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The use of transgenic mouse models to reveal the functions of Ca^{2+} buffer proteins in excitable cells

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Background: Cytosolic Ca^{2+} buffers are members of the large family of Ca^{2+}-binding proteins and are essential components of the Ca^{2+} signaling toolkit implicated in the precise regulation of intracellular Ca^{2+} signals. Their physiological role in excitable cells has been investigated *in vivo* by analyzing the phenotype of mice either lacking one of the Ca^{2+} buffers or mice with ectopic expression.
Scope of Review: In this review, results obtained with knockout mice for the three most prominent Ca^{2+} buffers, parvalbumin, calbindin-D28k and calretinin are summarized.
Major Conclusions: The absence of Ca^{2+} buffers in specific neuron subpopulations and for parvalbumin, additionally in fast-twitch muscles, leads to Ca^{2+} buffer-specific changes in intracellular Ca^{2+} signals. This affects the excitation–contraction cycle in parvalbumin-deficient muscles, and in Ca^{2+} buffer-deficient neurons, properties associated with synaptic transmission (e.g. short-term modulation), excitability and network oscillations are altered. These findings have not only resulted in a better understanding of the physiological function of Ca^{2+} buffers, but have revealed that the absence of Ca^{2+} signaling toolkit components leads to protein-and neuron-specific adaptive/homeostatic changes that also include changes in neuron morphology (e.g. altered spine morphology, changes in mitochondria content) and network properties.
General Significance: The complex phenotype of Ca^{2+} buffer knockout mice arises from the direct effect of these proteins on Ca^{2+} signaling and moreover from the homeostatic mechanisms induced in these mice. For a better mechanistic understanding of neurological diseases linked to disturbed/altered Ca^{2+} signaling, a global view on Ca^{2+} signaling is expected to lead to new avenues for specific therapies. This article is part of a Special Issue entitled Biochemical, biophysical and genetic approaches to intracellular calcium signaling.

1. Introduction

1.1. Ca^{2+} buffers are part of the large family of Ca^{2+}-binding proteins

The class of so-called Ca^{2+} buffers belongs to the large family of Ca^{2+}-binding proteins. According to their structure and function, Ca^{2+}-binding proteins can be separated into sub-families including the EF-hand proteins, annexins, pentraxins, C2-domain proteins and the vitamin K-dependent proteins, for details, see [1], as well as a family of low-affinity, high-capacity Ca^{2+}-binding proteins mostly localized to the lumen of organelles and thus named "organellar Ca^{2+} buffers" [2]. All the Ca^{2+} buffers described in this review are part of the largest family of Ca^{2+}-binding proteins, the EF-hand proteins [3]. The principal Ca^{2+}-binding motif consists of an alpha helix, a Ca^{2+}-binding loop of 12 amino acids (in the "classical" EF-hand) and a second alpha helix that is oriented perpendicularly to the first one. This motif was first identified in parvalbumin (PV) [4] and more than 240 proteins with identified EF-hand motifs are encoded in the human genome. Most EF-hand proteins have an even number (2, 4, 6) of EF-hand domains and thus the EF-hand pair (often showing co-operativity) is considered to be the functional physiological unit. There are some exceptions: parvalbumins (alpha and beta lineage) have 3 EF-hand domains, however the first (the AB domain) is non-functional with respect to Ca^{2+}-binding, but is necessary for protein structure and stability. The other exception is the family of penta EF-hand proteins [5]. For more details on EF-hand proteins, see [6,7]. The classification of EF-hand proteins in Ca^{2+} buffers and Ca^{2+} sensors is discussed next.

1.2. Ca^{2+} buffers vs. Ca^{2+} sensors

The effect of a cytosolic Ca^{2+} buffer on intracellular Ca^{2+} signals is dependent on A) the intracellular concentration of the Ca^{2+} buffer

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B) the Ca^{2+}-binding properties (affinity for Ca^{2+} and possibly other ions (e.g. Mg^{2+}, Zn^{2+}) and the kinetics of Ca^{2+}-binding ($k_{on,Ca}$) and Ca^{2+}-release ($k_{off,Ca}$) and C) the mobility of the Ca^{2+} buffer within the different cytosolic compartments. Detailed values for the proteins discussed in this review are found in [8]. Results from the last decade indicate that the kinetics of metal-binding and -release are very critical parameters determining the physiological function of a specific Ca^{2+} buffer. In comparison to the "slow" Ca^{2+} buffer parvalbumin (PV) with an on-rate of $\approx 6\times10^{6}\,\mu M^{-1}\,s^{-1}$ under physiological conditions, the "fast" buffer calbindin-D9k is 2–3 orders of magnitude faster ($\approx 1\times10^{9}\,\mu M^{-1}\,s^{-1}$). Typical hallmarks for pure Ca^{2+} buffers are the rather small Ca^{2+}-induced conformational changes and the lack of interaction with other proteins. Although named Ca^{2+} buffers, a better description would be Ca^{2+} signal modulators. Considering the basal (resting) intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of approximately 50 nM and the average Ca^{2+} buffer's dissociation constant ($K_{D,Ca}$) in the range of 0.3–1 μM, then these proteins are mostly Ca^{2+}-free inside a resting cell; the resting $[Ca^{2+}]_i$ is maintained through the balance between influx (Ca^{2+} channels) and efflux (Ca^{2+} pumps and exchangers). In combination with the mobility of Ca^{2+} buffers inside cellular compartments, in a neuron e.g. in the axon, soma or dendrites, these Ca^{2+} signal modulators not only affect the amplitude and kinetics of Ca^{2+} signals, but also spatial aspects such as spatially-restricted Ca^{2+} signals vs. global spreading of Ca^{2+} signals (see below).

Typical features of Ca^{2+} sensors are the rather pronounced Ca^{2+}-induced conformational changes allowing for the interaction with target proteins in a Ca^{2+}-regulated manner, thereby modulating the function of these targets; calmodulin (CaM) is considered as the most ubiquitous and prototypical Ca^{2+} sensor. However, Ca^{2+} sensors may also act as Ca^{2+} buffers when present at sufficiently high concentrations (see below). Although calbindin-D28k (CB-D28k) and calretinin (CR) are considered as Ca^{2+} buffers and results from Ca^{2+} buffer knockout mice confirm this notion (Sections 4 and 5), there are strong indications that the two proteins are likely to have also Ca^{2+} sensor functions. Both proteins display pronounced Ca^{2+}-induced conformational changes and targets for CB-D28k have been identified including *myo*-inositol monophosphatase [9], for more details see [8]. Recent experiments *in vitro* indicate that CaM is also an extremely fast Ca^{2+} buffer and it is hypothesized that Ca^{2+} entering via channels is first bound to CaM, which then passes on the Ca^{2+} ion to the "slower" Ca^{2+} buffers instead of reacting to the lower $[Ca^{2+}]_i$ caused by Ca^{2+} buffers [10]. Therefore the presence of the Ca^{2+} buffers PV, CB-D28k and CR would modulate the lifetime of Ca^{2+}-bound CaM. However, whether the sequential order is as postulated [10] critically depends on the concentrations of all proteins in a given cell (CaM, Ca^{2+} buffers) and further experiments need to be carried out to verify the hypothesis. In summary, novel findings start to obscure the previously clear distinction between Ca^{2+} buffers and Ca^{2+} sensors [8,11], which likely necessitates re-evaluation of this concept.

