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Dose-Response Effects of Lithium on Spatial Memory in the Black Molly Fish.

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by
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ABSTRACT

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Lithium continues to be widely prescribed for the management of bipolar disease, yet cognitive impairment-related side effects promote noncompliance of the treatment regimen. We have introduced a novel animal model, the black molly fish, to study dose-response effects of lithium on short-term (STM) and long-term (LTM) memories. We developed a method using capillary ion analysis (CIA), to measure plasma and brain lithium levels employed in our behavioral studies. We then developed an appropriate testing environment to ascertain learning capacities of these fish. We established that black mollies could adequately perform a forced-choice spontaneous alternation (SA) task used extensively in rodents as an index of spatial STM. Employing this paradigm we designed a dose-response experiment using chronic lithium regimens with a wide range of dosage groups to assess STM in the black molly. Results of the experiment indicated a robust effect in which performances of all dose groups were impaired in different degrees but not dose dependently. Using the same dosing regimen, we tested subjects in a place-learning task to assess dose-response effects of lithium on spatial LTM. A variety of performance measures were analyzed presenting a consistent theme implicating significant impairment with the high dose group. CIA results for the STM and LTM experiments revealed consistent linear relationships between mean plasma and brain lithium levels and lithium dosages. We have immunolocalized a 5-HT_{1A}-like receptor from the caudal midbrain of black mollies, an area structurally homologous to the mammalian raphe nuclei. This autoinhibitory receptor is considered to be involved in the regulation of firing of raphe serotonergic fibers and 5-HT release in terminal projection areas such as the hippocampus and frontal cortex. Downregulation of these receptors initiates excessive serotonin availability that may relieve symptoms of depression yet paradoxically impair cognition. It is unclear whether activity in the presynaptic raphe nuclei or the postsynaptic projection areas is responsible for these phenomena.

Because the black molly is not equipped with postsynaptic 5-HT_{1A} receptors, it offers a unique opportunity to study the effects of lithium on the presynaptic form of the receptor without compensating effects of the postsynaptic form exhibited in the mammal.

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LIST OF ABBREVIATIONS

ANOVA – analysis of variance

BD – bipolar disorder

CIA – capillary ion analysis

CV – coefficient of variation

IHC – immunohistochemistry

ITI – intertrial interval

LiCl – lithium chloride

LTM – long-term memory

mM – millimolar

p – probability value

PLSD – protected least significant difference, a post hoc ANOVA test

QC – quality control

R – replication number

R^2 – coefficient of determination, a typical regression analysis value

SA – spontaneous alternation

SE – standard error of the mean

STM – short-term memory

T1, T2 – Trial1, Trial 2

WAY100135 – selective 5-HT_{1A} antagonist

χ^2 = chi square value for contingency table results

5-HT – 5-hydroxytryptamine = serotonin

5-HT_{1A} – refers to the 5-HT_{1A} receptor subtype

8-OH-DPAT – (8-hydroxy-2-di-n-propylamino) tetralin, a selective 5-HT_{1A} agonist

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CHAPTER 1

INTRODUCTION

Treatment of bipolar disorder (BD) is limited not only by the scarcity of mood stabilizers available for pharmacological intervention but also by pervasive side effects induced by these drugs. This is particularly evident with patients being prescribed lithium. Lithium is one of only three mood stabilizers prescribed for the management of BD and is typically used as standard treatment for acute mania and maintenance of euthymic state in bipolar patients (Rosenthal & Goodwin, 1982). However, it has been successfully used in patients presenting with a wide variety of disorders including aggressive and self-mutilating behavior (Wickham & Reed, 1987) and cluster headaches (Ekbom, 1981).

Although lithium's efficacy in BD treatment is well established, a major problem with its therapy is patient noncompliance with the treatment regimen. One of the primary reasons for this noncompliance is patient perception of cognitive impairment. In a study of 27 side effects and their relation to compliance with a lithium treatment regimen, Gitlin, Cochran, and Jamison (1989) found that cognitive side effects and weight gain were the most disturbing to patients undergoing treatment and that noncompliance correlated most highly with coordination and cognitive side effects. In fact, the authors cite estimated rates of lithium noncompliance ranging from 10% to 47% (median = 29%). In assessing short-term memory (STM) and long-term memory (LTM) in normal humans subjected to either placebo or lithium (mean, 1569 mg/day) twice daily for three weeks, Stip, Dufresne, Lussier, and Yathan (2000) found no differences between the two groups in STM, assessed by an auditory recall test, but found differences in a long-term explicit memory word recall test. In another study, Glue, Nutt, Cowen, and Broadbent (1987) administered lithium to normal subjects for 22 days with doses adjusted to keep serum lithium levels between 0.5 and 0.8 mM followed by three computerized psychomotor tests (serial reaction time, semantic reasoning, and syntactic reasoning). The only significant effect was an impairment of semantic reasoning compared to controls. Their findings suggest a selective effect of lithium on associative mental tasks manifested as a mild form of nominal dysphasia or a slowing in naming objects. During one week of lithium administration to healthy volunteers (mean, 1225 mg/day with mean steady state serum lithium concentrations of 0.82 mM),

Weingartner, Rudorfer, and Linnoila (1985) evaluated several types of distinct cognitive processes. Their main finding was that lithium did not impair attention or vigilance but did impair the accuracy and clarity of remembered events.

The evidence is equivocal on the specific nature of chronic lithium-induced cognitive impairment, yet a wealth of studies demonstrate links between lithium use and cognitive deterioration ranging from slowing of information processing (Glue et al., 1987; Judd, Hubbard, Janowski, Huey, & Takahashi, 1977; Squire, Judd, Janowski, & Huey, 1980) to short-term memory (STM) (Kusumo & Vaughan, 1977) and long-term memory (LTM) (Karniol, Dalton, & Lader, 1978; Stip et al., 2000) deficits in both BD patients and healthy volunteers. Much of the discrepancy stems from differences in experimental design, such as the type of subjects tested, the neuropsychological tests employed, and the definitions used to differentiate various cognitive terms (Ananth, Ghadirian, & Engelsmann, 1987; Honig, Arts, Ponds, & Riedel, 1999). A central concern with studying cognitive side effects in mood disorder patients is the psychopathology itself may factor into actual or conceived impairment producing a potential confound in distinguishing effects induced by therapeutics or by the disorder (Stip et al., 2000). Nevertheless, studies with normal humans have led to some interesting, yet equivocal, findings generally acknowledging that chronic lithium treatments can induce subtle cognitive impairments. The studies with normal volunteers suggest that these effects likely do not characterize BD sequelae (Manji, Etcheberrigary, Chen, & Olds, 1993). Stoll, Locke, Vuckovic, and Mayer (1996) have reported that valproate (carbamazepine is the third mood stabilizer) substitutions alleviated much of the cognitive-related deficits experienced by their BD patients. The fact that lithium-induced cognitive complaints are significant enough for many BD patients to discontinue their treatment schedules should attest to the concern directed toward this side effect.

Our laboratory has introduced a novel animal model, the black molly fish, to study dose-response effects of lithium on STM and LTM as well as a possible mechanism of its action. The black molly (Poecilia latipinna) belongs to the family Poeciliidae of viviparous fishes within the order Atherinomorpha of a superorder of fishes, Teleostei, within the subclass, Actinopterygii or ray-finned fishes, in the major class of bony fishes, Osteichthya (Lauder & Liem, 1983). Fishes offer a number of advantages as animal models for studying the behavioral effects of lithium. Euryhaline fishes such as the black molly live naturally in a brackish ecosystem and thus have evolved to cope with rapidly changing ionic concentrations in their environments (Johnson,

1981). Because cyprinids are continuously active fishes with limited response repertoires, they acclimate well to a maze setting (Ingle, 1965) and provide uncomplicated observation for the experimenter. For example, fearful or apathetic fish are easily detected by a "freezing" behavior. Other specific advantages are related to our lab's interests in neuroreceptor-mediated mechanisms for lithium action that may be confounded in experiments with mammals that are endowed with a limbic system.

Although a correlation probably cannot be made between lithium therapeutic indices of humans and fish, it was important to establish dose-response effects for a number of behavioral parameters in the investigation. For this purpose we employed a capillary ion analysis technique for cation detection modified to accommodate analyses of plasma and brain lithium levels in the black molly. Capillary ion analysis (CIA) is an adaptation of capillary electrophoresis to a particular electrophoretic analysis of highly mobile, low molecular weight ions. The use of capillary electrophoresis for ion analysis was first reported by Hjerten (1967) for the separation of bismuth and copper cations. More recently, CIA has been applied to the analysis of ion concentrations in biological specimens (Creson, Monaco, Rasch, Hagardon, & Ferslew, 1998; Ferslew, Hagardon, Harrison, & McCormick, 1998; McClean, O'Kane, Coulter, McLean, & Smyth, 1998). Improvements in CIA techniques have made it a potentially desirable technique for analysis of minute ion concentrations in small biological specimens. Because the set-up procedures and sample times are short (generally five minutes per sample) the CIA technique is ideal for the large number of samples generated in our research.

Our laboratory has demonstrated in each of two large studies that a chronic lithium regimen impairs short-term working and long-term reference memories of the black molly fish in discrete-trials procedures utilizing two different spatial discrimination tasks in a four-arm maze situated in a spatially-constant environment. Working memory requires retention of a particular aspect of a task within a trial session and its subsequent exclusion from memory during the next trial session. Reference memory requires the subject to remember certain aspects of a task throughout an entire experiment. We implemented a forced-choice spontaneous alternation task to measure working memory and a place-learning task to measure reference memory. Most of the studies assessing lithium's effects on cognition have been conducted with humans as confirmed by several data base searches. Studies conducted with rats have largely been limited to conditioned taste aversion and other avoidance tests. Our studies are large, yet statistically

justified, systematic, studies utilizing spatial memory as an index of cognitive capacity. We opted for this approach because, during behavioral pilot studies with the black molly, we found they were well suited for maze-related learning tasks. A number of investigators have demonstrated that fishes can learn maze-related tasks (Aderman & Dawson, 1970; Bate & Kirby, 1977; Bitterman, 1965, 1975; Hughes, 1989; Ingle, 1965; Johnson, 1979). However, according to our data base searches, only one other investigator has measured behavioral effects of lithium in fishes (see Johnson, 1980). We did not use aversion paradigms because they can obscure results due to their nonselective effects on attention, arousal, or other processes not directly related to learning and memory (Meneses & Hong, 1991). The fact that our behavioral studies show no significant latency differences among controls and treated groups indicates that the lithium-treated animals were no less motivated to perform the tasks, nor were their locomotor abilities compromised relative to the controls.

Spontaneous alternation (SA) is the tendency for animals to travel in alternate directions with significant frequency at subsequent exposures to a choice point in discrete-trial procedures, typically in a T or Y maze. The moniker "spontaneous" implies that the subject has not been trained through differential reinforcement to alternate in a maze (Richman, Dember, & Kim, 1986/1987). The motivation to alternate in this nonreinforced task is considered to stem from an animal's intrinsic propensity to explore its environment in response to novelty, ultimately enhancing foraging strategies (Dember & Fowler, 1958; Hughes, 1998).

SA is a well-established behavioral phenomenon in rodents, yet studies in other species have yielded inconsistent findings. Although the long history of SA research affirms its existence in rodents, the etiology of the phenomenon has been encircled in a number of conceptual interpretations. Hull's response-oriented "reactive inhibition" behavior theory dominated American psychology in the 1940s (Dember, 1989a). Briefly, if a left-turning response is made in a two-choice situation, a certain amount of left-turning inhibition is generated which renders the right-turning response temporarily predominant (Dember, 1989b; see Dember & Fowler, 1958). Glanzer's (1953) diametrically opposed "stimulus satiation" construct suggested that an animal alternates stimuli rather than responses. Using a cross-shaped maze with two directionally-opposed start arms, Glanzer demonstrated that when rats are started from one of the start boxes (i.e., the north box) on one trial and started from the other start box (south) on the second trial, the rat repeats direction responses in favor of alternating stimuli as opposed to

responses. Montgomery's similar stimulus-oriented concept imparted a motivational basis to SA. She postulated " that an external stimulus situation evokes in the rat an exploratory tendency that leads to exploratory behavior, and that the strength of this tendency is a decreasing function of time of exposure to the situation" (Montgomery, 1951, 1952a, 1952b). Similarly, Estes and Schoeffler (1955) suggested that SA stems from a strategy-based behavior linked to motivational or survival techniques for exploration and food procurement in an organism's natural environment. Although these stimulus-oriented studies were instrumental in finally refuting Hull's reactive inhibition theory, they were based on a stimulus inhibition concept whereby the subject was said to have avoided the place to which it had previously visited. Further studies by Dember's group confirmed Glanzer's and Montgomery's reservations of Hull's theory. However, Dember argued against a stimulus inhibition concept in favor of a response to change or preference for novelty model (Richman et al., 1986/1987). By painting each of the choice arms of a T maze a different color, one black one white, on Trial 1 (T1), the subjects satiated to stimuli of both arms equally because even though glass partitions prevented them from making a choice, they were allowed to look down both alleys during their allotted 15 min start arm exploration. When both of the arms were changed to the same color on Trial 2 (T2), with the partitions removed, the subjects opted for the changed arm, thereby supporting Dember's hypothesis that SA was motivated through exploration of novel stimuli and not by repulsion of the familiar (Dember, 1956).

Pioneering studies of SA in rats by Tolman (1925) suggested that the rat utilizes a cognitive map to manage purposive behavior. Tolman suggested that the subject modifies its existing cognitive map in order to fulfill a purpose such as seeking a goal like acquiring food. Dennis (1939) considered SA to rely on a neurally-mediated memory process. He proposed that SA relies on a subject's retention of a previous choice that fades with time. In a more recent investigation Livesey, Livesey, and Syme (1981) advanced Dennis' concept to a "learning/memory hypothesis" of SA suggesting that a response on T2 is determined by the identity of the arm entered on T1 through memory of appropriate cues. Using a wide range of intertrial intervals (ITIs), the investigators demonstrated that the shorter the time between T1 and T2 the greater the rate of alternation. The results of the study support the idea that SA relies on a short-term working memory. The choice made on T2 is a result of the subject being able to remember the choice made on T1. The concept that memory traces fade with time has been

verified by a number of investigators notwithstanding their theoretical view of SA (Dennis, 1939; Grant, 1981; Rothkopf & Zeaman, 1952; Still, 1966). In a more encompassing view, Kleerekoper, Matis, Gensler, and Maynard (1974) have demonstrated that goldfish explore their environment systematically without repeating previously explored areas. The authors suggest that exploration in the goldfish involves a spatial parameter requiring a topographical memory and a temporal parameter shaped by the novelty of the environment. This notion supports both cognitive mapping and spatial working memory theories that have been in longstanding debate relative to the issue of hippocampal function in mammals (Schmajuk, 1984). Studies with organisms that lack a limbic system may provide insight for unraveling some of these unidentified mechanisms of SA.

Spontaneous alternation has been demonstrated, in one form or another, in a variety of higher vertebrate species other than mice and rats including rabbits (Baisden, Isaacson, Woodruff, & Hartesveldt, 1972), opossums (Doolittle, 1968), and hamsters (Kirby & Lackey, 1968) and lower vertebrate species, particularly fish (Ingle, 1965), as well as a wide variety of invertebrates such as cockroaches (Wilson & Fowler, 1976), woodlice (Hughes, 1985), planaria (Aderman & Dawson, 1970), paramecia, earthworms, and mealworms (see Lester, 1968). However, these studies are much fewer in number than rodent studies and use less standardized methods, so it may be expected that these studies have less consistent results. The results do identify a common theme of nonmammalian SA performance. Generally, these species require forced-choice trials with relatively short ITIs using highly discriminative choice arms to elicit SA behavior (Richman et al., 1986/1987) whereas rodents alternate at high percentage levels in both free-choice and forced-choice conditions with relatively long ITIs. A free-choice SA condition allows the subject to choose between two alternatives at a choice point during two consecutive trials whereas a forced-choice condition allows only one alternative during the first trial followed by choice options for both alternatives on the following trial. Bitterman (1965) and Gottlieb (1984) have suggested that phyletic differences in learning between so-called lower and higher life forms may stem from differences in amounts of behavioral flexibility or versatility. Through his phylogenetic filtration method, Bitterman (1965) has devised a rat-fish dichotomy scheme in which organisms from monkey to earthworm are placed in a hierarchy that reflects their abilities to solve spatial and visual problems. Warren (1957) has proposed a maze-learning

hierarchy among vertebrates and invertebrates that attempts to pattern a schema of metazoan evolution while shunning the notion of a unilinear evolutionary series of organisms.

Cue salience and choice discrimination have been longstanding issues in the quest to determine how organisms alternate. Until the mid 1960s behavioral investigators assumed laboratory animals solved maze-related tasks primarily through visual means. Douglas (1966) argued that rats alternate by using only an intramaze odor cue and an extramaze spatial direction cue. He claimed that rats did not use visual cues to alternate. However, a long list of discrimination-learning experiments has shown that rats do respond to visual cues. Later, Douglas, Mitchell, and Kentala (1972) employed different maze configurations in which the choice arms were situated at different angles from one another. They found huge differences in SA performance between subjects running a T maze and those running a parallel arm maze. This line of evidence suggests that the greater the spatial distinction between the choice arms the higher the SA percentage. However, in a series of maze environment manipulation experiments, Dudchenko (2001) demonstrates that rats can solve T maze tasks without using spatial relationships in the maze environment or the extramaze cues that are the source of the spatial relationships. He suggests that rats alternate by recalling their last action in a specific environment, independent of extramaze landmarks. It appears that rats will use highly distinctive cues if available to solve a maze-related task, yet they are flexible enough to resort to other strategies when the maze environment lacks cue salience. Although these issues have been addressed for animals other than the laboratory rat, behavioral studies suggesting that lower life forms use memory processes to solve maze tasks have not definitively characterized what cue forms these animals may be remembering.

Place learning is a type of discrimination learning in which subjects associate distinctive exteroceptive stimuli with a particular spatial location (Olton & Samuelson, 1976). Typically a subject is rewarded for remembering a fixed-goal location over a number of trials dispersed over time, a process that invokes the use of a long-term reference memory process. Rats are excellent place-learners whose rate of learning depends directly on the proportion of relevant, usable cues in the total set available (Restle, 1957). Restle further qualifies the proposal stating that the main factor in determining the outcome of a place-learning task is the amount of extra-maze visual stimulation that differentiates one goal area from another area. Similarly, Warburton (1990) demonstrated that goldfish learned food-patch placements more quickly and with greater

accuracy when visually distinct landmarks were positioned about the food patches than when they were absent. Although fishes perform qualitatively differently than higher vertebrates in discriminative learning paradigms (Bitterman, 1975), they are capable of solving maze-learning tasks that involve visual discrimination capacities requiring spatial memory (Churchill, 1916; Hughes & Blight, 1999, 2000; Ingle & Sahagian, 1973; Rodriguez, Duran, Vargas, Torres, & Salas, 1994; Roitblat, 1982). From an ecological standpoint it is critical to the survival of various fishes that they can utilize landmark memories within a spatial guidance framework for foraging and migratory purposes (Dodson, 1988).

Regardless of the mechanism proposed for solving allocentric reference frame problems, a subject of intense debate for nearly four decades, damage to the limbic system of higher vertebrates, particularly to the hippocampal formation, is generally considered to impair, i.e., place-learning tasks, which require encoding of relationships among multiple environmental features (Hollup, Kjelstrup, Hoff, Moser, & Moser, 2001; Jarrard, 1993; Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe, Nadel, Keightley, & Kill, 1975; Olton, Becker, & Handelmann, 1979; Rodriguez et al., 2002; Sutherland & Rudy, 1989). Fishes are not equipped with a limbic system like that of amniotes. However, a number of investigators contend there are structural homologies between various forebrain regions of ray-finned fishes and limbic system components of amniotes (Braford, 1995; Butler, 2000; Echteler & Saidel, 1981, Northcutt & Braford, 1980). Because telencephalons of ray-finned fishes go through a process of eversion during development, rather than evagination as with higher vertebrates, these two types of brains may appear radically different. However, comparative neuroanatomy strategies offer compelling cases for proposing, i.e., that the telencephalic dorsolateral region of ray-finned fishes shares homology with that of the amniotic hippocampus. Lopez, Bingman, Rodriguez, Gomez, and Salas (2000) and Salas, Rodriguez, Vargas, Duran, and Torres (1996) have demonstrated that telencephalic ablation in goldfish impairs performance of allocentric place-learning tasks but not egocentric cue tasks. The group has further revealed that selective lesions of the lateral, not the medial or dorsal, telencephalon impair place-learning performance (Rodriguez et al., 2002), consistent with aforementioned predictions concerning lower and higher vertebrate nervous system homologies. Neurohistochemical work in our lab confirms that the black molly possesses a central nervous system representative of the ray-finned fishes.

