Single Calcium Channel Nanodomains Drive Presynaptic Calcium Entry at Lamprey Reticulospinal Presynaptic Terminals

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Efficient and reliable neurotransmission requires precise coupling between action potentials (APs), Ca2+ entry and neurotransmitter release. However, Ca2+ requirements for release, including the number of channels required, their subtypes, and their location with respect to primed vesicles, remain to be precisely defined for central synapses. Indeed, Ca2+ entry may occur through small numbers or even single open Ca2+ channels, but these questions remain largely unexplored in simple active zone (AZ) synapses common in the nervous system, and key to addressing Ca2+ channel and synaptic dysfunction underlying numerous neurologic and neuropsychiatric disorders. Here, we present single channel analysis of evoked AZ Ca2+ entry, using cell-attached patch clamp and lattice light-sheet microscopy (LLSM), resolving small channel numbers evoking Ca2+ entry following depolarization, at single AZs in individual central lamprey reticulospinal presynaptic terminals from male and females. We show a small pool (mean of 23) of Ca2+ channels at each terminal, comprising N-(CaV2.2), P/Q-(CaV2.1), and R-(CaV2.3) subtypes, available to gate neurotransmitter release. Significantly, of this pool only one to seven channels (mean of 4) open on depolarization. High temporal fidelity lattice light-sheets imaging reveals AP-evoked Ca2+ transients exhibiting quantal amplitude variations of 0–6 event sizes between individual APs and stochastic variation of precise locations of Ca2+ entry within the AZ. Further, total Ca2+ channel numbers at each AZ correlate to the number of presynaptic primed synaptic vesicles. Dispersion of channel openings across the AZ and the similar number of primed vesicles and channels indicate that Ca2+ entry via as few as one channel may trigger neurotransmitter release.

Key words: active zone; nanodomain; neurotransmitter release; presynaptic; synaptic vesicle fusion; voltage-gated calcium channel

Significance Statement

Presynaptic Ca2+ entry through voltage-gated calcium channels (VGCCs) causes neurotransmitter release. To understand neurotransmission, its modulation, and plasticity, we must quantify Ca2+ entry and its relationship to vesicle fusion. This requires direct recordings from active zones (AZs), previously possible only at calyceal terminals containing many AZs, where few channels open following action potentials (APs; Sheng et al., 2012), and even single channel openings may trigger release (Stanley, 1991, 1993). However, recording from more conventional terminals with single AZs commonly found centrally has thus far been impossible. We addressed this by cell-attached recordings from acutely dissociated single lamprey giant axon AZs, and by lattice light sheet microscopy of presynaptic Ca2+ entry. We demonstrate nanodomains of presynaptic VGCCs coupling with primed vesicles with 1:1 stoichiometry.

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Introduction

Precise and reliable synaptic transmission requires rapid evoked synchronous neurotransmitter release caused by a transient depolarization-gated, voltage-gated calcium channels (VGCCs) mediated, increase in presynaptic Ca2+ (Katz and Miledi, 1967). To ensure fidelity of signaling and speed, this increase is in close spatial proximity to the release site and occurs with synaptic delays in the order of 60 μs (Sabatini and Regehr, 1996). Rapid and sequentially precise timing are critical between each signal, including onset of an action potential (AP), VGCC opening, Ca2+ binding to Synaptotagmin, and subsequent molecular
processes of vesicle fusion, leading to neurotransmitter release. Before fusion, vesicles dock by localizing to the active zone (AZ) membrane, and then prime by forming ternary SNARE structures at their site of fusion. During these processes, VGCCs tether or are tethered to the fusion machinery (Kaeser et al., 2011; Wong et al., 2014; Chen et al., 2017). This tethering ensures that Ca\(^{2+}\) enters very closely to its target to evoke release. Nevertheless, stoichiometry of opening VGCCs to primed vesicles remains undetermined, although this stoichiometry and the spatial relationships between VGCCs and their targets at the fusion machinery are vitally important to how synaptic transmission occurs (Stanley, 2016) and how it is subject to modulation and plasticity (Catterall and Few, 2008). Thus, it is imperative to precisely determine how much Ca\(^{2+}\) is required to trigger vesicle fusion and release, how many channels open and where they are located with respect to the vesicle fusion apparatus.

Two contrasting models have been proposed to explain the Ca\(^{2+}\) requirement for release. The microdomain model, wherein Ca\(^{2+}\) influx through the simultaneous opening of multiple clustered VGCCs, leads to μM [Ca\(^{2+}\)] in the terminal triggering release (Llinás et al., 1995). In contrast is the nanodomain model, where Ca\(^{2+}\) entry via few, possibly even a single open channel, could gate release (Stanley, 1993; Brandt et al., 2005; Gentile and Stanley, 2005; Shahrezaei et al., 2006; Bucurenciu et al., 2010). Ca\(^{2+}\) dye signals provide only indirect measurements of Ca\(^{2+}\) entry at the spatial and temporal scales that cause release. Nevertheless, Ca\(^{2+}\) imaging resolves variation in presynaptic events and using fluorescent dyes as Ca\(^{2+}\) buffers, provides insight into the total Ca\(^{2+}\) requirements of release at some central synapses (Cochilla and Alford, 1998; Sabatini and Regehr, 1998; Jackson and Redman, 2003; Brenowitz and Regehr, 2007; Hamid et al., 2019).

Precise measurement of the Ca\(^{2+}\) requirement for release requires direct recording of VGCC conductances at the release face membrane. Such recordings have been possible only at specialized calyceal synapses (Stanley, 1991, 1993; Sheng et al., 2012) where such characterizations have been achieved by acute separation of the presynaptic and postsynaptic structures. Recordings from the rat calyx of Held terminals have yielded a fairly homogenous 5–218 (mean of 42) calcium channels, of which 1–35 (mean of 7) channels open with a single AP (Sheng et al., 2012). Measurements from the chick ciliary ganglion terminals have shown calcium influx through a single open channel to be sufficient to drive release (Stanley, 1991, 1993). While these measurements have supported the idea that a small number of calcium channels can gate release, it remains unclear at conventional synapses how much Ca\(^{2+}\) and from how many channels is required to trigger vesicle fusion and, in lieu of this, how many channels open on depolarization.

We have recorded single channel openings of VGCCs at lamprey reticulospinal AZs by cell-attached patch clamp. We confirm conclusions from these findings using quantitative measurements of Ca\(^{2+}\) entry (Neher and Augustine, 1992; Hamid et al., 2019) and by performing high-fidelity imaging of fluorescent Ca\(^{2+}\) transients, using lattice light-sheet microscopy (LLSM; Chen et al., 2014) to image single AP-evoked fluorescent Ca\(^{2+}\) transients in situ. Our findings indicate equal numbers of presynaptic Ca\(^{2+}\) channels and primed vesicles, and that presynaptic Ca\(^{2+}\) entry at these synapses occurs through few open channels, possibly even one, localized closely to primed vesicles.

Materials and Methods

All procedures conformed to institutional (Animal Care Committee, University of Illinois at Chicago) and Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Spinal cords were dissected from ammocoete lampreys (Petromyzon marinus) of either sex, anesthetized with tricaine methanesulfonate (MS-222; 100 mg/l, Sigma), in ice cold (4°C) Ringer’s solution: 130 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl\(_2\), 1.8 mM MgCl\(_2\), 4 mM glucose, and 5 mM HEPES, pH 7.6, 270 mM Osm.

