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Temperature-dependent calcium-induced calcium release via InsP_3 receptors in olfactory ensheathing glial cells

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Summary

Cooling can induce Ca^{2+} signalling via activation of temperature-sensitive ion channels such as TRPM8, TRPA1 and ryanodine receptor channels. Here we have studied the mechanism of cooling-evoked Ca^{2+} signalling in mouse olfactory ensheathing cells (OECs), a specialized type of glial cells in the olfactory nerve layer of the olfactory bulb. Reducing the temperature from above 30°C to 28°C and below triggered Ca^{2+} transients that persisted in the absence of external Ca^{2+} , but were suppressed after Ca^{2+} store depletion by cyclopiazonic acid. Cooling-evoked Ca^{2+} transients were present in mice deficient of TRPM8 and TRPA1, two cold-activated ion channels, and were not inhibited by ryanodine receptor antagonists. Inhibition of InsP_3 receptors with 2-APB and caffeine entirely blocked cooling-evoked Ca^{2+} transients. Moderate Ca^{2+} increases, as evoked by flash photolysis of NP-EGTA (caged Ca^{2+}) and cyclopiazonic acid, triggered InsP_3 receptor-mediated Ca^{2+} release at 22°C, but not at 31°C. The results suggest that InsP_3 receptors mediate Ca^{2+} -induced Ca^{2+} release in OECs, and that this Ca^{2+} release is temperature-sensitive and can be suppressed at temperatures above 28°C.

1. Introduction

Ca^{2+} -induced Ca^{2+} release (CICR) is a key player in intracellular Ca^{2+} signalling in excitable cells such as muscle cells and neurons, where it contributes to excitation-contraction coupling and synaptic transmission [1,2]. CICR is defined as a Ca^{2+} release event from Ca^{2+} stores (endoplasmic reticulum) as a consequence of an initial, often moderate cytosolic Ca^{2+} elevation, caused by, for example, Ca^{2+} influx through voltage- or ligand-gated ion channels. In skeletal muscle cells, where CICR has first been shown, ryanodine receptors (RyR) mediate CICR [3-5], and RyR has been attributed to CICR in most other studies. In addition, activation of inositol 1,4,5-trisphosphate receptors (InsP_3Rs), the second major class of Ca^{2+} release channels in the ER, has been shown to be modulated by Ca^{2+} [1,6-8]. In glial cells, e.g., CICR is mediated by InsP_3Rs , but not RyRs [9,10].

RyRs are not only activated by Ca^{2+} , but have also been demonstrated to be cold-sensitive; a rapid drop in temperature from room temperature to near 0°C induces Ca^{2+} signalling and contraction in muscle cells [11,12]. Cooling can trigger Ca^{2+} signalling also via cold-sensitive transient receptor potential (TRP) channels such as TRPA1 and TRPM8 channels [13-16]. In the present study, we investigated cold-induced Ca^{2+} signalling in glial cells *in situ* of mouse olfactory bulbs. Decreasing the temperature of the bath solution from above 30°C to 28°C or below induced Ca^{2+} transients that were mediated by InsP_3Rs , but not by RyRs, TRPA1 or TRPM8. Furthermore, a Ca^{2+} increase evoked by laser photolysis of NP-EGTA (caged Ca^{2+}) or by inhibiting Ca^{2+} pumps with cyclopiazonic acid triggered InsP_3 -dependent CICR at room temperature, but not at temperatures above 30°C . The results show temperature-dependent CICR through InsP_3Rs and suggest that cooling evokes Ca^{2+} signalling by removing a temperature-dependent inhibition of CICR.

2. Material and methods

2.1 Solutions and reagents

Standard artificial cerebrospinal fluid (ACSF) consisted of (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 25 D-(+)-glucose, 1 MgCl₂, 2 CaCl₂ and 0.5 sodium-L-lactate, continuously gassed with carbogen (95 % O₂, 5 % CO₂) to maintain the pH of 7.4. For Ca²⁺-free solution, 2 mM CaCl₂ was replaced by 2 mM MgCl₂ and 1 mM EGTA was added. In solution containing caffeine, 20 mM NaCl was exchanged for 40 mM caffeine. Physiological saline used in Ca²⁺ imaging experiments with cultured cells contained (in mM): 145 NaCl, 5 KCl, 10 Glucose, 1 MgCl₂, 2 CaCl₂, 10 HEPES; pH adjusted to 7.4 with NaOH. EGTA, caffeine, menthol and ATP were obtained from Sigma-Aldrich (Taufkirchen, Germany). Cyclopiazonic acid (CPA) and dantrolene were purchased from Enzo Life Sciences (Loerrach, Germany). 3,5-Bis(trifluoromethyl)pyrazole derivative (BTP2) and 2-aminoethoxydiphenylborate (2-APB) were obtained from Calbiochem (Merck, Darmstadt, Germany). Icilin, 4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazine-carboxamide (BCTC), 4-(4-Chlorophenyl)-3-methyl-3-buten-2-one oxime (AP-18) and ryanodine were received from Biotrend Chemicals (Cologne, Germany). All reagents were stored as stock solutions corresponding to the manufacturers instructions and added to ACSF directly before the experiment. OECs are located in the nerve layer, the most superficial layer of the olfactory bulb, and hence are readily accessible for drugs applied with the perfusion system.

2.2 Animals and olfactory bulb *in-toto* preparation

NMRI and C57BL/6 mice of both genders (age: postnatal days 0-8; P0-P8) were obtained from the institutional animal facility at the University of Kaiserslautern.

TRPM8^{-/-} [17] and TRPA1^{-/-} [18] mice were reared at the animal facility at the University of Leuven. Animals were decapitated in accordance with the EU animal welfare guidelines. Both olfactory bulbs were dissected carefully from the opened head and transferred into chilled (4°C) ACSF. For recovery, bulbs were stored 45 min in carbogen-gassed ACSF at 30°C and 15 min at room temperature.

2.3 Calcium imaging and flash photolysis of caged compounds

Whole bulb hemispheres were glued with the median side down onto a cover slip and transferred into a recording chamber. For multicell bolus loading [19] a glass pipette with a resistance of ~2-4 mΩ was filled with 200 μM Fluo-4 AM (4 mM stock dissolved in DMSO and 20 % pluronic acid) and 1.6 μM sulforhodamine 101 (both dyes from Molecular Probes, Karlsruhe, Germany), which served as a control dye to visualize the injection. After inserting the pipette into the olfactory nerve layer, the dyes were pressure-injected into the tissue (0.7 bar for 20 s; Pneumatic Drug Ejection System, NPI, Tamm, Germany) followed by incubation for 30 min in ACSF at room temperature (Fig. 1A). Ca²⁺ signals of OECs were detected by the green fluorescence of Fluo-4 within the olfactory nerve layer in epifluorescence illumination (excitation 490 nm; Polychrome IV, TILL Photonics, Graefelfing Germany) or confocal microscopy (excitation 488 nm; Nikon eC1 plus). Images were achieved at an acquisition rate of 0.2 Hz. The temperature of the perfusion saline was controlled using a custom-made heating device with a feedback loop to control and change the bath temperature in the perfusion chamber.