The mechanisms of lithium action remains unknown, although there is strong evidence to suggest that it affects the phosphoinositide second messenger system in the brain (Jope & Williams, 1994) and indirectly affects the adenyly cyclase system. Both systems are mediated by one or more neurotransmitter systems. A principle mechanism by which chronic lithium administration may exert its therapeutic effect is through a downregulation of presynaptic serotonergic (5-HT) $_{1A}$ somatodendritic autoreceptors located in midbrain raphe nuclei. The resulting disinhibition promotes increased neuronal firing unchecked by negative feedback activity of these 5-HT_{1A} receptors. Paradoxically, the increased firing of these serotonergic neurons may contribute to the cognitive decline experienced by patients undergoing chronic lithium treatment. Nevertheless, this purported mechanism meets with considerable debate. Our laboratory has immunolocalized a 5-HT_{1A}-like receptor in the caudal midbrain of the black molly, an area structurally homologous to mammalian dorsal and medial raphe nuclei. However, our results suggest that the black molly may not be equipped with the postsynaptic form of this receptor that is typically found in terminal projection areas such as the mammalian hippocampus and frontal cortex. This situation presents an excellent opportunity to study the effects of lithium on the presynaptic 5-HT_{1A} receptor system without involvement of uncertain compensatory activities of the postsynaptic 5-HT_{1A} receptor system. Also, this approach should be able to answer questions concerning whether lower brain regions can influence cognitive behaviors. It is also important to tackle issues of drug action mechanisms for more appropriate treatment strategies that minimize drug side effects. Lithium is notorious for its side effects. Hence, if mechanisms that render lithium efficacious can be isolated from mechanisms contributing to its side effects, a cleaner mood stabilizer can be produced.

CHAPTER 2

CAPILLARY ION ANALYSIS OF LITHIUM CONCENTRATIONS IN BIOLOGICAL FLUIDS AND TISSUES OF THE BLACK MOLLY FISH (POECILIA LATIPINNA)

Introduction

Capillary ion analysis (CIA) is a form of capillary electrophoresis that uses the differential electrophoretic mobility of ions to perform a separation of an ionic mixture. Application of this technique for detection of lithium concentrations in plasma and tissues of the black molly fish (Poecilia latipinna) was the purpose of this investigation. CIA was performed using a 75 μm I.D. x 60 cm length fused silica capillary and a run electrolyte of 67.7 mg hydroxyisobutyric acid (HIBA), 52.8 mg 18-crown-6-ether and 64 μl UV-CAT-1 reagent (4-methylbenzylamine) in a volume of 100 ml water (18 MΩ) with a voltage of 20 kV using ultraviolet absorption detection at 214 nm. Migration times were: potassium, 2.98 min.; calcium, 3.48 min.; sodium, 3.60 min.; barium (internal standard), 4.15 min; and lithium, 4.26 min. Lithium and barium migration times were stable and reproducible. Correlation coefficients (r) between peak area ratios of lithium/barium for concentrations ranging from 0.1 to 2.0 mM were from 0.976 to 0.996. Coefficients of variation (CV) for lithium concentrations ranged from 4.07 to 15.71% between days and 4.38 to 7.76% within day. Application of this methodology for determination of lithium concentrations in the plasma, brains and livers of fish dosed with lithium for 23 days are presented. CIA is applicable to analysis of lithium concentrations in biological fluids and tissues of fish.

The purpose of this investigation was to assess the application of CIA methodology to the determination of lithium concentrations in the plasma and tissues of poeciliid fish. CIA has been established previously to detect highly mobile, low molecular weight cations including lithium in forensic, biological specimens (Ferslew et al., 1998). This investigation presents the application of this methodology for the determination of lithium ions in the plasma and tissues of fish after consistent administration of lithium to normal healthy fish.

Materials and Methods

Analytical Reagents

Run electrolyte was prepared by placing 100 ml of water (18 MΩ) in a 250 ml flask on a magnetic stirrer. Then 67.7 mg of hydroxyisobutyric acid (HIBA), 64 ml of UV-CAT-1 reagent (containing 4-methylbenzylamine, Waters Corporation, Milford, MA) and 52.8 mg of 18-crown-6-ether (Fisher Scientific Co., Fair Lawn, NJ) were added. Solution was stirred to dissolve and degassed under vacuum. Run electrolyte was prepared fresh daily. An internal standard (40 ppm is equivalent to 0.29 mM barium) was prepared by diluting 40 ml of barium (1000 ppm, Fisher Scientific Co., Fair Lawn, NJ) to 1 l with water (18 MΩ). A standard 500 ppm solution of the cations (potassium, calcium, sodium, barium and lithium) was prepared by dilution of 1000 ppm standards of each ion (Fisher Scientific Co., Fair Lawn, NJ) with water (18 MΩ).

Sample Preparation

The specimens used here, melanistic varieties of P. latipinna, a progenitor species of P. formosa, were relatively small, their standard body lengths usually ranged between 30 and 60 mm with total mass per animal approximately 1 g. Blood from 19 fish was collected via cardiac puncture into microhematocrit tubes and spun in a standard hematocrit centrifuge to collect and pool plasma into a single reservoir. Ten µl aliquots of plasma, 10 µl of 0.29 mM barium in 18 MΩ water and 10 µl of lithium were added to 30 µl of 18 MΩ deionized water and centrifuged at 15,000x g in 10,000 NMWL-Ultrafree Centrifugal Filter Unit tubes (Millipore Corporation, Bedford, MA) to remove high molecular weight components that might clog the electrophoretic capillary tube. The lithium concentrations were 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mM for development of standard curves or 0.375 and 0.75 mM to assess within and between day precision. Final sample assay volumes were 30 µl. Whole livers (490.1 mg) or brains (356.4 mg) from the same fish were pooled and homogenized 1:4 w/v with 18 MΩ water. Whole tissue homogenates were spun at 15,000 x g and supernates were collected. Ten µl aliquots of supernate, 10 µl of 0.29 mM barium in 18 MΩ water and 10 µl of lithium were added to 30 µl of

18 M□ deionized water were and centrifuged at 15,000 x g in the Millipore Ultrafree units. Final sample assay volumes were 30 □l.

Administration of Lithium and Analysis from Individual Specimens

Ten ml of 10 mM LiCl solution was administered to individual fish by gavage. Fish were handled in wet paper towels as lithium was administered with a 20 □l pipette. Seventeen doses over a 23 day period were administered. Immediately after receiving the last dose, fish were sacrificed. For analysis of lithium levels from tissues of individual fish specimens, livers and brains were separately homogenized in 100 □l of water. Supernates were collected and centrifuged with the Millipore units. Thirty □l aliquots of these filtered tissue homogenates were used for analysis. Five □l of plasma from individual fish was brought to a total volume of 30 □l in water for analysis.

Instrumentation

Samples were analyzed on a Waters Quanta 4000 Capillary Electrophoresis System with a Waters 745 Data Module (Waters Corporation, Milford, MA). Analytical conditions were: hydrostatic sampling time, 5 s for plasma and 10 s for liver and brain; run time, 5 min; voltage, 20 kV; auto purge between samples, 1 min with run buffer (the capillary is flushed with clean buffer to remove any contaminants between sample runs); current, approximately 5 A; detector polarity, negative; ultraviolet detection, 214 nm; absorbance range, 0.02 full scale; time constant, 0.3. A Waters Accusep Capillary (fused silica, 75 □m I.D. X 60 cm length) was used for these analyses. Carousel setup was performed by filtering 17 ml of capillary ion electrolyte through a 0.2 m Anotop 25 inorganic membrane filter (Whatman International, Ltd., Maidstone, England) using a 20 ml syringe (Fisher Scientific Co., Fair Lawn, NJ) into each of four electrolyte reservoirs. One reservoir was needed for each group of five samples. Maximum number of samples per carousel was 20. No preconditioning was performed on the capillary. Run electrolyte was allowed to equilibrate within the capillary for 10 minutes before analyses were performed. Stabilization produced a steadier baseline and better electropherograms with this procedure and integrator.

Linearity and Reproducibility Experiments

Standards of lithium were prepared in plasma and brain or liver homogenate over a lithium concentration range of 0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 mM for determination of linearity. Control specimens were analyzed at 0.375 and 0.75 mM lithium concentrations. Aliquoted samples of each concentration were analyzed to determine within day (n=5) and between day (n=5) coefficients of variation.

Calculations

Lithium concentrations were used in mM to make handling of the solutions easier. Concentrations for individual tissue samples were converted to ng Li/mg of tissue or ng Li/l plasma. Linear regressions for the standard curves of concentrations and lithium/barium peak area ratios as well as means, standard deviations and errors, and coefficients of variation were determined using Stat View Student Software (Abacus Concepts, Inc., Berkeley, CA) for a Power Macintosh computer (Apple Computer, Cupertino, CA). Specimen concentrations were determined by interpolation of their lithium/barium peak area ratios from those of the standard curves.

Results

A typical electropherogram for a mixture containing 500 ppm standards of cations is illustrated in Figure 1. Migration times were: potassium, 2.98 minutes; calcium, 3.48 minutes; sodium, 3.60 minutes; barium, 4.15 minutes; and lithium, 4.26 minutes. Lithium and barium migration times were stable and reproducible (mean relative migration time for lithium/barium from all standards was 1.0184 ± 0.0011, $n = 78$). A representative electropherogram of liver extract is illustrated in Figure 2. Electropherograms for plasma and brain extract (not shown) were similar. Standard curves of lithium/barium peak area ratios versus (vs.) lithium concentrations for plasma and for liver and brain tissue extracts are shown in Figure 3. Equations for linear regression and variances between peak area ratios and concentrations are also shown in Figure 3. Correlation coefficients ranged from 0.976 to 0.996. Both within day and between day coefficients of variation (CV) are given in Table 1. CVs for lithium concentrations ranged from 4.07 to 15.71% between days and 4.38 to 7.76% within day. Application of CIA lithium

methodology to determination of lithium from plasma and tissues of fish consistently dosed for 23 days is given in Table 2.

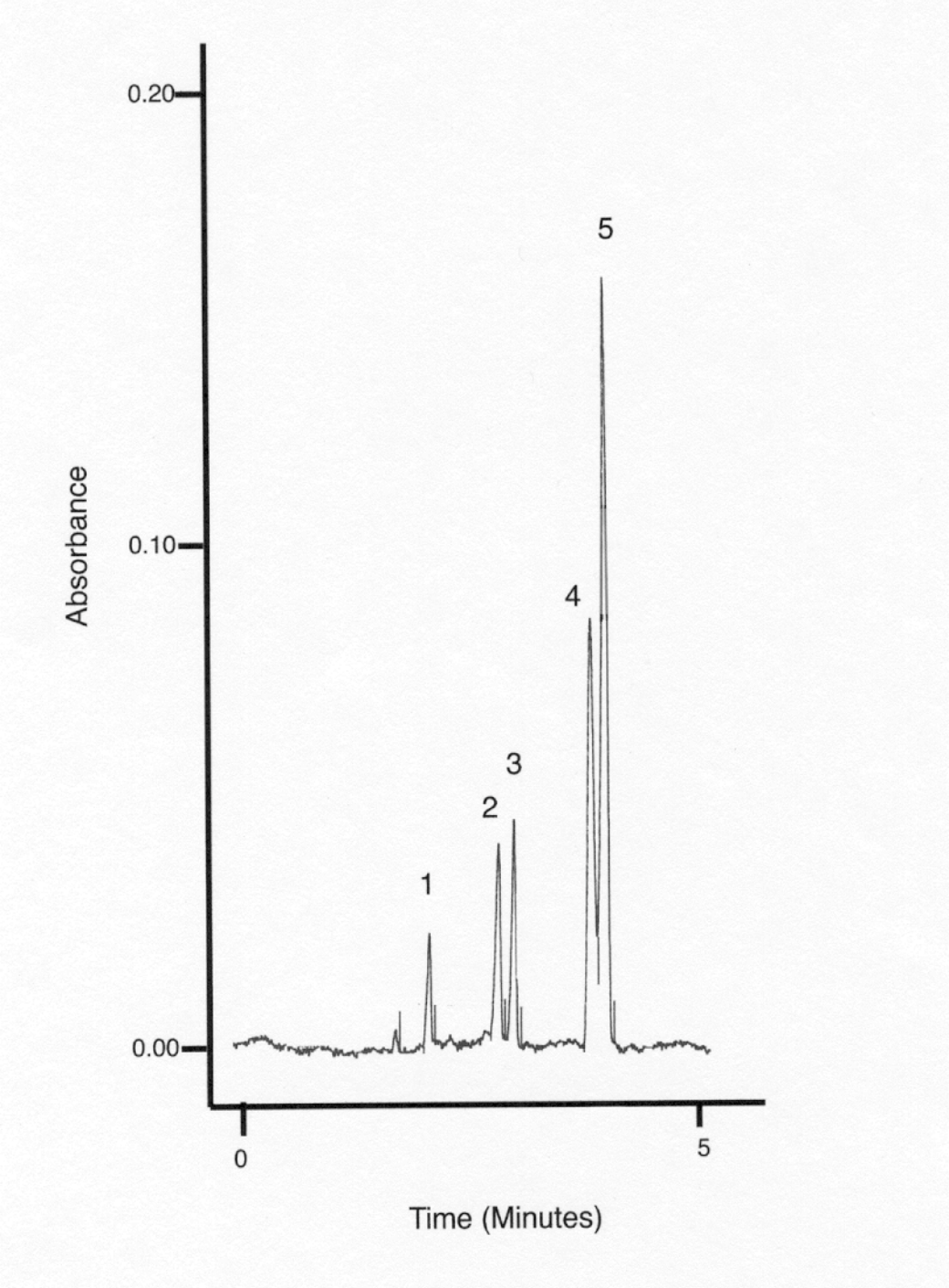

Figure 1. Electropherogram of standard cations (500 ppm of each ion). 1, Potassium; 2, calcium; 3, sodium; 4, barium (internal standard); and 5, lithium.

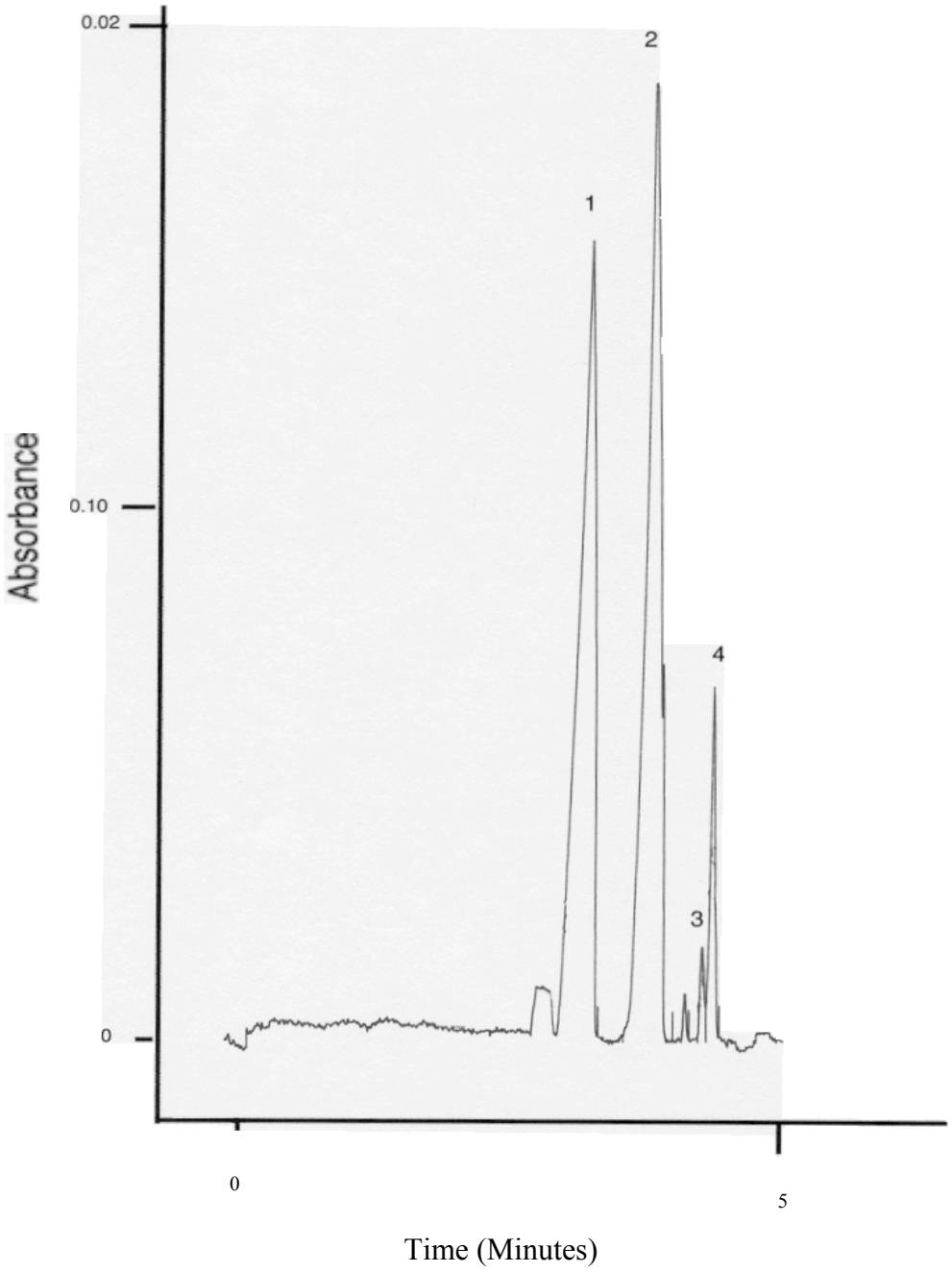

Figure 2. Typical fish liver electropherogram. 1, Potassium and calcium; 2, sodium; 3, barium (internal standard); 4, lithium.

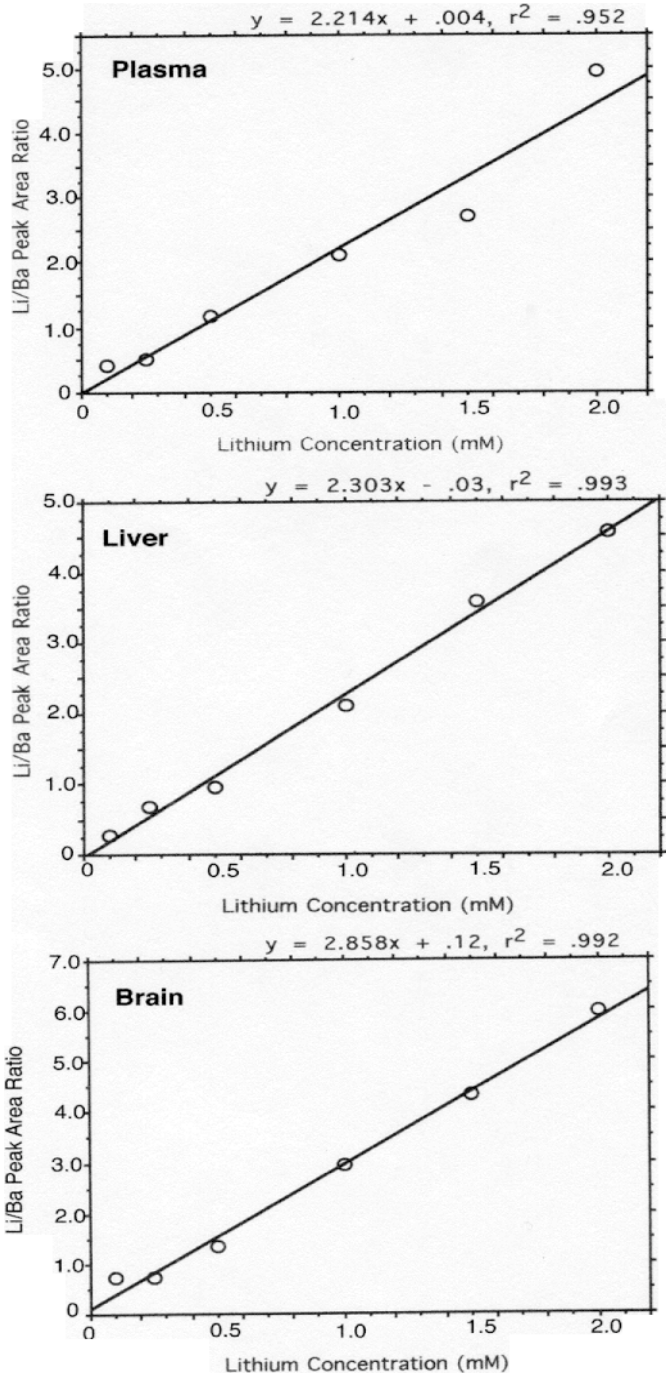

Figure 3. Correlation of lithium/barium peak area ratios to lithium concentrations for plasma, liver and brain. Regression equations and variances are shown for each graph.

Table 1

Within-Day and Between-Day Statistics - CIA

Lithium Sample	Within-day Mean (SE)	Within-day CV (%)	Between-day Mean (SE)	Between-day CV (%)
Concentration (mM)				
Plasma - 0.375	0.383 (0.01)	6.84	0.361 (0.03)	15.71
Plasma - 0.750	0.590 (0.02)	7.76	0.588 (0.01)	5.54
Liver - 0.375	0.356 (0.01)	4.38	0.369 (0.02)	13.81
Liver - 0.750	0.747 (0.03)	7.68	0.646 (0.03)	10.60
Brain - 0.375	0.330 (0.01)	5.48	0.334 (0.01)	4.07
Brain - 0.750	0.754 (0.02)	5.49	0.718 (0.02)	5.71

Note. Mean and standard errors are given in mM/l.

