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Calcium regulation of spontaneous and asynchronous neurotransmitter release

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Summary

The molecular machinery underlying action potential-evoked, synchronous neurotransmitter release, has been intensely studied. It was presumed that two other forms of exocytosis- delayed (asynchronous) and spontaneous transmission, were mediated by the same voltage-activated Ca^{2+} channels (VACCs), intracellular Ca^{2+} sensors and vesicle pools. However, a recent explosion in the study of spontaneous and asynchronous release has shown these presumptions to be incorrect. Furthermore, the finding that different forms of synaptic transmission may mediate distinct physiological functions emphasizes the importance of identifying the mechanisms by which Ca^{2+} regulates spontaneous and asynchronous release. In this article we will briefly summarize new and published data on the role of Ca^{2+} in regulating spontaneous and asynchronous release at a number of different synapses. We will discuss how an increase of extracellular [Ca^{2+}] increases spontaneous and asynchronous release, show that VACCs are involved at only some synapses, and identify regulatory roles for other ion channels and G protein-coupled receptors. In particular, we will focus on two novel pathways that play important roles in the regulation of non-synchronous release at two exemplary synapses: one modulated by the Ca^{2+}-sensing receptor and the other by transient receptor potential cation channel sub-family V member 1.

Introduction

At the chemical synapse the mechanisms by which action potentials and voltage activated Ca^{2+} channels (VACCs) trigger release of neurotransmitter packaged in single vesicles have been a major focus of investigation [1, 2]. Recently there has been a substantial increase in interest in two other forms of neurosecretion: spontaneous release that occurs in the absence of an action potential [3, 4] and asynchronous release that is only loosely time-locked to an action potential [5–8]. Just as for classical synchronous release, Ca^{2+} plays a key role in regulation of these two other forms of neurotransmission [6, 9–11]. It has been presumed that spontaneous and asynchronous release arise from the same vesicle pools as evoked exocytosis [12]. However, mounting data suggest that the situation is much more complex and that spontaneous and asynchronous pathways are unique and contrast in many ways with synchronous release in that they are regulated differently [6, 10], arise from distinct pathways [13, 14], are controlled by different synaptic machinery [8, 11, 15, 16], and

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potentially mediate different physiological functions [17]. In this article we will discuss the varied roles of Ca^{2+} in the regulation of spontaneous and asynchronous release at multiple synapses. We focus on three key aspects of synaptic transmission: alternative sources of Ca^{2+} mediating release, additional Ca^{2+} sensors, and independent vesicle pools for different modes of transmission.

Spontaneous and evoked release are physiologically different

Spontaneous release was originally described at the frog neuromuscular junction [18], with miniature end plate potentials (mEPPs) identified as small, subthreshold depolarizations in the postsynaptic muscle membrane. Miniatures had time courses similar to end-plate potentials (EPPs) and were similarly sensitive to curare, but unlike EPPs did not propagate beyond the immediate region of the synapse and were ~100 times smaller [18]. Increasing extracellular $[Mg^{2+}]$ ($[Mg^{2+}]_o$) and decreasing extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) reduced the EPP amplitude to the same size as the mEPP and a statistical approach indicated that synaptic transmission occurred via the release of a quantum of acetylcholine [19, 20]. Spontaneous transmission reflected fusion of a single vesicle whereas evoked release represented synchronized fusion from multiple nerve endings [19]. The discovery that some hippocampal neurons are connected by a single synapse suggested that communication via a single quantum, however it is triggered, must be physiologically important [21]. This idea was strengthened by work showing that firing patterns in high resistance cerebellar interneurons were also regulated by single quantal inputs [22]. Spontaneous neurotransmitter release also can impact network activity by regulating the strength of individual synapses. Synaptic release increases profoundly following only a few hours of synaptic blockade [23, 24], while spontaneous release alone is sufficient to maintain synaptic strength [25]. Thus spontaneous release has an important homeostatic role in preventing synaptic potentiation at times of reduced action potential-evoked activity. This result highlights both the physiological significance of spontaneous transmission and its differential role from action potential-evoked release.

Spontaneous and evoked release use different vesicle pools

In contrast to early assumptions that spontaneous and evoked release share the same vesicle population [12], the combination of electrophysiological and optical techniques have clarified that they use distinct vesicle pools [13, 14, 26]. At central synapses evoked and spontaneous vesicle pools have been distinguished by differences in their intracellular Ca^{2+} sensors for exocytosis, their sensitivity to phorbol esters, the spatial separation of the postsynaptic receptors that they target, and the mechanism by which endocytosis occurs [27–30]. Recent studies of the molecular machinery of spontaneous release indicate there are different pathways by which a nerve terminal could sort and regulate spontaneous and evoked vesicle pools [16].

