

ASCERTAINING THE LONG-TERM EFFECTS OF ACUTE SYSTEMIC NEONATAL PROTEIN SYNTHESIS INHIBITION ON COGNITION AND BEHAVIOR IN SPRAGUE-DAWLEY RATS (*RATTUS NORVEGICUS*)

Robert W. Flint, Jr., Heather Joppich, Christina L. Marino, Leslie A. Sandusky, Sarah Valentine and Jonathan E. Hill

The College of Saint Rose, Albany, NY 12203-1490

ABSTRACT

The early postnatal period represents a time of significant neural development. Neonatal exposure to stressors, environmental toxins, and many different pharmacological agents may have a significant impact on subsequent behavior and cognition in adulthood. The purpose of this study was to examine the effects of early administration of the protein synthesis inhibitor, cycloheximide, on a battery of behavioral and cognitive tests in adulthood. Twelve-day-old Sprague-Dawley rats were administered saline or .5 mg/kg of cycloheximide subcutaneously. Upon reaching adulthood, a battery of tests were administered in order to examine anxiety, locomotor activity, environmental habituation, spatial working memory, object recognition memory, long-term spatial memory, and conditioned taste aversion. Most tests failed to reveal any differences between postnatally exposed cycloheximide and saline animals. However, during the water maze probe test, cycloheximide exposed animals reached the original platform location significantly faster than saline controls. For the conditioned taste aversion test, during the initial exposure to a 10% sucrose solution (prior to the induction of the taste aversion) the cycloheximide animals consumed significantly more than saline controls, indicating an attenuation of neophobia. Although acquisition of a conditioned taste aversion was comparable in neonatally exposed cycloheximide and saline animals, there was a significant resistance to extinction by cycloheximide animals. These results suggest that animals exposed to cycloheximide on postnatal day 12 displayed enhanced long-term spatial memory, reduced neophobia, and enhanced memory for taste aversion conditioning as indicated by a resistance to extinction.

INTRODUCTION

Empirical investigation of the effects of early postnatal treatments on behavior and cognition represents an important area of research in developmental neuroscience. In the rat,

the first few weeks following birth embody a time period of immense development neurologically (Freeman and Nicholson, 2004; Vazquez, 1998), endocrinologically (Vazquez, 1998), behaviorally (Ba and Seri, 1995), and cognitively (Campbell, 1967; Campbell and Spear, 1972). Disruptions in these early processes may have a variety of significant and potentially permanent effects on behavior. The fact that the development of the mammalian nervous system is associated with a number of critical and limited developmental periods (mitosis, neuronal proliferation, neuronal organization, etc.) suggests that disruptions in the normal progression of protein synthesis may result in irreversible effects (Bertolini, Poggioli, Bernardi, Genedani, and Ferrari, 1980). The present study investigated the effects of acute protein synthesis inhibition at postnatal day 12, one such potential critical period associated with brain development, on a battery of behavioral and cognitive tests in adulthood.

The neonatal rat brain undergoes considerable development for at least three weeks following birth (Cotman, 1978; Davison and Dobbing, 1968; Dunlop, Bodony, and Lajtha, 1984; Lindholm and Khawaja, 1983; Mhaskar and Dunaway, 1995). For example, there appears to be a relatively constant rate of synapse addition between postnatal day 1 and postnatal day 15 (Steward and Falk, 1986) as well as an age-related increase in protein synthesis in neuronal perikarya between postnatal day 6 and postnatal day 18 (Lindholm and Khawaja, 1983). Dunlop et al. (1984) reported that the rate of protein synthesis [$(\% \text{ protein/h}) \times (\text{mg protein/g wet wt})$] decreased gradually from 1.25 at 2-days, to 1.15 at 8-days, to 1.00 at 16-days, finally to .70 in adult animals (see also Dunlop et al., 1977). These rates of protein synthesis were obtained from the cerebral hemispheres of rats and are reflective of developmental changes in RNA concentration (Dunlop, et al., 1984). Subsequent studies have examined the rates of protein synthesis in more specific regions of the brain, some of which are known to be heavily involved in cognition. Shahbazian, Jacobs, and Lajtha (1986) reported *in vivo* rates of hippocampal protein synthesis at 1.26 in 5-day-old rats and at 0.39 in 5-week-old rats. Levels also reportedly decreased in the striatum from 0.94 to 0.37, in the thalamus from 0.95 to 0.41, in the frontal cortex from 1.12 to 0.43, and in the parietal cortex from 1.26 to 0.44. These studies clearly indicate that levels of protein synthesis are elevated during the first few weeks of life and likely play an important role in the developmental maturation of the central nervous system.

While regions of the thalamus, striatum, frontal lobe, and parietal lobe are involved in cognition, the hippocampus is widely known to be involved in an array of tests of learning and memory. Phillips, Polack, and Steward (1990) examined the rates of protein synthesis in hippocampal slices from rat pups ranging in age from 4 to 21 days old and found that the levels peaked at postnatal day 12. However, selected subregions of the hippocampus revealed different rates of protein synthesis with peaks between 4 and 7 days for cell-body-enriched areas of the dentate gyrus and hippocampus proper and peaks at 12 days for molecular layers of the dentate gyrus. Given the substantial developmental changes in the neonate and the high levels of protein synthesis during this time, it follows that manipulations leading to alterations in protein synthesis have the potential to disrupt neural development resulting in behavioral or cognitive deficits later in life.

Research findings from developmental studies have revealed an enormous number of environmental (Cirulli, 2001; Greenough, 1975; Pryce and Feldon, 2003), nutritive (Dobbing, 1968; Resnick, 1979), and pharmacological/toxicological (Kabat, Buterbaugh, and Eccles, 1985; Kennedy, Birgis, Rakhra, and Nicholls, 1983) manipulations that affect subsequent behavior and cognition in adulthood. For example, studies examining the effects of

stimulating protein synthesis early in development have provided evidence of cognitive enhancement in adulthood (Clendinnen and Eayrs, 1961; Sara, Laxarus, Stuart, and King, 1974).

