Research Article

Insulin signaling mediates neurodegeneration in glioma

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Cell to cell communication facilitates tissue development and physiology. Under pathological conditions, brain tumors disrupt glia-neuron communication signals that in consequence, promote tumor expansion at the expense of surrounding healthy tissue. The glioblastoma is one of the most aggressive and frequent primary brain tumors. This type of glioma expands and infiltrates into the brain, causing neuronal degeneration and neurological decay, among other symptoms. Here, we describe in a Drosophila model how glioblastoma cells produce ImpL2, an antagonist of the insulin pathway, which targets neighboring neurons and causes mitochondrial disruption as well as synapse loss, both early symptoms of neurodegeneration. Furthermore, glioblastoma progression requires insulin pathway attenuation in neurons. Restoration of neuronal insulin activity is sufficient to rescue synapse loss and to delay the premature death caused by glioma. Therefore, signals from glioblastoma to neuron emerge as a potential field of study to prevent neurodegeneration and to develop anti-tumoral strategies.

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Significance Statement

Glioblastoma (GB) is among the most aggressive type of brain tumors, and currently there is no cure or effective treatment. Patients course with neurological deficit and previous data in animal models indicate that GB causes a neurodegenerative process. In a Drosophila model, we describe here a molecule named ImpL2 that is produced by GB cells and impacts on neighboring neurons. ImpL2 is an antagonist of the insulin pathway, and insulin signaling reduction in neurons causes mitochondrial defects and synapse loss. These mechanisms underlying GB-induced neurodegeneration play a central role in the premature death caused by this tumor. Restoration of insulin signaling in neurons prevents tumor progression and rescues the lethality caused by GB in Drosophila models.

Introduction

Cancer is one of the leading causes of mortality worldwide and is expected to be responsible for 15 million deaths in 2020 (65% in less developed countries) according to the World Health Organization. Notwithstanding recent advances in health treatments and extended lifespan of patients, some tumors still remain incurable. Among them, GB stands out because it is the most frequent and a very aggressive primary brain tumor. It is originated from glial cells and causes death within the first year after diagnosis (Bi & Beroukhim, 2014), despite standard treatments such as resection, radiotherapy, and chemotherapy. This is accompanied by broad neurological dysfunctions (Messaoudi et al, 2015). Brain tumors cause cognitive decline and neuronal dysfunction (reviewed in Gehrke et al [2013] and Bergo et al [2016]). These cognitive defects are consistent with typical neurodegenerative-associated symptoms such as synapse loss and mitochondrial alterations (Granholm et al, 2010; Levenson et al, 2014).

Drosophila melanogaster, the fruit fly, has emerged as a reliable animal system to mimic human diseases such as cancer (Sonoshita & Cagan, 2017). The aim is to model cellular and molecular mechanisms of human diseases, to identify targets for eventual diagnosis and treatments of patients. The power of Drosophila genetics allows genetic and pharmacological screens that may be translated to medicine, particularly for neurodegenerative disorders (Casas-Tinto et al, 2011; Dar et al, 2012; Burke et al, 2017; Held et al, 2019; Portela et al, 2019a). In fact, a Drosophila GB model that recapitulates most of the human disease features has been developed and validated (Read et al, 2009, 2013; Read, 2011; Portela et al, 2019a, 2019b). This model is based on two of the most frequent mutations in patients, a constitutively active form of the epidermal growth factor receptor (EGFR) and the phosphatidylinositol-3 kinase (PI3K) catalytic subunit p110α (PI3K92E) driven by the glial specific repo-Gal4 (Read et al, 2009). This animal model has brought novel understandings into GB molecular mechanisms (Read, 2011; Read et al, 2013; Weng et al, 2017; Portela et al, 2019a, 2019b).

miRNAs are short noncoding RNAs that control gene activity mainly through post-transcriptional mechanisms. Recently, they have been linked to almost all biological processes and diseases, particularly cancer (Peng & Croce, 2016; Sander & Herranz, 2019). Concerning glioma, the miR-200 family (which includes miR-200, miR-141, and miR-429) plays central roles in GB development, metastasis, therapeutic response, and prognosis (reviewed in Peng et al [2018]). Low levels of miR-200 are indicative of poor prognosis in GB (Men et al, 2014). In colorectal cancer and GB, low expression
levels of miRNAs correlates with up-regulation of insulin-like growth binding protein 7 (IGFBP7) (Jones et al., 2015). Likewise, in GB there is a transforming growth factor beta-2 (TGFβ2)–dependent increase in IGFBP7 protein levels (Pen et al., 2008). However, the mechanisms involved in IGFBP7 influence on GB progression and its regulation by mir-200 remains unsolved. In Drosophila, mir-200 and IGFBP7 are represented by mir-8 and Imaginal morphogenesis protein-late 2 (ImpL2), respectively (Honegger et al., 2008). In juvenile stages, mir-8 has been found to regulate glial cell growth and to promote synaptic growth at the neuromuscular junction (Morante et al., 2013; Loya et al., 2014).

