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Time Dependent Effects of Haloperidol and Ziprasidone on Nerve Growth Factor, Cholinergic Neurons, and Spatial Learning in Rats

Alvin V. Terry, Jr., Vinay Parikh, Debra A. Gearhart, Anilkumar Pillai, Elizabeth Hohnadel, Samantha Warner, Henry A. Nasrallah, and Sahebarao P. Mahadik

Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia, 30912 (AVT, DAG)

Small Animal Behavior Core, Medical College of Georgia, Augusta, Georgia, 30912 (AVT, EH, SW)

Department of Psychology, University of Michigan, 4032 East Hall, 525 East University Avenue, Ann Arbor, Michigan 48109 (VP)

Department of Psychiatry and Health Behavior, Medical College of Georgia, Augusta, Georgia, 30912 (AP, SPM)

Medical Research Service Line, VAMC, Augusta, Georgia, 30904 (AP, SPM)

Department of Psychiatry, University of Cincinnati, Cincinnati, Ohio, 45267 (HAN)

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Running Title: Neurobiological Comparisons of Haloperidol and Ziprasidone

Corresponding Author:

Alvin V. Terry Jr., Ph.D.
Professor of Pharmacology and Toxicology
Director, Small Animal Behavior Core
CJ-1020, Medical College of Georgia
1120 Fifteenth Street
Augusta, Georgia 30912-2450
Phone 706-721-4915
Fax 706-721-3994
e-mail: aterry@mail.mcg.edu

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Abbreviations:

AON=anterior olfactory nucleus,
APO, apomorphine
CA1=CA1 region of the hippocampus
Cg=cingulate cortex
ChAT, choline acetyltransferase
CP=caudate putamen
DIAZ, diazepam
DON, donepezil
FGA, first generation antipsychotic
HAL, haloperidol
[^{3}H]-EPB, [^{3}H]-epibatidine
[^{125}I]-BTX, [^{125}I]-α-bungarotoxin
[^{3}H]-PRZ, [^{3}H]-pirenzepine
[^{3}H]-AFX, [^{3}H]-AFDX-384
IP, intraperitoneal
mCPP, meta-chlorophenylpiperazine
NGF, Nerve Growth Factor
OB=olfactory bulbs
phospo- TrkA, phosphorylated TrkA
$p75^{NTR}$, p75 neurotrophin receptor

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PPI, prepulse inhibition
s.c., subcutaneous
SGA, second generation antipsychotic
SCOP, scopolamine
TrkA, tropomyosin-receptor kinase A
VAChT, vesicular acetylcholine transporter
VEH, vehicle control
ZIP, ziprasidone

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Abstract

In this rodent study we evaluated the effects of different time periods (7, 14, 45, and 90 days) of oral treatment with haloperidol (HAL, 2.0 mg/kg/day), or ziprasidone (ZIP, 12.0 mg/kg/day) on nerve growth factor (NGF) and choline acetyltransferase (ChAT) levels in the hippocampus, then subsequently assessed, water maze task performance, prepulse inhibition (PPI) of the auditory gating response, and several NGF-related proteins and cholinergic markers after 90 days of treatment. Seven and 14 days of treatment with either HAL or ZIP resulted in a notable increase in NGF and ChAT immunoreactivity in the dentate gyrus (DG), CA1 and CA3 areas of the hippocampus. After 45 days, NGF and ChAT, immunoreactivity had abated to control levels in ZIP treated animals, but was markedly reduced in HAL treated subjects. After 90 days of treatment, NGF and ChAT levels were substantially lower than controls in both antipsychotic groups. Furthermore, after 90 days of treatment and a drug free washout period, water maze performance (but not PPI) was impaired in both antipsychotic groups, although the decrement was greater in the HAL group. Several NGF-related and cholinergic proteins were diminished in the brains of subjects treated with either neuroleptic as well. These data support the premise that while ZIP (given chronically) appears somewhat superior to HAL due to less pronounced behavioral effects and a more delayed appearance of neurochemical deficits, both antipsychotics produce time-dependent, deleterious effects on NGF and cholinergic markers (i.e., important neurobiological substrates of memory) as well as cognitive function.

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Introduction

The newer pharmacological treatments for schizophrenia now commonly referred to as second generation antipsychotics (SGAs) offer several advantages over first generation antipsychotics (FGAs) such as greater improvements in negative symptoms, prevention of relapse, increased functional capacity and quality of life, and fewer movement-related side effects (reviewed, Miyamoto et al., 2005). It is also generally believed that SGAs are superior to FGAs when their effects on cognition are considered (see reviews, Keefe et al., 1999; Purdon, 1999), and some studies suggest that SGAs improve cognition in schizophrenia. This suggestion is of particular importance given that the degree of cognitive impairment in schizophrenia is recognized as an important predictor of social functioning, unemployment, and even relapse of psychiatric symptoms (reviewed, Castner et al., 2004). It should be noted, however, that such conclusions regarding antipsychotics and cognitive function rely primarily on meta-analyses and short clinical trials (i.e., they rarely exceed a few months to one year in length). Therefore, since schizophrenic patients are often treated with antipsychotics for decades, the aforementioned conclusions regarding SGAs may be premature. Since multi-year, prospective clinical studies designed specifically to identify neuroleptics that have optimal effects on cognition have not been conducted (and may be cost prohibitive), animal studies designed to investigate such issues may be especially important. Furthermore, animal studies allow for rigorous investigations of the effects of chronic neuroleptic treatment on the neurobiological substrates of cognitive function.

Previous work in our laboratories demonstrated that in contrast to certain SGAs such as risperidone and clozapine, chronic oral haloperidol (HAL) treatment in rats resulted in sustained impairments in spatial learning performance as well as decrements in an important cholinergic marker, choline acetyltransferase (ChAT) in brain regions such as the cortex and hippocampus

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(Terry et al., 2002; 2003). This finding is potentially very important, since cholinergic activity in these brain areas is well documented to modulate a number of cognitive processes (reviewed, Perry et al., 1999). Further work in our laboratories indicated that while HAL treatment was associated with decrements in brain levels of the endogenous neurotrophin, nerve growth factor (NGF) as well as ChAT, this did not appear to be the case with the SGAs, risperidone, clozapine, or olanzapine (Parikh, 2004a; 2004b) at least up to a treatment period of 90 days. More recently, however, we detected decrements in NGF associated with HAL, risperidone, and olanzapine treatment for 180 days (Pillai et al., 2006), leading to the conclusions that such growth factor changes, while dependent on the length of treatment, may be common to several antipsychotics. Since the survival and function of adult mammalian cholinergic neurons (particularly those projecting from the basal forebrain to the cortex and hippocampus) is dependent on NGF (reviewed, Counts and Mufson, 2005) we hypothesize that some of the unfavorable effects of HAL (and potentially other neuroleptics) on memory function may be related to time dependent impairments in cholinergic activity due to reduced levels of NGF and/or its receptors.

The novel SGA, ziprasidone (ZIP) has a unique pharmacological profile with high affinity at a number of neurotransmitter receptors including D_2, $5HT_{1A}$, $5HT_{2A}$, $5HT_{2C}$ as well as $5HT_{1B/1D}$ receptors (Seeger et al., 1995; Schmidt et al., 2001). It has proven efficacy in schizophrenia and related disorders (Goff et al., 1998; Daniel et al., 1999), as well as a low liability for certain adverse reactions such as extrapyramidal symptoms (EPS) and weight gain (reviewed, Weiden et al., 2003). However, as in the cases highlighted above, the effects of long-term treatment with ZIP (particularly on cognitive function and neurobiological substrates of cognitive function) have not been evaluated. The purpose of this study was, therefore, to compare ZIP to the archetypal FGA, HAL for effects on NGF and NGF receptors, key cholinergic proteins, and

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memory-related task performance in rats. The overall hypothesis was that chronic treatment with HAL or ZIP leads to sustained memory-related behavioral changes that are due (at least in part) to effects on NGF and/or its receptors and the regulation of key cholinergic marker proteins (i.e., for memory function). The two experimental approaches used to test this hypothesis were, 1) to investigate a time course of exposure to either HAL or ZIP for effects on the cholinergic marker protein, ChAT, and the neurotrophin, NGF and; 2) to measure the effects of the neuroleptics on memory-related behavioral tasks as well as the levels of NGF and cholinergic proteins after 90 days of treatment followed by a significant drug free washout period. The later experiments were designed to investigate the residual effects of prior chronic treatment with these agents (i.e., effects not associated with acute exposure).

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Methods

Test Subjects- Male albino Wistar rats (Harlan Sprague-Dawley, Inc.) 2-3 months old were housed individually in a temperature controlled room ($25^{O}C$), maintained on a 12-hour light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI).). Water was allowed ad libidum for the first week in all test animals, but then replaced with solutions that contained neuroleptics for the animals were placed in the chronic antipsychotic studies (see below). Table 1 provides the details for the all study cohorts, the numbers of animals tested per group, and the experiments conducted with each group. All procedures employed during this study were reviewed and approved by the Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain or discomfort in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised

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1996. Significant efforts were also made to minimize the total number of animals used while maintaining statistically valid group numbers.

Drug Dosing for Chronic Antipsychotic Experiments- Oral antipsychotic dosing was based several factors: 1) for HAL, previous rodent studies in our laboratory in which time dependent behavioral and neurochemical effects were detected and plasma drug levels were achieved that approximated those often associated with antipsychotic effects in humans (Terry et al., 2002, 2003; 2005a); 2) for ZIP, previous studies using oral dosing in rodents in which notable behavioral effects were observed (Mansbach et al., 2001). Furthermore, in both the case of the HAL and ZIP, the doses selected (see below) would be expected to achieve comparable (and therapeutically) relevant D_2 receptor occupancy values *in vivo* (i.e., in the range 65-80%, see Kapur et al., 2003) based on the recent work of Barth et al., 2006. Rats were thus treated with HAL (Sigma-Aldrich, St. Louis, MO), 2.0 mg/kg/day or ZIP (Pfizer, Inc, New York, NY), 12.0 mg/kg/day orally for periods of 7, 14, 45, or 90 days. The antipsychotics were dissolved in 0.1 M acetic acid and subsequently diluted (1:100) with distilled-deionized water for daily drug administration in drinking water. Drug dosing was based on the average daily fluid consumption and the weight of the animals. Animals that were evaluated for residual neuroleptic-related behavioral effects were administered antipsychotics at the doses described above (or vehicle) for 90 days, then given a one week, drug-free washout period (i.e., returned to normal drinking water), behaviorally tested for one week, and then sacrificed for neurochemical studies (i.e., two weeks after the last drug exposure).

Immunohistochemistry

In the first series of immunohistochemical experiments, rats were administered the antipsychotics (or vehicle) as described above and sacrificed at different time points (7, 14, 45,

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or 90 days of treatment, see Table 1) in order to determine if there are time dependent effects of antipsychotic treatment on NGF and ChAT.

NGF Rats were deeply anesthetized with ketamine/xylazine and perfused with cold 0.01M phosphate buffer saline (PBS) through the left cardiac ventricle to remove circulating blood elements. Brains were quickly removed and cryoprotected in embedding media. Coronal sections (20 μm in thickness) were cut at interaural 4.84mm, Bregma –4.16mm to obtain sections from hippocampus (dentate gyrus, DG; CA1 and CA3) (Paxinos and Watson 1998) using a cryostat microtome (Leica CM 3050S, Leica Microsystems Inc., Chantilly, VA, USA) at –20 ± 2^0 C. Fresh frozen sections were fixed in ice-cold acetone for 30 min and air-dried. Then sections were rinsed in 0.01M PBS containing Tween 20 (PBST). After blocking with 10% normal goat serum for 1 hr, sections were washed and incubated overnight at 4^0C with rabbit anti-mouse polyclonal NGF antibody (1:100) (Chemicon International Inc., Temecula, CA, USA). Endogenous peroxidase was blocked for 30 min with 0.1% H_2O_2 and 100% methanol. Sections were then washed and incubated for 2 hrs with biotinylated anti-rabbit IgG antibody made in goat (1:50) followed by incubation for 1 hr with avidin-biotin-horseradish peroxidase complex. The avidin-HRP complex was then detected with 3-3'-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.02% H_2O_2 and nickel chloride (Vectastain kit, Vector Laboratories, Burlingame, CA).

ChAT For ChAT immunohistochemistry, the method used has been described previously (see Parikh, 2004a; 2004b) . Briefly, rats were anesthetized and perfused with 100 ml of saline followed by 300 ml of ice-cold 4% paraformaldehyde in 0.1M PBS. After perfusion, all brains were post-fixed in paraformaldehyde for 2 hrs (with shaking) at 4^0C followed by storage at 30% sucrose solution in 0.1M PBS for 48 hrs. The tissues were embedded with OCT in liquid

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nitrogen and kept at –80^{0}C until further use. A cryostat was used to cut 40 μm coronal sections at specific anatomical landmarks: interaural 4.84mm, Bregma –4.16mm to obtain sections from hippocampus (Paxinos and Watson 1998). Cryoprotected fixed sections were washed 3 times in PBST, blocked with 10% normal horse serum for 1 hr, and then incubated with 10 μg/ml mouse monoclonal anti-ChAT antibody (Chemicon International Inc., Temecula, CA, USA) overnight at 4^{0}C. The sections were washed 3 times with PBST and then incubated for 2 hrs with 1:20 diluted rat adsorbed biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) containing 1% horse serum. After washing, the sections were incubated with avidin-HRP for 1 hr and the avidin-HRP complex was subsequently detected with DAB.

