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CHOLINERGIC MODULATION OF PAVLOVIAN FEAR CONDITIONING IN RATS: DIFFERENTIAL EFFECTS OF INTRAHIPPOCAMPAL INFUSION OF MECAMYLAMINE AND METHYLLYCACONITINE

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Abstract

The cholinergic system has consistently been implicated in Pavlovian fear conditioning. Considerable work has been done to localize specific nicotinic receptor subtypes in the hippocampus and determine their functional importance; however, the specific function of many of these subtypes has yet to be determined. An $\alpha 7$ nicotinic antagonist methyllycaconitine (MLA) (35 μ g), and a broad spectrum non- $\alpha 7$ nicotinic antagonist mecamylamine (35 μ g) was injected directly into the dorsal hippocampus or overlying cortex either 15 minutes pre-, 1 minute post-, or 6 hours post-fear conditioning. One week after conditioning, retention of contextual and cue (tone) conditioning were assessed. A significant impairment in retention of contextual fear was observed when mecamylamine was injected 15 minutes pre- and 1 minute post-conditioning. No significant impairment was observed when mecamylamine was injected 6 hours post-conditioning. Likewise, a significant impairment in retention of contextual fear was observed when MLA was injected 1 minute post-conditioning; however, in contrast, MLA did not show any significant impairments when injected 15 minutes pre-conditioning, but did show a significant impairment when injected 6 hours post-conditioning. There were no significant impairments observed when either drug was injected into overlying cortex. No significant impairments were observed in cue conditioning for either drug. In general, specific temporal dynamics involved in nicotinic receptor function were found relative to time of receptor dysfunction. The results indicate that the greatest deficits in long-term retention (1 week) of contextual fear are produced by central infusion of MLA minutes to hours post-conditioning or mecamylamine within minutes of conditioning.

Keywords

hippocampus; mecamylamine; methyllycaconitine; MLA; nicotine; nicotinic; acetylcholine; $\alpha 7$; non- $\alpha 7$

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1. Introduction

Classical or Pavlovian fear conditioning involves forming an association between a distinctive cue (i.e., tone) (conditioned stimulus [CS]) and an aversive event (i.e., footshock) (unconditioned stimulus [US]) (see Fanselow, 2000, & Maren, 2001, for review). Typically, this CS-US association occurs within a particular context and rats will acquire a conditioned response (CR) (i.e., freezing) to the context associated with the US delivery as well as to the cue. In contrast to the cue-shock association, the context-shock association has been found to be hippocampal dependent (Anagnostaras, Maren, & Fanselow, 1999a; Holland, & Bouton, 1999; Maren, 1998). That is, lesions to the dorsal hippocampus have been found to abolish the context-shock association with no effect on the cue-shock association (Anagnostaras, et al., 1999a; Fanselow, 2000; Maren, 2001). However, if a long training-to-lesion interval is allowed, then hippocampal lesions have little effect and result in smaller retention deficits (Anagnostaras, et al., 1999a; Fanselow, 2000; Kim, & Fanselow, 1992; Maren, Aharonov, & Fanselow, 1997; Maren, & Fanselow, 1997; Phillips, & Ledoux, 1992). This temporal gradient suggests that there is a limited amount of time during which the hippocampus is involved in acquisition and consolidation of contextual fear conditioning.

Adult rats also show impairments in contextual fear conditioning, but not in cue conditioning, with disruption of both muscarinic and nicotinic cholinergic neurotransmission (Anagnostaras, et al., 1999a; Anagnostaras, Maren, Sage, Goodrich, & Fanselow, 1999b; Caldarone, Duman, & Picciotto, 2000; Davis, & Gould, 2006; Gale, Anagnostaras, & Fanselow, 2001; Levin, 2002b; Paylor, Nguyen, Crawley, Patrick, Beaudet, & Orr-Urtreger, 1998; Rudy, 1996) or lesions of the fimbria-fornix, which has been shown to block cholinergic innervation of the hippocampus from the ventrolateral diagonal band (Maren, & Fanselow, 1997). This is consistent with the growing body of evidence implicating cholinergic neurotransmission in the hippocampus in many forms of context-dependent learning (Anagnostaras, Maren, & Fanselow, 1995; Anagnostaras, et al., 1999b; Barros, Ramirez, Dos Reis, & Izquierdo, 2004; Blokland, Honig, & Raaijmakers, 1992; Bovet-Nitti, 1966; Davis, & Gould, 2006; Gale, et al., 2001; Rudy, 1996; Wallenstein, & Vago, 2001). However, the temporal dynamics of muscarinic and nicotinic receptor systems along with functional deficits associated with broad-spectrum nAChR blockade and more specific nAChR subtype dysfunction (i.e., $\alpha 7$) are currently unclear and some results have been mixed.

