

1 **Title: Axonal CB1 receptors mediate inhibitory**
2 **bouton formation via cAMP increase**

3

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32

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34 The authors declare no competing financial interests.

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36

37 **Abstract**

38 Experience-dependent formation and removal of synapses are essential throughout
39 life. For instance, GABAergic synapses are removed to facilitate learning, and strong
40 excitatory activity is accompanied by formation of inhibitory synapses. We recently
41 discovered that active dendrites trigger the growth of inhibitory synapses via CB1
42 receptor-mediated endocannabinoid signaling, but the underlying mechanism
43 remained unclear. Using two-photon microscopy to monitor the formation of
44 individual inhibitory boutons, we found that CB1 receptor activation mediated
45 formation of inhibitory boutons and promoted their subsequent stabilization.
46 Inhibitory bouton formation did not require neuronal activity and was independent of
47 $G_{i/o}$ protein signaling, but was directly induced by elevating cAMP levels using forskolin
48 and by activating G_s proteins using DREADDs. Our findings reveal that axonal CB1
49 receptors signal via unconventional downstream pathways and that inhibitory bouton
50 formation is triggered by an increase in axonal cAMP levels. Our results demonstrate
51 a novel role for axonal CB1 receptors in axon-specific, and context-dependent,
52 inhibitory synapse formation.

53 – 155 words

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59 Introduction

60 Synaptic plasticity, the strengthening and weakening of existing synapses, is often
61 considered the physiological basis for learning and adaptation. In addition, the
62 experience-dependent formation and removal of synapses is equally important (Bailey
63 and Kandel, 1993; Caroni et al., 2012). Changes in the number of synaptic connections
64 have been shown to be critical during learning *in vivo* (Bailey and Chen, 1989; Caroni
65 et al., 2012; Hofer et al., 2009; Kozorovitskiy et al., 2012; Ruediger et al., 2011) and
66 strongly determine postsynaptic function (Scholl et al., 2020). Plasticity of GABAergic
67 synapses is particularly important for shaping and controlling brain activity throughout
68 life (Chiu et al., 2019; Flores and Méndez, 2014; Herstel and Wierenga, 2021; Maffei et
69 al., 2017) and GABAergic dysfunction is associated with multiple brain disorders,
70 including schizophrenia and autism (Lewis et al., 2005; Mullins et al., 2016; Tang et al.,
71 2021). For example, the number of inhibitory synapses is rapidly adjusted during
72 learning (Bourne and Harris, 2011; Chen et al., 2015; Donato et al., 2015, 2013) or
73 when sensory input is lost (Keck et al., 2011) to facilitate plasticity at nearby excitatory
74 synapses. Vice versa, potentiation of excitatory synapses can trigger the formation of
75 inhibitory synapses to maintain a local balance (Bourne and Harris, 2011; Hu et al.,
76 2019; Knott et al., 2002). The formation, stabilization and removal of synapses likely
77 requires local context-dependent signaling mechanisms (Hu et al., 2019; Kirchner and
78 Gjorgjieva, 2019; Kleindienst et al., 2011; Niculescu et al., 2018; Nishiyama and Yasuda,
79 2015; Oh et al., 2016), but our current understanding of these processes, especially at
80 inhibitory synapses, is far from complete.

81

82 We recently discovered that strong, clustered activation of excitatory synapses along
83 dendrites of hippocampal CA1 pyramidal neurons can trigger the formation of a new
84 inhibitory bouton onto the activated dendrite (Hu et al., 2019). We proposed that this
85 dendritic mechanism serves to maintain local balance between excitatory and
86 inhibitory inputs during ongoing synaptic plasticity. Inhibitory bouton formation
87 required dendritic endocannabinoid synthesis and activation of CB1 receptors (Hu et
88 al., 2019). Dendritic endocannabinoids are well-known to serve as retrograde signals
89 to regulate synaptic plasticity (Alger, 2002; Castillo et al., 2012; Chevaleyre and Castillo,
90 2003; Kano et al., 2009; Katona and Freund, 2012), but it is unclear how CB1 receptors
91 can trigger new inhibitory bouton formation.

92

93 CB1 receptors are G-protein coupled receptors and are widely abundant in the brain.
94 They are expressed in both excitatory and inhibitory neurons, as well as in glia cells
95 (Bonilla - Del Río et al., 2021; Hebert-Chatelain et al., 2016; Maroso et al., 2016;
96 Navarrete et al., 2014). Perhaps the most prominent CB1 expression is in a subset of

97 inhibitory axons in the dendritic layer of the hippocampal CA1 area (Bonilla-Del Río et
98 al., 2021; Dudok et al., 2015). Axonal CB1 signaling plays an important role during axon
99 guidance (Argaw et al., 2011; Berghuis et al., 2007; Njoo et al., 2015; Roland et al.,
100 2014), but axonal CB1 receptor expression remains high during adulthood. The best
101 described actions of CB1 receptors in adulthood is to suppress neurotransmitter
102 release (Alger, 2002; Castillo et al., 2012; Kano et al., 2009). However, CB1 receptors
103 are not enriched in boutons, but freely diffuse within the entire axonal membrane
104 (Dudok et al., 2015). It is possible that axonal CB1 receptors may function as
105 replacement pool for internalized synaptic receptors at boutons as recently suggested
106 for opioid receptors (Jullié et al., 2020), although synaptic enrichment would still be
107 expected. In addition, GABA release at dendritic inhibitory synapses is not strongly
108 modulated by CB1 receptors (Lee et al., 2015, 2010), and coupling between CB1
109 receptors and the active zone is weak (Dudok et al., 2015). This suggests that CB1
110 receptors in inhibitory axons serve an additional purpose. Interestingly, it was recently
111 described that CB1 receptors can also mediate synaptic potentiation (Cui et al., 2016;
112 Monday and Castillo, 2017; Wang et al., 2017). Although CB1 receptors typically signal
113 via $G_{i/o}$ -proteins, many additional downstream pathways, both dependent and
114 independent of G-proteins, have been described (Berghuis et al., 2007; Cui et al., 2016;
115 Flores-Otero et al., 2014; Glass and Felder, 1997; Roland et al., 2014; Zhou et al., 2019).
116
117 Here, we demonstrate that activation of axonal CB1 receptors can trigger the initial
118 formation of inhibitory synapses. Using two-photon time lapse imaging we observed
119 the formation of inhibitory boutons upon brief application of the CB1 receptor agonist
120 WIN. We demonstrate that this requires the presence of CB1 receptors on inhibitory
121 axons. Furthermore, we found that CB1-mediated inhibitory bouton formation is
122 independent of $G_{i/o}$ protein signaling and neuronal activity. We find that new inhibitory
123 boutons are formed in response to elevated cAMP levels or activation of G_s protein
124 signaling in inhibitory axons. Our data indicate that activation of axonal CB1 receptors
125 triggers inhibitory synapse formation via an atypical signaling pathway via G_s -proteins.
126 Furthermore, our data identify an increase in axonal cAMP as a crucial second
127 messenger for mediating inhibitory bouton formation.

128 Results

129 Repeated CB1 receptor activation increases functional presynaptic terminals

130 We previously demonstrated that new inhibitory boutons can form in response to brief
131 CB1 receptor activation (Hu et al., 2019). Newly formed boutons often did not persist
132 (Hu et al., 2019), suggesting that additional or repeated signaling is required to
133 eventually form functional inhibitory synapses (Frias et al., 2019; Wierenga, 2017). It
134 was recently reported that strong, but brief, CB1 receptor activation can induce

135 synaptic potentiation, while longer CB1 activation induces synaptic depression (Cui et
136 al., 2016, 2015). This suggests that CB1 activation pattern is an important factor in
137 determining its downstream signaling. We therefore sought to employ repeated, short
138 activation of CB1 receptors in order to induce the formation of inhibitory synapses. We
139 activated CB1 receptors in hippocampal slice cultures by repeated short exposure to
140 the CB1 receptor ligand 2-AG (100 μ M; 3 times 20 minutes with 2 hours interval) (Fig.
141 1A). We recorded miniature inhibitory postsynaptic currents (mIPSCs) in CA1
142 pyramidal neurons to assess functional inhibitory synapses 24 hours after the start of
143 the first 2-AG exposure (Fig. 1B). Repeated CB1 receptor activation resulted in an
144 increase of the mean mIPSC frequency by 38% (control: 3.9 ± 0.3 Hz; 2-AG: 5.5 ± 0.4
145 Hz, $p=0.013$; Fig 1C), while mIPSC amplitudes were not affected (Fig. 1D). Continuous
146 exposure to 2-AG for 24 hours did not alter frequency or amplitude of spontaneous
147 IPSCs (Fig. 1E,F), consistent with the notion that activation pattern determines CB1
148 downstream signaling. Interestingly, mIPSCs after repeated 2-AG exposure appeared
149 to have longer rise times (Fig. 1G), while decay times were not different (Fig. 1H). We
150 separated mIPSCs with slow and fast rise times based on a double Gaussian fit of the
151 distribution of rise times (Fig. 1I). When we then analyzed the interevent intervals of
152 fast and slow mIPSCs separately, we observed that the interevent intervals of slow
153 mIPSCs were decreased after repeated CB1 activation, while the interevent intervals
154 of fast mIPSCs were not affected (Fig. 1J,K). This analysis revealed that the observed
155 increase in mIPSC frequency was due to a specific increase in the frequency of slow
156 mIPSCs with long rise times (Fig. 1L). The rise time of mIPSCs depends on synaptic
157 maturation (Gonzalez-Burgos et al., 2015; Lazarus and Josh Huang, 2011; Pardo et al.,
158 2018), but is also strongly influenced by subcellular location, as dendritic filtering
159 attenuates mIPSCs originating from dendritic inhibitory synapses (Bekkers and
160 Clements, 1999; Rall, 1967; Wierenga and Wadman, 1999). This suggests that the
161 increased mIPSC frequency after CB1 receptor activation may reflect an increase of
162 inhibitory currents from dendritic locations, or from immature synapses.

163 To determine if the observed increase in mIPSCs was associated with an increase in
164 the number of inhibitory synapses, we analyzed presynaptic VGAT and postsynaptic
165 gephyrin puncta in the dendritic region of the CA1 area in parallel
166 immunohistochemistry experiments (Fig. 2A). We observed that the density of VGAT
167 puncta was slightly increased after repeated 2-AG application (Fig. 2B), while the VGAT
168 puncta size was decreased (Fig. 2C). Gephyrin puncta density and size were not
169 affected by repeated 2-AG exposure (Fig. 2D,E), and the density of inhibitory synapses,
170 defined as VGAT-gephyrin associations, was also not different from control slices (Fig.
171 2F,G). We therefore made a distinction between VGAT puncta that were associated
172 with gephyrin and VGAT puncta without gephyrin (Fig. 2A, last panel). We observed
173 that the increase in VGAT density was due to a specific increase in VGAT puncta that
174 were not associated with gephyrin (Fig. 2H). In contrast, the reduction in VGAT puncta
175 size was mostly due to a reduction in size of VGAT puncta with gephyrin association
176 (Fig. 2I). This suggests that repeated short activation of CB1 receptors has two

177 separable effect on inhibitory synapses: on the one hand it leads to shrinkage of VGAT
178 clusters at inhibitory synapses, possibly reflecting synaptic depression (Monday et al.,
179 2020), while at the same time new VGAT clusters are formed which are not associated
180 with the postsynaptic scaffold gephyrin. Live imaging experiments have shown that
181 VGAT is rapidly recruited when new boutons are formed in inhibitory axons, and that
182 gephyrin normally follows within a few hours (Dobie and Craig, 2011; Frias et al., 2019;
183 Wierenga et al., 2008). Our data suggest that repeated CB1 receptor activation induces
184 the formation of presynaptic VGAT clusters, likely reflecting immature inhibitory
185 synapses.

186 **Acute activation of CB1 receptors affects non-persistent boutons density only slightly**

187 To get further insight in the role of CB1 receptors in the formation of inhibitory
188 synapses, we performed two-photon live imaging in organotypic hippocampal slices to
189 monitor GFP-labeled inhibitory bouton dynamics in response to short activation of CB1
190 receptors. Here we used short applications (5 minutes) of CB1 receptor agonists to
191 mimic retrograde endocannabinoid signaling (Hu et al., 2019), but we wanted to avoid
192 inducing synaptic weakening (Monday et al., 2020). We used the endogenous CB1
193 receptor ligand 2-AG as well as the chemically synthesized agonist WIN552121-2 (WIN),
194 which is widely used because of its high affinity and stability (Chevalleyre et al., 2007;
195 Roland et al., 2014; Wang et al., 2017). We verified that brief WIN application only
196 transiently and mildly suppressed inhibitory currents (data not shown). As previously
197 reported (Frias et al., 2019), the majority of inhibitory boutons were present at all
198 timepoints during the 140 minutes imaging period (persistent boutons), but a
199 substantial fraction of inhibitory boutons appeared, disappeared, or reappeared,
200 during the imaging period (Fig 3A) (Frias et al., 2019; Schuemann et al., 2013). We will
201 refer to the latter as non-persistent (NP) boutons. Bath application of 100 μ M 2-AG (5
202 minutes) did not affect overall bouton density (control: 30.8 ± 1.7 boutons per 100 μ m;
203 2-AG: 29.8 ± 1.7 boutons per 100 μ m, $p=0.81$). The density of NP boutons appeared
204 slightly increased after 2-AG compared to DMSO control (Fig. 3B,C), but this was mainly
205 due to a large effect in a single axon. We calculated for each axon the average fraction
206 of NP boutons that are present over time (NP presence). In control slices there was a
207 small decrease in NP presence over time, possibly reflecting a decrease in network
208 activity level when the slices are transferred from the incubator to the microscope.
209 After 2-AG application NP presence appeared slightly more stable (Fig. 3D), but this
210 difference did not reach statistical significance. We assessed if this difference could be
211 traced back to a more specific effect in a particular subgroup of NP boutons (see
212 methods and (Frias et al., 2019)), but we could not detect any differences in the
213 densities of NP bouton subgroups in slices treated with control DMSO or 2-AG (Fig. 3E).
214 There was also no difference in bouton duration (data not shown).
215 The endocannabinoid 2-AG is rather unstable in solution and gets rapidly degraded in
216 biological tissue (Dócs et al., 2017; Savinainen et al., 2012). To exclude the possibility
217 that 2-AG gets degraded before it can activate CB1 receptors, we repeated these

218 experiments using 20 μ M WIN. Short activation (5 minutes) of CB1 receptors by bath
219 application of WIN slightly increased in NP bouton density (Fig. 3F,G). Although the
220 increase appeared more robust compared to the 2-AG-induced effect, the effect was
221 too small to reach statistical significance. Similar to 2-AG, the average NP presence
222 appeared slightly increased (Fig. 3H), but we could not detect any changes in specific
223 NP boutons subgroups (Fig. 3I). Together these observations indicate that short CB1
224 receptor activation by 2-AG or WIN leads to only a small (if any) increase in NP bouton
225 density in GFP-labeled inhibitory axons.

