Glioblastoma cancer stem cell lines express functional acid sensing ion channels ASIC1a and ASIC3

Yuemin Tian1, Pia Bresenitz2, Anna Reska1, Laila El Moussaoui3, Christoph Patrick Beier2,3 & Stefan Gründer1,1

Acidic microenvironment is commonly observed in tumour tissues, including glioblastoma (GBM), the most aggressive and lethal brain tumour in adults. Acid sensing ion channels (ASICs) are neuronal voltage-insensitive sodium channels, which are sensors of extracellular protons. Here we studied and functionally characterized ASICs in two primary glioblastoma stem cell lines as cell culture models. We detected transcripts of the ACCN2 and ACCN3 genes, coding for ASIC1 and ASIC3, respectively, but not transcripts of ACCN1 (coding for ASIC2). Available microarray data confirmed that ACCN1 is downregulated in glioma. Western blotting confirmed expression of ASIC1 and ASIC3, the most proton-sensitive ASICs, in both GBM cell lines. We characterized ASICs functionally using whole-cell patch clamp and detected different types of acid-sensitive currents. Some of these currents had kinetics typical for ASICs and were sensitive to specific toxin inhibitors of ASIC1a or ASIC3, demonstrating that the GBM cell lines express functional ASIC1a and ASIC3 that may enable GBM cells to sensitively detect extracellular pH in a tumour tissue. Microarray data revealed that expression of ACCN2 and ACCN3 is associated with improved survival of patients suffering from gliomas, suggesting that preserved susceptibility to extracellular pH may impair tumour growth.
non-canonical channel, the expression of ASICs has not been investigated in GBM. Moreover, ASIC expression has so far not been investigated in GSC lines containing cell populations that do grow in non-clonal spheres and better represent tumour tissue than clonal cell lines1.

Here we molecularly and functionally characterized ASICs in two established GSC lines – R54, a CD133+

proneural-like GSC line, and R8, a CD133−, mesenchymal-like GSC line22. We show that R54 and R8 GSC cell

lines express functional ASIC1a and ASIC3 that may serve as sensitive sensors of extracellular pH, and available microarray data suggest that expression of ASICs is associated with an improved survival.

Results

The GSC lines R54 and R8 express ASIC1a and ASIC3 mRNA and protein. To account for the molecular heterogeneity of GBM, we chose a GSC line with a proneural expression pattern (R54) and a GSC line with a more mesenchymal expression pattern (R8). First, we examined by RT-PCR the expression of the genes that contribute to proton-gated ASICs (ASIC1a, ASIC1b, ASIC2a, ASIC2b and ASIC3) in R54 and R8 cells. The ACCN2 gene (amiloride-sensitive cation channel neuronal 2) codes for ASIC1a and ASIC1b, ACCN1 for ASIC2a and ASIC2b and ACCN3 for ASIC3, respectively. We detected transcripts of ACCN2 variant a (ASIC1a) and b (ASIC1b) and of ACCN3 (ASIC3), but not of ACCN1 (ASIC2a and ASIC2b), in R54 and R8 cells (Fig. 1a, left). We quantified mRNA expression of ACCN genes by quantitative RT-PCR (Fig. 1a, right). While ACCN2 variant a showed the highest expression in both R54 and R8 cells, it was approximately 4.5-fold higher in R54 than R8. ACCN3 expression was lower but comparable in R54 and R8 cells, such that the ratio of ACCN2a/ACCN3 was different in the two GSC cell lines, being ~20 in R54 and ~4 in R8 cells. In whole brain, ASIC1a mRNA is approximately 7-fold more abundant than ASIC3 mRNA17. Thus, compared to whole brain the ratio of ACCN2a/ACCN3 is increased in R8 and decreased in R54 cells. ACCN2 variant b (ASIC1b) was weakly expressed in R54 and very weakly expressed in R8. Abundance of ACCN2 variant b was about 5% that of variant a in R54 cells and 6% in R8 cells. Thus, compared to whole brain, where abundance of ACCN2 variant b is about 2% that of variant a23, expression of variant b relative to variant a was slightly increased in the GSC lines, but still low. Real time PCR confirmed the absence of ACCN1 (ASIC2) transcripts in R54 and R8 cells (Fig. 1a, right).