Of particular relevance to the present study are those experiments where protein synthesis inhibitors, such as cycloheximide, are administered during infancy, and behavior is assessed in adulthood. Repeated administrations of cycloheximide between postnatal days 15 and 30 have been shown to significantly disrupt intraspecies aggressive behavior in adulthood (Rylov and Anokhin, 1992). Cycloheximide administered on postnatal day 7 significantly disrupted motor coordination and brain development present in adulthood (Sulc, Benesova, Kubik, Vavrova, and Pavlik, 1982). Sulc et al. found that these deficits remained, even when pyritinol (a nootropic drug) was administered daily between postnatal days 8 and 14. Pavlik and Jelinek (1979) reported that cycloheximide produced impairments in behavior that correlated with behavioral changes in adulthood when administered at postnatal day 7, whereas Bertolini et al. (1980) found that protein synthesis inhibition during the first three days of life disrupted hetero- and homo-sexual behavior in male rats and disrupted the estrous cycle and sexual receptivity in female animals. However, when protein synthesis was disrupted between postnatal days 7 and 21 these authors found a subsequent disruption of avoidance conditioning in adulthood.

The use of protein synthesis inhibitors to examine its impact on cognition is not new, as post-acquisition de novo protein synthesis is a well-established step in memory formation. Many studies have shown that protein synthesis inhibitors such as cycloheximide may significantly disrupt protein synthesis when administered systemically (Serova, Solov'ea, Lagutina, and Obukhova, 1996) or centrally (Kesner, Partlow, Bush, and Berman, 1981). Blocking protein synthesis in the brain has been shown to induce amnesia in memory consolidation and reconsolidation paradigms in rats (Berman and Dudai, 2001; Eisenberg, Kobil, Berman, and Dudai, 2003; Flint and Marino, 2007; Flint, Valentine, and Papandrea, 2007; Judge and Quartermain, 1982; Nader, Schafe, and LeDoux, 2000; Schmaltz and Clement-Forestier, 1977), snails (Gainutdinova et al., 2005; Kemenes, Kemenes, Michel, Papp, and Muller, 2006; Lukowiak, Fras, Smyth, Wong, and Hittel, 2007; Sangha, Scheibenstock, and Lukowiak, 2003), *Hermissenda* (Child, Epstein, Kuzirian, and Alkon, 2003), honeybees (Eisenhardt and Menzel, 2007; Stolhoff, Menzel, and Eisenhardt, 2005), *Caenorhabditis elegans* (Rose and Rankin, 2006), and crabs (Pedreira, Perez-Cuesta, and Maldonado, 2002). Taken together, the developmental literature and the evidence indicating an important function of protein synthesis in memory formation accentuate the critical role of protein synthesis in the central nervous system.

The purpose of the present study was to examine the impact of acute systemic cycloheximide administration at postnatal day 12, a period of significant hippocampal development, on a battery of behavioral and cognitive tests in adulthood. Tests were designed to provide assessments of anxiety, locomotor ability, environmental habituation, spatial working memory, object recognition memory, long-term spatial memory, neophobia and conditioned taste aversion. It was hypothesized that, if acute cycloheximide at postnatal day 12 is sufficient to disrupt neural development, performance on the behavioral and cognitive tests would be disturbed in adulthood.

METHODS

Subjects

Forty Sprague-Dawley rats were purchased from Hilltop Lab Animals (Scottsdale, PA) in litters of 10 pups each with a dam. Orders were placed with instructions for all pups to be male with no more than 1 pup per litter from the same dam. This precaution was taken to reduce any intralitter correlations that might exist among sibling littermates. Animals arrived at 8-days-of-age (DOB = day 0) and were allowed to acclimate in the colony room for 4 days before the study began. Mean bodyweight at 12 days-of-age (age of injection) was 21.5 g (SD = 3.14 g). Animals were maintained in their respective litters with food and water available *ad libitum* until 22 days-of-age, at which time they were weaned and housed in groups of 3 to 4 animals where each cage had at least one animal from each group (identifiable based on ear punch). Food and water were available *ad libitum* during post-weaning, except during the conditioned taste aversion portion of the study where animals were water deprived for a total of 22.5 hours a day for 6 days. Behavioral testing began at 68 days-of-age and was completed by 150 days-of-age. The colony room was maintained on a 12:12 hr light:dark cycle at approximately $72^{\circ}\pm 2$ °F, and all behavioral and cognitive testing took place during the light phase. All protocols were approved by the Institutional Animal Care and Use Committee prior to the onset of any empirical procedures.

Apparatus and Materials

Elevated Plus-Maze

The elevated plus-maze (EPM) was constructed from wood which was sealed with multiple coats of black paint. Each arm of the plus-maze measured 40.6 cm with a platform width of 8.9 cm. The sides and ends of the enclosed arms measured 40.6 cm and the junction where the 4 arms converged was 8.9 x 8.9 cm. Small 1 cm high edges were included on the open arms to prevent animals from slipping off the apparatus. The EPM was placed in a small research room and attached to a metal saw horse so that it was elevated 66.0 cm from the floor. White noise (68 dB) was generated to reduce the likelihood of noise-related disturbances. The room was illuminated with overhead fluorescent lighting and a small desk lamp with a 60 watt incandescent light bulb situated just above the maze to help illuminate the closed arms. A black/white video surveillance camera (Radio Shack) was suspended 152 cm from the maze and connected to a TV/VCR unit (JVC) which recorded each session.

Open Field Apparatus

The open field apparatus was constructed of clear Plexiglas painted black with dimensions of 61 x 61 cm and walls 61 cm high. The apparatus was placed into the same testing room described earlier. All testing occurred in the presence of 68 dB of white noise. The AnyMaze animal tracking system (Stoelting, Wood Dale, IL, USA) was used to monitor the animals. Using AnyMaze, the open field was divided into two zones. The center zone measured 31 cm x 31 cm located in the center of the open field, while the surround zone encompassed the 15 cm wide strip around the center zone. The room was illuminated with a

300 watt halogen lamp placed on a table in the far corner of the room which reduced glare and increased contrast between the animal and the apparatus for optimal tracking conditions.

T-Maze

The base of the T-maze was constructed of 1.3 cm wood, sealed with multiple coats of black paint. The sides were constructed of clear Plexiglas 30.5 to 44.5 cm high. Each alley of the T-maze measured 14 cm wide and 38.7 cm long. The center region of the maze where the 3 alleys came together measured 14 x 14 cm. The T-maze was set-up in a small test room on top of a stainless steel surgical table. The room was illuminated with overhead fluorescent lighting. Behavior was videotaped and examined for the number of arm entries and the order of arm entries from which the percent spontaneous alternation score was calculated.

