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Dopaminergic neuromodulation of prefrontal cortex activity requires the NMDA receptor co-agonist D-serine

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Short title: Dopamine tunes D-serine modulation of PFC functions

56 **Significance**

57 Dopamine and glutamate in the prefrontal cortex are important substrates of higher cognitive
58 functions, which are impaired in neuropsychiatric disorders. As regards glutamatergic
59 pathways, a role for the NMDA receptor co-agonist D-serine has been highlighted yet its
60 relationship to dopaminergic transmission remains unclear. In this study we reveal that D-
61 serine plays a pivotal role in the modulation by dopamine of NMDA receptor activity and
62 cognitive performance in the prefrontal cortex. Comprehensive evidence for this interaction is
63 provided at the synaptic, neuronal, network, and behavioral level. These observations are of
64 relevance to the pathophysiology and treatment of cognitive impairment in numerous disorders
65 involving disruption of the frontocortical dialogue between dopamine and glutamate.

66

67 **Abstract**

68 Prefrontal control of cognitive functions critically depends upon glutamatergic transmission
69 and NMDA receptors (NMDARs), the activity of which is regulated by dopamine. Yet, whether
70 the NMDA receptor co-agonist D-serine is implicated in the dopamine-glutamate dialogue in
71 prefrontal cortex (PFC) and other brain areas remains unexplored. Here, using
72 electrophysiological recordings, we show that D-serine is required for the fine-tuning of
73 glutamatergic neurotransmission, neuronal excitability and synaptic plasticity in the PFC
74 through the actions of dopamine at D₁- and D₃- receptors. Using *in vivo* microdialysis, we show
75 that D₁- and D₃- receptors exert a respective facilitatory and inhibitory influence on extracellular
76 levels and activity of D-serine in the PFC, actions expressed primarily *via* the cAMP/PKA
77 signalling cascade. Further, using fMRI imaging and behavioral assessment, we show that D-
78 serine is required for the potentiation of cognition by D₃R blockade as revealed in a test of
79 novel object recognition memory. Collectively, these results unveil a key role for D-serine in
80 the dopaminergic neuromodulation of glutamatergic transmission and PFC activity, findings
81 with clear relevance to the pathogenesis and treatment of diverse brain disorders involving
82 alterations in dopamine-glutamate cross-talk.

83

84 Introduction

85 The prefrontal cortex (PFC) supports the higher-order and top-down coordination of
86 complex behaviors, including executive function, working memory and social interactions (1).
87 Dopaminergic (DAergic) inputs exert a marked influence on the activity of frontocortical circuits
88 and the dynamic regulation of background dopamine (DA) levels allows for the optimization of
89 PFC cognitive performances (2). Interestingly, DA exerts opposite effects on glutamatergic
90 activity through activation of D₁-type (D₁- and D₅-) and D₂-type (D₂-, D₃-, and D₄-) receptors,
91 respectively (3, 4). Specifically, D₁ receptor (D₁R) activation increases neuronal excitability
92 and synaptic efficacy through modulation of *N*-methyl D-aspartate receptors (NMDARs), whilst
93 D₂ receptor (D₂R) signaling produces opposite effects (4). Furthermore, activation of D₁R
94 promotes Long-Term Potentiation (LTP) in the PFC (5, 6) whereas D₂R stimulation is required
95 for inducing Long-Term Depression (LTD) (6). These observations support the current view
96 that distinct cortical DA receptors differentially regulate cognitive functions through the
97 modulation of NMDARs. However, the underlying mechanisms still await clarification. While
98 D₁R activation in the PFC consistently facilitates working memory and executive function (7),
99 the significance of PFC-localized D₂-type receptors is more complex inasmuch as D₂R and
100 D₃R subtypes enact contrasting roles: activation of D₂R favors cognitive processes whereas
101 D₃R stimulation exerts a negative impact on cognition (8). Accordingly, blockade of D₃Rs
102 results in a broad-based positive influence upon cognition, including improvements in social
103 novelty discrimination and novel object recognition (NOR) and a reduction in the cognitive
104 deficits associated with a developmental model for schizophrenia (SCZ) (9, 10). Moreover, it
105 has been proposed that DAR and NMDARs can form clusters at the surface of neurons (11)
106 and that their reciprocal dialogue relies on intracellular signaling cascades involving protein
107 kinases (12). Whether functional DA-NMDAR cross-talk involves others mechanisms is not
108 known.

109 Activation of the canonical GluN1-GluN2 containing NMDARs requires binding of both
110 glutamate and a co-agonist, either glycine or D-serine (13). We have previously shown that
111 D-serine rather than glycine is the primary endogenous co-agonist of synaptic NMDARs at
112 glutamatergic synapses connecting pyramidal neurons in the PFC of adult rats (14). This is
113 important since genetic linkage and association studies show that the genes encoding the D-
114 serine producing and degrading enzymes serine racemase (SR) and D-amino acid oxidase
115 (DAAO), respectively, are risk genes for SCZ (15, 16). Accordingly, serum and cerebrospinal
116 fluid levels of D-serine are reduced in SCZ patients (16-18). Moreover, clinical trials of D-serine
117 supplementation or the administration of DAAO inhibitors have yielded encouraging results

118 (17, 18). An involvement of D-serine in SCZ is further supported by animal studies showing
119 that multiple risk pathways for SCZ converge in SR deficient mice (19). Yet, our understanding
120 of the significance of D-serine to the pathology and symptoms of SCZ remains limited, and the
121 question of how it interacts with DA signaling remains to be resolved. This issue is, moreover,
122 of broader pertinence in view of accumulating evidence that a malfunction of NMDAR is central
123 to the pathophysiology of multiple brain disorders (20).

124 In the current study, we sought to determine whether and how D-serine and DA interact
125 in the PFC. We show that D-serine is indeed required for the dopaminergic modulation of
126 NMDARs in the PFC, as expressed in measures of neuronal excitability, synaptic plasticity
127 and cognitive function, providing a novel framework for understanding PFC integrated DA-
128 glutamate crosstalk under physiological and pathological conditions.

129

130 **Results**

131 **Modulation of NMDAR and neuronal excitability by DA involves D-serine**

132 To test the hypothesis that D-serine may be implicated in DA modulation of NMDAR
133 functions, we first investigated whether DA modulations of isolated synaptic NMDAR-mediated
134 excitatory postsynaptic currents (NMDA-EPSCs) recorded at +40 mV and -70 mV in adult rat
135 prelimbic cortex (PrL) layer 5 pyramidal neurons (Fig.1 A-C) require D-serine. Low
136 concentrations of DA (1 μ M) potentiated NMDA-EPSCs by $73.9 \pm 14.1\%$ (Fig.1D, I, *SI*
137 *appendix* Fig.S8) while higher concentrations (10 μ M) decreased NMDA-EPSCs by $44.7 \pm$
138 6.7% (Fig.1D, I, *SI appendix* Fig.S8) confirming previous studies (21, 22). To determine if
139 these bidirectional modulations of NMDA-EPSCs by DA imply changes in the occupancy of
140 NMDARs by a co-agonist, we monitored the effects of DA after pre-saturation of the co-agonist
141 binding site by exogenous D-serine (100 μ M) (Fig.1E, I). This resulted in the occlusion of DA-
142 effected NMDA-EPSCs modulation. We then directly addressed the implication of D-serine in
143 the DA modulation of NMDA-EPSCs by treating the slices with *Rhodotorula gracilis* D-amino
144 acid oxidase (*RgdAAO*) (0.2 U/mL) to specifically deplete endogenous D-serine (14, 23)
145 (Fig.1F, I). Akin to the effect of saturating the D-serine binding site, removal of D-serine with
146 *RgdAAO* markedly reduced the potentiating and inhibitory effect of 1 μ M and 10 μ M DA,
147 respectively. As an important control we also confirmed, using enzymatic assays, that DA is
148 not a substrate or an inhibitor for *RgdAAO* (*SI appendix* Fig.S1). These data therefore indicate
149 that positive and negative modulations of NMDAR by exogenous DA requires D-serine.

