THE EFFECT OF INTRABASALIS OREXIN A INFUSION ON REVERSAL LEARNING PERFORMANCE IN RATS WITH 192 IGG-SAPORIN LESIONS OF THE NUCLEUS BASALIS MAGNOCELLULARIS

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(Psychology and Neuroscience)

Submitted as a St. Mary's Project
in Partial Fulfillment of the Graduation Requirements
for the Degree of Bachelor of Arts in Psychology

May, 2010

St. Mary's College of Maryland
St. Mary's City, Maryland

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Abstract

Alzheimer’s disease (AD) is characterized by hypofunction of the basal forebrain cholinergic system, which results in the memory and attentional deficits observed in individuals suffering from the disease. Progressive neurodegeneration renders the primary source of cortical acetylcholine (ACh), the nucleus basalis magnocellularis (nBM), unable to innervate the cortex at normal physiological levels. Recent research has implicated a group of hypothalamic neuropeptides, the orexins (orexin A and B, also known as hypocretin 1 and 2), in aiding in the efflux of endogenous ACh from the nBM to the cortex. Microdialysis administration of orexin A (OxA) to the nBM in rats has been shown to stimulate cortical ACh release and decrease feeding latency in response to an appetitive stimulus. No previous research has evaluated the impact of OxA administration on performance of a cortical dependent task in animals with selective cholinergic lesions of the nBM. The current study attempted to examine the effect of intrabasalis administration of OxA on olfactory discrimination reversal learning (ODRL) performance in rats with 192 IgG-saporin lesions of the nBM. It was hypothesized that OxA administration prior to reversal would ameliorate the reversal learning deficit characteristic of animals with nBM lesions. Results indicated that animals with cholinergic lesions trended towards impairment during reversal, although no effect of OxA was observed on performance during any stage of the ODRL task. Implications and possible confounds relating to cannula placement and lesion efficacy are discussed.
The effect of intrabasalis orexin A infusion on reversal learning performance in rats with 192 IgG-saporin lesions of the nucleus basalis magnocellularis

The basal forebrain is the neuroanatomical region that provides the majority of cholinergic input to the cerebral cortex, the amygdala, the hippocampus, and the olfactory bulb (Mesulam et al., 1983). In general, the basal forebrain cholinergic system (BFCS) is conceptualized as being composed of six anatomically distinct regions, classified as regions Ch1-Ch6 (Mesulam et al., 1983). The Ch4 region of the basal forebrain contains the nucleus basalis magnocellularis (nBM) and the substantia innominata (SI), nuclei that are closely related anatomically (Mesulam et al., 1983; Ezrin-Waters & Resch, 1989) as well as functionally (Schliebs, Roßner, & Bigl, 1996; Maddux et al., 2007). The nBM/SI complex is composed primarily of cholinergic neurons, which project to the cortex, although approximately 10% of the cholinergic neurons project to the amygdala (Mesulam et al., 1983; Mesulam, 2004).

Hypofunction of the BFCS is thought to result in the attentional, homeostatic, and memory deficits demonstrated by individuals suffering from Alzheimer’s disease (AD). Disruption of normal cholinergic functioning occurs as a result of the three traditional neuropathological hallmarks of AD: neurofibrillary tangles, amyloid plaques, and neuronal atrophy. Neuronal atrophy is relevant to the BFCS in particular, as degeneration of the magnocellular layers of the nucleus basalis of Meynert (the primate correlate of the nBM; Ezrin-Waters & Resch, 1989; Muir, 1997) results in the cognitive deficits associated with AD.

For the last four decades, much of the AD research has fit tightly under the “cholinergic hypothesis” umbrella, the central dogma suggesting that cholinergic dysfunction is the primary causal mechanism of the cognitive deficits associated with AD. Undeniably, voluminous amounts of both human and animal literature support this causal link. In humans, levels of
choline acetyltransferase (ChAT), an enzyme that functions to synthesize acetylcholine, were observed to be decreased in the cortex of individuals with AD (Bird et al., 1983). This decrease in ChAT levels was found to share a strong positive correlation with the severity of dementia symptoms, indicating that the severity of dementia may depend on the extent of cholinergic deinnervation (Perry et al., 1978; Geula & Mesulam, 1996). In addition to the lower levels of ChAT in the cortex, levels in the hippocampal formation, the area of the medial temporal lobe thought to be critical for learning and memory, as well as in the SI, were observed to be lower in individuals with AD (Bird et al., 1983). It is clear that ACh is critical for cortical processing, and the cholinergic system is disrupted in individuals with AD, but the extent to which other neurochemicals interact with these factors continues to be less clear.

As neuronal death in the nBM/SI is one of the primary neurological characteristics of AD, animal research has attempted to reproduce cognitive deficits similar to those manifested by individuals with AD by neurochemically damaging portions of the basal forebrain, including the nBM/SI (Wiley, Oeltmann, & Lappi, 1991; Baxter, Bucci, Gorman, Wiley, & Gallagher, 1995). Various lesion studies have established the role of the nBM/SI complex as being critical for cortical-dependent behaviors, including negative patterning (Butt, Noble, Rogers, & Rea, 2002), learning set acquisition, (Bailey, Rudisill, Hoof, & Loving, 2003) attention (Lehmann, Grottick, Cassell, & Higgins, 2003) and the encoding of feature binding (Botly & De Rosa, 2007). By utilizing 192 IgG-saporin (192 IgG-SAP), a choline-specific immunotoxin, to remove the cholinergic innervations from the nBM to the cortex, Butt et al. (2002) illustrated that the nBM was critical for the ability to attend to multiple stimuli in a negative patterning paradigm, but not the ability to make simple discriminations. This lack of simple discrimination impairment suggests that the nBM/SI may be particularly critical for more complex cognitive processes, but
not those that involve less cognitive effort. Similarly, disruptions in the formation and shifting of a learning set have been observed following nBM lesions (Bailey et al., 2003). Complex abilities that require attentional performance, such as feature binding, are also dependent on the cholinergic system (Botly & De Rosa, 2009). Cabrera, Chavez, Corley, & Butt (2006) propose that the general function of the nBM/SI complex is to maintain cognitive flexibility, which they defined as the ability to shift one's behavior in response to changes in the stimulus environment. Thus, animals with nBM/SI lesions typically exhibit deficits in tasks such as a reversal learning paradigm, where animals must shift from a previously reinforced rule to the previously unrewarded rule in order to again be rewarded.

In recent years, researchers have established a link between a discrete group of non-cholinergic neuromodulators in the basal forebrain and the stimulation of ACh release to the cortex. One family of neuropeptides originating in the lateral hypothalamus, known as the orexins (also termed the hypocretins), have been shown to form direct excitatory synapses on basal forebrain cholinergic neurons (Eggermann et al., 2001; Sakurai et al., 2005). The orexins are composed of two distinct neuropeptides, orexin A (OxA) and orexin B (OxB; Sakurai et al. 1998). Two types of orexin receptors are heavily expressed in the basal forebrain, the orexin-1 receptor (Ox1R), which binds OxA with high affinity, and the orexin-2 receptor (Ox2R), which binds both OxA and OxB with equal affinity (Sakurai et al., 1998). Microdialysis studies have revealed that administration of OxA to the basal forebrain results in increased cortical ACh release (Fadel, Pasumartthi, & Reznikov, 2005; Fadel & Frederick-Duus, 2008). In a novel experiment, Frederick-Duus, Guyton, & Fadel (2007) illustrated that the orexin/BFCS system is necessary for cortical ACh release in response to appetitive stimuli. Researchers eliminated the majority of orexin neurons in the BFCS using the toxin orexin-B-saporin, and observed that
lesioned animals exhibited severely diminished cortical ACh efflux in the prefrontal cortex (PFC) in response to an appetitive stimulus, as compared to sham animals (Frederick-Duus et al., 2007). Providing further support for the necessity of the orexins for cortical ACh efflux, animals given an orexin antagonist (SB 334867) had diminished cortical ACh efflux and increased feeding latency, as compared to saline-infused controls (Frederick-Duus et al., 2007).

The orexin system was thus hypothesized to be recruited in response to interoceptive cues (i.e. hunger), in order to focus attention on exteroceptive cues (i.e. food). This allocation of processes related to attention are manifested neurochemically as an increase in cortical ACh efflux, suggesting that the orexins may be able to influence cortical (and thus cholinergic)-dependent behaviors related to cognition. As of yet, there has been no empirical investigation of the possible impact of OxA administration on cortical-dependent behavior, such as those that are resultant from damage to the basal forebrain. The potential for the orexins to increase cortical ACh efflux has important clinical implications for individuals suffering from AD, particularly if ACh efflux is stimulated following the partial deafferentation of cholinergic neurons of the nBM.

The goal of the current study was to evaluate the impact of OxA infusion on performance of a cortical-dependant behavioral task in animals with 192 IgG-SAP lesions of the nBM/SI. The impact of intrabasalis administration of OxA on a cortical dependent cognitive task has not previously been evaluated in animals with lesions of the BFCS. We hypothesized that intrabasalis OxA administration would attenuate the reversal learning deficits typically observed in animals with lesion-induced cholinergic deafferentation.

Methods

Subjects
Twenty-four male Sprague Dawley rats were purchased for use in the study (Charles River, Raleigh, NC). Rats arrived at 60 days of age, and were randomly assigned to one of four treatment conditions: nBM lesion + OxA administration (n = 6), nBM lesion + artificial cerebrospinal fluid (aCSF) administration (n = 6), sham lesion + OxA administration (n = 6), or sham lesion + aCSF administration (n = 6). Rats were individually housed in standard 42.5 cm (length) x 21.0 cm (width) x 21.0 cm (height) polycarbonate cages. All animals were housed in a temperature controlled room, with a standard 12/12 hr light cycle (light from 9 AM – 9 PM). Rats were handled for approximately four days prior to surgery. During this pre-surgical time, rats were allowed access to food and water *ad libitum*. Following the onset of behavioral testing, all rats were limited to approximately 20g of food pellets (Prolab, Brentwood, MO) per day, until the cessation of behavioral testing. Study protocols were approved by the St. Mary’s College of Maryland Institutional Animal Care and Use Committee.

