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The long-lasting antidepressant effects of rapastinel (GLYX-13) are associated with a metaplasticity process in the medial prefrontal cortex and hippocampus

Jeffrey Burgdorf[1], **Xiao-lei Zhang**[2], **Craig Weiss**[3], **Amanda Gross**[4], **Steven R. Boikess**[5], **Roger A. Kroes**[4], **M. Amin Khan**[4], **Ronald M. Burch**[4], **Christopher S. Rex**[5], **John F. Disterhoft**[3], **Patric K. Stanton**[2], and **Joseph R. Moskal**[1,4,*]

[1]Falk Center for Molecular Therapeutics, Department of Biomedical Engineering, McCormick School of Engineering, Northwestern University, 1801 Maple Ave., Suite 4300, Evanston IL, 60201, USA

[2]Department of Cell Biology & Anatomy, Basic Sciences Bldg., Rm. 217, New York Medical College, Valhalla, NY 10595, USA

[3]Department of Physiology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Ward Building 7-140, Chicago, IL 6061, USA

[4]Naurex Inc., 1801 Maple Ave., Suite 4300, Evanston IL, 60201, USA

[5]Afraxis Inc. 11099 N. Torrey Pines Rd., Suite 290, La Jolla, CA 92037, USA

Abstract

Rapastinel (GLYX-13) is an N-methyl-D-aspartate receptor (NMDAR) modulator that has characteristics of a glycine site partial agonist. Rapastinel is a robust cognitive enhancer and facilitates hippocampal long-term potentiation (LTP) of synaptic transmission in slices. In human clinical trials, rapastinel has been shown to produce marked antidepressant properties that last for at least one week following a single dose. The long-lasting antidepressant effect of a single dose of rapastinel (3 mg/kg IV) was assessed in rats using the Porsolt, open field and ultrasonic vocalization assays. Cognitive enhancement was examined using the Morris water maze, positive emotional learning, and contextual fear extinction tests. LTP was assessed in hippocampal slices. Dendritic spine morphology was measured in the dentate gyrus and the medial prefrontal cortex. Significant antidepressant-like or cognitive enhancing effects were observed that lasted for at least one week in each model. Rapastinel facilitated LTP 1 day – 2 weeks but not 4 weeks post-dosing. Biweekly dosing with rapastinel sustained this effect for at least 8 weeks. A single dose of rapastinel increased the proportion of whole-cell NMDAR current contributed by NR2B-containing NMDARs in the hippocampus 1 week post-dosing, that returned to baseline by 4 weeks postdosing. The NMDAR antagonist CPP blocked the antidepressant-like effect of rapastinel 1

*Corresponding author. Address: Falk Center for Molecular Therapeutics, Northwestern University, 1801 Maple Ave., Suite 4300, Evanston, IL, 60201. Tel: +1-847-491-7438; fax +1-847-491-481; j-moskal@northwestern.edu.

week post dosing. A single injection of rapastinel also increased mature spine density in both brain regions 24 hrs post-dosing. These data demonstrate that rapastinel produces its long-lasting antidepressant effects via triggering NMDAR-dependent processes that lead to increased sensitivity to LTP that persist for up to two weeks. The data also suggest that these processes led to the alterations in dendritic spine morphologies associated with the maintenance of long-term changes in synaptic plasticity associated with learning and memory.

Keywords

NMDA Receptor; GLYX-13; Depression; LTP; Medial Prefrontal Cortex; Hippocampus

INTRODUCTION

NMDA receptors have recently become targets for drug development for the treatment of depression (Danysz and Parsons, 1998, Machado-Vieira et al., 2009, Skolnick et al., 2009, Krystal et al., 2013, Martinowich et al., 2013). *In vivo* imaging studies show decreased glutamate levels in the prefrontal cortex/anterior cortex of depressed patients (Hasler et al., 2007, Luykx et al., 2012) and postmortem data suggest that NMDAR protein levels are altered in the prefrontal cortex of depressed patients (Feyissa et al., 2009). Ketamine, a potent NMDA channel blocker, has been found to reduce depression scores in subjects with major depressive disorder (MDD) following a single dose (Berman et al., 2000, Zarate et al., 2006, aan het Rot et al., 2010, Martinowich et al., 2013), with efficacy apparent within a few hours and lasting approximately 1–2 weeks following a single dose (Berman et al., 2000, Zarate et al., 2006, aan het Rot et al., 2010). Similarly, CP-101,606, an NR2B subunit-selective NMDAR antagonist, reduced depression scores in another study, but was also associated with ketamine-like psychotomimetic side effects, though these were reduced when the dose level of CP-101,606 was reduced (Preskorn et al., 2008). Another NR2B-selective antagonist, MK-0657, also reduced secondary efficacy measures of depression by day 5 of oral administration in 5 subjects, reportedly without causing psychotomimetic side effects (Ibrahim et al., 2012). Lanicemine, a low-trapping NMDA channel blocker produces sustained antidepressant effects but with psychotomimetic side effects (Sanacora et al., 2014).

Rapastinel, an NMDA receptor modulator with glycine-site partial agonist properties and is currently in a phase II clinical development program as an adjunctive therapy for major depressive disorder (clinicaltrials.gov identifier NCT01684163). A single dose of rapastinel produces a robust antidepressant response in humans that lasts at least 1 week post-dosing (Moskal et al., 2014), and repeat weekly or bi-weekly dosing with rapastinel maintains this effect and produces further gains without evidence of tachyphylaxis (Moskal et al., 2014).

Rapastinel was derived from cloning a hypervariable region of a monoclonal antibody that was shown to be a NMDAR-specific glycine site partial agonist (Moskal et al., 2005). In rat hippocampal slices, rapastinel has been shown to preferentially enhance conductance of NR2B-containing NMDARs at rat Schaffer collateral-CA1 synapses *in vitro* (Zhang et al., 2008), and enhance the magnitude of long-term potentiation (LTP) of synaptic transmission

while simultaneously reducing that of long-term depression (LTD), which differentiates rapastinel from other NMDAR modulators such as D-cycloserine (Zhang et al., 2008, Burgdorf et al., 2011c). In animal studies, rapastinel has been shown to enhance performance in a variety of hippocampal-dependent learning tasks, including trace eyeblink conditioning and the Morris water maze, in both young adult and learning-impaired aged rats (Burgdorf et al., 2011c) and produces an antidepressant-like effect in Porsolt, learned helplessness, and novelty-induced hypophagia (NIH) tests in rats, without ketamine-like dissociative, addictive or sedative side effects (Burgdorf et al., 2013). Rapastinel facilitates positive emotional learning (PEL) and produces antidepressant-like effects when injected directly into the infralimbic or prelimbic medial prefrontal cortex (MPFC), but not into dorsal-lateral control sites (primary/secondary motor cortex; see (Burgdorf et al., 2011a, Burgdorf et al., 2013).

