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Efficacy of VMAT2 inhibition and synergy with antipsychotics in animal models of schizophrenia

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ABSTRACT

Antipsychotic medications function by blocking postsynaptic dopaminergic signaling in the central nervous system. Dopamine transmission can also be modulated presynaptically by inhibitors of vesicular monoamine transporter 2 (VMAT2), which inhibit loading of dopamine into presynaptic vesicles. Here we investigated the combination of these mechanisms in animal models of schizophrenia and weight gain (a primary side effect of antipsychotics). When dosed alone, the highly selective VMAT2 inhibitor RRR-dihydrotetrabenazine (RRR-DHTBZ, also known as [+]-α-HTBZ) elicited efficacy comparable to conventional antipsychotics in pre-pulse inhibition and conditioned avoidance models without eliciting weight gain. In combination experiments, synergy was observed: subthreshold doses of RRR-DHTBZ and risperidone or olanzapine produced robust efficacy, and in dose response experiments, RRR-DHTBZ increased the antipsychotic potency in the efficacy models but did not affect weight gain. The combinations did not affect plasma compound concentrations. The synergy is consistent with VMAT2 inhibition blocking the counterproductive presynaptic stimulation of dopamine by antipsychotics. These results suggest a therapeutic strategy of adding a VMAT2 inhibitor to lower the antipsychotic dose and reduce the side-effect burden of the antipsychotic while maintaining and potentially enhancing its therapeutic effects.

SIGNIFICANCE STATEMENT

Antipsychotics effectively reduce psychotic symptoms and are often necessary and life-changing medications; however, these benefits are at the expense of a high side effect burden. This study shows that combining these postsynaptic dopaminergic modulators with a presynaptic dopamine modulator (VMAT2 inhibitor) potentiates efficacy synergistically in animal models of schizophrenia without potentiating weight gain. Our data suggest that adding a VMAT2 inhibitor may be a viable therapeutic strategy for reducing antipsychotic side effects by lowering antipsychotic dose while maintaining therapeutic efficacy.

INTRODUCTION

Vesicular monoamine transporters (VMATs) are responsible for packaging monoamines into presynaptic vesicles that subsequently fuse with the presynaptic terminal membrane, thereby releasing those monoamines into the synaptic cleft (Erickson and Varoqui, 2000; Erickson et al., 1996). They belong to the superfamily of solute carrier (SLC) transporters (Jankovic, 2016; Lin et al., 2015). The VMAT2 subtype (SLC18A2) is the exclusive monoamine transporter in the central nervous system (CNS) (Erickson et al., 1996). VMAT2 is the site of action of the CNS drug tetrabenazine, which binds selectively to this subtype. First described and characterized in the 1950's (Pletscher, 1957; Quinn et al., 1959), tetrabenazine attenuates behavioral deficits in a variety of animal models and displays efficacy in a number of monoamine-mediated human disorders (Jankovic, 2016). Tetrabenazine and tetrabenazine analogues are currently in clinical use for treating movement disorders, specifically tardive dyskinesia (TD) and chorea associated with Huntington's disease. The ability of tetrabenazine to relieve these symptoms is believed to result from blockade of VMAT2 in the presynaptic terminals of striatal dopaminergic neurons, resulting in a reduction of synaptic dopamine levels (Erickson and Varoqui, 2000; Erickson et al., 1996; Grigoriadis et al., 2017; Vander Borght et al., 1995).

Tetrabenazine was originally developed as an antipsychotic, displaying efficacy for relieving the positive symptoms of schizophrenia (Lingjaerde, 1963; Malik and Balkoski, 2007). This effect can be explained by what has been known as "the dopamine hypothesis of schizophrenia" first adopted by Van Rossum in 1967 that resulted from the observations that amphetamine, a dopamine releaser, exacerbated psychotic symptoms while reserpine, a dopamine release inhibitor, reduced psychotic symptoms (Brill et al., 1967). Originally synthesized in the 1950s at Hoffmann-La Roche, tetrabenazine was identified as a reversible

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dopamine release inhibitor (Pletscher, 1957; Quinn et al., 1959). While a few well-controlled

clinical trials were conducted in psychotic patients at the time showing modest effects of

tetrabenazine, the coincident development of a class of selective high affinity dopamine receptor

antagonist molecules known as the phenothiazines demonstrated superior efficacy in psychotic

patients (Ashcroft et al., 1961). Today, the most commonly used antipsychotics are still those

that block central D2 dopamine receptors. This mode of treatment is the primary mechanism of

action of conventional antipsychotics such as haloperidol, risperidone and olanzapine (Kapur et

al., 1999; Seeman et al., 1976). The efficacy of tetrabenazine as an antipsychotic was likely

a result of reduced D2 dopamine receptor signaling resulting from reductions in synaptic

dopamine content following inhibition of VMAT2 activity. If this is the case, then two

independent mechanisms in the relevant dopaminergic signaling pathways can be targeted for

treating the positive symptoms of schizophrenia with the possibility of reducing the requirement

for high doses of the individual compounds working through the two mechanisms; postsynaptic

signaling (dopamine D2 receptor blockade) and reduced presynaptic dopamine packaging

(VMAT2).

The aim of this study was to use well-established animal models of schizophrenia and

antipsychotic efficacy to determine whether targeting the presynaptic pathway (VMAT2

inhibition) concurrently with the postsynaptic mechanisms (dopamine receptor blockade) results

in synergistic efficacy. In an effort to maintain unambiguous pharmacological effects for these

synergy studies, we selected the single isomer RRR-DHTBZ—the most active of the four

isomers generated from tetrabenazine and the only isomer generated from valbenazine

(also known as [+]-α-HTBZ) —which has exquisite specificity and selectivity for VMAT2 and

is devoid of any other off-target effects (Grigoriadis et al., 2017). Since the other isomers of

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tetrabenazine have multiple pharmacologic activities at monoamine receptors, including

blockade of D2 dopamine receptors, the selective nature of the RRR-DHTBZ isomer offers the

clearest interpretation of data associated with pre- and postsynaptic activity. Efficacy of VMAT2

inhibition alone in animal models of psychosis was first confirmed using RRR-DHTBZ in

prepulse inhibition (PPI) and conditioned avoidance assays and compared to efficacy of

antipsychotic-mediated postsynaptic dopamine inhibition. Once these independent baselines of

efficacy were determined in a dose-dependent manner for each compound, the potential synergy

of VMAT2 inhibition and D2-receptor blocking antipsychotics was evaluated by combining

individual subthreshold doses of RRR-DHTBZ and risperidone or olanzapine in the conditioned

avoidance assay. We further explored the effects of combining RRR-DHTBZ and olanzapine in

an antipsychotic-induced weight gain model, a surrogate for the metabolic syndrome side effects

of conventional antipsychotics, in an attempt to determine the extent to which conclusions could

be drawn on the translatability of these preclinical in vivo effects.

METHODS

Drug compounds and dosing information

Haloperidol, olanzapine and risperidone (all free base) were obtained from Millipore-Sigma (St. Louis, MO). RRR-DHTBZ (camphorsulfonic acid salt) and tetrabenazine hydrochloride were synthesized in-house at Neurocrine Biosciences, Inc. (San Diego, CA). For the behavioral tests, RRR-DHTBZ and tetrabenazine were dissolved in water and administered orally in a dose volume of 1 ml/kg, and olanzapine, risperidone and haloperidol were dissolved in 10% DMSO in water and administered intraperitoneally in a volume of 1 ml/kg. For the weight gain studies, RRR-DHTBZ was dissolved in water and administered orally in a volume of 3 ml/kg; olanzapine was dissolved in 1% methylcellulose and administered intraperitoneally in a volume of 3 ml/kg.

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Neurocrine Biosciences, Inc. and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Upon receipt, all animals remained housed in groups and acclimated to the colony room for at least 1 week prior to testing. During the period of acclimation, animals were examined on a regular basis, handled, and weighed to assure adequate health and suitability. Animals were maintained on a 12:12, light:dark cycle for the duration of the experiments and all behavioral assessments were conducted during the light phase. Room temperature was maintained between 20 and 23°C with a relative humidity between 30% and 70%. Chow and water were provided *ad libitum* for the duration of each study. In each study, animals were randomly assigned to treatment groups which were then balanced across the testing day to account for any potential differences in circadian response.

PPI of startle

RRR-DHTBZ was tested in the PPI of startle task, which is used as a measure of sensorimotor gating. The acoustic startle measures an unconditioned reflex response to external auditory stimulation. PPI, consisting of an inhibited startle response (reduction in amplitude) to an auditory stimulation following the presentation of a weak auditory stimulus, or prepulse, has been used as a tool for the assessment of deficiencies in sensorimotor gating, such as those seen in schizophrenia. It is well established that administration of either typical or atypical antipsychotic compounds increases the percent PPI typically displayed by C57BL/6J mice (Ouagazzal et al., 2001; Ralph and Caine, 2005). A pharmacological model of PPI was not used because the effectiveness of test compounds can be dependent on the mechanism of the compound used to induce PPI (Swerdlow et al., 2008).

Male C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME) were used in this study. Mice were received at 7 weeks of age, and upon receipt, mice were assigned unique identification numbers (tail marked) and group housed in OPTImice cages. The number of animals in each treatment group was between 9 and 11. C57BL/6J mice were pretreated with vehicle or test compound as described in "Drug compounds" above, and placed into holding cages prior to testing starting 30 minutes post-dose. Mice were placed in the PPI chambers (Med Associates, St. Albans, VT) for a 5-minute session of white noise (70 dB) habituation. After the acclimation period, the test session was automatically initiated. The session started with a habituation block of 6 presentations of the startle stimulus alone, followed by 10 PPI blocks of 6 different types of trials. Trial types were as follows: null (no stimuli), startle (120 dB), startle plus prepulse (4, 8 and 12 dB over background noise i.e., 74, 78 or 82 dB) and prepulse alone

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(82 dB). Trial types were presented at random within each block. Each trial started with a 50-millisecond null period during which baseline movements were recorded. There was a subsequent 20-millisecond period during which prepulse stimuli were presented and responses to the prepulse measured. After a further 100 milliseconds, the startle stimuli were presented for 40 milliseconds and responses recorded for 100 milliseconds from startle onset. Responses were sampled every millisecond. The inter-trial interval was variable with an average of 15 seconds (range from 10 to 20 seconds). In startle alone trials, the basic auditory startle was measured, and in prepulse plus startle trials, the amount of inhibition of the normal startle was determined and expressed as a percentage of the basic startle response (from startle alone trials) excluding the startle response of the first habituation block.

Conditioned avoidance response

The conditioned avoidance response (CAR) assay has been shown to be a very reliable animal model for screening antipsychotic drugs (Wadenberg and Hicks, 1999). In the CAR paradigm, an animal is trained to respond to a conditioned stimulus (auditory and visual) by negative reinforcement (foot shock). Numerous studies have shown that typical and atypical antipsychotic drugs selectively suppress a trained CAR, thus making it an ideal assay to screen potential antipsychotic compounds (Wadenberg and Hicks, 1999). The experimental paradigm and behavioral scoring method used was previously described (Kalinichev et al., 2013) and is shown in Fig. 1.

Male Wistar rats from Envigo (Indianapolis, IN) were used in the study. Upon receipt, rats were assigned unique identification numbers and were group housed with 3 rats per cage in polycarbonate cages with micro-isolator filter tops. On each training day, experimental

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animals were acclimated to the test room for at least 30 minutes prior to test. Rats were placed

in the CAR two-way shuttle box and the training period of 20 trials ensued. Each test trial

consisted of a conditioned stimulus (i.e., 10-second presentation of an 80 dB white noise)

immediately followed by a scrambled 0.6 mA foot shock lasting up to 20 seconds. The inter-

trial interval ranged from 20 to 60 seconds. Rats learned to avoid foot shocks by moving from

one compartment to the other when the cue was presented, and this was recorded as an

avoidance response. If the animal failed to move following cue presentation, it received

the foot-shock, during which it could then cross compartments to escape the full shock

duration. If the rat failed to leave the compartment, it endured the full 20-second foot-shock

and an escape failure was recorded. After 3 to 4 weeks of training in the CAR chambers,

rats that passed the testing criterion of performing 16 to 20 avoidance responses while also

successfully escaping the foot shock for 3 days in a row were included in the study.

CAR testing then followed the same paradigm as CAR training with the addition of test

compound dosing. The number of animals in each treatment group was between 7 and 12. In

some cases data from two treatment groups was combined, giving a final group size of up to

24 animals. On the test day, trained rats dosed with vehicle or compound, as described in

"Drug compounds" above. When RRR-DHTBZ and antipsychotic (risperidone or olanzapine)

formulations were administered in combination, they were dosed sequentially one immediately

after the other and rats were returned to their home cage. Following the 30-minute

pretreatment period, the animal was placed into the two-compartment shuttle box. Animals

were subsequently exposed to a series of conditioned stimuli. If the rat moved to the second

compartment upon presentation of the conditioned stimulus, this was scored as an avoidance

(Fig. 1). As mentioned above, an animal not leaving the chamber represents inhibition of

avoidance and is indicative of compound efficacy in the model. Inhibition of avoidance can result from either a true inhibition of the conditioned behavior or from a compound-induced inability of the animal to move from the chamber, however, these possibilities were differentiated by applying a mild foot shock to the test chamber. An animal leaving the chamber subsequent to the initiation of the foot shock (i.e., an escape) displayed a true inhibition of conditioned avoidance as the animal was physically capable of avoiding the shock (Fig. 1). Conversely, an animal not leaving the chamber upon initiation of the foot shock represents a compound-induced sedation or inhibition of movement rather than inhibition of the conditioned behavior and was scored as an escape failure (Fig. 1). Therefore, a potential antipsychotic compound will significantly decrease percent avoidance at a dose that does not significantly increase the number of escape failures. For inhibitors of dopaminergic signaling, escape failure likely results from sedation at lower doses and gross motor impairment at higher doses (Arnt, 1982; Wadenberg et al., 2001).

Catalepsy bar test

To further assess the potential for gross motor impairment, a separate cohort of animals were tested for compound-induced cataleptic effects using the bar test. Male Wistar rats from Envigo (Indianapolis, IN), age-matched to the rats used in the CAR experiments, were used in this study. On the day of testing, rats were brought to the experimental room for at least 1 hour to acclimate. Rats were dosed with vehicle or test compound, as described in "Drug compounds" above, and 30 minutes later cataleptic behavior was assessed. At the start of the assay, the front paws of the experimental subject were placed on a horizontal metal bar raised 6 inches above a Plexiglas platform and time was recorded for up to 60 seconds per trial at each observational time point.

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The assessment ended when the animal's front paws returned to the platform or after 60 seconds.

The test was repeated in triplicate and the average of the 3 trials is reported as the intensity index

of catalepsy. The number of animals in each treatment group was 4 or 5.

Ex vivo VMAT2 occupancy measurement

Occupancy of VMAT2 by RRR-DHTBZ in rats was assessed ex vivo using homogenates of

whole striatum. Male Wistar rats (satellite groups to those used in the CAR studies) were dosed

with RRR-DHTBZ, as described in "Drug compounds" above (n=4 to 5 per treatment group).

Thirty minutes later, animals were sacrificed and whole brain collected, which was then frozen

on aluminum foil on dry ice. On the day of assay, brains were partially thawed on wet ice and

whole striatum dissected and placed in 10 ml assay buffer in a 15 ml centrifuge tube on ice.

Assay buffer comprised Dulbecco's phosphate-buffered saline (DPBS): 1.5 mM KH_2PO_4,

8.1 mM Na_2HPO_4, 2.7 mM KCl, 138 mM NaCl (ThermoFisher Scientific, Waltham, MA),

supplemented with 10 mM $MgCl_2$, 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-

tetraacetic acid, pH 7.4, with NaOH. Assay buffer was maintained on ice throughout the

preparation procedure. Multiple samples (up to 48) were homogenized simultaneously using an

Autogizer (Tomtec Inc., Hamden, CT). Tubes containing the samples were maintained in an ice

bath throughout the homogenization procedure. The tissues were homogenized at 30,000 rpm

for 10 seconds. The samples were then centrifuged at $45,000 \times g$ for 20 minutes at 4°C.

The supernatant was discarded, and 2 ml of fresh assay buffer was added. The pellet was

dislodged by vortexing, and an additional 8 ml of buffer was added. The homogenization and

centrifugation steps were then repeated once. The final pellet was suspended in 2 ml assay buffer

and protein concentration determined using the Coomassie method (ThermoFisher Scientific,

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Waltham, MA) using bovine serum albumin as the standard. The sample volume was then adjusted to give a protein concentration of 400 μg/ml.

Occupancy of VMAT2 in the homogenate was then determined using a radioligand binding assay; RRR-DHTBZ administered to the animal which binds to VMAT2 will inhibit binding of a VMAT2 radioligand to the transporter. The radioligand used was [^{3}H]-RRR-DHTBZ, labeled with C^{3}H$_3$ at the 9 O-methyl position, custom-synthesized by Pharmeron (Irvine, CA) (formerly Quotient Bioresearch) to a specific activity of 75 Ci/mmol. The radioligand concentration used was 3 nM and the K_d was 0.6 nM. The assay was performed as described in (Grigoriadis et al., 2017). Binding was measured over 16 time points and the data analyzed to determine occupancy as described in (Malany et al., 2009). The maximal occupancy obtainable in the assay was approximately 80%, determined using maximally-occupying doses of RRR-DHTBZ (1 mg/kg) suggesting a pool of VMAT2 accessible in the homogenate in the binding assay but not in the intact tissue in the animal. Occupancy values were thus normalized to the maximal obtainable occupancy.

