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Nanodomain coupling between Ca^{2+} channels and sensors of exocytosis at fast mammalian synapses

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Abstract | The physical distance between presynaptic Ca^{2+} channels and the Ca^{2+} sensors that trigger exocytosis of neurotransmitter-containing vesicles is a key determinant of the signaling properties of synapses in the nervous system. Recent functional analysis indicates that in some fast central synapses, transmitter release is triggered by a small number of Ca^{2+} channels coupled to the Ca^{2+} sensors of exocytosis at the nanometer scale. Molecular analysis reveals that this tight coupling is generated by protein-protein interactions, involving Ca^{2+} channels, Ca^{2+} sensors, and various other synaptic proteins. Nanodomain coupling has several functional advantages, increasing the efficacy, speed, and energy efficiency of synaptic transmission.

Synaptic transmission involves a highly complex series of events. When an action potential invades a presynaptic terminal, Ca^{2+} inflow through voltage-gated Ca^{2+} channels leads to a rise in intracellular Ca^{2+} concentration. Next, Ca^{2+} binds to a presynaptic Ca^{2+} sensor, which subsequently triggers exocytosis of neurotransmitter-containing synaptic vesicles. Finally, the released transmitter diffuses across the synaptic cleft and binds to postsynaptic receptors. Thus, a voltage change in the presynaptic neuron (the action potential) is converted into two chemical signals (Ca^{2+} and transmitter), and finally converted into an electrical response in the postsynaptic cell. Remarkably, what sounds like a lengthy sequence of slow biophysical and biochemical events takes place in less than a millisecond[1,2,3,4,5].

How such short synaptic delays can be achieved is incompletely understood. According to the laws of physics, diffusion time is proportional to the square of distance[6]. Thus, high speed of synaptic transmission requires tight packing of the relevant molecules. The hypothesis of tight coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis received initial support from experiments on two "classical" synapses in the peripheral nervous system: the frog neuromuscular junction[7] (FIG. 1a) and the squid giant synapse[8] (FIG. 1b). At the frog neuromuscular junction, high resolution electron microscopy tomography revealed that the distance between putative Ca^{2+} channels and synaptic vesicles was only ~20 nm[9] and modeling combined with cooperativity measurements suggested that vesicle fusion results from the Ca^{2+} inflow through only one or two Ca^{2+} channels[10]. Similarly, at the squid giant synapse, functional analysis indicated that Ca^{2+} source and Ca^{2+} sensor are tightly coupled at nanometer distance[11] and only few Ca^{2+} channels are required for release[12,13]. Evidence for both tight coupling and involvement of a small number of channels has been also presented for ciliary ganglion calyx synapses of the chick[14,15]. In this uniquely accessible synaptic preparation, simultaneous electrophysiological recording from the release face and biochemical detection of

transmitter release demonstrated that the opening of a single presynaptic Ca^{2+} channel can trigger exocytosis[14].

Notably, all these synapses have highly specialized properties and belong to peripheral nervous systems of invertebrates or lower vertebrates. Does nanodomain coupling also occur at synapses in the mammalian CNS? This is an important question for several reasons. First, detailed knowledge about coupling is essential to understand the biophysical factors shaping efficacy and speed of synaptic transmission. Second, knowledge about coupling is necessary to correctly interpret the mechanisms of presynaptic forms of plasticity[16] and the action of Ca^{2+} buffers[17]. Finally, obtaining an answer is important to understand the mechanisms of information processing and coding in the brain. A definitive answer has been obtained only recently, after a variety of central synapses have been made accessible to quantitative biophysical analysis. These include the young and mature calyx of Held, a glutamatergic synapse in the auditory system[18,19] (FIG. 1c), as well as GABAergic synapses in the hippocampus and the cerebellum[20,21] (FIG. 1d, e), key synapses that mediate fast feedforward and feedback inhibition in neuronal microcircuits.

In this review, we will summarize recent evidence for tight coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis at central synapses, address the molecular mechanisms involved, and discuss the functional implications of this coupling configuration.

Tight coupling at fast synapses in the mammalian CNS

An ingenious method to probe the coupling distance between Ca^{2+} channels and Ca^{2+} sensors involves the intracellular application of two exogenous Ca^{2+} chelators with different binding rates (k_{on}), but comparable affinities (K_D; Table 1). The basic principle is simple[11] (Box 1). If the distance between Ca^{2+} channels and Ca^{2+} sensors of exocytosis is short (smaller than 100 nm), only the fast Ca^{2+} chelator BAPTA, but not the slow Ca^{2+} chelator EGTA will have enough time to capture the Ca^{2+} on its way from the Ca^{2+} channels to the Ca^{2+} sensors. In contrast, if the coupling distance is longer, both the fast and the slow Ca^{2+} chelator will be effective.

This approach has been applied to several synapses in the mammalian CNS, leading to surprising results. In the young calyx of Held and in neocortical glutamatergic synapses, evoked transmitter release is suppressed by millimolar concentrations of both BAPTA and EGTA[3,22,23,24,25] (Table 2). This implies that the distance between Ca^{2+} channels and Ca^{2+} sensors must be long. At the young calyx of Held, quantitative modeling suggests that the average coupling distance is

~100 nm, ranging from 30 - 300 nm[22]. Thus, evoked transmitter release at these synapses is triggered by "Ca^{2+} microdomains".

By contrast, at the output synapses of fast-spiking, parvalbumin-expressing GABAergic interneurons (basket cells) in the hippocampus, evoked transmitter release is inhibited by millimolar concentrations of intracellular BAPTA, but is largely unaffected by 30 mM intracellular EGTA[26] (Table 2; FIG. 2a - c). Furthermore, at the output synapses of inhibitory basket cells in the cerebellum, intracellular application of 1 mM EGTA has no effect on the proportion of synaptic failures[21]. Likewise, at cerebellar basket cell synapses, bath application of 20 μM of membrane-permeable EGTA acetoxymethylester (EGTA-AM) has only minimal effects on evoked transmitter release following a single presynaptic action potential[27]. Although in the latter case the concentration of intracellular EGTA is only roughly known[28], these results may suggest tight coupling between Ca^{2+} source and Ca^{2+} sensor. At the hippocampal basket cell–granule cell synapse, quantitative modeling suggests a uniform coupling distance in the range of 10 – 20 nm[26] (FIG. 2c). Thus, evoked transmitter release at fast hippocampal and cerebellar GABAergic synapses is triggered by "Ca^{2+} nanodomains".

Although the terms "nanodomain" and "microdomain" are widely used, their definition is not very precise and has undergone historic shifts. Originally, the term microdomain has been used for the high concentration of Ca^{2+} near an open Ca^{2+} channel[29,30,31,32]. This definition may be puzzling, since these microdomains have spatial dimensions of nanometers. More recently, the terms nanodomain and microdomain have been widely applied to distinguish tight and loose coupling regimes. This definition is also confusing, since 50 – 150 nm is often used as a criterion to separate between the two domains. Throughout this review, we pragmatically refer to nanodomain coupling if the mean coupling distance is < 100 nm, and to microdomain coupling coupling if the distance is larger (Box 1).

The Ca^{2+} chelator experiments not only suggest differences in the mean coupling distance, but also in the uniformity of source-sensor coupling between synapses. In the young calyx of Held, 1 and 10 mM EGTA are almost equally effective[3,22] . Accordingly, there is no single distance value that describes the concentration dependence of the chelator effects at this synapse[33]. This suggests substantial non-uniformity in the coupling distance[3,22]. In contrast, in the output synapses of hippocampal basket cells, a single distance can adequately describe the effects of BAPTA and EGTA over a wide concentration range (FIG. 2c)[26]. This suggests that the coupling is substantially more uniform. Thus, tightness and uniformity of coupling at different synapses seem to be related.