For photolysis of caged compounds, we used the photosensitive Ca²⁺ chelator nitrophenyl-EGTA-AM (NP-EGTA-AM; 630 μM; Molecular Probes, Karlsruhe, Germany) and caged ci-InsP₃/PM (40 μM; Mobitec, Goettingen, Germany), respectively. Experiments were performed on confocal laser scanning microscopes

(Zeiss LSM 700 and Nikon eC1plus). Cell loading of caged compounds together with Fluo-4 AM was realized by MCBL as described above. A small group of cells (3-5 cells) were irradiated by a 405-nm laser for 2 s, resulting in “uncaging” of the caged compounds and hence release of the bioactive compounds. Fluo-4 fluorescence was recorded immediately before and after photolysis, resulting in gap-free time series of Fluo-4 fluorescence.

2.4 Cell Culture

Astrocytic cell cultures from neonatal mice (P0-3) were obtained as previously described [20]. Astrocytes were loaded with Fluo-4 AM (1.5 μ M, 30 min) and imaged at an acquisition rate of 0.2 Hz using the Ca^{2+} imaging system as described above (TILL Photonics).

Purified cultures of OECs and Schwann cells were generated from 7-day old S100 β -DsRed mice [21]. In these mice both OECs and Schwann cells express DsRed fluorescent protein and are easily visualized in culture using fluorescent microscopy. To obtain OECs, the olfactory bulbs were removed from the cranial cavity and the nerve layer from the entire bulb was dissected out. The nerve layer tissue was incubated in plastic 24-well plates coated with Matrigel basement membrane matrix (10 mg/ml; BD Biosciences, San Jose, CA) and maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, G5 supplement (Gibco), gentamicin (Gibco, 50 mg/ml) and L-glutamine (200 μ M) at 37°C with 5% CO_2 for 3-5 days. Contaminating macrophages were removed by incubation with TrypLE Express (Gibco) for 2 min. OECs were incubated with TrypLE Express for a further 4-5 min and then replated in the same medium. These cultures routinely give purity of ~90% OECs which have previously been shown to be immunoreactive for S100 β and p75NTR which are markers of OECs [21,22]. Schwann cells were cultured from

dorsal root ganglia (DRG) as previously described [23] with minor modifications. Briefly, DRGs were excised and subjected to digestion with collagenase type I (1 mg/ml in phosphate buffered saline, Worthington) for 20 min. Ganglia were then transferred to Matrigel-coated plastic plates and maintained in the same medium as the OECs. When the Schwann cells had migrated out of the explants and reached ~75% confluency they were re-plated onto new matrigel-coated plates to obtain cultures with ~95 % purity.

For Ca^{2+} imaging, cells on cover slips were stained with 1.5 μM Fluo-4 AM (30 min) in an incubator at 37°C in a 5 % humidified atmosphere, transferred into a perfusion chamber and continuously superfused with CO_2 -independent medium (1x, Gibco, Invitrogen Corp, Melbourne, Australia). Time series were acquired using a Zeiss Axioobserver Z1 microscope with a CCD camera (AxioCam MRm, Zeiss, Goettingen, Germany) at a frequency of 0.2 Hz. Images were compiled using Zeiss Axiovision Rel 4.6.3. Only S100 β -DsRed-positive OECs and Schwann cells were selected and analyzed.

2.5 Data analysis

Ca^{2+} signals were analyzed by defining Fluo-4-loaded cells as a region of interest (ROI) with the software ImageJ 1.42I (W. Rasband, National Institute of Health, Maryland, USA). In confocal optical sections, each ROI represents a single cell. In measurements using epifluorescence, out-of-focus fluorescence might contaminate the fluorescence of the cell in focus, and hence a single ROI comprises one to 3 cells. Since OECs are located in the superficial layer of the olfactory bulb and, in addition, are much brighter labelled compared to cells in deeper layers [24] (see also Fig. 1B), fluorescence recordings originated almost exclusively from OECs. Ca^{2+} -dependent changes in fluorescence intensity were measured in each ROI and

calculated as relative fluorescence changes (ΔF) with respect to the basal fluorescence at resting conditions, which was normalized to 100%. The normalization reduces errors caused by different resting fluorescence values due to differences in Fluo-4 loading or tissue depth between cells. Since this kind of analysis is sensitive to artifacts generated by bleaching, we estimated bleaching effects by measuring the Fluo-4 fluorescence in OECs over a time range of up to two hours and could not detect a significant decrease in fluorescence, indicating that bleaching has no major impact on our measurements. Results are given as mean values $\pm$ SEM of the recorded amplitudes of Ca^{2+} peaks. The number of analyzed cells is indicated by n and pooled from at least three different animals for each experiment. When comparing two means, statistical significance ($P < 0.05$) was assessed by Student's t -test.

2.6 Immunohistochemistry

Immunohistochemistry was performed on olfactory bulbs of neonatal NMRI-mice (P8). After dissection, olfactory bulbs were kept in paraformaldehyde solution (PFA, 4% in phosphate buffered solution (PBS) containing (in mM): 130 NaCl, 14 Na_2HPO_4 , 6 NaH_2PO_4) overnight at 4°C. Subsequently, 200 μm thick sagittal slices were sectioned with a vibratome (VT1000S, Leica, Nussloch, Germany), washed three times for 5 min with PBS and incubated for 60 min in blocking solution (3% bovine serum albumin (BSA), 1% normal goat serum, 0.5% Triton X-100 in PBS) to mask unspecific binding sites. Afterwards, the following primary antibodies were incubated 24 h at 4°C: rabbit anti- $\text{InsP}_3\text{R-1}$ (1:250; Dianova, Hamburg, Germany), rabbit anti- $\text{InsP}_3\text{R-2}$ (1:20; Millipore, Eschborn, Germany), and mouse anti-S100B (1:50; Novus Biologicals, Cambridge, UK) diluted in PBS containing 1% BSA, 0.2% Triton X-100. After washing three times for 5 min in PBS, the secondary antibodies (goat-anti-rabbit-IgG-Alexa 488, 1:1000 in PBS, goat-anti-mouse-IgG-Alexa 546, 1:1000 in

PBS, Molecular Probes, Karlsruhe, Germany) and 5 μ M Hoechst (Molecular Probes) were incubated 2 h at room temperature followed by a repeated washing with PBS. Slices were mounted on slides and covered with self-hardening embedding medium (30 % glycerol, 12 % polyvinyl alcohol, 0.5 % phenol in 0.1 M Tris) and a cover slip and were analyzed with a confocal microscope (Zeiss LSM 700). Cerebellar slices were used as positive controls for InsP₃R-1 and InsP₃R-2 immunolabelling, in which the distribution of InsP₃Rs is well described [25,26]. Control stainings without primary antibody did not show fluorescence above background, indicating that there was no non-specific binding of the secondary antibody (not shown).

