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Effects of Subcutaneous Postnatal Choline Supplementation on Hippocampus-Mediated Learning and Memory in Rat Pups

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EFFECTS OF SUBCUTANEOUS POSTNATAL CHOLINE SUPPLEMENTATION ON HIPPOCAMPUS-MEDIATED LEARNING AND MEMORY IN RAT PUPS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jeremy Alan Moore ENTITLED Effects of Subcutaneous Postnatal Choline Supplementation on Hippocampus-Mediated Learning and Memory in Rat Pups BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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Abstract

Moore, Jeremy Alan. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2008. Effects of Subcutaneous Postnatal Choline Supplementation on Hippocampus-Mediated Learning and Memory in Rat Pups.

The effects of postnatal injections of choline on the acquisition of two variants of eyeblink conditioning were examined in developing Long-Evans rat pups. Choline administration on postnatal days (PND) 15-27 was followed by evaluation of trace eyeblink conditioning (Experiment 1) and delay eyeblink conditioning (Experiment 2) on PND 28-29. The results of these experiments show that choline produced greater improvements in learning and memory during the trace condition than in the delay condition, presumably due to its effect on the hippocampus. Trace eyeblink conditioning relies heavily on an intact hippocampus but delay eyeblink conditioning does not, and it is understood that choline has a positive influence on hippocampal function. This was the first study to examine the effects of choline on eyeblink conditioning and provides baseline data for future research into the beneficial effects of choline for this type of learning as well as its potential neuroprotective effects for the hippocampus.

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I. Introduction

This study provides baseline data for the influence of choline supplementation on learning and memory in young rats, as measured by eyeblink conditioning. In particular, the study aims to determine whether choline can improve learning using two variants of eyeblink conditioning procedures, trace vs. delay. Trace eyeblink conditioning is understood to involve the hippocampus, a structure which may be positively affected by choline supplementation. This study will provide critical pilot and control data for future work examining the potential for choline supplements to reverse deficits in eyeblink conditioning produced by impairments in hippocampal function.

Choline, an organic compound classified as a B-complex vitamin, is considered an essential nutrient for humans as well as other animals. Although minute amounts of choline are synthesized de novo by the body, choline-rich foods such as beef and chicken liver, eggs, wheat germ, bacon, dried soybeans, and pork are recommended by the National Academy of Sciences, USA, as part of a healthy diet (Zeisel, Mar, Howe, and Holden, 2003). The importance of choline is reiterated with its involvement in many vital biological functions. Choline is recognized as a precursor to phosphatidylcholine and sphingomyelin, phospholipids that serve as structural components in all mammalian cell membranes as well as precursors to the intracellular messenger molecules diacylglycerol and ceramide. Two other cell signaling molecules, platelet activating factor (PAF) and sphingophosphorylcholine, also originate from choline (Blusztajn and Wurtman, 1983). Choline participates in the prevention of fat and cholesterol accumulation in the liver as well as methyl group metabolism (Zeisel, 2000a).

Of particular interest to this study, choline serves as a precursor to the neurotransmitter acetylcholine and, therefore, is important in cholinergic neurotransmission in basal forebrain structures including the hippocampus (Blusztajn and Wurtman, 1983). It is believed that most of the acetylcholine in the neocortex originates in the forebrain and is used heavily by the hippocampus when forming memories. Evidence for such a relationship can be found in the brains of patients with Alzheimer's disease, where acetylcholine levels are severely diminished and the hippocampus is atrophied, contributing to the severe lack of memory.

Furthermore, it is understood that during pregnancy choline pools are reduced and the demand for dietary choline may exceed the amount in a normal diet (Zeisel, Mar, Zhou, and da Costa, 1995). Choline is transferred to the fetus in this gestational period via the placenta. Due to the immense amount of brain growth and development during this time, the developing fetus may be more sensitive to lower levels of choline.

Studies show that pregnant rats given prenatal supplementation of choline on embryonic days (ED) 11-17 have offspring with increased rates of cell division in the hippocampus (Garner, Mar, and Zeisel, 1995). There has also been evidence that peri- and postnatal dietary supplementation has cognitive enhancing effects on offspring, which may be due to improved cholinergic transmission and hippocampal function (reviewed in Meck and Williams, 2003). Choline, therefore, has been the target of many studies regarding the effects of pre-, peri-, and postnatal supplementation on cognitive development and enhancement, especially with learning tasks involving the hippocampus. These studies have primarily utilized spatial learning tasks described briefly below, which rely heavily on intact hippocampal function.

The Morris water maze is a spatial learning task in which a rat is placed in a pool of opaque water and has to find an escape platform placed in a certain location. The animal is set at different places during the trials, but the platform stays in the same location. The rat learns by using spatial cues located around the room to determine where the platform is positioned. It is understood that the hippocampus is integral in the acquisition of spatial learning tasks. Studies show that rats with lesions of the hippocampus demonstrate impaired spatial learning in the Morris water maze (Morris, Garrud, Rawlins, and O'Keefe, 1982; Bannerman et al., 2002; Gould et al., 2002; Ferbinteanu, Ray, and McDonald, 2003). Other studies found that alcohol exposure, too, can result in abnormal hippocampal development and function as evidenced with hyperactive animals that performed poorly in spatial learning tasks (Berman and Hannigan, 2000; Thomas, Biane, O'Bryan, O'Neill, and Dominguez, 2007). In contrast, choline-treated rat pups tested with the Morris water maze provided evidence for long-lasting enhancement of spatial working memory, presumably via beneficial effects on the hippocampus (Tees and Mohammadi, 1999).

An alternative spatial learning task is the radial arm maze. This apparatus has eight equally-spaced arms about four feet long radiating from a central platform, each with food located at its end which is not visible from the central platform. After checking for food at the end of each arm, the rat is forced to return to the central platform before making another choice. Learning is gauged by the animal's ability to remember which arms it has already traversed and by the latency to acquisition of the food located at the end of each arm. Pothuizen and colleagues (2004) showed that lesions specific to hippocampal regions are sufficient to cause impairments on the radial arm maze task.

The radial arm maze is also a viable spatial task to examine hippocampal deficits due to alcohol exposure (Berman and Hannigan, 2000). One such study utilizing the radial arm maze showed that only half of the alcohol-exposed offspring were able to complete the task, and those that did required almost twice as many training trials relative to the controls (Reyes, Wolfe, Savage, 1989). In contrast, Meck and Williams (1997b) found that rats treated perinatally with choline implemented a chunking strategy when traversing the radial arm maze. The treated rats had a greater ability to cluster like-food types to the number of maze arms, possibly due to a choline-induced increase in memory capacity via improved hippocampal function.