2. Genetic tools to study Ca^{2+} buffers *in vivo*

Within the last two decades, a wealth of techniques became available to manipulate protein expression levels *in vivo* [12]. These include conventional transgenic mice produced by heterologous recombination and knockout mice by homologous recombination and embrace antisense-knockout (KO), constitutive KO and also mice generated by methods based on small interference siRNA approaches including small hairpin shRNA. By various techniques (e.g. cre/lox) the transgene can be directed to specific cell populations and furthermore, ectopic expression or down-regulation can be induced by methods such as tetracycline (tet) and doxycholine (dox) regulatory systems. Expression levels *in vivo* can also be manipulated locally by virus-mediated (e.g. lentivirus) infection, also in an inducible way, if desired. In the list of reviews below, the different methods, their advantages and possible disadvantages are discussed in detail [12–16]. One concern is always the adaptations/modifications that may take place if protein levels are altered, especially if a component of the Ca^{2+} signaling toolkit [17] is affected (for details on the latter topic, see [18]).

3. Parvalbumin (PV)

3.1. Parvalbumin knockout and parvalbumin transgenic mice

The principal sites of PV expression comprise fast-twitch muscles in most species [19], a sub-population of neurons in the central and peripheral nervous system [20] and epithelial cells of the nephron in the kidney [21]. Thus, the effects of PV's absence or ectopic expression in transgenic mice are discussed in these three cell types. The first knockout mouse for PV (PV−/−) (mouse gene symbol: *Pvalb*; systematic strain name: $Pvalb^{tm1Swal}$) was reported in 1999, where the fast-twitch muscle phenotype was investigated [22]. In the absence of PV, in the tibialis anterior (TA) muscle, the decay in $[Ca^{2+}]_i$ after 20-ms stimulation is slower leading to a prolongation of the time required to attain peak twitch tension and furthermore, to an extension of the half-relaxation time. Since also the integral of $[Ca^{2+}]_i$ during the twitch is higher in PV−/−TA, the force generated during a single twitch is about 40% increased compared to TA from WT mice. Thus, in line with PV acting as a "slow-onset" Ca^{2+} buffer, the initial increase in $[Ca^{2+}]_i$ is not affected, but PV then accelerates the initial decay in $[Ca^{2+}]_i$. The increase in the rate of initial $[Ca^{2+}]_i$ decay by PV impinges on the force generated in TA that is repetitively stimulated at 20 Hz. In TA from WT mice, an almost complete relaxation occurs between twitches (Δt interval 50 ms), while the longer half-relaxation time in PV−/− TA causes a gradual build-up of force reaching 70% of tetanic force already after three 20 Hz stimuli. A similar property of PV, i.e. preventing/decreasing the building up of residual Ca^{2+} in presynaptic terminals and the connected consequences with respect to synaptic transmission is also seen PVergic neurons. Further evidence of PV's role on accelerating muscle relaxation comes from experiments where PV cDNA was injected in the rat slow-twitch muscle *soleus* (SOL). The presence of PV in transfected SOL muscles leads to a shortening of the twitch half-relaxation time without affecting the contraction time [23]. This effect is due to PV's Ca^{2+}-binding properties, because a mutant protein incapable of binding Ca^{2+} ions has no effect on the contraction/relaxation cycle. SOL and TA, or slow- and fast-twitch muscles in general, differ not only with respect to PV expression, but also have distinct protein isoforms of the contractile complex including myosin heavy chain (MHC), troponin I, troponin C and troponin T. Therefore, one cannot exclude the possibility that the forced expression of PV in the PV-injected SOL may induce changes in contractile protein isoforms that might be responsible, at least in part, for the observed effects. In PV−/− mice such an isoform switch does not occur, and all analyzed proteins are of the "fast" type, thus the observed effect on slowing of the contraction/relaxation cycle in PV−/− TA is not the result of isoform switching [22]. However, it cannot be entirely excluded that part of the effect is mediated, either blunted or exaggerated, by adaptation/compensation mechanisms occurring in PV−/− TA.

Several changes described as "homeostatic" mechanisms have been identified in "PVergic" cells of PV−/− mice. TA muscles from PV−/− mice are more resistant to muscle fatigue resulting from an upregulation of mitochondria [24]. The relative mitochondrial volume is almost doubled (185% of WT) in the PV−/− fast-twitch muscle extensor digitorum longus (EDL). The finding that $[Ca^{2+}]_i$ decline in the time period of 200–700 ms after a 50-ms EDL stimulation is faster in PV−/− than in WT mice indicates that mitochondria are likely involved in late $[Ca^{2+}]_i$ decrease. A similar effect of mitochondria on Ca^{2+} removal is also seen in a particularly large presynaptic terminal,

the calyx of Held [25]. Besides the increased mitochondrial volume, the increase of the density of capillaries in PV−/− EDL muscles is a likely factor co-contributing to the increased fatigue resistance of these muscles. Mitochondria are dynamic structures not only in relation to cellular mitochondrial content, but also with respect to organelle composition in different tissues (e.g. brain, heart, kidney, and liver) and they are "tailored to meet the metabolic and signaling needs of each cell" [26]. Thus, the mitochondrial protein composition in TA of PV−/− mice (e.g. mitochondrial DNA-encoded cytochrome oxidase c subunit I and nuclear DNA-encoded cytochrome oxidase c subunit Vb and F1-ATPase subunit beta) is almost identical to the one in SOL of WT mice, but not of WT TA [27]. It is hypothesized that the SOL-type mitochondria in PV−/− fast-twitch muscles may functionally replace PV. Unlike all other cytosolic Ca^{2+} buffers of the EF-hand family, mitochondria and PV share the slow-onset kinetic properties of Ca^{2+} removal/buffering. The inverse correlation between PV expression and mitochondria content is not restricted to fast-twitch muscles, but is equally seen in PVergic neurons, most notably in cerebellar Purkinje cells. In the soma of PV−/− Purkinje cells, the relative mitochondrial mass is increased by 40% and the additional mitochondria are selectively localized to a peripheral region of 1.5 μm width underneath the plasma membrane [28]. These mitochondria may be viewed as a "firewall" to prevent/restrict the spreading of Ca^{2+} signals from the somatic plasma membrane to the nucleus. Also in pancreatic acinar cells, perigranular mitochondria localized to the apical secretary pole, restrict $InsP_3$-mediated elevations in $[Ca^{2+}]_i$ to the apical region and prevent a global rise in $[Ca^{2+}]_i$ [29]. A function for PV and for slow-onset synthetic Ca^{2+} chelators (EGTA) in limiting the spreading of $InsP_3$-induced Ca^{2+} waves in oocytes is well documented [30,31]. In addition to the increase in mitochondria, a decrease in subplasmalemmal smooth endoplasmic reticulum (sPL-sER) in a zone of 0.5 μm thickness underneath the plasma membrane, the likely starting place for somatic Ca^{2+} waves [32] elicited by the opening of plasma membrane Ca^{2+} channels [33], is observed in PV−/− Purkinje cells [28]. Such global Ca^{2+} spreading involving Ca^{2+}-induced Ca^{2+} release (CICR) is likely to be reduced/blocked by PV, as shown for PV on the spreading of $InsP_3$-mediated Ca^{2+}signals [30]. While PV is probably involved in confining climbing fiber (CF)-mediated Ca^{2+} signals to a small sub-membrane shell, it is proposed that increased mitochondria volume and decreased sER in PV−/− Purkinje cell somata, functionally compensate for the absence of PV. Mature Purkinje cells not only express the "slow" Ca^{2+} buffer PV, but equally high levels of the "fast" Ca^{2+} buffer CB-D28k [20,34]. None of the changes seen in PV−/− Purkinje cell somata (increased mitochondria volume, decreased sPL-sER) are induced in mice deficient for CB-D28k. This indicates that each Ca^{2+} buffer has distinct properties and that its absence in CaBP-KO mice can't be simply substituted by another EF-hand family member. Thus, there is no indication that CB-D28k is elevated in Purkinje cells of PV−/− mice [35] and *vice versa* [36]. The specific adaptation/compensation mechanisms in Purkinje cells of CB-D28−/− mice are discussed in Section 4.

