

1 Microglia facilitate and stabilize the response to general 2 anesthesia

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33 **Abstract**

34 General anesthesia leads to a loss of consciousness and an unarousable state in patients.
35 Although general anesthetics are widely used in clinical practice, their underlying
36 mechanisms remain elusive. The potential involvement of nonneuronal cells is
37 unknown. Microglia are important immune cells in the central nervous system (CNS)
38 that play critical roles in CNS function and dysfunction. We unintentionally observed
39 delayed anesthesia induction and early anesthesia emergence in microglia-depleted
40 mice. We found that microglial depletion differentially regulates neuronal activities by
41 suppressing the neuronal network of anesthesia-activated brain regions and activating
42 emergence-activated brain regions. Thus, microglia facilitate and stabilize the
43 anesthesia status. This influence is not mediated by dendritic spine plasticity. Instead,
44 it relies on the activation of microglial P2Y12 and subsequent calcium influx, which
45 facilitates the general anesthesia response. Together, we elucidate the regulatory role of
46 microglia in general anesthesia, extending our knowledge of how nonneuronal cells
47 modulate neuronal activities.

48

49

50 **Introduction**

51 General anesthesia is a cornerstone of modern medical sciences. Upon the use of
52 anesthetics, patients lose consciousness and enter an unarousable state. Although
53 general anesthetics are widely used in clinical procedures, the mechanism of general
54 anesthesia remains elusive. Different anesthetics activate or inhibit specific receptors
55 in neurons, modulating neuronal activities across the entire network. However, the
56 involvement of nonelectrically active glial cells in anesthesia is poorly understood.
57 Microglia are yolk sac-derived glial cells in the central nervous system (CNS) (1). They
58 play critical roles in CNS development, function and dysfunction (2, 3). Previous
59 studies have found that neuronal activity in the CNS network modulates microglial
60 activity. Microglia exhibit elevated process motility, extension and territory
61 surveillance during anesthetization and sleep (4, 5). Suppression of neuronal activity
62 increases calcium signaling in microglial processes (6). Conversely, microglia also
63 modulate neuronal activity via multiple mechanisms (7-12). The microglial regulation
64 of neuronal activities thus raises the question of whether microglia can modulate the
65 general anesthesia response.

66

67 Microglial survival relies on colony-stimulating factor 1 receptor (CSF1R) signaling
68 (13). Pharmacological inhibition of CSF1R efficiently eliminates CNS microglia (14-
69 19). Previous studies have shown that acute microglial depletion does not induce
70 neuroinflammation (14, 15, 19, 20). The ablation of microglia in adulthood also does
71 not result in obvious general behavioral dysfunctions (14, 20), although this finding is
72 controversial (21). It seems that microglia are disposable under physiological
73 conditions. However, when we killed microglia with PLX5622, a CSF1R inhibitor (22),
74 we unintentionally observed robust resistance to anesthetic administration. This
75 suggests that microglia may facilitate general anesthesia by modulating neuronal
76 network activity.

77

78 To this end, we first quantified the influence of microglial depletion on the response to
79 general anesthesia. We utilized the loss of righting reflex (LORR) and recovery of
80 righting reflex (RORR) to evaluate anesthesia induction and emergence, respectively.
81 After microglial depletion, mice displayed a longer LORR time and a shorter RORR
82 time. The damped general anesthesia response was not dependent on specific
83 anesthetics or receptors, as this phenomenon was observed with three different agonists
84 of the GAGA_A receptor (pentobarbital, propofol and chloral hydrate) and one
85 antagonist of the NMDA receptor (ketamine). Electroencephalography (EEG) and
86 electromyography (EMG) findings further confirmed our initial observation. Different
87 brain regions diversely regulate anesthesia induction and emergence. Anesthesia
88 induction is positively correlated with anesthesia-activated brain regions (AABRs). In
89 contrast, anesthesia emergence is positively correlated with emergence-activated brain
90 regions (EABRs). We observed that microglia modulate brain network activity in a
91 brain region-specific manner rather than in a universal manner for all brain regions.
92 Based on c-Fos reactivity and patch clamp recordings, we demonstrated that microglial
93 depletion inhibits AABRs and activates EABRs. The divergent effects in different brain
94 regions orchestrate the status during general anesthesia use. Microglia-mediated
95 anesthesia modulation is not attributed to dendritic spine plasticity. We found that mice
96 with genetical knockout or pharmacological inhibition of microglial P2Y12 were more
97 resistant to general anesthesia. In addition, the contribution of microglial P2Y12 to
98 anesthesia response was further confirmed by the mice received microglia replacement,
99 in which the replaced microglia-like cells are P2Y12⁻ (18). On the other hand, the
100 intracellular Ca²⁺ concentration in microglia facilitates and stabilizes the response to
101 general anesthesia. Because purinergic activation of P2Y12 increases intracellular Ca²⁺
102 (23-25), our results reveal that the general anesthesia response is regulated through
103 P2Y12 to Ca²⁺ signaling in microglia.

104
105 In conclusion, our study demonstrates a regulatory role of microglia in the response to
106 general anesthesia and identifies the underlying mechanism of this process. This study
107 extends our knowledge of how nonelectrically active glial cells regulate the general

108 anesthesia response. It also sheds new light on how microglia contribute to maintaining
109 the status of the brain network. When we were preparing our manuscript, a paper
110 discussing a similar topic emerged (26).

111

112

113 **Results**

114 **Microglia regulate the induction of and emergence from general anesthesia**

115 We unintentionally observed that mice become more resistant to anesthesia after
116 microglia and macrophage depletion by the CSF1R inhibitor PLX5622. To
117 quantitatively study whether microglial depletion indeed influences the induction of
118 and emergence from general anesthesia, we first fed mice a PLX5622-formulated diet
119 (PLX5622 hereafter) to ablate CNS microglia and peripheral macrophages (16, 18, 19,
120 27). After 14 days of PLX5622 administration, we intraperitoneally injected
121 pentobarbital (80 mg pentobarbital sodium per kg of body weight) into the PLX5622-
122 treated mice (Figure 1A). Compared to naïve mice on day 0 (D0), the PLX5622-treated
123 mice at D14 displayed a longer time for LORR and shorter time for RORR (Figure 1B).
124 Pentobarbital is an agonist of the GABA_A receptor (28). We next examined whether
125 this microglia- and macrophage-mediated regulation of the anesthesia response is
126 restricted to pentobarbital or GABA_A receptor agonists. We assessed LORR and RORR
127 in PLX5622-treated mice by using other anesthetics, including two other GABA_A
128 receptor agonists (propofol, 200 mg/kg of body weight; chloral hydrate, 400 mg/kg of
129 body weight) (29-31) and one NMDA receptor antagonist (ketamine, 100 mg/kg of
130 body weight) (32). Similar trends were observed in propofol-, chloral hydrate- and
131 ketamine-induced LORR and RORR (Figure 1B). To exclude the possibility that
132 PLX5622-induced anesthesia resistance results from tolerance to repetitive anesthetic
133 injection, we sequentially treated mice with the same anesthetics 5 times at 7-day
134 intervals (Figure 2A). Both LORR and RORR were unchanged for pentobarbital,
135 propofol, chloral hydrate and ketamine, except for chloral hydrate-induced LORR after
136 D14 (the third dose) (Figure 2B). PLX5622-treated mice exhibited a resistant
137 phenotype (Figure 1B). In contrast, the third dose of chloral hydrate made mice more
138 susceptible to anesthesia (Figure 2B), an opposite trend from PLX5622 treatment.
139 Consequently, PLX5622-induced anesthesia resistance is not attributed to the tolerance
140 of repetitive anesthetic administration.

141

142 PLX5622 can simultaneously ablate brain microglia and peripheral macrophages (27).
143 To exclude the possibility that CSF1R-mediated general anesthesia regulation does not
144 result from peripheral macrophages, we utilized the blood-brain barrier-impermeable
145 CSF1R inhibitor PLX73086 to ablate peripheral macrophages without influencing
146 brain microglia (33). After administration of the PLX73086-formulated diet
147 (PLX73086 hereafter) for 14 days, macrophages in the liver, lung, spleen and kidney
148 were significantly ablated, while brain microglia were not influenced (Figure 3A-B).
149 PLX73086-treated mice (peripheral macrophage-depleted, brain microglia-unchanged)
150 displayed similar general anesthetic responses as CD-treated naïve mice, including
151 those treated with pentobarbital, propofol, chloral hydrate and ketamine, except for the
152 RORR in pentobarbital-treated mice (Figure 3C). Since PLX73086-treated mice
153 (peripheral macrophage-depleted, brain microglia-unchanged) exhibited a delayed
154 RORR with pentobarbital administration (Figure 3C), whereas PLX5622-treated mice
155 (peripheral macrophage-depleted, brain microglia-depleted) displayed an earlier RORR
156 (Figure 1B), the pentobarbital-induced early emergence in PLX5622-administered
157 mice is not attributed to peripheral macrophage ablation. Therefore, microglial
158 depletion, rather than macrophage depletion, leads to resistance to general anesthesia.

159
160 Next, we reasoned whether this anesthesia resistance is permanent or can be reversed
161 by microglial repopulation. To address this question, we ceased CSF1R inhibition by
162 treating the microglia-depleted mice with a control diet (CD) for 21 days to allow
163 microglia to repopulate the brain (Figure 1A), at which point repopulated microglia
164 recovered to the same density and similar transcriptional characteristics as those in
165 control mice (19). The LORR and RORR of microglia-repopulated mice at D35
166 recovered to the same level as those of naïve mice at D0 (Figure 1B), indicating that
167 fully repopulated microglia can reverse the anesthesia susceptibility of mice.

168
169 Together, our results indicate that microglial depletion by inhibiting CSF1R results in
170 delayed anesthesia induction and early anesthesia emergence, making the animals more
171 resistant to general anesthesia.

172

173 To further characterize the impact of microglial depletion throughout the
174 anesthetization window, we recorded EEG and EMG signals to monitor the anesthesia
175 state before and after pentobarbital administration (Figure 4A). Microglial depletion
176 showed no obvious influence on the EEG in the awake or conscious state before
177 pentobarbital administration (Figure 4B). In contrast, microglia-depleted mice
178 exhibited delayed anesthesia induction and early emergence in response to
179 pentobarbital (Figure 4B-C). In addition, microglial depletion significantly altered the
180 power spectrum during anesthesia induction and emergence but not consciousness
181 (Figure 4D). The EMG results showed that muscular activity in the conscious state was
182 unchanged upon microglial depletion. In contrast, microglia-depleted mice exhibited a
183 delayed loss and early recovery of muscular activity after pentobarbital injection
184 (Figure 4E). Moreover, the probability of being in the conscious state, predicted by an
185 algorithm combining EEG and EMG (34), triple confirmed delayed anesthesia
186 induction and early emergence after microglial depletion (Figure 4F). Similar results
187 were also observed for propofol (Figure 5) and ketamine (Figure 6). The EEG and EMG
188 results demonstrate that microglial depletion impedes the anesthesia process.

189

190 Together, our results demonstrate that brain microglia-depleted mice are resistant to
191 general anesthetics. In other words, microglia play important roles in facilitating and
192 stabilizing the status of general anesthesia response.

193

194 **Microglia facilitate the anesthesia response in a brain region-specific manner**

195 A previous study indicated that microglia negatively regulate neuronal activity through
196 the microglial catabolism of ATP and neuronal adenosine receptor A₁R. Microglial
197 depletion enhances neuronal activity in the striatum (12). Different brain regions
198 regulate anesthesia induction and emergence in diverse manners. If microglial depletion
199 indiscriminately influences neuronal activities among different brain regions, the
200 enhanced activities in AABRs and EABRs would be mutually antagonistic,
201 complicating the anesthetic effect. To investigate whether microglia regulate neuronal

202 activity in an indiscriminate or brain region-specific manner, we examined c-Fos
203 expression in AABRs and EABRs of CD- and PLX5622-treated mice (Figure 7A). We
204 first studied AABRs, including the lateral habenula (LHb) (35, 36), supraoptic nucleus
205 (SON) (37), ventrolateral preoptic nucleus (VLPO) (38) and thalamic reticular nucleus
206 (TRN) (39). The abundance of c-Fos⁺ cells was significantly reduced in the LHb and
207 SON of microglia-depleted mice (Figure 7B). VLPO exhibited a decreasing trend in the
208 abundance of c-Fos⁺ cells, although it did not reach a statistically significant level ($P =$
209 0.1592) (Figure 7B). The abundance of c-Fos⁺ cells was unchanged in the TRN (Figure
210 7B). We next examined EABRs, including the paraventricular thalamus (PVT) (40),
211 locus coeruleus (LC) (41), lateral hypothalamus (LH) (42, 43) and ventral tegmental
212 area (VTA) (44, 45). In contrast to a suppressed trend in AABRs, neuronal activity
213 exhibited an enhanced trend in EABRs. Microglial depletion significantly increased the
214 c-Fos⁺ cell number in the PVT and LC (Figure 7C). c-Fos⁺ cell numbers were also
215 increased in the LH and VTA, although the difference did not reach statistical
216 significance ($P = 0.0598$ and 0.1436, respectively) (Figure 7C). To exclude the
217 possibility that the different c-Fos⁺ cell numbers were attributed to animal handling, we
218 compared c-Fos expression in saline-injected and noninjected mice (Figure 8A). Our
219 results indicate that animal handling (saline injection) did not influence c-Fos
220 expression in the LHb, SON, VLPO, TRN, PVT, LC, LH or VTA (Figure 8B-C).
221 Therefore, we found that microglial depletion negatively regulates AABRs and
222 positively regulates EABRs, indicating that microglia regulate neuronal activity in a
223 brain region-specific manner. The suppressed neuronal activity in AABRs leads to
224 delayed anesthesia induction. In contrast, the elevated neuronal activity in EABRs
225 results in the early emergence of anesthesia.

