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Long-lasting synaptic regulation of dopamine neurons by astrocytes in the Ventral Tegmental Area

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23 **ABSTRACT**

24 The plasticity of glutamatergic transmission in the Ventral Tegmental Area (VTA) represents a fundamental mechanism in the modulation of dopamine neuron burst firing and the phasic 25 26 dopamine release at VTA target regions. These processes encode basic behavioral responses, including locomotor activity, learning and motivated-behaviors. Here we 27 28 describe a hitherto unidentified mechanism of long-lasting potentiation of glutamatergic 29 synapses on DA neurons. We found that VTA astrocytes respond to dopamine neuron bursts with Ca²⁺ elevations that require activation of endocannabinoid CB1 and dopamine 30 31 D2 receptors colocalized at the same astrocytic process. Astrocytes, in turn, release 32 glutamate that, through presynaptic metabotropic glutamate receptor activation coupled with 33 neuronal nitric oxide production, induces long-lasting potentiation of excitatory synapses on 34 adjacent dopamine neurons. Consistent with this finding, selective activation of VTA 35 astrocytes increases dopamine neuron bursts in vivo and induces locomotor hyperactivity. Astrocytes play, therefore, a key role in the modulation of VTA dopamine neuron activity. 36

37

38 INTRODUCTION

39 Dopamine (DA) neurons in the Ventral Tegmental Area (VTA) regulate a wide array 40 of physiological functions including locomotor activity, attention, motivation and reward-41 based learning^{1–3}. A fundamental step in these DA-dependent functions is the transition in 42 the spiking activity of VTA DA neurons from tonic, low frequency firing at rest, to high 43 frequency bursts that modulate the action of DA by determining the synaptic phasic release of DA at VTA target areas such as nucleus accumbens (NAc), medial prefrontal cortex, 44 hippocampus, and amygdala^{4–7}. This transition and the bursting activity of DA neurons are 45 46 under crucial control of glutamatergic afferent inputs to the VTA originating from various brain regions^{4,7,8}. Importantly, the enduring changes in the strength of these glutamatergic 47 synapses exert profound effects on DA neurons regulating their burst firing mode and the 48

release of dopamine at target regions^{1,9}. The plasticity of these glutamatergic synapses represents, therefore, a key mechanism in the modulation of DA transmission and DAdependent behaviors. While extensive studies highlighted the role of neuronal signals in the synaptic plasticity of VTA circuits^{1,10}, the role of astrocytes has been poorly investigated.

A recent study reported that optogenetic stimulation of channelrhodopsin-expressing VTA astrocytes alters glutamate transport, favoring DA neuron inhibition and avoidance behavior¹¹. However, this type of stimulation depolarizes astrocytes, leading to a significant increase in extracellular K⁺ and a subsequent neuronal excitation¹². Whether astrocytes are functionally recruited to the VTA circuitry by neuronal signals and influence the plasticity of glutamatergic synaptic transmission to VTA DA neurons remains totally unexplored.

Astrocytes are active components of brain circuits. Besides their support and 59 metabolic functions, they respond with Ca²⁺ elevations to neurotransmitters and, in turn, 60 61 release gliotransmitters that regulate synaptic transmission and plasticity^{13–15}. Astrocytes 62 are similarly activated by local signals, such as endocannabinoids (eCBs), released by 63 neurons at somatodendritic levels. In various brain areas, including the VTA, eCBs act as 64 retrograde signals that induce depression of synaptic neurotransmitter release upon presynaptic type-1 cannabinoid receptor (CB1R) activation^{16,17}. Studies in hippocampus and 65 dorsal striatum revealed that eCBs also target astrocytic CB1Rs evoking Ca²⁺ elevations 66 67 and glutamate release that potentiates distant excitatory synapses^{18–20}. Whether this lateral potentiation of synaptic transmission is also operative in the VTA is unknown. 68

Using *ex vivo* and *in vivo* approaches, we investigated whether eCBs released by bursting discharges of VTA DA neurons²¹ induce a potentiation of glutamatergic transmission to nearby DA neurons and whether this action is mediated by astrocytes. Because VTA DA neurons, beside eCBs, release DA at somatodendritic levels²², we investigated whether DA is also involved in DA neuron-to-astrocyte signalling. Finally, we evaluated the functional consequences of a specific activation of astrocytes *in vivo* at the

level of both VTA DA neuron firing and locomotor activity. Our results unveil a reciprocal
 functional signaling between DA neurons and astrocytes in VTA circuits.

77

78 **RESULTS**

79 Bursting activity of individual VTA DA neurons induces long-lasting potentiation of glutamatergic transmission in adjacent DA neurons. We investigated whether the 80 bursting activity in individual DA neurons evokes lateral potentiation of glutamatergic 81 82 synaptic transmission¹⁸. In VTA slices of postnatal day (P)14-17 female mice, we recorded from pairs of neurons exhibiting the typical features of DA neurons (Fig. 1a and Extended 83 84 Data Fig. 1a-d). In one neuron of the pair, we monitored excitatory post-synaptic currents (EPSCs) evoked by low frequency stimulation of the rostral glutamatergic afferents. To the 85 second neuron, located 70-120 µm apart, we imposed through intracellular current pulses 86 87 the burst firing mode that characterizes in vivo DA neuron activity (bursts of five action potentials at 20 Hz, 2 Hz interburst frequency, 5 min duration; Extended Data Fig. 1e)²³⁻²⁵. 88 89 Following this bursting activity, EPSC amplitude from the first DA neuron was significantly 90 increased and this potentiation was maintained for at least 45 min (Fig. 1b and 1d). The 91 long-lasting potentiation was not observed in age-matched male mice, in which EPSC 92 amplitude was only transiently increased 3 min after bursts (Fig. 1b and 1d). We defined 93 these two forms of lateral synaptic plasticity as burst-induced long- and short-term 94 potentiation (bLTP and bSTP, respectively). Evaluation of the paired-pulse ratio (PPR) in female mice revealed a significant PPR reduction at 30 and 45 minutes after bursts, 95 96 suggesting a presynaptic mechanism in bLTP generation (Fig. 1c). In contrast, the bSTP in 97 male mice occurred without PPR changes (Fig. 1c). Importantly, the induction of a tonic-like 98 discharge that mimics DA neuron activity at resting conditions (2 Hz action potential 99 frequency for 5 minutes, Extended Data Fig. 1e), failed to modify evoked EPSCs in adjacent

100 DA neurons at any time point tested (Fig. 1d), indicating that bLTP and bSTP are strictly 101 dependent on DA neuron bursting activity.

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Generation of bLTP requires Ca²⁺ elevations in astrocytes. We focused our study on 103 104 bLTP generation mechanism and asked whether astrocytes are involved. We performed experiments in type-2 inositol 1.4.5-trisphosphate receptor knock-out (IP₃R2^{-/-}) female mice 105 in which G-protein coupled-mediated astrocyte Ca²⁺ elevations are largely impaired^{19,26,27}. 106 107 In VTA slices from these mice, DA neuron bursts evoked transient, but not long-lasting potentiation of synaptic transmission (Fig. 1d), suggesting that bLTP induction depends on 108 IP₃R2-mediated astrocytic Ca²⁺ elevations induced by signals generated by the DA neuron. 109 This hypothesis was directly tested in VTA slices from wild type (wt) and IP₃R2^{-/-} mice loaded 110 with the Ca²⁺ fluorescent indicator Fluo-4 and the specific astrocytic marker SR101. To 111 monitor Ca²⁺ signals from astrocytes in proximity of soma and dendrites, through a patch 112 113 pipette we filled DA neurons with the fluorescence tracer neurobiotin (Fig. 1e). We observed 114 that the sustained bursting activity of the DA neuron evoked in astrocytes of female, but not 115 male mice, Ca²⁺ elevations that lasted for at least 25 min (Fig. 1f-h). Furthermore, DA neuron bursts failed to evoke Ca²⁺ elevations in astrocytes of IP₃R2^{-/-} female mice (Fig.1h). Overall, 116 these data suggest that astrocyte IP₃R2-mediated Ca²⁺ elevations are required for bLTP 117 118 generation.

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Generation of bLTP requires eCB and DA signalling to astrocytes, presynaptic mGluR1 activation and nitric oxide release from DA neurons. To gain further insights into the molecular mechanism of bLTP generation, we investigated whether CB1 and/or DA receptors (Rs), activated by eCBs and/or DA locally released by VTA DA neurons, are involved. We found that applications of either the CB1R antagonist AM251 (2 - 4 μ M) or the D2-type receptor antagonist eticlopride (1 μ M) prevented bLTP (but not bSTP), whereas the

D1-type receptor antagonist SCH-23390 hydrochloride (10 µM) was ineffective (Fig. 2a). We 126 127 also evaluated the role of the glutamatergic N-methyl-D-Aspartate receptor (NMDAR), which is a fundamental element in the plasticity of glutamatergic synaptic transmission in different 128 brain regions²⁸, including the VTA¹⁰. We found that in the presence of the NMDAR 129 antagonist D-AP5 (50 µM), bLTP was unchanged (Fig. 2a), indicating that NMDAR is not 130 involved in bLTP. We then hypothesized an involvement of type 1 metabotropic glutamate 131 receptor (mGluR1) activation, as previously observed for astrocyte-mediated synaptic 132 133 plasticity in hippocampal^{18,19} and striatal circuitries²⁰. We found that bLTP was abolished in the presence of the mGluR1 receptor antagonist LY-367385 (100 µM; Fig. 2a). Then, we 134 135 investigated whether nitric oxide (NO), which contributes to long-term synaptic plasticity in other brain circuits^{19,29}, is also involved. We found that blocking NO synthesis in the bursting 136 DA neuron using an NO synthase inhibitor (L-NAME)-containing patch pipette (100 μ M), 137 138 abolished bLTP (Fig. 2a). However, a transient EPSC potentiation, that was absent in the 139 presence of the other antagonists which block bLTP (Extended Data Fig. 2a), was observed 140 6 min after DA neuron bursts, suggesting that NO release by DA neurons contributes to the 141 sustained phase of bLTP.

