Coactivation of multiple tightly coupled calcium channels triggers spontaneous release of GABA

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Voltage-activated Ca2+ channels (VACCs) mediate Ca2+ influx to trigger action potential–evoked neurotransmitter release, but the mechanism by which Ca2+ regulates spontaneous transmission is unclear. We found that VACCs are the major physiological triggers for spontaneous release at mouse neocortical inhibitory synapses. Moreover, despite the absence of a synchronizing action potential, we found that spontaneous fusion of a GABA-containing vesicle required the activation of multiple tightly coupled VACCs of variable type.

Spontaneous and evoked neurotransmission, two forms of interneuronal communication, have been proposed to rely on different signaling mechanisms1 and to mediate physiologically distinct functions2. It is well-established that presynaptic action potentials activate VACCs, triggering Ca2+ influx and synchronous release of neurotransmitter, but the manner in which the external Ca2+ concentration ([Ca2+]o) is coupled to spontaneous release remains controversial. Although increasing [Ca2+]o enhances spontaneous neurotransmission at excitatory neocortical synapses, blocking VACCs or buffering the intracellular Ca2+ concentration ([Ca2+]i) has no effect on spontaneous release3. In contrast, mutation of Ca2+ sensors, such as synaptotagmin-1, at GABAergic cortical synapses affects spontaneous release, suggesting a major role for Ca2+ influx4. We found that, in contrast with regulation of excitatory synapses, spontaneous release from inhibitory synapses is dependent on VACCs and that single vesicle fusion requires coincident activation of multiple closely packed VACCs.

We examined the manner in which [Ca2+]o is coupled to spontaneous release of GABA by recording miniature inhibitory postsynaptic currents (mIPSCs) in cultured neocortical neurons. Changing [Ca2+]o from 1.1 mM altered mIPSC frequency in a reversible and concentration-dependent manner (n = 6; Fig. 1a–c). The steepness of the concentration-effect relationship was much lower (slope = 0.45) than that of evoked release5, but similar to that for miniature excitatory postsynaptic currents3. Application of Cd2+ (100 µM), a VACC blocker, with a [Ca2+]o of 1.1 mM reduced mIPSC frequency by 56 ± 7% (n = 6) from the basal level (Fig. 1d). At 6 mM [Ca2+]o, mIPSC frequency increased by 185 ± 46% of the basal level and was relatively reduced by 56 ± 8% following Cd2+ application. These data indicate that VACCs trigger spontaneous GABA release at both physiological and elevated [Ca2+]o. Stochastic VACC activity should be decreased by presynaptic hyperpolarization. Hyperpolarizing the nerve terminals by reducing the external K+ concentration from 4 to 1 mM reversibly reduced mIPSC frequency by 34 ± 8% (n = 6; Supplementary Fig. 1), but had little effect when VACCs were blocked by Cd2+. These data suggest that, in contrast with glutamatergic excitatory synapses3, VACCs regulate spontaneous GABA release at neocortical synapses.

Evoked GABA release is regulated by VACC subtypes at cortical synapses, in the order P/Q > N > R (ref. 6). To determine which VACCs regulate spontaneous GABA release, we applied specific channel-type blockers while recording mIPSCs. Blockade of N-type channels

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Received 1 February; accepted 19 June; published online 29 July 2012; doi:10.1038/nn.3162
with a saturating concentration\(^7\) of \(\omega\)-conotoxin-GVIA (GVIA, 1 \(\mu\)M) reduced mIPSC frequency by 32 \(\pm\) 7\% (\(n = 9\); Fig. 2a,b). Subsequent addition of a saturating concentration\(^8\) of the P/Q-type blocker \(\omega\)-Aga-toxin-Iva (Aga-Iva, 300 nM) reduced mIPSC frequency by 11 \(\pm\) 4\%, suggesting that N-type VACCs contribute more to GABA release than P/Q-type VACCs (\(P = 0.015\); Fig. 2a,b). The addition of Cd\(^2+\) reduced mIPSC frequency a further 23 \(\pm\) 8\% (Fig. 2a,b), indicating that either L- or R-type VACCs contribute to GABA release. On average, 33 \(\pm\) 6\% of mIPSCs were Cd\(^2+\) resistant, suggesting that other regulatory mechanisms also contribute to spontaneous GABA release\(^3\). Taken together, these data indicate that P/Q- and N-type VACCs are important triggers of mIPSCs.