226

227 The protein expression of c-Fos is relatively slow, peaking at a timepoint hours after
228 transcription (46). Mice in our study were quickly sacrificed after deep anesthesia,
229 typically within 5 to 10 min. The abundance of c-Fos protein seen with immunostaining
230 reflected neuronal activity during the consciousness stage (Figure 7). In contrast, the
231 mRNA expression of *Fos* (encoding c-Fos) is relatively fast, peaking at approximately

232 30 min after induction (46). We asked how microglia influence neuronal activity during
233 the anesthesia stage and whether microglia differentially influence neuronal activity
234 between the consciousness and anesthesia stages. To this end, we sacrificed microglia-
235 naïve and microglia-depleted mice 30 min after deep anesthetization by pentobarbital
236 and simultaneously labeled the c-Fos protein by immunostaining and *Fos* mRNA by
237 RNAscope (Figure 9A). The c-Fos⁺ cells represent activated neurons during the
238 consciousness stage, while *Fos*⁺ cells represent activated neurons during the anesthesia
239 stage (Figure 9A). We compared the c-Fos⁺ and *Fos*⁺ cells in AABRs and EABRs in
240 which neuronal activity was significantly altered in microglia-depleted mice, including
241 the LHb, SON, PVT and LC (Figure 7B-C and Figure 9B-C). After microglial depletion,
242 activated neurons in the anesthesia stage (*Fos*⁺) displayed similar trends as those in the
243 consciousness state (c-Fos⁺) in the LHb, SON and LC (Figure 9B-C). However, the
244 number of anesthesia-activated neurons (*Fos*⁺) was unchanged between the naïve and
245 microglia-depleted PVT, whereas the number of consciousness-activated neurons was
246 significantly increased upon microglial depletion (Figure 9C). Exploiting the c-Fos
247 protein and *Fos* mRNA dual labeling, we further compared consciousness-activated
248 anesthesia-activated (c-Fos⁺ *Fos*⁺), consciousness-activated anesthesia-nonactivated
249 (c-Fos⁺ *Fos*⁻) and consciousness-nonactivated anesthesia-activated (c-Fos⁻ *Fos*⁺)
250 neurons between naïve and microglia-depleted mice (Figure 9A). In the LHb and SON
251 of AABRs, consciousness-activated anesthesia-activated (c-Fos⁺ *Fos*⁺), consciousness-
252 activated anesthesia-nonactivated (c-Fos⁺ *Fos*⁻) and consciousness-nonactivated
253 anesthesia-activated (c-Fos⁻ *Fos*⁺) cell numbers exhibited a decreasing trend after
254 microglial depletion (Figure 9B). This indicates that microglial depletion influences
255 AABR neuronal activity at both the consciousness and anesthesia stages. In the PVT of
256 the EABR, consciousness-activated anesthesia-nonactivated (c-Fos⁺ *Fos*⁻) cell numbers
257 were significantly increased in microglia-depleted brains, whereas consciousness-
258 activated anesthesia-activated (c-Fos⁺ *Fos*⁺) and consciousness-nonactivated
259 anesthesia-activated (c-Fos⁻ *Fos*⁺) cell numbers were unchanged (Figure 9C). This
260 indicates that microglial depletion influences PVT neuronal activity at the
261 consciousness stage but not at the anesthesia stage. In the LC of the EABR, microglial

262 depletion significantly increased the cell numbers of consciousness-activated
263 anesthesia-activated ($c\text{-Fos}^+ Fos^+$) and consciousness-activated anesthesia-
264 nonactivated ($c\text{-Fos}^+ Fos^-$) neurons (Figure 9C). In contrast, the number of
265 consciousness-nonactivated anesthesia-activated ($c\text{-Fos}^- Fos^+$) cells was not altered
266 (Figure 9C). This finding indicates that microglial depletion does not influence the LC
267 neurons that are not activated in the consciousness stages.

268

269 The results indicate that microglia diversely regulate neuronal activity through a
270 sophisticated brain region-specific manner instead of via indiscriminately negative
271 feedback control as in the striatum (12). This may be due to microglial heterogeneity,
272 different neuronal cell types, and/or circuitries in different brain regions.

273

274 **Microglial depletion reduces the E/I ratio in AABR but enhances the E/I ratio in
275 EABR**

276 To understand how microglia reshape neuronal activity, we treated mice with CD or
277 PLX5622 for 14 days and performed whole-cell recordings in neurons of SON and LC
278 in acute brain slices, representing AABR and EABR with reduced and increased
279 neuronal activity upon microglial depletion, respectively (Figure 10A). We delivered
280 electrical pulses (0.1 ms in pulse duration, 10/20 Hz, 8 pulses) with a current intensity
281 increment of 10 μ A every 10 seconds to the neighboring tissue (approximately 50 μ m
282 from the recorded cell) to induce postsynaptic responses, including both evoked
283 excitatory postsynaptic currents (eEPSCs) and evoked inhibitory postsynaptic currents
284 (eIPSCs). In the SON of AABR, higher stimulation currents induced larger amplitudes
285 of both eEPSCs and eIPSCs (Figure 10B-C). The peak amplitudes of eEPSCs in
286 microglia-depleted mice were significantly smaller than those in naïve mice, while the
287 peak amplitudes of eIPSCs showed no significant difference (Figure 10B-C). As shown
288 in Figure 10C, the excitation received by SON neurons dominated in naïve mice. The
289 E/I ratio was also significantly decreased after microglial depletion, indicating
290 decreased neuronal excitability in the SON (Figure 10D). SON neurons with microglia
291 depletion exhibited a significantly increased paired-pulse ratio (PPR) of eEPSCs, while

292 the eIPSC PPR was similar between naïve and microglia-depleted mice (Figure 10E-F), indicating a reduction in presynaptic release probability in excitatory synapses.
293 Microglial depletion thus results in a more inhibitory state in the AABR SON. In the
294 LC of EABR, the eEPSC amplitudes induced by higher stimulation currents in
295 microglia-depleted mice were substantially greater than those in naïve mice (Figure
296 10G-H). In contrast, the eIPSC amplitudes showed no significant difference (Figure
297 10G-H). In contrast to that in the SON, the E/I ratio in the LC was significantly
298 enhanced in PLX5622-treated mice (Figure 10I), indicating an increase in the excitation
299 of LC neurons. Microglial depletion did not change the eEPSC PPR or eIPSC PPR in
300 the LC (Figure 10J-K), representing an unchanged presynaptic release probability in
301 both excitatory and inhibitory synapses. Microglial depletion thus results in a more
302 excitatory state in the EABR LC.
303

304
305 In conclusion, our results reveal that microglial depletion decreases AABR and
306 enhances EABR network activities, explaining delayed anesthesia induction and early
307 emergence.
308

309 **Microglia-mediated anesthesia modulation is not attributed to the influence of 310 dendritic spines**

311 Microglia play important roles in spine pruning (47, 48). We asked whether microglia-
312 mediated anesthesia regulation occurs through the alteration of dendritic spines. We
313 first quantified spine density after microglial depletion for 14 days (Figure 11A). Spine
314 density in both apical and basal dendrites of layer V pyramidal cells in the medial
315 prefrontal cortex (mPFC) was not changed in the relatively short period of 14 days
316 (Figure 11B), when anesthesia induction and emergence were already robustly
317 influenced (Figure 1). The ratios of spine categories of different shapes were altered in
318 microglia-depleted mice. In apical dendrites, the percentage of mature mushroom
319 spines was increased, whereas the percentage of filopodia spines was reduced (Figure
320 11B). In basal dendrites, mature mushroom spines were increased, while thin and
321 filopodia spines were decreased (Figure 11B). Thus, short-term microglial depletion for

322 14 days does not alter spine density but changes the percentage of different categories
323 of spines, even though general anesthesia is dramatically influenced. Next, we ask
324 whether the altered spine density influences general anesthesia response. Microglia
325 phagocytose dendritic spines via the C1q “eat me” signal (8, 47, 49). The dendritic
326 spine is remodeled in C1q-deficient mice with increased density (50). We thus examined
327 anesthesia induction and emergence in C1qa^{-/-} mice (Figure 11C). Even with the
328 alteration of spine density, the pentobarbital-, propofol- or ketamine-induced LORR
329 and RORR were not influenced in C1q-deficient mice (Figure 11D). Therefore, our
330 results reveal that even though microglia contribute to spine plasticity, microglia-
331 mediated anesthesia modulation does not result from spine pruning.

332

333 **Intracellular calcium in microglia regulates the anesthesia response through
334 P2Y12 signaling**

335 Microglial P2Y12 is a G protein-coupled receptor (GPCR) that modulates neuronal
336 activity (11). We thus asked whether microglia-mediated anesthesia modulation is
337 dependent on P2Y12. To address this question, we utilized the selective P2Y12
338 antagonist 2-MeSAMP (51) to block P2Y12 signaling by intracranial guide tube
339 implantation (Figure 12A). Ninety minutes after 2-MeSAMP administration, brain
340 microglia exhibited a more reactive morphology (Figure 12B), the consequence of
341 decreased P2Y12 signaling, as shown in previous studies (52). Pharmacological
342 inhibition of P2Y12 by 2-MeSAMP delayed the LORR and accelerated the RORR of
343 pentobarbital-induced anesthetization (Figure 12C). To further confirm the function of
344 microglial P2Y12 in the anesthesia response, we conditionally knocked out P2Y12 in
345 microglia in CX3CR1^{+/CreER}::P2Y12^{fl/fl} mice (Figure 12D). After 4 doses of tamoxifen,
346 the majority of P2Y12 was successfully knocked out in CX3CR1^{+/CreER}::P2Y12^{fl/fl} mice
347 (Figure 12E). CX3CR1^{+/CreER}::P2Y12^{fl/fl} mice exhibited delayed LORR and early
348 RORR in response to pentobarbital (Figure 12F), echoing the pharmacological
349 inhibition by 2-MeSAMP. Interestingly, when the conditional knockout of microglial
350 P2Y12 was induced at a lower efficacy in TMEM119^{CreER/CreER}::P2Y12^{fl/fl} mice (Figure
351 12G-H), as the recombinase activity of TMEM119-CreER is lower than that of

352 CX3CR1-CreER (53, 54), TMEM119^{CreER/CreER}..P2Y12^{f/f} mice displayed an early
353 RORR in response to pentobarbital, but the LORR was not affected (Figure 12I). The
354 results indicate that microglia regulate the anesthesia response through P2Y12 signaling
355 in a dose-dependent manner.

356

357 **The influence of microglia replacement to the general anesthesia**

358 In 2020, we first developed efficient strategies for microglia replacement and proposed
359 therapeutic applications for neurological disorders (18). Microglia replacement by bone
360 marrow transplantation (Mr BMT or mrBMT), one of the replacement strategies, can
361 induce bone marrow cells (BMCs) to differentiate into microglia-like cells and
362 efficiently replace endogenous microglia in the whole CNS (18, 55). Despite sharing
363 similar characteristics to endogenous microglia, the replaced cells are P2Y12⁻ (Figure
364 13A-B) as we previously reported (18). We thus reasoned that if microglial P2Y12
365 indeed influences the response to general anesthesia, Mr BMT-treated mice with
366 P2Y12⁻ microglia should display a dampened response to anesthetics. To this end, we
367 examined anesthesia induction and emergence in Mr BMT mice (Figure 13A). We
368 found that Mr BMT mice exhibited delayed LORR and early RORR (Figure 13C),
369 further echoing the important regulatory role of P2Y12 in the response to general
370 anesthesia.

371

372 **Microglial intracellular calcium regulates general anesthesia**

373 Purinergic activation of P2Y12 increases intracellular Ca²⁺ (23-25). We thus reasoned
374 that the modulation of the anesthesia response is mediated by downstream Ca²⁺
375 signaling. To this end, we ectopically expressed a chemogenetic receptor in microglia
376 by CX3CR1^{+/CreER}..:hM3Dq-YFP^{+/−} (Figure 14A-B). hM3Dq is a modified human M3
377 muscarinic (hM3) receptor that activates G_{aq} upon clozapine-N-oxide (CNO) and in
378 turn enhances its downstream Ca²⁺ concentration (56, 57). When we treated
379 CX3CR1^{+/CreER}..:hM3Dq-YFP^{+/−} with CNO to elevate the intracellular Ca²⁺ level in
380 microglia, the LORR to pentobarbital was accelerated, and RORR was delayed (Figure
381 14C). On the other hand, STIM1 is an endoplasmic reticulum Ca²⁺ sensor. The lack of

382 STIM1 results in impaired store-operated Ca^{2+} influx (58-60). To specifically disrupt
383 intracellular Ca^{2+} signaling in microglia, we conditionally knocked out STIM1 in
384 microglia in CX3CR1^{+/CreER}::STIM1^{fl/fl} mice. After tamoxifen induction, *Stim1* mRNA
385 was significantly reduced in the CX3CR1^{+/CreER}::STIM1^{fl/fl} mouse brain (Figure 14D-
386 E). We found that with impaired Ca^{2+} signaling in microglia, CX3CR1^{+/CreER}::STIM1^{fl/fl}
387 mice displayed delayed anesthesia induction and early emergence (Figure 14F). By
388 both enhancing and disrupting microglial Ca^{2+} , our results reveal that intracellular Ca^{2+}
389 in microglia facilitates the anesthesia process.

390

391 Purinergic activation of P2Y12 enhances intracellular Ca^{2+} (23-25). Our results thus
392 indicate that microglia regulate the anesthesia process through P2Y12 and its
393 downstream Ca^{2+} signaling.

394

395

396 **Discussion**

397 **The mutual interaction between microglia and neurons**

398 Previous studies have indicated that microglia exhibit increased process motility,
399 extension and territory surveillance during anesthetization and sleep (4, 5). However,
400 whether and how microglia regulate neuronal activity and contribute to anesthesia
401 response is largely unknown. Our study demonstrated an active role of microglia in
402 neuronal activity that facilitate and stabilize the anesthesia response by differentially
403 changing neuronal activity in the AABRs and EABRs. It relies on microglial P2Y12
404 and intracellular calcium, rather than the spines plasticity. Microglia and neurons
405 mutually interact with each other under both physiological and pathological conditions.
406 Previous studies have indicated that neurons can influence the morphology and function
407 of microglia through neurotransmitters and/or neuromodulators, such as GABA and
408 ATP (61, 62). On the other hand, microglia can regulate neuronal activity. Microglia in
409 the paraventricular nucleus are able to maintain the balance of sympathetic outflow and
410 suppress the pressor response under hypertensive insults (63). Chemogenetic
411 manipulations of microglia lead to a prostaglandin-dependent reduction in the
412 excitability of striatal neurons (64). This evidence reveals that although microglia are
413 resident immune cells in the brain, their functions are not limited to the immune
414 response. Our study found that during the process of general anesthesia, microglia serve
415 as an “anesthesia facilitator and stabilizer” through activating AABRs and inhibiting
416 EABRs. As a result, microglia-depleted mice are more resistant to general anesthesia.

417

418 **Microglial depletion diversely influences neuronal activities in different brain
419 regions**

420 Different nuclei are involved in the response to general anesthesia. The influences of
421 microglial depletion on neuronal activity among these nuclei are different. After
422 microglial ablation, c-Fos expression is decreased in AABRs but increased in EABRs.
423 Meanwhile, the electrophysiology results also show that the E/I ratio is differentially
424 regulated in different brain regions upon microglial depletion. However, the mechanism

425 behind brain region-specific regulation is unclear. Several hypotheses may explain the
426 microglia-mediated diverse regulations among different brain regions. First, it may be
427 due to microglial heterogeneity among brain regions. Nonetheless, recent study
428 indicated that the cross-regional heterogeneity in adulthood was over estimated in
429 previous studies (65). Second, different neuronal cell types may differentially respond
430 to microglial depletion. Taking the adenosine receptor as an example. The adenosine
431 concentration is reduced in the microglia-depleted cortex (66). Interference of P2Y12,
432 CD39 or CD73 in microglia disrupts the metabolism of extracellular adenosine in the
433 brain (67). Our results showed that inhibition or knockout of P2Y12 results in resistance
434 of general anesthesia, indicating that neurons in different brain regions, e.g., the AABRs
435 and EABRs, differentially respond to adenosine. Moreover, adenosine receptor
436 subtypes are discriminately distributed across different brain regions (68), suggesting
437 neurons of different adenosine receptors in the different brain regions may differentially
438 respond to adenosine. Third, the brain region-specific regulation may also rely on the
439 neural circuitry. Mutual innervations of the AABRs-EABRs and local circuitries within
440 specific brain region can result in the diverse neuronal response. The regulation of
441 neuronal activity is an overall effect that integrated with multiple variables. So does the
442 microglial contribution to the different brain regions.