We asked whether CB1 and D2R activation, which is required for bLTP generation, 142 is also required for DA neuron burst-induced astrocytic Ca²⁺ elevations. We found that the 143 144 astrocyte Ca²⁺ response to DA neuron bursts was abolished in the presence of either AM251 or eticlopride (Fig. 2b). We next asked whether mGluR1 activation, which is required for 145 bLTP generation, is mediated by astrocytic glutamate. In such a case, the mGluR1 146 antagonist LY-367385 should not block the astrocyte Ca²⁺ response to DA neuron bursts. 147 As expected, the Ca²⁺ response of astrocytes was unaffected by LY-367385 (Fig. 2b). 148 149 Therefore, mGluR1 activation plays a crucial role in bLTP generation downstream of astrocyte Ca²⁺ signals. Finally, we found that after blocking NO synthesis, DA neuron bursts 150 evoked in astrocytes only a transient Ca²⁺ response (Fig. 2b and Extended Data Fig. 2b), 151

indicating that NO release by DA neurons contributes to astrocyte Ca²⁺ signal dynamics, as
 previously reported³⁰.

In support of the role of astrocytic CB1 and D2-type receptors in bLTP generation, 154 155 pre-embedding electron microscope (EM) experiments showed that, besides neurons (Extended Data Fig. 3a, Table 1), astrocytes express CB1 and D2-type receptors (D2, D3) 156 and D4R, Fig. 2c, Extended Data Fig. 3b, Table 1 and 2). According to our post-embedding 157 quantitative EM analysis of CB1/D2R immunogold double-labelled astrocytic processes, 158 159 CB1 and D2Rs colocalize at perisynaptic processes at variable distances (Fig. 2d, e, mean distance: 532.2 ± 49.7 nm), indicating that the same astrocyte can sense both eCBs and 160 161 DA. Pre-embedding EM analysis also revealed that the mGluR1β isoform is expressed at axon terminals making asymmetric synaptic contacts (Fig. 2c; Extended Data Fig. 3c, Table 162 163 2 and 3), consistent with a presynaptic mechanism of bLTP, as suggested by PPR reduction. 164 Taking all these results together, we propose that bLTP in the VTA circuitry of female 165 mice is evoked by the following sequence of events (Fig. 2f). Firstly, DA neuron bursting 166 activity induces the somatodendritic release of eCBs, DA and NO; secondly, activation of 167 CB1 and D2Rs in astrocytes triggers IP₃R2-dependent Ca²⁺ elevations, modulated by neuronal NO, and a subsequent glutamate release; thirdly, presynaptic mGluR1 activation 168 by astrocytic glutamate, possibly coupled with NO actions on neurons, induces a sustained 169 170 increase in glutamate release probability leading to bLTP of excitatory transmission onto 171 adjacent DA neurons.

172

Differential expression of DA, CB1 and mGluR1 receptors in the VTA of young male and female mice. The bLTP was absent in the VTA of young male mice where astrocytes failed to response with Ca²⁺ elevations to DA neuron burst. We hypothesized that the lack of astrocyte Ca²⁺ responses is due to absence or lower levels of CB1 and/or D2Rs in male with respect to female mice. Our pre-embedding EM experiments showed that astrocytes

from male mice express CB1, D2, D3 and D4Rs (Fig. 3a and Extended Data Fig. 3a, d). 178 179 Quantitative evaluation revealed, however, that the expression of CB1 and D2Rs is higher in astrocytic processes of female than male mice (P < 0.0001 and P = 0.033, respectively; 180 181 Fig. 3a and Table 2), whereas that of D3Rs is higher in male than female mice (P < 0.0001, Extended Data Fig. 3d and Table 2). Furthermore, the percentage of axon terminals 182 expressing mGluR1 β in female mice is twice that observed in male mice (P = 0.016; Fig. 3a, 183 Extended Data Fig. 3c and Table 2). Therefore, the lack of bLTP in male mice is likely due 184 185 to a defective astrocyte Ca²⁺ response to DA neuron signalling caused by reduced expression of CB1Rs and different pattern and density of D2/D3Rs in the astrocytic 186 187 membrane, coupled to mGluR1^β reduction which affects the ability of astrocytic glutamate to regulate release probability at glutamatergic axon terminals. 188

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190 Selective chemogenetic activation of astrocytes rescues bLTP in young male mice. If the lack of bLTP in male mice is due, at least in part, to a lack of astrocyte Ca²⁺ responses 191 192 to DA neuron bursts, we expect bLTP to be rescued by stimulating astrocyte Ca²⁺ elevations. 193 As a specific stimulus, we used chemogenetic activation of Gq protein-coupled designer receptor exclusively activated by designer drugs (DREADDs, hM3D(Gq)) that were 194 195 selectively expressed in astrocytes (Fig. 3b, c and Extended Data Fig. 4). In VTA slices from 196 mice that express both DREADDs and GCaMP6f in VTA astrocytes, we observed that bath perfusion with the hM3D(Gq) agonist clozapine N-oxide (CNO, 10 µM) evoked transient 197 Ca²⁺ elevations in hM3D(Gg)-expressing astrocytes (Fig. 3d). In agreement with this 198 199 transient astrocyte response, parallel experiments performed in the presence of the CB1 200 and D2R antagonists, AM251 and eticlopride, revealed that CNO evoked in male mice 201 expressing hM3Dq in astrocytes, but not in non-injected control mice, a short-lasting potentiation of the excitatory transmission (Fig. 3e), which became a bLTP lasting at least 202 203 30 min after coupling CNO with DA neuron burst firing (Fig. 3f). These results further suggest

that NO is required for bLTP generation. Given that these experiments were performed in
the presence of CB1 and D2R antagonists, these results further support that bLTP
generation depends on astrocytic rather than neuronal CB1 and D2Rs.

207

208 Astrocytes induce bLTP in the VTA of both female and male adult mice. We next investigated whether the astrocyte-mediated bLTP observed in young mice is also present 209 210 in young adulthood. We found that in VTA slices from adolescent/adult mice (P30-70, to 211 simplify hereafter adult mice), DA neuron bursts evoked in adjacent DA neurons a bLTP that was maintained for at least 30 min after bursts (Fig. 4a). In contrast to data obtained 212 213 from young mice, the bLTP in adult mice was observed in both female and male (Fig. 4a). The presence of bLTP in adult male mice could be due to a developmentally regulated 214 expression of CB1, D2 and/or mGluR1ß receptors. Quantitative analysis from pre-215 216 embedded material of adult male mice showed that the levels of CB1Rs at astrocytic 217 processes and mGluR1ß at glutamatergic terminals are, indeed, higher in adult compared 218 to young male mice, while the levels of D2Rs are comparable (Fig. 4b, Table 4). In 219 agreement with the presence of bLTP in both female and male adult mice, quantitative evaluation of mGluR1_β, CB1 and D2 receptor expression revealed comparable levels in 220 221 these mice (Fig. 4b, Extended Data Fig. 5, Table 4). Together with data presented in figure 222 3f, these results suggest that the absence of bLTP in young male mice is mainly due to 223 CB1R low expression in astrocytic membranes.

The mechanism of bLTP generation in adult mice is similar to that in young female mice, because it was abolished by specific D2, CB1 or mGluR1 receptor antagonists (Extended Data Fig. 6). To further confirm the role played by astrocytic D2 and CB1Rs, we injected the AAV9/2-hGFAP-mCherry_iCre-WPRE-hGHp into the VTA of male mice carrying a "floxed" version of either the D2 or the CB1R genes, to express the Cre recombinase in VTA astrocytes (Fig. 4c). Immunohistochemical experiments showed that

230 the great majority of mCherry-Cre-immuno positive cells were also GFAP-positive and only 231 a very few mCherry-Cre-immunopositive cells were NeuN-positive (Fig. 4d, Extended Data Fig. 7a, b). As a control, we injected the same AAV vector in wt male mice. We found that 232 233 bLTP was abolished when the Cre recombinase was expressed in astrocytes containing the 234 D2 or CB1R floxed gene, whereas it could still be evoked when the recombinase was 235 expressed in wt astrocytes (Fig. 4e; see also specific comments on Discussion). Finally, as in young female mice, bLTP in adult male mice was abolished when the NO synthase 236 237 inhibitor L-NAME was included in the patch pipette (Extended Data Fig. 6), and only a reduced transient potentation lasting no more than 6 min (EPSC amplitude (%) t_{6min} = 112.7 238 239 \pm 4.4, p = 0.028, n = 7) was observed. Altogether, these data indicate that the astrocytemediated bLTP observed in young female mice is preserved in older female mice and is 240 also present in adult male mice with similar cellular and molecular mechanisms. 241

242

bLTP is abolished in adult IP3R2^{-/-} mice expressing a plasmatic Ca²⁺ pump in 243 244 **astrocytes.** To further explore the role of astrocyte Ca²⁺ signals in bLTP during adulthood, 245 we performed DA neuron-paired recording experiments from VTA slices of adult IP₃R2^{-/-} female and male mice. In both groups, the amplitude of evoked EPSCs showed an increase 246 at 30 min after DA neuron bursts that was not statistically significant (Extended Data Fig. 8). 247 248 However, after pooling results obtained from both female and male IP₃R2^{-/-} mice together, 249 we observed a statistically significant potentiation of the excitatory transmission at 30 min (Fig. 4g). Consistently, after pooling results obtained from female and male mice together 250 as wt or IP₃R2^{-/-} groups, the percentage of experiments showing bLTP was different (p < r251 252 0.05, Chi square test, 85.7 % female/male wt mice, n = 14, vs 44.4 % female/male IP₃R2^{-/-} mice, n = 18). These results suggest that the deletion of IP₃R2 has a lower impact on 253 astrocyte Ca²⁺ signal dynamics in adult than young mice and additional experimental tools 254 affecting Ca²⁺ signalling are needed to impair the Ca²⁺ dependent astrocytic actions in adult 255

mice. To validate this idea, we expressed in astrocytes from $IP_3R2^{-/-}$ mice the plasma membrane Ca²⁺ pump isoform hPMCA2w/b that significantly reduced Ca²⁺ signals in these cells³¹ (Fig. 4f and Extended Data Fig. 7c, d). We found that bLTP generation was fully impaired both in female and male $IP_3R2^{-/-}$ mice that expressed hPMCA2w/b in VTA astrocytes (Fig. 4g and Extended Data Fig. 8). Therefore, Ca²⁺ signaling is crucial for bLTP generation also in adult mice, but the impairment of the IP₃R2-mediated signalling in these mice is not sufficient to block astrocyte Ca²⁺-dependent actions.