Acutely dissociated reticulospinal axon preparation

Briefly, meninges were removed from 1 cm long spinal cord segments, and the dorsal column of the spinal cord removed on a silicone block in a vibrating slicer in ice-cold Ringer’s solution, leaving intact underneath reticulospinal tracts. The sliced tissue was incubated in a mixture of collagenase and protease in Ringer’s solution (1 mg/ml) at room temperature for 45 min. Lateral tracts of the spinal cord were cut and the tissue gently separated on poly-D-lysine (MW > 300000, Sigma-Aldrich) coated coverslips by applying mechanical tension along the rostral-caudal axis, until the reticulospinal axons were isolated from the spinal cord interior (Fig. 1B; Ramachandran and Alford, 2014).
Dissociated reticulospinal axons retain functional AZs. A, Representative images showing Alexa Fluor 488 Hydrazide filling of dissociated reticulospinal axon (left) by pressure injection of the dye through a patch pipette (orange asterisk) in whole-cell patch configuration. Note 30 min later, the dye integrity is maintained within the axon (left panel on dye loading, right panel after 30 min), indicating that the axons remain structurally intact during and after dissociation. B, Representative AP (n = 7 axons) elicited in a dissociated reticulospinal axon by stimulation with a sharp microelectrode containing 3 mM KCl. C, Representative image of a region in a dissociated reticulospinal axon showing punctate FM1-43-labeled recycling vesicle clusters, labeled by 30 mM KCl bath-perfusion stimulation. D, Antibody labeling against Syt-1. A luminal domain biotinylated anti-Syt-1 antibody (rabbit, anti-mouse amino acid residues 1–8, SYS 105 103BT) diluted 1:50 in Ringer was applied by pressure from a pipette over an isolated axon during 30 mM KCl stimulation of the axon. During subsequent stimulation, streptavidin-conjugated dye (Alexa Fluor 488 Hydrazide) was applied by pipette. Fluorescent puncta were again resolved. Control stimulation and application of streptavidin labeled dye but without prior application of antibody against Syt-1 revealed no puncta. E–G, Antibody labeling against N-type (CaV2.2; Ea), P/Q-type (CaV2.3; Fb), and R-type (CaV2.3) VGCC (Ga, left panels) demonstrating punctate distribution along the axon membrane. Note: primary antibodies against VGCC subtypes (rabbit, Alomone Labs), secondary antibody Alexa Fluor 633 Hydrazide-conjugated (goat, anti-rabbit, Alomone Labs). The primary anti-N-type antibody was against an intracellular epitope (C)ESQERSLDEGVSIDG, corresponding to amino acid residues 892–907 of rat CaV 2.2 (accession Q02294) located in the intracellular loop between Domains II and III of the VGCC. The primary anti-P/Q-type antibody was against an intracellular epitope (C)PSSPERAPGREGPYGRE, corresponding to amino acid residues 865–870 of rat CaV 2.3 (accession P54214) located in the intracellular loop between Domains II and III of the VGCC. The primary anti-R-type antibody was against an intracellular epitope (C)ASQERSLDEGVSIDG, corresponding to amino acid residues 952–967 of rat CaV 2.3 (accession P07482) located in the intracellular loop between Domains II and III of the VGCC. Presynaptic terminal locations were determined in the same axon by labeling presynaptic actin with Alexa Fluor 488 Hydrazide-conjugated phalloidin (Ea, Fa, Ga, center panels). Colocalization of VGCC labeling with locations of presynaptic terminals for each VGCC subtype characterized. Red indicates VGCC labeling, green indicates presynaptic actin labeling, and yellow colocalization (Ea, Fa, Ga, right panels). Scale bar in all images: 10 μm. n = 7 axons for each VGCC subtype: N-type 5 axons from 3 animals; P/Q-type 9 axons from 4 animals; R-type 3 axons from 2 animals. Representative images showing controls for the primary antibodies used for N-type, P/Q-type, and R-type channel staining. Primary antibody was preincubated with specific control antigen for 30 min before staining the preparation (Eb, Fb, Gb, left panels). Note the absence of any VGCC labeling in the controls. Eb, Fb, Gb, right panels, Presynaptic terminals location labeled by Alexa Fluor 488 Hydrazide-conjugated phalloidin labeling of presynaptic actin in same region of the axon as in the left panel. Scale bar in all images: 10 μm. n = 7 axons for each control: N-type control antigen 10 axons from 2 animals; P/Q-type control antigen 5 axons from 2 animals; R-type control antigen 3 axons from 2 animals. H, Representative image of control for secondary antibody (Ha) and presynaptic terminals location labeled by Alexa Fluor 488 Hydrazide-conjugated phalloidin labeling of presynaptic actin (Hb) in same region of the axon, n = 8 axons from 3 animals.

Immunohistochemistry
Dissociations were conducted in poly-D-lysine coated Petri dishes. Dissociated axons were allowed to recover for 30 min at 10°C. The dissociated preparation was fixed in 4% (w/v) paraformaldehyde for 20 min, washed with 0.1 M glycine (in PBS), permeabilized with 0.1% Triton X-100 (in PBS) for 10 min and washed with PBS for 20 min. All solution changes were performed by perfusion. Blocking was performed with 5% milk (in PBS) overnight at 4°C. Ca2+ channels were labeled by incubating with antibodies (host, rabbit; Alomone Labs), specific to N-type, P/Q-type, or R-type VGCCs (1:200 final equilibrated concentration in the dish) for 3 h at 4°C in dark. Further blocking was conducted with 1% bovine serum albumin (in PBS) for 20 min and washed with PBS for 20 min. Presynaptic AZs were
identified by co-labeling conducted by further incubating in Alexa Fluor 488 hydrazone-conjugated phalloidin (200 units/ml stock prepared in methanol, final working concentration of 10 units/ml, Life Technologies) for 20 min and washed with PBS for 20 min. Imaging was conducted on a custom-built upright confocal microscope with a 100× water immersion lens NA 1.0, at 488 and 633 nm. Image processing was conducted using ImageJ. Images were postfiltered through a smoothing filter, the two channels were merged, and co-localization was determined based on superimposed positioning of VGCC labeling and AZ labeling by phalloidin (Bleckert et al., 2012). Specificity of the antibody labeling was verified by preincubating each of the primary antibodies, before application, with the corresponding control antigen, for 30 min at room temperature.

Single channel recordings
Dissociations were conducted on poly-D-lysine coated coverslips, in Ringer’s solution, in a custom designed low-noise recording chamber, maintained at 10°C (Ramachandran and Alford, 2014). Dissociated axons were allowed to recover for 1 h and presynaptic terminals were labeled by phalloidin (Bleckert et al., 2012). Specificity of the antibody labeling was verified by preincubating each of the primary antibodies, before application, with the corresponding control antigen, for 30 min at room temperature.

Measurement of tail currents
Current from replicate sweeps at a given voltage in a patch recording (referred to as current records; Fig. 3D) was binned to generate amplitude histogram plots. These histograms were averaged (between 30 and 100 sweeps were analyzed) to generate a mean count per sweep amplitude histogram (Fig. 4A). Bin width for all amplitude histograms was 50 fA. Amplitude histograms were fitted with sum of Gaussian function in Igor Pro (Wavemetrics):

\[
\hat{f}(x) = \frac{A_1 e^{\left(-\frac{(x-C_1)^2}{2\sigma_1^2}\right)}}{C_1} + \frac{A_2 e^{\left(-\frac{(x-C_2)^2}{2\sigma_2^2}\right)}}{C_2} + \ldots + \frac{A_n e^{\left(-\frac{(x-C_n)^2}{2\sigma_n^2}\right)}}{C_n},
\]

where \(n\) is the maximum number of channels open (n_{channels,max}), \(A_i\) is the peak amplitude of open (0, noise) and \(C_i\) is the centroid of the noise Gaussian; \(A_j\) is the peak amplitude of the second Gaussian, \(C\) the centroid for both the Gaussians and \(A_i\) is the peak amplitude of the second Gaussian, \(C\) the centroid for both the Gaussians and \(\sigma\) is the standard deviation of the peak.
Fig. 3. Cell-attached recordings from AZs reveal multiple VGCC subtypes. **Aa**, Representative sweep from a cell-attached patch recording showing Ca$^{2+}$ currents recorded from a single AZ following a voltage step to 0 mV ($n = 5$ patches). For all Ca$^{2+}$ current traces shown, inward currents have a negative directionality. Solid line marked 0 indicates channel closed state, dashed lines indicate incremental channel opening events corresponding to the opening of 1, 2, or 3 channels. Patch pipette contains 10 mV [Ca$^{2+}$]_{external} as charge carrier. **Ab**, Amplitude histogram (gray bars) fitted by a sum of three Gaussian function (magenta fit line), indicates the opening of one to three Ca$^{2+}$ channels during the indicated recording. **Bb**, Similar cell-attached recordings, from single AZs, following a voltage step to 0 mV with 500 μM Ca$^{2+}$ and 10 mV [Ca$^{2+}$]_{external} in the patch pipette demonstrating absence of inward Ca$^{2+}$ currents. **Bb**, Amplitude histogram (gray bars; $n = 8$ patches) fitted with a single Gaussian function (magenta fit line) indicating only a current noise peak and no channel events. Inset, Tail currents measured, on repolarization to −80 mV from the voltage step during release of Ca$^{2+}$ block, plotted as amplitude histogram (gray bars). The amplitude histogram was fitted by a sum of three Gaussian function (magenta fit line), indicating the opening of one to three Ca$^{2+}$ channels on repolarization. **Ca**, Cell-attached recordings from nonpresynaptic terminal locations (Fig. 1C, white arrowhead), following a voltage step to 0 mV, demonstrating absence of inward Ca$^{2+}$ currents. **Cb**, Amplitude histogram (gray bars, $n = 3$) fitted with a single Gaussian function (magenta fit line) indicating only a noise peak and no channel events. **D**, Depolarization stimulus protocol, applied from a holding potential of −80 mV, to elicit VGCC currents in cell-attached patch recordings. Red bar at the holding potential indicates where the background current noise was measured (current noise record). Blue bars indicate the voltages at which analysis of single channel currents (current records) was conducted: step depolarizations to −30, 0, and 30 mV, and on repolarization to −80 mV (tail currents). **E**, Representative examples of N-type (CaV2.2), P/Q-type (CaV2.1), R-type (CaV2.3), and L-type (CaV1) currents, recorded at single AZs, following voltage steps to −30, 0, and 30 mV. Solid line marked 0 indicates channel closed state, dashed lines indicate incremental channel opening events, with maximum number of simultaneously open channels indicated in each representative trace. **Fa**, Cell-attached recordings from single AZs, following a voltage step to 0 mV, showing absence of depolarization evoked inward currents when all VGCC subtypes repolarization was determined from the number of Gaussians required to fit the tail current amplitude histogram (Fig. 4A).

