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Oral Haloperidol or Risperidone Treatment in Rats: Temporal Effects on Nerve Growth Factor Receptors, Cholinergic Neurons, and Memory Performance

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Abstract

First and second generation antipsychotics (FGAs and SGAs) ameliorate psychotic symptoms of schizophrenia, however, their chronic effects on information processing and memory function (i.e., key determinants of long term functional outcome) are largely unknown. In this rodent study the effects of different time periods (ranging from two weeks to six months) of oral treatment with the FGA, haloperidol (2.0 mg/kg/day), or the SGA, risperidone (2.5 mg/kg/day) on a water maze repeated acquisition procedure, the levels of nerve growth factor receptors, and two important cholinergic proteins, the vesicular acetylcholine transporter and the high affinity choline transporter were evaluated. The effects of the antipsychotics on a spontaneous novel object recognition procedure were also assessed during days 8-14 and 31-38 of treatment. Haloperidol (but not risperidone) was associated with impairments in water maze hidden platform trial performance at each of the time periods evaluated up to 45 days, but not when tested during days 83-90. In contrast, risperidone did not impair water maze task performance at the early time periods and it was actually associated with improved performance during the 83-90 day period. Both antipsychotics, however, were associated with significant water maze impairments during the 174-180 day period. Further, haloperidol was associated with decrements in short delay performance in the spontaneous novel object recognition task during both the 8-14 and 31-38 periods of treatment, while risperidone was associated with short delay impairment during the 31-38 day time period. Both antipsychotics were also associated with time dependent alterations in the vesicular acetylcholine transporter, the high affinity choline

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transporter, as well as TrkA, and p75 neurotrophin receptors in specific brain regions. These data support the notion that while risperidone may hold some advantages over haloperidol, both antipsychotics can produce time-dependent alterations in neurotrophin receptors and cholinergic proteins as well as impairments in the performance of tasks designed to assess spatial learning and episodic memory.

Keywords

schizophrenia, memory, cognition, antipsychotic; acetylcholine; growth factor

Introduction

There is considerable evidence to support the argument that second generation antipsychotics (SGAs) generally have a more benign side effect profile when compared to first generation antipsychotics (FGAs), particularly when movement-related effects (e.g., Parkinsonian effects, tardive dyskinesia) are considered (reviewed, Miyamoto et al., 2005). However, given the increasing reports of adverse (SGA-related) metabolic effects including weight gain, diabetes mellitus, and lipid abnormalities (reviewed, Gardner et al., 2005), combined with their enormous financial burden, arguments supporting the current widespread use of SGAs in lieu of moderate doses of FGAs may require more extensive experimentation. One argument favoring the preference for SGA over FGAs is based on reports of their superior effects on cognitive function (reviewed, Harvey et al., 2004), an important consideration given the widely recognized significance of cognition to the long-term functional outcome of schizophrenic patients (reviewed, Green et al., 2000). However, the empirical evidence to support this assertion is relatively limited (i.e., most of the cited studies were retrospective, open label in design and/or of short duration). Of the animal experiments conducted to date, many suggest 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 2006). Previous work in our laboratories indicated that while the magnitude of the deficits generally tended to be lower with SGAs, representatives of both antipsychotic classes (when administered chronically) were associated with impairments in some components of cognitive function (e.g., spatial learning). In addition, both FGAs and SGAs were associated with time-dependent alterations in levels of the neurotrophin, nerve growth factor (NGF) and the cholinergic marker protein, choline acetyltransferase (ChAT) in important memory-related brain regions such as the cortex and hippocampus (see Pillali et al., 2006, Terry et al., 2006). 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) and given the well documented importance of these neurons to mnemonic function (see review, Gold 2003), we have developed the hypothesis that some of the unfavorable effects that both FGAs and SGAs have on memory function may be related to time dependent impairments in cholinergic activity due to reduced levels of NGF and/or its receptors.

The purpose of the experiments described in this report was to further investigate such time-dependent (antipsychotic-related) effects. The effects of different time periods (ranging from two weeks to six months) of oral treatment with the archetypal FGA, haloperidol, or the commonly prescribed SGA, risperidone, on repeated acquisition of a water maze (spatial learning) procedure in rats were evaluated. Antipsychotic effects on performance of a (non-spatial) spontaneous novel object recognition task were also evaluated around the 14 day and 30 day time point. The water maze procedure was selected since it requires intact hippocampal function (which is commonly disrupted in schizophrenic patients), several processes that are important to human learning and memory such as information acquisition and encoding, consolidation, retention, and retrieval, and since it is sensitive to cholinergic dysfunction

(McNamara and Skelton, 1993; McDonald and White, 1995). The spontaneous novel object recognition test for rodents (Ennaceur and Delacour 1988), models some components of episodic and recognition memory (i.e., processes that are commonly impaired in schizophrenia, see Touloupoulou et al., 2003; and Aleman, et al., 1999, respectively), requires intact hippocampal function, and it is also sensitive to cholinergic dysfunction (Bartolini et al., 1996). We subsequently evaluated antipsychotic effects on the two plasma membrane receptors for NGF, the high affinity TrkA receptor and its activated form, phospho-TrkA, as well as the low affinity neurotrophin receptor p75^{NTR} (p75). NGF binding to TrkA has been observed to promote 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). Finally, we measured levels of the vesicular acetylcholine transporter (VACHT) and the high affinity choline transporter (CHT), proteins that are commonly assessed as cholinergic markers since they are only expressed by neurons that release acetylcholine (Arvidsson, et al., 1997; Okuda and Haga, 2003).

Methods

Test Subjects

Male albino Wistar rats (Harlan Sprague-Dawley, Inc.) 2-3 months old 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 antipsychotics for the remainder of the study (see below). Table 1 provides the details for all study cohorts, the numbers of animals tested per group, and the behavioral experiments conducted in 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 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 several factors: 1) previous rodent studies in our laboratory in which time dependent behavioral and neurochemical effects were detected; 2) plasma drug levels were achieved that approximated those often associated with antipsychotic effects in humans (Terry et al., 2002,2003; 2005); 3) 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 and colleagues (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 of 15 days up 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.

Stability of the Haloperidol and Risperidone As Concentrated Solutions and When Diluted in Rodent Drinking Water

In the initial portion of this study we conducted a series of experiments to ensure that the antipsychotic drugs evaluated were stable as concentrated solutions 0.1 M acetic acid and when diluted in tap water or deionized water (ultrapure water, Milli-Q[®] Ultrapure Water Purification

Systems Billerica, MA) at room temperature (i.e. to ensure that our method of administering the antipsychotics orally in drinking water was a valid approach).

