1 INTRODUCTION

1.1 Glial cells – Astrocytes

Apart from neurons, the central nervous system is composed of several other non-neuronal cell types, the “neuroglia” or “glial” cells, which actually outnumber neurons [1]. The term “neuroglia” was coined by Rudolph Virchow in 1856 for the interstitial, cement-like substance surrounding neurons in the brain. He did not use the term neuroglia for single cells, but to describe a connective tissue containing some cellular elements. Other scientists like Heinrich Müller, Otto Deiters, Michael von Lenhossek, Jakob Henle and Friedrich Merkel were involved in the discovery that the neuroglia consisted of single cells, which were thereafter referred to as neuroglia. Due to the development of metallic tissue staining techniques by Camillo Golgi in the late 19th century and their advancement and use by Ramon y Cajal and Pio del Rio-Hortega in the early 20th century, this view was confirmed and neuroglia could be subdevided into micro- and several types of macroglial cells [1;2]. As their name already implicates, the electrically not excitable glial cells were originally thought to be not more than neuronal glue, the filling material between the neurons, rendering them structural support. With time this passive view of glial cells regarding brain function has changed dramatically. The diversity among glial cells and the importance of glial cells, reaching far beyond structural support, has become more and more evident. Indicating their significance for brain function, the ratio of glia to neurons increases up the phylogenetic scale so that they account for about 50 percent of the higher vertebrate brain volume and up to 90 percent of the brain cell number. Furthermore the differentiation of glia is greatest in higher vertebrates [1;3].

Microglia are the immunocompetent cells of the central nervous system (CNS) and can undergo a graded transition from ramified resting cells into activated, cytotoxic and phagocytic cells [4;5]. They are believed to be of mesodermal origin and to stem from a monocytic lineage [6], although this is still debated [7]. Macroglia are of ectodermal origin and include myelinating glia and astroglia. The myelinating glia of the CNS are oligodendrocytes, which insulate neuronal axons by enwrapping them with a myelin sheath [8;9]. Astrocytes are the most common cell type within the human brain comprising 80 percent of all cells [1]. Their name was derived from their very often star-shaped, process-bearing appearance [10]. However, they can display diverse

morphologies (see below). They are characterized by the expression of the intermediate filament proteins glial fibrillary acidic protein (GFAP) and vimentin, the calcium binding protein S-100β, and glutamine synthetase (GS) [11]. Typical for astrocytes is their contact to neurons on one hand and the blood system or the pia mater on the other (Fig. 1) [1]. Another feature is their ability to form large syncytia by gap junctional coupling [12-16], mainly via connexin 43, but other connexins are expressed as well [17;18]. The extent of coupling appears to be brain region specific [19]. Astrocytes are not a homogeneous cell type, but can be differentiated in various ways. Morphologically, they were subdivided into fibrous or stellate astrocytes, mainly found in white matter, and protoplasmic astrocytes, predominantly located in gray matter [20-22]. Astrocytic sub-types, which have been traditionally distinguished, are the retinal Müller cells [23] and the cerebellar Bergmann glia [24]. Astrocytes were also found to have different properties within a given brain region like the hippocampus, where they exhibit different electrophysiological profiles [25;26].

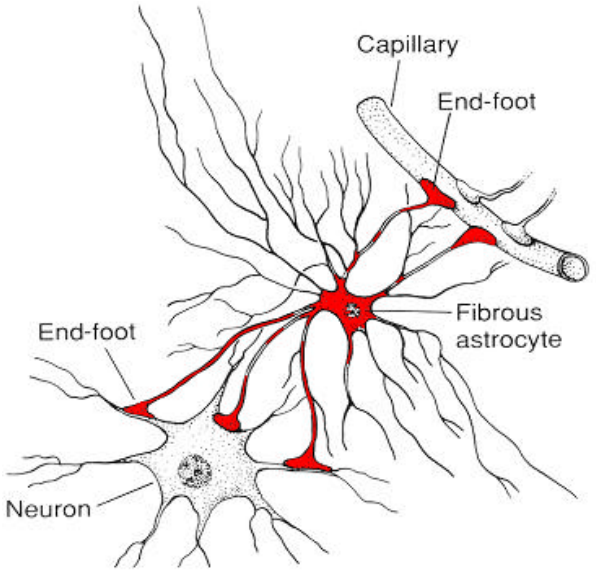

Fig. 1 Fibrous astrocyte
A fibrous white matter astrocyte is contacting neurons and capillaries with specialized structures at the end of its processes, the end feet. Figure is copied from E. Kandel, 1995 [3].

1.1.1 Role of astrocytes in the intact brain

It is now known that astrocytes have a variety of functions, apart from structural ones, including homeostatic, metabolic, trophic, signaling, and in pathology neuroprotective ones [1;27]. Astrocytes are involved in inducing and keeping up the blood-brain barrier. With structures at the tips of their processes termed “end feet”, they are in contact with endothelial

cells of the blood brain barrier and are inducing and maintaining the tight junctions between those cells [28]. They are also important in angiogenesis in development and during repair processes [29]. Latest research has demonstrated that astrocytes mediate the control of blood vessel diameter and therefore blood flow, as a consequence of neuronal activity [30-34]. Astrocytes also give nutritional support to neurons by providing the major glycogen store of the brain and are thought to be responsible for fast energy supply to neurons in form of lactate [35]. Another role is their up-take of glutamate [36;37] and its conversion to glutamine. Glutamate is taken up by the electrogenic glutamate transporters GLT-1 and GLAST [38;39]. Glutamine is then provided back to neurons, which are devoid of the enzyme glutamine synthetase. This is referred to as "glutamate-glutamine cycle" [40;41]. Enwrapping synapses with their processes, astrocytes are generally involved in neurotransmitter-clearance from the synaptic cleft and thus contribute to termination of neuronal signaling. Additionally to glutamate, astrocytes can take up γ-aminobutyric acid (GABA) [42], glycine [43] and taurine [44]. Furthermore they have been demonstrated to control the ionic composition of the cerebrospinal fluid, mainly with regard to K^+, Ca^{2+}, and H^+. Neuronal activity goes along with an accumulation of K^+ in the extracellular space. Astrocytes remove this excess K^+ and release it again after cessation of neuronal activity or redistribute it within their syncytium by "spatial K^+-buffering currents" to areas of lower neuronal activity [45;46]. Astrocytes express the electrophysiological properties to keep the extracellular K^+ concentration $[K^+]_o$ around 3 mM [47]. In line with this, astrocytes seem to be the main regulators of water homeostasis within the brain. They optimize interstitial distances by water up-take, which involves aquaporin 4, a water channel [48]. Under hypoosmolar conditions, astrocytes release the osmoregulatory amino acid taurine through volume regulated anion channels (VRACs) [49-51]. Astrocytes have further neuroprotective functions including the clearance of free radicals [52;53]. Being a source of extracellular matrix and adhesion proteins, as well as of a variety of trophic factors, astrocytes contribute to neuronal migration, formation of nuclei and neuronal differentiation during development [54-56]. In the adult brain they are required for neuronal survival, synapse formation and maintenance, and are even involved in regulating synaptic efficacy [54;57]. Within the past 15 years, evidence has accumulated that astrocytes can sense, respond to, modulate, and integrate synaptic activity locally and possibly over larger distances [1;58-60]. Therefore, they can directly take part in brain plasticity and

information processes. A model of bidirectional signaling between astrocytes and neurons, the model of the “tripartite synapse” evolved [61].

