Cytosolic Ca^{2+} Buffers Are Inherently Ca^{2+} Signal Modulators

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For precisely regulating intracellular Ca^{2+} signals in a time- and space-dependent manner, cells make use of various components of the “ Ca^{2+} signaling toolkit,” including Ca^{2+} entry and Ca^{2+} extrusion systems. A class of cytosolic Ca^{2+} -binding proteins termed Ca^{2+} buffers serves as modulators of such, mostly short-lived Ca^{2+} signals. Prototypical Ca^{2+} buffers include parvalbumins (α and β isoforms), calbindin-D9k, calbindin-D28k, and calretinin. Although initially considered to function as pure Ca^{2+} buffers, that is, as intracellular Ca^{2+} signal modulators controlling the shape (amplitude, decay, spread) of Ca^{2+} signals, evidence has accumulated that calbindin-D28k and calretinin have additional Ca^{2+} sensor functions. These other functions are brought about by direct interactions with target proteins, thereby modulating their targets’ function/activity. Dysregulation of Ca^{2+} buffer expression is associated with several neurologic/neurodevelopmental disorders including autism spectrum disorder (ASD) and schizophrenia. In some cases, the presence of these proteins is presumed to confer a neuroprotective effect, as evidenced in animal models of Parkinson’s or Alzheimer’s disease.

Since the publishing of “Cytosolic Ca^{2+} Buffers” (Schwaller 2010), new data on the structure, function, interactions, and regulation of this class of proteins has emerged, and is summarized here. As relatively little novel information was obtained in the intervening period on the basic concepts of how Ca^{2+} buffers function, the reader is advised to consult a previous article elsewhere (Schwaller 2010) on these topics. The relevance of Ca^{2+} buffers in certain diseases, and in particular the role of parvalbumin (PV), is discussed in more detail in this review.

DEFINITION OF A CYTOSOLIC Ca^{2+} BUFFER

Although several protein families localized in the cytosol (e.g., annexins, C2 domain-contain-

ing proteins, pentraxins; Schwaller 2004) might qualify as intracellular Ca^{2+} buffers, the term is generally restricted to members of the EF-hand protein family (for detailed information on EF-hand motifs, see Chazin 2011). The EF-hand (or EF-hand motif) is a structural domain consisting of an α -helix–loop– α -helix motif of ~ 30 amino acids. It may be represented by the right hand, where the index finger signifies the E-helix, the bent middle finger the Ca^{2+} -chelating loop and the thumb the F-helix. The motif was first discovered as the region embracing the fifth and sixth α -helix (thus, ABCDEF) of α PV (Kretsinger and Nockolds 1973). The canonical Ca^{2+} -binding loop of 12 amino acids contains a series of negatively charged residues (Glu, Asp) and/or backbone carbonyl groups providing the

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cage for the Ca^{2+} ion (Fig. 1). Functionally, EF-hand proteins were classified in two groups: Ca^{2+} buffers and Ca^{2+} sensors. The majority of the >600–800 family members (Kawasaki et al. 1998) from different species and kingdoms (≈ 250 in mice) (Girard et al. 2015) are described as Ca^{2+} sensors, with the prototypical Ca^{2+} sensor being calmodulin (CaM) (Chin and Means 2000). The distinction between these two groups is correlated with the Ca^{2+} -binding-induced conformational changes. In general, Ca^{2+} sensors undergo rather significant conformational changes between the Ca^{2+} -free (apo) and the Ca^{2+} -bound state, most often exposing hydrophobic patches required for the interaction with specific ligands, as exemplified by CaM (see Figure 2 in Chazin 2011) and most of the S100 family proteins. The exception is calbindin D-9k (also named calbindin-D9k or CB-D9k; human gene symbol: *S100G*), an atypical S100 protein functioning as the classical fast Ca^{2+} buffer. In CB-D9k, Ca^{2+} -induced conformational changes are minor, and the protein remains mostly in the closed, apo-like state (Chazin 2011). Other Ca^{2+} buffers include the EF-hand family members PV (α and β isoforms), calbindin-D28k (CB-D28k), and calretinin (CR).

The still currently used term Ca^{2+} buffer has created a large amount of confusion and misconception. In the chemical sense (e.g., pH buffers), a buffer system is tuned to clamp the pH to a predetermined value in such a way that the addition of an acid or a base elicits only a minor change of the pH. As a result, the buffering capacity of the system is highest when the pH is close to the pK value of the corresponding acid/base pair. This is profoundly different for the so-called cytosolic Ca^{2+} buffers. In resting cells, the steady-state (basal) intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is on the order of 20–100 nM, whereas the dissociation constants for Ca^{2+} ($K_{D,\text{Ca}}$, which is analogous to the pK value in an acid/base system) of essentially all Ca^{2+} buffers are one order of magnitude higher (≈ 200 –1500 nM; Table 1). Thus, at basal $[\text{Ca}^{2+}]_i$, Ca^{2+} buffers are mostly in their apo (Ca^{2+} -free) state, and ready to bind Ca^{2+} ions, whenever $[\text{Ca}^{2+}]_i$ is augmented. Depending on

the particular properties of a Ca^{2+} buffer, the spatiotemporal dynamics of an increase in $[\text{Ca}^{2+}]_i$ are modulated in a cell-type-dependent and protein-specific way. For these reasons, the name Ca^{2+} buffer ought to be replaced by the more precise description Ca^{2+} -signal modulator. Of note, if present at sufficiently high concentrations, EF-hand Ca^{2+} sensors may also function as Ca^{2+} signal modulators. Recent findings show that some Ca^{2+} signal modulators have additional functions as Ca^{2+} sensors (see sections on calretinin and calbindin-D28k).

When measuring the total Ca^{2+} -buffering capacity (κ_s) of a cell, often the distinction is made between mobile and immobile buffers (Zhou and Neher 1993; Lin et al. 2017). The latter buffers are defined as molecules capable of binding cytosolic Ca^{2+} and these mostly unidentified molecules are not washed out when, for example, the plasma membrane is patched with a pipette. Very little is known about the molecular identity of immobile Ca^{2+} buffers except their rather low Ca^{2+} affinity ($K_{D,\text{Ca}}$, 100–400 μM ; Lin et al. 2017). Thus, immobile buffers together with mobile Ca^{2+} buffers are responsible for the rather slow diffusion of Ca^{2+} ions inside a cell.

Functionally, two types of EF-hand Ca^{2+} -binding sites are discernable because of their different selectivity and affinity for Ca^{2+} and Mg^{2+} ions (Celio et al. 1996). The Ca^{2+} -specific sites display affinities for Ca^{2+} (K_{Ca}) on the order of 10^{-3} – 10^{-7} M, and significantly lower affinities for Mg^{2+} ($K_{\text{Mg}} = 10^{-1}$ – 10^{-2} M). The proteins CB-D28k and CR have four and five functional Ca^{2+} -binding sites of this type, respectively. The mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites bind Ca^{2+} with high affinity, and Mg^{2+} with moderate affinity, in a competitive manner (dissociation constants: $K_{\text{Ca}} = 10^{-7}$ – 10^{-9} M; $K_{\text{Mg}} = 10^{-3}$ – 10^{-5} M; Table 1). PV is the prototypical example of a protein with two mixed sites. Based on PV's affinities for Ca^{2+} and Mg^{2+} , and with $[\text{Ca}^{2+}]_i$ (≈ 50 nM) and $[\text{Mg}^{2+}]_i$ (0.5–1 mM), PV's two $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites are, to a large degree ($\geq 95\%$), occupied by Mg^{2+} under basal conditions.

