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Aberrant neuronal differentiation is common in glioma but is associated neither with epileptic seizures nor with better survival

Christoph Patrick Beier1,3, Tine Rasmussen1,2, Rikke Hedegaard Dahlrot4, Helene Broch Tenstad1,2, Julie Slinning Aarø1,2, Mai Froberg Sørensen1,2, Sólborg Berglind Heimisdóttir1,2, Mia Dahl Sørensen1,3, Per Svenningsen5, Markus J. Riemenschneider6, Dagmar Beier1,3 & Bjarne Winther Kristensen2,3

The mechanisms of glioma-associated seizures (GAS) have yet to be fully elucidated. Proneural subtype, isocitrate dehydrogenase 1 (IDH1) mutations, and epileptic seizures are closely associated suggesting that aberrant neuronal differentiation contributes to glioma-associated seizures. In a population-based cohort (n = 236), lack of stem cell marker expression (nestin, musashi) was significantly associated with IDH1 mutations and GAS at diagnosis. In vitro data suggested an association of IDH1 mutations and a more differentiated phenotype. Out of eight glioma stem cell (GSC) lines, seven revealed positivity for the synaptic marker protein synaptophysin. Three had synapse-like structures identified by electron microscopy and were either vGlut1 (glutamatergic) or GAD67 (GABAergic) positive. In vivo, >10% synaptophysin-positive tumour cells were present in >90% of all gliomas. Synaptophysin expression was associated with proneural subtype and vGlut1 expression, suggesting that most synapse-like structures in glioma are glutamatergic. However, we found null associations between vGlut1 protein/mRNA expression and survival, GAS at onset, development of GAS after resection, and refractory GAS. Synapse-like structures were neither functional nor activated by spontaneous action potentials or cellular networks. Thus, aberrant neuronal differentiation including glutamatergic synapse-like structures is detectable in glioma but is associated neither with epileptic seizures nor with better survival.

The treatment and prognosis of patients diagnosed with glioma of all grades has been improved in the last two decades, e.g. due to the introduction of temozolomide as standard of care for glioblastomas (GBM) and the combination of radiotherapy and chemotherapy with procarbacin/lomustine/vincristine in low-grade glioma. The number of long-term survivors has accordingly increased and the management of neurological symptoms will therefore become increasingly important in these patients.

Epileptic seizures substantially interfere with the quality of life of glioma patients, especially long-term survivors, irrespective of tumour grade, and are strongly associated with neuropsychological deficits. Glioma differs from other kinds of brain tumours due to its increased epileptogenicity with up to 80% of patients suffering from epileptic seizures. There is a substantial lack of valuable epidemiological data, but clinical experience and all available data clearly suggest that the risk of developing seizures is substantially higher in patients with glioma as compared to patients with other kinds of non-glial brain tumours, likely due to glioma-specific mechanisms of epileptogenesis. Although several mechanisms have been proposed to explain tumour-associated seizures, the imbalance between excitatory glutamatergic drive and impaired or defect GABAergic inhibition.

1Department of Neurology, Odense University Hospital, Odense, Denmark. 2Department of Pathology, Odense University Hospital, Odense, Denmark. 3Department of Clinical Research, University of Southern Denmark, Odense, Denmark. 4Department of Oncology, Odense University Hospital, Odense, Denmark. 5Department of Molecular Medicine, University of Southern Denmark, Odense, Denmark. 6Department for Neuropathology, Regensburg University Hospital, Odense, Germany. Correspondence and requests for materials should be addressed to C.P.B. (email: cbeier@health.sdu.dk)
Expression of markers indicating neuronal differentiation in GSC lines. We hypothesized that the more differentiated phenotype of IDH1 R132H glioma may contribute to their higher epileptogenicity. We therefore studied the spontaneous aberrant neuronal marker expression in seven primary GSC lines from two different laboratories (CPB and BWK). All GSC lines were cultured using serum-free medium supplemented with EGF and FGF. Cells from all GSC lines expressed markers of neuronal (NeuN, beta-3-tubulin) differentiation to a certain degree. In three GSC lines (R11, R28, T111) a subset of cells showed a strong and consistent neuronal marker expression pattern. They expressed markers for immature neurons (beta-3-tubulin), markers for mature neurons (NeuN) and markers indicative for synapses (synaptophysin, SV2A, Fig. 2, Supplementary Fig. 2, Table 1). The expression pattern was inconsistent in four GSC lines (Table 1).

