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Protracted Effects of Chronic Oral Haloperidol and Risperidone on Nerve Growth Factor, Cholinergic Neurons, and Spatial Reference Learning in Rats

Alvin V. Terry Jr^{1,2,3}, Debra A. Gearhart¹, Samantha Warner², Elizabeth J. Hohnadel³, Mary-Louise Middlemore³, Guodong Zhang⁴, Michael G. Bartlett⁴, and Sahebarao P. Mahadik^{5,6}

¹Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia, 30912

²Small Animal Behavior Core, Medical College of Georgia, Augusta, Georgia, 30912

³Program in Clinical and Experimental Therapeutics, University of Georgia College of Pharmacy, Augusta, Georgia, 30912

⁴Department of Pharmaceutical and Biomedical Sciences, University of Georgia College of Pharmacy, Athens GA 30607

⁵Department of Psychiatry and Health Behavior, Medical College of Georgia, Augusta, Georgia, 30912

⁶Medical Research Service Line, VAMC, Augusta, Georgia, 30904

Abstract

The primary therapeutic agents used for schizophrenia, antipsychotic drugs, ameliorate psychotic symptoms; however, their chronic effects on cognition (or the physiologic processes of the brain that support cognition) are largely unknown. The purpose of this rodent study was to extend our previous work on this subject by investigating persistent effects (i.e., during a 14 day drug-free washout period) of chronic treatment (i.e., ranging from 45 days to six months) with a representative first and second generation antipsychotic. Drug effects on learning and memory and important neurobiological substrates of memory, the neurotrophin, nerve growth factor (NGF) and its receptors, and certain components of the basal forebrain cholinergic system were investigated. Behavioral effects of oral haloperidol (2.0 mg/kg/day), or risperidone (2.5 mg/kg/day) were assessed in an open field, a water maze task, and a radial arm maze procedure and neurochemical effects in brain tissue were subsequently measured by enzyme linked immunosorbant assays (ELISAs). The results indicated that both antipsychotics produced time-dependent and protracted deficits in the performance of a water maze procedure when compared to vehicle-treated controls, while neither drug was associated with significant alterations in radial arm maze performance. Interestingly, haloperidol, but not risperidone, was detectable in the rodent brain in appreciable levels for up to two weeks after drug discontinuation. Both antipsychotics were also associated with reduced levels of NGF protein in the basal forebrain and prefrontal cortex and significant (or nearly significant) decreases in phospho-TrkA protein and the vesicular acetylcholine transporter (depending on the brain region analyzed). Neither antipsychotic markedly affected TrkA or p75^{NTR} levels. These data indicate that chronic treatment with either haloperidol or risperidone may be associated with protracted negative effects

Corresponding Author: Alvin V. Terry Jr., Ph.D., Professor of Pharmacology and Toxicology, CB-3618, Medical College of Georgia, 1120 Fifteenth Street, Augusta, Georgia 30912-2450, Phone 706-721-9462, Fax 706-721-2347, e-mail: aterry@mail.mcg.edu. **Section Editor:** Yoland Smith.

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on cognitive function as well as important neurotrophin and neurotransmitter pathways that support cognition.

Keywords

schizophrenia; memory; cognition; antipsychotic; acetylcholine; neurotrophin; water maze; radial arm maze

Introduction

Cognitive impairments in schizophrenia compromise the acquisition of social skills, success in rehabilitation programs, vocational achievements (reviewed, Green et al., 2000), as well as insight (Rossell et al., 2003), coping strategies (Wilder-Willis et al., 2002), and medication compliance (Donohoe et al., 2001). Therefore, it is imperative that the therapeutic agents most commonly used in schizophrenia (i.e., the antipsychotic drugs) that have optimal effects on cognition be distinguished, particularly when they are administered chronically (i.e., the more relevant clinical issue). There have been a number of reports suggesting that second generation antipsychotics (SGAs) have superior effects on cognitive function when compared to first generation antipsychotics (FGAs) and further, that SGAs actually improve cognitive function in schizophrenia (reviewed, Harvey et al., 2004). However, many of the studies cited to support this assertion were retrospective, open label in design, and/or of short duration (i.e., six months or less). Furthermore, the SGAs were commonly compared to relatively high doses of an FGA such as haloperidol (which has well documented adverse effects on cognition) and almost none of the studies with SGAs were performed in antipsychotic naïve subjects. A recent investigation that did not have these later limitations focused on the effects of risperidone on spatial working memory over a six month period in first episode (antipsychotic naïve) schizophrenia patients (Reilly et al., 2006). The conclusion of this study was that risperidone exacerbated working memory deficits in the study subjects.

The preponderance of the data from animal experiments conducted to date suggests that SGAs (similar to FGAs) are either inactive, or that they actually exert negative effects on memory-related task performance (see review, Terry and Mahadik 2007). In chronic antipsychotic studies (which are most relevant to the situation of human therapeutics), haloperidol, clozapine, and risperidone, impaired acquisition in an eight arm radial arm maze task in rats while olanzapine had no effect (Rosengarten and Quartermain, 2002). Chronic haloperidol has also been found to disrupt working memory in radial arm maze studies in rats in other laboratories (Levin et al., 1987; Levin 1997). We have observed that several antipsychotics from both drug classes including haloperidol, ziprasidone, and risperidone can impair spatial learning in rats and exert negative effects on the basal forebrain cholinergic system, as well as the neurotrophin that supports this system in adults, nerve growth factor (Terry et al., 2006; Terry et al., 2007). The negative neurochemical effects were time dependent and appeared to correlate with a decline in the performance of memory-related task performance over time. Such observations may have particular significance given the widely accepted role of the basal forebrain cholinergic system in cognitive function (reviewed, Gold 2003) and the growing interest in the role of neurotrophins in schizophrenia, especially their capacity to influence neuroplasticity in adult brain (reviewed Buckley et al., 2007).

