TRPC1- and TRPC3-dependent Ca\(^{2+}\) signaling in mouse cortical astrocytes affects injury-evoked astrogliosis in vivo

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Abstract

Following brain injury astrocytes change into a reactive state, proliferate and grow into the site of lesion, a process called astrogliosis, initiated and regulated by changes in cytoplasmic Ca\(^{2+}\). Transient receptor potential canonical (TRPC) channels may contribute to Ca\(^{2+}\) influx but their presence and possible function in astrocytes is not known. By RT-PCR and RNA sequencing we identified transcripts of Trpc1, Trpc2, Trpc3 and Trpc4 in FACS-sorted glutamate aspartate transporter (GLAST)-positive cultured mouse cortical astrocytes and subcloned full-length Trpc1 and Trpc3 cDNAs from these cells. Ca\(^{2+}\) entry in cortical astrocytes depended on TRPC3 and was increased in the absence of Trpc3. After co-expression of Trpc1 and Trpc3 in HEK-293 cells both proteins co-immunoprecipitate and form functional heteromeric channels, with TRPC1 reducing TRPC3 activity. In vitro, lack of Trpc3 reduced astrocyte proliferation and migration whereas the TRPC3 gain-of-function moonwalker mutation and Trpc1 deficiency increased astrocyte

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Authors contributions

T.B. designed and performed experiments and analyzed the data; A.N. and T.B. performed proliferation and migration assays; L.H., U.W. and T.B. performed Co-IPs and Western Blots; S.E.P. performed FACS; X.B. and A.S. performed initial stab wound experiments together with T.B.; E.M., P.L., C.B. and A.K. provided transcriptome and microarray data; L.B. provided mice; P.W. performed embryo transfer and mouse breeding; F.K., T.B. and V.F. edited the manuscript; A.B. conceived and designed the experiments, directed the project, analyzed data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.
migration. In vivo, astrogliosis and cortex edema following stab wound injury were reduced in
Trpc3−/− but increased in Trpc1−/− mice. In summary, our results show a decisive contribution of
TRPC3 to astrocyte Ca2+ signaling, which is even augmented in the absence of Trpc1, in particular
following brain injury. Targeted therapies to reduce TRPC3 channel activity in astrocytes might
therefore be beneficial in traumatic brain injury.

Keywords
glia; membrane currents; ion channels; proliferation; migration; stab wound injury

Introduction
The incidence of acute traumatic brain injuries (TBIs) was estimated to be 235 per 100,000
per year with a mortality rate of 15 per 100,000 per year in Europe (Tagliaferri,
Compagnone, Korsic, Servadei, & Kraus, 2006). In the United States annually an estimated
1.7 million people sustain a TBI, with 52,000 people dying from their injury (Faul, Xu,
Wald, Coronado, & Dellinger, 2010). TBIs are followed by changes in cerebral blood flow,
inflammation, alterations in oxygen delivery and metabolism and death of neural cells
(Dutton & McCunn, 2003). Damaged tissue, blood components and diverse messenger
molecules activate astrocytes into a reactive state, called astrogliosis, accompanied by
increased proliferation and growth towards the lesion (Bardehle et al., 2013; Sofroniew,
2009). The activated astrocytes then generate a glial scar, which is beneficial in covering the
damaged area, but hampers axonal regeneration (Windle, Clemente, & Chambers, 1952).
Thus, astrocytes can be both protective and hazardous to neurons, and manipulating their
function might be a valuable strategy for neuroprotection and regeneration after TBI. Cell
proliferation, growth and migration are among the cellular functions controlled by
cytoplasmic Ca2+, which is either released from intracellular stores after stimulation of Gq
protein-coupled receptors or receptor tyrosine kinases, or enters the cell via Ca2+-permeable
ion channels in the plasma membrane. Canonical transient receptor potential (TRPC)
channels may contribute to the Ca2+ influx mediating proliferation, growth and migration of
various cell types (Deliot & Constantin, 2015; Kuang et al., 2012; Zhao et al., 2012)
including astrocytes.

It has been shown that astrocytoma cells and astrocytes from different preparations express
diverse Trpc transcripts, including Trpc1 (Akita & Okada, 2011; Golovina, 2005; Li et al.,
2011; Malarkey, Ni, & Parpura, 2008; Reyes, Verkhratsky, & Parpura, 2013), Trpc3 (Akita
& Okada, 2011; Grimaldi, Maratos, & Verma, 2003; Miyano et al., 2010; Munakata et al.,
2013; Shirakawa et al., 2010; Streifel et al., 2014; Streifel, Miller, Mouneimne, & Tjalkens,
2013), Trpc4 (Song et al., 2005), Trpc5 (Malarkey et al., 2008) and Trpc6 (Beskina, Miller,
Mazzocco-Spezzia, Pulina, & Golovina, 2007), and that TRPC1 is important for
proliferation and chemotactic migration of human malignant glioma (astrocytoma) cells
(Bomben & Sontheimer, 2010; Bomben, Turner, Barclay, & Sontheimer, 2011), but there are
very few studies correlating TRPC activity with astrocyte function in vitro and in vivo.
In this study we wanted to identify Trpc expression in cortical astrocytes and to characterize the role of TRPCs for astrocyte function in vitro and, in a defined stab wound injury model, in vivo. With very few exceptions no specific agonists or antagonists are available for TRPC channels which would allow for specifically isolating TRPC currents and Ca\(^{2+}\) signals. Therefore, we made use of genetically modified mice with Trpc1 and Trpc3 deficiencies and mice harboring a heterozygous gain-of-function mutation within Trpc3, the moonwalker (mwk) mice (Becker, 2014; Becker et al., 2009). For in vitro studies cortical astrocytes from these mice were cultured to compare TRPC-dependent Ca\(^{2+}\) signaling. To prepare RNA to perform PCR and next generation sequencing we used pure GLAST-positive astrocytes sorted by FACS to avoid contribution of other cell types. We show, that i) TRPC3 and TRPC1 are present in cortical astrocytes, that ii) TRPC3 promotes astrocyte proliferation and migration in vitro as well as iii) astrogliosis and cortical edema after cortical stab wound injury in mice in vivo, whereas iv) TRPC1 significantly mitigates these TRPC3 activities.