443

444 **Molecular mechanisms of how microglia regulate neuronal activity**

445 The C1q-dependent spine pruning by microglia mediates memory forgetting (69). With
446 increased number of dendritic spines, C1q-deficient mice display enhanced synaptic
447 connectivity and seizure susceptibility (70). These indicate the importance of C1q in
448 maintaining the neural function. However, we did not observe the alteration of general
449 anesthesia response. Moreover, the spine density was not changed upon the short-term
450 microglial depletion for 14 days. Consequently, our results indicate the microglia-
451 mediated regulation of anesthesia response does not result from dendritic spine. In
452 striatum, microglia serve as a brake suppressing neuronal activity (12). P2Y12
453 knockout in microglia augments the epilepsy susceptible (71, 72). These evidences
454 indicate that P2Y12 signaling is critical to the stability of neuronal network. However,

455 when we compared neuronal activities by c-Fos staining and patch clamp recording
456 between the AABRs and EABRs, we observed different consequences to the microglial
457 depletion. With suppressed neuronal activity in AABRs and enhanced neuronal activity
458 in EABRs, microglial depletion results in delayed anesthesia induction and early
459 emergence. Our results also indicate that microglia sophisticatedly and diversely
460 contribute to orchestrating the CNS function, rather than play an indiscriminate role of
461 negative feedback control as they do in the striatum (12). Notably, P2Y12 is down
462 regulated in several neurological disorders (73). The consequences of P2Y12
463 downregulation in neurological disorders are largely unknown. It would be a potential
464 therapeutic target to harness neurological disorders.

465

466 **Microglia replacement and general anesthesia**

467 In 2020, we first developed three strategies to achieve efficient microglia replacement
468 (18), including Mr BMT (18, 55), microglia replacement by peripheral blood (Mr PB
469 or mrPB) (18, 74) and microglia replacement by microglia transplantation (Mr MT or
470 mrMT) (18, 75). We discussed a potential application for treating Alzheimer's disease
471 (AD) by replacing microglia deficient in TREM2, which is one of the major risk factors
472 in sporadic AD (76-84), with TREM2-normal microglia (18, 85, 86). Recent studies
473 verified this therapeutic effect of Mr BMT in an AD mouse model (87, 88),
474 demonstrating the clinical potential of microglia replacement. It also provides new and
475 clinically feasible strategies for treating other neurological disorders (18, 85, 86, 89).
476 Despite sharing similar characteristics with naïve microglia, Mr BMT cells are P2Y12-
477 (18). Whether P2Y12⁻ microglia-like Mr BMT cells influence the response to general
478 anesthesia is unknown. To this end, we tested the response to general anesthesia use in
479 mice treated with Mr BMT and found delayed anesthesia induction and early emergence.
480 This study not only demonstrates the role of P2Y12 signaling in regulating the response
481 to general anesthesia but also identifies an impeded general anesthesia response after
482 Mr BMT treatment.

483

484 **Optogenetic and chemogenetic manipulations in neuronal and nonneuronal cells**

485 The “activation” and “inhibition” of neurons are defined as the electrical activities (e.g.,
486 action potentials) by which neurons convey information and signals. Optogenetics and
487 chemogenetics are powerful tools widely used in manipulating neuronal electrical
488 activity to dissect neural circuitries. Optogenetics relies on light-sensitive ion channels,
489 pumps or enzymes. Channelrhodopsin-2 (ChR2) is an excitatory optogenetic tool of
490 light-sensitive cation channels from green algae (90). Ectopically expressed ChR2 in
491 neurons responds to blue light and undergoes a conformational change, which allows
492 the passive diffusion of Na^+ , Ca^{2+} , H^+ and K^+ . It thus depolarizes the membrane potential
493 and elicits action potentials in neurons (91, 92). Halorhodopsin (NpHR) is an inhibitory
494 optogenetic tool of archaeal light-driven chloride pumps. In response to yellow light,
495 the ectopically expressed NpHR in neurons actively pumps Cl^- into cells and
496 hyperpolarizes the membrane potential (93). Chemogenetic tools are based on designer
497 receptors exclusively activated by designer drugs (DREADDs) (56). hM3Dq is an
498 excitatory chemogenetic tool of genetically encoded tetracycline-sensitive G protein-
499 coupled receptor (GPCR) (94). hM3Dq responds to CNO and activates intracellular $\text{G}_{\alpha q}$.
500 Then, the elevated $\text{G}_{\alpha q}$ level enhances the intracellular Ca^{2+} concentration, thereby
501 inducing action potentials in neurons. hM4Di is an inhibitory chemogenetic tool (95).
502 hM4Di responds to CNO and engages the $\text{G}_{\alpha i}$ signaling pathway. $\text{G}_{\alpha i}$ in neurons reduces
503 intracellular Ca^{2+} (suppressing presynaptic transmitter release) and opens K^+ channels
504 (hyperpolarizing the membrane potential). Thus, the intracellular consequences from
505 optogenetic and chemogenetic manipulations can drive or suppress action potentials in
506 neurons, thus “activating” or “inhibiting” the neuron.

507
508 In contrast, the nonelectrically excitable cells, including microglia, have no action
509 potentials. The “activation” (or reactive state) and “inhibition” are not defined as
510 electrical activities. Instead, “activation” (or reactive state) and “inhibition” are defined
511 as responses to specific stimuli in diverse contexts in which nonneuronal cells
512 experience sophisticated alterations. The intracellular events of optogenetic and
513 chemogenetic tools are not directly associated with nonneuronal cell activation. Thus,
514 optogenetic and chemogenetic tools do not simply “activate” or “inhibit” nonneuronal

515 cells in the brain. In our study, we ectopically expressed hM3Dq in microglia. Upon
516 CNO administration, hM3Dq elevates intracellular Ca^{2+} levels. It does not result in
517 action potentials in microglia and thus does not “activate” microglia. Nonetheless, it is
518 a reliable chemogenetic tool for manipulating the Ca^{2+} level in microglia. We used this
519 approach to investigate the biological function of microglial Ca^{2+} in the response to
520 general anesthesia. Together, optogenetic and chemogenetic tools do not simply
521 “activate” or “inhibit” nonneuronal cells. Instead, they can be utilized to study the
522 function of nonneuronal cells regarding specific intracellular events.

523

524

525 **Methods**

526 **Animals**

527 C57BL/6J mice were purchased from SPF (Beijing) Vital River Laboratory Animal
528 Technology. P2Y12^{fl/fl} mice (72) were donated by Prof. Jiyun Peng at Nanchang
529 University. TMEM119-CreER mice (C57BL/6-Tmem119^{em1(cre/ERT2)Gfng}/J, Stock #:
530 031820) (96), CX3CR1-CreER mice (B6.129P2(C)-Cx3cr1^{tm2.1(cre/ERT2)Jung}/J, Stock#:
531 020940) (97), C1qa^{-/-} mice (B6(Cg)-C1qa^{tm1d(EUCOMM)Wtsi}/TennJ, Stock#: 31675) (98),
532 β-actin-GFP mice (C57BL/6-Tg (CAG-EGFP) 131Osb/LeySopJ, Stock#: 006567) (99)
533 and LSL-hM3Dq-YFP mice (B6N;129-Tg(CAG-CHRM3*, -mCitrine)1Ute/J, Stock#:
534 026220) (100) were purchased from Jackson Lab. STIM1^{fl/fl} mice (C57BL/6JGpt-
535 Stim1^{em1Cflo}/Gpt, Stock#: T013158) were purchased from GemPharmatech. All mice
536 were housed in the Animal Facility at the Department of Laboratory Animal Science at
537 Fudan University under a 12-hour light/dark cycle with food and water given *ad libitum*.
538 All animal experiments were conducted in accordance with the guidelines of the
539 Institutional Animal Care and Use Committee of the Department of Laboratory Animal
540 Science at Fudan University.

541

542 **Chemicals and reagents**

543 PLX5622 was formulated into the AIN-76A diet at a concentration of 1.2 g of PLX5622
544 per kilogram of diet by SYSE Bio (Cat#: D20010801). PLX73086 (Plexxikon) was
545 formulated into the AIN-76A diet at 0.2 g of PLX73086 per kilogram of diet by
546 Research Diet, Inc. (Cat#: D15180708i). The normal AIN-76A diet (control diet, CD)
547 was purchased from SYSE Bio (Cat#: PD1001). Chloral hydrate (Cat#: C104202) and
548 tamoxifen (Cat#: T137974) were purchased from Aladdin. The P2Y12 inhibitor 2-
549 MeSAMP (Cat#: HY-125989) and the DREADD agonist CNO (Cat#: HY-17366) were
550 purchased from MCE. Xylazine hydrochloride (Cat#: X1251) were purchased from
551 Sigma-Aldrich. Propofol (H20123318) was purchased from Xi'an Libang
552 Pharmaceutical. Ketamine (H20193336) was purchased from Shanghai Pharmaceutical.
553 Isoflurane (Lot#: 20230501) was purchased from RWD.

554

555 **Drug administration**

556 To pharmacologically ablate myeloid cells, mice were administered a PLX5622-
557 formulated AIN-76A diet (1.2 g PLX5622 per kilogram of diet, formulated by SYSE
558 Bio) *ad libitum* for 14 days. To pharmacologically ablate peripheral macrophages, mice
559 were administered a PLX73086-formulated AIN-76A diet (0.2 g PLX73086 per
560 kilogram of diet, formulated by Research Diet) *ad libitum* for 14 days. Control mice
561 were fed an AIN-76A control diet (CD). Since the microglial ablation efficiency by
562 CSF1R inhibition might be different between sexes (101), we utilized male mice for
563 this experiment. To efficiently induce CreER-dependent recombination, tamoxifen (150
564 mg per kg of body weight) dissolved in olive oil (Macklin, 0815210) was administered
565 via oral gavage for 4 consecutive days following our previously described procedures
566 (15-19). 2-MeSAMP (10 mM) was injected into the lateral ventricle 90 min before the
567 behavior test (52). CNO (100 µg/mL) was administered via intraperitoneal injection 90
568 min before the behavior test (102).

569

570 **Righting reflex**

571 First, the mice were placed in a box for 5 min for adaptation to the experimental
572 environment. Next, anesthesia was initiated, and the righting reflex of the mice was
573 checked every 15 s from the beginning of anesthesia. When the mice were in an
574 abnormal position (limbs up) and could not voluntarily return to the normal position,
575 this behavior was defined as the LORR. The mouse was kept in a position with its back
576 touching the ground and limbs facing upward during deep anesthesia. A thermostatic
577 heating pad (37 °C) was placed under the body to maintain body temperature. If the
578 mouse automatically returned to the normal position (all limbs touching the ground)
579 from the position where the righting reflex disappeared, it was considered to have
580 recovered. The time from the end of anesthesia to RORR was defined as the time of
581 emergence from anesthesia. All experiments were conducted between 20:00 and 4:00
582 the next day, in the same light-dark cycle of ZT 12:00 to 20:00.

583

584 **Brain tissue preparation**

585 Mice were deeply anesthetized with a mixture of ketamine hydrochloride (100 mg per
586 kg of body weight) and xylazine (10 mg per kg of body weight) by intraperitoneal
587 injection. For histological experiments, animals were sequentially transcranially
588 perfused with 0.01 M PBS and 4% paraformaldehyde (PFA) (Biosharp, Cat#: BL539A)
589 in 0.01 M PBS. Brains were then carefully harvested and postfixed in 4% PFA in 0.01
590 MPBS at 4 °C overnight.

591

592 **Cryosection preparation**

593 Brains and peripheral organs were dehydrated in 30% sucrose in 0.01 M PBS at 4 °C
594 for 3 days. After being embedded in optimal cutting temperature compound (OCT,
595 SAKURA, Cat#: 4583), brain and peripheral organ samples were frozen and stored at
596 -80 °C before sectioning. Tissue with regions of interest was cut by a cryostat (Leica,
597 CM1950) at a thickness of 35 µm.

598

599 **Immunohistochemistry and image acquisition**

600 Brain and peripheral organ sections were rinsed with 0.01 M PBS 3 times for 10 to 15
601 min, followed by blocking with 4% normal donkey serum (NDS, Jackson, Cat#: 017-
602 000-121) in 0.01 M PBS containing 0.3% Triton X-100 (Aladdin, Cat#: T109026)
603 (PBST) at room temperature (RT) for 2 hours. Then, the samples were incubated with
604 primary antibodies with 1% NDS in PBST at 4 °C overnight. After rinsing with PBST
605 for 3 changes, the samples were incubated with fluorescent dye-conjugated secondary
606 antibodies with 1% NDS in PBST with 4',6-diamidino-2-phenylindole (DAPI, 1:1000,
607 Sigma-Aldrich, D9542) at RT for 2 hours. Afterward, the samples were rinsed three
608 times before mounting with anti-fade mounting medium (SouthernBiotech, Cat#: 0100-
609 01).

610

611 Primary antibodies included rabbit anti-IBA1 (1:500, Wako, Cat#: 019-19741, Lot:
612 CAJ3125), goat anti-IBA1 (1:500, Abcam, Cat#: ab5076, Lot: GR3425808-1), rabbit
613 anti-GFP (1:1000, Invitrogen, Cat#: A11122, Lot: 2273763), rabbit anti-c-Fos (1:1000,

614 Abcam, Cat#: ab190289, Lot: GR3367372-1), and rabbit anti-P2Y12 (1:500, Sigma-
615 Aldrich, Cat#: S5768, Lot: 0000128079). Secondary antibodies included AF488
616 donkey anti-rabbit (1:1000, Jackson, Cat#: 711-545-152, Lot: 161527), AF568 donkey
617 anti-rabbit (1:1000, Invitrogen, A10042, Lot: 2433862), AF568 donkey anti-goat
618 (1:1000, Invitrogen, Cat#: A11057, Lot: 2160061), and AF647 donkey anti-goat
619 (1:1000, Jackson, Cat#: 705-605-003, Lot: 147708).

620

621 Confocal images were acquired by using an Olympus FV3000 confocal microscope
622 with a solid-state laser. Lasers with wavelengths of 405 nm, 488 nm, 561 nm and 640
623 nm were used to excite the fluorophores. 60X (oil), 40X (oil) and 20X objectives were
624 utilized. Some whole brain fluorescence images were acquired by an Olympus VS120
625 microscope equipped with a motorized stage. 10X objective was used. Z stacked focal
626 planes were acquired and maximally projected with Fiji. The brightness and contrast of
627 the image were adjusted with Fiji if necessary.

628

629 **c-Fos immunostaining and *Fos* RNAscope dual labeling**

630 Mice were fed a CD or PLX5622 for 14 days and placed alone in a quiet environment
631 for 2 h, and samples were taken 30 min after intraperitoneal injection of pentobarbital
632 sodium (80 mg/kg BW). Cryostat sections at 15 μ m were collected, and hybridizations
633 were carried out according to the manufacturer's instructions using RNAscope
634 Multiplex Fluorescent Detection Reagents V2 (Advanced Cell Diagnostics, Cat#:
635 323110, Lot: 2015636, 2019446). Briefly, sections were dehydrated in sequential
636 incubations with ethanol, followed by 30 min Protease Plus treatment and RNAscope
637 wash buffer wash. Mouse *Fos* probe (Cat#: 316921, Lot: 221048) was incubated for 2
638 h at 40 °C, followed by three amplification steps. After all these steps, general
639 immunostaining steps were performed as aforementioned.

