4 DISCUSSION

4.1 $NAADP^+$

A new intracellular Ca^{2+} releasing messenger, $NAADP^+$, was investigated with regard to astrocytes, since many physiological and pathological extracellular stimuli evoke a rise in the intracellular Ca^{2+} concentration of astrocytes; and inter-astrocytic communication, as well as their intercellular communication with neurons, critically depends on $[Ca^{2+}]_i$ changes.

In most studies carried out with intact cells, so far, $NAADP^+$ was injected into single cells [129] or released intracellularly, by flash photolysis of the caged compound [301]. In one case, application of extracellular $NAADP^+$ was tested on starfish oocytes, but a Ca^{2+} rise was not detected [302]. Aware of the fact that there was no physiological evidence for an extracellular action of $NAADP^+$, since it has been discovered as an intracellular second messenger, it was applied extracellularly, at relatively high concentration, assuming its uptake into the cells. $NAADP^+$ is effective at extremely low concentration, and therefore, only a small amount would need to reach the cytosol. This approach was prompted by the prior discovery that cADPR, the alternate product of the $NAADP^+$-synthesizing enzyme CD38, or its precursor NAD^+, could be applied extracellularly, and were effective in evoking Ca^{2+} responses in cultured astrocytes [157] and Müller cells [158].

4.1.1 $NAADP^+$ triggers Ca^{2+} signaling in all major brain cell types

Using cell cultures, it was demonstrated that extracellular application of $NAADP^+$ is able to induce diverse patterns of Ca^{2+} responses in all major cell types of the mammalian CNS: neurons, astrocytes, oligodendrocytes, and microglial cells (Fig. 9, *A*, *B* and *C,* and Fig. 16). Purified cultures were used (Fig. 9, *A*, *B* and *C*), and it can therefore be assumed that $NAADP^+$ acts directly on all these cell types. Cells in culture, however, may have properties distinct from those *in situ.* The work was expanded to brain slices with the focus on cortical astrocytes to study the mechanism of action of $NAADP^+$ (Fig. 7). Since the astrocytic responses in culture are similar to those *in situ,* it is assumed that $NAADP^+$ directly stimulated the astrocytes *in situ* as well and that their Ca^{2+} response is not due to an indirect effect mediated by another cell type. Moreover, effects mediated by neuronal activity could at least be partially excluded, since comparable

astrocytic responses were found after neuronal action potential propagation had been blocked by tetrodotoxin (Fig. 9, *D* and *E*).

4.1.2 Question of specificity and contribution of the purinergic and adenosine-mediated pathway

In contrast to other studies [129], it was found that the $NAADP^+$-precursors $NADP^+$ and NAAD did elicit similar Ca^{2+} responses as $NAADP^+$ itself (Fig. 10). Analytical HPLC (data not shown) excluded relevant contaminations for all three substances. One explanation of this observation would be that astrocytes are expressing more active enzymes for $NAADP^+$ synthesis than those cell types examined so far, allowing for immediate intracellular conversion to $NAADP^+$. In support of this, aytrocytes in brain slices were shown to express the enzyme CD38 (Fig. 15), which is possibly mainly responsible for synthesis of $NAADP^+$ from $NADP^+$ [303]. Furthermore extracellular conversion to $NAADP^+$ could be taken into consideration here, although CD38 is thought to synthesize $NAADP^+$ only in acidic milieu [120]. Whole rat brain extracts could produce $NAADP^+$ from $NADP^+$ and nicotinic acid; however, phosphorylation of NAAD could not be demonstrated [267]. Other alternatives would include the same (unspecific) extracellular actions as for $NAADP^+$, including degradation processes.

In the continuous presence of ATP, $NAADP^+$ can still trigger Ca^{2+} signals (Fig. 13). The response is reduced in comparison with the control, but a similar reduction was found for the metabotropic glutamatergic agonist tACPD. This speaks against an action of $NAADP^+$ via purinergic receptors. Moreover, GL 261 cells, a cell line with high affinity purinergic receptors [164], did not respond to $NAADP^+$, supporting the hypothesis that $NAADP^+$ does not act via purinergic receptors.

It was found that adenosine receptor activity is affecting $NAADP^+$-induced signaling. $NAADP^+$-induced Ca^{2+} signals were strongly reduced by the adenosine receptor inhibitor CGS-15943 (Fig. 14). Therefore, activation of adenosine receptors contributes strongly to the observed signal, whether by direct interaction of $NAADP^+$ with adenosine receptors or by its degradation to adenosine and the action of adenosine. However, adenosine receptor-mediated signaling may not directly account for part of the observed signal, but only modulate (enhance) it. Adenosine is a well-known modulator of several signaling pathways and has for instance been shown to modulate purinergic signaling [177] and dinucleotide signaling [304].

