December 1992

The Effect of Trimethyltin on the Cholinergic System of the Rat Hippocampus

Richard L. Cannon

East Tennessee State University

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The effect of trimethyltin on the cholinergic system of the rat hippocampus

Cannon, Richard Lee, Ph.D.
East Tennessee State University, 1992

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THE EFFECT OF TRIMETHYLTIN ON THE
CHOLINERGIC SYSTEM OF THE RAT HIPPOCAMPUS

A Dissertation Presented to
the Faculty of the Department of Anatomy
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Science

by
Richard Lee Cannon
December 1992
APPROVAL

This is to certify that the Graduate Committee of

Richard Lee Cannon

met on the

Twenty-ninth day of September, 1992

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Associate Vice-President for Research and Dean of the Graduate school, in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

[Signatures]

Approved on behalf of the Graduate Council

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean, School of Graduate Studies

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ABSTRACT
THE EFFECT OF TRIMETHYL Tin ON THE
CHOLINERGIC SYSTEM OF THE RAT HIPPOCAMPUS
by
Richard Lee Cannon

Trimethyl tin (TMT) is a neurotoxin occurring in the environment. Exposure to TMT is known to destroy specific neuronal components of the hippocampus in the rat and to cause clinical symptoms in exposed humans, including mnemonic deficits, that indicate hippocampal involvement. In addition to hippocampal cell loss TMT causes significant increases in cholinergic markers such as acetylcholinesterase (AChE) stain density and choline acetyltransferase (ChAT) activity in the hippocampus of rats. However, despite these observations the effect of TMT on hippocampal cholinergic system has not been investigated in detail. The purpose of the present study was to elucidate more fully the consequences of TMT administration on the rat cholinergic system. To this end the effects of increasing doses of TMT and time after TMT administration on choline acetyltransferase (ChAT) activity as well as TMT's effect on cholinergic muscarinic receptors was examined.

Results indicate that 4 and 6 mg/kg doses of TMT measurable neuropathological effects on pyramidal cells of the hippocampus. ChAT activity was increased in the hippocampus as a result of the 6 mg/kg dose. Six mg/kg TMT was observed to affect morphology differentially over time, with the various sub-fields examined being affected at different time intervals. The effect of time on increased ChAT activity after TMT-treatment was observed in the dentate gyrus prior to the CA1 region. The effect of 6 mg/kg TMT on muscarinic receptor distribution over time is first observed in CA1 and CA3c then CA3a-b of the subtype M2. The subtype M1 receptors are also affected in these regions but at later time intervals. The total distribution of muscarinic receptors is reduced in regions CA1 and CA3c. This is observed at similar time intervals as for M1 receptors.

The conclusions made as a result of these findings are: (1) That TMT's effect on ChAT activity and morphology of the rat hippocampus is both dose and time dependent; (2) That adverse effects of TMT on ChAT activity and morphology in sub-regions of the hippocampus are observed at different time intervals; and (3) That the distribution of the muscarinic receptors examined are affected by TMT in a regional manner dependent upon the time following administration of TMT.
DEDICATION

This dissertation is dedicated to Dawn S. Conner and her daughter Jami. In knowing and loving Dawn and Jami I have come to realize what true love for another person is all about.
ACKNOWLEDGMENTS

I would like to express my gratitude to the members of my committee; Dr. Donald Hoover for his guidance and assistance in the technical aspects of my project, Dr. Ronald Baisden for keeping my outlook in the proper perspective, Dr. Walter Isaacs for his understanding of the frustrations that a graduate student goes through, and to Dr. Richard Skalko, Chairman of the Anatomy Department, for his support and encouragement throughout my years as a graduate student.

I am most grateful to my major advisor, Dr. Michael Woodruff who inspired me to learn beyond that which is required and instilled in me the inquisitiveness needed to succeed in my studies and in the future.

I am also grateful to Dr. Ken Ferslew for teaching me to think about my results with a different perspective and being my friend.

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To the family of Dawn Conner: Norman and Jean Campbell; Robin and Eddie Hincke and their children, Jason, Justin, and Jacqui; Ken and Patty Campbell and their daughter Chantai. For the love and friendship you have shown to Rick and myself this all to short of time I have been here in Tennessee I will be eternally grateful. I love you all.
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A variety of compounds and elements found in the environment are known to cause permanent damage to the central nervous system. Whether naturally occurring, or the result of man's quest for new industrial technologies, the number of known neurotoxins continues to increase (U.S. Congress Office of Technology Assessment, 1990). For this reason, it is appropriate to delineate as clearly as possible the effects of environmental neurotoxins on the central nervous system.

The ability of some neurotoxic substances to cause damage to specific cell populations in discrete brain regions has led to proposals that the effect of these substances be used to model neuropathologies observed clinically in man. However, before a substance that creates a lesion in an infrahuman species can be accepted as a feasible model of a specific human disorder, it is necessary to describe as completely as possible the changes produced in the animal's central nervous system. One such neurotoxin is trimethlytin (TMT); an organometal neurotoxin found in both natural (Hallas et al., 1982) and man-made (Fortemps et al., 1978) environments. TMT neurotoxicity has been proposed as a model for Alzheimer's disease (AD), (Hagan et al., 1988) due to the selective neuropathology it produces in
both cortical and subcortical structures of the central nervous system which is similar to that seen in AD patients. The behavioral effects of TMT, particularly the mnemonic impairments observed in animals after exposure to TMT, also mimic at least some of the clinical signs and symptoms of AD.

Neuronal cytotoxic effects of TMT

The clinical consequences of TMT poisoning in humans suggest hippocampal involvement. For example, memory loss is a common complaint (Fortemps et al., 1978; Ross et al., 1981). Neuropathological findings in animals support this proposal. A brief overview of the structural components of the hippocampus, including the main afferent pathway, allows for greater appreciation of the effects of TMT on this brain region. The hippocampal formation consists of the entorhinal cortex, the subicular cortices, the dentate gyrus, and Ammon's horn (Figure 1). The entorhinal cortex receives input from virtually all neocortical and several subcortical areas of the brain. Its connection to the hippocampus makes it the main source of multi-modal input to the hippocampus. The axons from the entorhinal cortex to hippocampus are known as the perforant pathway and arise from cells in layer II of both the lateral and medial entorhinal areas. Fibers from the lateral entorhinal cortex terminate in the outer molecular layer of the dentate while those of the medial
Figure 1. An overview of the anatomical relationships among the various components of the hippocampal formation is presented in this low power photomicrograph of a horizontal section. The blade arrowhead indicates the point of transition between the pre-pyriform cortex and the entorhinal cortex (EC). The EC gives rise to the perforant pathway which terminates in the outer two-thirds of the molecular (M) layer of the dentate gyrus, where it synapses on dendrites of the dentate granule cells (G). The transition between the EC and hippocampus is continuous and extends through the parasubiculum (PS beginning at black arrow) and subiculum (S between open arrowheads) to field CA1 of Ammon's horn which contains pyramidal neurons. The pyramidal cells of field CA3 continue from CA1 and extend into the hilus (H) of the dentate gyrus. CA3 is divided into three parts labeled a, b and c. The subcortical outflow of the hippocampus goes through the fimbria (F). Abbreviation (hf) denotes the hippocampal fissure. Thionin stain. Scale bar = 495 μm.
entorhinal cortex terminate in the medial portion of the dentate molecular layer and in the stratum moleculare-lacunosum of Ammon's horn. The dentate granule cells that receive input from the entorhinal cortex send out their axons, referred to as mossy fibers, to contact dendrites of hippocampal pyramidal cells in all subsections of CA3. These cells in turn send out their axons, called Schaffer collaterals, to synapse in stratum lacunosum on pyramidal cell dendrites of field CA1. The integrity of the perforant pathway is essential for normal hippocampal function. TMT causes the death of cells in the entorhinal cortex which give rise to the perforant pathway (Brown et al., 1979; Chang et al., 1982a,b; Chang et al., 1983; Chang et al., 1984; Chang and Dyer, 1983).

The first reports of TMT-induced neuropathology in rats noted destruction of specific hippocampal neurons not observed with the control substances triethyltin and metacrylate. The alterations observed in the hippocampus included loss of pyramidal cells in the subfields CA1, CA2, CA3a-b, and CA3c in a dose-related basis. Higher doses of TMT, for example 4 mg/kg given once weekly for four weeks, induced a greater loss of pyramidal cells in these CA subfields and also destroyed neurons in the facia dentata (Milner, 1970; Brown et al., 1979) However, some neurons within the hippocampus resist the effects of TMT. Brown et al., (1979) indicated that pyramidal cells in CA3a and CA3b
were spared at even the highest doses. However the higher
doses of TMT did cause damage to the amygdala, piriform
cortex, and lateral neocortex.

The observation of TMT-induced hippocampal damage has
been replicated many times (Balaban et al., 1988; Bouldin et
al., 1981; Chang and Dyer, 1983; Chang et al., 1983; Dyer et
al., 1982a,b; Loullis et al., 1985; Valdes et al., 1983)
with the only apparent disagreement in these reports being
the pattern of cell loss seen in the subfields of the
hippocampus. Valdes et al., (1983) reported findings similar
to those of Brown et al., (1979) i.e. sparing of CA3a and
CA3b, but Dyer et al., (1982a,b) reported complete cell loss
in all of CA3 with sparing of CA1 and CA2. These
discrepancies were partially resolved by Chang and Dyer,
(1985) who reported that investigators had been examining
different anterior-posterior levels of the hippocampus.
Apparently the area of cell loss observed following
administration of TMT depends on the rostral-caudal level
being examined along the septo-temporal axis of the
hippocampus no matter what dose of TMT is involved. Chang
and Dyer, (1985) found heaviest damage to the fascia dentata
granule cells in the temporal regions of the hippocampus,
while the CA3a and CA3b pyramidal cells were most affected
at the septal pole, but preserved in the temporal
hippocampus. The difference in reports of damage to CA1 and
CA2 was not resolved by Chang and Dyer, although it has been
reported that cell loss occurs consistently in these areas after TMT poisoning (Hagan et al., 1988; Whittington et al., 1989).

Effects of TMT on Behavior

The behavioral effects observed in TMT-treated rats are similar to those reported in animals with hippocampal lesions. For example, either hippocampal lesions (Isaacson, 1982) or exposure to TMT (Loullis et al., 1985; Walsh et al., 1982a) impair passive avoidance acquisition. Radial arm maze (RAM) performance is also impaired in rats with surgical lesions of the hippocampus (Olton et al., 1978) and a single dose of 6 mg/kg TMT given to adult rats causes an impairment in this task (Walsh et al., 1982b). Further, neonatal rats given 0.3 mg/kg TMT on alternate days from postnatal day 3 to day 29 also show a deficit in acquisition and performance of the radial arm maze task (Miller et al., 1982).

Miller et al., (1982) reported that their animals were hyperactive as adults. Hyperactivity has often been reported as a consequence of TMT treatment and is observed beginning 1 to 3 days following exposure in the adult and continuing throughout the remainder of the animal’s life (Ruppert et al., 1982; Swartzwelder et al., 1981). Comparing these results suggest that TMT’s effect on the hippocampus is the causative factor for the hyperactivity. Finally, TMT
produces deficits in performance of rats on an operant delayed reinforcement (DRL) schedule (Mastropaolo et al., 1984). Deficient performance on this task by rats is a consistent consequence of hippocampal damage (Clark and Isaacson, 1965; Kearly et al., 1974).

Effects of TMT on Neurotransmitter Systems

The effects of TMT on neurotransmitter systems within the hippocampus have not been as well established as the effects of this neurotoxicant on hippocampal cell bodies. However, some experiments have found alterations in glutamic acid (Glu) and gamma-aminobutyric acid (GABA) uptake in the hippocampus of TMT treated rats at 7 and 14 days (Valdes et al., 1983) and 21 days (Naalsund et al., 1985; Patel et al., 1990) post-exposure. TMT exposure in rats has been reported to result in a concentration decrease of dopamine (DA) in nucleus accumbens and a decrease of serotonin (5-HT) in striatum, olfactory tubercle, septum, and frontal cortex while these neurotransmitters appear unaffected in the hippocampus of these same rats (DeHaven et al., 1984).

