

Reduction in Ventral Midbrain NMDA Receptors Reveals Two Opposite Modulatory Roles for Glutamate on Reward

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Glutamate is a major component of the reward circuitry and recent clinical studies suggest that new molecules that would target glutamate neurotransmission are most likely to constitute more effective medications for mood disorders. It is well known that activation of N-methyl-D-aspartate glutamate receptors (NMDARs) initiates dopamine burst firing, a mode associated with reward signaling; but NMDARs also contribute to the maintenance of an inhibitory drive to dopamine neurons. Such opposite modulatory functions imply that different subtypes of NMDARs are expressed on different ventral midbrain (VM) neurons and/or afferent inputs to dopamine neurons. By using the small interfering RNA (siRNA) technique, we studied the effects of VM downregulation of NMDAR subunits GluN1, GluN2A, and GluN2D on reward induced by dorsal raphe electrical stimulation. Reward thresholds were measured before and 24 h after each of three consecutive daily bilateral microinjections of siRNA for the targeted receptor subunit(s) or non-active RNA sequence. After the last measurement, reward thresholds were reassessed following a bilateral microinjection of the preferred GluN2A-NMDA antagonist, (2R,4S)-4-(3-Phosphopropyl)-2-piperidinecarboxylic acid (PPPA). Western-blot analysis showed that siRNAs reduced GluN1- and GluN2A-containing receptors whereas behavioral tests showed that only a reduction in GluN1 produced reward attenuation. Despite NMDAR reduction, reward-enhancing effect of PPPA remained unchanged. We conclude that VM glutamate relays the reward signal initiated by dorsal raphe electrical stimulation by acting on NMDARs devoid of GluN2A/2D subunits and exerts an inhibition on this reward signal by acting on GluN2A-containing NMDARs most likely located on afferent terminals.

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INTRODUCTION

Depression is the most common mental illness affecting around 350 million people worldwide. It is expected to become the second most common cause of disability worldwide by 2015 with obvious socio-economic implications (Strekalova *et al*, 2011). Traditional pharmacotherapy which focuses on increasing monoamines (norepinephrine or serotonin) neurotransmission has a relatively slow onset (Kupfer *et al*, 1989) of action and it has a limited effectiveness, between 10 and 20% of the treated patients do not respond to any antidepressant medication (Greenberg *et al*, 2004). Recent clinical studies have shown that a single subanesthetic dosage of the non-competitive N-methyl-D-aspartate receptor (NMDARs) antagonist, ketamine, produces a rapid and lasting antidepressant effect in patients who were resistant to the current treatment (Maeng *et al*, 2008). These findings suggest that glutamate receptors might

represent a very promising pharmacological approach to alleviate depressive symptoms.

Anhedonia, a core symptom of depression, is associated with a downregulation of the mesolimbic dopaminergic pathway (Nestler and Carlezon, 2006) and NMDARs antagonists may ameliorate mood disorders through the modulation of ventral midbrain (VM) dopamine (DA) neurons. Under resting condition, about half of DA neurons are under a strong tonic inhibitory drive that makes them hardly responsive to excitatory inputs (Grace and Bunney, 1984). The other half of DA neurons are spontaneously active and fire in two distinctive modes: an intrinsic sustained slow tonic firing or a fast phasic burst firing. The switch from tonic to phasic firing is controlled by glutamate signaling at its ionotropic receptors. Activation of glutamatergic afferents (Geisler *et al*, 2007; Grace and Bunney, 1984; Omelchenko and Sesack, 2009), and possibly VM VGluT2-containing interneurons (Dobi *et al*, 2010), control the transition from peacemaker tonic to phasic burst firing of DA neurons. In contrast, blockade of VM ionotropic glutamate receptors disrupts burst firing (Charley *et al*, 1991; Chergui *et al*, 1993). Dopamine burst firing signals the occurrence of salient unconditional and conditional stimuli (Schultz, 2010) and it is believed to convey motivationally relevant information to forebrain structures involved in motor execution

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(Overton and Clark, 1997). In addition DA burst firing is critical for learning of appetitive and aversive task (Zweifel *et al*, 2009) and it is believed to encode learning prediction errors (Schultz, 2010).

Besides its role in firing mode transition, glutamate also has a role in the maintenance of DA inhibitory drive (Grace and Bunney, 1984) and a reduction of this tonic inhibition allow an increased number of DA neurons to fire in a sustained slow tonic firing and in a fast phasic burst firing. These two opposite modulatory functions of glutamate imply that different subtypes of NMDARs are activated by different inputs to VM DA neurons, so that activation of one subtype of NMDAR initiates DA burst firing whereas activation of another enhances the inhibitory input to DA neurons. This idea is supported by data showing that either activation or blockade of VM NMDARs increases DA burst firing (French *et al*, 1993), accumbens DA release (Karreman *et al*, 1996; Kretschmer, 1999), and stimulates forward locomotion (Cornish *et al*, 2001; Kretschmer, 1999). Blockade of VM NMDARs also acts as a positive reinforcer and sustains self-administration behavior (David *et al*, 1998). Furthermore, VM microinjections of the PPPA and R-CPP, NMDAR antagonists for GluN2A-D subunits, potentiate the rewarding effect of electrical brain stimulation; such a potentiation was not seen with Ro 04-5595, a selective GluN2B antagonist, hence suggesting that glutamate exerts an inhibitory modulation on VM reward-relevant neurons by acting on NMDARs composed of GluN2A and/or GluN2D (Bergeron and Rompré, 2013). The present study was thus aimed at further characterizing the NMDARs that is/are responsible for this inhibitory modulation of reward. Using the curve-shift method, we studied the effects of VM downregulation of NMDAR subunits GluN1, GluN2A, GluN2D, and GluN2A + D, using a small interfering RNA (siRNA), on reward induced by electrical brain stimulation. Results show that VM glutamate relays the reward signal initiated by the electrical stimulation by acting on receptors devoid of GluN2A/2D subunits and exerts an inhibition on reward signaling by acting on GluN2A NMDARs that are most likely located on VM afferent terminals.