While mounting data support the idea of distinct vesicle pools for spontaneous and evoked release the question remains: what is the purpose of two presynaptic signaling pathways that employ the same type of postsynaptic receptor? While spontaneous release may be sufficient to substitute for evoked release in some experimental situations the low frequency of spontaneous release may mean that most of the time it does little more than increase noise at each synapse. Evidence identifying a physiological pathway for which spontaneous release is essential is in short supply since it is not as of yet possible to disrupt spontaneous fusion alone. However new data points to a functional role for which spontaneous release is necessary. BDNF-mediated antidepressant effects of ketamine have been proposed to result from the transient block of NMDA receptors activated by spontaneous glutamate release [17]. This work offers support to the intriguing hypothesis that the spontaneous pathway

mediates unique physiological messages using a parallel pathway employed by evoked neurotransmission, analogous to the residential telecommunications line that provides both telephone and internet connectivity.

The concentration-effect relationship for $[Ca^{2+}]_o$ and spontaneous exocytosis

Ca^{2+} entry is the initiation step for exocytosis at the synapse [31]. The fundamental observation by Dodge and Rahamimoff [32] that synaptic efficacy is dependent on the fourth power of $[Ca^{2+}]_o$ at the frog neuromuscular junction established the importance of Ca^{2+} in the exocytotic process and has since been confirmed in a number of preparations [33–38]. The steepness of the dose-response curve reflects the high intrinsic cooperativity of activation of the exocytotic machinery by calcium. Does the release mechanism for spontaneous release have the same high sensitivity to Ca^{2+}? At the neuromuscular junction the dependence of spontaneous release on $[Ca^{2+}]_o$ is relatively weak [39]. The slope of the log-log plot (n), a model- independent measure of Ca^{2+} cooperativity [40], was only 0.21–0.41, much lower than for evoked release [41–44]. At neocortical excitatory synapses spontaneous release of glutamate was also weakly dependent on external Ca^{2+} (n = 0.57–0.63, [10, 11]). In contrast to these observations glutamate and GABA release has been reported to be more strongly dependent on external Ca^{2+} by others (n = 1.47 and 1.49, respectively [9]). At the neuromuscular junction application of potassium increases the sensitivity to $[Ca^{2+}]_o$ presumably due to depolarization[45]. In these experiments the quantal events counted are a combination of release arising from spontaneous fusion and depolarization triggered fusion. This indicates that release occurring spontaneously at physiological and depolarized potentials may reflect different processes and that grouping them together may obscure understanding of the underlying mechanisms.

VACCs and spontaneous release

Evoked vesicle fusion is sensitive to changes in $[Ca^{2+}]_o$ [46] and is triggered by Ca^{2+} entry via N-, P/Q- or R-type VACCs [47, 48]. Release is remarkably sensitive and can be triggered by only 1 or 2 VACCs per vesicle at some synapses [49–52]. Thus stochastic activation of VACC could provide the mechanism for spontaneous release [41]. The central role of Ca^{2+} as a trigger for exocytosis and the number of apparent differences between evoked and spontaneous release raises the question: are both forms of release regulated similarly by external Ca^{2+}? Recently we showed that neocortical excitatory synapses do not require Ca^{2+} entry via VACC to trigger spontaneous release of glutamate[10]. Surprisingly both basal spontaneous release and the response to increased external Ca^{2+} were unaffected by VACC blockers. Increasing external Ca^{2+} did not affect $[Ca^{2+}]_i$ in the majority of the nerve terminals and spontaneous release was unaffected by the fast acting Ca^{2+} chelator BAPTA (control experiments showed that under these conditions BAPTA substantially attenuated evoked glutamate release). Similarly, in hippocampal and cerebellar neurons miniature excitatory postsynaptic current (mEPSC) frequency was unaffected by VACC blockers [53, 54]. Together these results indicate that Ca^{2+} influx via VACCs is not involved in spontaneous glutamate release at these synapses. In contrast, at inhibitory synapses the opposite is true: VACC blockers reduce spontaneous release of GABA in the hippocampus and neocortex [55, 56] (Chen, Williams, and Smith, unpublished observations). These findings indicate differential regulation of spontaneous release of GABA and glutamate in the cortex and point to an unappreciated degree of heterogeneity in the spontaneous pathway. Reasons for this could include differences in the number or type of VACCs, the association between Ca^{2+} source and release machinery, intracellular Ca^{2+} buffers, or resting membrane potential. In other parts of the nervous system spontaneous excitatory release is not always independent of VACC and miniature inhibitory postsynaptic currents

(mIPSCs) do not always require VACC activity. For instance, hair cells and retinal bipolar cells, which both use ribbon synapses, release glutamate in the absence of action potentials and hence can be considered to employ spontaneous forms of release [57–59]. At these synapses low-voltage-activated VACCs trigger glutamate release [60]. The left-shifted activation curve for these unusual L-type VACC ($V_{0.5}$= −40 mV as compared to −13 mV for N-type [61]), means that even at −40 mV 50% of the VACCs are activated. It follows that a modest depolarization can trigger ribbon synapses to release glutamate in a VACC-dependent fashion without needing the strong depolarization of an action potential [62, 63]. In the cerebellum mIPSCs are independent of VACC activity [54, 64] and may arise because of Ca^{2+} release from intracellular stores. This form of release occurs in the absence of action potentials at inhibitory and excitatory synapses [64, 65]. These characteristics are in contrast to basal spontaneous release that occurs in the absence of action potentials but also without Ca^{2+} release from internal stores and likely reflects another unidentified signaling pathway. These observations lead to several questions: what other Ca^{2+}-dependent mechanisms activate spontaneous glutamate release and how do unsynchronized VACCs trigger spontaneous vesicle fusion?