The cholinergic system is of special interest since it is thought to be adversely affected in human neuropathologies such as Alzheimer's disease. The cholinergic innervation of the hippocampus comes mainly from the septo-hippocampal pathway. The neurons of origin in this pathway are located in the medial septal nucleus and the
vertical limb of the diagonal band (Kuhar, 1975; Baisden et al., 1984). The fibers from these neurons terminate primarily in the stratum oriens of Ammon's horn. This layer of the hippocampus is located just below the pyramidal cells. The cholinergic fibers apparently synapse on the basilar dendrites of these cells (Kuhar, 1975). A small portion of the septo-hippocampal cholinergic fibers also terminate in the molecular layer of the dentate gyrus (Mosko et al., 1973). The pyramidal cells of Ammon's horn also receive cholinergic innervation of septal origin. Storm-Mathisen (1970) demonstrated the distribution of cholinergic activity associated with the pyramidal cell layer by histochemical staining. A lighter concentration was associated with the CA1 pyramidal cells than with those in CA3a-CA3c. TMT causes the death of pyramidal neurons that extend their dendrites into stratum oriens and because septo-hippocampal cholinergic fibers synapse on these dendrites, a reduction in cholinergic hippocampal afferents would be an assumed consequence of TMT exposure.

Studies which have examined the density of cholinergic muscarinic receptors in the hippocampus of TMT-treated rats (Loulilis et al., 1985) and mice (Ali et al., 1983, 1986) found decreases in receptor binding up to 14 days post-treatment. These results suggest a decrease in function within the hippocampal cholinergic system as a result of TMT exposure. However, observations by Hagan et al., (1988) and
Woodruff and Baisden, (1990) suggest the opposite effect. Acetylcholine (ACh) is degraded in the synaptic cleft by the enzyme acetylcholinesterase (AChE). Changes in ACh innervation may thus be reflected by changes in histochemical staining for AChE. Woodruff and Baisden, (1990) observed increased AChE staining in the outer molecular layer (OML) of the dentate gyrus in rats exposed to 6 mg/kg TMT and allowed to survive four months. A similar finding in rats treated with 7 mg/kg of TMT and killed four to six weeks later was previously reported (Hagan et al., 1988). Although the results of these two studies are suggestive of increased cholinergic activity, AChE may be present where ACh is not. A more specific and sensitive measure of cholinergic activity is that of the enzyme choline acetyltransferase (ChAT) which is responsible for the synthesis of acetylcholine in the presynaptic bouton of cholinergic neurons. A significant increase in ChAT activity in the hippocampus has been observed at relatively short intervals after TMT exposure (Naalsund et al., 1985) and at longer intervals (Cannon et al., 1991). The increased AChE staining and ChAT activity observed are suggestive of sprouting in the cholinergic septo-hippocampal innervation similar to that which has been observed after lesions of the entorhinal cortex (Lynch et al., 1972; Nadler et al., 1979; Stanfield and Cowan, 1982).

The term "sprouting" refers to the phenomenon of
"reactive synaptogenesis"; a plastic response of the central nervous system to cell or receptor loss due to injury or toxic insult. Surviving neurons either reorganize their remaining fibers and synapses or form new ones in an apparent attempt to compensate for the loss caused by the perturbation. For example, when Lynch et al., (1972) made unilateral surgical lesions of the entorhinal cortex in rats they observed a significant increase in AChE stain density in the outer molecular layer of the dentate gyrus. This observation was interpreted to indicate increased synaptic density consequent to expansion of the septo-dentate cholinergic pathway that innervates this area. Several subsequent studies support this contention, (see Cotman and Nadler, 1978 for review). Since TMT causes the death of a majority of neurons in the entorhinal cortex it would be assumed that a similar result would be observed.

In summary, TMT exposure induces a relatively selective permanent neuropathology without causing permanent damage to organ systems such as liver and kidney (Brown et al., 1979). The neuropathology observed is most prominent in the hippocampus, though a few neurons in some cortical and subcortical regions show necrotic changes. Hippocampal damage has been observed at TMT doses ranging from 2.9 mg/kg (expressed as the free base of TMT; Loullis et al., 1985) to a high of 16 mg/kg given in multiple doses (Brown et al., 1979; expressed as "mg of salt" by the authors,
approximately 11.2 mg/kg as free base weight). Severity of damage is dose dependent (Brown et al., 1979; Bouldin et al., 1981; Dyer et al., 1982a,b). Several behavioral and mnemonic deficits have been shown to result from TMT exposure (McMillan & Wenger, 1985). Finally, knowledge of the effect of TMT on neurotransmitter systems innervating the hippocampus is lacking the detail necessary to fully understand how these systems are affected by this neurotoxin. The experiments contained in this study were performed in order to more fully elucidate the reaction of the cholinergic system of the hippocampus to insult by TMT.

Knowing the minimal concentration of an environmental neurotoxin to which a subhuman species can be exposed before detrimental changes in the CNS are observed is a fundamental requirement of toxicologist in assessing possible effects to man. The first goal of this study was to test the hypothesis that the effect of TMT on the cholinergic system of the hippocampus is dose dependent (i.e. higher doses of TMT would produce greater cholinergic sprouting). The second goal of the study was to determine the relationship between the extent of cholinergic sprouting and amount of time following TMT administration. Experiments directed toward achieving these goals are presented in Chapter 2.

The experiments presented in Chapter 2 examine ChAT activity in the hippocampus of TMT treated rats. This biochemical marker is only found in the presence of
acetylcholine and is quantifiable with reasonable accuracy using established methodologies. The first experiment was undertaken to determine if a dose-response relationship between neurotoxicity caused by TMT and the reported increased activity of cholinergic markers in the dentate and CA1 of the hippocampus exist. The second experiment examined the effect of time on this phenomenon utilizing an effective dose as determined in experiment one.

The purpose of the final experiment was to further elucidate the changes in the cholinergic system of the hippocampus following TMT poisoning. This experiment is presented in Chapter 3. Using ligands specific for receptor subtypes I examined how cholinergic muscarinic receptors in Ammon’s horn (CA1, CA3a-b and CA3c) and the dentate gyrus are affected by TMT. The total distribution of all cholinergic muscarinic receptors was quantified, as well as the density and distribution of muscarinic receptor subtypes $M_1$ and $M_2$. 
CHAPTER 2

Choline Acetyltransferase Experiments

Experiment One: Dose Response

METHODS AND MATERIALS

The effect of a single treatment with 2 mg/kg, 4 mg/kg, or 6 mg/kg TMT (expressed as the free base weight of TMT chloride per rat body weight) on ChAT activity in CA1 of the hippocampus, the outer molecular layer of the dentate gyrus including the buried blade of the dentate, or the caudate nucleus was examined in this experiment. These doses were chosen because they produce a dose-related increase in locomotor activity and vertical rearings in an open field, as well as a dose-related decrease in the number of neurons in Ammon's horn and the dentate gyrus (Cannon et al., in press). These data indicate that these doses of TMT are effective both neurotoxically and behaviorally. Therefore, they are appropriate to indicate the dose-response relationship between TMT and changes in ChAT activity in the hippocampus.

Thirty-two adult male Long-Evans hooded rats served as subjects for this experiment, they were obtained from Charles River Farms and were maintained in the AAALAC-accredited colony facility of East Tennessee State University. The mean weight of the rats was 235 g at the
start of the study. The rats were housed individually in plastic cages with wood chip bedding and maintained on a 12h on 12h off lighting schedule with lights on at 0700 hours daily. Food (Wayne Lab Blox) and tap water were provided ad libitum throughout the experiment. The rats were randomly assigned to one of four groups with 8 animals per group. The various doses of TMT were dissolved in distilled water then administered via oral gavage to the rats in the appropriate treatment groups. Administration of the neurotoxin by gavage has been shown to cause less distress to the animals than intraperitoneal injections (Dyer et al., 1982b), and does not result in damage to other organ systems (Brown et al., 1979). To assess any possible effect of the administration technique on ChAT activity, four of the control rats were orally gavaged with an equivalent volume of distilled water. The remaining four rats served as untreated controls. Acronyms for the groups were consistent with treatment, TMT-2, TMT-4 and TMT-6 for the respective neurotoxin exposed groups and Control for the water gavaged and the untreated normal animals.

Between ninety and one hundred days following treatment the rats were each killed by decapitation and their brains quickly removed and frozen on a specimen plate on crushed dry ice. The brains were then serially sectioned in the coronal plane at -20°C on a microtome (Damon/IEC Division, Needham, Massachusetts) at 20, 40 and 300 μm.
The 20 \( \mu \text{m} \) sections were mounted on gelatin-subbed slides and stained with thionin for examination of morphology. The neuropathological changes caused by the various doses of TMT to the hippocampus were quantified by taking measurements of cell fields utilizing the method described by Dyer et al., (1982a). A Leitz Diaplan microscope and Optimas image analysis system were used to make the following measurements: (1) the length of the pyramidal cell line from the CA1 subiculum interface to the point where an organized pattern of pyramidal cells could no longer be detected (in normal tissue this would correspond to CA3c) (A-B in Figure 2); (2) the overall thickness of the dentate gyrus from the hippocampal fissure to the most ventral tissue of the dentate (C-D in Figure 2); (3) thickness of the dentate granule cell layer at the widest point of the buried blade (E-F in Figure 2); and (4) the thickness of the pyramidal cells in CA1 at its widest point (G-H in Figure 2). Measurements were made on three levels from each brain corresponding to -1.6, -2.6 and -3.0 in the atlas of Pellegrino et al., (1979). Measurements (1) and (2) above were made using a 4x objective with results recorded in millimeters while measurements (3) and (4) were made with the 25x objective and results recorded in microns.

The 40 \( \mu \text{m} \) sections were mounted on clean slides and processed for AChE histochemistry using the method described by Koelle (1955). To inhibit pseudocholinesterase activity,
Figure 2. Diagram of a coronal section at a medial level of a normal rat hippocampus illustrating the measurements made. (A-B) length of the pyramidal cell line; (C-D) thickness of the dentate gyrus; (E-F) thickness of the granule cell layer of the buried blade of the dentate; (G-H) thickness of CA1 Pyramidal cell field.
tetraisopropylpyrophosphoramide (10^{6} \text{ M}) was added to the preincubation solution. These slides were examined for changes in AChE density and served as a reference for the micropunch dissection of the 300 \( \mu \text{m} \) sections utilizing the technique of Palkovits, (1973) to collect samples for ChAT analysis. The 300 \( \mu \text{m} \) sections were freeze-mounted on slides maintained in the cryostat, allowed to thaw momentarily, and then refrozen on dry ice. These slides were then placed in a tight fitting slide box, wrapped with aluminum foil, and stored at -80° C until processing.

For analysis of ChAT activity, samples were collected from the 300 \( \mu \text{m} \) thick sections using a 300 \( \mu \text{m} \) circular micropunch cannula. Three rostral-caudal levels through the hippocampus, corresponding approximately to the AP levels -1.6, -2.6 and -3.4 shown in the atlas of Pellegrino et al., (1979) were examined. The first punch was taken at the crest of the granule cell layer. Subsequent punches were taken extending laterally along the hidden blade. Four or five bilateral micropunch samples were taken at the most septal end of the dentate gyrus. In the more caudal levels of the dentate eight to ten bilateral punches were taken from both the exposed and hidden blades. Three bilateral micropunches of the CA1 region were taken at each level. Additionally a single 1000 \( \mu \text{m} \) micropunch sample was taken from the caudate-putamen at the level of the anterior commissure. This substrate is high in ChAT activity and is not affected by
ChAT analysis was performed using the technique of Fonnum, (1975). Briefly, as the samples were collected they were placed in a polypropylene microtube containing 50 µl of 10 Mm EDTA-0.2% Triton X-100 (Ph 7.4) and homogenized by sonication. ChAT activity was measured in 2 µl aliquots of this homogenate. Acetyl-1-[3H] coenzyme A (1.3 Ci/mmol; Amersham) served as the labeled substrate, with incubations at 37° C lasting 20 min for caudate-putamen and 30 min for CA1 and dentate samples. Five (5) µl aliquots of the homogenate were taken for protein analysis using the technique described by Lowry et al., (1951). ChAT activity was expressed as pmol acetylcholine (ACh) formed/µg protein/hour.

