Neural precursor cells induce cell death of high-grade astrocytomas through stimulation of TRPV1

Kristin Stock1,16, Jitender Kumar1,16, Michael Synowitz1,2,16, Stefania Petrosino3, Roberta Imperatore4, Ewan St J Smith5,6, Peter Wend7,15, Bettina Purfürst8, Ulrike A Nuber9, Ulf Gurok10, Vitali Matyash1, Joo-Hee Wälzlein1, Sridhar R Chirasani5, Gunnar Dittmar11, Benjamin F Cravatt12, Stefan Momma13, Gary R Lewin5, Alessia Ligresti3, Luciano De Petrocellis4, Luigia Cristino4, Vincenzo Di Marzo3, Helmut Kettenmann1,16 & Rainer Glass1,14,16

Primary astrocytomas of grade 3 or 4 according to the classification system of the World Health Organization (high-grade astrocytomas or HGAs) are preponderant among adults and are almost invariably fatal despite the use of multimodal therapy. Here we show that the juvenile brain has an endogenous defense mechanism against HGAs. Neural precursor cells (NPCs) migrate to HGAs, reduce glioma expansion and prolong survival time by releasing endovanilloids that activate the vanilloid receptor (transient receptor potential vanilloid subfamily member-1 or TRPV1) on HGA cells. TRPV1 is highly expressed in tumor and weakly expressed in tumor-free brain. TRPV1 stimulation triggers tumor cell death through the branch of the endoplasmic reticulum stress pathway that is controlled by activating transcription factor-3 (ATF3). The antitumorigenic response of NPCs is lost with aging. NPC-mediated tumor suppression can be mimicked in the adult brain by systemic administration of the synthetic vanilloid arvanil, suggesting that TRPV1 agonists have potential as new HGA therapeutics.

Synergistic TRPV1 activation by AEA in combination with other fatty acid ethanolamides, such as oleoyl ethanolamide (OEA) or palmitoyl ethanolamide (PEA), has previously been reported15,16. The physiological role of the ion channel in non-neural tissues is largely unexplored16. Here we show that endovanilloid release from NPCs activates TRPV1 on HGA cells and thereby induces tumor-cell death.

RESULTS

NPCs induce HGA cell death through TRPV1

We investigated the signaling pathways that are activated in HGA cells after exposure to NPC-conditioned medium (NPC-CM). An analysis of gene expression changes (by microarrays of stimulated and control HGA cells; GSE37671) together with pharmacological studies using specific TRPV1 antagonists and molecular studies using specific TRPV1 knockdown in HGA cells suggested a role for NPC-derived endovanilloids as tumor suppressors. Furthermore, we observed by real time PCR that TRPV1 expression is positively correlated with grading in human primary brain tumors and that TRPV1 expression in tumors was higher than in human tumor-free brain (Supplementary Fig. 1a).

Received 12 November 2010; accepted 7 May 2012; published online 22 July 2012; doi:10.1038/nm.2827
We next explored the role of the endovanilloid system in NPC-mediated HGA suppression in an established mouse model\(^8,17\). We orthotopically implanted brain tumor cells (DsRed-expressing GL261 cells) in nestin-GFP mice, which are a model for the visualization of tumor-associated NPCs in humans (S. M., unpublished observations).\(^8\) We identified NPCs using colocalization of GFP with established immunocytochemical markers, such as polysialylated neuronal adhesion molecule (PSA-NCAM) (Fig. 1b) or Musashi\(^8,19,20\). PSA-NCAM is also a marker of tumor-associated NPCs in humans (S. M., unpublished observations). Notably, we found that the mouse HGAs had higher amounts of immunolabeling for TRPV1 (Fig. 1c) than tumor-free brain, whereas only a small fraction of tumor-associated nestin-GFP+ cells expressed TRPV1, and TRPV1 was absent from subventricular NPCs in the mice (Supplementary Fig. 1b).