640

641 **EEG/EMG surgery and recording**

642 The mice were initially anesthetized by 2% isoflurane and maintained under anesthesia
643 by 1% isoflurane during the surgery. Body temperature was monitored in real time and

644 kept at approximately 37 °C throughout the surgical procedure. For the EEG/EMG
645 recording experiment, two stainless steel screws were placed on the prefrontal cortex
646 (recording site) and cerebellar cortex (reference site) as EEG electrodes, and two other
647 thin stainless-steel wires were inserted into the bilateral neck muscles as EMG
648 electrodes.

649 Mice were allowed a minimum of 7 days of recovery following surgery. On the day of
650 recording, the mice were acclimated first for 20 min in a recording box, where the
651 temperature was kept at 25 °C, and the mice were allowed to move around freely. All
652 recordings were conducted between 20:00 and 24:00 Signals were amplified (Apollo I,
653 Bio-Signal Technologies, USA) and digitized at a sampling rate of 1000 Hz.

654

655 **EEG spectra analysis**

656 The raw EEG signals were down sampled to 250 Hz before analysis. The power
657 spectrum was computed using multitaper methods in the MATLAB Chronux toolbox
658 (version 2.1.2, <http://chronux.org/>), with 4 s data segments and 3-5 tapers (TW = 3, K
659 = 5). To normalize total power and to compare between groups, the power spectra were
660 normalized such that the total area under the spectra was unity (e.g., power spectral
661 density). Power spectral density analysis was performed on the data from the baseline
662 (20 min before injection), induction (slow oscillation appears for the first time and lasts
663 for more than 30 s after injection) and emergence (slow oscillation disappears for the
664 first time and lasts for more than 10 min after deep anesthesia) periods.

665 The time-frequency power spectrum (by the “cwt” function in the MATLAB wavelet
666 toolbox) was also computed using 80 Hz down sampled EEG to enhance the temporal
667 resolution.

668

669 **Root mean square of EMG**

670 The raw EMGs were further down sampled to 25 Hz. The root mean square (RMS) was
671 obtained using a 20 s moving window.

672

673 **Consciousness probability**

674 The vigilance states before and after injection of anesthetic were automatically
675 classified as *awake* and *nonawake* states by using artificial intelligence (AI)-driven
676 software Lunion Stage (<https://www.luniondata.com>, Shanghai, China) and were
677 checked manually (34). The awake probability was generated by 1,000 repeat bootstrap
678 analyses, e.g., for each repeat, we randomly selected 75% of the total animal data to
679 calculate the percentage of animals in the awake state at each time point. Any epochs
680 considered to contain significant movement artifacts were omitted from the data
681 analysis.

682

683 **Intracranial guide tube implantation and microinjection**

684 Briefly, mice were anesthetized with 3% isoflurane (RWD, Lot: 20230501) delivered
685 in 100% O₂ and then transferred to a stereotaxic frame with a mouse anesthesia mask
686 (RWD, China). The delivered isoflurane concentration was decreased to 1.5%. A
687 thermostatic heating pad (37 °C) was placed under the mouse to maintain body
688 temperature. Unilateral lateral ventricle cannulas were implanted in targeted
689 coordinates (anteroposterior: -0.5 mm; mediolateral: 1 mm; dorsoventral: -2.3 mm) in
690 8-week-old mice (103, 104). After a 7-day recovery from surgery, 5 µL of 2-MeSAMP
691 (10 mM) was injected via the guide cannula using a microsyringe pump at a rate of 0.5
692 µL/min according to the manufacturer's instructions. Behavioral tests were performed
693 90 min after the intraventricular injection.

694

695 **Microglia replacement by bone marrow transplantation (Mr BMT)**

696 Two approaches were used to achieve myeloid ablation/inhibition of Mr BMT in this
697 study. For Mr BMT by irradiation, 8-week-old recipient mice were fed PLX5622 from
698 day 0 to day 14. Then, the pretreated mice were exposed to 9 Gy X-ray irradiation on
699 day 14 (18, 55). For Mr BMT by busulfan, 8-week-old recipient mice were fed
700 PLX5622 from day 0 to day 14. Then, the mice received busulfan (25 mg/kg of body
701 weight for each day) from day 9 to day 12 by intraperitoneal injection. Afterward, 1 ×
702 10⁷ bone marrow cells harvested from the tibia and femur of the β-actin-GFP donor
703 mouse were immediately introduced into the recipient mice on day 14 via intravenous

704 injection. Then, the mice were fed a control diet. The mouse was fed neomycin (1.1 g/L)
705 in acidic water (pH 2-3) throughout the procedure of microglia replacement.

706

707 **Acute brain slice preparation for patch clamp recording and spine quantification**

708 Parasagittal slices containing the LC, SON and mPFC were obtained from mice aged
709 from postnatal day 60 (P60) to P70. Mice were deeply anesthetized with pentobarbital
710 sodium (80 mg/kg of body weight) before sacrifice by decapitation. The brain was
711 quickly removed and immersed in ice-cold sucrose-based ACSF (10 mM glucose, 213
712 mM sucrose, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 2 mM MgSO₄, 2 mM
713 CaCl₂). Acute brain slices with a thickness of 300 μ m were cut in sucrose-based ACSF
714 by a vibratome (Leica VT 1200S). Afterward, brain slices were immediately transferred
715 to an incubation chamber filled with 95% O₂ and 5% CO₂ equilibrated normal ACSF
716 (25 mM glucose, 126 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl,
717 2 mM MgSO₄ and 2 mM CaCl₂) at 34 °C for 45 min. Slices were then transferred to
718 95% O₂ and 5% CO₂ equilibrated normal ACSF at room temperature before recording.
719 Slices were then transferred to a recording chamber continuously perfused in 95% O₂
720 and 5% CO₂ equilibrated normal ACSF (approximately 60 mL/h) with the temperature
721 maintained at 34 ± 1 °C. An infrared-differential interference contrast (IR-DIC)
722 microscope (Olympus BX-51WI) was used for visualization of individual neurons.

723

724 **Patch clamp recording**

725 The intracellular solutions contained 138 mM CsCH₃SO₃, 3 mM CsCl, 2 mM MgCl₂,
726 0.2 mM EGTA, 10 mM HEPES, 2 mM ATP-Na₂ and 5 mM QX314. The pH was
727 appropriately adjusted to 7.3 by CsOH, and osmolarity was adjusted to 280-290 mOsm.
728 The electrode impedance was approximately 4 to 7 M Ω . When recording the evoked
729 EPSCs (eEPSCs), the membrane potential was held at -70 mV. After eEPSC recording,
730 the same cell was held at 0 mV to record evoked IPSCs (eIPSCs). The locations of SON
731 and LC were identified under an IR-DIC microscope based on their location and cell
732 density. The stimulating electrode was placed deep inside the nucleus and
733 approximately 50 μ m from the recorded cell. Membrane voltage and current were

734 sampled at 10-25 kHz and low-pass filtered at 2-10 kHz using the patch clamp amplifier
735 MultiClamp 700B (Molecular Devices, LLC), digitized and sampled by Micro 1401
736 with Spike2 software (Cambridge Electronic Design) or by Digidata 1440A with
737 pCLAMP 10.2 software (Molecular Devices, LLC). The evoked postsynaptic currents
738 were analyzed by MATLAB R2023a (MathWorks) and OriginPro9.1 (Originlab, Inc.).

739

740 **Biocytin filling and morphological reconstruction**

741 Coronal sections containing the mPFC, SON and LC were recorded using patch clamp
742 under a whole-cell configuration. The electrode was filled with the patch solution with
743 0.2% biocytin (Life Technologies, B1592). Neurons that maintained a stable membrane
744 potential for at least 20 min were included. Upon cessation of filling, the pipette was
745 slowly pulled out along the direction of recording until a membrane reseal was formed.
746 After a 10 min recovery, the slices were fixed in 4% paraformaldehyde overnight at
747 4 °C, cryoprotected in 30% sucrose solution (for 1 to 3 days) and incubated with AF488
748 streptavidin (1:1000, Invitrogen, Cat#: S11223, 2390711) in PBS containing 0.3%
749 Triton X-100 (Aladdin, T109026) overnight at 4 °C. To reconstruct the dendritic spines,
750 the coronal sections were resected at 70 µm thickness and coverslipped with the
751 mounting medium Fluoromount-G (Southern Biotechnology Associates). The images
752 were taken using an Olympus FV3000 confocal microscope equipped with a
753 UPLSAPO 60X oil-immersion lens (numerical aperture of 1.5). The 2048 × 2048 pixels
754 frame size was used without zooming. Serial Z-stack images with a step size of 0.7 µm
755 were collected. Dendritic segments located 30 µm away from the soma and 50 µm in
756 length were selected for analysis of spine density and category. For individual cells, 8
757 to 12 dendritic segments were chosen for analysis. Dendritic length and spines were
758 counted and categorized with ImageJ (NIH). Spines were classified into three subtypes:
759 thin, mushroom, and stubby based on previously described criteria (105). Briefly, thin
760 spines included a head-to-neck diameter ratio less than 1.1 and a length-to-spine head
761 ratio greater than 2.0. Mushroom spines had a head diameter larger than 0.5 mm and a
762 head-to-neck diameter ratio greater than 1.1. Stubby spines had no clear border between

763 the head and the attachment to the shaft. Filopodia had a long thin protrusion but
764 without a clear head shape.

765

766 **RNA extraction and qPCR**

767 Total RNA from brain tissue was extracted with TRIzol. cDNA was reverse transcribed
768 from total RNA using the Vazyme HIScript III RT SuperMix for qPCR kit according to
769 the manufacturer's instructions. Subsequently, a 20 μ L reaction system was prepared
770 for qPCR using Vazyme ChamQ Universal SYBR qPCR Master Mix kit with an ABI
771 StepOne Plus Real-Time PCR system. The relative cDNA concentrations of target
772 genes were normalized to Gapdh. The primers used in this study were synthesized by
773 Tsingke Biotechnology, including:

774 *Gapdh*-forward (TGAGGCCGGTGCTGAGTATG),

775 *Gapdh*-reverse (TGGTTCACACCCATCACAAACA),

776 *Stim1*-forward (CAGGTTCACTGAGACCCCTGTC),

777 *Stim1*-reverse (GCCCACCAAGATCTCCACAA).

778

779 **Statistics and reproducibility**

780 The statistical approaches are indicated in the figure legends. For the righting reflex
781 test, block randomization was performed on cages of mice such that an approximate
782 number of mice per cage were assigned to each experimental group. Collection of
783 behavior experiment data was double blinded. c-Fos<sup>+/Fos⁺ cell counting and spine
784 morphology analysis were evaluated independently by two blinded experienced
785 researchers by Fiji. No statistical methods were used to predetermine sample sizes, but
786 our sample sizes were similar to those reported in our previous publications (15-19,
787 106). The data distribution was assumed to be normal, but this assumption was not
788 formally tested. No data were excluded from the analyses. Data are shown as the mean
789 \pm SD or mean \pm SEM as specifically identified. The two-tailed unpaired t test, paired t
790 test and one-way or two-way repeated measures (RM) ANOVA followed by
791 Bonferroni's multiple comparisons test were used to assess statistical significance based
792 on GraphPad Prism 9.0 and MATLAB 2020b if necessary. Significance was defined as</sup>

793 P < 0.05. The layout of all of the figures was generated by Adobe Illustrator.

794

795

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812

813

814 **Author contributions**

815 B.P. accidentally observed an anesthetic-resistant phenotype in microglia-depleted mice
816 in 2015. B.P., Y.H. and Y.S. conceived and designed this study. B.P. and Y.S. supervised
817 and conceptualized this study. Y.H. performed most of the experiments and data
818 analysis unless specified. Y.H. and Q.H. conducted EEG and EMG experiments. T.L.,
819 W.K. and S.D. conducted patch clamp recordings. T.L. and Y.H. performed the spine
820 analysis. X.L. performed RNAscope. B.P., Y.S., Y.R., Y.M., F.G., and W.L. provided
821 necessary study support. All authors discussed the results and commented on this
822 manuscript.

823

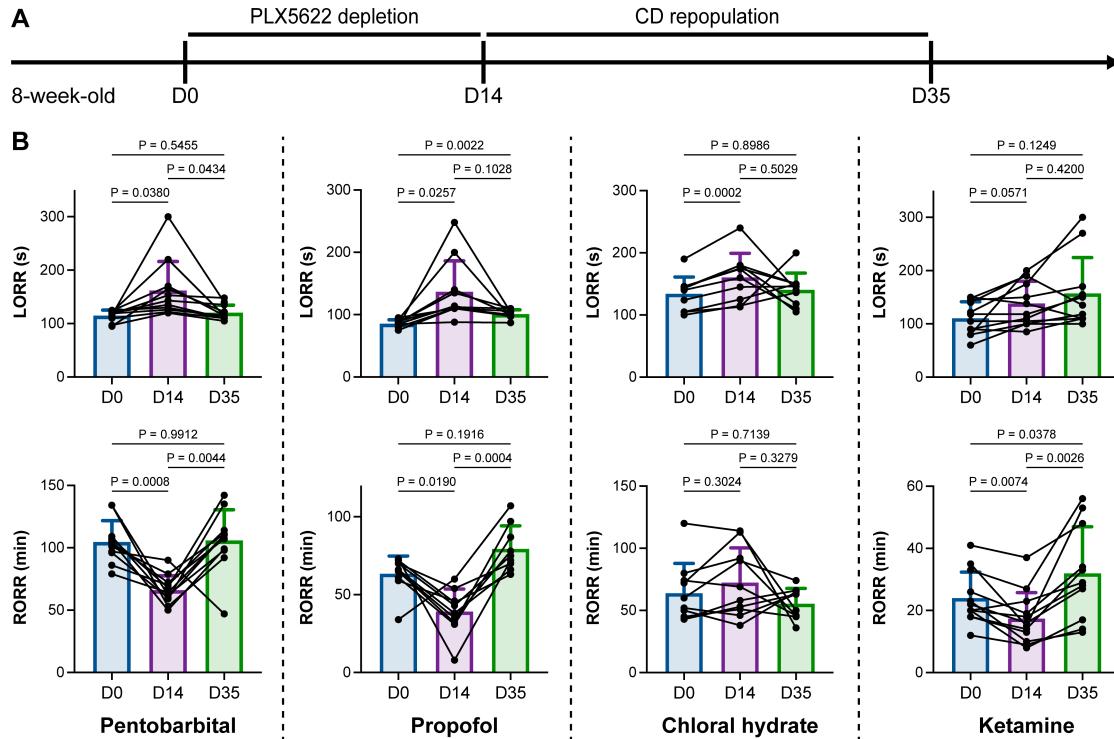
824

825 **Competing interests**

826 The authors declare no competing interests.

827 **Figures**

828



829

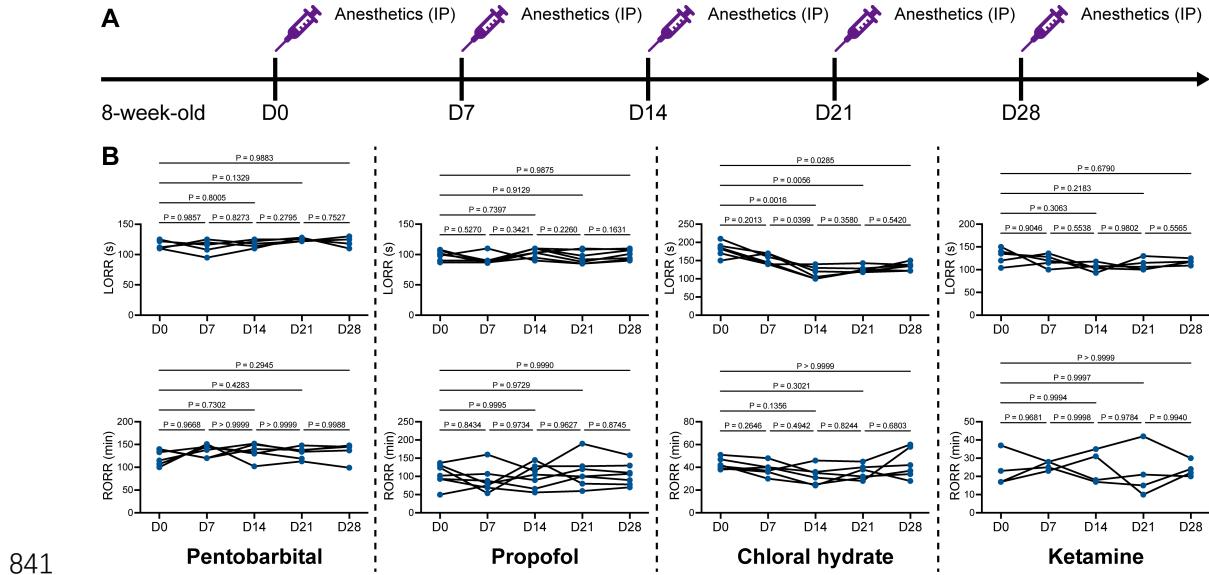
830 **Figure 1** Microglial depletion impedes anesthesia induction and accelerates emergence.