Results

Nestin is not an independent prognostic factor for epileptic seizures but associated with IDH1 R132H mutations. We studied the association of the stem cell markers nestin and musashi-1 with epileptic seizures at diagnosis in a cohort comprising 239 patients with gliomas (WHO II-IV, overview over patients: Supplementary Table 1, previously reported in26). IDH R132H mutation was strongly associated with seizures at onset (Fig. 1A). High nestin and high musashi-1 expression were associated with WHO grade (Fig. 1B, Supplementary Fig. 1A) and IDH R132H mutation (Fig. 1C, Supplementary Fig. 1B). IDH1 R132H mutation and low WHO grade showed a strong and significant association with epileptic seizures at diagnosis (Supplementary Table 2). In contrast, nestin and musashi-1 expression were associated inversely with epileptic seizures (Fig. 1D, Supplementary Fig. 1C, Supplementary Table 2). Due to the assumed interaction of IDH1 mutation, WHO grade and nestin expression, we performed a multi-variate analysis to identify crucial contributors among the three factors associated with epileptic seizures. Logistic regression showed that only IDH1 R132 mutations but neither nestin expression nor WHO grade remained independently associated with seizures at diagnosis (Supplementary Table 3).

IDH1 mutations in glioma are associated with a more differentiated phenotype in vitro. We studied the association of the stem cell markers nestin and musashi-1 with epileptic seizures at diagnosis in a cohort comprising 239 patients with gliomas (WHO II-IV, overview over patients: Supplementary Table 1, previously reported in26). IDH R132H mutation was strongly associated with seizures at onset (Fig. 1A). High nestin and high musashi-1 expression were associated with WHO grade (Fig. 1B, Supplementary Fig. 1A) and IDH1 R132H mutation (Fig. 1C, Supplementary Fig. 1B). IDH1 R132H mutation and low WHO grade showed a strong and significant association with epileptic seizures at diagnosis (Supplementary Table 2). In contrast, nestin and musashi-1 expression were associated inversely with epileptic seizures (Fig. 1D, Supplementary Fig. 1C, Supplementary Table 2). Due to the assumed interaction of IDH1 mutation, WHO grade and nestin expression, we performed a multi-variate analysis to identify crucial contributors among the three factors associated with epileptic seizures. Logistic regression showed that only IDH1 R132 mutations but neither nestin expression nor WHO grade remained independently associated with seizures at diagnosis (Supplementary Table 3).
Synapse-like structure within GSC lines in vitro. We found cell-cell contacts with strong positivity for the pan-synaptic marker protein synaptophysin suggesting synapse-like structures (Fig. 3A) in two GSC lines (R11 and R28 but not in the GSC line T111). Culture conditions known to foster neuronal differentiation in neuronal stem cells (growth factor deprivation, 1% serum, 0.1% retinoic acid), significantly increased the number of synaptophysin positive cell-cell contacts (Fig. 3B). To verify that these synaptophysin-positive cell-cell contacts correspond to synapse-like structures, we used electron microscopy of GSC cultured as spheres under standard conditions. The three GSC lines with substantial neuronal differentiation but none of the other GSC lines showed structures resembling synaptic vesicles. The GSC lines R11 and R28 that had spontaneous synaptophysin-positive cell-cell contacts also showed microstructures reminiscent to synapses (Fig. 3C, Table 1).
Synapse-like structures within GSC lines in vivo and in human glioma. To exclude that the synapse-like structures correspond to a cell culture artefact, we further analyzed tumour material from xenograft tumours from the GSC lines R8 (slightly synaptophysin positive) and the GSC line R28 (strongly synaptophysin positive). Both xenograft tumours showed substantial positivity for human synaptophysin mainly in the invasion zone and in the boundaries of the tumours (Fig. 4A). To further confirm the presence of synaptophysin-positive glioma cells in vivo, we used a tissue-microarray including three IDH1 positive gliomas. Three out of six tumours were strongly positive for synaptophysin, three were negative (Fig. 4B).