4.1.3 $NAADP^+$ potentially enters the cell to act

$NAADP^+$ responds with a delay when compared to a plasma membrane receptor-activating ligand (ATP). This again supports the hypothesis that $NAADP^+$ enters the cell and acts intracellularly. A similar observation was made for cADPR, the alternate product of the enzyme CD38. When added extracellularly, it elicited Ca^{2+} signals, and it was suggested to reach the cytosol via the enzyme CD38 itself [157], which can function as a cADPR transporter [283]. Moreover, nucleoside transporters were found to mediate cADPR transport across the cell membrane [305]. So far, however, $NAADP^+$ transporters have not been identified, and there may be no physiological requirement for its transport across the cell membrane, since it is produced in an intracellular acidic milieu. A potential entry route for $NAADP^+$ is via connexin hemichannels, which can function as regulated transporters of NAD^+ and related pyridine dinucleotides [284]. Hemichannels also permit the entry of the fluorescent dye Lucifer yellow in low divalent cation solution [306]. The result that a blocker of hemichannels (and gap junctions), carbenoxolone, reduced $NAADP^+$ signaling (Fig. 11, *A* and *B*) is consistent with hemichannel-mediated $NAADP^+$ entry, yet an additional entry route, which could account for the residual signaling during the block of hemichannels, could be pinocytosis followed by fusion of the $NAADP^+$-containing endosomes with lysosomes, the site of $NAADP^+$ action.

4.1.4 $NAADP^+$ signaling requires normal extracellular Ca^{2+} levels

Omission of Ca^{2+} from the superfusion solution led to a decrease in the number of $NAADP^+$-responsive cells and their signaling activity (Fig. 11). This effect was augmented by further decreasing extracellular Ca^{2+} levels in the slice by adding EGTA (Fig. 11). Under these conditions, only a few cells were able to respond. This indicates that either extracellular Ca^{2+} levels control the transport of $NAADP^+$ into the cell or that Ca^{2+} influx is required for a full Ca^{2+} response to $NAADP^+$. Similar findings were made in T-cells [290] or mature starfish oocytes [302], in which the response to $NAADP^+$ was strongly reduced or not even detected in Ca^{2+}-free buffer. In other cell types, Ca^{2+} influx plays merely a partial role. In human β-cells, the initiation of the $NAADP^+$-induced Ca^{2+} signal was not affected, but the second phase of the response was [291], in contrast to pancreatic acinar cells [280], where wash-out of Ca^{2+} after initiation of the response did not prevent the sustained phase, and in contrast to sea urchin eggs, where the

removal of extracellular Ca^{2+} abolished the first Ca^{2+} rise localized to the plasmamembrane but not the global Ca^{2+} response to $NAADP^+$ [307].

In this study, the L-type Ca^{2+} channel blocker verapamil reduced the response to $NAADP^+$ (Fig. 11, *A* and *B*). Although L-type Ca^{2+} channel expression has not been proven in astrocytes *in vivo*, primary cultured astrocytes are known to express VGCC [308-310]. Moreover, membrane depolarization of acutely isolated astrocytes by extracellular high K^+ was demonstrated to result in voltage-dependent Ca^{2+} influx [311;312], and astrocytes were shown to express subunits of VGCCs *in situ* by immunostaining [313;314]. The involvement of voltage-gated Ca^{2+} channels was also observed in other studies; the sperm-induced "cortical flash" at fertilization, which requires voltage-gated Ca^{2+} channels [315], is abolished by desensitization of the $NAADP^+$-system [307]. Also in mature starfish oocytes, the amplitude of the response to $NAADP^+$ could be strongly reduced by the L-type Ca^{2+} channel blockers verapamil and nifedipine [302]. Moreover, $NAADP^+$ induced a Ca^{2+}influx, as measured by the patch clamp technique, which could be significantly reduced by L-type Ca^{2+} channel blockers [281]. It has even been speculated about a physical link between lysosomally located $NAAPD^+$ receptors and Ca^{2+} channels in the plasma membrane [316] in analogy to the potential link of IP_3 receptors and capacitative Ca^{2+} entry channels [317].

4.1.5 $NAADP^+$ signaling requires both, lysosomes and thapsigargin-sensitive stores

The integrity and functionality of two intracellular compartments, the thapsigargin-sensitive and the GPN-sensitive ones, were essential for the ability of $NAADP^+$ to trigger Ca^{2+} signaling in astrocytes. GPN specifically destroys lysosomes, which are the suggested site of Ca^{2+} release by $NAADP^+$ and $NAADP^+$ receptor localization [95]. In addition, $NAADP^+$-triggered Ca^{2+} signaling crucially required the recruitment of thapsigargin-sensitive stores (Fig.12*A*). The Ca^{2+} release from the endoplasmic reticulum is mediated by IP_3 receptors, since the Ca^{2+} signal in response to $NAADP^+$ was almost completely eliminated by 2-APB, an IP_3 receptor blocker (Fig. 12*A*). Similarly, in starfish oocytes, the Ca^{2+} response was sensitive to thapsigargin [281], and thapsigargin also abolished the $NAADP^+$-stimulated secretion of insulin in human β-cells [291]. The fact that no massive Ca^{2+} release from the endoplasmic reticulum was detected after lysosomal disruption (Fig. 12), could be explained by the hypothesis that lysosomal activation by

$NAADP^+$ is a requirement for the subsequent activation of Ca^{2+} release from thapsigargin-sensitive stores. The link between lysosomal activation and activation of the endoplasmic reticulum could yet be provided by very restricted Ca^{2+} release from the lysosomes, too low or confined to too small compartments to be detected by the used recording system. Perfusion of slices with GPN itself induced Ca^{2+} responses in astrocytes (Fig. 12*B*) demonstrating that these organelles contain Ca^{2+} or that at least their disruption can trigger Ca^{2+} responses. Alternatively, the link could involve direct physical interaction of signaling cascade partners localised to lysosomes and to the endoplasmic reticulum.

