THE EFFECTS OF REPEATED NICOTINE EXPOSURE ON BRAIN PLASTICITY IN THE RAT

by

CATERINA MARIA HERNANDEZ

(Under the Direction of Alvin V. Terry, Jr., Ph.D.)

ABSTRACT

Introduction: To date, anticholinesterase therapy is the mainstay of treatment for Alzheimer's disease, but offers a limited effect on cognitive function. This therapeutic approach is based on extensive work conducted in the 1970's and 1980's that led to the cholinergic hypothesis, which focuses on the important role of the major neurotransmitter, acetylcholine. The central hypothesis of the following studies was that repeated exposure to nicotine, the prototypical nicotinic acetylcholine receptor agonist, would have a positive effect on (1) performance in a learning task, (2) the expression of central nicotinic acetylcholine receptors, (3) central nerve growth factor protein levels and (4) cell proliferation in the hippocampal formation with learning. The rationale for these studies was that a comprehensive investigation of how repeated nicotine exposure specifically affects brain plasticity would facilitate future studies in the design of nicotine analogs with enhanced specificity to targets mediating its beneficial effects and decreased specificity to targets mediating its adverse side effects. **Methods:** Male Wistar rats were exposed to nicotine (0.7 mg/kg/day) for 14 days and memory function was evaluated with two types of water maze methods, then brains were processed for receptor autoradiography, immunoblotting, ELISA and immunohistochemistry. **Results:** Rats treated with nicotine demonstrated: (1) improved water maze performance, (2) an increase in high affinity nicotinic and M2 muscarinic acetylcholine receptors, (3) an increase in the expression of hippocampal TrkA receptors, cholinergic markers and proliferating cells (with learning). **Discussion:** The results of these experiments suggest that in addition to improving memory task performance, nicotine's neurotrophic effects may be mediated through its interaction with the cholinergic and nerve growth factor system, and perhaps central proliferative zones. Conclusions: All together, the results of this doctoral dissertation add to a growing body of information about the positive effects of nicotine on brain plasticity. The significance of these positive changes is that many of the factors improved with nicotine exposure are also compromised and implicated in the pathogenesis of neurodegenerative diseases (i.e. Alzheimer's disease).

INDEX WORDS: Nicotine, Cholinergic, Choline acetyltransferase, Vesicular acetylcholine transporter, Nicotinic receptor, Muscarinic receptor, High affinity, Low affinity, Nerve growth factor, TrkA receptor, p75 receptor, Learning and memory, Cortex, Hippocampus, Cell proliferation, Bromodeoxyuridine

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DEDICATION

This dissertation is dedicated to my best friend Veronica Louise Haskamp (1973-1997).

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INTRODUCTION

Neurodegenerative diseases (e.g. Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, Motor Neuron disease, etc.) affect over 5 million people in the North America (Shoulson, 1998). AD, the most prevalent of the conditions affecting approximately 4 million (AD Association, 2002), is among one of the most extensively studied neurodegenerative diseases due to its increasing prevalence in the aging population in North America. One in 10 persons over 65 and nearly half of those over 85 have AD. The care of an Alzheimer's patient is marked by dementia and other problems with cognition and behavior (Shoulson, 1998). In a 1993 national survey, 19 million Americans said they had a family member with AD, and 37 million said they knew someone with AD. It is estimated that 14 million Americans will have AD by the middle of this century (2050) unless a cure or method of prevention is found (AD Association, 2002).

To date, anticholinesterase therapy is the mainstay of treatment for Alzheimer's disease, but offers a limited effect on cognitive function. This therapeutic approach is based on extensive work conducted in the 1970's and 1980's that led to the cholinergic hypothesis, which focuses on the important role of the major neurotransmitter, acetylcholine.

LITERATURE REVIEW

Acetylcholine and the Cholinergic Hypothesis

Acetylcholine (ACh) is an essential neurotransmitter for a variety of neurologic processes. In presynaptic nerve terminals, choline acetyltransferase (ChAT) synthesizes ACh and the vesicular acetylcholine transporter (VAChT) is responsible for the transport of ACh into synaptic vesicles for storage until exocytotic release into the synapse. ChAT and VAChT are considered markers for cholinergic neurons and have been localized to all major regions of the mammalian brain (e.g. septum, basal forebrain, basal ganglia, midbrain and hindbrain). Cholinergic neurons project fibers to many areas in the brain and participate in many complex functions such as learning and memory, arousal, sleep, attention and movement. With the discovery that cholinergic neurons degenerate in the forebrain of Alzheimer's patients and treatment with cholinergic antagonists disrupts learning and memory function (in humans, rodents, etc.), the cholinergic hypothesis of cognitive dysfunction was proposed by Bartus in 1982 (Bartus et al 1982; Bartus, 2000). Since this time, the contribution of this system to cognitive function has been under continuous and intense investigation and experimentation. Cholinergic basal forebrain neurons have long been known to be susceptible to marked atrophy and degeneration with aging in many mammalian species (Bartus, 2000; Finch, 1993). A large body of work has now established that the cholinergic basal forebrain system is also crucial in spatial learning in rodents (Hortnagl and Hellweg, 1997).

II. Acetylcholine Receptors

The actions of ACh on the central nervous system are mediated by nicotinic and muscarinic ACh receptors. Nicotinic ACh receptors (nAChR) are pentameric ligand-gated cation channels and categorized as α_7 or non-α_7, which are one of two subtypes: $\alpha_4\beta_2$ or $\alpha_3\beta_4$ (Dani 2001 for review). Of the two non--α_7 subtypes, the $\alpha_4\beta_2$ subtype is more widely distributed in the brain than $\alpha_3\beta_4$ (Dani 2001 for review). Muscarinic ACh receptors (mAChR) in contrast to nAChR's are classified as G-protein coupled receptors with M1 and M2 being the predominant subtypes in the brain (Felder et al, 2000). While both classes of AChR's are located in the mammalian brain (and may co-localize in certain brain regions), each individual subtype is associated with its own pattern of expression and distribution.

III. Nicotine

In agreement with the cholinergic hypothesis, patients suffering from AD have a marked reduction in cortical nicotinic acetylcholine receptors (Court et al, 2001). Therefore, the design of agents that stimulate (and perhaps upregulate) these receptors has become an additional therapeutic strategy (Newhouse et al, 2001). Nicotine is a natural alkaloid present in tobacco leaves and believed to be the principal substance responsible for tobacco dependence (Stolerman and Jarvis, 1995). Although nicotine exposure is also associated with some adverse side effects (e.g. addiction and cardiovascular and gastrointestinal side effects), it has been found to have a number of beneficial effects such as the enhancement of cognitive function (Rezvani and Levin, 2001), amelioration of anxiety (Picciotto et

al, 2001), and analgesia (Lloyd et al, 1998). Current drug discovery research is focusing on how nicotine exerts its beneficial effects, especially how nAChR subtypes mediate these effects (Levin and Rezvani, 2000; Marubio and Changeux, 2000; Picciotto et al, 2001). Thus, nicotinic analogs that demonstrate nicotine's beneficial neuromodulatory effects without the same adverse side effects are of interest.

IV. The cholinergic system and nerve growth factor

Previous work in our laboratory has also demonstrated that nicotine may have neurotrophic properties via its positive effect on nerve growth factor receptors (i.e. upregulation) (Terry and Clarke, 1994; Jonnala et al, 2002). This finding could have special significance for the therapeutic use of nicotine as a neurotrophic agent in the treatment of neurodegenerative diseases. Nerve growth factor (NGF) is a member of a family of proteins known collectively as neurotrophins (factors that promote and/or contribute to the maintenance, survival or growth of neurons). The effects of NGF on the survival and maintenance of central cholinergic neurons has been extensively studied since the late 1980's. Since it was discovered that experimentally-lesioned cholinergic neurons (i.e. to model the degeneration observed in AD) could be rescued from death with the administration of exogenous NGF (Montero and Hefti 1988; Junard et al, 1990, Hefti, 1986), another strategy for treatment of AD was introduced. In addition to its neurotrophic effects, NGF may also play a role in memory and attention as demonstrated in behavioral tasks that rely on the cholinergic septohippcampal pathway. For example, after NGF deprivation (an

additional method for experimentally lesioning cholinergic neurons), septal infusions of NGF have been shown to improve spatial memory task performance and restore cholinergic activity (Dekker et al, 1992; Janis et al, 1997). Endogenous NGF levels have also been found to correlate with an animal's capacity for spatial learning (Woolf et al, 2001; Ruberti et al, 2000).

V. Nerve growth factor receptors: TrkA and p75

The effects of NGF and other neurotrophins are mediated through binding to two types of receptors: the high-affinity tyrosine-receptor-kinase (Trk) receptors and the low-affinity p75 receptors. NGF interacts with TrkA (one of three Trk receptor subtypes) as well as p75. The binding of NGF to TrkA leads to the activation of its tyrosine-kinase domain, which leads to the autophosphorylation of the receptor and the induction of cascade of signal transduction events leading to neurite outgrowth and cell survival. Because it contains a death domain motif (a sequence associated with the induction of apoptosis), p75 (also a general neurotrophin receptor) is classified in the TNF-α superfamily of receptors. Both types of NGF receptors are located on the cells of areas in the basal forebrain, but display different functions depending on the presence of the other (Chao and Hempstead, 1995; Bresdesen and Rabizadeh, 1997): (1) neuronal survival, cell proliferation and axonal growth with TrkA expressed alone, (2) apoptosis when p75 is expressed alone (Barrett, 2000; Friedman, 2000). When TrkA is co-expressed with p75, its affinity for NGF is increased with subsequent enhanced neuronal survival (Barrett, 2000; Friedman, 2000).

VI. Learning and Secondary Neurogenesis

A decline in memory consolidation, a function mediated by the hippocampal formation (Suzuki and Clayton, 2000; Gilbert et al, 1998), has also been observed with patients suffering from neurodegenerative diseases with an underlying cholinergic dysfunction. With the discovery of secondary neurogenesis (the production of new neurons in adulthood) in the mammalian hippocampus (Temple and Alvarez-Buylla, 1999; Gross, 2000; Rakic, 1985), many studies have begun to focus on how to manipulate the proliferative activity of this region. Secondary (adult) neurogenesis has been found to be restricted to areas called “germinal zones” in the adult mammalian brain, one is the subventricular zone and the other is the subgranular zone (a layer between the granule cell layer and hilus of the dentate gyrus). The surviving cells generated within these regions are integrated into the olfactory bulbs (via migration through the rostral migratory stream) or granule cell layer of the hippocampus as interneurons, respectively. The neurogenic potential of these regions responds positively (i.e. increased cell proliferation) to environmental enrichment, physical activity and learning novel tasks and declines with aging and exposure to stress and/or stressful events (Kempermann et al, 1997; van Praag et al, 1999; Gould et al, 1999; Eisch and Nestler, 2002; Peterson, 2002). Currently, the design of therapeutics for the treatment of neurodegenerative diseases is aimed at directly or indirectly (1) promoting neuronal survival, (2) aiding in memory function or (3) enhancing protein receptor function. An additional strategy in the design of

agents for such diseases would be to enhance the proliferation and subsequent survival of these cells in the hippocampal formation.

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DISSERTATION OBJECTIVES

The objective of the studies presented in this manuscript was to evaluate nicotine as a prototypical agent for the therapeutics of neurodegenerative diseases by studying its effects on brain plasticity. Previous studies have evaluated the effects of both acute and repeated nicotine exposure on aspects of the central cholinergic system and how modulating the neurochemistry of this system can affect learning and memory function. The following studies were designed to gain insight into how repeated (low dose) nicotine exposure effects (1) the neurochemistry of the central cholinergic system and (2) the nerve growth factor system (which maintains neurons of the cholinergic system). In addition to evaluating the neurochemistry of both systems, learning and cell proliferation in central germinal zones were evaluated with exposure to nicotine.

The ***central hypothesis*** of the following studies was that repeated exposure to nicotine would have a positive affect on (1) performance in a learning task, (2) the expression of central nicotinic acetylcholine receptors, (3) central nerve growth factor protein levels and (4) cell proliferation in the hippocampal formation with learning. The ***rationale*** for these studies was that a comprehensive investigation of how repeated nicotine exposure specifically affects brain plasticity (memory function, cholinergic activity, growth factor expression and cell proliferation) would facilitate future studies in the design of nicotine analogs with enhanced specificity to targets mediating its beneficial effects and decreased specificity to targets mediating its adverse side effects.

The effects of repeated nicotine exposure on the adult male Wistar rat was evaluated with the three specific aims listed below and the design of the experiments are depicted in figures that follow this section:

Specific Aims I and II:

To evaluate the effects of repeated exposure to nicotine on:

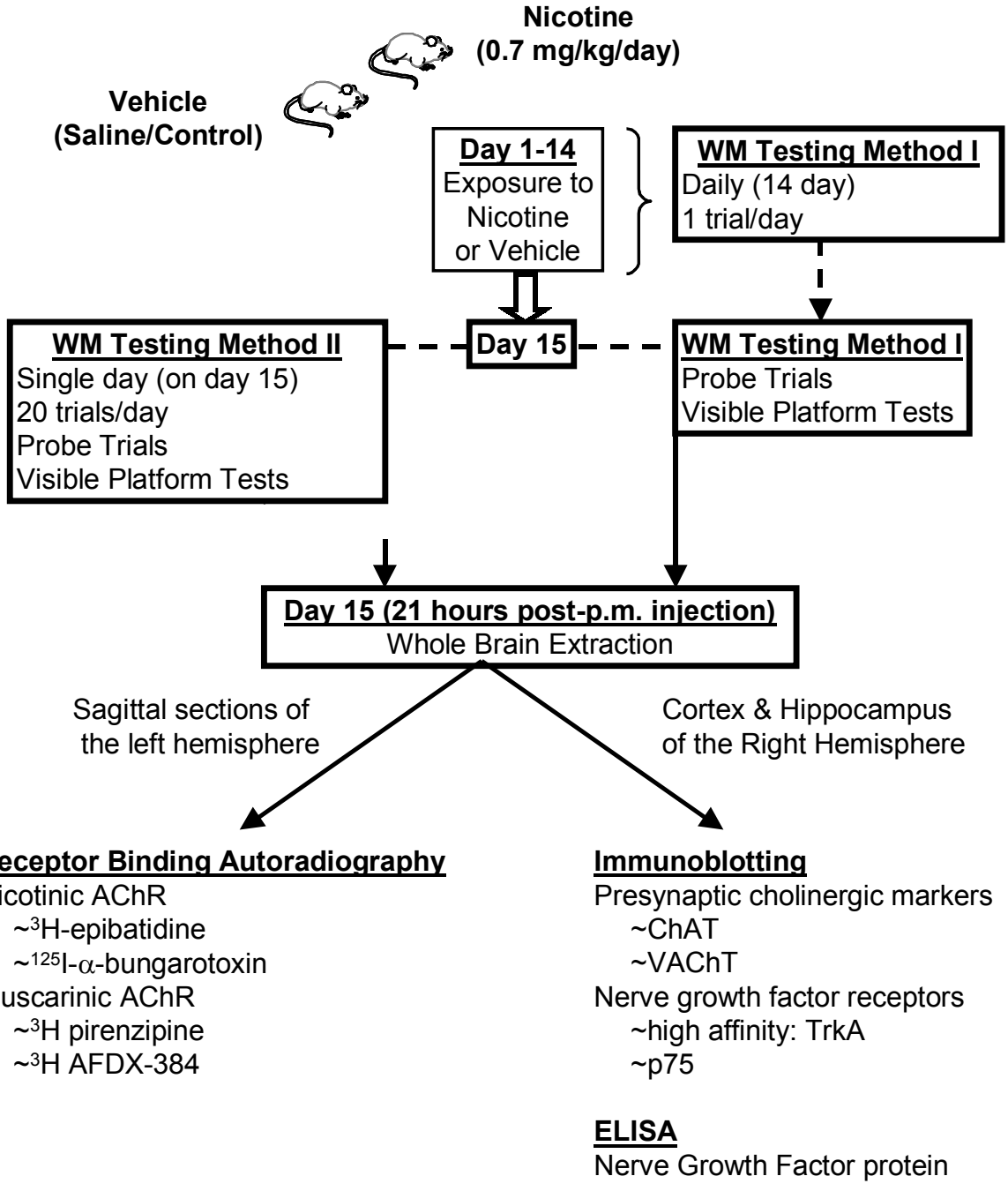
I. The expression of central acetylcholine receptor subtypes (muscarinic and nicotinic) and cholinergic markers (choline acetyltransferase and the vesicular acetylcholine transporter), and learning (water maze performance).

II. Cortical and hippocampal nerve growth factor protein levels using the ELISA technique as well as the expression of the high and low affinity nerve growth factors using immunoblotting techniques.

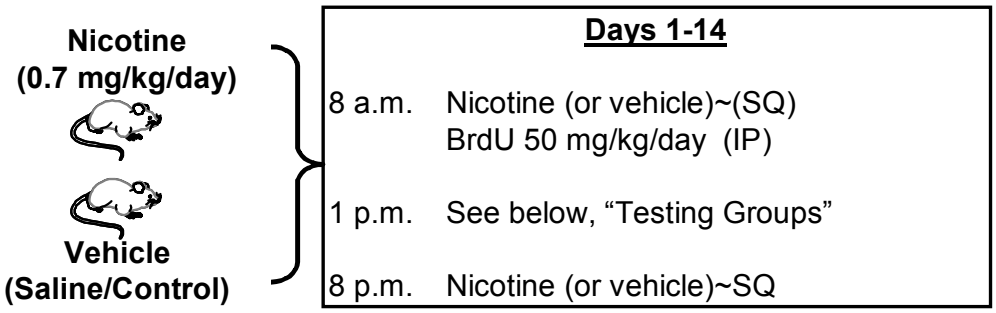
Specific Aim III:

To determine if the combination of both repeated nicotine exposure and learning increase cell proliferation in the hippocampal formation more than learning a novel task alone.