The nicotinic cholinergic system has been shown to be involved in several cognitive functions with convincing evidence of its involvement in short-term, long-term, and working memory function (Barros, et al., 2004; Decker, Brioni, Bannon, & Arneric, 1995; Decker, & Majchrzak, 1993; Felix, & Levin, 1997; Kim, & Levin, 1996; Levin, 2002a; Ohno, Yamamoto, & Watanabe, 1993). Considerable work has been done to localize the alpha and beta subunit receptors involved in hippocampal-dependent memory function (Barros, et al., 2004; Levin, 2002a; Levin, 2002b; Sargent, 1993). There is now strong evidence for specific nAChR subtypes (i.e., $\alpha 7$, $\alpha 4\beta 4$, $\alpha 4\beta 2$ and $\alpha 3\beta 4$) localized within the hippocampus and associated memory structures (Adams, Stitzel, Collins, & Freedman, 2001; Alkondon, & Albuquerque, 1993; Frotscher, & Leranth, 1985; Levin, & Simon, 1998). However, the role of these receptor subtypes in a variety of cognitive behaviors is currently incomplete (Levin, 2002b; Levin, & Simon, 1998) and results from the fear conditioning paradigm have been mixed (Gould, & Higgins, 2003; Wehner, Keller, Keller, Picciotto, Paylor, Booker, Beaudet, Heinemann, & Balogh, 2004). Previous fear conditioning studies using systemic administration of selective and non-selective nAChR antagonists, as well as studies involving nAChR subunit null mutant mice have failed to show profound deficits in 24 hr retention of contextual fear conditioning, but have shown subunit-specific modulatory effects (Davis, & Gould, 2006; Gould, & Higgins, 2003; Gould, & Wehner, 1999; Wehner, et al., 2004). Specifically, the $\alpha 4\beta 2$ nicotinic antagonist, dihydro-beta-erythroidine (DH β E), but not the $\alpha 7$ -specific antagonist

methylyaconitine (MLA) has shown disruptive effects on nicotine enhancement of conditioning (Davis, & Gould, 2006). Thus, the present study aimed to examine the time-dependent, functional differences between central blockade of broad-spectrum nAChRs and more specific nAChR blockade (e.g., $\alpha 7$) in long-term retention (i.e., 1 week) of contextual fear conditioning by infusing mecamylamine or MLA directly into the dorsal hippocampus. It has been found that mecamylamine acts as a more broad-spectrum nAChR antagonist (Chavez-Noriega, Crona, Washburn, Urrutia, Elliott, & Johnson, 1997; Debruyne, Sobrio, Hirschberger, Camsonne, Coquerel, & Barre, 2003; Martin, Onaivi, & Martin, 1989) making it a good candidate for affecting all nicotinic receptors; whereas, MLA has been found to inhibit $\alpha 6$ subunits at some concentrations, but selectively inhibit the $\alpha 7$ subunit in the hippocampus (Davies, Hardick, Blagbrough, Potter, Wolstenholme, & Wonnacott, 1999; Turek, Kang, Campbell, Arneric, & Sullivan, 1995). The data have implications for general and specific (i.e., $\alpha 7$) nAChR function in long-term memory consolidation and retention in contextual fear conditioning.

2. Materials and Methods

2.1. Subjects

Seventy-six male Long-Evans rats (300–365 g) were used in this experiment. All rats were individually housed and located in a colony room maintained on a 12:12 light: dark cycle. Each rat was handled prior to testing and had unrestricted access to food and water. All protocols conformed to the NIH Guide for the Care and Use of Laboratory Animals. The treatment of animals complied with all animal care guidelines that have been approved by the Institutional Animal Care and Use Committee (IACUC) and the University Animal Resource Center (ARC).