In contrast, Drosophila ImpL2 induces cachexia, a systemic effect characterized by anorexia and metabolic alterations induced by other malignant tumors (Petruzziello & Wagner, 2016). Secreted ImpL2 from epithelial tumor cells induces systemic organ wasting and insulin resistance by antagonizing insulin signaling (Figuerola-Clarevega & Bilder, 2015; Kwon et al., 2015). Interestingly, P3K and Drosophila Ras homolog enriched in brain (dRheb), two members of the insulin pathway, induce the formation of synapses between neurons (a process known as synaptogenesis) in the Drosophila larval brain (Martín-Peña et al., 2006). Actually, it has been shown that Akt, also involved in insulin signaling, acts as a pro-synaptogenic element (Jordán-Alvarez et al., 2017). These data strongly support a role for insulin signaling in the regulation of neuronal synaptic activity in Drosophila. In mammals, a similar effect of insulin signaling on synaptic plasticity has been described (Knafo & Esteban, 2017). Notably, synapse loss is an early step in neurodegeneration (Sephthon & Yu, 2015; Henstridge et al., 2016). We have recently re-evaluated GB as a tumor with degenerative disease, showing that GB reduces the number of synapses through wingless/frizzled 1 (wg/frz1) signaling (Portela et al., 2019b), equivalent to mammalian WNT pathway (Arnés & Casas Tintó, 2017). However, whether tumoral glial cells are able to modify insulin signaling in contiguous neurons, and consequently alter the number of synapses, is yet unknown.

To study the mechanisms of communication among malignant glial cells and neurons, we used a previously well-characterized Drosophila GB model that reproduces the oncogenic transformation of glial cells and lethal glial neoplasia in post-embryonic larval (Read et al., 2009, 2013) or adult brains (Portela et al., 2019a), leading to lethal glial neoplasia. We previously reported a reduction in the number of synapses in the neuromuscular junction (NMJ) of adult flies caused by GB progression (Portela et al., 2019b). This neurodegenerative process is consequence of genetic modifications caused in glial cells. This phenomenon suggests that signals originated in the glial tumor can impact on neighboring neurons. Synaptogenesis is tightly regulated by P3K, a main player in insulin signaling pathway (Jordán-Alvarez et al., 2017). Moreover, GB progression correlates with high levels of secreted molecules that decrease insulin pathway activity, such as ImpL2 (IGFBP7 in humans) (Pen et al., 2008; Jones et al., 2015).

In this report, we showed that secreted ImpL2 from glial-derived tumoral cells antagonizes insulin signaling in neighboring neurons, inducing a reduction in synapse number and consequently promoting neurodegeneration. ImpL2 expression in GB cells is regulated by mir-8, thus linking functionally miRNA pathway with insulin signaling in a GB model. We described the function of ImpL2 as a mediator in GB–neuron communication, responsible for the reduction in synapse number and neurological defects. Indeed, we propose the insulin pathway as a core signal in GB progression and neurological decay. Finally, we propose neurodegeneration as a relevant factor in the lethality induced by GB.

Results

ImpL2 mediates GB progression and the associated neurodegeneration

To determine ImpL2 mRNA expression levels in GB we performed qRT-PCR experiments. ImpL2 mRNA showed an increase in GB samples as compared with control brains (Fig 1A). To discriminate between ImpL2 expression in neuronal or glial (GB) cells, we used a MINIC GFP reporter that reproduced faithfully ImpL2 expression (Nagarkar-Jaiswal et al., 2015). Consistently, GB cells showed higher reporter GFP levels than control glial cells, which are restored to control levels upon ImpL2 knockdown (Fig 1B–D and G). Therefore, these results validate the ImpL2 RNAi tool and indicate that ImpL2 is up-regulated in GB cells.

In a previous work, we established that tumoral progression depended on the formation of a network of protrusions (i.e., an expansion of the membrane surface) named tumor microtubules (TMs), similarly to human GB (Osswald et al., 2015; Portela et al., 2019b). Besides, in mammals and flies, the TM network required the GAP43 and igloo gene functions, respectively (Osswald et al., 2015; Portela et al., 2019b). We also showed that GB progression requires c-Jun N-terminal Kinase (JNK) pathway activity (Portela et al., 2019b). The Drosophila JNK homolog Basket (Bsk) plays a central role in JNK signaling in normal and tumoral conditions (Fahey-Lozano et al., 2019). To determine if ImpL2 up-regulation in glial cells requires TMs network expansion, or JNK pathway activity in GB cells, we down-regulated igloo expression to prevent TMs formation, or over-expressed a dominant negative form of bsk (BSKDN) to block JNK activity. ImpL2 expression levels were reverted to normal levels in both cases, indicating that glial ImpL2 expression was dependent on TMs and JNK pathway activity, but not transcriptionally regulated by activated EGFR or Dp110 (Fig 1E–G).