Non VACC-dependent mechanisms impacting spontaneous release

The Na^{+}-Ca^{2+} exchanger (NCX) plays a prominent role in the removal of Ca^{2+} from nerve terminals [66–68]. If extracellular Ca^{2+} enhances spontaneous fusion by promoting reverse-mode NCX activity (Na^{+} efflux, Ca^{2+} influx), then direct inhibition of NCX-mediated Ca^{2+} transport should attenuate the enhancement of spontaneous fusion by elevation of $[Ca^{2+}]_o$. KB-R7943 inhibits forward- and reverse-mode NCX currents [69, 70] and basal mEPSC frequency is substantially increased by KB-R7943, consistent with the hypothesis that NCX modulates basal $[Ca^{2+}]_i$ and spontaneous fusion through it's forward transport mechanism at these synapses [10].

Surface charge screening was proposed to mediate the reduction in mEPP frequency seen at high $[Ca^{2+}]_o$ in the presence of high $[K^{+}]$ [41, 71](but see[72]). Such an effect would oppose the Ca^{2+}-dependent increase in mEPSC frequency observed in central neurons [10]. We hypothesized that elevation of $[Ca^{2+}]_o$ reduced channel activation, and thereby increased input resistance and extended the space-clamp, enhancing mEPSC detection from distal sites. However, input resistance and time constants for membrane capacitance were unchanged upon elevation of $[Ca^{2+}]_o$ from 1.1 to 6 mM. Thus, the changes in mEPSC frequency that accompany the change in $[Ca^{2+}]_o$ were not explained by surface charge screening of the neurons and altered detection of postsynaptic events.

GPCR activation increases spontaneous vesicle fusion

Extracellular Mg^{2+} decreases VACC currents and thereby inhibits spike-evoked synaptic transmission [35, 36, 73] but also increases spontaneous glutamate release in neocortical neurons [10]. This effect is attributed to Mg^{2+}-mediated activation of the G protein-coupled Ca^{2+} sensing receptor (CaSR). At inhibitory synapses magnesium increased mIPSC frequency at 6 mM but had little further effect at higher concentrations indicating spontaneous release at inhibitory synapses may depend on VACC and CaSR activation (Fig. 1). CaSR, is activated by increases in $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ [74] and functions at central nerve terminals [75, 76]. CaSR stimulation inhibits the activity of a nonselective cation channel in nerve terminals and impairs evoked excitatory transmission in necortical neurons [77, 78]. At the same synapses multiple lines of evidence indicate that CaSR activation strongly stimulates spontaneous glutamate release [10]. Gadolinium, also a CaSR agonist and VACC blocker, increases spontaneous release of glutamate [10]. CaSR null mutants have lower mEPSC frequency rates over the Ca^{2+} concentration range to which CaSR

responds. Since spontaneous release rates at inhibitory and excitatory synapses have contrasting sensitivities to VACC blockers we asked if CaSR similarly affected spontaneous release at excitatory and inhibitory synapses. Application of cinacalcet (10 μM), an allosteric CaSR agonist, increased both mEPSC and mIPSC frequency ([10] for methods; Fig. 2). However, the response to cinacalcet was substantially faster at inhibitory synapses (Fig. 2). One explanation is that the mechanism of cinacalcet action is different at glutamatergic and GABAergic synapses. CaSR typically couples to Gq and signals by activation of phospholipase-C (PLC), which in turn hydrolyses phosphatidylinositol-(4,5)-bisphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (for review see [79]). More work needs to be done to determine if this pathway is similarly used by glutamateric and GABAergic synapses to increase spontaneous release.

We found that signaling via CaSR accounted for about 30% of basal glutamate release but it remains unknown which pathways regulate the remaining 70% of spontaneous glutamate release. Some of these mechanisms must be Ca^{2+}-sensitive since spontaneous glutamate release from $CaSR^{-/-}$ neurons is still dependent on increases in $[Ca^{2+}]_o$ [10]. Perhaps other GPCRs play a role; multiple GPCRs modulate spontaneous release [3]. Group II mGluR agonists reduce spontaneous release but have no effect on evoked release of GABA onto Purkinje cells [80]. Likewise activation of $GABA_B$ or cannabinoid receptors reduces spontaneous release in the cerebellum [54, 81]. However, cannabinoids increase spontaneous release in the dentate gyrus indicating that there is brain region-specific variability in the mechanisms of regulation of spontaneous release [82].

