

38 **ABSTRACT**

39

40 Microcircuits in the neocortex are functionally organized along layers and columns,
41 which are the fundamental modules of cortical information processing. While the function of
42 cortical microcircuits has focused on neuronal elements, much less is known about the
43 functional organization of astrocytes and their bidirectional interaction with neurons. Here
44 we show that CB₁R-mediated astrocyte activation by neuron-released endocannabinoids
45 elevate astrocyte Ca²⁺ levels, stimulate ATP/adenosine release as gliotransmitters, and
46 transiently depress synaptic transmission in layer 5 pyramidal neurons at relatively distant
47 synapses (>20 μm) from the stimulated neuron. This astrocyte-mediated heteroneuronal
48 synaptic depression occurred between pyramidal neurons within a cortical column and was
49 absent in neurons belonging to adjacent cortical columns. Moreover, this form of
50 heteroneuronal synaptic depression occurs between neurons located in particular layers,
51 following a specific connectivity pattern that depends on a layer-specific neuron-to-astrocyte
52 signaling. These results unravel the existence of astrocyte-mediated non-synaptic
53 communication between cortical neurons, and that this communication is column- and layer-
54 specific, which adds further complexity to the intercellular signaling processes in the cortex.

55 INTRODUCTION

56 The neocortex is the most complex structure of the mammalian brain involved in higher
57 cognitive functions. The cellular organization of the cerebral cortex is well known since the work
58 of Cajal and his disciple Lorente de Nó, who proposed that cortical neurons form functional
59 modules that serve as the “elementary cortical unit of operation”^{1,2}. Cortical neurons are
60 organized horizontally in six layers and vertically in columns³. The columnar configuration of
61 the neocortex is a widely accepted idea explaining its functional organization^{4,5}. A great amount
62 of information has been provided regarding the neuronal elements involved in cortical circuits
63 and their synaptic microorganization⁶. However, the properties of non-neuronal cell types, like
64 astrocytes, and their functional interactions with neurons in this elementary module remain
65 largely unexplored.

66 Astrocytes have emerged as key regulatory elements of synapses, responding with Ca²⁺
67 elevations to synaptically-released neurotransmitters and releasing gliotransmitters that regulate
68 synaptic transmission in different brain areas⁷⁻⁹. In the cortex, sensory stimuli or direct neuronal
69 stimulation evoke astrocyte Ca²⁺ elevations¹⁰⁻¹⁷, which are topographically represented in the
70 primary somatosensory cortex S1¹⁸ and spatially restricted to the cortical columns in the
71 barrel cortex¹⁴. Cortical astrocyte Ca²⁺ elevations can, in turn, stimulate the release of
72 gliotransmitters, such as glutamate or D-Serine, that can regulate synaptic transmission^{13,15}, and
73 that can be responsible for the observed astrocyte-mediated regulation of the cortical network
74 function^{17,19-21}. Moreover, synaptic regulation by astrocytes may be exerted at synapses
75 relatively distant from the active synapses²²⁻²⁴. This phenomenon, termed lateral astrocyte
76 synaptic regulation²⁵, resembles the classical heterosynaptic modulation but is mechanistically
77 dissimilar because it involves astrocytes and may be crucial in brain circuits where spatial
78 signaling greatly influences neural network function, like the neocortical columns. However, the
79 spatial properties of astrocyte-neuron interaction and the consequent synaptic regulation in
80 defined cortical columns and layers remain unidentified.

81 Endocannabinoid (eCB) signaling has been proposed to mediate astrocyte-neuron
82 communication in different brain regions, including the neocortex. In particular, endogenous
83 activation of astroglial type-1 cannabinoid receptors (CB₁Rs) regulates hippocampal and
84 neocortical synaptic transmission and plasticity^{15,22,26,27}. Here, in order to decipher the regulatory
85 role of astrocytes in the synaptic physiology of cortical columns, we took advantage of this eCB
86 signaling to physiologically stimulate cortical astrocytes. We show that eCBs released from

87 layer 5 (L5) pyramidal neurons induce Ca^{2+} elevations in astrocytes and transiently depressed
88 synaptic transmission in adjacent pyramidal neurons. This form of heteroneuronal synaptic
89 depression required astrocytic cannabinoid receptor type (CB_1R) activation and was
90 mediated by presynaptic type 1 adenosine receptors (A_1Rs). Astrocyte-mediated
91 heteroneuronal synaptic depression was present between pyramidal neurons within a cortical
92 column and was absent in neurons belonging to adjacent cortical columns. Moreover, this
93 form of heteroneuronal synaptic depression occurred between neurons located in particular
94 layers, following a specific connectivity pattern that depends on a layer-specific neuron-to-
95 astrocyte signaling. These results reveal the existence of astrocyte-mediated non-synaptic
96 communication between cortical neurons, which is column- and layer-specific, and which
97 adds further complexity to the intercellular signaling processes in the cortex.

98 RESULTS

99

100 Endocannabinoid signaling induces homoneuronal and heteroneuronal synaptic 101 depression in S1

102 To investigate the spatial regulation of synaptic transmission in the primary
103 somatosensory cortex, we performed double patch-recordings of layer 5 (L5) pyramidal
104 neurons and monitored excitatory postsynaptic currents (EPSCs) evoked by electrical
105 stimulation of layer 2/3 (L2/3). We then stimulated one neuron by a depolarizing pulse and
106 recorded synaptic currents in both the “stimulated” neuron (homoneuronal synapses) and the
107 adjacent (70-270 μm apart) “nonstimulated” neuron (heteroneuronal synapses) (**Figures 1A**
108 **and 1B**). Stimulation of single L5 pyramidal neurons induced a transient synaptic depression
109 in 36 out of 93 (38.7%) homoneuronal synapses. Furthermore, in simultaneously recorded
110 heteroneuronal synapses, this neuronal depolarization (ND) also induced a transient
111 depression of synaptic transmission in 20 out of 72 (27.8%) heteroneuronal synapses
112 (**Figures 1C-E**). Both homoneuronal and heteroneuronal synaptic depressions could be
113 reliably induced by repeated stimulations (**Figure S1A and S1B**) and were associated with
114 changes in the paired-pulse ratio (PPR), which are consistent with presynaptic mechanisms
115 (**Figures S1C and S1D**).

116 ND is known to trigger the release of eCBs^{28,29} that can directly affect relatively close
117 synapses ($\sim 20 \mu\text{m}$)²⁹⁻³², a phenomenon called depolarization-induced suppression of
118 excitation (DSE)²⁹⁻³⁴, and indirectly regulate more distant synapses through stimulation of
119 astrocytes^{15,22,27}, a phenomenon called astrocyte-mediated lateral regulation of synaptic
120 transmission. Consistent with eCB-mediated synaptic regulation, homoneuronal and
121 heteroneuronal synaptic depressions observed under control conditions were abolished
122 following bath perfusion with the cannabinoid receptor type 1 (CB₁R) antagonist AM251 (2
123 μM ; n = 12 and 6), indicating that both phenomena were mediated by CB₁R activation
124 (**Figures 1D and 1E**).

125 We then tested whether these phenomena were present in other cortical layers by
126 performing paired recordings of neurons in L2/3 and L4. L2/3 or L4 ND induced both
127 homoneuronal (14 out of 38 and 8 of 32 pairs; 36.8% and 25%, respectively) and
128 heteroneuronal (14 out of 39 and 8 of 27 pairs; 35.9% and 29.6%, respectively) depression

129 (Figures 1F-I), indicating that eCB-induced homoneuronal and heteroneuronal synaptic
130 depressions are a general cortical phenomena.

131

132 **Heteroneuronal, but not homoneuronal, synaptic depression requires endocannabinoid** 133 **signaling in astrocytes**

134 We then investigated the role of astrocyte CB₁Rs on the eCB-induced homoneuronal and
135 heteroneuronal synaptic depression. We selectively deleted CB₁R expression in cortical
136 astrocytes by expressing Cre-recombinase under the control of the astroglial promoter GFAP,
137 using local injection of AAV8-GFAP-mCherry-Cre in S1 of CB₁R^{flox/flox} mice (Figure 2A).
138 These mice are herein termed aCB₁R^{-/-} mice, and their controls, termed aCB₁R mice, were
139 CB₁R^{flox/flox} mice injected with AAV8-GFAP-mCherry (i.e., lacking Cre). To assess the
140 efficacy of the approach, we monitored the CB₁R-mediated astrocyte Ca²⁺ responses to the
141 CB₁R agonist WIN 55,212-2 (300 μM) using two-photon microscopy and the genetically
142 encoded calcium indicator GCaMP6f selectively expressed in astrocytes via injection of
143 AAV5-gfaABC1D-cyto-GCaMP6f in S1 (Figure 2D). While the astrocyte Ca²⁺ activity,
144 quantified from the Ca²⁺ event probability, was increased by local application of WIN
145 55,212-2 in control aCB₁R mice (n = 190 astrocytes from 13 slices), the WIN-evoked
146 responses were significantly reduced in aCB₁R^{-/-} mice (n = 261 astrocytes from 14 slices;
147 Figure 2D-F), confirming the suitability of the viral approach to delete CB₁R signaling in
148 astrocytes.

149 Next, we tested the impact of astroglial deletion of CB₁Rs on the ND-evoked
150 homoneuronal and heteroneuronal synaptic depression in L5. Accordingly, the homoneuronal
151 depression was not affected in mice lacking CB₁Rs in astrocytes (12 out of 35 cells; 34.3%)
152 (Figures 2B and 2C). By contrast, the heteroneuronal synaptic depression was absent in
153 aCB₁R^{-/-} mice (0 out of 27 cells; 0%) (Figures 2B and 2C). These results indicate that eCB-
154 induced heteroneuronal, but not homoneuronal, synaptic depression involves CB₁R signaling
155 in astrocytes.