To study the neurodegeneration associated to GB progression, we quantified the number of synapses in the neuromuscular junction (NMJ) of the adult flies with GB. NMJ is a stereotyped structure that allows counting the number of synapses in the synaptic buttons of the motor neurons by using anti-bruchpilot, a specific antibody that recognizes synapses unambiguously (see the Materials and Methods section for details). To determine the contribution of glial ImpL2 to synapse loss, we knocked-down ImpL2 in GB cells and counted the number of synapses in adult NMJs. The results showed that ImpL2 reduction in GB cells counteracted the reduction in the number of synapses observed in GB samples (Fig 2A–C and E). Next, to determine if ImpL2 is sufficient to decrease the number of synapses or it requires further features of GB cells, we overexpressed ImpL2 in wild-type (wt) glial cells and quantified the number of synapses. The results show a decreased number of synapses in NMJs (Fig 2D and E), consistent with our previous
results, and suggest that ImpL2 expressed in gial cells is sufficient to cause synapse loss in neurons.

Besides, we studied the two additional and typical features of GB such as the increase in the number of gial cells and expansion of the TM network (Fig 2F, G, I, and J). The confocal images and quantifications showed that the down-regulation of ImpL2 RNA levels in GB caused a striking reduction in the number of gial cells (Fig 2H and I) and in the total tumor volume (Fig 2J). Thus, we concluded that GB cells up-regulated and secreted ImpL2, a necessary step to induce tumoral expansion and a reduction of the synapse number in surrounding neurons. These results suggest that GB progression and neurodegeneration are coupled events.

miRNAs levels inversely correlate with ImpL2 expression and GB progression

It has been described that low levels of miRNAs correlated with high levels of ImpL2 homolog in human GB, suggesting that ImpL2 regulation might be mediated by miRNAs (Jones et al, 2015). Accordingly, there is a down-regulation of the miRNA miR-200 family in GB samples (reviewed in Peng et al [2018]). Given that miR-8, the Drosophila homolog of miR-200 family, negatively regulates ImpL2 mRNA stability in the fat body (Lee et al, 2015), we hypothesized that miR-8 might play a role in GB progression and ImpL2 levels regulation. We used a miR-8 sensor to monitor miR-8 activity in GB brains. It includes miR-8 binding sites in the 3’UTR of the GFP gene (Kennell et al, 2012). Thus, high levels of GFP indicate low levels of miR-8 activity and vice versa (see the Materials and Methods section). GFP signal was increased in GB cells (Fig 3A–C), indicating that miR-8 levels were reduced. To determine if the increase in ImpL2 in GB depended on miR-8, we analyzed ImpL2 sensor upon miR-8 overexpression in that context. We observed a significant reduction in ImpL2 expression in GB cells in vivo upon miR-8 overexpression (Fig 3D–G). Consistently, miR-8 gain-of-function in GB rescued the loss of synapses, recapitulating the effect of ImpL2 loss of function in GB conditions (Fig 3H–I and L). These results are consistent with the effect of ImpL2 down-regulation in GB that also rescued the synapse number (Fig 2C and E), and suggest that miR-8 regulates ImpL2 expression in GB.

Furthermore, miR-8 overexpression in wt gial cells (with low ImpL2 levels) did not alter the number of synapses (Fig 3K and L). Intriguingly, GB cell number expansion was not prevented by miR-8 overexpression (Fig 3M–O and Q), and consistently miR-8 over-expression increased glial cell number in wild type conditions (Fig 3P and Q). However, we did observe a reduction in GB membrane volume upon miR-8 up-regulation, something that did not occur in normal gial cells (Fig 3R). Altogether, these results showed an inverse correlation between miR-8 and ImpL2 expression in GB cells and suggested that ImpL2 levels are regulated by miR-8 in vivo.

GB secreted ImpL2 reduces insulin signaling in neurons

To evaluate the impact of GB on insulin signaling in neurons, we used a fluorescent reporter (tGPH, composed by the fusion of a pleckstrin homology domain plus green fluorescent protein under the control of the tubulin promoter) that reports the activity of PI3K and, thus, is widely used as a monitor of insulin pathway (Britton et al. 2002). tGPH reporter activity in neurons is strongly reduced (although still detectable) upon GB induction compared with control neurons (Fig 4A and B). In addition, ImpL2 knockdown specifically in GB cells prevents tGPH reporter reduction in neurons, suggesting a normalization of insulin signaling levels (Fig 4C).

