Methylphenidate Exerts Dose-Dependent Effects on Glutamate Receptors and Behaviors

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Background: Methylphenidate (MPH), a psychostimulant drug used to treat attention-deficit/hyperactivity disorder, produces the effects of increasing alertness and improving attention. However, misuse of MPH has been associated with an increased risk of aggression and psychosis. We sought to determine the molecular mechanism underlying the complex actions of MPH.

Methods: Adolescent (4-week-old) rats were given one injection of MPH at different doses. The impact of MPH on glutamatergic signaling in pyramidal neurons of prefrontal cortex was measured. Behavioral changes induced by MPH were also examined in parallel.

Results: Administration of low-dose (.5 mg/kg) MPH selectively potentiated *N*-methyl-D-aspartate receptor (NMDAR)–mediated excitatory postsynaptic currents (EPSCs) via adrenergic receptor activation, whereas high-dose (10 mg/kg) MPH suppressed both NMDAR-mediated and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor–mediated EPSCs. The dual effects of MPH on EPSCs were associated with bidirectional changes in the surface level of glutamate receptor subunits. Behavioral tests also indicated that low-dose MPH facilitated prefrontal cortex–mediated temporal order recognition memory and attention. Animals injected with high-dose MPH exhibited significantly elevated locomotive activity. Inhibiting the function of synaptosomal-associated protein 25, a key SNARE protein involved in NMDAR exocytosis, blocked the increase of NMDAR-mediated EPSCs by low-dose MPH. In animals exposed to repeated stress, administration of low-dose MPH effectively restored NMDAR function and temporal order recognition memory via a mechanism dependent on synaptosomal-associated protein 25.

Conclusions: These results provide a potential mechanism underlying the cognitive-enhancing effects of low-dose MPH as well as the psychosis-inducing effects of high-dose MPH.

Key Words: AMPA receptors, methylphenidate, NMDA receptors, prefrontal cortex, SNAP-25, stress

Methylphenidate (MPH) is a psychostimulant widely used for the treatment of attention-deficit/hyperactivity disorder (ADHD) in adolescents and adults (1). Therapeutic dose of MPH effectively improves cognitive function and reduces hyperactivity in individuals with ADHD (2) as well as normal human subjects and animals (3,4). However, overdose of MPH produces agitation, restlessness, and hallucinations in humans (5) and hyperlocomotion and impaired cognition in animals (6). Intermediate-term administration of MPH in juvenile rodents was found to induce long-lasting behavioral adaptations (7,8). To achieve therapeutic benefit and minimal side effects, it is suggested that dosing of MPH should be titrated to an optimal level.

The biochemical action of MPH is well characterized. The dopamine transporter (DAT) and norepinephrine transporter (NET) are blocked by MPH, resulting in elevated concentration of dopamine and norepinephrine at synapses (3,9,10). However, the mechanisms by which therapeutic dose of MPH acutely improves cognitive functions and overdose of MPH induces psychosis are unclear.

The prefrontal cortex (PFC) is a key brain region mediating cognitive and executive functions, including working memory, sustained attention, inhibitory response control, and cognitive flexibility (11,12). A delayed maturation in the PFC (13), dysfunction of the frontostriatal circuitry (14), and hypoactivation in the frontal cortex (15,16) have been implicated in individuals with ADHD. Also, the PFC is identified as the primary target of MPH (17). The glutamatergic pyramidal neurons are one of the major cellular constituents in the PFC. Glutamatergic transmission that controls PFC activity is pivotal for cognitive function such as working memory (11,18). Disturbed glutamate receptors are implicated in cognitive dysfunction associated with many mental disorders (19). We speculated that glutamate receptors are potential targets of MPH critically involved in PFC-mediated cognitive functions. In this study, we examined the impact of low-dose versus high-dose MPH on glutamatergic transmission in PFC of adolescent rats and its relevance to behavioral outcomes.

Methods and Materials

Animals and Reagents

Male Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, Indiana). On arrival, animals were allowed 4–5 days to acclimate before the experiments. Rats at the early adolescent period (p25–30) (20) were paired-housed on a 12-hour light-dark cycle and provided ad libitum access to food and water. Rats from more than one litter were included in each treatment to avoid litter effects. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. See Supplementary Methods and Materials in Supplement 1 for details of reagents.

Animal Surgery

The delivery of peptides to the PFC was conducted as we described previously (22). See Supplementary Methods and Materials in Supplement 1 for details.

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Received Oct 24, 2013; revised Mar 17, 2014; accepted Apr 1, 2014.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2014.04.003

Electrophysiologic Recordings

Recordings of evoked synaptic currents in prefrontal cortical slices used standard whole-cell voltage-clamp technique as we described previously (23,24). The paired pulse ratio of *N*-methyl-D-aspartate receptor (NMDAR)–mediated excitatory postsynaptic currents (EPSCs) was calculated as described previously (25). See Supplementary Methods and Materials in Supplement 1 for details.

Biochemical Measurement of Surface and Total Proteins

Surface and total alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) and NMDARs were detected as we described previously (23,24). See Supplementary Methods and Materials in Supplement 1 for details.

Repeated Stress Paradigm

Repeated restraint stress was carried out as we previously described (24,26). Briefly, Sprague-Dawley rats were placed in air-accessible cylinders for 2 hours daily (10:00 AM-12:00 PM) for 5–7 days (starting at p21–23). The container size was similar to the animal size, which made the animal almost immobile in the container. Experiments were performed 24 hours after the last stressor exposure.