Table 2

Lithium Concentrations Detected in Fish after 23 Days of LiCl Administration

Tissue Sample	Lithium Concentration
Plasma	8.593 (0.616)
Liver	4.903 (0.991)
Brain	1.354 (0.127)

Note. Data represent the mean (and SE) from five fish.
Lithium concentrations are ng Li/□l plasma or ng Li/mg tissue. Fish were dosed once a day and sacrificed on day 23, immediately after LiCl administration.

Discussion

Lithium concentrations in biological specimens can be determined by a number of analytical techniques including flame emission spectrophotometry, atomic absorption spectrometry and potentiometry with an ion specific electrode (Tietz, Pruden, & Siggard-Anderson, 1997). The development of the CIA methodology reported herein offers a different qualitative and quantitative analytical technique.

The CIA separation was obtained with a short, small bore fused silica capillary column with minimal run electrolyte solution (<2 ml/analysis). Barium can be used as an internal standard to improve accuracy and precision as well as quality assurance with each analysis. Separation was obtained for the cations of interest (Figure 1) in under five minutes. Simple sample preparation (dilution, centrifugation and filtration of the plasma or homogenate) is a distinct advantage. The separation capabilities of CIA are demonstrated by the separation of potassium, calcium, sodium, barium and lithium on each specimen in a single analysis. The hydrostatic sampling and CIA separation produced accurate and precise quantitation (Table 1). The ultraviolet detection at 214 nm offered adequate limits of detection and quantitation (0.10 and 0.25 mM) and a linear determination of lithium concentrations within the concentration range of interest. Testing within our laboratory has proven that a linear response can be obtained to concentrations of as great as 32 mM.

Determinations of lithium in chronically-dosed fish specimens is shown primarily to validate that we can use CIA in our studies. The data suggest that lithium may accumulate in or is slowly cleared from fish tissues. Experiments are now underway to determine the kinetics of lithium absorption and clearance in poeciliid fishes. Data from these experiments will be essential as we develop a model to compare changes in behavioral modalities to chronic and acute lithium levels in fish as well as to monitor behavioral and physiologic changes in fishes that are rapidly removed from acutely toxic lithium. CIA offers a new analytical technique for analysis of minute lithium concentrations in small biological fluids and tissue specimens. Future applications of this method of analysis to determine the toxic effects of lithium in our black molly fish model appear promising.

CHAPTER 3

SPONTANEOUS ALTERNATION AS AN INDEX OF SPATIAL WORKING MEMORY IN THE BLACK MOLLY FISH (POECILIA LATIPINNA)

Introduction

Free-choice and forced-choice versions of the spontaneous alternation (SA) task were conducted in a + maze utilizing three intertrial interval (ITI) groups to assess spatial working memory in the black molly fish. Subjects alternated significantly above chance level (50%) in the forced-choice condition yet tended to perseverate in the free-choice condition. Performances were significantly different between the choice groups. Increasing ITIs compromised performance trends within both of the choice groups, yet differences among the ITI groups were not significant. In a second experiment, maze arms were obscured to assess subjects' reliance on extramaze visual cues in the forced-choice SA task. The control group alternated significantly above chance level yet the obscured group did not. Performance differences were significant between the groups. Results of the investigation suggest these fish use higher sensory functions such as visual cues within a working memory framework to judiciously explore their environment.

The purpose of the two experiments, then, is to demonstrate that the black molly does spontaneously alternate above chance level, that the phenomenon is limited to a forced-choice condition in these fish, and that the phenomenon decays over time as demonstrated by decreasing SA percentages with increasing ITIs. We also affirm that the black molly uses extramaze visual cues and therefore support the claim that SA is a stimulus driven phenomenon. Accordingly, in the black molly, SA can be considered as an index of spatial working memory, a form of short-term memory which requires retention of locations visited within a trial session and their exclusion from memory during subsequent trial sessions (Olton, 1979).

Experiment 1

Materials and Methods

Subjects. Sixty black mollies (melanistic varieties of P. latipinna), 4-6 cm in length, obtained from a local supplier were maintained in six groups (n = 10) in 15 "gallon" glass tanks with filtered water (Tap Water Filter, Aquarium Pharmaceuticals, Inc., Chalfont, PA) at 24EC ±1E. The tank water as well as all other water the fish inhabited at any time, i.e., the maze and holding tank water, was conditioned (Start Right and Fungus Guard, Jungle Laboratories Corporation, Cibolo, TX) and aerated for at least one week prior to the experiment. A 32 "gallon" container contained identical water for maintenance of water levels in the tanks, the maze, the holding tank, and the intertrial interval holding bowl. Thereafter, all water that the fish inhabited was monitored chemically for excess ammonia and nitrite levels, general and carbonate hardness, and for proper pH of 7.2 (Master Test Kit, Aquarium Pharmaceuticals, Inc., Chalfont, PA) as well as for temperature. Half of the fish were used for the free-choice condition, half for the forced-choice condition. The animals were further divided into 0, 5, and 10 min ITI groups (n =10). Each of the six groups was separated into two subgroups (n = 5) by aquarium dividers placed in each of the six home tanks for identification purposes. Subjects were distributed evenly yet randomly among groups on the basis of gender and size only. A 12:12 h light-dark cycle was automatically clocked by timed overhead fluorescent lights. The fish were fed once a day. On trial days, fish were fed after completion of trials.

Apparatus. The modifiable + maze (Figure 4) was made out of clear glass allowing for the incorporation of four spatially distinct T mazes without its reorientation. No intramaze cues were added other than the partitions. The intention was to keep the intramaze environment as uniform as possible. The side of the holding tank facing the maze was visually obscured. The ddH_2O in the holding tank was conditioned in the same manner as the maze and home tank water.

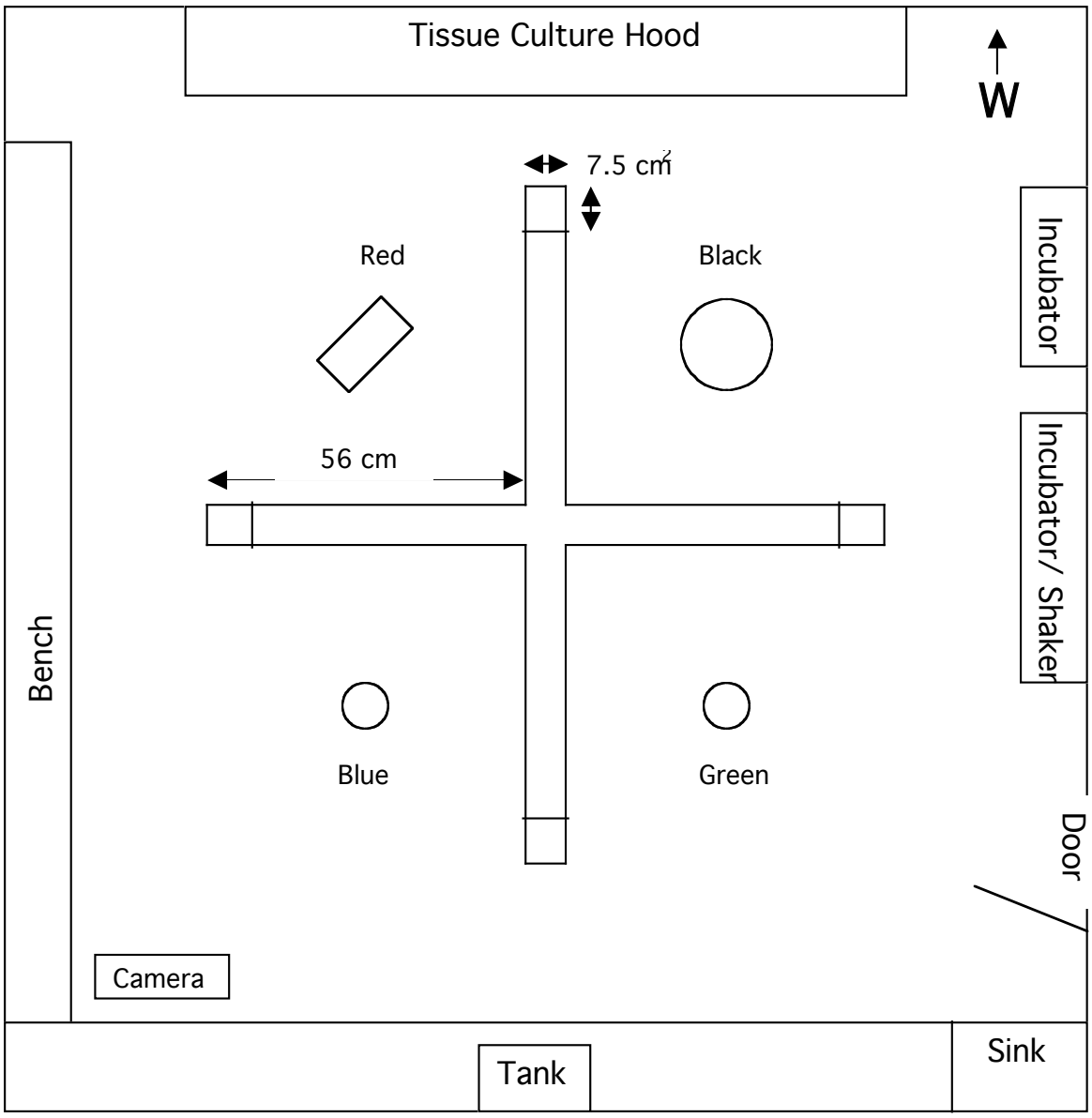

Figure 4. Maze schematic.

Note. The maze was mounted on an optically neutral platform atop a table (not shown) electrically equipped to aerate and heat the water, maintained at a depth of 12.5 cm. The maze walls were 15 cm high. A layer of gravel (1 cm) covered the entire floor of the maze. The central area included a 1.25 cm circular opening for drainage and slots at the entrances of each arm for placement of opaque partitions to selectively block arm entries. Start boxes were delimited at the distal ends of the arms by partitions extending 5 cm from the gravel surface. Red, black, blue, and green denote solid colors of 3-D objects, atop the platform, serving as immediate extramaze cues. The sizes and locations of these objects are drawn to scale relative to that of the maze. The

maze and room are drawn in relative scale. The maze room (5 x 3.5 x 3 m) was amply illuminated. The tank at the east end of the room served to collectively hold subjects of each dose group during their trials. A video camera, mounted on a tripod, was connected to a TV located just outside the maze room where behaviors were monitored.

Procedure. Subjects were given two successive trials per day for four consecutive days. Beginning each day's testing, the subjects of each ITI subgroup were collectively transferred from their home tanks to a small holding tank located in the maze room. A trial began when the subject was gently lowered into a designated start area of the maze with a small net. The experimenter immediately left the room to monitor trial performance. Direction and latency to choice were recorded once the animal was fully inside one of the two choice arms. The subject was allowed to remain in the choice arm approximately 30 sec before being gently removed. Each start area, designated E, N, W, and S, was used once for all of the subjects for days 1-4, respectively. After completion of the 0 min T1, the subject was returned to the start area to begin T2 and then returned to the holding tank after completing the trial. For the 5 and 10 min ITI groups, the subject was placed in a small bowl during the ITI. Location of the bowl directly behind (east of) the electrical outlet obscured sight of the maze during the ITI. After the elapsed ITI, the fish was returned to the start area for T2 and returned to the holding tank after completing the trial. Subgroups were collectively returned to their home tanks after completing their trials. All subjects were run during their light cycle.

During free choice trials, the arm opposite the start arm was blocked such that the subjects had access to either of the other two choice arms on both trials. During forced-choice trials, the arm opposite the start arm as well as one of the other choice arms was blocked on T1. Only the arm opposite the start arm was blocked on T2. The blocked choice arm of T1 was alternated with every other subject to control for potential laterality preferences. Testing order of subgroups and their subjects were preserved for each testing day so that each subject would be run at approximately the same time of day. However, because the first T1 blocked arm of each day was chosen randomly, subjects were not forced to the same T1 direction from day to day.

Statistics. An alpha level of .05 is considered significant for all statistical tests, which were performed using Statview (SAS, Cary, N.C).

Results

Contingency table analyses indicate that subjects in the free-choice condition perseverated significantly above chance level while the fish in the forced-choice condition alternated significantly above chance level for days 1-4 (Table 3). Table 3 also indicates that

these relationships are fairly strong for the 0 and 5 min groups yet disintegrate at the 10 min ITI level. Although the powers of the analyses diminish when broken down into individual days, it is noteworthy that the forced/0 min group alternated significantly above chance level for days 1 and 2 (χ^2s = 6.667 and 4.286, ps = .0098 and .0384, respectively) and near significance on day 3 (χ^2 = 3.600, p = .0578). By day 4, the forced/0 min group began to habituate to the task (χ^2 = 2.500, p = .1138). No other group on any of the four days of testing performed significantly above chance level for SA or perseveration. Table 4 values confirm the relationships among choice and ITI group conditions conveyed in Table 3. Using the values of Table 1 that represent SA (left-right and right-left turns for T1 and T2, respectfully) and pitting them against the values that represent perseveration (left-left and right-right turns), contingency table results in Table 4 indicate a highly significant difference in numbers of alternations and perseverations between the free- and forced-choice conditions for the 0 min groups and a significant difference for the 5 min groups. Again the relationship disintegrates at the 10 min level. A similar daily pattern of significance for turn directions is observed with SA versus perseveration performance. Contingency table analyses indicate significant differences between numbers of SAs and perseverations for free versus forced 0 min groups for days 1 and 2 (χ^2s = 5.495 and 5.051, ps = .0191 and .0246, respectively). Again, there is a trend toward this difference for day 3 (χ^2 = 3.333, p = .0679) and by day 4, the subjects appear to have habituated to the task. There are no other significant differences between SA and perseveration numbers for any of the other choice/ITI groups. However, there is a trend toward significant difference between free- and forced-choice groups within the 5 min group on day 3 (χ^2 = 3.333, p = .0679). Furthermore, contingency table analyses indicate no significant performance differences among the three ITI groups within either the free-choice or forced-choice group for days 1 to 4 (χ^2s = 1.749 and 4.301, ps = .4171 and .1164, respectively). Neither are there any significant differences within any of the four testing days. Note that in Experiment 1 the numbers of T1 left and right turns of the free choice groups were nearly equivalent, 62 and 58, respectfully, indicating these subjects had a negligible degree of cue or turn bias. With the schedule implemented for the forced-choice groups, the numbers of left and right T1 turns were again nearly equivalent, 58 versus 62, respectfully. The forced-choice condition schedule was implemented in order to anticipate a small bias for the free-choice groups.

Table 3

Contingency Table Comparisons of Numbers of Directional Turns as a Measure of Spontaneous Alternation (SA) or Perseveration (P) for Choice/Intertrial Interval (ITI) Groups during Four Testing Days

Choice/ITI Group	T2 (Free) Turn		χ^2 Test		SA vs P
T1 Turn	Left	Right	χ^2	p	
Free/0 min					
Left	13	7	3.600	.0578	P > SA
Right	7	13			
Free/5 min					
Left	12	9	3.879	.0489	P > SA
Right	5	14			
Free/10 min					
Left	9	12	0.150	.6982	SA ~ P
Right	7	12			
Forced/0 min					
Left	6	11	7.519	.0061	SA > P
Right	18	5			

table continues

Choice/ITI Group	T2 (Free) Turn		χ^2 Test		SA vs P
T1 Turn					
	Left	Right	χ^2	p	
Forced/5 min					
Left	7	10	2.283	.1308	SA > P
Right	15	8			
Forced/10 min					
Left	12	12	0 .000	>.999	SA ~ P
Right	8	8			
Total Free					
Left	34	28	5.925	.0149	P > SA
Right	19	39			
Total Forced					
Left	25	33	6.419	.0113	SA > P
Right	41	21			

Note. Forced-choice T1 turns are fixed by the experimenter. Free-choice T1 turns are not.

n = 10 for each of the six choice/ITI groups.

Table 4

Contingency Table Comparisons of Numbers of Spontaneous Alternations (SA) and Perseverations (P) between Free-Choice and Forced-Choice Groups within Intertrial Interval (ITI) Categories during Four Testing Days

ITI (min)		Choice group		χ^2 test	
		Free	Forced	χ^2	p
0	SA	14	29	11.314	.0008
	P	26	11		
5	SA	14	25	6.054	.0139
	P	26	15		
10	SA	19	20	0.050	.8230
	P	21	20		

n = 10 for each of the six choice/ITI groups.

Group mean latencies to choice for T1 and T2 for each choice/ITI group are presented in Figure 5 for days 1-4. The general trend for all six choice/ITI groups is shorter latencies to choice for T2 compared with T1. However, paired t tests indicate significant differences between T1 and T2 latencies for the free/0 min and forced/5 min groups only [t(18) = 6.180 and 7.814, ps = .0085 and .0044, respectively). Unpaired t tests comparing free- and forced-choice groups within each of the ITI groups reveals no significant differences in latencies to choice for T1 or T2. However, these analyses did disclose a near significant difference for T2 latencies between free- and forced-choice groups within the 5 min ITI group t(18) = 2.439, p = .0505) and a trend toward difference within the 0 min group t(18) = 2.139, p = .0762). A two-factor analysis of variance (ANOVA) comparing group mean latencies to choice for T1 for choice condition effect [F(1, 59) = 1.693, p = .2096], ITI effect [F(2, 57) = .335, p = .7198] or interactive effect between choice condition and ITI [F(2, 57) = .646, p = .5356] was insignificant. A similar ANOVA for T2 indicates a significant effect for choice condition [F(1,59) = 4.975, p = .0387] yet no significant differences for ITI effect [F(2,57) = 1.662, p = .2176] or interactive effect between choice condition and ITI [F (2, 57) = 2.828, p = .0855]. Fisher's PLSD post hoc tests reveal a significant difference between free- and forced-choice group T2 latencies (p = .0387), but no significant differences in T2 latencies between any of the ITI group pairings (p > .05).

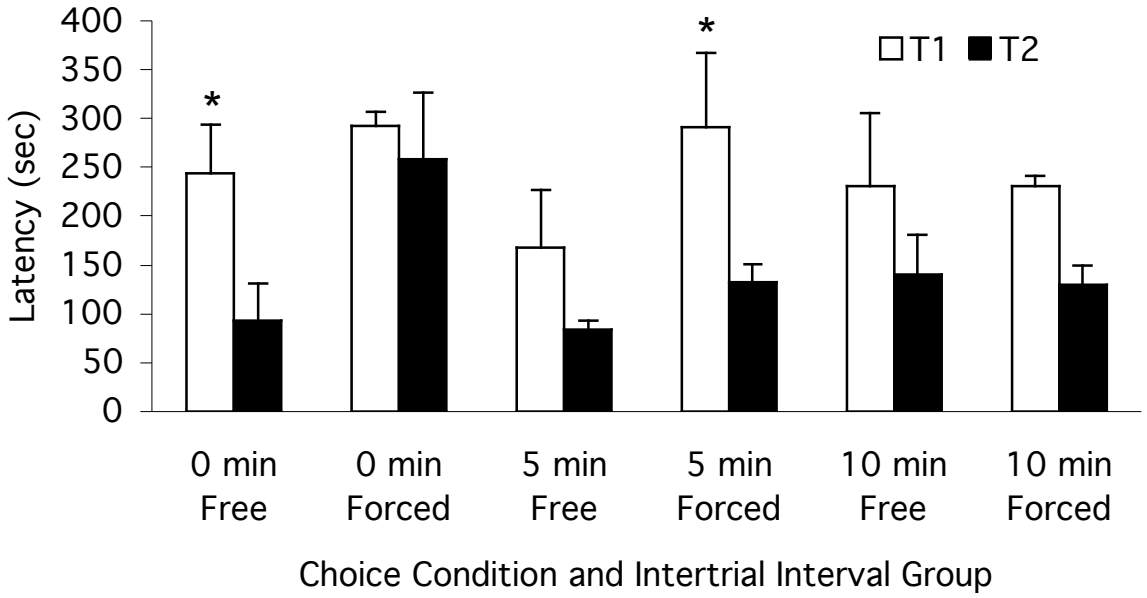

Figure 5. Trials 1 and 2 group mean latencies to choice) for each of six choice/ ITI groups) during four testing days of Experiment 1. Bars represent standard errors, n = 10).

Note. Paired t tests between T1(trial 1) and T2 (trial 2) within each of the six groups reveal significant differences for the free/0 min and forced/5 min choice/ITI groups only.
Two-factor ANOVA for T1: Choice condition effect - [F(1,59) = 1.693, p = .2096]
ITI effect - [F(2,57) = 0.335, p = .7198]
Two-factor ANOVA for T2: Choice condition effect - [F(1,59) = 4.975, p = .0387]
ITI effect - [F(2,57) = 2.828, p = .2176]
*p < .01.