To further analyze insulin-dependent FOXO activity, we used the ThorMIMIC line that bears a MiMIC transgene inserted in the...
The Thor gene encodes for a protein that is involved in translational control. It is regulated by FOXO, and its expression can hence be used as a surrogate of FOXO activity. In normal conditions, Thor transcription remains at low but detectable levels (Teleman et al., 2005). However, when insulin activity is compromised, Thor is highly transcribed, as reflected by LacZ or MIMIC lines (Galagovsky et al., 2014). Neurons exposed to GB had reduced insulin signaling, as shown by Thor MI09732GFP expression. This effect on insulin pathway was restored by down-regulating ImpL2 in GB cells (Fig 4D–G). All these results together suggested that the ImpL2 effect on synapsis was due to a deregulation of the insulin signaling in neurons.

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Figure 2. ImpL2 down-regulation in glioma cells rescues neurodegeneration and reduces tumor progression. (A, B, C, D) Confocal images of adult neuromuscular junction (NMJ) at 29°C from (A) repo>UAS-LacZ (Control), (B) repo>UAS-dEGFRλ, UAS-dp110CAAX (Glioma), (C) repo>UAS-dEGFRλ, UAS-dp110CAAX, UAS-impl2 RNAi (Glioma Impl2↓), and (D) repo>impl2 (Glia Impl2↑) 7-d-old animals. Active zones are marked in green (anti-NC82) (scale bar, 50 μm). (E) Quantification and statistical analysis of active zones in at least N = 13 per genotype (ANOVA, post hoc Bonferroni). (F, G, H) Confocal microscopy images of adult brains from (F) repo>UAS-LacZ, (G) repo>UAS-dEGFRλ, UAS-dp110CAAX and (H) repo>UAS-dEGFRλ, UAS-dp110CAAX, UAS-impl2 RNAi flies after 7 d at 29°C with the glial nuclei marked in green. (F′, G′, H′) Glial membrane is shown in red (scale bar, 100 μm). (I, J) Quantification of (I) glial cell number and (J) glial membrane volume for at least N = 7 per genotype (ANOVA, post hoc Bonferroni) (*P-value < 0.05, **P-value < 0.01, ***P-value < 0.001).
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Figure 3. Increasing miR-8 levels in gliomas impairs ImpL2 overexpression and synapse loss.

(A, B) Confocal microscopy images of adult brain showing the expression pattern of miR-8 in repo>UAS-LacZ control animals compared with repo>UAS-dEGFR, UAS-dp110CAAX flies using miR-8 sensor (see the Materials and Methods section). (A’, B’) Gial membrane is marked in red. (C) Quantification and statistical analysis of miR-8 expression based on the GFP pixel intensity in at least N = 3 per genotype (ANOVA, post hoc Bonferroni) (scale bar, 50 μm). GFP control levels were normalized to one and GFP increase in glioma brains was transformed into negative values to indicate the relation between the increase in GFP and the reduction in miR-8 levels of expression.

(D, E, F) Confocal microscopy images of 7–8–old adult brains from the following genotypes: (D) repo>UAS-LacZ (Control), (E) repo>UAS-dEGFR, UAS-dp110CAAX (Glioma), (F) repo>UAS-dEGFR, UAS-dp110CAAX, UAS-miR-8 (Glioma miR-8) animals, in all cases combined with ImpL2-MIMIC GFP transgene (see the Materials and Methods section). (D’, E’, F’) Merge with gial membrane in red and DAPI staining.

(G) Quantification and statistical analysis of co-localization rate between ImpL2-MIMIC GFP and gial membrane in at least N = 10 per genotype (ANOVA, post hoc Bonferroni).

(H, I, J, K) Adult neuromuscular junction (NMJ) from (H) repo>UAS-LacZ (Control), (I) repo>UAS-dEGFR, UAS-dp110CAAX (Glioma), (J) repo>UAS-dEGFR, UAS-dp110CAAX, UAS-miR-8 (Glioma miR-8), and (K) repo>miR-8 (Glia miR-8), active zones marked in green.

(L) Quantification and statistical analysis of active zones in at least N = 10 per genotype (ANOVA, post hoc Bonferroni) (scale bar, 50 μm).

(M, N, O, P) Confocal microscopy images of adult brains from 7–8–old flies of (M) repo>UAS-LacZ, (N) repo>UAS-dEGFR, UAS-dp110CAAX, (O) repo>UAS-dEGFR, UAS-dp110CAAX, UAS-miR-8 and (P) repo>UAS-miR-8 genotypes, gial nuclei marked in green (M’, N’, O’, P’) gial membrane is shown in red. (Q, R) Quantification of (Q) gial cells and (R) gial membrane volume for at least N = 9 per genotype (ANOVA, post hoc Bonferroni) (scale bar, 100 μm) (*P-value < 0.05**P-value < 0.01, ***P-value < 0.001).
Figure 4. Glioma-secreted ImpL2 inhibits insulin pathway activity in neurons.