Object Recognition

Object recognition testing was conducted in the open field apparatus described previously. Two identical white blocks measuring 8.57 cm long, 8.57 cm high, and 3.81 cm wide were used as the sample objects and one block with the same dimensions painted with alternating black and white stripes was used as the novel object. Training and test sessions were videotaped using the security camera and TV/VCR unit previously described. Videotape data of the testing sessions were examined for the amount of time spent exploring each object. Object exploration was indicated when the animal was facing and within 2 cm of the object, but not in direct contact with it.

Morris Water Maze

A plastic water tank (Terracon Corporation, Holliston, MA, USA) painted black with a diameter of 124.5 cm and a depth of 75 cm was used for the Morris Water Maze task. Water was added to the tank to approximately 17 cm from the top, allowing animals a good view of extra-maze cues when in the tank. Extra-maze cues in the test room included a wall-mounted faucet, window, shelving units, door, and a computer located on an elevated table. The maze was divided into four quadrants (NE, NW, SE, and SW). A black escape platform with a diameter of 11.4 cm was located in the center of the NE quadrant. Water level was maintained approximately 1.5 cm above the platform surface during the training and the probe test. For the visible platform test, water in the tank was drained to approximately 1.5 cm below the platform surface and a black probe approximately 14 cm long and .5 cm wide was inserted vertically into the center of the platform to enhance its visibility. The room was illuminated with a 300 watt halogen lamp and 68 dB of white noise was used to mask background noise. A standard laboratory stopwatch was used to record training latencies and the latency during the visible platform test. Probe test sessions were recorded on videotape for subsequent analysis of the latency to the original platform zone, number of crosses over that platform zone, and the time spent in the NE quadrant.

Taste Aversion Conditioning

Each phase of the taste aversion study (pre-training, training, testing, and extinction) took place in modified operant conditioning chambers. The dimensions of each apparatus were the same, measuring 21.5 cm wide, 19.0 cm high, and 23.5 cm deep. The two sides and the lid of the box were constructed of clear Plexiglas, while the front and back of the apparatus were constructed of aluminum. The floor of the box was comprised of 3.0 mm diameter steel rods

separated by 1.3 cm on center. A waste pan with woodchip bedding was located beneath the steel rod floor. Two holes in the front of the cage, 10.0 cm from the floor and 14.0 cm apart allowed for the simultaneous presentation of two bottles. Standard sipper tubes and stoppers were placed onto 100 ml graduated water bottles (Ancare, Inc., Bellmore, NY, USA) for monitoring fluid consumption. Standard table sugar was used to create a 10% sucrose solution. A .15 M solution of lithium chloride (Sigma Chemical, St. Louis, MO, USA) was used to induce malaise. Control animals received saline (Sigma Chemical).

Procedure

General Procedure

At 12 days-of-age, six animals from each litter were randomly selected, ear tagged, and administered a subcutaneous injection (sc; nape of the neck) of 0.50 mg/kg of the protein synthesis inhibitor cycloheximide (CXM; Oxoid Ltd., England) at a volume of 2 ml/kg. This dose was selected based on pilot research and prior studies with preweanling animals. The remaining 4 animals from each litter were given an equivalent volume of saline (Sigma Chemical, St. Louis, MO). Two animals were excluded due to experimental error during the injection procedure and three CXM animals died within 24-hrs of the injection. All animals that received CXM injections displayed some temporary inhibition of fur development on the back of their neck, head, and upper back around the injection site. Resulting group n's were 20 in the CXM group and 15 animals in the saline group. All behavioral testing was conducted during adulthood between 68- and 150-days-of-age, and all animals were tested on the same task at approximately the same age. Animals were transported to the laboratory in their home cages at least 30 minutes prior to any behavioral testing, with the exception of taste aversion training and testing. Each apparatus was thoroughly wiped down with disinfectant and dried following training and/or testing in the EPM, T-maze, open field, and object recognition apparatus. Researchers were blind to the experimental conditions during all training, testing, and video data coding. The battery of cognitive and behavioral tests was conducted in the order in which the procedures are presented below.

Elevated Plus-Maze Procedure

The EPM is a well-known and widely utilized test of anxiety (Hogg, 1996; Rodgers and Dalvi, 1997), known to be dependent upon the hippocampus (Degroot and Treit, 2004; File, Kenny, and Cheeta, 2000; Herrero, Sandi, and Venero, 2006; Rezayat, Roohbakhsh, Zarrindast, Massoudi, and Djahanguiri, 2005). Animals were placed individually into the center region of the EPM facing an enclosed arm, and were allowed to explore the maze for 5 minutes during which their behavior was videotaped. Subsequent data analysis involved recording the number of entries into and the time spent in the open arms, closed arms, and center zone. Ethological variables including the number of rearings, number of end explorations, and the time spent scanning were also recorded (Carobrez and Bertoglio, 2005; Cruz, Frei, and Graeff, 1994; File, Kenny, and Cheeta, 2000; Rodgers, Cao, Dalvi, and Holmes, 1997; Rodgers and Johnson, 1995).

Open Field Procedure

The open field tests were used to assess general locomotor activity, anxiety, and environmental habituation. Open field activity was assessed on two consecutive days at approximately the same time each day. Animals were placed individually into the center region of the open field and allowed to explore the environment for 5 minutes. Assessment of exploratory activity on day 1 was used to examine general locomotor activity and anxiety. Measures of locomotor activity on day 2 were compared with those from day 1 as indicators of habituation of exploratory activity. The AnyMaze tracking system recorded a number of dependent measures including the total distance traveled (m), overall average speed (m/s), and the time spent in the center zone (s) (anxiety measure, see Hinojosa et al., 2006).

T-Maze Procedure

The T-maze was used as a measure of spatial working memory. In a review of the neurobiological mechanisms of spontaneous alternation in the T-maze, Lalonde (2002) indicated that there are likely multiple limbic and non-limbic system pathways involved, including the hippocampus, prefrontal cortex, basal forebrain, and dorsal striatum. Animals were placed individually into the apparatus facing the back wall of one alley of the T-maze and were allowed to explore the apparatus for 5 min. The number and pattern of arm entries (4 paw criterion) were determined by videotape analysis. Prior to data analysis, a criterion was established where a minimum of 5 arm entries (3 possible alternations) were required for inclusion in the data analysis. An alternation was considered to be the entry into each of the three arms on three sequential entries (e.g., A, C, B, A, C; represents 3 alternations). The total number of alternations during the 5-min test was determined for each animal and divided by the total number of arm entries minus 2. This dividend was multiplied by 100 to provide the percent alternation score.