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In vivo activation of VTA astrocytes favors DA neuron bursts and induces long-lasting 264 motor hyperactivity. Glutamatergic synapses in the VTA circuitry modulate the firing 265 activity of DA neurons and their potentiation, mainly mediated by NMDARs, enhances the 266 burst firing in DA neurons thereby playing a key role in DA-dependent function and 267 268 dysfunction^{1,32}. Because astrocytes, as we show here, also induce a potentiation of these 269 glutamatergic synapses, we next investigated whether in vivo astrocyte activation increases 270 the burst firing mode of VTA DA neurons and eventually affects behavior. We injected AAV9-271 GFAP-hM3D(Gq)-mcherry or AAV5.GfaABC1D.cyto-tdTomato.SV40 in the VTA of adult male mice (Fig. 5a), specifically targeting astrocytes via the GFAP promoter (Extended Data 272 273 Fig. 9). Astrocytes were activated through brief pressure pulses applied to a CNO-containing 274 glass pipette, while recording the firing activity from individual VTA neurons that exhibit the 275 typical features of DA neurons (Fig. 5a, b). Consistent with the astrocyte-mediated enhancement of glutamatergic transmission to DA neurons observed in VTA slices, VTA 276 277 astrocyte activation by CNO increased the bursting discharges of all putative DA neurons 278 that persisted for at least 10 min (Fig. 5c, e-f) and it also increased the overall firing rate in 279 5 out of 7 DA neurons (Fig. 5g, h). In contrast, the firing mode of DA neurons in tdTomato-280 expressing mice was unaffected by CNO, in terms of percentage of spikes in bursts and

firing rate (Fig. 5d-h). These *in vivo* data show that VTA astrocytes exert a direct control on
DA neuron firing activity.

We then asked whether the selective activation of VTA astrocytes, that increases the 283 284 burst firing of VTA DA neurons, could influence DA-dependent physiological functions. We focused on motor activity, which is known to be controlled by DA. Notably, recent studies 285 revealed that DA neurons of the VTA, and not those of the Substantia Nigra (SN), play a 286 major role in the induction of motor hyperactivity^{3,33,34}. Locomotion in an open field arena 287 288 was tested in male mice given bilateral VTA injections of AAV9-GFAP-hM3D(Gg)-mCherry (hM3D) or AAV8-GFAP-GFP (GFP) (Fig. 6a, b). Thirty min after intraperitoneal injections, 289 290 CNO induced a locomotor hyperactivity in hM3D-injected mice as compared to GFP control 291 mice (Fig. 6c-e). The time spent at the center was similar in the two groups (Fig. 6f), suggesting no major effects on anxiety-like phenotypes. Interestingly, the sustained 292 293 locomotor hyperactivity was still observed in hM3D mice 48 hrs after the CNO treatment (Fig. 6c-e). Overall, these in vivo data show that activation of astrocytes in the VTA 294 295 enhances DA neuron firing activity and induces long-lasting motor hyperactivity.

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297

298 **DISCUSSION**

The present study shows that astrocytes of the VTA, a key area in DA-dependent functions, respond with Ca²⁺ elevations to eCBs and DA released by DA neurons and, in turn, induce a long-lasting potentiation of glutamatergic synapses onto adjacent DA neurons. The induction of this novel form of astrocyte-mediated plasticity is independent on NMDAR activation and requires astrocytic Ca²⁺ elevations, presynaptic mGluR1 activation and NO signalling. We also show that astrocyte activation *in vivo* increases the burst and the overall firing rate of DA neurons and induces locomotor hyperactivity. These results indicate that

astrocytes play a key role in the modulation of VTA DA neuron circuits that control DA dependent physiological functions.

Astrocytes have been shown to respond with Ca²⁺ elevations to synaptic 308 309 neurotransmitters and release gliotransmitters that contribute to sensory information 310 processing and behavioral responses^{13–15,35–38}. In different brain regions, including hippocampus, dorsal striatum and neocortex, eCBs released by neurons at the somato-311 dendritic level also recruit astrocytes evoking CB1R-mediated Ca2+ elevations and 312 313 gliotransmitter release that modulates synaptic transmission^{18–20,39}. The present results show that in the VTA, activation of both CB1 and D2Rs is required for astrocytic Ca²⁺ 314 elevations in response to DA neuron bursts and that these Ca²⁺ elevations in astrocytes are 315 crucial for bLTP generation. This is based on the following observations: 1) CB1 and D2Rs 316 are expressed and closely localized, in the same astrocyte; 2) astrocyte Ca²⁺ response to 317 318 DA neuron bursts and bLTP induction are abolished in the presence of specific antagonists 319 that block either the CB1 or the D2R; 3) DA neuron bursts fail to evoke bLTP following 320 deletion in VTA astrocytes of either the CB1 or the D2R; 4) bLTP could not be evoked after 321 the impairment of astrocytic Ca²⁺ elevations downstream the activation of CB1 and D2Rs; 5) bLTP can be rescued in young male mice upon selective chemogenetic activation of 322 astrocytes coupled to DA burst firing, even in the presence of the CB1 and D2R antagonists. 323 324 Notably, the results reported above in points 3-5, provide evidence that activation of 325 neuronal CB1 and D2Rs is not required for bLTP induction.

It is worth to further comment on results reported in point 3. In our experiments on mice carrying the "floxed" CB1 or D2 gene and injected in the VTA with AAV9/2-hGFAPmCherry_iCre, we observed that the great majority of Cre-positive cells were astrocytes and only about 5% were neurons (Extended Data Fig. 7). However, this approach may lead to undetectable expression levels of the Cre recombinase and result in CB1 or D2R deletion in a higher percentage of neurons⁴⁰. It is noteworthy, however, that neuronal CB1 and D2Rs

in the VTA are inhibitory. Indeed, activation of presynaptic CB1 or D2Rs inhibits excitatory transmission onto VTA DA neurons^{9,16} and activation of postsynaptic D2Rs induces a hyperpolarization that reduces VTA DA neuron excitability⁹. Furthermore, D2R activation in the VTA favors eCB-induced suppression of excitation¹⁶. These well-established inhibitory actions of neuronal CB1 and D2Rs in VTA circuitry are not consistent with the CB1 and D2R-dependent bLTP that we describe here and further support that activation of astrocytic rather than neuronal CB1 and D2Rs is required for bLTP generation.

339 Recent studies reported that astrocytes in different brain regions, including the VTA, express D1 and D2-type receptors and respond to bath applied DA stimuli with complex 340 Ca²⁺ dynamics including regulation of basal cytosolic Ca²⁺ and repetitive Ca²⁺ transients⁴¹⁻ 341 ⁴⁴. Most interestingly, astrocytes in the NAc respond to synaptically released DA with D1R-342 rather than D2R-mediated Ca²⁺ elevations⁴⁴. These data confirm that astrocytes of different 343 344 brain regions and synaptic circuitries express different receptors that match the specific signals generated by distinct neuronal activities¹³. Consistent with this view, through the low 345 346 affinity D1R, NAc astrocytes can sense the transient, high DA concentrations generated by 347 synaptic release of DA from VTA DA projections³². Conversely, through the high affinity D2R³², VTA astrocytes can sense the lower DA concentrations mainly generated in the VTA 348 349 by somatodendritic rather than synaptic release and thus be functional targets of DA volume 350 transmission⁴⁵.

An additional specificity of VTA astrocytes is that a cooperativity between CB1 and D2Rs is necessary for the Ca²⁺ response to DA neuron bursts, being activation of either CB1 or D2Rs alone insufficient to induce astrocytic Ca²⁺ elevations. Our EM immunogold experiments provide an ultrastructural background for this cooperativity revealing that CB1 and D2Rs are expressed in the same astrocytes, closely localized at astrocytic processes. Quantitative analysis from CB1/D2R double-labelled astrocytic processes also reveals that a group of couples exhibits an edge-to-edge separation \leq 50 nm, which suggests physical

interactions between these two receptors and possible formation of heterodimers. Notably, 358 previous studies reported that D2 and CB1R coactivation in neurons enhances the formation 359 of CB1/D2R heterodimers^{17,46}. Furthermore, we recently showed that coactivation of GABAb 360 361 and Somatostatin receptors in neocortical astrocytes confers signalling specificity between different interneuron subtypes and astrocytes⁴⁷. A cooperativity between different G-protein 362 coupled receptors may, therefore, be a general functional feature in the astrocyte response 363 to neuronal signals. Overall, CB1/D2R-expressing astrocytes in the VTA are fine-tuned to 364 365 sense eCB and dopamine releasing bursting neurons and extent excitation to neighboring DA neurons through lateral potentiation of glutamatergic transmission. These results provide 366 367 further evidence for circuit- and synapse-specificity of neuron-astrocyte reciprocal signalling in the brain¹³. 368

The astrocyte-mediated bLTP is absent in young male mice in which DA neuron 369 bursts fail to elevate Ca²⁺ in astrocytes. Our data suggest that this failure is mainly due to a 370 371 lower expression of astrocytic CB1Rs in young with respect to adult male mice showing 372 regular bLTP. Importantly, bLTP could be rescued in young male mice by coupling DA 373 neuron bursts with Ca²⁺ elevations evoked by CNO in hM3D-expressing astrocytes. These results further support a crucial role of astrocytic Ca²⁺ signals in bLTP induction mechanism. 374 It is noteworthy that the expression of mGlu1R in excitatory terminals is also significantly 375 376 lower in young with respect to adult male mice, suggesting that a defective neuronal 377 response to astrocytic glutamate may also contribute to the absence of bLTP in young male mice. 378

In hippocampus, transient enhancement of excitatory transmission induced by astrocytic glutamate becomes a long-lasting event when coupled with neuronal NO release¹⁹. A similar mechanism appears operative in the VTA where in the absence of NO only a transient (6 min) enhancement of glutamatergic transmission was observed.