To confirm the expression of ASIC1 and ASIC3 in GSC lines, we extracted proteins from R54 and R8 cells and subjected them to Western blot analysis with ASIC1- and ASIC3-specific antibodies, revealing the presence of ASIC1 and ASIC3 in both R8 and R54. While the protein abundance of ASIC1a was comparable in both cell lines, ASIC3 was significantly more abundant in R8 than in R54 cells (n = 3, P = 0.006, unpaired t-test; Fig. 1b). Thus, PCR and Western blot analysis showed that R54 and R8 cells express various ASICs, mainly ASIC1a and ASIC3, but also ASIC1b, with ASIC3 being more abundant in R8 than R54 cells.

Functional characterization of ASICs in proneural-like CD133+ R54 cells. We then functionally characterized ASICs in GSC lines by whole cell patch clamp. R54 cells responded to a rapid change of bath pH
from 7.4 to pH 6.8 or below with one of three kinetic patterns (Fig. 2a). Almost half of the cells (42%) responded with a typical transient ASIC inward current (type 1). A similar fraction of cells (39%) responded with a biphasic current, composed of a fast transient current and a sustained component that did not fully inactivate while the pH remained acidic (type 2). Relatively few cells (19%) had no transient current and responded with a small sustained inward current (type 3).

Robust transient currents were elicited by pH ≤ 6.5. Transient current amplitudes started to saturate around pH 6.0 and could be fitted with a Hill equation (Fig. 2a), revealing half-maximal activation of type 1 currents at pH 6.4 ± 0.1 (n = 8) and of type 2 currents at pH 6.5 ± 0.1 (n = 7), consistent with the presence of highly proton-sensitive ASIC1a or ASIC3. In all cells, the amplitudes of transient type 1 and type 2 currents decreased with repeated application of more acidic solutions (Fig. 2a). Such a tachyphylaxis is unique to homomeric ASIC1a/3, suggesting the presence of ASIC1a in these cells. In contrast to transient currents, sustained currents (type 2 and type 3) did not saturate but linearly increased with decreasing pH. We did not investigate sustained currents further.

Figure 2c shows the distribution of transient peak amplitudes at pH 6.0. In most cells the transient currents had peak amplitudes between 50–200 pA for both type 1 and 2, but in some cells type 2 currents were huge with amplitudes > 1,000 pA. Sustained currents of type 3 had maximal amplitudes < 100 pA. Desensitization time constant τ_{des} at pH 6.0 was 1.4 ± 0.1 sec for type 1 currents (n = 15) and 1.2 ± 0.1 sec for transient type 2 currents (n = 14; Fig. 2d), which are typical for ASIC1a but slower than desensitization of ASIC3.

So far, our results are consistent with the presence of ASIC1a and ASIC3 in R54 cells. These two ASICs can be differentiated by their sensitivity to animal toxins, PcTx1 and APETx2. PcTx1 potently inhibits homomeric ASIC1a and potentiates ASIC1b but has not effect on ASIC3. APETx2 inhibits ASIC3 but not ASIC1a. Heteromeric ASIC1a/ASIC3 are insensitive to PcTx1 and are inhibited by APETx1 with a lower potency than homomeric ASIC3. At conditioning pH 7.4, pre-application of PcTx1 for two min only slightly inhibited type 1 currents, while it potentiated type 2 currents (n = 4 for each; Fig. 3a), suggesting the presence of ASIC1b in some R54 cells.

Inhibition by PcTx1 is pH-sensitive, however, and steady-state desensitization of human ASIC1a is slightly shifted to more acidic pH compared to rat ASIC1a. Indeed, at conditioning pH 7.3, PcTx1 strongly inhibited ASIC currents in R54 cells (n = 5; Fig. 3b), indicating a high abundance of homomeric ASIC1a. The inhibition by PcTx1 was only partially reversible, probably due to tachyphylaxis of the ASIC1a current. Tachyphylaxis may slightly overestimate the inhibition by PcTx1. Pre-application of APETx2 (500 nM) for one min substantially reduced transient type 1 and 2 currents by approximately 40% (n = 5 and 6, respectively; Fig. 3c), demonstrating a considerable contribution of ASIC3 to ASIC currents in most R54 cells.