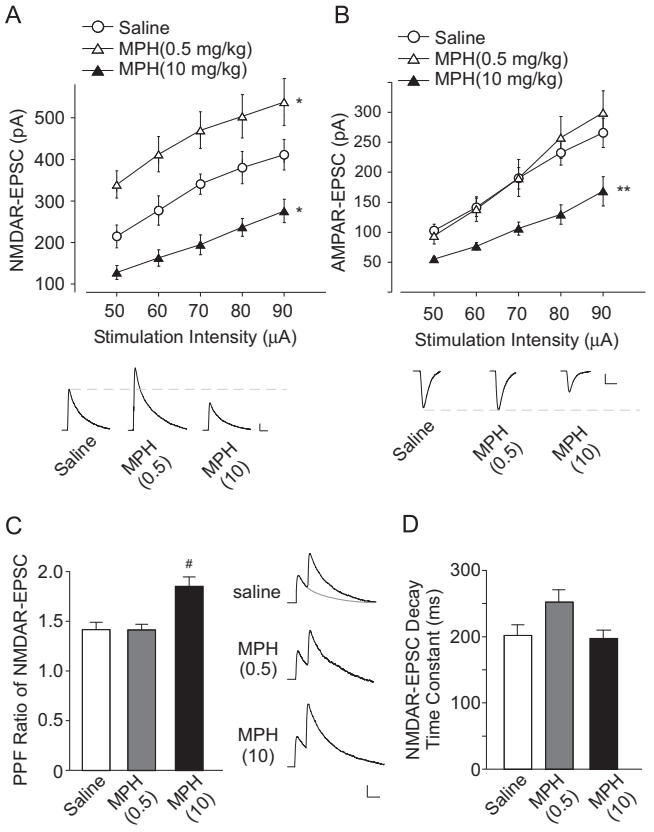

Figure 1. Low-dose methylphenidate (MPH) selectively enhances *N*-methyl-D-aspartate receptor–mediated excitatory postsynaptic currents (NMDAR-EPSC), whereas high-dose MPH reduces both NMDAR-EPSC and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor–mediated excitatory postsynaptic currents (AMPAR-EPSC). Input-output curves of NMDAR-EPSC **(A)** and AMPAR-EPSC **(B)** evoked by a series of stimulation intensities in prefrontal cortex pyramidal neurons from rats with a single intraperitoneal injection of saline, low-dose MPH (.5 mg/kg), or high-dose MPH (10 mg/kg). $^{*}p < .05$, $^{**}p < .01$. Inset shows representative EPSC traces. Scale bars = 50 pA, 100 msec **(A)**; 50 pA, 20 msec **(B)**. Bar graphs show the paired-pulse ratio (PPR) of NMDAR-EPSC (interstimulus interval, 100 msec) **(C)** and decay time constant of NMDAR-EPSC **(D)** in prefrontal cortex pyramidal neurons taken from animals injected with saline, low-dose MPH, or high-dose MPH. Inset shows representative NMDAR-EPSC traces evoked by paired pulses. $^{\#}p < .001$. Scale bar = 50 pA, 100 msec.

Behavioral Testing

Temporal order recognition memory (TORM), a cognitive behavior controlled by PFC (27); locomotor activity; and attentional set-shifting tasks were performed as previously described (24,26,28). See Supplementary Methods and Materials in Supplement 1 for details.

Statistics

Experiments with two groups were analyzed statistically using unpaired Student *t* tests. Experiments with more than two groups were subjected to one-way or two-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests.

Results

In Vivo Administration of Low-Dose MPH Enhances NMDAR-Mediated Synaptic Currents; High-Dose MPH Reduces Glutamatergic Transmission in Cortical Neurons

To investigate the impact of MPH on glutamate signaling, we examined the NMDAR-mediated and AMPAR-mediated EPSCs in the pyramidal neurons of PFC from adolescent male rats (4 weeks old) subjected to a single administration of low-dose (.5 mg/kg) or high-dose (10 mg/kg) MPH. As shown in Figure 1A and B, two-way ANOVA analysis revealed a significant main effect of MPH treatment on NMDAR-mediated or AMPAR-mediated EPSCs (NMDA [$F_{2,150} = 49.5$, $p < .001$]; AMPA [$F_{2,205} = 18.7$, $p < .001$]). Post hoc analysis indicated that low-dose MPH significantly potentiated NMDAR-mediated EPSCs (38%–57% increase, $n = 10$–13 cells/4 rats per group, $p < .05$) but not AMPAR-mediated EPSCs (<10% change, $n = 14$–21 cells/4 rats per group, $p > .05$). In contrast, high-dose MPH markedly reduced both NMDAR-mediated and AMPAR-mediated EPSCs (NMDA, 26%–48% decrease, $n = 10$ cells/4 rats per group, $p < .05$; AMPA acid, 36%–47% decrease, $n = 10$–21 cells/4 rats per group, $p < .01$). These results suggest that MPH exerts a dose-dependent effect on glutamatergic transmission in the PFC.

To test whether the effects of MPH on NMDAR-mediated EPSCs result from a presynaptic or postsynaptic mechanism, we measured the paired pulse ratio, a readout that is affected by the presynaptic transmitter release (29). As shown in Figure 1C, paired pulse ratio was unchanged by low-dose MPH but was significantly elevated by high-dose MPH (saline, 1.42 ± .07, $n = 12$; low-dose MPH, 1.41 ± .06, $n = 13$; high-dose MPH, 1.85 ± .09, $n = 12$ [$F_{2,36} = 11.24$, $p < .001$, ANOVA]). This finding suggests that low-dose MPH regulates glutamatergic transmission mainly via a postsynaptic mechanism, whereas high-dose MPH might affect presynaptic glutamate release or postsynaptic glutamate receptors. In addition, the decay time constant was not statistically changed in animals treated with MPH at low or high doses (saline, 202.0 ± 15.9, $n = 11$; low-dose MPH, 252.0 ± 18.8, $n = 15$; high-dose MPH, 197.4 ± 12.4, $n = 11$ [$F_{2,47} = .93$, $p > .05$], ANOVA), suggesting that elevated NMDAR-mediated EPSCs are mediated by both NR2A and NR2B subunits.

In Vivo Administration of Low-Dose MPH Increases Surface Level of NMDAR Subunits; High-Dose MPH Decreases Surface NMDAR and AMPAR Subunits

Because the surface expression of glutamate receptors could determine the strength of glutamatergic transmission, we performed

biotinylation and Western blotting to examine the surface level of NMDAR and AMPAR subunits in cortical slices from rats treated with saline or MPH. As shown in Figure 2A, low-dose MPH (.5 mg/kg) significantly enhanced the surface level of NMDAR subunits (NR1, 89.0% ± 15.3% increase; NR2A, 117.3% ± 18.4% increase; NR2B, 242.1% ± 47.0% increase; $n = 4$ pairs, $p < .001$, ANOVA) but increased only slightly (not significantly) the surface level of AMPAR subunits (GluR1, 39.0% ± 9.8% increase; GluR2, 36.1% ± 21.3% increase; $n = 4$ pairs, $p > .05$, ANOVA). Total protein levels of all of these glutamate receptor subunits were unchanged by low-dose MPH ($n = 5$ pairs, $p > .05$, ANOVA).

In animals injected with a medium dose of MPH (2.5 mg/kg) (30,31), only the surface NR1 level was modestly increased (36.2% ± 15.8% increase, $n = 4$ pairs, $p < .05$, ANOVA) (Figure 2A), whereas other subunits had no significant change in surface expression. However, a single administration of high-dose MPH (10 mg/kg) induced a substantial reduction of the surface levels of both NMDAR and AMPAR subunits (surface NR1, 45.0% ± 12.6% decrease; surface NR2A, 32.7% ± 7.8% decrease; surface NR2B, 21.9% ± 7.9% decrease; surface GluR1, 34.6% ± 6.3% decrease; surface GluR1, 37.5% ± 10.6% decrease; $n = 7$ pairs, $p < .05$, t test) (Figure 2B), without changing the total levels of glutamate receptors ($p > .05$, t test). Taken together, these results indicate that MPH exerts a dose-dependent bidirectional regulation of the surface expression of glutamate receptors, which may underlie the dual effects of MPH on NMDAR-mediated and AMPAR-mediated synaptic currents.