2.2. Implantation of Guide Cannulae

Rats were given an intraperitoneal injection of atropine sulfate (0.4 mg/kg, i.p.) to control for respiratory difficulties. Rats were then anesthetized with an intraperitoneal injection of sodium pentobarbital (65 mg/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was incised and retracted exposing bregma and lambda in the same horizontal plane. Small burr holes (2.0 mm diameter) were drilled bilaterally. Using stereotaxic coordinates, stainless steel guide cannula (28-gauge, stainless steel; Plastics One, Roanoke, VA) were then lowered bilaterally into 1) the dorsal hippocampus ($n=68$; 3.6 mm posterior to bregma, 2.5 mm lateral to the midsagittal suture, and 2.5 mm ventral from the brain surface) or 2) the overlying cortex ($n=8$; 3.6 mm posterior to bregma, 2.5 mm lateral to the midsagittal suture, and 1.8 mm ventral from the brain surface). These coordinates were chosen based on the histological analyses of Wallenstein and Vago (2001); however, in order to cover more of the dorsal hippocampus, the coordinates were changed 0.2 mm more lateral and 0.3 mm more ventral (see Fig. 1). The guide cannula were fixed with dental cement for which three small skull screws (1 mm) were previously screwed into the skull as anchors. Stainless steel stylets (33 gauge) with dustcaps were screwed into the top of the cannulae to prevent occlusion. Following surgery, the rats were allowed to recover on a heating pad before returning to their home cage. Rats were given acetaminophen (Children's Tylenol) (~150–200mg/kg) dissolved in drinking water for 2–3 days post-surgery as an analgesic.

2.3. Behavioral Apparatus

The training chamber (used for conditioning and context testing) consisted of two clear Plexiglas walls (rear and front door) and two aluminum side panels (28w $\times$ 21h $\times$ 22d cm; Colbourn Instruments, Allentown, PA). The floor of the chamber consisted of 18 steel rods (0.2 cm radius situated 1.5 cm apart) connected to a precision shock scrambler (Colbourn Instruments). A speaker was inserted in one of the side panels of the chamber to deliver the auditory stimuli. All stimuli were controlled by a computer software package (Graphic State,

Colbourn Instruments). The training chamber was placed in a room with fluorescent lighting, had a fixed arrangement of spatial cues surrounding it and was scented with artificial vanilla flavoring beneath the floor contributing to the particular context. Animals were continuously monitored on video screens by observers located in a separate room.

The cue (tone) test was completed in a chamber of exactly the same dimensions as the training chamber, but each side and top panel consisted of red, semi-translucent Plexiglas that prevented rats from seeing the external environment. A speaker was attached to a hole (2.5 cm diameter) made on one of the walls of the chamber to deliver the auditory stimuli. The tone test chamber was located in a novel, dimly lit room and the pan beneath the rods was scented with a 5% ammonia solution in order to maintain a novel context.

2.4. Intrahippocampal infusions

Approximately one week after cannulae implantation, each rat was given bilateral injections of either phosphate buffered saline (PBS), MLA, or mecamlamine (Sigma Chemicals, St. Louis, MO) into the dorsal hippocampus 15 minutes pre-, 1 minute post- or 6 hours post-conditioning. The times of administration were selected in order to test the pre- and post-conditioning effects of each drug and were based on similar times of administration in previous cue and contextual fear conditioning studies (Anagnostaras, et al., 1999b; Bernabeu, Bevilacqua, Ardenghi, Bromberg, Schmitz, Bianchin, Izquierdo, & Medina, 1997; Stewart, & Mckay, 2000; Wallenstein, & Vago, 2001; Wallenstein, Vago, & Walberer, 2002). Both drugs (35µg) were dissolved and delivered in a volume of 0.35µl of PBS. Both the concentration and volume of MLA and mecamlamine were selected from previous studies with similar doses that have shown significant deficits in various types of learning and memory and in which diffusion was found to be limited to the targeted area (Barros, et al., 2004; Bettany, & Levin, 2001; Blozovski, 1983; Decker, et al., 1995; Felix, & Levin, 1997; Levin, 2002b; Ohno, et al., 1993; Wallenstein, & Vago, 2001). A relatively high concentration was chosen to examine robust effects of each drug. Activity level was compared between drug and control groups by recording ambulatory crossovers (see **Results**).

After removing the stylets from the guide cannula, infusions into the dorsal hippocampus were administered through injector cannula (33 gauge) extending 1.0 mm below the guide cannula. Each injector cannula was attached to a 10µl Hamilton syringe (VWR, West Chester, PA) with polyethylene tubing. The syringe was then mounted on an infusion pump (Harvard Apparatus, Holliston, MA) and drug/vehicle was delivered bilaterally at a constant rate of 0.5µl/min for 42 seconds. Injector cannula were left in position for an additional 60 seconds following the infusion to allow for diffusion of the drug/vehicle and absorption of the infusion bolus by the brain tissue. The rate was chosen to reduce the tendency for the drug to diffuse up the cannula track (Keefe, & Gerfen, 1995).