The studies reported here were undertaken to determine if there is an association between metaplasticity and the long-lasting antidepressant effects in humans. The findings reported here showed that metaplasticity mechanisms associated with long term potentiation-like processes do play a key role in these processes and may be relevant for the long-lasting antidepressant effects observed with other NMDA receptor modulators as well (Burgdorf et al., 2013).

EXPERIMENTAL PROCEEDURES

Animals

Adult male (2–3 month old) Sprague-Dawley (SD) rats were purchased from Harlan (USA) for behavioral and dendritic spine morphology studies, or Charles River (USA) for electrophysiological studies. Rats were housed in lucite cages with aspen wood chip bedding, maintained on a 12:12 light:dark cycle (lights on at 5 AM), and given *ad libitum* access to Purina lab chow (USA) and tap water throughout the study. All experiments were approved by the Northwestern University or New York Medical College Animal Care and Use Committees.

Drugs

Rapastinel was synthesized in free base form by Sai Life Sciences (India), and was administered in 1 ml/kg 0.9% sterile saline vehicle. The dose of 3 mg/kg IV for rapastinel was chosen because it was the optimal antidepressant dose in Porsolt testing based on a previous dose-response (1–56 mg/kg IV) study (Burgdorf et al., 2013). The NMDA receptor antagonist (±)-CPP was purchased from Sigma (USA), and was also administered in 1 ml/kg 0.9% sterile saline vehicle. The dose of CPP (10 mg/kg IP) was chosen based on a previous report that this dose could block the antidepressant-like effects of a putative NMDAR modulator in the Porsolt test without exhibiting behavioral effects on its own in (Zhang et al., 2013).

Porsolt Test

The Porsolt forced swim test adapted for use in rats was performed as previously described (Page et al., 1999, Burgdorf et al., 2009). Animals were placed in a 46 cm tall × 20 cm in

diameter clear glass tube filled to 30 cm with tap water (23 ± 1 °C) for 15 min on the first day (habituation) and 5 min on the subsequent test day. Water was changed after every other animal. Animals were videotaped, and floating time as defined as the minimal amount of effort required to keep the animals head above water was scored offline by a blind experimenter with high inter-rater reliability (Pearson's r > .9).

Animals were tested 1 week post-dosing with rapastinel (3 mg/kg IV) or 0.9% sterile saline (1 ml/kg) vehicle (Figure 1A), or received a dose of CPP (10 mg/kg IP) 1 hr before the 1 week test point (Figure 4B). Alternatively, animals received pre-treatment with CPP (10 mg/kg IP) 1 hr before rapastinel administration and were tested 1 hr after rapastinel administration (Results Section). The broad spectrum NMDAR glutamate site antagonist CPP was chosen for these studies because it does not produce an antidepressant response in the Porsolt test (Zhang et al., 2013) unlike the NMDAR channel blockers like ketamine, MK-801 or the NR2B-specific antagonist Ro25-6981 (Maeng et al., 2008, Burgdorf et al., 2013).

Open Field Test

Open field testing was performed as previously described (Burgdorf et al., 2009). Time spent in the open compartment has been shown to be increased by some classes of anxiolytic/antidepressant compounds (Prut and Belzung, 2003). Testing consisted of placing an animal in a 40 cm × 40 cm × 20 cm high opaque plexiglas open field cage divided into 9 equally sized 13.3 cm × 13.3 cm sections under red lighting for 10 min. Between animals, boli and urine were removed from the apparatus. Animals were videotaped, and line crosses and time spent in the center chamber were scored offline by a blind experimenter with high inter-rater reliability (Pearson's r > .9).

Animals were tested 1 week post-dosing with rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg).

Ultrasonic Vocalization Testing

Heterospecific rough-and-tumble play was conducted as previously described (Burgdorf et al., 2011a). Frequency modulated 50-kHz USVs and 20-kHz USVs are validated behavioral indexes of positive and negative affect, respectively (Burgdorf et al., 2011b). Twenty-kHz USVs model the positive symptoms of anxiety and depression and have been shown to be reduced by both antidepressant and anxiolytic drugs (Sanchez, 2003). In contrast, 50-kHz USVs model the negative-anhedonic symptoms of depression (Burgdorf et al., 2011b). Heterospecific rough-and-tumble play stimulation was administered by the experimenter's right hand. The experimenter was blind to the drug condition. Animals received 2–3 min of heterospecific rough-and-tumble play consisting of alternating 15 sec blocks of heterospecific play and 15 sec of no-stimulation. Animals were not habituated to play stimulation before dosing and testing, and using this paradigm we have shown that the increase in 50-kHz USVs that occur across trial blocks reflects positive emotional learning (Burgdorf et al., 2011a).

High frequency recordings of ultrasonic vocalizations were captured using a condenser microphone amplified by a bat detector (D980, Pettersson Elektronik, Sweden) and recorded

with a Fostex FR2 field recorder (192 kHz sampling rate, 24 bit) onto compact flash cards (SanDisk, USA) as .wav files, as described previously (Burgdorf et al., 2008). Ultrasonic vocalizations were scored manually in a blind manner from sonograms generated by Avisoft SAS Laboratory Pro (Germany). Hedonic 50-kHz USVs, defined as having a peak frequency of greater than 40-kHz and a bandwidth greater than 18-kHz, were scored along with 20-kHz USVs (peak frequency 20–25 kHz, duration greater than 100 ms) as described in (Burgdorf et al., 2008). High inter- and intra-rater reliability for these measures (Pearson's r>.90) has been established for this method (Burgdorf et al., 2008). Rates of hedonic 50-kHz USVs during the interstimulus interval were reported.

Animals were tested 1 week post-dosing with rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg).

Contextual Fear Conditioning Test

Contextual fear conditioning and extinction testing was conducted as previously described (Akirav et al., 2009), and the first extinction tests occurred 1 hr post-dosing. On the contextual fear training day (D0), animals were placed in a Coulbourn instruments (USA) shock chamber (40 × 40 × 40 cm) for 400 seconds and received three 0.5 mA 1 sec footshocks delivered to the floor bars at 90, 210, and 330 second timepoints. During extinction, rats were subjected to daily 5 min non-reinforced (no shock) extinction trials for 6 days after training. Freezing was quantified via FreezeFrame software (Actimetrics, USA); at baseline (30–60 sec) on D0, and during the last 3 min of each extinction trial.

Animals received a single dose of rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg) 24 hrs before the first extinction session.