1.1.2 Role of astrocytes in pathology - Reactive astrogliosis and swelling

Astrocytes have been attributed a major role in pathological conditions [62-64]. In response to all types of brain injury, astrocytes become activated. This activated state, termed “reactive astrogliosis”, is accompanied by an increased GFAP expression and changes in proliferation and morphology. Astrogliosis results in the outgrowth of many cellular processes [65;66]. During reactive astrogliosis, astrocytes can release substances such as cytokines, arachidonic acid and prostaglandins or nitric oxide (NO), which contribute to inflammatory processes [67].

Soluble inflammatory factors [62], but also mechanical disturbances caused by head injuries or by secondary effects, such as vasogenic edema or astrocytic swelling, following many pathological conditions [64;68-71], can be a trigger for reactive gliosis. Signals, which cause astrocytic swelling, are high extracellular [K^+], acidification, ammonia, decrease in medium osmolarity, pathological states associated with energy depletion, glutamate, TNFα, arachidonic acid [72], and hypothermia [73-75]. Astrocytes are well prepared to compensate for changes in the extracellular osmotic pressure, since they are capable of regulatory volume decrease (RVD), an active mechanism for volume reduction after swelling [76]. In addition astrocytes are known to be mechanosensitive. They express stretch-activated ion channels, such as a stretch-activated K^+ channel [77;78] and volume regulated anion channels (VRACs) permeable for Cl^- [79]. Mechanical stress by direct mechanical stimulation [80;81] or by osmotic swelling of cultured astrocytes increases their cytosolic Ca^{2+} levels [76;82-84]. As a consequence of swelling, astrocytes release small excitatory amino acids (EAAs), such as glutamate, aspartate, glycine, and taurine, via VRACs [72;76;79;85;86] and possibly other substances via the mechanosensitive connexin hemichannels [87-89].

It is not entirely clear whether reactive astrocytes possess beneficial or detrimental properties, as both have been reported [64]. Moderate astrogliosis may exert neuroprotective functions and contribute to regenerative processes, while excessive astrogliosis may result in neuronal damage and loss.

1.2 Ca^{2+} signaling

Calcium ions (Ca^{2+}) are ubiquitously used as an intracellular second messenger. The basal level free Ca^{2+} concentration of the cytosol ($[Ca^{2+}]_i$) is kept very low (below 100-200 nM) in comparison to the extracellular concentration, which is around 20000-fold higher. Even upon stimulation, the cytosolic Ca^{2+} concentration does not exceed 10 μM [90]. Rises in cytosolic Ca^{2+} content constitute cellular signals, which are decoded and translated into a variety of cellular processes [90;91]. Some proteins are affected by binding Ca^{2+} directly, others by association with small Ca^{2+}-bound proteins, such as calmodulin (CAM). Cells contain many different types of proteins with Ca^{2+} binding sites or binding sites for the Ca^{2+}/CAM complex, including enzymes, ion channels and transcription factors [90-92].

The low $[Ca^{2+}]_i$ is maintained by a Na^+ gradient-driven Na^+/Ca^{2+}-exchanger and a plasma membrane Ca^{2+}-ATPase (PMCA), which pumps Ca^{2+} actively out of the cell. Moreover, Ca^{2+} is sequestered by the sarco-endoplasmatic reticulum Ca^{2+} ATPase (SERCA) into the major intracellular Ca^{2+} store, the endoplasmatic reticulum (ER) [90;91]. The ER Ca^{2+} ATPase can be inhibited by thapsigargin [93]. During Ca^{2+} signaling, mitochondria can also take up Ca^{2+} by the mitochondrial Ca^{2+} uniporter (MCU) located in the organelle's inner membrane [94]. Small acidic intracellular compartments, such as lysosome-like organelles [95] and secretory vesicles [96;97], have recently been shown to also function as targetable intracellular Ca^{2+} stores. Lysosome-like organelles can take up Ca^{2+} driven by the pH gradient, through a Ca^{2+}/H^+-exchanger [95]. Additionally, cells contain Ca^{2+} binding proteins, such as calbindin, calretinin, and parvalbumin, whose function it is to buffer cytosolic free Ca^{2+} [90;91].

Rises in intracellular Ca^{2+} concentration can be initiated in multiple ways (Fig. 2). Generally it is distinguished between Ca^{2+} influx from the extracellular space through ion channels in the plasmamembrane and Ca^{2+} release from intracellular stores. Ca^{2+} influx can occur via receptor-operated (ligand-gated) Ca^{2+} channels (ROCCs), also called ionotropic receptors, via voltage-gated Ca^{2+} channels (VGCCs), which allow Ca^{2+} influx upon membrane depolarisation, and via stretch-activated Ca^{2+} channels (SACCs), which open in response to mechanical stimuli. Furthermore cells possess store-operated Ca^{2+} channels (SOCCs), also named calcium release-activated channels (CRACs), which open after the depletion of internal Ca^{2+}-stores and operate their refill [90;91]. This replenishing phenomenon is consequently referred to as store-operated Ca^{2+} entry [98], capacitative calcium entry (CCE) [99], or calcium release-activated calcium

current (CRAC) [100;101]. SOCCs are thought to belong to the family of TRP channels, which also includes SACCs, VGCCs, and thermosensitive channels [102-104].