Measurement of weight gain in rats maintained on a high fat diet

It has been consistently demonstrated that repeated olanzapine treatment in rats elicits gender-specific increases in body weight compared to vehicle-treated counterparts (Castellani et al., 2019), with females regularly displaying treatment-induced body weight increases not always observed in males. Therefore, while males were utilized in the behavioral screening models, females were utilized for the body weight assessment studies as they represent a more translational model of human weight gain in response to olanzapine treatment. Female Sprague-Dawley rats from Charles River (Margate, Kent, UK) were individually housed in polypropylene

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cages with metal grid floors at a temperature of 21±4°C and 55±20% humidity. Animals were maintained on a reverse phase light-dark cycle (lights off for 8 h from 10:00-18:00 h) during which time the room will be illuminated by red light. Animals had free access to a high-fat powdered diet (VRF1, Special Diet Services, Witham, Essex, UK, plus 20% lard) and tap water at all times. The diet was contained in glass feeding jars with aluminum lids (Solmedia Laboratory Suppliers, Romford, Essex, UK). Each lid had a 3-4 cm hole cut in it to allow access to the food. Animals were accustomed to these conditions for at least two weeks before experimentation.

Animals underwent a 3-day baseline run-in period during which time all rats were dosed once a day with vehicle. Animals were weighed at the time of dosing. Towards the end of this baseline period, animals were weighed (to the nearest 0.1 g using an electronic top-pan balance) and allocated into 8 weight-matched treatment groups by a statistician. Rats were then dosed for 14 days with vehicle, olanzapine, or RRR-DHTBZ, as described in "Drug compounds" above. When olanzapine and RRR-DHTBZ were administered in combination, the latter was administered as soon as possible after the former. Dosing began at approximately 08:45 h each day (0h) (i.e. so the mid-point of dosing was approximately at the time of lights out). This strategy was taken to maximize the impact of olanzapine on food intake. Rats were weighed to the nearest 0.1 g every day at 0 h.

Plasma pharmacokinetics

For the CAR experiments, plasma compound pharmacokinetics were assessed in age-matched satellite groups of male Wistar rats and blood was collected via closed cardiac puncture under isoflurane anesthetic. Compounds were administered as described in "Drug compounds" above

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and blood collected 30 minutes later. Whole blood was collected into K3-EDTA–coated vials

and was then separated via centrifugation at 4°C at 10,000 rpm followed by transfer to

individually labeled sample vials that were flash frozen in liquid nitrogen and stored at -80ºC.

The number of satellite animals in each treatment group was 4 or 5. For the weight gain studies,

on the day following the last weight measurement, whole blood was collected by cardiac

puncture under CO_2 narcosis.

RRR-DHTBZ was quantified using liquid chromatography in tandem with a mass

spectrometric detector (LC-MS/MS). The plasma calibration standards were prepared by

a 12-point dilution series, from 2500 to 0.5 ng/ml. The lower limit of quantification in the assay

was 0.5 ng/ml. The plasma extraction procedure involved a protein precipitation extraction from

50 µl of calibration standards or study plasma samples with 175 µl acetonitrile containing

200 ng/ml internal standard (D6-RRR-DHTBZ). Samples were vortexed and centrifuged, and

75 µl of the supernatant was transferred to the sample injection plate and diluted with 225 µl

water for LC-MS/MS analysis.

Statistics

PPI, CAR, catalepsy and plasma concentrations

Single-factor analysis of variance (ANOVA) was used to compare effects of different treatments

in the PPI studies. In these experiments, the treatment groups compared were RRR-DHTBZ

(multiple doses), tetrabenazine (multiple doses), the antipsychotic haloperidol (single dose)

and the respective vehicles. Multiple comparisons were performed using the Tukey post-test.

This analysis was conducted using Prism 7.0 (GraphPad Software, Inc., La Jolla, CA) and the

results shown in Tables S1 and S2. This analysis was also performed for CAR assay data

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(escapes and escape failures) for the effect of multiple doses of RRR-DHTBZ and vehicle (Tables S3 and S4).

Conditioned avoidance treatment-combination experiments (i.e., experiments incorporating antipsychotic with and without RRR-DHTBZ) were designed to enable pairwise comparisons between the presence or absence of RRR-DHTBZ treatment groups and comparisons between doses of the antipsychotic (or a single dose and vehicle in one case for risperidone, Table S5). All the doses of antipsychotic tested alone were also treated with RRR-DHTBZ in the same experiment. This design enabled two-factor ANOVA and post-hoc testing using multiple comparisons. In all cases, an individual experiment was conducted on the same cohort of animals. In many cases, the treatment groups were split across 2 test sessions, necessitated by the large number of treatment groups. The session details are given in the Supplemental Information. The ANOVA compared the effect of the different antipsychotic doses and compared the effect of the antipsychotic in the absence or presence of RRR-DHTBZ. The interaction statistic of the ANOVA tested whether the effect of RRR-DHTBZ was dependent on the antipsychotic dose. The Tukey multiple comparisons post-test was then run to compare all treatment groups with one another. This design enabled multiple effects to be compared in the same experiment, specifically, 1) The effect of RRR-DHTBZ at each dose of antipsychotic; 2) The effect of RRR-DHTBZ in the absence of antipsychotic; 3) The effect of the different doses of antipsychotic and vehicle in the absence of RRR-DHTBZ. The post-hoc test reported significance at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, and these results are given in the Supplemental Information. Statistical analysis was performed using Prism 7.0. The effects tested in the conditioned avoidance test were the number of escapes and number of escape

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failures. If the highest number of escape failures among the treatment groups in an experiment was low (< 2 trials), the statistical test on escape failures was not performed.

The same two-factor ANOVA design was applied to the treatment-combination catalepsy bar test (time on bar data). The test was not performed if the highest effect among the treatment groups was small (time on bar < 10 seconds). In addition, plasma antipsychotic concentrations in the satellite groups of animals dosed with compound were compared using the two-factor ANOVA design. For the RRR-DHTBZ concentration, single-factor ANOVA was performed comparing the concentration of RRR-DHTBZ across the antipsychotic treatment groups (different antipsychotic doses and antipsychotic vehicle). In all cases, the p value from this test was > 0.05 so no post-test comparison between treatment groups was performed.

Outliers were assessed by calculating the standard deviation of each treatment group within an individual experiment. If a value was outside of the range of the mean ± 2 standard deviations, it was excluded.

Weight gain statistics

Statistical reports for the weight gain study are provided in the Supplemental Information (Tables S12 and S13). The statistical methods assume that data are normally distributed with equal variance in the groups. Body weight and body weight gain were analyzed by two-way analysis of covariance with treatment and cohort as factors and body weight on Day 1 as the covariate. Multiple comparisons were conducted as follows. In the experiment RS1046 (Fig. 8), multiple comparisons were by Williams' test to compare RRR-DHTBZ to vehicle, separate Dunnett's tests to compare olanzapine and the combination to vehicle, Williams' test to compare the combination to RRR-DHTBZ 0.15 mg/kg and the multiple t test to compare the combination

to the same dose of olanzapine. In the experiment RS1262, multiple comparisons were by separate Williams' tests to compare olanzapine and RRR-DHTBZ to vehicle, Dunnett's test to compare the combination to vehicle, Williams' test to compare the combination to olanzapine 0.3 mg/kg, and the multiple t test to compare the combination to the same dose of RRR-DHTBZ. The rationale for applying the Williams' and Dunnett's tests was as follows: Williams' test was used to compare increasing doses of a compound to vehicle control assuming that the dose-response relationship is monotonic (i.e., increasing the dose causes increased response). This assumption is reasonable for RRR-DHTBZ but not for olanzapine, which appears to have bell-shaped dose-response relationship (e.g., 3 mg/kg doesn't have as big an effect as 1.2 mg/kg) (Fig. 8). Williams' test was also appropriate to compare RRR-DHTBZ + olanzapine to RRR-DHTBZ 0.15 mg/kg because, again, it is reasonable to assume a monotonic dose-response relationship. Dunnett's test was used to compare several groups to a single group, making no assumptions about the dose-response relationship. It was also used to compare the combination to vehicle because a monotonic dose-response relationship would not be expected if olanzapine reversed the effect of RRR-DHTBZ.

RESULTS

Efficacy of the selective VMAT2 inhibitor RRR-DHTBZ in animal models of schizophrenia

Tetrabenazine has previously demonstrated efficacy in animal models of schizophrenia (Bocknik et al., 1968; Heise and Boff, 1960; Kuribara and Tadokoro, 1983; Levison and Freedman, 1967; Rigdon and Weatherspoon, 1992). These effects were originally presumed to be mediated solely by inhibition of VMAT2 activity. However, some of the metabolites of tetrabenazine also inhibit other receptors that modulate antipsychotic activity, albeit at lower potency. For example, the R,S,S isomer of DHTBZ binds the D2 receptor with a Ki of 53 nM and 5HT7 receptor with a Ki of 6 nM (Grigoriadis et al., 2017). Here we confirmed that VMAT2 inhibition can elicit antipsychotic-like efficacy using the highly selective tetrabenazine metabolite RRR-DHTBZ (VMAT2 Ki of 2 nM, >1,000-fold selective in a broad-panel selectivity screen, including monoamine receptors) (Grigoriadis et al., 2017). The compound was tested in 2 models: PPI of startle, a model of sensorimotor gating (Geyer et al., 2001); and CAR, a model of reinforcement learning (Wadenberg, 2010; Wadenberg and Hicks, 1999). PPI in rodent models has a direct translatability to humans in that deficits in PPI are observed in schizophrenic individuals, and drugs that are effective in this model are effective antipsychotics in humans (Geyer et al., 2001; Kumari and Sharma, 2002; Swerdlow et al., 2008). The CAR model also has excellent predictive validity. All clinically effective antipsychotics, but not most other classes of drugs, are effective in suppressing CAR at clinically-relevant doses (Arnt, 1982; Olsen et al., 2008; Wadenberg, 2010). In addition, pathways mediating CAR behavior have been invoked in the efficacy of antipsychotics for attenuating the positive symptoms of schizophrenia, notably the mesolimbic dopamine pathway (Wadenberg et al., 1990).

Prepulse inhibition

RRR-DHTBZ was tested in a genetic PPI model. The inbred C57BL/6J mouse strain displays a reduced level of PPI of acoustic startle compared with the parental C57BL line (Ralph et al., 2001). This reduction can be reversed by antipsychotics, i.e., the compounds increase PPI in C57BL/6J mice (Ouagazzal et al., 2001; Ralph and Caine, 2005). In the genetic PPI model, RRR-DHTBZ increased PPI in C57BL/6J mice in a dose-dependent manner at stimulus intensities of 74, 78 and 82 dB, with a statistically significant effect observed at 10 mg/kg (Fig. 2 [74 dB], Tables S1 and S2 [78 and 82 dB]). Likewise, tetrabenazine elicited a dose-dependent increase of PPI, with a statistically significant effect observed at 30 mg/kg (Fig. 2, Table S1). The increase of PPI observed at the highest doses of RRR-DHTBZ and tetrabenazine was not significantly different from the effect of haloperidol alone, used as a positive control (Fig. 2, Tables S1 and S2). These results indicate that selective inhibition of VMAT2 elicits efficacy in the PPI model equivalent to the antipsychotic haloperidol.

Conditioned avoidance

The effect of RRR-DHTBZ on conditioned avoidance was tested in rats. The experimental paradigm and behavioral scoring methods are shown in Fig. 1. CAR scoring was used as previously described (Kalinichev et al., 2013). On the test day, rats previously trained in a two-compartment shuttle box were exposed to the conditioned stimulus (a 10-second pulse of white noise at 80 dB). If the rat moved to the second compartment upon presentation of the conditioned stimulus, this was scored as an avoidance (Fig. 1). An animal not leaving the chamber represents inhibition of avoidance. Inhibition of avoidance can result from inhibition of the conditioned behavior (antipsychotic efficacy) or impairment of the ability of the animal

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to move from the chamber (sedation/motor impairment). These possibilities were differentiated

by applying a mild foot shock to the test chamber (Fig. 1). An animal leaving the chamber

subsequent to initiation of the foot shock represents inhibition of conditioned avoidance,

the behavior of interest. This event represents efficacy of the test compound in the model and

was scored as an escape (Fig. 1) (Kalinichev et al., 2013). The animal not leaving the chamber

represents an inhibition of movement rather than inhibition of the conditioned behavior.

This result was scored as an escape failure (Fig. 1). For inhibitors of dopaminergic signaling,

escape failure likely results from sedation at lower doses and gross motor impairment at higher

doses (Arnt, 1982; Wadenberg et al., 2001). To further assess the potential for gross motor

impairment, a separate cohort of animals were tested by measuring catalepsy using the bar test.

RRR-DHTBZ produced a dose-dependent suppression of conditioned avoidance,

manifest as an increase in the number of escapes (Fig. 3; Tables 1, S3 and S4). The lowest dose

eliciting a statistically significant effect was 0.3 mg/kg. At this dose, there was no detectable

catalepsy (Table S4) and there were no escape failures (Tables S3 and S4). The next highest dose

in the experiments (0.7 mg/kg [Table S3] or 0.6 mg/kg [Table S4]) elicited a greater number of

escapes, indicating an increased suppression of conditioned avoidance. At these doses,

a significant number of escape failures were recorded (Tables 1, S3 and S4), consistent with

mild sedation contributing to the overall suppression of avoidance. No significant catalepsy

was detected at these doses (Table S4). At a higher dose (1.2 mg/kg), catalepsy was observed

(Tables 1 and S4), indicating gross motor impairment, which is anticipated for a high dose of

an agent that suppresses dopaminergic transmission. (A high dose of risperidone [1 mg/kg] also

resulted in catalepsy [Tables 1 and S7], which is typically observed in rats treated with high

doses of antipsychotics) (Wadenberg et al., 2001). These findings imply that specific inhibition

of VMAT2 can suppress conditioned avoidance responding to the same extent as known antipsychotics.

The efficacy of RRR-DHTBZ in the CAR model was compared with tetrabenazine and with conventional antipsychotics. Tetrabenazine significantly increased the number of escapes at 3 mg/kg (Fig. 3, Table S3), consistent with previous studies (Bocknik et al., 1968; Heise and Boff, 1960; Kuribara and Tadokoro, 1983). The magnitude of the effect was not significantly different from that of 0.7 mg/kg RRR-DHTBZ (Table S3A). The plasma concentration of RRR-DHTBZ resulting from administration of 3 mg/kg tetrabenazine (34 ng/ml) was similar to that resulting from administration of 0.7 mg/kg RRR-DHTBZ (52 ng/ml, Table S3). These findings are consistent with the hypothesis that the effect of tetrabenazine in the CAR model results primarily from inhibition of VMAT2 activity. The conventional antipsychotics risperidone and olanzapine increased the number of escapes at a historically standard dose (0.3 mg/kg for risperidone [Fig. 3, Tables 1, S3, S6 and S7] and 3 mg/kg for olanzapine [Fig. 3, Tables 1, S8 and S11]) (Wadenberg et al., 2001). The magnitude of the effect was similar to that for 0.7 mg/kg RRR-DHTBZ (Fig. 3). (The effect of 0.3 mg/kg risperidone and 0.7 mg/kg RRR-DHTBZ were compared directly in 1 experiment and were not found to be significantly different [Table S3A]). These findings indicate inhibition of VMAT2 activity can be as effective as a conventional antipsychotic in inhibiting conditioned avoidance.

Relationship between RRR-DHTBZ plasma concentration, inhibition of conditioned avoidance and VMAT2 occupancy

The pharmacokinetic/pharmacodynamic relationships of conventional antipsychotics for inhibiting conditioned avoidance have been studied in detail. The effective plasma

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concentration for inhibiting conditioned avoidance closely matches the therapeutic plasma

concentration for treating schizophrenia (Olsen et al., 2008). D2 receptor occupancy of > 50%

is typically required for both therapeutic efficacy (Farde et al., 1988; Kapur et al., 1999) and for

half-maximal effect in CAR (Olsen et al., 2008; Wadenberg et al., 2001). Here we investigated

these relationships for VMAT2 inhibition using RRR-DHTBZ.

The plasma concentration-effect relationship of RRR-DHTBZ for suppressing CAR

is shown in Fig. 4. Significant suppression of CAR (manifest as an increase of escapes) was

observed at a plasma RRR-DHTBZ concentration of 19 ng/ml (Fig. 4, Table 1). The maximal

effect for suppressing CAR was observed in range of 100 ng/ml (Fig. 4). These plasma

concentrations are in the same range as the therapeutic RRR-DHTBZ plasma concentration in

humans for treating TD (20-48 ng/ml) resulting from administration of valbenazine (Skor et al.,

2017). VMAT2 occupancy was assessed in a dose-dependent manner by ex vivo binding in the

striatum in a satellite group of rats. Significant suppression of CAR was observed at 87%

VMAT2 occupancy and above. The dose dependence of the behavioral efficacy demonstrated

that there was no significant effect on conditioned avoidance below 59% occupancy (Fig. 4).

Window between dopaminergic behaviors

Dopaminergic inhibitors manifest numerous behaviors in animal models and the CAR assay

represents one preclinical model displaying high sensitivity to dopamine receptor antagonists

(Arnt 1982). In the CAR assay, antipsychotic compounds elicit an inhibition of a previously

trained conditioned avoidance response, a hallmark behavioral signature of both typical and

atypical antipsychotic treatment in rats (Fig. 1). It is well documented, however, that

antipsychotic compounds can also elicit on-target sedative effects or cataleptic behavior

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in preclinical models assessing antipsychotic-like activity. Therefore, a meaningful inhibition of the conditioned avoidance response in the CAR assay must be accompanied by the ability of the animal to move and escape the subsequent anticipated foot shock as reflected in a higher number of escape behaviors. If the animals show an inhibited avoidance response but fail to escape the foot shock, or if the compound itself elicits cataleptic behavior resulting from too robust of a decrease in striatal dopamine, interpretation of the CAR response is confounded at that dose and resultant exposure. We compared the window between these behaviors for RRR-DHTBZ and for the conventional antipsychotics risperidone and olanzapine. Figure 5 shows the behavior versus plasma concentration for the three compounds, and Table 1 includes the minimal effective plasma concentrations for each behavioral endpoint.

