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1 Electrical and synaptic integration of glioma into neural circuits

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30 **Abstract:**

31 High-grade gliomas are lethal brain cancers whose progression is robustly regulated by neuronal
32 activity. Activity-regulated growth factor release promotes glioma growth, but this alone is
33 insufficient to explain the effect that activity exerts on glioma progression. Here, we use single-
34 cell transcriptomics, electron microscopy, whole-cell patch-clamp electrophysiology and calcium
35 imaging to demonstrate that neuron-glioma interactions include electrochemical communication
36 through bona fide AMPA receptor-dependent neuron-glioma synapses. Neuronal activity also
37 evokes non-synaptic activity-dependent potassium currents that are amplified through gap
38 junction-mediated tumor interconnections forming an electrically-coupled network. Glioma
39 membrane depolarization assessed with in vivo optogenetics promotes proliferation, while
40 pharmacologically or genetically blocking electrochemical signaling inhibits glioma xenograft
41 growth and extends mouse survival. Emphasizing positive feedback mechanisms by which
42 gliomas increase neuronal excitability and thus activity-regulated glioma growth, human
43 intraoperative electrocorticography demonstrates increased cortical excitability in glioma-
44 infiltrated brain. Together, these findings indicate that synaptic and electrical integration in neural
45 circuits promotes glioma progression.
46

47 **Introduction**

48 High-grade gliomas are the leading cause of central nervous system (CNS) cancer-related death in
49 both children and adults. This clinical intractability indicates that current understanding of glioma
50 pathophysiology is insufficient. Gliomas infiltrate extensively within the brain and spinal cord, but
51 growth outside the CNS is exceedingly rare. Glioma progression is regulated not only by cell-
52 intrinsic mechanisms, but also by important microenvironmental dependencies. Neurons are a
53 critically important component of the glioma microenvironment and regulate malignant growth in
54 an activity-dependent manner[1,2]. Activity-regulated release of neuroligin-3 (NLGN3)[1,2] is required
55 for glioma progression[2], indicating a fundamental role in glioma pathophysiology incompletely
56 explained by stimulation of classical oncogenic signaling pathways alone[2]. We previously found
57 that neuroligin-3 induces glioma expression of numerous synaptic genes[2], raising the intriguing
58 possibility that glioma may engage in synaptic communication. Synapses exist between neurons

59 and normal oligodendroglial precursor cells (OPCs)[3,4], and electrochemical signaling can regulate
60 proliferation, differentiation or survival of OPCs and other neural precursor cells (NPCs)[5-9]. As
61 cellular subpopulations within gliomas closely resemble OPCs[10,11], we hypothesized that gliomas
62 may similarly engage in synaptic communication and that this integration into neural circuits may
63 be fundamental to glioma progression.
64

65 **Synaptic gene expression in glioma**

66 To examine synaptic gene expression in primary human glioma, we analyzed single cell
67 transcriptomic datasets generated from pre-treatment biopsy samples of the major classes of adult
68 and pediatric high-grade gliomas, including adult IDH-mutant glioma[11], adult IDH-WT glioma[10],
69 and pediatric histone-3 mutant (H3K27M) diffuse midline glioma[10]. We found broad expression
70 of glutamate receptor genes and post-synaptic structural genes in malignant glioma cells (Fig. 1a,
71 Extended Data Fig. 1a). Unsupervised principal component analysis revealed enrichment of
72 synaptic gene expression within distinct malignant cellular subpopulations (Fig. 1b). Gliomas are
73 comprised of cellular subpopulations that resemble various stages of astrocytic and
74 oligodendrocytic differentiation[10,11]. Synaptic gene enrichment was chiefly found in glioma cells
75 that resemble OPCs (Fig. 1b, Extended Data Fig. 2c), the only glial cell type that normally
76 functions as a post-synaptic cell[3,4]. These observations are consistent with the principle that
77 malignant cellular subpopulations assume distinct roles in the heterogeneous cancer ecosystem.
78 Concordant with these findings from primary biopsy tissue (Fig. 1b), single cell transcriptomics
79 of patient-derived H3K27M+ glioma xenografts demonstrated synaptic gene enrichment in the
80 OPC-like subpopulation (Extended Data Fig. 1b, Extended Data Fig. 2a-b).