Quantitative Image Analysis- Photomicrographs from each treatment group were obtained with a Zeiss Axioplan-2 microscope equipped with CCD camera, PC computer and Zeiss KS-300 image analyses software by an experimenter blinded to the study code. The analysis for NGF was performed on live acquired images of dimensions 582x455μm^2 in dentate gyrus (DG) granule cell layer and 582x228μm^2 each in CA1 and CA3 cell layer pyramidal neurons. For quantitation, densitometric assessments were made measuring the optical density (OD) of immunostained cells. The cells were considered positive if their OD values were higher than a defined threshold OD value above which only cell bodies and not processes were detectable. Three rectangles per section with dimensions 582x455μm^2 for DG and 582x228μm^2 for CA1 and CA3 region were delineated. NGF immunoreactive neurons were identified in these regions and staining intensity expressed as mean OD (MOD) was obtained by averaging OD values of all stained profiles in analyzed field and subtracting the background OD of each section. OD range 0-2 was divided into 256 (0-255) digitized values. ChAT immunoreactive nerve fibers in the hippocampus were analyzed as described previously (Parikh, 2004a; 2004b).

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Three rectangles per section for DG, CA1 and CA3 subfields with similar dimensions as described above were selected for analysis. All the digitalized images of ChAT immunoreactive nerve fibers were converted to gray scale and the brightness, contrast and masking was adjusted to enhance the visibility of fibers (Photoshop 5.0, Adobe System, Inc., San Jose, CA). Quantitative data for ChAT immunoreactive fibers are expressed as fiber pixel density.

Behavioral Experiments

In the behavioral studies, two series of experiments were conducted 1) behavioral task validation experiments; 2) comparisons of HAL and ZIP for residual effects on memory-related behavior performance after 90 days of treatment (i.e., a treatment period previously associated with HAL induced memory impairments) beginning one week into a drug free washout period.

Behavioral Task Validation Experiments- In each of the validation experiments, identical conditions were used in each procedure as that used for the antipsychotic behavioral studies (see methodological details below). Thus, in spatial learning experiments, the water maze task used was assessed for its sensitivity to cholinergic antagonism (a central issue in this study) with a reference dose of scopolamine (0.1 mg/kg, s.c. determined in preliminary experiments). Subsequently, two doses of the commonly used acetylcholinesterase inhibitor (AChEI) and cognitive enhancing agent, donepezil (see Table 1) were evaluated for their ability to attenuate the impairing effects of scopolamine. Subsequently, the anxiety test used, the light-dark box test, was evaluated for its sensitivity to published reference doses (see Table 1) of the anxiolytic agent diazepam and the anxiogenic agent meta-chlorophenylpiperazine (mCPP, see Chaouloff et al., 1997 and Bilkei-Gorzo et al., 1998, respectively). Finally, the prepulse inhibition (PPI) method used was evaluated for its sensitivity to known PPI impairing agents (and compounds known to attenuate the effects of such agents). Thus, dose-effect curves were established for the FGA,

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haloperidol, the SGA, clozapine, and the AChEI, donepezil to reverse the impairing effects of reference doses of the dopamine agonist apomorphine, the NMDA antagonist, MK801, and the muscarinic antagonist, scopolamine on PPI, respectively. Drug doses (see Table 1) were selected from previous work in our laboratory (Terry et al., 2005b) and other published PPI studies (Stanhope et al., 2001).

Water Maze Testing

Testing Apparatus. To determine the effects of the test drugs on spatial learning, water maze experiments were performed in a circular pool (diameter: 180 cm, height: 76 cm) made of black plastic. For the task validation experiments, reference compounds (see above) were administered by subcutaneous injection acutely and for the chronic (oral) neuroleptic studies testing was begun on day 7 of a drug free washout period (i.e., after the 90 days of oral drug dosing). The pool was filled to a depth of 35 cm of water (maintained at $25.0\pm1.0^{O}C$). The pool was located in a large room with a number of extra-maze visual cues including geometric images (squares, triangles, circles etc.) hung on the wall, ambient lighting, approximately 25-30 Lux (lumen/m^2), and black curtains used to hide the experimenter (visually) and the resting test subjects. Swimming activity of each rat was monitored via a television camera mounted overhead, which relayed information including latency to find the platform, total distance traveled, time and distance spent in each quadrant etc. to a video tracking system (Actimetrics, Evanston, IL).

Visible Platform Task- On the day prior to water maze hidden platform testing, a visible platform test was performed in order to assure that the study subjects were visually capable of performing the task and that they demonstrated normal search/escape behaviors. To accomplish this task, a highly visible (white) cover fitted with a small white flag was attached to the platform

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(dimensions with cover attached = 12 cm x 12 cm) which raised the surface approximately 1.0 cm above the surface of the water. Each rat was gently lowered into the water in the quadrant diametrically opposite to the platform quadrant and given one or more trials with a 90 sec time limit to locate and climb on to the platform. If unsuccessful after 90 sec, it was physically placed on the platform for 30 seconds then given a new trial. Once a rat was successful on its own accord it was then given a series of 4 additional trials (with a 1.0 min intertrial interval) and the latency (in sec) to locate the platform was recorded. The platform was moved on each trial to a different quadrant (the subject was always entered from the opposite quadrant) until the test was conducted once in all 4 quadrants.

Hidden Platform Task - For these experiments, an invisible (black) 10 cm x 10 cm square platform was submerged approximately 1.0 cm below the surface of the water and placed in the center of the northeast quadrant (which remained constant throughout hidden platform training). Each rat was given 2 trials per day for 6 consecutive days to locate and climb on to the hidden platform. A trial was initiated by placing the rat in the water directly facing the pool wall (i.e., nose approximately 2 cm from the wall) in one of the 4 quadrants. The daily order of entry into individual quadrants was pseudo-randomized such that all 4 quadrants were used once every two training days. For each trial, the rat was allowed to swim a maximum of 90 sec, in order to find the platform. When successful the rat was allowed a 30-sec rest period on the platform. If unsuccessful within the allotted time period, the rat was given a score of 90 sec and then physically placed on the platform and also allowed the 30-sec rest period. In either case the rat was given the next trial after an additional 1.5 min rest period (i.e., intertrial interval =2.0 min).

Probe Trials (Transfer Tests)- Twenty-four hours following the last hidden platform trial, a probe trial was conducted in which the platform was removed from the pool to measure

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spatial bias for the previous platform location. This was accomplished by measuring the percentage of time spent in the previous target quadrant and the number of crossings over the previous platform location, and provided a second estimate of the strength and accuracy of the memory of the previous platform location.

Locomotor Activity and the Light-Dark Preference Test. - To assess the effects test drugs on general locomotor activity as well as anxiety levels, a Light/Dark Preference Test (also referred to as light/dark exploration or emergence neophobia test) was conducted. In this test, we were interested in determining whether the neurolpetics (i.e., day 10 of a drug free washout) had significant motor effects that might have influenced performance in the memory-related tests. We were also interested to learn whether the neuroleptics (or reference compounds, see above) had any effects on anxiety levels (a factor that could at least theoretically influence performance in the water maze). The Light/Dark Preference Test is one of the most commonly-used rodent models of anxiety (see Holmes et al., 2001); and avoidance of the lighted portion of the chamber reflects elevated anxiety while significantly reduced time spent in the dark area reflects an anti-anxiety effect of a test drug. Med Associates (St Albans, VT) rat open field activity monitors (43.2 X 43.2 cm) were used for these experiments. They were fitted with dark box inserts (which are opaque to visible light) to cover one-half of the open field area thus separating the apparatus into two zones of equal area (i.e., a brightly lit zone and a darkened zone). Desk lamps located above the activity monitors were used to provide an illumination level of approximately 1000 lux in the brightly lit zone, whereas the illumination level in the darkened zone was approximately 5 lux. The following parameters were recorded for the 5 min test session: horizontal activity (horizontal photobeam breaks or counts), number of stereotypy movements, vertical activity (vertical photobeam breaks), as well as the time spent in the light

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and dark zones of the apparatus. Thus, spontaneous locomotor activity, olfactory activity (rearing and sniffing movements), stereotypical movements, and emergence neophobia were assessed.

Prepulse Inhibition (PPI) Procedure- To assess the effects of the reference compounds (see above) and previous neuroleptic exposure (i.e., day 12 of a drug free washout) on auditory gating (an important behavioral process that is often disrupted in schizophrenic patients), a PPI procedure was conducted as described previously (Terry et al., 2005a). Four startle chambers (San Diego Instruments, San Diego, CA) were used that consisted of a Plexiglas tube (diameter 8.2 cm, length 25 cm) placed in a sound–attenuated chamber, in which the rats were individually placed. The tube is mounted on a plastic frame, under which a piezoelectric accelerometer is mounted, which records and transduces the motion of the tube. Two days before drug testing the experimental subjects were each placed in one of the startle test chambers for a period of 10 minutes (without any startle stimuli) as an initial period of acclimation to the apparatus. One day before drug testing the animals were again placed in the test chamber and then exposed to 12 startle stimuli and to each prepulse level 3 times (see below). This procedure was done to reduce the highly variable responses to the initial exposures to the startle stimuli as well as to ensure that the prepulse stimuli (alone) had no significant effect on the startle response. On the day of drug testing, experimental subjects were transported to the startle chamber room and left undisturbed for at least 30 min. Afterwards, the rats were placed in the chamber, and then allowed to habituate for a period of 5 min, during which a 70 dB background white noise was present. After this period, the rats received 12 startle trials, 12 no-stimulus trials, and 12 trials of each of the prepulse/startle trials (see below) for a total of 60 trials. The intertrial interval ranged from 10 to

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30 s, and the total session lasted about 25-30 min. The startle trials consisted of single 120 dB white noise bursts lasting 20 ms.

The prepulse inhibition trials consisted of a prepulse (20 ms burst of white noise with intensities of 75, 80, or 85 dB) followed, 100 ms later, by a startle stimulus (120 dB, 20 ms white noise). During the no-stimulus trial, no startle noise was presented, but the movement of the rat was recorded. This represented a control trial for detecting differences in overall activity. The 60 different trials were presented pseudorandomly, ensuring that each trial was presented 12 times and that no two consecutive trials were identical. The resulting movement of the rat in the startle chamber was measured during 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer that calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 12 startle trials. Prepulse inhibition was calculated according to the formula: [100 - (startle amplitude on prepulse-pulse trials ÷ startle amplitude on pulse alone trials) x 100]. The mean level of PPI (i.e., averaged across the 3 prepulse intensities) was also analyzed.

ELISA Experiments- After behavioral testing, some of the test subjects (see Table 1) were anesthetized with KetaVed™ (ketamine hydrochloride injection; Vedco, Inc., St. Joseph, MO), intracardially-perfused with phosphate buffered saline (PBS, pH 7.4), and then decapitated. Brains were quickly harvested, immediately frozen in dry ice-cooled 2-methylbutane (isopentane), and stored at -70 °C until dissected for subsequent analyses. The basal forebrain, hippocampal formation, cortex, and prefrontal cortex, were dissected and the homogenized in RIPA buffer containing protease inhibitors and glycerol. Brain lysates were evaluated using ELISA methods to measure the relative levels of: choline acetyltransferase (ChAT); vesicular acetylcholine transporter (VAChT); p75 neurotrophin receptor ($p75^{NTR}$); TrkA (nerve growth

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factor receptor); phosphorylated-TrkA (P-TrkA); and nerve growth factor (NGF). The brain dissections, preparation of brain lysates, protein assay, and ELISA methods (except NGF) were performed according to Gearhart et al., 2005 except that in the present study, different quantities of brain protein were analyzed by ELISA (see Table 2 for details). As an internal control for day-to-day variation in the ELISA methods, brain lysates (same amount of protein per well) from vehicle-, HAL-, and ZIP-treated rats were always assayed at the same time on the same ELISA plate. NGF was measured using the NGF Emax® ImmunoAssay System (Promega catalog #G7631) according to the kit instructions. The brain lysates were not acid-treated before the NGF ELISA. On the day of the NGF assay, the brain lysates were diluted in sample buffer (SB) as follows: basal forebrain lysate (30 µl) + SB (90 µl); hippocampus lysate (30 µl) + SB (90 µl); cortex lysate (60 µl) + SB (30 µl); and prefrontal cortex lysate (60 µl) + SB (30 µl). The diluted lysates were mixed thoroughly, and then 100 µl of each diluted lysate was analyzed in the NGF ELISA. In addition, after preliminary experiments indicated that there were no significant differences in ELISA results between PBS perfused animals and those sacrificed by decapitation (see below), some half-brains left from the autoradiography experiments were dissected and used in increase the N in ELISA experiments.

Quantitative Receptor Autoradiography

In order to assess the effects of prior chronic antipsychotic administration on cholinergic receptor densities, autoradiographic analyses of brain tissues harvested from rats previously exposed to HAL, ZIP or vehicle were conducted with subtype specific cholinergic radioligands to nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), i.e., receptors which have been found to play important roles in learning and memory processes (van der Zee and Luiten, 1999; Rezvani and Levin 2001). After behavioral testing,

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some of the test subjects (N=6-9) were immediately sacrificed by decapitation, the brains flash frozen. Using a Microm® HM cryostat (-18ºC), the left hemisphere of each brain was serially sectioned (16 μm) up to the midline onto chrome alum/gelatin-coated slides. Low-affinity (homomeric α_7) nAChRs and high-affinity (heteromeric α/β subunit complexes) were labeled with [^{125}I]-α-bungarotoxin ([^{125}I]-BTX) and [^{3}H]-Epibatidine ([^{3}H]-EPB), respectively. The density of M_1 and M_2 mAChRs, i.e., the mAChRs expressed in highest quantities in mammalian brain (van der Zee and Luiten, 1999), were quantified using [^{3}H]-Pirenzipine ([^{3}H]-PRZ) and [^{3}H]-AFDX 384 ([^{3}H]-AFX), respectively. For each radioligand, the receptor subtype target, incubation time, concentration and duration of film exposure are listed in Table 3. Please refer to previous publications for more detailed descriptions of the autoradiographic procedures (Hernandez and Terry, 2005, Terry et al., 2005a).