(A, B, C) Confocal microscopy images of adult brain of 7-d-old flies from (A) repo>UAS-LacZ (Control), (B) repo>UAS-dEGFR, UAS-dp110CAAX (Glioma) and (C) repo>UAS-dEGFR, UAS-dp110CAAX, UAS-ImpL2 RNAi (Glioma ImpL2↓) genotypes, combined with tGPH transgene reporter of PI3K activity (green). (A′, B′, C′) Merge with glial nuclei in red and DAPI staining (scale bar 10 μm). White arrows point to non-glial cells with PI3K activity. The images are representative of at list N = 6 per genotype.

(D, E, F) Confocal microscopy images of 7-d-old adult fly brains of the genotypes: (D) repo>UAS-LacZ, (E) repo>UAS-dEGFR, UAS-dp110CAAX and (F) repo>UAS-dEGFR, UAS-dp110CAAX, UAS-ImpL2 RNAi, in all the cases combined with ThorMI09732 line. (D′, E′, F′) Neuronal nuclei are marked in blue. White arrows indicate nuclear localization of ThorGFP.

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that, in GB samples, mitochondria area is significantly reduced, and dRheb overexpression prevents this morphological defect (Fig 5N). Altogether, these results indicated that neurons showed mitochondrial disruption as a consequence of low insulin signaling. Increasing insulin pathway activity in neurons exposed to GB might be sufficient to recover functional organelles, as suggested by the synapse number.

GB brains with high insulin signaling in neurons also showed a reduction in the number of glial cells and in tumor volume (Fig 6A–E). More importantly, GB caused premature death, an effect that was significantly rescued by overexpressing dRheb in neurons (Fig 6F). These results suggested that restoration of neuronal insulin pathway activity improved the lifespan in animals with GB, thus linking synaptogenesis to a slower disease progression and functional protection. In conclusion, the communication between GB cells and neurons is proposed as a novel field of study for GB progression and provides a possible novel therapeutic target.

Discussion

GB is one of the most aggressive type of brain tumor (Bi & Beroukhim, 2014). During GB progression, tumoral cells extend a network of membrane projections that contribute to brain infiltration and results in poor prognosis for the patient (Osswald et al, 2015). GB courses with a neurological decay that includes sleep disturbances, speech difficulties and other typical symptoms of neurodegeneration (Bergo et al, 2016). For decades, the origin of this decay was attributed to the high pressure caused by the GB solid mass and the associated edema. Despite the solid mass of the GB being removed after surgery, the neurodegenerative process persists, mainly due to the GB remaining after surgery, the neurodegenerative process continues, more likely because of the diffuse GB progression. This indicates that mechanisms underlying neurological decay are not restricted to the intracranial pressure and edema.

Recent publications suggested an active communication between GB cells and the surrounding healthy tissue, including neurons. Experiments performed with human GB cells in mice xenografts revealed a physical interaction between GB cells and neurons as electrical and chemical synapses (Venkataramani et al, 2019; Venkatesh et al, 2019). In this case, neurons act as presynaptic structures whereas GB tumoral cells are postsynaptic elements. These so-called "synapses" are required for GB progression. Besides, we have recently described cellular mechanisms for GB to deplete Wingless (Wg)/WNT from neurons. GB cells project TMs that enwrap neurons and accumulate Frizzled1 (Fz1) receptor to vampirize Wg from neighboring neurons. This unidirectional mechanism facilitates GB proliferation and causes a loss of synapses in the neurons (Portela et al, 2019b). In addition to this, we here also describe a bidirectional communication system between GB cells and neurons. In contrast to what happen with Wg/Fz1, ImpL2 protein is originated in GB cells and target healthy neurons, but not vice versa. ImpL2 binds InR ligands and act as an antagonist of the pathway. In consequence, insulin signaling might be reduced in neurons which in turn caused synapse loss and lethality (Fig 7). Neuronal insulin signaling can be restored via dRheb up-regulation, and this is sufficient to extend the lifespan of GB animals. It is tempting to propose that ImpL2 impacts both tumor expansion (GB membrane) and neurodegeneration. The rescue of the neurodegenerative phenotype by ImpL2 knockdown could be caused by an autonomous GB signaling that impairs tumor progression, or by the restoration of insulin signaling in neurons. However, dRheb up-regulation in neurons prevented GB cells number increase, suggesting that GB progression is impaired when neuronal insulin signaling is active. These results suggest that reducing insulin signaling (and the subsequent neurodegeneration) is critical for GB progression and invasion, and ultimately for the lethality caused by GB. In conclusion, the GB is able to alter neuronal regular functions actively by at least two ways: vampirizing a required growth factor from neurons (like Wg) or secreting an antagonist of the pathway (such as ImpL2). In both cases, the consequence is similar, a signaling pathway essential for synaptogenesis in the healthy neuronal population is down-regulated.

