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Dissertation

THE EFFECTS OF TRANSCRIPTIONAL INHIBITION ON SPATIAL LEARNING AND DENSITY OF RECEPTORS IN RAT HIPPOCAMPUS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Ft. Collins CO

Spring 2000

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January 7, 2000

Colorado State University

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JEFFERSON WILLIAM KINNEY ENTITLED **THE EFFECTS OF TRANSCRIPTIONAL INHIBITION ON SPATIAL LEARNING AND DENSITY OF RECEPTORS IN RAT HIPPOCAMPUS** BE ACCEPTED AS FULLFILLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

The Effects of Transcriptional Inhibition on Spatial Learning and Density of Receptors in Rat Hippocampus

The investigation of learning and memory has focused considerable attention on LTP as a possible cellular mechanism responsible for consolidation. Several studies have provided evidence that inhibition of the activity of proteins involved in LTP has a detrimental affect on consolidation. In addition to the behavioral studies, considerable evidence has been gathered to determine the cellular mechanisms responsible for LTP. Of specific interest to the current study was the effect produced following the administration of a transcriptional inhibitor, which resulted in the ablation of the late phase of LTP. In the current study, Sprague-Dawley rats were given an infusion (50μM and 25μM) of a transcriptional inhibitor (Actinomycin D) directly into the CA1 field of the hippocampus. Following the infusion, rats were tested in a Morris water maze to determine if transcriptional inhibition caused any decrements in the acquisition of the spatial task.

The results indicated that the drug did disrupt subjects' performance compared to saline controls. The data gathered suggested that the difference between treatment groups was in the precision of localizing the platform in the target quadrant, not in the subjects' ability to locate the platform quadrant. No differences in swim speed or ability to locate a cued platform were observed.

It has been suggested that one of the cellular alterations responsible for LTP is an upregulation in the receptors responsible for the responsive properties of the postsynaptic neuron. The current study also examined differences in the densities of AMPA and NMDA receptor binding in CA1 following inhibition of transcription. In addition, the relative densities of the mRNA precursors for those receptors were examined. The results from the current study indicated that the administration of the transcriptional inhibitor to the CA1 field of the hippocampus altered the density of binding to both AMPA and NMDA receptors. The administration of the drug did not reliably affect the expression of mRNA for several subunits of glutamate receptors expressed in the CA1 field of the hippocampus.

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The Effects of Transcriptional Inhibition on Spatial Learning and Density of Receptors in Rat Hippocampus

I. Introduction:

The process by which organisms interact with their environment, specifically what allows animals to maintain the relevant information about the environment is of crucial importance for survival. For decades, researchers have painstakingly attempted to understand the behavioral, as well as physiological process by which animals are able to acquire information about their environment, as well as maintain that information. The basic research problems that have occupied so many scientists include investigations of the conditions that facilitate stimuli being encoded, and the regions of the brain responsible for storage and maintenance of that information. Of specific relevance to the current project were the mechanisms that are necessary and/or sufficient for the environmental stimuli to be consolidated into a relatively permanent memory.

The research project below investigated what role the administration of a transcriptional inhibitor to the CA1 field of the hippocampus had on rats' abilities to acquire a spatial task. A secondary goal of the research project was to investigate the effects of the transcriptional inhibitor on the density of binding to and production of two specific subtypes of glutamate receptors in the hippocampus. The two receptor subtypes that were investigated have been implicated in previous research to be involved in

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learning and memory, as well as long term potentiation (an alteration in synaptic plasticity). The results of the following study were intended to test the premise that learning involves an upregulation of these specific receptors. In addition, these experiments addressed the strength of the linkage of the neurophysiological mechanisms with the learning they are believed to underlie.

II Background Research:

The investigation of learning and memory in neuroscience has concentrated on the electrochemical functioning of neurons in several different regions of the brain, as well as on the special morphology of classes of neurons. In mammalian systems, experiments concerning the underlying neural substrates of learning and memory processes have focused primarily on the cerebellum and the hippocampus. Much of this research was initiated from case studies of patients who suffered injuries to one or both of these structures and exhibited selective deficits of learning and memory.

One case in particular served as a starting point for the investigation of the consolidation of memory (Bane, 1995). A patient identified as H.M. had severe lesions to his hippocampus as well as other temporal lobe structures. Following the lesions, H.M. suffered severe anterograde amnesia, and displayed specific problems in learning new spatial memories. The anomalies in his brain implicated the hippocampus as a possible site involved in the consolidation of memory, specifically spatial memory. The process of consolidation in the nervous system simply refers to the generation of a new long term memory. Thus, consolidation is the physiological process by which sensory information is altered into a relatively permanent association in the brain. Researchers

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have argued that the pairing of two or more pieces of sensory information in the nervous system is the means by which consolidation occurs.

Ablation of the hippocampus was the first technique used to study the role of the hippocampus in consolidation of memory. Electrolytic lesions were produced in animals' hippocampi; the animals were then tested in a variety of spatial behavioral tasks. One of the most frequently employed tasks was the radial arm maze. The maze consists of a circular platform from which several arms extend outward. Typically researchers would place a food reward at the end of some of the extending arms. The animal must remember which arms are baited based on their location in reference to distal spatial cues. In this task an animal explores the maze and retrieves food. The task allows for an assessment of an animals' working memory (i.e. remembering which arms an animal had already visited on that day) and reference memory (i.e. remembering which arms were baited every day). Accurate performance could occur only by an animal utilizing cues placed outside the maze, or, put more simply, navigating through the maze based on the position of cues in space. The results of such studies were uniform: animals whose hippocampi had been ablated prior to exposure to the task showed severe deficits in both reference memory and working memory. The inability of the ablated animals to learn the task has been argued to be a result of an inability to consolidate the appropriate stimuli, caused by the lack of hippocampal activity.

Research concerned with the hippocampus and its potential relationship to memory has also involved the recording of neuronal activity following high frequency stimulation of different regions of the hippocampus (see figure 1). Bliss and Lomo (1973) delivered electrical stimuli to neurons in the entorhinal cortex of rabbit's hippocampi and recorded

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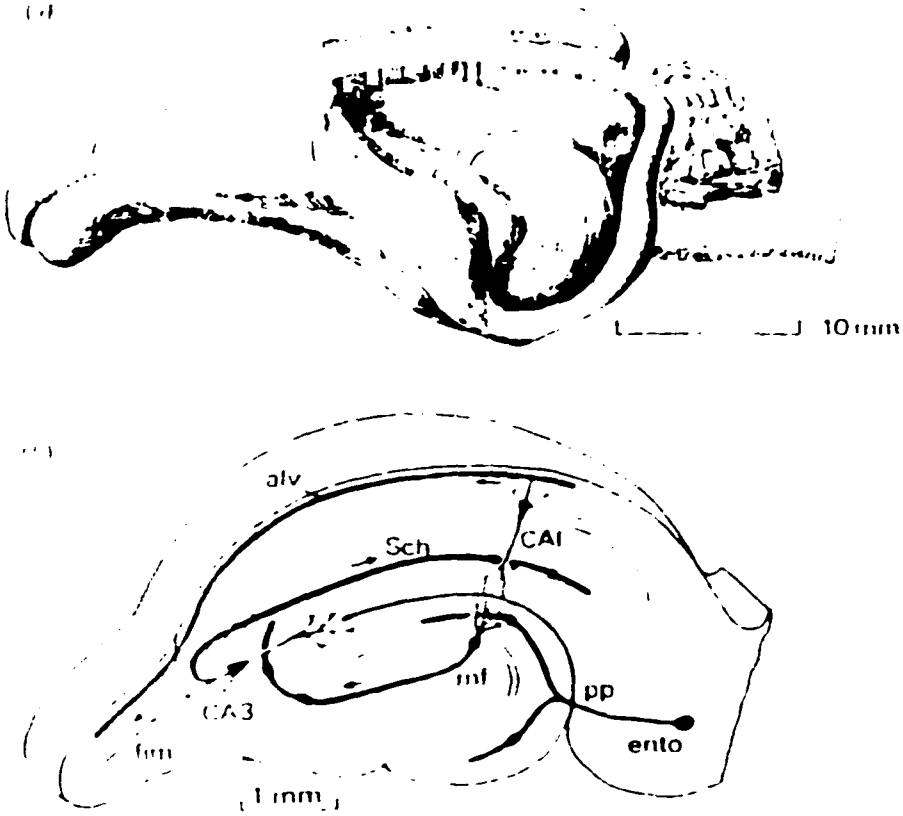

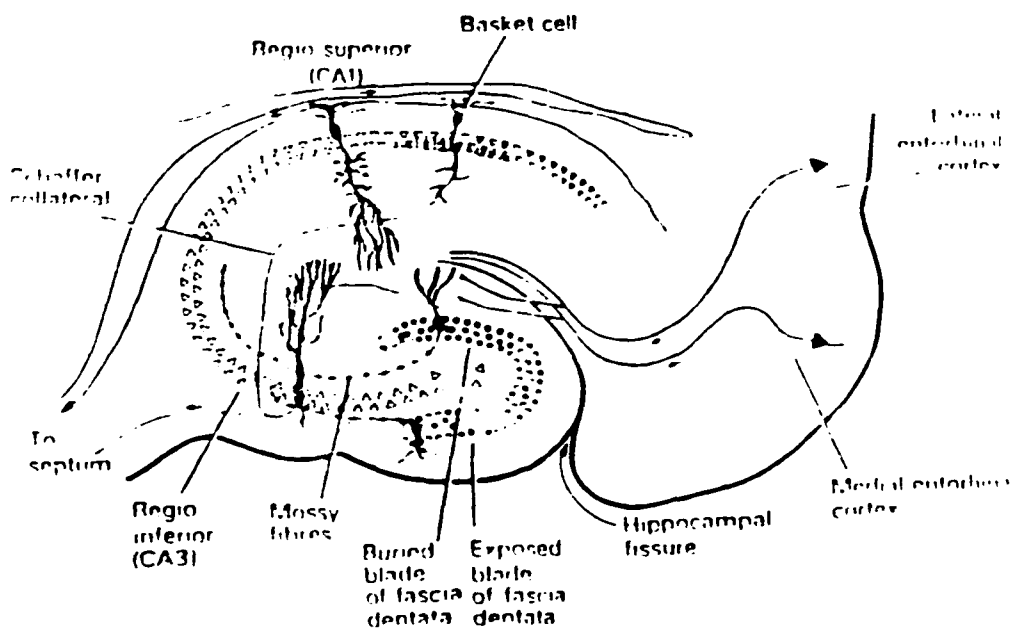

Figure 1 Location and organization of the hippocampus

(a) Lateral view of the rabbit brain with hippocampus exposed (b) Lamellar organization of the hippocampus. Alv= alveus (fiber bundle consisting of pyramidal cells); ento= enthorinal cortex; pp= perforant path; Sch= Schaffer collateral. (c) Schematic diagram of horizontal section of mouse hippocampus showing intrahippocampal connections. (Adapted from The Neuroscience of Animal Intelligence. Euan Macphail, 1993)

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the responses in the postsynaptic cells in the dentate gyrus (see figure 1). They discovered an unexpected change in the responses of the cells in the dentate gyrus following high frequency "tetanic" stimulation of the test pathway. The recorded responses of the postsynaptic cells to the same stimulus increased in strength following the tetanizing current. Bliss and Lomo termed this change in synaptic efficiency long-term potentiation (LTP), an increase in synaptic efficiency persisting long after the delivery of the tetanic stimulation. The researchers proposed that the change in synaptic strength might be an example of the Hebbian principle articulated twenty-three years earlier. According to Donald Hebb's (1949) postulate: " When some cell A excites some cell B and persistently or repeatedly takes part in firing it, some growth process or metabolic change occurs in one or both cells so that A's efficiency as one of the cells firing B is increased." This Hebbian rule served as an explanation of the synaptic change demonstrated by Bliss and Lomo. It also suggested that synaptic plasticity was possible well after developmental plasticity of the nervous system has declined. The idea of a synapse being plastic simply implies that it is alterable, or that the response obtained in a given synapse can be changed. The LTP that Bliss and Lomo observed demonstrated synaptic plasticity in the rabbit hippocampus.

Based on the advances in the above two areas; the involvement of the hippocampus in the consolidation of memory, and the discovery of a change in synaptic efficiency (LTP), researchers began to argue that LTP might be the physiological process that is necessary for consolidation. Thus, the research designed to better understand LTP and how it relates to behavior began. The connection between LTP and consolidation of

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memory is still largely theoretical. However, research has provided considerable evidence that LTP and consolidation are related.

III Current research:

Since the Bliss and Lomo study, researchers have extensively investigated the nature of hippocampal cells and the connections between those cells. The discovery of the pyramidal cells located in the CA3 and CA1 regions of the hippocampus have been of particular interest (for a review see Kandel, Schwartz, and Jessel; 1991). The pathway that connects the CA3 and the CA1 regions is referred to as the Schaffer collaterals (see figure 1). This pathway has unique properties and structures that may be involved in consolidation, specifically of spatial memories. CA1 pyramidal cells receiving signals from the Schaffer collaterals respond differently when an animal is in a specific location. Thus, these cells have been labeled "place cells". In animals that have had previous experience in a spatial task, these cells respond maximally when animals are in a specific location, and respond less vigorously the further the animals are from the specified location (Wilson and McNaughton, 1993). In addition, if an animal is placed in a novel environment, these space specific cells show no preference for discrete regions of space. The activity of these place cells is analogous to receptive fields in several sensory modalities. Of particular interest to researchers has been the specificity of the firing pattern of the place cells; they respond maximally to very discrete regions or positions. When an animal is exposed to a spatial task, it has been hypothesized that experience tunes the responses of the place cells; this results in the maximal response of a cell in a very discrete spatial region. Therefore, some researchers have proposed that

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consolidation of spatial information may be accomplished via the tuning of space specific cells.

Another interesting feature of these pyramidal cells is that they have a high concentration of a unique glutamate receptor known as the N-methyl-D-asparatate (NMDA) receptor (see figure 2a). NMDA receptors are named for a specific ligand that produces a strong agonistic effect (see figure 2b). These receptors are unique because they are both ligand gated (meaning they require neurotransmitter binding to function), and voltage gated (meaning they require a change in the cell's resting membrane potential to function). The ligand gating is the same as for other glutamate receptors. When glutamate binds to the receptors it causes a conformational change in the portion of the receptor that makes up the channel, which opens channels through the cell membrane, allowing the flow of cations and the depolerization of the neuron. However, the NMDA receptor can also have a magnesium ion which occupies the membrane channel (voltage gated). This receptor can function only if glutamate is bound to it, and only if there is sufficient depolarization of the cell to eject the magnesium ion from the channel. Therefore, the NMDA receptor is in fact a coincidence detector of simultaneous neurotransmitter release from a presynaptic site and depolarization of the postsynaptic cell. A second characteristic of the NMDA receptor that differentiates it from other glutamate receptors is the fact that it is permeable to calcium, as well as sodium and potassium. Thus, activation of the NMDA receptor allows an influx of calcium into the cell. Because calcium is a catalyst for several intra-cellular and extra-cellular events, the result of NMDA activation has the potential to alter cellular properties.

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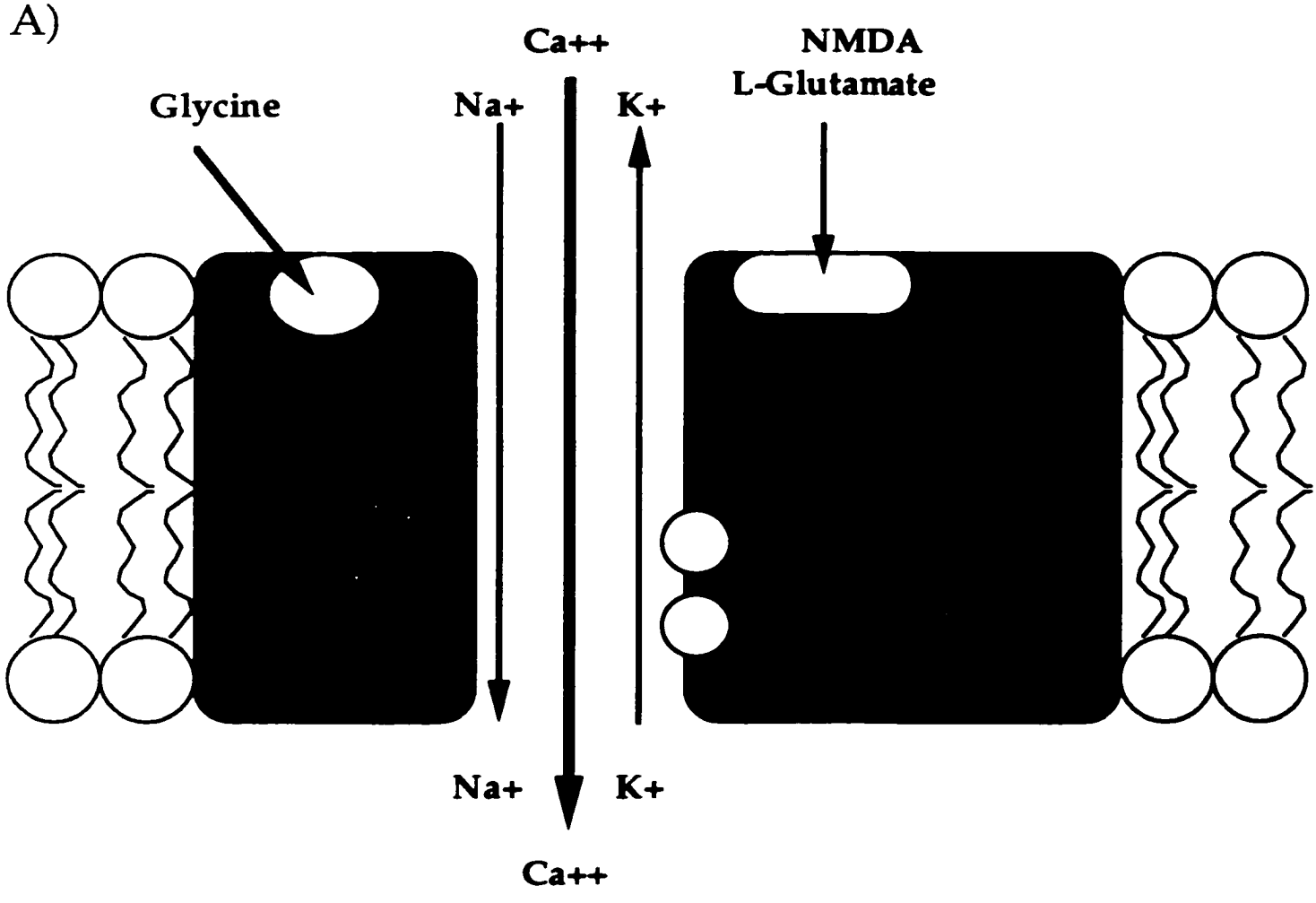
A)



B)

Ligand	Binding site	Agonist	Antagonist
NMDA	NMDA	XXX	
AP5	NMDA		XXX
MK-801	Channel protein		XXX
Glutamate	NMDA	XXX	
CPP	NMDA		XXX
Glycine	Glycine	XXX	
CGP	NMDA		XXX

Figure 2: A) Schematic representation of the NMDA receptor ion channel complex. Binding sites for glutamate, glycine, and magnesium are shown (From Wong and Kemp, 1991.) B) In addition, some pharmacological agents that are commonly used in studying the receptor are listed (From Bane, 1996).