226 Endocannabinoids are produced on demand in postsynaptic neurons (Alger and Kim,
227 2011; Hashimoto et al., 2013; Piomelli, 2014), but an ambient level of
228 endocannabinoids is always present, even in slices (Lee et al., 2015; Lenkey et al., 2015;
229 Szabó et al., 2014). Tonic CB1 receptor activation by endocannabinoids affects mostly
230 perisomatic inhibitory synapses, while dendritic inhibitory synapses are reported to be
231 less sensitive (Lee et al., 2015, 2010). To address if tonic activation of CB1 receptors
232 could have obscured the effects of CB1 receptor activation on inhibitory bouton
233 dynamics in our GFP-labeled axons (which mostly target dendrites (Wierenga et al.,
234 2010)), we applied 5 μ M AM251, an antagonist of CB1 receptors. However, AM251 had
235 no effect on NP bouton density (Fig. 3J,K), NP presence or NP bouton subgroups (Fig.
236 3L,M).

237 Together our experimental findings indicate that inhibitory bouton dynamics of the
238 GFP-labeled axons are not under strong tonic endocannabinoid control and that short
239 CB1 receptor activation by 2-AG or WIN only slightly increases NP inhibitory bouton
240 density.

241 **CB1 receptors regulate inhibitory bouton dynamics specifically in CB1R+ axons**

242 The expression of CB1 receptors largely overlaps with the expression pattern of CCK in
243 GABAergic interneurons (Katona et al., 2006, 1999). These interneurons are partially
244 labeled in the GAD65-GFP mice which we use for our experiments (Wierenga et al.,
245 2010). We previously estimated that ~50% of the GFP-labeled inhibitory axons express
246 CB1 receptors in our slices (Hu et al., 2019), and this may significantly dilute an effect
247 of CB1 receptor activation on bouton dynamics (Fig. 3). We therefore used *post-hoc*
248 immunostaining immediately after two-photon live imaging to distinguish between
249 axons with and without CB1 receptors (CB1R+ and CB1R- axons respectively; Fig. 4A,
250 B). In accordance with previous reports (Dudok et al., 2015; Mikasova et al., 2008), CB1
251 receptors covered the entire surface of CB1R+ inhibitory axons and individual CB1R+
252 axons could be easily traced from the CB1 immunostainings (Fig. 4A, B). In addition,
253 there was significant CB1 background staining, presumably reflecting CB1 receptors in
254 pyramidal cells and glia cells (Bonilla-Del Río et al., 2021). CB1R- axons had a higher
255 bouton density and higher bouton turnover compared to CB1R+ axons (Fig. 4C; see
256 below), supporting the notion that CB1R+ and CB1R- GFP-labeled axons belong to
257 separate subtypes of GABAergic cells.

258

259 We repeated the WIN application experiments, but now separately analyzed CB1R+
260 and CB1R- axons. In CB1R+ axons the density of NP boutons significantly increased
261 after WIN application (Fig. 5A,B). WIN also increased average NP presence compared
262 to control axons (Fig. 5C). When we analyzed the NP bouton subgroups we found a
263 specific increase in the density of new and stabilizing boutons (Fig. 5D,F), whereas
264 other NP subgroups were unaffected (Fig. 5D-H). New boutons reflect immature
265 synapses, which start to recruit pre- and postsynaptic proteins, while levels of VGAT
266 and gephyrin at stabilizing boutons at the end of the imaging period is comparable to
267 persistent boutons (Frias et al., 2019; Schuemann et al., 2013). In clear contrast, WIN
268 had no effect on bouton density or dynamics in CB1R- axons in the same slices (Fig. 5I-
269 P). These results indicate that axonal CB1 receptors are required for mediating the
270 WIN-induced changes in bouton dynamics in inhibitory axons, and exclude a role for
271 CB1 receptors on other cells. Our results indicate that short activation of axonal CB1
272 receptors leads to an increase in NP bouton density by specifically promoting the
273 formation and stabilization of inhibitory boutons.
274

275 **WIN-induced bouton formation does not require $G_{i/o}$ signaling and neuronal activity**

276 CB1 receptors are G-protein coupled receptors. Endocannabinoid signaling via CB1
277 receptors typically activates $G_{i/o}$ heterotrimeric proteins, resulting in a reduction of
278 neurotransmitter release at presynaptic terminals (Castillo et al., 2012; Lovinger, 2008).
279 We therefore tested whether WIN-induced bouton formation requires $G_{i/o}$ signaling.
280 We pretreated the slices with pertussis toxin (PTX) (1 μ g/ml) for 24 hours to eliminate
281 $G_{i/o}$ signaling (Campbell and Smrcka, 2018; Guo and Ikeda, 2004), and then performed
282 two-photon time-lapse live imaging as before. Axons with and without CB1R were
283 distinguished using *post-hoc* immunostaining (Fig. 6A-C). PTX pretreatment had no
284 major effect on CB1 receptor expression patterns.

285 Under control conditions, CB1R- axons had a higher bouton density compared to
286 CB1R+ axons (Fig. 6D), which was mainly due to a higher density of NP boutons (Fig.
287 6E,F). The density for all NP bouton subgroups was almost twice as high in CB1R- axons
288 compared to CB1R+ axons (Fig. 6G), showing that overall inhibitory bouton dynamics
289 were more pronounced in CB1R- axons compared to CB1R+ axons. Unexpectedly, we
290 observed that 24 hr pretreatment with PTX affected bouton density. PTX pretreatment
291 specifically downregulated bouton density in CB1R- axons, while bouton density in
292 CB1R+ axons was largely unaffected (Fig. 6D). PTX specifically reduced the density of
293 non-persistent boutons in CB1R- axons (Fig. 6E,F). After PTX pretreatment there was
294 no longer a difference in NP bouton subgroups between CB1R+ and CB1R- inhibitory
295 axons (Fig. 6H). This suggests that under normal conditions CB1R- axons have a higher
296 $G_{i/o}$ protein activity compared to CB1R+ axons in these slices. These data imply that
297 $G_{i/o}$ signaling is an important regulator of inhibitory bouton dynamics.
298

299 We then tested whether acute activation of CB1 receptors via WIN can induce changes

300 in inhibitory bouton dynamics in the absence of $G_{i/o}$ signaling. We observed that short
301 activation of CB1 receptors by WIN could still induce the formation of new inhibitory
302 boutons in CB1R+ axons after pretreatment with PTX (Fig. 7A). This indicates that the
303 formation of new inhibitory boutons by CB1 receptor activation is independent of $G_{i/o}$
304 signaling. However, in the absence of $G_{i/o}$ signaling WIN application no longer
305 promoted bouton stabilization (Fig. 7B; compare with Fig. 5F), suggesting that bouton
306 stabilization requires intact $G_{i/o}$ signaling. As before, other NP bouton subgroups were
307 not affected (Fig. 7C) and WIN application did not affect bouton formation (density of
308 new boutons was 81 ± 23 % of control; MW, $p = 0.51$) or bouton dynamics (data not
309 shown) in CB1R- axons. These data indicate that short activation of CB1 receptors on
310 inhibitory axons by WIN promotes the formation of new boutons via a $G_{i/o}$ -
311 independent signaling pathway.

312
313 $G_{i/o}$ protein signaling can hyperpolarize neurons via activation of K^+ channels (Bacci et
314 al., 2004; Guo and Ikeda, 2004). Blocking ongoing $G_{i/o}$ activity with PTX may therefore
315 enhance neuronal activity in our slices, which may by itself affect inhibitory bouton
316 dynamics. However, as enhancing neuronal activity is expected to promote overall
317 inhibitory bouton turnover (Frias et al., 2019; Schuemann et al., 2013), this does not
318 appear in line with the observed decrease in inhibitory bouton dynamics in CB1R-
319 axons after PTX. To address if WIN-induced inhibitory bouton formation is affected by
320 activity, we blocked network activity with TTX to reduce overall bouton dynamics (Frias
321 et al., 2019; Schuemann et al., 2013). We observed that in the presence of TTX, brief
322 activation of CB1 receptors with WIN still induced the specific increase in the density
323 of new boutons (Fig. 7D). However, WIN did no longer induce a change in the density
324 of stabilizing boutons (Fig. 7E), consistent with our earlier finding that inhibitory
325 bouton stabilization requires activity (Frias et al., 2019). Other NP bouton subgroups
326 were not affected (Fig. 7F) and WIN application did not significantly affect bouton
327 formation (179 ± 216 % of control; MW, $p = 0.11$) or other bouton dynamics (data not
328 shown) in CB1R- axons. Together these data demonstrate that CB1 receptor-mediated
329 inhibitory bouton formation does not require $G_{i/o}$ protein signaling and is independent
330 of neuronal activity.

331 **Acute elevation of cAMP levels promotes inhibitory bouton formation**

332 Besides the typical downstream signaling pathway via $G_{i/o}$ proteins, CB1R activation
333 can trigger several other signaling pathways, including via $G_{12/13}$ (Roland et al., 2014),
334 G_q (Lauckner et al., 2005) and G_s proteins (Finlay et al., 2017; Glass and Felder, 1997).
335 Intriguingly, a novel form of CB1 receptor-mediated synaptic potentiation was recently
336 reported, which was shown to depend on presynaptic PKA activity (Cui et al., 2016;
337 Wang et al., 2017). This raises the attention to CB1 receptor-mediated G_s signaling, as
338 G_s protein signaling enhances PKA activity via stimulation of cAMP production (Antoni,
339 2012; Taylor et al., 2013). We therefore tested if inhibitory bouton dynamics were
340 affected when we directly elevated cAMP levels via activation of adenylyl cyclase (AC)

341 by 25 μ M forskolin (5 minutes) (Fig. 8A). We observed that brief application of forskolin
342 induced the formation of new inhibitory boutons (Fig. 8B), while other NP subgroups
343 were not affected (Fig. 8C,D). This suggests that the inhibitory bouton formation that
344 we observed after CB1 receptor activation may be mediated by G_s signaling. The
345 increase in inhibitory bouton formation after forskolin application appeared much
346 stronger compared to WIN application (compare Fig. 8B to Fig. 3I), suggesting that
347 most, if not all, GFP-labeled inhibitory axons responded to forskolin. These data show
348 that the formation of new inhibitory boutons is promoted by increasing intracellular
349 cAMP levels via AC stimulation.

350 **G_s signaling in inhibitory axons promotes inhibitory bouton formation**

351 Bath application of forskolin strongly increases neuronal activity (Gekel and Neher,
352 2008; Mitoma and Konishi, 1996) and will raise cAMP levels in all cells in the slice. The
353 observed specific increase in new inhibitory bouton formation after forskolin, without
354 affecting overall bouton dynamics, is therefore quite remarkable. However, we cannot
355 conclude that the observed increase in inhibitory bouton formation is a direct effect
356 of elevated cAMP levels in the inhibitory axons. We made use of DREADDs (Designer
357 Receptors Exclusively Activated by Designer Drugs) (Roth, 2016; Urban and Roth, 2015)
358 to achieve cell-specific manipulation of presynaptic cAMP levels. G_s -DREADDs allow
359 the direct activation of the G_s -protein signaling pathway using the specific ligand CNO.
360 To achieve sparse expression restricted to inhibitory neurons we infected hippocampal
361 slices from VGAT-Cre mice with Cre-dependent AAVs. We used two AAVs: one
362 containing a HA-tagged G_s -DREADD construct and one containing GFP (Fig. 9A; see
363 methods for details). Infections with these two AAVs resulted in sparse GFP-labeling of
364 inhibitory cells and their axons, which partially overlapped with G_s -HA expression (Fig.
365 9B). *Post-hoc* immunostaining allowed us to identify GFP-labeled axons with and
366 without G_s -HA (HA+ and HA- axons) in the same slice (Fig. 9C,D). We performed two-
367 photon microscopy to monitor bouton dynamics in GFP-expressing HA+ and HA-
368 inhibitory axons (Fig. 9E). Bouton dynamics in VGAT-Cre slices were in line with
369 previous data (Frias et al., 2019), indicating that the AAV infections did not alter overall
370 bouton dynamics in inhibitory axons. After a 40-minute baseline period, G_s -DREADDs
371 were activated via bath application of CNO ligand. We found that CNO activation
372 strongly increased the density of new boutons in G_s -HA positive axons compared to
373 HA- axons (Fig. 9F). Other NP bouton subgroups were not affected, although the
374 density of stabilizing boutons appeared to be somewhat increased (Fig. 9G,H). These
375 data show that specific activation of G_s signaling in inhibitory axons mimics the WIN-
376 induced inhibitory bouton formation.

377 Together, our results indicate that inhibitory bouton formation after brief CB1 receptor
378 activation does not require $G_{i/o}$ -signaling, and that it is mimicked by activation of G_s
379 signaling in inhibitory axons. This suggests that CB1 receptors on inhibitory axons
380 couple to G_s proteins rather than the conventional $G_{i/o}$ effectors to trigger inhibitory
381 bouton formation.

382

383

384 **Discussion**

385 Here we examined the signaling pathway underlying the CB1 receptor-mediated
386 formation of new inhibitory synapses. We made several important observations. First
387 of all, repeated CB1 activation led to an increase in mIPSC frequency and an increase
388 in the density of presynaptic VGAT clusters, which were not associated with
389 postsynaptic gephyrin. Inhibitory synapses which do not contain gephyrin are
390 immature and show reduced transmission (Danglot et al., 2003; Nguyen et al., 2016;
391 Niwa et al., 2012; Patrizi et al., 2008; Yu et al., 2007). Our observations are in line with
392 the notion that pre- and postsynaptic signaling pathways during synapse formation are
393 largely independent (Jiang et al., 2021; Wierenga, 2017) and suggest that CB1
394 receptors act purely presynaptically. Second, brief activation of CB1 receptors
395 specifically triggered the formation of inhibitory synapses in CB1R+ axons. This
396 indicates that formation of inhibitory synapses is mediated by axonal CB1 receptors
397 and excludes a prominent role for CB1 receptors in astrocytes or postsynaptic neurons.
398 Third, bouton turnover in inhibitory axons was strongly reduced when $G_{i/o}$ protein
399 signaling was blocked by PTX pretreatment. This suggests that modulation of axonal
400 cAMP levels is an important regulator of bouton turnover in inhibitory axons. Fourth,
401 CB1 receptor-mediated inhibitory bouton growth was independent of ongoing $G_{i/o}$
402 signaling and activity, suggesting that signaling pathways downstream of axonal CB1
403 receptors differ from presynaptic CB1 receptors. Finally, inhibitory synapse formation
404 was induced in response to an increase in cAMP after forskolin application, and when
405 G_s signaling was activated via G_s -DREADDs, which were expressed exclusively in
406 inhibitory neurons. These findings revealed that an increase in cAMP is the key second
407 messenger signal for inhibitory bouton formation and suggest that axonal CB1
408 receptors trigger inhibitory bouton formation via G_s instead of $G_{i/o}$ protein signaling.