In Vivo Administration of Low-Dose MPH Facilitates Recognition Memory and Attention; High-Dose MPH Induces Hyperlocomotion

Because cortical glutamatergic transmission mediates many behavioral tasks, we examined the behavioral impact of MPH at different doses in adolescent rats. The TORM, a cognitive process controlled by medial PFC (24,27), was found to be significantly enhanced in animals with a single injection of low-dose (.5 mg/kg) MPH (discrimination ratio [DR] in saline, 29.1% ± 3.8%, $n = 6$; DR in low-dose MPH, 51.1% ± 8.4%, $n = 5$; $p < .05$, t test) (Figure 3A). In the test of perceptual attentional set-shifting, an aspect of attention mediated by medial frontal cortex (28), rats injected with low-dose MPH exhibited selective improvement in the extradimensional shift, taking fewer trials

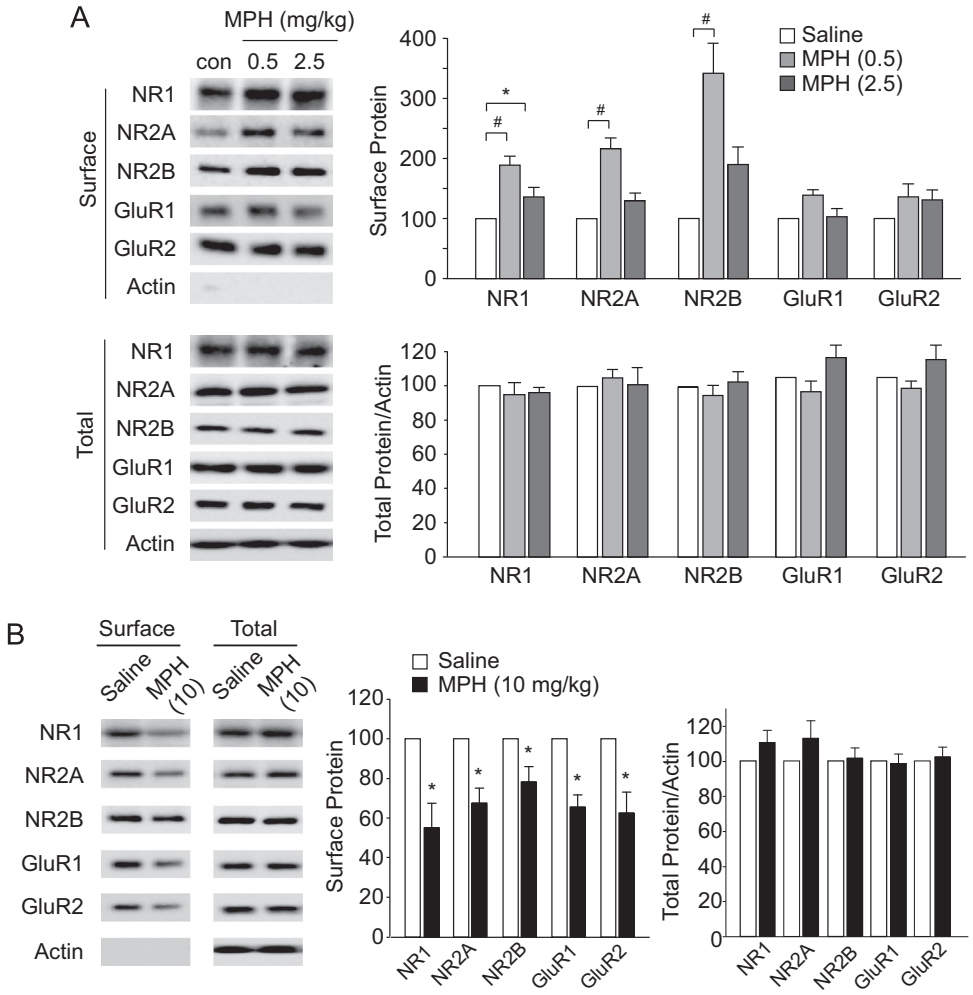

Figure 2. Low-dose methylphenidate (MPH) increases the surface level of *N*-methyl-D-aspartate receptor (NMDAR) subunits, whereas high-dose MPH decreases surface NMDAR and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) expression. **(A)** Immunoblots and quantification analysis of the surface and total NMDAR and AMPAR subunits in prefrontal cortex slices from rats injected with saline or MPH (.5 mg/kg or 2.5 mg/kg, intraperitoneal injection). $^{*}p < .05$, $^{\#}p < .001$. **(B)** Immunoblots and quantification analysis of the surface and total NMDAR and AMPAR subunits from the rats treated with saline or high-dose MPH (10 mg/kg, intraperitoneal injection). $^{*}p < .05$.

to learn the new discrimination (trials to criterion, saline, 13.8 ± .98, $n = 6$; low-dose MPH, 8.6 ± .4, $n = 5$; $p < .01$, t test) (Figure 3B). Locomotor activity was unchanged by the low-dose MPH injection (number of midline crossing, saline, 11.6 ± 1.7, $n = 11$; low-dose MPH, 12.1 ± 2.5, $n = 7$; $p > .05$, ANOVA) (Figure 3D).

A single injection of high-dose (10 mg/kg) MPH profoundly impaired the TORM (DR in saline, 32.0% ± 6.4%, $n = 4$; DR in high-dose MPH, −7.7% ± 14.2%, $n = 9$; $p < .05$, t test) (Figure 3C). A significant increase of locomotor activity was observed in rats injected with high-dose MPH (number of midline crossing, saline, 11.6 ± 1.7, $n = 11$; high-dose MPH, 34.0 ± 3.6, $n = 7$ [$F_{2,22} = 24.5$, $p < .001$, ANOVA]) (Figure 3D). Hyperlocomotion caused these animals to fail to complete the attentional set-shifting task.

Our results are consistent with previous animal and human subject studies showing the behavior changes by MPH at different doses (2,3,5,6). The potentiated NMDAR signaling by low-dose MPH may underlie the enhanced recognition memory (24,27), whereas the reduced glutamate signaling by high-dose MPH may underlie the increased locomotion because NMDAR antagonists profoundly stimulate locomotion in animals (32).