Object Recognition Procedure

The one-trial object recognition task was used as a measure of visual recognition memory (Aggleton, 1985) which likely involves a variety of neural substrates (Dere, Huston, and De Souza Silva, 2007). Animals were placed into the open field apparatus, where two identical sample objects were located in adjacent corners, and were allowed to explore their environment and the objects for 5 min. Four hours later, each animal was returned to the apparatus where one of the sample objects had been replaced with the novel object. Animals were given 5 minutes to explore their environment and the objects. The amount of time spent exploring each object (animal oriented toward object within 2 cm) was determined.

Morris Water Maze Procedure

The stationary submerged platform version of the Morris water maze was used to examine spatial learning and long-term spatial memory. Successful performance in the water maze requires an intact and functioning hippocampus (Morris et al., 2006; Nakazawa, McHugh, Willson, and Tonegawa, 2004). Acquisition and retention were assessed across three consecutive days where training/testing occurred at approximately the same time each day. On days 1 and 2, animals received 4 training trials each separated by approximately 8 minutes. On each training trial, the animal was placed into the water facing the side of the tank and allowed to swim for up to 120 seconds. If the animal discovered the hidden platform before the 120 second criterion it was allowed to remain there for 30 seconds and its latency

to the platform was recorded. If the animal did not find the platform within 120 seconds it was placed directly onto the platform for 30 second and a latency of 120 seconds was recorded for that trial. The start location (N, S, E, or W) was varied pseudo-randomly across trials/days so that each animal shared the same start location on each trial and so that the start location did not occur more than once on any given day. On day 3, the platform was removed from the tank for a probe test. Animals were placed into the apparatus at the S end and allowed to swim freely for 120 seconds during which their behavior was videotaped.

After each animal completed their probe test, the platform was returned to the apparatus but it was placed directly on the south line of the tank halfway between the edge and the center of the tank. Water was drained to approximately 1.5 cm below the platform level in order to expose the platform, and a black probe was inserted into the center to enhance the visibility. Animals were placed into the east end of maze and their latency to reach the visible platform was recorded as a control for sensorimotor ability.

RESULTS

Statistical analyses were conducted utilizing parametric tests and statistical significance was set at a probability of less than or equal to 0.05. Partial eta squared (η_p^2) has been reported as an indicator of effect size for all omnibus analyses and Cohen's *d* (*d*) has been reported for pairwise comparisons. Post hoc power is provided for those analyses that did not reach statistical significance.

Elevated Plus Maze

Recent reviews of behavioral tests using the elevated plus maze have suggested that a variety of traditional and ethological variables may be examined and subsequently divided into categorical groups indicative of different behaviors (Carobrez and Bertoglio, 2005; Cruz et al., 1994; File et al., 2000; Rodgers, Cao, Dalvi, and Holmes, 1997; Rodgers and Johnson, 1995). Utilizing this approach, the number of open arm entries (number of times animal entered an open arm with all four paws), percent open arm entries [(open arm entries / total arm entries) x 100], open arm time, and percent open arm time [(time on open arms / 300) x 100] were reported as measures of anxiety. The percent of time spent in the center zone [(time in center zone / 300) x 100] was recorded as a measure of decision making, and ethological measures of end exploring (number of times animal comes within 2.5 cm of the end of an open arm), time scanning (time spent dipping head over the side of an open arm and scanning beneath the maze), and number of rearings (number of times animal reared on 2 hind legs) were recorded as indices of exploration.

Anxiety

Mean scores for open arm entries, percent open arm entries, open arm time, and percent open arm time for the saline and cycloheximide groups are displayed in table 1. Visual inspection of the data suggests that cycloheximide and saline animals performed comparably on all dependent measures. Independent samples *t*-tests did not reveal any statistically

significant results for number of open arm entries [$t(33) = 0.09$, $p > .05$, $d = .03$, power = .06], percent open arm entries [$t(33) = 0.24$, $p > .05$, $d = .08$, power = .08], open arm time [$t(33) = .87$, $p > .05$, $d = .30$, power = .22], or percent open arm time [$t(33) = .87$, $p > .05$, $d = .30$, power = .22]. As indices of anxiety, these results suggest that acute protein synthesis inhibition at postnatal day 12 did impact performance in the elevated plus maze in adulthood.

Table 1. Elevated Plus Maze Dependent Measures (mean and standard error) for Cycloheximide and Saline Groups

	Saline		Cycloheximide	
	Mean	SE	Mean	SE
Number of Open Arm Entries	2.73	(.55)	2.80	(.48)
Percent Open Arm Entries	19.11	(14.20)	20.20	(12.27)
Open Arm Time	24.20	(4.97)	30.55	(5.12)
Percent Open Arm Time	8.07	(1.66)	10.18	(1.71)
Time in the Center Zone	23.47	(6.76)	22.85	(5.86)
Percent Center Zone Time	7.82	(2.11)	7.62	(2.04)
Time Scanning	11.87	(2.38)	12.35	(2.06)
Number of Rearings	12.33	(.73)	13.15	(.62)
Number of End Explorations	.73	(.33)	1.40	(.29)

Decision Making

An independent samples *t*-test for the percent of time spent in the center zone did not reveal any significant differences [$t(33) = .07$, $p > .05$, $d = .02$, power = .06] (see table 1) indicating that early protein synthesis inhibition did not alter decision making processes.

Exploration

Independent samples *t*-tests for the time scanning [$t(33) = .15$, $p > .05$, $d = .05$, power = .07], number of rearings [$t(33) = .85$, $p > .05$, $d = .36$, power = .27], and number of end explorations [$t(33) = 1.53$, $p > .05$, $d = .54$, power = .46] did not reveal any significant differences between groups (see table 1).