The purpose of this study was to further investigate the time-dependent effects of chronic treatment with a representative first and second generation antipsychotic (haloperidol and risperidone, respectively) on memory function (i.e., at time points ranging from 45 days to six months of treatment), and to determine if such effects were protracted (i.e., persistent during a drug free washout period). The washout approach was designed to mimic the clinical scenario

where poor drug compliance often results in periods where patients experience drug-free episodes and, at least theoretically, antipsychotic withdrawal effects could come into play (i.e., an area that has not been investigated to any significant extent to date). Behavioral effects of the antipsychotics were thus assessed in an open field, a water maze task, and a radial arm maze procedure. Subsequently, cholinergic marker proteins, NGF, and its receptors were measured by enzyme linked immunosorbant assays (ELISAs) after 90 days of treatment (the earliest time point associated with deficits in memory-related task performance). The expression and/or the intrinsic activities of protein markers found in basal forebrain cholinergic pathways (e.g., choline acetyltransferase and the vesicular acetylcholine transporter) have been used for decades to ascertain the consequence of disease or injury in these cells. In addition, NGF, through its interactions with high affinity TrkA receptors and low affinity p75 neurotrophin receptors (p75^{NTR}), is important for the maintenance and survival of basal forebrain cholinergic neurons (Li et al., 1995; Auld et al., 2001).

Methods

Test Subjects

Male albino Wistar rats (Harlan Sprague-Dawley, Inc.) 2-3 months old and 9 month old retired breeders (referred to as older rats in Table 1) were housed individually in a temperature controlled room (25°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 libitum* for the first week, but then replaced with solutions that contained neuroleptics for the remainder of the study (see below). Table 1 provides the details for the all study cohorts, the numbers of animals tested per group, and the experiments conducted in each group. For the animals that were used in the radial arm maze task (see below), food intake was restricted to approximately 85% of *ad libitum* consumption beginning one week prior to testing. Additional food was given on weekends and holidays (if necessary) to maintain the weight of each rat at approximately 85% of its freely fed weight. 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 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 on previous rodent studies in our laboratory in which time-dependent behavioral and neurochemical effects were detected (Terry et al., 2005; Terry et al., 2007). In these studies, the plasma drug levels achieved approximated those often associated with antipsychotic effects in humans (Terry et al., 2005; Terry et al., 2007). Moreover, the doses selected (see below) were expected to achieve comparable and (therapeutically) relevant D₂ 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 haloperidol (Sigma-Aldrich, St. Louis, MO), 2.0 mg/kg/day or risperidone (A&A Pharmachem, Ottawa, Ontario Canada), 2.5 mg/kg/day orally in drinking water for periods ranging from 45 to 180 days. The antipsychotics were dissolved in 0.1 M acetic acid and subsequently diluted (1:100) with ultrapure water for daily drug administration in drinking water. Drug dosing was based on the average daily fluid consumption and the weight of the animals. Control rats received the diluted acetic acid solution (0.001 M).

Plasma and Brain Antipsychotic Analysis

Plasma and brain samples were collected from rats (N=6) on day 45 of antipsychotic treatment, and from separate groups (N=6) on day 14 of a drug-free washout after 45 days of previous antipsychotic treatment. Subjects were anesthetized with isoflurane and 3.0 mL of blood was collected via cardiac puncture into a Vacutainer® tube containing potassium EDTA. The blood was centrifuged for 15 min at $2500 \times g$ at 4–5°C and the resulting plasma was frozen at –80°C until analyzed. Brains were removed from the same animals after perfusion with phosphate-buffered saline. Risperidone, 9-hydroxyrisperidone, and haloperidol levels in plasma were determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS) as described in detail previously (Zhang et al., 2007). Brain samples were assayed similarly to plasma; however, the higher lipid content of the brain required the following modifications to the sample preparation: 0.4 mL of phosphate buffer (pH = 10.69) was added to 0.2 ml of brain homogenate and the antipsychotics were subsequently extracted from the homogenate using 2×3 mL of isopropylether. The isopropylether was subsequently evaporated to dryness and the sample was reconstituted in 0.1 ml of mobile phase prior to injection in to the LCMS/MS.

Behavioral Experiments

All behavioral experiments were conducted in rooms equipped with white noise generators (San Diego Instruments, San Diego, CA) set to provide a constant background level of 70 dB and ambient lighting of approximately 25–30 Lux (lumen/m²).

Water Maze Testing—Water maze experiments were conducted as described in detail previously (Terry et al., 2006). Briefly, visible platform tests (4 trials/rat) were conducted on day 6 of the drug-free washout to measure visual acuity using a highly visible (white) cover fitted with a small white flag was attached to the platform. For the hidden platform test (beginning on day 7 of drug washout), rats were given 2 trials per day for 6 consecutive days to locate and climb on to the hidden platform. Probe trials were conducted twenty-four hours following the last hidden platform trial to measure spatial bias for the previous platform location.