*Stereotaxic Surgery*

Rats underwent either bilateral 192 IgG-SAP lesions of the nBM/SI (n = 12) or bilateral sham surgery with Dulbecco’s phosphate buffered saline administration (n = 12). On approximately postnatal day 70, all rats were anaesthetized using a combination of the alpha-2 agonist xylazine (6 mg/kg ip; Lloyd Laboratories, Shenandoah, IA) and ketamine (90 mg/kg ip; Phoenix Pharmaceutical Inc., St. Josephs, MO). Lesion coordinates followed those of Berger-Sweeney, Heckers, Mesulam, Wiley, Lappi, & Sharma (1994). Anaesthetized rats were placed on a stereotaxic apparatus (intraural bar set at -3.3 mm), and an incision was made along the midline of the skull, allowing for the removal of skin and muscle fibers. The stereotaxic arms were placed at the following coordinates according to bregma: AP = -0.9 mm, M/L = ±2.8 mm, and DV = -7.2 mm below dura (Berger-Sweeney et al., 1994). Bilateral infusions of a 0.2 µl (at
Orexin 7

0.375 µg/µl) 192 IgG-SAP (CHEMICON International, Temecula, CA) were conducted at a constant rate of 0.1 µl/min via a 10 µl Hamilton syringe. The cannula remained in place for 3 min to allow for diffusion of the toxin. Two 26-gauge guide cannulas (PlasticsOne, Roanoke, VA) were then inserted bilaterally, with the stereotaxic arms set at 10° angles, at the following coordinates according to bregma: AP = -0.9 mm, M/L = ±3.8 mm, and DV = -7.5 mm below dura. Each cannula was secured with dental cement (Dentsply International Inc., York, PA) and Duralay (Dental Mfg. Co., Worth, IL) to 4 stainless steel surgical screws, and a 31.5-gauge dummy cannula (PlasticsOne, Roanoke, VA) was inserted into each guide cannula. Sham surgical procedures were identical to the lesion procedures, except that the vehicle (0.2 µl of Dulbecco’s phosphate buffered saline; Sigma-Aldrich Inc, St. Louis, MO) were injected sans toxin.

Following the completion of surgery, all animals were given an injection of ketofen (5 mg/kg sc; Fort Dodge Animal Health, Fort Dodge, IA), which acts as an analgesic, as well as 3 cc of physiological saline (ip; Agri Laboratories Ltd., St. Josephs, MO). Rats were placed on a heating pad until they regained movement, at which point they were placed back into their standard single housed cages, accompanied by seven Froot Loop® rewards, which would later act as the appetitive reward during behavioral testing. Body weight, food and water intake, and wound health were monitored closely for 10 post-operative days. During post-operative recovery, dummy cannulas were routinely loosened and re-secured to the guide cannula to prevent complications such as blockage of the guide cannula. Body weight was recorded weekly for the duration of behavioral testing.

Apparatus
Testing occurred in a 42.5 cm (length) x 21.0 cm (width) x 21.0 cm (height) polycarbonate cage. A 11.4 cm (length) x 6.0 cm (width) x 1.0 cm (height) black wooden insert with two plastic food cups (5.7 cm (height) x 3.2 cm (diameter) plastic Dixie® cups) secured to one end was placed at the end of the cage. During testing, a mesh cage cover was placed over the apparatus. Before each trial, access to the food cups was prevented by a mesh insert. This mesh insert was further reinforced before each trial by an 18.5 cm (width) x 30 cm (height) wooden insert, which prevented visual cues during re-baiting of the food cups. Olfactory stimuli were composed of 2g of a pseudo-randomly selected spice (McCormick & Co. Inc., Hunt Valley, MD) and 220g of unscented sand (Lowes Home Improvement Warehouse). All testing occurred in the same temperature controlled room during the light portion of the rat’s light/dark cycle. Following completion of testing by each rat, the testing apparatus was thoroughly cleaned with quatricide (Pharmacal Research Laboratories Inc., Naugatuck, CT).

Behavioral Testing

Pretraining procedure. Rats underwent the pretraining stage of the behavioral task on approximately their 11th post-surgical day. Behavioral testing procedures followed those of Bailey et al. (2003). Each animal was food deprived (approximately 20g/day) one day prior to the onset of behavioral testing. All animals were fed immediately following the completion of behavioral testing each day.

On the first pretraining day, rats were exposed to the testing cage. A cup filled with unscented sand containing two partially buried Froot Loops® was placed into the right side of the wooden food cup insert, inside the testing cage. Each animal was allowed a maximum of 10 min to find and eat the Froot Loop® rewards. The animal was removed from the testing apparatus following consumption of all food rewards, or 10 min without finishing the food.
rewards. If the animal consumed the Froot Loop® rewards, they were placed back into their standard housing cage. If the animal reached the 10 min time limit, the animal was returned to its home cage and this procedure was repeated on the next testing day.

On the second and third pretraining days, rats were again presented with a cup of unscented sand with two partially buried Froot Loop® rewards. For these two pretraining days, the cup containing unscented sand and the Froot Loop® rewards was placed on the left side of the wooden food cup insert. During day four of pretraining, animals were presented with a cup of unscented sand containing a half- Froot Loop® reward buried slightly (approximately 2-3 cm) under the surface of the sand. Five trials of this stimulus were presented per day, with the location of the cup in the testing cage alternating according to a pseudorandom sequence (Fellows, 1967). This procedure was followed for days 4-8 of pretraining, with a different (pseudorandom) pattern of rewarded cup location on each day.

**Discrimination reversal learning.** Twenty-four hr after completion of pretraining, rats began acquisition of the olfactory discrimination reversal learning set (ODRL) procedure. On the first day, two cups of sand scented with two randomly selected spices were placed in the testing cage. One odor was randomly selected as the “correct” odor, and thus rats were allowed access to the reward (one half- Froot Loop®) only for digging in the food cup containing sand scented with the “correct” odor. Because only one scent combination (garlic powder / onion powder) was used for olfactory testing, the correct scent was counterbalanced across all groups. A dig was defined as a rat scraping at the sand with their paws. Rats were presented with 50 acquisition trials per day, with the location of the correctly scented cup alternating according to a pseudorandom sequence (Fellows, 1967). If the rat attempted to dig in the incorrect cup, the mesh and wooden insert were put back in place, and the trial began again. This continued until
the rat dug in the correctly scented cup, or completed 5 of these “correction trials”. Criterion for acquisition of the discrimination problem was defined as making 8 correct responses (no correction trials) in a row. Trials to criterion and the number of correction trials were recorded across all acquisition trials. Rats that failed to reach criterion after one testing day (50 trials) were tested again 24 hr later.

Twenty-four hr after reaching criterion on the ODRL acquisition, rats experienced a reversal of the previously rewarded contingency following OxA or aCSF infusion. Immediately following infusion (OxA or aCSF) the rat was placed into the testing cage, and the previously “correct” (rewarded) odor cue was reversed, forcing the rats to shift their behavioral response (i.e. dig in the cup containing the previously unrewarded olfactory stimulus). Rats were again expected to meet a criterion of 8 correct responses (no correction trials) in a row on the reversal task. A maximum of 50 trials were conducted in one day of testing. If an animal failed to reach criterion after 50 trials, they were tested again 48 hr later. Correction trials occurred in the same way as they did during acquisition.

*Orexin A administration*

All infusions were conducted prior to reversal of the simple olfactory discrimination in the behavioral testing room, using a microinfusion pump (Harvard Apparatus, South Natica, MA). Dummy cannulas were removed, and 31.5-gauge internal cannulas attached to 31.3-gauge cannula connectors (PlasticsOne, Roanoke, VA) were inserted bilaterally into the guide cannulas (extent of internal cannula flush with the guide). 0.25 nmol of OxA (Sigma-Aldrich Inc., St. Louis, MO) in a 250 nL vehicle (aCSF) solution were infused \((n = 12)\), or 250 nL of aCSF only \((n = 12)\) over a period of 2 minutes (Blanco-Centurion et al., 2006). The internal cannulas were
allowed to remain in place for an additional one minute, to allow the infusate to diffuse into the tissue. Internal cannulas were then replaced by the dummy cannulas, and DRLS testing began.

**Histology**

Following completion of behavioral testing, rats were anesthetized with a lethal dose of sodium pentobarbital (100 mg/kg i.p). Transcardial perfusions were preformed, with approximately 100 ml of a 0.9% saline solution introduced into the left ventricle and exiting via the right atrium, flushing the circulatory system of blood. Approximately 75 ml of a 0.4% paraformaldehyde (PFA) solution was then introduced to act as a fixant. Brains were excised and post-fixed overnight in 0.4% PFA, and then transferred after approximately 24 hr to a 30% sucrose solution.

In order to confirm cannula placement and lesion effectiveness, 60 µm coronal sections of the frozen brain were taken running through the rostrocaudal extent of the nBM/SI region using a freezing microtome (Microm, International GimbH; Walldorf, Germany). Sections were placed into wellplates containing a 0.1 M phosphate buffer solution, and refrigerated until staining. Sections were then selected for acetylcholinesterase (AChE) staining (frontal and parietal slices; to confirm lesion efficacy) or Nissl staining using the cresyl violet (Sigma-Aldrich Inc., St. Louis MO) method (nBM slices; to confirm cannula placement).

For AChE staining, a minimum of five slices from each animal were individually placed into mesh-bottom well plates and submerged for two hr in an incubation solution containing distilled water, cupric sulfate pentahydrate, glycine, sodium acetate trihydrota, acetylthiocholine iodide, ethopropazine (all Sigma-Aldrich Inc., St. Louis, MO) and glacial acetic acid (J.T. Baker Inc., Phillipsburg, NJ; incubation solution was titrated to a pH of 5.0). After two hr of incubation, the well plate was removed and submerged in separate solutions containing sodium
sulfide, silver nitrate, and sodium thiosulfate (twice; all Sigma-Aldrich Inc., St. Louis, MO), with rinses in distilled water occurring between each solution. Stained slices were refrigerated for 24-48 hr in a solution containing 0.1 M phosphate buffer until slices were mounted on glass slides (Fisher Scientific, Pittsburgh, PA). Slides were scanned into the computer using Nikon COOLSCAN IV, and analyzed for optical density using Image J Software in both the cortex and nBM.

Eight slices per animal running the rostrocaudal extent of the nBM were selected for Nissl staining using the cresyl violet staining method.