**Materials and Methods**

**Mice**

Trpc1\(^{-/-}\) (Dietrich et al., 2007), Trpc3\(^{-/-}\) (Hartmann et al., 2008), Trpc6\(^{-/-}\) (Tsvilovskyy et al., 2009) and Trpc3\(^{-/-}\)/Trpc6\(^{-/-}\) (Quick et al., 2012) as well as heterozygous TRPC3\(_{T635A}\) mutant moonwalker (mwk) mice (Becker et al., 2009) were kept in a specific pathogen-free animal facility and bred to a C57BL6/N or 129SvJ/C57Bl6/N background. Trpc1\(^{-/-}\)/Trpc5\(^{-/-}\) and Trpc3\(^{-/-}\)/Trpc6\(^{-/-}\) mice were generated by crossing the single gene-deficient strains. The heterozygous TRPC3\(_{T635A}\) mutant mwk mouse was purchased from Harwell Science and Innovation (Harwell, Oxfordshire, UK) and embryo-transferred into our mouse facility. All animal experiments were performed in accordance with the German legislation on the protection of animals and were approved by the responsible local ethics committee (AZ C1-2.4.2.2/11-2015).

**Preparation and culture of mouse cortical astrocytes**

Newborn (0–3 days) mice were decapitated. The cerebral cortices were isolated in ice cold PBS and the meninges were removed. The tissues were incubated with trypsin (5 g/l, Sigma) for 15 min at 37°C and digestion was stopped by adding Dulbecco’s Modified Eagle Medium (DMEM, Fisher Scientific) supplemented with 10% fetal calf serum (FCS, Fisher Scientific). The cortices were then triturated by pipetting up and down (10 times) using a 20G needle. The obtained suspension was filtered through a 40 μm cell strainer (BD Falcon) and centrifuged for 5 min at 200 g. Cells were re-suspended in DMEM containing 10% FCS, 1% GlutaMAX, 100 U/ml Penicillin and 100 μg/ml Streptomycin, plated in 75 cm\(^{2}\) poly-L-lysine (PLL, 0.1 mg/ml, Sigma) -coated flasks (BD Falcon; 3 brains per flask) and cultured at 37°C and 5% CO\(_{2}\) in a humidified incubator. After 2 to 3 days, the medium was changed to remove dead cells and debris. Afterwards the medium was changed twice a week until the cells reached confluency. For experiments microglial cells were removed by shaking, and astrocytes were trypsinized and subcultured either in 96-well plates for proliferation assays, 6-well plates for migration and proliferation assays or on PLL-coated glass coverslips for Ca\(^{2+}\) imaging or patch clamp experiments.
**Cell lines and transfection**

HEK-293 cells (ATCC, CRL 1573) were obtained from the American Type Culture Collection (Manassas, VA, USA). HEK cells stably expressing the human \textit{Trpc3} cDNA (Zhu, Jiang, & Birnbaumer, 1998) were kindly provided by Dr. M.X. Zhu (University of Texas Health Science Center, Houston, US). HEK cells stably expressing the mouse \textit{Trpc1} cDNA (accession no. NM-011643.3) or the tetracycline-inducible mouse moonwalker \textit{Trpc3\textsubscript{T635A}} cDNA were generated as described (Beck et al., 2013). For the latter, nucleotide A at position 1903 was replaced by G in the mouse \textit{Trpc3} cDNA (accession no. NM_019510) and the cDNA was subcloned into the pCDNA5/FRT/TO vector (Invitrogen). Cells were induced with 1 μg/ml tetracycline 2 h before the experiments, resulting in a strong expression of the mwk TRPC3. Wild-type HEK cells were cultured in Minimal Essential Medium (MEM, Fisher Scientific) and all other HEK cell lines in DMEM, supplemented with 10% FCS. Cells were kept in an incubator at 37°C and 5% CO\textsubscript{2}, and the medium was changed twice a week.

For Ca\textsuperscript{2+} imaging and patch clamp experiments cells were plated on glass coverslips coated with PLL. For transfection, HEK cells were cultured in 3.5 mm petri dishes until reaching 70% confluency and medium was renewed prior to transfection. The transfection mixture was prepared following the manufacturer’s recommendation using FuGENE HD Transfection Reagent (Promega). For every 3.5 mm petri dish, 2 μg of cDNA was mixed with 5 μl of FuGENE HD reagent and 100 μl of Opti-MEM medium (Thermo Scientific) and incubated at room temperature for 15 min. The transfection mixture was added onto the cells and incubated at 37°C and 5% CO\textsubscript{2} for 48 h. Then, cells were plated on PLL-coated glass coverslips and used for experiments for the next 24 h.

**Co-immunoprecipitation and western blot**

Co-immunoprecipitation, SDS-Page and western blots were performed as described previously (Zimmermann et al., 2014) using antibodies for mouse TRPC3 (ab 45/47 and ab 1378), mouse TRPC1 (F2-E4) and mouse Cav\textsubscript{3} (ab 828), all generated in-house. To quantify the expression of glial fibrillary acidic protein (GFAP) after stab wound injury (see below), we analyzed the injured cortical hemispheres from fixed brain slices of wild-type and \textit{Trpc3}\textsuperscript{−/−} mice. Total protein was extracted from the fixed brain slices using Qproteome FFPE Tissue Kit (Qiagene) according to the manufacturer’s guidelines and western blots were performed using antibodies for GFAP (rabbit anti-GFAP, 1:5000, SYnaptic SYstems) and α-tubulin (mouse anti-α-tubulin, 1:200, Santa Cruz Biotechnology). Proteins were detected with horse-radish peroxidase-coupled secondary antibodies and Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Original scans were saved as TIFF files from LAS 3000 (Fujifilm), which were further processed in Adobe Photoshop or Corel Draw. Images were cropped, resized proportionally, and brought to the resolution required for publication. To quantify GFAP, the background signals of the western blots were subtracted and the intensity of the GFAP staining was normalized to the corresponding intensity of the α-tubulin staining.
Preparative fluorescence-activated cell sorting (FACS)

2 to 3 weeks after preparation cortical cultures were trypsinized and suspended into culture medium, washed with phosphate-buffered saline (PBS) and re-suspended in 0.5% BSA in PBS buffer. Cells were incubated at 4°C for 10 min with anti-GLAST-PE (1:10, Miltenyi Biotec) and anti-CD11b-FITC (1:10, Miltenyi Biotec) in 0.5% BSA in PBS, and afterwards washed with 0.5% (w/v) bovine serum albumin (BSA, Sigma) in PBS, and loaded directly into the FACS (MoFlo, Beckman Coulter). 100 GLAST-positive CD11b-negative astrocytes or 50 GLAST-negative CD11b-positive microglial cells were sorted per RNase-free PCR tube. For RNA sequencing and microarray transcriptome analysis all cells from a culture flask were sorted in one vial. The tubes were immediately placed into liquid nitrogen and transferred to a −80°C freezer until further use.