Locomotor Activity and the Light-Dark Preference Test: To assess the effects of the test compounds on general locomotor activity and anxiety levels, a Light/Dark Preference Test (also referred to as light/dark exploration or emergence neophobia test) was conducted. In this test (conducted on day 9 of a drug free washout period), we were interested in determining whether the antipsychotics had residual motor effects that might have influenced performance in the memory-related tests. We were also interested to learn whether the antipsychotics 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 × 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 and vertical activity (lower and upper photobeam breaks or counts, respectively), number of stereotypical movements, and the time spent in the light and dark zones of the apparatus. Thus, spontaneous locomotor activity, olfactory activity (rearing and sniffing movements), stereotypical movements, and emergence neophobia were assessed.

Radial Arm Maze (RAM) Testing—RAM testing was conducted in a 12-arm radial maze (made of wood, painted black and sealed with a polyurethane coating) composed of a central octagonal platform (diameter 50 cm) with twelve arms extending radially from it (10 × 70 cm). Each arm contained a food cup 2-cm from the distal end. The maze was positioned approximately 90 cm above the floor in a testing room with a number of extra-maze cues (large geometrical shapes) attached to the wall.

Habituation: During the habituation phase, animals were acquainted with the radial arm maze apparatus, as well as the handling procedures associated with it. The maze contained pieces of food (Froot Loops®) scattered sparsely around the entire area. Animals were allowed to explore the entire maze for 15 min per day for two days (i.e., the last 2 days of the 90 day antipsychotic regimen).

Testing: After habituation (i.e., on day 1 of the drug free washout period) the animals were tested for 14 consecutive days (single daily sessions). For each session, a small piece of food (Froot Loops®) was placed in the hopper located at the distal end of eight of the twelve arms. The baited (and unbaited arms) arms were well kept consistent for individual animals throughout the 14 days of testing, but were different (pseudorandomly) for each animal. A session began after the rodent had been placed in the center of the maze and a circular barrier to the arms of the maze lifted by the experimenter. The session ended when all twelve food-hoppers had been investigated or the session timed out (15 min on Day 1; 5 min on Days 2-14). The number of arm entries was recorded, along with reference memory errors (entry to an arm that was never baited) or working-memory errors (repeat arm entries into any arm).

ELISA Methods

After behavioral testing, a subset of rats (N=7-12) from the 90 day treatment groups (i.e., after the 14 day drug-free washout period) was 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. Brain dissections, preparation of brain lysates, and ELISA methods are detailed in Gearhart *et al.* (2006). Briefly, the basal forebrain, hippocampal formation, cortex, and prefrontal cortex were dissected, homogenized in RIPA buffer (supplemented with protease inhibitors, phosphatase inhibitors and glycerol), and stored at -20 °C until analyzed. The Micro BCA™ Protein Assay Kit (Pierce Biotechnology; Rockford, IL) was used to determine the total protein concentration in brain lysates. Nerve growth factor (NGF) was measured in brain lysates (no acid treatment) using the NGF Emax® ImmunoAssay System (Promega, Madison WI) according to the kit instructions. ELISA methods were used to measure the following proteins (Gearhart *et al.*, 2006): vesicular acetylcholine transporter (VACHT); choline acetyltransferase (ChAT), p75 neurotrophin receptor (p75^{NTR}); TrkA (NGF receptor), and the phosphorylated-TrkA receptor (phospho-TrkA). The amount of protein analyzed per well was: *VACHT ELISA* – basal forebrain and hippocampus (1.0 µg), cortex (0.5 µg), prefrontal cortex (0.4 µg); *ChAT ELISA* – all regions (1.0 µg); *p75^{NTR} ELISA* – basal forebrain (0.8 µg), hippocampus, cortex, prefrontal cortex (0.4 µg); *TrkA ELISA* – all regions (0.4 µg); *phospho-TrkA ELISA* – all regions (70 µg). Brain lysates from vehicle-, haloperidol-, and risperidone-treated rats were assayed at the same time on the same ELISA plate, as an internal control for day-to-day variation in each ELISA.

Statistical Analyses

All statistical analyses were performed using SigmaStat 2.03 (SPSS Inc., Chicago, IL). A one or two-way analysis of variance (with repeated measures when indicated) was used for all treatment comparisons. A Student Newman Keuls multiple comparison procedure was used to

examine post hoc differences when indicated. Statistical significance was assessed using an alpha level of 0.05

Results

Plasma Antipsychotic Levels

Plasma and brain antipsychotic levels assessed on the last day of a 45 day oral administration period, and after a 14 day drug-free washout are provided in Table 2. Data on risperidone and the active 9-hydroxyrisperidone (9-OH-RISP) metabolite are provided since the combination of these two compounds is considered the most clinically relevant plasma measurement (Megens et al., 1994). Plasma drug levels were within the estimated therapeutic range for haloperidol (4-20 ng/ml) and the risperidone, 9-hydroxyrisperidone combination. (20-60 ng/ml) previously suggested (Baldessarini et al., 1988; Markowitz and Patrick, 1996; Coryell et al., 1998; Balant-Gorgia et al., 1999). Brain levels of haloperidol were markedly higher than plasma haloperidol levels (i.e., by over 28 fold) and they were also much higher than risperidone and the risperidone, 9-hydroxyrisperidone combination in the brain. After the 14 day washout, neither haloperidol nor risperidone (nor 9-hydroxyrisperidone) was detectable in plasma. Conversely, haloperidol was clearly detectable in brain tissue after the washout period while risperidone levels were minimal, i.e., above the method's limit of detection (0.05 ng/g), but below the validated limit of quantitation (0.2 ng/g). Therefore, the trace levels of risperidone should be regarded as approximate values.

