manich et al., 2019; G. J. Wright, Jones, Puklavec, Brown, & Barclay, 2001; Gavin J. Wright et al., 2003). Moreover, recent studies reveal an age-dependent relationship between orexin and microglial activation (Cayla M. Duffy et al., 2015; Xiong et al., 2013). Immunohistochemical studies reveal an age dependent increase in microglial activation (Harry, 2013; Ogura, Ogawa, & Yoshida, 1994a; Overmyer et al., 1999). This suggests a potential role for orexin receptors in mediating

inflammatory response (Ogura, Ogawa, & Yoshida, 1994b). Moreover, studies implicating the role of orexins in microglial regulation suggest that orexins may produce inflammatory changes which are neuroprotective against cognitive decline (C. M. Duffy et al., 2019; Cayla M. Duffy et al., 2015; Xiong et al., 2013). Studies also suggest a possible role for orexin receptor mediated microglial inflammation during aging and age-related cognitive decline (Frank et al., 2006; Ogura et al., 1994b; D. G. Walker, Dalsing-Hernandez, Campbell, & Lue, 2009). In our study, I propose that the loss orexin causes morphological changes in microglial indicative of a pro-inflammatory state.

5.2 Hypothesis

Previous work in our lab has demonstrated a potential role for orexin, in mediating physiological function, and in mediating attention as it relates to homeostatic status in the basal forebrain during aging. Our lab has demonstrated that the knock down of orexin expression in young rats, mimics similar cognitive and attentional deficits in age rats suggesting that the loss of orexin innervation seen in aged individuals is linked to the mild cognitive deficits manifested as one ages. My previous aims have targeted the relationship between attentional processing of salient stimuli and the BF afferent and BF local neuronal activation during aging. To assess the potential role of orexin-mediated glial regulation in the BF, I knockdown of orexin expression and examine the morphological changes in microglia and cytokine release in the BF and PFC, a major region which receives both LH and BF projections, of young rats. I hypothesize that study will demonstrate that the loss of orexin mediation on microglia produces a dysfunction inflammatory effect, normally seen in aged animals, in brain regions which

receive robust orexinergic input. Thus establishing a role for orexin induced neuroprotection.

5.3 Approach

All experiments were carried out in accordance with IACUC approved protocols. Experiments were carried out in two separate batches, one of which animals were entrained. All experiments were conducted using young (3-4 months, 250-300g) or aged (26-28 months, 450-550g) male Fisher 344/Brown Norway F1 hybrid rats (National Institutes of Aging Colony, Baltimore, Maryland, USA). All Animals received lentiviral injections following Habituation for 7 days and were given food ad libitum followed by an 80% diet restriction and kept on a regulated light:dark cycle (12:12 hr) during the duration of the experiment (Figure 5.1).

5.3.1 Stereotaxic Surgery and Lentiviral Injection

All animals were anesthetized using isoflurane (Piramal Critical Care Inc., Bethlehem, PA, USA) using E-Z anesthesia system (Euthanex Corps, Palmer, PA, USA). Animals were mounted on stereotaxic surgical device (Stoelting, Dale, IL, USA). Following sterilization, an incision is made, and a dremel tool was used to drill two holes for bilateral injection of the lentivirus (LV-preproorexin antisense) into the LH (Young: AP -2.5 mm, L ±1.2 mm, and DV -9.0 mm). This lentivirus vector was produced by transfecting human embryonic kidney (HEK) 293T cells with a transgene expression cassette (LV-PGK-ip-preproorexin-IRED-GFP-WPRE), an envelope cassette, a packaging cassette (pCMVpR8.92), and a vector cassette (HIV-1 Tat-dependent vector) which combined are able to transfect neuronal and glial cells with an antisense designed to knockdown preproorexin expression, a precursor to the orexin neuropeptides.

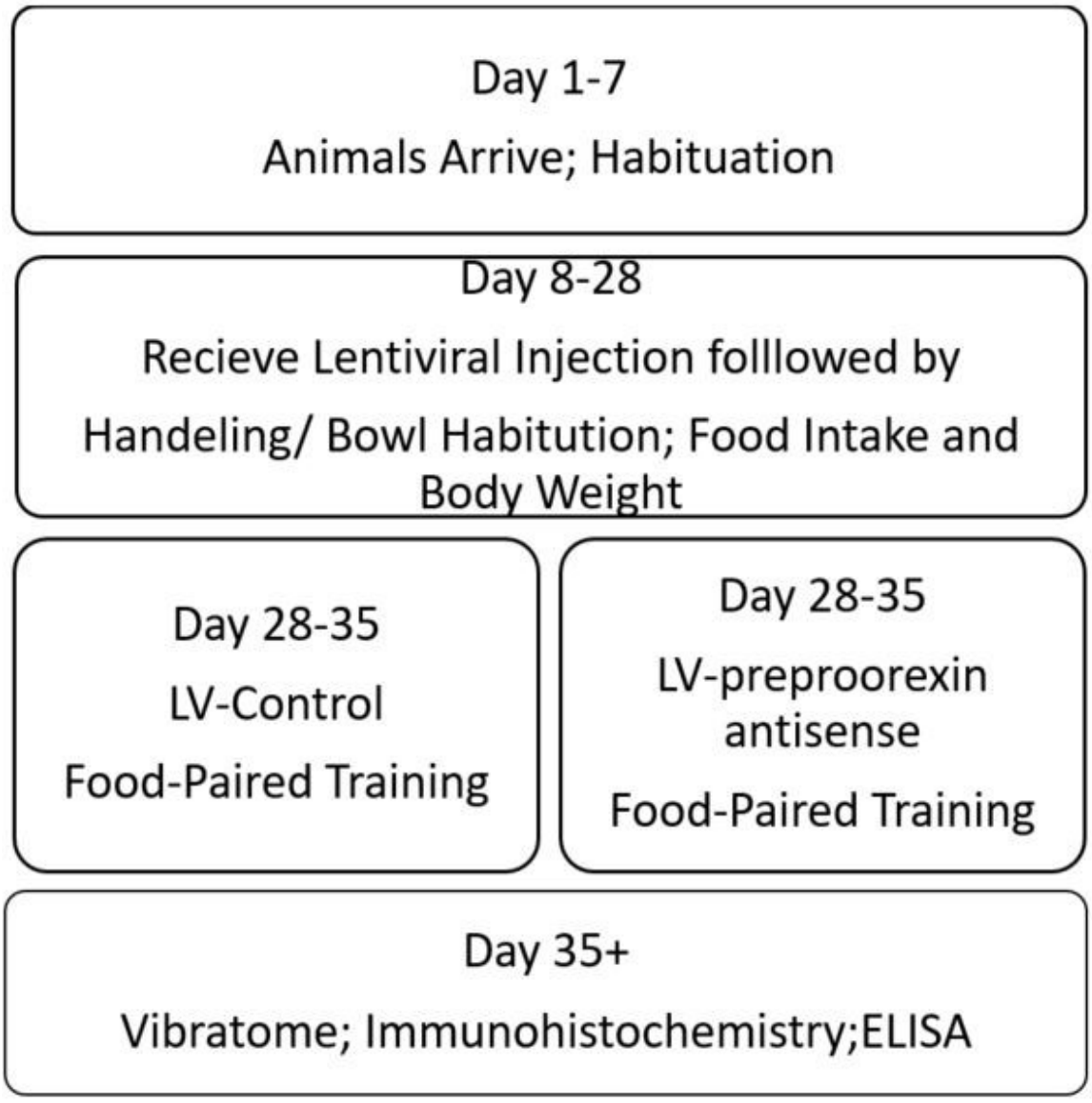

Figure 5.1 Timeline of lentiviral injection experiment on young rats (n=32). Rats were further divided into 2 subcategories based on lentiviral injection (control; LV-GFP) (SPWG-OX; LV-PPOX).

5.3.2 Entrainment

Following handling during day 8-14, rats were habituated in bowls for 1 hr per day until day 28 in which both controls (LV-control; n=8) and animals which received the preproorexin antisense (LV-preproorexin; n=8) were trained with a conditioned stimulus (bacon softie/darkness) and tested for feeding latency. During day 28-35 rats were tested daily at the same time while on an 80% food restricted diet. On day 35 animals were perfused with 4% paraformaldehyde 1 h after the completion of daily bowl habituation.

5.3.3 Trans-Cardial Perfusion and Tissue Collection

All experiments were completed with animals deeply anesthetized with isoflurane and sacrificed**.** Brains were extracted and half the brain was placed directly on dry ice and half post-fixed in a 4% paraformaldehyde solution. The flash frozen half of the brain was then stored at -80^{o} C to await further processing, and the post-fixed half of the brain was blocked into 3 sections and sliced using a vibratome (Electron Microscopy Sciences, OT-4000) into 50 micron thick slices. The slices of brain tissue were collected and free floated in six wells of the 1X Tris-buffered saline, then transferred to anti-freezing solution for long term storage.

5.3.4 Immunoperoxidase following Lentiviral Injection in the LH

Post-fixed brains stored in 4% paraformaldehyde for 24 hr were coronally sectioned (50 microns) using a vibratome. Rat brains injected with the lentivirus containing preproorexin antisense (LV-PPOX) or lentivirus containing GFP and only the GFP gene (LV-GFP) were double labeled for rabbit αIb1A (1:5000; Wako Pure Chemical Industries; Osaka, Japan; Cat# 019-19741; Lot# PTR2404), and either goat α-Orexin-A (OXA; 1:500; Santa Cruz Biotechnology Inc.; Dallas, TX, USA; Cat.# sc80263), goat α-

ChAT (1:3000; Millipore, Temecula, CA, USA;, Lot# 2762374, Cat#AB144), or mouse α-Parvalbumin (PV; 1:3000; Sigma, Saint Louis, Missouri, USA; Cat#P3088, Batch# 016MA847V) to identify microglia, and orexin neurons, cholinergic neurons, or parvalbumin positive neurons, respectively. Tissue was developed using DAB (Sigma-Aldrich co., St. Louis, MO, USA, Lot#MKCK2487) to produce microglial labeling along side either OXA, ChAT, or Parv neurons. For all immunoperoxidase slides, images were visualized using the Nikon Eclipse was fitted with CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA) and visualized using with the IP Labs Software (Scanalytics; Trenton, NJ, USA).

5.3.5 Assays

For brain homogenates, punches were taken using a freezing microtome and the rat brain atlas. Tissue was immediately weighed and placed in active lysis buffer solution on ice and homogenized using .5mm Zirconium Oxide beads (Next Advance; Troy, NY, USA; Cat# ZROB05) in a safe-lock Eppendorf tube which is then placed in a bullet blender (Next Advance; Troy, NY, USA; Cat# G14G15) for 5 minutes at speed 10 in 4^{o}C. Tubes are aliquoted into Eppendorf tubes (50ul each) and immediately placed in -80^{o}C freezer to await ELISA or BCA Protein Assay. BCA working reagent is determined and working reagent is prepared on a plate along with tissue samples which are diluted at 1:12.5 in HPLC grade water in Eppendorf tubes. To a sample plate, tissue sample, and working reagent is added to each well alongside a set of standards. Plate is then placed in oven, and then cooled for 15 minutes outside the oven before being read using a Biotek Synergy 2 Plate Reader (Biotek instruments Inc.; Winooski, VT, USA; Cat #7131000).

For the Elisa, brains homogenates of animals were analyzed using a 12-Plex Th1/Th2 Bio-Plex kit (Bio-Rad Laboratories Inc.; Cat # 171K1002M; USA). Brain Homogenates were thawed and diluted to 1:3 with diluent. To a plate, magnetic beads, standards and samples, the detection antibody and SAPE were added to each well following washes. The plate was read using a Luminex Bio-Plex Multiplex plate reader that uses high photomultiplier voltage to detect cytokines. Results were interpreted from a logarithmic standard curve.

5.4.6 Statistic analysis

Differences in latency to feed and immunohistochemistry were tested by a one-way ANOVA post hoc test with significance $p < .05$. All statistical tests were done using GraphPad Prism version 5.02 or 9 (GraphPad Software Inc.; San Diego, CA). BCA protein assays were analyzed using Excel and protein contents (ug/ml) were then interpolated in GraphPad Prism 9 using a standard curve. ELISA data (pg/ml) was normalized to protein content (ug/ml) and graphed using an analysis of variance (ANOVA) with significance set at $p < 0.05$. Cytokines were analyzed as one-way ANOVA or paired/unpaired t-test.

5.4 Results

5.4.1 Feeding Latency and Food intake

To test whether the administration of LV-PPOX changed food intake and body weight of animals, animals were weighed and food intake was taken daily at the same time (11:00hr). Previous research in our lab has shown that administering LV-PPOX has a significant effect on feeding latency and food intake (Danielle Frederick-Duus, 2008; Hagar et al., 2017). The administration of LV-PPOX into the LH, was confirmed to have

a significant change decrease in food intake (P <.0001; t=12.84, df= 30) (n=16) following an unpaired t-test (Figure 5.2). Analysis using multiple unpaired t-tests Similar increases in feeding latency has been shown following injection of LV-PPOX into the LH (n=8) (p=.0003; t= 7.977 df=14; Day 7; Figure 5.2 C, D).

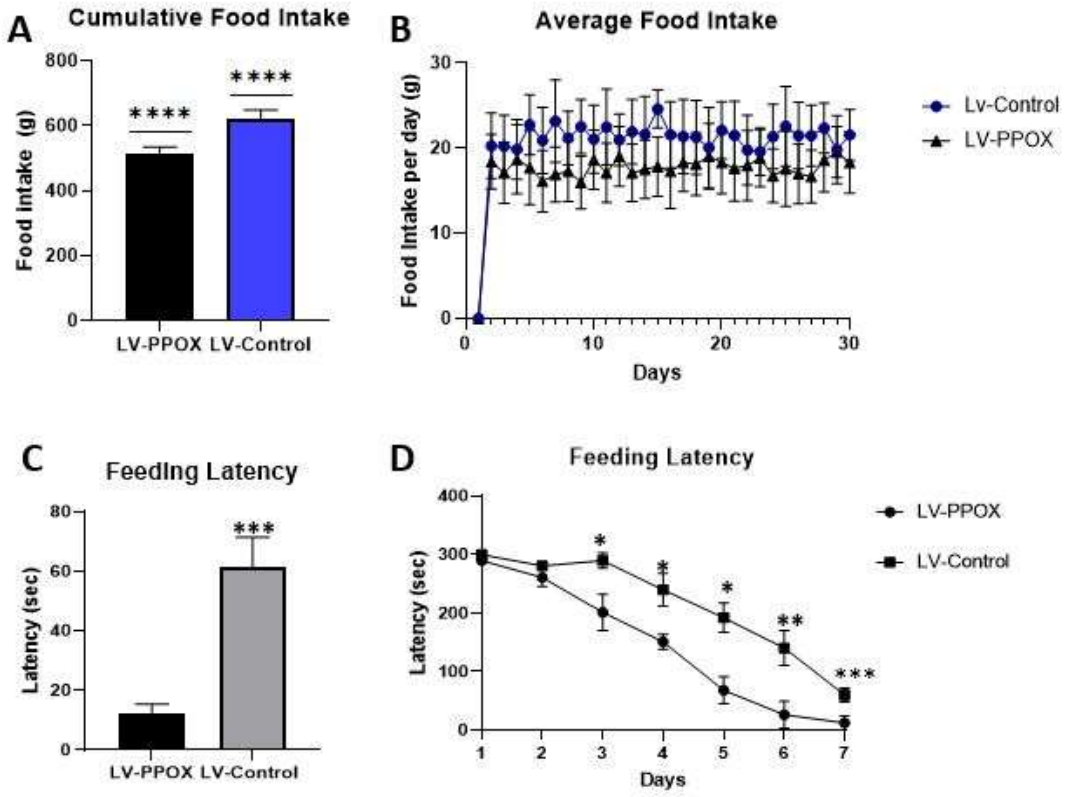

Figure 5.2 Food Intake and Feeding Latency following LV-PPOX
(A) A Significant difference between LV-PPOX and LV-Control groups (p < .0001). A graph showing average food intake between LV-PPOX and LV-Control administered animals reveals a defined decrease in food intake. (C) On day 7, following 6 day of food-paired stimulus entrainment, a significant difference between latency to approach and consume a bacon softie (feeding latency) of animals administered LV-PPOX and LV-Control (t-test; p=.003) into the LH and (D) a significant difference between treatment groups and time following a multiple t-tests where LV-PPOX significantly reduced latency to feed on Days 3 (p=.0043), 4 (p=.0122), 5 (p=.0348), 6 (p=.0094), and 7 (p=.0003).