MATERIALS AND METHODS

Subjects and Surgery

Fifty (50) male Long-Evans rats (Charles River, St Constant, QC, Canada) weighing between 350 and 400 g at the time of the surgery were used as experimental subjects. They were individually housed in a temperature and humidity controlled room with a 12-h light-dark cycle (lights on at 06:00 h) with *ad-libitum* access to food and water. After a minimum 7 days period of acclimation to the housing environment rats were anesthetized with isoflurane (2.5–3.5% O₂, 0.6 l/min) and stereotaxically implanted according to Paxinos and Watson (2007) coordinates with 26-gauge guided cannulae (Model C315G HRS Scientific, Montreal, Canada) aimed bilaterally above the VM (–5.5 mm AP, 3.2 mm ML at a 18° angle, 6.5 mm DV from the skull surface) and a movable monopolar electrode aimed at the dorsal raphe (–7.6 mm AP, 0.0 mm ML, 6.6 mm DV from the skull surface). See Bergeron and Rompré (2013) for further surgery details. All procedures were carried out in

accordance with established practices as described in the NIH Guide for Care and Use of Laboratory Animals. In addition, all procedures were reviewed and approved by the Animal Care and Use Committee of Université de Montréal.

Self-Stimulation Training

Each of the rats was shaped to nose-poke for a 0.4 s train of cathodal, rectangular, constant-current pulses, 0.1 ms in duration, delivered at a frequency of 98 Hz. For a detailed shaping procedure see Bergeron and Rompré (2013). Once the rat nose-poked consistently for currents between 125 and 400 μ A, a rate vs pulse-frequency curve was obtained by varying the stimulation frequency across trials over a range that drove the number of rewards earned from maximal to minimal levels. Each trial for obtaining the rate vs pulse-frequency sweep lasted for 55 s followed by a 15-s inter-trial interval during which stimulation was not available. The beginning of each trial was signaled by five trains of non-contingent priming stimulation delivered at a rate of 1 per second. Four sweeps were run daily and the first sweep was considered warmup and discarded from analysis. The data relating the rate to the pulse frequency was fitted to a sigmoid and the index of reward threshold defined as the pulse frequency sustaining a half-maximal rate of responding (M50) was obtained (see Supplementary Figure 1 for a diagram of the procedure).

Self-Stimulation Tests

Prior to the siRNA injections, sterile 0.9% saline was microinjected in the VM to habituate the animals to the injection procedure. Bilateral injections were made by inserting an injection cannula (Model C315I HRS Scientific, Montreal, Canada) that extended 2 mm beyond the guide cannula tip. The injection cannula was connected to a 5 μ l Hamilton micro-syringe via polyethylene tubing. A total volume of 0.5 μ l was injected in each hemisphere simultaneously over a 60-s period. The rate of delivery was controlled by an infusion pump (Harvard Instruments, Holliston, MA). To allow diffusion into the tissue the injection cannulae were left in place for additional 60 s. After the microinjection, the subjects were put into the operant boxes and allowed to self-stimulate. Results from this test were not included in the analysis. Baseline data were collected 1 week after this first saline microinjection and the self-stimulation behavior was considered stable when the M50 values varied less than one 0.1 log unit for three consecutive days. Once stable baseline was obtained siRNA (5 μ g per side) against GluN1, GluN2A, GluN2D, GluN2A + GluN2D, or the non-active RNA sequence was injected bilaterally into the VM for three consecutive days. Reward thresholds were measured 24 h after each injection. After 24 h the last threshold determination rats received a bilateral VM microinjection of the NMDA antagonist PPPA (0.825 nmol per 0.5 μ l per side) and reward thresholds were measured again immediately after the injection for 120 min.

siRNA and Drug

Downregulation of selective NMDAR subunits was achieved using a mixture of pre-validated siRNA sequences against

rat GluN1 (Cat. #4390816 ID s127804- s127806), GluN2A (Cat. #4390816 ID s127807- s127809), and/or GluN2D (Cat. #4390816 ID s127816- s127818) receptors. A non-active RNA sequence (Cat. #4390844) (scramble) was used as control. siRNA sequences and non-active RNA sequence were purchased from Ambion (Austin, TX) as control. The deprotected, duplexed, desalted siRNA was mixed with a cationic lipid transfection carrier N-[1-(2,3-Dioleoyloxy)-propyl]-N,N,N-trimethyl-ammonium methylsulfate (DOTAP) (Roche Applied Sciences, Indianapolis, IN) which showed high efficacy for *in-vivo* transfection (Salahpour *et al*, 2007). The final solution contained 10 μ g of the active or inactive siRNA and 1 μ g of DOTAP per μ l. NMDAR antagonist PPPA ((2R,4S)-4-(3-Phosphopropyl)-2-piperidinecarboxylic acid) was purchased from Tocris Bioscience (Ellisville, MI, USA). The drug was dissolved in sterile 0.9% saline and stored frozen in 40–50 μ l aliquots. Drug solutions were thawed just prior to testing and injected at a dose of 0.825 nmol per 0.5 μ l per side. Dose is expressed as salt.