RT-PCR

One-step RT-PCR was performed on 100 sorted GLAST-positive astrocytes and 50 sorted CD11b-positive microglial cells using SuperScript™ One-Step RT-PCR with Platinum Taq (Invitrogen). Each 25 μl reaction contained 0.5 μl sorted cells, 12.5 μl 2x Reaction Mix (0.4 mM dNTP, 24 mM MgSO₄), 0.5 μl RT/Platinum Taq DNA Polymerase, 0.75 μl forward primer (10 pmol/μl), 0.75 μl reverse primer (10 pmol/μl) and 10 μl sterile deionized H₂O. Cycle protocol: 30 min 50°C, 2 min 94°C, 60 cycles of 15 s 94°C + 15 s 62°C + 20 s (from the 16th cycle the time was increased by 2 s per cycle) 70°C, 5 min 72°C, 4°C.

Full length Trpc1 and Trpc3 cDNAs were amplified from sorted astrocytes as follows: Total RNA was extracted from GLAST-positive sorted astrocytes using RNeasy Mini Kit (Qiagen) and first strand cDNA was synthetized using the Maxima first strand cDNA synthesis kit (Thermo Scientific). The PCR was performed using KOD Xtreme™ Hot Start DNA Polymerase (Merck Millipore). Every reaction contained 25 μl of PCR mix (3 μl cDNA obtained from reverse transcription of 734.51 ng of RNA), 12.5 μl 2x Xtreme™ Buffer, 5 μl dNTPs (0.4 mM), 2 × 0.75 μl primers (10 pmol/μl each), 2.5 μl H₂O and 0.5 μl KOD-Xtreme™ polymerase (1 U/μl). Cycle protocol: 2 min 94°C, 40 cycles of 10 s 98°C + 30 s 62°C + 3 min 68°C, 5 min 68°C, 4°C. The amplified cDNAs were subcloned into pUC19 and sequenced on both strands. Table 1 shows the primers used for PCR.

Next generation sequencing

RNA probes from three independent astrocyte cultures were sequenced (paired-end, 75 bp) using an Illumina HiSeq sequencing system (Illumina Inc., San Diego, CA, US). Raw data were mapped against the mouse genome NCBI/build37.2 using TopHat (Trapnell, Pachter, & Salzberg, 2009). For the detection of differentially expressed genes and transcripts we followed the protocol of Trapnell et al. (Trapnell et al., 2012) closely using the tool Cuffdiff.

Oligonucleotide microarray analysis

The mRNA of probes from three independent astrocyte cultures each isolated from wild-type and Trpc3+/− mice were analyzed by Agilent. In brief, the raw intensity values were extracted from the image file using feature extraction software (Agilent Technologies). We used the gMedianSignal values as expression values for the probes and summarized the
probes belonging to the same transcript by taking the median of these values. We applied quantile normalization before using the expression values in downstream analysis.

**Calcium Imaging**

Astrocytes and HEK cells plated on PLL-coated glass coverslips were loaded with 5 μM Fura-2-AM in medium in the dark at 37°C for 30 to 40 min, then washed with bath solution (in mM: 140 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose, pH adjusted to 7.2 with NaOH). For nominal Ca²⁺-free solution CaCl₂ was replaced by MgCl₂. Measurements were performed on a monochromator (Polychrome V, TILL-Photonics) -equipped inverted microscope (Axiovert S100, Zeiss) using a 20x Fluar objective (Zeiss). Every 2 s Fura-2 was alternately excited at 340 nm and 380 nm for 30 ms each and the emitted fluorescence (F340 and F380, >510 nm) were recorded with a cooled charge-coupled device (CCD) camera (TILL Imago, TILL-Photonics, Germany). Ratio images were calculated from F340 and F380 pictures after background correction i.e. subtraction of the fluorescence intensity at 340 and 380 nm excitation from a cell-free area. Single cells were marked as regions of interest and F340/F380 was plotted versus time. Monochromator, camera, acquisition and analysis were controlled by TILLvisION software (TILL-Photonics).

**Electrophysiological recordings**

Membrane currents were recorded in the tight seal whole-cell patch clamp configuration using an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). Patch pipettes were pulled from glass capillaries GB150T-8P (Science Products, Hofheim, Germany) at a vertical Puller (PC-10, Narishige, Tokyo, Japan) and had resistances between 2 and 4 MΩ when filled with standard internal solution (in mM: 140 Cs-methanesulfonate, 8 NaCl, 1 MgCl₂, 10 HEPES, 10 Cs-BAPTA, 3.1 CaCl₂ (100 nM free Ca²⁺, calculated with WebMaxC http://www.stanford.edu/~cpatton/webmaxcS.htm) pH adjusted to 7.2 with CsOH. For Ca²⁺-free intracellular solution CaCl₂ was omitted. The compounds used for external application were diluted into the extracellular solution (in mM: 140 NaCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, pH adjusted to 7.2 with NaOH) to achieve the final concentration as indicated. For nominal Ca²⁺-free solution CaCl₂ was replaced by MgCl₂. All modified solutions were directly applied onto the patch-clamped cell via an air pressure-driven (MPCU, Lorenz Meßgerätebau, Katlenburg-Lindau, Germany) application pipette. Osmolality of all solutions ranged from 290 to 310 mOsm. Voltage ramps of 400 ms duration spanning a voltage range from −100 mV to +100 mV were applied every 2 s from a holding potential (Vₜₚ) of 0 mV using the PatchMaster software (HEKA). All voltages were corrected for a 10 mV liquid junction potential. Currents were filtered at 2.9 kHz and digitized at 400 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. Inward and outward currents were extracted from each individual ramp current recording by measuring the current amplitudes at −80 and +80 mV, respectively, and plotted versus time. Basal currents before an application were subtracted to get the net developing current. Current-voltage (IV) relationships were extracted at indicated time points. Currents were normalized to the initial size i.e. capacitance of the cell to obtain current densities (pA/pF).
Expansion/Migration Assay
The expansion and migration of astrocytes was studied using a scratch assay in which expansion and migration of cells was assessed by the rate of recovery of a defined scratched area on a confluent cell layer. Cells were plated in PLL-coated 6-well plates scratched from the outside bottom as markers to find the exact same regions for analysis again. Once cells reached confluency, two scratches were performed in every well (vertical to the outside bottom scratch) using a 200 μl pipette tip. Wells were washed with PBS to remove the detached cells and incubated at 37°C 5% CO₂ without FCS to inhibit proliferation. At 0, 4, 8 and 24 hours four pictures per well were taken at the crossing between the cellular scratch and the outside bottom marker scratch, using a phase-contrast light microscope (Axiovert 40C, Zeiss) equipped with a 10x A-Plan objective (Zeiss) and a camera (AxioCam, Zeiss). Expansion and migration were quantified as the average of the cell-free scratch area reoccupied by the recruited cells using Image J software (NIH). All measured areas were normalized to the “0 hour” area (100% cell-free scratch area).