In a series of in vitro experiments, we found that stimulation of mouse HGAs with factors released from mouse NPCs (mNPC-CM), but not with factors released from their fully differentiated progeny (astrocytes, oligodendrocytes and neurons) or from fibroblasts (scrc), strongly reduced the viability of HGA cells over a time-course of 3 d (Fig. 1d; the data for the conditioned medium from the control cells were obtained on day 3). In subsequent experiments, we stimulated the mouse HGA cells for 3 d, unless indicated otherwise. Stimulation with mNPC-CM reduced the viability of the HGA cells by inducing cell death, as indicated by TUNEL and cytotoxicity assays (Supplementary Fig. 1c). All cytotoxicity values are expressed as a percentage of the fully permeabilized cells (Supplementary Figs. 2 and 3).

HGAs in the GL261 mouse glioma model express TRPV1 and contain specific binding sites for a selective TRPV1 ligand (Supplementary Fig. 1d,e). In this model, HGA cell death induced by stimulation with mNPC-CM was greatly reduced by blocking TRPV1 with the selective antagonists\(^8\) I-RTX (10 nM; Fig. 1e) or capsaicin (CZP, 1 μM; Fig. 1f) and by knockdown of TRPV1 (TRPV1-KD; Fig. 1e). For the knockdown of TRPV1, we expressed five different TRPV1-directed shRNAs and control shRNA (scrambled) in GL261 cells and then selected for vectors that block TRPV1 function in GL261 cells. We subsequently quantified TRPV1 protein expression by quantitative proteomics (selected reaction monitoring, SRM) in knockdown and control GL261 cells (Supplementary Fig. 1d). SRM allowed for a direct, specific and well-controlled quantification of TRPV1 protein expression. We verified the efficiency and specificity of the TRPV1 knockdown (Supplementary Fig. 1d,e) by performing experiments with control shRNAs (scrambled shRNA) and with overexpression of a knockdown-resistant form of mouse TRPV1 (ref. 21) in TRPV1-KD HGA cells (Fig. 1e,f and Supplementary Fig. 2c). Exposure to mNPC-CM strongly induced cell death in various HGA cell lines (Fig. 1f) and in three different primary human GBM cultures (Fig. 1g), an effect that was blocked by the simultaneous application of either I-RTX (Supplementary Figs. 2b and 3a,b) or CZP. Notably, human NPC-CM also induced cell death in primary human GBMs after stimulation of TRPV1 (Fig. 1h).

Overall, our cell-culture experiments showed that human and mouse NPCs release TRPV1 agonists, which induce HGA cell death. Our data from a mouse model showed that, in the young brain, many NPCs accumulate at HGAs. An analysis of TRPV1 expression in...
HGAs and tumor-free brain from mice and humans indicated that HGAs have high expression of TRPV1, whereas we found low TRPV1 expression in non-neoplastic CNS.

NPCs constitutively release endovanilloids

We quantified the concentrations of AEA, NADA, OEA, PEA and the endocannabinoid arachidonoyl glycerol (2-AG) in samples from mNPCs, fully differentiated progeny from mNPCs and mouse HGA cells. Differentiation of NPCs was achieved by growth factor withdrawal and transient addition of fetal bovine serum. After differentiation, cells were alive and responsive. This figure shows data from representative experiments, and the number of similar experiments out of all experiments is given in parentheses. All data are mean ± s.d. *P < 0.05, **P < 0.005, ***P < 0.001 by Fisher’s exact test (a,b) or Student’s t test (c–e), and #P < 0.001 by Wilcoxon rank test.