156

157 **Heteroneuronal synaptic depression requires astrocyte Ca²⁺ signaling and activation of** 158 **presynaptic A1 receptors**

159 Ca^{2+} elevations in astrocytes evoked by different neurotransmitters, including
160 eCBs^{22,35,36}, are known to stimulate the release of gliotransmitters that regulate synaptic
161 function (e.g.,^{15,22,35–37}). Hence, we investigated whether the homoneuronal and
162 heteroneuronal synaptic depressions depended on the astrocytic Ca^{2+} signal. We depolarized
163 L5 pyramidal neurons using the approach that elicits homoneuronal and heteroneuronal
164 synaptic depression as indicated above and monitored the astrocyte Ca^{2+} activity using
165 GCaMP6f selectively expressed in astrocytes (**Figure 2D**). To isolate eCB-induced effects,
166 experiments were performed in the presence of a cocktail of antagonists of glutamatergic,
167 GABAergic, purinergic, and cholinergic receptors (see Material and Methods). Under these
168 conditions, ND elevated astrocyte Ca^{2+} fluctuations (n = 142 astrocytes of 6 slices), an effect
169 that was abolished in the presence of AM251, indicating that these responses were mediated
170 by CB₁R activation (n = 122 astrocytes of 6 slices; **Figures 3A and 3B**).

171 Moreover, ND-evoked astrocyte Ca^{2+} elevations were largely absent in inositol-1,4,5-
172 trisphosphate (IP₃)-receptor type 2-deficient mice (IP₃R₂^{-/-} mice), in which G protein-
173 mediated Ca^{2+} signal is selectively impaired in astrocytes^{27,38–40} (n = 164 astrocytes of 11
174 slices; **Figures 3A and 3B**). In these mice, the homoneuronal depression was preserved (13
175 out of 30 cases; 43.3%), but the heteroneuronal depression was absent (0 out of 17 cases;
176 0%) (**Figure 3C**). Collectively these results indicate that the astrocyte Ca^{2+} signal is required
177 for the heteroneuronal, but not the homoneuronal, synaptic depression.

178 We then investigated the gliotransmitter responsible for the heteroneuronal depression.
179 ATP and its metabolic product adenosine are known to be released by astrocytes⁸ and to
180 regulate synaptic transmission in several brain areas^{23,24,36,37,41,42}. Therefore, we hypothesized
181 that eCB-induced astrocyte calcium elevations would stimulate the release of ATP/adenosine
182 that acting on neuronal type 1 adenosine receptors (A₁Rs) would depress synaptic
183 transmission. To test this idea, we depolarized L5 pyramidal neurons and monitored the
184 homo- and heteroneuronal synaptic depression before and after bath application of the A₁R
185 antagonist CPT (5 μM). While the homoneuronal depression was unaffected (n = 8), the
186 heteroneuronal depression was abolished in the presence of CPT (n = 8; **Figure 3C**).
187 Consistent with results observed in L5, heteroneuronal depressions in L4 and L2/3 were also
188 abolished by the A₁R antagonist CPT (**Figure 3D**).

189 To further test the astrocyte involvement, we investigated if activation of G-protein-
190 mediated signaling in astrocytes depresses excitatory transmission in the S1 cortex by
191 directly and selectively activating astrocytes using designer receptors exclusively activated
192 by designed drugs (DREADDs). Astrocytes in the S1 cortex were targeted with AAV8-
193 GFAP-Gq-DREADD-mCherry and AAV5-gfaABC1d-GCaMP6f (**Figure 3E**). Activation of
194 Gq-DREADDs with the synthetic agonist clozapine-N-oxide (CNO, 1 mM) delivered from
195 a micropipette by pressure pulses (5 s) (**Figure 3E**) induced Ca^{2+} elevations in astrocytes (n
196 = 253 astrocytes from 14 slices, **Figure 3G**) and depressed synaptic transmission in L5
197 pyramidal neurons ($n = 11$; **Figure 3F**), which was associated with an increase in PPR
198 indicating a presynaptic mechanism ($n = 11$; **Figure S1E**). Moreover, in the presence of CPT,
199 CNO also induced Ca^{2+} elevations in astrocytes ($n = 87$ astrocytes from 7 slices, **Figure 3G**)
200 but failed to affect synaptic transmission ($n = 7$, **Figure 3F**). In slices from mice that were
201 injected with control AAV8-GFAP-mCherry virus (i.e., lacking DREADDs), CNO
202 application failed to affect both synaptic transmission ($n = 11$; **Figure 3F**) and astrocyte
203 Ca^{2+} dynamics ($n = 92$ astrocytes from 8 slices; **Figure 3G**). These results suggest that
204 astrocyte Ca^{2+} elevations are sufficient to regulate cortical synaptic transmission.

205

206 Taking together, these results indicate that neuron-released eCBs induce homeoneuronal
207 depression by directly acting on neuronal CB_1Rs . Concomitantly, eCBs activate CB_1Rs in
208 astrocytes, elevate their intracellular Ca^{2+} , and stimulate the release of ATP/Adenosine,
209 which acts on presynaptic A_1Rs triggering the heteroneuronal depression (**Figure 3H**).

210

211 **Astrocyte-mediated heteroneuronal depression is column-specific**

212 The functional properties of the somatosensory cortex rely on their modular organization,
213 comprising sub-circuits of layer connectivity within cortical columns^{3,5,6}. Thus, we
214 investigated whether the astrocyte-mediate heteroneuronal depression was also spatially
215 restricted to a single column. We analyzed this phenomenon in the somatosensory barrel
216 cortex, where cortical columns can be easily identified (**Figure 4A**). We performed paired
217 whole-cell recordings of L4 neurons located at a similar distance (70-270 μm) but either
218 within the same or in adjacent columns (**Figure 4A**). Depolarization of a single L4 neuron to
219 stimulate eCBs release induced heteroneuronal depression in the paired recorded neuron

220 located in the same column (10 out of 29 paired recordings; 35%, **Figure 4B**). Consistent
221 with the mechanistic interpretation described above, this phenomenon was blocked after CPT
222 perfusion (**Figure S1G**). In contrast to this intracolumn regulation, the heteroneuronal
223 regulation was absent in neurons located at a similar distance but in an adjacent cortical
224 column (0 out 15 paired recordings; 0%, **Figure 4C**). In both cases, intra- and intercolumn
225 recordings displayed homoneuronal synaptic depression (16 out 48 cells; 33%, **Figure S1F**).

226

227 Overall, these results indicate that the eCB-induced astrocyte-mediated heteroneuronal
228 synaptic regulation is column-specific, i.e., it is not a wide unspecific phenomenon but a
229 synaptic regulatory signaling that specifically occurs between cells located within a cortical
230 column.

231

232 **Astrocyte-mediated heterosynaptic depression is layer-specific**

233 Cortical information processing depends not only on the columnar organization but also
234 on the functional interaction across different layers⁶. Therefore, we examined the functional
235 organization of heteroneuronal and homoneuronal synaptic depression across different
236 cortical layers, i.e., between neurons located in L2/3, L4, and L5 (**Figure 5**). We performed
237 paired recordings of neurons in these layers, depolarized one neuron to stimulate eCB release,
238 and monitored EPSCs in the other neuron located in another layer. While depolarizing a
239 single L2/3 neuron did not affect synaptic transmission in L4 neurons (n = 11) (**Figures 5A**
240 **and 5B**), stimulation of a single L4 neuron induced heteroneuronal synaptic depression in
241 L2/3 neurons (5 out of 10 cases; 50%, **Figures 5A and B**). Likewise, stimulation of a single
242 L2/3 neuron did not alter synaptic transmission in L5 neurons (n = 8; **Figures 5C and 5D**),
243 but stimulation of L5 neurons induced heteroneuronal depression in L2/3 neurons (4 out of
244 10 cases; 40%, **Figures 5C and 5D**). Finally, stimulation of L4 neurons depressed
245 neurotransmission in L5 pyramidal neurons (4 out of 14 cases; 28.1%, **Figures 5E and 5F**),
246 but L5 neuron stimulation did not impact synaptic transmission in L4 neurons (n = 16;
247 **Figures 5E and 5F**). In summary, astrocyte-mediated heteroneuronal depression occurs
248 between neurons located in different layers, but following a specific pattern and not
249 necessarily reciprocally. For example, synapses in L2/3 neurons can be regulated by neurons

250 located in L4 or L5, but not vice versa; and L4 neurons can regulate neurons in L2/3 and L5
251 but are not regulated by them.

252 Together, these results indicate that eCB-induced astrocyte-mediated heteroneuronal
253 synaptic regulation is not an unspecific phenomenon, rather it is layer-specific, selectively
254 occurring among neurons following a layer-specific pattern (**Figure 5G**).

255 256 **Astrocytic calcium responses to eCBs are not homogeneous across cortical layers**

257 Because the heteroneuronal depression depends on the eCB-induced astrocyte
258 Ca^{2+} signals, its layer-specificity might be accounted for by layer-specificity of astrocyte
259 responsiveness to eCBs (**Figure 6A**). To test this idea, we examined the astrocyte
260 Ca^{2+} signals in different layers in response to eCBs released by depolarization of neurons.
261 To ensure that the astrocyte activation was due to eCBs, we performed the experiments in
262 the presence of TTX (1 μM) and the cocktail of neurotransmitter receptor antagonists (see
263 Material and Methods). Neuronal depolarization of L2/3, L4 or L5 neurons elevated
264 intracellular Ca^{2+} in astrocytes located within the same layer (n = 110 astrocytes from 5 slices;
265 n = 110 astrocytes from 4 slices; n = 142 from 6 slices respectively). These responses were
266 abolished by AM251, confirming to be the result of eCB signaling (n = 89 astrocytes from 5
267 slices; n = 79 astrocytes from 4 slices; n = 122 from 6 slices respectively, **Figures 6B-D**).

