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Prototypical ranitidine analog, JWS-USC-75-IX, improves information processing and cognitive function in animal models

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Abbreviations:

AChE, acetylcholinersterase
AD, Alzheimer's Disease
BChE, butyrylcholinseterase
CAR, Conditioned Avoidance Responding
DMTS, delayed match to sample
DMTS-D, delayed match to sample with distractor
5C-SRTT, five choice serial reaction time task
JWS, JWS-USC-75IX
PPI, Prepulse Inhibition
NOR, Spontaneous Novel Object Recognition Test

Abstract

This study was designed to further evaluate a prototypical ranitidine analog, JWS-USC-75-IX, (JWS), for neuropharmacologic properties that would theoretically be useful for treating cognitive and non-cognitive behavioral symptoms of neuropsychiatric disorders. JWS was previously found to inhibit acetylcholinesterase (AChE) activity, to serve as a potent ligand at muscarinic (M_2) acetylcholine receptors, and to elicit positive effects on spatial learning, passive avoidance, and working memory in rodents. In the current study, JWS was evaluated for binding activity at more than 60 neurotransmitter receptors, transporters, and ion channels, and for inhibitory activity at AChE and butyrylcholinseterase (BChE). The results indicated that JWS inhibits AChE and BChE at low (μM) concentrations and that it is a functional antagonist at M_2 receptors (K_B=320 nM). JWS was subsequently evaluated orally across additional behavioral assays in rodents (dose range 0.03 to 10.0 mg/kg) as well as non-human primates (dose range 0.05 to 2.0 mg/kg). In rats, JWS improved prepulse inhibition (PPI) of the acoustic startle response in non-impaired rats and it attenuated PPI deficits in three pharmacologic impairment models. JWS also attenuated scopolamine and MK-801-related impairments in a spontaneous novel object recognition task and a five choice serial reaction time task, respectively. In monkeys, JWS elicited dose-dependent improvements of a delayed match to sample (DMTS) task as well as an attention-related version of the task where randomly-presented (task-relevant) distractors were presented. Thus, JWS (potentially via effects at several drug targets) improves information processing, attention, and memory in animal models and may have potential to treat the cognitive and behavioral symptoms of some neuropsychiatric illnesses.

Introduction

It is now well documented that life expectancies are increasing worldwide and that this phenomenon is resulting in an unprecedented growth of elderly populations (United States Centers for Disease Control and Prevention, 2003; United Nations, 2007). However, the optimism surrounding this important trend is in many ways attenuated in older individuals by the fear of declining memory function and the specter of dementia. While there are therapies available to treat dementia such as acetylcholinesterase inhibitors (AChEI's) and the NMDA antagonist, memantine, these agents are limited by their modest levels of efficacy and/or adverse side effects. Furthermore, the cognitive deficits in those with dementia are often accompanied by other adverse behavioral symptoms (e.g., agitation, aggression, psychosis) that are (for the most part) left untreated by the currently available primary therapies (Minger et al., 2000). These "non-cognitive behavioral symptoms" significantly increase caregiver burden and the direct costs of care as well as result in earlier institutionalization of patients (reviewed Eustace et al., 2002). Unfortunately, to control these behavioral symptoms, patients are often treated with potent antipsychotic medications that are associated with movement disorders and/or a wide variety of metabolic abnormalities (e.g., weight gain, hyperglycemia, hyperlipidemia, reviewed, Miyamoto et al., 2005) as well as an increased mortality in older dementia patients (Ballard et al., 2009).

In diseases such as AD, it can be argued that a multiple drug target approach to therapy is necessary to address the varied pathological aspects of the disease and its diverse symptoms. However, even if the strategy of combining drugs with different therapeutic targets is feasible, the development of multi-functional compounds would circumvent the challenge of administering multiple drugs with potentially different degrees of bioavailability,

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pharmacokinetics, and metabolism (reviewed, Youdim and Buccafusco, 2005). An additional advantage to single drugs with multiple actions is the simplification of the therapeutic regimen and improved compliance (an important consideration, especially for individuals who suffer from disorders such as AD).

Several years ago, we synthesized a series of ranitidine analogs for the purpose of creating non-toxic AChEIs. The impetus for this work was the prior observation that ranitidine (a histamine H_2 antagonist commonly used to treat duodenal ulcers) had mild AChEI properties in vitro (Gwee and Cheah, 1986). Several of the compounds from this series of ranitidine analogs were subsequently found to possess potent AChEI properties as well as low toxicity profiles (Valli et. al., 1992). Of this series, one compound, (i.e., compound 26), JWS-USC-75-IX   (3-[[[2-[[(5-dimethylaminomethyl)-2-furanyl]methyl]thio]ethyl]amino]-4-nitropyridazine) was found to inhibit AChE activity *in vitro* ($IC_{50} \sim 470$ nM) and to bind M_2 muscarinic acetylcholine receptors with relative high affinity in rodent cerebral cortex ($IC_{50} \sim 60$ nM). Interestingly, it has been hypothesized that in the therapy of AD, an M_2-selective (autoreceptor) antagonist might be very useful given in conjunction with an AChEI to prevent the acetylcholine from decreasing its own release (Mash et. al., 1985; Quirion, 1993). JWS-USC-75-IX (JWS), thus, combined both features in a single molecule and offered a significant potential for improved reliability over presently available compounds for the treatment of diseases where cholinergic function is impaired (e.g., AD).

Later we evaluated JWS for effects in memory-related tasks in rodents (Terry et al., 1999) and observed JWS-related improvements in spatial learning, inhibitory avoidance, and working memory. One objective of the current study was to determine if JWS has the potential to improve memory-related function in additional cognitive domains in rodents (e.g., attention,

recognition memory) as well as to evaluate the compound in higher animals that more closely resemble humans (i.e., non-human primates). Interestingly, there are some data to suggest that M_2 receptor levels are elevated in the frontal and temporal cortex of AD patients who suffer from psychotic symptoms (i.e., compared with those without these symptoms, Lai et al., 2001). Such data, suggest that M_2 antagonists might have a role in ameliorating psychotic as well as cognitive symptoms in these AD patients. Thus, another objective of this study was to evaluate JWS for potential antipsychotic-like activity in rodents. Antipsychotic and pro-cognitive properties of JWS (if detected) would also suggest potential indications for neuropsychiatric disorders beyond AD (e.g., schizophrenia).

Since JWS was derived from the ranitidine molecule (a histaminic H_2 antagonist) it was conceivable that the compound might have activity at histaminic receptors (which had not previously been assessed) as well as additional drug targets. JWS was thus screened at a single concentration (10 μm) across more than 60 neurotransmitter receptors, transporters ion channels, etc. Subsequent ligand binding studies and functional assays were performed with multiple JWS concentrations to confirm activity at the targets identified in the screen.

Methods

All procedures employed during this study which involved animals 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.

Drugs

All drug doses were calculated based on the free base weight. The drugs other than JWS-USC-75-IX (see synthesis procedure below) were obtained from the following sources: apomorphine, MK-801, scopolamine hydrobromide, D-amphetamine sulfate, and haloperidol (Sigma Aldrich), donepezil (A&A Pharmachem, Ottawa, Ontario Canada) risperidone and galantamine (Janssen Pharmaceutica, Beerse, Belgium), rivastigmine (AK Scientific, Inc., Mountain View, CA). With the exception of JWS and the antipsychotic drugs risperidone and haloperidol, the vehicle for all drugs was normal (0.9%) saline. JWS was dissolved in μl amounts of dimethylsulfoxide (DMSO) then diluted in distilled water to the desired concentration. Risperidone was dissolved in 0.1N acetic acid, diluted to the desired concentration, and the pH of the solution was adjusted to 5.0 with 0.1N NaOH. Haloperidol was dissolved in a mixture of 0.1N acetic acid and 0.03% DMSO then diluted to the desired concentration and the pH of the solution was adjusted to 6.0 with 0.1N NaOH.

Synthesis of JWS-USC-75IX- JWS-USC-75-IX was synthesized as described previously (Valli et al., 1992). Briefly, commercially available 2-[(2-aminoethylthio)methyl]-5-[(N,N-dimethylamino)methyl]furan was reacted with 1,1-bis(methylthio)-2-nitroethylene to yield the

monothiomethyl intermediate. This remaining thiomethyl group was displaced by hydrazine and the resulting adduct cyclized with aqueous glyoxal to form the nitropyridazine, JWS-USC-75-IX. Structural characterization and purity of JWS-USC-75-IX was assessed utilizing 400mHz 1H-NMR, thin layer chromatography and elemental analysis (Atlantic Microlabs, Norcross, Ga).