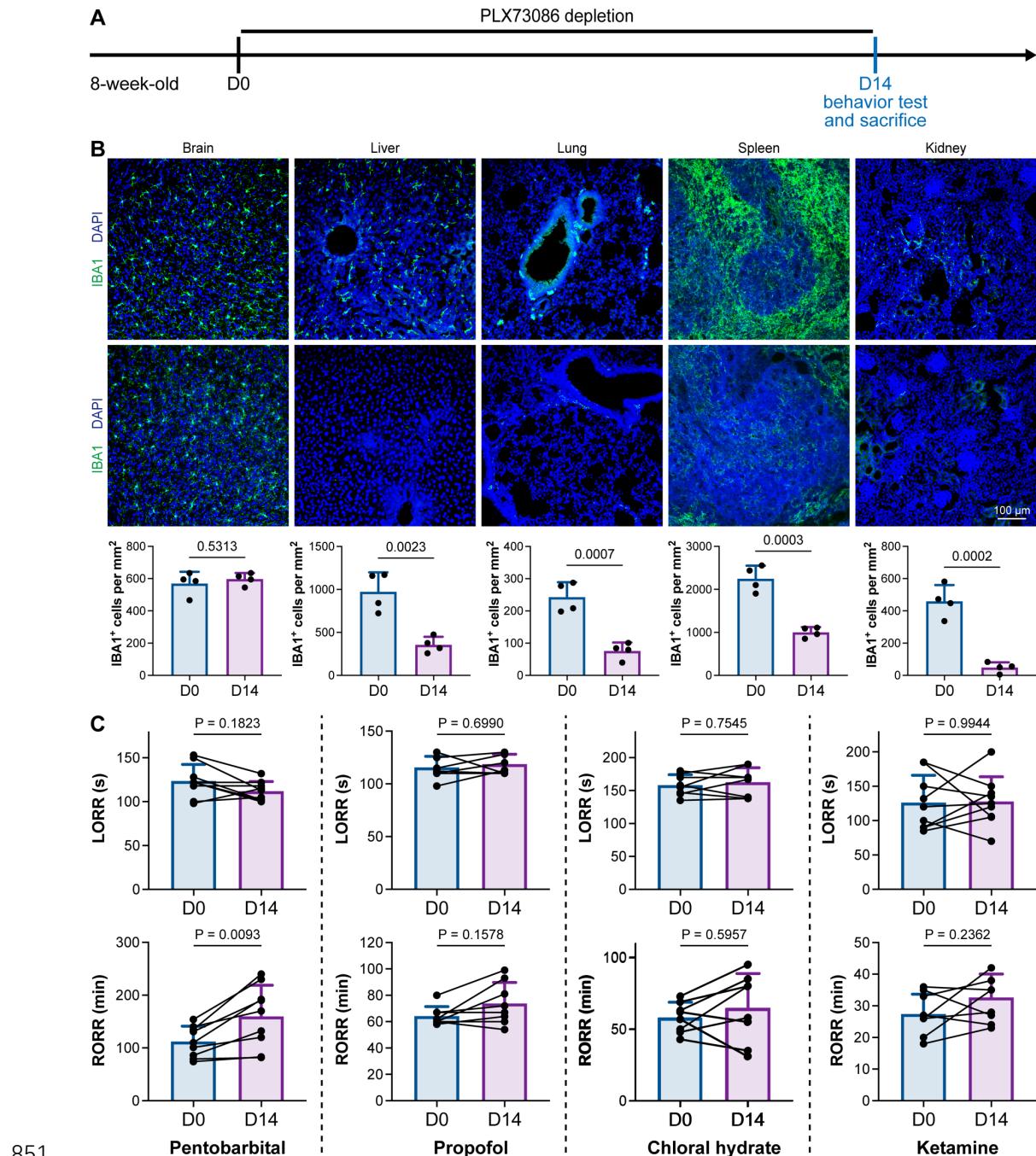
831 **(A)** Scheme of time points for microglial depletion and repopulation by PLX5622 and
832 CD.

833 **(B)** Mice exhibit delayed induction and early emergence in pentobarbital-, propofol-,
834 chloral hydrate- and ketamine-induced anesthesia. N = 11, 10, 10 and 12 mice for
835 pentobarbital, propofol, chloral hydrate and ketamine, respectively. Repeated measures
836 (paired) one-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple
837 comparison test.

838 Data are presented as mean \pm SD. PLX5622: PLX5622-formulated diet; CD: control
839 diet; LORR: loss of righting reflex; RORR: recovery of righting reflex.

840





852 **Figure 3** CSF1R inhibition-induced general anesthesia regulation is not due to the
 853 depletion of peripheral macrophages.

854 **(A)** Scheme of time points for peripheral macrophage depletion by PLX73086.

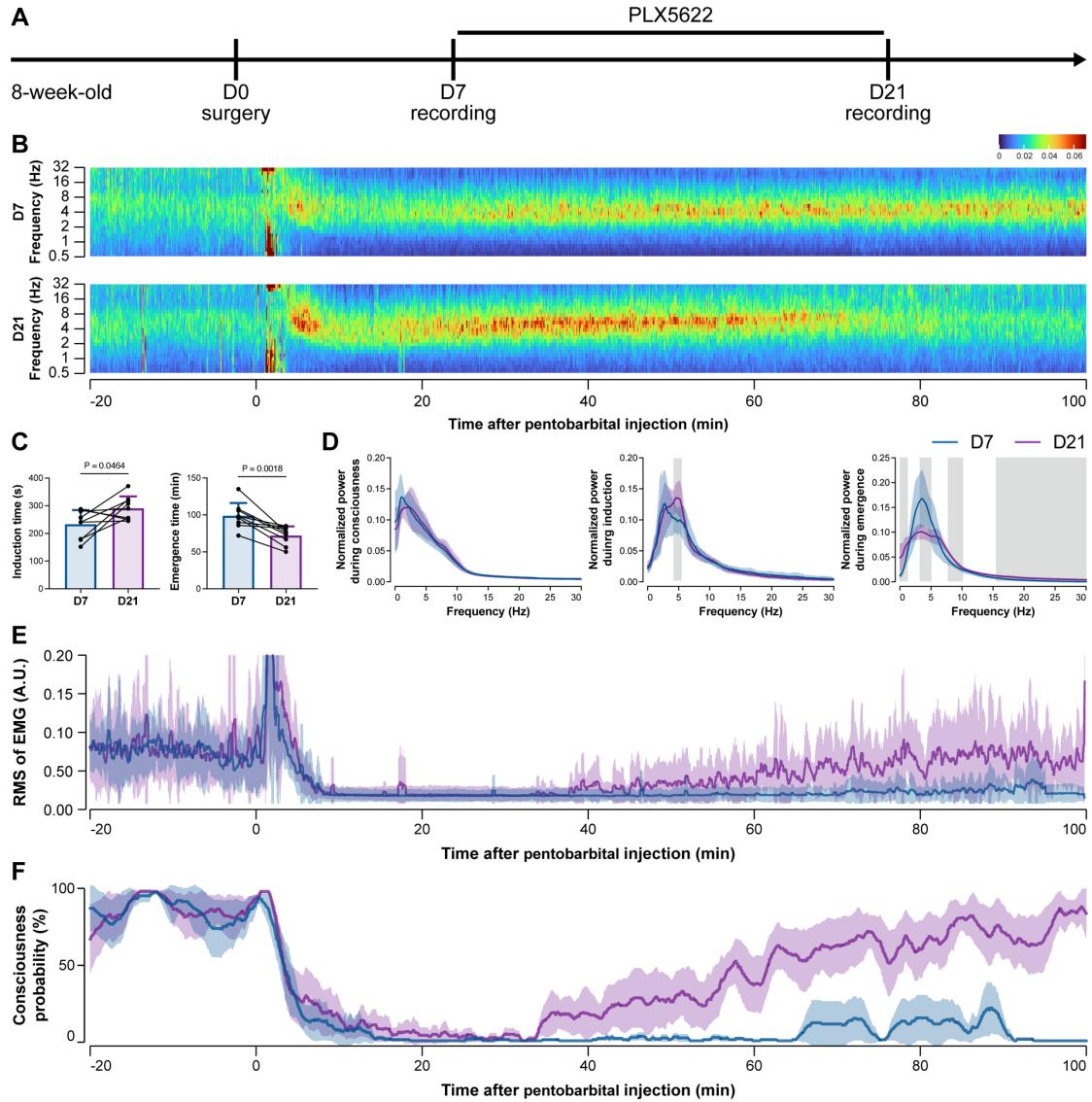
855 **(B)** CSF1R inhibition by PLX73086 dramatically ablates macrophages in the liver, lung,
 856 spleen and kidney and does not ablate brain microglia. N = 4 mice for each group. Two-
 857 tailed independent t test.

858 **(C)** Depletion of peripheral macrophages does not influence the anesthesia induction
 859 or emergence from

860 propofol, chlral hydrate and ketamine. However, it impedes anesthesia emergence
861 from pentobarbital. N = 9, 8, 8 and 9 mice for pentobarbital, propofol, chlral hydrate
862 and ketamine, respectively. Two-tailed paired t test.

863 Data are presented as mean \pm SD. PLX73086: PLX73086-formulated diet; CD: control
864 diet; LORR: loss of righting reflex; RORR: recovery of righting reflex.

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868 **Figure 4** EEG and EMG recordings reveal that mice with microglial depletion are
869 resistant to general anesthesia by pentobarbital.

870 **(A)** Scheme of time points for animal surgery, microglial depletion and EEG/EMG
871 recording.

872 **(B-D)** Microglial depletion shows no obvious change in EEG before the injection of
873 pentobarbital. Instead, it influences the EEG in anesthesia induction and emergence.
874 Two-tailed paired t test. The gray area in D indicates $P < 0.05$ between CD and
875 PLX5622.

876 **(E)** Microglial depletion does not change the EMG before the injection of pentobarbital.
877 Instead, it influences the EMG in the anesthesia process.

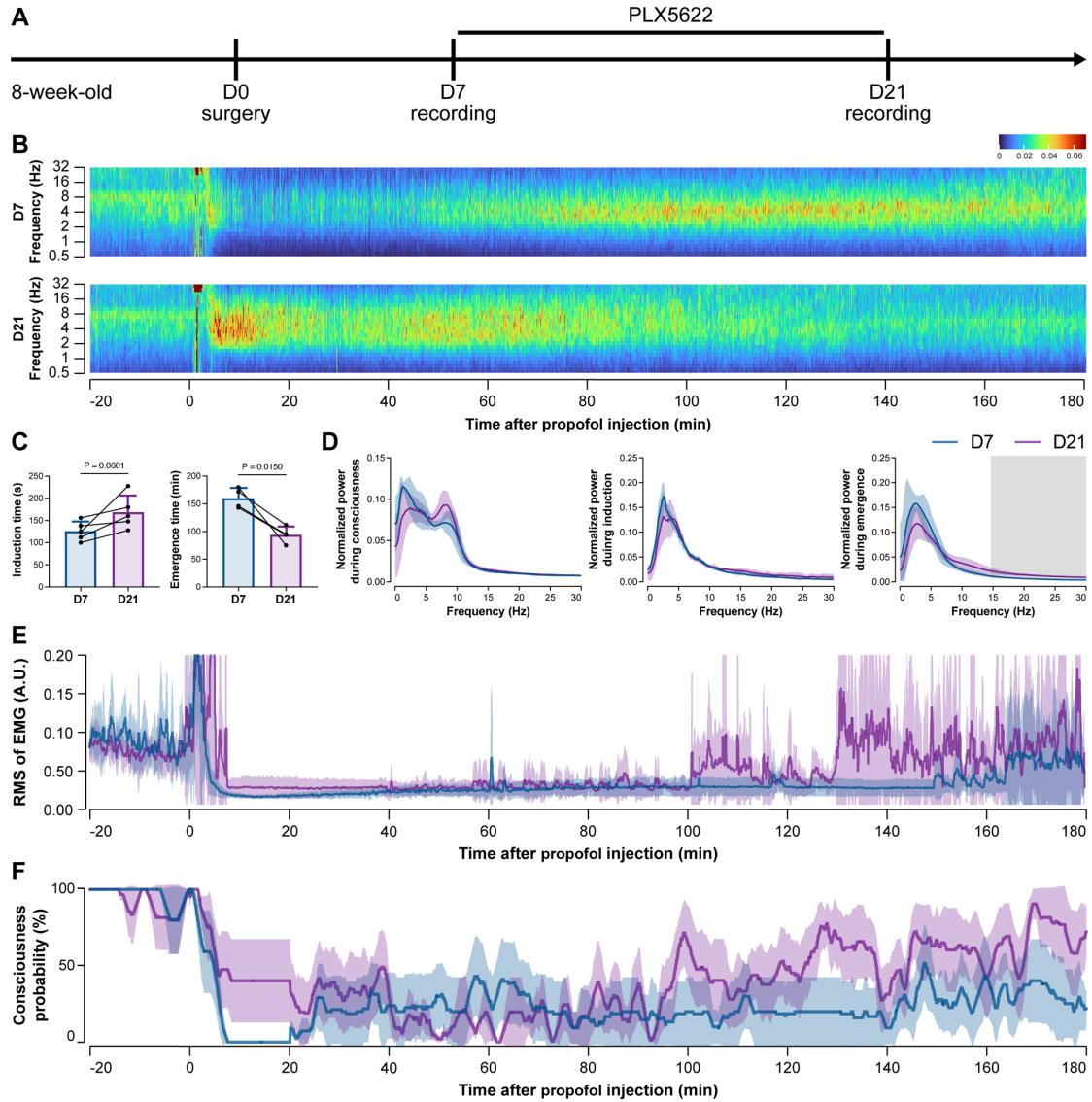
878 **(F)** Microglial depletion does not change the probability of consciousness before the

879 injection of pentobarbital. Instead, it influences the consciousness probability in the
880 anesthesia process.