Morris water maze testing

Methods were conducted as previously described (Burgdorf et al., 2009). Rats were tested for place learning in the hidden movable platform version of the Morris water maze (MWM). Each session consisted of four trials/session, with an inter-trial interval of 60 sec. Prior to the first training trial, rats were placed on the platform and allowed to remain there for 30 sec to familiarize themselves with its location. The platform was located in a novel location each test day, remaining constant over trials within a daily session. A matching to sample version of the MWM task was employed for this study because of the increased difficulty of this task over a standard reference memory MWM task. On each trial, rats were placed in the water, with their heads facing toward the tank wall, at one of four equally spaced positions around the tank (north, south, east, and west). Rats started from a different position each trial, and were allowed to swim until reaching the platform (10 cm square, submerged ~3 cm below the top of the water) and climbing on top, or until 60 sec had expired, at which time rats that did not find the platform were guided to it. Rats remained on the platform for 15 sec before being removed. The tank water was made opaque with a powdered, nontoxic white paint, and the tank water was maintained at 25±1°C. During testing, performance was recorded by a FC-82B video camera and wide-angle camera lens mounted above the center of the pool. The animal’s location in the tank was digitized with a VP200 tracker and data collected by HVS Water for Windows software from HVS Image

(United Kingdom). The tank (180 cm in diameter) was surrounded by curtains with detailed posters attached to them serving as extra-maze cues. The experimenter remained outside the curtains during trials and entered at the end of a trial to retrieve the rat. Placement of the extra-maze cues was consistent from day to day. Place learning was assessed by analyzing the distance in meters the animal traversed to locate the platform averaged across 4 trials for each of the consecutive 5 test days.

Animals received a single dose of rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg) 24 hrs before the first test session.

Electrophysiology

Animals were deeply anesthetized with isoflurane and decapitated. Brains were removed rapidly, submerged in ice-cold artificial cerebrospinal fluid (ACSF, 2–4 °C), which contained (in mM): 124 NaCl, 4 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose; at pH 7.4, gassed continuously with 95% O_2/5% CO_2). Brains were hemisected, the frontal lobes removed, and individual hemispheres glued using cyanoacrylate adhesive onto a stage immersed in ice-cold ACSF gassed continuously with 95% O_2/5% CO_2 during slicing. 400 μm thick coronal slices were cut using a Vibratome (Leica VT1200S), and transferred to an interface holding chamber for incubation at room temperature for a minimum of one hr before transferring to a Haas-style interface recording chamber continuously perfused at 3 ml/min with oxygenated ACSF at 32 ± 0.5°C.

For extracellular recordings, low resistance recording electrodes were made from thin-walled borosilicate glass (1–2 MΩ after filling with ACSF) and inserted into the apical dendritic region of the Schaffer collateral termination field in stratum radiatum of field CA1 region to record field excitatory postsynaptic potentials (fEPSPs). A bipolar stainless steel stimulating electrode (FHC Co.) was placed on Schaffer collateral-commissural fibers in CA3 stratum radiatum, and constant current stimulus intensity adjusted to evoke approximately halfmaximal fEPSPs once each 30 s (50–100 pA; 100 μs duration). fEPSP slope was measured before and after induction of LTP by linear interpolation from 20 to 80% of maximum negative deflection, and slopes confirmed to be stable to within ± 10% for at least 15 min before commencing an experiment. LTP was induced by stimulation of Schaffer collateral axons with four high frequency theta burst stimulus trains of 10 × 100 Hz/5 pulse bursts each, applied at an inter-burst interval of 200 ms. Each train was 2 s in duration, and trains were applied 15 s apart. Signals were recorded using a Multiclamp 700B amplifier and digitized with a Digidata 1322 (Axon Instruments, USA). Data were analyzed using pClamp software (version 9, Axon Instruments) on an IBM-compatible personal computer.

Whole-cell patch clamp recordings from CA1 pyramidal neurons were acquired as described previously (Burgdorf et al., 2013). Patch pipette resistance ranged from 6 to 6.5 MΩ when filled with intracellular solution that contained (in mM): 135 $CsMeSO_2$, 8 NaCl, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.3 Na-GTP, and 1 QX-314 [N-(2,6-dimethylphenylcarbamoylthyl)-triethylammonium bromide], 275 mOsm, pH 7.25 adjusted with $Cs(OH)_2$. CA1 pyramidal neurons were visualized by infrared imaging and patched using a 60× water-immerged objective mounted to a Zeiss microscope (Axioskop 2 Fs plus). After whole-cell voltage

clamp configuration was established, access resistance was carefully monitored, and only cells with stable access resistance (<5% change) were included in analyses. Excitatory postsynaptic currents (EPSCs) were recorded using a MultiClamp 700B (Molecular Devices, Union City, CA), with the low-pass filter setting at 1–3 kHz, series resistance was compensated in the voltage-clamp mode, and patched cells whose series resistance changed by >10% were rejected from analysis. Signals were filtered at 3 kHz and digitized at 10 kHz with a Digidata 1322A controlled by a Clampex (v9.2) (Molecular Devices, Union City, CA). A bipolar tungsten stimulating electrode (FHC, Bowdoin, ME) was placed in the Schaffer collateral-commissural fibers in CA3 stratum radiatum and stimulus pulses (800 μS duration) were delivered at 15–30 sec intervals. Neurons were voltage clamped at −70 mV to record EPSCs to assess input-output relations and paired-pulse facilitation. Neurons were clamped at −40 mV for recording NMDA currents, to relieve voltage-dependent magnesium block, and slices were perfused with ACSF containing 0 added magnesium, 3 mM calcium, 10 μM picrotoxin, and 10 μM CNQX, to isolate NMDA conductances.

All external recording pipette solutions were made with deionized distilled water (resistance > 18 MΩ cm^{-2}; Milli-Q system; Millipore, USA). Chemicals for making extra- and intracellular solutions were purchased from Sigma (USA) or Fluka (USA). Electrophysiological data were analyzed initially with Clampfit (v9) (Axon Instruments, USA), and further processed and presented with Origin 6.1 (Microcal Software, USA) and CorelDraw 10.0 (Corel, Canada) programs.

Animals received a single dose of rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg) 24 hrs, 1 week, 2 weeks, or 4 weeks before slice recordings. Alternatively, animals received repeated doses of rapastinel once every 2 weeks and tested 24 hrs after the final dose, or tested 4 weeks after the last dose.