VACC and spontaneous release coupling

If stochastic opening of VACCs is the portal for the Ca^{2+} entry that triggers spontaneous release, the low probability of activation of VACC implies that a single channel may initiate vesicle fusion [83]. Yet at many synapses Ca entry via more than one VACC is necessary to trigger vesicle fusion [84]. Are there enough VACC opening to account for the observed levels of spontaneous release? We estimated the frequency with which VACC channels open on inhibitory terminals synapsing with a single neocortical neuron (Fr_o) using equation 1.

$$Fr_o = N_T \times Pr_I \times N_{VACC} \times P_{VACC} / T \quad (1)$$

where N_T was the number nerve terminals per neuron, Pr_I was the proportion of synapses that are inhibitory, N_{VACC} was the number of VACC per terminal, P_{VACC} was the probability that a VACC was open and T was the average channel open time at the membrane potential. Values for N_T, Pr_I, N_{VACC}, and T were obtained from the references described (Table 1). We used the time constant of VACC current deactivation at −80 mV (T) as a measure of mean channel open time. We estimated P_{VACC} from VACC currents recorded from the somata of neocortical neurons in the presence of 1.1 mM external Ca^{2+} and Mg^{2+}. The VACC currents were pharmacologically isolated and activated by a voltage ramp protocol (Fig. 2a, −85 to 40 mV, 0.5 mV/ms) which generates an I–V relationship indistinguishable from voltage-step protocols [85]. The difference current, obtained by subtraction of the current recorded in the presence of the VACC blocker Cd^{2+} (100 μM) from the current recorded without Cd^{2+}, was converted into a conductance-voltage plot (see equation 1[10]) and then fit with a Boltzmann function (Fig. 3b). We obtained the fractional voltage-dependent activation of VACC at the resting membrane potential (−78±7 mV, mean ± standard deviation)[78] by dividing the conductance at that potential by the maximal conductance from the Boltzmann fit (Fig. 3c). The average fractional voltage-dependent activation of VACC was 0.009 ± 0.003% (n=4). The estimate of the average Fr_o was more

than 100 times greater than the average measured mIPSC frequency (513 s^{-1} versus 4.4 s^{-1}; Table 1) indicating that there are far more VACC openings than vesicle fusion events at inhibitory synapses.

If more than one VACC is required to trigger spontaneous fusion then what does this mean for the cell? An important physiological consequence of this would be a reduction in the basal rate of spontaneous GABA release due to random channel openings and therefore an increased signal-to-noise ratio at the synapse.

Asynchronous release – more than one mechanism

The delayed or asynchronous release of neurotransmitter following an action potential has been attributed to two distinct mechanisms. The more parsimonious view is that within a nerve terminal all vesicles employ the same core synaptic machinery and that the variable pattern of asynchronous release arises from collapse of Ca^{2+} microdomains, binding to slow Ca^{2+} buffers and Ca^{2+} extrusion [1]. Variability between synapses in the degree of asynchronous release could thus reflect differences in Ca^{2+} and vesicle handling [86]. Looser VACC-vesicle coupling leads to greater asynchronous release [5]. However, reduction of the slow endogenous Ca^{2+} buffer, parvalbumin, does not reduce asynchronous release as predicted [87, 88] indicating that the coupling between action potentials and asynchronous release may be complex. The other view is that there are distinct signaling pathways that govern asynchronous and synchronous release. Multiple lines of evidence support this hypothesis. The disruption of synchronous but not asynchronous release by deletion of synaptotagmin 1 in hippocampal neurons is an early demonstration that asynchronous and synchronous release do not share intracelleular Ca^{2+} sensors [89]. Similarly, at the calyx of Held synchronous release, but not asynchronous, is disrupted by synaptotagmin 2 deletion leading to the proposal that synapses that demonstrate asynchronous release possess two Ca^{2+} sensors [15]. Doc2 binds to target SNAREs in a Ca^{2+}-dependent manner and when knocked-down substantially reduces asynchronous release, supporting the hypothesis that Doc2 is the asynchronous Ca^{2+} sensor in hippocampal neurons [8]. In addition to distinct Ca^{2+} sensors it is emerging that vesicle pools involved in synchronous and asynchronous release may be distinctly labelled by synaptobrevin2 and VAMP4 respectively [6, 90]. Intriguingly, P/Q- and N-type VACCs, which trigger evoked release, will also activate sustained calcium entry following prolonged activation. This “asynchronous” Ca^{2+} current decays over hundreds of milliseconds, and may directly trigger asynchronous release [91]. TRPV1, the Ca^{2+} permeable non-selective cation channel, is another ion channel that may evoke asynchronous release but unlike P/Q- and N-type VACCs TRPV1 will not trigger synchronous release [6]. Below we discuss new research on the role of TRPV1 in glutamate release.

TRPV1 triggers asynchronous release

Second order neurons in the solitary tract nucleus (NTS) are innervated by either unmyelinated (C-type) or myelinated (A-type) afferents. C-type ST afferents comprise 80–90% of the total afferents innervating the NTS [92, 93].These ST afferents are the obligatory first stage of pathways regulating homeostasis involving the cardiovascular, respiratory and gastrointestinal systems [94, 95]. Recent work has shown that these afferents can be distinguished by their expression of TRPV1 and by the presence of asynchronous release [6, 95–97].

