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4 **Retrograde adenosine/A_{2A} receptor signaling mediates presynaptic**
5 **hippocampal LTP and facilitates epileptic seizures**
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46
47 **Abstract**
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51 A long-term change in neurotransmitter release is a widely expressed mechanism
52 controlling neural circuits in the mammalian brain. This presynaptic plasticity is commonly
53 mediated by retrograde signaling whereby a messenger released from the postsynaptic
54 neuron upon activity modifies neurotransmitter release in a long-term manner by targeting
55 a presynaptic receptor. In the dentate gyrus (DG), the main input area of the hippocampus,
56 granule cells (GCs) and mossy cells (MCs) form a recurrent excitatory circuit that is
57 critically involved in DG function and epilepsy. Here, we identified adenosine/A_{2A} receptor
58 (A_{2A}R) as a novel retrograde signaling system that mediates presynaptic long-term
59 potentiation (LTP) at MC-GC synapses. Using an adenosine sensor, we found that
60 neuronal activity triggered phasic, postsynaptic TrkB-dependent release of adenosine.
61 Additionally, epileptic seizures released adenosine *in vivo*, while removing A_{2A}Rs from DG
62 decreased seizure susceptibility. Thus, adenosine/A_{2A}R retrograde signaling mediates
63 presynaptic LTP that may contribute to DG-dependent learning and promote epilepsy.
64

65 INTRODUCTION

66
67 The dentate gyrus (DG), a major input area of the hippocampus, contains two types of
68 excitatory neurons: dentate granule cells (GCs) and hilar mossy cells (MCs). MCs and
69 GCs form an important but under-investigated associative circuit, that is proposed to play
70 a key role in DG-dependent cognitive functions^{1,2} and epilepsy³⁻⁵. Repetitive stimulation of
71 MC axons with physiologically relevant patterns of activity triggers robust presynaptic
72 long-term potentiation at MC-GC synapses (MC-GC LTP)⁶. Remarkably, recent evidence
73 supports that MC-GC LTP can be induced *in vivo* by enriched environment exposure⁷,
74 and following experimental epileptic activity⁸. Uncontrolled strengthening of MC-GC
75 transmission promotes seizures and contribute to the pro-epileptic role of MCs in early
76 epilepsy⁸. MC-GC LTP is mechanistically unique. Its induction is NMDA receptor-
77 independent but requires postsynaptic BDNF/TrkB and presynaptic cAMP/PKA signaling⁶,
78 strongly suggesting the involvement of a retrograde signal whose identity remains
79 unknown.

80
81 Retrograde signaling from the postsynapse to the presynapse is a well-established
82 mechanism implicated in presynaptic forms of long-term plasticity⁹. In this way, the target
83 neuron can strengthen or weaken the synaptic inputs it receives. Diverse messengers
84 have been identified, including lipids, gases, peptides and conventional
85 neurotransmitters¹⁰. Typically, these messengers are released upon neuronal activity and
86 target a presynaptic receptor (or other molecular target), thereby establishing a retrograde
87 signaling system. The following criteria must be satisfied to establish retrograde signaling
88 as a mechanism of presynaptic long-term plasticity. The retrograde messenger must be
89 synthesized and released from the postsynaptic compartment; interfering with the
90 synthesis and/or release of this messenger should prevent plasticity; the target for the
91 retrograde messenger must be present in the presynaptic bouton; interfering with the
92 presynaptic target should also prevent plasticity; and lastly, activation of the presynaptic
93 target by the retrograde messenger or some analogous molecule should mimic long-term
94 plasticity –although in some cases this activation alone may not be sufficient to mimic
95 plasticity.

96
97 In this study, we sought to determine the retrograde signal involved in presynaptic LTP at
98 MC-GC synapses. To our surprise, we found that most conventional retrograde
99 messengers were not implicated in this form of plasticity. Using single-cell manipulations
100 and selective pharmacology, we discovered that activation of G_s-coupled adenosine A_{2A}
101 receptors (A_{2A}Rs) are necessary and sufficient to induce MC-GC LTP. Interfering with
102 adenosine release from a single GC abolished LTP, and immunoelectron microscopy
103 revealed A_{2A}Rs at MC axon terminals. In addition, using a genetically encoded adenosine
104 sensor, we found that neuronal activity triggered phasic release of adenosine in a
105 postsynaptic TrkB-dependent manner. Furthermore, acutely induced epileptic seizures
106 released adenosine *in vivo*, while removing A_{2A}Rs from DG decreased seizure
107 susceptibility. Our findings not only establish adenosine/A_{2A}R as a novel retrograde
108 signaling system that mediates presynaptic plasticity but may also provide a mechanism
109 by which BDNF/TrkB and A_{2A}Rs may promote epileptic activity.

110 RESULTS

111

112 Theta burst firing of a single GC induces presynaptic LTP at MC-GC synapse

113

114 To investigate the identity of the retrograde signal mediating MC-GC LTP, we first tested
115 whether activation of a single GC, a manipulation previously used to characterize
116 endocannabinoid retrograde signaling in long-term plasticity¹¹, could trigger MC-GC LTP.
117 We found that GC theta-burst firing (TBF: 10 bursts at 5 Hz of 5 AP at 50 Hz, repeated 4
118 times every 5s, **Fig. 1a**) induced LTP selectively at MC-GC synapses but not at
119 neighboring medial perforant path (MPP) inputs (**Fig. 1b**). In addition, the group II mGluR
120 agonist DCG-IV (1 μ M), which selectively abolishes GC-MC but not MC-GC
121 transmission¹², did not affect the magnitude of TBF-induced LTP (**Fig. 1f** and **Fig. S1a**),
122 indicating this plasticity does not rely on the recruitment of MCs. Like synaptically-induced
123 MC-GC LTP^{6,7}, TBF-LTP is likely expressed presynaptically, as indicated by a significant
124 reduction in both paired-pulse ratio (PPR) and coefficient of variation (CV) (**Fig. 1c**). Like
125 synaptically-induced MC-GC LTP, TBF-LTP requires postsynaptic BDNF release,
126 postsynaptic but not presynaptic TrkB, and presynaptic cAMP/PKA signaling. Indeed,
127 TBF-LTP was abolished in the presence of the selective TrkB antagonist ANA-12 (15 μ M)
128 (**Fig. S1b** and **Fig. 1f**), and in postsynaptic but not presynaptic *TrkB* conditional knockout
129 (cKO) mice (**Fig. 1d,f** and **Fig. S1c**). TBF-LTP was also abolished in postsynaptic *Bdnf*
130 cKO mice, and in GC patch-loaded with botulinum toxin-B (Botox, 0.5 μ M; **Fig. S1d,e** and
131 **Fig. 1f**), which blocks BDNF release¹³. The PKA inhibitors, H89 (10 μ M, 40- to 60-min pre-
132 incubation and bath applied) (**Fig. S1f** and **Fig. 1f**) and PKI₁₄₋₂₂ myristoylated (1 μ M, 40-
133 to 60-min pre-incubation and bath applied) also abolished TBF-LTP, whereas loading the
134 membrane-impermeant PKI₆₋₂₂ (2.5 μ M) in GCs had no effect on this LTP (**Fig. 1e,f**). TBF-
135 LTP was also NMDAR-independent as it was normally induced in the presence of D-APV
136 (50 μ M) (**Fig. S1g** and **Fig. 1f**). Lastly, synaptically-induced MC-GC LTP —i.e. burst
137 stimulation (BS) of MC axons— and TBF-LTP occluded each other (**Fig. S2**), strongly
138 suggesting a common mechanism. Thus, the fact that a single-cell manipulation (GC TBF)
139 induced presynaptic LTP of MC-GC transmission with identical properties as synaptically-
140 induced MC-GC LTP, not only strengthens the involvement of retrograde signaling in this
141 form of plasticity, but also establishes a simple tool to investigate the identity of the
142 retrograde messenger as this induction method presumably does not recruit molecular
143 signals arising from neighboring cells.

144

145

146 A non-conventional retrograde signal likely mediates presynaptic LTP at MC-GC 147 synapses

148

149 Previous work indicates that retrograde signaling mediating MC-GC LTP does not involve
150 endocannabinoids, glutamate or GABA^{6,14}, and our current finding that TBF-LTP was
151 normally induced in presynaptic *TrkB* cKO (**Fig. S1c** and **Fig. 1f**), discards BDNF as
152 retrograde messenger. Thus, we tested the role of other conventional retrograde
153 messengers, such as nitric oxide (NO) and lipid-derived messengers¹⁰. Application of the
154 NO synthase inhibitor L-NAME (100 μ M, 50- to 90-min pre-incubation and bath applied)

155 did not impair TBF-LTP (**Fig. 2a**), but it significantly reduced LTP at CA3-CA1 synapses
156 (**Fig. 2b**), as previously reported¹⁵. Blockade of lipid-derived messengers with a cocktail
157 of inhibitors consisting of the FAAH and anandamide amidase inhibitor AACOCF3 (10 μ M,
158 50- to 80-min pre-incubation and bath applied), the lipoxygenases inhibitor baicalein (3
159 μ M, 50- to 80-min pre-incubation and bath applied) and the lipase inhibitor THL
160 (tetrahydrolipstatin or orlistat, 4 μ M in the recording pipette) did not impair TBF-LTP either
161 (**Fig. 2c**). As positive control, we found that both AACOCF3 and baicalein significantly
162 reduced LTD at CA3-CA1 synapses (**Fig. 2d**) and loading the lipase inhibitor THL (4 μ M)
163 in the recording pipette efficiently blocked CA1 inhibitory LTD (iLTD) (**Fig. 2e**)¹⁶. Lastly, as
164 for TBF-LTP, blocking NO signaling and lipid-derived messengers did not alter
165 synaptically-induced LTP either (**Fig. S3**). Altogether, our results strongly suggest the
166 involvement of a non-conventional retrograde messenger in presynaptic MC-GC LTP.

167 168 **Presynaptic A_{2A}Rs are required for activity-dependent strengthening of MC-GC** 169 **synapses**

171 Presynaptic PKA signaling is necessary and sufficient to induce LTP of MC-GC synaptic
172 transmission⁶, raising the possibility that the retrograde messenger induces LTP by
173 activating Gs-coupled GPCRs located on MC axon terminals. The Gs-coupled adenosine
174 A_{2A} receptor (A_{2A}R) is a good candidate as it has been implicated in BDNF-mediated
175 plasticity at other synapses¹⁷⁻¹⁹, and the endogenous ligand adenosine can be released
176 from neurons upon activity²⁰⁻²⁴. To test whether adenosine mediates MC-GC LTP by
177 activating presynaptic A_{2A}Rs, we first examined whether LTP induction requires A_{2A}Rs.
178 Remarkably, two different A_{2A}R selective antagonists, SCH 58261 (100 nM) and
179 ZM241385 (50 nM), abolished TBF-LTP (**Fig. 3a**). Moreover, bath application of SCH
180 58261 did not change basal transmission (**Fig. 3b**) but prevented the induction of LTP by
181 BDNF puffs (8 nM, 2 puffs of 3 s) delivered in the inner molecular layer (IML) (**Fig. 3c**)⁶,
182 suggesting that A_{2A}R activation is required downstream of the BDNF/TrkB cascade.
183 Postsynaptic A_{2A}Rs were not involved given that including the G-coupled protein inhibitor
184 GDP β s (1 mM) in the recording pipette did not affect TBF-LTP, (**Fig. 3d**), whereas it
185 abolished the change in holding current induced by the selective GABA_B receptor agonist
186 baclofen (10 μ M) (**Fig. 3d**). Likewise, BS-induced LTP was also abolished by SCH 58261
187 (**Fig. S4a**), but not by including GDP β s in the recording pipette (**Fig. S4b**). To demonstrate
188 the role of presynaptic A_{2A}Rs in MC-GC LTP, we also employed a conditional knockout
189 strategy combined with optogenetics which allow us to selectively activate A_{2A}R-deficient
190 MC axons expressing the fast opsin ChIEF (**Fig. 3e**). We found that presynaptic A_{2A}R cKO
191 abolished TBF-LTP (**Fig. 3f**). Taken together, these findings indicate that presynaptic but
192 not postsynaptic A_{2A}R activation was necessary for LTP at MC-GC synapses.

194 A_{2A}Rs are likely activated by adenosine released from GCs during LTP induction. As
195 adenosine can also target presynaptic adenosine type 1 receptors (A₁Rs), G_{i/o}-coupled
196 receptors whose activation typically suppress neurotransmitter release, we therefore
197 tested the role of A₁Rs in this LTP. Indeed, the selective A₁R antagonist DPCPX (100 nM)
198 significantly increased MC-GC LTP magnitude (**Fig. 3g**), whereas it had no effect on basal
199 transmission (**Fig. 3h**), indicating that A₁Rs negatively control MC-GC LTP.