Water Maze Testing

Visible Platform Test—This test was used to insure that the test subjects were not grossly impaired visually, and that they did not exhibit other (non-mnemonic) behaviors such as thigmotaxis that might have confounded the analyses. The averages over the 4 trials for the various treatment groups ranged from approximately 30 to 50 seconds. There were no significant treatment-related effects observed in this procedure (i.e., all p values for the comparisons were >0.05).

Swim Speeds—Swim speeds were also analyzed in an effort to further investigate treatment related differences in task performance. Average swim speeds ranged between 24-30 cm/sec across the groups for the 6 days of hidden platform testing. There were no significant treatment-related effects observed on swim speeds (i.e., all p values for the comparisons were >0.05).

Hidden Platform Test—Fig 1 illustrates the efficiency of each experimental group to locate a hidden platform in a water maze task on 6 consecutive days of testing during the drug-free washout period associated with the various treatment periods. Both the acquisition curves for swim distance (top of the figure), and the area under the curve for swim distances (bottom of figure) are depicted. Vehicle-treated rats progressively learned to locate the hidden platform with increasing levels of efficiency over the course of the 6 days as indicated by the decreasing slope of the acquisition curves. There was a highly significant treatment effect $F_{8,97} = 6.1$, $p < 0.001$, a significant day effect $F_{5,485} = 25.0$, $p < 0.001$, without a significant treatment $\times$ day interaction $F_{40,485} = 1.2$, $p = 0.18$. 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 haloperidol and risperidone animals after the 90 and 180 day (but not the 45 day) treatment periods. Post hoc analyses also indicated there were several individual days during testing (associated with the 90 and 180 day) treatment periods where there were significant impairments in the ability to locate the hidden platform ($p < 0.05$) as indicated by the longer swim distances.

In order to further assess the effects of the length of time of drug administration across the studies, the area under the distance learning curve for each animal was calculated and then group performances were compared statistically (two-way ANOVA for effects of treatment and time of administration). Comparing area under the curve (AUC) in water maze studies to provide a more simple comparison between groups has been previously published (Terry et al., 2007). In this analysis all factors were statistically significant: treatment ($p < 0.001$), time ($p = 0.02$), and treatment $\times$ time interaction ($p < 0.001$). Thus, as shown in the bottom half of Fig 1, there were no detectable antipsychotic effects on AUC at the 45 day time period; however, by 90 days, haloperidol and risperidone treatment was associated with impairments that were also present (or a significant trend toward impairment was observed) at the 180 day time period.

A concern with the water maze results described in the paragraph above was the possibility that an interaction between age and drug treatment could be a factor particularly at the 180 day assessment (since the age of the subjects had increased by 6 months by this time). To address this issue we conducted a separate set of experiments with retired breeder rats (i.e., 9 month, middle aged rats). We treated them with vehicle, haloperidol, or risperidone for 45 days, and then tested them in the water maze during the 14 day washout. The results (illustrated in the insets to Fig 1) clearly indicate that middle-aged rats were not more sensitive to these antipsychotics (the p values for all comparisons were > 0.05).

Probe Trials: Fig 2 illustrates the performance of probe trials by the various treatment groups after the different durations of antipsychotic treatment. There were statistically significant (treatment and time-related) effects on performance as indicated by the number of crossing over the previous 10 cm $\times$ 10 cm target area (treatment effect, $p < 0.02$; time effect, $p < 0.001$; treatment $\times$ time interaction, $p < 0.1$). Post hoc analysis indicated that performance was superior in the vehicle-treated animals compared to HAL-treated and RISP treated animals ($p < 0.01$ for both comparisons) at the 90 day time point. A separate analysis indicated that the older rats were less efficient than younger rats independent of treatment (age effect, $p < 0.001$), see Fig 2 inset.

Radial Arm Maze: Fig 3A and 3B illustrates the lack of residual effects of the antipsychotics on acquisition in a 12-arm radial arm maze task over 14 days of consecutive testing (Win-Shift Task). In the analysis of reference memory errors (Fig 3A) there was not a significant difference between the treatment groups, treatment effect, $F_{2,33} = 2.4$, $p = 0.11$; there was a highly significant effect of the day of testing, day effect $F_{13,401} = 7.9$, $p < 0.001$ (indicating that significant learning occurred over the 14 days of testing), and the treatment $\times$ day interaction was not significant $F_{26,401} = 1.3$, $p = 0.18$. A similar result was observed when working memory errors were analyzed (Fig 3B), treatment effect, $F_{2,33} = 0.30$, $p = 0.75$; day effect $F_{13,398} = 4.6$, $p < 0.001$; treatment $\times$ day interaction, $F_{26,398} = 0.79$, $p = 0.77$.