Norepinephrine Neurotransmission Mediates Potentiating Effect of Low-Dose MPH on NMDARs

Given the positive effects of low-dose MPH on NMDARs and cognitive behaviors, we next examined the molecular mechanisms underlying low-dose MPH. It is known that MPH blocks NET and DAT in the presynaptic terminals, resulting in elevated synaptic levels of these neurotransmitters (3,9,10). To determine whether dopaminergic or adrenergic neurotransmission is involved, we examined NMDAR-mediated EPSCs in animals treated with specific NET or DAT inhibitors. As shown in Figure 4A, animals injected with maprotiline (20 mg/kg, intraperitoneal injection), a highly selective NET inhibitor (33), exhibited enhanced NMDAR-mediated EPSCs (47%–57% increase, $n = 11$–12 cells/3 rats per group [$F_{1,84} = 42.6$, $p < .01$, ANOVA]), similar to what was found in animals injected with low-dose MPH. Animals injected with a higher dose of maprotiline (50 mg/kg) exhibited reduced NMDAR-mediated EPSCs (Figure S1 in Supplement 1). The dose-dependent effects of maprotiline are parallel with the effects of MPH. In contrast, animals injected with GBR-12909 (5 mg/kg, intraperitoneal injection), a highly selective DAT inhibitor (34), showed unaltered NMDAR-mediated EPSCs ($n = 6$–9 cells/3 rats per group [$F_{1,65} = 1.76$, $p > .05$, ANOVA]) (Figure 4B).

To confirm further that MPH regulates NMDAR responses by preferentially targeting adrenergic neurotransmission, we pretreated animals with prazosin, an antagonist of α_1-adrenergic receptor (35), and yohimbine, an antagonist of α_2-adrenergic receptor (36). As shown in Figure 4C, blocking adrenergic receptors with prazosin and yohimbine completely abolished the effect of low-dose MPH on NMDAR-mediated EPSCs (−12% to 11% increase, $n = 8$–10 cells/3 rats per group [$F_{1,160} = .29$, $p > .05$, ANOVA]). In contrast, when applying SCH 23390, a D_1-class receptor antagonist (37), and sulpiride, a D_2-class receptor antagonist (38), the enhancement of NMDAR-mediated EPSCs by low-dose MPH remained the same (29%–60% increase, $n = 8$–9 cells/3 rats per group [$F_{1,98} = 76.5$, $p < .01$, ANOVA]) (Figure 4D). These results suggest that low-dose MPH potentiates NMDAR-mediated EPSCs primarily by inhibiting norepinephrine transporter and activating adrenergic receptors.

Synaptosomal-Associated Protein 25 Mediates Enhancement of NMDARs and Cognition by Low-Dose MPH

The potentiated NMDAR currents by low-dose MPH are accompanied by elevated surface expression of NMDARs, suggesting that the membrane delivery of NMDARs might be affected. It is known that SNARE (soluble *N*-ethylmaleimide-sensitive factor [NSF] attachment protein receptor) proteins are the key protein family involved in the membrane fusion in eukaryotic cells (39). In particular, synaptosomal-associated protein 25 (SNAP-25), a SNARE protein, has been implicated in the incorporation of NMDARs to postsynaptic membrane (40,41). We examined the role of SNAP-25 in the potentiation of surface NMDARs by low-dose MPH. Because intravenous injection can reliably deliver TAT peptides into central nervous system neurons (22,42,43), we gave animals an intravenous injection of the SNAP-25 blocking peptide (.6 pmol/g) 30 min before MPH administration. This peptide mimics the N-terminal domain of SNAP-25 and disrupts the interaction of

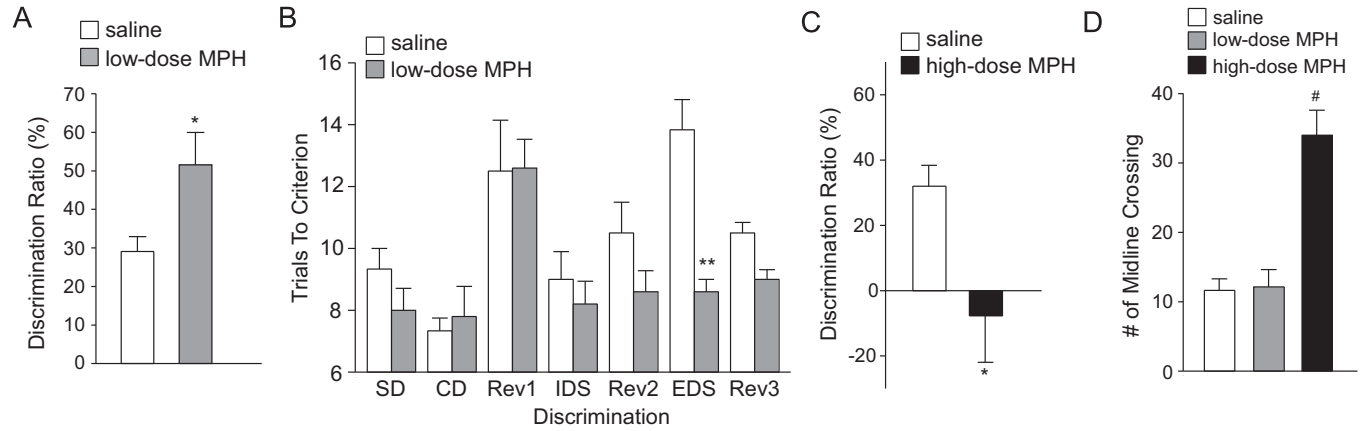

Figure 3. Low-dose methylphenidate (MPH) **(A, B)** enhances temporal order recognition memory and attentional set-shifting, whereas high-dose MPH **(C)** elevates locomotor activity. **(A, C)** Bar graphs (mean ± SEM) show the discrimination ratio of temporal order recognition memory tasks in animals treated with saline versus MPH, .5 mg/kg, intraperitoneal injection **(A)**; 10 mg/kg, intraperitoneal injection **(C)**. *$p < .05$. **(B)** Bar graph shows the number of trials to criterion (six consecutive correct trials) for each discrimination stage of the attentional set-shifting task in animals treated with saline or low-dose MPH (.5 mg/kg, intraperitoneal injection). **$p < .01$. **(D)** Bar graph shows the number of midline crossing in locomotion apparatus for animals injected with saline versus MPH (low or high dose). #$p < .001$. CD, compound discrimination; EDS, extradimensional shift; IDS, intradimensional shift; Rev, reversal discrimination; SD, simple discrimination.