200

201 **Activation of presynaptic A_{2A}Rs induces MC-GC LTP in a PKA-dependent manner**

202 Our results strongly suggest that MC terminals express functional A₁Rs and A_{2A}Rs (**Fig.**
203 **3**). To directly assess this possibility, we performed immunoelectron microscopy.
204 Immunoparticles for A₁Rs were mainly found presynaptically in the molecular layer of the
205 dentate gyrus at MC-GC and perforant path (PP)-GC synapses (**Fig. 4a-d**). Remarkably,
206 using an anti-A_{2A}R antibody previously validated in A_{2A}R-deficient mice²⁵, we found
207 strong A_{2A}R expression in the presynaptic membrane of asymmetric, putative MC-GC
208 synapses in the IML (**Fig. 4e,f,h**) and very few particles postsynaptically (**Fig. 4e,f,h**). In
209 contrast, a quasi-exclusive postsynaptic expression was detected at asymmetric PP-GC
210 synapses (**Fig. 4g-h**).

211 We next tested whether A_{2A}R activation was sufficient to induce LTP at MC-GC synapses.
212 Bath application of the A_{2A}R agonist CGS21680 (50 nM, 15 min) selectively enhanced MC
213 but not MPP EPSC amplitude (**Fig. 5a**). This synapse-specific potentiation was associated
214 with a significant decrease in both PPR and CV (**Fig. 5b**), suggesting a presynaptic
215 mechanism of expression, and supporting a presynaptic location of A_{2A}Rs. Adding the
216 selective A_{2A}R antagonist SCH 58261 (100 nM) during the washout of CGS21680 (50 nM)
217 did not impair the long-lasting potentiation, whereas continuous bath application of SCH
218 58261 (100 nM) abolished the CGS21680-induced potentiation (**Fig. 5c**). These results
219 indicate that activation of A_{2A}Rs was sufficient to induce LTP at MC-GC synapses.
220 Because the A_{2A}R is a G_s-coupled receptor, we next tested whether CGS21680-induced
221 LTP was PKA-dependent. Bath application of the selective, cell-permeant PKA inhibitor
222 myristoylated PKI₁₄₋₂₂ (1 μM) abolished CGS21680-induced LTP, whereas loading the
223 membrane-impermeant PKA inhibitor PKI₆₋₂₂ (2.5 μM) in GCs via the recording pipette had
224 no effect (**Fig. 5d**). Thus, A_{2A}Rs enhance glutamate release via presynaptic PKA
225 activation, consistent with previous findings showing that PKA activity is necessary and
226 sufficient for MC-GC LTP⁶.

227 Our results thus far indicate that both BDNF/TrkB and adenosine/A_{2A}R signaling are
228 involved in MC-GC LTP, and previous work demonstrated that A_{2A}Rs facilitate BDNF
229 signaling at some synapses¹⁷. We therefore sought to determine potential interactions
230 between these signaling cascades at the MC-GC synapse. The TrkB antagonist ANA-12
231 had no effect on CGS21680-induced LTP (**Fig. 5e**), whereas it blocked TBF-LTP in
232 interleaved experiments (**Fig. 5f**; see also **Fig. S1b**). These findings, together with our
233 previous results (**Fig. 3, 4**), strongly suggest that presynaptic adenosine/A_{2A}R signaling
234 mediates LTP downstream of BDNF/TrkB signaling.

235 MC axon terminals also express A₁Rs (**Fig. 4**) whose activation could trigger presynaptic
236 long-term depression²⁶. However, bath application of the selective A₁R agonist CCPA (50
237 nM, 15 min) reduced both MC and MPP-mediated transmission reversibly as the reduction
238 was washed out with the A₁R antagonist DPCPX (100 nM) (**Fig. S5a**). This transient A₁R-
239 induced depression was associated with a reversible increase in both PPR and CV (**Fig.**
240 **S5b,c**), suggesting a presynaptic mechanism. CCPA (50 nM, 15 min) failed to induce
241 synaptic depression in the presence of DPCPX (100 nM) (**Fig. S5,d**). Thus, although both

242 A₁ and A_{2A} receptors are expressed at MC axon terminals, only the latter engages long-
243 lasting synaptic plasticity.

244 **Passive adenosine release from GC is required for presynaptic MC-GC LTP**

245 Adenosine can be released from neurons upon activity^{20,22-24} via equilibrative nucleoside
246 transporters (ENTs)²¹. Our results strongly suggest that presynaptic A_{2A}Rs are activated
247 by endogenous adenosine during MC-GC LTP (**Fig. 3**). If adenosine is the retrograde
248 signal, interfering with adenosine release selectively from GCs should impair LTP. As a
249 first approach, we tested whether blocking ENTs can interfere with LTP induction. Bath
250 application of the ENTs inhibitors dipyridamole (20 μM) and NBMPR (10 μM) abolished
251 LTP (**Fig. 6a**) but did not affect basal MC-GC synaptic transmission (**Fig. 6b**). Because
252 ENTs are also implicated in adenosine reuptake, bath application of the ENT blockers
253 may significantly increase extracellular adenosine levels, which, by activating A_{2A}Rs may
254 potentiate MC-GC transmission and occlude LTP. The fact that ENT blockers had no
255 effect on basal MC-GC synaptic transmission (**Fig. 6b**) could be due to simultaneous
256 activation of A₁R, which might mask any potential A_{2A}R-mediated potentiation. To test this
257 possibility, we bath applied both the ENT blockers and DPCPX (100 nM) to prevent a
258 potential A₁R-mediated depression of MC-GC synaptic transmission. We found that co-
259 application of the ENT blockers and DPCPX (100 nM) increased MC-GC transmission,
260 and this effect was abolished in the presence of the A_{2A}R antagonist SCH 58261 (100 nM)
261 (**Fig. 6c**). These results indicate that increasing the extracellular concentration of
262 endogenous adenosine is sufficient to activate presynaptic A_{2A}R and trigger MC-GC
263 potentiation.

264 To avoid adenosine tonic activity due to extracellular accumulation, we employed a single-
265 cell approach and selectively blocked ENTs in a single GC. Similarly to previous
266 studies^{22,24}, we loaded inosine (100 μM) intracellularly, a competitive blocker of adenosine
267 efflux through ENTs²¹, *via* the recording pipette. We found that intracellular inosine
268 abolished TBF-LTP (**Fig. 6d**), whereas bath application of 100 μM inosine had no effect
269 on MC-GC synaptic transmission (**Fig. 6e**). Bath application of DPCPX increased MC
270 EPSC amplitude when ENTs inhibitors were continuously bath applied but not when
271 inosine (100 μM) was loaded in a single GC (**Fig. 6f**), confirming that intracellular inosine
272 did not increase adenosine tone. Altogether these findings indicate that adenosine,
273 passively released from GC through ENTs, likely acts as a retrograde messenger at MC-
274 GC synapses.

275 **Repetitive neuronal activity induces adenosine release via a BDNF/TrkB-dependent** 276 **mechanism**

277 To test whether adenosine is released during MC-GC LTP induction, we utilized the
278 genetically encoded sensor for adenosine GRAB_{Ado1.0m}²⁷ selectively expressed in
279 commissural MC axons (see Methods), and found a clear fluorescence signal in the
280 contralateral IML (**Fig. 7a,b**). The MC BS protocol that triggers MC-GC LTP in acute
281 hippocampal slices also induced a transient increase in the GRAB_{Ado1.0m} signal. As
282 expected²⁷, this enhancement was abolished when the MC BS protocol was delivered in

283 the presence of the $A_{2A}R$ antagonist SCH58261 (**Fig. 7b,c,f**). Consistent with $A_{2A}R$
284 signaling being engaged downstream of TrkB activation, we found no change in
285 GRAB_{Ado1.0m} fluorescence in the presence of the TrkB antagonist ANA-12 (15 μ M), and in
286 postsynaptic *TrkB* cKO mice (**Fig. 7d,e,f**). Altogether these results demonstrate that MC
287 repetitive activity releases adenosine in a postsynaptic TrkB-dependent manner.

288 Lastly, we aimed to determine whether neuronal activity induces adenosine release *in vivo*
289 and the functional impact of such release. We recently found that MC-GC LTP can also
290 be triggered by early epileptic activity induced with kainic acid (KA) intraperitoneal (i.p.)
291 administration, and this LTP further promotes seizure activity⁸. Therefore, we
292 hypothesized that epileptic activity should release adenosine, and interfering with
293 adenosine/ $A_{2A}R$ signaling should reduce seizures. To test these predictions, we
294 expressed GRAB_{Ado1.0m} in commissural MC axons and recorded the fluorescence signals
295 using fiber photometry in freely moving mice (**Fig. 8a,b**). We found a large increase in
296 GRAB_{Ado1.0m} fluorescence following i.p. injection of KA (**Fig. 8c,d**) but not saline (not
297 shown). Moreover, $A_{2A}R$ deletion from DG excitatory neurons, which blocked MC-GC LTP
298 *in vitro* (**Fig. 3**), reduced KA-induced seizure susceptibility and severity (**Fig. 8e-i**).
299 Altogether, these results not only demonstrate *in vivo* adenosine release following
300 neuronal activity that occur during initial seizures, but strongly suggest that adenosine
301 signaling promotes seizures via $A_{2A}Rs$.

302

303 **DISCUSSION (1,558 words)**

304
305 In this study, we discovered a novel retrograde signaling mechanism that mediates
306 presynaptic activity-dependent synaptic strengthening, at an important but long-
307 overlooked hippocampal circuit (**Fig. S6**). Specifically, we found that repetitive firing of a
308 single postsynaptic neuron was sufficient to trigger presynaptic LTP at MC-GC but not
309 neighboring MPP synapses. We demonstrated that functional A_{2A} receptors are
310 expressed at MC axon terminals but not at MPP axon terminals, and that $A_{2A}R$ activation
311 is necessary and sufficient to induce MC-GC LTP. During LTP induction GCs passively
312 release adenosine through ENTs, in a postsynaptic TrkB- and activity-dependent manner.
313 Moreover, we found that adenosine is released *in vivo* during initial seizures and $A_{2A}R$
314 cKO has an anti-epileptic effect. Our findings establish adenosine/ $A_{2A}R$ as a novel
315 retrograde signaling mechanism not only relevant to synaptic plasticity but also to early
316 epileptogenesis.

317

318 **GC TBF induces presynaptic LTP at MC-GC synapses**

319
320 We previously showed that MC bursts of activity powerfully induce MC-GC LTP⁶. The
321 present study revealed that direct activation of a single GC with physiologically relevant
322 pattern of activity²⁸⁻³³ was sufficient to induce presynaptic LTP at MC-GC synapses
323 without changing the strength of MPP neighboring inputs. Against the possibility that this
324 single cell-induced LTP could emerge from the activation of the recurrent GC-MC-GC
325 circuit are the following two observations. First, GC TBF induced normal LTP even in

326 continuous presence of the selective group II mGluR agonist DCG IV, which abolishes
327 GC-MC^{34,35} but not MC-GC transmission¹². Second, TBF LTP was normally induced at
328 optogenetically activated synaptic inputs arising from contralateral MC axons that are
329 separated from their cell bodies in hippocampal slices. Importantly, our results strongly
330 suggest that GC TBF and synaptically (MC BS)-induced LTP share a common mechanism
331 that involves postsynaptic BDNF/TrkB and presynaptic adenosine/A_{2A}R and cAMP/PKA
332 signaling. In addition, both potentiations are expressed presynaptically. We recently found
333 that synaptically-induced MC-GC LTP requires BDNF release from GCs⁷. It is therefore
334 likely that GC TBF induces LTP in part by releasing BDNF from the postsynaptic
335 compartment. Consistent with this possibility, we now report that GC TBF-induced LTP
336 requires both dendritic BDNF release via SNARE-dependent exocytosis as also reported
337 in cortical neurons and hippocampal slices^{13,36}.

338 339 **Adenosine is released from GC in an activity and TrkB-dependent manner**

340
341 Using the genetically encoded adenosine sensor GRAB_{Ado1.0m}²⁷, we detected a phasic
342 increase in extracellular adenosine following both LTP induction *ex vivo* and during
343 epileptic activity *in vivo*. Previous studies have shown that adenosine can be released
344 from neurons upon activity via ENTs²⁰⁻²⁴. In support of this mechanism, we found that
345 interfering with ENT-mediated release of adenosine from a single postsynaptic GC
346 abolished MC-GC LTP, indicating that passive release of adenosine from GCs is crucial
347 for this form of plasticity. However, we cannot discard a potential contribution of adenosine
348 arising from ATP extracellular conversion^{37,38} or from other cell types such as interneurons
349 and glial cells^{20,39}.