Pharmacological Activity of JWS-USC-75IX (in vitro)

JWS was screened at a single concentration (10 μm) across more than 60 neurotransmitter receptors, transporters, ion channels, and the enzyme acetylcholinesterase. by NovaScreen/Caliper Life Sciences (Hanover, MD). Details of each assay condition can be accessed through Caliper's web site at www.caliperls.com. Subsequent ligand binding studies were performed by NovaScreen/Caliper Life Sciences with three JWS concentrations to confirm activity at the targets identified in the screen (defined as $\geq$ 70% inhibition of binding). A functional assay was also conducted by CEREP (Celle l'Evesault, France) using standard protocols and procedures described on their website (www.cerep.com). Briefly, to identify agonist/antagonist activity of JWS-USC-75-IX at M_2 muscarinic receptors, a modification of the method of Michal et al. (2001) was used. Experiments were performed on CHO cells stably transfected with the human gene for muscarinic M_2 receptor subtype and agonist/antagonist activity of JWS was determined by monitoring the synthesis of cyclic AMP in response to 1.0 μM acetylcholine in the presence of various concentrations of JWS. The IC_{50} values (concentration causing a half-maximal inhibition of the control specific agonist response) were determined by non-linear regression analysis of the concentration-response curves generated with mean replicate values using Hill equation curve fitting $(Y = D + [(A - D)/(1 + (C/C_{50})nH)])$, where Y = specific response, D = minimum specific response, A = maximum specific response, C = compound concentration, and $C_{50} = IC_{50}$, and nH = slope factor). This analysis was

performed using a software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.). The apparent dissociation constants (K_B) were calculated using the modified Cheng Prusoff equation ($K_B = IC_{50}/(1+(A/EC_{50}A))$), where A = concentration of reference agonist in the assay, and $EC_{50}A = EC_{50}$ value of the reference agonist).

The inhibitory activity of JWS (compared to currently prescribed AChEIs, galantamine donepezil, and rivastigmine) on AChE and butyrylcholinesterase was determined using a modification of the method of Ellman et al. (1961) in a 24-well plate format at 37°C. Electric eel cholinesterase (EC 232-559-3) and equine butyrylcholinesterase (EC 232-579-2) purified from serum were purchased from Sigma. Assays were performed in 0.1 M sodium phosphate buffer (pH 8.0) containing 200 μM substrate (acetylthiocholine or butyrylthiocholine), 100 μM dithiobisnitrobenzoic acid, and 0.005 units enzyme in a final volume of 3000 μl. Following an eight-min pre-incubation with inhibitor and enzyme, the reaction was initiated by the addition of substrate. The 24-well plate was shaken for 30 sec using a Jitterbug plate shaker (Boekel Scientific, Feasterville, PA), before it was placed in a μQuant Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT). The formation of reaction product (yellow color) was monitored by measuring absorbance at 412 nm. Velocity was expressed as μM of substrate hydrolyzed/min/mg protein. All assays were performed at least 2-3 times. The IC_{50} values (concentration causing a half-maximal inhibition of the control response) were determined by non-linear regression analysis of the concentration-response curves generated.

Rodent Behavioral Studies

All rat 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/m^2). Animals were transferred (in their home cages) to the behavioral testing rooms each morning approximately 30 min before the beginning of experiments.

Test Subjects (Rats)- Male albino Wistar rats (Harlan, Indianapolis, IN) 2-3 months old were housed in pairs in a temperature controlled room (25°C), maintained on a reversed 12-hour light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI).

Amphetamine-Induced Locomotor Activity

Rat open field activity monitors (43.2 X 43.2 cm, Med Associates St. Albans, VT) were used for amphetamine-induced locomotor experiments and photobeam breaks (ambulatory counts) were assessed. Rats were habituated in the test chambers for 30 min and then vehicle, a dose of JWS, or risperidone (as a positive control) by oral gavage. At 60 min, all rats were injected subcutaneously with 1.0 mg/kg amphetamine and then monitored for an additional 120 min.

Catalepsy

Catalepsy was assessed via the bar test, as described in Sanberg et al (1988). The front limbs of the rats were placed over a 2-cm high horizontal bar. Catalepsy was measured by the time the rats remained in this position on 3 consecutive trials (maximum 120 seconds). An average of the 3 trials was used for the catalepsy score.

Rotarod

Rats were given 3 days of rotarod training consisting of 3-4 trials/day on an accelerating rod (0-13 rpm over 60 seconds) with a trial duration of 120 sec; the inter-trial interval was 30 min. On the test day (day 4), rats were given 3 additional training trials to ensure learned task

performance. Only animals that remained on the accelerating rod for 2 out of the 3 trials were used for testing. Test compounds were then administered (again by oral gavage) and at the appropriate pretreatment interval rotarod performance was assessed during 2 rotarod test trials.

Conditioned Avoidance Responding (CAR)

Conditioned avoidance training was conducted using commercially available shuttle boxes (Gemini Avoidance System, San Diego Instruments). The Plexiglas shuttle boxes were divided into two equally-sized compartments by a guillotine door. The shuttle boxes were fitted with a stainless steel grid rod floor and wired for presentation of an electric foot shock. Additionally, each side of the chamber was equipped with a houselight, speaker for delivering a tone, and multiple infrared photobeam detectors that tracked the location of the subject within the chamber. Training sessions consisted of a 2 min acclimation period followed by 11 trials presented on a variable interval schedule (intertrial interval of 25-40 sec). Each trial consisted of a 10 sec warning tone (85 dB) and illumination of the houselight (conditioned stimulus) followed by a 10 sec foot shock (0.7 mA; unconditioned stimulus) presented through the grid floor on the side where the animal was located. The shock was terminated when the animal crossed over to the other compartment or after 10 sec had lapsed. Active avoidance was defined as crossing over into the opposite compartment during the 10 sec conditioning stimuli (light and tone pairing) thereby preventing the delivery of the shock. Crossing over to the other compartment after the delivery of the shock terminated shock delivery and was recorded as an escape response. If the animal failed to cross over to the other compartment following termination of the shock the response was recorded as an escape failure. Training was continued until stable performance was obtained and a criterion of 80% avoidance responding for 3 consecutive days was achieved.

Drug testing occurred twice a week with training trials interspersed among the test trials and the animals had to maintain an 80% avoidance response accuracy to be included in the test.

Prepulse Inhibition (PPI)

To assess the effects of JWS on sensorimotor gating, a PPI procedure was conducted as described in detail previously (Hohnadel et al., 2007). Briefly, four startle chambers (San Diego Instruments, San Diego, CA) were used, the background white noise was set at 70 dB, and the PPI trials consisted of a prepulse (20 ms burst of white noise with intensities of 75, 80, or 85 dB) followed, 100 ms later, by a startle stimulus (120 dB, 20 ms white noise). PPI was calculated according to the formula: [100 - (startle amplitude on prepulse-pulse trials ÷ startle amplitude on pulse alone trials) x 100]. The mean level of PPI (i.e., averaged across the 3 prepulse intensities) was also analyzed. Rats were administered vehicle (VEH), PPI impairing agents, JWS, or reference doses of risperidone or donepezil (as positive controls), plus the PPI impairing agents before being evaluated in the PPI procedure. For the apomorphine and MK-801 reversal studies, test subjects were administered vehicle, JWS or risperidone dissolved in vehicle by oral gavage 30 minutes before testing followed by either saline, MK-801 0.1 mg/kg, or apomorphine 0.5 mg/kg s.c., 10 minutes before testing. For the scopolamine-reversal studies, scopolamine hydrobromide 0.33 mg/kg was administered i.p. 40 min before testing followed by vehicle, JWS, or donepezil (by oral gavage) 20 min before testing. The compounds used to disrupt PPI (and their doses) were based on earlier studies (Mansbach et al., 1988; Mansbach and Geyer, 1989) and recent work in our laboratory (Hohnadel et al., 2007).