4.1.6 Hypothetical mechanism of extracellularly applied $NAADP^+$-action

In the experimental setup used here, extracellular $NAADP^+$-triggered Ca^{2+} signaling in cortical astrocytes in slices is dependent on 1) connexin conductance, 2) extracellular Ca^{2+} and Ca^{2+} channels, 3) the integrity of lysosomal stores, and 4) Ca^{2+} release from thapsigargin-sensitive stores. At present, it can only be speculated how these different systems are interrelated (Fig. 29). The obtained results would be consistent with an intracellular action of $NAADP^+$, which enters into the cytoplasm via connexin hemichannels. However, this presumably applies merely to our simplified way of extracellular administration and may not be of physiological relevance, since $NAADP^+$ has so far only been recognized as an intracellular messenger. Moreover, there may be some purinergic but mainly adenosine receptor-mediated contribution. Therefore, the observed signal is most likely a superimposition or interplay of different components. Observed as a whole, activation of receptive sites, on or linked to lysosomes, seems to be an initial intracellular event prior to Ca^{2+} release from thapsigargin-sensitive stores and Ca^{2+} channel activation, which are both crucial for the observed Ca^{2+} response and may function as an amplifier. Under physiological conditions, lysosomes could be furthermore important as the site of $NAADP^+$ generation. The data generated here, indicate that Ca^{2+} release from thapsigargin-sensitive stores and Ca^{2+} influx by Ca^{2+} channel activation do not occur independently from each other.