The potency of RRR-D-HTBZ for inhibiting conditioned avoidance (increasing escapes) was higher than that for eliciting escape failures, which was in turn higher than that for causing catalepsy (Fig. 5), with minimally-effective plasma concentrations for the three behaviors of 19, 52 and 400 ng/ml, respectively. This magnitude of the potency difference between the activities was similar to that for risperidone (Fig. 5, Table 1; minimally-effective plasma concentrations of 45, 280 and 360 ng/ml for CAR inhibition, escape failures and catalepsy, respectively). This finding suggests that presynaptic dopaminergic inhibition with RRR-DHTBZ elicits a similar window between dopaminergic behaviors as postsynaptic inhibition with risperidone. For olanzapine, there was minimal apparent difference of the concentration-response relationship between CAR inhibition and escape failures (Fig. 5, Table 1). Catalepsy was not detected at the doses used (3 mg/kg and below) but has been detected at higher doses in other studies (Wadenberg et al., 2001).

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Effect of combining a VMAT2 inhibitor with a conventional antipsychotic on suppression of conditioned avoidance

Synergistic interaction revealed by subthreshold dose combination

The results above show that VMAT2 provides an additional site of action and mechanism to the D2 dopamine receptor for efficacy in animal models of schizophrenia. We next examined the effect of combining a VMAT2 inhibitor and a conventional D2-blocking antipsychotic using the CAR model. First, we tested for synergy by examining the effects of combining subthreshold doses of RRR-DHTBZ and risperidone or olanzapine. The threshold doses of these 3 compounds were established by assessing each compound in a dose-dependent manner. The lowest doses tested producing statistically significant suppression of CAR were 0.3 mg/kg, 0.3 mg/kg and 3 mg/kg for RRR-DHTBZ, risperidone and olanzapine, respectively (Tables 1, S3, S4, S6, S7, S8 and S11).

RRR-DHTBZ at half the threshold dose (i.e., 0.15 mg/kg) was combined with one third the threshold dose (i.e., 0.1 mg/kg) of risperidone. When administered alone, the individual treatments did not significantly suppress CAR (Fig. 6A, Tables S5 and S6). However, when combined, a profound and significant suppression of CAR was observed, manifest as an increase in the number of escapes (Fig. 6A, Tables S5 and S6). Remarkably, the magnitude of the effect produced by the combination was similar to the effect of the threshold dose of the compounds administered individually (6.5-7.1 escapes for the combination of 0.15 mg/kg RRR-DHTBZ and 0.1 mg/kg risperidone [Tables S5 and S6], 4.8-11.4 escapes for 0.3 mg/kg RRR-DHTBZ [Tables S3 and S4] and 7.2-8.0 escapes for 0.3 mg/kg risperidone [Tables S6 and S7]). The synergistic effect was not due to some drug-drug interaction potentially affecting the plasma concentration of the compounds. RRR-DHTBZ did not significantly affect the plasma

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concentration of risperidone and vice-versa in these experiments (Tables S5B, S5C, S6D and S6E, see legend to Fig. 6 for example). Furthermore, the synergistic effect did not result in the induction of catalepsy at the doses tested (Table S6).

Olanzapine at one fifth of its threshold dose (i.e., 0.6 mg/kg) was tested in combination with RRR-DHTBZ at half the threshold dose (i.e., 0.15 mg/kg). When administered alone, neither treatment significantly affected CAR (Fig. 6B, Tables S9 and S10). When combined, again, significant suppression of CAR was observed, evident as an increase in the number of escapes (Fig. 6B, Tables S9 and S10). Similar to risperidone, the magnitude of the effect of the combination (8.6-10.3 escapes, Tables S9 and S10) was similar to that of the threshold dose of the compounds administered individually (4.8-11.4 escapes for 0.3 mg/kg RRR-DHTBZ [Tables S3 and S4] and 8.6-12.3 escapes for 3 mg/kg olanzapine [Tables S8 and S11]). Once more, the synergistic effect was not due to a non-specific drug-drug interaction. RRR-DHTBZ did not significantly affect the plasma concentration of olanzapine and vice-versa in these experiments (Tables S9C, S9D, S10C and S10D, see legend to Fig. 6 for example). The synergistic effect did not result in the induction of catalepsy at the doses tested (Table S10). It is interesting to note that the synergy observed was independent of the antipsychotic used and that both studies reached the maximal efficacy observed with the high dose antipsychotics alone (compare Figs. 5 and 6).

RRR-DHTBZ increases the potency of risperidone and olanzapine in the CAR model
The effects of a subthreshold dose of RRR-DHTBZ on the potency of the conventional antipsychotics was tested by combining RRR-DHTBZ with a range of doses of risperidone or olanzapine. Conditioned avoidance suppression (number of escapes) was plotted versus the

plasma concentration of the compound (Fig. 7A and D). RRR-DHTBZ at its subthreshold dose

of 0.15 mg/kg increased the potency of both risperidone and olanzapine, evidenced as a clear

leftward shift of the response-versus-concentration curve for both antipsychotics (Fig. 7A and D,

Table 1). This finding confirms the synergistic interaction between the two classes of

dopaminergic modulator. The effect of the combination on the other dopaminergic behaviors,

escape failures and catalepsy, was also tested (Fig. 7B and E for escape failures, Fig. 7C and F

for catalepsy). RRR-DHTBZ at 0.15 mg/kg did not appear to affect the windows between the

behaviors for risperidone or olanzapine; RRR-DHTBZ potentiated escape failures and catalepsy

to a similar extent as it potentiated suppression of conditioned avoidance (Fig. 7, see Table 1 for

minimum effective plasma concentrations and doses). This finding was expected since escape

failures and catalepsy are both primarily mediated by dopaminergic inhibition for the compounds

tested.

Weight gain

The results above indicate that VMAT2 inhibition enhances the efficacy of the antipsychotics in

behavioral models predictive of therapeutic efficacy for relieving the positive symptoms of

schizophrenia. One of the primary side effects of antipsychotic drugs, however, is weight gain

and associated metabolic syndrome abnormalities. We therefore assessed whether VMAT

inhibition affected weight gain, alone or in combination with an antipsychotic, in an

antipsychotic-induced weight gain model. Olanzapine was selected as the test compound in

this model since it has been demonstrated to cause a particularly high degree of weight gain

in humans (Leucht et al., 2013; Lieberman et al., 2005). While there are mixed reports regarding

gender differences in olanzapine-induced weight gain in humans, preclinical studies have

consistently shown that female rats exhibit a greater olanzapine-induced weight gain than

do conspecific males when compared to vehicle-treated counterparts (Castellani et al., 2019).

Female rats were therefore utilized in the weight gain experiments as they better model the

adverse weight observed in humans following olanzapine treatment than do male rats.

In this female rat model, olanzapine yielded a bell-shaped dose response. The lowest dose

tested, 0.3 mg/kg, produced a small but not significant increase of body weight gain (Fig. 9,

Table S13, 39% increase). The next-highest doses, 0.6 and 1.2 mg/kg, produced statistically

significant 75% and 74% increases in weight gain, respectively (Fig. 8, Tables 1 and S12).

Increasing the dose to 3 mg/kg reduced the effect back to the same level observed with 0.3

mg/kg (Figs. 8 and 9, Tables S12 and S13). These results are in agreement with previous studies

(Heal et al., 2012).

The effect of VMAT2 inhibition alone was tested using RRR-DHTBZ. The dose range

used was based on the results of the CAR experiments: 0.15 mg/kg, the subthreshold dose for

suppressing CAR; 0.3 mg/kg, the threshold dose; and 0.5 mg/kg, representing a higher

efficacious dose (Fig. 3). RRR-DHTBZ did not significantly affect body weight (Figs. 8 and 9),

except at 0.5 mg/kg in Experiment 2 (Fig. 9). The plasma concentration versus weight gain

relationship is shown in Fig. 10. RRR-DHTBZ did not increase body weight over a plasma

concentration range effective for suppressing CAR (Fig. 10), or the therapeutic range for treating

TD (20-48 ng/ml) (Skor et al., 2017). At the highest plasma concentration tested, 210 ng/ml,

RRR-DHTBZ significantly increased weight gain (Fig. 10, Table 1). This plasma concentration

is 12-fold higher than the minimally-effective concentration for suppressing CAR (19 ng/ml,

Fig. 6, Table 1). In combination treatment groups, RRR-DHTBZ was administered at the

subthreshold dose previously shown to synergize with olanzapine and suppress the CAR

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response (0.15 mg/kg). This treatment did not significantly affect weight gain elicited by

olanzapine. This was observed for the sub-maximally efficacious dose of 0.3 mg/kg olanzapine

(Fig. 9) and the maximally efficacious doses of 0.6 and 1.2 mg/kg (Fig. 8). The weight gain data

were plotted as a function of olanzapine concentration in Fig. 11. The data for olanzapine in

combination with RRR-DHTBZ overlie those for olanzapine alone (Fig. 11, Table 1).

One potential explanation for the lack of synergy by RRR-DHTBZ in weight gain versus the

synergistic action in CAR is lower plasma concentration of the compounds in the weight gain

model; however, this was not observed. At the synergizing plasma concentration of olanzapine

in the CAR study, there was no synergy in weight gain (Fig. 11, concentration in CAR study

indicated by arrow was 42 ng/ml [average of the 0.6 mg/kg values in Tables S9 and S10]). At the

synergizing dose of RRR-DHTBZ (0.15 mg/kg), the concentration of RRR-DHTBZ was 24

ng/ml in the CAR study and 60 ng/ml in the weight gain study (average of the RRR-DHTBZ /

olanzapine combination values in Tables S12 and S13). In order to test this possibility further,

a higher dose of RRR-DHTBZ was examined; 0.3 mg/kg RRR-DHTBZ (plasma concentration of

110 ng/ml) also did not significantly alter weight gain elicited by 0.3 mg/kg olanzapine (Fig. 9).

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DISCUSSION

VMAT2 inhibitors such as tetrabenazine were first developed in the early 1960's as monoamine-depleting agents for the treatment of the positive symptoms of schizophrenia (Lingjaerde, 1963). Tetrabenazine was ultimately superseded by more specific pharmacological agents, the first-generation phenothiazine antipsychotics such as chlorpromazine, and later the butyrophenones such as haloperidol which directly, specifically and potently target the post-synaptic D2 dopamine receptor (Seeman et al., 1976). Subsequently, VMAT2 inhibitors have found utility in the treatment of involuntary hyperkinetic movement disorders (Huntington's chorea and TD, the approved drugs being tetrabenazine, deutetrabenazine and valbenazine) (Touma and Scarff, 2018; Yero and Rey, 2008). Given the existence of approved medications targeting pre- and postsynaptic dopamine mechanisms, particularly valbenazine with its improved pharmacokinetics allowing once-daily dosing, it is now possible to clinically evaluate the combination of these mechanisms on dopaminergic morbidities, such as schizophrenia. The goal of this study was to test the interaction between VMAT2 inhibition and classical antipsychotics in preclinical models of the positive symptoms of schizophrenia.

We first determined the efficacy of VMAT2 inhibition alone and, for the first time, compared it directly with that of conventional antipsychotics, using the highly selective VMAT2 inhibitor RRR-DHTBZ (also known as [+]-α-HTBZ). Previous studies have utilized tetrabenazine, which metabolizes into 4 independent and pharmacologically active metabolites that block receptors that have previously been hypothesized to play a role in treating psychosis (e.g., the D2 dopamine receptor and 5HT7 receptor) (Meltzer, 1999). RRR-DHTBZ displayed dose-dependent efficacy in both antipsychotic models (PPI and CAR) equivalent in magnitude to either typical or atypical antipsychotics (Figs. 2 and 3), confirming that inhibition of VMAT2

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and by extension, decreasing presynaptic dopamine release, can elicit efficacy in antipsychotic models. The effective plasma concentration of RRR-DHTBZ in CAR, an animal model with translational validity regarding effective concentration for traditional antipsychotics, was similar to that of RRR-DHTBZ in humans resulting from valbenazine dosing to treat TD (52 ng/ml at 0.7 mg/kg [Table S3] versus 48 ng/ml at 80 mg/kg in man) (Skor et al., 2017). The maximal detected efficacy of RRR-DHTBZ was similar to that of the antipsychotics, haloperidol (in PPI, Fig. 2), risperidone or olanzapine (in CAR, Fig. 3). This finding indicates that presynaptic modulation resulting from VMAT2 inhibition can be as effective as established antipsychotics in the behavioral models. Like the antipsychotics, VMAT2 inhibition elicited nonspecific motor effects at higher doses as anticipated for a dopaminergic inhibitor (Arnt, 1982; Wadenberg et al., 2001). At the higher doses, RRR-DHTBZ elicited escape failures (indicative of sedation, ≥ 0.6 mg/kg) and at the highest dose tested produced catalepsy (motor inhibition, 1.2 mg/kg) (Tables S3 and S4). The magnitude of these effects and the window between efficacy and motor disturbance was similar to that of risperidone and olanzapine in the CAR model (Fig. 5, Tables 1 and S5-S10). Collectively, if these findings translate to humans, selective VMAT2 inhibition as a stand-alone therapy might be as effective as conventional antipsychotics for treating the positive symptoms of schizophrenia.

We next investigated the effect of combining VMAT2 inhibition with antipsychotics on efficacy in the CAR model. A robust analytical design was implemented to enable the interaction to be rigorously quantified (Kalinichev et al., 2013) (Fig. 1). RRR-DHTBZ was observed to synergize with the antipsychotics risperidone or olanzapine in suppressing conditioned avoidance behavior (Figs. 6 and 7). This was shown first using the classical synergy test, that is, combination of subthreshold doses of the 2 agents. In this paradigm, the combination of

nonsignificant, subthreshold doses resulted in efficacy equivalent to that of maximally-effective

doses of the agents administered alone (Fig. 6, Tables S5, S6, S9 and S11). This was not due to a

drug-drug interaction; the combination did not significantly affect the exposure of either agent.

This provided confidence that the synergy that was observed was pharmacological or

mechanism-based for the dopamine system. In the next experiments, the effect of RRR-DHTBZ

on the dose-response relationship of the antipsychotics for suppressing CAR was tested (Fig. 7).

More precisely, the concentration-response relationship was evaluated for both risperidone and

olanzapine in the presence of subthreshold doses of the VMAT2 inhibitor. RRR-DHTBZ at

a subthreshold dose produced a leftward shift of the concentration-response curve of both

antipsychotics tested, olanzapine and risperidone (Fig. 7). This finding confirms that RRR-

DHTBZ increased the behavioral potency of the antipsychotics in the CAR model. If these

effects can be recapitulated in humans, this synergistic effect would allow a novel therapeutic

approach in which low-dose administration of a VMAT2 inhibitor could in turn lower the dose

of antipsychotic necessary to potentially produce efficacy in reducing positive symptoms.

Reciprocally, the synergy enables a low dose of VMAT2 inhibitor to be used. This could

potentially mitigate the side effects of VMAT2 inhibition noted in the early studies of

tetrabenazine's antipsychotic efficacy in man, such as somnolence and potentially depression

(Lingjaerde, 1963).

A plausible mechanism for the synergistic effect is presented in Fig. 12. This mechanism

is based on the known mechanics of striatal dopamine synaptic neurotransmission. In particular,

it is based on the presynaptic autoreceptor function of the D2 receptor (Ford, 2014). D2 receptors

in the postsynaptic membrane mediate dopaminergic signaling in the postsynaptic neuron. These

receptors are the therapeutic site of action of antipsychotics (Kapur and Mamo, 2003). However,

the D2 receptor is also expressed on the presynaptic terminal where it plays an autoreceptor role

in a negative feedback loop that prevents excessive dopaminergic transmission (Ford, 2014;

Kehr et al., 1972). In this mechanism, dopamine released into the synapse diffuses to the

presynaptic terminal where it activates presynaptic D2 receptors. This results in diminished

synthesis, release and uptake of dopamine. Activation of the autoreceptor decreases dopamine

synthesis by decreasing tyrosine hydroxylase activity (Kehr et al., 1972; Wolf and Roth, 1990).

A problem with antipsychotics is that they interfere with this feedback mechanism since they

block the autoreceptor with equal affinity (Carlsson et al., 1977; Kapur and Mamo, 2003).

This means antipsychotics stimulate dopamine presynaptically, counteracting their inhibitory

postsynaptic efficacy: The antipsychotic binds to the presynaptic D2 receptor, blocking the

negative feedback loop, resulting in elevated dopamine synthesis (Carlsson et al., 1977) and

extracellular concentration (Arbilla and Langer, 1981; Huang et al., 2020). A VMAT2 inhibitor

in principle would blunt the presynaptic action of the antipsychotic. We propose that the elevated

dopamine in the presynaptic terminal resulting from autoreceptor blockade can be prevented

from activating postsynaptic receptors by inhibiting its uptake into presynaptic vesicles via

inhibition of VMAT2[1]. As a result, the elevated synaptic dopamine is reduced. This reduces

competition for binding of the antipsychotic to postsynaptic receptors which in turn increases the

efficacy of the antipsychotic. This correction of the self-inhibitory action of the antipsychotic

explains the synergistic, as opposed to simply additive, action of VMAT2 inhibition on

antipsychotic efficacy. This hypothesis is consistent with recent results quantifying bulk

dopamine concentrations in the CNS by microdialysis (Huang et al., 2020). Risperidone and

olanzapine both elevated dopamine, presumably as a result of presynaptic D2 receptor blockade,

and coadministration of RRR-DHTBZ reduced this elevation, presumably by inhibiting

presynaptic packaging of dopamine. This hypothesis could be explored in future studies at higher

spatial and temporal resolution using recently-reported biosensors of synaptic dopamine

concentration (Patriarchi et al., 2018).

Clearly, reducing antipsychotic dose with the synergistic approach will only be beneficial

if there is no concomitant potentiation of side effects. Weight gain is associated with the use of

all conventional antipsychotics, as indicated on their labels. Consequently, we evaluated the

effect of VMAT2 inhibition in an antipsychotic-induced weight gain model, employing

olanzapine, the antipsychotic with the highest weight gain and metabolic syndrome liability

(Leucht et al., 2013; Lieberman et al., 2005). When dosed alone, RRR-DHTBZ did not

significantly affect body weight at doses and exposures effective in the rodent CAR model or

in man (Fig. 10). An increase was observed at the highest exposure (210 ng/ml), but this was

10-fold higher than the effective concentration for suppressing CAR. In the combination

experiment, RRR-DHTBZ did not change the effect of olanzapine, either at the efficacious doses

(0.6 and 1.2 mg/kg, Fig. 8) or sub-efficacious dose (0.3 mg/kg, Fig. 9) of olanzapine.

