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Cortical Modulation By Nucleus Basalis Magnocellularis Corticopetal Cholinergic Neurons During Anxiety-Like States Are Reflected By Decreases In Delta

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Abstract

Previous research has demonstrated that nucleus basalis magnocellularis (nbm) corticopetal cholinergic neurons modulate anxiety-like states, but cortical modulation by these neurons during anxiety-like states has not been characterized. In order to address this, we documented the effect of nbm corticopetal cholinergic lesions on cortical activity in direct (prefrontal cortex) and indirect (retrosplenial cortex) targets of nbm corticopetal cholinergic neurons during footshock induced operant suppression. The gamma/delta ratio and theta were used as indices of cortical activity, because these components of the electroencephalogram (EEG) are sensitive to basal forebrain corticopetal cholinergic modulation. During operant suppression, increases in the gamma/delta ratio and augmented theta were observed in both cortical EEGs. Lesions attenuated operant suppression and the gamma/delta ratio, but had no effect on increased theta. The effect of nbm corticopetal cholinergic lesions on the gamma/delta ratio was driven by the effect of the lesions on the delta band. The results of the study demonstrate that during anxiety-like states 1) decreases in delta reflect the action of nbm corticopetal cholinergic neurons, 2) nbm corticopetal cholinergic neurons alter neural processes in direct and indirect cortical targets, and 3) cortical theta is not dependent on the integrity of nbm corticopetal cholinergic neurons.

Section

Cognitive and Behavioral Neuroscience

Keywords

acetylcholine; fear; cortical activation; emotions; basal forebrain

2. Introduction¹

Previous reports have demonstrated that corticopetal cholinergic lesions in the nucleus basalis magnocellularis (nbm including the substantia innominata) attenuate conditioned operant

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suppression (Knox and Berntson, 2006; Stowell et al., 2000). Operant suppression is an animal model of anxiety (Gray and McNaughton, 2000; McNaughton and Corr, 2004), which suggests that nbm corticopetal cholinergic neurons are important for modulating anxiety-like states.

Nbm corticopetal cholinergic neurons modulate psychological processes by modulating cortical activity (Baxter and Chiba, 1999; Everitt and Robbins, 1997), but cortical modulation by nbm corticopetal cholinergic neurons during anxiety-like behavior has not been determined. Activation of basal forebrain corticopetal cholinergic neurons increases the ratio of fast wave (gamma) to slow wave (delta) components of the cortical electroencephalogram (EEG), and increases theta (Cape and Jones, 1998; Cape and Jones, 2000; Fournier et al., 2004; Jones, 2004; Manns et al., 2000a). These data suggest that increases in the ratio of gamma/delta and increases in theta are characteristic of cortical modulation by nbm corticopetal cholinergic neurons during anxiety-like states.

In order to test this hypothesis, we documented operant suppression induced by footshocks while simultaneously recording the prefrontal and retrosplenial EEGs in lesion and sham rats. Footshocks, as opposed to fear conditioned stimuli, were employed because they induce robust changes in the cortical EEG (laboratory observation). We recorded the EEG from the prefrontal and retrosplenial cortices, because previous studies have demonstrated that modulation of nbm corticopetal cholinergic neurons alters neural activity in these cortical substrates (Berntson et al., 2003; Berntson et al., 2002; Knox et al., 2004). Also, nbm corticopetal cholinergic neurons have direct projections to the prefrontal cortex, but not retrosplenial cortex (Gritti et al., 1997; Mesulam et al., 1983; Tengelsen et al., 1992; Woolf et al., 1983). Thus, sampling the EEG from these cortical regions allowed us to determine the direct and indirect effect of nbm corticopetal cholinergic lesions on cortical activity during anxiety-like states.

We predicted that exposure to footshocks would induce operant suppression, augment the gamma/delta ratio, and increase theta in the prefrontal and retrosplenial EEGs; and nbm corticopetal cholinergic lesions would attenuate these effects.