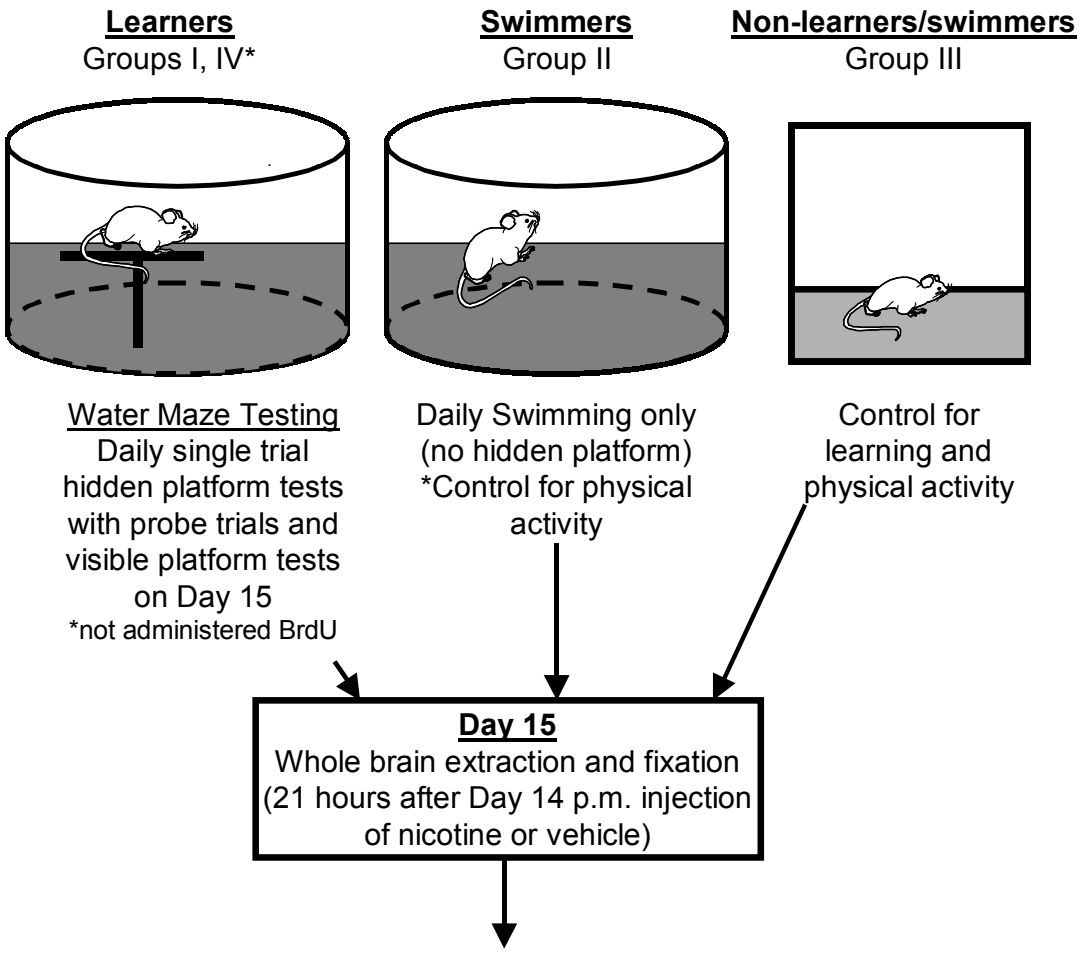
SPECIFIC AIMS I and II



SPECIFIC AIM III



TESTING GROUPS



Immunohistochemical localization, comparison and quantification of BrdU+ cells in the subventricular zone and hippocampal formation between all four testing groups

REPEATED NICOTINE EXPOSURE IN RATS: EFFECTS ON MEMORY FUNCTION, CHOLINERGIC MARKES AND NERVE GROWTH FACTOR[1]

[1] Hernandez, C.M. and A. V. Terry, Jr. To be submitted to the *Journal of Pharmacology and Experimental Therapeutics*.

Abstract

A substantial decrease in central nicotinic acetylcholine receptors is characteristic of several neurologic and psychiatric illnesses such as Alzheimer's and Parkinson's disease, dementia with Lewy Bodies and schizophrenia. These receptor deficits may contribute to both the cognitive deficits and degenerative changes observed in the brain. Chronic exposure to nicotine results in memory enhancement, an increase in central nicotinic acetylcholine receptors and the expression of high affinity nerve growth factor receptors, and therefore, could be used in the therapeutics of the illnesses highlighted above. However, to date, the effects of nicotine on specific acetylcholine receptor subtypes, other cholinergic markers, nerve growth factor protein or its high and low affinity receptors have not been fully characterized. In the present study, male Wistar rats were exposed to nicotine (0.7 mg/kg/day) for 14 days. Memory function was evaluated with two water maze paradigms: a 14-day single trial method and a 20 trial single day method. Following behavioral testing, whole brains were processed for receptor autoradiography and both immunoblotting and ELISA experiments to evaluate components of both the cholinergic and nerve growth receptor system. In water maze experiments, performance was significantly improved with nicotine treatment in both paradigms. Further, nicotine increased high affinity nicotinic acetylcholine receptors and M2 muscarinic acetylcholine receptors in neocortical areas as well as both high and low affinity nicotinic acetylcholine receptors in the amygdala. The expression of TrkA (phospho-dependent or independent), choline acetyltransferase and the vesicular acetylcholine transporter were all increased

in the hippocampus of nicotine-treated animals. However, nerve growth factor protein levels in the cortex and hippocampus were not significantly affected. The results of these experiments suggest that in addition to improving memory task performance, nicotine's neurotrophic effects may be mediated through its interaction with both nicotinic acetylcholine and high affinity nerve growth factor receptors.

A variety of postmortem studies have reported reductions in central nicotinic-acetylcholine receptors (nAChR's) in aged subjects and those who suffered from Alzheimer's Disease (AD) or other age-related neurodegenerative disease in which dementia was present (e.g., Lewy Body disease, Parkinson's disease, see Perry et al 2000). In AD, these findings have been confirmed in living patients via [^{11}C]-nicotine based positron emission tomography (PET) studies. These PET studies further suggested that nicotinic receptor deficits are in fact an early phenomenon in AD and that cortical nAChR deficits (in particular) significantly correlated with the level of cognitive impairment (Nordberg, 2001). Other studies indicate that high affinity α_4-containing nicotinic receptors are more significantly reduced than either α_3-or α_7-containing receptors, although decreases in α_7 binding sites have been observed in Lewy Body disease (Picciotto and Zoli, 2002 for review). These findings suggest that nAChR subtypes may be differentially reduced in different forms of dementia. The studies cited here also have provided the impetus for the design and development of compounds that stimulate (and perhaps upregulate) nAChR's as a therapeutic strategy for diseases such as AD (Newhouse et al, 2001).

A variety of studies have evaluated nicotine (NIC) (as a prototypical nAChR agonist) for its ability to increase the expression of nAChR's. The conventional treatment approaches have utilized twice daily subcutaneous injections of near maximal (i.e., sub-convulsive) doses of NIC or chronic infusion through controlled release osmotic pumps. Both types of administration are associated with increases in central nAChR's in areas known to involve memory

function (Pauly et al, 1991; Yates et al, 1995; Abdulla et al, 1996; Ulrich et al, 1997; Flores et al, 1997). It is important to note, however, that few studies have evaluated the subtype specific effects of NIC on *both* nAChR and muscarinic acetylcholine receptors (mAChR's) across all brain regions, particularly with intermittent dosing (which is more reminiscent of human exposure).

An additional positive effect associated with repeated NIC exposure is its ability to increase in the expression of nerve growth factor (NGF) mRNA (Rattray, 2001) and receptor protein (Terry and Clarke, 1994; Jonnala et al, 2002), as well as phosphorylation of factors such as CREB (Brunzell et al, 2003; Nakayama et al, 2001) downstream to the interaction of NGF with its high affinity receptor. These effects of NIC on NGF may be of particular importance from a potential therapeutic standpoint since the neurotrophin is required for the maintenance and survival of cholinergic neurons in the basal forebrain (i.e. neurons severely damaged in AD). To date, the effects of NIC on the expression of TrkA proteins have been assessed, but the experiments were unable to discern whether the activated (i.e., phosphorylated) form of the receptor or the low affinity p75 neurotrophin receptor was affected (Terry and Clarke, 1994; Jonnala et al, 2002; Rattray, 2001).

In the present study, therefore, water maze testing was performed to assess the effects of intermittent exposure to NIC (at a time point after injection when NIC is undetectable in the plasma; Hwa Jung et al, 2001) on spatial learning performance. After behavioral testing, subtype specific ligands and autoradiographic methods were utilized to label the predominant central nAChR

(α_7, $\alpha_4\beta_2$ and $\alpha_3\beta_4$) and mAChR (M1 and M2) subtypes in rodent brain. The expression of key presynaptic cholinergic markers, ChAT and VAChT, and both high and low affinity NGF receptors were measured via immunoblotting techniques and NGF protein levels were assessed with an ELISA method. Finally, phospho-specific antibodies to the TrkA receptor were utilized to determine if treatment with NIC had an effect on the activation (i.e. phosphorylation) of the high affinity receptor.

Materials and Methods

Animals

Male, Wistar rats (12 weeks of age) were purchased from Harlan (Indianapolis, IN) and housed in a temperature-controlled room (25°C) with a 12 h light-dark cycle. Each animal was provided with Harlan Teklad food (Madison, WI) and water *ad libitum*. All procedures employed during this study were consistent with AAALAC guidelines and reviewed and approved by the Medical College of Georgia Committee on Animal Use (CAURE) and the VA Medical Center (Augusta, GA) Subcommittee on Animal Use.

Chemicals and Antibodies

2-methylbutane, cresyl violet, (-)- Cytisine, (-)-nicotine hydrogen tartrate salt, atropine sulfate were obtained from Sigma-Aldrich (St. Louis, MO). [5,6-bicycloheptyl-^{3}H] (+/-)-Epibatidine, [^{125}I]-Tyr^{54}-α-Bungarotoxin [N-methyl-^{3}H] Pirenzipine and [2,3-dipropylamino-^{3}H] AF-DX 384 and [methyl-^{3}H] Choline

chloride were obtained from PerkinElmer Life Sciences (Boston, MA). Goat anti-choline acetyltransferase was obtained from Chemicon (Temecula, CA). Goat anti-p75 NTR, goat anti-vesicular acetylcholine transporter, horseradish peroxidase-conjugated bovine anti-goat were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cell lysis buffer, rabbit anti-TrkA, rabbit anti-phospho -TrkA (Tyr490), rabbit anti-phospho-TrkA (Tyr 674/675), horseradish peroxidase-conjugated anti-rabbit were obtained from Cell Signaling (Beverly, MA). Tris base, glycine, blocking grade nonfat dry milk and Bradford protein assay reagent were obtained from Biorad (Hercules, CA). NaCl, SDS and PMSF were obtained from Fisher Scientific (Pittsburgh PA). Pierce West Pico Supersignal ECL Substrate Reagent from Pierce Endogen (Rockford, IL).

Drug Administration

Beginning at 14 weeks of age, nicotine hydrogen bitartrate (1 mg/kg, equivalent to 0.7 mg/kg nicotine base) or saline (SAL, sterile 0.9% sodium chloride) were administered daily for 14 days to the rats (n=12 per group) as two subcutaneous injections every 12 hours.

Water Maze Testing

Animals were tested in one of two water maze (WM) testing methods: (1) A single day multiple trial method that began 12 hours after the last injection on day 14, and (2) A daily single trial method that began daily 4-5 hours following the morning administration of NIC or vehicle. Both WM testing (Morris 1984)

methods were executed in a circular (180 cm x 76 cm) black plastic pool (Bonar Plastics, Newnan, GA) filled with water (25.0 $\pm$ 1.0ºC). The walls surrounding the pool displayed reflective geometric shapes (i.e. visual cues) to aid in each rat's navigation of the WM. However, both the experimenter and other rats were blocked from the view of each rat (while in the maze) by black curtains. Once the rat was placed in the pool for testing, the swimming activity of each rat was recorded monitored via a television camera mounted over the pool that was connected to a Poly-Track video tracking system. Tracking of each rat was captured by the contrast of the white rat on a black background of the pool and diffuse lighting in the testing room. Both WM methods included three types of tests: hidden platform, probe trials and visible platform in which an escape platform was: 1) hidden (i.e. submerged underwater), 2) absent (i.e. removed from the pool) and 3) visible (i.e. above the water's surface), respectively.

Hidden Platform Tests: A black (10 cm^2) platform remained in a fixed position, submerged approximately 1 cm below the water's surface. Hidden platform trials were initiated by placing a (WM naïve) rat in the water facing the pool wall in one of 4 quadrants (designated NE, NW, SE, SW). The order of entry into each four individual quadrants was pseudo-randomized. For each trial, rats were given 90 sec to find the hidden platform. When successful the rat was allowed a 30 sec rest period on the platform. If unsuccessful within 90 sec, the rat was placed on the platform for a 30 sec rest period by the experimenter. Three parameters were recorded or calculated by the video tracking system: (1) latency (the number of sec required for the rat to locate and mount the hidden

platform), (2) distance (the total distance traveled in centimeters to reach the platform) and (3) swim speeds (the distance in cm/latency in sec). For the single day multiple trial method of WM testing, each rat had 1 trial every 30 minutes for 10 hours (total trials=20). For the daily single trial method of WM testing, each rat had 1 trial per day for 14 days.

Probe Trials: For both WM testing methods, a single 90 sec probe trial was conducted in which the platform was removed from the pool to measure a spatial bias for the previous platform location (Morris, 1984). For the single day method, the probe trial was conducted 30 minutes after the last hidden platform trial. For the daily single trial method, the probe trial was conducted on day 15, 18 hours after the last injection of NIC (or vehicle). The swim pattern of this trial was recorded by the video-tracking software to obtain the percentage of time and distance each rat spent in the quadrant where the platform was formerly located for the hidden platform tests.

Visible Platform Tests: Visible platform tests were conducted to determine if the rats had any visual problems. Immediately following probe trials, the platform was re-introduced into the pool covered with a highly visible (light-reflective) block that was approximately 1 cm above the water's surface. The curtains surrounding the maze were completely closed to prevent view of any previous visual cues in the testing room, which left the visible platform the only object in clear view. The visible platform was consecutively placed in each quadrant and each rat was given one trial to locate the platform in each quadrant of the maze. The time to locate the platform was recorded.

Tissue Preparation for Autoradiography and Immunoblot Analysis

On Day 15 (21 hours following the last injection of NIC (or vehicle) on day 14), rats were sacrificed by decapitation. Whole brain tissue was removed and flash frozen in 2-methylbutane for storage at –70°C. Using a Microm® HM cryostat (-18ºC), the left hemisphere (n=6 per group) was serially sectioned (16 μM) onto gelatin-coated slides up to the mid-line. After sectioning, the cortex and hippocampus were dissected and removed from the remaining right hemisphere. Tissue samples were homogenized in ice-cold 1X Cell Lysis Buffer with 1 mM PMSF. Protein concentrations for each homogenate were determined by the colorimetric method of Bradford (1976), using standards prepared from bovine serum albumin.

Quantitative Receptor Autoradiography

Preparation of Standards: To define the response of the radiosensitive films to increasing amounts of radioactivity, tissue paste standards containing increasing amounts of radioactivity were prepared (Terry et al, 2000; Hernandez et al, 2003) and included in all film exposures. Tissue paste standards were prepared from whole rat brains homogenized in ice-cold phosphate buffer. Depending on the radioligand evaluated, increasing amounts of [^{3}H]-choline chloride or [^{125}I]-α-bungarotoxin were added to individual aliquots of homogenized whole brain tissue. The specific activity range of each set of tissue

paste standards was 0.5-30.0 nCi/mg, as determined by a liquid scintillation or gamma counter depending on the radioligand used.

Radioligand Binding to Tissue Sections: The densities of both central nAChR and mAChR were measured with the following radioligands: [^{3}H]-epibatidine, [^{125}I] α-bungarotoxin, [^{3}H]-pirenzipine and [^{3}H]-AFDX-384. For all autoradiography experiments, slides were incubated with radioligands diluted in Kreb-Ringers-Hepes buffer (nicotinic radioligands) or 50 mM Tris-HCl buffer (muscarinic radioligands). Specific receptor subtype targets, radioligand incubation times, radioligand concentrations, types of buffers used, duration of film exposure, etc are listed in Table 1.1 and modified from the protocols of Terry et al (2000) and Hernandez et al (2003). Non-specific binding was determined by the addition of 300 μM or 1 mM nicotine hydrogen tartrate to the buffer prior to incubation with [^{3}H]-epibatidine and [^{125}I] α-bungarotoxin, respectively. Non-specific binding was determined by addition of 10 μM atropine sulfate to the buffer prior to the addition of [^{3}H]-pirenzipine or [^{3}H]-AFDX-384.

Film Exposure and Development: After incubation with designated radioligands, slides were stored overnight in a vacuum dessicator at room temperature. Autoradiograms were prepared by exposing the slides to radiosensitive film (Hyperfilm-3H or Bmax, Amersham Bioscience, Piscataway, NJ) for 1-10 weeks depending on the radioligand (see Table 1.I). All films were manually processed after exposure to slides with Kodak® D-19 Developer (5 min), Indicator Stop Bath (30 sec) and Rapid Fixer with hardener (10 min).