Open Field Activity and Habituation

The mean total distance traveled, overall average speed, number of center zone entries, and time spent in the center zone for each group on each day of testing are displayed in table 2. Two by two mixed ANOVAs (group x day) were conducted for each of these dependent measures. There were no main effects of group or group by day interactions for total distance traveled [$F(1,33) = 2.68$, $p > .05$, $\eta_p^2 = .08$, power = .36 and $F(1,33) = 1.11$, $p > .05$, $\eta_p^2 = .03$, power = .18], overall average speed [$F(1,33) = 2.71$, $p > .05$, $\eta_p^2 = .08$, power = .36 and $F(1,33) = 1.21$, $p > .05$, $\eta_p^2 = .04$, power = .19], number of center zone entries [$F(1,33) = .35$, $p > .05$, $\eta_p^2 = .01$, power = .09 and $F(1,33) = .05$, $p > .05$, $\eta_p^2 = .002$, power = .06], and time spent in the center zone [$F(1,33) = .00$, $p > .05$, $\eta_p^2 = .000$, power = .05 and $F(1,33) = 1.40$, $p > .05$, $\eta_p^2 = .04$, power = .21]. However, significant main effects of day were found for total distance traveled [$F(1,33) = 29.72$, $p < .001$, $\eta_p^2 = .47$] and overall average speed [$F(1,33) = 29.03$, $p < .001$, $\eta_p^2 = .47$], indicating that all animals displayed intact environmental habituation.

Table 2. Open Field and Habituation Dependent Measures (mean and standard error) for the Cycloheximide and Saline Groups on Days 1 and 2 of testing

		Saline		Cycloheximide	
		Mean	SE	Mean	SE
Total Distance Traveled (m)	Day 1 ^a	15.57	(1.00)	17.05	(.86)
	Day 2	10.84	(1.33)	13.85	(1.15)
Overall Average Speed (m/s)	Day 1 ^b	.052	(.003)	.057	(.003)
	Day 2	.036	(.004)	.046	(.039)
Number of Entries into Center Zone	Day 1	2.40	(.76)	2.85	(.66)
	Day 2	1.60	(.78)	2.25	(.68)
Time in the Center Zone	Day 1	6.33	(2.00)	4.83	(1.73)
	Day 2	2.55	(1.52)	4.21	(1.32)

Notes: ^aIndicates significant difference for total distance traveled between days 1 and 2. ^bIndicates significant difference for overall average speed between days 1 and 2.

T-Maze Arm Entries and Spontaneous Alternation

The mean number of arm entries and the percent spontaneous alternation score for each group is displayed in figure 1 (Panels A and B respectively). One animal was excluded from these analyses for failing to reach the 5-arm entry criterion. Independent samples *t*-tests for the number of arm entries [$t(32) = -.75, p > .05, d = .26, \text{power} = .18$] and percent spontaneous alternation [$t(32) = -.60, p > .05, d = .21, \text{power} = .15$] did not reveal any differences between the groups, suggesting that spatial working memory was not affected by early protein synthesis inhibition.

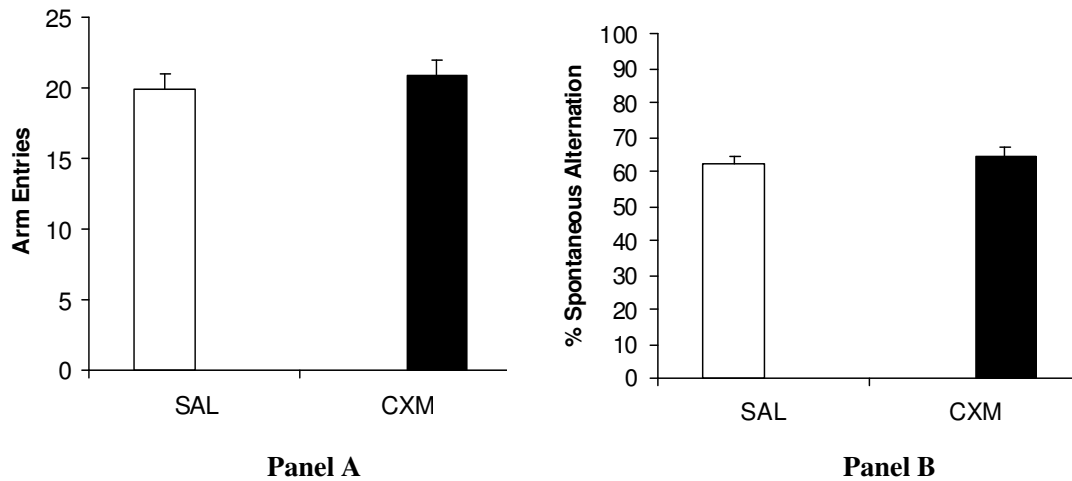


Figure 1. Panel A: Mean number of arm entries for the cycloheximide (CXM) and saline (SAL) groups during the 5-min T-maze test. Panel B: Mean percent spontaneous alternation scores for the cycloheximide (CXM) and saline (SAL) groups during the 5-min T-maze test. (Note: Error bars represent the standard error of the mean).

Object Recognition Memory

The amount of time spent exploring the novel object during the 5 minute recognition memory test was determined for each group. An independent samples *t*-test did not reveal any difference between the saline ($M=24.97$, $SE=3.77$) and cycloheximide ($M=22.53$, $SE=2.25$) group [$t(33)=.56$, $p>.05$, $d=.20$, $power=.14$].

Morris Water Maze

Acquisition performance in the Morris water maze was examined by conducting a $2 \times 2 \times 4$ (group \times day \times trial) mixed ANOVA on the training latencies (see figure 2). Results indicated a significant main effect of day [$F(1,33)=10.39$, $p<.01$, $\eta_p^2=.24$] and trial [Huynh-Feldt Correction $F(2.21,81.11)=15.04$, $p<.001$, $\eta_p^2=.31$]. There was no main effect of group [$F(1,33)=.11$, $p>.05$, $\eta_p^2=.003$, $power=.06$] and none of the interactions reached statistical significance.

Long-term spatial memory was assessed by examining the probe test latency, number of crosses over the original platform location, and time in the platform quadrant (see figure 3). Results of independent sample *t*-tests revealed a significant effect for latency [$t(33)=2.40$, $p<.05$, $d=.82$], but no significant effects for the number of crosses [$t(33)=.14$, $p>.05$, $d=.05$, $power=.09$], or the time spent in the quadrant where the platform had been located [$t(33)=.15$, $p>.05$, $d=.05$, $power=.07$]. Lastly, an independent sample *t*-test for the latency to reach the visible platform, as a measure of sensorimotor ability, did not reveal any difference between the groups [$t(33)=.20$, $p>.05$, $d=.07$, $power=.07$].