SNAP-25 with NSF, which is critical for the assembly and disassembly cycle of SNARE complexes (21,44). As shown in Figure 5A, two-way ANOVA analysis revealed a significant main effect on treatments [$F_{3,157} = 25.7$, $p < .001$]. Post hoc tests indicated that the enhancing effect of low-dose MPH on NMDAR-mediated EPSCs was blocked by the SNAP-25 blocking peptide (2%–9% increase, $n = 8$–10 cells/4 rats per group, $p > .05$) but not a scrambled peptide (43%–77% increase, $n = 8$–13 cells/4 rats per group, $p < .05$). Biotinylation assays also showed that the increasing effects of low-dose MPH on surface NMDAR subunits was abolished by SNAP-25 blocking peptide (surface NR1, 9.8% ± 10.4% decrease; surface NR2A, 27.4% ± 9.7% decrease; surface NR2B, 13.7% ± 21.0% decrease; $n = 4$ pairs, $p > .05$, ANOVA) (Figure 5B,C) but not the scrambled peptide (surface NR1, 73.3% ± 10.6% increase; surface NR2A, 117.2% ± 43.8% increase; surface NR2B, 218% ± 47.9% increase; $n = 4$ pairs, $p < .01$, ANOVA). Taken together, these results suggest that SNAP-25 mediates the enhanced exocytosis of NMDARs by low-dose MPH.

Next, we examined the role of SNAP-25 in MPH regulation of cognitive functions. As shown in Figure 5D, in rats injected with SNAP-25 peptide, low-dose MPH failed to enhance TORM (DR, SNAP-25 peptide + MPH, 29.4% ± 5.4%, $n = 5$, control peptide + MPH, 48.3% ± 5.3%, $n = 6$; $p < .05$, t test). Injection of SNAP-25 peptide blocked the beneficial effect of low-dose MPH in the attentional set-shifting task, resulting in more trials to achieve the criterion in extradimensional shift (trials to criterion, control peptide + MPH, 8.6 ± .7, $n = 5$; SNAP-25 peptide + MPH, 12.0 ± .8, $n = 5$; $p < .05$, t test).

To avoid potential nonspecific effects with the systemic administration of SNAP-25 peptide, we performed stereotactic injection of peptides to PFC bilaterally, followed by intraperitoneal MPH injection. Electrophysiologic recordings showed that PFC infusion of SNAP-25 peptide (3 pmol/side) blocked the increase of NMDAR-mediated EPSCs by low-dose MPH (SNAP-25 peptide, ~10% increase; control peptide, ~55% increase; $n = 16$–30 cells/4 rats per group [$F_{3,382} = 22.3$, $p < .001$, ANOVA]) (Figure 6A). Behavioral tests indicated that PFC infusion of SNAP-25 peptide blocked the increase of TORM by low-dose MPH (DR, SNAP-25 peptide, ~0-fold increase; control peptide, ~.8-fold increase; $n = 5$ pairs [$F_{3,20} = 5.89$, $p < .01$, ANOVA]) (Figure 6B).

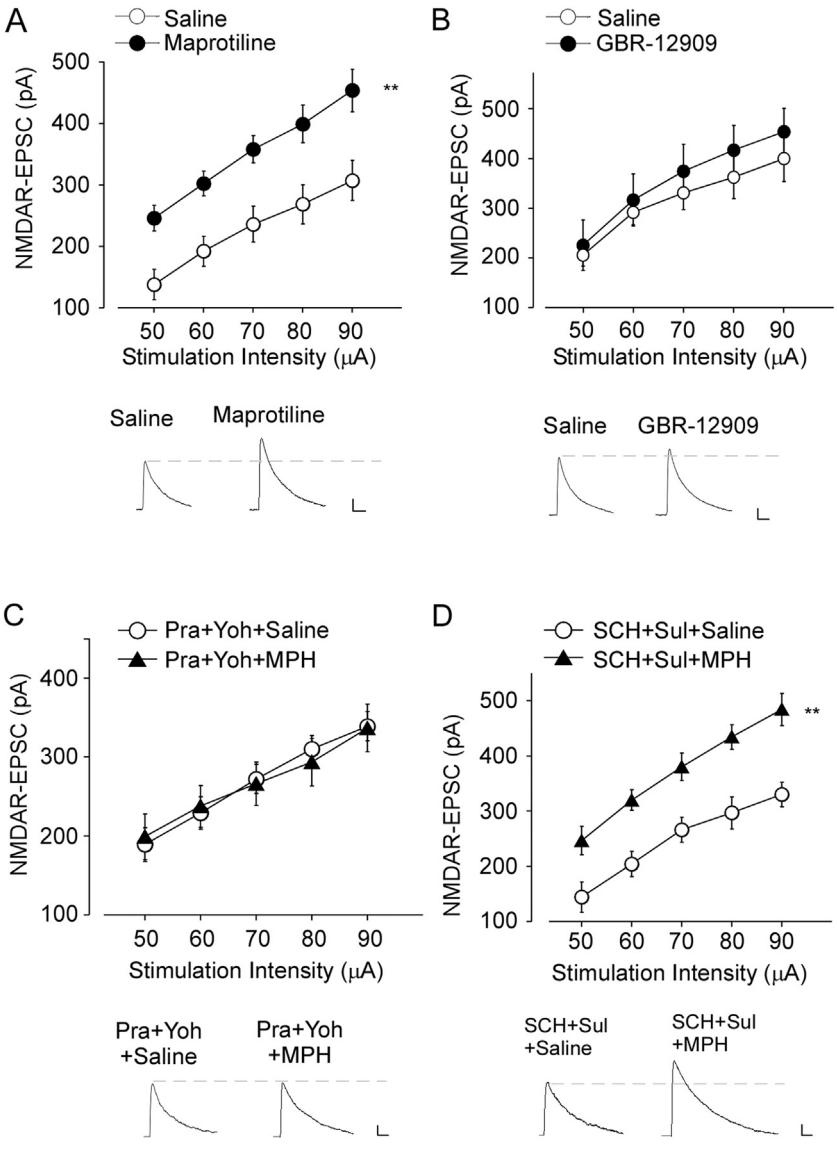

Figure 4. Low-dose methylphenidate (MPH) potentiates *N*-methyl-D-aspartate receptor–mediated excitatory postsynaptic currents (NMDAR-EPSC) via norepinephrine reuptake inhibition and adrenergic receptor activation. **(A, B)** Summarized input-output curves of NMDAR-EPSC in prefrontal cortex pyramidal neurons from rats treated with saline, maprotiline, 20 mg/kg, intraperitoneal injection **(A)**, or GBR-12909, 5 mg/kg, intraperitoneal injection **(B)**. Inset shows representative traces of NMDAR-EPSC. Scale bar = 50 pA, 200 msec. $^{**}p < .01$. **(C)** Summarized input-output curves of NMDAR-EPSC in saline-injected versus MPH-injected (.5 mg/kg, intraperitoneal injection) rats pretreated with prazosin (Pra) and yohimbine (Yoh) (Pra, 1 mg/kg, and Yoh, 5 mg/kg, intraperitoneal injection, injected .5 hour before MPH injection). Inset shows representative NMDAR-EPSC traces. Scale bar = 50 pA, 100 msec. **(D)** Summarized input-output curves of NMDAR-EPSC in saline-injected versus MPH-injected rats pretreated with SCH 23390 (SCH) and sulpiride (Sul) (SCH, 1 mg/kg, and Sul, 50 mg/kg, intraperitoneal injection, injected .5 hour before MPH injection). Inset shows representative traces. Scale bar = 50 pA, 100 msec. $^{**}p < .01$.