Dendritic Spine Morphology Analysis

Tissue preparation—Animals were dosed with rapastinel (3 mg/kg IV) or 0.9% sterile saline vehicle (1 ml/kg), and 24 hrs post-dosing they were deeply anesthetized (isoflurane) and brains fixed via cardiac perfusion using 4% paraformaldehyde. Brains were stored in ice cold 0.1 M phosphate buffer and stored at 4°C until sectioning. Brains were sectioned using a tissue vibratome (Leica VT1000) to collect sections (300 μm thick) from the anterior to posterior extremes of each brain. Ballistic dye labeling (DiI and DiO; 3 mg dissolved in methylene chloride and coated on tungsten particles) was performed using a commercially available gene gun (Bio-Rad) to label target neurons. Thick sections were mounted to slides with raised barriers using ProLong Gold (Life Technologies) and cover slipped. Laser-scanning confocal microscopy (Olympus FV1000) was performed using a 63× objective (1.42 NA) to scan individually labeled neurons at high resolution (0.103 × 0.103 × 0.33 μm voxels). Target neurons were identified in the brain regions of interest by anatomical location and cell morphology. Microscopy was performed blind to experimental conditions. A minimum of 5 cells per animal were sampled from each of the two regions. For dentate granule neurons, samples (50 μm) were collected from primary dendrites starting at 100 μm from the soma. For mPFC, samples (50 μm) were collected from proximal tufts.

Dendritic spine analysis and assessment of dendritic membrane integrity— Blind deconvolution (AutoQuant) was applied to raw three-dimensional digital images which were then analyzed for spine density and morphology by trained analysts. Individual spines were measured manually for (a) head diameter, (b) spine length, and (c) spine neck diameter from image Z-stacks using software custom-designed by Afraxis Inc. (USA). Each dendrite was analyzed by 3–4 independent analysts. Automated image assignment software (C++) distributed images to analysts in a randomized manner and ensured that each analyst performed measurements of near equal numbers of dendrites per group. Analysts were blinded to all experimental conditions (including treatment, brain region, and cell type). Statistical analysis of interanalyst variability for each dendrite was examined on-line and used to eliminate dendrites that did not meet interanalyst reliability criteria. For spine density and spine morphological classification, data across analysts were averaged to report data for each dendrite. Identification, measurement and classification of dendritic spines were performed as previously described (Ota et al., 2014). For the purposes of the current study, we have denoted thin spines as “immature” to underscore their vulnerability to modification and role in recent encoding processes relative to more “mature” classes (i.e. mushroom and stubby classes) which are more closely associated with long-term memory storage (Young et al., 2014). Data population values (N’s) were reported for dendrites collected equally from all rats.

RESULTS

As shown in figure 1, rapastinel produced an antidepressant like effect in the USVs test, as indexed by an increase in hedonic 50-kHz USVs [$F(1, 20) = 12.4$, $P < .05$] and a decrease in aversive 20-kHz USVs [$F(1, 20) = 6.8$, $P < .05$]. Rapastinel also produced an anxiolytic effect in the open field, as indexed by increased center time [$F(1, 20) = 19.2$, $P < .05$] without altering locomotor activity as measured by line crosses [$F(1, 20) = 0.0$, $P > .05$].

As shown in figure 2, rapastinel increased learning and memory in: the spontaneous alternation test, as indexed by increased proportion of alternating trials [$F(1, 17) = 9.7$, $P < .05$]; the positive emotional learning tests, as indexed by increases in hedonic USVs across trial blocks to a conditioned stimulus that predicts play [$F(1, 21) = 23.9$, $P < .05$]; the Morris water maze test, as measured by mean path length to find the hidden platform [$F(1, 14) = 23.8$, $P < .05$]; and the contextual fear conditioning test, as measured by freezing behavior in an environment previously paired with footshock [$F(1, 22) = 12.9$, $P < .05$].

As shown in figure 3, a single dose of rapastinel (24 hrs & 1 week post dosing) significantly enhanced the magnitude of long-term potentiation (LTP) of synaptic transmission in the hippocampus compared to vehicle treated controls [$F(4, 32) = 7.6$, $P < .05$; Fisher’s PLSD post hoc test vehicle vs. 24 hr rapastinel or 1 week rapastinel groups, $P < .05$; Figure 3A–D], and repeated doses of rapastinel once every two weeks facilitated LTP 24 hrs but not 4 weeks after the final dose [$F(4, 32) = 7.1$, $P < .05$; Fisher’s PLSD post hoc test Vehicle vs. 1–4 repeat doses 24 hrs the last dose, $P < .05$; Figure 3E–H].

As shown in figure 4A, a single dose of rapastinel increased the proportion of whole-cell NMDAR current elicited by NR2B-containing NMDAR in CA1 pyramidal neurons 1 week,

but not 4 weeks, post-dosing [$F(2,21) = 4.7$, $P < .05$; Fisher's PLSD post hoc test 1 week vs. all other groups, $P < .05$]. In contrast, total NR2A + NR2B current was not altered [$F(2,17) = 0.3$, $P > .05$; data not shown), indicating that NR2B-containing NMDAR were selectively upregulated. As shown in figure 4B, a silent dose of the NMDA receptor antagonist CPP blocked the antidepressant-like effect of rapastinel 1 week post rapastinel dosing as measured by floating time in the Porsolt forced swim test [$F(3, 32) = 132.5$, $P < .05$; Fisher's PLSD post hoc test rapastinel alone vs. all other groups, $P < .05$]. In addition, pretreatment with CPP (120 min before testing) blocked the acute antidepressant-like effect of rapastinel administered 60 min before testing [$F(3, 20) = 88.2$, $P < .05$; Fisher's PLSD post hoc test rapastinel alone vs. all other groups, $P < .05$; data not shown].

As shown in figure 5, rapastinel increased the proportion of mature spines [$F(1, 55) = 17.6$, $P < .05$; Fisher's PLSD post hoc test rapastinel vs. vehicle for both MPFC and DG] and the density of stubby spines [$F(1, 55) = 28.0$, $P < .05$; Fisher's PLSD post hoc test rapastinel vs. vehicle for both MPFC and DG] in both the distal dentate gyrus primary apical dendrites (100–150 μM from the soma) and MPFC layer V pyramidal neuron proximal tufts. Rapastinel decreased the proportion of immature spines [$F(1, 55) = 9.2$, $P < .05$; Fisher's PLSD post hoc test rapastinel vs. vehicle for DG] and density of thin spines [$F(1, 55) = 7.0$, $P < .05$; Fisher's PLSD post hoc test rapastinel vs. vehicle for DG] only in the dentate gyrus. Rapastinel did not alter the density of mushroom spines [$F(1, 55) = 0.9$, $P > .05$] or total spine density [$F(1, 55) = 0.2$, $P > .05$] in either region. We identified ~70% of total spines to be thin, a value that falls within the typical range observed for mPFC in adult rats (Dumitriu et al., 2011).