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Several behavioral research techniques have been used to examine the involvement of the Schaffer collaterals and the NMDA receptor in the consolidation of memory. Traditionally, in these experiments an NMDA antagonist was administered, then the disruption of performance in a spatial task was assessed. For example, Morris, Anderson, Lynch, and Baudry (1986) used this technique to investigate the necessity of the NMDA receptor complex for acquisition of a spatial task. The study utilized a specific NMDA antagonist aminophosphodovalerate (AP5) that selectively inactivated the receptor. Morris et. al. (1986) tested the animals in a large tank filled with milky opaque water, which is now referred to as the Morris water maze.

Inside the tank, a platform (painted white) was placed just beneath the water surface to insure that the animals would not be able to see it. The task consisted of placing the animals into the water at one of four locations; they were then required to navigate based on extra-maze cues to find the platform (rats appear to prefer standing on the platform rather than swimming in room temperature water). In the Morris (1986) study, the administration of an NMDA antagonist produced significant decrements in acquisition of the task compared to saline controls. Several more recent studies (Morris, 1989; Cleveland, 1991) have replicated these findings. Thus the NMDA receptor apparently plays a necessary role in the consolidation of spatial memory.

Researchers have also investigated the involvement of the NMDA receptor in LTP in the hippocampus. Administration of NMDA antagonists to hippocampal slices (e.g. AP5) severely inhibits the production of LTP in the Schaffer collaterals compared to control slices (Murphy, Reid, Trentham, and Bliss, 1997). These findings have led

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researchers to propose that LTP and the consolidation of spatial memories in the CA3 to CA1 pathway are dependent on the NMDA receptor complex, and by extension, are calcium dependent. Although the possibility exists that the results may be due to some factor relating to NMDA receptors in other regions of the hippocampus, researchers have shown that other hippocampal regions do not express the NMDA receptor to the same degree. Furthermore, LTP in other regions of the hippocampus is not dependent on NMDA receptors. However, the LTP produced in other hippocampal regions, as well as other regions of the brain do seem to be calcium dependent (Xie, Barrionuevo, and Berger, 1996).

In addition to the data gathered revealing the importance of the NMDA receptor in the induction of CA1 LTP, a second group of glutamate receptors must be included in describing hippocampal LTP. The AMPA family of glutamate receptors are expressed extensively throughout the mammalian brain; however, specific subtypes of AMPA receptors exist in different regions of the brain in different concentrations (Martin, Blackstone, Levey, Huganir, and Price, 1993; Takumi, Matsubara, Rinvik, and Ottersen, 1999). For the purposes of the current project the most highly expressed AMPA receptors in the hippocampus, specifically in CA1 are the AMPA subtypes Glutamate receptor 1 (A); (GluRA), Glutamate receptor 2 (B); (GluRB), glutamate receptor 3 (C); (GluRC), and glutamate receptor 4 (D); (GluRD). The above mentioned glutamate receptors function as ionotropic glutamate receptors that serve as the principle excitatory input in hippocampal pyramidal cells. These receptors serve as the gating mechanism for a channel that when opened is permeable to sodium and potassium. The activity of open AMPA receptors results in depolarization of the cell, and when a sufficient number of

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these channels are open. NMDA receptors can function. These four subtypes of AMPA receptors have been critically implicated in not only the induction of LTP, but also the maintenance of LTP (Kullmann and Asztely, 1998). The requirement of postsynaptic depolarization associated with presynaptic glutamate release for activation of the NMDA receptor elucidates the critical role that the AMPA receptor family plays in the induction of LTP. Thus the postsynaptic depolarization that is critical for NMDA activation is achieved via the AMPA receptors. In addition, the maintenance of LTP critically involves the AMPA family of receptors. The calcium activated second messengers that are initiated by calcium entry act on NMDA and on AMPA receptors to facilitate the efficiency of the AMPA and NMDA function, see below (Tingley, Ehlers, Kameyama, Doherty, Ptak, Riley, and Huganir, 1997). In addition to the alterations that have been proposed in intact AMPA and NMDA receptors, researchers have discovered that following LTP induction changes or additions to length and filapodia (growth sprouts that can form new synapses) appear in hippocampal pyramidal cells (Hosokawa, Rusakov, Bliss, and Fine, 1995). The implication of new synapse growth due to activity would obviously include increases in AMPA receptors to allow functional new synapses. Clearly the contribution to synaptic plasticity that the AMPA family of receptors contributes is a requisite for LTP induction and maintenance.

The investigation of LTP in the Schaffer collaterals has not been isolated to the receptors that are thought to be responsible. Researchers have also attempted to determine the cellular mechanisms that underlie the increase in synaptic plasticity. While it seems likely that the activation of the NMDA receptor is necessary for the induction of LTP in the CA1 field, as well as for the consolidation of new memories in a variety of

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behavioral tasks, the process by which these events occur is still a mystery. Researchers have investigated several avenues by which the influx of calcium could affect a cell sufficiently to alter its responsive properties. There is evidence that the increase in the efficiency of the synapse is due to a retrograde messenger released by the postsynaptic cell, which facilitates an increase in the neuro-transmitter released from the presynaptic cell (see Schuman & Madison; 1994). Other researchers have argued that the recruitment of several second messenger cascades in the post synaptic cell caused by an influx of calcium alters the properties of the post synaptic cell, potentiating the response of the cell (Chen and Tonegawa; 1997). For example, calcium calmodulin kinase II (CaMKII), a calcium sensitive second messenger has been implicated in phosphorylating AMPA and NMDA receptors. The phosphorylation of these receptors results in increased permeability and conductance of ions responsible for excitatory inputs in hippocampal cells. Several possible agents have been suggested as being involved in the production of CA1 LTP; each is dependent on the entry of calcium into the postsynaptic cell.

Until recently, the investigation of the NMDA receptor and the effects produced by the influx of calcium has, for the most part, relied on the administration of exogenous antagonists to inactivate receptors or inhibit possible second messengers in the postsynaptic and presynaptic cells. However, the substances administered may affect the cell in a different way, or affect some other process that has not yet been identified. Recent developments of several techniques in the molecular manipulations of cellular function allow for an assessment of cellular events at a genetic level.

One specific technique that has been used is gene "knockouts" for investigating the function of particular receptors and proteins in whole organisms. Specifically, the

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knockout procedure entails the selective replacement of a gene sequence that codes for a structure or protein, in effect removing that structure or protein from the cell (see Takahashi, Pinto, and Vitaterna; 1994). Researchers have utilized this procedure to investigate the role of a specific protein or structure in cellular function, as well as its role in behavior. In the investigation of synaptic plasticity and learning and memory, the selective knockout procedure has been used to assess the necessity of several protein kinases and receptors in the normal synaptic plasticity underlying learning and memory. Specifically, researchers have investigated the involvement of these proteins and receptors in LTP, as well as the learning the LTP is suggested to mediate.

While LTP obtained in CA1 pyramidal cells seems to be dependent on NMDA receptors, the means by which the activation of the receptor induces a synaptic change is still being investigated. Several researchers (Otani, Marshall, Tate, Goddard, and Abraham; 1989 and Takahashi, Pinto, and Vitaterna; 1994), have proposed that an influx of calcium through the NMDA receptor activates several biochemical cascades that could: alter the cell's sensitivity to glutamate, increase the number of receptors for glutamate, and/or activate a retrograde messenger that would facilitate the presynaptic release of glutamate (see figure 3). The net effect of any or all of the three possibilities would be an increase in the cell's response to stimulation by a presynaptic neuron, in short a Hebbian alteration in the synapse.

In order to investigate these cascades, recent workers (see Chen and Tonegawa, 1997) have used the selective knockout of several protein kinases or synthases to assess their involvement in an increase in synaptic plasticity. It has been proposed that the biochemical cascade that may underlie the change obtained in LTP occurs via several

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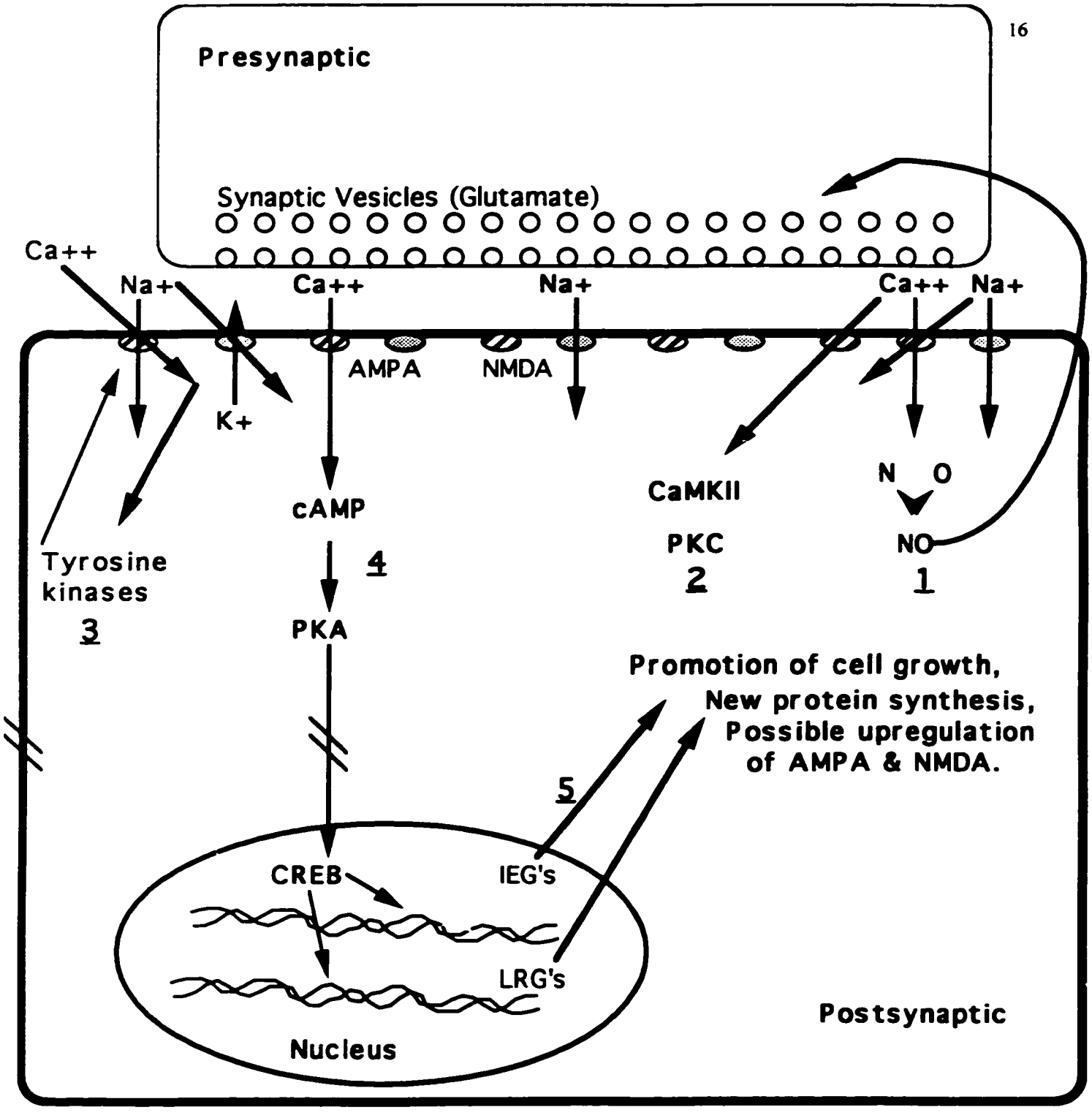

Figure 3 Overview of possible cellular mechanisms that underlie LTP in CA1. For all of the above events, calcium entry is directly or indirectly neccessary. (1) Synthesis of NO to facilitate presynaptic release. (2) Activation of PKC and CaMKII,both of which contribute to alteration in synapse. (3)Activation of tyrosine kinases (possibly via NO), which phosphorylate NMDA receptors. (4) Initiation and progression of PKA pathway, that may lead to the transcription of new proteins. (5) Upregulation of transcription.

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second messengers. The most likely second messengers in such a reaction would be calcium dependent, second messengers that exist in high concentrations in CA1 pyramidal cells. Calcium sensitive protein kinases have been discovered in the CA1 region, such as protein kinase C (PKC), calcium/calmodulin kinase II (CaMKII), and tyrosine kinases. The selective knockout procedure investigating these second messengers has illuminated their involvement in local and transitory effects in LTP. In addition, the data that has been gathered using the selective knockout animals has allowed the examination of behavioral and physiological mechanisms simultaneously. Typically the selective knockout of a specific second messenger reduces the extent of LTP induction without eliminating LTP, it also typically interferes with spatial learning without abolishing it.

All of the above possible second messengers could affect the synapse in such a way as to increase synaptic efficiency. The action of these proteins seems to be limited only to the specific region of the dendritic tree where NMDA activation is occurring. It also appears that the effect of these local modulatory mechanisms is limited to a specific time frame; eventually the second messengers will no longer facilitate the synapse. Long term alterations in a cell's response must not be due solely to such local effectors; there must also be some alteration in the proteins being produced by the nucleus. Studies investigating nuclear events involved in LTP have demonstrated that protein synthesis is necessary for sustained LTP (Silva, 1992). Therefore, a next step in attempting to understand the process that underlies the increase in synaptic plasticity may be an investigation of the effects produced following activation of calcium dependent non-local protein kinases. One question researchers have investigated is what effect a second

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messenger cascade progressing toward the nucleus produces in hippocampal neurons. It has been suggested that the cyclic AMP (cAMP)-dependent protein kinase A (PKA) may be involved in what has been termed the late phase of LTP (Chen and Tonegawa, 1997) (#4 in figure 3). Researchers have demonstrated two phases of LTP, short LTP (sLTP; approximately the initial 60 minutes following tetanization) and long LTP (lLTP; any LTP persisting more than 60 minutes). The sLTP is the immediate alteration in a cells excitability due to local effectors, for example the retrograde messenger and phosphorylation of channels (tyrosine kinases, CaMKII). The lLTP may be dependent on transcription of new genetic material, which results in the long lasting potentiation of a synapse. PKA is important in lLTP in that it is known that PKA mediates transcriptional events by phosphorylating transcription factors such as CREB (cAMP response element binding protein). It has been proposed that the phosphorylation of CREB induces the expression of several immediate early genes (IEG's) such as c-fos and c-jun (Robertson, 1991), which are thought to have a role in initiating or promoting cell growth. If the progression of activation of genetic systems is similar in mammals as those found in invertebrates (e.g. Aplysia), the IEG's may promote the transcription of late response genes that alter cellular properties indefinitely.

Several researchers have investigated the cellular events that are responsible for non-associative learning in Aplysia (see Alberini, Ghirardi, Metz, and Kandel, 1994). This has led to the discovery of several biochemical cascades that underlie learning in simple systems. One of the cellular events that has received considerable attention has been the cAMP dependent protein kinase A cascade. Generally the introduction of calcium into specific cells in invertebrates produces the activation of a cAMP second

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messenger. This second messenger, protein kinase A (PKA) alters protein synthesis in these cells. The PKA second messenger interacts with the cAMP responsive element binding protein (CREB), which leads to activation of what have been termed immediate early genes (IEG's). One proposed effect of the activation of IEG's is facilitation of the transcription of late response genes (LRG's). The late response genes are responsible for an alteration in the protein products that the cell then produces. Thus it is possible the initiation of the transcription of the late response genes is the method by which long lasting changes in the cell is carried out (see figure 3, #5).

To investigate the involvement of cAMP dependent PKA in mammalian Schaffer collateral LTP, Qi, Zhuo, Skalhegg, Brandon, Kandel, McKnight, and Idzerda (1996) introduced an inactivating mutation into the Cβ1 isoform subunit of PKA. PKA is composed of two subunits, a catalytic subunit (C) and a regulatory subunit (R). Qi et al. selectively inactivated one of the isoforms of the catalytic subunit (β1). The researchers found that CA1 LTP was initially identical following tetanic stimulation in the Cβ1 mutants and the wild type controls. However, the Cβ1 mutants were unable to maintain the potentiated response. The decrease in synaptic potentiation following induction of LTP in Cβ1 mutants supports the claim that the PKA cascade is involved in the late phase of LTP.

In another investigation of the activation of genetic material leading to the late phase of LTP, Bourtchuladze, Frenguelli, Blendy, Cioffi, Schultz, and Silva (1994) used gene targeting to disrupt the α- and δ- forms of CREB in mice. Bourtchuladze et. al. found that LTP was induced in both the wild type controls and the α- and δ- mutants. However, LTP decayed to baseline levels within 90 minutes in mice which were deficient

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in the two forms of CREB. The α- and δ- mutants ability to acquire a spatial task was also investigated. The results showed a decrement in performance for the mutant mice compared to controls; the CREB mutants were unable to acquire a spatial task to the same degree of accuracy as the wild type controls.

IV Research Project:

Of specific interest for the following experiment was the effect produced following the activation of CREB or other transcriptional factors, and their potential activation of the IEG's and LRG's. Involvement of the PKA pathway in the late phase of LTP has been argued to be a modification of the responsive properties of specific cells. In order to alter the responsive properties of such cells, some alteration or addition to the structures of the cells would be required. It was proposed that the involvement of new protein synthesis in the acquisition of a spatial task is necessary. Subjects were implanted with cannula into the apical layer of the CA1 region of the hippocampus and a transcriptional inhibitor (Actinomycin D) was administered through the cannula. The use of a non-specific transcriptional inhibitor in the present study was designed to block all new transcription, not just transcriptional activation via CREB. Recent work in synaptic plasticity has identified other intracellular transcriptional activators other than the PKA pathway (for example, the cyclic GMP pathway).

Previous research (Matthies, 1989) has investigated the involvement of new protein synthesis in simple light discrimination tasks. In these studies a transcriptional inhibitor (anisomycin) was administered and the subjects accuracy in avoiding a signaled shock was recorded. The subjects in these studies showed a decrement in avoiding the

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shock following the infusion of the inhibitor, compared to saline controls. The task employed in these studies however, was not sensitive to reference memory or working memory measures, as is a spatial task. In the present study the use of a spatial task allowed an evaluation of the effect of blocking transcription on reference and working memory. In addition, the use of a spatial task did not involve the administration of shocks to examine the subject's behavior; thus the present study did not involve pain transduction pathways.