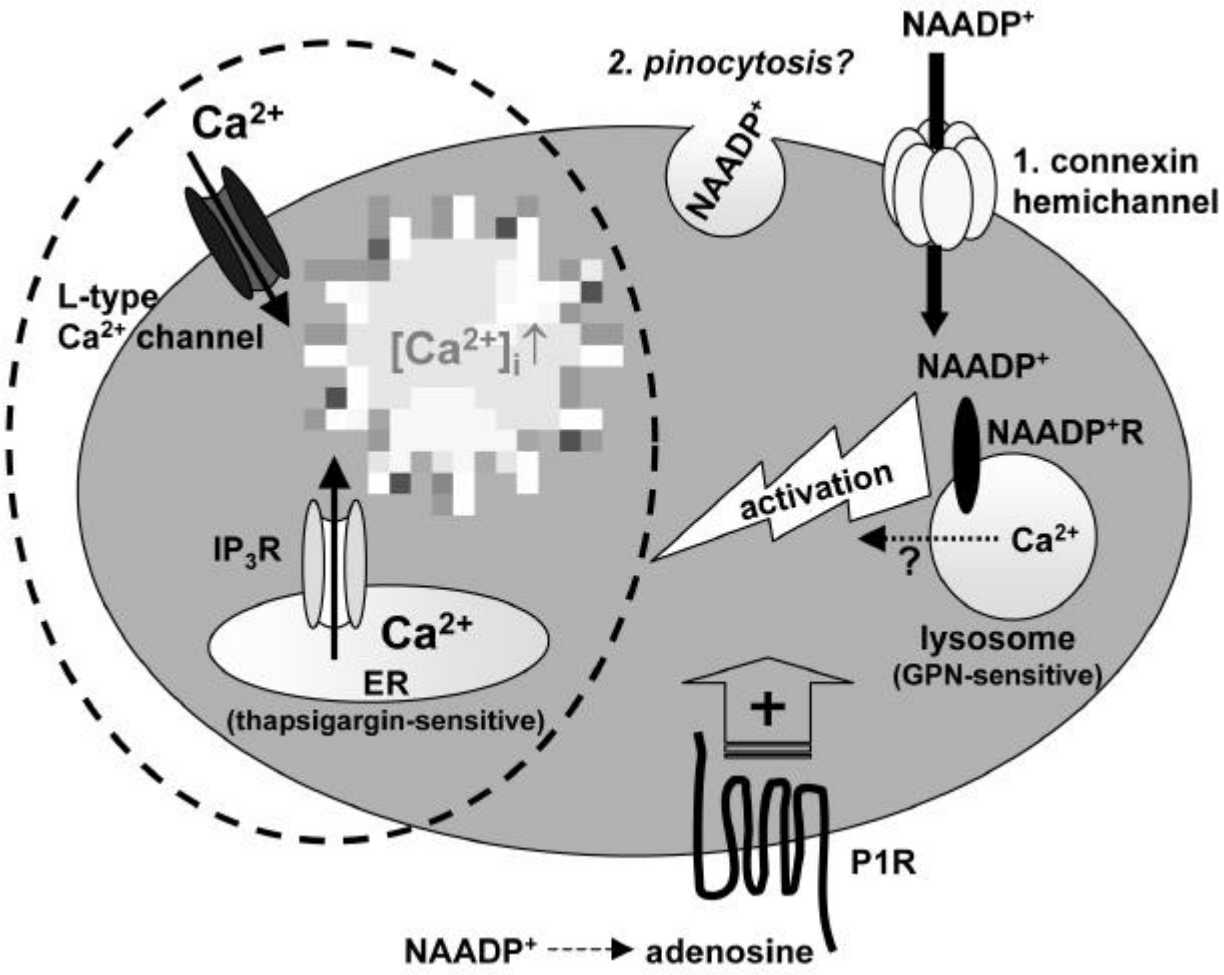

Fig. 29 Hypothetical mechanism of NAADP$^+$-action in astrocytes

This scheme illustrates a model of NAADP$^+$-action in cortical astrocytes, which is compatible with our experiments. NAADP$^+$ may at least partly enter the cell via connexin hemichannels. Inside the cell it first acts on receptors associated with lysosomes. This results in the activation of Ca^{2+} release from thapsigargin-sensitive stores (by IP_3 receptors) and Ca^{2+} influx (by L-type voltage-gated Ca^{2+} channels), which together leads to cytosolic Ca^{2+} increase. This signaling cascade could be modulated by adenosine, a potential degradation product of NAADP$^+$, acting on P1 receptors (P1R).

4.2 Stop of perfusion-induced Ca^{2+} signaling

4.2.1 Switching off of the perfusion induces Ca^{2+} signaling which is caused by the decrease in temperature

While carrying out other Ca^{2+} imaging experiments with brain slices at 30-33°C, it was observed that after switching the perfusion off, astrocytes start to exhibit Ca^{2+} signaling activity that is mainly oscillatory. This phenomenon was further investigated, since the astrocytes' translation of many extracellular stimuli into cellular responses, occurs through the second messenger Ca^{2+}, and oscillations in its free cytosolic concentration. Identification of circumstances inducing such Ca^{2+} oscillations, of their mechanisms and consequences, leads to a better understanding of how these cells interact with their environment. As astrocytes influence neuronal activity and play a role in many pathological processes, this knowledge contributes to a deeper insight into the information processing of the brain, as well as it provides possible medical targets. Moreover, it has methodological implications.

Prerequisite for observing this stop of perfusion-induced Ca^{2+} signaling in astrocytes were only low basal levels of spontaneous astrocytic Ca^{2+} signaling, which were registered during a perfusion of 31-33°C (Fig. 17, Fig. 18 and Fig. 20). It was realized that the stop of the perfusion was accompanied by a decrease in the temperature by several degrees Celsius in the perfusion chamber, as only the inflow tube was heated, but not the chamber (Fig. 19). The temperature decreased from 30-33°C to 22-24°C (RT). The collected data strongly suggest the drop in temperature to be the trigger for this phenomenon. First, switching off the heating of the inflow was able to mimic the stop of perfusion-induced signaling (Fig. 20). Second, the number of activated cells was strongly reduced when the perfusion chamber was heated to prevent the stop of perfusion-related drop in temperature (Fig. 21). Third, in all experiments the number of active cells was found to be higher at room temperature than at 31-34°C or 37-38°C, whether it was in the beginning of an experiment or during the course of it. Also, amplitude and frequency of the oscillations were higher at room temperature (Fig. 22, *C* and *D*, Fig. 23, and Fig. 24). Finally, at room temperature, switching off the perfusion did not clearly have the same effect as at 30-33°C. Activity, although relatively high before, increased when the perfusion was switched off, and pre-existing signaling patterns could be altered, but a considerable proportion of cells also remained

unaffected (Fig. 22, *A* and *B*). However, the activity increase often started before the perfusion was turned off (Fig. 22*A*). Moreover, at room temperature, the basal level activity of cells also increased within the first 30-180 seconds of a measurement, without any obvious stimulus (Fig. 22, *C*(*d*, *e*) and *D*(*d*, *e*), Fig. 23*D*, and Fig. 24*D*). Taken together with the observation that, at room temperature, it was not possible to evoke an effect following the second stop of perfusion, these results suggest that there are no major factors, other than the decrease in temperature, contributing considerably to the above-described phenomenon. The increase in Ca^{2+} activity, at constant room temperature, might be caused by phototoxic effects occuring during illumination of the preparation while imaging (see Discussion 4.2.4).

For the brain, it has not been demonstrated before that hypothermia directly affects cytosolic Ca^{2+} levels or signaling. Measuring at room temperature, "spontaneous" Ca^{2+} signaling activity in astrocytes, including oscillations, has been reported previously in acute slices of the ventrobasal thalamus (20-24°C) [224] and various other areas of the brain, including the neocortex (22-25°C) [226]. This spontaneous activity is thought to be intrinsically generated by astrocytes. However, the activity levels at room temperature were not compared with activity levels at higher temperature. In contrast, measuring at a slightly higher room temperature of ~25°C, it was found that this type of spontaneous activity occurs only in tissue under or pre-exposed to epileptiform conditions in the frontal, somatosensory and visual cortex [222]. "Rather quiescent $[Ca^{2+}]_i$ dynamics" during baseline anaesthesia were also observed in measurements *in vivo*, which increased by models of epilepsy [229]. Taken together, these findings are in line with the obtained data here, showing that astrocytic Ca^{2+} signaling activity is higher at lower temperature.