3. Results

3.1 Basal EEG

Nbm corticopetal cholinergic lesions augmented the relative contribution of slow wave frequencies and decreased the relative contribution of fast wave frequencies of the basal prefrontal cortical EEG. This effect was reflected by a significant surgery $\times$ band interaction on the cubic component [$F(1,10) = 9.82, p = .01$]. Lesions attenuate the gamma/delta ratio, and this difference approached significance [$t(10) = 2.111, p = .061$]. Nbm corticopetal cholinergic lesions did not have this simultaneous effect on slow and fast wave components of the basal retrosplenial cortical EEG [surgery $\times$ band interaction on the cubic component - $F(1,10) = .03, p = .88$]. Also, the gamma/delta ratio was not lower in lesion rats when compared to sham rats

¹AChE – acetylcholinesterase

AP – anterior-posterior

ChAT – choline acetyltransferase

DAB - 3, 3'-diaminobenzidine

DV – dorsoventral

EEG - electroencephalogram

FFT – Fast Fourier Transform

FR3 – fixed ratio schedule of 3

FR3-15 – 15 second time restricted fixed ratio schedule of 3

FR3-150 – 150 second time restricted fixed ratio schedule of 3

ML - mediolateral

NBM – nucleus basalis magnocellularis

PFC – prefrontal cortex

RSC – retrosplenial cortex

[$t(14) = 1.332, p = .204$]. These results are illustrated in figure 1, replicate a previous finding (Berntson et al., 2002), and are in accordance with previous studies that have demonstrated nbm cholinergic neurons innervate the prefrontal cortex, but not the retrosplenial cortex (Saper, 1984; Tengelsen et al., 1992; Torres et al., 1994; Woolf et al., 1983; Woolf et al., 1984).

3.2 Histology

Figure 2 displays AChE stained brain sections from representative rats in the study. Sham rats, in comparison to lesion rats, had considerably more AChE positive fibers in the frontoparietal cortex [lesion vs. sham: $t(12) = -4.38, p = .001$]. AChE fiber density was equivalent in the hippocampus and retrosplenial cortex of lesion and sham rats indicating that the lesion did not impact cholinergic neurons in the diagonal bands of Broca. This demonstrates that the lesion was restricted to nbm corticopetal cholinergic neurons. AChE fiber staining in the prefrontal cortex was equivalent between lesion and sham rats. This observation is consistent with a previous report (Holley et al., 1994), and may reflect the fact that the prefrontal cortex receives cholinergic projections from the diagonal bands of Broca and preoptic area, in addition to the nbm (Gritti et al., 1997; Torres et al., 1994; Woolf et al., 1983). Also, it has been proposed that the AChE stain may not be appropriate for documenting loss of cholinergic fibers in medial cortical regions, because ChAT and AChE fiber staining in these regions are not necessarily complimentary (Lysakowski et al., 1989; Stowell et al., 2000). Irrespective of the potential reasons, the electrophysiological data strongly suggest the loss of cholinergic input to the prefrontal cortex, which is in accordance with previous studies.

Figure 3 displays ChAT immunoreactive brain sections. ChAT immunoreactivity in the nbm, but not preoptic area, was attenuated in lesioned rats in comparison to sham rats. This suggests that the extent of the lesion was restricted to, at most, the preoptic area in the anterior direction; and further reinforces the assertion that the lesion procedures did not extend beyond the nbm.

3.3 Operant Suppression

Neither baseline operant performance [$t(22) = 1.255, p = .223$] nor trials required to reach criteria on the FR3-15 task [$t(22) = 1.451, p = .161$] was significantly altered by nbm corticopetal cholinergic lesions. The footshocks induced operant suppression during the shock and noshock FR3-150 trials. This was revealed by a significant one sample t-test analysis for the shock [$t(23) = 6.661, p < .001$], noshock 1 [$t(23) = 10.662, p < .001$], and noshock 2 [$t(23) = 7.089, p < .001$] trials. Operant suppression during all these trials were attenuated by nbm corticopetal cholinergic lesions as evidenced by the significant main effect of surgery [$F(1,22) = 5.041, p = .03$]. These results are illustrated in figure 4.

3.4 Prefrontal EEG

During the shock trials the ratio of gamma/delta was augmented, but returned to baseline levels during the noshock trials in control rats. Increases in the gamma/delta ratio during the shock trial were attenuated in lesion rats. These effects were reflected by a significant surgery $\times$ trial interaction [$F(2,20) = 11.519, p < .001$], and post hoc analysis [$t(10) = 5.006, p = .001$].

