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TITLE PAGE

Effects of ketamine and ketamine metabolites on evoked striatal dopamine release, dopamine receptors, and monoamine transporters

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RUNNING TITLE PAGE

Running title: *Lack of direct effects of ketamine on DAergic function*

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Non-standard abbreviations: MDD, major depressive disorder; DA, dopamine; NAc, nucleus accumbens; VTA, ventral tegmental area; NMDAR, *N*-methyl-*D*-aspartate glutamate receptor; DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; HNK, hydroxynorketamine; DHNK, dehydronorketamine; FSCV, fast scan cyclic voltammetry; AMPA, -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; $[DA]_{max}$, peak amplitude of evoked dopamine.

Recommended section: Neuropharmacology

ABSTRACT

Following administration at subanesthetic doses (*R,S*)-ketamine (ketamine) induces rapid and robust relief from symptoms of depression in treatment-refractory depressed patients. Previous studies suggest that ketamine's antidepressant properties involve enhancement of dopamine (DA) neurotransmission. Ketamine is rapidly metabolized to (*2S,6S*)- and (*2R,6R*)-hydroxynorketamine (HNK), which have antidepressant actions independent of NMDA receptor inhibition. These antidepressant actions of (*2S,6S;2R,6R*)-HNK, or other metabolites, as well as ketamine's side effects, including abuse potential, may be related to direct effects on components of the dopaminergic (DAergic) system. Here, brain and blood distribution/clearance and pharmacodynamic analyses at dopamine (DA) receptors (D_1-D_5), and the DA, norepinephrine, and serotonin transporters were assessed for ketamine and its major metabolites (norketamine, dehydronorketamine, and HNKs). Additionally, we measured electrically-evoked mesolimbic DA release and decay, using fast-scan cyclic voltammetry following acute administration of subanesthetic doses of ketamine (2, 10, 50 mg/kg, i.p.). Following ketamine injection, ketamine, norketamine, and multiple hydroxynorketamines were detected in the plasma and brain of mice. Dehydronorketamine was detectable in plasma, but concentrations were below detectable limits in the brain. Ketamine did not alter the magnitude or kinetics of evoked DA release in the nucleus accumbens in anesthetized mice. Neither ketamine's enantiomers nor its metabolites had affinity for DA receptors or the DA, noradrenaline and serotonin transporters (up to 10 uM). These results suggest that neither the side effects nor antidepressant actions of ketamine or ketamine metabolites are associated with direct effects on mesolimbic DAergic neurotransmission. Previously observed *in vivo* changes in DAergic neurotransmission following ketamine administration are likely indirect.

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INTRODUCTION

A single subanesthetic dose of (*R,S*)-ketamine (ketamine) produces rapid and sustained antidepressant effects in humans (Berman et al., 2000; Zarate et al., 2006; aan het Rot et al., 2010; Diazgranados et al., 2010; Zarate et al., 2012; Murrough et al., 2013). Although the discovery of ketamine's antidepressant efficacy was a major breakthrough, ketamine's clinical use is limited due to its serious side effects, which include dissociation and abuse potential (Krystal et al., 1994; Morgan and Curran, 2012).

Evidence suggests that ketamine's antidepressant actions might involve its actions on the dopaminergic (DAergic) system. Administration of ketamine reversed depression-related deficits in DA-dependent synaptic plasticity, which was associated with its ability to reverse helpless behavior in rats (Belujon and Grace, 2014). In addition, haloperidol, presumably acting as a D_2 receptor antagonist, blocked the antidepressant effects of ketamine in the mouse forced-swim test, while activation of D_2/D_3 receptors enhanced its antidepressant effects (Li et al., 2015). These findings, along with the well-established role of the dopaminergic (DAergic) system in the pathophysiology and treatment of depression (Willner et al., 2005; Papakostas, 2006; Dunlop and Nemeroff, 2007; Grace, 2016) suggest that ketamine might act via the regulation of the DAergic neurotransmission to exert its antidepressant effects. In fact, several agents that directly act on the DAergic system, including pramipexole (a D_2/D_3 receptor agonist), bupropion (DA reuptake inhibitor) and monoamine oxidase inhibitors have shown efficacy in the treatment of depression (Nieuwstraten and Dolovich, 2001; Zarate et al., 2004; Shulman et al., 2013). Since we have recently demonstrated that ketamine's antidepressant effects in mice are the result of the metabolite (*2S,6S;2R,6R*)-hydroxynorketamine (HNK) (Zanos et al., 2016), we hypothesize that direct effects of ketamine and/or its metabolites at DA receptors and monoamine transporters

might exist and contribute to these antidepressant properties or the mechanisms associated with ketamine's side effects.

The possible mechanistic link between ketamine, the DA system, and behavioral changes is further supported by previous studies in rodents. Haloperidol and destruction of catecholaminergic terminals with 6-hydroxydopamine both attenuated ketamine-induced hyperlocomotion in mice (Irifune et al., 1991). Additionally, ketamine reversed haloperidol-induced catalepsy in rats (Lannes et al., 1991) and enhanced D_2 agonist quinipirole-induced hyperlocomotion (Witkin et al., 2016). Haloperidol and the D_2 antagonist raclopride reversed the disruptive effect of ketamine on spatial delayed alternation performance (Verma and Moghaddam, 1996). Furthermore, pre-treatment with a D_1/D_5 receptor antagonist prevented ketamine-induced hippocampal synaptic depression and its associated spatial memory deficits in freely-moving rats (Duan et al., 2013). Administration of ketamine also enhanced the interoceptive stimulus properties of methamphetamine in a rat drug discrimination paradigm (Wooters et al., 2011). *In situ* receptor binding studies indicated that ketamine has a strong affinity for the rat D_2 receptor (Kapur and Seeman, 2002). However, positron emission tomography has not shown any effects of ketamine administration on these receptors in humans (Aalto et al., 2002).

Ketamine administration was recently shown to increase the number of spontaneously active DA neurons in the VTA (Belujon and Grace, 2014; Witkin et al., 2016), and to increase the firing rate and burst firing of these cells (Belujon and Grace, 2014). Similarly, in humans, ketamine enhances amphetamine-induced augmentation of striatal DA release (Kegeles et al., 2000). However, there exists conflicting data as well. Ketamine has been reported to increase (Irifune et al., 1991; Verma and Moghaddam, 1996; Witkin et al., 2016), to have no effect

(Lannes et al., 1991; Micheletti et al., 1992) or to decrease (Rao et al., 1989) striatal DA turnover, or extracellular DA dialysate levels. Stereoselective effects of ketamine on DA release in rat striatal slices have been reported (Hancock and Stamford, 1999; Tso et al., 2004), Thus, while overall changes in extracellular DA concentrations have been assessed previously, there is no consensus effect, and the low temporal resolution of microdialysis does not permit a determination of the relative contributions of DA release by axon terminals or the dynamics of DA reuptake.

Here, we utilized fast-scan cyclic voltammetry (FSCV) to assess the effects of ketamine treatment on the magnitude and temporal dynamics of DA release, and the reuptake of extracellular DA, in the NAc core *in vivo*. We also performed an *in vitro* pharmacological affinity screening of (S)- and (R)-enantiomers of ketamine and its principal metabolites, (R)- and (S)-norketamine, (*2S,6S*)-hydroxynorketamine (HNK), (*2R,6R*)-HNK, (*2S,6R*)-HNK and (*2R,6S*)-HNK, and (R)- and (S)-dehydronorketamines (DHNK) on DA D_1-D_5 receptors and monoamine transporters. Finally, we functionally examined possible agonist and antagonist actions of the stereoisomers of ketamine and metabolites on DA receptors and DA (DAT), norepinephrine (NET) and serotonin (SERT) transporters.

METHODS

Animals

Male C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME), age 11-12 weeks old at the time of the experiments, were housed five per cage in an animal room with a constant temperature (22 ± 1°C) and a 12-h light/dark cycle (lights on/off at 07.00/19.00), with food and

water provided *ad libitum.* Experiments were performed in the light phase of the cycle. All experimental procedures were approved by the University of Maryland, Baltimore Animal Care and Use Committee, and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs/Compounds

(R,S)-ketamine hydrochloride (ketamine; Sigma, St. Louis MO) was dissolved in 0.9% saline and injected intraperitoneally (i.p.) with an injection volume of 7.5 ml/kg, at doses of 2, 10, or 50 mg/kg. Quinpirole hydrochloride (Sigma, St. Louis MO) was dissolved in 7.5 ml/kg saline and injected i.p. at a dose of 0.5 mg/kg. For the binding and functional studies, (S)-ketamine, (R)-ketamine, (S)-norketamine, (R)-norketamine, (S)-DHNK, (R)-DHNK, (2S,6S)-HNK, (2R,6R)-HNK, (2R,6S)-HNK and (2S,6R)-HNK were synthesized as previously described (Moaddel et al., 2010; Zanos et al., 2016).

Tissue distribution and clearance measurements of ketamine and its metabolites

C57BL/6J male mice (n=3-4 for each time point) received a single i.p. injection of ketamine (10 mg/kg). 10, 30, 60, 240 or 480 minutes following ketamine injection mice were exposed to 3% isoflurane for a 30-sec exposure and decapitated. Trunk blood was collected in EDTA-containing tubes and centrifuged at 8000 rpm for 6 min at 4^{o}C. Plasma was collected and stored at −80°C until analysis. Whole brains were simultaneously collected, rinsed with phosphate-buffered saline, immediately frozen on dry ice and stored at −80°C until analysis.