In summary, the functional properties of proton-gated currents in R54 cells suggest the predominant presence of homomeric ASIC1a in these cells plus ASIC3-containing ASICs, which could be either heteromeric ASIC1a/3 or homomeric ASIC3.

**Functional characterization of ASICs in mesenchymal-like CD133⁺ R8 cells.** R8 cells did not have type 1 currents. As shown in Fig. 4, most cells (77%) had biphasic currents with a transient peak current and a sustained component (type 2). The remaining cells (23%) had sustained currents without any transient component. Transient currents could be fitted with a Hill equation. They saturated as pH was reduced with a half-maximal activity of type 1 currents at pH 6.4 ± 0.1 (n = 8) and of type 2 currents at pH 6.5 ± 0.1 (n = 7), consistent with the presence of highly proton-sensitive ASIC1a or ASIC3. In all cells, the amplitudes of transient type 1 and type 2 currents decreased with repeated application of more acidic solutions (Fig. 2a). Such a tachyphylaxis is unique to homomeric ASIC1a/3, suggesting the presence of ASIC1a in these cells. In contrast to transient currents, sustained currents (type 2 and type 3) did not saturate but linearly increased with decreasing pH. We did not investigate sustained currents further.

We tried to obtain further evidence for the presence of ASIC3-containing channels in R54 and R8 cells and pre-applied a recently discovered cone snail neuropeptide, RPRFa, that potentiates currents of both homomeric ASIC1a and ASIC3-containing channels, which could be either homomeric ASIC1a/3 or heteromeric ASIC1a/3.

We obtained robust transient currents with peak amplitudes between 50–200 pA for both type 1 and 2, but in some cells type 2 currents were huge with amplitudes > 1,000 pA. Sustained currents of type 3 had maximal amplitudes < 100 pA. Desensitization time constant τ_{des} at pH 6.0 was 1.4 ± 0.1 sec for type 1 currents (n = 15) and 1.2 ± 0.1 sec for transient type 2 currents (n = 14; Fig. 2d), which are typical for ASIC1a but slower than desensitization of ASIC3.

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Figure 2. Electrophysiological characterization of ASICs in R54 cells. (a) Left, whole cell current traces representative for type 1, type 2, and type 3 currents, activated by different acidic pH; conditioning pH was 7.4. Right, pH-response relationships. Peak currents from type 1 and type 2 cells were normalized to the current at pH 6.0. At pH ≥ 6.5, in most cases there was no transient current, and the amplitude of the inward current upon acidification was measured. The curves represent fits to the Hill equation. Amplitudes of sustained currents from type 2 and type 3 cells were determined just before stepping back to pH 7.4 and were connected by lines. (b) Pie chart illustrating the relative occurrence of type 1, type 2, and type 3 currents. (c) Frequency histogram of peak amplitude of type 1 (dark blue) and type 2 (light blue) transient currents. (d) Time constants of desensitization $\tau_{des}$ of transient type 1 and type 2 currents.
under these conditions application of pH 5.0 did not elicit transient ASIC-like current, neither in R54 nor R8 cells (Fig. 7b). Thus, expression of functional ASIC1b in R54 and R8 cells was too weak to be detected functionally.

Cells within GSC lines have no amiloride-sensitive constitutive current. It has been reported that primary cultures obtained from fresh brain tumour tissue as well as established clonal cell lines that were originally derived from human glioblastoma have a constitutive conductance that is sensitive to amiloride18,19 and that is presumably formed by a DEG/ENaC with unconventional composition, containing ASIC1a together with subunits of the epithelial Na⁺ channel20. To test whether R54 and R8 cells have a similar amiloride-sensitive basal current, we performed whole cell patch clamp experiments in which we changed the holding potential from −100 mV to +100 mV in 20 mV steps in the presence and absence of 100 μM amiloride. If anything, the basal conductance of R54 and R8 cells was increased in the presence of amiloride (P = 0.075 for R54 and P = 0.053 for R8, paired t-test; Fig. 8), indicating that GSC lines have no unconventional amiloride-sensitive constitutive conductance.

