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ENX-104: a selective and potent $D_{2/}D_3$ receptor antagonist enhances dopamine neurotransmission and reward responsiveness in translational rodent models

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Anhedonia, characterized by a diminished reactivity to pleasurable stimuli, is a core symptom across multiple neuropsychiatric disorders, including major depressive disorder. Disruptions in dopaminergic neurotransmission and dysfunction within the mesolimbic dopaminergic circuitry are key contributors to reward processing deficits. We hypothesized that low receptor occupancy–mediated antagonism of presynaptic inhibitory D_2/D_3 receptors could enhance dopaminergic neurotransmission and, in turn, improve reward responsiveness, a behavioral phenotype implicated in anhedonia. For this purpose, we developed ENX-104, a highly selective and potent D_2/D_3 receptor antagonist with favorable CNS pharmacokinetics, characterized by high brain penetrance and rapid plasma clearance. In preclinical studies, ENX-104 produced sustained increases in dopamine levels in the nucleus accumbens in rats. In the Probabilistic Reward Task (PRT), a preclinical model of reward responsiveness reverse-translated for rats from human studies designed to objectively quantify subdomains of anhedonia, ENX-104 enhanced reward responsiveness at low doses corresponding to approximately 10–50% D_2/D_3 receptor occupancy. Predictably, higher doses (65–80% receptor occupancy) were associated with antipsychotic-like effects in the rat conditioned avoidance response assay, while extrapyramidal side effects, such as catalepsy, emerged only at much higher occupancies (> 80% receptor occupancy). Our integrated pharmacokinetic-pharmacodynamic modeling suggests that a once-daily oral dosing regimen of ENX-104 could enable low D_2/D_3 receptor occupancies, potentially offering a novel therapeutic approach for the treatment of psychiatric conditions characterized by deficits in reward responsiveness.

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INTRODUCTION

Anhedonia, the lack of reactivity to previously rewarding stimuli, is a core symptom that is experienced by 60-70% of major depressive disorder (MDD) patients. It predicts poor disease course yet remains largely unresponsive to currently approved antidepressants [1, 2]. Anhedonia in MDD is, in part, characterized by reduced reward-related striatal activation and dysfunctional reward learning, reflecting deficient dopaminergic signaling [3, 4]. Indeed, depletion of dopamine can induce symptoms of depression and anhedonia [5], whereas increasing dopaminergic neurotransmission has been shown to alleviate symptoms of depression and anhedonia [6]. However, the construct of anhedonia, like depression, is heterogeneous and multifaceted in humans and encompasses both impaired hedonic capacity and motivational deficits as well as reduced reward learning [7, 8]. While dopamine may not directly mediate the experience of pleasure, per se, dopaminergic neurotransmission has been linked to reward-driven motivational processes and reward learning [9]. Dopamine plays a role in reward-related behavior and motor function, exerting its effects via distinct neural circuits [9]. The mesolimbic pathway includes projections from the ventral tegmental area (VTA) in the midbrain to the ventral striatum, including the nucleus accumbens. This pathway is also referred to as the reward pathway and is central to motivation and reward-related behavior and dopaminergic activation in it is associated with reward encoding and incentive motivation [10–12].

Dopamine exerts its effects by binding to G-protein coupled dopamine receptors, categorized into D_1-like (D_1, D_5) and D_2-like (D_2, D_3, D_4) families. D_1-like receptors are primarily Gs-coupled and excitatory, while D_2-like receptors are Gi-coupled and inhibitory. Both D_2 and D_3 receptors are expressed pre- and postsynaptically. At presynaptic sites, they function as autoreceptors regulating dopamine release through feedback inhibition. D_2 receptors exist in two isoforms, the long form (D_{2L}) and short form (D_{2S}) and of the two, D_{2L} is the most abundantly expressed in the brain [13]. D_{2L} receptors are found at both pre- and postsynaptic sites, whereas D_{2S} receptors show presynaptic enrichment with substantially lower overall expression [13]. Both D_2 and D_3 receptors are expressed at postsynaptic sites and at presynaptic terminals. D_2/D_3 receptors are particularly abundant at presynaptic sites, where they act as autoreceptors to mediate feedback inhibition of dopamine neuron activity, thereby modulating

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extracellular dopamine levels. Notably, D_3 receptors are enriched in the limbic system and striatal regions, areas central to reward processing and motivation, making them a target of growing interest in neuropsychiatric research.

D_2 receptor antagonists have long been used as antipsychotic agents, as they dopaminergic neurotransmission. Yet, studies indicate the potential of low doses of a prototypical D_2/D_3 receptor antagonist amisulpride, in the treatment of depression and anhedonia [14–16], and clinical proof-of-mechanism imaging studies show that a low dose of amisulpride rescued the blunted reward-related striatal activation in unmedicated depressed individuals [17–19]. Thus, we hypothesized that preferential antagonism of presynaptic inhibitory D_2/D_3 receptors, achieved through low receptor occupancy, could enhance dopaminergic neurotransmission and, in turn, improve reward responsiveness.

We developed ENX-104, for low-dose administration to preferentially block presynaptic D_2/D_3 autoreceptors and enhance dopaminergic neurotransmission in the reward circuitry. ENX-104 is structurally related to nemonapride, a substituted benzamide historically used as an antipsychotic in Japan and South Korea at relatively high doses and with a thrice-daily oral regimen [20, 21]. ENX-104 is a deuterated derivative of the (cis-S,S) enantiomer of nemonapride, designed for a more favorable pharmacokinetic profile. In this study, using preclinical in vitro and in vivo assays, we characterized the pharmacology, pharmacokinetic and pharmacodynamic profile of ENX-104. To assess its therapeutic potential as an anti-anhedonic agent, we utilized the probabilistic reward task (PRT). The PRT was originally developed to objectively quantify reward responsiveness in clinical populations with anhedonia [22]. In this computerized task, humans make rapid visual discriminations in which correct responses in the presence of one stimulus are three times more likely to result in reward (rich stimulus) than correct responses in the presence of the other (lean stimulus). As shown across numerous studies [23–27], heathy control participants readily develop the adaptive response bias for the more richly rewarded stimulus, whereas participants with anhedonia are often observed to have a blunted response bias which correlates with current and predicts future anhedonia [23, 25]. Given its utility, the PRT has been chosen as recommended task to probe the positive valence systems by the Research Domain Criteria (RDoC) in its most recent revision (NIMH, 2016). More recently, the PRT has been reverse-translated using touchscreen technology to study reward responsiveness in rodents and nonhuman primates [26]. In the present stud, we used the rat PRT [28] to determine the ability of ENX-104 to enhance reward responsiveness, which would be evident in drug-induced increases in response bias for the more richly rewarded stimulus [29, 30]. Additionally, we evaluated antipsychotic-like effects and potential motor side effects at higher doses, using the conditioned avoidance response and catalepsy assays in rodents, respectively. To contextualize the dose-dependent effects of ENX-104, we integrated pharmacokinetic-pharmacodynamic (PK-PD) data to identify a target D_2/D_3 receptor occupancy range associated with antianhedonic efficacy. Together, our data support a once-daily dosing regimen of ENX-104 at low receptor occupancies, offering a novel therapeutic approach for potentially treating psychiatric conditions such as major depressive disorder (MDD), in which deficits in reward responsiveness are prominent.

MATERIALS AND METHODS

In vitro pharmacology

Binding and functional activity of ENX-104 at human dopaminergic receptors were tested using recombinant mammalian cell lines expressing respective receptors. Binding assays were conducted using radiolabeled reference tracers and compared against reference comparators. ENX-104 was tested at eight concentrations in duplicate to test for radioligand binding, agonist, or antagonist functional activity. Agonist activity was expressed as a percent of the activity of the reference agonist at its EC_{100} concentration or percentage of the inhibition of the reference agonist at its EC_{80} concentration for antagonist activity. Data were averaged values derived from multiple experiments. Experimental details are listed in Supplemental Methods.

Pharmacokinetic studies

The pharmacokinetic (PK) distribution and brain penetration of ENX-104 were determined following oral administration to male Sprague Dawley rats (from Charles River) at Frontage laboratories. Rats ($n = 3$ per group) received a single oral (per os, po) dose of 2.5 mg/kg of ENX-104 and blood and brain samples were collected at 1, 2, 4, 8 and 24 h post-dose. ENX-104 plasma and brain concentrations were determined by liquid chromatography-tandem mass spectrometry. Experimental details on plasma/brain collection, tissue processing, and analyses are in Supplemental methods.

D_2/D_3 receptor occupancy studies

Sprague Dawley rats from Charles River (Margate) were obtained and on the day of testing, animals were dosed po with either vehicle (0.5% methylcellulose) or ENX-104 at a single dose (2.5 mg/kg) or the positive control olanzapine (10 mg/kg) ($n = 5$/group). Rats were euthanized 1, 2, 4, 8 or 24 h after ENX-104 administration or 1 h after vehicle or olanzapine administration.

A post-mortem blood sample (~5 ml) was taken by cardiac puncture and placed into K/EDTA tubes (32.332, Sarstedt) and processed and stored at −80 °C for analysis. Whole brains were removed, rinsed with saline and blot dried. Striata were dissected out and weighed before being frozen on dry ice.

The value for specific binding in disintegrations per minute (DPM) was generated by subtraction of mean non-specific binding from mean total binding for each animal. Data were calculated as mean specific binding, as a percentage of the vehicle-treated control taken as 100% corresponding to a mean receptor occupancy as 0%. All data were square root transformed and analyzed by one-way analysis of variance (ANOVA), and the sample size was determined to appropriately power the study. ENX-104 was compared to vehicle by Dunnett's test. Olanzapine was compared to vehicle by multiple t-test. Means were back-transformed to original units and adjusted for difference between vehicle and olanzapine groups. Experimental details can be found in Supplemental Methods.

In vivo microdialysis

Experiments were carried out in male Sprague Dawley rats (Charles River, UK) ($n = 7–8$/group). For the surgery, rats were anaesthetized with isoflurane in O_2 delivered via an anesthetic unit (Burtons Medical Equipment Ltd, UK). A dual-probe study was conducted whereby each rat had two concentric microdialysis probes (CMA 12 Elite probes, CMA Sweden) stereotaxically implanted into the prefrontal cortex (co-ordinates: AP + 3.2 mm; ML + /−2.5 mm relative to bregma; DV-4.0 mm relative to the skull surface, 2 mm tip) and nucleus accumbens (co-ordinates: AP + 2.2 mm; ML + /-1.5 mm relative to bregma; DV-8.0 mm relative to the skull surface, 2 mm tip). Coordinates were taken from [22]. Microdialysis experiments were performed the day after surgery. Microdialysate samples were collected from freely-moving rats at 30 min intervals for a baseline period of 120 min before administration of vehicle or drug. Samples were then collected for 8 h after drug treatment at intervals of 30 min.

The povehicle was 0.5% methylcellulose (400 cP, Sigma-Aldrich, Lot SLCB9094, pH 7.1) and a dose volume of 5 ml/kg po was used. The intraperitoneal (IP) vehicle was 0.9% saline (pH 5.6) and a dose volume of 2 ml/kg ip was used. All drug solutions were prepared on the day of use. ENX-104 (1 mg/kg and 2.5 mg/kg) was formulated in the po vehicle. D-Amphetamine sulfate (0.3 mg/kg) (Tocris, Batch No. 7 A/222903) formulation was prepared as a solution suitable for ip dosing.

Microdialysis data were log transformed. Baseline was defined as the geometric mean of the four pre-treatment samples (i.e., at −90, −60, −30 and 0 min). Data were log transformed and analysis was by robust regression using M estimation, Huber weighting, using the default parameter $c = 1.345$ with treatment as a factor and log(baseline) as a covariate. Each time was analyzed separately, together with means during each of the eight h after dosing and the overall 0-8 h after dosing. Comparisons to vehicle were by Williams' test for ENX-104 and by the multiple t-test for d-amphetamine, and ENX-104 + d-amphetamine. Comparisons to d-amphetamine alone and ENX-104 alone for ENX-104 +

d-amphetamine were by the multiple t-test and sample size was determined to appropriately power the study. One animal was excluded from the analysis for the nucleus accumbens due to poor chromatography. Experimental details are in Supplemental Methods.

