

1 **Remote neuronal activity drives glioma infiltration via Sema4f**

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3 Emmet Huang-Hobbs ¹⁻³, Yi-Ting Cheng ²⁻⁴, Yeunjung Ko ^{2,3,5,6}, Estefania Luna-
4 Figueroa ^{2,3}, Brittney Lozzi ^{2,3,6,7}, Kathryn R Taylor¹², Malcolm McDonald ^{2,3,8} , Peihao
5 He^{2,3,9}, Hsiao-Chi Chen ^{2,3,9}, Yuhui Yang ⁶, Ehson Maleki ^{2,3}, Zhung-Fu Lee ^{2,3,8},
6 Sanjana Murali ^{2,3,9}, Michael Williamson ^{2,3}, Dongjoo Choi ^{2,3}, Rachel Curry ^{1,2}, James
7 Bayley ⁶, Junsung Woo ^{2,3}, Ali Jalali ^{3,6}, Michelle Monje ¹²⁻¹⁴, Jeffrey L Noebels ¹⁰⁻¹¹,
8 Akdes Serin Harmanci ^{3,6}, Ganesh Rao ^{3,6}, and Benjamin Deneen ^{1-4,6,8,9*}

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10 ¹ The Integrative Molecular and Biomedical Sciences Graduate Program (IMBS), Baylor
11 College of Medicine, Houston TX 77030

12 ² Center for Cell and Gene Therapy, Baylor College of Medicine, Houston TX 77030

13 ³ Center for Cancer Neuroscience, Baylor College of Medicine, Houston, TX 77030

14 ⁴ Program in Developmental Biology, Baylor College of Medicine, Houston TX 77030

15 ⁵ Program in Immunology and Microbiology, Baylor College of Medicine, Houston, TX
16 77030

17 ⁶ Department of Neurosurgery, Baylor College of Medicine, Houston TX 77030

18 ⁷ Program in Genetics and Genomics, Baylor College of Medicine, Houston TX 77030

19 ⁸ Program in Development, Disease, Models and Therapeutics, Baylor College of
20 Medicine, Houston TX 77030

21 ⁹ Program in Cancer Cell Biology, Baylor College of Medicine, Houston TX 77030

22 ¹⁰ Department of Human and Molecular Genetics, Baylor College of Medicine, Houston,
23 TX, 77030

24 ¹¹ Department of Neurology, Baylor College of Medicine, Houston, TX 77030

25 ¹² Department of Neurology and Neurological Sciences, Stanford University, Stanford,
26 CA, USA.

27 ¹³ Department of Neurosurgery, Stanford University, Stanford, CA, USA

28 ¹⁴ Howard Hughes Medical Institute, Stanford University, Stanford, CA, USA.

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30 *Correspondence: deneen@bcm.edu

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47 **Abstract**

48 The tumor microenvironment (TME) plays an essential role in malignancy and neurons
49 have emerged as a key component of the TME that promotes tumorigenesis across a
50 host of cancers. Recent studies on glioblastoma (GBM) highlight bi-directional signaling
51 between tumors and neurons that propagates a vicious cycle of proliferation, synaptic
52 integration, and brain hyperactivity; however, the identity of neuronal subtypes and tumor
53 subpopulations driving this phenomenon are incompletely understood. Here we show that
54 callosal projection neurons located in the hemisphere contralateral to primary GBM
55 tumors promote progression and widespread infiltration. Using this platform to examine
56 GBM infiltration, we identified an activity dependent infiltrating population present at the
57 leading edge of mouse and human tumors that is enriched for axon guidance genes.
58 High-throughput, *in vivo* screening of these genes identified Sema4F as a key regulator
59 of tumorigenesis and activity-dependent infiltration. Furthermore, Sema4F promotes the
60 activity-dependent infiltrating population and propagates bi-directional signaling with
61 neurons by remodeling tumor adjacent synapses towards brain network hyperactivity.
62 Collectively, our studies demonstrate that subsets of neurons in locations remote to
63 primary GBM promote malignant progression, while revealing new mechanisms of tumor
64 infiltration that are regulated by neuronal activity.

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69 Glioblastoma (GBM) is the most aggressive and lethal form of brain tumor, featuring high
70 rates of proliferation and infiltration into surrounding brain tissue¹⁻⁴. Despite treatment,
71 recurrence is inevitable and tends to occur outside surgical margins or in locations remote
72 to the primary tumor⁵⁻⁷, highlighting the central role that tumor infiltration plays in this
73 malicious disease. GBM infiltration in the brain generally occurs along organized
74 anatomical structures such as blood vessels and white matter tracts, which contain
75 neuronal axons and suggests the involvement of neuronal populations⁸⁻¹¹. Previous
76 studies established correlations between the presence of GBM and heightened neuronal
77 activity in surrounding brain regions¹²⁻¹⁶. Moreover, it has been shown that increased
78 neuronal activity can promote optic nerve glioma progression and the growth of both
79 pediatric and adult forms of high-grade glioma through mechanisms involving activity-
80 regulated paracrine factors and neuron-to-glioma synaptic signaling¹⁶⁻¹⁹. This raises the
81 possibility that neuronal activity itself can promote tumor infiltration, a concept supported
82 by the recent discovery that direct synaptic signaling between neurons and glioma cells
83 can promote invasion^{20,21}. Whether neuronal activity promotes circuit-specific patterns of
84 glioma infiltration through paracrine signaling is unknown, and the underlying molecular
85 mechanisms driving GBM infiltration remain obscure. Furthermore, the brain contains a
86 plethora of neuronal subtypes, and which subtypes of neurons serve as the substrate for
87 driving GBM progression is also incompletely understood.

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92 **Results**

93 **Contralateral neuronal stimulation promotes glioma progression**

94 Neuronal activity promotes glioma proliferation, however whether activity promotes
95 transformation of low-grade glioma (LGG) to high-grade glioma (HGG) remains an open
96 question^{17,22}. Furthermore, these prior studies stimulated neurons in close proximity to
97 xenografted tumors, raising the question of whether long-range neuronal projections from
98 brain regions remote to the primary tumor also contribute to tumorigenesis. To determine
99 whether remote stimulation of neurons promotes LGG to HGG transformation, we used
100 the native RCAS/Ntva system that is driven by overexpression of PDGFB and generates
101 LGG^{23,24}. After initiating tumors in the cortex at P1, we injected the contralateral cortex
102 with AAV2/9 Syn1-hM3Dq-mCherry at P5 (**Fig.1a**). To stimulate contralateral neurons,
103 we treated mice with saline or 5 mg/Kg of clozapine N-oxide (CNO) two times a day, for
104 two months, starting at P20. To confirm that CNO treatment activates neurons
105 contralateral to the tumor, we performed slice recordings and found increased activity
106 upon CNO treatment (**Extended Data Figure 1a-b**). Strikingly, mice treated with CNO
107 exhibited a drastic decrease in median overall survival compared to the saline group
108 (51days CNO v. 95days saline) (**Fig.1b**). These changes in survival are complemented
109 by increased Ki67 expression in the CNO group and coupled with hallmark pathological
110 features of HGG, including microvascular proliferation and necrosis (**Fig.1c-red arrows**).
111 These observations indicate that stimulation of neuronal activity in regions remote to
112 primary LGG can promote progression to HGG.