3. Results

3.1. Cooling-evoked intracellular Ca²⁺ release in olfactory ensheathing cells.

Changes in the cytosolic Ca²⁺ concentration were monitored in olfactory ensheathing cells (OECs) in isolated olfactory bulbs loaded with Fluo-4 by multicell bolus loading [19] (Fig. 1A). In confocal optical sections, OECs can be easily identified by their bright Fluo-4 labelling and their position in the nerve layer (the most superficial layer of the olfactory bulb), which is bordered by the glomerular layer as indicated by the cell body-free glomeruli (Fig. 1B). Decreasing the temperature of the perfusion saline from 37°C to values of 28°C and below resulted in Ca²⁺ oscillations in OECs (Fig. 1C). The oscillations consisted of a large first Ca²⁺ transient followed by Ca²⁺ transients of decreasing amplitude at an average frequency of one Ca²⁺ transient per 5.3 ± 0.2 min (n= 25 animals). In some cases, the Ca²⁺ response consisted only of a single Ca²⁺ transient. For analysis, only the amplitude of the first Ca²⁺ transient was used.

The temperature was held at different levels to determine the temperature threshold at which Ca^{2+} signalling is triggered. Ca^{2+} signalling could not be measured at temperatures of 30°C and above, but was elicited by cooling at a threshold of $27.8^\circ\text{C} \pm 0.8^\circ\text{C}$ ($n = 9$ animals; Fig. 1C). In the following experiments, the temperature was held at 31°C and then dropped to approximately 22°C to induce Ca^{2+} signalling. The average amplitude of the initial Ca^{2+} peak following cooling was $184.8 \pm 8.9 \% \Delta F$ ($n = 122$) and decreased to $171.5 \pm 7.4 \% \Delta F$ ($n = 122$) and $155.0 \% \pm 5.6 \% \Delta F$ ($n = 122$), respectively, after a second and third cycle of heating and cooling (Fig. 1D). The analysis of the Ca^{2+} transients was hampered by shifts in the baseline which were caused by slight tissue swelling upon temperature changes, as seen, e.g. in Fig. 1D (arrows). Therefore, the fluorescence value immediately before the Ca^{2+} transient was taken as baseline value for analysis.

To identify the source of Ca^{2+} employed by cooling-evoked Ca^{2+} signalling, we tested the effects of external Ca^{2+} withdrawal and intracellular Ca^{2+} store depletion on the amplitude of Ca^{2+} transients following cooling. In Ca^{2+} -free, EGTA-buffered solution, cooling-evoked Ca^{2+} transients were partly reduced by $45.2 \pm 4.2 \%$ of the control ($n=81$) (Fig. 1E). In a second set of experiments, Ca^{2+} stores were depleted by application of 20 μM cyclopiazonic acid (CPA), which resulted in a slow sustained Ca^{2+} increase, attributable to Ca^{2+} leakage from internal stores (Fig. 1F). After Ca^{2+} stores were depleted, cooling failed to induce a Ca^{2+} transient ($n=76$). The results indicate that cooling-evoked Ca^{2+} signalling in OECs depends on intracellular Ca^{2+} stores, whereas Ca^{2+} influx from the extracellular space is not required to trigger cooling-dependent Ca^{2+} signalling.

3.2. Ca²⁺ signalling is not mediated by cold-sensitive TRP channels

The transient receptor potential (TRP) channel TRPM8 has been shown to be activated by cooling with a threshold of about 27°C [27], close to the temperature threshold of cooling-evoked Ca²⁺ transients found in the present study. While most of TRP channels are located in the plasma membrane, functional TRPM8 channels have also been found in the endoplasmic reticulum [28]. Therefore, we used TRPM8^{-/-} mice to study the contribution of TRPM8 to cooling-evoked Ca²⁺ signalling in OECs. Decreasing the temperature from above 30°C to 22°C still evoked Ca²⁺ transients or Ca²⁺ oscillations in OECs in isolated olfactory bulbs of TRPM8^{-/-} mice (Fig. 2A). In addition, cooling also triggered Ca²⁺ signalling in mice in which the TRPA1 channel was knocked out (Fig. 2B), another cold-sensitive TRP channel albeit with a lower temperature threshold of about 17°C [14]. Cooling-evoked Ca²⁺ transients in OECs of TRPM8^{-/-} and TRPA1^{-/-} mice were not significantly different from Ca²⁺ transients in wild type mice (Fig. 2C), indicating that both types of TRP channels are not involved in temperature-mediated Ca²⁺ signalling in OECs. This conclusion was confirmed by the lack of effect of BCTC and AP-18 (Fig. 2D-F), which block TRPM8 and TRPA1, respectively [29,30].

3.3. Cooling-evoked Ca²⁺ signalling is not mediated by ryanodine receptors

In muscle cells, a rapid drop in temperature can activate ryanodine receptors (RyR), and the resulting Ca²⁺ release from the sarcoplasmic reticulum triggers contraction [11,12]. To test the contribution of RyR to cooling-evoked Ca²⁺ signalling in OECs, we inhibited RyRs with 100 µM ryanodine or 100 µM dantrolene, respectively. Neither ryanodine nor dantrolene were able to reduce cooling-evoked Ca²⁺ signalling in OECs (Fig. 3A, B). When 40 mM caffeine, which can induce activation of RyRs, was applied to OECs, Ca²⁺ transients were not induced, suggesting the absence of RyR-

dependent Ca^{2+} signalling in OECs (Fig. 3C). Rather, cooling-evoked Ca^{2+} signalling was entirely suppressed by 40 mM caffeine. Fig. 3D summarizes the effects of ryanodine, dantrolene and caffeine on cooling-evoked Ca^{2+} transients in OECs.