Statistical Analyses

All statistical analyses were performed using either SigmaStat 2.03 (SPSS Inc., Chicago, IL) or GraphPad InStat® version 3.06 for Windows (GraphPad™ Software, San Diego CA). Statistical significance was assessed using an alpha level of 0.05. A one or two-way analysis of variance (ANOVA) (with repeated measures when indicated) was used for all treatment comparisons. In some cases ranked data were used when the particular data set was non-normally distributed. For post hoc analysis, the Bonferroni method was used for multiple comparisons and Dunnett's test was used for cases when comparisons were made against vehicle control only. Raw ELISA data (optical density @ 450 nm) were compared for all of the proteins with the exception of NGF in which pg NGF/mg protein were compared. For graphing purposes, the raw ELISA data were converted to percent of the vehicle-treated group. Specifically, each optical density (OD, N=7-10 per HAL or ZIP group) from each ELISA (ChAT, VAChT, $p75^{NTR}$,

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TrkA, and P-TrkA) was divided by the mean OD for the vehicle-treated group (N=6-12) that was tested on the same ELISA plate. Similar calculations were used for the NGF results, except that the units were pg NGF/mg protein (rather than OD). The resulting quotients were multiplied by 100 to yield percent of the vehicle-treated group, and then InStat® was used to determine the mean ± standard error of the mean (S.E.M.). For autoradiographic comparisons, a repeated measures two-factor ANOVA was used to examine differences in densitometry values between each treatment (HAL and ZIP) versus control within area of the brain. Animal was nested within the treatment and considered a random effect. Fixed effects were treatment and area of the brain. All two factor interactions were included in the model. An alpha level of 0.05 was used to examine post hoc differences on the adjusted least square means of the two-factor interaction between treatment from control and area of the brain.

Results

Immunohistochemistry

NGF- Treatment comparisons (i.e., for effects on NGF immunoreactivity) made by measuring the mean optical density (MOD) of immunostained cells are provided in Table 4. As indicated, NGF immunoreactivity was somewhat higher in the dentate gyrus (DG) when compared to the CA1 and CA3 regions of the hippocampus (which were similar). Interestingly, there was a notable increase in NGF immunoreactivity at 7 and 14 days of treatment with either HAL or ZIP in the DG, CA1, and CA3 areas of the hippocampus. After 45 days, however, animals treated with HAL had markedly reduced NGF immunoreactivity, whereas, immunoreactivity in ZIP treated subjects had returned to control levels. After 90 days of exposure to either HAL or ZIP, NGF levels were substantially lower than controls.

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ChAT - Varicose ChAT immunoreactive fibers were distributed in the granule cell layer of DG and in the pyramidal neurons of CA1 and CA3 subfields. The density of ChAT was highest in the hippocampal CA3 pyramidal cell layer (see Table 5). Representative images illustrating significant time dependent, treatment-related effects on ChAT immunoreactivity (fiber pixel density) are provided in Fig 1. The effects of the neuroleptics on ChAT immunoreactivity followed a similar pattern as the effects on NGF. Specifically, there was a significant increase in ChAT immunoreactivity at 7 and 14 days of treatment with either HAL or ZIP in the dentate gyrus (DG), CA1 and CA3 areas of the hippocampus. After 45 days, animals treated with HAL had markedly reduced ChAT immunoreactivity, whereas, immunoreactivity in ZIP treated subjects was similar to control levels. After 90 days of exposure to either HAL or ZIP, ChAT levels were substantially lower than controls.

Behavioral Test Validation Experiments

Water maze hidden platform test- Fig 2A illustrates the results of experiments in which a reference dose (0.1 mg/kg) of the muscarinic cholinergic receptor antagonist scopolamine was evaluated for it ability to impair spatial learning and for the effects of two doses of donepezil to reverse or attenuate these effects of scopolamine. The latency (number of seconds) of each experimental group to locate the hidden platform over 6 consecutive days of testing is depicted. There was a highly significant treatment effect $F_{3,45}$ =8.8, p<0.001, a significant day effect $F_{5,15}$ =14.1, p<0.001, without a significant treatment x day interaction $F_{225,293}$ =1.6, p=0.8. Post hoc analyses indicated that performance was clearly impaired by scopolamine and that the higher (but not the lower) dose of donepezil attenuated this impairment. Specifically, the performance of controls (vehicle + vehicle) and those administered scopolamine + donepezil (2.0 mg/kg) was

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superior ($p<0.05$) to performance of the animals administered scopolamine + vehicle on days 4, 5, and 6, and 4 and 6, respectively.

Light/Dark Box experiments- Fig 2B illustrates the results of experiments in which reference doses of the anxiolytic agent diazepam and the anxiogenic agent mCPP were evaluated in the light/dark box test. There was a highly significant differences in response to the different drugs, $F_{4,45}$ =13.1, $p<0.001$. Post hoc analyses indicated the following: 1) that both doses of diazepam significantly ($p<0.05$) increased the amount of time spent in the illuminated arena (and that there was a dose related difference in response) compared to vehicle controls, and 2) that the higher (but not the lower) dose of mCPP was associated with a strong trend ($p=0.06$) toward decreased time spent in the illuminated arena.

PPI experiments- Fig 2C-E illustrates the results of experiments in which known PPI impairing agents (and compounds known to attenuate the effects of such agents) were evaluated. The following results were observed (overall treatment effect, $p<0.001$ in all 3 PPI validation studies): 1) apomorphine, MK801, and scopolamine at the doses evaluated (0.5, 0.1, and 0.33 mg/kg s.c., respectively) clearly diminished the effects of the prepulse stimuli on the acoustic startle response; 2) the FGA, haloperidol (0.03, and 0.10 mg/kg, IP), clearly attenuated the effects of apomorhine, 3) the SGA clozapine (5.0 mg/kg, IP.) clearly attenuated the effects of MK-801, and 4) the AChEI, donepezil (1.0 mg/kg, s.c.) clearly attenuated the impairing effects of scopolamine (post hoc effects $p<0.05$ in all cases).

Water Maze (Chronic Antipsychotic Studies)

Visible Platform Test- Fig 3A illustrates the effect of the neurolpetics on the visible platform test (mean of 4 trials ± S.E.M.) in the water maze. This test was used as a method to insure that the test subjects were not impaired visually and that they did not exhibit other (non-

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mnemonic) behaviors such as thigmotaxis that might have confounded the analyses. There were no significant treatment-related effects observed in this procedure (i.e., all p values were >0.05).

Swim Speeds- Swim speeds were also analyzed (Fig 3B) in an effort to further investigate treatment related differences in task performance. Average swim speeds ranged between 15-20 cm/sec across the groups for the 6 days of hidden platform testing. Statistical analyses revealed that there was not an overall treatment (group) effect, $F_{2,33}$ =1.3, p=0.39; or day effect $F_{5,10}$ =1.4, p=0.25, however, there was a significant treatment x day interaction $F_{165,215}$ =2.1, p=0.02. Post hoc analyses indicated that the only treatment-related difference in swim speeds occurred on day two of testing, HAL-treated animals swam slightly (but significantly) faster than vehicle and ZIP-treated animals.

Hidden Platform Test- Fig 3C illustrates the efficiency of each experimental group to locate a hidden platform in a water maze task on 6 consecutive days of testing. For the latency comparisons, there was a highly significant treatment effect $F_{2,33}$ =8.0, p<0.001, a significant day effect $F_{5,10}$ =14.9, p<0.001, without a significant treatment x day interaction $F_{165,215}$ =1.6, p=0.1. Post hoc analyses (for overall treatment effect across all days of testing) indicated that performance was superior in the vehicle-treated animals compared to both the HAL and ZIP animals (p<0.001 and p<0.05 respectively). In the case of ZIP, all p values in the individual day comparisons were >0.05 (compared to vehicle controls), whereas in the case of HAL, there were several days where the performance was significantly different than controls (p<0.05, see Fig 2C).

Probe Trials- Figs 4A and 4B illustrate the performance of probe trials by the various treatment groups. There were statistically significant (treatment-related) effects on performance as indicated by the percentage of the total time spent in the previous target quadrant (treatment

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effect $F_{2,33}$ =3.4, p<0.05) and the number of crossing over the previous 10 cm x 10 cm target area (treatment effect $F_{2,33}$ =7.5, p<0.01). Post hoc analysis indicated that performance was superior in the vehicle-treated animals compared to HAL-treated animals in both measures (p<0.001 and p<0.01, respectively). In the case of ZIP, there was a not a significant difference (compared to control) although there was a trend toward a performance decrement in the platform crossing analysis (p=0.1).

Locomotor Activity and the Light-Dark Preference Test (Chronic Antipsychotic Studies)

Figs 5A-C illustrate the effects of the drug treatments on horizontal and vertical locomotor activity, stereotypical movements, as well as fear/anxiety-related behaviors (i.e., time spent in the lighted zone of the test apparatus). There were no significant treatment-related differences (p>0.05) in horizontal activity, vertical activity or stereotypical movements. There were also no significant drug related effects on the light-dark preference test, although there was a strong trend toward a reduction in anxiety-related behaviors in the ZIP group, treatment effect $F_{2,30}$ =3.3, p=0.053.

PPI Experiments (Chronic Antipsychotic Studies)

The effects of 90 days of prior exposure to the neuroleptics on PPI testing assessed on day 12 of the drug-free washout are presented in Figs 6A-C. There was a highly significant (prepulse intensity dependent) reduction in the startle response when the prepulse stimuli preceded the startle stimulus, prepulse level effect, F(2,4) = 41.1, p <0.001 (Fig 5A). Post hoc analysis indicated that a graded response to the increasing prepulse intensities was present (i.e., 75<80<85 dB) in all treatment groups. There were no significant differences in responses to the various drug treatments, however, F(2,27) = 0.9, p =0.44. Similarly, the treatment x prepulse level interaction was not significant F(54,89) = 0.9, p =0.49. Additional analyses revealed that

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the drugs also had no significant effects on the startle response, $F(2,27) = 0.4$, $p = 0.70$ (Fig 5B). Fig 5C depicts the overall effects of the treatments on PPI (i.e., averaged across prepulse intensity).

ELISA Experiments

Fig 7 summarizes ELISA results for ChAT, VAChT, $p75^{NTR}$, TrkA, phospho-TrkA, and NGF, in memory-associated brain regions from rats treated with HAL (Fig 7, A-D) or ZIP (Fig 7, E-H). Each plot is presented in the same format: ELISA results from drug-treated rats are presented as a percent of vehicle-treated rats; each bar is labeled with the name of the protein measured (sample number in parentheses); the left two bars show levels of the cholinergic-marker proteins, ChAT and VAChT; the middle three bars depict data for the NGF receptors ($p75^{NTR}$, TrkA, and phospho-TrkA); the far right bar represents the amount NGF protein; the horizontal dashed line is drawn at 100% of the vehicle treated group (i.e., bars near this line indicate similar protein levels between vehicle- and drug- treated groups).

HAL-Treated Rats. HAL treatment did not change ChAT levels in any of the four brain regions tested; however, VAChT protein was significantly decreased in the hippocampus (-25%, Fig 7B) and prefrontal cortex (-10%, Fig 7D). HAL-treatment had modest effects on the levels of NGF-receptors ($p75^{NTR}$, TrkA, and phospho-TrkA), except for a ~50% decrease in phosphorylated-TrkA protein in the hippocampus (Fig 7B). Notably, HAL-treated rats had significantly less NGF protein in the basal forebrain (-25%, Fig 7A), cortex (-20%, Fig 7C), and prefrontal cortex (-20%, Fig 7D).

ZIP-Treated Rats. In contrast to HAL, ZIP treatment significantly decreased (by 15-25%) ChAT and VAChT in the hippocampus (Fig 7F) and prefrontal cortex (Fig 7H). ZIP-treatment had little to no effect on the levels of NGF-receptors ($p75^{NTR}$, TrkA, and phospho-TrkA) in the

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basal forebrain (Fig 7E) and cortex (10% *increase* in TrkA, Fig 7D). In the hippocampus from ZIP-treated rats (Fig 7F), $p75^{NTR}$ and TrkA were slightly decreased, while phospho-TrkA protein decreased by 35%. In the prefrontal cortex from ZIP-treated rats (Fig 7H), $p75^{NTR}$ and TrkA were reduced by ~25% and 10%, respectively. Like HAL, ZIP-treated rats had significantly less NGF protein in the basal forebrain (-30%, Fig 7A), and prefrontal cortex (-35%, Fig 7D).

Autoradiographic Data

Autoradiographic results are depicted in Tables 6 and 7. Representative autoradiograms are illustrated in Fig 8. For each of the radioligands employed in this study, the pattern of binding site distribution was similar to that observed in previous studies (see Hernandez and Terry, 2005). Statistical analyses revealed that for each of the ligands there were highly significant regional binding differences (area effect $p<0.001$) as expected, however there were no significant, overall treatment-related differences or treatment x brain area interactions (i.e, all p values were >0.05). Post hoc analyses on individual brain areas did reveal a few select, treatment-related differences however (see below).