Changes in the gamma/delta ratio were driven by the effect of nbm corticopetal cholinergic lesions on the delta band. Delta in the prefrontal cortical EEG was decreased during the shock trial, and this effect rebounded above baseline during the noshock trials. This was revealed by a significant main effect of trial on the linear trend component [$F(1,10) = 35.59, p < .001$]. Nbm corticopetal cholinergic lesions attenuated decreases in delta during the shock trials. This effect was revealed by a significant surgery $\times$ trial interaction [$F(2,20) = 5.672, p = .01$], and post hoc analysis [$t(10) = 4.792, p = .001$]. Gamma in the prefrontal cortex was not altered during the shock trials, but decreases in gamma were observed during noshock trials 1 and 2.

This was revealed by a significant main effect of trial on the linear trend component [$F(1,10) = 7.398$, $p = .022$]. These effects were not altered in lesion rats [$F(1,10) = .037$, $p = .851$].

Theta was augmented during the shock trial and this effect decreased across noshock trials 1 and 2. This was revealed by a significant main effect of trial on the linear trend component [$F(1,10) = 9.016$, $p = .013$]. Theta remained elevated during noshock trial 1 [$t(11) = 3.099$, $p = .02$], but decreased to baseline levels during noshock trial 2 [$t(11) = 1.254$, $p = .236$]. Changes in theta during the shock and noshock trials were not altered by nbm corticopetal cholinergic lesions [$F(2,20) = .326$, $p = .726$]. These results are illustrated in figure 5.

3.5 Retrosplenial EEG

During the shock trials the ratio of gamma/delta was augmented, but returned to baseline levels during the noshock trials in control rats. Increases in the gamma/delta ratio during the shock trial were attenuated in lesion rats. These effects were reflected by a significant surgery $\times$ trial interaction [$F(2,28) = 9.34$, $p = .001$], and post hoc analysis [$t(14) = 4.285$, $p = .001$]. Changes in the gamma/delta ratio were driven by the effect of nbm corticopetal cholinergic lesions on the delta band. In control rats, decreases in delta in the retrosplenial EEG was observed and rebounded above baseline levels during the noshock trials. This effect was attenuated in lesion rats, and was reflected by the significant surgery $\times$ trial effect on the linear trend component [$F(1,14) = 7.285$, $p = .017$]. This finding was reinforced by post hoc analysis [$t(14) = .428$, $p = .001$]. Gamma in the retrosplenial cortex was not significantly altered during any of the trials [$F(2,28) = 1.786$, $p = .186$].

Theta was augmented during the shock trials and this effect decreased over noshock trials 1 and 2. This was revealed by a significant main effect of trial on the linear trend component [$F(1,14) = 5.375$, $p = .036$]. Theta remained elevated during noshock trial 1 [$t(14) = 2.53$, $p = .046$] and returned to baseline during noshock trial 2 [$t(14) = .995$, $p = .762$]. Even though theta was attenuated in lesion rats, this effect was not statistically significant [$F(1,14) = 2.132$, $p = .16$]. These results are illustrated in figure 6.

4. Discussion

Nbm corticopetal cholinergic lesions attenuated operant suppression generated by the footshocks, and prior research has demonstrated that these lesions attenuate conditioned operant suppression (Knox and Berntson, 2006; Stowell et al., 2000). In previous reports (Knox and Berntson, 2006; Stowell et al., 2000) and in this study (see results) nbm corticopetal cholinergic lesions did not affect operant performance. Taken together, these findings demonstrate that nbm corticopetal cholinergic lesions attenuate operant suppression induced by conditioned and unconditioned aversive events, which reinforces the assertion that these lesions attenuate anxiety-like states.

Nbm corticopetal cholinergic lesions attenuated the ratio of gamma/delta in the prefrontal and retrosplenial cortices during the shock trials; an effect that was driven by changes in the delta band. This strongly suggests that decreases in delta during anxiety-like states reflect the action of nbm corticopetal cholinergic neurons on the cortex. Decreases in delta were not observed during the noshock trials in sham rats even though operant suppression remained high in these rats. This suggests that decreases in delta may reflect neural processes crucial for the initiation, but not maintenance, of anxiety-like states. Since nbm corticopetal cholinergic lesions attenuated operant suppression during the noshock trials it is likely that these lesions alter other neural processes that are not reflected by changes in the EEG, but critical for the maintenance of anxiety-like states.