RRR-DHTBZ did not change the plasma concentration-effect relationship of olanzapine in the

weight gain model (Fig. 11), indicating it did not affect the potency of olanzapine to elicit weight

gain in contrast to its ability to enhance the efficacy of olanzapine in the CAR model. These

findings indicate the synergizing effect of VMAT2 inhibition in the CAR model is not manifest

in the weight gain model at the doses tested. One explanation for the lack of synergy on weight

gain is the hypothesis that weight gain is mediated by targets other than the D2 dopamine

receptor alone. Implicated targets include the M3 muscarinic acetylcholine receptor,

5HT2 serotonin receptor subtypes, and the H1 histamine receptor (Roerig et al., 2011).

In summary, these findings demonstrate VMAT2 inhibition is effective in animal models of schizophrenia and potentiates the behavioral potency of antipsychotics in these models without affecting weight gain. If translatable to man, these effects could provide new treatment modalities for schizophrenia with a reduced side effect burden while maintaining and potentially enhancing therapeutic efficacy.

Authorship Contributions

Participated in research design: Hoare, Kudwa, Luo, Grigoriadis

Conducted experiments: Hoare, Kudwa, Luo

Performed data analysis: Hoare, Kudwa, Luo

Wrote or contributed to the writing of the manuscript: Hoare, Kudwa, Luo, Grigoriadis

Conflicts of Interest

The studies included in this report were supported by Neurocrine Biosciences, Inc. (San Diego, CA). All authors are current or former full-time employees of Neurocrine and may hold stock or other interest in the company.

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FOOTNOTES

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Some findings of these studies were previously presented as follows: Grigoriadis D, Hoare S, Kudwa A, Boikess, S. Can presynaptic and postsynaptic monoaminergic mechanisms work synergistically? Current findings and future directions. 56th Annual Meeting of the American College of Neuropsychopharmacology; 2017 Dec 3-7; Palm Springs, CA and Grigoriadis D, Hoare S, Kudwa A, Boikess, S. VMAT2 inhibition – A novel mechanism for maintained efficacy at lowered antipsychotic doses. Society of Biological Psychiatry Annual Meeting; 2017 May 18-20; San Diego, CA.

Please send reprint requests to Dimitri E. Grigoriadis, Neurocrine Biosciences, Inc., 12780 El Camino Real, San Diego, California 92130, email: dgrigoriadis@neurocrine.com.

[1]Uncontrolled cytoplasmic buildup of dopamine resulting from increased synthesis but blocked vesicular packaging is likely blunted by a second known feedback mechanism: Dopamine is a competitive inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis Daubner, S. C., Le, T., Wang, S., 2011. Tyrosine hydroxylase and regulation of dopamine synthesis. Arch Biochem Biophys 508, 1-12, doi:10.1016/j.abb.2010.12.017.

FIGURE LEGENDS

Fig. 1. CAR model experimental paradigm and response quantification. The scoring system is that used in (Kalinichev et al., 2013). Rats were trained in a two-chamber shuttle box to escape to the second chamber on presentation of the conditioned stimulus (a 10-second pulse of white noise at 80 dB) to avoid a mild foot shock. In the experiment, test compound was administered to the rat and after the appropriate pretreatment time the conditioned stimulus was applied. The rat could either escape to the second compartment or remain in the test compartment. The former represents the conditioned avoidance response (and would be observed for an untreated rat). The latter represents inhibition of avoidance, which could in principle be due either to inhibition of the conditioned response by the test compound or inhibition of movement (typically by sedation or gross motor inhibition). These mechanisms can be discriminated in the second stage of the experiment. A mild foot shock is applied to the test chamber after presentation of the white noise pulse. If the rat moves to the second chamber it is assumed the inhibition of avoidance was a result of inhibition of the conditioned response. This represents efficacy of the drug for inhibiting conditioned avoidance and was scored as an escape. If the rat failed to move, this implicated failure of movement and was scored as an escape failure. Twenty of these trials were performed for each rat and the mean number of escapes and escape failures quantified. The maximum score is therefore 20.

Fig. 2. VMAT2 inhibition reverses PPI deficit in C57BL/6J mice. This inbred mouse strain displays a deficit in PPI of acoustic startle. The compounds tested were the selective VMAT2 inhibitor RRR-DHTBZ (also known as [+]-α-HTBZ), tetrabenazine and haloperidol (the positive control) administered 30 minutes prior to measurement of PPI. PPI was measured at a stimulus intensity of 74 dB (shown in figure), and 78 and 82 dB (data in Table S1). The experiments were performed twice with similar results (Tables S1 and S2), except the experiment for tetrabenazine which was performed once (Table S1). Data in the figure are from Table S1. The number of animals in each treatment group was 10 or 11. Statistical significance of PPI between treatment groups was tested by single-factor ANOVA followed by Tukey's Multiple Comparison post-test. The asterisks denote significant difference between test-compound treatment and the corresponding vehicle. * $p < 0.05$, *** $p < 0.001$.

Fig. 3. VMAT2 inhibition suppresses conditioned avoidance responding in rats.

The compounds tested were the selective VMAT2 inhibitor RRR-DHTBZ, tetrabenazine, and the

conventional antipsychotics risperidone and olanzapine. Inhibition of conditioned avoidance

was quantified as the number of escapes, as described in Fig. 1. Data in the figure are from

Experiment 1 (Table S3), except the data for olanzapine which are from Experiment 3

(Table S8). The experiments were performed multiple times with similar results (twice for

RRR-DHTBZ, Tables S3 and S4; 3 times for risperidone, Tables S3, S6 and S7; twice for

olanzapine, Tables S8 and S11), except for tetrabenazine which was performed once (Table S3).

The number of animals in each group was between 9 and 24. Statistical significance of the

number of escapes between treatment groups was tested by single-factor ANOVA followed by

Tukey's Multiple Comparison post-test. The asterisks denote significant difference between test-

compound treatment and the corresponding vehicle. $*$ $p < 0.05$, $***$ $p < 0.001$.

Fig. 4. Concentration-response for CAR suppression and VMAT2 occupancy by RRR-DHTBZ. The pink bar is the range of VMAT2 occupancy resulting in statistically significant suppression of CAR (87-100%). CAR suppression was quantified as the number of escapes, as described in Fig. 1 (n = 9 or 10 animals in each treatment group). VMAT2 occupancy was measured in the striatum using an ex vivo occupancy assay (n = 4 or 5). Plasma concentration and VMAT2 occupancy were measured in satellite groups of animals (n = 4 or 5). Each point on the graph is a treatment group from an individual experiment, and the data points from all the experiments are shown on the graph (see Tables S3 and S4 for data values). CAR data are included for RRR-DHTBZ doses which did not cause catalepsy.

Fig. 5. Window between dopaminergic behaviors in the conditioned avoidance model.

Dopaminergic inhibition produces multiple behaviors in the CAR test (Fig. 1). At low doses inhibition of conditioned avoidance predominates (manifest as an increase in the number of escapes). At higher doses, sedation can occur, evident as an increase in escape failures. Further increases in dose results in gross motor impairment (catalepsy), measured using the bar test. The plasma concentration versus effect for these behaviors was assessed for RRR-DHTBZ (A), risperidone (B) and olanzapine (C). Data for RRR-DHTBZ are from Tables S3 and S4, data for risperidone from Tables S5-S7, and data for olanzapine from Tables S8-S11, with group sizes of 7-24 animals for escapes and escape failures, and 4-5 animals for catalepsy.

Fig. 6. Synergy between RRR-DHTBZ and antipsychotics for suppressing CAR at subthreshold doses. A. Risperidone. B. Olanzapine. Data in the figure are from the experiment in Table S6 for risperidone and Table S9 for olanzapine. Statistical analysis was performed using two-factor ANOVA followed by Tukey's Multiple Comparison post-test (Table S6A for risperidone and S9A for olanzapine). The compounds dosed alone did not significantly affect the number of escapes compared with vehicle ($p > 0.05$). The effect of the combination was significantly different from vehicle (asterisks in figure) and from the compounds administered alone ($p < 0.001$). Escape failure and catalepsy were minimal in all treatment groups. RRR-DHTBZ did not significantly affect the plasma concentration of the antipsychotic, or vice-versa (Tables S6D and S6E for risperidone, Tables S9C and S9D for olanzapine). The experiments were performed twice with similar results (Tables S5 and S6 for risperidone, and Tables S9 and S10 for olanzapine), with group sizes of between 7 and 24 animals.

Fig. 7. CAR suppression, escape failures, and catalepsy vs. plasma concentration for combination of conventional antipsychotics with RRR-DHTBZ. The subthreshold dose of RRR-DHTBZ (0.15 mg/kg) was combined with a range of doses of risperidone (A, B, C) or olanzapine (D, E, F) and the data plotted versus plasma concentration of the antipsychotic (suppression of CAR, indicative of antipsychotic efficacy [A, D]; escape failures, indicative of sedation [B, E]; catalepsy, indicative of motor impairment [C, F]). CAR suppression (number of escapes) and escape failures were scored as described in Fig. 1, and catalepsy assessed using the bar test. The plasma concentration for risperidone is "Risperidone total," the sum of the concentrations of risperidone and its fully active metabolite 9-OH-risperidone. Each point on the graph is a treatment group from an individual experiment, and the data points from all the experiments are shown on the graph (see Tables S5-S7 for risperidone data, and Tables S8-S11 for olanzapine data), with group sizes of 7-24 animals for escapes and escape failures, and 4-5 animals for catalepsy.

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Fig. 8. Body weight gain in female rats fed a high-fat diet treated with olanzapine and RRR-DHTBZ alone and in combination: maximally effective olanzapine doses. In this experiment (RS1046), RRR-DHTBZ was combined with maximally effective doses of olanzapine for increasing weight gain (0.6 and 1.2 mg/kg). Body weight gain was determined over 14 days, the data values calculated as body weight on Day 14 minus body weight on Day 1. The asterisks above the bars denote significant difference ($p < 0.05$) between the treatment group and vehicle. The numbers below the x-axis are p values of statistical comparison between the indicated treatment groups. Data and statistical analysis are in Tables S12 and S12A, respectively. The group size was 10 animals.

Fig. 9. Body weight gain in female rats fed a high-fat diet treated with olanzapine and RRR-DHTBZ alone and in combination: sub-maximally effective olanzapine dose. In this experiment (RS1262), RRR-DHTBZ was combined with a sub-maximally effective dose of olanzapine for increasing weight gain (0.3 mg/kg). Body weight gain was determined over 14 days, with data values calculated as body weight on Day 14 minus body weight on Day 1. The asterisks above the bars denote significant difference ($p < 0.05$) between the treatment group and vehicle. The numbers below the x-axis are p values of statistical comparison between the indicated treatment groups. Data and statistical analysis are in Tables S13 and S13A, respectively. The group size was 10 animals.

Fig. 10. Body weight gain and CAR suppression versus RRR-DHTBZ concentration.

Body weight gain was measured over 14 days in female rats fed a high-fat diet. CAR data are

from Fig. 4. Weight gain data are in Tables S12 and S13. The dashed red line is body weight

gain of vehicle-treated rats. The asterisk denotes significant difference ($p < 0.05$) between the

treatment group and vehicle for weight gain. (See Tables S12A and S13A for statistical analysis.)

The group size was 9-10 and 10 animals for CAR and weight gain, respectively.

Fig. 11. Olanzapine-induced elevation of weight gain in the absence and presence of RRR-DHTBZ, plotted against olanzapine plasma concentration. The dose of RRR-DHTBZ was 0.15 mg/kg. Body weight gain was measured over 14 days in female rats fed a high-fat diet. The arrow indicates synergizing plasma concentration of olanzapine in the CAR study (42 ng/ml, average of the 0.6 mg/kg values in Tables S9 and S10). Data values are given in Tables S12 and S13. The group size was 8-10 and 10 animals for CAR and weight gain, respectively.

Fig. 12. Proposed mechanism of synergy between VMAT2 inhibition and D2 dopamine receptor blockade in antipsychotic efficacy. The D2 dopamine receptor is located postsynaptically where it mediates antipsychotic efficacy (Kapur and Mamo, 2003) and presynaptically where it mediates negative feedback mechanisms by functioning as an autoreceptor (Ford, 2014). A. Untreated dopamine synapse. Dopamine released into the synapse activates postsynaptic receptors to modulate postsynaptic signaling (1). Dopamine also activates presynaptic receptors (2), which results in diminished dopamine synthesis by a reduction of tyrosine hydroxylase activity (3). This negative feedback loop maintains control of dopaminergic neurotransmission. B. Antipsychotic-treated synapse. The antipsychotic blocks postsynaptic D2 receptors (4). However, blockade of the autoreceptor (5) ultimately reduces the amount of block achieved, limiting efficacy. This results from elevated dopamine, a consequence of blocking negative feedback by inhibiting the action of dopamine at the autoreceptor. Specifically, antipsychotic treatment increases tyrosine hydroxylase activity (6) (Carlsson et al., 1977), increasing dopamine synthesis and presynaptic dopamine. This dopamine is subsequently released into the synapse where it competes with antipsychotic for binding to postsynaptic receptors (7). C. VMAT2 inhibitor-treated synapse. This mechanism inhibits postsynaptic transmission indirectly, by reducing synaptic dopamine release by blocking dopamine packaging into presynaptic vesicles (8). D. Combination of both treatments. The VMAT2 inhibitor relieves the presynaptic dopaminergic effect of the antipsychotic by blocking the packaging of the extra dopamine into presynaptic vesicles (9), attenuating the elevated synaptic dopamine resulting from antipsychotic binding to the autoreceptor (10). This reduces competition for binding of the antipsychotic to postsynaptic receptors, increasing occupancy (11). This in turn decreases dopamine signaling and increases the efficacy of the antipsychotic. Runaway elevation of

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cytoplasmic dopamine is likely prevented by feedback inhibition of synthesis; dopamine is

a competitive inhibitor of tyrosine hydroxylase (12) (Daubner et al., 2011).

Table 1. Minimum effective plasma concentration and dose for dopaminergic behaviors and weight gain in rats

Compound	CAR escapes (number of trials)	Escape failures (number of trials)	Catalepsy	Weight gain
RRR-DHTBZ	19 (0.3) [A]	52 (0.7) [A]	400 (1.2) [B]	210 (0.5) [C]
Risperidone	45 (0.3) [A]	280 (1.0) [D]	360 (1.0) [E]	Not tested
Risperidone with 0.15 mg/kg RRR-DHTBZ	20 (0.1) [F]	59 (0.3) [D]	190 (1.0) [D]	Not tested
Olanzapine	390 (3.0) [G]	390 (3) [G]	ND (> 3.0)	17 (0.6) [H]
Olanzapine with 0.15 mg/kg RRR-DHTBZ	30 (0.6) [I]	140 (1.0) [J]	ND (> 3.0)	14 (0.6) [H]

The minimum plasma concentration and dose required to elicit statistically significant activity was determined (see Supplementary Tables and footnotes below). CAR escapes and escape failures and catalepsy were measured in male Wistar rats 30 min after a single dose of the compounds. Weight gain was measured in female Sprague-Dawley rats and the plasma concentration measured 30 minutes following the final dose of a once-daily dosing regimen for 15 days. Upper values are plasma concentration in ng/ml and lower values in parentheses are dose in mg/kg. [A]Table S3; [B]Table S4; [C]Table S13; [D]Table S6; [E]Table S7; [F]Table S5; [G]Table S11; [H]Table S12; [I]Table S9; [J]Table S8. ND=not done.