As described above, cells posses several intracellular Ca^{2+} stores. There are two major pathways leading to Ca^{2+} release from the ER. One involves activation of inositol-1,4,5-trisphosphate receptors (IP_3R) [105], the other activation of ryanodine receptors (RYR), which were named after an exogenous agonist, the plant alkaloid ryanodine [106]. The inositol-1,4,5-trisphosphate (IP_3) pathway is thought to be mainly regulated by activation of metabotropic receptors (mRs), which are coupled to G-protein-activated signaling cascades, or by activation of receptor tyrosine kinases (RTKs). Both receptor types can lead to the activation of phospholipase C (PLC) isoforms; the former is linked to PLCβ, the latter to PLCγ [107]. However, there is also indication that receptors connected to adenosine cyclase activity can lead to PLCε activation mediated by a cyclic AMP-activated rap GTPase [108]. Another PLC isoform, PLCδ, is Ca^{2+} activated [109]. PLC hydrolizes phosphatidylinositol-4,5-bisphosphate (PIP_2), into inositol-1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3 consequently leads to Ca^{2+} efflux through IP_3Rs on the ER [110;111]. Ca^{2+} itself further modulates Ca^{2+} release by these receptors. At low concentrations Ca^{2+} is stimulatory, at high ones inhibitory [112-114]. Recently, Ca^{2+} binding proteins in the Ca^{2+}-bound forms were shown to activate IP_3Rs independently of IP_3 [115]. The circumstances, which induce the opening of the RYRs are not equally transparent. It is generally consented that RYRs are directly activated by Ca^{2+} [116;117]. RYRs are moreover positively modulated by cyclic ADP ribose (cADPR) [118;119]. Mammalian cells express a type-II transmembrane glycoprotein ADPR-cyclase, termed CD38, and a soluble cytosolic ADPR-cyclase [120;121]. ADPR-cyclase activity was observed to be coupled to metabotropic receptors in a G-protein-dependent way [122][123]. NO in turn activates ADPR-cyclase via phosphorylation by protein kinase G (PKG), which is switched on by NO-dependent 3',5'-cyclic guanosine monophosphate (cGMP) production. Additionally, NO seems to be able to directly activate RYRs by nitrosylation of regulatory thiol groups [124]. As for IP_3Rs [125], kinases and phosphatases, and the redox state and energy charge of cells are further modulators of RYRs [126]. Therefore, RYRs may also be stimulated directly without prior elevation in cytosolic Ca^{2+}. Due to their properties of being Ca^{2+}-modulated, both receptors can account for a phenomenon termed Ca^{2+}-induced Ca^{2+} release (CICR), in which previously released Ca^{2+} amplifies Ca^{2+} release from the ER [116;127;128]. Additionally, Ca^{2+} can indirectly enhance Ca^{2+} release from

both receptor types, since some isoforms of PLC, AMP cyclase and nitric oxide synthase (NOS) are Ca^{2+} activated [90;91].

Another intracellular Ca^{2+} releasing messenger, nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) [129], has been found to mainly release Ca^{2+} from the acidic organelle Ca^{2+} stores mentioned above [95-97]. $NAADP^+$ is synthesized by the same enzyme as cADPR [120], but unlike for cADPR, its synthesis has not yet been directly linked to metabotropic receptor activation. $NAADP^+$ acts on a hypothetical nicotinic acid adenine dinucleotide phosphate receptor ($NAADP^+R$) connected to a Ca^{2+} channel, whose protein has been isolated, but which has not been cloned, yet [130;131].

These different means of elevating the intracellular cytosolic Ca^{2+} level can and have been shown to interact on many levels. There are several ways by which those mechanisms can feed back on each other and can amplify each other. Due to the complexity of the machinery in cells, which controls the cytosolic Ca^{2+} concentration, complex temporal and spatial patterns of rises in intracellular Ca^{2+} concentration can be evoked [132]. Ca^{2+} elevations can remain very locally restricted, termed "Ca^{2+} puffs" or "Ca^{2+} sparks", or propagate wave-like throughout the whole cell causing a global Ca^{2+} signal. The signals can be transient spikes, oscillatory or reach plateau levels, moreover combinations of all these types [133].

Elevations in Ca^{2+} can initiate a whole array of the most diverse biological processes, such as fertilization, proliferation, differentiation, cell motility, muscle contraction, secretion, neurotransmission, and apoptosis [90;134]. Therefore, it is evident that Ca^{2+} signals must convey some specificity. The spatiotemporal pattern of Ca^{2+} elevations seems to encode this specific information [132;133;135]. Cells posses an equally elaborate machinery to decode these specific signals. Many components of intracellular signaling cascades are sensitive to Ca^{2+} directly or to the Ca^{2+}/CAM complex. Ca^{2+} signals can evoke immediate effects via the activation of protein kinases, but also lead to gene transcription via the activation of transcription factors [132;133;135]. The main known proteins decoding frequency-modulated Ca^{2+} signals are CaMKII [136;137] and PKC [138-140]. Spatial decoding is achieved by clustering of signaling cascade components and their effector molecules in large complexes near microdomains of Ca^{2+} release or influx [141].