383 Therefore, NO released by burst firing DA neurons is required for the sustained phase of384 bLTP.

Astrocytic Ca²⁺ signalling is required for bLTP generation in both young female and adult mice. However, while the mechanism downstream the astrocyte Ca²⁺ signaling remains similar, IP₃R2 deletion in adult mice is insufficient to abolish bLTP. This finding reveals an increased complexity in the regulatory mechanisms of astrocytic Ca²⁺ dynamics during development with contribution of signalling pathways other than the IP₃R2-mediated pathway^{27,48}. From these observations it also follows that negative results on the role of astrocytic Ca²⁺ signaling in IP₃R2^{-/-} adult mice must be interpreted with caution.

392 Transient and/or persistent potentiation of glutamatergic synapses, fundamentally mediated by NMDAR activation, regulate the burst firing mode of VTA DA neurons that plays 393 a pivotal role in DA-dependent behaviors^{1,32}. The novel form of astrocyte-mediated 394 395 potentiation described here may integrate with these other forms of NMDAR-dependent 396 plasticity that favor the burst firing of DA neurons. Consistent with this view, we report that 397 in vivo astrocyte activation enhances DA neurons bursts and leads to a long-lasting 398 locomotor hyperactivity that recent studies revealed to depend on VTA rather than SN DA 399 neuron activity^{3,33,34}. Present results also suggest that activation of astrocytes by burst firing 400 DA neurons and the consequent lateral potentiation of glutamatergic synapses, may 401 represent a strategy used by individual DA neurons to expand the burst firing mode to 402 neighboring DA neurons. Hence, it is possible that, through the fundamental recruitment of astrocytes, an isolated, high bursting DA neuron favors the formation of spatially defined 403 404 clusters of coactive DA neurons that have been proposed to convey essential information 405 about a specific subset of behavioral variables to target regions⁴⁹. Further specifically 406 designed experiments are necessary to validate this hypothesis.

407 Present results show that in the VTA circuitry, astrocyte signaling induces a long-408 lasting potentiation of glutamatergic synapses to DA neurons, increases the burst firing

409 mode of DA neurons and favors locomotor hyperactivity thereby revealing an astrocyte-410 mediated mechanism in the control of DA neuron activity and DA-dependent behaviors. Our 411 study also paves the way to future investigations examining whether dysregulations of DA 412 neuron-astrocyte reciprocal communication within the VTA may contribute to the 413 development of psychiatry disease states including motivation disorders, psychiatric 414 disorders with a strong motor component, such as attention-deficit/hyperactivity disorder, 415 and drug addiction.

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418

419 **METHODS**

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Animals and brain slice preparation. Animal care, handling and procedures were carried 421 422 out in accordance with National (D.L. n.26, March 14, 2014) and European Community Council (2010/63/UE) laws, policies, and guidelines, and were approved by the local 423 424 veterinary service. Horizontal VTA slices (240 µm) were obtained from both male and female 425 C57BL/6J wild type (wt) mice and inositol 1,4,5-triphosphate-type 2 receptor (IP₃R2) knockout mice (IP₃R2^{-/-})⁵⁰, and from male CB1R-⁵¹ and D2R-flox⁵² mice, at P14-17 (young 426 mice) or P30-70 (adolescent/adult mice, to simplify adult mice). Animals were anesthetized 427 428 with isofluorane, the brain removed and transferred into an ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 25 NaHCO₃, 429 430 1.25 NaH₂PO₄, pH 7.4 with 95% O₂-5% CO₂. Slices were cut with a vibratome (Leica Vibratome VT1000S Mannhein, Germany) in the ice-cold solution described in Dugue at al. 431 2005⁵³ containing (in mM): 130 KGluconate, 15 KCl, 0.2 EGTA, 20 HEPES, 25 glucose, 2 432 433 Kynurenic acid. Slices were then transferred for 1 minute in a room-temperature solution 434 containing (in mM): 225 D-mannitol, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 25 glucose, 0.8 CaCl₂, 8 MgCl₂, with 95% O₂-5% CO₂. Finally, slices were transferred in ACSF at 32°C for 435 15-20 minutes and then maintained at room temperature for the entire experiment. 436

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Electrophysiological recordings and extracellular stimulation. Brain slices were continuously perfused in a submerged chamber with recording solution containing (in mM): NaCl 120; KCl 2; NaH₂PO4 1; NaHCO₃ 26; MgCl₂ 1; CaCl₂ 2; glucose 10; pH 7.4 (with 95% O₂-5% CO₂). Picrotoxin (50 μ M) was added to block GABA_A receptor currents. When indicated, other antagonists were bath applied with the recording solution. Cells were visualized with an Olympus FV1000 microscope (Olympus Optical, Tokyo, Japan). Conventional VTA DA neurons were recorded in the lateral part of the region medial to the

medial terminal nucleus of the accessory optical tract (mt, Fig. 1)^{5,54}. DA neurons from the 445 446 lateral VTA were identified on the basis of their distinct morphology characterized by a large 447 and elongated soma with no particular dendritic orientation and the presence of the following 448 electrophysiological properties: a low-frequency tonic firing, a large lh current elicited by 449 hyperpolarizing steps¹⁶ and a slow depolarizing potential during current step injections⁵⁵ 450 (Extended Data Fig. 1 b-d). Simultaneous electrophysiological whole-cell patch-clamp recordings from two DA neurons (distance of the somata, 70-120 µm) were made. Patch 451 452 electrodes (resistance, 3-4 M Ω) were filled with an internal solution containing (in mM): Kgluconate, 135; KCl, 70; Hepes, 10; MgCl₂, 1; Na₂ATP 2 (pH 7.4 adjusted with KOH, 280-453 454 290 mOsm/L). Recordings were obtained using a multiclamp-700B amplifier (Molecular Device, Foster City, CA, USA). Signals were filtered at 1 kHz and acquired at 10 KHz 455 sampling rate with a DigiData 1440A interface board and pClamp 10 software. Series and 456 457 input resistances were monitored throughout the experiment using a 5 mV pulse. 458 Recordings were considered stable when the change of series and input resistances were 459 below 20%. Cells that do not meet these criteria were discharged. Theta capillaries filled 460 with recording solution were used for bipolar stimulation. To stimulate glutamatergic afferents, electrodes were connected to an S-900 stimulator through an isolation unit and 461 462 placed 100-200 µm rostral to the recording electrode (Extended Data Fig. 1a). Paired pulses 463 (50 ms intervals) were delivered at 0.33 Hz. Excitatory post-synaptic currents (EPSCs) were recorded while holding the membrane potential at -70 mV. Stimulus intensity was adjusted 464 to evoke 30-50 % maximal EPSCs. The EPSC amplitude was measured as the peak current 465 466 amplitude (2-9 ms after stimulus) minus the mean baseline current (100 ms before stimulus). 467 To illustrate the mean EPSCs time course, values were grouped in 3-min bins (i. e. mean 468 EPSCs from 60 stimuli). Changes in mean EPSCs in the first DA neuron were monitored 469 after imposing a burst or a tonic firing pattern to the second DA neuron (70-120 mm apart). 470 Burst firing pattern was imposed in current-clamp mode, through injections of intracellular

current pulses, with 5-pulse 20 Hz burst, every 500 ms for 5 min (Extended Data Fig. 1e)²³. 471 Tonic firing was imposed with individual current pulses applied at 2 Hz for 5 minutes²³ 472 473 (Extended Data Fig. 1e). During the burst/tonic firing the extracellular stimulation was 474 switched off. In electrophysiological experiments, time 0 indicates the end of the burst/tonic firing. For statistical analysis of short-term effects, mean EPSCs from 120 stimuli applied 475 before (basal) and mean EPSCs from 60 stimuli applied during the first 3 min after the firing 476 protocol (burst or tonic firing pattern) were compared. For statistical analysis of long-term 477 478 effects, mean EPSCs from 120 stimuli applied before (basal), 24-30 min (indicated as 30 479 min time point in the bar chart) or 39-45 min (indicated as 45 min time point in the bar chart) 480 after the firing protocol were compared. In young adult mice only the long-term effect at the 481 time point of 30 min was analyzed due to the difficulty of obtaining long-lasting recordings in tissues from these mice. Paired-pulse ratio (PPR) was calculated as 2nd EPSC/1st EPSC 482 483 and evaluation of the PPR before and after the burst firing protocol was used to identify the 484 pre- or postsynaptic locus of the bLTP.

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486 Dye loading and Ca²⁺ imaging experiments. Slices were loaded with the astrocyte specific 487 marker Sulforhodamine 101 (SR101) (0.3 µM, Sigma Aldrich, Milano) in ACSF at 32°C for 15 minutes. Then, slices were loaded for 45 min at room temperature with the Ca²⁺ sensitive 488 489 dye Fluo4-AM (7,6 µM, Life Technologies, Monza, IT) in an ACSF solution containing 490 pluronic F-127 (0.0067%, Sigma Aldrich, Milano, IT) and bubbled with 95% O₂-5% CO₂. 491 Ca²⁺ imaging experiments were conducted with a confocal laser scanning microscope TCS-492 SP5-RS (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with two solid state 493 lasers tuned at 448 nm and 543 nm (to image Fluo-4 and SR101 fluorescence, respectively) 494 and a 20x objective (NA, 1.0). Images were acquired with a 0.5 Hz frame rate for 90s, with 495 time intervals of 5 min between recordings. Image sequences were processed with ImageJ. 496 Regions of interest (ROIs) were drawn around cellular somata using the red SR101 signal.