A one-way analysis of variance (ANOVA) was performed to statistically analyze the data collected from the ChAT activity of the caudate-putamen samples. For the CA1 and dentate data a repeated measures ANOVA was performed on each with one factor being treatment and the other factor being anatomical level. Data analysis for the results of histological measurements consisted of a repeated measures ANOVA conducted on each cell field measured as one factor and anatomical level as the second. When significance was indicated by ANOVA results a Least Significant Difference (LSD) post hoc test was applied to the data to determine where significance lie. There were no differences observed
Figure 3. Photomicrographs of coronal sections through the brains of rats exposed to varying doses of TMT. The regions of the hippocampus that may be damaged by TMT are labeled in the section from a control rat brain (A). Two mg/kg TMT had no obvious effect on Ammon's horn (CA1 & CA3a,b,c in A) or the dentate gyrus. However, 4 mg/kg of TMT produced obvious loss of pyramidal cells in the CA3c portion of Ammon's horn (between arrows in C). Six mg/kg of TMT produced more extensive loss of CA3 pyramidal neurons (between arrows in D) than was found after 4 mg/kg TMT and also caused loss of CA1 pyramidal neurons (between arrowheads in D). All sections stained with thionin. Abbreviations: g - granule cells of the dentate gyrus; h - hilar region of the dentate gyrus; mb - molecular layer of the buried blade of the dentate gyrus; me - exposed blade of the dentate gyrus; T - thalamus. Scale bars (in μm): (A) 510, (B) 470, (C) 270 and (D) 350.
Figure 4. High power photomicrographs of CA1 pyramidal cells from the sections in Figure 3. The tightly packed cells seen in the control rat (A) are changed little in the TMT-2 group (B) but are reduced significantly in the TMT-4 group (C). CA1 is reduced to only one or two layers in the TMT-6 animals (D) with necrotic figures still evident over 100 days after dosing (arrows). Scale bars (in $\mu$m): (A) 45, (B) 40, (C) 33 and (D) 59.
Figure 5. These high power photomicrographs are of the CA3c pyramidal cell region from the animals in Figure 3. The normal distribution of cells can be seen in a control rat (A). Two mg/kg TMT had little effect on this area (B), while a reduction in pyramidal cells can be seen in the TMT-4 group (C). Few cells survive in the animal from the TMT-6 group (D) with the remaining cells showing signs of necrosis (arrows). Scale bars (in μm): (A) 229, (B) 213, (C) 301, and (D) 258.
histologically or biochemically within the control group between those gavaged with water and those that remained untreated so they were combined into one group for statistical analysis.

RESULTS

Histological examination of thionin stained sections from all rats indicated a dose-related reduction in CA1 and CA3 pyramidal cell fields with group TMT-6 most affected by the neurotoxin (Figure 3D) when compared to the Control (Figure 3A) and TMT-2 groups (Figure 3B). A significant reduction in pyramidal cells in the CA3c subfield of Ammon's horn can also be appreciated in the TMT-4 group (Figure 3C). The necrotic changes are obvious under higher magnification of CA1 (Figure 4D) from a TMT-6 rat. CA1 is also reduced to a thickness of one or two cells throughout its extent compared to this same cell field in the Control and TMT-2 rats (Figure 4A and 4B respectively). Thinning in CA1 can also be appreciated in the TMT-4 animal (Figure 4C). The pyramidal cells of CA3c are obviously absent in the TMT-4 and TMT-6 groups when viewed with high magnification (Figure 5C and 5D respectively).

Cholinesterase-stained sections revealed an apparent increase in stain density in a dose-related manner in the OML of the dentate and to a lesser extent above and below CA1 (Figure 6A-D). The AChE stain density increase in CA1 is
obvious in both the TMT-4 and TMT-6 groups compared to Control and TMT-2 groups when examined at a higher magnification (Figure 7). In viewing the OML of cholinesterase-stained sections at higher magnification the increased density of staining is most evident in the TMT-4 and TMT-6 groups (Figure 8).

The repeated measures ANOVA indicated that the length of the pyramidal cell body layer of Ammon's horn was greatly reduced. A large statistically significant overall effect was found in this measure of morphological change ($F(3,28)=72.88, p<0.001$). Post hoc results revealed that the 6 mg/kg dose reduced the length of the CA cell field compared to all other groups at all levels examined. The 4 mg/kg dose caused a reduction in the length of the CA cell field compared to controls and the 2 mg/kg group at all levels (Figure 9). The ANOVA applied to measurement results of the width of CA1 was also significant ($F(3,28)=66.79, p<0.001$). When a multiple comparisons post hoc test was applied to these data, it revealed that the 6 mg/kg dose of TMT significantly reduced the width of CA1 compared to all other groups at all levels. The 4 mg/kg dose reduced this measurement significantly compared to controls at all levels and from the 2 mg/kg group at the medial level (Figure 10).

The measurement data from the dentate indicated that this region was not affected to the extent that the CA cell
Figure 6. Cholinesterase-stained sections from representative animals showing the effect of TMT dose on density of staining. The Control rat in (A) appears to have a greater density of staining in the outer molecular layer than the TMT-2 rat (B). In the TMT-4 rat (C) the density of staining can be seen to be increased in both the OML and area CA1 (arrows). Stain density is intensified in all areas of the hippocampus in the section from a TMT-6 animal (D). Scale bars (in μm): (A) 550, (B) 400, (C) 385 and (D) 350.
Figure 7. These photomicrographs are a higher magnification of area CA1 from the sections in Figure 6. In the section from the Control rat (A) staining is seen both above and below (arrows) the pyramidal cell layer (py). The representative of the TMT-2 group (B) shows stain density covering the pyramidal cells, while in the representative of the TMT-4 group (C) the stain density increases and is again restricted to above and below the pyramidal cells (arrows). In the TMT-6 animal (D) the stain density is increased and is found mainly above the few remaining cells (arrow). Scale bars (in μm): (A) 109, (B) 90, (C) 87 and (D) 65.
Figure 8. High power photomicrographs of the buried blade of the dentate gyrus taken from the sections presented in Figure 6. The increased density of AChE reaction is obvious in the outer molecular layer of the dentate in the rat given 6 mg/kg TMT (D). Abbreviations: G - granule cell layer; HF - hippocampal fissure; IML - inner molecular layer; OML - outer molecular layer; SG - supragranular layer. Scale bars (in μm): (A) 101, (B) 94, (C) 87 and (D) 65.
field had been. When an ANOVA was performed on the data from the granule cell layer of the buried blade, (E-F in Figure 2), no significant differences emerged. The ANOVA applied to the overall width of the dentate (C-D in Figure 2) was significant ($F(3,28)=10.38, p<0.001$). A multiple comparisons test performed on these data indicated that the 6 mg/kg TMT differed significantly from Control and 2 mg/kg TMT groups at all anatomical levels examined. The 4 mg/kg TMT group differed significantly from the Control group at the anterior and medial levels and from the 2 mg/kg TMT group at the posterior anatomical level (Figure 11).

The results of the one-way ANOVA for data collected from micropunch samples of the caudate-putamen revealed no significant effect at any dose of TMT compared to controls ($F(3,31)=0.39, p>0.05$) (Figure 12). A repeated measures ANOVA was applied to the data from samples dissected from CA1 with one factor being treatment and the second factor being anatomical level. Significant effects of treatment ($F(3,28)=14.56, p<0.01$) and level ($F(2,56)=14.38, p<0.01$) were found. The interaction was not significant. A post hoc multiple comparison test applied to these data revealed that the 6 mg/kg TMT group differed significantly from all other groups at each of the anatomical levels (Figure 13).

Additionally, it can be seen that all groups demonstrated significantly higher ChAT activity at the posterior level when compared to the anterior and medial levels. This
Figure 9. The effect of TMT dose on length of the CA pyramidal cell field of Ammon's horn (A-B in Figure 2) is presented in this figure. The means and standard error of the means of data collected at each level for each group are shown. The length of the CA cell field is significantly reduced in group TMT-6 compared to all other groups and in the TMT-4 group compared to the Control and TMT-2 rats.
EFFECT OF TMT DOSE
ON WIDTH OF CA1 CELL FIELD

Figure 10. The means and standard error of the means for data collected from all rats showing the effect of increasing dose of TMT on width of the pyramidal cell field CA1 (G-H in Figure 2) is presented in this graph. A significant reduction in the width of CA1 in the TMT-6 group compared to the other treatment groups and controls at each anatomical level was observed. CA1 width in the treatment group TMT-4 also was significantly reduced compared to the Control group rats at each level and the TMT-2 group at the middle level.
Figure 11. This graph presents the means and standard error of the means for measurement data collected from all treated and control rats of the overall width of the dentate gyrus (C-D in Figure 2). The width of the dentate was significantly reduced in the TMT-4 group compared to the Control group at the anterior and middle levels and from TMT-2 rats at the posterior level. The TMT-6 group differed significantly from Control and TMT-2 rats at all anatomical levels but did not differ from the TMT-4 rats.
EFFECT OF TMT DOSE ON ChAT ACTIVITY IN CAUDATE

Figure 12. The means and standard error of the means for the data from ChAT samples taken from the Caudate-Putamen from all rats are presented in this graph. ChAT activity is expressed as pmol of acetylcholine formed per µg protein per hour. The lack of significant difference in this region was expected since TMT does not affect this region of the brain.
EFFECT OF TMT DOSE
ON ChAT ACTIVITY IN CA1

Figure 13. The means and standard error of the means amount of ChAT activity for each level of CA1 sampled in the experimental and control rats is presented above. ChAT activity was significantly higher for the TMT-6 treatment group compared to Control, TMT-2 and TMT-4 groups at each anatomical level examined.
EFFECT OF TMT DOSE
ON ChAT ACTIVITY IN DENTATE

Figure 14. The means and standard error of the means presented in this graph are for the ChAT data collected from micropunch samples dissected out of the dentate gyrus of experimental and control rats. ChAT activity was significantly higher in the TMT-6 group at the anterior and middle levels compared to the Control rats and the other treatment groups.
would account for the significant level effect indicated by the ANOVA results and the lack of interaction reported.

A repeated measures ANOVA was also applied to data from samples dissected from the dentate gyrus with treatment and anatomical level being the factors. A significant treatment effect was found, \( F(3,28)=13.84, p<0.01 \), but no significant effect of anatomical level emerged \( F(2,56)=2.34, p>0.05 \). Applying the post hoc multiple comparisons test to these data indicated that the 6 mg/kg TMT group differed significantly from all other groups at anterior and medial anatomical levels, but not at the posterior level (Figure 14). No interaction effect was detected between treatment and anatomical level in the dentate gyrus.

**Summary of Experiment 1 Results**

In summary, the effect of increasing doses of TMT on the cytoarchitecture of the hippocampus is apparent at a lower dose than that observed for increased ChAT activity. A reduction in length of the pyramidal cell line of Ammon's horn, in width of pyramidal cell layer CA1 and the overall width of the dentate gyrus was observed in the TMT-4 group compared to the Control and TMT-2 groups without a concomitant increase in ChAT activity. A significant reduction was observed in these same morphological measurements in the TMT-6 group compared to those given lower doses of TMT and the Control group. No difference in
ChAT activity was observed between any of the groups in the micropunch samples collected from the caudate-putamen. An increase in ChAT activity was observed in the TMT-6 group compared to the TMT-2 and TMT-4 groups and the Control group at all anatomical levels examined in samples collected from CA1. In the micropunch samples from the dentate gyrus a significant increase in ChAT activity was observed in the TMT-6 group compared to the lower dose animals and the controls.