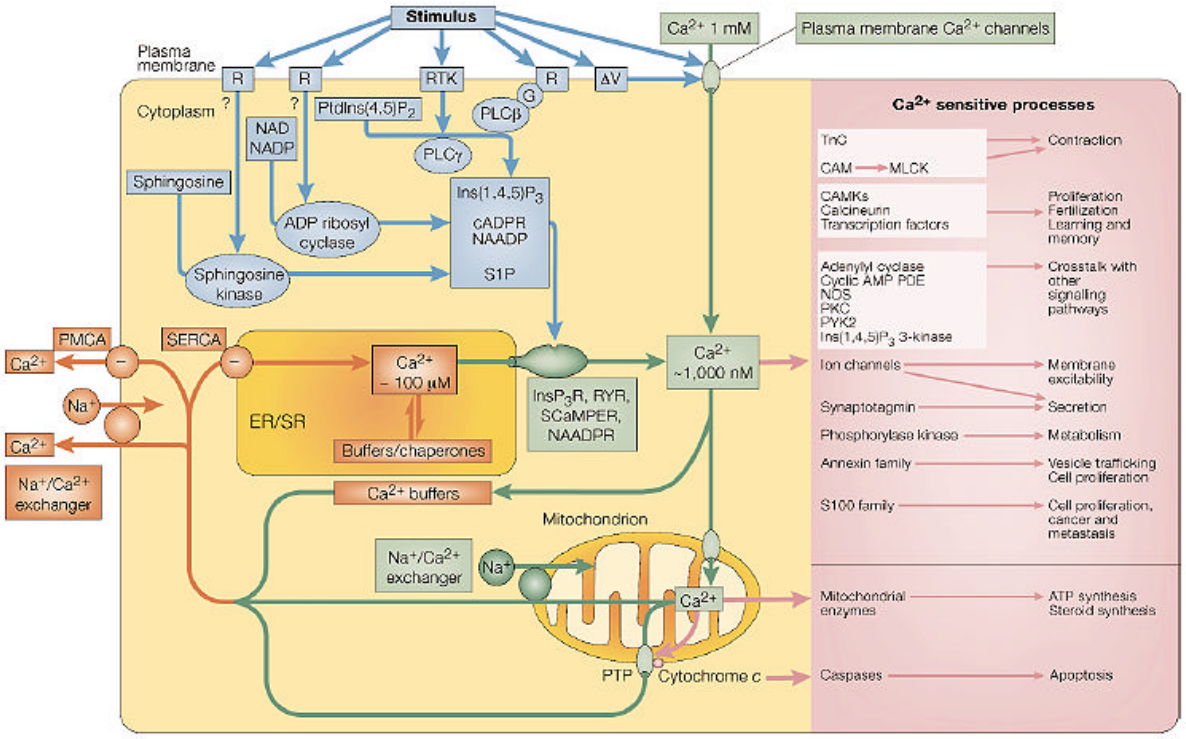

Fig. 2 Elements of the Ca^{2+} signaling toolkit

Cells have an extensive signaling toolkit that can be mixed and matched to create Ca^{2+} signals of widely different properties. Ca^{2+}-mobilizing signals (*blue*) are generated by stimuli acting through a variety of cell-surface receptors (R), including G-protein (G)-linked receptors and receptor tyrosine kinases (RTK). The signals generated include: inositol-1,4,5-trisphosphate (Ins(1,4,5)P3), generated by the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) by a family of phospholipase C enzymes (PLCâ, PLCã); cyclic ADP ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP), both generated from nicotinamide-adenine dinucleotide (NAD) and its phosphorylated derivative NADP by ADP ribosyl cyclase; and sphingosine 1-phosphate (S1P), generated from sphingosine by a sphingosine kinase. ON mechanisms (*green*) include plasma membrane Ca^{2+} channels, which respond to transmitters or to membrane depolarization (ÄV), and intracellular Ca^{2+} channels — the Ins(1,4,5)P3 receptor (InsP3R), ryanodine receptor (RYR), NAADP receptor and sphingolipid Ca^{2+} release-mediating protein of the ER (SCaMPER). The Ca^{2+} released into the cytoplasm by these ON mechanisms activates different Ca^{2+} sensors (purple), which augment a wide range of Ca2+-sensitive processes (purple), depending on cell type and context. OFF mechanisms (*red*) pump Ca^{2+} out of the cytoplasm: the Na^{+}/ Ca^{2+} exchanger and the plasma membrane Ca^{2+} ATPase (PMCA) pumps Ca^{2+} out of the cell and the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps it back into the ER/SR. (TnC, troponin C; CAM, calmodulin; MLCK, myosin light chain kinase; CAMK, Ca^{2+}/calmodulin-dependent protein kinase; cyclic AMP PDE, cyclic AMP phosphodiesterase; NOS, nitric oxide synthase; PKC, protein kinase C; PYK2, proline-rich kinase 2; PTP, permeability transition pore.) Figure and legend are cited from Berridge et al., 2000 [90].

1.3 Ca^{2+} signaling in astrocytic communication

1.3.1 Ca^{2+} excitability of astrocytes

Astrocytes were found to express a variety of neurotransmitter receptors [142;143] and ion channels [144] comparable to neurons. Since astrocytic processes ensheath synapses [145-147] they can sense neuronal activity by spillover of neutrotransmitters from the synaptic cleft. Recently, direct synapse-like connections from neurons to macroglial NG2-expressing cells, which have some, but not all features in common with astrocytes, and which may be astrocytic or oligodendrocytic precursor cells, were discovered [148;149]. Moreover, astrocytes can be coupled to neurons by gap junctions [150;151].

Unlike for neurons, activation of neurotransmitter receptors does not lead to the formation of action potentials. Instead, most receptors on astrocytes are coupled to a transient intracellular rise of the second messenger Ca^{2+} [142;143]. Apart from classical neurotransmitters, many other substances, such as adenosine, ATP, various hormones, neuropetides, growth factors, chemokines and prostaglandins and NO are able to evoke a glial rise in intracellular Ca^{2+} [142;143].

The intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in resting condition shows a great variability in glial cells and was determined from 30 up to 400 nM. After activation, it can rise to levels of 1000 nM. Free cytoplasmatic Ca^{2+} comprises only a very small fraction (<0.001%) of the total intracellular Ca^{2+}, which is mainly either bound to cytosolic Ca^{2+} buffering proteins (like S-100β, which is unique for astrocytes) or actively accumulated by and stored in intracellular organelles. Astrocytes were demonstrated to posses a low Ca^{2+} buffering capacity, which means that even minor Ca^{2+}-influx or release of Ca^{2+} from intracellular stores leads to a substantial rise in $[Ca^{2+}]_i$ [152]. Astrocytes very likely posses the complete Ca^{2+} signaling machinery described earlier. Their resting Ca^{2+} level was found to be mainly controlled by a genistein-sensitive Na^+/Ca^{2+} exchanger [153]. The presence of RYRs in astrocytes is still debated, but they have been shown to express a specific brain ryanodine receptor isoform (RYR3) [154;155], which is positively modulated by cyclic ADP ribose (cADPR) [156]. In this way, stimuli inducing ADP ribosyl cyclase activity may also directly induce a Ca^{2+}-rise alternatively to the IP_3-pathway in astrocytes. Extracellular application of cADPR was shown to induce a Ca^{2+} rise in astroctes [157] and Müller cells [158]. The functionality of the $NAADP^+$ system had not yet been tested in astrocytes. As in other cell types, Ca^{2+} controls several basic cellular functions in astrocytes. It

regulates glycolysis [159], triggers, as in neurons, vesicular release of neurotransmitters [160-162], and is especially important in inter-glial [163;164] and neuron-glia communication [58-60].

Astrocytes can express agonist-specific and concentration-dependent patterns of Ca^{2+} reactions [143]. Several factors, such as PKC [139] and growth factors via a MEK-regulated mechanism [165], were shown to promote oscillatory Ca^{2+} responses to glutamate in astrocytes, whereas proinflammatory cytokines suppressed them [165]. Repeated application of an oscillation-stimulating concentration of glutamate resulted in an increase in oscillatory frequency [166]. In conclusion, responses of astrocytes to their environment, but also intrinsic activity can be most readily detected by observing changes in the intracellular Ca^{2+} concentration with the Ca^{2+} imaging method. Referencing electrical excitability of neurons, whose activity is recorded with electrodes, the term "Ca^{2+} excitability" has been coined for astrocytes.