Data analysis

Data were analyzed using the PASW (Predictive Analytics SoftWare, SPSS Inc.) statistics package. Four individual 2 (lesion type) x 2 (infusion type) univariate analyses of variance (ANOVA) were conducted on each of the four dependent measures: trials to criterion during acquisition of the simple olfactory discrimination task, correction trials during acquisition, trials to criterion during reversal of the simple olfactory discrimination task, and correction trials during reversal. Post-hoc independent sample t-tests were conducted where necessary to establish the impact of the nBM lesion on the behavioral dependent measures of interest. In addition, two 3-way mixed ANOVAs were conducted, one with trials to criterion during acquisition and reversal as the within-subjects variable, and the other with error type (perseverative or non-perseverative) as the within-subjects variable. Surgical procedure and infusion type acted as the between-subjects variable in both mixed ANOVAs. A 2-way mixed ANOVA was used to evaluate lesion effectiveness in the nBM region, with brain hemisphere as the within-subjects variable, and lesion type as the between-subjects variable.

Results
Correction trial data from one animal (sham + aCSF group) was not recorded due to an error in the administration of the task, and thus data from this animal was not included in all correction trial analyses. A separate animal (sham + aCSF group) received only unilateral infusions of aCSF due to a blockage of the right guide cannula impeding the attachment of the internal.

**Trials to Criterion During Acquisition**

Results of the 2 (surgical procedure) x 2 (infusion type) univariate ANOVA on the number of trials to criterion during acquisition were as follows, $n = 12$ for all groups analyzed. Sham-operated animals ($M = 31.5$, $SD = 14.23$) did not differ significantly from lesioned animals ($M = 36.42$, $SD = 12.70$), $F(1,20) = 0.82$, $p = .38$ in terms of trials to criterion during acquisition of the simple olfactory discrimination task. Similarly, animals that would later receive OxA infusion before reversal testing ($M = 37.08$, $SD = 13.69$) did not differ significantly from animals who would later receive aCSF infusion ($M = 30.83$, $SD = 12.99$), $F(1,20) = 1.32$, $p = .26$. There was no significant surgical procedure by infusion type interaction, $F(1,20) = 1.25$, $p = .28$ (see Figure 1).

To further elucidate the impact of surgical procedure on simple olfactory discrimination performance, the data set was split by infusion type ($n = 6$ for all groups analyzed), and an independent samples t-test was conducted on the number of trials to criterion during acquisition of the simple olfactory discrimination task. Animals with nBM lesions that would receive aCSF infusion before reversal of the olfactory discrimination task ($M = 36.33$, $SD = 13.94$) did not differ from sham-operated animals that would receive aCSF infusion before reversal ($M = 25.33$, $SD = 10.21$), $t(10) = -1.56$, $p = .15$ (see Figure 1).

**Correction Trials During Acquisition**
Results of the 2 (surgical procedure) x 2 (infusion type) univariate ANOVA on the number of correction trials during acquisition of the simple olfactory discrimination were as follows, \( n = 12 \) for all groups analyzed. Lesioned animals (\( M = 24.00, SD = 10.90 \)) did not require more correction trials than sham-operated animals (\( M = 18.36, SD = 10.43 \)), \( F(1,19) = 1.846, p = .19 \). There was no significant difference between the group that would later receive aCSF (\( M = 20.36, SD = 11.25 \)) and the group that would later receive OxA (\( M = 22.17, SD = 10.85 \)), \( F(1,19) = .284, p = .60 \). Again, there was no significant interaction between surgical procedure and infusion type, \( F(1,19) = 2.06, p = .17 \) (see Figure 2).

An independent samples t-test with the data split by infusion type (\( n = 6 \) for all groups) was conducted. These results indicated that sham animals that would later receive aCSF infusion prior to ODRL testing (\( M = 13.60, SD = 6.99 \)) required fewer correction trials during acquisition than lesion animals who would later receive aCSF infusion (\( M = 26.00, SD = 11.40 \)), at levels approaching significance, \( t(10) = -2.11, p = .06 \) (see Figure 2). Estimates of effect size suggest that surgical procedure explained 33% of the variance in the number of correction trials required between animals that would receive aCSF infusions prior to reversal of the simple olfactory discrimination, \( \eta^2 = 0.33 \).

**Trials to Criterion During Reversal**

Results of the 2 (surgical procedure) x 2 (infusion type) ANOVA on the number of trials to criterion during reversal of the simple olfactory discrimination task were as follows, \( n = 12 \) for all groups analyzed. Animals in the lesion group (\( M = 46.50, SD = 19.58 \)) did not differ significantly from animals in the sham group (\( M = 35.42, SD = 11.32 \)), \( F(1,20) = 2.798, p = .11 \). Despite the lack of significant effect, estimates of effect size indicated that surgical procedure explained greater than 10% of the variance observed among trials to criterion during reversal,
partial $\eta^2 = .12$. There was no difference between animals that received aCSF infusion prior to reversal ($M = 39.92, SD = 17.71$) and animals who received OxA infusion prior to reversal ($M = 42.00, SD = 16.20$) on the number of trials to criterion during reversal of the simple olfactory discrimination, $F(1,20) = 0.10, p = .77$. There was no interaction between surgical procedure and infusion type, $F(1,20) = 1.25, p = .28$ (see Figure 3).

The data set was again split by infusion type ($n = 6$ for all groups) and the number of trials to criterion on the reversal of the simple olfactory discrimination problem were analyzed. Results indicated that lesioned animals who received aCSF infusion ($M = 49.17, SD = 21.37$) required more trials to criterion than sham-operated animals who received aCSF infusion ($M = 30.67, SD = 5.28$) prior to reversal, at levels approaching significance, $t(10) = -2.06, p = .07$ (see Figure 3). Estimates of effect size indicated that surgical procedure explained 30% of the reversal trials to criterion variance between animals that received aCSF infusions during reversal of the olfactory discrimination task, $\eta^2 = 0.30$.

To evaluate the change over time among treatment groups from acquisition to reversal, a 3-way mixed ANOVA was conducted, with surgical procedure and infusion type as the between-subjects variables, and trials to criterion during acquisition and reversal as the within-subjects variable. Results are displayed in Figure 4, and illustrate that there was no difference between trials to criterion from acquisition to reversal for any of the treatment groups, as well as no interactions, all $p$-values $> .05$. Additionally, animals did not perform significantly worse from acquisition ($M = 33.96, SD = 13.43$) to reversal ($M = 40.96, SD = 16.63$), $F(1,20) = 2.49, p = .13$.

**Correction Trials During Reversal**
Results of the 2 (surgical procedure) x 2 (infusion type) ANOVA on the number of correction trials during reversal of the simple olfactory discrimination task indicated that animals with nBM lesions \((M = 56.58, SD = 48.44)\) did not differ significantly from animals in the sham surgery group \((M = 30.45, SD = 10.81)\), \(F(1,19) = 2.876, p = .11\) (see Figure 5). Effect size estimates indicated that surgical procedure explained greater than 10% of the variance for correction trials during reversal of the ODRL task, partial \(\eta^2 = .11\). There again was no effect of infusion type on the number of correction trials during reversal, with aCSF animals \((M = 40.18, SD = 34.04)\) performing no different than OxA animals \((M = 47.67, SD = 41.55)\), \(F(1,19) = 0.31, p = .59\). No significant interaction between surgical procedure and infusion type was observed, \(F(1,19) = 0.13, p = .99\) (see Figure 5).

In order to evaluate the effect of lesion on reversal learning correction trials, the data set was again split by infusion type \((n = 6\) for all groups), and a independent samples t-test was conducted. Animals in the lesion group who received aCSF infusions \((M = 52.17, SD = 42.09)\) did not differ significantly from sham animals who received aCSF infusions \((M = 25.80, SD = 14.43)\), \(t(9) = -1.33, p = .22\) (see Figure 5).

**Evaluation of Error Type**

In an attempt to determine the nature of the difference between nBM lesioned animals and sham surgical animals, errors made during the reversal of the simple olfactory discrimination problem were coded as perseverative (i.e. responding to the previously correct rule during reversal testing) if the rat was performing at a worse-than-chance level (less than 2 of 10 correct responses) in accordance with Bailey & Thomas (2001). Errors occurring at greater than 2 out of 10 correct responses were defined as non-perservative errors. Data were analyzed using a 3-way
mixed ANOVA, with surgical procedure and infusion type as the between-subjects variables, and error type (perseverative or non-perseverative) during reversal as the within-subjects variable.

Results for these analyses are reported in Figure 6. There was no significant difference between the sham-operated ($M = 8.75, SD = 13.59$) and lesioned ($M = 28.50, SD = 44.38$) animals in terms of number of perseverative errors, $F(1, 20) = 1.98, p = .17$. Similarly, there was no significant difference between the number of non-perseverative errors committed by sham-operated ($M = 22.41, SD = 11.68$) and lesioned ($M = 20.67, SD = 16.18$), $F(1, 20) = 0.09, p = .77$. There was no significant effect of infusion type, as well as no significant surgical procedure by infusion type interaction, all $p > .05$.

**Histology**

*Acetylcholinesterase:* Sample AChE stained tissue are displayed in Figure 7. AChE-containing cells were evident in the globus pallidus and caudate putamen of both sham-operated (Fig. 7 A, C) and lesioned (Fig. 7 C, D) animals, as well as ventral areas of the basal forebrain (horizontal nucleus of the diagonal band, magnocellular preoptic nucleus; Fig 7. A & B). Tissue at approximately -0.30 mm from Bregma (Fig. 7: A & B) were intended to be utilized for cortical AChE analysis, but dark striations of the 6 cortical layers did not appear following staining.

Tissue at approximately -1.30 mm from Bregma (Fig. 7: C & D) were analyzed for AChE content in the nBM. Results indicated that sham-operated rats ($M = 46.80, SEM = 5.34$) did not differ from lesioned animals ($M = 46.71, SD = 5.11$), $p = .82$, in terms of optical density (AChE concentration) in the nBM/SI. Similarly to the proposed cortical analysis, tissue utilized for nBM analysis were universally stained lightly.

*Nissl staining:* Nissl staining via the cresyl violet method revealed that a number of the guide cannulas were implanted in locations that were ventral to the nBM/SI. Figure 8 illustrates
correct placement of the guide cannula inside the dorsal and ventral reaches of the nBM/SI (black arrows) as well as typical ventral locations of incorrectly placed cannula (red circles). Data relating to infusion type must thus be considered in light of the incorrect dorsal-ventral locations of guide cannula in a number of animals, across all treatment groups.