The finding that the calyx of Held uses microdomain signaling for transmitter release[3,22] was puzzling for several reasons. First, it may be difficult to accept that

two synapses with calyx morphology (the calyx of Held and the ciliary ganglion calyx) would differ fundamentally in the coupling configuration. Second, if tight coupling served the purpose of speed and precision of transmitter release, it may be surprising that it is not utilized in the auditory system where the timing of signaling is critically important. Indeed, analysis of coupling at the auditory hair cell ribbon synapse, the first station in the auditory pathway, revealed that transmitter release was blocked by intracellular BAPTA, but not EGTA, suggesting nanodomain coupling[34]. Similar results were obtained at ribbon synapses in the visual system[35,36]. A resolution of this apparent paradox was provided when coupling at the calyx of Held was examined at different developmental stages[37,38]. In the mature calyx of Held, release is suppressed by millimolar concentrations of intracellular BAPTA, but unaffected by 10 mM intracellular EGTA[23,38] (Table 1). Modeling indicated that the coupling distance decreased to ~20 nm during development[39], similar to that at the hippocampal basket cell synapses. Thus, transmitter release at fast synapses in the mature auditory pathway is also triggered by Ca^{2+} nanodomains.

Synapse specificity and dynamics of nanodomain coupling

The results described above suggest that certain synapses in neuronal microcircuits (e.g. fast GABAergic output synapses of hippocampal or cerebellar basket cells) use nanodomain coupling, whereas others (e.g. glutamatergic synapses between layer 5 pyramidal neurons[24]) involve microdomain coupling. These results raise two important questions. First, what are the rules that lead to the use of nanodomain signaling in one case and microdomain signaling in the other case? Second, is the coupling distance regulated dynamically?

Several lines of evidence suggest that synapses formed by different presynaptic neurons on the same target cell can use different coupling configurations. One example is provided by the diametrically opposite properties of synapses of parvalbumin- and cholecystokinin (CCK)-expressing interneurons onto hippocampal granule cells[40,41]. The fast-spiking, parvalbumin-expressing interneurons exhibit tight coupling, as confirmed by the lack of effects of external EGTA-AM, whereas the interneurons expressing the peptide CCK show loose coupling, as demonstrated by the large effects of EGTA-AM on evoked release under identical experimental conditions[26,40] (Table 2).

Furthermore, synapses formed by the same presynaptic neuron on different postsynaptic target cells can differ in their coupling configuration. The diverging output from layer 2 / 3 pyramidal neurons in the neocortex onto two types of interneurons provides a clear example[25]. Layer 2 / 3 pyramidal neuron synapses on multipolar (presumably parvalbumin-expressing) interneurons show a smaller

sensitivity to EGTA than synapses on bipolar (presumably somatostatin-expressing) interneurons (Table 2). These results may imply that a retrograde signaling mechanism regulates the tightness of the coupling in the presynaptic terminals.

Finally, the available results suggest that the use of nanodomain versus microdomain coupling may be pathway-specific. For example, both the input and the output synapses of parvalbumin-expressing interneurons use relatively tight coupling to trigger transmitter release[25,26]. Likewise, both hair cells and mature calyces in the auditory system rely on nanodomain coupling[23,42]. Thus, the tightness of coupling appears to be regulated in a pathway-specific manner. This regulation may be activity-dependent[43], but a more systematic analysis of different synapses, microcircuits, and conditions will be needed to test this hypothesis.

An intriguing possibility is that the coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis is not static, but regulated dynamically. Recent results suggest that the induction of presynaptic long-term potentiation at distal perforant path synapses on CA1 pyramidal neurons is associated with an altered reliance of transmitter release on P/Q- and N-type Ca^{2+} channels, i.e. an increased contribution of N-type Ca^{2+} channels after potentiation[44]. It is possible that these changes in reliance on the Ca^{2+} channel type are connected to changes in channel-sensor coupling. Thus, dynamic regulation of the coupling distance may contribute to presynaptic forms of plasticity at central synapses[44].

In conclusion, the available evidence indicates that nanodomain coupling is regulated by both pre- and postsynaptic neurons, probably in a pathway-specific manner. Furthermore, recent results suggest that coupling configuration is not static, but regulated dynamically during presynaptic forms of synaptic plasticity. Further experiments will be needed to directly probe the dynamics of the coupling during presynaptic forms of plasticity.

How many open Ca^{2+} channels initiate release?

Nanodomain coupling between Ca^{2+} channels and Ca^{2+} sensors places structural and functional constraints on the number of Ca^{2+} channels that can be involved in transmitter release. As voltage-gated Ca^{2+} channel proteins have a diameter of ~10 nm[45], the highest physically possible channel density is ~10000 μm^{-2}. Accordingly, the number of Ca^{2+} channels involved in transmitter release in nanodomain coupling regimes must be small. For example, only ~12 channels can be placed on a planar presynaptic membrane within 20 nm distance from a synaptic vesicle. Furthermore, if coupling is tight, only a small number of Ca^{2+} channels may be needed to reach effective Ca^{2+} concentrations at the sensor.

How can one experimentally determine the number of open Ca^{2+} channels necessary for transmitter release? A classical approach is based on an analysis of the relationship between presynaptic Ca^{2+} inflow and transmitter release during an experimental reduction in the number of active Ca^{2+} channels. Such a reduction of Ca^{2+} channel number can be achieved either by application of slow Ca^{2+} channel blockers, such as peptide toxins[46], or by modifying the presynaptic voltage waveform that triggers exocytosis. The basic idea is relatively simple (Box 2). If several open Ca^{2+} channels jointly trigger the release of a synaptic vesicle, the progressive reduction of Ca^{2+} inflow will lead to a supralinear reduction in transmitter release. This results from the "biochemical" cooperativity of the Ca^{2+} sensor synaptotagmin, which has five binding sites for Ca^{2+} [47,48] and is expressed in multiple copies on each synaptic vesicle[49]. By contrast, in the extreme case when only a single open Ca^{2+} channel triggers release of a synaptic vesicle, the slow blocker will reduce Ca^{2+} inflow and release proportionally.

This approach has been recently applied to a variety of central synapses. At the young calyx of Held, the relationship between evoked transmitter release and presynaptic Ca^{2+} currents during slow Ca^{2+} channel block is highly supralinear, with a power coefficient (m) greater than 3, suggesting the involvement of a large number of open Ca^{2+} channels[23,38,50]. By contrast, at the output synapses of hippocampal basket cells, the relationship is only slightly supralinear, with a power coefficient of 1.6[51] (FIG. 2d - f). Modeling of experimental data with a binomial model of Ca^{2+} channel block suggested that two or three open Ca^{2+} channels trigger transmitter release at this synapse. Furthermore, in the mature calyx of Held, the power coefficient is significantly smaller than in the young calyx[23,52]. Modeling suggested the involvement of a small number of open Ca^{2+} channels in the mature calyx[39]. Finally, in both auditory hair cell ribbon synapses and retinal ribbon synapses, the relation between evoked transmitter release and presynaptic Ca^{2+} during slow Ca^{2+} channel block shows a power coefficient of 1.1 - 1.4, also suggesting the involvement of a small number of open Ca^{2+} channels[36,42,53].

The involvement of a small number of open Ca^{2+} channels may be explained by two different configurations. In the first scenario, only a small number of Ca^{2+} channels are physically present at each active zone, but these channels are activated effectively by presynaptic action potentials. In the second scenario, the total Ca^{2+} channel number is large, but the efficacy of activation is low. In fast CNS synapses, the high efficacy of activation of P/Q and N-type Ca^{2+} channels by action potentials (relative open probability 0.35 - 0.88 in different mammalian presynaptic terminals, including the calyx of Held) [54,55,56,57] argues in favor of first scenario. In contrast, in the auditory hair cell synapses the lower efficacy of activation of L-type Ca^{2+} channels would be more consistent with the second scenario[42].

These results converge towards a quantitative picture of signaling at fast central synapses. If an action potential invades a presynaptic structure, two or three Ca^{2+} channels near any given vesicle will open, generating a Ca^{2+} nanodomain. The Ca^{2+} concentration is high in the center of the nanodomain, but steeply declines as a function of distance (Box 3). Thus, the Ca^{2+} sensor on the vesicle membrane would "see" a Ca^{2+} transient with a high peak concentration and a fast time course, leading to vesicle fusion with high probability, short delay, and high temporal precision. In this scenario, a "release site"[58] would correspond to a channel-vesicle nanocomplex.