It has been suggested that the simultaneous activation of multiple VACCs is necessary at most synapses to trigger single vesicle fusion during evoked release, whereas it has been proposed that spontaneous release results from the activation of a single VACC\(^9\)\(^\,\,11\). If each fusion event depends on the opening of a single channel, the effects of blocking P/Q- or N-type VACCs with slowly dissociating toxins should be independent of each other. Conversely, if multiple channels are involved, cooperativity should result in proportionately smaller reductions in release probability as the total fraction of VACC blocked is increased\(^13\). Consistent with multiple channel involvement, the relative effectiveness of 300 nM Aga-Iva and 1 \(\mu\)M GVIA was reversed when the order of toxin application was switched (Aga-Iva, 32 \(\pm\) 7\%; GVIA, 3 \(\pm\) 3\%; \(P = 0.050\); Fig. 2c,d). This was not a result of interneuronal variability in the proportion of mIPSCs independent of VACCs, as the reversal of the apparent effectiveness of GVIA and Aga-Iva was also evident when we compared the toxin’s actions on the Cd\(^2+\)-sensitive fraction (Fig. 2e). In other words, GVIA and Aga-Iva were more effective at reducing mIPSC frequency when the neuron had not already been exposed to saturating doses of the other blocker (GVIA, \(P = 0.008\); Aga-Iva, \(P = 0.018\); Fig. 2e). At higher doses, Aga-Iva cross-reacts with N-type channels\(^11\). To determine whether cross-reactivity was responsible for the reduced effects of the second toxin applications on mIPSC frequency, we directly measured VACC current block in these neurons. The percentages of the total VACC currents carried by 1 \(\mu\)M GVIA- and 300 nM Aga-Iva-sensitive fractions were unchanged by the order of toxin application (Supplementary Fig. 2), suggesting that cross-reactivity was not responsible for the

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**Figure 2** Pharmacological dissection of the identity of VACCs mediating spontaneous GABA release. (a) Plot of mIPSC frequency versus time from an exemplary experiment indicating the effects of GVIA (1 \(\mu\)M), Aga-Iva (300 nM) and Cd\(^2+\) (100 \(\mu\)M). (b) Histogram indicating average effects on mIPSC frequency of application of Aga-Iva, GVIA and Cd\(^2+\). (c) Plot of mIPSC frequency versus time from an exemplary experiment indicating effects of Aga-Iva, GVIA and Cd\(^2+\). (d) Histogram showing the average effects on normalized mIPSC frequency of application of Aga-Iva, GVIA and Cd\(^2+\). (e) Effect of varying the order of application of Aga-Iva and GVIA on the Cd\(^2+\)-sensitive fraction of mIPSC frequency. The second application was always in the presence of a saturating dose of the other blocker at steady state. The frequencies of mIPSCs were reduced differently by the first and second applications of GVIA (first, 48 \(\pm\) 8\%; second, 5 \(\pm\) 5\%, \(n = 4\); \(P = 0.008\)) and Aga-Iva (first, 51 \(\pm\) 9\%; second, 19 \(\pm\) 6\%, \(n = 9\); \(P = 0.018\)). * \(P < 0.05\) and ** \(P < 0.01\). Error bars indicate s.e.m. in all panels.

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**Figure 3** VACC-vesicle coupling is attenuated by BAPTA-AM, but not by EGTA-AM. (a) Application of 50 \(\mu\)M BAPTA-AM (\(n = 9\)), but not 50 \(\mu\)M EGTA-AM (\(n = 6\)), decreased the response to 6 mM Ca\(^2+\). Shown is a plot of normalized average mIPSC frequency versus time. The thick black line indicates chelator application. (b,c) Histograms of steady-state mIPSC frequency for the experiments in a in 1.1 or 6 mM Ca\(^2+\) and in the absence and presence of BAPTA-AM (b) or EGTA-AM (c). * \(P < 0.05\), ** \(P < 0.01\) and *** \(P < 0.001\); n.s., not significant (\(P > 0.05\)). (d) Histogram of normalized steady-state mIPSC frequency (200–500 s) in either BAPTA-AM (50 \(\mu\)M, \(n = 14\)) or EGTA-AM (50 \(\mu\)M, \(n = 17\)). Cd\(^2+\) inhibition of the mIPSC frequency following application of BAPTA-AM (\(n = 7\)) or EGTA-AM (\(n = 7\)) exposure is also shown. (e) Plot of normalized average mIPSC frequency versus time for the experiments in d showing the affect of BAPTA-AM (open circles) and EGTA-AM (filled circles) on basal mIPSC frequency. The broken red lines indicate the average mIPSC frequency between 100 and 400 s of chelator application. (f) Plot of normalized average mIPSC frequency showing the action of Cd\(^2+\) after 400 s of chelator application and 100-s wash for the experiments in e. Error bars indicate s.e.m. in all panels.
reduced effect of the second toxin application (Fig. 2). Collectively, these data indicate that each fusion event is dependent on multiple VACCs and that different VACC types cooperate to trigger fusion of a single vesicle.