3.4. Cooling-evoked Ca^{2+} signalling depends on InsP_3 receptors

Caffeine not only activates RyR, but also inhibits InsP_3 Rs [31]. Since cooling was not able to evoke Ca^{2+} transients in the presence of 40 mM caffeine, we studied the contribution of InsP_3 Rs to cooling-evoked Ca^{2+} signalling in OECs. Like caffeine, the InsP_3 R blocker 2-APB (100 μM) entirely suppressed Ca^{2+} signalling upon cooling (Fig. 4A). 2-APB has also been shown to block store-operated Ca^{2+} (SOC) channels, thus reducing Ca^{2+} oscillations [32]. Hence, 2-APB might suppress cooling-evoked Ca^{2+} signalling by blocking SOC channels. To test the involvement of SOC channels, we blocked these channels by 100 μM BTP2 (Fig. 4B), another SOC channel blocker that does not interfere with InsP_3 Rs [33]. In the presence of BTP2, cooling-evoked Ca^{2+} transients were reduced in amplitude by 34.9 ± 1.4 % of the control ($n=96$), but not completely blocked. Thus, SOC channels are not required to trigger cooling-evoked Ca^{2+} signalling in OECs, but contribute to it. To confirm the presence of functional InsP_3 Rs, we loaded OECs with caged InsP_3 and released InsP_3 by illumination with a 405-nm laser diode for 2 s. At 22°C, photolysis of caged InsP_3 resulted in a Ca^{2+} transient of 349.2 ± 18.5 % ΔF ($n=76$). Since the kinetics of ion fluxes through ion channels becomes faster at increasing temperature, we expected a larger amplitude of caged InsP_3 -dependent Ca^{2+} transients at 31°C. However, photolysis of caged InsP_3 evoked a Ca^{2+} transient of 164.1 ± 15.4 % ΔF ($n=51$) at 31°C, which was significantly smaller than at 22°C ($p < 0.005$), suggesting a temperature-dependent inhibition of InsP_3 R-mediated Ca^{2+} signalling in OECs. Ca^{2+} transients upon photolysis of caged InsP_3 were largely reduced by 100 μM 2-APB to

16.1 $\pm$ 5.5 % ΔF (n=12, Fig. 4D), confirming the efficacy of 2-APB as an InsP₃R blocker. Immunohistochemical stainings revealed that InsP₃R1 colocalizes with S100B-expression in OECs in the olfactory bulb (Fig. 4E), while InsP₃R2 could not be detected in OECs (SFig. 1).

3.5. InsP₃R-mediated Ca²⁺-induced Ca²⁺ release is inhibited at high temperatures

The results show, that in OECs InsP₃-induced Ca²⁺ signalling is reduced at high temperatures, and that cooling evokes InsP₃R-mediated Ca²⁺ transients. However, cooling *per se* is not expected to increase InsP₃ levels, raising the question how cooling activates InsP₃R-mediated Ca²⁺ signalling. InsP₃Rs are Ca²⁺-dependent, and increases in Ca²⁺ support the activation of InsP₃Rs leading to Ca²⁺ release from internal stores, a mechanism termed Ca²⁺-induced Ca²⁺ release (CICR). We studied the effect of rather moderate Ca²⁺ increases (i.e. much smaller than the cooling-evoked Ca²⁺ transients) on InsP₃R-dependent Ca²⁺ signalling in OECs. As shown in Fig. 1D, application of CPA induced a slow Ca²⁺ rise of 32.7 $\pm$ 1.6 % ΔF (n=76) at 31°C (see also Fig. 5A, upper trace). In contrast, when cells were incubated at 22°C and CPA was applied after the first cooling-evoked Ca²⁺ transient, the slow CPA-induced Ca²⁺ rise was superimposed by a fast transient Ca²⁺ peak similar to the cooling-evoked Ca²⁺ transient (Fig. 5A, lower trace). The average amplitude of this Ca²⁺ peak was 64.3 $\pm$ 3.6 % ΔF (n = 59). The fast CPA-induced Ca²⁺ peak at 22°C persisted in Ca²⁺-free solution, demonstrating that it was due to Ca²⁺ release from internal stores (Fig. 5B). When InsP₃Rs were blocked by 100 μ M 2-APB or 40 mM caffeine, CPA evoked only a slow, small Ca²⁺ rise of 10.8 $\pm$ 0.6 % ΔF (n=68) and 15.3 $\pm$ 1.1 % ΔF (n=69), respectively, indicating that the fast Ca²⁺ peak is mediated by InsP₃Rs (Fig. 5C, D). Hence, the Ca²⁺ rise induced by Ca²⁺ leakage from internal

stores upon application of CPA triggers InsP₃R-dependent Ca²⁺ release at 22°C, but not at 31°C. To verify that a moderate Ca²⁺ rise is sufficient to trigger intracellular Ca²⁺ release in a temperature-dependent manner, we elicited Ca²⁺ transients by photolysis of caged Ca²⁺ in NP-EGTA-loaded OECs at different temperatures. At 22°C, illumination of NP-EGTA-loaded OECs with 405 nm resulted in a Ca²⁺ transient of $56.2 \pm 7.6 \% \Delta F$ (n=29; Fig. 5E). At 31°C, the Ca²⁺ transient was significantly smaller and amounted to $30.4 \pm 3.4 \% \Delta F$ (n=27). A similar reduction in amplitude of the caged Ca²⁺-induced Ca²⁺ transients was measured when InsP₃Rs were blocked with 100 μ M 2-APB at 22°C (Fig. 5F), with Ca²⁺ transients having a mean amplitude of $25.8 \pm 3.8 \% \Delta F$ (n=24, Fig. 5G). This value presumably reflects the actual amount of Ca²⁺ that is released by photolysis of caged Ca²⁺ without a contribution of Ca²⁺ release mediated by InsP₃Rs. To check whether the efficacy of the photolysis or the response properties of Fluo-4 could account for the differences in caged Ca²⁺-induced Ca²⁺ transients between 22°C and 31°C, we released caged Ca²⁺ also at 31°C in the presence of 100 μ M 2-APB (not shown). This elicited a Ca²⁺ response of $19.4 \pm 3.1 \Delta F$ (n=28), which was not significantly different from Ca²⁺ responses in 2-APB at 22°C (Fig. 5G), indicating that temperature does not considerably influence photolysis of caged Ca²⁺ or the sensitivity of Fluo-4. Together with the finding that photolysis of caged InsP₃ elicited Ca²⁺ release both at 22°C and 31°C, the results suggest that the reduction of caged Ca²⁺-induced Ca²⁺ release at 31°C compared to 22°C is due to a temperature-dependent inhibition of the Ca²⁺-mediated InsP₃R activation.

To further analyze the temperature dependency of CICR, we photoreleased caged Ca²⁺ at different temperatures ranging from 16°C to 31°C (Fig. 5H). Increasing the temperature from 16°C to 22°C did not significantly alter the amplitude of the Ca²⁺ transients. Further increase in temperature to 25°C led to a decrease in the

amplitude of the caged Ca^{2+} -induced Ca^{2+} transients, suggesting that this is close to the threshold for temperature-dependent inhibition of InsP_3R -mediated CICR.

To confirm that the cooling-evoked Ca^{2+} signalling in OECs depends on free cytosolic Ca^{2+} , we studied the effect of buffering cytosolic Ca^{2+} (Fig. 5I). NP-EGTA not only is a photoactivatable Ca^{2+} donor, it also is a highly efficient Ca^{2+} buffer ($K_d=50$ nM) [34]. In NP-EGTA-loaded OECs, cooling failed to induce Ca^{2+} transients, while 405-nm illumination was still able to trigger Ca^{2+} signalling (Fig. 5I). These results demonstrate that both, temperatures below 25-28°C and free cytosolic Ca^{2+} are required to activate InsP_3R -mediated CICR.