Ca^{2+} chelator experiments and cooperativity measurements provide additional constraints for the topographical arrangement of Ca^{2+} channels and vesicles in presynaptic terminals. First, they indicate that these nanocomplexes are sufficiently separated from their nearest neighbors so that their Ca^{2+} nanodomains do not overlap. Second, they suggest that nanocomplexes must be sufficiently far away from isolated Ca^{2+} channels that are not coupled to any synaptic vesicles. Finally, they imply that nanocomplexes are far away from isolated fusion competent vesicles that are not coupled to any Ca^{2+} channels[59]. How could this segregation of Ca^{2+} channel-vesicle nanocomplexes be achieved? In basket cell synapses, which have small boutons with often a single active zone (AZ)[26], nanocomplexes could be allocated to different boutons. At mature calyx synapses, which have ~600 AZs[37,60], or in auditory hair cells, which have ~15 AZs[42,61], nanocomplexes could be placed into different active zones of the same presynaptic terminal. However, sufficient separation may be also possible if nanocomplexes are located in different subregions of the same AZ. AZs have a mean area of ~0.1 μm^2 (0.094 ± 0.01 μm^2 in hippocampal basket cells, n = 11, A. Kulik, personal communication, Bucurenciu et al.[26]; 0.0996 μm^2 in the young calyx[60]; 0.0548 μm^2 in the mature calyx[37]; 0.06 μm^2 in auditory hair cells[61]), corresponding to a circle with ~150 nm radius. If channel-vesicle nanocomplexes would be preferentially placed in the periphery, several of these complexes could be accommodated in a single AZ.

Do nanodomains care about endogenous Ca^{2+} buffers?

The defining feature of nanodomain coupling is that the fast *exogenous* buffer BAPTA interferes with release at millimolar concentrations, whereas the slow *exogenous* buffer EGTA is ineffective[17]. This raises the question of how *endogenous* buffers act in nanodomain coupling regimes. A large number of Ca^{2+} buffers are present in presynaptic terminals of fast signaling synapses. These include parvalbumin in GABAergic synapses in hippocampus, GABAergic synapses in cerebellum, and the calyx of Held[62,63,64,65], calretinin in the mature calyx of Held and auditory or vestibular hair cells[66,67], and calbindin in auditory hair cells[62,68]. However,

being confronted with all these "famous" Ca^{2+} binding proteins, one should not forget that several proteins in the active zone also have binding sites for Ca^{2+}. These include Munc13s, RIMs, and the Ca^{2+} sensor synaptotagmin itself[47,48]. Furthermore, several proteins enriched in the active zone contain binding sites for ubiquitously expressed Ca^{2+}-binding proteins. For example, P/Q-type Ca^{2+} channels have binding sites for calmodulin[69,70]. Collectively, all these proteins may contribute to the high endogenous buffer capacity of fast signaling neurons[64,71].

Can these endogenous Ca^{2+}-binding proteins affect nanodomain coupling? To address this question, information about concentration and Ca^{2+}-binding rate is required[17]. Recent evidence suggests that endogenous Ca^{2+} buffers can reach high (i.e. millimolar) concentrations. Calibrated immunohistochemistry revealed that cerebellar basket cells express parvalbumin at a concentration of ~0.6 mM[72]. Furthermore, single-cell protein content analysis demonstrated that vestibular hair cells contain calretinin at a concentration of ~1.2 mM[67]. Finally, experiments with recombinant Ca^{2+} channels and tethered calmodulin mutants suggested a local concentration as high as 2.5 mM[70]. As these Ca^{2+}-binding proteins have 2 – 4 EF hand Ca^{2+} binding sites per molecule, this results in high millimolar buffer concentrations in nanodomains. Recent results further suggest that the Ca^{2+}-binding rate (k_{on}) of endogenous buffers may be faster than previously thought. For several Ca^{2+}-binding proteins, the k_{on} values have now been quantified in Ca^{2+} uncaging experiments[73,74,75]. For both calretinin (relaxed form) and calbindin, k_{on} values are comparable to those of BAPTA[74] (Table 1). For the calmodulin C-lobe (relaxed form), k_{on} is intermediate between BAPTA and EGTA, whereas for the calmodulin N-lobe, k_{on} is 100-fold higher than that of BAPTA[75] (Table 1). Finally, Ca^{2+} uncaging experiments suggest that k_{on} of the Ca^{2+} sensor synaptotagmin is comparable to that of BAPTA[76,77,78].

Taken together, these results indicate that many endogenous buffers are present at millimolar concentrations and have BAPTA-like binding properties, suggesting that they may interfere with nanodomain signaling. Several functional consequences are conceivable. First, fast endogenous buffers may reduce the amplitude of the Ca^{2+} transient, offering a mechanism to regulate the efficacy of synaptic transmission via regulation in buffer expression. Second, fast endogenous buffers will shorten the length constant of the buffer system, focusing the nanodomain in space. This effect may be particularly pronounced for fixed buffers, which will be saturated in the nanodomain, but unsaturated in the surround[79]. Finally, buffers may contribute to use-dependency of presynaptic Ca^{2+} signaling[25,79,80,81,82,83]. If presynaptic Ca^{2+} inflow during a first action potential saturates the buffer, the peak amplitude of a subsequent second Ca^{2+} transient will be facilitated relative to that of the first. Although facilitation of the Ca^{2+} transient is generally small, it will be

amplified into a much larger facilitation of transmitter release, because of "biochemical" cooperativity[23,50,51,84]. Hence, endogenous Ca^{2+} buffers may regulate amplitude, spatial extent, and dynamics of Ca^{2+} nanodomains.

Amongst all Ca^{2+}-binding proteins, parvalbumin appears to be a special case, because its EF sites bind both Ca^{2+} and Mg^{2+} [85,86,87]. Ca^{2+} binding shows fast on rate and high affinity, whereas Mg^{2+} binding is characterized by slower on rate and lower affinity. As the physiological cytoplasmic concentration of Mg^{2+} is high, Mg^{2+} has to unbind before Ca^{2+} can bind. Thus, parvalbumin may act as a slow buffer, similar to the exogenous Ca^{2+} chelator EGTA[86,87]. Furthermore, PV shows a higher mobility than other Ca^{2+}-binding proteins[88,89]. With all of these properties in mind, the tight correlation of parvalbumin expression with nanodomain signaling[63,64,65,66] is highly perplexing. In some rapidly signaling synapses, the high total concentration of parvalbumin may provide a resolution to this apparent paradox. Although the fraction of free parvalbumin (i.e. the non Mg^{2+}-bound, non Ca^{2+}-bound state) under physiological conditions is < 10%, the absolute concentration of the free buffer becomes significant. This may have two consequences. First, PV may not exclusively act as a slow buffer (like EGTA)[86], but also like a fast buffer (like BAPTA) under physiological conditions. This explains how parvalbumin can affect synaptic transmission in tight coupling regimes[21,64,71]. Second, the Mg^{2+}-bound parvalbumin fraction will not primarily slow the effective Ca^{2+}-binding rate, but rather contribute to regeneration of free buffer. Therefore, Mg^{2+} binding / unbinding may establish a "metabuffering" (i. e. buffering of buffering) mechanism, maintaining the concentration of free parvalbumin during repetitive activity in fast spiking neurons. In parallel, the high mobility of PV will contribute to buffer regeneration in the nanodomain by rapid diffusion of free buffer from the periphery to the center[88,89]. Both experimental approaches and realistic modeling of parvalbumin effects in nanodomain coupling regimes (Box 3) will be needed to further test these ideas.

From Ca^{2+} nanodomains to protein complexes

A distance between Ca^{2+} channels and sensors of exocytosis of ~20 nm[23,26,27] would be consistent with the idea that tight coupling is achieved by protein-protein interactions. Active zones are comprised of several evolutionarily conserved proteins, including SNARE (*N*-ethylmaleimide-sensitive-factor attachment protein receptor) proteins, Rab3 interacting molecules (RIMs), glutamic acid (E), leucine (L), lysine (K), and serine (S)-rich proteins (ELKS) / cytomatrix of the active zone-associated structural proteins (CASK), and septins[90]. Recent results show that several of these proteins play a role in nanodomain coupling (FIG. 3).