81 **Structural neuron-to-glioma synapses regulated by neuroligin-3**

82 Having established that primary glioma cells express a repertoire of synaptic genes, we
83 next assessed whether structural synapses form between glioma cells and neurons in the tumor
84 microenvironment. Examination of primary glioblastoma tissue ultrastructure using electron
85 microscopy (EM) revealed clear synaptic structures (Extended Data Fig. 2d). To confirm that
86 glioma cells participate in such putative neuron-glioma synapses, we performed immuno-EM in
87 GFP-labeled, patient-derived glioma xenografts. Immuno-EM analyses unambiguously identified
88 GFP+ glioma cells on the post-synaptic side of synaptic structures, with synapses on ~10% of
89 GFP+ glioma processes (Fig. 1c,d and Extended Data Fig. 2e-g). To test the contribution of
90 microenvironmental NLGN3 to neuron-glioma synaptogenesis, we next co-cultured glioma cells
91 expressing fluorescently-tagged PSD95 with WT or $NLGN3^{-/-}$ neurons. We found marked
92 reduction in the co-localization of neuronal presynaptic puncta (synapsin) with glioma post-
93 synaptic puncta (PSD95-RFP) in co-cultures with $NLGN3^{-/-}$ neurons compared to WT neurons
94 (Fig.1e-f, Extended Data Fig. 2h). This further implicates NLGN3 in glioma synapse formation, a
95 function distinct from its role stimulating classical oncogenic signaling pathways (Extended Data
96 Fig.3a-c).

97 **Electrophysiologically functional neuron-to-glioma synapses mediated by AMPA Receptors**

98 Focusing on pediatric gliomas, we next tested if neurons and glioma cells establish
99 electrophysiologically functional synapses using four distinct patient-derived orthotopic xenograft
100 models (Extended Data Table 1). GFP-labeled glioma cells were stereotactically xenografted into
101 the CA1 region of the hippocampal circuit. Following a period of engraftment and growth, acute
102 hippocampal slices were prepared for whole cell patch clamp recordings of GFP+ glioma cells.
103 Stimulation of Schaffer collateral/commissural afferent axons arising from CA3 while patch
104 clamping CA1 region glioma cells enables measurement of the glioma response to axonal activity

105 (Fig. 2a-b). Voltage-clamp recordings revealed stimulation-evoked fast (<5 ms; 25-50 pA) inward
106 currents consistent with excitatory postsynaptic currents (EPSCs, Fig. 2c). Current-clamp
107 recordings demonstrated that these inward currents were depolarizing (Fig. 2d). Glioma EPSCs
108 were blocked by the voltage-gated sodium channel blocker tetrodotoxin (TTX; Fig. 2e), illustrating
109 dependence on neuronal action potentials. Measuring the current-voltage relationship (I-V curve)
110 illustrated reversal at approximately 0 mV (Fig. 2f) and glioma EPSCs displayed facilitation in
111 response to paired stimuli (Fig. 2g), electrophysiological characteristics suggesting synaptic
112 communication through AMPA receptors (AMPAR, a type of ionotropic glutamate receptor).
113 Concordantly, glioma EPSCs were blocked by NBQX, an AMPAR antagonist (Fig. 2g-i) and
114 decreased by NASPM, an antagonist of calcium-permeable AMPARs (Extended Data Fig. 3d-e).
115 AMPARs lacking GluA2 or containing GluA2 that has not undergone RNA editing of its Q/R site
116 are calcium-permable[12,13]. GluA2 is broadly expressed in gliomas (Fig. 1a); examination of RNA
117 editing of the Q/R site in pediatric glioma demonstrated GluA2 under-editing (~50-70% edited;
118 Extended Data Fig. 3f-g). Taken together, these results indicate that axon stimulation-evoked,
119 millisecond timescale glioma cell currents require action potentials and are mediated by AMPARs,
120 properties consistent with the conclusion that subpopulations of glioma cells form bona fide
121 synapses with neurons.