Nicotinic Receptor Expression

[^{125}I]-BTX: Binding of [^{125}I]-BTX was widely distributed across all regions of the brain, with the exception of the striatum and cerebellum (Table 6 and Fig. 8). The highest [^{125}I]-BTX 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. There were no significant differences in [^{125}I]-BTX binding associated with the different antipsychotic treatments, however (all p values were >0.05 in post hoc analyses).

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[^{3}H]-EPB: The highest [^{3}H]-EPB binding densities were observed in the medial habenular nuclei, interpeduncular nuclei, and pineal gland (Table 6 and Fig. 8). Moderate binding was observed in the anterior thalamus and subicular complex while lower binding densities were observed in the cerebral cortex and individual cortical layers. Of the 19 areas measured, antipsychotic-treated animals exhibited a statistically significant difference from vehicle controls (in post hoc analyses) in two areas, the cingulate cortex and the anteroventral thalamic nucleus. In the cingulate cortex both HAL and ZIP were associated with a decrease in binding sites, whereas in the anteroventral thalamic nucleus binding was significantly increased by HAL (compared to vehicle controls).

Muscarinic Receptor Expression

[^{3}H]-AFX: The highest [^{3}H]-AFX binding densities were observed in olfactory areas, the caudate putamen, and accumbens nuclei, while moderate binding was found in the cortex, basolateral amygdala, and hippocampal formation (Table 7 and Fig. 8). Of the 24 areas measured, antipsychotic-treated animals exhibited a statistically significant difference from vehicle controls (in post hoc analyses) in only one area. Specifically, binding was higher in the HAL treated rats in the pontine nuclei compared to vehicle-treated rats.

[^{3}H]-PRZ: [^{3}H]-PRZ binding was widely distributed in the cortex and hippocampal formation and minimally represented in the thalamus, hypothalamus, and midbrain (Table 7 and Fig. 8). The highest [^{3}H]-PRZ binding densities were observed in the telencephalic regions such as CA1 region of the hippocampus, dentate gyrus, nucleus accumbens, basolateral amygdala, neocortex caudate putamen, and anterior olfactory nuclei. There were no significant differences in [^{3}H]-PRZ binding associated with the different antipsychotic treatments (all p values were >0.05).

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Discussion

In the initial phase of this study we observed that during the early time points of treatment (i.e., 7 and 14 days), both HAL and ZIP were associated with marked increases in NGF and ChAT immunoreactivity in the dentate gyrus (DG), CA1 and CA3 regions of the hippocampus. Such increases in NGF protein in the hippocampus have been previously observed in association with 14 days of HAL treatment (Ozaki, 2000). After 45 days, however, a very different pattern was observed; NGF and ChAT levels had abated to control levels in the ZIP-treated animals and had dropped significantly below control in HAL-treated animals. Moreover, after 90 days, NGF and ChAT levels were substantially lower than controls in both antipsychotic groups. The basis for this time-dependent (biphasic), growth factor and cholinergic response to the neuroleptics in the hippocampus is unclear, since such observations have not been reported previously. The pattern could reflect some compensatory (but unsustainable) growth factor response to a neurotoxic effect of the antipsychotics or alternatively, some time dependent reaction to their inhibitory D_2 receptor effects on cholinergic neurons. Interestingly, observations of time-dependent (biphasic), cholinergic responses to FGAs in the striatum of animals (similar to our observations in the hippocampus) have been discussed as a potential mechanism of their adverse motor effects in humans. Namely, an increase in the activity of cholinergic interneurons in the striatum in response to FGAs initially appears to parallel extrapyamidal side effects in humans, whereas longer treatment periods are associated with decreases in cholinergic activity below baseline (i.e., effects that correspond with the emergence of tardive dyskinesia, see Miller and Chouinard, 2003; Kelley and Roberts, 2004). This phenomenon may reflect the inhibitory D_2 receptor effects on cholinergic interneurons by FGAs which results in excessive neuronal activity, intracellular accumulation of calcium, and

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subsequent cell damage. Since D_2 receptors are relatively sparse in the septohippocampal pathway, it is unclear whether an analogous process would occur here. However, dopamine regulation (via the D_1 receptor) of septohippocampal cholinergic activity has been described (Day and Fibiger, 1994) and both HAL and ZIP have significant antagonist activity at D_1 receptors (see Miyamoto et al., 2005). Our detection of sustained (HAL and ZIP-related) decreases in cholinergic markers in the cortex (described further below) may reflect the involvement of other dopamine-acetylcholine interactions. For example, it has been hypothesized that dopamine in the nucleus accumbens inhibits the activity of GABAergic projections to the basal forebrain thus modulating the excitability of cholinergic neurons that project to the cortex (see Sarter and Bruno, 1999). Accordingly, chronic exposure to drugs that antagonize mesolimbic dopamine receptors could indirectly lead to imbalances in cholinergic activity in basal forebrain neurons (and thus projection areas such as the cortex).

In the second (behavioral testing) phase of the study, the task validation experiments indicated that: 1) the water maze task used was sensitive to cholinergic manipulation (a central issue in this study), 2) reference anxiolytic and anxiogenic agents were active in the light/dark box procedure, and 3) the PPI task was sensitive to the effects of 3 known PPI impairing agents and their antagonism by reference antipsychotic and cholinergic agents. In the chronic neuroleptic studies, rats previously treated with HAL were impaired in water maze hidden platform tests and probe trials (i.e., spatial learning/acquisition and retention). ZIP treated animals were also impaired during task acquisition, although the magnitude of the deficits was lower than with HAL. The absence of HAL or ZIP-related effects on swim speeds, visible platform tests, or in light/dark box/activity monitor experiments, argues against the premise that residual drug effects on locomotor activity, visual acuity, or anxiety levels underlie the observed

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deficits in water maze performance. The final behavior experiments were conducted to assess the residual effects of the antipsychotics on PPI. While a number of neuroleptic drugs (administered acutely) reverse or attenuate PPI deficits in pharmacologic and neurodevelopmental models of schizophrenia (reviewed, Geyer and Ellenbroek, 2003), the effects of chronic antipsychotic treatment (i.e., similar to the situation in schizophrenia) have not been evaluated. Several neurotransmitters including dopamine, serotonin, and glutamate are known to regulate PPI, however, observations that decreased cholinergic activity results in PPI disruption (Stanhope et al., 2001; Jones et al., 2005), were of particular interest to us in light of our earlier findings of neuroleptic-associated decreases in ChAT (Terry et al., 2003). In the present study, neither HAL nor ZIP (i.e., in a normal, non-impaired animal model) was associated with significant alterations in PPI or startle amplitude, even though these agents were associated with quite notable (negative) effects on cholinergic markers, see below).

The residual effects of chronic HAL and ZIP treatment on the neurotrophin NGF (and its receptors) and cholinergic proteins in memory-related brain areas were then assessed. NGF interacts with two plasma membrane receptors, the high affinity TrkA receptor and the neurotrophin receptor $p75^{NTR}$ (p75) and provides the primary trophic support to the cholinergic basal forebrain and its projections to cortex and hippocampus. NGF binding to TrkA promotes TrkA autophosphorylation which activates pathways that enhance cholinergic neuron survival, while NGF signaling via $p75^{NTR}$ typically (but not exclusively) activates pathways leading to cell death (see reviews, Sofroniew et al., 2001; Counts and Mufson 2005). ChAT and VAChT are commonly assessed as cholinergic markers since only neurons that release acetylcholine express these proteins (Arvidsson, et al., 1997; Wu and Hersh, 1994). While there were some regional differences in drug responses, in general, both HAL and ZIP tended to decrease the levels of

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cholinergic proteins (i.e., either ChAT or VAChT or both) and decrease NGF (and/or its receptors). Surprisingly, in contrast to the immunohistochemical results described earlier, we did not detect a decrease in ChAT protein by ELISAs performed with hippocampal lysates from HAL-treated rats. While ChAT immunoreactivity in the hippocampus was clearly demarcated within the hippocampal subfields, the ELISAs measured ChAT protein in heterogynous tissue lysates that included the dentate gyrus (DG) combined with the CA1 and CA3 subfields. Thus, the sensitivity to HAL- associated decreases in ChAT in the hippocampus may be lower when the tissues were combined. The differential finding could also be explained by the fact that ChAT immunoreactivity averaged across the hippocampal subfields decreased by ~75% in ZIP-treated rats (i.e., at the 90-day time point) whereas the decrease was ~55% in HAL-treated rats, and, therefore, the deficits may have simply been more easily detectable in the ZIP-treated animals by ELISA. An alternative explanation is that there are differential antipsychotic-related neurochemical responses to the 2-week washout.

The changes that were most striking and shared by both drugs in the ELISAs were decreases in NGF in the basal forebrain and prefrontal cortex and a decrease in phospho-TrKA in the hippocampus (i.e., brain areas known to affect acquisition and retention in a number of behavioral tasks, see Kesner and Rogers, 2004). Surprisingly, $p75^{NTR}$ was decreased by both HAL and ZIP in the prefrontal cortex as well. Given the commonly described negative role of $p75^{NTR}$ on neurons, the finding was a bit perplexing, although, in memory-related illnesses such as Alzheimer's disease, TrkA depletion more reliably correlates with deteriorated cognitive ability than alterations in $p75^{NTR}$ (Counts et al., 2004) even though basal forebrain cholinergic neurons (commonly damaged in Alzheimer's Disease) express both types of NGF receptors.

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In the final portion of the study, we investigated whether chronic HAL or ZIP treatment resulted in persistent changes in cholinergic receptor densities. Given the aforementioned decreases in ChAT and VAChT (i.e., presynaptic proteins), we expected to observe reduced levels of presynaptic cholinergic receptors (i.e., nicotinic, and M_2, muscarinic). Surprisingly, there were only a few minor changes (e.g., decreases in [^{3}H]-EPB binding in the cingulate cortex of rats administered HAL or ZIP). This observation appears to indicate that the antipsychotics affect proteins that are more directly influenced by NGF and involved in metabolic processes such as acetylcholine synthesis and storage (i.e., ChAT and VAChT) as opposed to cholinergic receptors.

In summary, the results of this study indicate that while ZIP (given chronically) appears somewhat superior to HAL due to less pronounced behavioral effects and a more delayed appearance of neurochemical deficits, both agents may be associated with deleterious time-dependent (and persistent) effects on the neurotrophin, NGF and cholinergic neurons, as well as spatial learning. Due to the fact that single doses of the antipsychotics were evaluated in this study, these data should be viewed with caution until more extensive dose-effect relationships for chronic treatment are established. Further, it is not entirely clear if the behavioral results observed in this study reflect some persistent neurochemical adaptation to chronic dosing or an antipsychotic withdrawal effect. Either scenario would likely have relevance to the therapeutics of schizophrenia given that chronic treatment periods are standard practice and that drug withdrawal periods are common (i.e., from poor compliance). It is also important to note that, similar to our water maze results, chronic treatment with FGAs such as HAL (Levin et al., 1987), as well as SGAs such as clozapine, and risperidone (Rosengarten and Quartermain, 2002) has been associated with impaired acquisition in radial arm maze tasks as well. Collectively, these

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animal data thus suggest that there are potential limitations to extended therapy with both FGAs and SGAs especially when cognitive function is considered.

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Footnotes:

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Figure Legends

Fig 1. Series of photomicrographs illustrating the time-dependent effects of the antipsychotics, haloperidol and ziprasidone on ChAT immunoreactivity in the CA1 region of the hippocampus. VEH=vehicle controls; HAL, =haloperidol treated; ZIP=ziprasidone treated. Bar represents approximately 100 μm. N=6 rats/group.

Fig 2. Behavioral Task Validation Studies A: Water maze hidden platform studies in which a reference dose (0.1 mg/kg) of the muscarinic receptor antagonist scopolamine was evaluated for it ability to impair spatial learning, and two doses of the acetylcholinesterase inhibitor, donepezil were evaluated for their ability to attenuate the effects of scopolamine. The latency (mean number of seconds ± S.E.M) of each experimental group to locate a hidden platform over 6 consecutive days of testing is depicted. * significantly different than scopolamine-vehicle treated animals ($p<0.05$). B: Light/Dark Box experiments in which 2 doses of the anxiolytic agent diazepam and the anxiogenic agent mCPP were evaluated. Bars indicate the mean time (sec) spent in the lighted zone (± S.E.M). * significantly different than vehicle controls ($p<0.05$). # dose-dependent effect of diazepam. C-E: results of experiments in which reference doses of known PPI impairing agents (apomorphine, MK801, and scopolamine) and compounds known to attenuate the effects of these agents (haloperidol, clozapine, and donepezil, respectively) were evaluated. Bars represent the mean (± S.E.M) percentage of prepulse inhibition averaged across the three prepulse intensities (5, 10, and 15 dB above background). * significantly different ($p<0.05$) than vehicle control response; + =significantly different ($p<0.05$) than vehicle + PPI antagonist related response. VEH=vehicle, SCOP = scopolamine, DON= donepezil, APO =apomorphine. N=8-15 (see Table 1).

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Fig 3. Water maze tests beginning 7 days after the last day of a 90 day treatment period with either haloperidol (HAL) or ziprasidone (ZIP). A: Visible platform test (mean of 4 trials ± S.E.M.). B: Daily swim speeds (mean ± S.E.M. cm/sec) during water maze hidden platform trials C: Hidden platform test (mean ± S.E.M), 2 trials/day over 6 consecutive days of testing. Both HAL and ZIP treated animals performed less efficiently than controls. * significantly different than vehicle controls ($p<0.05$). N=12 rats/group.