The first presynaptic proteins shown to be involved in protein-protein interactions with presynaptic Ca^{2+} channels were the t-SNARE proteins, syntaxin and SNAP-25. Both biochemical experiments (yeast two-hybrid experiments, coimmunoprecipitation, and proteomic screens) and functional coexpression studies indicated that syntaxin and SNAP-25 directly interact with voltage-gated Ca^{2+} channels at the intracellular loop between domains II and III of the channel protein, the so called "synprint" site[91,92,93,94,95]. Synaptotagmin, the Ca^{2+} sensor that triggers exocytosis, also interacts with the synprint site in a Ca^{2+}-dependent manner[91,94]. Intriguingly, the interactions between Ca^{2+} channels and SNARE proteins also affect Ca^{2+} channel function. Coexpression of syntaxin and SNAP-25 with Ca^{2+} channels reduces the channel open probability, whereas additional coexpression of synaptotagmin reverses this effect[94]. These results suggest a dual function for protein-protein interactions between Ca^{2+} channels and SNAREs in nanodomain coupling. First, they will link the individual molecular elements within the nanodomain. Second, they will establish a regulatory switch by which presynaptic Ca^{2+} channels bound to Ca^{2+} sensors are functionally selected, whereas Ca^{2+} channels decoupled from Ca^{2+} sensors are disabled.

Another protein relevant for the Ca^{2+} channel-sensor coupling is the *Drosophila* protein Bruchpilot. Bruchpilot is a ~200 kD active zone protein containing several coiled-coil domains[96]. In the neuromuscular junctions of Bruchpilot knockout flies, synaptic efficacy is reduced and sensitivity to EGTA-AM is increased, suggesting a conversion from nanodomain to microdomain coupling[96]. In mammalian synapses, two proteins homologous to Bruchpilot, ELKS/CAST 1 and 2, are expressed. However, genetic elimination of ELKS/CAST in mice has only moderate effects on synaptic function[97,98]. Further studies will be required to clarify the exact role of ELKS/CAST proteins in the regulation of coupling at mammalian synapses.

α-Neurexins also appear to be involved in the regulation of coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis[99]. Neurexins are 200 kDa polymorphic cell surface proteins with several EGF and laminin-neurexin-sex hormone binding globulin (LNS) domains. They are encoded by three genes and expressed in ~1000 isoforms. α-Neurexins interact with neuroligins on the postsynaptic membrane and with both ELKS/CAST and synaptotagmin within the presynaptic terminal[99,100]. Deletion of all three neurexin genes reduces evoked transmitter release and the contribution of N-type Ca^{2+} channels to release at synapses in brainstem and cortex[99], consistent with a role of α-neurexins in the regulation of Ca^{2+} channel-sensor coupling. Neurexin-neuroligin interactions may potentially explain the target cell specificity of coupling[25]. Ca^{2+} chelator experiments in neurexin knockout synapses will be needed to test this idea.

Recent results suggest that the Rab3-binding protein RIM plays a central organizing role in regulating the coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis[101,102] (FIG. 3b - d). RIMs are multidomain proteins that contain a PDZ domain that selectively interacts with the C terminus of P/Q- and N-type channels. RIM also contains a binding site for the RIM-binding protein (RIM-BP), which in turn binds to several Ca^{2+} channel subtypes[103]. Thus, RIM establishes two links to voltage-gated Ca^{2+} channels: a direct and specific link and an indirect and unselective link via RIM-BP. In inhibitory hippocampal synapses in culture, genetic elimination of RIM1 and RIM2 reduces the amplitude of evoked inhibitory postsynaptic currents, desynchronizes release, accelerates the onset of the blocking effects of EGTA-AM, and shifts the dependence of release on extracellular Ca^{2+} concentration to higher values[101] (FIG. 3c, d). Taken together, these results suggest that the coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis is disrupted in RIM1 / RIM2 double knockout synapses. Similarly, in the calyx of Held genetic elimination of RIM1 and RIM2 reduces both the presynaptic Ca^{2+} channel density and the amplitude of the Ca^{2+} transient at the Ca^{2+} sensor[102]. Additionally, RIM1 / RIM2 knockout may also affect the number of docked and primed vesicles[101,102]. Thus, at both inhibitory hippocampal synapses and the calyx of Held, RIMs seem to be critically involved in the regulation of the coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis.

Finally, the presynaptic GTP/GDP- and syntaxin-binding protein septin regulates the coupling between Ca^{2+} channels and Ca^{2+} sensors[104,105]. Septins are ~35 kDa proteins that form oligomers and higher order structures, such as filaments, rings, and gauzes. Septins may form filaments between synaptic vesicles and active zones[106]. In the young calyx of Held, genetic elimination of septin 5 reduces the sensitivity to EGTA, suggesting a conversion from microdomain to nanodomain coupling[105]. Two aspects of the function of septin 5 are remarkable. First, unlike other presynaptic proteins, septin 5 *increases* the coupling distance, suggesting antagonistic control of coupling by presynaptic proteins. Second, the expression of septin 5 is downregulated during development, suggesting an involvement in the developmental switch from microdomain to nanodomain coupling at the calyx[105].

Intriguingly, the tightness of the coupling not only depends on various release machinery proteins, but also on the Ca^{2+} channel subtype. In basket cell output synapses of hippocampus and cerebellum, as well as in the mature calyx of Held, tight coupling goes hand-in-hand with the nearly exclusive use of P/Q-type Ca^{2+} channels for transmitter release[40,51,107,108,109]. In contrast, loose coupling is often correlated with the involvement of N- or R-type Ca^{2+} channels[40,50]. Additionally, there is evidence that P/Q- and N-type Ca^{2+} channels populate partially non-overlapping “slots” within the active zone of glutamatergic synapses[110]. Finally, L-type Ca^{2+}

channels (rather than P/Q-, N-, or R-type) are tightly coupled to their Ca^{2+} sensors in auditory hair cells[42]. Clearly, this coupling specificity cannot be mediated by the synprint site, which follows an efficacy sequence of N > P/Q > L[111,112]. Thus, the molecular mechanisms underlying this specificity remain unclear.

Nanodomain coupling – advantage, bug, or feature?

Nanodomain coupling offers several functional advantages, but may also have disadvantages. The long list of obvious advantages includes the efficacy and speed of synaptic transmission (FIG. 4a - c). First, tight coupling reduces the synaptic delay[22,26]. Although the reduction in delay is small for a monosynaptic connection (~100 µs), it will accumulate in polysynaptic chains. Second, tight coupling reduces the duration of the release period, as the time course of the Ca^{2+} transient "seen" by the Ca^{2+} sensor is faster in nanodomain than in microdomain coupling regimes. Third, tight coupling increases the ratio of peak Ca^{2+} to residual Ca^{2+} and hence the ratio of synchronous to asynchronous release[26,40,113]. Therefore, in relative terms, tight coupling *reduces* asynchronous release. This effect may be particularly important in small boutons, in which residual Ca^{2+} concentration after an action potential is higher than in large presynaptic terminals. Finally, another advantage of nanodomain coupling is that release outside the active zone ("ectoptic release") is minimized[114,115].

As tight coupling of a small number of channels to the Ca^{2+} sensors reduces the total Ca^{2+} inflow into presynaptic terminals, this configuration is also favorable for the energetics of synaptic transmission (FIG. 4d). Ca^{2+} extrusion from the presynaptic terminal involves either Na^{+} / Ca^{2+} exchangers or Ca^{2+} ATPases[116]. In both cases, the extrusion of one Ca^{2+} ion requires the hydrolysis of ~ 1 ATP molecule. A coupling configuration in which a small number of Ca^{2+} channels are tightly coupled to presynaptic Ca^{2+} sensors therefore reduces the metabolic cost of synaptic transmission. Such an energy saving mechanism may be important at both GABAergic synapses in the cortex and glutamatergic synapses in the auditory pathway, which are active at high frequency under physiological conditions *in vivo*.