The addition of fatty acid amide hydrolase (FAAH), which degrades endocannabinoids, blunts the HGA cell death induced by the combination treatment with AEA, OEA and PEA (Fig. 2c). GL261 HGA cells were stimulated with the vanilloid I-RTX (10 nM). I-RTX cooperatively induced cell death in HGAs. (Fig. 2b) More specifically, mNPC-CM plus FAAH blunted the HGA cell death induced by the subthreshold concentration of AEA alone. I-RTX blunted the HGA cell death induced by the combination treatment with AEA, OEA and PEA. (Fig. 2c) Figure 2 NPC-released fatty acid ethanolamides induce cell death in HGAs. (a) Quantification of AEA, OEA, PEA and 2-AG in mNPCs, differentiated NPCs (Diff. NPC) and GL261 HGAs. (b) Quantification of AEA in conditioned medium from mNPCs, differentiated NPCs and GL261 HGA cells. (c) GL261 HGA cell death is cooperatively induced after the simultaneous application of AEA, PEA and OEA but not by a subthreshold concentration of AEA alone. I-RTX blunted the HGA cell death induced by the combination treatment with AEA, OEA and PEA. (d) Cytotoxicity of GL261 cells exposed to nonconditioned medium (Ctrl.), mNPC-CM or mNPC-CM plus FAAH. (e) GL261 HGA growth in Faah–/– mice with control (Ctrl.) or TRPV1-KD tumors (n ≥ 7 mice per experimental group, containing male and female mice). (f) Ca2+ responses in wild-type (WT-GL261), control shRNA (scrambled), TRPV1-KD and TRPV1 rescue GL261 HGA cells (TRPV1-resc.) stimulated with mNPC-CM. Ca2+ responses to mNPC-CM were also recorded from three different primary human GBM cultures (middle graph) and HGA cell lines (right graph). CZP blocked the Ca2+ signals evoked by mNPC-CM in all cases. The dashed red line indicates the baseline values for each experiment. ATP (1 mM) induced a Ca2+ response in all experiments, indicating that all cells were alive and responsive. This figure shows data from representative experiments, and the number of similar experiments out of all experiments is given in parentheses. All data are mean ± s.d. *P < 0.05, **P < 0.005, ***P < 0.001 by Fisher’s exact test (a,b) or Student’s t test (c–e), and #P < 0.001 by Wilcoxon rank test.

TRPV1 is abundantly expressed in dorsal root ganglion neurons (DRGs)25, and opening of the TRPV1 channel induces a cation influx, which can be measured, for example, as a rise in the intracellular concentration of free calcium ions. To assay the activity of endovanilloids released from NPCs, we designed a bioassay using DRGs from wild-type and TRPV1–/– mice26. We stimulated the DRGs from both groups with mNPC-CM and measured the responses using Fura-2–based calcium imaging. We observed that 5.4% of the wild-type DRGs responded to both an initial stimulation with NPC-CM and to a subsequent stimulation with the selective TRPV1 agonist.
TRPV1 induces cell death through ER stress

We next investigated the gene expression pattern in mouse tumor cells after incubation with nonconditioned medium (controls) or with mNPC-CM by microarrays (GSE37671). We found that ER stress genes, such as the activating transcription factor-3 gene (ATF3), were robustly upregulated in mouse HGA cells treated with mNPC-CM compared to controls. Immunocytochemical labeling and reporter gene assays in GL261 cells treated with mNPC-CM showed that the expression of ATF3 was higher in both the cytoplasm and nucleus of these cells than in controls and that mNPC-CM activates an ATF3-responsive element in a gene promoter (Fig. 3a,b). Forced expression of ATF3 reduced the number of GL261 cells in culture and increased the number of TUNEL+ tumor cells (Fig. 3c). Notably, siRNA-mediated downregulation of ATF3 expression (Supplementary Fig. 6a) in mouse HGA cells prevented the tumor cell death induced by mNPC-CM (Fig. 3d). Therefore, ATF3 is necessary and sufficient for mediating the HGA cell death induced by mNPC-CM. Administering the TRPV1 antagonist CZP blocked the mNPC-CM–induced nuclear strand breaks in GL261 HGA cells. All data are mean ± s.d. *P < 0.05, **P < 0.005, ***P < 0.001 by Student’s t test.

Figure 3 TRPV1 agonists released by NPCs trigger the ATF3 pathway in HGAs. (a) ATF3 expression in HGAs (GL261 cells expressing GFP, GL261-GFP) exposed to nonconditioned medium (Ctrl.) or mNPC-CM; notably, the cell morphology changed after exposure to NPC-CM, and the small and rounded GL261 cells (asterisk) seem damaged. Scale bar, 10 μm. (b) A wild-type form of the ATF3 gene promoter (WT) was activated in GL261 cells after stimulation with NPC-CM, whereas a mutant control of the ATF3 gene promoter (mutant) was not. A gene promoter with an ATF3 binding site (ATF3 function) is also induced after stimulation with mNPC-CM. (c) Overexpression of ATF3 reduced GL261 cell density and induced DNA fragmentation (determined by TUNEL) as compared to GL261 cells expressing empty vector (control). (d) siRNA for ATF3 prevents mNPC-CM–induced nuclear strand breaks in GL261 HGA cells. All data are mean ± s.d. *P < 0.05, **P < 0.005, ***P < 0.001 by Student’s t test.