5.4.2 Age-related Changes in Microglia Morphology in the BF

Preliminary work our lab using aged and young animals has confirmed work which suggests age-related microglial change. Single Ib1A-labeled (microglia) cells were counted under 20X magnification using two different sections of young and aged (n=8) BF sections. In addition, the labeled cells where manually analyzed for morphological differences in which M1 is categorized by short processes and large cell bodies and M2 is categorized by long processes and medium to small cell bodies. Unpaired t-test on the average number of both sections in the BF revealed no significant changes in total microglia amount (unpaired; p= .5248; df= 14; t= 2.549), but do exhibit a change in morphology, particularly an increase in M1 phenotype microglia, characterized by short processes and large cell bodies (unpaired; p= .0718; df= 14; t= 1.491) (Figure 5.3). This data is supported by previous research which has identified a change in microglial morphology between young and aged animals (Spittau, 2017; VanGuilder et al., 2011).

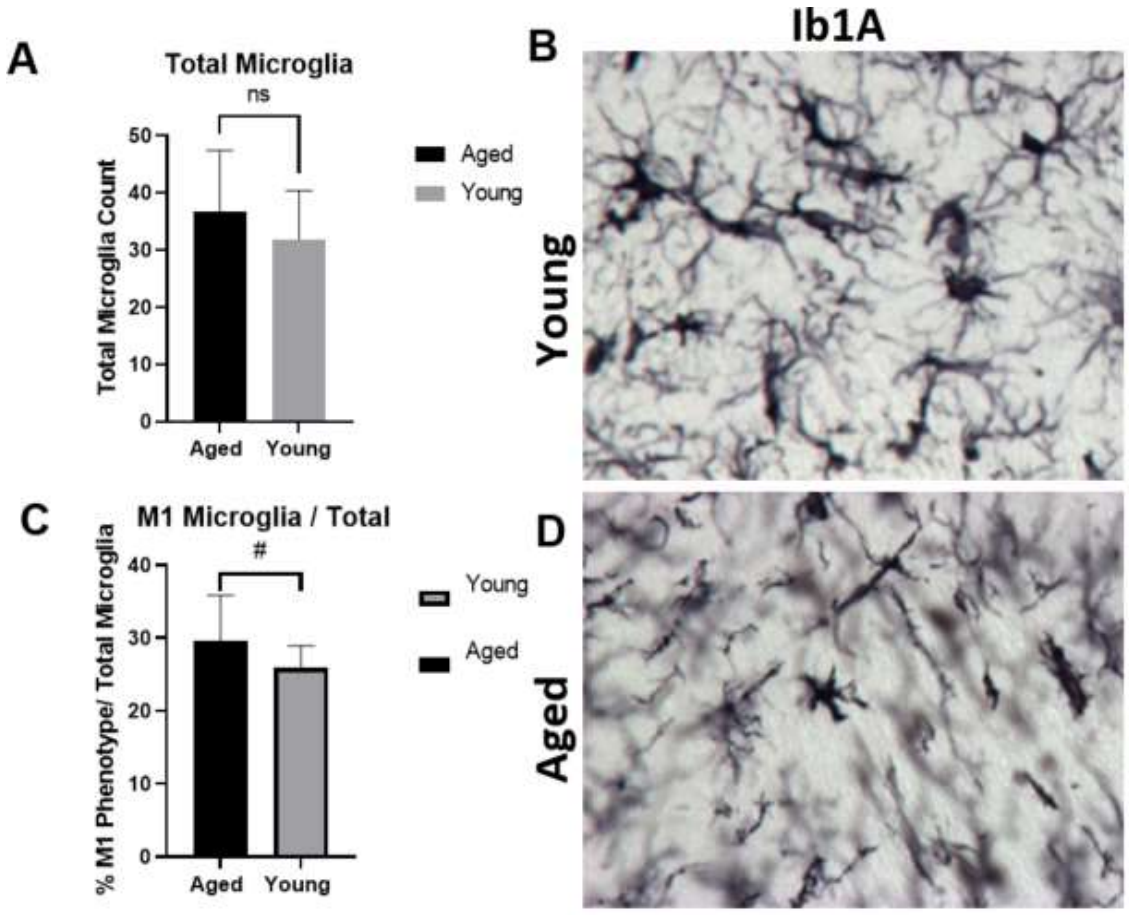

Figure 5.3 Changes in Microglial Morphology in Young and Aged Rats
(A) No Significant difference between total count of microglia in aged (26-28 month) and young (3-4 month) groups (p =.5248). (C) A small trend in M1 morphology between aged (26-28 month) and young (3-4 month) groups (p =.0718). (B)(D) A graph showing microglia morphology via single labeled (Ib1A) cells in aged and young animals reveals exemplifies a difference in microglia morphology.

5.4.3 Changes in Microglia Morphology in the BF following LV-PPOX

Double labeling for OX-A-Ib1A (Orexin and microglia) cells were analyzed to count microglia amount and Orexin presence/proximity under 20X magnification using two different sections of young and aged (n=16) BF sections (Figure 5.4). The orexin (OX-A) labeled cells revealed a decrease in orexin fibers. The decrease in Orexin fibers following LV-PPOX administration confirms the lentiviruses ability to downregulate orexin expression in young animals. The Ib1A (microglia) labeled cells where analyzed morphologically in which M1 is categorized by short processes and large cell bodies and M2 is categorized by long processes and medium to small cell bodies. Unpaired t-test on the average number of both sections in the BF revealed no significant changes in total microglia amount following LV-PPOX (unpaired; $p= .2412$; $df= 30$; $t= 1.196$). However, following the analysis of Ib1A cell morphology, microglia were found to exhibit a significant increase in M1 phenotype characteristics, including larger cell body and shorter processes (unpaired; $p= .0280$; $df= 30$; $t= 2.310$). This data is similar to aged animals which exhibited an increase in M1 phenotype suggesting that administration of LV-PPOX into the LH of young animals can create similar phenotypic changes in microglia morphology as aged animals.

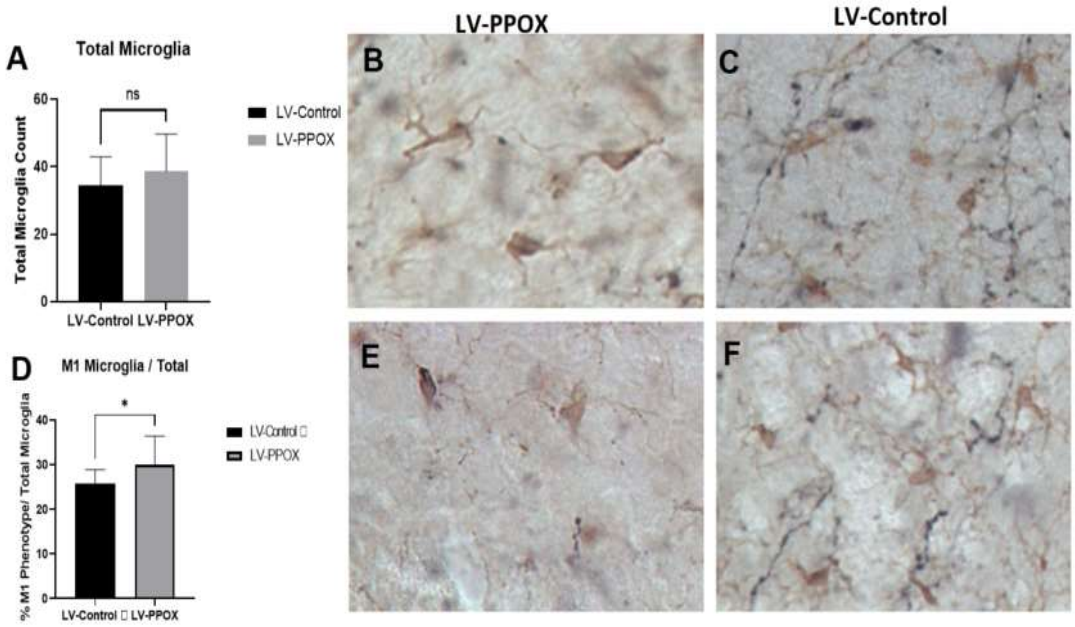

Figure 5.4 Changes in Microglial Morphology following LV-PPOX
(A) No Significant difference between total count of microglia in LV-Control and LV-PPOX groups in young animals (3-4 months) (p =.2412). (C) A significant increase in M1 morphology between microglia in LV-Control and LV-PPOX groups in young animals (3-4 months) (p =.0280). (B)(E) Two pictographs showing microglia morphology and orexin fiber labeling in LV-PPOX (C)(F) and LV-Control groups reveals LV-PPOX downregulates orexin expression of young animals 4 weeks after administration into the LH.

5.4.4 Cytokine changes in the BF following LV-PPOX

Four weeks after LV-PPOX or LV-Control injection of young rats, the BF was homogenized and contents of the pro-inflammatory and anti-inflammatory cytokines was observed using an ELISA assay (Figure 5.5). Following a t-test, the expression of TNFα (unpaired; p= .0691; df= 14; t= .5614) showed a trend of increased release in the BF of young animals administered LV-PPOX compared to controls. Analysis of other pro-inflammatory cytokines, such as IL1β, IL-6, IFN-γ, and IL-12 did not show any significant changes in expression. Additionally, analysis of the anti-inflammatory cytokine, IL-10, showed no significant changes between LV-Control and LV-PPOX group of young animals.

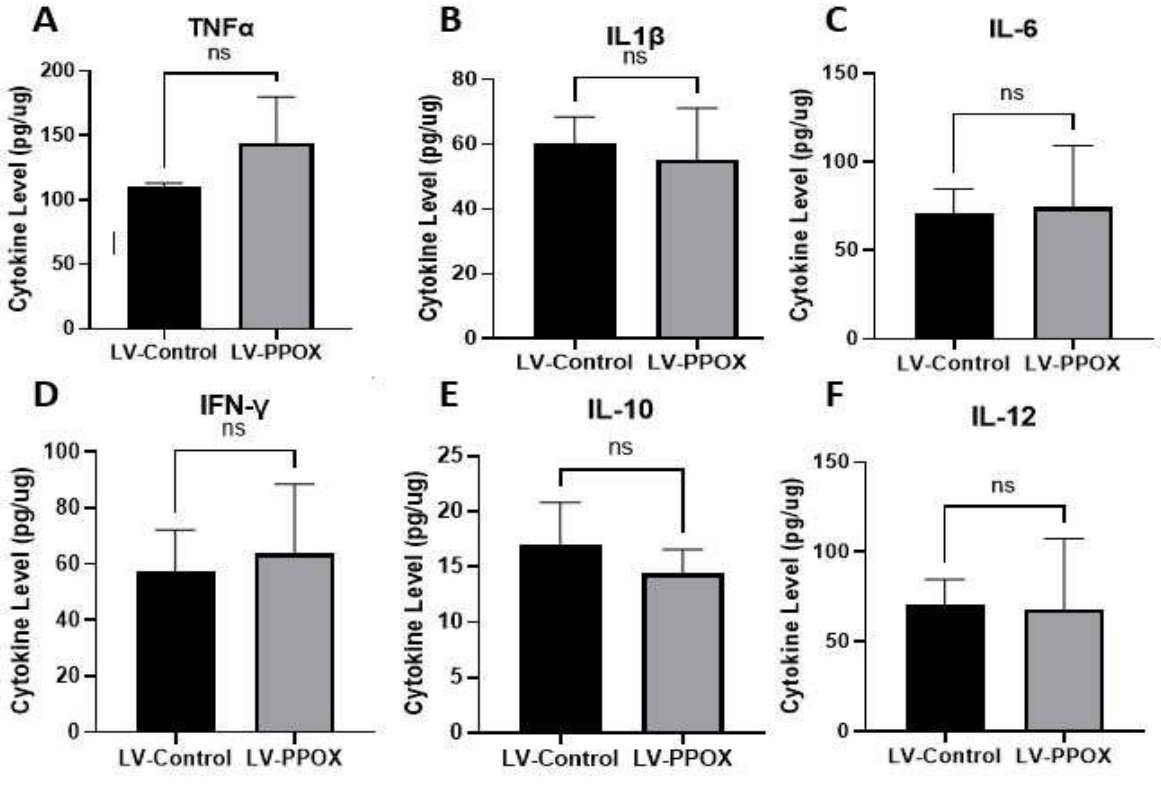

Figure 5.5 Cytokine changes in the BF following LV-PPOX
(A) A significant increase in TNFα between LV-Control and LV-PPOX groups in young animals (3-4 months) (p =.0691). (B) IL1β (C) IL-6 (D) IFN-γ (E) IL-12 (F) IL-10 did not show significant changes between LV-Control and LV-PPOX groups in young animals (3-4 months) in the BF 4 weeks after administration into the LH.

5.4.5 Cytokine changes in the PFC following LV-PPOX

Four weeks after LV-PPOX or LV-Control injection of young rats, the PFC was homogenized and contents of the pro-inflammatory and anti-inflammatory cytokines was observed using an ELISA assay (Figure 5.6). Following a t-test, the expression of TNFα (unpaired; p= .0040; df= 14; t= 3.484) and IL-6 (unpaired; p= .0016; df= 14; t= 3.902) showed a significant increase in release in the BF of young animals administered LV-PPOX compared to LV-Controls. Analysis of other pro-inflammatory cytokines, such as IL1β, IFN-γ, and IL-12 did not show any significant changes in expression. Additionally, analysis of the anti-inflammatory cytokine, IL-10, showed no significant changes between LV-Control and LV-PPOX group of young animals.

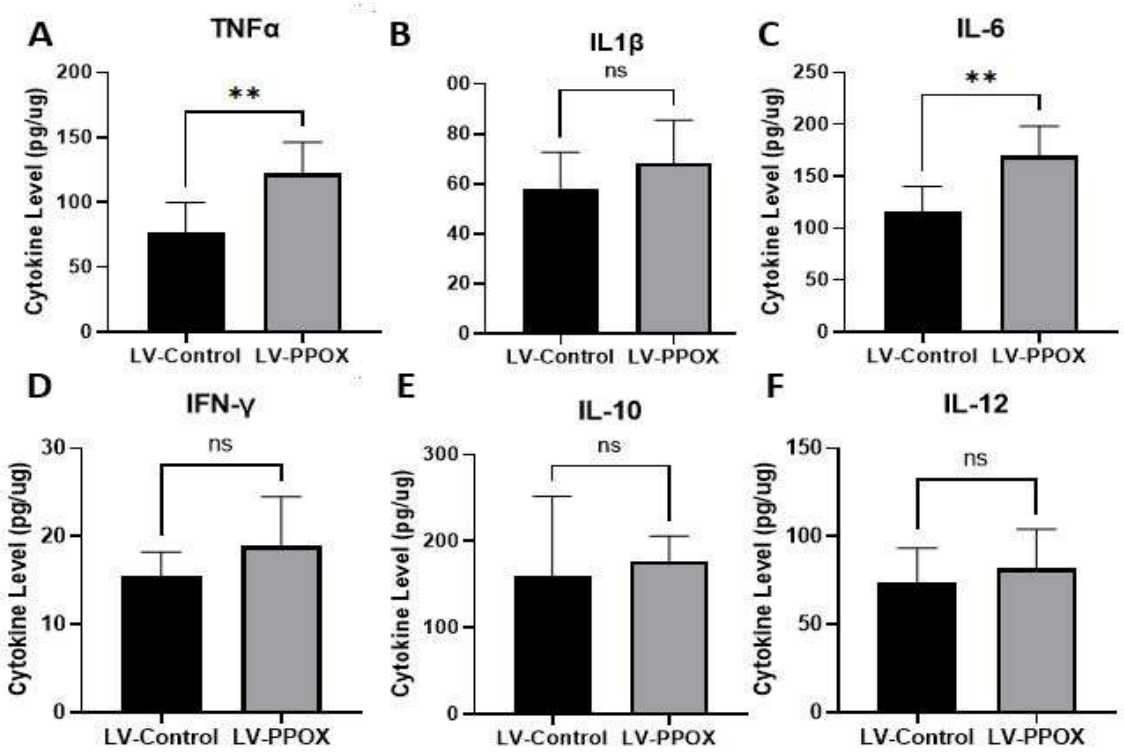

Figure 5.6 Cytokine changes in the PFC following LV-PPOX
(A)(C) A significant increase in TNFα and IL-6 between LV-Control and LV-PPOX groups in young animals (3-4 months) (p =.004). (B) IL1β (D) IFN-γ (E) IL-12 (F) IL-10 did not show significant changes between LV-Control and LV-PPOX groups in young animals (3-4 months) in the PFC four weeks after administration into the LH.