A potential disadvantage of nanodomain coupling with a small number of Ca^{2+} channels could be an additional jitter of evoked transmitter release caused by the stochastic opening of presynaptic Ca^{2+} channels[15,22] (FIG. 4e). However, whereas the opening and closing of Ca^{2+} channels is stochastic, the rising phase of the corresponding Ca^{2+} transient evoked by an overshooting action potential is largely deterministic, governed by the increase in driving force during the repolarization phase[51] (FIG. 4e; see Ribrault et al.[117]). Thus, transmitter release remains tightly

synchronized, even if evoked release is triggered by only a small number of Ca^{2+} channels.

Another potential disadvantage of nanodomain coupling is that spontaneous openings of Ca^{2+} channels at rest might trigger transmitter release[15]. However, recent results in dentate gyrus granule cells suggest that block of these P/Q-type Ca^{2+} channels by ω-agatoxin IVa has no effect on miniature IPSC frequency, although evoked release at basket cell–granule cell synapses exclusively relies on P/Q-type Ca^{2+} channels (FIG. 4f)[118]. Furthermore, BAPTA-AM and EGTA-AM reduce miniature IPSC frequency to the same extent, suggesting that microdomains rather than nanodomains trigger spontaneous release[118]. Thus, the high activation threshold and the steep voltage dependence of P/Q-type Ca^{2+} channels and the use of two or three open Ca^{2+} channels rather than a single channel may protect the synapse form excessive spontaneous release generated by stochastic Ca^{2+} channel opening[51,54].

Nanodomain coupling also has substantial implications for synaptic dynamics, promoting synaptic depression over facilitation for two reasons. First, it increases release probability and thus enhances depression due to depletion of the releasable pool of synaptic vesicles. Second, it reduces facilitation by decreasing the relative weight of residual Ca^{2+} [119]. Consistent with these effects, the fast signaling synapses that rely on nanodomain coupling often show depression during high-frequency stimulus trains, albeit to a different extent[19,20,21].

Finally, nanodomain coupling will have implications for how neuromodulators affect release and interact with synaptic dynamics. Previous studies suggested that presynaptic G-protein coupled receptors (such as presynaptic $GABA_B$ receptors) reduce the activity of P/Q- and N-type Ca^{2+} channels via binding of G-protein beta/gamma subunits to Ca^{2+} channels[120]. In nanodomain coupling regimes, this will have two consequences. First, the reduction in transmitter release will be largely proportional to the degree of presynaptic receptor activation. This may allow a more precise regulation of synaptic efficacy than a highly supralinear relationship. Second, as presynaptic receptor activation will reduce the number of Ca^{2+} channel-vesicle nanocomplexes, but will not affect release probability, the neuromodulators will not affect short-term dynamics, resulting in scaling of synaptic responses during repetitive stimulation[121].

Conclusions

Twenty years after the original finding of nanodomain coupling at the squid giant synapse[11], and after a subsequent decade of accumulating evidence for microdomain coupling at central synapses[122], it has become clear that synapses in the mammalian CNS also make extensive use of nanodomain coupling for fast transmitter release. In particular, GABAergic interneuron output synapses and

glutamatergic synapses in the auditory pathway rely on nanodomain coupling. Nanodomain coupling provides several functional advantages, including efficacy, speed, and energy efficiency of synaptic transmission. How abundantly nanodomain coupling is used by different synapses in the mammalian CNS remains to be addressed. Furthermore, the rules of synapse specificity of nanodomain coupling remain to be determined. Finally, it will be interesting to see whether nanodomain coupling between Ca^{2+} channels and Ca^{2+} sensors of exocytosis is disrupted in neurological or psychiatric diseases[123].

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Competing interests statement

The authors declare no competing financial interests.

Boxes

Box 1 | **Probing the distance between Ca^{2+} source and Ca^{2+} sensor with exogenous Ca^{2+} chelators.**

A classical approach to probe the distance between Ca^{2+} source and Ca^{2+} sensor is the use of Ca^{2+} chelators with different Ca^{2+}-binding rates (k_{on}) but comparable affinities (K_D)[11]. The basic idea is that chelators suppress synaptic transmission by intercepting the Ca^{2+} on its way from the Ca^{2+} source to the Ca^{2+} sensor in a way dependent on both the source-sensor distance and the binding rate of the chelator. If the coupling distance is short, only the fast Ca^{2+} chelator will have an effect at millimolar concentrations. If the coupling distance is long, both fast and slow Ca^{2+} chelators will be effective, according to their affinity at equilibrium. This approach was first applied to the squid giant synapse[11], using the fast chelator BAPTA and the slow chelator EGTA. BAPTA has an on rate of $4\ 10^8\ M^{-1}\ s^{-1}$, whereas EGTA has an on rate of $1\ 10^7\ M^{-1}\ s^{-1}$ at near-physiological pH[22,33,73,124] (Table 1). In contrast, both chelators have comparable affinity values (220 nM versus 70 nM).

The concentration dependence of the BAPTA and EGTA effects provides information about the average coupling distance between Ca^{2+} channels and Ca^{2+} sensors. Such data may be used to distinguish between nanodomain and microdomain coupling regimes. The concentration dependence of the chelator effects also provides information about the uniformity of the coupling distance. For example, at the young calyx of Held, the concentration dependence determined experimentally can be only described by theoretical models if a certain extent of non-uniformity in the coupling distance is assumed (30 – 300 nm[22]).

Although the terms nanodomain and microdomain are widely used, they are not very precisely defined. Where is the distance limit between nanodomains and microdomains? One approach is to use the spatial extent of the regimes dominated by diffusion and buffering as a criterion. For example, one may choose a distance where buffering reduces the Ca^{2+} concentration to 50%. This can be roughly estimated from the length constant (λ) of endogenous buffers. With $\lambda = \sqrt{D_{Ca} / (k_{on}[B])}$, and $D_{Ca} = 220\ \mu m^2\ s^{-1}$ [17], $k_{on} = 10^8\ M^{-1}\ s^{-1}$ (an on rate representative for endogenous buffers, Table 1), and [B] = 100 µM, $[Ca^{2+}]_{50\%}$ is reached at a distance of 100 nm. Alternatively, the limit may be set according to vesicle size and active zone size. In this scenario, the radius of synaptic vesicles would be ~20 nm[60], the diameter of active zones would be ~150 nm[26,37,60,61], and the limit between nanodomain and microdomain should be located in between. Throughout this review, we pragmatically define the border between nanodomain and microdomain at a distance of ~100 nm.

Box 2 | **Counting the number of open Ca^{2+} channels by analysis of the relation between transmitter release and presynaptic Ca^{2+} transients.**

Another clever method can be used to determine the number of open Ca^{2+} channels required for transmitter release from the shape of the relationship between release

and presynaptic Ca^{2+} inflow[12,13,51]. In synapses where presynaptic voltage clamp is possible, the number of open channels can be changed by varying the amplitude and duration of the depolarization[23]. In this scenario, the presynaptic Ca^{2+} current can be directly recorded. In other synapses where presynaptic voltage clamp is not possible, the number of Ca^{2+} channels can be changed by application of channel blockers[46,51]. Under these conditions, presynaptic Ca^{2+} inflow is quantified by Ca^{2+} imaging. The results from these measurements then give the relationship between transmitter release and presynaptic Ca^{2+} inflow. If a large number of open Ca^{2+} channels are required for transmitter release, the relationship will be supralinear, approaching the "biochemical" cooperativity of the Ca^{2+} sensor, because the blocker will reduce the amplitude of the Ca^{2+} transient at each site. In contrast, if a single open Ca^{2+} channel is sufficient to trigger transmitter release, the relationship will be linear, because the blocker will sequentially eliminate channel – vesicle complexes. If the number of channels is small, but > 1, the shape of the relation will be intermediate between these two extremes.