Synaptophysin expression was also common in IDH^{wildtype} glioblastoma. In a cohort of 40 samples, 29 out of 40 IDH^{wildtype} tumours expressed synaptophysin (Fig. 4C). Immunostaining with histological markers indicative for the proneural and mesenchymal subtype (described in29) showed, a significant association with the proneural subtype but synaptophysin was also detectable in mesenchymal GBM (Fig. 4D). There was no association of epileptic seizures with proneural phenotype or synaptophysin expression (Fig. 4E,F).
Synapse-like structures within GSC lines may be glutamatergic or GABAergic. Glutamate (excitatory) and GABA (inhibitory) are the two most important neurotransmitters in the human brain. To detect glutamatergic synapses, we used the well-established marker protein vGlut1 (SLC17A7), which is a vesicular protein that transports glutamate into the synaptic vesicle. In our panel of seven GSC lines, three expressed vGlut1. Another four were positive for GAD67, the crucial protein in the biosynthesis of GABA and an established marker for GABAergic neurons (Fig. 5A). One of the GAD67 positive GSC lines was also positive for vGlut1, however, none of the GAD67-positive GSC lines studied had detectable synapse-like structures when studied using electron microscopy.

Table 1. Markers of neuronal differentiation in a panel of GSC lines. Legend: Immunocytochemistry: – No staining, (+) few cells/low staining intensity + clear positivity ++ = +++ strong/very strong staining intensity. Electronmicroscopy: – No structures reminiscent to synapses. (+) vesicles + synapses ++ many synapses.

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Figure 3. Synapse-like structures within GSC lines. (A) DAPI, synaptophysin (high and low magnification), phasecontrast and β-III tubulin staining of GSC line R28 is shown. (B) Number of synaptophysin-positive cell-cell contacts under standard culture conditions (serum free medium supplemented with EGF/FGF/B27) and under conditions favoring neuronal differentiation (no growth factors, 1% serum, 0.1% retinoic acid, p < 0.005, student’s-t-test). (C) Electronmicroscopy of GSC spheres cultured under standard conditions. Synapse-like structures are shown in higher magnification on the right side.
Figure 4. Synapse-like structures in vivo. (A) Immunostaining detecting human synaptophysin in xenograft tumours (R8, R28). (B) Synaptophysin immunostaining (left side) and IDH1 immunostaining (right side) of a tissue microarray containing WHO grade 2 tumours (2) and WHO grade 3 tumour (3), cerebellum as positive control (C), and intestine as negative control (IT) for the synaptophysin staining. (C) Representative synaptophysin immunostaining of human IDH wildtype glioblastoma samples with high (left) and low synaptophysin expression (right). (D) Correlation of synaptophysin expression with markers indicative for proneural (proneural-mesenchymal score >0) and the mesenchymal subtype (proneural-mesenchymal score <0, Pearson r = 0.3, p = 0.05). (E) Association of epileptic seizures and markers indicative for proneural (proneural-mesenchymal score >0) and the mesenchymal subtype (proneural-mesenchymal score <0 (p = 0.3, Mann-Whitney U-test). (F) Association of synaptophysin expression with epileptic seizures (p = 0.9, Mann-Whitney U-test).
microscopy (Table 1). Notably, all GSC lines expressed the synaptic protein SV2A, which binds to levetiracetam, to some extent. SV2A was expressed in both glutamatergic and GABAergic synapses and varied substantially between the GSC lines. The classical, serum cultured glioma cells lines like T98G or U87 as well as the breast cancer cell lines MCF7 did not express SV2A (Fig. 5B).

In vivo, the vesicular transporter vGlut1 co-localized with synaptophysin in both double-positive GSC lines (Fig. 5C). In vivo, vGlut1 mRNA expression correlated very strongly with synaptophysin expression in microarrays available to the RemBraNDT (REpository for Molecular BRAin Neoplasia DaTa) database (Pearson r = 0.92, p < 0.001, Fig. 5D). In contrast, expression of GAD67 mRNA was less associated with synaptophysin mRNA expression (Pearson r = 0.36, p = 0.001, Fig. 5E). Analysis of the TCGA database available at the same repository gave similar results (Synaptophysin/vGlut1: Pearson r = 0.85, synaptophysin/GAD67: Pearson r = 0.38).

**Lack of association of vGlut1 expression and epileptic seizures.** Given that glutamate is the strongest and most common excitatory neurotransmitter in the human brain, we hypothesized that neuronal differentiation with a "glutamateric phenotype" may contribute to GAS. We therefore studied a large, representative

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**Figure 5.** Glutamatergic and GABAergic glioma cells. (A,B) Westernblot of GSC lines and control samples (breast cancer cell line MCF7, classical glioma cell lines T98G, U87). Blots were probed with antibodies against GAD67, synaptophysin, SV2A and vGlut1. Actin was used as loading control. (C) Immunocytochemistry against vGlut1 and synaptophysin. DAPI was used to label the nuclei. (D,E) Correlation of synaptophysin expression with vGlut1 (Pearson r = 0.92, p < 0.001) and GAD67 (Pearson r = 0.36, p = 0.001) using mRNA expression data from the RemBraNDT database (accessed: 09 January 2018).
cohort of 224 patients for vGlut1 expression and seizures at onset (Patients characteristics: Supplementary Table 5). However, the expression of vGlut1 was neither associated with IDH1 status nor with tumour grade (data not shown). Further, we found no association with seizures at onset (Fig. 6A), development of seizures at any time point (Fig. 6B), or survival (Fig. 6C). vGlut1 expression was associated with MGMT methylation pattern (Fig. 6D).