The regulation of ImpL2 expression in GB cells seems to depend on miRNAs, at least in part. In cancer, miRNAs have emerged as general regulators key for tumoral progression, including GB (Sander & Herranz, 2019). Interestingly, miR-200 family (miR-8 in Drosophila) plays a key role in human GB. We have described a correlation between miR-8 levels, ImpL2 expression and GB progression. However, there are no predicted miR-8 binding sites in ImpL2 sequence (StarMiRSoftware for Statistical Folding of Nucleic Acids and Studies of Regulatory RNAs- (Rennie et al, 2014) or TargetScanFly- http://www.targetscan.org/), therefore, it is unlikely that miR-8 regulates directly ImpL2 mRNA stability. One possibility is the existence of mediator proteins that depend directly on miR-8. The most evident possibility is a transcription factor whose mRNA stability is sensitive to miR-8 and acts as a transcriptional regulator of ImpL2. However, the regulation of ImpL2 and its association to miRNAs is a matter for future studies. Another intriguing point is the differential effects that both miR-8 overexpression and ImpL2 down-regulation have on GB growth. Whereas an excess of miR-8, which in turn reduces ImpL2, is unable to reduce GB cell number, direct down-regulation of ImpL2 significantly reduces the growth of GB cells. Nevertheless, it is known that most of miRNAs control several mRNAs, thus miR-8 overexpression might affect to different extent other miRNAs than just ImpL2 mRNA, which may account for such differences.

Altogether, our data suggest that the progression of brain tumors in Drosophila depends not only on the intrinsic properties of the tumoral cells, but also on the physiological condition of the surrounding cells (Fig 7). The potential relationship between Wg/WNT and insulin pathway has been proposed under physiological or tumoral conditions (Desbois-Mouthon et al, 2001; Yi et al, 2008;
Figure 5. Rheb expression in neurons rescues active zone loss and mitochondrial alterations.

(A) qRT-PCR of 7-d-old adult brains from repo>UAS-dEGFR, UAS-dp110CAAX; Elav-LexA>lexAop-CD8GFP (glioma) and repo>UAS-dEGFR, UAS-dp110CAAX; elav-LexA>lexAop-Rheb (glioma+Elav>Rheb) flies show Rheb expression in glioma brains, and Rheb up-regulation by ectopic expression of Rheb in neurons (t test). (B, C, D) Adult neuromuscular junction (NMJ) from (B) repo>UAS-LacZ; elav-LexA (C) repo>UAS-dEGFR, UAS-dp110CAAX; elav-LexA and (D) repo>UAS-dEGFR, UAS-dp110CAAX; elav-LexA>lexAop-Rheb after 7 d at 29°C (active zones are marked in green). (E) Quantification and statistical analysis of active zones in at least n = 20 per genotype (ANOVA, post hoc Bonferroni) (scale bar, 50 μm). (F, F', G, G', H, H') Confocal microscopy images of adult brains (detail of Kenyon cells) from (F) repo>UAS-LacZ;
Palsgaard et al, 2012) and represent a potential issue of interest to study in GB-host biology. We described recently (Portela et al, 2019b) the positive feedback loop established with wingless/JNK/IMP3s and tumor microtubes that promote GB progression. We do not have evidences that miR-8/melP2 regulation is directly controlled by EGFR and/or PI3K signaling pathways. However, the results included in Fig 1 suggest that a reduction in JNK pathway (BSKTM) or the knockdown of iglao (prevention of TMs formation) reduces melP2 expression. It has been described that miR-8-mutant animals activate JNK signaling, but there is no evidence that JNK pathway can regulate miR-8. However, miRNA regulation has the tendency to establish reciprocal feedback loops and networks (Herranz & Cohen, 2010), so it might be plausible that JNK signaling and miR-8 would have such a reciprocal regulation. These results suggest that melP2 up-regulation is sensitive to TMs formation and JNK, and one could speculate that it might be also dependent on Wg/WNT signaling pathway. Our observations in Drosophila suggest that both pathways (insulin and Wg) participate in the equilibrium between GB cells and neurons. The relations among all different pathways and the mutual regulation should be matter of study of future projects.

GB patients respond differently to the progression of the GB: some patients survive for a few months, whereas others survive for years. If we accept that the coordinated effect in GB and neurons result in differential tumor progression, the vast differences in how patients respond to GB could be, in part, dependent on genetic or epigenetic conditions related to InR signaling genes in neurons, and probably other pathways such as WNT or Hedgehog.

Materials and Methods

Fly stocks and genetics

All fly stocks were maintained at 25°C (unless otherwise specified) on a 12/12 h light/dark cycles at constant humidity in a standard room.
medium. The stocks used from Bloomington Stock Center were tub-Gal80<sup>T</sup> (BL-7019), Repo-Gal4 (BL-7415), D42-Gal4 (BL-8816), UAS-InR DN (BL-2852), UAS-myr-RFP (BL-7119) UAS-LacZ (BL-8529), UAS-impL2 RNAi (BL-55855), ImpL2-MIMIC (BL-59296), (lexAop-mito-Cherry (BL-66530), tGPH (BL-8164), and Thor<sup>56505752</sup> (BL-53159). Other fly stocks used were miR-8 sensor (Kennell et al, 2012), UAS-miR-8-RFP (Lu et al, 2014), Elav-LexA (BL52676), UAS-HRP-CD2 (gifted by L Luo), UAS-deGFR<sup>δ</sup>,UAS-dp110<sup>CAAX</sup> (gift from R Read; Read et al, 2009) UAS-impL2 (gift from Hugo Stocker), and lexAop-Rheb (gift from Nuria Romero).