5.5 Discussion

Orexin has been described as a key neuropeptide which regulates fundamental brain functions, including arousal, attention, appetite, and cognition. The dysregulation of orexin signaling plays an important role in aging and age-related diseases, such as AD (Liguori, 2017). Research has shown that excessive or inhibition of orexinergic signaling leads to a disruption in cognitive decline, sleep disturbance, dysregulation of microglial signaling, and interrupts Aβ deposition and aggregation (Um & Lim, 2020). This suggests that the imbalances of orexin signaling can contribute to the pathology seen in aged individuals and AD patients. In models of aged animals, our lab has previously shown that orexin is lost which contributes to age-related cognitive decline (Kessler et al., 2011). Additionally, we have demonstrated that the orexin system plays an important role in regulating cognitive function and that aging is associated with dramatic reductions in the number of orexin neurons and deficits in activation of cortical, LH and circuitry by homeostatically-relevant stimuli. (Calva et al., 2018, 2020; J. Fadel & Burk, 2010b; Hagar et al., 2017; Kessler et al., 2011).

Recent studies have reported that orexin may exert an important role in neuroprotection by reducing apoptosis and inflammation (Irving et al., 2002; Sokołowska, Urbańska, Namiecińska, Biegańska, & Zawilska, 2012). Studies using transgenic mouse model of neurodegeneration, orexin/ataxin-3 mice, link orexin loss to neurodegeneration, memory and cognitive deficits, and neuroinflammation. In conjunction, studies describing age-related changes in microglial morphology may undergo age-dependent degeneration due to their loss of neuroprotective function there by contributing sporadic AD (Streit, Braak, Xue, & Bechmann, 2009; Streit et al., 2004).

Moreover, aged microglia have been characterized have been characterized by their reduced processes and increased proinflammatory cytokines, such as TNFα, IL1β and IL-6 (Sierra, Gottfried-Blackmore, Mceven, & Bulloch, 2007). Altered microglia morphology and reduced arborization have been reported in the human brain during aging and in patients diagnosed with AD (D. S. Davies, Ma, Jegathees, & Goldsbury, 2017).

The activation of BF circuitry by orexinergic neurons, and their role in arousal and cognition, particularly attention, are relatively well known. Recent reports have shown that orexin-deficient mice under a high fat diet have impaired cognition and increased microglial activation in the cortex (C. M. Duffy et al., 2019). Orexinergic neurons and both orexin receptor subtypes are distributed in the BF and cortex, and orexin has been shown to modulate the BFCS. Studies have shown an upregulation in microglia in the cortex in response to inflammation caused by impairment (Mihara et al., 2011). This suggests a potential role for orexin-glial communication in the BF and cortex which may be disrupted with age. Following the inhibition of orexin using LV-PPOX, we demonstrated changes in microglial morphology similar to that of aged animals and orexin reduction will contribute to an increase inflammatory cytokines, particularly in the PFC.

It should be noted that the approach, ELISA assay of brain homogenates, used did not distinguish the origin of cytokine release. Other glia, including astrocytes, are also capable of contributing to the cytokine increase in the PFC and BF. Thus, further studies defining the role of astrocytes in this mechanism are necessary. Additionally, I expected to see greater activated microglial expression in the BF of LV-PPOX treated rats

compared to LV-Controls. However, considering that inputs into the PFC include BF efferents and orexinergic efferents from the LH, it is not surprising to see a greater effect in the PFC (Figure 5.7). Further studies will be essential in order to broaden our understanding of the role of aged microglia in the relationship between the LH originating orexin neurons and microglia residing in brain regions with heavy orexinergic input, such as the PFC and BF, to elucidate the impact of aging on microglial functions.

CHAPTER 6. GENERAL CONCLUSIONS

Aging is characterized by mild neuronal degeneration which leads to mild memory loss, attentional deficits, homeostatic imbalance and sleeping abnormalities. BF neurons are particularly vulnerable to dysfunction and degeneration in aged humans and, more dramatically, in diseases such as AD. This suggests that dysfunctional anatomical correlates of the BF, specifically in the SI/NBM, may be a key component in the spread and development of mild cognitive impairments and, in more severe cases, AD. This study investigates age-related changes in basal forebrain afferent neuronal and cell populations to identify vulnerable populations involved in age-associated cognitive decline and dysfunction. Specifically, my study compared neuronal afferent activation, BF neuronal activation and glial populations to identify anatomical factors susceptible to age-related homeostatic dysfunction in the BF.

I first looked at changes in activation of BF inputs from several brain regions including the PFC, ILC, Cl, CeA, LH, NAc, and VTA. The BF plays a significant role in the information processing and integrating signals from different afferent regions. These projections make contact with several populations in the BF including BFCS, glutamatergic neurons, GABAergic neurons and glial populations, such as microglia. We know the loss of BF neurons can effect neural networks which rely on BF input to provide necessary communication attributed to the cognitive and homeostatic balance necessary for proper function. It is also possible that the dysfunctional signaling contacting the local BF neurons may contribute in some way to neuronal dysregulation and degeneration. Regions which send inputs to the BF may be involved in the circuits which seem to manifest the neuronal dysfunction and degeneration in aging and age-

related diseases, such as AD. However, it is still not known how these regions are effected during normal age relate changes and there is still much to learn about afferent neuronal populations which make contacts with the BFCS and local cell populations. We hypothesize that age-related attentional deficits related to the attentional processing of homeostatically-relevant stimuli is associated with dysfunctional neuronal activation of specific BF afferents. We have revealed several circuit motifs vulnerable to age-related dysfunction, including the PFC, LH, NAc, and VTA, which will enable future anatomical studies to define specific cell signaling mechanisms that are effected by aging and age-related diseases (Figure 1.1). We have also show that a deregulation in activation of PV+, GABAergic neurons, is associated with processing of the homeostatically-relevant stimulus which is attenuated with aging revealing a potential role of GABAergic neurons in age-related cognitive decline. PV+ GABAergic neurons have been attributed to behavioral state control and arousal (S. C. Lin, Brown, Shuler, Petersen, & Kepecs, 2015). Our data supports a role for PV+ GABAergic neurons in arousal and attention related to a homeostatically relevant stimulus, and presents a novel age-dependent deficit in PV activation of BF neurons upon the presentation of a food-paired conditioned stimulus.

An interesting hypothesis is that the manifestation of age-related deficits may share common underlying neurobiological mechanism, possibly through an anatomical region involved in homeostasis which regulates the activity of brain regions which mediate behavioral and cognitive responses to physiological imbalances, and these interactions may be impacted in aging. Our lab has shown that orexin neuropeptides, which have been shown to degrade during aging, play a pivotal role in maintaining

homeostatic balance and exert influence over the PFC, NAc, BF, and VTA. Moreover, orexin may exert an important role in neuroprotection exerted by microglia by reducing apoptosis and inflammation, and that during aging microglial morphology may undergo age-dependent degeneration contributing to their loss of neuroprotective function (Irving et al., 2002; Streit et al., 2009, 2004). We have shown that downregulation of orexin via LV-PPOX can mimic similar changes, including the presence of microglia with reduced processes and increased proinflammatory cytokines, such as TNFα, and IL-6. This suggests a potential role for orexin-glial communication in the BF and cortex which may be disrupted with age. Thus the orexin system has been shown to influence changes in response to a homeostatically-relevant stimuli which is then attenuated with age. BF inputs which have an intimate relationship with the orexin system as it relates to processing/activation in response to homeostatically relevant salient cues show attenuation during aging. The loss of orexin mimics feeding latencies, activational changes, and microglial changes seen in aging. Thus, the orexin system and the BF play a pivotal role in the age-related cognitive and functional deficits. Further studies will be essential in order to broaden our understanding of the role orexin neurons during aging as it relates to BF neuronal and glial dysfunction. However, the results from this study have contributed toward the efforts to identify targets of age-related dysfunction which may play a role in age associated MCI and homeostatic dysfunction exacerbated during age-related neurodegenerative diseases, such as AD.

BIBLIOGRAPHY

Adcock, R. A., Thangavel, A., Whitfield-Gabrieli, S., Knutson, B., & Gabrieli, J. D. E. (2006). Reward-Motivated Learning: Mesolimbic Activation Precedes Memory Formation. *Neuron*, *50*(3), 507–517. https://doi.org/10.1016/j.neuron.2006.03.036

Afif, A., & Mertens, P. (2010). Description of sulcal organization of the insular cortex. *Surgical and Radiologic Anatomy*, *32*(5), 491–498. https://doi.org/10.1007/s00276-009-0598-4

Afridi, R., Lee, W. H., & Suk, K. (2020). Microglia Gone Awry: Linking Immunometabolism to Neurodegeneration. *Frontiers in Cellular Neuroscience*. https://doi.org/10.3389/fncel.2020.00246

Aguzzi, A., Barres, B. A., & Bennett, M. L. (2013). Microglia: Scapegoat, saboteur, or something else? *Science*. https://doi.org/10.1126/science.1227901

Aitta-Aho, T., Phillips, B. U., Pappa, E., Hay, Y. A., Harnischfeger, F., Heath, C. J., … Apergis-Schoute, J. (n.d.). *Accumbal Cholinergic Interneurons Differentially Influence Motivation Related to Satiety Signaling*. https://doi.org/10.1523/ENEURO.0328-16.2017

Allen, T. G. J., Abogadie, F. C., & Brown, D. A. (2006). Simultaneous release of glutamate and acetylcholine from single magnocellular “cholinergic” basal forebrain neurons. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.3979-05.2006

Ammoun, S., Holmqvist, T., Shariatmadari, R., Oonk, H. B., Detheux, M., Parmentier, M., … Kukkonen, J. P. (2003). Distinct recognition of OX1 and OX2 receptors by orexin peptides. *Journal of Pharmacology and Experimental Therapeutics*, *305*(2), 507–514. https://doi.org/10.1124/jpet.102.048025

Anand, B. K., & Brobeck, J. R. (1951). Localization of a "Feeding Center" in the Hypothalamus of the Rat. *Proceedings of the Society for Experimental Biology and Medicine*, *77*(2), 323–325. https://doi.org/10.3181/00379727-77-18766

Arends, Y. M., Duyckaerts, C., Rozemuller, J. M., Eikelenboom, P., & Hauw, J. J. (2000). Microglia, amyloid and dementia in Alzheimer disease: A correlative study. *Neurobiology of Aging*. https://doi.org/10.1016/S0197-4580(00)00094-4

Arendt, T., Bigl, V., Tennstedt, A., & Arendt, A. (1985). Neuronal loss in different parts of the nucleus basalis is related to neuritic plaque formation in cortical target areas in alzheimer's disease. *Neuroscience*, *14*(1), 1–14. https://doi.org/10.1016/0306-4522(85)90160-5

Armstrong, R. A. (2013). What causes Alzheimer's disease? *Folia Neuropathologica*. https://doi.org/10.5114/fn.2013.37702

Arnold, H. M., Fadel, J., Sarter, M., & Bruno, J. P. (2001). Amphetamine-stimulated cortical acetylcholine release: Role of the basal forebrain. *Brain Research*, *894*(1), 74–87. https://doi.org/10.1016/S0006-8993(00)03328-X

Auerbach, J. M., & Segal, M. (1996). Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus. *Journal of Physiology*, *492*(2), 479–493. https://doi.org/10.1113/jphysiol.1996.sp021323

Augustine, J. R. (1985). The insular lobe in primates including humans. *Neurological Research*, *7*(1), 2–10. https://doi.org/10.1080/01616412.1985.11739692

Augustine, J. R. (1996, October). Circuitry and functional aspects of the insular lobe in primates including humans. *Brain Research Reviews*, Vol. 22, pp. 229–244. https://doi.org/10.1016/S0165-0173(96)00011-2

Auld, D. S., Kornecook, T. J., Bastianetto, S., & Quirion, R. (2002). Alzheimer's disease and the basal forebrain cholinergic system: Relations to β-amyloid peptides, cognition, and treatment strategies. *Progress in Neurobiology*. https://doi.org/10.1016/S0301-0082(02)00079-5

Avila, I., & Lin, S. C. (2014). Motivational Salience Signal in the Basal Forebrain Is Coupled with Faster and More Precise Decision Speed. *PLoS Biology*, *12*(3). https://doi.org/10.1371/journal.pbio.1001811

Ball, M., Braak, H., Coleman, P., Dickson, D., Duyckaerts, C., Gambetti, P., … Khachaturian, Z. (1997). Consensus recommendations for the postmortem diagnosis of Alzheimer's disease. *Neurobiology of Aging*. https://doi.org/10.1016/S0197-4580(97)00057-2

Ballinger, E. C., Ananth, M., Talmage, D. A., & Role, L. W. (2016). Basal Forebrain Cholinergic Circuits and Signaling in Cognition and Cognitive Decline. *Neuron*. https://doi.org/10.1016/j.neuron.2016.09.006

Barragán Martínez, D., García Soldevilla, M. A., Parra Santiago, A., & Tejeiro Martínez, J. (2019). Alzheimer's disease. *Medicine (Spain)*. https://doi.org/10.1016/j.med.2019.03.012

Bartus, R. T., Fleming, D., & Johnson, H. R. (1978). Aging in the rhesus monkey: Debilitating effects on short-term memory. *Journals of Gerontology*. https://doi.org/10.1093/geronj/33.6.858

Bartus, Raymond T. (1979). Physostigmine and recent memory: Effects in young and aged nonhuman primates. *Science*, *206*(4422), 1087–1089. https://doi.org/10.1126/science.227061

Bartus, Raymond T. (2000). On neurodegenerative diseases, models, and treatment strategies: Lessons learned and lessons forgotten a generation following the cholinergic hypothesis. *Experimental Neurology*. https://doi.org/10.1006/exnr.2000.7397

Bartus, Raymond T., Dean, R. L., Beer, B., & Lippa, A. S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science*. https://doi.org/10.1126/science.7046051

Bassareo, V., & Di Chiara, G. (1999). Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. *Neuroscience*, *89*(3), 637–641. https://doi.org/10.1016/S0306-4522(98)00583-1

Baxter, M. G., & Chiba, A. A. (1999). Cognitive functions of the basal forebrain. *Current Opinion in Neurobiology*. https://doi.org/10.1016/S0959-4388(99)80024-5

Beach, T. G., Walker, R., & McGeer, E. G. (1989). Patterns of gliosis in alzheimer's disease and aging cerebrum. *Glia*. https://doi.org/10.1002/glia.440020605

Beier, K. T., Steinberg, E. E., Deloach, K. E., Xie, S., Miyamichi, K., Schwarz, L., … Luo, L. (2015). Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic Input-Output Mapping. *Cell*, *162*(3), 622–634. https://doi.org/10.1016/j.cell.2015.07.015

Berridge, K. C. (2007, April). The debate over dopamine's role in reward: The case for incentive salience. *Psychopharmacology*, Vol. 191, pp. 391–431. https://doi.org/10.1007/s00213-006-0578-x