Probabilistic reward task

Adult male Sprague Dawley rats (n = 8/group) obtained from Charles River Laboratories (Wilmington, MA) weighing between 250 and 300 g were used. Rats in the PRT study were group housed as a form of environmental enrichment; however, to match previous studies using this protocol, nothing else was provided. Utilizing touchscreen apparatus and previously published protocols rats were trained to discriminate a long versus short time via differential responding on one of two virtual levers (see Fig. 4A) [28, 31, 32]. Following line-length discrimination training, 3:1 rich/lean probabilistic reinforcement schedules were introduced such that 60% of correct responses to one of the line lengths (e.g., long line = rich stimulus) and 20% of correct responses to the other line length (e.g., short line = lean stimulus) were rewarded. Rich/lean line assignment was counterbalanced across subjects and 50 trials of each trial type were presented in a quasi-random sequence.

Following the establishment of response biases under probabilistic contingencies, an acute drug testing protocol was arranged that included intermittent maintenance sessions in which correct responses on all trials were reinforced, control sessions in which 3:1 (60%:20%) rich/lean probabilistic contingencies were arranged and, no more than once per week, a drug testing session in which vehicle or a dose of ENX-104 (0.5, 1, or 2.5 mg/kg) was tested by administering po, 4 h prior to a 3:1 (60%:20%) probabilistic session. Doses of ENX-104 were tested in a mixed order across subjects using a Latin Square design. Vehicle (0.5% methylcellulose) and all doses of ENX-104 were tested in all 8 rats. ENX-104 doses and pretreatment interval were chosen based on pharmacokinetic data. Prior to ENX-104, amisulpride (1, 5, 50 mg/kg sc) was tested in an independent sample under otherwise identical conditions in the PRT.

Data analysis

The PRT yields two primary dependent measures: response bias and discriminability. These can be quantified by examining the number of correct and incorrect responses in rich and lean trial types using, respectively, log *b* and log *d* equations derived from signal detection theory [26]:

$$\log b = 0.5 * \log\left(\frac{(Rich_{Correct} + 0.5) * (Lean_{Incorrect} + 0.5)}{(Rich_{Incorrect} + 0.5) * (Lean_{Correct} + 0.5)}\right)$$

$$\log d = 0.5 * \log\left(\frac{(Rich_{Correct} + 0.5) * (Lean_{Correct} + 0.5)}{(Rich_{Incorrect} + 0.5) * (Lean_{Incorrect} + 0.5)}\right)$$

High bias values are produced by high numbers of correct responses during rich trials and incorrect responses during lean trials, which increase the log *b* numerator. High discriminability values are produced by high numbers of correct responses during both rich and lean trials, which increase the log *d* numerator. (0.5 is added to all parameters to allow log transformation in cases of no errors). Given the a priori hypotheses discussed above of low doses of ENX-104 increasing reward responsiveness (putatively due to presynaptic autoreceptor blockade) but high doses decreasing reward responsiveness (putatively due to postsynaptic blockade), log *b* data were subjected to general linear model tests of within-subjects contrast in which the quadratic term was specifically evaluated (an inverted U-function was expected). All other data (log *d*) were subject to repeated measures ANOVA. For accuracy and reaction time, the repeated measures factor trial type (rich vs. lean) was added to the model. When appropriate, ANOVAs were followed by post-hoc tests (one-way: Dunnett's multiple comparisons test; two-way: Bonferroni multiple comparisons test) to evaluate the statistical significance of stimulus type and drug treatment. Given a priori hypotheses (see below), paired t-tests and Cohen's *d* were also used to evaluate, respectively, the statistical significance of differences in log *b* values and effect sizes following vehicle treatment and each individual dose of ENX-104. Effect sizes (Cohen's *d* values) were interpreted using established conventions: small ($d = 0.20$), medium ($d = 0.50$), and large effect ($d > 0.80$). Sample size was determined to appropriately power the study and all statistical analyses were conducted using GraphPad Prism 10 Software (San Diego, CA, USA). Further details on testing apparatus, experimental procedure, and data analyses are in Supplemental Methods.

Conditioned avoidance response

The Conditioned Avoidance Response (CAR) Test has been shown to be a very reliable animal model for screening antipsychotic drugs. 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 avoidance response in CAR, thus making it one the preferred assay to screen potential antipsychotic compounds. Adult, male Wistar rats from Envigo (Indianapolis, IN) were used in this study (n = 10/group) and randomly assigned across the treatment groups. The experiments were conducted during the animal's light cycle phase. ENX-104 (0.5, 2.5 and 5 mg/kg) was formulated in 0.5% Methyl Cellulose in water and administered orally at a dose volume of 1 ml/kg 4 h prior to test.

The CAR apparatus consists of a two-way shuttle box with infra-red (I/R) detection housed in sound-attenuating chambers (Med Associates). The two-way shuttle boxes have stainless steel grid floors and are partitioned by a guillotine door. Programs are run through Med-PC version IV software. Rats were trained to avoid a foot shock following presentation of a light or tone. Rats were placed in the CAR two-compartment shuttle box and presented with a conditioned stimulus (CS; light and tone), followed by an aversive unconditioned stimulus (US; foot-shock of 0.65 mA). Each rat goes through 20 trials with a variable ITI (20 – 60 sec). After several weeks of training in the CAR chambers, rats that passed the testing criterion of performing 16 -20 avoidance responses for three days in a row are included in the study and testing commenced. CAR testing consists of the same procedure as CAR training. Baseline was measured over three days prior to drug testing. Following the test, the rats were given one week washout between tests. The measures obtained from this test are:

Avoidance response. If the rat moved from one compartment to the other during the cued stimulus (CS) presentation and prior to foot-shock delivery. Decreased avoidance responding is the typical signature of an efficacious dose of an antipsychotic.

Escape failure. If the rat failed to move into the other compartment during the 20 s footshock.

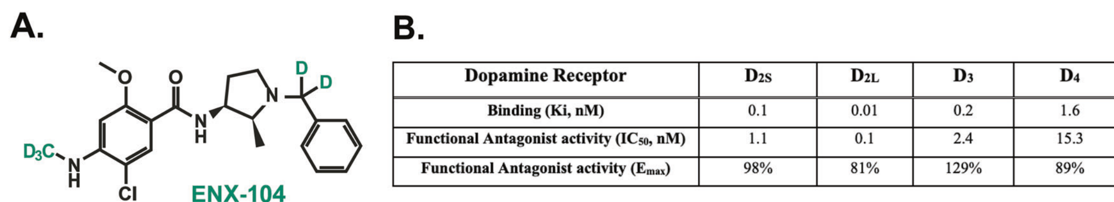
Avoidance response data are presented as percent of avoidance responses based on the three baseline responses prior to drug test. Escape failures were expressed as the total number of failures during the test session. Sample size was determined to appropriately power the study and Dunnett's post-hoc comparisons when appropriate. Escape failures were analyzed by Kruskal-Wallis nonparametric analysis followed by Dunnett's post-hoc comparisons when appropriate. Results are reported as mean ± SEM. An effect was considered significant if $p < 0.05$.

Catalepsy

Adult Sprague-Dawley male rats were obtained from Charles River (Margate) and assigned randomly across treatment groups (n = 8/group): Vehicle, haloperidol (positive control; Tocris, Product Code 0931; Lot: 4B/263582), and ENX-104 at 1, 2.5, or 10 mg/kg. ENX-104 was prepared in 0.5% methylcellulose and dosed orally. All compounds were formulated on the day of dosing and administered using a dose volume of 5 ml/kg.

On the day of the test, animals were dosed with vehicle (po), ENX-104 (1, 2.5 and 10 mg/kg, po) or haloperidol (0.82 mg/kg, ip; ED_{75} dose). Animals were tested individually for catalepsy at 90, 180 (data not shown) and 240 min post-dose by gently placing each paw in turn on a large rubber bung (42 mm high, 45 mm wide at upper surface). A score of 1 was given for each paw which remained in position for 15 s, giving each rat a maximum score of 4. The duration the paw remained in position was recorded (maximum latency score 15 s on each trial) and total latency for paw withdrawal was determined giving each rat a maximum total latency of 60 s. Sample size was determined to appropriately power the study and for statistical analyses, the total catalepsy score and total latency were compared to vehicle and haloperidol by exact Wilcoxon rank sum tests. Raw means and standard errors were calculated. $p < 0.05$ was considered statistically significant.

Statement of ethics for animal experimentation. All animal experimentation was performed in strict accordance with ethical standards of the respective institutions as well as guidelines provided for humane and ethical treatment of animals. In vivo microdialysis experiments were performed in accordance with Home Office Guidelines and licensed under the Animals (Scientific Procedures) Act 1986 (Project License P58B4DA70).



Dopamine Receptor	D_{2S}	D_{2L}	D_3	D_4
Binding (Ki, nM)	0.1	0.01	0.2	1.6
Functional Antagonist activity (IC_{50}, nM)	1.1	0.1	2.4	15.3
Functional Antagonist activity (E_{max})	98%	81%	129%	89%

Fig. 1 ENX-104 in vitro pharmacology at human receptors. A Structure of ENX-104 with deuterium (D) substitutions shown in green. **B** ENX-104 in vitro pharmacology at human dopamine D_{2S} (short form), D_{2L} (long form), D_3 and D_4 receptors showing binding affinity (Ki), potency as a functional antagonist (IC_{50}) and efficacy as a functional antagonist (E_{max}).

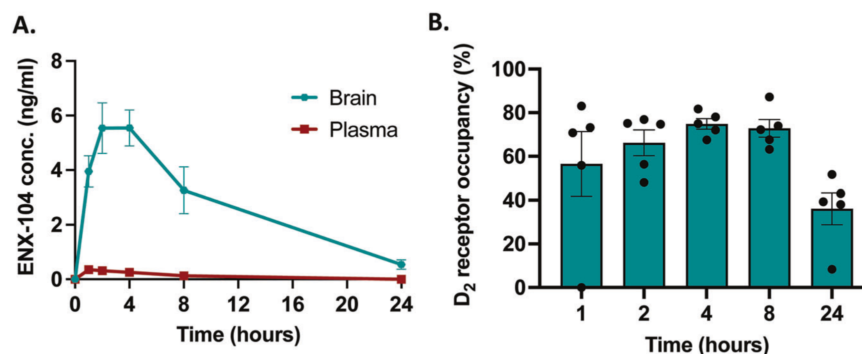


Fig. 2 Pharmacokinetic profile and receptor occupancy of ENX-104. A Pharmacokinetic profile of ENX-104 in the rat after a single 2.5 mg/kg oral dose. Shown are ENX-104 levels (ng/ml) in the brain (green) and plasma (red) at five time points tested, over 24 h (n = 3/group). **B** D_2/D_3 receptor occupancy time course data at five time points over 24 h (n = 5/group) after a single oral dose of ENX-104 (2.5 mg/kg); dots representing data from individual animals. Data are presented as means ± SEM (error bars).

The PRT protocol was approved by the Institutional Animal Care and Use Committee at McLean Hospital and in accordance with guidelines provided by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, Commission on Life Sciences (National Research Council, 2011).

RESULTS

ENX-104 is a selective and potent D2/D3 receptor antagonist

ENX-104 ($C_{21}H_{21}D_5ClN_3O_2$) is a deuterated molecule with the molecular weight of 392.94 g/mol (Fig. 1A). ENX-104 displayed potent binding at all the D_2-like inhibitory dopamine receptors tested (D_{2S}, D_{2L}, D_3, and D_4). The most potent binding and full antagonist activity was observed for D_{2L}, affinity of 0.01 nM (Ki), and 0.1 nM (half-maximal inhibitory concentration [IC_{50}]). ENX-104 also bound strongly to and displayed potent antagonist activity at D_{2S} (Ki = 0.1 nM, IC_{50} = 1.1 nM) and D_3 (Ki = 0.2 nM, IC_{50} = 2.4 nM) receptors. Compared to D_{2L}, ENX-104 showed weaker antagonist activity at the D_4 receptor (Ki = 1.6 nM, IC_{50} = 15.3 nM) (Fig. 1B). ENX-104 exhibited over a hundred-fold weaker affinity for other receptors evaluated in a broad in vitro screening assay (data not shown). Thus, ENX-104 displayed selective and potent antagonism at D_2/D_3 receptors with limited off-target activity.

ENX-104 displays brain enrichment, retention, and target engagement

Following administration of an oral dose of 2.5 mg/kg, ENX-104 exhibited rapid peripheral metabolism, with a short plasma half-life and significant brain enrichment and retention over the course of 24 h. For rat PK studies, we focused on 2.5 mg/kg dose levels to gain accurate insights especially at 24 h post dose. Given the rapid metabolization of ENX-104, at lower dose levels (0.5 mg/kg) plasma drug levels drop below the detection limit at the 24 h time point (data not shown), even though brain levels are detectable. To more accurately calculate drug and ratios of brain:plasma levels over 24 h, we chose the 2.5 mg/kg dose for pharmacokinetic and receptor occupancy studies. Although plasma concentrations started decreasing by 2 h, brain concentrations were at maximum approximately 2-4 h post-dose. ENX-104 demonstrated excellent penetration into the central nervous system. Brain ENX-104 levels were higher than plasma levels at 1, 4, 8, and 24 h after dosing, with an approximately 10-fold enrichment at the 4 h timepoint (Fig. 2A).