Ca²⁺ events were estimated as changes of the Fluo-4 fluorescence signal over baseline 497 $(\Delta F/F_0 = (F(t)-F_0)/F_0)$. A fluorescence increase was considered a significant event when it 498 exceeded two times the standard deviation from the baseline. Astrocyte Ca²⁺ responses 499 were quantified by analyzing the probability of occurrence of Ca²⁺ spike by detecting the 500 501 onset of Ca²⁺ elevations (Ca²⁺ spikes) during the recording period. To investigate the 502 astrocyte response to the burst firing of DA neurons, a DA neuron was patch with an intracellular solution (see details before) containing the fluorescent tracer Neurobiotin 488 503 (65 µM, Vector Laboratories, Inc, USA) to visualize neuronal soma and dendrites. To obtain 504 the time course of the Ca²⁺ spike probability index reported in figures 1 and 2, the number 505 of astrocytic Ca²⁺ spikes for each recording period was divided by the number of SR101-506 positive astrocytes in proximity (around 50 µm) to Neurobiotin 488-filled DA neuron soma 507 508 and dendrites. After three basal recordings, a burst firing pattern was imposed to the DA 509 neuron (in current-clamp mode, through injections of intracellular current pulses, with 5pulse 20 Hz burst, every 500 ms for 5 min) and the quantification of the Ca²⁺ spike probability 510 was resumed 4.5 min after the initiation of the burst firing. In Ca²⁺ imaging experiments, time 511 0 indicates the start of the burst firing. For statistical analysis, a mean value of the Ca²⁺ spike 512 513 probability/min per slice was calculated at basal conditions (mean of the three basal 514 recordings) and after DA neuronal burst firing (mean of 4 consecutive recordings after the 515 burst firing, the first at a time point of 4.5 min after the burst firing and the last at a time point of 24 min after the burst firing). When indicated, Ca²⁺ imaging experiments were performed 516 in slices expressing the genetically encoded Ca²⁺ indicator GCaMP6f and the Gg protein-517 coupled designer receptor exclusively activated by designer drugs (DREADDs) hM3D in 518 astrocytes (for AAVs delivery details see below). In these experiments, Ca²⁺ elevations were 519 520 evoked by bath perfusion of the hM3D agonist clozapine N-oxide CNO (10 µM) 2.5 min after the start of the burst firing. A mean time course of the Ca²⁺ response to CNO was calculated 521

522 by plotting the $\Delta F/F_0$ of a ROI drawn around the entire recording field. Then, plots were 523 aligned for the Ca²⁺ peak to calculate the mean time course of the Ca²⁺ response to CNO. 524

AAVs delivery. We bilaterally injected in the VTA of C57BL6J wild-type male mice, at 525 postnatal days 0-2 or 28-30, the viral vector ssAAV-9/2-hGFAP-hM3D(Gg) mCherry-526 WPRE-hGHp(A) (VVF-UZH, Zurich, Switzerland, 4.6 x 10E12 viral genomes (vg)/ml), that 527 contains the astrocytic promoter to selectively express in astrocytes the mCherry-tagged, 528 529 Gq-coupled Designer Receptors Activated Only by Designer Drugs (DREADD) hM3D. Twothree weeks after injection, we performed electrophysiological experiments in brain slices 530 531 from juvenile mice (P14-17) and in vivo single unit recordings in adult mice (P45-50). As control for single unit recording experiments, in the VTA of wild-type male mice we bilaterally 532 injected the viral vector AAV5.GfaABC1D.cyto-tdTomato.SV40 (Addgene, USA, 2.4x 10E13 533 534 vg/ml), that carries the astrocytic promoter GfaABC1D to express selectively in astrocytes the tdTomato marker. In a group of P0-P2 mice, both ssAAV-9/2-hGFAP-535 536 hM3D(Gq)_mCherry-WPRE-hGHp(A) and AAV5.GfaABC1D.cytoGCaMP6f.SV40 537 (Addgene, USA, 1.81 x 10E13 genome copies/ml) viral vectors were injected together to assess the Ca²⁺ responses evoked in astrocytes after activation of hM3D, using the Ca²⁺ 538 indicator GCaMP6f. To selectively express the Cre recombinase in astrocytes, injections of 539 540 the viral vector ssAAV9/2-hGFAP-mCherry iCre-WPRE-hGHp(A) (VVF-UZH, Zurich, 541 Switzerland, 5.2 x 10E12 vector genomes/ml) carrying the astrocytic promoter GfaABC1D were bilaterally performed in the VTA of P 28-30 male mice containing the D2R or the CB1R 542 543 floxed gene. As control mice, the same viral vector was injected in age-matched C57BL6J 544 wild-type mice. Brain slice electrophysiological experiments were performed 4 weeks after 545 injection. AAV5.GfaABC1D.mCherry.hPMCA2w/b.SV40 (Penn Vector Core, USA, 1.14 x 546 10E13 genome copies/ml) was bilaterally injected in the VTA of both female and male IP₃R2⁻ ⁻ mice, at postnatal days 28-30, to selectively express in astrocytes the Ca²⁺ pump 547

548 hPMCA2w/b³¹. Brain slice electrophysiological experiments were performed 4 weeks after 549 injection. AAV5.GfaABC1D.mCherry.hPMCA2w/b.SV40 was injected without previous dilution. The other viral vectors were diluted to 50% in ACSF before injection. When two viral 550 551 vectors were injected, both vectors were present at a 1:1 ratio. The coordinates for viral injections were (in mm): AP 0.1, ML ± 0.15, DV -3.8 from lambda for P0-2 mice; AP -3.0, ML 552 ± 0.5, DV -4.4 from Bregma for P28-30 mice. For injections in P0 mice, animals were 553 554 anesthetized by hypothermia for three minutes and fixed into a modeled platform. Using a 555 manually graduated pull glassed pipette, connected to a costume-made pressure injection system, we punch the skull bilaterally and injected a total volume of 350 nl containing the 556 557 viral vector. After injection, the skin was sutured and mice were revitalized under a heat lamp before returning to their cage. For injections in P28-30 mice, animals were 558 559 anesthetized with isoflurane (induction 4-5%, maintenance 1-2%). Depth of anesthesia was 560 assured by monitoring respiration rate, eyelid reflex, vibrissae movements, and reactions to pinching the tail and toe. After drilling two holes into the skull over the VTA, we injected a 561 562 total volume of 500 nl by using a pulled glass pipette connected to a peristaltic pump, at a 563 rate of 100 nl/min. Pipette was kept for 10 minutes in the tissue before slow withdrawal. After injections, the skin was sutured and mice were revitalized under a heat lamp and returned 564 565 to their cage.

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Immuhistochemistry and cell counting. For the evaluation of the number of mCherry expressing astrocytes and neurons we prepared 70 µm thick brain slices from young and adult animals injected with ssAAV-9/2-hGFAP-hM3D(Gq)_mCherry-WPRE-hGHp(A), ssAAV9/2-hGFAP-mCherry_iCre-WPRE-hGHp(A) or AAV5.GfaABC1D.mCherry.hPMCA2 w/b.SV40. Mice were euthanized with 5% isoflurane and transcardially perfused with PBS followed by ice-cold 4% PFA in PBS. Brains were removed and postfixed overnight at 4°C in the same fixative solution. Horizontal brain slices were obtained with a VT1000S

vibratome (Leica), collected as floating sections and blocked for 1 hr in the Blocking Serum 574 575 (BS: 1% BSA, 2% goat serum and 1% horse serum in PBS) and 0,2% TritonX-100. After blocking, sections were incubated (overnight at 4°C) with the following primary antibodies in 576 577 BS plus 0,02% Tx-100: anti-NeuN (RRID:AB 2298772, 1:200 mouse, Thermofisher Millipore fibrillary 578 MAB377), anti-glial acidic protein (GFAP, 579 RRID:AB 10013382, 1:400 rabbit, Dako Agilent , Z0334), anti-S100B (RRID:AB 2315306, 580 1:400 rabbit, Dako Agilent, Z031129), anti-glutamate transporter 1 (GLT1, RRID:AB_90949, 1:400 guinea pig, Abcam, AB1783); prefixed in 50% methanol in PBS per 15 minutes). After 581 582 washing with PBS, slices were incubated for 2 hr at room temperature with secondary 583 antibodies conjugated with AlexaFluor-488 (1:500; A21202 donkey anti-mouse; A21206 584 donkey anti-rabbit; A11073 goat anti-guinea pig, Invitrogen Thermo-Scientific). To evaluate 585 mCherry positive cells, we directly evaluate the red fluorescence of infected slices. Only for IP3R2-/- mice injected with AAV5.GfaABC1D.mCherry.hPMCA2w/b.SV40, we performed 586 587 double immunofluorescence of NeuN (or GLT1) together with anti-RFP (RRID:AB 2209751, 588 1:1000 rabbit, Rockland, 600-401-379). In this case, secondary antibodies were anti-mouse 589 (or anti-guinea pig) AlexaFluor-488 conjugated together the anti-rabbit AlexaFluor-555 590 conjugated (A21430 donkey anti-rabbit, Invitrogen Thermo-Scientific, 1:500). Slices were 591 then washed and nuclei were stained with Top-Ro3 (Invitrogen Thermo-Scientific, 1:1000). Negative controls were performed in the absence of the primary antibodies. We used a TCS-592 593 SP5-RS laser scanning microscope (Leica, Germany; 20x NA1x/W objective) to acquire 594 sequential three channels, confocal image z-stacks (1 µm z-step, 456,33x456,33 µm) and ImageJ for double-labeled cell counting. We counted mCherry-positive cells and then we 595 596 evaluated the percentage of mCherry-positive cells that were neurons (mCherry+/NeuN+) 597 or glial cells. VTA from both hemispheres of injected animals was evaluated in at least two 598 mice for each group.