150 We next assessed whether D-serine is involved in the regulation of NMDA-EPSCs by
151 endogenous DA. To this end, we first studied the effects of the DA reuptake inhibitor
152 GBR12909 on NMDA-EPSCs. Bath applied GBR12909 (0.5 μ M) potentiated NMDA-EPSCs
153 (Fig.1G, I) similarly to exogenous low DA (1 μ M). Remarkably, performing this experiment
154 under D₁R blockade by addition of the antagonist SCH39166 (1 μ M) not only abolished the
155 potentiating effect of GBR12909, but also unmasked a depressant action on NMDA-EPSCs
156 (Fig. 1G, I). To directly address whether the potentiating effect of GBR12909 requires D-serine
157 acting on NMDARs, we next recorded NMDAR currents on PFC slices isolated from mice
158 lacking the D-serine producing enzyme serine racemase (SR^{-/-}), in which D-serine content is
159 ~10% of wild-type (SR^{+/+}) mice (24). Bath application of GBR12909 (0.5 μ M) potentiated
160 NMDA-EPSCs in SR^{+/+} control slices and depressed these currents upon blockade of D₁Rs.
161 In contrast, in slices from SR^{-/-} mice, GBR12909 failed to induce any changes in NMDA-EPSCs
162 (Fig.1H, I). Together, these results indicate that D-serine is required for appropriate DA
163 modulation of NMDA-EPSCs.

164 As DA is known to regulate PFC activity through modulation of pyramidal neurons
165 excitability (4) and modulation of NMDARs also influences PFC neuronal firing activity (25,
166 26), we next investigated whether D-serine is also involved in the DA modulation of layer 5
167 pyramidal neurons excitability. To this end, cell firing was induced by incremental square
168 depolarizing current pulses during whole-cell current-clamp recordings. DA concentrations of
169 1 μ M and 10 μ M applied to rat PFC slices respectively increased and decreased neuronal
170 excitability (*SI appendix* Fig.S2A,B) without altering membrane properties (*SI appendix* Table
171 1). Depletion of D-serine with RgdAAO prevented these changes in excitability (*SI appendix*
172 Fig.S2C,D). We also undertook these experiments in PFC slices from SR^{-/-} mice and SR^{+/+}
173 mice. DA at 1 μ M and 10 μ M induced changes in SR^{+/+} mice cell excitability similar to those
174 observed in rats, without altering membrane properties (*SI appendix* Table 2; Fig.S2E,F).
175 Absence of D-serine in SR^{-/-} mice resulted in a complete loss of these DA modulations,
176 mirroring the effect observed with RgdAAO (*SI appendix* Fig.S2G,H). These results thus
177 indicate that D-serine is required for DA-induced changes in NMDAR-dependent
178 neurotransmission and excitability at PFC pyramidal neurons.

179 **Opposite influence of D₁R and D₃R activation on NMDAR**

180 Positive DA modulation on PFC pyramidal neurons is classically attributed to D₁-type
181 receptor activation whereas negative modulation is thought to be mediated by the D₂-type
182 receptor (3). We then tested whether modulation of NMDA-EPSCs by selective D₁-type and
183 D₂-type agonists required D-serine. As D₃Rs were recently found to play an important

184 regulatory role in the PFC and in SCZ (27), we ensured that we could detect these receptors
185 in patched PFC neurons using single cell RT-PCR (Fig.2A) and particularly focused on this
186 subtype of receptor. Bath application of the preferential D₃R agonist PD128907 (1 μ M)
187 reduced NMDA-EPSCs whereas the D₁R agonist SKF81297 (10 μ M) induced a reliable
188 potentiation of NMDA-EPSCs (Fig.2C,E). As with DA, actions of these two agonists were
189 strongly impaired by saturating the NMDARs co-agonist binding site with exogenous D-serine
190 (100 μ M, Fig.2D,E). Furthermore, application of S33084 (0.1 μ M), a selective D₃R antagonist
191 (10), fully abolished the inhibitory effect of PD128907 on NMDA-EPSCs but did not alter the
192 potentiating effect of the D₁R agonist SKF81297 (*SI appendix* Fig.S3).

193 We next analyzed the effects of D₁- and D₃R agonists on NMDA-EPSCs in SR^{-/-} mice.
194 Mirroring rat PFC slices, application of SKF81297 and PD128907 respectively increased and
195 decreased NMDAR-EPSCs in SR^{+/+} slices (Fig.2F,H) and these effects were markedly
196 compromised in SR^{-/-} slices (Fig.2G,H). To further confirm that synaptic deficits in SR^{-/-} mice
197 were specifically attributable to a lack of D-serine and not to other putative modulators such as
198 glycine or D-aspartate, we restored its levels in SR^{-/-} mice by chronic subcutaneous
199 administration ([D-serine]=150 mg/kg) for 21 days, as previously described (19). While levels
200 of D-serine, D-aspartate and glycine in the medial PFC (mPFC) were respectively decreased
201 by ~89%, ~35%, and ~69% in saline-treated SR^{-/-} mice, treatment with D-serine selectively re-
202 established levels of D-serine alone to near control values in mutant mice (*SI appendix* Fig.S4).
203 Importantly, this resulted in the normalization of the modulatory actions of D₁R and D₃R
204 agonists on NMDA-EPSCs in D-serine-treated but not in saline-treated SR^{-/-} mice (Fig.2I-K).
205 Thus, these rescue experiments confirm that in SR^{-/-} mice, D-serine is the missing element
206 affecting DAergic modulation of NMDARs. Collectively, these experiments demonstrate that
207 D-serine is a critical factor for DA modulation of NMDARs *via* D₁- and D₃-Rs.

208 **D-serine mediates DA modulations of synaptic plasticity**

209 Activity-dependent changes of synaptic strength in the PFC rely on NMDARs (6, 14)
210 and are strongly regulated by DA (3, 5, 6). In particular, D₁-type receptor stimulation enhances
211 LTP whilst LTD requires the activation of both D₁- and D₂-type receptors (5, 6). To specifically
212 determine the contribution of D-serine to DA modulation of NMDAR-dependent long-term
213 changes in synaptic plasticity, we investigated the effect of DA receptor stimulation on
214 excitatory post-synaptic field potential (fEPSP) plasticity in the PrL of SR^{+/+} and SR^{-/-} mice.
215 Analyses of SR^{-/-} mice synaptic function revealed that basal synaptic transmission was
216 reduced by ~26% (Fig.3A), a decrease most likely attributable to post-synaptic mechanisms
217 as paired-pulse experiments did not show any changes in release probability (Fig.3B). These

218 results are reminiscent of previously reported NMDAR hypofunction in SR^{-/-} mice (19, 24).
219 Although no difference in LTP magnitude between SR^{+/+} and SR^{-/-} mice was detectable
220 (Fig.3C), D₁-type receptor activation by SKF81297 (10 μM) enhanced LTP in SR^{+/+} (Fig.3D),
221 consistent with previous investigations (5, 6), but not SR^{-/-} mice (Fig.3E), thereby indicating
222 that D-serine is required for DAergic modulation of PrL LTP.

223 We then investigated LTD. As previously reported (6), low-frequency stimulation (LFS)
224 could only induce LTD when performed in conjunction with D₁- and D₂-type receptor activation
225 (*SI appendix* Fig.S5, Fig.3F). Indeed, combining SKF81297 (10 μM) and quinpirole (1 μM) with
226 LFS elicited LTD (*SI appendix* Fig.S5D). Strikingly the latter effect was abolished in D₃R knock-
227 out (D₃R^{-/-}) mice (*SI appendix* Fig.S5D), indicating that D₃Rs play a critical role in the DA-
228 dependent generation of LTD in the PrL. Accordingly, co-stimulation of D₁R and D₃R with
229 SKF81297 and PD128907 combined with LFS reliably induced LTD in SR^{+/+} mice (Fig.3F).
230 Importantly, this DA-dependent LTD was markedly reduced by ~ 62% in SR^{-/-} mice (Fig.3F).
231 Collectively, these data indicate that D-serine is critical for the dopaminergic modulation of
232 long-term synaptic plasticity in the PrL *via* both D₁R and D₃R.