Ca²⁺ imaging revealed the heterogeneity of GSC lines. As expected, our electrophysiological analysis revealed that cells within GSC lines are heterogeneous. To quickly gain a more comprehensive picture of the heterogeneity of GSC lines, we performed Ca²⁺ imaging. It has previously been reported that TRPV1 is highly expressed in brain tumour33 and that TRPM8 is expressed in a human glioblastoma cell line34; both appear to be upregulated in patients diagnosed with glioblastoma35. We therefore examined whether pH 6.0, the TRPV1 agonist capsaicin, the TRPM8 agonist menthol or ATP were able to elicit a Ca²⁺ response in R54 and R8 cells.

Moreover, cells were depolarized by 30 mM K⁺ to test whether they have voltage-gated Ca²⁺ channels (VGCCs). Ionomycin was used as positive control. The cells of GSC lines indeed showed different responses to the same stimulations. Figure 9a shows examples of pseudo-coloured images of Ca²⁺ signals during applications of different compounds to R54 cells and Fig. 9b summarizes the mean responses of 61 R8 cells and 97 R54 cells. pH 6 elicited a slight Ca²⁺ response in only 1–2% of the cells from R54 and R8. Since Ca²⁺ permeability of ASICs is low36, cells need to have VGCCs that are activated by the depolarization due to ASIC activation, to secondarily raise the Ca²⁺ concentration after pH 6 stimulation. Indeed, none of the 97 R54 cells responded with a Ca²⁺ increase by a depolarization with high K⁺, suggesting that R54 cells are not excitable. In contrast, 16% of the R8 cells showed various increases in intracellular Ca²⁺ concentration in high K⁺, showing that R8 cells contain a relatively small fraction of excitable cells. As these cells did not respond to pH 6 with a Ca²⁺ signal, they either did not express an ASIC at sufficiently high amounts (perhaps type 3 currents) or the depolarization by ASIC activation was not strong enough to activate VGCCs. Capsaicin did not elicit any Ca²⁺ response, neither in R54 nor in R8 cells, suggesting that they do not express TRPV1. In strong contrast, menthol elicited in 67% of the R54 cells and in all R8 cells a Ca²⁺ response, showing that the majority of R54 and all R8 cells express TRPM8. 100μM ATP elicited a Ca²⁺ response in 97% of R54 cells and in 95% R8 cells, demonstrating that they either express P2Y or P2X receptors or both. The results from calcium imaging are summarized in Table 1.

Expression of ACCN2 or ACCN3 is associated with improved survival. To confirm the expression of ASICs in human GBM samples, we analysed the expression levels in Repository of Molecular Brain Neoplasia...
Figure 4. Electrophysiological characterization of ASICs in R8 cells. (a) Left, whole cell current traces representative for type 2 and type 3 currents, activated by different acidic pH; conditioning pH was 7.4. Right, pH-response relationships. Peak currents from type 2 cells were normalized to the current at pH 6.5. The curves represent fits to the Hill equation. Amplitudes of sustained currents from type 2 and type 3 cells were normalized to pH 5 and were connected by lines. (b) Pie chart illustrating the relative occurrence of type 2 and type 3 currents. (c) Frequency histogram of peak amplitude of type 2 transient currents. (d) Time constants of desensitization $\tau_{des}$ of transient type 2 currents.
Data (Rembrandt), a cancer clinical genomics database and a Web-based data mining and analysis platform, established by the National Cancer Institute\textsuperscript{37}. Rembrandt contains data from different glioma subtypes, including glioblastoma multiforme (WHO grade IV), astrocytoma (WHO grade II), and oligodendroglioma (WHO grade II). In line with our \textit{in vitro} results, ACCN1 (ASIC2) was downregulated in all types of glioma samples as compared to the non-tumour samples used as control (\( P < 0.001 \), two-sided t-test; Fig. 10a). In contrast, ACCN2 and ACCN3 showed similar expression levels as compared to non-tumour groups irrespective of tumour grade (Fig. 10a). The Kaplan-Meier analyses unveiled a significant survival benefit of glioma patients with high ACCN2 (\( P < 0.001 \), log-rank test; Fig. 10b) or high ACCN3 expression (\( P = 0.003 \), log-rank test; Fig. 10c).