The concentrations of ketamine and its metabolites in plasma and brain tissue were determined by achiral liquid chromatography-tandem mass spectrometry using a previously described protocol with slight modifications (Paul et al., 2014; Moaddel et al., 2015). For plasma

samples, the calibration standards for (R,S)-ketamine, (R,S)-norketamine, (2R,6R;2S,6S)-HNK and (R,S)-DHNK ranged from 10,000 ng/ml to 19.53 ng/ml. The quantification of (R,S)-ketamine, (R,S)-norketamine, (R,S)-DHNK, and the HNK stereoisomers was accomplished by calculating area ratios using D_4-ketamine (10 µl of 10 µg/ml solution) as the internal standard. Whole brains were suspended in 990 µl of water:methanol (3:2, v/v), D_4-ketamine (10 µl of 10 µg/ml) added and the resulting mixture homogenized on ice with a polytron homogenizer and centrifuged at 21,000 x g for 30 min. The supernatant was collected and processed using 1 ml Oasis HLB solid phase extraction cartridges (Waters Corp., Waltham, MA). The cartridges were preconditioned with 1 ml of methanol, followed by 1 ml of water and then 1 ml ammonium acetate [10 mM, pH 9.5]. The supernatants were added to the cartridges, followed by 1 ml of water and the compounds were eluted with 1 ml of methanol. The eluent was transferred to an autosampler vial for analysis. Quality control standards were prepared at 78.125 ng/ml, 625 ng/ml and 2500 ng/ml.

Fast-scan cyclic voltammetry

Electrodes for measuring extracellular DA concentration were constructed by inserting a carbon fiber (7 µm diameter, Goodfellow, UK) into a glass capillary tube (1.2mm diameter o.d., A-M Systems, Sequim, WA), pulled with a micropipette puller (Narishige, Japan). Carbon fibers were then cut at approximately 100 µM past the glass tip (Heien et al., 2004). Mice were anesthetized with urethane (1.5 g/kg, i.p.) and their heads were positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Body temperature was continuously regulated with a rectal thermoregulator (CMS Instruments) and maintained at 37 ℃ during surgery. Burr holes were drilled in the skull for the implantation of three electrodes (recording, stimulating, and reference) in the brain. The recording electrode was placed at the level of the NAc core (+1.2 AP,

+1.1 ML, and -3.4 DV). A bipolar steel stimulation electrode (Plastics1, Roanoke, VA) was positioned ipsilaterally in the VTA (-3.1 AP, +0.7 ML, and +4.8 DV). An Ag/AgCl reference electrode (0.5mm diameter; Acros, NJ) was placed in the cortex contralateral to the recording and stimulating electrodes. Both recording and stimulating electrodes were slowly lowered into target locations until evoked DA release was maximized. Electrode placements were finalized once maximal evoked DA output was reached and the locations of electrodes were kept unchanged throughout the remainder of the experiment. Recording electrodes were conditioned by applying an inverted V waveform (-0.4V to +1.3V to -0.4V, 400 V/s) at 60 Hz for 10 minutes, after which the frequency of the waveform was changed to 10 Hz and kept constant during the subsequent procedures. In all experiments, we recorded the "background currents" produced by the inverted V waveform applied to the recording electrode (Heien et al., 2004). This background current was subtracted from the "faradic currents" recorded after each VTA stimulation, to derive the current attributable to DA release.

Electrical stimulation (60 Hz, 60 biphasic square pulses, 2ms/phase, 300 μA) was applied with a constant-current isolator (A-M Systems, Sequim, WA) to evoke DA release every three minutes for thirty minutes. Baseline recordings of stimulation-evoked DA was collected every three minutes until the peak amplitude was stabilized with less than 15% variance. Immediately following recording of the last baseline response, each mouse received by i.p. injection the single drug and dose combination that it was assigned to. Cyclic voltammograms were recorded and analyzed with TarHeel CV and Demon Voltammetry software (UNC, Chapel Hill, NC and Wake Forest University, Winston-Salem, NC, respectively). The peak amplitude of evoked DA ($[DA]_{max}$) obtained in the last pre-drug stimulation was used as baseline and the $[DA]_{max}$ following all subsequent stimulations was calculated as a percentage change from this baseline

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for each individual mouse. For each stimulation, the duration of the rising phase of the response was calculated by measuring the time that it took to reach $[DA]_{max}$ starting from the initiation of electrical stimulation. The time constant of decay (tau) was calculated by fitting the falling phase of each $[DA]_{max}$ to a single exponential decay function (Yorgason et al., 2011). These release and decay values were then normalized to the average pre-drug baseline for each individual animal and reported as percentages.

Radioligand binding assays

Binding profiles of (S)-, and (R)-ketamine, as well as (S)-norketamine, (R)-norketamine, (S)-DHNK, (R)-DHNK, (2S,6S)-HNK, (2R,6R)-HNK, (2R,6S)-HNK and (2S,6R)-HNK for DA receptors and monoamine transporters were performed by the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH PDSP; University of North Carolina, Chapel Hill, NC, USA). For all radioligand receptor assay protocols, see the Psychoactive Drug Screening Program web site (http://pdsp.med.unc.edu) and previously published protocols (Besnard et al., 2012). The data were utilized to calculate binding affinities expressed as K_i values.

Monoamine transporters and dopamine receptor functional assays

Monoamine transporter functional assays were performed by the NIMH PDSP (for details see http://pdsp.med.unc.edu) using Molecular Devices' (Sunnyvale, CA, USA) neurotransmitter transporter uptake assay kit. Agonist and antagonist activity at DA receptors (D_1-D_5) was determined using GPCR Tango assays, also performed by the NIMH PDSP, using previously published protocols with modifications (Kroeze et al., 2015). Briefly, HTLA cells were transfected with DA receptor Tango constructs (D_1 to D_5) overnight and plated in Poly-L-Lys coated 384-well white clear bottom cell culture plates using DMEM supplemented with 1%

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dialyzed FBS, at a density of 15,000 cells in 40 µl per well. Compounds (concentration-responses) were then added to cells 6 hours after plating. For antagonist activity, reference agonist at about EC_{80} concentration was added 30 min after testing compounds. After overnight stimulation, medium and drug solutions were removed and BrightGlo reagents (Promega) were added to cells (20 µl/well). Luminescence was counted on a luminescence counter after plates were incubated for 20 min in dark at room temperature.

Agonist activity: EC_{50} values were determined using an agonist dose-response formula: response = basal activity + [(E_{max} - basal activity)/ (1 + $10^{(LogEC_{50} - \text{agonist concentration}) \times \text{Hill slope}}$)]

Antagonist activity: antagonist responses were measured at a fixed EC_{80} concentration to the corresponding agonist in the presence of antagonist dilutions. IC_{50} values were determined using a concentration-response formula: response=basal activity + [(Emax - basal activity)/ (1 + $10^{(LogIC_{50} - \text{agonist concentration}) \times \text{Hill slope}}$)]. IC_{50} was then converted to K_i using the Cheng-Prusoff equation: $K_i = IC_{50}/ [1 + (L/ EC_{50})]$ in which K_i is the binding affinity derived from an antagonist concentration-response assay; IC_{50} is the antagonist concentration where 50% of inhibition is reached; L is the reference agonist concentration used in the assay (i.e., EC_{50} to EC_{80} concentration of the reference agonist); EC_{50} is the known potency of the reference agonist (Cheng and Prusoff, 1973).

Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed by Graphpad Prism *v*6. FSCV data were analyzed with two-way repeated-measures ANOVA. Correction for multiple comparisons was done by Holm-Šídák *post-hoc* test. The criterion for statistical significance was $p<0.05$.

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RESULTS

Plasma and brain tissue distribution and clearance of ketamine and major metabolites

Ketamine is extensively and stereoselectively transformed by multiple hepatic cytochrome P450 isoforms into multiple metabolites, (Adams et al., 1981; Desta et al., 2012). We first sought to quantify and compare brain and plasma concentrations of ketamine and ketamine's major metabolites in the C57BL/6J mouse strain that would be used subsequently for FSCV. As shown in the representative chromatographic trace quantifiable plasma concentrations of (*R,S*)-ketamine, (*R,S*)-norketamine, (*2S,6S;2R,6R*)-HNKs, (*2S,6R;2R,6S*)-HNK and (*R,S*)-DHNK were identified within 10 min of an i.p. injection of ketamine (10mg/kg) (Figure 1A). The plasma concentration – time curves are presented in Figure 1B. The maximum plasma concentration of (R,S)-ketamine (4.07±0.3 nmol/ml) was observed at the 10 min time point and the concentration rapidly declined reaching 0.03±0.03 nmol/ml at 120 min post-injection. The maximum plasma concentration of (*R,S*)-norketamine (6.90±0.5 nmol/ml) was also observed at the 10 min time point, indicating the rapid and extensive N-demethylation of (*R,S*)-ketamine. The clearance of (*R,S*)-norketamine was slower than (*R,S*)-ketamine as the minimum quantifiable concentration (0.07±0.07 nmol/ml) was measured in the plasma sample obtained at 240 min post-administration. The peak plasma concentration of (*2S,6S;2R,6R*)-HNK (3.16±0.4 nmol/ml) was observed at 30 min following ketamine injection and then declined to 0.32±0.1 nmol/ml in the 240 min plasma samples. The longer time to peak concentration and slower clearance of (*2S,6S;2R,6R*)-HNK is consistent with the primary formation of the metabolite from ring hydroxylation of (*R,S*)-norketamine (Desta, et al., 2012). For (*2S,6R; 2R,6S*)-HNK, maximum plasma levels were observed 30 min following ketamine injection (0.66±0.2 nmol/ml) and was rapidly cleared within 60 min (0.23±0.2 nmol/ml). The maximum plasma concentration of (*R,S*)-

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DHNK (0.74±0.08 nmol/ml) was also reached at 30 min post-ketamine administration and declined to a concentration of 0.04±0.04 nmol/ml in the plasma 120 min plasma sample. Plasma levels of HNK metabolites (*2S,5S;2R,5R*)-HNK, (*2S,4S;2R,4R*)-HNK, (*2S,4R;2R,4S*)-HNK, and (*2S,5R;2R,5S*)-HNK were below quantification limits at every time point.