Bettcher, B. M., Johnson, S. C., Fitch, R., Casaletto, K., Heffernan, K. S., Asthana, S., … Kramer, J. H. (2017). [P2-208]: CSF AND PLASMA LEVELS OF INFLAMMATION DIFFERENTIALLY RELATE TO CNS MARKERS OF ALZHEIMER'S DISEASE PATHOLOGY AND NEURONAL DAMAGE. *Alzheimer's & Dementia*. https://doi.org/10.1016/j.jalz.2017.06.860

Blitzer, R. D., Gil, O., & Landau, E. M. (1990). Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. *Neuroscience Letters*, *119*(2), 207–210. https://doi.org/10.1016/0304-3940(90)90835-W

Bloem, B., Schoppink, L., Rotaru, D. C., Faiz, A., Hendriks, P., Mansvelder, H. D., … Wouterlood, F. G. (2014). Topographic mapping between basal forebrain cholinergic neurons and the medial prefrontal cortex in mice. *Journal of Neuroscience*, *34*(49), 16234–16246. https://doi.org/10.1523/JNEUROSCI.3011-14.2014

Bobinski, M., De Leon, M. J., Wegiel, J., Desanti, S., Convit, A., Saint Louis, L. A., … Wisniewski, H. M. (1999). The histological validation of post mortem magnetic resonance imaging- determined hippocampal volume in Alzheimer's disease. *Neuroscience*. https://doi.org/10.1016/S0306-4522(99)00476-5

Bossert, J. M., Stern, A. L., Theberge, F. R. M., Marchant, N. J., Wang, H. L., Morales, M., & Shaham, Y. (2012). Role of projections from ventral medial prefrontal cortex to nucleus accumbens shell in context-induced reinstatement of heroin seeking. *Journal of Neuroscience*, *32*(14), 4982–4991. https://doi.org/10.1523/JNEUROSCI.0005-12.2012

Boswell, R. G., & Kober, H. (2016). Food cue reactivity and craving predict eating and weight gain: A meta-analytic review. *Obesity Reviews*, *17*(2), 159–177. https://doi.org/10.1111/obr.12354

Bowen, D. M., Smith, C. B., White, P., & Davison, A. N. (1976). Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain*. https://doi.org/10.1093/brain/99.3.459

Brog, J. S., Salyapongse, A., Deutch, A. Y., & Zahm, D. S. (1993). The patterns of afferent innervation of the core and shell in the "Accumbens" part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. *Journal of Comparative Neurology*, *338*(2), 255–278. https://doi.org/10.1002/cne.903380209

Bromberg-Martin, E. S., Matsumoto, M., & Hikosaka, O. (2010, December 9). Dopamine in Motivational Control: Rewarding, Aversive, and Alerting. *Neuron*, Vol. 68, pp. 815–834. https://doi.org/10.1016/j.neuron.2010.11.022

Brueggen, K., Dyrba, M., Barkhof, F., Hausner, L., Filippi, M., Nestor, P. J., … Teipel, S. J. (2015). Basal forebrain and hippocampus as predictors of conversion to Alzheimer's disease in patients with mild cognitive impairment-a multicenter DTI and volumetry study. *Journal of Alzheimer's Disease*. https://doi.org/10.3233/JAD-150063

Brugg, B., Dubreuil, Y. L., Huber, G., Wollman, E. E., Delhaye-Bouchaud, N., & Mariani, J. (1995). Inflammatory processes induce β-amyloid precursor protein changes in mouse brain. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.92.7.3032

Bucci, D. J., Holland, P. C., & Gallagher, M. (1998). Removal of cholinergic input to rat posterior parietal cortex disrupts incremental processing of conditioned stimuli. *Journal of Neuroscience*, *18*(19), 8038–8046. https://doi.org/10.1523/jneurosci.18-19-08038.1998

Buchman, A. S., Wilson, R. S., Bienias, J. L., Shah, R. C., Evans, D. A., & Bennett, D. A. (2005). Change in body mass index and risk of incident Alzheimer disease. *Neurology*. https://doi.org/10.1212/01.wnl.0000176061.33817.90

Budzynska, B., Boguszewska-Czubara, A., Kruk-Slomka, M., Skalicka-Wozniak, K., Michalak, A., Musik, I., & Biala, G. (2015). Effects of imperatorin on scopolamine-induced cognitive impairment and oxidative stress in mice. *Psychopharmacology*. https://doi.org/10.1007/s00213-014-3728-6

Burgos-robles, A., Bravo-rivera, H., & Quirk, G. J. (2013). *Prelimbic and Infralimbic Neurons Signal Distinct Aspects of Appetitive Instrumental Behavior*. *8*(2), 1–7. https://doi.org/10.1371/journal.pone.0057575

Cacquevel, M., Lebeurrier, N., Cheenne, S., & Vivien, D. (2005). Cytokines in Neuroinflammation and Alzheimers Disease. *Current Drug Targets*. https://doi.org/10.2174/1389450043345308

Cagnin, A., Brooks, D. J., Kennedy, A. M., Gunn, R. N., Myers, R., Turkheimer, F. E., … Banati, R. B. (2001). In-vivo measurement of activated microglia in dementia. *Lancet*. https://doi.org/10.1016/S0140-6736(01)05625-2

Calva, C. B., & Fadel, J. R. (2020). Intranasal administration of orexin peptides: Mechanisms and therapeutic potential for age-related cognitive dysfunction. *Brain Research*. https://doi.org/10.1016/j.brainres.2018.08.024

Calva, C. B., Fayyaz, H., & Fadel, J. R. (2018). Increased acetylcholine and glutamate efflux in the prefrontal cortex following intranasal orexin-A (hypocretin-1). *Journal of Neurochemistry*. https://doi.org/10.1111/jnc.14279

Calva, C. B., Fayyaz, H., & Fadel, J. R. (2020). Effects of Intranasal Orexin-A (Hypocretin-1) Administration on Neuronal Activation, Neurochemistry, and Attention in Aged Rats. *Frontiers in Aging Neuroscience*. https://doi.org/10.3389/fnagi.2019.00362

Chaves-Coira, I., Martín-Cortecero, J., Nuñez, A., & Rodrigo-Angulo, M. L. (2018). Basal Forebrain Nuclei Display Distinct Projecting Pathways and Functional Circuits to Sensory Primary and Prefrontal Cortices in the Rat. *Frontiers in Neuroanatomy*, *12*. https://doi.org/10.3389/fnana.2018.00069

Chaves-Coira, I., Rodrigo-Angulo, M. L., & Nuñez, A. (2018). Bilateral pathways from the basal forebrain to sensory cortices may contribute to synchronous sensory processing. *Frontiers in Neuroanatomy*, *12*. https://doi.org/10.3389/fnana.2018.00005

Chieffi, S., Carotenuto, M., Monda, V., Valenzano, A., Villano, I., Precenzano, F., … Messina1, A. (2017). Orexin system: The key for a healthy life. *Frontiers in Neurology*, *8*(May), 1–9. https://doi.org/10.3389/fphys.2017.00357

Chrzanowska, G., & Beben, A. (1973). Weight of the brain and body height in man between the ages of 20 and 89 years. *Folia Morphologica*.

Chung, H. Y., Cesari, M., Anton, S., Marzetti, E., Giovannini, S., Seo, A. Y., … Leeuwenburgh, C. (2009, January). Molecular inflammation: Underpinnings of aging and age-related diseases. *Ageing Research Reviews*, Vol. 8, pp. 18–30. https://doi.org/10.1016/j.arr.2008.07.002

Clark, R. E., & Editors, S. J. M. (n.d.). *Current Topics in Behavioral Neurosciences 37 Behavioral Neuroscience of Learning and Memory*.

Cockrell, A. S., & Kafri, T. (2007, July). Gene delivery by lentivirus vectors. *Molecular Biotechnology*, Vol. 36, pp. 184–204. https://doi.org/10.1007/s12033-007-0010-8

Cole, S., Hobin, M. P., & Petrovich, G. D. (2015). Appetitive associative learning recruits a distinct network with cortical, striatal, and hypothalamic regions. *Neuroscience*, *286*, 187–202. https://doi.org/10.1016/j.neuroscience.2014.11.026

Cole, Sindy, Keefer, S. E., Anderson, L. C., & Petrovich, G. D. (2020). Medial prefrontal cortex neural plasticity, orexin receptor 1 signaling, and connectivity with the lateral hypothalamus are necessary in cue-potentiated feeding. *Journal of Neuroscience*, *40*(8), 1744–1755. https://doi.org/10.1523/JNEUROSCI.1803-19.2020

Colomb, J., Kluger, A., De Leon, M. J., Ferris, S. H., Mittelman, M., Cohen, J., & George, A. E. (1996). Hippocampal formation size predicts declining memory performance in normal aging. *Neurology*. https://doi.org/10.1212/wnl.47.3.810

Contestabile, A. (2011). The history of the cholinergic hypothesis. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2009.12.044

Cova, I., Clerici, F., Rossi, A., Cucumo, V., Ghiretti, R., Maggiore, L., … Caracciolo, B. (2016). Weight loss predicts progression of mild cognitive impairment to Alzheimer's disease. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0151710

Craig, A. D. (2003). Interoception: The sense of the physiological condition of the body. *Current Opinion in Neurobiology*, Vol. 13, pp. 500–505. https://doi.org/10.1016/S0959-4388(03)00090-4

Craig, A. D. (2013). Cooling, pain, and other feelings from the body in relation to the autonomic nervous system. In *Handbook of Clinical Neurology* (Vol. 117, pp. 103–109). https://doi.org/10.1016/B978-0-444-53491-0.00009-2

Craig, A. D. B. (2011). Significance of the insula for the evolution of human awareness of feelings from the body. *Annals of the New York Academy of Sciences*, *1225*(1), 72–82. https://doi.org/10.1111/j.1749-6632.2011.05990.x

Davies, D. S., Ma, J., Jegathees, T., & Goldsbury, C. (2017). Microglia show altered morphology and reduced arborization in human brain during aging and Alzheimer's disease. *Brain Pathology*, *27*(6), 795–808. https://doi.org/10.1111/bpa.12456

Davies, P., & Maloney, A. J. F. (1976). SELECTIVE LOSS OF CENTRAL CHOLINERGIC NEURONS IN ALZHEIMER'S DISEASE. *The Lancet*. https://doi.org/10.1016/S0140-6736(76)91936-X

De Lecea, L., Kilduff, T. S., Peyron, C., Gao, X. B., Foye, P. E., Danielson, P. E., … Sutcliffe, J. G. (1998). The hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(1), 322–327. https://doi.org/10.1073/pnas.95.1.322

De Lecea, Luis, Sutcliffe, G. J., & Fabre, V. (2002). Hypocretins/orexins as integrators of physiological information: Lessons from mutant animals. *Neuropeptides*, *36*(2–3), 85–95. https://doi.org/10.1054/npep.2002.0892

Dekaban, A. S., & Sadowsky, D. (1978). Changes in brain weights during the span of human life: Relation of brain weights to body heights and body weights. *Annals of Neurology*. https://doi.org/10.1002/ana.410040410

Dekker, A. D. J. A. M., Connor, D. J., & Thal, L. J. (1991). The role of cholinergic projections from the nucleus basalis in memory. *Neuroscience and Biobehavioral Reviews*, *15*(2), 299–317. https://doi.org/10.1016/S0149-7634(05)80008-9

Desarnaud, F., Murillo-Rodriguez, E., Lin, L., Xu, M., Gerashchenko, D., Shiromani, S. N., … Shiromani, P. J. (2004). The diurnal rhythm of hypocretin in young and old F344 rats. *Sleep*. https://doi.org/10.1093/sleep/27.5.851

Diniz, B. S., Teixeira, A. L., Ojopi, E. B., Talib, L. L., Mendonça, V. A., Gattaz, W. F., & Forlenza, O. V. (2010). Higher serum sTNFR1 level predicts conversion from mild cognitive impairment to Alzheimer's disease. *Journal of Alzheimer's Disease*. https://doi.org/10.3233/JAD-2010-100921

Dorszewska, J., Prendecki, M., Oczkowska, A., Dezor, M., & Kozubski, W. (2016). Molecular Basis of Familial and Sporadic Alzheimer's Disease. *Current Alzheimer Research*. https://doi.org/10.2174/1567205013666160314150501

Drachman, D. A., & Leavitt, J. (1974). Human Memory and the Cholinergic System: A Relationship to Aging? *Archives of Neurology*. https://doi.org/10.1001/archneur.1974.00490320001001

Duffy, C. M., Butterick, T. A., & Nixon, J. P. (2019). High fat diet increases cognitive decline and neuroinflammation in a model of orexin loss. *Psychological Bulletin*, *126*(1), 21. https://doi.org/.1037//0033-2909.I26.1.78

Duffy, Cayla M., Yuan, C., Wisdorf, L. E., Billington, C. J., Kotz, C. M., Nixon, J. P., & Butterick, T. A. (2015). Role of orexin A signaling in dietary palmitic acid-activated microglial cells. *Neuroscience Letters*. https://doi.org/10.1016/j.neulet.2015.08.033

Düzel, S., Münte, T. F., Lindenberger, U., Bunzeck, N., Schütze, H., Heinze, H. J., & Düzel, E. (2010). Basal forebrain integrity and cognitive memory profile in healthy aging. *Brain Research*. https://doi.org/10.1016/j.brainres.2009.10.048

Ebert, U., & Kirch, W. (1998). Scopolamine model of dementia: Electroencephalogram findings and cognitive performance. *European Journal of Clinical Investigation*. https://doi.org/10.1046/j.1365-2362.1998.00393.x

Eggermann, E., Serafin, M., Bayer, L., Machard, D., Saint-Mleux, B., Jones, B. E., & Mühlethaler, M. (2001). Orexins/hypocretins excite basal forebrain cholinergic neurones. *Neuroscience*. https://doi.org/10.1016/S0306-4522(01)00512-7

Espana, R. A., Baldo, B. A., Kelley, A. E., & Berridge, C. W. (2001). Wake-promoting and sleep-suppressing actions of hypocretin (orexin): Basal forebrain sites of action. *Neuroscience*, *106*(4), 699–715. https://doi.org/10.1016/S0306-4522(01)00319-0

Espiritu, J. R. D. (2008). Aging-Related Sleep Changes. *Clinics in Geriatric Medicine*. https://doi.org/10.1016/j.cger.2007.08.007

Everitt, B. J., & Robbins, T. W. (1997). CENTRAL CHOLINERGIC SYSTEMS AND COGNITION. *Annual Review of Psychology*. https://doi.org/10.1146/annurev.psych.48.1.649

Evrard, H. C. (2019, April 16). The organization of the primate insular cortex. *Frontiers in Neuroanatomy*, Vol. 13, p. 43. https://doi.org/10.3389/fnana.2019.00043

Fadel, C., Digiaro, M., Choueiri, E., El Beaino, T., Saponari, M., Savino, V., & Martelli, G. P. (2005). On the presence and distribution of olive viruses in Lebanon. *EPPO Bulletin*, *35*(1), 33–36. https://doi.org/10.1111/j.1365-2338.2005.00776.x

Fadel, J., & Burk, J. A. (2010a). Orexin/hypocretin modulation of the basal forebrain cholinergic system: Role in attention. *Brain Research*. https://doi.org/10.1016/j.brainres.2009.08.046

Fadel, J., & Burk, J. A. (2010b). Orexin/hypocretin modulation of the basal forebrain cholinergic system: Role in attention. *Brain Research*, *1314*, 112–123. https://doi.org/10.1016/j.brainres.2009.08.046

Fadel, J., Pasumarthi, R., & Reznikov, L. R. (2005). Stimulation of cortical acetylcholine release by orexin A. *Neuroscience*, *130*(2), 541–547. https://doi.org/10.1016/j.neuroscience.2004.09.050

Fadel, J. R., Jolivalt, C. G., & Reagan, L. P. (2013). Food for thought: The role of appetitive peptides in age-related cognitive decline. *Ageing Research Reviews*, Vol. 12, pp. 764–776. https://doi.org/10.1016/j.arr.2013.01.009