At this dose, ENX-104 exhibited significant striatal D_2/D_3 receptor occupancy at 1, 2, 4, and 8 h (averages at 1 h, ~59%; 2 h, ~67%; 4 h, ~75%; and 8 h, ~73%) (Fig. 2B). Maximum receptor occupancy was observed 4 h (~ 75%) post dosing. Notably, while D_2/D_3 receptor occupancy was also observed 24 h post-dosing but lower relative to peak levels, indicating the reversibility of ENX-104 binding to the D_2/D_3 receptor within 24 hours. Thus, striatal D_2/D_3 receptor occupancy for ENX-104 mirrored the brain concentrations observed across the study. In contrast, plasma concentrations of ENX-104 reached a plateau in advance of peak of D_2/D_3 receptor occupancy. ENX-104 showed 4 to 40-fold brain:plasma ratios over the course of 24 h. Thus, ENX-104, with rapid plasma clearance and excellent blood-brain-barrier penetration after oral dosing, exhibits a favorable PK profile for a CNS drug.

ENX-104 increased dopamine levels in the reward circuitry

To test whether ENX-104 treatment enhanced dopamine in the reward circuit (presumably, via blockade of D_2/D_3 autoreceptors), dopamine release was directly measured using in vivo microdialysis in freely moving rats following oral administration. As hypothesized, ENX-104 significantly increased extracellular dopamine in both the nucleus accumbens and the prefrontal cortex (Fig. 3, Supplemental Fig. S1). In the nucleus accumbens, both doses (1 and 2.5 mg/kg) resulted in a significant and sustained increase averaged over the entire 8-hour sampling period (Fig. 3A, B). ENX-104 (1 mg/kg) produced a maximal increase (159% compared to controls; $p < 0.001$). ENX-104 at 2.5 mg/kg produced a similar pattern with a maximal increase (146% compared to controls; $p < 0.001$) observed at 90 min post-dose. In the prefrontal cortex, only the 2.5 mg/kg dose level resulted in a transient but

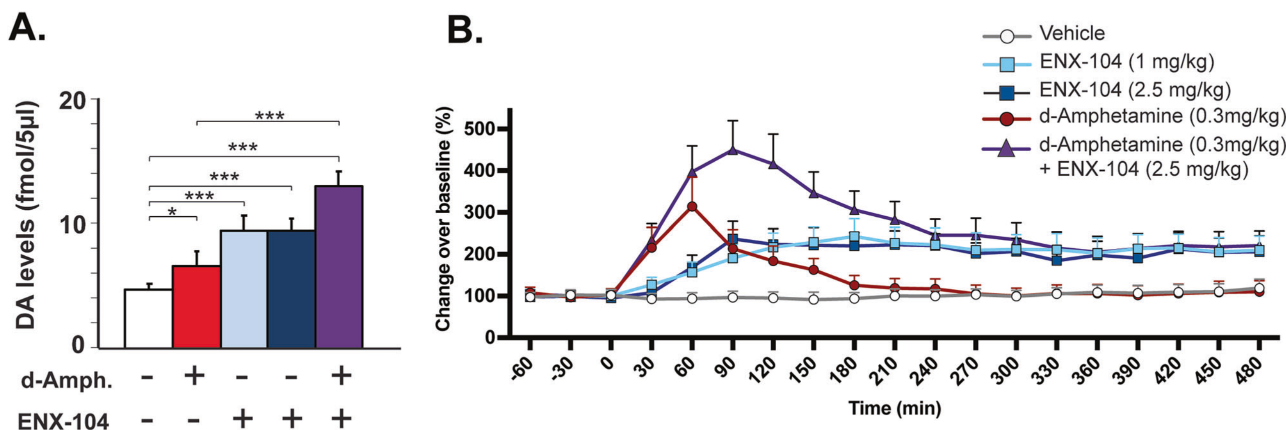


Fig. 3 Dopamine levels in the nucleus accumbens. Animals were administered vehicle (white, ip and po), d-amphetamine (positive control, 0.3 mg/kg ip; red), ENX-104 (1 mg/kg po, light blue), ENX-104 (2.5 mg/kg po, dark blue), d-amphetamine+ENX-104 (2.5 mg/kg po) (purple) and dialysate collected at 30 min intervals, up to 8 h post dose (n = 7-8/group). **A** Shown are average dopamine levels over an 8-hour period post dosing ± SEM, *p < 0.05, ***p < 0.001. **B** Time course of dopamine reveals significant and sustained effects of ENX-104 (1 and 2.5 mg/kg dose levels) up to 8 h post dose. Data are presented as adjusted means (from log-transformed data) ± SEM. Statistically significant differences between groups per time point in are shown in Supplementary Fig. S1.

significant change in dopamine levels (Supplemental Fig. S1). d-Amphetamine (0.3 mg/kg ip), used as a positive control, significantly increased dopamine in the nucleus accumbens as expected (Fig. 3A, B) and transiently in the prefrontal cortex (Supplemental Fig. S1). When ENX-104 (2.5 mg/kg, po) was administered together with d-amphetamine (0.3 mg/kg, ip), the increases in dopamine in the nucleus accumbens were significantly greater as compared to controls (366%, $p < 0.001$). At the time of peak effect (1-2 h), average dopamine levels in the combination group were significantly greater than ENX-104 alone (126%, $p < 0.001$) or d-amphetamine alone (118%, $p < 0.001$) (Fig. 3B). This provides mechanistic proof that blockade of autoreceptors together with a dopamine releasing agent like d-amphetamine results in further increase in extracellular dopamine levels. Further, in comparison to d-amphetamine, which only transiently increased dopamine (~2.5 h post dose), ENX-104 increased dopamine to physiologically relevant levels and over a period of ~8 h within the reward circuit.

ENX-104 enhanced reward responsiveness in the PRT

Impact of ENX-104 on reward responsiveness was quantitatively characterized in rats using the PRT (Fig. 4) [23, 25, 28, 31]. Prior to testing ENX-104, the assay was validated with amisulpride, which has been historically used at low doses for the treatment of dysthymia in patients[14, 19, 33, 34]. As predicted, following 13.5 (±1.5) sessions of training, PRT test sessions following administration of low doses of amisulpride (1 and 5 mg/kg) were characterized by enhancement of response bias (log *b*) as compared to vehicle. Specifically, analyses showed that 1 mg/kg was significantly different than vehicle (paired t-test analyses: $t[7] = 2.20$; $p = 0.03$; $d = 0.79$), as was 5 mg/kg of amisulpride ($t[7] = 2.08$; $p = 0.04$; $d = 0.74$) (Fig. 4B). Because low (1 and 5 mg/kg) but not higher doses (50 mg/kg) of amisulpride increased log *b*, general linear model tests with within-subjects contrast (quadratic term) were conducted, which yielded a trend towards statistical significance ($F[1,7] = 4.80$; $p = 0.065$).

Next, the impact of ENX-104 at three dose levels (0.5, 1, and 2.5 mg/kg) was examined in the rat PRT. Following 15.3 (±0.6) sessions of training, PRT test sessions following administration of low dose levels, 0.5 and 1 mg/kg ENX-104 significantly increased reward responsiveness (log *b*) to 0.44 ± 0.11 and 0.48 ± 0.1, respectively as compared to vehicle treatment 0.27 ± 0.05 (Fig. 4C). Because the higher dose of ENX-104 (2.5 mg/kg) was not hypothesized to increase reward responsiveness, a one-way ANOVA was not an appropriate statistical analysis of dose-response function. Instead, general linear model tests with within-subjects contrast (quadratic term) were conducted and yielded statistical significance across the dose-response function ($F[1,7] = 5.69$; $p = 0.048$). Additionally, given the a priori hypotheses, paired t-test analyses between vehicle and ENX-104 dose groups enabled separate appraisal of statistical significance for dose-related effects. These analyses showed that 0.5 mg/kg was significantly greater than vehicle ($t[7] = 3.4$; $p = 0.006$; $d = 1.206$), as was 1 mg/kg ENX-104 ($t[7] = 2.13$; $p = 0.04$; $d = 0.753$), but not 2.5 mg.kg ENX-104 ($t[7] = 1.41$; $p = 0.10$; $d = 0.498$). It is important to note that this effect was revealed by substantive increases in log *b* without reductions in task discriminability (log *d*) for ENX-104 or amisulpride (Supplementary Fig. S2).

Data from a receptor occupancy study with amisulpride helped predict D_2 receptor occupancy at different dose levels. Using the model derived from our empirical data (data not shown), we chose these three doses of amisulpride to help cover an receptor occupancy range from 20-70%, enabling a direct comparison of the behavioral effects of ENX-104 and amisulpride. Based on this, the 50 mg/kg dose corresponded to 55-75% D_2 receptor occupancy range, in a range similar to the higher dose of ENX-104 (2.5 mg/kg). Cohen's *d* values were computed to compare effect sizes across treatments. Effect sizes for ENX-104-treated groups at 0.5, 1, and 2.5 mg/kg versus the vehicle-treated group were 1.2, 0.75, and 0.5, respectively. Whereas Cohen's *d* values for amisulpride-treated groups at 1, 5, and 50 mg/kg dose levels versus the vehicle-treated group were 0.79, 0.74, and 0.19, respectively (Fig. 4B, C). Notably, computation of Cohen's *d* values across experiments revealed that the 0.5 mg/kg ENX-104 dose was linked to the largest effect across all dose levels tested (Supplementary Fig. S2) with an effect size greater than that of low dose amisulpride at equivalent estimated D_2/D_3 receptor occupancy (Fig. 4B, C). Highlighting specificity, ENX-104 did not affect discriminability (Supplementary Fig. S2), suggesting that changes in task difficulty did not confound the response bias findings.

Integrated model of ENX-104 efficacy at various D_2/D_3 receptor occupancies

D_2/D_3 antagonists are known to display antipsychotic efficacy and higher doses are associated with motor side effects including extrapyramidal symptoms (EPS). Among the multiple assays available for testing antipsychotic efficacy, such as amphetamine