Current–voltage relationships (I-V plots)
For each of the VGCC subtypes, current–voltage (I-V) plots (Fig. 3G) were plotted using the mean single channel amplitude $i$ calculated at voltages of −30, 0, 30 mV and on repolarization to −80 mV (tail current). I-V plots were fitted with a linear function (Eq. 4) and the single channel conductance ($pS$) for each channel subtype was determined by the inverse of the slope. Reversal potential ($E_{rev}$) for each channel subtype was obtained from I-V plots. The X intercept of the linear fit to the I-V data indicates the reversal potential:

$$y = a + bx,$$

where $a$ is the intercept and $b$ is the slope.

**Determination of open probability ($P_{open}$)**
Current records were integrated using a trapezoidal algorithm in Igor Pro and open probability (Fig. 3H) was calculated using Equation 5 (Jackson, 1998).

$$P_{open} = \frac{1}{ND} \int_0^D I(t) dt$$

where $P_{open}$ is the open probability for a single channel at a given voltage, $i$ is the single channel current in Amperes, $N$ is number of channels in the current record, $D$ is the duration of the current record, and $t$ is time.

In situ calcium imaging (molar quantitation of calcium entry)
Ca$^{2+}$ binding properties of fura-2, Oregon Green BAPTA1 and Fluo-5F were determined using Ca$^{2+}$ standards (Invitrogen) at 10°C (the temperature at which experiments were performed) and at a pH of 7.2 (to which the intracellular solutions were buffered). Log plots of these data points were used to determine $K_f$. For fura-2, this plot was of log([R$^{-}$ − $R_{min}$]/($R_{max}$ − R)) versus log [Ca$^{2+}$]$_{free}$, where R is the Fluorescence ratio measured from excitation at 350 and 380 nm, $R_{max}$ and $R_{min}$ are the minimum and maximum

(N, P/Q, R and L were blocked. **Fb**, Amplitude histogram (gray bars, $n = 4$) fitted with a single Gaussian function (magenta fit line) indicating only a current noise peak and no channel events. **G**, Current–voltage relationships plotted for each of the indicated Ca$^{2+}$ channel subtypes. For each channel subtype, mean single channel amplitude measured at corresponding voltages is shown along with the linear fit (N-type green, P/Q-type purple, R-type orange, L-type blue). Error bars indicate SEM. Linear fits have been extrapolated to intersect the x-axis indicating the reversal potential ($E_{rev}$). **H**, Mean open probability (mean $P_{open}$ plotted against membrane voltage (mV) for each of the indicated Ca$^{2+}$ channel subtypes (N-type green, P/Q-type purple, R-type orange, L-type blue).
ratios obtained at 0 and saturating $[\text{Ca}^{2+}]$. Free $[\text{Ca}^{2+}]$ is applied from EGTA buffered free $\text{Ca}^{2+}$ standards (Invitrogen) corrected for temperature (10°C; Harrison and Bers, 1987). This was calculated at 326 nM. For Oregon Green BAPTA1 and Fluo-5F, the plots were of $\log((F - F_{\text{min}})/(F_{\text{max}} - F))$ versus $\log[\text{Ca}^{2+}]_{\text{free}}$, where $F$ is the value of fluorescence obtained at each $\text{Ca}^{2+}$ standard concentration, and $F_{\text{min}}$ and $F_{\text{max}}$ are the minimum and maximum values at 0 and saturating $\text{Ca}^{2+}$. Values of $K_d$ were 450 nM for Oregon Green BAPTA1 and 1.12 mM for Fluo-5F (Fig. 5A).

Images were obtained from axons loaded with $\text{Ca}^{2+}$ dye by injection from a microelectrode. Spinal cords were isolated, pinned in a custom chamber and perfused with oxygenated Ringer's solution (10°C) either under an upright epifluorescence or a confocal microscope (Fig. 5B). Reticulospinal axons were impaled with microelectrodes containing the ratiometric dye fura-2 or either of the nonratiometric dyes Fluo-5F or Oregon Green BAPTA1 (Life Technologies) in buffered 1M KCl solution (pH 7.2 with HEPES, 5 mM; Fig. 5C,D). Axons were filled with dyes from different initial electrode concentrations (0.1–1 mM) and with dyes of differing affinities to obtain a range of dye buffering capacities in the axons. Filling was achieved by application of 200 ms pulses of pressure (up to 140 kPa) using a picospritzer (General Valve). Imaging of fura-2 fluorescence was performed on a conventional epifluorescence microscope using excitation from a Xenon source through bandpass filters of 20-nm bandwidth centered at 350 and at 380 nm. Fluorescence was

<table>
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<th>Voltage (mV)</th>
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<th>0</th>
<th>30</th>
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<td>N-type</td>
<td>0.58 ± 0.01</td>
<td>0.35 ± 0.03</td>
<td>0.28 ± 0.01</td>
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<td>P/Q-type</td>
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<tr>
<td>R-type</td>
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<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>L-type</td>
<td>0.53 ± 0.003</td>
<td>0.31 ± 0.01</td>
<td>0.28 ± 0.02</td>
<td>0.27 ± 0.01</td>
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Mean single channel current measured for each VGCC subtype. Single channel conductance (pS) for each VGCC subtype was obtained from the slope of current–voltage relationship plots (I-V plots). Data represented as mean ± SE (n = 6 for patch recordings) for each channel subtype: N-type ($n$ = 6), P/Q-type ($n$ = 5), R-type ($n$ = 6), L-type ($n$ = 6).

Table 1. Single channel properties of characterized N-type, P/Q-type, R-type, and L-type channels

Figure 4. Few open channels from a small available VGCC pool mediate $\text{Ca}^{2+}$ influx. A, Gaussian fits for the amplitude histograms (gray bars) for the indicated $\text{Ca}^{2+}$ channel subtypes (10 mM $[\text{Ca}^{2+}]_{\text{external}}$) following voltage steps to $-30$, 0, and 30 mV. Tail currents were measured, within a window of 20 ms immediately after repolarization to $-80$ mV, and plotted as amplitude histograms (gray bars). Inset (in N-type $-80$ mV) shows representative example of tail current channel opening events. Sum of Gaussian fit to the amplitude histogram is shown as solid line (N-type green, P/Q-type purple, R-type orange, L-type blue). Insets in histogram plots provide magnified view of the histogram (note expanded y-axis) showing bin values for higher current amplitudes that are not discernable from the full scaled histogram. B, Representative current traces, from cell-attached patch clamp recordings at single AZs with 90 mM [Ba$^{2+}$]$_{\text{external}}$ in the patch pipette, following voltage steps to $-50$, $-30$, 0, and 30 mV. Solid line in all the representative traces indicates the closed state of the channel. Dashed lines indicate incremental channel opening events, with maximum number of simultaneously open channels indicated in each representative trace. Amplitude histograms are shown in gray and the sum of Gaussian fit line in blue. Note the absence of any channel openings at a membrane voltage of $-50$ mV, one to two channels at $-30$ mV, and one to three channels at 0 and 30 mV.
Figure 5. Quantifying AP-evoked molar Ca$^{2+}$ entry to AZs. **A**, Dyes from the lots used from the experiments were calibrated using the same light path, temperature, and pH of solution as during experiments. Graph shows plots of log$_{10}[(R - R_{\min})/(R_{\max} - R)]$ for ratiometric fura-2 or log$_{10}[(F - F_{\min})/(F_{\max} - F)]$ for single wavelength Fluo-5F and Oregon Green BAPTA1 versus log$_{10}[\text{Ca}^{2+}]$ from EGTA buffered standards (Life Technologies). $K_d$s obtained from these plots shown in corresponding colors. **B**, Imaging was performed in axons in superfused intact spinal cords impaled with microelectrodes containing fura-2 (Life Technologies), Fluo-5F or Oregon Green BAPTA1. Dyes were in buffered KCl solution (pH 7.2). Imaging used either an upright epifluorescence microscope (ratiometric imaging) or confocal microscope (single wavelength dyes). In both cases the arrangement of objective (Olympus LUMPLF $40\times C$ 0.8 NA), spinal cord, and recording/injection microelectrodes were identical. Axons were stimulated with AP trains via current injection through the microelectrode. **Ca**, Reticulospinal axons were loaded with dye by pressure injection through the recording microelectrode. Representative Ca$^{2+}$ responses (**Ca**) show the effect of increased dye concentration on peak Ca$^{2+}$ amplitude and decay rate (**Ca**) from single exponential fits (black traces) to traces at lower (red) and higher (blue) concentration of dye. **Cd**, Alexa Fluor 594 Hydrazide-conjugated phalloidin (Life Technologies) labeled presynaptic AZs imaged after subsequent injection into the axon with a second electrode revealing their numbers in the imaged region. **D**, Dye concentrations in fura-2 filled axons were calculated by comparing fluorescence intensity of dye at known concentration in the microelectrode with dye labeled axons at the same depth in tissue. **Da**, For fura-2, images were taken with electrode diameter the same as the axon to allow imaging from the same volume exciting at the isosbestic point. **Db**, For nonratiometric dyes, Alexa Fluor 594 Hydrazide was imaged confocally at the same depth in tissue in adjacent axons and electrodes uses images substantially larger than the microscope point spread function. **E**, The rate of decay of Ca$^{2+}$ transients ($\tau$) was plotted versus buffering capacity of the dye ($K_d$) from exponential fits (Ga) over a range of $K_d$s. These data were well fit ($r^2 = 0.88$) with a linear function (dark red line, 95% confidence interval of 0.79 to 0.90) yielding an estimate of the endogenous buffering capacity ($K_\text{end}$) of $17.3 \pm 11.1$ nM per stimulus. **F**, $\Delta$[Ca$^{2+}$] was plotted against a range of $K_d$s and fitted with Equation 11 (dark red line, 95% confidence interval of 31.6 $\pm$ 5.2 nM per stimulus) yielding a peak $\Delta$[Ca$^{2+}$] of 31.6 $\pm$ 5.2 nM per stimulus. **Ga**, $\Delta$[Ca$^{2+}$] was plotted against $K_d$ and fitted by Equation 12 to yield a $\Delta$[Ca$^{2+}$] of 31.6 $\pm$ 5.2 nM per stimulus. **Gb**, total Ca$^{2+}$ entering bound to dye ($\Delta$[Ca$^{2+} = \Delta$[Ca$^{2+}$] $\times K_\text{dye}$) for each AP was plotted against $K_\text{dye}$.
captured using a dichroic cutoff of 405 nm and through a long pass filter from 450 nm. Fluorescence of Oregon Green BAPTA1 and Fluo-3F were obtained using a confocal microscope with 488-nm excitation and captured in long pass from 510 nm.