In-vitro experiments using Actinomycin D have demonstrated that it blocks the late phase of LTP (Nguyen, Abel, and Kandel. 1994). The researchers investigating the effects of Actinomycin D on LTP did not however discuss what the cellular consequences were following the administration of the drug, or the behavioral effects following lLTP elimination. It seems likely that the inhibition of protein synthesis in the CA1 field of the hippocampus would interfere with any long term alterations in the cell that might be due to learning, or even underlie learning. In the present experiment administration of the transcriptional inhibitor prior to an animal's exposure to a spatial task was hypothesized to interfere with the acquisition of that task, if production of new cellular proteins is necessary for learning to occur. One of the possible alterations in a cell that might occur during LTP or the acquisition of a spatial task, is an upregulation of receptors responsible for the responsive properties of the cell. In the CA1 region of the hippocampus the receptors responsible for the responsiveness of the cell, as well as LTP are the AMPA and NMDA receptors. The present experiment attempted to determine if antagonism of new protein synthesis does in fact influence the receptors expressed by the pyramidal cells in the CA1 region.

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Five groups of rats were used to examine the effects produced by administration of Actinomycin D (ACT D) prior to being exposed to a spatial task. Three separate solutions were used: (1) high ACT D (50μM/10μl), (2) low ACT D (25μM/10μl), and (3) vehicle only (10μl) administration. The selected concentrations were designed to allow a dose dependent evaluation of the effects produced behaviorally as well as effects in density of receptor binding following a spatial task. All five groups of animals were exposed to a Morris water maze task. Three of the behavioral groups had an escape platform placed at various locations within the maze (contingent groups). The three contingent groups received each of the separate concentrations of the drug. Two of the experimental groups (a second saline infusion group, and a second high dose group), i.e., non-contingent groups, were matched to their counterparts in the same treatment in the contingent groups and allowed to swim in the maze for the same amount of time. However, the animals in the non-contingent groups did not have access to the escape platform. These non-contingent behavior groups were then used as a baseline comparison with the contingent behavior groups for receptor density differences due to learning in the maze. The use of the high dose non-contingent group was to determine the amount of inhibition of receptor production that the drug alone was producing. This group provided information relevant to the amount of normal receptor production when compared to the saline non-contingent group, separate from the receptor production due to learning while engaging in the same behavior.

Following the completion of the spatial task, all of the animals from each group were sacrificed for histology of their brains. Quantitative autoradiography was performed on brains to determine if there were any reliable differences in the

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concentrations of binding to NMDA and AMPA (glutamate) receptors in the CA1 field of the hippocampus. Additionally *in-situ* hybridization was performed on these brains to determine if any differences in the density of mRNA for NMDA and AMPA receptors existed among the experimental groups. The *in-situ* hybridization assays labeled the mRNA (precursor for the structure) for the essential subunits of the NMDA receptor complex, and four subtypes of AMPA receptors. The use of non-contingent behavioral experimental groups was necessary to determine if any differences obtained in receptors or mRNA was actually due to the drug administration, or simply due to learning the spatial task independent of the behavior of swimming in the maze.

The results were expected to support the hypothesis that (1) the acquisition of a new spatial task requires new protein synthesis. This would potentially underlie an upregulation of the receptors responsible for excitatory inputs in the CA1 field of the hippocampus. By extension, it was hypothesized that (2) there would be reliable differences in the latencies to escape by the animals receiving the transcriptional inhibitor compared to the subjects receiving the saline only infusion. In addition the assays on the brains of the animals run in the water maze would (3) show an increased density of binding to the receptors and of mRNA for those receptors in the saline infusion group compared to the groups administered the drug. It was also hypothesized that (4) there would be additional distinguishable differences between the expression of those receptors in animals exposed to the contingent behavioral task compared to animals exposed to the non-contingent behavioral task.

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V Methods:

Method

Behavioral Testing:

Subjects:

Fifty adult male Sprague-Dawley rats that were approximately 90 days old and weighed approximately 300 grams were purchased from Zivic-Miller laboratories with cannula implanted unilaterally into the apical surface of the CA1 region of the hippocampus. The animals were given free access to food and water in their home cages for the entire experiment. Animals were individually housed in hanging plastic cages containing wood shavings as bedding material. The colony room that housed the animals was maintained at a constant temperature (24^0 C $\pm$ 0.5^0 C), and was on a twelve-hour light/dark cycle.

Apparatus:

A Morris water maze (MWM) was used as the behavioral task. The maze was a 183 cm in diameter steel trough approximately 6 cm deep that was filled to a depth of 30 cm with room temperature water. The sides of the maze were painted black, as was an escape platform that was placed in the maze 1 cm below the surface of the water. The escape platform for all trials except for the cued phase was an 8.8 cm in diameter X 27 cm in height piece of PVC tubing fitted to a 14 cm PVC base. The tube was filled with gravel to ensure that it would not float in the maze. A PVC end cap was glued to the top of the PVC tubing that was 10 cm in diameter. The platform was then placed in the maze in the center of one of four 90^0 quadrants, approximately 45 cm from the outer wall of the

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maze. A second platform used was identical to the above platform, with the exception that the PVC tube was 2 cm longer. The second platform was painted white and protruded 1 cm out of the water. This platform was only used for the cued trials. The water in the maze was colored black to conceal the platform with Dry Temp powder tempera paint. A closed circuit camera connected to a Macintosh Quadra computer was used to track the rats while in the water maze. The programming application Dynamic Animal Movement Analyzer (DAMA) allowed the computer to track and record the path of the animals, as well as record speed of swimming, and latency to find the platform.

Drug:

The drug used in this study, Actinomycin D was purchased from Sigma pharmaceuticals in powder form. It was then mixed with saline (0.9% NaCl) to achieve a low concentration of 25 μM, and a high concentration of 50 μM. The drug was infused via a microsyringe in a 10 μL volume into the cannula implanted in the animal.

Procedure:

Prior to arrival in the laboratory, the 50 rats underwent stereotaxic surgery in which a cannula was implanted unilaterally into the dorsal surface of the CA1 region of the hippocampus. The coordinates of the implantation were relative to Bregma: 3.6 mm posterior, 2.0 mm lateral, and 2.5 mm ventral. Half of the cannula were implanted to the left of bregma and the other half were placed to the right of bregma; the sites were selected randomly. Following the implantation of the cannula the animals were given four days of post-operative care prior to being shipped to CSU.

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Upon arrival to the laboratory, all animals were weighed and given free access to food and water in their home cages. The subjects were then divided into five randomly assigned experimental groups. Three of the experimental groups were used for the contingent behavioral testing (experimental contingent behavior groups), and given access to the escape platform in the water maze in all experimental sessions. Two other groups of subjects were matched to subjects in the high dose contingent group and the saline contingent groups to control for time in the maze. The non-contingent behavioral groups were allowed to explore the maze for an equivalent amount of time as the contingent behavioral groups used that day, however, the escape platform was removed from the water in all experimental sessions. The non-contingent groups were not able to escape the water until the experimenters removed them from the maze. One group of animals in both the contingent behavior group, and non-contingent behavior group received a daily infusion of saline (10 μl) into the CA1 field of the hippocampus via the cannula. A single group of animals in the contingent behavior group was given a daily infusion of the low dose of Actinomycin D (10 μl of a 25 μM solution) into the CA1 field of the hippocampus via the cannula. The two remaining groups for both the contingent behavior and non-contingent behavior animals received a daily infusion into the CA1 field of the hippocampus of the high dose of Actinomycin D (10 μl of a 50 μM solution). Following the infusion of the solutions the non-contingent behavior groups were placed in the maze for a period of time equivalent to their matched animals in the contingent behavior group.

The procedure for the water maze experiment was divided into four separate phases. The first phase consisted of place trials for the animals in the contingent

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behavioral group (sessions 1-6). Each animal was given three trials each session, and the place trials were conducted for a total of six sessions. Subjects were removed from their home cages and given the infusion specified by the treatment group to which they were assigned. Five minutes following the infusion subjects were placed in the maze individually. The place trial phase consisted of the escape platform being placed in one of four randomly selected quadrants of the water maze. The remaining three quadrants were numbered sequentially 1-3 counter-clockwise from the platform. The platform location was consistent for the entire place trial phase for each animal; however, the platform location for all subjects was not the same to ensure platform location did not affect performance.

During the experimental sessions the animal was placed directly along the outer wall of the maze in the center of one of the three numbered quadrants selected randomly. The subjects were placed in the maze facing the outer wall, following which the experimenter would exit the area of the maze to ensure the experimenter was not serving as a spatial cue. Once the animal had been placed in the maze it was given a maximum time of sixty seconds in which to locate the hidden platform. If after sixty seconds the animal had not found the platform the experimenter would place the subject on the platform. Once the animal had either found the platform, or been placed on the platform it was given thirty seconds to orient itself within the maze. Following the thirty seconds on the platform the animal was placed under a heat lamp for a period of thirty seconds to dry. When the thirty seconds of drying had elapsed the animal was placed in the maze for the second and third trial in exactly the same manner as the first trial, with the starting

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quadrant location being randomly selected. After each animal completed the third trial, it was towel dried before being returned to its home cage.

The data collected during the place trials consisted of several measures. The latency to find the platform was collected for every trial, as well as the average speed of swimming, and the distance traveled. The data collected allowed an assessment of the working memory capabilities and the reference memory capabilities of the rats. The data collected on the first trial of each session gave a measure of the subjects reference memory to locate the hidden platform; any improvements across sessions must be due to consolidation of the memory to find the platform. The remaining latencies for the two trials the animal was placed in the maze were used to determine if any working memory deficits were produced due to the drug. A previous researcher using the MWM has argued that any improvements within a session must be due to working memory activity (Morris, 1986).

The first experimental session following the place trials phase was a probe phase (session 7) to determine what the animals had learned during the place trials. The first trial of the first session the platform was removed and the animal was placed in the maze for sixty seconds. The amount of time the animal spent in the quadrant (target quadrant) that the platform was located during the place trials was collected. In addition to the time in the quadrant, the number of times the animals' paths crossed over the location that the platform was located (annulus crossings) were determined from the tracking system. Two additional trials were carried out in this session identical to the place trials phase, with the platform returned to the same location in the maze.

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The third phase of the behavioral experiment was a reversal phase (sessions 8-11): the procedure was identical to the place trials phase, except that the platform was moved 180 degrees from its original location. All other characteristics of the MWM procedure remained the same. The data collected in the reversal phase were also consistent with the data from the place trails phase.

The last phase of the behavioral experiment was a cued phase (sessions 12-13). For two sessions with three trials each session, the black hidden platform was replaced with a white platform that protruded above the water surface. Each trial within a session involved the platform being moved to a new quadrant location, and the animal placed into the maze and run in the same way as the previous phases. The order of platform location was randomly selected and was identical for all the animals. The latency to find the platform was collected for all of the cued trials to determine if the drug produced any sensory or motor deficits.

Following the last trial of the Cued Phase in the water maze, all of the animals were euthanized via a CO_2 overdose. Once the animals had expired, their brains were removed immediately and placed in dry ice and kept frozen for the autoradiography and in-situ hybridization assays.

The only measure that was recorded from the non contingent behavioral subjects during the experiment was the speed of swimming. This data was collected to ensure that there were not large differences in the behavior of the contingent animals and the non contingent animals in the maze.

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Tissue Materials and Methods:

Animals and Tissue Preparation:

All fifty brains were utilized for the autoradiography and *in situ* hybridization, ten rats from each of the groups from the behavioral experiment. The brains were removed following euthanasia via CO_2 overdose, and frozen in dry ice. The brains were stored in a -70°C freezer until sectioning, then were gradually warmed to sectioning temperature (-15° C). The brains were sectioned using a Zeiss cryostat to twelve micron thick coronal slices which were cold mounted onto gelatin-coated slides. One section from each of the groups was placed on each slide. The positions that the tissue was placed on the slides were randomly selected and varied between cutting groups to ensure that position did not affect binding density.

Ligand Binding:

[^{3}H]L-glutamate binding to NMDA receptor sites:

[^{3}H]L-glutamate purchased from New England Nuclear (Boston, MA) was used to label the NMDA receptor complex. The binding procedure was in accordance with procedures currently used in Dr. Kathy Magnusson's laboratory (Magnusson, 1995). Slides were preincubated in cold (4°C) 50 mM Tris acetate (TA) buffer (pH 7.0) for 30 min., followed by 2 X 10 min. incubations in warm TA buffer (30° C). The sections were then incubated in a solution of 100 nM [^{3}H]L-glutamate, 1 μM kainate, 5 μM α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA), and 100 μM 4-acetamido-4'-isothiocyanotostilbene-2,2'-disulfonic acid (SITS) for 10 min. at 4°C. The slides were then rinsed in 4 changes of TA buffer for a total of 30 seconds at 4°C and dried by a

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stream of compressed air at room temperature. Unlabeled 200 μM NMDA was included in the incubation solution to determine nonspecific binding.

[^{3}H]AMPA binding:

Binding was performed similarly to NMDA binding, with some exceptions. [^{3}H]AMPA purchased from New England Nuclear was used to label AMPA receptors. Slides were preincubated in 50 mM TA buffer (pH 7.2) containing 2.5 mM $CaCl_2$ and 30mM KSCN for 30 min. at 4°C followed by 10 min. in TA buffer at 30° C. The tissue was then incubated in a solution containing 50 nM [3H]AMPA and 100 μM potassium thiocyanate (KSCN) for 10 min. at 4° C for total binding. Nonspecific binding was determined by the addition of 100 μM unlabeled AMPA to the incubation solution. Slides were then rinsed in four changes of TA buffer containing 2.5 mM $CaCl_2$ for 30 seconds at 4° C, then dried by a stream of compressed air at room temperature.

Autoradiography:

Following ligand binding the slides were allowed to dry overnight then apposed to tritium-sensitive film (Hyperfilm, Amersham) for 10 days along with autoradiographic standards (tritium standards, Amersham). The film was developed in D-19 (Kodak) developer and Rapid Fix (Kodak). The films were then uniformly illuminated via a fluorescent light box (MCID, St. Lawrence, Canada), and captured using a Panasonic CCD camera interfaced by a Quick capture board to a Macintosh IIci computer. Quantitative densitometry was performed using NIH image software (public domain software) on 4 slides for total binding, and 2 slides for non-specific binding for both the NMDA-labeled and AMPA-labeled tissue. The tritium standards were used to convert the optical density measurements to fmol/mg protein of binding.

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In situ Hybridization Materials and Methods:

An oligonucleotide (45 nucleic acids) was commercially prepared (Macromolecular Resources, Colorado State University) for the NR1 (complementary to nucleotide residues GCA CAG CGG GCC TGG CTC TGG GTT GCG CGA GTG CGA CGA CCT CGC) subunit of the rat NMDA receptor. A second group of oligonucleotides were prepared to label four different subtypes of the rat AMPA glutamate receptor. Four separate oligonucleuotide probes (45 nucleic acids) were commercially prepared (Macromolecular Resources, Colorado State University) for the different subtypes (GluR A (1), GluR B (2), GluR C (3), GluR D (4)) of the AMPA receptor. The four receptor subtype oligonucleuotides were as follows; for GluR A base number range 1167-1212, (complementary to nucleotide residues CCC CGC AGC CAC CGA CGC TCA GGC TGG AGG GGA CAA CTC AAG CGT), for GluR B base number range 1188-1233, (complementary to nucleotide residues TGT CAC CCT AAC TGA GCT CCC ATC AGG AAA TGA CAC GTC TGG GCT). The sequence for GluR C base number range 1200-1245, (complementary to nucleotide residues GCC CTT CTC AGA TCA ACA AAT CAG CAA TGA CAG CTC ATC CTC AG), and for GluR D base number range 1191-1236, (complementary to nucleotide residues CTT GAT TCA AGA TAT GCC TAC TCT GGG CAA TGA CAC AGC AGC TAT). All oligonucleotides were labeled with ^{33}P-dATP using terminal deoxyribonucleotidyl transferase (New England Nuclear) and NENSORB20 purification cartridges (New England Nuclear). The *in situ* hybridization was performed according to the procedures currently used in Dr. Kathy Magnusson's laboratory. All solution steps were performed with gentle rotation on a

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rotating table, with the exception of the fixation and hybridization steps. Slides prepared as described above were thawed, air dried, fixed in 4% paraformaldehyde / phosphate buffered saline (PBS; pH 7.2, 25°C) for 15 minutes, then placed in 2 mg/ml glycine in PBS (pH 7.2, 25°C) for 20 minutes, and then placed in 0.25% acetic anhydride/ 0.1 M triethanolamine (pH 8.0, 25°C) for 10 minutes. Slides were then placed in coplin jars for 2 hours in a prehybridization solution, which consisted of 50% formamide, 0.1 M Tris-HCl (pH 7.5), 4 X SSC (1 X SSC = 150mM NaCl and 15 mM sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2.0% sarkosyl, and 250 mg/ml salmon testes DNA. Slides were then successively washed for 5 minutes each in 2 X SSC, 70% and 100% ethanol, then they were air dried for 15 minutes. Hybridization was then performed by placing 150 µl of prehybridization solution with 10% dextran sulfate and 1 X 10^6 dpm of ^{33}P-labeled oligonucleotide probe added onto the slides, coverslipping the slides with parafilm and incubating them for 18 hours in a 42°C oven humidified with 5 X SSC. Coverslips were then removed and the slides were rinsed for 40 minutes each in 2 X SSC and 0.1% sarkosyl (25°C) and twice in 0.1 X SSC and 0.1% sarkosyl (55°C) and air dried. Nonspecific binding was determined by the addition of a 20-fold excess of unlabeled oligonucleotide to the hybridization solution on some slides. Sections were then exposed to Hyperfilm-β max (Amersham) for 3-5 days along with brain paste standards. The standards were prepared by homogenizing whole rodent brain and mixing in measured amounts of ^{33}P-dATP. Brain and standard images were captured using the same procedure as specified for the autoradiography. Quantitative densitometry was then performed on images from four slides for total binding and two slides for nonspecific binding from each animal. The standards were

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used to convert optical density to cpm/mg tissue, and the specific signal was determined by subtracting nonspecific binding from total binding.