In most proteins, EF-hands are paired in domains; two α -helix– Ca^{2+} -binding loop–

Figure 1. Conserved amino acids in the Ca^{2+} -binding loop and structure of selected EF-hand proteins. (A) Consensus sequence of the canonical EF-hand Ca^{2+} -binding loop of 12 amino acids. Amino acids X, Y, Z, and -Z provide side-chain oxygen ligands, * provides the backbone carbonyl oxygen, and at -X a water molecule is hydrogen-bonded to a loop residue. Amino acids most often present at a given position are shown *below* and shaded residues are the most conserved ones (Marsden et al. 1990). At positions X and -Z, Asp (D) and Glu (E) are generally present, respectively. The seven oxygen ligands coordinating the Ca^{2+} ion are located at the seven corners of a pentagonal bipyramid and the Ca^{2+} ion (not shown) is in the center (*right*). (B) Solution structure of Ca^{2+} -bound human α parvalbumin (PV) (PDB: 1RJV). Both, the CD domain (green) and EF domain (yellow/red) bind one Ca^{2+} ion each (green spheres) in canonical Ca^{2+} -binding loops of 12 amino acids. The orthogonally oriented helices E and F (gray-shaded) are connected by the Ca^{2+} -binding loop. Both Ca^{2+} -binding sites in PV are of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ mixed type. The amino-terminal AB domain (blue) is necessary for protein stability. (C) Nuclear magnetic resonance (NMR) solution structure of bovine CB-D9k (PDB: 1B1G). The shown structure takes into account the Ca^{2+} ions and explicit solvent molecules. The amino-terminal domain EF1 is a pseudo (Ψ) EF-hand with a larger loop of 14 amino acids, whereas the second domain (EF2) has a canonical Ca^{2+} -binding loop of 12 amino acids. In both loops, the Glu residue at the position -Z with the two carboxyl oxygen atoms (red) serves as a bidentate ligand representing two corners of the pentagonal bipyramid. This residue, most often Glu (rarely Asp), is a critical determinant for the Ca^{2+} affinity of the entire loop; Ca^{2+} ions are shown as green spheres. The two Ca^{2+} -binding loops are in close proximity and stabilized via short β -type interactions (gray-shaded area). (D) 3D NMR structure of CB-D28k (PDB: 2G9B). CB-D28k has a relatively compact structure comprising three Ca^{2+} -binding units, each unit consisting of a pair of EF-hands. Ca^{2+} -binding is restricted to the Ca^{2+} -binding loop 1 in the amino-terminal unit (blue), to both loops 3 and 4 in the middle unit (green) and to loop 5 in the carboxy-terminal pair (yellow/red). EF-hands 2 and 6 are nonfunctional with respect to Ca^{2+} -binding. The Ca^{2+} -binding loops flanked by two almost perpendicular α -helical regions are numbered from 1 to 6. (Images B–D were generated with PDB ProteinWorkshop 1.50; Moreland et al. 2005. From Schwaller 2010; reprinted, with permission, from the author.)

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α -helix regions are connected by a short stretch of 5–10 amino acid residues and form a functional unit, also termed domain (Chazin 2011). Besides providing structural stability to EF-hand domains, because of the close apposition also of the Ca^{2+} -binding loops in such a tandem domain (Fig. 1), binding of a Ca^{2+} ion to one loop may allosterically affect the other: both with respect to affinity and binding kinetics (Nelson et al. 2002; Faas et al. 2007). As a result of this building principle, the majority of EF-hand proteins have an even number of EF-hand motifs (two in CB-D9k, four in CaM, six in CB-D28k and CR); the uneven number (three) for PV (α and β) is, together with the group of penta-EF-hand Ca^{2+} -binding proteins (Maki et al. 2012), rather the exception.

It is the kinetics of Ca^{2+} binding to the various Ca^{2+} signal modulators that strongly affect the spatiotemporal aspects of Ca^{2+} signals. This is particularly true for excitable cells in which $[\text{Ca}^{2+}]_i$ transients can be brief, typically lasting for tens of milliseconds to several hundred milliseconds, and thus are differently modulated by kinetically distinct Ca^{2+} buffers. The on rates for Ca^{2+} binding (k_{on}) to various Ca^{2+} buffers vary by more than two orders of magnitude. Typical fast buffers with Ca^{2+} -specific sites (e.g., CB-D9k) have k_{on} rates $>10^8 \text{ M}^{-1} \text{ sec}^{-1}$ comparable to the on-rate of the fast synthetic Ca^{2+} buffer BAPTA. At the other end of the scale, on-rates for the slow-onset buffer PV are $\approx 3 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ under physiological conditions with $[\text{Mg}^{2+}]_i$ of $\approx 0.5\text{--}1.0 \text{ mM}$ (Table 1). All parameters for the Ca^{2+} signal modulators PV, CB-D9k, CB-D28k, and CR, as well as for EGTA and BAPTA are listed in Table 1. Besides binding kinetics, the mobility of Ca^{2+} signal modulators determines the proteins' range of affecting intracellular Ca^{2+} signals (for details on mobility, see Schwaller 2010 and Table 1). In summary, the observed slow diffusion of Ca^{2+} ions in a resting cell ($[\text{Ca}^{2+}]_i \approx 50 \text{ nM}$) is caused by slowly mobile and immobile buffers acting like Velcro for Ca^{2+} ions, thereby limiting the effective range of an unbuffered free Ca^{2+} ion to $\approx 0.1 \text{ }\mu\text{m}$. Thus, the range of Ca^{2+} can be increased by buffered diffusion, that is, mobile Ca^{2+} buffers acting as shuttles transporting Ca^{2+} through

the mesh of immobile buffers (Schmidt et al. 2007a).

PARVALBUMINS (α AND β LINEAGE)

Structural Aspects of Parvalbumins

The atypical EF-hand protein PV ($M_r \approx 12 \text{ kDa}$; PVALB) has an uneven number (three) of EF-hands and the two functional ones are mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites (Table 1). The amino-terminal AB site, which does not bind Ca^{2+} ions, is necessary for PV's stability (Fig. 1; Cox et al. 1999). The CD- and EF-hands form a functional pair (or domain). In both sites, the Ca^{2+} ion in the center of the loop is coordinated by seven ligands sitting in the corners of a pentagonal bipyramid (Swain et al. 1989). The solution structures of α PV and β PV (in humans and rodents named oncomodulin [OM]; OCM) (Babini et al. 2004) in the Ca^{2+} -loaded and the apo (metal-free) form are summarized here: (1) PV's Ca^{2+} -loaded EF-hands, the linker region connecting the CD- and EF-hands, as well as amino and carboxyl termini of PV (Baldellon et al. 1998) are rather rigid structures. (2) Differences in the structure of apo- and Ca^{2+} -loaded forms of rat PV are small, mostly confined to the loop region. Thus, Ca^{2+} -binding does not induce major structural rearrangements (Henzl and Tanner 2008). (3) The first two points hold also true for the $\text{Ca}^{2+}/\text{Mg}^{2+}$ (EF) site in rat β PV, whereas the noncanonical CD site undergoes significant structural alterations when Ca^{2+} is removed from β PV (Henzl and Tanner 2007). Thus, the global rigidity of α PV favors this molecule to serve as a simple Ca^{2+} signal modulator, whereas the Ca^{2+} -dependent conformational changes in β PV may provide β PV also with a Ca^{2+} sensor function. PV concentrations may reach millimolar ranges in fast-twitch muscles and are highly correlated with the speed of muscle relaxation (Heizmann et al. 1982). In neurons, PV concentration is on average one order of magnitude lower ($10\text{--}150 \text{ }\mu\text{M}$), and is rather variable in some neuron populations. For example, in mouse cerebellar interneurons, the PV concentration ranges from 55 to $1788 \text{ }\mu\text{M}$ (mean $563 \pm 66 \text{ }\mu\text{M}$) (Eggermann and Jonas 2012).

Table 1. Properties of selected Ca^{2+} -binding proteins

	α PV	β PV (OM)	CB-D9k	CB-D28k	CR	EGTA	BAPTA
Ca^{2+} -binding sites (functional)	3 (2)	3 (2)	2 (2)	6 (4)	6 (5)	1 (1)	1 (1)
Ca^{2+} specific/mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites	0/2	1/1	2/0	4/0 ^a	5/0	1/0	1/0
$K_{D,\text{Ca}}$ (nM)	4–9 ^b	Mixed: 42–45 ^d	$K_{D1} \approx 200$ –500 ^e $K_{D2} \approx 60$ –300	High affinity (h) ^f $K_{D1} \approx 180$ –240	$K_{D(T)} 28 \mu\text{M}^g$ $K_{D(R)} 68$ $K_{D(\text{app})} 1.4 \mu\text{M}$	70–550 ^p	160 ^q
		Ca^{2+} -specific: 590–780 ^d		Medium affinity (m)			
$K_{D,\text{Mg}}$	$\approx 30 \mu\text{M}$	160–250 μM^d	n/a	$K_{D2} \approx 410$ –510 714 μM^a	EF5: 36 μM 4.5 mM ^h		
$K_{D,\text{Ca}}(\text{app})$ (nM) at $[\text{Mg}^{2+}]$ of 0.5–1 mM	150–250 ^c	230–310 ^c					
$k_{\text{on},\text{Ca}}$ ($\mu\text{M}^{-1} \text{sec}^{-1}$)	6 ^b	Up to 1000 ⁱ		h sites $\approx 12^f$ m sites ≈ 82	T sites: 1.8 ^g R sites: 310 Site EF5: 7.3	3–10 ^p	100–1000 ^q
$k_{\text{on},\text{Mg}}$ ($\mu\text{M}^{-1} \text{sec}^{-1}$)	0.1–1 ^c						
Cooperativity	No ^j	? Yes ^k	Yes ^l	Yes	Yes	No	No
$D_{\text{Ca buffer}}$ ($\mu\text{m}^2 \text{sec}^{-1}$)	37–43 ^m sec^{-1}	n/a	n/a	$n_H \approx 1.1$ –1.2 ^a >100 ⁿ sec^{-1}	$n_H \approx 1.3$ –1.9 ^g $\approx 25^o$	200–300 ^r	200–300 ^r

Data in table modified from Schwaller (2009, 2010).