Western-Blot

Rats were decapitated immediately after the last behavioral test. Brains were removed and immediately placed on an ice-cold brain matrix, and sectioned coronally. The VM was dissected on an ice-cooled plate from a 0.75–1-mm slice using a 15-gauge tissue punch. The tissue was then immediately frozen at -80°C until biochemical experiments were performed. VM samples were mechanically homogenized in lysis buffer (10 mM Tris-HCl (pH 6.8), 2% SDS, and a cocktail protease inhibitor (Roche)). The protein concentrations of the tissue samples were measured using BCA protein assay kit (Pierce, USA). Equal amounts of protein (10–50 μ g) were dissolved into 25 μ l lysis buffer (which contained 4.5 μ l $5\times$ loading buffer and 0.5 β -mercaptoethanol), boiled at 95°C for 5 min, and then subjected to SDS-PAGE with 8% polyacrylamide gels and transferred to PVDF membrane (Bio-Rad Laboratories). The membranes were blocked for 1 h in TBST with 5% dry milk and were incubated with mouse anti- β -actin (Millipore) at 1:10000 with either rabbit anti-GluN1 (Novus Biological) at 1:500, anti-GluN2A (Cayman) at 1:1000, anti-GluN2B (Novus Biological) at 1:1000, or anti-GluN2D (Santa Cruz Biotechnology) at 1:100 overnight at 4°C . After rinsing four times with TBST for 5 min, the membranes were incubated in HRP-conjugated goat anti-Rabbit IgG (Millipore) at 1:6000 (to detect GluN1, GluN2A, GluN2B, GluN2D) or HRP-conjugated goat anti-Mouse IgG (Millipore) at 1:6000 (to detect β -actin, GluN2D) for 1 h. The membrane protein bands were detected with ECL western blotting system (PerkinElmer) and visualized on an X-ray film. The band densities were measured with ImageJ (NIH) and were normalized to β -actin. The protein levels of the different subunits of the NMDAR were expressed as a percentage of control.

Data Analysis

Levels of protein were contrasted using Student's *t*-test. Fit of the rate-frequency data and M50 value were obtained using Matlab (Natick, MA). Differences in M50 values were assessed by using a repeated measures analysis of variance

(ANOVA). Differences in protein levels were assessed using a one-way ANOVA. Tukey's honestly significant difference (HSD) was used as a post-hot test. Correlation analysis was carried out between protein levels and M50 values. Data analysis was performed using Statistica v12 (Tulsa, OK), and graphics were done in Origin v9 (Northampton, MA).

RESULTS

Validation of siRNA Effects on NMDA Receptor Subunit Protein Levels

Figure 1 shows VM protein levels of NMDAR subunit GluN1 and GluN2A from total homogenates of rats injected (intra-VM) with, GluN1, GluN2A, GluN2A + GluN2D, or control (scramble) siRNA. Microinjections of siRNA against GluN1 produced a significant 41% (SEM = 3.22) decrease in the expression of NMDAR GluN1 (Figure 1a) when compared against control ($t_{(20)} = 5.38$; $p < 0.0001$). Similarly, a significant 39% (SEM = 5.26) reduction of NMDAR GluN2A expression was observed in rats injected with siRNA against GluN2A (Figure 1b) ($t_{(21)} = 4.48$; $p < 0.0001$). For rats injected with GluN2A and GluN2D siRNA combination, a significant 42% (SEM = 4.67) decrease of NMDAR GluN2A expression (Figure 1c) ($t_{(14)} = 3.78$; $p < 0.001$), but no specific signal was detected for the GluN2D expression in any of the present experimental conditions (control or in the presence of the Glu2D siRNA). Although there is evidence that GluN2D protein might be expressed at low levels in the VM (Dunah *et al*, 1996), similar difficulties had been experienced by others (Schilström *et al*, 2006). This is not related to the lack of protein detection by the antibody used, because a strong GluN2D immunosignal was detected in the hippocampus and thalamus brain areas enriched of this NMDAR subunit (Supplementary Figure 2). To test the selectivity of the siRNA for GluN2A, GluN2D, or their combination we tested the expression of GluN2B NMDAR in a subset of subjects. The results show no effect ($F_{(3,12)} = 0.315$; $p > 0.05$) on the expression of GluN2B receptor (Supplementary Figure 3).

VM Downregulation of the GluN1 subunit, but not GluN2A, Strongly Reduced Electrically Evoked Reward

Figure 2 shows for representative subjects the behavioral effects observed 24-h after the last siRNA injection for the different sequences used. Intra-VM injections of the inactive RNA sequence did not produce significant changes of the curve that relates the nose-poke rate as function of pulse frequency (Figure 2a). In contrast, intra-VM injections of the siRNA for GluN1 produced a rightward displacement of this curve. This displacement implies that the manipulation produced a reduction of the rewarding effectiveness of the stimulation (Figure 2b). Figure 2c–e shows that intra-VM injection of GluN2A, GluN2D alone, or their combination, did not produce any significant changes in the rate vs pulse-frequency curves. Intra-VM injections of PPPA produced, regardless of the siRNA manipulation, a leftward displacement of the curve that relates the nose-poke rate as a function of pulse frequency, so less stimulation was necessary to obtain a given level of performance (Figure 2a–e).