DISCUSSION

The studies presented here were undertaken to further elucidate the mechanisms responsible for the long lasting antidepressant effects seen in humans after a single dose of rapastinel. In each of the seven different paradigms tested, rapastinel was found to have a significant effect that lasted for at least one week. These included tests associated with depression/anxiety including the forced swim, open field, and ultrasonic vocalization tests in which rapastinel had a robust antidepressant-like and/or anxiolytic-like effect as well as in tests associated with learning, including alternating + maze, positive emotional learning, Morris water maze and contextual fear extinction. Interestingly, the dose of rapastinel found to give optimal antidepressant- /anxiolytic-like effects (3 mg/kg IV) was also found to produce optimal cognitive enhancement. This suggests a mechanistic link between the long-lasting antidepressant-like effects of rapastinel and its cognitive enhancing effects and lends support to the idea that the antidepressant- /anxiolytic-like effects of rapastinel operate through a mechanism shared with the mechanisms associated with learning and memory.

Since rapastinel is an NMDA receptor modulator, it is also reasonable to assume that its long-lasting antidepressant- /anxiolytic-like effects involve an NMDAR mediated process akin to LTP; particularly since we have previously reported that rapastinel does in fact enhance the magnitude of LTP in rat hippocampal slice preparations (Zhang et al., 2008), and that the NMDAR-specific, glutamate site antagonist CPP also prevented the antidepressant-like effects of rapastinel. It should also be noted that the increase in the

fraction of mature dendritic spines and stubby spine densities in the dentate gyrus and in layer V of the medial prefrontal cortex as well as the increase in NMDA receptor expression seen at 24 hrs have been shown to be causally linked to LTP formation (Bosch and Hayashi, 2012, Burgdorf et al., 2013, Bosch et al., 2014). Mushroom spines and stubby spines are both functionally classed as large, mature spines that express NMDAR-dependent Ca^{2+} conductances and activity-dependent structural stability, with stubby spines producing the greatest net influx of NMDAR dependent Ca^{2+} (Noguchi et al., 2005, Hasegawa et al., 2015).

Thus, the most appealing way to couch these data as a whole is in the framework of metaplasticity (Abraham and Bear, 1996). This the only model that posits that modulation of the ability to induce synaptic plasticity in an already established neural circuit can be affected by prior stimulation and is typically measured by changes in the threshold for induction of LTP or LTD (Abraham, 2008). Metaplasticity is now a well-established phenomenon having been shown in a variety of brain regions and induced by a variety of stimuli (Wexler and Stanton, 1993, Stanton, 1995, Abraham, 2008, Hulme et al., 2013). Of particular interest here, Richter-Levin and Maroun (Richter-Levin and Maroun, 2010) have characterized an NMDA receptor-dependent, emotionally modulated form of metaplasticity in the medial prefrontal cortex. This resonates very well with the studies reported here in that rapastinel treatment showed a long lasting behavioral effect in many models associated with synaptic plasticity as well as in physiology studies showing marked increases in the magnitude of LTP induced by a sub-maximal stimulus that lasted at least two weeks with a single dose of rapastinel and at least 8 weeks with repeat dosing. Rapastinel has also been shown to produce a long-lasting facilitation of LTP in the MPFC (Burgdorf et al., 2015). In addition to producing a long-lasting selective increase in the contribution of NR2B-containing NMDAR to total NMDA current, rapastinel also produced a long-lasting increase in both NR2B NMDAR and GLUR1 AMPAR cell surface expression, with both receptors necessary for long-lasting behavioral effects of rapastinel (Burgdorf et al., 2013). In contrast, a study by Hall et al. (2007) that examined synapse development in embryonic cortical neurons in culture, found that enhanced NR2B NMDAR activation can suppress LTP. If their observations extend to intact adult synapses, this would suggest that GLYX-13 increases NR2B and AMPAR expression via different mechanisms, which could explain why repeat dosing is necessary to maximally enhance magnitude of LTP.

It is now clear that the regulation of metaplasticity at the receptor level (e.g., by changes in channel kinetics, receptor expression in both number and type, and trafficking) and biochemically (late stage and thus persistent transcription and translation-dependent metaplasticity) can have significant effects on synaptic activity associated with learning and memory. Thus, a plausible hypothesis for how rapastinel displays long-term antidepressant properties in humans is that it induces an NMDA receptor-triggered, AMPA receptor-dependent, facilitation of metaplasticity processes associated with an LTP-like mechanism (Moskal et al., 2005, Zhang et al., 2008, Burgdorf et al., 2013, Moskal et al., 2014). This is different from the antidepressant effects induced by NMDA receptor antagonists and suggests a novel aspect of the mechanistic underpinnings of the antidepressant effects of glutamatergic modulators.

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Abbreviations

ACSF	artificial cerebrospinal fluid
(±)-CPP	(±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
fEPSPs	field excitatory postsynaptic potentials
LTD	long-term depression
LTP	long-term potentiation
MDD	major depressive disorder
MPFC	medial prefrontal cortex
MWM	Morris water maze
NIH	novelty-induced hypophagia
NMDAR	N-methyl-D-aspartate receptor
PEL	positive emotional learning

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Highlights

- GLYX-13 produces antidepressant-like effects for ≥ 1 week following a single dose
- GLYX-13 facilitates learning and memory for ≥ 1 week following a single dose
- GLYX-13 enhances hippocampal metaplasticity 24 hrs - 2 wks following a single dose
- Repeat doses of GLYX-13 maintained the metaplasticity effects for at least 8 wks
- GLYX-13 increased the number of mature dendritic spines 24 hrs after a single dose

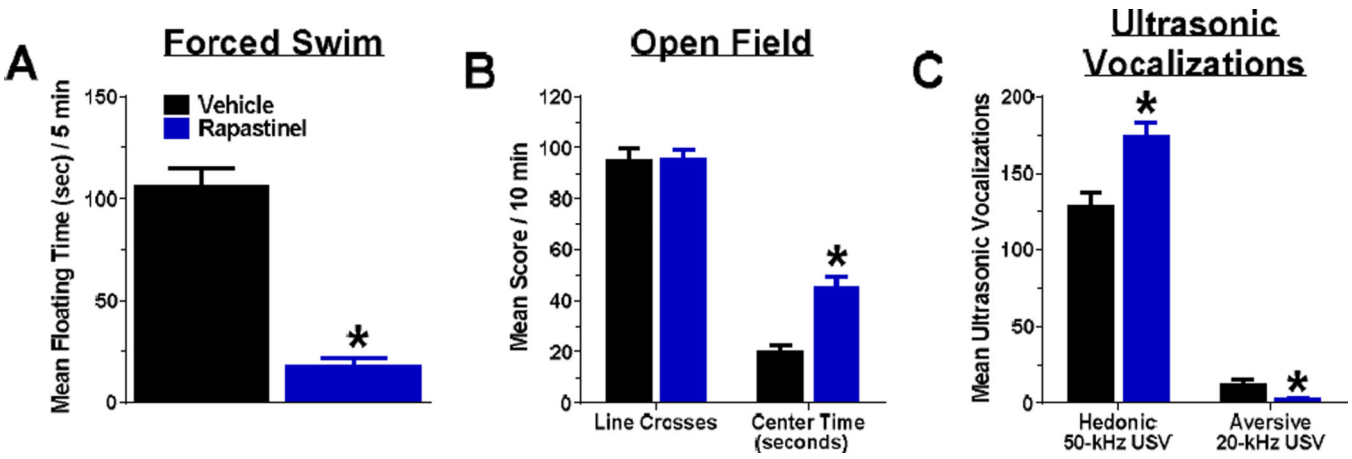

Fig 1. A single dose of rapastinel (3 mg/kg IV) produces antidepressant-like effects in multiple models 1 week post-dosing