Nbm corticopetal cholinergic neurons have direct projections to the prefrontal, but not retrosplenial, cortex (see Introduction). This demonstrates the effect of lesions on delta in the retrosplenial cortex during the shock trials reflect the action of nbm corticopetal cholinergic lesions on cortical-cortical circuits. To the knowledge of the authors this data is the first to demonstrate that a restricted basal forebrain corticopetal cholinergic lesion can impact cortical activity in a region of the cortex that does not receive input from these restricted set of neurons. This indirect action (i.e. changes in retrosplenial cortical activity) was not observed during baseline operant performance, which strongly suggests that only during periods of augmented cortical processing (e.g. operant suppression) do nbm corticopetal cholinergic neurons engage cortical-cortical circuits.

The prefrontal cortex has extensive efferents to cortical substrates (Sesack et al., 1989), and in this study, nbm corticopetal cholinergic lesions attenuated changes in prefrontal cortical activity during operant suppression. Via the prefrontal cortex, nbm corticopetal cholinergic neurons may modulate neural activity in cortical substrates (e.g. retrosplenial cortex) that do not receive afferent input from nbm corticopetal cholinergic neurons. This assertion is consistent with a previous report that demonstrates cholinergic modulation of neural activity in the prefrontal cortex induces changes in neurotransmitter release in the parietal cortex; but cholinergic modulation of neural activity in the parietal cortex does not induce changes in neurotransmitter release in the prefrontal cortex (Nelson et al., 2005). However, this assertion remains to be empirically evaluated.

During the shock trials increased gamma was not observed in the prefrontal or retrosplenial cortices, which contrasts with previous studies that have demonstrated cortical activation is characterized by increases in gamma (Fournier et al., 2004; Jones, 2003; Jones, 2004). During baseline operant performance the relative power in the gamma band was already high. As a result, a ceiling effect may have occurred whereby further increases in gamma, induced by activation of nbm corticopetal cholinergic neurons or otherwise, could not be observed. This argument would account for the observation that nbm corticopetal cholinergic lesions decreased gamma during operant performance (i.e. baseline EEG), but activation of nbm corticopetal cholinergic neurons by footshocks did not increase gamma. Neither during operant performance nor operant suppression did nbm corticopetal cholinergic lesions alter theta in the prefrontal and retrosplenial cortices. Even though magnocellular preoptic corticopetal cholinergic neurons play a prominent role in modulating theta in the cortical mantle (Cape and Jones, 1998; Cape and Jones, 2000; Manns et al., 2000a); the results of this study demonstrate that nbm corticopetal cholinergic neurons are not necessary for the generation of theta in the cortical mantle.

Anxiety is associated with increased vigilance and/or sustained attention of cues within the immediate environment (Berntson et al., 1998; Eysenck, 1991; Gray and McNaughton, 2000; McNaughton and Corr, 2004); and nbm corticopetal cholinergic neurons may be critical for this attentional processing. This assertion is consistent with the role of nbm corticopetal cholinergic neurons in attentional processing (Baxter et al., 1999; McGaughy et al., 1996; Sarter and Bruno, 1997) and data that demonstrates nbm corticopetal cholinergic lesions do not induce general deficits in anxiety-like states. For example, nbm corticopetal cholinergic lesions have no effect on anxiety-like states in the elevated plus maze or open field (Knox and Berntson, 2006; Pizzo et al., 2002). Rats can avoid the aversive contexts' (i.e. open arms) of the elevated plus maze by entering the closed arms and aversive context (i.e. center region) of the open field by moving along the walls of the open field. These paradigms may not require sustained activation of attentional systems in the brain, because rats can use a behavioral strategy to attenuate the anxiety-like state generated by open space. During operant suppression rats have to perform an operant response to receive a reward in a threatening environment they cannot avoid. This feature of the operant suppression paradigm may result in sustained

activation of attentional systems; because rats attend to aversive stimuli, while simultaneously adjusting operant performance based on aversive stimulus presentation.

Even though it has been hypothesized that increases in the fast wave components of the EEG reflect increased attentional processing during aversive states (Davis and Whalen, 2001; Kapp et al., 1992), the results of this study suggest that changes in the ratio of fast wave/slow wave components of the EEG may be a better index of attentional processing during these anxiety-like states.

5. Experimental Procedures

5.1 Subjects

Twenty four male Sprague-Dawley rats, weighing between 400–450 g, were obtained from Harlan Industries (Indianapolis IN) and singly housed on a 12 hour light-dark cycle with lights on at 6:00a.m. and off at 6:00p.m. Rats had ad lib access to standard lab chow, but were put on a regulated water regime where rats had access to water for 15 minutes a day during operant training schedules. This water regime was used because it causes minimal weight loss over an extended period of time (Stowell et al., 2000). All experiments were approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and pursued in facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care.