2.5. Behavioral training

The training and testing schedule of Wallenstein and Vago (2001) was adopted and modified for comparison. One group of rats received intra-hippocampal injections of either MLA (n=8) or mecamlamine (n=8) in their home cages 15 minutes before being placed in the fear conditioning chamber. Another group of rats received intra-hippocampal injections of either MLA (n=8) or mecamlamine (n=8) approximately 1 minute after being taken out of the fear conditioning chamber. A control group of rats received injections into the overlying cortex of either MLA (n=4) or mecamlamine (n=4) approximately 1 minute after removal from the fear conditioning chamber. This anatomical control was necessary to assess the effect of the drugs in areas near to the intended injection site and where the drug may have diffused up the cannula track. The next group received intra-hippocampal injections of either MLA (n=8) or mecamlamine (n=8) 6 hours after removal from the fear conditioning chamber. Another

control group was given PBS (n=8) at all time intervals and all drug groups were injected with PBS into the hippocampus at every time interval in which they were not given a drug treatment. In order to control for any state-dependent effects, a final two groups of rats received mecamlamine (n=6) or MLA (n=6) infusions fifteen minutes before conditioning as well as fifteen minutes before testing. This condition was important to assess whether learning under the influence of either drug would act as a state-dependent cue affecting retrieval during testing.

After the appropriate injections were made, each rat was placed into the training chamber. After a 2-minute acclimation period, a 30-second, 2 KHz, 85 dB (A-scale) tone was presented and co-terminated with a 2-second, ~1 mA footshock. This pairing was repeated five times every 60 seconds. Approximately one minute after the final tone/shock pairing, each animal was removed from the chamber and given the appropriate injections into their respective cannulae and returned to their home cages. Six hours later, each group of rats was given their respective injections (i.e., PBS, mecamlamine, or MLA) and returned once again to their home cages.

2.6. Behavioral Testing

Retention testing of both contextual and cue conditioning was assessed drug-free. This avoided any possible confound of hyperactivity or other behavioral change due to infusion of the drug. The state-dependent control also received either MLA or mecamlamine 15 min pre-testing. Testing of retention of contextual fear conditioning (context test) began one week after conditioning (drug-free). Each animal was returned to the original conditioning chamber with the same contextual cues for a period of 16 minutes. During this time, freezing behavior (an index of conditioned fear – (Fanselow, & Bolles, 1979) was scored blind using an 8-second sampling procedure (Anagnostaras, et al., 1995). Average freezing during the first 8 minutes was compared among all groups. Animals begin to habituate to the same level of exploration after 8 minutes of testing, hence, only the first 8 minutes were analyzed.

Retention testing of cued fear conditioning (tone test) was completed approximately 24 hours after the context test (drug-free). After a 2-minute acclimation period, a 16-minute, 2 kHz, 85-dB (A-scale) tone was presented from a speaker in the side of the tone test chamber. During this time, freezing behavior was scored blind using an 8-second sampling procedure (Anagnostaras, et al., 1995). Average freezing during the first 8 min was compared among all groups.

2.7. Histology

After all behavioral testing was completed rats were anesthetized with a lethal dose of Nembutal (sodium pentobarbital, 70 mg/kg, i.p.) and perfused intracardially with saline solution followed by a 10% formalin solution. In order to verify cannula placements and get an estimate of drug diffusion, a bilateral infusion of 4% Chicago blue in PBS (.35µl) was performed prior to nembutal injection using the same procedure as infusion protocol mentioned earlier. Each brain was extracted and stored in a 10% formalin/30% sucrose solution at 4 °C for 5 days. A tissue-block from bregma-2.0 to bregma-4.0 was frozen (−8 °C) and cut perpendicularly at 40µm section on a cryostat, and every third section was collected on a glass slide, producing 16 sections collected throughout the dorsal hippocampus for each brain. The sections were stained with Cresyl Violet.

3. Results

3.1. Histology

Injector cannula were found to be located bilaterally within the dorsal hippocampus (n=68) or overlying cortex (n=8) (Fig. 1). For the rats in which our aim was to target the entire dorsal hippocampus, the dye (Chicago blue) appeared to spread through the majority of the

hippocampal subfields with traces of dye detected in the majority of the pyramidal and molecular cell layers. It should be noted, however, that the diffusion of the dye does not reflect the diffusion of the drug precisely due to the various differences in absorption, molecular weights, and pharmacokinetics of the drugs/dye.