Male 2–3 month old Sprague Dawley rats were pretreated with a single dose of rapastinel (TPPT-NH_2; 3 mg/kg IV) or sterile saline vehicle (0.9%, 1 ml/kg) and were tested 1 week post-dosing in the following behavioral paradigms: (A) Porsolt forced swim test with floating time (sec) quantified during the 5 min test; (B) open field test with total number of line crosses and time (sec) spent in the center compartment of the open field being measured during the 10 min test; (C) ultrasonic vocalization (USVs) test with total hedonic and aversive USVs measured during the 3 min test session. N = 11 rats per group. Mean ± SEM
* $P < .05$ ANOVA, rapastinel vs. vehicle.

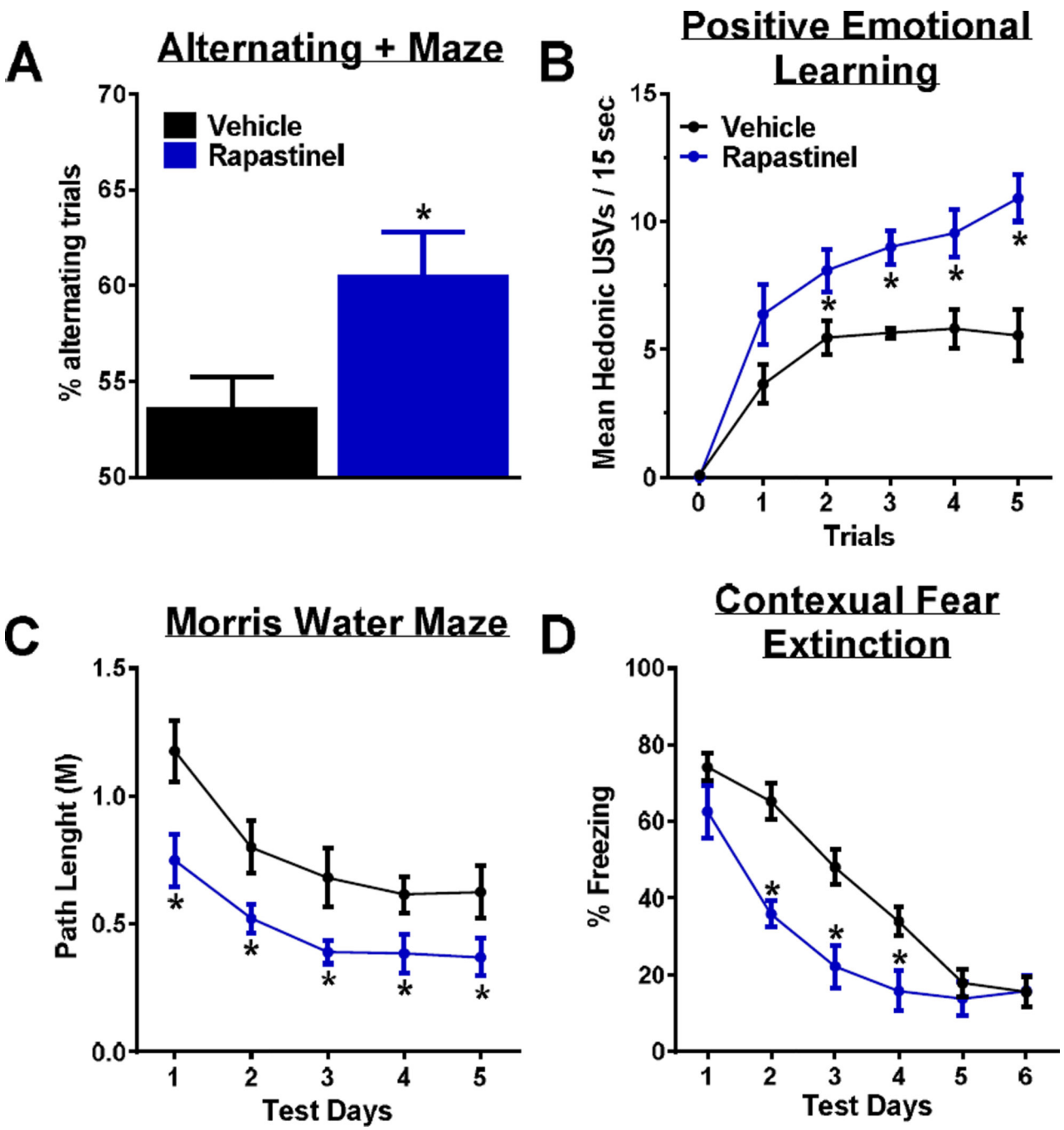

Fig 2. A single dose of rapastinel (3 mg/kg IV) facilitates learning and memory in multiple models 1 day to 1 week post-dosing

Male 2–3 month old Sprague Dawley rats were pretreated with a single dose of rapastinel (3 mg/kg IV) or sterile saline vehicle (0.9%, 1 ml/kg) and received a single tests session 1 week post dosing (A–B) or were dosed 24 hrs before the first of 5 (C) or 6 (D) daily test sessions: (A) % of alternating trials in the spontaneous alternating closed arm plus maze test; (B) hedonic ultrasonic vocalizations in response to a conditioned stimuli that predicts heterospecific play in the USVs test; (C) path length to find the hidden platform in the movable platform version of the Morris water maze test; (D) % freezing during contextual

fear extinction. N = 8–21 rats per group. Mean ± SEM (a) * P < .05 within subjects t-test, 2 tailed, (B–D) * P < .05 Fisher's PLSD post hoc test rapastinel vs. vehicle.