409

410 Our present study has limitations that are important to mention here. First of all, we
411 use transgenic mice in which several inhibitory neuron subtypes are labeled with GFP
412 (Wierenga et al., 2010). This unspecific labeling diluted and hampered the detection
413 of axon-specific effects (Fig. 2). However, we used it to our advantage by performing
414 posthoc immunostaining to distinguish between different inhibitory axon types. This
415 allowed comparison between CB1R+ and CB1R-, or HA+ and HA- axons in the same
416 slice and avoided comparison between slices from different GFP-labeled mouse lines.
417 Another limitation of our study is that we have used bath application of CB1 agonist
418 WIN to trigger inhibitory bouton formation. Under physiological conditions,
419 endocannabinoid signals are likely transient and highly localized (Hashimotodani et al.,
420 2007; Hu et al., 2019; Monday and Castillo, 2017), providing spatial and temporal
421 control over inhibitory synapse formation. We elevated cAMP levels to trigger
422 inhibitory bouton formation by bath application of forskolin or by activation of G_s -

423 DREADDs in inhibitory cells. While this allowed separation of formation and
424 stabilization of inhibitory boutons, it likely abolished spatial modulations. Axons
425 contain several phosphodiesterases, which rapidly degrade cAMP and provide
426 spatiotemporal compartmentalization of cAMP signaling (Argyrousi et al., 2020; Baillie,
427 2009). Pretreatment with PTX will disturb these cAMP modulations and this strongly
428 reduced inhibitory bouton dynamics and abolished the difference between CB1R+ and
429 CB1R- axons (Fig. 6L). This indicates that CB1R- axons have higher $G_{i/o}$ baseline activity
430 compared to CB1R+ axons and suggests that cAMP modulation is an important factor
431 regulating inhibitory bouton dynamics. Future research should further assess the
432 relationship between cAMP signaling and inhibitory bouton turnover.

433

434 Synapse formation is a multistep process, with each step regulated by specific signaling
435 pathways (Jiang et al., 2021; Wierenga, 2017). Our detailed two-photon analysis allows
436 dissecting these steps and addressing the involved signaling pathways. Inhibitory
437 synapse formation starts with the growth of a new bouton at an axonal location where
438 the inhibitory axon is in close proximity to a dendrite (Dobie and Craig, 2011; Hu et al.,
439 2019; Villa et al., 2016; Wierenga et al., 2008). Our data indicate that axonal CB1
440 receptors can trigger bouton formation, which does not require neuronal activity. We
441 observed that CB1 receptor-mediated inhibitory bouton formation was not affected in
442 the presence of TTX (Fig. 7D). In addition, we observed that forskolin, which strongly
443 raises neuronal activity (data not shown), did not affect overall bouton turnover (Fig.
444 8D). This was unexpected given our previous observations that inhibitory bouton
445 turnover is enhanced by neuronal activity (Frias et al., 2019; Schuemann et al., 2013).
446 On the other hand, we observed that blocking $G_{i/o}$ signaling strongly affected bouton
447 turnover (Fig. 6F,H), which appeared independent of activity. These data suggest that
448 axonal cAMP is the primary second messenger affecting inhibitory bouton formation,
449 which is indirectly modulated by activity, possibly via changes in neuromodulatory
450 signals.

451 Our data indicate that axonal CB1 receptors can directly trigger bouton formation via
452 an increase in cAMP, while subsequent bouton stabilization and postsynaptic assembly
453 requires additional signaling. WIN-induced bouton stabilization was prevented when
454 $G_{i/o}$ signaling was blocked by PTX (Fig. 7B), and bouton stabilization was not altered by
455 increasing cAMP levels with forskolin (Fig. 8C), although it may be facilitated with
456 longer elevations (Fig. 9H). These data suggest that after the initial formation, CB1
457 receptors may also promote bouton stabilization via a more indirect pathway. We
458 previously showed that bouton stabilization requires neuronal activity and involves
459 local actin remodeling via a reduction in ROCK activity (Frias et al., 2019). Interactions
460 between CB1 receptor signaling and ROCK activity (Berghuis et al., 2007) and actin
461 remodeling (Njoo et al., 2015; Zhou et al., 2019) have been reported, but future
462 research should further clarify the precise nature of these interactions.

463

464 CB1 receptors are highly versatile and are involved in many neuronal processes via

465 multiple downstream pathways, including axon guidance and synaptic plasticity
466 (Araque et al., 2017; Berghuis et al., 2007; Monday and Castillo, 2017; Njoo et al., 2015;
467 Roland et al., 2014). There are multiple factors, including interacting proteins
468 (Guggenhuber et al., 2016), which determine which downstream signaling pathway is
469 activated after CB1 receptor activation (Flores-Otero et al., 2014; Nogueras-Ortiz and
470 Yudowski, 2016) and this functional selectivity of CB1 receptors may have important
471 clinical relevance (Ibsen et al., 2017; Laprairie et al., 2017; Sholler et al., 2020). It was
472 recently reported that the duration of CB1 receptor activation determines the
473 direction of plasticity at corticostriatal synapses with brief activation inducing LTP,
474 while prolonged activation induces LTD (Cui et al., 2016, 2015). Our data suggest that
475 brief activation of axonal CB1 receptors promotes the formation of new inhibitory
476 boutons via G_s -mediated elevation of cAMP levels, but we have not extensively tested
477 longer activations or different ligand concentrations. It is possible that the subcellular
478 location of CB1 receptors affects downstream signaling pathway: CB1 receptors at
479 presynaptic terminals couple to $G_{i/o}$ to affect GABA release (Guo and Ikeda, 2004; Lee
480 et al., 2015), while CB1 receptors in the axonal shaft of the same inhibitory axons may
481 couple to G_s proteins. Even though CB1 receptors prefer coupling to G_i -proteins, they
482 can switch to G_s when G_i -proteins are not available or already occupied (Caballero-
483 Florán et al., 2016; Eldeeb et al., 2016; Finlay et al., 2017; Glass and Felder, 1997). This
484 may suggest that G_i proteins are only available at presynaptic terminals, while G_s
485 protein coupling could be dominant in axons.

486
487

488 Our experiments do not address which effectors are downstream of cAMP to trigger
489 inhibitory bouton formation. The most prominent effector of cAMP is protein kinase A
490 (PKA). Presynaptic PKA activity is involved in CB1-mediated synaptic plasticity
491 (Chevalleyre et al., 2007; Cui et al., 2016) and also is therefore a strong candidate for
492 regulating presynaptic bouton formation. PKA may for instance alter local clustering
493 and inter-bouton exchange of synaptic vesicles (Chenouard et al., 2020; Patzke et al.,
494 2019). PKA resides close to the plasma membrane and preferably phosphorylates
495 membrane proteins in its close proximity (Tillo et al., 2017). However, potential PKA
496 targets to mediate inhibitory bouton formation remain yet to be identified. In addition,
497 there are important PKA-independent pathways downstream of cAMP signaling, most
498 importantly via Epac2 (Kawasaki et al., 1998). Epac2 activity can strongly increase
499 synaptic transmission (Fernandes et al., 2015; Gekel and Neher, 2008), yet a role in
500 synapse formation has not been reported. Interestingly, Epac2 was recently found to
501 be downstream of G_s -coupled β adrenergic receptors to mediate presynaptic LTP at
502 parallel fiber synapses to Purkinje cells (Martín et al., 2020). cAMP signaling via PKA,
503 Epac2 or Rho GTPases may affect the axonal cytoskeleton. Actin is important in the
504 formation, stabilization and maintenance of presynaptic terminals (Bednarek and
505 Caroni, 2011; Chenouard et al., 2020; Chia et al., 2014, 2013; Frias et al., 2019; Pielage
506 et al., 2011) and cAMP fluctuations may drive local modifications in the actin

507 cytoskeleton (Bernier et al., 2019) underlying structural presynaptic changes.
508
509 Our findings suggest that axonal CB1 receptors serve an important role in local, on
510 demand synapse formation. Our observation that inhibitory bouton formation was
511 more prominent after cAMP elevation than after WIN application (compare Fig. 8B and
512 9F to 3I) suggests that axonal cAMP signaling is an important second messenger signal
513 mediating bouton formation not only in CB1R+, but perhaps in all, inhibitory axons.
514 Intriguingly, our observations are reminiscent of cAMP-mediated bouton formation in
515 zebrafish (Yoshida and Mishina, 2005), *Aplysia* (Bailey and Kandel, 1993; Nazif et al.,
516 1991; Upreti et al., 2019) and *Drosophila* axons (Koon et al., 2011; Maiellaro et al.,
517 2016; Zhong et al., 1992). This raises the possibility that axonal cAMP signaling is a
518 universal second messenger system for regulating structural plasticity in axons.
519 Activation of CB1 receptors via dendritic endocannabinoid signaling (Hu et al., 2019)
520 then represents one specific way to trigger cAMP-mediated bouton formation in
521 CB1R+ axons in response to strong excitatory synaptic activity. Other axons may
522 employ different axonal receptors to mediate bouton formation. Indeed, GABAergic
523 interneurons express many different G-proteins (Cox et al., 2008; Helboe et al., 2015;
524 Puighermanal et al., 2017), which often provide neuromodulatory context signals from
525 other brain areas (Hattori et al., 2017). Our findings raise the intriguing possibility that
526 neuromodulatory receptors on the axonal surface provide the opportunity to build a
527 new inhibitory bouton on demand, triggered by axon-specific and context-dependent
528 signaling.
529
530
531

532 Figure legends

533 **Figure 1. Repeated CB1 receptor activation results in increased mIPSC frequency.**

534 **(A)** Organotypic hippocampal cultures were treated 3 times with culturing medium
535 containing 100 μ M 2-AG or DMSO (control) for 20 minutes with 2 hour intervals. After
536 24 hours, slices were used for electrophysiology and immunostaining experiments.

537 **(B)** Example traces of miniature inhibitory postsynaptic currents (mIPSCs) recordings
538 from control (black) and 2-AG treated slice (red).

539 **(C, D)** Mean frequency (C) and amplitude (D) of mIPSCs in control and 2-AG treated
540 slices (MW, $p = 0.013$ in C and $p = 0.16$ in D). Data from 22 cells in 6 control slices and
541 19 cells in 6 2-AG treated slices.

542 **(E, F)** Mean frequency (E) and amplitude (F) of sIPSCs in control and 2-AG treated
543 slices, when 2-AG was continuously present for 24 hr ($p = 0.99$ in E and $p = 0.95$ in F;
544 MW). Data from 11 cells in 5 control slices and 11 cells in 6 2-AG treated slices.

545 **(G)** Mean rise time of mIPSCs in control and 2-AG treated slices (MW, $p = 0.073$).

546 **(H)** Mean of mIPSC decay time in control and 2-AG treated slices (MW, $p = 0.19$).

547 **(I)** The distribution of rise times of mIPSCs was fitted with a double Gaussian to
548 separate fast and slow mIPSCs.

549 **(J,K)** Cumulative distribution of interevent intervals of mIPSCs with fast (J) and slow
550 (K) rise times (KS, $p = 0.65$ in J, and $p < 0.0001$ in K).

551 **(L)** Mean frequency of mIPSCs with fast and slow rise times (2w ANOVA Sidak, fast:
552 $p = 0.14$; slow: $p = 0.0095$).

553 Data in G-L and C,D are from the same data set.

554

555 **Figure 2. Repeated CB1 receptor activation induces the formation of partial** 556 **inhibitory synapses.**

557 **(A)** Representative immunostaining images showing the presynaptic VGAT (blue)
558 and postsynaptic gephyrin (purple) in control (upper) and 2-AG (lower) slices.
559 Individual VGAT puncta were identified using watershed segmentation and these were
560 color coded to distinguish VGAT puncta associated with gephyrin (blue) and VGAT
561 puncta without gephyrin (red).

562 **(B, C)** Normalized density (B) and size (C) of VGAT puncta in control and 2-AG slices
563 (MW, $p = 0.0061$ in B; $p = 0.004$ in C).

564 **(D, E)** Normalized density (D) and size (E) of gephyrin puncta in control and 2-AG
565 slices (MW, $p = 0.54$ in D; $p = 0.64$ in E).

566 **(F, G)** Normalized density (F) and size (G) of VGAT/gephyrin colocalizations in control
567 and 2-AG slices (MW, $p = 0.76$ in F; $p = 0.099$ in G).

568 **(H, I)** Normalized density (F) and size (G) of VGAT puncta with and without gephyrin
569 (2w ANOVA Sidak, $p = 0.55$ and $p = 0.003$ in H; $p = 0.017$ and $p = 0.65$ in I).

570 Data from 13 image stacks in 7 slices per group.

571

572

573 **Figure 3. Brief activation of CB1 receptors slightly increases NP bouton density**

574 **(A)** Representative two-photon time lapse images of GAD65-GFP labelled inhibitory
575 axons in the dendritic region of the hippocampal CA1 area (maximal projections of 17
576 z-sections). After a baseline of five time points (40 minutes), CB1 receptor agonist or
577 DMSO was washed in for 5 minutes. Imaging was continued for another ten time
578 points (total imaging period is 140 minutes). Persistent boutons (blue) and non-
579 persistent (NP) boutons (orange) are indicated by arrow heads. Empty arrow heads
580 reflect a NP bouton which was absent at the time point. Scale bar is 2 μm .

581 **(B)** CB1 receptors were activated by bath application of 100 μM 2-AG for 5 minutes.
582 Normalized NP bouton density over time in control (black) slices and after 2-AG (red)
583 application (2w ANOVA, $p = 0.33$).

584 **(C)** Maximum change in NP bouton density in control slices and after 2-AG
585 application (MW, $p = 0.54$).

586 **(D)** Normalized NP presence over time in control and 2-AG treated slices. P1= time
587 points 1 to 5, P2= time points 6 to 10, and P3= time points 11 to 15) in control and 2-
588 AG treated slices (2w ANOVA, $p = 0.61$).

589 **(E)** Mean density of NP bouton subgroups in control slices and after 2-AG application.
590 N – new boutons (MW, $p = 0.35$); L – lost boutons (MW, $p = 0.44$); S – stabilizing boutons
591 (MW, $p = 0.21$); D – destabilizing boutons (MW, $p = 0.91$); I – intermittent boutons (MW,
592 $p = 0.87$).

593 **(F)** CB1 receptors were activated by bath application of 20 μM WIN for 5 minutes.
594 Normalized NP bouton density over time in control (black) slices and after 2-AG (green)
595 application (2w ANOVA, $p = 0.20$).

596 **(G)** Maximum change in NP bouton density in control slices and after WIN
597 application (MW, $p = 0.11$).

598 **(H)** Normalized NP presence over time in control slices and after WIN application
599 (2w ANOVA, $p = 0.20$).

600 **(I)** Mean density of NP bouton subgroups in control slices and after WIN application
601 (MW, $p = 0.40$ (N); $p = 0.06$ (L); $p = 0.79$ (S); $p = 0.70$ (D); $p = 0.10$ (I)).

602 **(J)** Slices were treated with the CB1 receptor antagonist AM251 (5 μM) after time
603 point 5. Normalized NP bouton density over time in control (black) slices and during
604 AM251 (blue) application (2w ANOVA, $p = 0.66$).

605 **(K)** Maximum change in NP bouton density in control slices and during AM251
606 application (MW, $p = 0.6$).

607 **(L)** Normalized NP presence over time in control slices and during AM251 application
608 (2w ANOVA, $p = 0.56$).