Preparation of Standard Solutions—Stock solutions of haloperidol and risperidone were prepared in 0.1 M acetic acid at concentrations of 5.0 and 6.25 mg/ml, respectively and kept in glass bottles in a refrigerator at 4°C for up to 4 weeks. Dilutions of the concentrated solutions in tap water or deionized water (final concentrations of 20 µg/ml and 22.5 µg/ml for haloperidol and risperidone, respectively) were also prepared and transferred into standard rodent drinking water bottles with rubber stoppers and then stored for up to 96 hours at room temperature.

Instrument Conditions for Drug Stability Study—Separations were carried out at ambient temperature on an Agilent Eclipse XBD C-8 column (4.6×150 mm, 5µm) with a Phenomenex Security Guard C-8 guard column (4.0mm×2.0mm). An Agilent 1100 series HPLC consisting of a degasser, quaternary pump, autosampler and variable wavelength ultraviolet detector was used (Palo Alto, CA, USA). The mobile phase consisted of a gradient of 30 mM ammonium acetate containing 0.05% (v/v) triethylamine, 0.025% (v/v), acetic acid, and acetonitrile. The mobile phase ratio began at 68% aqueous and changed linearly to 60% over 16 minutes. The aqueous percentage was then lowered to 20% for 6 minutes to flush the column and then reequilibrated for 8 minutes at the initial mobile phase conditions. The flow rate was 1.0 ml/min and the injection volume was 20 µL. Risperidone was monitored at 277 nm and haloperidol was monitored at 245 nm. The retention time for risperidone was 4.3 minutes and haloperidol was 8.5 minutes.

Plasma Antipsychotic Analysis

Plasma Collection—Plasma samples were collected at days 15, 90, and 180 days of treatment in selected rats that were to be used for neurochemical analyses. Rats were anesthetized with isoflurane and 3.0 mL of blood was collected via cardiac puncture in sodium heparin. The blood was centrifuged for 15 min at 2500 × g at 4-5°C and the resulting plasma was frozen until analyzed.

Sample Preparation—To a 250 µl rat plasma sample, 25 µl of internal standard (40 ng/ml midazolam) and 0.2 ml 0.5 M Na₂HPO₄ (pH = 10.7) were added. The samples were briefly mixed and extracted in 3 ml isopropyl ether for 10 min. After centrifugation at 2000 g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 100 µl methanol: 20 mM ammonium formate (pH = 3.9) (70:30) was added, sonicated, vortexed, and centrifuged at 16000 × g for 10 min. Fifteen µl of the aqueous phase was injected for LC-MS/MS. Water samples, taken to test the stability of the compounds in the animal's drinking water, were run without any sample pretreatment.

LC-MS/MS Procedure—Separations were carried out at 25°C on a Waters AtlantisTM dC-18 (2.1×30 mm, 3µm) with a Phenomenex Security Guard C-18 guard column (4.0mm×2.0mm). An Agilent 1100 series HPLC consisting of a degasser, binary pump, autosampler, and thermostated column compartment was used (Palo Alto, CA, USA). The mobile phase consisted of a gradient of 5 mM ammonium formate buffer and acetonitrile. The first minute is maintained at 15% acetonitrile and then increasing linearly from 15 to 50% acetonitrile over the next 4.0 min, and to 80% over the next 7 min. The flow rate was 0.3 ml/min. The mass spectrometer utilized for this work was a Waters Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization source. The capillary voltage was 3500 volts and the cone voltages ranged from 30-35 volts depending on the analyte. The collision energies ranged from 21-27 eV and the collision cell pressure was 2.4×10^{-3} mbar. The collision gas was argon. The instrument was operated using multiple reaction monitoring (MRM) following the transitions from the protonated molecular ion to a diagnostic

fragment ion for risperidone (411→191), 9-hydroxyrisperidone (427→207), haloperidol (325→165) and the internal standard midazolam (326→291).

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). Animals were transferred (in their home cages) to the behavioral testing rooms each morning approximately 30 min before the beginning of experiments. Table 1 provides the details for all study cohorts, including the behavioral experiments performed in each group, the time period of drug treatment when behavioral testing was conducted, and the numbers of animals tested per group.

Water Maze Repeated Acquisition

At various times during drug treatment, test subjects were evaluated for performance of a water maze procedure. Sessions (which included hidden platform tests and probe trials) were conducted at the following time points of drug treatment: Session 1 = days 8-14; Session 2 = days 22-28; Session 3 days = 39-45; Session 4 = days 84-90; Session 5; days 174-180. For each session the hidden platform was moved to a new quadrant location in the pool.

Test Apparatus—Water maze experiments were performed in a circular pool (diameter: 180 cm, height: 76 cm) made of black plastic and filled to a depth of 35 cm of water (maintained at 25.0±1.0°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, 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).

Hidden Platform Task—For these experiments, an invisible (black) 10 cm × 10 cm square platform was submerged approximately 1.0 cm below the surface of the water and placed in the center of a quadrant (one-fourth of the total pool area defined via the tracking software). For each test session rats were 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 of each test session, probe trials were conducted in which the platform was removed from the pool to measure spatial bias for the previous platform location. This was accomplished by measuring 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.

Visible Platform Task—After probe trials on sessions 4 and 5, a visible platform test was performed as a general estimate of visual acuity. To accomplish this task, a highly visible (white) cover fitted with a small white flag was attached to the platform (dimensions with cover attached = 12 cm × 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. When a rat was successful (on its own accord without assistance) 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.

Spontaneous Novel Object Recognition Test (OR)

Subjects (including additional cohorts of animals) were evaluated in the OR test during the 814 and 31-38 day treatment periods (see Table 1).

Testing Apparatus and Objects Used—Each test apparatus consisted of black plastic (rectangular) open field box (length $\times$ width $\times$ height = 65.5 $\times$ 41.0 $\times$ 36.5 cm, respectively) with normal rodent bedding material (Sani-Chips[®], Harlan Teklad Madison, WI) covering the floor. The objects discriminated were made of glass, ceramic, clay, or plastic. Each object was available in four identical copies, and the weights were such that the rat could not move the objects. Objects varied in height from 4.5 to 15 cm and in width from 4 to 10 cm.

Procedure—Habituation to the test apparatus consisted of two daily 10-min. sessions in which the animals were allowed to freely explore the open field box. Video-recorded OR testing began on the third day and ended on day 5. Each test day began with a 3.0 min (A/A) information session, in which each rat was placed in the box and exposed to two identical sample stimuli. The objects were placed near two adjacent corners (i.e., 10 cm from each wall) of the box and the rat was placed into the box equi-distant from each object during a given test session. The (A/A) information session was followed by a 1.0, 15.0 or 60 min delay period (administered in a pseudorandom order for the test subjects), during which time the animal was returned to its home cage. Subsequently, a 3.0 min dissimilar stimuli (A/B) session followed, in which the rat was placed into the box in the same location as in the previous session (bedding material on floor was rearranged to disrupt olfactory cues) and exposed to the familiar object (A) and a novel object (B). These objects were placed in the same locales as the two objects in the previous session. The familiar object was identical in appearance to the two objects from the (A/A) information session but was not physically either of those objects. The total amount of time spent with each object was recorded, where “time spent” was operationally defined as the animal directing its nose to the object at a distance of less than 2.0 cm and/or by the animal touching the object with its nose or mouth. Animals that did not investigate both objects for at least 5 seconds each during the A/A portion of the test were eliminated from the trial. The proportion of the total exploration time that the animal spent investigating the novel object was the index of recognition memory. A recognition index calculated for each animal was expressed as the ratio $TB/(TA+TB)$; [TA=time spent exploring object A (familiar object), TB = time spent exploring object B (novel object)] according to Schroder et al., 2003.