Spontaneous Novel Object Recognition Test (NOR)

NOR tests were conducted as described in detail previously (Terry et al., 2007). Briefly, 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-minute information session (i.e., the A/A session with identical objects) followed by a 1, 15, or 60 min delay period (administered in a pseudorandom order), and a subsequent 3-min dissimilar stimuli (A/B) session. The objects discriminated were made of glass, ceramic, clay, or plastic. The total exploration time that the subjects spent investigating the each object was recorded. A discrimination index (d2) was calculated on each A/B trial and was defined as the difference in time spent exploring the novel and familiar objects divided by the total exploration time for both objects: d2 index = (novel-familiar)/(novel + familiar). This measure is considered an index of recognition memory and takes into account individual differences in the total amount of object exploration time. For the evaluation of JWS in the scopolamine impairment model, scopolamine hydrobromide was administered subcutaneously 40 min before the A/A session; donepezil, as a positive control, or JWS were administered by oral gavage 20 min before testing.

Five-Choice Serial Reaction Time Task (5C-SRTT)

5C-SRTT Training and testing was conducted using six ventilated, sound attenuated operant chambers (Med Associates, St. Albans, VT, USA) as we have described previously (Middlemore-Risher et al., 2010). Each operant chamber consisted of 9 nose pokes/apertures, 4 of which were closed off with metal inserts, leaving every other nose poke available (2.5cm wide, 4cm deep). The apertures were arranged on a curved panel 2 cm above the floor and were equipped with a photocell beam to detect nose pokes. There was a lamp (2.8W) on the rear wall of each aperture that could be illuminated randomly and for varying durations. Food pellets were delivered automatically to a magazine on the opposite wall to the nose pokes. A light inside the food magazine was also turned on to indicate that a pellet (45mg chow pellet, BioServ,

Frenchtown, NJ, USA) had been dispensed. The food magazine was equidistant from all nose poke apertures. There was a house light that remained on for the entire session unless an error or omission occurred; the light was located towards the roof of the operant chamber above the magazine. The apparatus was controlled using MedPC software (Med Associates, St. Albans, VT, USA).

Week one consisted of handling animals to reduce stress and anxiety in preparation for training and testing. Week 2 consisted of handling and food restriction. Week 3 consisted of food restriction, habituation to the apparatus and preparation for training. Specifically, on days 1 and 2 of week 3 subjects were placed in the operant chambers for 15 minutes with the house light and magazine light on and 10 pellets in the magazine dispenser. On days 3-5 of week 3 animals performed a non-spatial training program. Briefly, the animals were placed in the 5C-SRTT operant chamber with the house light illuminated. A pellet was released into the food magazine which was simultaneously illuminated for a maximum of 5 sec or until the animal collected the pellet. After a 10 sec interval another pellet was released from the simultaneously illuminated food magazine. This continued until the 15 min habituation period expired.

Spatial training in the task began on day 1 of week 4. Animals began with the stimulus duration of 10 sec, each session being 100 trials or 30 min in duration. An initial pellet was delivered to the magazine to facilitate the start of each session. One of the 5 nose poke apertures was illuminated randomly for 10 sec after which the light was extinguished. The animal was then required to respond correctly by nose poking the previously illuminated aperture within 5 sec of the light being extinguished. A correct response in the previously established time frame (5 sec) resulted in a pellet being dispensed into the magazine that was simultaneously illuminated for a maximum of 5 sec or until the animal retrieved the pellet. Collection of this pellet initiated

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the intertrial interval, a delay of 5 sec, before the next trial began. An incorrect response, premature response, or failure to respond (omission) resulted in a 5 sec timeout, marked by the extinction of the house light for 5 sec and no food reward, after which the animal initiated the next trial by a nose poke into the magazine. Animals were trained 5 days a week until they reached stable performance levels at the 10 sec stimulus duration (stable performance criterion defined as 3 consecutive days at >80% accuracy, <20% omissions and completion of all 100 trials). Once this criterion was achieved the animals were moved to the next, more challenging stimulus duration and the same performance criterion was applied before the next progression. The following stimulus durations were employed: 5 → 2.5 →1.25 → 1.0 → 0.8 → 0.6 → 0.5 seconds. The animals were required to meet criterion for a minimum of 3 days at the 0.5 second stimulus duration prior to being moved to the testing groups. Reaching criterion at the 0.5 second stimulus duration required on average 42±1.36 sessions.

The following parameters were measured to assess performance: *% correct* = [# correct /(# correct + # incorrect)]x100; *% omissions* = [# omissions/(# trials completed)]x100; *% premature responses (impulsivity)* = [(premature responses/trials initiated)] x 100 (specifically, the % of responses made after the trial began, but before onset of the light stimulus, i.e., during the 5 second intertrial interval); *% perseverative responses (compulsivity)* = [(perseverative responses/correct responses)] x 100 (specifically, the % of nose pokes made after the correct response has been made but before collecting the reward). Responding could occur in the aperture where the responding has just earned a food reward or at another location. *Trials completed* = (# correct + # incorrect + # omissions); *Latency to correct* = time elapsed from the onset of the light stimulus to making the correct nose poke response; *Latency to incorrect* = time elapsed from the onset of the light stimulus to making the incorrect nose poke response; *Latency*

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to reward = time elapsed from making a correct nose poke response to retrieving the food reward from the magazine.

Non-human Primate Studies

Test Subjects- Non-human primate subjects were 5 male and 4 female pigtail macaques (Macaca nemestrina). Subject information is summarized in Table 4. Each animal was well trained (>100 individual sessions) in the delayed matching-to-sample (DMTS) task. The animals were maintained on tap water (unlimited) and standard laboratory monkey chow (Harlan Teklad Laboratory monkey diet, Madison, WI) supplemented with fruits and vegetables. Food was removed from cages at about 0630 hours, and replaced after the completion of testing of all subjects for the day (at about 1630 hours). Additional nourishment was derived from 300 mg reinforcement food pellets (commercial composition of standard monkey chow and banana flakes, Noyes Precision food pellets, P.J. Noyes Co., Lancaster, NH) obtained during experimental sessions. On weekends animals were fed without time restrictions. Room temperature and humidity were maintained at $22\pm0.6^{o}C$ and $52\pm2\%$, respectively.

Each test subject had previously participated in one or more short-term studies assessing the effects of reversible drugs on DMTS performance. Prior drug experience produced no observable untoward effects in the animals, and each subject received at least a 4-week washout period (with continued weekday DMTS testing) prior to the start of this study.

Drug Administration - JWS was dissolved in μl amounts of dimethylsulfoxide (DMSO), subsequently diluted in commercial fruit juice and administered orally 30 min before behavioral testing. Five doses of JWS, 0.05, 0.10, 0.50, 1.0, and 2.0 mg/kg were evaluated. Control sessions consisted of oral administration of fruit juice/DMSO only.

Delayed Match to Sample (DMTS) Testing

DMTS testing was conducted using a modification of the procedure we have described previously (Terry et al., 2005). Test panels attached to each animal's home cage presented the task by using a computer-automated system. The test system included a touch-sensitive screen (15 inch AccuTouch LCD Panelmount TouchMonitor) and pellet dispenser units (Med Associates) mounted in light-weight aluminum chasses. The stimuli included red, blue, and yellow rectangles presented against a black background. A trial was initiated by presentation of a sample stimulus composed of one of the three colors. The sample stimulus (located above and centered between the two choice stimuli) remained in view until the monkey touched the screen within the borders of the sample rectangle to initiate a pre-programmed delay interval. Touching a stimulus provided the illusion that the figure was actually depressed. Following the delay interval, the two choice stimuli were presented. One of the two choice colors was presented so that the color of one stimulus matched the color of the sample stimulus. A correct (matching) choice was reinforced. Non-matching choices were neither reinforced nor punished. The inter-trial interval was 5 sec and each session consisted of 96 trials. The presentation of stimulus color, choice colors, and choice position were fully counterbalanced so as to relegate non-matching (mediating) strategies to chance levels of accuracy. Three to five different presentation sequences were rotated through each daily session to prevent the subjects from memorizing the first several trials. Delay (memory retention) intervals were established during several non-drug or vehicle sessions prior to initiating the study. The duration for each delay interval was adjusted for each subject until three levels of group performance accuracy were approximated: zero delay (85-100% of trials answered correctly); short delay interval (75-84% correct); medium delay interval (65-74% correct); and long delay interval (55-64% correct). For this subject population, the average delay intervals for each category are provided in Table 4. The assignment of delay

intervals was necessary to avoid ceiling effects in the most proficient animals during drug studies, and to insure that each animal began testing at relatively the same level of task difficulty. Failure to respond during a trial initiated the next trial in the sequence. Task accuracy (% trials correct) was determined only from the total number of trials actually completed.