268 We then examined the astrocyte responses to neuron-released eCBs across cortical
269 layers. Stimulation of L2/3 neurons increased the astrocyte Ca^{2+} event probability in L2/3 (n
270 = 185 astrocytes from 10 slices) but failed to increase Ca^{2+} signaling in L4 (n = 62 astrocytes
271 from 5 slices) or L5 (n = 129 astrocytes from 6 slices) astrocytes (**Figure 6E**). Likewise,
272 stimulation of L4 neurons elevated Ca^{2+} in L4 (n = 163 astrocytes from 8 slices), L5 (n = 87
273 astrocytes from 7 slices) and L2/3 (n = 71 astrocytes from 6 slices) astrocytes (**Figure 6F**).
274 Finally, stimulation of L5 neurons produced an increase in the Ca^{2+} event probability in L5
275 (n = 267 astrocytes from 13 slices) and L2/3 (n = 202 astrocytes from 8 slices) astrocytes but
276 not in L4 (n = 144 astrocytes from 8 slices) astrocytes (**Figure 6G**). These results indicate
277 that astrocyte Ca^{2+} increases mediated by eCBs signaling obeys a layer-specific pattern in
278 agreement with the astrocyte-mediated heterosynaptic regulation produced by neuronal
279 depolarization (**Figure 6H**).

280

281 In summary, eCB-mediated neuron-to-astrocyte signaling is a form of communication
282 that occurs between cells that can be located in different layers, but following a specific
283 connectivity pattern. Like the heteroneuronal depression, this specific pattern does not
284 necessarily involve reciprocal signaling between layers. Notably, the specific neuron-to-
285 astrocyte connectivity pattern mirrors the heteroneuronal depression pattern, suggesting that
286 the selective neuron-astrocyte signaling between layers is responsible for the astrocyte-
287 mediated non-synaptic communication between neurons in different cortical layers.

288 **DISCUSSION**

289

290 Present results show that astrocytes regulate cortical synaptic function in a layer- and
291 column-specific manner and that the functional interaction between cortical astrocytes and
292 synapses is highly spatially organized. We show that activation of astrocytes by endogenous
293 stimuli –eCBs physiologically released from cortical neurons–, induced astrocyte Ca^{2+}
294 elevations and transiently depressed synaptic transmission in neurons located in the same and
295 distinct cortical layers. This heteroneuronal synaptic depression requires astrocytic CB_1R
296 activation, is mediated by activation of presynaptic A_1 receptors, and can be mimicked by
297 astrocyte-specific chemogenetic stimulation. Additionally, our results also show that neuron-
298 released eCBs can depress synaptic transmission by directly activating CB_1Rs in
299 homoneuronal synapses, a phenomenon known as DSE^{29–34}.

300 The neocortex is highly organized in layers and columns with precisely neuronal
301 connectivity. Our results indicate that eCB-mediated astrocyte-neuron signaling is also
302 exquisitely organized. First, the astrocyte-mediated heteroneuronal depression was found to
303 be column-specific because it only occurred between neurons located within the same
304 column and not between neurons located at similar distances but in adjacent columns (**Figure**
305 **4**). Second, astrocyte-mediated heteroneuronal regulation occurred between neurons located
306 in different layers, but according to a specific connectivity pattern (**Figure 5**). Third, the
307 eCB-mediated neuron-to-astrocyte signaling was also layer-specific because astrocytic
308 calcium responses to eCBs released by neurons in different cortical layers were not
309 homogeneous across the cortical layers; rather neuron-to-astrocyte signaling occurred
310 according to particular signaling patterns (**Figure 6**).

311 Several previous studies have shown that astrocytes stimulated by eCBs lead to
312 regulation of synaptic transmission in diverse brain areas, including the hippocampus,
313 amygdala, and neocortex^{15,22,26,27}. In the neocortex, synapses onto layer 2/3 neurons undergo
314 spike-timing long-term depression (LTD) mediated by glutamate released from astrocytes¹⁵.
315 By contrast, we found (**Figures 1, 2, and 3**) that eCB-induced astrocyte activation transiently
316 depresses synapses through ATP/adenosine release as gliotransmitters. Different neuronal
317 stimulating paradigms used in these studies may account for such discrepancies. As a matter
318 of fact, astrocytes are competent to release distinct gliotransmitters depending on the pattern
319 of neuronal stimulation as demonstrated in the hippocampus, where astrocytes can release

320 glutamate upon low frequency stimulation of neighboring interneurons or glutamate and
321 ATP/adenosine upon high frequency stimulation⁴³.

322 The eCB-induced astrocyte-mediated heteroneuronal depression was found to be
323 restricted within a single cortical column, supporting the idea of a highly organized signaling
324 between astrocytes and neurons at a modular level. These results agree with previous reports
325 showing that astrocyte Ca²⁺ signal is spatially restricted in astrocytes located within the
326 columns of the barrel cortex^{14,44,45}. The column-specific astrocyte-mediated synaptic
327 regulation also indicates that astrocyte-neuron networks are functionally organized obeying
328 the columnar organization of the neuronal connectivity pattern.

329 In conclusion, the present data indicate that astrocytes modulate cortical synaptic
330 transmission in a column and layer-specific manner, obeying the structural and functional
331 organization of the cortex, which suggests that they are an integral part of the cortical
332 modules. Moreover, astrocytes, by providing layer-specific signaling pathways of non-
333 synaptic communication between neurons, add further complexity to the signaling
334 mechanisms underlying cortical network function. This finely controlled astrocyte-synapse
335 interaction is particularly significant in the neocortex, where the spatial integration of
336 synaptic signals is highly relevant for cortical information processing.

337 **METHODS**

338

339 **Ethics statement**

340 All of the procedures for handling and sacrificing animals were approved by the
341 University of Minnesota Institutional Animal Care and Use Committee (IACUC) in
342 compliance with the National Institutes of Health guidelines for the care and use of laboratory
343 animals.

344

345 **Animals**

346 Mice were housed under 12/12-h light/dark cycle, up to five animals per cage, at
347 temperatures between 68–74°F at 30–70% humidity with freely available food and water.
348 The following animals (males and females) were used for the present study C57BL/6J, IP₃R₂⁻
349 ⁻ (generously donated by Dr. J Chen), and CB₁R^{fl/fl} 46,47. Adult (≥8 weeks) mice were used.

350

351 **Somatosensory Cortex Slice Preparation**

352 Mice were euthanized by decapitation and brains were rapidly removed and placed in
353 ice-cold artificial cerebrospinal fluid (ACSF). Three-hundred and fifty-micrometer coronal
354 brain slices containing the somatosensory cortex were prepared via a Leica VT1200
355 vibratome in a 4°C ACSF solution. Following cutting, slices were allowed to recover in
356 ACSF containing (in mM): NaCl 124, KCl 2.69, KH₂PO₄ 1.25, MgSO₄ 2, NaHCO₃ 26, CaCl₂
357 2 and glucose 10, gassed with 95% O₂/5% CO₂ (pH = 7.3) at 31°C for 30 min followed by
358 30 min at 20–22°C before recording. After a 1 h recovery period, slices were kept at 20–22°C
359 for the rest of the recording day. Slices were then transferred to an immersion recording
360 chamber and superfused at 2 ml/min with gassed ACSF and the temperature of the bath
361 solution was kept at 34°C with a temperature controller TC-324B (Warner Instruments Co.).
362 Cells were visualized using infrared-differential interference contrast optics (Nikon Eclipse
363 E600FN, Tokyo, Japan) and 40x water immersion lens. L2/3, L4, and L5 from the forelimb
364 and hindlimb somatosensory cortex and the barrel subfields were identified with a 10x
365 objective.

366

367 **Electrophysiology**

368 Neurons were selected based on their location, morphology, and firing pattern.
369 Simultaneous dual electrophysiological recordings from layers 2/3, 4, and 5 pyramidal
370 neurons were made using the whole-cell-patch-clamp technique. When filled with an internal
371 solution containing (in mM): KGlucuronate 135, KCl 10, HEPES 10, MgCl₂ 1, ATP-Na₂ 2 (pH
372 = 7.3) patch electrodes exhibited a resistance of 3-10 MΩ. All recordings were performed
373 using PC-ONE amplifiers (Dagan Instruments, Minneapolis, MN). Fast and slow whole-cell
374 capacitances were neutralized, and series resistance was compensated (~70%), and the
375 membrane potential was held at -70 mV. Intrinsic electrophysiological properties were
376 monitored at the beginning and the end of the experiments. Series and input resistances were
377 monitored throughout the experiment using a -5 mV pulse. Recordings were considered
378 stable when the series and input resistances, resting membrane, and stimulus artifact duration
379 did not change > 20%. Furthermore, I-V curves and firing patterns at the beginning and the
380 end of the experiments were similar. Recordings that did not meet these criteria were
381 discarded. Signals were fed to a Pentium-based PC through a DigiData 1322A interface
382 board. Signals were filtered at 1 kHz and acquired at a 10 kHz sampling rate using a DigiData
383 1322A data acquisition system and pCLAMP 10.3 software (Molecular Devices, San Jose,
384 CA). Distance of the somas of the paired recorded neurons within a layer varied from, 70-
385 270 μm. In paired recordings across layers 2/3, 4, and 5, neurons were selected following the
386 same vertical axis.