Densitometry: Images of each section were captured from autoradiograms for the densitometry of individual brain regions using NIH Image Software and an imaging station (Macintosh PowerPC 8100/100I computer, Data Translation QuickCapture imaging board, Sony SC-77 CCD camera and a Northern Lights Precision Desktop Illuminator). Receptor binding was quantified as optical density in all brain areas that had a signal greater than background. Optical densities of the tissue paste standards (with known nCi/mg concentrations) were obtained and a sigmoidal calibration curve (standard [nCi/mg] vs. optical density) was generated using Table Curve 2D software (Systat, Richmond, CA). After films were developed, sections were stained with 0.5% cresyl violet (pH 4.0) in order to better visualize and discriminate between structures and boundaries of individual brain area. Brain nuclei were identified using Paxinos and Watson's Rat Atlas 4th Edition (1998).

Immunoblot Analysis of Cortical and Hippocampal Samples

Gel electrophoresis and Protein Transfer: Cortical and hippocampal tissue homogenates (25-200 μg total protein) were size fractionated in 7.5-12% SDS-polyacrylamide gels, then transferred onto PVDF membranes (30 V/overnight/4°C). Membranes were blocked in 5.0% nonfat blocking grade milk prepared in TBS, then probed overnight at 4ºC with the following primary antibodies prepared in TBS with 0.05% Tween: goat anti-choline acetyltransferase (1:500), goat vesicular acetylcholine transporter (1:1000), goat-anti-p75 NTR (1:1000), rabbit anti-TrkA, phospho-TrkA (Tyr490) (TrkAY490) or

phospho-TrkA (Tyr674/675) (TrkAY674/5) (1:1000). Anti-TrkA antibodies were detected with an anti-biotin/rabbit anti-horseradish peroxidase (HRP) complex all others with bovine anti-goat HRP. HRP activity was revealed with the enhanced chemiluminescence (ECL) procedure using Pierce West Pico Supersignal Substrate according to manufacturer's instructions. Chemiluminescent signal was detected and visualized using Amersham Hyperfilm-ECL film developed in an automatic Kodak® X-OMAT processor. Films were captured using the same imaging station cited for the autoradiograms. The optical densities of bands corresponding to the target proteins (verified with a molecular weight marker on each blot) were obtained and compared between NIC-treated rats and controls.

Measurement of NGF levels by ELISA

Acidification of Tissue: Twenty-one hours following the last injection of NIC (or vehicle) on day 14, rats were anesthetized with a cocktail of ketamine (200 mg/kg, i.m.) and xylazine (7.2 mg/kg, i.m.) and then sacrificed by transcardial perfusion with phosphate-buffered saline. Brains were extracted and the cortex and hippocampus were removed. Tissue samples were homogenized in an ice-cold high salt/high detergent buffer with protease inhibitors (100 mM Tris-HCl, 1 M NaCl, 2% BSA, 4 mM EDTA, 2.0% Triton X-100, 0.02% NaN_3, 0.1 μg/mL pepstatin A, 5 μg/mL aprotonin, 1 mM sodium orthovanadate and 1 mM PMSF) modified from Zettler et al (1996). Samples were then centrifuged for 30 minutes at 13,0000 x *g* at 4ºC to obtain the supernatant. Supernatant protein concentrations were determined by the colorimetric method of Bradford (1976),

using bovine serum albumin as a standard. Samples were then diluted in Dulbecco's PBS and acid treated with 1N HCl for 15 minutes to solubilize NGF and dissociate it from its receptors (Zetteler et al, 1996). Following acidification, samples were neutralized to pH 7.4-7.6 with 1N NaOH.

Detection of NGF by ELISA: ELISA's were performed using NGF E_{max} Immunoassay System Kit (Promega, Madison, WI) according to package instructions. Nunc Maxisorp 96-well plates (Nalge Nunc International, Rochester, NY) were coated with polyclonal sheep anti-NGF diluted in 0.025 M sodium carbonate/bicarbonate buffer (pH 9.7) and incubated overnight at 4°C. Prior to treatment with antibodies listed below, plates were thoroughly rinsed with wash buffer (Tris-buffered saline with 0.05% Tween-20). Plates were blocked with buffer supplied by the kit for one hour at room temperature. Dilutions NGF standard and acid-treated supernatants from cortex and hippocampus were added to the plates and incubated at room temperature on a plate shaker for 6 hours. Monoclonal anti-NGF was added to each well and plates were incubated overnight at 4°C on a shaker. Wells were then treated with anti-rat IgG HRP conjugate for 2.5 hours at room temperature on a plate shaker. The plates were incubated in tetramethylbenzidine solution for 20 minutes on a plate shaker to produce a color reaction, which was stopped with the addition of 1N HCl. The plates were read on microplate reader (MWG Biotech, High Point, NC) equipped with a 450nm filter. Sample absorbances were converted to units of pg NGF/mL using standard curves generated with Table Curve 2D software. Values were

corrected for total amount of protein in the sample. The minimum sensitivity of the NGF assay was 15.6 pg/mL.

Statistical Analyses

Comparisons between treatment groups were made using analysis of variance (with repeated measures when necessary) followed by the Student-Newman-Keuls method for post hoc analysis or two-sample t-tests where applicable. Statistical significance was assessed at an alpha level of 0.05. In all studies, the investigator performing experiments was blind to the treatment group. All data sets are expressed as the mean + S.E.M.

Results

Water Maze Testing

Single day method

Both NIC-treated rats and controls progressively swam less distance ($F=19.19$, $p<0.0001$) and took less time (latency) ($F=20.93$, $p<0.0001$) to locate the hidden platform over the course of 20 trials. The distances swam to locate the position of the hidden platform (Fig. 1.1A) were not significantly different between NIC and SAL-treated animals over the course of the first 20 trials. In the subsequent probe trials, NIC-treated animals spent a higher percentage of time and swam further in the target quadrant (i.e. quadrant where hidden platform was stationed) than controls ($p<0.03$) (Fig. 1.2A). No statistical difference in the time

to locate a visible platform was observed between NIC-treated rats and controls (data not shown).

Daily single trial method

The daily mean distances swam to locate the hidden platform for NIC-treated rats and controls are depicted in Fig. 1.1B. Performance of NIC-treated animals in the hidden platform tests was significantly better than SAL-treated controls (i.e. NIC-treated animals used overall less distance and time to locate the hidden platform): distance ($F=16.42$, $p=0.002$), latency ($F=9.45$, $p=0.01$). Both groups of animals progressively learned to locate the platform more quickly over the course of 14 days (distance: $F=16.52$, $p<0.0001$, latency: $F=18.69$, $p<0.0001$). Comparison of the probe trials between groups revealed that the mean percentage of total time spent and distance swam in the target quadrant (that previously held the hidden platform) was not significantly different between NIC-treated rats and controls. (Fig. 1.2B). The effect of NIC treatment on vision was evaluated by comparing the time to locate a visible platform to controls. No statistical difference in the time to locate a visible platform was observed between NIC-treated rats and controls (data not shown).

Receptor Autoradiography

[^{3}H]-Epibatidine: The highest [^{3}H]-Epibatidine binding densities were observed in the medial habenular nuclei, interpeduncular nuclei and pineal gland (Fig. 1.3D). Moderate binding was observed in the caudate putamen, thalamus, subicular complex, tectum and nuclei of the solitary tract. Lower binding

densities were observed in the cerebral cortex and individual cortical layers. These findings are in agreement with the distributions observed in previous studies performed in rodents (Perry and Kellar, 1995 and Marks et al, 1998). Out of the 50 areas measured for [^{3}H]-epibatidine, NIC-treated animals exhibited statistically higher binding densities than controls in 14 regions. The highest differences in the magnitude of binding densities were in the neocortical (e.g. frontal cortex) and rhinencephalic (e.g. anterior olfactory nucleus) regions of the telencephalon ($p<0.003$) (Table 1.2) in which increases of up to 20% were detected in the brains of NIC-treated animals.

[^{3}H]-epibatidine + 150 nM cytisine: In order to quantify the density of α_3-containing nicotinic acetylcholine receptors (Marks et al, 1998), sections were incubated with [^{3}H]-epibatidine and 150 nM cytisine. The autoradiographic distributions of [^{3}H]-epibatidine + cytisine binding to central α_3-containing nAChR's in were similar between NIC treated rats and controls (Fig. 1.3E). The highest [^{3}H]-epibatidine + cytisine binding densities were observed in the medial habenular nuclei, interpeduncular nuclei and pineal glad, while moderate binding densities were found in the fasciculus retroflexi, superior colliculus and tectum. Lower [^{3}H]-epibatidine + cytisine binding densities were found in the geniculate nuclei and substantia nigra. This pattern of [^{3}H]-epibatidine + cytisine binding is agreement with previous studies (Marks et al, 1998). Out of the 19 areas measured, no significant differences were observed between treatment groups (Table 1.3).

[^{125}I]-α-bungarotoxin: Binding of [^{125}I]-α-bungarotoxin was widely distributed across all regions of the brain, with the exception of the striatum and cerebellum (Fig. 1.3C). The highest [^{125}I]-α-bungarotoxin binding densities were observed in the accessory olfactory bulb, supraoptic nuclei, mammillary nuclei, dorsal raphe and medial vestibular nuclei. Moderate binding was observed in the superior colliculus, hippocampus, hypothalamus and tegmental nuclei. Lower binding densities were observed in the cerebral cortex and amygdala. The distribution of [^{125}I]-α-bungarotoxin binding was in agreement with previous studies (Terry et al, 2000). Out of the 53 areas measured for [^{125}I]-α-bungarotoxin, NIC-treated animals exhibited significantly higher binding densities in three areas: ventromedial hypothalamus, posterior cortical amygdala and medial vestibular nuclei, whereas one area was higher in the control group, the accessory olfactory bulb (Table 1.4).

[^{3}H]-Pirenzipine: [^{3}H]-pirenzipine binding was widely distributed in the neocortex and hippocampal formation and minimally represented in the thalamus, hypothalamus and midbrain (Fig. 1.3B). The highest [^{3}H]-pirenzipine binding densities were observed in the CA1 region of the hippocampus, dentate gyrus, nucleus accumbens and the basolateral amygdala. Moderate binding was observed in the cortex, caudate putamen and anterior olfactory nuclei. The lowest [^{3}H]-pirenzipine binding densities were found in the subicular complex, lateral septal nuclei, and thalamus. The distribution of [^{3}H]-pirenzipine binding was in agreement with previous studies in rodents (Gattu et al, 1997, Hernandez

et al, 2003). No significant differences were observed between treatment groups for [[^{3}H]-pirenzipine autoradiography experiments (Table 1.5).

[^{3}H]-AFDX 384: Like [^{3}H]-pirenzipine binding, [^{3}H]-AFDX-384 binding was widely distributed in the cortex and hippocampal formation. Unlike [^{3}H]-pirenzipine binding, [^{3}H]-AFDX-384 binding was also distributed across the thalamus, hypothalamus and hindbrain. The highest [^{3}H]-AFDX-384 binding densities were observed in the caudate putamen, accumbens nuclei and olfactory tubercle. Moderate binding was found in the cortex, basolateral amygdala and hippocampal formation. Lower [^{3}H]-AFDX-384 binding densities were found in the hypothalamus, thalamus and hindbrain. The distribution of [^{3}H]-AFDX-384 sites was in general agreement with previous autoradiographic studies in rodents (Gattu et al, 1997; Hernandez et al, 2003). NIC-treated animals demonstrated a higher density of [^{3}H]-AFDX-384 binding sites in the cerebral cortex, both anterior and posterior thalamic nuclei and rostroventrolateral medulla (Fig. 1.3A).

Immunoblotting and ELISA experiments

ChAT and VAChT: The antibodies to both ChAT and VAChT recognized a 70 kD band in the cortex and hippocampus of NIC-treated animals and control. Rats treated with NIC exhibited a significant increase in both hippocampal ChAT and VAChT immunoreactivity versus control (Fig. 1.4B). No differences in ChAT or VAChT immunoreactivity in the cortex were observed between NIC-treated animals and controls (Fig. 1.4A).

NGF protein and receptors: After samples of cortex and hippocampus were treated with acid to dissociate NGF from its receptors, the concentration of NGF protein was measured by the ELISA method. Treatment related changes in NGF protein were not found in either brain region (Fig. 1.6).

Using immunoblotting methods, antibodies to both phospho-dependent and independent TrkA recognized a 140 kD band in the cortex and hippocampus of NIC-treated animals and control. Rats treated with NIC exhibited a significant increase in cortical TrkA (Y674/675) immunoreactivity ($p<0.02$) (Fig. 1.5A), but not phospho-independent TrkA or TrkA (Y490). Immunoreactivity of both phospho-independent and dependent TrkA was also increased in the hippocampus of NIC-treated rats versus controls (Fig. 1.5B). The antibody to p75 recognized an 80 kD band in the cortex and hippocampus of NIC-treated animals and controls. No treatment related differences in p75 immunoreactivity were observed in the cortex or hippocampus (data not shown).

Discussion

The pharmacological effects of the tobacco alkaloid NIC have been extensively studied for decades, yet there are many effects of this agent that remain unclear or not fully characterized. Examples include the underlying basis for the variety of neuroprotective actions demonstrated in both *in vitro* and *in vivo* models of neural toxicity (Kihara et al, 1997) and the protracted effects on memory function which are detectible long after the compound has cleared from plasma (Terry et al, 1993). Several years ago we hypothesized that one

mechanism for these effects with NIC might lie in its ability to enhance the expression of the receptors for neurotrophins such as nerve growth factor (Terry and Clarke, 1994; Jonnala et al, 2002). This endogenous compound is responsible for the maintenance and function of adult basal forebrain cholinergic neurons (cells know to be important for memory function and reproducibly damaged in AD). We demonstrated both *in vitro* and *in vivo* that exposure to NIC enhanced the expression of NGF receptors (Terry and Clarke, 1994; Jonnala et al, 2002). The purpose of the studies described in this report was to further investigate these effects of NIC on NGF as well as to further characterize the compound's effects on memory function and cholinergic markers (i.e., factors dependent of NGF). We thus focused on the effects of intermittent exposure to NIC on: 1) memory function (after it has cleared the plasma, thus non-acute effects), as opposed to the majority of studies which have studied acute effects (Popke et al, 2000), 2) the effects of this exposure method on the major acetylcholine receptor subtypes that are expressed in the brain, 3) the expression of other key cholinergic markers (i.e., ChAT and VAChT) and, finally, 4) the effects on nerve growth factor protein and its receptors.

In a similar fashion to other published studies of the effects of NIC on maze learning in rodents (Arendash et al, 1995; Socci et al, 1995; Attaway et al, 1999), rats repeatedly exposed to NIC in our study demonstrated enhanced efficiency when compared to controls. This was evident in the hidden platform tests of the daily method and in probe trials for the single day (multiple trial) method. For the latter method, we did not detect significant improvements in the

hidden platform trials, as did Abdulla et al (1996) (in a similar single day multiple trial testing method) after repeated NIC exposure. There were subtle differences in our study compared to the study cited above, however, such as the14 day exposure period to NIC in our study (versus 10 days of exposure), our 90 sec maximum time allowed to find the platform (versus 60 sec), and our 30 sec time allowed on the platform (versus 15 sec). Furthermore, they did not conduct probe trials after the hidden platform tests.

After memory testing, autoradiographic analyses were conducted with subtype specific radioligands to both nAChR's and mAChR's, both of which have been implicated in learning and memory processes (van der Zee and Luiten, 1999; Dani, 2001; Rezvani and Levin, 2001). High affinity (heteromeric α/β subunit complexes) and low affinity (homomeric α_7) nAChR's are transmitter gated ion channels that were localized with [^{3}H]-epibatidine and [^{125}I]-α-bungarotoxin, respectively. Of the high affinity nAChR's, the $\alpha_4\beta_2$ subtype is the predominant receptor subtype that is more widely distributed in mammalian brain (i.e., compared to $\alpha_3\beta_4$). We detected an increase in $\alpha_4\beta_2$ nAChR density in prefrontal cortex, frontal cortex and parietal cortex, as well as the different lamina of the cortex. These results are in general agreement with previous studies (Ulrich et al, 1997) and support the potential of using NIC as a prototype for AD therapy. Specifically, several post mortem studies of the cortex of AD brains have demonstrated that the $\alpha_4\beta_2$ nAChR is the major cholinergic receptor subtype that is diminished (Warpman and Nordberg, 1995; Sihver et al, 1999). These studies are supported by other data indicating that α_4 and β_2 subunit

mRNA levels are decreased in AD patients versus age-matched controls (Tohgi et al, 1998). Animal data further suggest an important role of $\alpha_4\beta_2$ nAChR's in mammalian brain. For example, mice lacking the β_2 subunit of the nAChR (thus deficient in high affinity nAChR's) show accelerated structural and cognitive degeneration with age (Zoli et al, 1999).

Another notable finding in the autoradiographic studies were the binding results in the amygdala, which demonstrated upregulation of both high and low-affinity nAChR. Recent studies suggest that the amygdala (specifically the basolateral amygdaloid nucleus) is an important area that mediates NIC's actions on memory function (Addy et al, 2003). We detected a 25%+ increase in [^{3}H]-epibatidine binding when the whole amygdala was analyzed (which includes the basolateral amygdala) and an 18+% increase in [^{125}I]-α-bungarotoxin binding in the posterior cortical amygdala. Although the posterior cortical amygdala is not part of the basolateral amygdala, and is generally associated with the processing of pheremonal information in animals, recent studies have suggested that it may modulate memory processing through its connections to the basal forebrain and hippocampal formation (Kemppainen et al, 2002).

The density of (G-protein coupled) M1 and M2 receptors (i.e., the muscarinic receptors expressed in highest quantities in mammalian brain (van der Zee and Luiten, 1999) were quantified using [^{3}H]-pirenzipine and [^{3}H]-AFDX-384, respectively. Post-synaptic M1 mAChR's stimulate phosphatidyl-inositol turnover while M2 mAChR's are thought to primarily exist as presynaptic autoreceptors that inhibit the release of adenylate cyclase (Caufield 1993).