Proliferation Assay
Astrocytes proliferation was quantified using the colorimetric MTS cell proliferation assay (Promega). 5000 cells per well were plated in a 96-well plate in triplicate and incubated at 37°C with medium containing 10% FCS. The amount of viable cells was determined after incubating the desired wells with 20 μl of MTS for 1 hour at 37°C by measuring the absorbance of the MTS metabolite formazan at 490 nm using a 96-well plate reader (infinite M200 TECAN). Measurements were performed at day 0 (right after plating), 3, 5, 6 and 7. The absorbance of the medium was subtracted as background.

The proliferative activity of astrocytes after injury was studied by performing a scratch assay (see above). Cells were plated on PLL-coated glass coverslips in 24-well plates and after reaching confluency, scratches were made using a 200 μl pipette tip. Coverslips were washed with PBS to remove the detached cells and incubated at 37°C 5% CO₂ in DMEM with 10% FCS. After 24 h cells were fixed and incubated with 4′6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) and rabbit anti-Ki-67 antibody (1:200, Thermo Fisher Scientific), a cellular marker for proliferation. The anti-Ki-67 antibody was stained by a donkey anti-rabbit Alexa 555 antibody (1:500, Invitrogen). Images were taken at a fluorescent microscope (Zeiss Axio Scan.Z1) equipped with the appropriate filter sets. The Ki-67-positive cells were counted in a defined area around the scratch and normalized to the total number of cells.

Cortical stab wound and immunohistochemistry
8 and 11 week-old male Tpc3<sup>−/−</sup> and Tpc1<sup>−/−</sup>/Tpc5<sup>−/−</sup> mice, respectively, and their corresponding wild-type mice, were anaesthetized using ketamine (87 mg/kg body weight) and xylazine (13 mg/kg body weight) via intraperitoneal injection and placed on a stereotactic instrument. A 1 cm long incision in the skin was made and, using an electric drill, the skull was thinned (3 to 4 mm in length) in the right hemisphere region located 2 mm from the sagittal suture and 4 mm from the olfactory lobe, followed by an insertion with a 3 mm wide scalpel 2 mm deep into the cortex, controlled by a stereotactic arm. After injury the skin was sutured and animals received a subcutaneous buprenorphine injection (0.05 mg/kg body weight). Mice were kept and monitored for three days and then
anaesthetized (see above) and sacrificed by perfusion with 4% paraformaldehyde (PFA). Brains were incubated overnight in 4% PFA, washed with PBS, trimmed with a scalpel and mounted caudal side-down on a vibratome (Microm HM 650V, Thermo Scientific) stage using tissue glue (Loctite Super Glue). 35 μm thick coronal sections were made in the wound region and four representative sections were taken from each brain for immunostaining. Slices were permeabilized in 3% (v/v) TritonX (Roth) / 5% (v/v) donkey serum in PBS and incubated with anti-GFAP antibody (from rabbit, 1:1000, DAKO). Donkey anti-rabbit Alexa 555 antibody (1:1000, Invitrogen) was used to visualize the primary anti-GFAP antibody and nuclei were stained with DAPI. Slices were mounted on glass slides with mounting medium (Immu-Mount, Thermo Scientific) and pictures were taken as mosaic images with 10% overlap using a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a 20x Plan-Apochromat (Zeiss) and a color camera (AxioCam, Zeiss), using the AxioVision Rel. 4.7 software. Images were analyzed with AxioVision by measuring three parameters: the area of reactive astrocytes (GFAP-stained cells), cortex edema as the increase in cortex thickness, and distribution of reactive astrocytes measured from the fluorescence intensity of the GFAP staining in defined squares along the cortex normalized to the mean fluorescence in the non-injured side (see Figure 7E).

**Statistical Analysis**

Data were analyzed using TILLvisION (Till Photonics), PatchMaster or Fitmaster (HEKA), Microsoft Excel, Igor Pro 5.1 (WaveMetrics), AxioVision Rel. 4.7 (Zeiss) and ImageJ (NIH). Data are shown as mean ± SEM with n indicating the number of patch-clamped cells, cells measured from × Ca^{2+} imaging experiments (n/x), scratches (migration) or wells (proliferation) measured in × independent cultures (n/x), or brain slices analyzed from × mice (n/x). To estimate the significance of differences, Student’s t-test for two groups and one-way ANOVA followed by Bonferroni test for more than two groups were performed. The differences were assigned as significant if the P-value was < 0.05 (*), <0.01 (**) or < 0.001 (***)

**Results**

**TRPC transcript expression in cultured mouse cortical astrocytes**

Astrocytes and microglia were sorted by FACS (Supplementary Figure 1) into PCR tubes from cortical cell cultures prepared from 0–3 days old mice two to three weeks after isolation. TRPC transcripts were amplified by RT-PCR using 100 GLAST-positive astrocytes and 50 CD11b-positive microglial cells per tube. Consistently, *Trpc3* expression was identified in thirteen independent reactions using astrocytes from five independent sortings; *Trpc1* and *Trpc2* were identified in 10 reactions, *Trpc4* in 11 of 13 reactions, whereas *Trpc5, Trpc6* and *Trpc7* were not detectable (Figure 1A). In contrast, microglial cells did express *Trpc4* in three PCRs from three independent sortings, whereas *Trpc1, Trpc3, Trpc5, Trpc6* and *Trpc7* were not detectable (Figure 1B). The clear difference between the *Trpc* expression profile from sorted astrocytes and microglial cells shows the accuracy of the sorting. All *Trpc* transcripts were detectable using mouse brain RNA (Figure 1C) as control. The expression of *Trpc3 and Trpc1* transcripts was confirmed by RNA sequencing (Supplementary Figure 2). Similar expression levels as for *Trpc3 and Trpc1* were
identified for transcripts of NMDA receptors (Grin1, Grin2d and Grin3a), metabotropic glutamate receptors (Grm5) or IP3 receptors (Itprl and Itpr2; Supplementary Figure 2), known to be important for astrocyte function. The TRPC3 and TRPC1 proteins are present in mouse cortex (Figure 1D, E) and ultimate proof of Tprc3 and Tprcl expression in astrocytes was obtained by amplifying the full-length cDNAs from the sorted cells (Figure 1F, G).