881 N = 9 mice for each group. Data are presented as mean \pm SD. RMS: root mean square;
882 A.U.: arbitrary unit; PLX5622: PLX5622-formulated diet.

883

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885

886 **Figure 5** EEG and EMG recordings reveal that mice with microglial depletion are
887 resistant to general anesthesia by propofol.

888 **(A)** Scheme of time points for animal surgery, microglial depletion and EEG/EMG
889 recording.

890 **(B-D)** Microglial depletion does not change the EEG before the injection of propofol.
891 Instead, it influences the EEG in anesthesia induction and emergence. Two-tailed paired
892 t test. The gray area in D indicates $P < 0.05$ between CD and PLX5622.

893 **(E)** Microglial depletion does not change the EMG before the injection of propofol.
894 Instead, it influences the EMG in the anesthesia process.

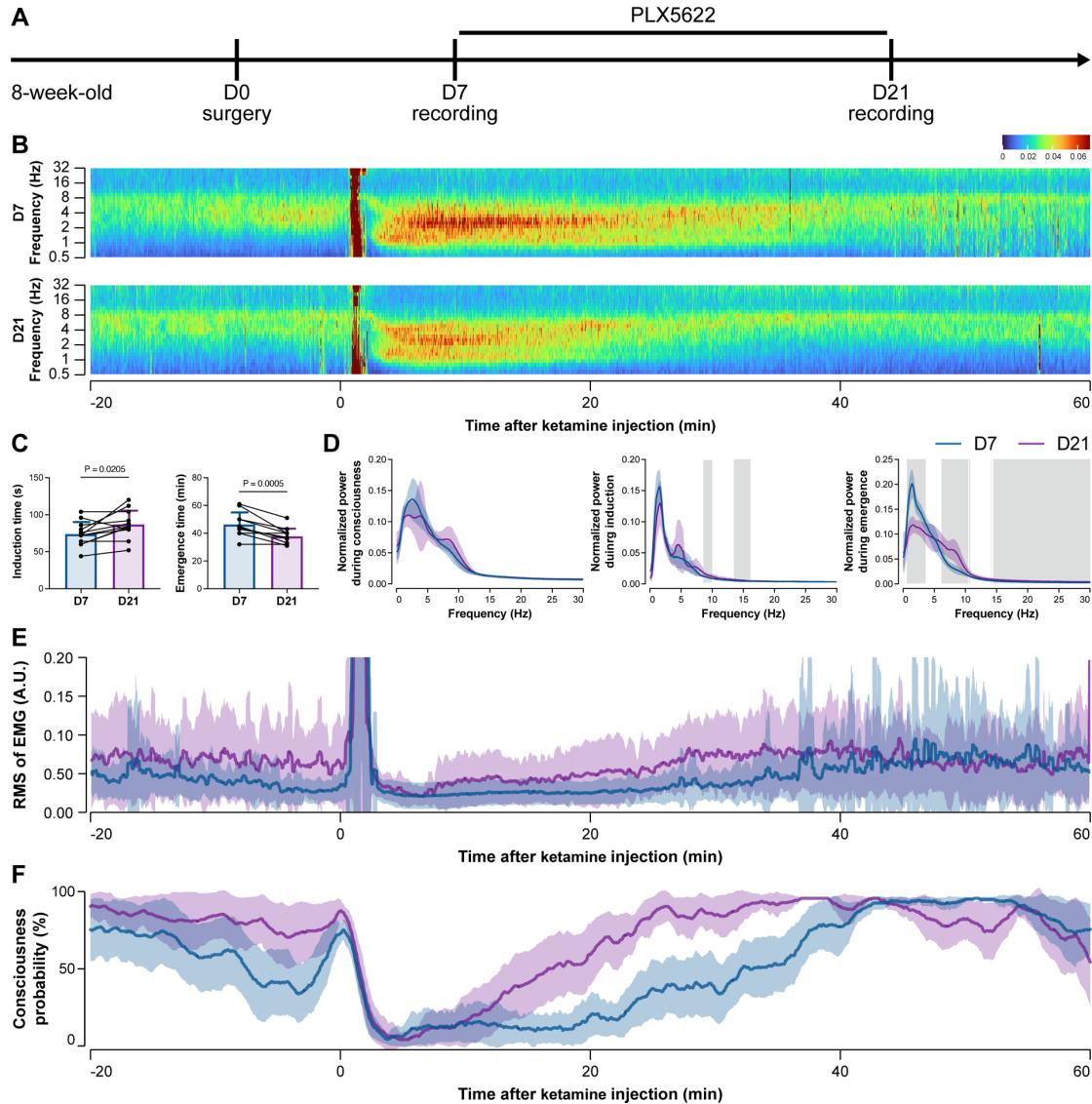
895 **(F)** Microglial depletion does not change the probability of consciousness before the
896 injection of propofol. Instead, it influences the consciousness probability in the

897 anesthesia process.

898 N = 5 mice for each group. Data are presented as mean \pm SD. RMS: root mean square;

899 A.U.: arbitrary unit; PLX5622: PLX5622-formulated diet.

900



901

902 **Figure 6** EEG and EMG recordings reveal that mice with microglial depletion are
903 mouse resistant to general anesthesia by ketamine.

904 **(A)** Scheme of time points for animal surgery, microglial depletion and EEG/EMG
905 recording.

906 **(B-D)** Microglial depletion does not change the EEG before the injection of ketamine.
907 Instead, it influences the EEG in anesthesia induction and emergence. Two-tailed paired
908 t test. The gray area in D indicates $P < 0.05$ between CD and PLX5622.

909 **(E)** Microglial depletion does not change the EMG before the injection of ketamine.
910 Instead, it influences the EMG in the anesthesia process.

911 **(F)** Microglial depletion does not change the probability of consciousness before the
912 injection of ketamine. Instead, it influences the consciousness probability in the

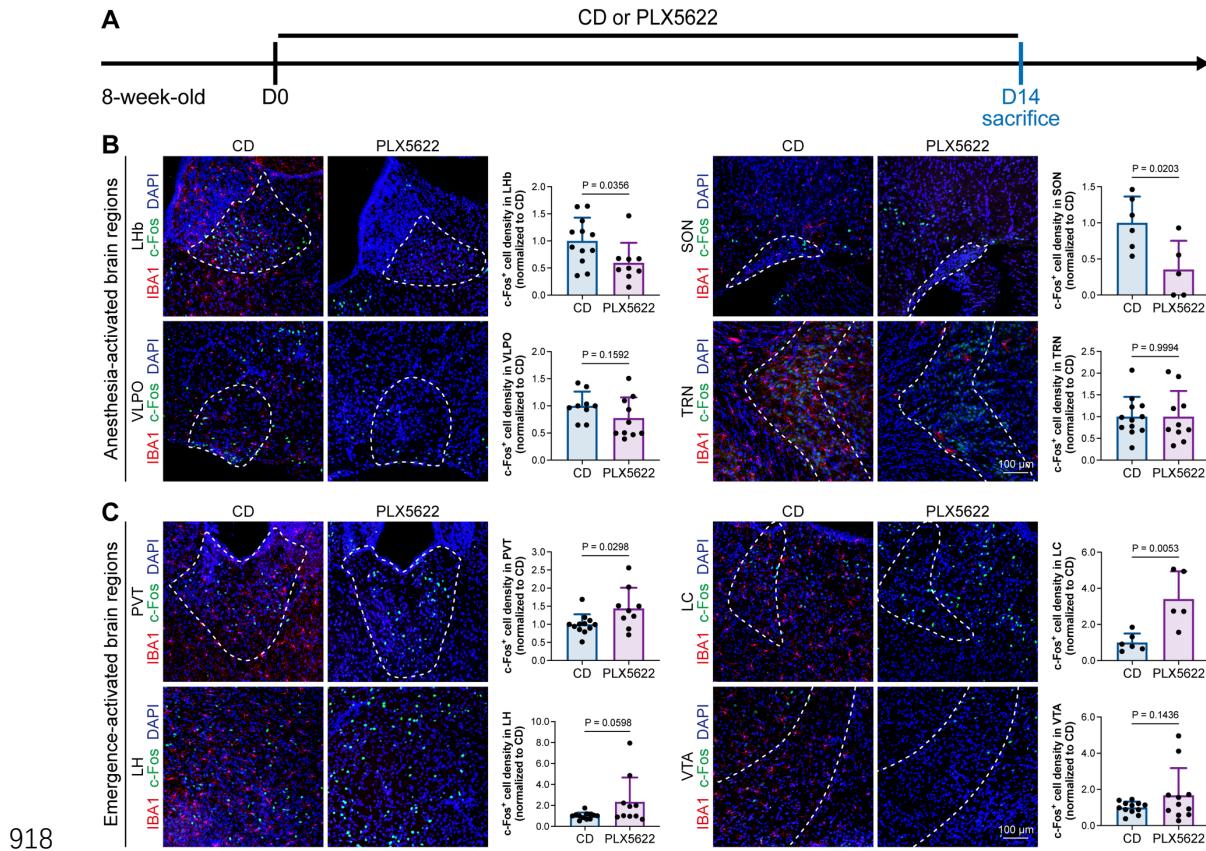
913 anesthesia process.

914 N = 12 mice for each group. Data are presented as mean \pm SD. RMS: root mean square;

915 A.U.: arbitrary unit; PLX5622: PLX5622-formulated diet.

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918 **Figure 7** Microglial depletion diversely influences neuronal activity in different
919
920 anesthesia-related brain regions.

921 **(A)** Scheme of time points for microglial depletion and examination time points.
922
923 **(B)** Influence of microglial depletion in anesthesia-activated brain regions. Microglial
924 depletion reduces neuronal activity in the LHB ($P = 0.0356$), SON ($P = 0.0203$) and
925 VLPO ($P = 0.1592$) and does not influence neuronal activity in the TRN ($P = 0.9994$).
926 N = 12, 6, 9 and 12 mice for LHB, SON, VLPO and TRN in the CD group, respectively.
927 N = 9, 5, 10 and 10 mice for LHB, SON, VLPO and TRN in the PLX5622 group,
928 respectively.

929 **(C)** Influence of microglial depletion in emergence-activated brain regions. Microglial
930 depletion enhances neuronal activity in the PVT ($P = 0.0298$), LC ($P = 0.0053$), LH (P
931 = 0.0598) and VTA ($P = 0.1436$). N = 12, 6, 12 and 12 mice for PVT, LC, LH and VTA
932 in the CD group, respectively. N = 9, 5, 10 and 11 mice for PVT, LC, LH and VTA in
933 the PLX5622 group, respectively.

934 Two-tailed independent t test. Data are presented as mean \pm SD. PLX5622: PLX5622-
935 formulated diet; CD: control diet; LHB: lateral habenula; SON: supraoptic nucleus;

935 VLPO: ventrolateral preoptic nucleus; TRN: thalamic reticular nucleus; PVT:
936 paraventricular thalamus; LC: locus coeruleus; LH: lateral hypothalamus; VTA: ventral
937 tegmental area.
938

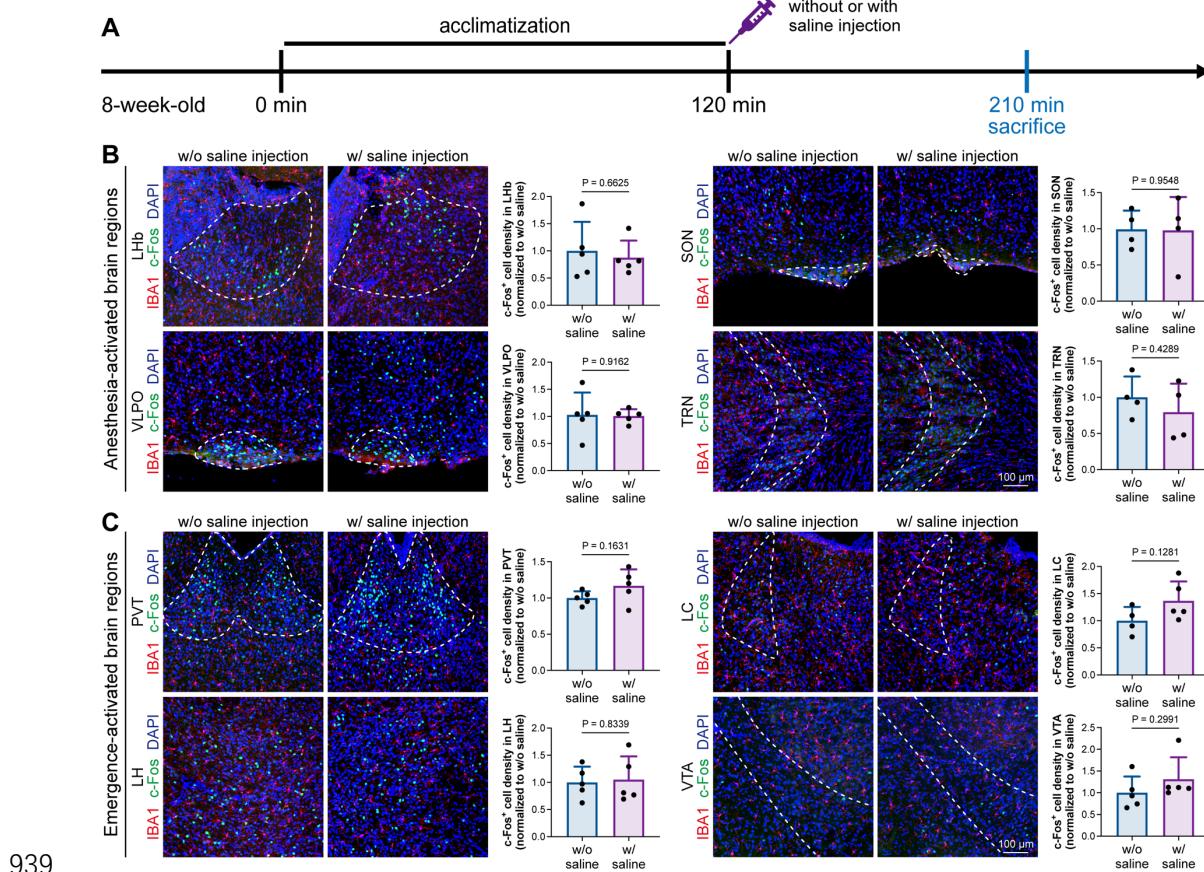


Figure 8 Animal handling and intraperitoneal injection do not influence neuronal activity in anesthesia-related brain regions.