Experiment Two: Effect of Time

The effect of time on ChAT activity in the hippocampal pyramidal cell-field CA1 and in the dentate gyrus was examined in this experiment. Studies in which observations of changes in cholinergic markers have been made have not examined the relationship of time to the observed changes in AChE or ChAT (Hagan et al., 1988; Naalsund et al., 1985; Woodruff & Baisden, 1990; Cannon et al., 1991). The behavioral sequelae to TMT poisoning in rats (Dyer et al., 1982a) are observed starting from one to three days following exposure and continue up to three weeks after dosing. Hyperactivity in rats as a result of TMT poisoning can also be observed soon after exposure to TMT and continues throughout the remainder of the animal’s life (Miller et al., 1982; Ruppert et al., 1982; Swartzwelder et al., 1981). Additionally, it has been observed that some
fiber degeneration continues in the hippocampal formation for extended periods after treatment with 6 mg/kg TMT in rats (Whittington et al., 1989). Studies which have examined reactive synaptogenesis in the hippocampus after entorhinal lesions report the first detectable increase in cholinergic markers in the hippocampus 4 to 5 days following the lesions (Cotman and Nadler, 1978). These results suggest that changes in ChAT activity might be observed early after exposure to TMT. Therefore the range of days chosen for this experiment included both early and late times after exposure without duplicating the periods covered in the first experiment.

Methods and Materials

Forty-two adult male Long-Evans rats weighing between 225 and 250 g at the beginning of the experiment served as subjects. The rats were obtained from Charles River Farms and maintained as described for experiment 1. Thirty-six of these rats were orally gavaged with TMT chloride dissolved in distilled water at a dose of 7.69 mg/kg (6.0 mg/kg of the TMT free base). The remaining 6 rats served as untreated controls. Behavioral observations were made and noted on the day of sacrifice for all animals. ChAT activity in the dentate gyrus and CA1 were examined on 1, 3, 7, 21, 35 and 60 days after exposure. At each time point 6 of the TMT rats and one control rat were killed by decapitation, their
brains quickly removed and frozen to a specimen plate on crushed dry ice. The brains were then processed using the procedures as in experiment one with the following exceptions. Since the results of Experiment 1 revealed no significant effect of TMT on ChAT activity in the caudate-putamen this region was not examined. Further, due to the lack of significant difference between the various levels examined in Experiment 1, a single dorsal level corresponding approximately to the AP level -2.6 shown in the atlas of Pellegrino et al., (1979) was examined. Additionally, since no effect of water gavage was observed in Experiment 1, all control rats remained untreated. To ensure the consistency of assay results slides containing the thaw-mounted sections for ChAT assay were placed in tight-fitting slide boxes, wrapped with aluminum foil, and maintained at -80°C until all sections could be processed together. The effect of time on the various cell fields of the hippocampus was quantified by taking the series of measurements outlined in Figure 2. Since all three anatomical levels examined in experiment one with these measurements were equally effected by TMT, I decided to examine only the middle level used for the ChAT assay in this experiment. In addition, the thionin stained sections were examined for signs of neuropathology in the various regions of the hippocampus at all time points.

Statistical analysis was conducted using a one-way
analysis of variance (ANOVA) on results of ChAT activity taken from either the CA1 or dentate, and for each cell field measured. When significance was detected, the least significant difference (LSD) post hoc test was applied to the data in order to determine at what time points significant changes occurred.

Results

The behavioral effects of TMT administration were consistent with that previously reported (Dyer et al., 1982b). The Day 1 animals did not demonstrate any behavioral anomalies resulting from the neurotoxin. When the Day 3 animals were sacrificed they appeared dehydrated and lethargic. These rats were unable to eat or drink without the food and water being presented to them. On the seventh day the rats had recovered from the visceral effects of the toxin (Ally et al., 1986; Opacka and Sparrow, 1985), but demonstrated an extreme aggressiveness. By day 21 the only behavioral abnormalities observed in the rats were hyperactivity and occasional spontaneous motor seizures. These characteristics were observed in the TMT rats at all subsequent time points examined.

There were no differences between Control and Day 1 rats in any of the sections examined histologically. To avoid duplication, photomicrographs of sections from control animals are not included in the figures representing the
Figure 15. Photomicrographs of the dorsal hippocampus from rats representative of the different post-TMT time intervals are presented in this figure. The Day-1 rat (A) differs little from Day-3 (B) and Day-7 (C) animals. Pyramidal cell loss can be appreciated in the CA3c region (between arrows in D) of the Day-21 rat (D). Thinning of the CA1 region (arrows in E and F) in addition to cell loss in CA3c is observed in the Day-35 (E) and Day-60 (F) rats. Thionin stain. Scale bars (in μm): (A) 520, (B) 515, (C) 498, (D) 407, (E) 378 and (F) 297.
Figure 16. High power photomicrographs of area CA1 from the thionin-stained sections in Figure 15 are presented above. No apparent necrotic effects of TMT are discernable in this representative section from a Day-1 rat (A). At Day-3 (B) the pyramidal cells appear less well organized and a few necrotic figures can be seen (arrows). On Day-7 (C) thinning of the cell layer is apparent. Thinning of the cell layer continues at Day-21 (D) with several viable cells still present. On Day-35 (E) neuronal death continues and at Day-60 (F) only a few scattered necrotic cells remain. Scale bars (in μm): (A) 58, (B) 56, (C) 43, (D) 38, (E) 21 and (F) 18.
Figure 17. This figure presents high power photomicrographs of area CA3c from the thionin sections in Figure 15. Sections from Day-1 rats (A) did not differ from Controls. Loss of cells in this region becomes apparent in the Day-7 rat (C). By Day-21 (D) few cells remain in CA3c. At Day-35 (E) and Day-60 (F) the region is devoid of viable pyramidal cells. Scale bars (in μm): (A) 186, (B) 232, (C) 135, (D) 145, (E) 133 and (F) 116.
Histological examination of thionin-stained coronal sections at low magnification (1.6x) reveal the first cell loss occurring in CA3c in the Day 21 rats. Cell loss in CA1 is first observed at this magnification in the Day 35 rats and both areas are significantly reduced in the Day 60 animals (Figure 15). At higher magnification (40x) of CA1, cell loss is evident in the Day 7 rat (Figure 16C). Cell loss continues to increase at the remaining time points. In the Day 60, rat necrotic figures and cell ghosts are about all that remain in this region (Figure 16F). A reduction in pyramidal cells can be appreciated at Day 7 in higher magnification (10x) of the CA3c region of the thionin sections (Figure 17C). The cell loss is greater at Day 21 (Figure 17D) and the area is nearly void of pyramidal cells at Days 35 and 60 (Figure 17E and F respectively).

Examination of cholinesterase-stained sections at low magnification (1.6x) reveal a time-dependent increase in stain density first observed at Day 3 (Figure 18B). In the Day 7 rat, stain density increases so that the granule cells of the dentate stand out due to their comparative lack of staining (Figure 18C). At subsequent time points, stain density continues to increase and by Day 60 the OML and CA fields appear black in the photomicrograph (Figure 18F). When the CA1 area of the cholinesterase-stained sections was examined at a higher power (10x), an increase in stain density compared to sections from rats in Control and
Figure 18. Representative photomicrographs of AChE-stained coronal sections of the dorsal hippocampus are presented in this figure. The pattern of staining 1 day after TMT administration (A) did not differ from the pattern in control rats. On Day-3 (B) and Day-7 (C) a small increase in stain density is observed. The first significant increase in stain density in the CA fields and dentate is apparent in the Day-21 (D) rat. The Day-35 (E) and Day-60 (F) rats both have very dense staining associated with the CA fields and OML of the dentate. Scale bars (in μm): (A) 826, (B) 605, (C) 814, (D) 748, (E) 737 and (F) 726.
Figure 19. This figure presents high power photomicrographs of area CA1 from the sections in Figure 18. At this magnification it can be observed that stain density increases substantially in the Day 7 rat (C) which was not apparent at low power. The increase in density is more obvious in each of the subsequent time points. Day 21 (D), Day 35 (E) and Day 60 (F). Abbreviations: PY - pyramidal cell bodies; SO - stratum oriens; SR - stratum radiatum. Scale bars (in μm): (A) 56, (B and C) 54, (D) 52, (E) 50 and (F) 39.
Figure 20. High power photomicrographs of the molecular layer of the dentate gyrus from sections shown in Figure 18 are presented above. The increased stain density in the outer molecular layer (OML) equals that observed in CA1 (Figure 19). It is apparent from the intense stain density observed that AChE activity is enhanced significantly. Abbreviations: SG - supragranular layer; GC - granule cell layer. Scale bars (in μm): (A) 98, (B and C) 116, (D) 99, (E) 101 and (F) 95.
Day 1 groups (Figure 19A) sections was appreciated at all time points beginning at Day 3 (Figure 19B). The stain density above and below the pyramidal cell field continues to increase at each subsequent time examined until Day 60, when the density covers the entire field (Figure 19F). Examination of the dentate OML at higher magnification (10x) reveals an increase in cholinesterase stain density dependent upon time after exposure to TMT (Figure 20).

The effect of time on the morphology of the hippocampus was reflected in the results of ANOVAs applied to the measurement data. The ANOVA for the measurements obtained for the length of the CA pyramidal field (A-B in Figure 2) was significant ($F(6,35) = 21.69, p < 0.001$). An LSD post hoc test applied to these data reveal a significant decline in length of the CA field at days 21, 35, and 60 compared to all earlier time groups and the Control group (Figure 21). The results of the ANOVA performed on the measurements taken of CA1 width (G-H in Figure 2) were even more robust ($F(6,35) = 28.52, p(0.001)$. The LSD test applied to these data indicated a significant decline in CA1 width at Day 3 compared to the width of this field in rats in the Control and Day 1 groups. On Day 7, 21, and 35 the width of CA1 was reduced compared to its width in Control group rats. Day 60 measurements differed significantly from all other time points and control values (Figure 22). ANOVA results for the overall width of the dentate gyrus measurements were also
Figure 21. The means and standard error of the means of measurement data for length of the CA cell field in time course animals is presented in this graph. A significant reduction in the length of Ammon’s horn CA cell field compared to the control rats and the rats on days 1, 3 and 7 was observed in rats on Day 21 and remains constant at days 35 and 60.
TIME COURSE EFFECT OF 6mg/kg TMT ON WIDTH OF CA1 CELL FIELD

Figure 22. Analysis of measurement data obtained for width of the CA1 cell field indicates that thinning of this region as a result of TMT exposure is significantly different from control values beginning on Day 3. Days 7, 21 and 35 differed significantly from control values and from values for Day 1 and Day 3 rats. The Day 60 rats were significantly different from all other groups. Data are presented as means and standard error of the means.
TIME COURSE EFFECT OF 6mg/kg TMT ON WIDTH OF DENTATE GYRUS

Figure 23. Measurement data obtained for overall width of the dentate gyrus is presented in this graph. A significant reduction in dentate width was observed on days 21, 35 and 60 as compared to control values and to days 1, 3 and 7. Data shown are means and standard error of the means.
TIME COURSE EFFECT OF 6mg/kg TMT ON ChAT ACTIVITY IN CA1

Figure 24. The means and standard error of the means for ChAT activity for data obtained from micropunch samples of CA1 from control and TMT rats at increasing time points after exposure to TMT are presented in this graph. A significant increase in ChAT activity compared to control and Day 1 rats is observed at days 7, 21, 35 and 60. A significant increase in ChAT activity compared to Day 3 rats was observed at days 7 and 35.
TIME COURSE EFFECT OF 6mg/kg TMT ON ChAT ACTIVITY IN DENTATE

Figure 25. This graph shows the means and standard error of the means for ChAT activity in the dentate gyrus at different time intervals after exposure to TMT. A significant increase in ChAT activity is observed in TMT rats at days 3, 7, 21, 35 and 60 as compared to ChAT activity in controls. A significant increase compared to Day 1 rats was seen at days 7, 21, 35 and 60. Day 21, 35 and 60 rats were significantly higher in ChAT activity than the Day 3 rats. A significant increase in ChAT activity compared to Day 7 rats was observed on days 21 and 60.
significant ($F(6,35)=4.31, p<0.002$). An LSD test applied to these data revealed that days 21, 35 and 60 differ significantly from the earlier time points and control values (Figure 23).