In other cell types (e.g. rabbit renal tubules, hepatocytes, parenchymal, endothelial, and Kupffer cells of the liver), it has been shown that acute hypothermia affects the intracellular Ca^{2+} homeostasis and goes along with a rise in cytosolic Ca^{2+} levels [318-320].

Mild hypothermia (systemic as well as brain-selective) is generally considered to be neuroprotective [294-296]. It has clinical relevance, and has been used effectively (with core temperatures as low as 32-34°C) as medical treatment, after many types of CNS injuries and during CNS surgery, to attenuate neuronal damage [293].

In the course of the present work, it was shown that the increase in astrocytic Ca^{2+} signaling activity, after a drop in temperature, first observed for a decrease from 30-33°C down to room

temperature (22-24°C), also occurs at higher temperature (drop from 37-38°C to 31-34°C). Therefore, it is relevant in the temperature range for clinical use of mild hypothermia, although in this range, the effect is less pronounced (Fig. 24).

4.2.2 The mechanism of hypothermia-induced Ca^{2+} signaling

4.2.2.1 Ca^{2+}-influx is mainly involved in hypothermia-induced Ca^{2+} signaling

There is not much known about the mechanisms by which hypothermia causes a general rise in intracellular Ca^{2+} concentration or increases Ca^{2+} signaling activity. The Ca^{2+} signaling described here, seemed to mainly result from Ca^{2+} influx, since Ca^{2+}-free buffer reduced the number of responding cells (Fig. 25). The previously reported spontaneous astrocytic Ca^{2+} oscillations, observed at room temperature, required both intra- and extracellular Ca^{2+}, however, extracellular Ca^{2+} was required mainly for store refill [224;226].

Although in the experiments carried out here, the number of responding cells was only slightly reduced, after the depletion of intracellular stores, by prolonged administration of thapsigargin (Fig. 25*A*), Ca^{2+} from intracellular stores may play a role in modulation of the response with regard to the oscillatory pattern. The Ca^{2+} signals were oscillatory in the majority of cases, and since PKC can be involved in the termination of astrocytic Ca^{2+} oscillations [34;139], a role of IP_3 in the drop in temperature-induced signaling is not unlikely. Furthermore, the thapsigargin treatment may not have sufficed to completely empty the intracellular stores, even though it did with respect to the Ca^{2+} responses to SNOG (NO donor; see Discussion 4.2.2.5) and to $NAADP^+$ (see Discussion 4.1.5).

A partial necessity for extracellular Ca^{2+}, in acute hypothermia-induced signaling, has been shown before in other cell types, such as parenchymal liver cells [320]. In rabbit renal tubules, hypothermia induces a typical biphasic Ca^{2+} response, requiring both, intra- and extracellular Ca^{2+}, for the initial spike and the prolonged $[Ca^{2+}]_i$ elevation, respectively [318]. In hepatocytes, the increase in cytosolic Ca^{2+} concentration in response to hypothermia is dependent on mitochondrial Ca^{2+} and Ca^{2+} from ryanodine-sensitive stores in the ER [319]. Therefore, the mechanism of hypothermia-dependent Ca^{2+} signaling appears to be rather cell type dependent, more than to be common to all cell types, but is very likely also determined by the degree of hypothermia.

Based on the general consequences of hypothermia, one could imagine that factors, such as altered membrane fluidity, changes in metabolic activity and cellular kinetics and consequently deregulated ion homeostasis, might account for the induction of Ca^{2+} signaling.

In cultured astrocytes, mild hypothermia leads to intracellular accumulation of Na^+, partially by activation of the Na^+-H^+ antiporter [73], which may cause [75] an inhibition or even reversal of the Na^+/Ca^{2+}-exchanger. This in turn may result in a rise of the intracellular Ca^{2+} concentration, since the basal cytosolic Ca^{2+} concentration of cultured astrocytes has been shown to mainly depend on a Na^+/Ca^{2+}-exchanger [153]. The hypothermia-induced ion shifts in astrocytes may also lead to membrane depolarization, which may activate VGCCs (see Discussion 4.1.4), as it was proposed for osmotic swelling-induced membrane depolarisation [76].

A mathematical model, based on several studies of hypothermia-evoked cellular ion fluxes, confirmed increased specific and inspecific Ca^{2+} fluxes across both, the plasmalemma and organelle membranes, in addition to a suppression of Ca^{2+} removal from the cytosol [321].

4.2.2.2 Hypothermia-induced swelling may contribute to the Ca^{2+} signaling

Another consequence of mild hypothermia, specifically shown for astrocytes and glioma cells [73-75], but also for neurons [322], is swelling. An indication of swelling upon a drop in temperature was observed as well in this study. Along with switching off the perfusion (30-33°C), usually a minimal movement of the tissue was observed. It was reversible after the perfusion was switched back on. This effect was due to the change in temperature, as the same effect occurred, when the heating was switched off and on.