Discussion

Results of Experiment 1 indicate black mollies perseverate, that is alternate significantly below chance level, in a free-choice SA condition, yet alternate significantly above chance levels in a forced-choice condition provided that ITIs between T1 and T2 are relatively short in length. The forced-choice finding is consistent with those of other investigators employing other fish species (Aderman & Dawson, 1970; Bate & Kirkby, 1977; Fidura & Leberer, 1974; Ingle, 1965; Neiberg, Dale, & Grainger, 1970). However, the free-choice finding of Experiment 1 is unique. There may indeed be phyletic differences in learning capacities as suggested by Bitterman (1965). Ample evidence exists to suggest that limbic system structures of the mammalian brain, such as the hippocampus and septum, are intimately involved in spatial working memory. Although there may be some degree of brain homology between lower and higher vertebrates, lower vertebrates do not possess a discrete limbic system as do higher vertebrates. Herein is a potential source for a phyletic difference in learning.

The black molly alternates around 70 % in the forced/0 min condition for days 1-3, which is well below typical free-choice SA performance of around 80% (Douglas, 1966) or higher for rats depending upon the maze configuration (Douglas et al., 1972). This SA percentage for the black molly in our lab has been a consistent figure in a number of pilot studies as well as with control subjects in large-scale psychopharmacological studies using the SA behavioral assay. A common feature between rodent SA performance and that of the black molly is the phenomenon of habituation to the task, which occurs at around day 4. Clarification of this term is in order. We are not referring to the classic operant definition involving unconditioned stimulus and response. Rather we refer to habituation as a decrement from significantly above chance level performance to chance level performance that might be expected to occur in a nonreinforced task. That is, when exploration of a novel environment is completed, and further responding does not yield reinforcement, response strength declines (Kokkinidis, 1989). Habituation to the SA task appears dramatically by day 4 of testing in this study as well as the pilot studies and other large-scale studies conducted in our lab with the black molly.

Overall, contingency table analyses indicate significant performance differences between the free-choice and forced-choice groups within the 0 min and 5 min groups but not for the 10 min groups, which neither alternated nor perseverated above chance levels. Results suggest that the black molly's working memory for the choice arm entered during T1 fades after about 10

min outside of the maze. This is, indeed, a much shorter ITI than those reported for the rodent. However, the ITI lengths involving extinction of memory traces in SA tasks are widely variable even among rodent studies and may depend on variables such as maze configuration and degree of discrimination or salience among various cues of a particular maze environment.

Considerable debate has centered on the issue of the legitimacy of equivalently comparing free-choice and forced-choice SA using the same probability ratio of chance-level performances, i.e., 50%. Dember and Fowler (1958) and scores of other investigators have haggled this point incessantly during the long history of SA research. These arguments are based on considerations that T1 and T2 behaviors are not independent of one another and that preferences for one choice arm or the other are not always equal during free-choice T1s. Rodrigues, Gomez, Alonso, and Afonso (1992), for instance, have suggested that most animals choose the right side more often than the left side in T-maze tests. Our results indicate that the number of T1 left and right turns of the free-choice group were nearly equivalent. Our decision to use contingency table analyses with careful scheduling of forced-choice T1 choice-arm blocking should control for these potential disparities between the free- and forced-choice conditions. Still and MacMillan (1977) have stated that the contingency table index provides a measure of alternation that is statistically independent of response bias. Furthermore, scheduling of T1 blocked arms in Experiment 1 should have prevented any possibility that the subjects could predict to what arm they would be forced; therefore, they would not be able to perform the task through a learned series of turns. As well, the experiment was only four days long with only two trials per day in which a spatially distinct start area was employed for each testing day. Therefore, the likelihood of the subject's reliance on internal turning strategies or invertebrate correcting behaviors is slim. Therefore, it is justified to compare the free- and forced-choice groups equivalently.

Figure 5 clearly illustrates a general trend among the six choice/ITI groups. All six groups opted for the T2 choice more readily than their respective T1 choice. However, paired t tests indicate that only the free/0 min and the forced/5 min groups differed significantly between T1 and T2 latencies to choice. There are no significant between group differences for T1 or T2 latencies to choice. This rather unremarkable pattern of latencies to choice among the six groups cannot account for the differences in the free- and forced-choice groups' performances. This suggests that the time to completion of T1 does not influence direction of choice during T2.

However, the one obvious caveat illustrated in Figure 5, although not statistically different from the T2 latencies of the other five groups, is the T2 forced/0 min latency to choice. Standing alone, the suggestion is apparent. The superior SA performance of the forced/0 min group is directly related to the extra time taken during T2 to make a choice. If this is so, we have a contradiction to resolve.

Experiment 2

If perseveration is the diametric opposite of SA and between group differences in latencies cannot explain the dichotomous difference in behavior between the free- and forced-choice groups, what might account for the difference? We had designed the maze to minimize intramaze cue differences. Although the introduction of the partition to the blocked choice arm during T1 of the forced-choice condition is an obvious intramaze cue, in Experiment 2, we wanted to know if the black molly actually was attending to any sort of extramaze cue. If the difference in free-choice versus forced-choice behavior is based primarily on recognition of this one intramaze cue, then there should be little difference in behavior between two forced-choice groups, one that has visual access to extramaze cues (control group) and one that does not (visually obscured (VO) group). If there is a difference in behavior between the control and the VO groups, then we have to assume the difference is based on the effect of the extramaze cues.

Materials and Methods

Subjects. Another group of 50 black mollies from the same local supplier were housed in four 15 “gallon” glass tanks. The fish and water were maintained under the same conditions as in Experiment 1. Half of the subjects served as the control group. The other half served as the VO group. Each of these groups was further subdivided, 12 and 13 subjects per group, to control for laterality preferences during T1. Group selection of subjects was evenly, yet randomly, chosen on the basis of gender and size as in Experiment 1.

Apparatus. The same apparatus, including maze, extramaze cues and orientation of the maze, used in Experiment 1 was preserved for Experiment 2. Home tanks, the holding tank, and the maze were cleaned and replenished with fresh water, treated and maintained as in

Experiment 1. For the VO group's trials, start areas and arms were lined with white opaque plastic that could be easily and quickly removed for control group trials.

Procedure. The fish were given two successive trials per day for four consecutive days in the forced-choice condition/0 min ITI condition as in Experiment 1. The trials were run in the same manner as in Experiment 1. However, the order of T1 blocked choice arms was somewhat different from that in Experiment 1 to accommodate equal numbers of T1 left and right forced turns for each group over days 1-4 of testing. Twelve subjects of the control and VO groups were forced to swim to one of the choice arms during T1 while the other 13 subjects of each group were forced to the other arm. The left-right order of T1 blocked choice arms was switched for each group for each successive day of testing.

Statistics. All p values are considered significant at the .05 level. Statistical tests were conducted using Statview (SAS, Cary, N. C.)

Results

Table 5 compares numbers of T2 left and right turns contingent upon forced T1 left and right turns as a measure of degree of SA or perseveration for the control and VO groups for days 1-4. Similar to the forced/0 min group of Experiment 1, the control group of Experiment 2 alternated decidedly above chance level. However, the VO group alternated nearly at chance level. Table 6 indicates a significant difference in alternation performances between the two groups. When the number of T2 left and right turns of the control group are compared on individual days, a pattern common with the forced/0 min group of Experiment 1 is noted, namely significant or nearly signficant above chance levels of alternation for days 1-3 with concomitant habituation to the task by day 4. Contingency table analyses designate the following results for days 1,2,3, and 4, respectively (χ^2s = 4.812, 3.381, 5.235, and 0.051, ps = .0283, .0660, .0221, and .8213). This pattern is not seen with the VO group (χ^2s = 4.996, 0.520, 0.037, and 0.371, ps = .0254 (P > SA), .4710, .8475, and .5425 for days 1,2,3, and 4, respectively). Note that the VO group perseverated above chance level on day 1.

Group mean latencies to choice are presented for both groups in Figure 6. Paired t tests comparing T1 versus T2 latencies to choice within each of the two groups reveals a significant difference for the VO group for days 1-4 [$t(49) = 3.035$, $p = .0031$] but not for the control group [$t(49) = 1.214$, $p = .2278$]. Unpaired t tests between the control and VO groups for T1 and for T2 for days 1-4 were both highly significant [$ts(49) = 8.652$ and 5.133, $ps < .0001$, respectively. Note that the T2 latency to choice for the VO group is greater than the T1 latency for this group. This result is unlike that of the control group or any of the other groups in Experiment 1 where T1 latencies to choice were consistently longer than their T2 counterparts.

Discussion

The difference in behavioral performances between the two groups is remarkable. Clearly the two groups are operating under entirely different circumstances. The VO group basically performed at chance level, indicative of random behavior. As in Experiment 1, the control group alternated at a highly significant level. Differences in group mean latencies to choice for both T1 and T2 between the two groups is also remarkable. The longer latencies of the control group suggest that these fish were spending time attending to visual cues to which the VO did not have access. Unrecorded observations of the VO group suggest that these subjects were exhibiting a random-like behavior devoid of a choice component. Unlike subjects of the VO group that tended to swim directly to a choice arm, control group subjects tended to retrace the start arm, presumably gathering cue information, then, hesitated at the choice point, and finally made a choice. Black mollies are fast moving animals, and their frequent retracings of the start arm may reflect consolidation about their environment. With access to visual cues, the control group, then, appeared to have been making choices in T2 that reflected the establishment of a working memory in T1.

Table 5

Numbers of Directional Turns as a Measure of Spontaneous Alternation (SA) or Perseveration (P) for Control and Visually Obscured (VO) Groups during Four Testing Days

Group	T1 (Forced) Turn	T2 (Free) Turn		χ^2 test		SA vs P
		Left	Right	χ^2	p	
Control						
	Left	18	32	10.256	.0014	SA > P
	Right	34	16			
VO	Left	27	23	0.160	.6889	SA ~ P
	Right	25	25			

Note. Both groups were run in the forced-choice/0 min condition, thus all T1 turns are fixed by the experimenter.

n = 25 for each of the two groups.

Table 6

Contingency Table Comparison of Numbers of Spontaneous Alternations (SA) and Perseverations (P) between Control and Visually Obscured (VO) Groups during Four Testing Days

SA / P	Group		χ^2 Test	
	Control	VO	χ^2	p
SA	66	48	6.610	.0101
P	34	52		

Note. Both groups were run in the 0 min/forced-choice condition.

n = 25 for each of the two groups.

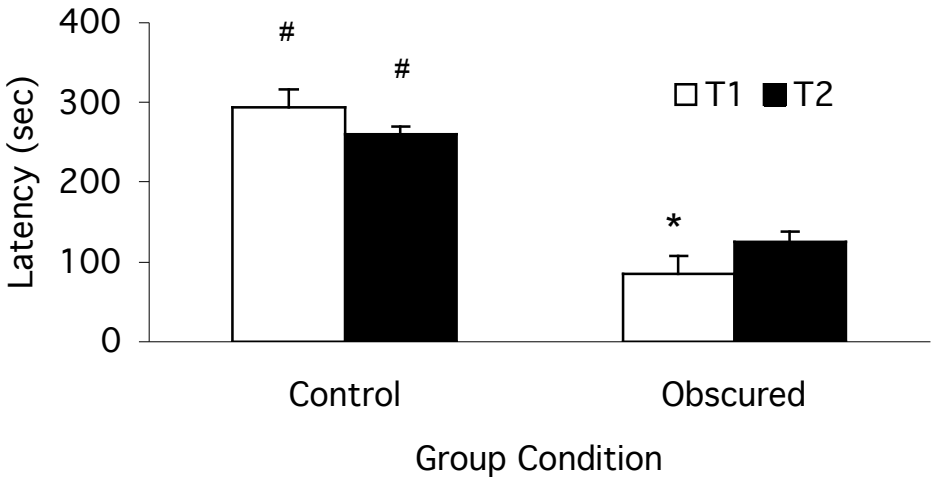

Figure 6. Trials 1 and 2 group mean latencies to choice (+SE) for each of two conditions during four testing days of Experiment 2. Bars represent standard errors.

Note. Paired t tests between T1 (trial 1) and T2 (trial 2) within each of the two groups reveal a significant difference for the obscured group, not for the control group. Unpaired t tests within T1 and within T2 reveal highly significant differences between the two groups.

n = 25 for each condition

*$p < .01$. Denotes significance of paired t test.
#$p < .0001$. Denotes significances of unpaired t tests.

General Discussion

Dember and Fowler (1959) and Thompson (1960) have independently demonstrated that rats alternate at significantly higher frequencies in a forced-choice condition than in a free-choice condition; however, differences in short and long ITIs within each of the conditions were not significant. In both of these experiments, a single free-choice trial followed a single forced-choice trial. Calvin, Schulz, and Clifford (1956) compared SA performances between a group of rats given 10 forced trials a day for four days versus a group given one forced trial a day for 40 days. Both groups were given a single free trial on the 41st trial. The first group showed significant alternation while the second group did not. However, Hughes (1966) was not able to experimentally substantiate performance differences between free- and forced-choice conditions.

Results of the present investigation indicate that there is a natural tendency for black molly fish to perseverate during free-choice SA conditions. However, in forced-choice SA conditions, these fish alternate well above chance level. Although it would be improper to statistically compare the results of Experiment 1 and Experiment 2, the similarities are noteworthy, reflecting consistencies in experimental subject population and methodology. For example, both of the forced/0 min group of Experiment 1 and the control group of Experiment 2 alternated decidedly above chance levels (ps = .0061 and .0014). As well, the group mean latencies to choice for T1 and T2 for both the forced/0 min and the control group were nearly identical. Recall that both groups of Experiment 2 were run in the forced/0 min condition. Although not presented in the results as such, percentage SA in the 0 min/forced group was 72 % for days 1-4 and 66 % for the control group. As well, both groups appeared to habituate to the task by day 4. SA percentages for day 4 were 40 % for the forced/0 min and 52 % for the control group.

These findings are in stark contrast to those involving the rat, which may alternate at higher frequencies in the forced-choice condition yet still alternate significantly above chance level in the free-choice condition with relatively longer ITIs. However, other studies do support our forced-choice findings. For example, in Neiberg et al.'s (1970) SA paradigm, comparing free-choice and forced-choice conditions in fish, rat, and man, blue gourami fish alternated above chance level only in the forced-choice condition, whereas only the rat alternated above chance level in both conditions. Aderman and Dawson (1970) compared SA performance between goldfish and planaria in forced-choice conditions with five differently sized mazes. Both species

alternated significantly higher with the shorter-armed mazes, lending credence to the idea that the experimental apparatus should be appropriate for the species studied. Ingle (1965) and later Fidura and Leberer (1974) found that percentage alternation in goldfish is directly proportional to the number of forced-choice trials given prior to the free-choice trial. Note that all of these studies used 0 min ITIs.

Although to our knowledge, sources of phyletic differences in SA have not been discussed, Bitterman (1965) has offered an intriguing set of behavioral comparisons of phylogenetically diverse organisms. Comparing strategies of habit reversal and probability learning, Bitterman devised a scheme that places organisms in one of four classes of problem solvers within a so-called rat-fish dichotomy. In a series of behavioral studies with decorticated rats, Bitterman discovered that the behavior of these rats was indistinguishable from that of the normal rat in spatial problems, yet in visual problems the decorticated rats behaved like a fish. Bitterman suggests "that as we ascend the phyletic scale new modes of adjustment appear earlier in spatial than in visual contexts". The inference is that higher order organisms are equipped with a greater degree of neural complexity that is manifested as greater behavioral flexibility, i.e., to solve problems.

Proper functioning of the mammalian limbic system, and particularly the hippocampus, has been implicated as being crucial in solving spatial working memory problems such as SA. In an early study, which demonstrated that hippocampal-damaged rats were severely impaired in a free-choice SA condition, Roberts, Dember, and Brodwick (1962) ascertained that the function of the hippocampus involves the recent memory of familiar stimuli. Later studies by Dalland (1974, 1976) demonstrated that rats with septal or hippocampal lesions perseverate in free-choice SA conditions yet alternate like normals in forced-choice conditions. This is precisely what occurred with the black mollies in Experiment 1. We have, then, identified a potential source for a phyletic difference between the fish and the rat. Fishes are not endowed with discrete limbic systems as are mammals. However, a good deal of research has suggested that the dorsolateral region of the teleost telencephalon (bony fish "forebrain") is homologous to the mammalian hippocampus (i.e., Echteler & Saidel, 1981). Therefore, fishes' capacities for memory should not be discounted. However, these neuroanatomical differences might account for the shorter ITI requirements necessary for lower vertebrate SA.

Species-specific performance may depend on preferential use of a select set of inherent sensory cues utilized in a particular task environment. The experimenter's task, then, is to understand the subjects' cue preferences as well their capacity for behavioral flexibility. Douglas (1966), Douglas et al. (1972), and Richman (1989) have argued that the design of an apparatus used in a task dictates what the subject can attend to, and that performance is related to appropriately matching cue salience afforded by the apparatus to a particular subject. Neiberg et al. (1970) suggest that differences in SA frequencies among species reflect failures in developing methodologies that are equivalent across species. The maze for the present investigation was built with relatively large dimensions compared with those designed for rodents because black molly fish are continuously active, spending time traversing the start arm in what appears to be an information gathering process (Bate & Kirkby, 1977; Ingle, 1965). T mazes for rats are typically designed such that the animal is prevented from turning around in the maze thereby minimizing potential directional displacements that may disorganize perceived relative placements of start and choice arms. A design of this nature is precluded for fishes due to the obvious stress it would impart. Tacit, then, to a discussion of SA in fishes is the manner in which they consolidate spatial information. Although the experimenters cannot guarantee absolute similarities among the arms of the maze, we purposely designed the maze so that it would have few, if any, discriminable intramaze cues in order to diminish potential turn or cue biases so that the subjects would concentrate on the rich set of extramaze cues provided.

Nevertheless, a critical difference between the free- and forced-choice conditions was the introduction of a white opaque partition blocking one of the choice arms during T1 of the forced-choice trials. The novelty afforded by the absence of the partition during T2 seems to be a reasonable explanation for the highly significant level of alternation of the forced/0 min group. The difficult part of this investigation to explicate is the differences in behavior between the free-choice groups and the VO group. It appears logical that the subjects of the VO group would perform randomly because there are no cues to negotiate other than a possible internal correcting behavior suggested by Lester (1968) and Hughes (1985, 1989) to explain invertebrate response alternation. Possibly if enough of these trials were given, the black molly might be able to adapt to this strategy. However, it is obvious that this is not a preferred cue mode for the black molly in this study's environment. So, why do the free/0 min and free/5 min groups perseverate? We have offered a comparison with septal- and hippocampal-damage rats that seems logical; however,

this does not answer why the black molly perseverates in the free-choice condition rather than, say, randomly alternates. If the free-choice situation did not instill a sense of novelty in the fish during T2, it would seem logical that this group would alternate randomly like the VO group. The motive of the black mollies' perseverative strategy eludes us.

In general, the fish appears to be a suitable subject to study behavioral phenomena such as SA that incorporates exploration and memory because many fish, including the black molly, are continuously active, necessitating formations and subsequent erasures of memory traces to efficiently negotiate their environments (Ingle, 1965).

CHAPTER 4

DOSE-RESPONSE EFFECTS OF A CHRONIC LITHIUM REGIMEN ON SPATIAL WORKING MEMORY IN THE BLACK MOLLY FISH

Introduction

Lithium has long been used in the management of bipolar disorder; however, a serious side effect of the drug is memory impairment. To assess the effects of lithium on spatial working memory we have employed a + maze using a spontaneous alternation (SA) paradigm with the black molly fish. Four treatment groups were gavaged with 20 μl of a 10, 100, or 1000 mM LiCl solution or ddH_2O vehicle every 12 hours for 22 days. On day 15, subjects began an eight-day SA task. Results indicate that there is a significant difference in SA performance among the treatment groups for days 1, 2, and 3. Lack of significance beyond day 3 is attributed to habituation to the task by the controls. Capillary ion analysis determinations of plasma and brain lithium levels illustrate linear dose-response relationships to doses administered. Regression analyses indicate that there is a relationship between SA performance and plasma/brain lithium levels during the initial part of testing. Collectively, the results indicate that chronic lithium administration impairs spatial working memory. This study and other work in our lab suggest that the black molly may be an appropriate animal model subject for further psychopharmacological studies in the field of cognition. To our knowledge only one study (Johnson, 1980) has investigated the effects of lithium on SA behavior in fishes. The purpose of the present study is to establish a dose-response effect of lithium on spatial working memory in a fish animal model.

Materials and Methods

Subjects

One hundred thirty- one black mollies (melanistic varieties of Poecilia latipinna), 4-6 cm in length, obtained from a local supplier were used in three consecutive replications (Rs) of an eight-day SA task. Subjects were randomly yet evenly assigned to one of four dose groups based on gender and size only: ddH_2O control ($n = 34$), 10 mM LiCl ($n = 31$), 100 mM LiCl ($n = 33$),

and 1 M LiCl (n = 33). Each dose group was fed and maintained as described in Chapter 3. Each tank was cleaned and replenished with conditioned and aerated water after each repetition.