When an ANOVA was applied to the ChAT data from caudate samples no significant differences emerged. The results of the ANOVA applied to the ChAT data from CA1 indicated a significant effect of time on the increased activity of ChAT due to TMT treatment ($F(6,35)=5.39, p<0.001$) compared to controls (Figure 24). When the LSD post hoc test was performed on these data it revealed that a significant increase in ChAT activity was first observed in CA1 at 7 days post-exposure. An apparent plateau was reached at this time and remained consistent throughout the remainder of time points examined. The LSD results showed that values from control animals differed significantly from those of TMT-exposed rats on days 7, 21, 35 and 60. Day 1 rats also differed significantly from days 7, 21, 35 and 60, while Day 3 differed only from days 7 and 35. The ANOVA results for the dentate data were even more robust ($F(6,35)=13.67, p<0.001$). As can be seen in Figure 25, the plateau of increased ChAT activity apparent at day 7 in the samples from CA1 was not observed until day 21 in the dentate. When the LSD test was applied to the dentate data a significant increase in ChAT activity compared to activity in the control group was found on days 3, 7, 21, 35 and 60. Day 1
rats differed from rats killed on post-exposure days 7, 21, 35 and 60. Day 3 rats differed from those killed on days 21, 35 and 60, while animals killed on Day 7 differed from those killed on days 21 and 60. Although an increase in ChAT activity was evident one day after exposure to 6 mg/kg TMT, it is interesting to note that the data from Day 1 failed to reach statistical significance, with a mean difference from controls of 28.03, while the LSD value needed for significance is 28.56.

**Summary of Experiment 2 Results**

In summary, the microscopic measurements taken indicate that the earliest significant effects of 6mg/kg TMT on the morphology of the hippocampus are found in the CA1 region of Ammon’s horn. This region was significantly reduced in width at Day 3, while the measurements for the full length of the pyramidal cell field and the overall width of the dentate gyrus were not observed to be significantly changed by the neurotoxin until Day 21. No significant effects of TMT on ChAT activity were observed at any time point in samples taken of the caudate-putamen region. The effect of time after exposure to 6 mg/kg TMT on ChAT activity in the micropunch samples collected from the CA1 region indicate a significant increase compared to control values in this enzyme’s activity at Day 7. The results for the samples taken from the dentate gyrus at all time points show this
region begins to express increased ChAT activity on the third day following treatment with TMT.

The results for ChAT activity obtained in Experiments 1 and 2 presented in this chapter will be discussed with the results of the autoradiographic binding of cholinergic muscarinic receptors experiment detailed in Chapter 3.
Chapter 3

Effect of TMT on Muscarinic Receptors

Introduction

The increase in ChAT activity observed in the first two experiments suggests that the cholinergic innervation of the hippocampus is not adversely affected by the TMT, but may possibly be enhanced. This finding is compatible with the results of several published experiments which suggest that the consequences of TMT exposure for the hippocampus exceed the simple destruction of some of its principal neurons. For example, Miller and O’Callaghan (1984) observed that TMT reduced hippocampal levels of synapsin I when this phosphoprotein associated with synaptic vesicles was measured 17 days after 5 day-old rat pups were exposed to the neurotoxicant, but that synapsin I levels were at least normal 61 days post-exposure. A similar observation was made in adult rats by Brock and O’Callaghan, (1987). O’Callaghan and his co-workers interpret the decrease in synapsin I at 17 days to reflect the TMT-induced loss of cell bodies and processes from the hippocampus and the later return to normal levels by synapsin I to reflect reactive synaptogenesis of axon terminals within the hippocampus that survive the toxic insult. The experiments from O’Callaghan’s laboratory suggest that reactive synaptogenesis occurs
following TMT exposure, but does not indicate which systems might be involved. One brief observation by Hagan et al. (1988) made in a paper otherwise devoted to behavioral effects of TMT suggested that at least some of the reactive synaptogenesis might be attributable to the septo-dentate cholinergic pathway. If an increase in septo-dentate cholinergic fibers does occur as a result of TMT poisoning then it would be assumed that this phenomenon would correlate to an increase in cholinergic synapses in the various regions of the hippocampus. Further, the density of cholinergic receptors might increase over time in response to reactive synaptogenesis.

Previous studies which have examined the density of cholinergic muscarinic receptors in the hippocampus of TMT treated rats are in apparent disagreement as to the effect of the neurotoxin on this system (Loullis et al., 1985; Summer and Hirsch, 1982; Cohen et al., 1984). Loullis et al., (1985) found decreases in total muscarinic receptor binding at several time points of less than 30 days post-treatment. In the study by Cohen et al., (1984) it was reported that a significant decrease in total muscarinic receptor binding in the hippocampus was observed up to 5 months after dosing with TMT. These results differ from the observation by Summer and Hirsch, (1982) in which a transient decrease in total muscarinic receptor binding was noted at 4 days post exposure, but was not present in rats
14 days after dosing. The results of Summer and Hirsch, (1982) are similar to those from studies using mice in which observations of an initial decrease in total muscarinic receptor binding followed by a return toward normal levels at two weeks post exposure have been made (Ali et al., 1983, & 1986; Slikker et al., 1985).

The above cited studies all examined muscarinic receptor binding in homogenates of hippocampal tissue. Several questions remain concerning the effects of TMT on muscarinic cholinergic receptors within the hippocampus. How TMT effects the distribution of muscarinic receptors on cells in various regions in the hippocampus has not been examined. Long-term time course studies of the effects of TMT on cholinergic muscarinic receptors associated with specific cell fields have not been performed, nor has any effort been made to explain the effects of TMT on muscarinic receptor subtypes within the hippocampus. The purpose of the experiment described in this chapter was to answer the questions that arise from the absence of these data.

The distribution of cholinergic muscarinic receptors in specific subfields of the normal hippocampus, as determined by [³H]Quinuclidinyl benzilate ([³H]QNB) autoradiography, is homogeneous in both the anteroposterior and dorsoventral aspects of the hippocampal formation (Kuhar and Yamamura, 1976; Palacios and Mengold, 1989). The normal distribution of cholinergic muscarinic receptors as labeled with [³H]-QNB
Figure 26. Reverse image photograph of the distribution of cholinergic muscarinic receptors as labeled with $[^3H]$-QNB in a normal rat hippocampus. Abbreviations: CC - corpus collosum; DML - dentate gyrus molecular layer; PY - pyramidal cell layer (darker strip at end of arrows) of Ammon’s horn; SO - stratum oriens of Ammon’s horn; SR - stratum radiatum of Ammon’s horn.
is shown in Figure 26. The CA1 region has a density of muscarinic receptors visible in photographs of the region, which appears uniform from the alveus above the pyramidal cell layer to stratum radiatum moleculare below the pyramidal cell field. This wide band of receptors ends abruptly at the beginning of CA2. A narrow band of receptors is then visible in CA3a-b, which appears to be associated with fibers just above and below the pyramidal cell layer with little direct contact to the cells themselves. This dual band of receptors continues, associated with the pyramidal cells of CA3c, into the hilus of the dentate. Both the buried and exposed blades of the dentate gyrus show a dense distribution of muscarinic receptors associated with the granule cells of this structure.

There are at least two subtypes of muscarinic cholinergic receptors known to be present in the hippocampus of normal rats (Potter et al., 1984). The designation $M_1$ was given to a receptor that has a high affinity for the radioligand $^3$H-pirenzepine ($^3$H-PZ) and $M_2$ to a receptor that has a high affinity for $^3$H-oxotremorine-M in the presence of unlabeled pirenzepine ($[^{3}H]\text{-OXO-M/PZ}$). The subtype $M_1$ is most abundant, having a density nearly equivalent to that observed when all receptors are labeled with $[^{3}H]QNB$. The highest densities of $M_1$ receptors are associated with the pyramidal cells of CA1 and the granule cell layers of the dentate gyrus (Figure 27B. The subtype $M_2$ is located
primarily in two layers of Ammon's horn, the pyramidal cell field, and the molecular layer. The highest density of M₂ receptors is in the CA2-CA3 region and is represented by a much lower density of receptor sites than the M₁ subtype. There are no reports of M₂ receptors being localized to the dentate gyrus in rats (Figure 27C). The differential distribution of receptor subtypes suggests that they differ in synaptic location, with M₁ being postsynaptic and M₂ being presynaptic (Mash and Potter, 1986; Spencer et al., 1986). There is also evidence of presynaptic localization for both these receptor subtypes (Watson et al., 1983). The effect of TMT in destroying the cells which contain the postsynaptic receptors could possibly be appreciated in a reduction of the M₁ muscarinic receptor subtype. If the presynaptic muscarinic receptors are of the subtype M₂ as Mash and Potter (1986) and Spencer et al., (1986) have suggested, then reactive synaptogenesis of the septo-hippocampal cholinergic pathway might be appreciated by an increase in this receptor subtype following exposure to TMT.

Methods and Materials

Subjects for this experiment were 49 adult male Long-Evans hooded rats weighing between 220 and 255 g at the start of the experiment. They were obtained from the same supplier and housed in the same colony space as were the rats used in the previous experiments. The rats were housed
individually in plastic cages with wood chip bedding and maintained on a 12h on 12h off lighting schedule with lights on at 0700 hours daily. Food (Wayne Lab Blox) and tap water were provided ad libitum throughout the experiment. The rats were randomly assigned to one of two groups, a treatment group (n=42) and a control group (n=7). Treatment consisted of each rat being orally gavaged with 6 mg/kg (freebase weight) of TMT chloride dissolved in distilled water. The remaining seven rats served as untreated controls.

The time points utilized were 1, 3, 7, 14, 21, 35 and 60 days post exposure. The 14 day time point was included in the receptor study due to the significant change in ChAT activity observed between the 7 day and 21 day time points in the preceding experiment. At each time point six TMT rats, chosen randomly from the treatment group, and one control animal were killed by decapitation. The brain was quickly removed and frozen to a specimen plate on dry ice. The brains were then sectioned serially in the coronal plane at -20°C in a freezing microtome (IEC Minot Custom Microtome, Damon/IEC Division, Needham, Massachusetts) beginning at the first morphological signs of the hippocampus to the most caudal aspect of the hippocampus (corresponding approximately -1.0 and -4.6 respectively in the atlas of Pellegrino et al., 1979). The sections were cut at 20 μm for thionin stain and autoradiography, and at 40 μm for AChE staining. The sections were thaw-mounted onto
gelatin coated slides in the following serial order; first a 20 μm section for thionin stain, second a 40 μm section for AChE stain, third a pair of adjacent sections at 20 μm for specific and non specific binding of [3H]QNB, fourth a pair of adjacent sections at 20 μm for specific and nonspecific binding of [3H]OXO-M/PZ and fifth an adjacent pair of 20 μm sections for specific and nonspecific binding of [3H]PZ. This series was repeated throughout the extent of the hippocampus.