387

388 **Synaptic Stimulation**

389 Theta capillaries (2-5 μm tip) filled with ACSF were used for bipolar stimulation. The
390 electrodes were connected to a stimulator S-900 through an isolation unit and placed in L2/3.
391 When indicated, the stimulation electrode was placed in L4. Paired pulses of 1 ms duration
392 and 50 ms interval were continuously delivered at 0.33 Hz. Excitatory postsynaptic currents
393 (EPSCs) were isolated using picrotoxin (50 μM) and CGP5462 (1 μM) to block GABA_AR
394 and GABA_BR, respectively. EPSC amplitude was determined as the peak current amplitude
395 (2–20 ms after stimulus) minus the mean baseline current (10-30 ms before stimulus). The
396 paired-pulse ratio (PPR) was estimated as $PPR = (2^{nd} \text{ EPSC} / 1^{st} \text{ EPSC})$.

397 To induce eCB release, pyramidal neurons were depolarized from -70 mV to 0 mV for
398 5 s (ND)^{28,29}. Synaptic parameters were determined from 60 stimuli before (basal) and

399 following ND. Baseline mean EPSC amplitude was obtained by averaging mean values
400 obtained within 3 min of baseline recordings and mean EPSC amplitudes were normalized
401 to baseline. The ND was applied 2.5 s after the last basal delivered pulse, and no pulses were
402 presented during the ND. Immediately after the ND was finished, the 0.33-Hz pulse protocol
403 was restarted. To illustrate the time course of ND-induced effects, synaptic parameters were
404 grouped in 15 s bins. Three consecutive responses to ND were averaged. A response was
405 considered a depression if the amplitude of the current was < 2 times the standard deviation
406 of the baseline current during the first 45 s after ND and was verified by visual inspection.

407 The effects of pharmacological agents (CPT 5 μM and AM251 2 μM) were tested after
408 10 min bath perfusion and in the same neurons that previously were depolarized in control
409 conditions. In all cases the effects of pharmacological agents were tested at < 40 min after
410 entering whole-cell mode in the stimulating neuron.

411

412 **Ex vivo two-photon calcium fluorescence imaging and electrophysiology**

413 Two-photon microscopy imaging was performed using a Leica SP5 multi-photon
414 microscope (Leica Microsystems, USA) controlled by the Leica LAS software and adapted
415 to perform electrophysiological recordings. C57BL/6J, $\text{IP}_3\text{R}_2^{-/-}$ and aCB_1R mice injected into
416 S1 with AAV5-GfaABC1d-GCaMP6f and AAV8-GFAP-mCherry were used (for $\text{aCB}_1\text{R}^{-/-}$
417 instead AAV8-GFAP-mCherry we used AAV8-GFAP-mCherry-Cre). All Ca^{2+} experiments,
418 except those in which synaptic transmission was recorded, were performed in the presence
419 of TTX (1 μM) and a cocktail of neurotransmitter receptor antagonists containing: CNQX
420 (20 μM), AP5 (50 μM), MPEP (50 μM), LY367385 (100 μM), picrotoxin (50 μM), CGP5462
421 (1 μM), atropine (50 μM), CPT (5 μM), flupenthixol (30 μM), and suramin (100 μM).

422 Videos were obtained at 512×512 resolution with a sampling interval of 1 s. Red and
423 green fluorescence was obtained in parallel to match red mCherry-stained astrocyte structure
424 with green GCaMP6f astrocyte calcium. A custom MATLAB program (Calsee:
425 <https://www.araquelab.com/code/>) was used to quantify fluorescence level measurements in
426 astrocytes. Ca^{2+} variations recorded at the soma and processes of the cells were estimated as
427 changes of the fluorescence signal over baseline ($\Delta F/F_0$), and cells were considered to show
428 a Ca^{2+} event when the $\Delta F/F_0$ increase was at least two times the standard deviation of the
429 baseline.

430 The astrocyte Ca^{2+} signal was quantified from the Ca^{2+} event probability, which was
431 calculated from the number of Ca^{2+} elevations grouped in 5 s bins recorded from 8-50
432 astrocytes per field of view (layer 2/3, 4 or 5 of S1). The time of occurrence was considered
433 at the onset of the Ca^{2+} event. For each astrocyte analyzed, values of 0 and 1 were assigned
434 for bins showing either no response or a Ca^{2+} event, respectively, and the Ca^{2+} event
435 probability was obtained by dividing the number of astrocytes showing an event at each time
436 bin by the total number of monitored astrocytes²². All the astrocytes that showed a Ca^{2+} event
437 during the experiment were used for the analysis. The calcium event probability was
438 calculated in each slice, and for statistical analysis, the sample size corresponded to the
439 number of slices as different slices were considered as independent variables. To examine
440 the difference in Ca^{2+} event probability in distinct conditions, the basal Ca^{2+} event probability
441 (mean of the 30 s before a stimulus) was averaged and compared to the average Ca^{2+} event
442 probability (5 s after a stimulus). For ND experiments, each layer was recorded 1 minute
443 before and after the ND. Three consecutive responses to ND were averaged in each layer.
444 For WIN application, a micropipette was filled with 300 μM WIN solution and placed 100–
445 150 μm away from the tissue (layer 5), and a pressure pulse at 1 bar (PMI-100 DAGAN,
446 Minneapolis, MN) was applied for 5 s. The absence of mechanical movement of the tissue
447 was confirmed in every case. For acute application of CNO, a micropipette was filled with 1
448 mM CNO solution and placed 100–150 μm away from the recording neuron, and a pressure
449 pulse was applied for 5 s. The absence of mechanical movement of the tissue was confirmed
450 in every case. Stimulus effects on EPSCs were statistically tested comparing the normalized
451 EPSCs recorded 1 min before and 30 s after the stimulus to assess changes in EPSC amplitude
452 and PPR. Astrocytic Ca^{2+} events were recorded at the same time. The changes on the Ca^{2+}
453 event probability after CNO application were statistically tested comparing the basal Ca^{2+}
454 event probability 1 minute before and 5 s after the stimulus.

455 The effects of pharmacological agents (CPT 5 μM and AM251 2 μM) were tested after
456 10 min bath perfusion in the same region and same astrocytes recorded in control conditions.
457 In the cases when Ca^{2+} imaging and electrophysiology were performed at same time the
458 effects of pharmacological agents were tested at < 40 min after entering whole-cell mode in
459 the stimulating or recorded neuron.

460

461 **AAV viral surgeries**

462 Animals were anesthetized using a ketamine (10 mg/mL) xylazine (1 mg/mL) mixture
463 and placed on a heating pad to maintain body temperature and faux tears were applied to the
464 cornea. Animals (8 weeks of age) were placed in a stereotaxic apparatus and an incision was
465 made down the midline of the scalp to expose the skull. A hole was drilled over the forelimb
466 and hindlimb somatosensory cortex (S1: -0.4_{a-p} , 1.9_{m-l}), and a Hamilton syringe was lowered
467 to (in mm from bregma: -0.7_{d-v}) and viruses were injected bilaterally at 100 nL/min⁴⁸. Mice
468 were then sutured and left to heal for 2–3 weeks.

469 AAV5-pZac2.1-gfaABC1d-cyto-GCaMP6f (Addgene), AAV8-GFAP-hM3D(Gq)-
470 mCherry (UMN vector core), AAV8-GFAP-mCherry (UMN vector core) and AAV8-GFAP-
471 mCherry-Cre (UMN vector core) viral constructs were used. For CNO experiments,
472 C57BL/6J mice were injected with AAV8-GFAP-hM3D(Gq)-mCherry virus. In control
473 conditions, a virus of AAV8-GFAP-mCherry was injected instead. For CB₁R^{fl/fl} mice
474 experiments, AAV8-GFAP-mCherry-Cre was injected to delete CB₁R from astrocytes
475 (aCB₁R^{-/-}). AAV8-GFAP-mCherry was used as a control (aCB₁R).

476

477 **Immunohistochemistry**

478 The animals were anesthetized with Avertin (2,2,2 tribromoethanol, 240 mg/kg, i.p.) and
479 intracardially perfused with ice cold phosphate buffered saline (PBS) and subsequently with
480 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (pH 7.4). The brain was
481 removed, and 100 um coronal sections were made using a Leica VT1000S vibratome.
482 Vibratome sections were incubated for one h in blocking buffer (0.1% Triton X-100, 10%
483 Donkey or Goat serum in PBS) at room temperature. The primary antibodies were diluted in
484 the blocking solution and the sections were incubated for two days at 4°C. The following
485 primary antibodies were used: Rabbit anti-GFAP (Sigma, 1:500) Mouse anti-NeuN
486 (Millipore, 1:500). The slices were then washed three times for fifteen minutes each in PBS.
487 The secondary antibodies were diluted in the secondary antibody buffer (0.1% Triton X-100,
488 5% Donkey or Goat serum in PBS) and incubated for 2 days at room temperature. The
489 following secondary antibodies were used: 488 goat anti-rabbit (Invitrogen, 1:1000), 405
490 goat anti-mouse (Invitrogen, 1:500). The sections were then washed 3 times with 1xPBS for
491 10 min each and mounted using Vectashield Mounting media (Vector laboratories). The

492 slides were imaged using a Leica SP5 multiphoton confocal microscope and Olympus
493 FluoView FV1000.

494 The cellular specificity of Cre viral vectors was tested by immunohistochemical analysis of
495 randomly selected areas of the S1. Out of the 784 cells expressing mCherry from the AAV8-
496 GFAP-mCherry-Cre viral vector, 86.7% were astrocytes (identified by GFAP) and 11.3%
497 were neurons (identified by NeuN) (**Figures S2A and S2B**).