350
351 Intracellular accumulation of adenosine can be the consequence of sequential
352 dephosphorylation of ATP or the hydrolysis of S-adenosylho-mocysteine⁴⁰. During MC-
353 GC LTP induction, formation of intracellular adenosine in GCs likely results from robust
354 postsynaptic TrkB activation, which presumably causes sequential dephosphorylation of
355 ATP. In support of this scenario are the following observations. First, MC-GC LTP
356 induction releases BDNF that activates TrkB receptors on GCs^{6,7}. Second, activity-
357 induced adenosine release in the IML is postsynaptic TrkB-dependent, as it is abolished
358 in the presence of the TrkB selective antagonist ANA-12 and by genetically removing *TrkB*
359 from GCs. Third, adenosine/A_{2A}R signaling is engaged downstream of TrkB activation, as
360 indicated by the fact that BDNF-induced LTP was abolished by A_{2A}R antagonism, whereas
361 TrkB antagonism had no effect on A_{2A}R agonist-induced LTP. Lastly, adenosine acts via
362 presynaptic A_{2A}Rs, whereas BDNF activates postsynaptic TrkB^{6,7}, discarding physical
363 interaction or transactivation, a process whereby A_{2A}R activation can induce TrkB
364 phosphorylation in the absence of neurotrophins⁴¹.

365
366 To our knowledge, we provide the first direct evidence that adenosine release can occur
367 in a TrkB-dependent manner. Such a mechanism could explain, at least in part, BDNF
368 and adenosine signaling interactions reported at other synapses in the brain. Several
369 studies showed that activation of the G_s-coupled A_{2A}R and PKA are required for BDNF-
370 induced facilitation of synaptic transmission and plasticity. Although such observations

371 have been extensively reported in the hippocampus^{17-19,42,43}, the precise mechanism of
372 this crosstalk remains elusive. Consistent with our findings at the hippocampal MC-GC
373 synapse, BDNF-induced facilitation at the neuromuscular junction was abolished in
374 presence of A_{2A}R antagonists but also by PKA inhibitors, while A_{2A}R agonist-induced
375 increase in neurotransmitter release is TrkB-independent^{17,42,43}. In conclusion, our
376 findings support a model whereby neuronal activity releases adenosine in TrkB-dependent
377 manner. Further work is required to determine the generalizability of this model in other
378 brain areas and to identify the precise mechanisms whereby TrkB activation leads to
379 adenosine release.

380

381 **Retrograde adenosine/A_{2A}R cascade mediates presynaptic LTP at MC-GC** 382 **synapses**

383

384 Retrograde signaling is a common mechanism in presynaptic forms of long-term
385 plasticity⁹. While several retrograde signals have been identified throughout the brain¹⁰;
386 here we uncovered adenosine/A_{2A}R as a novel form of retrograde signaling in the
387 hippocampus. Single cell manipulations revealed that interfering with adenosine release
388 from a single postsynaptic neuron completely abolished MC-GC LTP, induced by GC firing
389 alone. Furthermore, by using pharmacology, A_{2A}R presynaptic cKO and electron
390 microscopy, we demonstrated that activation of presynaptic A_{2A}R was necessary and
391 sufficient for MC-GC LTP. However, we cannot discard potential role of other cell-types,
392 including glial cells, as potential source or target of adenosine. Despite the relatively low
393 expression levels of A_{2A}R in the hippocampus⁴⁴, previous work has demonstrated that
394 A_{2A}R signaling can regulate hippocampal synaptic plasticity^{45,46}. To our knowledge, our
395 study provides the first evidence that retrograde adenosine signaling can mediate
396 presynaptic LTP. As Gs-coupled receptors, A_{2A}R on MC axon terminals likely activate
397 the cAMP/PKA cascade, and as for other forms of presynaptic LTP⁹, this activation
398 engages a long-lasting increase of glutamate release whose downstream mechanism is
399 poorly understood. Whether retrograde adenosine/A_{2A}R signaling can mediate activity-
400 dependent strengthening at other synapses in the brain remain to be investigated.

401

402 **Role of A₁R in controlling MC-GC synaptic transmission and LTP**

403

404 As previously reported at other synapses^{42,47}, our findings strongly suggest that
405 endogenous adenosine simultaneously activate A₁R and A_{2A}R localized at MC terminals.
406 While there is evidence that A₁R and A_{2A}R can localize at the same nerve terminal⁴⁸ and
407 even form heteromers⁴⁹, our results cannot deny or support these possibilities. The
408 induction of MC-GC LTP was associated with a surge of extracellular adenosine that
409 triggered LTP via A_{2A}R activation, but dampened LTP via A₁R activation. While A₁R
410 agonism transiently inhibited glutamate release, A_{2A}R agonism increased glutamate
411 release in a long-lasting manner. Consistent with these pharmacological observations, the
412 net effect mediated by transient release of endogenous adenosine during LTP induction
413 was a long-lasting enhancement of glutamate release. Similar A₁R-mediated dampening
414 of LTP has previously been observed at other synapses^{50,51}. A possible explanation for
415 this dampening is that presynaptic A₁Rs, which are G_{i/o}-coupled, decrease the level of

416 cAMP required for MC-GC LTP⁶. We recently reported that the type 1 cannabinoid
417 receptor, another G_{i/o}-coupled receptor that is highly expressed at MC axon terminals,
418 also dampens MC-GC LTP¹⁴. Thus, the induction of this form of plasticity is tightly
419 controlled by two distinct retrograde signals, adenosine and endocannabinoids, which by
420 activating presynaptic G_{i/o}-coupled receptors dampen LTP induction. In this way, both
421 retrograde signals could prevent runaway activity of the MC-GC-MC recurrent excitatory
422 circuit. Notably, we did not find any tonic regulation of basal transmission by A₁R nor A_{2A}R
423 at MC-GC synapse.

424 425 **Physiological and pathological relevance of A_{2A}R-mediated LTP in the dentate** 426 **gyrus** 427

428 Growing evidence indicates that MCs are critically involved in hippocampal-dependent
429 learning². For example, silencing MCs, selectively, impairs retrieval of spatial memory⁵²
430 and novelty-induced contextual memory acquisition⁵³. MC-GC LTP is a robust form of
431 presynaptic plasticity that can be elicited in vivo upon experience⁷ and, by changing
432 information flow in the DG⁶, it may contribute significantly to learning and memory. While
433 GCs exhibit extremely sparse and selective firing in single place field³⁰, we found that MC-
434 GC LTP can be triggered by GC firing alone, suggesting that this plasticity could be
435 implicated in experience-driven refinement of DG circuit and memory. Previous work
436 indicates that adenosine/A_{2A}R signaling may contribute to hippocampal-dependent
437 memory^{46,49}. Our findings demonstrating a key role for adenosine/A_{2A}R signaling in MC-
438 GC LTP could explain, at least in part, memory impairments found in both A_{2A}R
439 antagonist-injected animals⁵⁴ and hippocampal A_{2A}R cKO⁵⁵. Demonstrating the precise
440 role of adenosine/A_{2A}R retrograde signaling at MC-GC synapses will necessitate selective
441 manipulation of A_{2A}R at MC axon terminals, a task requiring tools that are not currently
442 available.

443
444 Adenosinergic signaling is critically involved in epilepsy⁵⁶. Global A_{2A}R genetic deletion
445 and selective A_{2A}R antagonism attenuates both seizures progression^{57,58} and seizures-
446 induced neuronal damage⁵⁹⁻⁶¹, whereas A_{2A}R activation lowers seizures' threshold⁶². In
447 addition, decreased A₁R signaling and increased A_{2A}R⁶³ and BDNF/TrkB signaling⁶⁴ occurs
448 in epilepsy. Remarkably, we found that MC-GC LTP required TrkB-dependent
449 adenosine/A_{2A}R signaling while its induction was negatively regulated by adenosine A₁R
450 activity. Although the precise mechanism by which A_{2A}R participate in temporal lobe
451 epilepsy is poorly understood, presynaptic A_{2A}R at MC axons may explain, at least in
452 part, the pro-epileptic role of A_{2A}R and the antiepileptic role of A₁R. This notion is
453 consistent with our recent work demonstrating that initial seizures trigger MC-GC LTP
454 broadly in the DG and that uncontrolled LTP is sufficient to promote subsequent seizures⁸.
455 Our new study demonstrating adenosine/A_{2A}R-dependent strengthening of MC-GC
456 synapses provides not only a novel mechanism whereby MC activity may contribute to
457 early stages of temporal lobe epilepsy^{2,5,8,65}, but also highlights a potential target for the
458 treatment of this disorder.

459
460

461 **METHODS**

462

463 **Experimental Model and Subject Details**

464 Sprague-Dawley rats P19-P30 and P50-P70 C57BL/6, floxed TrkB (*TrkB^{fl/fl}*), floxed BDNF
465 (*Bdnf^{fl/fl}*) and floxed A_{2A}R (*A_{2A}R^{fl/fl}*) mice were used. Males and females were equally used.
466 All animals were group housed in a standard 12 hr light/12 hr dark cycle and had free
467 access to food and water. Animal handling and use followed a protocol approved by the
468 Institutional Animal Care and Use Committee of Albert Einstein College of Medicine, in
469 accordance with the National Institutes of Health guidelines. *TrkB^{fl/fl}* and *Bdnf^{fl/fl}* mice
470 generated by Dr. Luis Parada, were kindly donated by Dr. Lisa Monteggia (University of
471 Texas, Southwestern Medical Center). *Adora2^{fl/fl}* were obtained from Jackson Laboratory
472 (B6;129-Adora2atm1Dyj/J, Jax 010687).

473

474 **Hippocampal slice preparation**

475 Acute transverse hippocampal slices were prepared from Sprague-Dawley rats (400 μm
476 thick) and mice (300-μm thick). Animals were anesthetized with isoflurane and
477 euthanized in accordance with institutional regulations. The hippocampi were then
478 removed and cut using a VT1200s microslicer (Leica Microsystems Co.) in a cutting
479 solution. Hippocampal slices from rats were prepared using a cutting solution containing
480 (in mM): 215 sucrose, 2.5 KCl, 26 NaHCO₃, 1.6 NaH₂PO₄, 1 CaCl₂, 4 MgCl₂, 4 MgSO₄
481 and 20 D-glucose. 30 min post-sectioning, the cutting medium was gradually switched to
482 extracellular artificial cerebrospinal (ACSF) recording solution containing (in mM): 124
483 NaCl, 2.5 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄ and 10 D-glucose. Slices
484 were incubated for at least 40 min in the ACSF solution before recording. Mouse
485 hippocampal slices were prepared using a NMDG-based cutting solution containing (in
486 mM): 93 N-Methyl-d-glucamin, 2.5 KCl, 1.25 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 D-
487 glucose, 2 Thiourea, 5 Na-Ascorbate, 3 Na-Pyruvate, 0.5 CaCl₂, 10 MgCl₂. These slices
488 were then transferred to 32°C ACSF for 30 min and then kept at room temperature for at
489 least 1h before recording. All solutions were equilibrated with 95% O₂ and 5% CO₂ (pH
490 7.4).

491

492 **Electrophysiology**

493 Recordings were performed at 28 ± 1°C, otherwise stated, in a submersion-type recording
494 chamber perfused at 2 mL/min with ACSF supplemented with the GABA_A and the GABA_B
495 receptor antagonists, picrotoxin (100 μM) and CGP55845 hydrochloride (3 μM). Whole-
496 cell patch-clamp recordings using a Multiclamp 700A amplifier (Molecular Devices) were
497 obtained from GCs voltage clamped at -60 mV using patch-type pipette electrodes (~3-4
498 MΩ) containing (in mM): 135 KMeSO₄, 5 KCl, 1 CaCl₂, 5 NaOH, 10 HEPES, 5 MgATP,
499 0.4 Na₃GTP, 5 EGTA and 10 D-glucose, pH 7.2 (288-291 mOsm). For IPSC recording in
500 CA1, whole cell voltage clamp recordings were obtained from CA1 pyramidal neurons
501 voltage clamped at V_h = 10 mV using a cesium-based internal solution containing (in mM):
502 131 cesium gluconate, 8 NaCl, 1 CaCl₂, 10 EGTA, 10 D-glucose and 10 HEPES, pH 7.2
503 (285-290 mOsm). IPSCs were evoked using an electrical stimulating pipette placed in CA1
504 *stratum radiatum*, and recordings were performed in the continuous presence of ionotropic
505 glutamate receptor antagonists NBQX (10 μM) and D-APV (50 μM). LTD of inhibitory

506 inputs onto CA1 pyramidal neurons (iLTD) was induced using theta burst-stimulation
507 protocol (TBS, 10 bursts at 5 Hz of 5 pulses at 100 Hz, repeated every 5 s, 4 times). Series
508 resistance (~6-25 M Ω) was monitored throughout all experiments with a -5 mV, 80 ms
509 voltage step, and cells that exhibited a significant change in series resistance (> 15%)
510 were excluded from analysis. Botox experiments were performed at 32 °C. Botox was
511 supplemented with 5 mM dithiothreitol (DTT) in the intracellular solution, and interleaved
512 control experiments included DTT only.