Figure 4 TRPV1 agonists released by NPCs induce ER-stress–mediated cell death. (a) Ultrastructure of GL261 HGA cells after incubation with mNPC-CM or control medium. The arrowheads point to the ER membrane, and the asterisk indicates an inflated ER lumen. Scale bar, 500 nm. (b) The relative increase in ER size after incubation with mNPC-CM quantified in various HGA cell lines. (c,d) ER size quantified in primary human GBM cells after incubation with mNPC-CM (c) and human NPC-CM (d). (e) Vanilloids and pharmacological ER stress inducers (tunicamycin, Tunicam.; thapsigargin, Thapsig.) have cooperative effects: subthreshold concentrations of the combined substances induce ER enlargement. (f) Illustration of NPCs constitutively releasing endovanilloids (fatty acid ethanolamides such as AEA, PEA, and OEA), which traverse the plasma membrane of HGAs and stimulate TRPV1 by docking to an intracellular receptor binding site. NPC-induced TRPV1 activation (mainly located in the ER; Supplementary Fig. 5) triggers the ATF3-dependent ER stress pathway in HGAs, which includes activation (phosphorylation, indicated by the P within a circle) of eukaryotic initiation factor 2 α (eIF2-α) and ATF4 (ref. 28; Supplementary Fig. 6). Increased expression of ATF3 is necessary and sufficient to mediate HGA cell death. All data are mean ± s.d. *P < 0.05, **P < 0.005, ***P < 0.001 by Student’s t test.
Electron microscopy revealed that GL261 cells treated with mNPC-CM had an enlarged ER as compared to controls (Fig. 4a and Supplementary Fig. 6d), which is a morphological hallmark of ER stress. We quantified the effect of mNPC-CM (with or without the addition of CZP) on the ER in primary human GBM and in human, rat and mouse HGA cell lines using ER-tracker (Fig. 4b,c).

We then determined the effect of human NPC-CM on ER size in primary human GBMs (Fig. 4d). In all HGA cells studied, we detected a very robust increase in ER size after stimulation with human or mouse NPC-CM, which was always attenuated by the addition of CZP (Fig. 4b–d and Supplementary Fig. 7). Additionally, we used synthetic AEA and inducers of ER stress, such as tunicamycin or thapsigargin, at concentrations that were below the threshold for ER stress induction when applied alone. We found that the simultaneous application of AEA and either tunicamycin or thapsigargin led to strong increases in ER size in GL261 cells (Fig. 4e).

The combined substances had a clear cooperative effect on the rise in ER size, confirming that vanilloid-induced signaling and ER stress are part of the same signal transduction pathway in HGA cells. These data show that human and mouse NPC-derived endovanilloids induce HGA cell death through the ER stress pathway (Fig. 4f).

**Age dependency of NPC-induced tumor suppression**

To investigate whether NPC-derived endovanilloids can suppress HGAs in vivo, we performed orthotopic implantation of HGAs into nestin-GFP mice of different ages. Implantation of GL261 cells (TRPV1-KD or controls, which express a nontargeting shRNA) into young (30-day-old) mice resulted in the association of many endogenous NPCs to the tumor (Fig. 5a). Nestin-GFP NPCs accumulated at GL261 control HGAs and TRPV1-KD HGAs in equal densities. Notably, we found that young mice injected with TRPV1-KD cells had significantly larger (70%) tumors compared to young mice injected with GL261 control cells (Fig. 5b). We found no difference in tumor size in 90-day-old adult mice administered GL261 control cells and those administered TRPV1-KD cells (Fig. 5b). In another set of experiments, we used a transgenic mouse model to manipulate the numbers of endogenous NPCs independently of aging. Therefore, we used cyclin D2 knockout (Ccnd2−/−) mice, which have substantially lower amounts of adult neurogenesis than do wild-type mice. In these Ccnd2−/− mice and their wild-type littermates, we orthotopically implanted GL261 cells (control or TRPV1-KD) and measured the tumor size 14 days after implantation. We found that the tumor size in the wild-type mice receiving control HGAs was at least 63% smaller than the tumors in the wild-type mice receiving TRPV1-KD tumor cells or Ccnd2−/− mice receiving either control HGAs or TRPV1-KD tumor cells (Fig. 5c). We then measured HGA cell death in vivo by systemically delivering propidium iodide (Fig. 5d). We noted that TRPV1-KD HGAs had much lower levels of HGA cell death than control HGAs.