Prenatal choline supplementation is normally administered by way of choline-enhanced diets to the pregnant dams. One such study showed that the offspring of rat dams supplemented with choline during pregnancy (ED 11-17) were better able to make use of relational cues to navigate during a water maze task when compared to offspring of dams fed a normal diet. Further, this supplementation to the mother rat increased the concentration of choline in the blood and the brains of the rat fetuses (Garner et al., 1995). Prenatal dietary choline supplementation has also been shown to result in long-term changes in hippocampal function that may last into adulthood, including decreased acetylcholinesterase activity and promotion of excitatory synaptic efficacy in hippocampal circuits (Montoya et al., 2000).

Perinatal choline supplementation has also been found to positively change brain development in offspring, in turn improving memory function when tested in adulthood (Brandner, 2002; Meck, Smith and Williams, 1988, 1989; Tees and Mohommadi, 1999). Supplementation begins prenatally via the mother's diet and continues to reach the pups

postnatally either through the dam's milk supply, or in some cases, through esophageal cannulas or subcutaneous injections. Perinatally choline-treated rats have demonstrated improvement in spatial capacities presumably due to a choline-induced modification of relevant components used for spatial learning, such as the hippocampus (Brandner, 2002). Another study showed enhancement of temporal processing produced by perinatal choline supplementation and related these effects to modifications in cholinergic function (Meck and Williams, 1997a). Meck and colleagues (1988) found that rats given supplemental choline perinatally performed more accurately on the radial arm maze when they reached adulthood than controls. Long-term facilitative effects of perinatal choline supplementation have been found on working and reference memory in rats (Meck et al., 1989).

Exclusively postnatal supplementation of choline in rat pups and adults yields less robust and contradictory results. Dietary administration of choline in adult rats increases acetylcholine synthesis and decreases its degradation in the brains of rats whose choline pools were reduced (Reviewed in Meck and Williams, 2003; Wecker, 1986). Under normal brain conditions, however, it was shown that short-term and long-term choline supplementation in adult rats does not alter the steady-state concentration of acetylcholine (Wecker, 1986). Yet, postnatal choline supplementation is understood to be most effective when administered during a sensitive period in rat brain development on postnatal days (PND) 16-30, as evidenced by an improvement in performance during a spatial memory task (Zeisel, 2000b; Meck et al., 1989). Postnatal dietary supplementation with choline (PND 5-18) showed no effects on spatial short-term memory in artificially-reared rats (Wainwright, et al., 2007). In normal rats, choline

produces some sexually dimorphic effects, including increased locomotor activity in females and decreased choline acetyltransferase activity in the hippocampus of males. Moreover, this study found increased retention on a passive avoidance learning task in choline-treated rats (Ricceri and Berger-Sweeney, 1998). More recently, there have been studies showing the neuroprotective characteristics of postnatal choline supplementation (Thomas, Garrison, and O'Neill, 2004a; Thomas, O'Neill, and Dominguez, 2004b). It was demonstrated that postnatal choline supplementation to young rats attenuated the adverse behavioral and developmental effects of prenatal alcohol exposure (Thomas et al., 2004a). Another study looking at the effects of prenatal alcohol exposure showed improvement on a visuospatial discrimination learning task following choline administration on PND 2-21 (Thomas, La Fiette, Quinn, and Riley, 2000). Later choline supplementation on PND 10-30 also reduced the severity of overactivity and spatial learning deficits resulting from an alcohol-induced insult (Thomas, et al., 2007). A study utilizing trace fear conditioning, which also uses the hippocampus and central cholinergic systems, showed that postnatal choline supplementation reversed alcohol-induced impairments on this task.

Other than the recent data on visual discrimination (Thomas et al., 2000) and fear conditioning (Wagner and Hunt, 2006), little is known about the potential effects of choline on non-spatial tasks that engage the hippocampus during learning. Eyeblink conditioning is a type of Pavlovian conditioning that has been used for over 30 years and is very well understood at both behavioral and neural levels (Stanton, Freeman, and Skelton, 1992). Research with adult rabbits established that eyeblink conditioning is mediated by a neural circuit located in the brain stem and cerebellum and is modulated by

forebrain structures such as the hippocampus (Thompson, 1986). Developing rats, however, are an ideal species in which to study changes in associative learning that may parallel underlying changes in postnatal hippocampal and cerebellar development as well as learning capacities (Stanton and Freeman, 1994). Eyeblink conditioning can be designed to employ different neural circuits by using an assortment of paradigms, including delay and trace conditioning. Delay conditioning does not fully engage the hippocampus whereas trace conditioning is highly dependent on hippocampal function.

Delay eyeblink conditioning is the simplest form of associative learning and involves a conditioned stimulus (CS- tone or light) and an unconditioned stimulus (US- periocular shock or air puff) that overlap and coterminate (see Figure 1). The US results in an eyeblink response which, over time, becomes conditioned to the CS. This eyeblink response is termed the conditioned response (CR). The delay paradigm utilizes a basic brain stem–cerebellar circuit (Thompson, 1986). By examining the effects of hippocampal lesions on delay and trace conditioning, several studies have reported that this lower-order conditioning is not entirely dependent on hippocampal function (e.g., Ivkovich and Stanton, 2001; Weiss, Bouwmeester, Power, and Disterhoft, 1999).

By contrast, trace eyeblink conditioning is a higher-order form of conditioning that relies heavily on the hippocampus (Ivkovich and Stanton, 2001; Moyer, Deyo, and Disterhoft, 1990; Weiss et al., 1999) and the prefrontal cortex (Plakke, Freeman, and Poremba, 2007). During trace conditioning, the tone or light (CS) and the periocular shock or air puff (US) are separated by a stimulus-free period during which the subject must hold a memory "trace" of the CS long enough to form an association with the

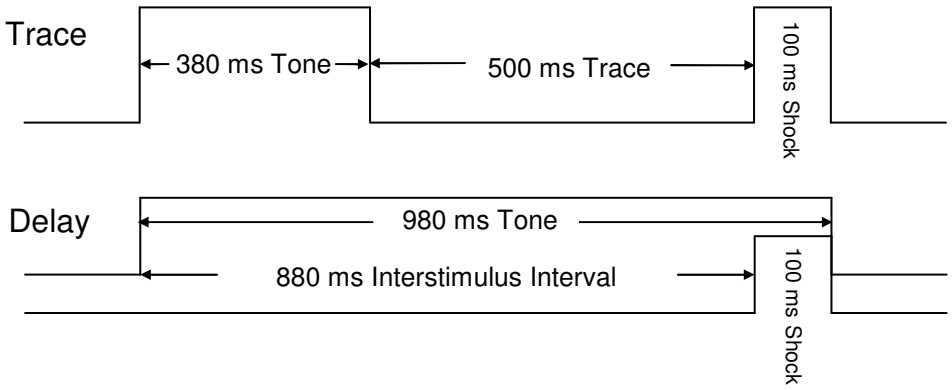

Figure 1. Animals were tested with one of two conditioning paradigms: trace and delay. For the trace conditioning, there is a 380-ms tone followed by a stimulus-free period of 500-ms (deemed the trace period) which terminates with a 100-ms shock. In delay conditioning, the tone is 980-ms and it overlaps and coterminates with a 100-ms shock. Note that both conditions have an 880-ms interstimulus interval.

subsequent US (see Figure 1; Ivkovich and Stanton, 2001). Because of the role of the hippocampus in this paradigm, trace eyeblink conditioning affords the ability to study and further understand the interactions between the hippocampus and the cholinergic system.