498

499 **Biocytin-stained neurons**

500 Pair of neurons were recorded with patch pipettes and filled with internal solution
501 containing 0.5% biocytin. Slices were fixed in 4% PFA in 0.1 PBS (pH 7.4) at 4°C. Slices
502 were washed three times in 1xPBS (10 min each). To visualize biocytin slices were incubated
503 with Alexa488-Streptavidin (RRID: AB 2315383; 1:500) for 48 h at 4°C. Slices were then
504 washed for 3 times with 1xPBS (10 min each) and mounted with Vectashield mounting media
505 (Vector laboratories). All mounted slices were imaged using a Leica SP5 multi-photon
506 microscope. Also, pair of neurons were filled with biocytin through whole-cell recording, the
507 slices were fixed using 4% paraformaldehyde. Then the slices were washed with PBS (100
508 mM sodium phosphate, pH 7.2). Endogenous peroxidases were then quenched by incubation
509 with 1% H₂O₂. The slices were subsequently rinsed in PBS. Slices were conjugated with
510 avidin-biotinylated horseradish peroxidase following the manufacturer's instructions (ABC-
511 Elite, Vector stains). Slices were then washed, and subsequently, biocytin-stained neurons
512 were visualized under a reaction with 0.5 mg/ml DAB and 0.01% H₂O₂. When the neuronal
513 processes were visible, the reaction was stopped by washing with PBS.

514

515 **Drugs and Chemicals**

516 4-[3-[2-(Trifluoromethyl)-9H-thioxanthen-9-ylidene]propyl]-1-piperazineethanol
517 dihydrochloride (flupenthixol dihydrochloride), [S-(R*,R*)]-[3-[[1-(3,4-
518 Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid (CGP
519 54626 hydrochloride), 8,8'-[Carbonylbis[imino-3,1-phenylenecarbonylimino(4-methyl-3,1-
520 phenylene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (suramin
521 hexasodium salt), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-
522 1H-pyrazole-3-carboxamide (AM251), D-(-)-2-Amino-5-phosphonopentanoic acid (D-

523 AP5), 6-Cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX disodium salt), (S)-(+)-a-
524 Amino-4-carboxy-2-methylbenzeneacetic acid (LY367385), and 2-Methyl-6-
525 (phenylethynyl)pyridine hydrochloride (MPEP hydrochloride), Octahydro-12-
526 (hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d] pyrimidine-
527 4,7,10,11,12-pentol (Tetrodotoxin: TTX) were purchased from Tocris Bioscience. Endo-(±)-
528 α-(Hydroxymethyl)benzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester (atropine)
529 and 8-Cyclopentyl-1,3-dimethylxanthine (CPT) were from Sigma. Picrotoxin from Indofine
530 Chemical Company (Hillsborough, NJ). All other drugs were purchased from Sigma.

531

532 **Statistical analysis**

533 Number of neurons was used as a sample size for electrophysiology comparisons and
534 number of slices for Ca²⁺ signal comparisons. At least 2 mice per experimental group were
535 used. Data are expressed as mean ± standard error of the mean (SEM). Data normality was
536 tested using a Shapiro-Wilk test. Results were compared using a two-tailed Student's t test
537 (Paired, before-after stimulus-treatment; Unpaired between groups). A full report of the
538 statistics used in every case is detailed in **Table S1**. Statistical differences were established
539 with $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)).

540

541

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555 **AUTHOR CONTRIBUTIONS**

556 A.M.B. performed experiments and analyzed data. L.B. and P.K. performed
557 immunohistochemistry. P.K., A.A., G.M., C.M., S.M. and A.M.B. conceived the study and
558 wrote the manuscript.

559 **COMPETING FINANCIAL INTERESTS**

560 The authors declare no competing financial interests.

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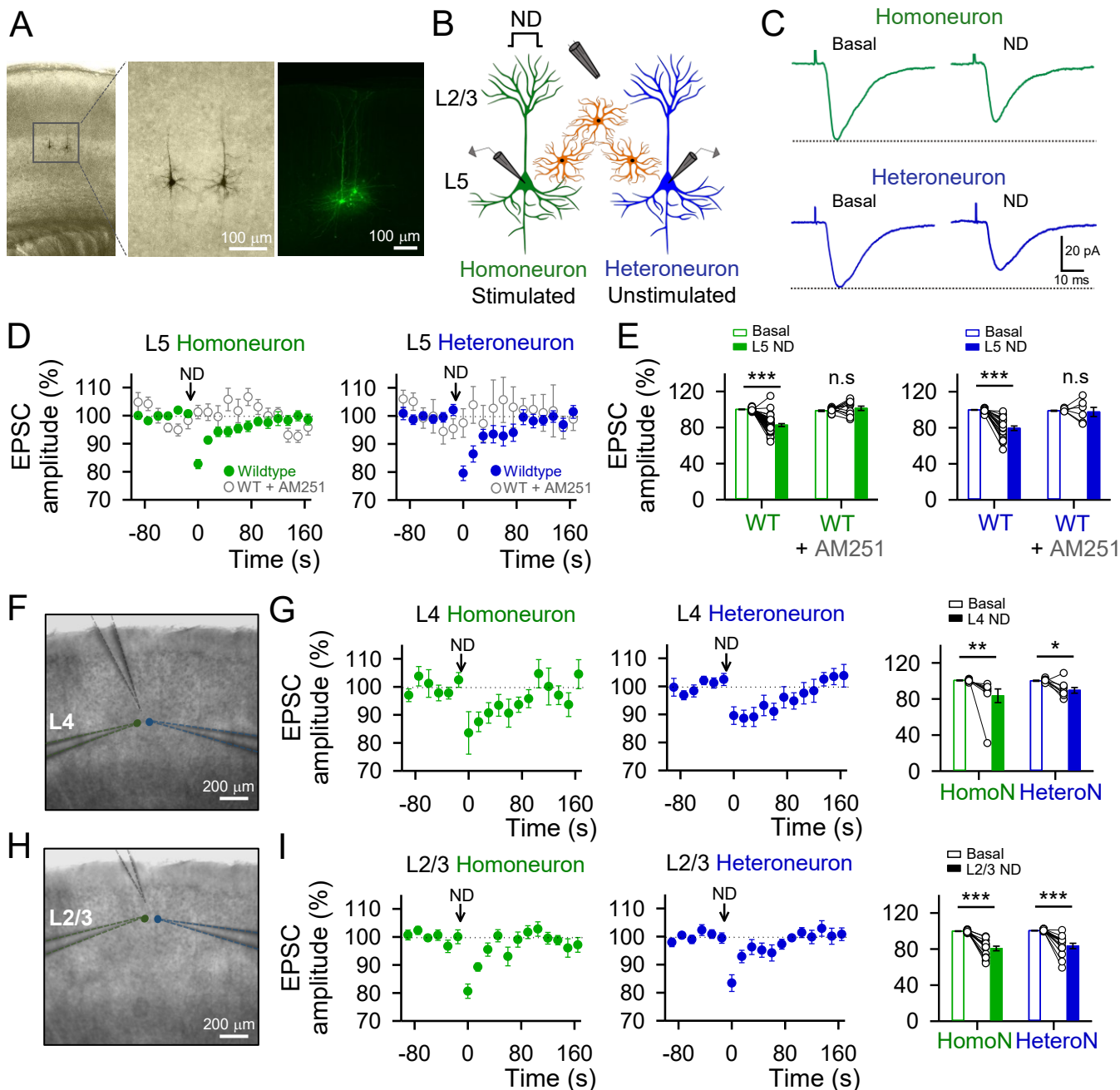


Figure 1. Endocannabinoid signaling induces homoneuronal and heteroneuronal synaptic depression in S1

(A) Biocytin loading S1 L5 pyramidal neurons image. (B) Schematic drawing depicting double patch-recordings from L5 pyramidal neurons and the stimulating electrode in L2/3. (C) Averaged EPSCs ($n = 20$ stimuli) before (control) and after neuronal depolarization (ND) in wildtype mice. (D) EPSCs amplitude *versus* time before (basal) and after ND in control (green or blue) and in the presence of AM251 ($2 \mu\text{M}$; open grey) in the homoneuron (left) and heteroneuron (right) from layer 5. (E) Relative changes in EPSC amplitude in control and with AM251 ($2 \mu\text{M}$). Two-tailed Student's paired t test. (F) Representative infrared differential interference contrast image of the experimental configuration with the stimulation pipette in layer 4 and the homoneuronal and heteroneuronal neurons located in layer 4. (G) EPSCs amplitude *versus* time before (basal) and after ND in the homoneuron (left, green) and heteroneuron (middle, blue) in the experimental conditions represented in panel F. Right: Relative changes in EPSC amplitude. (H) Representative infrared differential interference contrast image of the experimental configuration with the stimulation pipette in L2/3 and the homoneuronal and heteroneuronal neurons located in L2/3. (I) EPSCs amplitude *versus* time before (basal) and after ND in the homoneuron (left, green) and heteroneuron (middle, blue) in the experimental conditions represented in panel H. Right: Relative changes in EPSC amplitude. Two-tailed Student's paired t test. Data are expressed as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