113 Infiltration throughout the brain is another key facet of HGG progression, which we
114 examined using our native CRISPR/Cas9-based in utero electroporation (IUE) model of

115 HGG. Using IUE-based approaches we introduced gRNAs to *NF1*, *PTEN*, and *p53* into a
116 single cortical ventricle at e16.5, which initiates glioma tumorigenesis in a single cortical
117 hemisphere and eventually infiltrates across the corpus callosum to the contralateral
118 hemisphere (**Fig. 1a and Extended Data Figure 1c-d**). To examine whether stimulation
119 of neurons from brain regions remote to the primary tumor promotes infiltration, we
120 injected the contralateral cortex with AAV2/9 Syn1-hM3Dq-mCherry at P5 (**Fig.1a**) and
121 treated with CNO (or saline controls) starting at P20. Using migration across the corpus
122 callosum, into the contralateral cortex as an index of glioma infiltration, we found that
123 CNO treated tumors exhibited a dramatic increase in infiltration as early as P30 (i.e., 10
124 days post-CNO) and was also observed at P50 (**Fig.1d-e**). This acceleration of glioma
125 infiltration was complemented by an increase in Ki67 expression in the CNO group
126 (**Fig.1e and Extended Data Figure 1e**); critically CNO-only controls (without hM3Dq) did
127 not demonstrate any effects on either tumor proliferation or infiltration (**Fig.1e and**
128 **Extended Data Figure 1f-h**). To ascertain whether these effects on infiltration are
129 specific to stimulation of contralateral neurons, we generated tumors and activated
130 neurons in the ipsilateral cortex, finding an increase in proliferation, but no significant
131 changes in infiltration at P30 when compared to saline controls. (**Fig.1f-g and Extended**
132 **Data Figure 2a**). Moreover, direct comparison between contralateral and ipsilateral
133 stimulation groups revealed a significant increase in infiltration after contralateral
134 stimulation (**Fig.1g**). Together, these findings suggest that neuronal activity in the
135 hemisphere contralateral to the primary tumor specifically promotes precocious tumor
136 infiltration across cortical hemispheres.

137 To confirm that stimulation of contralateral neurons promotes glioma infiltration,
138 we employed mathematical modeling^{25,48} finding that tumor infiltration width (IW) is
139 increased relative to tumor mass (TM) in the CNO treated group compared to the saline
140 group and non-treated control tumors at P30 (**Fig.1h**). To independently validate that
141 neuronal activity-regulated paracrine factors promotes glioma infiltration, we used patient-
142 derived glioma cell cultures in conjunction with an established three-dimensional spheroid
143 culture system to measure infiltration^{26,27}. These cultures were treated with conditioned
144 media (CM) from cortical explants with spontaneously active neurons, optogenetically
145 stimulated neurons, unconditioned control media (artificial cerebrospinal fluid, ACSF), or
146 CM from cortical explants treated with TTX to silence neuronal activity. These studies
147 revealed that treatment of glioma spheroids with CM from cortical explants containing
148 active neurons promoted glioma infiltration (**Fig.1i**). Collectively, these data indicate that
149 neuronal activity promotes tumor infiltration through secreted factors and that neurons
150 contralateral to the primary tumor specifically drive this phenomenon during the early
151 stages of tumor progression.

152

153 **Callosal projection neurons promote glioma progression**

154 The preceding observations raise the question of which neuronal populations in the
155 contralateral cortex are driving glioma infiltration and progression. Given that the axons
156 of callosal projection neurons (CPN's) cross cortical hemispheres, we reasoned that this
157 population in the contralateral cortex is contributing to activity dependent infiltration²⁸.
158 CPN axons cross the cortical hemisphere along the corpus callosum, a white matter tract
159 that also serves as a major route of glioma infiltration. Therefore, to test whether CPN's

160 are necessary for driving activity dependent tumor infiltration in our system, we severed
161 the corpus callosum in the context of stimulation of contralateral neurons. Using our
162 established paradigm (**Fig.1a**), we severed the corpus callosum at P10 and initiated CNO
163 and saline treatments at P20 (along with non-severed controls), followed by harvesting
164 tumor bearing brains at P30. Analysis of these tumors revealed that severing the corpus
165 callosum abolished the activity-dependent acceleration of infiltration that was observed
166 with the intact control (**Fig.2a-b**). Moreover, we observed that activity-dependent
167 increases in Ki67 expression were also lost after severing the corpus callosum (**Fig.2b**
168 **and Extended Data Figure 2b**). These results indicate that an intact corpus callosum is
169 necessary for contralateral neurons to promote glioma progression, implicating CPNs in
170 this phenomenon.

171 To examine whether CPNs are sufficient to accelerate tumor progression we
172 utilized the *Rasgrf2*-dCre line, which marks layers 2/3 of the cortex and from which ~80%
173 of CPN's are derived (**Fig.2c**)^{28,29}. To achieve selective activation of *Rasgrf2*-dCre
174 expressing neurons, we utilized a double-floxed inverse orf (DIO) construct (pAAV-Syn1-
175 DIO-hM3D-2A-mCherry), while inducing dCre with Trimethorpim, at 100ng/g body weight
176 (**Fig.2d**). Induction of dCre and activity of *Rasgrf2-Cre*; *ROSA-floxed-tdTomato* in cortical
177 layer 2/3 was confirmed (**Extended Data Figure 3a**), enabling us to use this mouse line
178 in our IUE-based, glioma-activity paradigm (**Fig.1a**). Here, we injected the AAV-DIO virus
179 in the contralateral cortex at P5, followed by dCre induction at P15, which enabled
180 expression of hM3Dq in layer 2/3 neurons. After these manipulations, we treated mice
181 with saline or CNO at P20 and harvested tumor bearing brains at P30. Strikingly, selective
182 stimulation of *Rasgrf2*-Cre expressing neurons with CNO in the contralateral hemisphere

183 promoted both tumor infiltration and Ki67 expression when compared to saline controls
184 **(Fig.2e-f and Extended Data Figure 2c)** and at levels comparable to pan-neuronal
185 activation controls. As a control for the specificity of this manipulation we activated
186 inhibitory neurons, which are distinct from CPNs, in the contralateral hemisphere using
187 AAV2/9 Dlx5/6-hM3Dq-mCherry in our paradigm. These studies revealed no changes in
188 tumor cell infiltration or proliferation at P30, further supporting the specificity of Layer2/3
189 neurons from the contralateral hemisphere **(Extended Data Figure 3b-c)**. Collectively,
190 these data indicate that CPN's play a critical role in driving tumor progression, while
191 highlighting the contributions of neurons in remote brain regions to glioma progression.

192

193 **Identification of activity-dependent, infiltrating glioma populations**

194 Infiltrating glioma cells play a central role in progression and eventual recurrence. Our
195 activity-driven paradigm of glioma progression offers a venue in which to examine the
196 cellular and molecular properties of these critical, yet poorly defined populations. To
197 achieve this, we performed single-cell RNA-sequencing on P50 glioma tumors generated
198 in the presence of contralateral stimulation (and saline controls) and using GFP as a
199 marker of tumor cells we were able to distinguish host microenvironmental populations
200 from tumor populations **(Fig.3a)**. This analysis revealed widespread changes in the
201 immune microenvironment in the presence of increased neuronal activity **(Fig. 3a and**
202 **Extended Data Figure 3d-e and Extended Tables 1-2)**, coupled with changes in the
203 cellular constituency of the tumor. Focusing on the GFP+ tumor populations, we
204 performed additional analysis and identified several prospective subpopulations that are
205 enriched in the CNO, stimulated tumors **(Fig.3b)**. We performed Gene Ontology (GO)

206 analysis on the most enriched subpopulation in this CNO-enriched clusters (**Fig.3b-red**
207 **arrow**) and identified a host of unique GO terms, including genes associated with
208 glutamatergic synapses and axon guidance (**Fig.3b and Extended Table 3**). Next, we
209 sought to localize this activity-dependent subpopulation within the tumor, hypothesizing
210 that it likely resides at the leading edge in the contralateral hemisphere. Using spatial
211 transcriptomics of P50 activity-driven glioma, we localized the gene signatures associated
212 with the activity-dependent cluster in infiltrating tumor cells in the cortex contralateral to
213 the primary tumor (**Fig.3c-d**). Furthermore, the cells at the leading edge were also
214 enriched for genes associated with axon guidance (**Extended Data Figure 3f and**
215 **Extended Table 4**). These observations in mouse models led us to examine whether this
216 infiltrating population is also present in the leading edge of human GBM. Therefore, we
217 cross-correlated the gene signature associated with the infiltrating mouse population with
218 the IVY-GAP database, which has transcriptomic data for distinct anatomical structures
219 of GBM, including the leading edge³⁰. This analysis revealed a similar and highly specific
220 enrichment of this infiltrating signature at the leading edge of human GBM (**Fig.3e**).
221 Together these observations indicate that neuronal stimulation drives the generation of
222 infiltrating populations, and these populations correspond to the leading edge of GBM
223 tumors.