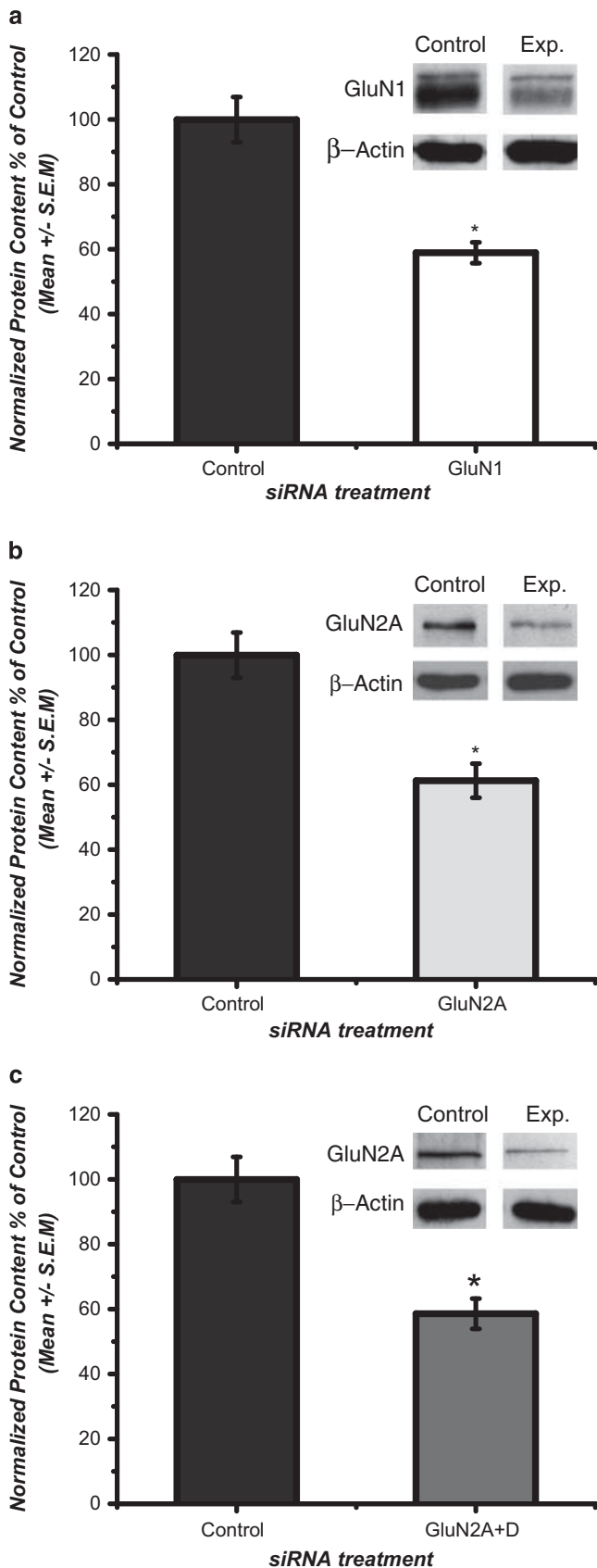


Figure 3 shows the average changes in M50 values (Figure 3a) and in maximal response (Figure 3b), expressed as % of baseline, for all the subjects that were injected with the inactive (control) and active siRNA. Analysis of variance confirms a significant effect of treatment ($F_{(4,45)} = 8.45$, $p < 0.0001$). *Post-hoc* test shows that intra-VM injection of GluN1 produced a significant 25.5% (SEM = 3.67) increase in M50 values when compared against the rats that were injected with the non-active RNA. Although the maximum response rate (Figure 3b) was lower after rats received siRNA microinjections against GluN2A, GluN2D, and GluN2A + 2D, this reduction was not statistically significant ($F_{(4,45)} = 1.26$; $p = 0.299$).

Figure 4 shows the correlation between the levels of increase in M50 observed in those rats which GluN1 receptor was reduced and the amount of protein down-regulation measured in the VM. This result indicates a positive significant correlation between the maximum percentage change in reward threshold and the percentage change in protein reduction ($r = 0.652$; $p < 0.05$). The increase in the reward threshold was directly related to the level of downregulation in GluN1 receptor; the reduction in GluN1 expression explains 36% of the increase in reward threshold.

Pharmacological GluN2A-Containing NMDAR Blockade Enhanced Brain Stimulation Reward Regardless of the siRNA Treatment

We tested if reduction of the expression of the different NMDARs would impact intra-VM injections of PPPA. Despite the fact that PPPA displays only a modest pharmacological selectivity for GluN2A- vs GluN2B-containing NMDARs, we have previously shown that enhancement of electrically evoked reward following a wide range of intra-VM PPPA infusion is associated with GluN2A-containing NMDA (Bergeron and Rompré, 2013; Ducrot et al, 2013). As expected, PPPA enhanced the rewarding effectiveness of the electrical stimulation and produced an increase in the maximum response rate. The magnitude of the leftward and upward displacement of the rate vs pulse-frequency function can be appreciated in Figure 2. The maximum displacement of the M50 was obtained in average 19 min (SEM = 1.76) after the PPPA injection, whereas the

Figure 1 siRNA microinjections against NMDARs produce a decrease in GluN1 and GluN2A subunit immunoreactivity in the VM. (a) The inset of the figure shows samples of reactivity obtained for 10 μ g of VM homogenate from those rats treated with the control scramble siRNA, or against GluN1. The bar histogram shows the normalized levels of NMDA GluN1 receptor against control. (b) The inset of the figure shows samples of reactivity obtained for 20 μ g of VM homogenate from those rats treated with the control scramble siRNA, or against GluN2A. The bar histogram shows the normalized levels of NMDA GluN2A receptor against control. (c) The inset shows samples of reactivity obtained for 20 μ g of VM homogenate from those rats treated with the control scramble siRNA, or against GluN2A and GluN2D. Only GluN2A was detected in our experiment. The bar histogram shows the normalized levels of NMDA GluN2A receptor against control. The levels of GluN1 and GluN2A were detected using specific antibodies and normalized to β -actin. Asterisks indicate significant differences ($*p < 0.001$; Student's *t*-test).