^aAlthough considered as Ca^{2+} specific, at physiological $[\text{Mg}^{2+}]$ the apparent Ca^{2+} affinity is $\approx$ twofold lower (Berggård et al. 2002a).^bThis represents the $K_{D,\text{Ca}}$ value in the absence of Mg^{2+} ions (Eberhard and Erne 1994).^c $K_{D,\text{Ca}}$ and k_{on} for PV are $[\text{Mg}^{2+}]$ dependent; calculated values are estimates at $[\text{Mg}^{2+}]$ 0.6–0.9 mM.^dCox et al. (1990).^eLinse et al. (1991).^fCB-D28k has high-affinity (h) and medium-affinity (m) sites, the stoichiometry h/m is either 2/2 or 3/1 (Nägerl et al. 2000).^gFor details, see text and Faas et al. (2007).^hStevens and Rogers (1997).ⁱThe value represents the diffusion limit, assuming a maximal Ca^{2+} diffusion rate of $\approx 200 \mu\text{m}^2 \text{sec}^{-1}$ (Martin et al. 1990).^jPV has two essentially identical Ca^{2+} -binding sites with n_H close to 1.^kYin et al. (2006).^lAkce et al. (1991).^mThe diffusion coefficients $D_{\text{Ca buffer}}$ for PV in muscle myoplasm ($37 \mu\text{m}^2 \text{sec}^{-1}$ [Maughan and Godt 1999]) and Purkinje cell (PC) dendrites ($43 \mu\text{m}^2 \text{sec}^{-1}$ [Schmidt et al. 2003a]); smaller values are measured in PC soma and axons ($\approx 12 \mu\text{m}^2 \text{sec}^{-1}$ [Schmidt et al. 2007a]).ⁿCB-D28k's mobility in PC dendrites is $26 \mu\text{m}^2 \text{sec}^{-1}$ (Schmidt et al. 2005), clearly slower than in water (Gabso et al. 1997) and also slower than PV in PC dendrites.^oEstimation based on the similar size of CB-D28k and CR.^pThe lower value (70 nM) is derived from experiments in vitro (cuvette recordings); (data adapted from Nägerl et al. 2000). The rather low affinity of $\approx 550 \text{ nM}$ is based on experiments measured in the calyx of Held and is mostly the result of a higher than previously assumed dissociation rate constant ($2.38 \pm 0.20 \text{ sec}^{-1}$) (Lin et al. 2017) versus 0.95 sec^{-1} (Lee et al. 2000b).^qPethig et al. (1989).^rValues for EGTA and BAPTA are based on the reported mobility ($283 \mu\text{m}^2 \text{sec}^{-1}$) of InsP_3 (Allbritton et al. 1992).

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Functional Aspects and Regulation of Oncomodulin (OM) and Parvalbumin

Still little is known about OM's specific function. OM is present in the organ of Corti (Thalmann et al. 1995), that is, in cochlear outer hair cells (Sakaguchi et al. 1998) in gerbil, rat, and mouse. An extracellular role for OM in retinal ganglion cell regeneration (Yin et al. 2006) was reported (but also see Hauk et al. 2008 and Schwaller 2009). An up-regulation of OM occurs in $PV^{-/-}$ mice in a sparse subpopulation of neurons in the thalamus and in the dentate gyrus (DG) (Csillik et al. 2010). The functional significance of ectopic OM expression in $PV^{-/-}$ mice remains to be shown.

PV is assumed to function in a similar way as the slow synthetic buffer EGTA (Eggermann and Jonas 2012). Inhibitory postsynaptic current (IPSC) recordings in Purkinje cells revealed that PV-deficiency in molecular layer interneurons (MLIs) of $PV^{-/-}$ mice may be functionally compensated by interneuron loading with sufficiently high concentrations (1 mM) of EGTA; for example, with respect to EGTA's effect on reducing paired-pulse facilitation (PPF) at interspike intervals (ISIs) of 20–100 msec, an effect exerted by PV in MLI of $PV^{+/+}$ wild-type (WT) mice (Caillard et al. 2000). Modeling studies suggest that high PV concentrations may additionally “exert BAPTA-like effects, modulating nanodomain coupling via competition with local saturation of endogenous fixed buffers” (Eggermann and Jonas 2012). High PV-expressing cells include a subset of mostly GABAergic neurons (hereafter called Pvalb neurons, irrespective of PV protein expression levels), fast-twitch muscle fibers, and epithelial cells in the kidney. In neurons and muscle fibers, PV expression is strongly activity dependent. Muscle denervation causes rapid PV down-regulation (Leberer and Pette 1986). Decreased neuronal activity or oxidative stress in Pvalb neurons results in a decrease in PV-immunoreactive (PV^{+}) neurons, *PVALB* messenger RNA (mRNA), and/or PV protein levels (Gonzalez-Burgos and Lewis 2012; Berridge 2014; Steullet et al. 2017). 17β -estradiol (E2) is an inducer of PV expression; E2 treatment of ovariectomized rats

subjected to middle cerebral artery occlusion has a neuroprotective effect and prevents PV down-regulation. E2 also blocks a decrease of PV in hippocampal neurons subjected to glutamate toxicity, suggesting that the neuroprotective effects are exerted via E2-mediated PV up-regulation (Koh 2014). E2 not only up-regulates PV expression in arcuate nucleus POMC neurons (Sotonyi et al. 2010), but in most-to-all Pvalb neurons with $\approx 50\%$ reduced PV levels in $PV^{+/-}$ mice (Filice et al. 2018).

PV's slow-onset Ca^{2+} -binding properties affect Ca^{2+} transients in a particular way; the rate of $[Ca^{2+}]_i$ increase is hardly affected, but the initial rate of $[Ca^{2+}]_i$ decay is increased. In the later phase of the decay, the unbinding of Ca^{2+} from PV prolongs the late phase of the $[Ca^{2+}]_i$ decay. Thus, PV's hallmark is the conversion of a mono-exponential $[Ca^{2+}]_i$ decay into a bi-exponential one (Lee et al. 2000b; Collin et al. 2005). In $PV^{-/-}$ fast-twitch muscles, twitch relaxation speed is slowed down, while barely affecting the contraction phase (Schwaller et al. 1999). Conversely, PV overexpression in the rat slow-twitch muscle soleus significantly increases the speed of relaxation, without affecting contraction (Muntener et al. 1995). *Pvalb* gene delivery in rat heart in vivo increases the rate of relaxation in normal hearts and in an animal model of slowed cardiac muscle relaxation (Szatkowski et al. 2001). Thus, PV or genetically tuned PV variants are discussed as potential tools to enhance cardiac diastolic function (Rodenbaugh et al. 2007; Wang and Metzger 2008; Wang et al. 2013b; Asp et al. 2016). By acting as transient Ca^{2+} sources Ca^{2+} buffers, prolong the $[Ca^{2+}]_i$ decay in fast-twitch muscles (Raymackers et al. 2000). The slow decay component mediated by Ca^{2+} -bound PV also leads to a robust, PV-dependent, delayed transmitter release at cerebellar MLI-MLI synapses subsequent to presynaptic bursts of action potentials (Collin et al. 2005).

The PV-mediated, bi-exponential $[Ca^{2+}]_i$ decay is observed in (1) PV-injected chromaffin cells (Lee et al. 2000b), (2) Pvalb hippocampal interneurons (Lee et al. 2000a), (3) Pvalb MLI in the cerebellum (Collin et al. 2005), (4) presynaptic terminals of the calyx of Held (Muller