A representative chromatographic trace from the analysis of brain tissue obtained after an i.p. injection of ketamine (10mg/kg) is presented in Figure 1C. The relationship between time following injection and measured concentrations of (*R,S*)-ketamine, (*R,S*)-norketamine, (*2S,6S;2R,6R*)-HNK and (*2S,6R;2R,6S*)-HNK are presented in Figure 1D. The peak brain tissue concentration of (*R,S*)-ketamine (7.03±0.4 nmol/g) was observed 10 min following (*R,S*)-ketamine administration and then declined to 0.09±0.05 nmol/g in the 240 min samples. The maximum brain tissue concentration of (*R,S*)-norketamine (3.51±0.17 nmol/g) and (*2S,6S;2R,6R*)-HNK (1.72±0.25 nmol/g) were also observed at the 10 min time point and then decreased to 0.14±0.03 nmol/g and to 0.09±0.02 nmol/g, respectively, at 240 min. Peak brain levels of the (*2S,6R;2R,6S*)-HNK metabolite were observed 10 min following ketamine administration (0.8±0.1 nmol/g) and decreased to 0.20±0.04 nmol/g within 60 min. The maximum brain tissue concentration of (*R,S*)-ketamine was 73% higher than the corresponding plasma concentration, while the brain tissue concentrations of (*R,S*)-norketamine and (*2S,6S;2R,6R*)-HNK were 49% and 45% lower than the corresponding maximum plasma concentrations. These data are consistent with previous observations that (*R,S*)-ketamine rapidly accumulates in the brain of rats (Moaddel et al., 2015). While (*R,S*)-DHNK was also detected in brain tissues, its levels were below quantification. This may in part be due to circulating plasma levels of (*R,S*)-DHNK that are significantly (4-8 times) lower than the other metabolites, but also poor crossing of the blood-brain-barrier. A potential explanation for the low circulating and brain

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tissue concentrations of (R,S)-DHNK was provided by a recent study reporting that (R,S)-DHNK rapidly and irreversibly partitions into rat red blood cells reducing the available plasma concentrations to less than 20% of the spiked values (Moaddel et al., 2016). The same effect was not observed with (R,S)-ketamine, (R,S)-norketamine or (2S,6S;2R,6R)-HNK (Hijazi et al., 2001; Moaddel et al., 2016). Brain levels of (*2S,5S;2R,5R*)-HNK, (*2S,4S;2R,4R*)-HNK, (*2S,4R;2R,4S*)-HNK, and (*2S,5R;2R,5S*)-HNK were below quantification at every time point.

Effects of ketamine administration on the kinetics of electrically-evoked dopamine release

We utilized FSCV to assess the magnitude and temporal dynamics of DA release and reuptake at subsecond temporal resolution. We electrically stimulated the VTA and recorded the resulting changes in extracellular DA concentrations in the NAc core once every three minutes for a thirty-minute period. Individual animals were either administered (i.p.) vehicle (saline) or ketamine at 2, 10, or 50 mg/kg, which represent the range of subanesthetic doses that produce antidepressant actions in rodent behavioral models (Browne and Lucki, 2013). Representative voltammograms from mice of each treatment group are presented in Figure 2. Ketamine did not significantly modify DA release at any of the doses that were tested. In contrast, quinpirole administration (0.5 mg/kg) induced a marked decrease in the peak amplitude of evoked DA ($[DA]_{max}$) values from the baseline (Figure 2), as previously reported (Stamford et al., 1991; Maina and Mathews, 2010), and consistent with its actions on D_2 autoreceptors. Quantification group data for $[DA]_{max}$ are presented in Figure 3A. A two-way repeated measures ANOVA revealed a significant main effect of 'time' ($F_{9,216} = 16.25$, $p<0.0001$) and an interaction between 'time' x 'drug treatment' ($F_{36,216} = 2.52$, $p<0.0001$) but no significant 'drug treatment' effect ($F_{4,24} = 1.88$, $p=0.146$). Holm-Šídák *post-hoc* comparisons on the effects of quinpirole administration indicated that $[DA]_{max}$ values were significantly lower compared to saline, starting

at the 18th minute and lasting until the end of data collection (Figure 3A). No statistically significant differences between saline- and ketamine-treated groups were observed at any time point (p>0.05). Furthermore, ketamine administration did not significantly alter $[DA]_{max}$ values at any time point after injection (Figure 3A).

A two-way repeated measures ANOVA performed on changes in rise time values (time that it takes for evoked DA concentrations to reach their maximal values ($[DA]_{max}$)) after the start of each electrical stimulation) indicated no main effect of drug treatment ($F_{4,24}$ = 2.01, p>0.05), but a significant main effect of time ($F_{9,216}$ = 3.08, p<0.01) and no interaction between these factors ($F_{36,216}$ = 0.91, p >*0.05*) (Figure 3B). Two-way repeated measures ANOVA on decay constants revealed no main effect of 'time' ($F_{9,216}$ = 1.82, p>0.05), but there was a main effect of 'drug treatment' ($F_{4,24}$ = 4.04, p<0.05) (Figure 3C). Although the ANOVA interaction between these variables was not statistically significant ($F_{36,216}$ = 1.2, p>0.05), Holm-Šídák *post-hoc* pairwise comparisons between saline and all other treatment groups were performed to assess whether decay constants were differentially altered between treatment groups. These comparisons revealed that the decrease in decay constants were significantly higher in the quinpirole treatment group compared to saline group at all times from the 9th minute onward, whereas there was no significant difference in change in decay constants in ketamine treatment groups and the saline group (Figure 3C).

Overall, our results reveal that ketamine does not change electrically-evoked DA release or alter DA release and decay kinetics in the NAc core. However, quinpirole, a D2 receptor agonist, decreases DA release, and led to a significant decrease in decay constants as expected from activation of pre-synaptic D_2 autoreceptors (Stamford et al., 1991; Maina and Mathews, 2010).

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Dopamine receptor binding affinity and functional activity

An *in vitro* affinity screening was performed, to assess affinity of (S)-, (R)-ketamine, or their metabolites (*S*)-norketamine, (*R*)-norketamine, (*S*)-DHNK, (*R*)-DHNK, (*2S,6S*)-HNK, (*2R,6R*)-HNK, (*2R,6S*)-HNK and (*2S,6R*)-HNK on DA D_1, D_2, D_3, D_4 or D_5 receptors. In the primary receptor screening, at 10 μM concentrations, none of ketamine's enantiomers or metabolites showed inhibition of any of the DA receptor subtypes more than 50% (Table 1). Thus, no K_i determinations were performed.

In vitro agonist and antagonist functional assays were performed at DA D_1, D_2, D_3, D_4 and D_5 receptors for (*S*)-, (*R*)-ketamine, and their metabolites, using the GPCR Tango assays to measure agonist mediated β-arrestin translocation (Kroeze et al., 2015). No agonist (Figure 4) or antagonist (Figure 5) actions on DA receptors were observed.

Binding affinity and functional activity at monoamine transporters

In vitro binding assays were performed to assess binding affinity of (S)-, (R)-ketamine, and their metabolites to the monoamine transporters. No inhibition of binding by ketamine enantiomers and metabolites were observed at concentrations up to 10 μM at DAT, NET or SERT (Figure 6). We also determined effects of ketamine enantiomers and metabolites at DAT, NET and SERT and found that they showed no inhibitory effect on neurotransmitter transporter activity (Figure 7) at up to 10 μM.