For ratiometric measurements using fura-2, Ca\(^{2+}\) concentration can be expressed as in Equation 6:

\[
[Ca^{2+}] = K_d Q \left( \frac{R - R_{min}}{(R_{max} - R)} \right),
\]

where \(Q\) is the ratio of \(F_{max}/F_{min}\) for fluorescence for for excitation. Nonratiometric calculations of Ca\(^{2+}\) concentrations can also be made using Equation 7:

\[
[Ca^{2+}] = K_d \left( \frac{F - F_{min}}{F_{max} - F} \right)
\]

For fura-2 experiments, we obtained transient Ca\(^{2+}\) concentrations by measuring fluorescence only at 380-nm excitation during the evoked transient to enable sufficient temporal resolution, but after obtaining the resting Ca\(^{2+}\) concentration ratiometrically with excitation at both 350 and 380 nm. Absolute values of resting Ca\(^{2+}\) were calculated from Equation 6 after measurements of values of R obtained by sequentially exciting fluorescence at 350 and 380 nm. Values of \(F_{max}\) and \(F_{min}\) for excitation at 380 nm expressed as \(\Delta F/F\) can be obtained from the value of \(Q\) (\(Q = 5.45\)) from standard measurements and because the absolute value of resting [Ca\(^{2+}\)] was obtained. Additionally, values of \(F_{max}\) were obtained for comparison at the end of experiments by stimulation until saturation of the dye signal was recorded. For nonratiometric dyes the absolute values of Ca\(^{2+}\) concentration during transients were obtained using Equation 7. \(F_{max}\) for each transient was determined by intense stimulation to saturation after the transient was measured. \(F_{max}\) was calculated as a ratio of this value from measurement of Ca\(^{2+}\) standards. Values of F calculated throughout the transient as \(\Delta F/(F + 1)\) could then be used to calculate [Ca\(^{2+}\)] from Equation 7.

Fluorescence analysis of Ca\(^{2+}\) entry at AZs with LLSM
To analyze variance of presynaptic Ca\(^{2+}\) entry through few channels we must resolve Ca\(^{2+}\) responses, spatially and temporally at individual AZs, evoked by single APs. Wide field fluorescence causes high fluorescence background noise and rapid photobleaching from excitation light. Background noise can be avoided by confocal or two-photon approaches, but high excitation intensities cause rapid photobleaching, making this to line-scanning limits the field of view. This, and the limited number of repeated exposures feasible because of photobleaching, make interpretations of variations in responses between AZs or within single AZs not feasible. To overcome these limitations, we built a custom LLSM (Chen et al., 2014). The LLSM generates a convergent lattice of 30 Bessel beams (Chen et al., 2014), confined to a plane (\(-0.4 \mu m\) deep) as they self-reinforce and propagate in situ, providing substantially better resolution than conventional light sheet imaging. The LLSM beam is viewed orthogonally to its projection allowing low excitation intensity illumination of planes to be viewed rapidly in sequence with little photobleaching, allowing many repeated measurements of AP-evoked transients.

Spinal cords were pinned ventral side up in a cooled (10°C) perfused chamber using a perfusion medium of 30 Bessel beams (Chen et al., 2014). Confined to a plane, LLSM allows low excitation intensity illumination of planes to be repeatedly recorded. Peak amplitudes of values of \(\Delta F/F\) were calculated and plotted as histograms. Multiple Gaussian fits were applied to this using Equation 8:

\[
f(F) = A_{noise} e^{-\frac{(\frac{F-F_1}{W_1})^2}{2}} + A_{1} e^{-\frac{(\frac{F-F_2}{W_1})^2}{2}} + A_{2} e^{-\frac{(\frac{F-F_3}{W_1})^2}{2}}......
\]

where \(A_{noise}\) = peak number of failures at \(F_{noise}\) value of \(\Delta F/F\) of failure; \(A_{1}, A_{2}, A_{3}\) to \(A_{1}\) = peak numbers of event at \(F_{1}\) – the transient peak \(\Delta F/F\), and its multiples. \(W_{1}\) = variance of the unitary amplitude.

**Paired recordings to determine the size of the primed vesicle pool**
Paired cell recordings were made between reticulospinal axons recorded with sharp microelectrodes and postsynaptic spinal ventral horn neurons with whole-cell patch clamp as previously described (Gerachshenko et al., 2005). Single APs were stimulated at 30-s intervals in the presynaptic axons by applying 2-ms depolarizing currents. This evoked EPSCs at up to 12 AZs between individual reticulospinal axons and their postsynaptic ventral horn target. Peak amplitudes of these EPSCs were measured during over 200 repeated stimuli. From these data, amplitude histograms were constructed and fitted with multiple Gaussians. For experiments to determine the size of the primed vesicle pool, similar paired recordings were made but BoNT/B was included in the presynaptic electrode solution and pressure injected into the axon after obtaining control EPSCs. Light-chain BoNT/B (Calbiochem; 50 μg/ml), was stored at –20°C in 20 mM sodium phosphate, 10 mM NaCl, and 5 mM DTT at pH 6.0. The buffered toxins were diluted 2:5 with 2M potassium methyl-sulfate and 5 mM HEPES for inclusion in the presynaptic electrode. Asynchronous activity was also recorded using similar paired recordings, except the presynaptic microelectrode contained 100 mM CaCl2, 100 mM EGTA, 5 mM HEPES, and 1 mM KCl. The solution was buffered to pH 7.2 by titrating with KOH. This gave a free Ca\(^{2+}\) concentration of \(-200 \muM\).

**Antibody labeling against synaptotagmin-1 (Syt-1)**
A lumenal domain biotinylated anti-Syt-1 antibody (rabbit, anti-mouse amino acid residues 1–8; SY5 105 103BT) diluted 1:50 in Ringer was applied by pressure from a pipette over an isolated axon during 30 mM KCl stimulation of the axon. During subsequent stimulation, streptavidin-conjugated dye (Alexa Fluor 488) was applied by pipette and fluorescent puncta resolved. Control stimulation and application of streptavidin-labeled dye were performed similarly without prior application of antibody against Syt-1.

**Experimental design and statistical analysis**
Quantitative imaging fits to datasets for a number of equations or multi-Gaussian fits to channel opening were performed in Igor Pro. Error intervals from fitted data represent the 90% confidence limits of those fits. Fits to equations were considered valid if residuals were randomly distributed showing no systematic pattern. Throughout the manuscript errors are reported as the SEM. Significance was tested with two-tailed Student’s t test or two-factor ANOVAs where appropriate. We used an α level of 0.05 for significance for statistical tests.