609 **(M)** Mean density of NP bouton subgroups in control slices and during AM251
610 application (MW, $p = 0.46$ (N); $p = 0.23$ (L); $p = 0.94$ (S); $p = 0.29$ (D); $p = 0.10$ (I)).

611 Data in A from 24 axons in 6 control slices and 23 axons in 6 2-AG slices. Data in B from
612 24 axons in 7 control slices and 22 axons in 7 WIN slices. Data in C from 20 axons in 5
613 control slices and 20 axons in 5 AM251 slices.

614

615 **Figure 4. Distinction between CB1R+ and CB1R- axons using post hoc**
616 **immunohistochemistry**

617 **(A)** Z-projection of representative two-photon image of GFP-labeled inhibitory axons.
618 After two-photon live imaging, the slice was immediately fixated and further
619 processed for immunohistochemistry to assess CB1R expression.

620 **(B)** Confocal images of the same area after post hoc immunohistochemistry, showing
621 the same GFP-labeled axons (B1) as in A (indicated with solid and dashed red boxes).
622 Immunostaining against CB1 receptors (B2) show a clear distinction between CB1R+
623 axons (solid red box), which express CB1 receptors, which cover the entire axonal
624 surface, and CB1R- axons (dashed red box). Which do not express CB1 receptors.

625 **(C)** Two-photon time lapse imaging of bouton dynamics in the CB1R+ and CB1R-
626 axons indicated in A and B. Arrow heads indicate P (blue) and NP (orange) boutons as
627 in Fig. 3A.

628 Scale bars are 10 μm in A,B and 2 μm in C,D.

629

630 **Figure 5. WIN promotes formation and stabilization of inhibitory boutons only in**
631 **CB1R+ axons**

632 **(A)** Normalized NP bouton density in CB1R+ axons over time in control (black) slices
633 and after WIN (green) application (2w ANOVA, $p = 0.018$; interaction $p = 0.026$).

634 **(B)** Maximum change in NP bouton density in CB1R+ axons in control (black) slices
635 and after WIN (green) application (MW, $p = 0.047$).

636 **(C)** Normalized NP presence in CB1R+ axons over time in control slices and after WIN
637 application (2w ANOVA, $p = 0.022$; interaction $p = 0.045$).

638 **(D-H)** Mean density of NP bouton subgroups in CB1R+ axons in control slices and
639 after WIN application. D, new boutons (MW, $p = 0.002$); E, lost boutons (MW, $p = 0.39$);
640 F, stabilizing boutons (MW, $p = 0.005$); G, destabilizing boutons (MW, $p = 0.87$); H,
641 intermittent boutons (MW, $p = 0.16$).

642 **(I)** Normalized NP bouton density in CB1R- axons over time in control (black) slices
643 and after WIN (green) application (2w ANOVA, $p = 0.27$).

644 **(J)** Maximum change in NP bouton density in CB1R- axons in control (black) slices
645 and after WIN (green) application (MW, $p = 0.21$).

646 **(K)** Normalized NP presence in CB1R- axons over time in control slices and after WIN
647 application (2w ANOVA, $p = 0.37$).

648 **(L-P)** Mean density of NP bouton subgroups in CB1R- axons in control slices and after
649 WIN application. L, new boutons (MW, $p = 0.77$); M, lost boutons (MW, $p = 0.46$); N,
650 stabilizing boutons (MW, $p = 0.50$); O, destabilizing boutons (MW, $p = 0.99$); P,
651 intermittent boutons (MW, $p = 0.34$).

652 Data from 25 CB1R+ and 16 CB1R- axons in 4 control slices and 20 CB1R+ and 15 CB1R-
653 axons in 4 slices with WIN application.

654

655

656 **Figure 6. $G_{i/o}$ signaling is an important regulator of inhibitory bouton dynamics**
657 **(A)** Z-projection of representative two-photon image of GFP-labeled inhibitory axons
658 after PTX pretreatment.
659 **(B)** Confocal images of the same area after post hoc immunohistochemistry, showing
660 the same GFP-labeled axons (B1) as in A (solid and dashed red boxes indicate CB1R+
661 and CB1R- axons).
662 **(C)** Two-photon time lapse imaging of bouton dynamics in the CB1R+ and CB1R-
663 axons indicated in A and B after PTX pretreatment. Arrow heads indicate P (blue) and
664 NP (orange) boutons as in Fig. 3A.
665 **(D)** Average bouton density during baseline in CB1R+ and CB1R- axons in control
666 slices and after PTX pretreatment. Comparisons between CB1R+ and CB1R- axons: p
667 = 0.0056 for control, $p = 0.79$ after PTX; between control and PTX: $p = 0.11$ for CB1R-
668 axons, $p > 0.99$ for CB1R+ axons; between CB1R+ (control) and CB1R- (PTX): $p = 0.86$
669 and between CB1R- (control) and CB1R+ (PTX): $p = 0.0057$ (2w ANOVA Sidak).
670 **(E)** Average density of persistent (P) boutons during baseline in CB1R+ and CB1R-
671 axons in control slices and after PTX pretreatment ($p=0.057$ for axon type, 2w ANOVA
672 Sidak).
673 **(F)** Average density of non-persistent (NP) boutons during baseline in CB1R+ and
674 CB1R- axons in control slices and after PTX pretreatment. Comparisons between
675 CB1R+ and CB1R- axons: $p = 0.0007$ for control, $p = 0.99$ after PTX; between control
676 and PTX: $p < 0.0001$ for CB1R- axons, $p > 0.99$ for CB1R+ axons; between CB1R+
677 (control) and CB1R- (PTX): $p = 0.93$ and between CB1R- (control) with CB1R+ (PTX):
678 $p = 0.0008$ (2w ANOVA Sidak).
679 **(G)** Mean density of NP bouton subgroups in CB1R+ and CB1R- axons in control slices.
680 N – new boutons (MW, $p = 0.035$); L – lost boutons (MW, $p = 0.037$); S – stabilizing
681 boutons (MW, $p = 0.002$); D – destabilizing boutons (MW, $p = 0.47$); I – intermittent
682 boutons (MW, $p = 0.010$).
683 **(H)** Mean density of NP bouton subgroups in CB1R+ and CB1R- axons after PTX
684 pretreatment (MW, $p = 0.45$ (N); $p = 0.41$ (L); $p = 0.36$ (S); $p = 0.88$ (D); $p = 0.40$ (I)).
685 Data from 23 CB1R+ and 16 CB1R- axons in 4 control slices, and 18 CB1R+ and 21 CB1R-
686 axons in 4 PTX-pretreated slices. Scale bars are 10 μm in A,B and 2 μm in C,D.

687

688 **Figure 7. CB1-mediated bouton formation does not require $G_{i/o}$ signaling and is**
689 **independent of activity.**

690 **(A)** Mean density of new boutons in CB1R+ axons after control (black) and WIN
691 (green) application in PTX-pretreated slices (MW, $p = 0.047$).

692 **(B)** Mean density of stabilizing boutons in CB1R+ axons after control and WIN
693 application in PTX-pretreated slices (MW, $p = 0.93$).

694 **(C)** Mean density of other NP bouton subgroups in CB1R+ axons after control and
695 WIN application in PTX-pretreated slices. L – lost boutons (MW, $p = 0.82$); D –
696 destabilizing boutons (MW, $p = 0.37$); I – intermittent boutons (MW, $p = 0.59$).

697 **(D)** Mean density of new boutons in CB1R+ axons after control (black) and WIN

698 (green) application in the presence of TTX (MW, $p = 0.013$).
699 **(E)** Mean density of stabilizing boutons in CB1R+ axons after control and WIN
700 application in the presence of TTX (MW, $p = 0.61$).
701 **(F)** Mean density of other NP bouton subgroups in CB1R+ axons after control and
702 WIN application in the presence of TTX. L – lost boutons (MW, $p = 0.23$); D –
703 destabilizing boutons (MW, $p = 0.56$); I – intermittent boutons (MW, $p = 0.16$).
704 Data in A-C from 18 axons in 4 slices with DMSO (control) application and 18 axons in
705 4 slices with WIN application. Data in D-F from 14 axons in 4 slices with DMSO (control)
706 application and 15 axons in 5 slices with WIN application.

707

708 **Figure 8. Inhibitory bouton formation is promoted by increasing intracellular cAMP**
709 **levels with forskolin**

710 **(A)** Representative two-photon time lapse images of bouton dynamics in GFP-
711 labeled axons after control or forskolin application. Arrow heads indicate P (blue) and
712 NP (orange) boutons as in Fig. 3A. Scale bar is 2 μm .

713 **(B)** Mean density of new boutons in control (black) slices and after forskolin (blue)
714 application (MW, $p = 0.007$).

715 **(C)** Mean density of stabilizing boutons in control slices and after forskolin
716 application (MW, $p = 0.67$).

717 **(D)** Mean density of other subgroup of NP boutons in control slices and after
718 forskolin application. L – lost boutons (MW, $p = 0.46$); D – destabilizing boutons (MW,
719 $p = 0.37$); I – intermittent boutons (MW, $p = 0.81$).

720 Scale bars are 2 μm .

721

722 **Figure 9. Specific activation of Gs at inhibitory axons induce new bouton formation.**

723 **(A)** Experimental design. Hippocampal slice cultures are prepared from P7 VGAT-Cre
724 mouse pups. At DIV1 (days *in vitro*), AAV5-hSyn-DIO-EGFP and AAV5-hSyn-DIO-Gs-HA
725 viruses are applied to the VGAT-Cre slice cultures. After 2-3 weeks (DIV 14-21) slices
726 were used for two-photon live imaging and *post hoc* immunostaining to reveal Gs-HA
727 expression.

728 **(B)** Representative example of VGAT-Cre slice culture at DIV20 showing sparse
729 expression of GFP and Gs-HA in GABAergic cells. Right images (zoom from white box)
730 show Gs-HA and EGFP co-expression in a subset of neurons (red arrow heads).

731 **(C)** Z-projection of representative two-photon image of GFP-labeled inhibitory axons
732 in VGAT-Cre slice.

733 **(D)** Confocal images of the same area in C after *post hoc* immunohistochemistry
734 against HA, showing the same GFP-labeled axons as in A (solid and dashed red boxes
735 indicate HA+ and HA- axons).

736 **(E)** Two-photon time lapse imaging of bouton dynamics in the HA+ and HA- axons
737 indicated in C and D. Gs-DREADDs were activated by bath application of 10 μM CNO
738 after the 40 minutes baseline period. Arrow heads indicate P (blue) and NP (orange)
739 boutons as in Fig. 3A.

740 **(F)** Mean density of new boutons at HA+ and HA- axons in response to Gs-DREADD
741 activation (MW, $p = 0.003$).

742 **(G)** Mean density of stabilizing boutons at HA+ and HA- axons in response to Gs-
743 DREADD activation (MW, $p = 0.10$).

744 **(H)** Mean density of other subgroup of NP boutons at HA+ and HA- axons in response
745 to Gs-DREADD activation. L – lost boutons (MW, $p = 0.30$); D – destabilizing boutons
746 (MW, $p = 0.44$); I – intermittent boutons (MW, $p = 0.85$)

747 Data from 11 HA+ and 11 HA- axons in 4 slices. Scale bars are 200 μm (overview) and
748 20 μm (zoom) in B, 10 μm in C,D and 2 μm in E.

749

750 **Materials and Methods**

751 **Animals**

752 All animal experiments were performed in compliance with the guidelines for the
753 welfare of experimental animals issued by the Federal Government of The Netherlands.
754 All animal experiments were approved by the Animal Ethical Review Committee (DEC)
755 of Utrecht University.

756

757 **Mouse hippocampal slice culture**

758 Organotypic mouse hippocampal slices were acquired from female and male GAD65-
759 GFP mice at 6-7 days after birth. In these mice, ~20% interneurons are labelled by GFP
760 from early embryonic developmental stage into adulthood (López-Bendito et al., 2004).
761 Most GFP-labelled interneurons target dendrites of CA1 pyramidal cells and express
762 VIP or reelin, while parvalbumin and somatostatin-positive neurons are not labelled
763 (Wierenga et al., 2010). Slice culture preparation details are described previously (Frias
764 et al., 2019; Hu et al., 2019). Mice were sacrificed and the isolated hippocampus was
765 placed in ice-cold HEPES-GBSS (containing 1.5 mM CaCl₂·2H₂O, 0.2 mM KH₂PO₄, 0.3
766 mM MgSO₄·7H₂O, 5 mM KCl, 1 mM MgCl₂·6H₂O, 137 mM NaCl, 0.85 mM Na₂HPO₄ and
767 12.5 mM HEPES) supplemented with 12.5 mM HEPES, 25 mM glucose and 1 mM
768 kynurenic acid (pH set around 7.2, osmolarity set around 320 mOsm, sterile filtered).
769 Slices were vertically chopped along the long axis of hippocampus at thickness of 400
770 µm. They were then quickly washed with culturing medium (consisting of 48% MEM,
771 25% HBSS, 25% horse serum, 30 mM glucose and 12.5 mM HEPES, pH set at 7.3-7.4
772 and osmolarity set at 325 mOsm), and transferred to Millicell cell culture inserts
773 (Milipore) in 6-well plates. Slices were cultured in an incubator (35 °C, 5% CO₂) until
774 use. Culturing medium was completely replaced twice a week. Slices were used after
775 2 to 3 weeks *in vitro*, when the circuitry is relatively mature and stable (De Simoni et
776 al., 2003).

777

778 **Pharmacological treatments**

779 The following drugs were used: 20 µM WIN 55212-2 (WIN; Tocris Bioscience), 100 µM
780 2-AG (Tocris Bioscience), 25 µM forskolin (Abcam), Pertussis toxin 1 µg/ml (Tocris
781 Bioscience), 10 µM CNO (Tocris Bioscience), 5 µM AM251 (Tocris Bioscience). For acute
782 treatments, ACSF containing the drug or 0.1% DMSO vehicle was bath applied for 5
783 minutes. AM251 and CNO were bath applied after a baseline period (5 time points)
784 and continued until the end of the experiment. Pertussis toxin was added to the slice
785 culture medium and a small drop was placed on top of the slice 24 hours before the
786 start of the experiment. Treated slices were kept in the incubator.

787 CB1 receptor activation can result in different downstream signaling pathways, which
788 depend on ligand concentration and duration (Cui et al., 2016, 2015). We used a
789 relatively high concentration of WIN (20 µM) to aim for strong activation of CB1

790 receptors. We used short applications to mimic CB1 receptor activation under
791 physiological conditions (Hu et al., 2019) and to avoid the induction of synaptic
792 depression (Monday et al., 2020).

793 For repeated treatment of 2-AG, normal culturing medium was replaced by medium
794 containing 100 μ M 2-AG or 0.1% DMSO for 20 minutes. This was repeated 3 times with
795 2 hours intervals. At the start of each medium replacement, a small drop was placed
796 on top of the slices to facilitate exchange. A treatment duration of 20 minutes (rather
797 than 5) was chosen to ensure penetration in the entire slice as solution exchange may
798 be slower in the incubator compared to the microscope bath. After the last treatment,
799 the medium was replaced 3 times with fresh medium to ensure wash out. During and
800 after the treatment slices were kept in the incubator and experiments
801 (immunocytochemistry or electrophysiology) were performed 24 hours after the start
802 of the first treatment.