The rather large number of subjects used in the study was justified by the following sample size determination equation: $N > (\square) (P_1Q_1 + P_2Q_2 / (P_1 - P_2)^2$ where N represents the minimum number of subjects needed for statistical difference in an experiment with four groups. The $\square$ value reflects $\square$ ($p < .05$) and B (90 % power) values. P and Q represent proportional values obtained from pilot study data. Some mortality was expected during the course of the experiment; therefore, a few more fish per group were included to insure a reliable subject number. Fortunately, most of the mortality occurred before trial days. Only three subjects (in the 100 mM group of R1) died during the experiment yet were included in data analyses for the days they lived. No animals were run if they appeared sickly.

Apparatus

The modifiable + maze, holding tank, and maze room environment were the same as in the previous SA experiment and are depicted in Figure 4.

Procedure

Dosing Regimen. One week after commencement of acclimation to home tanks, subjects began a two-week dosing regimen. Every 12 h subjects were gavaged via micropipette with 20 $\square$l of one of three doses of lithium chloride (Purified LiCl, Fisher Scientific, Fair Lawn, NJ) dissolved in ddH_2O as vehicle. The control group was dosed with vehicle only. For each dosing session, with gloved hands, the fish were individually handled while a micropipette tip was inserted past the throat and the solution dispensed. Dosing times were 9 am and 9 pm at the beginning and end of the subjects' light period.

Spontaneous alternation test. On day 15, subjects were given two successive trials per day for eight consecutive days in a 0 min ITI/forced-choice SA task. Beginning each day's testing, the subjects of each dose group were collectively transferred from their home tanks to the small holding tank located in the maze room. A trial began when the subject was gently lowered into a designated start area of the maze with a small scoop. The experimenter immediately left

the room to monitor trial performance. Direction and latency to choice were recorded once the animal was fully inside one of the two choice arms. The subject was allowed to remain in the choice arm approximately 30 sec before being gently removed. Subjects were given 10 min to make a choice. Subjects were eliminated from the study if they froze in the start arm for over two min. Each start area, designated E, N, W, and S, was used once for all of the subjects for days 1-4 and days 5-8, respectively. After completion of T1 the subject was returned to the start area to begin T2 and returned to its home tank after completion of T2. All subjects were run during their light cycle.

The arm opposite the start arm as well as one of the other choice arms was blocked during T1. Only the arm opposite the start arm was blocked during T2. During T1 half of the subjects of each group were forced to the left arm while the other half were forced to the right arm. The blocked choice arm of T1 was alternated for all subjects for each consecutive testing day to control for potential laterality preferences. Testing order of dose groups and their subjects were preserved for each testing day so that each subject would be run at approximately the same time of day. These measures also prevented any potential exposure of the control group to any LiCl excreted in the holding tank or maze by the other groups. Water in the holding tank and maze were exchanged daily for freshly conditioned and aerated ddH_2O.

Lithium measurement. The last dose was administered the following morning after SA testing was completed. Two hours after the last dose was given half of the subjects from each dose group were sacrificed for plasma and brain lithium levels. Subjects were euthanized in a 0.1 % methanesulfonate salt solution (MS222, 3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St Louis, MO). After 2 min in the solution, when opercular movements had ceased, cardiac punctures were performed for blood collection and whole brains were extracted, weighed, and frozen at –80^{o} C until time of processing for lithium measurement. The blood was immediately spun for plasma and frozen with the brains. Plasma and brain lithium levels were determined using the capillary ion analysis technique specifically developed for measurement of cations in the black molly fish as described in Chapter 2.

Data Analyses. Contingency table analyses were used to compare numbers of left and right T2 turns for determinations of SA levels of performance within dose groups and to compare

numbers of alternations versus perseverations among dose groups. Dose group comparisons of day block SA percentages were analyzed using the Kruskall Wallis test. Latencies to choice were analyzed using a three-factor repeated-measures ANOVA. Plasma and brain lithium levels were compared among groups using a one-factor ANOVA. Regression analyses were conducted to determine whether a relationship existed between plasma and brain lithium levels and to infer linear relationships between doses administered and lithium levels obtained. Regression analyses were also conducted to determine whether relationships existed between SA performances and lithium levels.

Results

Contingency table analyses (Table 7) indicate that chronic LiCl regimens impair SA performance. Contingency table analyses of left versus right T2 turns, contingent upon forced T1 turns, for each day of testing indicate that the controls alternated significantly above chance level for days 1, 2, and 3 (χ^2s = 7.556, 6.103, and 5.765 and ps = .0060, .0135, and .0164, respectively) but not for any day thereafter. Although the 100 mM LiCl group significantly perseverated for days 1-4, no significant values were achieved for individual days (χ^2s = 0.259, 2.496, 2.637, and 0.776; ps = .6109, .1142, .1044, .3785, respectfully for days 1, 2, 3, and 4). However, a trend toward perseveration is indicated for days 2 and 3. The control groups of each of the three Rs alternated significantly above chance level for days 1-4 (χ^2s = 4.912, 7.056, and 7.056; ps = .0267, .0079, and .0079, for R1, R2, and R3, respectfully). However, the 100 mM group perseverated during days 1-4 only in repetition 2 (χ^2s = 0.444, 6.481, and 0.100; ps = .5050, .0109, and .7515, for R1, R2, and R3, respectfully). Higher mortality rates of the R1 100 mM group (n = 3) during acclimation to home tanks may justify results seen in R1 but not in R3. Significance in the R2 100 mM group is likely due to the larger group size (n = 19) employed to compensate for increased pretest mortality rate of the R1 100 mM group. All other dose group sizes per repetition conformed to n = 10 $\pm$ 2. Only one significant difference in numbers of left versus right turns within any of the repetitions was indicated for any dose group for days 5-8. R2 controls alternated significantly above chance level (χ^2 = 4.148; p = .0417). All subjects were forced to the left and right during T1 an equal number of times for days 1-4 and days 5-8 in order to justify 50 % SA as chance level.

Table 7

Degree of Spontaneous Alternation or Perseveration within Each Dose Group for Testing Day Blocks 1-4 and 5-8

Day Block / Dose Group	T1 Turn	T2 Turns		χ^2 Test		SA vs P
		Left	Right	χ^2	p	
1-4/Control	Left	21	47	18.386	< .0001	SA >> P
	Right	46	22			
1-4/10 mM	Left	27	35	0.033	.8560	SA ~ P
	Right	26	36			
1-4/100 mM	Left	35	29	5.345	.0208	P > SA
	Right	22	42			
1-4/1 M	Left	38	28	3.030	.0817	P > SA
	Right	28	38			
5-8/Control	Left	39	29	0.000	> .9999	SA ~ P
	Right	39	29			
5-8/10 mM	Left	29	33	0.525	.4688	SA ~ P
	Right	25	37			
5-8/100 mM	Left	30	30	0.301	.5834	SA ~ P
	Right	33	27			
5-8/1 M	Left	33	33	0.030	.8618	SA ~ P
	Right	32	34			

Note. Trial 1 turns are fixed by the experimenter. T1 = Trial 1; T2 = Trial 2; SA = spontaneous alternation; P = perseveration. > greater than; >> much greater than; ~ approximately equal.

Significant differences among dose groups in numbers of alternations and perseverations within days 1, 2, and 3 (Table 8) are generally attributed to superior SA performance by the controls as indicated by contingency table analyses for all six possible combinations of groups within each of these days. Values for control versus 10 mM groups for days 1, 2, and 3 are χ^2s = 1.111, 4.317, and 5.429; ps = .2919, .0377, and .0198. For control versus 100 mM groups, χ^2s = 5.486, 7.890, and 8.041; ps = .0192, .0050, and .0046, for days 1, 2, and 3, respectfully. For control versus 1 M groups, χ^2s = 7.949, 4.347, and 3.401; ps = .0048, .0370 .0652, for days 1, 2, and 3 respectfully. None of the other possible group combinations (10 mM vs. 100 mM, 10 mM vs. 1 M, or 100 mM vs. 1 M) yielded remotely significant contingency table values for any of the three days except 10 mM versus 1 M groups on day 1 (χ^2 = 3.065; p = .0800). Contingency table analyses revealed no significant differences among the three repetitions for numbers of alternations within any of the dose groups for days 1-4 or days 5-8 (ps > .05).

Figure 7 depicts dose group mean percent alternations for each day of testing. Standard errors are not indicated because the data are represented categorically. That is, subjects either alternated or perseverated during a trial session. The Kruskall-Wallis test indicates a significant difference in alternation percentages among the dose groups for days 1-4 (H = 9.568; p = .0226) but not for days 5-8 (H = 1.301; p = .7288). Mann-Whitney tests comparing SA percentages among the six different dose group combinations again reflect superior SA performance by the controls during days 1-4 (controls vs. 10 mM: U = 1; p = .0433, controls vs. 100 mM and 1M: Us = 0; ps = .0209, respectfully, 10 mM vs. 100 mM: U = 2.5; p = .1124, and 10 and 100 mM vs. 1 M: Us = 5; ps = .3865, respectfully). No significant differences in SA percentages were indicated between any combinations of groups for days 5-8 (p > .05). Contingency table analyses specify no significant differences in SA percentages among the three repetitions for days 1-4 (χ^2 = 13.970; p = .9944) or for days 5-8 (χ^2 = 30.611; p = .4347).

Table 8

Dose Groups Comparisons of Numbers of Alternations and Perseverations

Testing Day(s)	χ^2	p
1	9.673	.0216
2	8.775	.0324
3	9.207	.0242
4	3.724	.2907
5	0.802	.8489
6	1.621	.6546
7	1.910	.5912
8	0.457	.9283
1-4	26.719	< .0001
5-8	0.815	.8458

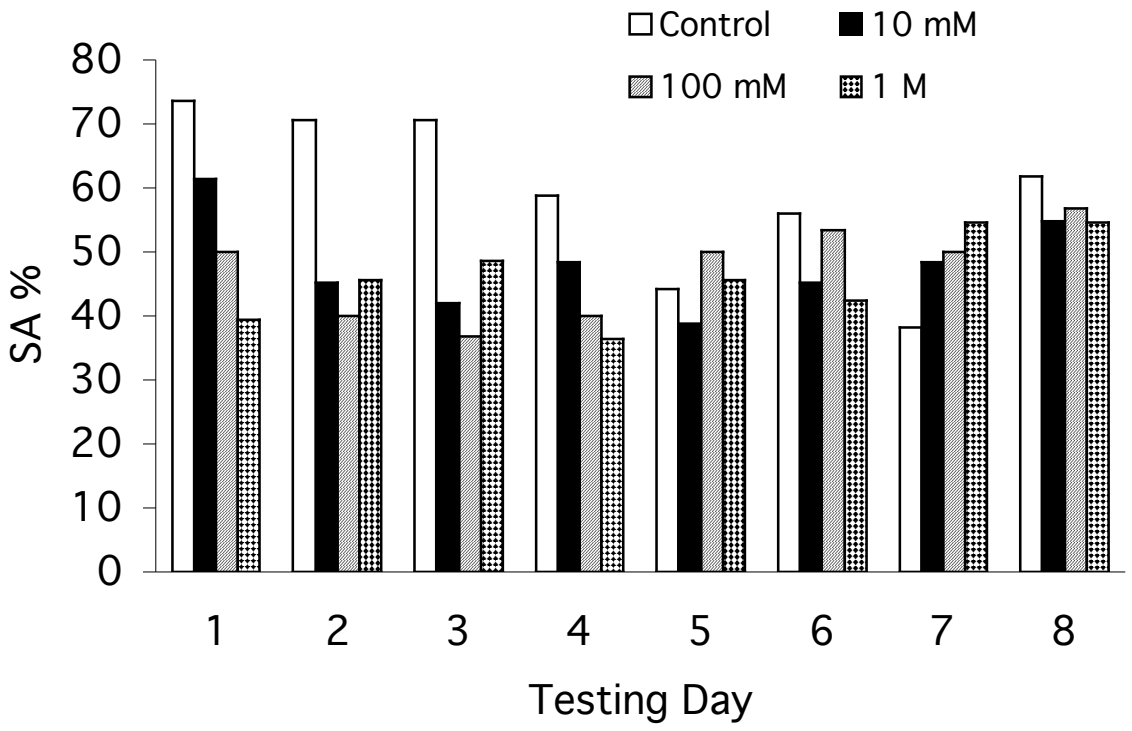

Figure 7. Spontaneous alternation (SA) percentage means for each dose group during each testing day.

Figure 8 depicts T1 and T2 latencies to choice for each testing day. A three-factor repeated-measures ANOVA revealed significant main effects for latency [$F(1,1031) = 11.663$; $p = .0007$], testing day [$F(7,1025) = 5.357$; $p < .0001$], and replication [$F(2,1030) = 4.149$; $p = .0161$], but not for dose group [$F(3,127) = 0.731$; $p = .5335$]. There were no significant interactive effects between dose group and testing day [$F(21,1001) = .680$; $p = .8555$], testing day and replication number [$F(14,1009) = 1.348$; $p = .1725$], nor dose group, testing day, and repetition number [$F(42,937) = 0.961$; $p = .5439$]. There was a significant interaction between dose group and replication number [$F(6,1021) = 2.311$; $p = .0320$]. There were no significant interactions between latency and any of the other factors or combinations of factors ($ps > .05$).

Figure 9 illustrates near linear representations of plasma and brain lithium levels relative to administered lithium doses. One-factor ANOVAs indicate significant differences in plasma lithium levels among the four dose groups [$F(3,55) = 6.0485$; $p = .0012$] but not for brain lithium levels [$F(3,59) = 1.4232$; $p = .2450$]. Fisher's Protected Least Significant Difference (PLSD) tests indicate significant plasma lithium level differences between the control and 100 mM groups ($p = .0224$), the control and 1 M groups ($p = .0002$), and the 10 mM and 1 M groups ($p = .0029$). Regression analysis verifies a significant linear relationship between plasma and brain lithium levels among dose group mean values [$R^2 = .959$; $F = 47.351$; $p = .0205$]. Regression analyses indicate significant relationships between each of the plasma and brain mean lithium levels, respectively, and mean SA percentages for days 1 and 4, respectively [R^2s = .986 and .989; Fs = 141.865 and 187.424; $ps = .0070$ and .0053, for day 1, and R^2s = .921 and .991; Fs = 23.224 and 213.926; $ps = .0405$ and .0046, for day 4]. These relationships failed to reach significance for days 2 or 3, yet tended toward significance for days 1-4 for plasma and brain levels, respectively [R^2s = .710 and .857; Fs = 4.895 and 12.001; $ps = .1574$ and .0742].

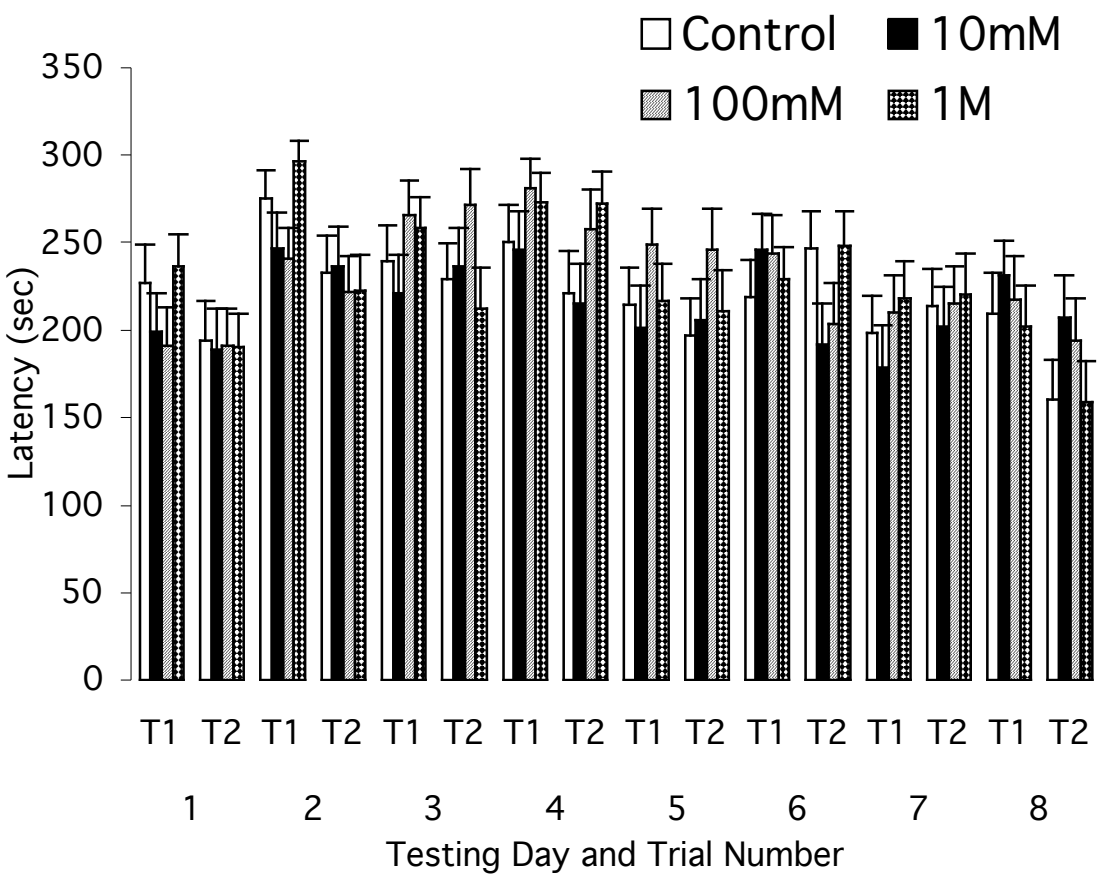

Figure 8. Trials 1 and 2 dose group mean latencies to choice for each testing day. Bars represent standard errors.

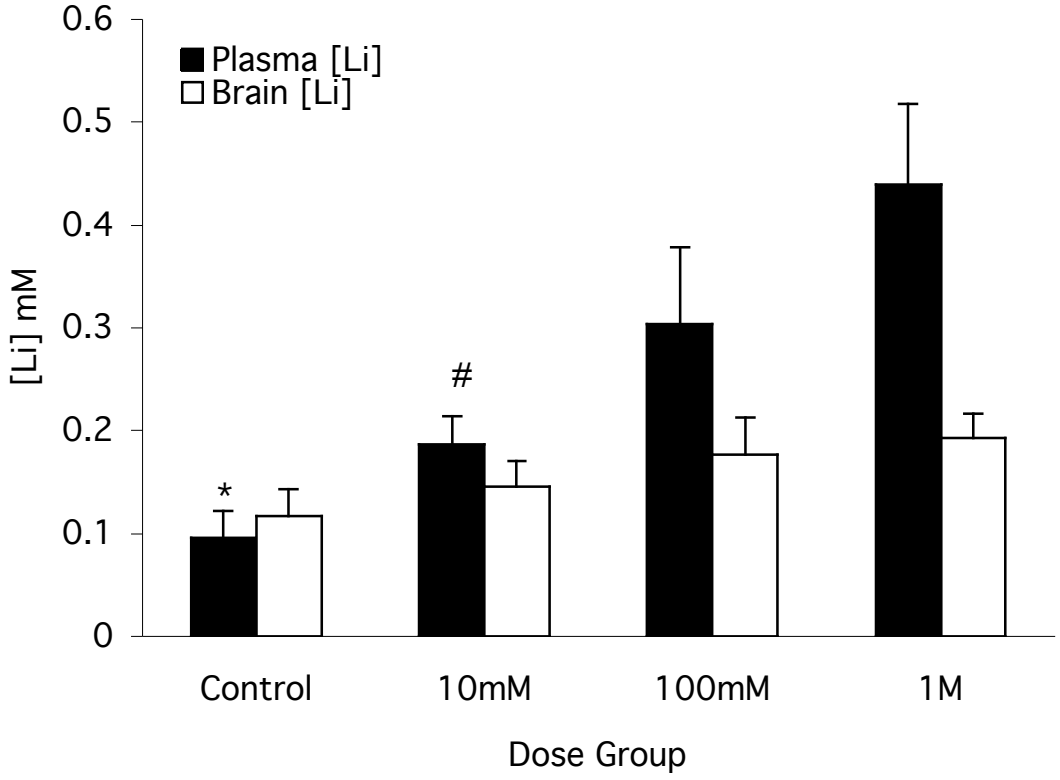

Figure 9. Dose group mean plasma and brain lithium levels from chronically dosed black molly fish. Bars represent standard errors.

Note. Plasma and brain samples were analyzed for lithium levels via a capillary ion analysis (CIA) technique using the Waters Quanta 4000 Capillary Electrophoresis System (Milford, MA). CIA was performed using a 75 □l internal diameter x 60 cm length fused silica capillary and a run electrolyte of 67.7 mg hydroxybutyric acid, 53.8 mg 18-crown-6-ether and 64 □l UV-CAT reagent (4-methylbenzylamine) in a volume of 100 ml ddH_2O (18 M□) with a voltage of 20 kV using indirect UV absorption detection at 214 nm.

*p < .05. Fisher's PLSD for control vs. 100 mM and control vs. 1 M groups.
#p < .05. Fisher's PLSD for 10 mM vs. 1 M groups.