These results indicate that animals in each group acquired the ability to locate the submerged platform at an equal rate across trials and days. However, animals that had been exposed to cycloheximide at 12-days-old had significantly shorter latencies to the platform location during the probe test, indicating enhanced spatial long-term memory as adults.

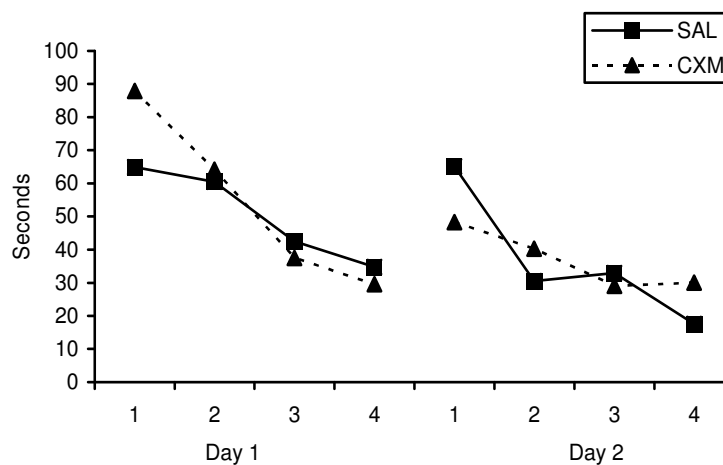


Figure 2. Mean latency to reach the hidden platform for the 4 training trials on each day of the Morris water maze training for the cycloheximide (CXM) and saline (SAL) groups.

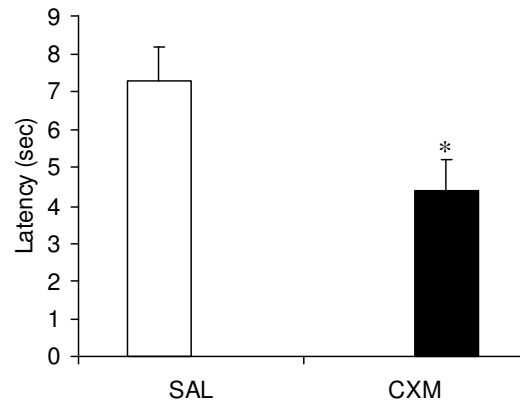
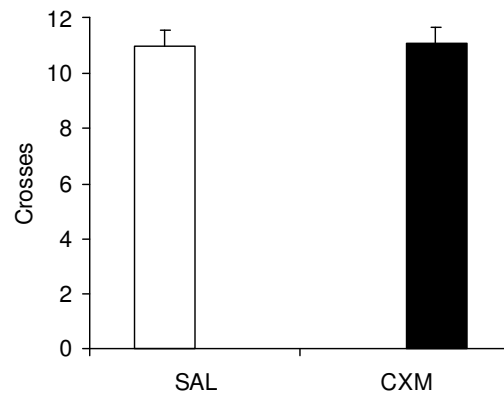
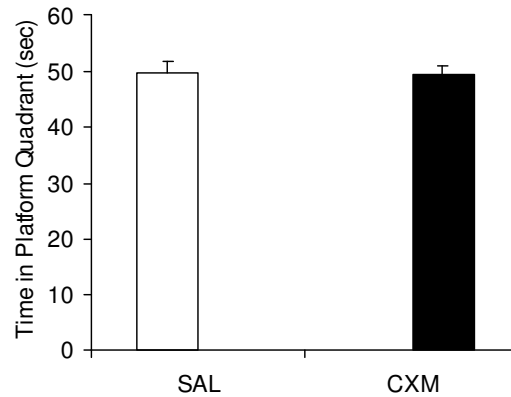
**Panel A****Panel B****Panel C**

Figure 3. Panel A: Mean latency to the original location of the submerged platform during the Morris water maze probe test. Panel B: Mean number of crosses over the original platform location during the Morris water maze probe test. Panel C: Mean time spent in the NE quadrant, where the platform had been located, during the Morris water maze probe test. (Note: Error bars represent the standard error of the mean).

Conditioned Taste Aversion

The milliliters of sucrose solution consumed during the training session were calculated for each group (see figure 4) and an independent samples *t*-test was conducted in order to examine neophobia. Animals given saline at 12-days-of-age consumed significantly less sucrose solution during their first exposure [$t(33)=3.45$, $p<.01$, $d=1.20$] in comparison with those that were given cycloheximide at 12-days-of-age. These results indicate that early protein synthesis inhibition resulted in a reduction of neophobia to a 10% sucrose solution in adulthood.

A one-way ANOVA was also conducted using these same training data with the saline and cycloheximide groups divided into their CTA conditioning subgroups (saline/saline, $n=10$; CXM/saline, $n=7$; saline/LiCl, $n=9$; CXM/LiCl, $n=9$) in order to ensure that there were no differences in consumption that might impact the development of a taste aversion. Results indicated that there were no differences among the four CTA conditioning groups [$F(3,35)=1.07$, $p>0.05$, $\eta_p^2=.09$, $\text{power}=.26$] prior to conditioning.

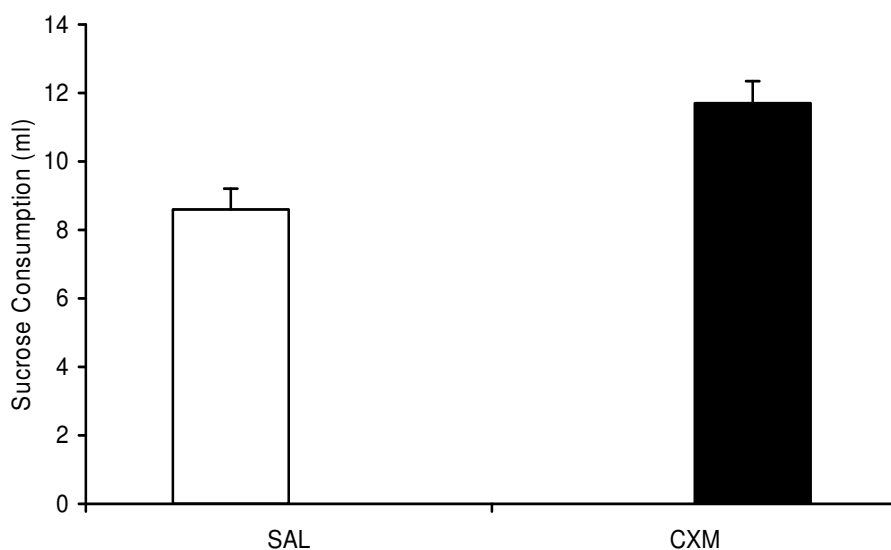


Figure 4. Mean sucrose consumption for cycloheximide (CXM) and saline (SAL) groups during animal's initial exposure to the sucrose solution and prior to LiCl injection. (Note: Error bars represent the standard error of the mean).