233 **Activation of D₁R and D₃R modulates PFC D-serine levels *in vivo***

234 To gain insights into how D-serine participates in the DAergic modulations of NMDAR
235 function, we tested whether activation of D₁- and D₃Rs could regulate extracellular levels of
236 this NMDAR co-agonist. To this end, we performed bilateral *in vivo* microdialysis in the mPFC
237 of freely moving mice measuring basal extracellular levels of D- and L-serine for 1 h in response
238 to perfusion of artificial cerebrospinal fluid (aCSF) as a control, the D₁R agonist SKF81297
239 (10 μM), the D₃R agonist PD128907 (1 μM) or a combination of both (Fig.4A-C). The basal
240 levels of D-serine and L-serine in the mPFC were 812.80 ± 22.97 nM and 1514.00 ± 126.30
241 nM respectively, in the range of previous studies (28, 29). SKF81297 increased extracellular
242 levels of D-serine level by ~17%, providing a mechanism for the D-serine dependent activation
243 of NMDARs by D₁Rs reported herein. Although activation of D₃Rs with PD128907 alone did
244 not induce any change in extracellular D-serine levels, it blunted the increase in D-serine levels
245 elicited by D₁R activation (Fig.4C, left) indicating functional cross-talk between D₁R and D₃R.
246 Interestingly, local infusion of SKF81297 also increased levels of L-serine, the precursor of D-
247 serine, and this effect was also blunted by D₃Rs activation (Fig.4C, right). These data therefore
248 indicate that D₁R and D₃R activation modulate extracellular levels of D-serine *via* direct release
249 as well as increased biosynthesis by SR and/or decreased degradation by DAAO. Since we
250 found that chronic supplementation of D-serine was sufficient to restore D₁R and D₃R

modulations of NMDARs in SR^{-/-} mice, we conclude that release of D-serine is the primary process at play.

To ascertain whether the inhibitory influence of D₃R on D₁R is indeed functionally relevant at NMDARs, we recorded NMDA-EPSCs in the presence of PD128907 (1 μM) and under these conditions applied SKF81297 (10 μM). Instead of potentiating NMDA-EPSCs as occurs when applied alone (Fig.2F), SKF81297 in the presence of PD128907 induced no change (Fig.4D), thus corroborating the inhibitory action that D₃R activation exerts on D₁R function. These data suggest that D-serine function at synaptic NMDARs can be fine-tuned by DAergic input *via* the balanced activation of D₁- and D₃Rs (Fig.4D).

The balance of D₁ vs D₃R activation regulates D-serine functions *via* the cAMP/PKA pathway

We next sought to determine the signaling cascade underlying the D₁/D₃R cross-talk regulating D-serine function. To this end, we focused on the adenylyl cyclase (AC) – cAMP – protein kinase A (PKA) pathway because AC activity is differentially influenced by both D₁- and D₃R (3) and PKA is known to regulate exocytosis (30, 31). We posited that D₁R activation leads to an upregulation of AC activity to induce an increase in D-serine release whereas D₃Rs activation inhibits this process by suppression of AC. To test this hypothesis, we inhibited AC with SQ22536 (20 μM) (Fig.5A) or PKA with H89 (10 μM) (Fig.5B) in SR^{+/+} PFC slices and under these conditions activated D₁Rs with SKF28197 (10 μM). Blocking AC or PKA yielded a similar blunting effect on D₁R-induced facilitation of NMDA-EPSCs (Fig.5A-B) as activation of D₃Rs (Fig.4D), thus corroborating our hypothesis. Accordingly, blockade of PKA also occluded the effect of D₃R activation (Fig.5C). To directly test whether this signaling pathway is indeed underlying DAergic modulation of D-serine functions, we assessed the effect of AC activation with forskolin (50μM) on SR^{+/+} and SR^{-/-} NMDA-EPSCs. While, akin to D₁R stimulation, AC activation induced a robust increase in NMDA-EPSCs amplitude in SR^{+/+} mice (+57.60 ± 10.94%), this effect was abolished in SR^{-/-} mice (Fig.5D). Together these data identify the AC-cAMP-PKA signaling cascade as a key intracellular pathway transducing the opposing actions of D₁R and D₃R on D-serine function.

Dopaminergic modulation of PFC cognitive functions involves D-serine

To evaluate the implication of this newly identified neuromodulatory interplay on cognition, we next investigated the role of D-serine in D₃R modulation of PFC-dependent cognitive processes. We previously showed that antagonizing D₃Rs with systemic administration of S33084 (0.63 mg/kg) enhances rat short-term memory in the novel object

284 recognition (NOR) task (10). Here, we further established that this effect was attributable to
285 changes in PFC function by testing NOR memory in rats after intra-PFC administration of
286 S33084 5 min prior to the acquisition phase (T1, *SI appendix* Fig.S6A). As with systemic
287 administration, treatment resulted in the retention of the novel object 4h after T1, while in
288 control vehicle-treated rats NOR memory was lost at this inter-trial interval (ITI) (10), *SI*
289 *appendix* Fig.S6B), thus confirming the PFC origin of the pro-cognitive effect of D₃R blockade
290 on recognition memory.

291 To test the involvement of NMDARs in this pro-cognitive effect, the non-competitive
292 NMDAR channel blocker MK801 (0.05 mg/kg, i.p.) or the selective and competitive NMDAR
293 antagonist CPP (10 mg/kg, i.p) were administered 15 min prior to S33084 (0.63 mg/kg, s.c).
294 Both NMDAR blockers prevented the pro-cognitive effect of D₃R antagonism on NOR memory
295 (Fig.6A). We also studied the contribution of the co-agonist modulatory binding site using the
296 selective D-serine/glycine binding site antagonist, L701,324 (5 mg/kg, i.p.) (32), which also
297 abolished the reversal of delay-induced impairment in NOR memory by S33084 (Fig.6A).
298 These results indicate that the pro-cognitive action of S33084 depends on NMDAR function
299 and activation of the co-agonist site, possibly *via* D-serine.

300 To further explore this possibility, we assessed NOR memory in SR^{+/+} and SR^{-/-} mice.
301 The two genotypes displayed similar baseline NOR performances, being intact at 2 min and
302 1 h after the acquisition trial but lost after a 2 h ITI (*SI appendix* Fig.S6C). Thus, a deficit in D-
303 serine does not affect NOR performance *per se*. However, S33084 applied systemically
304 (0.63 mg/kg, s.c) 30 min before T1 or by local intra-PFC injection (2.5 µg, 0.5 µL) 5 min prior
305 to T1 reversed the 2 h delay-induced NOR impairment in SR^{+/+} mice (Fig.6B, *SI appendix*
306 Fig.S6D). This result indicates that, in mice, D₃R antagonism also yields a pro-cognitive action
307 that relies on PFC neuromodulation. Strikingly, S33084 (0.63 mg/kg, s.c.) was ineffective in
308 SR^{-/-} mice (Fig.6B), thus demonstrating the pivotal role of D-serine in the DA modulation of
309 PFC-dependent cognitive functions. We here further ensured that this impairment was indeed
310 attributable to the lack of D-serine in SR^{-/-} mice by performing rescue experiments in which D-
311 serine supplementation of SR^{-/-} mice reversed this deficit (Fig. 6B). Taken together, these data
312 demonstrate that, in the PFC, modulation of D-serine function *via* D₃Rs impacts cognition.

313 **Dopaminergic regulation of PFC global activity is impaired in SR knock-out mice.**

314 We finally tested *in vivo* whether D₃R modulation of PFC activity is impaired in SR^{-/-}
315 mice by pharmacological magnetic resonance imaging (phMRI). Since antagonizing D₃Rs
316 potentiates the cerebral blood volume response (rCBV) to the DA-releasing compound D-

317 amphetamine in cingulate cortex (33), we reasoned that if D-serine is required for optimal
318 DAergic modulation of PFC activity, the rCBV change in response to D-amphetamine in the
319 presence of S33084 (0.63 mg/kg, s.c.) should be impaired in SR^{-/-} mice. As predicted, SR^{-/-}
320 mice displayed a blunted rCBV response to D-amphetamine (1 mg/kg, i.p.), as compared to
321 control SR^{+/+} mice (*SI appendix* Fig.S7A-C). These data therefore confirm, *in vivo*, that DA
322 modulation of PFC activity occurs through regulation of D-serine.

323

324 Discussion

325 The present study reveals a novel role for D-serine in driving PFC dopamine-glutamate
326 interactions at the cellular, network, and behavioral level. The underlying signaling pathway
327 involves differential regulation of AC activity by D₁- and D₃-R, most likely resulting in PKA-
328 mediated changes in D-serine release. The pharmacological tools employed in this study were
329 in general used at a single dose which may perhaps be a limitation. Yet we selected doses
330 selective for their respective targets and the use of several agents, together with other
331 complementary approaches, reinforces our conclusions.