Data Analysis:

All data collected from the MWM procedure were analyzed via the SPSS statistical package. The mean latency to find the platform was analyzed across all three trials of the place trails phase, and the reversal phase. In addition the mean latency on each of the individual three trials was averaged for each treatment group and analyzed. A repeated measures analysis of variance was performed to determine the differences between session and trials within a treatment, as well as between treatments. The average speed of swimming and mean distance traveled were also subjected to repeated measures analysis of variance for the place trials and the reversal phase. Tukey post hoc comparisons were performed when any significant differences were obtained within groups or between groups from the analysis of variance. The data collected on the probe trial (annulus crossings and persistence in the platform quadrant) were analyzed via an analysis of variance. The mean latencies on the cued trials were averaged for each of the treatment groups and analyzed in the same fashion as the data from the place trials. Tukey post hoc comparisons were performed when any significant differences were obtained.

The data collected from the tissue assays were analyzed via repeated measures analysis of variance as well. The fmol/mg protein of binding for each of the receptor assays obtained in the autoradiography and the cpms/mg tissue obtained in each of *the in-situ* hybridization assays were compared among treatment groups, as well as between the

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infused hippocampus and non-infused hippocampus. Any significant differences obtained in these analyses resulted in Tukey post hoc comparisons being performed to determine where the differences were.

Results

Behavioral Data:

The data from the place trials' phase revealed no reliable differences in the mean latency to find the hidden platform among the treatment groups. The three trial mean for each session indicated no reliable differences among the treatments (see figure 4) $F_{(2,27)} = .925$ $p=.409$, however, there were reliable differences among experimental sessions $F_{(5,135)} = 40.159$ $p=.001$. No statistically significant interactions of treatment and day were observed. There were small differences observed in the mean latency to locate the hidden platform on the first trial of each session (see figure 5), however, the differences were not statistically reliable $F_{(2,27)} = 1.488$ $p=.244$. The data gathered for the first trial mean latency also showed a significant improvement across session $F_{(5,135)} = 31.215$ $p=.001$. No significant interactions between treatment and day were found for the first trial mean latency data. In addition to the mean latency to find the platform, the mean swimming speed for all three treatment groups were collected (see figure 6). The mean speeds of swimming, in pixels/second were not reliably different among treatment groups $F_{(2,27)} = .914$ $p=.413$. There was a reliable increase in speed of swimming across sessions throughout the place trials, $F_{(5,135)} = 7.472$ $p=.001$. No significant interaction

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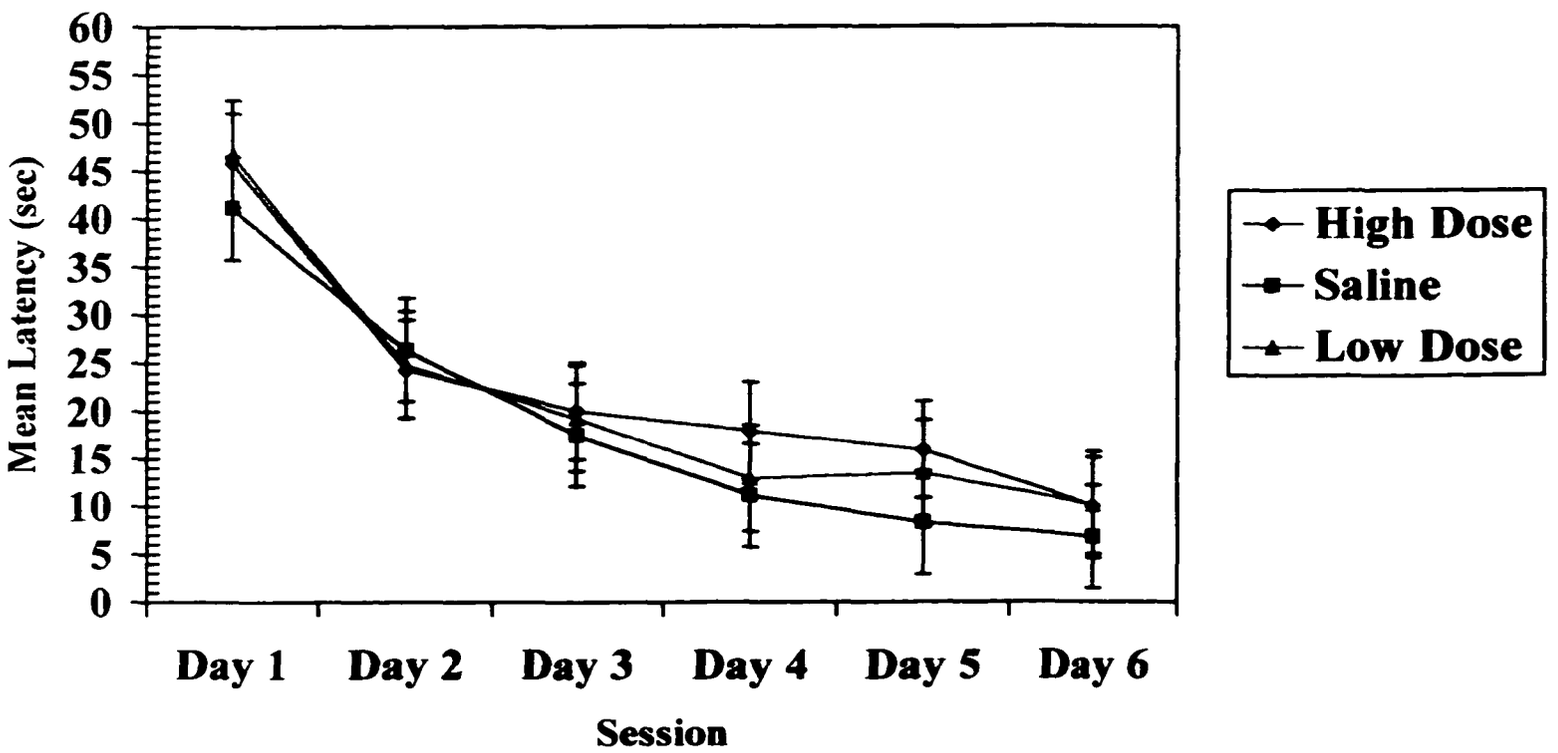

Figure 4: Mean latency in seconds (±SEM) for all three trials each session of the place trials to find the hidden platform in the Morris water maze. Each of the treatment groups are depicted; Saline (10μl infused), Low Dose (25μM ACT D/ 10 μl infused) and High Dose (50μM ACT D/ 10μl infused).

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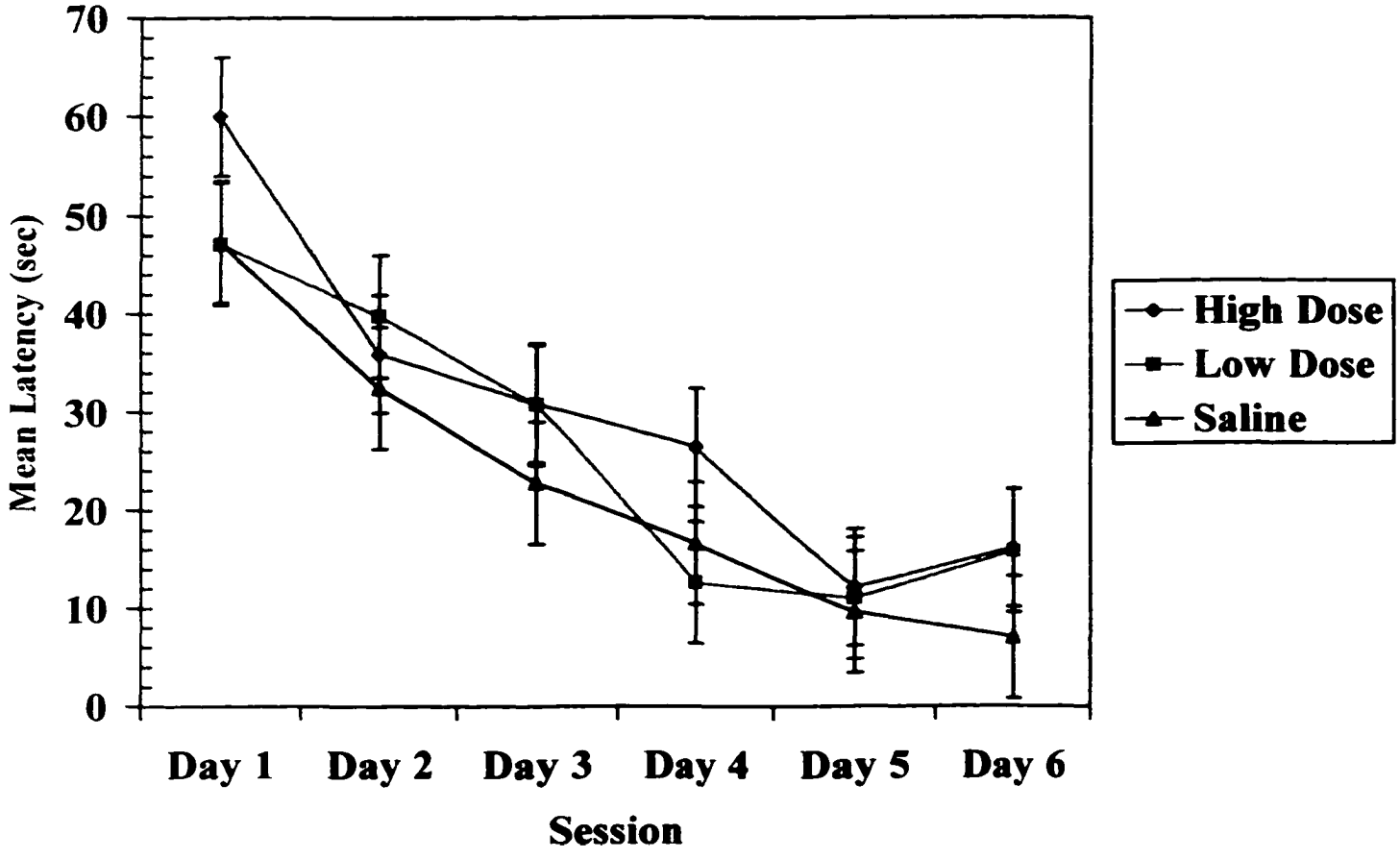

Figure 5: Mean latency in seconds (±SEM) for the first trial of each session of the place trials to find the hidden platform in the Morris water maze. Each of the treatment groups are depicted; Saline (10µl infused), Low Dose (25µM ACT D/ 10 µl infused) and High Dose (50µM ACT D/ 10µl infused).

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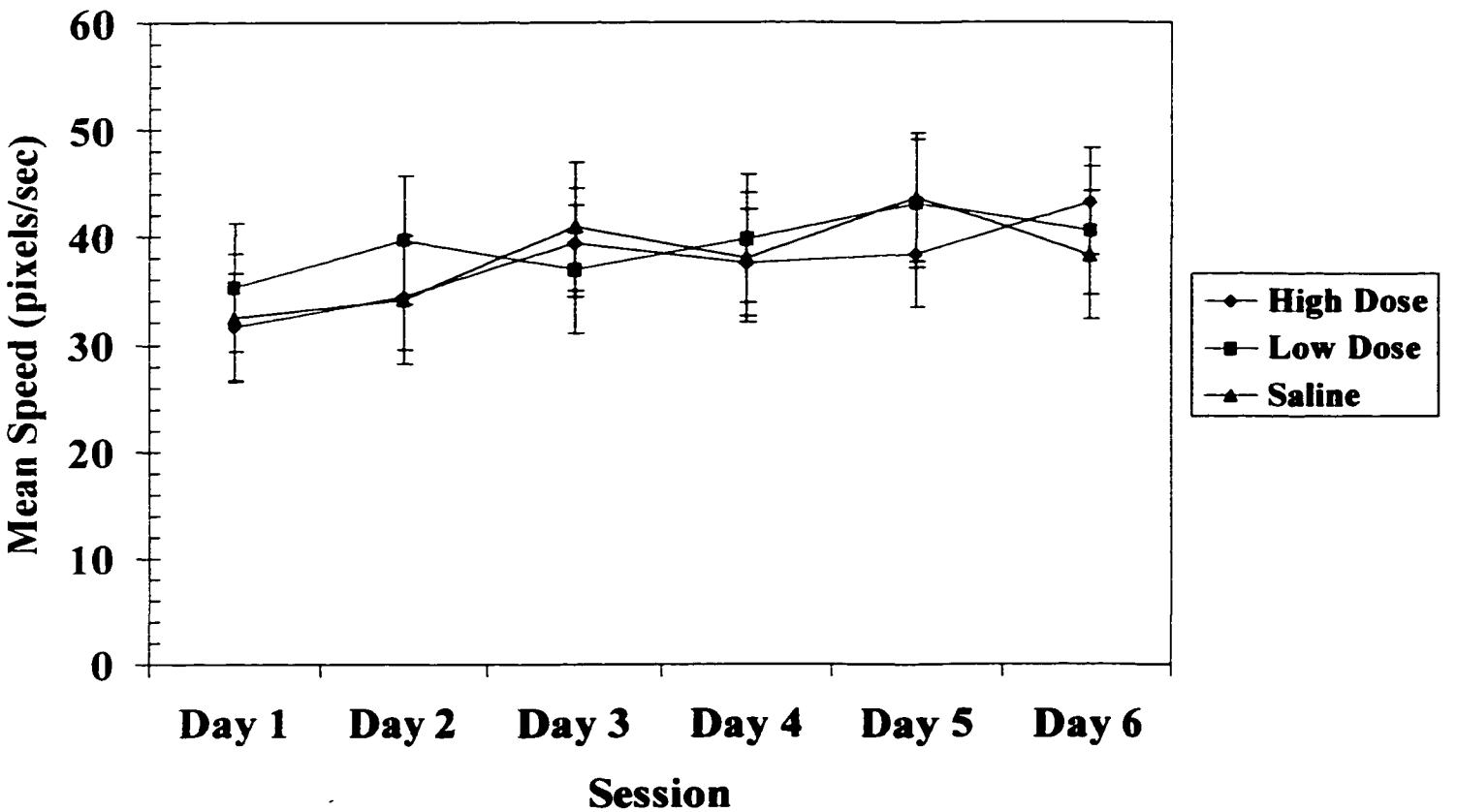

Figure 6: Mean swim speed (±SEM) across all three trials throughout the place trials. Each of the treatment groups are depicted; Saline (10μl infused), Low Dose (25μM ACT D/ 10 μl infused) and High Dose (50μM ACT D/ 10μl infused).

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of session and treatment was found for speed of swimming. An additional measure recorded throughout the place trials was the average distance in pixels the animals swam in the maze. The data collected for the distance traveled was very similar to the mean latency data. Because there were no significant differences in speed of swimming or mean latency to find the platform. the same trend in the data was observed for distance traveled. There were no reliable differences in the mean distance traveled across all three trials among the treatment groups (see figure 7), $F_{(2,27)} = 1.500$ p=.241. There were however, reliable differences across sessions for the distance traveled $F_{(5,135)} = 26.022$ p=.001. No interaction between treatment and session was found for distance traveled. The data for the first trials of each session for mean distance were very similar to the data from the three trial mean (see figure 8). There were no reliable differences in the mean distance traveled among treatment groups $F_{(2,27)} = 1.948$ p=.162. A significant difference was observed across sessions for mean distance traveled on the first trial of each session of the place trials, $F_{(5,135)} = 19.334$ p=.001. The final data gathered throughout the place trials were the mean latencies to locate the hidden platform for each of the treatment groups on each trial regardless of session. These data were designed to determine if any differences in working memory was detectable among the treatment groups. The data indicated that there were no reliable differences between the treatment groups on each of the individual trials $F_{(2,15)} = .137$ p=.873. There were reliable differences however, when the mean latencies to find the platform on each trial were compared (see figure 9), $F_{(2,30)} = 13.722$ p=.001.

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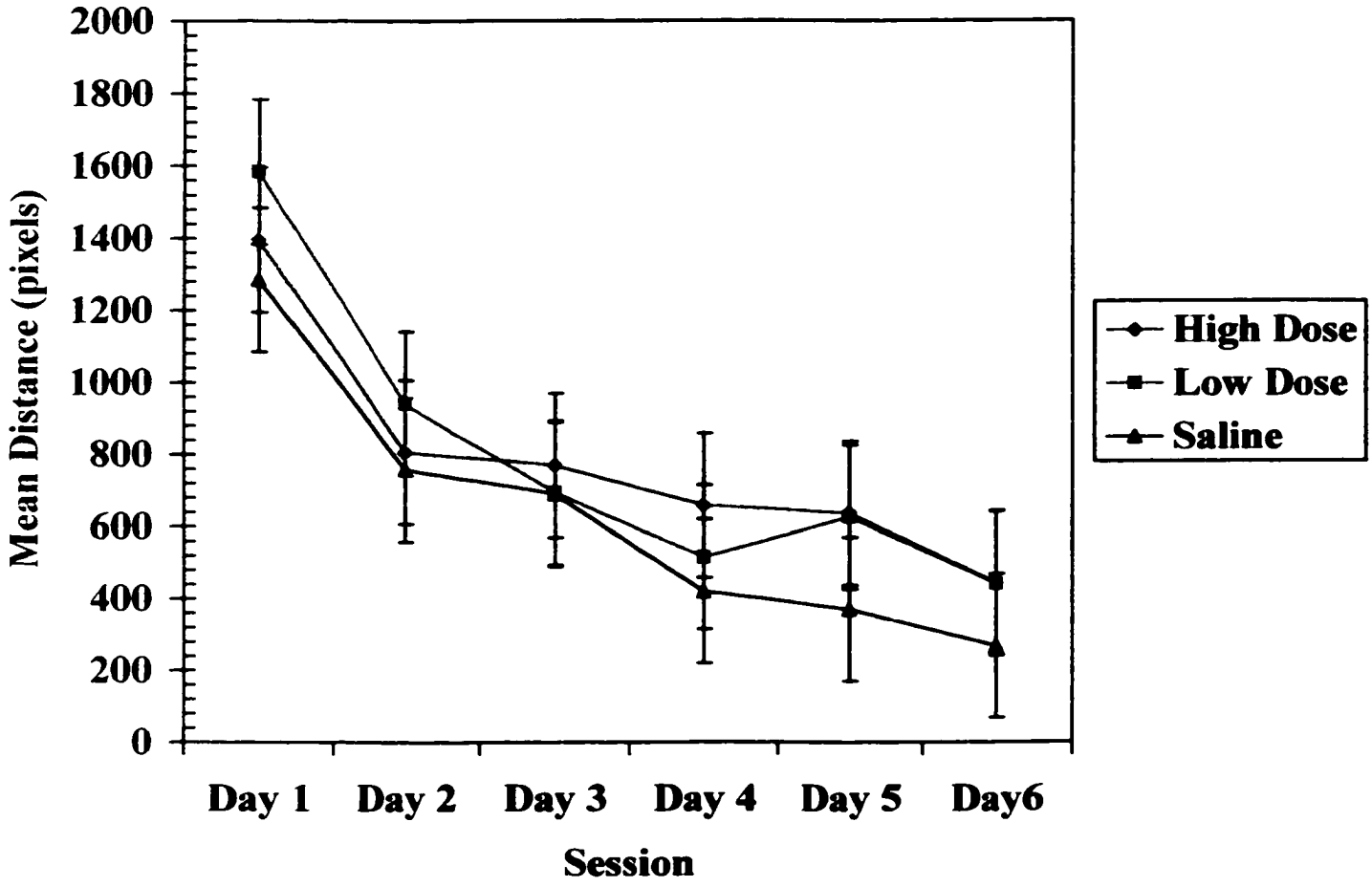