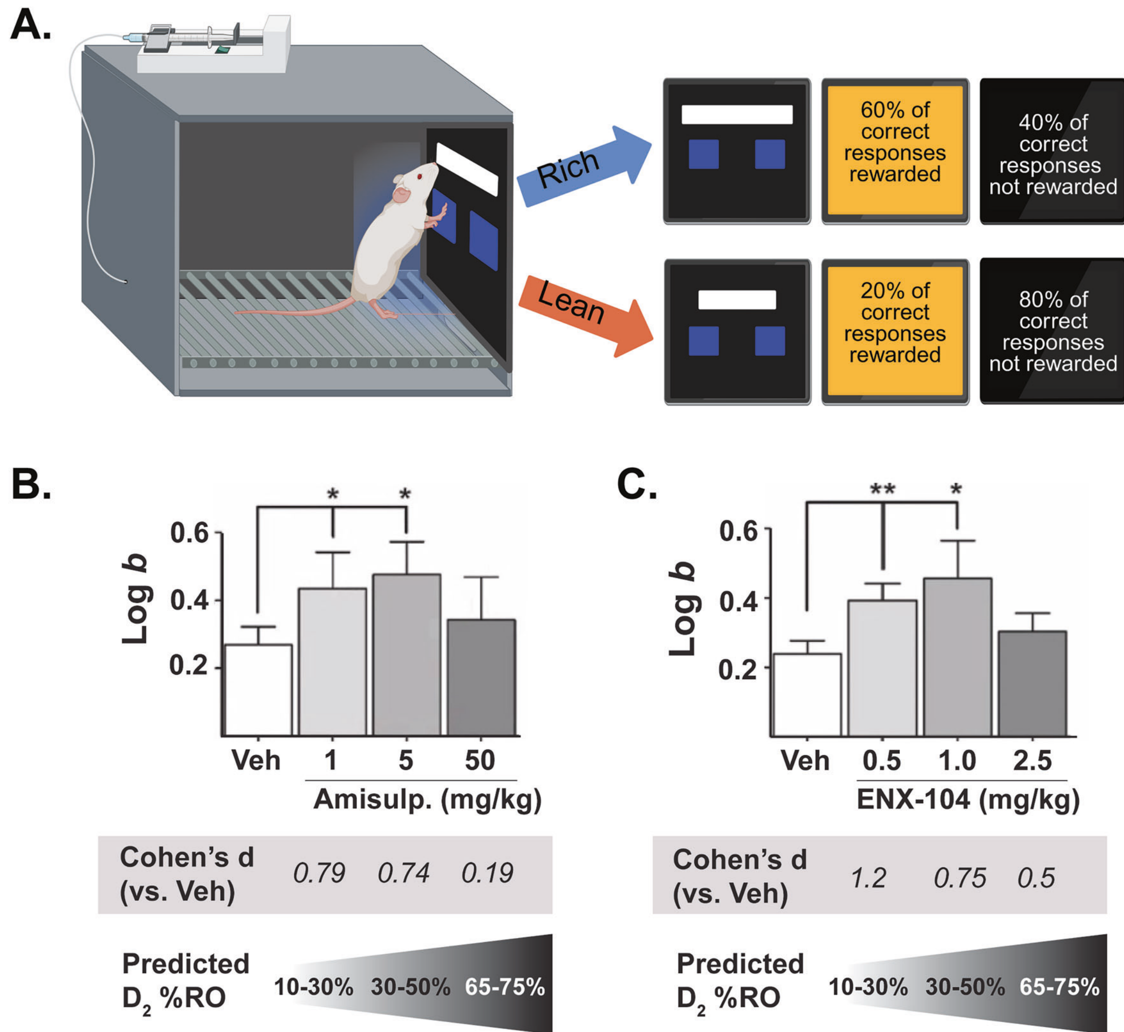


Fig. 4 Anti-anhedonic effects in the rodent Probabilistic Reward Task (PRT). A Schematic of the touchscreen PRT testing conditions. In the PRT, doses of amisulpride and ENX-104 targeting low but not high $D_{2/3}$ receptor occupancy (RO) significantly increased reward responsiveness. This was revealed by significant increase in response bias (log *b*). **B** Response bias with low doses of amisulpride was significantly higher compared to vehicle. **C** Response bias with ENX-104 at low doses was significantly greater than vehicle. **B**, **C** Log *b* effect size comparison to respective vehicle controls revealed larger effect size of low dose ENX-104 (0.5 mg/kg: Cohen's *d* ~ 1.2) as compared to amisulpride (1 mg/kg: Cohen's *d* ~ 0.8) at corresponding low doses. Data are presented as means ± SEM. *p < 0.05, **p < 0.01, n = 8/group.

induced hyperlocomotion (modeling psychotic symptoms) and prepulse inhibition (modeling sensorimotor deficits), we utilized the conditioned avoidance response (CAR) assay for broader sensitivity to antipsychotic efficacy [35]. ENX-104, specifically at higher doses tested (2.5 and 5 mg/kg), showed significant positive effects in the rat CAR ($F[4,49] = 49.1$; $p < 0.001$), with mean values of 66.2 ± 8.9% and 17.5 ± 3.61% avoidance response, respectively, compared to vehicle treated controls (98.4 ± 1.57%). In contrast, the lower dose of 0.5 mg/kg did not impact avoidance response in the CAR (84.6 ± 7.08%) as compared to controls (Supplementary Fig. S3). In addition, ENX-104 at dose levels of 0.5 and 2.5 mg/kg did not increase (0 escape failures at these dose levels) the number of escape failures which is associated with motor impairment. Only the higher dose level tested, 5 mg/kg, showed a significant increase in escape failures compared to vehicle (median of 2.5 escape failures as compared to 0 in vehicle treated controls) (Supplementary Fig. S3). Notably, ENX-104 at dose levels (0.5 mg/kg) associated with enhanced reward response did not show antipsychotic efficacy, nor motor impairment in the CAR assay.

The induction of catalepsy in rodents is an assay used for detecting antipsychotic drugs that induce extrapyramidal symptoms (EPS). The effects of ENX-104 at three dose levels (1, 2.5, and 10 mg/kg, po) on catalepsy were assessed at 4 h post-dose in rats. ENX-104, at 1 and 2.5 mg/kg dose levels (mean catalepsy of 0 out of 4 for each dose group), did not induce catalepsy compared to vehicle-treated controls (mean catalepsy score of 0). Only at the highest dose level of 10 mg/kg, ENX-104 induced catalepsy at 4 h post-dose (increased mean catalepsy score of 2.8 + 0.25 ($p < 0.001$) and latency for paw withdrawal 53.6 + 1.66 ($p < 0.001$) (Supplementary Fig. S4). Notably, no catalepsy was observed at dose levels associated with enhanced reward response (1 mg/kg) or antipsychotic like effects (2.5 mg/kg).

By integrating ENX-104 brain exposure data from the PRT, CAR and catalepsy assays, it can be predicted that D_2/D_3 receptor occupancy of ~10–50% is associated with enhanced reward responsiveness, ~65-80% receptor occupancy is associated with antipsychotic activity and >80% receptor occupancy is associated with catalepsy (Fig. 5). Integrating data from brain ENX-104 levels, at the 1 mg/kg dose we predict sustained D_2/D_3 receptor

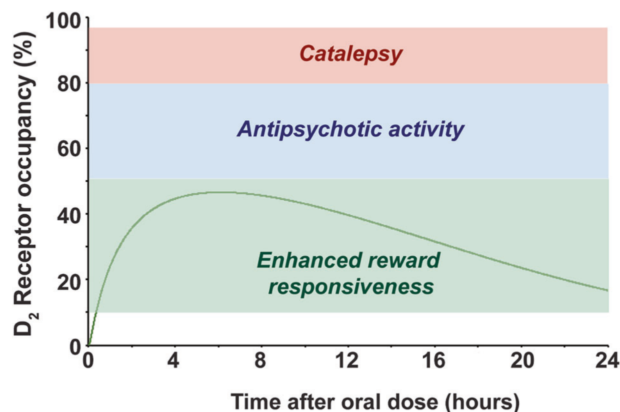


Fig. 5 Integrated model of ENX-104 efficacy. An integrated model of ENX-104 efficacy indicates that D_2/D_3 receptor occupancy of ~10–50% is the target range for enhancing reward responsiveness which may correlate with anti-anhedonic effects (green shade), and antipsychotic effects at 50-80% (blue shade) and catalepsy emerging only at receptor occupancy (RO) greater than ~80% (red shade). A model incorporating pharmacokinetics and pharmacodynamic effects of ENX-104 indicates that a single oral dose (1 mg/kg in rat) would produce sustained target engagement over 24 h within the target range for potential anti-anhedonic activity (green line).

occupancy in the reward-response enhancing receptor occupancy range over the course of 24 h (Fig. 5).

DISCUSSION

We hypothesized that a selective D_2/D_3 receptor antagonist, administered at low doses (corresponding to low D_2/D_3 receptor occupancy) would preferentially block presynaptic autoreceptors, increase dopamine in reward-related circuits and enhance reward responsiveness. In support of this, our microdialysis studies revealed that while the dopamine releasing agent amphetamine increased dopamine levels, co-administration with ENX-104 resulted in an even greater and more sustained elevation of striatal dopamine, consistent with enhanced dopamine release via blockade of feedback inhibition. Under physiological conditions, ENX-104 increased dopamine levels in the nucleus accumbens at doses of 1 mg/kg and 2.5 mg/kg, corresponding to approximately 40% and 70% D_2/D_3 RO, respectively. Interestingly, while both doses elevated dopamine, their behavioral effects in the PRT and CAR diverged.

In the rat PRT, a translational preclinical assay to objectively quantify enhancement of reward responsiveness [31], only low doses (0.5 and 1 mg/kg, ~10–50% RO) increased response bias for the richly rewarded stimulus. Consistent with our hypotheses, higher doses (2.5 mg/kg, ~65–80% RO) did not affect reward responsiveness in the rat PRT but, rather, showed antipsychotic efficacy in the conditioned avoidance response (CAR) test, a validated rodent model of antipsychotic activity [35]. As expected, 0.5 mg/kg dose showed no effect in the rat CAR, indicating functional specificity at different D_2 receptor occupancy thresholds.

ENX-104 also demonstrated a favorable side effect profile preclinically. Catalepsy, a rodent marker for EPS often associated with high-dose D_2 receptor antagonism, was only observed at the highest tested dose (10 mg/kg), but not at doses producing both increases in reward responsiveness and antipsychotic effects (1 mg/kg and 2.5 mg/kg, respectively). Based on these preclinical data, ENX-104 is anticipated to carry low risk of motor side effects at doses that enhance reward responsiveness, suggesting a potentially favorable therapeutic window. However, this would have to be verified empirically in clinical trials. PK profiling in rats demonstrated rapid plasma clearance alongside robust brain enrichment, supporting the rationale for a once-daily dosing regimen. This PK profile underscores the compound's potential safety and tolerability, though confirmation will require clinical evaluation. Importantly, all experiments in the present study were conducted in rodents, therefore, clinical translation will necessitate single- and multiple-ascending-dose PK studies combined with D_2/D_3 receptor PET imaging to establish safety and target engagement in humans. Although amisulpride is approved in several European countries for dysthymia, a chronic depressive condition marked by persistent low mood [18], there remains an unmet need for a selective and brain penetrant molecule for the treatment of reward response deficits.

ENX-104, as a brain-penetrant, highly selective D_2/D_3 receptor antagonist offers a promising new modality for treating reward-related deficits in MDD at low doses, meeting a critical need in depression therapeutics. As reviewed above, anhedonia is a complex heterogeneous construct. As such, deficits in reward responsiveness may reflect not only impaired hedonic capacity but also broader motivational or cognitive deficits. This distinction is particularly important in considering the role of dopamine, which primarily supports motivational processes such as incentive salience and reward learning, rather than the hedonic experience, per se [8, 11]. This nuance has further implications for translational research. In schizophrenia, for example, anhedonia (diminished experience of pleasure) can be dissociated from avolition (diminished goal-directed behavior) [36], while in depression the two often overlap [37]. Accordingly, animal models that show blunted responsiveness to reinforcers may capture reward learning abnormalities rather than subjective hedonic states. With the above caveats in mind, the PRT has nonetheless emerged as a useful measure of reward responsiveness, a behavioral phenotype associated with anhedonia. Thus, reduced response bias in MDD, specifically among MDD individuals with elevated anhedonic symptoms or the melancholic subtype of MDD [6, 23–25, 38–40], has been found to correlate with current [22, 41–43] and predict future [22, 25] anhedonic symptoms. Critically, and consistent with the notion that the PRT probes specific subdomains of reward processing (reward responsiveness and reward learning) rather than the full spectrum of anhedonic phenotypes, response bias has been found to explain 11-15% of the variance of anhedonic symptoms [22, 41, 43]. Nevertheless, in the context of drug development, the PRT offers a valuable translational tool for assessing whether pharmacological agents, such as compounds that modulate dopaminergic transmission, can enhance reward responsiveness, and thereby target a critical component of anhedonia.

DATA AVAILABILITY

Raw or processed data from the manuscript are available upon request.

REFERENCES

1. Pizzagalli DA. Toward a better understanding of the mechanisms and pathophysiology of anhedonia: are we ready for translation?. Am J Psychiatry. 2022;179:458–69. https://doi.org/10.1176/appi.ajp.20220423.
2. Kelly CA, Freeman KB, Schumacher JA. Treatment-resistant depression with anhedonia: Integrating clinical and preclinical approaches to investigate distinct phenotypes. Neurosci Biobehav Rev. 2022;136:104578. https://doi.org/10.1016/j.neubiorev.2022.104578.
3. Mizuno Y, Ashok AH, Bhat BB, Jauhar S, Howes OD. Dopamine in major depressive disorder: A systematic review and meta-analysis of in vivo imaging studies. J Psychopharmacol. 2023;37:1058–69. https://doi.org/10.1177/02698811231200881.
4. Peciña M, Sikora M, Avery ET, Heffernan J, Peciña S, Mickey BJ, et al. Striatal dopamine D2/3 receptor-mediated neurotransmission in major depression: Implications for anhedonia, anxiety and treatment response. Eur Neuropsychopharmacol. 2017;27:977–86. https://doi.org/10.1016/j.euroneuro.2017.08.427.
5. Hasler G, Fromm S, Carlson PJ, Luckenbaugh DA, Waldeck T, Geraci M, et al. Neural response to catecholamine depletion in unmedicated subjects with major depressive disorder in remission and healthy subjects. Arch Gen Psychiatry. 2008;65:521–31. https://doi.org/10.1001/archpsyc.65.5.521.