How close are these VACCs to the vesicle? One hypothesis is that VACCs are not tightly associated with vesicles, but cooperate to raise bulk \([\text{Ca}^{2+}]_i\), which increases mIPSC frequency. BAPTA and EGTA have similar affinities for Ca\(^{2+}\), but BAPTA has a ~40-fold faster rate of binding so that, at millimolar concentrations, BAPTA will affect signaling if the mean diffusion distance for Ca\(^{2+}\) is as short as 10–20 nm, whereas EGTA will only have an effect if the path length is relatively long (>100 nm)\(^{11}\). Application of cell-permeant BAPTA-AM (50 \(\mu\)M, 600 s) substantially reduced mIPSC frequency \((P = 0.007, n = 8\); Fig. 3a,b), indicating that this Ca\(^{2+}\) buffer attenuated the \([\text{Ca}^{2+}]_i\) transient when \([\text{Ca}^{2+}]_o\) was 6 mM. In contrast, EGTA-AM (50 \(\mu\)M, 600 s) did not change the response to increases in \([\text{Ca}^{2+}]_o\) \((P = 0.391, n = 6\); Fig. 3a,c and Supplementary Fig. 3). At physiological \([\text{Ca}^{2+}]_o\), BAPTA reduced mIPSC frequency by 24 ± 7\% \((n = 14)\), whereas EGTA was ineffective \((1 ± 6\%, n = 16, P = 0.009\); Fig. 3d,e). Application of Cd\(^{2+}\) after BAPTA exposure reduced mIPSC frequency further \((65 ± 7\%, n = 7\); Fig. 3d,f and Supplementary Fig. 3). Given that the final intracellular concentrations of the two buffers are likely to have been similar, these data strongly indicate that VACCs trigger spontaneous GABA release via tightly coupled vesicles and not by changing bulk \([\text{Ca}^{2+}]_o\)\(^{11,14}\). Notably, action potential–evoked release of a single GABA-containing vesicle relies on a vesicle-VACC coupling distance of 10–20 nm and activation of up to three VACCs\(^{10}\).

An important physiological consequence of the requirement that multiple VACC openings combine to trigger each mIPSC will be a lower basal rate of spontaneous GABA release resulting from the low probability of coincident VACC openings. The mechanism synchronizing the activation of multiple VACCs remains unclear. On the basis of somatic VACC currents, stochastic synchronization seems unlikely, although it cannot be ruled out, as the membrane potential \((-78 ± 2 \text{ mV}, n = 17)\) sits at the foot of the VACC current activation curve (Supplementary Fig. 4). Another possibility is that nerve terminal VACCs are linked via their C termini, leading to coupled gating similar to the mechanism proposed to synchronize L-type VACC activity\(^{15}\). The importance of VACCs as triggers for spontaneous GABA release is surprising in the light of our earlier findings that spontaneous glutamate release is not initiated by Ca\(^{2+}\) influx\(^{3}\), and indicates a substantial difference between the regulation of GABAergic and glutamatergic synapses in the neocortex. Further research is required to identify the constituents at the active zone responsible for the differential regulation of inhibitory and excitatory spontaneous release and to determine whether this phenomenon extends to other regions of the brain.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank M. Andreassen and K. Khodakhah for helpful comments. The work was supported by the US National Institutes of Health (DA027110 and GM097433) and OCTRI. C.W. and N.P.V. were supported by a grant from the National Heart, Lung, and Blood Institute (T32HL033808).