**Discussion**

Our results show the expression of ASICs in two molecularly distinct GSC lines. We detected mRNA and protein expression for ASIC1a and ASIC3 (Fig. 1), the two most proton-sensitive ASICs\textsuperscript{7}. We did not find a substantial expression of ASIC2 in the GSC lines studied and inspection of published microarray data\textsuperscript{37} revealed a significant downregulation in tumour samples as compared to non-tumour samples. This supports the previously described anti-migratory and therefore anti-oncogenic effects of ASIC2 in glioma cells\textsuperscript{38}. The expression of either ACCN2 or ACCN3 was associated with a significant survival benefit in glioma samples, underscoring their clinical relevance. Electrophysiological characterization revealed clear evidence for the presence of functional homomeric ASIC1a and homo- and/or heteromeric ASIC3-containing ASICs (summarized in Table 2), demonstrating that the ACCN2 and ACCN3 mRNA is also translated in GSC cell lines. Tachyphylaxis of transient ASIC currents in most cells indicated the presence of homomeric ASIC1a\textsuperscript{24,25}, the half-maximal activation around pH 6.5 is characteristic for both ASIC1a\textsuperscript{6,15} and ASIC3\textsuperscript{16,32}, and the sensitivity of most currents to PcTx1 or APETx1 is indicative of homomeric ASIC1a and ASIC3-containing channels, respectively\textsuperscript{26,29}. Time constants of desensitization \( \tau_{\text{des}} \) in R54 cells were more similar to ASIC1a (Table 2), but showed a rather large range, compatible with mixed populations of ASIC1a and other more rapidly desensitizing ASICs. In R8 cells, \( \tau_{\text{des}} \) was more homogenous and similar to ASIC1a (Table 2), but was still compatible with mixed populations of ASIC1a and other ASICs. The slight potentiation of ASIC currents in R54 and R8 cells by the cono-RFamide RPRFa also indicates the presence of ASIC3-containing channels in these cells\textsuperscript{31}. Thus, to our knowledge, this is the first time that ASIC3 was characterized in native human cells. This is the more important as it is generally considered to be a specific receptor of the peripheral nervous system.

We detected also low amounts of ASIC1b mRNA in R54 and R8 cells (Fig. 1a) but had no clear electrophysiological evidence for the presence of functional ASIC1b. ASIC1b is less sensitive to protons than either ASIC1a or ASIC3\textsuperscript{15,23,39} with a half-maximal activation at pH 5.9 (Table 2). Since transient currents saturated at pH 6.0, no substantial amount of ASIC1b was present in these cells. Only the slight potentiation of type 2 currents when PcTx1 was applied at a conditioning pH of 7.4 (Fig. 3a) suggested the presence of functional ASIC1b in these cells\textsuperscript{28}. 

![Figure 5. Toxin sensitivity of ASICs in R8 cells.](image)
Thus, it is clear that in both cell types, R54 and R8, homomeric ASIC1a and homo- and/or heteromeric ASIC3-containing ASICs were present. We obtained no unequivocal results concerning the relative ratios of these two channel populations, however. For example, in R54 cells at conditioning pH 7.3 > 60% of the cells were sensitive to PcTx1 (Fig. 3b), indicative of homomeric ASIC1a 26, but ~60% of the cells were also sensitive to APETx1 (Fig. 3c), indicative of ASIC3-containing channels 29. This discrepancy might be due to the heterogeneity of the GBM cells that we analysed. Moreover, although we avoided long-time passaging of the cells, we cannot completely exclude that the GBM cell population changed its composition over time. The presence and co-existence of ASIC1a and ASIC3 in both R54 and R8 cells was a consistent finding, however.

In Ca\(^{2+}\) imaging, only around 1% of cells responded to acidic pH (pH 6.0) with a calcium signal (Table 1) even though ~80% of the cells express ASICs (Figs 2 and 4). Although homomeric ASIC1a is known to be slightly permeable to Ca\(^{2+}\) 6, its Ca\(^{2+}\) permeability is low 36,40. Thus, voltage-gated Ca\(^{2+}\) channels (VGCCs) are necessary to mediate a Ca\(^{2+}\) influx upon depolarization by ASIC activation. In agreement with the inability of pH 6.0 to produce an intracellular Ca\(^{2+}\) signal, only 16% of the R8 cells and none of the R54 cells responded to the strong depolarization induced by 30 mM K\(^{+}\) with a Ca\(^{2+}\) signal (Table 1), demonstrating that R54 cells do not express VGCCs and only a small subpopulation of R8 cells does so.