600 Pre- and post-embedding electron microscopy. Animals. Seven P16 and four P50 601 C57BL/6 mice (3 females, 4 males for P16; 2 females and 2 males for P50) were used. Mice were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) and 602 603 perfused transcardially with a flush of saline solution followed by 4% freshly depolymerized 604 paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed, post-fixed in the same fixative (for 48 hrs), and cut on a Vibratome in 50 µm 605 serial horizontal sections from the midbrain which were collected in PB until processing⁵⁶. 606 607 Horizontal sections were through the dorso-ventral extent of ventral tegmental area (VTA) 608 resulting in 7-8 sections per series. In order to verify the dorso-ventral extension of VTA, a 609 pilot series of sections from a male mouse, were immuno-processed for tyrosinehydroxylase (primary antibodies from Millipore, AB1542, RRID:AB 90755⁵⁷; 1:500) and for 610 611 Nissl staining. In immunoreacted sections (see data collection and analysis) lateral VTA was 612 identified as the region medial to the medial terminal nucleus of the accessory optical tract^{5,54} 613 (MT). Information on antibodies used for electron microscopy are reported in Table 1.

614 Immunoperoxidase and pre-embedding procedures. Sections were treated with H₂O₂ 615 (1% in PB; 30 min) to remove endogenous peroxidase activity, rinsed in PB and pre-616 incubated in 10% normal goat serum (NGS, 1 hr; for mGLUR1α, mGlur1β, D2, D3, D4) or in 10 % normal donkey serum (NDS, 1 hr; for CB1). Sections were then incubated in a 617 solution containing primary antibodies (see Table 1 for dilutions; 2 hrs at room temperature 618 619 [RT] and overnight at 4°C). The following day, sections were rinsed 3 times in PB and 620 incubated first in 10% NGS or 10% NDS (15 min) and then in a solution containing secondary biotinylated secondary antibodies (see Table 1 for dilutions; 1.5 hr at RT). 621 622 Sections were subsequently rinsed in PB, incubated in avidin-biotin peroxidase complex (ABC Elite PK6100, Vector), washed several times in PB, and incubated in 623 3,3'diaminobenzidine tetrahydrochloride (DAB; 0.05% in 0.05 M Tris buffer, pH 7.6 with 624 625 0.03% H2O2). Method specificity was verified by substituting primary antibodies with PB or

NGS. As previously described⁵⁶ after completion of immunoperoxidase procedures, 626 627 sections were post-fixed in 1% osmium tetroxide in PB for 45 m and contrasted with 1% uranyl acetate in maleate buffer (pH 6.0; 1 h). After dehydration in ethanol and propylene 628 629 oxide, sections were embedded in Epon/Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA), flattened between Aclar sheets (Electron Microscopy Sciences) and 630 polymerized at 60°C for (48 hrs). Chips including lateral VTA were selected by light-631 microscopic inspection, glued to blank epoxy and sectioned with an ultramicrotome (MTX; 632 633 Research and Manufactoring Company Inc., Tucson, AZ, USA). The most superficial ultrathin sections (~60 nm) were collected and mounted on 300 mesh nickel grids, stained 634 635 with Sato's lead and examined with a Philips EM 208 and CM10 electron microscopes coupled to a MegaView-II high resolution CCD camera (Soft Imaging System). To minimize 636 637 the effects of procedural variables, all material from P16 and P50 females and males was processed in parallel. 638

639 Post-embedding procedures. Sections were processed for an osmium-free 640 embedding method⁵⁸. Dehydrated sections were immersed in propylene oxide, infiltrated with a mixture of Epon/Spurr resins, sandwiched between Aclar films, and polymerized at 641 60 °C for 48 h. After polymerization, chips were cut from the wafers, glued to blank resin 642 blocks and sectioned with an ultramicrotome. Thin sections (60-80 nm) were cut and 643 mounted on 300 mesh nickel grids and processed for immunogold labeling^{58,59}. In brief, after 644 645 treatment with 4 % para-phenylenediamine in Tris-buffered saline [0.1 M Tris, pH 7.6, with 0.005 %Tergitol NP-10 (TBST)], grids were washed in TBST (pH 7.6), transferred for 15 min 646 647 in 0.25% NDS in TBST (pH 7.6) and then incubated overnight (26°C) in a solution of TBST 648 (pH 7.6) containing a mixture of anti-D2 and anti-CB1 primary antibodies (see Table 1 for 649 dilutions). Grids were subsequently washed in TBST (pH 8.2), transferred for 10 min in 0.5% NDS in TBST (pH 8.2), incubated for 2 hrs (26°C) in TBST (pH 8.2) containing secondary 650 651 antibodies conjugated to 18 and 12 nm gold particles, washed in distilled water, and then

stained with uranyl acetate and Sato's lead. The optimal concentration of antibodies to D2 and CB1Rs was sought by testing several dilutions; the concentration yielding the lowest level of background labeling and still immunopositive elements was used to perform the final studies. Gold particles were not detected when primary antiserum was omitted. When normal serum was substituted for immune serum, sparse and scattered gold particles were observed, but they did not show any specific relationship to subcellular compartments.

Data collection and analysis. All data were obtained from lateral VTA of immunoreacted sections^{5,54}.

For pre-embedding electron microscopy, mGluR1 α , mGluR1 β , CB1, D2, D3, D4R immunoreactive profiles were studied in ultrathin sections from the surface of the embedded blocks. Quantitative data derived from the analysis of microscopic fields of lateral VTA (10– 12 ultrathin sections/animal) selected and captured at original magnifications of 12,000x-30,000x. Microscopical fields from females and males containing positive processes were randomly selected. Acquisition of microscopical fields and analysis of female and male mice were performed under blinded conditions.

For the analysis of the distribution of mGluR1 α , mGluR1 β , CB1, D2, D3, D4R positive 667 668 profiles, subcellular compartments were identified according to well-established criteria⁶⁰ 669 (Extended Data Figures and Tables 1 and 2). For quantifying mGluR1 α or mGluR1 β in P16 670 VTA, and mGluR1β in P50 VTA at axon terminals, synapses exclusively characterized by a presynaptic terminal with clear and round vesicles nearby the presynaptic density, a synaptic 671 672 cleft displaying electron dense material, pre- and postsynaptic membranes defining the 673 active zone and the post-synaptic specialization, and finally by a prominent postsynaptic density the asymmetric synapses^{60,61} were sampled (axon terminals making asymmetric 674 synaptic contacts containing one or more dense core vesicles more likely representative of 675 co-release of glutamate and others neurotransmitters^{62–64} were not included in this group; 676 677 Tables 2 and 4).

678 For quantifying CB1, D2, D3, D4R at astrocytic processes in P16 VTA, and CB1 and 679 D2R in P50 VTA, astrocytic profiles were identified based on their typical irregular outlines and the paucity of cytoplasmic components (with the exception of ribosomes, glycogen 680 granules and various fibrils⁶⁰. For post-embedding electron microscopy, ultrathin sections 681 682 (20 ultrathin sections/animal) were examined at 50,000-85,000x and fields that included at 683 least 1 double immunolabeled astrocytic profile were selected. For determining the relative 684 density of D2 and CB1Rs at the membranes of double labeled astrocytic profiles, pyramidal 685 cell nuclei were also identified: gold particles within labeled structures were counted and areas were calculated using ImageJ (NIH, Bethesda, MD, USA). Background was calculated 686 687 by estimating labeling density over pyramidal cell nuclei^{59,65}. Particle densities were counted and compared with background labeling. Gold particles were considered associated with 688 689 plasma membrane if they were within 20 nm of the extracellular side of the membrane. To 690 determine the degree of nearness of D2 and CB1R at the membrane of double-labeled 691 profiles the edge-to-edge distances between immunogold labeled D2 and CB1R were 692 measured along the membrane using ImageJ and the distribution of the separation 693 distances between D2 and CB1R was determined^{47,59,66–69}. In the cases in which multiple paths connecting particles gave different inter-distance values, the shortest inter-distance 694 695 was selected and used for distribution analysis. Given that gold particles with edge-to-edge 696 separation distance ≤50 nm are highly suggestive of physical interactions of two detected proteins (i.e., a physical coupling complex^{47,59,66–69}) distribution analysis of the interdistance 697 698 between particles was based on bin of 50 nm.

- For all microscopy data, normality tests and statistical analyses were performed using
 GraphPad Prism Software v.7.0a (San Diego, CA, USA).
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Protein	Source	Primary antibody	Host	Characterized in	Dilution	Secondary antibody	Source	Dilution
mGLUR1a	Frontier Institute	Rb-Af811 (RRID:AB_257179)	Rb	Ohtani et al. 2014 ^a	1:200	Biotinylated	Jackson	1:500
mGLUR1β	Frontier Institute	Rb-Af250 (RRID: AB_2616586)	Rb	Ohtani et al. 2014 ^b	1:100	Biotinylated	Jackson	1:500
CB1	Frontier Institute	GP-Af530 (RRID: AB_2571593)	GP	Garcia-Ovejero et al. 2013 ^c	1:200 IP 1:25 IG	Biotinylated 18 nm gold	Jackson Jackson	1:500 1:20
D2	Millipore	AB5084P (RRID:AB_2094980)	Rb	Stojanovic et al. 2017 ^d	1:100 IP 1:10 IG	Biotinylated 12 nm gold	Jackson Jackson	1:500 1:20
D3	Alomone	ADR-003 (RRID:AB_2039830)	Rb	Solís et al. 2017 Castro-Hernandez et al. 2015 ^e	1:100	Biotinylated	Jackson	1:500
D4	Millipore	AB324405 (RRID:AB_564550)	Rb	Barili et al. 2000 ^f	1:1000	Biotinylated	Jackson	1:500

707 708

709 Abbreviations: IP, immunoperoxidase for pre-embedding electron microscopy; IG,

710 immunogold for post-embedding electron microscopy.