If the upregulation of mitochondria in PV−/− fast-twitch muscles and Purkinje cells is considered to be a homeostatic mechanism, then it is expected to function in both directions. In transgenic mice ectopically expressing PV in all neurons (Thy-PV; systematic name: B6.Tg (Thy1-Pvalb)^{2Mbch}), the mitochondrial volume of PV-expressing striatal medium spiny neurons (MSN), i.e. a neuron sub-population devoid of PV in WT mice, is reduced to nearly 50% compared to the same neuron population of WT mice [37]. This strongly indicates that I) the regulation of PV and mitochondrial volume is not restricted to cells with endogenous PV expression (e.g. fast-twitch muscles, Purkinje cells) and II) the inverse regulation hints towards a homeostatic mechanism. The elucidation of signaling pathways for this antagonistic regulation is expected to shed some light on the functioning of the Ca^{2+} homeostasome [8,38]. Some unexpected results obtained with the Thy-PV mice are discussed in Section 6.

The role of PV in neurons has been addressed in several studies using PV−/− mice. In line with PV's function as a slow-onset Ca^{2+} buffer, the absence of PV in cerebellar molecular layer interneurons (MLI) has no effect on the initial inhibitory postsynaptic currents (IPSC) recorded from Purkinje cells, but modulates short-term synaptic plasticity evidenced by paired-pulse protocols. In WT mice at interspike intervals (ISI) of 30–300 ms, paired-pulse depression (paired-pulse ratio; PPR<1) is observed, while paired-pulse facilitation is seen at ISI of 30–100 ms in PV−/− mice [39]. The effect is presynaptic and specific for a Ca^{2+} buffer with slow kinetics, since paired-pulse depression in PV−/− Purkinje cells can be restored by adding EGTA to the presynaptic "PVergic" MLIs. Direct measurement of action potential-evoked $[Ca^{2+}]_i$ in axons (single varicosities) of MLI revealed the decay in $[Ca^{2+}]_i$ to be dependent on PV. As shown for the $[Ca^{2+}]_i$ decay in PV-loaded chromaffin cells [40], the presence of PV converts a mono-exponential decay into a bi-exponential one, i.e. PV accelerates the initial decay (Ca^{2+}-binding to PV), followed by a slowing of the decay due to the release of Ca^{2+} from PV [41]. This slower component in PV's presence becomes more important after repetitive stimulation (10 pulses at 50 Hz), where it is hypothesized that the increased residual Ca^{2+} prolongs the delayed transmitter release at MLI–MLI synapses following the action potential (AP) train; it lasts for about 2400 ms in WT cells in comparison to a duration of approximately 400 ms in PV−/− cells. However, in another study using paired recordings (in contrast to extracellular stimulation of presynaptic axons [41]), such an effect of PV on delayed (asynchronous) transmitter release between basket cells and Purkinje cells is not observed [42]. In hippocampal PVergic interneurons innervating perisomatic regions of pyramidal (CA1) cells, neither single IPSCs nor paired-pulse modulation are different between WT and PV−/− neurons under the selected experimental conditions (reduced release probability and minimal presynaptic suppression). However, in trains of stimuli (>20 Hz), IPSCs are more facilitated in neurons from PV−/− mice and the largest relative difference between genotypes occurs at 33 Hz, within the range of gamma frequencies (30–80 Hz). In hippocampal slices, the power of kainate-induced rhythmic IPSC-paced neuronal oscillations at gamma frequencies is higher (3–4-fold elevation) in PV−/− than WT slices (determined with dual field potential recordings in CA3 region). Of note, neither the dominant frequency (≈35 Hz) nor coherence of oscillations is affected by PV deficiency. Thus, it was proposed that "PV deficiency, due to an increased short-term facilitation of GABA release, enhances inhibition by high-frequency burst-firing PV-expressing interneurons and may affect the higher cognitive functions associated with gamma oscillations" [43]. Behavioral experiments to test these predictions on cognitive functions are under way (B. Schwaller, unpublished).

While most PV-expressing neurons are GABA-ergic, neurons within the superior olivary complex (SOC) of the mammalian brainstem ending at a large excitatory nerve terminal in the auditory brainstem, the calyx of Held, use glutamate as transmitter. This large synapse allows to directly address the role of presynaptic PV in short-term plasticity of synaptic transmission. In PV−/− mice, the $[Ca^{2+}]_i$ decay and facilitation are slowed down approximately two-fold, similar to what is observed in rat calyces of Held during prolonged whole-cell recordings leading to a washout of mobile, highly diffusible Ca^{2+} buffers [44]. As expected from a slow-onset Ca^{2+} buffer, the amplitude of the $[Ca^{2+}]_i$ transient is not different in calyces from WT and PV−/− mice. Besides the discussed PVergic interneuron populations in the cerebellum and the hippocampus, the PVergic chandelier and basket cells of the cortex are part of a network of specific inhibitory interneurons that regulate principal cell firing in several forms of neocortical activity. The fast-spiking (FS) PV-expressing interneurons are efficiently self-inhibited by GABAergic autaptic transmission. In acute cortical slices, the synchronous and asynchronous release during and after a spike train in these autapses is differently affected by fast- and slow-onset synthetic Ca^{2+} buffers such as BAPTA and

EGTA, but also by the endogenous PV [45]. As in hippocampal and cerebellar PVergic interneurons, peak amplitudes of single-spike-evoked unitary autaptic IPSCs are not different in neurons from WT and PV−/− mice. The absence of PV mostly slows down the decay of the activity-dependent asynchronous release in the time period 300–700 ms following a high-frequency stimulation (500-ms train, 200 Hz). The initial magnitude of the asynchronous release (calculated 100 ms after the spike trains) is the same in WT and PV−/− neurons. This finding suggests that asynchronous autaptic release is the result of increased intra-terminal Ca^{2+} build-up in the absence of PV. Nonetheless, other mechanisms can't be entirely excluded, also based on significant adaptive/compensatory/homeostatic changes in PV−/− neurons. The principal function of this FS-mediated asynchronous release is thought as a mechanism that decreases FS-cell spike reliability and furthermore reduces the ability of pyramidal neurons to integrate incoming stimuli into precise firing. This may be important to desynchronize a large portion of the local network and prevent/disrupt excessive synchronized activity [45].