332 Activation of D₁R enhances NMDAR-dependent functions to influence
333 neurotransmission, synaptic plasticity and cell excitability through D-serine. Conversely,
334 activation of D₃R exerts an opposite and inhibitory action. Although contrasting effects of D₁-
335 and D₂-type receptors on NMDAR currents have been reported to partly act *via* distinct
336 excitatory and inhibitory microcircuits (34), such a regulatory mode can be ruled-out since in
337 this study all electrophysiological recordings were performed in the presence of the GABA_A
338 receptor antagonist picrotoxin to block GABAergic inhibitory transmission. Still, the question
339 remains of whether the DA modulations of D-serine functions unveiled herein are cell-
340 autonomous. We found that activation of D₁Rs increased extracellular levels of D-serine and
341 in consequence NMDAR-dependent functions in the PFC, an effect counteracted by activation
342 of D₃Rs, indicating functional interactions between the two receptors. Our data further indicate
343 that the underlying mechanism involves differential regulation of the AC-PKA pathway,
344 consistent with a cell-autonomous effect. As previous studies showed that D₁- and D₃R
345 positive pyramidal neurons are particularly abundant in L5 of mPFC and that the two receptors
346 may reside on the same neurons (35), such a scenario is indeed conceivable.

347 Intriguingly, we found that modulation of L-serine levels by D₁- and D₃Rs mirrored their
348 influence on D-serine. Although these observations might suggest intensification of the D-
349 serine biosynthesis pathway through activation of the glia-to-neuron serine shuttle (36), the

fact that we could rescue DAergic control of synaptic and cognitive functions by D-serine supplementation in SR^{-/-} mice indicates that modulation of D-serine biosynthesis is not the limiting step in DA regulation. Rather, DA-induced changes in neuronal D-serine release are most likely central to these processes, while L-serine shuttling and D-serine synthesis by SR would be secondarily upregulated as a homeostatic loop to support the metabolic demands imposed on neuronal circuitry.

Modulation of D-serine levels upon activation of D₁- or D₃R offers a multifaceted signaling system for DA fine-tuning of NMDAR function, thereby optimizing synaptic and network activity. Tonic, spontaneous DA release may increase NMDAR function through D₁-type receptors whilst the higher extracellular level of DA attained during phasic firing of VTA-derived mesocortical pathways would recruit D₃Rs. By inhibiting the AC-PKA pathway, D₃R would counteract D₁R activation to lower D-serine levels and as a consequence the operation of NMDAR. In line with this notion, enhancement and reduction of NMDAR activity by respectively low and high concentrations of DA has previously been reported (21, 22) and is coherent with the U-shaped function of the influence of DA receptors activation upon cognition (37, 38). In the case of PFC, such effects have been attributed to the contrasting level of expression of D₁- and D₂-type receptors (39) as well as the differential localization of these receptors (3, 39). In addition, there is compelling evidence for a differential facilitatory and suppressive influence upon cognitive function of D₁ and D₃R respectively (27, 38).

The striking defects in D₁- and D₃R-dependent modulation of neurotransmission and PFC function in SR^{-/-} mice suggest that DA modulation specifically involves NMDAR co-agonism by D-serine but not glycine. It might then be enquired why SR^{-/-} mice did not exhibit baseline deficits in the NOR procedure which requires functionally-intact NMDARs (40). We propose that, under conditions of a persistent lack of D-serine in the mPFC, glycine can, to some extent, assume a compensatory role at NMDARs. Possible reasons for the specific role of D-serine rather than glycine as a mediator of DA modulation of PFC functions include differential actions related to their spatial segregation. Indeed, we previously found that under physiological conditions synaptic pools of D-serine but not glycine gate synaptic NMDARs in the mPFC (14), whereas glycine preferentially activates extrasynaptic NMDARs (41).

As for potential pathophysiological relevance, by identifying D-serine as a novel substrate for the influence of DA upon glutamatergic transmission in PFC, the current observations are relevant to several neuropsychiatric disorders, such as SCZ where there is a well-established link between a dysfunction of D-serine-mediated NMDAR signaling and both cognitive and positive symptoms (19). Our findings are also consistent with a previous report

384 by Nomura and colleagues showing that neonatal D-serine supplementation of mice displaying
385 a knockout for the SCZ susceptibility gene *pick1* rescues DA modulation of NMDAR-mediated
386 control of pyramidal cell excitability (42). Interestingly, the atypical antipsychotic clozapine
387 enhances D-serine release in rat frontal cortex (43) and elevates plasma levels of D-serine and
388 L-serine in patients with SCZ (44). As clozapine notably acts on DA receptors, in light of our
389 findings, one interpretation might be that clozapine actually changes the balance between D1-
390 and D2-type receptors activation, thereby leading to an increase in D-serine levels. The
391 present study may also offer a cellular explanation for previous studies showing that D-serine
392 plays a major role in cocaine-induced sensitization (45, 46) and to studies suggesting that the
393 novel antipsychotics cariprazine and blonaneserin strengthen cognitive functions by virtue of
394 their attenuation of D₃R activity in the PFC (47,48). In addition, there is increasing interest in
395 the potential utility for ligands at NMDAR in the management of other disorders from autism to
396 Alzheimer's disease (49,50).

397 Finally, gender is clearly a factor related to the prevalence, clinical picture and treatment
398 of many psychiatric disorders, so it should be pointed out that only males were used in the
399 present study, representing a potential limitation to any clinical extrapolation. Nonetheless the
400 present study provides a novel framework for an improved understanding and, ultimately,
401 management of the diversity of brain disorders involving disruption of the frontocortical DA-
402 glutamate interface (51,52).

403

404 ***Methods and Materials***

405

406 A detailed description of all methods and materials can be found in the *SI Appendix*.

407 All experiments were conducted in accordance with European and French directives on animal
408 experimentation and with local ethical committee approval. Electrophysiological recordings
409 and behavioral analyses were performed on adult Wistar and Lister hooded rats as well as
410 serine racemase knock-out mice (SR^{-/-}) and control littermates (SR^{+/+}) while *in vivo*
411 microdialysis and pHMRI experiments were performed only on mice. Only males were used.
412 All quantitative data are expressed as mean ± SEM. Statistical analyses performed are
413 detailed in the *SI Appendix and* in figure legends.

414

415 ***Acknowledgments***

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GD, XL, PL, NM, SS, RA, THA, BP, DJGW, GL, PF, AB, JTC, GC, LP, MG, KCFF, AMG, TF, AC, and JPM declare no conflict of interest. MJ Millan and JM Rivet respectively were and are full time employees of Institut de Recherche Servier and have no other interests to declare. S Schmidt and J Kehr are employees of Pronexus Analytical AB but declare no conflicts of interests.

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Figures legends

Fig.1. D-serine is required for dopamine modulations of NMDA-EPSCs. A: Diagram showing the slice and electrodes placements for recording NMDA-EPSCs at pyramidal neurons in layer 5 of the prelimbic (PrL) cortex. B-C: Pharmacologically isolated NMDA-EPSCs can be recorded in PrL neurons from rat slices at +40mV and at -70 mV in the presence of GABA-receptor blocker PTX (50 μ M) and AMPA-receptor blocker NBQX (10 μ M) and are blocked by D-AP5 (50 μ M). D: Bath application of low (light green, 1 μ M, n=6) or high (dark green, 10 μ M, n = 7) concentrations of DA respectively up- (+73.9 \pm 14.1%) or downregulate (-44.7 \pm 6.7%) NMDA-EPSCs. E-F: Pre-incubation with 100 μ M D-serine (triangle symbols) or treatment with RgDAAO (square symbols) opposes occluding action on the positive (DA₁ μ M+D-Ser: +22.5 \pm 4.6% n=7; DA₁ μ M+RgDAAO: +5.4 \pm 5.93%, n=7) and negative (DA₁₀ μ M+D-Ser: -18.7 \pm 6.7%, n=6; DA₁₀ μ M+RgDAAO: -16.8 \pm 6.2%, n=5) regulations of NMDA-EPSCs by exogenous DA. G: Accumulation of endogenous DA by application of the DA reuptake inhibitor GBR12909 (0.5 μ M) increases NMDA-EPSCs in control rat slices (filled light green circles, +73.78 \pm 21.35%, n=11) and decreases NMDA-EPSCs in the presence of the D1R antagonist SCH39166 (1 μ M) (filled dark green circles, -35.68 \pm 1.45%, n=13). H: GBR12909 (0.5 μ M) increases NMDA-EPSCs in mouse SR^{+/+} control slices (filled light green circles, +41.73 \pm

12.12%, n=5) and decreases NMDA-EPSCs in the presence of the D1R antagonist SCH39166 (1 μ M) (filled dark green circles, $-22.84 \pm 1.79\%$, n=5). GBR12909 (0.5 μ M) induces no change in slices from SR^{-/-} mice (empty green circles, $+0.93 \pm 4.22\%$, n=6). Top traces scale bars: 50 pA, 100 ms. Bottom traces scale bars: 50 pA, 500 ms. H: Histograms summarizing the DA modulations of NMDA-EPSCs and the opposing action of D-serine binding site occlusion, RgDAAO treatment and serine racemase deletion. * p<0.05, ** p<0.01, ***p<0.001.