3.6. Cooling slows down Ca^{2+} regulation

To check whether cooling could evoke a rise in the cytosolic Ca^{2+} concentration by slowing down Ca^{2+} regulation, we measured the time constant of Ca^{2+} extrusion in OECs at different temperatures. Ca^{2+} transients were induced by photolysis of caged Ca^{2+} , and the Ca^{2+} decay was fitted with the equation of an exponential decay with a single time constant. At 31°C, the time constant was 8.0 ± 0.5 s ($n=90$; Fig. 6A). This value increased to 15.1 ± 0.6 s ($n=81$) at 22°C, and to 30.9 ± 1.3 s ($n=91$) at 16°C. When Ca^{2+} ATPases of the endoplasmic reticulum (SERCA) were inhibited by CPA at 31°C, the time constant of Ca^{2+} decay increased to 17.2 ± 1.9 s ($n=37$). This time constant was not significantly different from the time constant measured at 22°C without CPA, suggesting that cooling the cells from 31°C to 22°C has a similar effect on Ca^{2+} regulation as inhibiting SERCAs and thus might produce a Ca^{2+} rise sufficient to trigger CICR. The results also show that fast SERCA-mediated Ca^{2+} uptake into the endoplasmic reticulum is largely responsible for cytosolic Ca^{2+} regulation.

3.7 Effect of cooling on calcium signalling in cultured glial cells

We also studied this temperature-sensitive mechanism of Ca^{2+} release in different types of cultured glial cells (Fig. 7A-C). At 22°C, application of 100 μM ATP for 30 s evoked Ca^{2+} transients in cultured OECs with an amplitude of $67.4 \pm 10.8 \% \Delta F$ ($n=57$; Fig. 7D). Increasing the temperature to 31°C reduced the amplitude of ATP-evoked Ca^{2+} transients to $27.1 \pm 2.0 \% \Delta F$ ($n=46$). In Schwann cells, increasing the temperature from 22°C to 31°C reduced the amplitude of ATP-evoked Ca^{2+} transients from $205.1 \pm 5.6 \% \Delta F$ ($n=361$) to $25.8 \pm 1.8 \% \Delta F$ ($n=40$), while it had no effect on ATP-evoked Ca^{2+} signalling in cultured astrocytes ($n=138$ and 136 ; Fig. 7D, E).

4. Discussion

In the present study, we have investigated cytosolic Ca^{2+} signalling during cooling and Ca^{2+} -dependent activation of InsP_3Rs at different temperatures in olfactory ensheathing glial cells. Our results show that lowering the temperature from above 30°C to 28°C and below triggers Ca^{2+} release from intracellular stores via InsP_3R activation. We did not find evidence for the involvement of known temperature-sensitive ion channels such as ryanodine receptors, TRPM8 and TRPA1 in this Ca^{2+} signalling in OECs.

4.1 Mechanism of cooling-evoked Ca^{2+} signalling

The experiments were performed in the nominal absence of receptor ligands; hence, InsP_3R activation appears to occur without increasing the InsP_3 concentration by activation of $G_{q/11}$ protein-coupled receptors such as neurotransmitter receptors. In fact, an increase in cytosolic Ca^{2+} not only is sufficient to open InsP_3Rs at resting InsP_3 concentrations, since “uncaging” of caged Ca^{2+} triggered InsP_3R -mediated

Ca^{2+} release, but also is required for cooling-evoked Ca^{2+} transients, since buffering cytosolic Ca^{2+} entirely suppressed the Ca^{2+} rise upon cooling (Fig. 5I). A Ca^{2+} -dependent activation of InsP_3Rs at low levels of InsP_3 could be induced by multiple mechanisms. InsP_3Rs themselves have a Ca^{2+} binding site, and increasing cytosolic Ca^{2+} from resting levels around 100 nM to 1-2 μM supports InsP_3R activation, while Ca^{2+} increases above 10 μM reduces InsP_3R activation [35,36]. In addition, InsP_3Rs have binding sites for Ca^{2+} binding proteins such as CaBPs, CIB1 and NCS-1 which modulate the open probability of InsP_3Rs , some of them even in the absence of InsP_3 [37-40]. Another Ca^{2+} -binding protein, calmodulin, has been shown to inhibit InsP_3Rs [41-42]. The inhibitory effect of Ca^{2+} and/or Ca^{2+} binding proteins such as calmodulin on InsP_3Rs at high Ca^{2+} concentrations might cause the termination of the cooling-evoked Ca^{2+} transients. As a consequence, InsP_3R activation at moderate Ca^{2+} concentrations alternates with InsP_3R inhibition at high Ca^{2+} concentrations, which results in Ca^{2+} oscillations as frequently found in the present study.

Most of the studies analyzing Ca^{2+} -dependent activation of InsP_3Rs were performed at room temperature and did not investigate the influence of temperature on InsP_3R -mediated CICR. We found that a moderate increase in Ca^{2+} was able to trigger CICR only below 28°C, while InsP_3R -dependent CICR was inhibited at higher temperatures. The inhibition of CICR at high temperatures appeared to be due to inhibition of the Ca^{2+} -dependent activation of the InsP_3R . InsP_3 (photoreleased from caged InsP_3) was still able to activate InsP_3Rs at 30°C and above, albeit with a reduced amplitude. This reduction in amplitude at high temperatures could result from the lack of CICR, although we cannot exclude that in addition the InsP_3 sensitivity or the conductance of the InsP_3R is depressed by temperatures above 30°C.

Thermosensitive Ca^{2+} release mediated by InsP_3Rs was also observed in HeLa cells. Tseeb et al. [43] postulated a reduced activation of SERCA pumps, which we also found in OECs (Fig. 6), and a high open probability of InsP_3Rs at temperatures below 30°C , leading to Ca^{2+} transients upon cooling. Although cooling-evoked Ca^{2+} transients in OECs depend on Ca^{2+} release from intracellular stores and were entirely suppressed when Ca^{2+} stores were depleted or InsP_3Rs were inhibited, the amplitude of the Ca^{2+} transients was reduced in Ca^{2+} -free saline. This indicates that Ca^{2+} influx from the extracellular space contributes to cooling-evoked Ca^{2+} transients. A reduction in amplitude of the Ca^{2+} transients similar to that mediated by external Ca^{2+} withdrawal was measured in the presence of BTP2, a blocker of SOC channels. SOC channels are Ca^{2+} -permeable ion channels that are activated upon depletion of Ca^{2+} stores [44,45] and has been found in a variety of cell types, including glial cells [46]. In astrocytes, Ca^{2+} entry via SOC channels has been shown to be sensitive to BTP2 [20]. The inhibitory effect of BTP2 on cooling-evoked Ca^{2+} transients suggests the involvement of SOC channels in Ca^{2+} signalling in OECs, too.