The present study applied classical eyeblink conditioning as a means to measure the potential effects of choline supplementation on learning and memory in developing rats with regards to both trace and delay eyeblink conditioning. This study is partially based on previous work showing deficits on trace eyeblink conditioning using glucocorticoids, which may affect cognitive ability due to its action on the hippocampus (Claflin, Hennessy, and Jensen, 2005). In the aforementioned study, corticosterone administered on PND 15 impaired trace but not delay conditioning on PND 28 and 29 presumably through effects on the hippocampus. It is possible that choline may serve to protect the hippocampus from the deleterious effects of corticosterone. For this reason, it is important to know what effects, if any, choline has on eyeblink conditioning in normal rats. This study consisted of two experiments. Experiment 1 examined the effect of choline on trace eyeblink conditioning and Experiment 2 looked at the effect of choline on delay eyeblink conditioning. We expected to see improved learning in choline-treated animals relative to the controls for the trace paradigm, which engages the hippocampus, as opposed to similarly treated rats trained with the delay paradigm.

II. Experiment 1: Trace Conditioning

The purpose of this experiment was to examine if postnatal subcutaneous choline supplementation would enhance trace eyeblink conditioning in young rats. The developing rat brain, including the hippocampus, may have especially high demands for choline (Meck and Williams, 2003). Trace eyeblink conditioning is a type of learning that incorporates the hippocampus and, therefore, may be affected by choline supplementation. Previous studies showed that bilateral hippocampal lesions impaired trace eyeblink conditioning as opposed to delay in adult rabbits and rats (Kim, Clark, and Thompson, 1995; Moyer et al., 1990; Weiss et al., 1999). Developmental studies in rats have shown similar results (Ivkovich and Stanton, 2001). Moreover, impairments in trace conditioning were observed in male (but not female) rats administered glucocorticoids subcutaneously through time-release pellets starting on PND 15 (Claflin, Hennessy, and Jensen, 2005). Because of interest in choline as a putative treatment for such impairments, the following study also used PND 15 for the beginning of choline treatment and evaluated the possibility of differential effects depending on sex.

Methods

Subjects

Timed-pregnant (E12) female Long-Evans rats were ordered from Charles River Laboratories (Raleigh, NC). Born litters were culled by laboratory personnel on postnatal Day (PND) 4 to an ideal split of 5 males and 5 females, when possible, to ensure balance of sex and similar developmental opportunities across litters. Pups resided with their

lactating dams until PND 21 when they were weaned and housed with same-sex littermates. The mothers were then adopted or euthanized as determined by Wright State University's animal resources staff. Twenty-five rat pups were in the final dataset, 13 female and 12 male. These were taken from 10 litters and no more than 1 male and 1 female from the same litter were assigned to the same experimental condition, to control for litter effects. The pups were assigned to one of four groups based on drug and sex: choline male (n = 6), choline female (n = 7), saline male (n = 6), and saline female (n = 6). Weight was monitored every other day throughout the experiment. Animals were maintained on a 12:12-h light-dark cycle with lights on at 07:00 h and were housed in a colony room accredited by the American Association for the Accreditation of Laboratory Animal Care and the Animal Care Committee of Wright State University in Dayton, Ohio. Pups were provided with *ad libitum* access to food and water throughout with exception during eyeblink conditioning.

Design and Procedure

Animals were subject to one of two drug treatments: choline (experimental) or saline (control), and similar numbers of males and females were assigned to each treatment so that possible sex differences could be considered. Trace eyeblink conditioning took place over 2 days with 3 sessions on each day, thus creating a repeated-measures design: 2 [choline vs. saline] x 2 [male vs. female] x 2 [day] x 3 [session].

From PND 15-27, subjects received daily subcutaneous injections of choline or saline following procedures reported by Thomas et al. (2004) and Wagner and Hunt (2006). Rats were given 0.10 ml of an 18.8 mg/ml solution of choline chloride (Sigma-

Aldrich) or the equivalent of a saline vehicle. Injections occurred daily at the same time (1400 h) and generally in the same environment. On PND 27, pups underwent eyeblink electrode surgery (see detail below) and were then housed individually for the remainder of the experiment. Eyeblink conditioning took place on PND 28-29 and animals were euthanized on PND 30.

For trace conditioning, a 380-ms tone (CS) and a 100-ms shock (US) were separated by a stimulus-free period of 500-ms (see Figure 1). The conditioning period consisted of 6 sessions over a 2 day period (3 sessions/day). Each session had 100 trials, 90 of which were paired (both CS and US) and 10 of which were tone-alone trials (only CS). Responses on paired trials were evaluated for analysis of learning

Eyeblink Electrode Surgery

A surgical procedure developed by Stanton et al. (1992) for eyeblink conditioning in rat pups was used. On PND 27, one day before eyeblink conditioning, all rat pups underwent surgery to implant a bipolar stimulating electrode and a differential electromyographic (EMG) recording electrode. The bipolar delivered a mild periocular shock to elicit the eyeblink reflex. The EMG electrode was used to record the muscle activity of the upper eyelid muscle (*orbicularis oculi*) during the eye closure.

Each animal was anesthetized with an intraperitoneal injection of ketamine (75mg/kg) and xylazine (5mg/kg). If during surgery a pup began to show signs of awakening, the pup was given an additional half of the original dose of ketamine/xylazine originally used to induce anesthesia. Once the pup was fully anesthetized, as determined

by a toe pinch, the hair on top of the skull was shaved and the exposed skin was disinfected with a Betadine soaked 2x2 inch gauze pad.

A midline incision, approximately 0.5 inches in length, was then made on the scalp. One to three drops of Bupivicane, a topical/local analgesic, was applied to the exposed skull. Connective tissue was cleared from the skull surface using a scalpel and cotton-tipped applicator to scrape the periosteum. A 26-gauge needle point was used to drill four small holes in the soft skull: two bilaterally immediately posterior to the bregma and two bilaterally anterior to the lambda. Two triangular-shaped sterilized 24-gauge stainless-steel wire "skull hooks" were inserted in both pairs of holes located at the front and the back of the skull and serve as anchors for the dental acrylic.