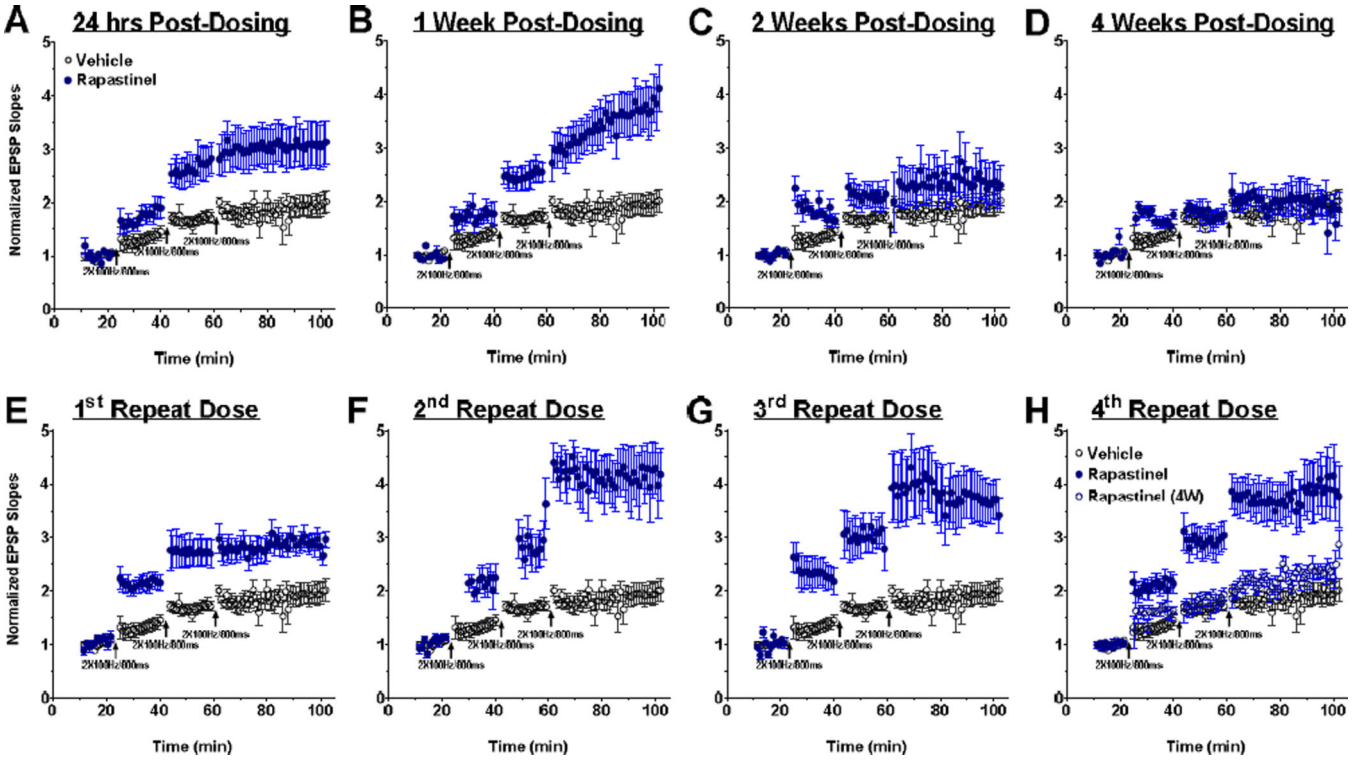

Fig 3. Rapastinel enhances hippocampal LTP 24 h and 1 week following a single dose, and persistently enhances LTP following multiple doses every 2 weeks

A single in vivo dose of rapastinel (3 mg/kg IV; filled blue circles) in 2–3 month old male SD rats significantly enhanced the magnitude of long-term potentiation (LTP) of synaptic transmission compared to vehicle treated controls (open black circles), tested in vitro (A) 24 hrs and (B) 1 week post-dosing at Schaffer collateral-CA1 synapses after 1, 2 and 3 sub-maximal high-frequency stimulus trains (2×100Hz/800ms, arrows, $P < .05$, Fisher's PLSD post hoc test), but not (C) 2 or (D) 4 weeks post-dosing ($P > .05$). In contrast, short-term potentiation (STP) 5 min after the first high frequency stimulus was significantly increased at all timepoints from 24 hrs to 4 weeks post-dosing ($P < .05$, Fisher's PLSD post hoc test). Repeated dosing once every 2 weeks for up to 8 weeks with rapastinel produced sustained enhancement of both LTP and STP measured 24 hrs after the final dose (E–H, filled blue circles, $P < .05$; Fisher's PLSD post hoc test), which reversed by 4 weeks following the final dose (H, open blue circles, $P > 0.05$, Fisher's PLSD post hoc test). N = 5–9 slices per group.

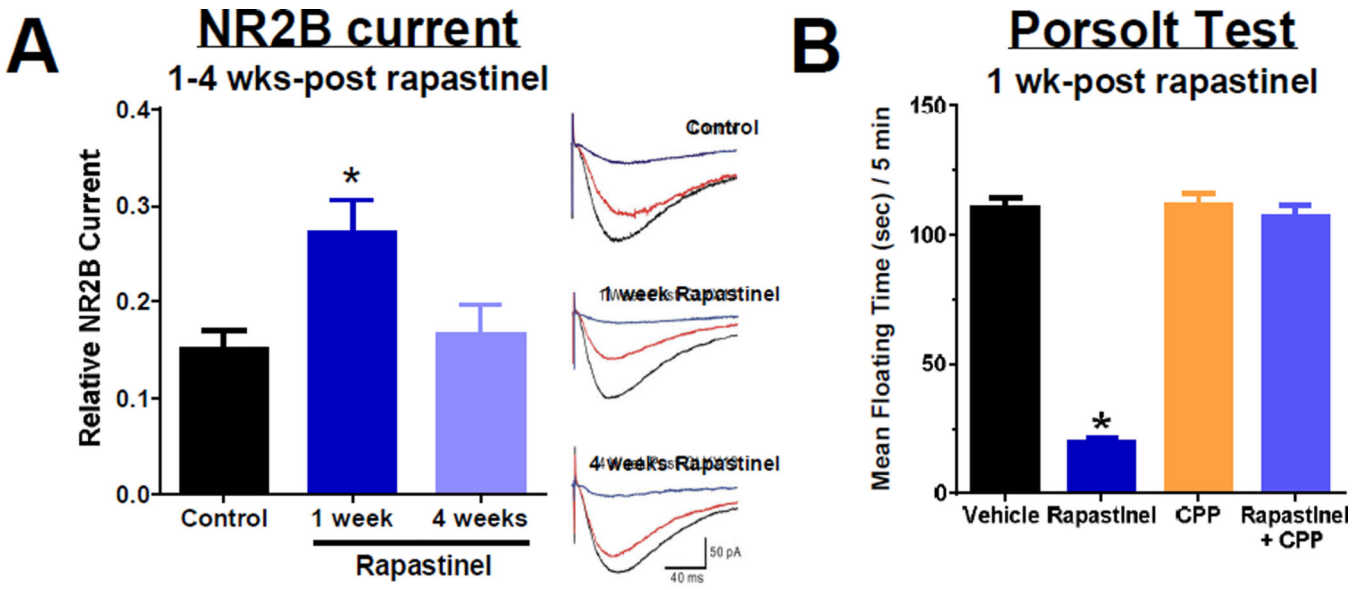

Fig 4. Rapastinel persistently increases the NR2B component of NMDAR conductances for 1 week post-dosing, and the NMDAR antagonist CPP blocks the antidepressant-like effect of rapastinel 1 week post-dosing