224

225 **Axon guidance genes drive glioma progression**

226 The enrichment of axon guidance genes in the activity-driven, infiltrating glioma
227 population (**Fig.3b**), led us to investigate their contributions to glioma infiltration. To
228 examine their roles in this context, we performed a bar-coded, overexpression screen by

229 generating a PiggyBac-based, barcoded library of 43 axon guidance-associated genes in
230 our IUE-HGG model (**Fig.4a and Extended Table 5**). Following introduction of the axon
231 guidance library, we harvested tumor bearing mice at P90 and dissected tumors based
232 on ipsilateral- (primary) and contralateral- (secondary) locations, with the contralateral
233 population likely enriched in infiltrating populations. Barcode sequencing was performed
234 on samples from both sites, and we compared barcode enrichment between primary and
235 secondary sites, seeking to identify those barcodes that are enriched in secondary sites,
236 as those are candidate drivers of infiltration (**Fig.4a-b**). This analysis nominated a series
237 of candidates enriched in secondary sites, including *Unc5B*^{31,32}, *Sema7A*^{33,34}, and
238 *Sema3C*^{35,36}, which have been previously implicated in tumor invasion (**Fig.4b**). Next, we
239 evaluated the expression of genes enriched at secondary sites in the IVY-GAP database,
240 finding that Semaphorin-4F (*Sema4F*), EphrinA6 (*EphA6*), and EphrinA7 (*EphA7*) are
241 enriched in the leading edge of human GBM (**Extended Data Figure 4a-c**), while
242 validating protein expression in primary GBM tumor samples (**Fig.4f**). Furthermore, the
243 roles of *EphA6*, *EphA7*, and *Sema4F* in glioma infiltration are undefined, prompting us to
244 further examine their contribution to tumorigenesis.

245 To determine the roles of *Sema4F*, *EphA6*, and *EphA7* in glioma tumorigenesis
246 and infiltration, we performed gain-of-function (GOF) overexpression and CRISPR-Cas9
247 based loss-of-function (LOF) studies in our IUE-HGG model (**Extended Data Figure 4d**).
248 Using overall survival as a proxy for tumor burden, we found that LOF studies with *EphA6*
249 and *Sema4F* extended mouse survival, while GOF studies with *Sema4F* decreased
250 overall survival (**Fig.4c**). To determine how these manipulations impacted tumor
251 infiltration we generated LOF and GOF tumors from each gene and harvested tumors at

252 P30, P50, and P70, measuring contralateral infiltration. Consistent with our overall
253 survival studies, we found that GOF manipulations with *Sema4F* accelerated infiltration,
254 while LOF manipulations impaired infiltration (**Fig.4d-e**). Analysis of LOF of *EphA6*
255 revealed impaired infiltration, while the remainder of the manipulations with *EphA6* and
256 *EphA7* had relatively modest impacts on infiltration while retaining high-grade glioma
257 histopathology (**Fig.4d-e and Extended Data Figure 5a-c**). Focusing on *Sema4F*, we
258 generated human glioma cell lines that overexpress *Sema4F* (GOF) or have shRNA-
259 knockdown of *Sema4F* (LOF) (**Extended Data Figure 5d**). We implanted these cell lines
260 (and control cell lines) into the mouse brain and found that knockdown of *Sema4F*
261 resulted in a significant extension of overall survival (**Fig.4h**). To evaluate glioma cell
262 infiltration, we performed transwell assays, finding that *Sema4F*-GOF resulted in
263 enhanced infiltration, while *Sema4F*-LOF suppressed infiltration (**Fig.4g**). Together,
264 these studies indicate that our *in vivo* screening approach can identify new regulators of
265 glioma infiltration, highlighting the role of *Sema4F* in glioma tumorigenesis.

266

267 ***Sema4F* is required for activity dependent infiltration**

268 The central role of *Sema4F* in glioma infiltration raises the question of whether it is
269 required for activity-dependent infiltration. To address this question, we used our activity-
270 driven paradigm of glioma progression (**Fig.1a**), in combination with LOF of *Sema4F*. As
271 before, we stimulated with CNO and employed saline controls starting at P20, harvesting
272 tumor bearing brains at P30 and assessed contralateral infiltration, as well as Ki67
273 expression. Our analysis revealed that that loss of *Sema4F* in the context of CNO-based
274 stimulation abolished activity-dependent infiltration when compared to controls containing

275 **Sema4F (Fig.5a-b)**. Additionally, Sema4F LOF abolished the effect of neural stimulation
276 on tumor proliferation as measured by Ki67 staining, which demonstrated no significant
277 differences between CNO and saline treated tumors (**Fig.5b and Extended Data Figure**
278 **6a-b**). Next, we examined whether the Sema4F contributes to glioma tumorigenesis via
279 cell extrinsic mechanisms by overexpressing the Sema4F-ectodomain (S4E) in our IUE-
280 HGG model, finding that it promotes infiltration and proliferation at P30 (**Extended Data**
281 **Figure 6c-f**). These findings prompted us to examine whether S4E can rescue the deficits
282 manifest in Sema4F-LOF tumors. Therefore, we overexpressed S4E in the context of
283 Sema4F-LOF, finding that expression of S4E can rescue both infiltration and proliferation
284 at P30 (**Extended Data Figure 6c-f**). Together, these results indicate that Sema4F is a
285 key mediator of activity-dependent glioma and that it promotes tumor infiltration through
286 its ectodomain, suggesting that it regulates this phenomenon through interactions with
287 the brain microenvironment.

288

289 **Sema4F promotes synaptic remodeling and brain hyperactivity**

290 The foregoing data also suggest that Sema4F itself promotes the generation of the
291 activity-dependent, infiltrating population (**Fig.3**). To test this, we performed scRNA-Seq
292 on Sema4F-GOF tumors at P50 and cross-compared these data with our activity-driven
293 scRNA-Seq datasets (**Fig.5c and Extended Data Figure 7a**). This analysis revealed that
294 the same cluster enriched in our activity-dependent dataset, was also enriched in our
295 Sema4F-GOF dataset and contains the corresponding axon guidance gene signature
296 (**Fig.5c-d; Extended Tables 2, 6**). These data suggest that Sema4F expression is
297 capable of generating activity-dependent infiltrating glioma populations. Further analysis