Unlike in most mammalian central synapses, where transmitter release is triggered by Ca^{2+} microdomains ("loose coupling"), in presynaptic terminals of hippocampal PV-expressing basket cells contacting dentate gyrus granule cells, Ca^{2+} channels are highly concentrated in the active zone ("tight" coupling). GABA release from these terminals is sensitive to the fast Ca^{2+} buffer BAPTA, but insensitive to EGTA; experiments with PV−/− mice suggest that these terminals are also insensitive to endogenous PV indicative of nanodomain coupling of transmitter release [46]. Such a tight coupling is also expected to exist in cerebellar basket cells. This is consistent with the finding that the initial MLI-mediated IPSCs in Purkinje cells ($IPSC_1$) are not different in WT and PV−/− neurons [39]. Moreover, unitary IPSCs evoked by single presynaptic AP between pairs of basket cells and Purkinje cells are not different between genotypes [42]. Based on PV's well-described, slow-onset Ca^{2+}-buffering kinetics, spike-triggered averaged somatic Ca^{2+} signals in basket cells from cerebellar slices were not expected to differ between WT and PV−/− mice. However, not only the $[Ca^{2+}]_i$ decay is 7-fold slower in MLIs, but also the amplitude is considerably larger in PV−/− MLIs indicating that PV may also function as a fast Ca^{2+}-buffer similar to BAPTA [47]. How can we reconcile such a notion that PV may act in both ways, as a slow and a fast buffer? Inside a cell with a resting $[Ca^{2+}]_i$ of 50–100 nM and $[Mg^{2+}]_i$ of 0.5–1 mM, PV molecules exist in three states: Ca^{2+}-PV, Mg^{2+}-PV and metal-free (apo)-PV. While the majority of molecules (>70%) are present as Mg^{2+}-PV acting as a slow Ca^{2+} buffer, due to the slow unbinding of Mg^{2+} ($\approx 1\ s^{-1}$ [40]), the small fraction of apo-PV ($\approx$5% at the above conditions) acts as a high-affinity, fast Ca^{2+} buffer ($K_{D,Ca} \approx 10$ nM, $k_{on,Ca} \approx 1 \times 10^8\ M^{-1}\ s^{-1}$), i.e. Ca^{2+}-binding is almost as fast as with BAPTA. Since the intracellular concentration of PV is estimated to be in the order of 80–100 μM in Purkinje cells [34,48] and even less in hippocampal interneurons ($\approx$50 μM), the small fraction of apo-PV was thought to have no significant effect on $[Ca^{2+}]_i$ transients. Most recent measurements of intracellular PV concentrations revealed those to be much higher in cerebellar basket cells, up to 1 mM, i.e. at least one order of magnitude higher than in dentate gyrus basket cells [42]. Thus, in the former neurons, the concentration of apo-PV is around 25–50 μM, a concentration high enough to also affect the amplitude of small $[Ca^{2+}]_i$ elevations (spike-triggered averages) in the soma of cerebellar MLIs [47]. Moreover, modeling studies on apo-PV with its BAPTA-like properties suggest that the fraction of metal-free PV also affects nanodomain coupling, while Mg^{2+}-PV contributes to buffer regeneration under physiological conditions. It is suggested that in neurons with high PV expression levels (e.g. cerebellar basket cells), apo-PV has "a substantial impact on synaptic transmission, preventing facilitation in nanodomain coupling regimes" [42]. The most surprising conclusions from these two recent studies [42,47] is that unlike EGTA, i.e. the "classical" slow-onset Ca^{2+} buffer used in many studies, PV has a dual role, mostly acting as slow-onset Ca^{2+} buffer, but with the possibility of apo-PV functioning as fast-onset Ca^{2+} buffer. If present at high enough concentration, apo-PV's effect on Ca^{2+} signals closely resembles the action of BAPTA. A similar dual role for CR, a presumably fast Ca^{2+} buffer is observed in *Xenopus* oocytes. Unlike with the synthetic fast buffer BAPTA, the spreading of $InsP_3$-induced Ca^{2+} signals is differently affected by small and large increases in $InsP_3$ (and associated elevations in $[Ca^{2+}]_i$) in the presence of CR. In case of small $[Ca^{2+}]_i$ raises, CR shows a similar behavior like EGTA or PV, i.e. a fragmentation of global responses into discrete localized events (Ca^{2+} puffs), while a large $InsP_3$ release causes 'globalization' of spatially uniform Ca^{2+} signals [30]. The variable effect of CR on the spatiotemporal aspect of Ca^{2+} signals arises from the strong co-operativity between EF-hand domains [49].

What are the visible genotypic consequences of genetic ablation of PV expression and what are the possible underlying mechanisms? Since many models of behavioral testing are based in a way or another on locomotor activity, PV−/−, but also CB-D28k−/− and mice deficient for both proteins (PV−/−CB-D28k−/−) were analyzed. The spontaneous locomotor activity repeatedly recorded during 18–20 days shows a somewhat increased activity in mice with one or the other gene ablated, and a decrease in activity in the double knockout mice [50]. The characteristic movement speed is increased during the first 8 days in PV−/− mice, while the percentage of fast movements is lower in both strains without CB-D28k expression. In addition, the fastest speed is lower in the double mutant mice. A strong correlation exists between these alterations in locomotor activity and the impairment in locomotor coordination. Both, on the runway assay and the rotarod assay, strains without CB-D28k are more impaired than mice lacking PV and the level of impairment is qualitatively correlated with alterations in Purkinje cell firing [51]. Initially detected and described in alert knockout mice for CR and CB-D28k [52], these mice show 160-Hz oscillations in the cerebellum, a brain region generally not showing pronounced oscillatory activity. Since the principal features of these local field potential (LFP) oscillations are identical for mice lacking either CR, CB-D28k, PV or combinations thereof, the most important findings are summarized here. In all mutant mice, Purkinje cells fire simple spikes (SS) phase locked to the oscillations and synchronized along the parallel fiber (PF) axis. By either blocking NMDA or $GABA_A$ receptors or blocking gap junctions (mostly dendro-dendritic gap junctions between MLIs), the oscillations can be reversibly blocked/attenuated. Oscillations are also temporarily suppressed by air puffs on the whiskers or during spontaneous muscle activity, but the latter only if the SS firing is also modulated [53]. Thus, the oscillations are the result of a network mechanism that involves I) the excitatory CR-expressing granule cells acting via the PF, II) the PV- and CB-D28k-expressing Purkinje cells and III) the network of PV-expressing MLIs; changes in the properties (e.g. excitability, short-term plasticity) of any of the three neuron populations finally lead to strong synchronization of Purkinje cell assemblies. With respect to the magnitude of Purkinje cell firing alterations, i.e. decreased complex spike (CS) duration and pause, increased SS firing rate, the effects are always larger in CB-D28k−/− than in PV−/− mice and CS alterations are cumulative in PV−/−CB-D28k −/− Purkinje cells [51]. These neurons from double knockout mice show also a dual-frequency (110 and 240 Hz) oscillation and the instantaneous spectral densities of the slow and fast component are inversely correlated. In summary, the absence of the Ca^{2+} buffers PV, CB-D8k and CR disturbs the regulation of Purkinje cell firing rate and rhythmicity *in vivo*. Thus, "apparently" small changes in the temporal aspects of $[Ca^{2+}]_i$ transients in cerebellar neurons caused by elimination of Ca^{2+} buffers from any of the relevant neuron population (granule cells, Purkinje cells, MLIs) significantly perturb the normal spontaneous arrhythmic and asynchronous firing pattern of Purkinje cells. With respect to PV, the increased synchronization of networks in the absence of PV not only occurs in the cerebellum, but may also provide a likely explanation for the increased susceptibility to epileptic seizures [35]. Unlike in the

cerebellum, networks of cortical GABAergic interneurons, most notably the network of PVergic, fast-spiking (FS) neurons are of vital importance in generating and promoting synchronous activity. These networks are involved in creating coherent oscillations, rhythms that emerge during performance of cognitive tasks [54,55]. PV-deficient mice subjected to the epileptogenic drug pentylenetetrazole (PTZ) show more severe seizures than WT control mice, albeit with a slightly later onset of the initial phase, i.e. onset of clonic–tonic seizures (CTS). The absence of PV in the hippocampus of knockout mice facilitates the $GABA_A$-ergic current reversal caused by high-frequency stimulation and this mechanism is implied in the generation of epileptic activity [35].