DISCUSSION

We used FSCV to study, *in vivo*, the effects of acute, subanesthetic doses of ketamine on the magnitude and kinetics of evoked DA release in the NAc core of anesthetized mice. We also conducted a comprehensive *in vitro* pharmacological screening of the binding and functional activity of ketamine and its metabolites on DA receptors and monoamine transporters. We observed no significant effects of acute, systemic administration of ketamine on the magnitude or kinetics of the electrically-evoked DA concentrations. This contrasts with the finding of Hancock and Stamford (1999), who observed that a 100μM concentration of ketamine (and in particular (*S*)-ketamine) increased NAc DA release, as measured by FSCV in rat slices. However, the concentration of ketamine used in this previous study is higher than brain exposure under our conditions relevant to the antidepressant actions of ketamine. Our observed lack of direct effects of ketamine on DA release *in vivo* is supported by the pharmacological/functional activity profile of ketamine and its metabolites, which also showed no significant affinity or agonist/antagonist activity on DA $D_{1\text{-}5}$ receptors or DAT, NET, SERT

Previous studies showed that ketamine has an affinity (K_i = 0.05-0.5μM) for DA D_2 type receptors *in vitro* (Kapur and Seeman, 2002; Seeman et al., 2005), which was not identified in our study (Table 1). We also did not find any agonist or antagonist functional activity at the D_2 receptor (Figures 4 and 5). Nishimura and colleagues have reported that ketamine inhibits NET (66.8 μM), DAT (62.9 μM), and SERT (162 μM) transporters expressed in HEK-293 cells (Nishimura et al., 1998). In a follow-up study they reported that (*S*)-ketamine shows a greater inhibition at DAT than (*R*)-ketamine (K_i = 46.9 μM *vs.* 390 μM) (Nishimura and Sato, 1999). In contrast, ketamine exhibited no stereoselectivity for NET and SERT (Nishimura and Sato, 1999). Consistent with these results we did not observe any binding of ketamine's enantiomers or metabolites to DAT, NET or SERT when tested up to 10 μM. Moreover, we demonstrate no

functional inhibition of ketamine's enantiomers or primary metabolites on DAT, NET or SERT (up to a maximum concentration of 10 μM). Our FSCV studies confirmed the lack of activity of ketamine on monoamine transporters and DA receptors, since we did not observe a statistically significant effect of different doses of ketamine on extracellular NAc DA kinetics, whereas the D_2 receptor agonist quinpirole significantly decreased the evoked release of DA. Our pharmacological/functional screening similarly did not provide any evidence for direct effects of ketamine and its metabolites on DA receptor function. Taken together, our data indicate that ketamine does not affect electrically-evoked DA release in the NAc of anesthetized mice, and that ketamine and its metabolites exert no significant effect on the DAergic receptors and transporters. Additionally, no functional effects on any of these receptors and transporters were observed *in vitro* at concentrations that are relevant to the antidepressant effects of ketamine.

Ketamine is a non-competitive *N*-methyl-*D*-aspartate glutamate receptor (NMDAR) antagonist (K_i = 0.2-1.6 μM, with (*S*)-ketamine approximately 4 fold more potent than (*R*)-ketamine (Parsons et al., 1995; Ebert et al., 1997; Moaddel et al., 2013)). (*S*)-and (*R*)-Norketamine are less potent NMDAR antagonist (K_i = 1.7-2.25 μM, and 13-26 μM respectively) than ketamine, while (*2S,6S*)-HNK and (*2R,6R*)-HNK show limited if any *in vitro* binding or *in situ* functional activity at the NMDAR (Moaddel et al., 2013; Zanos et al., 2016). We have recently shown that the antidepressant actions of (*2R,6R*)-HNK involves potentiation of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Zanos et al., 2016). Our current findings cannot preclude indirect effects of ketamine administration on DA neurotransmission *in vivo,* since glutamatergic signaling plays an important role in the regulation of the mesolimbic DAergic system. In fact, DAergic neurons in the VTA express both NMDAR and AMPARs, and these neurons receive direct glutamatergic inputs from numerous cortical

(prefrontal cortex) and subcortical (laterodorsal tegmental and pedunculopontine tegmental nuclei, the bed nucleus of the stria terminalis and the superior colliculus) brain regions (Morikawa and Paladini, 2011). Activation of NMDARs in the VTA increases DA neuron burst firing rates (Overton and Clark, 1992; Chergui et al., 1993), whereas NMDAR antagonists decrease spontaneous bursts of VTA DAergic neurons after the activation of glutamatergic inputs to the VTA (Overton and Clark, 1992; Chergui et al., 1994; Overton and Clark, 1997; Morikawa and Paladini, 2011). In a similar fashion, in mutant mice lacking NMDARs specifically on DAergic neurons, burst firing of VTA DAergic neurons and striatal DA release are attenuated after electrical stimulation of the pedunculopontine tegmental nucleus, which projects glutamatergic inputs to the VTA (Zweifel et al., 2009). Ketamine-induced VTA neuronal activation *in vivo* has been shown to be glutamate-dependent, since administration of the AMPA receptor antagonist NBQX blocked this effect of ketamine (Witkin et al., 2016). Moreover, indirect modulation of the DAergic neurotransmission in other brain regions by ketamine might also exist. The results of previous studies indicate that systemic subanesthetic doses of ketamine increase both DA and glutamate release in the prefrontal cortex as measured by microdialysis or DA turnover quantification (Rao et al., 1989; Verma and Moghaddam, 1996; Lindefors et al., 1997; Moghaddam et al., 1997; Lorrain et al., 2003). Stereoselective effects of (*S*)- and (*R*)-ketamine on increased electrically-evoked DA release in the caudate putamen and bed nucleus of stria terminalis in rat brain slices have also been reported (Tso et al., 2004). However, this occurred at a ketamine concentration of 100 μM, much higher than concentrations (<10nmol/g) found in the brain following an antidepressant effective dose (Figure 1).

Other NMDAR antagonists have been shown to modulate the midbrain DAergic system as well. In particular, systemic administration of MK801, a selective non-competitive NMDAR

antagonist increases extracellular levels of DA and DA metabolism in the striatum and prefrontal cortex (Liljequist et al., 1991; Loscher et al., 1991; Bristow et al., 1993; Wolf et al., 1993). Similarly, phencyclidine (PCP), another non-competitive NMDAR antagonist increases extracellular levels of DA or DA metabolism in the NAc, amygdala and prefrontal cortex (Rao et al., 1989; Bristow et al., 1993; Hondo et al., 1994). Although these findings show that inhibition of the NMDAR induces changes in the DA system, they do not clarify whether these effects are due to indirect effects of NMDAR antagonists via glutamatergic signaling or direct effects over DA receptors and transporter. For example, Carlsson and Carlsson, (1989) showed that MK801-induced hyperlocomotion is not affected by monoamine depletion in mice, indicating that this behavioral response does not depend on the DAergic system. We also note that haloperidol did not reduce ketamine-induced perceptual changes, negative symptoms, or euphoria in healthy subjects (Krystal et al., 1999).

In addition to NMDAR activity, ketamine has reported effects on other receptors including μ-opioid receptors (K_i=26.8μM), σ-opioid receptors (K_i=26.8μM), κ-opioid receptors (K_i=85.2μM), δ-opioid receptors (K_i=101μM) (Smith et al., 1987), M_1 muscarinic receptors (K_i=200μM; (Durieux, 1995)), serotonin 5-HT_2 receptors (K_i=15μM; (Kapur and Seeman, 2002)) and nicotinic receptors (Yamakura et al., 2000; Weber et al., 2005; Moaddel et al., 2013). While the binding or uptake of ketamine, or its metabolites, to these membrane constituents might contribute to its clinical profile (Mathew et al., 2012), these K_i values are higher than peak concentrations found in the brain or plasma (~8 nmol/g or 8 nmol/ml respectively) following an antidepressant effective dose (Figure 1).

These results extend our knowledge of ketamine's actions on mesolimbic DA release and highlight the need for the study of potential indirect interactions between glutamatergic and

DAergic neurotransmitter systems in order to obtain a complete picture of ketamine's actions relevant to both therapeutic actions and side effects (see (Grace, 2016) for a recent review).

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FOOTNOTES

A. Can and P. Zanos contributed equally to the manuscript

Ruin Moaddel and Irving W. Wainer are listed as co-inventors on a patent for the use of (*2R,6R*)-hydroxynorketamine, (*S*)-dehydronorketamine and other stereoisomeric dehydro- and hydroxylated metabolites of (*R,S*)-ketamine metabolites in the treatment of depression and neuropathic pain. Panoz Zanos, Ruin Moaddel, Irving W. Wainer, and Todd D. Gould are listed as co-inventors on a patent application for the use of (*2R,6R*)-hydroxynorketamine and (*2S,6S*)-hydroxynorketamine in the treatment of depression, anxiety, anhedonia, suicidal ideation and post-traumatic stress disorders. Ruin Moaddel and Irving W. Wainer have assigned their patent rights to the U.S. government but will share a percentage of any royalties that may be received by the government. Panoz Zanos and Todd D. Gould have assigned their patent rights to the University of Maryland Baltimore but will share a percentage of any royalties that may be received by the University of Maryland Baltimore. All other authors declare no competing interests.

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LEGENDS FOR FIGURES

Figure 1. ***Plasma and brain concentrations of ketamine and its metabolites following systemic ketamine administration.*** Representative chromatograms from the 10-minute time point from **(A)** plasma and **(C)** brain. Concentration *versus* time relationship for **(B)** Plasma and **(D)** brain tissue concentrations of (*R,S*)-ketamine, (*R,S*)-norketamine, (*R,S*)-dehydronorketamine, (*2S,6R;2R,6R*)-hydroxynorketamine, and (*2S,6S;2R,6R*)-hydroxynorktamine after intraperitoneal administration of (*R,S*)-ketamine (10 mg/kg). The measured analyte concentrations in the brain were normalized according to tissue weight and are reported as umol/g of tissue. Data are the mean ± SEM (n=4/time point). *Abbreviations*: KET, ketamine; DHNK, dehydronorketamine; HNK, hydroxynorketamine.