Fig.2. Modulations of NMDA-EPSCs by D₁- and D₃-receptors involves D-serine. A-B: Single-Cell multiplex RT-PCR showing the expression of D1 and D2 receptors subtypes in L5 excitatory neurons of the PrL area. C: Bath application of D₁R agonist SKF81297 10 μ M increases NMDA-EPSCs ($93.93 \pm 14.05\%$, n=11) while the D₃R specific agonist PD128907 1 μ M has an opposing action ($-39.67 \pm 8.1\%$ n=8). D: Occluding the co-agonist binding site of NMDAR with 100 μ M D-serine markedly impairs NMDAR-EPSCs modulations by DA receptors activation (SKF81297+D-ser: $+17.04 \pm 2.48\%$, n=6; PD128907+D-ser: $-21.7 \pm 5.6\%$, n=6). E: Histograms summarizing the effects of D₁R and D₃R agonists in the absence and presence of occluding D-serine. F: Modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation in SR^{+/+} mice (SKF81297: $+92.66 \pm 23.5\%$, n=8; PD128907: $-34.92 \pm 4.95\%$, n=7). G: Modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation in SR^{-/-} mice (SKF81297; $+51.03 \pm 10.91\%$, n=7; PD128907: $-10.36 \pm 11.75\%$, n=7). H: Histograms showing that modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation is impaired in SR^{-/-} mice. I: Modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation in SR^{-/-} mice chronically supplemented with D-serine (SKF81297; $+86.86 \pm 30.01\%$, n=7; PD128907: $-26.46 \pm 4.19\%$, n=14). J: Modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation in SR^{-/-} mice chronically supplemented with control saline (SKF81297; $+11.98 \pm 10.32\%$, n=6; PD128907: $-3.70 \pm 4.56\%$, n=14). H: Histograms showing that chronic D-serine supplementation rescues modulations of NMDAR-EPSCs by D₁Rs and D₃Rs activation in SR^{-/-} mice. * p<0.05; ** p<0.01. Top traces scale bars: 50 pA, 100 ms. Bottom traces scale bars: 50 pA, 500 ms.

Fig.3. D-serine mediates DA regulations of synaptic plasticity. A: Input/output relationship in wild-type vs SR deficient mice (SR^{+/+} n=44; SR^{-/-} n=21). B: Paired-pulse ratio profiles in wild-type vs SR deficient mice (SR^{+/+} n=44; SR^{-/-} n=21). C: LTP of the field potential in wild-type vs SR deficient mice (SR^{+/+}: $+48.88 \pm 9.20\%$, n=9; SR^{-/-}: $+48.13 \pm 14.6\%$, n=8). D: Effect of the D₁R-type agonist SKF81297 (10 μ M) on LTP in SR^{+/+} mice ($+83.66 \pm 10.72\%$, n=10) E: Effect of SKF81297 (10 μ M) on LTP in SR^{-/-} mice ($+61.64 \pm 14.66\%$, n=9). F: LTD induced by concomitant application of SKF81297, PD128907 and LFS in wild-type vs SR deficient mice (SR^{+/+}: $-32.39 \pm 7.58\%$, n=6; SR^{-/-}: $-12.20 \pm 5.44\%$, n=6). * p<0.05. Scale bars: 0.1 mV, 2 ms.

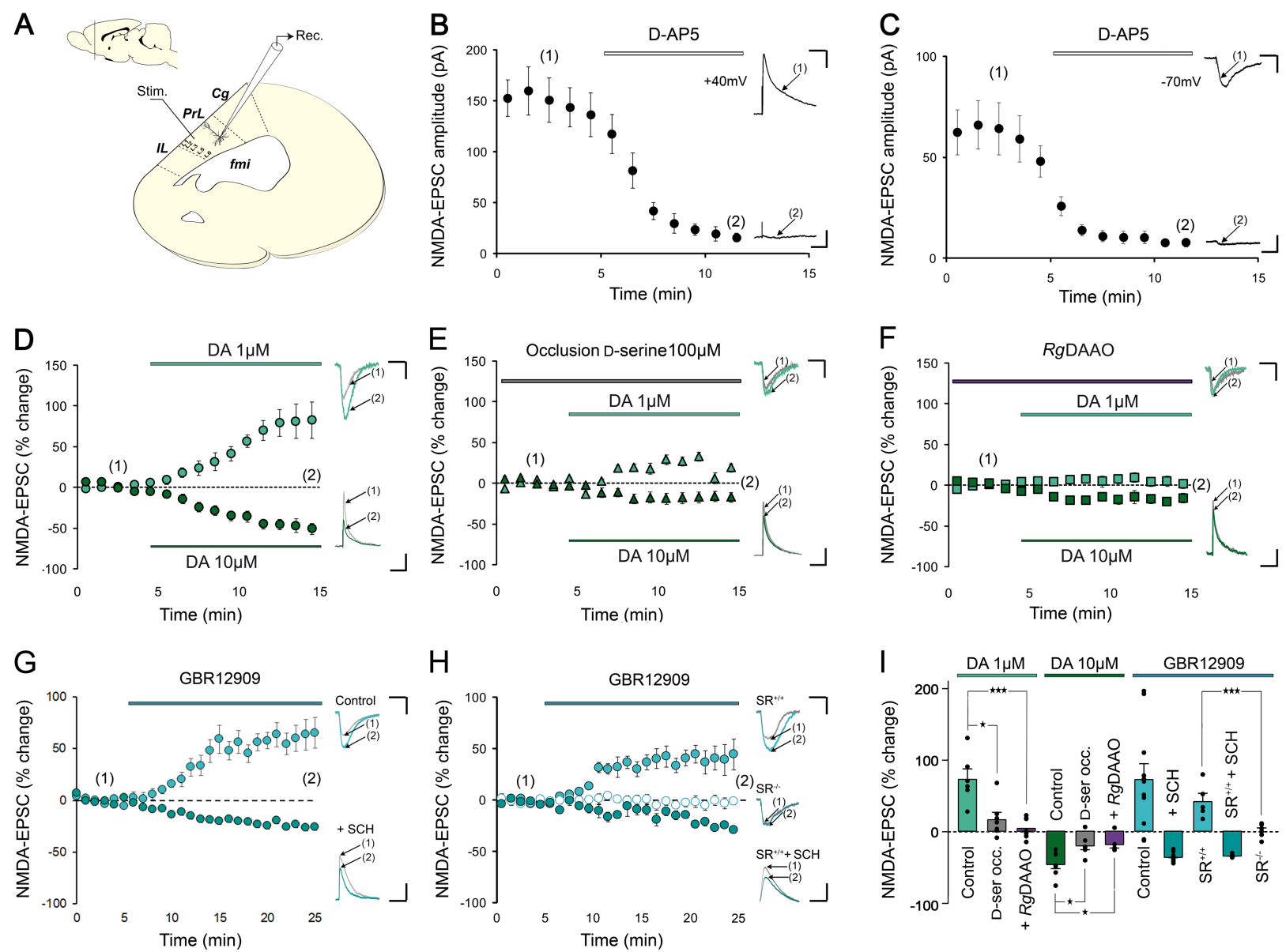
Fig.4. DA receptors activation regulates extracellular mPFC D-serine levels in freely moving mice. A: Mice were implanted with two microdialysis probes placed in the left and right mPFC. Microdialysates were collected every 20 min and D- and L-serine were quantified using UHPLC-MS/MS. B: Left chromatogram shows separation of a standard mixture of D-serine at LLOQ (20 nM) and L-serine in AAS18 in aCSF (black line) and D,L-serine-d3 (230 nM, internal standard, gray line). Right chromatogram shows a typical microdialysis sample in basal conditions (vehicle, black line) and D,L-serine-d3 (gray line). C: Changes in extracellular D-serine and L-serine levels from 20 to 60 min during drug infusion in the mPFC. Histograms represent values for area under the curve (AUC). Left, the D₁R agonist SKF81297 increased the extracellular levels of D-serine by $17.40 \pm 4.73\%$ (n=8 samples, 6 mice) while D₃R agonist PD128907 on its own had no effect (vehicle: $-0.25 \pm 0.49\%$ change, n=10 samples from 6 mice vs PD128907: $-1.90 \pm 2.4\%$ change, n=10 samples from 6 mice) but blocked the potentiating effect of SKF81297 ($-0.08 \pm 3.13\%$ change, n=13 samples from 7 mice). Right, SKF81297 also increased the extracellular levels of L-serine by $33.10 \pm 15.05\%$ while D₃R agonist PD128907 had no effect on its own (vehicle: $-1.31 \pm 1.15\%$ change vs PD128907: $0.00 \pm 8.90\%$ change) but blocked the potentiating effect of SKF81297 ($-9.15 \pm 6.32\%$ change). * p<0.05, **p<0.01, ***p<0.001. D: Recordings of NMDA-EPSCs in the mPFC of mice showing that in the presence of PD128907, SKF81297 fails to potentiate NMDA responses ($-3.15 \pm 6.16\%$ change, n=6).