The bipolar electrode was inserted under the skin of the left side of the face such that the tips of the wires terminated in the periocular region immediately lateral to the left eye. The EMG electrode was positioned through the superior portion of the *orbicularis oculi* muscle of the left eyelid with the end of the wire terminating at the outer surface of the eyelid in a 1-2 mm section that was bent up and away from the eye. Petrolatum ophthalmic ointment was administered to keep the eyes of the anesthetized rats moist. The ground lead from the EMG was placed subcutaneously toward the back of the neck. Both electrodes terminated in plugs at the other end and were secured using dental acrylic, which also served to seal the wound.

Immediately following surgery, rats were given a subcutaneous injection of buprenorphine (.05 mg/kg), a partial opiate agonist used to manage post-surgical comfort. The pups were placed in a heated recovery chamber and monitored by laboratory personnel until the anesthetic dissipated. Once the rats recovered from surgery, as noted

by movement around the recovery chambers, they were placed in individual cages with *ad libitum* food and water.

Eyeblink Conditioning Apparatus

Animals were allowed to move freely in a Plexiglas test chamber (12 x 9.5 x 11.5 cm, L x W x H) with a stainless steel grid floor contained within a sound attenuating chamber. The chamber was equipped with a fan (background noise level 65-70 dB, dim light (15 W), and two speakers (2-12 kHz ranged), one of which delivers the tone (CS). The shock stimulus (US), was given by a constant-current, 60 Hz square wave stimulator set to deliver a 1.5 mA, 100 ms shock. The electrodes secured to the rats' skulls were connected to wire leads that passed through an opening in the conditioning chamber to a commutator located above the chamber. A custom-built Eyeblink Conditioning System (JSA Designs, Raleigh, NC) controlled stimulus presentations and processed EMG records by integrating the stimulus and storing it to a computer.

Data Analysis

For all repeated-measures ANOVAs, where assumptions of sphericity were violated, Huynh-Feldt corrected degrees of freedom and *F*-values are reported. Post-hoc Tukey *t*-tests were performed on data that demonstrated a significant main effect on a between-groups variable. Interactions or significant effects on within-groups variables were analyzed using a simple means analysis with Bonferroni's adjustment. Data were analyzed with SPSS software. Results meet the .05 significance level unless otherwise stated.

Learning Measures. Percentage of CR (CRP), CR amplitude (CMA), onset latency of CR (CL), and latency to the peak of CR (CML) were all analyzed over 6 training sessions for trace eyeblink conditioning using a between-groups, repeated-measures ANOVA [2 (drug) x 2 (sex) x 2 (day) x 3 (session)]. CRs were defined as any muscle response exceeding threshold (0.5 arbitrary EMG units above baseline in pre-CS period) during the allotted learning window (adaptive vs. non-adaptive). Data were calculated from the paired CS-US trials. CRP demonstrated the frequency of responses in anticipation of the US, whereas CMA revealed the magnitude of the learned response in anticipation of the US. An increase in CRP or CMA over the course of training is an indication of learning. CL and CML reflect the timing of CRs in anticipation of the imminent US. CL and CML were measured for responses occurring anytime after the 80-ms startle period through the end of the trial. A decrease in CL but increase in CML (peak of CR close to US onset) are signs of learning.

CRP and CMA were analyzed in both adaptive and non-adaptive time windows. Adaptive learning was measured by responses occurring 680-ms after the tone onset. Therefore, these are timed well as they occur within the 200-ms period immediately preceding the US (adaptive CR). Non-adaptive learning still reflected the animals' anticipation of the impending US, but was sampled within a larger 800-ms window preceding the US (non-adaptive CR). CL and CML are analyzed throughout the whole trial so as not to exclude early responses.

Control Measures. The percentage of startle responses (SRP) and amplitude of unconditioned responses (UMA) were analyzed to make certain there were no group differences in learning due to differences in stimulus sensitivity. The SR was measured

during the first 80-ms after the CS. SRP was analyzed by the same method as the learning measures, using a between-groups, repeated-measures ANOVA [2 (drug) x 2 (sex) x 2 (day) x 3 (session)]. Unconditioned responses (UR) were measured in the 140-ms following the US. The UMA was analyzed using a one-way ANOVA for only Session 1 to capture initial US sensitivity since continued training will ultimately cause response habituation.

Results

Trace CRP

Adaptive. A 2 [drug] x 2 [sex] x 2 [day] x 3 [session] repeated-measures ANOVA for CRP in the adaptive period concluded there were effects of both session, $F(2, 42) = 10.68$, $p < .001$, and day, $F(1, 21) = 41.63$, $p < .001$. Examination of the data revealed that CR percentage significantly increased across the two days and increased across the 3 sessions on both of these days. Significances in day and session were expected as they reflect continuous learning regardless of drug treatment. However, there were no significant differences between the choline and saline groups for trace conditioning in this time period.

Non-adaptive. The non-adaptive analysis yielded a similar effect of day, $F(1, 21) = 9.02$, $p = .007$, where CR percentages increased from Day 1 to Day 2, but no session effect. Perhaps more importantly, there was a main effect of drug, $F(1, 21) = 11.5$, $p = .003$. This effect suggests that animals administered choline in the trace paradigm learned better than those given saline, as demonstrated by a significantly higher percentage of conditioned responses (see Figure 2). A significant interaction of Session x