It has previously been reported that ASIC1 and ASIC2 are present in different cell lines derived from glioblastoma patients 18,19. But rather than mediating typical transient ASIC currents, it was found that they assemble in a non-canonical mixed ASIC/ENaC channel that mediates a constitutive amiloride-sensitive Na\(^{+}\) conductance 20. We found no evidence for such a conductance in GSC lines, however (Fig. 8), which may be due to the use of GSC lines cultured in serum-free medium that more closely mirror the phenotype and genotype of primary tumours than do serum-cultured cell lines 1. We therefore conclude that the unconventional constitutive amiloride-sensitive Na\(^{+}\) conductance does at least not play a general role in GBMs.

Our data clearly show that glioma cells express functional ASIC1a and ASIC3, with substantial impact on patient survival. The molecular mechanisms behind the putative anti-oncogenic effects of ASICs expression is, however, unknown. Data from in vivo and in vitro studies clearly indicate that the average pH is ~0.2 pH units lower in GBM (pH 6.97) than in normal brain tissue (pH 7.2) 41. Anti-angiogenic treatment with e.g. bevacicumab may further decrease pH. If low pH alone is beneficial or detrimental for glioma cells is currently unknown. In vitro, low pH impairs proliferation of GSC lines 4, however low pH induces stem cell properties and GSC lines challenged with low pH become significantly more aggressive as compared to GSC lines cultured under standard condition 5.
Figure 7. There is no alkalization-sensitive ASIC3 current and no ASIC1b current in R54 or R8 cells. (a) Current traces from R54 and R8 cells, showing either no current when stepping from pH 6.6 to pH 8.0 or a small outward current. Traces are representative for 4 cells, each. Stepping from acidic pH 6.6 to alkaline pH 8.0 did never elicit inward currents. (b) Current traces from R54 and R8 cells, showing either no current when stepping from conditioning pH 6.7 to pH 5.0 or a small sustained inward current. Traces are representative for 8 cells (R8) or 4 cells (R54), respectively. Stepping from pH 6.7 to pH 5.0 did not elicit typical transient ASIC currents.

Figure 8. There is no constitutive amiloride-sensitive Na$^+$ conductance in R54 or R8 cells. (a) Left, representative whole cell current traces from R54 cells without and with amiloride. Cells were clamped in 20 mV steps for 800 ms to holding potentials ranging from −100 to +100 mV. Right, I/V curves do not reveal a decrease of the conductance by amiloride. $n = 6$. (b) as in (a) but for R8 cells. $n = 6$. 
Although ASICs desensitize in the continued presence of protons, it has been shown that ASIC3 can also detect sustained acidification in the pH range around 7.0, which might enable this channel to detect the acidification that accompanies heart ischemia. In addition, human ASIC1a is less sensitive to protons than its rodent orthologues, such that it will not be desensitized at pH 7.0. Thus, pH sensitivity of ASIC1a and ASIC3 might just be tuned such as to endow GBMs with the capacity to detect and signal the acidic environment surrounding it. One might speculate that this capacity increases the susceptibility of glioma cells to extracellular acidosis. Our data allow, however, no conclusion on the precise mechanisms involved. Moreover, ASICs may have different impact on different tumours, because breast cancer cells also express ASIC1 and alterations in ACCN2 (including amplification, mutations and upregulation) are associated with poor prognosis. In addition, ASIC1 and ASIC3...
contribute to epithelial-mesenchymal transition of pancreatic cancer cells. Future studies need to clarify the exact role of ASIC1 and ASIC3 in different tumours.

In summary, we identify and functionally characterize ASICs as sensitive proton sensors in glioma cells. ASICs endow glioma cells with the capacity to sense the tumour-surrounding pH, which may be associated with an improved prognosis.