Fig 4. Water Maze Probe Trials conducted 14 days after the last day of a 90 day treatment period with either haloperidol (HAL) or ziprasidone (ZIP). A: % of total time spent swimming in the previous target quadrant (mean ± S.E.M). B: Platform area crossings (mean ± S.E.M.). * significantly different than vehicle controls ($p<0.05$). N=12 rats/group.

Fig 5. Effects of prior chronic oral treatment with haloperidol (HAL) or ziprasidone (ZIP) on locomotor activity and the Light-Dark Box Test (day 10 of a drug free washout). **A:** Vertical activity measured as the mean number of photobeam breaks/5 min); **B:** Horizontal activity measured as the mean number of photobeam breaks/5 min); C. Stereotypical movements (repetitive photobeam breaks/ 5 min); **D:** Fear/anxiety related behavior (emergence neophobia) measured as the time spent in a brightly lit zone of the activity monitor. Bars represent the mean ± S.E.M. N=12.

Fig 6. A. Effects of prior chronic oral treatment with haloperidol (HAL) or ziprasidone (ZIP) (i.e., testing on day 12 of a drug free washout) on the percentage of prepulse inhibition in rats for three prepulse intensities (5, 10, and 15 dB above background). B. Drug effects on the mean

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startle amplitude to 120-dB, 20-ms noise burst. C. Drug effects on the percentage of prepulse inhibition averaged across the three prepulse intensities (5, 10, and 15 dB above background). Bars represent mean ± S.E.M. for each treatment (N=12). There were no significant treatment-related effects (i.e., all p values were >0.05).

Fig 7. ELISA results (expressed as percent of vehicle-treated group) for memory-related brain regions from rats treated with haloperidol (A-D) or ziprasidone (E-H). Rats were euthanized 14 days after 90 days of antipsychotic treatment, and then the dissected brain regions – basal forebrain (A & D), hippocampus (B & E), cortex (C & F), and prefrontal cortex (D & H) – were processed for ELISA methods. Proteins detected by ELISA were: choline acetyltransferase (ChAT); vesicular acetylcholine transporter (VAChT); neurotrophin receptor ($p75^{NTR}$); nerve growth factor receptor (NGF); the NGF receptor (TrkA); phosphorylated TrkA (P-TrkA). For each bar, (#) is the number of rats per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 = significantly different from vehicle control.

Fig 8. Representative autoradiograms illustrating nAChR and mAChR subtypes in rats labeled by the following radioligands: nAChRs, [^{125}I]-α-bungarotoxin (α_7), [^{3}H]-epibatidine (predominantly $\alpha_4\beta_2$) and mAChRs [^{3}H]-pirenzipine (M_1/M_4) and [^{3}H]-AFDX384 (predominantly M_2). Experiments were conducted using 16 μM sagittal sections of brains from adult male Wistar rats treated with antipsychotics or vehicle for 90 days followed by a 14 day drug-free washout period. Abbreviations are as follows: CON=vehicle controls; HAL, =haloperidol treated; ZIP=ziprasidone treated, AON=anterior olfactory nucleus, CA1=CA1 region of the hippocampus, Cg=cingulate cortex, OB=olfactory bulbs.

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Table 1: Study Protocol Details

Study Title and Test Cohort	Group	N	Treatment (mg/kg)	No. of Days of Treatment	Procedure (s)
Chronic Antipsychotic-Immunohistochemistry					
A	1	6	VEH	7	Sacrifice-ChAT Staining
	2	6	HAL 2.0	7	Sacrifice-ChAT Staining
	3	6	ZIP 12.0	7	Sacrifice-ChAT Staining
B	4	6	VEH	14	Sacrifice-ChAT Staining
	5	6	HAL 2.0	14	Sacrifice-ChAT Staining
	6	6	ZIP 12.0	14	Sacrifice-ChAT Staining
C	7	6	VEH	45	Sacrifice-ChAT Staining
	8	6	HAL 2.0	45	Sacrifice-ChAT Staining
	9	6	ZIP 12.0	45	Sacrifice-ChAT Staining
D	10	6	VEH	90	Sacrifice-ChAT Staining
	11	6	HAL 2.0	90	Sacrifice-ChAT Staining
	12	6	ZIP 12.0	90	Sacrifice-ChAT Staining
	13	6	VEH	7	Sacrifice-NGF Staining
E	14	6	HAL 2.0	7	Sacrifice-NGF Staining
	15	6	ZIP 12.0	7	Sacrifice-NGF Staining
	16	6	VEH	14	Sacrifice-NGF Staining
F	17	6	HAL 2.0	14	Sacrifice-NGF Staining
	18	6	ZIP 12.0	14	Sacrifice-NGF Staining
	19	6	VEH	45	Sacrifice-NGF Staining
G	20	6	HAL 2.0	45	Sacrifice-NGF Staining
	21	6	ZIP 12.0	45	Sacrifice-NGF Staining
H	22	6	VEH	90	Sacrifice-NGF Staining
	23	6	HAL 2.0	90	Sacrifice-NGF Staining

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	24	6	ZIP 12.0	90	Sacrifice-NGF Staining
Behavioral Task Validations					
I	1	18	VEH-VEH	1	WM Hidden Platform
	2	9	SCOP 0.1 -VEH	1	WM Hidden Platform
	3	9	SCOP 0.1-DON 1.0	1	WM Hidden Platform
	4	9	SCOP 0.1-DON 2.0	1	WM Hidden Platform
J	5	15	VEH	1	Light/Dark Box
	6	8	DIAZ 1.0	1	Light/Dark Box
	7	10	DIAZ 3.0	1	Light/Dark Box
	8	9	mCPP 1.0	1	Light/Dark Box
	9	8	mCPP 2.0	1	Light/Dark Box
K	10	12	VEH-VEH	1	PPI
	11	12	VEH-APO	1	PPI
	12	10	HAL 0.01-APO 0.5	1	PPI
	13	15	HAL 0.03-APO 0.5	1	PPI
	14	15	HAL 0.10-APO 0.5	1	PPI
L	15	10	VEH-VEH	1	PPI
	16	8	VEH-MK801 0.1	1	PPI
	17	10	CLOZ 1.0- MK801 0.1	1	PPI
	18	10	CLOZ 2.0- MK801 0.1	1	PPI
	19	10	CLOZ 5.0- MK801 0.1	1	PPI
M	20	10	VEH-VEH	1	PPI
	21	10	SCOP 0.33-VEH	1	PPI
	22	9	SCOP 0.33-DON 1.0	1	PPI
	23	8	SCOP0.33-DON 1.0	1	PPI
	24	8	SCOP 0.33-DON 2.0	1	PPI
Chronic Antipsychotic-Behavior and					

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Neurochemistry					
N	1	12	VEH	90+ 7 day washout	WM (washout-days 8-14-AM)- Light/Dark Box (day 10-PM)- PPI (day 12-PM)-Sacrifice (Day 14 of washout)-ELISA/Autoradiography
O	2	12	HAL 2.0	90+ 7 day washout	WM (washout-days 8-14-AM)- Light/Dark Box (day 10- PM)- PPI (day 12-PM)-Sacrifice (Day 14 of washout)-ELISA/Autoradiography
P	3	12	ZIP 12.0	90+ 7 day washout	WM (washout-days 8-14-AM)- Light/Dark Box (day 10- PM)- PPI (day 12-PM)-Sacrifice (Day 14 of washout)-ELISA/Autoradiography

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Table 2. Quantity of Brain Protein Analyzed in Each ELISA

	Micrograms (μg) Total Protein per Microwell by Brain Region			
ELISA Method	Basal Forebrain	Hippocampus	Cortex	Prefrontal Cortex
ChAT	1.0	1.0	1.0	1.0
VAChT	1.0	1.0	0.5	0.4
$p75^{NTR}$	0.8	0.4	0.4	0.4
TrkA	0.4	0.4	0.4	0.4
P-TrkA	70	70	70	70

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Table 3. Conditions for Autoradiography on Sagittal Sections from Rat Brain

Ligand	Epibatidine	Alpha-Bungarotoxin	Pirenzepine	AFDX-384
Receptor Subtype Target	Nicotinic non-alpha 7	Nicotinic alpha 7	Muscarinic M_1/M_4	Muscarinic M_2
Catalog #	NET-1102	NEX-1265	NET-780	NET-1041
Isotope	^{3}H	^{125}I	^{3}H	^{3}H
Specific Activity	55.5 Ci/mmole	17.0 µCi/µg	86 Ci/mmole	120 Ci/mmole
Radioligand Incubation Concentration (nM)	0.8	1.0	3.0	1.8
Pre-Incubation Time in Buffer (min)	none	20	15	15
Radioligand Incubation Time (min)	40	120	90	90
Film Exposure Time	13 weeks	160 hours	6 weeks	16 weeks

Radioligands from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA)
Film = BioMax MR-2 film (35 x 43 cm, Eastman Kodak Company #865 7232)
Films were manually processed using Kodak GBX developer and fixer according to the manufacturer's protocol.

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TABLE 4

Drug Effects on NGF Immunoreactivity the Hippocampus

NGF (Mean Optical Density ± S.E.M.)

Treatment	Region: DG	CA1	CA3
		7 Days	
VEH	77.8 ± 5.3	60.8 ± 3.5	59.2 ± 3.8
HAL	108.2 ± 7.5**	82.2 ± 5.1**	81.0 ± 4.8**
ZIP	105.8 ± 5.7**	81.0 ± 4.9**	80.0 ± 4.4**
		14 Days	
VEH	75.5 ± 6.3	59.7 ± 3.9	58.8 ± 3.5
HAL	103.2 ± 6.5**	80.8 ± 5.3**	81.3 ± 5.7**
ZIP	100.1 ± 4.9*	79.9 ± 3.2**	79.4 ± 4.5**
		45 Days	
VEH	79.7 ± 5.8	61.5 ± 3.9	60.8 ± 4.1
HAL	58.2 ± 5.9*	44.8 ± 3.2**	35.3 ± 3.6***
ZIP	76.2 ± 5.1	60.4 ± 3.4	59.0 ± 3.4
		90 Days	
VEH	75.3 ± 3.3	58.1 ± 1.9	60.45 ± 2.2
HAL	32.4 ± 3.0***	24.8 ± 1.0***	29.2 ± 2.0***
ZIP	51.3 ± 1.9***	40.4 ± 1.3***	41.7 ± 2.5***

*p<0.05, **p<0.01, ***p<0.001 vs vehicle control, ANOVA (N=5-6/group)

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TABLE 5

Drug Effects on ChAT Immunoreactivity the Hippocampus

ChAT (Fiber Pixel Density ± S.E.M.)

Treatment	Region: DG	CA1	CA3
		7 Days	
VEH	0.078 ± 0.002	0.082 ± 0.003	0.100 ± 0.003
HAL	0.098 ± 0.003*	0.107 ± 0.003**	0.131 ± 0.004**
ZIP	0.096 ± 0.004*	0.105 ± 0.003**	0.130 ± 0.004**
		14 Days	
VEH	0.080 ± 0.003	0.084 ± 0.002	0.103 ± 0.003
HAL	0.100 ± 0.005*	0.108 ± 0.004**	0.129 ± 0.004**
ZIP	0.098 ± 0.003*	0.106 ± 0.006*	0.124 ± 0.005*
		45 Days	
VEH	0.077 ± 0.004	0.079 ± 0.002	0.101 ± 0.002
HAL	0.058 ± 0.003*	0.059 ± 0.002**	0.078 ± 0.003**
ZIP	0.075 ± 0.003	0.076 ± 0.004	0.098 ± 0.004
		90 Days	
VEH	0.079 ± 0.003	0.081 ± 0.003	0.101 ± 0.003
HAL	0.039 ± 0.002***	0.048 ± 0.003***	0.054 ± 0.005***
ZIP	0.052 ± 0.002***	0.065 ± 0.001***	0.081 ± 0.002***

*p<0.05, **p<0.01, ***p<0.001 vs vehicle control, ANOVA (N=5-6/group)

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TABLE 6. Nicotinic Receptors: α_7 (^{125}I-Bungarotoxin) and α_4/β_2 (^{3}H-Epibatidine)

	^{125}I-BUNGAROTOXIN			^{3}H-EPIBATIDINE		
BRAIN REGION	**Vehicle**	**Haloperidol**	**Ziprasidone**	**Vehicle**	**Haloperidol**	**Ziprasidone**
Cerebral Cortex						
Lamina I				1.00 ± 0.02	0.95 ± 0.02	1.01 ± 0.05
Lamina II				1.67 ± 0.03	1.61 ± 0.06	1.68 ± 0.03
Lamina III-VI				1.29 ± 0.02	1.23 ± 0.05	1.29 ± 0.03
Lamina I-V (medial)	11.37 ± 0.55	10.97 ± 0.60	12.31 ± 0.64			
Lamina I-V (lateral)	11.30 ± 0.62	11.05 ± 0.67	12.62 ± 0.67			
Lamina VI (medial)	14.39 ± 0.67	14.16 ± 0.57	15.47 ± 0.61			
Lamina VI (lateral)	14.75 ± 0.54	14.86 ± 0.62	16.41 ± 0.86			
Entire Cortex				1.29 ± 0.02	1.24 ± 0.04	1.29 ± 0.02
Entire Cortex (medial)	12.48 ± 0 .57	12.19 ± 0.58	13.53 ± 0.59			
Entire Cortex (lateral)	12.51 ± 0.58	12.28 ± 0.63	13.85 ± 0.78			
Prefrontal	14.02 ± 0.70	13.92 ± 0.39	15.31 ± 0.79	1.51 ± 0.03	1.47 ± 0.03	1.48 ± 0.04
Prefrontal (medial)	16.26 ± 0.81	16.23 ± 0.47	18.18 ± 1.14			
Prefrontal (outer)	12.2 6 ± 0.67	11.88 ± 0.35	12.80 ± 0.50			
Cingulate				2.05 ± 0.19	**1.75 ± 0.03***	**1.83 ± 0.06***
Entorhinal (lateral)	19.21 ± 1.02	20.02 ± 1.06	18.11 ± 1.01			
Entorhinal (medial)	15.79 ± 0.45	15.15 ± 0.42	16.00 ± 1.08			
Entorhinal (entire)				1.17 ± 0.03	1.09 ± 0.03	1.20 ± 0.03
Retrosplenial Angular				1.53 ± 0.03	1.39 ± 0.07	1.51 ± 0.04
Hippocampal Formation						
CA1 (lateral)	13.44 ± 0.60	13.6 3± 0.70	14.14 ± 0.74			