122 To test this hypothesis further, we replaced extracellular calcium with strontium, a
123 manipulation that facilitates asynchronous presynaptic vesicle release[14] (Fig. 2j). In the presence
124 of strontium we detected small, fast inward currents consistent with miniature EPSCs (mEPSCs),
125 indicating quantal responses to synaptic vesicles[14] in glioma cells. Quantal glioma mEPSCs were
126 similarly blocked by NBQX (Fig. 2j). No fast, large currents reminiscent of action potentials were
127 observed in any of >640 glioma cell recordings. Taken together, these results indicate that synaptic

128 transmission occurs between neurons and a subset of xenografted human glioma cells, exhibiting
129 properties similar to synapses formed with normal OPCs[3,4].

130 Further exploring the consequences of activity-dependent currents in glioma, we
131 performed *in situ* two-photon calcium imaging of xenografted glioma expressing the genetically
132 encoded calcium indicator GCaMP6s. Glioma-specific expression of the calcium indicator was
133 validated by co-staining for human nuclear antigen (Extended Data Fig. 4a). Spontaneous calcium
134 transients were consistently observed (Extended Data Fig. 4b-d). Stimulation of Schaffer
135 collateral/commissural afferents elicited calcium transients in glioma cells located in the CA1
136 target area of the stimulated axons (Fig. 2k-l), providing additional evidence that endogenous
137 circuit activity may exert functionally relevant effects on glioma cells. These evoked calcium
138 transients were blocked by TTX (Fig. 2m, Extended Data Fig. 4e).

139 **Neuronal activity-dependent potassium currents in glioma cells**

140 A longer duration electrophysiological response to neuronal activity was found in a subset
141 of glioma cells (Fig. 3a). Distinct from the classical EPSCs (<5ms) described above, these
142 prolonged currents (>1 sec) exhibited kinetics inconsistent with a synaptic response and are instead
143 reminiscent of the neuronal activity-evoked currents observed in normal astrocytes. Supporting
144 the idea that these prolonged currents are distinct from the synaptic responses described above, the
145 calcium-permeable AMPAR inhibitor NASPM had no effect (Extended data Fig. 5a-b). These
146 prolonged glioma currents were blocked by TTX (Fig. 3b-c). Further illustrating a response
147 coupled to neuronal population firing, the morphology of the prolonged currents revealed spike-
148 like waveforms phase-locked to neuronal field potential waveforms that scaled with increased
149 axonal stimulation intensity (Extended Data Fig. 5c-d). Simultaneous whole cell current clamp
150 and field potential recordings reveal that the prolonged current amplitude scaled directly with field

151 potential, meaning that prolonged glioma current amplitude increases with increasing neuronal
152 activity (Extended Data Fig. 5e-f). In normal astrocytes, activity-dependent currents are
153 attributable to glutamate transporter currents and inward potassium currents due to a rise in
154 extracellular potassium from neurons[15-17]. Consistent with a direct role for increases in
155 extracellular potassium in generating these prolonged currents, similar prolonged glioma currents
156 were elicited by application of potassium alone with neuronal activity pharmacologically blocked
157 (Fig. 3d, Extended Data Fig. 5g). Further, activity-dependent prolonged currents were largely
158 blocked by barium, an ion that occludes potassium pores (Fig.3e-f). In contrast, the glutamate
159 transporter antagonist TBOA had a negligible effect (Extended Data Fig. 5h). Taken together,
160 these results support the interpretation that non-synaptic, prolonged glioma currents chiefly reflect
161 potassium flux attributable to a rise in extracellular potassium with neuronal activity.