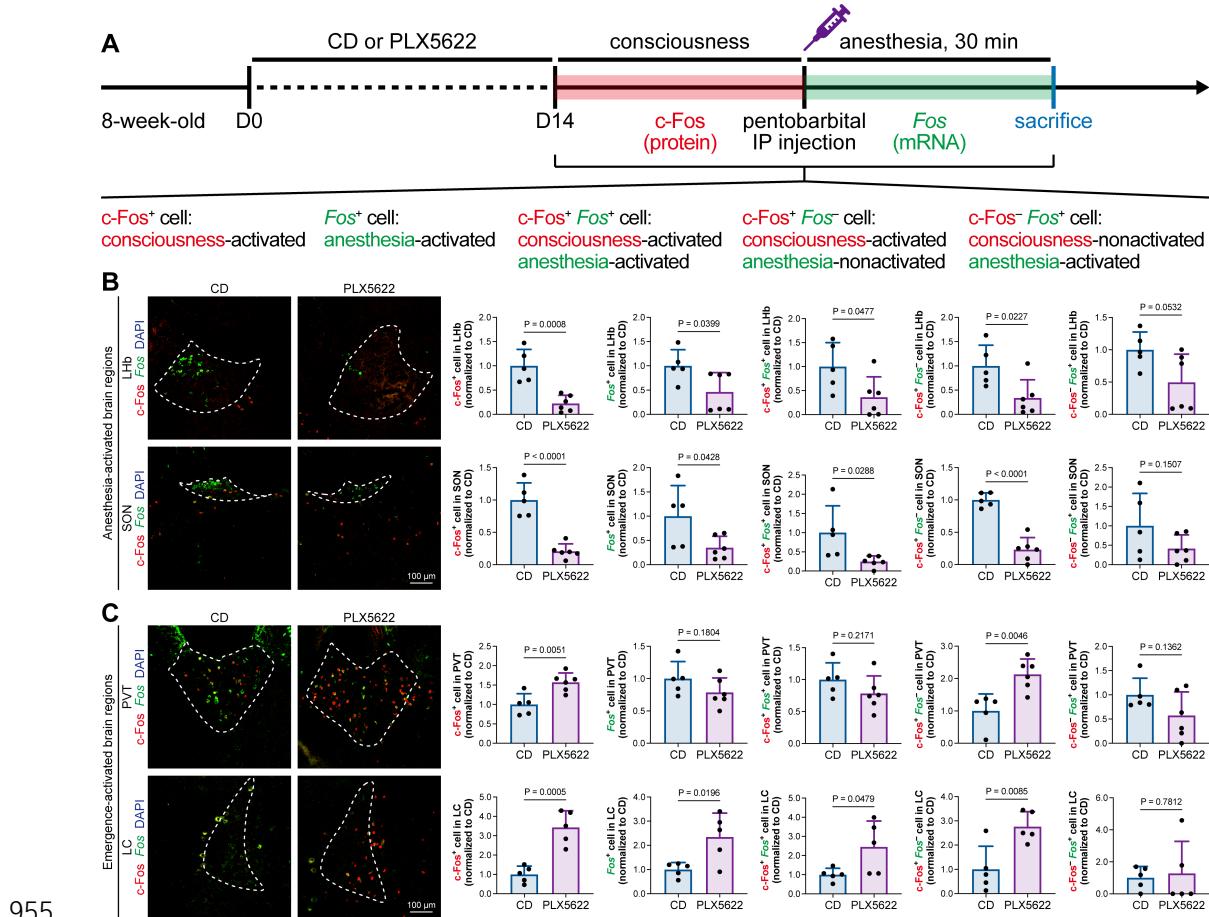
(A) Scheme of time points for microglial depletion and examination time points.

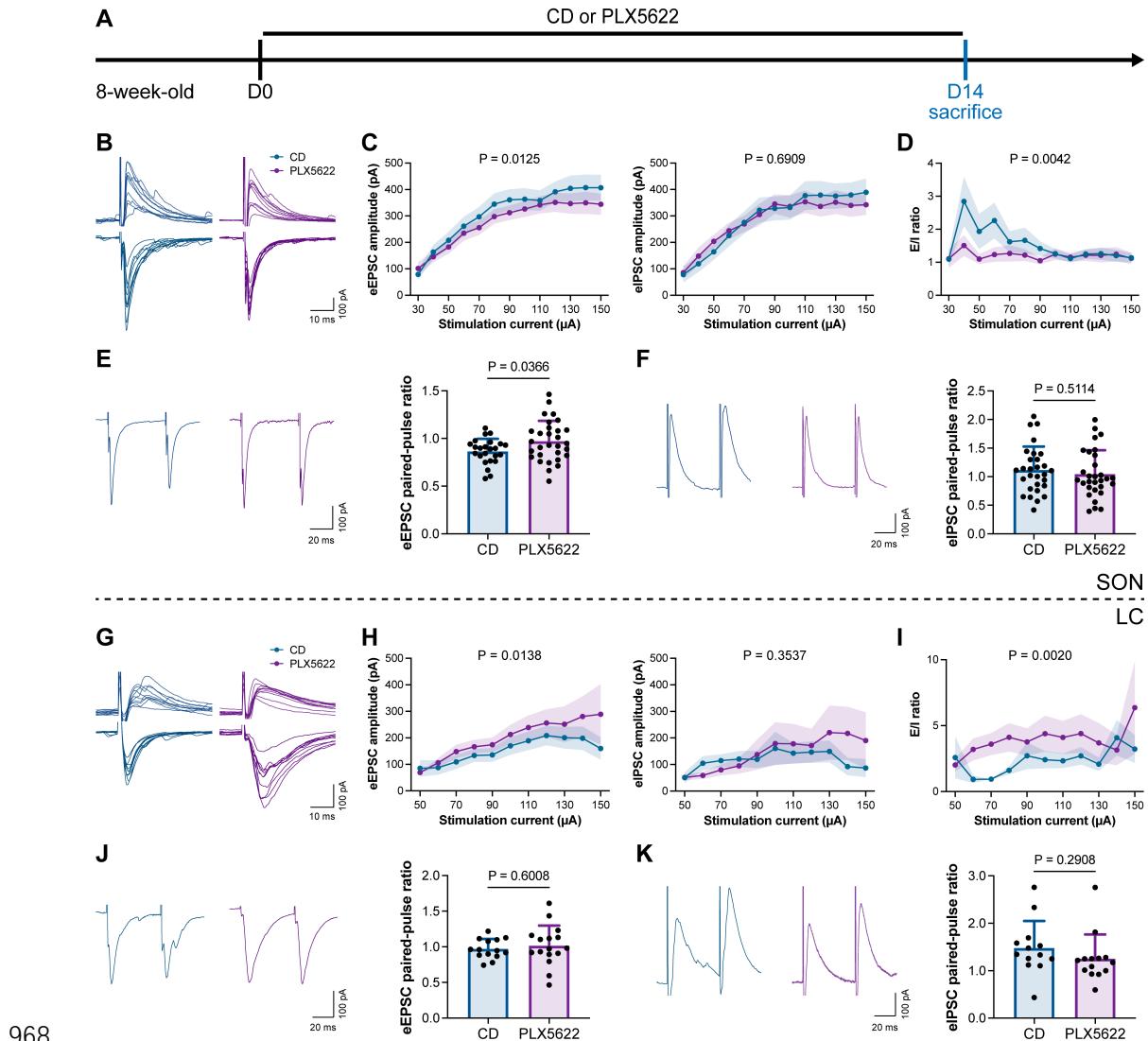
(B) Microglial depletion does not influence neuronal activity in the LHB, SON, VLPO or TRN. N = 5, 4, 5 and 4 mice for LHB, SON, VLPO and TRN in the CD group, respectively. N = 5, 4, 5 and 4 mice for LHB, SON, VLPO and TRN in the PLX5622 group, respectively.

(C) Microglial depletion does not influence neuronal activity in the PVT, LC, LH or VTA. N = 5, 4, 5 and 5 mice for PVT, LC, LH and VTA in the CD group, respectively.

N = 5, 5, 5 and 5 mice for PVT, LC, LH and VTA in the PLX5622 group, respectively.

Two-tailed independent t test. Data are presented as mean \pm SD. PLX5622: PLX5622-formulated diet; CD: control diet; LHB: lateral habenula; SON: supraoptic nucleus; VLPO: ventrolateral preoptic nucleus; TRN: thalamic reticular nucleus; PVT: paraventricular thalamus; LC: locus coeruleus; LH: lateral hypothalamus; VTA: ventral tegmental area.





969 **Figure 10** Microglial depletion reduces the E/I ratio in SON but enhances the E/I ratio
970 in LC.

971 **(A)** Scheme of time points for microglial depletion by PLX5622.
972 **(B)** Representative traces for evoked postsynaptic currents in the SON to 10 increasing
973 stimulation currents.
974 **(C)** Amplitudes of evoked postsynaptic currents in the SON in response to increasing
975 electrical stimulation intensities. Two-way ANOVA. Data are presented as mean \pm SEM.
976 **(D)** E/I ratios with different stimulation intensities in the SON. N = 21 cells from 5 mice
977 for each group. Two-way ANOVA. Data are presented as mean \pm SEM.
978 **(E)** Representative traces (left) and quantitative results (right) show that PLX5622-
979 treated mice exhibited a higher eEPSC PPR in SON. N = 24 (CD) and 30 (PLX5622)

980 cells from 5 mice for each group. Two-tailed independent t test. Data are presented as
981 mean \pm SD.

982 **(F)** Representative traces (left) and quantitative results (right) show that PLX5622-
983 treated mice exhibited a similar eIPSC PPR in SON. N = 29 (CD) and 30 (PLX5622)
984 cells from 5 mice for each group. Two-tailed independent t test. Data are presented as
985 mean \pm SD.

986 **(G)** Representative traces for evoked postsynaptic currents in the LC in response to 10
987 increasing stimulation currents.

988 **(H)** Amplitudes of evoked postsynaptic currents in the LC in response to increasing
989 electrical stimulation intensities. in response to the electrical stimulation. N = 15 (EPSC
990 CD), 18 (EPSC PLX5622), 15 (IPSC CD) and 18 (IPSC PLX5622) cells from 5 mice
991 for each group. Two-way ANOVA. Data are presented as mean \pm SEM.

992 **(I)** E/I ratios with different stimulation currents in the LC. N = 15 (EPSC CD), 18
993 (EPSC PLX5622), 15 (IPSC CD) and 18 (IPSC PLX5622) cells from 5 mice for each
994 group. Two-way ANOVA. Data are presented as mean \pm SEM.

995 **(J)** Representative traces (left) and quantitative results (right) show that PLX5622-
996 treated mice exhibited a similar eEPSC PPR in the LC. N = 14 (CD) and 16 (PLX5622)
997 cells from 5 mice for each group. Two-tailed independent t test. Data are presented as
998 mean \pm SD.

999 **(K)** Representative traces (left) and quantitative results (right) show that PLX5622-
1000 treated mice exhibited a similar eIPSC PPR in the LC. N = 13 (CD) and 14 (PLX5622)
1001 cells from 5 mice for each group. Two-tailed independent t test. Data are presented as
1002 mean \pm SD.

1003 PLX5622: PLX5622-formulated diet; CD: control diet. eEPSC: evoked excitatory
1004 postsynaptic current; eIPSC: evoked inhibitory postsynaptic current.

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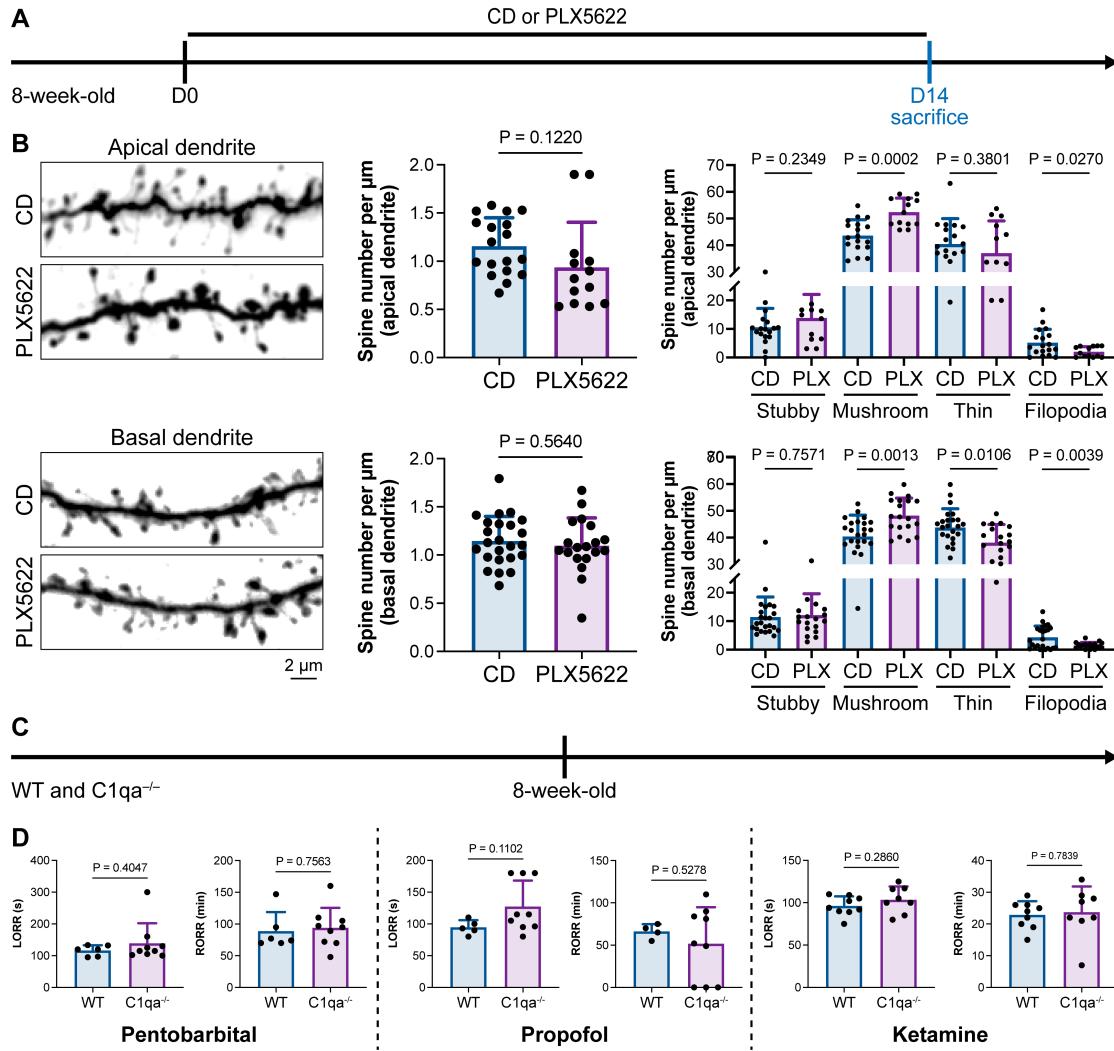


Figure 11 Interruption of the spine “eat me” signal by $\text{C1qa}^{-/-}$ does not influence the anesthesia process and microglial depletion alters the proportion of spine categories.