These data suggest that SNAP-25 in the PFC is critical for the potentiation of NMDARs and cognition by low-dose MPH.

Because protein kinase C (PKC) phosphorylation of SNAP-25 could affect the surface expression of NMDARs (40), we also examined the involvement of PKC in MPH effects. Low-dose MPH failed to enhance NMDAR-mediated EPSCs in the presence of a PKC inhibitor, chelerythrine (3 mg/kg, intraperitoneal injection) (45) (~7% increase, $n = 10–12$ cells/3 rats per group [$F_{1,100} = .59$, $p > .05$, ANOVA]) (Figure S2 in Supplement 1). These results suggest that PKC, which may be activated by low-dose MPH, is important for facilitating SNAP-25-dependent NMDAR surface delivery.

Low-Dose MPH Rescues Impaired NMDAR and Cognitive Function in Animals Exposed to Repeated Stress

Because low-dose MPH enhances NMDAR function and memory processes in naïve animals, we examined whether low-dose MPH restores impaired NMDAR and cognitive function in animals exposed to repeated stress (24). A significant main effect was found in treatment groups [$F_{5,277} = 159.8$, $p < .001$, two-way ANOVA] (Figure 7A). Post hoc tests indicated that NMDAR-mediated EPSCs were markedly decreased in PFC pyramidal neurons from young male rats exposed to repeated (7 days) restraint stress (76%–96% reduction, $n = 13–17$ cells/4 rats per

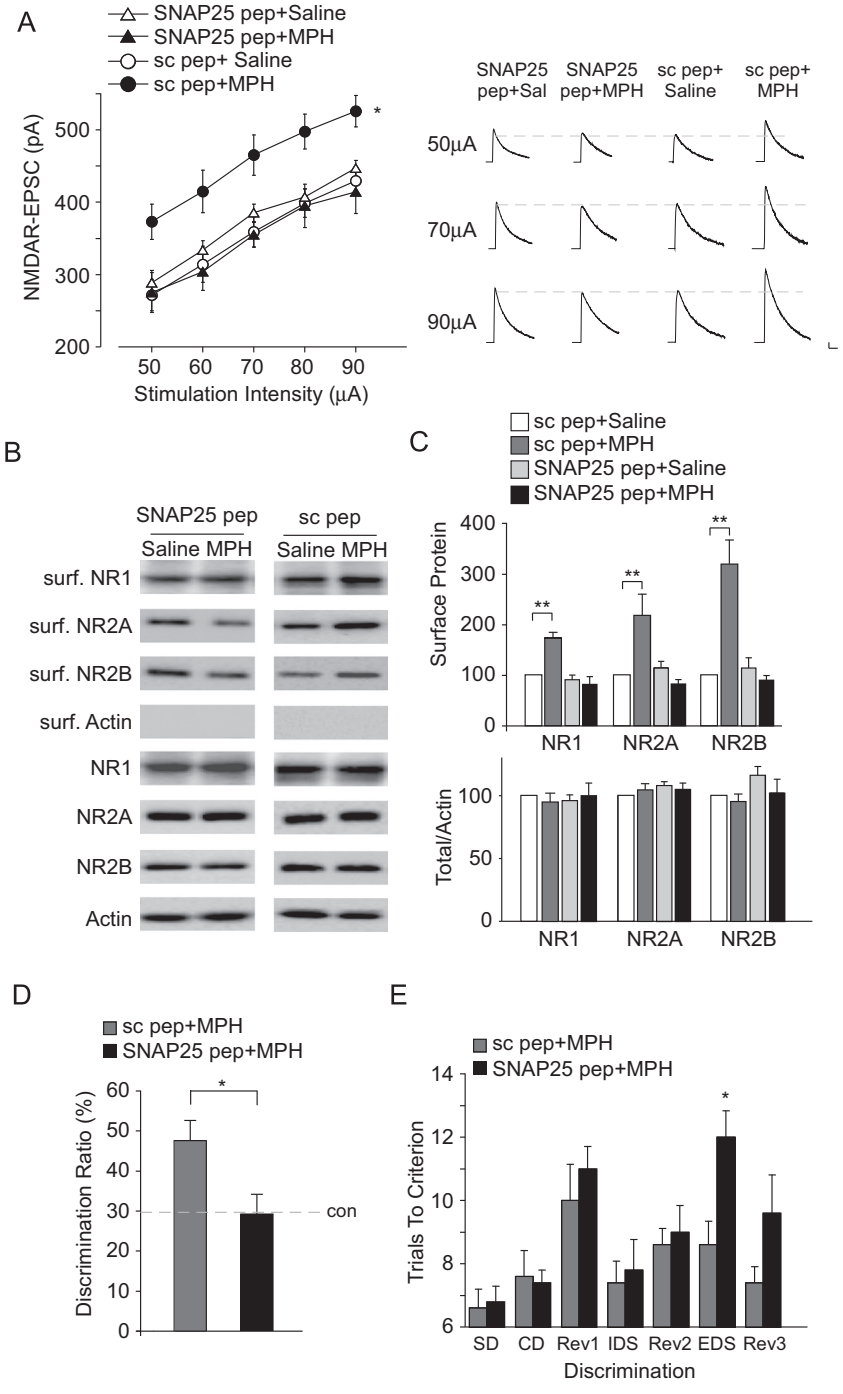

Figure 5. Synaptosomal-associated protein 25 (SNAP-25) participates in the potentiation of *N*-methyl-D-aspartate receptor–mediated excitatory postsynaptic currents (NMDAR-EPSC) and cognitive functions by low-dose MPH. **(A)** Summarized input-output curves of NMDAR-EPSC in saline-injected versus MPH-injected (.5 mg/kg, intraperitoneal injection) rats pretreated with SNAP-25 blocking peptide (SNAP-25 pep, .6 pmol/g, intravenous injection) or a scrambled peptide (sc pep, .6 pmol/g, intravenous injection). Inset shows representative EPSC traces. Scale bar = 50 pA, 200 msec. $^*p < .05$. Immunoblots **(B)** and quantification analysis **(C)** of the surface and total NMDAR subunits in rat prefrontal cortex slices from saline-injected versus MPH-injected rats pretreated with SNAP-25 blocking peptide or a scrambled peptide. $^{**}p < .01$. Bar graphs show the discrimination ratio of temporal order recognition memory tasks **(D)** and number of trials to criterion at each discrimination stage of the attentional set-shifting task **(E)** in MPH-injected (.5 mg/kg, intraperitoneal injection) animals pretreated with a scrambled peptide or SNAP-25 blocking peptide. CD, compound discrimination; EDS, extradimensional shift; IDS, intradimensional shift; Rev, reversal discrimination; SD, simple discrimination. $^*p < .05$.

group, $p < .001$), consistent with our previous results (29,30). A single injection of low-dose MPH (.5 mg/kg, intraperitoneal injection) after the repeated stress exposure restored NMDAR-mediated EPSCs to the control level ($n = 13–17$ cells/4 rats per group, $p > .05$). The recovery was blocked in animals pretreated with SNAP-25 blocking peptide (.6 pmol/g, intravenous injection, 37%–81% reduction, $n = 8–18$ cells/3 rats per group, $p < .001$).