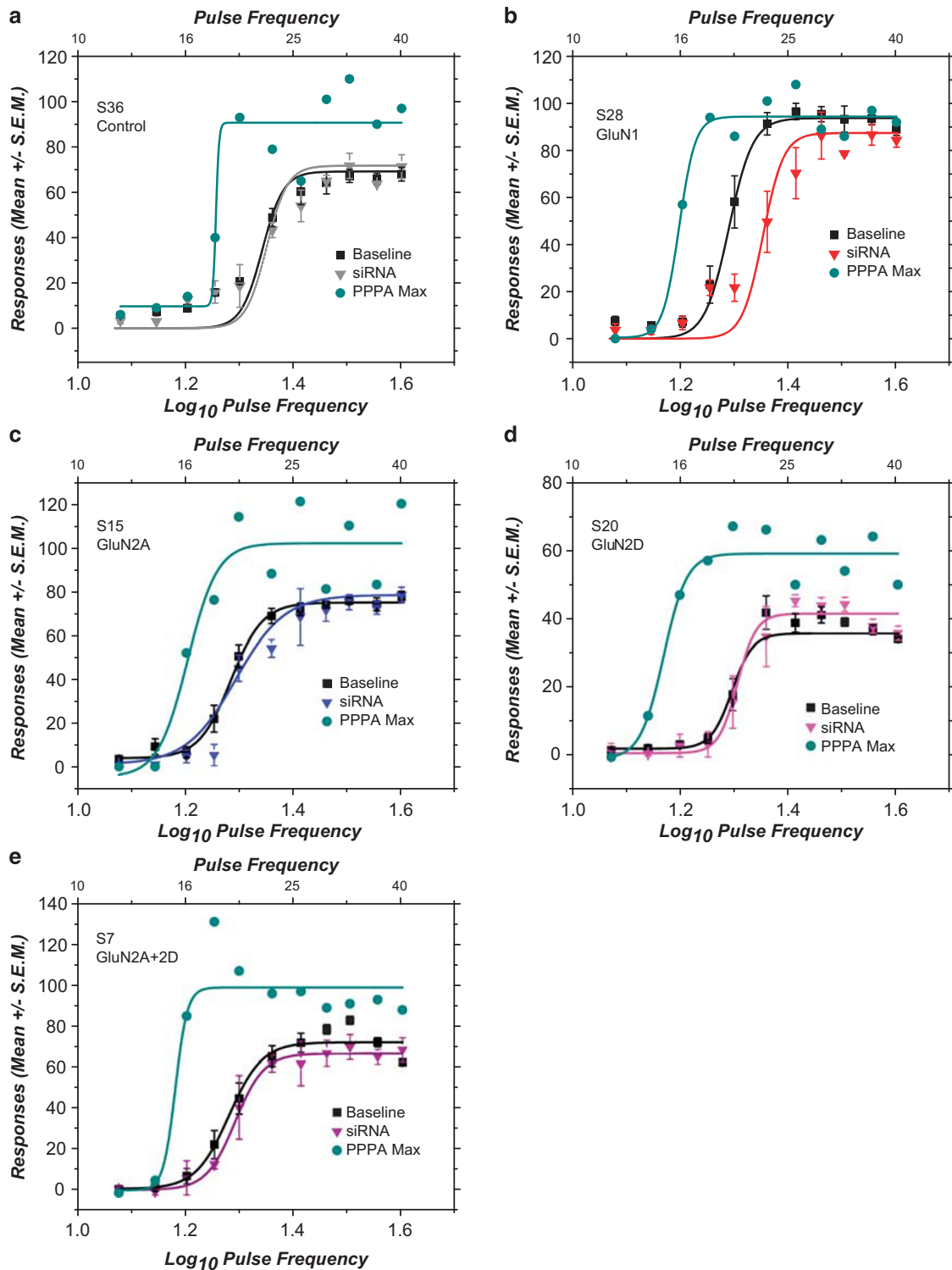


Figure 2 Rate-frequency curves for selected subjects show the effect of the different siRNA treatment and intra-VM injection of PPPA (0.825 nmol per 0.5 μ l per side). (a) Treatment with scramble siRNA (0.5 μ g per side) produced no effect on the curve that relates the nose-poke rate with the electrical pulse trains. The baseline curve and the one obtained 24-h after the last siRNA injection overlapped. Intra-VM injection of PPPA produced a leftward and upward shift of rate-frequency curve. (b) Treatment with siRNA (0.5 μ g per side) against GluN1 produced a decrease of the rewarding effects that arise from the electrical stimulation; the red curve obtained 24-h after the last siRNA injection is displaced to the right of the baseline curve that is shown in black. The siRNA treatment did not alter PPPA's effectiveness to produce an enhancement in reward. (c) Treatment with siRNA (0.5 μ g per side) against GluN2A was ineffective in altering the rewarding effects that arise from the electrical stimulation; the blue curve obtained 24-h after the last siRNA injection overlapped with that obtained during baseline. Intra-VM injection of PPPA produced a leftward and upward shift of rate-frequency curve. (d) Treatment with siRNA (0.5 μ g per side) against GluN2D and (e) the combination of GluN2A and GluN2D were ineffective in altering the rewarding effects that arise from the electrical stimulation, respectively, the magenta and dark magenta curves overlapped with those obtained during baseline. siRNA treatment did not alter the effectiveness of intra-VM injection of PPPA, this drug produced a leftward and upward shift of rate-frequency curves.