Discussion

The fact that the current study was unable to show a robust effect of lesion both histologically and behaviorally is difficult to interpret. There is no indication as to why AChE staining was universally too light to evaluate lesion efficacy, although it is possible one or more of the chemicals used in staining were not effective, possibly due to age. Despite the lack of histological and behavioral support for lesion effectiveness, a number of the dependent measures expected to be sensitive to cholinergic deinnervation trended in that direction. Specifically, surgical procedure accounted for greater than 10% of the variance on both trials to criterion and correction trials needed on the reversal portion of the ODRL task. Generally, a single variable that explains greater than 10% of the total variance between a dependent measure is described as having between a medium and large effect size (Cohen, 1988). Thus, despite the lack of histological support, we can be reasonably sure that nBM lesions played a role in producing the trends towards ODRL task deficits. This was most evident behaviorally in the nearly significant effect of lesion on trials to criterion during reversal in animals that received aCSF (and thus did not experience OxA) infusion prior to reversal of the simple olfactory discrimination task. As the impact of infusion type on behavior is somewhat in question, the most accurate way in the current study to assess lesion effectiveness was to compare the effect of surgical procedure between animals that received aCSF prior to reversal only. These results generally supported a
lesion-induced impairment, with lesioned animals requiring more trials to criterion than sham-operated animals.

While this trend towards reversal learning impairment supports some of the previous literature (Cabrera et al., 2006), it is in direct contradiction to the findings of others (Tait & Brown, 2007). Tait & Brown (2007) did not observe any deficits in animals with 192 IgG-SAP lesions of the nBM during acquisition or reversal of a set-shifting task. Interestingly, lesions of the nBM via the unselective excitotoxin ibotenic acid did produce deficits in reversal learning, suggesting that non-cholinergic neurons of the nBM may be critical in reversal learning performance. Despite this, there is research to suggest that 192 IgG-SAP lesions of the nBM do impact reversal learning performance (Cabrera et al., 2006). Cabrera et al. (2006) utilized the reversal of a simple operant discrimination task to evaluate the effect of selective cholinergic de-innervation of the nBM. Their results indicated that nBM lesioned rats were not impaired during acquisition of the simple discrimination task, but were impaired during the first reversal (Cabrera et al., 2006). Due to the nature of the task (rewarded stimuli were temporal associations between a light and a tone), the deficit observed by Cabrera et al. (2006) may have been a result of impaired attentional functioning. The deficit observed in the present study is less likely to be a result of attentional deficits, as olfactory discrimination reversal learning does not tax attention to the same extent as the operant discrimination utilized by Cabrera et al. (2006). Thus, the trend towards a reversal learning deficit observed in the present study may be a direct result of cholinergic deinnervation of the nBM, or an indirect result of communication disruption between damaged cholinergic neurons and intact non-cholinergic neurons.

The result that lesioned + aCSF prior to ODRL testing animals were not impaired in the number of trials to criterion during acquisition, but were impaired at nearly significant levels in
terms of the number of correction trials during acquisition, is in partial support with previous literature. Bailey et al. (2003) observed that animals with 192 IgG-SAP lesions of the nBM were able to acquire a simple olfactory learning set, but did so at a slower rate than sham-operated animals. The subset of lesioned aCSF infused animals, which again removes the impact of OxA infusion on the results, reached criterion in a comparable time to sham aCSF animals, as evidenced by no significant difference in overall trials to criterion. However, lesion + aCSF animals required a greater amount of correction trials during acquisition to reach criterion than sham + aCSF animals, at levels that approached significance. This may represent a deficit in the ability of animals with cholinergic lesions to “learn to learn” during acquisition of a simple discrimination problem, as they require greater amounts input before making the correct behavioral choice. Thus, nBM animals displayed impaired behavioral flexibility during acquisition and reversal of a simple olfactory discrimination task.

The primary research hypothesis, that OxA infusions may be able to impact reversal learning performance in the absence of cholinergic neurons, was not supported. Nissl staining revealed that a number of the surgically implanted guide cannulas were beyond the most ventral extent of the nBM/SI. Unfortunately, there was not adequate time to reassess all statistical analyses in lieu of the misplaced cannula, and thus interpretation of results relating to infusion type must take this into consideration. Regardless, the results of the current study relating to infusion type may suggest that complete cholinergic innervation of the nBM is necessary for OxA-mitigated cognitive processing. This result is in contradiction with research indicating that OxA infusion can impact wakefulness in the absence of nBM cholinergic innervations (Blanco-Centurion, Shiromani, Winston, & Shiromani, 2006). Blanco-Centurion et al. (2006) observed that OxA infusion stimulated wakefulness and decrease REM sleep in animals with selective
cholinergic lesions of the nBM. Despite both being dependent on cholinergic input, the pathways implicated in wakefulness and reversal learning are likely neurologically different. One action of the orexins in the basal forebrain is to stimulate local glutamate release (Frederick-Duus & Fadel, 2008). Previous research has determined that stimulation of glutamate receptors (both AMPA and NMDA) in the nBM of rats results in increased wakefulness (Manfridi, Brambilla, & Mancia, 1999). Thus, it is possible that the impact of the orexins relating to arousal is more strongly tied to glutamatergic activation than cholinergic activation. This may explain the orexin-mitigated increase in arousal observed in cholinergically depleted rats by Blanco-Centurion et al. (2006). As cognition is heavily tied to the cholinergic system, orexin-mitigated local glutamate release likely would have little effect on reversal learning performance.

Recent research conducted by Boschen, Fadel, & Burke (2009) at least partially supports the necessity of cholinergic neurons in orexin signaling relating to cognition. Researchers observed that intrabasalis administration of an OxR1 antagonist impaired attentional performance on a two-lever sustained attention task, providing evidence for the effect of the orexins on cognition. Because many orexin neurons synapse on ChAT-immunoreactive neurons in the basal forebrain, it is possible that the impact of the orexins on cognition is primarily mitigated by cholinergic neurons. In observing that intrabasalis OxA infusion was not able to mitigate lesion-induced reversal learning deficits, the current study provides support for the necessity of cholinergic innervations to the cortex in orexin signaling related to cognition. This conclusion is made more difficult by the histological results indicating that a number of the OxA guide cannula were implanted in areas ventral to the nBM/SI.

As this study was a fairly exploratory venture, a number of considerations should be made for future research in this area. Primarily, the study was limited by a number of
methodological failures. In replicating the current study, an explanation for the low depth of the guide cannula needs to be determined. Pilot surgeries to determine effective lesion coordinates should be conducted. It would be advisable to utilize Long Evans rats in the future, as the majority of research relating to nBM immunotoxic lesions are conducted in that particular strain. Similarly, a review of the AChE staining protocol and chemicals should be conducted, with time allocated for testing the time required for maximal staining effectiveness. A number of control methods relating to behavioral testing should ideally be taken into consideration as well. First, testing should be conducted with the researcher blind to the particular treatment group that the rat belongs to. Secondly, behavioral testing should be conducted in a dark environment, utilizing a red light (a wavelength that the rat is not able to see) so that visual cues are further limited. These controls should limit the amount of researcher bias inherent in the behavioral testing protocol.

In summary, the current study illustrates that cholinergic deafferentation of the nBM produces acquisition and reversal learning impairments characterized by a lack of behavioral flexibility, at levels that approached significance. No effect of infusion type was observed across any dependent measure, which could be explained by a necessity for cholinergic neurons in orexin signaling related to cognition, or by the apparent incorrect location of some guide cannula. Regardless, future research should continue to evaluate the impact of OxA on cognition in cholinergically-challenged animals. If OxA proves capable of stimulating cortical ACh efflux in the absence of the majority of nBM cholinergic neurons, evaluation of OxA as a possible AD treatment should be conducted.
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Figure 1. Mean (±SD) trials to criterion during acquisition of the simple olfactory discrimination task ($n = 6$ for all groups). There was no effect of lesion or infusion type, and no interaction, all $p$-values $> .05$. 
Figure 2. Mean (±SD) number of correction trials during acquisition of the simple olfactory discrimination task. There was no effect of lesion or infusion type, and no interaction, all $p$-values $> .05$. 

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Figure 3. Mean (±SD) number of trials to criterion during reversal of the simple olfactory discrimination task. No effect of surgical procedure, infusion type, or interaction was observed, all $p$-values > .05.
Figure 4. Mean (±SD) trials to criterion during acquisition and reversal of the olfactory discrimination task. There was no significant difference between the amount of trials to criterion during acquisition and reversal, $p = .13$. There were also no significant interactions between surgical procedure, infusion type, or surgical procedure and infusion type, on the amount of trials to criterion during acquisition and reversal of the olfactory discrimination task, all $p$-values $> .05$. 
Figure 5. Mean (±SD) correction trials during reversal of the simple olfactory discrimination problem. There was no effect of lesion ($n = 12$ for both groups) or infusion type ($n = 6$ for all groups with the exception of sham + aCSF, $n = 5$) as well as no interaction, all $p$-values $> .05$. 
Figure 6. Mean (±SEM) number of perseverative errors and non-perseverative errors committed during reversal of the simple olfactory discrimination task. There was no difference between sham-operated ($n = 12$) and lesion animals ($n = 12$) on the number of perseverative errors ($p = .17$) and non-perseverative errors ($p = .77$).
Figure 7. A & B: Representative AChE stained tissue approximately -0.30 from Bregma, A = sham-operated rat, and B = nBM lesioned rat. Cortical striation did not become apparent, thus statistical analysis was not possible. C & D: Representative AChE stained tissue approximately -1.30 from bregma, C = sham-operated rat, D = nBM lesioned rat. Approximate areas selected for nBM analysis are labeled in black. There was no significant difference between nBM density measurements in sham-operated animals ($M = 46.80$, $SEM = 5.34$) and lesioned animals ($M = 46.71$, $SD = 5.11$), $p = .82$. Staining was likely not dark enough in the nBM / basal forebrain region to accurately assess AChE levels.
Figure 8. Representative Nissl stained (cresyl violet method) slice revealing guide cannula placement (approximately -1.00 mm from bregma; Paxinos & Watson, 2005). Arrows indicate the most dorsal portion of the guide cannula track. Red dots represent cannula locations that were too ventral in relation to the nBM/SI.
Appendix

The effect of intrabasalis orexin A infusion on reversal learning performance in rats with 192 IgG-saporin lesions of the nucleus basalis magnocellularis

Introduction

Alzheimer’s disease

Alzheimer’s disease (AD) is a neurodegenerative disease characterized by progressive and debilitating cognitive impairments primarily relating to memory and attention, as well as a disruption of homeostatic functions (Gallagher & Pelleymounter, 1988; Hall et al., 2008; Muir, 1997; Traissard et al., 2007). AD is generally conceptualized as having three distinct neurological hallmarks: neurofibrillary tangles, amyloid plaques, and neuronal atrophy. These neuropathological symptoms manifest themselves behaviorally as deficits related to memory encoding and recall, attentional capability, and the ability to regulate the body’s various homeostatic mechanisms. AD differs from the normal cognitive decline associated with aging in that the disease results in the inability to function in one’s typical social or occupational environment (Muir, 1997). This difference is manifested in both the severity of the neurological damage, and the ramifications of this damage on behavior. Retrospective studies indicate that the cognitive decline associated with AD begins years prior the appearance of symptoms that are clinically significant, and then proceeds rapidly from the time symptoms become diagnosable (Grober et al., 2008). This fact in particular provides hope for a future pre-clinical assessment and treatment for AD.