513
514 A broken tip (~10–20 μ m) stimulating patch-type micropipette filled with ACSF was placed
515 in the inner molecular layer (IML, < 50 μ m from the border of the GC body layer) to activate
516 MC axons, and in the middle third of the molecular layer to activate MPP inputs. To elicit
517 synaptic responses, paired, monopolar square-wave voltage or current pulses (100–200
518 μ s pulse width, 4–27 V) were delivered through a stimulus isolator (Digitimer DS2A-MKII).
519 Typically, stimulation intensity was adjusted to obtain synaptic responses comparable in
520 amplitude across experiments; e.g., 30–70 pA EPSCs (V_h -60 mV). For the optogenetic
521 experiments, EPSCs were evoked using 1–3 ms pulses of blue (470-nm) light, provided
522 by a collimated LED (Thorlabs, M470L3-C5, 470 nm, 300mW) and delivered through the
523 microscope objective (40X, 0.8 NA). GC TBF-induced LTP was typically induced with 10
524 bursts at 5 Hz of 5 AP at 50 Hz, repeated 4 times every 5s, while the membrane potential
525 was held at -60 mV in current clamp. Extracellular field recordings were performed using
526 patch-type pipettes filled with 1M NaCl, placed in CA1 *stratum radiatum*. fESPS were
527 evoked with an ACSF-containing broken tip patch pipette in CA1 *stratum radiatum*. LTP
528 at CA3-CA1 synapses, was triggered using high frequency stimulation protocol (4 HFS:
529 100 pulses at 100 Hz repeated 4 times every 10 s) and recordings were performed at 25
530 \pm 1°C. LTD was induced with low frequency stimulation protocol (LFS: 900 pulses at 1
531 Hz).

532
533 Electrophysiological data were acquired at 5 kHz, filtered at 2.4 kHz, and analyzed using
534 custom-made software for IgorPro (Wavemetrics Inc.). Paired-pulse ratio (PPR) was
535 defined as the ratio of the amplitude of the second EPSC (baseline taken 1–2 ms before
536 the stimulus artifact) to the amplitude of the first EPSC. Coefficient of variation (CV) was
537 calculated as the standard deviation of EPSC amplitude divided by mean EPSC
538 amplitude. Both PPR and CV were measured 10 min before and 20–30 min after LTP
539 induction protocol or CGS21680-induced potentiation. The magnitude of LTP/LTD was
540 determined by comparing 10 min baseline responses with responses 20–30 min (or 30–40
541 min for **Fig. 1b**, 40–50 min for **Fig. 2b**, 45–55 min for **Fig. 2d**) after induction protocol.
542 Averaged traces include 20 consecutive individual responses.

543
544 **Postsynaptic TrkB and BDNF conditional KO**
545 Adeno-associated virus AAV₅.CamKII.eGFP (control virus) or AAV₅.CamKII.GFP-CRE
546 (University of Pennsylvania Vector Core) was injected (1 μ L at a flow rate of 0.1 μ L/min)
547 unilaterally into the dorsal blade of the dentate gyrus (2.06 mm posterior to bregma, 1.5
548 mm lateral to bregma, 1.8 mm ventral from dura) of *TrkB^{fl/fl}* or *Bdnf^{fl/fl}* mice (4–5 week old).
549 Animals were placed in a stereotaxic frame and anesthetized with isoflurane (up to 5% for

550 induction and 1%–3% for maintenance). Both male and female mice were used with a
551 similar ratio for the two types of viruses. Slices for electrophysiology were prepared from
552 injected animals 3–5 weeks after injection. For each animal, the absence of GFP-
553 expressing cells in the hilus of the entire ipsilateral hippocampus was verified.

554
555 **Presynaptic TrkB and A_{2A}R conditional KO**
556 A mix with (1:2 ratio, 1.2 μL at 0.1 μL/min) of Cre recombinase-containing AAV
557 (AAV₅.CamKII.Cre-mCherry, UNC Vector) and Cre-dependent ChIEF
558 (AAV_{DJ}.Flx.ChIEF.Tdtomato) was injected into the dentate gyrus (relative to bregma: 1.9
559 mm posterior, 1.25 mm lateral, 2.3 ventral) of adult *TrkB^{fl/fl}*, *A_{2A}R^{fl/fl}* or WT control mice (4-
560 5 week old). We then performed electrophysiology experiments 4–5 weeks post-injection
561 in contralateral hippocampal slices. This allowed us to optically activate Cre-expressing
562 MC axons.

563
564 **Pharmacology**
565 Reagents were bath applied following dilution into ACSF from stock solutions stored at
566 –20°C prepared in water, DMSO or ethanol, depending on the manufacturer’s
567 recommendation. BDNF puffs (8 nM, 2.5-3 PSI, 3 s puffs repeated twice, 5 s interval) were
568 applied using a Picospritzer III (Parker) connected to a broken patch pipette. The tip of the
569 puffer pipette was positioned above the IML while monitoring MC-GC transmission. For
570 experiments requiring postsynaptic loading PKI₆₋₂₂, LTP was induced at least 20 min after
571 establishing the whole-cell configuration. Time of LTP induction protocol was matched in
572 interleaved controls.

573
574 **Electron Microscopy**
575 Animal care and handling prior to and during the experimental procedures were in
576 accordance with Spanish (RD 1201/2005) and European Union (86/609/EC) regulations,
577 and the protocols were approved by the University’s Animal Care and Use Committee.
578 Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (ratio 1:1, 0.1
579 mL/kg) and transcardially perfused with ice-cold fixative containing 4% paraformaldehyde,
580 with 0.05% glutaraldehyde and 15% (v/v) saturated picric acid made up in 0.1 M
581 phosphate buffer (PB, pH 7.4). Brains were then removed and immersed in the same
582 fixative for 2 hours or overnight at 4°C. Tissue blocks were washed thoroughly in 0.1 M
583 PB. Coronal sections (60-μm thick) were cut using a vibratome (Leica V1000). Pre-
584 embedding immunohistochemical analyses was performed as described previously⁶⁶.
585 Free-floating sections were incubated in 10% (v/v) NGS diluted in TBS. Sections were
586 then incubated in, 3-5 μg/mL diluted in TBS containing 1% (v/v) NGS, anti-A_{2A}R [guinea
587 pig anti-A_{2A}R polyclonal (AB_2571656; Frontier Institute co., Japan)] or anti-A₁R [rabbit-
588 anti-A₁R antibody (2 mg/ml; Affinity Bioreagents, Labome, USA)] antibodies, followed by
589 incubation in goat anti-guinea pig IgG coupled to 1.4 nm gold or in goat anti-rabbit IgG
590 coupled to 1.4 nm gold (Nanoprobes Inc., Stony Brook, NY, USA), respectively. Sections
591 were postfixed in 1% (v/v) glutaraldehyde and washed in double-distilled water, followed
592 by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes Inc.).
593 Sections were then treated with osmium tetroxide (1% in 0.1 m phosphate buffer), block-
594 stained with uranyl acetate, dehydrated in graded series of ethanol and flat-embedded on

595 glass slides in Durcupan (Fluka) resin. Regions of interest were cut at 70-90 nm on an
596 ultramicrotome (Reichert Ultracut E, Leica, Austria) and collected on single slot pioloform-
597 coated copper grids. Staining was performed on drops of 1% aqueous uranyl acetate
598 followed by Reynolds's lead citrate. Ultrastructural analyses were performed in a Jeol-
599 1010 electron microscope. Quantitative analysis of the relative abundance of A_{2A}R or A₁R,
600 in the molecular layer of the dentate gyrus, was performed from 60 μm coronal slices as
601 described⁶⁶, in the area for MC-GC synapses and the area of PP-GC synapses. For each
602 of three animals, three samples of tissue were obtained (nine total blocks). Electron
603 microscopic serial ultrathin sections were cut close to the surface of each block because
604 immunoreactivity decreased with depth. Randomly selected areas were captured at a final
605 magnification of 45,000X, and measurements covered a total section area of ~5000 μm².
606 Dendritic shafts, dendritic spines and axon terminals were assessed for the presence of
607 immunoparticles. The percentage of immunoparticles for A_{2A}R or for A₁R at
608 postsynaptic and presynaptic sites was calculated. To establish the relative frequency of
609 A_{2A}R in axon terminals, immunoparticles identified in this compartment were counted. The
610 perimeter of each axon terminal was measured in reference areas totaling ~2,000 μm².
611 All axon terminals establishing excitatory synapses were counted and assessed from
612 single ultrathin sections.

613
614 **Two-photon live imaging of GRAB_{Ado} in acute hippocampal slices**
615 AAV₉.hSyn.GRAB.Ado1.0m (WZ Biosciences Inc) was injected (1 μL at 0.1 μL/min)
616 unilaterally into the hilus of WT mice or *TrkB^{fl/fl}* mice (3 - 4 week old). Slices were prepared
617 3 - 4 weeks post-injection and expression of GRAB_{Ado1.0m} was confirmed in MCs of the
618 ipsilateral hippocampus for each injected mouse. Hippocampal slices from GRAB_{Ado1.0m}-
619 injected mice were prepared using an ice-cold dissection buffer maintained in 5%
620 CO₂/95% O₂ and containing (in mM): 25 NaHCO₃, 1.25 NaH₂PO₄, 2.5 KCl, 0.5 CaCl₂, 7
621 MgCl₂, 25 D-glucose, 110 choline chloride, 11.6 ascorbic acid, 3.1 pyruvic acid. Slices
622 were transferred to 32°C ACSF for 30 min and kept at room temperature for at least 45
623 min before imaging. Slices were then transferred to an imaging chamber under an Ultima
624 2-photon microscope (Bruker Corp.) equipped with a 60X NA 1.0 water-immersion
625 objective and InSight DeepSee laser (Spectra-Physics). A 920-nm laser was used to
626 excite GRAB_{Ado1.0m}, and the emission signal was acquired using a 525–570 nm band-pass
627 filter. The field of view (512 x 512 pixels per frame) was chosen in the IML of contralateral
628 hippocampal slices where MC commissural axons projected onto GCs and expressed
629 GRAB_{Ado1.0m}. A broken tip stimulating patch-type micropipette filled with ACSF was
630 positioned in the IML ~150 μm from the imaging region to activate MC axons. The region
631 of interest (ROI) was magnified to 2X and at least 40 consecutive images (at 0.25 Hz) as
632 a baseline using a PrairieView 5.4 (Bruker Corp.). Following baseline acquisition, MC
633 burst stimulation was applied and at least additional 100 images (at 0.25 Hz). To verify
634 reactivity of the ROI adenosine (100 μM) was added at the end of the imaging session.
635 The fluorescence intensity of the ROI was measured and the ΔF/F₀ of
636 the GRAB_{Ado1.0m} signal was calculated using ImageJ software. All recordings were
637 performed at 28 ± 1°C in a submersion-type recording chamber perfused at 2 mL/min with
638 ACSF.
639

640 **Seizure induction and monitoring**

641 Epileptic seizures were induced acutely in 2-3-month-old mice. Intraperitoneal (IP)
642 injection of 20-30 mg/kg of kainic acid (KA, HelloBio HB0355) prepared in saline solution
643 the same day were performed. For behavioral seizure scoring, mice were monitored
644 during 120 min post-injection and behavioral seizures were scored, by an experimenter
645 blind to condition (control vs A_{2A}R cKO), using a modified Racine scale as follows: stage
646 0: normal behavior, stage 1: immobility and rigidity, stage 2: head bobbing, stage 3:
647 forelimb clonus and rearing, stage 4: continuous rearing and falling, stage 5: clonic-tonic
648 seizure, stage 6: death. The maximum Racine score was recorded every 10 minutes and
649 the cumulative seizure score was obtained by summing these scores across all 12 bins of
650 the 120 min experiment.

