THE BEHAVIORAL SIGNIFICANCE OF NITRIC OXIDE IN A PRIMARY OLFACTORY NETWORK: INSIGHTS INTO LEARNING AND MEMORY IN THE ANTENNAL LOBE OF MANDUCA SEXTA

By

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ABSTRACT

Nitric oxide (NO) is a gaseous, unconventional chemical messenger suggested to play a fundamental role in olfaction. This thesis focuses on the role of NO in a primary olfactory center, the antennal lobe (AL) of the moth, *Manduca sexta* (*M. sexta*), to understand how NO affects olfactory-guided behavior. Studies in *M. sexta* report that NO is produced upon odor stimulation and has profound effects at the physiological level, but little is known about its significance to behavior. The central hypothesis examined in this thesis is that NO functions as a neuromodulator of olfactory-guided behavior in a circadian fashion. This hypothesis is examined in the following three studies:

The first study questions whether basal levels of NO fluctuate with the light cycle. *M. sexta* are nocturnal animals that actively engage in odor-seeking behaviors at night. Using an NO sensor, NO concentrations were measured in the AL, optic lobe, and the remainder of the brain during subjective day and subjective night. NO concentrations are higher in the AL and optic lobes at night, suggesting that NO is likely involved in olfactory-guided behavior.

The second inquiry focuses on developing a technique to manipulate NO levels in the AL and whether a specific behavior is affected. Using the proboscis extension reflex, olfactory conditioning is used to ask three questions: (1) does NO affect odor detection, (2) does NO affect discrimination between odorants, and (3) does NO affect learning and memory? Results indicate that NO affects short-term memory but does not affect odor detection, or discrimination between dissimilar odorants.

The third inquiry examines the role of NO in memory and circadian time. It asks: (1) is there an optimal time of day for learning and memory, and (2) does the role of NO in memory change depending on the time of olfactory conditioning? Results indicate that NO in memory is modulated by circadian time.

Taken together, these results suggest a unique functional role for NO in olfactory-guided behavior with two main conclusions: (1) NO modulates short-term memory in the AL, and (2) NO may be important for the circadian regulation of memory.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 AN INTRODUCTION TO NITRIC OXIDE

Nitric oxide (NO) is a hydrophobic diatomic gas that affects a wide variety of biological functions throughout the animal kingdom and also in plants. NO's unique history, spanning nearly two centuries, continues to unfold as exciting new functional roles for NO are discovered.

The role of NO as a chemical messenger within the nervous system is perhaps the most intriguing. NO functions as an unconventional neurotransmitter and neuromodulator that has revolutionized the way scientists think about cell-to-cell communication. This cellular communication lies at the heart of a functioning nervous system and ultimately enables animal behavior.

The unique properties of NO as a gaseous messenger allow for its unconventional signaling in the nervous system. Unlike classical neurotransmitters that require synaptic machinery and membrane-bound receptors, NO can freely pass through cell walls and interact with targets that are not membrane-bound. This flexibility allows NO to affect cells in many ways through signal transduction cascades. NO signaling is especially prominent in the olfactory system and has been found in every species in which it has been looked for. This widespread presence suggests that NO signalling is fundamental to how animals process odors. The goal of this thesis is determine how NO affects olfactory-guided behavior.

The following topics will be reviewed to introduce the findings in this thesis: The history and discovery of NO (section 1.2), NO synthase and NO signaling (section 1.3), NO and synaptic plasticity (section 1.4), NO in the olfactory system (section 1.5), NO in olfactory memory (section 1.6), *M. sexta* as a model system (section 1.7), and the significance of the work (section 1.8).

1.2 THE HISTORY AND DISCOVERY OF NITRIC OXIDE

The history and discovery of NO began with the discovery of nitroglycerine by Ascanio Sobrero (1812-1888) in 1847 (Marsh and Marsh, 2000; Wilson, 2005). Sobrero, a talented Italian chemist, traveled to Paris to study with the famous chemist Theophile-Jules Pelouze. Pelouze was interested in vegetable acids, and was the first to use glycerol in various synthetic pathways. Sobrero experimented with glycerol using a mixture of nitric and sulfuric acids that resulted in a highly exothermic reaction. He found that this reaction, if not cooled, led to the detonation of what he called "pyroglycerine." Sobrero demonstrated its explosive effects during his famous lecture given at Turin in 1847 by detonating a small amount of nitroglycerine. Later on, in the grand tradition of mad scientists, he tasted his creation and described it as, "sweet, pungent and aromatic, [but] . . . great precaution should be used, for a very minute quantity put upon the tongue produces a violent headache for several hours." (Marsh and Marsh, 2000). As it would turn out, this report would have profound repercussions decades later.

In 1860, a young Alfred Nobel (1833-1896) was introduced to Pelouze. The Nobel family provided the young Nobel with a first-class education, and though Nobel did not show an early predilection for chemistry, he was tutored by University-level chemistry professors, one of whom introduced him to Pelouze. Fueled by the entrepreneurial spirit, Nobel became fascinated with nitroglycerine and garnered a loan to test its properties and capabilities. He recognized that the main problem with this substance was how to control its detonation. Soon after, his epic invention was realized in 1863—with the emergence of the Nobel patent detonator, "dynamite." This invention ushered in a series of events ingrained into common folklore, including the development of this "new" class of explosives, the success of the Nobel family, and the creation of the best known prizes in the world (Marsh and Marsh, 2000).

Alongside the discovery of the explosive nature of nitrates, the medical field was also experimenting with therapeutic uses. In 1859, Frederick Guthrie experimented with the medicinal use of amly nitrite. He famously wrote:

" . . . one of the most prominent of its properties is the singular effect of its vapour when inhaled, upon the action of the heart. If a piece of [absorbent] paper, moistened with two drops of the nitric of amyl be held to the nostrils . . . after a lapse of 50s, a sudden throbbing of the arteries of the neck is felt, immediately followed by a flushing of neck, temples, and forehead and an accelerated heart."—Frederick Guthrie, 1859

Guthrie's observation was the first of its kind to report the effect of nitrate inhalation on the heart. He suggested it could be used as a "resuscitative… in drowning, suffocation and protracted fainting." What he did not realize was that amyl nitrite caused a drop in blood pressure.

Guthrie would later become affiliated with a group in Edinburgh, Scotland, that would expand upon his discovery and propel the use of nitrates in medicine. Benjamin Richardson was the first to take amyl nitrite and test its function in an animal model. He found that amyl nitrite caused the dilatation of capillaries in a frog's foot (Richardson, 1864). Arthur Gamgee then found it lowered blood pressure in humans and other animal models (Marsh and Marsh, 2000). Thomas Lauder Brunton, a pivotal figure in the development of pharmacology, was also involved at Edinburgh. After his precocious thesis examining digitalis and urine output at 22, he studied angina pectoris. He noted that digitalis was ineffective in treating the condition, but "small bleedings of 3 or 4 ounces produced temporary relief (Marsh and Marsh, 2000)." He attributed the limited effectiveness of the bloodletting to a reduction in blood pressure. He began experimenting with nitrate-containing compounds. His major breakthrough occurred during an experiment conducted on a patient presenting severe symptoms of angina pectoris (Brunton, 1908). All other treatments had failed, and the patient agreed to an experimental procedure using amyl nitrite. The patient was instructed to inhale five to ten drops given to him on a cloth. After 30 seconds, the pain subsided, and the procedure was considered a tremendous success. Amyl nitrite was effective, but only briefly. Realizing the potential, but the imperfect nature of amyl nitrite, Brunton and his colleagues looked for similar compounds with a longer-lasting effect.

Inspired by nitroglycerine and the headaches Sobrero reported, Constantin Hering became another important figure in the history of NO. Hering, a German physician who converted to homeopathic medicine, was particularly intrigued by the reports of a substance causing a massive headache. He administered nitroglycerine to healthy individuals and documented the incidence of headaches. He wrote in 1849 that "there is nothing known which in small quantities and with such precision causes headaches. Every substance with certainty of effect, ought also to be considered as important to the physician." He reasoned that a substance that produces particular symptoms in a normal patient would be efficacious to a patient exhibiting those symptoms. In effect, "like cures like." This reasoning was met with skepticism by the medical community, and in turn, the homeopaths were skeptical of the efficacy of nitric compounds to treat angina pectoris. Remarkably, however, it was this homeopathic study with nitroglycerine that prompted William Murrell, a London physician, to first use nitroglycerine for angina pectoris in 1878. He noted that "a dose of medicine [nitroglycerine] taken during an attack would cut it short (Murrel, 1879)." Within four years, nitroglycerine was hailed as the remedy *par excellence* for treating angina pectoris (Marsh and Marsh, 2000).

Though it was the go-to treatment for angina pectoris, the mechanism behind nitroglycerine's efficacy would have to wait 100 years until the 1970s. Ferid Murad and colleagues were looking into the action of various vasodilator molecules, including nitroglycerine. They wanted to see how these molecules affected guanylate cyclase activity. The found that nitric-containing compounds activated soluble

guanylate cyclase, which in turn increased cyclic guanosine monophosphate (cGMP) and led to vascular relaxation (Katsuki et al., 1977). They reasoned that it was possible that NO could be the contributing factor because it was known to increase guanyl cyclase activity. Fascinated by the idea that a gas could cause smooth muscle relaxation, Murad speculated further that hormones and other endogenous factors may also act through NO (Marsh and Marsh, 2000). At the same time, Robert Furchgott and colleagues also recognized that soluble guanylyl cyclase could be activated by NO. His group proposed that relaxation was due to bradykinin, histamine, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) that was propelled by "the same unstable relaxing substance (Cherry et al., 1982)." They named this substance endothelial-derived relaxing factor (EDRF). It was then Louis Ignarro and colleagues that demonstrated that NO was EDRF (Ignarro et al., 1987).

Quite fittingly, the 1998 Nobel Prize in Physiology or Medicine was awarded to Murad, Furchgott, and Ignarro for their discoveries concerning the role of NO as a signaling molecule in the cardiovascular system. In a twist of irony, the discovery of dynamite—that was marred in destruction and loss of life—would then result in the institution awarding the Nobel Prize to the discovery of NO in human health. While dynamite has largely been replaced as a demolition device, nitroglycerine continues to be a leading treatment today in angina pectoris (Marsh and Marsh, 2000).

1.3 NITRIC OXIDE SYNTHASE AND NITRIC OXIDE SIGNALING

In addition to its role in cardiac function, the involvement of NO continues to expand into all areas of physiology. For example, NO has been notably recognized as an inhibitor of platelet aggregation (Radomski et al., 1987a&b), and implicated in the control of respiration (Kline et al., 1998), islet hormone release (Akesson and Lundquist, 1999), cancer progression and cell death (Burke et al., 2013), immune response (Moncada et al., 1991), and neurotransmission (Jaffrey, 1995).

These biological roles of NO are enabled through the activation of NO-producing enzyme nitric oxide synthase (NOS). NOS exists in three isoforms in vertebrates: neuronal NOS (nNOS; also referred to as Type I, NOS-1, NOS-I), macrophage-inducible NOS (iNOS; Type II, NOS-2, NOS-II), and endothelial NOS (eNOS; NOS-3, NOS-II). The most abundant isoform in the brain is nNOS. The activity of nNOS is regulated by calcium-calmodulin, and higher concentrations of calcium result in more NO being produced (Schmidt and Walter, 1994). iNOS is rarely present under typical conditions, but it can be expressed by numerous cell types when subjected to an immune response (Nathan, 1992; Garthwaite, 2008). iNOS is found predominantly in macrophages and in microglia in the central nervous system (Garthwaite, 2008). eNOS, the NOS isoform most responsible for the effects of NO in cardiac function, is found in vascular endothelial cells. When activated by calcium, eNOS produces NO and relaxes blood vessels (Knowles and Moncada, 1992). In less complex organisms such as insects, only one form of NOS is found that most closely resembles nNOS in

sequence and activation (Regulski and Tully, 1995; Yuda et al., 1996; Nighorn, et al., 1998).

The diverse biological range of effects that result from NO signaling are likely attributed to its properties as a free-radical gas. Upon NOS activation by calcium, L-Arginine is converted to NO and citrilline. NO can then freely pass through cell walls without the need for membrane receptors or exocytosis machinery. It can also diffuse in any direction, unlike classic synaptic transmission that travels in one direction. This signal property of NO revolutionized the way neuroscientists think about cell-to-cell communication. NO has a short half-life (three to five seconds), and its effects are likely limited by its diffusion range of approximately 100 microns (O'Dell, et al., 1991; Schuman and Madison, 1994).

The best-characterized target of NO is soluble guanylyl cyclase (sGC). sGC is a heme-containing heterodimer consisting of alpha and beta subunits. Through studies of NO in vascular function, it is known that NO activation of sGC increases the levels of cGMP. cGMP can affect a number of downstream targets. These targets include protein kinase G (PKG), ion channels such as cyclic nucleotide-gated channels (CNGCs), hyperpolarization-activated cyclic nucleotide-modulated channels (HCN—reviewed in Craven and Zagotta, 2006), and phosphodiesterases (PDEs) (Garthwaite, 2008). It has been suggested that the sGC/cGMP appears to be the predominant form of NO signaling (Garthwaite, 2008). The most striking example of this claim occurs in the vascular system. When NO-activated guanylyl cyclases are genetically deleted, there is a complete loss of NO-mediated vascular relaxation (Friebe et al., 2007).

It is also important to note that NO exerts non-sGC/cGMP effects in the body. NO can exert actions through s-nitrosylation. S-nitrosylation occurs when NO binds with a sulfur atom on a cysteine-thiol residue to form S-nitrothiols (Ahern et al., 2002). This results in protein modification that can affect many proteins, including ion channels and nuclear receptors. S-nitrosylation has been found to occur with higher levels of NO, and these effects occur at a slower rate (Ahern et al., 2002). Along with sulfur atoms, NO can also stimulate the ADP-ribosylation of proteins. ADP-ribosylation can affect ion channel subunits or regulatory proteins that tightly associate with the channel (Schuman et al., 1994).

The inactivation of NO is also unique with respect to other signaling molecules. The activation of NO is limited by its diffusion and short half-life, but also by well-known oxidative mechanisms. Unlike classical neurotransmitters, NO's inactivation is dependent on non-enzymatic chemical reactivity with other molecules. The primary mechanism by which the diffusion and concentration of NO are controlled *in vivo* is the interaction with oxyhemoglobin or oxymyoglobin. This reaction produces nitrate (NO_3^-). Due to high levels of oxyhemoglobin in the body, its reaction with NO is likely the primary metabolic and detoxification mechanism for NO (Ignarro, 2000). NO is also capable of reducing Fe (III) to Fe (II) resulting in the formation of nitrite (NO_2^-), deoxyhemoglobin, or deoxymyoglobin. NO can also be inactivated by the formation of peroxynitrite. Peroxynitrite has very little biological activity compared with NO. (Ignarro, 2000).

1.4 NITRIC OXIDE AND SYNAPTIC PLASTICITY

"When an axon A . . . excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased." –Donald Hebb, 1949

One of the most significant biological effects of NO is its role in synaptic plasticity. Synaptic plasticity refers to a change in strength, or connectivity, between two neurons. It is believed that this fundamental process underlies an organism's capacity to learn and store memory. It has now been established that the NO/cGMP pathway affects synaptic plasticity at many synapses throughout the CNS and even at the neuromuscular junction (Garthwaite, 2008). In this section, the role of NO in long-term potentiation (LTP) and long-term depression (LTD) will be reviewed, alongside the behavioral evidence that highlights the importance of NO in learning and memory.

In the mammalian brain, there are two structures that are critical for encoding and storing explicit memories: the prefrontal cortex and the hippocampus (Kandel et al., 2013). The prefrontal cortex is implicated in working memory, or the ability to store information for short periods of time. The ability to remember a phone number only long enough to dial it is an example of working memory. Persistent neural firing appears to be key for working memory (Kandel et al., 2013). The hippocampus, on the other hand, stores long-term information that may last for hours, days, weeks, or even a lifetime. This longer form of memory depends on long-lasting changes in the strength of synaptic connections.

The search for the biological existence of memory spans decades, with major breakthroughs in the 1970s. In 1973, Timothy Bliss and Terje Lomo revealed that cells in the hippocampus show remarkable sensitivity to previous activity. They found that a brief, high-frequency train of stimuli, or tetanus, gives rise to LTP; or a long-lasting increase in the amplitude of the excitatory post-synaptic potentials (EPSPs) in the dentate granule neurons. This finding suggested that the strength of the synapse increased. This effect can last for days or weeks when induced in an intact animal using an implanted electrode.