162 **Gap junction coupling amplifies potassium currents in glioma**

163 Cells with prolonged currents exhibit strikingly low input resistance (Extended Data Fig.
164 6a), reminiscent of astrocytes. Extensive gap-junctional coupling is partially responsible for low
165 membrane resistance in astrocytes[18]. Gap junctions couple adult glioma cells through long
166 processes called tumor microtubes[19], which we demonstrated in primary pediatric glioma tissue
167 (Extended Data Fig. 5j-o). Following biocytin dye-filling of single cells exhibiting prolonged
168 currents, biocytin diffused to a network of glioma cells (Extended Data Fig. 6b), supporting the
169 existence of a gap junction-coupled network. To test this conclusion, we applied the gap junction
170 blockers carbenoxolone (CBX) or meclofenamate, which reduced the amplitude of prolonged
171 glioma currents (Fig. 3g, Extended Data Fig. 6c-e, 6h-i) while simultaneously increasing glioma
172 input resistance (Extended Data Fig. 6f-g, 6j-k). Together, these observations strongly suggest that
173 activity-regulated increases in extracellular potassium concentrations cause glioma depolarization

174 and that a gap junction-coupled glioma network amplifies the consequences of activity-induced
175 changes in the extracellular ionic environment.

176 Two-photon calcium imaging further revealed distinct synchronous network calcium
177 transients that both occur spontaneously (Fig. 3h, Extended Data Fig. 5i, Supplementary Video 1)
178 and are elicited by afferent stimulation (Fig. 3i, Supplementary Video 2). This synchronicity could
179 be explained by gap-junction coupling, and accordingly was blocked by application of CBX
180 (Extended Data Fig. 6l-o, Supplementary Video 3), further indicating a functional glioma network
181 through which depolarizing currents propagate. Demonstration of glutamatergic chemical
182 synapses (Fig. 2) and activity-dependent, non-synaptic potassium currents (Fig. 3) build upon early
183 work illustrating glutamate-dependent currents in glioblastoma[20] to underscore the surprising
184 observation that this cancer is an electrically active tissue (see Supplementary Videos 1, 2, and 3).

185 **Intratumoral electrophysiological heterogeneity**

186 Gliomas exhibit intratumoral and intertumoral cellular heterogeneity, with subpopulations
187 of cancer cells assuming particular roles and even very small cellular fractions proving essential
188 for cancer progression[21]. Considering all pediatric glioma cells examined (n=643), we find that
189 ~5–10% of glioma cells exhibit synaptic EPSCs, ~40% exhibit prolonged currents in response to
190 neuronal activity. While all four patient-derived xenograft models exhibited neuronal activity-
191 evoked inward currents, the proportion of cells displaying fast EPSCs or prolonged currents varies
192 between patient-derived models. This intertumoral heterogeneity is evident even within a
193 molecularly-defined subtype such as H3K27M+ glioma and predicted by the varied composition
194 of OPC-like and astrocyte-like compartments in individual tumors (Extended Data Fig. 2b, 7a).

195 **Membrane depolarization promotes glioma proliferation**

196 Depolarization can profoundly affect cellular behavior[5-9], and we have found two distinct
197 mechanisms by which neuronal activity induces glioma cell membrane depolarization. To test if
198 glioma cell membrane depolarization promotes proliferation, we used *in vivo* optogenetic
199 techniques to depolarize xenografted glioma cells expressing the blue light-sensitive cation
200 channel channelrhodopsin-2 (ChR2; Extended Data Fig. 7b). Glioma cells expressing ChR2-YFP
201 were xenografted to the cortex, and after a period of engraftment and growth blue light was
202 delivered to depolarize the glioma xenograft. Compared to mock-stimulated control groups, we
203 found that glioma depolarization robustly promoted glioma xenograft proliferation (Fig. 4a-d).
204 Blue light exposure alone had no effect on proliferation nor apoptosis in control glioma xenografts
205 (Extended Data Fig. 7c-e).