The examination of taste aversion conditioning using the two bottle test was done by subtracting the number of milliliters of sucrose solution consumed from the number of milliliters of water consumed. Using this method, negative scores would indicate preference for the sucrose solution (preference for sucrose), a score of 0 would indicate equal consumption of sucrose and water (equivalent preference for sucrose and water), and positive scores would indicate avoidance of the sucrose solution (strong taste aversion). The mean difference score for each of the groups is displayed in figure 5. A one-way ANOVA revealed

significant differences among the groups [$F(3,35)=12.20$, $p<.01$, $\eta_p^2=.54$]. Post-hoc pair-wise comparisons using Tukey's HSD revealed that the saline/saline group consumed significantly more sucrose than the saline/LiCl group [MD=-7.93, $p<.05$, $d=1.27$]. Similarly, the CXM/saline group also consumed significantly more sucrose than the CXM/LiCl group [MD=-12.38, $p<.01$, $d=3.25$]. These results indicate that LiCl produced a strong taste aversion regardless of early postnatal exposure to the protein synthesis inhibitor. Additional post-hoc comparisons revealed no differences between the saline/saline and CXM/saline [MD=2.69, $p>.05$, $d=.67$, power=.61] or between the saline/LiCl and CXM/LiCl [MD=-4.44, $p>.05$, $d=.65$, power=.59] groups, indicating that cycloheximide did not differentially impact consumption at testing for the taste aversion control or treatment groups, respectively.

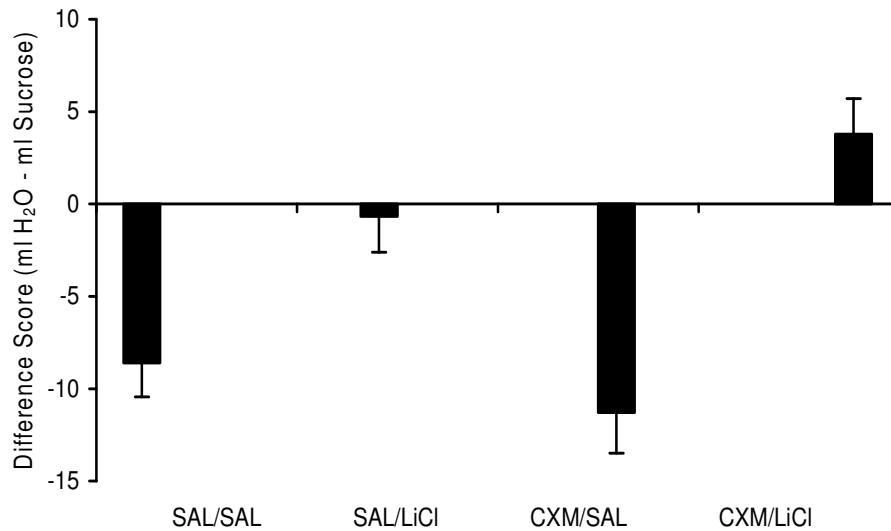


Figure 5. Mean difference scores (ml H₂O – ml sucrose) for animals administered cycloheximide at postnatal day 12 and injected with lithium chloride (CXM/LiCl) or saline (CXM/SAL) during taste aversion training, and animals administered saline at postnatal day 12 and injected with lithium chloride (SAL/LiCl) or saline (SAL/SAL) during taste aversion training. (Note: Error bars represent the standard error of the mean).

Given that a strong taste aversion was demonstrated in all animals administered LiCl, it is possible that any effects of early protein synthesis inhibition might be masked, and thus more evident through extinction testing. In order to examine extinction, the total number of milliliters of sucrose solution consumed was calculated on each of the three consecutive extinction tests, and a 3 x 4 mixed ANOVA (3 extinction tests x 4 groups) was conducted (see table 3). Results revealed a significant main effect for extinction test [Huynh-Feldt Correction $F(1.88,58.20)=65.25$, $p<.001$, $\eta_p^2=.68$] and a significant main effect for group [$F(3,31)=3.55$, $p<.05$, $\eta_p^2=.26$]. There was no group by extinction test interaction [Huynh-Feldt Correction $F(5.63,58.20)=1.77$, $p>.05$, $\eta_p^2=.15$, power=.60]. A subsequent one-way ANOVA for extinction day 1 revealed significant differences among the groups [$F(3,35)=5.52$, $p<.01$, $\eta_p^2=.35$], and post-hoc LSD tests for pairwise comparisons revealed significant differences between all saline groups (saline/saline and CXM/saline) and all LiCl

groups (saline/LiCl and CXM/LiCl). The one-way ANOVA for extinction day 2 also revealed a significant effect among groups [$F(3,35)=2.96$, $p<.05$, $\eta_p^2=.22$], but post-hoc LSD tests revealed that the CXM/LiCl was significantly different from both the saline/saline and CXM/saline groups. There were no differences among the groups for extinction day 3 [$F(3,35)=.24$, $p>.05$, $\eta_p^2=.02$, power=.09]. These results suggest that the cycloheximide group that was given taste aversion conditioning demonstrated (i.e., enhanced memory of training) some mild resistance to extinction which was attenuated by the third day of extinction testing.

Table 3. Mean and Standard Error for Sucrose Consumption of each Day of the CTA Extinction Testing for each Group

Group	Extinction Day 1		Extinction Day 2		Extinction Day 3	
	Mean	SE	Mean	SE	Mean	SE
Saline/Saline	12.70	(1.23)	17.30	(1.19)	18.40	(1.24)
Saline/LiCl	7.67	(1.30)	14.11	(1.25)	17.56	(1.31)
CXM/Saline	12.29	(1.47)	16.57	(1.42)	17.71	(1.49)
CXM/LiCl	6.78	(1.30)	12.67 ^a	(1.25)	16.89	(1.31)

Notes: ^aIndicates a significant difference between CXM/LiCl group and all other groups on extinction day 2.