In both ST afferents N-type Ca^{2+} channels are the dominant Ca^{2+} portal for glutamate release during all evoked synchronous EPSCs, which are electrophysiologically indistinguishable [6, 98]. However, only C-type afferent terminals of the ST express the Ca^{2+}-permeable Transient Receptor Potential Vanilloid Type 1 (TRPV1) receptor [96, 99–

101]. TRPV1 selective agonist capsaicin (CAP) depolarizes C-type afferents and, at central ST terminals, activates glutamate release [6, 99]. With sustained exposure, CAP blocks EPSCs evoked by ST shocks – a finding consistent with depolarization-induced block of action potentials [102].

TRPV1+ and TRPV1− fibers show similar synchronized release

Detailed studies of ST-evoked EPSCs indicate that the synaptic transmission process is remarkably reliable with minimal variation in latency across repeated trials (jitter). These synapses use monosynaptic transmission to evoke highly synchronous release of glutamate (Figure 4) that is indistinguishable for TRPV1+ and TRPV1− ST afferents [6, 99, 103]. The probability of glutamate release estimated from variance-mean analysis of ST-evoked transmission averages ~90% in 2 mM Ca^{2+} and failures to ST shocks averaged well less than 1% [104]. High frequency activation produces substantial but similar frequency dependent depression in both subtypes. Together such observations suggest that action potentials in both myelinated and unmyelinated afferents evoke a similar release process using a similar readily releasable pool (RRP) of docked vesicles with a high release probability. Changes in external Ca^{2+} modify the probability of release in a relation well-fit by parabolic functions that are similar across TRPV1+ and TRPV1− ST cells [103–105]. Unitary afferent-evoked EPSCs reflect an average of about 20 release sites[105]. Together these data indicate a surprisingly uniform process regulating the evoked RRP despite substantial differences in excitability between these two classes of afferents [101]. Notwithstanding this similarity in synchronous release, the rate of spontaneous release from TRPV1+ ST afferents greatly exceeds that of TRPV1− ST afferents.

TRPV1 links vesicles to a pool specific to asynchronous release

Spontaneous release from TRPV1+ neurons of the ST generate roughly 10x greater rates of mEPSCs than from TRPV1− neurons [101] (Figure 4). Thus despite similar evoked synchronous release processes, the regulation of spontaneous release differs substantially. In TRPV1+ neurons, but not TRPV1−, CAP dramatically increases spontaneous release of glutamate [100] and bursts of ST shocks evoke substantial asynchronous EPSCs that extended for 1–3 seconds after the synchronized EPSCs (Figure 4). Accordingly, neurons in which ST activation failed to trigger asynchronous EPSCs were also completely unresponsive to CAP. Interestingly, the threshold shock intensity for triggering the ST-synced EPSCs was identical to that needed to trigger asynchronous EPSCs, and asynchronous release increased in proportion to EPSC magnitude, the number of ST shocks and the number of active ST inputs [6, 96]. This suggests that both types of release arise from the same afferent axon. However, during prolonged, high frequency stimulation, when synchronous release was depressed, asynchronous release was unchanged. Additionally, TRPV1-selective antagonists decreased the asynchronous frequency but did not affect the synchronous activity. These data support the novel hypothesis that there is a TRPV1-linked pool of vesicles responsible for asynchronous but not synchronous release.

Thermal modulation of TRPV1 operated glutamate release

We had shown that TRPV1 agonists increase mEPSC frequency and TRPV1 antagonists decrease mEPSC frequency. Therefore, we hypothesized that Ca^{2+} ions entering through TPRV1 modulate spontaneous glutamate release. However, we did not know what gates TRPV1 in this system. We hypothesized that ambient temperature activates the temperature-sensitive TRPV1 and supplies a unique source of tonic Ca^{2+} entry to regulate spontaneous glutamate release. We found that changes in temperature between 30 and 39° C rapidly altered ST mEPSC rates while the amplitude of synchronized EPSCs showed little change [6]. Although VACCs contribute to release triggered by CAP [100], CAP- or temperature-

triggered increases in mEPSC frequency persist in the presence of the VACC blocker cadmium [97, 100, 106]. Together these observations support the hypothesis that TRPV1 operates spontaneous release from a pool of vesicles that is independent of VACCs and the release mechanisms that trigger evoked synchronous EPSCs. This TRPV1-mediated release mechanism is also uniquely sensitive to changes in temperature.

Physiological implications of TRPV1-mediated release

Recent investigations of a model of sudden infant death syndrome (SIDS) demonstrated that modest temperature elevation (+2° C, a major risk factor in SIDS) of the medial region of caudal NTS prolongs the activation of the laryngeal chemoreflex[107]. This reflex is C-fiber dependent and regulates patterned breathing and can lead to profound apnea. Excitingly, this apnea was relieved by application of TRPV1 antagonist to NTS [108, 109]. Such observations suggest that the full implications of the TRPV1 regulation of glutamate release may be most pronounced in pathological states and may affect homeostatic regulation such as cardiorespiratory control mechanisms.