Figure 2. ***Representative fast scan cyclic voltammetry recordings and color plots from each treatment group***. Mice received saline, ketamine (2, 10, or 50 mg/kg), or quinpirole (0.5 mg/kg). Electrical stimulation (60Hz, 300 µA, 1sec, biphasic, 2ms/phase) was applied to the VTA at every three minutes. Black trace and upper color plot show a representative baseline recording from each group. Red trace and the color plots in the lower panel show the last recording from the same animal at the 30th minute after the drug treatment. Time scale on the X-axis of the first row also applies to X-axis of the color plots. For the color plots the Y-Axis indicates applied potential. The pseudo-color plot scale (Z-axis) indicates the percentage change from the baseline DA concentration, where DA signals are represented by the color change in the approximate center (~0.6 V) of the rising phase of the voltage ramp. *Abbreviations*: KET, ketamine; QNP, quinpirole.

Figure 3. ***Effects of ketamine on parameters of dopamine release*** **(A)** Percentage changes in the $[DA]_{max}$ of stimulation-evoked DA release at each stimulation after the drug treatment (pre-

drug baseline=100). **(B)** Percentage changes in rise time (the amount of time that it takes to reach peak DA concentrations) at each stimulation during the release of DA (pre-drug baseline=100). **(C)** Percentage changes in decay constants (tau) values at each stimulation during the falling phase of evoked DA (pre-drug baseline=100). Two-way repeated measures ANOVA; *p<0.05, **p<0.01 compared to the saline group, Holm-Šídák *post hoc* test. Data are the mean ± SEM (n=4-8/group in each experiment). *Abbreviations:* KET, ketamine; QNP, quinpirole; $[DA]_{max}$, peak amplitude of evoked DA.

Figure 4. Functional agonist activity of ketamine and ketamine metabolites at dopamine D_{1-5} receptors compared to positive control compounds. Functional assays of (*S*)-ketamine, (*R*)-ketamine, (*S*)-norketamine, (*R*)-norketamine, (*S*)-DHNK, (*R*)-DHNK, (*2S,6S*)-HNK, (*2R,6R*)-HNK, (*2R,6R*)-HNK and (*2S,6R*)-HNK at DA receptors D_{1-5} indicated lack of agonist action. Positive control data for each experiment are indicated by colored lines. Data are the mean ± SEM (n=9; 3 independent experiments and 3 replicates per experiment). *Abbreviations*: DHNK, dehydronorketamine; HNK, hydroxynorketamine; KET, ketamine; M, Molar.

Figure 5. Functional antagonist activity of ketamine and ketamine metabolites at dopamine D_{1-5} receptors compared to positive control compounds. Functional assays of (S)-ketamine, (R)-ketamine, (S)-norketamine, (R)-norketamine, (S)-DHNK, (R)-DHNK, (2S,6S)-HNK, (2R,6R)-HNK, (2R,6R)-HNK and (2S,6R)-HNK at DA receptors D_{1-5} indicated lack of antagonistic action. Data are the mean ± SEM (n=9; 3 independent experiments and 3 replicates per experiment). *Abbreviations*: DHNK, dehydronorketamine; HNK, hydroxynorketamine; KET, ketamine; M, Molar.

Figure 6. Competition binding profiles for ketamine's enantiomers and metabolites against the dopamine (DAT), norepinephrine (NET) and serotonin transporters (SERT). Binding displacement by (*S*)-ketamine, (*R*)-ketamine, (*S*)-norketamine, (*R*)-norketamine, (*S*)-DHNK, (*R*)-DHNK, (*2S,6S*)-HNK, (*2R,6R*)-HNK, (*2R,6R*)-HNK and (*2S,6R*)-HNK was determined at DAT using ^{3}H-Win35428 with GBR12909 as a reference ligand, at NET using ^{3}H-Nisoxetine with Desipramine as a reference ligand, and at SERT using ^{3}H-Citalopram with Amitriptyline as a reference ligand. Data are the mean ± SEM (n≥9; ≥3 independent experiments and 3-4 replicates per experiment). *Abbreviations*: DHNK, dehydronorketamine; HNK, hydroxynorketamine; KET, ketamine; M, Molar.

Figure 7. ***Functional activity of ketamine and ketamine metabolites at monoamine transporters compared to positive control compounds.*** Functional assays of (S)-ketamine, (R)-ketamine, (S)-norketamine, (R)-norketamine, (S)-DHNK, (R)-DHNK, (2S,6S)-HNK, (2R,6R)-HNK, (2R,6S)-HNK and (2S,6R)-HNK at DAT, NET and SERT indicated lack of inhibitory activity. Positive control data for each experiment are indicated by colored lines. Data are the mean ± SEM (n=11; 3 independent experiments and 3-4 replicates per experiment). *Abbreviations*: DHNK, dehydronorketamine; HNK, hydroxynorketamine; KET, ketamine; M, Molar. .

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TABLES

Table 1. Binding Potency of ketamine's enantiomers and metabolites at dopamine receptors.

	(S)-KET	(R)-KET	(S)-norKET	(R)-norKET	(S)-DHNK	(R)-DHNK	(2S,6S)-HNK	(2R,6R)-HNK	(2R,6S)-HNK	(2S,6R)-HNK
Target	*% Inhibition*									
D_1R	*12%*	*4%*	*0%*	*7%*	*0%*	*0%*	*9%*	*0%*	*26%*	*0%*
D_2R	*1%*	*12%*	*0%*	*2%*	*5%*	*17%*	*0%*	*0%*	*9%*	*0%*
D_3R	*10%*	*8%*	*0%*	*2%*	*0%*	*3%*	*0%*	*0%*	*0%*	*0%*
D_4R	*4%*	*0%*	*0%*	*0%*	*8%*	*0%*	*0%*	*0%*	*6%*	*0%*
D_5R	*0%*	*0%*	*0%*	*0%*	*0%*	*0%*	*0%*	*0%*	*4%*	*2%*

% values are the mean of 2-5 experiments, with 4 replicates each, and were derived from a single concentration (10μM). Negative values are represented as 0%. *Abbreviations*: D_1R, dopamine receptor D_1; D_2R, dopamine receptor D_2; D_3R, dopamine receptor D_3; D_4R, dopamine receptor D_4; D_5R, dopamine receptor D_5; DHNK, dehydronorketamine; HNK, hydroxynorketamine; KET, ketamine; norKET, norketamine