5.2 Operant Task

In the operant task employed in this study, time to perform an operant response was used to measure operant suppression as opposed to the rate of operant responses. In our experience when time, as opposed to rate, is used to index operant suppression the training protocols are considerably shorter and operant suppression induced by aversive events can be observed (laboratory observation). Furthermore, the operant procedure described here has been successfully employed to study the effects of nbm corticopetal cholinergic lesions on operant suppression induced by a classical fear conditioned stimulus (Knox and Berntson, 2006).

Rats were subjected to training schedules in isolated operant boxes equipped with retractable levers and a shock grid within a sound attenuated chamber (Med Associates, St. Albans VT). Initially, rats were placed on an FR1 schedule where a bar press resulted in a 50 μ L water reward. Rats were then progressed up to an FR3 schedule until they depressed the lever greater than 50 times in 30 minutes. After this, rats were put on a time restricted fixed ratio schedule where rats had 15 s to hit the lever three times in order to obtain a water reward (FR3-15 trial). After rats had attained a 75% accuracy rate over nine trials a day for two days, they were subjected to surgical procedures. All training was conducted with the house light of the operant box on.

5.3 Surgery

Rats were administered xylazine (12 mg/kg) subcutaneously, maintained at a surgical plane using isoflurane, and placed in a stereotaxic apparatus (David Kopf, Tujunga CA). In order to induce cholinergic lesions a 5 μ L Hamilton syringe with a 26 gauge needle was used to deliver 0.5 μ L of the selective immunotoxin 192-IgG saporin (ATS, San Diego CA) into the nbm at a concentration of 0.2 μ g/ μ L. We previously demonstrated that this batch of 192-IgG saporin induced selective corticopetal cholinergic lesions in the nbm (Knox and Berntson, 2006). We targeted cholinergic neurons in the nbm because 1) the nbm contains a large quantity of corticopetal cholinergic neurons (Mesulam et al., 1983); 2) nbm cholinergic neurons do not have appreciable projections to the hippocampus (Tengelsen et al., 1992; Torres et al., 1994; Woolf et al., 1984), thus, targeting these neurons allow for selective manipulation of the

corticopetal cholinergic system without directly altering the septohippocampal cholinergic system; 3) previous studies have demonstrated that targeting cholinergic neurons in this region of the basal forebrain attenuate epinephrine primed cortical activation and aversive states induced by conditioned aversive events (see Introduction). The coordinates used to target the nbm area were AP = -1.4 mm, ML = $\pm$ 3.1 mm, DV = -7.0 mm, referenced from bregma, and in accord with the atlas of Paxinos and Watson, (1998). Sham lesions were accomplished by infusion of an equivalent volume of Dulbecco saline (Sigma, St. Louis MO) into the nbm.

Skull trephine holes were then drilled over the retrosplenial cortex (AP = -4.5 mm, ML = 0.5 mm) and frontal sinus (AP = 10 mm) for implantation of a surface recording electrode and reference electrode respectively. Both kinds of electrodes were stainless steel screws (length = 2 mm, diameter = 1 mm); and these electrodes were threaded into the trephine holes over the retrosplenial cortex and frontal sinus (~0.5 mm). Leads were then attached to the electrodes for connection to a single channel bio-telemetric device (Data Sciences International, St. Paul MN). This device was implanted subcutaneously in the dorsum of rats between the shoulder blades.

A subset of rats had a depth recording electrode placed into the prefrontal cortex (AP = 2 mm, ML = 0.6 mm, DV = -3.6 mm) in addition to the surface electrodes placed over the retrosplenial cortex and frontal sinus. Depth electrodes were made of stainless steel, 300 μ m in diameter, and coated with formvar. The tip of all depth electrodes were stripped of formvar (~0.5 mm) before being inserted into the prefrontal cortex. All electrodes were then connected to leads and attached to a dual channel bio-telemetric device implanted subcutaneously in the dorsum of rats. The ground for all telemetrics was placed in the body of rats. Two more screws were then inserted into the skull of rats to serve as anchors for the head stage which was made of dental acrylic. All electrode placements were made relative to bregma and referenced from the atlas of Paxinos and Watson (1998). Following completion of surgery, rats received the antibiotic amoxicillin (subcutaneously, 30–100 mg/kg) and topical application of a local anesthetic over all wounds. Antibiotic treatment continued for two days post surgery or as necessary. The rats were then placed back into their home cages and had ad lib access to food and water. A time period of ten days was allowed to pass before the remainder of the experiment continued in order to permit surgical recovery and degeneration of cholinergic fibers.