298 of our Sema4F-GOF scRNA-Seq dataset identified the upregulation of several synaptic
299 signaling pathways in tumor cells (**Fig.5d**), including glutamatergic synapse genes. Bulk
300 RNA-Seq of Sema4F-GOF human glioma cell lines revealed an analogous enrichment in
301 synaptic signaling and axon guidance pathways, suggesting conserved function of
302 Sema4F across these model systems (**Extended Figure 7c-d; Extended Tables 7-8**).
303 Prior studies have shown that Sema-family members and their PlexinB receptors, which
304 are expressed in neurons from our scRNA-Seq data (**Extended Data Figure 7b**), can
305 engender synapse formation ³⁷. These observations, coupled with the role of the
306 Sema4F-ectodomain in tumorigenesis (**Extended Data Figure 6c-f**) prompted us to
307 assess excitatory- and inhibitory- synapses in neurons outside the tumor margins in
308 Sema4F-GOF tumors. These studies revealed a marked decrease in inhibitory synapses
309 (VGAT-Gephyrin), coupled with an increase in excitatory synapses (Vglut2-PSD95) in
310 mouse Sema4F-GOF tumors (**Fig.5e-f**), which we also observed in mice bearing
311 Sema4F-GOF tumors derived from human glioma cell lines (**Extended Data Figure 8**).
312 Together these data indicate extensive synaptic remodeling towards hyperactive states.
313 Next, we examined whether these alterations in the synaptic milieu influence brain
314 network activity by performing serial electroencephalograms (EEG) on mice bearing
315 Sema4F GOF tumors¹⁵. As shown in **Figure 5g-h**, mice bearing Sema4F-GOF tumors
316 exhibit an early onset of brain network hyperactivity, featuring increased spiking,
317 compared to mice bearing control tumors. These EEG data indicate that synaptic
318 remodeling by Sema4F promotes brain network hyperactivity and in conjunction with the
319 scRNA-Seq data suggests that Sema4F itself drives the generation of these activity
320 dependent, infiltrating glioma populations.

321

322 **Discussion**

323 Neuronal activity has emerged as a key component of the TME that engenders malignant
324 growth in gliomas and across a host of cancers^{15–21,38–40}, however the nature of tumor-
325 neuron interactions remains incompletely understood. In this study, we use CPN
326 activation to demonstrate that long-range projections from neuronal populations remote
327 to primary glioma can drive progression and infiltration. Neuronal axons can extend
328 across relatively long distances from their cell bodies. Therefore, our findings suggest that
329 brain tumors receive inputs from a host of brain regions which implies a broader
330 relationship between brain tumors and resident neurons than previously thought²⁸.
331 Glioma and neurons make direct synaptic connections¹⁶. Given our findings it is likely that
332 circuit disruption is not limited to regions where the primary tumor resides but is more
333 widespread throughout the brain. Furthermore, glioma tumors remodel local neuronal
334 synapses towards hyperactivity^{15–20}, raising the possibility that synapses from these long-
335 range projections are also remodeled by the tumor resulting in deleterious effects on brain
336 circuits in remote regions. Interestingly, recent studies from mouse glioma models
337 revealed spreading depolarization and hyperactivity across cortical hemispheres⁴¹,
338 suggesting dysregulation of circuits remote to the primary tumor.

339

340 Despite its central role in glioma recurrence, the cellular and molecular mechanisms
341 regulating tumor infiltration remain elusive. We identified an activity-dependent infiltrating
342 glioma population, which indicates that glioma tumors utilize neuronal signals to drive
343 progression and widespread infiltration. The precocious emergence of this population is

344 facilitated by neuronal activity, however our identification of this population in Sema4F-
345 GOF tumors and at later stages of progression suggest that its emergence is a core
346 feature of infiltration. Glioma tends to use white matter tracts as routes of infiltration.
347 These myelinated axonal structures are populated by nodes of Ranvier, which are
348 sources of dynamic ion flux during activity and could serve as an infiltrative cue^{9,11,42–44}.
349 Mechanistically, we found that the infiltrating population is enriched for axon guidance
350 genes and our *in vivo* screen identified Sema4F as a key driver of glioma progression
351 and activity-dependent infiltration. Despite playing key roles in responding to
352 environmental cues during development, roles for axon guidance genes in glioma remain
353 poorly defined and our studies highlight their central role in activity-dependent glioma
354 progression^{45–47}. An intriguing line of future investigation is to decipher how Ephrin- and
355 Sema- family members cooperate to regulate glioma infiltration and progression. Further
356 analysis revealed that Sema4F promotes synaptic remodeling in neurons adjacent to
357 glioma, which is consistent with prior models suggesting that tumors in the CNS generate
358 a positive feedback loop of receiving and promoting synaptic signaling to tumor
359 populations^{15–17,22,41}. When put together, a model emerges where neurons provoke
360 expression of genes from glioma tumors that subsequently drive their own synaptic
361 activity.

362

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364

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382
383 **Authors Contributions**

384
385 EHH and BD conceived the project and designed the experiments; EHH, YTC, YK, ELF,
386 YY, KRT, MMc, PH, HCC, EM, ZFL, SM, MW, and DC performed the experiments; JW
387 executed the electrophysiology studies; RNC, MM, AJ, JLN, GR provided essential
388 reagents; EHH, BL, ASH, and JB designed and executed the bioinformatics analyses.
389 KRT and MM designed and performed in vitro glioma migration experiments. EHH and
390 BD wrote the manuscript.

391
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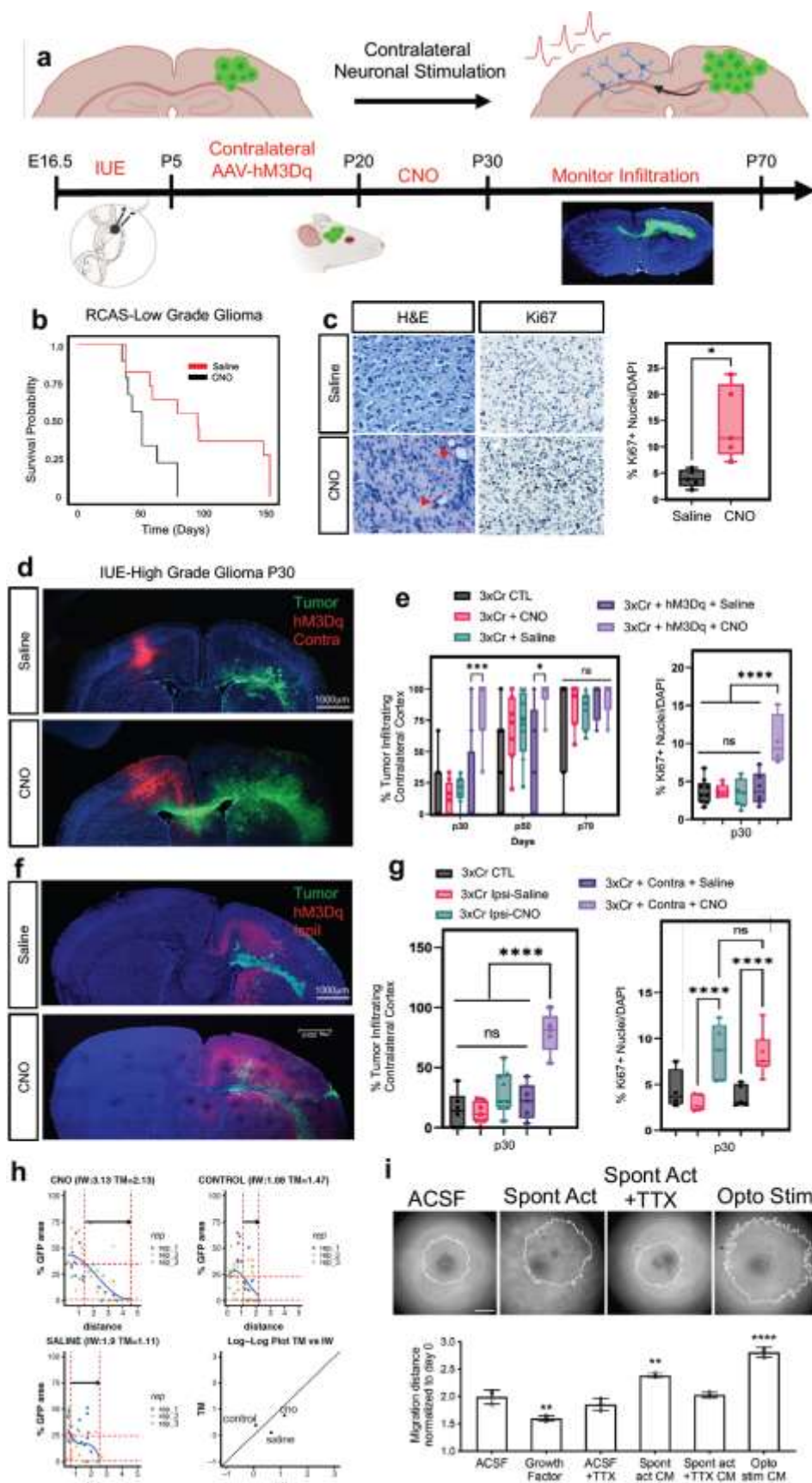
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508 **Figures and Legends**