**OAG-mediated cytosolic Ca2+ signals in cortical astrocytes depend on TRPC3**

To study whether TRPC3 and TRPC1 are involved in Ca2+ imaging experiments in cultured astrocytes from wild-type mice and mice lacking the Tprc3 and Tprcl genes. Intracellular Ca2+ stores were depleted in the absence of extracellular Ca2+ by thapsigargin (TG) or cyclopiazonic acid (CPA) followed by addition of extracellular Ca2+, but no significant contribution of TRPC3 (and TRPC6, Figure 2A) or TRPC1 (Figure 2B) was observed. TRPC3 has been shown to be activated by 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Hofmann et al., 1999), an analogue of diacylglycerol (DAG). In the presence of external Ca2+ bath application of 100 μM OAG induced Ca2+ oscillations in astrocytes (Figure 3A), but not in microglial cells (Figure 3C). Some OAG-mediated Ca2+ oscillations occurred in the absence of extracellular Ca2+ (Figure 3B), indicating that Ca2+ release from intracellular stores in addition to Ca2+ influx is affected by OAG. To separate Ca2+ influx from Ca2+ release the intracellular Ca2+ stores were depleted by 10 μM CPA in the presence of extracellular Ca2+ and OAG was applied on top. As shown in Figure 3D 100 μM OAG still induced significant Ca2+ oscillations which under these conditions depend on Ca2+ influx. Among Tprc3+/− astrocytes, less cells responded to OAG and those cells which responded showed less peaks compared to wild-type cells (Figure 3E, G, H, I).

In some experiments we used astrocytes from Tprc6−/− and Tprc3+/−/Tprc6−/− mice. Since Tprc6 is not detectable in the astrocytes (Figure 1A and Supplementary Figure 2), astrocytes from Tprc6−/− mice showed a very similar response as wild-type astrocytes, whereas OAG-induced Ca2+ influx is not significantly different in cells from Tprc3+/−/Tprc6−/− and Tprc3−/− mice (Figure 3G, H, I). In contrast, the numbers of OAG-responding cells and of Ca2+ peaks per responding cell were higher among Tprc1−/− astrocytes compared to wild-type astrocytes (Figure 3F, G, H, I). These data indicate, that the OAG-mediated Ca2+ oscillations in astrocytes depend on TRPC3-mediated Ca2+ influx. Compared to wild-type, Tprc3-deficiency reduced and Tprcl-deficiency increased Ca2+ influx.

**Functional interaction of TRPC3 and TRPC1**

In the absence of Tprcl TRPC3 activity is increased (Figure 3F, G, H, I) and the apparent interaction of TRPC3 and TRPC1 was confirmed by co-immunoprecipitation of TRPC1 and TRPC3 by antibodies for both proteins (Figure 4A). Next the conditions to record TRPC3 currents in HEK cells by whole-cell patch clamp were optimized and a maximal OAG-induced TRPC3 current amplitude was obtained in the absence of extra- and intracellular Ca2+ (Figure 4B, C). However, under this condition the success rate of measuring OAG-mediated currents in astrocytes isolated from wild-type mice was very low, and only two cells out of more than 200 revealed a TRPC3-like current (Supplementary Figure 3). The following patch clamp experiments were therefore performed in the absence of intra- and
extracellular Ca$^{2+}$ in HEK-293 cells. The amplitude of OAG-mediated TRPC3 currents were significantly reduced after co-expression of $Tpc1$ (Figure 4D, E, F). Non-transfected HEK cells (control) and HEK cells only expressing $Tpc1$ did not reveal any current upon OAG application (Figure 4D, E, F).

Moonwalker mice (mwk) harbor the TRPC3 T635A gain-of-function mutation. In astrocytes isolated from heterozygous mwk mice we detected a significantly higher basal Ca$^{2+}$ level in the presence of extracellular Ca$^{2+}$ (Figure 5A, C) and a significantly higher Ca$^{2+}$ influx upon addition of external Ca$^{2+}$ (Figure 5B, C) compared to astrocytes isolated from wild-type littermates. These data confirm that Ca$^{2+}$ influx in astrocytes depends on TRPC3. To study the interaction with TRPC1 we generated a tetracycline-inducible TRPC3$_{T635A}$ HEK cell line. The spontaneous currents recorded two hours after induction were significantly reduced in the presence of $Tpc1$ (Figure 5D, E, F, G). Non-induced (control) cells did not reveal a spontaneous current (Figure 5D, E, F, G). The latter results from patch clamp experiments were confirmed by Fura-2 Ca$^{2+}$ imaging. Here the Ca$^{2+}$ influx upon addition of extracellular Ca$^{2+}$ and the basal Ca$^{2+}$ level in the presence of extracellular Ca$^{2+}$ were significantly higher in induced TRPC3$_{T635A}$ cells compared to these cells co-expressing $Tpc1$ or cells which were not induced (control) by tetracycline (Figure 5H, I).

**TRPC3 and TRPC1 are involved in expansion and migration of astrocytes in vitro**

As shown in vivo, astrocytes rather proliferate and grow towards a lesion than actively migrate into the injury site (Bardehle et al., 2013). However, after performing a scratch in a confluent astrocyte layer in vitro astrocytes recover the cell-free area by proliferation, growth and migration (Zhan et al., 2016). The TRPC3-induced Ca$^{2+}$ signals might translate into changes in proliferation, growth and migration of astrocytes. Astrocytes isolated from $Tpc3^{-/-}$ mice proliferated significantly slower compared to their corresponding wild-type cells, whereas astrocytes from heterozygous mwk and $Tpc1^{-/-}$ mice revealed a similar proliferation rate as wild-type controls (Supplementary Figure 4A). Supplementary figure 5 shows that astrocytes expand, migrate and recover the cell-free area from the boundary of the scratch and isolated cells invading the cell-free area are primarily microglial cells. Astrocytes lacking $Tpc3$ or both $Tpc3$ and $Tpc6$ recovered a cell-free scratch area significantly slower than their corresponding wild-type cells, whereas astrocytes lacking only $Tpc6$ responded, as expected, like wild-type, and astrocytes from heterozygous mwk and $Tpc1^{-/-}$ mice filled the scratched area of the cell monolayer significantly faster than the corresponding wild-type cells (Figure 6A, B). Accordingly, expansion and migration of astrocytes in vitro depends on TRPC3 and TRPC1. To study the proliferative activity of wild-type and $Tpc3^{-/-}$ astrocytes after injury, we performed the scratch assay in the presence of 10% FCS and quantified the percentage of proliferating cells using the proliferation marker anti-Ki-67. 24 hours after performing the scratch wild-type astrocytes revealed a significantly higher percentage of Ki-67-positive cells compared to $Tpc3^{-/-}$ astrocytes (Supplementary Figure 4B, C).