Fadel, J., Sarter, M., & Bruno, J. P. (1999). Age-related attenuation of stimulated cortical acetylcholine release in basal forebrain-lesioned rats. *Neuroscience*, *90*(3), 793–802. https://doi.org/10.1016/S0306-4522(98)00515-6

Fadel, Jim, Bubser, M., & Deutch, A. Y. (2002). Differential activation of orexin neurons by antipsychotic drugs associated with weight gain. *Journal of Neuroscience*. https://doi.org/10.1523/jneurosci.22-15-06742.2002

Fadel, Jim, & Frederick-Duus, D. (2008). Orexin/hypocretin modulation of the basal forebrain cholinergic system: Insights from in vivo microdialysis studies. *Pharmacology Biochemistry and Behavior*. https://doi.org/10.1016/j.pbb.2008.01.008

Fadel, Jim, Moore, H., Sarter, M., & Bruno, J. P. (1996). Trans-synaptic stimulation of cortical acetylcholine release after partial 192 IgG-saporin-induced loss of cortical cholinergic afferents. *Journal of Neuroscience*, *16*(20), 6592–6600. https://doi.org/10.1523/jneurosci.16-20-06592.1996

Fadel, Jim, Sarter, M., & Bruno, J. P. (2001). Basal forebrain glutamatergic modulation of cortical acetylcholine release. *Synapse*. https://doi.org/10.1002/1098-2396(20010301)39:3<201::AID-SYN1001>3.0.CO;2-3

Fein, J. A., Sokolow, S., Miller, C. A., Vinters, H. V., Yang, F., Cole, G. M., & Gylys, K. H. (2008). Co-localization of amyloid beta and tau pathology in Alzheimer's disease synaptosomes. *American Journal of Pathology*. https://doi.org/10.2353/ajpath.2008.070829

Ferguson, B. R., & Gao, W. J. (2018, May 16). Pv interneurons: critical regulators of E/I balance for prefrontal cortex-dependent behavior and psychiatric disorders. *Frontiers in Neural Circuits*, Vol. 12. https://doi.org/10.3389/fncir.2018.00037

Fernández-Cabello, S., Kronbichler, M., Dijk, K. R. A. Van, Goodman, J. A., Spreng, R. N., Schmitz, T. W., & Initiative, A. D. N. (2019). Basal forebrain volume selectively and reliably predicts the cortical spread of Alzheimer's degeneration. *BioRxiv*. https://doi.org/10.1101/676544

Fleischman, D. A., Leurgans, S., Arfanakis, K., Arvanitakis, Z., Barnes, L. L., Boyle, P. A., … Bennett, D. A. (2014). Gray-matter macrostructure in cognitively healthy older persons: associations with age and cognition. *Brain Structure and Function*. https://doi.org/10.1007/s00429-013-0622-7

Flynn, F. G., Benson, D. F., & Ardila, A. (1999). Anatomy of the insula - Functional and clinical correlates. *Aphasiology*, Vol. 13, pp. 55–78. https://doi.org/10.1080/026870399402325

Frank, M. G., Barrientos, R. M., Biedenkapp, J. C., Rudy, J. W., Watkins, L. R., & Maier, S. F. (2006). mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiology of Aging*. https://doi.org/10.1016/j.neurobiolaging.2005.03.013

Frederick-Duus, D., Guyton, M. F., & Fadel, J. (2007). Food-elicited increases in cortical acetylcholine release require orexin transmission. *Neuroscience*. https://doi.org/10.1016/j.neuroscience.2007.07.061

Frederick-Duus, Danielle. (2008). Age-related loss of orexin innervation contributes to cortical cholinergic decline.

Fremeau, R. T., Voglmaier, S., Seal, R. P., & Edwards, R. H. (2004). VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends in Neurosciences*, Vol. 27, pp. 98–103. https://doi.org/10.1016/j.tins.2003.11.005

Freund, T. F., & Meskenaite, V. (1992a). γ-Aminobutyric acid-containing basal forebrain neurons innervate inhibitory interneurons in the neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(2), 738–742. https://doi.org/10.1073/pnas.89.2.738

Freund, T. F., & Meskenaite, V. (1992b). γ-Aminobutyric acid-containing basal forebrain neurons innervate inhibitory interneurons in the neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(2), 738–742. https://doi.org/10.1073/pnas.89.2.738

Gamage, R., Wagnon, I., Rossetti, I., Childs, R., Niedermayer, G., Chesworth, R., & Gyengesi, E. (2020, October 15). Cholinergic Modulation of Glial Function During Aging and Chronic Neuroinflammation. *Frontiers in Cellular Neuroscience*, Vol. 14. https://doi.org/10.3389/fncel.2020.577912

Gaykema, R. P.A., Gaal, G., Trader, J., Hersh, L. B., & Luiten, P. G. M. (1991). The basal forebrain cholinergic system: efferent and afferent connectivity and long-term effects of lesions. *Acta Psychiatrica Scandinavica*, *83*(366 S), 14–26. https://doi.org/10.1111/j.1600-0447.1991.tb03105.x

Gaykema, Ronald P.A., Van Weeghel, R., Hersh, L. B., & Luiten, P. G. M. (1991). Prefrontal cortical projections to the cholinergic neurons in the basal forebrain. *Journal of Comparative Neurology*, *303*(4), 563–583. https://doi.org/10.1002/cne.903030405

Gaykema, Ronald P.A., & Zaborszky, L. (1997). Parvalbumin-containing neurons in the basal forebrain receive direct input from the substantia nigra-ventral tegmental area. *Brain Research*. https://doi.org/10.1016/S0006-8993(96)01309-1

Gielow, M. R., & Zaborszky, L. (2017). The Input-Output Relationship of the Cholinergic Basal Forebrain. *Cell Reports*. https://doi.org/10.1016/j.celrep.2017.01.060

Gritti, I., Henny, P., Galloni, F., Mainville, L., Mariotti, M., & Jones, B. E. (2006). Stereological estimates of the basal forebrain cell population in the rat, including neurons containing choline acetyltransferase, glutamic acid decarboxylase or phosphate-activated glutaminase and colocalizing vesicular glutamate transporters. *Neuroscience*. https://doi.org/10.1016/j.neuroscience.2006.09.024

Gritti, Ivana, Mainville, L., & Jones, B. E. (1993). Codistribution of GABA- with acetylcholine-synthesizing neurons in the basal forebrain of the rat. *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.903290403

Gritti, Ivana, Mainville, L., Mancia, M., & Jones, B. E. (1997a). GABAergic and other noncholinergic basal forebrain neurons, together with cholinergic neurons, project to the mesocortex and isocortex in the rat. *Journal of Comparative Neurology*, *383*(2), 163–177. https://doi.org/10.1002/(SICI)1096-9861(19970630)383:2<163::AID-CNE4>3.0.CO;2-Z

Gritti, Ivana, Mainville, L., Mancia, M., & Jones, B. E. (1997b). GABAergic and Other Noncholinergic Basal Forebrain Neurons, Together With Cholinergic Neurons, Project to the Mesocortex and Isocortex in the Rat. In *J. Comp. Neurol* (Vol. 383). https://doi.org/10.1002/(SICI)1096-9861(19970630)383:2

Gritti, Ivana, Manns, I. D., Mainville, L., & Jones, B. E. (2003). Parvalbumin, calbindin, or calretinin in cortically projecting and GABAergic, cholinergic, or glutamatergic basal forebrain neurons of the rat. *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.10505

Groenewegen, H. J., der Zee, E. V. Van, te Kortschot, A., & Witter, M. P. (1987). Organization of the projections from the subiculum to the ventral striatum in the rat. A study using anterograde transport of Phaseolus vulgaris leucoagglutinin. *Neuroscience*, *23*(1), 103–120. https://doi.org/10.1016/0306-4522(87)90275-2

Groenewegen, Henk J., Wright, C. I., Beijer, A. V. J., & Voorn, P. (1999). Convergence and segregation of ventral striatal inputs and outputs. *Annals of the New York Academy of Sciences*, *877*, 49–63. https://doi.org/10.1111/j.1749-6632.1999.tb09260.x

Grothe, M., Heinsen, H., & Teipel, S. (2013). Longitudinal measures of cholinergic forebrain atrophy in the transition from healthy aging to Alzheimer's disease. *Neurobiology of Aging*. https://doi.org/10.1016/j.neurobiolaging.2012.10.018

Grothe, M. J., Heinsen, H., Amaro, E., Grinberg, L. T., & Teipel, S. J. (2016). Cognitive Correlates of Basal Forebrain Atrophy and Associated Cortical Hypometabolism in Mild Cognitive Impairment. *Cerebral Cortex*. https://doi.org/10.1093/cercor/bhv062

Gu, Z., Wortwein, G., Yu, J., & Perez-Polo, J. R. (2000). Model for aging in the basal forebrain cholinergic system. *Antioxidants and Redox Signaling*. https://doi.org/10.1089/15230860050192215

Gutiérrez, H., Gutiérrez, R., Ramírez-Trejo, L., Silva-Gandarias, R., Ormsby, C. E., Miranda, M. I., & Bermúdez-Rattoni, F. (1999). Redundant basal forebrain modulation in taste aversion memory formation. *Journal of Neuroscience*. https://doi.org/10.1523/jneurosci.19-17-07661.1999

H. Ferreira-Vieira, T., M. Guimaraes, I., R. Silva, F., & M. Ribeiro, F. (2016). Alzheimer's disease: Targeting the Cholinergic System. *Current Neuropharmacology*. https://doi.org/10.2174/1570159x13666150716165726

Hä, W., Stieler, J., Boerema, A. S., Wolf, J., Schmidt, U., Weißfuß, J., … Arendt, T. (n.d.). *Hibernation model of tau phosphorylation in hamsters: selective vulnerability of cholinergic basal forebrain neurons-implications for Alzheimer's disease*. https://doi.org/10.1111/j.1460-9568.2006.05250.x

Hagar, J. M., Macht, V. A., Wilson, S. P., & Fadel, J. R. (2017). Upregulation of orexin/hypocretin expression in aged rats: Effects on feeding latency and neurotransmission in the insular cortex. *Neuroscience*. https://doi.org/10.1016/j.neuroscience.2017.03.021

Hampel, H., Mesulam, M. M., Cuello, A. C., Farlow, M. R., Giacobini, E., Grossberg, G. T., … Khachaturian, Z. S. (2018). The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. *Brain*. https://doi.org/10.1093/brain/awy132

Hangya, B., Ranade, S. P., Lorenc, M., & Kepecs, A. (2015). Central Cholinergic Neurons Are Rapidly Recruited by Reinforcement Feedback. *Cell*, *162*(5), 1155–1168. https://doi.org/10.1016/j.cell.2015.07.057

Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's disease: Progress and problems on the road to therapeutics. *Science*. https://doi.org/10.1126/science.1072994

Haring, J. H., & Wang, R. Y. (1986). The identification of some sources of afferent input to the rat nucleus basalis magnocellularis by retrograde transport of horseradish peroxidase. *Brain Research*, *366*(1–2), 152–158. https://doi.org/10.1016/0006-8993(86)91290-4

Harry, G. J. (2013). Microglia during development and aging. *Pharmacology and Therapeutics*. https://doi.org/10.1016/j.pharmthera.2013.04.013 Associate Editor: C. Pope

Hasselmo, M. E., & Sarter, M. (2011). Modes and models of forebrain cholinergic neuromodulation of cognition. *Neuropsychopharmacology*, *36*(1), 52–73. https://doi.org/10.1038/npp.2010.104

Hauss-Wegrzyniak, B., Dobrzanski, P., Stoehr, J. D., & Wenk, G. L. (1998). Chronic neuroinflammation in rats reproduces components of the neurobiology of Alzheimer's disease. *Brain Research*, *780*(2), 294–303. https://doi.org/10.1016/S0006-8993(97)01215-8

Hebert, L. E., Beckett, L. A., Evans, D. A., Scherr, P. A., Albert, M. S., Pilgrim, D. M., … Harris Funkenstein, H. (1995). Age-Specific Incidence of Alzheimer's Disease in a Community Population. *JAMA: The Journal of the American Medical Association*. https://doi.org/10.1001/jama.1995.03520410048025

Hebert, L. E., Weuve, J., Scherr, P. A., & Evans, D. A. (2013). Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology*. https://doi.org/10.1212/WNL.0b013e31828726f5

Hedreen, J. C., Struble, R. G., Whitehouse, P. J., & Price, D. L. (1984). Topography of the magnocellular basal forebrain system in human brain. *Journal of Neuropathology and Experimental Neurology*. https://doi.org/10.1097/00005072-198401000-00001

Heimer, L., & Alheid, G. F. (1991). Piecing together the puzzle of basal forebrain anatomy. *Advances in Experimental Medicine and Biology*, Vol. 295, pp. 1–42. https://doi.org/10.1007/978-1-4757-0145-6_1

Henny, P., & Jones, B. E. (2008). Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons. *European Journal of Neuroscience*, *27*(3), 654–670. https://doi.org/10.1111/j.1460-9568.2008.06029.x

Heppner, F. L., Ransohoff, R. M., & Becher, B. (2015, June 26). Immune attack: The role

of inflammation in Alzheimer disease. *Nature Reviews Neuroscience*, Vol. 16, pp. 358–372. https://doi.org/10.1038/nrn3880

Herman, A. M., Ortiz-Guzman, J., Kochukov, M., Herman, I., Quast, K. B., Patel, J. M., … Arenkiel, B. R. (2016). A cholinergic basal forebrain feeding circuit modulates appetite suppression. *Nature*. https://doi.org/10.1038/nature19789

Herzog, E., Bellenchi, G. C., Gras, C., Bernard, V., Ravassard, P., Bedet, C., … El Mestikawy, S. (2001). The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(22). https://doi.org/10.1523/jneurosci.21-22-j0001.2001

Hickman, S. E., Allison, E. K., & El Khoury, J. (2008). Microglial dysfunction and defective β-amyloid clearance pathways in aging alzheimer's disease mice. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.0616-08.2008

Hickman, S., Izzy, S., Sen, P., Morsett, L., & El Khoury, J. (2018). Microglia in neurodegeneration. *Nature Neuroscience*. https://doi.org/10.1038/s41593-018-0242-x

Hopp, S. C., Lin, Y., Oakley, D., Roe, A. D., DeVos, S. L., Hanlon, D., & Hyman, B. T. (2018). The role of microglia in processing and spreading of bioactive tau seeds in Alzheimer's disease. *Journal of Neuroinflammation*. https://doi.org/10.1186/s12974-018-1309-z

Hopperton, K. E., Mohammad, D., Trépanier, M. O., Giuliano, V., & Bazinet, R. P. (2018). Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: A systematic review. *Molecular Psychiatry*. https://doi.org/10.1038/mp.2017.246

Hu, H., Gan, J., & Jonas, P. (2014, August 1). Fast-spiking, parvalbumin+ GABAergic interneurons: From cellular design to microcircuit function. *Science*, Vol. 345. https://doi.org/10.1126/science.1255263

Hu, R., Jin, S., He, X., Xu, F., & Hu, J. (2016). Whole-Brain Monosynaptic Afferent Inputs to Basal Forebrain Cholinergic System. *Frontiers in Neuroanatomy*, *10*(OCT), 98. https://doi.org/10.3389/fnana.2016.00098

Hur, E. E., & Zaborszky, L. (2005). Vglut2 afferents to the medial prefrontal and primary somatosensory cortices: A combined retrograde tracing in situ hybridization. *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.20444

Ikonomovic, M. D., Klunk, W. E., Abrahamson, E. E., Mathis, C. A., Price, J. C., Tsopelas, N. D., … DeKosky, S. T. (2008). Post-mortem correlates of in vivo PiB-PET amyloid imaging in a typical case of Alzheimer's disease. *Brain*. https://doi.org/10.1093/brain/awn016

Inglis, F. M., Day, J. C., & Fibiger, H. C. (1994). Enhanced acetylcholine release in hippocampus and cortex during the anticipation and consumption of a palatable meal. *Neuroscience*, *62*(4), 1049–1056. https://doi.org/10.1016/0306-4522(94)90342-5