206 **Targeting mechanisms of electrochemical communication reduces glioma growth**

207 As membrane depolarization promotes glioma proliferation, we next tested the relative
208 functional contributions of each mechanism of electrochemical communication, beginning with
209 AMPAR-mediated EPSCs. We over-expressed either WT-GluA2 subunit fused to GFP, GFP
210 alone, or a dominant-negative GluA2 (GluA2-DN-GFP) in glioma and confirmed decreased
211 conductance in GluA2-DN-GFP-expressing glioma cells (Extended Data Fig. 7f-g). Mice bearing
212 xenografts overexpressing WT-GluA2-GFP survived a shorter time than GFP-only xenografts,
213 while mice bearing GluA2-DN-GFP xenografts exhibited improved survival and decreased tumor
214 burden compared to GFP-only controls (Fig. 4e-j, Extended Data Fig. 8a-b). To test for a possible
215 *in vivo* growth advantage of GluA2-expression, we xenografted a mixture of 80% GluA2-DN-GFP
216 construct-expressing:20% non-expressing cells and found that tumors were composed almost
217 entirely of non-GluA2-DN-GFP expressing cells at the survival endpoint (Fig. 4g-h).

218 Similar to *in vivo* experiments, co-culture of glioma cells with neurons markedly increased
219 proliferation. NBQX partially reduced this effect (Extended Data Fig. 8c-d); neuronal secreted
220 factors such as NLGN3 accounts for residual elevated proliferation[1]. Given the stark effect of
221 AMPAR function on glioma growth *in vivo* and in co-culture, we next tested the relative
222 contributions of cell-intrinsic glutamate signaling mechanisms. While paracrine/autocrine AMPA
223 signaling may promote adult glioblastoma growth[22,23], NBQX had no effect on pediatric glioma
224 proliferation in the absence of neurons (Extended Data Fig. 8c-d). Testing cell-intrinsic effects of
225 GluA2-DN expression, we similarly found no difference in growth rate or apoptosis in pediatric
226 glioma cells outside of the neuronal microenvironment (Extended Data Fig. 8e-f). In contrast,
227 pediatric glioma cell migration and invasion were influenced by GluA2-DN expression in a cell-
228 intrinsic manner (Extended Data Fig. 8g-j), consistent with previous reports in adult gliomas[24].
229 Taken together, these findings indicate that glioma AMPAR activation promotes pediatric glioma
230 growth chiefly through microenvironmental interactions such as neuron-to-glioma synaptic
231 transmission.

232 Next, we sought to therapeutically target glioma currents using existing drugs. Using an
233 AMPAR-blocking anti-epileptic drug (perampanel), we found a ~50% decrease in pediatric glioma
234 proliferation in perampanel-treated mice compared to vehicle-treated controls (Fig. 4k, Extended
235 Data Fig. 8k). We then targeted gap junction-mediated amplification of neuronal activity-
236 dependent potassium currents using the brain-penetrant gap-junction blocker meclofenamate[25].
237 Meclofenamate treatment similarly decreased pediatric glioma xenograft proliferation and growth
238 (Fig. 4l, Extended Data Fig. 8l).