3.2. Reporter strains targeted at PVergic neurons

The PVergic (cortical) interneurons exert a powerful inhibition onto pyramidal cells and are thus critically involved in several aspects of network functions including oscillations. As a consequence, this neuron subpopulation has attracted interest from several fields of Neuroscience. With the aim to manipulate expression levels of proteins of interest specifically in the "PV neurons", several transgenic mouse lines have been generated and are available from The Jackson Laboratory (Bar Harbor, MA, USA). In the strain B6;129P2-Pvalb$^{tm1(cre)Arbr}$, the Cre recombinase is expressed from the endogenous *Pvalb* gene locus. An IRES-Cre-pA targeting cassette is placed in the 3′UTR region of the murine *Pvalb* gene [56]. The recombination in a mouse strain containing *loxP* sites flanking the sequence of interest occurs at >90% of PV neurons. A similar strategy was used to create the line B6.Cg-Pvalb$^{tm1.1(tTA2)Hze}$; the mice contain a synthetic modified tetracycline-regulated transactivator ($tTA2^S$) gene just downstream of the translational stop codon of the *Pvalb* gene. When these mice are crossed with a strain carrying the gene of interest under the control of a tetracycline-responsive promoter element (TRE, TetRE or tetO) the expression of the target gene in PV neurons can be attenuated/blocked with administration of doxycycline (dox), a tetracycline analog. In another knockout/knockin mouse line, B6(Cg)-Pvalb$^{tm1(cre/ERT2)Zjh}$ the transgene is targeted to the *Pvalb* locus and disrupts endogenous PV expression as in PV−/− mice. The creERT2 fusion protein consists of a Cre recombinase fused to a triple mutant form of the human estrogen receptor. This protein doesn't bind the natural ligand 17β-estradiol, but the synthetic ligand 4-hydroxytamoxifen (OHT or tamoxifen), thus allowing activation of the Cre recombinase by tamoxifen. Such activation in a *loxP* strain leads to a tamoxifen-inducible Cre activity selectively in the PV-expressing neurons. A different strategy was chosen in mouse strains, where the transgene is inserted randomly by heterologous recombination using large bacterial artificial chromosome (BAC) transgenes resulting in Cre expression under the control of this transgene containing a large region comprising the *Pvalb* gene locus [57]. A similar strategy was also used to express the enhanced green fluorescent protein (EGFP) in PV neurons [58]. Independently, another group produced a line of PV-Cre mice [59]. By crossing this PV-Cre line with a *loxP* GFP mouse line revealed the GFP to be expressed not only in the PV interneurons, but also in many excitatory neurons in the neocortex and the thalamus. In contrast, in the other reported PV-Cre strain, EGFP expression is primarily restricted to PV-ir neurons [58].

4. Calbindin-D28k (CB-D28k)

The first report to manipulate (down-regulate) CB-D28k *in vivo* was published in 1996 [60]. For the generation of this transgenic line produced by heterologous recombination, the plasmid pNF-L/CBas2, containing I) the human neurofilament L promoter and 5′ flanking sequences controlling neuronal-specific transcription, II) the mouse CB-D28k cDNA (from nt 419 to nt 1459) in reverse (antisense) orientation and III) SV40 splicing and polyadenylylation signals was injected into the pronuclei of mouse fertilized oocytes. The expression of CB-D28k antisense RNA leads to an almost complete loss of CB-D28k mRNA in most brain regions including the hippocampal formation, with the exception of Purkinje cells, where a still rather strong signal is seen in transgenic mice. In these CB-D28k antisense transgenic mice, LTP in the hippocampal CA1 area is impaired, and it is proposed that CB-D28k may, at least in part, modulate the activation of NMDA receptors [61]. However, the fundamental biophysical properties of NMDA receptors and their number are not different in the CB-D28k-deficient mice [62]. The impairment in LTP is linked to augmented tetanic-induced $[Ca^{2+}]_i$ elevations in the postsynaptic CA1 pyramidal cells, since a weaker tetanus stimulation leads to LTP induction and maintenance also in mice with reduced/absent levels of CB-D28k. Also BAPTA-AM loading or blocking of NMDA receptors by D-APV, both restricting elevations in $[Ca^{2+}]_i$ restore LTP induction in the transgenic mice. As in the CB-D28k−/− mice (see below), elevations in $[Ca^{2+}]_i$ that are effected by NMDA- or K^+-induced depolarization are prolonged in neurons from slices of CB-D28k antisense mice [63]. Initial effects caused by deletion of the functional mouse *Calb1* gene coding for CB-D28k were reported in 1997 [36] and in this section, the focus is on results obtained in CB-D28k−/− mice within the last few years, for more details on initial studies, see [38,64]. The global phenotype observed in the null-mutant CB-D28k −/− mice (systematic name: Calb1^{tm1Mpin}) is rather discrete, similar to other Ca^{2+} buffer knockout strains: general brain development is normal, no obvious morphological changes are discernible on histological sections and under normal housing conditions, no behavioral phenotype is evident [36]. CB-D28k expression is found in many brain regions and various cell types, for details in the rodent brain, see [20]. In the rodent hippocampal formation, CB-D28k expression in principal cells is restricted to dentate gyrus granule cells and CA1 pyramidal cells [65]. In addition, CB-D28k-ir interneurons are present within all subdivisions. The other brain region with a very limited distribution of CB-D28k is the cerebellum, where expression is confined to Purkinje cells. Thus, the physiological role of CB-D28k and the effects caused by its absence in CB-D28k−/− mice were mostly investigated in the hippocampus and the cerebellum.

In acutely dissociated dentate gyrus granule cells of humans with chronic temporal lobe epilepsy (TLE), neurons characterized by significantly reduced levels of CB-D28k [66], show strong Ca^{2+}-dependent inactivation of high-voltage activated (HVA) Ca^{2+} conductances (I_{Ca}) [67]. Based on these results, it was proposed that the loss of CB-D28k from these neurons might increase the Ca^{2+}-dependent I_{Ca} inactivation and thus decrease the total Ca^{2+} load. Thus, in contrast to the "classical" model of Ca^{2+} buffers having a neuroprotective role, here the absence of a Ca^{2+} buffer with fast binding kinetics is protective by limiting/decreasing Ca^{2+} entry during prolonged AP trains that occur e.g. during epileptic seizures [66]. This counter-intuitive hypothesis was tested in CB-D28k−/− mice. CA1 pyramidal neurons, a population extremely vulnerable to ischemia in WT mice, "recovered significantly faster and more completely in CB-D28k−/− mice, after a transient oxygen-glucose deprivation *in vitro*, and sustained less cellular damage following a 12 min carotid artery occlusion *in vivo*" [68]. Also additional experiments on synaptic properties, e.g. altered adaptation of AP firing, altered paired-pulse and frequency potentiation, are in line with the removal of a fast Ca^{2+} buffer causing these effects. In this setting, containing a fast Ca^{2+} buffer increases the vulnerability of neurons, thus the down-regulation might be viewed as a neuroprotective mechanism.