Conclusions

Increasingly, experimental data point to differences between the regulation of the core exocytotic machinery for synchronous, asynchronous, and spontaneous release. Here we discussed recent work identifying how Ca^{2+} differentially regulates these various types of release via different synaptic proteins. CaSR is a major regulator of spontaneous release at both excitatory and inhibitory synapses. VACCs trigger spontaneous and synchronous release at inhibitory synapses, whereas at excitatory synapses Ca^{2+} influx triggers only synchronous release. And finally, Ca^{2+} entry via TRPV1 is an important trigger for asynchronous, but not synchronous release. It appears likely that these different forms of release are regulated differently and serve diverse physiological roles.

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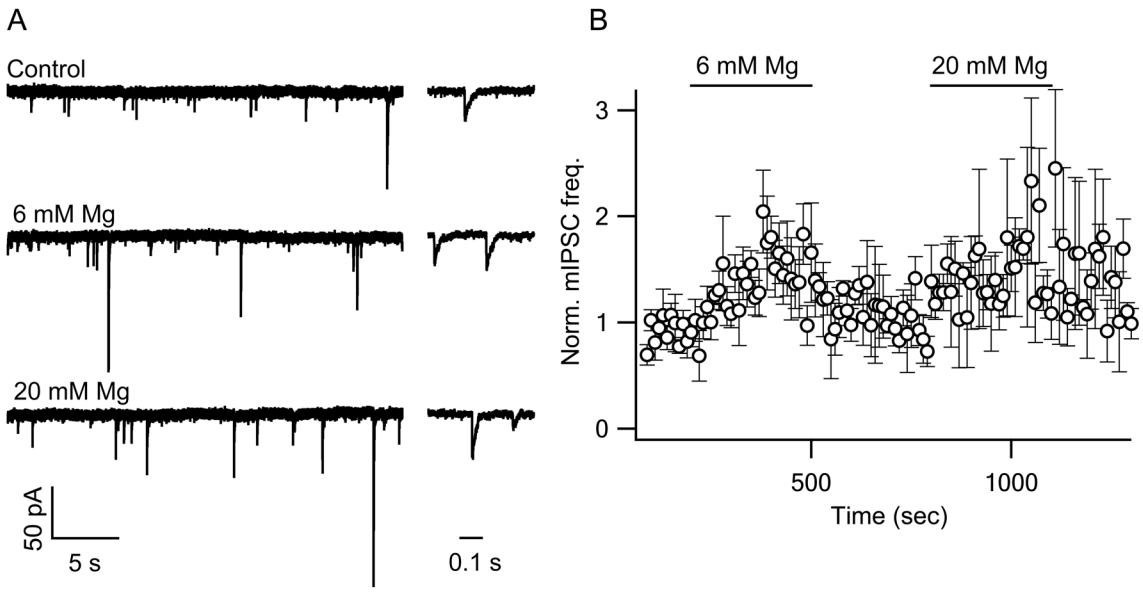

Figure 1. Magnesium reversibly increases mIPSC frequency

a, Representative traces (left) with expanded time course (right) showing the effect of step changes in $[Mg^{2+}]_o$ on mIPSC frequency. b, normalized average diary plot showing that that subsequent step changes in $[Mg^{2+}]_o$ from 1.1 mM to 6 and 20 mM reversibly increase mIPSC frequency (n=3).