AUTHOR CONTRIBUTIONS

C.W. conducted the calcium chelation, specific toxin block of mIPSC and VACC currents, and VACC gating experiments, and helped write the manuscript. W.C. conducted the calcium chelation, Cd\(^{2+}\) and specific toxin block of mIPSC and VACC currents, and hyperpolarization experiments. C.-H.L. conducted the calcium concentration effect experiments. D.Y. conducted the Cd\(^{2+}\) block of mIPSC and hyperpolarization experiments. N.P.V. provided cell cultures and helped write the manuscript. S.M.S. designed the experiments, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Neuronal cell culture. Neocortical neurons were isolated from postnatal day 1–2 mouse pups as described previously\(^{16}\). All animal procedures were approved by Oregon Health and Science University’s Institutional Animal Care and Use Committee in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were decapitated following general anesthetic with isoflurane and the cerebral cortices were removed. Cortices were incubated in trypsin and DNase and then dissociated with a heat-polished pipette. Dissociated cells were cultured in MEM plus 5% fetal bovine serum (vol/vol) on glass coverslips. Cytosine arabinoside (4 µM) was added 48 h after plating to limit glial division. Cells were used after a minimum of 14 d in culture.

Electrophysiological recordings. Cells were visualized with an Olympus IX70 inverted microscope. Recordings were made in whole-cell voltage-clamp mode in neurons voltage-clamped at −70 mV. Voltages were corrected for liquid junction potentials\(^{17}\). In general and except where stated in the text, extracellular solution contained 150 mM NaCl, 4 KCl, 10 mM HEPES, 10 mM glucose and 1.1 mM MgCl\(_2\) (pH 7.33 with NaOH). CaCl\(_2\) was 1.1 mM unless otherwise indicated. Recordings of mIPSCs were made in the presence of tetrodotoxin (1 µM) and CNQX (10 µM) to block Na\(^+\) channels and AMPA receptors, respectively. Recordings of mIPSCs were made using a potassium chloride–rich intracellular solution containing 118 mM KCl, 9 mM EGTA, 10 mM HEPES, 4 mM MgCl\(_2\), 1 mM CaCl\(_2\), 4 mM NaATP, 0.3 mM NaGTP and 14 mM creatine phosphate (pH 7.2 with KOH). Electrodes had resistances of 3–7 MΩ. VACC currents were isolated using cesium methane sulfonate–rich solution as described previously\(^3\). Currents were recorded with a HEKA EPC9/2 amplifier and filtered at 1 kHz using a Bessel filter and sampled at 10 kHz. Series resistance (\(R_s\)) was monitored and recordings were discarded if \(R_s\) changed significantly during the course of a recording. \(R_s\) was compensated to −70% in recordings of VACC currents.

Solution application. Solutions were gravity fed through a glass capillary (1.2-mm outer diameter) placed ~1 mm from the patch pipette tip. Toxin (Alomone Labs) stock solutions were all made at 1,000× concentration with distilled water and stored at ~20 °C. Cytochrome C (0.1 mg ml\(^{-1}\)) was also added to Aga-I\(\alpha\)- containing solutions to minimize nonselective toxin binding to the apparatus. BAPTA-AM (Invitrogen) was dissolved in DMSO at 50 mM stock concentration. Before use, extracellular solution was incubated at 30 °C while undergoing ultrasonic agitation for ≥30 min to ensure BAPTA-AM dissolved completely. EGTA-AM (Invitrogen) was dissolved in DMSO at 50 mM stock concentration.

Analysis. Data were acquired on a desktop computer and analyzed with IgorPro (WaveMetrics) and Minianalysis (Synaptosoft) software using a threshold-crossing algorithm. mIPSC data were normalized to the basal level by dividing the mIPSC frequency measured over each 10-s interval by the average mIPSC frequency over 100–200 s at the beginning of the experiment. Steady-state mIPSC frequency changes were the averages measured over ≥60 s as a percentage of the basal level. In some experiments (Fig. 2e), reductions in mIPSC frequency were described as a percentage of the Cd\(^{2+}\)-sensitive component by comparing the response to blockers as a fraction of the difference between the basal mIPSC rate and the mIPSC rate following application of Cd\(^{2+}\) (100 µM). Exemplar plots of mIPSC frequency versus time are shown (Supplementary Fig. 4) to illustrate the variability of basal mIPSC frequency which presumably reflect differences in the number of synapses and release probability. The average basal mIPSC frequency was 5.3 ± 0.6 s\(^{-1}\) (n = 61).

Statistical analysis. Data values are reported as mean ± s.e.m. Pairwise comparison of data were performed using Student’s t test or Mann-Whitney U test if the data were not normally distributed (Microsoft Excel or Sigmaplot). We used two-way repeated-measures ANOVA to examine the effect of BAPTA-AM and EGTA-AM on the mIPSC frequency at different external calcium levels. Subsequent pairwise comparisons were performed with the Holm-Sidak method (Sigmaplot). Curve fitting was carried out using IgorPro (WaveMetrics).

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