(A) Mean ± SEM fraction of blockade of NMDAR-mediated EPSCs by the NR2B-selective blocker Ro25-6981 (1μM), in control (0 mg/kg; N=8) CA1 pyramidal neurons, versus neurons in slices from rats treated one week (1 W; N=7) or 4 weeks (4 W; N=10) earlier with 3 mg/kg Rapastinel. *Right panel:* Representative EPSCs recorded from CA1 pyramidal neurons in slices prior to drug treatment (black traces), in the presence of the NR2B-selective NMDAR antagonist 1μM Ro25-6981 (red traces) and in the presence of 1μM Ro25-6981 plus the NR2A-selective NMDAR antagonist 10μM TCN-213 (blue traces). Ro25-6981 + TCN-213 blocked 79% of all current and did not differ between groups (results section). **(B)** Mean (±SEM) floating time in the Porsolt forced swim test in 2–3 month old male SD rats pretreated with rapastinel (3 mg/kg IV; or saline vehicle IV) 1 week before administration of the NMDAR receptor antagonist CPP (10 mg/kg IP; or saline vehicle IP) and tested 1 hr post CPP administration. Testing consisted of a single 5 min swim session, and the animals were given a 15 min habituation swim session 24 hrs before testing (N = 9 per group. * P < .05 Fisher's PLSD post hoc test vs. all other groups).

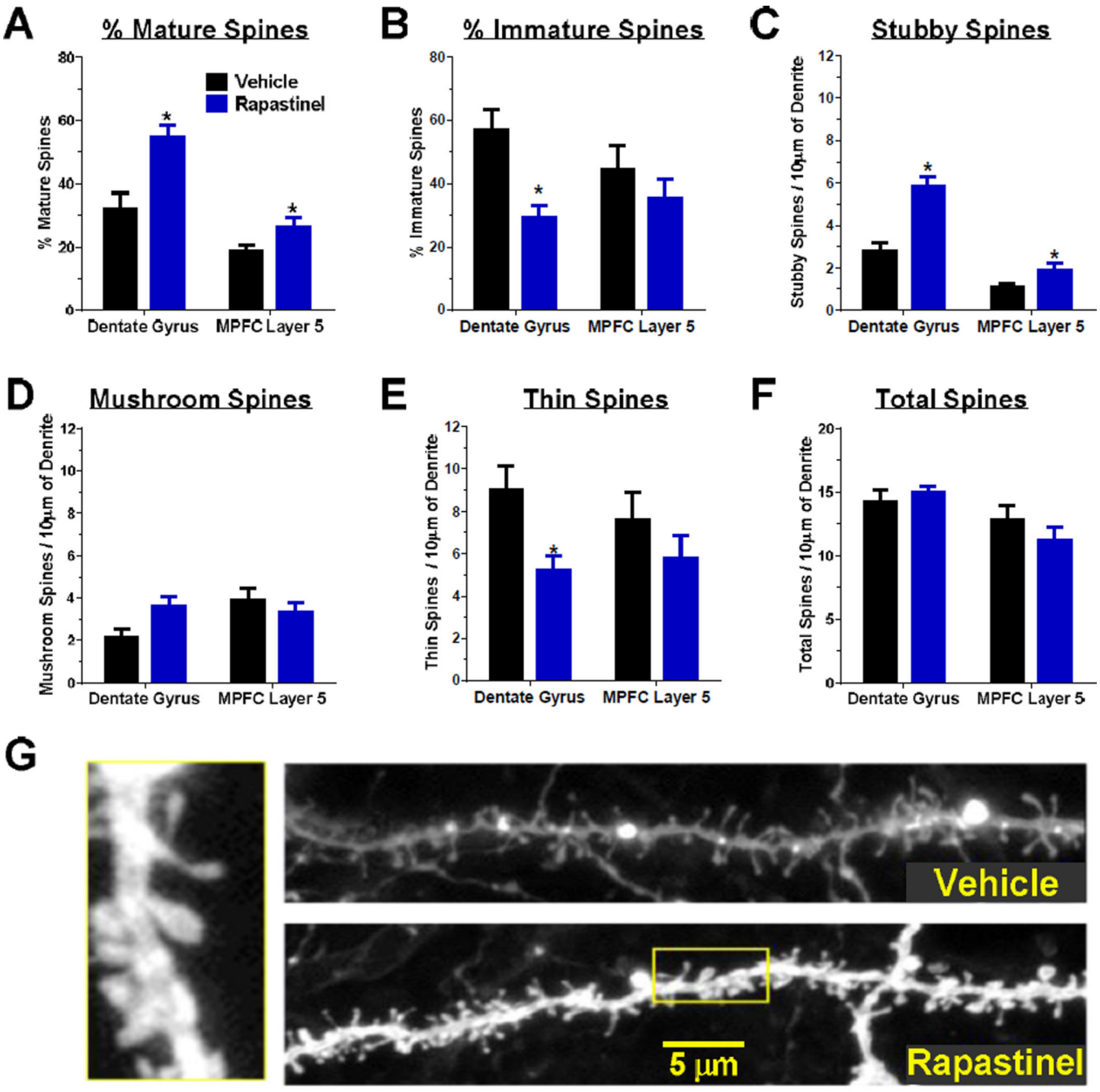

Fig 5. A single dose of rapastinel (3 mg/kg IV) induces increases in mature spines in distal dendrites of the dentate gyrus and Layer 5 of the MPFC 24 hrs post-dosing

In male 2–3 month old rats, rapastinel (3 mg/kg IV, in blue) significantly increased (A) the proportion of mature dendritic spines in the dentate gyrus (primary apical, 100–150 µM from the dendrite) or MPFC layer 5 tufts, and increased (C) the density (spines/10 µM of dendrite) of stubby spines in both regions. Rapastinel also decreased (B) % of immature spines (thin spines) and (E) the density of thin spines only in the dentate gyrus. Rapastinel did not alter (D) mushroom spines or (F) total spine density in these same regions. Representative laser-scanning confocal micrographs of layer 5 MPFC dendrites from

rapastinel and vehicle treated animals are shown in panel (G) Mean ± SEM. N = 15 cells/per group (n = 3 rats per group; ~ 13,125 dendrites/group), * P < .05 Fisher's PLSD post hoc test.