Up to date, it has been experimentally proven, first in mixed cultures [167] and in organotypic slices [168], later in acute brain slices [169] that astrocytes can react with increases of $[Ca^{2+}]_i$ to closeby neuronal activity [58;60]. Astrocytes in culture were shown to react with different Ca^{2+} signaling patterns to different transmitter concentrations [143;166]. Their type of reaction can therefore even reflect the degree of neuronal activity. In response to from low up to high neuronal activity, the signals can remain locally confined to single astrocytic processes [146;147] or spread throughout the whole soma of the astrocyte accordingly [166;170;171]. Also according to the strength of neuronal stimulation, the rise in astrocytic Ca^{2+} can be transient with the amplitude dependent on the extent of neuronal activity [172], oscillatory with the oscillatory frequency rising along with synaptic activity [170;173] or even propagate to neighboring astrocytes, which is termed intercellular Ca^{2+} wave [168]. In the hippocampus, astrocytic responses to neuronal activity have been shown to be mediated by glutamate acting on metabotropic glutamate receptors [169;170;172], GABA via GABA(B) receptors [171] and ACh via muscarinic ACh receptors [172;174]. Moreover, one single astrocyte can sense and differentiate activity of different types of neurons [175]. In the cortex, neuronal activity has been shown to induce Ca^{2+} increases in astrocytes by mGluR receptor activation [30;31;173]. In the cerebellum, electrically stimulated parallel fibers signal to Bergmann glia cells via release of NO [146;147;176]. In the retina, light-evoked neuronal activity in ganglion cells led to ATP-mediated and largely adenosine-augmented Ca^{2+} elevations in Müller cells, the astrocyte-like glia of the retina [177].

1.3.2 Ca^{2+}-dependent astrocytic feedback on neuronal activity

Astrocytes can release glutamate [178-181], D-Serine [182;183] and other neuromodulatory substances, such as ATP (and adenosine) [89;184-188], chemokines [67], prostaglandins [31;178] and NO [101] upon stimulation, many of which in a Ca^{2+}-dependent manner. Glutamate is released from vesicles comparable to release from neuronal presynaptic terminals [161;162;179;181;189-194]. Also, controlled release of substances by volume-regulated anion channels (VRACs) [195;196], ATP-binding cassette transporters [197], the cystic fibrosis transmembrane conductance regulator [198;198], connexin hemichannels [88;89;187] and the purinergic ionotropic receptor subtype P2X7 [199;200] are discussed.

The oscillatory frequency rather than the amplitude of Ca^{2+} oscillations controls the Ca^{2+}-dependent release of substances [30;170]. Given the fact that the frequency is determined by the strength of the neuronal activity, the amount of substance release by astrocytes is adjusted. Thus, astrocytes cannot only detect neuronal activity, but they can also signal back and modify neuronal signaling. This implies that they may actively contribute to short and long term synaptic plasticity.

Astrocytes can modulate the activity of apposed neurons in culture [151;201-205] as well as in acute brain slices [170;171;206-208]. Astrocytes can both weaken and or strengthen closeby synapses. In mixed cultures, the specific activation of astrocytes can reduce the amplitude of evoked excitatory (EPSCs) and inhibitory postsynaptic currents (IPSCs) [201], it can enhance the frequency of spontaneous miniature EPSCs and miniature IPSCs [202] and it can evoke depolarizing “slow inward currents” (SICs) in postsynaptic neurons [167;203;209]. All these phenomena are caused by astrocytic glutamate release, after which glutamate acts on presynaptic neuronal mGluRs, NMADRs or postsynaptic AMPARs and NMDARs repectively.

In the hippocampus, astrocytes mediate activity-dependent homo- and heterosynaptic suppression of the glutamatergic Schaffer collateral by releasing ATP, which degraded to adenosine acts on presynaptic terminals [210]. Astrocytes also mediate activity-dependent potentiation of gabaergic, hippocampal interneurons by their Ca^{2+}-dependent release of glutamate, which acts on interneuronal AMPA and NMDARs to potentiate transmitter release [171].

1.3.3 Astrocytic intercellular Ca^{2+} waves involved in long-range communication

Different processes of a single astrocyte can contact different synapses of the same or distinct neurons and Ca^{2+} waves can spread intracellularly from one process across the cell body to another [210]. Moreover, Ca^{2+} waves can not only spread throughout a single cell, but can be propagated to closeby astrocytes and in that manner spread over larger distances in culture [80;211] and in brain slices [163]. These intercellular Ca^{2+} waves can be elicted by mechanical, electrical and chemical stimulation in culture as well as in acute brain slices. They are at least partially regenerative [212;213] and are thought to occur via two main mechanisms depending on the brain area [164]. The first mechanism is by intracellular diffusion of the Ca^{2+} releasing messenger IP_3 through gap junctions [214;215], the second is by the release of ATP and its extracellular diffusion and subsequent action on astrocytic purinergic receptors [186;216]. Glutamate is also released during an intercellular Ca^{2+} wave, but does not contribute to the spread of the wave [180;212;217]. These intercellular waves provide a type of long-range communication system by which astrocytes could coordinate and spread information over whole assemblies of neurons and other cell types. Possessing these properties, astrocytes can act as coordinators and integrators of neuronal activity.

1.3.4 "Spontaneous" Ca^{2+} oscillations in astrocytes as a generator of neuronal activity

Although involved in the functioning of the synapse, astrocytes are viewed as reactive elements, which sense neuronal activity and signal back. However, it has been shown that astrocytes show intrinsic, "spontaneous", $[Ca^{2+}]_i$-signaling in culture [204;218-220] and *in situ* in acute brain slices of most brain regions so far examined, which are independent of neuronal activity [34;221;222]. The observed regions include the rat ventrobasal thalamus [223;224], the rat [225] and the mouse hippocampus [221], and the neocortex, hippocampus, entorhinal cortex, striatum, cerebellum, thalamus, hypothalamus, and spinal cord of GFAP/GFP transgenic mice [226]. Interestingly, reactive astrocytes surrounding stab wounds have been shown not to be spontaneously active in the neocortex [226]. However, in another study, oscillations were only observed in the mouse neocortex under epileptiform conditions [222]. This astrocytic Ca^{2+} signaling activity has been found at all ages, but is highest during the first ten days to two weeks

after birth and then gradually decreased to a lower level during adulthood [224;226]. Astrocytes being able to autonomously generate Ca^{2+} activity may therefore also initiate synaptic signaling events or changes in synaptic strength and not only react to neural activity [227].

All studies observed variable Ca^{2+} signals including random profiles, rhythmic oscillations, as well as bursting activity, with a wide range of periods from 10 to 300 seconds. In the thalamus, a small subset of so-called “pacemaker cells” was found, which displayed very rhythmic oscillatory patterns of 0.019 Hz [223]. Ca^{2+} signals can establish not only throughout the whole cell, but also in microdomains. These locally restricted elevations can spread along processes, and oscillations can occur independently of each other in different processes of a single cell [228]. For most brain regions, the trigger for the spontaneous astrocytic $[Ca^{2+}]_i$ signaling has not yet been clearly identified. In the hippocampus, it is glutamate-driven [225]. Generally, the Ca^{2+} seems to be derived mainly from intracellular stores, but extracellular Ca^{2+} appears to be of essential importance for store refill, and VGCC were implicated in this. Most studies point out a role for IP_3Rs [221;224], one claims RyRs are relevant [222].