**Results**
**Dissociated axons retain functional terminals**
Precise measures of evoked presynaptic Ca\(^{2+}\) influx requires direct recordings from the release face membrane of individual presynaptic terminals (Fig. 1A). Such recordings impeded in vivo at more conventional central synapses with single AZs because of the close apposition between the presynapse and postsynapse have, thus far, been possible only at acutely isolated specialized calyceal-type terminals (Stanley, 1991, 1993; Sheng et al., 2012).
Using a similar approach to accessing the presynaptic VGCCs, we developed an acute dissociation of the lamprey spinal cord (Ramachandran and Alford, 2014), by applying a combination of enzymatic digestion of spinal cord tissue and acute mechanical dissociation, enabling isolation of reticulospinal axons with functional presynaptic terminals without any postsynaptic processes (Fig. 1B). This permits direct recordings from single AZs. Presynaptic terminals maintained evoked synaptic vesicle cycling via exo-endocytosis visualized by FM1-43 staining of recycling vesicle clusters during 30 mM KCl depolarization (Fig. 1C; Betz and Bewick, 1992; Photowala et al., 2005) enabling targeted recordings of Ca$^{2+}$ currents from fluorescently identified single terminals (Fig. 1C, D). Excess FM dye was washed off with Advasep-7 (Kay et al., 1999). Notably, we recorded only from presynaptic terminals with simple AZs (diameter 0.4–2.8 μm, mean 1.6 ± 0.2 μm), allowing for correlations of Ca$^{2+}$ measures presented in this work to the fusion requirements of a single synaptic vesicle. Dissociated axons retained structural integrity, verified by patching the axons in whole-cell patch clamp configuration and gently pressure loading the axons with the fluorescent dye Alexa Fluor 488 hydrazide. Loaded axons were monitored over 30 min for dye leakage, demonstrating uniform loading throughout the axon with no decrease in fluorescence over time (Fig. 2A). Dissociated axons retained physiological membrane potentials (–58 ± 1.6 mV, n = 17 axons) and the capacity to fire APs (n = 7 axons; Fig. 2B). They also retained evoked compensatory endocytosis shown by labeling of synaptic vesicle clusters with FM1-43 following stimulation with high K$^+$ containing solution (Fig. 2C) and could be

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**Figure 6.** Recording presynaptic Ca$^{2+}$ transients with LLSM. A, Lamprey spinal cords were isolated in a custom chamber (Aa) designed to allow superfusion, recording and stimulation of giant axons under two lenses of the LLSM (Ab). Axons filled with Ca$^{2+}$ dye were imaged in single LLSM planes over time (Ac). B, Imaging single planes at 300 Hz during a single AP reveals Ca$^{2+}$ hotspots. Stimulation of the axons with an extracellular stimulation electrode evoked Ca$^{2+}$ transients at multiple discrete locations at the axon surface. An example of one hotspot (Ba), in response to single APs in the plane of focus of the recording, is shown when recording the same plane repeatedly at intervals indicated. Individual planes are shown as ΔF/F in pseudocolor and intensity mapped to the vertical dimension to emphasize the resolution of the hotspot and are averages of responses from five stimuli. Other hotspots not in focus are also seen. The summed Ca$^{2+}$ entry from multiple hotspot locations diffuses throughout the axons within 10 ms of the stimulus. This diffusion is shown across the axon with a profile centered on the hotspot. Bb, A profile plot (between white lines of pseudocolor image at left). This profile is displayed for the same frames shown in Ba. C, The intensity of the center 1-μm diameter (green) and the surrounding 5-μm (red) across all frames is expressed as ΔF/F along with the nonhotspot whole axon response (black). The latter representing diffusion from all hotspots. These responses are overlaid (Ca). The diffusional background is subtracted (Cb) to reveal just the hotspot Ca$^{2+}$ signal and its time course (green and red from the same locations as Ca). D, In repetitive stimulation (five stimuli at 50 Hz), the slower, whole axon diffusional signal was subtracted from the hotspot signal isolating Ca$^{2+}$ hotspots (Da; blue and green, hotspots shown in insets; black, whole axon signal). This approach isolated responses even during bursts of stimuli (Db; colors as for Da).
similarly labeled with an antibody against Synaptogamin which demonstrated punctate localization (Fig. 2D).

Immunohistochemical characterization of multiple VGCC subtypes at reticulospinal AZs

We validated the retention of VGCC localization, after dissociation, at single presynaptic terminals using antibodies specifically labeling three different Ca\(^{2+}\) channel subtypes, N-type (CaV2.2), P/Q-type (CaV2.1), and R-type (CaV2.3), previously implicated in synaptic transmission from lamprey reticulospinal axon (Krieger et al., 1999; Büssges et al., 2000; Photowala et al., 2005). For each of the VGCC subtypes tested, we were able to observe distinct punctate labeling by VGCC antibodies along the axons at discrete locations (Fig. 2E–G), consistent with a restricted localization of VGCCs along the axonal membrane surface, similar to the distribution of synapses on these axons in situ. We verified that these punctate distributions localized to presynaptic terminals by labeling presynaptic AZs in the fixed axons, post VGCC antibody labeling, with Alexa Fluor 633-conjugated phalloidin (Fig. 2E–G; Photowala et al., 2005; Bleckert et al., 2012). Specificity of the antibody labeling was verified by pre-incubating each of the primary antibodies, before application, with the corresponding control antigen. No antibody labeling was observed in the controls; nevertheless, we were still able to observe individual presynaptic terminals clearly identified by Alexa Fluor 633-conjugated phalloidin labeling (Fig. 2E–G). Similarly, controls for the secondary antibody did not result in any nonspecific labeling (Fig. 2H).

Characterization of VGCC subtype specific currents at individual presynaptic terminals

The acutely dissociated preparations provide unhindered access to direct recording at the release face membrane of single presynaptic terminals. Presynaptic terminals were identified by fluorescent labeling with FM1-43 during evoked depolarization by perfusing 30 mM KCl. This allowed us to target individual AZs for cell-attached patch clamp recordings using fluorescence microscopy (Fig. 1C). We recorded Ca\(^{2+}\) currents at the release face membrane of individual fluorescently labeled AZs, with 10 mM [Ca\(^{2+}\)]\(_{\text{external}}\) in patch pipette (Fig. 3A, n = 5 patches), using custom low-noise single channel cell-attached patch clamp recordings (Ramachandran and Alford, 2014). Single AZs were patched in cell-attached configuration, and the holding potential was set to −88 mV (adjusted from the known intracellular membrane potential determined from intracellular sharp electrode recording). The patch solution contained channel specific blockers, blocking all other known ionic currents (Materials and Methods), permitting the isolation of Ca\(^{2+}\) currents. We validated that the currents recorded represented Ca\(^{2+}\) influx through VGCCs by additionaly incorporating Cd\(^{2+}\) in the patch solution (Swandulla and Armstrong, 1989). Pharmacological block of all VGCCs with Cd\(^{2+}\) (Fig. 3B, n = 8 patches) eliminated all currents, leaving the Gaussian distribution of the amplitude histogram indistinguishable from background noise. Voltage dependent Cd\(^{2+}\) block was reversed on repolarization shown by tail currents at the end of the pulse (Fig. 3Bb). Additionally, patch recordings obtained from nonpresynaptic terminal locations, identified by absence of fluorescent FM1-43 puncta along the axon membrane, demonstrated no VGCC currents (Fig. 3C, n = 3 patches), corroborating our previous findings of VGCCs being restricted to presynaptic AZs at these synapses (Photowala et al., 2005; Bleckert et al., 2012).

Multiple channel subtypes have been implicated in depolarization-evoked Ca\(^{2+}\) influx for vesicle fusion and neurotransmitter release (Wheeler et al., 1994; Mintz et al., 1995; Poncer et al., 1997; Wu et al., 1999; Cao and Tsien, 2010; Sheng et al., 2012). In lamprey, this is supported by our immunohistochemical analysis as well as previous imaging studies in the intact spinal cord in situ (Photowala et al., 2005). Therefore, we isolated VGCC subtype-specific currents pharmacologically (Materials and Methods), corresponding to each of the four different VGCC subtypes: N-type (CaV2.2, n = 6 patches), P/Q-type (CaV2.1, n = 5 patches), R-type (CaV2.3, n = 6 patches), and L-type (CaV1, n = 4 patches) with 10 mM [Ca\(^{2+}\)]\(_{\text{external}}\) in patch pipette, eliciting Ca\(^{2+}\) currents (Fig. 3D,E). Gaussian fits to amplitude histograms of current recordings, on combined selective blocking of all four VGCC subtypes and other known ionic currents, demonstrated no additional currents with a distribution similar to background noise (Fig. 3F, n = 5 patches). Channel openings were observed only on depolarization to a membrane voltage of −30 mV (Figs. 3E, 4A), indicating the absence of any low-voltage-activated T-type (CaV3) channels at these presynaptic terminals. We measured single channel amplitude (1), from current recordings for each channel subtype that demonstrated the opening of only a single channel, at depolarized voltages of −30, 0, 30 mV and on repolarization to −80 mV (tail currents; Fig. 3D). Single channel amplitudes at 0 mV were 0.28 ± 0.01 (N-type), 0.29 ± 0.03 (P/Q-type), 0.28 ± 0.02 (R-type), and 0.28 ± 0.02 pA (L-type; Fig. 3G; Table 1). Single channel conductance was calculated to be 2.97 ± 0.06 pS (N-type), 1.82 ± 0.36 pS (P/Q-type), 1.94 ± 0.41 pS (R-type), and 2.4 ± 0.75 pS (L-type; Fig. 3G; Table 1). Mean channel opening probability (P\(_{\text{open}}\)) at 0 mV was 0.26 ± 0.02 (N-type), 0.29 ± 0.03 (P/Q-type), 0.22 ± 0.02 (R-type), and 0.24 ± 0.02 (L-type; Fig. 3H; Table 2).