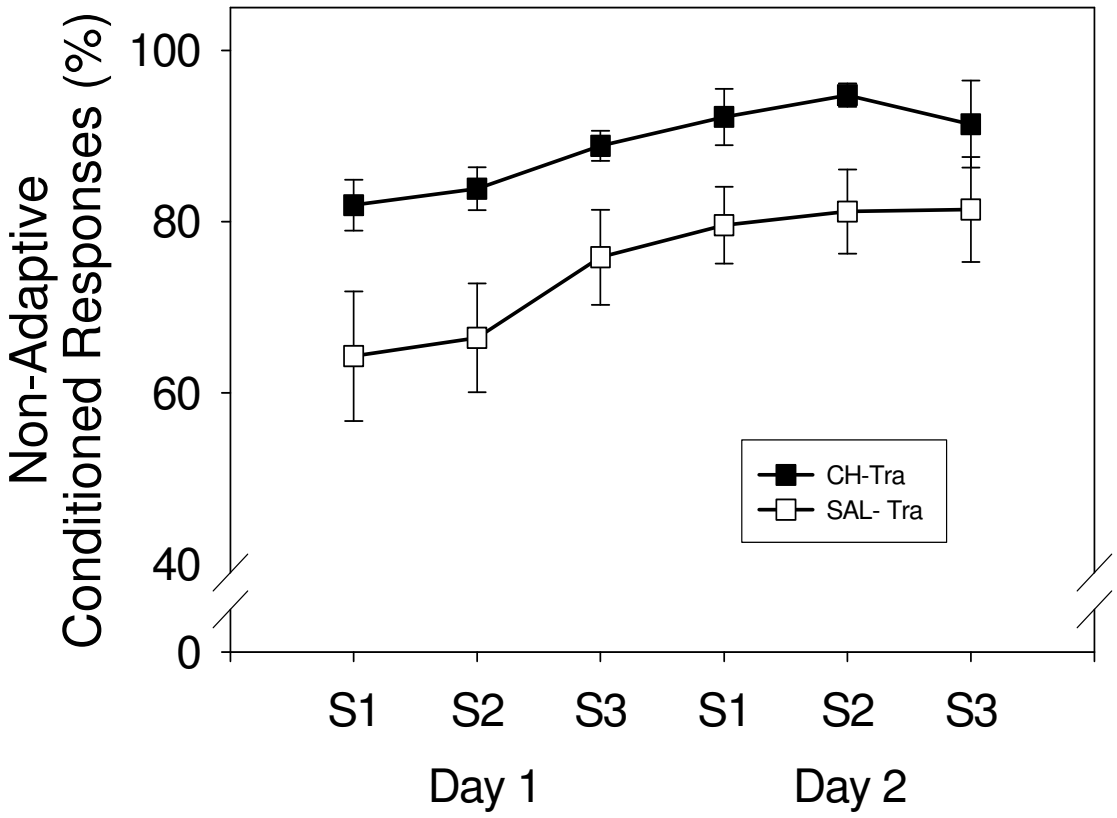

Figure 2. Choline increases the percentage of conditioned responses in trace conditioning (Main effect of drug: $F(1, 21) = 11.5$, $p = .003$). Error bars are the standard error of the mean.

Sex x Drug, $F(2, 42) = 3.39$, $p = .043$, was obtained. This interaction signified an increased number of CRs during certain sessions depending on sex for the choline groups. A simple means analysis showed that in Session 1, regardless of which day of training, male choline animals produced significantly more conditioned responses than male saline animals ($p = .011$). In contrast, female choline animals generated significantly more conditioned responses than female saline animals during Sessions 2 and 3 ($p = .004$ and $p = .022$, respectively).

Trace CMA

Adaptive. The only significant effects for CMA in the adaptive period were a main effect of day, $F(1, 21) = 23.18$, $p < .001$, and session, $F(1.82, 38.12) = 7.66$, $p = .002$. Consistent with the increased adaptive CRP analysis, this significance in CMA is due to an increase in CR amplitude and frequencies across the two days of training as well as across sessions.

Non-Adaptive. A main effect of day was observed for the amplitude of non-adaptive CRs, $F(1, 21) = 21.69$, $p < .001$. There was an increase in CR amplitude from Day 1 to Day 2, consistent with the increase of CR percentages seen in the non-adaptive period. There is also an interaction effect of Day x Drug approaching significance, $F(1, 21) = 4.25$, $p = .052$. Further analysis of simple means showed that rat pups supplemented with choline had significantly higher CR amplitudes on Day 2 than those with saline on Day 2 ($p = .048$). The average CR amplitude for saline-treated animals was 1.7 on Day 1 and 2.3 on Day 2 (measured in arbitrary EMG units). For choline-treated animals, it was 2.2 on Day 1 and 3.6 on Day 2. There was also a significant two-

way interaction of Day x Sex, $F(1, 21) = 5.09$, $p = .035$, and a significant three-way interaction of Day x Session x Sex, $F(1.89, 39.65) = 3.82$, $p = .033$. Males had a significantly greater increase in amplitudes from Day 1 to Day 2 when compared to females

Trace CL

A 2 [drug] x 2 [sex] x 2 [day] x 3 [session] repeated-measures ANOVA for onset latency revealed a significant effect of day, $F(1, 21) = 5.54$, $p = .028$. Further analysis showed the significance was due to decreased average onset latency from Day 1 (375.6 ms) to Day 2 (332.5 ms), indicating learning based on earlier anticipation of the US. As with the CRP analysis, there was also an overall effect of drug, $F(1, 21) = 11.58$, $p = .003$. Choline-treated animals in the trace condition had significantly reduced onset latencies when compared to saline-treated animals (see Figure 3). Several interaction effects involved the administration of choline, including: Day x Sex x Drug, $F(1, 21) = 4.84$, $p = .039$; Session x Drug, $F(2, 42) = 3.75$, $p = .032$; and a Day x Drug interaction that was approaching significance, $F(1, 21) = 4.25$, $p = .052$. Simple means analysis indicated the first effect involving sex was due to female choline subjects that had, on average, much shorter onset latencies than female saline subjects just on Day 2 (295.4-ms and 455.8-ms respectively). However, saline-treated females did not show a decrease in onset latency across days as did all other groups. The onset latencies of all subjects supplemented with choline became shorter over the course of training, whereas the onset latencies in the saline group increased or showed no change (see Table 1). Consistent with this finding, the conditioned responses of choline-treated rat pups had significantly