**GSC lines with marked neuronal differentiation are not excitable and do not show spontaneous action potentials.** To further understand why these vesicular structures filled with glutamate did not influence the odds for developing epileptic seizures, we studied if glioma cells with neuronal differentiation are able to release glutamate. In mature neurons, synaptic release of glutamate is triggered by calcium influx due to voltage-dependent calcium channels. We therefore studied intracellular calcium levels using Fluo4. Time-lapse imaging in a cell culture incubator with stable temperature and oxygen concentrations revealed that five out of six GSC lines studied showed spontaneous calcium changes in intracellular calcium concentrations that were not seen in the breast cancer cell lines MCF7 (Fig. 6E, Supplementary Film 1). The membrane potentials in single R28 cells were depolarized (−28 ± 5 mV, n = 4) and stable, indicating that the changes in intracellular calcium were not due to spontaneous action potentials (data not shown). Moreover, the changes in intracellular calcium were not consistently inhibited or induced by high potassium concentration (data not shown) and the analysis of close adjacent cells clearly indicated that glioma cells did neither create cellular networks nor consistently modified calcium levels in neighbouring cells (Supplementary Film 2).

**Figure 6.** Association of vGlut1 expression with clinical characteristics. (A,B) Risk of seizures at onset (A) and at any time point during disease (B) in patients with high, intermediate, low and no vGlut1 expression. (C) Survival of patients with high, intermediate, low and no vGlut1 expression (p = 7., Log-Rank test). (D) MGMT methylation status in patients with high, intermediate, low and no vGlut1 expression. (E) Proportion of cells with spontaneous changing intracellular calcium concentrations within 5 min periods.
Discussion

Aberrant neuronal marker expression - alone or in combination with glial markers - is well established in GSC lines and was also observed in vivo13,24,30. We here provide evidence that a subset of glioma cells display more advanced neuronal differentiation in vitro and in vivo that went beyond aberrant marker expression but included synapse-like structures with the ability to generate non-functional synapse-like direct cell-cell contacts among glioma cells.

Our data did not substantiate the hypothesis that the (pro-)neural differentiation was crucial for or at least contributed to GAS. We challenged the hypothesis using several approaches (e.g. association of proneural phenotype and seizures in IDH\textsuperscript{wildtype} GBM, correlation of synaptophysin and seizures in IDH\textsuperscript{wildtype} GBM). None of the results supported the hypothesis. We therefore only focused on gliomas with a glutamatergic differentiation but did not find any association with seizures either. Given that the proneural phenotype is associated with the polymethylator-phenotype19, the identified association of neuronal differentiation, glutamatergic synapse-like structures, and MGMT methylation was expected. In summary, the provided data falsified the working hypothesis. For obvious reasons, it is impossible to exclude a yet unknown mechanism associated with neuronal differentiation and contributing to GAS in minor subgroups within the different cohorts studied. However, because we did not find consistent or suggestive trends in our data in support of the working hypothesis, we do not assume having overlooked a biological relevant effect.