The situation is different in the aging brain, where decreases in CB-D28k (and PV) expression levels are observed. CB-D28k−/− mice served as a model to test, whether the diminished levels of CB-D28k contribute to age-related hippocampal dysfunction. Basal hippocampal metabolism was estimated from steady-state relative cerebral blood volume (rCBV) measurements, an fMRI variant providing the highest spatial resolution. Normal aging in WT mice is characterized by an age-dependent decline in dentate gyrus metabolism and

CB-D28k−/− mice show accelerated age-dependent decline of rCBV-estimated metabolism in this hippocampal region, where strong expression of CB-D28k exists in WT granule cells [69]. In hippocampal regions with relatively low CB-D28k expression (e.g. CA3 region), no significant differences exist with respect to rCBV-estimated metabolism. Unexpectedly, structural changes also take place in the CB-D28k−/− mice: an enlarged hippocampus and neocortex and moreover, global brain hypertrophy are seen in CB-D28k−/− mice [69]. Additional subtle morphological changes in the cerebellum caused by the absence of CB-D28k are discussed below. These functional and structural changes are also manifest at the level of behavior. In a hippocampus-dependent learning task (active place avoidance) CB-D28k−/− mice more often enter the "shock zone", thus the observed fMRI alterations (accelerated age-dependent decline) correlate with a deficit in hippocampus-dependent learning.

Returning to the cellular mechanism(s) how Ca^{2+} buffers affect Ca^{2+} signaling and associated synaptic plasticity: use-dependent Ca^{2+} buffer saturation is responsible for the short-term plasticity (facilitation) between CB-D28k-expressing multipolar bursting cell (an interneuron type) and pyramidal cells [70]. In patch clamp experiments, washout of cytosolic constituents including mobile Ca^{2+} buffers increases the amplitude of the first response ($IPSP_1$) and decreases paired-pulse facilitation. The situation is reverted by either adding CB-D28k or the fast Ca^{2+} buffer BAPTA to the multipolar bursting cell. Unlike in the situation caused by PV, i.e. preventing/attenuating an increase in $IPSP_2$ [39], the rapid binding kinetics of presynaptic CB-D28k causes a decrease in $IPSP_1$ and the mechanism is named facilitation by buffer saturation. The mechanism is not restricted to the multipolar bursting cell-pyramidal cell synapse, but it is equally seen in the hippocampus at CB-immunoreactive (CB-ir) facilitating excitatory mossy fiber (MF)-CA3 pyramidal cell synapses; experiments in slices from CB-D28k mice have established buffer saturation of a fast Ca^{2+} buffer to be responsible for this presynaptic mechanism of synaptic plasticity, a mechanism insensitive to the action of the slow-onset buffer EGTA [70].

Although initially reported as "Ataxia and altered dendritic calcium … calbindin D28k gene" [36], the cerebellar phenotype of CB-D28−/− mice is better described as impairment of motor coordination. Under standard conditions in a home cage, the motor phenotype is not visible, even to an experienced observer. However, in tests of motor coordination including the runway assay, where the mice have to cross a narrow bar with regularly spaced small obstacles, the motor coordination impairment is easily detected [36,50]. Results from CB-D28k−/− mice indicate that the motor phenotype is linked to the emergence of 160-Hz oscillations in the cerebellum of null-mutant mice [51,52], for more details see Section 3 on PV. To verify that the motor phenotype is Purkinje cell-specific, transgenic mice were generated, where CB-D28k expression is selectively ablated in Purkinje cells [71]. For this a transgenic line expressing Cre recombinase under the control of the L7/pcp-2 gene promoter [72] was bred with a line containing *loxP* sites within the *Calb1* gene in such a way that the recombination leads to a Purkinje cell-specific inactivation of the *Calb1* gene. The motor coordination phenotype of mice with a Purkinje cell-specific deletion of CB-D28k is indistinguishable from that of "full" CB-D28k−/− mice validating the Purkinje cell specificity of this particular motor phenotype [73]. Already in the initial CB-D28k−/− strain, the hallmark of eliminating a fast Ca^{2+} buffer from Purkinje cell dendrites was identified. Several characteristic changes in the shape of synaptically evoked postsynaptic Ca^{2+} transients (by either PF or CF stimulation) are observed in CB-D28k−/− Purkinje cells: the amplitude is approximately 2-fold increased and the fast, but not the slow $[Ca^{2+}]_i$ decay component is larger compared to the shape of Ca^{2+} transients in WT mice [36]. Identical results are also seen in Purkinje cell dendrites and spines from the Purkinje cell-selective knockout mice [73]. Interestingly, the delayed metabotropic glutamate receptor (mGluR)-mediated Ca^{2+} transients, characterized by a slow rising phase lasting for up to 0.5 s, are not different than those in WT animals [73]. This points again to the importance of the type (kinetics) of Ca^{2+} signals that are/are not altered by the elimination of a Ca^{2+} buffer. Nonetheless, it is possible that the adaptive/compensatory/homeostatic mechanisms induced by the absence of a Ca^{2+} buffer are sufficient to completely compensate for Ca^{2+} signals elicited by one mechanism (slow mGluR-mediated) but not the other (fast PF- or CF-induced). Long-term depression (LTD) of Purkinje cell-PF synaptic transmission is considered as a critical determinant of normal cerebellar function, however, the absence of CB-D28k in Purkinje cells has no effect on this type of LTD [73]. In contrast mice lacking the type 1 inositol 1,4,5-trisphosphate receptor, necessary for (mGluR)-mediated/$InsP_3$-dependent Ca^{2+} signaling, show severe ataxia that is associated with impairment of LTD [74].

As discussed in the chapter on PV, alterations in Purkinje cell morphology are induced by the absence of either Ca^{2+} buffer. While changes in the Purkinje cell somata (increased subplasmalemmal mitochondria, decreased sER) are PV-specific [28], morphology of Purkinje cell spines is altered selectively by the absence of CB-D28k and is present in CB-D28k−/− and PV−/− CB-D28k−/− mice [75]. The spine length, as well as the spine head volume is increased in the absence of CB-D28k. The only spine parameter that is specifically affected in the double-KO mice is spine density (number of spines/μm of dendrite), which is increased by 40–50%. Spine density in Purkinje cells lacking either PV or CB-D28k is not different than that of WT neurons. Analysis of CF-induced Ca^{2+} signals in Purkinje cell dendrites form PV−/− and double-KO mice allowed to determine in a quantitative manner the contribution of CB-D28k [34]. Based on the experimental data, the roles and contribution of either PV or CB-D28k on dendritic Ca^{2+} signals was modeled. While the $[Ca^{2+}]_i$ decay in dendrites from WT Purkinje cells shows marked bi-exponential characteristics, the biphasic nature is less pronounced in PV−/− neurons, while the peak amplitude is not affected. This is exactly the hallmark of the slow-onset Ca^{2+} buffer as also seen in fast-twitch muscles [22], in PV-loaded chromaffin cells [40] and cerebellar interneurons [41]. On the other hand, CB-D28k reduces the peak amplitude, but slows $[Ca^{2+}]_i$ decay resulting in an almost monophasic decay. In the study of Schmidt et al. [34], the biphasic $[Ca^{2+}]_i$ decay kinetics was proposed to derive not only from PV's slow-onset properties, but also, in part, from the high-affinity, "slow-onset" Ca^{2+}-binding sites of CB-D28k. The kinetics of CB-D28k had been determined *in vitro* by flash photolysis of caged Ca^{2+} [76]. However, most recent experiments using the same, but improved methodology indicate that all four functional Ca^{2+}-binding sites in CB-D28k are identical [10]. The experimental data on dendritic Ca^{2+} signals together with the determination of PV's and CB-D28k's mobility in Purkinje cells [9,77,78] permitted modeling of the role of the two Ca^{2+} buffers in mediating buffered diffusion of Ca^{2+}, i.e. these proteins serving as shuttles for transporting Ca^{2+} ions from spines to the dendritic tree [79]. Evidently the spine neck geometry is a critical parameter for this spino-dendritic crosstalk [80]. Results indicate that CB-D28k in spines of Purkinje cells has an important role in this spino-dendritic coupling, while the effect of PV is minute. It is proposed that "this represents a central mechanism for activating calmodulin in dendritic shafts and therefore a novel form of signal integration in spiny dendrites" [79]. Besides the morphological changes caused by CB-D28k's absence, also changes at the level of Ca^{2+} signaling toolkit components are observed. Voltage-gated Ca^{2+} channels, in particular $Ca_V2.1$ (P/Q type) regulate Ca^{2+} signaling and excitability of Purkinje cells. Upon Ca^{2+} entry, they undergo a dual feedback regulation, Ca^{2+}-dependent facilitation (CDF) and inactivation (CDI) and the latter is also dependent on mobile Ca^{2+} buffers such as PV and/or CB-D28k [81]. Unlike *in vitro*, where PV and CB-D28k show the expected effect, i.e. reducing CDI, in PV−/−CB-D28k−/− Purkinje cells net CDI is similar as in WT Purkinje cells. However, in these neurons $Ca_V2.1$ currents show increased