Although no differences in pirenzipine binding were noted after NIC exposure, a significant increase in cortical M2 muscarinic receptors was observed. Recent studies have demonstrated a persistent increase in cortical ACh efflux over time with repeated NIC exposure (Arnold et al, 2002), an effect that could result in a compensatory upregulation of M2 autoreceptors (as observed in our study).

The enhanced expression ChAT and VAChT levels in the hippocampus are intriguing, particularly since these factors are known to be regulated by NGF. In cholinergic presynaptic terminals, ChAT facilitates acetylcholine synthesis and VAChT facilitates the transport of acetylcholine into synaptic vesicles for regulated exocytotic release. The reason for the lack of a similar increase in ChAT and VAChT in the cortex is unclear at present. Another interesting observation was the lack of effect of the repeated NIC exposure method on NGF levels in the brain which conflicts with other studies that found an increase in NGF mRNA associated with NIC exposure (Rattray, 2001). It should be noted that in the study of Rattray (2001) NIC was administered directly in the brain as opposed to our study in which peripheral injections were administered repeatedly over 14 days and NGF levels were assessed after a 21-hour washout period.

The increased expression of the phosphorylated (activated) form of TrkA in the present study may have important therapeutic ramifications. Phosphorylation of TrkA at TYR 490 is an upstream event in the signaling cascade responsible for the activation of CREB (Sweatt, 2001). It is also important to note that in the context of AD, there does not appear to be a deficiency in the synthesis or availability of NGF protein in the hippocampus or

neocortex. Whereas, substantial evidence suggests that signal transduction via the high affinity TrkA receptor is compromised (Mufson et al, 1997). In addition, we did not detect any significant changes in the in the p75 (low affinity) NGF receptor which has commonly been associated with apoptosis.

Collectively, the results summarized above indicate that repeated exposure to NIC results in lasting beneficial effects on memory function as well several important components of the cholinergic system. The positive effects on NGF receptor phosphorylation suggest one possible mechanism for these effects. Further, (as might be expected if NGF plays a role) NIC's actions on cholinergic neurons appear to be presynaptic as indicated by the effects on nAChR's, M2AchRs, ChAT and VAChT, but not M1 receptors. Interestingly, studies by Sabbagh et al (1998) have demonstrated that the majority of the nAChR's lost in AD are presynaptic. Evaluation of the frontal cortex of AD patients revealed a significant correlation between decreases in [^{3}H]-epibatidine binding and decreased expression of synaptophysin, a presynaptic marker. Other studies have also demonstrated presynaptic losses in cholinergic function: (1) a loss in ChAT activity. (Paterson and Nordberg, 2000), and (2) a decrease in the binding of both [^{3}H]-epibatidine, [^{3}H]-nicotine, [^{3}H]-cytisine and [^{3}H]-vesamicol binding temporal cortex (Sihver et al, 1999). The results of our study further support the potential use of nicotine-like compounds for the therapeutics of diseases in which cholinergic function is compromised.

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Table 1.1 Specific methods for autoradiography with both nicotinic and muscarinic radioligands on whole brain sagittal sections

Ligand	**Epibatidine**	**α-Bungarotoxin**	**Pirenzipine**	**AFDX-384**
Isotope	^{3}H	^{125}I	^{3}H	^{3}H
Receptor Subtype Target	Nicotinic non-α7*	Nicotinic α7	Muscarinic M1	Muscarinic M2
Radioligand Incubation Concentration (nM)	0.45	1.5	5.0	10.0
Incubation Time (min)	60	150	90	90
Film Exposure Time (weeks)	10	1	2	4

*α4-containing nAChR's are targeted with 3H-epibatidine alone, α3-containing nAChR's are targeted with [^{3}H]-epibatidine with 150 nM cytisine (see Perry et al1995, Marks et al 1998 and Hernandez et al 2003)

Table 1.2 Comparison of [^{3}H]-epibatidine binding site densities in selected brain regions from male Wistar rats (16 weeks old) treated with nicotine (0.7 mg/kg/day) for 14 days versus control (saline). Binding is expressed as nCi of bound ^{3}H per mg wet tissue. Each value represents mean ± S.E.M. (n=6). Significant differences where treatment with nicotine was greater than controls are denoted by a "*". *p<0.05, **p<0.01, ***p<0.001. Abbreviations used in this table are as follows: Nucleus/I (N.), Solitary Tract (S.T.).

BRAIN AREA	SALINE	NICOTINE
TELENCEPHALON		
Neocortex		
Cerebral Cortex		
Entorhinal*	1.049±0.028	1.213±0.068
Frontal***	1.087±0.027	1.338±0.024
Parietal**	1.174±0.028	1.323±0.025
Piriform***	0.521±0.026	0.734±0.036
Prefrontal**	1.315±0.026	1.468±0.032
Lamina I***	0.988±0.032	1.197±0.022
Lamina II**	1.399±0.049	1.647±0.027
Lamina III-V**	1.068±0.037	1.278±0.031
Rhinencephalon		
Anterior Olfactory N.***	0.721±0.025	0.929±0.017
Hippocampus	0 436±0.024	0.442±0.018
Septum, Lateral	0.585±0.029	0.607±0.022
Subicular Complex		
Postsubiculum	2.645±0.122	2.635±0.058
Presubiculum	2.345±0.064	2.455±0.083
Subiculum	1.534±0.047	1.421±0.043
Amygdala		
Amygdala***	0.570±0.029	0.770±0.025

Basal Ganglia		
Accumbens N. **	1.173±0.050	1.345±0.029
Bed N. of Stria Terminalis	0.675±0.031	0.657±0.019
Caudate Putamen	1.347±0.027	1.428±0.033
Globus Pallidus (Lateral)	0.537±0.020	0.518±0.010
DIENCEPHALON		
Epithalamus		
Habenular N., Medial	11.578±0.065	11.678±0.040
Metathalamus		
Anterior Pretectal N.		
Dorsal	1.366±0.052	1.317±0.033
Ventral	0.941±0.058	0.897±0.033
Geniculate N.		
Medial	1.901±0.048	1.928±0.067
Ventrolateral	2.024±0.008	2.069±0.072
Dorsolateral	2.798±0.140	2.694±0.106
Olivary Pretectal N.	1.588±0.026	1.649±0.033
Thalamus		
Thalamic N.		
Anteromedial	2.934±0.127	2.888±0.125
Anteroventral	4.537±0.500	3.766±0.170
Central Medial	2.146±0.083	2.209±0.091
Lateral Posterior	2.166±0.106	2.084±0.061
Laterodorsal	2.560±0.092	2.466±0.066
Mediodorsal	2.370±0.072	2.383±0.035
Paraventricular	1.884±0.052	1.982±0.018
Posterior	2.186±0.111	2.248±0.043
Reticular	1.630±0.052	1.674±0.063
Reuniens	2.364±0.056	2.468±0.084
Ventral Posterolateral	1.710±0.055	1.813±0.064

Subthalamus		
Zona Incerta	0.718±0.028	0.766±0.016
Hypothalamus		
Hypothalamus	0.611±0.022	0.629±0.012
MESENCEPHALON		
Interpeduncular N.	11.183±0.056	11.462±0.051
Periaqueductal Gray*	0.751±0.037	0.854±0.021
Substantia Nigra	0.671±0.017	0.642±0.015
Superior Colliculus*	3.398±0.086	3.122±0.064
Tegmental N., Dorsal	1.142±0.059	1.247±0.052
METENCEPHALON		
Cerebellum		
Cerebellum		
Purkinje Layer	0.910±0.032	0.866±0.018
Medulla		
N. of the S.T.	1.888±0.230	1.989±0.103
Vestibular N., Medial	0.999±0.024	1.035±0.030
Pons		
Pontine N.	0.785±0.023	0.841±0.029
OTHER		
Fasciculus Retroflexus*	3.197±0.138	4.117±0.980
Pineal Gland	11.436±0.057	11.355±0.021

Table 1.3 Comparison of [^{3}H]-epibatidine (with 150 nM cytisine) binding site densities in selected brain regions from male Wistar rat (16 weeks old) treated with nicotine (0.7 mg/kg/day) for 14 days versus control (saline). Binding is expressed as nCi of bound ^{3}H per mg wet tissue. Each value represents mean ± S.E.M. (n=6). Abbreviations used in this table are as follows: Nucleus/I (N.), Solitary Tract (S.T.).

BRAIN AREA	SALINE	NICOTINE
TELENCEPHALON		
Neocortex		
Cerebral Cortex	0.268±0.015	0.291±0.003
Rhinencephalon		
Postsubiculum	0.584±0.027	0.576±0.021
Presubiculum	0.633±0.058	0.656±0.040
Basal Ganglia		
Caudate Putamen	0.319±0.020	0.323±0.004
DIENCEPHALON		
Epithalamus		
Medial Habenular N.	10.843±0.124	10.875±0.120
Metathalamus		
Pretectal N.	1.046±0.088	1.049±0.049
Geniculate N.		
Dorsolateral	0.803±0.018	0.825±0.018
Medial	0.386±0.032	0.363±0.011
Ventrolateral	0.827±0.069	0.818±0.022
Thalamus		
Thalamic N., Anterior	0.670±0.025	0.656±0.018
MESENCEPHALON		
Collicular N.		
Central Inferior	0.405±0.017	0.337±0.031
Superior	1.464±0.024	1.555±0.071
Interpeduncular N.	9.952±0.217	9.962±0.156

Substantia Nigra	1.234±0.047	1.090±0.098
METENCEPHALON		
N. of the S.T.	1.718±0.159	1.661±0.121
Vestibular N., Medial	0.465±0.050	0.426±0.013
Cerebellum	0.197±0.015	0.177±0.005
OTHER		
Fasciculus Retroflexus	2.716+0.258	2.754±0.191
Pineal Gland	10.226±0.256	10.306±0.065

Table 1.4 Comparison of [^{125}I]-α-bungarotoxin binding site densities in selected brain regions male Wistar rats (16 weeks old) treated with nicotine (NIC), 0.7 mg/kg/day, for 14 days versus controls treated with saline (SAL). Binding is expressed as nCi of bound ^{125}I per mg wet tissue. Each value represents mean $\pm$ S.E.M. (n=6). Significant differences between groups are denoted by the following symbols: "$*$" when SAL<NIC ($*p<0.05$ and $**p<0.01$) or "ϕ." when SAL>NIC ($\phi p<0.05$). Abbreviations used in this table are as follows: Nucleus/I (N.), Posterior Cortical (Post.-Cort.), Anterior (Ant.), Ventrolateral (VL), Ventromedial (VM), Magnocellular N. of the Post. Commissure, Gigantocellular (Gigantocell.), Microcellular (Microcell.), Tegmental (Tegmtl.), Pedunculopontine (Pedunculopon.), Principal Trigeminal Sensory (Princ. Trigeminal Sens.)

BRAIN AREA	SALINE	NICOTINE
TELENCEPHALON		
Neocortex		
Cerebral Cortex		
Entorhinal	0.088±0.004	0.089±0.010
Insular	0.074±0.003	0.070±0.003
Motor	0.059±0.005	0.059±0.004
Orbital	0.084±0.007	0.074±0.004
Parietal Association	0.166±0.009	0.180±0.008
Piriform	0.049±0.004	0.052±0.003
Somatosensory	0.057±0.004	0.058±0.003
Visual	0.052±0.004	0.053±0.002
Lamina I-IV	0.047±0.004	0.048±0.001
Lamina V	0.080±0.003	0.084±0.004
Rhinencephalon		
Accessory Olfactory Bulbϕ	0.605±0.028	0.514±0.010
Olfactory Bulbs	0.015±0.003	0.022±0.004
Anterior Olfactory N.		
Dorsoventrolateral	0.139±0.009	0.133±0.007
Posterior	0.149±0.009	0.147±0.007

Hippocampal Formation		
CA1 region	0.093±0.004	0.094±0.005
CA2 and 3 region	0.150±0.006	0.152±0.006
Dentate Gyrus	0.497±0.021	0.497±0.033
Polymorphic Layer	0.231±0.011	0.252±0.010
Subicular Complex		
Presubiculum	0.168±0.010	0.164±0.009
Subiculum	0.145±0.008	0.132±0.009
Postsubiculum	0.044±0.002	0.040±0.001
Amygdala		
Ant. Amygdaloid Area	0.143±0.004	0.140±0.004
Post-Cort. Amygdaloid N.*	0.241±0.011	0.294±0.016
Basal Ganglia		
Dorsal Endopiriform N.	0.175±0.010	0.183±0.005
DIENCEPHALON		
Epithalamus		
Habenular N., Lateral	0.078±0.007	0.073±0.006
Metathalamus		
Pretectal N.	0.146±0.005	0.149±0.009
Geniculate N. (VL)	0.257±0.016	0.255±0.013
Subthalamus		
Subthalamic N.	0.359±0.023	0.396±0.033
Zona Incerta	0.082±0.002	0.081±0.004
Hypothalamus		
Hypothalamic Area		
Anterior	0.080±0.010	0.068±0.005
Posterior	0.096±0.005	0.095±0.003
VM Hypothalamic N. **	0.133±0.003	0.162±0.007
Medial Preoptic Area	0.140±0.007	0.414±0.003
Supraoptic N.	0.456±0.037	0.515±0.022
Mammillary Peduncle	0.290±0.010	0.296±0.005

MESENCEPHALON		
Collicular N.		
Central Inferior	0.328±0.011	0.307±0.004
Superior	0.350±0.011	0.349±0.006
Dorsal Raphe N.	0.614±0.039	0.686±0.028
MnPC	0.175±0.010	0.189±0.019
Periaqueductal Gray	0.095±0.003	0.098±0.004
Substantia Nigra	0.083±0.002	0.077±0.002
Medulla		
Cochlear N.	0.063±0.005	0.071±0.004
Gigantocell. Reticular N.	0.055±0.003	0.055±0.003
Inferior Olive	0.201±0.020	0.192±0.010
Medial Vestibular N. *	0.487±0.004	0.526±0.029
Pons		
Dorsal Tegmtl. N.	0.562±0.062	0.484±0.027
Lateral Leminiscus	0.190±0.015	0.183±0.012
Lateral Superior Olive	0.226±0.008	0.194±0.013
Microcell. Tegmtl. N.	0.226±0.016	0.216±0.007
Parabrachial N.	0.163±0.005	0.168±0.005
Pedunculopon. Tegmtl. N.	0.144±0.005	0.148±0.004
Princ. Trigeminal Sens. N.	0.145±0.011	0.140±0.006
Spinal Trigeminal N.	0.100±0.004	0.105±0.005

Table 1.5 Comparison of [^{3}H]-Pirenzipine binding site densities in selected brain regions from male Wistar rats (16 weeks old) treated with nicotine (0.7 mg/kg/day) for 14 days versus control (saline). Binding is expressed as nCi of bound ^{3}H per mg wet tissue. Each value represents mean ± S.E.M. (n=6). Abbreviations used in this table are as follows: Nucleus/i (N.), Rostroventrolateral (RVL).

BRAIN AREA	SALINE	NICOTINE
TELENCEPHALON		
Neocortex		
Cerebral Cortex		
Cingulate	10.691±0.132	10.794±0.066
Entorhinal	10.218±0.158	10.217±0.102
Frontal	9.868±0.190	9.822±0.092
Insular	10.878±0.080	10.771±0.056
Piriform	10.874±0.119	10.863±0.074
Retrosplenial	7.538±0.311	7.556±0.120
Lamina I	11.256±0.117	11.164±0.071
Lamina II-VI	9.691±0.212	9.481±0.136
Rhinencephalon		
Anterior Olfactory N.	11.015±0.126	10.962±0.091
Hippocampal Formation		
CA1 region	12.068±0.140	12.032±0.091
CA2 and 3 regions	10.068±0.122	9.997±0.052
Dentate Gyrus		
Inner Blade	9.897+0.129	9.843±0.070
Outer Blade	11.962±0.129	12.003±0.104
Olfactory Bulbs	7.226±0.575	7.678±0.214
Subicular Complex		
Presubiculum	8.182±0.215	8.051±0.139
Parasubiculum	6.989±0.269	7.390±0.300
Subiculum	6.929±0.190	7.062±0.172

Postsubiculum	5.536±0.273	5.319±0.075
Lateral Septal N.	3.603±0.093	3.816±0.097
Substantia Innominata	3.197±0.037	3.252±0.065
Amygdala		
Anterior Amygdaloid Area	9.780±0.124	9.621±0.187
Basolateral Amygdaloid N.	11.234±0.160	10.852±0.205
Basal Ganglia		
Accumbens N.	11.348±0.131	11.349±0.077
Bed N. of Stria Terminalis	4.458±0.055	4.312±0.162
Caudate Putamen	10.736±0.136	10.737±0.027
Globus Pallidus (Lateral)	2.231±0.022	2.344±0.051
DIENCEPHALON		
Metathalamus		
Geniculate N.		
Dorsolateral	2.955±0.051	2.996±0.105
Medial	3.174±0.100	3.352±0.077
Olivary Pretectal N.	11.383±0.158	11.324±0.081
Thalamus		
Thalamic N., Anterior	3.764±0.109	3.842±0.088
Posterior	3.720±0.115	3.529±0.091
Ventral Posterior	10.608±0.096	10.644±0.066
Hypothalamus	1.737±0.033	1.700±0.042
MESENCEPHALON		
Mesencephalon		
Collicular N.		
Central Inferior	2.068±0.112	2.179±0.064
Superior	2.457±0.115	2.312±0.055
Periaqueductal Gray	1.611±0.055	1.601±0.030
Substantia Nigra	2.156±0.077	2.134±0.035

METENCEPHALON		
Medulla		
RVL reticular N.	3.765±0.209	3.745±0.156
Pons		
Parabrachial N.	2.218±0.123	2.143±0.129
Pontine N.	1.687±0.054	1.641±0.062

Table 1.6 Comparison of [^{3}H]-AFDX-384 binding site densities in selected brain regions from male Wistar rats (16 weeks old) treated with nicotine (0.7 mg/kg/day) for 14 days versus control (saline). Binding is expressed as nCi of bound ^{3}H per mg wet tissue. Significant differences ($p<0.05$) when treatment with nicotine was greater than controls are denoted by a "∗". Abbreviations used in this table are as follows: Nucleus/I (N.), Amygdaloid (Amy.), Solitary Tract (S.T.), Rostroventrolateral (RVL).