**TRPC3 promotes astrogliosis and cortical edema in mice after cortical stab wound injury**

To monitor astrogliosis as a measure of astrocyte function in vivo, defined cortical stab wounds were applied to $Tpc3^{-/-}$ and wild-type mice. Three days after lesion, the injured
areas were analyzed by fluorescence microscopy (see Figure 7A and E). The level of astrocyte activation was determined by immunostaining for GFAP (Figure 7A, C, E, F). Cortical edema were quantified in respect to the contralateral side of the injury (Figure 7A, E, G). To measure the distribution of GFAP-positive staining we defined squares along the cortex, determined the fluorescence intensity and normalized it to the fluorescence intensity of the non-injured hemisphere (Figure 7C, D, E). Brain slices from mice lacking Tpc3 revealed significantly less edema and less GFAP-staining, i.e. less astrogliosis compared to wild-type mice (Figure 7A, C, F, G). To prove the results from GFAP staining, we quantified the amount of GFAP protein in injured hemispheres from the brain slices of wild-type and Tpc3−/− mice by western blot analysis: the injured hemisphere in Tpc3−/− mice revealed significantly less GFAP protein as compared to wild-type mice (Figure 7H, I). As already observed for the OAG-mediated cytosolic Ca^{2+} oscillations in isolated astrocytes (Figure 3) and in vitro migration experiments (Figure 6), the lack of Tpc3 resulted in the opposite effects as the lack of Tpc1: The area of reactive astrocytes (Figure 7B, D, F) and of the cortical edema (Figure B, G) are significantly increased in mice lacking Tpc3 compared to the corresponding wild-type mice.

**Discussion**

Upon brain injury, signaling molecules stimulate astrocyte proliferation, growth and their recruitment to the injured tissue (Burda & Sofroniew, 2014). These processes significantly depend on changes of the intracellular Ca^{2+} concentration (Gao et al., 2013; Kanemaru et al., 2013). In astrocytes signaling molecules as diverse as ATP, glutamate, thrombin and endothelin stimulate phospholipase C-coupled pathways resulting in IP3-mediated Ca^{2+} release and mobilization of DAG. Among the cellular targets of DAG and its analogue OAG is the Ca^{2+}-permeable TRPC3 channel. In this study we show that OAG-induced Ca^{2+} oscillations depend on the presence of TRPC3 in cultured astrocytes, that TRPC3 promotes astrogliosis and cortical edema in vivo, and these TRPC3 activities are attenuated by TRPC1, both in vitro and in vivo.

TRPC3 and TRPC1 have been suggested to be present in diverse astrocytes and astrocytoma cells. OAG- and thrombin-mediated Ca^{2+} oscillations as well as ATP-, substance P- and bradykinin-induced Ca^{2+} signals in cultured rat cortical and spinal cord astrocytes, in 1321N1 human astrocytoma cells and in primary mouse striatal and cultured cortical astrocytes were suggested to depend on TRPCs (Akita & Okada, 2011; Grimaldi et al., 2003; Miyano et al., 2010; Nakao et al., 2008; Shirakawa et al., 2010; Streifel et al., 2014; Streifel et al., 2013) which presumably are involved in Na^+ and Ca^{2+} influx including store-operated Ca^{2+} entry in these cells (Golovina, 2005; Pizzo, Burgo, Pozzan, & Fasolato, 2001; Reyes et al., 2013). Using the appropriate Tpc-deficient mice as controls we could show that neither TRPC3 nor TRPC1 significantly contribute to Ca^{2+} entry after store-depletion in cultured cortical astrocytes (Figure 2).

The sparse data on TRPC3 function in astrocytes (see above) essentially rely on the use of the compound Pyrazolium 3 (Pyr3), which was first published as a specific TRPC3 antagonist (Kiyonaka et al., 2009). Munakata and colleagues (Munakata et al., 2013) showed that Pyr3 significantly reduced astrogliosis and brain edema in a mouse model of...
intracerebral hemorrhage and they concluded that this effect was mediated by blocking TRPC3 function. Meanwhile, it has been shown that Pyr3 inhibits store-operated Orai1 channels with a similar potency as TRPC3 (Schleifer et al., 2012). Considering the expression levels of orai1, stim1 and stim2 in astrocytes (Supplementary Figure 2) the effects of Pyr3 on astrogliosis upon intracerebral hemorrhage might have been mediated by Orai/STIM channels.

Whereas Peters et al. (Peters et al., 2012) observed no significant change in the spontaneous recovery after closed head injury in Tipc1- or Tipc3-deficient mice compared with wild-type mice, Shirakawa et al. (Shirakawa et al., 2010) described a contribution of TRPC3 to pathological activation of astrocytes after intracortical injection of thrombin, and Nakao et al. (Nakao et al., 2008) suggested that thrombin-induced Ca\(^{2+}\) oscillations in 1321N1 human astrocytoma cells depend on Ca\(^{2+}\) release from intracellular stores, and that Ca\(^{2+}\) influx via TRPC3, itself not visible, refills the stores to sustain the oscillatory activity. However, in the present study the OAG-mediated TRPC3-dependent Ca\(^{2+}\) oscillations occur although the intracellular Ca\(^{2+}\) stores remain depleted by the continuous presence of CPA. These OAG-induced Ca\(^{2+}\) oscillations are increased in the absence of TRPC1.

TRPC1 and TRPC3 may form heterotetrameric TRPC1/TRPC3 channels (Cheung et al., 2011; Lintschinger et al., 2000; Liu, Bandyopadhyay, Singh, Groschner, & Ambudkar, 2005; Schaefer, 2005; Storch, Forst, Philipp, Gudermann, & Mederos y Schnitzler, 2012; Wang, Wang, & Li, 2016; Woo, Lee, Huang, Cho, & Lee, 2014; Wu, Zagranichnaya, Gurda, Eves, & Villereal, 2004). Heterotetrameric TRPC1/TRPC4 and TRPC1/TRPC5 channels reveal altered current-voltage relations and cation selectivity compared to homomeric TRPC4 and TRPC5 channels (Storch et al., 2012; Strubing, Krapivinsky, Krapivinsky, & Clapham, 2001), but although TRPC1/TRPC3 currents had reduced current amplitudes compared to TRPC3 currents their current-voltage relations and cation selectivity were apparently not different (Figure 4). So far, we did not address the mechanism of TRPC1-dependent inhibition of TRPC3 in astrocytes, but TRPC1 might be an inhibitory ion conducting subunit of heterotetrameric TRPC1/TRPC3 channels, act as an auxiliary inhibitory and non-conducting beta subunit for TRPC3 channels or inhibit the plasma membrane targeting of TRPC3. TRPC1 does significantly mitigate constitutive mwk-induced Ca\(^{2+}\) entry; apparently it does not discriminate between wild-type TRPC3 and its gain-of-function moonwalker mutation.