4.2 Involvement of CICR in glial Ca^{2+} signalling

InsP_3R -mediated Ca^{2+} release is a key player in the Ca^{2+} signalling machinery in all types of glial cells [47,48]. OECs respond to glutamate and ATP released from axons of sensory neurons in the olfactory nerve layer with rises in the cytosolic Ca^{2+} concentration [24,49]. InsP_3R -dependent CICR is an important pathway to enhance ligand-evoked Ca^{2+} signalling in glial cells. We also demonstrated this in cultured OECs and Schwann cells, in which ATP-induced Ca^{2+} transients were greatly reduced when InsP_3R -mediated Ca^{2+} signalling was suppressed by increasing the temperature. Surprisingly, changes in the temperature had no effect on ATP-induced Ca^{2+} transients in astrocytes, indicating either the absence of InsP_3R -mediated CICR

or lack of temperature sensitivity of this type of CICR. In general, InsP₃R-mediated CICR is present in astrocytes, and moderate Ca²⁺ rises are able to trigger CICR via InsP₃Rs in olfactory bulb astrocytes *in situ* [10,50]. The difference in the temperature sensitivity of CICR in astrocytes versus OECs may originate from different types of InsP₃Rs expressed by astrocytes and OECs. Astrocytes highly express InsP₃R-2 [51], while we did not find InsP₃R-2-like immunolabelling, but rather an expression of InsP₃R-1 in OECs, an InsP₃R type that is highly sensitive to Ca²⁺ [7]. In addition, differences in InsP₃ metabolism may also account for differences in the temperature sensitivity of CICR in different cell types.

4.3 Conclusion

Our results show that cooling is able to trigger Ca²⁺ signalling via InsP₃Rs without an external stimulus that increases the InsP₃ concentration. Rather, cooling appears to release a temperature-dependent attenuation of the Ca²⁺-sensitivity of the InsP₃Rs and simultaneously causes a moderate Ca²⁺ increase by slowing down Ca²⁺ transport mechanisms, which, together, result in CICR at a certain temperature threshold.

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6. References

1. M. J. Berridge, The endoplasmic reticulum: a multifunctional signaling organelle, *Cell Calcium*. 32 (2002) 235-249.
2. A. Verkhratsky, Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons, *Physiol Rev*. 85 (2005) 201-279.
3. T. Imagawa, J.S. Smith, R. Coronado, K.P. Campbell, Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+} -permeable pore of the calcium release channel, *J Biol Chem*. 262 (1987) 16636-16643.
4. M. Inui, a Saito, S. Fleischer, Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures, *J Biol Chem*. 262 (1987) 15637-15642.
5. F.A. Lai, H.P. Erickson, E. Rousseau, Q.Y. Liu, G. Meissner, Purification and reconstitution of the calcium release channel from skeletal muscle, *Nature*. 331 (1988) 315-319.
6. M. D. Bootman, L. Missiaen, J. B. Parys, H. De Smedt, R. Casteels, Control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release by cytosolic Ca^{2+} , *Biochem J*. 306 (1995) 445-51.
7. J.K. Foskett, C. White, K.-H. Cheung, D.-O.D. Mak, Inositol trisphosphate receptor Ca^{2+} release channels, *Physiological Reviews*. 87 (2007) 593-658.
8. E. Sammels, J.B. Parys, L. Missiaen, H. De Smedt, G. Bultynck, Intracellular Ca^{2+} storage in health and disease: a dynamic equilibrium, *Cell Calcium*. 47 (2010) 297-314.
9. A. Beck, R. Zur Nieden, H.P. Schneider, J.W. Deitmer, Calcium release from intracellular stores in rodent astrocytes and neurons in situ, *Cell Calcium*. 35 (2004) 47-58.

10. M. Doengi, D. Hirnet, P. Coulon, H.-C. Pape, J.W. Deitmer, C. Lohr, GABA uptake-dependent Ca^{2+} signaling in developing olfactory bulb astrocytes, *Proc Natl Acad Sci U S A.* 106 (2009) 17570-17575.
11. S. Talon, C. Huchet-Cadiou, C. Léoty, Rapid cooling-induced contractures in rat skinned skeletal muscle fibres originate from sarcoplasmic reticulum Ca^{2+} release through ryanodine and inositol trisphosphate receptors, *Pflügers Arch Eur J Physiol.* 441 (2000) 108-117.
12. F. Protasi, A. Shtifman, F.J. Julian, P.D. Allen, All three ryanodine receptor isoforms generate rapid cooling responses in muscle cells, *Am J Physiol Cell Physiol.* 286 (2004) C662-C670.
13. A.M. Peier, A. Moqrich, A.C. Hergarden, et al., A TRP channel that senses cold stimuli and menthol, *Cell.* 108 (2002) 705-715.
14. G.M. Story, A.M. Peier, A.J. Reeve, et al., ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures, *Cell.* 112 (2003) 819-829.
15. K. Talavera, B. Nilius, T. Voets, Neuronal TRP channels: thermometers, pathfinders and life-savers, *Trends Neurosci.* 31 (2008) 287-295.
16. A. Dhaka, V. Viswanath, A. Patapoutian, TRP Ion Channels and Temperature Sensation, *Annu Rev Neurosci.* 29 (2006) 135-161.
17. A. Dhaka, A.N. Murray, J. Mathur, T.J. Earley, M.J. Petrus, A. Patapoutian, TRPM8 is required for cold sensation in mice, *Neuron.* 54 (2007) 371-378.
18. K.Y. Kwan, A.J. Allchorne, M.A. Vollrath, et al., TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction, *Neuron.* 50 (2006) 277-289.
19. C. Stosiek, O. Garaschuk, K. Holthoff, A. Konnerth, In vivo two-photon calcium imaging of neuronal networks, *Proc Natl Acad Sci U S A.* 100 (2003) 7319-7324.