et al. 2007), and (5) in Purkinje cell dendrites (Schmidt et al. 2007b). PV's acceleration of the early phase of $[\text{Ca}^{2+}]_i$ decay limits or slows down the buildup of residual $[\text{Ca}^{2+}]_i$ in presynaptic terminals, thus affecting short-term plasticity. The effect is most pronounced at time points when $[\text{Ca}^{2+}]_i$ decay curves in the presence or absence of PV show the largest differences. PV's effect on decreasing/preventing PPF at synapses between MLI and Purkinje cells is most noticeable at an ISI of ≈ 30 msec (Caillard et al. 2000), as is also seen in the presynaptic terminals of the calyx of Held (Muller et al. 2007). In hippocampal Pvalb neurons, differences in facilitation between WT and $\text{PV}^{-/-}$ mice are apparent only when trains at 33, 50, and 100 Hz are delivered (Vreugdenhil et al. 2003), which is likely the result of more efficient presynaptic Ca^{2+} extrusion mechanisms. The largest relative effect of PV in preventing facilitation is also seen at ~ 33 Hz, within the range of γ frequency (30–80 Hz) oscillations. A similar effect of PV on preventing facilitation is observed in striatal Pvalb neurons. PV decreases PPF of inhibitory postsynaptic potentials at intervals < 50 msec, but additionally PV-deficient Pvalb neurons fire more regularly and are more excitable than control FSIs (fast spiking interneurons) by a mechanism in which Ca^{2+} buffering is linked to spiking activity as a result of the activation of small conductance Ca^{2+} -dependent K^+ channels (Orduz et al. 2013), the latter effect supported by modeling studies (Bischof et al. 2012). This indicates “PV is crucial for fine-tuning of the temporal responses of the FSI network and for the orchestration of MSN populations” (Orduz et al. 2013). In the mouse reticular thalamic nucleus (RTN) consisting of a majority of Pvalb neurons, PV deficiency results in changes in the proportion of neurons with particular firing properties. Compared with WT mice, medium bursting (type II) neurons are more frequent in $\text{PV}^{-/-}$ brains and ISIs within the burst are longer without affecting the number of spikes, indicating that “PV may affect the firing properties of RTN neurons via a mechanism associated with the kinetics of burst discharges” (Albéri et al. 2013). These alterations are

associated with changes in the localization of $\text{Ca}_v3.2$ channels.

In accordance with the hypothesis that changes in the inhibitory activity of Pvalb neurons in the neocortex—often critically involved in strong perisomatic inhibition—may be a major mechanism underlying epileptic seizures (Mihaly et al. 1997), $\text{PV}^{-/-}$ mice have a lower threshold for pentylenetetrazole (PTZ)-induced seizures (Schwaller et al. 2004). The subpopulation of Pvalb neurons is critically involved in controlling the output of principal neurons (Freund 2003); moreover, PV contributes to controlling the network activity. The absence of PV in the cerebellar circuitry leads to the emergence of 160-Hz oscillations in vivo sustained by synchronous, rhythmic-firing Purkinje cells (Servais et al. 2005), likely linked to altered Purkinje cell-firing properties. Complex spike duration and spike pause are decreased and the simple spike firing rate is increased. These changes are the probable cause for the mild locomotor phenotype, that is, a slight impairment of motor coordination/motor learning in mice deficient for PV (Farre-Castany et al. 2007).

Parvalbumin and Mitochondria

Cytosolic Ca^{2+} buffers act as transitory Ca^{2+} sinks/stores and operate together with plasmalemmal extrusion systems, Ca^{2+} uptake into endoplasmic reticulum (ER) and mitochondria as a cell's “ Ca^{2+} off mechanisms” (Berridge et al. 2003). Besides their most essential role in energy metabolism (ATP production), mitochondria have important roles as regulators and decoders of Ca^{2+} signals. Mitochondria are capable of taking up large amounts of Ca^{2+} ions by the mitochondrial Ca^{2+} uniporter (MCU) complex present in the inner mitochondrial membrane and can thus be viewed as (partially mobile) bulk cytosolic Ca^{2+} -buffering systems with relatively slow kinetics (for details, see Giorgi et al. 2018). The effect of PV and mitochondria on the shape of $[\text{Ca}^{2+}]_i$ transients and decreasing PPF in neurons are quite similar. In presynaptic terminals of the calyx of Held, mitochondria contribute to increase the rate in $[\text{Ca}^{2+}]_i$ decay, when peak $[\text{Ca}^{2+}]_i$ is $> 2.5 \mu\text{M}$ (Kim et al. 2005). This

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decreases PPF (or more precisely recovery from depression; Billups and Forsythe 2002), similar to PV's effects (Muller et al. 2007), an effect that can be reverted by the addition of 1 mM EGTA. In analogy, at MLI synapses release of Ca^{2+} from Ca^{2+} -bound PV increases delayed transmitter release after an AP train (Collin et al. 2005; Franconville et al. 2011). The increased removal of $[\text{Ca}^{2+}]_i$ by PV prevents the build-up of residual $[\text{Ca}^{2+}]$ and thus reduces PPF at the calyx of Held and in MLI axon terminals from $\text{PV}^{-/-}$ mice (Collin et al. 2005; Muller et al. 2007).

Not only do PV and mitochondria show similarities in their (slow-onset) kinetics of Ca^{2+} binding and Ca^{2+} uptake, respectively, but in all investigated models are regulated in an antagonistic way, that is, an increase in one leads to a decrease in the other. This is the case in fast-twitch muscle (Chen et al. 2001) and Purkinje cells (Chen et al. 2006) of $\text{PV}^{-/-}$ mice, as well as in cell models, that is, C2C12 myotubes (Ducieux et al. 2012) and MDCK kidney cells (Henzi and Schwaller 2015), in vitro cell models, in which PV levels can be increased or lowered experimentally. Both, in vivo and in vitro, PV down-regulation induces an increase in the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α), which is considered as a master regulator of mitochondria biogenesis, and moreover of its positive regulator, the deacetylase sirtuin 1 (SIRT1). In epithelial cells of the distal convoluted tubule in the mouse kidney, several genes implicated in mitochondrial Ca^{2+} uptake are increased in $\text{PV}^{-/-}$ mice (Henzi and Schwaller 2015), whereas mitochondrial genes linked to ATP synthesis are not affected, again indicating that mitochondria up-regulation is likely aimed to increase the slow Ca^{2+} -buffering capacity provided by PV in WT cells. In PV-overexpressing MDCK cells, the consequent smaller mitochondrial volume is the result of reduced fusion rates and augmented mitophagy, paralleled by lower cell mobility in vitro (Lichvarova et al. 2018). The opposite occurs if PV in the overexpressing cells is decreased by an shPvalb approach, thus mimicking the situation occurring in $\text{PV}^{-/-}$ mice. This shows that the antagonistic PV/mitochondria

mechanism operates in a bidirectional, likely homeostatic manner.

This regulation is a prototypical example of how the different components making up a cell's Ca^{2+} -signaling toolkit (Berridge et al. 2003) are interconnected (physically and/or functionally) and are able to regulate the expression of its own components necessary for accurate and cell-specific Ca^{2+} signaling. This functional concept is called the Ca^{2+} homeostasome (for details, see Schwaller 2009, 2012) and is not limited to individual cells, but also operating at a larger scale, for example, in regulating global Ca^{2+} homeostasis in an entire organism evidenced in mice. Some details are mentioned in the next section.

Parvalbumin, Pvalb Neurons, and Neuropsychiatric Diseases

Tremendous progress has been made in understanding the function of Pvalb neurons: "Thanks to the massive use of subcellular patch-clamp techniques, simultaneous multiple-cell recording, optogenetics, in vivo measurements, and computational approaches, our knowledge about PV^+ interneurons became more extensive than for several types of pyramidal neurons" (Hu et al. 2014). Pvalb neurons are in the spotlight with respect to their involvement in neuropsychiatric diseases, in particular in schizophrenia and bipolar disorder and in neurodevelopmental disorders including autism spectrum disorder (ASD) (Ferguson and Gao 2018). Of note, most studies have focused on the Pvalb neurons in general, while less attention was devoted to deciphering the role of PV itself, evidently the identification marker of Pvalb neurons.

Pvalb neuron impairment in ASD is well documented (for review, see Marín 2012; Ferguson and Gao 2018) based on several mouse ASD models (Gogolla et al. 2009; Wöhr et al. 2015). Although the observed impairment (less PV^+ cells) was previously mostly described/ implied as loss of Pvalb neurons (see Table 1 in Gogolla et al. 2009 and supplementary Table S1 in Wöhr et al. 2015), this view was recently challenged. In $\text{PV}^{-/-}$ and $\text{PV}^{+/-}$ mice showing a robust ASD-like behavioral phenotype, the num-

ber of Pvalb neurons is unaltered, although the number of PV^+ neurons is either zero or $\approx 65\%$ of WT control, respectively (Filice et al. 2016). This decrease/absence in $\text{PV}^{+/-}$ and $\text{PV}^{-/-}$ mice is the result of PV down-regulation by $\sim 50\%$ in $\text{PV}^{+/-}$ mice, such that about one-third of Pvalb neurons fall below the PV immunohistochemistry (IHC) signal's detection limit. Of relevance, cortical, hippocampal, striatal, and cerebellar molecular layer Pvalb neurons are surrounded by perineuronal nets (PNNs) consisting of particular extracellular matrix components (e.g., aggrecan) that can be visualized by lectin staining (Vicia villosa agglutinin [VVA] and Wisteria floribunda agglutinin [WFA]) (Härtig et al. 1992). Thus, PNN staining allows for the identification of Pvalb neurons irrespective of PV expression levels and the number of VVA^+ cells is unchanged in $\text{PV}^{-/-}$ and $\text{PV}^{+/-}$ mice (Wöhr et al. 2015). This is also observed in other validated mouse ASD models, either genetic ($\text{Shank1}^{-/-}$, $\text{Shank3B}^{-/-}$ [Filice et al. 2016], $\text{Cntnap2}^{-/-}$ [Laubert et al. 2018], or “environmental” [in utero treatment with valproic acid; Laubert et al. 2016]). Thus, PV down-regulation (either global or brain region-specific) in Pvalb neuron populations seems a point of convergence with respect to ASD. In agreement with the finding on the positive regulation of PV by E2 reported as an increase in PV^+ POMC neurons after E2 administration (Sotonyi et al. 2010), PV protein levels in PND25 $\text{PV}^{+/-}$ mice treated with E2 from PND5–15 almost reach PV levels as present in WT PND25 mice (Filice et al. 2018). In tests assessing sociability, that is, the direct reciprocal social interaction and the three-chamber social approach assay, ASD-associated deficits observed in sham-treated $\text{PV}^{+/-}$ mice are clearly attenuated in the E2-treated group and also repetitive behaviors, the second core ASD symptom are decreased after E2 treatment.