Figure 1
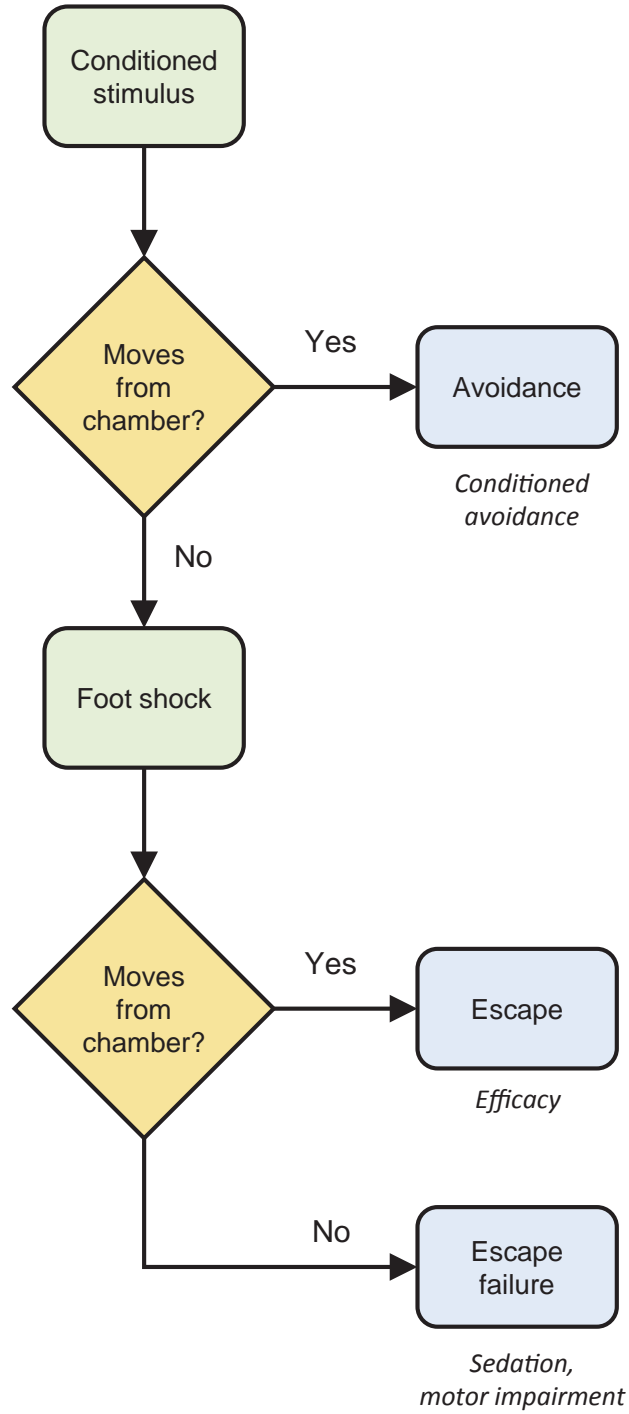
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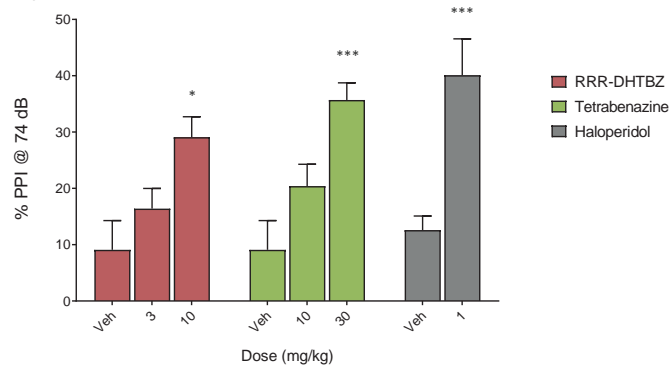
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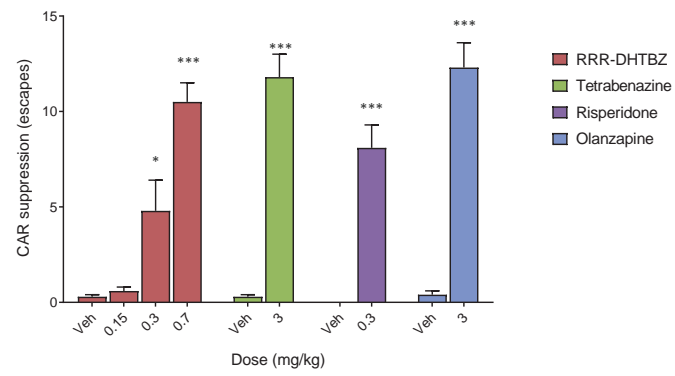
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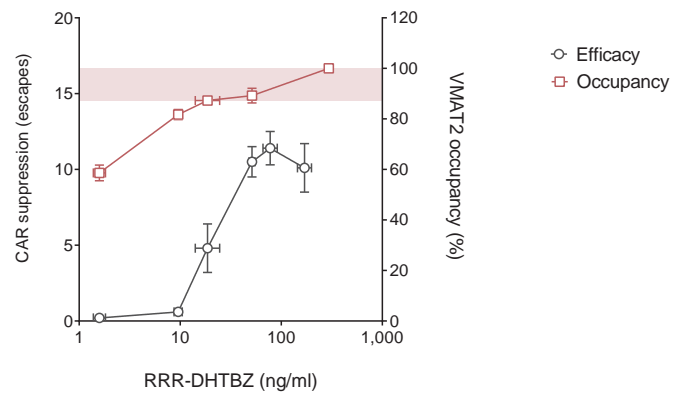
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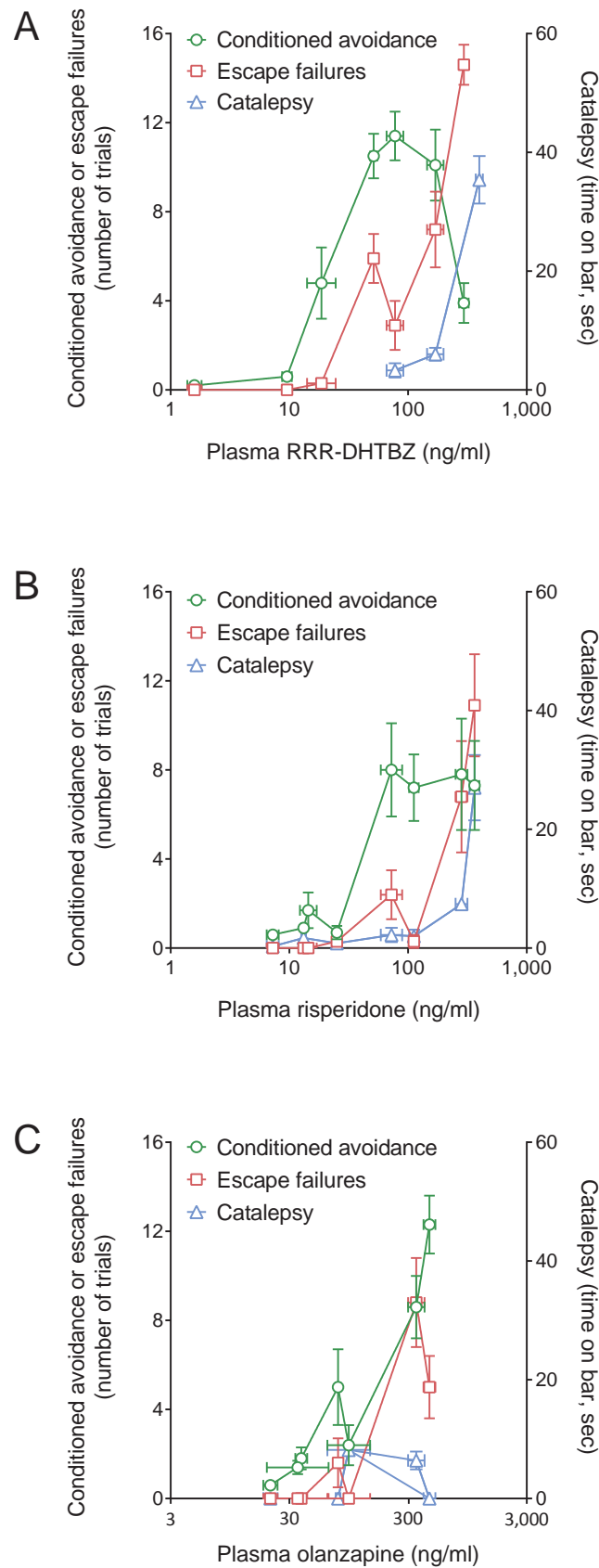
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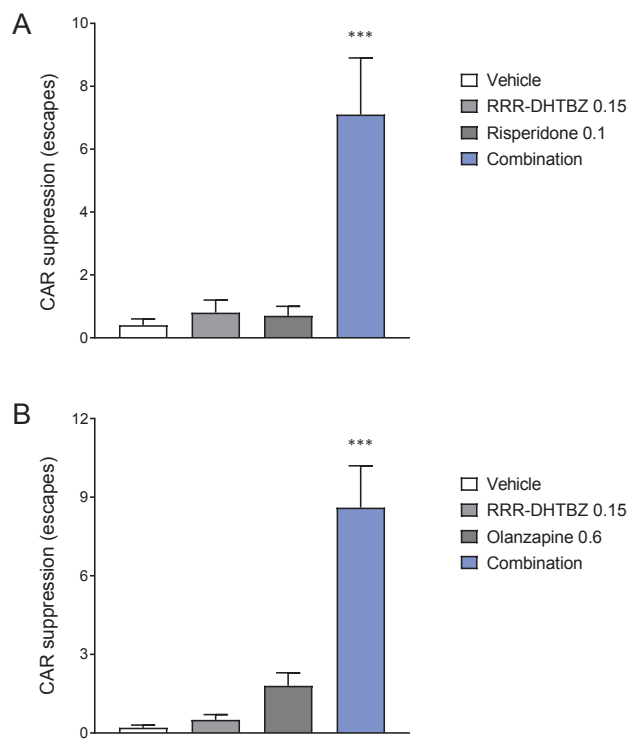
Figure 7

Figure 8

Legend:
- Vehicle
- Olanzapine 0.6
- Olanzapine 1.2
- Olanzapine 3
- RRR-DHTBZ 0.15
- RRR-DHTBZ 0.5
- Olanzapine 0.6 + RRR-DHTBZ 0.15
- Olanzapine 1.2 + RRR-DHTBZ 0.15

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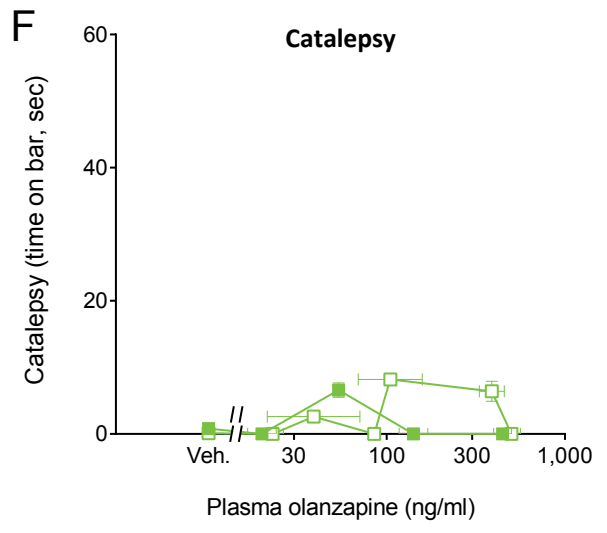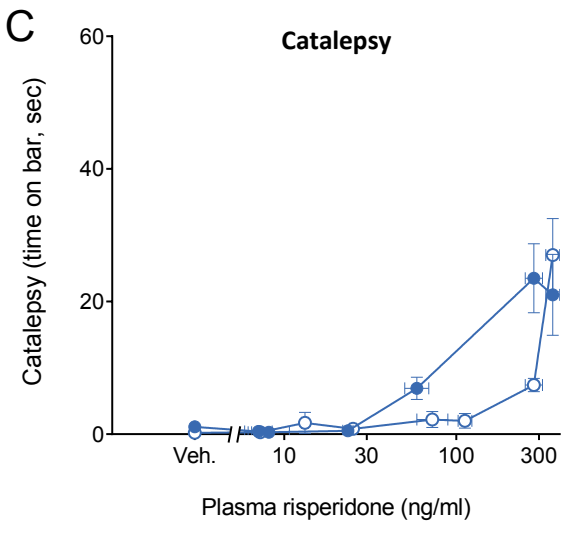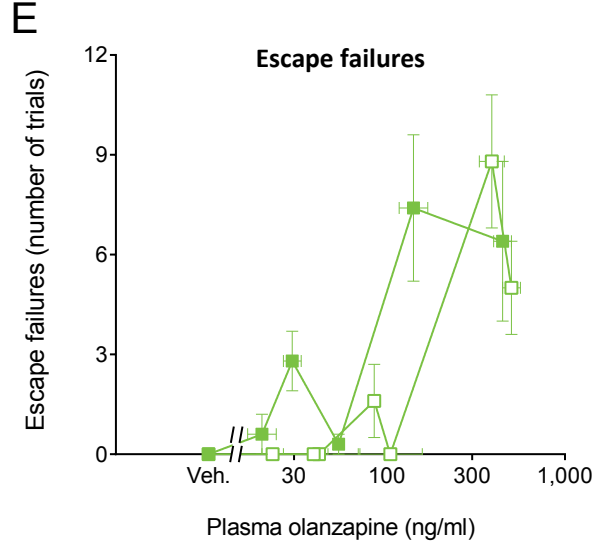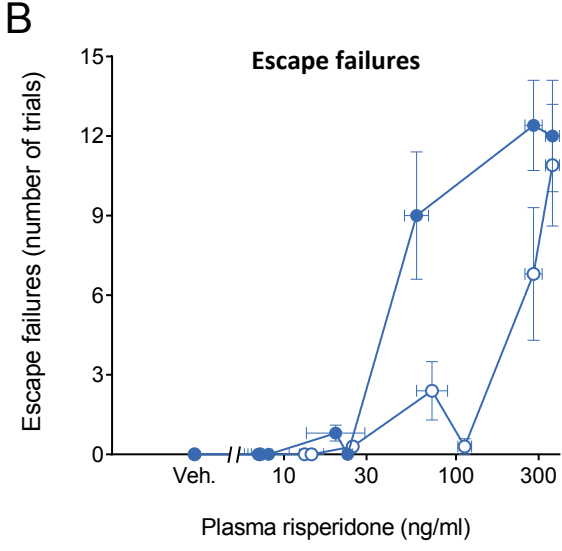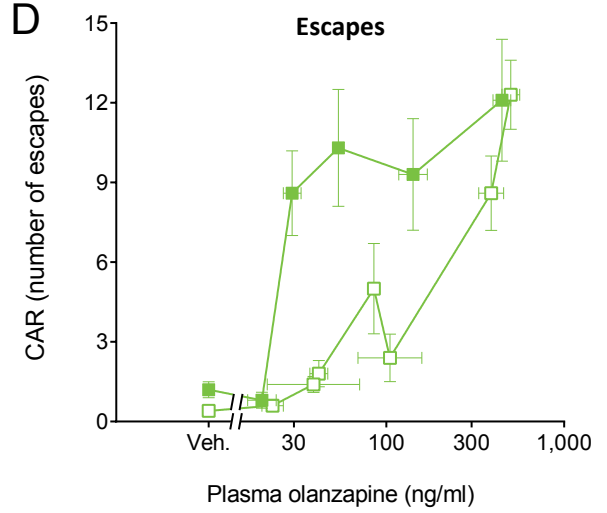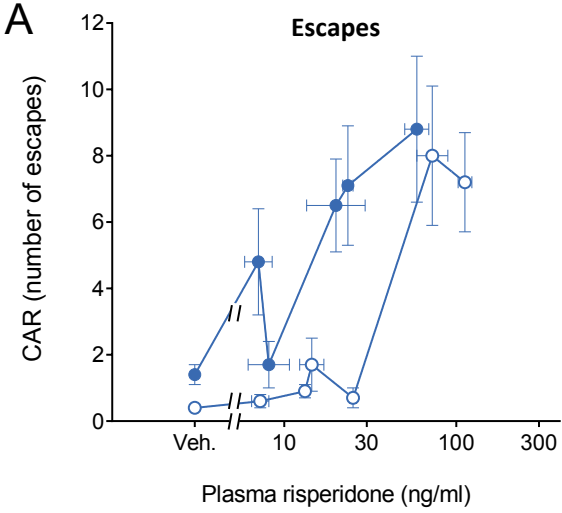
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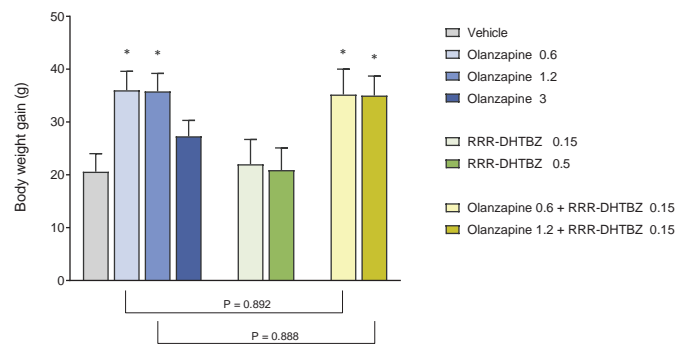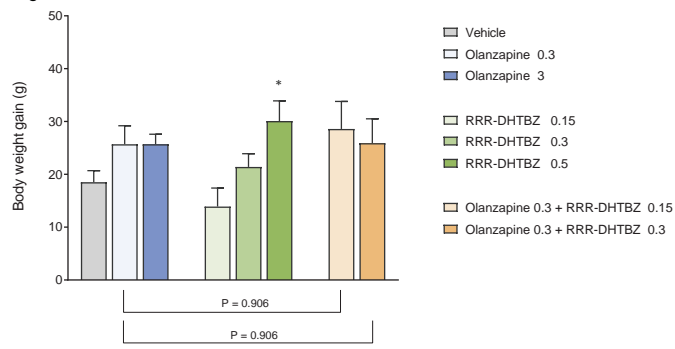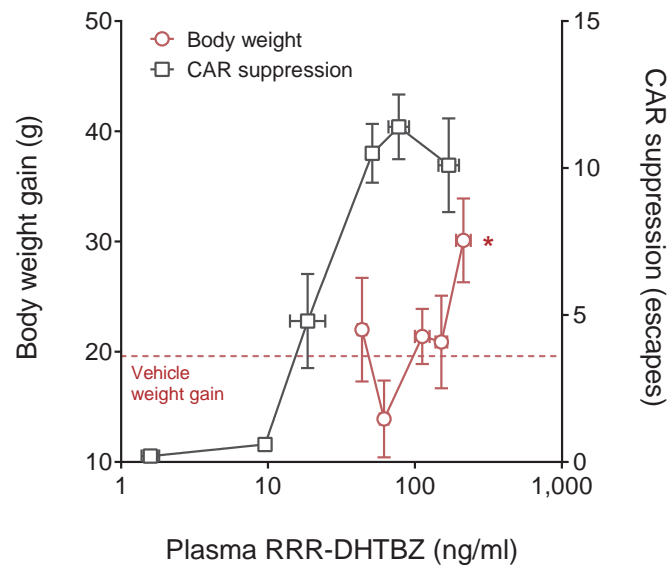
Figure 9