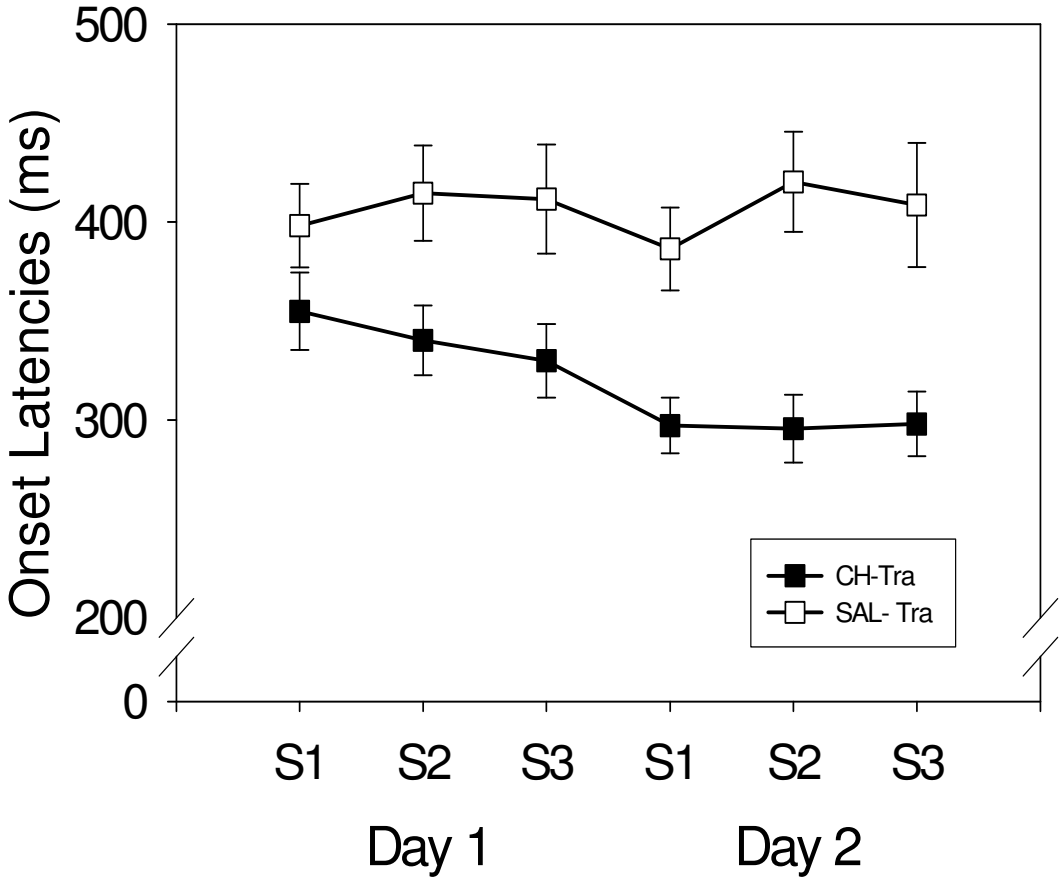

Figure 3. Choline reduces the onset latencies of conditioned responses in the trace condition (Main effect of drug: $F(1, 21) = 11.58$, $p = .003$). Error bars are the standard error of the mean.

Drug	**S1**	**S2**	**S3**
Choline	326.9 ± 16.9	318.3 ± 17.8	313.9 ± 20.7
Saline	392.3 ± 17.6	417.5 ± 18.4	410.1 ± 21.4

Table 1. Average onset latencies of conditioned responses (ms) (Interaction effect of Session x Drug, $F(2, 42) = 3.75$, $p = .032$). The onset latencies of rat pups supplemented with choline decreases across sessions (S1, S2, and S3 combined from both testing days) while the saline-treated animals have a net increase in onset latencies across sessions.

lower onset latencies on Day 2 than on Day 1. The repeated-measures ANOVA also showed an effect of Day x Sex, $F(1, 21) = 8.99$, $p = .007$.

Trace CML

A repeated-measures ANOVA of the onset latency to the peak of the CR showed an effect of day, $F(1, 21) = 10.67$, $p = .004$, and session, $F(2, 42) = 16.61$, $p < .001$. The rats tested in the trace condition, regardless of drug, had significantly increased peak latencies for conditioned responses on Day 2 than Day 1 and increased peak latencies across sessions. There was also a significant interaction of Day x Sex, $F(1, 21) = 4.48$, $p = .046$, which may reflect a greater change in latency for females (~70 ms) versus males (~20 ms; Day 1: Female- 525.4 ms, Male- 555.4 ms; Day 2: Female- 596.9 ms, Male- 570.7 ms).

Trace SRP

A significant main effect of day, $F(1, 21) = 9.52$, $p = .006$, was observed for the percentages of startle responses. There were also several interaction effects, including Session x Sex, $F(2, 42) = 3.9$, $p = .028$ and Day x Session, $F(1.7, 35.77) = 5.8$, $p = .009$. A main effect of drug, $F(1, 21) = 4.49$, $p = .046$, was also present. Choline-treated rat pups had more startle responses than saline animals throughout sessions; however, this finding could also be attributed to the peculiarly low startle rate of saline females throughout training (see Table 2). A significant four-way interaction existed as well, Day x Session x Sex x Drug, $F(1.7, 35.77) = 4.18$, $p = .029$. This interaction was due to

	DAY 1			DAY 2		
	S1	**S2**	**S3**	**S1**	**S2**	**S3**
Female Choline	30.3 ± 12.9	25.4 ± 10.3	32.4 ± 12.7	39.1 ± 22.5	40.4 ± 20.9	28.4 ± 17.1
Female Saline	**15.3 ± 12.8**	**8.3 ± 6.6**	**6.5 ± 4.4**	**10.5 ± 13.6**	**10.5 ± 12.3**	**8.8 ± 11.1**
Male Choline	40.7 ± 31.4	37.0 ± 30.3	38.0 ± 29.0	45.0 ± 29.5	49.2 ± 19.8	48.0 ± 27.7
Male Saline	28.5 ± 24.5	22.2 ± 20.5	32.2 ± 25.6	32.3 ± 19.6	39.7 ± 28.4	34.3 ± 25.3

Table 2. Percentages of startle responses for rat pups tested in the trace condition (Interaction effect of Day x Session x Sex x Drug, $F(1.7, 35.77) = 4.18$, $p = .029$). Note the unusually low percentages of startle responses of the saline-treated female subjects.

female saline-treated rats having a significantly low percentage of startle responses on Day 1, Session 3; and on Day 2, Sessions 1 and 2 (see Table 2).

Trace UMA

Analysis of the amplitude of the unconditioned response in the trace condition showed a main effect of drug, $F(1, 21) = 4.99$, $p = .036$. The mean UMA (measured in arbitrary EMG units) for choline-treated animals was 9.65 and the mean for saline-treated animals was 9.73.

Discussion

The findings of this experiment show that subcutaneous supplementation of choline on PND 15-27 improved trace eyeblink conditioning on PND 28-29. These findings are based on data analyzed in the non-adaptive window; adaptive CR data were not significantly different for choline versus control treatments. The adaptive and non-adaptive time-periods for analysis are frequently applied to eyeblink conditioning paradigms that have a long ISI, which was 880-ms in this study. These time-periods allow the researcher to more accurately examine the timing of conditioned responses occurring in anticipation of the US.

Choline-treated animals averaged significantly higher percentages of conditioned responses, greater CR amplitudes, and earlier onset latencies, when compared to saline animals. CML was not different for the drug treatment groups, but did increase over the course of training for all animals as expected when learning occurs. Animals supplemented with choline prepared for the impending US more often than those with

saline. A reduction in onset latency suggests that those animals supplemented with choline were giving earlier conditioned responses, indicating a learned association between the CS and the US. It is important to note, however, that these CRs were not so early as to be considered short-latency CRs that are typically associated with damage of the hippocampus (Port, Romano, Steinmetz, Mikhail, and Patterson, 1986).

Choline-treated animals also had significantly higher percentages of startle responses when compared to saline-treated animals. Although a higher sensitivity to the CS may lead to improved conditioning (Grice and Hunter, 1964), we believe this SRP effect was due to performance of the non-drug group. Saline-treated females had unusually low SRPs and we did not see similar patterns of drug-related sex differences in learning measures to suggest that SRP explains learning differences. As such, we do not believe the SRP to have affected learning outcomes. The range of startle percentages observed in choline-treated males and females and for the saline-treated males in the trace condition is similar to what has been previously reported (Ivkovich, Packzkowski, and Stanton, 2000).

Another potential concern is that unconditioned responses differed between the choline and saline groups at the start of conditioning. Interestingly, however, it was the saline-treated animals that had higher unconditioned response amplitudes than choline-treated animals. This may mean that saline animals were slightly more sensitive to the periocular shock than the choline group, although the difference is minute (9.65 and 9.73 EMG units). A higher sensitivity to the shock can lead to increased strength and frequency of conditioned responses. Therefore, the larger and more frequent conditioned

responses demonstrated by the choline-treated animals, all indicators of better learning, are independent of differences associated with sensitivity to the shock.