Inutsuka, A., Inui, A., Tabuchi, S., Tsunematsu, T., Lazarus, M., & Yamanaka, A. (2014). Concurrent and robust regulation of feeding behaviors and metabolism by orexin neurons. *Neuropharmacology*. https://doi.org/10.1016/j.neuropharm.2014.06.015

Irle, E., & Markowitsch, H. J. (1987). Basal forebrain—lesioned monkeys are severely impaired in tasks of association and recognition memory. *Annals of Neurology*, *22*(6), 735–743. https://doi.org/10.1002/ana.410220610

Irving, E. A., Harrison, D. C., Babbs, A. J., Mayes, A. C., Campbell, C. A., Hunter, A. J., … Parsons, A. A. (2002). Increased cortical expression of the orexin-1 receptor following permanent middle cerebral artery occlusion in the rat. *Neuroscience Letters*, *324*(1), 53–56. https://doi.org/10.1016/S0304-3940(02)00176-3

Johnson, D. K., Wilkins, C. H., & Morris, J. C. (2006). Accelerated weight loss may precede diagnosis in Alzheimer disease. *Archives of Neurology*. https://doi.org/10.1001/archneur.63.9.1312

Kawas, C., Gray, S., Brookmeyer, R., Fozard, J., & Zonderman, A. (2000). Age-specific incidence rates of Alzheimer's disease: The Baltimore longitudinal study of aging. *Neurology*. https://doi.org/10.1212/WNL.54.11.2072

Kerbler, G. M., Fripp, J., Rowe, C. C., Villemagne, V. L., Salvado, O., Rose, S., & Coulson, E. J. (2015). Basal forebrain atrophy correlates with amyloid β burden in Alzheimer's disease. *NeuroImage: Clinical*, *7*, 105–113. https://doi.org/10.1016/j.nicl.2014.11.015

Kessler, B. A., Stanley, E. M., Frederick-Duus, D., & Fadel, J. (2011). Age-related loss of orexin/hypocretin neurons. *Neuroscience*. https://doi.org/10.1016/j.neuroscience.2011.01.031

Kilimann, I., Grothe, M., Heinsen, H., Alho, E. J. L., Grinberg, L., Amaro, E., … Teipel, S. J. (2014). Subregional basal forebrain atrophy in alzheimer's disease: A multicenter study. *Journal of Alzheimer's Disease*. https://doi.org/10.3233/JAD-132345

Kim, S. U., & De Vellis, J. (2005). Microglia in health and disease. *Journal of Neuroscience Research*. https://doi.org/10.1002/jnr.20562

Kim, T., Thankachan, S., McKenna, J. T., McNally, J. M., Yang, C., Choi, J. H., … McCarley, R. W. (2015). Cortically projecting basal forebrain parvalbumin neurons regulate cortical gamma band oscillations. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(11), 3535–3540. https://doi.org/10.1073/pnas.1413625112

Kiss, J., Patel, A. J., Baimbridge, K. G., & Freund, T. F. (1990). Topographical localization of neurons containing parvalbumin and choline acetyltransferase in the medial septum-diagonal band region of the rat. *Neuroscience*, *36*(1), 61–72. https://doi.org/10.1016/0306-4522(90)90351-4

Knox, D. (2016, September 1). The role of basal forebrain cholinergic neurons in fear and extinction memory. *Neurobiology of Learning and Memory*, Vol. 133, pp. 39–52. https://doi.org/10.1016/j.nlm.2016.06.001

Knox, D., & Keller, S. M. (2016). Cholinergic neuronal lesions in the medial septum and vertical limb of the diagonal bands of Broca induce contextual fear memory generalization and impair acquisition of fear extinction. *Hippocampus*, *26*(6), 718–726. https://doi.org/10.1002/hipo.22553

Koellhoffer, E. C., McCullough, L. D., & Ritzel, R. M. (2017). Old maids: Aging and its impact on microglia function. *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms18040769

Kondo, H., & Zaborszky, L. (2016). Topographic organization of the basal forebrain projections to the perirhinal, postrhinal, and entorhinal cortex in rats. *Journal of Comparative Neurology*, *524*(12). https://doi.org/10.1002/cne.23967

Kukkonen, J. P., Holmqvist, T., Ammoun, S., & Åkerman, K. E. O. (2002, December 1). Functions of the orexinergic/hypocretinergic system. *American Journal of Physiology - Cell Physiology*, Vol. 283, pp. 1567–1591. https://doi.org/10.1152/ajpcell.00055.2002

Lahiri, D. K., Farlow, M. R., Greig, N. H., & Sambamurti, K. (2002). Current drug targets for Alzheimer's disease treatment. *Drug Development Research*. https://doi.org/10.1002/ddr.10081

Lammers, F., Borchers, F., Feinkohl, I., Hendrikse, J., Kant, I. M. J., Kozma, P., … Winterer, G. (2018). Basal forebrain cholinergic system volume is associated with general cognitive ability in the elderly. *Neuropsychologia*. https://doi.org/10.1016/j.neuropsychologia.2018.08.005

Lamour, Y., Dutar, P., Rascol, O., & Jobert, A. (1986). Basal forebrain neurons projecting to the rat frontoparietal cortex: Electrophysiological and pharmacological properties. *Brain Research*, *362*(1), 122–131. https://doi.org/10.1016/0006-8993(86)91405-8

Liguori, C. (2017). Orexin and Alzheimer's disease. In *Current Topics in Behavioral Neurosciences* (Vol. 33, pp. 305–322). https://doi.org/10.1007/7854_2016_50

Lin, S.-C., Brown, R. E., Hussain Shuler, M. G., Petersen, C. C. H., & Kepecs, A. (2015). Optogenetic Dissection of the Basal Forebrain Neuromodulatory Control of Cortical Activation, Plasticity, and Cognition. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.2590-15.2015

Lin, S. C., Brown, R. E., Shuler, M. G. H., Petersen, C. C. H., & Kepecs, A. (2015). Optogenetic dissection of the basal forebrain neuromodulatory control of cortical activation, plasticity, and cognition. *Journal of Neuroscience*, *35*(41), 13896–13903. https://doi.org/10.1523/JNEUROSCI.2590-15.2015

Lin, S. C., Gervasoni, D., & Nicolelis, M. A. L. (2006). Fast modulation of prefrontal cortex activity by basal forebrain noncholinergic neuronal ensembles. *Journal of Neurophysiology*, *96*(6), 3209–3219. https://doi.org/10.1152/jn.00524.2006

Lin, S. C., & Nicolelis, M. A. L. (2008). Neuronal Ensemble Bursting in the Basal Forebrain Encodes Salience Irrespective of Valence. *Neuron*, *59*(1), 138–149. https://doi.org/10.1016/j.neuron.2008.04.031

Lipman, R. D., Chrisp, C. E., Hazzard, D. W. G., & Bronson, R. T. (1996). Pathologic characterization of Brown Norway, Brown Norway x Fischer 344, and Fischer 344 x Brown Norway rats with relation to age. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*. https://doi.org/10.1093/gerona/51A.1.B54

Lista, S., O'Bryant, S. E., Blennow, K., Dubois, B., Hugon, J., Zetterberg, H., & Hampel, H. (2015). Biomarkers in Sporadic and Familial Alzheimer's Disease. *Journal of Alzheimer's Disease*. https://doi.org/10.3233/JAD-143006

Loewi, O. (1921). Über humorale übertragbarkeit der Herznervenwirkung - I. Mitteilung. *Pflügers Archiv Für Die Gesamte Physiologie Des Menschen Und Der Tiere*. https://doi.org/10.1007/BF01738910

Mandrekar-Colucci, S., & Landreth, G. E. (2012). Microglia and Inflammation in Alzheimers Disease. *CNS & Neurological Disorders - Drug Targets*. https://doi.org/10.2174/187152710791012071

Mangialasche, F., Solomon, A., Winblad, B., Mecocci, P., & Kivipelto, M. (2010). Alzheimer's disease: clinical trials and drug development. *The Lancet Neurology*. https://doi.org/10.1016/S1474-4422(10)70119-8

Manich, G., Recasens, M., Valente, T., Almolda, B., González, B., & Castellano, B. (2019). Role of the CD200-CD200R Axis During Homeostasis and Neuroinflammation. *Neuroscience*, *405*, 118–136. https://doi.org/10.1016/j.neuroscience.2018.10.030

Manns, I. D., Mainville, L., & Jones, B. E. (2001). Evidence for glutamate, in addition to acetylcholine and GABA, neurotransmitter synthesis in basal forebrain neurons projecting to the entorhinal cortex. *Neuroscience*, *107*(2), 249–263. https://doi.org/10.1016/S0306-4522(01)00302-5

Marcus, J. N., Aschkenasi, C. J., Lee, C. E., Chemelli, R. M., Saper, C. B., Yanagisawa, M., & Elmquist, J. K. (2001). Differential expression of Orexin receptors 1 and 2 in the rat brain. *Journal of Comparative Neurology*, *435*(1), 6–25. https://doi.org/10.1002/cne.1190

Marschallinger, J., Iram, T., Zardeneta, M., Lee, S. E., Lehallier, B., Haney, M. S., … Wyss-Coray, T. (2020). Lipid-droplet-accumulating microglia represent a dysfunctional and proinflammatory state in the aging brain. *Nature Neuroscience*. https://doi.org/10.1038/s41593-019-0566-1

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports*, *6*. https://doi.org/10.12703/P6-13

Martone, A. M., Onder, G., Vetrano, D. L., Ortolani, E., Tosato, M., Marzetti, E., & Landi, F. (2013). Anorexia of aging: A modifiable risk factor for frailty. *Nutrients*. https://doi.org/10.3390/nu5104126

Massoud, F., & Gauthier, S. (2010). Update on the Pharmacological Treatment of Alzheimers Disease. *Current Neuropharmacology*. https://doi.org/10.2174/157015910790909520

Matsumura, T., Nakayama, M., Nomura, A., Naito, A., Kamahara, K., Kadono, K., … Sekizawa, K. (2002). Age-related changes in plasma orexin-A concentrations. *Experimental Gerontology*. https://doi.org/10.1016/S0531-5565(02)00092-X

Mawuenyega, K. G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J. C., … Bateman, R. J. (2010). Decreased clearance of CNS β-amyloid in Alzheimer's disease. *Science*. https://doi.org/10.1126/science.1197623

McCormick, D. A. (1993). Actions of acetylcholine in the cerebral cortex and thalamus and implications for function. *Progress in Brain Research*, *98*(C), 303–308. https://doi.org/10.1016/S0079-6123(08)62412-7

McGaughy, J., Kaiser, T., & Sarter, M. (1996). Behavioral vigilance following infusions of 192 IgG-saporin into the basal forebrain: Selectivity of the behavioral impairment and relation to cortical AChE-positive fiber density. *Behavioral Neuroscience*. https://doi.org/10.1037/0735-7044.110.2.247

McGaughy, J., & Sarter, M. (1998). Sustained attention performance in rats with intracortical infusions of 192 IgG-saporin-induced cortical cholinergic deafferentation: Effects of physostigmine and FG 7142. *Behavioral Neuroscience*. https://doi.org/10.1037/0735-7044.112.6.1519

McKinney, M., Coyle, J. T., & Hedreen, J. C. (1983). Topographic analysis of the innervation of the rat neocortex and hippocampus by the basal forebrain cholinergic system. *Journal of Comparative Neurology*. https://doi.org/10.1002/cne.902170109

Mesulam, M.-M., & Mufson, E. J. (1981). NEURAL INPUTS INTO THE NUCLEUS BASALIS OF THE SUBSTANTIA INNOMINATA (Ch4) IN THE RHESUS MONKEY. In *Brain* (Vol. 107).

Mesulam, M. -Marsel, Mufson, E. J., Levey, A. I., & Wainer, B. H. (1983). Cholinergic innervation of cortex by the basal forebrain: Cytochemistry and cortical connections of the septal area, diagonal band nuclei, nucleus basalis (Substantia innominata), and hypothalamus in the rhesus monkey. *Journal of Comparative Neurology*, *214*(2), 170–197. https://doi.org/10.1002/cne.902140206

Mesulam, M. M. (1976). A horseradish peroxidase method for the identification of the efferents of acetyl cholinesterase containing neurons. *Journal of Histochemistry and Cytochemistry*. https://doi.org/10.1177/24.12.826585

Mesulam, M. M. (1996). The systems-level organization of cholinergic innervation in the human cerebral cortex and its alterations in Alzheimer's disease. *Progress in Brain Research*, *109*, 285–297. https://doi.org/10.1016/s0079-6123(08)62112-3

Mesulam, M. M., Guillozet, A., Shaw, P., Levey, A., Duysen, E. G., & Lockridge, O. (2002). Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. *Neuroscience*. https://doi.org/10.1016/S0306-4522(01)00613-3

Mesulam, M. Marsel. (2004). The cholinergic innervation of the human cerebral cortex. *Progress in Brain Research*. https://doi.org/10.1016/S0079-6123(03)45004-8

Mesulam, M. Marsel, & Mufson, E. J. (1984). Neural inputs into the nucleus basalis of the substantia innominata (ch4) in the rhesus monkey. *Brain*, *107*(1), 253–274. https://doi.org/10.1093/brain/107.1.253

Mihara, Y., Dohi, K., Yofu, S., Nakamachi, T., Ohtaki, H., Shioda, S., & Aruga, T. (2011). Expression and localization of the orexin-1 receptor (OX1R) after traumatic brain injury in mice. *Journal of Molecular Neuroscience*, *43*(2), 162–168. https://doi.org/10.1007/s12031-010-9438-6

Minter, M. R., Taylor, J. M., & Crack, P. J. (2016). The contribution of neuroinflammation to amyloid toxicity in Alzheimer's disease. *Journal of Neurochemistry*. https://doi.org/10.1111/jnc.13411

Misra, S., & Medhi, B. (2013). Drug development status for Alzheimer's disease: Present scenario. *Neurological Sciences*. https://doi.org/10.1007/s10072-013-1316-x

Monosov, I. E., Leopold, D. A., & Hikosaka, O. (2015). Neurons in the primate medial basal forebrain signal combined information about reward uncertainty, value, and punishment anticipation. *Journal of Neuroscience*, *35*(19), 7443–7459. https://doi.org/10.1523/JNEUROSCI.0051-15.2015

Moore, Holly, Sarter, M., & Bruno, J. P. (1992). Age-dependent modulation of in vivo cortical acetylcholine release by benzodiazepine receptor ligands. *Brain Research*, *596*(1–2), 17–29. https://doi.org/10.1016/0006-8993(92)91527-L

Moore, Hooly, Sarter, M., & Bruno, J. P. (1995). Bidirectional modulation of cortical acetylcholine efflux by infusion of benzodiazepine receptor ligands into the basal forebrain. *Neuroscience Letters*, *189*(1), 31–34. https://doi.org/10.1016/0304-3940(95)11444-2

Morales, M., & Margolis, E. B. (2017, February 1). Ventral tegmental area: Cellular heterogeneity, connectivity and behaviour. *Nature Reviews Neuroscience*, Vol. 18, pp. 73–85. https://doi.org/10.1038/nrn.2016.165

Morgan, A. J., Harrod, S. B., Lacy, R. T., Stanley, E. M., & Fadel, J. R. (2013). Intravenous prenatal nicotine exposure increases orexin expression in the lateral hypothalamus and orexin innervation of the ventral tegmental area in adult male rats. *Drug and Alcohol Dependence*, *132*(3), 562–570. https://doi.org/10.1016/j.drugalcdep.2013.04.003

Morley, J. E. (2001). Decreased food intake with aging. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *56*(SPEC. ISS. 2), 81–88. https://doi.org/10.1093/gerona/56.suppl_2.81

Morley, John E., & Thomas, D. R. (1999, June). Anorexia and aging: Pathophysiology. *Nutrition*, Vol. 15, pp. 499–503. https://doi.org/10.1016/S0899-9007(99)00057-X

Morrison, S. D., & Mayer, J. (1957). Adipsia and aphagia in rats after lateral subthalamic lesions. *The American Journal of Physiology*, *191*(2), 248–254. https://doi.org/10.1152/ajplegacy.1957.191.2.248