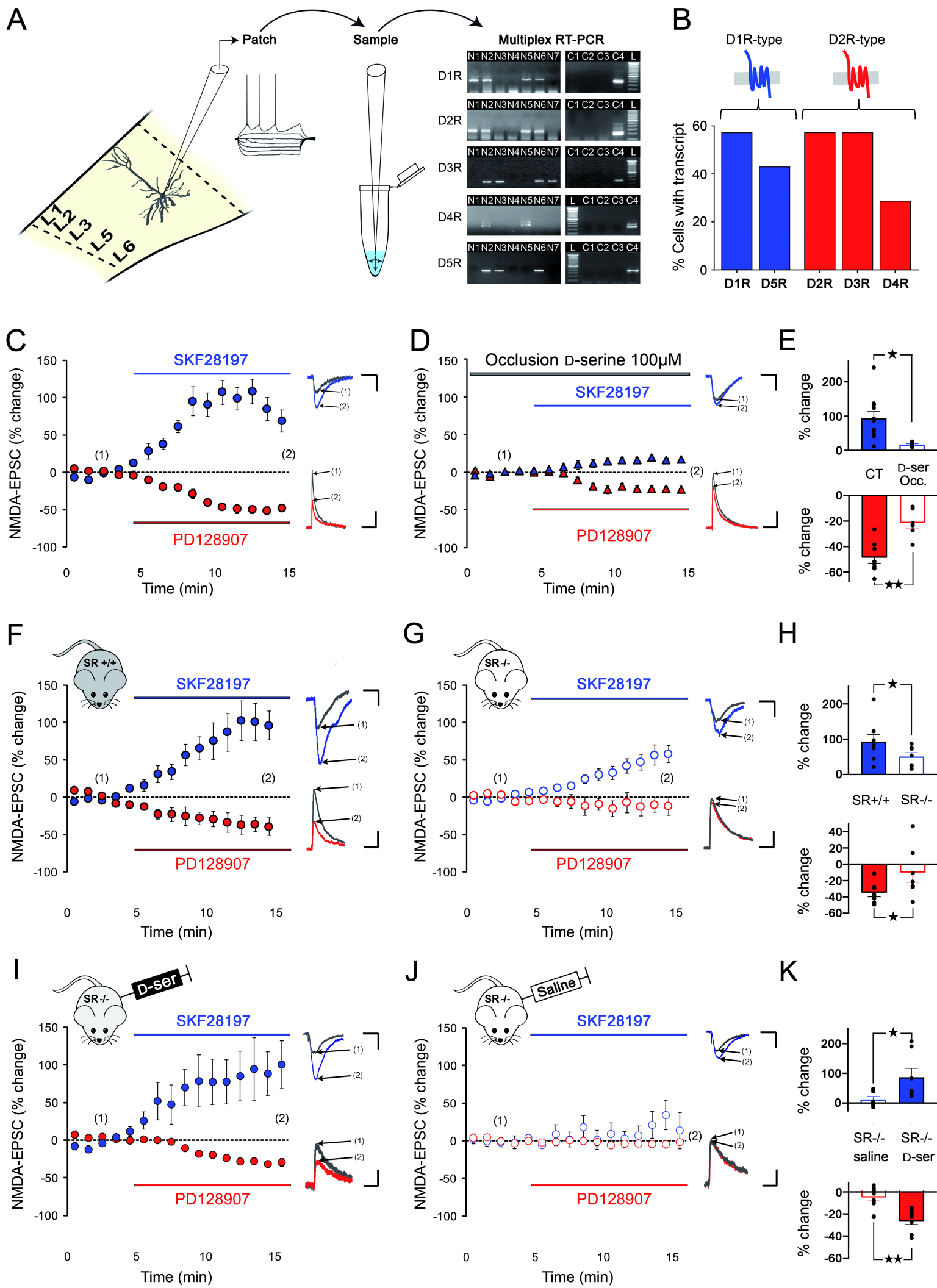
Fig.5: DA modulations of D-serine NMDAR co-agonist functions occurs through cAMP/PKA signaling. A: The facilitating effect of D₁R activation on NMDA-EPSCs is abolished by blocking production of cAMP by the adenylate cyclase enzyme (AC) ($-4.52 \pm 3.21\%$, n=12). B: Similarly, blocking downstream the PKA enzyme prevents the facilitating effect of D₁R activation on NMDA-EPSC ($+6.18 \pm 3.27\%$, n=12). C: Downregulation of NMDAR-EPSCs by D₃R activation is also abolished by H89 blockade, which is indicative of an occlusion. D: Activating AC increased NMDA-EPSCs in SR^{+/+} mice ($+51.61 \pm 11.62\%$, n=10) but not in SR^{-/-} mice ($-0.63 \pm 2.01\%$, n=9; p<0.001). Scale bars: 20 pA, 100 ms.