**Methods**

**Cell culture.** GSC lines were grown, as previously described, in DMEM:F12 (Gibco) supplemented with 2% B27 supplement, 1% glutamine, 1% vitamin, 1% penicillin-streptomycin, 0.1% fibroblast growth factor (FGF) and 0.1% epidermal growth factor (EGF) at 37°C in a humidified atmosphere with 5% CO₂.

|        | R54   | R8    |
|--------|-------|-------|
| pH 6   | 1% positive | 2% positive |
| 30 mM K⁺ | 0% positive | 16% positive |
| capsaicin | 0% positive | 0% positive |
| menthol | 67% positive | 100% positive |
| ATP    | 97% positive | 95% positive |
| ionomycin | 100% positive | 100% positive |

Table 1. Ca²⁺ responses in R54 and R8 cells. Percentages of cells responding with an increase in intracellular Ca²⁺ concentration are indicated. Total number of cells was 97 for R54 and 61 for R8.
Experiments were repeated with RNA from at least n negative control. qPCR was performed in a Rotor-Gene Q (Qiagen), starting with a long denaturation phase. 

|    | Type 1       | Type 2       | pHi50 act. |
|----|--------------|--------------|------------|
| R54| 1.4 ± 0.1 (15)| 1.2 ± 0.1 (14)| 6.4 ± 0.1 (8)|
| R8 | 1.2 ± 0.1 (17)| 1.2 ± 0.1 (17)| 6.5 ± 0.1 (7)|
| hASIC1a | 1.1–3.0^1^2  | 6.45–6.55^1^2 |          |
| hASIC1b | 0.55^1^      | 5.9^1^      |          |
| hASIC3 | 0.5 ± 0.04 (7) at pH 6 | 6.51^1^ |          |

Table 2. Functional properties of ASIC currents in R54 and R8 cells compared to properties of recombinant human ASICs in heterologous expression systems. Values for R54 and R8 cells are mean ± SEM; the number n of measurements is indicated in parentheses. Values for recombinant ASICs are from the literature; references are indicated in superscripts and are as follows: ^1^Gunthorpe, 2001, ^2^Sherwood, 2008, ^3^Hoagland, 2010, ^4^Delaua, 2012. n.d., not determined. τdes for hASIC3 was determined in HeLa cells with a recombinant clone.

Reverse transcription and quantitative real-time PCR. Total RNA was isolated from GSC lines using RNeasy minikit (Qiagen, Venlo, The Netherlands). Concentration and quality of the RNA was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific). RNAs with a 260 nm/280 nm ratio > 2.00 and a 260 nm/230 nm ratio > 1.80 were used for reverse transcription. First-strand cDNA was synthesized from 1 μg total RNA using QuantiTect Reverse Transcription Kit (Qiagen), yielding 20 μl cDNA. All kits were used according to the manufacturer’s instructions. Contamination with genomic DNA was controlled by RT-PCR using intron-spanning primers for the reference gene hypoxanthine–phosphoribosyl-transferase (HPRT).

1 μl cDNA was used for each PCR reaction. Sequences of primers for standard PCR were as follows: hHPRT-sense, 5′-GGA CCC CAC GAA GTG TTG GAT ATG AG-3′, hHPRT-antisense, 5′-GTC AAG GCC ATA TCC TAG AAC AAA TTT GAT ATA AG-3′; hASIC1a-sense, 5′-CCT CGA AGC TCT CTC GGG-3′, hASIC1a-antisense, 5′-GCC GCT CCA CCT GAG-3′; ASIC2a-sense, 5′-CCG GCT CCA CCT GAG-3′, ASIC2a-antisense, 5′-GCC GTA TCT CCA GCT G-3′; ASIC1b-sense, 5′-CCA TCA CGA GCA GGA C-3′, ASIC1b-antisense, 5′-GAC AGC CGC ACA ACA GGA TTA G-3′; ASIC2-sense, 5′-CTG TTT ACA GCA TCA CCG-3′, ASIC2-antisense, 5′-CCA AGG AGG TCT AAT AGC-3′; 3′′-GSC lines were lysed with RIPA buffer (25 mM Tris Cl pH 7.6, 0.1% SDS, 150 mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, 1% PMSF, and 1% proteinase inhibitor cocktail; Roche). Samples were loaded for 15 min at 37 °C with 2 μM Fura-2-AM (Molecular Probes) in bath solution. Fura-2 was excited at 340/380 nm, and the emission was recorded between 470 and 550 nm using a sensicam CCD camera (PCO imagine). Acquisition and data analysis were done using Till Vision software. Ionomycin, capsaicin, menthol and ATP were purchased from Sigma-Aldrich.