3.2. Ambulatory Crossovers

As a measure of general activity, ambulatory crossovers (number of midline crossovers) in the training chamber 4 minutes prior to conditioning were recorded for animals receiving drug into the dorsal hippocampus before conditioning, and compared with animals receiving PBS (Fig. 2). Crossovers were defined as both of the rat's hind paws crossing the midline of the training chamber. A one-way analysis of variance (ANOVA) was performed with drug type (PBS-control, mecamlamine, MLA) which revealed that there were no significant differences in the average amount of general activity prior to conditioning.

3.3. Behavioral Data Analysis

As shown in Fig. 3, both the pre- and post-infusion of nicotinic antagonists, MLA and mecamlamine into the dorsal hippocampus impaired contextual but not cued fear conditioning. Interestingly, each drug produced distinctive deficits depending on the time the drug was administered.

3.4. Contextual Retention Test

For purposes of analysis, percent freezing was averaged across the first 8 minutes of context testing and compared across time of drug administration. A two-way ANOVA was performed with each drug type (mecamlamine, MLA, and PBS-control) and time of drug administration (pre-15 min, post-1 min, and post-6 hr). The analysis revealed a significant main effect for type of drug, $F(2, 63) = 22.86, p < 0.001$, but not for time of drug administration. The analysis also revealed a significant interaction between type of drug and time of drug administration, $F(4, 63) = 6.27, p < 0.001$.

A post-hoc test (Tukey-HSD) performed on the main effect for type of drug revealed that both the mecamlamine and MLA drug groups were significantly different from the control (PBS) group, $p < 0.001$. To explore the interaction between drug type and time of administration into the dorsal hippocampus, a post-hoc test (Tukey-HSD) was performed. The analysis revealed that rats administered mecamlamine 15 minutes pre-conditioning froze significantly less than rats administered the PBS control vehicle, $p < 0.001$, whereas rats administered mecamlamine or MLA 1-minute post-conditioning froze significantly less than rats administered the PBS control, $p < 0.05$, and were not significantly different from each other. Rats administered MLA 6 hours post-conditioning froze significantly less than rats administered the PBS control, $p < 0.01$. Rats administered mecamlamine or MLA 15 minutes pre-conditioning displayed significantly different levels of freezing, $p < 0.01$, whereas only a trend was apparent in the difference between the two groups administered drug 6 hours post-conditioning, $p = 0.072$. Interestingly, a temporal gradient was significant for mecamlamine, in that the pre-15 min group froze significantly less than the post-6 hr group, $p < 0.01$, while only a trend was apparent for the gradient in the opposite direction representing the effects of MLA, such that the pre-15 minute group displayed higher levels of freezing than the post-6 hour group, $p = 0.098$ (see Fig. 3a).

3.5. Cue (Tone) Retention test

Baseline activity was measured and compared between each drug type and across time of drug administration. A two-way ANOVA was performed with each drug type (mecamlamine, MLA, and PBS-control) and time of drug administration (pre-15 min, post-1 min, and post-6

hr). The analysis revealed no significant effects for baseline levels of freezing across groups of drug and time of administration. For purposes of analysis, the data were averaged across the first 8 minutes of tone testing and compared across time of drug administration. A two-way analysis of variance was performed with each drug type and time of drug administration. The analysis revealed no significant effects. Central infusions of nicotinic antagonist, mecamlamine or MLA into the dorsal hippocampus did not affect conditioning to the tone cue (Fig. 3b).

3.6. State-dependent Control—Contextual and Cued (Tone) Retention Test

A two-way analysis of variance was performed with each drug type (mecamlamine, MLA, and PBS-control) and time of drug administration (pre-15 min and preconditioning/pre-test) for the state-dependent control groups of rats that received drug/control vehicle before conditioning and before testing and groups of rats receiving drug/control vehicle before conditioning only. The analysis for the context test revealed a significant main effect for type of drug, $F(2, 38) = 41.01, p < 0.001$, but not for time of drug administration. The analysis revealed no significant interactions. A post-hoc test (Tukey-HSD) was performed for the main effect of drug and revealed that mecamlamine but not MLA significantly impairs retention of contextual fear conditioning compared to control when injected either before conditioning or both before conditioning and testing, $p < 0.001$ (see Fig. 4a). There were no significant differences between drug groups injected with drug before conditioning and drug groups injected both before conditioning and testing.

A two-way analysis of variance was performed for drug type and time of administration to compare baseline levels of freezing before tone presentation. No significant effects were found in baseline levels of freezing across drug types or time of administration (Fig. 4b). A two-way analysis of variance was performed with each drug type and time of drug administration to assess any state-dependent differences to conditioning of the cue with the aversive stimulus (i.e., shock). The analysis revealed no significant effects (Fig. 4b).