Astrocytes are strongly involved in maintaining ion and water homeostasis within the brain [48], which causes them to swell under many conditions, especially in pathology [72]. Moreover, astrocytes are known to be highly mechanosensitive and to react with Ca^{2+} elevations to mechanical stress [80;81] including osmotic swelling [76;82-84]. Therefore, cold-induced swelling could also contribute to the observed Ca^{2+} signaling. Consequently, the effect of hyperosmolar mannitol was tested on the stop of perfusion/drop in temperature-induced Ca^{2+} signaling. Mannitol was chosen for its clinical relevance in anaesthesia and critical care medicine, by reducing brain swelling and edema, after many types of CNS injuries [296;298-300]. Moreover, it has been used for preincubation of brain slices to suppress swelling during drug

applications [165;297]. Here, under conditions where extra- and intracellular concentrations of mannitol equilibrate [323], the stop of perfusion-induced Ca^{2+} signaling was impaired in that Ca^{2+} elevations could occur, but no oscillations (Fig. 26, *A*, *B* and *C*). That mannitol itself induced Ca^{2+} signaling (Fig. 26, *A*, *B* and *D*) could be explained by the hypertonic mannitol solution causing a rapid volume recovery and rebound swelling after an initial shrinkage [323], followed by swelling evoked Ca^{2+} signaling. Taken together with the largely overlapping population of cells, in which mannitol and switching off the perfusion induced similar Ca^{2+} responses (Fig. 26*D*), these findings suggest a contribution of swelling to the drop in temperature-induced signaling.

Mechanistically, swelling upon exposure to hypotonic medium is best investigated in astrocytes, but results are diverse and not completely consistent. Hypoosmotic swelling is thought to induce an intracellular Ca^{2+} rise, consisting of an initial Ca^{2+} peak from intracellular stores followed by a plateau, which is partially due to influx through VGCCs and to CCE [76;82;83]. Hypotonic medium leads to a membrane depolarization by opening of non-specific cation channels [324], which is sufficient to induce voltage-gated Ca^{2+} influx [325]. However, the role of VGCCs in the Ca^{2+} influx is debated [84] (see Discussion 4.1.4). Recently, NMDA receptor-mediated Ca^{2+} influx alone has been suggested to be responsible for the Ca^{2+} rise triggered by hypoosmotic medium [264], which is in line with the predominant contribution of extracellular Ca^{2+} observed here. Generally, however, glutamate is believed to cause Ca^{2+} oscillations in astrocytes mainly by its action on mGluRs [225], and the requirement of those receptors was excluded here (Fig. 27; also see Discussion 4.2.2.4). Inhibition of mGluRs even enhanced the signaling, though not significantly. Therefore, stimulation of mGluRs may attenuate Ca^{2+} influx via NMDARs. Further experiments are required to evaluate a role of NMDA receptor-mediated Ca^{2+} entry in the hypothermia-induced signaling.

4.2.2.3 The Ca^{2+} signaling is not caused by neuronal activity or fluxes of substances through connexin channels

The drop in temperature and the associated cellular stress and swelling of the tissue could result in the release of substances, including neurotransmitters [72;76;79;85;86;88;264;265;326;327;327]. These substances may induce or enhance Ca^{2+} signaling in astrocytes. They may also affect neuronal activity, which is preserved at the

temperatures used here [328]; and astrocytes can respond with Ca^{2+} signals to closeby neuronal activity [58;329]. Connexin hemichannels can be involved in the release of "Ca^{2+}-active" and "neuro-active" substances, and they open under conditions, which may occur during hypothermia and swelling [87;330-333]. Connexin hemichannel-mediated autocrine astrocytic Ca^{2+} responses were demonstrated before [88;89;157;333]. In the here-conducted experiments, neither induction of neuronal activity nor the efflux of substances from connexin hemichannels seems to play a role for the astrocytic Ca^{2+} responses, as TTX and carbenoxolone, respectively, did not have any significant effects (Fig. 27). Carbenoxolone also blocks the gap-junctional conductance, which plays a role for the spread of Ca^{2+} signals in inter-astrocytic communication [214;215]. Therefore, astrocytes do not rely on their properties as a syncytium for the observed phenomenon either.

4.2.2.4 Hypothermia-mediated release of Ca^{2+} elevating substances from the tissue – A role for NO

Substances known to be released during cerebral stress and by swelling, which can induce Ca^{2+} signaling in astrocytes, include glutamate, ATP, adenosine, aspartate, GABA, NO, PGE_2, and endothelin [143;176;178;326;334]. Among the substances glutamate, ATP, adenosine, GABA and NO, for which receptor antagonists or inhibitors of synthesis were tested, only NO is involved in the Ca^{2+} signaling, because only after inhibition of NOS activity, the number of cells responding was largely reduced (Fig. 27). More evidence, for a role of NO in the Ca^{2+} activity recorded here, is that perfusion with the NO donor, SNOG, could mimic the stop of perfusion-induced signaling (Fig. 28, *A, B* and *D*).