[3H]Quinuclidinyl benzilate ([3H]QNB) (50 Ci/m mole, Amersham Corporation, Arlington Heights, Illinois) was used for general autoradiographic localization by binding all types of muscarinic receptors in the hippocampus. Muscarinic binding sites were labeled with [3H]QNB as described by Wamsley et al., (1981). Due to the high specific activity of the ligand used in this experiment the concentration of [3H]QNB was reduced from 1 nM, as in Wamsley's paradigm, to 0.5 nM plus 0.5 nM unlabeled QNB (UL-QNB) to achieve the 1 nM concentration called for. Briefly, adjacent sections were incubated for 2 hours at room temperature in phosphate buffered saline (PBS) at Ph 7.5 containing 1 nM [3H]QNB/UL-QNB for total binding or 1 nM [3H]QNB/UL-QNB plus 1 μM atropine sulfate for nonspecific binding. Atropine sulfate has a greater affinity for muscarinic receptors than any of the ligands used in this experiment and blocks the tritiated ligands from binding. The slides were then rinsed in Coplin
jars of cold (4°C) PBS for 5 min two times then dipped in cold distilled water for a final rinse. The sections were then air dried and the slides placed in X-ray cassettes and opposed to Hyperfilm-\(^3^H\) autoradiographic film (Amersham). The cassettes were stored at 4°C for 5 weeks then the film developed in Kodak D-19 (1:1).

The visualization of \(M_1\) muscarinic receptors was conducted using the method described by Wamsley et al., (1984). Briefly, sections were incubated for 60 min at room temperature in Tris-HEPES buffer with 10 mM magnesium (pH 7.6) containing 10 nM \([^3H]PZ\) (83.1 Ci/mmole, New England Nuclear, Boston Massachusetts) for total binding. The slides were then rinsed in ice cold buffer 2 times for 5 min each and dipped in cold distilled water for a final rinse then air dried. For nonspecific binding, adjacent sections were incubated simultaneously, except the 10 nM \([^3H]PZ\) solution also contained 1 \(\mu\)M atropine sulfate. The slides were loaded into X-ray cassettes and opposed to Hyperfilm-\(^3^H\) (Amersham) and stored at 4°C for 5 weeks to expose the film. The film was developed in Kodak D-19 (1:1).

The visualization of \(M_2\) receptors was carried out using \([^3H]\)-oxotremorine-M (87.5 Ci/mmole, New England Nuclear, Boston Massachusetts) as the ligand (Vogt and Burns, 1988). For total binding the sections were incubated for 60 min in 20 mM Tris-HEPES buffer with 10 mM magnesium (pH 7.6), containing 0.5 nM \([^3H]\)-Oxotremorine-M with 50 nM
Figure 27. This diagram indicates the circular areas sampled for the various regions examined densitometrically for the binding of muscarinic receptors. Three areas were sampled in each region except the dentate gyrus where three areas were examined in each of the buried and exposed blades then combined for one reading of this region.
pirenzepine. Nonspecific binding was determined by a co-incubation of adjacent sections in buffer containing 0.5 nM [\(^3\)H]OXO/PZ and 1 \(\mu\)M atropine sulfate. The sections were washed in cold buffer, 2 times for 5 min each and dipped in cold distilled water for a final rinse and allowed to air dry. The slides were loaded into X-ray cassettes with Hyperfilm-\(^3\)H (Amersham) and stored at 4°C for 6 to 8 weeks to expose the film. The film was developed in Kodak D-19 (1:1).

Binding of total cholinergic muscarinic receptors with \([\(^3\)H]-QNB, muscarinic receptor subtype M\(_2\) with \([\(^3\)H]-OXO-m, and muscarinic receptor subtype M\(_1\) with \([\(^3\)H]-PZ was quantified from the X-ray films densitometrically using an MCID video-based image analysis system (Imaging Research, St. Catherines, Ont.). Values were determined on a femto mole per milligram of tissue basis by comparison with 20\(\mu\)m thick standards (Microscales, Amersham, Arlington Heights, Illinois) containing known amounts of tritium (\(^3\)H) which were mounted on clean slides and apposed to each individual X-ray film. Adjustments in \(^3\)H decay for each ligand were made to assure accuracy in the standard values entered into the computer at the time densitometry was performed. Binding values were recorded from a single middle level coronal section corresponding approximately to the level -2.6 in the atlas of Pelligrino et al., (1969) and to the level used for ChAT assay in the previous experiments. The diagram in
Figure 27 shows the areas of the hippocampus from which recordings were made. Briefly, using a circular area of approximately 150μm in diameter, three adjacent areas were taken for each recording made of receptor density in regions CA1, CA3 a-b, and CA3c. Due to the existence of a buried and exposed blade of the dentate gyrus, three circular areas taken of each were utilized in making a single recording of binding for this structure. In this system, any overlapping of circular areas is ignored by the computer when taking a measurement therefore eliminating any possible duplication in reading receptor density. Statistical analysis was performed on the separate ligands by subjecting data from each region to a one-way ANOVA. Criteria for significance was set at an alpha level of less than .05. When significance was indicated by ANOVA results an LSD post hoc test was performed on the data to indicate at which time points significance was found.

RESULTS

Histological examination of thionin-stained sections from all rats revealed cell loss in the various regions of the hippocampus in the TMT-treated rats as a result of time which was consistent with that observed in the ChAT time course experiment in the previous chapter. Sections examined at low magnification (1.6x) from the Day 14 rats included in this experiment did not demonstrate any difference from Day
7 rats. At high magnification (40x) the presence of a few necrotic figures not present in the Day 7 rats could be observed. Examination of AChE-stained sections indicated that an increase in stain density as a result of time after exposure to TMT was also consistent with the observations of similarly stained sections from animals in the time course ChAT experiment. When AChE-stained sections from the Day 14 rats were examined they did not differ from Day 7 rats in stain density.

In the hippocampus of control rats the autoradiographic distribution of cholinergic muscarinic receptors in bound by [³H]-QNB was consistent with previous findings (Kuhar and Yamamura, 1976; Palacios and Mengold, 1989). Compare the photomicrograph showing the distribution of [³H]-QNB bound cholinergic muscarinic receptors from a control group rat (Figure 28A) to that of a Day 60 TMT exposed rat shown in Figure 28D. An obvious reduction in receptor binding is observed in the subicular cortices and continues throughout the entire length of Ammon's horn. Little receptor binding can be seen associated with the regions CA1 through CA3a-b. While the receptor distribution normally found in the CA3c region is nearly absent. The distribution of muscarinic receptors in the dentate gyrus appears to be less effected by TMT, as the appearance of this region in Figure 28D differs little from that of the Control rat in Figure 28A.
Figure 28. These reverse image photographs present the autoradiographic images of hippocampal cholinergic muscarinic receptors bound by \[^3H\]-QNB in control rats (A), M₁ subtype bound by \[^3H\]-PZ (B), and the M₂ subtype bound by \[^3H\]-OXO-M (C). Compare the distribution in the control rat (A) to a Day 60 TMT rat labeled with \[^3H\]-QNB (D). A reduction in total cholinergic muscarinic receptor binding is seen from the subicular cortices throughout Ammon's horn, while no apparent loss of receptors is appreciated in the dentate gyrus. CA3c of Ammon's horn shows a complete absence of receptor binding in the Day 60 TMT rat (D). The reduced distribution of subtype M₁ receptors in a Day 60 TMT rat (E) appears identical to total binding of all receptors (D). The distribution of subtype M₂ receptors in a control rat is represented by a dual band above and below the CA cell field and is heaviest in CA3a-b (C). In a TMT Day 60 rat the distribution of M₂ receptors is limited to a band above the pyramidal cells of Ammon's horn (F).
The distribution of high affinity $M_1$ receptors (as revealed by $[3^H] \cdot PZ$) and high affinity $M_2$ receptors (as revealed by $[3^H] \cdot OXO-M$) were also consistent with the literature (Mash and Potter., 1986; Spencer et al., 1986) and are shown in Figure 28B and C, respectively. The effect of 6mg/kg TMT in reducing the distribution of the muscarinic receptor subtype $M_1$ (Figure 28E) was nearly identical to that observed for the binding of all cholinergic muscarinic receptors with $[3^H] \cdot QNB$ (Figure 28D). The only difference being the distribution of $[3^H] \cdot PZ$ labeled subtype $M_1$ receptors was greater in the CA3a-b region. TMT’s effect in reducing the observable distribution of the muscarinic receptor subtype $M_2$ differed from that observed for the $M_1$ subtype. A band of $M_2$ muscarinic subtype receptors can be seen associated with what would appear to be the region where apical dendritic processes of pyramidal cells would normally be found from the subicular cortices to the beginning of region CA3c (Figure 28F). When compared to the binding of this receptor subtype in CA3c of the control animal shown in Figure 28C, it is obvious that no binding of this receptor subtype could be appreciated in the CA3c region of the TMT-treated rat killed 60 days following gavage.

The densitometry data gathered by examining the autoradiographic images on the X-ray films for each ligand utilized in this experiment were subjected to a series of
TIME COURSE EFFECT OF 6mg/kg TMT ON TOTAL BINDING OF MUSCARINIC RECEPTORS IN CA1 OF THE HIPPOCAMPUS

Figure 29. The means and standard error of the means amount of [³H]-quinuclidinyl benzilate bound to cholinergic muscarinic receptors in area CA1 of the hippocampus as determined by autoradiographic densitometry are presented in this graph. Bound receptors are expressed as fmol per mg of tissue. Receptor binding was significantly lower in Day 14, 21, 35 and 60 rats compared to controls. Days 1, 3, 7 and 35 also differed from Day 60 rats significantly in amount of receptors bound.
TIME COURSE EFFECT OF 6mg/kg TMT ON TOTAL BINDING OF MUSCARINIC RECEPTORS IN CA3c OF THE HIPPOCAMPUS

![Graph showing the time course effect of 6mg/kg TMT on total binding of muscarinic receptors in CA3c of the hippocampus.](image)

Figure 30. This graph presents the means and standard error of the means for cholinergic muscarinic receptors bound by \([^3H]-quinuclidinyl benzilate\) in the CA3c region of the hippocampus. The amount of bound receptors is presented as fmol per mg tissue. Control rats differed significantly from TMT-treated rats at days 7, 14, 21, 35 and 60. Day 1 rats were significantly different from days 14, 21 and 35. The Day 7 animals also differed from these time intervals and from the Day 60 animals as well.
analysis of variance tests for significance. The results of the one-way ANOVA performed on the data obtained from the densitometry measurements of CA1 in sections bound with \[^3\text{H}]-\text{QNB} were significant, \( F(7,41)=3.53, p<0.005 \). A post hoc LSD test applied to these data indicate that a significant reduction in total cholinergic muscarinic receptor binding is observed at days 14, 21, 35 and 60 compared to controls. Days 1, 3, 7 and 35 also demonstrated a significant reduction in total receptor binding in CA1 compared to Day 60 rats (Figure 29). The ANOVA performed on densitometry data obtained from CA3c for receptors bound with \[^3\text{H}]-\text{QNB was also significant}, \( F(7,41)=5.16, p<0.001 \). An LSD test applied to these data indicate the receptor binding in this region is effected by TMT treatment at an earlier time point than the CA1 region. In the CA3c region a significant reduction is first observed in the Day 7 group compared to controls. Days 14, 21, 35 and 60 also differed significantly from controls in CA3c total receptor binding. Day 1 animals differed from days 14, 21 and 35, while the Day 3 rats differed from days 14, 21, 35 and 60 in this measure (Figure 30). No significant difference as a result of TMT exposure was detected for CA3a-b or the dentate gyrus for total cholinergic muscarinic receptor binding with \[^3\text{H}]-\text{QNB}.

A series of ANOVAs was also performed on the densitometry data obtained for each cell field labeled with \[^3\text{H}]-\text{PZ} for the muscarinic receptor subtype \( M_4 \). The results
TIME COURSE EFFECT OF 6mg/kg TMT ON BINDING OF THE M1 MUSCARINIC RECEPTOR SUBTYPE IN CA1 OF THE HIPPOCAMPUS

![Graph showing the binding of muscarinic receptor subtype M1 with [3H]-pirenzepine in the CA1 region of the rat hippocampus. The control, Day 1 and Day 3 rats differed significantly from days 14, 21, 35 and 60. Day 7 rats were significantly different from days 14, 35 and 60. The rats in groups Day 21 and Day 35 differed from Day 60 rats also.]