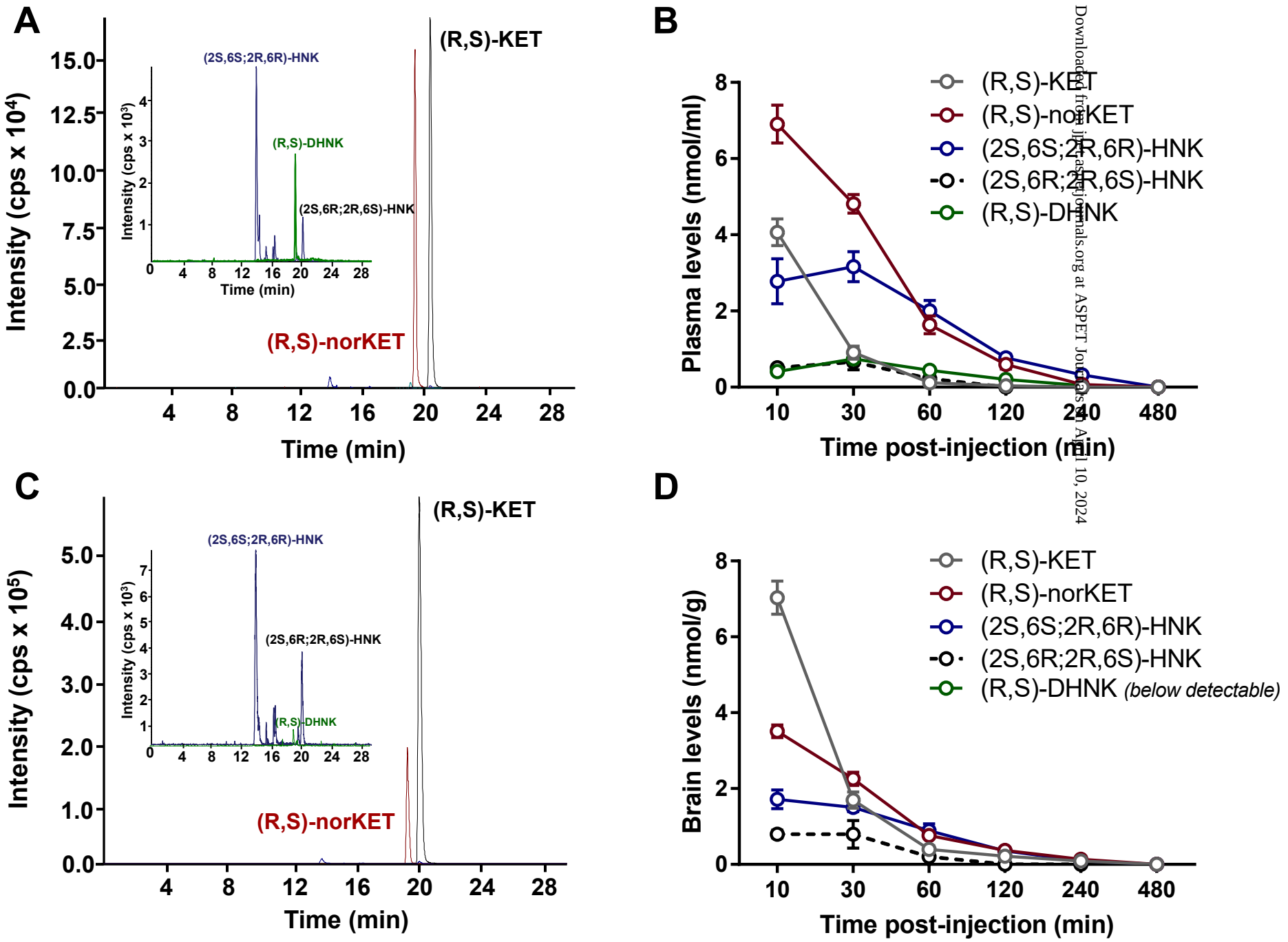

Figure 1

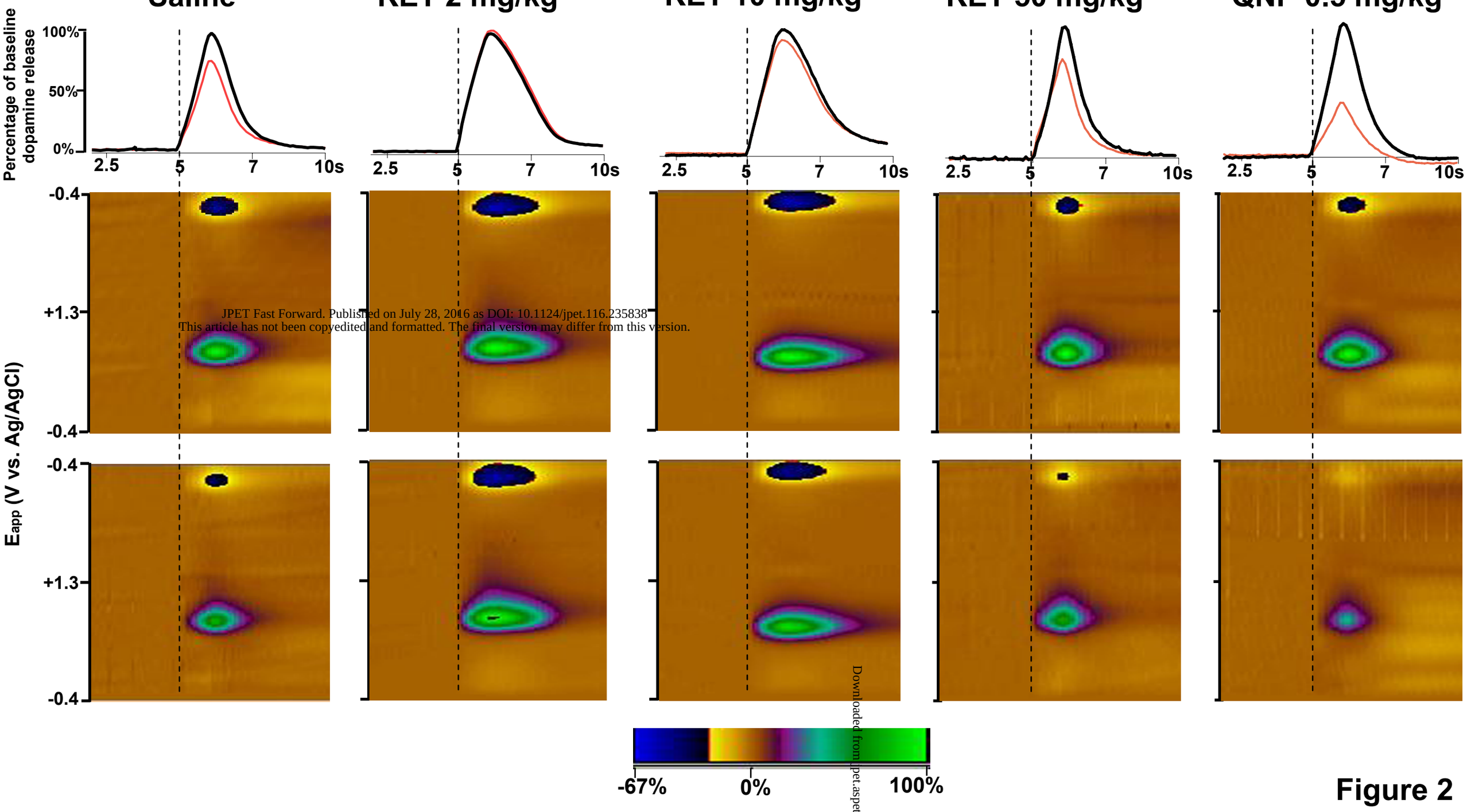

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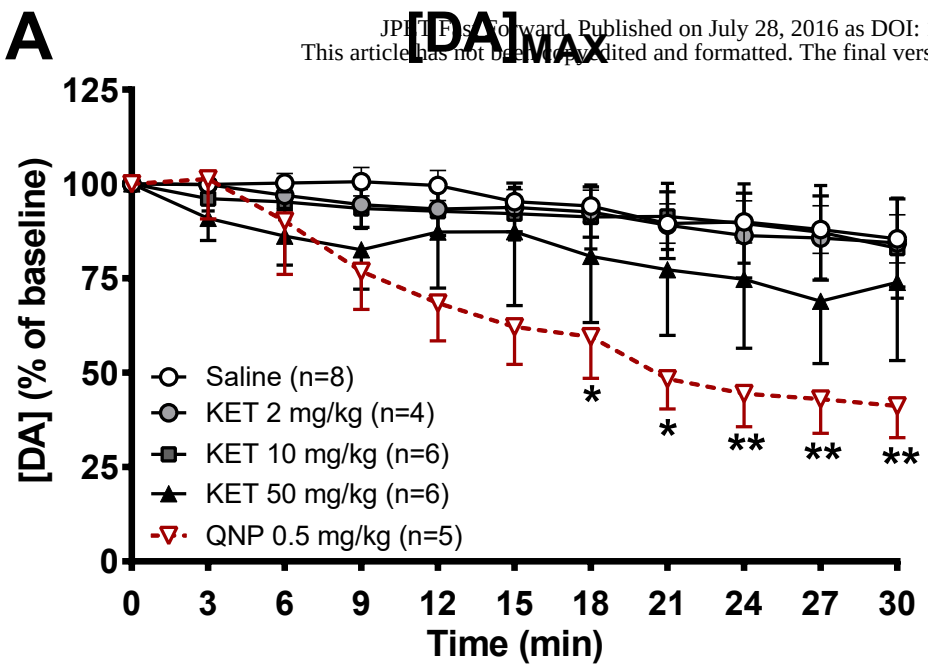
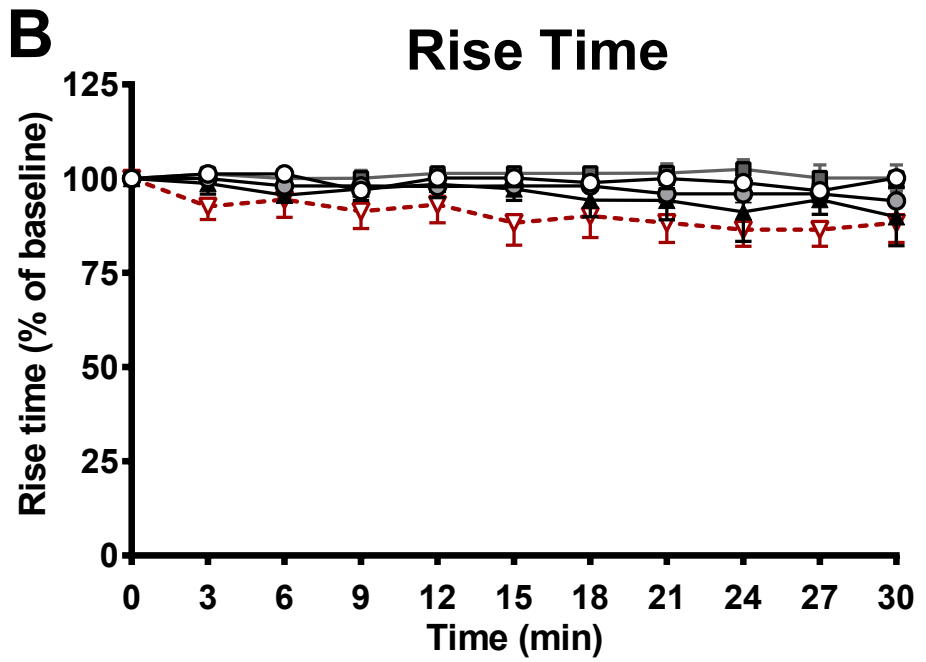
Figure 2