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CA1 (dorsal)	16.13 ± 0.69	16.44 ± 0.91	18.53 ± 1.49			
CA1 (ventral)	25.14 ± 0.92	25.73 ± 0.78	26.63 ± 0.92			
CA2 (dorsal)	17.95 ± 0.67	17.93 ± 0.83	20.06 ± 1.11			
CA2 (ventral)	23.21 ± 0.68	23.42 ± 0.78	23.76 ± 0.89			
CA2/C3 (medial)	15.44 ± 0.46	14.97 ± 0.67	16.45 ± 0.79			
CA3 (dorsal)	19.13 ± 0.39	19.11 ± 0.52	20.30 ± 0.67			
Dentate Gyrus (dorsal)	14.43 ± 0.44	14.43 ± 0.63	15.19 ± 0.71			
Dentate Gyrus (lateral)	21.24 ± 0.65	21.56 ± 0.76	22.44 ± 0.92			
Dentate Gyrus (ventral)	43.81 ± 1.09	44.67 ± 0.93	42.18 ± 0.81			
Polymorph Layer of Dentate Gyrus (dorsal)	27.00 ± 0.45	26.09 ± 0.79	27.40 ± 0.57			
Polymorph Layer of Dentate Gyrus (lateral)	41.84 ± 1.74	43.67 ± 1.28	43.83 ± 2.00			
Dentate Gyrus (entire)				0.80 ± 0.03	0.74 ± 0.02	0.76 ± 0.02
Molecular Layer of Dentate Gyrus				0.65 ± 0.02	0.65 ± 0.02	0.65 ± 0.01
Subicular Complex						
Presubiculum	19.43 ± 0.48	19.51 ± 0.45	21.21 ± 1.17	2.70 ± 0.04	2.76 ± 0.09	2.76 ± 0.11
Postsubiculum				2.30 ± 0.04	2.17 ± 0.12	2.33 ± 0.06
Subiculum				1.65± 0.03	1.53 ± 0.06	1.61 ± 0.04
Subiculum (medial)	13.38 ± 0.59	13.43 ± 0.49	13.77 ± 0.71			
Subiculum (lateral)	20.60 ± 0.67	20.62 ± 1.02	21.57 ± 1.15			
Thalamic Nuclei						
Medial Dorsal				3.64 ± 0.08	3.48 ± 0.13	3.64 ± 0.12

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Antereoventral				4.49 ± 0.17	**4.26 ± 0.19***	4.60 ± 0.24
Olfactory Nuclei						
Accessory Olfactory Bulb	28.35 ± 1.63	28.84 ± 1.72	29.15 ± 0.87			
Anterior Olfactory Nucleus	13.12 ± 0.54	13.18 ± 0.47	14.72 ± 0.76	0.59 ± 0.03	0.68 ± 0.02	0.66 ± 0.01
Olfactory Bulb	7.48 ± 0.36	7.35 ± 0.46	8.12 ± 0.42			
Other Nuclei						
Basal Nucleus				0.52 ± 0.02	0.46 ± 0.01	0.50 ± 0.02
Lateral Septal				0.49 ± 0.03	0.50 ± 0.02	0.48 ± 0.03
Triangular Septal Nucleus				0.67 ± 0.08	0.73 ± 0.08	0.75 ± 0.08
Posterior Cortical Amygdaloid Nucleus	25.80 ± 0.69	23.50 ± 0.68	23.97 ± 1.24			
Medial Amygdala	17.72 ± 0.69	17.31 ± 0.62	18.50 ± 0.61			
Basolateral Amygdala (medial)	18.71 ± 1.67	18.57 ± 0.73	19.64 ± 1.14			
Basolateral Amygdala (lateral)	16.69 ± 0.66	17.92 ± 0.59	18.95 ± 0.77			
Mammillary Nuclei	20.75 ± 0.93	20.82 ± 1.10	19.83 ± 0.87			
Mammillary Peduncles	24.21 ± 1.53	22.81± 1.79	24.34 ± 1.99			
Substantia Innominata	16.25 ± 0.68	16.47 ± 0.57	17.60 ± 0.41			

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Amersham Autoradiographic [^{125}I] or [^{3}H]-Microscales were placed in each film cassette, and then analyzed to generate standard curves (OD versus nCi of ligand/mg tissue equivalents). Binding values above are expressed as nCi/mg tissue equivalents. Each value represents the mean ± S.E.M. of 6-9 rats. * and bolded text =significantly different ($p<0.05$) than vehicle associated value.

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TABLE 7. Muscarinic Receptors: M_2 Receptor (^{3}H-AFDX-384) and M_1/M_4 Receptor (^{3}H-Pirenzepine)

	^{3}H-AFDX-384			^{3}H-PIRENZEPINE		
BRAIN REGION	**Vehicle**	**Haloperidol**	**Ziprasidone**	**Vehicle**	**Haloperidol**	**Ziprasidone**
Cerebral Cortex						
Lamina I	9.29 ± 0.31	9.78 ± 0.51	9.98 ± 0.28			
Lamina I (medial)				12.63 ± 0.69	13.28 ± 1.17	12.93 ± 0.70
Lamina I (lateral)				12.38 ± 0.87	13.53 ± 1.10	13.32 ± 0.81
Lamina II (medial)				10.36 ± 0.49	11.00 ± 0.82	10.78 ± 0.55
Lamina II (lateral)				9.83 ± 0.55	10.66 ± 0.69	10.43 ± 0.50
Lamina VI	9.59 ± 0.31	10.23 ± 0.51	10.36 ± 0.30			
Lamina I-V	9.15 ± 0.34	9.85 ± 0.50	9.82 ± 0.29			
Entire Cortex				11.03 ± 0.51	11.84 ± 0.79	11.43 ± 0.52
Prefrontal	9.36 ± 0.35	9.71 ± 0.55	9.68 ± 0.26	12.39 ± 0.68	12.98 ± 0.92	12.48 ± 0.68
Entorhinal	7.14 ± 0.27	7.65 ± 0.41	7.73 ± 0.32	11.87 ± 0.61	11.20 ± 0.76	11.82 ± 0.84
Piriform	6.94 ± 0.30	7.72 ± 0.42	7.40 ± 0.26			
Retrosplenial				10.33 ± 0.52	10.67 ± 0.93	10.46 ± 0.69
Hippocampal Formation						
CA1 Region (lateral)	8.03 ± 0.27	8.51 ± 0.48	8.39 ± 0.49			
CA1 Region (dorsal)	8.50 ± 0.27	9.11 ± 0.51	9.10 ± 0.23			
CA1 Region (entire)				17.52 ± 1.09	18.51 ± 1.76	18.40 ± 1.74
CA2 Region (entire)				11.63 ± 0.79	11.99 ± 0.98	12.35 ± 0.93
CA2/C3 Region (lateral)	6.26 ± 0.26	6.87 ± 0.41	7.02 ± 0.32			
CA2/C3 Region (dorsal)	6.88 ± 0.26	7.29 ± 0.40	7.35 ± 0.22			
CA2/C3 Region (entire)				10.27 ± 0.46	10.69 ± 0.83	10.77 ± 0.63

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CA3 Region (entire)				10.10 ± 0.55	10.72 ± 0.89	10.56 ± 0.67
Dentate Gyrus (dorsal)	5.60 ± 0.18	5.92 ± 0.44	5.89 ± 0.26			
Dentate Gyrus (lateral)	6.17 ± 0.22	6.72 ± 0.40	6.85 ± 0.33			
Dentate Gyrus (entire)				17.46 ± 1.26	17.79 ± 1.67	16.74 ± 0.99
Polymorph Layer of Dentate Gyrus				13.04 ± 0.54	13.48 ± 0.72	13.45 ± 0.54
Thalamic Nuclei						
Medial Dorsal	8.49 ± 0.27	8.75 ± 0.51	9.06 ± 0.18			
Antereoventral	12.59 ± 0.40	13.22 ± 0.79	13.47 ± 0.32			
Olfactory Nuclei						
Anterior Olfactory Nucleus	7.71 ± 0.29	8.17 ± 0.51	8.05 ± 0.22	13.52 ± 0.83	13.90 ± 1.13	13.70 ± 0.83
Olfactory Bulb (external plexiform layer)	16.01 ± 0.58	16.88 ± 0.95	15.87 ± 0.91			
Olfactory Bulb (internal plexiform layer)	10.36 ± 0.38	10.54 ± 0.43	9.90 ± 1.03			
Olfactory Bulb				6.84 ± 0.50	7.38 ± 0.69	6.45 ± 0.62
Olfactory Tubercle	10.44 ± 0.63	10.71 ± 0.67	10.52 ± 0.78	13.12 ± 0.92	13.97 ± 1.27	13.97 ± 1.05
Other Nuclei						
Amygdala	6.42 ± 0.33	6.63 ± 0.41	6.93 ± 0.33			
Substantia Innominata	7.02 ± 0.26	7.37 ± 0.42	7.27 ± 0.17			
Medial Septal	7.17 ± 0.53	7.81 ± 0.58	7.75 ± 0.43			
Pontine	10.30 ± 0.49	**12.03 ± 0.78***	11.57 ± 0.65			
Subiculum	7.53 ± 0.24	7.96 ± 0.43	8.22 ± 0.26	6.96 ± 0.46	7.00 ± 0.47	7.25 ± 0.52
Basal Nucleus of Stria Terminalis	6.18 ± 0.27	6.38 ± 0.41	6.60 ± 0.22			

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Basal Nucleus				2.88 ± 0.36	3.02 ± 0.25	2.74 ± 0.34

Amersham Autoradiographic [^{3}H]-Microscales were placed in each film cassette, and then analyzed to generate standard curves (OD versus nCi of ligand/mg tissue equivalents). Binding values above are expressed as nCi/mg tissue equivalents. Each value represents the mean ± S.E.M. of 8-9 rats.

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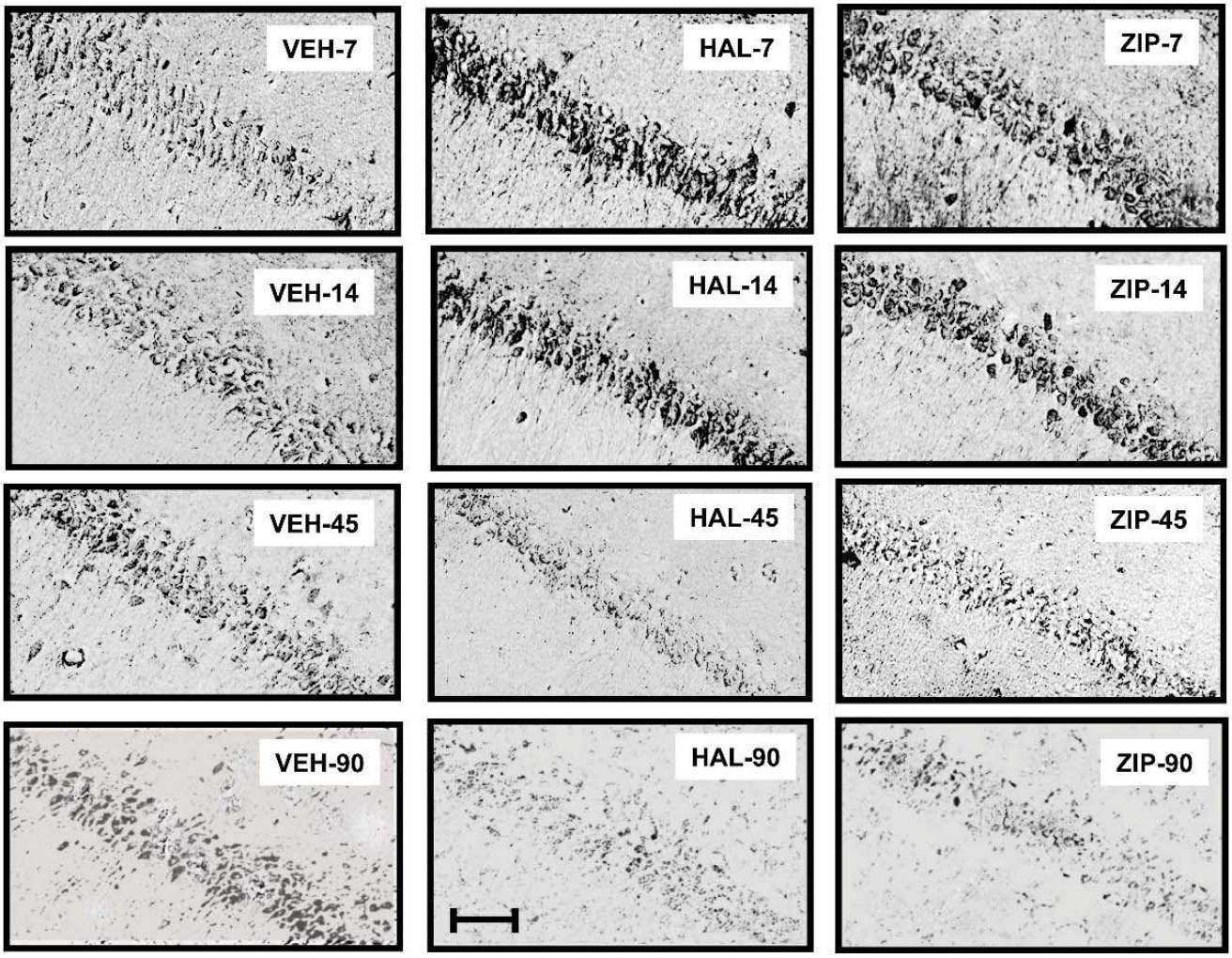
Fig 1