Discussion

Results of this study indicate that lower doses of a chronic lithium regimen (i.e., 10 mM) impair short-term memory in the black molly as indexed by SA, a spatial working memory task. Perseverative tendencies, indicated with higher doses, may require an additional or possibly different interpretation of lithium's effects on SA. The overall effect of lithium on SA was not linear throughout the range of administered lithium concentrations, however there is a clear pattern of deterioration of SA performance from controls to the 100 mM group for days 1-4. Control group performance in this experiment is consistent with that of forced-choice/0 min ITI group performances in previous experiments in this lab (See Chapter 3). As well, controls in both studies habituated to the SA task by day 4. Although the LiCl dosages were relatively high compared with those of humans, according to CIA results, plasma and lithium levels were at or below therapeutic levels. Interspecies comparisons may have little bearing on what constitutes therapeutic indices for lithium in fish; however, black molly plasma and brain lithium levels reflect linear relationships to the doses administered.

Granted the lithium dosages were relatively high, the insignificant main effect in latencies to choice for dose groups suggests that neither motivation to perform the task nor locomotor ability were compromised by the lithium treatments. Although not statistically relevant, T1 and T2 latencies values are consistent with those of the forced-choice/0 min groups of previous experiments in our lab (See Chapter 3). T2 latencies were generally shorter in duration than T1 latencies, and this, too, is consistent with earlier work in our lab. The significant findings tied with the repetition factor are likely due to the unequal subjects numbers of the 100 mM groups of R1 and R2 discussed previously.

A number of interpretations have been presented to account for lithium's effects on exploratory-based behaviors but none that directly address the mechanism by which lithium affects short-term memory. Johnson has conducted a number of studies examining different aspects of lithium action in rats and goldfish. His hypothesis states that the effects of lithium on animal behavior may result from an impairment of central analysis of sensory input such that treated animals become less responsive to their surroundings (Johnson, 1983). In one of these studies, Johnson (1980) demonstrated that goldfish kept in a solution of 10 mM LiCl for two days prior to testing in a forced-choice SA condition alternated significantly above chance level yet alternated significantly less than the 10 mM NaCl controls. Johnson's theory is consistent

with clinical results suggesting that lithium may induce slowing of information processing (Glue et al.,1987, Judd et al., 1977; Squire et al., 1980). This line of reasoning may explain the results of the 10 mM group in the present experiment, which alternated at chance level, yet may not be consistent with the perseverative tendencies of the higher dose groups.

Perseveration in SA behavior is a hallmark result of limbic system damage (Roberts et al., 1962; Thomas, 1972) and drug-induced, i.e., amphetamine, toxicosis in rats (Kokkinidis, 1989). Because, at least, the hippocampus receives input from all sensory modalities, manipulations of this system will affect cue salience and thus performances in spatial tasks. Although the debate is long running, there is considerable evidence that hippocampal activity may be important in the regulation of spatial working memory (Olton et al., 1979) as well. Disputing claims that SA impairments after hippocampectomy are due to loss of internal inhibition, Isseroff (1979) demonstrated that, while operated rats were able to perform as well as controls in an SA task with minimal ITI delay, operated rats were significantly impaired relative to controls when a 10 sec ITI was introduced. Using ITIs ranging from 50 sec to 5 h, Livesey et al. (1981) demonstrated that, in untreated rats, SA rates were indirectly proportional to ITI lengths. Dalland (1976) has demonstrated that hippocampal-damaged rats perseverate body turns in a two-trial free-choice SA condition but alternate like controls in a forced-choice modification of the task. This phenomenon is precisely what our lab has demonstrated in the untreated black molly (See Chapter 3). The black molly alternates significantly above chance level in a forced-choice SA condition but perseverates in a free-choice condition. Dalland (1976) suggested that hippocampal-damaged rats are unable to shift to another response once a turn has been made. These animals appear to abandon normal use of relevant extramaze cues for navigation and to be impaired in their ability to inhibit certain types of responses related to choice. So the argument could be made that higher lithium dosages may not just interfere with hippocampal function but turn its function off more completely such that the subject is forced to rely on more primitive forms of navigation such as proprioceptive feedback proposed for insects (Lester, 1968) or, similarly, Hughes' (1985) BALM (bilaterally asymmetrical leg movements)-induced compensatory mechanism for woodlice that corrects for previously forced turns. Nevertheless, Wilson and Fowler (1976) have developed a compelling experimental arrangement utilizing a forced-choice SA paradigm to measure short-term memory in the cockroach. Alternatively, if animals perseverate on the basis of stimulus factors as opposed to response feedback (Kokkinidis

& Anisman, 1976), at least for lower LiCl dosages, then Johnson's (1979) stimulus significance hypothesis of lithium action is favored. That is, lithium may impair stimulus processing by reducing the significance with which the animal attaches to incoming stimuli. Cappeliez and Moore's (1988) hypothesis similarly states that lithium narrows the breadth of attention onto stimuli of high salience at the expense of the processing of stimuli of low salience. These altered processes, in turn, compromise the animal's short-term memory of stimulus placement.

Teleosts (bony fishes) such as the black molly lack the typical limbic system apparatus found in mammals. However, evidence suggests the dorsolateral regions of the teleost telencephalon are homologous with that of the mammalian hippocampus (Echteler & Saidel, 1981). Ohnishi (1997) has shown that telencephalic-ablated goldfish are impaired relative to controls in a short-term memory task utilizing a Y-maze training paradigm. Salas et al. (1996) have demonstrated that telencephalic-ablated goldfish are impaired in place learning strategies. The same group (Rodriguez et al., 2002) has recently shown that goldfish with lateral, but not medial or dorsal, telencephalic ablations are impaired in similar place learning strategies.

Our working hypothesis with the black molly model suggests that chronic lithium administration downregulates 5-HT_{1A} receptors leaving serotonergic neuronal firing unchecked in caudal midbrain raphe nuclei. In turn, forebrain terminal areas are bombarded with excess 5-HT release, which is thought to impair cognition. Considerable debate has been generated whether lithium and other mood stabilizers adversely affect cognition through either a presynaptic or postsynaptic 5-HT_{1A} receptor downregulation mechanism. Our animal model may offer an advantage in addressing this question because teleosts are not equipped with a postsynaptic 5-HT_{1A} receptor system typically found in limbic systems and frontal cortical regions of the mammal. Future studies will compare the effects of lithium and combinations of 5-HT_{1A} agonists and antagonists on 5-HT_{1A} receptor numbers in the black molly brain and their relation to short- and long-term memory impairments.

CHAPTER 5

DOSE-RESPONSE EFFECTS OF CHRONIC LITHIUM REGIMENS ON SPATIAL REFERENCE MEMORY IN BLACK MOLLY FISH

Introduction

Lithium is widely used in the management of bipolar disorder, yet memory impairment is a serious side effect. Previous research in our lab indicates that chronic lithium regimens differentially impair working memory in the black molly fish as assessed by a forced-choice spontaneous alternation task. To assess the behavioral effects of lithium on reference memory, we employed a + maze using a place learning task. Four treatment groups (N=140) were gavaged with 20 µl of one of three different concentrations of lithium chloride (10 mM, 100 mM, 1 M) or vehicle (ddH_2O) for controls every 12 hours for 25 days. On day 15, subjects began the 40 trials, four/day, task. Several measures of performance were analyzed to evaluate reference memory. Results indicate that the 1 M dose group needed significantly more trials to reach criterion and made significantly fewer correct first choices than the other dose groups. Capillary ion analysis determinations of plasma and brain lithium levels illustrate linear dose-response relationships to doses administered. Collectively, the results indicate that chronic lithium administration impairs spatial reference memory.

Materials and Methods

Subjects

One hundred forty black mollies (melanistic varieties of Poecilia latipinna), 4-6 cm in length, weighing 1.00-3.30 g, obtained from a local supplier, were used in four consecutive replications (Rs) of a four trials per day, 10-day place-learning task. Subjects were randomly yet evenly assigned to one of four dose groups based on gender and size only: ddH_2O control (n = 31), 10 mM LiCl (n = 37), 100 mM LiCl (n = 34), and 1 M LiCl (n = 38). Subject numbers for R1, R2, R3, and R4 were 29, 30, 40,and 41, respectively. Each dose group was maintained as described in Chapter 3. On trial days, fish were fed half their usual amount after completion of trials.

The large number of subjects used in the study was justified as described in Chapter 4. Again, some mortality was expected during the course of the experiment; therefore, a few more fish per group were included to insure a reliable subject number. Fortunately, most of the mortality occurred before trial days. Six subjects (two controls, two 100 mM, two 1M) died during the experiment and were not included in data analyses. No animals were run if they appeared sickly. Animals that froze in the maze for more than 2 min at the initiation of testing were eliminated from the experiment.

Apparatus

Other than the usual cleaning of the tanks and mazes, the behavioral testing environment was unmodified from the previous experiments. It should be noted that each dose group tank, the holding tank, and the maze were resituated to a common locale throughout all of the behavioral experiments conducted in this investigation. As well, the placements of maze room objects were left intact.

Procedure

Dosing Regimen. One week after commencement of acclimation to home tanks, subjects began a 25-day dosing regimen that lasted throughout the testing procedure. Every 12 h subjects were gavaged via micropipette with 20 μl of one of three doses of lithium chloride (Purified LiCl, Fisher Scientific, Fair Lawn, NJ) dissolved in ddH_2O as vehicle. The control group was dosed with vehicle only. For each dosing session, with gloved hands, the fish were individually handled while a micropipette tip was inserted past the throat and the solution dispensed. Dosing times were 9 am and 9 pm at the beginning and end of the subjects' light period.

Place-learning test. Because this task involved food reinforcement, subjects were conditioned to food procurement in the maze 15 min per day for three successive days prior to commencement of the task. On the first pretraining day, food flakes (normal staple) were sprinkled onto the water surface of the entire maze. Subjects of each dose group were collectively transferred from their home tanks to the choice point and allowed to feed in and explore all four arms of the maze. During the second and third pretraining days, food was placed

only at the distal ends of the arms behind partitions now set into place. No other food was provided thereafter. On day 15, subjects began a 10-day task, consisting of four trials per day, separated by approximately 30 min ITIs, conducted on an individual basis. The arm opposite the start arm was always closed with a white opaque partition while the two other arms were always open. A single large food flake was located in only one goal area for each subject throughout the task. Each dose group was subdivided equally for designated goal areas. Start positions were alternated 180^{o} after T1 and T3 for each day of testing. T1 start positions were switched for each subsequent day of testing. These measures controlled for any turn preferences. Subjects that had not obtained food within 10 trials of testing were eliminated from the experiment.

Several measures of performance were analyzed to evaluate reference memory assessed by the place-learning task as detailed in the following list:

Number of trials to criterion - Subjects that swam to their designated goal areas before any others and obtained food in five out of six consecutive trials were deemed to have reached criterion or acquisitioned the task.

Percent correct first choices - The percent of correct first choices to reward within each 10-trial block were recorded.

Number of errors - Subjects made errors when they fully entered undesignated goal arms. An error was not made if the subject swam in its designated goal arm but did not obtain food.

Number of omissions - One omission was recorded when subjects failed to obtain food within the five minute framework allowed for each trial.

Latencies to reward - The time taken to leave the start area and obtain food was recorded to assess lithium's effects on motivation and locomotion.

Left turn preference – Because percent correct first choices were lower than anticipated, we wanted to know if this was due to turn preferences.

Lithium measurement. The last dose was administered the following morning after the last day of trials for each repetition was completed. Two hours after the last dose was given subjects from each dose group were sacrificed for plasma and brain lithium levels. Subjects were euthanized in a 0.1 % methanesulfonate salt solution (MS222, 3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St Louis, MO). After 2 min in the solution, when opercular movements had ceased, body weights of each subject was determined. Cardiac punctures were performed for

blood collection and whole brains were extracted, weighed, and frozen at –80^{o} C until time of processing for lithium measurement. The blood was immediately spun for plasma and frozen with the brains. Plasma and brain lithium levels were determined using a capillary ion analysis technique specifically developed for measurement of cations in the black molly fish (Creson et al., 1998).

Data analyses. A series of two-factor ANOVAs and post hoc Fisher's PLSD tests where appropriate were conducted to evaluate each of the different levels of performance measures as well as plasma and brain lithium levels.

Results

The high dose group (1 M LiCl) took significantly longer to learn the task than the rest of the groups. Figure 10 illustrates the mean number of trials to criterion for each dose group. A two-factor ANOVA specified no significant main effects for dose group [$F(3,136) = 2.222$; $p = .0890$] or replication number [$F(3,136) = 0.316$; $p = .8140$], or the interactive effect between these two factors [$F(9,123) = 1.877$; $p = .0615$]. However, Fisher's PLSD tests indicated significant differences between the 10 mM and 1 M groups ($p = .0360$) and the 100 mM and 1 M groups ($p = .0376$) and a virtually significant difference between the controls and 1 M groups ($p = .0541$). No other post hoc test differences between other dose group or replication number pairings were designated ($p > .05$).

In addition, the high dose group firstly chose their designated goals significantly fewer times during T11-20 than the other dose groups. T11-20 appears to be a period of intense learning. Other than the controls, all other groups maximized first-choice performance during T21-30. Figure 11 depicts the mean percentages of correct first choices to reward within each trial block for each dose group. A two-factor ANOVA indicated no significant main effects during T1-11 for dose group [$F(3,136) = 1.285$; $p = .2826$], replication number [$F(3,136) = 1.357$; $p = .2590$], or the factors' interactive effects [$F(9,123) = 1.507$; $p = .1527$]. A significant main effect is indicated for dose group during T11-20 [$F(3,136) = 3.160$; $p = .0271$] but not for replication number [$F(3,136) = 0.601$; $p = .6156$] or their interaction [$F(9,123) = 1.168$; $p = .3212$]. Fisher's PLSD tests revealed significant differences between the 10 mM and 1 M groups ($p = .0360$) and between the 100 mM and 1 M groups ($p = .0066$) as well as a near significant

difference between controls and the 1 M group (p = .0560) during T11-20. No other significant between-group differences were designated with these post hoc tests during T11-20. Two-factor ANOVAs computed respectively for T21-30 and T31-40 disclose no significant main effects for dose group [Fs(3,136) = 1.175 and 1.178; ps = .3222 and .3211], replication number [Fs(3,136) = 2.158 and 0.568; ps = .0964 and .6369], or their interaction [Fs(9,123) = 0.180 and 0.648; ps = .9958 and .7539].

Figure 12 illustrates mean dose group numbers of errors committed before making a correct choice for each dose group during each of the four trial blocks. Again the 1 M LiCl group appears to perform more poorly than the other groups during T11-20. A two-factor ANOVA for T1-10 indicates no main effect for dose group [F(3,136) = 1.063; p = .3673] yet a significant main effect for replication number [F(3,136) = 3.254; p = .0241]. No significant interactive effect was shown [F(3,136) = 1.355; p = .2158]. Fisher's PLSD tests indicate one significant difference between replication pairings, R2 and R4 (p = .0021). No significant main or interactive effects were noted for T11-20: dose group [F(3,136) = 2.223; p = .0888], replication number [F(3,136) = 1.009; p = .3915], interaction [F(9,123) = 1.003; p = .4411] or T31-40: dose group [F(3,136) = 1.135; p = .3376], replication number [F(3,136) = 1.155; p = .3300], interaction [F(9,123) = 1.115; p = .3569]. Nevertheless, Figure 12 illustrates an obvious difference in number of errors committed by the high dose group during T11-20. Yet, only the 100 mM group significantly differs from the 1 M group (p = .0130). Post hoc tests also reveal a significant difference in errors committed during T31-40 between the controls and the 1 M group. During T21-30 there was no significant main effect for dose group [F(3,136) = 0.236; p = .8712] but there was for replication number[F(3.136) = 2.839; p = .0408]. No significant interaction was seen for T21-30 [F(9,123) = 0.393; p = .9365]. Significant T21-30 differences in errors between R2 and R3 (p = .0098) and between R2 and R4 (p = .0233) are denoted by Fisher's PLSD tests.

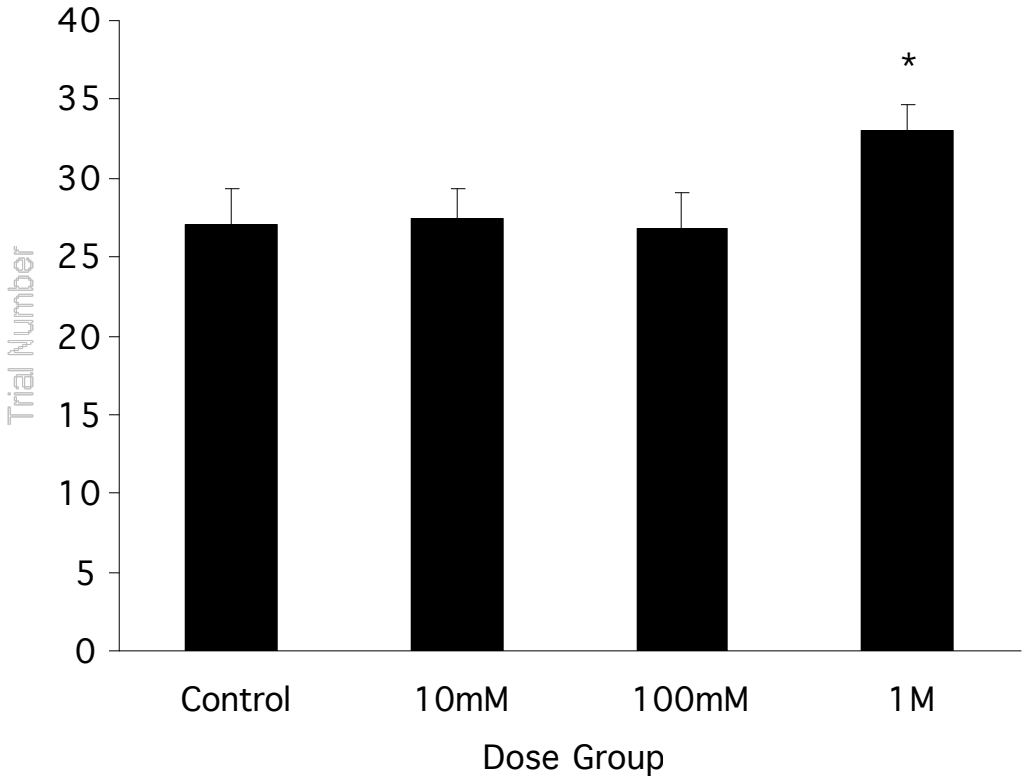

Figure 10. Dose group mean trials to criterion. Bars represent standard errors.

*p < .05: 1 M group vs. 10 mM and 100 mM groups.

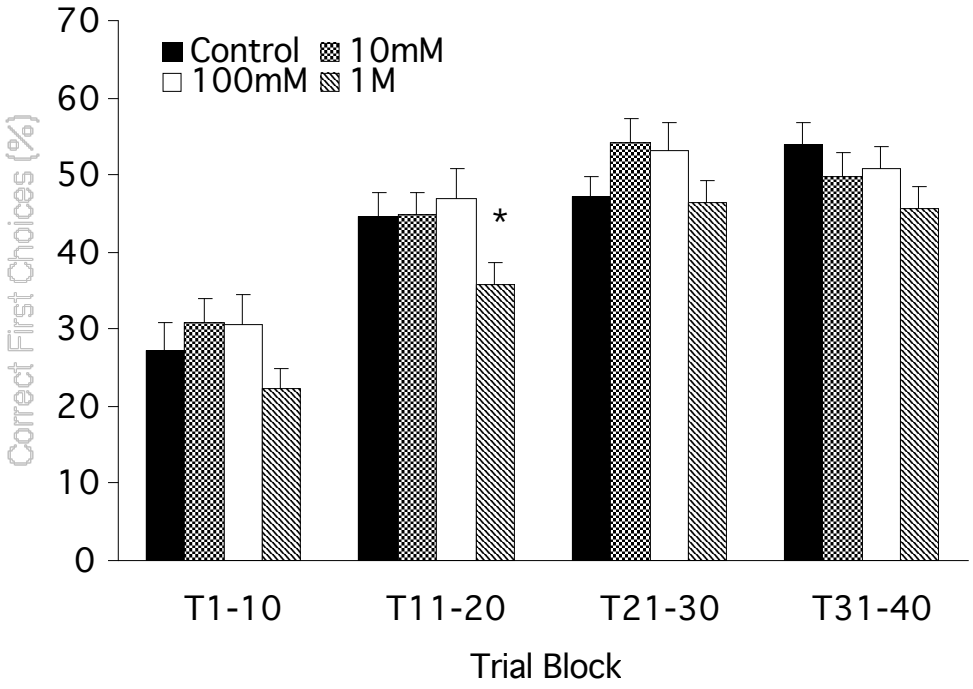

Figure 11. Dose group mean correct first choice percentages during each of four trial blocks. Bars represent standard errors.

*p < .05: 1 M group vs. 10 mM and 100 mM groups.