In summary, CRP, CMA, and CL showed positive and beneficial changes in response to choline. Since these improvements were observed in trace eyeblink conditioning, a type of learning that relies on hippocampal function, choline's effects on acquisition of trace eyeblink conditioning may be mediated by the hippocampus.

III. Experiment 2: Delay Conditioning

The purpose of this experiment was to determine whether the results observed in Experiment 1 were unique to trace conditioning. This study used delay eyeblink conditioning in order to evaluate the effects of choline on the basic neural circuit for eyeblink conditioning, the brainstem and cerebellum. The delay conditioning procedures used here are typically referred to as long-delay because the 880-ms interstimulus interval (ISI) is designed to match that of trace conditioning (see Design and Procedure below) rather than the simpler delay interval of 280-ms, which has become standard in similar studies. Note, it is understood that the hippocampus is involved but not critical for this lower-order form of conditioning (Ivkovich and Stanton, 2001). If the effects of choline are mediated by the hippocampus, then it might be expected that treatment with choline, which improved trace eyeblink conditioning in Experiment 1, would not have as robust of effects on delay eyeblink conditioning.

Methods

Subjects

Twenty-seven Long-Evans rat pups were in the final dataset, 16 female and 11 male. As in Experiment 1, pups were taken from 10 litters, limiting 1 male and 1 female from a litter to any given condition. The pups were assigned to one of four groups based on drug and sex: choline male (n = 4), choline female (n = 8), saline male (n = 7), and saline female (n = 8). Animals were housed and maintained as in Experiment 1.

Design and Procedure

The design and procedure were as described for Experiment 1 [2 (drug) x 2 (sex) x 2 (day) x 3 (session)], except that animals received training on delay eyeblink conditioning. In this experiment, delay conditioning consisted of a 980-ms tone (CS) that overlapped and co-terminated with a 100-ms shock (US). The delay paradigm was standardized to the trace paradigm such that both had an 880-ms ISI between the tone onset and the shock onset (see Figure 1). As with Experiment 1, conditioning took place 3 times a day over 2 days. Again, each session had 100 trials, 90 of which were paired (both CS and US) and 10 of which were tone-alone trials (only CS). Eyeblink Surgery, Apparatus, and Data Analysis were all as described in Experiment 1.

Results

Delay CRP

Adaptive. A 2 [drug] x 2 [sex] x 2 [day] x 3 [session] repeated-measures ANOVA for adaptive CRs showed that there were effects of day, $F(1, 23) = 80.85$, $p < .001$, and session, $F(1.95, 44.75) = 7.01$, $p = .002$. Analysis of the data shows that adaptive CR percentage, regardless of drug, significantly increased across the two days and increased across the 3 sessions in both of these days.

Non-Adaptive. For non-adaptive CRs, an effect of day was observed, $F(1,23) = 27.16$, $p < .001$. Consistent with adaptive CRs and learning, animals demonstrated more non-adaptive CRs on Day 2 than on Day 1. There were no effects of drug as seen in Experiment 1 (see Figure 4).

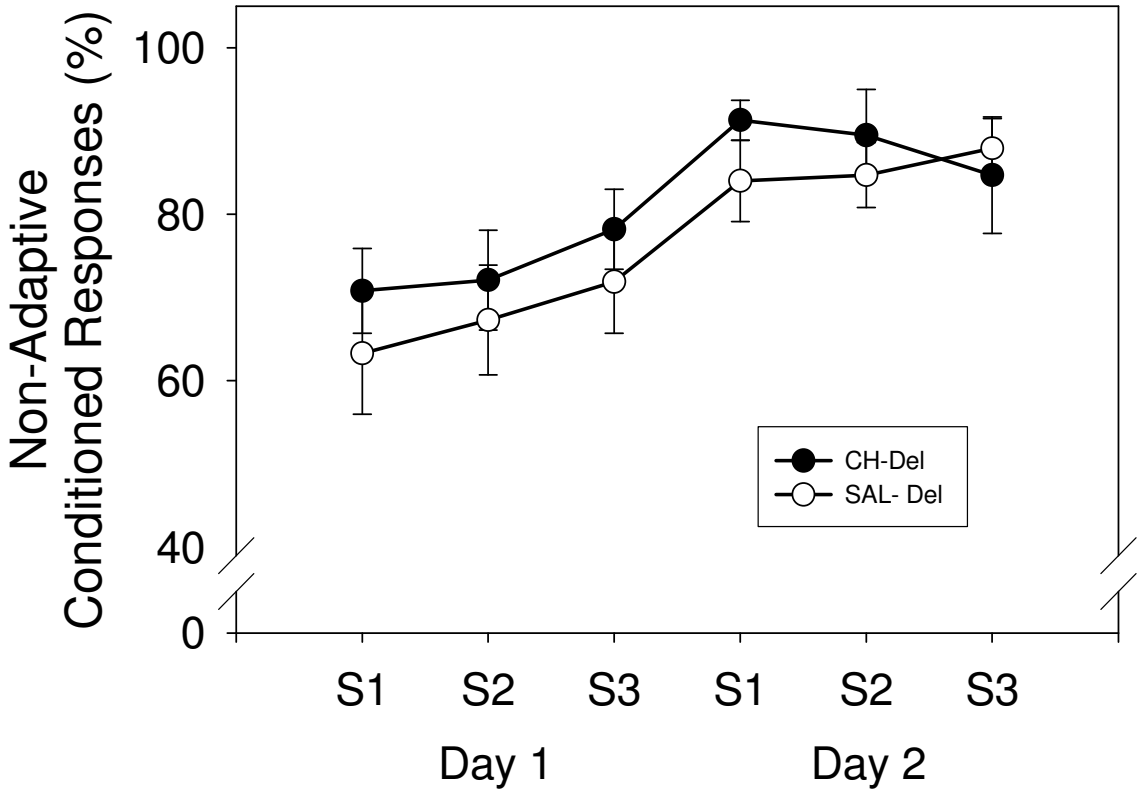

Figure 4. Choline does not significantly influence the amount of non-adaptive conditioned responses in delay. Error bars are the standard error of the mean.

Delay CMA

Adaptive. A 2 [drug] x 2 [sex] x 2 [day] x 3 [session] repeated-measures ANOVA for adaptive CR amplitude gave an effect of day, $F(1, 23) = 45.72$, $p < .001$ and an effect of session, $F(1.83, 42.86) = 10.3$, $p < .001$. This significance is due to increased CR amplitude across the two days of training as well as across sessions on these days. There was also a significant three-way interaction effect of Day x Sex x Drug, $F(1, 23) = 5.61$, $p = .027$. A simple means post-hoc analysis indicated that only saline-treated males did not demonstrate a significant change in amplitudes from Day 1 to Day 2. However, amplitude did show a continual increase across sessions.