Figure 31. The results presented in this graph are for the binding of muscarinic receptor subtype M1 with [3H]-pirenzepine in the CA1 region of the rat hippocampus. The means and standard error of the means are shown, with the amount of binding presented as fmol per mg tissue. The Control, Day 1 and Day 3 rats differed significantly from days 14, 21, 35 and 60. Day 7 rats were significant compared to days 14, 35 and 60. The rats in groups Day 21 and Day 35 differed from Day 60 rats also.
TIME COURSE EFFECT OF 6mg/kg TMT ON BINDING OF THE M1 MUSCARINIC RECEPTOR SUBTYPE IN CA3a-b OF THE HIPPOCAMPUS

Figure 32. The densitometry results for the binding of muscarinic receptor subtype M₁ in the CA3a-b region of the rat hippocampus with [³H]-pirenzepine is presented in this graph. The amount of receptors bound by this ligand were measured in fmol per mg tissue. For this receptor subtype the control rats and the TMT-treated rats on days 1 and 7 differed significantly from Day 14 rats only. The Day 3 rats were significantly different from days 14 and 35. The means and standard error of the means are shown.
TIME COURSE EFFECT OF 6mg/kg TMT ON BINDING OF THE M1 MUSCARINIC RECEPTOR SUBTYPE IN CA3c OF THE HIPPOCAMPUS

Figure 33. This graph presents the means and standard error of the means for results of densitometry performed on the CA3c region of the rat hippocampus measuring the amount of $[^3H]$-pirenzepine bound to muscarinic receptor subtype M1 in control and TMT treated rats. In this region a significant reduction in receptor binding was observed at days 7, 14, 21, 35 and 60 compared to controls and days 1 and 3.
of the ANOVA for CA1 data was significant $F(7,41)=8.99, p<0.001$ (Figure 31). When an LSD post hoc test was applied to these data, it revealed that control, Day 1, and Day 3 rats differed significantly from days 14, 21, 35 and 60. The Day 7 animals differed significantly from days 14, 35 and 60, while the Day 21 and Day 35 rats were significantly different from Day 60 rats. The ANOVA results for CA3a-b data were also significant $F(7,41)=2.9, p<0.015$. The results of an LSD test applied to these data show a different pattern of significance than observed in the other regions examined (Figure 32). In CA3a-b, a significant reduction in $M_1$ receptor binding compared to controls and days 1 and 7 is only observed at Day 14. The Day 3 rats also were significant from the Day 14 animals and additionally the Day 35 rats. ANOVA results from densitometry data garnered for the binding of muscarinic receptor subtype $M_1$ in the CA3c region was highly significant $F(7,41)=11.34, p<0.001$. When an LSD post hoc test was applied to these data significance was observed at an earlier time point for this receptor subtype in CA3c than in the other regions examined similar to the results of total binding. Control, Day 1 and Day 3 rats differed significantly from days 7, 14, 21, 35 and 60. No significant effect of TMT on binding of the muscarinic receptor subtype $M_1$ was detected in the dentate gyrus.

Densitometry results for the binding of muscarinic
Figure 34. Densitometry results quantifying the amount of [3H]-oxotremorine-m bound to muscarinic receptor subtype M2 in the CA1 region of control and TMT rats is presented in this graph. The means and standard error of the means amount are shown as fmol of ligand bound per mg tissue. A significant reduction in this receptor subtype compared to Control rats was observed on day 1 and all subsequent time intervals. The Day 60 rats differed significantly from controls and all other time points except day 21.
TIME COURSE EFFECT OF 6mg/kg TMT ON BINDING OF THE M2 MUSCARINIC RECEPTOR SUBTYPE IN CA3a-b OF THE HIPPOCAMPUS

Figure 35. The means and standard error of the means amount of [3H]-oxotremorine-m bound to muscarinic receptor subtype M2 in region CA3a-b of control and TMT-treated rats are presented in this graph. A significant reduction in receptor binding compared to Control and Day 3 rats was observed at days 7, 21, 35 and 60. The Day 60 rats differed significantly from all other TMT rats and controls.
TIME COURSE EFFECT OF 6mg/kg TMT ON BINDING OF THE M2 MUSCARINIC RECEPTOR SUBTYPE IN CA3c OF THE HIPPOCAMPUS

Figure 36. This graph presents the means and standard error of the means for results of densitometry performed on region CA3c of the rat hippocampus quantifying the amount of [3H]-oxotremorine-m bound to muscarinic receptor subtype M2. A significant reduction in M2 receptor binding was observed in TMT-treated rats compared to Control animals at every time point examined. The Day 3 rats were significantly different from days 14, 21, 35 and 60. The Day 60 rats were significantly different from controls and all other TMT rats except Day 14 animals.
receptor subtype $M_2$ with $[^3]H$-oxotremorine-M in the various regions examined were also subjected to a series of one-way analysis of variance tests for significance. The ANOVA results for $M_2$ receptors bound in autoradiograms of CA1 of Ammon's horn were highly significant $F(7,41)=11.46, p<0.001$. A post hoc LSD test applied to these data indicate that a significant reduction in this subtype of receptor is first observed on the first day after exposure to TMT and continues to decline at all subsequent time points (Figure 34). The Day 60 rats also differed significantly from all time points except the Day 21 animals. When an ANOVA was applied to the densitometry results for $M_2$ receptor binding in region CA3a-b, significance also emerged $F(7,41)=5.49, p<0.001$. An LSD test performed on these data indicate that the control and Day 3 rats differed significantly from days 7, 21, 35 and 60. The Day 60 rats were significantly lower than all others (Figure 35). ANOVA results for the binding data obtained for the $M_2$ receptor subtype in the CA3c region was also significant $F(7,41)=10.06, p<0.001$. Results of a post hoc LSD test applied to these data indicate that control rats differed significantly from TMT exposed animals at every time point examined. The LSD results also show that the Day 3 rats differed from days 14, 21, 35 and 60 while the Day 60 rats were significantly different from all groups except Day 14.
Summary of Receptor Binding Results

In summary; TMT's effect on the density and distribution of all cholinergic muscarinic receptors bound with $[^3H]$-quinuclidinyl benzilate, the muscarinic receptor subtype $M_1$ bound with $[^3H]$-pirenzepine and the muscarinic receptor subtype $M_2$ bound with $[^3H]$-oxotremorine-m as a result of time after TMT administration was measured in the hippocampus of the rat in Ammon's horn (CA1, CA3a-b and CA3c) and the dentate gyrus.

The densitometry results for binding of $[^3H]$-QNB in the various hippocampal regions were subjected to statistical analysis. The results indicate a significant reduction in binding of this receptor ligand in both CA1 and CA3c regions compared to controls. The CA3c region was observed to be significantly affected by TMT poisoning at Day 7 compared to control rats while the CA1 region did not exhibit a significant decrease in receptor binding until the Day 14 time point. By Day 60 no appreciable quantity of muscarinic receptors could be detected in the CA3c region of the TMT treated rats hippocampus. No significant effect of TMT administration was detected in densitometry results for total muscarinic receptor binding in the CA3a-b region of Ammon's horn or for the dentate gyrus.

The densitometry results for the muscarinic receptor subtype $M_1$ bound with $[^3H]$-PZ were also subjected to
statistical analysis with the results indicating this receptor subtype is affected by TMT exposure to a greater extent than the reduction in total cholinergic muscarinic receptors were. A significant reduction in muscarinic receptor subtype M₁ in region CA1 compared to controls was observed on Day 14 as with the total receptor binding in this region. As with the binding of all cholinergic muscarinic receptors with \([^3H]-QNB\) in region CA3c of the hippocampus, the receptor subtype M₁ was observed to be reduced in density at 7 days post-treatment with TMT and totally absent by Day 60. The addition of a significant reduction in binding of M₁ subtype muscarinic receptors observed in the CA3a-b region of TMT exposed rats on the 14th day after treatment compared to controls differs from the results for total binding of all muscarinic receptors in this region.

When the densitometry results for the muscarinic receptor subtype M₂ bound with \([^3H]-OXO-M\) were analyzed, it was observed that this subtype of muscarinic receptor is adversely affected at a much earlier time by TMT treatment than is the case for overall cholinergic muscarinic receptor binding or the binding of the receptor subtype M₁ in all regions examined. In the CA1 region of the hippocampus a significant reduction in binding of M₂ subtype muscarinic receptors was observed on the first day after TMT exposure and continued to decline in density at subsequent time
points examined. This early reduction in binding of $M_2$
receptors at a level of significance compared to control
rats was also observed in the CA3c region. The binding of
receptor subtype $M_2$ in the CA3a-b region of Ammon's horn was
significantly reduced compared to controls at 7 days post-
treatment with TMT and this reduction was still observed in
the Day 60 rats. These results will be discussed in Chapter
4.
Chapter 4

Discussion

Effects of Varying Doses of TMT on Principal Neurons of the Hippocampus

The primary purpose of the experiments conducted in the present investigation was to determine the effects of TMT on the cholinergic septo-hippocampal system. In order to provide adequate data to accomplish this goal, however, various measurements were made to quantitate TMT-induced loss of the principal neurons of the hippocampus, i.e. the pyramidal cells of Ammon's horn and the granule cells of the dentate gyrus. Comparison of the results of these measurements to similar data reported by others indicates some variations. For example, the results of measurement of hippocampal field CA1 obtained in Experiment 1 indicate a significant decrease in the width of this pyramidal cell layer in rats exposed to doses of 4 mg/kg or 6 mg/kg TMT. This finding is in disagreement with the results of Dyer et al., (1982) in which no significant thinning of CA1 was observed 30 days after administration of 5, 6, or 7 mg/kg TMT. One possible explanation for this discrepancy is survival time following exposure to TMT. In the present experiment the rats survived for more than 90 days, compared to the 30 day survival used by Dyer's group.
A second possible explanation for the discrepancy may be in the thickness of sections examined in the two experiments. Dyer and his colleagues took their measurements using 80 \( \mu m \) thick frozen sections while in this study I used 20 \( \mu m \) thick frozen sections. The added density of overlapping cells in the thicker sections examined by Dyer could easily account for the apparent lack of thinning in CA1 reported at the doses of TMT utilized in his experimental paradigm. Although the phenomenon of overlapping cell bodies is observed in 20 \( \mu m \) sections it is not as obvious as with the thick sections studied by Dyer.

The measurement data for CA1 from Experiment 1 also disagrees with previous observations from our laboratory (Cannon et al., in press) in which cell counts were made in this sub-field. Compared to control rats we found a significant reduction in the number of cells counted in CA1 in rats receiving 2 mg/kg TMT. This dose of TMT was not found to produce measurable cell loss in this study. Two factors may account for this discrepancy. First, TMT affects individual rats differently and sample selection between the two experiments may account for the differences. Even within the present study these individual differences may be exemplified by examining the range of measurements taken of CA1 for the control group and the 2 mg/kg TMT group. At the anterior level the control group range was from 58.7 \( \mu m \) to 77.8 \( \mu m \), while the TMT-2 group had a low of 41.7 \( \mu m \) and a
high of 77.7 μm. Measurements from both the middle and posterior levels revealed a similar distribution. It appears from these data that some rats have an inherent ability to combat the neurotoxic effects of TMT at this low dose, which was not observed at the higher doses used in this study. Some rats may have the ability to metabolize TMT before it has an opportunity to cause necrotic damage.

The second factor that might account for the difference in results is that different techniques were employed to quantitate cell loss in the 2 experiments. Actual cell counts were conducted by Cannon et al., (in press), while the technique of Dyer et al., (1982) in which the width of the various cell fields is made, was used in the present experiment. Actual counting of cells may be a more sensitive index of cell loss and therefore, the minimal effect of 2 mg/kg TMT was detected in the previous, but not the present experiment. This contention is supported by the failure to find a significant difference in the width of the granule cell layer between any of the groups in this study, but a significant reduction in the number of cells counted in this same region in the previous experiment by Cannon et al., (in press), at doses of 6 and 7 mg/kg of TMT.