The glioma-inducing line contains the UAS-deGFR<sup>δ</sup>, UAS-dp110<sup>CAAX</sup> transgenes that encodes for the constitutively active forms of the human orthologs PI3K and EGFR, respectively (Read et al, 2009). Repo–Gal4 line drives the Gal4 expression to glial cells and precursors (Lee & Jones, 2005; Casas-Tintó et al, 2017) and combined with the UAS-deGFR<sup>δ</sup>, UAS-dp110<sup>CAAX</sup> line allow us to generate a glia thanks to the Gal4 system (Brand & Perrimon, 1993). Elav-LexA line drives the expression to neurons, allowing us to manipulate neurons in a glia containing LexA and Gal4 expression systems (Lai & Lee, 2006).

Gal80<sup>T</sup> is a repressor of the Gal4 activity at 18<sup>°</sup>C, although at 29<sup>°</sup>C is inactivated (McGuire et al, 2003). The tub-Gal80<sup>T</sup> construct was used in all the crosses to avoid the lethality caused by the glioma development during the larval stage. The crosses were kept at 17<sup>°</sup>C until the adult flies emerged. To inactivate the Gal80<sup>T</sup> protein and activate the Gal4/UAS system to allow the expression of different reporters, we used the appropriate control and glioma genotypes that include them and performed the experiment in parallel for each grouped panel (at least three times): Figs 1B–F, 2, 3A, B, D–F, and –P, 4A–C and F–H, 5A–E and J–L, and 6.

Immunostaining and image acquisition

Adult brains were dissected and fixed with 4% formaldehyde in phosphate-buffered saline for 20 min whereas adult NMJ were fixed 10 min; in both cases, samples were washed 3 × 15 min with PBS+0.4% triton, blocked for 1 h with PBS+0.4% triton+ BSA 5%, incubated overnight with primary antibodies, washed 3 × 15 min, incubated with secondary antibodies for 2 h, and mounted in Vectashield mounting medium, with DAPI in the case of the brains. The primary antibodies used were anti-repo mouse (1/200; DSHB) to recognize glial nuclei, anti-bruchpilot-NC82-mouse (1/50; DSHB) to recognize the presynaptic protein bruchpilot, anti-HRP rabbit (1/400; Cell Signalling) to recognize membranes, anti-GFP mouse (1/50; DSHB) to recognize the presynaptic protein bruchpilot, and repo mouse (1/200; DSHB) to recognize glial nuclei. The secondary antibodies used were Alexa 488 or 647 (1/500; Life Technologies). Images were taken by a Leica SP5 confocal microscopy.

RNA extraction, reverse transcription and qRT-PCR

For RNA extraction, 1- to 4-d-old male adults were entrained to a 12:12 h LD cycle for 7 d at 29<sup>°</sup>C and then collected on dry ice at ZT 6. Total RNA was extracted from 30 heads of adult males of the Control (repo>LacZ), Glioma (repo>UAS-deGFR<sup>δ</sup>, UAS-dp110<sup>CAAX</sup>), and repo>UAS-deGFR<sup>δ</sup>, UAS-dp110<sup>CAAX</sup>, elav-LexA, lexAop-Rheb genotypes after 7 d of glioma developement. RNA was extracted with TRIzol and phenol chloroform. Total RNA concentration was measured by using NanoDrop ND-1000. cDNA was synthesized from 1 mg of total RNA using M-MLV RT (Invitrogen). cDNA samples from 1: 5 dilutions were used for real-time PCR reactions. Transcription levels were determined in a 14-µl volume in duplicate using SYBR Green (Applied Biosystem) and 7500 qPCR (Thermo Fisher Scientific). We analyzed transcription levels of Impl2, dRheb, and Rp49 as housekeeping gene reference.

Sequences of primers were RP49 F: GCATAACAGCCCACAGATCGT, Rp49 R: AACCGATGTGGGCATCAGA, ImpL2 F: CGGAGATCACGTGGTGAAAT, ImpL2 R: AGGTATCGGCCTGATCCCTT, dRheb F: CGAGCTTATGCGGCAAGAAAT, and dRheb R: CAAGACAACCGCCTCTTCC.

After completing each real-time PCR run, outlier data were analyzed using 7500 software (Applied Biosystems). Ct values of duplicates from three biological samples were analyzed calculating 2DDCt and comparing the results using a t test with GraphPad (GraphPad Software).