In another set of experiments, we tested the effect of endovanilloids released from NPCs on the overall survival of a cohort of wild-type mice with HGAs. First, we orthotopically implanted control or TRPV1-KD GL261 cells into young mice and compared the cumulative survival times of the two groups. We observed that young wild-type tumor-bearing mice (Fig. 5e) had a significantly longer survival time after implantation than the older mice. However, when we...
implanted young mice with TRPV1-KD HGA cells they survived, on average, the same amount of time as older mice. These data show that younger mice have an intrinsic protective mechanism against HGAs that is dependent on endovanilloid signaling. In a second study, we investigated whether the survival-promoting effect that we observed could be attributed specifically to NPCs. Therefore, we implanted adult mice with exogenously cultivated NPCs and control HGAs or TRPV1-KD tumor cells (Fig. 5f). We found that implantation of control HGAs always generated tumors but that co-implantation of mNPCs together with HGAs prolonged survival (Fig. 5e,f; $P < 0.001$). We also found that the survival-promoting effect of NPCs in adult mice was absent after implantation with TRPV1-KD cells (Fig. 5f).

Our data suggest that NPCs release endovanilloids in vivo in a way similar to NPCs in vitro. Consistently, the extent of the NPC-mediated antitumor response depended on the amount of adult neurogenesis.

**Synthetic vanilloids as therapeutics for HGAs**

We next investigated the therapeutic potential of the synthetic, non-pungent, blood-brain-barrier–permeable vanilloid arvanil $^{12,33}$. We allowed control HGAs to develop for 5 d in organotypic brain slice cultures obtained from wild-type mice. The addition of temozolomide $^{14}$ (200 μM, which is the current standard of care for the treatment of patients with GBM) or arvanil (50 nM) strongly reduced the size of the HGAs as compared to vehicle-treated controls (Fig. 6a,b). Furthermore, arvanil induced a TRPV1-dependent Ca$^{2+}$ signal and TRPV1-dependent cell death in the HGAs (Supplementary Fig. 8).

In further experiments, we orthotopically implanted TRPV1-KD or control HGAs into wild-type mice and treated both groups with arvanil as described above. We compared the survival of these arvanil-treated mice to a cohort of mice receiving control HGAs and no arvanil treatment. We found that the mice receiving the control HGAs plus arvanil had significantly longer survival time than the mice receiving TRPV1-KD tumor cells plus arvanil (Fig. 6c; $P < 0.001$) or control tumors without arvanil. These data indicate that arvanil elicits its therapeutic effect as a TRPV1 agonist. To determine whether arvanil would also lead to longer survival in other HGA models, we implanted primary human GBM cells (GBM1 and GBM2) into severe combined immunodeficient (SCID) mice. At 1 week after implantation, we examined the tumor development in the mice and administered arvanil (a total of four intraperitoneal injections of 1 mg kg$^{-1}$ body weight each) or vehicle. Treatment with arvanil substantially prolonged survival as compared to treatment with vehicle (Fig. 6d-f; $P < 0.001$). We then compared the effects of the application of arvanil and temozolomide (alone and in combination) on survival after implantation of a third primary human HGA culture (GBM3) in immune deficient (SCID) mice. We found that arvanil prolonged survival in a cohort of SCID mice that received GBM3 cells that did not respond to temozolomide (given once daily for 5 d at 100 mg kg$^{-1}$ body weight (ref. 35); Fig. 6f; $P < 0.001$). These data show the potential clinical value of an experimental HGA therapy using vanilloids, which may also offer a new therapeutic option for temozolomide-resistant HGAs $^{36}$.

**DISCUSSION**

We have shown that HGAs have high expression of TRPV1 and that TRPV1 stimulation induces tumor cell death. Neural stem and precursor cells home in on HGAs and release antitumorigenic TRPV1 agonists (endovanilloids). Endogenous and exogenous NPCs show extensive tropism for brain tumors $^{6-10}$. However, the number of endogenous NPCs accumulating at HGAs depends on the proliferative activity in the stem cell niche and declines before the onset of adulthood $^{9}$. Hence, the recruitment of large numbers of NPCs to a tumor, and the concomitant antitumorigenic release of endovanilloids, is mostly restricted to the young brain. Additionally, other age-related changes in neural stem cell physiology may also impinge on the ability of NPCs to suppress tumors $^{27,38}$.