BRAIN AREA	SALINE	NICOTINE
TELENCEPHALON		
Neocortex		
Cerebral Cortex∗	4.352±0.224	6.161±0.661
Entorhinal∗	3.511±0.116	4.336±0.333
Piriform	3.842±0.212	4.470±0.228
Lamina I	6.798±0.686	8.187±0.671
Lamina II	4.065±0.253	5.283±0.492
Rhinencephalon		
Anterior Olfactory N.	5.562±0.511	6.353±0.749
Hippocampal Formation		
CA1 region	6.734±0.671	7.409±0.797
CA2 and 3 region	3.093±0.093	3.304±0.218
Dentate Gyrus	3.908±0.198	4.245±0.284
Polymorphic Layer	3.635±0.108	4.061±0.321
Olfactory Bulb Layers		
Granular Cell	9.045±0.087	9.213±0.175
External Plexiform	10.706±0.155	10.906±0.099
Olfactory Tubercle	10.355±0.159	10.788±0.13
Subiculum	2.917±0.098	3.246±0.147
Amygdala		
Amy. Hippocampal Area	3.196±0.238	3.794±0.416
Centrobasolateral Amy. N.	3.272±0.222	5.146±0.891

Basal Ganglia		
Accumbens N.	9.997±0.155	10.266±0.149
Bed N. of Stria Terminalis	2.681±0.102	3.110±0.177
Caudate Putamen	9.587±0.127	10.071±0.191
Septum		
Septal N.		
Lateral	2.982±0.102	3.468±0.229
Medial	2.440±0.059	2.560±0.167
DIENCEPHALON		
Metathalamus		
Anterior Pretectal N.	2.505±0.082	2.839±0.182
Geniculate N.		
Dorsolateral	2.400±0.131	2.792±0.134
Medial	2.234±0.052	2.538±0.137
Ventrolateral	2.245±0.14	2.136±0.085
Thalamus		
Thalamic N.		
Anterior*	4.293±0.165	5.989±0.628
Laterodorsal	2.745±0.09	3.043±0.176
Lateroposterior	2.648±0.145	3.050±0.205
Mediodorsal	2.691±0.078	3.098±0.167
Ventroposterior	1.989±0.044	2.220±0.096
Posterior*	2.768±0.076	3.182±0.163
MESENCEPHALON		
Mesencephalon		
Collicular N.		
Central Inferior	2.025±0.054	2.223±0.153
Superior	2.98 ±0.175	3.439±0.361
Deep Layer	2.374±0.087	2.689±0.219
Periaqueductal Gray	2.225±0.096	2.354±0.151

METENCEPHALON		
Medulla		
N. of the S.T.	3.508±0.088	4.150±0.247
RVL Reticular N. *	3.216±0.08	3.787±0.211
Pons		
Parabrachial N.	3.147±0.2	3.461±0.185
Pontine N.	2.895±0.19	4.514±0.420
OTHER		
Pineal Gland	1.909±0.013	1.989±0.032

Figure Legends

Fig. 1.1 Water maze testing: Hidden Platform Tests. The distances swam to locate the platform in the hidden platform tests are depicted in (A) for the single day multiple trial method (n=12) and (B) for the daily single trial method (n=6) water maze testing. Filled circles=nicotine-treated rats, empty circles=saline-treated controls.

Fig. 1.2 Water maze testing: Probe Trials. The percentage of total distance swam in the target quadrant for probe trials are depicted for the (A) single day multiple trial and (B) daily single trial methods. Filled bars=nicotine-treated rats, empty bars=saline-treated controls.

Fig. 1.3 Representative autoradiograms illustrating muscarinic and nicotinic acetylcholine receptor subtypes labeled by both muscarinic radioligands (A) [^{3}H]-AFDX-384 (M2) and (B) [^{3}H]-pirenzipine (M2), and nicotinic radioligands (C) [^{125}I]-α-bungarotoxin (α_7) and non-α_7 (D) [^{3}H]-epibatidine (predominantly $\alpha_4\beta_2$) or (E) [^{3}H]-epibatidine + 150 nM cytisine (predominantly $\alpha_3\beta_4$) in sagittal sections of brains from male Wistar rats (16 weeks old) after 14 days of repeated nicotine (0.7 mg/kg/day) exposure vs. control (saline). Selected areas that are characteristic of the binding distribution of these radioligands are indicated with arrows and numbered as follows: (1) caudate putamen, (2) dentate gyrus, (3) accessory olfactory bulb, (4) prefrontal cortex and (5) dorsolateral geniculate nucleus.

Fig. 1.4 Comparison of the immunoreactivity of two presynaptic cholinergic markers, choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT), in the (A) cortex and (B) hippocampus of male Wistar rats (16 weeks old) after 14 days of exposure to nicotine (NIC) or vehicle (saline) (n=6 rats per group). Immunoreactivity of both anti-ChAT and anti-VAChT are presented as optical density values in arbitrary units (AU) with samples of representative immunoblots below each bar. "∗"=Saline<NIC ($p<0.05$). Filled bars=nicotine-treated rats, empty bars=saline-treated controls.

Fig. 1.5 Comparison of the immunoreactivity of the high affinity NGF receptor, TrkA (phospho-independent and dependent) in the (A) cortex and (B) hippocampus of male Wistar rats (16 weeks old) after 14 days of exposure to nicotine (NIC) or vehicle (saline) (n=6 rats per group). Each set of plots is presented as follows: anti-TrkA is far left, anti-phospho-TrkA (Y674/675) is center and anti-phospho-TrkA (Y490) is far right. The immunoreactivities of anti-TrkA (phospho-independent and dependent) are presented as optical density values in arbitrary units (AU) with samples of representative immunoblots below each bar. Significant differences where treatment with NIC was greater than controls are denoted by a "*". *$p<0.05$, **$p<0.01$, ***$p<0.001$. Filled bars=nicotine-treated rats, empty bars=saline-treated controls.

Fig. 1.6 NGF levels in the cortex and hippocampus collected from male Wistar rats (16 weeks old) after 14 days of exposure to nicotine or vehicle (saline) (n=6 rats per group). Filled bars=nicotine-treated rats, empty bars=saline-treated controls.

Fig 1.1

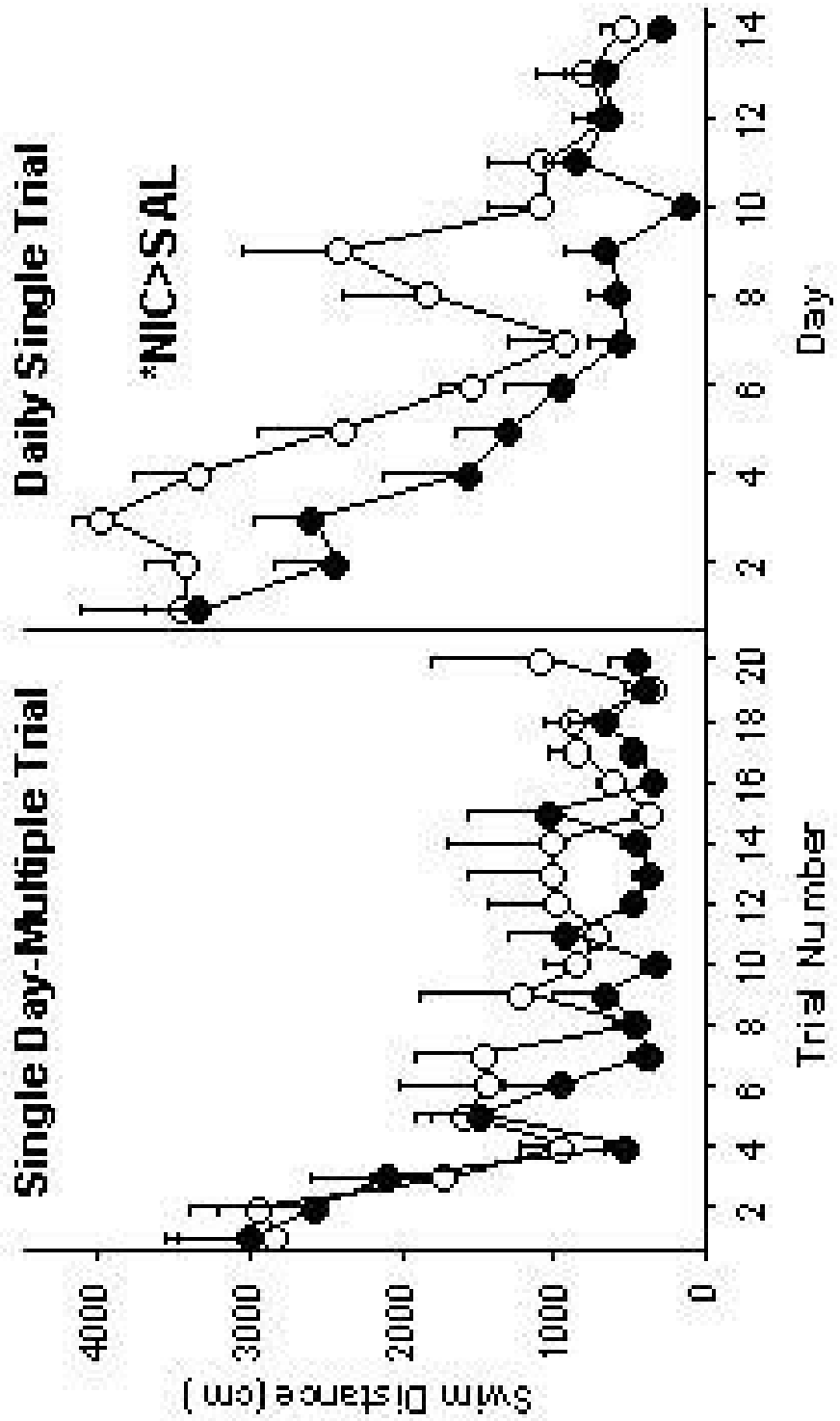

Single Day-Multiple Trial
Daily Single Trial
*NIC>SAL
Swim Distance (cm)
0
1000
2000
3000
4000
2
4
6
8
10
12
14
16
18
20
Trial Number
2
4
6
8
10
12
14
Day

Fig. 1.2

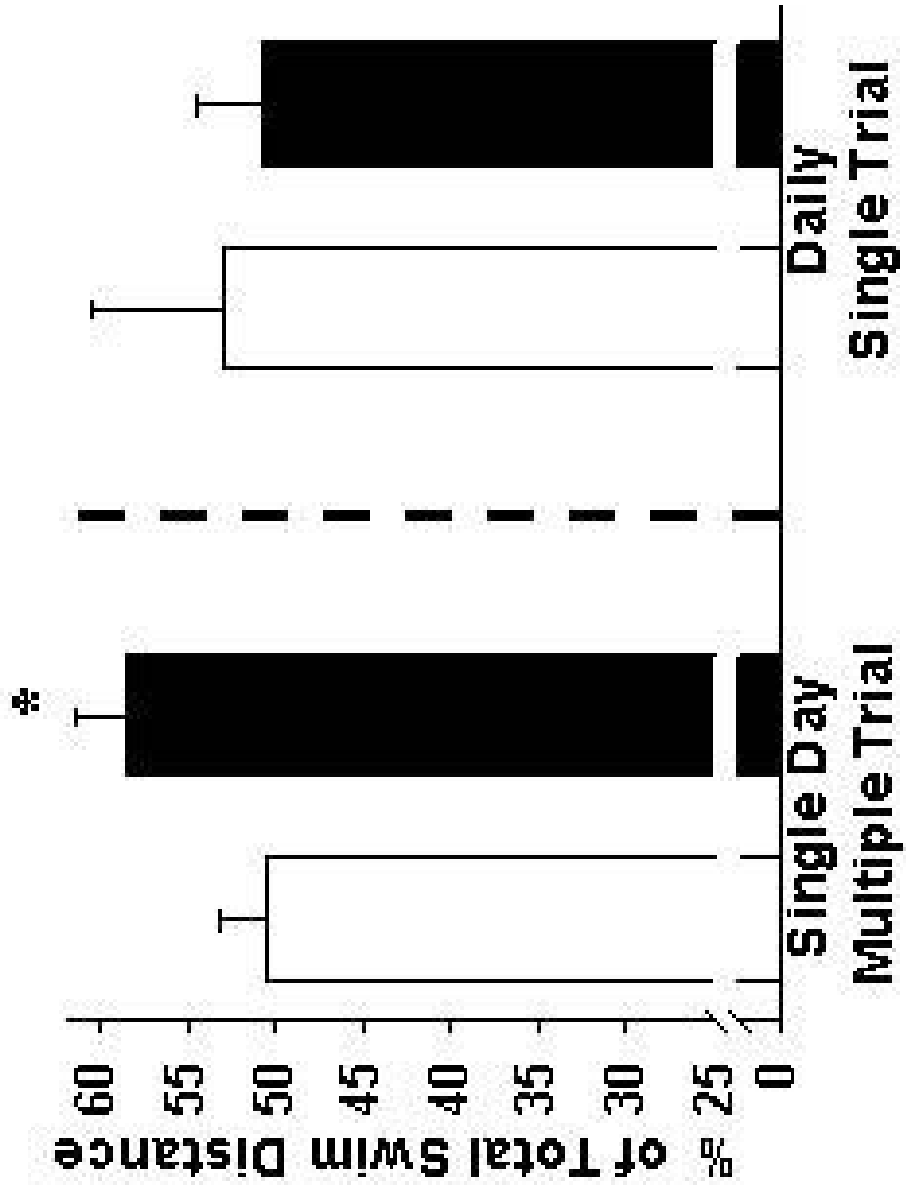

% of Total Swim Distance
60
55
50
45
40
35
30
25
0
*
Single Day
Multiple Trial
Daily
Single Trial

Fig. 1.3

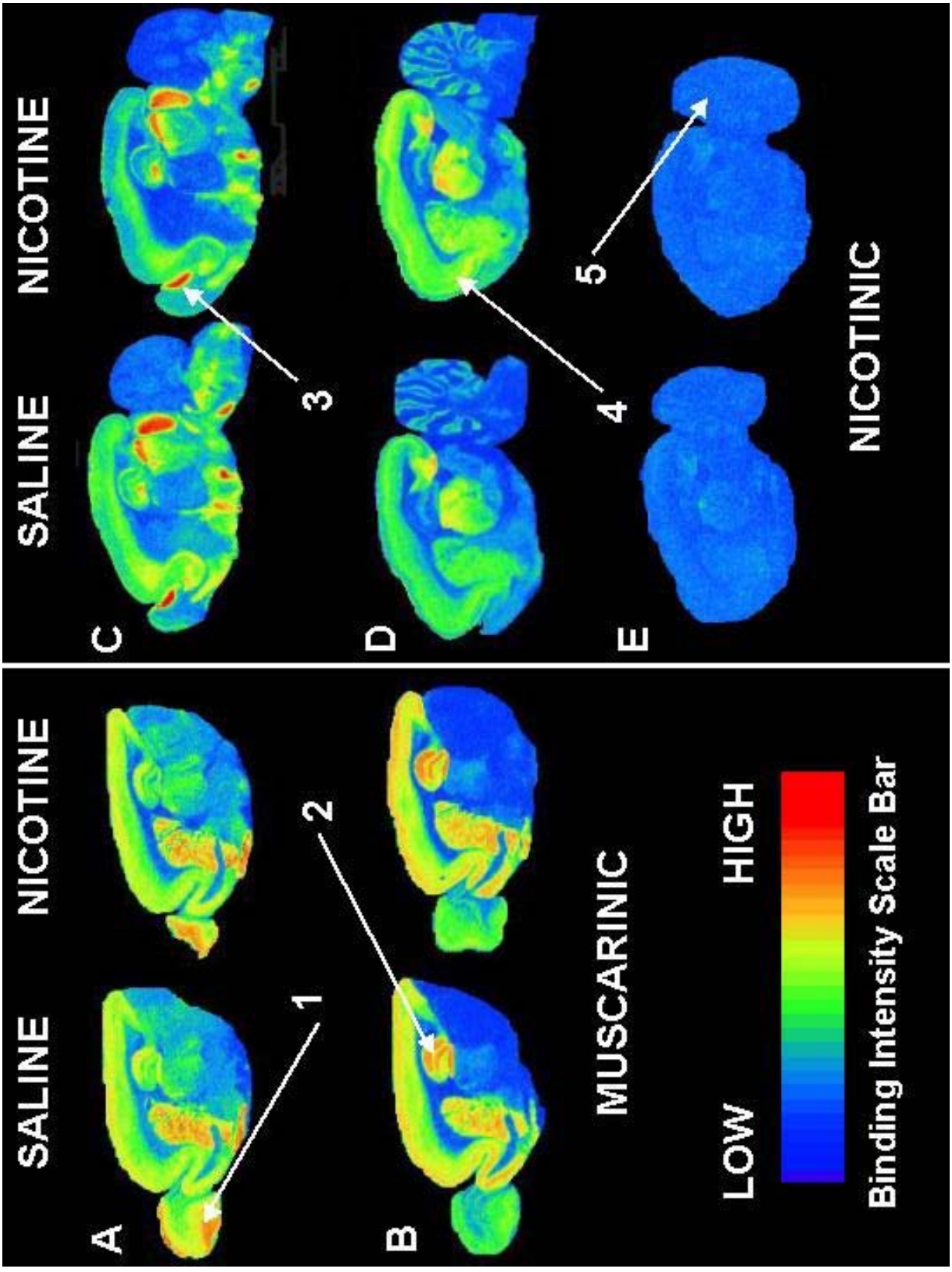

SALINE
NICOTINE
SALINE
NICOTINE
A
B
C
D
E
1
2
3
4
5
MUSCARINIC
NICOTINIC
LOW
HIGH
Binding Intensity Scale Bar