Table 2. Open probability for characterized channel subtypes

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>N-type</th>
<th>P/Q-type</th>
<th>R-type</th>
<th>L-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>−30</td>
<td>0.17 ± 0.04</td>
<td>0.19 ± 0.04</td>
<td>0.14 ± 0.04</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>0</td>
<td>0.26 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.22 ± 0.02</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>30</td>
<td>0.24 ± 0.02</td>
<td>0.24 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

Mean open probability (P\(_{\text{open}}\)) calculated for each VGCC subtype at −30, 0, and 30 mV. P\(_{\text{open}}\) was calculated by integrating current data from current records (Fig. 5), at each analyzed voltage (−30, 0, and 30 mV), clearly demonstrating the opening of only a single channel. Data reported as mean ± SE (patch recordings) for each channel subtype: N-type (n = 6), P/Q-type (n = 5), R-type (n = 6), L-type (n = 4).

Few open channels from a small available VGCC Pool mediate Ca\(^{2+}\) influx

We next enumerated Ca\(^{2+}\) channels at single presynaptic AZs for each Ca\(^{2+}\) channel subtype. The current data from replicate sweeps, for each analyzed voltage, in a patch recording were binned to generate amplitude histograms, which were averaged and fitted with a sum of Gaussians function. Using this approach, we enumerated the maximum number of channels, visually confirmed by inspection of the channel opening events, that simultaneously opened at each analyzed voltage during a patch recording. A maximum count of 10 (4–10, mean 5) N-type, 9 (3–9, mean of 6) P/Q-type, 32 (4–32, mean 12) R-type, and 17 (3–17, mean 10) L-type channels yielded a total maximum VGCC count of 68 channels (14–68 channels, mean 33; Fig. 4A; Table 3) at a presynaptic AZ. Restricting the count to only N + P/Q + R channel subtypes, that are implicated in evoked synaptic transmission at lamprey reticulospinal synapses,
Table 3. Maximum number of VGCCs observed to open at individual presynaptic AZs

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>−30</th>
<th>0</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I_{max} (pA)</td>
<td>1.6</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>i (pA)</td>
<td>0.35</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4.57</td>
<td>6.07</td>
<td>10.4</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>N_{chpatch.max}</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P/Q-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I_{max} (pA)</td>
<td>1.35</td>
<td>2.85</td>
<td>2.15</td>
</tr>
<tr>
<td>i (pA)</td>
<td>0.32</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>3.97</td>
<td>9.83</td>
<td>7.96</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>3</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>N_{chpatch.max}</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I_{max} (pA)</td>
<td>1.3</td>
<td>5.35</td>
<td>8.35</td>
</tr>
<tr>
<td>i (pA)</td>
<td>0.32</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4.06</td>
<td>19.11</td>
<td>32.12</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td>N_{chpatch.max}</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I_{max} (pA)</td>
<td>1.3</td>
<td>5.35</td>
<td>8.35</td>
</tr>
<tr>
<td>i (pA)</td>
<td>0.32</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4.06</td>
<td>19.11</td>
<td>32.12</td>
</tr>
<tr>
<td>N_{chopen.max}</td>
<td>4</td>
<td>19</td>
<td>32</td>
</tr>
<tr>
<td>N_{chpatch.max}</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total N_{chpatch.max} (N + P/Q + R + L)</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total N_{chpatch.max} (N + P/Q + R)</td>
<td>51</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Channel numbers enumerated from patch recordings at single presynaptic AZs, with 10 mM [Ca^{2+}]_{in external} in the patch pipette, under conditions where currents specific to a single VGCC subtype were pharmacologically isolated. For each indicated channel type, measurements were consolidated from all current records in all obtained patch recordings (Figs. 3F, 4A). N_{chopen.max} is the maximum number of channel openings for a given voltage is the maximum number of channels simultaneously opening in n patches. N_{chpatch.max} is the maximum number of channels simultaneously opening at a presynaptic terminal for each VGCC subtype. N_{chpatch.mean} is the mean number of channels at a presynaptic terminal from n patches.

we obtained a maximum total of 11–51 channels (mean 23; Fig. 4A; Table 4) that may contribute Ca^{2+} to drive release at a single AZ.

Having determined the available pool of VGCCs at a single AZ, we next asked how many VGCCs open on depolarization, when all channel subtypes are available for opening. We used voltage steps with the same voltage range as seen during presynaptic APs recorded directly in situ (Cochilla and Alford, 1997) and thus the same voltage range to when an AP evokes presynaptic Ca^{2+} influx. We conducted cell-attached recordings from single presynaptic AZs (Fig. 1C,D), with 90 mM [KCl]_{in}; 90 mM [Ba^{2+}]_{in} external (in patch pipette). As for our previously described recordings with 10 mM Ca^{2+}, Ba^{2+} currents were evoked only on depolarization to −30 mV. Notably, on depolarization to 30 mV, which is close to the peak of APs, we observed the opening of only one to seven (mean 4) channels, out of the available pool of a maximum of 68 (mean 33) channels at an AZ (Fig. 4B; Table 5, n = 5 patches), suggesting very few out of the available small pool of channels mediate Ca^{2+} influx at depolarization voltages that are equivalent to peaks of APs.

Molar quantitation corroborates Ca^{2+} entry through few open VGCCs

Evoked openings of VGCCs in cell-attached recordings were determined after acute isolation. A similar approach has been used in calyceal synapses (Sheng et al., 2012). However, acute isolation may alter channel activity. To confirm that evoked Ca^{2+} entry remains the same in isolated axons to those in situ we used quantitative imaging to determine molar quantities of Ca^{2+} that entered each AZ during each AP. We used Ca^{2+} dyes as buffers to quantify total molar quantities of AP-evoked Ca^{2+} entry to the reticulospinal axons maintained in the intact spinal cord in situ as an independent measure of axonal evoked Ca^{2+} entry with no dissociation. Ca^{2+} dyes with calibrated binding properties were injected directly into axons at known concentrations, before recording AP-evoked Ca^{2+} transients (Materials and Methods). Ca^{2+} binding properties of the Ca^{2+} dyes, fura-2, Oregon Green BAPTA1, and Fluo-5F from the lots used experimentally, were determined using Ca^{2+} standards (Invitrogen). Log plots of these data points were used to determine K_d as well as fluorescent maximum/minimum ratios (Fig. 5A). Microelectrodes were filled with Ca^{2+} dyes and reticulospinal axons were impaled to drive them with dyes of differing affinities; either the ratiometric dye fura-2 or the nonratiometric dyes Fluo-5F or Oregon Green BAPTA1 (Life Technologies) in buffered 1M KCl solution (pH 7.2 and 10°C). Microelectrodes were injected directly into axons at known concentrations, before recording AP-evoked Ca^{2+} transients (Materials and Methods) and imaged under epifluorescence or confocal microscopy (Fig. 5B). Microelectrodes were filled with Ca^{2+} dyes and reticulospinal axons were impaled to drive them with dyes of differing affinities; either the ratiometric dye fura-2 or the nonratiometric dyes Fluo-5F or Oregon Green BAPTA1 (Life Technologies) in buffered 1 M KCl solution (pH 7.2 with 5 mM HEPES; Fig. 5C,D). Dye (from 0.1 to 1 mM in electrode) was pressure injected into the axon to obtain a range of dye buffering capacities (k_dyeb, see Eq. 9) in the axons. After
loading with dye, the axon was stimulated with a train of APs. The axon was allowed to recover between trials for 3–5 min.

Images were acquired for fura-2 fluorescence on a conventional epifluorescence microscope. Fluorescence transients from excitation at 380 nm were obtained after capturing baseline images from excitation at 350 and 380 nm and absolute Ca$^{2+}$ concentrations calculated from Equation 6 (Materials and Methods). Examples of responses (Fig. 5Ca,Cb) evoked by 10 APs (Fig. 5Cc) at two different axonal fura-2 concentrations are shown to demonstrate that as the concentration, and therefore $\kappa_{\text{dye}}$, rises, the peak amplitude of free Ca$^{2+}$ decreases and the time course ($\tau$) of the decay increases. AP-evoked fluorescence transients were also obtained using Oregon Green BAPTA1 and Fluo-5F. For these, imaging was confocal with 488-nm excitation captured in long pass from 510 nm. Fluorescence maxima were measured during repetitive stimulation at 50 Hz until intensity reached an asymptotic maximum. Absolute Ca$^{2+}$ concentrations were obtained from these data and Equation 7 (Materials and Methods). To obtain the amount of Ca$^{2+}$ entering each AZ, we counted AZ numbers within the region of measurement. To achieve this, the Ca$^{2+}$ dye/buffer electrode was withdrawn, and the same axon was re-impaled with an electrode filled with Alexa Fluor 594 Hydrazide-conjugated phalloidin. Phalloidin was pressure injected into the axon and images captured after 10–15 min allowing for the phalloidin to label presynaptic actin (Bleckert et al., 2012). From these images the number of phalloidin labeled puncta were counted (Fig. 5Cd). The mean number of AZs per axon region imaged was 79 ± 7 (0.40 ± 0.04 synapses per linear micrometer of axon).