We show here that NPCs are a primary source of endogenous TRPV1 and cannabinoid receptor agonists such as AEA $^{15,39}$. This finding was supported by our detection of high amounts of AEA and related acylethanolamides in undifferentiated NPCs, as well as our findings that factors released by NPCs evoke TRPV1-dependent Ca$^{2+}$ responses in DRGs and HGAs, that the tumor-suppressive effect of NPC-CM is lost after the addition of FAAH and that NPC-induced HGA cell death is dependent on TRPV1 in vivo and in vitro. These data are in agreement with previous reports indicating that synthetic AEA induces HGA cell death $^{40}$.

A role for TRP channels in tumor suppression was previously suggested by us and others $^{41-45}$, but the present study is the first, to our knowledge, to identify NPCs as a cellular source for
tumor-suppressive endovanilloids and uncover the role of TRPV1 agonists and modulators released by NPCs on HGA cell death. Overall, our study suggests that endovanilloids are intrinsic tumor suppressors in the brain and that synthetic vanilloid compounds may have clinical potential for the treatment of brain tumors.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data are deposited in Gene Expression Omnibus under the accession code GSE37671.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank G. Gargiulo, O. Daumke and J. Kurrek for discussion of the manuscript, S. Kitajima (Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan) and H.V. J. (Warsaw, Poland) for providing Cendza–/– mice. We acknowledge funding from the Helios Clinics (HeFo-ID1148) and the US National Institutes of Health (DA-009789 to V.D.M.).

AUTHOR CONTRIBUTIONS

S.P., R.L., A.L., D.P., U.C. and S.R.C. designed and conducted the experiments, and interpreted the data. K.S., J.K., E.S.S., J.P.W., B.P., U.A.N., V.M., B.F.C., S.M., V.D.M., J.-H.W., G.D. and L.C. contributed to manuscript preparation. G.R.L., V.D.M. and H.K. designed the experiments, supervised the project and the data contributed to manuscript preparation. M.S. provided brain tumor resections and provided tumor samples. M.S. and R.G. designed and conducted the experiments, contributed to manuscript preparation. M.S. performed brain tumor resections and H.K. designed the experiments, supervised the project, interpreted the data and prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nm.2827. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Sanai, N., Alvaraz-Buylla, A. & Berger, M.S. Neural stem cells and the origin of gliomas. Nat. Eng. J. Med. 353, 811–822 (2005).
2. Ohgaki, H. & Kleihues, P. Genetic pathways to primary and secondary glioblastoma. Am. J. Pathol. 170, 1445–1453 (2007).
3. Knott, R. et al. Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. Proc. Natl. Acad. Sci. USA 104, 17169–17173 (2007).
4. Assanah, M. et al. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor–expressing retroviruses. J. Neurosci. 25, 2637–2646 (2005).
5. Walzlein, J.H., Assanah, M.C. & Kleihues, P. Visualization of neuronal and glial progenitor cell lines infecting intracranial gliomas. Proc. Natl. Acad. Sci. USA 97, 12846–12851 (2000).
6. Suzuki, T. et al. Inhibition of glioma cell proliferation by neuronal stem cell factor. J. Neurosci. Res. 74, 233–239 (2005).
7. Staflin, K. et al. Neural stem cells contribute to glioma pathogenesis in vivo. Oncogene 23, 5347–5354 (2004).
8. Staflin, K., Zuchner, T., Honeth, G., Darabi, A. & Lundberg, C. Identification of proteins involved in neural progenitor cell targeting of gliomas. BMC Cancer 9, 206 (2009).
9. Visnyei, K. et al. A molecular screening approach to identify and characterize inhibitors of glioblastoma stem cells. Mol. Cancer Ther. 10, 1818–1828 (2011).
10. Zjaic, J.H., Assanah, M.C. & Kleihues, P. Genetic pathways to primary and secondary glioblastoma. Nat. Med. 11, 133–137 (2005).
11. Sanai, N., Alvaraz-Buylla, A. & Berger, M.S. Neural stem cells and the origin of gliomas. Nat. Eng. J. Med. 353, 811–822 (2005).
12. Ohgaki, H. & Kleihues, P. Genetic pathways to primary and secondary glioblastoma. Am. J. Pathol. 170, 1445–1453 (2007).
13. Knott, R. et al. Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. Proc. Natl. Acad. Sci. USA 104, 17169–17173 (2007).
14. Assanah, M. et al. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor–expressing retroviruses. J. Neurosci. 25, 2637–2646 (2005).
15. Walzlein, J.H., Assanah, M.C. & Kleihues, P. Visualization of neuronal and glial progenitor cell lines infecting intracranial gliomas. Proc. Natl. Acad. Sci. USA 97, 12846–12851 (2000).
16. Suzuki, T. et al. Inhibition of glioma cell proliferation by neuronal stem cell factor. J. Neurosci. Res. 74, 233–239 (2005).
17. Staflin, K. et al. Neural progenitor cell lines inhibit rat tumor growth in vivo. Cancer Res. 64, 5347–5354 (2004).
18. Staflin, K., Zuchner, T., Honeth, G., Darabi, A. & Lundberg, C. Identification of proteins involved in neural progenitor cell targeting of gliomas. BMC Cancer 9, 206 (2009).
ONLINE METHODS