Delayed Match to Sample with Distractor (DMTS-D) Testing

In a separate experimental series, distractor stimuli (interference trials) were presented on 24 of the 96 trials completed during distractor DMTS sessions. The stimuli were presented simultaneously on the sample and choice keys for 3 sec and they consisted of a random pattern of the three colored rectangles flashing in an alternating manner. The distractor rectangles were comprised of the same three colors used for sample and choice stimuli presentation. The total duration of presentation for a given colored light was 0.33 sec. Distractor stimuli were presented an equal number of times on trials with short, medium, and long delay intervals. The distractor sequence was initiated 1 sec into the delay interval. Three response latencies also were measured: the "sample latency", which is the time between presentation of the sample color and the animal pressing in sample rectangle; and the "choice latency" which is the time between presentation of the choice colors and the animal pressing one of the choice rectangles. Choice latencies were divided into those associated with correct and incorrect responses.

Statistical Analyses

For one and two factor comparisons, analysis of variance (with repeated measures when indicated) was used followed by the Student Newman Keuls or Dunnett's method (for comparisons to vehicle controls only) for post hoc analysis (SigmaPlot 11.2). When multiple factors were analyzed (NOR), a multi-factorial analysis of variance (ANOVA) with repeated measures (SAS, JMP statistical software package) was used. For post hoc comparisons, an

orthogonal multi-comparison t-test (Bonferroni corrected) was used to compare individual means. For each figure presented error values denoted by ± indicates the standard error of the mean. Differences between means from experimental groups were considered significant at the p<0.05 level. Trends toward significance were considered at the *p*<0.10.

Results

Pharmacological Activity of JWS-USC-75IX (in vitro)

The results of the initial pharmacological screen for JWS activity across more than 60 neurotransmitter receptors, transporters, ion channels and the enzyme AChE are provided in Table 1. Significant activity at any of these sites was defined as the inhibition of ligand binding (or AChE activity) by $\geq$ 70% (indicated by the bolded text in Table 1). Using this criterion significant activity was observed at muscarinic M_1, M_2 and M_4 receptors, at serotonin $5HT_4$ receptors, and at sigma 1 receptors. To further investigate activity at the targets identified in the initial screen, additional ligand binding experiments were conducted using 3 concentrations of JWS (100.0 nM, 1.0 μM, and 10.0 μM). The results of these experiments are provided in Table 2. The most notable observation from these experiments was the >96% inhibition of ligand binding at M_2 receptors by 1.0 μM JWS. Based on these results, IC_{50} and K_I values were determined in pharmacological displacement assays and subsequent functional assays were conducted to determine agonist/antagonist activity (and the K_B value). The results of these assays indicated that JWS is a functional antagonist at M_2 receptors with a K_B of approximately 320 nM. Inhibitory activity of JWS at AChE was also observed in the initial pharmacological screen (see Table 1). Subsequent enzyme assays using multiple concentrations of JWS indicated that JWS inhibits both AChE and BChE at low (μM) concentrations (see Table 2). The commonly prescribed AChEIs, galantamine, rivastigmine, and donepezil were also evaluated in these assays as positive controls. The IC_{50} values determined for galantamine, rivastigmine, and donepezil agree well with published values (Schott et al., 2006; Zhang et al., 2009) and indicate that donepezil is the most potent AChEI followed by galantamine and rivastigmine, and that

rivastigmine is the most potent inhibitor of BChE. In these experiments, JWS was found to be a modest AChEI with an IC_{50} value that is similar to galantamine.

Rodent Behavioral Studies

Effects of JWS-USC-75IX (JWS) on locomotor activity and motor function

Amphetamine-Induced Locomotor Activity- Fig 1A illustrates the dose-effect relationship for risperidone and JWS (compared to vehicle) in the amphetamine-induced locomotor activity test. There was a highly significant difference between the study groups, main effect for treatment, $F(7,80) = 7.9$, $p<0.001$. Post hoc analysis indicated (as expected) that amphetamine (1.0 mg/kg) produced a robust increase in locomotor activity ($p<0.001$ versus the vehicle response) and that risperidone attenuated the effects of amphetamine in a dose-dependent manner ($p<0.05$ for the 0.3 and 1.0 mg/kg dose compared to amphetamine alone). In contrast, the amphetamine-induced increase in locomotor activity was not attenuated by any of the JWS doses that were evaluated (1.0-10.0 mg/kg).

Catalepsy- Fig 1B illustrates the dose-effect relationship for haloperidol and JWS (compared to vehicle) as well as the combination of JWS with haloperidol (0.5 mg/kg) in the catalepsy test. There was a highly significant difference between the study groups, main effect for treatment, $F(12,156) = 26.2$, $p<0.001$. Post hoc analysis indicated that haloperidol dos-dependently increased the mean catalepsy score, that JWS (when administered alone) did not significantly affect the catalepsy score (compared to vehicle), and finally, that JWS (at the doses evaluated) was unable to attenuate the catalepsy response induced by haloperidol 0.5 mg/kg.

Rotarod- Fig 1C illustrates the dose-effect relationship for JWS (compared to a reference dose of haloperidol and vehicle) in the rotarod test. There was a highly significant difference between the study groups, main effect for treatment, $F(4,25)= 24.1$, $p<0.001$. Post hoc analysis

indicated that latencies (to fall off of the rotarod) in rats administered haloperidol were significantly lower than that associated with vehicle administration (p<0.001) and that animals administered JWS were not significantly different from vehicle-controls.

Conditioned Avoidance Responding (CAR)- Two separate vehicle-controlled studies were conducted to evaluate the effects of oral administration of haloperidol and JWS on CAR. The results of these experiments are presented in Fig 1D. In the haloperidol experiments, there was a highly significant main effect of dose, $F_{(4,48)}= 20.9$, p<0.001, behavioral response (avoidance, escape or escape failure), $F_{(2,96)}= 187.8$, p<0.001, and there was a significant dose x behavioral response interaction $F_{(8,96)}= 61.9$, p<0.001. Post-hoc comparisons indicated that haloperidol significantly reduced avoidance responding and increased escape responses at doses of 0.3, 0.6 and 1.2 mg/kg (*p*<0.001 for these 3 doses). Haloperidol did not elicit escape failure responding (*p*>0.05 for all doses compared to vehicle). In the JWS experiments, there was a highly significant behavioral response interaction $F_{(2,80)}= 423.3$, p<0.001 (most all subjects exhibited avoidance responding and very few exhibited escape responses or escape failures), however there was neither a significant main effect of dose of JWS, nor a significant dose x behavioral response interaction (p>0.05).

Prepulse Inhibition (PPI) Experiments

In all of the PPI studies described below, there was a highly significant reduction in the startle response which was dependent upon the magnitude of the prepulse stimulus (i.e., prepulse level difference p<0.001 in all studies-see the open bars in the A insets of Figs. 2-5).

Effects of JWS alone on Startle and PPI

As indicated in Fig. 2A, JWS improved the PPI response when compared to vehicle, main effect for treatment, $F_{(5,112)}=2.5$, p=0.034, however, the treatment x prepulse level

interaction was not significant. Post hoc analyses indicated that JWS (0.3 and 1.0 mg/kg) significantly (p<0.05) improved PPI at the 75 and 80dB levels. The positive effect of JWS was also apparent when the data were averaged across the prepulse levels (see Fig. 2C). There were no significant effects of JWS on startle amplitude (Fig. 2B).

Effects of the JWS on Pharmacological Inhibitors of PPI

Attenuation of apomorphine - As indicated in Fig. 3A, there were significant differences in responses to the various drug treatments in the apomorphine-reversal study, main effect for treatment, $F(7,107)=6.3$, $p=<0.001$; and the treatment x prepulse level interaction was statistically significant as well, $F(2,214)=2.4$, $p=0.004$. Post hoc analyses indicated that apomorphine (0.5 mg/kg) significantly (p<0.05) diminished PPI at all three prepulse levels when the effect was compared to the vehicle-associated response. Risperidone 0.1 mg/kg (as a positive control) significantly antagonized the effects of apomorphine on PPI at all 3 prepulse levels. The effect of risperidone on the APO associated response was also significant when the data were averaged across prepulse intensity (Fig 3C). Post hoc analyses further indicated that doses of JWS ranging from 0.1 to 3.0 mg/kg (depending on the prepulse level) significantly (p<0.05) or nearly significantly (p<0.09) attenuated the deficits in PPI produced by apomorphine (Fig 3A). The positive effects of JWS on PPI were also apparent when the data were averaged across the prepulse levels (see Fig. 3C). There were no significant effects of apomorphine, risperidone, or the JWS-apomorphine combinations on startle amplitude (Fig. 3B).