239 **Gliomas increase neuronal excitability in humans**

240 Neuron-glioma interactions are bidirectional; neuronal activity increases glioma growth[1],
241 and gliomas are thought to increase neuronal activity. In preclinical adult glioblastoma models,
242 glioma cells induce neuronal hyperexcitability and seizures[26,27] through non-synaptic glutamate
243 secretion[26,28], and through secretion of synaptogenic factors[27]. To assess neuronal hyperexcitability
244 in primary human glioblastoma, we performed intraoperative electrocorticography in three awake
245 adult human subjects with cortical high-grade gliomas (IDH WT) prior to surgical resection (Fig.
246 5). High gamma frequency (70-110 Hz) power, a measure that correlates with neuronal firing rate
247 and local field potential[29] and that is elevated by cortical hyperexcitability[30], was sampled over a
248 3-minute period while the subjects were in a resting state. Outside of the necrotic nodular core of
249 the tumor, we found markedly increased high gamma power in tumor-infiltrated brain compared
250 to normal-appearing brain (Fig. 5a-b, Extended Data Fig. 9a). Concordantly, we found neuronal
251 hyperexcitability in the microenvironment of pediatric glioma xenografts (Fig. 5c). These findings
252 support the concept[26,27] of hyperexcitable neurons in the glioma microenvironment, which would
253 potentiate mechanisms of activity-regulated glioma progression. As neuronal action potentials
254 result in extracellular potassium rise[31,32], neuronal hyperexcitability in the glioma
255 microenvironment would promote non-synaptic prolonged glioma potassium currents, as well as
256 synaptic neuron-to-glioma EPSCs (Extended Data Fig. 9b).

257 **Discussion**

258 Membrane depolarization and depolarization-induced calcium transients promote normal
259 NPC development, in part through voltage-gated calcium channel signaling[5-9]. Non-synaptic
260 neurotransmitter release mediates synchronous calcium transients in gap-junction-coupled NPCs
261 in many contexts[33-35], but synapses between presynaptic neurons and post-synaptic NPCs are also
262 well-described, including transient synapses onto migrating neuroblasts during corticogenesis[36]

and synapses onto OPCs in the developing and adult brain[3,4]. High-grade gliomas integrate into electrical networks and that depolarizing current promotes glioma progression similarly to the well-established effect in normal NPCs. In glioma, we have demonstrated bona fide neuron-to-glioma synapses, reminiscent of the synapses found on normal OPCs. As well, we have shown neuronal activity-evoked potassium currents in glioma cells, reminiscent of activity-dependent currents in normal astrocytes[37]. Neuronal activity-induced glioma membrane depolarization by either mechanism promotes glioma proliferation and growth, through voltage-sensitive signaling pathway(s) to be fully elucidated in future work.

Neuronal activity is emerging as a critical regulator of glioma progression. The electrochemical communication described here joins activity-regulated secretion of growth factors[1,2] as mechanisms that mediate this important microenvironmental interaction. Appreciating the crosstalk between neurons and the glioma cells invading and integrating into neural circuitry elucidates promising therapeutic targets, including activity-regulated secreted growth factors[1,2], neuron-to-glioma neurotransmission, ion channels and gap-junction coupling. Modulating the influence of glioma on neuronal excitability represents an important opportunity to dampen the magnitude of activity-regulated glioma growth. Taken together, the findings presented here identify synaptic neurotransmission and activity-dependent potassium currents as mechanisms driving glioma growth and elucidate the previously unexplored potential to target glioma circuit dynamics for therapy of these lethal cancers.

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Author Contributions: H.S.V. and M.M. designed, conducted, and analyzed experiments. W.M. conducted electrophysiology experiments. L.N. and H.V. contributed to electron microscopy data acquisition and analyses. S.H.J. performed intraoperative electrocorticography, D.B. conducted high gamma frequency power computational analyses. D.S. and S.M.G. contributed to single cell transcriptomic analysis, S.M.G. and M.A contributed to synaptic puncta and tumor microtube confocal imaging, A.G. and L.T.T. contributed to optogenetic experiments. A.A. and D.E.B. provided GluA2 dominant negative construct. A.P, K.R.T., and P.J.W. contributed to in vitro and in vivo data collection and analyses. C.E. contributed to quantitative imaging analyses. R.C.M., D.E.B., S.M.G., W.M., H.S.V. and M.M. contributed to manuscript editing. H.S.V. and M.M. wrote the manuscript. M.M. supervised all aspects of the work.

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