Behavioral studies found that the repeatedly stressed rats had impaired TORM, which was recovered by a single injection of low-dose MPH (DR, stress + saline, 6.6% ± 7.0%, $n = 7$; stress + MPH, 56.3% ± 11.4%, $n = 9$ [$F_{2,23} = 5.7$, $p < .01$, ANOVA]) (Figure 7B). The recovering effect of low-dose MPH was abolished by pretreatment with SNAP-25 blocking peptide (DR, stress + SNAP-25, 3.7% ± 10.9%, $n = 4$; stress + SNAP-25 + MPH, 1.2% ± 5.8%, $n = 6$, $p > .05$). The total exploration time in the two sample phases and the subsequent test trial was unchanged by any of these treatments ($p > .05$, ANOVA) (Figure 7C). These results suggest that low-dose MPH is capable of rescuing the impaired NMDAR function and cognitive deficits in stressed animals through a mechanism involving SNAP-25.

Discussion

Despite the widespread use of MPH as a cognitive enhancer, little is known about the causal mechanism underlying its behavioral actions. The dopamine and adrenergic system has been primarily studied for MPH; however, considering that the glutamatergic system is critically involved in synaptic plasticity and cognitive processes (16,25), regulation of glutamate signaling might underlie the neuronal mechanism of MPH. Because MPH is commonly prescribed for the treatment of ADHD in children and adolescents, it is important to use adolescent rats to study the effect of MPH exposure in early life. In the present study, we found that a low dose of MPH that yields clinically relevant plasma levels (3) remarkably potentiated NMDAR-mediated synaptic responses and the surface expression of NMDARs in adolescent rats. We also found that a high dose of MPH substantially decreased glutamatergic transmission, via a mechanism involving both decreasing presynaptic glutamate release probability and reducing postsynaptic glutamate receptor surface expression. In contrast, a previous study showed that 1 hour after a single injection of MPH (1 mg/kg, intraperitoneal injection), NMDAR-mediated currents and NMDAR total protein levels were decreased in the PFC of juvenile rats (p15–25) (46). We have not seen such reducing effects with MPH (1 mg/kg) injection.

In parallel with the dose-dependent bidirectional effects of MPH on PFC glutamatergic signaling, our behavioral studies found that low-dose MPH enhanced TORM and attentional set-shifting, whereas high-dose MPH impaired TORM and elevated locomotor activity. These results are consistent with previous work in animals and human subjects showing that the therapeutic dose of MPH effectively improves cognitive functions (2,3), whereas overdose of MPH is associated with aggression and hyperactivity (4). Given that children with ADHD exhibit prefrontal hypoactivity (15,16), the elevated NMDAR function by low-dose MPH might underlie its beneficial effects on memory, attention, and other cognitive aspects. However, because NMDAR antagonists, such as phencyclidine or ketamine, can lead to the formation of psychotic symptoms, including hyperlocomotion (32,47), the reduced glutamate signaling by high-dose MPH might underlie its psychosis-inducing effects.

It is known that MPH acts as a NET and DAT inhibitor, and our data indicate that low-dose MPH potentiates NMDAR functions mainly through the norepinephrine system. Consistently, MPH is shown to have higher affinity for NET than DAT in vitro (48), to affect norepinephrine preferentially at low doses in vivo (49), and to occupy NET significantly at clinically relevant doses in humans (10). The norepinephrine system has been implicated in many PFC functions, including working memory, attention, and emotional control (50,51). An in vitro study suggested that the enhancement of NMDAR-mediated EPSCs by bath application of MPH (50 μmol/L) in PFC slices is mediated by sigma-1 receptors instead of adrenergic or dopamine receptors (31). The inconsistency may be due to different routes of drug administration and different MPH concentrations.

Because low-dose MPH increases NMDAR surface expression, we have examined the potential molecule downstream of adrenergic receptors that is involved in NMDAR exocytosis. The SNARE proteins, comprising SNAP-25/23, syntaxins, and synaptobrevin/vesicle-associated membrane proteins, form SNARE complexes in the late stage of synaptic vesicle exocytosis mediating vesicle docking and fusion (39). A key component of SNARE complex expressed in excitatory neurons (52), SNAP-25 participates in the delivery of NMDAR vesicles at postsynaptic sites (21,40,41). More importantly, dysfunction of SNAP-25 is linked to various human mental disorders, such as schizophrenia, ADHD,

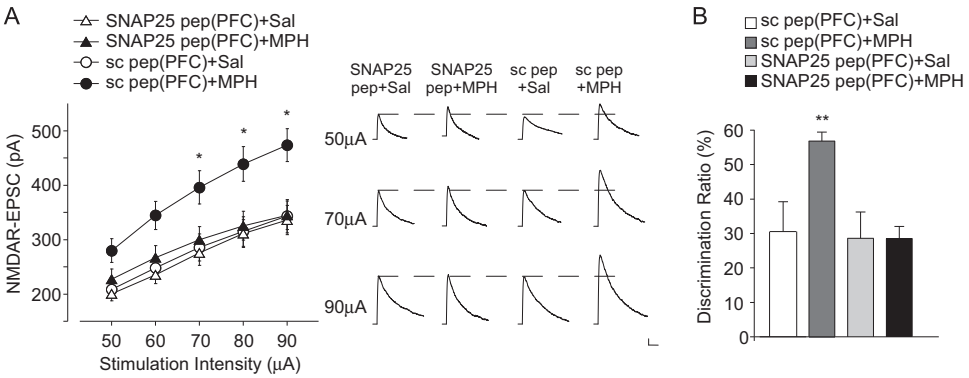

Figure 6. Prefrontal cortex (PFC) infusion of synaptosomal-associated protein 25 (SNAP-25) blocking peptide abolished low-dose methylphenidate (MPH)–induced enhancement of *N*-methyl-D-aspartate receptor–mediated excitatory postsynaptic currents (NMDAR-EPSC) and temporal order recognition memory. Summarized input-output curves of NMDAR-EPSC **(A)** and bar graph (mean ± SEM) show the discrimination ratio of temporal order recognition memory tasks **(B)** in saline (Sal)-injected versus MPH-injected (.5 mg/kg, intraperitoneal injection) animals with PFC infusion of a scrambled peptide (sc pep) or SNAP-25 blocking peptide (pep) (3 pmol/site). Inset shows representative excitatory postsynaptic current traces. Scale bar = 50 pA, 200 msec. $^*p < .05$, $^{**}p < .01$.