Figure 7: Mean distance in pixels (± SEM) for all three trials during the place trials. All three treatment infusions are depicted; saline (10μl infused), low dose (25μM/ 10μl infused), and high dose (50μM/ 10μl infused)

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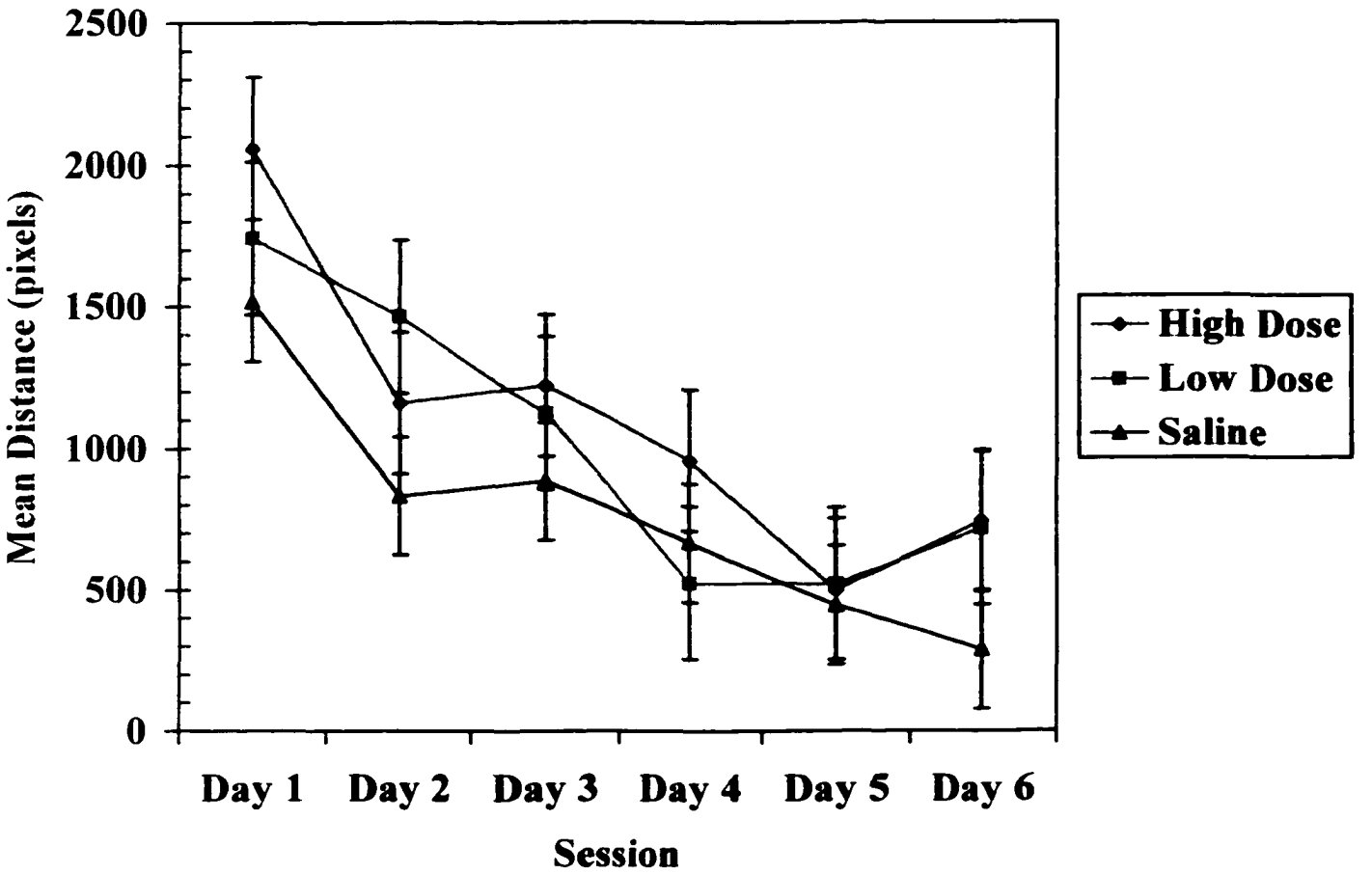

Figure 8: Mean distance traveled (± SEM) in pixels for the first trial during the place trials phase. The treatment groups infused are depicted, Saline (10μl infused), Low Dose (25μM ACT D/ 10 μl infused) and High Dose (50μM ACT D/ 10μl infused).

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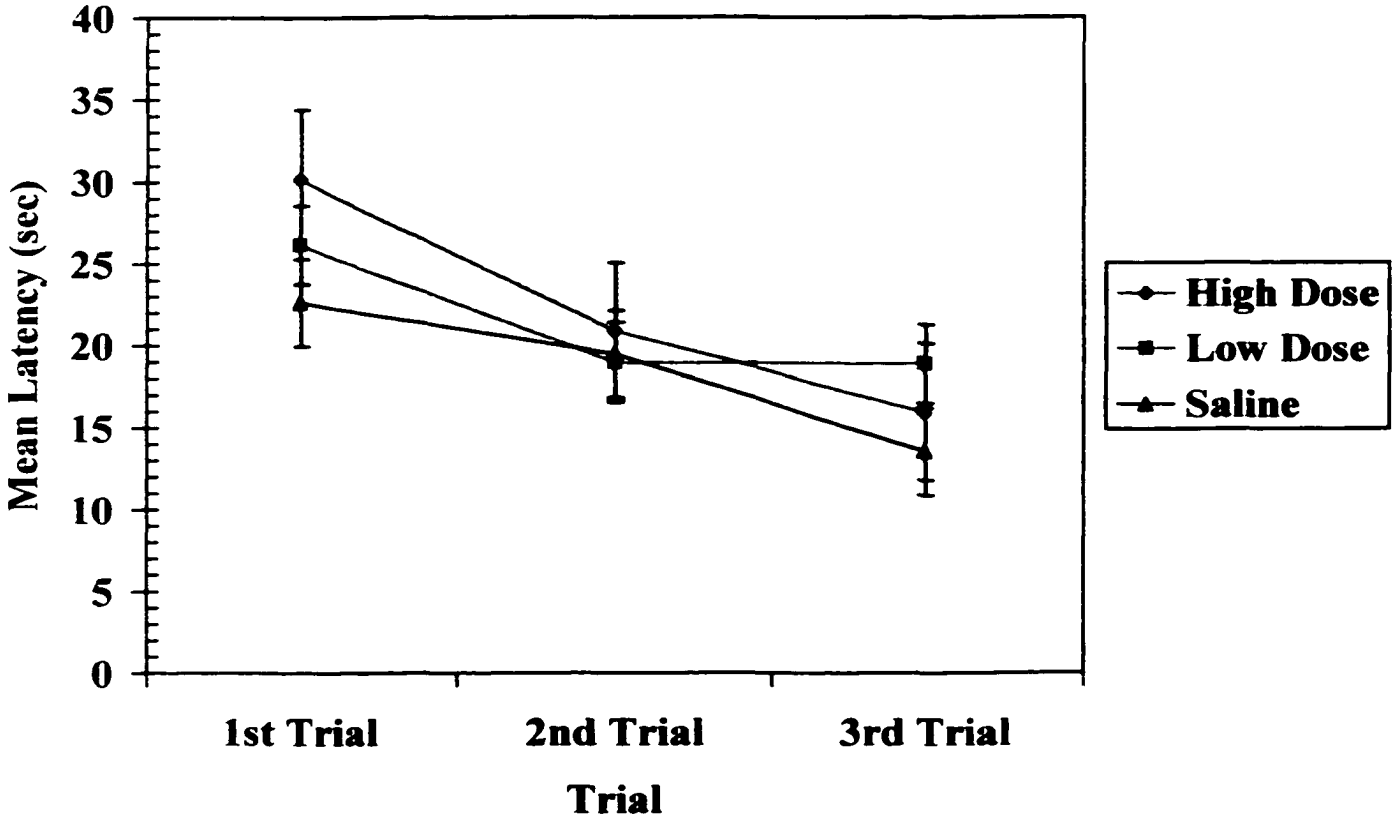

Figure 9: Mean latency in seconds (± SEM) to locate the hidden platform for each trial throughout the place trials. Each treatment is represented; saline (10µl infused), Low dose (25µM/ 10µl), and the High dose (50µM/ 10µl).

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The first session following the place trials was a probe trial in which the platform was removed from the maze to determine what the animals had learned. The two measures recorded for the probe trial were the mean persistence in the target quadrant by each of the treatment groups and the mean number of annulus crossings for each treatment group. As can be seen in figure 10, all three treatment groups spent significantly more time in the target quadrant than chance would produce (15 seconds). One way T-tests were performed comparing mean persistence for each group versus fifteen seconds (chance). The results were for the high dose (50μM ACT D) a mean difference of 13.866 seconds ($p = .001$). The results for the low dose group (25μM ACT D) showed a mean difference of 14.878 seconds ($p = .001$), and the results for the saline infused group produced a mean difference of 17.483 ($p = .001$). There were not any reliable differences between the treatment groups in mean persistence $F_{(2,27)} = .681$ $p=.515$. The data collected for mean number of annulus crossings during the probe trial (see figure 11) revealed a significant difference among the treatment groups, $F_{(2,27)} = 7.243$ $p=.003$. Tukey post-hoc comparisons were performed on these data and revealed a significant difference between the saline infused group and low dose infusion (25 μM ACT D) and the high dose infusion groups (50 μM ACT D), $p = .038$. These data reliably demonstrated that the saline animals crossed the former platform location more than the drug infused subjects.

The data gathered from the reversal phase was consistent with data produced in the place trials phase. There were no significant differences between the treatment groups for the three trial mean latency to find the platform (see figure 12) $F_{(2,27)} = .110$

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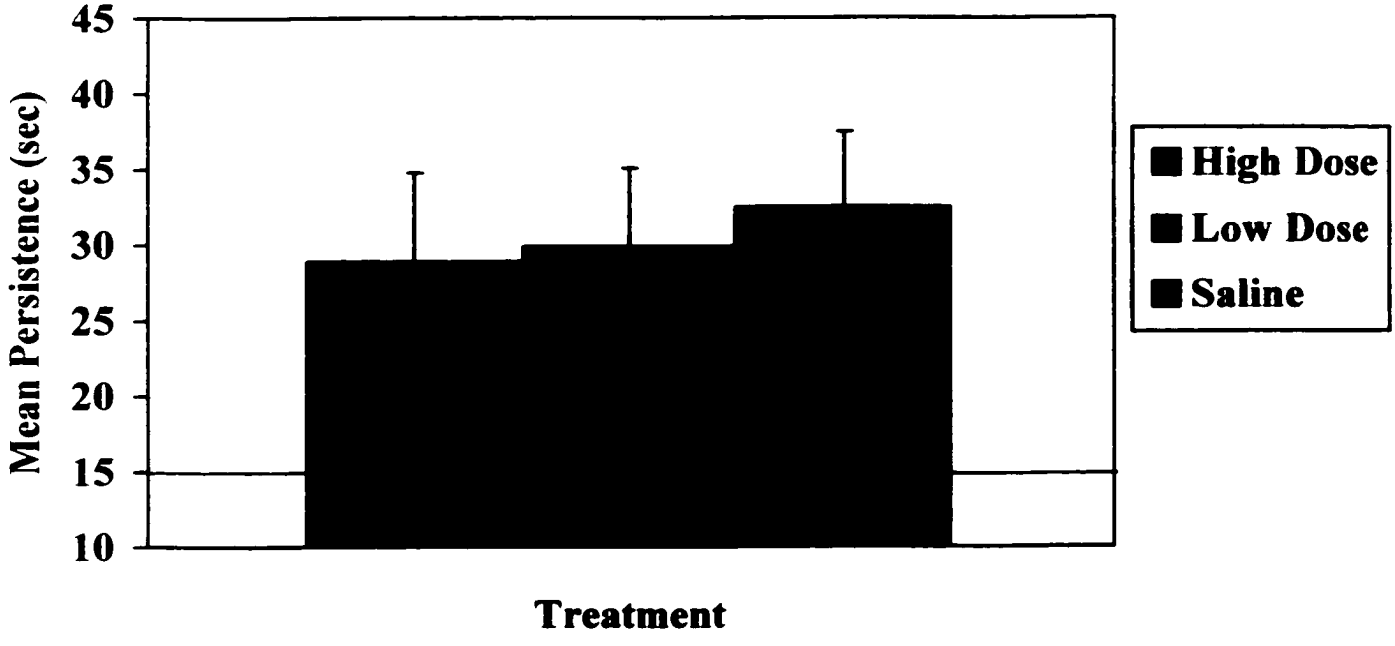

Figure 10: Mean persistence (±SEM) in the target quadrant during the sixty second probe trial for each of the infused groups; Saline (10µl infused), Low Dose (25µM ACT D/ 10 µl infused) and High Dose (50µM ACT D/ 10µl infused). The solid line at 15 seconds represents chance persistence on probe trial.

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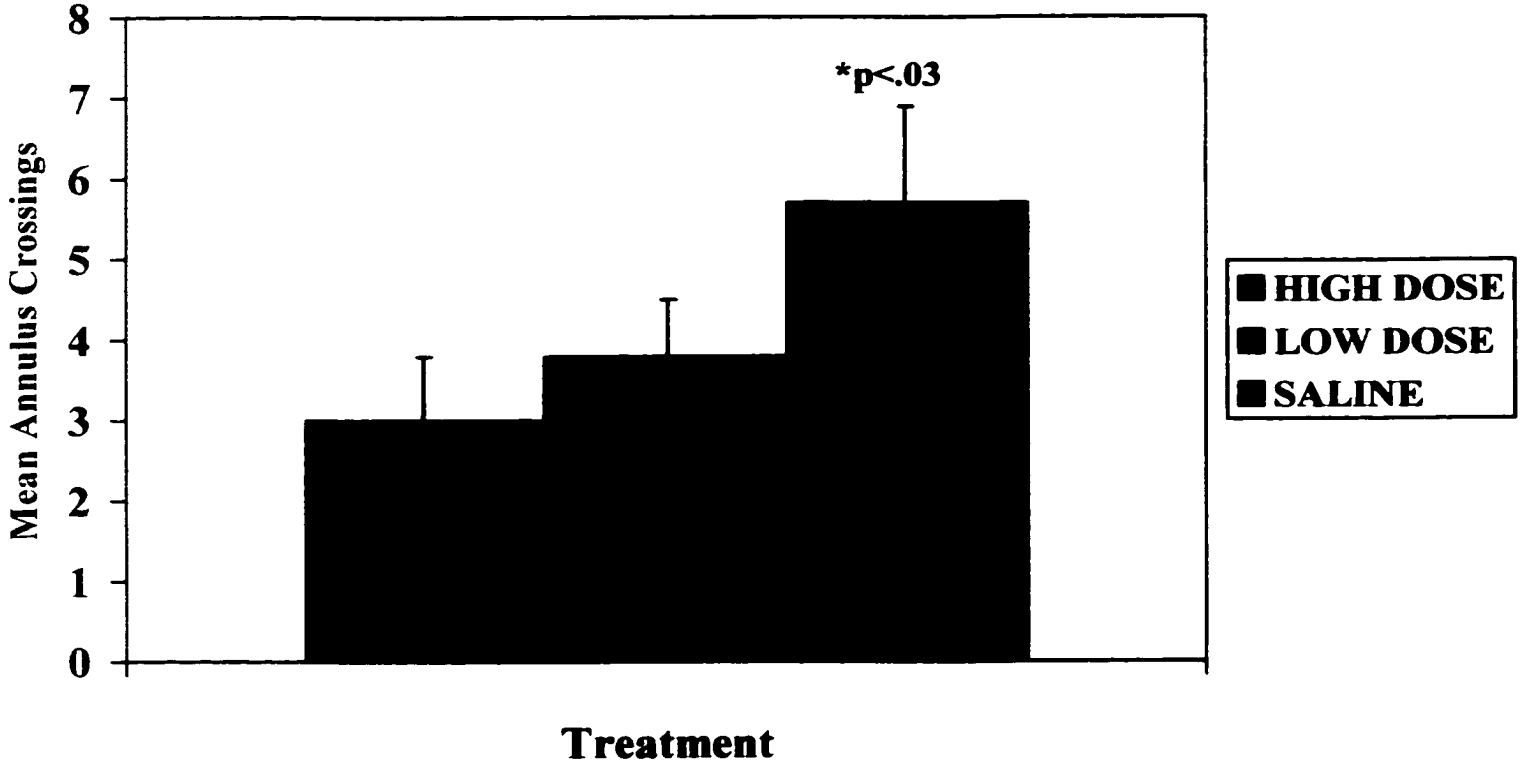

Figure 11: Mean number of annulus crossings (crossing over the location the platform was located during place trials) (±SEM) for each of the treatment groups; Saline (10µl infused), Low Dose (25µM ACT D/ 10 µl infused) and High Dose (50µM ACT D/ 10µl infused). * Represents significant difference between saline and both drug infused groups.