Mosher, K. I., & Wyss-Coray, T. (2014). Microglial dysfunction in brain aging and Alzheimer's disease. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2014.01.008

Muir, J. L., Dunnett, S. B., Robbins, T. W., & Everitt, B. J. (1992). Attentional functions of the forebrain cholinergic systems: effects of intraventricular hemicholinium, physostigmine, basal forebrain lesions and intracortical grafts on a multiple-choice serial reaction time task. *Experimental Brain Research*. https://doi.org/10.1007/BF00229886

Nagai, M., Kishi, K., & Kato, S. (2007, September). Insular cortex and neuropsychiatric disorders: A review of recent literature. *European Psychiatry*, Vol. 22, pp. 387–394. https://doi.org/10.1016/j.eurpsy.2007.02.006

Nagai, Michiaki, Scheper, V., Lenarz, T., & Förster, C. Y. (2021, April 1). The insular cortex as a vestibular area in relation to autonomic function. *Clinical Autonomic Research*, Vol. 31, pp. 179–185. https://doi.org/10.1007/s10286-020-00744-8

Nelson, C. L., Sarter, M., & Bruno, J. P. (2005). Prefrontal cortical modulation of acetylcholine release in posterior parietal cortex. *Neuroscience*, *132*(2), 347–359. https://doi.org/10.1016/j.neuroscience.2004.12.007

Nilsson, L., Nordberg, A., Hardy, J., Wester, P., & Winblad, B. (1986). Physostigmine restores3H-acetylcholine efflux from Alzheimer brain slices to normal level. *Journal of Neural Transmission*. https://doi.org/10.1007/BF01243353

Nyakas, C., Granic, I., Halmy, L. G., Banerjee, P., & Luiten, P. G. (2010). The basal forebrain cholinergic system in aging and dementia. Rescuing cholinergic neurons from neurotoxic amyloid-Î²42 with memantine. *Behavioural Brain Research*, *221*, 594–603. https://doi.org/10.1016/j.bbr.2010.05.033

Ogura, K. I., Ogawa, M., & Yoshida, M. (1994a). Effects of ageing on microglia in the normal rat brain: Immunohistochemical observations. *NeuroReport*, *5*(10), 1224–1226. https://doi.org/10.1097/00001756-199406020-00016

Ogura, K. I., Ogawa, M., & Yoshida, M. (1994b). Effects of ageing on microglia in the normal rat brain: Immunohistochemical observations. *NeuroReport*. https://doi.org/10.1097/00001756-199406020-00016

Overmyer, M., Helisalmi, S., Soininen, H., Laakso, M., Riekkinen, P., & Alafuzoff, I. (1999). Reactive microglia in aging and dementia: An immunohistochemical study of postmortem human brain tissue. *Acta Neuropathologica*, *97*(4), 383–392. https://doi.org/10.1007/s004010051002

Ownby, R. L. (2010). Neuroinflammation and cognitive aging. *Current Psychiatry Reports*. https://doi.org/10.1007/s11920-009-0082-1

Paasila, P. J., Davies, D. S., Kril, J. J., Goldsbury, C., & Sutherland, G. T. (2019). The relationship between the morphological subtypes of microglia and Alzheimer's disease neuropathology. *Brain Pathology*, *29*(6), 726–740. https://doi.org/10.1111/BPA.12717

Packer, A. M., & Yuste, R. (2011). Dense, unspecific connectivity of neocortical parvalbumin-positive interneurons: A canonical microcircuit for inhibition? *Journal of Neuroscience*, *31*(37), 13260–13271. https://doi.org/10.1523/JNEUROSCI.3131-11.2011

Pais, M., Martinez, L., Ribeiro, O., Loureiro, J., Fernandez, R., Valiengo, L., … Forlenza, O. V. (2020). Early diagnosis and treatment of Alzheimer's disease: New definitions and challenges. *Brazilian Journal of Psychiatry*. https://doi.org/10.1590/1516-4446-2019-0735

Pappas, B. A., Bayley, P. J., Bui, B. K., Hansen, L. A., & Thal, L. J. (2000). Choline acetyltransferase activity and cognitive domain scores of Alzheimer's patients. *Neurobiology of Aging*. https://doi.org/10.1016/S0197-4580(00)00090-7

Pavlov, V. A., & Tracey, K. J. (2005). The cholinergic anti-inflammatory pathway. *Brain, Behavior, and Immunity*, Vol. 19, pp. 493–499. https://doi.org/10.1016/j.bbi.2005.03.015

Paxinos, G., & Watson, C. (1998). *The Rat Brain in Stereotaxic Coordinates - George Paxinos, Charles Watson - Google Books* (4th ed.). Science.

Pepeu, G., Giovannini, M. G., & Bracco, L. (2013). Effect of cholinesterase inhibitors on attention. *Chemico-Biological Interactions*, *203*(1), 361–364. https://doi.org/10.1016/j.cbi.2012.09.016

Perlmutter, L. S., Barron, E., & Chui, H. C. (1990). Morphologic association between microglia and senile plaque amyloid in Alzheimer's disease. *Neuroscience Letters*. https://doi.org/10.1016/0304-3940(90)90748-X

Perry, E. K., Curtis, M., Dick, D. J., Candy, J. M., Atack, J. R., Bloxham, C. A., … Tomlinson, B. E. (1985). Cholinergic correlates of cognitive impairment in Parkinson's disease: Comparisons with Alzheimer's disease. *Journal of Neurology Neurosurgery and Psychiatry*. https://doi.org/10.1136/jnnp.48.5.413

Perry, Elaine K., Gibson, P. H., Blessed, G., Perry, R. H., & Tomlinson, B. E. (1977). Neurotransmitter enzyme abnormalities in senile dementia. Choline acetyltransferase and glutamic acid decarboxylase activities in necropsy brain tissue. *Journal of the Neurological Sciences*. https://doi.org/10.1016/0022-510X(77)90073-9

Peyron, C., Tighe, D. K., Van Den Pol, A. N., De Lecea, L., Heller, H. C., Sutcliffe, J. G., & Kilduff, T. S. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. *Journal of Neuroscience*, *18*(23), 9996–10015. https://doi.org/10.1523/jneurosci.18-23-09996.1998

Porkka-Heiskanen, T., Alanko, L., Kalinchuk, A., Heiskanen, S., & Stenberg, D. (2004). The effect of age on prepro-orexin gene expression and contents of orexin A and B in the rat brain. *Neurobiology of Aging*. https://doi.org/10.1016/S0197-4580(03)00043-5

Pratt, W. E., Will, M. J., Kelley, A. E., & Baldo, B. A. (n.d.). *KelleyetalPhys&Beh2005 Stress and Drug Reward View project Hedonic and Homeostatic Feeding Regulation View project Corticostriatal-hypothalamic circuitry and food motivation: Integration of energy, action and reward*. https://doi.org/10.1016/j.physbeh.2005.08.066

Qi, J., Zhang, S., Wang, H. L., Barker, D. J., Miranda-Barrientos, J., & Morales, M. (2016). VTA glutamatergic inputs to nucleus accumbens drive aversion by acting on GABAergic interneurons. *Nature Neuroscience*, *19*(5), 725–733. https://doi.org/10.1038/nn.4281

Quan, N., Sundar, S. K., & Weiss, J. M. (1994). Induction of interleukin-1 in various brain regions after peripheral and central injections of lipopolysaccharide. *Journal of Neuroimmunology*. https://doi.org/10.1016/0165-5728(94)90188-0

Ransohoff, R. M. (2016). How neuroinflammation contributes to neurodegeneration. *Science*. https://doi.org/10.1126/science.aag2590

Raskin, J., Cummings, J., Hardy, J., Schuh, K., & Dean, R. (2015). Neurobiology of Alzheimer's Disease: Integrated Molecular, Physiological, Anatomical, Biomarker, and Cognitive Dimensions. *Current Alzheimer Research*. https://doi.org/10.2174/1567205012666150701103107

Rea, I. M., Gibson, D. S., McGilligan, V., McNerlan, S. E., Denis Alexander, H., & Ross, O. A. (2018, April 9). Age and age-related diseases: Role of inflammation triggers and cytokines. *Frontiers in Immunology*, Vol. 9, p. 1. https://doi.org/10.3389/fimmu.2018.00586

Rebec, G. V., Grabner, C. P., Johnson, M., Pierce, R. C., & Bardo, M. T. (1996). Transient increases in catecholaminergic activity in medial prefrontal cortex and nucleus accumbens shell during novelty. *Neuroscience*, *76*(3), 707–714. https://doi.org/10.1016/S0306-4522(96)00382-X

Release, T. E. (2020). *Longitudinal basal forebrain degeneration interacts with TREM2 / C3 biomarkers of inflammation in pre-symptomatic Alzheimer ' s disease*. (December 2019).

Riedel, W. J., & Jolles, J. (1996). Cognition enhancers in age-related cognitive decline. *Drugs and Aging*. https://doi.org/10.2165/00002512-199608040-00003

Roberson, E. D., & Mucke, L. (2006). 100 Years and counting: Prospects for defeating Alzheimer's disease. *Science*. https://doi.org/10.1126/science.1132813

Roberts, R., & Knopman, D. S. (2013). Classification and epidemiology of MCI. *Clinics in Geriatric Medicine*. https://doi.org/10.1016/j.cger.2013.07.003

Rodríguez-García, G., María, X., & Miranda, I. (2016). *Opposing Roles of Cholinergic and GABAergic Activity in the Insular Cortex and Nucleus Basalis Magnocellularis during Novel Recognition and Familiar Taste Memory Retrieval*. https://doi.org/10.1523/JNEUROSCI.2340-15.2016

Rosin, D. L., Weston, M. C., Sevigny, C. P., Stornetta, R. L., & Guyenet, P. G. (2003). Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. *Journal of Comparative Neurology*, *465*(4), 593–603. https://doi.org/10.1002/cne.10860

Rossini, P. M., Rossi, S., Babiloni, C., & Polich, J. (2007). Clinical neurophysiology of aging brain: From normal aging to neurodegeneration. *Progress in Neurobiology*. https://doi.org/10.1016/j.pneurobio.2007.07.010

Russchen, F. T., Amaral, D. G., & Price, J. L. (1985). The afferent connections of the substantia innominata in the monkey,Macaca fascicularis. *The Journal of Comparative Neurology*, *242*(1), 1–27. https://doi.org/10.1002/cne.902420102

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R. M., Tanaka, H., … Yanagisawa, M. (1998). Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell*, *92*(4), 573–585. https://doi.org/10.1016/S0092-8674(00)80949-6

Sakurai, T., & Mieda, M. (2011). Connectomics of orexin-producing neurons: Interface of systems of emotion, energy homeostasis and arousal. *Trends in Pharmacological Sciences*. https://doi.org/10.1016/j.tips.2011.03.007

Salamone, J. D., & Correa, M. (2012, November 8). The Mysterious Motivational Functions of Mesolimbic Dopamine. *Neuron*, Vol. 76, pp. 470–485. https://doi.org/10.1016/j.neuron.2012.10.021

Salgado, S., & Kaplitt, M. G. (2015, March 24). The nucleus accumbens: A comprehensive review. *Stereotactic and Functional Neurosurgery*, Vol. 93, pp. 75–93. https://doi.org/10.1159/000368279

Sarter, M., & Bruno, J. P. (2002). The neglected constituent of the basal forebrain corticopetal projection system: GABAergic projections. *European Journal of Neuroscience*. https://doi.org/10.1046/j.1460-9568.2002.02004.x

Sarter, M., Bruno, J. P., & Turchi, J. (n.d.). *Basal Forebrain Afferent Projections Modulating Cortical Acetylcholine, Attention, and Implications for Neuropsychiatric Disorders*.

Sarter, M., Bruno, J. P., & Turchi, J. (1999). Basal forebrain afferent projections modulating cortical acetylcholine, attention, and implications for neuropsychiatric disorders. *Annals of the New York Academy of Sciences*, *877*, 368–382. https://doi.org/10.1111/j.1749-6632.1999.tb09277.x

Sarter, M. F., & Bruno, J. P. (1994). Cognitive functions of cortical ACh: lessons from studies on trans-synaptic modulation of activated efflux. *Trends in Neurosciences*, *17*(6), 217–221. https://doi.org/10.1016/0166-2236(94)90001-9

Sarter, M., Givens, B., & Bruno, J. P. (2001). The cognitive neuroscience of sustained attention: Where top-down meets bottom-up. *Brain Research Reviews*. https://doi.org/10.1016/S0165-0173(01)00044-3

Scammel, T. E., & Winrow, C. J. (2011). Orexin receptors: Pharmacology and therapeutic opportunities. *Annual Review of Pharmacology and Toxicology*, *51*, 243–266. https://doi.org/10.1146/annurev-pharmtox-010510-100528

Schlachetzki, J., & Hull, M. (2009). Microglial Activation in Alzheimers Disease. *Current Alzheimer Research*. https://doi.org/10.2174/156720509790147179

Schliebs, R., & Arendt, T. (2011). The cholinergic system in aging and neuronal degeneration. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2010.11.058

Schmitz, T. W., Mur, M., Aghourian, M., Bedard, M. A., & Spreng, R. N. (2018). Longitudinal Alzheimer's Degeneration Reflects the Spatial Topography of Cholinergic Basal Forebrain Projections. *Cell Reports*. https://doi.org/10.1016/j.celrep.2018.06.001

Schmitz, T. W., & Nathan Spreng, R. (2016). Basal forebrain degeneration precedes and predicts the cortical spread of Alzheimer's pathology. *Nature Communications*. https://doi.org/10.1038/ncomms13249

Schmitz, T. W., Soreq, H., Judes Poirier, X., & Nathan Spreng, X. R. (2020). Longitudinal basal forebrain degeneration interacts with TREM2/C3 biomarkers of inflammation in presymptomatic Alzheimer's disease. *Journal of Neuroscience*, *40*(9), 1931–1942. https://doi.org/10.1523/JNEUROSCI.1184-19.2019

Sesack, S. R., Deutch, A. Y., Roth, R. H., & Bunney, B. S. (1989). Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: An anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *Journal of Comparative Neurology*, *290*(2), 213–242. https://doi.org/10.1002/cne.902900205

Sierra, A., Gottfried-Blackmore, A. C., Mceven, B. S., & Bulloch, K. (2007). Microglia derived from aging mice exhibit an altered inflammatory profile. *GLIA*, *55*(4), 412–424. https://doi.org/10.1002/glia.20468

Smart, D., & Jerman, J. C. (2002). The physiology and pharmacology of the orexins. *Pharmacology and Therapeutics*, Vol. 94, pp. 51–61. https://doi.org/10.1016/S0163-7258(02)00171-7

Sokołowska, P., Urbańska, A., Namiecińska, M., Biegańska, K., & Zawilska, J. B. (2012). Orexins promote survival of rat cortical neurons. *Neuroscience Letters*, *506*(2), 303–306. https://doi.org/10.1016/j.neulet.2011.11.028

Solito, E., & Sastre, M. (2012). Microglia function in Alzheimer's disease. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2012.00014

Sparkman, N. L., & Johnson, R. W. (2008). Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. *NeuroImmunoModulation*. https://doi.org/10.1159/000156474

Spittau, B. (2017). Aging microglia-phenotypes, functions and implications for age-related neurodegenerative diseases. *Frontiers in Aging Neuroscience*, *9*(JUN). https://doi.org/10.3389/fnagi.2017.00194

Stamatakis, A. M., Jennings, J. H., Ung, R. L., Blair, G. A., Weinberg, R. J., Neve, R. L., … Stuber, G. D. (2013). A Unique Population of Ventral Tegmental Area Neurons Inhibits the Lateral Habenula to Promote Reward. *Neuron*, *80*(4), 1039–1053. https://doi.org/10.1016/j.neuron.2013.08.023

Streit, W. J., Braak, H., Xue, Q. S., & Bechmann, I. (2009). Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. *Acta Neuropathologica*, *118*(4), 475–485. https://doi.org/10.1007/s00401-009-0556-6