Figure 10

Figure 11
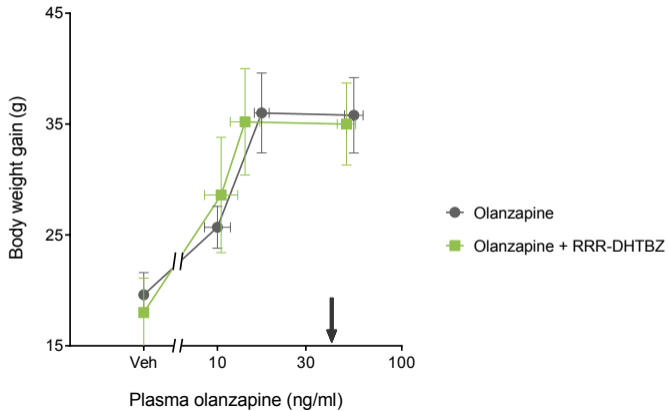
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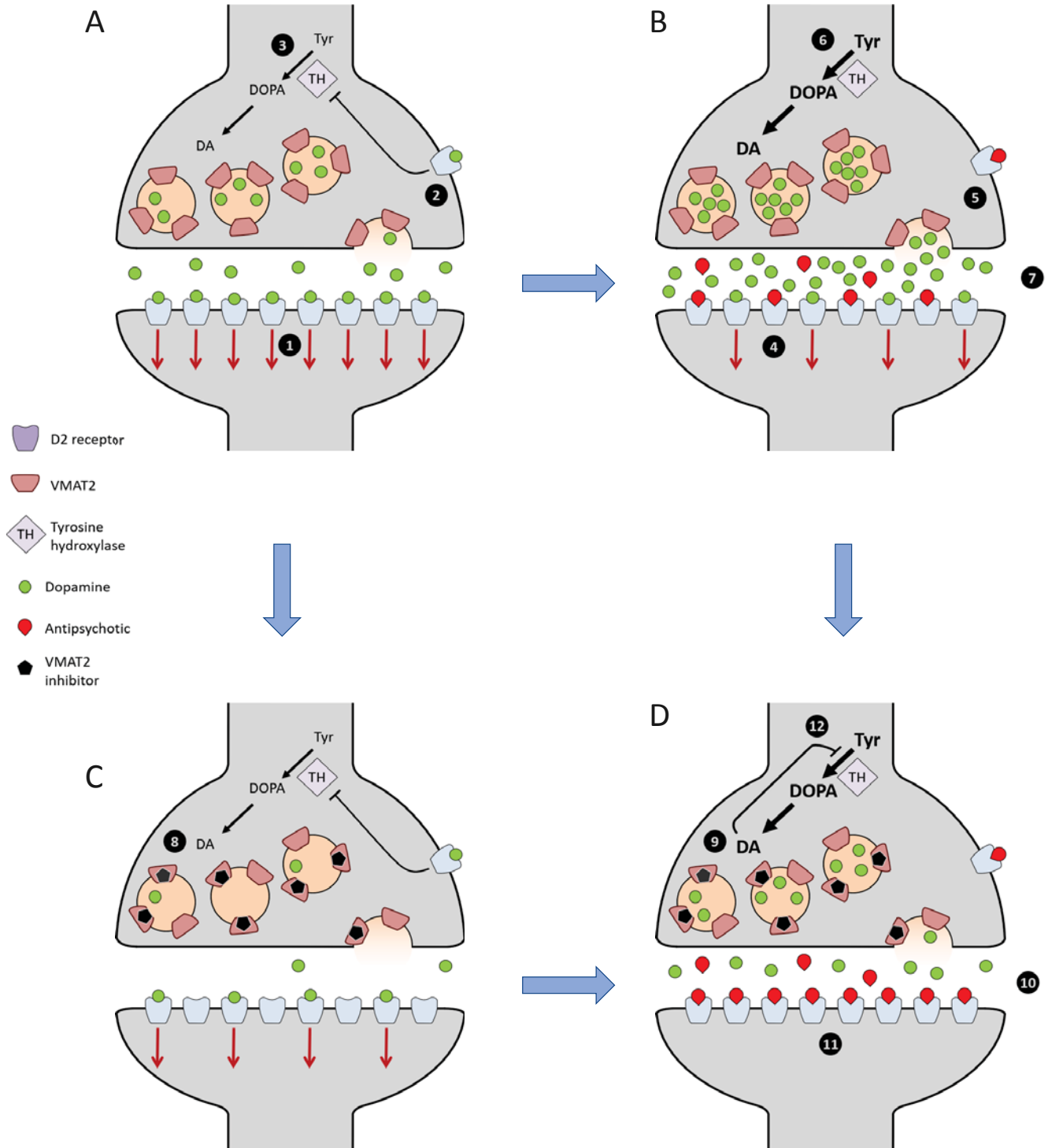
Efficacy of VMAT2 inhibition and synergy with antipsychotics in animal models of schizophrenia

JPET-AR-2021-000979R1

Sam R. J. Hoare, Andrea E. Kudwa, Rosa Luo, Dimitri E. Grigoriadis

Prepulse inhibition experiments

Table S1. PPI Expt 1: RRR-DHTBZ dose response, TBZ and haloperidol data

Treatment	% PPI, 74 dB		% PPI, 78 dB		% PPI, 82 dB		Startle		n
	mean	sem	mean	sem	mean	sem	mean	sem	
Vehicle for VMAT2 ligands – water	9.1	5.2	19.0	4.2	24.9	4.8	641	37	11
RRR-DHTBZ, 3 mg/kg	16.4	3.6	25.3	4.8	27.5	4.3	629	46	11
RRR-DHTBZ, 10 mg/kg	29.1	3.6	42.3	4.7	52.7	4.9	392	53	11
TBZ, 10 mg/kg	20.4	3.9	26.4	4.0	30.5	4.2	650	76	11
TBZ, 30 mg/kg	35.7	3.0	47.8	1.1	61.5	2.8	507	73	10
Vehicle for haloperidol – 10% DMSO	12.6	2.5	15.3	3.2	23.4	5.0	737	68	10
Haloperidol, 1 mg/kg	40.1	6.5	50.8	4.7	55.2	3.6	372	51	10

% PPI at 74 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	8712	6	1452	F (6, 67) = 7.691	< 0.0001
Residual (within columns)	12650	67	188.8		
Total	21362	73			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	TBZ 10	TBZ 30	10% DMSO	Haloperidol 1
water							
RRR-DHTBZ 3	NS						
RRR-DHTBZ 10	*	NS					
TBZ 10	NS	NS	NS				
TBZ 30	***	*	NS	NS			
10% DMSO	NS	NS	NS	NS	**		
Haloperidol 1	***	**	NS	*	NS	***	

* p < 0.05; ** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg.

% PPI at 78 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	12685	6	2114	F (6, 67) = 12.22	< 0.0001
Residual (within columns)	11592	67	173.0		
Total	24277	73			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	TBZ 10	TBZ 30	10% DMSO	Haloperidol 1
water							
RRR-DHTBZ 3	NS						
RRR-DHTBZ 10	**	NS					
TBZ 10	NS	NS	NS				
TBZ 30	***	**	NS	NS			
10% DMSO	NS	NS	***	NS	***		
Haloperidol 1	***	***	NS	**	NS	***	

** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg.

% PPI at 82 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	16598	6	2766	F (6, 67) = 13.88	< 0.0001
Residual (within columns)	13355	67	199.3		
Total	29954	73			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	TBZ 10	TBZ 30	10% DMSO	Haloperidol 1
water							
RRR-DHTBZ 3	NS						
RRR-DHTBZ 10	***	**					
TBZ 10	NS	NS	**				
TBZ 30	***	***	NS	***			
10% DMSO	NS	NS	***	NS	***		
Haloperidol 1	***	***	NS	**	NS	***	

** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Startle response, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	1.219e+006	6	203162	F (6, 67) = 5.588	< 0.0001
Residual (within columns)	2.436e+006	67	36358		
Total	3.655e+006	73			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	TBZ 10	TBZ 30	10% DMSO	Haloperidol 1
water							
RRR-DHTBZ 3	NS						
RRR-DHTBZ 10	*	NS					
TBZ 10	NS	NS	*				
TBZ 30	NS	NS	NS	NS			
10% DMSO	NS	NS	**	NS	NS		
Haloperidol 1	*	**	NS	*	NS	**	

* $p < 0.05$; ** $p < 0.01$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Table S2. PPI Expt 2: RRR-DHTBZ dose response and haloperidol data

Treatment	% PPI, 74 dB		% PPI, 78 dB		% PPI, 82 dB		startle		n
	mean	sem	mean	sem	mean	sem	mean	sem	
Vehicle for RRR-DHTBZ – water	9.2	4.7	16.6	3.4	23.4	5.2	648	76	11
RRR-DHTBZ, 3 mg/kg	11.9	3.1	19.2	3.9	27.3	3.4	720	56	10
RRR-DHTBZ, 10 mg/kg	29.6	4.2	50.0	4.0	58.6	3.8	463	58	10
Haloperidol, 1 mg/kg	35.0	3.1	43.2	4.8	57.0	2.8	301	54	9

% PPI at 74 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	4845	3	1615	F (3, 36) = 10.41	< 0.0001
Residual (within columns)	5587	36	155.2		
Total	10432	39			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	Haloperidol 1
water				
RRR-DHTBZ 3	NS			
RRR-DHTBZ 10	**	*		
Haloperidol 1	***	**	NS	

* p < 0.05; ** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg.

% PPI at 78 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	8599	3	2866	F (3, 36) = 17.93	< 0.0001
Residual (within columns)	5755	36	159.8		
Total	14353	39			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	Haloperidol 1
water				
RRR-DHTBZ 3	NS			
RRR-DHTBZ 10	***	***		
Haloperidol 1	***	**	NS	

** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg.

% PPI at 82 dB, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	10679	3	3560	F (3, 36) = 21.75	< 0.0001
Residual (within columns)	5891	36	163.7		
Total	16570	39			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	Haloperidol 1
water				
RRR-DHTBZ 3	NS			
RRR-DHTBZ 10	***	***		
Haloperidol 1	***	***	NS	

*** $p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Startle response, comparing treatment groups: Single-factor ANOVA followed by Tukey's Multiple Comparison post-test

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	1.025e+006	3	341521	F (3, 36) = 8.638	0.0002
Residual (within columns)	1.423e+006	36	39538		
Total	2.448e+006	39			

Multiple comparisons

	water	RRR-DHTBZ 3	RRR-DHTBZ 10	Haloperidol 1
water				
RRR-DHTBZ 3	NS			
RRR-DHTBZ 10	NS	*		
Haloperidol 1	**	***	NS	

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

RRR-DHTBZ Dose Response Conditioned Avoidance Experiments

Table S3: RRR-DHTBZ Dose Response

From Experiment 1 (Cohort 1, Sessions 1 and 2)

Treatment	Conditioned avoidance (number of trials, out of 20)					Plasma concentration (log ng/ml)			
	Escapes		Escape failures		n	mean	sem	n	mean ng/ml
	mean	sem	mean	sem					
Vehicle for VMAT2 ligands – water	0.3	0.1	0.0	0.0	22				
RRR-DHTBZ, 0.03 mg/kg	0.2	0.1	0.0	0.0	10	0.20	0.06	5	1.6
RRR-DHTBZ, 0.15 mg/kg	0.6	0.2	0.0	0.0	21	0.98	0.04	10	9.5
RRR-DHTBZ, 0.3 mg/kg	4.8	1.6	0.3	0.1	12	1.27	0.12	5	19
RRR-DHTBZ, 0.7 mg/kg	10.5	1.0	5.9	1.1	20	1.71	0.03	10	52
RRR-DHTBZ, 3 mg/kg [C]	1.5	0.3	14.6	0.9	10	2.47	0.04	5	290
TBZ, 3 mg/kg	11.8	1.2	6.3	1.0	9	1.83 [A]	0.02	5	34
Vehicle for risperidone – 10% DMSO	0.0	0.0	0.0	0.0	10				
Risperidone, 0.3 mg/kg	8.1	1.2	4.1	1.2	24	1.65 [B]	0.11	5	45

[A]This is the RRR-DHTBZ concentration resulting from exposure to TBZ. Under the conditions of the analyte detection procedure, both RRR-DHTBZ and SSS-DHTBZ metabolites of TBZ are detected. The concentration of RRR-DHTBZ in the plasma samples was assumed to be 50% of the total signal detected.

[B]For the risperidone concentration, risperidone was measured but the active metabolite 9-OH risperidone was not (in contrast to all the other studies). For the purposes of comparison between studies, the risperidone concentration values are interpolated risperidone total values (i.e., the sum of risperidone and 9-OH risperidone), assuming a ratio of 9-OH risperidone : risperidone of 1.62 (the average of the ratio in all samples where both analytes were measured).

[C]Conditioned avoidance data not included in statistics because escape failures were very high.

Table S3A. Escapes in response to RRR-DHTBZ, TBZ and risperidone

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	2605	7	372	F (7, 120) = 24.7	<0.0001
Residual (within columns)	1809	120	15.1		
Total	4414	127			

Multiple comparisons

	Water	RRR-DHTBZ 0.03	RRR-DHTBZ 0.15	RRR-DHTBZ 0.3	RRR-DHTBZ 0.7	TBZ 3	10% DMSO	Risperidone 0.3
Water								
RRR-DHTBZ 0.03	NS							
RRR-DHTBZ 0.15	NS	NS						
RRR-DHTBZ 0.3	*	NS	NS					
RRR-DHTBZ 0.7	***	***	***	**				
TBZ 3	***	***	***	**	NS			
10% DMSO	NS	NS	NS	NS	***	***		
Risperidone 0.3	***	***	***	NS	NS	NS	***	

$* \ p < 0.05; ** \ p < 0.01; *** \ p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Table S3B. Escape failures in response to RRR-DHTBZ

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	860	7	112	F (7, 120) = 11.7	<0.0001
Residual (within columns)	1260	120	10.5		
Total	2120	127			

Multiple comparisons

	Water	RRR-DHTBZ 0.03	RRR-DHTBZ 0.15	RRR-DHTBZ 0.3	RRR-DHTBZ 0.7	TBZ 3	10% DMSO	Risperidone 0.3
Water								
RRR-DHTBZ 0.03	NS							
RRR-DHTBZ 0.15	NS	NS						
RRR-DHTBZ 0.3	NS	NS	NS					
RRR-DHTBZ 0.7	***	***	***	***				
TBZ 3	***	**	***	**	NS			
10% DMSO	NS	NS	NS	NS	***	**		
Risperidone 0.3	**	*	**	*	NS	NS	*	

$* \ p < 0.05; ** \ p < 0.01; *** \ p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Table S4: RRR-DHTBZ Dose Response

From Experiment 7 (Cohort 4, Session 3)

| Treatment | Conditioned avoidance (number of trials, out of 20) | | | | | Plasma concentration (log ng/ml) | | | | Catalepsy (Time on bar, sec) | | |
| | Escapes | | Escape failures | | n | | | | | | | |
	mean	SEM	mean	SEM		mean	SEM	n	mean ng/ml	mean	SEM	n
Vehicle – water	0.4	0.2	0.0	0.0	9					0.1	0.1	5
RRR-DHTBZ 0.3 mg/kg	11.4	1.1	2.9	1.1	9	1.89	0.07	5	77	3.3	1.2	5
RRR-DHTBZ 0.6 mg/kg	10.1	1.6	7.2	1.7	10	2.23	0.07	5	170	6.0	1.1	5
RRR-DHTBZ 1.2 mg/kg	10.8	1.4	7.8	1.5	10	2.6	0.03	5	400	35.4	4.0	5

Table S4A. Escapes in response to RRR-DHTBZ

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	658	2	329	F (2, 25) = 26.5	< 0.0001
Residual (within columns)	311	25	12.4		
Total	969	27			

Multiple comparisons

	Vehicle	RRR-DHTBZ 0.3	RRR-DHTBZ 0.6
Vehicle			
RRR-DHTBZ 0.3	***		
RRR-DHTBZ 0.6	***	NS	

*** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg. Data for 1.2 mg/kg RRR-DHTBZ were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 10 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.

Table S4B. Escape failures in response to RRR-DHTBZ

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	250	2	125	F (2, 25) = 8.71	0.0013
Residual (within columns)	359	25	14.4		
Total	610	27			

Multiple comparisons

	Vehicle	RRR-DHTBZ 0.3	RRR-DHTBZ 0.6
Vehicle			
RRR-DHTBZ 0.3	NS		
RRR-DHTBZ 0.6	***	NS	

*** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg. Data for 1.2 mg/kg RRR-DHTBZ were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 20 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.

Table S4C. Catalepsy in response to RRR-DHTBZ

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	3992	3	1331	F (3, 16) = 57.3	<0.0001
Residual (within columns)	372	16	23.2		
Total	4363	19			

Multiple comparisons

	Vehicle	RRR-DHTBZ 0.3	RRR-DHTBZ 0.6	RRR-DHTBZ 1.2
Vehicle				
RRR-DHTBZ 0.3	NS			
RRR-DHTBZ 0.6	NS	NS		
RRR-DHTBZ 1.2	***	***	***	

*** p < 0.001; NS – not significant, p > 0.05. Numbers are dose in mg/kg.

Risperidone–RRR-DHTBZ Combination Conditioned Avoidance Experiments

Table S5: Risperidone – RRR-DHTBZ Combination

From Experiment 1 (Cohort 1, Sessions 1 and 2)

Treatment	Conditioned avoidance (number of trials, out of 20)					Plasma concentration (log ng/ml)			
	Escapes		Escape failures		n				
	mean	SEM	mean	SEM		mean	SEM	n	mean ng/ml
Vehicle for RRR-DHTBZ – water	0.3	0.1	0.0	0.0	22				
RRR-DHTBZ, 0.15 mg/kg	0.6	0.2	0.0	0.0	21	0.98	0.04	10	9.5
Risperidone, 0.1 mg/kg	1.7	0.8	0.0	0.0	12	1.16 [A]	0.07	5	14
Risperidone, 0.1 mg/kg + RRR-DHTBZ, 0.15 mg/kg	6.5	1.4	0.8	0.3	12	1.30 [A] 1.08	0.17 0.07	4 4	20 12

For the risperidone + RRR-DHTBZ experiment plasma concentration column, the upper value is the risperidone concentration and the lower value the RRR-DHTBZ concentration. For the risperidone concentration, risperidone was measured but the active metabolite 9-OH risperidone was not (in contrast to all the other studies).

[A]For the purposes of comparison between studies, the risperidone concentration values are interpolated risperidone total values (i.e., the sum of risperidone and 9-OH risperidone), assuming a ratio of 9-OH risperidone : risperidone of 1.62 (the average of the ratio in all samples where both analytes were measured).

Table S5A. Comparing escapes in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	10.6	0.0004	***	Yes	
Risperidone	27.8	<0.0001	****	Yes	
RRR-DHTBZ	13.6	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	78	1	78	F (1, 63) = 13.8	0.0004
Risperidone	205	1	205	F (1, 63) = 36.4	<0.0001
RRR-DHTBZ	100	1	100	F (1, 63) = 17.8	<0.0001
Residual	355	63	5.64		

Multiple comparisons

	water	RRR-DHTBZ, 0.15	Risperidone, 0.1	RRR-DHTBZ, 0.15 + Risperidone, 0.1
water				
RRR-DHTBZ, 0.15	NS			
Risperidone, 0.1	NS	NS		
RRR-DHTBZ, 0.15 + Risperidone, 0.1	***	***	***	

*** $p < 0.001$; NS – not significant, $p > 0.05$. Numbers are dose in mg/kg.

Comparing escape failures in response to risperidone in the absence and presence of RRR-DHTBZ

No statistical test was performed because the mean escape failures value was minimal in all treatment groups (< 1 trial).