Dye concentrations in the axons were also measured. For fura-2, at the end of the experiment, fluorescence at the isosbestic point (350- to 360-nm excitation) was obtained from the axon and the electrode in which the dye concentration was known (Fig. 5D). Because the conventional epifluorescence imaging captured out of focus light, the electrode was positioned adjacent to the imaged axon and such that the imaged portion was the same diameter as the imaged axon, to give the same imaged volumes. Axon dye concentrations were calculated as a fraction of the known concentration measured in profile plots (Fig. 5Da). A similar method was used to estimate Oregon Green BAPTA1 and Fluo-5F concentrations. For these confocal images, a known concentration of Alexa Fluor 594 Hydrazide was included in the electrode and co-injected into the axons. Electrodes and axons at the same depth were imaged using 560-nm excitation. The electrode was positioned such that its diameter was greater than the electrode in which the dye concentration was known and the electrode in which the dye concentration was known.

The buffering capacity ($\kappa_{\text{end}}$) of the axon ($\kappa_{\text{dye}}$) during each stimulated transient was calculated using Equation 9 (Augustine and Neher, 1992; Jackson and Redman, 2003; Hamid et al., 2019):

$$\kappa_{\text{dye}} = \frac{\Delta[C_{\text{dye}}]}{\Delta[Ca^{2+}]} = \frac{\text{Dye}_{\text{total}}}{k_i \left( 1 + \frac{[Ca^{2+}]}{k_i} \right) \left( 1 + \frac{[Ca^{2+}]}{k_i} \right)}$$, (9)

where $[Ca_{\text{dye}}]$ is the concentration of Ca$^{2+}$ bound dye, $\Delta[Ca^{2+}]_i$ is the evoked change in Ca$^{2+}$ concentration, and $\text{Dye}_{\text{total}}$ is the total dye concentration. $[Ca^{2+}]_i$ and $[Ca^{2+}]-[Ca^{2+}]_i$ are the resting Ca$^{2+}$ and peak Ca$^{2+}$ concentrations before and during stimulation respectively (Fig. 5Ca,Cb).

The endogenous buffering capacity of the axon ($\kappa_{\text{end}}$) was determined from the dependency of the rate of decay of the dye recorded Ca$^{2+}$ transient to the buffering capacity of the dye at that time. The recovery of the stimulus evoked Ca$^{2+}$ transient (Fig. 5Ca) was fitted with an exponential function and the decay rate ($\tau$) measured, over a range of values of $\kappa_{\text{dye}}$ calculated from Equation 9. Values of $\tau$ were plotted against $\kappa_{\text{dye}}$ and fitted with a linear function (Fig. 5E, $r^2 = 0.88$) yielding an estimate of the endogenous buffering capacity ($\kappa_{\text{end}} = 13.1 \pm 11.1$) from Equation 10, which describes the decay rate ($\tau$) of a Ca$^{2+}$ signal in a cell compartment (Neher and Augustine, 1992):

$$\tau = \tau_{\text{end}} (1 + \kappa_{\text{end}} + \kappa_{\text{dye}})$$, (10)

This value of $\kappa_{\text{end}}$ gives an estimate independent of the absolute measure of Ca$^{2+}$ transient amplitudes and thus provides a reasonable measure of validity for the remaining calculations. We may also calculate the free Ca$^{2+}$ concentration evoked by stimulation and the total molar quantity of Ca$^{2+}$ that enters the axon. By measuring the peak amplitude of the free Ca$^{2+}$ transient throughout the variance over a range of values of $\kappa_{\text{dye}}$ (Neher and Augustine, 1992; Hamid et al., 2019), we may state:

$$\Delta[Ca^{2+}]_i = \frac{\Delta[Ca_{\text{total}}]}{(1 + \kappa_{\text{dye}} + \kappa_{\text{end}})}$$, (11)

where $\Delta[Ca^{2+}]_i$ varies with $\kappa_{\text{dye}}$. Values of $\Delta[Ca^{2+}]_i$ are computed from our data using Equation 6 or Equation 7 (Materials and Methods) and values of $\kappa_{\text{dye}}$ from Equation 9. Peak $\Delta[Ca^{2+}]_i$, per AP in the volume of axon, was plotted for a range of calculated $\kappa_{\text{dye}}$ and fitted with Equation 11 (Fig. 5F) yielding a peak free $\Delta[Ca^{2+}]$ of 31.6 ± 5.2 nm throughout the axon per stimulus from the intercept of the ordinate axis. This extrapolation is the true value of peak $\Delta[Ca^{2+}]_i$, in the axons when no dye is present. This fit also yielded the value of total Ca$^{2+}$ concentration, bound and unbound ($\Delta[Ca_{\text{total}}]$) of 109.9 ± 19.2 nm that entered the whole axon volume and a $\kappa_{\text{end}}$ of 2.5 ± 1.1. Equation 11 is hyperbolic, therefore the inverse of evoked free Ca$^{2+}$ was plotted against $\kappa_{\text{dye}}$ giving a linear relationship. The straight line fit to this gives $r^2 = 0.9$. We can also calculate the total Ca$^{2+}$ bound to dye for each stimulus against $\kappa_{\text{dye}}$. At higher dye concentrations the dye/buffer captures a greater proportion of entering Ca$^{2+}$ reaching an asymptote as all entering Ca$^{2+}$ is bound to dye. Thus, we plotted change in Ca$^{2+}$ bound dye concentration ($\Delta[Ca_{\text{dye}}]$) against $\kappa_{\text{dye}}$ and fitted this with Equation 12:

$$\Delta[Ca_{\text{total}}] = \Delta[Ca_{\text{dye}}] \left( \frac{(\kappa_{\text{end}} + 1)}{\kappa_{\text{dye}}} + 1 \right)$$, (12)

This fit yields $\kappa_{\text{end}}$ of 5.2 ± 2.2, and an asymptotic value of $\Delta[Ca_{\text{total}}]$ (the total stimulated Ca$^{2+}$ entry) representing full dye binding of all Ca$^{2+}$ entry of 116.9 ± 13.2 nm (Fig. 5G). Values obtained were similar from fits to Equations 11, 12 (mean values, Table 6). Note that during Ca$^{2+}$ entry that occurs only at AZs, the local concentration of Ca$^{2+}$ which evokes neurotransmission is transiently at much higher concentrations, but this high concentration almost immediately disperses throughout the larger axonal volume from which it was imaged. We calculated axon volumes from images, assuming the axons are cylindrical, to determine the molar quantity of Ca$^{2+}$ that entered the axon per AP and the mean molar amount entering at each terminal can...
Potcoava et al., 2021

Table 6. Quantitation of molar calcium entry into axons and AZs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
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<tbody>
<tr>
<td>$\kappa_{ax}$</td>
<td>6.9</td>
<td>Mean from fits to Fig. 5</td>
</tr>
<tr>
<td>Resting free $[Ca^{2+}]_i$ (nM)</td>
<td>$8150 \pm 21$</td>
<td>Mean of fura-2 measurements</td>
</tr>
<tr>
<td>Peak free $\Delta [Ca^{2+}] / \text{stimulus}$ (nM)</td>
<td>$36.8 \pm 5.2$</td>
<td>Fig. 5</td>
</tr>
<tr>
<td>Number of presynaptic terminals</td>
<td>79 $\pm$ 7</td>
<td>Phalloidin labeling</td>
</tr>
<tr>
<td>Mean axon volume $\mu m^3$</td>
<td>$28,100 \pm 2200$</td>
<td>Diameter of axons</td>
</tr>
</tbody>
</table>

Total axonal $[Ca^{2+}] / \text{stimulus}$ (nM) | $116.9 \pm 13.2$ | Fig. 5 |

Total molar quantity $Ca^{2+}$ entering (n) | $4.14 \times 10^{-6} \pm 0.46 \times 10^{-9}$ | Calculated |

Total # ions entering | $24,900 \pm 2800$ |

Total charge (C) | $1.60 \pm 0.23$ |

Current over 5 ms (pA) | | |

Table summarizes the quantitation of AP-evoked entry of calcium into presynaptic axons and individual AZs. This enables an estimate to be made of the current carried by calcium at each AP.

then be given by dividing this total by the number of synaptic terminals imaged using phalloidin (Fig. 5Gf). From these calculations (Table 6), we conclude a total charge entering the axon at each AZ during one AP is just $7.98 \pm 0.90$ fC representing a Ca$^{2+}$ current of $1.6 \pm 0.3$ pA over the duration of the AP (2–3 ms of depolarization and 2 ms of estimated tail current). Mean channel currents over this voltage range were about $0.4$ pA (from the integral of the slope between $-80$ and $+20$ mV in Fig. 3G), indicating opening of a mean of four channels per AZ per AP. This is very similar to results from our cell-attached recordings.