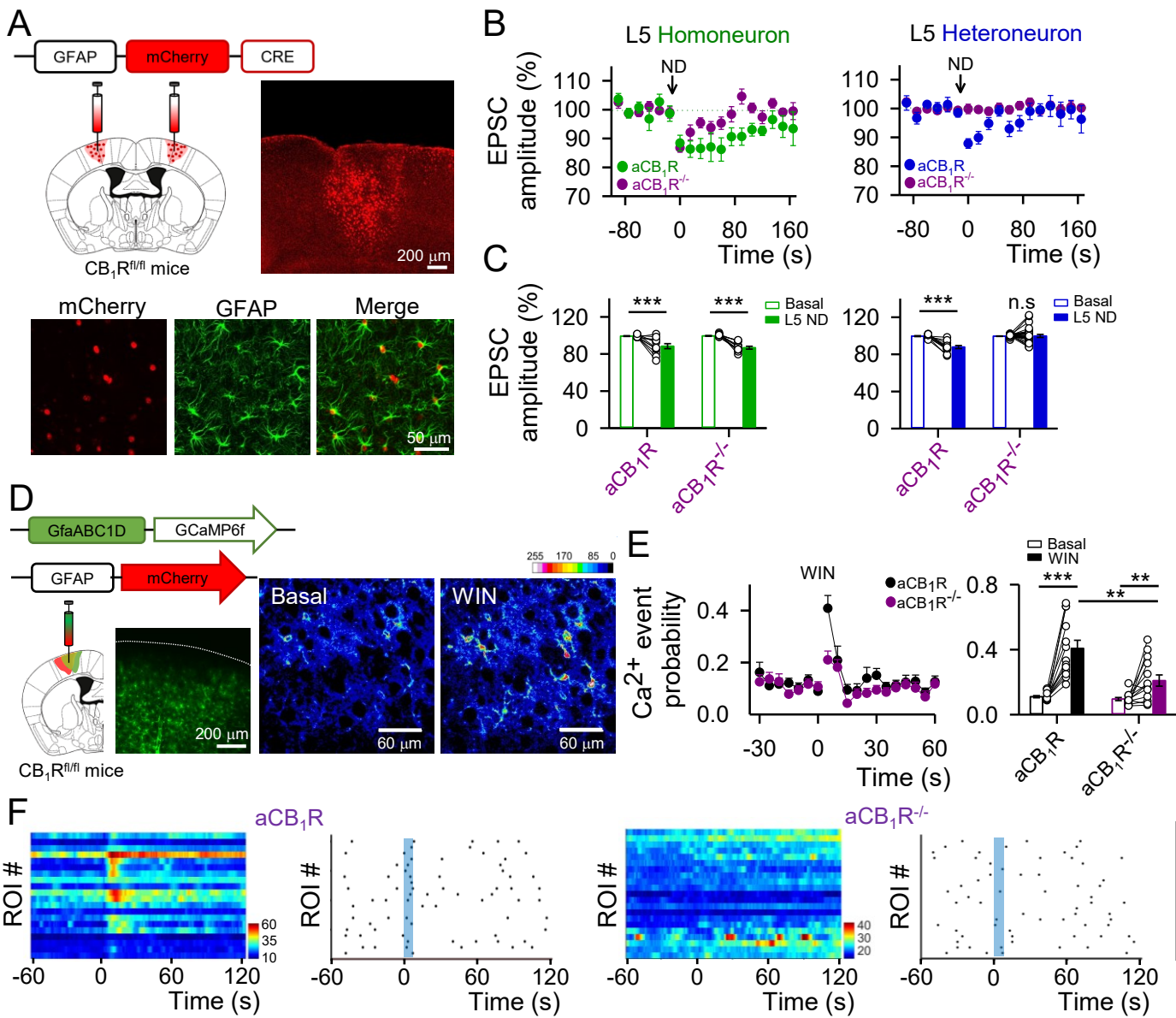


Figure 2. Heteroneuronal, but not homoneuronal, synaptic depression requires endocannabinoid signaling in astrocytes

(A) Viral vector injected into the S1 of CB₁R^{fl/fl} mice and fluorescence image showing mCherry-Cre expression in the S1 (top), and immunohistochemistry images showing co-localization between mCherry-cre and GFAP (bottom). (B) EPSCs amplitude *versus* time before (basal) and after ND in CB₁R mice injected with AAV8-GFAP-mCherry (aCB₁R; green or blue) or with AAV8-GFAP-mCherry-Cre (aCB₁R^{-/-}; purple) in the homoneuron (left) and heteroneuron (right) from L5. (C) Relative changes in EPSC amplitude in aCB₁R and aCB₁R^{-/-} mice in the homoneuron (left) and heteroneuron (right). Two-tailed Student's paired t test. (D) Viral vector injected into the S1 of CB₁R^{fl/fl} mice, fluorescence image showing GCaMP6f expression in the S1 and pseudocolor images showing the fluorescence intensities of GCaMP6f-expressing astrocytes before and after WIN (300 μ M) application in L5. (E) Ca²⁺ event probability over time (left) and Ca²⁺ event probability before (basal) and after WIN application in aCB₁R (black) and aCB₁R^{-/-} (purple) mice (right). Blue shadow indicates 5s WIN application. Two-tailed Student's paired t test (before and after) and two-tailed Student's unpaired t test (between groups). (F) Raster plots and heat maps showing the Ca²⁺ events recorded from all ROIs including astrocyte somas and processes in aCB₁R (left) and aCB₁R^{-/-} (right) mice before and after WIN stimulation. Blue shadow indicates 5s WIN application. Data are expressed as mean \pm SEM, **p < 0.01, ***p < 0.001.

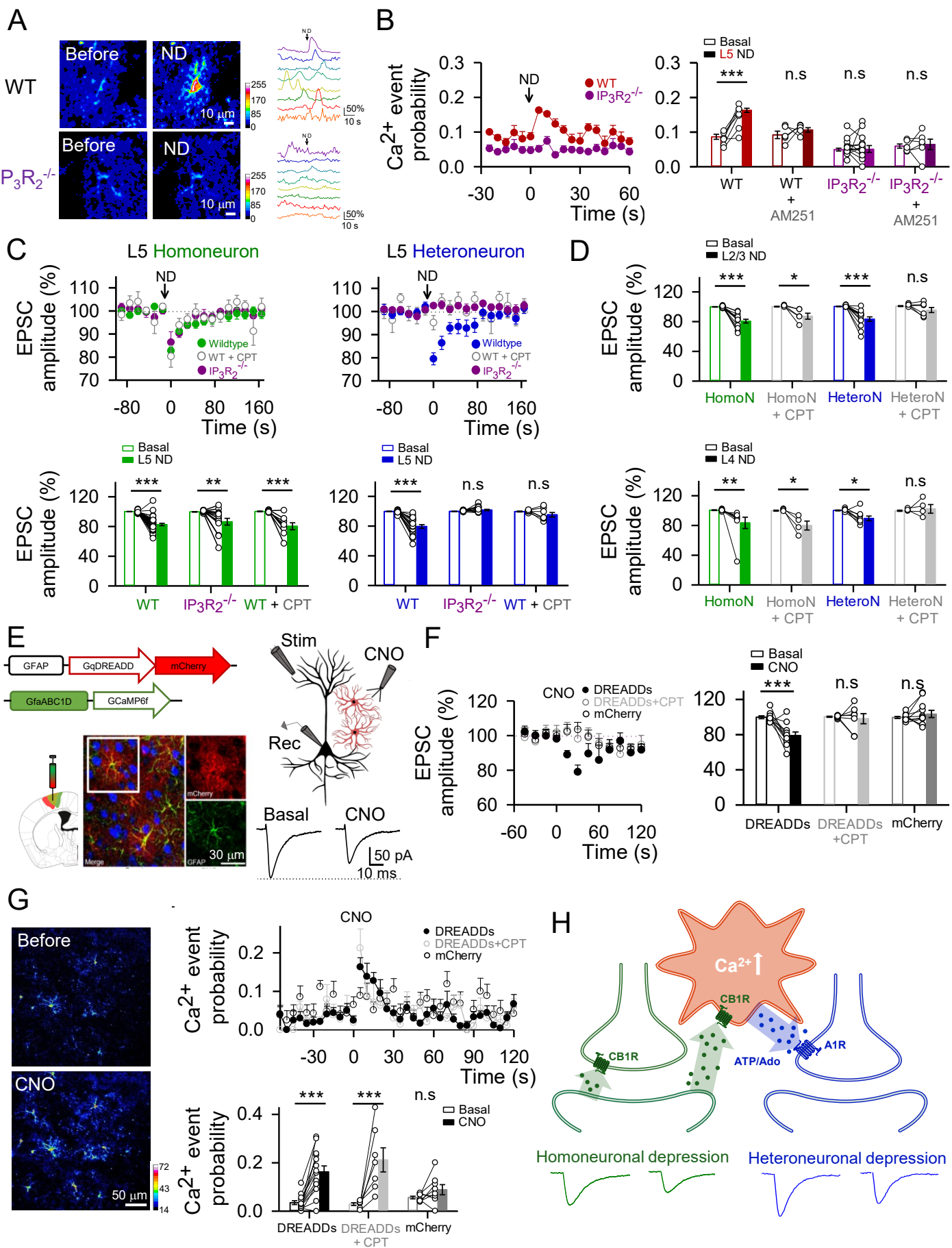
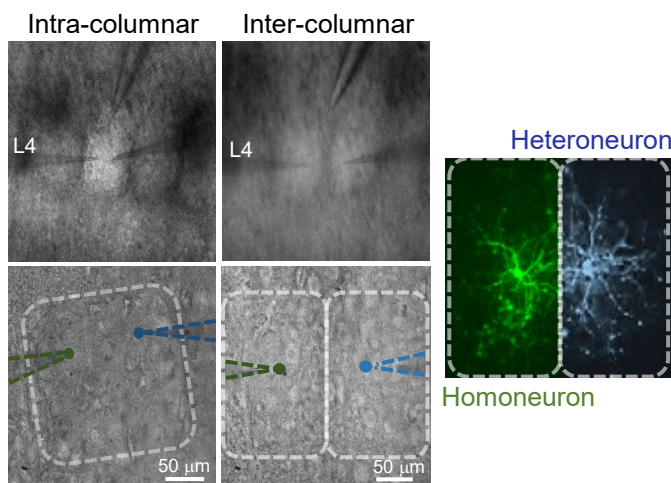