Motor Function Tests

Tests of motor function were conducted in animal cohorts 4 and 5 during days 50-53 of drug exposure (see Table 1).

Open Field Activity—Rat open field activity monitors (43.2 $\times$ 43.2 cm, Med Associates St Albans, VT) were used for these experiments. The following parameters were recorded for the 5 min test session: horizontal activity (horizontal photobeam breaks or counts), number of stereotypical movements, and vertical activity (vertical photobeam breaks). Thus, spontaneous locomotor activity, olfactory activity (rearing and sniffing movements) and stereotypical movements were assessed. In light of previous reports indicating that risperidone (in contrast

to haloperidol) has anxiolytic activity in rats (Nowakowska et al., 1999) we also recorded the time spent in the central and peripheral zones of the apparatus (defined areas represented approximately 75% and 25% of the total floor area, respectively). Drugs that possess anxiolytic activity tend to decrease the amount of time the rat spends in close proximity to the walls when placed into novel open field environments (thigmotaxis) and to increase exploratory activity in the center compartment (Treit and Fundytus, 1988).

Accelerating Rotarod—Motor coordination, balance, and motor learning were evaluated with an accelerating rotarod (Rotor-Rod System[®], San Diego Instruments, San Diego, CA). Individual rats were assessed for their ability to maintain balance on a rotating bar that accelerated from 4 to 40 rpm over a 5.0 min period. The amount of time elapsed before each subject fell from the rod was recorded. Each test subject was given four trials per day for two consecutive days with an intertrial interval of 30 min.

Grip Strength—Forelimb grip strength was measured with a digital grip strength meter (Animal Grip Strength System[®], San Diego Instruments, San Diego, CA) by holding the rat by the nape of the neck and by the base of the tail. The forelimbs were placed on the tension bar and the rat was pulled back gently until it released the bar. Each animal was assessed three times and mean grip strength (measured in kg of resistance) $\pm$ S.E.M. calculated.

ELISA Experiments

After behavioral testing the rats were sacrificed and the brains were used for neurochemical analyses and blood was collected for measurements of plasma antipsychotic levels (described above in the Plasma Antipsychotic Analysis section of the methods). Euthanasia of rats, brain dissections, preparation of brain lysates, and ELISA methods were performed according to Gearhart et al. (2006), except as noted below. Briefly, the basal forebrain (BF), hippocampal formation (HIPPO), cortex (CTX), and prefrontal cortex (PRF) were dissected and then homogenized in RIPA buffer containing protease inhibitors and glycerol. The Micro BCA[™] Protein Assay Kit (Pierce Biotechnology; Rockford, IL) was used to determine the total protein concentration in each brain lysate. ELISA methods were used to evaluate the brain lysates for relative levels of the following proteins: vesicular acetylcholine transporter (VACHT); high affinity choline transporter (CHT), p75 neurotrophin receptor (p75^{NTR}); TrkA (high affinity nerve growth factor receptor); and phosphorylated-TrkA (P-TrkA). The amount of protein analyzed per well in each ELISA varied by antigen (VACHT, p75^{NTR}, TrkA, or phospho-TrkA) and brain region (BF, HIPPO, PRF, or CTX). As an internal control for day-to-day variation in the ELISA methods, brain lysates from vehicle-, haloperidol-, and risperidone-treated rats were assayed at the same time on the same ELISA plate. The quantity of protein analyzed per well was: *VACHT ELISA* - all brain regions (0.4 μ g); *CHT ELISA* - all brain regions (0.25 μ g); *p75^{NTR} ELISA* - BF and PRF (0.4 μ g), HIPPO and CTX (0.5 μ g); *TrkA ELISA* - BF (0.5 μ g), PRF and HIPPO (0.4 μ g), CTX (0.2 μ g); *phospho-TrkA ELISA* - BF (30 μ g), HIPPO, CTX, PRF (70 μ g).

Note that for the phospho-TrkA ELISA, we did not use the anti-phosphotyrosine, recombinant 4G10 HRP-conjugate (Upstate #16-184) that was used in previous experiments (Gearhart et al., 2006) due to problems with high-background. Coating with anti-TrkA (capture antibody), blocking, and sample incubation were the same as our published ELISA protocol for measuring phospho-TrkA (Gearhart et al., 2006). However, the captured phospho-TrkA was detected using the following three steps (in order): (1) a two hour incubation with mouse anti-phosphotyrosine (Upstate #05-521, diluted 1:1000 in blocking buffer, 50 μ l/well); (2) a one hour incubation with goat anti-mouse IgG-HRP (Jackson ImmunoResearch #115-035-145, diluted 1:10,000 in blocking buffer, 50 μ l/well); (3) the TMB incubation and

spectrophotometry were done as described by Gearhart et al. (2006). All incubations (1-3 above) and washes were done according to Gearhart et al. (2006).

We designed an indirect ELISA for the high affinity choline transporter (CHT). Brain lysates were adsorbed to microwells according to Gearhart et al. (2006). The next morning, microwells were washed one time with 300 μ l of PBS containing 0.05 % Triton-X100 (PBST). Protein-Free Blocking Buffer (Pierce catalog #37572; 300 μ l per well) was used to block the wells; blocking was done at room temperature for four hours at slow speed on an orbital shaker. Wells were washed one time with 300 μ l of PBST, before adding 50 μ l of diluted (1:500 blocking buffer) CHT antibody (Chemicon catalog #MAB5514) to each well. The plate was sealed with parafilm, placed in a zip-lock bag with a wet paper towel, and incubated overnight on an orbital shaker (slow speed) in a refrigerator. The next morning, the plate was washed with PBST (300 μ l/well x three times), and then incubated for one hour with goat anti-mouse IgGHRP (Jackson ImmunoResearch #115-035-145, diluted 1:10,000 in blocking buffer, 50 μ l/well). The plate was washed five times with PBST (300 μ l/well); TMB incubation and spectrophotometry were done as described by Gearhart et al. (2006).

Statistical Analyses

All statistical analyses were performed using SAS 9.1.3. Statistical significance was assessed using an alpha level of 0.05. Post hoc tests were performed using a Bonferroni adjustment to the overall alpha level for the number of post hoc comparisons performed.