CONCLUSION

Relatively few studies have been conducted examining the long-term effects of early neonatal protein synthesis inhibition, and still fewer have examined performance on tests of anxiety, learning, and memory. The purpose of this study was to administer a battery of behavioral tests in order to assess locomotor behavior, anxiety, learning, and memory in adult animals that had received a single injection of the protein synthesis inhibitor cycloheximide at postnatal day 12.

In our study, cycloheximide had no effect on 1) anxiety as measured in the elevated plus-maze and open field, 2) locomotor activity in the open field and plus-maze, 3) environmental habituation in the open field, 4) short-term working memory as assessed with spontaneous alternation in the T-maze, and 5) object recognition memory. Acquisition of the Morris water maze task was equivalent for the saline and cycloheximide groups, indicating that animals had equivalent rates of learning on the task. Similarly, cycloheximide and saline postnatal groups performed comparably when saline was administered during CTA conditioning. The same was true for animals administered LiCl during CTA conditioning. Cross-group comparisons revealed the expected aversion of sucrose solution for those animals that had received LiCl during CTA conditioning in comparison to those that received saline. Interestingly, the significant results that were revealed in association with cycloheximide on the water maze probe test and CTA extinction suggested that its effects enhanced or strengthened memory as opposed to impairing it.

In the Morris water maze, the latency measure from the probe test indicated that cycloheximide exposed animals reached the location where the submerged platform had been significantly faster than animals in the saline group. Other probe test dependent measures

failed to reveal a difference between the groups, but the latency measure is well-known as the most reliable measure of long-term spatial memory in the water maze. Immediately following the probe test a visible platform test was administered in order to assess sensorimotor abilities that might confound spatial memory performance. Unfortunately, this manipulation prevented subsequent extinction tests, as the visible platform test involved navigating to a visible platform in a novel location of the maze. Future studies might investigate extinction of spatial memory in the water maze, especially considering the results from extinction testing for taste aversion training discussed below.

Analyses of performance on the CTA extinction tests revealed that on the second day, cycloheximide animals that received LiCl consumed less sucrose solution (continued aversion to sucrose) than the other groups. This finding suggests that these animals were more resistant to extinction. Krejci (1982) reported a similar finding using an appetitive instrumental learning task. In their study, animals were administered saline or 0.6 mg/kg of cycloheximide on postnatal day 7. The cycloheximide animals were divided into 3 groups given either saline, pyritinol, or piracetam daily between 8- and 14-days-of-age. Animals were weaned on day 28 and tested on the appetitive instrumental learning task between 100- and 200-days-of-age. Those animals administered cycloheximide on day 7 and saline on days 8 through 14 showed a resistance to extinction of the instrumental learning test (indicating a better memory of training), which is consistent with the resistance to extinction of a taste aversion we report in the present study.

Despite the fact that only three significant cycloheximide effects were revealed, each of these findings suggest facilitating or enhancing effects on cognition. The reason for these facilitating effects are not clear, although the resistance to extinction is consistent with Krejci (1982). The Morris water maze is considered a classic test of spatial long-term memory known to be heavily dependent on the hippocampus (Morris et al., 2006; Nakazawa et al., 2004), neophobia is believed to involve the basolateral nucleus of the amygdala (Reilly and Bornovalova, 2005), and extinction of a conditioned taste aversion has been shown to involve the basolateral nucleus (Bahar, Dorfman, and Dudai, 2004), insular cortex (Berman and Dudai, 2001), and the ventromedial prefrontal cortex (Akirav et al., 2006).

In the present study, histological analyses of the brains were not conducted. Therefore whether or not any of these neural substrates were affected by acute systemic neonatal cycloheximide administration is unknown. It is possible that cycloheximide selectively disrupted one or more neural substrates or neuronal connections between substrates. The suggestion that neonatal brain damage alters neuronal maturation in other areas of the brain is not novel. O'Donnell, Lewis, Weinberger, and Lipska (2002) lesioned the ventral hippocampus of 7-day-old rats in a model of schizophrenia. They found that intracellular recordings from the medial prefrontal cortex were significantly altered in response to electrical stimulation in the midbrain. Thus, it is conceivable that our manipulations may have resulted in a protein synthesis inhibitor-induced developmental lesion, or may have led to abnormal neural development of those neural processes occurring during and after postnatal day 12. Histological analyses of neural tissues in future studies will help determine the cause of the behavioral and cognitive effects revealed in this study.

The fact that other studies involving neonatal cycloheximide administration have found long-term alterations in the central nervous system suggests that such an occurrence in the present study is likely. While brain damage or manipulations of neuronal circuitry is traditionally considered to result in behavioral and/or cognitive deficits, a number of studies

have reported that the destruction of neural tissues or reversible inactivation of neural tissues may produce enhanced performance. Reversible inactivation of the median raphe nucleus has been shown to enhance working memory in the Morris water maze (Sarihi, Motamedi, Naghdi, and Rashidy-Pour, 2000), while reversible inactivation of the hippocampus may enhance acquisition of a response task in the water maze (Chang and Gold, 2003; Schroeder, Wingard, and Packard, 2002) and enhance taste aversion learning (Stone, Grimes, and Katz, 2005). Rhodes and Killcross (2004) have more recently found that lesions of the infralimbic cortex in rats enhanced performance on an appetitive Pavlovian task. Thus, it seems plausible that some of the memory facilitating effects we observed during adulthood may have resulted from cycloheximide-induced disruptions during neuronal development at 12-days-of-age. Future studies involving histology and potentially correlating the volumetric size of different brain regions with behavioral outcomes should help to reveal such relationships.

In conclusion, the data presented here suggest that de novo protein synthesis at postnatal day 12 is important for normal long-term spatial memory, neophobia, and extinction of taste aversion conditioning in adulthood. Acute cycloheximide-induced disruption of protein synthesis at this age appears to lead to a reduction in neophobia, enhanced spatial memory, and enhanced taste aversion memory as evidenced by a resistance to extinction.

AUTHOR NOTES

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Reviewed by Mitchell M. Metzger, Ph.D., Associate Professor of Psychology at Ashland University.