510 **Figure 1. Remote neuronal stimulation accelerates glioma progression**

511 **a.** Schematic of DREADD-based activation of neurons contralateral to tumor in both
512 RCAS-Ntva and IUE models. **b.** Kaplan-Meier survival analysis of RCAS-Ntva tumors
513 treated with saline (median_{saline} = 95 days, n = 11) or CNO (median_{CNO} = 51 days, n = 9)
514 showing significantly faster morbidity in CNO treated RCAS tumors (Log-rank (Mantel-
515 Cox) test, Chisq = 6.456, df = 1, p-value = 0.0111, CNO/Saline HR_{log-rank} = 2.768, 95% CI
516 = 0.9770 to 7.945). **c.** H&E staining of RCAS-Ntva tumors samples revealed high grade
517 characteristics in CNO treated tumor groups (red arrows). Ki67 staining proliferation in
518 CNO treated mice versus saline treated mice. Quantification is derived from n=5 mice
519 from CNO (mean = 14.51%, SD = 7.076%) and Saline (mean = 4.017%, SD = 2.179%)
520 groups and determined by Welch's unpaired t-test (p-value = 0.0276, t = 3.228, df =
521 4.441). **d.** Representative images from IUE-HGG tumors at P30 demonstrating
522 infiltration; green is tumor, red is AAV-DREADD virus. **e.** Quantification of infiltration and
523 Ki67 expression across the P30-P70 timecourse. Infiltration was quantified based on the
524 presence of tumor cells in contralateral cortex and analyzed via two-way ANOVA; data
525 derived from p30 CTL n = 8, p50 CTL n = 7, p70CTL n = 7, p30+CNO n = 8, p50+CNO n
526 = 7, p70+CNO n = 7, p30+Saline n = 8, p50+Saline n = 7, p70+Saline n = 7,
527 p30+AAV+Saline n = 8, p50+AAV+Saline n = 5, p30+AAV+Saline n = 4, p30+AAV+CNO
528 n = 8, p50+AAV+CNO n = 6, p30+AAV+CNO n = 5 samples. Ki67 staining was performed
529 at the p30 time point, from CTL n = 9, CNOonly n = 8, Salineonly n = 7, AAV+Saline n =
530 5, AAV+CNO n = 4 samples. **f.** Representative images from IUE-HGG tumors at P30
531 demonstrating the extent of infiltration after activation of neurons in the cortex ipsilateral
532 to the tumor (CNO) and saline treated controls; green is tumor, red is AAV-DREADD

533 virus. **g.** Quantification of tumor infiltration and Ki67 expression at P30 (3xCr CTL n = 5
534 mean = 4.329%, 3xCr+IpsilAAV+Saline n=5, mean = 2.975%, 3xCr+IpsilAAV+CNO n=5,
535 mean = 8.144%). Significant difference was found in Ki67+ nuclei in ipsilateral CNO
536 stimulated tumors vs saline control (p-value <0.0001) and vs 3xCr only controls (p-value
537 = 0.0020); Infiltration was quantified based on the presence of tumor cells in contralateral
538 cortex and analyzed via one-way ANOVA (3xCr CTL n = 6, 3xCr+IpsilAAV+Saline n=7,
539 3xCr+IpsilAAV+CNO n=9) with CNO stimulated brains showing no statistical difference
540 to Saline treated (p-value = 0.0649) or control tumors (p-value = 0.1504). Direct
541 comparison between ipsilateral-CNO and contralateral-CNO groups revealed a
542 statistically significant difference (p-value <0.0001). **h.** Mathematical modeling of glioma
543 infiltration as a function of tumor mass. Blue line is the smoothed data points using
544 piecewise-cubic splines; red horizontal dashed lines are the $0.8 \rho_{\max}$ and $0.02 \rho_{\max}$ glioma
545 cell density of the maximum smoothed cellular density (ρ_{\max}). Red vertical lines are the
546 intersecting distance points of the red horizontal lines with smoothed blue line, which is
547 used in calculating infiltrating width (IW). Black arrow shows the IW. Log-log plot shows
548 the dependence of IW and tumor mass (TM). Analysis was performed at the p30 timepoint
549 on CTL n = 3, Saline n = 3, CNO n = 3; samples from individual biological replicates are
550 color coded. **i.** Glioma 3D spheroid migration assay, measuring glioma infiltration after
551 treatment with growth factor media, conditioned media (CM) from spontaneously active
552 cortical explants, spontaneously active cortical explants silenced with TTX ($10\mu\text{m}$) or
553 optogenetically stimulated cortical explants (channelrhodopsin-2 (ChR2)-expressing
554 deep layer cortical projection neurons), in comparison to ACSF control. Scale bar is
555 $500\mu\text{m}$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, log-rank(**b**), unpaired Welch's

556 t-test (**c**), two-way analysis of variance (ANOVA) (**e**), one-way analysis of variance
557 (ANOVA) (**c, e**).

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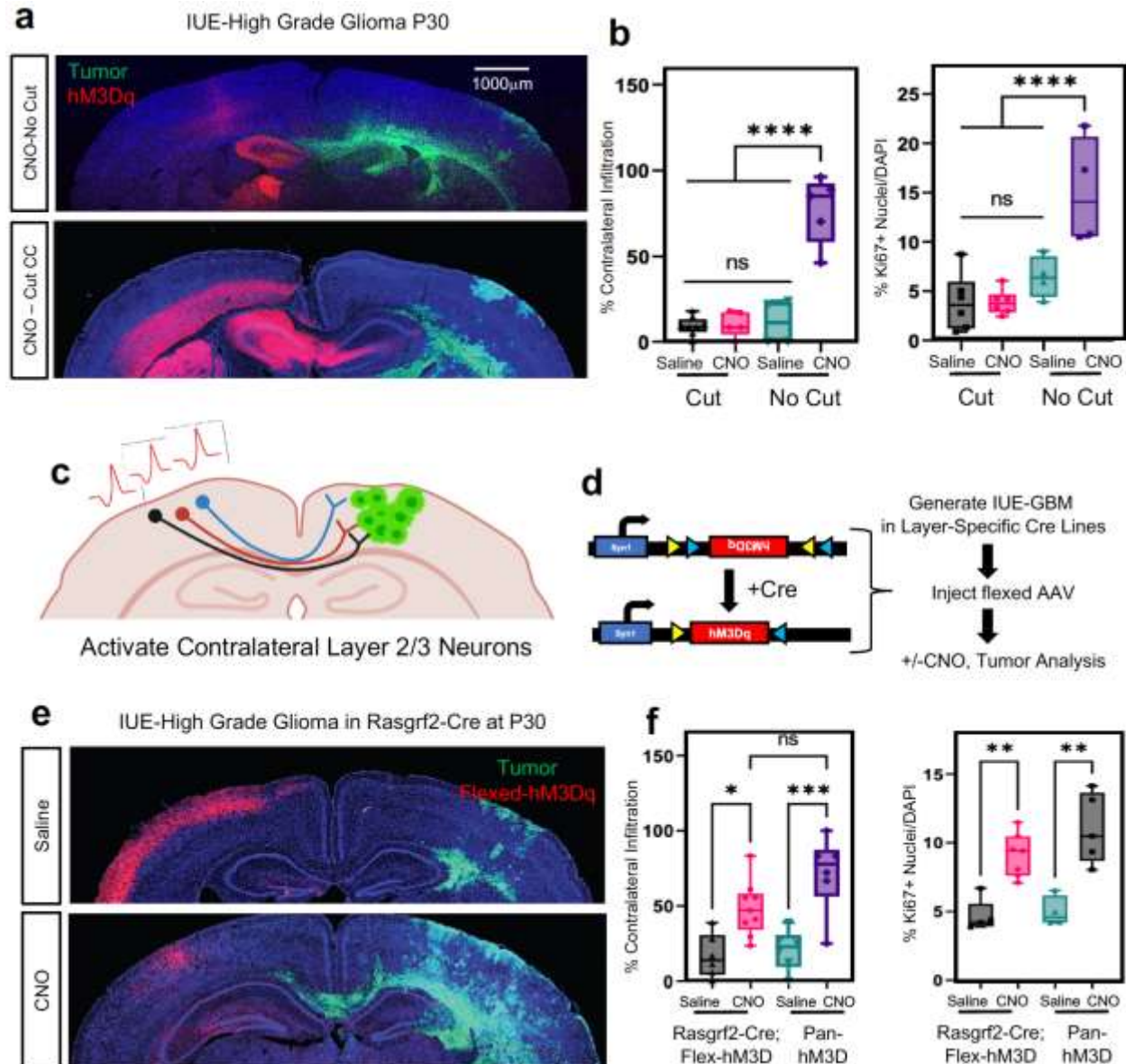
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578 **Figure 2. Callosal projection neurons promote glioma infiltration**