20. K. Singaravelu, C. Lohr, J.W. Deitmer, Regulation of store-operated calcium entry by calcium-independent phospholipase A2 in rat cerebellar astrocytes, *J Neurosci.* 26 (2006) 9579-9592.
21. L.C.E. Windus, C. Claxton, C.L. Allen, B. Key, J.A. St John, Motile membrane protrusions regulate cell-cell adhesion and migration of olfactory ensheathing glia, *Glia.* 55 (2007) 1708-1719.
22. L.C.E. Windus, K.E. Lineburg, S.E. Scott, C. Claxton, A. Mackay-Sim, B. Key, J.A. St John, Lamellipodia mediate the heterogeneity of central olfactory ensheathing cell interactions, *Cell Mol Life Sci.* 67 (2010) 1735-1750.
23. H. Chi, H. Horie, N. Hikawa, T. Takenaka, Isolation and age-related characterization of mouse Schwann cells from dorsal root ganglion explants in type I collagen gels, *J Neurosci Res.* 35 (1993) 183-187.
24. A. Rieger, J.W. Deitmer, C. Lohr, Axon-glia communication evokes calcium signaling in olfactory ensheathing cells of the developing olfactory bulb, *Glia.* 55 (2007) 352-359.
25. P. Koulen, T. Janowitz, L.D. Johnston, B.E. Ehrlich, Conservation of localization patterns of IP₃ receptor type 1 in cerebellar Purkinje cells across vertebrate species, *J Neurosci Res.* 61 (2000) 493-499.
26. J. Oberdorf, M.L. Vallano, R.J. Wojcikiewicz, Expression and regulation of types I and II inositol 1,4,5-trisphosphate receptors in rat cerebellar granule cell preparations, *J Neurochem.* 69 (1997) 1897-1903.
27. D.D. McKemy, W.M. Neuhausser, D. Julius, Identification of a cold receptor reveals a general role for TRP channels in thermosensation, *Nature.* 416 (2002) 52-58.

28. L. Zhang, G.J. Barritt, Evidence that TRPM8 is an androgen-dependent Ca^{2+} channel required for the survival of prostate cancer cells, *Cancer Res.* 64 (2004) 8365-8373.
29. H.-J. Behrendt, T. Germann, C. Gillen, H. Hatt, R. Jostock, Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay, *Br J Pharmacol.* 141 (2004) 737-745.
30. M. Petrus, A.M. Peier, M. Bandell, et al., A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition, *Molecular Pain.* 3 (2007) 40.
31. Y. Sei, K.L. Gallagher, J.W. Daly, Multiple effects of caffeine on Ca^{2+} release and influx in human B lymphocytes, *Cell Calcium.* 29 (2001) 149-160.
32. K. Singaravelu, C. Lohr, J.W. Deitmer, Calcium-independent phospholipase A2 mediates store-operated calcium entry in rat cerebellar granule cells, *Cerebellum.* 7 (2008) 467-481
33. C. Zitt, B. Strauss, E.C. Schwarz, N. Spaeth, G. Rast, A. Hatzelmann, M. Hoth. Potent inhibition of Ca^{2+} release-activated Ca^{2+} channels and T-lymphocyte activation by the pyrazole derivative BTP2, *J Biol Chem.* 279 (2004) 12427-12437.
34. G. C. Faas, K. Karacs, J. L. Vergara, I. Mody, Kinetic properties of DM-nitrophen binding to calcium and magnesium, *Biophys J.* 88 (2005) 4421-4433.
35. I. Bezprozvanny, J. Watras, B.E. Ehrlich, Bell-shaped calcium-response curves of $\text{Ins}(1,4,5)\text{P}_3$ - and calcium-gated channels from endoplasmic reticulum of cerebellum, *Nature.* 351 (1991) 751-754.

36. D.O. Mak, S. McBride, J.K. Foskett, Inositol 1,4,5-trisphosphate activation of inositol trisphosphate receptor Ca^{2+} channel by ligand tuning of Ca^{2+} inhibition, *Proc Natl Acad Sci U S A.* 95 (1998) 15821-15825.
37. J. Yang, S. McBride, D.-O.D. Mak, et al., Identification of a family of calcium sensors as protein ligands of inositol trisphosphate receptor Ca^{2+} release channels, *Proc Natl Acad Sci U S A.* 99 (2002) 7711-7716.
38. C. White, J. Yang, M.J. Monteiro, J.K. Foskett, CIB1, a ubiquitously expressed Ca^{2+} -binding protein ligand of the InsP3 receptor Ca^{2+} release channel, *J Biol Chem.* 281 (2006) 20825-20833.
39. C. Schlecker, W. Boehmerle, A. Jeromin, et al., Neuronal calcium sensor-1 enhancement of InsP3 receptor activity is inhibited by therapeutic levels of lithium, *J Clin Invest.* 116 (2006) 1668-1674.
40. C.U. Choe, B.E. Ehrlich, The inositol 1,4,5-trisphosphate receptor (IP3R) and its regulators: sometimes good and sometimes bad teamwork, *Sci STKE.* 363 (2006) re15.
41. S. Patel, S. a Morris, C.E. Adkins, G. O'Beirne, C.W. Taylor, Ca^{2+} -independent inhibition of inositol trisphosphate receptors by calmodulin: redistribution of calmodulin as a possible means of regulating Ca^{2+} mobilization, *Proc Natl Acad Sci U S A.* 94 (1997) 11627-11632.
42. W. Taylor, A. J. Laude, IP₃ receptors and their regulation by calmodulin and cytosolic Ca^{2+} , *Cell Calcium* 32 (2002) 321-334.
43. V. Tseeb, M. Suzuki, K. Oyama, K. Iwai, S. Ishiwata, Highly thermosensitive Ca^{2+} dynamics in a HeLa cell through IP₃ receptors, *HFSP journal.* 3 (2009) 117-123.
44. L. Vaca, SOCIC: the store-operated calcium influx complex, *Cell Calcium* 47 (2010) 199-209.

45. A.B. Parekh, Store-operated CRAC channels: function in health and disease, *Nat Rev Drug Discov.* 9 (2010) 399-410.
46. V. Parpura, V. Grubisic, A. Verkhratsky, Ca^{2+} sources for the exocytotic release of glutamate from astrocytes. *Biochim Biophys Acta.* 1813 (2011) 984-991.
47. M. Nedergaard, J.J. Rodríguez, A. Verkhratsky, Glial calcium and diseases of the nervous system, *Cell Calcium.* 47 (2010) 140-149.
48. C. Lohr, J.W. Deitmer, Calcium signaling in invertebrate glial cells, *Glia.* 54 (2006) 642-649.
49. A. Thyssen, D. Hirnet, H. Wolburg, G. Schmalzing, J.W. Deitmer, C. Lohr, Ectopic vesicular neurotransmitter release along sensory axons mediates neurovascular coupling via glial calcium signaling, *Proc Natl Acad Sci U S A.* 107 (2010) 15258–15263.
50. M. Doengi, J.W. Deitmer, C. Lohr, New evidence for purinergic signaling in the olfactory bulb: $\text{A}_{2\text{A}}$ and P2Y_1 receptors mediate intracellular calcium release in astrocytes, *FASEB J.* 22 (2008) 2368-2378.
51. C. A. Sheppard, P.B. Simpson, A. H. Sharp, et al., Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca^{2+} release sites that support Ca^{2+} waves in cultured astrocytes, *J Neurochem.* 68 (1997) 2317-2327.