The term “Alzheimer’s disease” was coined by Alois Alzheimer in 1906 to describe the condition he observed during examinations of the autopsied brains of two individuals with dementia (Muir, 1997). Current estimates suggest that approximately 2-5% of aged individuals
suffer from dementia, with about 50-70% of demented individuals having AD (Muir, 1997).
Multi-infarct (vascular) dementia (VaD), a common form of dementia, involves the degeneration of multiple vascular areas, including a softening of the brain tissue in cortical and subcortical structures (Mathias & Burke, 2009; Muir, 1997). It is typically difficult to clinically differentiate between VaD and AD, as the primary diagnostic tools, cognitive tests, do not appear to distinguish between the two diseases well (Mathias & Burke, 2009). There is some evidence that this difficulty may be due to the diseases having similar neurological etiologies. An analysis of the literature relating to similarities between the neuropathological symptoms of AD and VaD conducted by Kallaria and Ballard (1999) suggests that 30% of AD cases have co-occurring VaD symptomology, and a substantial amount of those diagnosed with VaD will demonstrate AD pathology following autopsy. It is important to recognize that there is likely some overlap in VaD and AD diagnoses, as a result of similar cognitive deficits, as well as similar neurological symptoms.

1. Mild cognitive impairment (MCI)

Research indicates that AD is the end-stage of a disease that progresses through multiple stages, from preclinical, to mild cognitive impairment (MCI), and finally to clinical AD (Babiloni et al., 2007; Burrachio & Kaye, 2009). MCI can be conceptualized as a “transition state”, consisting of subjective complaints of mild memory and cognitive impairment (Burrachio & Kaye, 2009). Individuals with MCI are much more likely to develop AD than those who do not, although demonstrating MCI is not necessarily deterministic of an eventual AD diagnosis (Burrachio & Kaye, 2009). Deficits resulting from MCI can be difficult to assess with standard cognitive measures, as the functional impairments are often not great or consistent enough to be detected (Burrachio & Kaye, 2009). In addition, many of the characteristic behavioral symptoms
of AD, such as apathy or disruption of homeostatic functions (i.e. dressing and eating), are not displayed by individuals with MCI (Burrachio & Kaye, 2009; Schroeter et al., 2009).

Despite the relatively mild cognitive deficits, research indicates that there are neurological abnormalities that predict AD development in individuals with MCI (Burrachio & Kaye, 2009; Ray et al., 2007; Schroeter et al., 2009). In a meta-analysis of the results from numerous imaging studies related to AD and MCI, Schroeter et al. (2009) determined that damage in the transentorhinal area and the hippocampal body (including neurofibrillary tangle development) and the inferior parietal lobules and precuneus (amyloid deposits) predicts the progression from MCI to AD. The promise of a possible pre-clinical AD diagnosis prompted Ray et al. (2007) to analyze differences in signaling proteins in blood plasma samples of individuals at all stages of the AD spectrum (preclinical through clinical) and age matched, non-demented controls. Ray et al. (2007) first identified 19 signaling proteins that discriminated AD plasma samples from non-demented controls. Researchers then analyzed the plasma samples of individuals with MCI, and used predictive analysis microarray (PAM) to predict AD development of the MCI individuals (Ray et al., 2007). Using plasma samples taken when individuals only displayed MCI, PAM was able to correctly classify 20 of 22 individuals who would develop AD 2-5 years later as illustrating the “Alzheimer’s” phenotype (Ray et al., 2007). Additionally, individuals who did not develop AD following a MCI diagnosis were classified correctly as non-Alzheimer’s via their blood plasma samples (Ray et al., 2007). Preliminary analysis of the function of the 18 signaling proteins identified as distinguishing between AD stage suggests that they may play roles in apoptosis or hematopoiesis (Ray et al., 2007). As AD is characterized by cell death, it would be unsurprising that differential expression of these particular signaling proteins would reliably predict AD development. Results such as these support the view of MCI representing an
early step in the AD continuum, as well as provide hope for a possible preclinical diagnostic mechanism for AD.

2. **Role of Acetylcholine**

The role of acetylcholine (ACh) in AD pathology has been well documented (Davies, 1979; Mesulam, 1983; Ezrin-Waters & Resch, 1986). Although many neurotransmitter systems are impacted by AD development, ACh is one of the most critical due to its role in stimulating the cortex, the hippocampus, and the amygdala (Mesulam, 1983). Early research into the impact of AD on cholinergic systems indicated that choline acetyltransferase (ChAT), an enzyme that synthesizes ACh, is decreased in individuals with AD (Bird et al., 1983). Lower levels of ChAT in the cortex have been correlated with the more severe dementia (Geula & Mesulam, 1996; Perry et al., 1978). ChAT levels are decreased in the hippocampi, the area of the brain critical for learning and memory, of individuals with late onset AD, as compared to age matched controls (Bird et al., 1983). Levels of ChAT in the cortex and substantia innominata (a nucleus of the basal forebrain) are decreased in individuals with early onset AD (Bird et al., 1983). The brain area primarily involved in providing ACh to the cortex is the basal forebrain (Mesulam et al., 1983). Degeneration of this basal forebrain cholinergic system (BFCS) has also been correlated with dementia severity (Perry et al., 1981).

There are two primary types of ACh receptors, the muscarinic ACh receptor (mAChR) and the nicotinic ACh receptor (nAChR).

a. **nAChRs – nicotinic acetylcholine receptors**

nAChRs are ionotropic receptors composed of numerous α and β subunits which can be combined to create distinct receptor subtypes (Wevers & Schroder, 1999; Rubio, Perez, & Avila, 2006). The most common nAChRs in the mammalian brain are the α4β2 and α7 subtypes, which
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are located throughout the cortex in both presynaptic and postsynaptic locations (Wevers et al., 2000; Wevers & Schroder, 1999). These receptor subtypes have been implicated in AD dysfunction, and are located in brain areas critical to dementia development, including the BFCS (Rubio et al., 2006; Wevers et al., 1999). Wevers et al. (1999) analyzed the distribution and density of α4 and α7- positive neurons at the mRNA level, as well as at the protein level. Results indicated that there was no difference in cortical nAChR expression at the mRNA level between AD individuals and age-matched controls, but there was a significant loss in density of neurons stained for the α4 (28% loss) and α7 (32%) subunits (Wevers et al., 1999). Other research has identified α4, α3, and α7 subunit density decreases specific to the hippocampus and temporal lobes of AD patients (Guan et al., 2000). Using western blot analysis, researchers identified significant losses of α3, α4 and α7 nAChR subunits in the hippocampus, and losses of α3 and α4 subunits in the temporal lobes of individuals with AD (Guan et al., 2000). The discrepancy between normal nAChR mRNA levels and low protein concentrations, suggests a possible impact of AD on the translation of nAChR proteins from mRNA (Wevers et al., 1999). Support for this hypothesis is provided by ligand binding studies, such as those conducted by Whitehouse et al. (1985), in which high affinity nACh binding sites in the cerebral cortex of tissue samples from AD patients and age-matched controls were radio-labeled and compared. Large decreases in the density of cortical nAChRs were found in AD samples, as compared to age-matched controls (Whitehouse et al., 1985). It is clear that the cortical densities of nAChRs are diminished in AD as compared to normal aging brains.

b. mAChRs – muscarinic acetylcholine receptors

mAChRs are metabotropic G-protein coupled receptors that are found throughout the central and peripheral nervous system (Caufield, 1993; Rubio et al., 2006). These receptors have
five subtypes, classified pharmacologically as M1-M5 (Caufield, 1993). The M1, M3, and M5 mACHR subtypes act through stimulation of the second messenger phospholipase C, while the M2 and M4 receptors act to inhibit adenylyl cyclase activity (Caufield, 1993; Jope, 1999). The most influential mACHR subtypes in relation to AD are the M1 and M2 receptors (Jope, 1999). The M1 receptor has been shown to be relatively preserved postsynaptically in the brains of AD patients (Eckelman, 2006; Fisher, 2007; Rubio et al., 2006). These receptors are highly concentrated in the cortex and hippocampus, suggesting a primary function related to attention and memory (Fisher, 1999). Treatment of AD using selective M1 agonists has been shown to produce significant improvement in typical AD symptoms, including a decrease in Aβ levels and tau hyperphosphorylation (Fisher, 2007). M1 agonists may continue to have a therapeutic effect after traditional AChE inhibitors stop working due to the progressive cholinergic degeneration (Fisher, 1999). Antagonism of the M1 receptor in 3xTG-AD mice (a mouse model of AD) results in exacerbated AD symptomology, while agonism of the M1 receptor subtype eliminates cholinergic lesions typical of AD (Fisher, 2007). Fisher (2007) hypothesizes that the pronounced therapeutic effect of mACHR agonists is produced through the stimulation of a MAP kinase signaling pathway that imitates the effect of ACh without input from nACHRs. In response to endogenous ACh, nACHRs or other mACHR subtypes may have mitigating effect on the associated physiological response, but selective agonism of the M1 mACHR receptor bypasses this additional input. This is particularly important given the fact that M1 mACHRs remain intact despite cholinergic degeneration, while the density of nACHRs decreases throughout the course of AD. M1 selective agonists, such as AF267B, have proven to be the most effective in alleviating the debilitating effects of numerous AD hallmarks, and are likely to undergo clinical trials in the future (Fisher, 2007).
The M2 mAChR subtype has been shown to be significantly decreased in the brains of AD patients, and is known to be located presynaptically, functioning as an autoreceptor for ACh (Caufield, 1993; Jope, 1999). mAChRs in the hippocampus appear particularly important in learning and memory, specifically short term potentiation (STP) and long term potentiation (LTP; Seeger et al., 2004). Using M2 and M4 knockout mice, Tsavara et al. (2003) illustrated that both subtypes are critical for ACh regulation in the hippocampus. Basal ACh levels were increased in combined M2 and M4 mAChR knockout rats, suggesting that the function of these subtypes is to inhibit synaptic ACh levels (Tsavara et al., 2003). Researchers then evaluated the performance of these knockout rats on a passive avoidance task, with results indicating that M2 mAChR subtype knockout rats showing impaired memory retention on the task (Tsavara et al., 2003). In M4-KO mice, basal levels of ACh were increased, while basal levels of ACh remained unchanged in M2-KO mice, suggesting that the M4 receptor subtype is particularly important for tonic autoregulation of ACh release (Tsavara et al., 2003). Interestingly, a combined M2/M4-KO mouse had even greater basal ACh deficits than M4-KO mice did, implicating the M2 receptor in presynaptic ACh regulation (Tsavara et al., 2003). Thus, M2 mAChR appears to be important in the autoregulation of ACh in response to pharmacological or physiological induced ACh release (Tsavara et al., 2003). ACh release was attenuated in M2-KO mice in response to scopolamine (a non-selective muscarinic antagonist) administration, as well as exposure to a novel environment (Tsavara et al., 2003).