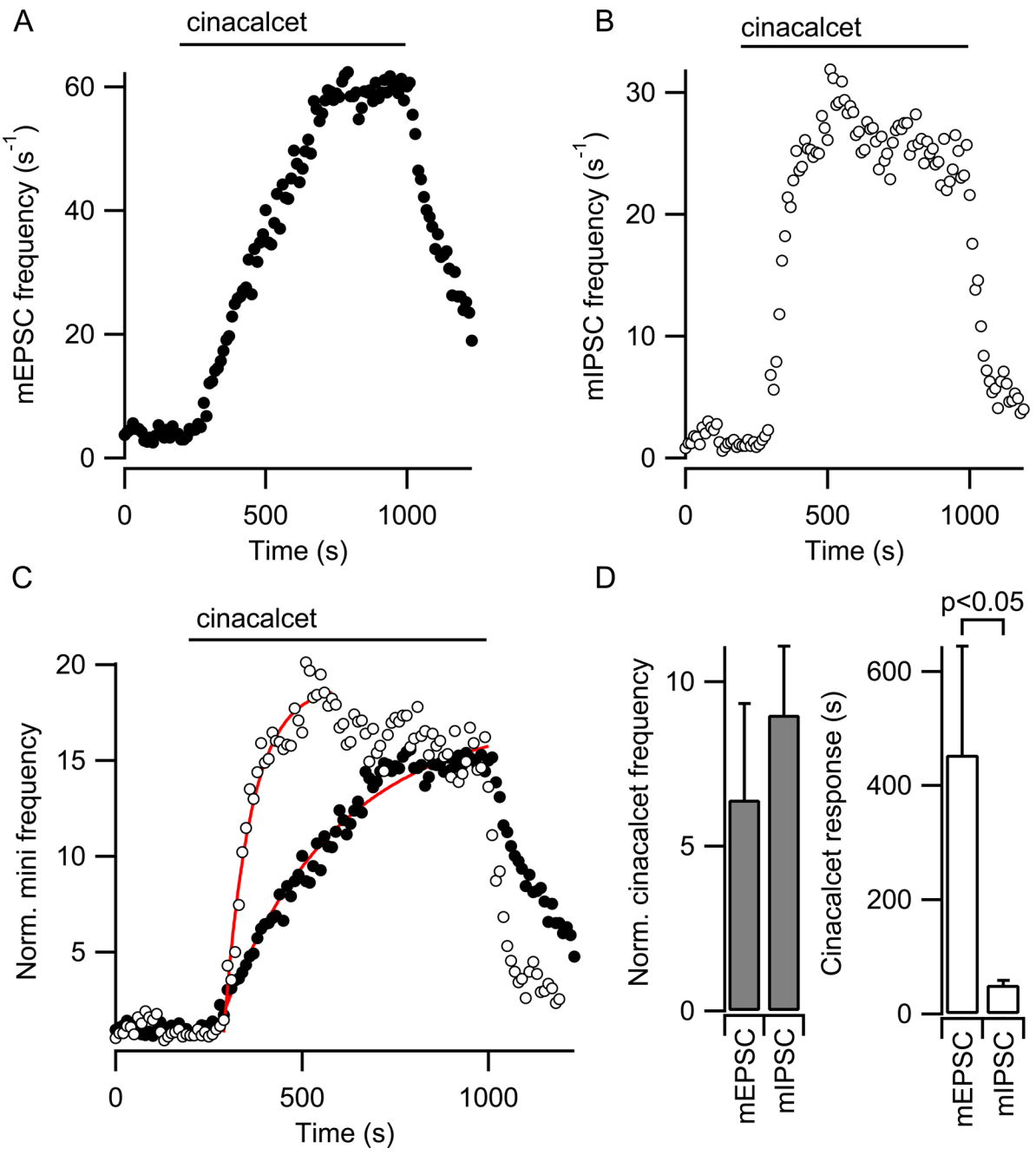

Figure 2. CaSR activation enhances spontaneous release of glutamate and GABA

a, example normalized diary plot of mEPSC frequency versus time recorded in 1 μM TTX and 10 μM CNQX showing that cinaclacet (10 μM) reversibly increases the rate of spontaneous glutamate release. b, example normalized diary plot of mIPSC frequeny versus time recorded in 1 μM TTX and 10 μM bicuculine showing that cinacalcet (10 μM) reversibly increases spontaneous release of GABA. c, Overlay of exemplar normalized diary plots from a (closed circles) and b (open circles) illustrate rate of cinacalcet action on mEPSC and mIPSC. Red curves describe exponential fits to data. d, Histograms of maximum normalized frequency response (gray bars) and time constant of response (open

bars) to cinacalcet (normalized to 200 s before application) for mEPSC (n=4) and mIPSC (n=5).

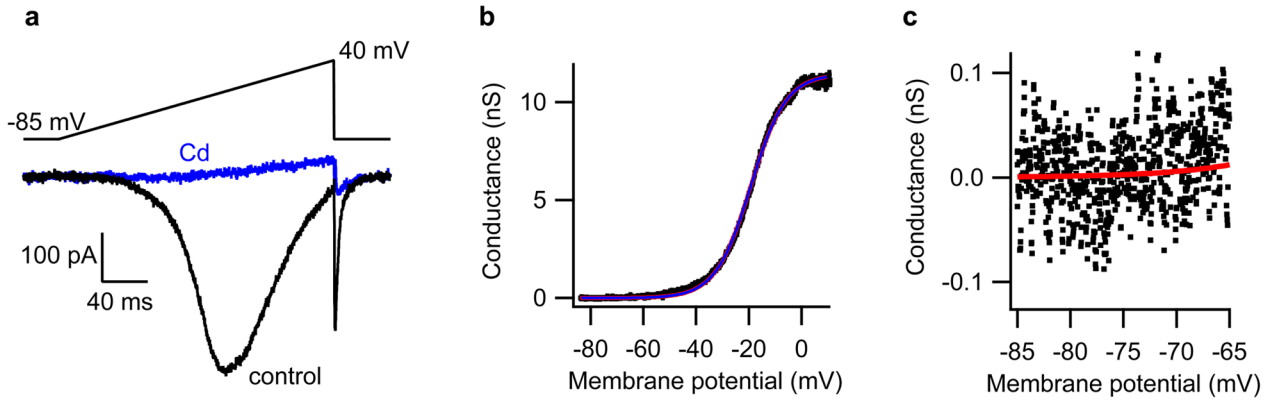

Figure 3. The voltage-dependent activation and inactivation of VACC currents

a, Representative recording of VACC currents activated by voltage ramp depolarization (−85 to 40 mV at 0.5mV/ms, upper panel) in the absence (black trace) and presence of 100 μM Cd^{2+} (blue) in neocortical cultured neurons. Traces are averages of at three consecutive sweeps. Currents remaining in 100 μM Cd^{2+} were subtracted from the average traces. b, Plot of conductance versus membrane potential for Cd^{2+}-sensitive current in a. The red curve represents best fit of the Boltzmann function to the data ($G = 1/ (1 - \exp^{(V1/2 - V)/k})$) where $V_{1/2}$ is half-maximal activation voltage, and k is the slope factor. Values for this recording were −16 mV and 5.4 mV respectively. c, Expansion of b to indicate the conductance at resting voltages.