voltage-dependent inactivation, which results from a decrease in the expression of the auxiliary Ca_Vbeta2a subunit compared to WT Purkinje cells [82]. Thus, it is likely that the increased VDI due to a switch in $Ca_V2.1$ beta subunits preserves "normal", WT-like activity-dependent Ca^{2+} signals in the absence of the two major Ca^{2+} buffers in the double-KO mice. The contribution of each of the two Ca^{2+} buffers to the VDI effect was not investigated in this study.

5. Calretinin (CR)

The hexa-EF-hand protein calretinin (CR) was initially discovered as a Ca^{2+}-binding protein in the retina, thus the name calcium + retina = calretinin. Prominent neuronal subpopulations expressing CR include specific neurons in the cortex [83], in the hippocampus and the cerebellar granule cells [84] as well as specific neurons of the peripheral nervous system [85]. In 1997, the first report on CR−/− mice (gene symbol: *Calb2*; systematic strain name: $Calb2^{tm1Map}$) was published [86] and was focused on the function of CR in the neurons of the hippocampal formation. There, CR is expressed in a subset of GABAergic interneurons in all subfields and in glutamatergic hilar mossy cells of the dentate gyrus. In CR−/− mice, basal synaptic transmission is unaffected, but LTP is impaired between the perforant pathway and granule cells, however, not at the Schaffer commissural input to CA1 pyramidal neurons. At the former, LTP is restored in the presence of the $GABA_A$ receptor antagonist bicuculline, indicating that the absence of CR in hilar mossy cells increases the drive onto GABAergic interneurons, which then indirectly impairs LTP. The reduction in LTP, however does not affect the performance of CR−/− mice in a spatial memory task. This study still represents the only one published on the putative role of CR in hippocampal-related learning and memory. Based on CR's prominent expression in granule cells, the effects of its absence in CR−/− mice on cerebellar function were investigated. The intrinsic electroresponsiveness of CR−/− granule cells is altered: APs are faster and granule cells generate repetitive spike discharge characterized by an enhanced frequency increase with injected currents [87]. The increased excitability *in vitro* is reverted by loading the fast Ca^{2+} buffer BAPTA (150 μM) into CR−/− granule cells and a mathematical model links the increased granule cell excitability with the decreased cytosolic Ca^{2+}-buffering capacity [88]. How do these changes correlate with synaptic transmission between PF and Purkinje cells and moreover the firing of Purkinje cells? In alert CR−/− mice several changes in the firing behavior are observed: simple spike (SS) firing rate is increased, the complex spike (CS) duration is decreased, and SS pause is shorter [89]. These alterations are also indirectly reflected in CR−/− Purkinje cells evidenced by immunohistochemical stainings. The staining intensity for CB-D28k is stronger despite similar CB-D28k expression levels in the cerebellum of WT and CR−/− mice. This hints at a modification in the Ca^{2+} homeostasis in CR−/− Purkinje cells leading to a higher (steady-state) Ca^{2+}-loading status (relative saturation) of CB-D28k. The fact that the alterations in Purkinje cell firing are not seen in slices from CR−/− mice and furthermore, transmission at PF- and CF-Purkinje cell synapses is unaltered in slices, demonstrates CR's major physiological role at the network level. Not only Purkinje cell firing properties are different in CR−/− mice *in vivo*, but these mice show 160-Hz local field potential oscillations in the cerebellum that reach a maximum amplitude in the Purkinje cell layer, similar as mice deficient for CB-D28k [52]. Other details about these oscillations are discussed in Section 3.1 on PV−/− mice and for more details on CR−/− mice, see [90]. The consequences of those oscillations in mice deficient for CR, CB-D28k or PV are manifested as impairments in tests of motor coordination [36,50,89]. The motor phenotype, Purkinje cell firing behavior and the associated 160 Hz-oscillations in CR−/− mice are rescued, when CR is selectively expressed in granule cells of CR−/− mice [53]. For this, CR is expressed in transgenic mice via the $GABA_A$ receptor α6 promoter on a CR−/− background. Is the decreased Ca^{2+}-buffering capacity of granule cells in the absence of CR responsible for the cerebellar phenotype of CR−/− mice? Unfortunately, the experiments carried out so far do not allow this question to be answered. The electroresponsiveness of CR−/− granule cells can be restored by increasing the "fast" Ca^{2+}-buffering capacity *in vitro*, but whether or not additional CR-mediated functions in granule cells *in vivo* are responsible for the granule cell-specific rescue remains to be shown. Perhaps expressing another fast Ca^{2+} buffer with BAPTA-like properties (e.g. CB-D9k) selectively in granule cells of CR−/− mice might address this question. In summary, CR expression in granule cells is required for the physiological function (correct computation) of the cerebellum and furthermore, granule cell excitability is dependent on the Ca^{2+}-buffering capacity. A more detailed phenotypic analysis, e.g. on CR's role in the subpopulation of neocortical interneurons is still lacking, possibly as a consequence of the rather discrete neuronal/neurological phenotype of CR−/− mice.