A similar study conducted by Seeger et al., (2004) illustrated the function of M2 subtype mAChRs in working memory, behavioral flexibility, and hippocampal synaptic plasticity. M2-KO mice were shown to have impaired memory (increased escape latency and increased perseveration as compared to control mice) on the Barnes circular maze test, as well as a t-maze
delayed spatial alternation task (less correct responding after a longer delay, as compared to control animals; Seeger et al., 2004). As M2 receptors are highly concentrated in the hippocampus, memory impairments following their removal would be expected. In addition, hippocampal slices from M2-KO mice were less able to potentiate STP and LTP in the CA1 region of the hippocampus proper when the Schaffer collaterals were stimulated (Seeger et al., 2004). Taken together, these results suggest a critical role for M2 mAChRs in physiological and behavioral memory function. As M2 receptor loss has been correlated with AD development, it is possible that they are causally involved in the progressive memory impairments seen in individuals with AD.

3. The “cholinergic hypothesis” of AD

The overwhelming amount of research linking ACh to the cognitive dysfunction seen in AD has lead to the development of what is commonly dubbed the “cholinergic hypothesis” of AD development. The majority of cholinergic input to the cortex, the amygdala, the hippocampus, and the olfactory bulb originates in a brain region known as the basal forebrain (Mesulam et al., 1983). The basal forebrain can be described as being divided into sections, Ch1-Ch6, with each section corresponding to cholinergic innervations in specific forebrain nuclei (Mesulam et al., 1983).

Cholinergic projections from the basal forebrain originate in section Ch4, and terminate in a number of areas, including the cortex, the amygdala, the hippocampus, and the entorhinal cortex (Mesulam et al., 1983; Mesulam, 2004). One particular nuclei of the basal forebrain cholinergic system (BFCS), the nucleus basalis magnocellularis (nBM), has been linked to the dysfunction associated with AD (Ezrin-Waters & Resch, 1986; Schliebs et al., 1996; Mesulam, 2004). The mammalian nBM is located inside the area known as the substantia innominata (SI),
forming what is commonly known as the nBM/SI complex (Hedreen et al., 1984). Both anatomical areas are located just under the most ventral portion of the globus pallidus in the Ch4 region of the basal forebrain, consisting primarily of magnocellular cholinergic neurons that project to the neocortex (Bigl, Woolf, & Butcher, 1982; Mesulam et al., 1983; Hedreen et al., 1984). In primates, the nBM is typically referred to as the nucleus basalis of Meynert, as compared to the nucleus basalis magnocellularis in rats and other mammals (Ezrin-Waters & Resch, 1986; Hedreen et al., 1984; Mesulam et al., 1983). Compared to other portions of the basal forebrain, the nBM is the most impacted by evolution (Mesulam, 1983). Thus, the basal forebrain in primate systems is much larger and more easily differentiated than in other mammalian species such as the rat (Mesulam, 1983). Ninety-percent of neurons in this Ch4-nBM/SI complex are cholinergic in both the monkey and the rat brain (Mesulam, 2004). Overall, the SI contains a majority of cholinergic neurons, deinnervation of which results in cognitive deficits typically associated with AD (Mesulam, 1983; Ezrin-Waters & Resch, 1986; Cabrera et al., 2006; Maddux et al., 2007). Cholinergic fiber deinnervation in the cortex of AD patients is extensive, with one study indicating an average loss of approximately 55% (Geula & Mesulam, 1996).

Projections from the basal forebrain to the hippocampus originate in the Ch1-Ch3 sections (Mesulam, 1983). The medial septum and the vertical limb nucleus of the diagonal band form what is known as the setptohippocampal pathway, and contain a substantial amount of non-cholinergic fibers (Mesulam, 1983). Disruption of the septohippocampal cholinergic pathway results in memory deficits characteristic of AD (Berger-Sweeney et al., 1994; Torres et al., 1994; Lehmann, Grottick, Cassel, & Higgins, 2003; Fitz, Gibbs, & Johnson, 2008). The Ch5 and Ch6 sections of the basal forebrain form the pontomesencephalic reticular formation, which
provides the majority of cholinergic input to the thalamus (Mesulam, 1983). This region is likely involved in relating the reticular activating system to cortical activation levels.

**AD Symptoms**

1. **Amyloid-β plaque formation**

Amyloid-β plaques arise from the amyloid-β peptides 1-42 (Aβ42) and 1-40 (Aβ40) becoming surrounded by two types of glial cells, astrocytes and microglia (D’Andrea et al., 2004; Rubio et al., 2006; Wevers et al., 1999). These specific peptides are formed through the cleavage of amyloid precursor protein (APP; Rubio et al., 2006). Insoluble forms of amyloid-β accumulate in an area, forming dense plaques, which can result in the death of surrounding cells (Galvan & Bredesen, 2007; Rubio et al., 2006). Aβ can also have a direct neurotoxic effect, producing cell death and cognitive impairments when administered (Choi et al., 2007). Research indicates that plasma levels of Aβ42 and Aβ40 increase with age, and are elevated in individuals with AD as compared to elderly controls (Mayeaux et al., 2004). Similar research evaluating the presence of Aβ42 in first generation offspring of AD patients indicates that levels of Aβ42 are elevated in offspring, regardless of age (Abdullah et al., 2009). These results illustrate a genetic link between extracellular Aβ levels and familial AD development, with higher Aβ levels occurring prior to AD development.

Intracellular Aβ also appears to influence plaque development (Knobloch et al., 2007). Using transgenic mice expressing the human form of APP, Knobloch et al. (2007) observed that deposits of intracellular Aβ occur prior to extracellular deposits become apparent. These intracellular Aβ deposits also co-occur with the onset of cognitive deficits, suggesting a role of intracellular Aβ in the early stages of neurological and cognitive impairment (Knobloch et al., 2007).
2. Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are the result of the microtubule associated protein (MT) $\tau$ (tau) becoming hyperphosphorylated (Grundke-Iqbal et al., 1986). Tau is the major protein subunit that forms paired helical filaments (PHFs), which are part of NFTs (Grundke-Iqbal et al., 1986). Tau must be phosphorylated by multiple kinases in order to reach a neuropathological, hyperphosphorylated level (Wang, Grundke-Iqbal, Iqbal, 2007). Hyperphosphorylated tau self-assembles into tangles of PHFs in AD (Wang, Grundke-Iqbal, Iqbal, 2007). Research indicates that acetylcholine receptors are related to tau phosphorylation, with activation of nAChRs increasing tau phosphorylation, and mAChRs decreasing tau phosphorylation (Rubio et al., 2006).

NFTs have been correlated with the severity of dementia symptoms, with greater amount of NFTs correlated with more severe dementia symptoms (Rubio et al., 2006). NFTs first become evident in the transentorhinal cortex, followed by the pre-$$\alpha$$ layer of the entorhinal cortex (Brion, 1998). NFT development in these areas do not result in observable cognitive deficits at this early stage (Brion, 1998). Cognitive deficits associated with AD begin to be manifested when NFTs accumulate in the hippocampus and neocortical association areas (Brion, 1998). Late-stage AD is characterized by NFT formation in the entorhinal cortex, the basal forebrain, and the hippocampus, areas critical for attention, memory, and other integral cognitive functions (Brion, 1998; Rubio et al., 2006).

3. Neuronal death

Cholinergic cell degeneration is a widely accepted neuropathological symptom of those suffering from AD (Geula & Mesulam, 1995; 1996), although the mechanisms that underlie this cell death and it’s timeline are both points of contention in the literature. The hypothesized
mechanisms of AD cholinergic degeneration include the dysfunction of programmed cell death pathways, the overproduction of neurotoxic forms of Aβ, as well as the interaction of neurofibrillary tangles and amyloid plaques.

Research indicates that, in familial forms of AD, there are mutations in two genes that code for presenilins, which play an integral role in the production of Aβ (Popescu & Ankarcrona, 2004; Jellinger & Stadelmann, 2001). These mutations increase the production of Aβ, which results in an increase in amyloid plaque formation, as well as amyloid-induced neurotoxicity (LeBlanc, 2005; Popescu & Ankarcrona, 2004). Numerous researchers hypothesize that the primary insult that results in the neuronal death that characterizes AD is the increase in extracellular and intracellular Aβ (Culmsee & Landshamer, 2006; LeBlanc, 2005). The neurotoxic effect of Aβ is believed to occur through activation of an apoptopic pathway, although the specific mechanisms are numerous (Popescu & Ankarcrona, 2004). Regardless, it is clear that elevated levels of Aβ both directly and indirectly result in the neuronal death that characterizes AD.

To further study the relationship of apoptosis to AD, a model using the mechanisms of ischemic neural cell death has been developed (Hayashi, Shoji, Abe, 2006). Results indicate that, following ischemic stroke, there is a loss of adenosine triphosphate (ATP), the major fuel for cellular function (Hayashi et al., 2006). This results in an ion imbalance in glutamatergic neurons that causes an increase in Ca²⁺ influx presynaptically, resulting in an excess of glutamate release (Hayashi et al., 2006). This increase in excitation could result in the neurotoxicity seen in AD. Additionally, it has been shown that fluctuations in neurochemical pH is a major factor in ischemic cell death. To evaluate the impact of pH on cholinergic neural death, Pirchl, Marksteiner, and Humpel (2006) exposed cholinergic neurons to a medium at a pH of 6, below
the pH of 7.4 that they naturally exist in. They observed marked damage to the neurons after one day of exposure, and almost complete loss of cholinergic neurons after four days (Pirchl et al., 2006). The researchers suggest that, because Aβ aggregation is increased at more acidic pH levels, indicating a link between pH fluctuations, and AD neuropathology (Pirchl et al., 2006).