6. Whitton AE, Reinen JM, Slifstein M, Ang YS, McGrath PJ, Iosifescu DV, et al. Baseline reward processing and ventrostriatal dopamine function are associated with pramipexole response in depression. Brain. 2020;143:701–10. https://doi.org/10.1093/brain/awaa002.
7. Rizvi SJ, Pizzagalli DA, Sproule BA, Kennedy SH. Assessing anhedonia in depression: Potentials and pitfalls. Neurosci Biobehav Rev. 2016;65:21–35. https://doi.org/10.1016/j.neubiorev.2016.03.004.
8. Webber HE, Lopez-Gamundi P, Stamatovich SN, de Wit H, Wardle MC. Using pharmacological manipulations to study the role of dopamine in human reward functioning: A review of studies in healthy adults. Neurosci Biobehav Rev. 2021;120:123–58. https://doi.org/10.1016/j.neubiorev.2020.11.004.
9. Berridge KC, Kringelbach ML. Pleasure systems in the brain. Neuron. 2015;86:646–64. https://doi.org/10.1016/j.neuron.2015.02.018.
10. Alcaro A, Huber R, Panksepp J. Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. Brain Res Rev. 2007;56:283–321. https://doi.org/10.1016/j.brainresrev.2007.07.014.
11. Berridge KC, Kringelbach ML. Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacol (Berl). 2008;199:457–80. https://doi.org/10.1007/s00213-008-1099-6.
12. Richard JM, Castro DC, Difeliceantonio AG, Robinson MJ, Berridge KC. Mapping brain circuits of reward and motivation: in the footsteps of Ann Kelley. Neurosci Biobehav Rev. 2013;37:1919–31. https://doi.org/10.1016/j.neubiorev.2012.12.008.
13. Lindgren N, Usiello A, Goiny M, Haycock J, Erbs E, Greengard P, et al. Distinct roles of dopamine D2L and D2S receptor isoforms in the regulation of protein phosphorylation at presynaptic and postsynaptic sites. Proc Natl Acad Sci USA. 2003;100:4305–9. https://doi.org/10.1073/pnas.0730708100.
14. Boyer P, Lecrubier Y, Stalla-Bourdillon A, Fleurot O. Amisulpride versus amineptine and placebo for the treatment of dysthymia. Neuropsychobiology. 1999;39:25–32. https://doi.org/10.1159/000026556.
15. Papp M, Wieronska J. Antidepressant-like activity of amisulpride in two animal models of depression. J Psychopharmacol. 2000;14:46–52. https://doi.org/10.1177/026988110001400106.
16. Rittmannsberger H. Amisulpride as an augmentation agent in treatment resistant depression: a case series and review of the literature. Psychiatr Danub. 2019;31:148–56. https://doi.org/10.24869/psyd.2019.148.
17. Admon R, Kaiser RH, Dillon DG, Beltzer M, Goer F, Olson DP, et al. Dopaminergic enhancement of striatal response to reward in major depression. Am J Psychiatry. 2017;174:378–86. https://doi.org/10.1176/appi.ajp.2016.16010111.
18. Zangani C, Giordano B, Stein HC, Bonora S, D'Agostino A, Ostinelli EG. Efficacy of amisulpride for depressive symptoms in individuals with mental disorders: a systematic review and meta-analysis. Hum Psychopharmacol. 2021;36:e2801. https://doi.org/10.1002/hup.2801.
19. Lecrubier Y, Boyer P, Turjanski S, Rein W. Amisulpride versus imipramine and placebo in dysthymia and major depression. Amisulpride Study Group J Affect Disord. 1997;43:95–103. https://doi.org/10.1016/s0165-0327(96)00103-6.
20. Kondo T, Mihara K, Yasui N, Nagashima U, Ono S, Kaneko S, et al. Therapeutic spectrum of nemonapride and its relationship with plasma concentrations of the drug and prolactin. J Clin Psychopharmacol. 2000;20:404–9. https://doi.org/10.1097/00004714-200008000-00003.
21. Satoh K, Someya T, Shibasaki M. Effects of nemonapride on positive and negative symptoms of schizophrenia. Int Clin Psychopharmacol. 1996;11:279–81. https://doi.org/10.1097/00004850-199612000-00010.
22. Pizzagalli DA, Jahn AL, O'Shea JP. Toward an objective characterization of an anhedonic phenotype: a signal-detection approach. Biol Psychiatry. 2005;57:319–27. https://doi.org/10.1016/j.biopsych.2004.11.026.
23. Fletcher K, Parker G, Paterson A, Fava M, Iosifescu D, Pizzagalli DA. Anhedonia in melancholic and non-melancholic depressive disorders. J Affect Disord. 2015;184:81–88. https://doi.org/10.1016/j.jad.2015.05.028.
24. Pizzagalli DA, Iosifescu D, Hallett LA, Ratner KG, Fava M. Reduced hedonic capacity in major depressive disorder: evidence from a probabilistic reward task. J Psychiatr Res. 2008;43:76–87. https://doi.org/10.1016/j.jpsychires.2008.03.001.
25. Vrieze E, Pizzagalli DA, Demyttenaere K, Hompes T, Sienaert P, de Boer P, et al. Reduced reward learning predicts outcome in major depressive disorder. Biol Psychiatry. 2013;73:639–45. https://doi.org/10.1016/j.biopsych.2012.10.014.
26. Luc OT, Pizzagalli DA, Kangas BD. Toward a quantification of anhedonia: unified matching law and signal detection for clinical assessment and drug development. Perspect Behav Sci. 2021;44:517–40. https://doi.org/10.1007/s40614-021-00288-w.
27. Pizzagalli DA, Goetz E, Ostacher M, Iosifescu DV, Perlis RH. Euthymic patients with bipolar disorder show decreased reward learning in a probabilistic reward task. Biol Psychiatry. 2008;64:162–8. https://doi.org/10.1016/j.biopsych.2007.12.001.
28. Kangas BD, Wooldridge LM, Luc OT, Bergman J, Pizzagalli DA. Empirical validation of a touchscreen probabilistic reward task in rats. Transl Psychiatry. 2020;10:285. https://doi.org/10.1038/s41398-020-00969-1.
29. Adam AS, LaMalfa KS, Razavi Y, Kohut SJ, Kangas BD. A multimodal preclinical assessment of MDMA in female and male rats: prohedonic, cognition disruptive, and prosocial effects. Psychedelic Med. 2024;2:96–108. https://doi.org/10.1089/psymed.2023.0049.
30. Jenkins AR, Radl DB, Kornecook TJ, Pizzagalli DA, Bergman J, Buhl DL, et al. Environmental determinants of ketamine's prohedonic and antianhedonic efficacy: Persistence of enhanced reward responsiveness is modulated by chronic stress. J Pharm Exp Ther. 2025;392:103572. https://doi.org/10.1016/j.jpet.2025.103572.
31. Kangas BD, Der-Avakian A, Pizzagalli DA. Probabilistic reinforcement learning and anhedonia. Curr Top Behav Neurosci. 2022;58:355–77. https://doi.org/10.1007/7854_2022_349.
32. Kangas BD, Bergman J. Touchscreen technology in the study of cognition-related behavior. Behav Pharm. 2017;28:623–9. https://doi.org/10.1097/FBP.0000000000000356.
33. Ravizza L. Amisulpride in medium-term treatment of dysthymia: a six-month, double-blind safety study versus amitriptyline. AMILONG investigators. J Psychopharmacol. 1999;13:248–54. https://doi.org/10.1177/026988119901300307.
34. Natesan S, Reckless GE, Barlow KB, Nobrega JN, Kapur S. Amisulpride the 'atypical' atypical antipsychotic-comparison to haloperidol, risperidone and clozapine. Schizophr Res. 2008;105:224–35. https://doi.org/10.1016/j.schres.2008.07.005.
35. Wadenberg ML, Hicks PB. The conditioned avoidance response test re-evaluated: is it a sensitive test for the detection of potentially atypical antipsychotics?. Neurosci Biobehav Rev. 1999;23:851–62. https://doi.org/10.1016/s0149-7634(99)00037-8.
36. Strauss GP, Horan WP, Kirkpatrick B, Fischer BA, Keller WR, Miski P, et al. Deconstructing negative symptoms of schizophrenia: avolition-apathy and diminished expression clusters predict clinical presentation and functional outcome. J Psychiatr Res. 2013;47:783–90. https://doi.org/10.1016/j.jpsychires.2013.01.015.
37. Treadway MT, Zald DH. Reconsidering anhedonia in depression: lessons from translational neuroscience. Neurosci Biobehav Rev. 2011;35:537–55. https://doi.org/10.1016/j.neubiorev.2010.06.006.
38. Liu WH, Chan RC, Wang LZ, Huang J, Cheung EF, Gong QY, et al. Deficits in sustaining reward responses in subsyndromal and syndromal major depression. Prog Neuropsychopharmacol Biol Psychiatry. 2011;35:1045–52. https://doi.org/10.1016/j.pnpbp.2011.02.018.
39. Costi S, Morris LS, Collins A, Fernandez NF, Patel M, Xie H, et al. Peripheral immune cell reactivity and neural response to reward in patients with depression and anhedonia. Transl Psychiatry. 2021;11:565. https://doi.org/10.1038/s41398-021-01668-1.
40. Xiao J, Adkinson JA, Myers J, Allawala AB, Mathura RK, Pirtle V, et al. Beta activity in human anterior cingulate cortex mediates reward biases. Nat Commun. 2024;15:5528. https://doi.org/10.1038/s41467-024-49600-7.
41. Reilly EE, Whitton AE, Pizzagalli DA, Rutherford AV, Stein MB, Paulus MP, et al. Diagnostic and dimensional evaluation of implicit reward learning in social anxiety disorder and major depression. Depress Anxiety. 2020;37:1221–30. https://doi.org/10.1002/da.23081.
42. Chevallier C, Tonge N, Safra L, Kahn D, Kohls G, Miller J, et al. Measuring social motivation using signal detection and reward responsiveness. PLoS One. 2016;11:e0167024 https://doi.org/10.1371/journal.pone.0167024.
43. Bogdan R, Pizzagalli DA. Acute stress reduces reward responsiveness: implications for depression. Biol Psychiatry. 2006;60:1147–54. https://doi.org/10.1016/j.biopsych.2006.03.037.

AUTHOR CONTRIBUTIONS

KCV, JS, DAP, BDK, DSG, WB, VS, KEV conceptualized and designed experiments and contributed to the manuscript. KCV, BDK, DAP, and KEV executed experiments, and/or analyzed results, and/or interpreted the data and/or wrote the manuscript. All authors reviewed the final manuscript.

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COMPETING INTERESTS

KCV, JS, WB, KEV and VS are/were employees of Engrail Therapeutics at the time of this research and may own stock and/or stock options in the company. Over the past 3 years, BDK has received sponsored research agreements from BlackThorn Therapeutics, Compass Pathways, Delix Therapeutics, Engrail Therapeutics, Neurocrine Biosciences, and Takeda Pharmaceuticals. Over the past 3 years, DAP has received consulting fees from Abbvie, Arrowhead Pharmaceuticals, Boehringer

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ADDITIONAL INFORMATION

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SUPPLEMENTARY MATERIAL

SUPPLEMENTAL METHODS

In vitro pharmacology

Binding and functional activity of ENX-104 at human dopaminergic and serotonergic receptors were tested using recombinant cell lines (Chinese Hamster Ovary; [CHO]-K1) expressing respective receptors. Binding assays were conducted using radiolabeled reference tracers for each receptor and compared against reference comparators. ENX-104 was tested at eight concentrations in duplicate to test for radioligand binding, agonist, or antagonist activity. Agonist activity is expressed as a percent of the activity of the reference agonist at its EC_{100} concentration. Antagonist activity of the test compound is expressed as a percentage of the inhibition of the reference agonist at its EC_{80} concentration. Data was averaged values derived from multiple experiments.
For determining binding potency and functional activity assays, radiolabeled tracers/reference comparators were used as listed below.

Binding potency:

Receptor	Cell line	Reference tracer	Reference competitor
D2L	CHO-K1	[^{3}H]-Spiperone	Risperidone
D2S	CHO-K1	[^{3}H]-Spiperone	Risperidone
D3	CHO-K1	[^{3}H]-R-(+)-7-OH-DPAT	R-(+)-7-OH-DPAT
D4.4	CHO-K1	[^{3}H]-Spiperone	Haloperidol
human D2L	CHO-K1	[^{3}H]-Spiperone	Risperidone

Functional activity:

Receptor	Assay	Cell line	Reference agonist	Reference antagonist
D2L	cAMP	CHO-K1	Quinpirole	Haloperidol
D2S	cAMP	CHO-K1	Quinpirole	Haloperidol
D3	GTP	CHO-K1	Dopamine	GR103691
D4.4	cAMP	CHO-K1	Dopamine	Spiperone

Pharmacokinetic studies

The PK and brain penetration of ENX-104 was determined following oral administration to male Sprague Dawley rats. Rat (n = 3 per dose group) received a single PO dose of 5 mg/kg of ENX-104 and serial blood samples were collected at 5, 10, and 30 minutes and at 1, 2, 4, and 8 hours post-dose. Terminal blood and brain tissue samples were collected at 24 hours post-dose. Two additional groups of animals (n = 3 per dose group / time point) received a single PO dose 5 mg/kg of ENX-104 and terminal blood and brain tissue were collected from three animals per time point

in each dose group at 1, 4, and 8 hours post-dose. Plasma was obtained by centrifugation and brain tissue samples were homogenized in water. Concentrations of ENX-104 in plasma and brain were determined by LC-MS/MS. The PK parameters were determined using a NCA of Phoenix WinNonlin (v8.1) software. Plasma PK parameters for ENX-104 from the serial sampling (n = 3/timepoint).