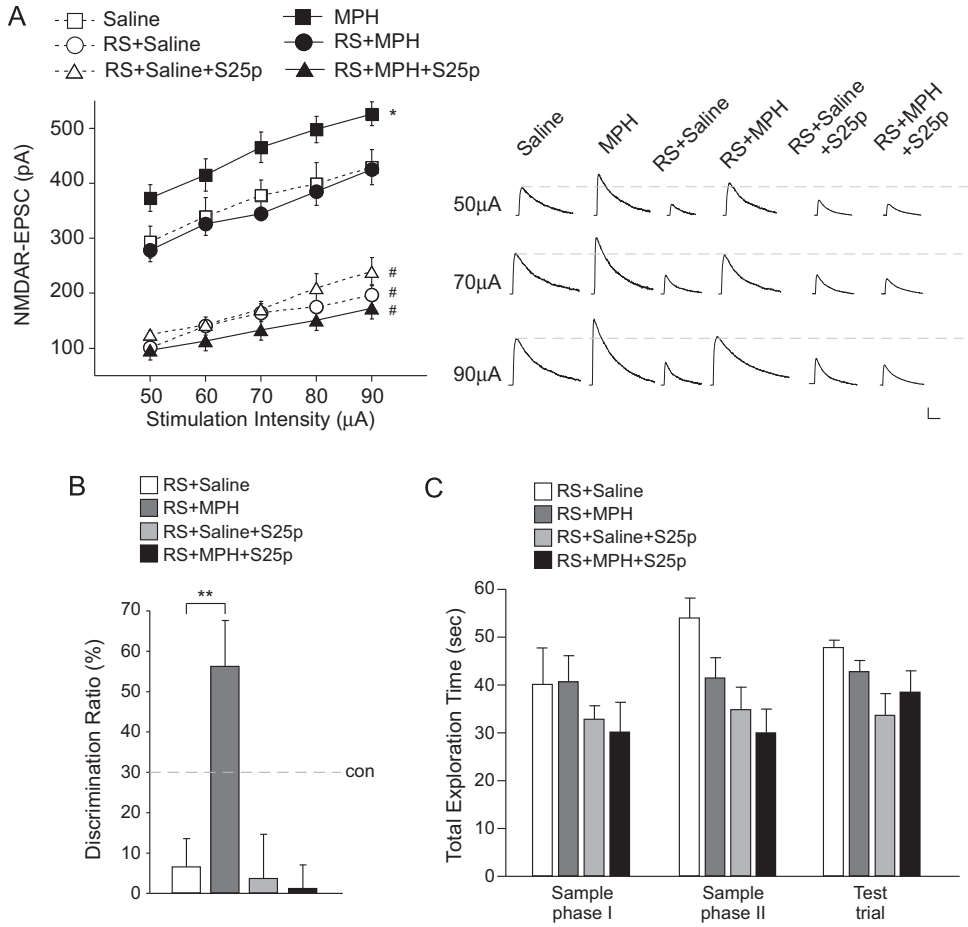

Figure 7. Low-dose methylphenidate (MPH) restores impaired *N*-methyl-D-aspartate receptor function and recognition memory in animals exposed to repeated stress. **(A)** Summarized input-output curves of *N*-methyl-D-aspartate receptor–mediated excitatory postsynaptic currents (NMDAR-EPSC) in control or repeatedly stressed (RS) rats treated with saline or MPH (.5 mg/kg, intraperitoneal injection) without or with pretreatment with synaptosomal-associated protein 25 (SNAP-25) peptide (S25p, .6 pmol/g, intravenous injection). $^{*}p < .05$, $^{\#}p < .001$. Inset shows representative NMDAR-EPSC traces. Scale bar = 50 pA, 200 msec. **(B, C)** Bar graphs (mean ± SEM) show the discrimination ratio **(B)** and total exploration time **(C)** of temporal order recognition memory tasks in repeatedly stressed animals injected with saline or MPH without or with pretreatment with SNAP-25 peptide. $^{**}p < .01$.

and early-onset bipolar disorder (53–55). Mice carrying a deletion of SNAP-25 gene have been used as an ADHD animal model (56). In the present study, we demonstrate that SNAP-25 mediates the increase of NMDAR exocytosis by low-dose MPH.

In addition to enhancing cognitive function, MPH is able to combat stress (57). Chronic or severe stress is a trigger for many mental illnesses (58). Previous studies have found that repeated stress suppresses PFC glutamatergic signaling, resulting in cognitive impairment (24,26,59,60). In this study, we found that low-dose MPH restored impaired NMDAR function and object recognition memory in animals exposed to repeated stress through a mechanism dependent on SNAP-25-mediated exocytosis of NMDARs. This study provides a molecular mechanism for MPH to be used as a potential therapeutic strategy for stress treatment.

A remaining question is the long-term effect of MPH on glutamatergic transmission and PFC-dependent cognitive function. Previous studies suggested that glutamatergic pathways are involved in short-term and long-term MPH regulation of locomotion in adult rats (61), and exposing rats to MPH during the adolescent period results in increased stress reactivity (7). Whether PFC network activity is altered after long-term exposure to different doses of MPH is a subject for future study.

In conclusion, the present study shows that administration of low-dose MPH potentiates NMDAR trafficking and function, enhances PFC-mediated cognition, and counteracts the detrimental effects of repeated stress in adolescent rats via a mechanism involving adrenergic receptors and SNAP-25. In contrast, administration of high-dose MPH suppresses PFC glutamatergic transmission and induces hyperlocomotion. This study provides a potential mechanism underlying the cognitive-enhancing effects of low-dose MPH and the psychosis-inducing effects of high-dose MPH.

This work was supported by the National Institutes of Health (Grant Nos. DA037618, MH085774, MH101690 to ZY) and the National Natural Science Foundation of China (Grant Nos. 81220108010, 81171197 to GJ-C). We thank Xiaoqing Chen for her excellent technical support. Dr. Yulei Deng provided kind help in some experiments.

The authors report no biomedical financial interests or potential conflicts of interest.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2014.04.003.

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