Non-Adaptive. An effect of day, $F(1,23) = 57.45$, $p < .001$, was observed for the non-adaptive CR amplitudes. A significant three-way interaction consistent with the adaptive CMA analysis was Day x Sex x Drug, $F(1, 23) = 4.33$, $p = .049$ as well as an interaction of Day x Session x Sex, $F(2, 46) = 3.69$, $p = .033$. A simple means post-hoc analysis showed that, as seen in the adaptive analysis above, saline-treated males did not have a significant increase in amplitudes from Day 1 to Day 2 as seen with the other groups.

Delay CL

A 2 [drug] x 2 [sex] x 2 [day] x 3 [session] repeated-measures ANOVA for CR latency revealed a significant effect of day, $F(1, 23) = 6.78$, $p = .016$. On Day 1 the average onset to latency was 357.58-ms and on Day 2 the average onset to latency was 324.41-ms. There was also a significant interaction of Day x Sex, $F(1, 23) = 6.09$, $p = .021$. Further analysis of simple means revealed that female subjects, on Day 2, had

significantly shorter onset latencies for conditioned responses than did male subjects. Female rats began their conditioned responses, on average, 280.57-ms after onset of the tone while male rats' onset latency was significantly later at 368.26-ms.

Delay CML

Effects of both day, $F(1, 23) = 45.11$, $p < .001$, and session, $F(2, 46) = 12.65$, $p < .001$, were observed for peak CR latencies in the delay condition. CML increased from Day 1 to Day 2 and increased across sessions. There were also significant three-way interactions of Day x Sex x Drug, $F(1, 23) = 6.68$, $p = .017$, and Session x Sex x Drug, $F(2, 46) = 5.75$, $p = .006$. A simple means post-hoc analysis showed that all groups showed a significant increase in onset latency to the peak of the CR (moving closer to the US) except for the choline-treated females. All groups, however, were moving in the same direction, suggesting a weak interaction.

Delay SRP

Using the same repeated-measures ANOVA as in Experiment 1, we found a significant effect of day, $F(1,23) = 4.9$, $p = .037$, and a main effect of sex, $F(1,23) = 5.37$, $p = .030$. Males, on average, produced less startle responses than females. An interaction of Day x Session, $F(1.66, 38.11) = 6.08$, $p = .008$, was due to a decrease in startle response percentages on Day 1 from Session 1 to 2.

Delay UMA

The amplitude of the unconditioned response was analyzed for the first session on Day 1 and there were no significant differences between groups related to drug or sex. Sensitivity to the periocular shock (US) is, therefore, not a significant factor in the differences of learning rates demonstrated by subjects in the delay condition.

Discussion

There were no overall effects of drug found for any of the learning measures in Experiment 2. This indicates that choline alone, did not significantly affect learning in delay conditioning. The percentage and amplitude of conditioned responses increased across days and sessions, as would be expected when learning is observed. Choline, however, did not have an influence on the number or size of conditioned responses given.

The only drug interaction (CMA) was due to saline-treated males not increasing amplitudes to the extent of the other groups. Therefore, no clear choline effects were observed in this experiment.

Moreover, drug was not a significant factor in either of the control measures: startle responses or amplitudes of the unconditioned response. Females produced more startle responses than the males, and there was a decrease in startle percentages on Day 1; however, these differences do not correspond to any group differences in learning outcomes.

IV. General Discussion

The effects of choline supplementation on acquisition of trace eyeblink conditioning (Experiment 1) and delay eyeblink conditioning (Experiment 2) were examined in young rats. The focus of these studies was to analyze drug-related changes in hippocampus-mediated learning and to see if choline had any preferential effects on a certain sex.

Together, these experiments showed that rat pups administered choline postnatally (PND 15-27) demonstrated better learning than saline-treated pups in trace conditioning as evidenced by higher CRP and CMA and decreased onset latencies of CRs. This was not the case in the delay conditioning. Seeing positive effects on trace but not delay conditioning is consistent with the idea that choline is acting to promote hippocampal function. It is important to note that this is one of few studies examining the effects of postnatal choline supplementation on a non-spatial task (reviewed in Meck and Williams, 2003).

Choline did not appear to preferentially improve learning for one sex over the other in either experiment. Small differences for onset latency of the CRs in female choline subjects were found in the trace conditioning; however, this group did not stand out in other learning measures. Therefore, it can be assumed that choline does not benefit one sex more than the other.

It may be that postnatal choline supplementation in this study was successful because it occurred within a time window (PND 16-30) where the rat pups seem to have a higher sensitivity to choline supplementation (Meck et al., 1989; Zeisel, 2000b). Studies

examining the effects of postnatal choline supplementation on hippocampus-dependent spatial tasks are contradictory. This may be a direct result of the time period in which the choline was administered. Wainwright et al. (2007) found that postnatal dietary supplementation (PND 5-18) with choline had no effect on spatial short-term memory in artificially-reared rats. However, supplementation of choline in this study did not occur during the effective time-period described above. Other studies that supplemented choline during this sensitive time-period have shown the positive effects of postnatal choline in its ability to ameliorate the negative effects of prenatal and postnatal alcohol exposure (Thomas et al., 2007; Thomas et al., 2004a). In these studies, choline was supplemented on PND 10-30 (Thomas et al., 2007) and on PND 4-30 (Thomas, et al., 2004a). Therefore, the fact that we see improvements in trace conditioning is consistent with this effective PND 16-30 time window.

The significant data in both experiments were primarily recorded in the non-adaptive, as opposed to the adaptive, time window. While this may raise questions, it is important to note that the CR latencies were not so early and short (short-latency CRs) to suggest that the hippocampus in these animals was immature or damaged (Port et al., 1986). It may be that eyeblink studies with long ISIs require longer adaptive periods (i.e. > 200-ms before US). More work needs to be done to understand the difference between adaptive and non-adaptive time periods, and future studies may examine and designate optimal periods for long ISI studies.

This is the first we know of choline's beneficial effects on a non-spatial task such as eyeblink conditioning. However, since trace conditioning is also mediated by the hippocampus, this study provides further evidence that postnatal choline administration

can produce beneficial effects on tasks that require an intact hippocampus. Further studies of eyeblink conditioning may yield more information regarding the uses of choline in facilitating this form of learning in normally developing animals. Moreover, based on other studies where choline actually reversed or protected vulnerable brain regions from functional impairment due to alcohol, it may be that choline has similar protective effects against exposure to high levels of glucocorticoids (Claflin et al., 2005). This study showed that choline is beneficial to normal rats and, therefore, suggests that future studies regarding the neuroprotective ability of choline should include a normal, choline-supplemented control group. These findings further broaden the uses of eyeblink conditioning as a means to study the hippocampus and its influences.

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