3.7. Anatomical Control—Contextual and Cue (Tone) Retention Test

As shown previously, (i.e., *Contextual Retention Test*), the control group froze significantly more than the animals in either group that received drug approximately 1 minute post-conditioning and animals receiving either MLA or mecamlamine approximately 1 minute post-conditioning were not significantly different from each other; thus both drug groups were combined for the purpose of this analyses and only different injection sites were compared. Animals administered mecamlamine ($n=8$), or MLA ($n=8$) injections into the dorsal hippocampus approximately 1 minute after conditioning were compared to animals administered mecamlamine ($n=4$) or MLA ($n=4$) injections into the overlying cortex approximately 1 minute after conditioning. Freezing behavior was measured in the original context for 16 minutes 1 week post-conditioning drug-free. Average freezing during the first 8 minutes of testing was compared. A one-way ANOVA was performed for drug group and revealed a significant main effect for type of drug, $F(3, 32) = 7.86, p < 0.01$. A post-hoc test (Tukey-HSD) was performed to explore the main effect of drug and revealed a significant difference between PBS-control and MLA, $p < 0.01$, and between PBS-control and mecamlamine, $p < 0.01$, but not for either mecamlamine or MLA administered into the overlying cortex. An independent sample one-tailed t test was performed to explore the differences between injection sites of drug only. As predicted, freezing levels in animals administered drug (i.e., mecamlamine and MLA) into the overlying cortex ($M = 81.50, SD = 13.01$) were significantly greater than freezing levels in animals administered drug (i.e., mecamlamine and MLA) into the dorsal hippocampus ($M = 55.48, SD = 25.68$), $t(22) = -2.68, p < 0.007$, one-tailed.

As shown in previous analyses (i.e., *Cue (Tone) Retention test*), no significant differences in the amount of freezing to the cue were found among groups of animals injected with mecamlamine, MLA, or PBS control vehicle. It was therefore predicted that injections into the overlying cortex would not be significantly different from injections into the dorsal hippocampus. Approximately 24 hours after the context test and a 2-minute baseline (BL) period, the original tone was played continuously for 16 minutes in a novel context. Average freezing levels during the first 8 minutes of testing for animals administered mecamlamine (n=8), or MLA (n=8) injections into the dorsal hippocampus approximately 1 minute after conditioning were compared to animals administered mecamlamine (n=4) or MLA (n=4) injections into the overlying cortex approximately 1 minute after conditioning. An independent sample one-tailed t test was performed separately for baseline freezing levels and for freezing during the presentation of the tone with each area of drug injection (hippocampus, overlying cortex). No differences were observed between groups injected with drug into either the dorsal hippocampus or overlying cortex during the BL or during the first 8 minutes of tone.

4. Discussion

The central purpose of this study was to examine the effects of $\alpha 7$ and broad-spectrum non- $\alpha 7$ NACHR dysfunction on long-term retention of contextual and cued fear conditioning. From the results of this experiment, it is clear that there is a significant role for nicotinic cholinergic systems within the CNS, specifically in the dorsal hippocampus as assessed by contextual fear conditioning of rats. Interestingly, the results demonstrated individualized effects specific to the receptor subtype suggesting there are specific temporal dynamics involved with nAChR activation. Specifically, the $\alpha 7$ nicotinic receptor appears to influence contextual memory acquisition and consolidation processes minutes to hours after conditioning, whereas broad-spectrum non- $\alpha 7$ nicotinic receptors appear to influence the earlier stages of memory acquisition and consolidation processes during initial exposure and immediately thereafter with no influence hours after conditioning.

Although there were no significant differences between the pre-15 minute and post-1 minute groups for mecamlamine, the deficits associated with mecamlamine between the pre-15 minute and post-6 hour groups resembled a temporal gradient typically observed in hippocampal damage or dysfunction. In other words, mecamlamine appeared to influence the association made between the context and aversive stimulus (i.e., shock) at the initial exposure of the association and directly after the conditioning took place. Mecamlamine appeared to have no influence in disrupting further consolidation of the association hours after conditioning. A temporal gradient was apparent for MLA as well; however, the trend representing the difference between the pre-15 minute and post-6 hour time points was only marginally significant. MLA appeared to influence the context-shock association during later time points with no significant influence over the initial exposure. The opposing gradients support very distinct temporal dynamics for $\alpha 7$ and more broad-spectrum non- $\alpha 7$ specific nicotinic acetylcholine receptors in modulating the susceptibility of contextual memory traces in the dorsal hippocampus.