ChAT Immunostaining of Hippocampus (CA1 Region)



Bar = approx. 100 μm

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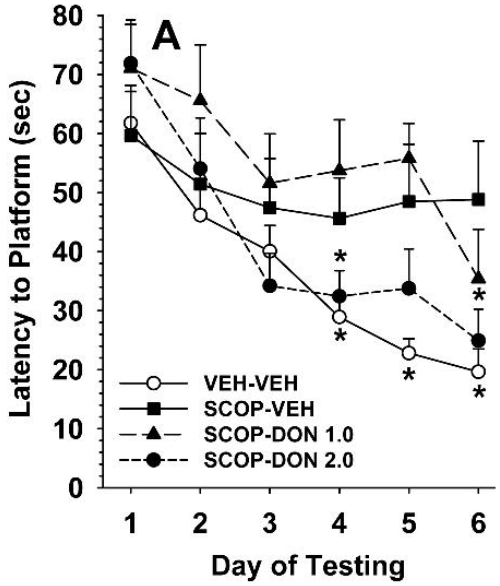
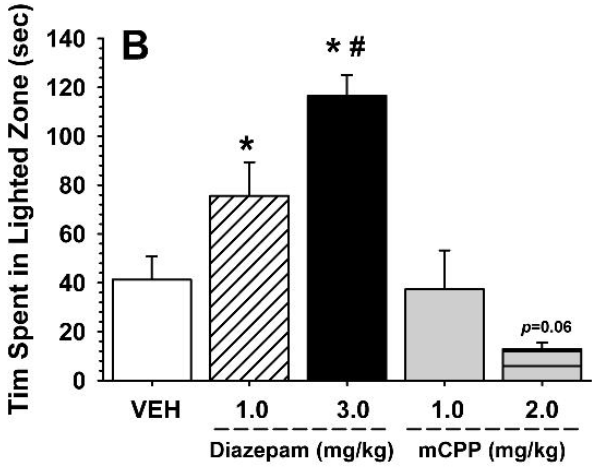
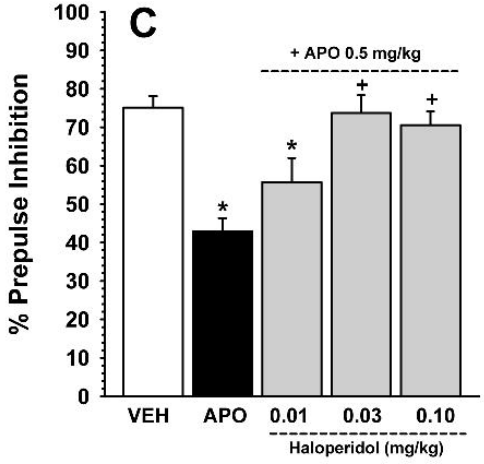
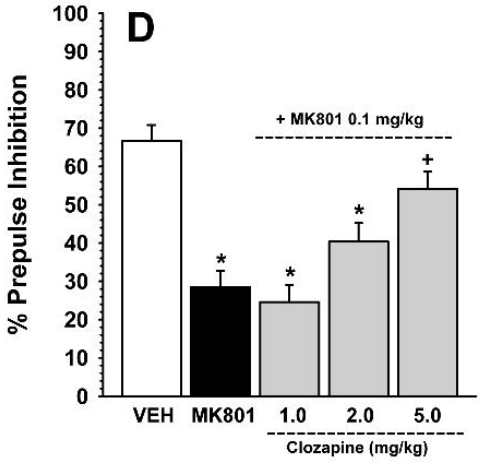
Fig 2