Our data suggests that proneural differentiation and GAS are both secondary to IDH1 R132H gain-of-function mutations. The most plausible explanation for GAS in IDH\textsuperscript{wildtype} glioma is directed stimulation of NMDA receptors by D2HG16. We assume that neuronal differentiation is likely secondary to the epigenetic changes resulting from D2HG induced hypermethylation as suggested by data from Noushmehr et al.16. In line with other studies, we did not find a prognostic relevance of neuronal differentiation as indicated by vGlut1 expression21,22. Although our data consistently falsify the working hypothesis, the amount and degree of neuronal differentiation within glioma was surprising. Here we describe and characterize for the first time synapse-like structures within glioma cells in vitro and in vivo. The GSC lines with consistent neuronal differentiation used for our experiments have been derived from typical primary GBM and were able to form GBM-like lesions after transplantation into nude mice23,29,31. By choosing GSC lines from two laboratories, we reduced the risk of contamination or other technical artefacts. The biological significance of this neuronal differentiation remains unknown and the results of several experiments were unanticipated (e.g. expression of synaptophysin in the invasion zone of xenotransplants only). It may represent an epiphenomenon that indicates that the cells of origin of GBM as well as the stem cell-like tumour cells (“cancer stem cells”) driving tumour growth are pluripotent with a variable degree of neuronal differentiation. This would be in line with a recent study using a murine glioma model describing that all glioma derive from proneural progenitors27. From a broader perspective, it is likely that we characterized the cellular correlate to the proneural/neural glioma subtype identified in the context of several large-scale attempts to subclassify glioma19,20,28,33–35. As in our study, the translational significance of the different subtypes identified in the last decade remains vague despite evidence suggesting some importance for treatment response26–28. Although we did not assess the molecular status of the glioma samples used in detail, it appears likely that the assumed higher incidence of seizures in proneural glioma is secondary to IDH mutations and therefore adds GAS to the list of clinical features not associated with the molecular well-defined subtypes.

In summary, our study provides evidence suggestive for the existence of an advanced neuronal differentiation of glioma cells and showed that neuronal differentiation of glioma cells is associated neither with survival nor with seizures at onset.

Material and Methods

Patient cohorts. Cohort Fig. 1 and Supplementary Fig. 1: This retrospective study examined all adult patients with newly diagnosed glioma in the Region of Southern Denmark between 2005 and 2009 (n = 433). For 239 of these patients, sufficient tumour tissue was available. The histological diagnosis was made using the 2007 WHO criteria40. Three patients with WHO grade 1 glioma were excluded. Data on seizures at onset were available for all patients. The patient cohort and nestin staining has been published previously28 (Ethic Committee approval: S2DO90080, Danish Data Protection Agency: 2009-41-3070). All experiments in this paper with patient material/data were performed in accordance with relevant national and local guidelines and regulations.

Cohort Fig. 4D: This retrospective study examined patients diagnosed with adult IDH\textsuperscript{wildtype} GBM at Odense University Hospital between January 2009 and December 2012 (n = 40). The GBM were chosen consecutively by starting at the last patient diagnosed in 2012 and going backwards until we had a total of 40 patients (Ethic committee approval: S-20110022, Danish Data Protection Agency: 16/32269).

Cohort Fig. 6: The cohort includes all consecutive glioma samples resected in the Department of Neurosurgery, Odense University Hospital, with sufficient high quality material for tissue microarrays. The histological diagnosis was made using the 2007 WHO criteria40. Demographic and survival data, data on seizures at onset, development of seizures at any time point, data on molecular markers (methyl-guanidin-methyl-transferase (MGMT) promoter, IDH1 status) were systematically assessed based on the patients records. In the same cohort, expression of xCT was determined, which was published elsewhere41 (Ethic committee approval: S-20150148, Danish Data Protection Agency: 16/32269).

Generation of GSC lines. Between 2005 and 2010, >70 GSC lines have been established and characterized using tumour samples from glioma WHO grade 2-4,23,27,28. This was approved by the local ethics committee (no: 05/105, University of Regensburg, Germany). The preparation of GSC lines was previously described in3,25. In brief, fresh tumour material was dissociated into a single cell suspension and the cells were seeded in serum-free medium supplemented with 20 ng/ml EGF and FGF in addition to LIF and B27 supplement. GSC lines were cultured at least 4 weeks and the growth pattern was analyzed at least once a week. The growth pattern described refers to the initial growth pattern of GSC lines during the first 5 passages. Generation of GSC lines T111 and T78
were previously described. All other experiments, GSC lines were cultured in serum-free medium supplemented with 20 ng/ml EGF, 20 ng/ml FGF, and 1% B27 supplement.

**Histology.** The IDH1 status of the GSC lines was determined by sequencing as described in. In all other experiments, the IDH R132H mutation was detected as previously described using the mIDH1R132H antibody (mIDH1R132H, clone H14, Dionova, 1:100).

Nestin and musashi-1 positive tumour cells were identified as previously described (nestin 196908 antibody, R&D systems (1:100) and musashi-1 clone 14H1, MBL International, 1:200). Slides were stained on the AutostainerPlus platform (Dako, Glostrup, Denmark) and DyLight 650 (1 + 100 Fisher Scientific) was used for detection of nestin. The fraction of nestin positive tumour cells was identified using quantitative image analysis with continuous estimates of staining in the Visiomorph module of the Visiopharm Integrator System (Visiopharm, Hørsholm, Denmark).