Similarly to previous fear conditioning studies, an impairment in retention due to drug infusions before conditioning can be translated as an encoding or acquisition deficit; whereas, dysfunction due to drug infusions long after conditioning can be translated as a consolidation deficit (Anagnostaras, et al., 1995; Bernabeu, et al., 1997; Rogers, & Kesner, 2004; Wallenstein, & Vago, 2001). These conclusions assume that acquisition processes can be differentiated from consolidation processes that have long been thought to persist for a long period of time after initial learning has taken place. With state-dependent effects ruled out, the pre-15 minute infusion of mecamlamine produces either a generalized dysfunction in hippocampal circuitry or secondary modulatory effects that interfere with acquiring the contextual memory trace and/

or the subsequent association with the aversive shock. Some research suggests high doses of mecamylamine disrupts NMDA receptor function (O'dell, & Christensen, 1988); however, it remains unclear how nACh and NMDA receptor pharmacology interact in terms of contextual memory consolidation. The post-6 hour infusion of MLA most likely interferes with the translation of the shock-context association into a stable memory trace. Due to the lack of significance between the post-1 min groups for MLA and mecamylamine, the associated retention deficit does not add much relevance to the encoding/consolidation dissociation; however, because it is difficult to dissociate the processes at a theoretical level, it is likewise difficult to disambiguate the data.

We are making the assumption that at the volume and rate of infusion, neither drug diffuses further than the dorsal hippocampus. We maintain that the infusion of 4% Chicago blue dye is a gross approximation of the diffusion of either drug; however, there are many nicotinic receptors localized within the dorsal hippocampus and it is likely they are directly influenced. It remains unclear whether the drug infusion volume covered the entire dorsal hippocampus, remained close to the injection site, or spread outside the borders of the hippocampus, but we must assume that the observed effects are working at some level within the hippocampus. Any drug reaching the ventricles or beyond the hippocampus is still a better approximation of nicotinic receptor dysfunction at the level of the hippocampus compared to the alternative peripheral administration that can be working at multiple levels with much greater variability. In order to further control for the anatomical specificity of the observed effect, eight animals were injected 1 minute post-conditioning with either MLA or mecamylamine into the overlying cortex. The time point was chosen because of the observed effect for both drugs at 1 minute post-conditioning. Animals receiving injections of drug into the overlying cortex displayed significantly less freezing during the context test compared with animals receiving injections of drug into the dorsal hippocampus. Thus, both MLA and mecamylamine appeared to produce significant effects that were specific to the dorsal hippocampus.

Although mecamylamine and MLA have been used extensively in the experimental literature, there are relatively few pharmacokinetic studies that have determined the extent to which they block nicotinic receptors in the hippocampus. Debruyne and colleagues (2003) determined the hippocampal kinetics of mecamylamine by intravenous (iv) injections (5 mg/kg body wt) of a solution of mecamylamine (5 mg/mL) in 0.9 % saline and sacrificed the animals within 1 hr after injection. A plateau followed an increase of mecamylamine concentration after 15 minutes. Mecamylamine was found in high concentrations in the hippocampus and colliculi with a lack of lipophilic metabolites that would disturb specific binding to nAChRs. The apparent half life of mecamylamine is 1.2 hrs. Although the differences in pharmacokinetics between local and peripheral administration of the drug must be taken into consideration, the pre-15 and post-1 minute infusions may be supporting a continuous disruption of general non- $\alpha 7$ nAChRs during a time in which a stable memory trace would be predicted to be formed.

Additionally, an autoradiographic study by Davies and colleagues (1999) characterized the high selectivity of MLA to $\alpha 7$ nAChRs in the hippocampus. The study demonstrated rapid binding kinetics of MLA with a half life of 12.6 minutes. This study may account for the absence of observed impairments when MLA was infused 15 minutes before conditioning. Although the central infusion of MLA may have very different binding kinetics, the rapid dissociation kinetics of MLA must be taken into consideration when interpreting the findings of the reported results. In any case, it remains unclear as to why there are significant differences between the mecamylamine and MLA data at the post-6 hour time point. There has been ample evidence for the differential effects of $\alpha 7$ and non- $\alpha 7$ nAChRs on a cellular level and by no means has this behavioral analysis been able to disambiguate such findings. However, the observed effect of $\alpha 7$ and non- $\alpha 7$ nACh receptor dysfunction has been made evident in this particular context.