Neuroscientists have come to learn that LTP is not a single form of synaptic plasticity, but rather a family of processes that strengthen synaptic transmission through distinct cellular and molecular processes (Kandel et al., 2013). Even at a single synapse, different forms of LTP can be induced by different patterns of synaptic activity. Even though the different forms of LTP are distinct processes, they share similarities. For example, all forms of LTP are induced by synaptic activity in the pathway that is being potentiated. They differ based on the relative contribution of different receptors and ion channels that may initiate second-messenger signaling pathways in the pre-synaptic cell or in the post-synaptic cell. For example, LTP can increase transmitter release from the pre-synaptic cell, or affect the sensitivity of the post-synaptic cell to the released transmitter, or affect both simultaneously.

LTP has been most extensively studied in the Schaffer collateral pathway in the hippocampus. This pathway focuses on the synaptic connections made between pyramidal and CA1 cells. This synapse is glutamatergic, and the CA1 post-synaptic

cells have both AMPA and NMDA receptors. LTP in this pathway heavily depends on the NMDA glutamate receptor and the presence of calcium. A mild stimulus in these cells will result in the flow of current through AMPA receptors. However, the NMDA receptor requires two key factors to open: (1) glutamate binding, and (2) significant depolarization to expel Mg^{++} from the pore. A strong stimulus, such as a flood of current through the AMPA receptors, can generate a depolarizing EPSP sufficient to expel the Mg^{++} from the NMDA receptor. When open, the NMDA receptor gates calcium and other cations. Calcium is vital to the induction of LTP. As a second messenger, calcium can activate calcium/calmodulin-dependent protein kinase (CamKII), protein kinase C (PKC), and tyrosine kinases. These pathways may result in the insertion of new AMPA receptors in the post-synaptic membrane, thereby increasing the sensitivity of the post-synaptic cell to glutamate. With higher levels of calcium, calcium binds to adenlyl cyclase increasing the levels of cyclic adenosine monophosphate (cAMP) and the activation of PKC. Ultimately, this signaling pathway leads to the activation of CREB-1, the transcription factor thought to lead to the growth of new synaptic connections.

The pre-synaptic neuron also undertakes changes in synaptic strength. Until recently, a lingering question was how changes in the post-synaptic cell triggered a change in the pre-synaptic cell. Diffusible messengers like NO, that affect signaling in multiple directions, seemed like good candidates. NO is activated by calcium, and because it does not require synaptic machinery, it could easily diffuse in a retrograde fashion back to the pre-synaptic cell. Despite several conflicting early results

(Williams, et al., 1993; Boulton et al., 1994), recent evidence suggests that NO is the diffusible messenger in question. In CA1 cells of the hippocampus, NO produced in the post-synaptic cell generated an increase in glutamate release in the pre-synaptic cell (Neitz et al., 2013). It was found that NO exerts this action through the cGMP activation of HCN channels located in the pre-synaptic membrane (Neitz et al., 2013).

The Schaffer collateral pathway underscores how synaptic plasticity can affect learning and memory (reviewed in Kandel et al., 2013). The pathway itself has three features important to learning and memory. The first feature is *cooperativity*, a term used when LTP is dependent upon near-simultaneous activation of many afferent axons, and stems from the fact that the NMDA receptor requires significant depolarization. In essence, this ensures that only events of high significance will result in memory storage. The second feature is the *associative* nature of the pathway. A weak input can be paired with a strong synaptic input that will open the NMDA channel and allow LTP to occur. This is necessary for Pavlovian conditioning. This allows an event that may not have a consequence in and of itself (like a conditioned stimulus), to take on a higher degree of meaning if that event occurs just before a more significant event (like an unconditioned stimulus). The last feature refers to synaptic *specificity*. For example, if a synapse is not activated during a period of high tetanic stimulation, it will not undergo LTP. This ensures that inputs that are not related to a particular event do not get strengthened and participate in a given memory.

It is now established that the NO-cGMP pathway plays a role in LTP at many synapses throughout the CNS and even at the NMJ (Garthwaite, 2008). Several behavioral studies directly looking at the role of NO further confirm its widespread implications in learning and memory. NO affects contextual fear learning in mice (Kelley et al., 2010&2011), delayed visual recall in non-human primates (Prendergast et al., 1997a), negative patterning in turtles (Yeh and Powers, 2005), spatial navigation in rats and mice (Prendergast et al., 1997b; Mutlu et al., 2011), and olfactory conditioning in both vertebrates and invertebrates (Daly et al., 2000; Kendrick et al., 1997; Samama and Boehm, 1999; Yabumoto et al., 2008;Yamada et al., 1995; Muller, 1996; Gage et al., 2013).

NO also plays a well-documented role in long-term depression (LTD) in the cerebellum (reviewed in Garthwaite, 2008). Unlike LTP, LTD weakens connections between two neurons. NO is generated pre-synaptically or in interneurons and acts on post-synaptic cells in the cerebellum. This is in contrast to the retrograde action in the hippocampus. The current model suggests that NO acts post-synaptically at parallel fiber synapses to raise cGMP and activate PKG. Because Purkinje cells are enriched in the PKG substance, G-substrate, upon phosphorylation, PKG functions as a phosphatase inhibitor (Endo et al., 1999). This action, combined with persistent PKC activity, leads to persistent phosphorylation of AMPA receptors. This action favors AMPA receptor endocytosis—the removal of AMPA receptors from the membrane. Knocking down PKG in Purkinje cells impaired LTD and introduced a deficit in motor learning behavior (Feil et al., 2003). LTD, while seemingly contradictory, is

very important for memory to function correctly. The nervous system needs to remain plastic for the formation of new memories.

1.5 NITRIC OXIDE IN THE OLFACTORY SYSTEM

In evolutionary terms, NO is one of the oldest chemical messengers (Ignarro, 2000; Garthwaite, 2008). It may not be coincidence that the olfactory system, which enables our primitive sense of smell, expresses high levels of NO. The enzyme that generates NO is found in every olfactory system examined (Kishimoto et al., 1993; Zhao et al., 1994; Dellacorte et al., 1995; Elphick al., 1995; Hopkins et al., 1996; Homberg, 2002; Muller and Hildebrandt, 1995; Nighorn et al., 1998; Collmann et al., 2004; Regulski and Tully, 1995; Kendrick et al., 1997), and this widespread presence suggests that NO likely plays a significant role in olfaction. These roles are only beginning to be understood. This section will review insights about NO's roles from localization studies, roles in development, and physiological actions.

The primary olfactory network refers to the olfactory bulb in vertebrates and AL in invertebrates. It is the area of the olfactory system where the expression of NOS and NO target molecules appears highest (Collmann et al., 2004). The structural organization of the primary olfactory network suggests that diffusible messengers like NO could be fundamental to odor processing. Sensory afferents innervate dense spheroidal neuropils called glomeruli, which constitute the first synaptic relay in olfactory processing. Sensory afferents synapse with secondary cells that facilitate

signaling between and within olfactory glomeruli. Due to the structure of a glomerulus, which is roughly spheroidal in shape, NO is suggested to be a key neuromodulator in these processes. NO may modify signaling within a glomerulus because of its limited diffusion and the barrier provided by glial cells. Glomeruli are often surrounded by several layers of glial processes (Tolbert and Oland, 1990), and this barrier of cellular tissue is believed to function as an additional NO inactivation method (Garthwaite, 2008). In essence, if NO is produced anywhere within the glomerulus, its diffusion has the capacity to modulate the activity of any cell within that space.

Localization studies:

The presence of NO signaling in the olfactory system has been mostly determined using two immuno-staining methods that express either NOS, or nicotinamide adenine dinucleotide phosphate (NADPH). The expression of NOS and NADPH was first found in the mouse olfactory bulb (Kishimoto et al., 1993). It was later found in the rat and salamander olfactory bulb and epithelium (Zhao et al., 1994). Since then, NOS was found in the catfish olfactory epithelium (Dellacorte et al., 1995); the antennal lobes of the locust, bee, moth, and fly (Elphick al., 1995; Homberg, 2002; Muller and Hildebrandt, 1995; Nighorn et al., 1998; Collmann et al., 2004; Regulski and Tully, 1995); and the sheep olfactory bulb (Kendrick et al., 1997).

The localization of NOS varies depending on the species, but NO is suggested to function in similar ways (Breer and Shepherd, 1993). For example, in adult

mammals, NOS is localized in periglomerular, granule, and short axon cells in the rat (Hopkins et al., 1996). In mice, NOS is found in interneurons including the periglomerular cells, granule cells, and short axon cells, but not in the mitral and tufted cells (Kishimoto et al., 1993). There are variances in strains of mice, however (Weruaga, et al.,1998). Sheep express NOS in mitral, tufted cells, periglomerular, and granule cells (Kendrick et al., 1997). In invertebrates, NOS localization also varies. Locusts have NADPH staining only in local interneurons in the AL (Elphick et al., 1995; Homberg, 2002); and in the moth, NOS immunocytochemistry is most pronounced in the olfactory receptor neurons (Nighorn et al., 1998; Collmann et al., 2004).

NO target molecules—sGC, cGMP, PKG—are also expressed in the olfactory system closely opposing NOS-expressing cells in several species (Gibb and Garthwaite, 2001; Bicker et al., 1996&2001; Hopkins et al., 1996; Kendrick et al., 1997; Elphick and Jones, 1998; Nighorn et al., 1998; Collmann et al., 2004; Fujie et al., 2005). Similar to NOS expression, sGC and cGMP expression also vary, but sGC and cGMP are often located within neurons or glial cells comprising glomeruli (Hopkins et al., 1996; Bicker et al., 1996; Collmann et al., 2004).

Development studies:

NO appears to regulate cell migration and synapse formation during development. NO plays a role in the developing NMJ of the locust (Truman et al., 1996), the visual

system of *Drosophila melanogaster* (Gibbs and Truman, 1998), and in vertebrate and invertebrate olfactory systems, (Schachtner, 1999; Gibson et al., 2000&2001; Roskams et al., 1994; Arnhold et al., 1997). The morphogen sonic hedgehog, which is widely distributed in many tissues during embryonic development, also appears to be modulated by NO (Loulier et al., 2005).

The localization of NOS in the olfactory system can vary with the stage of development. In the rat for example, NOS is expressed in the olfactory receptor neurons during development, but later expressed only in secondary cells (periglomerular, granule, and short axon cells) (Hopkins et al., 1996). In the moth, NOS immunoreactivity is present in olfactory receptor axons, and its distribution changes during development that coincides with the position of migrating peripheral glia (Gibson et al., 2000; Tolbert et al., 2004). Inhibition of NO results in the growth of abnormal ALs, whereby the glia associated with glomeruli fail to migrate (Gibson, 2001). The serotonin immunoreactive neuron also exhibited abnormal dendritic arborization in the moth (Gibson, 2001). In the mouse, where olfactory bulb neurons continue to regenerate through adulthood, NO was found to be necessary to affect neuronal differentiation in the subventricular zone (Moreno-Lopez et al., 2004).

Physiology Studies:

NO is produced in the primary olfactory system in response to electrical and odor stimulation (Fujie et al., 2002; Collmann et al., 2004; and Lowe et al., 2008). The first study to examine NO in relation to evoked stimuli was performed by Fujie et al., in

2002. This group inserted a NO sensor into the procerebral lobe of the mollusk and showed that electrical stimulation increased the levels of NO. This method did not allow for discrete measurements of NO, and the procerebral lobe of the mollusk is unusual with its lack of glomeruli, but it did open up intriguing possibilities for the physiology of NO. In 2004, the Nighorn group first demonstrated that odors evoke an increase in NO in the antennal lobe of the moth (Collmann et al., 2004). Using an NO-sensitive dye, odor-evoked NO was spatially focused and concentration-dependent. In 2008, these effects were reproduced in the mouse olfactory bulb and the concentration of NO in response to odor-stimulation was measured and found to be in the nanomolar range (Lowe et al., 2008).

In 2007, a comprehensive electrophysiological study of NO effects on AL neurons was conducted on the moth, *Manduca sexta* (Wilson et al., 2005&2007). NOS inhibition affects the resting membrane conductance in local interneurons and projection neurons resulting in drastic changes in firing patterns. These effects are mediated by both sGC and non-sGC related mechanisms. This finding also suggests NO is present at basal levels in the AL. The whole-cell effects of NO were measured *via* patch-clamp recording (Higgins et al., 2012). These studies found that NO increases inward currents in projection neurons and exerts variable effects on local interneurons consistent with morphological type.

1.6 NITRIC OXIDE IN OLFACTORY MEMORY

NO's unusual signaling capabilities may underlie its roles in olfactory learning and memory. In this section, the behavioral evidence for NO's role in odor learning and memory will be discussed as well as the molecular and cellular evidence for distinct memory traces in the olfactory system.

Numerous behavioral studies indicate that NO is necessary for olfactory learning and memory (for review see Susswein et al., 2004). One of the most interesting studies comes from the sheep olfactory bulb (Kendrick et al., 1997). *Post-partum* sheep form olfactory memories soon after birth which enables them to distinguish their kin from other lambs. Kendrick and colleagues used a microdialysis probe to deliver different drugs into the olfactory bulbs. What they found was that a NOS inhibitor and an sGC inhibitor both impaired the ability of the ewe to recognize her young. These mothers interacted with their young and strange lambs equally, indicating that NO signaling prevented this kin-recognition memory formation. Moreover, if an NO donor was applied alongside the NOS inhibitor, the memory was restored. Once the olfactory memory was formed, the NO drugs did not affect kin recognition.

This study highlights an important theme found in behavioral studies of NO in learning and memory. Once a memory is formed, NO does not affect the retrieval of this memory. Similar findings were reported in newborn rat pups associating peppermint with a tactile stimulus (Samama and Boehm, 1999), rats learning a radial

arm maze and habituation tasks (Yamada et al., 1995), turtles in a negative patterning task (Yeh and Powers, 2005), and honeybees in associative olfactory learning (Muller, 1996).

Muller's 1996 study in honeybees is a pivotal paper that presents NO as a signaling model involved in distinct memory traces. Honeybees and moths have a feeding reflex, where they extend their proboscis to consume nectar (Takeda, 1961; Daly et al., 2000). This reflex, called the proboscis extension reflex (PER) can be used in olfactory conditioning, where an odor can be presented to the antenna followed by sucrose, and the animal will extend its proboscis to that odor. Muller found that a NOS inhibitor injected into the haemolymph affected a specific form of long-term memory. This effect was found through multi-trial conditioning, or multiple pairings. Interestingly, NOS inhibition did not have an effect with only a single pairing. This finding would suggest that the modulatory role for NO in learning and memory may be conditioning specific, and the parameters of the conditioning paradigm likely affect its role in memory. Moreover, this study highlights the developing evidence that multiple forms of memory are formed during the time of conditioning. This suggests that distinct molecular mechanisms acting in parallel may underlie different forms of memory.

Evidence for multiple forms of memory was found in the olfactory system of *D. melanogaster*. Studies using functional cellular imaging have identified six memory traces that form in response to conditioned odors (Davis, 2011). These traces are the result of molecular substrates. Imaging studies of memory traces have been identified

through increases in calcium or synaptic release in response to the conditioned odor. These memory traces are formed during the time of conditioning and span different temporal windows. It is believed that these memory traces underlie different forms of memory, such as short-term, intermediate-term, and long-term forms of memory. Short-term memory is generally defined as covalent modifications of proteins that are already present, such as those generated by second messengers, protein kinases, and phosphorylation of specific proteins (Susswein et al., 2004). Intermediate-term memory has been characterized in *Aplysia* as dependent upon mitogen-activated kinase (MAPK) activity and protein synthesis (Lyons et al., 2008). Long-term memory is generally characterized by changes in gene expression (Lyons, 2011).