Fig. 1.4

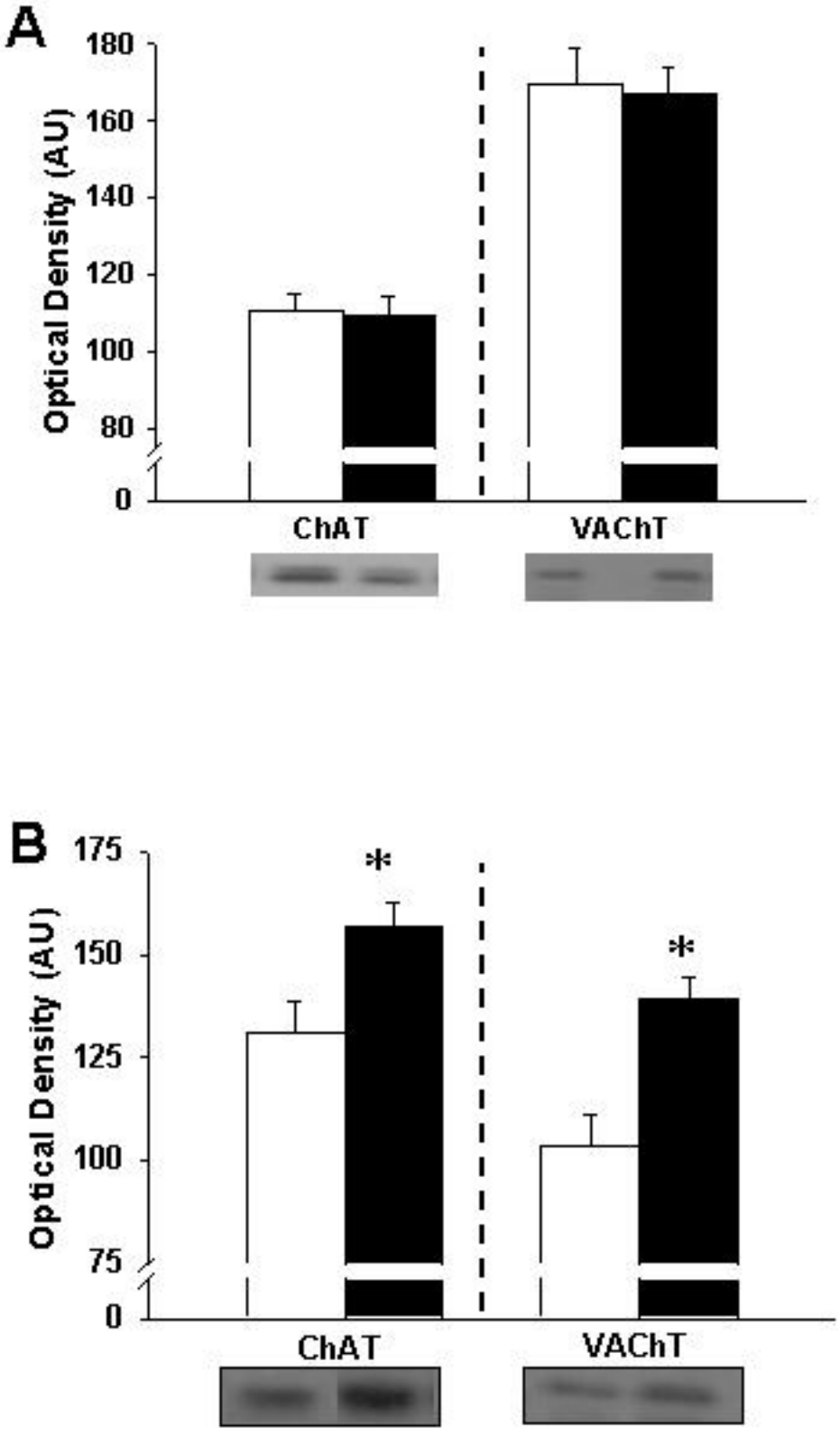

A
180
160
140
120
100
80
0
Optical Density (AU)
ChAT
VAChT
B
175
150
125
100
75
0
Optical Density (AU)
*
*
ChAT
VAChT

Fig. 1.5

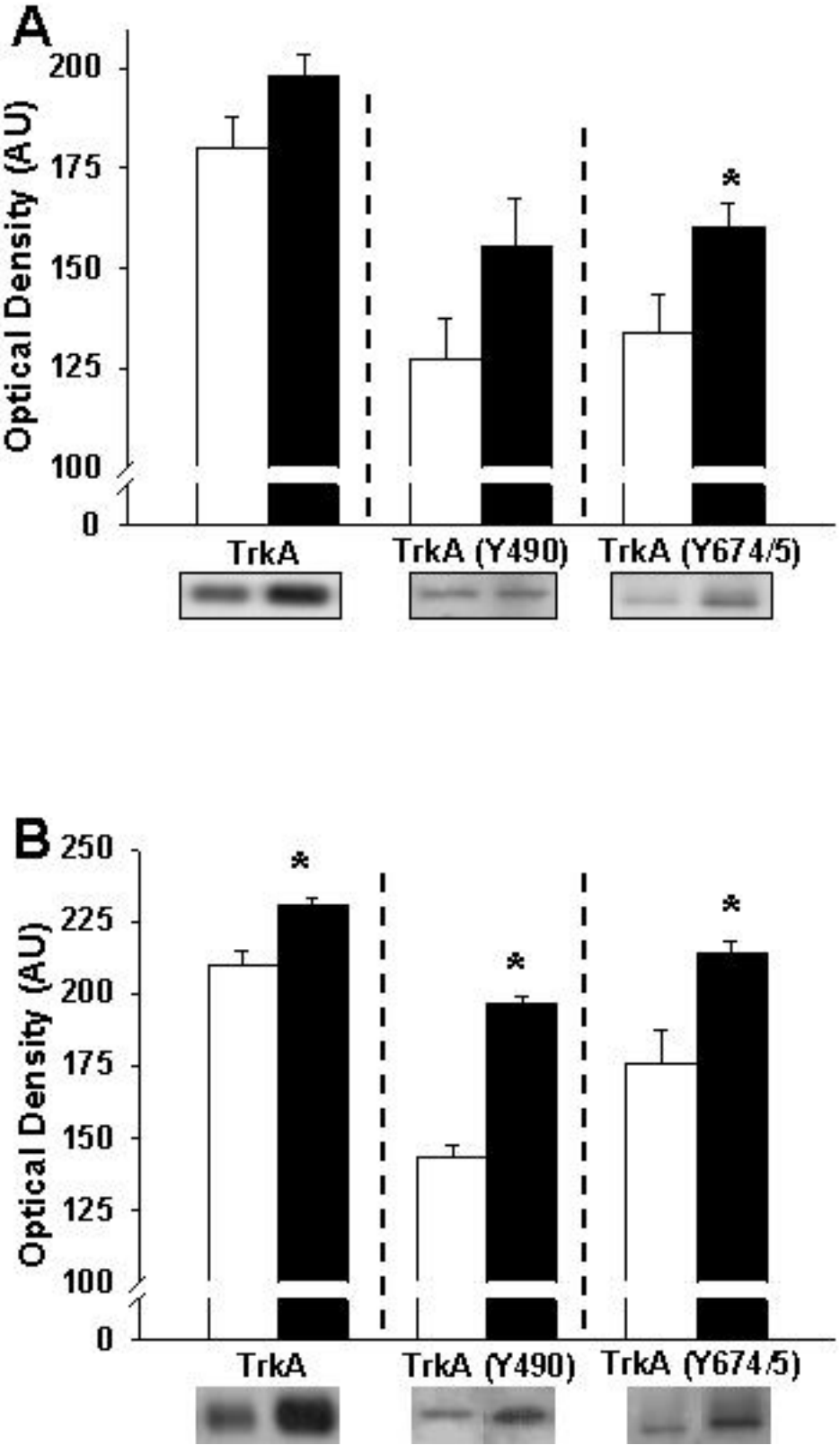
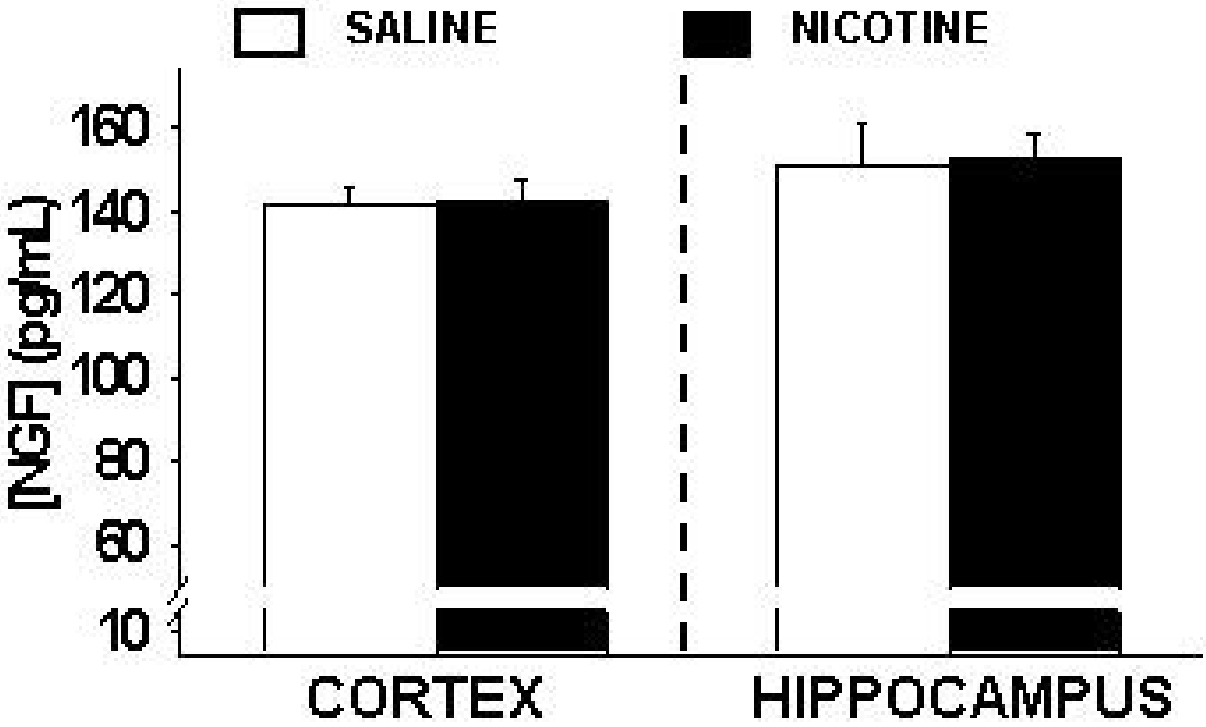

A
Optical Density (AU)
200
175
150
125
100
0
*
TrkA
TrkA (Y490)
TrkA (Y674/5)
B
Optical Density (AU)
250
225
200
175
150
125
100
0
*
*
*
TrkA
TrkA (Y490)
TrkA (Y674/5)

Fig. 1.6


SALINE
NICOTINE
[NGF] (pg/mL)
160
140
120
100
80
60
10
CORTEX
HIPPOCAMPUS

THE EFFECTS OF REPEATED NICOTINE EXPOSURE AND LEARNING ON CELL PROLIFERATION IN THE HIPPOCAMPUS[1]

[1] Hernandez, C.M., Schreihofer, A.M. and A.V. Terry, Jr. To be submitted to the *Journal of Pharmacology and Experimental Therapeutics.*

Abstract

Nicotine binds nicotinic acetylcholine receptors in a number of regions in the mammalian brain including the hippocampal formation, an area of the brain important for the formation of new memories that will eventually be consolidated into long-term memory storage. The hippocampus also contains a zone with the potential to produce new neurons into adulthood, a process known as secondary neurogenesis. Recently, it has been discovered that learning a novel task promotes neurogenesis in this region. Combining information that of what we know about the effects of learning on hippocampal neurogenesis with what is known about the positive effects of nicotine on learning, it was hypothesized that nicotine could modulate the proliferative potential of this region. Our objective was to evaluation the combined effects of nicotine and learning on cell proliferation (the initial step of neurogenesis) in the adult rat hippocampus. Rats were exposed to nicotine then trained in the water maze (a hippocampal-dependent learning task). Throughout the course of the study, rats were treated with bromodeoxyuridine to localize proliferating cells. The number of new cells in the hippocampal formation and subventricular zone were quantified using immunohistochemical localization of anti-BrdU. The combination of nicotine treatment and participation in a learning task resulted in a significant increase in cell proliferation in the hippocampus, but not subventricular zone, when compared to inactive controls. The next step in evaluating nicotine and a neurogenic agent with learning will be to track and follow the long-term survival

and differentiation of these cells into particular phenotypes, especially if they are permanently incorporated into the hippocampus.

Nicotine (the prototypic nicotinic agonist) binds nicotinic acetylcholine receptors in a number of regions in the mammalian brain including the hippocampal formation, an area of the brain important for the formation of new memories that will eventually be consolidated into long-term memory storage (Suzuki and Clayton 2000; Gilbert et al 1998). In addition to its role in memory, the hippocampus is one of five different areas of the brain recently discovered to produce new neurons into adulthood, a process called neurogenesis (Gross 2000; Eisch 2002). Hippocampal neurogenesis has been found to be increased by environmental enrichment (e.g. exercise) (Kempermann et al 1997; van Praag et al 1999) as well as learning (Gould et al 1999). Since previous studies have demonstrated that nicotine improves performance in learning tasks, we hypothesized that nicotine may enhance cell proliferation with water maze training (a hippocampal-dependent learning task).

Our objective was to evaluate the combined effects of nicotine and learning on cell proliferation (the initial step of neurogenesis) in the adult rat hippocampus. In the following experiments, rats were exposed to nicotine then trained in a hippocampal-dependent learning task (e.g. Morris Water Maze (MWM)). Animals were also treated with bromodeoxyuridine (BrdU) (a thymidine analog that is incorporated into the DNA newly divided cells) to localize proliferating cells after repeated exposure to nicotine and a maze learning method. At the end of behavioral testing, animals were sacrificed for whole brain removal. The number of new cells in the hippocampal formation and

subventricular zone were quantified using immunohistochemical localization of anti-BrdU.

Materials and Methods

Animals

Ten to twelve week old male albino Wistar rats (350-450 grams) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in a temperature-controlled room (25°C) with a 12-hour light/dark cycle. Upon arrival, each animal was provided with tap water and food (Teklad) ad libitum. All rats were handled daily until testing began at fourteen weeks of age. All procedures employed during this study were consistent with AAALAC guidelines and reviewed and approved by the Medical College of Georgia Committee on Animal Use (CAURE) and the VA Medical Center (Augusta, GA) Subcommittee on Animal Use.

Chemicals and Antibodies

Bromodeoxyuridine, (-)-nicotine hydrogen tartrate salt, paraformaldehyde, bis-benzimide and sodium citrate were purchased from Sigma Aldrich (St. Louis, MO). Ketamine (Ketaved®) and xylazine (TranquiVed®) were purchased from Phoenix Scientific (St. Joseph, MO). Sodium phosphate monobasic, sodium phosphate dibasic, paraffin, Triton-X-100 and xylene were purchased from Fisher-Scientific (Pittsburgh, PA). Vectashield aqueous mounting media and biotinylated anti-mouse IgG were purchased from Vector Laboratories

(Burlingame, CA). The sheep anti-BrdU was purchased from Biodesign International (Saco, ME). The mouse anti-neuronal nuclear antigen was purchased from Chemicon (Temecula, CA). The streptavidin-AlexaFluor488 was purchased from Molecular Probes (Eugene, OR). The donkey anti-sheep Cy3 was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Drugs and Administration

At fourteen weeks of age, animals were administered nicotine twice daily and bromodeoxyuridine once daily for 14 days. BrdU was prepared in saline (0.9% sodium chloride in sterile water) at a concentration of 33.3 mg/mL, then administered intraperitoneally at a dose of 50 mg/kg. Nicotine hydrogen tartrate was prepared in saline at a concentration of 1 mg/mL, then subcutaneously administered twice daily at a dose of 1 mg/kg. The dose of nicotine (equivalent to 0.7 mg/kg/day of nicotine base) was chosen to obtain plasma levels that have measurable biochemical and/or behavioral effects in rats, but undetectable in plasma during behavioral testing (Hwa Jung et al 2001). Rats were treated with nicotine (or the vehicle, saline) twice daily (8 a.m., 8 p.m.) and BrdU once daily (8 a.m.). The dosing schedule ensured that nicotine was cleared from plasma at the time of maze testing or swimming and enough time had elapsed for BrdU to be incorporated into the DNA of mitotic cells (Miller and Nowakowski 1988). An additional group was treated with nicotine (or vehicle), but not BrdU (a negative control for BrdU staining in immunohistochemistry experiments).

Behavioral Testing

To test the combined effect of nicotine administration and learning on cell proliferation in the hippocampal formation and subventricular zone, rats were divided into three different groups (n=6 each): learners, swimmers and non-learners/non-swimmers (see introduction section).