Next, a mathematical model describing the relationship between release and presynaptic Ca^{2+} inflow has to be established. If blockers are used, a simple binomial model of Ca^{2+} channel block can be chosen. However, there are several factors to be considered. The properties of the blocker are critical: the ideal blocker should have slow kinetics and block Ca^{2+} channels uniformly throughout the presynaptic terminal. Fast blockers that generate a flicker block[125] or blockers that reduce single-channel conductance cannot be used. The techniques for measuring presynaptic Ca^{2+} inflow and transmitter release have to be quantitative and linear. The modeling is based on several assumptions, such as uniform coupling distance and independent block of channels, which may not be valid in all cases. It must also be kept in mind that the method measures the number of **open** channels, not the **total** number of Ca^{2+} channels present. These two numbers can substantially differ, because the open probability of Ca^{2+} channels during presynaptic action potentials is significantly smaller than unity[54,55,56,57]. Finally, the power coefficient of the release - Ca^{2+} inflow relationship is not identical to the number of open Ca^{2+} channels necessary for transmitter release. The upper bound of the power coefficient is given by the power coefficient of "biochemical" cooperativity[51,126].

This approach has been successfully applied to synapses where transmitter release exclusively relies on a single type of Ca^{2+} channel, such as the P/Q-type Ca^{2+} channel in GABAergic synapses[40,51] or the L-type Ca^{2+} channel in auditory hair cell ribbon synapses[42]. At synapses where transmitter release relies on the concerted action of P/Q-, N-, and R-type channels[50,127,128,129] very careful interpretation of the results is required. If release-[Ca^{2+}] relationships are separately measured for blockers of two or multiple Ca^{2+} channels, the results will provide information about

channel location rather than number. If channels are loosely coupled, they will contribute little to release (low power coefficient), whereas if they are tightly coupled, they will contribute more (high power coefficient[130]). Thus, the power coefficients, although informative, are entirely unrelated to channel numbers. By contrast, if the additivity of Ca^{2+} channel blocker effects is measured, this can provide indirect information about channel number. Evidence for nonlinear blocker effects was reported at the young calyx of Held[50], glutamatergic synapses in hippocampus[127,128,131], and glutamatergic parallel fiber synapses in cerebellum[129].

Box 3 | **Modeling the effects of buffers in realistic coupling regimes – a cookbook.**

A lot of insight into the mechanisms of coupling between Ca^{2+} channels and Ca^{2+} sensors can be obtained by modeling the diffusion of Ca^{2+} and its reaction with buffers. As in other fields of Neuroscience, the Hopfield quote "build it, and you understand it" perfectly applies. How can one model the Ca^{2+} transient?

In a simplifying scenario, the steady-state solution to the linearized reaction-diffusion problem is obtained analytically[17,132]. In this framework, the Ca^{2+} concentration ($[Ca^{2+}]$) can be described by a simple equation, which is comprised of a 1 / r term (representing diffusion) and an exponential term (representing buffering):

$$[Ca^{2+}] = \frac{i_{Ca}}{4\pi F D_{Ca}} \; 1 / r \;\; \exp(-r / \lambda) \qquad \text{(Eq. 1)}$$

with $\lambda = \sqrt{D_{Ca}} / (k_{on} [B])$,

where i_{Ca} is the Ca^{2+} current, F is the Faraday constant, D_{Ca} is the diffusion coefficient of Ca^{2+}, r is radial distance from a source, λ is the length constant, k_{on} is the Ca^{2+}-binding rate of the buffer, and [B] is the concentration of the buffer[17].

Although the linear approach represents a useful approximation for short distances from the source, it does not account for the time course of the Ca^{2+} transient, the phenomenon of buffer saturation, and the presence of fixed and mobile buffers[17].

The limitations can be overcome by obtaining the time-dependent solution to the full reaction-diffusion equations[26,51,80,133,134]. This can be done by numerically solving a set of partial differential equations, containing the Ca^{2+} and buffer concentrations as a function of space and time, as well as several partial derivatives.

Everything starts from Fick's first and second law of diffusion[135]. Fick's first law relates the diffusive flux to the concentration field. In the simplest possible form in one spatial dimension, the first law is

$$J = D_{Ca} \frac{\partial [Ca^{2+}]}{\partial x}, \qquad \text{(Eq. 2)}$$

where J is the flux in units mol s^{-1} m^{-2}. From the law of mass conservation and Fick's first law, Fick's second law can be obtained[132].

$$\frac{\partial [Ca^{2+}]}{\partial t} = \frac{\partial J}{\partial x} = \frac{\partial}{\partial x}\left(D_{Ca} \frac{\partial [Ca^{2+}]}{\partial x} \right) \qquad \text{(Eq. 3)}$$

Equation 3 gives the partial differential equation that has to be solved. Equation 2 gives the boundary condition near the source. In addition, a second boundary condition has to be implemented remote from the source. This is usually a reflective boundary condition, which is given as $\partial [Ca^{2+}] / \partial x = 0$ for $x \rightarrow x_{max}$. As there is no gradient at this distance, Ca^{2+} cannot escape beyond this point. Furthermore, initial conditions have to be appropriately chosen. For example, $[Ca^{2+}]$ at t = 0 is set to the resting value. The partial differential equations can be solved numerically, e.g. using NDSolve of Mathematica[26,51,136].

Finally, the effect of the Ca^{2+} transient on transmitter release has to be simulated, using models of transmitter release derived from Ca^{2+} uncaging experiments[76,77,78,137,138] . Based on a 6- to 8-state reaction scheme, a set of ordinary differential equations can be formulated, which can be solved numerically.

The cookbook recipe (Eq. 1 – 3) describes the backbone of the simulations, defining the Ca^{2+} transients from a point source in the absence of buffers. For a more realistic simulation, several extensions have to be made. In the presence of buffers, the right hand side of equation 2 has to be extended by the sum of reaction terms. To simulate Ca^{2+} transients originating from Ca^{2+} channel clusters or other distributed sources, the one-dimensional simulations have to be extended into two or three dimensions[80,133,134].

Early studies have used several different approximations, such as the steady-state excess buffer approximation (EBA; buffer concentration is so high that it changes little during Ca^{2+} inflow) and rapid buffer approximation (RBA; buffers are so fast that they are in chemical equilibrium with Ca^{2+} at every point in time and space[134]). As computer power has increased, these approximations are now obsolete.

Glossary (alphabetical order)

Basket cell. A type of perisomatic inhibitory GABAergic interneuron in hippocampus and cerebellum. The name was given as the axon forms “baskets” around somata of postsynaptic target cells.

Buffer saturation. Reduction of the concentration of free buffer, for example after presynaptic Ca^{2+} inflow. Buffer saturation can occur locally (in the vicinity of Ca^{2+} channels), or globally (in the entire presynaptic terminal).

Ca^{2+} chelator. A chemical substance that binds Ca^{2+}. In synaptic physiology, BAPTA (Ethylenedioxybis-(o-phenylenenitrilo)tetraacetic acid) and EGTA (ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) are widely used Ca^{2+} chelators. Both chelators are also available in membrane-permeable acetoxymethylester (AM) forms.

“Biochemical” cooperativity: Nonlinear dependence of transmitter release on the intracellular Ca^{2+} concentration, presumably due to multiple Ca^{2+}-binding sites on the Ca^{2+} sensor synaptotagmin and multiple synaptotagmins on individual synaptic vesicles.

Ca^{2+} nanodomain. A domain of elevated Ca^{2+} concentration that extends over less than 100 nanometers (1 nm = 10^{-9} m).

Ca^{2+} microdomain. A domain of elevated Ca^{2+} concentration that extends over more than 100 nanometers. Note that this definition does not imply that the distance is in the micrometer range (1 µm = 10^{-6} m).

Mobile buffers. Mobile Ca^{2+} buffers can move in space, with a diffusion coefficient near that in aqueous solution. Mobile buffers are replenished easily by diffusion from compartments remote from the Ca^{2+} source.

Fixed buffers. Fixed buffers always remain at the same location. In contrast to mobile buffers, fixed buffers can be only regenerated by Ca^{2+} unbinding, not by diffusion.

Ordinary differential equations. Ordinary differential equations (ODEs) describe the rate of change of a function of a single independent variable (e.g. Ca^{2+} concentration versus time).

Partial differential equations. Partial differential equations (PDEs) describe the rate of change of a function with respect to multiple independent variables (e.g. Ca^{2+} concentration versus time and space). Partial differential equations are used to model several diverse processes that change in both time and space, such as diffusion, heat conduction, and propagation of voltage signals in cables.