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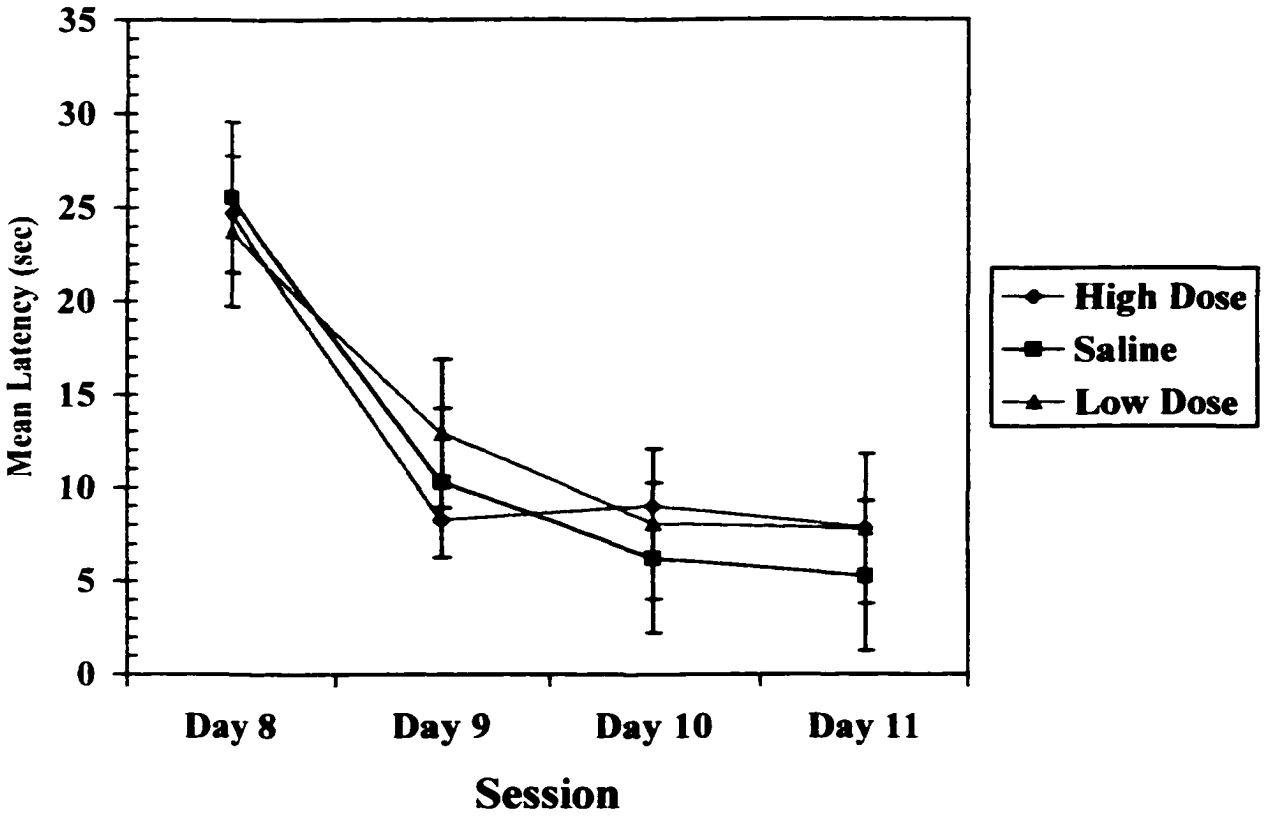

Figure 12: Mean latency (±SEM) for all three trials during the reversal phase. The three treatments depicted were saline (10μl infused), low dose (25μM/ 10μl infused), and high dose (50μM/ 10μl infused).

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p=.953, or the first trial mean latency to find the platform (see figure 13) $F_{(2,27)} = .372$ p=.693. There was a significant effect of session during the reversal phase for both the three trial mean latency $F_{(3,81)} = 31.386$ p=.001, and the first trial mean latency $F_{(3,81)}$ =54.336 p=.001. No reliable interactions of treatment and session were obtained in the reversal phase. Since there were no reliable differences in mean latency to find the platform in the reversal phase, or in the mean speed of swimming during the place trials, no analysis was performed on the mean distance data.

The data from the final phase, the cued trials, were consistent with data collected from the previous phases. There were no reliable differences in the mean latency to locate the cued platform (see figure 14), $F_{(2,27)} = .176$ p=.840. There was a significant difference between sessions during the cued phase $F_{(1,27)} = 28.51$ p=.001. No interaction was obtained between the treatment and session.

Assay data:

The data collected from the assay procedures consisted of mean densities for the infused and non-infused hippocampus from each animal for each of the five treatment groups. High dose contingent (HDC; 50 μM ACT D infused), high dose non-contingent (HDNC; 50 μM ACT D infused), low dose (LD; 25 μM ACT D infused), saline contingent (SC; saline infused), and saline non-contingent (SNC; saline infused).

Quantitative Autoradiography:

The data from the AMPA quantitative autoradiography produced a significant difference in mean density of binding, $F_{(9,90)} = 4.467$ p=.001 (see figure 15). Tukey post- hoc comparisons revealed significant differences in mean density of binding (in

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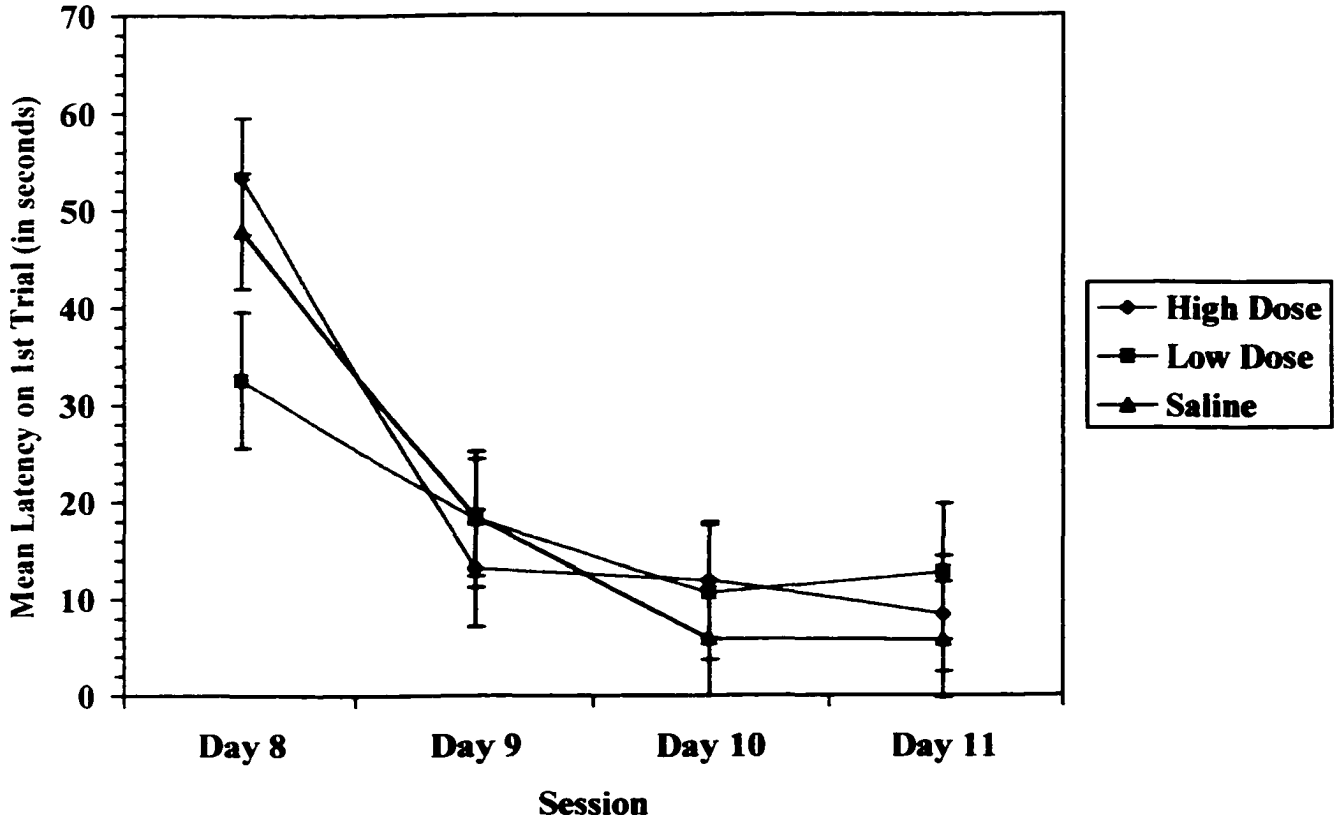

Figure 13: Mean latency to find the platform (±SEM) for the first trial of the reversal phase. The three treatments depicted were saline (10μl infused), low dose (25μM/ 10μl infused), and high dose (50μM/ 10μl infused).

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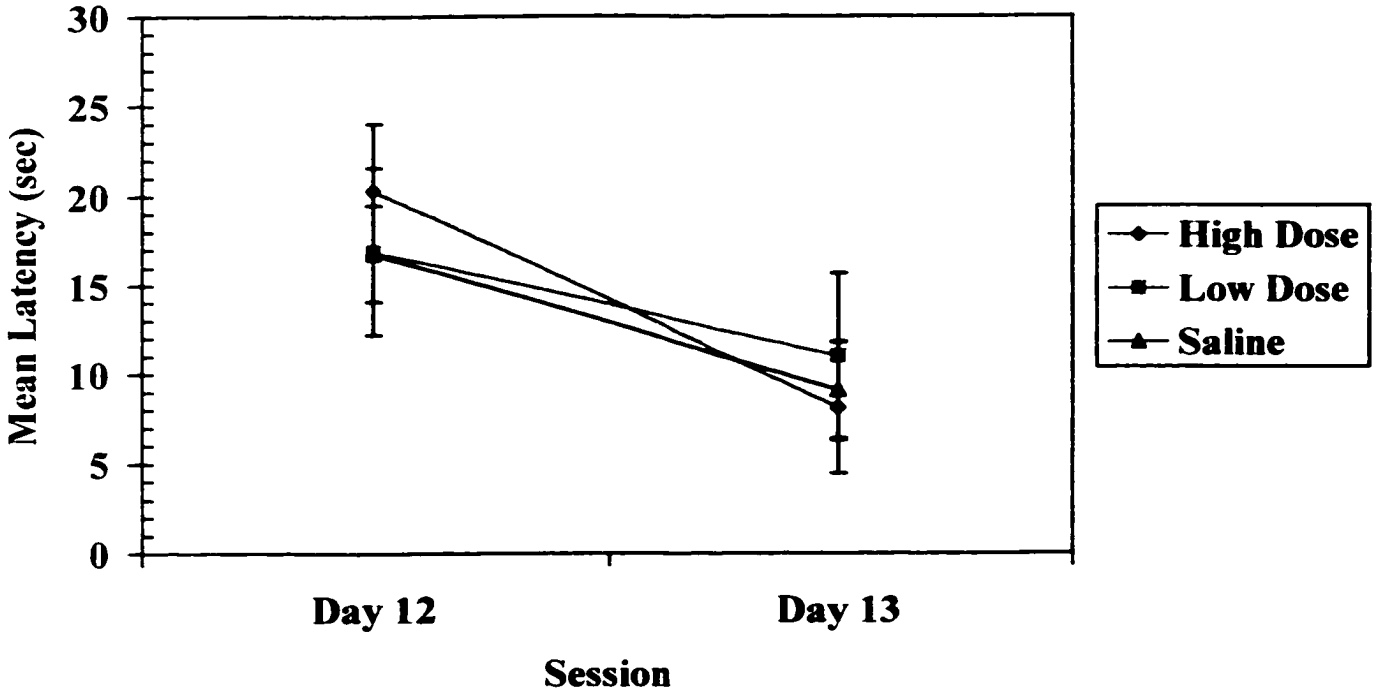

Figure 14: Mean latency in seconds (±SEM) for all three trials throughout the cued sessions. The hidden platform was replaced with a visible platform that protruded above the water surface for all three treatment groups; Saline (10μl infused), Low Dose (25μM ACT D/ 10 μl infused) and High Dose (50μM ACT D/ 10μl infused).

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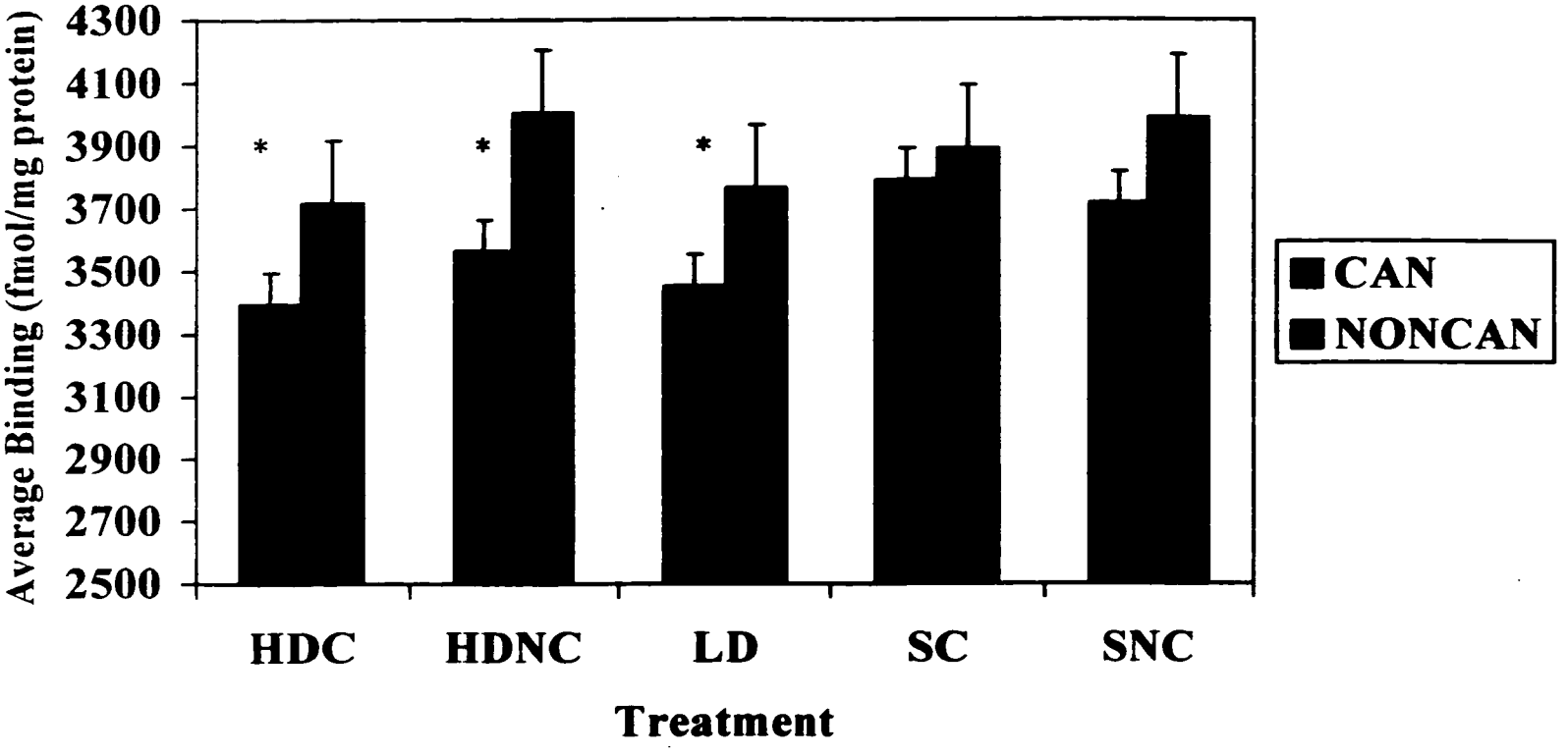

Figure 15: Average binding (fmol/mg protein) (±SEM) for all treatment groups for the quantitative AMPA autoradiography assays. Each of the infused groups of subjects are represented; High Dose Contingent (HDC-50μM ACT D/ 10μl infused), High Dose Non-contingent (HDNC-50μM ACT D/ 10μl infused), Low Dose (LD-25μM ACT D/ 10 μl infused), Saline Contingent (SC-10μl infused), and Saline Non-contingent (SNC-10μl infused). In addition the cannulated hippocampus (CAN) and non-cannulated hippocampus (NONCAN) are represented. * Indicate significant differences between drug infused hippocampi and saline infused as well as non infused hippocampi.

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mean fmol/mg protein) between the HDC infused hippocampus and the HDNC non-infused hippocampus (p=.007), the SC non-infused hippocampus (p=.029), and SNC non-infused hippocampus (p=.008). Significant post-hoc comparisons were also obtained between the mean density of binding to AMPA receptors for the HDNC infused hippocampus and HDNC non-infused hippocampus. Additional significant differences in mean binding were obtained between the LD infused hippocampus and the HDNC non-infused hippocampus (p=.009), SC non- infused hippocampus (p=.039), and SNC non-infused hippocampus (p=.012).

Data from the NMDA quantitative autoradiography also revealed a significant difference in mean binding to NMDA receptors. $F_{(9,90)} = 4.639$ p=.001 (see figure 16). Tukey post-hoc comparisons revealed significant differences in mean density of binding (in mean fmol/mg protein) between the HDC infused hippocampus and the HDNC non-infused hippocampus (p=.05), the SC non-infused hippocampus (p=.015), and the SNC non- infused hippocampus (p=.032). Tukey post-hoc's also revealed significant differences in mean density of binding (in mean fmol/mg protein) between HDNC infused hippocampus and HDNC non-infused hippocampus (p=.019), the LD non-infused hippocampus (p=.043), the SC non-infused hippocampus (p=.005), the SNC infused hippocampus (p=.05), and the SNC non-infused hippocampus (p=.011). One additional significant post-hoc comparison revealed a difference between the mean fmol binding/mg protein for the LD infused hippocampus and the SC non-infused hippocampus (p=.037).

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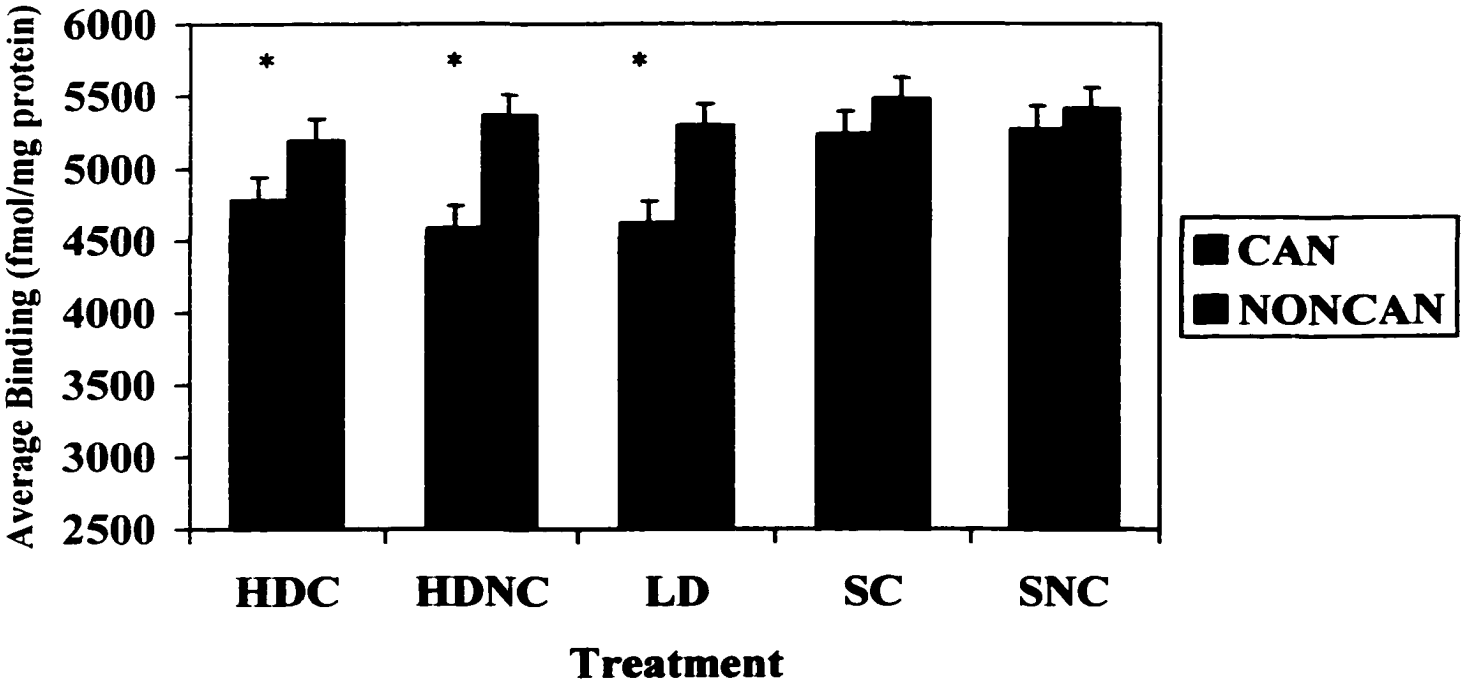

Figure 16: Average density (fmol/mg tissue) (±SEM) for all treatment groups for the quantitative NMDA autoradiography assays. Each of the infused groups of subjects are represented; High Dose Contingent (HDC-50μM ACT D/ 10μl infused), High Dose Non-contingent (HDNC-50μM ACT D/ 10μl infused), Low Dose (LD-25μM ACT D/ 10 μl infused), Saline Contingent (SC-10μl infused), and Saline Non-contingent (SNC-10μl infused). In addition the cannulated hippocampus (CAN) and non-cannulated hippocampus (NONCAN) are represented. * Indicate significant differences between drug infused hippocampus and non infused hippocampi and saline infused hippocampi.