Attenuation of MK801- As indicated in Fig. 4A, there were significant differences in response to the various drug treatments in the MK-801-reversal study, main effect for treatment, $F(7,128)=13.8$, $p<0.001$ and the treatment x prepulse level interaction was nearly significant, $F(2,256)=1.6$, $(p<0.08)$. Post hoc analyses indicated that MK801 (0.1 mg/kg) significantly

(p<0.05) diminished PPI (at all prepulse levels) when compared to the vehicle-associated response. Risperidone 0.1 mg/kg (as a positive control) significantly antagonized the effects of MK-801 on PPI at all 3 prepulse levels. Post hoc analyses further indicated that doses 0.1, 0.3 and 10.0 mg/kg (primarily at the 75 and 80 dB prepulse level) significantly (p<0.05) or nearly significantly (p<0.07) attenuated the deficits in PPI produced by MK-801. The positive effects of JWS on PPI were limited to the 0.3 mg/kg dose of JWS when the data were averaged across the prepulse levels (see Fig. 4C). There were significant treatment-related effects on startle amplitude in the MK-801 reversal study (Fig. 4B). Specifically, MK-801 was associated with a significant increase in startle amplitude, an effect that was reversed by risperidone. There were no significant effects of the JWS-MK-801 combination on startle amplitude when compared to the other treatment groups.

Attenuation of scopolamine - As indicated in Fig. 5A, there were also significant differences in response to the various drug treatments in the scopolamine-reversal study, main effect for treatment, $F(8,37)=2.6$, $p=0.01$, however, the treatment x prepulse level interaction was not significant. Post hoc analyses indicated that scopolamine (0.33 mg/kg) significantly (p<0.05) diminished PPI (at all prepulse levels) when compared to the vehicle-associated response. Donepezil 3.0 mg/kg (as a positive control) significantly antagonized the effects of scopolamine on PPI at all 3 prepulse levels. Post hoc analyses further indicated that only the 10 mg/kg dose of JWS significantly (p<0.05) attenuated the deficits in PPI produced by scopolamine. When the data were averaged across the prepulse levels (see Fig. 5C), there was also a trend (p<0.07) toward attenuation of the scopolamine-related response associated with the 3.0 mg/kg dose. There were also significant treatment-related effects on startle amplitude in the scopolamine reversal study (Fig. 5B). Specifically, in all groups in which scopolamine was administered

(with the exception of the subjects also administered the 1.0 and 10.0 mg/kg doses of JWS) a significant (p<0.05) decrease in startle amplitude was observed (compared to the vehicle-control group.

Spontaneous Novel Object Recognition Test (OR)

Fig 6 illustrates the effects of JWS treatment on performance in the OR task in a scopolamine-impairment model (only the A/B sessions are illustrated). In statistical analyses the most notable results were the significant main effects of treatment $F(7,297)=10.2$, $p<0.0001$, object type (i.e., novel versus familiar), $F(1,297)=89.2$, $p<0.0001$, the treatment x delay interaction, $F(14,297)=2.5$, $p<0.003$, and the treatment x object type interaction, $F(7,297)=3.6$, $p<0.001$. Post hoc analysis indicated that the significant preference for the novel object was lost in animals treated with scopolamine at all three delay intervals (i.e, no * over the black-filled bars) and that donepezil 2.0 mg/kg (as a positive control) restored the preference (note the * over the black-filled bars) at all three delay intervals. In addition, depending on the dose and delay interval, JWS restored the preference for the novel object. The positive effects of donepezil and JWS were further exemplified by the analysis of the d2 index for the overall response (averaged across delay interval, see Fig 6 inset).

Five-Choice Serial Reaction Time Task (5C-SRTT)

In the variable stimulus duration version of the 5C-SRTT (Fig 7A), JWS was associated with improvements in accuracy, main effect of dose $F(3,15)=5.0$, $p=0.013$, stimulus duration ($F_{(2,29)}= 30.90$, $p<0.001$), dose by stimulus duration interaction ($F(6,29)= 0.33$, $p=0.92$. Post hoc analysis indicated (as expected) that accuracy in all subjects was reduced with each decrease in stimulus duration and, further, the 1.0 mg/kg dose of JWS was associated with an increase in accuracy compared to vehicle controls ($p=0.035$). This improvement in accuracy was not

dependent on stimulus duration (p<0.05 versus control for all 3 SDs). In addition, there were no significant effects of JWS on the number of premature or perseverative responses (Fig 7A, middle and far right) or on the mean latencies associated with correct responses, incorrect responses, and reward collection (Table 3, Top section). Likewise, there were no significant effects on the number of omissions, or the number of trials completed.

In the standard version of the 5C-SRTT (single 0.5 sec SD), JWS was evaluated for its ability to attenuate impairments induced by the glutamate NMDA antagonist, MK-801. The results of these experiments are provided in (Fig 7B) and the lower portion of Table 3. In the accuracy assessment, there was a significant main effect of treatment $F(4,44)=6.5$, $p<0.001$. Post hoc analyses indicated that MK-801 impaired accuracy of the task (p<0.05 versus vehicle) and the 0.3 mg/kg dose of JWS attenuated this impairment (p<0.05 versus MK-801). Likewise, MK-801 increased the number of premature responses, an effect that was attenuated by all three doses of JWS, main effect of treatment $F(4,44)=13.8$, $p<0.001$, post hoc, p<0.05 for JWS versus MK-801 for all three doses. The other notable observation was that MK-801 decreased the number of trials completed and, again, all three doses of JWS attenuated this effect, $F(4,44)=5.4$, $p=0.001$, post hoc results, p<0.05 for JWS versus MK-801 all three doses (see Table 3 bottom). MK-801 also slightly (but significantly) decreased magazine latencies and the number of perseverative responses (see Table 3 and Fig 7B respectively), but this outcome was not affected by JWS.

Non-Human Primate Studies

Standard DMTS- Fig 8 illustrates the dose-effect relationship for JWS in nine adult pigtail monkeys 1 hr after drug administration. The data are presented for each drug dose associated with each of the four retention intervals. Accuracies in the standard DMTS task after vehicle administration approximated the criteria set forth above in the methods section: Zero

delay, 98.6%, Short delay 84.2%, Medium delay 69.6%, and Long delay, 59.5% correct. There was not a significant main effect of dose $F_{(5,35)}=1.22$, $p=0.32$, however, there was significant effect of delay $F_{(3,102)}=232.6$, $p<0.001$ and a significant dose $\times$ delay interaction $F_{(15,102)}=2.29$, $p=0.008$. Post-hoc analyses indicated that the 1.0 mg/kg dose significantly improved DMTS accuracy ($p<0.05$) at the medium delay interval and that the 0.5 and 2.0 mg/kg doses improved accuracy at the long delays.

DMTS with Distractor (DMTS-D)-The effects of JWS administration on the performance by nine adult monkeys in the distractor version of the DMTS task are presented in Fig 9. Three data sets (designated as "treatments") were compared in the analysis of the non-distractor trial component of the Distractor-DMTS task. The first data set included baseline sessions of the Standard DMTS task run both before and after Distractor-DMTS sessions. The Standard DMTS sessions were included to allow evaluation of any carryover effect of the distractor to the non-distractor trials in subsequent sessions. The second data set included sessions in which vehicle preceded non-Distractor-DMTS sessions. The third data set included sessions in which JWS preceded non-Distractor-DMTS sessions. In the non-Distractor Trials (Fig 9 Top) the main effect of treatment was statistically significant, $F_{(6,42)}=5.85$, $p<0.001$), there was significant effect of delay $F_{(3,126)}=83.42$, $p<0.001$, however, the treatment $\times$ delay interaction was not significant, $F_{(18,126)}=1.04$, $p=0.42$. Post hoc analysis indicated that the distractor significantly ($p<0.05$) impaired performance at the short delays (compared to standard DMTS-vehicle trials), but there were no significant effects of JWS.