One proposed mechanism of action for TMT in causing cell death in the hippocampus (Chang, 1986) states that excessive stimulation beginning in the entorhinal cortex results in a cascade of hyperexcitatory responses. This
cascade beginning in the entorhinal cortex then damaging the dentate gyrus followed by CA3 and CA1,2 of Ammon's horn follows the pattern of cell necrosis observed by Chang and others using several doses of TMT. The studies examined in Chang's effort to establish a pattern of neuropathology for TMT included time course studies all of which suggest a loss of cells in CA3c prior to CA1. Though the measurement results reported here differ from Chang's hypothesis, i.e. the width of CA1 was reduced before cell loss was observed in CA3c, the cellular pathology observed in the various regions examined in this study are in agreement with Chang. Actual cell death in CA3c can be observed prior to the loss of neurons in CA1 (Figures 16 and 17). One possible explanation for the reduction in CA1 width observed in Day 3 rats is an initial reduction in fibers innervating the area from the entorhinal/subicular cortices (Cotman and Nadler, 1978; Whittington et al., 1989) resulting in shrinkage of the width of the sub-field prior to actual cell death being observed.

Effects of TMT on Choline Acetyltransferase Activity

The dose-response curve for the TMT-induced increase in ChAT activity for both the CA1 and dentate gyrus is essentially asymptotic, with a significant increase found only at the highest dose administered (Figures 13 & 14). In Experiment 1 the increasing doses of TMT were observed to
change ChAT activity in CA1 compared to the dentate gyrus differently at the various levels examined. The results for ChAT activity in CA1, shown in Figure 13, indicate that all levels examined have a significant increase in ChAT activity in the 6 mg/kg group compared to all other groups, with the largest increase observed in samples from the posterior level. This differs from the results for ChAT activity in the dentate gyrus, shown in Figure 14, where the greatest increase in ChAT activity is observed at the anterior level with no significant change in the activity of this enzyme at the posterior level. Furthermore, when the width of the CA1 region is compared to the amount of ChAT activity in CA1 it is apparent that the greatest reduction in the width of CA1 at the posterior level corresponds to the highest increase in this enzyme’s activity. This phenomenon was also observed in the dentate gyrus where the largest increase in ChAT activity was found in samples from the anterior level, and this increase corresponds to the greatest reduction in the overall width of the dentate gyrus. This finding would indicate that more severe damage in a specific region of the hippocampus could possibly be directly related to greater increases in ChAT activity in these regions.

However, the results of Experiment 2 indicate that subfield width and intensity of ChAT activity may not necessarily be inversely related, but that this reciprocal relationship may only emerge at long survival periods. In
Experiment 2 the decrease in overall width of the dentate gyrus or width of the CA1 region did not correspond to the observations of increased ChAT activity. A significant reduction in the width of CA1 compared to the control rats was observed 3 days post-exposure, while a significant increase in ChAT activity in this region was not observed until the 7 day time interval. In the dentate gyrus the increase in ChAT was observed prior to a significant reduction in width of the dentate being observed. In the dentate, a significant increase in ChAT activity was observed at 3 days following TMT-treatment, while a significant decrease in the overall width of this region was not observed until 21 days after TMT exposure. These data indicate that enhanced ChAT activity is not some artifact of cell loss in CA1 or the dentate and may be interpreted to indicate that terminals of the cholinergic hippocampal afferents react to TMT-induced changes in the brain before any appreciable cell death is produced by the toxin. The cause of this reaction may be loss of perforant path afferents originating from the entorhinal cortex, an area which Chang (1986) proposed to be first affected by TMT. Loss of these afferents would set the occasion for reactive synaptogenesis in the septo-hippocampal afferents that overlap terminal distribution with the perforant path.

The significant increase in ChAT activity observed in CA1 at 7 days following exposure to TMT differs from results
of a previous study (Naalsund et al., 1985). Naalsund et al. first detected increased ChAT activity in CA1 of the hippocampus at 35 days following the first of three weekly injections of 3 mg/kg TMT. A possible explanation for the difference between Naalsund's group and those of the present experiment may be the different dosing paradigms utilized. The single dose of 6 mg/kg TMT in Experiment 2 resulted in observable neuropathology in the hippocampus 7 days post-exposure which was comparable to the neuropathological changes reported by Naalsund et al., (1985) at 35 days following the first weekly dose of 3 mg/kg in their paradigm.

The appearance of increased ChAT activity in the dentate at 3 days following exposure also appears to be inconsistent with the fiber degeneration pattern caused by TMT observed in this cell field (Whittington et al., 1989). Fiber degeneration was observed in the dentate on the 6th day following an acute dose of 6 mg/kg TMT chloride by Whittington and his colleagues. In the pyramidal cell fields degeneration was first observed in the hippocampus on the 3rd day. This observation would suggest that the pyramidal cell fields of Ammon's horn should show histochemical signs of reactive synaptogenesis before those of the dentate gyrus.

This discrepancy may be resolved by examining the results of experiments in which reactive synaptogenesis is
studied following discrete lesions of the entorhinal cortex. Cotman and Nadler, (1978) reported that discrete lesions of the entorhinal cortex result in an earlier expansion of septo-hippocampal fibers than more extensive lesions. In their series of studies a small priming lesion of the most medial entorhinal cortex was followed either 4 or 13 days later by removal of the entire entorhinal cortex. They observed that the priming lesion induced a minimal septo-hippocampal fiber growth into the denervated area, but that the time to completion of this growth was accelerated. They noted that normal reactive fiber growth is detected from 4 to 5 days post-lesion in animals given large entorhinal lesions initially, but with the priming lesions this fiber growth could be detected at 2 days following the lesions. Although the cytotoxic effects of TMT do eventually virtually eliminate the perforant pathway from the entorhinal cortex to the hippocampus, it may be that limited early reduction of this pathway which went undetected by Whittington et al., (1989) produced the rapid increase in ChAT activity in CA1 and the dentate observed in this experiment.

Effects of TMT on Cholinergic Muscarinic Receptors

The results of Experiment 3 show that a reduction in binding of $[^3]H$-QNB to cholinergic muscarinic receptors in specific sub-fields of the hippocampus does occur as a
result of TMT exposure in rats. However, of the 4 regions of the hippocampus examined densitometrically, only CA1 and CA3c of Ammon's horn had a significant reduction in total binding of muscarinic receptors. The CA3a-b region of Ammon's horn and the dentate gyrus granule cell layers did not have a significant reduction in total binding of \[^{3}H\text{-QNB}].

The regional differences in total cholinergic muscarinic receptor binding observed in this experiment may be responsible for the apparent discrepancies in previous studies which examined the effect of TMT on cholinergic muscarinic receptors in homogenates of this brain region (Summer and Hirsch, 1982; Cohen et al., 1984 and Loullis et al., 1985). Cohen et al., (1984) and Loullis et al., (1985) reported muscarinic receptor loss in the hippocampus of rats up to 5 months after exposure to TMT, while Summer and Hirsch (1982) reported a transient loss of muscarinic receptors evident at 4 days, but absent at 14 days in rats injected with 2.5 mg/kg TMT. The regional variation produced by TMT in hippocampal muscarinic receptor binding revealed by densitometric analysis used in the present experiment suggests the possibility that previous investigators may have sampled different hippocampal regions. Further, the method of examining changes in receptor density following neurotoxic insult used in the present experiments may be preferable to the homogenate methods used in the previous
study by Summer & Hirsch and others.

The results of densitometry performed on autoradiograms of sections from control rats incubated with either [³H]-QNB or [³H]-PZ indicate that the distribution of receptors labeled by these ligands are nearly identical. The densitometric data gathered from the time course of receptor binding for these two ligands following TMT-administration indicate that the distribution of the M₁ receptor subtype is affected differently by TMT than that for total muscarinic receptor binding. The binding of [³H]-QNB to cholinergic muscarinic receptors showed a significant reduction in CA1 and CA3c regions of the hippocampus compared to the control group rats, while binding of [³H]-QNB was not significantly reduced in the other regions of the hippocampus examined. The cholinergic muscarinic receptor subtype M₁ labeled with [³H]-PZ was also significantly reduced compared to control rats in the CA1 and CA3c regions of the hippocampus and additionally, a significant reduction in M₁ receptors was observed in the CA3a-b region. Although the distribution of muscarinic receptor subtype M₂ differs greatly from the subtype M₁, a similar result for regional reduction of the M₂ receptor subtype was observed.

In addition to the regional differences observed in how TMT effects the distribution of the muscarinic receptors in the hippocampus, there is also a difference in the effect of time following TMT administration on receptor binding. In
CA1 a significant reduction in total binding of muscarinic receptors and for the receptor subtype M₁ was first observed 14 days after TMT-treatment. However, the muscarinic receptor subtype M₂ is significantly lower than control values the 1st day following TMT-exposure. A similar pattern is observed in the CA3c region of the hippocampus. A significant reduction in total binding of muscarinic receptors and receptor subtype M₁ compared to control values in CA3c was first observed on the 7th day following TMT-treatment. While the distribution of receptor subtype M₂ was observed to be reduced significantly compared to controls 1 day after TMT administration. This differential effect of TMT on these receptor subtypes regionally over time is interpreted as evidence for differences in synaptic location for the M₁ and M₂ receptors as proposed by reports from earlier studies (Mash and Potter, 1986; Spencer et al., 1986). The significant loss of M₂ subtype receptors 1 day after TMT-exposure in CA1 and CA3c would indicate that these receptors are associated with afferents to the hippocampus first affected by TMT. This contention also supports the hypothesis of Chang (1986). Increased excitation of afferents from the entorhinal cortex could result in the early damage to the cholinergic system observed in this experiment.

The effect of TMT administration on the binding of muscarinic receptors over time does not correspond to the
enhanced ChAT activity observed in various regions of the hippocampus of TMT-treated rats. ChAT activity in the dentate gyrus of rats given 6 mg/kg TMT is observed to be significantly increased at 3 days post-exposure. In this same region of the hippocampus, no significant change in receptor density was observed following gavage with 6 mg/kg TMT at any time interval examined. This is interpreted to be an indication of reactive synaptogenesis of the septo-hippocampal cholinergic pathway innervating the molecular layer of the dentate gyrus, in response to TMT acting on afferent fibers of the perforant pathway. The failure to see a significant reduction in receptor density in the dentate, while an increase in ChAT activity in the dentate is observed is believed to support this contention.

**In Summary**

The effects of increasing doses of TMT on morphological measurements of hippocampal sub-regions was not directly related to observations of increased AChE stain density or ChAT activity in these same regions following TMT administration in Experiment 1. While the 4 mg/kg dose of TMT had an effect in reducing the length of Ammon's horn CA pyramidal cell field, the width of the CA1 pyramidal cell field, and overall width of the dentate gyrus, this dose did not result in a change in AChE stain density or an increase in ChAT activity. Therefore, in assessing the effects of TMT
on the cholinergic system of the hippocampus a minimum dose of 6 mg/kg is required to get observable results in these cholinergic markers.

The cholinergic system innervating the OML of the dentate gyrus is affected by 6 mg/kg of TMT before that of the CA1 pyramidal cell field as revealed in time course ChAT assay results. Furthermore, this increase in ChAT activity in the dentate continues to rise at time intervals after the enhanced ChAT activity in the CA1 region levels off. TMT administration affected the M2 muscarinic receptor subtype before the other muscarinic receptors of the hippocampus examined. The density of cholinergic muscarinic receptors associated with the CA cell fields are reduced as a result of TMT poisoning. The lack of significant effect of TMT on muscarinic receptors in the dentate gyrus granule cell layer indicates TMT has little effect on this region. Although changes in behavior, histology, cholinergic histochemistry, cholinergic biochemistry, and cholinergic muscarinic receptor density observed in the hippocampal formation after 6mg/kg of TMT may be directly related to each other, the results of this series of experiments indicate that these changes are not observed at times where one phenomenon can be directly correlated to another.
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