Assessments of Open Field Locomotor Activity and Motor Function

In these experiments we were interested in determining whether the antipsychotics had significant effects on motor function that might have influenced performance in the memory-related tests. Fig 4 illustrates the effects of the antipsychotic treatments on vertical and horizontal locomotor activity, stereotypical movements, as well as fear/anxiety-related behaviors (i.e., time spent in the lighted zone of the test apparatus). Activity data for animals tested on day 9 of the 14 day washout after 90 or 180 days of antipsychotic treatment, respectively, are illustrated. There were no statistically significant effects observed in this analysis associated with 90 days of prior antipsychotic treatment, however, there were several notable findings in the animals previously treated with vehicle or antipsychotic for 180 days. Vertical and horizontal locomotor activity, as well as stereotypical movement was reduced in the 180 day vehicle-treated animals compared to the 90 day vehicle treated animals, indicative

of an age-associated effect. Further, compared to vehicle-controls, 180 days of haloperidol treatment was associated with increased vertical and horizontal locomotor activity, as well as increased stereotypical movements, while risperidone was associated with increased vertical activity. Finally, 180 days of risperidone treatment was associated with a marked increase in the time spent in the lighted portion of test apparatus (i.e., compared to 180-vehicle controls) that was not evident at the 90 day time point (indicating a time dependent anxiolytic effect).

ELISA Experiments

Figs 5-7 depict ELISA results for ChAT, VAcHT, p75^{NTR}, NGF, TrkA, and phospho-TrkA, in memory-associated brain regions from rats 14 days after treatment with vehicle, haloperidol, or risperidone for 90 days.

VAcHT and ChAT—VAcHT protein was significantly decreased in the basal forebrain (i.e., by ~25%) and the prefrontal cortex (i.e., by ~10%) in animals previously treated with risperidone (compared to vehicle control animals), while haloperidol was only associated with a modest decrease (~12%) in the prefrontal cortex (Fig 5A). Neither antipsychotic was associated with significant changes in ChAT protein in any of the 4 brain regions analyzed (Fig 5B).

p75^{NTR} and NGF—The only significant antipsychotic-related effect on the p75^{NTR} was a modest (~10%) decrease in the prefrontal cortex associated with haloperidol (Fig 6A). There were several notable findings associated with antipsychotic treatment on NGF levels, however (Fig 6B). In haloperidol-treated animals, NGF levels were diminished (relative to vehicle controls) in the basal forebrain, cortex and prefrontal cortex. Risperidone had a similar effect on NGF in these same three brain regions; in fact, NGF was reduced by approximately 50% in the basal forebrain.

TrkA, and phospho-TrkA: In the analysis of TrkA (Fig 7A) and phospho-TrkA (Fig 7B) protein levels, there was only one notable finding, a significant decrease in phospho-TrkA in the hippocampus in animals previously treated with haloperidol (i.e., by ~42%) and a nearly significant decrease in animals previously treated with risperidone ($p < 0.1$).

Discussion

Some years ago we observed that the antipsychotics haloperidol and olanzapine had little effect on spatial learning after 45 days of chronic treatment, whereas 90 days of treatment was associated with substantial and persistent negative behavioral effects (Terry et al., 2002; Terry et al., 2003). Further, there was a decrease in NGF and ChAT immunostaining in memory-related brain regions in rats treated with haloperidol (cortex, hippocampus) that was more pronounced at the 90 day time point. In a more recent study, we observed similar (negative) effects on spatial learning, NGF, and ChAT levels after 90 days of treatment with ziprasidone (Terry et al., 2006). These results lead to the hypothesis that both representative FGAs and SGAs are capable of exerting temporally dependent (and protracted) impairments in spatial learning as well as alterations in neurotrophin and cholinergic function in mammalian brain. In the present study, a longer period of continuous treatment (180 days) was evaluated and a longer drug free washout period was utilized (two weeks) in an effort to learn more about the temporal dependence of the antipsychotic effects as well as the persistence of these effects. We chose a commonly used water maze procedure for rodents (see Morris 1984) as one of the behavioral tasks because it is sensitive to cholinergic alterations (McNamara and Skelton 1993) in the brain (i.e., an important factor in these studies) and since it allows for an assessment of information encoding and retrieval capacity in rodents, capacities that are commonly impaired in schizophrenia (Ragland et al., 2001; Cairo et al., 2006).

As described above in the results, rats previously treated with haloperidol or risperidone were not impaired in water maze hidden platform tests or probe trials (i.e., spatial learning/acquisition and retention) after 45 days of treatment, whereas they were significantly impaired at the 90 and 180 day time points in the hidden platform test (and at the 90 day time point in probe trials). The absence of protracted haloperidol or risperidone-related effects on swim speeds or visible platform tests, argues that gross drug effects on locomotor activity or visual acuity were unlikely to have explained the deficits in water maze performance. Further, the experiments conducted in middle-aged rats administered antipsychotics for 45 days, support the argument that the apparent time-dependent deficits in water maze performance were not simply due to increased drug sensitivity within age.

Interestingly, there were some decreases in locomotor activity in vehicle-treated animals with age, however, horizontal activity was actually increased in the risperidone and haloperidol treated animals. In addition, there was a clear anxiolytic effect of risperidone at the 180 day time point (i.e., as indicated by the increase time in the lighted portion of the chamber in the light-dark box experiments). Again, such effects would not be expected to underlie deficits in water maze performance. It should be noted that risperidone has been observed to have anxiolytic effects in other rodent studies (Nowakowska et al., 1999).