Because neuronal damage, specifically cholinergic cell death, is such an integral part of AD pathology, animal models have focused on replicating this cell death to expand the understanding of the disorder, as well as to evaluate possible pharmacological treatments.

Basal forebrain lesion models of AD

1. Mechanical lesions

The two primary types of mechanical lesions used in AD research are radiofrequency lesions and electrolysis (Schliebs, Roßner, Bigl, 1996). These lesions suffer from what is known as the fibers of passage problem, in that they are not selective to cholinergic neurons but lesion all fibers in a specific area. Dubois et al. (1985) observed that radiofrequency lesions of the nBM resulted in similar deficits to an excitotoxic lesion, although only the excitotoxic lesion preserved catecholamine (epinephrine and norepinephrine) function in the lesioned area. Similar patterns of cell death have been observed following electrolysis of the nBM (Miyamoto et al., 1985). There is also evidence for glutamatergic deficits in mechanically lesioned rats (Schliebs et al., 1994). Rats given mechanical lesions exhibit less NMDA activity, and increased AMPA receptor binding in cortical areas, reinforcing the potential impact of the fibers of passage problem (Schliebs et al., 1994). Because of the fibers of passage problem, the majority of current research relies on chemical methods, so as to isolate only cholinergic dysfunction.

2. Excitotoxic lesions
Excitotoxic lesions include all toxins that act as agonists on glutamatergic receptors, causing excitation for a longer period of time, which increases Cl\(^-\) and Ca\(^{2+}\) ion influx (Schliebs, Roßner, Bigl, 1996). The two primary excitotoxins, quisqualate acid and ibotenic acid, bind preferentially to different glutamatergic receptors (Schliebs et al., 1996). Quisqualate acid preferentially binds to AMPA receptors, while ibotenic acid preferentially binds to NMDA receptors (Schliebs et al., 1996). This differential in binding affinity has major implications for the effectiveness of lesions, as the concentration of glutamatergic receptors in the lesioned area must be taken into account in order to choose the maximally effective toxin. NMDA lesions reduce ChAT activity in relatively diffuse areas throughout the cortex, destroying cholinergic input in the Ch4 region (nBM) of the basal forebrain (Roberts et al., 1992). Overall, AMPA-induced lesions of the BFCS results in greater ChAT reductions, and greater behavioral impairments, than other excitotoxic methods (Muir, 1997; Schliebs et al., 1996). Similar to mechanical lesions, excitotoxic lesions are not specific to cholinergic systems, as they create deficits in glutamatergic systems that impact cholinergic ones. Thus, specific cholinergic toxins have been developed to more closely mimic the effects of AD on cholinergic systems.

3. **Immunolesioning**

Immunolesioning using 192 IgG-saporin (192 IgG-SAP) has been shown to be much more effective than other lesion types in selectively destroying cholinergic fibers, without damaging other neural inputs (Torres et al., 1994; Wiley et al., 1991). 192 IgG is a monoclonal antibody that binds to the p75 nerve growth receptor and is selectively taken up by cholinergic neurons (Wiley et al., 1991). 192 IgG is paired with a ribosome inactivation protein, saporin, resulting in a toxin that destroys cholinergic neurons specifically (Wiley et al., 1991). 192 IgG-SAP lesions impair a number of behavioral and cognitive tasks, in a lesion-target specific way.
nBM lesions produce a 75-90% loss of ChAT in the neocortex. 192 IgG-SAP lesions of the medial septum results in septohippocampal cholinergic cell loss, with 65-72% ChAT loss in the hippocampus (Torres et al., 1994). Additionally, intraparenchymal injections directly into the basal forebrain result in more specific damage, at lower dosage, than intracerebroventricular injections (Torres et al., 1994; Wiley et al., 1991). In studies where diffuse damage is ideal, intracerebroventricular injections of 192 IgG-SAP can create deficits that are related to a number of basal forebrain systems (Garcia-Alloza et al., 2006). For example, a study by Garcia-Alloza et al. (2006) illustrated that intrabasalis infusions of 192 IgG-SAP do not effect septohippocampal projections, while intracerebroventricular infusions result in cholinergic damage in the hippocampus, producing deficits in working memory.

Lesions of the medial septum disrupt the function of the septohippocampal pathway, which appears to produce deficits related to memory (Craig et al., 2008; Cutuli et al., 2009; Fitz, Gibbs, Johnson, 2008; Garcia-Alloza et al., 2006). Similarly, 192 IgG-SAP lesions of the nBM result in impairment of attentional function (Butt et al., 2002).

a. Septohippocampal damage

Lesion models that target the septohippocampal pathway mimic the cholinergic deinnervation that is believed to result in memory deficits characteristic of individuals with AD (Mesulam et al., 1983; Lehmann et al., 2003; Gallagher & Pelleymounter, 1987). While memory is characteristically deficient in individuals with late stage AD, there is evidence that memory decline begins years prior to AD development (Grober et al., 2008). As the disease progresses, damage to the BFCS innervations of the hippocampus cause memory performance to become disrupted. Research indicates that intraparenchymal injections of 192 IgG-SAP directly to the medial septum results in impairment of working memory (Fitz et al., 2008; Johnson, Gabon,
Gibbs, 2002). Fitz et al. (2008) examined the effectiveness of 192 IgG-SAP lesions of the medial septum in impairing acquisition of a delayed matching to position task, which is a measure of working memory. On average, rats that were given 192 IgG-SAP lesions took longer to reach criterion and showed more perseveration of the response strategy than control rats (Fitz et al., 2008). These results support the hypothesis that decreasing ACh efflux into the hippocampus by lesioning the medial septum can result in memory deficits.

A common test of working memory is the Morris water maze (MWM). Despite illustrating that medial septum lesions using a specific cholino-toxin results in impairments of working memory, spatial working memory (as measured by performance on the MWM) remains intact (Fitz et al., 2008; Garcia-Alloza et al., 2006). This discrepancy points to the interaction of other neurotransmitter systems in influencing performance on spatial memory tasks. Research has shown that combining 192 IgG-SAP medial septum lesions with stress can result in impairment of spatial working memory on the MWM (Craig et al., 2008). Rats who were exposed to stress or immunotoxin alone did not display spatial working memory deficits, but rats in the combined group were impaired during acquisition, learning the location of a new platform, and re-acquisition of the original location, on the MWM task (Craig et al., 2008). The researchers hypothesize that, during the course of AD, cholinergic neurons in the septohippocampal pathway may become susceptible to stress related damage, causing the observed memory deficits in individuals with AD (Craig et al., 2008).

b. Nucleus basalis damage

Deficits in performance on cortical-dependent behavioral tasks are typically observed following damage to the cholinergic fibers of the nBM (Butt, Noble, Rogers, & Rea, 2002; Bailey, Rudisill, Hoof, & Loving, 2003; Cabrera et al., 2006). A number of different cortical-
dependent tasks have been developed to assess the impact of cholinergic deinnervation. In the set shifting paradigm, animals are required to acquire a specific learning set (i.e. learn to respond to the task using a specific rule), which is then shifted, forcing the animal to change the rule that they were previously rewarded for responding with. Variations of this task are used to study impairments caused by lesions of the nBM. Set-shifting paradigms have been designed to evaluate numerous cognitive processes, including learning and attention.

Impairment during acquisition of an olfactory learning set have been observed in rats following selective cholinergic lesions of the nBM (Bailey, Rudisill, Hoof, & Loving, 2003). Rats with 192 IgG-SAP lesions demonstrated impairments during olfactory discrimination learning set acquisition, performing at chance level until the final acquisition trial block, while control animals performed significantly better than chance after only block three (Bailey et al., 2003). While these results suggest a learning impairment, nBM lesioned animals did eventually acquire the olfactory learning set, indicating that nBM lesions may not impair the ability to form simple olfactory learning sets (Bailey et al., 2003). Interestingly, rats with nBM lesions displayed no deficits in reversal learning performance, although the difference between performance by lesioned rats as compared to sham-operated animals approached significance on the first reversal (Bailey et al., 2003). Results such as these illustrate that cholinergic deficits impair the ability to “learn to learn”, although deficits may involve multiple neurotransmitter systems (Bailey et al., 2003).

More complex tests of attention have been developed to further elucidate the functions of the nBM/SI complex. In contrast to the research by Bailey et al. (2003), Butt et al. (2002) found that there was no difference in performance on a simple discrimination task (responding more to a rewarded stimulus than to an unrewarded stimulus) in 192 IgG-SAP nBM lesioned rats.
However, when the task difficulty was increased, requiring rats to respond when two pairs of stimuli were presented individually, but not when they were presented together, rats with nBM lesions performed significantly worse than sham-operated rats (Butt et al., 2002). Similar results were observed by Cabrera, Chavez, Corley, Kitto, & Butt (2006), although they employed a reversal learning paradigm as opposed to the negative patterning task utilized by Butt et al. (2002). On a simple discrimination task, nBM lesioned rats did not take significantly more trials to reach criterion, as compared to sham-operated rats (Cabrera et al., 2006). However, rats with selective cholinergic lesions of the nBM had significantly greater trials to criterion on the first reversal task than sham-operated animals, illustrating that they were unable to perseverate their responding to the previously acquired rule (Cabrera et al., 2006). Lesioned animals did not perform differently than sham-operated animals on subsequent reversals, again indicating that the lesion-induced impairment is only present during initial learning (Cabrera et al., 2006). On tasks that require significant cognitive effort, such as reversal learning, rats with nBM/SI lesions take longer to reach criterion on reversals, although they eventually will reach it (Bailey & Thomas, 2001; Cabrera et al., 2006). Cabrera et al. (2006) hypothesized that the underlying cognitive deficits observed following nBM/SI lesions are related to deficits in cognitive consistency, which they define as the ability to shift one’s behavior in response to stimulus environment or reinforcement contingency changes related to that environment.