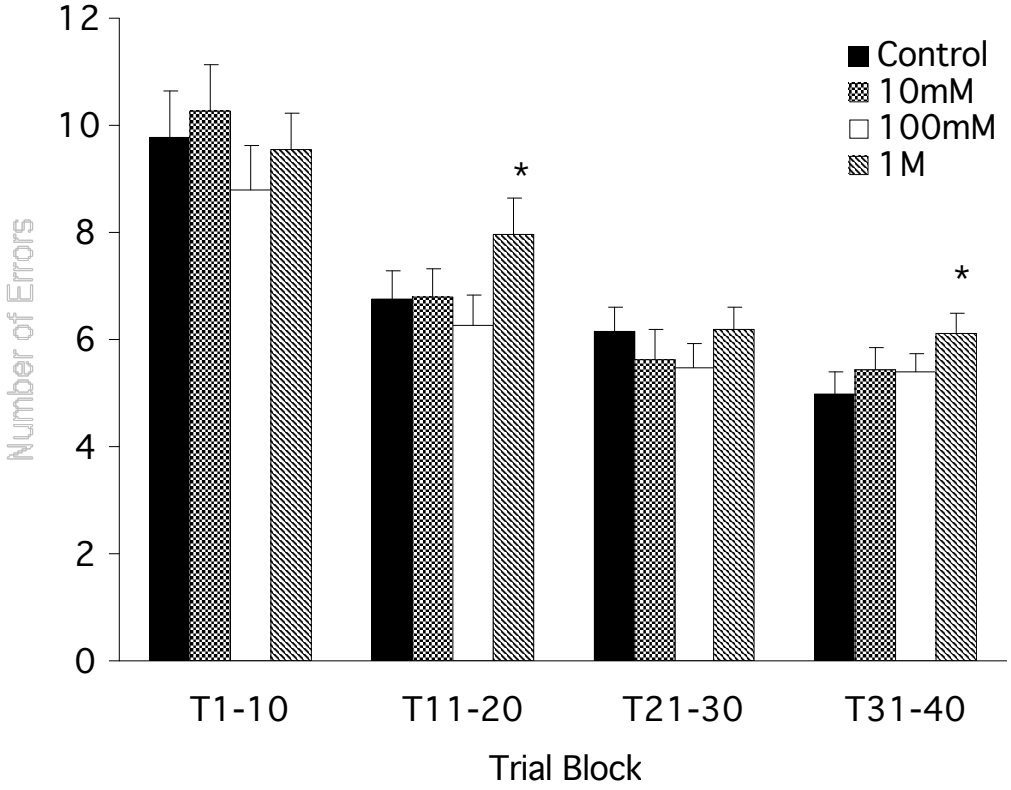

Figure 12. Dose group mean number of errors committed before acquiring reward during each of four trial blocks. Bars represent standard errors.

*p < .05: 1 M group vs. 100 mM group during T11-20; 1 M vs.controls during T31-40.

Evaluation of the omission number parameter illustrated in Figure 13 plainly indicates inferior place-learning performance for the 1 M group for each of the trial blocks. A two-factor ANOVA for T1-10 revealed no significant main effect for dose group [$F(3,136) = 2.280$; $p = .0827$], yet a significant main effect for replication number [$F(3,136) = 5.070$; $p = .0024$] and a significant interactive effect [$F(9,123) = 2.196$; $p = .0266$] was found. However, Fisher's PLSD tests indicated significant T1-10 dose group differences between the 10 mM and 1 M groups ($p = .0096$) and a closely significant difference between the 10 mM and 100 mM groups ($p = .0606$). Post hoc tests also revealed significant differences during T1-10 between R1 and R3 ($p = .0020$), R1 and R4 ($p = .0284$), and R2 and R3 ($p = .0058$). Two-factor ANOVAs for the other three trial blocks specified no other significant main or interactive effects for dose group and replication number: T11-20, T21-30, and T31-40 dose groups [$Fs(3,136) = 1.686$, 0.474, and 1.033; $ps = .1735$, .7012, and .3804], T11-20, T21-30, and T31-40 replications [$Fs(3,136) = 1.705$, 1.342, and 2.253; $ps = .1695$, .2638, and .0855], T11-20, T21-30, and T31-40 interactions [$Fs(3,136) = 1.733$, 1.658, and 1.001; $ps = .7163$, .4050, and .2349].

Generally, the wide range of lithium dosages used in this investigation did not impair the subjects' motivation to perform the task nor did it compromise their locomotor abilities. Figure 14 designates mean latencies to reward for each dose group during each trial block. A two-factor ANOVA indicates no significant dose group main effect for latency during T1-10 [$F(3,136) = 1.057$; $p = .3701$], yet a significant main effect for replication was found [$F(3,136) = 7.297$; $p = .0002$]. No significant interactive effect is noted during T1-10 [$F(9,123) = 1.401$; $p = .1949$. Fisher's PLSD tests for the replication number factor indicate significant differences between R1 and R3 ($p = .0002$), R1 and R4 ($p = .0158$), R2 and R3 ($p = .0006$), and between R2 and R4 ($p = .0297$). A two-factor ANOVA indicates significant main effects for dose group [$F(3,136) = 2.932$; $p = .0362$] and replication number [$F(3,136) = 4.349$; $p = .0060$] during T11-20. However, Fisher's PLSD reveal no significant differences between any of the possible dose group pairings ($p > .05$). Significant replication number differences were detected between R1 and R3 ($p = .0019$), R2 and R3 ($p = .0248$), and R3 and R4 ($p = .0440$). No significant interactive effect was detected for T11-20 [$F(9,123) = 1.442$; $p = .1773$]. No significant main or interactive effects were designated for T21-30 and T31-40, respectively: dose group [$Fs(3,136) = 1.164$ and 0.896; $p = .3263$ and .4455], replication number [$Fs(3,136) = 1.465$ and 1.618; $ps = .2274$ and .185], interaction [$Fs(9,123) = 1.201$ and 1.530; $ps = .3006$ and .1445.

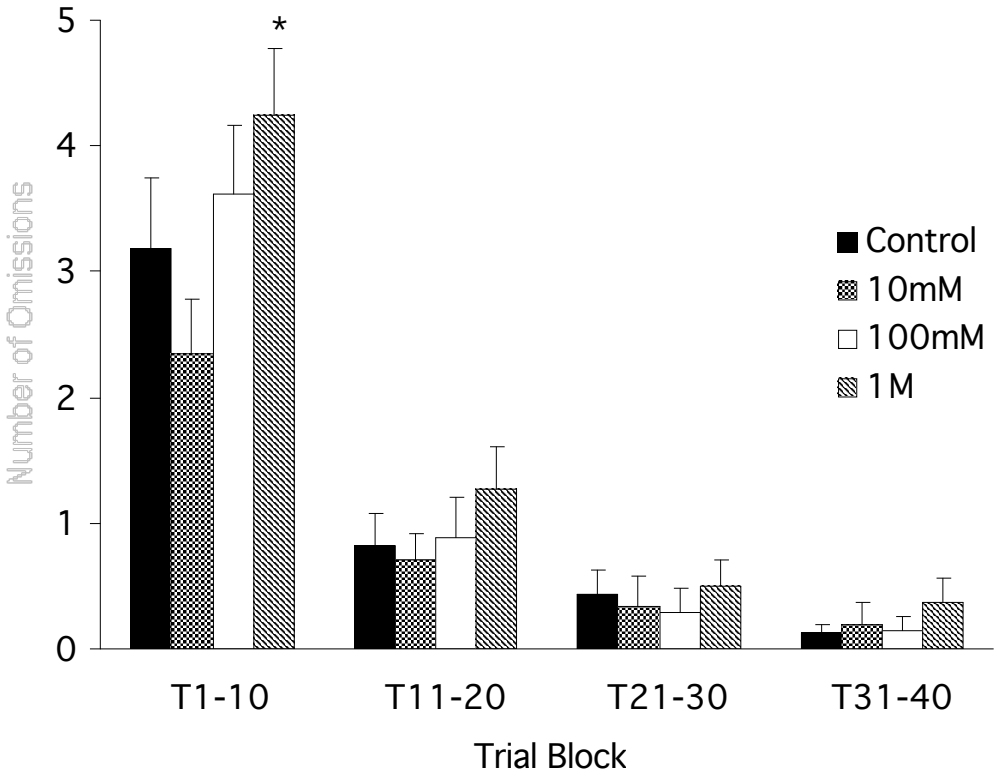

Figure 13. Dose group mean numbers of omissions during each of four trial blocks. Bars represent standard errors.

*p < .01: 1 M vs. 10 mM groups.

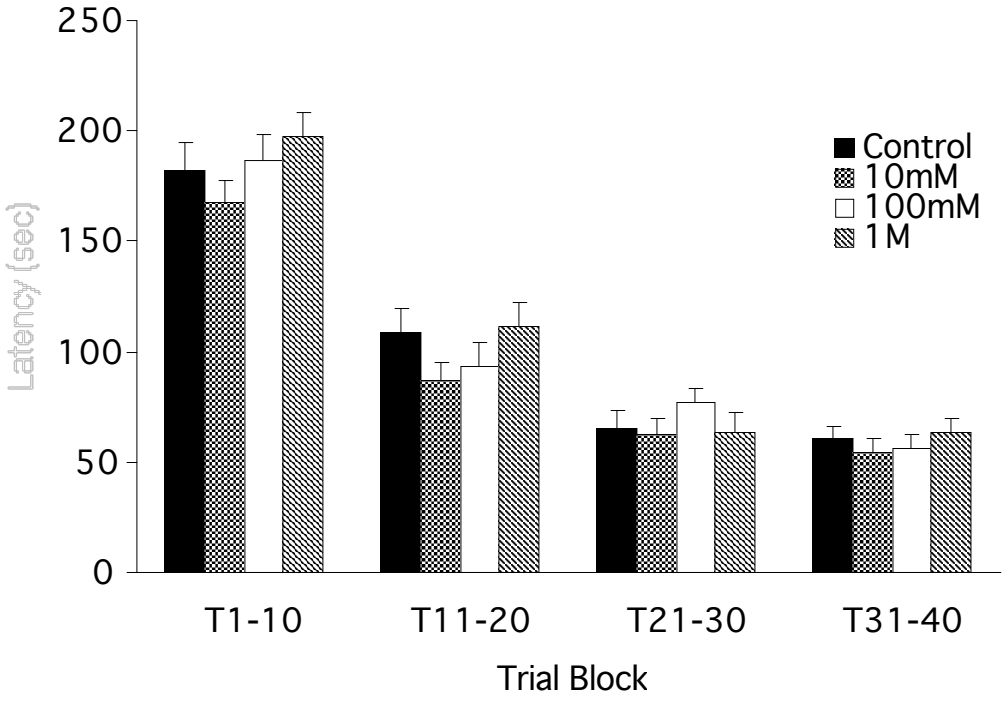

Figure 14. Dose group mean latencies to reward for each of four trial blocks. Bars represent standard errors.

Note. No significant differences between dose group pairings in latencies to reward were found within any of the four trial blocks suggesting that the lithium dosages used did not interfere with subject motivation to perform the task nor did they compromise subject locomotor ability.

Because percentages of first correct choices were lower than expected, we were interested in whether the subjects were exhibiting turn preferences. Left turns were arbitrarily chosen for analysis. Results indicate the subjects did not exhibit turn preferences. Figure 15 illustrates mean percent left turns negotiated at the central choice area of the maze for each dose group during each trial block as a measure of directional turn preference, i.e., left or right turns from the central area. Results of these analyses indicate none of the dose groups exhibited a turn preference for any of the trial blocks. For T1-10, T11-20, T21-30, and T31-40, respectively, no significant differences among dose groups [Fs(3,136) = 1.364, 0.182, 0.642, and 0.049; ps = .2570, .9083, .5896, and .9857], replication numbers [Fs(3,136) = 0.880, 0.486, 1.206, and 1.631; ps = .4534, .6927, .3104, and .1856] or their interactions [Fs(9,123) = 1.051, 0.812, 0.832, and 0.541; ps = .4040, .6061, .5882, and .8422].

Both plasma and brain lithium levels assume linear dose-response effects for the doses administered. Further analyses indicated neither body weight nor gender influenced plasma or brain levels therefore these factors were not accounted for during performance measure analyses. Figure 16 depicts the linear relationship between plasma and brain lithium concentrations and dosages used in this investigation. Two-way ANOVAs of plasma lithium levels designate significant main effect differences for dose group [$F(3,136) = 15.538$; $p < .0001$] but not for replication number [$F(3,136) = 2.189$; $p = .0953$]. No interactive effect is indicated [$F(9,123) = 0.667$; $p = .7366$]. Fisher's PLSD tests show significant differences between controls and the 10 mM group ($p = .0005$), controls vs. the 100 mM and 1 M groups, and the 10 mM vs. the 1 M groups ($p < .0001$). There was also a significant difference between the 100 mM and 1 M groups ($p = .0238$) but not between the 10 mM and 100 mM groups ($p = .0896$). Two-way ANOVAs conducted for brain lithium levels indicate significant main effect differences for dose group [$F(3,136) = 6.550$; $p = .0005$] and replication number [$F(3,136) = 9.138$; $p < .0001$] and a significant interactive effect [$F(9,123) = 2.566$; $p = .0116$]. Fisher's PLSD tests indicate significant differences between the controls and the 10 mM group (.0219), controls vs. 100 mM group ($p = .0022$), controls vs. 1 M group ($p < .0001$), 10 mM vs. 100 mM groups ($p = .0026$), and 100 mM vs. 1 M groups ($p = .0454$) but, as with the plasma levels, no significant difference between the 10 mM and 100 mM groups. Post hoc tests for replications indicate significant differences between R1 and R2, R3, and R4 ($p < .0005$) and between R2 and R3 ($p = .0196$).

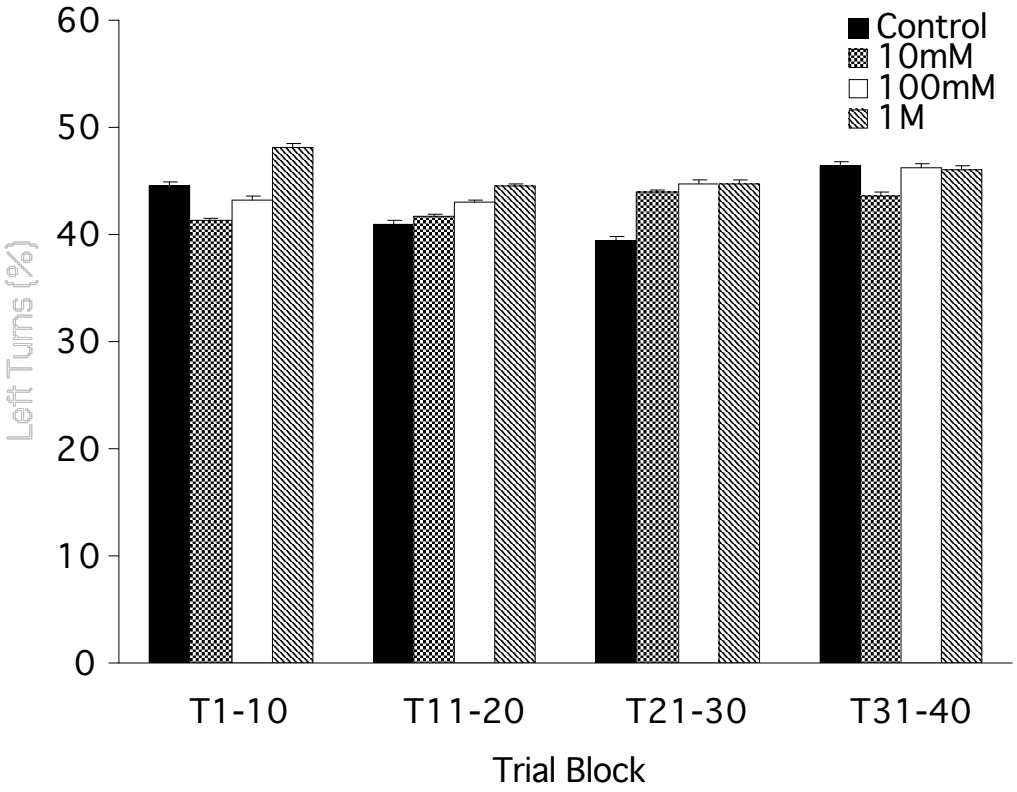

Figure 15. Dose group mean percentages of left turns committed at the choice area. Bars represent standard errors.

Note. Analyses indicate that, collectively, none of the dose groups exhibited a turn preference during the experiment. No significant differences between any dose group pairings within any of the trial blocks were found. Standard error values were minimal.

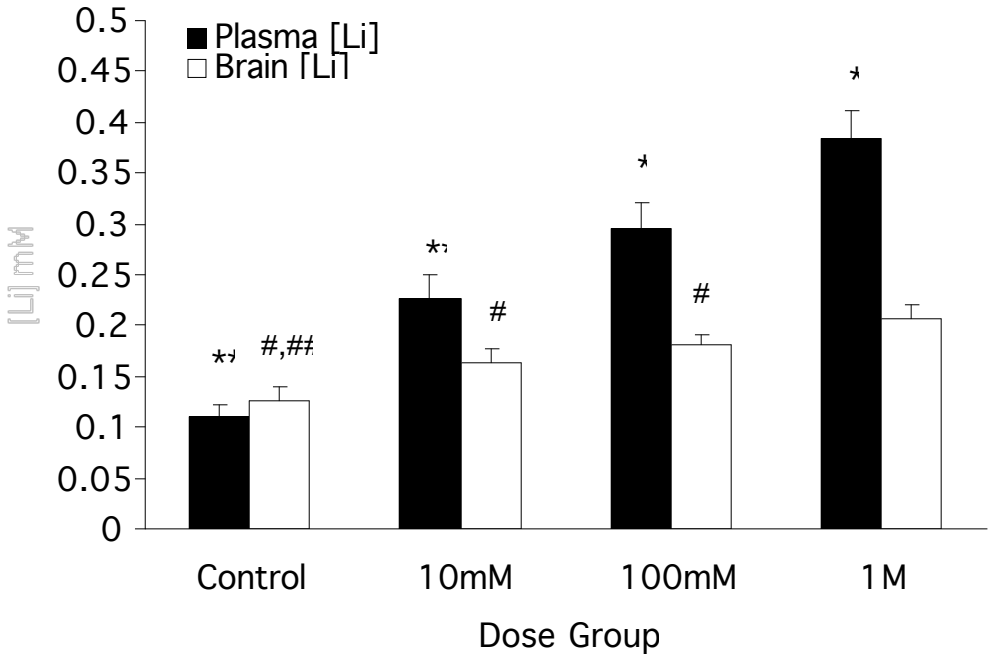

Figure 16. Dose group mean plasma and brain lithium concentrations representing a chronic (25 day) lithium regimen. Bars represent standard errors.

Note. Plasma and brain samples were analyzed for lithium levels via a capillary ion analysis (CIA) technique using the Waters Quanta 4000 Capillary Electrophoresis System (Milford, MA). CIA was performed using a 75 μl internal diameter x 60 cm length fused silica capillary and a run electrolyte of 67.7 mg hydroxybutyric acid, 53.8 mg 18-crown-6-ether and 64 μl UV-CAT reagent (4-methylbenzylamine) in a volume of 100 ml ddH_2O (18 MΩ) with a voltage of 20 kV using indirect UV absorption detection at 214 nm.

*$p < .05$: 100 mM vs. 1 M groups.
**$p < .001$: Controls vs. 10 mM, 100 mM and 1 M groups; 10 mM vs. 1 M groups.
#$p < .05$: Control vs. 10 mM groups; 100 mM vs. 1 M groups.
##$p < .005$: Control vs. 100 mM and 1 M groups; 10 mM vs. 1 M groups.

Subjects used in this investigation had a relatively wide range of body weights (1.00 – 3.30 g; mean = 1.747 g). Results indicate that a uniform dose quantity (20 μl) was appropriate for all subjects. A two-way ANOVA comparing plasma lithium levels among three different body weight groups (1.00-1.50 g, 1.51-2.00 g, and > 2.00) according to dose group indicated no significant main effect for body weight group [$F(2,137) = 0.343$; $p = .7102$] or an interactive effect between dose group and body weight group [$F(6,118) = 0.931$; $p = .4756$]. For brain lithium level comparisons, the two-factor ANOVA revealed a significant main effect for body weight group [$F(2,137) = 3.160$; $p = .0470$] and an interactive effect [$F(6,94) = 2.407$; $p = .0330$]. However, according to Fisher's PLSD tests there were no significant differences between the body weight group pairings within respective dose groups ($p > .05$).

Two-factor ANOVAs comparing lithium levels between genders (male and female) of each dose group reveal no significant main gender effects for plasma [$F(1,138) = 0.154$; $p = .6950$] or brain [$F(1,138) = 0.004$; $p = .9522$] or the interactive effects between dose group and gender for plasma [$F(3,122) = 0.241$; $p = .8677$] or brain [$F(3,122) = 1.057$; $p = .3711$.

Discussion

Performance trends measured in this investigation suggest high dosages of lithium may impair long-term memory. Results from a previous study (see Chapter 4) suggest a more robust linear dose-response effect of lithium dosage on short-term memory as assessed by a forced-choice spontaneous alternation.