Three traces have been found to associate with short-term memory, one with intermediate memory, and two with long-term memory. These olfactory memory traces occur in both the AL and in the mushroom bodies. The first memory trace was found in the AL in both bees and in *D. melanogaster* (Faber et al., 1999; Yu et al., 2004). The trace found in *D. melanogaster* is considered a short-term memory trace and only lasts for seven minutes (Yu et al., 2004). The short-term memory trace recruits projection neurons into the representational space of the conditioned odor (Yu et al., 2004). Wang et al., 2008 reported another early forming memory trace in the lobes of the mushroom bodies. The mushroom bodies receive axons from projection neurons in the AL via the antennal cerebral tract. This trace is imaged as an increase in calcium to the conditioned odor and disappears after 60 minutes. An intermediate-term memory trace was also found in the mushroom bodies, and

specifically the dorsal paired medial (DPM) neurons that innervate the vertical and horizontal lobes (Yu et al., 2005). This phase of memory forms in flies after olfactory conditioning and follows short-term memory. This phase forms within the first hour after conditioning and persists for a few hours, but the role of intermediate-term memory is unclear. Davis, 2011 speculates this may be an independent trace, or it may be a consolidating force. Long-term memory in *D. melanogaster* is also dependent upon spaced trials similar to the honeybee (Davis, 2011). A long-term memory trace is found in the αβ neurons of the mushroom bodies. This trace results in a calcium influx to the conditioned odor and occupies the 9- to 24-hour window after olfactory conditioning. This particular memory trace is dependent upon cAMP response element-binding protein CREB activation and CamKII activity.

In the last decade, several reports reveal the importance of the primary olfactory center as a source for learning and memory. Evidence of change as a result of learning and memory in the antennal lobe has been found in bees (Rath et al., 2011; Faber et al., 1999; Sandoz et al., 2003; Arenas et al., 2009, Fernandez et al., 2009, Denker et al., 2010), flies (Yu et al., 2004), moths (Daly et al., 2004), and the mammalian olfactory bulb (Kay and Laurent, 1999; Doucette and Restrepo, 2008). The organization of the primary olfactory center suggests that gaseous, diffusible messengers like NO could be fundamental in odor processing and recognizing an odor as rewarding. The glomerulus is suggested to function as a unit that encompasses synaptic connections between olfactory receptor neurons, local interneurons, and projection neurons. Olfactory learning and memory is strongly

correlated to the plastic changes that occur at these synapses (Yu, 2004; Lyons and Roman, 2008; Rath et al., 2011).

In conclusion, learning and memory do not appear to be a unitary process. Many studies have shown that short-term memory and long-term memory represent different processes (Susswein et al., 2004). In theory, NO could be affecting learning by enabling those initial biochemical processes to occur, or through different forms of memory, or both.

1.7 MANDUCA SEXTA AS A MODEL ORGANISM

For decades, the moth, *Manduca sexta* (Lepidoptera:Sphingidae, *aka* the tobacco horn worm), has been a model organism for olfaction research. *M. sexta* are almost entirely olfactory-guided, heavily relying on their sense of smell to locate mates, feed, and find sources on which to lay eggs. Great efforts have been taken to understand the biology of the olfactory system. *M. sexta's* dependency on olfactory processing, large size, and presence as an agricultural pest propelled *M. sexta* into being a leading olfactory model. In addition, the AL of *M. sexta* is comparable to the olfactory bulb in vertebrates (Hildebrand and Shepherd, 1997). This knowledge of *M. sexta's* olfactory processing, in combination with the detailed behavioral repertoire of these animals, forms a powerful model to investigate NO in olfactory-guided behavior.

M. sexta perceive odors through the binding of odorants to the olfactory receptor neurons (ORNs) located in the sensilla of the antennae. Odor-ligand binding is both

metabotropic and ionotropic and depolarizes ORNs. ORNs converge into the antennal nerve and project to chemo-specific glomeruli in the AL. ORNs that express the same receptor genes, and therefore respond to structurally similar odorants, converge into a single glomerulus in the AL (Hildebrand et al.,1997; Rössler et al., 1999; Vosshall et al., 2000). The AL contains 63±1 glomeruli that are arranged around a region of dense neuropil. In a glomerulus, ORN axon terminals synapse with projection neurons and local interneurons. Projection neuron dendrites generally innervate a single glomerulus and send projections to higher brain regions in the mushroom bodies and lateral horn. The local interneurons, on the other hand, arborize in many glomeruli, forming an inhibitory network influencing both projection neurons and other local interneurons. It has been found that local interneurons affect the temporal firing of projection neurons (Christensen and Hildebrand, 1997) and also inhibit the activity of neighboring glomeruli unaffected by the odor (Hansson et al., 1991; Reisenman et al., 2004&2005). It is important to note that connections between these cell types vary. For example, not all projection neurons receive monosynaptic input from ORNs (Christensen et al., 1998a), and some projection neurons may activate local interneurons (Sun et al., 1997). Taken altogether, interactions between and within olfactory glomeruli comprise unique, combinatorial activity patterns in the AL that ultimately enable olfactory processing and the ability to follow odor cues in the environment.

This circuitry is modulated by a suite of neurotransmitters and modulators. These include, but are not limited to, acetylcholine released by ORNs (Stengl et al., 1990),

and GABAergic inhibition in local interneurons (Hoskins, 1986). In addition, each AL also contains several amines including serotonin, dopamine, and octopamine. The sole source of serotonin in the AL is provided by a single serotonin-immunoreactive neuron (Kent et al., 1987). The cell body of this neuron is located in the lateral cell cluster of each AL and projects contralaterally to the opposing lobe and innervates every glomerulus (Dacks, 2006a&2007). Serotonin fluctuates with the light cycle in the AL and is suggested to be a circadian modulator of olfactory processing (Kloppenburg et al., 1999; Dacks, 2007; Kloppenburg and Mercer, 2008). The ALs are also innervated by two pairs of dopamine neurons (Dacks et al., 2012). Dopamine and tyrosine hydroxylase immunoreactivity are observed in every glomerulus, and play a role in aversive learning (Dacks et al., 2012). Octopamine, the insect analog of norepinephrine, is necessary for maintaining motor control of flight behavior (Claassen and Kammer,1986). Both the ALs and the mushroom bodies receive octopamine innervation (Dacks et al., 2006b).

In addition to these classical transmitters, gaseous NO has been carefully studied for expression and its effects on physiology. *M. sexta* nitric oxide synthase (MsNOS) and sGC (MsGCα1&MsGCβ1) have been cloned, and their locations have been identified (Nighorn et al., 1998; Collmann et al., 2004). NOS is expressed in the ORNs (Nighorn et al., 1998; Collmann et al., 2004); and sGCs are expressed in projection neurons, some GABAergic local interneurons, and the serotonin-immunoreactive neuron (Nighorn et al., 1998; Collmann et al., 2004). NO is produced in response to an odor and is spatially focused and concentration-dependent

(Collmann et al., 2004). NO maintains resting membrane conductance in AL neurons and influences whole-cell currents (Wilson et al., 2007; Higgins et al., 2012).

M. sexta also perform distinct, highly reproducible behaviors. Like many moths, *M. sexta* are nocturnal and use their olfactory systems to identify mates, food sources, and sites to lay eggs. *M. sexta* behavior has been studied in nature for decades, and these behaviors can be reproduced in the laboratory (Gregory, 1963; Yamamoto, 1969; Grant, 1983; Raguso and Willis, 2005). Moths can fly upwind in a wind tunnel to locate a pheromone source on a piece of filter paper. In a classic "zig-zag" pattern, male moths will hover over the source and curl their abdomen in attempt to mate (Willis and Arbas, 1991). The wind-tunnel can also be used to understand host-plant foraging interactions. *M. sexta* commonly feed from the Solanacae family, notably from *Datura wrightii*, and will extend their proboscis into a paper flower exuding those volatiles (Riffell et al., 2009). *D. wrightii* is the preferred host-plant of *M. sexta* and is known to illicit innate responses in proboscis extension (Raguso and Willis, 2002&2005; Riffell et al., 2009). It is suggested that responses to *D. wrightii* is somehow "hardwired" in the AL. Oviposition, or egg-laying, is also most effective when using *D. wrightii* plants or extract (Reisenman et al., 2009&2013). Female moths will curl their abdomen to deposit eggs on the underside of *D. wrightii* leaves or to a piece of cardboard with the appropriate volatiles.

All of these behaviors rely on olfactory learning and memory. *M. sexta* are easily conditioned to associate odors with either a rewarding stimulus or an aversive stimulus (Daly et al., 2000 & 2004; Dacks et al., 2012). Similar to honeybees,

olfactory conditioning uses the PER (Takeda, 1961). This feeding reflex results in an extension of the proboscis to a conditioned odor. Odors can be conditioned by puffing an odor to the antenna and immediately applying a sucrose award to the proboscis. *M. sexta* can then be tested for olfactory learning and memory by presenting the conditioned odor alone. Moths learn and remember odor associations readily, and will often extend their proboscis after one or two pairings (Gage et al., 2013).

It may seem counterintuitive that learning and memory are important for *M. sexta* when *D. wrightii* are known to illicit innate proboscis extensions (Raguso and Willis, 2002&2005; Riffell et al., 2009). *D. wrightii*, however, are only present at a specific time of the year in locations like Southern Arizona (Riffell et al., 2008). It then becomes necessary for *M. sexta* to learn to feed from other food sources. The *Agave* species is particularly foraged by *M. sexta* in Southern Arizona. *Agave palmeri* exhibits a completely different odor profile from *D. wrightii* and contains a higher caloric value (Riffell et al., 2008). These studies by Riffell et al., 2008 highlight that while *M. sexta* are foraging specialists, they can adapt and learn to feed from other species. This behavior further underscores the importance of olfactory learning and memory.

1.8 SIGNIFICANCE OF THE WORK

Considerable evidence suggests that NO signaling is a fundamental feature of olfactory processing. While progress has been made at the physiological level, it is still unclear how NO signaling in the olfactory system is behaviorally meaningful. NO is implicated in learning and memory in some species, and if this is the case in *M. sexta*, questions still remain about which processes are involved. The olfactory system of *M. sexta* provides a unique opportunity to understand the behavioral significance of NO to olfaction. The AL is well described, similar to vertebrates (Hildebrand and Shepherd, 1997), and NO has been carefully characterized in terms of receptors, location, and physiology. Roles of NO and behavior identified in *M. sexta* are likely common to many or all olfactory systems.

This thesis addresses the central hypothesis that *nitric oxide modulates olfactory-guided behavior in a circadian fashion*. In the following sections, evidence will be presented supporting this hypothesis and describing further roles for NO in a primary olfactory network. This thesis contains one appendix that includes published work (A: Nitric Oxide Affects Short-Term Olfactory Memory in Manduca Sexta) and work prepared for publication (Chapter 3: The Role of Nitric Oxide in Memory is Modulated By Circadian Time).

1.9 SPECIFIC CONTRIBUTION OF THE AUTHOR

The specific contribution of the author of this thesis to this work is in accordance with the thesis guidelines provided by the Graduate College of the University of Arizona. For the appendix work, the author was principally involved in the conception of the proposed experiments, developing the methods, performing and analyzing the experiments, and drafting the manuscripts.

CHAPTER 2: PRESENT STUDY

NITRIC OXIDE AFFECTS SHORT-TERM MEMORY IN THE ANTENNAL LOBE OF MANDUCA SEXTA

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2.1 INTRODUCTION

The background, methods, results, and conclusions are presented in the manuscript appended to the thesis (Appendix A). The following is a summary of the most important findings in this document.

2.2 SUMMARY

This study first examines the possibility of nitric oxide (NO) involvement in olfactory-guided behavior in the antennal lobe (AL) of *Manduca sexta* (*M. sexta*). This study uses chemical detection of NO and olfactory conditioning assays to begin testing roles of NO in behavior. Outlined below are the three main findings of this study: (1) NO fluctuates with the light cycle in the AL and optic lobes, (2) NO affects short-term memory traces, and (3) NO does not affect odor detection or discrimination between dissimilar odorants.

The author first questioned whether basal levels of NO can be measured, and if so, do levels of NO fluctuate with the light cycle/activity phase? To accomplish this, a commercial NO sensor (from Innovative Instruments, Tampa, FL, USA) was used and adapted to measure NO concentration in isolated regions of the *M. sexta* brain, which include the ALs, optic lobes, and the remainder of the brain. NO was measured in each

region in the nanomolar range. Interestingly, NO concentration significantly fluctuates in the antennal and optic lobes during *M. sexta's* nocturnal active phase. Circadian increases in NO in these sensory areas lends support to the hypothesis that NO modulates olfactory-guided behavior.

This second question focused on the role of NO in a specific behavior. The author first addressed whether NO affects odor detection. To do so, a microinjection surgery was developed (originally published in Lei et al., 2009) and further refined. Briefly, this surgery allows for a substance, such as a NOS inhibitor, to be pressure-injected into each AL and have the animal recover within minutes to participate in behavioral assays. Once the surgery is performed with the necessary controls, the animal participates in a forward-paired, appetitive associative conditioning assay using the proboscis extension reflex (PER). An odor is puffed onto the antenna and paired with a sucrose reward. This pairing is performed six times, spaced four minutes apart, which is considered a robust learning and memory paradigm allowing for the encoding of long-term memory (Menzel et al., 2001). The animal is then tested by presenting the conditioned odor alone, and the presence or absence of a proboscis extension is recorded. To test the role of NO in odor detection, a synthetic *Datura wrightii* blend was used as the conditioned stimulus. Animals injected with a NOS inhibitor demonstrated a significant decrease in PER to the conditioned odor. To test whether this effect was due to a disruption in odor detection, or the learning and memory process, odor conditioning was performed first and the NOS inhibition surgery second. The results reveal odor detection remains intact, but learning and memory is affected by NO. These results were confirmed using a novel odor,

hibiscus, to insure that these findings were not the result of a confound with preferred host-plant, *D. wrightii*.

The ability to distinguish odors in the environment is another critical feature of the olfactory system. Some neuromodulators, like serotonin, are suggested to enhance contrast between different chemical classes of odorants (Dacks et al., 2008). NO was tested in this capacity to discriminate between linalool (a monoterpenoid) and methyl salicylate (an aromatic). Animals successfully distinguish between these odorants even in the presence of a NOS inhibitor, which suggest that NO does not play a major role in the discrimination between dissimilar odorants.

The role of NO in olfactory memory was further examined. Recent findings in the field of learning and memory suggest that traces of memory occur in parallel (Muller, 1996; Davis, 2011). In other words, the molecular substrates underlying different forms of memory, such as short-term, intermediate-term, and long-term, occur in parallel at the time of conditioning. To test to see if NO signaling was important for specific memory traces, learning and memory of the conditioned odor was tested at multiple time windows after conditioning to account for different forms of memory. The results indicate that NO affects short-term memory processes.

CHAPTER 3: PRESENT STUDY

THE ROLE OF NITRIC OXIDE IN OLFACTORY MEMORY IS MODULATED BY CIRCADIAN TIME

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(*Note: in preparation for Frontiers in Neuroscience Special Topics: Sleep and Circadian Rhythms in Plasticity and Memory*)

3.1 ABSTRACT

Nitric oxide (NO) is thought to play an important neuromodulatory role in the olfactory system. This modulation has been suggested to be necessary for olfactory learning and memory in the antennal lobe (the primary olfactory network in invertebrates). We are using the hawkmoth, *Manduca sexta*, to further investigate the role of NO in olfactory memory. Recent findings suggest that NO affects short-term memory traces. NO concentration also appears to fluctuate with the light cycle. These new lines of evidence give rise to our hypothesis that NO may be involved in the connection between memory and circadian rhythms. In this study, we explore the role of circadian time and NO in memory by altering the time of day when associative-olfactory conditioning is performed. We find a strong effect of NO on short-term memory, and two surprising effects of circadian time. We find that (1) at certain time points, NO affects longer traces of memory in addition to short-term memory, and (2) when conditioning is performed close to the light cycle switches—both from light to dark and dark to light—NO does not significantly affect memory at all. These findings may suggest an intriguing functional role for NO in the connection between memory and circadian rhythms.

3.2 INTRODUCTION

Nitric oxide (NO) is a gaseous, unconventional neurotransmitter and modulator suggested to be fundamental to odor processing. In every species investigated, NO is highly expressed in the primary olfactory center in both vertebrates (olfactory bulb) and invertebrates (antennal lobe) (Bredt et al., 1991; Muller and Hildebrandt, 1995; Elphick et al., 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004). Given this widespread prominence, NO likely plays a significant functional role in olfactory processing and behavior, yet the significance of this role is only beginning to be understood. The Gage et al., 2013 study in the hawkmoth, *Manduca sexta,* suggests that NO is necessary for short-term olfactory memory in the AL. These moths are nocturnal and heavily depend on their olfactory systems to find mates, feed, and find sites to lay eggs. During the nocturnal active period, NO levels are significantly higher in the antennal and optic lobes, suggesting that NO signaling is heightened at night and may play a phase-dependent role. These findings have led to the hypothesis that NO may be involved in the connection between olfactory memory and circadian rhythms.