NO has previously been shown to be involved in stretch-, Ca^{2+}-, and astrocyte-related signaling by several studies. There is a body of evidence that NO is produced by astrocytes upon mechanical stress, including swelling; and NO can induce Ca^{2+} signaling in astrocytes (see Introduction 1.4.1 and 1.4.2). According to recent findings [101;253;256;257;264], apart from neurons and endothelial cells, also astrocytes are capable of fast NO production and may be the source of NO, which may in turn act in an autocrine way, or rather as an intracellular messenger. The collected data give no direct information about whether NO is the initial trigger of the drop in temperature-induced Ca^{2+} signaling, or merely has a secondary role in its amplification. NO has already been suggested to function as an autocrine factor for astrocytes [264;265;327] and

even to be an endogenous Ca^{2+} influx factor, responsible for the refill of internal stores [101]. This, together with the fact that NOS is mainly activated by Ca^{2+} [335;335;336], supports the possibility of an amplifying role for NO. In the signaling observed here, NO synthesis might only be initiated after Ca^{2+} influx is caused by other mechanisms. However, in that case, a reduction in signaling amplitude, rather than in cell number, would have been expected. Though, indetectable Ca^{2+} influx appears to be sufficient to activate secondary amplifying processes, resulting in a full cellular Ca^{2+} signal, which can be detected [281]. Taking this possibility into account, the number of cells with detectable Ca^{2+} responses could be dependent on NO, despite its function as a signal amplifier.

Hypoosmotic swelling was found to result in NMDAR-mediated Ca^{2+} influx that led to activation of NO synthesis and to increased protein tyrosine nitration in astrocytes [264]. Direct modification of proteins by tyrosine nitration or S-nitosylation are two ways, by which NO can regulate the activity of Ca^{2+} channels [254]. However, in neurons, NO production upon Ca^{2+} influx through NMDA receptors leads to its tyrosine nitration, resultig in a reduction of Ca^{2+} influx, not an augmentation, as suggested by the present study [255]. Therefore, the mechanism involved here is likely to be different.

4.2.2.5 NO (SNOG) induces oscillatory Ca^{2+} responses in astrocytes in acute cortical slices

So far, in astrocytes, NO-induced Ca^{2+} signaling has only been shown in acute cerebellar [176] and organotypic hippocampal slices [257]. Here, it was demonstrated, for the first time, that NO can trigger Ca^{2+} signaling in astrocytes in acute cortical brain slices (Fig. 28*A*). Moreover, NO-evoked oscillatory Ca^{2+} elevations (Fig. 28*B*) have never been described before. The response caused by NO was due to both, Ca^{2+} influx and Ca^{2+} release from intracellular stores, since nominally Ca^{2+}-free buffer and preincubation with thapsigargin reduced the number of responsive cells significantly (Fig. 28*C*). However, the effect of thapsigargin was much more pronounced, as it nearly abolished SNOG-induced responses (Fig. 28*C*). Prior reports about the Ca^{2+} elevating mechanism of NO are not consistent. In some studies, it is suggested that NO exclusively causes Ca^{2+} influx [101;176], others claim a contribution of both, influx and intracellular stores, similarly to the observations made here [258] (also see Introduction 1.4.1) [256;257]. Even

though Ca^{2+} influx was involved in this study, SOCCs, as suggested before [101], were not the mediating channels, since La^{3+} did not have any effect (Fig. 28*C*).

With regard to the role of NO in the hypothermia-induced Ca^{2+} signaling, the findings might appear contradictory, as this signaling was mainly due to Ca^{2+} influx, but NO applied directly, caused both influx and an even more prominent release. Considering NO as an intracellular messenger in astrocytes, a possible explanation would be that in intrinsic NO signaling, the subcellular localization of NO synthesis determines the effect of NO [207;243]. NOS, localized at the cell membrane, may be selectively activated by Ca^{2+} influx, and may lead to further, NO-dependent Ca^{2+} influx. Whereas, for exogenously applied excess NO, an effect on intracellular stores may be dominant.

4.2.2.6 Phototoxicity may contribute to Ca^{2+} signaling at RT

The observation of Ca^{2+} signaling starting or increasing, without further stimulus after 30-180 seconds at room temperature (Fig. 22, C(*d*, *e*) and D(*d*, *e*), Fig. 23*D*, and Fig. 24*D*), may be explained by phototoxic effects. These are caused by the light-induced excitation and subsequent disintegration of the Ca^{2+}-sensitive dye, accompanied by the production of free radicals and oxidative stress. Photoinstabiliy of Fluo-4 was demonstrated previously [337] and it has been reported before that imaging-related illumination of Fluo-4 loaded cells can evoke Ca^{2+} responses by itself [338]. Increasing basal level activity was not seen at higher temperatures of 30-33°C (Fig. 18 and Fig. 20) or 37-38°C (Fig. 22, *C*(*f*, *g*) and *D*(*f*, *g*)). This was probably not due to increased decomposition of the dye at room temperature, but to an elevated astrocytic sensitivity to external stimuli at lower temperature. In support, hypoosmotic swelling of astroglioma cells increased their susceptibility to oxidative stress [339]. Consequently, phototoxicity may contribute to the higher signaling levels observed at room temperature and to the switch-off of the perfusion/drop in temperature-induced signaling. Switching off the perfusion at room temperature may have an additional effect due to the accumulation of reactive oxygen and nitrogen species. Their accumulation could be responsible for the change in the signaling pattern in already active cells at the time point of switching the perfusion off (Fig. 6*B*).

4.2.3 Possible functions of hypothermia-induced Ca^{2+} signaling

The results obtained here, do not allow drawing a final conclusion about whether the induced Ca^{2+} signaling activity has protective functions or is damaging. Ca^{2+} is a second messenger shared by many cellular signaling cascades, leading to the most diverse cellular processes such as the onset of protective mechanism and cell death [134].