711

^a Raised against a synthetic peptide corresponding to AA 945-1127 of mouse mGLUR1α.
 Immunoreactivity verified in mGluR1 knock-out mice⁷⁰.

^b Raised against a synthetic peptide corresponding to AA 899-906 of mouse mGLUR1β.
 Immunoreactivity verified in mGluR1 knock-out mice⁷⁰.

^c Raised against the C-terminal 31 AA of mouse CB1. Lack of immunoreactivity in CB1
 knock-out mice⁷¹.

^d Raised against a peptide corresponding to 28 AA within the third cytoplasmic loop of
 human D2 receptor. Lack of immunoreactivity in D2 knock-out mice⁷².

^e Raised against a synthetic peptide corresponding to AA 15-29 within the extracellular N terminus sequence of rat D3 receptor. Lack of immunoreactivity in D3 knock-out mice^{73,74}.

^f Raised against a synthetic peptide corresponding to AA 176-185 of human D4 receptor.
 Immunoreactivity totally abolished by preadsorption of D4 antibodies with immunogen peptide⁷⁵.

726 In vivo single unit recordings. C57BL/6J wild-type male mice, injected 2-3 weeks before with ssAAV-9/2-hGFAP-hM3D(Gq) mCherry-WPRE-hGHp(A) or AAV5.GfaABC1D.cyto-727 728 tdTomato.SV40, were anesthetized using chloral hydrate (400 mg/kg i.p.), supplemented as 729 required to maintain optimal anesthesia throughout the experiment, and placed in the 730 stereotaxic apparatus (Kopf, Germany). Their body temperature was maintained at 36 ± 1 °C using a feedback-controlled heating pad. For the placement of a recording electrode, the 731 732 scalp was retracted and one burr hole was drilled above the parabrachial pigmented nuclei 733 of the posterior VTA (AP: - 3.0-3.5 mm from bregma; L: 0.4-0.6 mm from midline; V: 4-5 mm 734 from the cortical surface) according to the Paxinos and Franklin atlas (2004). Extracellular 735 identification of putative DA neurons was based on their location as well as on the set of 736 electrophysiological features that characterize these cells in vivo: (1) a typical triphasic 737 action potential with a marked negative deflection; (2) an action potential width from start to 738 end > 2.5 ms; (3) a slow firing rate (< 10 Hz). VTA putative DA neurons were selected only when all the already published criteria were fulfilled^{76–79}. Single unit activity of putative DA 739 740 neurons was recorded extracellularly using glass micropipettes filled with 2% Chicago sky 741 blue dissolved in 0.5 M sodium acetate (impedance 3-7 MΩ). An injection pipette (20-40 μm 742 in diameter attached ~150-100 µm above the recording tip) was used for simultaneous local 743 microinjection of CNO (1 mM). Signal was pre-amplified, amplified (Neurolog System, 744 Digitimer, UK), filtered (band-pass 500-5000 Hz), and displayed on a digital storage 745 oscilloscope. Experiments were sampled on- and off-line by a computer connected to CED 746 Power 1401 laboratory interface (Cambridge Electronic Design, Cambridge, UK) running 747 the Spike2 software (Cambridge Electronic Design, Cambridge).

Single units were isolated and the spontaneous activity recorded for a minimum of 3 min before local application of CNO (1 mM). A total volume of 30-100 nl was infused using brief (10-100 ms) pressure pulses (40 psi, Picospritzer, Narishige, Japan). One injection maximum per hemisphere was given. For statistical analysis, we calculated the mean firing

752 rate (number of spikes/s) and the percentage of spikes in burst (SiB), before and after CNO 753 application (in 2-min bins or in the 10 min of recording after CNO application). Bursts were defined as the occurrence of two spikes at an inter-spike interval of < 80 ms, and terminated 754 when the inter-spike interval exceeded 160 ms⁸⁰. At the end of the experiment, negative 755 DC (15 mA for 5 minutes) was passed through the recording electrode to eject Pontamine 756 757 sky blue, which allowed the anatomical location of the recorded neuron. Mice were then 758 euthanized and brains were rapidly removed and fixed in 4% paraformaldehyde solution. 759 The position of the electrodes was identified with a microscope in coronal sections (100 µm). Only recordings in the correct area were considered for analysis. 760

761

Behavioral test. Viral Injection. C57BL/6J mice were naïve and two month-old at the time 762 of surgery. All mice were anesthetized with a mix of isoflurane/oxygen 2%/1% by inhalation 763 764 and mounted into a stereotaxic frame (Kopf). Brain coordinates of viral injections in the VTA were chosen in accordance with the mouse brain atlas: AP: -3 mm; ML: \pm 0.50 mm; DV: -765 4.7 mm. The volume of AAV injection (AAV9-GFAP-hM3D(Gg)-mcherry or AAV8-GFAP-766 GFP) was 100 nl per hemisphere. We infused virus through a glass micropipette connected 767 768 to a 10-µL Hamilton syringe. After infusion, the pipette was kept in place for 6 min and then slowly withdrawn. 769

Locomotor Activity. Mice were tested during first two hours of the dark phase in an experimental apparatus consisting of 4 grey, opaque open field boxes (40x40x40 cm) evenly illuminated by overhead lighting (5 ± 1 lux). Each session was video recorded with ANYmaze tracking software (Stoelting Co.) for one hour. In the first day of locomotor activity all animal received an injection of CNO (3mg/kg) 30 minutes before the beginning of the test; 48 hours later, the animals have been tested for a second time in the same apparatus with a saline injection.

777

Drugs. Picrotoxin 50 μ M (SIGMA, Aldrich, Milano, IT); AM251 2-4 μ M (Abcam, Cambridge, UK); eticlopride hydrochloride 1 μ M (Abcam, Cambridge, UK); SCH-23390 hydrochloride 10 μ M (Abcam, Cambridge, UK); D-AP5 50 μ M (Abcam, Cambridge, UK); LY-367385 100 μ M (Abcam, Cambridge, UK); clozapine-N-oxide (CNO) 10 μ M (MedChemExpress, USA) and L-741,626 10 μ M (Tocris, Bristol, UK) were bath applied. L-NAME 100 μ M (SIGMA, Aldrich, Milano, IT) was included in the patch pipette.

784

Data analysis. Data analysis was performed with Clampfit 10.5, Origin 8.0 (Microcal
 Software), Microsoft Excel 2010, ImageJ (NHI), Sigma Plot 11, ANY-maze tracking software
 and GraphPad Prism 7.0a.

788

789 Statistical analysis. No statistical methods were used to predetermine sample size. Data 790 are expressed as mean ± standard error of the mean (SEM). Normality test (Shaphiro-Wilk 791 test) was applied to the data before running statistical tests. Based on the normality test 792 result, data were analyzed using either parametric (two-tailed Student's t-test) or 793 nonparametric tests (Wilcoxon signed-rank test) as appropriate. For electron microscopy 794 data analysis, Mann-Whitney test and contingency Fisher's test were used. For *in vivo* single 795 unit recordings and behavioral tests, two-way RM ANOVA and Bonferroni's multiple 796 comparison test was used. Statistical differences were established with p < 0.05 (*), p < 0.01797 (**), p < 0.001 (***) and p < 0.0001 (****).

798

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811

812 AUTHOR CONTRIBUTIONS

LMR, MGG and GC designed the study. LMR and MGG performed the electrophysiological and Ca²⁺ imaging experiments in brain slices. AC performed the immunohistochemistry experiments. FM, GP and FP performed the behavioral experiments. MC and ALM performed the in vivo single unit recordings. MM, AP and FC performed the electron microscopy experiments. GM provided the CB1-floxed mice. All authors discussed the results. MGG and GC wrote the paper with inputs from all the authors.

819

820 COMPETING INTERESTS

821 The authors declare no competing interests.

822

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Figure 1. Astrocyte recruitment by DA neuron burst firing induces LTP of excitatory synapses onto adjacent DA neurons in young female mice. a) a1, low-magnification from a horizontal brain slice with a caudal-rostral orientation. Cx, cortex, Hc, hippocampus. Scale bar, 1 mm. a2, high magnification of the area indicated in a1. mt, medial terminal nucleus of the accessory optical tract; SN, substantia nigra (lateral to mt); VTA, ventral tegmental area (medial to mt), c, caudal; r, rostral. Dashed square, lateral part of the VTA where conventional DA neurons were recorded. Scale bar, 200 µm. a3, schematic of the experimental design showing a DA neuron pair, two recording pipettes, the burst firing imposed to a DA neuron (left) and the EPSC evoked on the other DA neuron (right) by extracellular stimulation of rostral glutamatergic afferents (Stim). b) Left, evoked EPSCs at basal conditions (bsl) and at different time points after bursts, from slices of female (upper traces) and male (lower traces) mice. Stimulus artifacts were removed. Scale bars, 50 pA, 20 ms. Right, EPSC amplitude in female (n = 11) and male ($n \ge 6$) mice after the burst firing protocol (arrowhead). In this and the other figures reporting EPSC amplitude vs time, t = 0indicates the end of the burst firing. c) Left, representative pair of evoked EPSCs (time interval, 50 ms) from a DA neuron of a female mouse, before (bsl, black traces) and after bursts (red traces). Scale bars, 20 pA, 10 ms. Right, paired-pulse ratio (ppr) values vs time in female (n = 11) and male (n = 12) mice. d) Left, EPSC amplitude after tonic and burst firing protocol in wt female mice (n = 8) and IP₃R2^{-/-} female mice (n = 12), respectively. Right, EPSC amplitude at time points indicated after burst/tonic firing in the different groups. e) Schematic and fluorescence image of a neurobiotin-488 filled DA neuron (dotted line, patch pipette) and SR101-loaded astrocytes (white asterisks). Scale bar, 30 µm. See next page.