Plasma samples processing:

Aliquots (30 µL) of each plasma sample were mixed with 30 µL of ACN: water [50:50, v/v] (diluent) and then extracted using 200 µL of IS (200 ng/mL verapamil) solution in ACN. The samples were vortexed vigorously for 10 minutes and then centrifuge at 4000 rpm for 10 minutes at 15°C. After centrifugation, supernatant aliquots of 100 µL were diluted with water (100 µL) and aliquots of 5 µL were injected for LC-MS/MS analysis.

Brain tissue processing:

The pre-weighed brain tissue samples were transferred to separate polypropylene tubes and three volumes of water and one ceramic bead were added for homogenization. The samples were homogenized with a FastPrep-24™ 5G Tissue and Cell Homogenizer (MP Biomedical, LLC, Santa Ana, CA) set to a speed of 4.0 m/sec for 20 sec × 3. An aliquot (15 µL) of each brain tissue sample homogenate was mixed with 15 µL of control rat plasma to yield samples with a total dilution of 8. The diluted samples were mixed with 30 µL of diluent and then extracted using 200 µL of IS solution. The samples were then processed as described above.

Aliquots of each working stock were serially diluted with diluent to yield separate calibration standard solutions at concentrations of 20.0, 10.0, 5.00, 2.00, 1.00, 0.500, 0.200, 0.100, 0.0500, 0.0200 and 0.0100 ng/mL for ENX-104. Aliquots (30 µL) of each calibration standard solution were mixed with 30 µL of control rat plasma and then extracted using 200 µL of IS solution. The standards were then processed as described above.

Concentrations of ENX-104 in quality control, plasma and brain tissue samples were determined by LC-MS/MS analysis using verapamil as the IS. Separations were performed with a Shimadzu LC pump and autosampler (Shimadzu Scientific Instruments, Columbia, MD) using an ACE 5 Excel C18, 2.1 × 50 mm, 5 µm column (Advanced Chromatography Technologies Ltd, Aberdeen, Scotland) maintained at ambient temperature. The mobile phase consisted of 0.1% aqueous formic acid (eluent A) and 0.1% formic acid in ACN (eluent B). A SCIEX Triple Quad™ 6500+ LC-MS/MS system (SCIEX, Framingham, MA) equipped with an electrospray ionization (ESI) source was used as the detector. The instrument was operated in positive ion mode using multiple reaction monitoring (MRM) with specific precursor-product ion pairs for ENX-104 and verapamil.

Non-compartmental analyses were performed to calculate the pharmacokinetics of ENX-104 in male SD rats. The maximum plasma concentration (C_{max}) and time to maximum plasma concentration (t_{max}) were obtained directly from the measured plasma concentrations of ENX-104. The area under the concentration-time curve (AUC) was calculated from zero to the last quantifiable concentration using the linear trapezoidal method. Phoenix® WinNonlin® (version 8.4) (Certara USA, Inc., Princeton, NJ) was used to generate pharmacokinetic data.

Receptor occupancy studies

Sprague-Dawley rats from Charles River (Margate) were obtained and group housed (2 or 3 to a cage) at an ambient temperature of 21±2°C on a normal 12-hour light/dark cycle (lights on 07:00). Relative humidity was typically 55±15% with prolonged periods below 40% RH (Relative Humidity) avoided as detailed in the UK Code of Practice. As a refinement, each cage contained a red plastic tunnel and chew stick. Standard pelleted diet and filtered water were available ad libitum. Upon arrival, rats were weighed and given wet mash overnight (standard maintenance diet mixed with water) to aid recovery from transport.

Animals were allocated to treatment groups based on body weight and cage so animals in the same cage received the same treatment. On the day of testing, animals were dosed orally with either vehicle (0.5% methylcellulose) or ENX-104 at a single dose (2.5 mg/kg) or the positive control olanzapine (10 mg/kg) (n = 5 / group). Rats were humanely killed by a Schedule 1 method (increasing exposure to CO2 with confirmation of death by cervical dislocation) at 1, 2, 4, 8 or 24 hours after ENX-104 administration or 1 hour after vehicle or olanzapine administration.

A post-mortem blood sample (~ 5 ml) was taken by cardiac puncture and placed into K/EDTA tubes (32.332, Sarstedt). The post-mortem blood samples were gently inverted, centrifuged (1900 g for 5 minutes at 4°C) and 1 ml of plasma from each animal was placed into a screwcap microtube (CP5915, Alpha Laboratories) for PK determination. All plasma samples were frozen and stored at -80°C.

Whole brains were removed, rinsed with saline and blot dried. The left striatum (~35-50 mg) and right striatum (approx. 35-50 mg) were dissected out and weighed before being frozen on dry ice. The striata from each hemisphere were frozen separately. The tissue was wrapped in aluminum placed in bags and stored at -20°C until the day of the appropriate study's binding assay. The remaining brain tissue was weighed, frozen on dry ice, then wrapped in aluminum foil, placed in bags and stored at -80°C prior to shipping along with the plasma samples for bioanalysis.

The striata were homogenized individually in ice-cold 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2 and 10 µM pargyline using a tight-fitting glass/Teflon homogeniser equivalent to 6.25 mg wet weight of tissue/ml and used immediately in the binding assay. Striatal homogenates (400 µl, equivalent to 2.5 mg wet weight tissue/tube) were incubated with 50 µl of 1.6 nM [^{3}H]raclopride and either 50 µl assay buffer (total binding) or 50 µl of 1 µM (-)Sulpiride (to define non-specific binding) for 30 minutes at 23°C. The assay buffer consisted of 50 mM Tris, pH 7.4, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2 and 10 µM pargyline. The was buffer consisted of 50 mM Tris, pH 7.4. There were two tubes for the determination of total binding and two tubes for the determination of non-specific binding. Membrane bound radioactivity was recovered by filtration under vacuum through Skatron 11731 filters, pre-soaked in 0.5% polyethyleneimine (PEI) using a Skatron cell harvester. Filters were rapidly washed with ice-cold buffer (was setting 9, 9, 0) and radioactivity was determined by liquid scintillation counting (1 ml Packard MV Gold scintillator).

The value for specific binding in disintegrations per minute (DPM) was generated by subtraction of mean non-specific binding (DPM) from mean total binding (DPM) for each animal. Data was presented as mean specific binding (DPM), mean specific binding as a percentage of the vehicle-treated control taken as 100% and as mean receptor occupancy as a percentage of control taken as 0%. All data was square root transformed and analyzed by

one-way ANOVA. ENX-104 was compared to vehicle by Dunnett's test. Olanzapine was compared to vehicle by multiple t-test. Means were back transformed and adjusted for difference between vehicle and olanzapine groups between studies. P value < 0.05 was considered significant.

In vivo microdialysis

Experiments were carried out in male Sprague Dawley rats (~26-384 g in weight or approximately 8-10 weeks old at the time of the experiment; Charles River, UK). Animals were housed in groups of three (PK study) and four (microdialysis study) on a 12 h/12 h light/dark cycle (lights on at 07.00 h), at an average ambient temperature of 19.96 23.04°C. Average relative humidity was 40.42 66.92%. Standard pelleted rat diet (Teklad Certified Global 18% Protein Rodent Diet; Envigo) and filtered tap water were available ad libitum. Rats were weighed on the day of their arrival and provided with wet mash overnight to negate any weight loss during transportation, in addition to their diet pellets. The wet mash consisted of their standard rodent diet in powdered form mixed with water. They were weighed again the day after arrival and the mash was removed. Animals were allowed to acclimatize to these conditions for at least 1 week and were weighed/handled for three days prior to use (excluding weekends). These experiments were performed in strict accordance with Home Office Guidelines and licensed under the Animals (Scientific Procedures) Act 1986 (Project Licence P58B4DA70).

For the surgery, rats were anaesthetised with isoflurane (5% to induce, 2% to maintain) in O_2 (1 litre/min) delivered via an anaesthetic unit (Burtons Medical Equipment Ltd, UK). A dual-probe study was conducted whereby each rat had two concentric microdialysis probes (CMA 12 Elite probes, CMA Sweden) stereotaxically implanted into the prefrontal cortex (co-ordinates: AP+3.2 mm; ML+/-2.5 mm relative to bregma; DV-4.0 mm relative to the skull surface, 2 mm tip) and nucleus accumbens (co-ordinates: AP+2.2 mm; ML+/-1.5 mm relative to bregma; DV-8.0 mm relative to the skull surface, 2 mm tip). Co-ordinates were taken from Paxinos and Watson atlas. The upper incisor bar was set at 3.3 mm below the interaural line so that the skull surface between bregma and lambda was horizontal. Additional burr holes were made for skull screws (stainless steel) and the probes were secured using dental cement. Carprofen (Rimadyl, Zoetis) was administered for pain relief at least 30 min prior to animals regaining consciousness following surgery (5 mg/kg s.c.). Following surgery, animals were individually housed in microdialysis round-bottom bowls (245 mm internal diameter at base of bowl, 360 mm wall height, BASi) with the microdialysis probes connected to liquid swivels and a counter-balanced arm to allow unrestricted movement. Rats were allowed a recovery period of at least 16 h with food and water available *ad libitum*. During this time, the probes were continuously perfused at a flow rate of 1.2 µl/min with an artificial cerebrospinal fluid (aCSF; Harvard Apparatus, UK) of the following electrolyte composition (in mM): sodium 150; potassium 3.0; magnesium 0.8; calcium 1.4; phosphate 1.0; chloride 155.0. Microdialysis experiments were performed the day after surgery with 8 rats per experimental session. Microdialysate samples were collected from freely-moving rats at 30 min intervals for a baseline period of 120 min prior to the administration of vehicle or drug. Samples were then collected for a further 8 hours (20 samples in total) after drug treatment at intvervals of 30 minutes (n =8/group). Throughout the experiment, probes were perfused at a flow rate of 1.2 µl/min. Samples (36 µl) were collected into Eppendorf vials (300 µl volume) containing 7.5 µl of

preservative to prevent oxidation of the monoamines (thus total sample volume was 43.5 µl). Samples were frozen in dry ice immediately after collection and after the completion of each microdialysis experiment all samples were stored at -80ºC until analysis.

At the end of the experiments, rats were killed by a UK Home Office approved Schedule 1 method (Euthatal [pentobarbital sodium] overdose IP) and their brains rapidly removed and fixed in a 10% v/v formalin saline solution for a minimum of 5 days. If data were considered statistical outliers, the actual location of the probe was verified by comparing the tract of the probe against the desired position, using a stereotaxic atlas. If probes were found to be incorrectly located, the data from the rat in question was discarded from the study.

Drugs

Each rat was administered vehicle or drug by the oral (p.o.) route (via gavage) immediately followed by vehicle or drug via the intraperitoneal (IP) route. The oral vehicle was 0.5% methylcellulose (400 cP, Sigma-Aldrich, Lot SLCB9094, pH 7.1) and a dose volume of 5 ml/kg p.o. was used. The IP vehicle was 0.9% saline (pH 5.6) and a dose volume of 2 ml/kg IP was used. All drug solutions were prepared on the day of use.

ENX-104 was stored at ambient temperature and weighed into glass vials (Group E/F) or glass Duran bottles. Vehicle was added to the receptacles, which were then sonicated (15-20 min) forming uniform suspensions suitable for p.o. administration. The suspensions were protected from light and stirred for 15 min prior to and throughout the dosing period. This resulted in solutions which were suitable for p.o. administration.

d-Amphetamine sulfate (Tocris, Batch No. 7A/222903) was weighed into glass Duran bottles and vehicle was added. The bottles were briefly shaken (few sec) resulting in clear solutions suitable for IP dosing (pH 6.1). The solutions were shaken well prior to administration. A correction factor of 1.36 was used to correct from salt to base and the dose stated is for the free base.