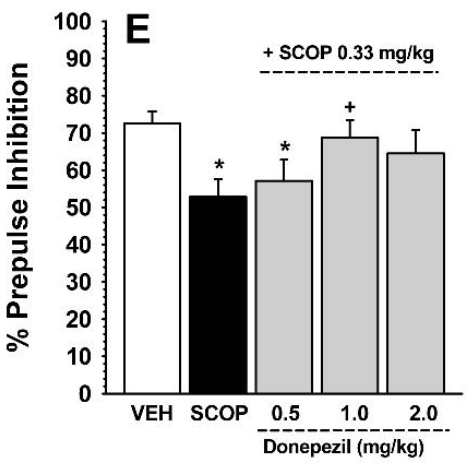


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Fig 3

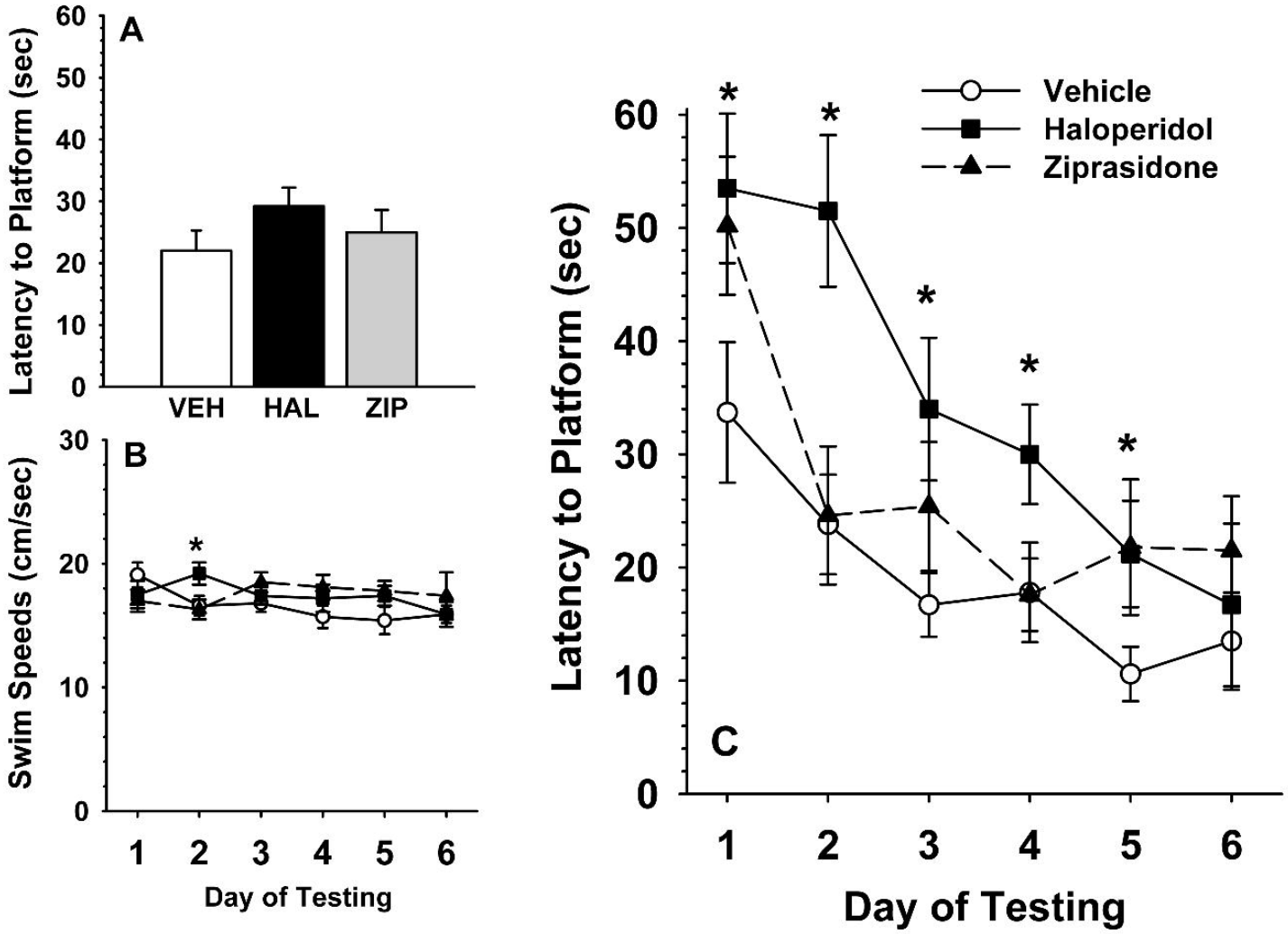

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Fig 4

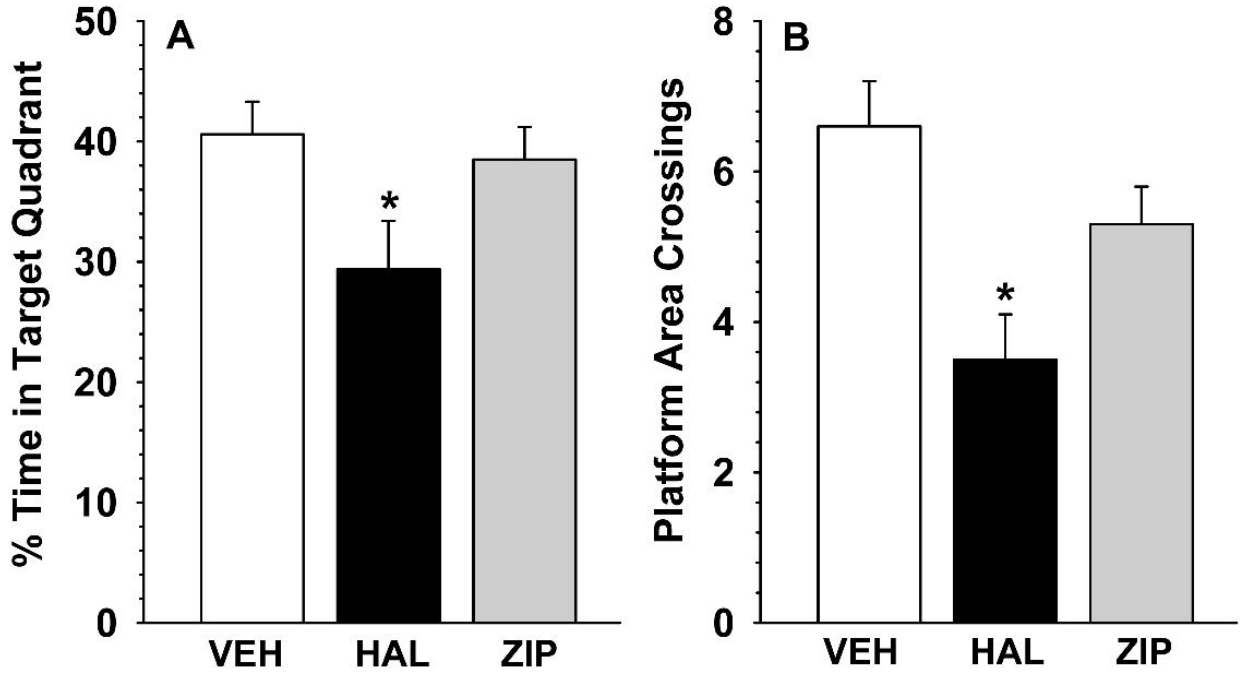

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Fig 5

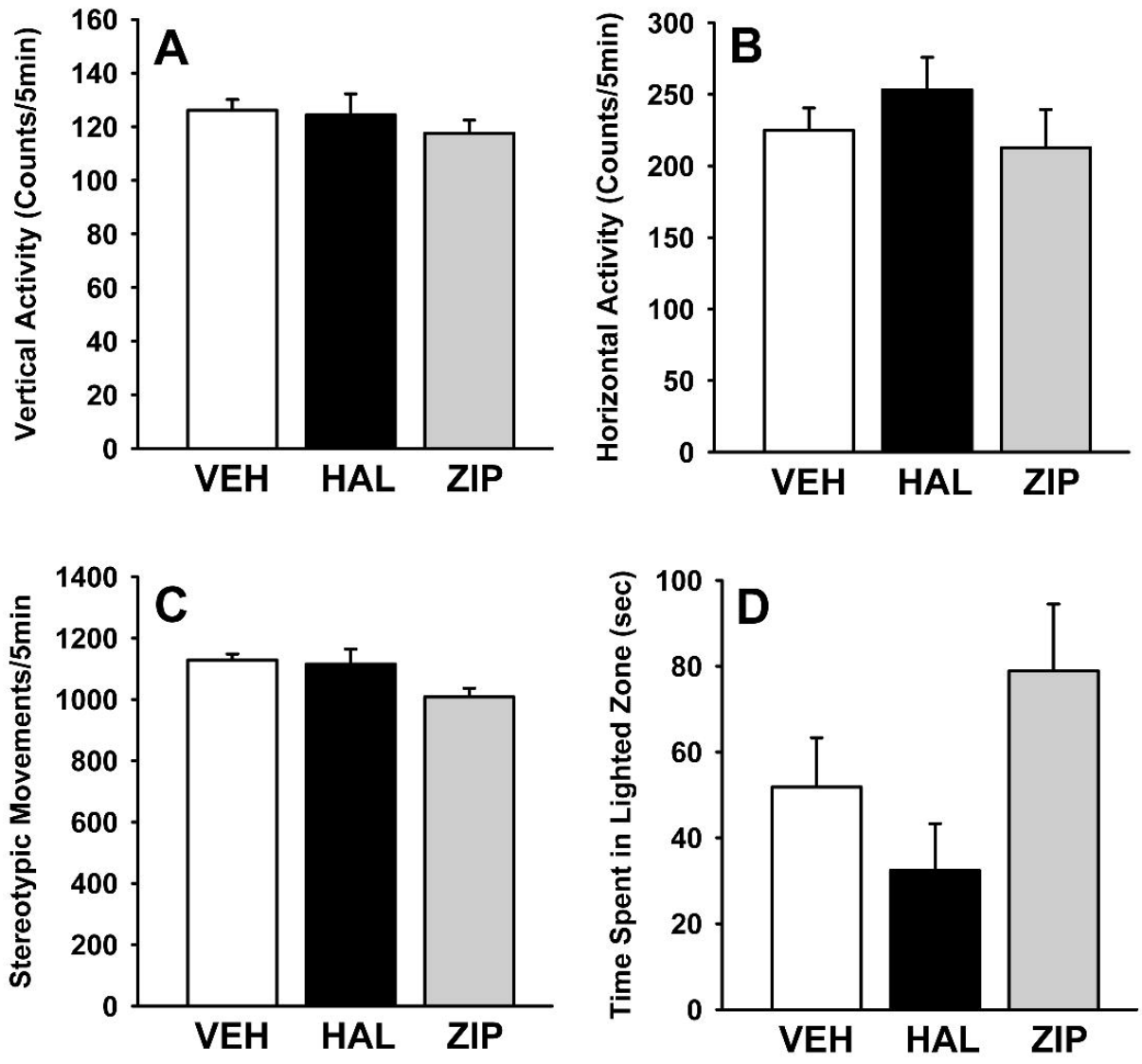

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Fig 6

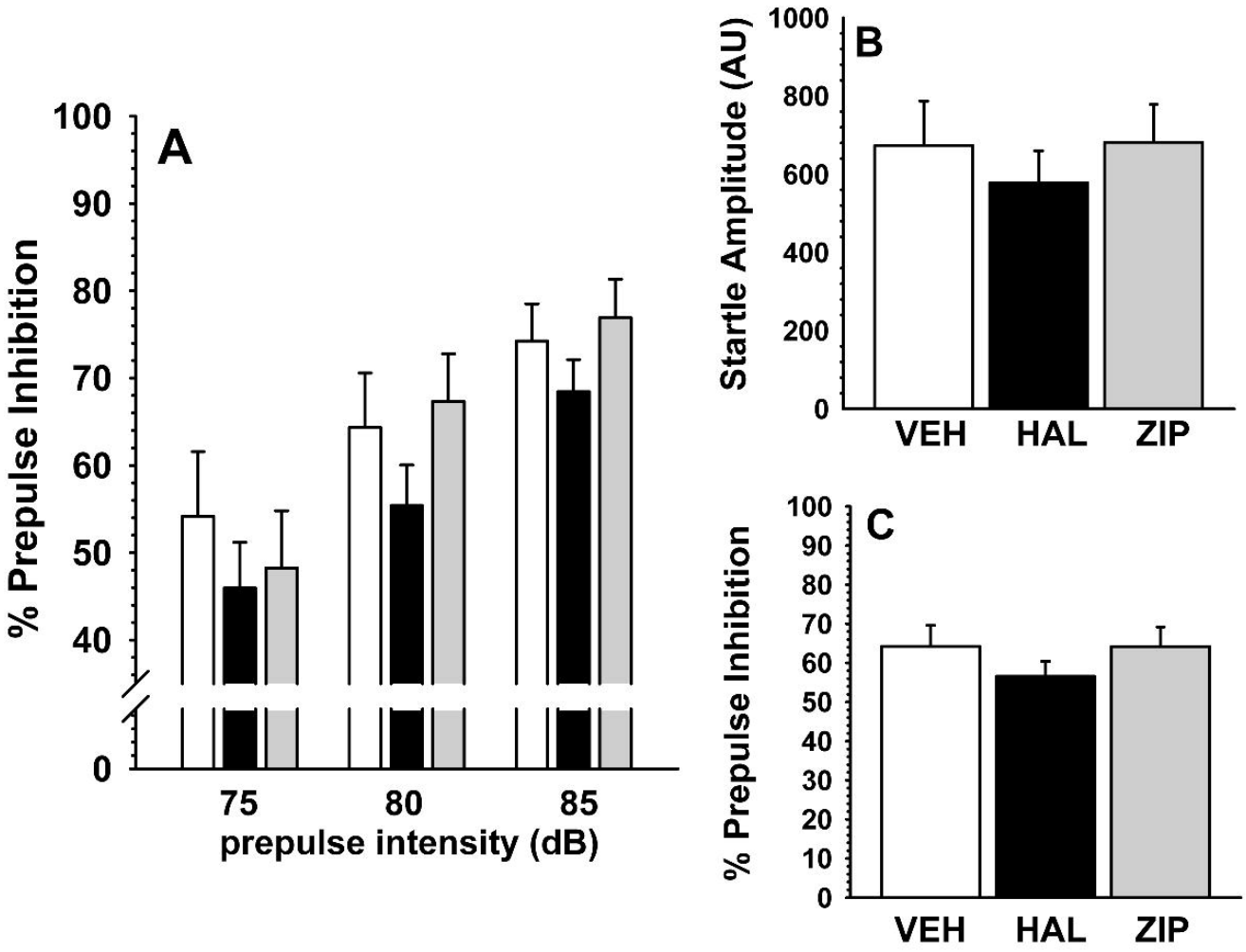

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Fig 7

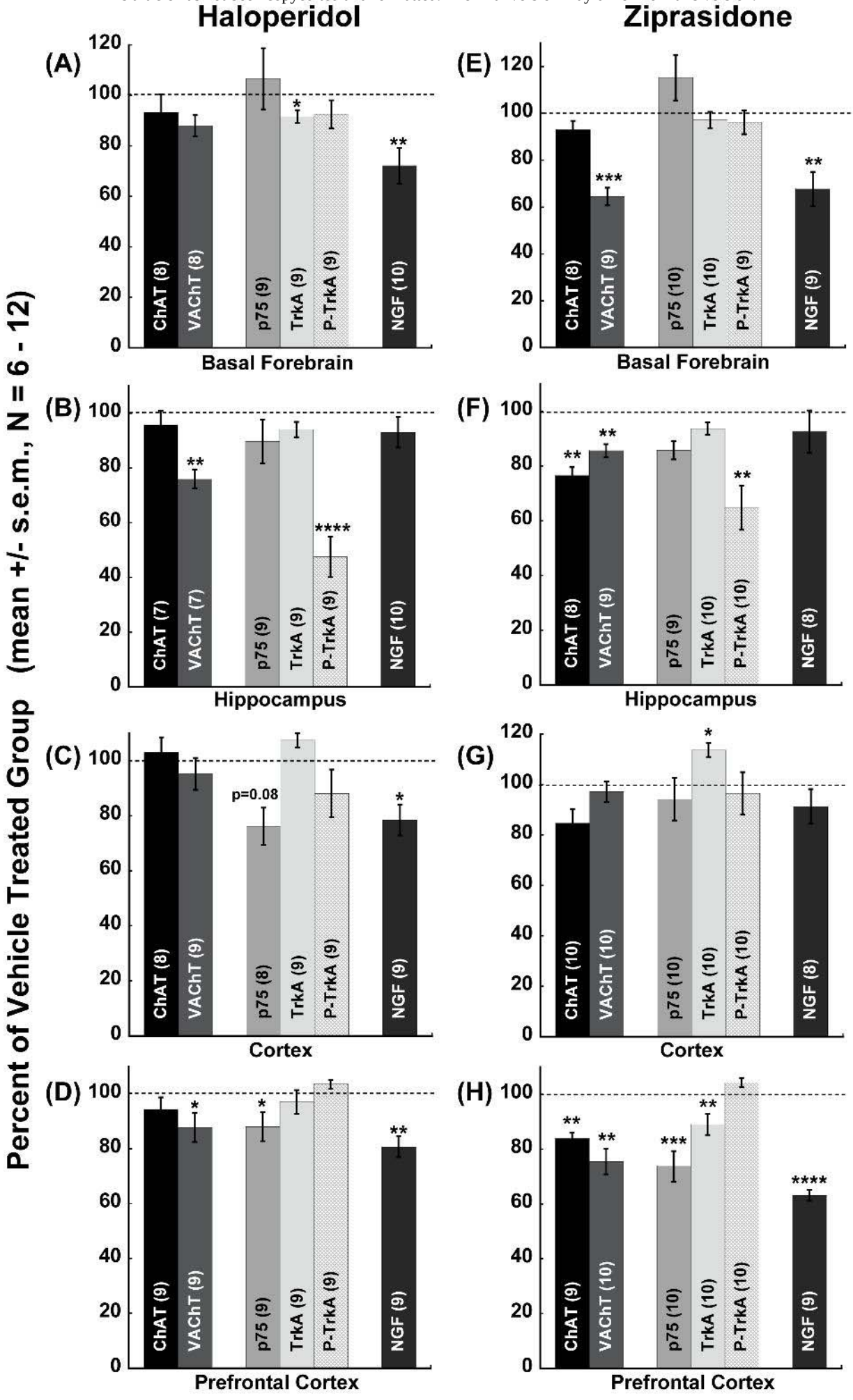

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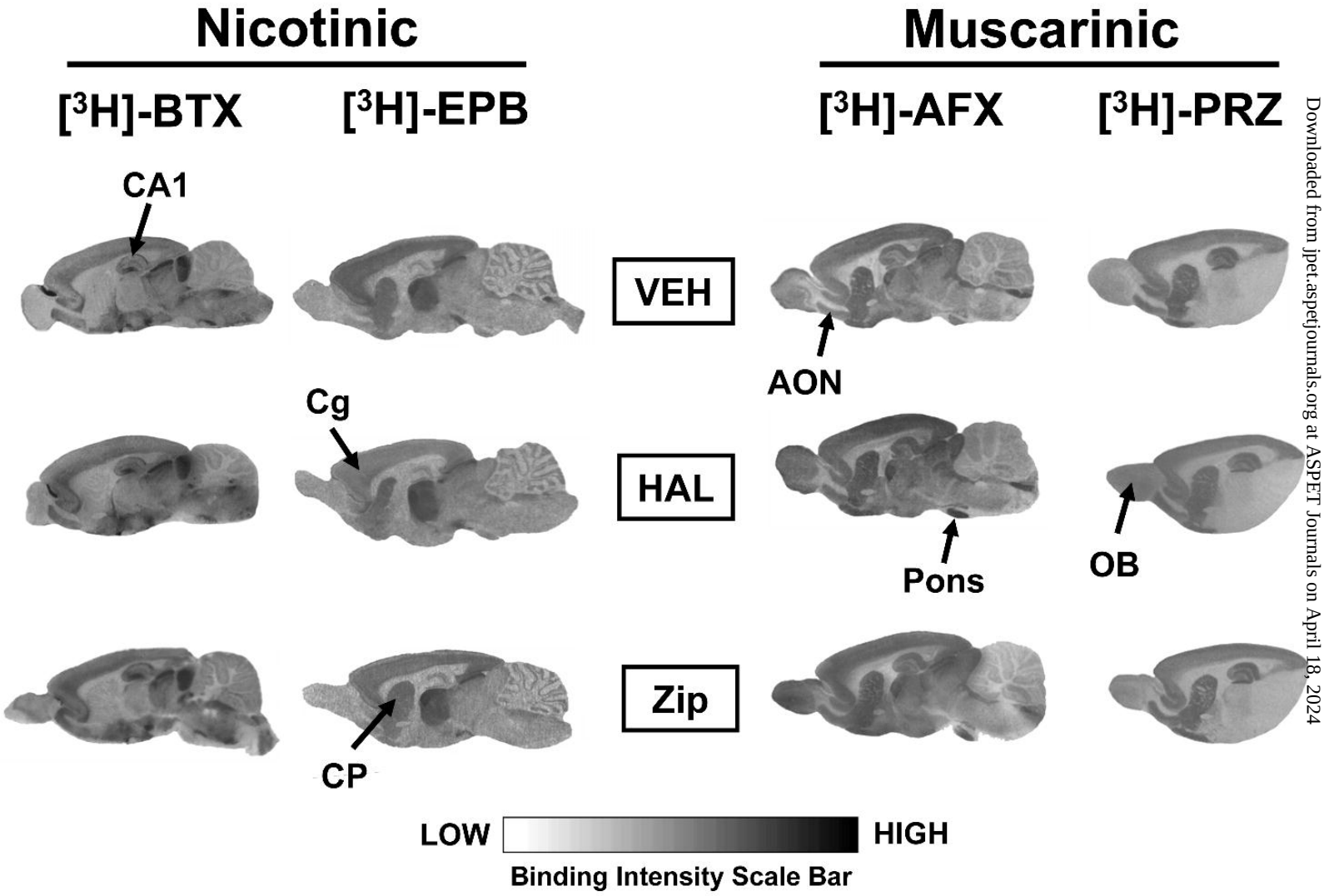
Fig 8



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