Figure 1. f) Upper panel, time course of Ca²⁺ levels from astrocytes shown in (e) at basal conditions, 4.5 min and 24 min after burst firing. Scale bars, 100 %, 20 s. Lower panel, Fluo-4 fluorescence images of the dashed square shown in e). Arrows, two DA neuron dendrites at different focal planes; yellow asterisks, astrocytes displaying Ca²⁺ transients (upper traces) at the time points indicated (dashed lines). Scale bar, 20 μ m. g) Raster plot reporting the onset of Ca²⁺ transients from 84 astrocytes, at basal conditions and after DA neuron burst firing. In this and the other figures reporting the time course of the astrocytic Ca²⁺ spike probability/min, t = 0 indicates burst firing onset. h) Left, time course of astrocytic Ca²⁺ spike probability/min in female, male and IP₃R2^{-/-} female mice (n = 6), before and after burst firing. Right, Ca²⁺ spike probability/min in basal conditions and after burst firing. In this and the other figures reporting the time course of astrocytic Ca²⁺ spike probability/min in basal conditions and after burst firing. In this and the other figures reporting the time (n = 6), before and after burst firing. Right, Ca²⁺ spike probability/min in basal conditions and after burst firing. In this and the other figures, data are represented as mean ± SEM; *, < 0.05; **, < 0.01; ***, <0.001; ****, < 0.001.



Figure 2. bLTP generation in female mice requires both eCB and DA signaling to astrocytes, mGluR1 activation and NO signaling. a) Time course and bar chart of EPSC amplitude in the presence of different antagonists (AM251 (CB1R), n = 7; eticlopride (D2type R), n = 10; SCH-23390 (D1-type R), n = 10; D-AP5 (NMDAR), n = 11; LY-367385 (mGluR1), n = 12; L-NAME (NO synthase), n = 12). b) Time course and bar chart of astrocytic Ca²⁺ spike probability/min in the presence of antagonists that impaired bLTP generation ($n \ge 6$). c) Pre-embedding electron microscopy images from lateral VTA of a young female mouse of CB1, D2 and mGluR1ß receptors. Green arrows, immunopositive products in AsP (AsP+) and AxT (AxT+) forming asymmetric synaptic contacts (arrowheads) with a dendrite (Den). Scale bar, 300 nm. d) Post-embedding electron microscopy images of CB1/D2R immunogold double-labelled astrocytic processes (AsP) in lateral VTA (CB1R, 18 nm gold particles; D2R, 12 nm gold particles). Upper panel, a double-labelled AsP expressing CB1 and D2R (arrows) in close apposition to an asymmetric synapse (AxT, axon terminal; Den, dendrite; PSD, post-synaptic density). Lower panel, an edge-to-edge separation distance between these receptors \leq 50 nm (arrowhead. Scale bar, 300 nm. e) Upper panel, CB1 and D2R immunogold densities at the membranes of astrocytic processes (AsP, n = 79 from two P16 females; 30.1 ± 2.6 gold particles/ μ m² for D2Rs (D2 mem), and 24.9 \pm 2.0 particles/µm² for CB1Rs (CB1 mem)) and at neuronal nuclei (b, background values, n = 10; 0.82 ± 0.12 for 12 nm gold particles; 0.49 ± 0.07 for 18 nm gold particles, Mann-Whitney test). Lower panel, distribution of the edge-to-edge interdistances (bin, 50 nm) between membrane D2 and CB1R immunogold couples. f) Schematic of the proposed cellular and molecular mechanism for bLTP generation (see text for details).



Figure 3. Chemogenetic selective activation of astrocytes rescues bLTP in young male mice. a) Representative pre-embedding electron microscopy in the lateral VTA from a young male mouse of CB1 and D2R expression at AsP and mGlurR1ß expression at AxT forming asymmetric synaptic contact (arrowheads) with dendrite (Den). Blue arrows, immunopositive products in AsP (AsP+) and AxT. AsP-, AsP without immunoreactivity. Scale bar, 300 nm. Lower panel, quantification and comparison (contingency Fisher's test) of CB1, D2 and mGlu1ßR expression in female and male mice. b) Schematic of the AAV-9/2-hGFAP-hM3D(Gq) mCherry-WPRE-hGHp(A) injection in the VTA of a neonatal male mouse and fluorescence image of a brain slice two weeks after injection (yellow, mCherryhM3D expression). c) Confocal images of the VTA from a mouse injected with AAV-9/2hGFAP-hM3D(Gg) mCherry-WPRE-hGHp(A), showing the fluorescence of mCherry-hM3D (red), the nuclear Top-Ro3 (blue) and the specific green fluorescence for either neurons (α -NeuN staining) or astrocytes (α -S100 β staining). Scale bars, 50 µm. d) GCaMP6f fluorescence images of astrocytes in basal conditions and after CNO perfusion. Scale bar, 50 µm. Lower panels, time course of Ca²⁺ elevations evoked by CNO in these astrocytes (scale bars, 100%, 30 s) and mean change of total Ca^{2+} levels in slices (n = 9) expressing GCaMP6f and hM3D in astrocytes in response to CNO (scale bars, 2%, 30 s). e) Upper panel, schematic of the experimental design. Lower panel, CNO transiently increases EPSC amplitude of DA neurons in male mice expressing hM3D in astrocytes (n = 9), but not in non-injected mice (n = 8). These experiments were performed in the presence of AM251 and eticlopride. f) Upper panel, schematic of the experimental design. Lower panel, pairing of burst firing protocol and CNO application partially rescues bLTP in male mice expressing hM3D in astrocytes (n = 9), but not in non-injected mice (n = 6). These experiments were performed in the presence of AM251 and eticlopride.



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Figure 5. Chemogenetic activation of astrocytes in vivo favors bursting and overall firing activity in VTA DA neurons. a) Schematic of the experimental design showing the pipettes for recording (blue) and CNO application (gray). b) Left, representative action potential (start to end > 2.5 ms) from a putative VTA DA neuron. Right, location of a recorded putative VTA DA neuron. PSB; pontamine sky blue; SNR, susbtantia nigra pars reticulata; RPC, red nucleus. c, d) Upper panels, representative firing rate (Spikes/10 s) histograms and percentage of spikes in burst (SiB/20 s) trends over time of VTA DA neurons from mice expressing hM3D (c) or tdTomato (d) in VTA astrocytes. Lower panel, examples of raw spike traces of the same neurons before and after local CNO applications. e) Time course of the bursting activity (after local CNO applications (hM3D, n = 7; tdTomato n = 10). Two-wav RM ANOVA and Bonferroni's multiple comparison test: main effects are not indicated; hM3D 2 minutes vs. hM3D basal **p < 0.01; hM3D 4, 6, 8, 10 minutes vs. hM3D basal **** p < 0.0001. f) Percentage of spikes in bursts before and after local CNO applications (n hM3D = 7: n tdTtomato = 10). Two-way RM ANOVA and Bonferroni's multiple comparison test: main effects are not indicated; hM3D after CNO vs. hM3D basal ** p < 0.01. g) Time course of the firing rate after local CNO applications (hM3D, n = 7; tdTomato, n = 10). Two-way RM ANOVA and Bonferroni's multiple comparison test: main effects are not indicated; hM3D 2, 10 minutes vs. hM3D basal ***p < 0.001; hM3D 4, 6, 8 minutes vs. hM3D basal **** p < 0.0001. h) Firing rate before and after local CNO applications. Two-way RM ANOVA and Bonferroni's multiple comparison test: main effects are not indicated; hM3D after CNO vs. hM3D basal ** p < 0.01.



Figure 6. Chemogenetic activation of astrocytes *in vivo* induces a long-lasting motor hyperactivity. a) Schematic of the experimental design for testing locomotion in mice expressing in VTA astrocytes either GFP or hM3D, 30 min and 48 hrs after the intraperitoneal CNO application (3mg/kg). b) Fluorescence image of hM3D expression in VTA; fr, fasciculus retroflexus. c) Representative examples of locomotion in the open field test. d, e) Locomotor activity (d) and total distance (e) traveled by GFP- and hM3D-injected mice 30 min or 48 hrs after the intraperitoneal CNO injection (3 mg/kg, n = 4-6 mice/group). f) Time spent in the open field center of GFP- and hM3D-injected mice, at different time points after the intraperitoneal CNO injection.

Figures



Figure 1

Astrocyte recruitment by DA neuron burst firing induces LTP of excitatory synapses onto adjacent DA neurons in young female mice. a) a1, low-magnification from a horizontal brain slice with a caudal-rostral orientation. Cx, cortex, Hc, hippocampus. Scale bar, 1 mm. a2, high magnification of the area indicated in a1. mt, medial terminal nucleus of the accessory optical tract; SN, substantia nigra (lateral to mt); VTA, ventral tegmental area (medial to mt), c, caudal; r, rostral. Dashed square, lateral part of the VTA where conventional DA neurons were recorded. Scale bar, 200 µm. a3, schematic of the experimental design showing a DA neuron pair, two recording pipettes, the burst firing imposed to a DA neuron (left) and the EPSC evoked on the other DA neuron (right) by extracellular stimulation of rostral glutamatergic afferents

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а



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Figure 6

Chemogenetic activation of astrocytes in vivo induces a long-lasting motor hyperactivity. a) Schematic of the experimental design for testing locomotion in mice expressing in VTA astrocytes either GFP or hM3D, 30 min and 48 hrs after the intraperitoneal CNO application (3mg/kg). b) Fluorescence image of hM3D expression in VTA; fr, fasciculus retroflexus. c) Representative examples of locomotion in the open field test. d, e) Locomotor activity (d) and total distance (e) traveled by GFP- and hM3D-injected mice 30 min or 48 hrs after the intraperitoneal CNO injection (3 mg/kg, n = 4-6 mice/group). f) Time spent in the open field center of GFP- and hM3D-injected mice, at different time points after the intraperitoneal CNO injection.

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