Circadian influence on memory and behavior is highly conserved among species (Gerstner et al., 2009). The repetitive nature of the light cycle that coincides with the availability of vital resources has led to a "timed" physiological environment (Gerstner et al., 2012). In this way, organisms experience physiological changes at the cellular and molecular level that are both circadian and seasonal, and ultimately lead to timed variations in behavior. These behavioral responses are often coordinated with regular and

predictable stimuli present in the environment. For example, bees and moths forage at the time of day when pollen and sucrose are at peak levels (Baker, 1961; Guerenstein et al., 2004). Memory, which is intricately intertwined in behavior, has also evolved in a circadian, time-dependent manner. Learning and memory are metabolically expensive, and it is widely believed that these mechanisms are conserved and function optimally when predictable resources are available (Gerstner, 2012; Lyons, 2011; Dukas, 2008). In essence, there is a "plasticity in plasticity."

Though behavior and memory are controlled by the circadian clock, the nervous system also encodes for variation (Gerstner et al., 2012). This variation is believed to exist to help animals adapt to a changing environment, such as the change in daylight hours throughout the year. This ability to adapt is suggested to be regulated by neuromodulators (Gerstner et al., 2012). Neuromodulators adjust sensory circuitry to account for changing conditions and are thought to optimize energy use in finding resources. NO could be an important neuromodulator in this process. NO is demonstrated to affect memory in many species and paradigms (Kelley et al., 2010; Prendergast et al., 1997a; Yeh and Powers, 2005; Prendergast et al., 1997b; Mutlu et al., 2011; Kendrick et al., 1997; Samama and Boehm, 1999; Yabumoto et al., 2008; Yamada et al., 1995; Muller, 1996), and some reports also find direct effects of NO in the superchiasmatic nucleus (Ignarro, 2000) and in peripheral pacemakers (Bullmann and Stevenson, 2008).

These effects likely result from NO's unconventional signaling. NO freely passes through cell walls and can affect cells in many ways. These mechanisms include the

activation of protein kinases such as PKG, phosphodiesterases, and cyclic nucleotide gates channels. The production of NO in the nervous system is mediated by the calcium activation of nitric oxide synthase (NOS). NO may affect the soluble guanylyl cyclase /cyclic guanosine monophosphate (sGC/cGMP) pathway, or initiate *s*-nitrosylation or ADP-ribosylation. Interestingly, NO signaling via the sGC/cGMP pathway, the best-characterized target for NO, may play a role in the connection between memory and circadian rhythms. The molecular mechanisms suggested to gait circadian rhythms and memory are reported to involve calcium, cGMP/PKG and the cAMP/MapK/CREB cascades (Gerstner, 2012). Therefore, NO may be an important modulator in the circadian regulation of memory.

The olfactory system provides an excellent opportunity to investigate this possibility. The primary olfactory center is organized similarly across phylogeny (Hildebrand and Shepherd, 1997), and NO is highly expressed in every primary olfactory center in which it has been examined (Bredt et al., 1991; Muller and Hildebrandt, 1995; Elphick et al., 1995; Hopkins et al., 1996; Kendrick et al., 1997; Nighorn et al., 1998; Fujie et al., 2002; Collmann et al., 2004). Olfactory learning and memory, especially in insects, is well studied, and much is known about the behavior and molecular components (for reviews see Dukas, 2008; Davis, 2011; Guirfa, 2012). Olfactory memory also appears to be regulated by the circadian clock. Several reports reveal a circadian-dependent change in memory using olfactory conditioning. These effects have been demonstrated in the cockroach, *Leucophaea maderae* (Decker et al. , 2007); in the soil dwelling nematode, *Caenorhabditis elegans* (Olmedo et al., 2012); and in the fruit

fly, *D. melanogaster* (Lyons and Roman, 2008). It appears that the circadian clock regulates memory rather than olfactory responsiveness (Lyons and Roman, 2008; Lyons, 2011). Studies in rodents show that olfactory bulb neurons express functional and entrainable circadian rhythms that operate independently of the superchiasmatic nucleus (Granadas-Fuentes, 2004). These rhythms in olfactory activity in both vertebrates and invertebrates appear to depend on *BMAL1* and *period* genes (Lyons and Roman, 2009).

In this study, we utilize the olfactory system of *M. sexta* to study the role of NO in memory in relation to circadian time. *M. sexta* demonstrate robust learning and memory in classical conditioning paradigms using the proboscis extension reflex (Daly and Smith, 2000; Dacks et al., 2012; Gage et al., 2013). The olfactory behavior and ecology in the hawkmoth is well described and can be useful when interpreting olfactory memory with light/activity phase effects (Baker, 1961; Grant, 1983; Riffell et al., 2008a). Although *M. sexta* is not a traditional model used in circadian rhythm biology, *period* expression is found in the photoreceptors in the compound eye, neurons in the optic lobe, and glial cells in the AL (Wise et al., 2002). We know that NOS is localized in the olfactory receptor neurons and sGC is expressed in all projection neurons, some local interneurons, and the serotonin immunoreactive neuron (Collmann et al., 2004). NO also exerts substantial effects at the physiological level in *M. sexta* that include: (1) a spatially focused increase in NO during odor stimulation (Collmann et al., 2004), (2) persistent basal levels in olfactory neurons that affect resting membrane conductance (Wilson et al., 2007), and (3) whole-cell current modulation (Higgins et al., 2013).

We ask two main questions in this study: (1) is there an optimal time of day for learning and memory in *M. sexta*, and (2) does the role of NO in memory change depending on the time of conditioning? To do so, we pair a microinjection surgery to manipulate NO levels in the AL with an appetitive, odor-associative conditioning paradigm. Conditioning is performed at different times around the circadian clock that include 12 hours of subjective day, followed by 12 hours of subjective night. The proboscis extension reflex is used to measure memory of the conditioned odor (CS). We tested olfactory memory at four time points after conditioning to account for both short-term, intermediate-term, and long-term memory traces. We present findings that suggest a unique role for NO in memory that is modulated by circadian time.

3.3 MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were reared in the Department of Neuroscience at the University of Arizona. Animals were raised on an artificial diet and maintained under a long-day photoperiod regimen (17hr light: 17hr dark) at 25°C at 50-60% relative humidity. Females at pupae stage 16 were transferred into a biological incubator (Model I-36 VL; Percival Scientific, Perry, IA, USA) and placed under a 12 hour light: 12 hour dark cycle and kept at 25°C at 50-60% relative humidity. Five-day-old females were unfed after eclosion and used for all experiments.

Pharmacology and Microinjection Surgery

NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), was dissolved into physiological saline (150mmol 1^{-1} NaCl, 3 mmol1^{-1} KCl, 10 mmol1^{-1} TES, pH 6.9) and used at a 15 mmol 1^{-1} concentration. In *M. sexta,* this concentration was found to be the minimal effective dose in extracellular recording (Wilson et al., 2007) and also found to affect odor learning and memory (Gage, et al., 2013).

Drug delivery into the ALs was accomplished via a microinjection surgery (Lei et al., 2009; Gage et al., 2013). Animals were restrained in a plastic tube, and an hourglass window was cut into the head capsule. The ALs were visualized by gently moving connective tissue with fine forceps. Quartz pipettes (o.d. 1.0 mm, i.d. 70 mm; Sutter Instruments, San Diego, CA, USA) were pulled with a Model P-2000 puller (Sutter Instruments) and clipped to allow solution passage. The pipettes were filled with either L-NAME or saline and manually injected into each AL (for visual see Gage et al., 2013) using a General Valve Picospritzer II (East Hanover, NJ, USA). The cut window was resealed with myristic acid (Sigma-Aldrich). The identity of the drug *versus* saline control was blind to both the experimenter performing the surgery and the experimenter observing behavior in all experiments.

Olfactory stimulation and appetitive conditioning

Hibiscus oil blend (diluted 1:1000 in mineral oil; Select Oils, Tulsa, OK, USA) was the odor used for appetitive conditioning. Hibiscus is not a reported host plant of hawkmoths and serves as a novel odor to gauge odor-associative learning and memory.

Hibiscus was delivered by a solenoid-controlled air stream into an odor-containing glass syringe. Each syringe contained 10 uL of the odor on a piece of filter paper.

Appetitive conditioning was performed utilizing the proboscis extension reflex. This is a feeding reflex that was originally discovered in honeybees (Takeda, 1961) and has since been adapted for *M. sexta* (Daly and Smith, 2000). Moths trained to associate an odor with a sucrose reward will extend their proboscis to the rewarded odor. This measure can be used in a number of paradigms and is especially useful to gauge odor learning and memory. In these experiments, moths were restrained in a plastic tube prior to surgery and conditioning. After surgery, a clear plastic tube was situated over the proboscis to secure a uniform position both to apply a sucrose reward (1 uL, 25% sucrose solution) and to observe maximum pumping motion and extension. Five-day-old moths were trained in a forward conditioning paradigm to associate hibiscus with the sucrose reward (Figure 1). The hibiscus-containing syringe was positioned approximately 5 cm from the antenna and delivered via a 5-s odor pulse. Three seconds into the pulse, sucrose was delivered to the tip of the proboscis using a pipette. This conditioning sequence was repeated six times, spaced four minutes apart. Multiple, spaced trials is a very robust form of conditioning that was employed to test shorter and longer forms of memory (Menzel et al., 2001).

Learning and Memory

Proboscis extension to the conditioned odor was tested at four time points after conditioning: 5 min, 1hr, 4hr, and 24hr (Figure 1). These time points approximate

memory traces that underlie short-term memory, short-term/intermediate-term memory, intermediate-term/long-term memory, and long-term memory, respectively (Davis, 2011).

Circadian time in learning and memory

We sought to test how an animal's physiological time of day affects learning and memory and whether the role of NO in memory is affected. To do so, we chose six time points over a 24-hour period divided into photophase (subjective day; 00:00-12:00hr) and scotophase (subjective night; 12:00-24:00hr) (Figure 1). Three time points in photophase were chosen: 02:30hr, 07:30hr, and 11:30hr; and three points in scotophase were chosen: 14:30hr, 19:30hr, 23:30hr.

These time points were chosen for three main reasons: (1) 14:30 was chosen because this time approximates the hours after dusk (2.5) when *M. sexta* are actively using their olfactory systems to find mates, feed, and find sites for oviposition (Gregory, 1963; Yamamoto et al., 1969). 14:30 was the time point found in the Gage et al., 2013 study that showed a robust effect of NO in short-term memory. 02:30 was used as a photophase counterpoint to examine memory 2.5 hours after subjective photophase/sunrise. (2) 11:30 and 23:30 were chosen because each preceded the light cycle switch (from photophase to scotophase and from scotophase to photophase) by thirty minutes, potentially illuminating an association between memory and the impending light cycle change. (3) 07:30 and 19:30 were chosen as mid-phase time points, both 7.5 hours into photophase and scotophase.

Statistical Analysis

All statistical analyses were performed using JMP 9.0.1 (SAS Institute, Cary, NC, USA). Proboscis extension reflexes were scored with a 1 or a 0 to employ parametric tests. A One-Way ANOVA was performed with a *post-hoc* Tukey-Kramer HSD test. In all tests, $\alpha = 0.05$, and a 95% confidence level was used. Data are expressed as means ± s.e.m.

3.4 RESULTS

The role of nitric oxide in memory changes with the time of conditioning

The time of olfactory conditioning influences the role of NO signaling in memory. Six conditioning times were chosen throughout the day governed by a 12hr light: 00:00 – 12:00/ 12hr dark: 12:00 – 24:00 cycle. At each conditioning time, animals were tested at 5 min, 1hr, 4hr, and 24 hr after conditioning. What we found was a circadian, time-dependent change of the role of NO signaling in memory. Figure 2 encompasses all six time points discussed below:

Photophase 02:30: This time point was chosen to mimic the physiological time of day 2.5 hours after sunrise in light conditions. Under light conditions, or photophase, *M. sexta* are at rest. When conditioned at 02:30, L-NAME-injected animals (L-NAME is a NOS inhibitor) show a significant decrease in proboscis extension when tested at 5 min ($F_{1,67}$ = 7.09, p = 0.009, N = 23), 1 hr ($F_{1,67}$ = 18.92, p < .0001, N = 23), and 4 hr ($F_{1,67}$ = 7.61, p

= 0.008, N = 23). There was no significant effect of L-NAME *versus* saline controls at 24 hours after conditioning ($F_{1,67}$ = 0.32, p = 0.57, N = 23).

Photophase 07:30: This time point was chosen to assess learning and memory mid-photophase, or 7.5 hours after sunrise. At this time, *M. sexta* are at rest. When conditioned at 07:30, L-NAME-injected animals show a significant decrease in proboscis extension only at 1 hr ($F_{1,73}$ = 34.51, p < 0.0001, N = 25). Unlike at photophase 02:30, memory tested at 5 min ($F_{1,73}$ = 2.78, p = 0.09, N = 25) and 4 hr ($F_{1,73}$ = 1.68, p = 0.19, N = 25) did not show significant differences with saline controls. There was no significant effect of L-NAME *versus* saline controls at 24 hours ($F_{1,73}$ = 0.01, p = 0.91, N = 25).

Photophase 11:30: This time point mimics the physiological time of day 30 minutes prior to dusk and the active evening period. When conditioned at 11:30, L-NAME-injected animals are not significantly different in proboscis extension than saline controls at any of the four time points tested (5 min: $F_{1,67}$ = 1.02, p = 0.32, N = 23; 1 hr: $F_{1,67}$ = 0.88, p = 0.35, N = 23; 4 hr: $F_{1,67}$ = 2.22, p = 0.14, N = 23; 24 hr: $F_{1,67}$ = 2.63, p = 0.11, N = 23).

Scotophase 14:30: This time point mimics the physiological time of day 2.5 hours after dusk. This time period is highly active. *M. sexta* can be found seeking mates, food, and sources to lay eggs. This time point was the time of conditioning in the Gage et al., 2013 study that reported the NO effects on short-term memory. When conditioned at 14:30, L-NAME injected animals show a significant decrease in proboscis extension at 5 min ($F_{1,64}$ = 5.07, p = 0.028, N = 22) and 1 hr post-conditioning ($F_{1,64}$ = 13.09, p = 0.0006, N

= 22). There was no significance found at 4 hours ($F_{1,64}$ = 2.21, p = 0.14, N = 22) or 24 hours ($F_{1,64}$ = 0.02, p = 0.89, N = 22).

Scotophase 19:30: This time point was chosen to assess learning and memory mid-scotophase. At this time, *M. sexta* are still active, but peak activity has begun to taper off (Gregory, 1963). When conditioned at 19:30, L-NAME-injected animals show a significant decrease in proboscis extension with saline controls at 5 min ($F_{1,67}$ = 9.80, p = 0.003, N = 23), 1 hr ($F_{1,67}$ = 10.36, p = 0.002, N = 23), and 4 hr post-conditioning ($F_{1,67}$ = 9.81, p = 0.002, N = 23). There was no effect of L-NAME *versus* saline controls at 24 hours ($F_{1,67}$ = 0.003, p = 0.96, N = 23).

Scotophase 23:30: This time point was chosen to assess learning and memory 30 minutes prior to sunrise. At this time, *M. sexta* are finding locations to hide and rest for the impending daytime hours. Similar to photophase 11:30 (just prior to the light switch to scotophase), L-NAME-injected animals conditioned at 23:30 do not show significant differences with saline controls at any time post-conditioning (5 min: $F_{1,58}$ = 1.07, p = 0.30, N = 20; 1 hr: $F_{1,58}$ = 0.04, p = 0.84, N = 20; 4 hr: $F_{1,58}$ = 0.42, p = 0.52, N = 20; 24 hr: $F_{1,58}$ = 0.07, p = 0.79, N = 20).

Individual memory windows are affected by circadian time

In addition to determining the treatment effect with L-NAME, it is necessary to consider individual memory traces collected over the six conditioning times. For example, we ask if there is an effect of conditioning time on the five minute memory trace. Figure 3 examines each of the four time points (5 min, 1hr, 4 hr, and 24 hr) in both

saline controls and L-NAME-injected animals to determine whether conditioning time is significant.

5 minutes after conditioning: The 5 min memory trace was examined separately in both saline- and L-NAME-treated animals. Saline-injected animals do not show a significant difference in proboscis extension with different conditioning times ($F_{5,192}$ = .51, p = 0.77). L-NAME treated moths, however, show a significant effect of conditioning time ($F_{5,204}$ = 2.74, p = 0.02). L-NAME-injected moths appear to have a peak 5 min memory trace prior to both light cycle switches, with a trough in between time points (Figure 2).