Viability and survival assays

Lifespan was determined under 12:12 h LD cycles at 29<sup>°</sup>C conditions. Three replicates of 30 1- to 4-d-old male adults were collected in vials containing standard Drosophila media and transferred every 2–3 d to fresh Drosophila media.
Electron microscopy

Adult brains of repo>UAS-dEGRF, UAS-dp110GAS, and repo>UAS-dEGRF, UAS-dp110GAS, elav-LexA>lexAop-Rheb animals expressing CD2-HRP in glial membranes were dissected after 7 d of glioma development and fixed with 4% formaldehyde in phosphate-buffered saline for 30 min. The samples were washed twice with PBS and incubate with R.T.U. VECTASTAIN kit (VECTOR) for 30 min at RT and washed once with PBS. Followed by an incubation in dark with SIGMA FAST 3,3’-Diaminobenzidine Tablet SETS (Sigma-Aldrich) for 75 min at RT, washed once with PBT, and incubate with 4% formaldehyde + 2% glutaraldehyde for 1 h and stored at 4°C. Following fixation samples were washed three times in 0.1 M phosphate buffer. Post-fixation was performed in 1% osmium tetroxide + 1% potassium ferrocyanide for 1 h at 4°C, three washes in H2O2dd and incubated in PBS 0.1M + 0.15% tonic acid for 1 min, washed once in PBS 0.1M, and twice in H2O2dd. After incubation in 2% uranil acetate, it was incubated for 1 h at RT in darkness and washed three times in H2O2dd. Dehydration was done in ethanol series (30%, 50%, 70%, 90%, and 3 × 100%). The samples were infiltrated with increasing concentrations of epoxy resin TAAB-812 (TAAB Laboratories) in propilenoxid and encapsulated in BEEM capsules to polymerize 48 h at 60°C. Ultrathin sections of 70–80 nm were cut using Ultracut E microtome (Leica) and stained with 2% uranyl acetate solution in water and lead Reynols citrate. Grids were examined with JeolJEM1400Flash electron microscope at 80 kV. Images were taken with a OneView (4K × 4K) CMOS camera (Gatan).

Quantification

Fluorescent reporter-relative Impl2 and Thor signals within brains were determined from images taken at the same confocal settings avoiding saturation. For the analysis of co-localization rates, “co-localization” tool from LAS AF Lite software (Leica) was used taking the co-localization rate data for the statistics analyzing the co-localization between green signal (both cases) and signal coming from glial tissue (for Impl2 levels) or neuronal nuclei (for Thor levels) from three slices per brain in similar positions of the z axis.

Average pixel intensity from miR-8 sensor and mito-Cherry was measured using measurement log tool from Adobe Photoshop CS5.1. Average pixel intensity of miR-8 sensor was analyzed quantifying the green sensor signaling glial tissue with green signal that did not overlap with glial cells to generate a ratio. Measurements were taken from similar localizations in three slices per brain with equivalent positions of the z axis. Average pixel intensity of mito-Cherry was analyzed taking measurements in the red signal from the reporter from at least five points of the NM.

Glial network was marked by a UAS-myrirstoylated-RFP reporter specifically expressed under the control of repo-Gal4. The total volume was quantified using Imaris surface tool (Imaris 6.3.1) software. Glial nuclei were marked by staining with the anti-Repo (DSHB). The number of Repo+ cells and number of synapses (anti-nc82; DSHB) were quantified by using the spots tool in Imaris 6.3.1 software. We selected a minimum size and threshold for the spot in the control samples of each experiment: 0.5 μm for active zones and 2 μm for glial cell nuclei. Then we applied the same conditions to the analysis the corresponding experimental sample.

For electron microscopy images quantification, we used FIJI (Image 1.52v) software. After manually selecting the perimeter of each mitochondrion, we measured the area, major and minor axis. All measurements were taken blind. In total, at least 15 mitochondria were measured from three different animals per genotype.

Statistics

The results were analyzed using the GraphPad Prism 5 software (www.graphpad.com). Quantitative parameters were divided into parametric and nonparametric using the D’Agostino and Pearson omnibus normality test, and the variances were analyzed with F test. t test and ANOVA test with Bonferroni’s post hoc were used in parametric parameters, using Welch’s correction when necessary. To the nonparametric parameters, Mann–Whitney test and Kruskal–Wallis test with Dunn’s post hoc were used. The survival assays were analyzed with Mantel–Cox test. The P limit value for rejecting the null hypothesis and considering the differences between cases as statistically significant was P < 0.05 (*). Other P-values are indicated as ** when P < 0.01 and *** when P < 0.001.

Supplementary Information

Supplementary information is available at https://doi.org/10.26508/lsa.202000693.

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FA Martín: conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, and writing—original draft, review, and editing.
S Casas-Tintó: conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, project administration, and writing—original draft, review, and editing.
Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References