Mice. Mouse experiments were carried out in compliance with the German laws on animal welfare, and the mouse protocols were approved by the Landesamt für Gesundheit und Soziales (LaGeSo) in Berlin. Wild-type C57BL/6 mice, nestin-GFP mice, Trpv1–/– mice, Cndd2–/– mice, Faah–/– mice and SCID mice (B6.CB17-Pkd1<sup>−/−</sup>,Sj) were purchased from Charles River Breeding Laboratories (Schöneiche, Germany). All were of both sexes and were housed in a 12 h light, 12 h dark cycle and received food ad libitum.

SVZ specimens, tumor specimens, GBM complementary DNA (cDNA) arrays and normal brain cDNA arrays. Normal SVZ specimens from human brains were obtained as part of planned resections during anterior temporal lobectomy for the treatment of intractable epilepsy from mesial temporal sclerosis. Tumor samples were obtained from otherwise untreated primary GBMs from patients undergoing planned tumor resections at the Charité University Clinics. We obtained ethical approval for the human studies from the ethics committee of the Charité University clinics (EA112/2001, EA3/023/06 and EA2/101/08). According to German governmental and internal (Charité University) rules and regulations, all patients gave informed consent to use the material for scientific experimentation; cDNA samples and tissue arrays from human brain tumors and from tumor-free brain were obtained from OriGene.

Cell culture. All GBM cells were maintained as previously described for neurospheres. Mouse, rat and human HGA cell lines and 293T cells were obtained from the National Cancer Institute, the Frederick National Laboratory for Cancer Research and from American Type Culture Collection. Mouse NPCs were obtained from the SVZ of wild-type mice; DRG neurons were prepared from both wild-type and Trpv1<sup>–/–</sup> adult mice, as described previously.

shRNA experiments. The pLKO.1 shRNA vector was from Bio-Cat. The validity of the shRNA-mediated knockdown was confirmed on the protein level by western blotting and fluorescence-activated cell sorting, as previously described, as well as on the functional level (by calcium imaging and cytotoxicity assays). The TRPV1 rescue construct was mutated in the seed region of the shRNA knockdown construct.

Cytotoxicity assays. CytoTox-Fluor Cytotoxicity Assays (Promega) were measured (485 nm/520 nm) with the fluorometer (Tecan).

TUNEL assay. TUNEL+ cells were quantified using the DELFIA Cell-Based Fragmentation Assay (PerkinElmer).

Measurement of ER size. HGA cells were seeded in poly-L-lysine (PLL)-coated 96-well plates and treated with NPC-conditioned medium with or without TRPV1 antagonist. The ER stress inducer thapsigargin (30 ng ml<sup>−1</sup>) was added to the positive control wells for 6 h. For live-cell ER labeling, ER tracker solution (500 nM) was added to the cells for an incubation time of 30 min at 37 °C. Subsequently, the stained cells were fixed with 4% PFA for 15 min at room temperature. The staining was evaluated using a DAPI longpass filter. The relative increase in ER size after incubation with NPC-conditioned medium was quantified by confocal microscopy z-stacks (nonconditioned medium was used as the control; incubation with thapsigargin was set as 100%). For antagonist treatment, the cells were preincubated with antagonists for 3 h in control medium. Afterward, the medium was exchanged with medium containing agonist and antagonist.