Independent of neuronal activity, the astrocytic activity still influences and is influenced by neurons; spontaneous Ca^{2+} transients in astrocytes drive NMDAR-mediated neuronal excitation [224] and neuronal activity synchronizes astrocytic network activity [226]. Induced epileptic activity increased the correlated network activity as well as the number of astrocytes showing spontaneaous Ca^{2+} signals [226].

Ca^{2+} signaling activity has also been observed *in vivo* [229]. A large proportion (~60%) of astrocytes were active, although with a rather low frequency in comparison to the astrocytes *in situ.* Induced neuronal activity by local application of bicuculline enhanced the number of active astrocytes and their synchronization.

Several roles have been ascribed to this intrinsic spontaneous Ca^{2+} signaling activity of astrocytes. Ca^{2+} oscillations are generally known to play a role in development, differentiation and maturation of tissues. Especially in the nervous system, it is established that spontaneous oscillatory electrical activity acccompanied by Ca^{2+} oscillations, underlies the establishment of connections in the CNS [230;231],[232-234] and the astrocytic activity was hypothesized to drive those neuronal oscillations [235;236]. In the epileptic model, the astrocytic oscillations were suggested to mediate the development of astrogliosis, which occurs in epileptic syndromes. Therefore, with time, astrocytes would contribute to the persistence of chronic epilepsy [226].

Ca^{2+} oscillations in astrocytes were moreover demonstrated to mediate neuronal activity-dependent vessel relaxation by regulation of prostaglandin release [30;31;33]. In general, astrocytic Ca^{2+} oscillations are known to regulate release of substance, such as the exocytosis of glutamate [160;178;180;208;237;237]. Local Ca^{2+} oscillations in human U87 astrocytoma cells were related to disassembly of focal adhesions by the Ca^{2+} dependent self-phosphosphorylation of FAK and therefore to cell motility [238;239].

1.4 NO in the CNS

The gaseous free radical nitric oxide (NO) is an important messenger within the CNS, playing a role in many physiological and pathophysiological processes, such as synaptic plasticity and neurotoxicity [240]. First discovered as an intracellular messenger in the CNS [241], it has since also been established as an extracellular messenger, even co-neurotransmitter [240].

NO is synthesized by nitric oxide synthase (NOS), which catalyzes the NADPH-dependent oxidation of L-arginine with O_2, to form L-citrulline and NO. There are three isoforms of NOS, the neuronal, the endothelial, and the inducible form (nNOS, eNOS, and iNOS) [242]. NOS is controlled by different mechanisms on several levels transcriptionally, translationally and posttranslationally [207;243]. Two of the isoforms, nNOS and eNOS, are mainly activated by the Ca^{2+}/CAM complex, whereas iNOS is less Ca^{2+}-sensitive [242]. While the former two isoforms are constitutively expressed and produce low amounts of NO, iNOS activity, in contrast, is mainly transcriptionally regulated and produces larger amounts of NO. iNOS is thought to be upregulated predominantly in pathology upon proinflammatory stimuli [244].

nNOS and neuronally located eNOS are implicated in learning and memory underlying long term potentiation (LTP) and long term depression (LTD) and thought to act as retrograde messenger co-ordinating pre- and postsynapse. eNOS expressed in endothelial cells of cerebral vessels has generally shown to be the source of NO employed in vasodilation [240]. While neurons are thought to be the main source for NO under physiological conditions, glial cells, especially microglia, are presumed to release NO primarily in pathology [245-247]. Astrocytes in culture express all three forms of NOS [247]. *In vivo*, however, their constitutional expression is still debated. Astrocytic NOS activity was in most cases ascribed to induction of protein synthesis of iNOS, which is connected with their transition to reactive astrocytes [248-250] or nNOS [251;252]. However, latest results indicate that iNOS can be responsible for fast NO production

related to neuronal cell death in acute brain slices [253]. There is recent evidence that fast NO production by astrocytes may also play a role under non-pathological conditions. Astrocyte-derived NO was suggested to mediate astrocytic memory for glutamatergic stimulation [166]. Moreover, ATP stimulation induced rapid Ca^{2+}-dependent NO synthesis in cultured astrocytes, which is partly responsible for intracellular Ca^{2+} store refill [101].

1.4.1 NO and Ca^{2+} homeostasis

NO signaling is tightly connected with Ca^{2+} signaling. Apart from the Ca^{2+}-dependent activation of nNOS and eNOS, NO has been shown to affect many components involved in the regulation of the cellular Ca^{2+} homeostasis (Fig. 3). NO can affect Ca^{2+} release from intracellular stores, Ca^{2+} influx, as well as the plasmalemmal Na^{+}/Ca^{2+} exchanger and the plasmalemmal and endoplasmatic reticular Ca^{2+} pumps [124].

A main pathway, by which NO can trigger an intracellular Ca^{2+} rise is the “cGMP/PKG pathway”. NO activates guanylate cyclase, which synthesizes cGMP. The cGMP can directly modulate Ca^{2+} channels or activate PKG that regulates proteins involved in Ca^{2+} homeostasis by phosphorylation [254]. This pathway is connected to the production of cADPR, a possible activator or at least positive modulator of RYRs [118;119;124]. Additionally, NO can also directly modulate proteins, which control the cytosolic Ca^{2+} level, by S-nitrosylation or tyrosine nitration, including the RyR as an example for the former and the NMDAR as an example for the latter [124;254;255]. Generally, the action of NO is considered cell type-dependent and of modulatory fine tuning nature [124].

NO has been shown to induce transient $[Ca^{2+}]_i$ responses in cultured astrocytes [176;256;257], in organic hippocampal slice cultures [257] and in Bergmann glial cells in acute brain slices [176], and even to trigger intercellular Ca^{2+} waves [256;257]. Regarding the mechanism, there is evidence for influx [101;176;257;258], as well as for intracellular release pathways [256]. The “cGMP/PKG/cADPR pathway” has been implicated in the release from intracellular stores [256], membrane depolarization activated VGCCs [257] and SOCCs [101;176;258] in the influx.