All reagents used in high-performance liquid chromatography (HPLC) analysis were of HPLC grade. Phosphoric acid, EDTA and methanol were obtained from Fisher Scientific (UK). 1-Octane sulphonic acid was purchased from Sigma Aldrich (UK). Dopamine hydrochloride (DA; Batch No. BCCH5705) and serotonin creatinine sulfate monohydrate (5-HT; Batch No. BCCD2035) were purchased from Sigma-Aldrich (UK). Neutral buffered formalin (10%) was obtained in 20 ml CellStor™ pots from CellPath Ltd (UK). All solvents used for LC-MS were LCMS grade from Fisher Scientific except the water which was deionised and filtered before use.

HPLC

Detection and subsequent quantification of DA in the microdialysis samples was based on reversed-phase, ion-pair HPLC coupled with electrochemical detection and involved the use of an ALEXYS™ monoamine analyser (Antec Scientific, The Netherlands). The system consisted of two separate analytical columns with precolumn filters that shared a dual loop autosampler allowing one sample to be analysed simultaneously by two systems optimised for different neurotransmitters. Two solvent delivery pumps (LC 110) were used to circulate the respective mobile phases and an Antec in line degassing unit was used to remove air. Samples (10 µl total) were injected onto the columns via an autosampler (AS 110) with a cooling tray set at 4 ºC. Antec DECADE II™ electrochemical detectors were used

and Antec micro VT 03 cells employing a high density, glassy carbon working electrode combined with a salt bridge reference electrode. The electrode signal was integrated using Antec's CLARITY™ data acquisition system. Individual standard stock solutions of DA (10.0 mM) were prepared by dissolution in a mixture of equal quantities of deionised water and preservative (in order to prevent oxidation) and stored at 4 ºC. A working solution of the standard solution containing neurotransmitters was prepared daily by dilution in aCSF.

Data analysis

Microdialysis data were log transformed. Baseline was defined as the geometric mean of the four pre-treatment samples (i.e. those collected at -90 min, -60 min, -30 min and 0 min). Data were log transformed and analysis was by robust regression using M estimation, Huber weighting, using the default parameter c=1.345 with treatment as a factor and log(baseline) as a covariate. Each time was analysed separately, together with means during each of the eight hours after dosing, 0-2 and 0-4 hours and the overall 0-8 hours after dosing. For calculation of hourly and 8 hourly means, missing data were imputed to be the geometric mean of the previous and subsequent values (if the 450-480 min value was missing, it was imputed to be equal to the 420-450 min value).

Comparisons to vehicle were by Williams' test for ENX-104 and by the multiple t test for d-amphetamine, and ENX-104 + d-amphetamine. Comparisons to d-amphetamine alone and to ENX-104 alone for ENX-104 + d-amphetamine were by the multiple t test. A p value of < 0.05 was considered statistically significant. One animal was excluded from the anlaysis for the nucleus accumbens due to poor chromatography.

Probabilistic Reward Task

Eight adult male Sprague Dawley rats obtained from Charles River Laboratories (Wilmington, MA) weighing between 250 and 300 grams were used in the present study. Animals were housed in a climate-controlled vivarium with a 12-h light/dark cycle (lights on at 7am). Animals were maintained at approximately 80% of their free-feeding weight via post-session portions of rodent chow and had unrestricted access to water in their home cage. Experimental sessions were conducted 5 days a week (Mon-Fri). The protocol for the present studies was approved by the Institutional Animal Care and Use Committee at McLean Hospital and in accordance with guidelines provided by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Resources, Commission on Life Sciences (National Research Council, 2011).

Details of the apparatus can be found in Kangas and Bergman (2017). Briefly, a custom-built Plexiglas chamber (25x30x35 cm) was situated in a sound- and light-attenuating enclosure (40x60x45 cm). A 17" touch-sensitive screen (1739L, ELO TouchSystems, Menlo Park, CA) comprised the inside right-hand wall of the enclosure. An infusion pump (PHM-100-5, Med Associates, St. Albans, VT) outside the enclosure was used to deliver sweetened condensed milk solution into the shallow reservoir of a custom-designed aluminum receptacle. The receptacle was mounted 3 cm above the floor bars and centered on the left-hand inside wall. Both touchscreen and fluid reservoir were easily accessible to the subject. A speaker bar (NQ576AT, Hewlett-Packard, Palo Alto, CA) mounted above the touchscreen was used to emit audible feedback. All experimental events and data collection were programmed in E-Prime Professional 2.0 (Psychology Software Tools, Inc., Sharpsburg, PA).

Initial training

Modified response-shaping techniques were used to train rats to engage with the touchscreen26. A 5x5 cm blue square on a black background was presented in different sections of the touchscreen (left, right, or center), with the proviso that its lower edge always was 10 cm above the floor bars. This required the rat to rear on its hind legs to reach the screen and make a touchscreen response with its paw. Each response was reinforced with 0.1 mL of 30% sweetened condensed milk and the delivery was paired with an 880 ms yellow screen flash and 440 Hz tone and followed by a 5-sec intertrial interval (ITI) blackout period. After responses were reliably observed with latencies <5 sec following stimulus presentation, line-length discrimination training commenced.

Line-length discrimination training

Discrete trials began with concurrent presentation of a white line presented 5 cm above left and right response boxes. The width of the line was always 7 cm, but the length of the line was either 30 cm or 15 cm and varied in a quasi-random fashion across 100-trial sessions (50 trials of each length). Subjects learned to respond to the left or right response box depending on the length of the white line (i.e., long line = respond left, short line = respond right, or vice versa).

Response box designation was counter-balanced across subjects. A correct response was reinforced as described above and was followed by a 5 sec ITI, whereas an incorrect response immediately resulted in a 5 sec ITI. A correction procedure27 was implemented during initial discrimination training—each incorrect trial was repeated until a correct response was made—and was discontinued after session-wide trial repeats were <5 in each trial type. Discrimination sessions continued without correction until accuracies for both line lengths were >75% correct for 3 consecutive sessions.

Probabilistic Reward Task

Following line-length discrimination training, probabilistic reinforcement schedules were introduced. Based on the human task protocol, a 3:1 rich/lean probabilistic schedule was arranged such that 60% of correct responses to one of the line lengths (e.g., long line = rich alternative) and 20% of correct responses to the other line length (e.g., short line = lean alternative) were rewarded. rich/lean line assignment was counterbalanced across subjects and 50 trials of each trial type were presented in a quasi-random sequence. These probabilistic contingencies were assessed across 5 consecutive sessions prior to initiation of drug testing.

PRT Drug Tests. Following the establishment of probabilistic contingencies, an acute drug testing protocol was arranged that included intermittent maintenance sessions in which correct responses on all trials were reinforced, control sessions in which 3:1 (60%:20%) rich/lean probabilistic contingencies were arranged and, no more than once per week, a drug testing session in which vehicle or a dose of ENV-104 (0.5, 1, or 2.5 mg/kg) was tested by administering it per os (p.o.) 4 hours prior to a 3:1 (60%:20%) probabilistic session. Doses of ENX-104 were tested

in a mixed order across subjects using a Latin Square design. Vehicle (0.5% methylcellulose) and all doses of ENX-104 were tested in all 8 animals (n = 8 / group).

Data Analysis

The implementation of probabilistic contingencies yields two primary dependent measures: response bias and task discriminability. These can be quantified by examining the number of Correct and incorrect responses in rich and lean trial types using, respectively, log *b* and log *d* equations derived from signal detection theory.

$$log\ b = 0.5 * log\left(\frac{(Rich_{Correct}+0.5)*(Lean_{Incorrect}+0.5)}{(Rich_{Incorrect}+0.5)*(Lean_{Correct}+0.5)}\right) \qquad log\ d = 0.5 * log\left(\frac{(Rich_{Correct}+0.5)*(Lean_{Correct}+0.5)}{(Rich_{Incorrect}+0.5)*(Lean_{Incorrect}+0.5)}\right)$$

High bias values are produced by high numbers of correct responses during rich trials and incorrect responses during lean trials, which increase the log *b* numerator. High discriminability values are produced by high numbers of correct responses during both rich and lean trials, which increase the log *d* numerator. (0.5 is added to all parameters to avoid instances where no errors are made on a given trial type, which would make log transformation impossible.) Given the *a priori* hypotheses discussed above of small doses of ENX-104 increasing reward responsiveness (putatively due to presynaptic autoreceptor blockade) but high doses decreasing reward responsiveness (putatively due to postsynaptic blockade), log *b* data were subjected to general linear model tests of within-subjects contrast in which the quadradic term was specifically evaluated (an inverted U-function was expected). All other data (log *d*, accuracy, reaction time) were subject to repeated measures analysis of variance (ANOVA). For accuracy and reaction time, the repeated measures factor trial type (rich vs. lean) was added to the model. When appropriate, ANOVAs were followed by post-hoc tests (one-way: Dunnett's multiple comparisons test; two-way: Bonferroni multiple comparisons test) to evaluate the statistical significance of stimulus type and drug treatment. Given *a priori* hypotheses (see below), paired t-tests and Cohen's *d* were also used to evaluate, respectively, the statistical significance of differences in log *b* values and effect sizes following vehicle treatment and each individual dose of ENX-104. The criterion for significance was set at $p<0.05$. Effect sizes (Cohen's *d* values) were interpreted using established conventions31: small (d=0.20), medium (d=0.50), and large effect (d>0.80). All statistical analyses were conducted using GraphPad Prism 9 Software (San Diego, CA, USA).

Conditioned avoidance response

Adult, male Wistar rats from Envigo (Indianapolis, IN) were used in this study. Rats were received at approximately 100-150g, assigned unique identification numbers and group housed 2-3 per cage in ventilated cages. Animals were maintained in a 12/12-hour light/dark cycle with room temperature maintained at 22 ± 1°C and with the relative humidity maintained at approximately 50%. Food and water were provided ad libitum. All rats were examined, handled, and weighed prior to initiation of the study to assure adequate health and to minimize the non-specific stress associated with testing. Each animal was randomly assigned across the treatment groups. The experiments were conducted during the animal's light cycle phase. ENX-104 (0.5, 2.5 and 5 mg/kg) was formulated in 0.5% Methyl Cellulose in water and administered orally at a dose volume of 1 ml/kg 4 hours prior to test.

The Conditioned Avoidance Response (CAR) Test has been shown to be a very reliable animal model for screening antipsychotic drugs. 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 avoidance response in CAR, thus making it one the preferred assay to screen potential antipsychotic compounds. The CAR apparatus consists of a two-way shuttle box with infra-red (I/R) detection housed in sound-attenuating chambers (Med Associates). The two-way shuttle boxes have stainless steel grid floors and are partitioned by a guillotine door. Programs are run through Med-PC version IV software. Rats were trained to avoid a foot shock following presentation of a light or tone. Rats were placed in the CAR two-compartment shuttle box (Med Associates) and presented with a conditioned stimulus (CS; light and tone), followed by an aversive unconditioned stimulus (US;foot-shock of 0.65 mA). Each rat goes through 20 trials with a variable ITI (20 – 60 sec). After several weeks of training in the CAR chambers, rats that passed the testing criterion of performing 16 -20 avoidance responses for three days in a row are included in the study and testing commenced. CAR testing consists of the same procedure as CAR training. Baseline was measured over three days prior to drug testing. Following the test, the rats were given one week washout between tests. The measures obtained from this test are:

Avoidance response: If the rat moved from one compartment to the other during the cuedstimulus (CS) presentation and prior to foot-shock delivery. Decreased avoidance responding is the typical signature of an efficacious dose of an antipsychotic.

Escape Failure: If the rat failed to move into the other compartment during the 20-sec footshock.

Avoidance response data were expressed as the number of avoidance responses as well as percent of avoidance responses based on the three baseline responses prior to drug test. Escape failures were expressed as the total number of failures during the test session. Dunnett's post-hoc comparisons when appropriate. Escape failures were analyzed by Kruskal-Wallis nonparametric analysis followed by Dunnett's post-hoc comparisons when appropriate. Results are reported as mean ± SEM. An effect was considered significant if $p<0.05$ ($n = 10$ / group).

Catalepsy

Sprague-Dawley male rats (~161- 210 g body weight on arrival) were obtained from Charles River (Margate). Rats were group housed (2-4 animals/cage) on a 12 h/12 h light/dark cycle (lights on at 07.00h) at an ambient temperature of 21±3°C. Relative humidity was typically 55±15% with prolonged periods below 40% RH or above 70% RH avoided as detailed in the UK Code of Practice. As a refinement, each cage contained a red plastic tunnel and a chew stick. Teklad 2018C pelleted diet and filtered water was available *ad libitum*.