651 **Fiber photometry in freely behaving mice**

652 AAV₉.hSyn.GRAB.Ado1.0m (1.2 μ L at 0.1 μ L/min) was unilaterally injected into the DG
653 (relative to bregma: 1.9 mm posterior, 1.25 mm lateral, 2.3 ventral) of WT mice (5-6 week
654 old). An optic fiber (200 μ m diameter, NA = 0.37, Neurophotometrics) was implanted
655 unilaterally, into the contralateral DG above the IML (relative to bregma: 1.9 mm posterior,
656 1.25 mm lateral, 1.8 mm ventral) to record GRAB_{Ado1.0m} fluorescence *in vivo*. 3-5 weeks
657 after surgery, the fluorescence signal was monitored before and after KA IP injection (30
658 mg/kg). To record GRAB_{Ado1.0m} fluorescence, a three-channel multi-fiber photometry
659 system (Neurophotometrics v1 Ltd) was used. 470 nm and 415 nm out of phase excitation
660 lights were bandpass filtered and directed via a 20X objective (power: 30 μ W). A single
661 patch cord connected to the optic fiber implant was used to deliver light and collect the
662 emitted fluorescence, which was filtered, and projected on a CMOS camera sensor. The
663 open-source software Bonsai was used for data acquisition (40 frames/s rate). The
664 fluorescence intensity profile of each channel was calculated as the mean pixel value of
665 the region of interest. To calculate $\Delta F/F_0$, the 470 nm-evoked signal was normalized by the
666 isosbestic signal (415 nm) to correct for photobleaching and potential artefacts. F_0
667 corresponds to the average of the last three-minute (baseline) before KA injection.
668

669 **Post-hoc analysis of AAV expression and optic fiber location**

670 Viral expression and optic fiber location were verified *post hoc*, at the end of the
671 experiments (**Fig. 8**). Mice were anesthetized with isoflurane (3-5%) and transcardially
672 perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PBS). 50
673 μ m-thick brain coronal sections were prepared using a DSK Microslicer (DTK-1000),
674 stained with DAPI (1:1000) to label cell nuclei, and mounted with Prolong diamond
675 antifade reagent montant (ThermoFisher) onto microscope slides. Zeiss LSM 880
676 Airyscan Confocal microscope with Super-Resolution and ZEN (black edition) software
677 and a 25X oil-immersion objective were used for images acquisition.
678

679 **Quantification and Statistical Analysis**

680 The normality of distributions was assessed using the Shapiro-Wilk test. In normal
681 distributions, Student's unpaired and paired two-tailed t tests were used to assess
682 between-group and within-group differences, respectively. One way ANOVA and One way
683 ANOVA repeated measure (RM) were used when more than two groups were compared.
684

685 The non-parametric paired sample Wilcoxon signed rank test and Mann-Whitney's U test
686 were used in non-normal distributions. Statistical significance was set to $p < 0.05$ (***)
687 indicates $p < 0.001$, ** indicates $p < 0.01$, and * indicates $p < 0.05$). All values are reported
688 as the mean \pm SEM. Statistical results are summarized in **Table S1**. All experiments
689 included at least three animals per condition. Statistical analysis was performed using
690 OriginPro software (OriginLab).

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692
693

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711
712

712 **Conflict of interest**

713 Authors declare no conflict of interest.

714
715

715 **Author contributions**

716 K.N. and P.E.C. designed studies and wrote the manuscript. K.N. performed experiments and
717 analyzed all data except for the *in vitro* adenosine measurements that were designed, performed,
718 and analyzed by C.B.. R.L. performed and analyzed immunoelectron microscopy experiments.
719 M.G. tested the role of A1 receptors. Y.H and A.C designed, performed, and analyzed early
720 experiments assessing conventional retrograde signals. All authors read and edited the
721 manuscript.

722

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- 912
- 913

914 **FIGURE LEGENDS**

915

916 **Figure 1: Theta-burst firing of a single postsynaptic GC induces presynaptic LTP at**
917 **MC-GC synapse**

918 **(a)** *Left*, Diagram illustrating the recording configuration. MC and MPP EPSCs were
919 recorded from the same GC and evoked with stimulation electrodes placed in the inner
920 and middle molecular layer, respectively. *Right*, Current clamp recording showing GC
921 theta-burst firing (GC TBF). LTP induction protocol (GC TBF) was composed of 10 bursts
922 at 5 Hz of 5 action potentials at 50 Hz, repeated 4 time every 5 s.

923 **(b)** *Left*, Representative traces before (1) and after (2) GC TBF delivery. *Right*, Time-
924 course plot showing that GC TBF induced LTP at MC-GC but not at MPP-GC synapses.

925 **(c)** GC TBF-induced LTP was associated with significant reduction in PPR and CV ($n =$
926 13 cells).

927 **(d)** LTP was blocked when TrkB was conditionally knocked out from from GCs (Post TrkB
928 cKO, *TrkB^{fl/fl}* mice injected with AAV5.CaMKII.Cre.GFP). LTP was unaffected in control
929 animals (Control, *TrkB^{fl/fl}* mice injected with AAV5.CaMKII.eGFP).

930 **(e)** LTP was normally induced when loading PKI₆₋₂₂ (2.5 μ M) in GCs via the recording
931 pipette but completely blocked when the cell-permeant PKA inhibitor PKI₁₄₋₂₂ myristoylated
932 (1 μ M) was bath applied.

933 **(f)** Summary bar graph showing the magnitude of GC TBF induced LTP in the presence
934 of DGC-IV (1 μ M), when TrkB was conditionally knocked out from MCs (Pre TrkB cKO),
935 when loading the PKI₆₋₂₂ (2.5 μ M) in GCs and in presence of D-APV (50 μ M). LTP was
936 abolished in presence of the TrkB antagonist ANA-12 (15 μ M), Botox (0.5 μ M loaded
937 postsynaptically), postsynaptic BDNF and TrkB cKO, and bath application of the PKA
938 inhibitors H89 (10 μ M) or PKI₁₄₋₂₂ myristoylated (1 μ M).

939 Numbers in parentheses indicate the number of cells. Data are presented as mean \pm SEM.

940

941

942 **Figure 2: MC-GC LTP does not require conventional retrograde messengers**

943 **(a)** Bath application of the NO synthase inhibitor L-NAME (100 μ M, 50- to 90-min pre-
944 incubation and bath applied) did not impair MC-GC LTP.

945 **(b)** Positive controls for L-NAME. Extracellular recordings in CA1 *stratum radiatum*

946 showing that L-NAME blocked LTP at SC-CA1 synapses induced by 4 HFS (100 pulses
947 at 100 Hz repeated 4 times every 10 s) at 25 °C.

948 **(c)** Application of cocktail of lipase inhibitors AACOCF3 (10 μ M, 50- to 90-min pre-
949 incubation and bath applied), Baicalein (3 μ M, 50- to 90-min pre-incubation and bath
950 applied), and THL (4 μ M loaded in the recording pipette) did not affect MC-GC LTP.

951 **(d)** Positive controls for AACOCF3 and Baicalein. Extracellular recordings in CA1 *stratum*
952 *radiatum* showing that application of AACOCF3 and Baicalein significantly reduced LFS
953 (900 pulses at 1 Hz)-induced LTD at SC-CA1 synapses.

954 **(e)** Positive control for the lipase inhibitor THL showing that loading THL in CA1 pyramidal
955 neuron via the recording pipette blocked iLTD induced by TBS (10 bursts at 5 Hz of 5
956 pulses at 100 Hz, repeated every 5 s, 4 times).

957 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

958
959 **Figure 3: MC-GC LTP requires activation of presynaptic A_{2A}Rs whereas A₁Rs**
960 **dampen LTP.**

961 **(a)** Bath application of the A_{2A}R selective antagonists SCH 58261 (100 nM) and
962 ZM241385 (50 nM) blocked GC TBF-induced LTP.

963 **(b)** SCH 58261 (100 nM) did not change basal EPSC amplitude.

964 **(c)** SCH 58261 (100 nM) abolished LTP induced by BDNF puffs (8 nm, 2 puffs of 3 s in
965 the IML), as compared with interleaved controls.

966 **(d)** *Left*, loading GDP β S in the recording pipette did not affect MC-GC LTP. *Right*, positive
967 control showing that replacing GTP by 1 mM GDP β S in the internal solution efficiently
968 abolished the GABA_B receptor agonist (baclofen, 10 μ M)-induced increase in holding
969 current.

970 **(e, f)** Presynaptic A_{2A}R cKO abolished MC-GC LTP. **(e)** *Left*, A mix of AAV5.CamKII.Cre-
971 mCherry and AAV_{DJ}.hSyn.Flex.ChIEF.Tdtomato was injected unilaterally into the DG of
972 A_{2A}R^{fl/fl} (cKO) or WT (control) mice. MC EPSCs were light-evoked and recorded in
973 contralateral DG. *Right*, Infrared/differential interference contrast (IR/DIC, left images) and
974 fluorescence (right) images showing that ChIEF-TdTomato was expressed in MC soma in
975 ipsilateral DG (top) and selectively in putative MC axons (bottom) of contralateral IML. **(f)**
976 MC-GC LTP was abolished in presynaptic A_{2A}R conditional knockout mice as compared

977 with controls.

978 **(g)** MC-GC LTP magnitude was increased in the presence of the A₁R selective antagonist
979 DPCPX (100 nM) as compared to interleaved control experiments.

980 **(h)** Application of DPCPX (100 nM) did not change basal EPSC amplitude at MC-GC and
981 MPP-GC synapses.

982 Numbers in parentheses represent number of cells. Data are presented as mean ± SEM.

983
984 **Figure 4: Subcellular localization of the A₁ and A_{2A} adenosine receptors in the**
985 **molecular layer of the dentate gyrus.**
986

987 **(a-c, e-g)** Electron micrographs of the molecular layer of the dentate gyrus showing
988 immunoreactivity for A₁Rs and A_{2A}Rs revealed by pre-embedding immunogold methods.

989 **(a-c)** Both at MC-GC **(a and b)** and PP-GC **(c)** putative synapses, immunoparticles for
990 A₁R were mainly observed on the presynaptic plasma membrane (arrowheads) of axon
991 terminals (at), with very low frequency in postsynaptic sites (arrows) of spines (s) or
992 dendritic shafts (Den).

993 **(d)** Quantitative analysis of the relative number of immunoparticles found in the
994 presynaptic membrane for A₁R at MC-GC and MPP-GC putative synapses. From the total
995 number of immunoparticles detected (n = 490 for MC-GC; n = 419 for MPP-GC synapses,
996 N = 3 mice), 468 (95.5%) and 402 (95.9%) were present in presynaptic sites of MC-GC
997 and MPP-GC putative synapses, respectively.

998 **(e-h)** Electron micrographs **(e-g)** and quantitative analysis **(h)** showing that, at putative
999 MC-GC synapses **(e, f and h)**, immunoparticles for A_{2A}R were mainly detected
1000 presynaptically (91.0%) whereas they were mainly found on the postsynaptic plasma
1001 membrane (92.0%) of putative PP-GC synapses **(g and h)**. Total number of
1002 immunoparticles: n = 668 for MC-GC; n = 690 for MPP-GC synapses, N = 3 mice.

1003 Scale bars: 200 nm.

1004
1005 **Figure 5: A_{2A}R activation is sufficient to trigger PKA-dependent LTP at MC-GC but**
1006 **not at MPP-GC synapses.**

1007 **(a)** Representative traces *(left)* and time-course summary plot *(right)* showing that bath
1008 application of the A_{2A}R selective agonist CGS21680 (50 nM) potentiated MC-GC but not

1009 MPP-GC synaptic transmission.

1010 **(b)** CGS21680-induced potentiation at MC-GC synapse was associated with a significant
1011 reduction of both PPR and CV. ** $p < 0.01$; $n = 9$ cells.

1012 **(c)** CGS21680 induced long-lasting potentiation even when the $A_{2A}R$ selective antagonist
1013 SCH 58261 (100 nM) was included during CGS21680 washout. CGS21680-induced LTP
1014 was completely abolished in continuous presence of SCH 58261.

1015 **(d)** Loading the selective PKA blocker PKI₆₋₂₂ (2.5 μ M) in GCs via the recording pipette
1016 did not impair CGS21680-induced LTP while bath application of the cell-permeant PKA
1017 inhibitor PKI₁₄₋₂₂ myristoylated (1 μ M) completely blocked LTP.

1018 **(e)** Bath application of ANA-12 (15 μ M) did not impair CGS21680-induced LTP.

1019 **(f)** Positive control in interleaved experiments showing that ANA-12 (15 μ M) efficiently
1020 blocked GC TBF-induced LTP.