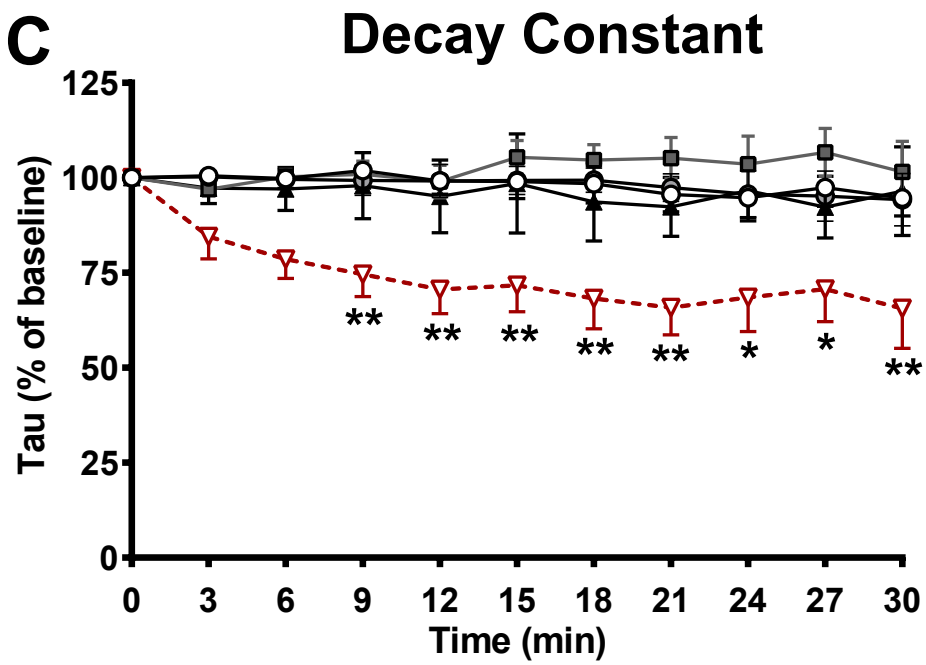


Figure 3

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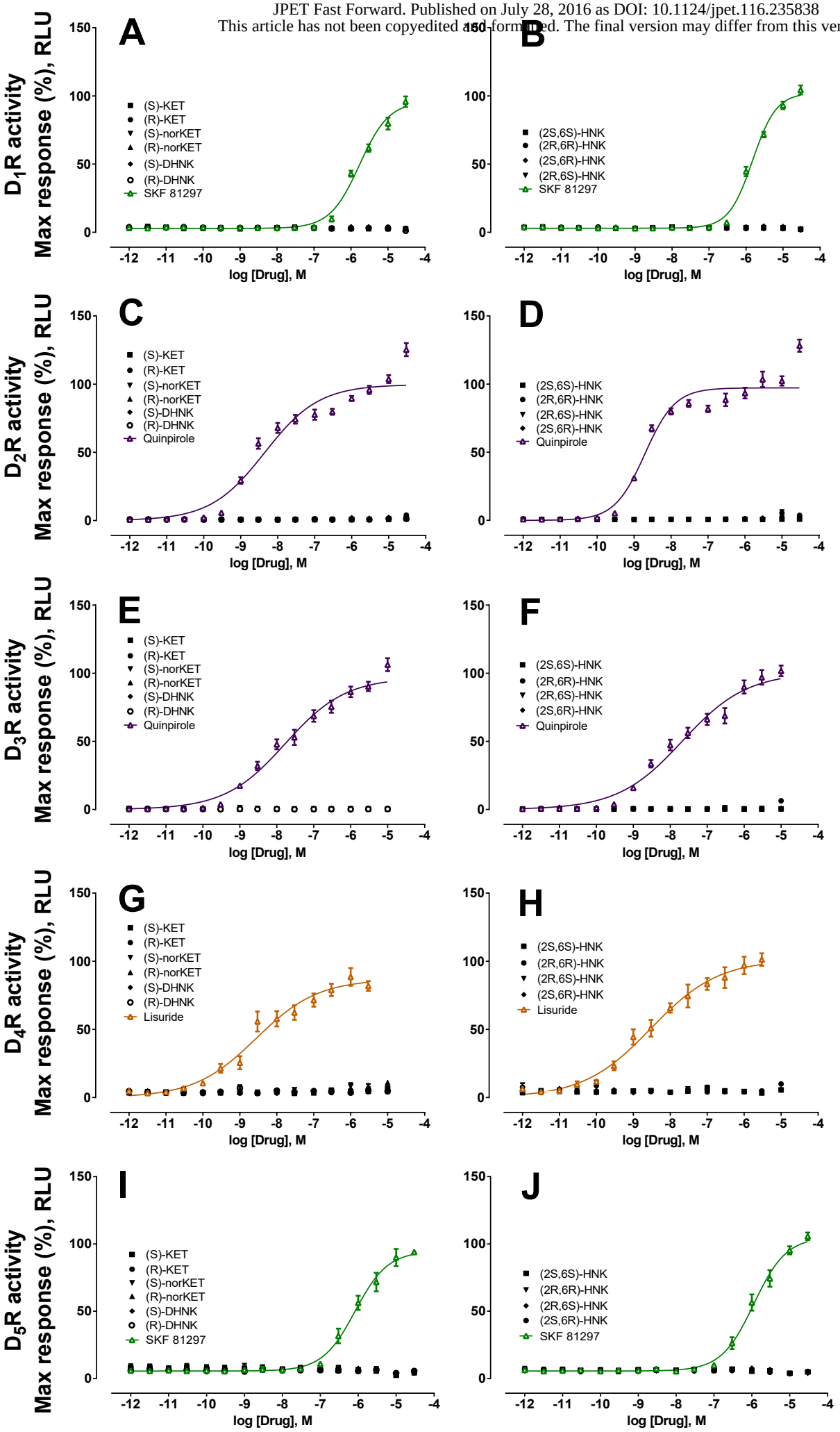