The next series of experiments was designed to determine antipsychotic effects on spatial working (as well as reference) memory in a radial arm maze test. Given that impairments in water maze task acquisition were evident by 90 days of drug treatment in the studies described above, the 90-day time period of drug administration was selected for these studies. Performance of the radial arm maze (RAM) test in rodents relies heavily on “spatial working memory” (Olton and Papas, 1979, Levin et al., 1996) which is commonly disrupted in schizophrenic patients across a variety of test procedures (Keefe et al., 1995; Park et al., 1999). Performance of the rodent task is thought to reflect normal foraging strategies used in their natural environments (and is thus ethologically relevant); however, in contrast to the water maze, the RAM is a positive reinforcing (i.e., appetitively motivated) paradigm. As in the case of the water maze task, successful performance of the RAM procedure is known to depend on intact cholinergic function (reviewed, Decker and McGaugh, 1991). In the present study, RAM testing (beginning on day 1 of the drug free washout) was conducted in a 12-arm radial maze for 14 consecutive days (single daily sessions) that was set up so that working and reference memory errors could be assessed (i.e., in a win-shift method with 8 arms baited and 4 arms never baited). Test results indicated that there were no significant effects of the antipsychotic drugs on working or reference memory errors in any portion of the study. Therefore, as opposed to protracted negative effects on spatial reference learning, prior chronic treatment with neither haloperidol nor risperidone appears to significantly affect performance of an appetitively-motivated spatial working memory task.

The basis of the differential effects in the water maze and RAM is unclear, although disparate water maze and RAM results have been encountered in other, previously published studies (e.g., in rats exposed to ischemic brain injury). In fact, the results (reviewed in Hodges, 1996) were in many ways similar to ours (impaired water maze but not RAM performance). In the Hodges review, a number of factors were discussed regarding potential explanations for the performance differences in the tasks including the free versus restrained search, differential availability and use of intra and extramaze cues, motivational issues (aversive search versus food reinforcement), and thus ostensibly similar spatial tasks may in fact be looking at different behavioral processes. It is also important to reiterate that the animals tested in the water maze had seven days of drug-free washout before the initiation of testing, whereas for the radial arm maze, testing began on the following day after drug termination. This was done (i.e., after 14 days of consecutive RAM testing) so that we could sacrifice the animals at the same time point after the last drug exposure as the water maze subjects. Therefore it may be possible that a

carry over (i.e., residual) effect of the antipsychotics in the RAM studies could have prevented the deficits in task acquisition that were observed in the water maze studies.

The potential mechanism of the negative behavioral effects of haloperidol and risperidone in water maze experiments was the focus of subsequent neurochemical studies. We sought to determine whether the protracted behavioral deficits could be related to alterations in the expression of proteins that have important roles in cholinergic function. Interestingly, several lines of evidence suggest that cholinergic deficits in the brains of schizophrenic patients may contribute to the cognitive dysfunction as well as other symptoms of the illness. In fact, early observations led to the suggestion that alterations in the cholinergic–dopaminergic balance in the CNS might play a key role in the pathophysiology of schizophrenia (Tandon and Greden, 1989). A considerable amount of experimental evidence to support a significant role of cholinergic abnormalities in schizophrenia has accrued since these earlier studies (reviewed Terry and Mahadik, 2007). However, the extent to which antipsychotic drugs contribute to these abnormalities over time is unknown (i.e., an important question given that the brains of antipsychotic naïve schizophrenic patients have rarely been analyzed).

In the present study we measured the levels of proteins (i.e., VACHT and ChAT) that are commonly assessed as cholinergic markers, since they are only expressed by neurons that release acetylcholine (Arvidsson, et al., 1997; Wu and Hersh, 1994). We also assessed antipsychotic effects on the neurotrophin NGF (which is known to support adult cholinergic neurons) and its receptors, the high affinity TrkA receptor, its activated form, phospho-TrkA, as well as the low affinity neurotrophin receptor p75^{NTR} (p75). NGF binding to TrkA promotes TrkA autophosphorylation which activates pathways that enhance cholinergic neuron survival, while NGF signaling via p75^{NTR} typically activates pathways leading to cell death (see reviews, Sofroniew et al., 2001; Counts and Mufson 2005).

The decreases in VACHT observed in the prefrontal cortex associated with haloperidol and risperidone in this study are consistent with the protracted impairment in spatial learning, although we were surprised that there were no antipsychotic effects on VACHT in the hippocampus (a structure generally considered more critical to water maze performance). Cholinergic function in prefrontal cortex (specifically medial prefrontal cortex) has been suggested to be important for egocentric but not allocentric spatial memory in water maze tasks (Nieto-Escámez et al., 2002). The basis for the differential effects of the antipsychotics on ChAT versus VACHT was perplexing; however, we have encountered similar results in a previous study (Terry et al., 2007). As noted in that study, VACHT and ChAT share a common gene locus as well as regulatory elements for gene transcription (reviewed, Eiden, 1988), however, there appears to be a variety of factors that could influence the expression of these proteins (i.e., factors that could at least theoretically be influenced by drug treatments). For example, Schutz et al., 2001 suggested that separate transcriptional start sites within the cholinergic gene locus control VACHT and ChAT transcription, while additional elements are responsible for the specific transcriptional control of the entire locus in cholinergic versus non-cholinergic neurons. Furthermore, earlier neurodevelopmental studies (Holler et al., 1996) and more recent culture studies (Castell et al., 2002) suggest the possibility that VACHT and ChAT are independently regulated either through separate mechanisms that control the activity of specific promoters, or through posttranscriptional mechanisms.