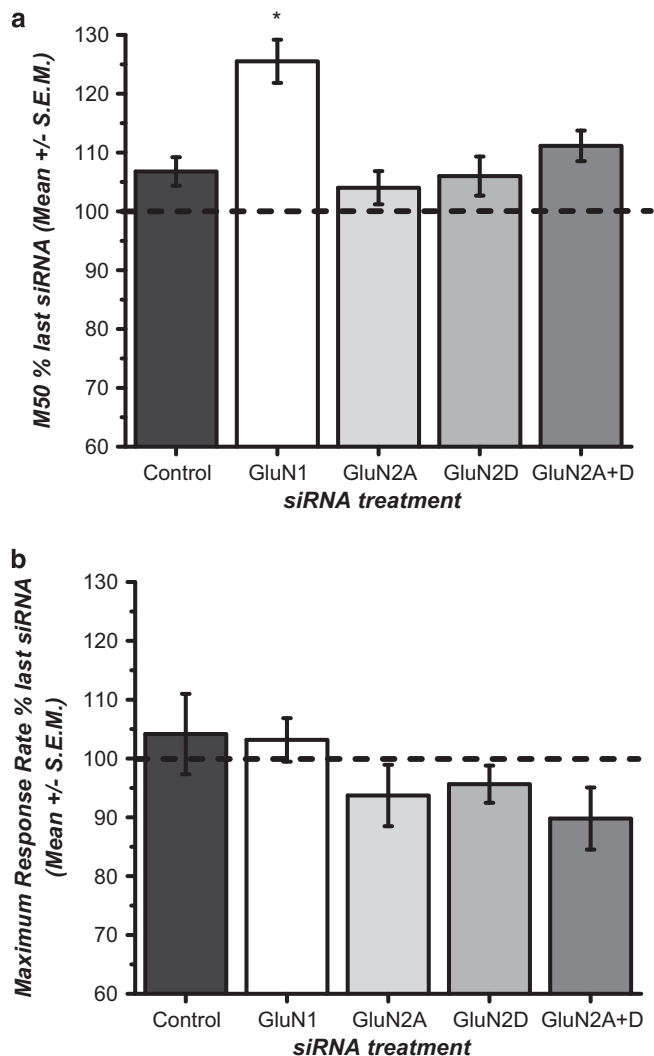


Figure 3 Bar graphs representing the averages of maximum change in stimulation threshold and maximum response rate for all siRNA treatments. (a) Treatment with siRNA against GluN1 receptor produce a significant increase in the stimulation threshold ($F_{(4,45)} = 8.45$; $p < 0.0001$). (b) None of the siRNA treatments produced a significant change in maximum response rate ($F_{(4,45)} = 1.26$; $p = 0.299$).

change in maximum rate was obtained after 25 min ($SEM = 3.13$). There was no significant difference between the groups in their timing for M50 displacement ($F_{(4,43)} = 1.58$; $p > 0.05$) or maximum response rate displacement ($F_{(4,43)} = 0.97$; $p > 0.05$). Figure 5 shows the effect of PPPA for each of the treatment groups on the maximum decrease of M50 (Figure 5a) and the maximum increase in response rate (Figure 5b). PPPA produced an average 25.5% ($SEM = 1.37$) reduction in M50 value. The ANOVA yielded a significant effect of treatment ($F_{(5,44)} = 14.88$, $p < 0.0001$) and *post-hoc* test showed that the M50 values, when compared with subjects that received saline, were significantly reduced regardless of the siRNA treatment. PPPA also produced comparable increases in the maximum response rate regardless of the siRNA treatment group, an average of 42% ($SEM = 5.12$). The ANOVA yielded a significant effect of treatment ($F_{(5,44)} = 2.58$, $p < 0.039$) and

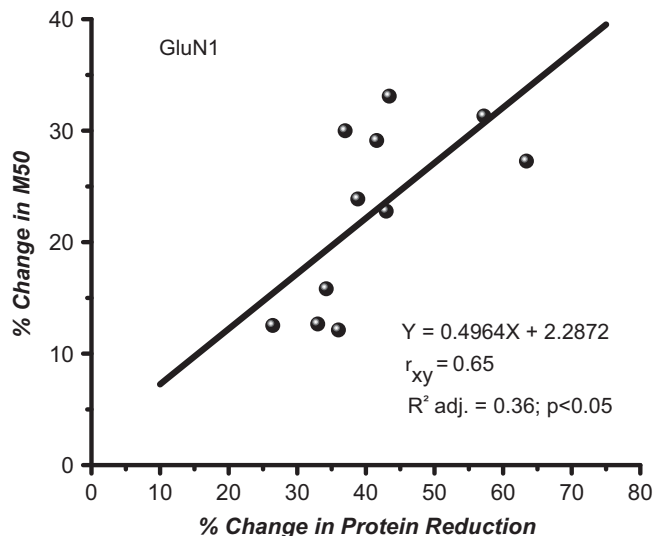


Figure 4 Scatterplot showing the positive relation between the change in stimulation threshold and the change in protein reduction for those rats that were injected with siRNA against GluN1. There is a positive correlation ($r_{(x,y)} = 0.652$; $p < 0.05$) between the reduction in GluN1 and the amount of change in M50, so that the greatest the protein reduction the biggest was the observed increase in stimulation threshold. The line represents the fit of the linear equation shown in the graphs and the R^2_{adj} shows the proportion of explained variance in M50 by the changes in GluN1 expression.

post-hoc test showed that maximum response rate, when compared with subjects who received saline, was significantly increased regardless of the siRNA treatment.

DISCUSSION

The main finding of the present study is that down-regulation of VM NMDAR following repeated microinjections of siRNA targeting the GluN1 subunit produces an attenuation of the reward signal induced by DR electrical stimulation. This attenuation is inferred by a rightward displacement of the rate *vs* pulse-frequency curve and the absence of a significant change in the maximum response rate, a measure that has been traditionally associated with changes in performance capacity (Miliaressis *et al*, 1986). This attenuation effect is most likely because of a decrease in the glutamatergic excitability onto DA neurons. Rewarding electrical stimulation produces an increase in DA cell firing and DA release (Hernández and Shizgal, 2009; Moisan and Rompre, 1998) and a large body of evidence shows that reward induced by brain electrical stimulation is highly sensitive to changes in VM DA neurotransmission. Drugs that enhance synaptic DA levels such as DA transporter blocker GBR12909 produces a leftward displacement of the curve that relates response rate to stimulation frequency (Rompré and Baucó, 1990). Opposite effects are obtained with DA receptor antagonists such as haloperidol and raclopride (Nakajima and Patterson, 1997). In further support for this hypothesis are the results showing that glutamate-containing terminals establish synaptic contacts with DA neurons (Carr and Sesack, 2000;