Possibly the most remarkable results of the experiment was the slower acquisition of the task and the fewer correct first choices to reward by the high dose (1 M) group. Although there was not a significant main dose group effect for the trials-to-criterion parameter, there was a significant or near significant difference in the number of trials the high dose group took to reach criterion compared with each of the other dose groups. A significant main effect for dose group was found with the correct first choices parameter during 11-20, and, again, the high dose group was responsible for this effect. These results are more impressive when considering they were repeatable. For instance, there were no significant replication differences among the groups for either of these two parameters.

Perusing the prepared figures of the experiment it appears that the high dose group performed at a slower rate committing more errors and omissions in nearly every trial block.

This is particularly evident within T11-20 when major improvements in performance are indicated. As in the SA experiment there were generally no significant differences in latencies to reward among the dose groups for the place-learning experiment. A main effect for dose group during T11-20 was found yet the result bears minimal weight when considering there were no individual between-group differences. Latency results were encouraging, as with the SA experiment, because they indicate that the dose ranges chosen for dose-response studies were appropriate for these subjects. That is, the dose range was outside the toxic range. As well, once lithium administrations began, mortality rates were very low. In fact, the animals appeared to thrive under the conditions provided. In light of the rather low correct first choice values of the experiment, we conducted the left turn percentage analysis to determine whether the fish were simply using a turn bias to perform the task. Results clearly indicate there were no such turn preferences. Because we set the experiment up in such a way that subjects would need to make about the same amount of left and right turns, we feel confident that the subjects were using spatial cues to perform the task.

As with the SA task, results of the lithium level analyses indicate linear relationships between both plasma and brain lithium levels and doses administered. As well, both mean plasma and brain lithium levels were consistent for each group between the two experiments. Again, CIA results indicate that the black molly contains endogenous low-level concentrations of lithium independent of their environment. CIA analyses of tank waters before and after each replication of the experiment indicated no detectable levels of lithium.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Results of the present investigations suggest that lithium impairs STM and LTM in the black molly fish in a manner analogous to that of higher vertebrates. The effects of lithium on LTM are subtler than those on STM in the black molly. Although the dose-response effects were not linear, there were clear differences in performances among the four dose groups. This was particularly evident in the forced-choice SA task where different degrees of impairment were seen among the dose groups. Controls alternated significantly above chance. The low dose (10 mM) group operated at chance level, and the mid dose (100 mM) and high dose (1 M) groups perseverated. Only the high dose group was significantly impaired in the place-learning task suggesting that lithium marginally affects LTM. Latency parameter results for both STM and LTM indicated no significant differences among the dose groups. Latency values were also consistent between the two investigations. As well, control group latencies were consistent among all of the SA experiments here reported. These are important findings given the narrow therapeutic index of lithium in humans. Fishes may be equipped with different ionic pump systems than higher vertebrates given that they are able to adapt to a wide range of ionic concentrations in their environment (Maetz, 1976). This may be particularly relevant to euryhaline fishes such as the black molly that can live in waters with a wide range of salinity. The fact that plasma levels for the high-dose group were just below the lower end of the human therapeutic plasma level (0.5 mM) supports this notion. It appears, then, that the range of lithium dosages employed in these investigations was appropriate. The dosages did not compromise motivations to perform the tasks nor did it impair locomotion. As well, mortality rates during experimentation were very low (i.e., 4.1 % during the place learning experiment) and were evenly spread among the dose groups.

Plasma and brain lithium level values, as assessed by CIA, were consistent between SA and place learning experiments. Because a barium internal standard of known concentration was used to calculate lithium concentrations in plasma and brain samples, standard samples were run at the beginning of each batch of plasma and brain samples in order to indicate relative migration times for barium and lithium peaks. The relative migration times verified the identities of the barium and lithium peaks in the electropherogram characterizing each sample. Using known

barium and lithium concentrations in the standards allowed for the generation of an F value that was used to calculate the unknown lithium concentrations in the plasma and brain samples for both SA and place learning experiments. Quality control (QC) samples, with known amounts of lithium (5 mM and 10 mM), were run at the end of each batch of samples to establish experimenter precision with sample processing for each batch of samples. Coefficients of variation (CVs) were utilized to measure precision in sample processing throughout both SA and place learning experiments. The CV value for the standards was 5.06 %. The CV value for the 5 mM QC was 10.35 %, and the CV value for the 10 mM QC was 10.90%. These values indicate acceptable levels of precision. In all, these measures provide confidence in the accuracies of the unknown plasma and brain values reported.

In each of the behavioral experiments we used distilled water that was treated with a water conditioner and a fungal prophylactic, neither of which contained lithium in any form. We abandoned the use of tap water long ago because we found, through CIA testing, that it did contain about 0.1 mM lithium. Notwithstanding, we have implemented as standard practice the measuring of tank waters for lithium before and at the end of each replication. No lithium peaks were identified in any of the home tank water samples.

Fishes are the phylogenetically oldest vertebrate group including more species than all other vertebrates combined (Arevalo et al., 1992). With thousands of different species of fishes still in existence, with variable brain morphologies, the general organization of ascending and descending serotonergic (5-HTergic) systems of teleost brain is similar to that of mammals suggesting an early evolutionary significance for this neurotransmitter system (Kah & Chambolle, 1983). Presence of 5-HT neurons in the midline raphe region is one of the most constant features of the monoamine systems in vertebrates (Parent, Dube, Braford, & Northcutt, 1978; Parent, 1983). Several studies indicate that numerous large-sized 5-HT immunoreactive perikarya are present within the teleost raphe region between the medial longitudinal fasciculi (MLF) in a column extending in a mediosaggital plane from the caudal midbrain to the anterior medulla oblongata (Parent et al., 1978; Parent, 1981, 1983). The studies also indicate that these perikarya are confined to the raphe region and are not found in teleost telencephalon, which is in contrast to their presence in mammalian frontal cortex and limbic system. However, numerous 5-HT-immunoreactive varicosities are found in teleost telencephalon (Parent, 1981). In concert with the results of these investigators, we have immunolocalized a 5-HT_{1A}-like receptor in the

black molly raphe system. Fishes have no limbic system or frontal cortex, yet they do have a telencephalon where 5-HT_{1A} receptors might be expected to reside. Our immunohistochemical (IHC) work shows that this is not the case for black molly fish. However, Khan et al. (1996) has revealed the presence of high-affinity [^{3}H] 5-HT binding sites in telencephalon and diencephalon of rainbow trout. Spiperone and 8-OH-DPAT (8-hydroxy-2-di-n-propylamino) tetralin), both 5-HT_{1A} agonists, inhibit this binding suggesting the presence of 5-HT1A receptors in these brain regions. In addition to structural similarities of 5-HT systems between mammals and teleosts, fishes may be of particular value in behavioral pharmacology studies because their blood brain barrier is less developed compared with higher vertebrates, thus less protected, and consequently more penetrable by exogenous chemicals (Levine, Chengappa, & Reddy, 1999; Ferriere, Khan, Meyniel, & Deschaux, 1997). Yet our studies with the black molly demonstrate that these fish thrive when subjected to chronic administrations of a wide range of lithium levels. Our prime interest in the use of a fish animal model, however, stems from work in our lab that suggests the lack of a postsynaptic 5-HT_{1A} receptor system in the black molly.

What is unclear is the selective role of the 5-HT_{1A} receptor in either presynaptic 5-HTergic neurotransmission or postsynaptic function in cognition as well as depression. Presynaptic 5-HT_{1A} receptors occupy somatodendritic positions where they function as autoreceptors that inhibit firing of serotonergic neurons in dorsal and median raphe nuclei of mammalian caudal midbrain (Buhot, Martin, & Segu, 2000). Presynaptic terminal 5-HT_{1B} autoreceptors located in the different projecting areas such as the septum and hippocampus or the frontal and entorhinal cortices inhibit 5-HT release when stimulated (Buhot Martin, & Segu, 2000). 5-HT_{1A} postsynaptic heteroreceptors are located on pyramidal neurons within these projection areas that they inhibit when stimulated (Buhot Martin, & Segu, 2000). Although the radioligand profiles of presynaptic and postsynaptic 5-HT_{1A} receptors are similar, there is good evidence that these receptors are functionally distinct (de Montigny, Chaput, & Blier, 1993)). All known $5HT_1$ receptor subtypes are negatively coupled to adenylyl cyclase (AC) via a pertussis toxin-sensitive G_i protein and are thus inhibitory in nature (Saudou & Hen, 1994). However, Clarke, Yocca, and Maayani (1996) have demonstrated that, in the rat hippocampus, 5-HT_{1A} receptors are coupled to AC and K^+ channels yet, in the raphe, the receptors are not coupled to AC. These couplings may be independent of one another thereby functionally differentiating presynaptic from postsynaptic 5-HT_{1A} receptors. Yet, Evans, Cropper, Berg, and Clarke (2001)

have recently suggested that presynaptic 5-HT_{1A} receptors may not lack AC coupling. Instead, the coupling may be sensitive to the activation state of AC, which is influenced by consequences of activation of the phospholipase A and C effector pathways. For example, lithium may produce inactivation of G_i through a crosstalk mechanism with the phospholipase C pathway (Manji, Potter, & Lenox, 1995). Lithium, via its inhibition of inositol 1-phosphatase, produces an elevation of diacylglycerol levels which activates protein kinase C, which in turn stabilizes the inactive phosphorylated state of G_i. Lithium may also interfere with G_i dephosphorylation through its direct inhibition of protein phosphatases 1 and 2A. These findings, downstream of the receptor, are beyond the scope of this dissertation, yet they illustrate potential sources of functional differences between presynaptic and postsynaptic 5-HT_{1A} receptors and will be the subject of future studies in our laboratory.

The ultimate purpose of this line of work is to identify a cellular entity that is responsible for a mechanism by which lithium-induced cognitive impairment is manifested in the fish, an animal model that appears not to have a postsynaptic 5-HT_{1A} receptor system like that of mammals. Although it would be presumptuous to consider lithium to operate solely on such a limited mechanistic scope, there is ample evidence supporting the claim that lithium downregulates 5-HT_{1A} receptors (Odagaki, Koyama, Matsubara, & Yamashita, 1990; Price, Charney, Delgado, & Heninger, 1990; Goodwin, 1989; Friedman & Wang, 1988; Hotta & Yamawaki, 1988; Goodwin, DeSouza, Wood, & Green, 1986; Hotta, Yamawaki, & Segawa, 1986). As well, a large body of evidence supports the existence of a link between cognition and the 5-HT_{1A} receptor (Bertrand, Lehmann, Lazarus, Jeltsch, & Cassel, 2000; Buhot, 1997; Meneses & Hong, 1997; Warburton, Harrison, Robbins & Everitt, 1997; Sirvio, Riekkinen, Jakala, & Riekkinen, 1994; Ohno, Yamamoto, & Watanabe, 1993; Winter & Petti, 1987). Acute activation of the 5-HT_{1A} receptor is achieved by an increased availability of 5-HT. The increased availability can be induced via an SSRI (selective serotonin receptor inhibitor) reuptake mechanism or by MAO (monoamine oxidase) inhibition, or with an exogenous agonist, such as lithium or 8-OH-DPAT. These actions suppress 5-HT neuronal firing activity (Blier, Pineyro, El Mansari, Bergeron, & de Montigny, 1998). As the treatment is prolonged, the firing of 5-HT neurons gradually recovers as a result of the desensitization of their 5-HT_{1A} autoreceptors. Desensitization followed by downregulation of these receptors disinhibits 5-HT firing, which ultimately enhances 5-HT transmission. Electrophysiological results of Haddjeri, Blier, and de

Montigny (1998) suggest that long-term treatments with 5-HT_{1A} agonists desensitize presynaptic, not postsynaptic, 5-HT_{1A} receptors. Using in vivo microdialysis, Kreiss and Lucki (1994) have shown that 5-HT release in terminal fields of frontal cortex and hippocampus is regulated by somatodendritic 5-HT_{1A} receptors located respectively in the dorsal and median raphe nuclei. In a multi-experiment study, Santucci, Knott, and Haroutunian (1996) have demonstrated that rats given *p*-chloroamphetamine, which promotes 5-HT release, increase working and reference memory errors in a well-learned radial arm maze task. It is reasonable, then, that 5-HT_{1A} receptor downregulation could account for performance declines in cognitive-related tasks. Inherent with the immunohistochemical and behavioral investigations conducted, we propose that lithium, as a 5-HT_{1A} agonist, may indeed impair spatial learning in these fish through disruption of a "neurochemical brake" (see Benkelfat, 1993) on behavior provided by normal serotonergic neurotransmission. Fishes, and possibly other lower vertebrates, provide a unique opportunity to manipulate the presynaptic 5-HT_{1A} receptor system in vivo without possible postsynaptic 5-HT_{1A} receptor compensatory mechanisms entering into the equation of the study.

We have spent a considerable amount of time developing a specific IHC protocol that would best display these receptors in the black molly brain. Because we used a rodent antibody to the receptor in another animal, namely, the black molly, we set ourselves up for background staining problems. However, using available antibody and various modifications of a free-floating frozen-section IHC protocol, we were able to eliminate a significant amount of background without compromising clarity or quantity of antibody staining. Figure 17 depicts two adjacent sections at a caudal midbrain level from an untreated molly. The upper panel includes low power photographs (10X) of sections stained with and without the primary antibody, from left to right, respectively. The lower panel displays the same section sequence at a higher power (40X).

Our investigations of the 5-HT_{1A} receptor system in the black molly are at an early stage of development. Analyses of receptor density differences among the dose groups has not been completed, so conclusions regarding lithium effects on 5-HT_{1A} downregulation in the black molly brain are inappropriate at this time. As well, we will need to carry out comparative behavioral and IHC tests between lithium and 5-HT_{1A} agonist (i.e., 8-OH-DPAT) and antagonist (i.e., WAY 100135) to support our claims. This will require a number of preliminary dose-response experiments employing a wide range of agonist and antagonist concentrations to

establish appropriate concentrations to be used in the comparative analyses. To confirm that the positively stained cells in our IHC experiments are endowed specifically with 5-HT_{1A} receptors, we would conduct autoradiographic studies using appropriately determined concentrations of, i.e., tritiated versions of 8-OH-DPAT and WAY100135 as outlined by Waeber and Palacios (1993). We have put forth a concerted effort in detailing these studies, which we have lumped together for these purposes as “future directions”.

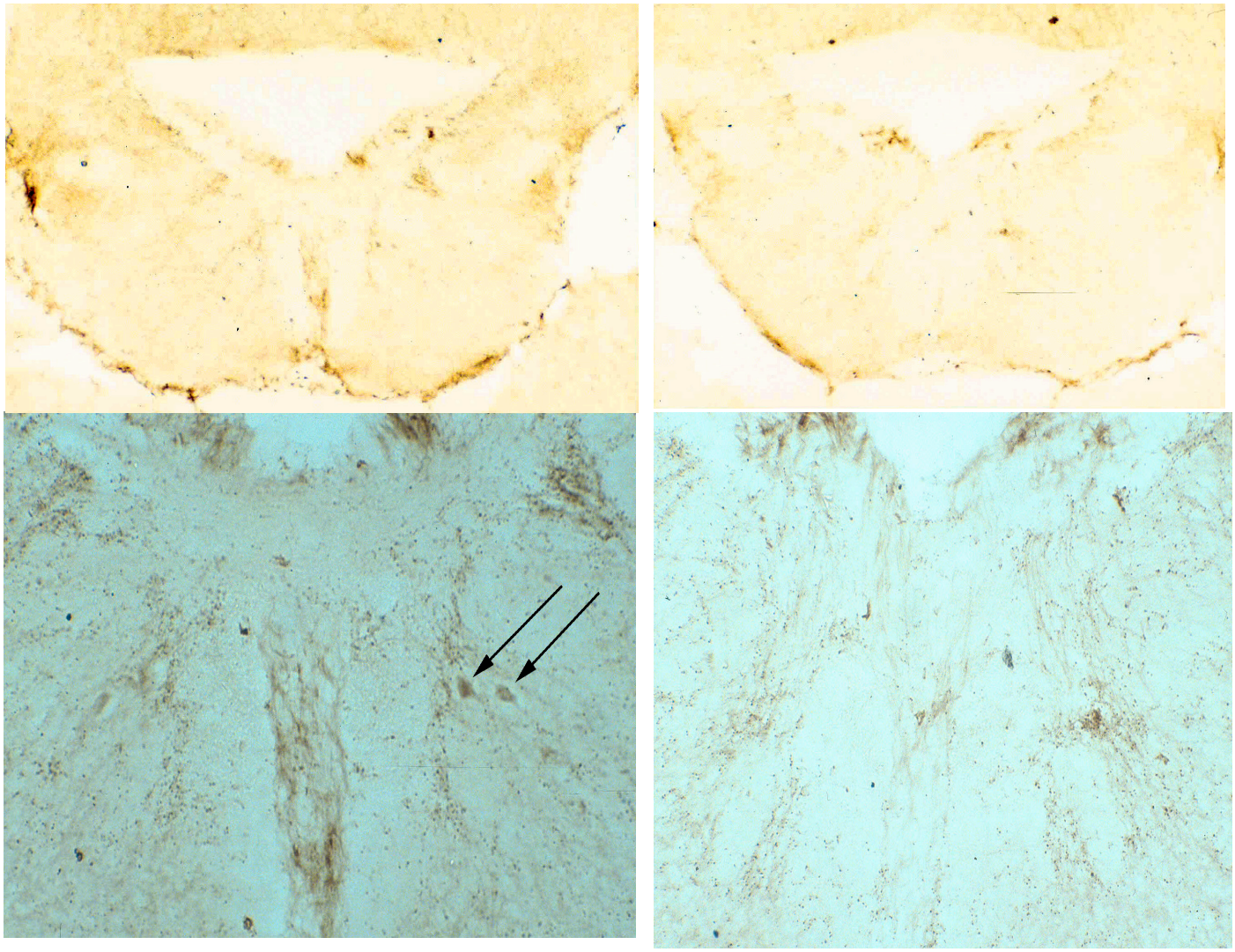

Figure 17. 5-HT_{1A} receptor immunohistochemistry of black molly caudal midbrain.

Note. Arrows denote two presynaptic cell bodies positively stained for the 5-HT_{1A} receptor.

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APPENDIX

APPENDIX A

Protocol For A 5-HT_{1A}-Like Receptor in the Caudal Midbrain of the Black Molly Fish

Our immunohistochemical (IHC) protocol for a 5-HT_{1A}-like receptor in the black molly brain is fairly standard for frozen free-floating sections. A few additions or modifications were included to deal with the problem of background staining. All brains sectioned to this date are from untreated fish. All rinse cycles involved three 15 min rinses in PBS (pH = 7.1) @ room temperature (RT) on a shaker. The third rinse of each cycle contained 0.5% BSA and 0.4% Triton X-100 in PBS. The molly brain is roughly 2.5 mm from anterior tip of telencephalon to anterior spinal cord yielding about 50 sections (50 μ each) per brain. Our protocol follows:

- Subject placed in 0.1% solution of 3-aminobenzoic acid ethyl ester (methanesulfonate salt) for 2 min; body weighed; cardiac puncture for plasma prep followed by extraction of whole brain into 4% paraformaldehyde for 5.5 hrs. Brains frozen at –80 ^{o}C until sectioned.
- Brains warmed to –20 ^{o}C in cryostat. Transverse sections cut 50 μ thick and placed in PBS in individual wells of culture dishes (Costar).
- Initial Blocking Step: 10% gelatin from cold water fish skin (Sigma-Aldrich) + 0.5% BSA + 0.4% Triton X-100 in PBS for 1 hr at room temperature (RT) on slow shaker followed by rinse cycle.
- Peroxidase Quenching Step: 1% H_2O_2 in 70% MetOH in PBS for 30 min followed by rinse cycle.
- Blocking Step: 10% normal goat serum + 0.5% BSA + 0.4% Triton X-100 in PBS for 30 min followed by rinse cycle.
- Primary Ab Incubation: Rabbit polyclonal to amino acids 294-312 of the rat 5-HT_{1A} receptor (Diasorin) 1 : 500 in PBS + 0.5% BSA + 0.4% Triton X-100 for 30 min @ RT followed by overnight incubation @ 4 ^{o}C.
- Secondary Ab Incubation: 1% biotinylated secondary Ab (Vector Elite Kit) + 10 % fish serum neat (Seablock ^{TM} – East Coast Biologics, Inc.) in PBS + 0.5% BSA + 0.4% Triton X-100 for 30 min @ RT on shaker followed by rinse cycle.

- Tertiary Reagent Incubation: Vector Elite Avidin-Biotin Complex reagents (1%) in PBS + 0.5% BSA + 0.4% Triton X-100 + 2% NaCl for 30 min on shaker followed by rinse cycle.
- Enzyme Substrate Step: 2% 3,3’ diaminobenzidine (DAB) in ddH_2O for 1 min followed by ddH_2O rinse.
- Sections placed on chrom alum slides, air dried, dehydrated through graded alcohol series into xylene and mounted for viewing and photography.

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