Microarray analyses. cDNA microarrays were generated using ~20,000 mouse cDNA clones (ArrayTag clone collection) from LION Bioscience, and six arrays were used in total. Image acquisition and data analyses were done as previously described.

HPLC and mass spectrometry. Lipids were purified using open-bed chromatography on silica gel, and AEA, 2-AG, PEA, OEA and NADA were analyzed by isotope dilution–liquid chromatography/ atmospheric pressure chemical ionization/mass spectrometry.

Peptides for the development of a selected reaction monitoring (SRM) method were selected. Cells were lysed, digested using protease, purified, separated by HPLC and electrosprayed into the mass spectrometer (AB SCIEX QTRAP 4000). For the data analyses, the MultiQuant (AB SCIEX) and R software packages (http://www.r-project.org/) were used.

Calcium measurements. Cells were loaded with Fura-2-acetoxyethyl ester (TEFLabs), excited at 340 nm and 380 nm, and imaged with a 510-nm long-pass filter; the results are presented as the ratio between the emission signals acquired using the two excitation wavelengths.

Real-time PCR. Real-time PCR was performed on the iCycler IQ 5 multicolor real-time detection system (Bio-Rad) using absolute SYBR Green/Fluorescein (Abgene). Oligonucleotides were purchased from Invitrogen.

Western blot. Membranes were incubated with specific antibodies, and western blots were developed using the chemiluminescence method (GE Healthcare).

Tumor implantation. Surgical procedures were performed as previously described: anesthetized mice received tumors (2 × 10<sup>4</sup> HGA cells per 1 μl); this applies to all HGA cells used for in vivo experimentation in this study, G261, GBM1, GBM2 or GBM3 alone or in combination with exogenously cultivated NPCs (6 × 10<sup>4</sup> precursor cells in 4 μl).

Immunofluorescence and microscopy. All stainings and microscopies for NPC and HGA markers were carried out as described previously.

Electron microscopy. For the ER visualization, ultrathin cryosections (70 nm) of fixed HGA cells were contrasted, stabilized and examined with a Zeiss 910 electron microscope. For immunogold labeling before embedding, HGA cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde and incubated with TRPV1-specific antibody.

Cell counting and unbiased stereology. In every twelfth axial section, we sampled the area that was primarily infiltrated by the tumor in an unbiased approach using the optical fractionator procedure (Stereo Investigator, MicroBrightField Inc.). Tumor volume was quantified according to Cavalieri’s principle.

Statistical analyses. Survival statistics were analyzed using MatLab software (Natick, MA). Bar diagrams are shown as means ± s.d. Comparisons among the groups were performed with the Student’s t-test, Fisher’s exact test and the Wilcoxon rank test (as indicated).

46. Lee, J. et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9, 391–403 (2006).
47. Grüneweller, A. et al. Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-O-methyl RNA, phosphorothioates and small interfering RNA. Nucleic Acids Res. 31, 3185–3193 (2003).
48. Gurok, U. et al. Gene expression changes in the course of neural progenitor cell differentiation. J. Neurosci. 24, 5982–6002 (2004).
49. Devane, W.A. et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949 (1992).
50. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L. & Di Marzo, V. Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. J. Biol. Chem. 272, 3315–3323 (1997).
51. Marsicano, G. et al. The endogenous cannabinoid system controls extinction of aversive memories. Nature 418, 530–534 (2002).
52. de Godoy, L.M. et al. Comprehensive mass-spectrometry–based proteome quantification of hippocampal versus dextrin diet. Nature 455, 1251–1254 (2008).
53. Kempenmann, G., Gast, D., Kronenberg, G., Yamaguchi, M. & Gage, F.H. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130, 391–399 (2003).
54. Reimer, T.A. et al. Reevaluation of the 22–1-1 antibody and its putative antigen, EBAG9/RCAS1, as a tumor marker. BMC Cancer 5, 47 (2005).