1021 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

1022

1023 **Figure 6: MC-GC LTP requires passive release of adenosine from GCs, via ENTs.**

1024 **(a)** Representative traces (*left*) and time-course summary plot (*right*) showing that GC TBF
1025 failed to induce LTP in presence of the ENT blockers (20 μ M of dipyridamole and 10 μ M
1026 of NBMPR) as compared with interleaved controls.

1027 **(b)** Bath application of the ENT blockers (20 μ M of dipyridamole and 10 μ M of NBMPR)
1028 did not change basal MC-GC EPSC amplitude.

1029 **(c)** Co-application of the ENT blockers (20 μ M of dipyridamole and 10 μ M of NBMPR) and
1030 the A_1R antagonist DPCPX (100 nM) increased EPSC amplitude in control condition but
1031 not in presence of the $A_{2A}R$ antagonist SCH 58261 (100 nM).

1032 **(d)** Including inosine (100 μ M) in the recording solution completely prevented GC TBF-
1033 induced LTP.

1034 **(e)** Bath application of inosine (100 μ M) did not change basal MC-GC EPSC amplitude.

1035 **(f)** Bath application of DPCPX (100 nM) increased EPSC amplitude when the ENT
1036 blockers (20 μ M of dipyridamole and 10 μ M of NBMPR) were included in the bath but not
1037 when inosine (100 μ M) was loaded in the postsynaptic neuron *via* the patch pipette.

1038 Numbers in parentheses represent number of cells. Data are represented as mean \pm
1039 SEM.

1040

1041 **Figure 7: Induction of MC-GC LTP triggers a transient TrkB-dependent increase in**
1042 **extracellular adenosine level**

1043 **(a)** AAV9.hSyn.GRAB.Ado1.0m (GRAB_{Ado}) was injected unilaterally in the DG of WT mice.
1044 Two-photon image (*bottom*) showing GRAB_{Ado} was selectively expressed in putative MC
1045 axons in contralateral IML.

1046 **(b, c)** Two-photon images **(b)** showing GRAB_{Ado} fluorescence intensity increased during
1047 burst electrical stimulation of MC axon terminals (MC BS) in normal ACSF (control) but
1048 not in the presence of A_{2A} receptor antagonist SCH58261 (100 nM). Time-course
1049 summary plot of the average fractional fluorescence changes ($\Delta F/F_0$) with time are shown
1050 in **c**.

1051 **(d, e)** Two-photon images **(d)** and time-course summary plot **(e)** showing how MC BS
1052 failed to increase GRAB_{Ado} fluorescence in presence of the TrkB receptor antagonist ANA-
1053 12 (15 μ M) or when TrkB was conditionally knocked out from GCs (Post TrkB cKO).

1054 **(f)** Quantification of the averaged responses during burst stimulation of MCs (15-25 s) * p
1055 < 0.05, ** p < 0.01, *** p < 0.001; one-way ANOVA.

1056 Number of slices are shown between parentheses. Data are represented as mean \pm SEM.

1057

1058 **Figure 8: *In vivo* release of adenosine during epileptic seizures likely promotes**
1059 **seizures by activating A_{2A}Rs**

1060 **(a)** AAV9.hSyn.GRAB.Ado1.0m (GRAB_{Ado}) was injected unilaterally in the DG of WT mice.
1061 An optic fiber was implanted above the contralateral IML. Fiber photometry was performed
1062 3-5 weeks later to assess GRAB_{Ado} fluorescence intensity before and after acute seizure
1063 induction with kainic acid (KA, 30 mg/kg).

1064 **(b)** Confocal image showing fiber tract and GRAB_{Ado} expression in the contralateral IML.

1065 **(c, d)** Time-course of representative experiment **(c)** and histogram **(d)** showing how KA
1066 (30 mg/kg) administration increased the average fractional fluorescence ($\Delta F/F_0$) of
1067 GRAB_{Ado}. * p < 0.05, n = 5, paired t test.

1068 **(e)** Representation of the experimental timeline. AAV5-CaMKII-Cre-mCherry was injected
1069 bilaterally into ventral and dorsal DG of WT (control) and A_{2A}R^{fl/fl} (cKO) mice. Mouse
1070 behavior was assessed for 120 min following KA (20 mg/kg) administration.

1071
1072 **(f)** Confocal images showing the viral expression in the DG of WT (top) and $A_{2A}R^{fl/fl}$
1073 (bottom) mice.

1074
1075 **(g-i)** Deletion of $A_{2A}R$ from DG excitatory neurons ($A_{2A}R$ cKO, $A_{2A}R^{fl/fl}$ mice injected with
1076 AAV₅-CaMKII-Cre-mCherry) significantly increased latency to convulsive seizures **(g, h)**
1077 but did not affect seizure severity **(i)**. ** $p < 0.01$, unpaired t test.

1078
1079 Numbers in parentheses represent number of mice. Data are presented as mean \pm SEM.
1080

1081

1082 SUPPLEMENTAL FIGURE LEGENDS

1083 **Figure_S1 (related to Figure 1): GC TBF-induced LTP properties**

1084 **(a)** GC TBF induced a normal LTP in presence of the group II mGluR agonist DCG-IV (1
1085 μ M) as compared to interleaved controls.

1086 **(b)** TBF-induced LTP was blocked in presence of the TrkB selective antagonist ANA-12.

1087 **(c)** Presynaptic TrkB cKO did not impair GC TBF-induced LTP. $TrkB^{fl/fl}$ mice were
1088 unilaterally injected with a mix of AAV₅.CaMKII.Cre and
1089 AAV_{DJ}.hSyn.Flex.ChiEF.Tdtomato. EPSCs were recorded in contralateral DG in response
1090 to light stimulation of Cre⁺ and ChiEF⁺ MC axons (lacking TrkB).

1091 **(d)** GC TBF-induced LTP was blocked in GFP-Cre⁺ GCs (BDNF cKO, $Bdnf^{fl/fl}$ mice
1092 injected with AAV₅.CaMKII.Cre.GFP) as compared with GFP⁺ GCs (control, $Bdnf^{fl/fl}$ mice
1093 injected with AAV₅.CaMKII.eGFP).

1094 **(e)** Loading Botox (0.5 μ M) in the postsynaptic neuron via the patch pipette completely
1095 abolished MC-GC LTP.

1096 **(f)** GC TBF failed to induce LTP in presence of the PKA inhibitor H89 (10 μ M, 40- to 60
1097 min pre-incubation and bath applied, black circles).

1098 **(g)** Bath application of the selective NMDAR antagonist D-APV (50 μ M, black circles) did
1099 not affect GC TBF-induced LTP, as compared with interleaved control experiments (white
1100 circles). Representative traces are shown on the left and time-course summary plot on the
1101 right.

1102 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

1103

1104 **Figure_S2 (related to Figure 1): GC TBF- and MC BS-induced LTP occlude each**

1105 **other**

1106 **(a)** Representative single cell experiments showing how GC TBF induced a normal LTP
1107 in naive slices (top) but not when MC BS was previously applied (bottom). Vertical dashed
1108 line indicated the time point when stimulation intensity was reduced in order to avoid
1109 potential ceiling effect.

1110 **(b)** Representative traces and time course summary plot showing how MC BS occluded
1111 GC TBF -induced LTP.

1112 **(c)** Representative single cell experiments showing how MC BS induced a normal LTP in
1113 naive slices (top) but not when GC TBF was previously applied (bottom). Vertical dashed
1114 line indicated the time point when stimulation intensity was reduced in order to avoid
1115 potential ceiling effect.

1116 **(d)** Pre-application of the GC TBF protocol occluded MC BS-induced LTP (black circles)
1117 as compared with interleaved controls (naive, white circles).

1118 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

1119
1120 **Figure_S3 (related to Figure 2): MC-GC LTP does not require conventional**
1121 **retrograde messengers**

1122 MC BS induced a normal LTP in presence of L-NAME **(a)**, AACOCF3 **(b)**, Baicalein **(c)** or
1123 when THL was loaded **(d)** in the postsynaptic GC, as compared with interleaved controls.

1124 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

1125
1126 **Figure S4 (related to fig 4): MC BS-induced LTP requires A_{2A}R activation.**

1127 **(a)** Bath application of the A_{2A}R selective antagonist SCH 58261 (100 nM) abolished LTP
1128 induced by MC BS (5 pulses at 100 Hz repeated 50 times every 0.5 s).

1129 **(b)** *Left*, loading GDP β s (1 mM) in the recording pipette did not affect LTP induced by MC
1130 BS. *Right*, interleaved positive controls for GDP β s showing that replacing GTP by 1 mM
1131 GDP β s in the recording pipette abolished baclofen (10 μ M)-induced increase in holding
1132 current. Baclofen is a GABA_B receptor selective agonist.

1133 Numbers in parentheses represent number of cells. Data are presented as mean \pm SEM.

1134
1135 **Figure_S5 (related to Figure 4): Activation of A₁R induces a short-term depression**

1136 **at both MC-GC and MPP-GC synapses**

1137 **(a)** Bath application of CCPA (50 nM, 15 min), a selective adenosine A₁R agonist, induced
1138 a short-term depression at both MC-GC and MPP-GC synapses. The selective A₁R
1139 antagonist DPCPX (100 nM) was included in the washout of CCPA.

1140 **(b, c)** CCPA-induced depression was associated with a significant and reversible increase
1141 in both PPR and CV, *p < 0.05, n = 7, one-way ANOVA RM.

1142 **(d)** Bath application of the A₁R antagonist DPCPX (100 nM) completely blocked CCPA-
1143 induced depression.

1144 Numbers in parentheses represent number of cells. Data are presented as mean ± SEM.

1145
1146 **Figure S6 (related to Figures 1-8). Adenosine is a retrograde messenger mediating**
1147 **activity-dependent presynaptic LTP at the MC-GC synapse**
1148

1149 Scheme illustrating the emerging model for the mechanism of activity-induced MC-GC
1150 LTP. MC (MC BS) and GC bursts (GC TBF) of activity trigger BDNF release and
1151 subsequent TrkB activation in GCs (1). Activation of postsynaptic TrkB leads to
1152 intracellular accumulation of adenosine, which is passively released from GCs via ENTs
1153 (2). Adenosine then activates presynaptic A₁Rs and A_{2A}Rs (3). A_{2A}R activation induces a
1154 long-lasting PKA-dependent increase in glutamate (glu) release (4), whereas A₁R
1155 activation dampens LTP.
1156