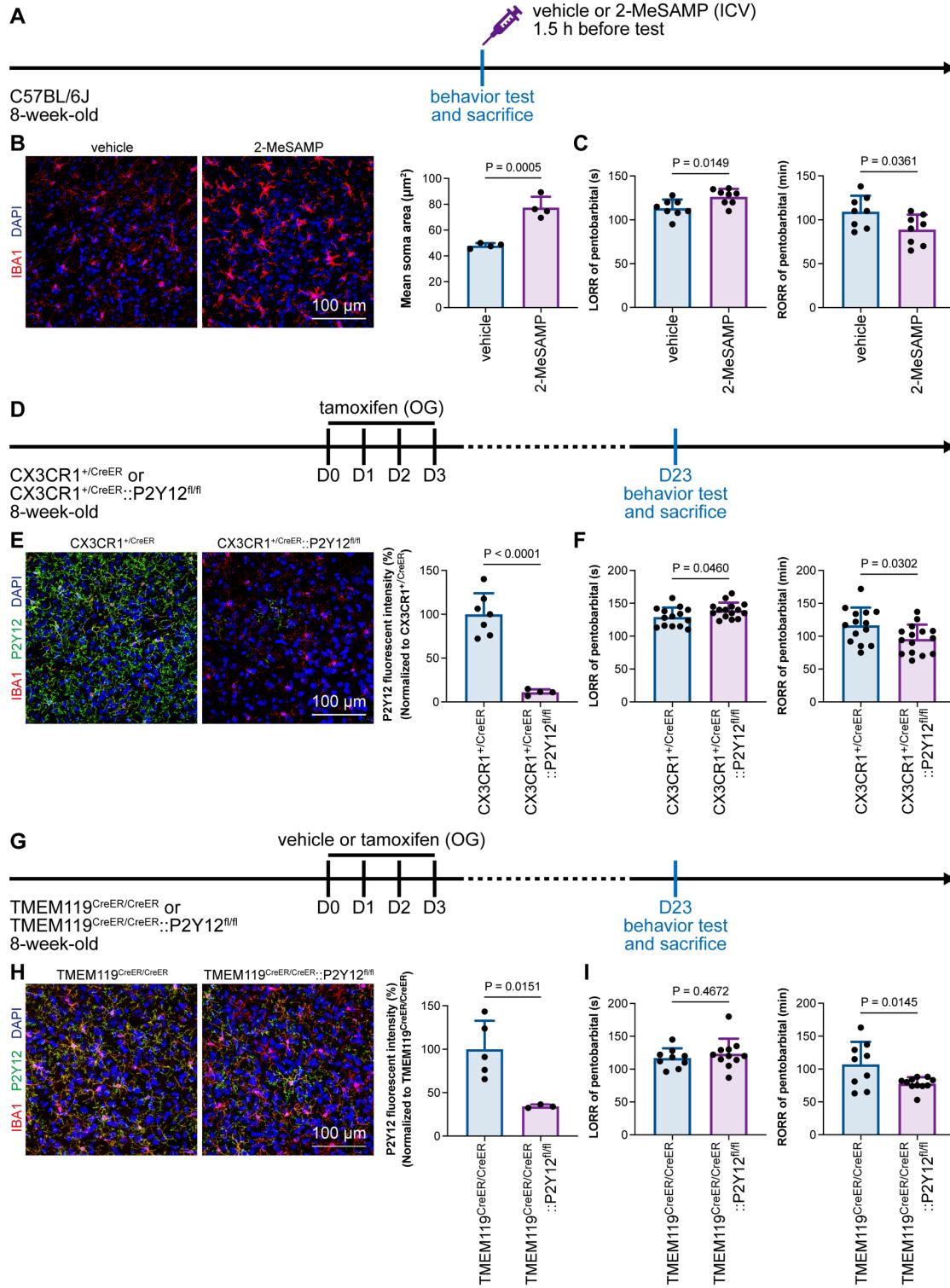
(A) Scheme of time points for microglial depletion and examination time points.

(B) CSF1R inhibition for 14 days does not influence spine density but changes the proportion of spine subtypes. $N = 18$ and 13 cells from 5 mice for each group of apical spines, $N = 24$ and 19 cells from 5 mice for each group of basal spines.

(C) Scheme of LORR and RORR tests in wild-type and $\text{C1qa}^{-/-}$ mice.

(D) C1q knockout does not influence anesthesia induction and emergence in response to pentobarbital, propofol and ketamine. $N = 6$ (pentobarbital WT), 9 (pentobarbital $\text{C1qa}^{-/-}$), 5 (propofol WT), 9 (propofol $\text{C1qa}^{-/-}$), 9 (ketamine WT) and 8 (ketamine $\text{C1qa}^{-/-}$).

1020 Two-tailed independent t test. Data are presented as mean \pm SD. PLX5622: PLX5622-
1021 formulated diet; CD: control diet; LORR: loss of righting reflex; RORR: recovery of
1022 righting reflex.



1023

1024 **Figure 12** Microglial P2Y12 regulates the induction and emergence of anesthesia.

1025 **(A)** Scheme of 2-MeSAMP administration and behavior tests for anesthesia.

1026 **(B)** P2Y12 inhibition by 2-MeSAMP drives microglia to a more reactive state. N = 4
1027 mice for each group.

1028 **(C)** P2Y12 inhibition by 2-MeSAMP results in delayed anesthesia induction and early
1029 emergence. N = 8 mice for each group.

1030 **(D)** Scheme of animal treatment and examination time points for CX3CR1^{+/CreER} and
1031 CX3CR1^{+/CreER}::P2Y12^{fl/fl} mice.

1032 **(E)** Tamoxifen induces efficient P2Y12 knockout in CX3CR1^{+/CreER}::P2Y12^{fl/fl} mice. N
1033 = 7 mice for the CX3CR1^{+/CreER} group and 4 mice for the CX3CR1^{+/CreER}::P2Y12^{fl/fl}
1034 group.

1035 **(F)** Efficient knockout of P2Y12 significantly elongates the LORR and shortens the
1036 RORR. N = 14 mice for the CX3CR1^{+/CreER} group and 15 mice for the
1037 CX3CR1^{+/CreER}::P2Y12^{fl/fl} group.

1038 **(G)** Scheme of animal treatment and examination time points for TMEM119^{CreER/CreER}
1039 and TMEM119^{CreER/CreER}::P2Y12^{fl/fl} mice.

1040 **(H)** Tamoxifen induces relatively low efficiency of P2Y12 knockout in
1041 TMEM119^{CreER/CreER}::P2Y12^{fl/fl} mice. N = 5 mice for the TMEM119^{CreER/CreER} group
1042 and 3 mice for the TMEM119^{CreER/CreER}::P2Y12^{fl/fl} group.

1043 **(I)** Low-efficiency knockout of P2Y12 does not affect anesthesia induction but
1044 significantly shortens the emergence time. N = 9 mice for the TMEM119^{CreER/CreER}
1045 group and 11 mice for the TMEM119^{CreER/CreER}::P2Y12^{fl/fl} group.

1046 Two-tailed independent t test. Data are presented as mean \pm SD. ICV:
1047 intracerebroventricular; OG: oral gavage; LORR: loss of righting reflex; RORR:
1048 recovery of righting reflex.

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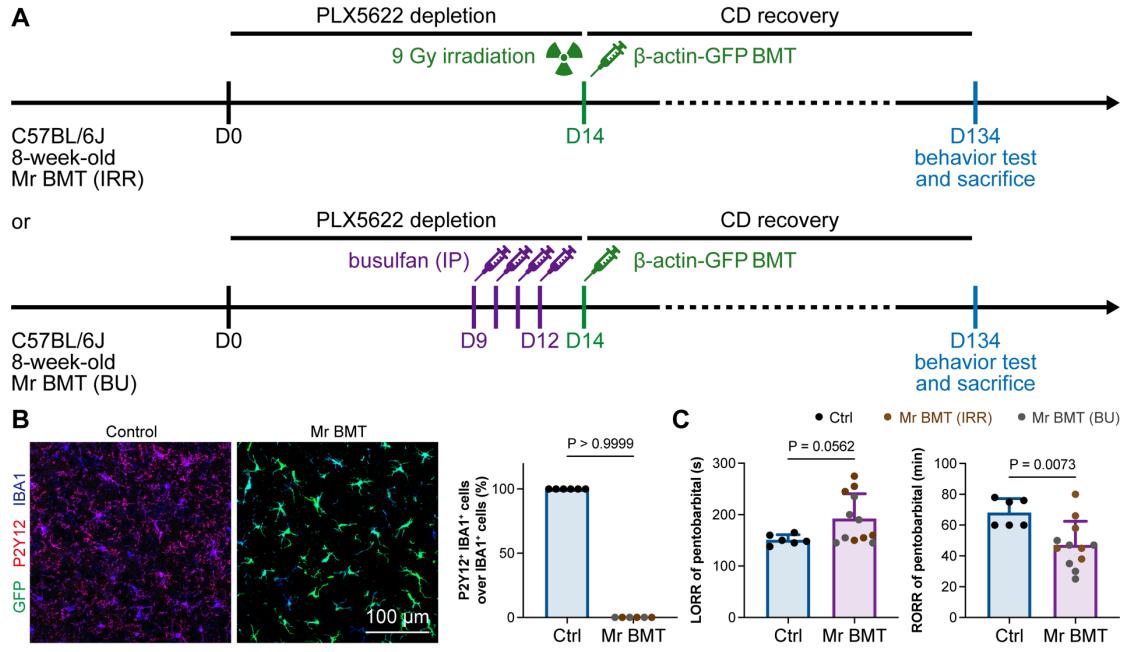


Figure 13 Mice with P2Y12⁻ Mr BMT cells display delayed anesthesia induction and early emergence.

(A) Scheme of microglia replacement by Mr BMT and behavior tests for anesthesia.

(B) Mr BMT cells exhibit a P2Y12⁻ phenotype. N = 6 mice for each group.

(C) P2Y12⁻ microglia lead to delayed anesthesia induction and early emergence. N = 6 mice for the control group, 6 mice for the Mr BMT (IRR) group and 6 mice for the Mr BMT (BU) group.

Two-tailed independent t test. Data are presented as mean \pm SD. IP: intraperitoneal injection; Mr BMT: microglia replacement by bone marrow transplantation; BMT: bone marrow transplantation; Ctrl: control; IRR: irradiation; BU: busulfan; LORR: loss of righting reflex; RORR: recovery of righting reflex.

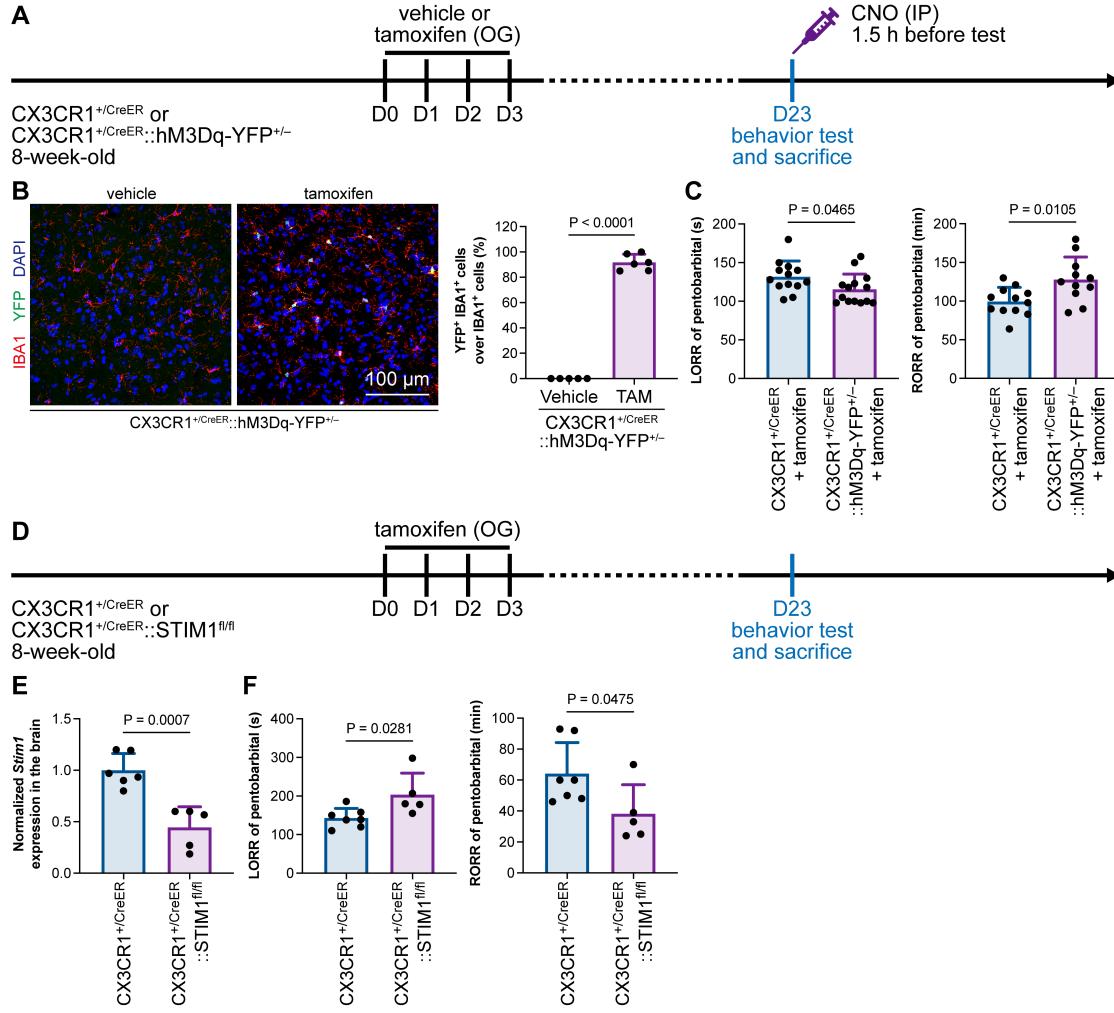


Figure 14 General anesthesia is regulated by intracellular calcium in microglia.

(A) Scheme of animal treatment and examination time points for CX3CR1^{+/CreER} and CX3CR1^{+/CreER}::hM3Dq-YFP⁺⁻ mice.

(B) Tamoxifen induces high Cre-dependent recombination in CX3CR1^{+/CreER}::hM3Dq-YFP⁺⁻ mice. N = 5 mice for the vehicle group and 6 mice for the tamoxifen group.

(C) Elevation of microglial intracellular Ca²⁺ results in a shorter anesthesia induction time and longer emergence time. N = 13 (LORR CX3CR1^{+/CreER}), 14 (LORR CX3CR1^{+/CreER}::hM3Dq-YFP⁺⁻), 12 (RORR CX3CR1^{+/CreER}) and 11 (RORR CX3CR1^{+/CreER}::hM3Dq-YFP⁺⁻) mice per group.

(D) Scheme of animal treatment and examination time points for CX3CR1^{+/CreER} and CX3CR1^{+/CreER}::STIM1^{fl/fl} mice.

1076 **(E)** qPCR results reveal decreased *Stim1* transcription in CX3CR1^{+/CreER}::STIM1^{fl/fl}
1077 mouse brains. N = 6 mice for the CX3CR1^{+/CreER} group and 5 mice for the
1078 CX3CR1^{+/CreER}::STIM1^{fl/fl} group.

1079 **(F)** Downregulation of microglial intracellular Ca²⁺ results in longer anesthesia
1080 induction time and shorter emergence time. N = 7 CX3CR1^{+/CreER} and 5
1081 CX3CR1^{+/CreER}::STIM1^{fl/fl}) mice per group.

1082 Two-tailed independent t test. Data are presented as mean ± SD. OG: oral gavage; IP:
1083 intraperitoneal injection; TAM: tamoxifen; LORR: loss of righting reflex; RORR:
1084 recovery of righting reflex.

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