Water Maze—Repeated measures analysis of variance (ANOVA) using mixed models was used to examine differences in the area under the latency curve (determined across the 6 days of each session), average swim speed, and platform area crossing between treatment groups (haloperidol or risperidone versus vehicle control) across sessions (1-5). Repeated measures ANOVA using mixed models was performed within session to examine differences in latency and distance across the 6 days of testing between treatment groups (haloperidol or risperidone versus vehicle control).

Spontaneous Novel Object Recognition—To examine differences in preference for the objects and the retention index within trial type (A/A or A/B) and between treatments (haloperidol or risperidone versus vehicle control) and delays (short, medium and long), repeated measures analysis of variance using mixed models was used with the delay being considered the repeated measure. For all mixed models, a compound symmetry covariance structure was assumed between the repeated measures. The animal was considered a random effect.

Locomotor Activity and Grip Strength—One-way ANOVA was used to examine differences in horizontal activity, number of stereotypy movements, and vertical activity in activity monitors, as well as grip strength. A two-way ANOVA with repeated measures was used for daily rotarod performance.

ELISA Data—Repeated measures ANOVA using mixed models was used to examine differences across time between brain regions on the mean normalized OD values. Each OD value was normalized to the mean of the vehicle controls on each plate by subtracting the mean of the vehicle control from each OD value. There were two treatments that were examined, haloperidol and risperidone, but differences between treatments were not of interest. As well, there were five ELISAs used, P-TrkA, TrkA, VACHT, CHT, and p75, but differences between ELISAs were, likewise, not of interest. Thus each repeated measures ANOVA was performed within treatment and ELISA. The repeated measures ANOVA model for each treatment and ELISA combination included fixed effects of length of drug treatment and region of the brain

as well as the two-factor interaction between length of drug treatment and region. The animal nested within length of drug treatment was included in the model and considered a random effect. The repeated measure was region as several regions of each animal's brain were evaluated.

Results

Stability of the Antipsychotics in Aqueous Solution

The stability of the concentrated antipsychotic solutions in 0.1M acetic acid immediately after preparation (time zero) and after storage at 4°C in a refrigerator for 1, 2, and 4 weeks is provided in Table 2A. The data indicate no significant degradation for up to 4 weeks. The stability of the diluted compounds in tap water or deionized water at room temperature at time zero (just after dilution) and at various time points up to 96 hrs after dilution is indicated in Table 2B. The data indicate no significant degradation for up to 96 hrs.

Plasma Antipsychotic Levels

Plasma antipsychotic concentrations assessed after 15, 90, and 180 days of continuous oral administration of either haloperidol or risperidone are provided in Table 3. The combination (i.e., summed levels) of risperidone and the active 9-hydroxyrisperidone (9-OH-RISP) metabolite is also provided since this combination is considered the most clinically relevant measurement (Megens et al., 1994). As indicated, plasma drug levels were within the proposed therapeutic range for haloperidol and the risperidone, 9-hydroxyrisperidone combination. (see Baldessarini et al., 1988; Balant-Gorgia et al., 1999) at each of the 3 time points that were assessed.

Behavioral Experiments

Water Maze Testing

Hidden Platform Test Fig 1 illustrates the efficiency of each experimental group to locate a hidden platform in the water maze task on 6 consecutive days during each of 5 different training sessions. The figures present both the acquisition curves (Fig 1A-mean latencies $\pm$ SEM to locate the hidden platform), the area under the curve (Fig 1A inset-AUC) for latencies, as well as the swim distances (Fig 1B). Under vehicle control conditions, rats progressively learned to locate the hidden platform with increasing levels of efficiency over the course of the 6 days during each testing session as well as over the course of the five different sessions. The latter observation was evident in both the decreasing slope of the acquisition curves over time as well as diminishing areas under the latency curve. In comparisons of haloperidol versus vehicle treated rats there were a number of significant differences detected. In the latency and AUC comparisons, the effects of treatment, session and the treatment x session interaction were all highly significant (all p values were <0.01). Thus, haloperidol was associated with early impairments of acquisition that could be overcome by repeated testing up to 90 days of treatment. However, impairments returned when the longer period of treatment (6 months) were analyzed. In the case of risperidone versus vehicle treated rats, there were two notable findings. First, performance in the risperidone-treated animals was superior to controls ($p<0.03$ for latency and $p<0.06$ for distance) during session 4 and second, performance in the risperidone-treated animals was inferior to controls in session 5 ($p<0.06$ for latency and $p<0.03$ for distance). This finding was also evident in the latency AUC comparisons (noted by a + and a * respectively when $p<0.05$).

Probe Trials Fig 2 illustrates the performance of probe trials by the various treatment groups at the end of sessions 1-5. There were no statistically significant treatment-related effects on performance as indicated by the number of crossings over the previous 10 cm $\times$ 10 cm target

area (haloperidol treatment effect, $p=0.23$; risperidone treatment effect, $p=0.68$) however, there was a trend toward inferior performance in the haloperidol treated animals in session 1 (Bonferroni corrected $p=0.08$).

Visible Platform Test After probe trials on sessions 4 and 5 visible platform tests (4 trials per session per group) were conducted to ensure that the test subjects did not exhibit crude deficits in visual acuity that might have confounded the water maze hidden platform and probe trial analyses. The latencies for the 3 groups to find the visible platform (i.e., the mean of the 4 trials per session) ranged from 5.5 to 6.9 sec. There were no significant treatment-related effects observed in this procedure (i.e., all p values were >0.05 , data not shown).

Spontaneous Novel Object Recognition Test (OR)

Fig 3 illustrates the effects of haloperidol and risperidone on performance of the OR task in separate cohorts of animals (only the A/B sessions are illustrated) at 2 treatment periods (days 8-14 and 31-38). The * in Fig 3 also indicate the preference for the novel object ($p<0.05$) at most of the A/B sessions. At each of the treatment periods (under vehicle control conditions) short delays were generally associated with a higher retention index than long delays (statistically significant only for the 31-38 day treatment period, $p=0.004$). For both treatment periods (i.e., for both antipsychotic drugs), the two factor interaction between treatment and delay was not statistically significant for trial type A/A (i.e., identical objects). In haloperidol treated animals, the two factor interaction between treatment and delay was statistically significant for trial type A/B (different objects) at both days 8-14 and 31-38. Post hoc analysis indicated a highly significant decrease in retention index compared to vehicle control at the short delay ($p<0.05$ and $p=0.003$, for the two time periods, respectively). In the case of risperidone, the two factor interaction between treatment and delay was statistically significant for trial type A/B at days 31-38 only. Again, post-hoc analysis indicated a highly significant decrease in retention index compared to vehicle control at the short delay ($p=0.0007$). The results of all other post hoc tests (i.e., all Bonferroni corrected p values) were non significant ($p>0.05$).

Assessments of 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, particularly the water maze experiments. The results of these experiments are provided in Fig 4.