Learners participated in water maze testing using a daily single trial method (1 trial/day for 14 days) that began daily 4-5 hours following the morning administration of nicotine or vehicle. Maze testing was executed in a circular (180 cm x 76 cm) black plastic pool (Bonar Plastics, Newnan, GA) filled with water (25.0 + 1.0ºC) (Morris 1984). The walls surrounding the pool displayed reflective geometric shapes (i.e. visual cues) to aid in each rat's navigation of the water maze. However, both the experimenter and other rats were blocked from the view of each rat (while in the maze) by black curtains surrounding three-quarters of the pool's diameter. Once the rat was placed in the pool for testing, swimming activity of each rat was recorded monitored via a television camera mounted over the pool that was connected to a Poly-Track video tracking system (San Diego Instruments, San Diego, CA). Tracking of each rat was captured by the contrast of the white rat on a black background of the pool and diffuse lighting in the testing room. Maze testing included three types of tests: hidden platform, probe trials and visible platform in which an escape platform was: 1) hidden (i.e. submerged underwater), 2) absent (i.e. removed from the pool) and 3) visible (i.e. above the water's surface), respectively.

Hidden Platform Tests: A black (10 cm^2) platform remained in a fixed position, submerged approximately 1 cm below the water's surface. Hidden platform trials were initiated by placing a (water-maze naïve) rat in the water facing the pool wall in one of 4 quadrants (designated NE, NW, SE, SW). The order of entry into each four individual quadrants was pseudo-randomized. For each trial, rats were given 90 sec to find the hidden platform. When successful the rat was allowed a 30 sec rest period on the platform. If unsuccessful within 90 sec, the rat was placed on the platform for a 30 sec rest period by the experimenter. Three parameters were recorded and calculated by the video tracking system: (1) latency, the time (sec) to locate and mount the hidden platform, (2) distance, the distance swam (cm) to reach the platform and (3) swim speeds (the distance (cm) /latency (sec)).

Probe Trials: On day 15, a single 90 sec probe trial was conducted where the platform was removed from the pool to measure a spatial bias for the previous platform location (Morris 1984). The swim pattern of this trial was recorded by the tracking system. The percentages of total time spent and distance swam by each rat in the target quadrant where the platform was formerly located for the hidden platform tests) was calculated.

Visible Platform Tests: On day 15, visible platform tests were conducted after the probe trials in order to determine if the rats had any visual problems from combined treatment with nicotine and/or BrdU (which could potentially affect maze learning). The platform was re-introduced into the pool covered with a highly visible (light-reflective) block that was approximately 1 cm above the

water's surface. The curtains surrounding the maze were completely closed to prevent view of any previous visual cues in the testing room, which left the visible platform the only object in clear view. The visible platform was consecutively placed in each quadrant and each rat was given one trial to locate the platform in each quadrant of the maze. The time to locate the platform was recorded.

"Swim-yoked controls", a control group for the physical activity component of the water maze, was included because increased physical activity had been found to increase neurogenesis (van Praag et al 1999). In parallel with the learners participating in maze testing, swim-yoked controls swam in the water maze pool with the hidden platform removed. Since learners took progressively less time to locate the hidden platform, the length of time the swimmers were given in the maze pool was adjusted. The amount of time swimmers swam in the maze pool over the course of 14 days was matched by day to the mean amount of time learners (both nicotine and saline treated combined) took to locate the hidden platform each day of testing.

Inactive (i.e. non-learning/non-swimming) rats, a control group for learning and physical activity components of the water maze testing, were also included Inactive controls were handled daily, but remained in their individual vivariums and did not participate in daily maze learning tests or swimming trials.

A fourth group of rats was not administered BrdU, but participated in maze testing, in order to determine if the combination of nicotine and BrdU had any negative effects on maze learning. Additionally, the brain tissue of these "non-

BrdU"-learners were used as negative controls in BrdU immunohistochemistry experiments.

Preparation of tissue for immunohistochemical experiments

After a washout period (21 hours), animals were prepared for whole brain extraction on day 15. Rats were deeply anesthetized with ketamine (200 mg/kg, i.m.) and xylazine (7.2 mg/kg, i.m.), then transcardially-perfused with phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde (PFA). Whole brains were extracted, post-fixed overnight in 4% PFA and stored in 70% ethanol.

A single (3 mm) sagittal block was sectioned from the right hemisphere (3 mm lateral to the center line and 0.00 mm interaural, (using Paxinos and Watson 1998 as a guide) and fixed in paraffin. Both serial and adjacent sagittal sections (10 mm) were collected: (1) every tenth section was designated for BrdU immunohistochemistry and (2) every eleventh section was stained with hematoxylin and eosin (H&E). Collecting every tenth section ensured that the same BrdU-positive (BrdU+) cell was not counted more than once. H&E staining was used to verify that the orientation and stereological location of the hippocampal formation and subventricular zone was matched between sections of individual rats.

Immunohistochemical localization of BrdU

After deparaffinization with xylene and ethanol and repermeabilization in PBS with 0.1% Triton-X-100, sections were boiled in 0.01M sodium citrate (pH 6.5). Once slides had cooled to room temperature, sections were rinsed with PBS then blocked for 30 minutes in 2% normal serum in PBS. Both primary and secondary antibodies utilized in these experiments were diluted in PBS.

The dual labeling method for localizing cells positive for BrdU or nuclear antigen (NeuN) were as follows: Sections were incubated overnight at 4°C with mouse anti-NeuN (1:2000). To detect anti-NeuN, sections were incubated (25°C) for 30 minutes with biotinylated anti-mouse IgG (1:200) and streptavidin-AlexaFluor488 (1:400), respectively. Sections were then incubated for 48 hours at 4°C with sheep anti-BrdU (1:200). To detect anti-BrdU, sections were incubated for 1 hour with donkey anti-sheep Cy3 (1:400). All sections were counterstained with bis-benzimide (1:12,000, a.k.a. the Hoescht nuclear stain), then coverslipped (using Vectashield mounting media). Coverslips were stabilized with clear nail polish.

Quantification of BrdU positive cells

At both 0.9mm and 1.40 mm lateral to the center (as verified by parallel H&E-stained sections), all BrdU-positive cells were localized and quantified in the subventricular zone (1.5 mm>bregma>-1.5 mm) and hippocampal formation (Fig. 2.3). Sections were examined by epifluorescence using an Olympus BX51 microscope equipped with filters (Chroma Technology, Brattleboro, VT) for

visualizing Cy3, AlexaFluor488 and the Hoescht stain, and connected to a Lucivid camera and Ludl motor-driven microscope stage. Before counting the number of BrdU+ cells in each section, the localization of anti-NeuN binding and/or the Hoescht stain in the hippocampal formation and subventricular zone was outlined utilizing Neurolucida software (Microbrightfield, Williston, VT) to ensure and verify regions were compared accurately between rats. Cells that were positive for both BrdU and the Hoescht stain were plotted with Neurolucida, then quantified using Neuroexplorer software (Microbrightfield).

Statistical Analyses

Water Maze Testing: Two-way repeated measures analysis of variance (ANOVA) tests were used to compare: (1) daily water maze performance for hidden platform tests between learners administered nicotine (or vehicle), with and without the administration of BrdU, and (2) daily swim distances and speeds between learners and swim-yoked controls. All post hoc multiple comparison tests were made according to the Student-Newman-Keuls method with NCSS software (Number Crunchers Statistical Systems, Kaysville, UT). One-way ANOVA was used to compare group performance for probe trials and visible platform tests.

BrdU Immunohistochemistry: One-way ANOVAs were used to compare the number of BrdU+ cells between learners and swim-yoked or inactive controls. All post hoc multiple comparison tests were made according to the Fishers LSD method with NCSS software.

Results

Water Maze Testing

Learners administered BrdU: The effects of repeated nicotine exposure on the distances swam to locate a hidden platform for a daily single trial method of water maze testing are depicted in Fig. 2.1, however no differences in performance were noted versus controls. However, a day effect was observed where both nicotine-treated rats and controls spent less time ($F=10.4$, $p<0.0001$) and swam less distance ($F=10.42$, $p<0.0001$) to locate the hidden platform across 14 days of testing. No significant difference was noted between nicotine-treated rats and controls for probe trials. For visible platform tests (results not shown in the figures), the mean time to locate the visible platform was not significantly different between nicotine-treated rats (13 ± 6 sec) and controls (17 ± 4sec).

Learners not administered BrdU ("non-BrdU"): The daily means for the distances swam and latencies to locate the hidden platform for non-BrdU nicotine-treated rats (and controls) are presented in Fig. 2.1 (insets). "Non-BrdU" rats treated with nicotine learned to locate the hidden platform more efficiently than controls; distances swam: group effect ($F=16.42$, $p=0.002$) and day effect ($F=16.52$, $p<0.001$) and latencies: group effect ($F=9.45$, $p=0.0118$) and day effect ($F=18.69$, $p<0.001$). No significant difference in performance was observed for probe trials or visible platform tests between the "non-BrdU" groups (results not shown).

BrdU vs. "non-BrdU" Learners: An inset plot for Fig. 2.1 depicts the differences in the distance traveled to locate the hidden platform between rats with and without (as an inset) the administration of BrdU. Rats that were exposed daily to both nicotine and BrdU performed worse in hidden platform tests, than animals only administered nicotine (Fig. 2.1). There was no difference in water maze performance between saline-treated controls, with or without daily exposure to BrdU (Fig. 2.1). When the distances swam to locate the hidden platform were compared between both groups (+/-BrdU) of nicotine learners, a significant group effect ($F=5.31$, $p=0.007$) and group by day effect ($F=1.51$, $p<0.03$) was observed (Fig. 2.1). Significant differences in latency to locate the hidden platform were noted as well: group effect ($F=4.55$, $p<0.01$ and group by day effect ($F=1.54$, $p<0.03$).

Learners vs. Swim-Yoked Controls: Daily swim speeds were significantly different between learners and swimmers. Regardless of treatment, nicotine-treated/swim-yoked controls swam faster than maze learners ($F=1.27$, $p<0.01$) with (regardless of treatment). As expected, rats swam less distance each day ($F=42.5$, $p<0.0001$) (i.e. as maze learners took less distance to locate the hidden platform) (data not shown).

BrdU Immunohistochemistry

Both BrdU and NeuN staining were localized in distinct and non-overlapping populations of cells (Fig. 2.2, 2.3). This was expected since newly generated cells have been found to take up to 4 weeks to express proteins that

indicated the cell phenotype (i.e. neural stem cell, mature neuronal cell, glial cell, etc) and rats in this study were exposed to BrdU for 2 weeks then sacrificed. Negative controls exhibited no BrdU-positive cells. Figures 2.2 and 2.3 include images of sections of the hippocampal formation (2) and subventricular zone (3) stained for the BrdU (Cy3, red), Hoescht stain (blue), and NeuN (AlexaFluor488, green).

The number of BrdU-positive cells in the hippocampus was not significantly different between learners and swimmers, regardless of treatment with nicotine or vehicle. There was also no significant difference between nicotine and saline learners. However, nicotine-treated learners demonstrated a significant increase in BrdU-positive cells when compared to inactive controls (Fig. 2.4B) with or without the administration of nicotine, but not saline-treated learners. It should be noted that no significant difference was observed between swimmers and inactive controls.

Regardless of treatment with nicotine or vehicle, the number of BrdU-positive cells in the subventricular zone (Fig. 2.4A) was not significantly different between learners and both control groups: swimmers and inactive non-learners/non-swimmers. (Fig. 2.5A)

DISCUSSION

Until recently, it was generally assumed that adult mammalian brains lacked the potential to generate new neurons. However it is now widely known that stem cells remain in the adult central nervous system and retain the potential to

differentiate into new neurons that can be incorporated into specific regions within the mammalian brain (Cayre et al 2002). These findings have generated considerable excitement and new research focused on the possibility of enhancing neurogenesis (via pharmacological methods or other means) as a method of treating neurodegenerative disease or other forms of neuronal injury or loss. Secondary (adult) neurogenesis (i.e., the focus of such experiments) involves two stages: neuroblast proliferation and neuron differentiation and these stages have been found to be regulated by a variety of factors: hormones (Tanapat et al 1999), neurotransmitters (Cameron et al 1995; Brezun and Daszuta 1999), growth factors (Craig et al 1996; Kuhn et al 1997; Wagner et al 1999; Aberg et al 2000) and environmental cues (See Cayre et al 2002; Eisch et al 2002).

In disorders of learning and memory (e.g., Alzheimer's Disease) one brain target for enhancing neurogenesis is the hippocampus (an area well known to suffer considerable neuronal loss in the disease). This brain area is important for learning and memory function (Gilbert et al 1998; Suzuki et al 2000) and is of key importance because cells generated in this zone are known to: (1) increase with learning (Gould et al 1999) and (2) be incorporated into the dentate gyrus (permanently) if they survive apoptosis (Altman and Das 1965). Previous studies demonstrating that nicotine has neuroprotective properties (Rattray 2001) possibly mediated through positive effects on the neurotrophin, nerve growth factor (Rattray 2001) as well as learning and memory may be of particular importance from a drug development standpoint for neurodegenerative disease.

Like neurogenesis, the production of nerve growth factor has also been found to increase with learning (Woolf et al 2001) and environmental enrichment (Cayre et al 2002) leading to the strong possibility that the processes (i.e., NGF activity and neurogenesis) are in some way associated. Further, nicotine, since it has been shown to increase the expression of NGF receptors could in fact affect the process of neurogenesis.

The hypothesis tested in the present experiments was, therefore, that repeated exposure to nicotine would improve performance in a hippocampal-dependent learning task (Rezvani and Levin 2001), thus resulting in increases in neurogenesis (Shors et al 2002) in the hippocampus. The combined effects of nicotine and learning on the initial stages of secondary neurogenesis (cell proliferation) were assessed using the studies of van Praag et al (1999) as a general model. Cell proliferation was evaluated in the hippocampus and subventricular zone utilizing bromodeoxyuridine to localize newly divided cells. The subventricular zone was evaluated as a control, since its neurogenic potential has not been associated with events that have been shown to affect the plasticity of the hippocampus. Nicotine-treated rats were tested in a daily single trial water maze method for 14 days with controls for: (1) treatment, by running parallel studies in saline-treated rats and rats not administered BrdU, (2) the physical component of water maze training (i.e. swim-yoked controls) (van Praag et al, 1999), and (3) the learning component of water maze training (i.e. inactive non-learning/non-swimming controls).

As expected, repeated exposure to nicotine improved water maze performance compared to controls (Attaway et al, 1999). Unexpectedly, the combination of nicotine and BrdU significantly impaired maze learning (an effect that was not observed in the saline-BrdU group) suggesting that the combination of nicotine and BrdU is toxic or otherwise associated with undesirable side effects.

Although the combination of nicotine and BrdU had a negative effect on maze learning, an increase in the number of BrdU positive cells was observed in the hippocampus (but not in the subventricular zone) versus inactive controls. Swim-yoked controls did not exhibit a significant difference in the number of BrdU+ cells when compared to learners or inactive controls. This suggests that the cells generated in the hippocampus are the result of some learning component of water maze testing and not simply an increase in physical activity.

In contrast to our findings, recent studies evaluating the effects of nicotine on neurogenesis in a self-administration paradigm found that increased nicotine administration correlated with a decrease in hippocampal neurogenesis and an increase in apoptosis. It should be noted the results obtained from animals tested in a self-administration paradigm (which focuses on the addictive properties of nicotine and not an enriched environment or intermittent, peripheral administration as conducted in our study) might not be directly comparable to the results of studies such as ours. Nicotine in self administration paradigms (which are associated with considerable stress to the animal resulting from withdrawal)

is associated with a rise in corticosterone levels, which has been shown to decrease neurogenesis (Gould et al 1992)

The results of this study indicate that nicotine and learning increase cell proliferation, the initial step of secondary neurogenesis in the hippocampus. Future experiments will be designed to further evaluate the effects of nicotine and learning on the plasticity of this region specifically by evaluating the survival and phenotype of the newly generated cells as well as whether these surviving cells are incorporated into the hippocampus.

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Figure Legends

Fig. 2.1 Water maze testing: Hidden Platform tests. The effects of repeated nicotine exposure on daily single trial water maze testing. The distances swam to locate the platform in the hidden platform tests are depicted in (A), with the results of non-bromodeoxyuridine controls inset.