Drugs

Haloperidol (Product Code 0931; Lot: 4B/263582) was purchased from Tocris, Abingdon, UK. Haloperidol was prepared in saline and dosed intraperitoneally as a clear solution. ENX104 was prepared in 0.5% methylcellulose and dosed orally as a uniform fine suspension and all ENX-104 dosing preparations were stirred on a magnetic plate prior to and throughout dosing and protected from light. All compounds were formulated on the day of dosing and administered using a dose volume of 5 ml/kg. Drug doses are expressed as free base.

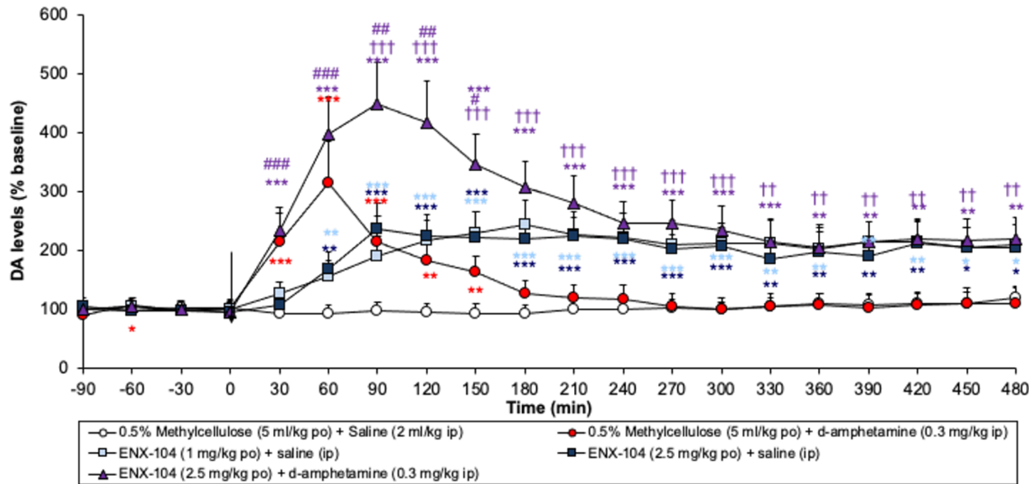
On the day of the test, animals were dosed with vehicle (po), ENX-104 (1, 2.5 and 10 mg/kg, po) or haloperidol (0.82 mg/kg, ip; ED_{75} dose). Animals were tested individually for catalepsy at 90, 180 (data not shown) and 240 minutes post-dose by gently placing each paw in turn on a large rubber bung (42 mm high, 45 mm wide at upper surface). A score of 1 was given for each paw which remained in position for 15 seconds, giving each rat a maximum score of 4. The duration the paw remained in position was recorded (maximum latency score 15 seconds on each trial) and total latency for paw withdrawal was determined giving each rat a maximum total latency of 60 seconds.

Statistical analysis was performed by a qualified statistician. The total catalepsy score and total latency were compared to vehicle and haloperidol by exact Wilcoxon rank sum tests. Raw means and standard errors were calculated. $p<0.05$ was the level used for statistical significance (n = 8/group).

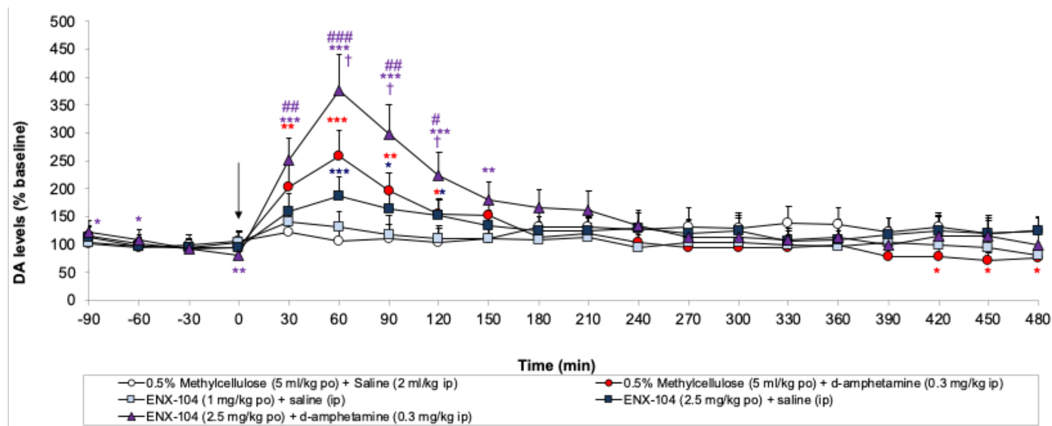
SUPPLEMENTAL FIGURES

Figure S1. Dopamine levels in freely moving rats

A. Nucleus accumbens



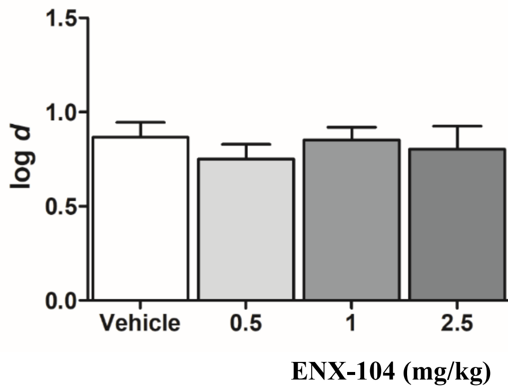
B. Prefrontal cortex



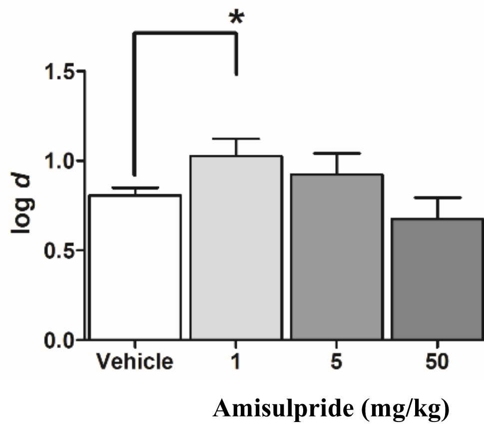
Timecourse of dopamine (DA) levels in the (A) nucleus accumbens and (B) prefrontal cortex of freely moving rats. ENX-104 was orally administered at 1 mg/kg (light blue squares) and 2.5 mg/kg (dark blue squares) dose levels and compared to vehicle (0.5% Methylcellulose) treated animals (white cirlces). Arrow denotes start of dose (timepoint 0). D-amphetamine (0.3 mg/kg IP, red circles) served as a positive control. Results are adjusted means (from log-transformed data) ; n=7-8/group. SEMs are calculated from the residuals of the statistical model. ENX-104 treated groups were compared to vehicle by William's test, other comparisons to vehicle were using the multiple t test. Differences vs. vehicle (per time point) were considered statistically significant at *p < 0.05, **p < 0.01, ***p < 0.001 ; vs. ENX-104 at #p < 0.05, ##p < 0.01, ###p < 0.001 ; vs. d-amphetamine at †p < 0.05, ††p < 0.01, †††p < 0.001.

Figure S2. Probabilistic reward task

A. Discriminability (log *d*) in the PRT



B. Discriminability (log *d*) in the PRT

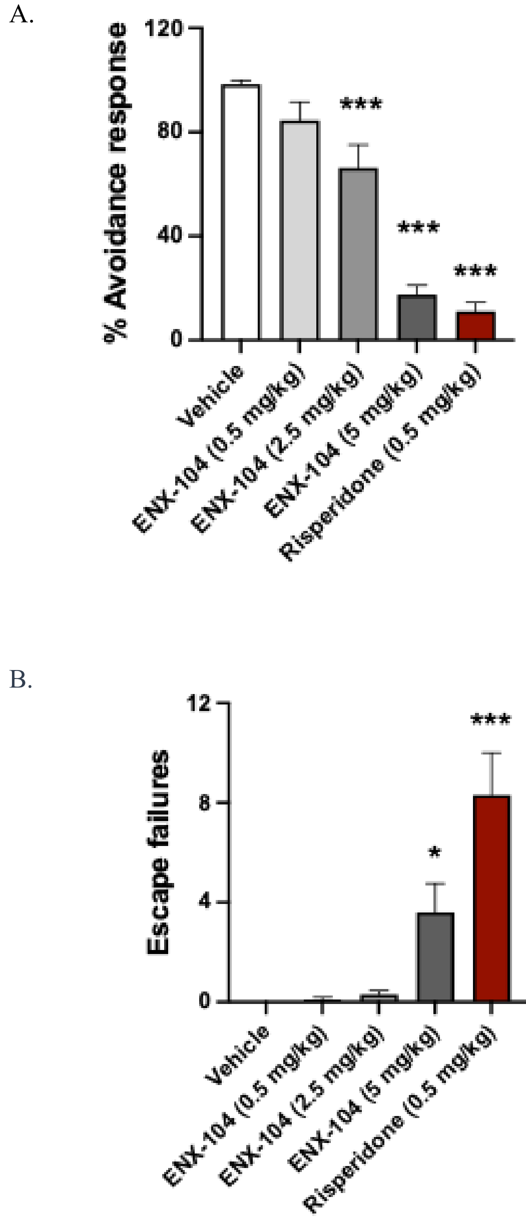


C. Effect sizes for response bias (log *b*) in ENX-104 treated groups in the PRT

	Vehicle	0.5 mg	1 mg	2.5 mg
Vehicle		1.206	0.753	0.498
0.5 mg			0.138	-0.497
1 mg				-0.468
2.5 mg				

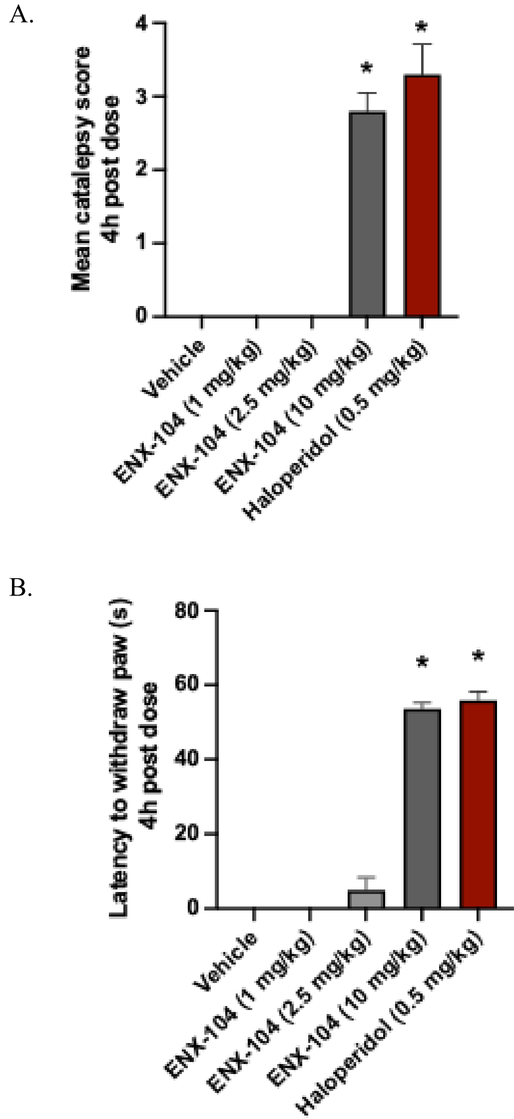
(A, B) Log d values in the PRT from ENX-104 and amisulpride treated animals. Data are presented as means ± SEM. *p<0.05. (C) Log *b* effect sizes (Cohen's *d*), pairwise comparisons of Vehicle vs. ENX-104-treated groups (at listed doses).

Figure S3. Conditioned avoidance response assay



(A) Percent avoidance response (associated with antipsychotic activity) and (B) escape failures (associated with potential motor impairment) in the CAR in orally administered ENX-104 (gray bars) (0.5 mg/kg, 2.5 mg/kg, 5 mg/kg) and control groups, administered vehicle (white bar) or risperidone (0.5 mg/kg ip, red bar). Data are presented as means $\pm$ SEM. * $p<0.05$, *** $p<0.001$.

Figure S4: Catalepsy



Catalepsy in rats was measured as (A) mean catalepsy score calculated as a sum of observations in each limb (maximum score possible is 4) and (B) latency to withdraw. Animals were orally administered ENX-104 (gray bars) at three dose levels (0.5 mg/kg, 2.5 mg/kg, 5 mg/kg, testing at 4h post dose) and control groups, administered vehicle (white bar) or haloperidol (0.5 mg/kg ip, red bar). Data are presented as means ± SEM. * $p<0.05$.