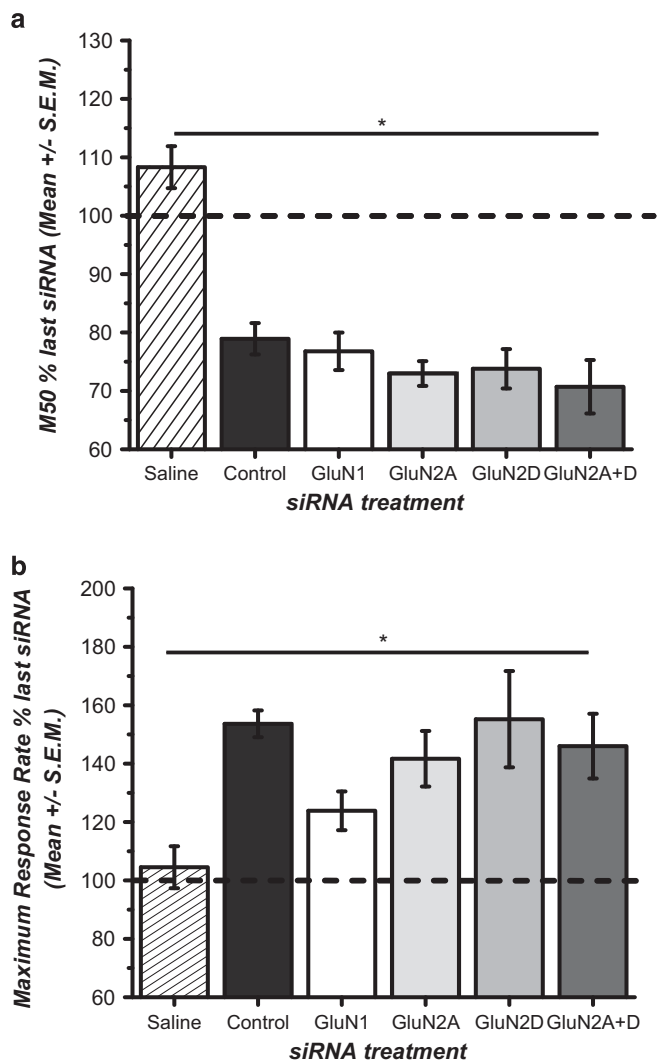


Figure 5 Bar graphs representing the Averages of maximum change in stimulation threshold and maximum response rate after intra-VM injection of saline or PPPA (0.82 pmol per 0.5 μ l per side) for all subject that received the different siRNA treatments. (a) PPPA injection, when contrasted against those subjects that received saline produced a significant reduction in self-stimulation threshold. This reduction was similar regardless of the NMDAR(s) targeted by the siRNA treatment ($F_{(5,44)} = 14.88$, $p < 0.0001$). (b) Similarly, PPPA when contrasted against those subjects that received saline produced a significant increase in maximum response rate, and such increase was indifferent to the siRNA treatment ($F_{(5,44)} = 2.58$, $p < 0.039$).

Omelchenko and Sesack, 2009) and that rewarding electrical stimulation is associated with an increase in VM glutamate release (You *et al*, 2001).

Both serotonergic and non-serotonergic DR neurons project to the VM and a recent study has shown that selective activation of DR non-serotonergic cell bodies, or their VM terminals, sustains operant responding and induces place preference (McDevitt *et al*, 2014). McDevitt *et al*, (2014) also showed that stimulation of this DR to VM pathway generates glutamatergic excitatory postsynaptic current in VM dopamine neurons, and that the rewarding effect associated with its activation is blocked by a selective dopamine D1 antagonist. A substantial number of DR

neurons express VGluT3, which constitutes a major part of the DR efferent projection to the ventral midbrain (Hioki *et al*, 2010; Qi *et al*, 2014; Watabe-Uchida *et al*, 2012). Dorsal raphe glutamatergic neurons also establish asymmetrical synaptic connections with VM dopamine neurons (Qi *et al*, 2014). Altogether, these findings strongly suggest that reward can be induced by activation of DR glutamatergic afferent inputs to VM DA neurons. Consistently, two recent optogenetic studies have shown that selective activation of DR glutamatergic releasing neurons or glutamatergic VM terminals is rewarding (Liu *et al*, 2014; Qi *et al*, 2014).

The hypothesis that the rewarding signal is transmitted to the VM by glutamate raises the possibility that the enhanced glutamate release produced by repeated electrical stimulations alter the expression of the NMDARs. It was shown for instance that repeated treatments with drugs of abuse known to enhance brain stimulation reward alter the expression of GluN2A and GluN2B, and their respective mRNA, in different limbic brain regions (Barr *et al*, 2014; Gipson and Reissner, 2013; Kindlundh-Högberg *et al*, 2008). Carlezon *et al*, (2001) compared the expression of GluN1 in the ventral tegmental area between rats that self-stimulated for one hour consecutively and rats that received no stimulation and found no difference between the two groups. This result suggests that repeated rewarding stimulations do not alter VTA NMDARs expression.