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In situ Hybridization assay's:

All data gathered for the *in situ* hybridization assays consisted of tissue groups identical to the quantitative autoradiography assays. The data from the NR1 *in situ* assay revealed no significant differences in the mean density (cpms/mg tissue) of mRNA (see figure 17) $F_{(9,90)} = .585$ $p=.806$. Data gathered from the remaining *in situ* assays examining the mRNA mean densities (cpms/mg tissue) of the four subtypes of glutamate receptors revealed no significant differences among treatment and/or the hippocampus infused (see figure 18); GluRA $F_{(9,90)} = 1.438$ $p=.184$, GluRB $F_{(9,90)} = .272$ $p=.981$, GluRC $F_{(9,90)} = .554$ $p=.826$, and GluRD $F_{(9,90)} = .770$ $p=.644$.

Discussion

The data gathered from the behavioral procedure clearly indicated that the local perfusion of the transcriptional inhibitor to a single hippocampus was not sufficient to reliably alter the animals' ability to acquire the spatial task. The subjects' performance in all three of the contingent treatment groups improved significantly in the time necessary to locate the hidden platform. It was presumed that decreases in escape latency over successive sessions indicated the animals' ability to use extra-maze cues for navigation to a particular location (Morris, 1981). Further, any improvement in performance over successive sessions should represent the animals' reference memory capabilities. Presumably, the improvement over successive sessions is due to more specific responses by the space specific pyramidal cells. While there were small differences obtained in the mean latencies to find the hidden platform between the treatment groups, the variability

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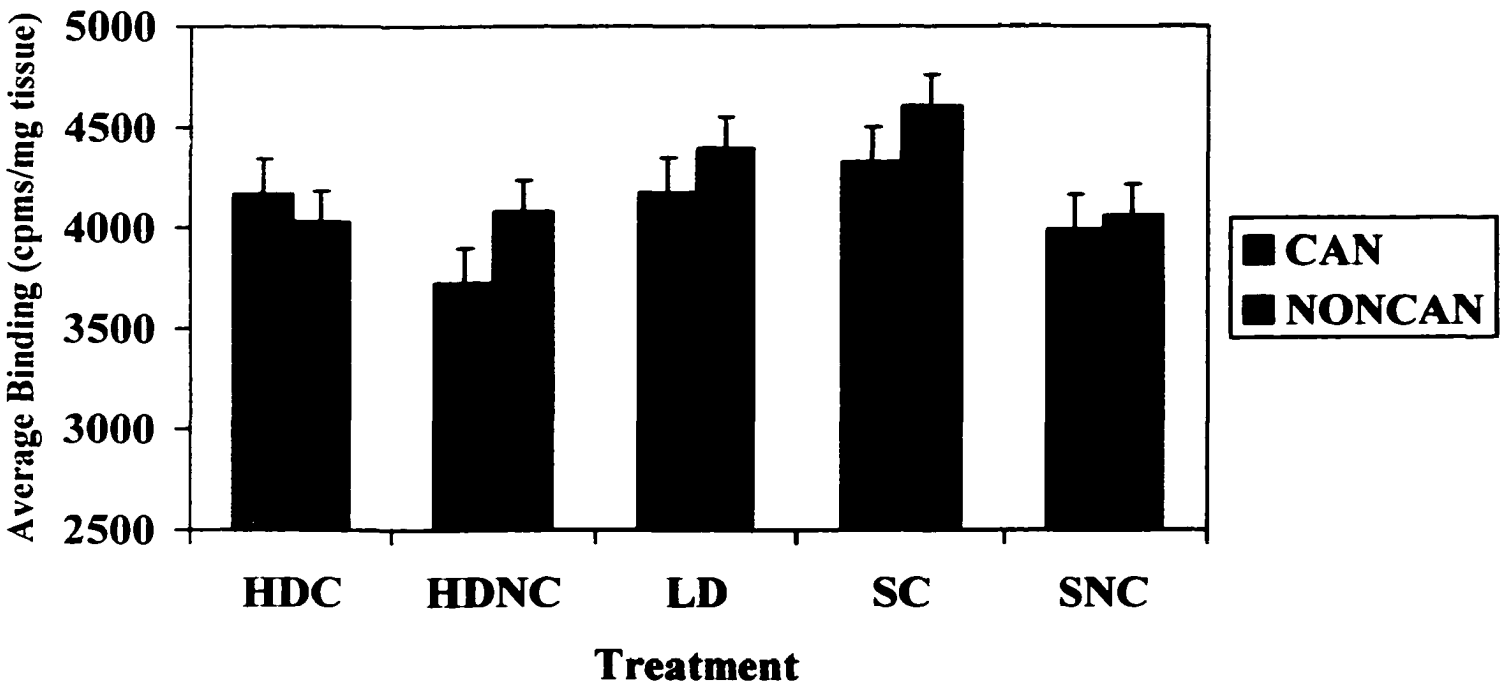

Figure 17: Average density (cpms/mg tissue) (±SEM) for all treatment groups for the NR1 quantitative *in situ* hybridization assays. Each of the infused groups of subjects are represented; High Dose Contingent (HDC-50μM ACT D/ 10μl infused), High Dose Non-contingent (HDNC-50μM ACT D/ 10μl infused), Low Dose (LD-25μM ACT D/ 10 μl infused), Saline Contingent (SC-10μl infused), and Saline Non-contingent (SNC-10μl infused). In addition the cannulated hippocampus (CAN) and non-cannulated hippocampus (NONCAN) are represented.

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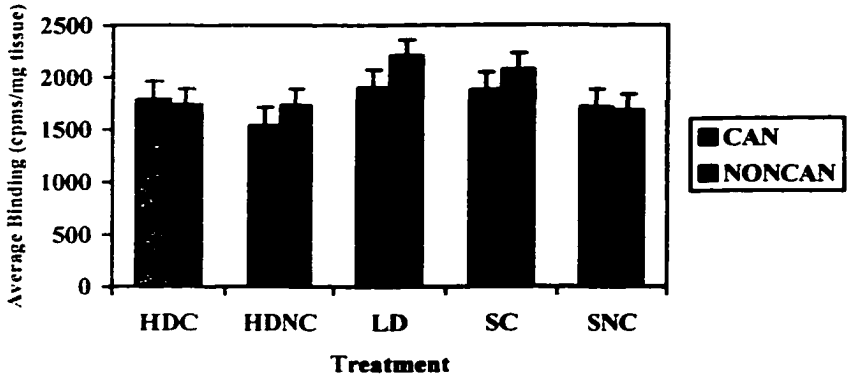
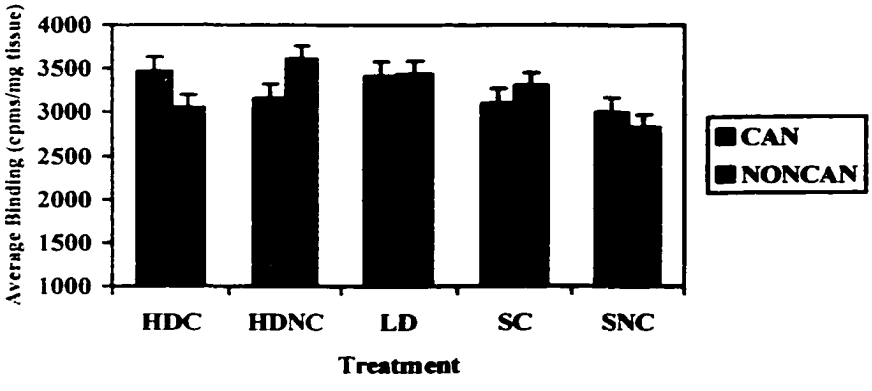
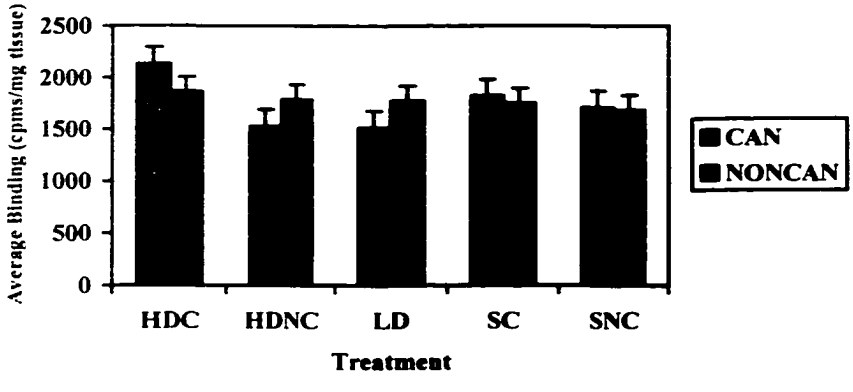
A) GLURA *In situ*



B) GLURB *In situ*



C) GLURC *In situ*



D) GLURD *In situ*

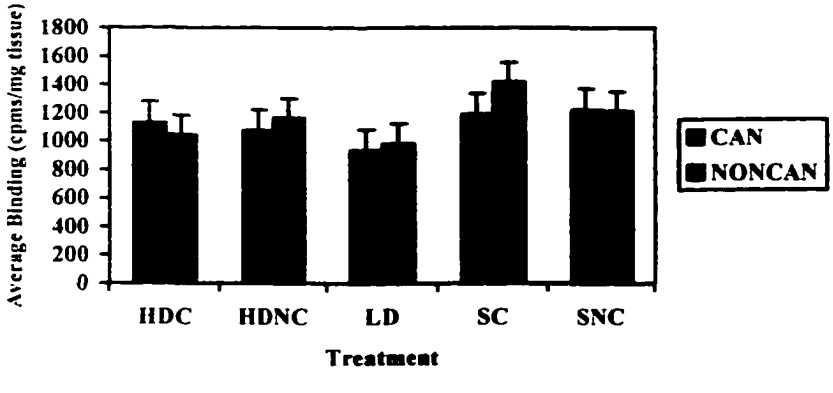


Figure 18: Average density (cpms/mg tissue) (±SEM) for all treatment groups for the quantitative *in situ* hybridization assays for AMPA receptor subtypes. Each of the infused groups of subjects are represented; High Dose Contingent (HDC-50μM ACT D/ 10μl infused), High Dose Non-contingent (HDNC-50μM ACT D/ 10μl infused), Low Dose (LD-25μM ACT D/ 10 μl infused), Saline Contingent (SC-10μl infused), and Saline Non-contingent (SNC-10μl infused). In addition the cannulated hippocampus (CAN) and non-cannulated hippocampus (NONCAN) are represented. A) Glutamate receptor subtype A *in situ* hybridization assay results. B) Glutamate receptor subtype B *in situ* hybridization assay results. C) Glutamate receptor subtype C *in situ* hybridization assay results. D) Glutamate receptor subtype D *in situ* hybridization assay results.

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in latencies eliminated a statistically significant difference (see figure 5). The data gathered from the mean latency to find the platform on the first trial of each session did suggest some differences between treatment groups, however, the individual subject variability eliminated any significant differences. Further evidence of this can be seen in the comparison of mean latency to find the platform for each trial regardless of session. There were no reliable differences between treatments, however, the differences between treatments was much larger on the first trial than the subsequent two trials. This effect suggested that there were slight, although not statistically significant deficits in the reference memory abilities of the drug infused animals. The comparison of each trial's mean latency data also indicated that there were no differences between the treatment groups working memory ability. The ability of subjects' performances to improve within a specific session was used as a measure of working memory capability in the water maze. As can be seen by the data on the second and third trials collapsed across sessions (see figure 9), all treatment groups located the hidden platform on the second and third trials in an equivalent amount of time. Because no differences were observed in the mean swim speed between any of the treatment groups the differences observed on the first trial were not attributable to motor deficits produced by the drug infusion.

The data gathered from the probe trial further indicated that the infusion of the drug did not reliably alter the animals' ability to locate the target quadrant of the maze. These findings clearly indicated that the administration of the drug did not affect very diffuse (i.e. general) learning of the platform quadrant. All three groups spent significantly more time in the target quadrant than chance alone would predict. The second measure of specific importance recorded during the probe trial was the annulus

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crossing data. Significant differences were obtained between the saline infused group and both of the ACT D infused groups. The animals receiving either concentration of the drug crossed over the former platform location significantly less than the saline infused animals. These data were interpreted to indicate that although there were not differences in the persistence data, the differences in annulus crossings suggested that the specificity of what was learned was different based on the solution infused. The animals given a local perfusion of the transcriptional inhibitor to the hippocampus did not learn the specific location of the hidden platform as well as the saline infused animals. If learning the location of the hidden platform is in part due to the fine-tuning of the space specific cells in CA1, the annulus crossing data suggested that the drug infusion did in fact interfere with the specificity of place field formation. These data are increasingly important in that the infusion of the drug did not interfere with learning the general location of the platform.

The remaining behavioral data using the hidden platform was consistent with the data gathered during the place trials. No reliable differences were observed among the treatment groups throughout the reversal phase. These data suggested that the animals receiving the drug were as capable as saline controls to learn a new platform location. No differences in perseverating in the previous target quadrant were observed among the treatment groups. Subjects in all three of the contingent behavioral groups displayed an increase in mean latency to find the hidden platform on the first session of the reversal phase. Following the first session, all of the treatment groups given access to the hidden platform located the platform in its new location as rapidly as the last session of the place

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trials. These data suggested that the administration of the drug did not reliably interfere with the animals' ability to alter their behavior to find the platform in the water maze.

The data collected during the cued phase further established that there were no sensory or motor deficits caused by the administration of the drug. During the cued phase the use of a visible platform in the water at several different locations in the maze provided a measure of general sensory and motor ability of the subjects. Because the cued platform does not require the subjects to use spatial navigation to escape the water, any differences produced would have been independent of the working and reference memory components necessary throughout the other water maze phases.

A final behavioral observation needs to be included in the description of the performance of the subjects receiving the infusion of ACT D. It appeared to the researchers that throughout the place trials phase subjects receiving the infusion of the drug engaged in a different search behavior compared to the saline controls. It appeared that the search strategy that the drug subjects utilized consisted of a sweeping movement laterally as they swam through the maze. In contrast it appeared that the saline infused subjects swam in a more linear single direction while in the maze. The above differences observed were too subtle to be detected by the tracking system consistently; however, the small differences in mean distance on the first trial appeared to be larger than the mean latency data would predict. Because the mean swim speed for each of the treatment groups was nearly identical, the discrepancy observed, although not statistically significant, seemed to support the contention that the animals receiving the drug searched for the platform using a sweeping strategy. If the animals receiving the drug were not able to learn the platform location as specifically as the saline infused subjects, as the

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probe data indicated, then the drug infused subjects could have learned to solve the task from very general spatial information coupled with a different search strategy once in the target quadrant. Such a behavioral adaptation could account for the lack of differences in mean latency to find the platform, while reliable differences appeared in the annulus crossing data.

The combined behavioral data suggested that a 50 μM and 25 μM infusion to locally perfuse a single hippocampus were not sufficient to abolish learning the hidden platform version of the morris water maze. The behavioral data also indicated that there were differences in the specificity of what was learned based on the administration of the drug. This data was specifically relevant to previous work investigating place field firing properties in hippocampal CA1 pyramidal cells. It has been suggested that the means by which an organism learns a spatial environment is the tuning of the space specific cells. It has been further argued that the tuning of the space specific cells is accomplished via LTP. In the current study the administration of a transcriptional inhibitor that had been shown to abolish the late phase of LTP did not abolish learning; however, the drug did interfere with the learning of the platform's specific location. These findings are consistent with the idea that the drug interfered with the tuning of space specific cells in the infused hippocampus, and thus the differences observed on the probe trial. The most crucial follow up study may be to examine the activity of the space specific cells following the same behavioral study. Several researchers have utilized multi-unit recordings to examine space specific firing of CA1 pyramidal cells while an animal engages in a spatial task. If the interpretation of the behavioral data in the current study is accurate, recording from place cells should indicate that the place field formation in the

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drug infused subjects was more diffuse than the saline control place fields. The ability of the drug infused subjects to localize the target quadrant during the probe trial was consistent with the interpretation that the place field formation in the drug infused subjects was more diffuse than the saline infused subjects'. In addition the lack of differences in the place trials latency data between the drug infused groups and the saline infused group may reflect the ability of a single non-infused hippocampus and a modified search strategy to efficiently allow some retention of the spatial task. However, as the probe data indicated, the degree to which the drug animals remembered the task was significantly lower.

The data from the quantitative autoradiography tissue assays clearly demonstrated that the infusion of the drug at either concentration decreased the density of binding to AMPA and NMDA receptors in the infused hippocampus. The mean densities of binding to AMPA receptors in the infused hippocampus for both concentrations of the drug were significantly lower than the mean densities of binding in the non-infused or saline infused hippocampi. The data collected from the NMDA autoradiography assays were consistent with the AMPA data. The differences observed between the drug infused hippocampi and the non-infused hippocampi and the saline infused hippocampi was similar for NMDA and AMPA. The data clearly indicated that the infusion of the drug decreased the amount of binding to AMPA and NMDA receptors in the infused hippocampus. There were no differences in AMPA and NMDA receptor binding in the non-infused hippocampi, indicating the infusion procedure did not affect the receptor binding density.