Synchronous and asynchronous release. Synchronous release directly follows the presynaptic action potential (within a few milliseconds), whereas asynchronous release follows presynaptic action potentials with longer latencies (within several hundreds of milliseconds). Asynchronous release is particularly pronounced when synapses are stimulated repetitively.

Synaptic delay. The time interval between the presynaptic action potential and the postsynaptic response. The synaptic delay is comprised of several components: opening of presynaptic Ca^{2+} channels, diffusion of Ca^{2+} from the channels to the Ca^{2+} sensors, activation of Ca^{2+} sensors, exocytosis, diffusion of transmitter across the synaptic cleft, and activation of postsynaptic receptors.

Synaptic depression. Reduction of transmitter release during repetitive stimulation of the presynaptic neuron. Synaptic depression is often interpreted as a depletion of the releasable pool of synaptic vesicles, although additional mechanisms such as changes in presynaptic action potential shape and inactivation of presynaptic Ca^{2+} channels may also contribute.

Synaptic facilitation. A short-lasting increase of efficacy of synaptic transmission during repeated stimulation. Traditionally, different forms of enhancement are classified according to the kinetics of decay. At the neuromuscular junction, the fastest form is facilitation, followed by augmentation, followed by potentiation.

Legends to Figures

Figure 1 | **"Model synapses" used for the analysis of Ca^{2+} channel-sensor coupling: Advantages and disadvantages.**

a | The frog neuromuscular junction, a classical preparation for the analysis of synaptic transmission[7]. A technical advantage is the 1 : 1 innervation, in which a single motoaxon (blue) selectively innervates a single muscle fiber (black). Furthermore, the structure of this synapse has been studied extensively[9]. Presynaptic access, however, is not possible.

b | The squid giant synapse, another classical preparation[8]. A technical advantage is that presynaptic terminals can be recorded directly with sharp microelectrodes.

c | The calyx of Held in the auditory brainstem[18,19].

A technical advantage of this synapse is that presynaptic terminals can be recorded directly with patch-clamp techniques. Furthermore, postsynaptic currents can be measured under ideal voltage-clamp conditions, since the synapse is located perisomatically. However, a limitation is that recordings typically have to be made from relatively young animals (often postnatal day 8 – 10).

d | The hippocampal dentate gyrus basket cell synapse[20]. Left, confocal stack projection. Right, double labeling of presynaptic terminals with the intracellular morphological tracer (biocytin, green) and an antibody against the Ca^{2+}-binding protein parvalbumin (right, red).

e | The cerebellar basket cell synapse[21]. Left, confocal stack projection. Right, expanded view of the presynaptic terminals. Note the formation of pericellular baskets around somata and Pinceau structures (arrowheads) around axons of Purkinje cells.

In hippocampal and cerebellar basket cell synapses, paired recordings between presynaptic neurons and postsynaptic cells can be obtained with high success rate, because of the relatively high connectivity. A minus point of these synapses is that presynaptic terminals cannot be routinely recorded.

Image in **a** from Nicholls et al.[139], p. 192, based on data from U.J. McMahan; image in **b** from Bullock and Hagiwara[140]; image in **c** from von Gersdorff and Borst[19]; images in **d** and **e** from E.E. and P.J[71].

Figure 2 | **Experimental determination of the coupling distance and the number of open Ca^{2+} channels that mediate transmitter release.**

a | Ca^{2+} chelators with different on rates are used to probe the distance between Ca^{2+} channels and sensors. In a tight coupling regime (upper), only the fast Ca^{2+} chelator BAPTA, but not the slow Ca^{2+} chelator EGTA will capture the Ca^{2+} on its way from the source to the sensor. By contrast, in a loose coupling regime (lower), both chelators will be effective, according to their affinity values, which are comparable.

b | Effects of 10 mM BAPTA (upper traces) and 30 mM EGTA (lower traces) on unitary IPSCs at hippocampal basket cell output synapses under steady-state

conditions. Red traces, presynaptic action potentials; black traces, IPSCs; green traces, averages. Note that millimolar concentrations of BAPTA, but not EGTA block transmitter release at these synapses.

c | Concentration dependence of the effects of BAPTA and EGTA at the hippocampal basket cell – granule cell synapse. The chelators were delivered to presynaptic sites by pipette perfusion. Lines represent predictions of a reaction – diffusion model simplified by linearization (continuous lines, predictions for a single Ca^{2+} channel; dashed lines, predictions for a cluster of multiple Ca^{2+} channels). The best description of the experimental data was obtained assuming a coupling distance of 12 nm.

d | In a multiple channel scenario (upper), blocking Ca^{2+} channels with a slow blocker scales the Ca^{2+} transient at the vesicular Ca^{2+} sensor, reducing transmitter release supralinearly. In a single-channel coupling scenario (lower), blocking Ca^{2+} channels sequentially eliminates channel – vesicle nanocomplexes, reducing transmitter release linearly. Inset shows ω-agatoxin IVa, which selectively blocks P/Q-type Ca^{2+} channels at hippocampal basket cell output synapses.

e | Ca^{2+} transients and IPSCs before (top) and after (bottom) application of ω-agatoxin IVa. In each pair of graphs, the upper traces represent the Ca^{2+} transients measured as relative fluorescence changes ($\Delta F / F_0$) and the lower traces represent IPSCs; corresponding scale bars at the bottom. Note that the toxin reduces Ca^{2+} transients and IPSCs to a comparable extent. Presynaptic Ca^{2+} transients were measured with the Ca^{2+} indicator dye Oregon Green BAPTA1.

f | Plot of peak amplitudes of synaptic currents as a measure of exocytosis against $\Delta F / F_0$ as a measure of Ca^{2+} inflow. The blue curves show the predictions of a binomial model of Ca^{2+} channel block with different numbers of open Ca^{2+} channels (N = 1, 2, or 10). The red curve shows free fit with a power function. Note that the best fit of the experimental observations can be obtained with a model assuming two or three Ca^{2+} channels. Data in **b**, **c** from Bucurenciu et al.[26]; data in **e**, **f** from Bucurenciu et al.[51]

Figure 3 | **Molecular mechanisms of tight coupling.**

a | Space filling models of protein complexes in the active zone. A synaptic vesicle (SV) is surrounded by several proteins. Only a single copy of each protein is depicted[88].

b | Schematic illustration of the proposed function of RIM as a tether in the active zone. CaV, voltage-gated calcium channel; Syt, synaptotagmin; Rab3A, small G protein localized on synaptic vesicles; RIM, Rab3A-interacting molecule; RIM-BP, RIM binding protein; Munc 13, mammalian homolog of unc = uncoordinated. Note

that both RIM and RIM-BP bind to the C-terminus of the Ca^{2+} channel and that the N-terminus of RIM binds to Rab3A. As Rab3A is a vesicular protein, the complex links Ca^{2+} channels to synaptic vesicles.

c | Genetic elimination of RIMs changes the dependency of IPSC amplitudes on extracellular Ca^{2+} concentration at GABAergic synapses. Left, dose-effect curves in control synapses, RIM double knockout synapses, and after rescue with recombinantly expressed RIM1. Right, summary bar graph of EC_{50} values in the three conditions.

d | Genetic elimination of RIMs changes the coupling between Ca^{2+} source and Ca^{2+} sensor at GABAergic synapses. Left, IPSCs in control synapses (top) and in RIM double knockout synapses (bottom) at different times during application of EGTA-AM. Center, time course of inhibitory effects of EGTA-AM at control synapses (gray) and double knockout synapses (black). Right, time constants of the onset of the effects of EGTA-AM. EGTA-AM acts more rapidly in the RIM double knockout mouse, suggesting a looser coupling between Ca^{2+} channels and sensors of exocytosis[101].

Although the experiments were performed at cultured hippocampal inhibitory synapses, it is likely that at least a subset includes output synapses from parvalbumin-expressing fast spiking interneurons.