In general, the behavioral results assessed in the present experiment are consistent with other experiments reporting similar deficits with lesions of the dorsal hippocampus, and hippocampal models based at the cellular level that predict impairments in encoding and consolidating new contextual information with blockade of muscarinic and nicotinic cholinergic receptors in the hippocampus (e.g. (Anagnostaras, et al., 1999b; Barros, et al., 2004; Gale, et al., 2001; Hasselmo, Wyble, & Wallenstein, 1996; Kim, & Fanselow, 1992; Phillips, & Ledoux, 1992; Rudy, 1996; Wallenstein, & Hasselmo, 1997; Wallenstein, & Vago, 2001). It is unlikely, however, that muscarinic and nicotinic antagonists are working at the same level in terms of contextual memory acquisition and long-term retention. There are apparently unique time-dependent characterizations for specific nAChRs in affecting retention one week post-conditioning that may account for differences observed in previous studies that used both systemic pharmacology and genetic disruption of nAChRs and assessed retention 24 hr post-conditioning. One may speculate further on the exact mechanism that differentiates the receptor subtypes on a behavioral level by integrating the cellular and molecular findings, which have suggested specific modulatory roles for nAChRs over other neurotransmitter systems (e.g., dopaminergic, glutamatergic) (Decker, & Mcgaugh, 1991; Fanselow, Kim, Yipp, & De Oca, 1994; O'dell, & Christensen, 1988). Future studies are also needed to clarify how these and other specific subpopulations of muscarinic and nicotinic receptors map onto the cellular and molecular mechanisms posited for associative learning mediated by the hippocampal region.

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Figure 1.

Histological verification of cannulae placement (n=76). a. A transverse brain section through the dorsal hippocampus stained with cresyl violet. The section represents the approximate site for cannulae placement for targeted dorsal hippocampus. b. A transverse brain section through the dorsal hippocampus representing approximate site for cannulae placement in targeted overlying cortex. c. Approximate spread of drug assessed by dye (Chicago blue) infusion is indicated by circular shaded area. Each closed dot/open square represents the approximate point in which the cannulae were positioned for each animal. Stereotaxic images adapted from (Paxinos, 1998).

Figure 2. General activity level. Mean number of ambulatory crossovers during a 4-min period preceding first conditioning trial.

Figure 3.

Context and Tone test. a. Context test. Average freezing level ($\pm$ SEM) of each drug type (mecamylamine, MLA, or PBS) at each time of drug administration [pre-15 min, post-1 min, and post-6 hr] collapsed across the first 8 minutes of context testing. Animals received either mecamylamine, MLA, or PBS 15 minutes before conditioning, 1 minute or 6 hours post-conditioning; one week post-conditioning, they were placed back in the original context. Freezing was scored for 16 minutes and reported as an average percentage value (see text for details). Animals begin to habituate to the same level of exploration after 8 minutes of testing, hence, only an average of the first 8 minutes are shown. b. Animals were placed in a novel context approximately 24 hours after the context test. b. Tone test. After a 2-minute baseline period, the original tone was played continuously for 16 minutes; freezing was scored and reported as an average percentage value during the first 8 minutes after baseline. No differences were observed between any of the groups during the baseline or during the first 8 minutes of tone. (* = $p < 0.05$)

Figure 4.

Context and Tone test - State-dependent control. Animals administered mecamlamine (n=6) or MLA (n=6) injections 15 minutes before conditioning as well as 15 minutes before testing were compared to the groups of animals given mecamlamine (n=8) and MLA (n=8) 15 minutes before conditioning only. All animals were also compared to the PBS control. a. Context test. Freezing behavior was measured in original context for 16 minutes 1 week post-conditioning and 15 minutes after drug/control injections. Average freezing during the first 8 minutes of testing is shown. There were no significant differences between drug groups injected with drug before conditioning and drug groups injected both before conditioning and testing. Additionally, only mecamlamine significantly differed from control. b. Tone test.

Approximately 24 hours after the context test and 15 min after drug/control injections and a 2-minute baseline (BL) period, the original tone was played continuously for 16 minutes; freezing behavior was scored and reported as average percent freezing during the first 8 minutes. Animals administered mecamlamine (n=4) or MLA (n=4) injections 15 minutes before conditioning as well as 15 minutes before testing were compared to the groups of animals given mecamlamine (n=8) and MLA (n=8) 15 minutes before conditioning only. All animals were also compared to the PBS control. There were no significant differences among drug groups or PBS control during the baseline or during the presentation of the tone. (* = $p < 0.001$)