Our observation that lack of Tipc3 reduced astrocyte proliferation and migration, whereas the TRPC3 moonwalker mutant and the lack of Tipc1 increased migration are compatible with TRPC3 activity promoting proliferation and migration of cortical astrocytes. Table 2 lists several genes which are involved in proliferation and migration in diverse other cell types and which are significantly downregulated in Tipc3-deficient astrocytes compared with the same genes in astrocytes from wild-type mice. The microarray analysis on independent mRNA extracted from sorted wild-type and Tipc3-deficient astrocytes was performed in biological triplicates and revealed no significant change of expression of other Tipc genes or genes of other Ca\(^{2+}\)-permeable ion channels.
Astrocytes in culture mostly reveal a prominent expression of GFAP (see e.g. Supplement Figure 1A), whereas astrocytes in the intact brain usually exhibit low GFAP expression (see e.g. Figure 7A - non-injured hemisphere). Thus, astrocytes in vitro seem to be in a more reactive state compared to astrocytes in vivo, i.e. conditions in vitro and in vivo might be different. In addition, properties and functions of astrocytes in different brain areas and at different ages are also versatile. However, our data on cultured cells isolated from the cortex of newborn mice and the stab wound data on adult mice both suggest significant functions of TRPC3 and TRPC1 in the cortical astrocytes.

In summary, TRPC3-induced $Ca^{2+}$ entry promotes astrocyte proliferation and migration i.e. astrocyte activity in vitro which is attenuated by the presence of TRPC1. Following brain injury, the absence of TRPC3 results in a significant reduction of astrogliosis and cortical edema in vivo, suggesting that a targeted therapy to reduce TRPC3 channel activity, for example by increasing formation of heteromeric TRPC3/TRPC1 channels, might be beneficial in traumatic brain injury.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


Beskina O, Miller A, Mazzocco-Spezzia A, Pulina MV, Golovina VA. Mechanisms of interleukin-1beta-induced Ca2+ signals in mouse cortical astrocytes: roles of store- and receptor-


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Main points

- Cortical astrocytes functionally express Ca\(^{2+}\)-permeable TRPC3 channels.
- TRPC3 promotes astrogliosis and cortical edema following a cortical stab wound injury.
- The decisive contribution of TRPC3 to astrocyte Ca\(^{2+}\) signaling is mitigated by TRPC1.
Figure 1. Cortical astrocytes express Trpc3 and Trpc1

(A–C) RT-PCRs performed from 100 FACS-sorted GLAST-positive astrocytes (A), 50 FACS-sorted CD11b-positive microglial cells (B) and mouse brain RNA (C). The frequencies of amplified Trpc transcripts in astrocytes in 13 independent RT-PCRs from 5 independent sortings and in microglia in 3 independent RT-PCRs from 3 independent sortings are shown below the blots. Hprt served as positive control. First and last lanes in A, B and C show marker DNAs. (D, E) Immunoblot analysis of TRPC3 (D) and TRPC1 (E) proteins in lysates from mouse cerebellum and cortex (100 μg/lane) extracted from wild-type and Trpc3−/− mice or Trpc1−/− mice revealed the loss of TRPC3 and TRPC1 proteins in Trpc3−/− and Trpc1−/− mice, respectively. To confirm equal loading of samples independent blots running in parallel were probed with a Cavβ3 antibody. (F, G) Full-length amplification of Trpc3 (F) and Trpc1 (G) cDNAs from cultured cortical Glast-positive FACS-sorted astrocytes.
Figure 2. Ca$^{2+}$ influx after store depletion does not depend on TRPC3, TRPC6 or TRPC1 in cortical astrocytes

(A, B) Ca$^{2+}$ release induced by 2 μM thapsigargin (TG, A) or 10 μM cyclopiazonic acid (CPA, B) in the absence of extracellular Ca$^{2+}$ and subsequent Ca$^{2+}$ entry upon Ca$^{2+}$ readdition in astrocytes from Trpc3$^{-/-}$/Trpc6$^{-/-}$ (Trpc3/c6 dko, A) and Trpc1$^{-/-}$ mice (Trpc1 ko, B) and their corresponding wild-types (wt), measured as Fura-2 ratio F340/F380. Data represent means ± S.E.M. of n cells from × experiments (n/x).
Figure 3. OAG-mediated Ca^{2+} influx depends on Trpc3.

(A, B) OAG-induced cytoplasmic Ca^{2+} increase in astrocytes in the presence (A) and absence of extracellular Ca^{2+} (B), and upon addition of Ca^{2+} to the bath as indicated (B).

(C) Microglial cells do not reveal a change of cytoplasmic Ca^{2+} upon OAG application. (D–F) OAG-induced Ca^{2+} entry in the presence of extracellular Ca^{2+} (2 mM) at sustained store depletion by cyclopiazonic acid (CPA, 10 μM) present throughout the experiment in astrocytes from wild-type (wt, D), Trpc3^{−/−} (Trpc3 ko, E) and Trpc1^{−/−} (Trpc1 ko, F) mice.