Streit, W. J., Mrak, R. E., & Griffin, W. S. T. (2004). Microglia and neuroinflammation: A pathological perspective. *Journal of Neuroinflammation*. https://doi.org/10.1186/1742-2094-1-14

Stuber, G. D., & Wise, R. A. (2016, January 27). Lateral hypothalamic circuits for feeding and reward. *Nature Neuroscience*, Vol. 19, pp. 198–205. https://doi.org/10.1038/nn.4220

Takeda, S., Commins, C., DeVos, S. L., Nobuhara, C. K., Wegmann, S., Roe, A. D., … Hyman, B. T. (2016). Seed-competent high-molecular-weight tau species accumulates in the cerebrospinal fluid of Alzheimer's disease mouse model and human patients. *Annals of Neurology*. https://doi.org/10.1002/ana.24716

Takeda, S., Wegmann, S., Cho, H., Devos, S. L., Commins, C., Roe, A. D., … Hyman, B. T. (2015). Neuronal uptake and propagation of a rare phosphorylated high-molecular-weight tau derived from Alzheimer's disease brain. *Nature Communications*. https://doi.org/10.1038/ncomms9490

Tarkowski, E., Andreasen, N., Tarkowski, A., & Blennow, K. (2003). Intrathecal inflammation precedes development of Alzheimer's disease. *Journal of Neurology, Neurosurgery and Psychiatry*. https://doi.org/10.1136/jnnp.74.9.1200

Tashakori-Sabzevar, F., & Ward, R. D. (2018). Basal Forebrain Mediates Motivational Recruitment of Attention by Reward-Associated Cues. *Frontiers in Neuroscience*, *12*, 786. https://doi.org/10.3389/fnins.2018.00786

Teipel, S. J., Cavedo, E., Hampel, H., Grothe, M. J., Aguilar, L. F., Babiloni, C., … Cummings, J. L. (2018). Basal forebrain volume, but not hippocampal volume, is a predictor of global cognitive decline in patients with alzheimer's disease treated with cholinesterase inhibitors. *Frontiers in Neurology*. https://doi.org/10.3389/fneur.2018.00642

Teipel, S. J., Flatz, W. H., Heinsen, H., Bokde, A. L. W., Schoenberg, S. O., Stöckel, S., … Hampel, H. (2005). Measurement of basal forebrain atrophy in Alzheimer's disease using MRI. *Brain*. https://doi.org/10.1093/brain/awh589

Teipel, S. J., Meindl, T., Grinberg, L., Grothe, M., Cantero, J. L., Reiser, M. F., … Hampel, H. (2011). The cholinergic system in mild cognitive impairment and Alzheimer's disease: An in Vivo MRI and DTI study. *Human Brain Mapping*. https://doi.org/10.1002/hbm.21111

Tejera, D., Mercan, D., Sanchez-Caro, J. M., Hanan, M., Greenberg, D., Soreq, H., … Heneka, M. T. (2019). Systemic inflammation impairs microglial Aβ clearance through NLRP 3 inflammasome . *The EMBO Journal*. https://doi.org/10.15252/embj.2018101064

Terao, A., Apte-Deshpande, A., Morairty, S., Freund, Y. R., & Kilduff, T. S. (2002). Age-related decline in hypocretin (orexin) receptor 2 messenger RNA levels in the mouse brain. *Neuroscience Letters*, *332*(3), 190–194. https://doi.org/10.1016/S0304-3940(02)00953-9

Thompson, M. D., Xhaard, H., Sakurai, T., Rainero, I., & Kukkonen, J. P. (2014, May 6). OX1 and OX2 orexin/hypocretin receptor pharmacogenetics. *Frontiers in Neuroscience*, Vol. 8, p. 57. https://doi.org/10.3389/fnins.2014.00057

Torres, E. M., Perry, T. A., Blokland, A., Wilkinson, L. S., Wiley, R. G., Lappi, D. A., & Dunnett, S. B. (1994). Behavioural, histochemical and biochemical consequences of selective immunolesions in discrete regions of the basal forebrain cholinergic system. *Neuroscience*. https://doi.org/10.1016/0306-4522(94)90010-8

Tuppo, E. E., & Arias, H. R. (2005). The role of inflammation in Alzheimer's disease. *International Journal of Biochemistry and Cell Biology*. https://doi.org/10.1016/j.biocel.2004.07.009

Um, Y. H., & Lim, H. K. (2020, July 1). Orexin and alzheimer's disease: A new perspective. *Psychiatry Investigation*, Vol. 17, pp. 616–626. https://doi.org/10.30773/pi.2020.0136

Unal, C. T., Pare, D., & Zaborszky, L. (2015). Impact of Basal Forebrain Cholinergic Inputs on Basolateral Amygdala Neurons. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.2706-14.2015

Van Der Staay, F. J., & Blokland, A. (1996). Behavioral differences between outbred Wistar, inbred Fischer 344, Brown Norway, and hybrid Fischer 344 × Brown Norway rats. *Physiology and Behavior*. https://doi.org/10.1016/0031-9384(95)02274-0

Van Zessen, R., Phillips, J. L., Budygin, E. A., & Stuber, G. D. (2012). Activation of VTA GABA Neurons Disrupts Reward Consumption. *Neuron*, *73*(6), 1184–1194. https://doi.org/10.1016/j.neuron.2012.02.016

VanGuilder, H. D., Bixler, G. V., Brucklacher, R. M., Farley, J. A., Yan, H., Warrington, J. P., … Freeman, W. M. (2011). Concurrent hippocampal induction of MHC II pathway components and glial activation with advanced aging is not correlated with cognitive impairment. *Journal of Neuroinflammation*, *8*. https://doi.org/10.1186/1742-2094-8-138

Vertes, R. P. (2003). *Differential Projections of the Infralimbic and Prelimbic Cortex in the Rat*. https://doi.org/10.1002/syn.10279

Viggiano, A., Chieffi, S., Tafuri, D., Messina, G., Monda, M., & De Luca, B. (2014). Laterality of a second player position affects lateral deviation of basketball shooting. *Journal of Sports Sciences*, *32*(1), 46–52. https://doi.org/10.1080/02640414.2013.805236

Villano, I., Messina, A., Valenzano, A., Moscatelli, F., Esposito, T., Monda, V., … Messina, G. (2017). Basal forebrain cholinergic system and orexin neurons: Effects on attention. *Frontiers in Behavioral Neuroscience*, Vol. 11. https://doi.org/10.3389/fnbeh.2017.00010

Visvanathan, R. (2015). Anorexia of Aging. *Clinics in Geriatric Medicine*. https://doi.org/10.1016/j.cger.2015.04.012

Voytko, M. Lou. (1996). Cognitive functions of the basal forebrain cholinergic system in monkeys: Memory or attention? *Behavioural Brain Research*, Vol. 75, pp. 13–25. https://doi.org/10.1016/0166-4328(95)00143-3

Voytko, M. Lou, Olton, D. S., Richardson, R. T., Gorman, L. K., Tobin, J. R., & Price, D. L. (1994). Basal forebrain lesions in monkeys disrupt attention but not learning and memory. *Journal of Neuroscience*, *14*(1), 167–186. https://doi.org/10.1523/jneurosci.14-01-00167.1994

Walker, D. G., Dalsing-Hernandez, J. E., Campbell, N. A., & Lue, L. F. (2009). Decreased expression of CD200 and CD200 receptor in Alzheimer's disease: A potential mechanism leading to chronic inflammation. *Experimental Neurology*. https://doi.org/10.1016/j.expneurol.2008.09.003

Walker, D., & Lue, L.-F. (2007). Anti-inflammatory and Immune Therapy for Alzheimers Disease: Current Status and Future Directions. *Current Neuropharmacology*. https://doi.org/10.2174/157015907782793667

Wang, H., Yu, M., Ochani, M., Amelia, C. A., Tanovic, M., Susarla, S., … Tracey, K. J. (2003). Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. *Nature*, *421*(6921), 384–388. https://doi.org/10.1038/nature01339

Wang, J., Li, J., Yang, Q., Xie, Y. K., Wen, Y. L., Xu, Z. Z., … Xu, H. (2021). Basal forebrain mediates prosocial behavior via disinhibition of midbrain dopamine neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(7). https://doi.org/10.1073/pnas.2019295118

Ward, A., Tardiff, S., Dye, C., & Arrighi, H. M. (2013). Rate of Conversion from Prodromal Alzheimer's Disease to Alzheimer's Dementia: A Systematic Review of the Literature. *Dementia and Geriatric Cognitive Disorders Extra*. https://doi.org/10.1159/000354370

Watabe-Uchida, M., Zhu, L., Ogawa, S. K., Vamanrao, A., & Uchida, N. (2012). Whole-Brain Mapping of Direct Inputs to Midbrain Dopamine Neurons. *Neuron*, *74*(5), 858–873. https://doi.org/10.1016/j.neuron.2012.03.017

Wenk, G. L., McGann, K., Mencarelli, A., Hauss-Wegrzyniak, B., Del Soldato, P., & Fiorucci, S. (2000). Mechanisms to prevent the toxicity of chronic neuroinflammation on forebrain cholinergic neurons. *European Journal of Pharmacology*, *402*(1–2), 77–85. https://doi.org/10.1016/S0014-2999(00)00523-9

Westin, K., Buchhave, P., Nielsen, H., Minthon, L., Janciauskiene, S., & Hansson, O. (2012). CCL2 is associated with a faster rate of cognitive decline during early stages of Alzheimer's disease. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0030525

Whitehouse, P. J., Price, D. L., Clark, A. W., Coyle, J. T., & DeLong, M. R. (1981). Alzheimer disease: Evidence for selective loss of cholinergic neurons in the nucleus basalis. *Annals of Neurology*. https://doi.org/10.1002/ana.410100203

Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., & DeLong, M. R. (1982). Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain. *Science*. https://doi.org/10.1126/science.7058341

Wilcock, G. K., Esiri, M. M., Bowen, D. M., & Smith, C. C. T. (1982). Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. *Journal of the Neurological Sciences*. https://doi.org/10.1016/0022-510X(82)90045-4

Willard, B., Hauss-Wegrzyniak, B., & Wenk, G. L. (1999). Pathological and biochemical consequences of acute and chronic neuroinflammation within the basal forebrain cholinergic system of rats. *Neuroscience*, *88*(1), 193–200. https://doi.org/10.1016/S0306-4522(98)00216-4

Wolf, D., Grothe, M., Fischer, F. U., Heinsen, H., Kilimann, I., Teipel, S., & Fellgiebel, A. (2014). Association of basal forebrain volumes and cognition in normal aging. *Neuropsychologia*. https://doi.org/10.1016/j.neuropsychologia.2013.11.002

Wong, L. F., Goodhead, L., Prat, C., Mitrophanous, K. A., Kingsman, S. M., & Mazarakis, N. D. (2006, January). Lentivirus-mediated gene transfer to the central nervous system: Therapeutic and research applications. *Human Gene Therapy*, Vol. 17, pp. 1–9. https://doi.org/10.1089/hum.2006.17.1

Wood, P. L., Etienne, P., Lal, S., Nair, N. P. V., Finlayson, M. H., Gauthier, S., … Bird, E. D. (1983). A post-mortem comparison of the cortical cholinergic system in Alzheimer's disease and Pick's disease. *Journal of the Neurological Sciences*. https://doi.org/10.1016/0022-510X(83)90200-9

Wright, G. J., Jones, M., Puklavec, M. J., Brown, M. H., & Barclay, A. N. (2001). The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. *Immunology*, *102*(2), 173–179. https://doi.org/10.1046/j.1365-2567.2001.01163.x

Wright, Gavin J., Cherwinski, H., Foster-Cuevas, M., Brooke, G., Puklavec, M. J., Bigler, M., … Barclay, A. N. (2003). Characterization of the CD200 Receptor Family in Mice and Humans and Their Interactions with CD200. *The Journal of Immunology*, *171*(6), 3034–3046. https://doi.org/10.4049/jimmunol.171.6.3034

Xiong, X., White, R. E., Xu, L., Yang, L., Sun, X., Zou, B., … Xie, X. S. (2013). Mitigation of murine focal cerebral ischemia by the hypocretin/orexin system is associated with reduced inflammation. *Stroke*, *44*(3), 764–770. https://doi.org/10.1161/STROKEAHA.112.681700

Záborsky, L., & Cullinan, W. E. (1992). Projections from the nucleus accumbens to cholinergic neurons of the ventral pallidum: a correlated light and electron microscopic double-immunolabeling study in rat. *Brain Research*. https://doi.org/10.1016/0006-8993(92)90568-T

Záborszky, L., Carlsen, J., Brashear, H. R., & Heimer, L. (1986). Cholinergic and GABAergic afferents to the olfactory bulb in the rat with special emphasis on the projection neurons in the nucleus of the horizontal limb of the diagonal band. *Journal of Comparative Neurology*, *243*(4), 488–509. https://doi.org/10.1002/cne.902430405

Zaborszky, L., Cullinan, W. E., & Braun, A. (1991). Afferents to basal forebrain cholinergic projection neurons: An update. *Advances in Experimental Medicine and Biology*, Vol. 295, pp. 43–100. https://doi.org/10.1007/978-1-4757-0145-6_2

Zaborszky, L., Gaykema, R. P., Swanson, D. J., & Cullinan, W. E. (1997). Cortical input to the basal forebrain. *Neuroscience*, *79*(4), 1051–1078. https://doi.org/10.1016/S0306-4522(97)00049-3

Zaborszky, Laszlo, Csordas, A., Mosca, K., Kim, J., Gielow, M. R., Vadasz, C., &

Nadasdy, Z. (2015). Neurons in the basal forebrain project to the cortex in a complex topographic organization that reflects corticocortical connectivity patterns: An experimental study based on retrograde tracing and 3D reconstruction. *Cerebral Cortex*. https://doi.org/10.1093/cercor/bht210

Záborszky, Laszlo, Gombkoto, P., Varsanyi, P., Gielow, M. R., Poe, G., Role, L. W., … Chiba, A. A. (2018). Specific basal forebrain–cortical cholinergic circuits coordinate cognitive operations. *Journal of Neuroscience*, *38*(44), 9446–9458. https://doi.org/10.1523/JNEUROSCI.1676-18.2018

Zaborszky, Laszlo, Pang, K., Somogyi, J., Nadasdy, Z., & Kallo, I. (1999). The basal forebrain corticopetal system revisited. *Annals of the New York Academy of Sciences*, *877*, 339–367. https://doi.org/10.1111/j.1749-6632.1999.tb09276.x

Zaborszky, Laszlo, van den Pol, A. N., & Gyengesi, E. (2012). The Basal Forebrain Cholinergic Projection System in Mice. In *The Mouse Nervous System* (pp. 684–718). https://doi.org/10.1016/B978-0-12-369497-3.10028-7

Zahm, D. S. (2000). An integrative neuroanatomical perspective on some subcortical substrates of adaptive responding with emphasis on the nucleus accumbens. *Neuroscience and Biobehavioral Reviews*, *24*(1), 85–105. https://doi.org/10.1016/S0149-7634(99)00065-2

Zahm, D. S., & Trimble, M. (2008). The dopaminergic projection system, basal forebrain macrosystems, and conditioned stimuli. *CNS Spectrums*, Vol. 13, pp. 32–40. https://doi.org/10.1017/S1092852900016138

Zhang, J. H., Sampogna, S., Morales, F. R., & Chase, M. H. (2002). Age-related changes in hypocretin (orexin) immunoreactivity in the cat brainstem. *Brain Research*. https://doi.org/10.1016/S0006-8993(02)02240-0

Zhang, J. H., Sampogna, S., Morales, F. R., & Chase, M. H. (2005). Age-related ultrastructural changes in hypocretinergic terminals in the brainstem and spinal cord of cats. *Neuroscience Letters*. https://doi.org/10.1016/j.neulet.2003.08.085

Zhang, J., Rane, G., Dai, X., Shanmugam, M. K., Arfuso, F., Samy, R. P., … Sethi, G. (2016). Ageing and the telomere connection: An intimate relationship with inflammation. *Ageing Research Reviews*. https://doi.org/10.1016/j.arr.2015.11.006