5.4 Basal EEG Acquisition and Re-training

After this ten day period, rats were placed back on the water restricted diet, re-trained on the FR3-15 schedule (see above), and had to complete nine FR3-15 trials in one day at an accuracy rate of 75% a day over three days before moving on to the next phase of the study. During these re-training trials, the EEG was recorded in the prefrontal and retrosplenial cortices for 30s at the start of each trial in order to determine the effect of nbm corticopetal cholinergic lesions on basal cortical EEG. The EEG recorded from the prefrontal and retrosplenial cortices were transmitted to a PhysioTel Receiver (Data Sciences International, St. Paul MN), which was coupled to a Biopac recording system (Biopac, Goleta CA) via an analog adaptor. Signals were amplified by a factor of 100, digitized at 1 kHz (12 bit), displayed on a computer monitor, and stored for offline processing.

5.5 Operant Suppression

Twenty four hours after the end of basal EEG acquisition, procedures were employed to measure operant suppression induced by footshocks. All rats had to perform FR3-150 trials (150 s to depress a lever three times to obtain a water reward) that were separated by an inter-trial interval of 60 s. The time to complete the FR3 schedule was changed from 15 s to 150 s in order to allow sufficient time to capture operant suppression induced by the footshocks. Electrophysiological data were obtained during all FR3-150 trials in an identical manner to

that already described. For all FR3-150 trials, operant suppression was defined as the latency to perform the FR3 operant schedule. All trials were conducted in the same operant box in which rats were trained.

After a five minute adaptation period, rats had to perform three FR3-150 trials which were used to obtain baseline behavioral and electrophysiological measures. At the start of the fourth FR3-150 trial, a 1.5mA footshock lasting for 500 ms was delivered through the floor of the operant box (shock trial). This was followed by two FR3-150 trials (noshock 1 and noshock 2 trials) during which time there was no footshock presentations. This sequence of trials (i.e. shock, noshock 1, noshock 2) was repeated one more time. At the end of the last noshock trial rats were returned to their home cages.

5.6 Histochemistry

We used AChE histochemistry to quantify the extent of the cortical cholinergic lesion induced in this study. Choline acetyltransferase (ChAT) immunohistochemistry was performed in a subset of rats in order to demonstrate the anterior extent of the cholinergic lesion induced by our surgical procedures. At the conclusion of the study, nineteen of the rats were deeply anesthetized and transcardially perfused with heparinized saline followed by a 10% formalin solution. The other five rats were transcardially perfused with heparinized saline followed by a 4% paraformaldehyde solution in order to visualize ChAT. The brains were removed and maintained in the respective fixative for at least 24 hours after which they were placed in a 30% sucrose/phosphate buffer until they sank. Brains were then maintained at -19°C in a cryostat (Leica, Bannockburn IL) and sections were taken at $50\text{ }\mu\text{m}$ through the frontoparietal cortex, prefrontal cortex, retrosplenial cortex, and hippocampus for visualization of AChE. Sections were also taken at $30\text{ }\mu\text{m}$ through the preoptic area and nbm for visualization of ChAT. Procedures used to visualize and quantify AChE are detailed elsewhere (Berntson et al., 2002; Stowell et al., 2000; Tago et al., 1986) and are only briefly described here.

After incubation of sections in .1 % H_2O_2 , sections were incubated for 45 minutes in a solution consisting of 5 mg of acetylthiocholine iodide, .112 g sodium citrate, .025 g copper sulfate, and .0164 g potassium ferricyanide in .1 M maleate buffer. After a rinse in 50 mM Tris buffer (pH 7.6) AChE was visualized by incubating sections in a .4 % 3, 3'-diaminobenzidine (DAB) solution. Finally, sections were mounted on gelatin coated slides, dehydrated in ethanol, and coverslipped using DPX mountant (Electron Microscopy Sciences, Hatfield, PA). Sections were visually inspected to determine if an AChE lesion could be observed. If a lesion was observed in a particular brain region, then an AChE fiber counting method was adopted from Stowell JR et al. (2000) and Connor JM et al. (2003) to count AChE fibers in that brain region. Only rats that exhibited cortical AChE fiber loss, without hippocampal AChE fiber loss, were included in the lesion group.