803

804

805 **Electrophysiology recording and analysis**

806 Slices were transferred to a recording chamber which was continuously perfused with
807 carbogenated artificial cerebrospinal fluid (ACSF; containing 126 mM NaCl, 3 mM KCl,
808 2.5 mM CaCl₂, 1.3 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 20 mM glucose and
809 1 mM Trolox) at 32 °C. Whole-cell patch clamp measurements were recorded with a
810 MultiClamp 700B amplifier (Molecular Devices) and stored using pClamp 10 software.
811 Recordings were filtered with a 3 kHz Bessel filter. Thick-walled borosilicate pipettes of
812 4–6 M Ω were filled with pipette solution containing (in mM): 70 K-gluconate, 70 KCl,
813 0.5 EGTA, 10 HEPES, 4 MgATP, 0.4 NaGTP, and 4 Na₂-phosphocreatine. Cells were
814 discarded if series resistance was above 35 M Ω or if the resting membrane potential
815 exceeded -50 mV. Recordings were excluded when the series resistance after the
816 recording deviated more than 30% from its original value. To isolate miniature
817 inhibitory postsynaptic currents (mIPSCs) TTX, AP5 and DNQX were added to the ACSF.
818 The mIPSCs were analyzed in pClamp and Matlab with homemade scripts (Ruiter et al.,
819 2020). Rise time of mIPSCs were determined as the time between 10% and 90% of the
820 peak value. The distribution of the rise times of mIPSCs recorded in control conditions
821 (generated from 150 mIPSCs per cell) were fitted with two Gaussians and their crossing
822 point determined the separation between fast and slow mIPSCs (Ruiter et al., 2020). A
823 double Gaussian fit for the rise time distribution in 2-AG conditions gave a similar
824 separation value (control: 0.9 ms; 2-AG: 1.1 ms) and we verified that our conclusions
825 did not change by taking the 2-AG separation value.

826

827

828 **Two-photon time lapse imaging**

829 Time-lapse two-photon imaging was performed in carbogenated, continuously
830 perfused ACSF at 32 °C. Slices were transferred in a 3 cm dish containing ACSF. Two-
831 photon imaging was performed on a customized two-photon laser scanning

832 microscope (Femto2D, Femtonics, Budapest, Hungary) with a Ti-Sapphire
833 femtosecond pulsed laser (MaiTai HP, Spectra-Physics) with a 60x water immersion
834 objective (Nikon NIR Apochromat, NA = 1.0). A 4x objective (Nikon Plan Apochromat)
835 was used to determine the location of the dendritic layer of the CA1 region. GFP was
836 excited at 910 nm to visualize GFP-labelled axons. 3D image stacks were acquired at a
837 size of 93.5 μm x 93.5 μm (1124 x 1124 pixels) with 50-63 z-steps (0.5 μm step size).
838 Acquisition time per image stack was \sim 7 minutes. We acquired image stacks every 10
839 minutes, with a total of 15 time points (140 minutes). After a baseline of 5 time points,
840 drugs were bath applied.

841 For slices in which we performed post-hoc immunostaining, an overview of the
842 imaging region was made after the last time point (203 μm x 203 μm , \sim 50 z-steps of
843 1.0 μm step size), and a line scar was made using high intensity laser power at 910 nm
844 at the edge of the zoomed out imaging area to facilitate alignment with post-hoc
845 confocal microscopy.

846

847

848 **Two-photon image analysis**

849 Individual axons with at least 50 μm length were traced using the CellCounter plugin
850 imbedded in Fiji for all time points (TPs). Individual boutons were identified with
851 custom-built semi-automatic Matlab software, as described previously (Frias et al.,
852 2019). In short, a 3D axonal intensity profile was reconstructed at each TP and
853 individual boutons were selected based on a local threshold (0.5 standard deviation
854 above mean axon intensity). Only boutons containing at least 5 pixels above threshold
855 were included. All boutons at all time points were visually inspected and manual
856 corrections were made if deemed necessary.

857

858 Persistent (P) boutons were defined as boutons which were present at all TPs. Non-
859 persistent (NP) boutons were absent at one or more TPs. Boutons which were present
860 for only one time point were considered transport events and were excluded (Frias et
861 al., 2019; Schuemann et al., 2013). Based on their presence or absence during baseline
862 (first 5 TP) and after treatment, NP boutons were further classified into five subgroups
863 (Frias et al., 2019; Ruitter et al., 2021): new boutons (only present after baseline), lost
864 boutons (only present during baseline), stabilizing boutons (non-persistent during
865 baseline, persistent after treatment), destabilizing boutons (persistent during baseline,
866 non-persistent after treatment), intermittent boutons (non-persistent during baseline
867 and after treatment).

868 Bouton density was calculated per axon as the average number of boutons at each TP
869 divided by the 3D axon length. NP bouton density was determined for each TP as the
870 number of NP boutons that were present divided by the 3D axon length. NP bouton
871 densities were normalized to the average baseline value (first 5 TP) to allow
872 comparison between axons. The maximum change in NP bouton density change was
873 calculated as the maximum NP bouton density (average over 3TPs) divided by the

874 baseline NP bouton density (average over TP2-4). NP presence was determined as the
875 fraction of NP boutons that were present at each time point and these values were
876 averaged for the first, second and third period of 5 TPs each. Changes in NP presence
877 reflect changes in the density of NP bouton subgroups, as well as in bouton duration.
878 However, differences in bouton duration (% of TPs present) of NP bouton subgroups
879 were never observed in any of the conditions and we therefore only report NP bouton
880 densities.

881

882 **Immunocytochemistry and confocal microscopy**

883 Fixation of the slices was performed in 4% paraformaldehyde (PFA) for 30 minutes at
884 room temperature covered by aluminum foil. Following washing with phosphate-
885 buffered saline (PBS; 3x 10 minutes), slices were permeabilized with 0.5% Triton X-100
886 (15 minutes), followed by PBS washing (3x 5 minutes), and 1 hour incubation in
887 blocking solution (0.2% Triton X-100 and 10% goat serum). Application of primary
888 antibodies in blocking solution was performed overnight at 4 °C. After PBS washing (3x
889 15 minutes), secondary antibodies were applied for 4 hours. After PBS washing (2x 15
890 minutes), slices were mounted in Vectashield solution.

891

892 We used the following primary antibodies: rabbit α -VGAT (Synaptic Systems, 131 003;
893 1:1000), mouse α -gephyrin (Synaptic Systems, 147 011; 1:1000), mouse α -CB1R
894 (Synaptic Systems, 258 011; 1:1000), rat α -HA (Roche, 11 867 423 001; 1:500), and the
895 following secondary antibodies: anti-mouse Alexa647 (Life Technologies, A21241, 1:500)
896 and anti-rabbit Alexa405 (Life Technologies, A31556, 1:250) for VGAT and gephyrin
897 staining, anti-mouse Alexa647 (Life Technologies, A21236, 1:500) and anti-mouse
898 Alexa568 (Life Technologies, A11031, 1:500) for CB1R staining, and anti-rat Alexa568
899 (Life Technologies, A11077, 1:500) for HA staining.

900

901 Confocal imaging was performed using a Zeiss LSM-700 microscope system with a 63x
902 oil-immersion objective. A 20x objective was used to find back the two-photon imaging
903 area based on the line scar. Image size was 101.3 μ m x 101.3 μ m (1024 x 1024 pixels)
904 with 0.3 μ m z steps for synapse quantification, and up to 203 μ m x 203 μ m for post-
905 hoc axon identification.

906 Confocal images were analyzed in Fiji and corresponding axons in the confocal and
907 two-photon images were identified using the line scar as a guide. Expression of CB1R
908 or HA was determined by visual inspection. In some cases, the image was mirrored to
909 confirm or reject positive staining. Negative axons were always chosen close to positive
910 axons in the same imaging area, assuring that the absence of CB1R or HA expression
911 was not due to low immunostaining quality. In addition, we verified that CB1R
912 expression or staining levels did not affect our conclusion as we found the same results
913 when we split CB1R+ axons in two separate groups with high and low CB1R levels. Per
914 slice, 2-6 axons per group were included in the analysis.

915 For synapse quantification images were analyzed in Fiji using a custom macro (Ruiter

916 et al., 2020). An average projection image was made from 5 z-planes, images were
917 median filtered (1 pixel radius) and individual puncta were identified using watershed
918 segmentation. VGAT and gephyrin puncta were analyzed separately and overlap was
919 determined afterwards. Four independent experiments were performed with 1 or 2
920 image areas per slice. To compare between treatments, data were normalized per
921 experiment.

922

923

924 **VGAT-Cre slice preparation and AAV virus injection**

925 Hippocampal slice cultures were prepared as described above from VGAT-Cre mice
926 (JAX stock #028862) at 6-7 days after birth. Floxed AAV5 viruses (DIO-EGFP, #v115-1,
927 DIO-Gs-HA, #v111-1; Viral Vector Facility, Zurich University) were applied at DIV1 on
928 top of the hippocampal CA1 region by a microinjector (Eppendorf, FemtoJet) aided by
929 a stereoscopic microscope (Leica, M80). This resulted in widespread, but sparse GFP
930 and Gs-HA expression in GABAergic neurons, which partially overlapped. Two-photon
931 time lapse imaging was performed when slices were kept 2 to 3 weeks *in vitro*. After a
932 baseline period (5 time points), Gs signaling was activated by bath application of 10
933 μM CNO (Tocris Bioscience), which was continued until the end of the experiment.
934 *Post hoc* immunostaining was performed using rat anti-HA primary antibodies (Roche,
935 #11 867 423 001) and anti-rat Alexa568 (Life Technologies, A11077, 1:500) as
936 secondary antibodies. We selected slices with good GFP labeling in the dendritic layer
937 for the two-photon experiments and in 4 out of 13 slices we were able to identify up
938 to 5 axons of each type within the imaging area. Identification of HA+ and HA- axons
939 was performed in Fiji, bouton dynamics analysis in Matlab as described above.

940

941 **Statistical Analysis**

942 All experiments were performed and analyzed blindly. Live imaging experiments for
943 bouton dynamics analysis were performed in paired slices from the same animal and
944 the same culture. Statistical analysis was performed using GraphPad Prism software.
945 Data are reported as mean \pm standard error unless stated otherwise. The variance
946 between axons was larger than the variance between slices, indicating that individual
947 axons are independent measurements. Results from treatment and control
948 experiments were compared using the nonparametric Mann-Whitney U test (MW).
949 Distributions were compared with Kolmogorov-Smirnov test (KS). Multiple
950 comparisons were made using two-way ANOVA (2w ANOVA) followed by Sidak's test.
951 Repeated two-way ANOVA analysis was used for comparing NP bouton density and NP
952 presence over time. P-values (not adjusted for multiplicity) are indicated in the figure
953 legends. Differences were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$,
954 *** $p < 0.001$).

955

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Figure 1

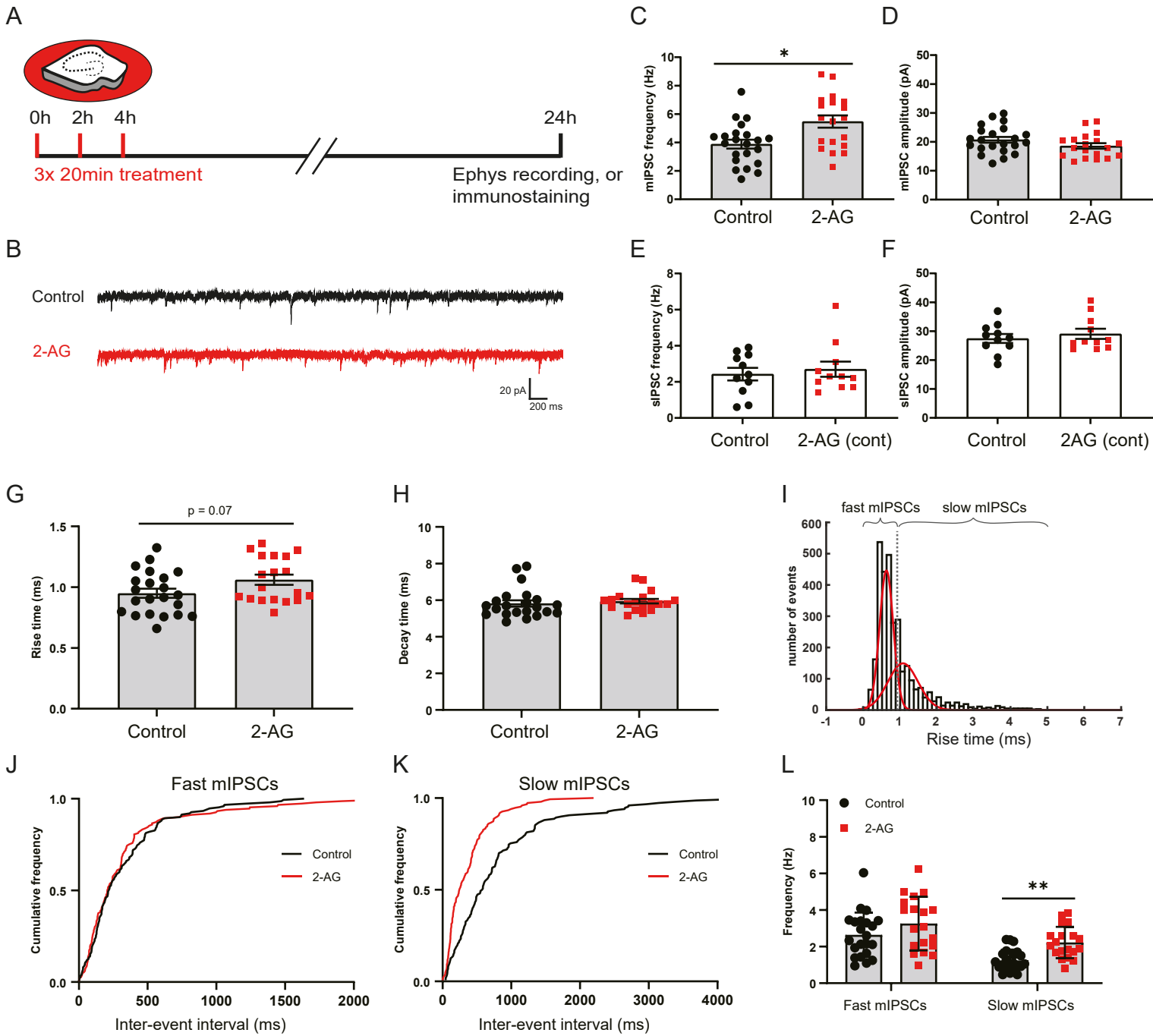


Figure 1. Repeated CB1 receptor activation results in increased mIPSC frequency.

(A) Organotypic hippocampal cultures were treated 3 times with culturing medium containing 100 μ M 2-AG or DMSO (control) for 20 minutes with 2 hour intervals. After 24 hours, slices were used for electrophysiology and immunostaining experiments.