It is not clear from the autoradiography assays however, if the altered binding densities were due to a reduction in the number of glutamate receptors, or if the

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differences reflected a decrease in the affinity of the examined receptors. The use of a single concentration of labeled ligand did not allow an examination of the specific changes in binding density the administration of the drug produced. The collected data from the autoradiography assays did support the hypothesis that the administration of the transcriptional inhibitor decreased the binding of ligand to glutamate receptors in the CA1 pyramidal cells. It is likely that the reduction in glutamate receptor binding density reflected the importance of new protein synthesis during and immediately following learning a spatial task. The most plausible means of the altered densities of these receptors would be an increase in the production of the receptor precursors, which the administration of the transcriptional inhibitor interfered with, thus the reduced receptor binding densities. It is however, possible that the administration of the drug reduced the binding affinity of glutamate receptors in the infused hippocampus. Such an alteration would decrease the efficiency of NMDA and AMPA receptors without altering the number of receptors in CA1. It would be possible to determine if the altered binding densities were due to a change in receptor production, or a change in the affinity of the examined receptors. If several concentrations of labeled ligand were used in the autoradiography procedures, changes in the number or the affinity of those receptors would be delineated. The most likely explanation of the differences in receptor binding density observed was that the density of receptors was altered due to the site of action of the drug infused. There has been no evidence gathered that the transcriptional inhibitor used acts outside of the nucleus.

The data gathered from the *in situ* hybridization assays did not indicate any reliable differences in the density of the message precursor (mRNA) for NMDA (NR1),

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or the four subtypes of AMPA receptors (GluRA – GluRD) examined. These data suggest that the administration of the transcriptional inhibitor did not affect the production of the mRNA of the crucial subunits that form intact glutamate receptors in CA1. The NR1 subunit examined is the necessary subunit of a functioning NMDA receptor, while the AMPA mRNA represents the precursors for the four most highly expressed AMPA receptors in CA1. It is possible; however, that the examination of the density of the mRNA occurred too long after any alteration in production of mRNA was produced. The possibility exists that there was an alteration in the production of new transcriptional products for AMPA and NMDA due to the drug early on in administration. However, in the thirteen days the administration of the drug was carried out any changes in mRNA synthesis may have returned to baseline levels.

Taken together the data from the tissue assays suggest that the administration of the drug reliably affected the density of binding of ligand to intact glutamate receptors without affecting the concentration of the mRNA precursors for those receptors. This was interpreted to indicate that if the differences were due to a change in mRNA the alteration in the density of the receptor binding was made in a very short time frame early in the behavioral procedure. It seemed likely that whatever alteration in the expression of the receptors responsible for excitatory inputs in the CA1 field would have been initiated as soon as the animals were exposed to a novel environment. It was hoped that any alteration in the production of the mRNA for the glutamate receptors would be maintained throughout the thirteen days, however, the data from the *in situ* assays did not indicate any differences after the thirteen day procedure. If the observed reductions in the density of AMPA and NMDA receptor binding in the drug infused groups was due to

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a decrease in the mRNA production in the nucleus, the changes or lack thereof in either the drug infused hippocampi, or the non-infused and saline hippocampi may have occurred very rapidly and then returned to comparable levels. An important follow up procedure could be performed examining the expression of the mRNAs examined in the current study over the time course of the behavioral procedure. Instead of examining the mRNA levels following the behavioral procedure, harvesting tissue on each day and examining the density of the mRNA over time would delineate the explanation that changes in AMPA and NMDA receptor precursors occurred more rapidly than the current study could examine. Much of the current work in synaptic plasticity (Matthies, 1989; Abraham, Mason, Demmer, Williams, Richardson, Tate, Lawlor, and Dragunow, 1993) has focused on the expression of IEG's in LTP. These studies have examined not only the proteins produced due to high frequency stimulation, but also the time course of gene expression. The alterations in proteins happens in a very short time frame, thus examining mRNA at periodic intervals in the current study might determine if changes in proteins synthesized in the nucleus was involved in the differences seen in receptor binding density. For a review of this material see Thomas and Hunt (1996).

Another interpretation of the tissue data was that the results were due to alterations in receptors in CA1 without the involvement of changes in mRNA. This interpretation would suggest that the insertion of functional AMPA and NMDA receptors at specific synapses were affected by the administration of the transcriptional inhibitor, even though the production of the mRNA was not. This interpretation also suggests that the changes in receptor binding density were not regulated by changes in nuclear events, rather the receptor binding was being modified at the synapse. The theory of receptor

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modifications at the synapse not requiring nuclear events has recently been explored (McNamera, Vandongen, and Vandogen, 1999). These researchers have explored protein synthesis at the dendrites, and have discovered that some synthesis related to LTP does occur at the specific synapses in CA1. Electron micrographs have found substantial cellular machinery (ribosome's, glogi, ect...) in the dendridic tree of the pyramidal cells in the molecular layer of CA1. The advantages of such a mechanism would be a very specific alteration in the synapses in a very short period of time following increased activity.

An additional component of the increased AMPA and NMDA receptors in CA1 without being mediated by nuclear events may be found in some recent work investigating cellular alterations not involving IEG's or LRG's. Several studies (Engert and Bonhoeffer, 1999) have explored the changes in synaptic structure following high frequency stimulation to induce LTP specifically at the synapse. A relatively new hypothesis in this literature has focused on the phenomenon of what has been termed a "silent synapse" (Nicoll and Malenka, 1999; Malenka, 1999). The silent synapse theory involves NMDA receptors in the membrane of the neuron without functional AMPA receptors nearby. Without functional AMPA receptors the NMDA channels can not be activated. In theory, the high frequency stimulation of specific cells recruits functional AMPA receptors into the membrane, thus allowing NMDA to be activated. This alteration would increase the excitatory input at the synapse substantially. The research to date has discovered the insertion of AMPA receptors following the induction of LTP; in addition the receptors that have been inserted seem to be already located in the dendritic tree. These increases in AMPA receptors observed in hippocampal CA1

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pyramidal cells were accomplished not only faster than could have occurred if mediated by nuclear events, but the mRNA precursors for the AMPA receptors were localized in the synapses prior to LTP induction. It is not completely clear if these changes in receptor density are sustained for long time periods, or if the alteration is a relatively temporary alteration that is accomplished until such time as nuclear alterations in the synapse can be performed in order to sustain the LTP. The evidence that has been gathered suggests that there may be AMPA receptors that are held in reserve until the appropriate stimuli elicit their insertion into the membrane of the neuron. This insertion would clearly facilitate the responsiveness of the postsynaptic cell to presynaptic excitation. Furthermore, if non-inserted AMPA receptors exist near the synapse then a change in density can occur without a change in mRNA production.

In addition to alterations in the current procedure for examining the time course of mRNA expression, an additional procedural inclusion could elucidate the differences in receptor binding density observed. Current research (Shi, Hayashi, Esteban, Piccini, Poncer, and Malinow, 1999) has discovered a possible role for CaMKII as a marker for AMPA receptor insertion into the membrane of a neuron. Not only has CaMKII been implicated in phosphorylating intact glutamate receptors, the recent work has suggested that active CaMKII may serve as a marker for synapses engaged in a high degree of activity. Recent work has discovered a region of the GluR 1 (A) that may serve as a site for CaMKII driven delivery of AMPA receptors to specific synapses (Shi, Hayashi, Esteban, Piccni, Poncer, and Malinow, 1999). One of the problems with much of the current theory on an upregulation of specific receptors being responsible for lLTP has been the means by which the cell inserts receptors at specific synapses. Using a

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procedure for imaging or localization of the density of CaMKII in CA1 cells, it would be interesting to determine if the differences observed in the current study in NMDA and AMPA receptors would be consistent with any changes in active forms of CaMKII. If the CaMKII second messenger is in fact a marker for the location undergoing synaptic modification, it seems plausible that regions showing increased CaMKII activity would also show increased density of AMPA and/or NMDA receptors. It would also be of interest to determine from electrophysiological studies if the specific stimulated synapses when inducing LTP showed differences in CaMKII and glutamate receptors. Additionally, the insertion of receptors into those regions would interact with CaMKII to alter the signal, a possible feedback mechanism. This feedback could be used to examine the involvement of transcriptional activation if coupled to the methods employed in the current project, with the inclusion of determining a time course of mRNA expression, as well as CaMKII activity over time.

Taken together the data gathered from the current experiment elucidated a role for the activation of transcription for specific learning of a spatial task. The differences observed behaviorally clearly demonstrate that transcriptional inhibition of a single hippocampus is not sufficient to eliminate spatial learning; however, the data does suggest that the administration of the transcriptional inhibitor did interfere with the specificity of learning. In addition the data from the tissue assays indicated that the administration of the transcriptional inhibitor did affect the density of ligand binding to AMPA and NMDA receptors in the hippocampus, without affecting the mRNA of those receptors as examined after the thirteen day procedure. Several researchers (Chen and Tonegawa, 1997) have suggested that consolidation is in fact carried out via an LTP

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mechanism. in addition the administration of ACT D has been found to interfere with the production of the late phase of LTP (Nguyen. Abel. and Kandel. 1994). The data from the present experiment was interpreted as furthering the connection between protein synthesis and the consolidation of a spatial task. as well as possibly the late phase LTP and consolidation. It is interesting to note that the infusion of the drug at a concentration sufficient to abolish the late phase of LTP in hippocampal slices was not sufficient to eliminate the subjects' abilities to acquire the spatial task. This result may simply reflect the lateralization of function between the hippocampi; i.e. the ablation of the late phase of LTP in a single hippocampus is not sufficient to abolish learning with the contralateral hippocampus functioning normally. Alternatively, the data gathered may indicate that new protein synthesis represents only a portion of the cellular activity necessary for consolidation of spatial memories. The work to date has only begun to determine the numerous mechanisms involved in the neural activity responsible for learning behavior. It seems clear that the physiological process that underlies the ability of organisms to interact and retain information about their environment is a very complicated series of events.

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References

Abelovich, A., Paylor, P., Chen, C., Jeansok, J., Wehner, J., and Tonegawa, T. (1993). PKC mutant mice exhibit mild deficits in spatial and contextual learning. Cell, Vol. 75, 1263-1271

Abraham, W.C., Mason, S.E., Demmer, J., Williams, J.M., Richardson, C.L., Tate, W.P., Lawlor, P.A., and Dragunow, M. (1993). Correlation's between immediate early gene induction and the persistence of long-term potentiation. Neuroscience, Vol. 56 No. 3, 717-727.

Alberini, C., Ghirardi, M., Metz, R., and Kandel, E.R., (1994). C/EBP is an immediate-early gene required for the consolidation of long-term facilitation in aplysia. Cell, Vol. 76, 1099-1114.

Bane, A. (1995). The effects of antioxidant treatment on spatial learning and the N-methyl-D-asparatate receptor. Unpublished doctoral dissertation. Colorado State University, Department of Psychology.

Bliss, T., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthesized rabbit following stimulation of the preforant path. Journal of Physiology. 232, 331-356.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schultz, G., and Silva, A. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element binding protein. Cell, Vol. 79, 59-68.

Chen, C. and Tonegawa, S. (1997). Molecular genetic analysis of synaptic plasticity, activity-dependant neural development, learning, and memory in the mammalian brain. Annual Review of Neuroscience, Vol. 20, 157-184.

Engert, F., Bonhoeffer, T. (1999). Dendridic spine changes associated with hippocampal long-term synaptic plasticity. Nature, Vol. 399, 66-70.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Grant, S. (1996). Analysis of NMDA receptor mediated synaptic plasticity using gene targeting: roles of Fyn and FAK non-receptor tyrosine kinases. Journal of Physiology, Vol. 90. 337-338.

Hebb, D.O., (1949). The Organization of Behavior. New York: Wiley Press.

Hosokawa, T., Rusakov, D.A., Bliss, T.V.P., and Fine, A. (1995). Repeated confocal imaging of individual dendritic spines in the living hippocampal slice: evidence for changes in length and orientation associated with chemically induced LTP. The Journal of Neuroscience, Vol. 15 (8), 5560-5573.

Kandel, E., Schwartz, J., and Jessell, T. (1991). Principles of Neural Science (third edition). Elsevier Science Publishing Co., Inc. New York, New York.

Kullmann, D.M. and Asztely, F., (1998). Extrasynaptic glutamate spillover in the hippocampus: evidence and implications. Trends in Neuroscience, Vol. 21 (1), 8-14.

Magnusson, K.R., (1995). Differential effects of aging on binding sites of the activated NMDA receptor complex in mice. Mechanisms of Aging and Development, Vol. 84 (3), 227-243.

Malenka, R.C. (1999). Special Lecture: LTP and LTD: A Decade of Progress? Annual Meeting; Society for Neuroscience, Miami FL.

Martin L.J., Blackstone, C.D., Levey, A.L., Huganir, R.L., and Price, D.L. (1993). AMPA glutamate receptor subunits are differentially distributed in rat brain. Neuroscience, Vol. 53 (2), 327-358.

Matthies, H. (1989). In search of cellular mechanisms of memory. Progress in Neurobiology, Vol. 32, 277-349.

McHugh, T., Blum, K., Tsien, J., Tonegawa, S., and Wilson, M. (1996). Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. Cell, Vol. 87, 1339-1349.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

McNamera, J.O., Vandongen, H.M.A., and Vandongen, A.M.J. (1999). Dynamics of protein synthesis visualized in dendrites of living hippocampal neurons. Annual Meeting: Society for Neuroscience, Abstract 183.10.

Morris, R.G., (1989). Synaptic plasticity and learning: selective impairment of learning rats and blockade of long-term potentiation in-vivo by the N-methyl-D-asparatate receptor antagonist AP5. Journal of Neuroscience, Vol. 9 (9), 3040-3057.

Morris, R.G., Anderson, E., Lynch, G., and Baudry, M. (1986). Selective impairment of learning and blockade of long term potentiation by N-methyl-D-asparatate receptor antagonist, AP5. Nature, Vol. 319, 774-776.

Morris, R.G., Garrad, P., Rawlins, J.N., and O'Keefe, J., (1982). Place navigation impaired in rats with hippocampal lesions. Nature, Vol. 297 (5868), 681-683.

Murphy, K.P., Reid, G.P., Trentham, D.R., and Bliss, T.V. (1997). Activation of NMDA receptors is necessary for the induction of associative long-term potentiation in area CA1 of the rat hippocampal Slice. Journal of Physiology, Vol. 504 (2), 379-385.

Nguyen, P.V., Abel, T., Kandel, E.R., (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. Science, Vol. 265, 1104-1107.

Nicoll, R.A. and Malenka, R.C. (1999). Expression mechanisms underlying NMDA receptor-dependant long-term potentiation. Annals of the New York Academy of Sciences, Vol. 30: 868, 515-525.

Otani, S., Marshall, C., Tate, W., Goddard, G., and Abraham, W. (1989). Maintenance of long term potentiation in rat dentate gyrus requires protein synthesis but not messenger RNA synthesis immediately post-tetanization. Neuroscience, Vol. 28, (3), 519-526.

Qi, M., Zhuo, M., Skalhegg, B., Brandon, E., Kandel, E., McKnight, S., and Idzerda, R. (1996). Impaired hippocampal plasticity in mice lacking the Cβ1 catalytic subunit of cAMP-dependant protein kinase. Proceedings of the National Academy of Science of the United States of America, Vol. 93, 1571-1576.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Robertson, H. (1991). Immediate-early genes, neuronal plasticity, and memory. Biochemistry and Cell Biology Vol. 70, 729-737.

Schuman, E. and Madison, D. (1991). A requirement for the intracellular messenger nitric oxide in long term potentiation. Science, Vol. 254, 1503-1506.

Schuman, E. and Madison, D. (1994). Locally distributed synaptic potentiation in the hippocampus. Science, Vol. 263, 532-536.

Shi, S.H., Hayashi, Y., Esteban, J.A., Piccini, A., Poncer, J.C. and Malinow, R. (1999). Sites on GluR1 carboxyl terminus controlling CaMKII driven delivery of AMPA receptors to synapses. Annual Meeting: Society for Neuroscience, Miami FL. Abstract 117.9.

Silva, A., Stevens, C., Tonegawa, S., and Wang, Y. (1992a). Deficient hippocampal long-term potentiation in calcium-calmodulin kinase II-mutant mice. Science, Vol. 257, 201-206.

Silva, A., Paylor, R., Wehner, J., and Tonegawa, S. (1992b). Impaired spatial learning in calcium-calmodulin kinase II-mutant mice. Science, Vol. 257, 206-211.

Son, H., Hawkins, R., Martin, K., Kiebler, M., Huang, P., Fishman, M., and Kandel, E. (1996). Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. Cell, Vol 87, 1015-1023.

Takahashi, J., Pinto, L., and Vitaterna, M. (1994). Forward and reverse genetic approaches to behavior in the mouse. Science, Vol. 264, 1724-1733.

Takumi, Y., Matsubara, A., Rinvik, E., and Ottersen, O.P., (1999). The arrangement of glutamate receptors in excitatory synapses. Annals of the New York Academy of Sciences, Vol. 868, 474-482

Tingley, W.G., Ehlers, M.D., Kameyama, K., Doherty, C., Ptak, J.B., Riley, C.T., and Huganir, R.L., (1997). Characterization of protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. The Journal of Biological Chemistry, Vol. 272, No. 8, 5157-5166.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Thomas, K.L., and Hunt, S.P. (1996). Postsynaptic gene expression and long-term potentiation in the hippocampus. In: Cortical Plasticity LTP and LTD, 103-136. Edited by Fazeli, M.S., and Collingridge, G.L., Bios Scientific Publishers.

Tsien, J., Huerta, P., and Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependant synaptic plasticity in spatial memory. Cell, Vol. 87, 1327-1338.

Wilson, M. and McNaughton, B.L. (1993), Dynamics of the hippocampal ensemble code for space. Science, Vol. 261(5124), 993-994.

Wilson, M. and Tonegawa, S. (1997). Synaptic plasticity, place cells and spatial memory: study with second generation knockouts. Trends in Neuroscience, Vol. 20, No. 3, 102-106.

Wong, E., and Kemp, J. (1991). Sites for antagonism on the N-methyl-D-asparatate receptor channel complex. Annual Review of Pharmacology and Toxicology, Vol. 31, 401-425.

Xie, X., Barrionuevo, G., and Berger, T.W., (1996). Differential expression of short-term potentiation by AMPA and NMDA receptors in dentate gyrus. Learning and Memory, Vol. 3(2-3), 115-123.

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