Studies with humans further support the animal model results linking PV and ASD. Transcriptomic coexpression network analyses (Voineagu et al. 2011; Parikshak et al. 2013) have identified networks centered on Pvalb neurons, for example, a neuron-specific ASD-linked module containing the *PVALB* gene. RNA-seq and RT-qPCR analyses of postmortem samples of

prefrontal cortex (PFC), temporal cortex, and cerebellum revealed *PVALB* gene transcripts to be among the most strongly down-regulated ones in the ASD group (Parikshak et al. 2016). This in line with the decreased number of PV^+ neurons reported in postmortem neocortical tissue in prefrontal Brodmann areas (BA) BA46, BA47, and BA9 in ASD subjects, when compared with age-matched controls (Hashemi et al. 2017). In summary, many points hint that the impairment of Pvalb neurons in ASD is associated with or possibly even causally linked to PV down-regulation, the latter effect suggested as a homeostatic compensation mechanism to increase synaptic output of Pvalb neurons (Wöhr et al. 2015). Evidently, other pathophysiological hypotheses exist on mechanisms leading to ASD. A unifying view centers on autoregulatory feedback loops linked to altered synaptic structure and function, for example, alterations in “neuronal electrical activity, the collective strength of synapses onto a dendritic branch, the local concentration of a signaling molecule, or the relative strengths of synaptic excitation and inhibition” (Mullins et al. 2016). PV as part of the Ca^{2+} homeostasome (Schwaller 2012) does fulfill these criteria for sensors (homeostats) “that operate as negative feedback elements to keep the biological variable tightly confined” (Mullins et al. 2016).

Also, in the field of schizophrenia research, PV down-regulation is a well known and often reported finding. The down-regulation of PV in Pvalb neurons caused by, for example, NMDA receptor hypofunction (Hashimoto et al. 2003) and the importance of fast inhibitory transmission of Pvalb neurons in the generation of γ oscillation (Bartos et al. 2002) and its impairment in schizophrenia, together with molecular alterations in Pvalb neurons of postmortem human brains are well accepted in the field of schizophrenia (Gonzalez-Burgos et al. 2015). Oxidative stress causing Pvalb neuron dysfunction associated with PV down-regulation might represent a point of convergence in the development of schizophrenia (Steullet et al. 2017). In the dorsolateral prefrontal cortex (dlPFC) of schizophrenia subjects, densities of Pvalb neurons and PNN-ensheathed neurons are not

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altered. Yet, the decreased intensity of PV immunofluorescence is indicative of PV protein down-regulation, in addition to the weaker staining of PNNs (Enwright et al. 2016). Importantly, this decrease in the number of PV⁺ neurons indicates no loss of Pvalb neurons, as was reported in previous studies. In line with the NMDA-R hypofunction hypothesis in schizophrenia, the number of putative excitatory synapses (puncta with overlap of presynaptic VGlut1 and postsynaptic PSD95) are 18% lower in dlPFC Pvalb neurons of schizophrenia subjects (Chung et al. 2016). This decrease in synapse density and the likely associated decrease in neuronal activity might cause the neuronal activity-dependent down-regulation of PV and GAD67. Transcriptome analysis of dlPFC layer 3 Pvalb neurons in schizophrenia subjects revealed that among the ≈ 800 differentially expressed transcripts, genes associated with mitochondrial function pathways are enriched (Enwright et al. 2018). These data provide an interesting link between Pvalb neurons, PV expression, and mitochondria dysfunction/hyperfunction and oxidative stress in schizophrenia, but also in ASD. In Purkinje cells of PV^{-/-} mice, PV's absence results in the up-regulation of mitochondria, assumed to functionally replace PV based on similar (slow) Ca²⁺-buffering/uptake kinetics (Chen et al. 2006; for review, see Schwaller 2012). Such an antagonistic regulation, as discussed earlier, appears universal, as it is also seen in PV-expressing fast-twitch muscles and epithelial cells.

In the schizophrenia mouse model *Gclm*^{-/-} with reduced glutathione (GSH) synthesis, Pvalb neuron maturation is delayed in the anterior cingulate cortex (ACC) of *Gclm*^{-/-} mice (Cabungcal et al. 2013). If preweaning or pubertal *Gclm*^{-/-} mice are subjected to an additional oxidative challenge the number of PV⁺ neurons is decreased, possibly linked to PV down-regulation; the decrease in PV⁺ neurons is prevented with the antioxidant *N*-acetylcysteine (NAC). A decrease in the number of PV⁺ neurons is also seen in *Gclm*^{-/-} RTN neurons and postmortem brains of schizophrenia and bipolar disorder (BD) subjects (Steullet et al. 2018). The observed changes in the RTN firing properties (as seen in

PV^{-/-} mice; Albéri et al. 2013) may hint that the decrease in PV⁺ RTN neurons being the result of PV down-regulation. Yet, another process leading to PV down-regulation is the methylation of the *PVALB/Pvalb* promoter. Subchronic administration of phencyclidine, an animal model of psychosis, leads to hypermethylation of the *Pvalb* promoter in the PFC and hippocampus of rat brain, possibly contributing to PV deficits (Fachim et al. 2016). The same is seen in methamphetamine-dependent human subjects (Veerasakul et al. 2017) and is present in the hippocampus of postmortem brain of schizophrenia subjects (Fachim et al. 2018). Thus, decreased neuronal activity may lead to *PVALB* promoter hypermethylation resulting in PV down-regulation seen in schizophrenia subjects. Thus, also in schizophrenia there appears to exist a link between phenotype and decreased PV levels. A comparison of the presumed mechanisms leading to Pvalb neuron impairment associated with PV down-regulation in ASD and schizophrenia is shown in Figure 2. The main difference between ASD and schizophrenia is the time point of PV down-regulation. In mouse ASD models, the down-regulation occurs during early development (before PND25), a period when essential functional and structural maturation (e.g., increases in PV and PNN and closing of developmental critical periods) of Pvalb neuron networks take place. PV down-regulation in schizophrenia models appears to occur later. This is in good agreement with the situation in humans, in which ASD is normally diagnosed before the age of 3, whereas first symptoms in schizophrenia are generally observed during early adulthood.

In conclusion, although Pvalb neuron impairment in ASD and schizophrenia is widely accepted, the hypothesis that PV down-regulation and not Pvalb neuron loss might be prominently implicated in the phenotype should be investigated in further detail.