579 **a.** Representative images from IUE-HGG tumors at P30 demonstrating the extent of
 580 infiltration with a severed corpus callosum (cut) or control (no cut); green is tumor, red is
 581 AAV-DREADD virus. **b.** Quantification of tumor infiltration and Ki67 expression at P30,
 582 data derived from CTL n = 4, AAV+CNO n = 5, AAV+Saline n = 4, CCcut+AAV+CNO n =
 583 6, CCcut+AAV+Saline n = 6, Infiltration was quantified based on the presence of tumor
 584 cells in contralateral cortex and analyzed via ordinary one-way analysis of variance

585 (ANOVA); CTL n=8, CC-Cut+AAV+CNO n=5, CC-cut+AAV+Saline n=8 (≥ 12 coronal
586 sections assessed per brain). **c.** Schematic of callosal projection neuron activation
587 experiment. **d.** Schematic of combined Rasgrf2-dCre mouse line with Cre-inducible DIO-
588 hM3D-2a-mCherry DREADD to selectively activate layer2/3 neurons in contralateral
589 hemisphere. **e.** Representative images from IUE-HGG tumors, injected with AAV-DIO-
590 hM3D-2a-mCherry in Rasgrf2-Cre mice. Mice were harvested at P30 and the extent of
591 tumor infiltration was evaluated **f.** Quantification of tumor infiltration and Ki67 expression
592 at P30; Infiltration was quantified based on the presence of tumor cells in contralateral
593 cortex and analyzed via ordinary one-way analysis of variance (ANOVA);
594 Rasgrf2+AAV+CNO n=9, Rasgrf2+AAV+Saline n=6, 3xCr+AAV+CNO n=6,
595 3xCr+AAV+Saline n=6 (≥ 12 coronal sections assessed per brain). Proliferation samples
596 were analyzed via one way analysis of variance; Rasgrf2+AAV+CNO n=7,
597 Rasgrf2+AAV+Saline n=5, 3xCr+AAV+CNO n=5, 3xCr+AAV+Saline n=4. * $P < 0.05$, ** P
598 < 0.01 , *** $P < 0.001$, **** $P < 0.0001$, one-way analysis of variance (ANOVA) (**b, f**).

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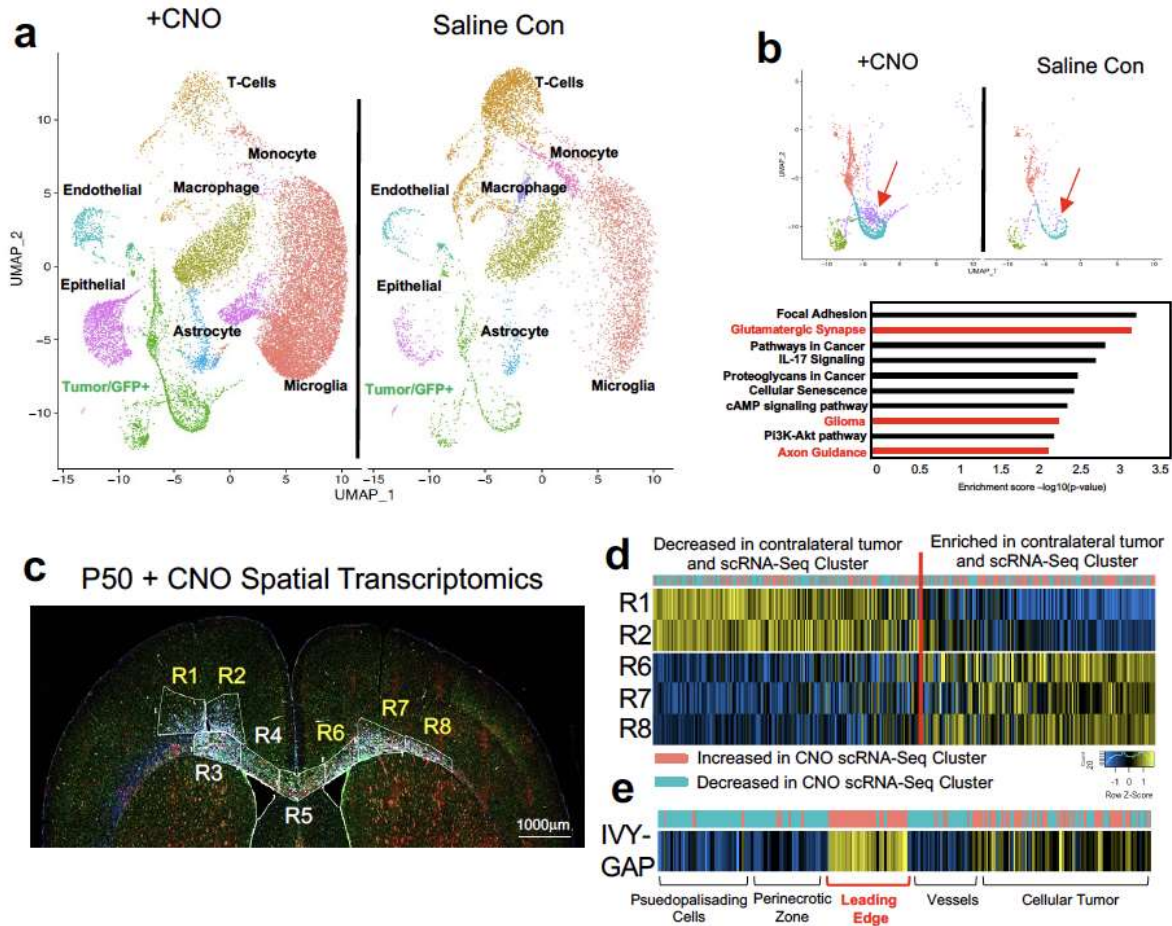
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609 **Figure 3. Identification of activity-dependent infiltrating glioma population**