Three-micrometer sections of paraffin-embedded GBM samples were stained using synaptophysin antibody from NovoCastra (NCL-SYNAP-299, clone 27G12, 1:50) on an automated immunostainer (BenchMark Ultra IHC/ISH staining system, Ventana Medical Systems, USA). OLIG2, NeuN, DLL3, YKL40, CD44, and VEGF were stained as described in detail in. All samples were scored blinded (MFS, SBH). Synaptophysin was evaluated with respect to antigen expression alone, and scored from 0–100 according to percent positivity. OLIG2, NeuN, DLL3, YKL40, CD44, and VEGF was scored as described in. In brief, 5 positions per slide were scored from each marker: (0: no expression; 1: <10% expression; 2: 10%–49% expression; 3: 50%–90%; 4: >90%). The sum of the means of the mesenchymal markers was subtracted from the sum of the means of the proneural markers.

**Immunocytochemistry.** Immunochemistry of glioma cells plated on cover slips coated with poly-ornithine and laminin was performed as described in. The following antibodies were used in a dilution of 1:100 if not otherwise specified: beta-3-tubulin: Promega G7121 (1:1000), GFAP: DAKO Z 0334, Nestin; Chemicon MAB5326 (1:500), NeuN: Chemicon MAD377 (1:50), SVA2: Abcam ab49572 (1:100), GAD67: ThermoFisher PA1-37733 (1:1000), vGlut1: Abcam ab193595 (1:1000), synaptophysin: Novocastra NCL-SYNAP-299 (1:50).

**Electronmicroscopy.** Electronmicroscopy was performed according to standard procedures in the Department of Pathology, Odense University Hospital. In brief, cells were centrifuged, washed using 0.1 phosphatebuffer and fixed using 2% glutaraldehyd. After fixation, pellets were stained using 1% osmiumtetraoxid and embedded in epoxy and analyzed using JEOL JEM-1400 electronmicroscope.

**Westernblot.** Westernblots were performed according standard procedures. The following antibodies were used for probing: beta-actin: Sigma A5441 (1:1000), Promega G7121, SVA2: Abcam ab49572 (1:1000), GAD67: ThermoFisher PA1-37733 (1:1000), vGlut1: Abcam ab193595 (1:1000), synaptophysin: Novocastra NCL-SYNAP-299 (1:1000).

**Current Clamp.** Membrane potentials in GSC cells were recorded using the current-clamp mode of the Axopatch-amplifier (Axon Instruments, Foster City, CA, USA) and the Clampex 9.2 data acquisition software (Axon Instruments). Membrane potentials were measured after obtaining the whole-cell configuration and measured for minutes to test for spontaneous changes. The solution in the pipette was (in mmol/l): 135 K-glutamate; 10 KCl; 10 NaCl; 1 MgCl2; 10 HEPES; 0.3 NaGTP; 0.5 MgATP and 2 EGTA. Bath solution was (in mmol/l): 10 HEPES-NaOH, 140 NaCl, 2.8, KCl, 1 MgCl2, 2 CaCl2, 11 glucose, and 10 sucrose. Experiments were performed at room temperature.

**Microarray analysis.** We used the publically available Repository for Molecular BRAin Neoplasia DaTa (REMBRANDT, http://www.betastasis.com/glioma/rembrandt/) that is based on 524 Affymatrix U133 2.0 plus microarrays to investigate the correlation of vGlut1 and synaptophysin expression. The databased was accessed on 09 January 2018 and data was analysed using SPSS 24.0.

**Statistics.** All statistical analyses were performed used SPSS 24.0. The statistical test used is given in the legend to the respective Figures.

**References**

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Author Contributions
C.P.B. drafted designed and coordinated the study and drafted the manuscript, T.D.R. performed the experiments shown in Figs 2–3, R.H.D. collected data for Fig. 1A–D, H.B.T. and J.S.A. collected and analysed data shown in Fig. 4C–F, M.F.S. and S.B.H. collected and analysed data shown in Fig. 6A–D, P.S. performed the electrophysiological experiments, M.D.S. provided the clinical data used in Figs 4 and 6. M.J.R. performed the I.D.H. analyses of G.S.C. lines, D.B. performed the experiments shown in Figs 4A, 1E–G. All authors edited and approved the final manuscript. B.W.K. provided the pathological samples used in this study, supervised the pathological and histological experiments in this manuscript, and coordinated the study.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-33282-5.

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