STRUCTURE, FUNCTION, AND REGULATION OF CALBINDIN-D9k (CB-D9k)

CB-D9k (human gene symbol: *S100G*) is the smallest protein ($M_r \approx 9$ kDa) with four α -heli-

Figure 2. Comparison of mechanisms hypothesized to lead to PV down-regulation and Pvalb neuron impairment in autism spectrum disorder (ASD) (light blue) and schizophrenia (violet). Both in ASD and schizophrenia genetic (G, orange) and environmental (E, green) factors are likely involved. Identified human ASD risk genes and mouse models based on these risk genes are listed. Full names of the proteins encoded by these ASD- and schizophrenia-associated genes and additional details are found in the literature. G factors, E factors, and/or G x E interactions disturb normal neurodevelopment evidenced as excitation/inhibition (E/I) imbalance, impaired synaptic transmission, as well as hyper-/hypoconnectivity of short- or long-range neuronal circuits (yellow box). This is assumed to result in PV down-regulation (red box). In both disorders, PV down-regulation is proposed as the hub or point-of-convergence. The differences lie in the different kinetics of PV down-regulation. A decrease in PV expression during early neurodevelopment, that is, detectable already at PND25 in mice, as shown for mice deficient for PV, SHANK1 and 3 (Filice et al. 2016), CNTNAP2 (Lauber et al. 2018), and VPA mice (Lauber et al. 2016) is correlated with an ASD-like phenotype previously observed in mice or in the case of VPA in rats (Schneider et al. 2008; Peça et al. 2011; Peñagarikano et al. 2011; Wöhr et al. 2011, 2015). In mouse schizophrenia models, in which a phenotype is most often apparent in adult mice (>3 months old), it is hypothesized that a slower PV down-regulation leads to a compensatory/adaptive increase in mitochondria volume and subsequently to augmented ROS production in Pvalb neurons impairing their function. This Pvalb neuron dysfunction will precipitate at the behavioral level, as a schizophrenia-like phenotype. The precise order of events, that is, PV down-regulation-mediated increased oxidative stress leading to Pvalb neuron dysfunction or oxidative stress-mediated Pvalb neuron impairment leading to PV down-regulation is currently unknown and under investigation (adapted from data in Steullet et al. 2016, 2017b).

cal motifs forming two EF-hands: one consisting of a canonical EF-hand comprising a Ca^{2+} -binding loop of 12 amino acids (EF2), and the other EF-hand with a noncanonical/pseudo (Ψ) or S100-specific (EF1) Ca^{2+} -binding loop of 14 amino acids joined by a linker region of 10

amino acids and forming the EF-hand domain (Fig. 1). S100 family members (Marenholz et al. 2004) contain a loop of 14 amino acids with an inside-out conformation compared with the canonical loop; that is, Ca^{2+} -coordination is preferentially provided by backbone carbonyl

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groups (Nelson et al. 2002). The domain is stabilized by a short β -type interaction between the two Ca^{2+} -binding loops (Kördel et al. 1993). CB-D9k is monomeric, whereas the larger S100 family members mostly form dimers (homo- or heterodimers). $K_{D,\text{Ca}}$ values are almost identical for both sites (Table 1) and the two Ca^{2+} ions bind with positive cooperativity (Linse et al. 1991). CB-D9k undergoes Ca^{2+} -induced conformational changes; they are, however, less pronounced than in the prototypical sensor CaM (Chazin 2011). This together with no identified binding partners indicates that CB-D9k most likely functions as a Ca^{2+} signal modulator and/or a Ca^{2+} shuttle (Skelton et al. 1994). The high affinity, together with the very fast on-rate of Ca^{2+} binding, show striking similarities of CB-D9k with the fast synthetic buffer BAPTA (Table 1).

CB-D9k is expressed in nonexcitable cells, yet including cells undergoing regulated exocytosis that are in most cases of epithelial origin. CB-D9k was first discovered in the intestine and kidney, as a protein strongly up-regulated in vitamin D-deficient rats after vitamin D supplementation, thus its name: a calcium-binding protein, up-regulated by 1,25(OH) $_2$ vitamin D3 (D3) with a relative molecular mass of 9 kDa (Kallfelz et al. 1967). In rat kidney, CB-D9k is expressed in the loop of Henle, the distal convoluted tubule and in intercalated cells of the collecting duct (Bindels et al. 1991). In mice, CB-D9k expression is present in late distal convoluted tubules (Lee et al. 2006) and connecting tubules. Strong expression of CB-D9k in the mouse intestine is restricted to enterocytes present within the first 2 cm of the duodenum (Huybers et al. 2007). In these cells, expression of CB-9k is mediated via a vitamin D-responsive element (VDR) present in the *S100g* gene promoter and it is assumed that CB-D9k, besides its transcellular Ca^{2+} -shuttling function, limits the amount and/or the amplitude of intracellular Ca^{2+} signals that otherwise might lead to the activation of cell death pathways (Ca^{2+} cytotoxicity; for details, see Hong and Jeung 2013). In the epithelial cells of both kidney and intestine, CB-D9k is a freely mobile molecule in the

cytoplasm and implicated in transcellular Ca^{2+} transport.

Much concerning the regulation of CB-D9k was obtained either from in vitro cell models, or more recently, through the availability of CB-D9k knockout mice (Lee et al. 2007). In the rat pituitary cell line GH3, CB-D9k expression is regulated by E2 via the classical estrogen receptor α (ER α)-mediated pathway and a putative ERE (Vo et al. 2011), but also via the nongenomic pathway shown by the effects of membrane-impermeable estrogen (Dang et al. 2010). Also, in epithelial cells of the uterus, CB-D9k expression is regulated by E2 (Darwish et al. 1991). In the colon cancer cell line Caco-2, CB-D9k expression is induced by short chain fatty acids (SCFAs) such as propionate or butyrate (Fukushima et al. 2012). Whether this regulation occurs also via butyrate-responsive elements (BREs) putatively present in the promoter of *S100g*, as was shown for the regulation of the *Calb2* promoter (coding for CR) in colon cancer cells (Häner et al. 2010) remains to be shown. In pancreatic β cells of CB-D9k $^{-/-}$ mice, insulin secretion is reduced and serum glucose levels are increased, indicating that CB-D9k is part of the insulin-secreting/ Ca^{2+} signaling pathway (Ahn et al. 2016). In the mouse lung, CB-D9k is present in mucin-producing Clara cells and alveolar type 2 cells (An et al. 2018); mucin secretion is regulated via glucocorticoid-induced modulation of CB-D9k. High expression of CB-D9k is present in the fetal part of murine placenta indicative of a role in controlling Ca^{2+} transport across the placenta (Koo et al. 2012). In summary, CB-D9k is implicated in the regulation of Ca^{2+} transport processes across epithelial cells (Bindels et al. 1991; Nijenhuis et al. 2003), acts as a Ca^{2+} buffer/shuttle optimally tuned for transcellular Ca^{2+} transport (Choi and Jeung 2008) and protects cells from high Ca^{2+} -induced cytotoxicity.

CALBINDIN-D28k (CB-D28k)

Structural and General Aspects of CB-D28k

CB-D28k ($M_r \approx 29$ kDa; human gene symbol: *CALB1*) contains six EF-hand motifs arranged

in three domains; four EF-hands bind Ca^{2+} with medium/high affinity (Cheung et al. 1993). EF-hands 2 and 6 are nonfunctional in physiological conditions. The four medium/high-affinity sites (Nägerl et al. 2000) are considered Ca^{2+} -specific, albeit displaying low affinity Mg^{2+} binding ($K_{D,\text{Mg}} \approx 700 \mu\text{M}$) to the same sites at physiological $[\text{Mg}^{2+}]_i$ of $\sim 1 \text{ mM}$, decreasing the apparent Ca^{2+} affinity $\approx$ twofold; in addition, Mg^{2+} binding increases the cooperativity of Ca^{2+} binding (Berggård et al. 2002a). The NMR solution structure of Ca^{2+} -bound rat CB-D28k reveals that it consists of a single, almost globular (ellipsoid) fold with six distinguishable EF-hands (Fig. 1; Kojetin et al. 2006). The on rates of CB-D28k's fast binding sites ($k_{\text{on}} \approx 8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$) are fast enough to affect the early rising phase of Ca^{2+} transients; the peak $[\text{Ca}^{2+}]_i$ amplitude is significantly decreased in WT Purkinje cells when compared with Purkinje cells from CB-D28k $^{-/-}$ mice (Airaksinen et al. 1997). The fact that the time to peak is not significantly different in CB-D28k $^{-/-}$ and WT Purkinje cells indicates that the initial increase in $[\text{Ca}^{2+}]_i$ is principally governed by the properties (density, kinetics) of the Ca^{2+} channels. In Purkinje cell dendrites, CB-D28k acts as a fast Ca^{2+} buffer for the first $\sim 100 \text{ msec}$ reducing the peak $[\text{Ca}^{2+}]_i$ amplitude to about one half, while later on prolonging the decay by acting as a Ca^{2+} source (Schmidt et al. 2003b). Estimated concentrations of CB-D28k are $150\text{--}360 \mu\text{M}$ in Purkinje cells, $40\text{--}50 \mu\text{M}$ in mature hippocampal DG granule cells (GCs), CA3 interneurons, and in CA1 pyramidal cells (Muller et al. 2005). Although principally considered as a freely mobile protein, CB-D28k binds to several identified target proteins including Ran-binding protein (RanBP) M (Lutz et al. 2003), caspase-3 (Bellido et al. 2000), 3',5'-cyclic nucleotide phosphodiesterase (Reisner et al. 1992), plasma membrane ATPase (Morgan et al. 1986), L-type Ca^{2+} channel α subunit (CANAC1C) (Christakos et al. 2007), *myo*-inositol monophosphatase (IMPase) (Berggård et al. 2002b), and TRPV5 in the kidney (Lambers et al. 2006). In most cases, binding studies were performed in vitro and the physiological implications of these interactions are not clear yet. In dendrites and spines of

Purkinje cells, $\sim 20\%$ of CB-D28k molecules are temporarily, that is, for several seconds, immobile by their binding to IMPase, a key enzyme of the InsP_3 signaling cascade and the fraction of immobilized CB-D28k increases by climbing fiber stimulation (Schmidt et al. 2005). In summary, the above findings together with CB-D28k's Ca^{2+} -dependent conformational changes (Berggård et al. 2002a) indicate additional Ca^{2+} sensor functions.