1 hour after conditioning: The 1 hr memory trace seems especially prominent in *M. sexta* and most affected by circadian time. Saline-injected animals show a significant difference in proboscis extension with respect to the time of conditioning ($F_{5,192}$ = 2.99, p = 0.01). Likewise, L-NAME animals also exhibit a significant difference in proboscis extension ($F_{5,204}$ = 5.08, p = 0.0002).

4 hours after conditioning: At 4 hours after conditioning, saline-treated animals show a borderline statistical significance between time points ($F_{5,192}$ = 2.15, p = 0.06, Tukey-Kramer *post-hoc* shows significance between the 14:30 and 23:30 time periods: p = .03). L-NAME-treated animals showed a significant difference in proboscis extension among conditioning times ($F_{5,204}$ = 6.12, p < .0001).

24 hours after conditioning: At 24 hours after conditioning, widely viewed as the time frame for long-term memory formation (Davis, 2011), the saline-injected animals do not show changes in proboscis extension among conditioning times ($F_{5,192}$ = 1.29, p = 0.27).

L-NAME-injected moths, however, surprisingly show an effect of conditioning time ($F_{5,204}$ = 2.51, p = 0.03).

3.5 DISCUSSION

There are two main findings in this study that further illustrate the role of NO in memory: (1) NO can affect multiple traces of memory, and (2) memory is unaffected by NO at both light cycle switches. Memory in normal animals is also affected by time of conditioning, especially at 23:30. In this discussion, we speculate about these results using an ecological and behavioral perspective.

NO affects longer memory windows in addition to short-term memory

At the 02:30 (photophase) and 19:30 (scotophase) time points, NO affects the 4 hr memory window (Figure 2). This time window compares with memory traces in *D. melanogaster* that reflect borderline intermediate-term, or long-term memory. It is not clear why NO would affect this longer trace at the 02:30 and 19:30 time points, but fluctuating basal NO levels may play a role. We know that at 02:30, basal NO averages approximately 50 nM; and at peak activity time 12 hours later at 14:30, basal NO is approximately 120 nM (Gage et al., 2013). These results may indicate that effects of NO in memory formation are concentration-dependent. For example, high levels of NO at 14:30 may form memory in the short-term while inhibiting longer forms of memory. At 02:30 when NO levels are lower, there are short-term memory effects as well as long-term. Another point to consider is the effect of L-NAME on basal NO levels. It would be useful to know how much L-NAME reduces basal NO. Perhaps at lower NO levels such

as at 02:30, L-NAME exerts a stronger decline in NO that affects a 4 hr memory window. In other words, NO may affect memory traces in a concentration-specific manner. In moving forward, it would helpful to determine basal NO levels at each conditioning time tested and also when 15mM L-NAME is applied.

We can also consider the importance of a 4 hr memory window to the animal. At 19:30, moths are still active, but the activity period is winding down. For example, four hours after 19:30, approximately 23:30-24:00 when the memory is retrieved, moths are looking for places to hide and rest. Sucrose levels in *Datura Wrightii*—the preferred, night-blooming host-plant of *M. sexta*—are also low. This may be a time period in which formation of intermediate- and long-term memory can be conserved. Likewise at 02:30, moths are at rest, and four hours later (06:30-07:30), *D. Wrightii* flowers have closed and activity is minimal. The role of other neurotransmitters and modulators should also be considered here. Intermediate- and long-term memory depend on multiple signal cascades. It could be that at high activity times when memory formation is crucial, many other signaling mechanisms are at work and we may not see an effect of NO. In contrast, when activity is low and memory is less crucial, we may see an effect of NO in these longer forms of memory.

NO does not affect memory at the light cycle switches

The second intriguing finding suggests that NO does not affect memory at the light cycle switches. This finding was surprising given the robust role of NOS inhibition in short-term memory. At both light cycle switches, from light to dark (11:30) and from

dark to light (23:30), NOS inhibition does not produce a significant change in memory compared with the saline controls (Figure 2). One interpretation may be that significant physiological changes are happening at these times to prepare for the light cycle/phase shift. The role of NO in memory may be overshadowed by other forms of neuromodulation happening here. The 11:30 time point, which precedes the nocturnal activity period, could be especially dominated by heightened physiological activity. This activity may be modulated by several neuromodulators. The ability to form and consolidate memories is also very important at this time. *M. sexta* are especially active one to two hours after dusk, and perhaps this crucial time is too important to be dominated by one neurotransmitter.

Five minute and one hour memory windows represent distinct memory traces

From the Gage et al., 2013 study, it was not clear whether short-term memory encompassed one memory trace that spanned from five minutes to an hour, or two memory traces. The 07:30 conditioning time suggests that these time windows represent two memory traces. At 07:30, NO significantly affects the 1 hr window, but not the 5 min window. If we compare these post-conditioning times with memory traces found in *D. melanogaster*, it would appear that different forms of memory may be occurring. Five minutes is considered to be short-term memory, which is characterized by modifications of already existing proteins such as those generated by second messengers, protein kinases, and phosphorylation (Susswein et al., 2004). The 1 hr window, however, borders short-term and intermediate-term memory. Intermediate-term memory has been

suggested to appear 30 to 70 minutes after conditioning and persist for a few hours (Davis, 2011). This form of memory depends on protein synthesis, but not transcription (Sutton et al., 2001). It is not clear what the function of intermediate-term memory is. It could be independent trace or a consolidating force (Davis, 2011). Sensitization studies in *Aplysia* suggest that intermediate memory affects mitogen-activated protein kinase (MAPK) activity and protein synthesis (Lyons et al., 2008). It could be that these time windows underlie different forms of memory, and therefore we can speculate that the distinction made between 5 min and 1 hr is the result of different molecular substrates. The results at 07:30 also highlight the robust role of NO in the formation of the 1 hr memory window.

Individual memory traces are affected by the circadian time of conditioning

In L-NAME-treated moths, conditioning time significantly affects all four memory windows (5 min, 1 hr, 4 hr, and 24 hr). These results support the hypothesis that NO in memory is modulated by circadian time. These results also suggest that olfactory memory in *M. sexta* may be regulated by the circadian clock, and NO may be directly or indirectly involved.

It is notable that the 24 hr memory window in L-NAME animals is affected by conditioning time. This time window is widely believed to represent long-term memory (Davis, 2011). The 24 hr trace, which did not show a significant difference compared with saline controls at any time point, is significantly affected by conditioning time. This may suggest that NO plays a small role in long-term memory as well. Perhaps under a

less robust conditioning paradigm, effects of NO in long-term memory can be seen. In addition, it may also reveal a more global effect of circadian regulation of long-term memory. There was not a significant difference found in saline-injected animals, but perhaps the effect noted in NOS-inhibited animals underscores a masked effect.

There is also a clear pattern among all memory windows. At the 5 min, 1 hr, 4 hr, and 24 hr windows, memory peaks prior to active scotophase (Figure 3). This might suggest that there are other mechanisms in place that promote memory at this important time. Serotonin, for example, may be a likely candidate. The serotonin immunoreactive neuron in *M. sexta* highly expresses NO target sGC, suggesting that these transmitter systems work in tandem. This interaction could account for the Ca^{2+}, cGMP/PKG and cAMP/MAPK/CREB cascades underlying circadian regulation of memory (Gerstner, 2012). A study examining both of these transmitters in circadian memory would be especially powerful.

Saline-injected animals illuminate memory and circadian time in M. sexta

The saline-injected controls show how memory is affected by conditioning time in *M. sexta* aside from the effect of NO. We saw a significant effect of conditioned time at the 1 hr memory window and a borderline effect at the 4 hr window, but not at 5 min and 24 hr.

The 5 min short-term memory window does not significantly differ with conditioning time. This finding is similar to other animal models examining short-term memory, including the well-studied *Aplysia* models (Lyons, 2011). It has been postulated

that short-term memory is needed throughout the circadian day to meet immediate survival needs, such as escaping from a predator (Lyons, 2011). The Lyons and Roman, 2008 study, however, does show that short-term memory is regulated by the circadian clock in *D. melanogaster*. This memory is dependent on *timeless* and *period* genes and seems to be controlled centrally, rather than by peripheral pacemakers. Our results suggest either that short-term memory in *M. sexta* is unregulated by the circadian clock, or that our paradigm using appetitive conditioning in multiple-spaced trials masks an effect on short-term memory.

In contrast, the 1 hr window—and possibly the 4 hr memory window—are significantly affected by conditioning time in saline-injected animals. The 1 hr memory window appears to be especially important in *M. sexta*. Bordering short- and intermediate-term memory, the 1 hr memory window is depressed at nearly all time points under NOS inhibition, and the single trace affected by conditioning time in saline-injected animals. The 1 hr trace is significantly depressed at the 23:30 time point in saline-injected animals, just before the light cycle switch to photophase. At this time, *M. sexta* are entering the rest phase. *D. wrightii* flowers have lower sucrose levels and are about to close. It is therefore likely, as suggested before, that energy could be conserved here. The formation of a 1 hr memory trace is unlikely to be beneficial during the rest period and may be regulated by the circadian clock. Similarly, the 4 hr memory window is borderline significant and is also depressed at the 23:30 conditioning time (Figure 2). The One-Way ANOVA test reveals a p-value equal to 0.06. The *post-hoc* Tukey-Kramer test, however, reveals a significant difference at the 23:30 time point. Similar to the 1 hr

memory trace, it may not be energetically favorable to form a memory trace when resources are low.

Many studies indicate that long-term memory, represented by the 24 hr memory window, is most under the control of the circadian clock. We would have predicted a difference with conditioning time among saline-injected animals, but no significant difference was found. Several reasons could explain this. Most studies use negative reinforcement paradigms (such as an odor paired with an electric shock) when considering circadian rhythms, which are thought to produce the most robust forms of memory (Lyons, 2011). Sucrose is highly rewarding in *M. sexta* but may not be as effective as negative reinforcement. Secondly, the conditioning protocol used is considered very robust. Six trials, spaced four minutes apart, were used to condition an animal. It could be that the strength of the conditioned association masked an effect on long-term memory. The 24 hr trace in the L-NAME-injected animals *did* show significance with circadian time. It would be interesting to see these results with fewer conditioning trials.

3.6 CONCLUSION

This study sought to shed light on two questions: (1) is there an optimal time of day for learning and memory in *M. sexta*, and (2) is the role of NO in memory modulated by the time of conditioning? Regarding the former, there does not seem to be a specific time of day in which learning and memory is optimal, but there is variation that appears phase-dependent. Prior to active scotophase, NOS inhibition does not affect olfactory

memory, which is evident by the high levels of proboscis extensions that are comparable with saline-injected animals. Prior to inactive photophase, however, there is a significant depression in proboscis extensions at the 1 hr and 4 hr memory windows in both saline and NOS-inhibited animals. These results suggest that there is a phase dependency in a least some forms of memory. Regarding the second question, NO also appears to be an important neuromodulator involved in memory, which is potentially circadian-regulated. Circadian time modulates the effect of NO in multiple memory formations, suggesting a role for NO in short-term, intermediate-term, and long-term memory. Moreover, NO's relationship with memory may be modulated by the circadian clock. It would be interesting to examine a connection between *period* and NO. *Period* is found in the glial cells surrounding olfactory glomeruli (Wise et al., 2002) and could possibly influence NO circadian activity and function within a glomerulus. NO activity may directly affect these pacemaker cells or be a downstream result. Taken altogether, NO may be of special interest for studies examining the circadian regulation of memory.

3.7 FIGURE LEGENDS AND FIGURES

Figure 1: Protocols outlining the sequence of injection, conditioning, and testing. Above: The first line, CS: Hibiscus, denotes the presentation of the conditioned stimulus (CS), hibiscus odor blend, through an air puff. Each raised step on this line refers to the presentation of hibiscus. For example, during conditioning, hibiscus was presented a total of six times to the antenna. The second line, US: sucrose, represents when sucrose, the unconditioned stimulus (US), was applied to the tip of proboscis relative to the CS odor puff. For example, sucrose was applied three seconds into the odor puff. Below: the protocol for testing learning and memory at different times over a 24-hour period. Each square represents the time of conditioning in six separate experiments. Scotophase, represented by the black bar, denotes subjective night; and photophase, represented by the white bar, denotes subjective day.

Figure 2: The time of conditioning changes the effect of nitric oxide in memory. Six separate experiments were performed to test the role of circadian time in olfactory learning and memory and the role of NO. Six time points were chosen: three in photophase (subjective day) and three in scotophase (subjective night). Conditioning began at the start of the time point (e.g. 14:30). After conditioning, animals were tested for their response to the CS (hibiscus odor) by observing the proboscis extension reflex at 5 min, 1 hr, 4 hr, and 24 hr. Asterisks denote significance between treatment groups (saline-injected and L-NAME-injected) using a One-Way ANOVA test. Total N = 136.

Figure 3: Examination of memory by treatment and conditioning time throughout the circadian day. Memory was tested by observing the proboscis extension reflex to the conditioned odor (CS, hibiscus) at 5 minutes, 1 hour, 4 hours, and 24 hours after conditioning. This sequence was repeated at six time points throughout the circadian day. Figure 3 shows the effects of circadian time on a single memory test (e.g. 5 minutes after conditioning). For example, the top left graph examines the 5 min results during each of the six time points tested around the circadian day. The black bar on each x-axis denotes the light cycle change (12hr light; 12hr dark) from photophase to scotophase beginning at 12:00. Asterisks denote a significant effect of conditioning time between at least one group/time point using a One-Way ANOVA test. Total N = 136.

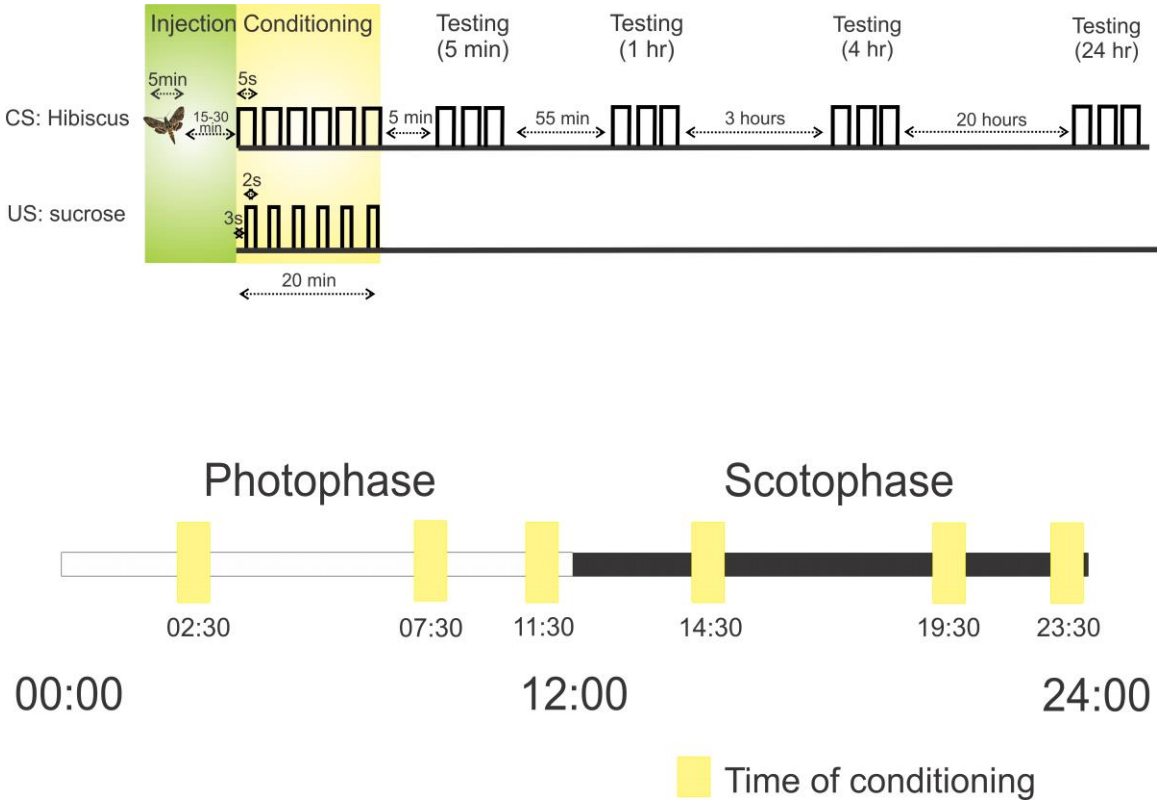

Figure 3.1: Protocols outlining the sequence of injection, conditioning, and testing.