Image in **a** from Müller et al.[88]; scheme in **b** from Pernía-Andrade and Jonas[141]; data in **c**, **d** from Kaeser et al.[101]

Figure 4 | **Functional consequences of nanodomain coupling.**

a | Tight coupling increases the ratio of synchronous to asynchronous release by increasing the ratio of peak Ca^{2+} to residual Ca^{2+}. Traces show normalized action potential-evoked Ca^{2+} transients at distances between 20 nm and 200 nm (step size 20 nm). The fast component of the Ca^{2+} transient will drive synchronous release, whereas the slow component will initiate asynchronous release. The red dashed line represents the presynaptic action potential.

b | Tight coupling reduces the component of the synaptic delay that is caused by diffusion of Ca^{2+} (circles, red curve, “delay”) and, in parallel, increases the temporal precision of release in relation to the presynaptic action potential (squares, blue curve, “half-duration”).

c | Tight coupling increases release probability and thus synaptic efficacy (circles, red curve) and, in relative terms, decreases asynchronous release (squares, blue curve).

d | Tight coupling reduces the presynaptic Ca^{2+} load and thus introduces energetic advantages. Na^+ / K^+ -ATPase, Na^+ / Ca^{2+} exchanger, and Ca^{2+}-ATPase are depicted schematically. Na^+ / Ca^{2+} exchanger and Ca^{2+}-ATPase are the main Ca^{2+} extrusion mechanisms in the presynaptic plasma membrane. The Ca^{2+}-ATPase is primary

active, i.e. directly dependent on the hydrolysis of ATP. The Na^+ / Ca^{2+} exchanger is secondary active. It exploits the Na^+ ion gradient previously generated by the Na^+ / K^+ -ATPase, another primary active transport. Thus, both Ca^{2+} extrusion pathways require hydrolysis of ~1 ATP for the extrusion of 1 Ca^{2+} ion.

e | Use of a small number of Ca^{2+} channels introduces stochastic components in Ca^{2+} channel opening and closing, without affecting the rising phase of corresponding Ca^{2+} transients. Main plot, simulated Ca^{2+} concentration 12 nm away from a single Ca^{2+} channel activated by an action potential. Inset, open probability of the single Ca^{2+} channel. 10 individual openings are shown superimposed. Red curves, regime with an infinite number of Ca^{2+} channels shown for comparison. Note that the rising phase of the Ca^{2+} transient is the same, despite stochastic Ca^{2+} channel opening. Thus, the opening of the Ca^{2+} channels is stochastic, whereas the rising phase of the Ca^{2+} transients is largely deterministic.

f | Use of a small number of Ca^{2+} channels does not lead to excessive miniature release due to stochastic Ca^{2+} channel opening. Left, miniature IPSCs in control conditions (top three traces) and after application of ω-agatoxin IVa (bottom three traces) in dentate gyrus granule cells. Right, corresponding cumulative histogram of interevent interval (black, control; red, agatoxin). Note that the frequency of miniature IPSCs is not different in the two conditions.

Data in **a** - **c** from Bucurenciu et al.[26]; data in **e** from Bucurenciu et al.[51]; data in **f** from Goswami et al.[118] In **b**, **c**, and **e**, transmitter release was simulated using a previously established release model[78].

Table 1 | **Physicochemical properties of exogenous and endogenous Ca^{2+} buffers**.

Chelator / Ca^{2+}-binding protein	**Ca^{2+}-binding rate (k_{on})**	**Ca^{2+}-unbinding rate (k_{off})**	**Affinity (K_D)**	**Reference**
BAPTA	4 10^8 M^{-1} s^{-1}		220 nM	Naraghi[124]; Naraghi and Neher[33]; Meinrenken et al.[22]
EGTA	1 10^7 M^{-1} s^{-1}		70 nM	Nägerl et al.[73]; Meinrenken et al.[22]

Calbindin	$7.5\ 10^{7}\ M^{-1}\ s^{-1}$	$29.5\ s^{-1}$		Nägerl et al.[73]; Faas et al.[75]
Calretinin	$1.8\ 10^{6}\ M^{-1}s^{-1}$ (T) $3.1\ 10^{8}\ M^{-1}\ s^{-1}$ (R)	$1.29\ s^{-1}$ (T) $1.73\ s^{-1}$ (R)		Faas et al.[74]
Calmodulin N-lobe	$7.7\ 10^{8}\ M^{-1}\ s^{-1}$ (T) $3.2\ 10^{10}\ M^{-1}\ s^{-1}$ (R)	$1.6\ 10^{5}\ s^{-1}$ (T) $2.2\ 10^{4}\ s^{-1}$ (R)		Faas et al.[75]
Calmodulin C-lobe	$8.4\ 10^{7}\ M^{-1}\ s^{-1}$ (T) $2.5\ 10^{7}\ M^{-1}\ s^{-1}$ (R)	$2.6\ 10^{3}\ s^{-1}$ (T) $6.5\ s^{-1}$ (R)		Faas et al.[75]

For the exogenous chelators, the Ca^{2+}-binding rate (on rate) is 40-times higher for BAPTA than for EGTA. In contrast, the affinity values are comparable; in fact the affinity is 3-fold **lower** for BAPTA than for EGTA.

For the Ca^{2+}-binding proteins calretinin and calmodulin, Ca^{2+} binding is highly cooperative. Therefore, rates are given separately for tense (T) and relaxed (R) conformations of the protein. Either Ca^{2+} unbinding rates (k_{off}) or affinity values are given, because the two values are directly related ($K_D = k_{off} / k_{on}$).

Table 2 | **Differential coupling of Ca^{2+} channels and Ca^{2+} sensors at different synapses.**

Synapses with "nanodomain" coupling	**Age**	**BAPTA IC_{50} / amplitude PSC**	**EGTA IC_{50} / amplitude PSC**	**Reference**
Squid giant synapse	Adult		>>80 mM	Adler et al.[11]
Mature calyx of Held	P16-18	1.3 mM	35.4 mM	Fedchyshyn and Wang[23]
Hippocampal basket cell – granule cell synapses	P18-21	1.6 mM	61.5 mM	Bucurenciu et al.[26]
Dto	P19-22	63.9 ± 4.3 % in 100 µM BAPTA-AM	No effect in 100 µM EGTA-AM	Hefft and Jonas[40]
Cerebellar molecular layer	P14-20		97.5 ± 4.8 % 82.8 ± 11.3 % in	Christie et al.[27]

interneuron – interneuron synapses			20 µM EGTA-AM	
Cerebellar climbing fiber-Purkinje cell synapses	P8-20		103 ± 5 % in 20 µM EGTA-AM	Matsui and Jahr[114]
Auditory hair cell ribbon synapse	P14-40		>> 5 mM	Moser and Beutner[34]
Retinal bipolar cell synapse	Adult	2.2 mM	>> 5 – 10 mM	Mennerick and Matthews[35]; Singer and Diamond[142]
Synapses with "microdomain" coupling				
Young calyx of Held	P8-12	1.3 mM	7.5 mM	Fedchyshyn and Wang[23]
Young calyx of Held	P8 – 10	0.61 mM	13.3 mM	Borst et al.[143]; Borst and Sakmann[3]
Layer 5 – layer 5 neocortical synapses	P14-16	0.7 mM	7.9 mM	Ohana and Sakmann[24]
Layer 2/3 pyramidal cell synapse on bitufted interneurons	P14-15	0.1 mM	1 mM	Rozov et al.[25]
Layer 2/3 pyramidal cell synapse on multipolar interneurons	P14-15	0.5 mM	7 mM	Rozov et al.[25]
CCK interneuron – granule cell synapses	P19-22		6.8 ± 3.8 % in 100 µM EGTA-AM	Hefft and Jonas[40]

Cerebellar climbing fiber synapses, ectopic release on Bergmann glial cells	P8-20		67 ± 11 % in 20 µM EGTA-AM	Matsui and Jahr[114]

IC_{50} values were either directly taken from references or calculated from the amount of block according to a Hill equation.

AM-ester forms of EGTA permeate cell membranes easily. Once the intracellular compartment is reached, the AM residue is cleaved by endogenous esterases, and the Ca^{2+} chelator is trapped intracellularly. Although the precise EGTA concentration is not known, it is thought that this trapping mechanism leads to a ~100-fold enrichment in comparison to the extracellular concentration (compare Figs. 5b and 10a in Atluri and Regehr[28]).