Water Maze Swim Speeds—Average swim speeds $\pm$ SEM at each of the water maze testing sessions are depicted in the top row of Fig 4. In the case of haloperidol there was a significant treatment effect ($p<0.01$), indicative of slightly slower swim speeds (compared to vehicle controls) however, the two factor interaction between treatment and session was not statistically significant indicating that the effect of haloperidol was similar in the different sessions. There were no significant effects of risperidone on swim speeds.

Open Field Activity—The middle row of Fig 4 illustrates 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 peripheral versus central zone of the test apparatus). There were no significant treatment-related effects ($p>0.05$ for all differences) on any of these measures.

Grip Strength—The effects of haloperidol and risperidone on forelimb grip strength are illustrated at the lower left portion of Fig 4. There was a statistically significant overall

treatment effect ($p=0.004$) and a trend toward increased grip strength in the haloperidol-treated animals (post hoc analysis, $p=0.09$ versus vehicle control).

Rotarod Performance—The effects of haloperidol and risperidone on the performance of the rotarod task are illustrated at the bottom right of Fig 4. There was a significant effect of treatment ($p<0.03$), trial ($p<0.001$), and a significant treatment $\times$ trial interaction ($p<0.01$). Post hoc analysis indicated that during the first training day (depending on the trial) both haloperidol and risperidone were associated with superior performance ($p<0.05$).

ELISA Experiments

Figures 5-9 depict the ELISA data from the four selected brain regions in rats treated with haloperidol or risperidone for time periods ranging from 15 to 180 days. Results from drug-treated rats are presented as a percent of control (vehicle-treated rats); 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). Statistically significant time $\times$ brain region interactions were observed for all comparisons with the exception of haloperidol and TrkA, which had a p -value for the interaction of 0.054. The statistically significant interaction indicates that the effect of time on the normalized OD values was different in the different brain regions.

VACHT (Fig 5)—In the basal forebrain, a biphasic (time dependent) effect was evident in both haloperidol and risperidone-treated rats. Specifically, at the earlier time points (15 and 45 days) VACHT protein levels were significantly higher than vehicle controls (i.e., as high as 40% above control), whereas at the longer (180 day) time period, VACHT levels were lower than control compared to vehicle controls in the case of risperidone ($p<0.05$) and a significant trend toward lower levels in the haloperidol treated animals, $p<0.08$). This biphasic effect was also observed in the prefrontal cortex in the risperidone-treated rats. In contrast, a distinctly different treatment-related pattern was observed in the hippocampus and cortex. VACHT levels in the haloperidol-treated rats were significantly higher than vehicle controls at 15 and 45 days of treatment, whereas at the longer treatment periods, no significant effect was evident. In the cortex of haloperidol-treated rats, as well as in the hippocampus and cortex of risperidone-treated animals, VACHT levels were similar to (or slightly below) control levels at all time points except the 180 day point where VACHT levels were elevated.

CHT (Fig 6)—as observed in the case of VACHT, there was an increase in CHT protein in the basal forebrain at the 15 and 45 day time points of treatment with both haloperidol and risperidone, whereas, at the longer time periods, the effect had abated toward control levels or dropped below baseline levels (e.g., at the 180 time points $p\sim 0.1$ for both compounds). There were no statistically significant drug effects detected in the other brain regions, although a trend toward a biphasic drug effect was evident in the case of the risperidone-treated animals in the prefrontal cortex.

TrkA (Fig 7)—In the basal forebrain, a biphasic effect (i.e., as observed in the VACHT experiments) on TrkA levels was evident in both haloperidol and risperidone-treated rats. Specifically, at the earlier time points TrkA protein levels were significantly higher than vehicle controls, whereas at the 180 day time point they were significantly lower than control. Interestingly, in the hippocampus a pattern emerged in which the 90 day treatment was associated with a decrease in TrkA in both antipsychotic treatment groups while (with the exception of a slight increase at the 180 day time point in the risperidone -treated rats) there were no significant drug effects at the other time points. In the cortex, only the 90 day risperidone treatment was associated with a significant drug-related effect (i.e., a decrease in TrkA).

Phospho-TrkA (Fig 8)—There was no particular time-related pattern of drug effects evident in phospho-TrkA experiments and both increases and decreases in phospho-TrkA protein levels were observed depending on the time point assessed. Interestingly, there were several cases in which phospho-TrkA levels were increased by as much as 30-35% above control (e.g., prefrontal cortex at 90 days with haloperidol, basal forebrain at 45 days, and hippocampus at 180 days (in association with risperidone))

p75^{NTR} (Fig 8)—In the basal forebrain (as observed in the case with VAcHT and TrkA) there was a significant increase (i.e., by as much as 40% over vehicle controls) in the p75^{NTR} associated with both haloperidol and risperidone at the shorter (15 and 45 day) treatment periods. However, at the 90 and 180 days time points, p75^{NTR} levels had abated to control in both antipsychotic treatment groups. There were only two other notable findings in these experiments, 1) a significant increase in hippocampal p75^{NTR} levels in rats treated with haloperidol for 45 days and, 2) a significant increase in p75^{NTR} levels in the prefrontal cortex of rats treated with risperidone for 15 days.

Discussion

The results of this study can be summarized as follows: 1) the method used for administering antipsychotic drugs orally in drinking water is a valid approach as exemplified by the fact that the drugs were stable (i.e., diluted in tap water or deionized water) for at least 96 hours at room temperature, and that the plasma antipsychotic values generated in the rat approximated those generally considered therapeutic in humans; 2) while risperidone appeared to have some advantages over haloperidol particularly during early periods of treatment, both antipsychotic drugs produced impairments in a water maze spatial learning task; 3) both haloperidol and risperidone were associated with decrements in short delay performance in a spontaneous novel object recognition procedure at approximately one month of treatment; 4) both haloperidol and risperidone were associated time dependent alterations (i.e., either increases or decreases depending on the length of drug exposure) in the cholinergic marker proteins VAcHT and HCT, the neurotrophin receptors, TrkA, phospho-TrkA and p75^{NTR} in memory-related regions of the brain.