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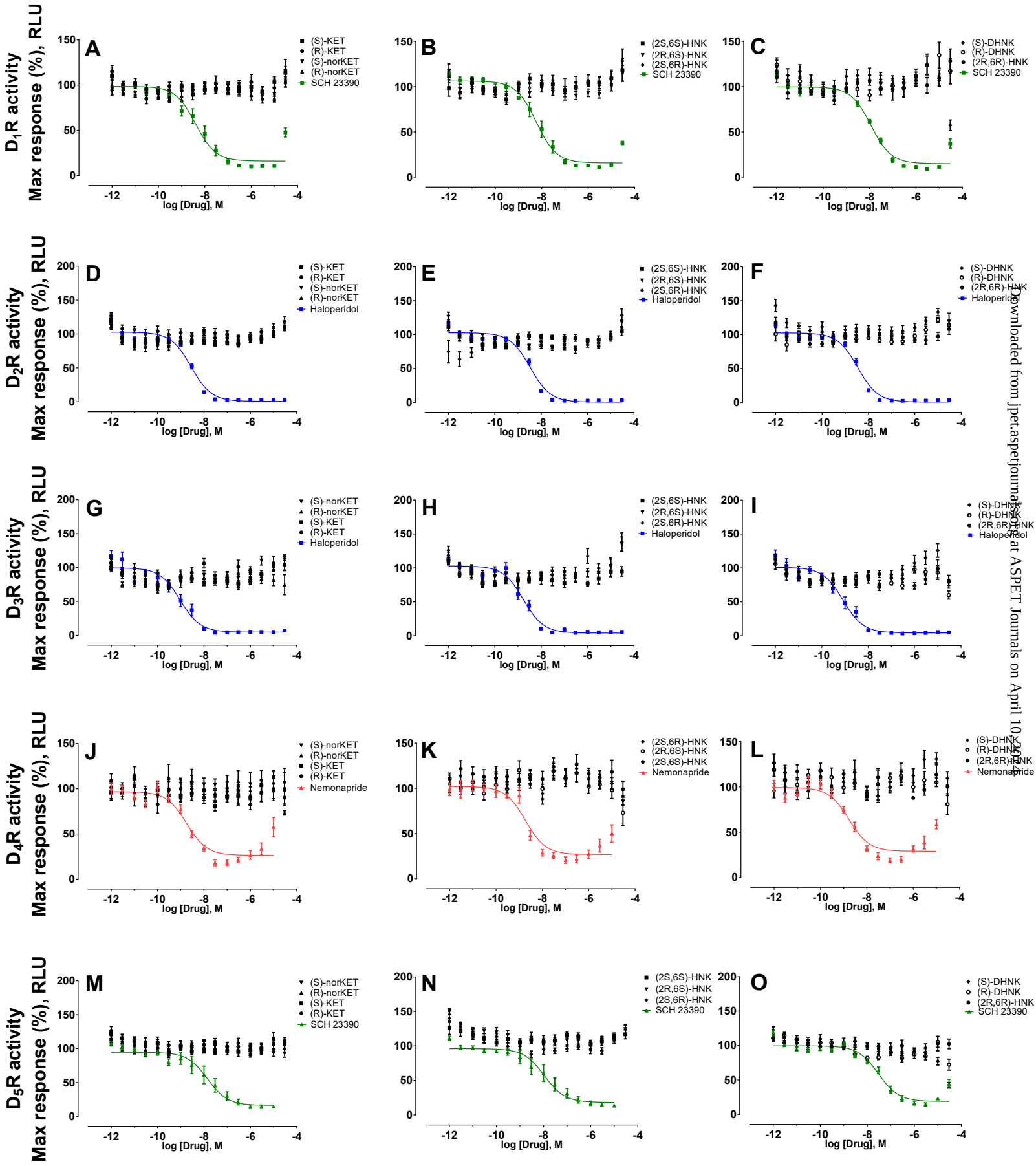
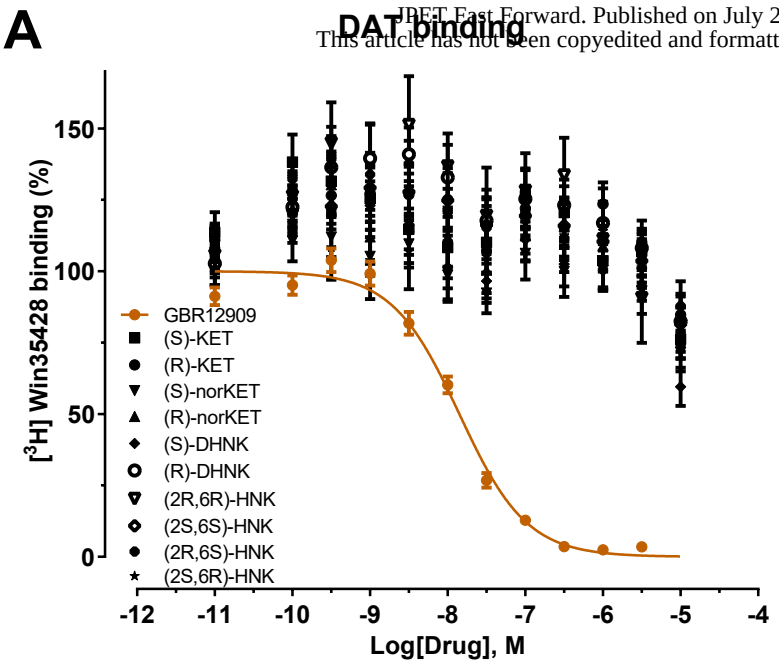
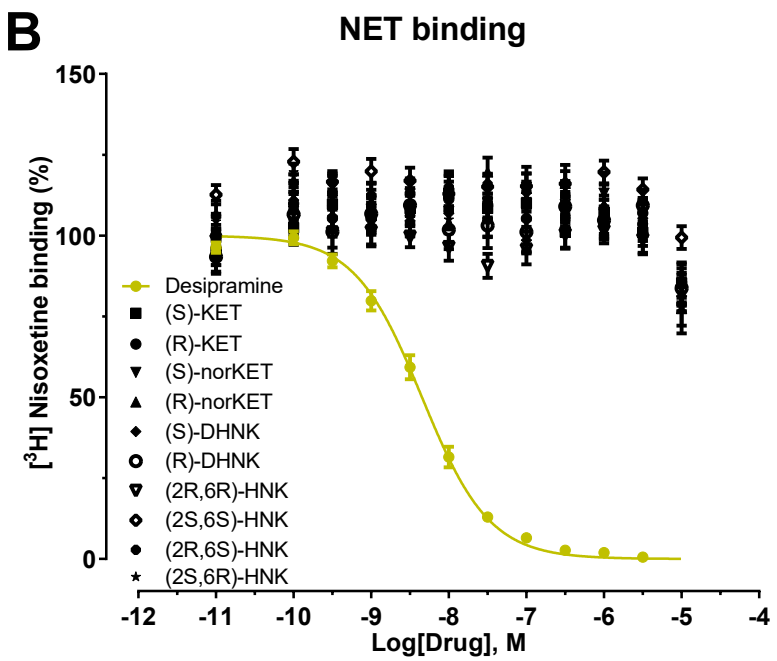


Figure 5





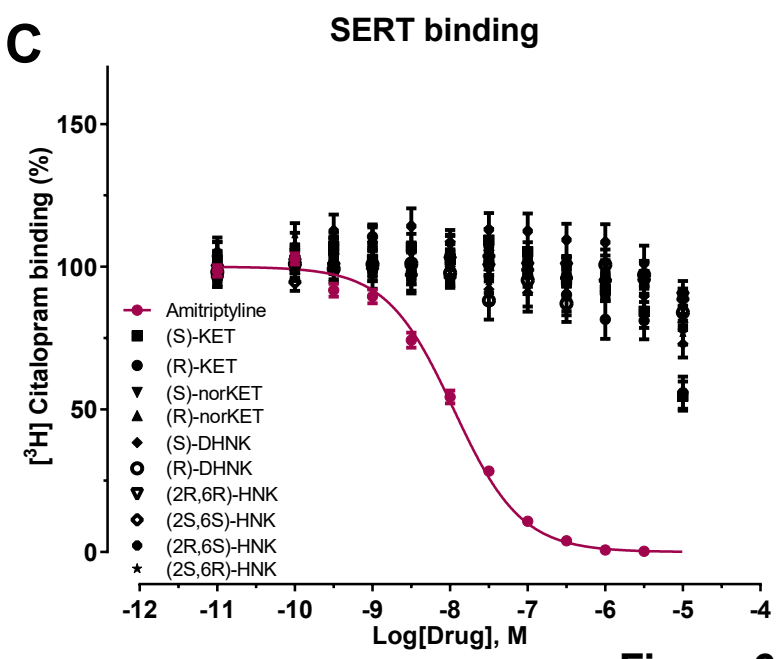


Figure 6

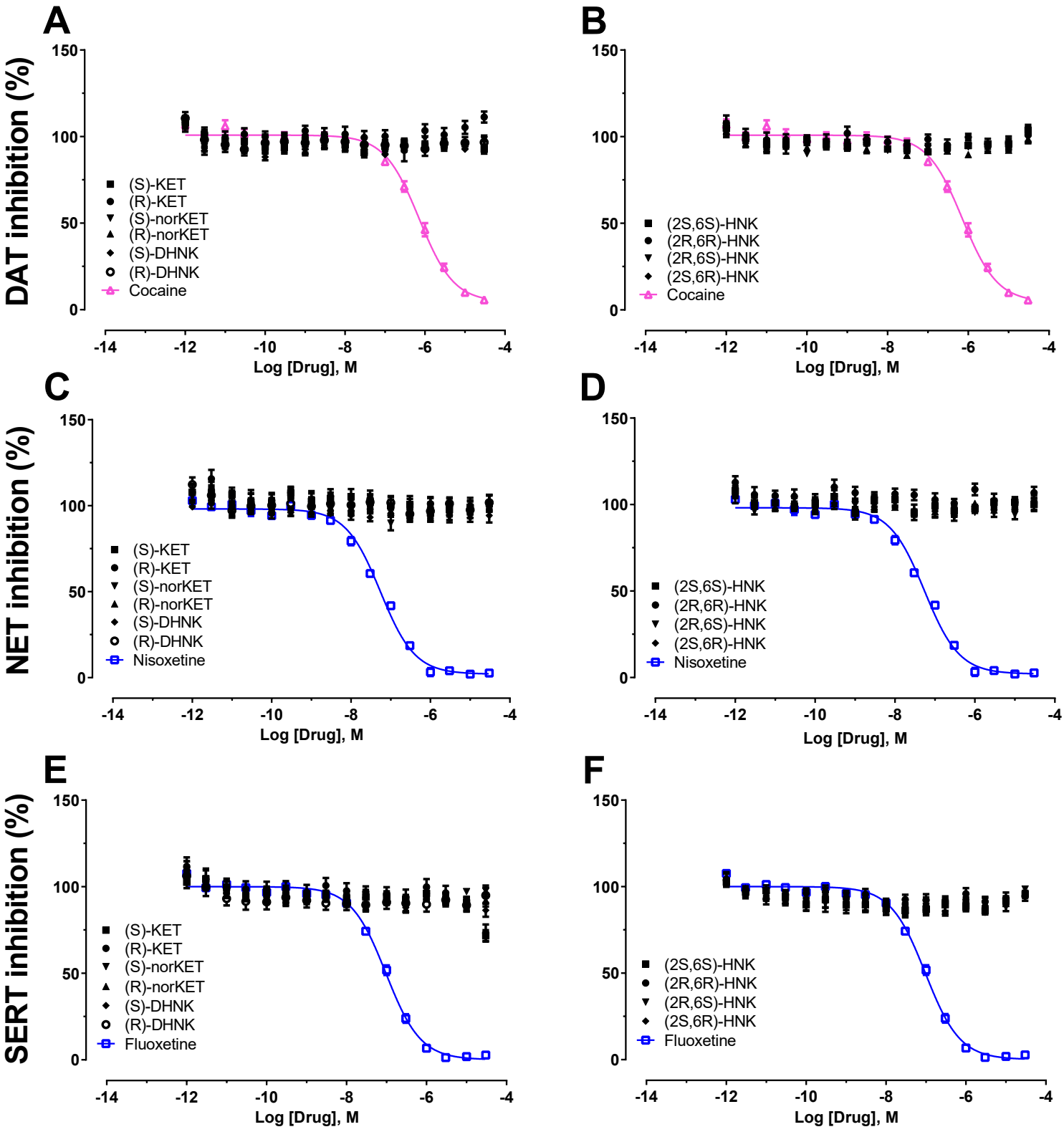


Figure 7