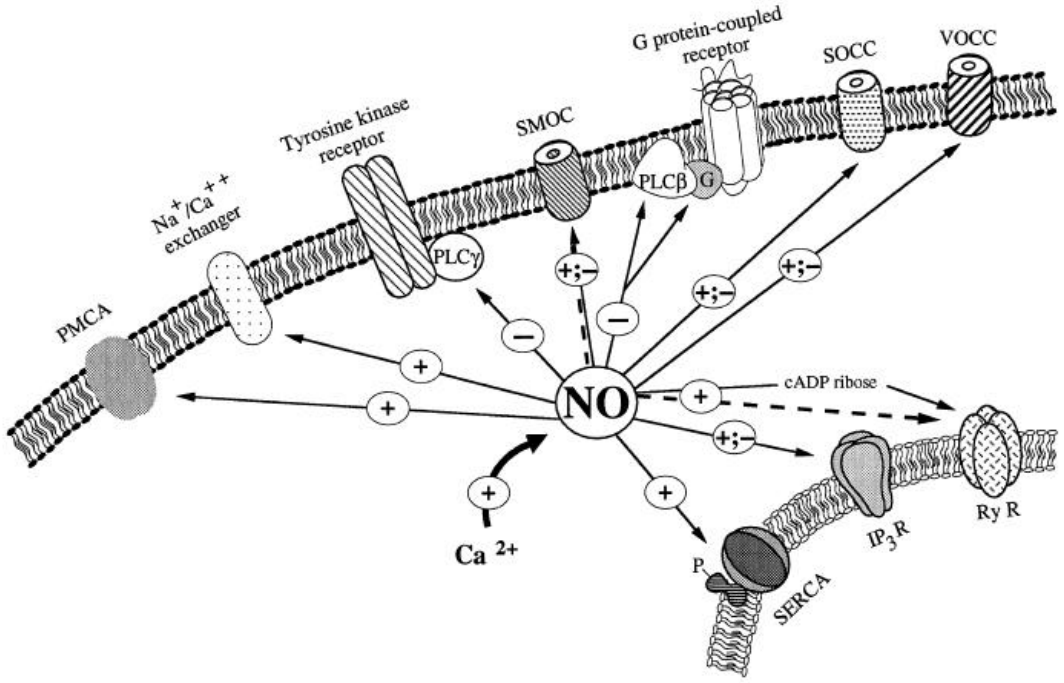

Fig. 3 Molecular targets and mechanisms of NO modulatory actions on Ca^{2+} homeostasis

The diagram shows in a schematic way a portion of plasmalemmal and endo/sarcoplasmic reticulum membranes that are endowed with those components of the Ca^{2+} homeostasis machinery whose function is modulated by NO. Arrows refer to the effects of NO, which are mediated via either cGMP/G kinase-dependent (*solid line*) or -independent (*dashed line*) pathways. The *signs* + and - refer to stimulation or inhibition, respectively. When both signs are present, the effect may be either stimulatory or inhibitory, depending on the cell type investigated. The acronyms that have not already been described in the text are as follows: G, heterotrimeric G protein; VOCC, voltage-operated Ca^{2+} channels, and PMCA and SERCA, plasma membrane and sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPases. Figure and legend are cited from E. Clementi, 1998 [124].

With regard to astrocytes, NO has been demonstrated to take part in several physiological processes. It plays a role in propagation of mechanically stimulated astrocytic Ca^{2+} waves in mixed glial-neuron cultures and acute hippocampal slices [256;257]. It can signal neuronal activity to Bergmann glia [176] and it induces Ca^{2+}-dependent, vesicular glutamate and ATP release in cultured astrocytes [258].

1.4.2 Mechanical strain-related NO signaling

NO can be produced upon mechanical strain and plays a role in stretch-related cellular signaling. It was shown that in the guinea pig heart stretch-activated ion channels mediate NO-release [259]. In vascular smooth muscle cells, cyclic stretch induces their proliferation and alignment via NO signaling [260]. Furthermore, in rat cardiomyocytes, stretch leads to NO-dependent Ca^{2+} signaling [261]. Cortical spreading depression, which goes along with massive changes in cellular volume and astrocytic Ca^{2+} signaling, is accompanied by NO release [262;263]. NO moreover plays a role in propagation of mechanically stimulated astrocytic Ca^{2+} waves in mixed glial-neuron cultures and acute hippocampal slices [256;257]. Direct mechanical stimulation [256] and hypoosmotic swelling [264;265] result in NO production in astrocytes.

1.5 $NAADP^+$ and the CNS

Almost a decade ago, a novel intracellular Ca^{2+} releasing second messenger was identified in sea urchin eggs, nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) [129], which binds to an unknown receptor. Since its discovery, there is increasing evidence that $NAADP^+$ also has a physiological role in Ca^{2+} signal transduction in vertebrates, including mammalian cells [266]. In the vertebrate nervous system, intracellular Ca^{2+} release by $NAADP^+$ has only been demonstrated from rat brain microsome preparations [267] and in frog motoneurons [268]. $NAADP^+$-specific binding sites are found in grey and white matter of rat brain, but the cellular specificity of the binding site has not yet been determined [269].

The major way of $NAADP^+$ synthesis in mammalian tissue takes place under acidic conditions and in the presence of nicotinic acid from $NADP^+$ by the type-II transmembrane glycoprotein ADP-ribosyl cyclase CD38 (Fig. 4). In neutral or alkaline environment CD38 exclusively catalyses the conversion of NAD^+ into cADPR [120]. Apart from cell membrane localisation, CD38 has been located to various intracellular organelle membranes and can moreover be internalised by endocytosis [270]. Therefore $NAADP^+$ synthesis takes most likely place in membranes of intracellular acidic organelles such as lysosomes or late endosomes, whereas CD38 in the plasma membrane synthesises cADPR. Regarding the mammalian CNS, CD38 expression was demonstrated in the rat cerebral and cerebellar cortex for neurons and astrocytes by immunoelectron microscopy [271]. Also, cultured rat hippocampal astrocytes are immunopositive for CD38, with plasmamembrane staining and staining in the perinuclear Golgi

region [157]. Furthermore, CD38 activity could be detected in rat cortical astrocytic cultures and their membrane preparations, where activity was also found intracellularly [272], in rat hippocampal astrocytic cultures [157], and in mouse [273] and rat brain homogenates [274].

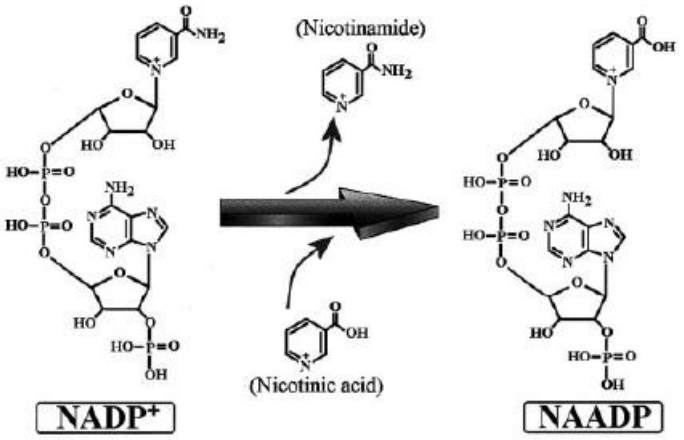

Fig. 4 Base-exchange reaction
The nicotinamide group of nicotinamide adenine dinucleotide phosphate ($NADP^+$) is exchanged for nicotinic acid, resulting in nicotinic acid adenine dinucleotide phosphate (NAADP). Figure and legend are cited from Chini and De Toledo, 2002 [275].