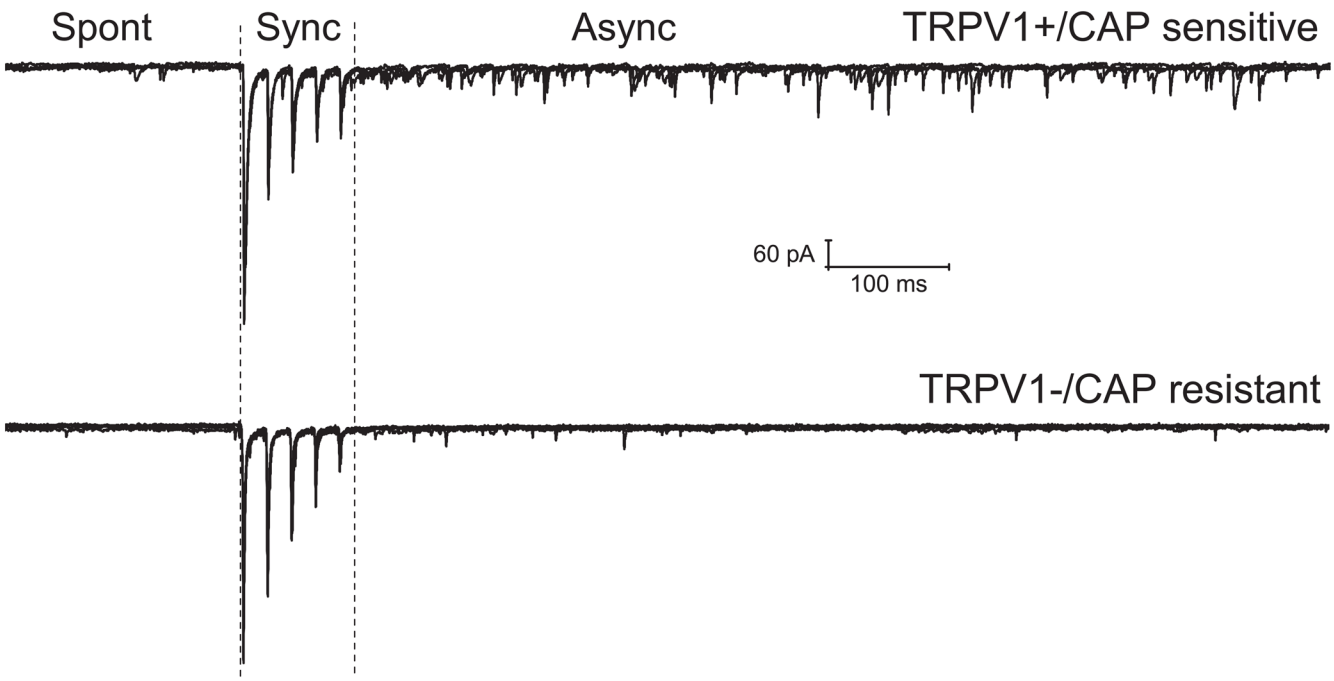

Figure 4. Synchronous and asynchronous vesicle pools are kinetically distinct

Representative traces show spontaneous, synchronous, and asynchronous events in C-type (top) sensitive to the TRPV1 agonist capsaicin (CAP-sensitive, not shown) and a neuron receiving myelinated afferents, TRPV1− (bottom, CAP-resistant) recorded from neurons in acute rat NTS slice preparations. Synchronous response was evoked with bursts of five stimuli at 50 Hz and showed similar depression in amplitude in both phenotypes of ST afferent, TRPV1+ −. TRPV1+ afferents show elevated spontaneous release and the asynchronous responses following the burst stimulus compared to TRPV1− afferents. Four individual sweeps from each recording are superimposed in each trace.

Table 1

Values used to estimate frequency of VACC openings at the resting mebrane potential at inhibitory terminals. We estimated lower and upper limits for Fr_o using minimum and maximum published values and estimates of P_{VACC} at −85 and −71 mV. These voltages represent the mean ± one standard deviation for the membrane potential of these neurons

	Average	Min	Max	References
N_T	152	63	569	[110]
Pr_I	0.31	0.27	0.35	[111, 112]
N_{VACC}	21	6	36	[113, 114]
P_{VACC}	9.3×10^{-5} @−78 mV	3.9×10^{-5} @−85 mV	2.2×10^{-4} @−71 mV	Fig. 3
T (μs)	180	160	200	[113, 115]
Fr_o (s^{-1})	**513**	**20**	**9992**	
mIPSC frequency (s^{-1})	**4.4 ± 0.6; n=67**	**0.1**	**22.3**	