Figure legends

Fig. 1: Cooling evokes Ca^{2+} release from intracellular stores in olfactory ensheathing cells (OECs). A) Illustration of the experimental setup. The dotted line indicates the focal plane in B. B) Confocal image of the olfactory bulb loaded with Fluo-4. Arrows point to brightly labelled OECs, asterisks indicate glomeruli. Scale bar: 100 μm . C) Cooling the perfusion saline from 37°C to 24°C, as measured in the experimental chamber (upper trace), evokes Ca^{2+} oscillations (lower trace) in a representative individual OEC. D) Repetitive induction of cooling-evoked Ca^{2+} signalling. E) Effect of withdrawal of external Ca^{2+} on cooling-evoked Ca^{2+} transients. F) Cooling-evoked Ca^{2+} is suppressed after Ca^{2+} store depletion by cyclopiazonic acid (CPA).

Fig. 2: Cooling-evoked Ca^{2+} transients are not mediated by cold-sensitive TRP channels. A) Cooling-evoked Ca^{2+} signalling in OECs of a TRPM8 knockout mouse and B) a TRPA1 knockout mouse. C) Ca^{2+} signalling in TRPM8 knockout and TRPA1 knockout mice were not significantly different from wildtype mice (WT). D) The TRPM8 antagonist BCTC and E) the TRPA1 antagonist AP-18 had no effect on cooling-evoked Ca^{2+} signalling in wildtype mice. F) Statistical analysis of TRP channel antagonists on cooling-evoked Ca^{2+} signalling in wildtype OECs. n.s, not significantly different (see also following figures).

Fig. 3: Ryanodine receptors (RyR) do not mediate cooling-evoked Ca^{2+} signalling. A) Effect of 100 μM ryanodine and B) 100 μM dantrolene on cooling-evoked Ca^{2+} transients. C) The RyR agonist caffeine (40 mM) did not induce Ca^{2+} transients in

OECs, but blocked cooling-evoked Ca^{2+} transients. D) Statistics of RyR ligands on cooling-evoked Ca^{2+} signalling. ***, $p < 0.005$ (also in following figures).

Fig. 4: InsP_3 receptors mediate cooling-evoked Ca^{2+} signalling. A) The InsP_3R antagonist 2-APB entirely inhibits cooling-evoked Ca^{2+} signalling. B) The store-operated Ca^{2+} channel blocker BTP2 had only a minor effect on cooling-evoked Ca^{2+} transients. C) Ca^{2+} transients evoked by photolysis of caged InsP_3 were reduced at 31°C as compared to 22°C . D) 2-APB blocked Ca^{2+} transients evoked by photolysis of caged InsP_3 . E) $\text{InsP}_3\text{R-1}$ immunoreactivity colocalizes with S100B immunoreactivity, a marker for OECs; scale bar: $10\ \mu\text{M}$.

Fig. 5: InsP_3Rs mediate temperature-sensitive Ca^{2+} -induced Ca^{2+} release. A) Store-depletion by CPA is reflected by a slow Ca^{2+} rise in OECs at 31°C (A1). When CPA is applied at 22°C after the cooling-evoked Ca^{2+} transient (dotted line), the slow CPA-induced Ca^{2+} rise triggers a fast Ca^{2+} transient (arrow) (A2). B) The fast Ca^{2+} transient upon CPA application persists in Ca^{2+} -free saline and hence is mediated by release from intracellular Ca^{2+} stores. C) The fast CPA-induced Ca^{2+} transient is suppressed after blockage of InsP_3Rs with 2-APB ($100\ \mu\text{M}$) and D) caffeine ($40\ \text{mM}$). E) Photolysis ($405\ \text{nm}$) of caged Ca^{2+} (NP-EGTA) results in a Ca^{2+} transient at 22°C that is significantly reduced at 31°C and F) to a similar extent by 2-APB ($100\ \mu\text{M}$). G) Effect of rising the temperature from 22°C to 31°C and of 2-APB ($100\ \mu\text{M}$) on Ca^{2+} transients evoked by photolysis of caged Ca^{2+} in the same cells. H) Amplitudes of Ca^{2+} transients evoked by photolysis of caged Ca^{2+} at different temperatures. Results were pooled from different imaging setups and were normalized to the response at

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Supplementary figure 1: InsP_3R -like immunoreactivity (red) in the olfactory bulb and cerebellum. Nuclei were counterstained with Hoechst (blue). A) InsP_3R -2 immunoreactivity in the olfactory bulb. In the glomerular layer (GL), arrows point to InsP_3R -2-positive periglomerular cells, presumably astrocytes, and asterisks indicate glomeruli. In the olfactory nerve layer (ONL), arrowheads point to InsP_3R -2-positive

structures, which can be identified as blood vessels (arrowhead) as shown in the magnified panel B), whereas OECs appear not to be labelled by the antibody. C-D) As a control, cerebellar slices were labelled with the antibodies. Anti-InsP₃R-2 antibodies highlighted Bergmann glial cells (arrowheads in C), while the anti-InsP₃R-1 antibodies labelled Purkinje neurons (arrow in D).

Figures

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Fig. 6: Effect of temperature on cytosolic Ca^{2+} regulation. A) Normalized time course of Ca^{2+} regulation after a Ca^{2+} rise due to photolysis of caged Ca^{2+} at different temperatures and after inhibition of SERCA by CPA at 31°C. B) Time constant of regulation at different temperatures and in the presence of CPA at 31°C.

Fig. 7: Temperature-dependent Ca^{2+} -induced Ca^{2+} release in cultured glial cells. A) Fluorescent image of DsRed-labelled cultured OECs. B) Fluorescent image of DsRed-labelled cultured Schwann cells. C) Fluorescent image of Fluo-4-loaded cultured astrocytes; scale bar: 50 μm . D) ATP (100 μM , 30 s) evoked Ca^{2+} transients at 22°C that were reduced at 31°C in cultured OECs and Schwann cells, but not in astrocytes. E) Amplitudes of ATP-induced Ca^{2+} transients at 22°C and 31°C in cultured OECs, Schwann cells and astrocytes, respectively.

Supplementary figure 1: InsP₃R-like immunoreactivity (red) in the olfactory bulb and cerebellum. Nuclei were counterstained with Hoechst (blue). A) InsP₃R-2 immunoreactivity in the olfactory bulb. In the glomerular layer (GL), arrows point to InsP₃R-2-positive periglomerular cells, presumably astrocytes, and asterisks indicate glomeruli. In the olfactory nerve layer (ONL), arrowheads point to InsP₃R-2-positive structures, which can be identified as blood vessels (arrowhead) as shown in the magnified panel B), whereas OECs appear not to be labelled by the antibody. C-D) As a control, cerebellar slices were labelled with the antibodies. Anti-InsP₃R-2 antibodies highlighted Bergmann glial cells (arrowheads in C), while the anti-InsP₃R-1 antibodies labelled Purkinje neurons (arrow in D).