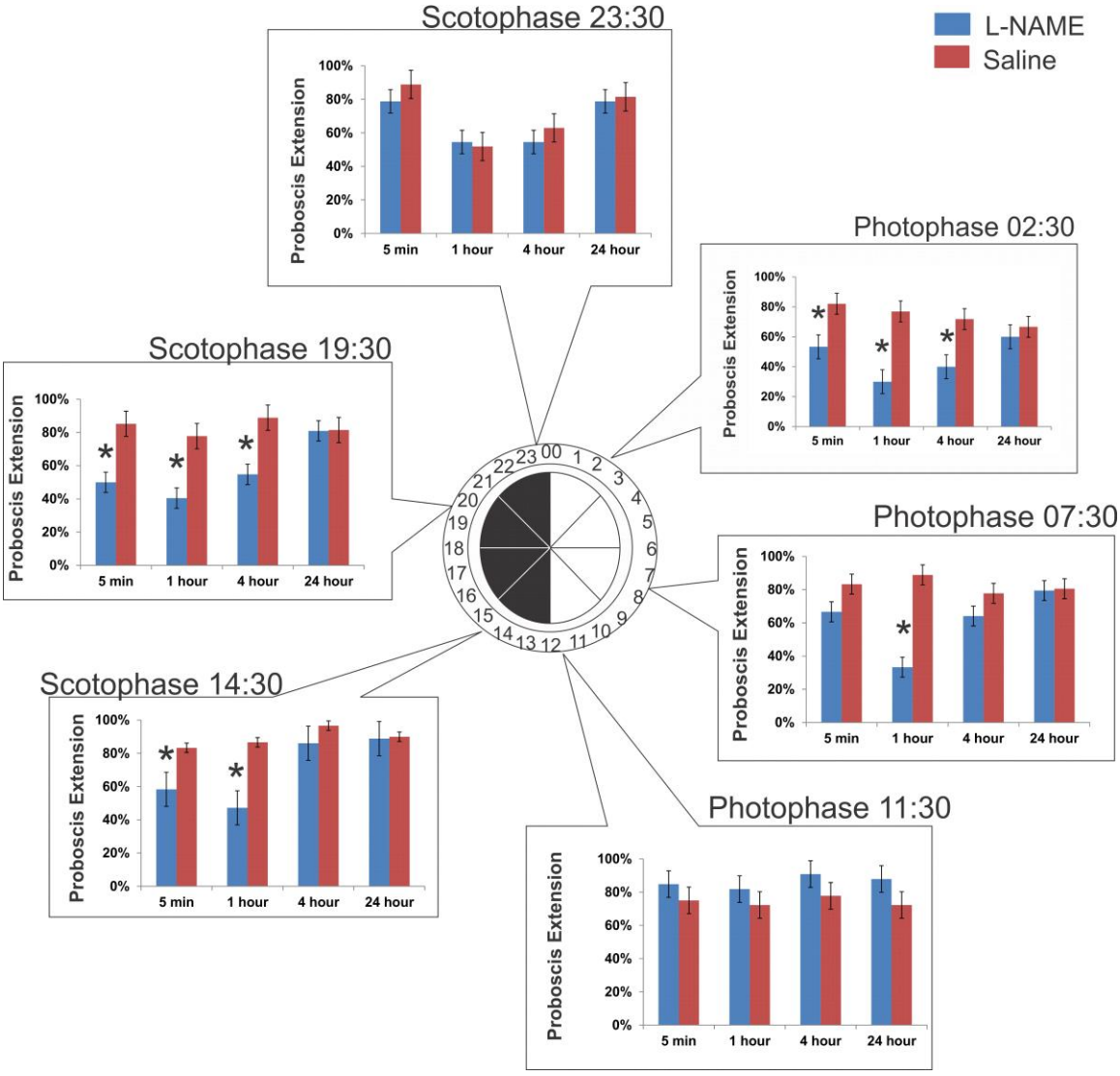

Figure 3.2: The time of conditioning changes the effect of nitric oxide in memory.

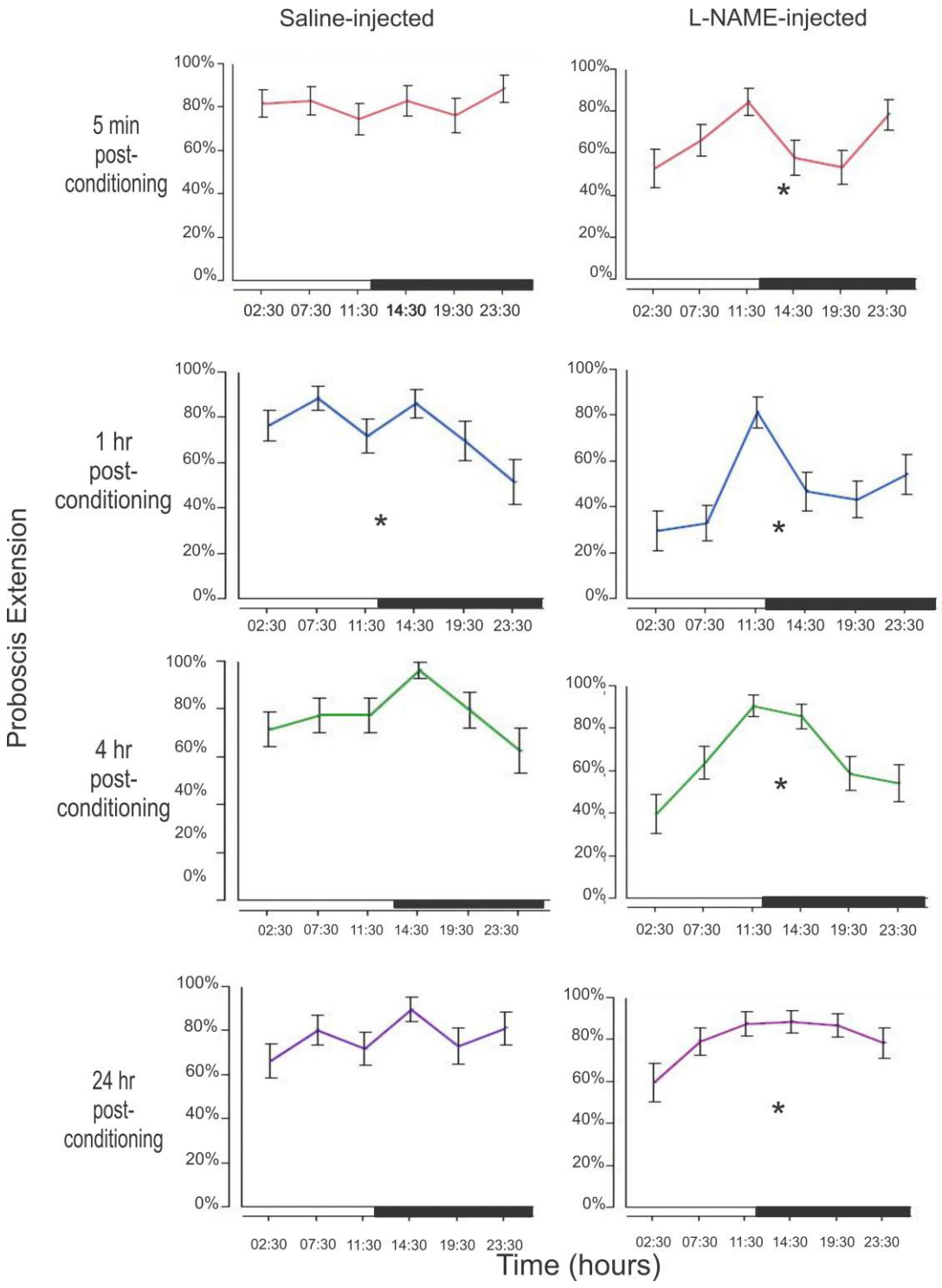

Figure 3.3: Examination of memory by treatment and conditioning time throughout the circadian day.

CHAPTER 4: CONCLUSIONS

4.1 SUMMARY OF RESULTS

This thesis sought to examine the central hypothesis that NO affects olfactory-guided behavior. Due to its widespread presence in primary olfactory networks, the behavioral significance of NO in odor processing is suggested to function similarly among species. In this thesis, methods were developed and tested to begin this investigation in the moth, *M. sexta*. Using a NO sensor, concentrations of NO basal levels were measured in isolated areas of the brain that include the antennal and optic lobes, and the remainder of the brain. NO levels are significantly higher in the sensory processing areas of the AL and optic lobe. This was the first study to measure basal NO levels in regions of the insect brain and the first to find a phase-dependent change in NO levels. These findings that there are phase-dependent changes in NO levels that roughly correspond to phase-dependent activities in the moth provide evidence that NO may indeed play a role in olfactory-guided behavior. The next step was to see how NO affected a specific behavior. A microinjection surgery technique was refined to manipulate levels of NO specifically in the AL, and this surgery was paired with olfactory conditioning assays modified to test olfactory learning and memory, odor detection, and odorant discrimination. NO plays a role in short-term memory and does not affect odor detection or discrimination between dissimilar odorants. Given these results, it was speculated that NO may affect the emerging phenomenon of circadian-regulated memory. Olfactory conditioning was performed at six time points around the

circadian clock to see (1) if memory in *M. sexta* is affected by the time of day, and (2) if the role of NO memory changes. Two unexpected results were found: NO affects longer forms of memory at certain times, and NO does not significantly affect memory at all just before the light cycle switches. This suggests that the role of NO in memory is mediated by circadian time and sheds light on the possibility that NO may be a key modulator in the circadian regulation of memory.

4.2 FUTURE STUDIES

The implications of better understood olfaction, memory, and circadian rhythms are numerous. The fact that NO may affect all of these processes makes its function especially intriguing. In this section, I will outline three areas of study that I think merit further attention.

Defining the role of NO in olfactory memory

The olfactory conditioning studies in this thesis all use six trials or pairings of the odor with the sucrose reward. These trials are spaced four minutes apart, which allows for longer forms of memory to develop during the time of conditioning (Menzel et al., 2001). It is very possible that the conditioning paradigm affects the observed role of NO in memory, similar to the finding in honeybees (Muller, 1996). One caveat of this study is that differences in paradigms were not performed. In moving forward, it would be beneficial to observe NO in memory with only one or two pairings of an odor and sucrose

reward. Then I would predict that we would see more variation in memory performance when examining the effect of circadian time in NOS-treated animals and in saline-treated animals especially.

Another point to consider is fluctuating basal NO. Only two time points were considered in in this study, yet the effect of the light cycle is robust. An important next step would be to determine whether NO fluctuation is entrained by the light cycle and fluctuates independently of exogenous stimuli. There are at least two ways to go about this question. First, one could sample NO in the *M. sexta* brain over a 24 to 48 period under a normal light/dark cycle to determine a pattern of fluctuation. Then that fluctuation can be determined under a dark/dark cycle. If the rhythm persists in dark/dark, this would support NO being an endogenous, circadian modulator of olfactory activity. Second, one could also test this hypothesis using the behavioral assays described in this thesis. Since a pattern of NO in olfactory memory has been established, one can then test the effect of the light cycle on memory. For example, animals can be entrained to a light cycle for several days, then on the day of memory testing, a dark/dark light cycle could be imposed. If NO and memory follows a similar pattern, it would support the role of NO as an endogenous modulator. It would also be interesting, however, if NO functions not as an endogenous circadian modulator, but as a dependent molecule on an exogenous light cue. This effect would result in a more rapid modulatory effect that presumably would occur in changing environmental conditions. Light is a strong oxidizer and could potentially influence NO directly. It seems noteworthy that these fluctuations of NO with the light cycle occurred in both sensory areas—the ALs and optic lobes.

NO and memory also need to be addressed at the level of the synapse. How is the circuitry of olfactory receptor neurons, projection neurons, and interneurons affected by memory formation? It would be interesting to look at changes in whole-cell current of AL neurons or calcium imaging during olfactory conditioning and at various temporal windows after. It should be possible to investigate this relationship in a semi-intact *M. sexta* prep.

Serotonin and NO Interaction in Learning and Memory

There could be a unique relationship between NO and serotonin in the AL. The strongest evidence for interaction is that the serotonin immunoreactive neuron highly expresses NO-target sGC in *M. sexta* (Collmann et al., 2004). It is also known that NOS inhibition affects the morphology of the serotonin immunoreactive neuron during development (Gibson et al., 2001). In theory as described below, NO has the potential to affect serotonin signaling and serotonin has the capacity to effect NO signaling. This interaction could be especially involved in olfactory learning and memory and the connection with circadian rhythms.

Serotonin can trigger the cAMP/PKA second messenger signaling cascade. This pathway has been well documented in *Aplysia* models of learning and memory (Kandel, 2013), LTP, and also in circadian regulation of memory (Gerstner, 2012). Manipulation of the cAMP cascade has been shown to cause a reduction in short-term memory (Nighorn et al., 1991; Han et al., 1992) and may be a mechanism by which NOS-inhibition affects short-term memory. Serotonin has been known to enhance, but not

mediate, olfactory learning and memory in behavior assays (McLean et al., 1993; Langdon et al., 1997), suggesting a smaller, modulatory role. Serotonin is also suggested to be a modulator of circadian activity that fluctuates with the light cycle (Kloppenburg et al., 1999; Dacks, 2007). Interaction with serotonin could be a potential mechanism for the observed effects of circadian time in olfactory memory, and could explain the non-effect of NO at the light cycle switches. I hypothesize that studies of learning and memory examining both serotonin and NO simultaneously will find a synergistic interaction between the two, suggesting they work in tandem.

NO and Glial Cells

Another intriguing study could be examining the relationship between glial cells and NO in the adult moth. We know that NO affects glial cell migration and formation of the AL in development (Gibson et al., 2001&2002), but not much is known in the adult. Two key findings of glial cells in the AL shed light on the possibility of neuron-glia communication *via* NO. Glial cells border the olfactory glomeruli (Oland and Tolbert, 1990), and it is suggested that these glial cells halt the diffusion of NO by functioning as an NO sink. Diffusion into cellular tissue is a suggested mechanism of inactivation of NO (Garthwaite, 2008), but perhaps there is a more involved mechanism. In addition, the glial cells surrounding AL glomeruli are the only AL cells found to express *period* (Wise et al., 2002). Given the recent evidence of NO and circadian time, interaction with *period*-expressing glia could be an underlying mechanism.

4.3 IMPLICATIONS AND CLINICAL SIGNIFICANCE

The study of olfaction not only benefits our understanding of how animals interact with the environment, but also our understanding of human health and disease. Olfaction is currently emerging as an early indicator in neurodegenerative diseases (Hawkes et al., 1999; George et al., 2013; Johansen et al., 2013; Poddighe et al., 2013; Seki et al., 2013). The most prominent example is in Parkinson's disease. Nearly 80% of Parkinson's disease patients classify as anosmic, having an impaired sense of smell (Hawkes et al., 1999). Studies have shown that these olfactory symptoms represent one of the earliest indicators of Parkinson's disease that substantially precedes the progression of motor symptoms. It has even been suggested that environmental contaminants entering through the nose may even trigger certain forms of Parkinson's disease (Hawkes et al., 1999). It would appear that perhaps we should consider the olfactory system more carefully in terms of disease progression and even as a point of entry. In addition, under specific conditions, NO appears to be produced in excess and may play a causative role in Parkinson's disease (Virarkar et al., 2013). If we have a better understanding of the olfactory system as a whole, including the role of NO, we can begin to understand disease pathology in the olfactory system, potentially diagnose a disease like Parkinson's earlier, and halt its progression.

The role of memory in human health and disease should also be considered further. Memory loss is another earlier indicator of several diseases. Memory loss is implicated in diseases such as dementia, Alzheimer's disease, and Huntington's disease.

These diseases further necessitate the need to better understand different forms of memory. For example, in Huntington's disease, short-term memory is particularly affected (American Speech-Language-Hearing Association, 2013). Multiple species exhibit temporal windows for various forms of memory (Gerstner, 2009), and if the scientific community can further pinpoint the substrates underlying individual memory traces, better therapies can be developed to improve specific memory impairments. Again, perhaps we should be thinking about memory loss not as a symptom, but rather as an important component in disease pathology.