(B) Example traces of miniature inhibitory postsynaptic currents (mIPSCs) recordings from control (black) and 2-AG treated slice (red).

(C, D) Mean frequency (C) and amplitude (D) of mIPSCs in control and 2-AG treated slices (MW, $p = 0.013$ in C and $p = 0.16$ in D). Data from 22 cells in 6 control slices and 19 cells in 6 2-AG treated slices.

(E, F) Mean frequency (E) and amplitude (F) of sIPSCs in control and 2-AG treated slices, when 2-AG was continuously present for 24 hr ($p = 0.99$ in E and $p = 0.95$ in F; MW). Data from 11 cells in 5 control slices and 11 cells in 6 2-AG treated slices.

(G) Mean rise time of mIPSCs in control and 2-AG treated slices (MW, $p = 0.073$).

(H) Mean of mIPSC decay time in control and 2-AG treated slices (MW, $p = 0.19$).

(I) The distribution of rise times of mIPSCs was fitted with a double Gaussian to separate fast and slow mIPSCs.

(J,K) Cumulative distribution of interevent intervals of mIPSCs with fast (J) and slow (K) rise times (KS, $p = 0.65$ in J, and $p < 0.0001$ in K).

(L) Mean frequency of mIPSCs with fast and slow rise times (2w ANOVA Sidak, fast: $p = 0.14$; slow: $p = 0.0095$).

Data in G-L and C,D are from the same data set.

Figure 2

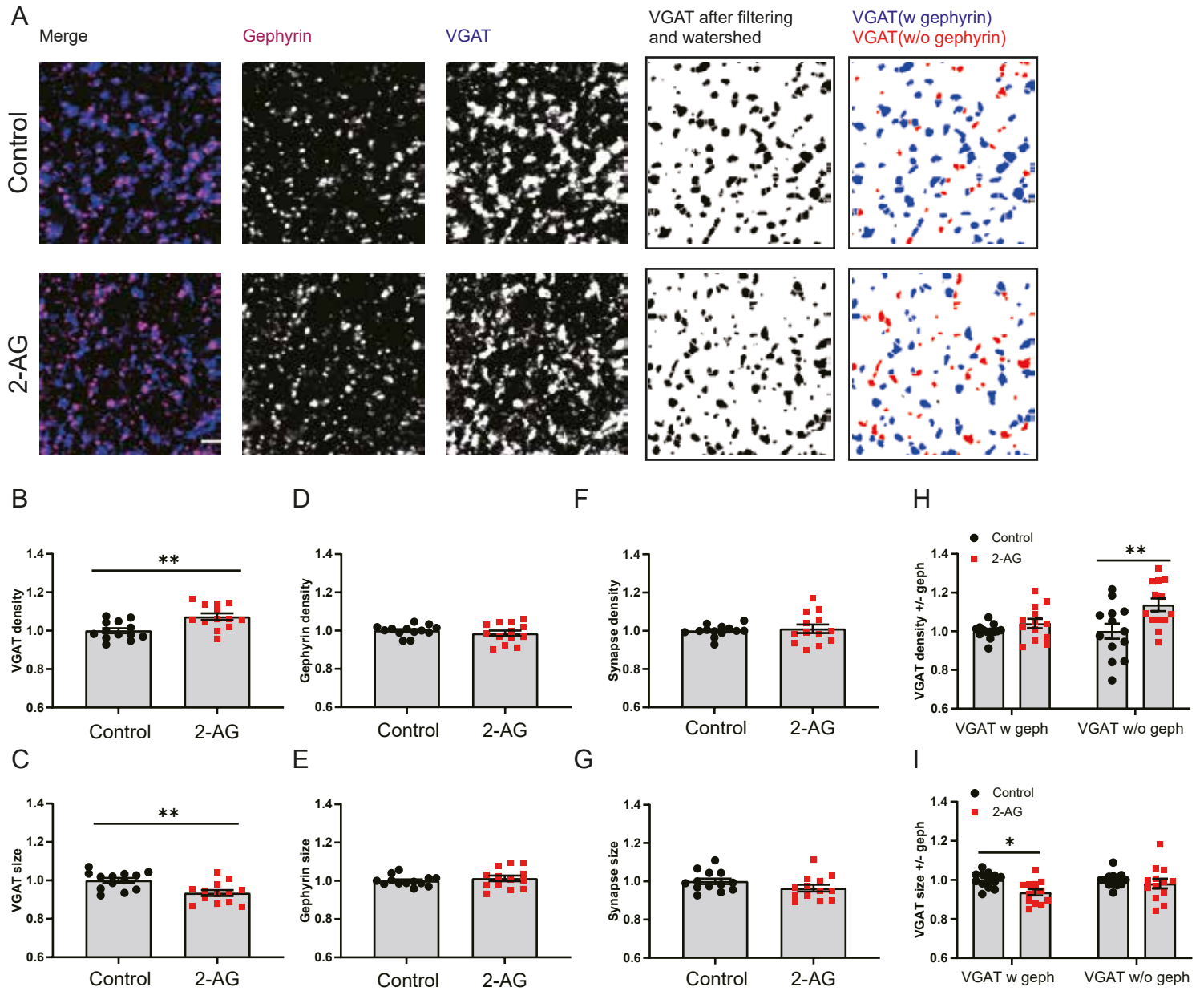


Figure 2. Repeated CB1 receptor activation induces the formation of partial inhibitory synapses.

(A) Representative immunostaining images showing the presynaptic VGAT (blue) and postsynaptic gephyrin (purple) in control (upper) and 2-AG (lower) slices. Individual VGAT puncta were identified using watershed segmentation and these were color coded to distinguish VGAT puncta associated with gephyrin (blue) and VGAT puncta without gephyrin (red).

(B, C) Normalized density (B) and size (C) of VGAT puncta in control and 2-AG slices (MW, $p = 0.0061$ in B; $p = 0.004$ in C).

(D, E) Normalized density (D) and size (E) of gephyrin puncta in control and 2-AG slices (MW, $p = 0.54$ in D; $p = 0.64$ in E).

(F, G) Normalized density (F) and size (G) of VGAT/gephyrin colocalizations in control and 2-AG slices (MW, $p = 0.76$ in F; $p = 0.099$ in G).

(H, I) Normalized density (F) and size (G) of VGAT puncta with and without gephyrin (2w ANOVA Sidak, $p = 0.55$ and $p = 0.003$ in H; $p = 0.017$ and $p = 0.65$ in I).

Data from 13 image stacks in 7 slices per group.

Figure 3

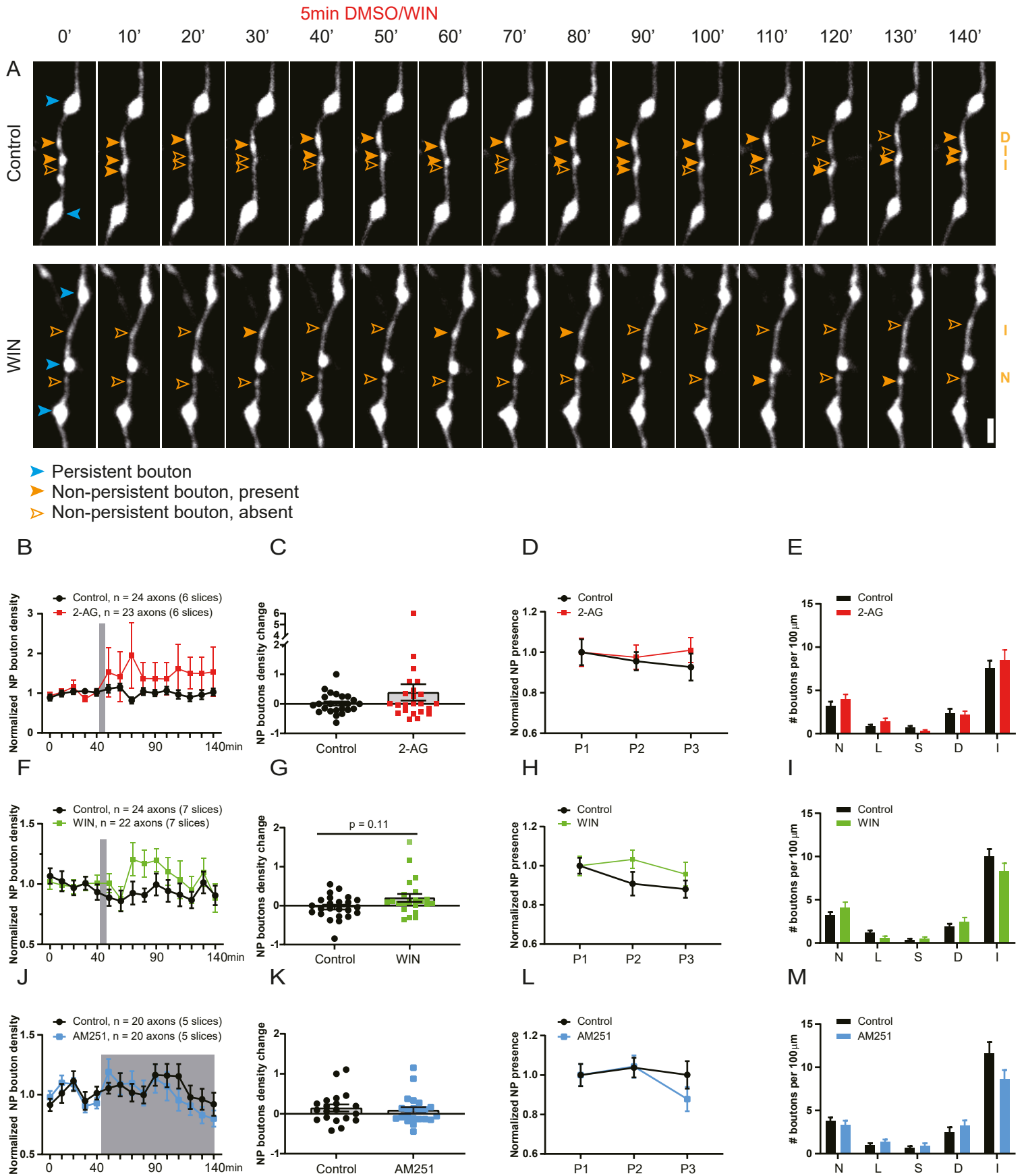


Figure 3. Brief activation of CB1 receptors slightly increases NP bouton density

(A) Representative two-photon time lapse images of GAD65-GFP labelled inhibitory axons in the dendritic region of the hippocampal CA1 area (maximal projections of 17 z-sections). After a baseline of five time points (40 minutes), CB1 receptor agonist or DMSO was washed in for 5 minutes. Imaging was continued for another ten time points (total imaging period is 140 minutes). Persistent boutons (blue) and non-persistent (NP) boutons (orange) are indicated by arrow heads. Empty arrow heads reflect a NP bouton which was absent at the time point. Scale bar is 2 μm .

(B) CB1 receptors were activated by bath application of 100 μM 2-AG for 5 minutes. Normalized NP bouton density over time in control (black) slices and after 2-AG (red) application (2w ANOVA, $p = 0.33$).

(C) Maximum change in NP bouton density in control slices and after 2-AG application (MW, $p = 0.54$).

(D) Normalized NP presence over time in control and 2-AG treated slices. P1= time points 1 to 5, P2= time points 6 to 10, and P3= time points 11 to 15) in control and 2-AG treated slices (2w ANOVA, $p = 0.61$).

(E) Mean density of NP bouton subgroups in control slices and after 2-AG application. N – new boutons (MW, $p = 0.35$); L – lost boutons (MW, $p = 0.44$); S – stabilizing boutons (MW, $p = 0.21$); D – destabilizing boutons (MW, $p = 0.91$); I – intermittent boutons (MW, $p = 0.87$).

(F) CB1 receptors were activated by bath application of 20 μM WIN for 5 minutes. Normalized NP bouton density over time in control (black) slices and after 2-AG (green) application (2w ANOVA, $p = 0.20$).

(G) Maximum change in NP bouton density in control slices and after WIN application (MW, $p = 0.11$).

(H) Normalized NP presence over time in control slices and after WIN application (2w ANOVA, $p = 0.20$).

(I) Mean density of NP bouton subgroups in control slices and after WIN application (MW, $p = 0.40$ (N); $p = 0.06$ (L); $p = 0.79$ (S); $p = 0.70$ (D); $p = 0.10$ (I)).

(J) Slices were treated with the CB1 receptor antagonist AM251 (5 μM) after time point 5. Normalized NP bouton density over time in control (black) slices and during AM251 (blue) application (2w ANOVA, $p = 0.66$).

(K) Maximum change in NP bouton density in control slices and during AM251 application (MW, $p = 0.6$).

(L) Normalized NP presence over time in control slices and during AM251 application (2w ANOVA, $p = 0.56$).

(M) Mean density of NP bouton subgroups in control slices and during AM251 application (MW, $p = 0.46$ (N); $p = 0.23$ (L); $p = 0.94$ (S); $p = 0.29$ (D); $p = 0.10$ (I)).

Data in A from 24 axons in 6 control slices and 23 axons in 6 2-AG slices. Data in B from 24 axons in 7 control slices and 22 axons in 7 WIN slices. Data in C from 20 axons in 5 control slices and 20 axons in 5 AM251 slices.