(G, H) Percentage of OAG-responding cells (G) and number of OAG-mediated Ca^{2+} peaks per responding cell (H) in astrocytes from wild-type, Trpc6^{−/−}, Trpc1^{−/−}, Trpc3^{−/−} and Belkacemi et al. Page 21 Glia. Author manuscript; available in PMC 2018 September 01.
$Tpc3^{-/-} / c6^{-/-}$ mice. Data represent means ± S.E.M. (n, number of cells from x, number of experiments (n/x)) with asterisks in G and H marking significant differences compared to the wild-type (** P<0.01, *** P<0.001). (I) P-values for comparisons in G and H were obtained by one-way ANOVA followed by Bonferroni test.
Figure 4. Interaction of TRPC3 and TRPC1

(A) Co-immunoprecipitation of TRPC3 and TRPC1 and vice versa from lysates of HEK cells transfected with the *Trpc1* and *Trpc3* cDNAs. (B) 100 μM OAG-induced TRPC3 inward and outward currents at −80 and +80 mV in the absence and presence of extracellular (ec) Ca$^{2+}$ (1 mM) or intracellular (ic) Ca$^{2+}$ (100 nM), in HEK cells stably expressing human (h) TRPC3. (C) Corresponding current-voltage relationships of currents in B. (D–F) OAG-induced inward and outward currents in HEK wild-type cells and HEK cells stably expressing *Trpc1* without (control and C1) and with transient expression of *Trpc3* cDNA (C3 and C1+C3). Current voltage relationships and differences (* P<0.05) of current amplitudes are shown in E and F, respectively. Inward and outward currents are means ± S.E.M. of n cells.
Figure 5. Constitutive activity of the TRPC3<sub>T635A</sub> moonwalker (mwk) mutant is antagonized by Trpc1 expression

(A, B) Changes of cytoplasmic Ca<sup>2+</sup> (F340/F380) in cultured cortical astrocytes isolated from wild-type (wt) and heterozygous TRPC3<sub>T635A</sub> moonwalker (mwk) mice in the presence (A), absence (B, 0–100 s) and after addition (B, application bar) of extracellular Ca<sup>2+</sup>. (C) Basal cytoplasmic Ca<sup>2+</sup> (F340/F380) in A and ΔF340/F380 upon Ca<sup>2+</sup> readdition in B. (D–G) Spontaneous inward and outward currents at −80 mV and +80 mV in the tetracycline-inducible HEK TRPC3<sub>T635A</sub> (mwk) cell line. Control, no tetracycline induction; mwk, after tetracycline induction; mwk+C1, after tetracycline induction and transfection with the Trpc1 cDNA. Current-voltage relationships at break in (E) and at 150 s (F) and corresponding current amplitudes at −80 mV and +80 mV (G), plotted as means ± S.E.M. (n, number of cells). (H, I) Basal cytoplasmic Ca<sup>2+</sup> and increase of cytoplasmic Ca<sup>2+</sup> upon addition of 2 mM extracellular Ca<sup>2+</sup>; Control, no tetracycline induction; mwk, after tetracycline induction; mwk+C1, after tetracycline induction and transfection with the Trpc1 cDNA. Cytoplasmic Ca<sup>2+</sup> at 40 s and at 400 s (I) plotted as mean ± S.E.M. (n, number of cells from x, number of experiments (n/x)). Asterisks assign significant differences (*P<0.05, **P<0.01, ***P<0.001, ns = not significant).
Figure 6. *In vitro* migration of astrocytes depends on **Trpc3** and **Trpc1**

(A) Representative images of scratched areas in cultured astrocytes isolated from wild-type (wt), **Trpc3**⁻/⁻ (**Trpc3** ko), **Trpc6**⁻/⁻ (**Trpc6** ko), **Trpc3**⁻/⁻/**Trpc6**⁻/⁻ (**Trpc3/c6** dko), **Trpc1**⁻/⁻ (**Trpc1** ko) and heterozygous TRPC3<sub>T635A</sub> mutant (mwk) mice directly (0 h) and 4, 8 and 24 hours after scratching. (B) Data as in A, plotted as percentage of scratch area recovered by migrating astrocytes 4, 8 and 24 hours after scratching. Data in B represent means ± S.E.M. (n, number of experiments from x, number of cultures (n/x)). Asterisks assign the significant differences compared to the corresponding wt at the same hour (* P<0.05, ** P<0.01, *** P<0.001).
Figure 7. Distribution of reactive astrocytes and cortical edema after brain injury in wild-type, Trpc3<sup>−/−</sup> and Trpc1<sup>−/−</sup> mice

(A, B) Fluorescence images of GFAP (red)-stained brain slices after cortical stab wound injury in wild-type (wt, A, B, top) and Trpc3<sup>−/−</sup> (Trpc3 ko, A, bottom) and Trpc1<sup>−/−</sup>/Trpc5<sup>−/−</sup> (Trpc1/c5 dko, B, bottom) mice. Since Trpc5 was not detectable in the cortical astrocytes (Figure 1A and Supplementary Figure 2) we regarded the Trpc1<sup>−/−</sup>/Trpc5<sup>−/−</sup> mice as single Trpc1<sup>−/−</sup> mice in respect of the astrocytes. (C, D) Analysis of the distribution of reactive astrocytes (GFAP-staining) after stab wound injury in Trpc3<sup>−/−</sup> (Trpc3 ko, C) and Trpc1<sup>−/−</sup>/Trpc5<sup>−/−</sup> (Trpc1/c5 dko, D) mice and the corresponding wild-types. The mean

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fluorescence intensity in the squares along the cortex were measured, and normalized to the non-injured hemisphere. The scheme in (E) represents the brain slice and symbolizes the location of the squares analyzed in C and D. (F, G) Summary of the GFAP-stained area as measure for reactive astrocytes (F) and the cortical edema (percentage of increase in the injured cortex thickness, G) after cortical stab wound injury in Trpc3−/− (Trpc3 ko) and Trpc1−/−/Trpc5−/− (Trpc1/c5 dko) mice and in the corresponding wild-types. Data represent means ± S.E.M. of n, number of analyzed brain slices and x, number of stab wound experiments i.e. x, number of mice (n/x). (H, I) Immunoblot of GFAP (top) and α-tubulin (bottom) in lysates from non-injured wild-type brain (lane 1, brain) and from injured hemispheres extracted from the brain slices (see A) of 4 wild-type (lane 2–5) and 4 Trpc3−/− mice (lane 6–9; H). Quantification of intensity of GFAP protein stain normalized to the corresponding α-tubulin intensity from background subtracted blots (I, data represent means ± S.E.M. of 12 lanes from lysates from brain slices of 6 wild-type and 6 Trpc3−/− animals). Significant differences of Trpc3−/− and Trpc1−/− compared to the corresponding wild-types are marked with asterisks (* P<0.05, ** P<0.01, *** P<0.001).
Table 1

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<td><strong>Hprt</strong></td>
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<td><strong>Trpc1 full-length</strong></td>
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<td><strong>Trpc3 full-length</strong></td>
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Table 2

Genes downregulated in astrocytes from *Trpc3<sup>−/−</sup>* mice compared to expression levels in astrocytes from wild-type mice. Calculated from the means of microarray analysis from three independent cultures each.

<table>
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<th>Fold change</th>
<th>Related function</th>
<th>Reference</th>
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<td>Enah</td>
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