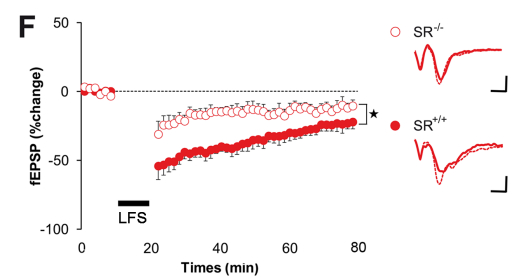
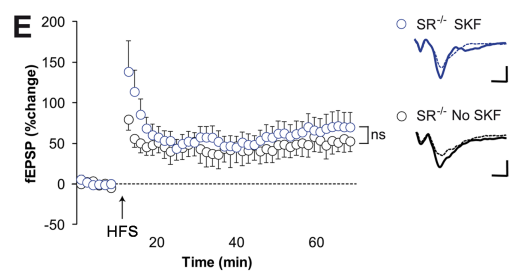
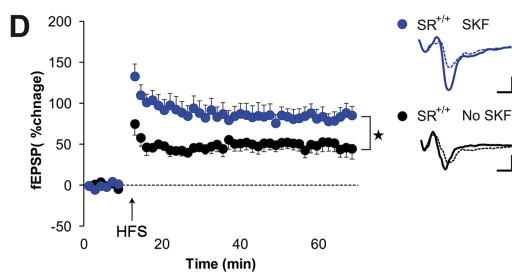
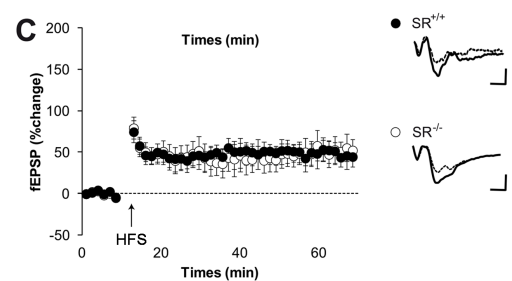
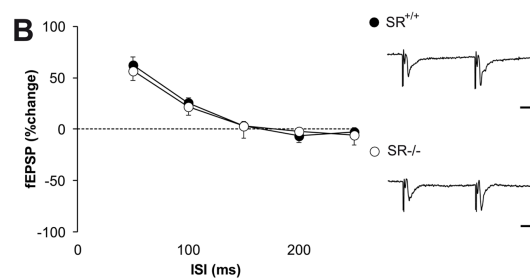
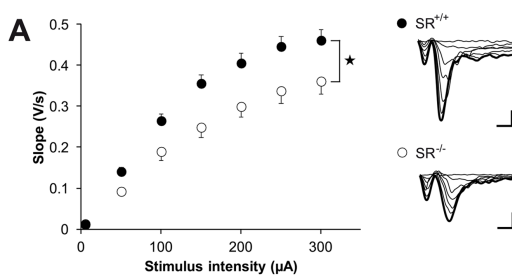
Fig.6. Pro-cognitive action of D₃R antagonist S33084 relies on D-serine modulation of NMDARs. A: Administration of the D₃R antagonist S33084 (0.63 mg/kg, s.c.) reverses the impairment in NOR memory induced by a 4 hours inter-trial interval (ITI) in rats (n=12). Systemic administration of the NMDAR antagonists, MK801 (B, n=12) and CPP (C, n=12) as well as the glycine/D-serine binding site blocker L701,324 (C, n=12) all prevented the enhancement of NOR memory induced by S33084. B: Administration of the D₃R antagonist S33084 (0.63 mg/kg, s.c.) reversed the impairment in novel object recognition (NOR) memory

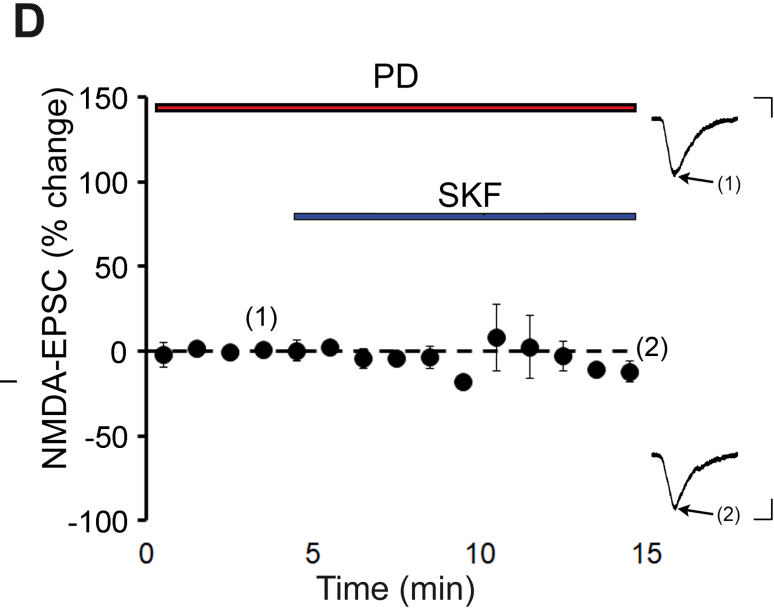
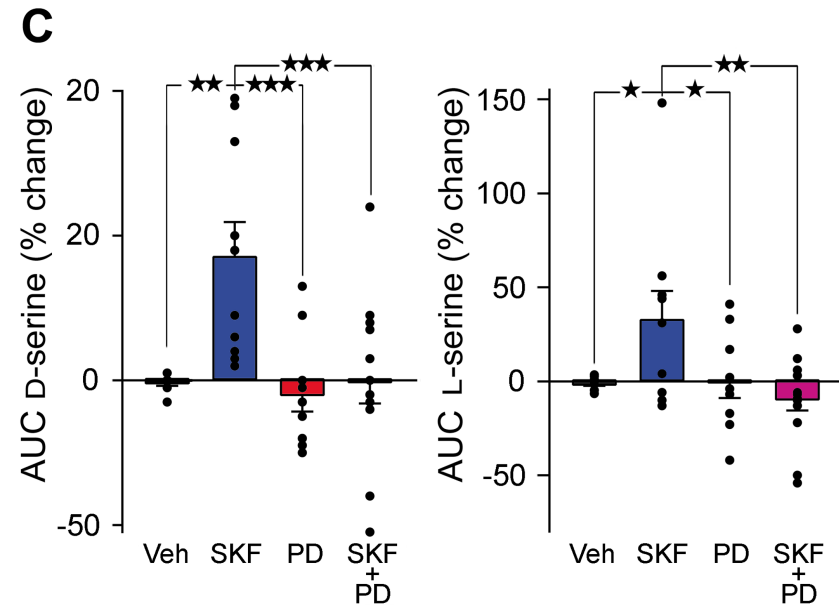
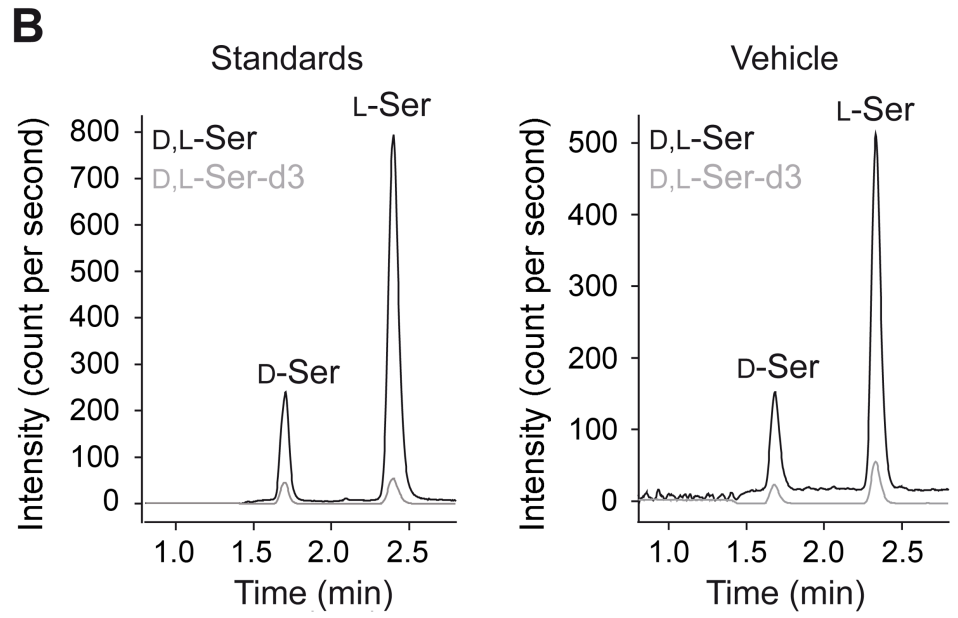
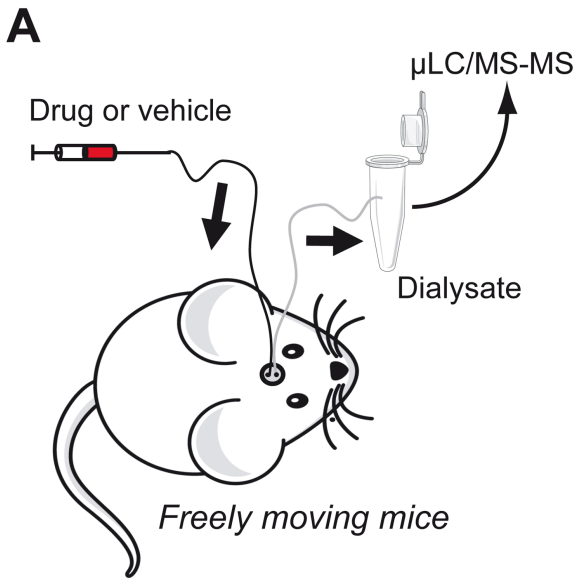
658 induced by a 2 hours ITI in SR^{+/+} mice (vehicle, n=14; S33084, n=18) but not in SR^{-/-} mice
659 (vehicle: n=9; S33084, n=10). The pro-cognitive effect of S33084 is rescued in SR^{-/-} mice by
660 chronic supplementation of D-serine (SR^{+/+}+saline, n=7; SR^{-/-}+D-ser, n=8) performed by s.c.
661 administration with 300 mg/kg D-serine (or control saline) on day 1 and then 150 mg/kg D-
662 serine (or control saline) daily for 20 days. **p<0.01, ***p<0.001 compared to control SR^{+/+}
663 mice treated with vehicle. **p<0.01, ***p<0.001 compared to SR^{+/+} mice treated with S33084.