Figure 4

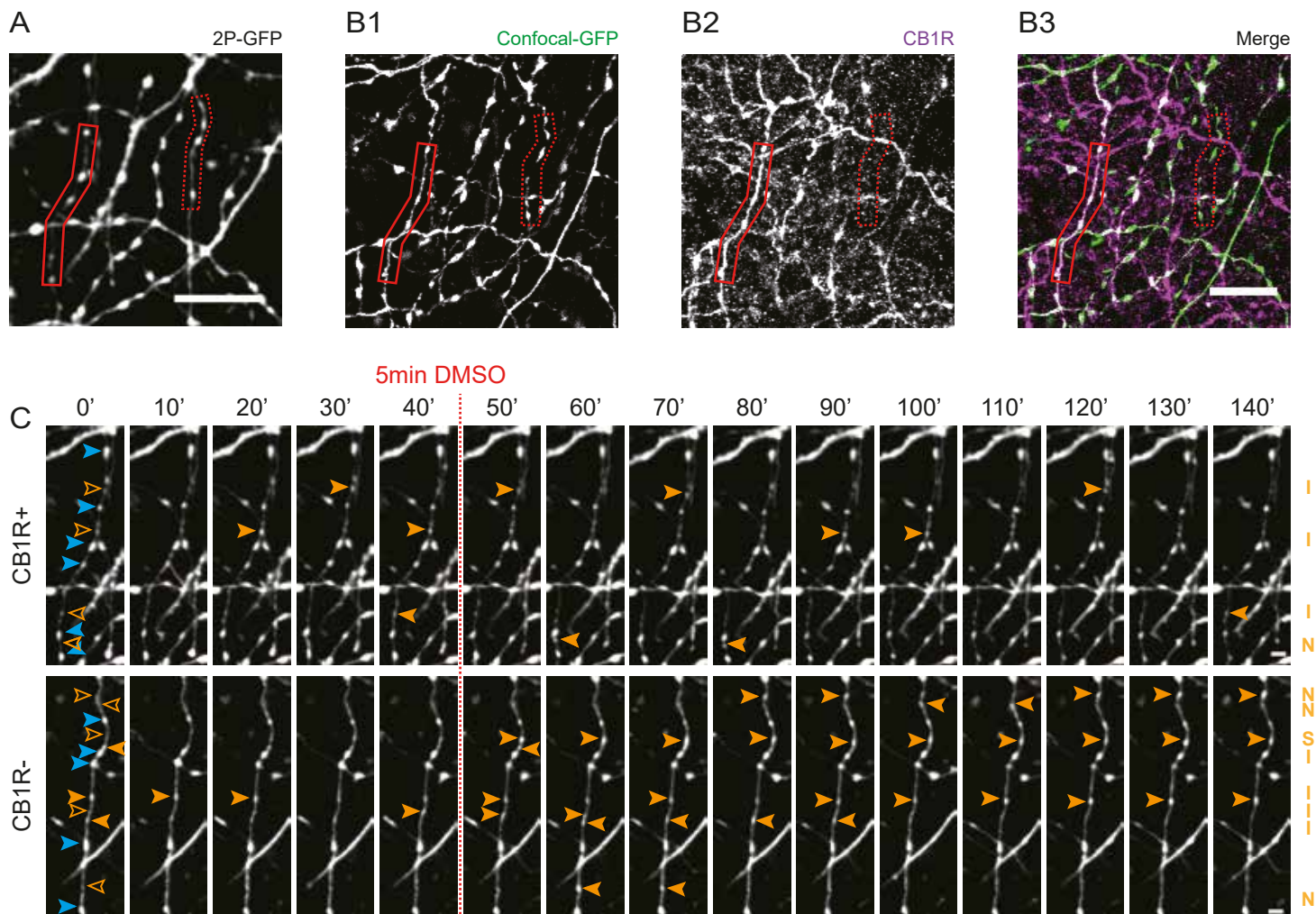


Figure 4. Distinction between CB1R+ and CB1R- axons using post hoc immunohistochemistry

(A) Z-projection of representative two-photon image of GFP-labeled inhibitory axons. After two-photon live imaging, the slice was immediately fixated and further processed for immunohistochemistry to assess CB1R expression.

(B) Confocal images of the same area after post hoc immunohistochemistry, showing the same GFP-labeled axons (B1) as in A (indicated with solid and dashed red boxes). Immunostaining against CB1 receptors (B2) show a clear distinction between CB1R+ axons (solid red box), which express CB1 receptors, which cover the entire axonal surface, and CB1R- axons (dashed red box). Which do not express CB1 receptors.

(C) Two-photon time lapse imaging of bouton dynamics in the CB1R+ and CB1R- axons indicated in A and B. Arrow heads indicate P (blue) and NP (orange) boutons as in Fig. 3A.

Scale bars are 10 μm in A,B and 2 μm in C,D.

Figure 5

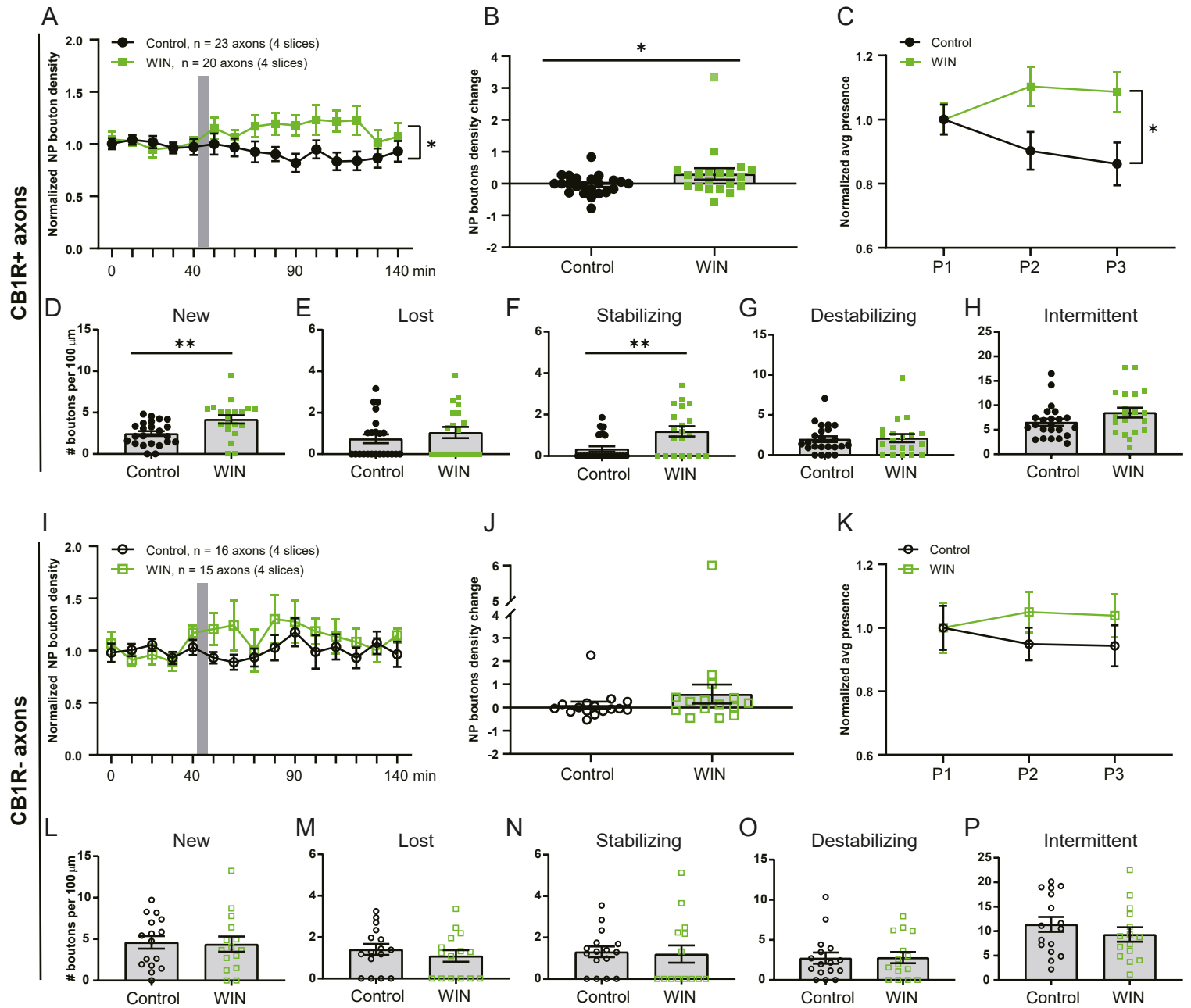


Figure 5. WIN promotes formation and stabilization of inhibitory boutons only in CB1R+ axons

(A) Normalized NP bouton density in CB1R+ axons over time in control (black) slices and after WIN (green) application (2w ANOVA, $p = 0.018$; interaction $p = 0.026$).

(B) Maximum change in NP bouton density in CB1R+ axons in control (black) slices and after WIN (green) application (MW, $p = 0.047$).

(C) Normalized NP presence in CB1R+ axons over time in control slices and after WIN application (2w ANOVA, $p = 0.022$; interaction $p = 0.045$).

(D-H) Mean density of NP bouton subgroups in CB1R+ axons in control slices and after WIN application. D, new boutons (MW, $p = 0.002$); E, lost boutons (MW, $p = 0.39$); F, stabilizing boutons (MW, $p = 0.005$); G, destabilizing boutons (MW, $p = 0.87$); H, intermittent boutons (MW, $p = 0.16$).

(I) Normalized NP bouton density in CB1R- axons over time in control (black) slices and after WIN (green) application (2w ANOVA, $p = 0.27$).

(J) Maximum change in NP bouton density in CB1R- axons in control (black) slices and after WIN (green) application (MW, $p = 0.21$).

(K) Normalized NP presence in CB1R- axons over time in control slices and after WIN application (2w ANOVA, $p = 0.37$).

(L-P) Mean density of NP bouton subgroups in CB1R- axons in control slices and after WIN application. L, new boutons (MW, $p = 0.77$); M, lost boutons (MW, $p = 0.46$); N, stabilizing boutons (MW, $p = 0.50$); O, destabilizing boutons (MW, $p = 0.99$); P, intermittent boutons (MW, $p = 0.34$).

Data from 25 CB1R+ and 16 CB1R- axons in 4 control slices and 20 CB1R+ and 15 CB1R- axons in 4 slices with WIN application.

Figure 6

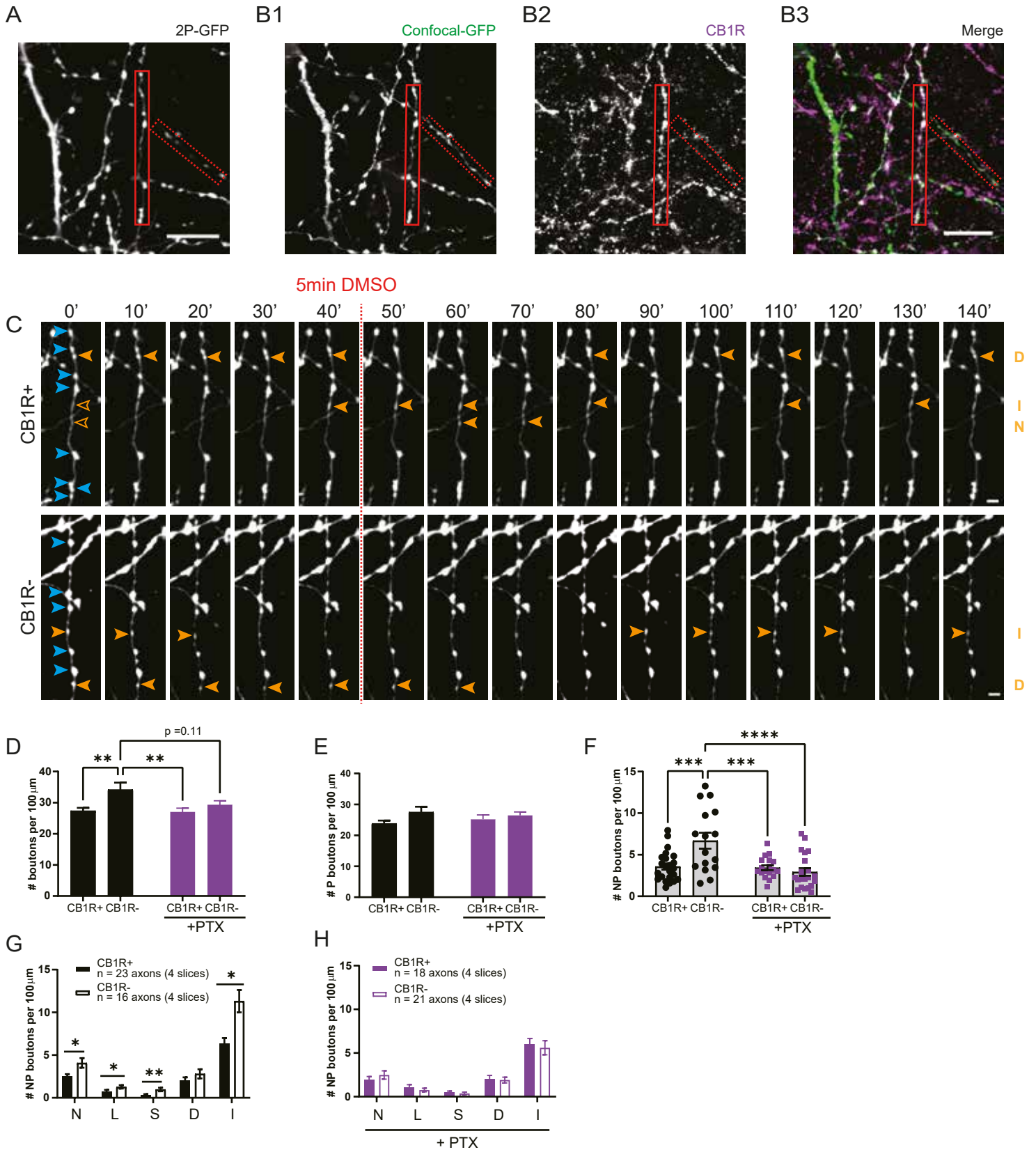


Figure 6. $G_{i/o}$ signaling is an important regulator of inhibitory bouton dynamics

(A) Z-projection of representative two-photon image of GFP-labeled inhibitory axons after PTX pretreatment.

(B) Confocal images of the same area after post hoc immunohistochemistry, showing the same GFP-labeled axons (B1) as in A (solid and dashed red boxes indicate CB1R+ and CB1R- axons).

(C) Two-photon time lapse imaging of bouton dynamics in the CB1R+ and CB1R- axons indicated in A and B after PTX pretreatment. Arrow heads indicate P (blue) and NP (orange) boutons as in Fig. 3A.

(D) Average bouton density during baseline in CB1R+ and CB1R- axons in control slices and after PTX pretreatment. Comparisons between CB1R+ and CB1R- axons: $p = 0.0056$ for control, $p = 0.79$ after PTX; between control and PTX: $p = 0.11$ for CB1R- axons, $p > 0.99$ for CB1R+ axons; between CB1R+ (control) and CB1R- (PTX): $p = 0.86$ and between CB1R- (control) and CB1R+ (PTX): $p = 0.0057$ (2w ANOVA Sidak).

(E) Average density of persistent (P) boutons during baseline in CB1R+ and CB1R- axons in control slices and after PTX pretreatment ($p=0.057$ for axon type, 2w ANOVA Sidak).

(F) Average density of non-persistent (NP) boutons during baseline in CB1R+ and CB1R- axons in control slices and after PTX pretreatment. Comparisons between CB1R+ and CB1R- axons: $p = 0.0007$ for control, $p = 0.99$ after PTX; between control and PTX: $p < 0.0001$ for CB1R- axons, $p > 0.99$ for CB1R+ axons; between CB1R+ (control) and CB1R- (PTX): $p = 0.93$ and between CB1R- (control) with CB1R+ (PTX): $p = 0.0008$ (2w ANOVA Sidak).

(G) Mean density of NP bouton subgroups in CB1R+ and CB1R- axons in control slices. N – new boutons (MW, $p = 0.035$); L – lost boutons (MW, $p = 0.037$); S – stabilizing boutons (MW, $p = 0.002$); D – destabilizing boutons (MW, $p = 0.47$); I – intermittent boutons (MW, $p = 0.010$).

(H) Mean density of NP bouton subgroups in CB1R+ and CB1R- axons after PTX pretreatment (MW, $p = 0.45$ (N); $p = 0.41$ (L); $p = 0.36$ (S); $p = 0.88$ (D); $p = 0.40$ (I)).

Data from 23 CB1R+ and 16 CB1R- axons in 4 control slices, and 18 CB1R+ and 21 CB1R- axons in 4 PTX-pretreated slices. Scale bars are 10 μm in A,B and 2 μm in C,D.