As noted above in the Methods section, the oral antipsychotic dosing approach used in this study (i.e., drugs delivered in drinking water and dosed on a mg/kg/24 hour basis) was based on previous studies in our laboratory (e.g., Terry et al., 2005) where the plasma drug levels achieved approximated those generally considered therapeutic in humans. The haloperidol plasma levels detected in the present study (i.e., a range of 6.4-12.4 ng/ml) were somewhat higher than those detected in previous chronic studies (e.g., 2.1 ng/ml at 45 days of treatment, Terry et al., 2005), but well within the general range of 4.0-18.0 ng/ml that has been estimated as therapeutic in humans (see reviews, Baldessari et al., 1988; Markowitz and Patrick, 1996; Coryell et al., 1998). The range of plasma levels of the risperidone, 9-hydroxyrisperidone combination (27.1-37.5 ng/ml) detected would fit well within the therapeutic range of 20-60 ng/ml suggested by Balant-Gorgia et al., 1999. While there are no published pharmacokinetic and/or D₂ occupancy studies using our specific method of drug administration in rats, the plasma antipsychotic levels achieved would be expected to reflect steady state levels observed in humans with doses of ranging from approximately 2.0-5.0 (or possibly 6.0) mg/day and 2.0-6.0 mg/day for haloperidol and risperidone, respectively (see Kapur et al., 2003). Based on the acute oral dosing study in rats by Barth et al., 2006 (and extrapolations from Fig 1 in their study) and the study of Kapur et al., 2003 (cited above) we would expect that our antipsychotic dosing approach generated D₂ occupancy levels roughly in the range of 65-80 for risperidone and 75-90 (or possibly higher) for haloperidol. The higher estimate for haloperidol could be surmised from the Kapur study, where the peak plasma level of haloperidol (11 nM or 4.1 ng/ml) detected in rats after multiple subcutaneous injections was

associated with a D₂ occupancy value of was greater than 90%. Although the dosing approach in our study was quite different from those described above, such data indicate that some caution should be exercised when interpreting the behavioral results discussed below especially when making direct comparisons between risperidone and haloperidol.

In the behavioral studies, the repeated acquisition approach to water maze testing was chosen specifically for the purpose of evaluating the effects of antipsychotics on learning/encoding and retrieval (repeatedly) over time. Impaired information encoding and retrieval capacity are commonly reported in schizophrenia (Gur, et al., 2000;Cairo et al., 2006), and further, learning potential (i.e., as determined in multiple administrations of neurocognitive tests) predicts work skill attainment in schizophrenia, a factor known to predict the rehabilitation outcome (Sergi et al., 2005). Accordingly, the data obtained in this study appear to suggest that both haloperidol and risperidone (depending on the duration of treatment) can adversely affect information encoding and as a result, negatively affect learning potential. It is important to note, however, that risperidone had more favorable effects on water maze acquisition when compared to haloperidol, and it was even associated with an improvement in performance at the 3 month time point. Such data may indicate that there is a limited window where risperidone may have beneficial effects on cognition. We have observed a similar effect of risperidone in previous water maze studies in which rats were evaluated in a water maze task after 3 months of treatment (Terry et al., 2003). The basis for the decrements in performance in the object recognition experiments associated with haloperidol and risperidone specifically at the short delays is unclear. Since the short delay was only one minute in duration, impairment could be indicative of drug-related disorientation, distractibility, or an attention-related deficit. Attentional deficits associated with haloperidol and risperidone have been observed by other investigators (Rezvani and Levin, 2004).

The absence of haloperidol or risperidone-related effects on the visible platform tests in the water maze, horizontal, vertical or stereotypical activity (or time spent in the peripheral zones) in the open field experiments, argues against the premise that gross drug effects on locomotor activity, visual acuity, or anxiety levels underlie the observed deficits in water maze or object recognition task performance. A lack of motor-related impairments was further evident in the grip strength studies and surprisingly, in the improvements in rotarod performance by animals treated with either haloperidol or risperidone on day one of the rotarod test. However, the consistently (albeit modestly) slower swim speeds across all time points in the water maze task in animals treated with haloperidol could indicate of some type of confounding psychomotor impairment. Haloperidol is well-documented to induce motor impairments acutely in both animals and humans. In Wistar rats (i.e., the same strain used in this study), for example, haloperidol can induce catalepsy acutely at oral doses that are relevant to this study (e.g., 1.0-4.0 mg/kg) however, marked tolerance to the effect develops in a few days and is maximal at 16 days (Ezrin-Waters and Seeman, 1977). It is thus unlikely that catalepsy-like motor effects would underlie the water maze deficits observed in this study (other than possibly in Session 1), particularly since the doses in our study were delivered over a 24 hr period (i.e., not a bolus dose as in the aforementioned study). Furthermore, the reduced swim speeds in the haloperidol-treated animals also occurred at time points when latencies to locate the platform were not significantly different from vehicle controls (e.g., session 4).

Regarding the memory-related behavioral findings in this study, there are both similarities and differences in the pharmacology of haloperidol and risperidone that could be important. Both antipsychotics bind with similar high (i.e., nM) affinity as antagonists at dopamine D₂ receptors *in vitro* (Schotte et al., 1993) and, interestingly, selective D₂ antagonists (e.g., raclopride) have been observed to induce a number of detrimental effects on spatial memory, and sustained attention (Von Huben et al., 2006). Risperidone, in contrast to haloperidol, (which had more favorable effects on water maze acquisition in the present study) is characterized by a high

affinity (i.e., as an antagonist) for 5HT_{2A} receptors (Schotte et al., 1996). Importantly, antagonist activity at 5HT_{2A} receptors has been associated with improved performance of memory-related tasks in rats and monkeys (Winstanley et al., 2003; Terry et al., 2005). Such observations regarding cognitive effects of D₂ and 5HT_{2A} antagonists described above represent acute drug effects, however, and it is unclear if such observations would persist in during chronic treatment.

The memory-related behavioral data obtained in this study are in general agreement with an increasing body of animal evidence (i.e., both in acute and chronic studies) suggesting that both SGAs and FGAs can exert negative effects on cognition (reviewed, Terry and Mahadik 2006). In other chronic studies (which are relevant to this study and the more common clinical use of antipsychotics in schizophrenia), haloperidol, clozapine, and risperidone impaired acquisition in a radial arm maze task in rats (Rosengarten and Quartermain, 2002). Furthermore, both risperidone and haloperidol impaired working memory in a cross maze task (Karl et al., 2006), while Didriksen and colleagues (2006) observed impairments in water maze performance in association with chronic olanzapine treatment. In addition to the present study, we have observed spatial learning deficits in rats previously treated chronically with haloperidol, chlorpromazine, risperidone, olanzapine, and ziprasidone (Terry et al., 2002; Terry et al., 2006; Terry and Mahadik, 2006).