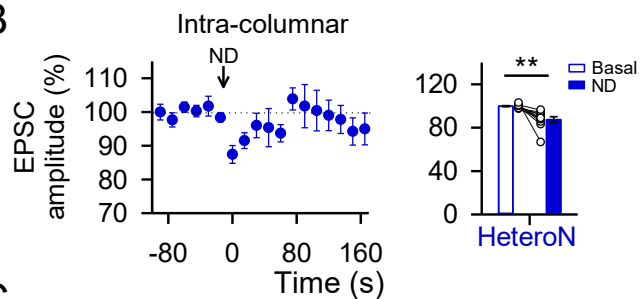
Figure 3. Heteroneuronal synaptic depression requires astrocyte calcium signaling and activation of presynaptic A1 receptors

(A) Left: Pseudocolor images showing the fluorescence intensities of GCaMP6f-expressing astrocytes in L5 before and after L5 ND in wildtype (top) and $IP_3R_2^{-/-}$ mice (bottom). Right: representative Ca^{2+} traces of astrocytes (arrow indicates ND). (B) Left: L5 astrocytes Ca^{2+} event probability over time before (basal) and after L5 ND in wildtype (red) and $IP_3R_2^{-/-}$ (purple) mice. Right: Relative changes in Ca^{2+} event probability in wildtype and $IP_3R_2^{-/-}$ mice in control and with AM251 (2 μ M). All experimental conditions were performed in TTX (1 μ M) and in a cocktail of neurotransmitter receptor antagonists (see Material and Methods). Two-tailed Student's paired t test. (C) Top: EPSCs amplitude *versus* time before (basal) and after ND in wildtype mice in control (green or blue), in presence of CPT (5 μ M) (open gray) and in $IP_3R_2^{-/-}$ mice (purple) in the homoneuron (left) and heteroneuron (right) from layer 5. Bottom: Relative changes in EPSC amplitude in wildtype mice in control, in presence of CPT (5 μ M) and in $IP_3R_2^{-/-}$ mice. Two-tailed Student's paired t-test. (D) Top: Relative changes in EPSC amplitude before (basal) and after L2/3 ND in control and with CPT (5 μ M) in the homoneuron (green) and heteroneuron (blue) from layer 2/3. Bottom: Relative changes in EPSC amplitude before (basal) and after L4 ND in control and with CPT (5 μ M) in the homoneuron (green) and heteroneuron (blue) from layer 4. Two-tailed Student's paired t test. (E) Left: Viral vectors injected into the S1 of wildtype mice and immunohistochemistry images showing the expression of NeuN (blue), mCherry (red) and GFAP (green) in the somatosensory cortex slices of a DREADDs injected mouse. Note the selective expression of hm3D-mCherry in astrocytes. Right: Scheme of the experimental approach and representative EPSC traces before (basal) and after CNO (1mM) application in L5. (F) Left: EPSCs amplitude *versus* time before (basal) and after CNO application in AAV8-GFAP-Gq-DREADD-mCherry injected mice in control (black, close) and in presence of CPT (gray, open), and in AAV8-GFAP-mCherry injected mice (black, open). Blue shadow indicates 5s CNO application. Right: Relative changes in EPSC amplitude in DREADDs injected mice in control and in presence of CPT, and in mCherry injected mice. Two-tailed Student's paired t test. (G) Left: Pseudocolor images showing the fluorescence intensities of GCaMP6f-expressing astrocytes before and after CNO application in L5. Top right: Ca^{2+} event probability over time of L5 astrocytes before (basal) and after CNO application in AAV8-GFAP-Gq-DREADD-mCherry injected mice in control (black, close) and in presence of CPT (gray, open), and in AAV8-GFAP-mCherry injected mice (black, open). Blue shadow indicates 5s CNO application. Bottom right: relative changes in Ca^{2+} event probability in DREADDs injected mice in control and in presence of CPT, and in mCherry injected mice. Two-tailed Student's paired t test. (H) Schematic summary depicting the signaling pathways involved in eCBs-induced heteroneuronal synaptic depression. Data are expressed as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

A



B



C

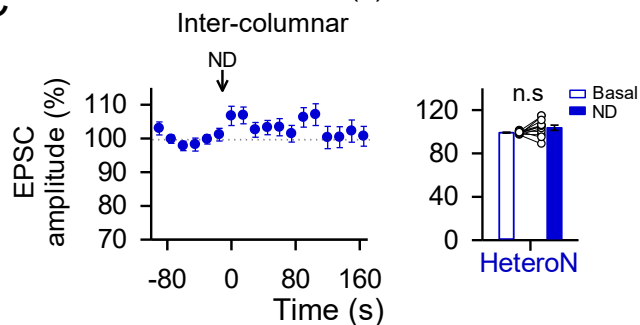


Fig. 4

Figure 4. Astrocyte-mediated heterosynaptic depression is column specific.

(A) Representative infrared differential interference contrast images of the barrel field in the primary somatosensory cortex showing intracolumnar (left) and intercolumnar (middle) pair of neurons patched and the stimulation electrode. False-color biocytin loading barrel cortex intercolumnar pair of neurons image (right). (B) Left: EPSCs amplitude *versus* time before (basal) and after ND in the intracolumnar heteroneuron. Right: Relative changes in EPSC amplitude in the intracolumnar heteroneuron. (C) Left: EPSCs amplitude *versus* time before (basal) and after ND in the intercolumnar heteroneuron. Right: Relative changes in EPSC amplitude in the intercolumnar heteroneuron. Two-tailed Student's paired t test. Data are expressed as mean \pm SEM, ** $p < 0.01$.

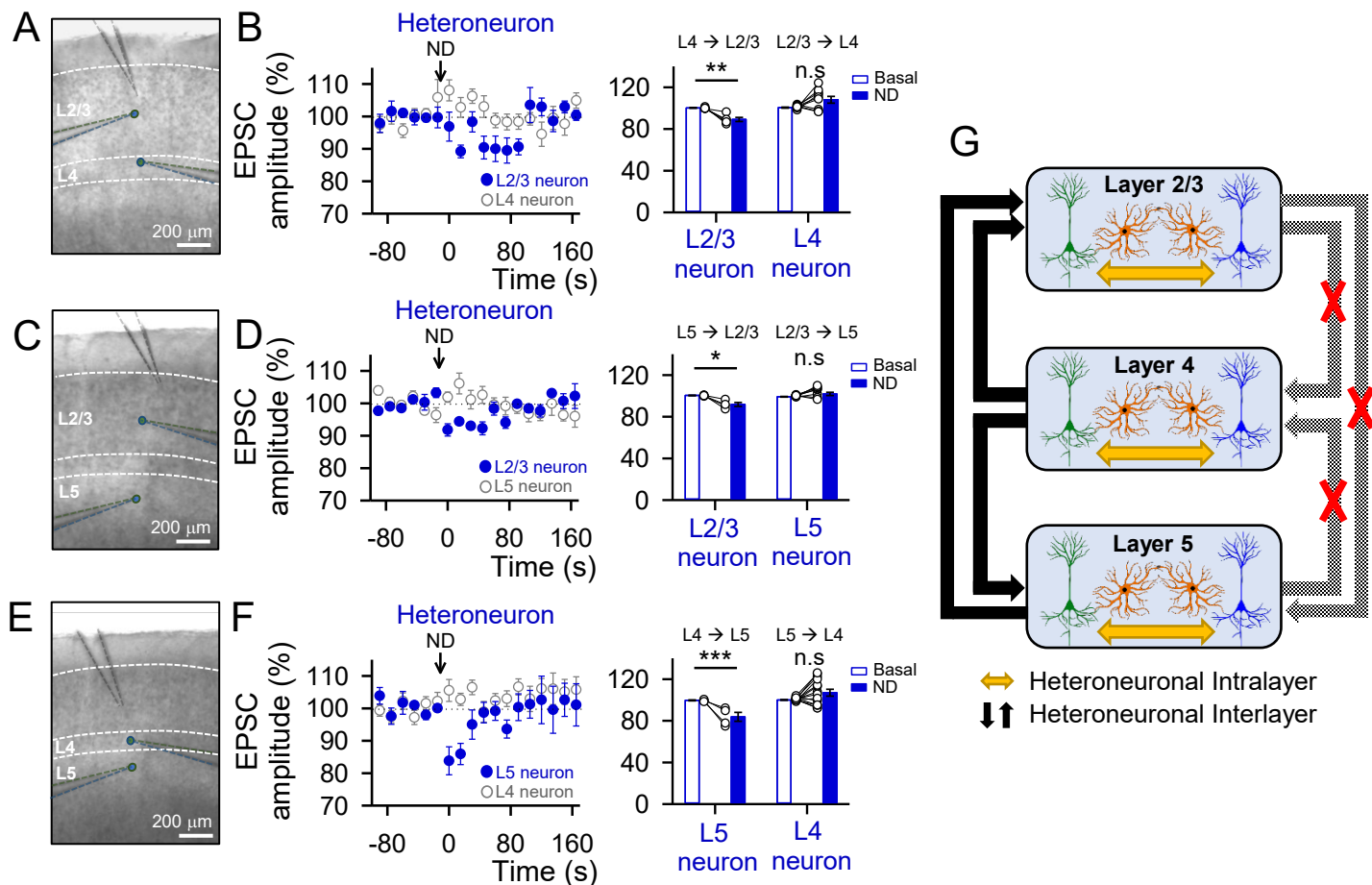


Figure 5. Astrocyte-mediated heterosynaptic depression is layer specific.