Mild hypothermia (systemic and brain-selective) is generally considered to be neuroprotective [294-296] and has clinical relevance within the temperature range used here [293], although its mechanisms of action are not completely understood [293-296]. With regard to astrocytic cell cultures, hypothermia was found to decrease their injury following glucose-oxygen deprivation [340]. In neuronal cell cultures, *in situ* in brain slices, and in animal studies it was shown to reduce many of the cytotoxic consequences of brain injury and pathology [341-343]. In clinical studies, the protective effect of hypothermia has been mainly ascribed to the reduction of brain swelling. In turn, hypothermia itself has been shown to induce swelling in neurons and astrocytes, which is supported by this study. Moreover, hypothermia did not prevent astrocytic swelling caused by other factors thought to be involved in brain pathologies and after injury [73;74;74;344]. Astrocytic swelling is generally assumed to be detrimental [72;339] and suggested to be a signal for proliferation and thus to play a role in the induction of gliosis [64;71;345]. Astrocytic swelling after ischemia and trauma is mainly thought to be damaging, by the swelling-activated excitatory amino acid (EEA) release through VRACs [72;85]. On the other hand, tissue swelling can be caused in two ways, by vasogenic (extracellular) or intracellular edema, and hypothermia rather leads to a reduction of vasogenic than cellular edema [346]. Astrocytic swelling appears to generally lead to the gene expression of aquaporins [347-349], which are involved in the reabsorption of excess fluid in vasogenic brain edema [346]. The here observed Ca^{2+} signaling may play a role in aquaporin induction [82;350], thereby counteracting vasogenic swelling in clinically applied hypothermia [351]. Another protective effect of the described Ca^{2+} signaling may be its role in regulatory volume drecrease (RVD), an active way of cells to reduce their volume after swelling. In astrocytes this RVD was shown to be dependent on Ca^{2+}-influx [76].

Even though here, mild hypothermia was observed to induce increased Ca^{2+} signaling activity, mild hypothermia (33 and 29°C) was shown to decrease the Ca^{2+} overload occurring and involved in causing damage following reperfusion after glucose-oxygen deprivation [352-354]. In

a trauma model, pharmacologically induced Ca^{2+} oscillations were able to prevent the detrimental cytosolic Ca^{2+} increase usually following trauma [355]. Therefore, controlled Ca^{2+} oscillations may be involved in counteracting deleterious cellular Ca^{2+} overloads. This may apply for the Ca^{2+} oscillations induced by mild hypothermia investigated here. Hypothermia-triggered Ca^{2+} signaling may therefore be an unacknowledged contributor to the beneficial effects of hypothermia and may play a role in reducing edema.

On the other hand, during cold preservation of organs, the disturbance of Ca^{2+} homeostasis caused by hypothermia is generally considered a detrimental factor. Ca^{2+} signaling is also involved in triggering reactive astrogliosis [143;222], which could make secondary adverse contributions to pathology, while reactive astrocytes have not been found to show spontaneous Ca^{2+} elevations in a study carried out at room temperature [226].

To summarize, it was shown that the observed stop of perfusion-induced Ca^{2+} signaling in astrocytes in acute cortical brain slices is caused by a temperature decrease. This is also relevant at temperatures clinically used for mild hypothermia. The Ca^{2+} signaling resulted mainly from Ca^{2+} influx. Hypothermia-induced swelling and NO, most likely as an autocrine amplifier, contributed to the signaling. The acquired data do not allow to completely differentiate between an effect induced by a change (a decrease) in temperature and absolute temperatures. Two effects may be observed in parallel; a rapid drop in temperature may induce a rather fast and transient signal, while at lower temperatures, the signaling activity is generally higher in addition. Also, phototoxicity may contribute to the elevated activity level at room temperature. Exogenous NO (SNOG) was identified as Ca^{2+} oscillation-inducing stimulus for cortical astrocytes *in situ*, which mainly causes Ca^{2+} release from intracellular stores.

More experiments will be needed to distinguish between effects of temperature changes and absolute temperatures, to further evaluate the mechanism by which NO is involved, and to identify other temperature-dependent factors contributing. Also, the extent of contribution of phototoxicity should be further quantified. Most importantly, the cellular consequences of this type of hypothermia-induced signaling need to be investigated.

A deeper knowledge about the cellular effects of hypothermia should ultimately contribute to a more specific clinical application, and a better combination with other therapies. It would, moreover, be important in improving cryopreservation of immature neurons for subsequent therapeutic intracerebral transplantation, and cryopreservation of organized adult cerebral tissue slices of potential interest for pharmaceutical drug development [356].

In addition, the above findings are of methodological relevance. Many studies on astrocytic cultures or brain slices are carried out at room temperature [224;226]. The results obtained here, demonstrate that astrocytes show altered Ca^{2+} signaling behavior at room temperature, in comparison with more physiological temperatures. Therefore, any measurements on astrocytes at room temperature are prone to artifacts, as Ca^{2+} signaling can account for many cellular processes, possibly influencing the measured parameters. Since astrocytic Ca^{2+} signaling was demonstrated to affect neuronal activity, the same applies for measurements of neuronal parameters, in brain slices or mixed cultures.