Measurement of intracellular Ca^2+ concentration. For cell fluorescence measurements, GSC lines were grown on glass coverslips mounted in a cell chamber and perfused with bath solution at room temperature. Fluorescence was measured continuously on an inverted microscope (IX71, Olympus, Chromaphor) using a Fluar 20 ×/0.75 objective (Olympus) and TillVision real-time imaging software (Till Photonics). Cells were loaded for 15 min at 37 °C with 2 μM Fura-2-AM (Molecular Probes) in bath solution. Fura-2 was excited at 340/380 nm, and the emission was recorded between 470 and 550 nm using a sensiscan CCD camera (PCO imaging). Acquisition and data analysis were done using Till Vision software. Ionomycin, capsaicin, menthol and ATP were purchased from Smartox biotechnology.

Western blot. GSC lines were lysed with RIPA buffer (25 mM Tris-Cl pH 7.6, 0.1% SDS, 150 mM NaCl, 1% Triton-X-100, 1% sodium deoxycholate, 1% PMSF, and 1% proteinase inhibitor cocktail; Roche). Samples were...
quantified using a protein assay (Micro BCA; Thermo Fisher Scientific) and the same amount of protein was separated using SDS-PAGE (10%). Proteins were transferred to PVDF membranes (Roche, Mannheim, Germany), and probed overnight at 4 °C with the following primary antibodies: mouse monoclonal anti-ASIC1 (NeuroMab), rabbit polyclonal anti-ASIC2 (Abcam), or mouse monoclonal anti-actin (Sigma-Aldrich). Blots were visualized using secondary HRP-conjugated anti-rabbit or anti-mouse antibodies and SuperSignal ELISA Femto Substrate (Thermo Fisher Scientific).

**Microarray analysis.** We used the publically available REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT, http://www.betastasis.com/glioma/rembrandt/) to investigate expression level and prognostic significance of ACCN2 and ACCN3 expression. REMBRANDT is based on a total of 524 microarrays (Affymetrix U133 2.0. plus), of which 329 are included in the online database. The database was accessed September 4, 2017. Data shown have been generated using the analysis tools provided at the REMBRANDT website. For Kaplan-Meier analysis, we set the median of ACCN expression as threshold, such that half of the samples were defined as “low expression” and the other half as “high expression”. Statistical analysis was done with a log-rank test available at the REMBRANDT website.

**Statistical analysis.** Data are reported as mean ± s.e.m. Student’s t-test was used for paired or unpaired samples. P ≤ 0.05 was considered as significant. Proton-response curves were fit with a Hill function:

\[ I(x) = a + \frac{(I_{\text{max}} - a)}{1 + (x_{\text{half}}/x)^h} \]

where \( a \) represents the baseline current and \( I_{\text{max}} \) the maximal normalized current, \( x_{\text{half}} \) is the concentration to elicit a half-maximal effect, and \( h \) represents the Hill coefficient. Expression level of ACCN genes in tumour and control samples was analysed by a two-sided t-test assuming equal variance with Bonferroni correction, using SPSS Statistics 24 (IBM).

**Data availability.** All data generated or analysed during this study are included in this published article, except the datasets on expression levels and prognostic significance of ACCN2 and ACCN3 expression in patients, which are available in the REMBRANDT repository, http://www.betastasis.com/glioma/rembrandt/.

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Author Contributions
Y.T. carried out most Western blots, most of the electrophysiological analysis of R8/R54 cells and the calcium imaging, P.B. did the qPCR and some Western blots, A.R. did some of the electrophysiological analysis, L.E.M. cultured R8/R54 cells, and C.B. provided R8/R54 cells and analysed the REMBRANDT database. Y.T., C.B. and S.G. participated in data analysis. Y.T., L.E.M. and C.B. participated in the design of the study. S.G. conceived of the study, designed the study and coordinated the study. Y.T. and S.G. drafted the manuscript and all other authors edited and approved the final manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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