Regarding the antipsychotic effects on NGF and NGF-responsive receptors, the most notable finding was that both haloperidol and risperidone were associated with significant deficits in NGF levels, depending on the brain region analyzed. NGF levels were particularly low in the basal forebrain after treatment with these antipsychotics. While there were other areas in which significant alterations were observed (e.g., a decrease in phospho-TrkA in the hippocampus, and a decrease in p75^{NTR} in the prefrontal cortex) the region-specific effects (i.e., without a

commensurate decrease in cholinergic markers in the same brain regions) were a bit difficult to interpret. It should be noted that p75^{NTR} and TrkA receptors are not exclusively expressed on cholinergic neurons (Vega et al., 2003; Gentry et al., 2004); therefore, contributions from non-cholinergic cell types may confound the interpretations of ELISA data for TrkA, phospho-TrkA, and p75^{NTR}.

Finally, analysis of the plasma and brain antipsychotic levels from the test subjects treated for 45 days, and the approximately 28-fold higher brain to plasma ratio detected for haloperidol, clearly indicates that this antipsychotic sequesters in rodent brain. Interestingly (and in agreement with our results), haloperidol concentrations were also found to be 10-30 times higher than optimum serum levels in postmortem human brain from psychiatric patients who were treated with therapeutic doses of haloperidol (Kornhuber et al., 1999). Furthermore, our results in rats given a 14 day drug-free washout (i.e., after 45 days of continuous oral treatment) indicated that haloperidol persists in rodent brain for many days after it has been withdrawn. This finding is in general agreement with Cohen and colleagues (Cohen et al., 1988; Cohen et al., 1992) who reported a terminal half-life of haloperidol in rodent brain tissue of 6.6-16.7 days. It is unclear at present what effect the residual levels of haloperidol had on behavioral testing in our study since risperidone was clearly associated with water maze performance deficits even though only trace levels of the compound remained in the brain. This may indicate that risperidone treatment can lead to untoward withdrawal effects when it is discontinued after chronic treatment (a potentially important issue given the treatment compliance challenges in psychiatric patients).

The results of this study can thus be summarized as follows: 1) prior chronic exposure to haloperidol or risperidone was associated with time-dependent decrements in the acquisition of a spatial reference learning (water maze) procedure; 2) middle-aged rats were not more sensitive than young rats to treatment with haloperidol or risperidone in this task; 3) prior chronic exposure to haloperidol or risperidone was not associated with impairments in the acquisition of a spatial working memory task, the radial Arm maze; 4) there was no evidence that gross impairments in locomotor activity, visual acuity, or elevated anxiety levels contributed to the impairments in water maze performance; 5) both haloperidol and risperidone were associated with alterations in the neurotrophin, NGF, and its activated receptor phospho-TrkA as well as the vesicular acetylcholine transporter (depending on the brain region analyzed); 6) haloperidol remained in the rodent brain in appreciable levels for up to two weeks after drug discontinuation.

In conclusion, these data indicate that chronic treatment with commonly used first and second generation antipsychotics may be associated with protracted, negative effects on spatial learning and recall, as well as on important neurobiological substrates of cognition including the key cholinergic protein, VACHT, and the neurotrophin, NGF (and its receptors). Additional experiments will be necessary to further delineate important differences in the available antipsychotic agents to identify optimal therapeutic compounds.

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Abbreviations

ChAT	choline acetyltransferase
CON	vehicle control

HAL	haloperidol
RISP	risperidone
NGF	Nerve Growth Factor
TrkA	tropomyosin-receptor kinase A
phospho-TrkA	phosphorylated TrkA
p75^{NTR}	p75 neurotrophin receptor
VACht	vesicular acetylcholine transporter

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Fig 1.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on the performance of a water maze hidden platform procedure in young rats. Testing began 7 days after the last day of a 45, 90, or 180 day antipsychotic treatment period. Top row: acquisition curves, each point represents the mean swim distance to the hidden platform ($\pm$ S.E.M) of 2 trials/day over 6 consecutive days of testing. Bottom row: area under the distance curve for acquisition over the six consecutive days of testing. The insets illustrate performance in 9 month old rats treated with antipsychotic or vehicle for 45 days. * significantly different than vehicle controls ($p < 0.05$). † = trend toward significance ($p < 0.1$). N=12 rats/group.

Fig 2.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on the performance of water maze probe trials in young rats. Testing was conducted on the fourteenth day after the last day of a 45, 90, or 180 day antipsychotic treatment period. The number of crossings over the previous (10 × 10 cm) platform area (mean ± S.E.M.) are depicted. The inset illustrates performance in 9 month old rats treated with antipsychotic or vehicle for 45 days. * significantly different than vehicle controls (p < 0.05). N = 12 rats/group.

Fig 3.