Three similar data sets to that described above were also compared with the distractor trial component of the Distractor-DMTS task. In the distractor trials (Fig 9 bottom) the main effect of treatment was also statistically significant, $F_{6,42}=3.57$, $p<0.01$, there was significant

effect of delay $F(2,84)=3.91$, $p<0.05$, however, the treatment $\times$ delay interaction was not significant, $F(12,84)=1.15$, $p=0.33$. Post hoc analysis indicated that the distractor significantly ($p<0.05$) impaired performance at the short and medium delay intervals (compared to standard DMTS-vehicle trials), and that there was trend ($p<0.08$) toward an attenuation of the distractor effect at short delays at the 1.0 mg/kg dose of JWS. A separate analysis was conducted in which the data were averaged across delays. In this analysis (one-way repeated measures ANOVA), the 1.0 mg/kg dose was associated with a significant ($p<0.05$) attenuation of the effects of the distractor (see the inset at the bottom of Fig 9).

Discussion

The results of this study can be summarized as follows: 1) The ranitidine analog JWS inhibits AChE and BChE at low (µM) concentrations and it is a functional antagonist at M_2 receptors, 2) JWS does not exhibit the classic features often observed in currently marketed antipsychotic drugs such as the ability to attenuate amphetamine-enhanced locomotor activity, induce catalepsy, alter conditioned avoidance responding, or impair motor function. 3) JWS does appear to have the ability to improve information processing (specifically sensorimotor gating) as indicated by its ability to improve PPI in non-impaired adult rats and to attenuate deficits in three pharmacologic models of PPI impairment. 4) JWS also attenuated scopolamine-related impairments in an NOR task and improved 5C-SRTT performance in rats (in both a variable stimulus duration version of the task and an MK-801-impairment model), indicating its ability to improve recognition memory and sustained attention, respectively. 5) Likewise, in monkeys, JWS (depending on the dose) was associated with modest improvements in the performance of a DMTS (working/short term memory) task and a distractor (attention-related) version of the DMTS task. Collectively, these data (combined with the previously published results) indicate that JWS improves information processing and cognitive function in both rodent and non-human primate models.

The results of the pharmacological screen confirmed earlier work where the drug was found to have high affinity at M_2 receptors, and moderate affinity for AChE. Interestingly, we also observed some (albeit modest) activity at the Sigma 1 receptor. This may be important since this receptor is also now considered a viable therapeutic target for both cognitive and psychiatric disorders (see Espallergues, et al., 2007 and review, Collier et al., 2007). In addition, there were no potent effects on any receptors that would normally be considered a major concern

from an adverse side effect standpoint (α_1, H_1, prostaglandins, gut peptides, etc). Accordingly, it

is also important to note that in the rodent studies conducted to date, we have observed no

adverse effects with intraperitioneal (i.p.) doses up to 10 mg/kg in behavioral studies, and in

earlier studies (Valli et al., 1992) the lethal dose in mice was found to be greater than 160 mg/kg,

i.p. (i.e., approximately 8-fold less toxic than tacrine and 200 fold less toxic than physostigmine).

In the initial set of behavioral experiments JWS was evaluated for effects on locomotor

activity and motor function, specifically in tasks that have commonly been used to screen

compounds for potential antipsychotic activity. Amphetamine-induced hyperlocomotion is

commonly used as a pharmacological model of psychosis in animals (Geyer and Ellenbroek,

2003). The model is thought to mimic the hyperdopaminergic tone thought to be present in

many schizophrenic patients (Kapur and Mamo, 2003). While compounds with significant D_2

receptor antagonist activity (i.e., such as both first and second generation antipsychotics) are

active in this model (as might be expected), several agents from different drug classes have

demonstrated activity in this model as well (e.g., H_3 receptor antagonists, phosphodiesterase 10A

inhibitors, see Akhtar et al., 2006; Schmidt et al., 2008, respectively). As noted above, JWS was

not active in this model. JWS was subsequently evaluated in a catalepsy procedure to assess its

potential for inducing extrapyramidal symptoms (a major liability of most of the currently

marketed antipsychotic drugs). Catalepsy refers to an inability of the test subject to modify a

body posture imposed by the experimenter and is generally interpreted as similar to the

extrapyramidal symptoms observed in humans (e.g., such as acute dystonia, akathisia, and

parkinsonism, see Burki, 1979). JWS did not induce catalepsy on its own or potentiate

haloperidol-induced catalepsy (like PDE10A inhibitors can), nor did it attenuate catalepsy

induced by haloperidol. JWS also did not affect the ability of rats to maintain balance on a

rotarod. Finally, JWS was also not active in the conditioned avoidance response task, another method commonly used as a preclinical screen for potential antipsychotic (reviewed, Wadenberg and Hicks, 1999). Effective (currently marketed) antipsychotics appear to have the unique ability to selectively suppress CAR behavior, specifically, in a CAR task where animals are trained to respond to a stimulus within a certain time by moving from one place to another (avoidance), an antipsychotic agent produces a selective suppression of the avoidance response.

The observation that JWS was effective in three pharmacological impairments models of PPI and even improved PPI in non-impaired rats was particularly interesting. Tests of PPI (defined as the reduction in startle response produced by a low-intensity stimulus presented before a high-intensity, startle-producing stimulus, see Graham et al., 1975) are useful for identifying novel therapeutic agents with the ability to improve sensory information-processing deficits, a common feature in several neuropsychiatric conditions. Auditory (sensory) gating deficits in neuropsychiatric conditions are in fact thought to contribute to the deficits in attention, cognitive impairment, and even hallucinations in conditions like schizophrenia (Adler et al., 1998). The pharmacological impairment models used in this study (apomorphine, MK-801, and scopolamine) have been previously used to provide evidence that the neurotransmitters dopamine (Mansbach et al., 1988), glutamate (Mansbach and Geyer, 1989), and acetylcholine (Jones and Shannon, 2000), are each likely to play an important role in normal sensory gating and PPI as well as disorders of these processes. The positive effects of JWS in all three pharmacologic impairments models suggest that JWS (via its multi-target effects) may be able to influence the activity of all three neurotransmitters.

The observation that JWS attenuated scopolamine-related decreases in PPI and OR performance was not altogether surprising since it is an AChEI, an effect that would presumably

result in elevated synaptic acetylcholine levels that could overcome scopolamine antagonism at muscarinic receptors. However, the data do support the premise that cholinergic function is important for optimal OR performance and PPI (and thus a viable target in conditions where it is impaired). We recently reported that the AChEIs galantamine and donepezil can also attenuate scopolamine-related impairments in PPI (Hohnadel et al., 2007). In addition, PPI deficits induced by immunolesions of cholinergic neurons of the nucleus basalis were reversed by the AChEI, rivastigmine (Ballmaier et al., 2002). Additional data to support the role of muscarinic receptors in PPI (and that these receptors could serve as targets for drug development in schizophrenia) have been reported in which, xanomeline, an M_1/M_4 AChR agonist (Jones et al., 2005; Stanhope et al., 2001), normalized apomorphine-related impairments in PPI.

The positive effects of JWS observed in the spontaneous novel object recognition (OR) test complement the results of previous studies in which JWS improved water maze learning and probe trial performance, passive avoidance, and delayed stimulus discrimination (Terry et al., 1999). OR (Ennaceur and Delacour 1988) is a rodent model of recognition memory, which by definition consists of two components, a recollective (episodic) component and a familiarity component (Squire et al., 2004). Recognition memory is demonstrated in the OR task when subjects explore a novel object more than a familiar one.

The JWS-related improvements in performance of the 5C-SRTT and the ability to attenuate impairments in the distractor version of the DMTS indicate the potential of JWS to improve attention and decrease distractibility, attractive features that could be useful for the treatment of multiple neurologic and psychiatric disorders. Performance of the rodent 5C-SRTT is considered to be analogous to the various versions of the human continuous performance test (CPT) of sustained attention (Robbins 2002). Attention, as indexed by the accuracy

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measurement in addition to inhibitory response control (thought to be a form of executive functioning) is assessed. In the current study JWS was able to improve accuracy (a variable stimulus duration version of the task) as well as to attenuate both the accuracy deficits and the elevated premature responses induced by MK-801. Premature responses, which occur during the inter-trial interval before the target stimulus has been presented, are generally interpreted as a form of impulsive behavior. Thus, improvements in accuracy and a decrease in impulsive-like behaviors in a NMDA antagonist model could have special relevance for neuropsychiatric conditions such as schizophrenia. Finally, the positive effects of JWS in the DMTS task were encouraging since it can be used to assess behaviors that are relevant to human cognition such as attention, strategy formation, and working memory (Paule et al. 1998).