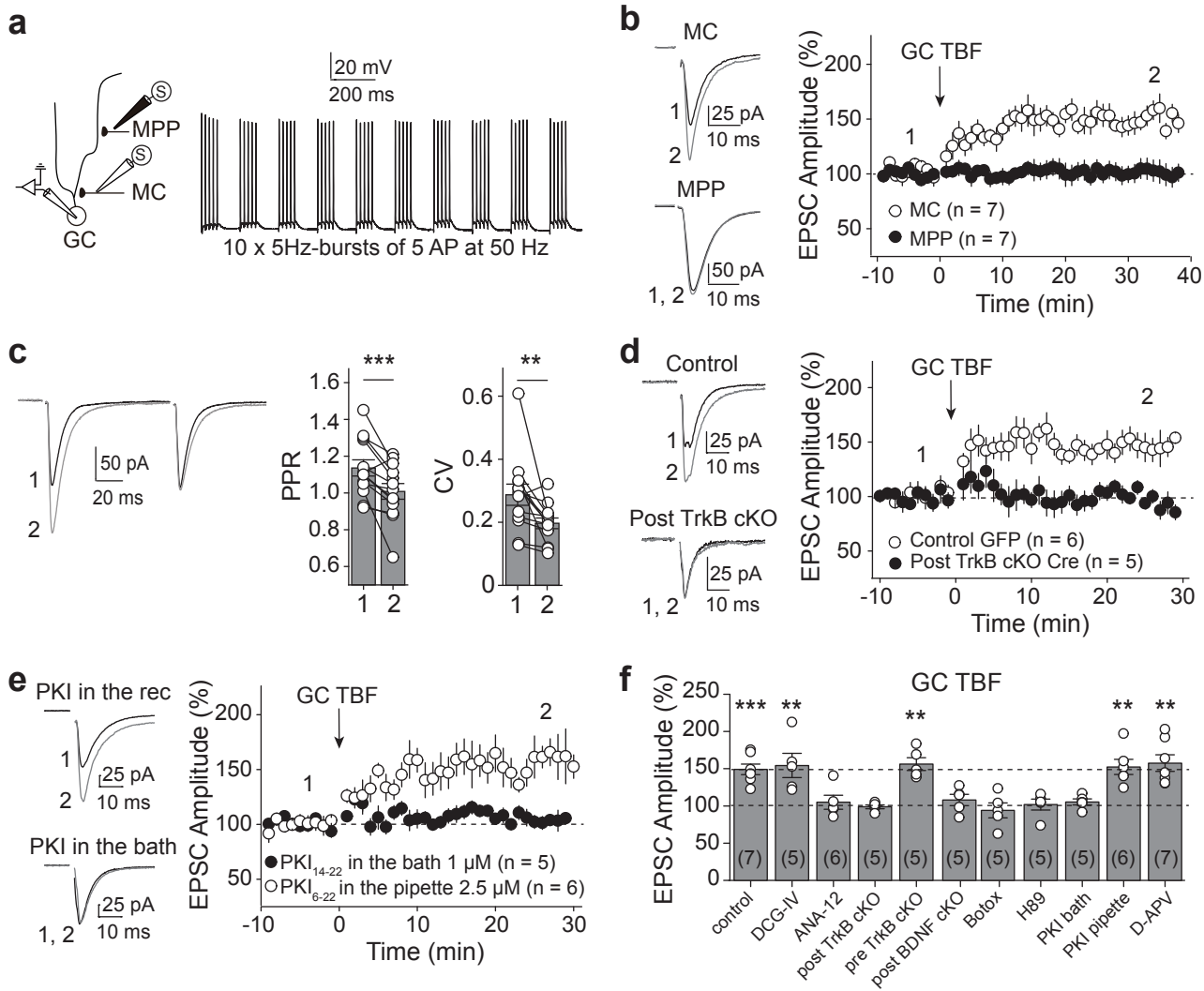


Figure 1

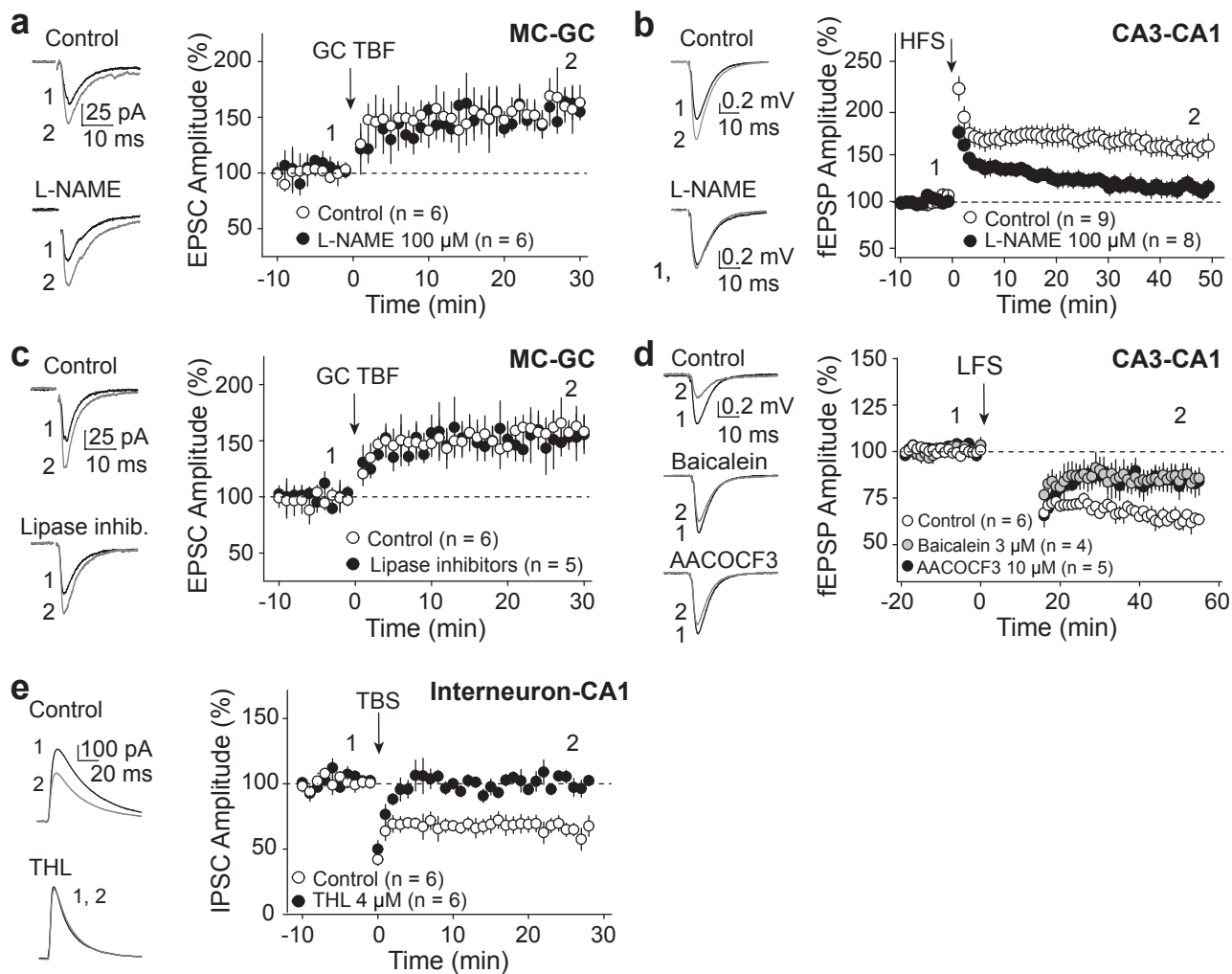


Figure 2

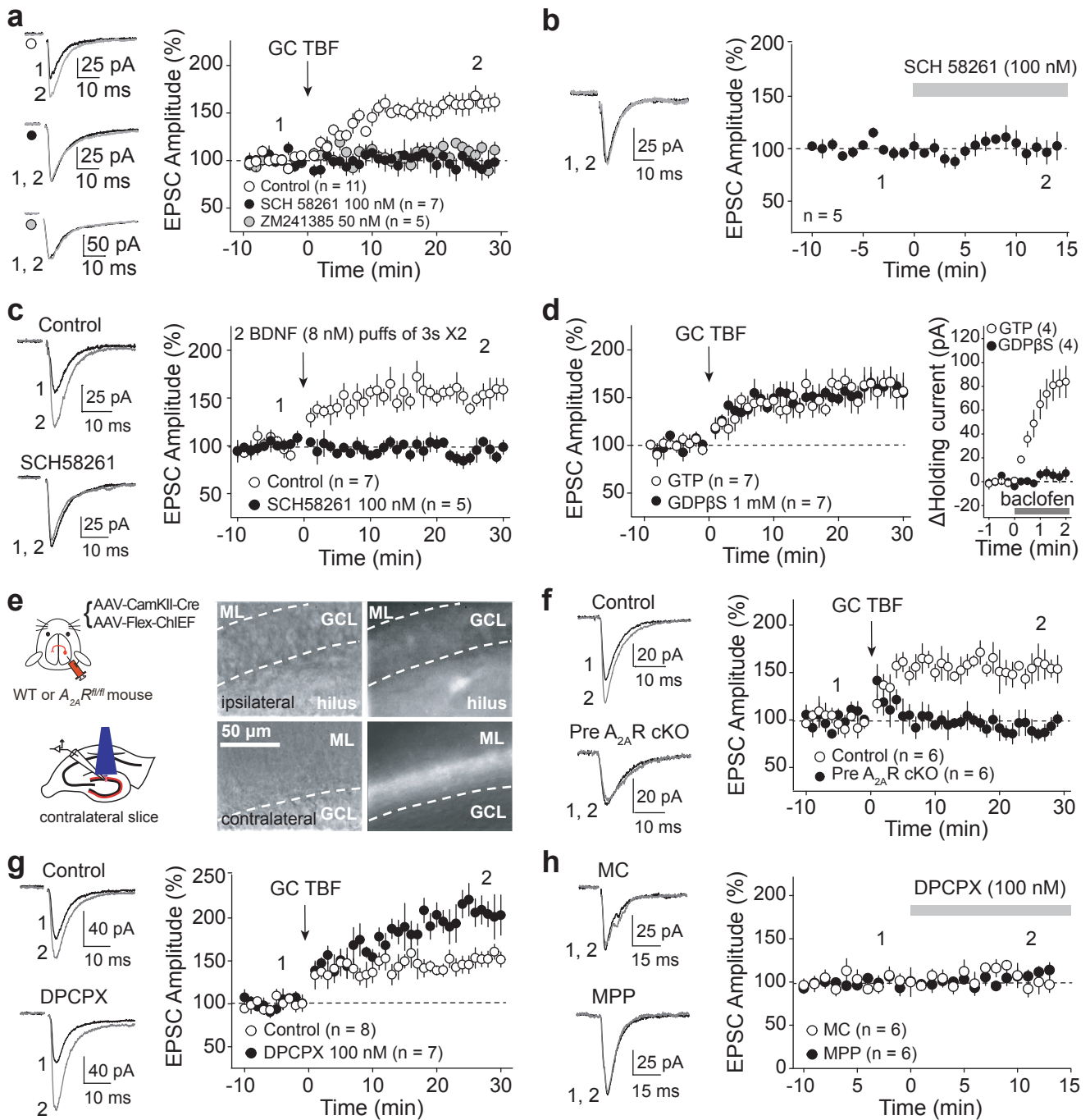


Figure 3

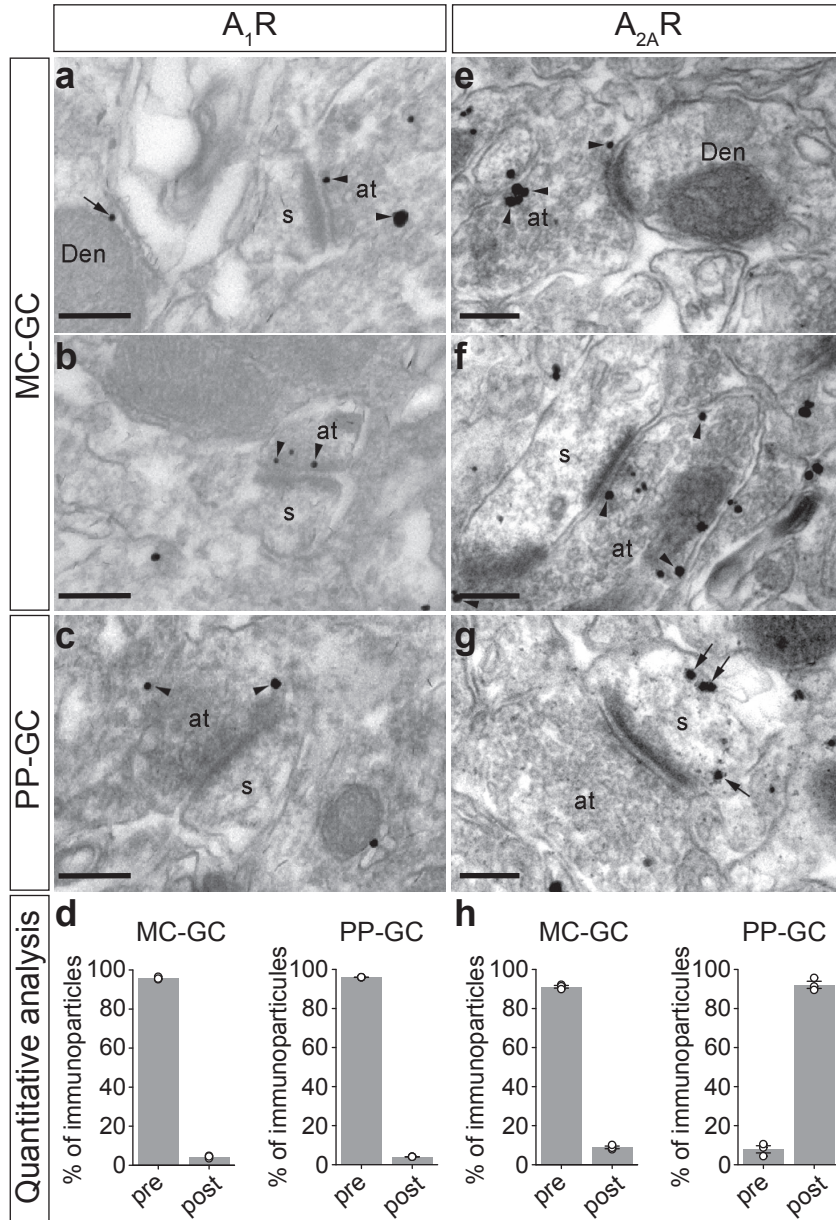


Figure 4