In the neurochemistry experiments, the most notable observation was the biphasic effect of the antipsychotics on VACHT, CHT, and the neurotrophin receptors (TrkA, and p75^{NTR}) that was limited to the basal forebrain and prefrontal cortex. While such findings are difficult to interpret, we also observed similar biphasic alterations in NGF (protein) and the cholinergic marker, ChAT, in association with haloperidol and the SGA ziprasidone in a previous study (Terry et al., 2006). Specifically, both haloperidol and ziprasidone were associated with a significant increase in NGF and ChAT levels in the hippocampus during early time points of drug exposure (i.e., up to 14 days) followed by a significant decrease below baseline at longer time points (90 days). It should be noted, however, that the effects on NGF receptors, VACHT, and CHT described above in the basal forebrain and prefrontal were not detected in the hippocampus in the present study. There may be several potential reasons for the differences in the two studies 1) different antipsychotics may have distinct effects on NGF protein versus NGF receptors 2) different methods were used in the two studies (i.e., ELISA analysis of whole hippocampal homogenates in the present study versus layer specific immunohistochemical analysis in the previous study may be important, 3) the levels of ChAT, VACHT, and CHT may be independently altered by antipsychotic treatment. The latter possibility could also be relevant to another particularly confounding observation in the present study; the approximately 40% increase of VACHT (but not CHT) in the prefrontal cortex at the early time points after risperidone treatment. This observation is even more intriguing when combined with recent findings in another study in our laboratory (unpublished data) in which risperidone and haloperidol appear to differentially affect VACHT and ChAT levels in rodent brain. 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. Earlier neurodevelopmental studies (Holler et al., 1996) and more recent culture studies (Castell et al., 2002) suggest the possibility of independent regulation of VACHT and ChAT, either through separate mechanisms that control the activity of specific promoters, or through posttranscriptional mechanisms. Such mechanisms could be involved in differential antipsychotic effects on VACHT and CHT levels as well, although the brain region-specific effects of a single drug (as observed in several cases

in this study) remain perplexing. Finally, regarding the (sometimes difficult to interpret) antipsychotic effects on the NGF responsive receptors, TrkA and p75^{NTR}, it should be noted that these proteins 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}, and the effect of these changes on cholinergic function.

Observations of time-dependent (biphasic), cholinergic responses to FGAs (somewhat similar to our observations in basal forebrain and prefrontal cortex) in the striatum of animals have been discussed as a potential mechanism of their adverse motor effects in humans (Miller and Chouinard, 2003). Such a phenomenon may reflect the inhibitory D₂ receptor effects on cholinergic interneurons in the striatum by FGAs which results in excessive neuronal activity, intracellular accumulation of calcium, and subsequent cell damage. Our detection of haloperidol and risperidone-related decreases in cholinergic markers in the prefrontal cortex in this study may reflect the involvement of other dopamine-acetylcholine interactions. For example, it has been hypothesized that dopamine in the nucleus accumbens may inhibit 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). While these GABAergic projections from the nucleus accumbens may only represent a small fraction of the total, chronic exposure to drugs that antagonize dopamine receptors in the accumbens could indirectly lead to imbalances in cholinergic activity in basal forebrain neurons (and thus projection areas such as the prefrontal cortex). It also is interesting to speculate that the initial increase in cholinergic markers associated with the blockade of inhibitory D₂ receptors (i.e., on cholinergic neurons in a number of brain regions) by antipsychotics such as haloperidol and risperidone could eventually overcome by an upregulation of the D₂ receptor after chronic inhibition, thus, leading to the biphasic cholinergic effects observed in this study.

Regarding a relationship between functional (behavioral) outcomes in this study and neurochemical measurements, the deficits in VAcHT observed in association with chronic antipsychotic treatment could have relevance to the deficits in water maze and object recognition performance since both of these tasks requires intact cholinergic function. It is also interesting to note that mice bred to express reduced levels of VAcHT are deficient in object recognition at both short and long retention intervals (Prado et al., 2006).

In conclusion, the results of this study in rats support the notion that while risperidone may hold some advantages over haloperidol particular during the earlier periods of treatment, both antipsychotics can produce time-dependent alterations in NGF receptors and cholinergic proteins as well as impairments in the performance of tasks designed to assess spatial learning and episodic memory. The time dependent (antipsychotic-related) alterations in NGF receptors and cholinergic markers observed in this study add to the accruing body of evidence which suggests that chronic exposure to FGAs or SGAs can have a significant impact on neurotrophin function as well as cholinergic neurons. Such observations may be significant given the important role of neurotrophins in adult synaptic plasticity, and the role of NGF in supporting cholinergic neurons, particularly those originating in the basal forebrain. These neurons have been implicated in almost every step of information processing from attention to encoding to memory consolidation and retrieval (see review, Gold 2003).

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List of Abbreviations

ChAT, choline acetyltransferase; CON, vehicle control; HAL, haloperidol; CHT =, high affinity choline transporter; RISP, risperidone; NGF, Nerve Growth Factor; TrkA, tyrosine kinase A; phospho- TrkA, phosphorylated TrkA; p75^{NTR}, p75 neurotrophin receptor; VACHT, vesicular acetylcholine transporter.

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Fig 1.

Antipsychotic effects on a water maze repeated acquisition procedure. S refers to the testing session, hidden platform tests were conducted during the first 6 days of each session. S1 = days 8-14; S2= days 22-28; S3 =days 39-45; S4 = days 84-90; S5= days 174-180 of drug treatment. For each session, the hidden platform was moved to a new quadrant location in the pool. **A.** Latency to find a hidden platform. Each point represents the mean latency in seconds $\pm$ SEM for each testing day. **Inset.** Latency Area Under the Curve (AUC). Each bar represents the mean latency AUC $\pm$ SEM. **B.** Distance swam to find a hidden platform. Each point represents the mean distance in centimeters $\pm$ SEM for each testing day. VEH (or V) =Vehicle; HAL (or H) =haloperidol; RISP (or R) =risperidone. N=12 for all treatment groups except for S5, where

N=6 for each group. Within each session, treatment effects (across the session) are noted when significant ($p < 0.05$) or nearly significant ($p < 0.06$). Post hoc differences are indicated as follows: * = significantly ($p < 0.05$) inferior performance when compared to vehicle control. + = significantly ($p < 0.05$) superior performance when compared to vehicle controls.

Fig 2.

Antipsychotic effects on a water maze probe trial performance as assessed by the number of platform area crossings (mean $\pm$ S.E.M.). S refers to the testing session; the probe trial was conducted on the last day of each of the sessions. S1= days 8-14; S2= days 22-28; S3=days 39-45; S4 = days 84-90; S5= days 174-180 of drug treatment * significantly different than vehicle controls ($p < 0.05$). N=12 for all treatment groups except for S5, where N=6 for each group. * = sig. different ($p < 0.05$) from vehicle control.

Fig 3.

Antipsychotic effects on the performance of a spontaneous novel object recognition task conducted (in separate groups of animals) during (A) days 8-14 or (B) days 31-38 of treatment. The illustrations at the left of each figure indicate the preference for the novel object compared with the familiar object (*= $p < 0.05$) at each of the 3 delays. The insets at right of each figure illustrate drug effects on the "Recognition Index" which refers to the proportion of the total exploration time the animal spent investigating the novel object (see Methods); + = significantly different ($p < 0.05$) from vehicle control performance. Data are expressed as the mean $\pm$ S.E.M. N=6 for each group in (A) and N=18-30 animals per group (B).

Fig 4.