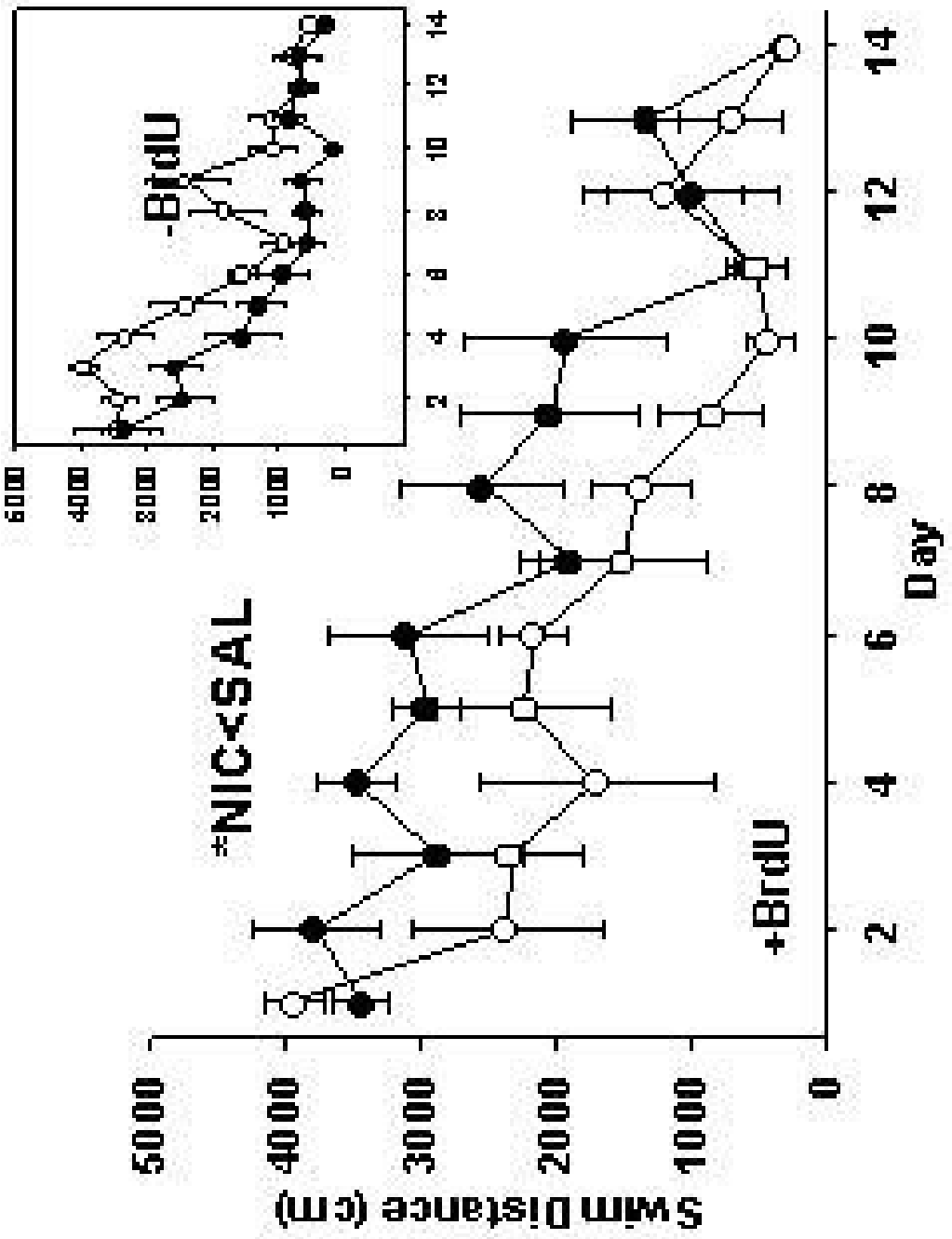
Fig. 2.2 BrdU immunohistochemistry in the subventricular zone. Images demonstrate the distribution of binding for the (A) Hoescht stain, (C) anti-BrdU (visualized with Cy3) and (E) anti-NeuN (visualized with AlexaFluor488) are depicted at 4X magnification. The subventricular border along with portions of choroid plexus (a positive control region) the same sections are depicted at 40X magnification as well (B, D and F, respectively).

Fig. 2.3 BrdU immunohistochemistry in the hippocampus. Images demonstrate the distribution of binding for the (A) Hoescht stain, (C) anti-BrdU (visualized with Cy3) and (E) anti-NeuN (visualized with AlexaFluor488) are depicted at 4X magnification. The hilar regions of the same sections are depicted at 40X magnification as well (B, D and F, respectively).

Fig. 2.4 A comparison of the combined effects of repeated nicotine exposure and learning on the number of BrdU+ cells in the (A) subventricular zone and (B) hippocampus between maze learners and both swim-yoked controls and inactive (non-learning/non-swimming controls.). Empty bars correspond to controls (i.e.

saline-treated rats). Shaded bars correspond to nicotine-treated rats. "*"=significantly different than nicotine-treated learners.

Fig. 2.1


Swim Distance (cm)
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40000
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20000
10000
0
+BrdU
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Day
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-BrdU
6000
4000
3000
2000
1000
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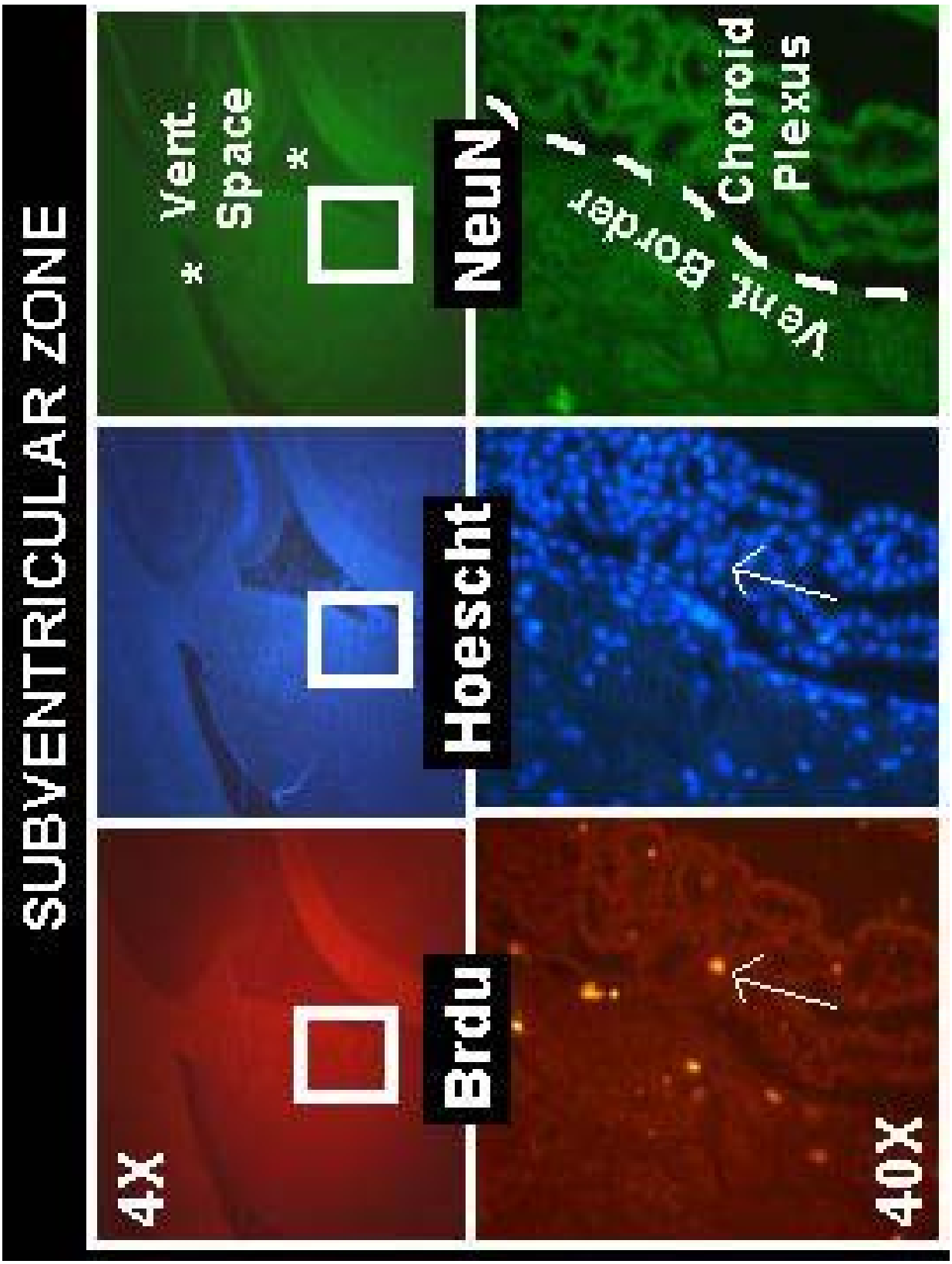
Fig. 2.2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Vent. Space
NeuN
Vent. Border
Choroid Plexus
Hoescht
Brdu
4X
40X

Fig. 2.3

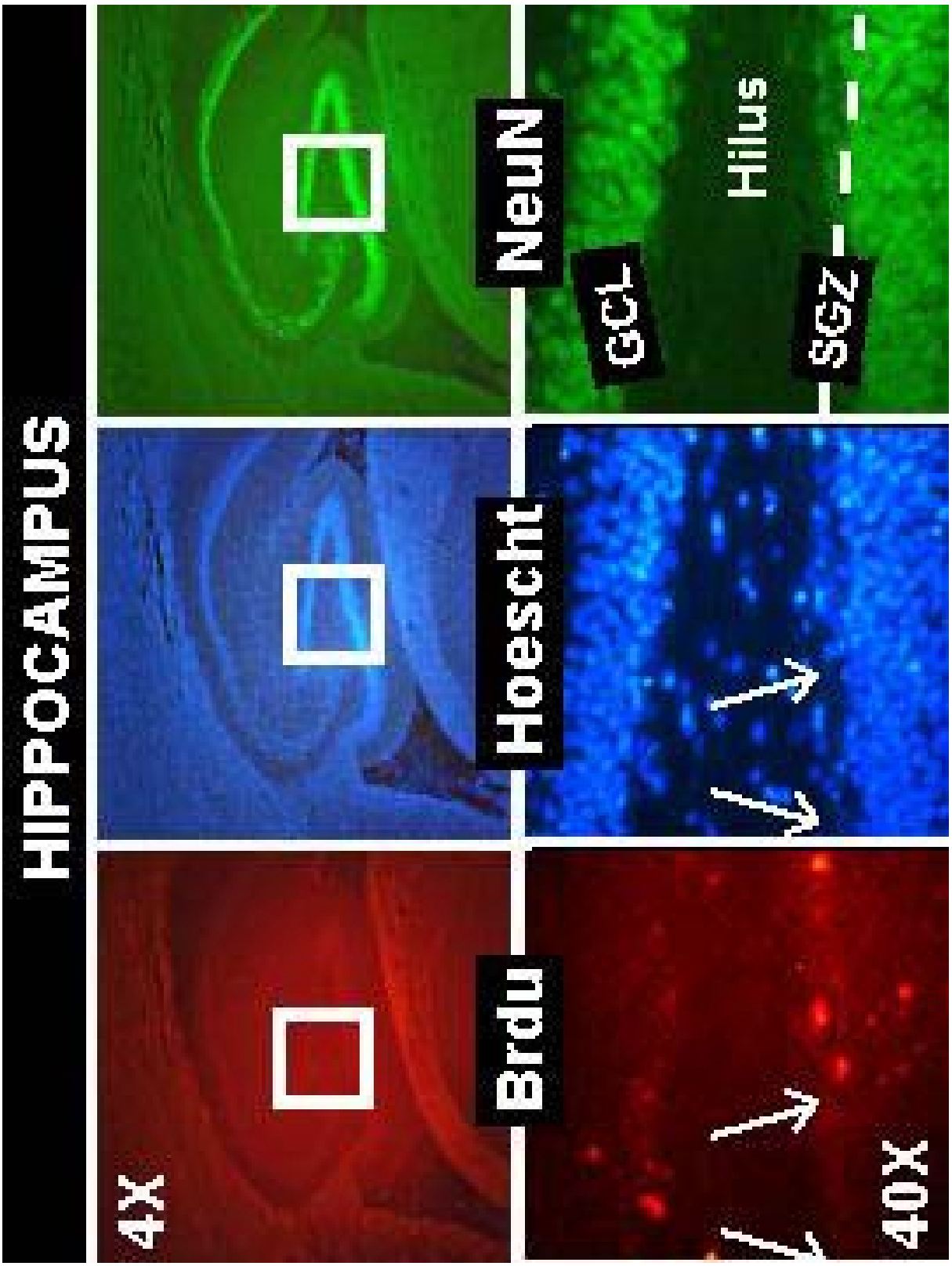
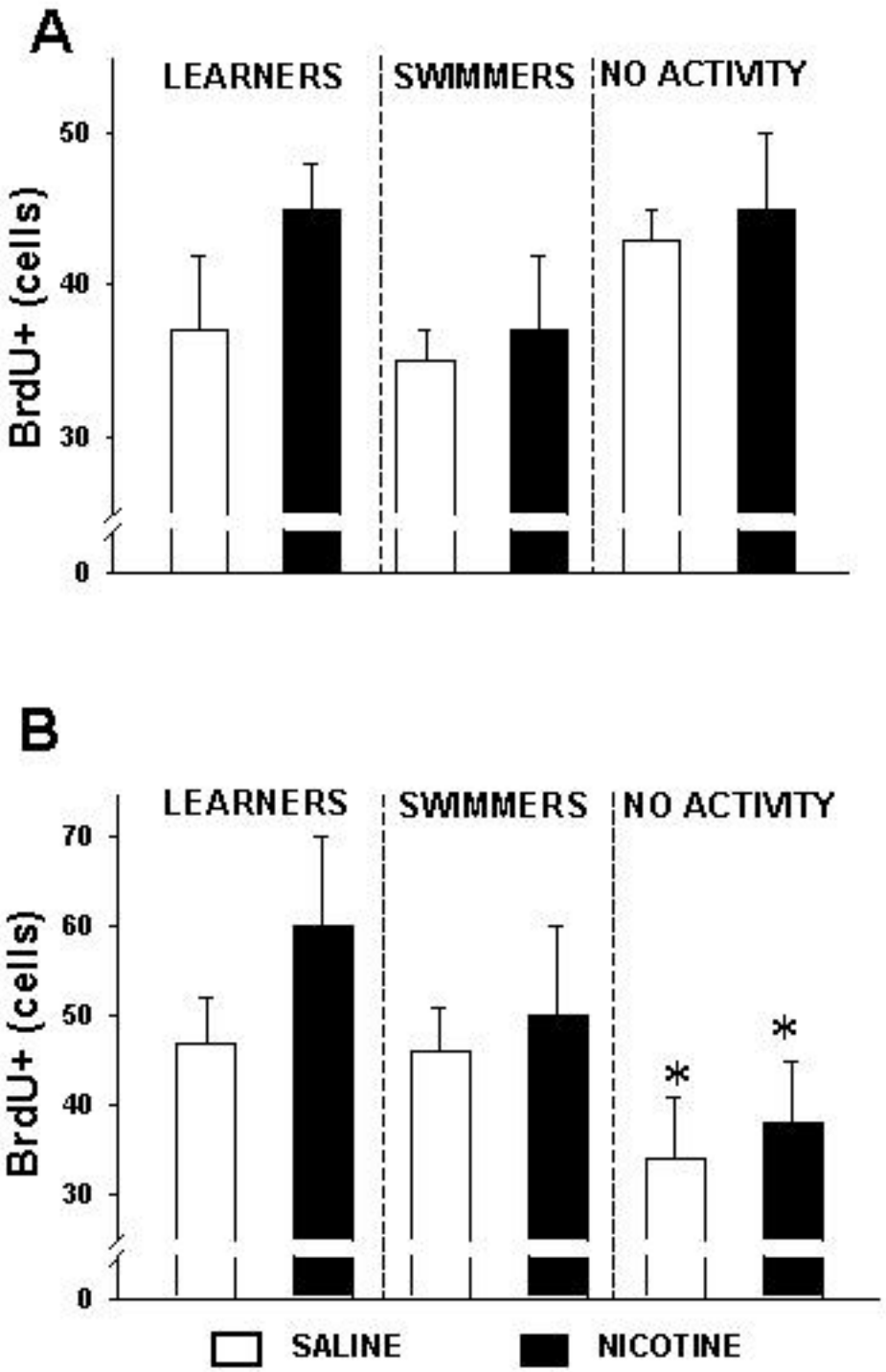


Fig. 2.4


A
LEARNERS
SWIMMERS
NO ACTIVITY
BrdU+ (cells)
50
40
30
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B
LEARNERS
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NO ACTIVITY
BrdU+ (cells)
70
60
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*
SALINE
NICOTINE

Concluding Remarks

The experiments to accomplish test the specific aims (see Dissertation Objectives, page 13) of this doctoral dissertation, "The effects of repeated nicotine exposure on brain plasticity" were completed as proposed in October 2002. Below is a summary of the most remarkable results with repeated nicotine exposure:

- Water maze learning: The time and distance swam to locate the hidden platform was more efficient in the daily (single trial) method. In addition, the percentage of time and distance swam in the target quadrant was increased in probe trials for the single day (multiple trial) method.
- Central acetylcholine receptor subtypes: Receptor autoradiography experiments with subtype specific radioligands revealed an upregulation of the high affinity ($\alpha4\beta2$) nicotinic and the presynaptic inhibitory (M2) muscarinic acetylcholine receptors in the cerebral cortex (an area important for learning and memory function). Both high ($\alpha4\beta2$) and low ($\alpha7$) affinity nicotinic acetylcholine receptors were upregulated in the amygdala, another area important for learning and memory function.
- Presynaptic cholinergic markers: Immunoblotting experiments revealed that the expression of both choline acetyltransferase and the vesicular acetylcholine transporter were increased in the hippocampus (but not cortex)

- Nerve growth factor (NGF) protein levels: Using the ELISA technique, it was determined that no change occurred in either cortical or hippocampal NGF protein levels.
- Nerve growth factor receptors: Using immunoblotting techniques, it was determined that the expression of the phosphorylated (i.e. activated) high affinity TrkA NGF receptors was increased in the hippocampus. No notable changes were observed in the expression of the low affinity p75 NGF receptor in the cortex or hippocampus.
- Cell proliferation in the hippocampus and subventricular zone with learning: Using immunohistochemical techniques to localize the incorporation of bromodeoxyuridine into newly divided cells, the number of bromodeoxyuridine-positive cells in the hippocampus was increased with the combination of nicotine treatment and maze learning when compared to inactive (non-learning/swimming) controls. No notable changes with nicotine or maze learning were observed in the subventricular zone.

All together, these results of this doctoral dissertation add to a growing body of information about the positive effects of nicotine on brain plasticity. Nicotine had a marked effect on (1) the cholinergic system and the receptors that mediate the survival of cholinergic neurons and (2) learning efficiency and learning-dependent cell proliferation in the hippocampus (i.e. the initial step of secondary neurogenesis). The significance of these positive changes is that many of the factors improved with nicotine exposure are also compromised and

implicated in the pathogenesis of neurodegenerative diseases (i.e. Alzheimer's disease).