610 **a.** Single Cell RNA-Seq DimPlots of P50 IUE-HGG from CNO and saline controls. Cell
611 types were mapped in SingleR with celldex as expression profile reference. **b.** Sub-
612 clustering analysis of GFP+ tumor+ cells shown in **a.** red arrow indicates the cluster of
613 interest we chose to investigate. GO-term analysis on this prospective cell cluster was
614 performed using EnrichR and the KEGG 2021 Human dataset. **c.** Spatial transcriptomics
615 on P50 IUE-HGG from the CNO group were performed with GeoMx Nanostring Digital
616 Spatial Profiling. Regions were selected based on the expression of elevated GFAP and
617 vimentin in the section and the presence of GFP in those regions on adjacent sections;
618 R1-R2 denote ipsilateral tumor, R3-R5 denote tumor within corpus callosum, R6-R8

619 denote infiltrating tumor in contralateral hemisphere. **d.** Heatmap depicting the
620 expression of markers associated with the single cell cluster of interest and their relative
621 expression across the tumor regions displayed in **c.** Fold Change of markers from the
622 scRNA-Seq data are mapped in pink and blue. **e.** Heatmap depicting the enrichment of
623 markers associated with the single cell cluster of interest and their relative expression
624 across various anatomical locations in human GBM, derived from the IVY-GAP database.
625 These enrichment scores were generated with AUCCell analysis (depicted in red and blue),
626 or ssGSEA analysis (depicted in yellow and blue) and plotted as a heatmap

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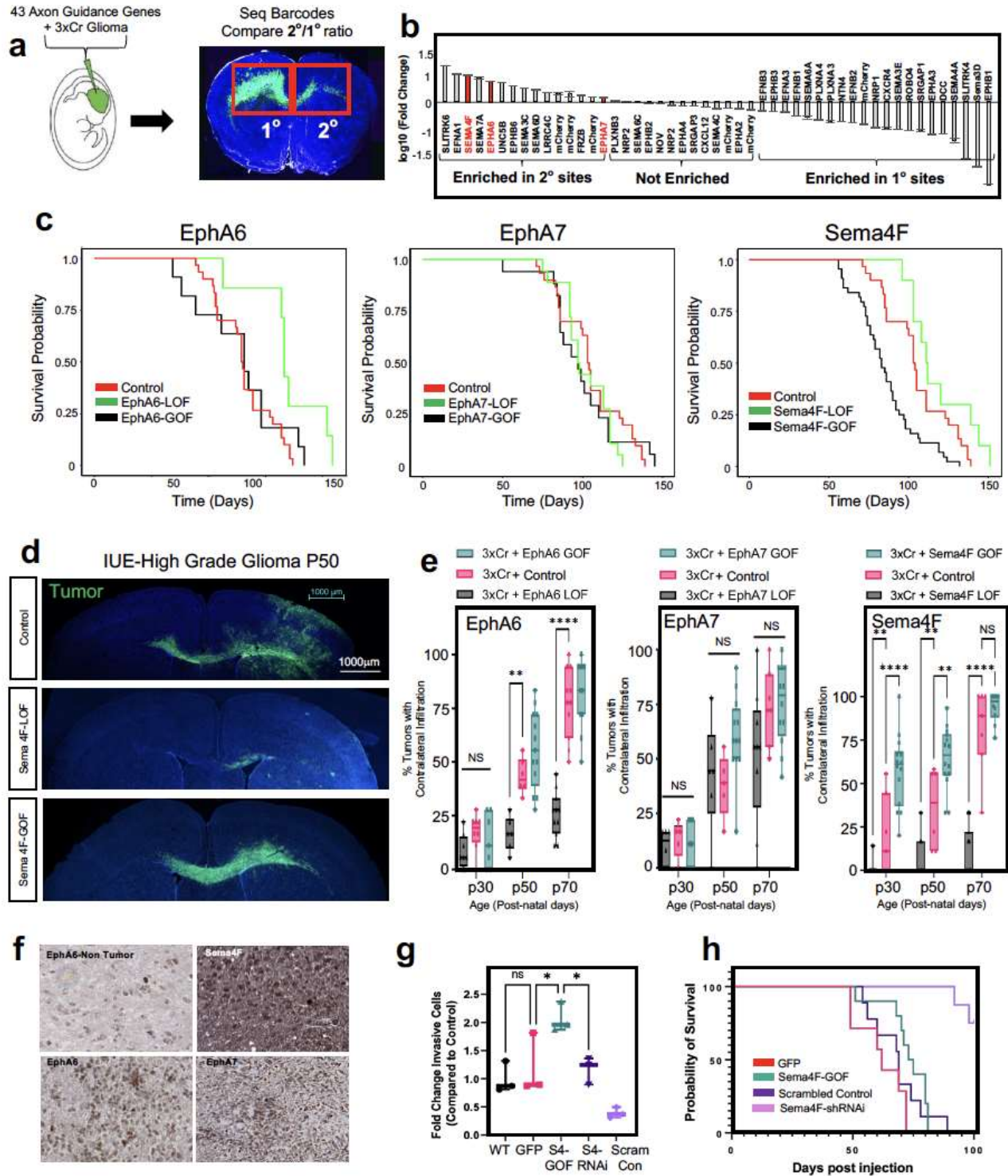
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643 **Figure 4. In vivo screen identifies Sema4F as a driver of glioma infiltration**

644 **a.** Schematic of barcoded screen of 43 axon guidance genes and 7 internal mCherry

645 controls. Tumors were harvested from ipsilateral primary and contralateral secondary

646 tumor sites and barcode sequencing performed (n=4 tumors with paired sites). **b.** Next-
647 generation sequencing for barcode amplification, fold-change was calculated with library
648 input control and relative enrichment in primary or secondary site was determined. **c.**
649 Kaplan-Meier survival curve of individual gain-of-function and loss-of-function validation
650 studies for EphA6, EphA7 and Sema4F. EphA6-GOF (median_{A6GOF} = 105 days, Chisq =
651 0.7, df=1, p-value =0.4, n=11), EphA6-LOF (median_{A6LOF} = 133days, Chis =7.2, df=1, p-
652 value=0.007, n = 7), EphA7-GOF (median_{A7GOF}= 97 days, Chisq=0, df=1 p-value=0.9,
653 n=15), EphA7-LOF (median_{A7LOF}= 97.5 days, Chisq=1.3, df=1, p-value=0.3), Sema4F-
654 GOF (median_{S4FGOF}=83 days, Chisq = 14.8, df = 1 p-value = 0.0001, n = 44), Sema-LOF
655 (median_{S4FLOF}=112 days, Chisq = 3.9, df = 1 p-value = 0.05, n = 10), controls (n=18) **d.**
656 Representative images from IUE-HGG tumors at P50 from Sema4F-GOF, Sema4F-LOF,
657 or control groups demonstrating infiltration; green is tumor. **e.** Quantification of infiltration
658 from these tumors across the P30-P70 timecourse. Infiltration was quantified based on
659 the presence of tumor cells in contralateral cortex and analyzed via two-way analysis of
660 variance (ANOVA). Error bars represent standard deviation, data derived from EphA6-
661 GOF p30 n=7, EphA6-GOF p50 n=13, EphA6-GOF p70 n=11, EphA6 CTL p30 n=8,
662 EphA6 CTL p50 n=6, EphA6 CTL p70 n=11, EphA6-LOF p30 n=8, EphA6-LOF p50 n=6,
663 EphA6-LOF p70 n=11, EphA7-GOF p30 n=9, EphA7-GOF p50 n=12, EphA7-GOF p70
664 n=12, EphA7 CTL p30 n=5, EphA7 CTL p50 n=5, EphA7 CTL p70 n=7, EphA7-LOF p30
665 n=6, EphA7-LOF p50 n=6, EphA7-LOF p70 n=9, Sema4F-GOF p30 n=14, Sema4F-GOF
666 p50 n=14, Sema4F-GOF p70 n=10, Sema4F CTL p30 n=7, Sema4F CTL p50 n=6
667 Sema4F CTL p70 n=7, Sema4F-LOF p30 n=17, Sema4F-LOF p50 n=16, Sema4F-LOF
668 p70 n=19. **f.** Representative immunostainings of EphA6, EphA7, and Sema4F human