There are some limitations to this study that should be discussed. While the salient pharmacological properties of JWS (M_2 and AChE antagonist activity) would suggest efficacy in conditions like AD, we did not evaluate JWS in AD animal models specifically. While the scopolamine impairment procedure (used in this study) is a commonly employed pharmacological model with relevance to AD (especially the cholinergic impairments), it would be interesting to evaluate JWS in transgenic mouse models of AD as well as aged animals. In addition, given that only specific doses of JWS were associated with statistically significant effects on DMTS performance in monkeys, it is unclear whether the positive effects would be potent enough to be observed clinically.

In conclusion JWS (potentially via effects at several drug targets) improves information processing, attention, and memory-related task function in animal models. The data support the role of JWS as a prototypical representative of a novel class of therapeutic agents for disorders of cognition such as AD. The positive effects observed in the PPI and 5C-SRTT studies in rats, and

the DMTS-D studies in monkeys, suggest that JWS might be useful in neuropsychiatric conditions not necessarily associated with advanced aged such as schizophrenia. The results of this study also support the potential value of developing single molecular entities with multiple therapeutic targets for neuropsychiatric disorders. Additional research is currently underway to further explore the multi-receptor targeting properties and therapeutic potential of JWS analogues.

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Authorship Contribution

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Footnotes:

Legends for Figures

Fig 1. **A**: Effects of JWS-USC-75IX (JWS) on locomotor activity and motor function. **A**: Effects of oral administration of JWS (compared to the second generation antipsychotic risperidone) on amphetamine (1.0 mg/kg injected subcutaneously)-induced locomotor activity (total photobeam breaks). **B.** Effects of oral administration of the first generation antipsychotic haloperidol, JWS alone, and JWS combined with haloperidol on the mean catalepsy score. **C.** Effects of oral administration of JWS (compared to haloperidol) in the rotarod task. **D.** Effects of oral administration of haloperidol compared to JWS (N=11-14) on conditioned avoidance responding. The figure illustrates decreases in avoidance responses and increases in response failures produced by haloperidol and the lack of effects of JWS in rats where behavior was maintained under a discrete-trial avoidance schedule.

Fig 2. **A**: Effects of oral administration of JWS-USC-75IX (JWS) on the percentage of prepulse inhibition (PPI) in rats for three prepulse intensities (5, 10, and 15 dB above background). **B**: JWS effects on the mean startle amplitude to 120-dB, 20-ms noise burst. **C**: JWS effects on the percentage of prepulse inhibition averaged across the three prepulse intensities. VEH = vehicle; JWS = JWS-USC-75IX. Bars represent mean ± S.E.M. for each treatment. * = significantly different from vehicle controls ($p<0.05$); † = $p<0.09$. N=12-18 rats/group.

Fig 3 (A) Effects of apomorphine (0.5 mg/kg injected subcutaneously) and several oral doses of JWS-USC-75IX (JWS) on apomorphine-induced deficits in prepulse inhibition in rats associated with three prepulse intensities (75, 80, and 85 dB). A reference dose of risperidone (0.3 mg/kg) was included as a positive control for attenuating the effects of apomorphine. **(B)** Effects of

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apomorphine and JWS combined with apomorphine on startle amplitude. **(C)** Effects of

apomorphine (0.5 mg/kg) and several doses of JWS on apomorphine-induced deficits in prepulse

inhibition averaged across prepulse level. Bars represent mean ± S.E.M. for each treatment

(N=8-10). VEH = vehicle; APO = apomorphine; JWS = JWS-USC-75IX; RISP = risperidone. #

= significantly different ($p < 0.05$) than the vehicle associated response. * = significantly different

($p < 0.05$) than the apomorphine-associated response.

Fig 4 (A) Effects of MK801 (0.1 mg/kg injected subcutaneously) and several oral doses of JWS-

USC-75IX (JWS) on MK-801-induced deficits in prepulse inhibition in rats associated with three

prepulse intensities (75, 80, and 85 dB). A reference dose of risperidone (0.3 mg/kg) was

included as a positive control for attenuating the effects of MK801. **(B)** Effects of MK801 and

JWS combined with MK801 on startle amplitude. **(C)** Effects of JWS on MK801-induced

deficits in prepulse inhibition averaged across prepulse level. Bars represent mean ± S.E.M. for

each treatment (N=10). VEH = vehicle; MK= MK801; JWS = JWS-USC-75IX; RISP =

risperidone. # = significantly different ($p < 0.05$) than the vehicle associated response. * =

significantly ($p < 0.05$); † = nearly significantly ($p < 0.08$) different from the MK801-associated

response.

Fig 5 (A) Effects of scopolamine (0.33 mg/kg injected intraperitoneally) and several oral doses

of JWS-USC-75IX (JWS) on scopolamine-induced deficits in prepulse inhibition in rats

associated with three prepulse intensities (75, 80, and 85 dB). A reference (oral) dose of

donepezil (2.0 mg/kg) was included as a positive control for attenuating the effects of

scopolamine. **(B)** Effects of scopolamine and JWS combined with scopolamine on startle

amplitude. **(C)** Effects of JWS on scopolamine-induced deficits in prepulse inhibition across prepulse level. Bars represent mean ± S.E.M. for each treatment (N=8-10). VEH = vehicle; SCOP = scopolamine; DON= donepezil. JWS = JWS-USC-75IX. # = significantly different (p<0.05) than the vehicle associated response. * = significantly (p<0.05); † = nearly significantly (p<0.08) different from the scopolamine-associated response.

Fig 6. Effects of scopolamine (0.33 mg/kg injected peritoneally) and several oral doses of JWS-USC-75IX (JWS) on scopolamine-induced deficits in the performance of a spontaneous novel object recognition task. A reference (oral) dose of donepezil (2.0 mg/kg) was included as a positive control for attenuating the effects of scopolamine. The illustrations at the left indicate the preference for the novel object compared with the familiar object (*= p<0.05) at each of the 3 delays. The inset at right illustrates drug effects (averaged across delays) on the "Discrimination Index" (d2) which refers to the proportion of the total exploration time the animal spent investigating the novel object (see Methods); + = significantly different (p<0.01) from vehicle control performance. Data are expressed as the mean ± S.E.M. N=12-18 rats/group

Fig 7. A. Effects of JWS-USC-75IX (JWS) on the performance of a variable stimulus duration version of the five choice serial reaction time task (5C-SRTT). Rats (N=6) were trained to meet specific performance criterion (described in the Methods section) at stimulus duration (SD) of 0.5 sec. Subsequently, shorter SDs (0.10 and 0.25 sec) were presented pseudorandomly along with the 0.5 sec SD. Vehicle and three doses of JWS (administered orally 30 min before testing) were evaluated for effects on task accuracy (% correct), the % of premature responses and the %

of perseverative responses. **B.** Effects of JWS on MK-801-related impairments in the standard version of 5C-SRTT with a 0.5 sec stimulus duration (N=12). Vehicle and several doses of JWS (administered orally 30 min before testing) were evaluated for their ability to attenuate the negative effects of MK-801 (administered s.c., 10 min before testing). Each bar represents the mean ± SEM for each test group. *=significantly different (p<0.05) compared to vehicle-associated performance level. +=significantly different (p<0.05) compared to MK-801-associated performance level (ANOVA).

Fig 8. Effects of JWS-USC-75IX (JWS) on Delayed Match to Sample (DMTS) performance in monkeys. The figure illustrates the dose-effect relationship for each delay in the DMTS task in 9 adult pigtail macaques, 30 min after the oral administration of JWS. Each bar represents the mean (% correct) ± SEM over 96 trials per session. The baseline (Vehicle) was determined from the average of all vehicle sessions run throughout the study. *p<0.05 compared to vehicle baseline levels of DMTS accuracy.

Fig 9. The effect of JWS-USC-75IX (JWS) on the performance in the distractor version of the Delayed Match to Sample task (DMTS-D) in monkeys. The figure illustrates the dose-effect relationship for each delay in the DMTS-D task in 9 adult pigtail macaques, 30 min after the oral administration of JWS. **Top**: Non-distractor-related accuracies (72 trials) are plotted as a function of dose for each of four task delay intervals. **Bottom:** Distractor-related accuracies (24 trials) are plotted as a function of dose for each of three task delay intervals. Mean accuracies associated with the standard DMTS task (DMTS) are included for comparison. **Bottom Inset**: Distractor-related accuracies averaged across delays. Each bar represents the mean ± S.E.M.

*Significantly different ($p<0.05$) compared with respective Vehicle DMTS (non-distractor) mean; + = significantly different ($p<0.05$) compared to Vehicle DMTS-D (distractor) mean. † = p<0.09 compared to Vehicle DMTS-D mean.