6. Ca^{2+} buffer transgenic mice as models for specific pathologies and diseases?

The Thy-PV mice had been initially produced in order to test a rather "old" hypothesis, i.e. the putative neuroprotective role of intracellular Ca^{2+} buffers in neurodegenerative diseases including ischemia, Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). Motor neurons, a neuron population characterized by low Ca^{2+} buffering capacity [91], are highly vulnerable in ALS patients, possibly linked to Ca^{2+} overload induced by excitotoxic stimuli. In cultured PV-expressing spinal motor neurons from Thy-PV mice, the increase in $[Ca^{2+}]_i$ induced by kainic acid (KA) and mediated by Ca^{2+}-permeable AMPA receptors is smaller and furthermore, these neurons are also better protected from KA-induced death *in vitro* as compared to those isolated from WT littermates [92]. Interestingly, the increase in $[Ca^{2+}]_i$ caused by a short K^+-mediated depolarization is the same in motor neurons with or without PV indicating that the way and kinetics of Ca^{2+} entry also determines the (neuroprotective) effect of a Ca^{2+} buffer. PV-overexpressing neurons are also better protected *in vivo* against neonatal axotomy-induced motor neuron death. Eight weeks after crushing the sciatic nerve in one hindlimb, 47% motor neurons survive in comparison to WT mice, where the survival rate of injured motor neurons is only 20% [93]. Unfortunately, this impressive increase in motor survival in Thy-PV mice is not reflected in a significant improvement in muscle function; 8 weeks after injury neither maximal twitch and tetanic force is increased nor are muscle weights higher in the transgenic mice. In the same Thy-PV mice, the putative neuroprotective role against Ca^{2+} mediated glutamate excitotoxicity has been investigated. Injection of the glutamate agonist ibotenic acid (IBO) into the striatum of adult mice, a brain region particularly vulnerable to excitotoxicity, leads to local neuron loss, reactive astrogliosis and parenchymal microcalcification. Unexpectedly, the neurodegenerative process is increased, not decreased in Thy-PV mice [37]. As described in Section 6, the mitochondrial volume in striatal neurons from Thy-PV mice is reduced to almost half. This hints in the direction that not the increased PV-mediated Ca^{2+}-buffering capacity, but the adaptive/compensatory/homeostatic decrease in mitochondria volume is the likely cause for the increased susceptibility to glutamate toxicity. In IBO-treated Thy-PV mice, the microcalcification is enhanced and accelerated compared to WT mice [94]. Neuronal mitochondria appear to be the nucleators of the hydroxyapatite deposits, suggesting that increased mitochondrial Ca^{2+} accumulation in the fewer mitochondria of Thy-PV neurons are responsible for the increased damage caused by IBO.

The putative neuroprotective role of Ca^{2+} buffers was also investigated in another model, the kainate (KA) model of mesial temporal lobe epilepsy. Unilateral intrahippocampal KA injection induces acute epileptic seizures and long-term alterations of the hippocampal formation including bilateral induction of neuropeptide Y in granule cells and an enlargement and dispersion of dentate gyrus granule

cells. None of the investigated parameters are different in single knockout mice for PV, CB-D28k and CR as well as in double knockout mutants compared to WT mice [95]. This includes the vulnerability to acute kainate-induced excitotoxicity, the long-term effects of recurrent focal seizures, the global cytoarchitecture of the hippocampal formation, as well as the number, distribution, and morphology of interneurons. This indicates that either the tested proteins are not protective against kainate-induced excitotoxicity or that the adaptive/compensatory mechanisms induced in the mice with constitutively-deleted Ca^{2+} buffers are sufficient to cope with the kainate-induced insult similar as WT mice expressing the respective Ca^{2+} buffer proteins. In favor of the latter hypothesis, a rapid increase in Ca^{2+} buffering following an acute ischemic stroke protects neurons from cell death [96], while adeno-associated virus (AAV)-mediated overexpression of CB-D28k in striatal and cortical brain regions for 12 weeks blunts the initially protective effect of CB-D28k [97]. The authors suggest that a long-term adaptation process to the ectopic expression of CB-D28k obliterates the acutely protective effect, thus shedding serious doubts on a clinical applicability of this putatively preventive approach.

A marked neuronal loss is a characteristic of prion diseases, but not all neuron subpopulations are equally sensitive. In contrast to the rather resistant CB-D28k-ir neurons, PVergic neurons are selectively destroyed early in the course of human and experimental prion diseases. The question of an involvement of CB-D28k and PV in resistance against and/or vulnerability to nerve cell death was addressed in 139A scrapie-inoculated PV−/− and CB−/− mice [98]. Histological lesion profiles are not different between WT and the two null-mutant strains. The number of isolectin B4-decorated perineuronal nets [99] surrounding PVergic cells is equally diminished in PV−/− and WT brains and the number of Purkinje cells is not different between brains from CB−/− and WT mice. Thus, also in this model neither the presence of CB-D28k nor PV confers resistance towards scrapie-mediated nerve cell death. Of interest, survival times of CB−/− mice are slightly reduced, however no satisfactory explanation has so far been presented for this observation.

7. Transgenic mice for other Ca^{2+} buffers

7.1. Oncomodulin (beta parvalbumin)

Until now, no report has been published on a transgenic mouse line in which oncomodulin levels, the mammalian beta parvalbumin (mouse gene symbol: *Ocm*) have been modulated. For more details on oncomodulin expression and function, see [8]. A single indirect study reports that in partly varicose axons in the diencephalon, mainly in the lamina medullaris externa surrounding the thalamus and in a sparse subpopulation of neurons in the thalamus and in the dentate gyrus of PV−/− mice, ectopic expression of oncomodulin is observed [100]. However, the functional consequences of this particular oncomodulin expression are unknown. In the nervous system of WT rodents, the expression is restricted to outer hair cells in the organ of Corti.

7.2. Secretagogin

Secretagogin (murine gene symbol: *Scgn*) is the third and least explored member of the hexa EF-hand family of Ca^{2+}-buffers; no transgenic mouse lines have been reported and the current knowledge about this protein has not been reviewed in detail. Initially secretagogin was discovered as a protein expressed in neuroendocrine cells [101] and in specific neurons including I) MLIs (stellate and basket cells), II) a subpopulation of neurons in the frontal and parietal neocortex and hypothalamus and III) in pyramidal cells of the hippocampus [102]. In P301L tau transgenic mice, which show marked and widespread tau pathology, the increased levels of tau down-regulate secretagogin [103]. Nonetheless, secretagogin-ir hippocampal neurons are better able to resist tau-induced pathology in AD, better than the majority of other hippocampal neurons. It remains to be shown if the increased resistance is a direct (neuroprotective) effect mediated by secretagogin.

7.3. Calbindin-D9k (CB-D9k)

Calbindin-D9k (murine gene symbol: *S100g*) is not expressed in excitable cells including muscle cells, heart cells (myocytes) or neurons, for more details on CB-D9k distribution and function, see [104]. CB-D9k is implicated in Ca^{2+} resorption in the intestine and kidney. Studies with two independently generated CB-D9k−/− mouse strains have been reported [105,106].

8. Summary

Genetically modified mice (knockout and transgenic) with targeted ablation or ectopic expression of Ca^{2+} buffer proteins have clearly served to better understand the physiological function of these proteins *in vivo*. However, the importance of *in vitro* results obtained on these proteins should not be underestimated and such findings remain essential for a better comprehension of their role(s) in a living organism; it is the synergistic combination of the two approaches that has advanced the field.

In any biological system, removal or ectopic expression of a protein leads to an adaptation of the system to the altered situation. In particular with respect to Ca^{2+} signaling, where the various components are functionally highly interconnected, removal of a Ca^{2+} signaling toolkit component induces specific adaptive/homeostatic/compensatory mechanisms. The phenotype of Ca^{2+} buffer protein knockout mice can be fully understood only when such mechanisms are taken into consideration. Future studies with these genetically modified mice, but also with all other transgenic mice with altered expression of Ca^{2+} signaling components (e.g. channels, pumps) should be designed such as to obtain results with a global focus on Ca^{2+} signaling/homeostasis. I am convinced that this will eventually result in a better mechanistic understanding of the many neurological diseases linked to altered Ca^{2+} signaling, and will possibly lead to new avenues for specific therapies.

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