Lack of protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on win-shift acquisition in a 12-arm radial arm maze over 14 consecutive days of testing. **A.** reference memory errors (entries into arms that were never baited) **B.** working memory errors (repeat entries into arms that were previously baited in the same session). N=12 rats per group

Fig 4.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on locomotor activity and the light-dark preference test. Top to Bottom: activity data for animals tested on day 9 of a 14 day washout after 90 or 180 days of antipsychotic treatment, respectively Left to right: vertical activity measured as the mean number of photobeam breaks/ 5 min; horizontal activity measured as the mean number of photobeam breaks/5 min; stereotypical movements (repetitive photobeam breaks/ 5 min); fear/anxiety related behavior (emergence neophobia) measured as the time spent in a brightly lit zone of the activity monitor. Bars represent the mean $\pm$ S.E.M. N=12 rats/group. * significantly different ($p < 0.05$) than vehicle controls; + significant difference between the haloperidol and risperidone-related response; # significant effect of the length of time (i.e., 180 versus 90 days) with the same treatment.

Fig 5.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on levels of **TOP:** the vesicular acetylcholine transporter (VACHT) and **Bottom:** choline acetyltransferase (ChAT) measured by indirect ELISA. For each ELISA, samples from one brain region for each treatment group were analyzed in the same 96-well ELISA plate, and equal amounts of total protein were analyzed across treatment groups. Data are expressed as relative levels (absorbance at 450 nm). Significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$. $N = 7-12$.

Fig 6.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on levels of **TOP:** the low affinity neurotrophin receptor, p75^{NTR} and **Bottom:** nerve growth factor, measured by ELISA. For each p75^{NTR} ELISA, samples from one brain region for each treatment group were analyzed in the same 96-well ELISA plate, and equal amounts of total protein were analyzed across treatment groups. Data for p75^{NTR} are expressed as relative levels (absorbance at 450 nm). Significant differences are indicated as follows: *p<0.05; **p<0.01, ***p<0.001. N=7-12.

Fig 7.

Protracted effects of chronic treatment with haloperidol or risperidone (compared to vehicle controls) on levels of the NGF receptors, **TOP:** TrkA and **Bottom:** phosphorylated-TrkA (p-TrkA), measured by indirect and sandwich ELISA, respectively. For each ELISA, samples from one brain region for each treatment group were analyzed in the same 96-well ELISA plate, and equal amounts of total protein were analyzed across treatment groups. Data are expressed as relative levels (absorbance at 450 nm). Significant differences are indicated as follows: **p<0.01; † = trend toward significance (p<0.1).

N=7-12.

Table 1

Rat Testing Protocol

Cohort	Group	N	Treatment	Days of Drug Exposure-Procedures Conducted	90	180
1	A	6	HAL	SAC-Plasma & Brain Analysis (LCMS)		
	B	6	RISP	SAC-Plasma & Brain Analysis (LCMS)		
	C	6	VEH	Washout-WM-SAC		
2	D	6	HAL	Washout-WM-SAC-Plasma & Brain Analysis (LCMS)		
	E	6	RISP	Washout-WM-SAC-Plasma & Brain Analysis (LCMS)		
	F	6	VEH	Washout-WM-SAC		
	G	6	HAL	Washout-WM-SAC		
4	H	6	RISP	Washout-WM-SAC		
	I	12	VEH	Washout-WM-SAC-Older Rats		
	J	12	HAL	Washout-WM-SAC-Older Rats		
5	K	12	RISP	Washout-WM-SAC-Older Rats		
	L	12	VEH		Washout-WM-Motor-Anxiety-SAC-ELISA	
	M	12	HAL		Washout-WM-Motor-Anxiety-SAC-ELISA	
	N	12	RISP		Washout-WM-Motor-Anxiety-SAC-ELISA	
6	O	12	VEH			Washout-WM-Motor-Anxiety-SAC
	P	12	HAL			Washout-WM-Motor-Anxiety-SAC
7	Q	12	RISP			Washout-WM-Motor-Anxiety-SAC
	R	12	VEH			Washout-WM-Motor-Anxiety-SAC
T	S	12	HAL		RAM-SAC-ELISA	
		12	HAL		RAM-SAC-ELISA	
	T	12	RISP		RAM-SAC-ELISA	

VEH = vehicle; HAL = haloperidol; RISP = risperidone; WM = water maze; RAM = radial arm, maze; Motor-Anxiety = horizontal activity, vertical activity, stereotypical movements, light-dark box test; SAC = sacrifice; LCMS = liquid chromatography mass spectrometry; ELISA = enzyme-linked immunosorbent assays.

Table 2

Plasma and Brain Antipsychotic Levels (45 Day Time Point)

Cohort	Antipsychotic	Dose/24 hr	Compound Measured	Plasma Levels (ng/ml) ± S.E.M.	Brain Levels (ng/g) ± S.E.M.
1	haloperidol	2.0 mg/kg	haloperidol	19.7 ± 3.8	562.0 ± 116.1
	risperidone	2.5 mg/kg	risperidone	10.9 ± 5.7	5.2 ± 1.8
			9-OH-risperidone	31.0 ± 10.2	4.9 ± 0.9
2	haloperidol + washout	2.0 mg/kg	haloperidol	ND	3.0 ± 0.3
	risperidone + washout	2.5 mg/kg	risperidone	ND	0.1 ± 0.1
			9-OH-risperidone	ND	ND

ND = not detected; N=6 for all groups