669 tumor micro-array. **g.** Quantification of transwell migration of human glioma cell lines;
670 infiltrating cells were counted after 48 hours incubation (n=3 wells per condition). **h.**
671 Kaplan-Meier survival curve for human glioma cell lines transplanted into mouse brain.
672 Samples were analyzed via log-rank (Mantel-Cox) test. WT median survival=66 days,
673 n=11; GFP median=62 days n=7; Sema4f-GOF median=74 days, n=10, Chi = 0.4120, p-
674 value = 0.5209; shSCR median=69 days, n=9; shSema4F median = undefined after 100
675 days, n=8, Chi = 14.08 p-value = 0.0002. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P <$
676 0.0001, two-way analysis of variance (ANOVA) (**e**), one-way analysis of variance
677 (ANOVA) (**f**), Log-rank (Mantel-Cox) test (**c,g**)

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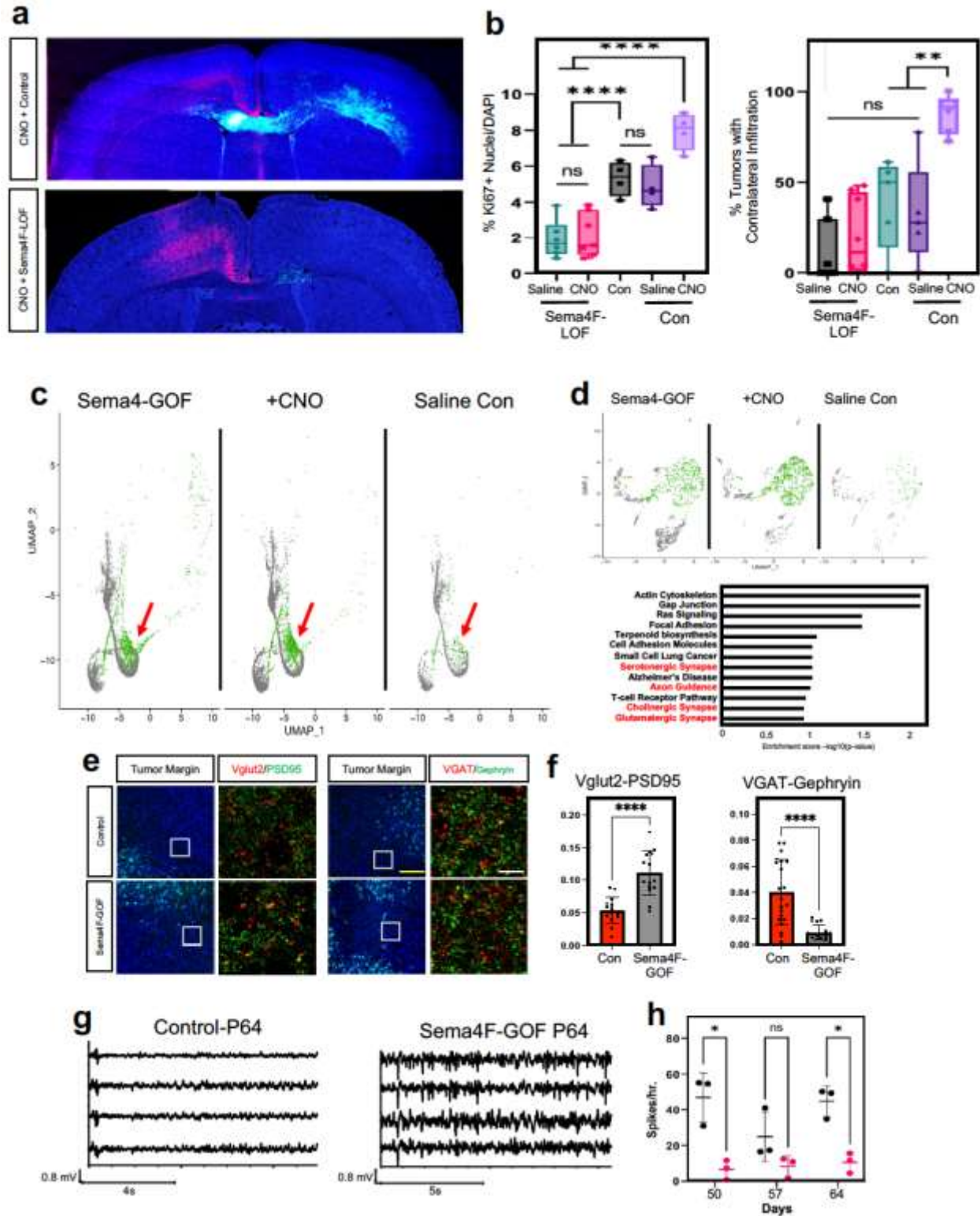
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693 **Figure 5. Sema4F promotes synaptic remodeling and brain hyperactivity**

694 **a.** Representative images from IUE-HGG tumors at P30 demonstrating the extent of
695 infiltration with combined Sema4F-LOF and neuronal activation (CNO) or neuronal
696 activation control; green is tumor, red is AAV-DREADD virus. **b.** Quantification of tumor
697 infiltration and Ki67 expression at P30; Infiltration was quantified based on the presence
698 of tumor cells in contralateral cortex and analyzed via one-way analysis of variance
699 (ANOVA). Error bars represent standard error, infiltration data derived from Sema4F-
700 KO+AAV+Saline n=7, Sema4F-KO+AAV+CNO n=8, 3xCr CTL n=5, 3xCr+AAV+Saline
701 n=5, 3xCr+AAV+CNO n=6. Proliferation data derived from Sema4F-KO+AAV+Saline
702 n=6, Sema4F-KO+AAV+CNO n=7, 3xCr CTL n=4, 3xCr+AAV+Saline n=4,
703 3xCr+AAV+CNO n=4. **c.** Single Cell RNA-Seq DimPlots of P50 IUE-HGG from Sema4F-
704 GOF, CNO, and saline controls. Cell types were mapped in SingleR with celldex as
705 expression profile reference; shown is the GFP+ tumor cluster. Infiltrating tumor
706 subpopulation is highlighted in green and denoted by red arrow. **d.** Sub-clustering
707 analysis of tumor cells shown in GO-term analysis on the unique prospective cell
708 population (in green) was performed using EnrichR and the KEGG 2021 Human dataset.
709 **e.** Antibody staining of excitatory (Vglut2-PSD95) and inhibitory synapses (VGAT-
710 Gephyrin) P50 mouse brains at peritumoral margins from Sema4F and control tumors;
711 box denotes zoomed in region in adjacent panel (10X and 200X magnification left to right;
712 white scale bar is 12.5 μ m and yellow scale bar is 200 μ m). **f.** Quantification of synaptic
713 staining derived from 3 separate tumors for each condition. Error bars represent standard
714 error, data derived from 3 tumors derived from 3 mice with n \geq 15 fields analyzed and
715 quantified, per condition. **g.** Sample EEG traces from mice bearing control or Sema4F-
716 GOF tumors. **h.** Quantification of spikes/hr over a 24-hr period at one-week intervals from

717 P50 to P64. Spikes were recorded in 3 mice per condition and analyzed via one-way
718 analysis of variance (ANOVA). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, one-
719 way analysis of variance (ANOVA) (f,h)