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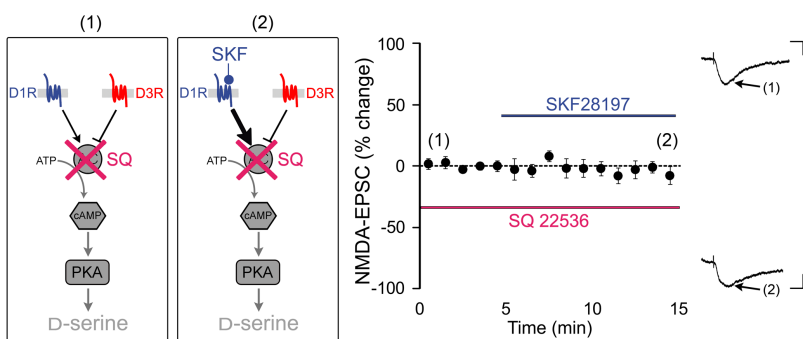




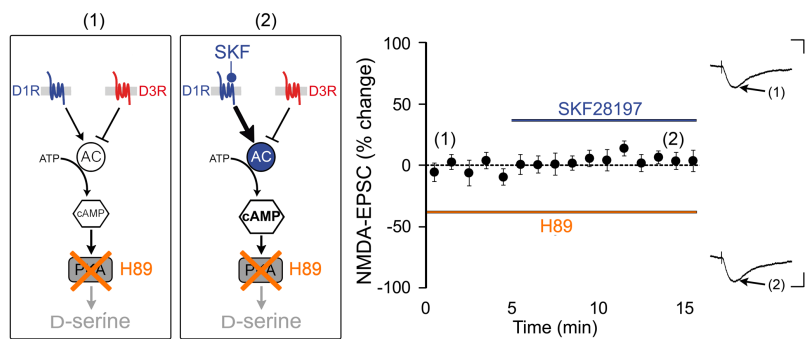




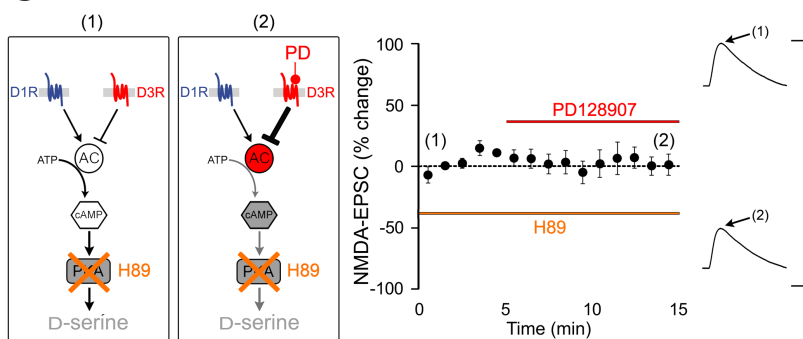
A



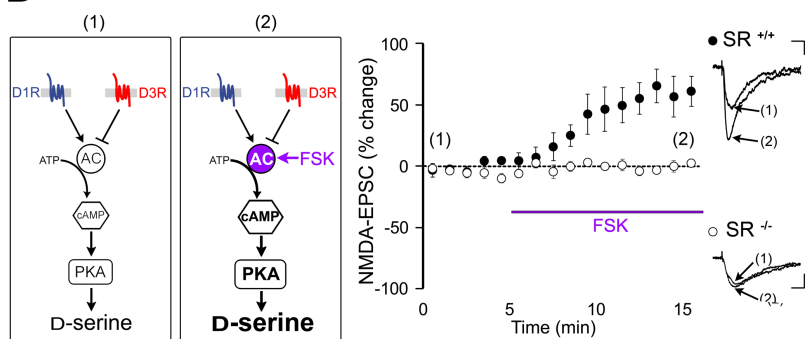
B



C

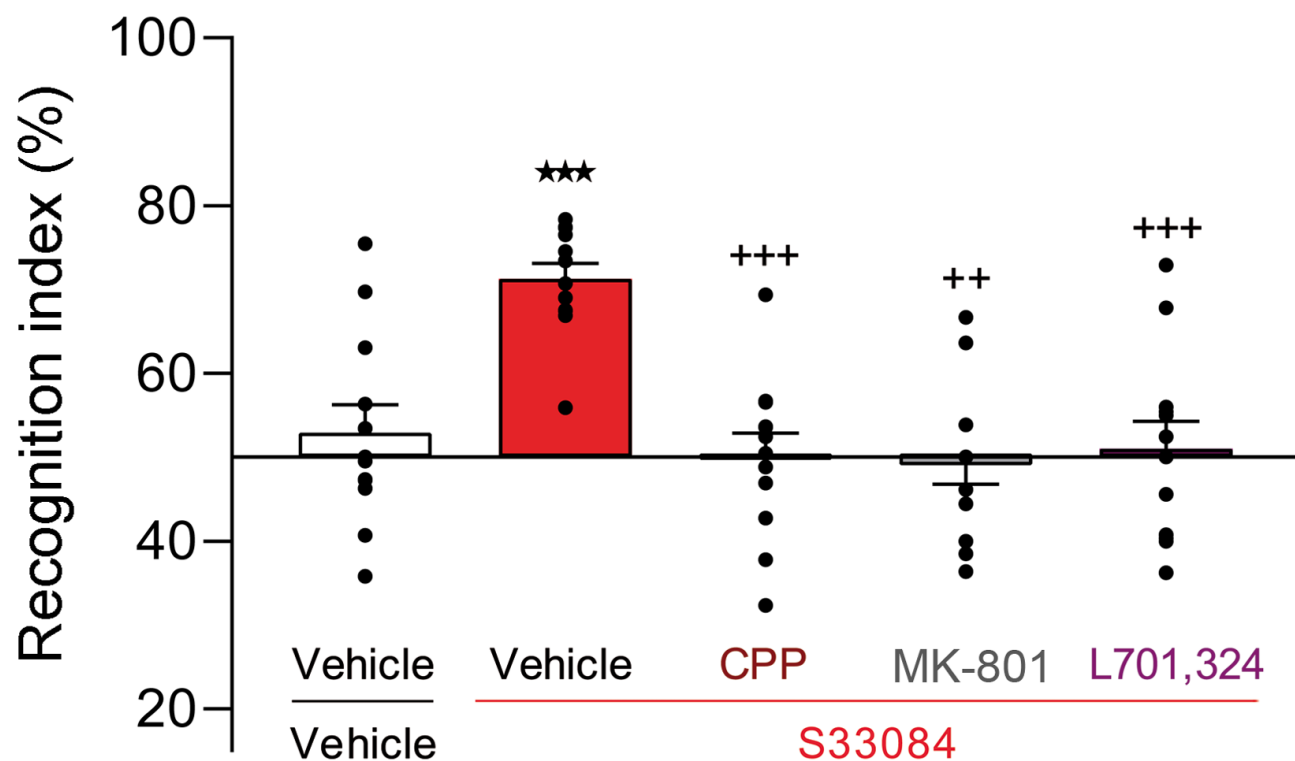


D



A

NMDAR antagonist or vehicle $\xrightarrow{15 \text{ min}}$ S33084 or vehicle $\xrightarrow{30 \text{ min}}$ T1: acquisition $\xrightarrow{4 \text{ h}}$ T2: retention

**B**

S33084 or vehicle $\xrightarrow{30 \text{ min}}$ T1: acquisition $\xrightarrow{2 \text{ h}}$ T2: retention