Function and Regulation of CB-D28k

Reported functions for CB-D28k in nonexcitable cells include a role in Ca^{2+} resorption in the kidney (Boros et al. 2009) and modulation of insulin production and secretion in pancreatic β cells (Reddy et al. 1997; Sooy et al. 1999). CB-D28k blocks the up-regulation of apoptosis-related genes and ER stress genes in the endometrium of the murine uterus, indicating a role for CB-D28k in decreasing the expression of proapoptotic genes (Jung et al. 2012). Besides the well-documented regulation of CB-D28k by D3 in kidney and mammalian intestine, NFAT- and TFII-I-binding elements exist in the *Calb1* promoter that are activated in kidney and β cells in response to serum deprivation leading to increased CB-D28k levels. This is viewed as a protective mechanism in vivo, possibly during ischemia and growth factor withdrawal (Hajibeigi et al. 2015).

In the brain, CB-D28 expression is regulated by neuronal activity. Consistent whisker removal in mice, resulting in the inactivation of the main sensitive tactile system in rodents leads to CB-D28k down-regulation in hippocampal DG GCs paralleled by a loss of cells positive for the activity-driven (and Ca^{2+} -dependent) immediate early gene *c-Fos* (Gonzalez-Perez et al. 2018). The changes coincide with an increase in apoptosis and a decrease in the proliferation of neural DG precursor cells, further indicating that CB-D28k has an antiapoptotic function. The hippocampal aberrations are associated with a significant impairment of spatial memory and navigation skills. In line, CB-D28k $^{-/-}$ mice reveal accelerated age-dependent decline of steady-state relative cerebral blood

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volume (rCBV) in the DG, a proxy measure for brain metabolism (Moreno et al. 2012). The metabolic (and structural changes) correlate with a deficit in hippocampus-dependent learning in the active place avoidance task indicating that CB-D28k helps to delay/prevent age-related hippocampal metabolic decline. Similarly, early-life stress-induced decreases in CB-D28k in CA1 pyramidal and DG neurons or direct CB-D28k down-regulation leads to impaired long-term memory making CB-D28k “a molecular target of early-life stress and an essential substrate for memory” (Li et al. 2017). At the cellular level, short-term plasticity between cortical multipolar bursting cells and pyramidal cells is affected by CB-D28k (Blatow et al. 2003). The rapid saturation of presynaptic CB-D28k transiently decreases the Ca^{2+} buffering capacity leading to enhanced facilitation (Blatow et al. 2003) by a mechanism called “facilitation by buffer saturation” (Neher 1998; Maeda et al. 1999). Absence of CB-D28k results in a consistent failure to maintain long-term potentiation (LTP) in DG and CA1 (Westerink et al. 2012). Following tetanic stimulation, amplitudes of CA1 population spikes increase in WT mice, but steadily decrease in CB-D28k^{-/-} mice. Thus, these alterations are a likely origin for the deficit in hippocampus-dependent learning and memory described above (Moreno et al. 2012; Li et al. 2017). Absence of CB-D28k affects additional brain functions linked to fear memory and social behavior in male CB-D28k^{-/-} mice and decreases anxiety in both sexes (Harris et al. 2016). This clearly links CB-D28k with affective and social behaviors in rodents and a decline in CB-D28k might be associated with neuropsychiatric disorders, for example, schizophrenia (Eyles et al. 2002) and/or impaired social behaviors.

CB-D28k^{-/-} mice show no striking phenotype related to development, the general morphology of the nervous system, the visual (Wässle et al. 1998), and auditory (Airaksinen et al. 2000) systems and behavior under standard housing conditions (Airaksinen et al. 1997). They show a mild impairment in motor coordination/motor learning (Airaksinen et al. 1997; Farre-Castany et al. 2007) likely resulting

from the 160-Hz oscillations in the cerebellum (Cheron et al. 2004; Servais et al. 2005). CB-D28k also affects Ca^{2+} -dependent inactivation (CDI) of voltage-dependent Ca^{2+} currents (I_{Ca}). In DG, GCs with low or absent CB-D28k expression resulting from Ammon’s horn sclerosis (AHS) in humans (Nägerl and Mody 1998) or in mice with *Calb1* gene ablation (Klapstein et al. 1998), CDI is increased compared with CB-D28k-expressing GCs, thereby decreasing the total Ca^{2+} load. Increased CDI in the absence of CB-D28k may be viewed as a protective/homeostatic mechanism to limit Ca^{2+} influx to augment the resistance against excitotoxicity and to protect the surviving neurons.

Earlier data on a putative neuroprotective role of CB-D28k had resulted in an inconsistent picture (for review, see Schwaller et al. 2002, 2009). Newer results indicate that CB-D28k has a neuroprotective effect on dopaminergic (DA) neurons in several models of Parkinson’s disease. In the *Calb1*-transfected DA cell line, MN9D treatment with the neurotoxin 6-hydroxydopamine greatly reduces the apoptosis rate linked to increased levels of phosphorylated AKT (p-AKT) (Sun et al. 2011). Thus, PI3K/AKT signaling pathway-mediated increase in CB-D28k is suggested to protect DA neurons. In physiological conditions, E2 increases tyrosine hydroxylase and CB-D28k expression in DA neurons of midbrain substantia nigra pars compacta in a Parkinson mouse model (treatment with 1-methyl-4-phenyl-1,2,3,6-tetrapyridine [MPTP]). At the behavioral level, E2 treatment improves swimming and hanging behaviors (Wang et al. 2013a). Additional data on the most pertinent roles of CB-D28k functioning as a (1) Ca^{2+} signal modulator (or Ca^{2+} buffer), (2) Ca^{2+} shuttle/transporter, and (3) noncanonical Ca^{2+} sensor are summarized in Schmidt (2012).

CALRETININ (CR)

Structural and General Aspects of CR

Human CR ($M_r \approx 31$ kDa; gene symbol: *CALB2*) consists of 271 amino acids and contains six EF-hands, five of which (nonfunction-

al: EF6) are able to bind Ca^{2+} ions (Schwaller et al. 1997; Stevens and Rogers 1997). Structural NMR data of the amino-terminal 100 amino acids of rat CR comprising EF hands 1 and 2 (Palczewska et al. 2001) show that they form a relatively tight structure. CR's Ca^{2+} -dependent conformational changes together with results from other in vitro studies are indicative of CR also having Ca^{2+} -sensor functions (Billing-Marczak and Kuznicki 1999) (see below). Based on CR's Ca^{2+} -binding properties including kinetics determined in vitro (Faas et al. 2007), a modeling study of CR's effects in cerebellar GCs predicted that CR would reduce AP-associated increases in $[\text{Ca}^{2+}]_i$ even at a distance as short as 30 nm from a single Ca^{2+} channel (Saftenku 2012). Despite buildup of residual Ca^{2+} , maximal $[\text{Ca}^{2+}]_i$ levels should remain almost constant during repetitive channel openings at frequencies <80 Hz. This was hypothesized to be the result of CR's particular properties, that is, accelerated Ca^{2+} binding as CR binds more Ca^{2+} ions. The effect of CR on short-term plasticity was experimentally determined at parallel fiber (PF)-to-Purkinje cell synapses (Brachtendorf et al. 2015). In presynaptic PF terminals from $\text{CR}^{-/-}$ mice, maximal $[\text{Ca}^{2+}]_i$ is increased and moreover, the initial vesicular release probability (p_r) is almost doubled. Unexpectedly, this doesn't lead to changes in PPF at an ISI of 10 msec. Analysis of the frequency- and Ca^{2+} -dependence of PPF at this synapse revealed that PPF is definitively smaller in $\text{CR}^{-/-}$ than in WT mice; yet, rapid vesicle replenishment and recruitment of additional release sites dominate the synaptic efficacy of the second response. "These Ca^{2+} -driven processes operate more effectively in the absence of CR, thereby, explaining the preservation of robust PPF in the mutants" (Brachtendorf et al. 2015). Of note, although the Ca^{2+} -binding properties of CR's fast binding sites are very similar to BAPTA, and moreover that $150\ \mu\text{M}$ BAPTA is able to restore $\text{CR}^{-/-}$ cerebellar GC excitability (Gall et al. 2003), "buffering of regional Ca^{2+} signals by CR can never be mimicked by a certain concentration of BAPTA, under all different experimental conditions" (Saftenku 2012). With respect to functional consequences caused by