Table S5B. Comparing risperidone plasma concentration; risperidone dosed alone or in combination with 0.15 mg/kg RRR-DHTBZ

t test summary

Unpaired t test	
p value	0.4207
p value summary	ns
Significantly different (p < 0.05)?	No
One- or two-tailed p value?	Two-tailed
t, df	t=0.855 df=7

Risperidone dosed at 0.1 mg/kg

Table S5C. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with 0.1 mg/kg risperidone

t test summary

Unpaired t test	
p value	0.2264
p value summary	ns
Significantly different (p < 0.05)?	No
One- or two-tailed p value?	Two-tailed
t, df	t=1.28 df=12

RRR-DHTBZ dosed at 0.15 mg/kg

Table S6: Risperidone – RRR-DHTBZ Combination

From Experiment 2 (Cohort 2, Sessions 1 and 2)

Treatment	Conditioned avoidance (number of trials, out of 20)					Plasma concentration (log ng/ml)				Catalepsy (Time on bar, sec)		
	Escapes		Escape failures		n	mean	SEM	n	mean ng/ml	mean	SEM	n
	mean	SEM	mean	SEM								
Vehicle for RRR-DHTBZ + Vehicle for risperidone	0.4	0.2	0.0	0.0	15					0.3	0.3	4
RRR-DHTBZ, 0.15 mg/kg	0.8	0.4	0.0	0.0	9	0.93	0.05	5	8.5	0.3	0.3	5
Risperidone, 0.03 mg/kg	0.6	0.2	0.0	0.0	8	0.86	0.05	5	7.3	0.3	0.2	4
Risperidone, 0.03 mg/kg + RRR-DHTBZ 0.15 mg/kg	4.8	1.6	0.0	0.0	9	0.85 1.09	0.08 0.03	2 5	7.1 12	0.4	0.3	5
Risperidone, 0.1 mg/kg	0.7	0.3	0.3	0.2	7	1.40	0.02	5	25	0.8	0.1	4
Risperidone, 0.1 mg/kg + RRR-DHTBZ 0.15 mg/kg	7.1	1.8	0.0	0.0	9	1.37 0.98	0.03 0.07	5 5	23 9.5	0.5	0.2	5
Risperidone, 0.3 mg/kg	8.0	2.1	2.4	1.1	8	1.86	0.09	5	72	2.2	1.2	4
Risperidone, 0.3 mg/kg + RRR-DHTBZ 0.15 mg/kg	8.8	2.2	9.0	2.4	8	1.77 0.86	0.07 0.12	5 5	59 7.3	6.9	1.7	5
Risperidone, 1 mg/kg	7.8	2.5	6.8	2.5	9	2.45	0.05	5	280	7.4	1.0	4
Risperidone, 1 mg/kg + RRR-DHTBZ 0.15 mg/kg	6.6	1.7	12.4	1.7	9	2.29 0.95	0.17 0.05	4 4	190 8.8	23.5	5.2	5

For the risperidone + RRR-DHTBZ experiment plasma concentration column, the upper value is the risperidone concentration and the lower value the RRR-DHTBZ concentration. The plasma concentration given for risperidone is risperidone-total, the sum of risperidone and 9-OH risperidone concentrations. Vehicle for RRR-DHTBZ – water. Vehicle for risperidone – 10% DMSO.

Table S6A. Comparing escapes in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	5.96	0.0766	ns	No	
Risperidone	31.6	<0.0001	****	Yes	
RRR-DH-TBZ	8.44	0.0022	**	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	107	3	35.7	F (3, 65) = 2.39	0.0766
Risperidone	568	3	189	F (3, 65) = 12.7	<0.0001
RRR-DH-TBZ	152	1	152	F (1, 65) = 10.2	0.0022
Residual	971	65	14.9		

Data for 1 mg/kg risperidone were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 10 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.1	Risp 0.1 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ
Vehicle								
Vehicle + RRR-DHTBZ	NS							
Risp 0.03	NS	NS						
Risp 0.03 + RRR-DHTBZ	NS	NS	NS					
Risp 0.1	NS	NS	NS	NS				
Risp 0.1 + RRR-DHTBZ	**	*	*	NS	*			
Risp 0.3	***	**	**	NS	*	NS		
Risp 0.3 + RRR-DHTBZ	***	**	**	NS	**	NS	NS	

* p < 0.05; ** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S6B. Comparing escape failures in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	14.5	0.0002	***	Yes	
Risperidone	41.2	<0.0001	****	Yes	
RRR-DHTBZ	4.26	0.0107	*	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	147	3	49	F (3, 65) = 7.84	0.0002
Risperidone	418	3	139	F (3, 65) = 22.3	<0.0001
RRR-DHTBZ	43.2	1	43.2	F (1, 65) = 6.92	=0.0107
Residual	406	65	6.25		

Data for 1 mg/kg risperidone were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 10 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.1	Risp 0.1 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ
Vehicle								
Vehicle + RRR-DHTBZ	NS							
Risp 0.03	NS	NS						
Risp 0.03 + RRR-DHTBZ	NS	NS	NS					
Risp 0.1	NS	NS	NS	NS				
Risp 0.1 + RRR-DHTBZ	NS	NS	NS	NS	NS			
Risp 0.3	NS	NS	NS	NS	NS	NS		
Risp 0.3 + RRR-DHTBZ	***	***	***	***	***	***	***	

*** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S6C. Comparing catalepsy in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	15.8	0.0008	***	Yes	
Risperidone	54.5	<0.0001	****	Yes	
RRR-DHTBZ	6.83	0.0027	**	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	437	4	109	$F_{(4, 35)} = 6.04$	0.0008
Risperidone	1505	4	376	$F_{(4, 35)} = 20.8$	<0.0001
RRR-DHTBZ	189	1	189	$F_{(1, 35)} = 10.4$	0.0027
Residual	632	35	18.1		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Risp 0.03	Risp 0.03+ RRR-DHTBZ	Risp 0.1	Risp 0.1 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ	Risp 1	Risp 1 + RRR-DHTBZ
Vehicle										
Vehicle + RRR-DHTBZ	NS									
Risp 0.03	NS	NS								
Risp 0.03+ RRR-DHTBZ	NS	NS	NS							
Risp 0.1	NS	NS	NS	NS						
Risp 0.1 + RRR-DHTBZ	NS	NS	NS	NS	NS					
Risp 0.3	NS	NS	NS	NS	NS	NS				
Risp 0.3 + RRR-DHTBZ	NS	NS	NS	NS	NS	NS	NS			
Risp 1	NS	NS	NS	NS	NS	NS	NS	NS		
Risp 1 + RRR-DHTBZ	***	***	***	***	***	***	***	***	***	

*** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S6D. Comparing risperidone-total plasma concentration; risperidone dosed alone or in combination with 0.15 mg/kg RRR-DHTBZ

Two-factor ANOVA summary

Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	0.256	0.7737	ns	No	
Risperidone	92.9	<0.0001	****	Yes	
RRR-DHTBZ	0.394	0.2008	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	0.0281	3	0.00935	F (3, 28) = 0.372	0.7737
Risperidone	10.2	3	3.39	F (3, 28) = 135	<0.0001
RRR-DHTBZ	0.0431	1	0.0431	F (1, 28) = 1.72	0.2008
Residual	0.704	28	0.0251		

Multiple comparisons

	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.1	Risp 0.1 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ	Risp 1	Risp 1 + RRR-DHTBZ
Risp 0.03								
Risp 0.03 + RRR-DHTBZ	NS							
Risp 0.1	***	**						
Risp 0.1 + RRR-DHTBZ	***	*	NS					
Risp 0.3	***	***	**	***				
Risp 0.3 + RRR-DHTBZ	***	***	*	**	NS			
Risp 1	***	***	***	***	***	***		
Risp 1 + RRR-DHTBZ	***	***	***	***	**	***	NS	

* p < 0.05; ** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg. Risperidone-total concentration is the sum of risperidone and 9-OH risperidone concentrations.

Table S6E. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with risperidone

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	0.141	4	0.0353	F (4, 19) = 1.33	0.2956
Residual (within columns)	0.505	19	0.0266		
Total	0.646	23			

RRR-DHTBZ dosed at 0.15 mg/kg. Risperidone dosed at 0.03, 0.1, 0.3 or 1 mg/kg.

Table S7: Risperidone – RRR-DHTBZ Combination

From Experiment 5 (Cohort 4, Session 1)

| Treatment | Conditioned avoidance (number of trials, out of 20) | | | | | Plasma concentration (log ng/ml) | | | | Catalepsy (Time on bar, sec) | | |
| | Escapes | | Escape failures | | n | mean | SEM | n | mean ng/ml | mean | SEM | n |
	mean	SEM	mean	SEM								
Vehicle for RRR-DHTBZ + Vehicle for risperidone [A]	0.8	0.2	0.0	0.0	9					0.1	0.1	5
RRR-DHTBZ 0.15 mg/kg [A]	3.1	1.0	0.0	0.0	8	1.55	0.06	5	36	1.9	0.5	5
Risperidone, 0.03 mg/kg	0.9	0.2	0.0	0.0	9	1.12	0.02	5	13	1.7	1.6	5
Risperidone, 0.03 mg/kg + RRR-DHTBZ 0.15 mg/kg	1.7	0.7	0.0	0.0	9	0.91 1.59	0.12 0.06	5 5	8.1 39	0.3	0.2	5
Risperidone, 0.3 mg/kg	7.2	1.5	0.3	0.3	9	2.05	0.04	5	110	2.0	1.1	5
Risperidone, 0.3 mg/kg + RRR-DHTBZ 0.15 mg/kg	15.6	0.4	1.2	0.3	9	1.95 1.42	0.14 0.05	5 5	89 27	1.2	0.5	5
Risperidone, 1 mg/kg	7.3	2.0	10.9	2.3	9	2.56	0.04	5	360	27.0	5.5	5
Risperidone, 1 mg/kg + RRR-DHTBZ 0.15 mg/kg	5.9	1.9	12.0	2.1	9	2.68 1.40	0.05 0.09	5 5	470 25	21.0	6.1	5

[A]Group from Session 2 of the same cohort (Experiment 6).

For the risperidone + RRR-DHTBZ experiment plasma concentration column, the upper value is the risperidone concentration and the lower value the RRR-DHTBZ concentration. The plasma concentration given for risperidone is risperidone-total, the sum of risperidone and 9-OH risperidone concentrations. Vehicle for RRR-DHTBZ – water. Vehicle for risperidone – 10% DMSO.

Table S7A. Comparing escapes in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	6.63	0.0034	**	Yes	
Risperidone	52.9	<0.0001	****	Yes	
RRR-DHTBZ	15	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	132	2	66.1	F (2, 49) = 6.38	0.0034
Risperidone	1055	2	527	F (2, 49) = 50.9	<0.0001
RRR-DHTBZ	299	1	299	F (1, 49) = 28.8	<0.0001
Residual	508	49	10.4		

Data for 1 mg/kg risperidone were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 10 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	*					
Risp 0.03	NS	*				
Risp 0.03 + RRR-DHTBZ	NS	NS	NS			
Risp 0.3	**	NS	**	**		
Risp 0.3 + RRR-DHTBZ	***	***	***	***	***	

* p < 0.05; ** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Comparing escape failures in response to risperidone in the absence and presence of RRR-DHTBZ

No statistical test was performed because the escape failure value was minimal for all treatment groups (highest value of 1.2 trials). (Data for 1 mg/kg risperidone were excluded from the analysis because catalepsy time-on-bar was greater than the criterion of 10 seconds. It was assumed that this extent of catalepsy would interfere with escape and escape failure behavior.)

Table S7B. Comparing catalepsy in response to risperidone in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	1.45	0.6323	ns	No	
Risperidone	71.5	<0.0001	****	Yes	
RRR-DHTBZ	0.469	0.4581	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	79	3	26.3	F (3, 32) = 0.58	0.6323
Risperidone	3901	3	1300	F (3, 32) = 28.7	<0.0001
RRR-DHTBZ	25.6	1	25.6	F (1, 32) = 0.564	0.4581
Residual	1452	32	45.4		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ
Vehicle								
Vehicle + RRR-DHTBZ	NS							
Risp 0.03	NS	NS						
Risp 0.03 + RRR-DHTBZ	NS	NS	NS					
Risp 0.3	NS	NS	NS	NS				
Risp 0.3 + RRR-DHTBZ	NS	NS	NS	NS	NS			
Risp 0.3	***	***	***	***	***	***		
Risp 0.3 + RRR-DHTBZ	***	**	**	***	**	**	NS	

** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S7C. Comparing risperidone-total plasma concentration; risperidone dosed alone or in combination with 0.15 mg/kg RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	1	0.1501	ns	No	
Risperidone	92.9	<0.0001	****	Yes	
RRR-DHTBZ	0.213	0.3587	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	0.141	2	0.0706	F (2, 24) = 2.05	0.1501
Risperidone	13.1	2	6.55	F (2, 24) = 191	<0.0001
RRR-DHTBZ	0.0301	1	0.0301	F (1, 24) = 0.876	0.3587
Residual	0.824	24	0.0344		

Risperidone-total concentration is the sum of risperidone and 9-OH risperidone concentrations.

Multiple comparisons

	Risp 0.03	Risp 0.03 + RRR-DHTBZ	Risp 0.3	Risp 0.3 + RRR-DHTBZ	Risp 1	Risp 1 + RRR-DHTBZ
Risp 0.03						
Risp 0.03 + RRR-DHTBZ	NS					
Risp 0.3	***	***				
Risp 0.3 + RRR-DHTBZ	***	***	NS			
Risp 1	***	***	***	**		
Risp 1 + RRR-DHTBZ	***	***	***	***	NS	

** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S7D. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with risperidone

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	0.133	3	0.0443	F (3, 16) = 1.96	0.1601
Residual (within columns)	0.361	16	0.0226		
Total	0.494	19			

RRR-DHTBZ dosed at 0.15 mg/kg. Risperidone dosed at 0.03 or 1 mg/kg.

Olanzapine – RRR-DHTBZ Combination Conditioned Avoidance Experiments

Table S8: Olanzapine – RRR-DHTBZ Combination

From Experiment 3 (Cohort 2, Session 3)

Treatment	Conditioned avoidance (number of trials, out of 20)					Plasma concentration (log ng/ml)				Catalepsy (Time on bar, sec)		
	Escapes		Escape failures		n							
	mean	SEM	mean	SEM		mean	SEM	n	mean ng/ml	mean	SEM	n
Vehicle for RRR-DHTBZ + Vehicle for olanzapine	0.4	0.2	0.1	0.1	8					0.0	0.0	4
RRR-DHTBZ 0.15 mg/kg [A]	0.8	0.4	0.0	0.0	9	0.93	0.05	5	8.5	0.3	0.3	5
Olanzapine, 1 mg/kg	5.0	1.7	1.6	1.1	8	1.93	0.02	4	86	0.0	0.0	4
Olanzapine, 1 mg/kg + RRR-DHTBZ 0.15 mg/kg	9.3	2.1	7.4	2.2	9	2.15 1.10	0.08 0.04	5 5	140 12	0.0	0.0	5
Olanzapine, 3 mg/kg	12.3	1.3	5.0	1.4	8	2.70	0.05	4	500	0.0	0.0	4
Olanzapine, 3 mg/kg + RRR-DHTBZ 0.15 mg/kg	12.1	2.3	6.4	2.4	8	2.65 0.87	0.05 0.20	5 5	450 7.4	0.0	0.0	5

[A]Group from Session 1 of the same cohort (i.e., Cohort 2).

For the olanzapine + RRR-DHTBZ experiment plasma concentration column, the upper value is the olanzapine concentration and the lower value the RRR-DHTBZ concentration. Vehicle for RRR-DHTBZ – water. Vehicle for olanzapine – 10% DMSO.

Table S8A. Comparing escapes in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	2.38	0.3018	ns	No	
Olanzapine	53.8	<0.0001	****	Yes	
RRR-DHTBZ	1.34	0.2444	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	49.6	2	24.8	F (2, 44) = 1.23	0.3018
Olanzapine	1124	2	562	F (2, 44) = 27.9	<0.0001
RRR-DHTBZ	28	1	28	F (1, 44) = 1.39	0.2444
Residual	886	44	20.1		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 1	Olanz 1 + RRR-DHTBZ	Olanz 3	Olanz 3 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 1	NS	NS				
Olanz 1 + RRR-DHTBZ	**	**	NS			
Olanz 3	***	***	*	NS		
Olanz 3 + RRR-DHTBZ	***	***	*	NS	NS	

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS – not significant, $p > 0.05$. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S8B. Comparing escape failures in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	6.08	0.1420	ns	No	
Olanzapine	22.9	0.0014	**	Yes	
RRR-DHTBZ	5.43	0.0627	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	78.1	2	39.1	F (2, 44) = 2.04	0.1420
Olanzapine	294	2	147	F (2, 44) = 7.69	0.0014
RRR-DHTBZ	69.8	1	69.8	F (1, 44) = 3.65	0.0627
Residual	842	44	19.1		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 1	Olanz 1 + RRR-DHTBZ	Olanz 3	Olanz 3 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 1	NS	NS				
Olanz 1 + RRR-DHTBZ	*	*	NS			
Olanz 3	NS	NS	NS	NS		
Olanz 3 + RRR-DHTBZ	NS	*	NS	NS	NS	

* $p < 0.05$; NS – not significant, $p > 0.05$. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Comparing catalepsy in response to olanzapine in the absence and presence of RRR-DHTBZ

No statistical test was performed because the time on bar was less than 10 seconds for all treatment groups.