$NAADP^+$ as an intracellular messenger acts at very low concentrations. The cytoplasmic concentration has been determined for human red blood cells to be as low as ~60 nM [276]**.** In contrast to invertebrate cells where NAADP binding is irreversible and the receptor is inactivated even at subthreshold concentrations [277], $NAADP^+$ binding is reversible in mammalian cells and the receptor is only inactivated at high concentrations (~50-100 μM) [266]. The intracellular Ca^{2+} stores sensitive to $NAADP^+$ were in most studies in cell free systems different from the thapsigargin-sensitive stores, which respond to IP_3 and cADPR [278], although there is one report in which $NAADP^+$ appears capable to release Ca^{2+} directly from the nuclear envelope by acting on ryanodine receptors, a process which is thapsigargin-sensitive [279]. $NAADP^+$-induced Ca^{2+} signaling can be abolished by glycyl-L-phenylalanine 2-naphthylamide (GPN), a substrate of lysosomal cathepsin C whose hydrolysis leads to the specific osmotic lysis of lysosomes**,** which can also function as Ca^{2+} stores [95]. In intact cells, both types of stores interact and it was suggested that the $NAADP^+$-activated stores serve to prime the other stores and are involved in initiating Ca^{2+} oscillations then maintained by IP_3 and cADPR [280]. In addition there is evidence for Ca^{2+} influx in response to $NAADP^+$ [281].

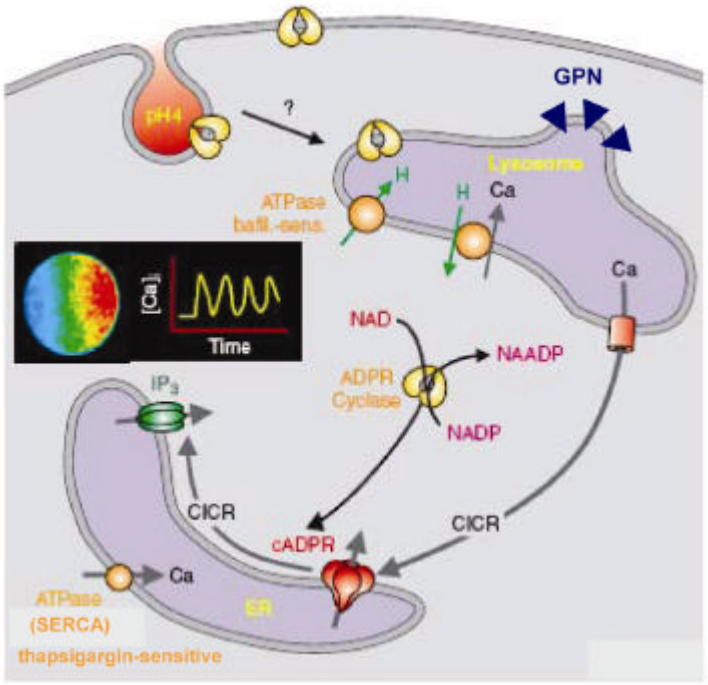

Fig. 5 Multiple Ca^{2+} stores in cells

$NAADP^+$ targets lysosome-like organelles, whereas the cADPR- and IP_3-sensitive Ca^{2+} stores are co-localized in the endoplasmic reticulum (ER). Ca^{2+} transport in the lysosomal stores is mediated by the combined action of a bafilomycin-sensitive (bafil. sens.) proton pump and a Ca^{2+}/H^+ exchanger. The Ca^{2+}-pump in the ER is sensitive, instead, to thapsigargin. In several cell types, Ca^{2+} release activated by $NAADP^+$ serves as triggering signals that are amplified and/or propagated by the cADPR- and IP_3-mechanisms via Ca^{2+}-induced Ca^{2+} release (CICR). This interaction between the two Ca^{2+} pools can produce Ca^{2+} oscillation as well as Ca^{2+} wave propagation. Both $NAADP^+$ and cADPR are synthesized by a single enzyme, ADP-ribosyl cyclase (ADPR cyclase), using, respectively, $NADP^+$ and NAD as substrates. The synthesis of $NAADP^+$ has an acidic pH optimum. CD38 is a mammalian homolog of ADPR cyclase, which in some cells, is expressed on the surface and can be internalized by endocytosis. $NAADP^+$-mediated Ca^{2+} release can be interfered with by GPN, which leads to the osmotic burst of lysosomes. Figure and legend are modified from Lee, 2003 [282].

cADPR, the alternate product of 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]. An autocrine loop was proposed [157], in which NAD^+ reaches the extracellular space via connexin hemichannels, is extracellularly converted to cADPR by CD38, and gets back into the cell either via CD38 [283] or connexin hemichannels [284].

1.6 Aims

The overall aim of this thesis was to investigate aspects of astrocytic Ca^{2+} signaling. Astrocytes express a variety of receptors that couple to an increase in the intracellular Ca^{2+} concentration, which controls the release of neuro- and vasoactive, as well as inflammatory substances. To produce specific signals in response to different receptor inputs by use of one single messenger, Ca^{2+}, several second messenger pathways and different sources of Ca^{2+} need to be involved. It is thus important, to decipher the astrocytic "Ca^{2+} code", since this should lead to further understanding of how these cells can react to, and influence, the neuronal network in physiology and pathology. To unravel this code it is necessary to identify new substances and conditions that can elicit Ca^{2+} signals in astrocytes, as well as to investigate the variety and interplay of Ca^{2+} entry and intracellular Ca^{2+} release pathways.

The first aim of this thesis was to investigate whether the newly discovered $NAADP^+$-dependent Ca^{2+} release system was functional in cortical astrocytes *in situ.* Although so far only investigated in a few cell types, $NAADP^+$ is thought to be the third second messenger able to induce Ca^{2+} release from intracellular stores, alongside IP_3 and cADPR. It releases Ca^{2+} from stores other than the ER, such as small acidic cellular compartments and is more potent than the other two messenger molecules. There is some evidence that this system is active in the mammalian nervous system, however, no cell type-specific or mechanistic investigations have been undertaken.

The second aim of this work was to gain further insight into a previously unexplained phenomenon of Ca^{2+} signaling that occurs in the same acute cortical slice preparations used for the experiments with $NAADP^+$ [285]. Perfused cortical slices exhibit an increase in Ca^{2+} signaling activity in astrocytes when the flow of perfusate is suspended, that normalizes when perfusion is restored. Any paradigm that induces Ca^{2+} oscillations in astrocytes is of interest, since it could potentially be linked to altered astrocytic properties *in vivo*, influencing neuronal activity and blood flow. Unacknowledged astrocytic Ca^{2+} signaling could also cause artifacts in measurements that are conducted in brain slices.