the absence of CR, $\text{CR}^{-/-}$ mice show impaired LTP in the hippocampus (Schurmans et al. 1997; Gurden et al. 1998), yet caused by an indirect effect (see Figure 5 in Schurmans et al. 1997). The uniform expression of CR in cerebellar GC together with the stereotypic cerebellar organization has allowed for a detailed investigation of CR in vivo. $\text{CR}^{-/-}$ GC, in addition to increased excitability, show faster APs and under conditions generating repetitive spike discharges show enhanced increases in frequency with injected currents (Gall et al. 2003). This leads to impaired Ca^{2+} homeostasis in Purkinje cells. The firing properties of Purkinje cells are altered in alert $\text{CR}^{-/-}$ mice: the simple spike-firing rate is increased; the complex spike duration and the spike pause are shorter (Schiffmann et al. 1999). As in $\text{PV}^{-/-}$ and $\text{CB-D28k}^{-/-}$ mice, alert $\text{CR}^{-/-}$ mice show 160 Hz oscillations (Cheron et al. 2004) that appear phenotypically as an impairment of motor coordination. In alert rescue mice, in which CR is selectively re-expressed in GC of $\text{CR}^{-/-}$ mice, GC excitability as well as Purkinje cell firing resembles that in WT mice. As a consequence, neither 160 Hz oscillations nor motor coordination impairment are detected in the rescue mice (Bearzatto et al. 2006). The similarity of the oscillations and motor coordination deficits in mice deficient for either one of the three CaBPs (PV, CR, CB-D28) points toward an effect at the cerebellar network level. In all three knockout strains, the oscillations are temporarily reduced by blocking of (1) gap junctions between interneurons, (2) *N*-methyl-D-aspartate receptors, or (3) GABA_A receptors. This indicates that oscillations emerge via a mechanism that synchronizes assemblies of Purkinje cells (mediated by parallel fiber excitation) and the network of chemically coupled MLI. In addition, recurrent Purkinje cell collaterals (Orduz and Llano 2007) may be implicated in these oscillations (de Solages et al. 2008).

Function and Regulation of CR

Besides CR's expression in specific neuron subpopulations in the central and peripheral nervous system, CR is also expressed in some nonexcitable cells in physiological conditions

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including the testosterone-producing Leydig cells in testis, Sertoli cells, and mesenchymal cells during embryonic development. In the Leydig cell line MLTC-1 overexpressing CR, testosterone levels are significantly higher, whereas in CR-expressing R2C cells CR down-regulation decreases progesterone levels. The increased levels of proteins implicated in the phospholipase C-Ca²⁺-protein kinase C pathway including phospholipase C, phosphorylated protein kinase C μ (PKD), phosphorylated myristoylated alanine-rich C-kinase substrate (p-MARCKS), and cAMP response element-binding protein (CREB) in CR-overexpressing cells and the decreased expression of the same proteins after CR-down-regulation strongly indicates the involvement of CR in this pathway implicated in steroidogenesis (Xu et al. 2018). Moreover, in Leydig cells, CR increases viability and proliferation, possibly via ERK1/2 and AKT pathways, and suppresses apoptosis; in Leydig cells possibly via the mitochondria-related apoptotic pathway (Xu et al. 2018). Under pathologic conditions, CR is also expressed in certain colon cancers and derived cell lines (Gotzos et al. 1996a), as well as in malignant mesothelioma (MM) (Gotzos et al. 1996b). In CR-expressing MM cell lines (mostly of the epithelioid subtype), CR is essential for MM cell growth/survival. CR down-regulation via lentiviral-mediated short-hairpin RNA substantially decreases MM cells viability and proliferation in vitro. CR-depleted MM cells undergo apoptosis within 72 h postinfection via activation of the intrinsic caspase 9-dependent pathway (Blum and Schwaller 2013). Although treatment of CR-positive colon cancer cells (e.g., HT-29, CaCo-2) with butyrate decreases CR levels via BREs acting as repressors in the *CALB2* promoter and possibly linked to butyrate's chemopreventive activity (Häner et al. 2010), similar BREs further upstream of the *CALB2* promoter act as MM-specific transcriptional activators, thus increasing CR expression in MM cells (Blum et al. 2018). Septin 7 generally considered as a cytoskeletal protein is one of the proteins binding to the BRE-containing *CALB2* promoter region, thereby inhibiting CR expression. Interestingly, CR overexpression in MM cells

decreases septin 7 levels, indicative of a bidirectional regulation. CR and septin 7 also directly interact with each other, in line with CR's additional sensor function. CR interacts with the $\alpha_{1.1}$ subunit of the voltage-dependent Ca²⁺ channel Ca_v2.1 (P/Q type) and regulates Ca²⁺-dependent inactivation and facilitation (Christel et al. 2012). This interaction is also involved in reducing CR's mobility in cerebellar GC (Arendt et al. 2013), although based on modeling, likely other CR-binding partners besides Ca_v2.1 exist in these neurons. Other binding partners in GC might include huntingtin (Dong et al. 2012) or the Na⁺/Ca²⁺ exchanger NCX1, both shown to interact with CR. The latter interaction was shown to occur in human SHSY-5Y cells, a neuronal cell line with constitutive CR expression (Boscia et al. 2016).

Additional identified promoter elements are implicated in CR regulation in MM cells. Paralleled *CALB2* transcript and CR protein levels in human MM cells are indicative of a transcriptional control (Kresoja-Rakic et al. 2016) and the sequence in the human *CALB2* promoter spanning the -161/+80 bp region sustains transcriptional activity. *cis*-regulatory elements include binding sites for NRF-1 and E2F2 and siRNA knockdown of NRF-1 decreases CR expression in MM cells. Posttranscriptional *CALB2* regulation is mediated by the presence of binding sites for miR-30a/b/c/d/e, as well as by an adenine/uridine-rich element (ARE) that binds human antigen R (HuR) in the 3' untranslated region (3'UTR) (Kresoja-Rakic et al. 2017). Although HuR-binding to the ARE results in *CALB2*-3'UTR stabilization, miR-30e-5p is implicated in the posttranscriptional negative *CALB2* regulation resulting in decreased CR expression via interaction with the *CALB2*'s 3'UTR. DA neurons in substantia nigra pars compacta from ovariectomized (OVX) mice show decreased CR staining that is reversed by treatment with E2 (Yi et al. 2016). In parallel, the OVX-induced decrease in p-AKT is also abrogated by E2. It is assumed that E2-regulated increase in CR confers neuroprotection in DA neurons, possibly related to the activation of the PI3K/AKT signaling pathway.

This is reminiscent of the condition prevailing in CR-positive immortalized mesothelial cells. In the mesothelial cell line Met-5A, CR overexpression confers protection against asbestos-induced acute cytotoxicity also by the activation of PI3K/AKT signaling (Henzi et al. 2009).

A role for CR in neuroprotection against glutamate toxicity was postulated, but evidence is most often indirect or obtained in model systems in vitro (Lukas and Jones 1994; Pike and Cotman 1995; D'Orlando et al. 2001; for more details, see Schwaller 2009). In pain-sensing peripheral sensory neurons, dorsal root ganglion (DRG) neurons, overstimulation of TRPV1 channels by capsaicin leads to Ca^{2+} -dependent cell death. CR is also expressed in a subpopulation of TRPV1-positive neurons. In line with CR acting as a fast Ca^{2+} buffer, CR decreases the amplitude, but slows down the decay of Ca^{2+} signals evoked by ionomycin. Importantly, cells coexpressing TRPV1 and CR are less sensitive to TRPV1-mediated, capsaicin-induced volume increases (swelling) causing formation of membrane blebs and cell death, revealing a protective effect of CR (Pecze et al. 2015). CR is also implicated in murine adult hippocampal neurogenesis. In CR^{-/-} mice, CR's absence "from immature early postmitotic granule cells causes an early loss in proliferative capacity of the SGZ that is maintained into adult age, when it has a further impact on the migration/survival of newborn granule cells" (Todkar et al. 2012). Thus, transient CR expression was proposed to have two functions (Todkar et al. 2012): (1) to serve as a self-maintenance signal for the pool of cells at the same stage of neurogenesis contributing to their survival/differentiation, and (2) it may contribute to retrograde signaling required for maintenance of the progenitor pool.

To summarize:

1. Cytosolic EF-hand Ca^{2+} buffers are best described as Ca^{2+} signal modulators;
2. Calbindin-D28k and CR have dual functions as Ca^{2+} buffers and Ca^{2+} sensors, the latter by directly interacting and modulating the function/activity of target proteins; and

3. Decreases in PV expression levels in neurons are associated with neurodevelopmental diseases including autism spectrum disorder and schizophrenia.

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