Sections taken for visualization of ChAT were incubated in .01 % Triton and rinsed in .1 M phosphate buffer. Sections were then incubated in a diluent for one hour. Diluent consisted of .1 M phosphate buffer, .1 % Triton, and 3 % rabbit serum. Sections were rinsed in phosphate buffer again and incubated in diluent and a primary goat anti-ChAT polyclonal antibody in a dilution of 1:1000 for 24 hours at 4°C . Afterward, sections were rinsed in phosphate buffer and incubated with diluent and a secondary rabbit anti-goat antibody mixed to a dilution of 1:2000 for 1 hour at 4°C . Sections were rinsed with phosphate buffer and incubated in diluent and a goat peroxidase-antiperoxidase soluble immune complex at a concentration of $40\text{ }\mu\text{g}/\mu\text{L}$ at 4°C . Sections were then rinsed in Tris buffer and incubated in a .05 % DAB solution for 10 minutes in order to visualize the immunoprecipitate. Sections were then mounted on gelatin coated glass slides, dehydrated in ethanol, and coverslipped using DPX mountant (Electron Microscopy Sciences, Hatfield, PA). All antibodies used for the ChAT histological procedure

were obtained from Chemicon (Temecula CA). All other reagents were obtained from Sigma-Aldrich (St. Louis MO).

5.7 Data and Statistical Analysis

EEG signals were tapered through a Hamming window, subjected to a Fast Fourier Transform (FFT), analyzed in one second epochs using Mindware (Columbus, OH), and averaged to obtain a single measure of each frequency band of the EEG during every FR3-150 trial. We defined the EEG components as follows: delta (1–4 Hz), theta (4.5–9 Hz), gamma (30.5–58 Hz). Raw values of the EEG were converted to relative scores using the following equation:

$$\text{Relative power} = (\text{band power} / \text{total power}) * 100 \quad (1)$$

From this point onward, unless stated otherwise, the use of a frequency band (e.g. delta) refers to the relative contribution of the band to the EEG and not the absolute power within that band.

AChE positive fiber counts were subjected to a t-test (surgery: lesion vs. sham). The prefrontal and retrosplenial EEG were averaged during the re-training and baseline trials to obtain a single measure of the basal EEG for each cortical region. A two (surgery: lesion vs. sham) $\times$ five (band: delta, theta, alpha, beta, gamma) factorial design was used to analyze the basal prefrontal and retrosplenial EEGs. Gamma/delta ratios were calculated for the prefrontal and retrosplenial EEGs and subjected to t-test (surgery). Pairs of behavioral and electrophysiological data during the two sequences of shock, noshock 1, and noshock 2 trials were averaged to obtain single behavioral and electrophysiological measures for these trials. Baseline behavioral measures were subtracted from their respective values during the shock and noshock trials and subjected to a two (surgery) $\times$ three (trial: shock, noshock 1, noshock 2) factorial design.

Electrophysiological measures during the shock and noshock trials were expressed as a percent change from baseline and subjected to a two (surgery) $\times$ three (trial: shock, noshock 1, noshock 2) factorial design. Main and simple effects were analyzed using analysis of variance and main and simple comparisons were analyzed using one and independent sample t-tests with a Bonferroni correction applied where necessary. For one sample t-test the reference value was set at 0. The criteria level for all statistical tests was set at .05. The data from an individual rat was discarded from the study if datum within a level of any factor was more than two standard deviations away from the respective mean.

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Figure 1. The effect of nbm corticopetal cholinergic lesions on the basal cortical EEG

a) Lesions augmented the relative contribution of slow wave frequencies and attenuated the relative contribution of fast wave frequencies, of the basal PFC EEG. The greatest difference in slow wave frequencies between lesion and sham rats was within the delta band, and the greatest difference in fast wave frequencies was in the gamma band. b) Lesions attenuated the gamma/delta ratio in the PFC EEG, and this difference approached significance. Lesion = 7, Sham = 5. c) Nbm corticopetal cholinergic lesions did not alter components of the basal RSC EEG. Delta in lesion rats was attenuated in the RSC EEG but this was not statistically significant. d) Lesions did not alter the gamma/delta ratio in the RSC EEG. Lesion = 8, Sham = 8. PFC – prefrontal cortex, RSC – retrosplenial cortex. * indicates a significant comparison with an alpha levels set at .05.