Additionally, the circadian regulation of memory carries consequences for human health and performance. Circadian influence on memory is highly conserved among species (Gerstner, 2009). Studies in humans describe changes in cognitive performance based on the internal circadian clock and interactions with sleep-wake homeostasis (Gerstner, 2009; Ellenbogen et al., 2006; Ruby et al., 2008). Our understanding of short-term memory and its regulation by the circadian clock are especially important for cognitive performance (Lyons and Roman, 2008). These findings have implications for shift-workers, the medical profession, and the transportation industries (Lyons and Roman, 2008). The importance of short-term memory in olfaction also cannot be over stated. Short-term memory is intricately intertwined with a healthy olfactory system. Humans and other animals sniff or perceive an odor and make instantaneous conclusions that allow us to detect whether a particular odor is a dangerous warning, indicative of something pleasant, or even a trigger for a memory of a time and place in early childhood.

REFERENCES

Ahern, G. P., Klyachko, V. A., & Jackson, M. B. (2002). cGMP and S-nitrosylation: Two routes for modulation of neuronal excitability by NO. *Trends Neurosci., 25*(10), 510-517.

Akesson, B., & Lundquist, I. (1999). Nitric oxide and hydroperoxide affect islet hormone release and ca(2+) efflux. *Endocrine, 11*(1), 99-107.

American Speech-Language-Hearing Association. (2013). *Huntington's disease.* Retrieved May/2013, 2013, from http://www.asha.org/public/speech/disorders/HuntingtonsDisease.htm

Arenas, A., Fernandez, V. M., & Farina, W. M. (2009). Associative learning during early adulthood enhances later memory retention in honeybees. *PLoS One, 4*(12), e8046.

Arnhold, S., Andressen, C., Bloch, W., Mai, J. K., & Addicks, K. (1997). NO synthase-II is transiently expressed in embryonic mouse olfactory receptor neurons. *Neurosci.Lett., 229*(3), 165-168.

Bicker, G. (2001). Sources and targets of nitric oxide signalling in insect nervous systems. *Cell Tissue Res., 303*(2), 137-146.

Bicker, G., Schmachtenberg, O., & De Vente, J. (1996). The nitric oxide/cyclic GMP messenger system in olfactory pathways of the locust brain. *Eur.J.Neurosci., 8*(12), 2635-2643.

Bliss, T. V., & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J.Physiol., 232*(2), 331-356.

Boulton, C. L., Irving, A. J., Southam, E., Potier, B., Garthwaite, J., & Collingridge, G. L. (1994). The nitric oxide--cyclic GMP pathway and synaptic depression in rat hippocampal slices. *Eur.J.Neurosci., 6*(10), 1528-1535.

Bredt, D. S., Glatt, C. E., Hwang, P. M., Fotuhi, M., Dawson, T. M., & Snyder, S. H. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron, 7*(4), 615-624.

Breer, H., & Shepherd, G. M. (1993). Implications of the NO/cGMP system for olfaction. *Trends Neurosci., 16*(1), 5-9.

Brunton, L. (1908). *Therapeutics of the circulation. murray, london. 1908.* London, Murray:

Bullmann, T., & Stevenson, P. A. (2008). Nitric oxide as an efferent modulator of circadian pacemaker neurones in the eye of the marine mollusc bulla gouldiana.*1*, 18-28.

Burke, A. J., Sullivan, F. J., Giles, F. J., & Glynn, S. A. (2013). The yin and yang of nitric oxide in cancer progression. *Carcinogenesis*, *34*(3), 503-512.

Cherry, P. D., Furchgott, R. F., Zawadzki, J. V., & Jothianandan, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. .*72*, 2106-10.

Christensen, T. A., & Hildebrand, J. G. (1997). Coincident stimulation with pheromone components improves temporal pattern resolution in central olfactory neurons. *J.Neurophysiol.*, *77*(2), 775-781.

Christensen, T. A., Waldrop, B. R., & Hildebrand, J. G. (1998). Multitasking in the olfactory system: Context-dependent responses to odors reveal dual GABA-regulated coding mechanisms in single olfactory projection neurons. *J.Neurosci.*, *18*(15), 5999-6008.

Claassen, D. E., & Kammer, A. E. (1986). Effects of octopamine, dopamine, and serotonin on production of flight motor output by thoracic ganglia of manduca sexta. *J.Neurobiol.*, *17*(1), 1-14.

Collmann, C., Carlsson, M. A., Hansson, B. S., & Nighorn, A. (2004). Odorant-evoked nitric oxide signals in the antennal lobe of manduca sexta. *J.Neurosci., 24*(27), 6070-6077.

Craven, K. B., & Zagotta, W. N. (2006). CNG and HCN channels: Two peas, one pod. *Annu.Rev.Physiol., 68*, 375-401.

Dacks, A. M., Christensen, T. A., & Hildebrand, J. G. (2008). Modulation of olfactory information processing in the antennal lobe of manduca sexta by serotonin. *J.Neurophysiol., 99*(5), 2077-2085.

Dacks, A. M., Christensen, T. A., & Hildebrand, J. G. (2006a). Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain. *J.Comp.Neurol., 498*(6), 727-746.

Dacks, A. M., Dacks, J. B., Christensen, T. A., & Nighorn, A. J. (2006b). The cloning of one putative octopamine receptor and two putative serotonin receptors from the tobacco hawkmoth, manduca sexta. *Insect Biochem.Mol.Biol., 36*(9), 741-747.

Dacks, A. M., Riffell, J. A., Martin, J. P., Gage, S. L., & Nighorn, A. J. (2012). Olfactory modulation by dopamine in the context of aversive learning. *J.Neurophysiol., 108*(2), 539-550.

Dacks, A. M. (2007). *Serotonergic modulation of olfactory processing in the antennal lobe of the tobacco hawkmoth, manduca sexta*. Tucson, Arizona: University of Arizona.

Daly, K. C., Christensen, T. A., Lei, H., Smith, B. H., & Hildebrand, J. G. (2004). Learning modulates the ensemble representations for odors in primary olfactory networks. *Proc.Natl.Acad.Sci.U.S.A., 101*(28), 10476-10481.

Daly, K. C., & Smith, B. H. (2000). Associative olfactory learning in the moth manduca sexta. *J.Exp.Biol., 203*(Pt 13), 2025-2038.

Davis, R. L. (2011). Traces of drosophila memory. *Neuron, 70*(1), 8-19.

Decker, S., McConnaughey, S., & Page, T. L. (2007). Circadian regulation of insect olfactory learning. *Proc.Natl.Acad.Sci.U.S.A., 104*(40), 15905-15910.

Dellacorte, C., Kalinoski, D. L., Huque, T., Wysocki, L., & Restrepo, D. (1995). NADPH diaphorase staining suggests localization of nitric oxide synthase within mature vertebrate olfactory neurons. *Neuroscience,66*(1), 215-225.

Denker, M., Finke, R., Schaupp, F., Grun, S., & Menzel, R. (2010). Neural correlates of odor learning in the honeybee antennal lobe. *Eur.J.Neurosci., 31*(1), 119-133.

Doucette, W., & Restrepo, D. (2008). Profound context-dependent plasticity of mitral cell responses in olfactory bulb. *PLoS Biol., 6*(10), e258.

Dukas, R. (2008). Evolutionary biology of insect learning. *Annu.Rev.Entomol., 53*, 145-160.

Ellenbogen, J. M., Payne, J. D., & Stickgold, R. (2006). The role of sleep in declarative memory consolidation: Passive, permissive, active or none? *Curr.Opin.Neurobiol., 16*(6), 716-722.

Elphick, M., Rayne, R., Riveros-Moreno, V. V., Moncada, S., & Shea, M. (1995). Nitric oxide synthesis in locust olfactory interneurones. *J.Exp.Biol., 198*(Pt 3), 821-829.

Elphick, M. R., & Jones, I. W. (1998). Localization of soluble guanylyl cyclase alpha-subunit in identified insect neurons. *Brain Res., 800*(1), 174-179.

Endo, S., Suzuki, M., Sumi, M., Nairn, A. C., Morita, R., Yamakawa, K., et al. (1999). Molecular identification of human G-substrate, a possible downstream component of the cGMP-dependent protein kinase cascade in cerebellar purkinje cells. *Proc.Natl.Acad.Sci.U.S.A., 96*(5), 2467-2472.

Faber, T., Joerges, J., & Menzel, R. (1999). Associative learning modifies neural representations of odors in the insect brain. *Nat.Neurosci., 2*(1), 74-78.

Feil, R., Hartmann, J., Luo, C., Wolfsgruber, W., Schilling, K., Feil, S., et al. (2003). Impairment of LTD and cerebellar learning by purkinje cell-specific ablation of cGMP-dependent protein kinase I. *J.Cell Biol., 163*(2), 295-302.

Fernandez, P. C., Locatelli, F. F., Person-Rennell, N., Deleo, G., & Smith, B. H. (2009). Associative conditioning tunes transient dynamics of early olfactory processing. *J.Neurosci., 29*(33), 10191-10202.

Friebe, A., Mergia, E., Dangel, O., Lange, A., & Koesling, D. (2007). Fatal gastrointestinal obstruction and hypertension in mice lacking nitric oxide-sensitive guanylyl cyclase. *Proc.Natl.Acad.Sci.U.S.A., 104*(18), 7699-7704.

Fujie, S., Aonuma, H., Ito, I., Gelperin, A., & Ito, E. (2002). The nitric oxide/cyclic GMP pathway in the olfactory processing system of the terrestrial slug limax marginatus. *Zoolog Sci., 19*(1), 15-26.

Gage, S. L., Daly, K. C., & Nighorn, A. (2013). Nitric oxide affects short-term olfactory memory in the antennal lobe of manduca sexta. *J.Exp.Biol.,216*, 3294-3300.

Garthwaite, J. (2008). Concepts of neural nitric oxide-mediated transmission. *Eur.J.Neurosci., 27*(11), 2783-2802.

George, J., Jose, T., & Behari, M. (2013). Use of indian smell identification test for evaluating olfaction in idiopathic parkinson's disease patients in india. *Neurol.India, 61*(4), 365-370.

Gerstner, J. R. (2012). On the evolution of memory: A time for clocks. *Front.Mol.Neurosci., 5*, 23.

Gerstner, J. R., Lyons, L. C., Wright, K. P.,Jr, Loh, D. H., Rawashdeh, O., Eckel-Mahan, K. L., et al. (2009). Cycling behavior and memory formation. *J.Neurosci., 29*(41), 12824-12830.

Gibb, B. J., & Garthwaite, J. (2001). Subunits of the nitric oxide receptor, soluble guanylyl cyclase, expressed in rat brain. *Eur.J.Neurosci., 13*(3), 539-544.

Gibbs, S. M., & Truman, J. W. (1998). Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of drosophila. *Neuron, 20*(1), 83-93.

Gibson, N. J., & Nighorn, A. (2000). Expression of nitric oxide synthase and soluble guanylyl cyclase in the developing olfactory system of manduca sexta. *J.Comp.Neurol., 422*(2), 191-205.

Gibson, N. J., Rossler, W., Nighorn, A. J., Oland, L. A., Hildebrand, J. G., & Tolbert, L. P. (2001). Neuron-glia communication via nitric oxide is essential in establishing antennal-lobe structure in manduca sexta.*Dev.Biol., 240*(2), 326-339.

Giurfa, M., & Sandoz, J. C. (2012). Invertebrate learning and memory: Fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn.Mem., 19*(2), 54-66.

Granados-Fuentes, D., Prolo, L. M., Abraham, U., & Herzog, E. D. (2004). The suprachiasmatic nucleus entrains, but does not sustain, circadian rhythmicity in the olfactory bulb. *J.Neurosci., 24*(3), 615-619.

Grant, V. (1983). Behavior of hawkmoths on flowers of datura meteloides.*144*(2), pp. 280-284.

Gregory, D. P. (1963). Hawkmoth pollination in the genus oenothera.*5*, 357-384.

Guerenstein, P. G., A Yepez, E., Van Haren, J., Williams, D. G., & Hildebrand, J. G. (2004). Floral CO(2) emission may indicate food abundance to nectar-feeding moths. *Naturwissenschaften, 91*(7), 329-333.

Guthrie, F. (1859). Contributions to the knowledge of the amyl group 1. nitryl of amyl and its derivatives. *.11*, 245-52.

Han, P. L., Levin, L. R., Reed, R. R., & Davis, R. L. (1992). Preferential expression of the drosophila rutabaga gene in mushroom bodies, neural centers for learning in insects. *Neuron, 9*(4), 619-627.

Hansson, B. S., Christensen, T. A., & Hildebrand, J. G. (1991). Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth manduca sexta. *J.Comp.Neurol.,312*(2), 264-278.

Hawkes, C. H., Shephard, B. C., & Daniel, S. E. (1999). Is parkinson's disease a primary olfactory disorder? *Qjm, 92*(8), 473-480.

Hebb, D. O. (1949). *The organization of behavior.* New York: Wiley & Sons.

Hering, C. (1849). Glonoine, a new medicine for headache etc. *.4*(3)

Higgins, M., Miller, M., & Nighorn, A. (2012). Nitric oxide has differential effects on currents in different subsets of manduca sexta antennal lobe neurons. *PLoS One, 7*(8), e42556.

Hildebrand, J. G., & Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. *Annu.Rev.Neurosci., 20*, 595-631.

Homberg, U. (2002). Neurotransmitters and neuropeptides in the brain of the locust. *Microsc.Res.Tech., 56*(3), 189-209.

Hopkins, D. A., Steinbusch, H. W., Markerink-van Ittersum, M., & De Vente, J. (1996). Nitric oxide synthase, cGMP, and NO-mediated cGMP production in the olfactory bulb of the rat. *J.Comp.Neurol., 375*(4), 641-658.

Hoskins, S. G., Homberg, U., Kingan, T. G., Christensen, T. A., & Hildebrand, J. G. (1986). Immunocytochemistry of GABA in the antennal lobes of the sphinx moth manduca sexta. *Cell Tissue Res., 244*(2), 243-252.

Ignarro, L. J., Byrns, R. E., Buga, G. M., & Wood, K. S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *.61*, 866-79.

Ignarro, L. J. (2000). Introduction and overview. In L. J. Ignarro (Ed.), *Nitric oxide: Biology and PathobiologyI* (pp. 3) Academic Press.

Jaffrey, S. R., & Snyder, S. H. (1995). Nitric oxide: A neural messenger. *Annu.Rev.Cell Dev.Biol., 11*, 417-440.

Johansen, K. K., Waro, B. J., & Aasly, J. O. (2013). Olfactory dysfunction in sporadic parkinson's disease and LRRK2 carriers. *Acta Neurol.Scand.,*

Kandel, E. R., Schwartz, J. H., Jessell, T. M., Siegelbaum, S. A., & Hudspeth, A. J. (2013). *Principles of neural science* (5th ed.). New York: McGraw-Hill Companies.

Katsuki, S., Arnold, W., Mittal, C., & Murad, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine.*3*, 32-5.

Kay, L. M., & Laurent, G. (1999). Odor- and context-dependent modulation of mitral cell activity in behaving rats. *Nat.Neurosci., 2*(11), 1003-1009.

Kelley, J. B., Anderson, K. L., Altmann, S. L., & Itzhak, Y. (2011). Long-term memory of visually cued fear conditioning: Roles of the neuronal nitric oxide synthase gene and cyclic AMP response element-binding protein. *Neuroscience, 174*, 91-103.

Kelley, J. B., Anderson, K. L., & Itzhak, Y. (2010). Pharmacological modulators of nitric oxide signaling and contextual fear conditioning in mice. *Psychopharmacology (Berl), 210*(1), 65-74.

Kendrick, K. M., Guevara-Guzman, R., Zorrilla, J., Hinton, M. R., Broad, K. D., Mimmack, M., et al. (1997). Formation of olfactory memories mediated by nitric oxide. *Nature, 388*(6643), 670-674.

Kent, K. S., Hoskins, S. G., & Hildebrand, J. G. (1987). A novel serotonin-immunoreactive neuron in the antennal lobe of the sphinx moth manduca sexta persists throughout postembryonic life. *J.Neurobiol., 18*(5), 451-465.

Kishimoto, J., Keverne, E. B., Hardwick, J., & Emson, P. C. (1993). Localization of nitric oxide synthase in the mouse olfactory and vomeronasal system: A histochemical, immunological and in situ hybridization study. *Eur.J.Neurosci., 5*(12), 1684-1694.