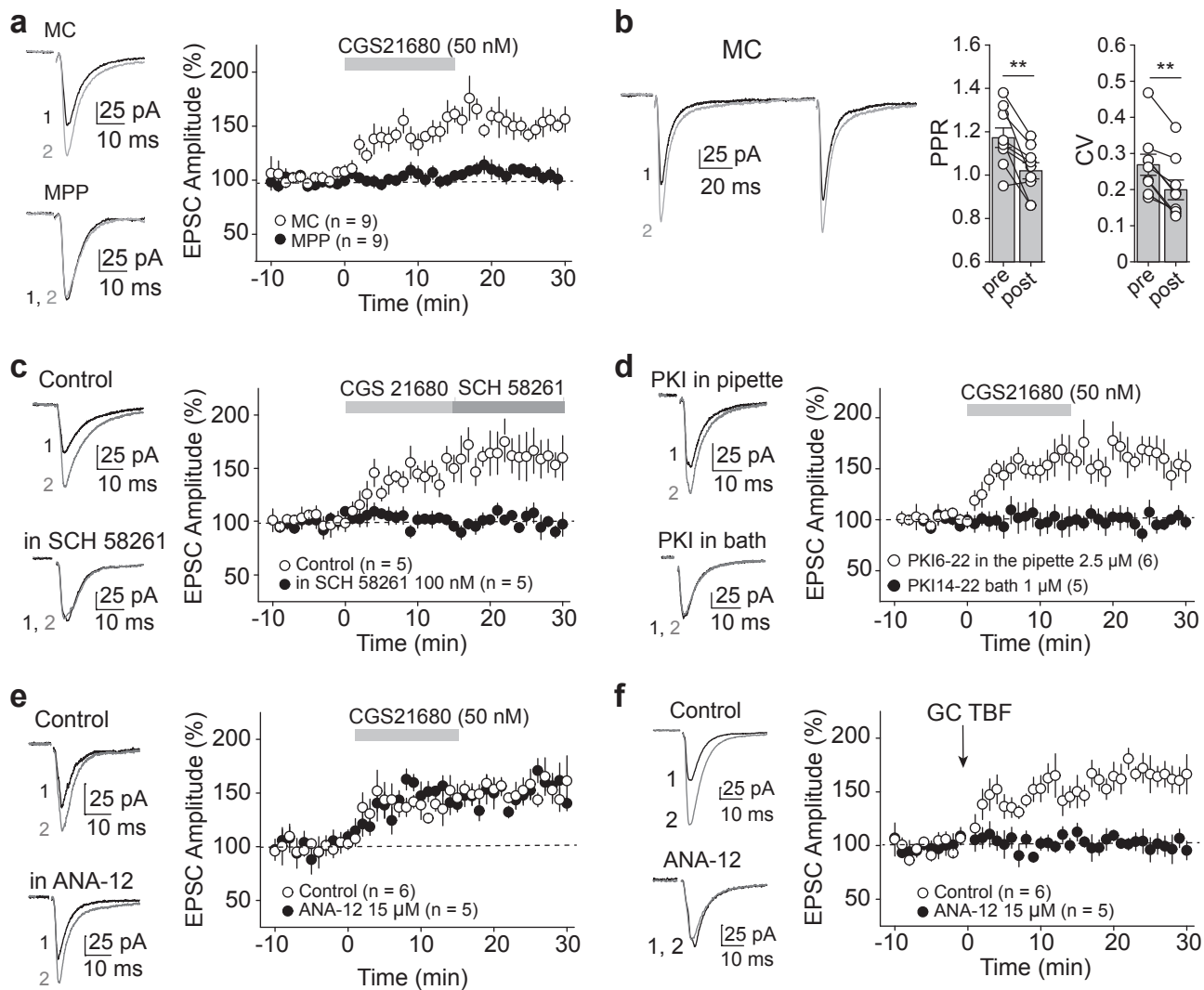


Figure 5

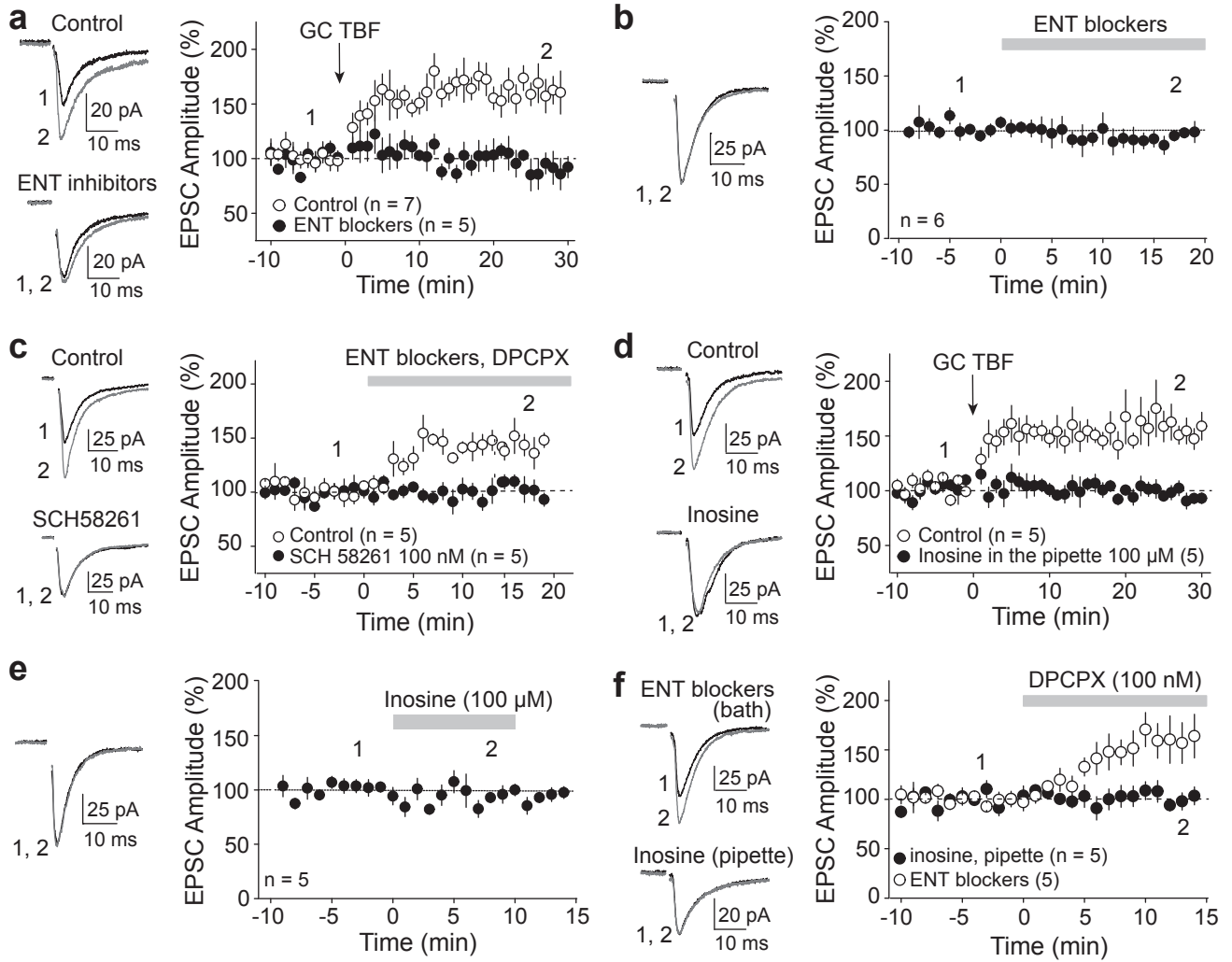


Figure 6

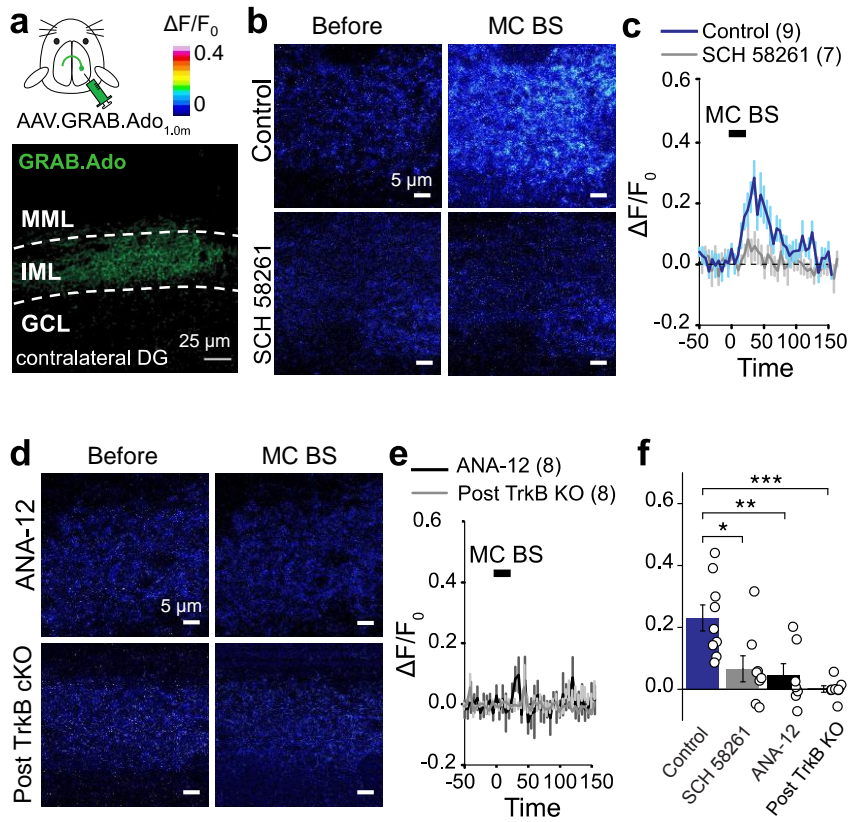


Figure 7

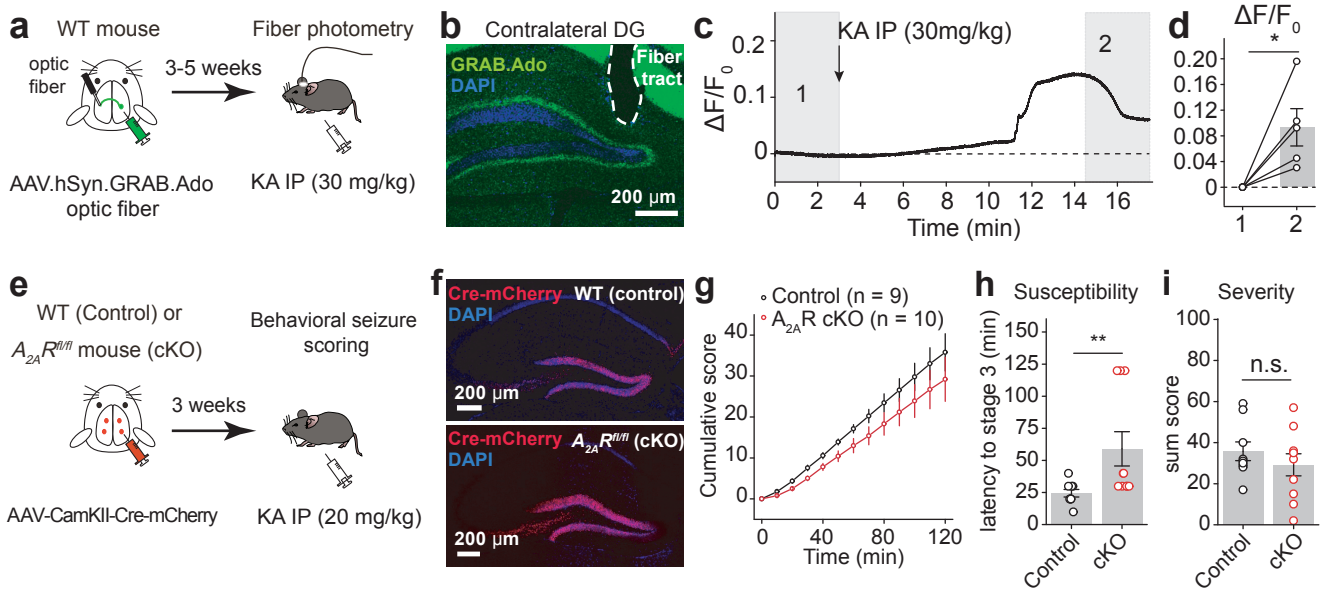


Figure 8