Table 1: *in vitro* Binding Profile of JWS-USC-75IX

Target	% Inhibition (10.0 µM)	Target	% Inhibition (10.0 µM)
Adenosine Transporter	-11.25	Nicotinic, Neuronal (a-BnTx insensitive)	52.02
A1 *(h)*	4.25	NE transporter *(h)*	7.73
A2A *(h)*	0.09	Opioid, Delta 2 *(h)*	-0.80
α1A	55.89	Opioid, Mu *(h)*	14.66
α1B	59.63	5-HT Transporter	67.83
α2A *(h)*	16.65	5-HT1A *(h)*	21.51
α2B	53.20	5-HT1D	37.59
α2C *(h)*	22.12	5-HT2A *(h)*	16.67
β1 *(h)*	15.80	5-HT2C *(h)*	14.12
β2 *(h)*	13.88	5-HT3 *(h)*	17.59
DA Transporter	26.35	**5-HT4**	**77.81**
D1 *(h)*	28.40	5-HT5A *(h)*	1.41
D2S *(h)*	13.16	5-HT6 *(h)*	25.36
D3 *(h)*	59.10	5-HT7 *(h)*	35.19
D4.4 *(h)*	-3.00	σ^1	**90.01**
GABA A, Agonist site	-15.06	σ^2	19.92
GABA A, BDZ, α1 site	38.74	Ca^{2+} channel (L-type, Dihydropyridine site)	2.30
GABA B	20.94	Ca^{2+} channel (N-type)	-10.36
Glutamate, AMPA site	-0.90	GABA, Chloride, TBOB site	12.98
Glutamate, Kainate site	5.50	K^+ channel, ATP sensitive	9.41
Glutamate, MK-801 site	-7.92	K^+ channel, Ca^{2+} Act., VI	19.82
Glutamate, NMDA agonist site	-11.47	K^+ channel, I[Kr] (hERG) *(h)*	20.02
Glutamate, NMDA, PCP site	11.83	Na^+ channel (site 2)	6.50
Glutamate, NMDA, glycine (Stry-insens site)	8.06	NO, NOS (neuronal binding)	8.17
Glycine, Strychnine sensitive	6.47	Leukotriene, LTB4 (BLT)	13.83
Histamine, H1	10.48	Leukotriene, LTD4 (CysLT1)	-8.58
Histamine, H2	**92.72**	Thromboxane A2 *(h)*	-7.54
Histamine, H3	63.43	Angiotensin II, AT1 *(h)*	0.25
Muscarinic, M1 *(h,r)*	**80.24**	Bradykinin, BK2	24.55
Muscarinic, M2 *(h)*	**96.69**	Endothelin, ET-A *(h)*	-11.04
Muscarinic, M3 *(h)*	32.00	Neurokinin, NK1	21.31
Muscarinic, M4 *(h)*	**73.80**	Neuropeptide, NPY2 *(h)*	0.10
Muscarinic, M5 *(h)*	44.33	**Acetylcholinesterase *(h)***	**87.98**

Values are expressed as the percent inhibition of specific binding and represent the average of replicate tubes at each of the concentrations tested. Bolded text highlights ≥ 70% inhibition. *h* = human

Table 2: Additional *in vitro* Pharmacology of JWS-USC-75IX

Receptor Binding Target	% Inhibition		
	100.0 nM	1.0 μM	10.0 μM
Muscarinic, M1 (hr)	1.00	64.80	86.71
Muscarinic, M2 (h)	49.50	96.28	99.62
Muscarinic, M4 (h)	1.57	46.39	90.55
serotonin, 5-HT4	-5.68	32.83	94.82
sigma 1 (σ 1)	12.59	54.23	97.89

IC_{50}/K_I Determination — Muscarinic, M_2 (h)

Drug	IC_{50} (nM)	K_I (nM)	Slope
JWS-USC-75IX	166.0	56.3	-1.2
(-) Scopolamine MeBr	1.2	0.41	-1.0

Functional Agonist/Antagonist Determination — Muscarinic, M_2 (h)

Drug	IC_{50} (nM)	K_B (nM)
JWS-USC-75IX	3300.0	320.0
Methoctramine	210.0	20.0

Cholinesterase Inhibitor Properties	AChE IC_{50} (μM)	BChE IC_{50} (μM)
Drug		
JWS-USC-75IX	1.67	4.78
Galantamine	1.12	8.29
Rivastigmine	12.75	1.01
Donepezil	0.05	6.43

Table 3. Effects of JWS USC-75IX in the Five Choice Serial Reaction Time Task (5C-SRTT)

A. Latencies, Omissions, and Trials Completed in the Variable Stimulus Duration Version of the 5C-SRTT (N=6)

Treatment	Stimulus Duration (s)	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle	0.10	0.82±0.08	1.56±0.15	1.59±0.12	2.67±0.99	
	0.25	0.70±0.06	1.64±0.15	1.41±0.16	3.50±0.80	(total)
	0.5	0.73±0.05	1.83±0.18	1.43±0.14	2.58±0.89	100.0±0.0
JWS-USC-75IX 0.3 mg/kg	0.10	0.86±0.11	1.55±0.06	1.37±0.11	2.42±1.08	
	0.25	0.74±0.08	1.67±0.08	1.36±0.14	2.58±0.96	(total)
	0.5	0.82±0.11	1.83±0.20	1.34±0.12	1.75±0.76	100.0±0.0
JWS-USC-75IX 1.0 mg/kg	0.10	0.82±0.10	1.35±0.29	1.48±0.12	3.83±0.87	
	0.25	0.64±0.04	1.26±0.26	1.43±0.12	3.17±1.01	(total)
	0.5	0.72±0.06	1.34±0.42	1.56±0.15	3.17±1.47	100.0±0.0
JWS-USC-75IX 3.0 mg/kg	0.10	0.81±0.11	1.58±0.18	1.39±0.20	1.83±1.17	
	0.25	0.69±0.07	1.38±0.15	1.30±0.14	2.17±1.33	(total)
	0.5	0.64±0.07	1.31±0.27	1.34±0.18	2.00±0.82	100.0±0.0

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B. Effects of MK-801 alone and MK-801 combined with JWS-USC-75IX on Latencies, Omissions, and Trials Completed in the standard version of the 5C-SRTT (0.5 sec stimulus duration) N=12
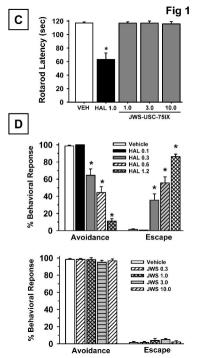
Treatment	Latency Correct (s)	Latency Incorrect (s)	Magazine Latency (s)	% Omissions	Trials Completed
Vehicle + Vehicle	0.71±0.04	1.72±0.07	1.56±0.10	5.06±2.19	99.70±0.16
Vehicle + MK-801	0.68±0.03	1.56±0.16	1.10±0.16*	7.90±0.86	71.50±6.64*
JWS-USC-75IX 0.1 mg/kg + MK-801	0.66±0.02	1.84±0.16	1.07±0.05*	6.70±1.63	87.08±4.55#
JWS-USC-75IX 0.3 mg/kg + MK-801	0.70±0.04	1.72±0.21	1.22±0.13*	6.21±1.44	92.83±5.09#
JWS-USC-75IX 1.0 mg/kg + MK-801	0.69±0.03	1.48±0.17	1.05±0.05*	6.04±1.41	85.88±4.57#

Data are presented as the mean ± SEM. MK-801 dose = 0.05 mg/kg. * = statistically significant difference ($p<0.05$) from vehicle - treated group; # = statistically significant difference ($p<0.05$) from the Vehicle-MK-801 Treated group.

Table 4: Monkey Subject Information

Subject ID	Gender	Age	Wt (kg)	Delay Intervals (sec)		
				Short	Medium	Long
119	Female	20	8.6	5	10	20
146	Male	25	9.6	5	15	30
c8r	Male	11	12.2	10	15	45
p18	Female	14	7.8	15	40	80
pa1	Male	17	17.0	30	30	45
tp8	Female	19	7.2	10	15	35
797	Male	19	15.2	10	20	30
V6t	Male	11	17.0	5	10	20
	Mean	17.00	11.83	11.25	19.38	38.13
	SEM	1.70	1.45	2.95	3.71	6.88

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Fig 8

Fig 9