(A) Representative infrared differential interference contrast image of the experimental configuration with the stimulation pipette in layer 2/3 and a pair of neurons patched one in L4 and the other in L2/3. (B) Left: Heterosynaptic EPSCs amplitude versus time in a pair of neurons patched one in L4 and the other in L2/3 before (basal) and after ND of L4 (blue) or L2/3 neuron (open grey). Right: Relative changes in EPSC amplitude in L2/3 (when L4 neuron is stimulated) and L4 (when L2/3 neuron is stimulated) neuron. Two-tailed Student's paired t-test. (C) Representative infrared differential interference contrast image of the experimental configuration with the stimulation pipette in layer 2/3 and a pair of neurons patched one in L5 and the other in L2/3. (D) Left: Heterosynaptic EPSCs amplitude versus time in a pair of neurons patched one in L5 and the other in L2/3 before (basal) and after ND of L5 (blue) or L2/3 neuron (open grey). Right: Relative changes in EPSC amplitude in L2/3 (when L5 neuron is stimulated) and L5 (when L2/3 neuron is stimulated) neuron. Two-tailed Student's paired t-test. (E) Representative infrared differential interference contrast image of the experimental configuration with the stimulation pipette in layer 2/3 and a pair of neurons patched one in L4 and the other in L5. (F) Left: Heterosynaptic EPSCs amplitude versus time in a pair of neurons patched one in L4 and the other in L5 before (basal) and after ND of L4 (blue) or L5 neuron (open grey). Right: Relative changes in EPSC amplitude in L5 (when L4 neuron is stimulated) and L4 (when L5 neuron is stimulated) neuron. Two-tailed Student's paired t test. (G) Schematic summary depicting the astrocyte-mediated heterosynaptic regulation pathways into the same layer (intralayer) and between layers (interlayer). Data are expressed as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

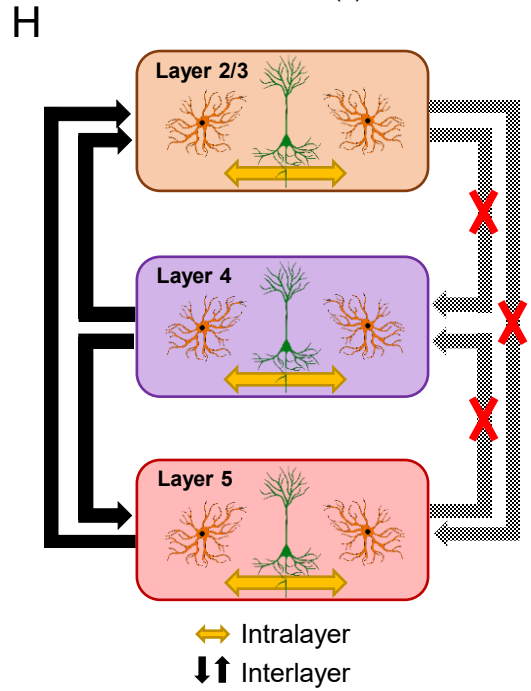
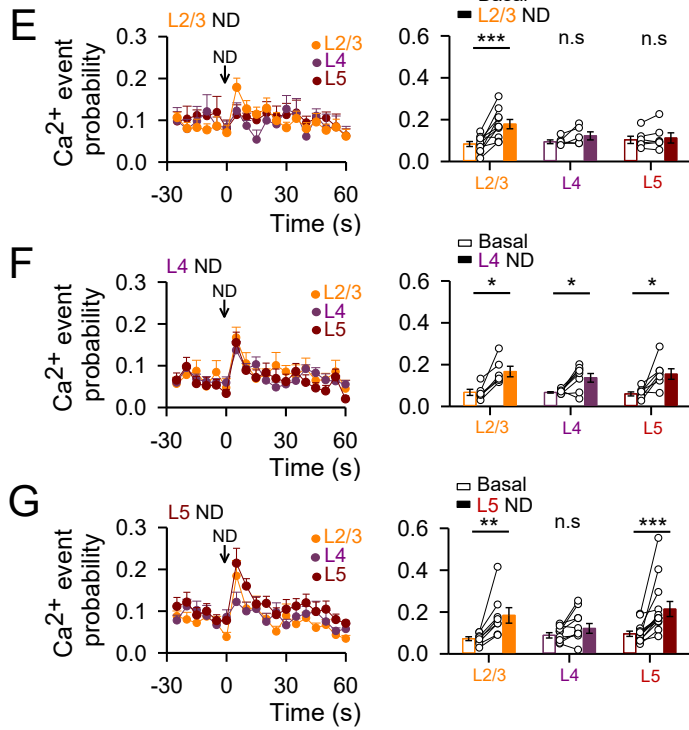
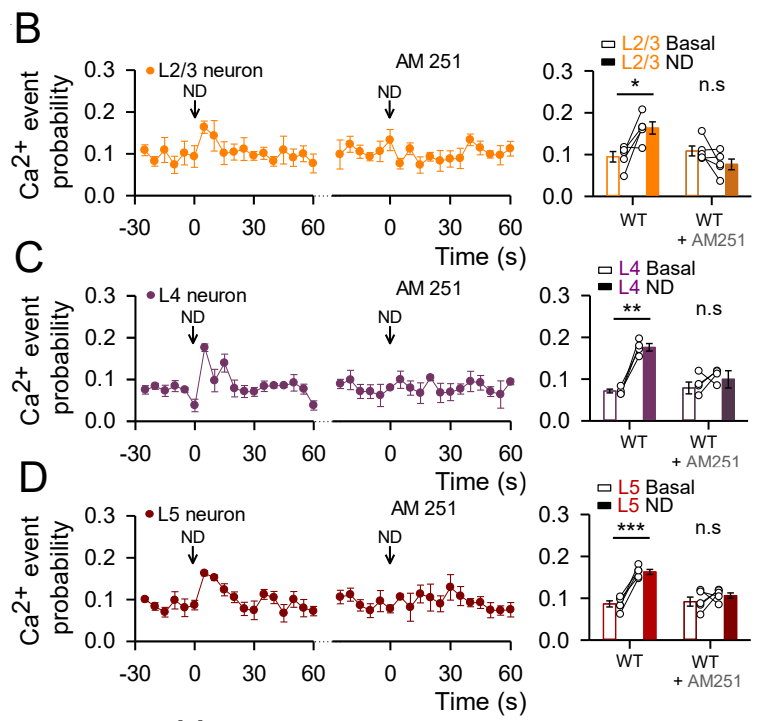
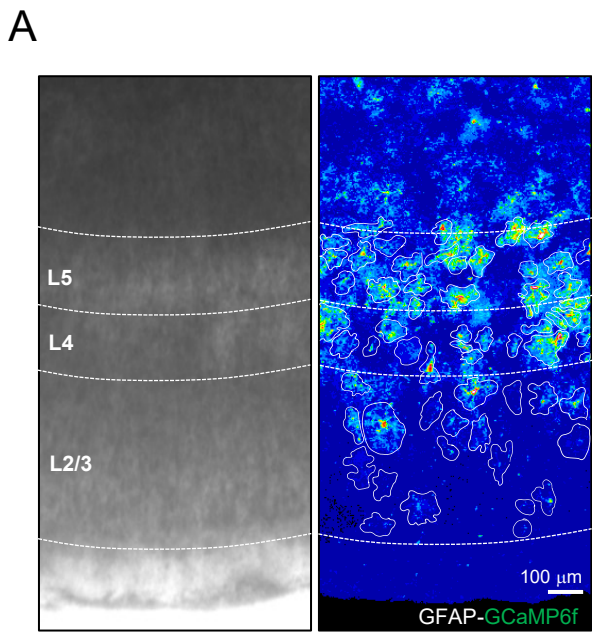


Figure 6. Astrocytic calcium responses to eCBs are not homogeneous across cortical layers.

(A) Representative infrared differential interference contrast image and pseudocolor image representing fluorescence intensities of GCaMP6f-expressing astrocytes in the different layers of the primary somatosensory cortex. (B-D) Left: Ca^{2+} event probability over time before (basal) and after ND in control and in presence of AM251 (2 μM) when patched and recorded in L2/3 (B, orange), L4 (C, purple) or L5 (D, red). Right: relative changes in Ca^{2+} event probability in control and in presence of AM251 (2 μM) when patched and recorded in L2/3 (B, orange), L4 (C, purple) or L5 (D, red). Two-tailed Student's paired t test. (E) Left: Ca^{2+} event probability over time of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red) before (basal) and after L2/3 neuron depolarization. Right: relative changes in Ca^{2+} event probability of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red). Two-tailed Student's paired t test. (F) Left: Ca^{2+} event probability over time of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red) before (basal) and after L4 neuron depolarization. Right: relative changes in Ca^{2+} event probability of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red). Two-tailed Student's paired t test. (G) Left: Ca^{2+} event probability over time of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red) before (basal) and after L5 neuron depolarization. Right: relative changes in Ca^{2+} event probability of astrocytes of layer 2/3 (orange), 4 (purple) and 5 (red). Two-tailed Student's paired t test. All experimental conditions were performed in TTX (1 μM) and in a cocktail of neurotransmitter receptor antagonists (see Material and Methods). (H) Schematic summary depicting the calcium responses of astrocytes located in the same (intralayer) or different (interlayer) layers to the endogenous mobilized eCBs from neurons located in the same or different layers. Data are expressed as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.