Figure 2. AChE stained sections for lesion and sham rats

Cortical sections were taken at a magnification of 40 $\times$. a) Brain sections displaying the frontoparietal cortex, prefrontal cortex, retrosplenial cortex, and hippocampus. AChE fiber depletion was observed in the frontoparietal cortex, but not hippocampus or retrosplenial cortex, of lesion rats indicating cholinergic lesions did not encroach on cholinergic neurons in the diagonal bands of Broca. There was no difference between AChE fiber staining in the prefrontal cortex of lesion and sham rats. b) *Title: AChE fiber counts in the frontoparietal cortex.* Of the cortical areas sampled, the frontoparietal cortex was the only region that displayed AChE fiber deafferentation. As a result, the counting method described was only applied to this cortical region. AChE – acetylcholinesterase. * indicates a significant comparison with an alpha levels set at .05.

3**Preoptic Area****Nbm****Lesion** **Sham**

Figure 3. ChAT immunoreactive sections through the nbm, and preoptic area

The inset pictures show a magnification of the respective areas at 40× while the larger pictures show sections photographed at 100×. ChAT immunoreactivity was attenuated in the nbm, but not the preoptic area of lesion rats. This suggests that the lesion procedures employed in this study did not encroach on cholinergic neurons in the diagonal bands of Broca. ChAT was conducted in two lesion and three sham rats. ac – anterior commissure, ChAT – choline acetyltransferase, ic – internal capsule, nbm – nucleus basalis magnocellularis.

Figure 4. The effect of nbm corticopetal cholinergic lesions on operant performance and suppression

a) The number of trials required to attain criteria in the FR3 task, and b) time to perform the operant task during the re-training and baseline trials was equivalent in lesion and sham rats. c) Time to perform the FR3 schedule during baseline was subtracted from respective values during the shock and noshock trials and graphed as difference scores. Operant suppression was observed during the shock and noshock trials. These effects were attenuated by nbm corticopetal cholinergic lesions. Identical results were obtained when rate was used (i.e. no. of responses/ 150s) as opposed to time (data not shown). Operant suppression for one lesion rat during the shock trial was greater than two standard deviations above the mean. As a result,

this rat was dropped from the study. Lesion = 11, Sham = 12. * indicates a significant comparison with an alpha levels set at .05.

Figure 5. The effect of nbm corticopetal cholinergic lesions on changes in measures of cortical activation in the prefrontal cortex

Electrophysiological measures during the shock and noshock trials were expressed as a percent change from baseline values. a) The gamma/delta ratio was augmented during the shock trials and this effect was attenuated in lesion rats. b) Decreases in delta were induced by the footshocks during the shock trials, and these effects were attenuated by nbm corticopetal cholinergic lesions, indicating that changes in delta primarily contributed to the effect lesions had on the gamma/delta ratio. c) Footshock presentation had no effect on gamma during the shock trials, but gamma was attenuated during the noshock trials. This effect was not attenuated by nbm corticopetal cholinergic lesions. d) Footshock presentation augmented theta during the

shock trial and no shock trial 1. These effects were not attenuated by nbm corticopetal cholinergic lesions. Lesion = 7, Sham = 5. * indicates a significant comparison with an alpha levels set at .05.

Figure 6. The effect of nbm corticopetal cholinergic lesions on changes in measures of cortical activation in the retrosplenial cortex

Electrophysiological measures during the shock and noshock trials were expressed as a percent change from baseline values. a) The gamma/delta ratio was augmented during the shock trials and this effect was attenuated in lesion rats. b) Decreases in delta were induced by the footshocks during the shock trials, and these effects were attenuated by nbm corticopetal cholinergic lesions, indicating that changes in delta primarily contributed to the effect lesions had on the gamma/delta ratio. c) Neither footshock presentation nor nbm corticopetal cholinergic lesions had any effect on gamma during the shock and noshock trials. d) Footshock presentation augmented theta during the shock and noshock trial 1. While these effects were

attenuated in lesions rats, this difference was not statistically significant. Lesion = 8, Sham = 7. * indicates a significant comparison with an alpha levels set at .05.