NMDARs are heterodimers composed of two obligatory GluN1 subunits with GluN2 and/or GluN3 subunits. Since GluN1 subunits are common to all NMDARs, the siRNA directed toward GluN1 should have produced a non-selective downregulation of NMDAR expression. Because other siRNA manipulations produced a significant reduction of GluN2A without any reliable change in self-stimulation behavior, we are forced to conclude that the NMDARs mediating the reward signal are not composed of GluN2A subunits. They are also unlikely composed of GluN2B, since no reward enhancement was observed in a previous pharmacological study with a selective GluN2B antagonist injected into the VM (Bergeron and Rompré, 2013). One hypothesis then is that the relevant NMDARs are heterodimers composed of GluN1 and GluN3 subunits. In support to this possibility, Yuan *et al*, (2013) recently identified GluN3A mediated current in VM DA neurons and showed that GluN3A-containing NMDARs mediate DA neurons plasticity induced by cocaine reward. Alternative hypotheses are that the reduction in GluN2A receptor was not sufficient to modify the excitatory drive of glutamate or that the behavioral change was not reliably detectable by the curve-shift paradigm and some other more sensitive paradigm, like the reward-mountain paradigm (Hernandez *et al*, 2010) would be necessary to detect such changes. The likelihood of these hypotheses, however, is very low given that a comparable downregulation in the GluN1 receptor produced a significant reduction in reward pursuit that was readily detectable by the behavioral paradigm that we used.

In accordance with previous results, ventral midbrain microinjection of the NMDA antagonist, PPPA, produced a leftward and upward shift of the pulse-frequency curve reflecting an enhancement of reward and performance (see Bergeron and Rompré, 2013; Ducrot *et al*, 2013). This shows that VM glutamate also exerts a negative modulation on

reward. The main idea to account for the opposite effects of glutamate is that they are mediated by different subtypes of NMDARs localized on different afferent inputs to VM DA neurons or on DA cells themselves (see Supplementary Figure 4). If inhibitory interneurons that maintained a strong inhibitory drive over DA neurons express GluN2A, blockade of these receptors would then allow DA neurons to pass from a silent state into a tonic firing state; this state will increase the probability that a glutamate signal initiates burst firing. Glutamate participates in this form of feed-forward inhibition, altering the plasticity of local inhibitory neurons and dopamine cell firing as *in-vitro* electrophysiological recordings had shown (Bonci and Malenka, 1999; Nugent and Kauer, 2008). But how can we explain the fact that VM microinjection of PPPA, a preferred GluN2A antagonist, still enhanced reward in animals that had a reduction in NMDARs, and particularly in GluN2A subunits? A tantalizing explanation is that downregulation of NMDARs was not sufficient to alter the reward-enhancing effect of the drug. Again, this is rather unlikely as the reduction in GluN1 was sufficient to reduce reward but not the reward-enhancing effect of PPPA. The cellular machinery required for silencing the targeted RNA is primarily located in the nucleus of the cell. Unlike pharmacological manipulations, local brain infusion of siRNA only downregulates receptors of those cells that have their cell bodies located in the VM; it is ineffective at reducing receptors located on afferent terminals. Inhibitory interneurons establish connection with dendritic shafts which render their physiological capacity to be a stronger inhibitory input but make them very important in coordinating neighboring cell activity. This characteristic contrasts with that of inhibitory afferents which establish somatic connections and therefore believed to establish a stronger inhibition (Omelchenko and Sesack, 2009). Thus, our siRNA manipulation might have altered the neural coordination within the VM, but did not dramatically alter the inhibition of DA neurons. If the main inhibitory source comes from VM afferents then PPPA blockade of GluN2A occurs on presynaptic receptors. There is evidence that presynaptic NMDARs are localized in GABAergic terminals of several brain areas including the VM (Paquet and Smith, 2000) and their role seems to modulate the strength of synaptic transmission by varying the likelihood of neurotransmitter release (Pinheiro and Mulle, 2008). In most brain areas, the presence of presynaptic NMDARs contribute to release of inhibitory neurotransmitter in the cerebral cortex (Mathew and Hablitz, 2011; Ren *et al*, 2007), lateral amygdala (Humeau *et al*, 2003), cerebellum (Bidoret *et al*, 2009), entorhinal cortex (Chamberlain *et al*, 2008), and visual cortex (Li *et al*, 2008). In general these studies demonstrate that inhibition can be potentiated by axo-axonic contacts without the need for somatic interneuron firing; such mechanism alters the weight between excitatory and inhibitory inputs and therefore alters the output of the neuronal circuit. The idea of a presynaptic control of GABA release is also supported by our PPPA results where pharmacological blockade of GluN2A NMDARs produced a similar enhancement in reward as the one observed in the control subjects and in previous studies (Bergeron and Rompré, 2013; Ducrot *et al*, 2013), whereas intra-VM downregulation of its mRNA remained without effect.

The rapid effects of ketamine on mood deficit at a dose that acts primarily as NMDAR antagonist (Berman *et al*, 2000) supports the importance of glutamate and its regulation on mood disorders. Although the clinical effect of ketamine is mainly attributed to blockade of GluN2B NMDAR (Fond *et al*, 2014; McGirr *et al*, 2014), our results show that GluN2A-NMDARs located on afferent terminals of the VM are the most likely source of inhibition that modulates reward seeking, suggesting a complex circuitry that was not fully appreciated in prior pharmacological studies. Our results suggest that a dysregulation or an enhancement of VM GluN2A-mediated neurotransmission can lead to severe alteration of mood characterized by anhedonia. It would be important to identify with the use of new technologies, like optogenetics and designer receptors exclusively activated by a designer drug (DREADD), that offers temporal and regional specific control over targeted neural cell types and receptors which GABAergic afferents to the VM regulate brain reward function. One likely candidate is the ventral pallidum which sends dense GABAergic connections to the VM (Kalivas *et al*, 1993), regulates DA neurons firing (Floresco *et al*, 2003) and affects reward-relevant tasks (see (Smith *et al*, 2009) for a review). These strategies may provide important insights into the neural circuitry underlying reward pursuit and would shed light on disorders in which the reward signal is not adequately integrated or weighted as it the case in depression.

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The authors declare no conflict of interest.

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