Antipsychotic effects on motor function conducted during days 50-53 of treatment. **Top:** Water maze swim speeds measured in cm/sec. S refers to the testing session; S1= days 8-14; S2= days 22-28; S3 =days 39-45; S4 = days 84-90; S5= days 174-180 of drug treatment. **Middle:** Open Field Activity, left to right: horizontal activity measured as the number of photobeam breaks/5 min; Vertical activity measured as the number of photobeam breaks/5 min; stereotypical movements (repetitive photobeam breaks/ 5 min); fear/anxiety related behavior measured as the time spent in the central (C) versus the peripheral (P) zone of the activity monitor. **Bottom Left:** Forelimb Grip strength measured in Kg of resistance. **Bottom Right:** Accelerating Rotarod performance expressed as time maintained on a rotating bar that accelerated from 4 to 40 rpm over a 5-min period. The bars or points represent the mean $\pm$ S.E.M. N=12 rats/group. * = sig. different ($p < 0.05$) from vehicle control.

Fig 5.

ELISA results for VACHT protein (expressed as percent of vehicle-treated group) for the brain regions indicated from rats treated with haloperidol or risperidone. Rats were euthanized at each of the time points indicated and then the dissected brain regions were processed for ELISA methods. *p<0.05, **p<0.01, ***p<0.001, = significantly different from vehicle control. N=5-6.

Fig 6.

ELISA results for CHT protein (expressed as percent of vehicle-treated group) for the brain regions indicated from rats treated with haloperidol or risperidone. Rats were euthanized at each of the time points indicated and then the dissected brain regions were processed for ELISA methods. *p<0.05, **p<0.01, = significantly different from vehicle control. N=5-6.

Fig 7.

ELISA results for TrkA receptor protein (expressed as percent of vehicle-treated group) for the brain regions indicated from rats treated with haloperidol or risperidone. Rats were euthanized at each of the time points indicated and then the dissected brain regions were processed for ELISA methods. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, = significantly different from vehicle control. $N = 5-6$.

Fig 8.

ELISA results for phospho-TrkA receptor protein (expressed as percent of vehicle-treated group) for the brain regions indicated from rats treated with haloperidol or risperidone. Rats were euthanized at each of the time points indicated and then the dissected brain regions were processed for ELISA methods. *p<0.05, **p<0.01, ***p<0.001, = significantly different from vehicle control. N=5-6.

Fig 9.

ELISA results for p75^{NTR} receptor protein (expressed as percent of vehicle-treated group) for the brain regions indicated from rats treated with haloperidol or risperidone. Rats were euthanized at each of the time points indicated and then the dissected brain regions were processed for ELISA methods. *p<0.05, **p<0.01, ***p<0.001, = significantly different from vehicle control. N=5-6.

Table 1

Rat Behavioral Testing Protocol

Cohort	Group	N	Treatment	Day of Drug Exposure/Procedure Conducted									
				8-14	15	22-28	31-38	39-45	45	50-53	83-90	90	174-180
1	A	6	VEH	OR	SAC								180
	B	6	HAL	OR	SAC								
	C	6	RISP	OR	SAC								
2	D	12	VEH				OR		SAC				
	E	6	HAL				OR		SAC				
	F	6	RISP				OR		SAC				
3	G	12	VEH				OR						
	H	6	HAL				OR						
	I	6	RISP				OR						
4	J	6	VEH	WM		WM	OR	WM		Motor	WM	SAC	
	K	6	HAL	WM		WM	OR	WM		Motor	WM	SAC	
	L	6	RISP	WM		WM	OR	WM		Motor	WM	SAC	
5	M	6	VEH	WM		WM		WM		Motor	WM		SAC
	N	6	HAL	WM		WM		WM		Motor	WM	WM	SAC
	O	6	RISP	WM		WM		WM		Motor	WM	WM	SAC

Grp=Group; Trt=treatment; VEH = vehicle; HAL = haloperidol; RISP = risperidone; OR = object recognition; WM = water maze; SAC= sacrifice; Motor = motor function test (open field, rotor-rod, and grip strength)

Table 2

Stability of Haloperidol and Risperidone

A: Stability as Concentrated Solutions in 0.1 M acetic acid at 4°C									
Haloperidol			Risperidone						
Time (weeks)	Concentration (mg/ml)	SD	R.S.D. (%)	Time (weeks)	Concentration (mg/ml)	SD	RSD. (%)		
0	4.7	0.03	0.70	0	5.6	0.08	1.33		
1	4.9	0.04	0.71	1	5.4	0.08	1.40		
2	4.9	0.16	3.24	2	5.5	0.06	1.06		
4	4.8	0.01	2.20	4	5.7	0.19	3.28		
B: Stability of Dilutions in Rodent Drinking Water									
Haloperidol (Tap water)			Haloperidol (distilled/deionized Water)						
Time (hours)	Concentration (µg/ml)	SD	R.S.D. (%)	Time (hours)	Concentration (µg/ml)	SD	RSD. (%)		
0	20.1	0.26	1.31	0	20.5	0.22	1.06		
6	20.1	0.16	0.81	6	20.4	0.09	0.44		
24	19.7	0.11	0.57	24	19.9	0.13	0.68		
48	19.6	0.30	1.50	48	20.0	0.03	0.16		
72	20.3	0.10	0.48	72	20.5	0.02	0.11		
96	20.1	0.07	0.34	96	20.5	0.09	0.44		
Risperidone (Tap water)			Risperidone (distilled/deionized Water)						
Time (hours)	Concentration (µg/ml)	SD	R.S.D. (%)	Time (hours)	Concentration (µg/ml)	SD	RSD. (%)		
0	22.2	0.33	1.47	0	22.8	0.04	0.18		
6	22.5	0.27	1.19	6	22.8	0.22	0.95		
24	22.1	0.08	0.34	24	22.2	0.15	0.68		
48	22.5	0.36	1.59	48	22.3	0.02	0.08		
72	22.6	0.16	0.70	72	22.3	0.39	1.73		
96	22.6	0.22	0.07	96	22.7	0.22	0.98		

N=3 samples per determination; SD= Standard Deviation; RSD= relative standard deviation

Plasma Antipsychotic Levels

Table 3

Test Group	Dose/24 hr	Compound Measured	Plasma Concentration (ng/mL) ± S.E.M. N=6-12			Estimated Human Therapeutic Plasma Range (ng/mL)*
			15 Days	90 days	180 days	
haloperidol risperidone	2.0 mg/kg	haloperidol	6.4 ± 0.9	11.5 ± 2.4	12.4 ± 2.8	4.0-18.0
	2.5 mg/kg	risperidone	4.7 ± 1.3	7.4 ± 2.8	4.3 ± 1.6	
		9-OH risperidone	22.5 ± 3.5	30.2 ± 8.8	22.8 ± 7.4	20.0-60.0
		risperidone + 9-OH risperidone	27.2 ± 4.6	37.5 ± 11.5	27.1 ± 8.9	

* Estimated human therapeutic plasma ranges listed are based on Baldessarini et al., 1988;Markowitz and Patrick, 1996;Coryell et al., 1998;Balanti-Gorgia et al., 1999.