Further support for the belief that nBM damage impairs performance on more challenging cognitive tasks is provided by deficits in feature binding illustrated by Botly & De Rosa (2007; 2008; 2009). Feature-binding refers to the ability to integrate neural inputs from diverse brain regions that occur in response to multi-modal stimuli (Botly & De Rosa, 2009). While it is known that regions of the frontoparietal cortices control the attentional mechanisms
critical to the ability to bind features, downstream neurological structures that modulate the
cortical response have not been well established. Botly & De Rosa (2007) first observed that,
following application of scopolamine, a selective mAChR antagonist, rats were unable to
perform at greater than chance levels on a forced choice (FC) task that required feature binding.
Scopolamine-infused rats were not impaired on a FC task that did not require feature binding,
suggesting that a fully functional cholinergic system is required for feature binding (Botly & De
Rosa, 2007). To expound on this result, researchers then evaluated at what stage of learning
intact ACh functioning was required for. Utilizing the same FC task, results indicated that
scopolamine rats were only impaired during initial encoding of the FC task requiring feature
binding, but not at any other stage of learning (Botly & De Rosa, 2008). Interestingly,
researchers correlated feature binding performance of the scopolamine-infused rats with humans
who were cholinergically-challenged by dividing attention during a FC task that required feature
binding (Botly & De Rosa, 2008). Results indicated remarkably similar patterns of impairment,
with cholinergically-challenged humans and rats suffering deficits during encoding of a FC task
requiring feature binding (Botly & De Rosa, 2008). As the nBM is known to provide cholinergic
innervations to the majority of the cortex, Botly & De Rosa (2009) the utilized the same FC task
to evaluate feature binding performance in 192 IgG-SAP lesioned rats. Rats with 192 IgG-SAP
lesions of the nBM were impaired during acquisition of the FC task requiring feature binding, as
compared to sham-operated animals. Thus, the nBM provides cholinergic innervations to the
cortex that are integral to both acquisition and encoding of feature binding.

In addition to impacting attention related to feature binding, Lehmann, Grottick, Cassel
& Higgins (2003) observed nBM-mitigated visuospatial attention deficits in rats following 192
IgG-SAP lesions of the nBM. Researchers compared the performance of rats with nBM lesions,
medial septum (MS) lesions, combined nBM and (MS) lesions on the 5-choice serial reaction time task (5-CSRTT) and the eight arm radial arm maze (RAM). Their findings were generally consistent with the literature, with nBM lesioned animals performing worse on the 5-CSRTT, and MS lesioned animals being impaired on the RAM (Lehmann et al., 2003). Combined nBM/MS lesions impaired attention to a lesser extent than nBM lesions alone, and also impaired radial arm performance (Lehmann et al., 2003). These results support an integral role for the nBM in attentional functioning, while the MS is more closely related to memory. Given that the nBM provides ACh to the cortex and the MS provides ACh to the hippocampus, these deficits are unsurprising. Interestingly, lesions of the central nucleus of the amygdala (CEA), which receives input from the SI/nBM, also have been shown to cause performance deficits on the 5-CSRTT (Maddux et al., 2007). Rats who were lesioned in an area (posterior parietal cortex) that does not receive input from the nBM did not show any deficits on the 5-CSRTT. This implies a potential circuit between the CEA and the nBM that regulates attention (Maddux et al., 2007).

In summary, research seems to indicate that the nBM is critical for behavioral flexibility, which can be understood as the ability to modify behavior in response to changes in the environment, as well as complex cognitive processing.

*The role of the orexins/hypocretins*

The orexins (also known as hypocretins) are a family of hypothalamic neuropeptides that originate in the lateral hypothalamus and project to numerous brain areas, including the basal forebrain (Sakurai et al., 2005). Research indicates that there are two distinct orexin receptor subtypes in the basal forebrain, orexin-1 (Ox1R) and orexin-2 (Ox2R), both of which are G-protein coupled (Eggermann et al., 2001; Fadel, Pasumarthi, Reznikov, 2005; Sakurai et al., 2005; Sakurai, 2007). Similarly, there are two types of orexins, which have different affinities
for the separate types of orexin receptors (Eggerman et al., 2001; Thakkar et al., 2001). Both orexin A (OxA; also known as hypocretin-1) and orexin B (OxB; also known as hypocretin-2) bind with high affinity to Ox2Rs, while only OxA binds to Ox1Rs with high affinity (Eggerman et al., 2001; Thakkar et al., 2001). Although there is a greater density of Ox2Rs in the basal forebrain as compared to Ox1Rs, OxA is typically favored in the literature because it has high affinity for both receptor subtypes (Eggermann et al., 2001).

The orexins have been generally implicated in the sleep-wake irregularities observed in individuals suffering from narcolepsy-cataplexy (Chemelli et al., 1999; Liu et al., 2008; Peyron et al., 2000; Thannickal et al., 2000). Narcolepsy-cataplexy is characterized by sudden onset of sleep, constant sleepiness, increased rapid eye movement (REM) sleep stages, and hypnagogic hallucinations (Aldrich, 1993). Nishino et al. (2000) found that, as compared to age matched controls, human narcoleptics had far lower levels of orexin in the CSF. This suggested that a disruption of the orexin system is correlated with the development of a narcoleptic phenotype, and may even represent a causal relationship. To further elucidate this connection, research conducted by Thannickal et al. (2000) revealed that humans with narcolepsy have between 85-95% fewer orexinergetic neurons in the dorsomedial and perifornical hypothalamus, as well as evidence of gliosis in the hypothalamus. Gliosis occurs following cell death, and is known to be an indicator of neurodegeneration. Related research was conducted by Murillo-Rodriguez, Liu, Blanco-Centurion, & Shiromani (2008), in which the neurotoxin HCRT-2-saporin (HCRT-2-SAP) selectively destroyed the majority of orexin fibers in the lateral hypothalamus. It was observed that rats with reduced orexin innervations slept more. In addition to the hypothalamus, loss of orexinergetic neurons has been shown to occur in numerous other areas throughout the central nervous system, particularly those associated with the sleep-wake cycle (Peyron et al.,
It is now commonly accepted that narcolepsy-cataplexy is a neurodegenerative disorder characterized by progressive loss of the Ox2R subtype in brain areas critical to arousal and wakefulness (Sakurai, 2007). Research linking the basal forebrain cholinergic system with orexin signaling suggests a possible role for the orexins in maintaining arousal and attention (Thannickal et al., 2000).

The orexins have been shown to stimulate wakefulness when applied to the basal forebrain (Eggermann et al., 2001; Thakkar et al., 2001; Blanco-Centurion et al., 2006; Boschen, Fadel, Burk, 2009). In rats, OxA levels exhibit a diurnal pattern, with OxA building up during the active period, and falling during the rest period (Yoshida et al., 2001). This finding fits well with the observed neurodegeneration of orexinergic neurons in narcoleptics resulting in a difficulty maintaining wakefulness, attentional deficiencies while awake, as well as dissociated REM sleep (Nishino et al., 2000). Support for the hypothesis that the orexins stimulate wakefulness are provided by studies in which researchers administer orexin to rats and observe multiple indices of arousal (Thakkar et al., 2001; Blanco-Centurion et al., 2006). Thakkar et al. (2001) utilized microdialysis probes to directly infuse OxA into two regions of the basal forebrain, the SI and the horizontal band of Broca. Results indicated that intrabasalis OxA administration produced a dose-dependent increase in wakefulness and decreases in non-REM and REM sleep, suggesting that OxA works in the BFCS to stimulate wakefulness (Thakkar et al., 2001). This result raises the question of whether complete cholinergic innervation of the basal forebrain is required for orexinergic neurons to stimulate wakefulness. Blanco-Centurion, Shiromani, Winston, & Shiromani (2006) attempted to answer this question by selectively lesioning the BFCS using intracerebroventricular administration of 192 IgG-SAP to remove the majority of cholinergic innervations in the basal forebrain, before giving intrabasalis infusions of
OxA. Following lesions of the BFCS, researchers infused one of three doses of orexin into the basal forebrain of rats, and then monitored their sleep patterns using a sleep-recording microelectrode (Blanco-Centurion et al., 2006). Researchers found that lesioned rats given orexin infusions exhibited increased wakefulness and decreased REM sleep, even in the absence of the vast majority of cholinergic fibers in the BFCS (Blanco-Centurion et al., 2006). These results indicate that the orexins stimulate arousal.

The neurobiological mechanisms by which the orexins may interact with the BFCS are only beginning to be understood. Orexin neurons have been shown to make direct, functional synapses on basal forebrain cholinergic neurons both the SI and the ventral pallidum (VP; Fadel, Pasumarthi, & Reznikov, 2005). As previously noted, the primary function of the BFCS is to provide the cortex with ACh, contributing to cognitive functioning. Fadel, Pasumarthi, and Reznikov (2005) also observed that infusions of OxA via microdialysis increased cortical ACh release in a dose-dependent manner. This effect is not observed following direct infusions of OxA into the prefrontal cortex (PFC), implying that orexinergic neurons in the basal forebrain are integral for cortical ACh efflux (Fadel et al., 2005). Because of orexinergic projections originate in the LHA, commonly described as the brain’s “feeding center”, Frederick-Duus, Guyton, & Fadel (2007) chose to examine the role of the orexins in appetitive-induced cortical ACh efflux and feeding latency. Using in vivo microdialysis, Frederick-Duus, Guyton, and Fadel (2007) examined the effect of OxA administration on cortical ACh efflux in response to an appetitive stimulus. Results indicated that rats with orexin-B-saporin lesions of the basal forebrain exhibited significantly less cortical ACh efflux in response to a salient food stimulus (Frederick-Duus et al., 2007). Administration of an Ox1R-selective antagonist also reduced cortical ACh efflux in response to the food stimulus, as compared to vehicle-infused controls.
By stimulating cortical ACh release from the basal forebrain, the orexins indirectly impact the ability of an animal to attend to biologically relevant stimuli (Fadel & Frederick-Duus, 2008; Frederick-Duus, Guyton, Fadel, 2007). Orexin innervations of the BFCS appear to play a role in the ability to maintain homeostasis through interoceptive input, which is disturbed in individuals with AD. In many individuals with AD, dramatic weight loss occurs shortly before death (White, Pieper, & Schmader, 1998). While the neurobiological mechanisms underpinning this weight loss have not been discovered, it is possible that the decrease in cortical ACh caused by BFCS neuronal degeneration results in disrupted-food related homeostasis. The role of the orexins in mitigating this effect should be illuminated. Studies such as those conducted by Fadel et al. (2005) provide a better understanding of the mechanisms by which homeostatic disregulation may occur in AD.