Table S8C. Comparing olanzapine plasma concentration; olanzapine dosed alone or in combination with 0.15 mg/kg RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	3.83	0.0350	*	Yes	
Olanzapine	84.8	<0.0001	****	Yes	
RRR-DHTBZ	1.52	0.1636	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	0.081	1	0.081	F (1, 14) = 5.45	0.0350
Olanzapine	1.79	1	1.79	F (1, 14) = 121	<0.0001
RRR-DHTBZ	0.0321	1	0.0321	F (1, 14) = 2.16	0.1636
Residual	0.208	14	0.0149		

Multiple comparisons

	Olanz 1	Olanz 1 + RRR-DHTBZ	Olanz 3	Olanz 3 + RRR-DHTBZ
Olanz 1				
Olanz 1 + RRR-DHTBZ	NS			
Olanz 3	***	***		
Olanz 3 + RRR-DHTBZ	***	***	NS	

*** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S8D. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with olanzapine

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	0.142	2	0.0712	F (2, 12) = 0.921	0.4245
Residual (within columns)	0.927	12	0.0773		
Total	1.07	14			

Table S9: Olanzapine – RRR-DHTBZ Combination

From Experiment 4 (Cohort 3, Sessions 1 and 2)

| Treatment | Conditioned avoidance (number of trials, out of 20) | | | | | Plasma concentration (log ng/ml) | | | | Catalepsy (Time on bar, sec) | | |
| | Escapes | | Escape failures | | n | mean | SEM | n | mean ng/ml | mean | SEM | n |
	mean	SEM	mean	SEM								
Vehicle for RRR-DHTBZ + Vehicle for olanzapine	0.2	0.1	0.0	0.0	8					0.4	0.2	4
RRR-DHTBZ 0.15 mg/kg	0.5	0.2	0.0	0.0	9	1.09	0.07	10	12	0.2	0.1	4
Olanzapine, 0.3 mg/kg	0.6	0.2	0.0	0.0	9	1.36	0.06	5	23	0.0	0.0	4
Olanzapine, 0.3 mg/kg + RRR-DHTBZ 0.15 mg/kg	0.8	0.3	0.6	0.6	8	1.30 / 1.05	0.08 / 0.06	5 / 5	20 / 11	0.2	0.1	5
Olanzapine, 0.6 mg/kg	1.8	0.5	0.0	0.0	9	1.62	0.05	5	41	ND		
Olanzapine, 0.6 mg/kg + RRR-DHTBZ 0.15 mg/kg	8.6	1.6	2.8	0.9	9	1.47 / 1.24	0.05 / 0.10	5 / 5	30 / 18	ND		

For the olanzapine + RRR-DHTBZ experiment plasma concentration column, the upper value is the olanzapine concentration and the lower value the RRR-DHTBZ concentration. Vehicle for RRR-DHTBZ – water. Vehicle for olanzapine – 10% DMSO. ND – not done.

Table S9A. Comparing escapes in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	20.3	<0.0001	****	Yes	
Olanzapine	39.4	<0.0001	****	Yes	
RRR-DHTBZ	11.6	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	157	2	78.4	F (2, 67) = 23.7	<0.0001
Olanzapine	304	2	152	F (2, 67) = 45.9	<0.0001
RRR-DHTBZ	89.4	1	89.4	F (1, 67) = 27	<0.0001
Residual	222	67	3.32		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 0.3	Olanz 0.3 + RRR-DHTBZ	Olanz 0.6	Olanz 0.6 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 0.3	NS	NS				
Olanz 0.3 + RRR-DHTBZ	NS	NS	NS			
Olanz 0.6	NS	NS	NS	NS		
Olanz 0.6 + RRR-DHTBZ	***	***	***	***	***	

*** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S9B. Comparing escape failures in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	15.7	0.0002	***	Yes	
Olanzapine	15.7	0.0002	***	Yes	
RRR-DHTBZ	13.9	0.0001	***	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	23.1	2	11.6	F (2, 67) = 9.56	0.0002
Olanzapine	23.1	2	11.6	F (2, 67) = 9.56	0.0002
RRR-DHTBZ	20.5	1	20.5	F (1, 67) = 17	0.0001
Residual	81	67	1.21		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 0.3	Olanz 0.3 + RRR-DHTBZ	Olanz 0.6	Olanz 0.6 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 0.3	NS	NS				
Olanz 0.3 + RRR-DHTBZ	NS	NS	NS			
Olanz 0.6	NS	NS	NS	NS		
Olanz 0.6 + RRR-DHTBZ	***	***	***	**	***	

** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Comparing catalepsy in response to olanzapine in the absence and presence of RRR-DHTBZ

No statistical test was performed because the time on bar was less than 10 seconds for all treatment groups.

Table S9C. Comparing olanzapine plasma concentration; olanzapine dosed alone or in combination with 0.15 mg/kg RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	1.7	0.4734	ns	No	
Olanzapine	38.7	0.0029	**	Yes	
RRR-DHTBZ	9.24	0.1059	ns	No	
ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Interaction	0.0101	1	0.0101	F (1, 16) = 0.539	0.4734
Olanzapine	0.231	1	0.231	F (1, 16) = 12.3	0.0029
RRR-DHTBZ	0.0551	1	0.0551	F (1, 16) = 2.94	0.1059
Residual	0.3	16	0.0188		

Multiple comparisons

	Olanz 0.3	Olanz 0.3 + RRR-DHTBZ	Olanz 0.6	Olanz 0.6 + RRR-DHTBZ
Olanz 0.3				
Olanz 0.3 + RRR-DHTBZ	NS			
Olanz 0.6	*	**		
Olanz 0.6 + RRR-DHTBZ	NS	NS	NS	

* p < 0.05; ** p <0.01; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg. Numbers are dose in mg/kg.

Table S9D. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with olanzapine

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	0.105	2	0.0527	F (2, 17) = 1.21	0.3213
Residual (within columns)	0.737	17	0.0434		
Total	0.843	19			

RRR-DHTBZ dosed at 0.15 mg/kg. Olanzapine dosed at 0.3, 0.6, 1 or 3 mg/kg.

Table S10: Olanzapine – RRR-DHTBZ Combination

From Experiment 6 (Cohort 4, Session 2)

| Treatment | Conditioned avoidance (number of trials, out of 20) | | | | | Plasma concentration (log ng/ml) | | | | Catalepsy (Time on bar, sec) | | |
| | Escapes | | Escape failures | | n | | | | | | | |
	mean	SEM	mean	SEM		mean	SEM	n	mean ng/ml	mean	SEM	n
Vehicle for RRR-DHTBZ + Vehicle for olanzapine	0.8	0.2	0.0	0.0	9					0.1	0.1	5
RRR-DHTBZ 0.15 mg/kg	3.1	1.0	0.0	0.0	8	1.55	0.06	5	36	1.9	0.5	5
Olanzapine, 0.6 mg/kg	1.4	0.3	0.0	0.0	10	1.59	0.26	5	39	2.6	0.5	5
Olanzapine, 0.6 mg/kg + RRR-DHTBZ 0.15 mg/kg	10.3	2.2	0.3	0.3	9	1.73 1.47	0.03 0.05	4 5	54 30	6.6	1.1	5
Olanzapine, 1 mg/kg	2.4	0.9	0.0	0.0	8	2.02	0.18	5	100	8.2	0.7	5
Olanzapine, 1 mg/kg + RRR-DHTBZ 0.15 mg/kg [A]	12.9	10	2.4	0.8	9	1.30 1.53	0.15 0.04	2 2	20 34	3.7	0.8	5

[A]Group from Session 3 of the same cohort (i.e., Cohort 4).

For the olanzapine + RRR-DHTBZ experiment plasma concentration column, the upper value is the olanzapine concentration and the lower value the RRR-DHTBZ concentration. Vehicle for RRR-DHTBZ – water. Vehicle for risperidone – 10% DMSO.

Table S10A. Comparing escapes in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	4.11	0.0905	ns	No	
Olanzapine	9.54	0.0053	**	Yes	
RRR-DHTBZ	46.4	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	78.8	2	39.4	F (2, 49) = 2.52	0.0905
Olanzapine	183	2	91.3	F (2, 49) = 5.85	0.0053
RRR-DHTBZ	890	1	890	F (1, 49) = 57	P<0.0001
Residual	765	49	15.6		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 0.6	Olanz 0.6 + RRR-DHTBZ	Olanz 1	Olanz 1 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 0.6	NS	NS				
Olanz 0.6 + RRR-DHTBZ	***	NS	***			
Olanz 1	NS	NS	NS	**		
Olanz 1 + RRR-DHTBZ	***	**	***	NS	***	

** p <0.01; *** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg.

Table S10B. Comparing escape failures in response to olanzapine in the absence and presence of RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	26.5	<0.0001	****	Yes	
Olanzapine	26.5	<0.0001	****	Yes	
RRR-DHTBZ	14.6	<0.0001	****	Yes	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	237	2	118	F (2, 50) = 20.4	<0.0001
Olanzapine	237	2	118	F (2, 50) = 20.4	<0.0001
RRR-DHTBZ	131	1	131	F (1, 50) = 22.5	<0.0001
Residual	290	50	5.8		

Multiple comparisons

	Vehicle	Vehicle + RRR-DHTBZ	Olanz 0.6	Olanz 0.6 + RRR-DHTBZ	Olanz 1	Olanz 1 + RRR-DHTBZ
Vehicle						
Vehicle + RRR-DHTBZ	NS					
Olanz 0.6	NS	NS				
Olanz 0.6 + RRR-DHTBZ	NS	NS	NS			
Olanz 1	NS	NS	NS	NS		
Olanz 1 + RRR-DHTBZ	***	***	***	***	***	

*** p < 0.001; NS – not significant, p > 0.05. RRR-DHTBZ dose was 0.15 mg/kg.

Comparing catalepsy in response to olanzapine in the absence and presence of RRR-DHTBZ

No statistical test was performed because the time on bar was less than 10 seconds for all treatment groups.

Table S10C. Comparing olanzapine plasma concentration; olanzapine dosed alone or in combination with RRR-DHTBZ

Two-factor ANOVA summary

Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	p value	p value summary	Significant?	
Interaction	21.9	0.0734	ns	No	
Olanzapine	2.33e-029	>0.9999	ns	No	
RRR-DHTBZ	9.95	0.2105	ns	No	
ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	p value
Interaction	0.643	1	0.643	F (1, 12) = 3.85	0.0734
Olanzapine	6.86e-031	1	6.86e-031	F (1, 12) = 4.1e-030	>0.9999
RRR-DHTBZ	0.293	1	0.293	F (1, 12) = 1.75	0.2105
Residual	2.01	12	0.167		

Multiple comparisons

No multiple comparisons were performed because there was no significant treatment effect in the ANOVA.

Table S10D. Comparing RRR-DHTBZ plasma concentration; RRR-DHTBZ dosed alone or in combination with olanzapine

Single-factor ANOVA summary

ANOVA table	SS	DF	MS	F (DFn, DFd)	p value
Treatment (between columns)	0.0173	2	0.00867	F (2, 12) = 0.554	0.5885
Residual (within columns)	0.188	12	0.0156		
Total	0.205	14			

Table S11: Olanzapine 3 mg/kg

From Experiment 8 (Cohort 5 Session 3)

Treatment	Conditioned avoidance (number of trials, out of 20)					Plasma concentration (log ng/ml)				Catalepsy (Time on bar, sec)		
	Escapes		Escape failures		n							
	mean	SEM	mean	SEM		mean	SEM	n	mean ng/ml	mean	SEM	n
Water + Vehicle for olanzapine	0.5	0.3	0.0	0.0	8					0.0	0.0	5
Olanzapine, 3 mg/kg	8.6	1.4	8.8	2.0	8	2.59	0.03	5	390	6.4	1.5	5

Table S11A. Comparing escapes in response to olanzapine and vehicle

t test summary

Unpaired t test	
p value	0.0221
p value summary	*
Significantly different (p < 0.05)?	Yes
One- or two-tailed p value?	Two-tailed
t, df	t=2.57 df=14

Table S11B. Comparing escape failures in response to olanzapine and vehicle

t test summary

Unpaired t test	
p value	0.0006
p value summary	***
Significantly different (p < 0.05)?	Yes
One- or two-tailed p value?	Two-tailed
t, df	t=4.37 df=14

Comparing catalepsy in response to olanzapine and vehicle

No statistical test was performed because the time on bar was less than 10 seconds for both treatment groups.

Antipsychotic-induced weight gain experiments

Table S12: Experiment 1

Treatment	Weight gain (grams)				Plasma concentration (log ng/ml)		
	Mean	SEM	Diff. from veh.	% increase over veh.	Mean	SEM	Mean ng/ml
Vehicle for RRR-DHTBZ + Vehicle for olanzapine	20.6	3.4					
Olanzapine, 0.6 mg/kg	36.0	3.6	15.4	75	1.24	0.04	17
Olanzapine, 1.2 mg/kg	35.8	3.4	15.2	74	1.74	0.05	55
Olanzapine, 3 mg/kg	27.3	3.0	6.7	33	2.23	0.08	170
RRR-DHTBZ, 0.15 mg/kg	22.0	4.7	1.4	7	1.64	0.03	44
RRR-DHTBZ, 0.5 mg/kg	20.9	4.2	0.3	1	2.18	0.04	150
Olanzapine, 0.6 mg/kg + RRR-DHTBZ, 0.15 mg/kg	35.2	4.8	14.6	71	1.15 1.79	0.08 0.02	14 62
Olanzapine, 1.2 mg/kg + RRR-DHTBZ, 0.15 mg/kg	35.0	3.7	14.4	70	1.70 1.81	0.05 0.02	50 65

Weight gain was calculated as body weight on Day 14 minus body weight on Day 1.

For the olanzapine + RRR-DHTBZ experiment plasma concentration column, the upper value is the olanzapine concentration and the lower value the RRR-DHTBZ concentration.

The treatment group size was n = 10 in all cases.

Vehicle for RRR-DHTBZ – water. Vehicle for olanzapine – 1% methylcellulose.

Table S12A. Comparing weight gain between treatment groups

Treatment 1	Treatment 2	p value
Comparison with vehicle		
Vehicle	Olanzapine 0.6 mg/kg	0.021
Vehicle	Olanzapine 1.2 mg/kg	0.023
Vehicle	RRR-DHTBZ 0.15 mg/kg	> 0.999
Vehicle	Olanzapine 3 mg/kg	0.500
Vehicle	RRR-DHTBZ 0.3 mg/kg	> 0.999
Vehicle	Olanzapine, 0.6 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.021
Vehicle	Olanzapine, 1.2 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.023
Effect of RRR-DHTBZ on olanzapine		
Olanzapine 0.6 mg/kg	Olanzapine, 0.6 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.892
Olanzapine 1.2 mg/kg	Olanzapine, 1.2 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.888
Effect of olanzapine on RRR-DHTBZ		
Olanzapine, 0.6 mg/kg + RRR-DHTBZ, 0.15 mg/kg	RRR-DHTBZ 0.15 mg/kg	0.025
Olanzapine, 1.2 mg/kg + RRR-DHTBZ, 0.15 mg/kg	RRR-DHTBZ 0.15 mg/kg	0.025

Multiple comparisons were made using Williams' test to compare RRR-DHTBZ to vehicle, separate Dunnett's tests to compare olanzapine and the combination to vehicle, Williams' test to compare the combination to RRR-DHTBZ 0.15 mg/kg, and the multiple *t* test to compare the combination to the same dose of olanzapine. Yellow-shading, $p < 0.05$.

Table S13: Experiment 2

Treatment	Weight gain (grams)				Plasma concentration (log ng/ml)		
	Mean	SEM	Diff. from veh.	% increase over veh.	Mean	SEM	Mean ng/ml
Vehicle for RRR-DHTBZ + Vehicle for olanzapine	18.5	2.2					
Olanzapine, 0.3 mg/kg	25.7	3.5	7.2	39	1.00	0.07	10
Olanzapine, 3 mg/kg	25.7	1.9	7.2	39	2.79	0.03	620
RRR-DHTBZ, 0.15 mg/kg	13.8	3.5	-4.7	-25	1.79	0.03	62
RRR-DHTBZ, 0.3 mg/kg	21.4	2.5	2.9	16	2.05	0.05	110
RRR-DHTBZ, 0.5 mg/kg	30.1	3.8	11.6	63	2.33	0.05	210
Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.15 mg/kg	28.6	5.2	10.1	55	1.02 1.73	0.09 0.05	10 54
Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.3 mg/kg	25.8	4.6	7.3	40	1.17 2.05	0.07 0.05	15 110

Weight gain was calculated as body weight on Day 14 minus body weight on Day 1.

For the olanzapine + RRR-DHTBZ experiment plasma concentration column, the upper value is the olanzapine concentration and the lower value the RRR-DHTBZ concentration.

The treatment group size was n = 10 in all cases.

Vehicle for RRR-DHTBZ – water. Vehicle for olanzapine – 1% methylcellulose.

Table S13A. Comparing weight gain between treatment groups

Treatment 1	Treatment 2	p value
Comparison with vehicle		
Vehicle	Olanzapine 0.3 mg/kg	0.188
Vehicle	Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.091
Vehicle	Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.3 mg/kg	0.261
Vehicle	RRR-DHTBZ 0.15 mg/kg	>0.999
Vehicle	RRR-DHTBZ 0.3 mg/kg	0.682
Vehicle	Olanzapine 3 mg/kg	0.188
Vehicle	RRR-DHTBZ 0.5 mg/kg	0.030
Effect of RRR-DHTBZ on olanzapine		
Olanzapine 0.3 mg/kg	Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.15 mg/kg	0.906
Olanzapine 0.3 mg/kg	Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.3 mg/kg	0.906
Effect of olanzapine on RRR-DHTBZ		
Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.15 mg/kg	RRR-DHTBZ 0.15 mg/kg	0.005
Olanzapine, 0.3 mg/kg + RRR-DHTBZ, 0.3 mg/kg	RRR-DHTBZ 0.3 mg/kg	0.385

Multiple comparisons were by separate Williams' tests to compare olanzapine and RRR-DHTBZ to vehicle, Dunnett's test to compare the combination to vehicle, Williams' test to compare the combination to olanzapine 0.3 mg/kg and the multiple *t* test to compare the combination to the same dose of RRR-DHTBZ. Yellow-shading, $p < 0.05$.