Figure 7

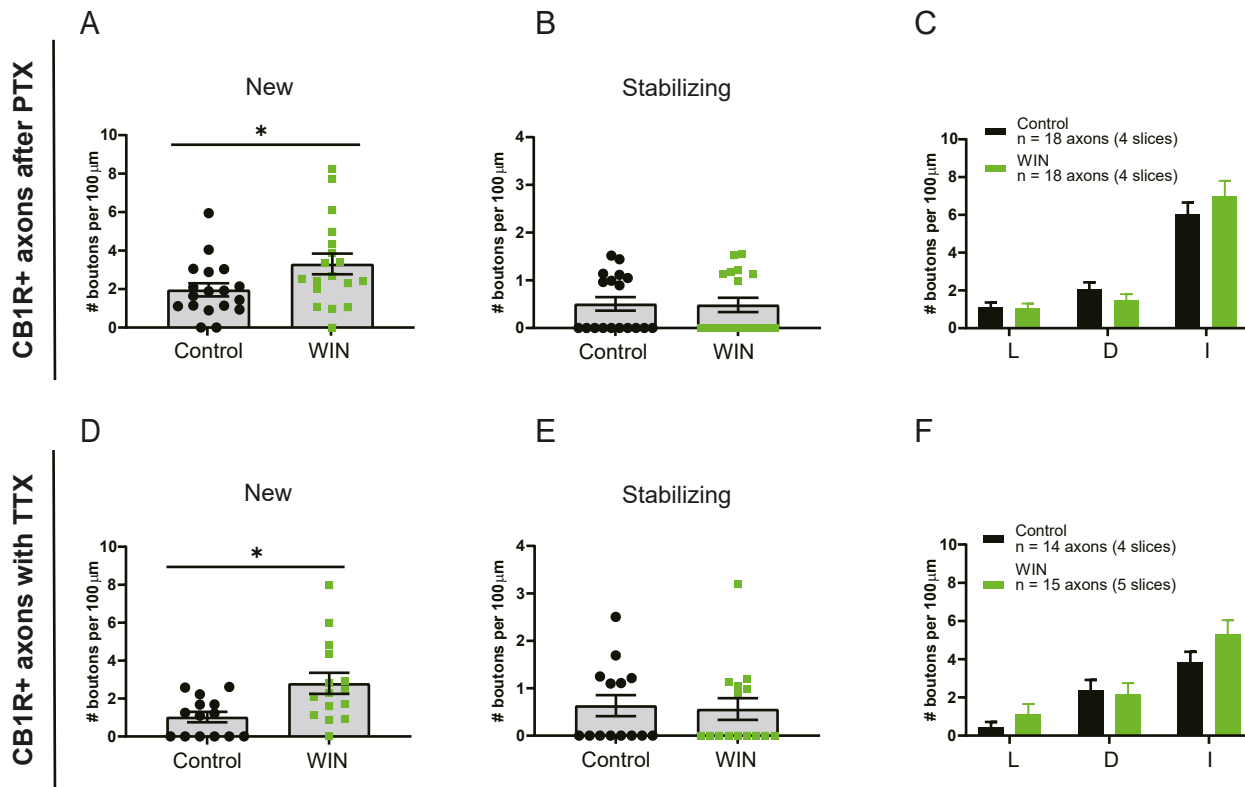


Figure 7. CB1-mediated bouton formation does not require $G_{i/o}$ signaling and is independent of activity.

(A) Mean density of new boutons in CB1R+ axons after control (black) and WIN (green) application in PTX-pretreated slices (MW, $p = 0.047$).

(B) Mean density of stabilizing boutons in CB1R+ axons after control and WIN application in PTX-pretreated slices (MW, $p = 0.93$).

(C) Mean density of other NP bouton subgroups in CB1R+ axons after control and WIN application in PTX-pretreated slices. L – lost boutons (MW, $p = 0.82$); D – destabilizing boutons (MW, $p = 0.37$); I – intermittent boutons (MW, $p = 0.59$).

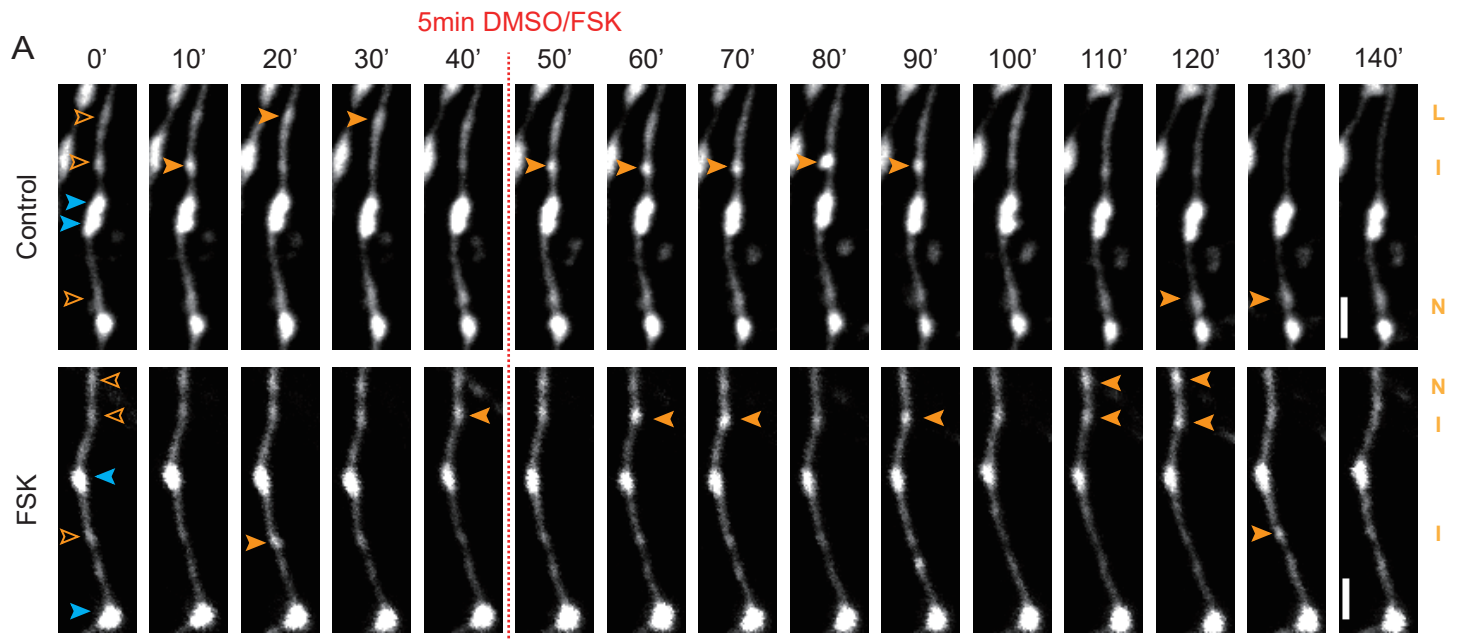
(D) Mean density of new boutons in CB1R+ axons after control (black) and WIN (green) application in the presence of TTX (MW, $p = 0.013$).

(E) Mean density of stabilizing boutons in CB1R+ axons after control and WIN application in the presence of TTX (MW, $p = 0.61$).

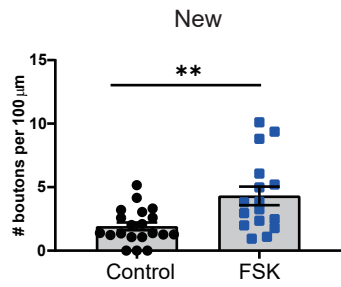
(F) Mean density of other NP bouton subgroups in CB1R+ axons after control and WIN application in the presence of TTX. L – lost boutons (MW, $p = 0.23$); D – destabilizing boutons (MW, $p = 0.56$); I – intermittent boutons (MW, $p = 0.16$).

Data in A-C from 18 axons in 4 slices with DMSO (control) application and 18 axons in 4 slices with WIN application. Data in D-F from 14 axons in 4 slices with DMSO (control) application and 15 axons in 5 slices with WIN application.

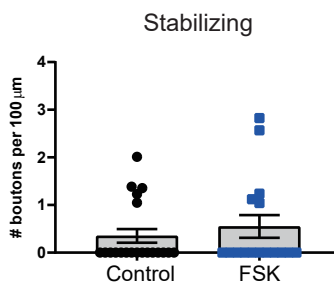
Figure 8



B



C



D

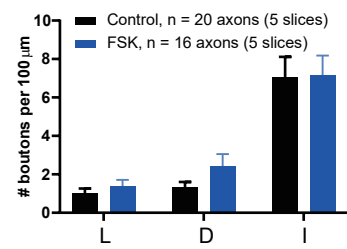


Figure 8. Inhibitory bouton formation is promoted by increasing intracellular cAMP levels with forskolin

(A) Representative two-photon time lapse images of bouton dynamics in GFP-labeled axons after control or forskolin application. Arrow heads indicate P (blue) and NP (orange) boutons as in Fig. 3A. Scale bar is 2 μ m.

(B) Mean density of new boutons in control (black) slices and after forskolin (blue) application (MW, $p = 0.007$).

(C) Mean density of stabilizing boutons in control slices and after forskolin application (MW, $p = 0.67$).

(D) Mean density of other subgroup of NP boutons in control slices and after forskolin application. L – lost boutons (MW, $p = 0.46$); D – destabilizing boutons (MW, $p = 0.37$); I – intermittent boutons (MW, $p = 0.81$).

Scale bars are 2 μ m.

Figure 9

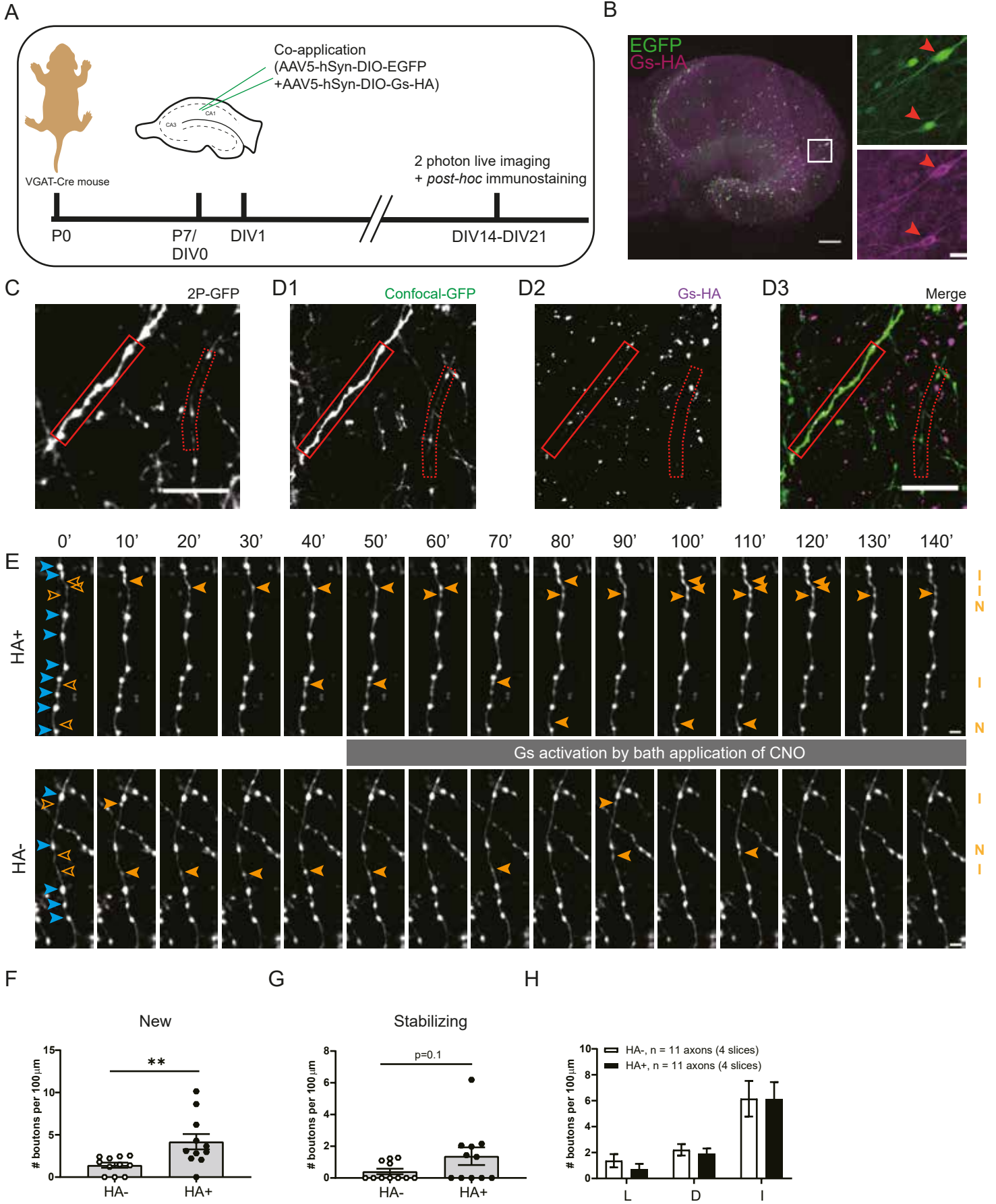


Figure 9. Specific activation of Gs at inhibitory axons induce new bouton formation.

(A) Experimental design. Hippocampal slice cultures are prepared from P7 VGAT-Cre mouse pups. At DIV1 (days *in vitro*), AAV5-hSyn-DIO-EGFP and AAV5-hSyn-DIO-Gs-HA viruses are applied to the VGAT-Cre slice cultures. After 2-3 weeks (DIV 14-21) slices were used for two-photon live imaging and *post hoc* immunostaining to reveal Gs-HA expression.

(B) Representative example of VGAT-Cre slice culture at DIV20 showing sparse expression of GFP and Gs-HA in GABAergic cells. Right images (zoom from white box) show Gs-HA and EGFP co-expression in a subset of neurons (red arrow heads).

(C) Z-projection of representative two-photon image of GFP-labeled inhibitory axons in VGAT-Cre slice.

(D) Confocal images of the same area in C after *post hoc* immunohistochemistry against HA, showing the same GFP-labeled axons as in A (solid and dashed red boxes indicate HA+ and HA- axons).

(E) Two-photon time lapse imaging of bouton dynamics in the HA+ and HA- axons indicated in C and D. Gs-DREADDs were activated by bath application of 10 μ M CNO after the 40 minutes baseline period. Arrow heads indicate P (blue) and NP (orange) boutons as in Fig. 3A.

(F) Mean density of new boutons at HA+ and HA- axons in response to Gs-DREADD activation (MW, $p = 0.003$).

(G) Mean density of stabilizing boutons at HA+ and HA- axons in response to Gs-DREADD activation (MW, $p = 0.10$).

(H) Mean density of other subgroup of NP boutons at HA+ and HA- axons in response to Gs-DREADD activation. L – lost boutons (MW, $p = 0.30$); D – destabilizing boutons (MW, $p = 0.44$); I – intermittent boutons (MW, $p = 0.85$)

Data from 11 HA+ and 11 HA- axons in 4 slices. Scale bars are 200 μ m (overview) and 20 μ m (zoom) in B, 10 μ m in C,D and 2 μ m in E.