Kline, D. D., Yang, T., Huang, P. L., & Prabhakar, N. R. (1998). Altered respiratory responses to hypoxia in mutant mice deficient in neuronal nitric oxide synthase. *J.Physiol., 511 (Pt 1)*(Pt 1), 273-287.

Kloppenburg, P., Ferns, D., & Mercer, A. R. (1999). Serotonin enhances central olfactory neuron responses to female sex pheromone in the male sphinx moth manduca sexta. *J.Neurosci., 19*(19), 8172-8181.

Kloppenburg, P., & Mercer, A. R. (2008). Serotonin modulation of moth central olfactory neurons. *Annu.Rev.Entomol., 53*, 179-190.

Knowles, R. G., & Moncada, S. (1992). Nitric oxide as a signal in blood vessels. *Trends Biochem.Sci., 17*(10), 399-402.

Langdon, P. E., Harley, C. W., & McLean, J. H. (1997). Increased beta adrenoceptor activation overcomes conditioned olfactory learning deficits induced by serotonin depletion. *Brain Res.Dev.Brain Res., 102*(2), 291-293.

Lei, H., Riffell, J. A., Gage, S. L., & Hildebrand, J. G. (2009). Contrast enhancement of stimulus intermittency in a primary olfactory network and its behavioral significance.*8*

Loulier, K., Ruat, M., & Traiffort, E. (2005). Analysis of hedgehog interacting protein in the brain and its expression in nitric oxide synthase-positive cells. *Neuroreport, 16*(17), 1959-1962.

Lowe, G., Buerk, D. G., Ma, J., & Gelperin, A. (2008). Tonic and stimulus-evoked nitric oxide production in the mouse olfactory bulb. *Neuroscience, 153*(3), 842-850.

Lyons, L. C. (2011). Critical role of the circadian clock in memory formation: Lessons from aplysia. *Front.Mol.Neurosci., 4*, 52.

Lyons, L. C., Green, C. L., & Eskin, A. (2008). Intermediate-term memory is modulated by the circadian clock. *J.Biol.Rhythms, 23*(6), 538-542.

Lyons, L. C., & Roman, G. (2008). Circadian modulation of short-term memory in drosophila. *Learn.Mem., 16*(1), 19-27.

Marsh, N., & Marsh, A. (2000). A short history of nitroglycerine and nitric oxide in pharmacology and physiology. *Clin.Exp.Pharmacol.Physiol., 27*(4), 313-319.

McLean, J. H., Darby-King, A., Sullivan, R. M., & King, S. R. (1993). Serotonergic influence on olfactory learning in the neonate rat. *Behav.Neural Biol., 60*(2), 152-162.

Menzel, R., Manz, G., Menzel, R., & Greggers, U. (2001). Massed and spaced learning in honeybees: The role of CS, US, the intertrial interval, and the test interval. *Learn.Mem., 8*(4), 198-208.

Moncada, S., Palmer, R. M., & Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol.Rev., 43*(2), 109-142.

Moreno-Lopez, B., Romero-Grimaldi, C., Noval, J. A., Murillo-Carretero, M., Matarredona, E. R., & Estrada, C. (2004). Nitric oxide is a physiological inhibitor of neurogenesis in the adult mouse subventricular zone and olfactory bulb. *J.Neurosci., 24*(1), 85-95.

Muller, U. (1996). Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, apis mellifera. *Neuron, 16*(3), 541-549.

Muller, U., & Hildebrandt, H. (1995). The nitric oxide/cGMP system in the antennal lobe of apis mellifera is implicated in integrative processing of chemosensory stimuli. *Eur.J.Neurosci., 7*(11), 2240-2248.

Murrell, W. (1879). Nitro-glycerine as a remedy for angina pectoris. .(80-1)

Mutlu, O., Ulak, G., & Belzung, C. (2011). Effects of nitric oxide synthase inhibitors 1-(2-trifluoromethylphenyl)--imidazole (TRIM) and 7-nitroindazole (7-NI) on learning and memory in mice. *Fundam.Clin.Pharmacol.,25*(3), 368-377.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *Faseb j., 6*(12), 3051-3064.

Neitz, A., Mergia, E., Imbrosci, B., Petrasch-Parwez, E., Eysel, U. T., Koesling, D., et al. (2013). Postsynaptic NO/cGMP increases NMDA receptor currents via hyperpolarization-activated cyclic nucleotide-gated channels in the hippocampus. *Cereb.Cortex,*

Nighorn, A., Gibson, N. J., Rivers, D. M., Hildebrand, J. G., & Morton, D. B. (1998). The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurons in the antennal lobe of manduca sexta. *J.Neurosci., 18*(18), 7244-7255.

Nighorn, A., Healy, M. J., & Davis, R. L. (1991). The cyclic AMP phosphodiesterase encoded by the drosophila dunce gene is concentrated in the mushroom body neuropil. *Neuron, 6*(3), 455-467.

O'Dell, T. J., Hawkins, R. D., Kandel, E. R., & Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: Evidence for nitric oxide as a possible early retrograde messenger.*Proc.Natl.Acad.Sci.U.S.A., 88*(24), 11285-11289.

Olmedo, M., O'Neill, J. S., Edgar, R. S., Valekunja, U. K., Reddy, A. B., & Merrow, M. (2012). Circadian regulation of olfaction and an evolutionarily conserved, nontranscriptional marker in caenorhabditis elegans.*Proc.Natl.Acad.Sci.U.S.A., 109*(50), 20479-20484.

Poddighe, S., Bhat, K. M., Setzu, M. D., Solla, P., Angioy, A. M., Marotta, R., et al. (2013). Impaired sense of smell in a drosophila parkinson's model. *PLoS One, 8*(8), e73156.

Prendergast, M. A., Terry, A. V.,Jr, Jackson, W. J., & Buccafusco, J. J. (1997a). Nitric oxide synthase inhibition impairs delayed recall in mature monkeys. *Pharmacol.Biochem.Behav., 56*(1), 81-87.

Prendergast, M. A., Buccafusco, J. J., & Terry, A. V.,Jr. (1997b). Nitric oxide synthase inhibition impairs spatial navigation learning and induces conditioned taste aversion. *Pharmacol.Biochem.Behav., 57*(1-2), 347-352.

Radomski, M. W., Palmer, R. M., & Moncada, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet, 2*(8567), 1057-1058.

Radomski, M. W., Palmer, R. M., & Moncada, S. (1987). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem.Biophys.Res.Commun., 148*(3), 1482-1489.

Raguso, R. A., & Willis, M. A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, manduca sexta.*69*, 407-418.

Raguso, R. A., & Willis, M. A. (2002). Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, manduca sexta.*64*, 685-695.

Rath, L., Giovanni Galizia, C., & Szyszka, P. (2011). Multiple memory traces after associative learning in the honey bee antennal lobe. *Eur.J.Neurosci., 34*(2), 352-360.

Regulski, M., & Tully, T. (1995). Molecular and biochemical characterization of dNOS: A drosophila Ca2+/calmodulin-dependent nitric oxide synthase. *Proc.Natl.Acad.Sci.U.S.A., 92*(20), 9072-9076.

Reisenman, C. E., Christensen, T. A., Francke, W., & Hildebrand, J. G. (2004). Enantioselectivity of projection neurons innervating identified olfactory glomeruli. *J.Neurosci., 24*(11), 2602-2611.

Reisenman, C. E., Christensen, T. A., & Hildebrand, J. G. (2005). Chemosensory selectivity of output neurons innervating an identified, sexually isomorphic olfactory glomerulus. *J.Neurosci., 25*(35), 8017-8026.

Reisenman, C. E., Riffell, J. A., Duffy, K., Pesque, A., Mikles, D., & Goodwin, B. (2013). Species-specific effects of herbivory on the oviposition behavior of the moth manduca sexta. *J.Chem.Ecol., 39*(1), 76-89.

Reisenman, C. E., Riffell, J. A., & Hildebrand, J. G. (2009). Neuroethology of oviposition behavior in the moth manduca sexta. *Ann.N.Y.Acad.Sci., 1170,* 462-467.

Richardson, B. (1864). Report of the physiological action of nitrite of amyl. .*34*, 120-9.

Riffell, J. A., Alarcon, R., & Abrell, L. (2008). Floral trait associations in hawkmoth-specialized and mixed pollination systems: Datura wrightii and agave spp. in the sonoran desert. *Commun.Integr.Biol., 1*(1), 6-8.

Riffell, J. A., Lei, H., Christensen, T. A., & Hildebrand, J. G. (2009). Characterization and coding of behaviorally significant odor mixtures. *Curr.Biol., 19*(4), 335-340.

Roskams, A. J., Bredt, D. S., Dawson, T. M., & Ronnett, G. V. (1994). Nitric oxide mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons. *Neuron, 13*(2), 289-299.

Rossler, W., Oland, L. A., Higgins, M. R., Hildebrand, J. G., & Tolbert, L. P. (1999). Development of a glia-rich axon-sorting zone in the olfactory pathway of the moth manduca sexta. *J.Neurosci., 19*(22), 9865-9877.

Rossler, W., Randolph, P. W., Tolbert, L. P., & Hildebrand, J. G. (1999). Axons of olfactory receptor cells of transsexually grafted antennae induce development of sexually dimorphic glomeruli in manduca sexta.*J.Neurobiol., 38*(4), 521-541.

Ruby, N. F., Hwang, C. E., Wessells, C., Fernandez, F., Zhang, P., Sapolsky, R., et al. (2008). Hippocampal-dependent learning requires a functional circadian system. *Proc.Natl.Acad.Sci.U.S.A., 105*(40), 15593-15598.

Samama, B., & Boehm, N. (1999). Inhibition of nitric oxide synthase impairs early olfactory associative learning in newborn rats, *Neurobiol.Learn.Mem., 71*(2), 219-231.

Sandoz, J. C. (2003). Olfactory perception and learning in the honey bee (apis mellifera): Calcium imaging in the antenna lobe. [Perception et apprentissage olfactifs chez l'Abeille domestique (Apis mellifera): imagerie calcique dans le lobe antennaire] *J.Soc.Biol., 197*(3), 277-282.

Schachtner, J., Homberg, U., & Truman, J. W. (1999). Regulation of cyclic GMP elevation in the developing antennal lobe of the sphinx moth, manduca sexta. *J.Neurobiol., 41*(3), 359-375.

Schmidt, H. H., & Walter, U. (1994). NO at work. *Cell, 78*(6), 919-925.

Schuman, E. M., & Madison, D. V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science, 254*(5037), 1503-1506.

Schuman, E. M., Meffert, M. K., Schulman, H., & Madison, D. V. (1994). An ADP-ribosyltransferase as a potential target for nitric oxide action in hippocampal long-term potentiation. *Proc.Natl.Acad.Sci.U.S.A., 91*(25), 11958-11962.

Seki, K., Tsuruta, K., Inatsu, A., Fukumoto, Y., & Shigeta, M. (2013). Classification of reduced sense of smell in women with parkinson's disease. *Nihon Ronen Igakkai Zasshi., 50*(2), 243-248.

Stengl, M., Homberg, U., & Hildebrand, J. G. (1990). Acetylcholinesterase activity in antennal receptor neurons of the sphinx moth manduca sexta. *Cell Tissue Res., 262*(2), 245-252.

Sun, X. J., Tolbert, L. P., & Hildebrand, J. G. (1997). Synaptic organization of the uniglomerular projection neurons of the antennal lobe of the moth manduca sexta: A laser scanning confocal and electron microscopic study. *J.Comp.Neurol., 379*(1), 2-20.

Susswein, A. J., Katzoff, A., Miller, N., & Hurwitz, I. (2004). Nitric oxide and memory. *Neuroscientist, 10*(2), 153-162.

Sutton, M. A., Masters, S. E., Bagnall, M. W., & Carew, T. J. (2001). Molecular mechanisms underlying a unique intermediate phase of memory in aplysia. *Neuron, 31*(1), 143-154.

Takeda, K. (1961). Classical conditioned response in the honey bee.*6*, 168-179.

Tolbert, L. P., & Oland, L. A. (1990). Glial cells form boundaries for developing insect olfactory glomeruli. *Exp.Neurol., 109*(1), 19-28.

Tolbert, L. P., Oland, L. A., Tucker, E. S., Gibson, N. J., Higgins, M. R., & Lipscomb, B. W. (2004). Bidirectional influences between neurons and glial cells in the developing olfactory system. *Prog.Neurobiol., 73*(2), 73-105.

Truman, J. W., De Vente, J., & Ball, E. E. (1996). Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects. *Development, 122*(12), 3949-3958.

Virarkar, M., Alappat, L., Bradford, P. G., & Awad, A. B. (2013). L-arginine and nitric oxide in CNS function and neurodegenerative diseases. *Crit.Rev.Food Sci.Nutr., 53*(11), 1157-1167.

Vosshall, L. B., Wong, A. M., & Axel, R. (2000). An olfactory sensory map in the fly brain. *Cell, 102*(2), 147-159.

Wang, Y., Mamiya, A., Chiang, A. S., & Zhong, Y. (2008). Imaging of an early memory trace in the drosophila mushroom body. *J.Neurosci., 28*(17), 4368-4376.

Weruaga, E., Crespo, C., Porteros, A., Brinon, J. G., Arevalo, R., Aijon, J., et al. (1998). NADPH-diaphorase histochemistry reveals heterogeneity in the distribution of nitric oxide synthase-expressing interneurons between olfactory glomeruli in two mouse strains. *J.Neurosci.Res., 53*(2), 239-250.

Williams, J. H., Li, Y. G., Nayak, A., Errington, M. L., Murphy, K. P., & Bliss, T. V. (1993). The suppression of long-term potentiation in rat hippocampus by inhibitors of nitric oxide synthase is temperature and age dependent. *Neuron, 11*(5), 877-884.

Willis, M. A., & Arbas, E. A. (1991). Odor-modulated upwind flight of the sphinx moth, manduca sexta L. *J.Comp.Physiol.A., 169*(4), 427-440.

Wilson, C. H. (2005). An examination of the effects and possible targets of nitric oxide in olfactory neurons in the moth, manduca sexta . (PhD, University of Arizona).

Wilson, C. H., Christensen, T. A., & Nighorn, A. J. (2007). Inhibition of nitric oxide and soluble guanylyl cyclase signaling affects olfactory neuron activity in the moth, manduca sexta. *J.Comp.Physiol.A.Neuroethol Sens.Neural Behav.Physiol., 193*(7), 715-728.

Wise, S., Davis, N. T., Tyndale, E., Noveral, J., Folwell, M. G., Bedian, V., et al. (2002). Neuroanatomical studies of period gene expression in the hawkmoth, manduca sexta. *J.Comp.Neurol., 447*(4), 366-380.

Yabumoto, T., Takanashi, F., Kirino, Y., & Watanabe, S. (2008). Nitric oxide is involved in appetitive but not aversive olfactory learning in the land mollusk limax valentianus. *Learn.Mem., 15*(4), 229-232.

Yamada, K., Noda, Y., Nakayama, S., Komori, Y., Sugihara, H., Hasegawa, T., et al. (1995). Role of nitric oxide in learning and memory and in monoamine metabolism in the rat brain. *Br.J.Pharmacol., 115*(5), 852-858.

Yamamoto, R. T., Jenkins, R. Y., & McClusky, R. K. (1969). Factors determining the selection of plants for oviposition by the tabacco hornworm. *Ent. Exp. & Appl, 12*, 504-508.

Yeh, C. I., & Powers, A. S. (2005). Effects of blocking nitric oxide on learning in turtles (chrysemys picta). *Behav.Neurosci., 119*(6), 1656-1661.

Yu, D., Keene, A. C., Srivatsan, A., Waddell, S., & Davis, R. L. (2005). Drosophila DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning. *Cell, 123*(5), 945-957.

Yu, D., Ponomarev, A., & Davis, R. L. (2004). Altered representation of the spatial code for odors after olfactory classical conditioning; memory trace formation by synaptic recruitment. *Neuron, 42*(3), 437-449.

Yuda, M., Higuchi, K., Sun, J., Kureishi, Y., Ito, M., & Chinzei, Y. (1997). Expression, reconstitution and characterization of prolixin-S as a vasodilator--a salivary gland nitric-oxide-binding hemoprotein of rhodnius prolixus. *Eur.J.Biochem., 249*(1), 337-342.

Zhao, H., Firestein, S., & Greer, C. A. (1994). NADPH-diaphorase localization in the olfactory system. *Neuroreport*, *6*(1), 149-152.