Resolving presynaptic Ca$^{2+}$ channel transients with LLSM

Ca$^{2+}$ entry to lamprey reticulospinal axons is limited to synaptic AZs (Photowala et al., 2005), and we have demonstrated that few channels open at each AZ on presynaptic depolarization. Thus, variability of channel activation between APs may contribute to the stochastic nature of quantal release. To investigate these properties of presynaptic VGCCs in intact synapses physiologically activated by APs, we visualized Ca$^{2+}$ transients at single presynaptic terminals in spinal cords in the intact spinal cord. Prior methods that provide sufficient temporal resolution to image single transients such as confocal line scanning (Takahashi et al., 2001) suffer from insufficient signal-to-noise characteristics to resolve variations in signal between single APs at each AZ. They also cause photodamage because high excitation powers are necessary to resolve the responses. To overcome these difficulties, we used a custom built LLSM (Chen et al., 2014; Potcoava et al., 2021; Fig. 6A). This eliminated out of focus noise and enabled optical slicing at sufficient rates to resolve presynaptic Ca$^{2+}$ transients. It reduced the light dose compared with confocal imaging by about 1000-fold. This enabled variations in AZ Ca$^{2+}$ signaling to be compared between APs. Additionally, the near absence of photobleaching (Chen et al., 2014) allowed recording of many repetitions of stimulated transients (Chen et al., 2014) allowing us to investigate the stochastic nature of channel dependent presynaptic Ca$^{2+}$ entry at single presynaptic AZs.

Lamprey giant synapses are uniquely suited for this study, because they have simple AZs (Gustafsson et al., 2002) and a large axon into which Ca$^{2+}$ can disperse (Coehilla and Alford, 1998; Takahashi et al., 2001), enabling resolution of Ca$^{2+}$ transients from single AZs. To label reticulospinal axons with Ca$^{2+}$-sensitive dye, axons in intact lamprey spinal cords were impaled with microelectrodes containing 5 mM Fluo-5F and pressure injected via the microelectrode (final concentration in axon $\sim 4 \mu M$ (Takahashi et al., 2001; Materials and Methods). We used LLSM (Fig. 6A) to image AP-evoked Ca$^{2+}$ transients at AZs in situ in these axons at 330–800 Hz at fixed z-axis (Fig. 6Ac). Superficially, Ca$^{2+}$ transients appear as single hotspots (Fig. 6Ba), that prior studies demonstrate colocalize to AZs (Photowala et al., 2005), although LLSM gave substantially greater signal-to-noise (mean LLSM rms peak signal-to-noise ratios = 41.1 $\pm$ 14.2 n = 10, compared with 4.0 $\pm$ 1.3 with line scanning n = 6). LLSM reveals readily resolved transients at single AZs during each AP. We characterized this Ca$^{2+}$ entry and its distribution in 10 axons. To illustrate the response to single presynaptic AP average, the response to 10 sequential stimuli applied at 1 min intervals is shown (coded in color LUTs and the intensity profile represented in the z-axis of selected frames) from before and after stimulation (Fig. 6Ba). Frames were analyzed by subtracting the background signal outside the axon and expressing data as ΔF/F. In the recording shown (Fig. 6Ba), the LLSM lattice plane was placed over a single AZ (circled in red, frame at 3.2 ms), although other slightly out of focus hotspots also contributed to this initial signal (red arrows) whereby many AZs arranged around the axon plasmalemma contribute to the total Ca$^{2+}$ entry. Ca$^{2+}$ entered at AZs during stimulation and diffused throughout the 10-μm diameter structure within 10 ms [mean rise time (τ) of the Ca$^{2+}$ signal at the center of the axon was 9.1 $\pm$ 1.6 ms]. This is shown graphically by calculating the profile signal in a region of interest (ROI) spanning the width of the optical section (Fig. 6Bb, white box) and plotting the profile before, immediately after and in subsequent frames (Fig. 6Bb).

To visualize the time course of the evoked responses, the transient taken from $0.5 \times 0.5$ μm ($5 \times 5$ pixels) at the hotspot center is graphed (Fig. 6Ca, green) as well as the signal from the whole ROI in red (from ROI looped the hotspot at 3.2 ms; Fig. 6Ba). The mean signal from the axon, excluding hotspots is also shown (Fig. 6Ca, marked in black). It originates from the summed Ca$^{2+}$ diffusing from all hotspots around the periphery of the axon, both in and out of focus. From phalloidin labeling data (Fig. 5A), a mean of $0.40 \pm 0.04$ nanospecks is present per linear micrometer of axon, thus on average, 20 AZs are present in this 50-μm region of axon. To demonstrate the amplitude and time course of Ca$^{2+}$ entry to hotspots this overall Ca$^{2+}$ signal derived from Ca$^{2+}$ diffusion from all hotspots in this region of the axon was subtracted from the hotspot signals (Fig. 6Cb). We sought to investigate the variability of Ca$^{2+}$ entry between single stimuli, and therefore used the same subtraction approach during bursts of repetitive stimulation. An example is shown for two hotspots (Fig. 6Da, blue and green) in the same axon (Fig. 6D, insets). Individual hotspots showed diffuse Ca$^{2+}$ accumulation. The nonhotspot signal was again subtracted from this to reveal the time course of local Ca$^{2+}$ entry and its amplitude (Fig. 6Db). We then analyzed these individual hotspot signals to determine variation within subregions of each hotspot and between individual AP-evoked hotspots.

Table 7. Quantal release of neurotransmitter

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
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<tbody>
<tr>
<td>Number of AZ release sites between pairs of axons and target neurons</td>
<td>$7 \pm 0.5$</td>
<td></td>
</tr>
<tr>
<td>Quantal amplitude (pA)</td>
<td>$8.1 \pm 2.7$</td>
<td>Fig. 9</td>
</tr>
<tr>
<td>Probability of release at each release site</td>
<td>$0.22 \pm 0.02$</td>
<td>Fig. 9</td>
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Quantal analysis of evoked responses from paired recordings between individual axons and their target neurons enabled calculation of probability of release at each AZ.
Figure 7. Variation in Ca\(^{2+}\) within hotspots. A, Ca\(^{2+}\) transient intensity was recorded following single APs in regions within the hotspot to demonstrate variation in responses between APs. Aa, Example of a single un-averaged response to one AP, expanded in Ab to outline a grid of analyzed regions (each 5 × 5 pixels = 0.5 × 0.5 μm) within a ROI encompassing the whole hotspot. B, Stimuli, three trains of five APs shows variations in amplitudes in the whole region (large yellow ROI in Ab) between individual stimuli. C, Variation within the smaller subregions is much greater. Two subregions are compared in the traces (top) and all subregions compared with color coding of peak amplitudes of hotspot. B, responses expressed as shown on the right of the figure confirms this lack of correlation. Between each subregion and all others in this AZ for 20 stimuli coding positive correlation of 1 as red, negative correlation of zero correlation as black. Correlation between subregions was very low (mean correlation coefficient = −0.09). For this one hotspot, data are shown in the left of the figure. Mean correlations for similar analyses in AZs from five axons is shown on the right of the figure confirms this lack of correlation.

We analyzed subregions within AP-evoked Ca\(^{2+}\) transients. Peak evoked Ca\(^{2+}\) hotspot transients from just one AP are shown for a section of axon (Fig. 7Aa) and the highlighted hotspot (Fig. 7Aa). The larger yellow ROI encompassing the whole hotspot was imaged during sequential bursts of 5 evoked APs (Fig. 7B, blue). Transients were isolated to just hotspots by removing the overall axon signal using the approach described above (Fig. 6Cb,Db). While the whole region showed variability in Ca\(^{2+}\) transient peak amplitudes, variations between stimuli were greater between subregions. This was assessed by measuring the amplitudes in a grid of ROIs, each of 5 × 5 pixels (0.5 × 0.5 μm) within the hotspot (Fig. 7Ab, small yellow ROIs). Two of these responses from different subregions during the same stimulus train are overlaid (Fig. 7C, top regions e and g) and peak amplitudes from all the subregions are expressed as a color-coded grid (Fig. 7C, bottom). Each region shows variability, but they do not co-vary in amplitude. Peak amplitudes of the two regions (from Fig. 7Ce,g) were also directly compared for each of 40 stimuli (Fig. 7D). There was no discernible correlation. This was true for all pairs of locations tested at this synapse and in a further four synapses with similar high signal-to-noise recording.

To determine whether these local rapid signals showed correlation in variation because of proximity, correlation coefficients between the peak values ΔF/F of Ca\(^{2+}\) transients each subregion and the other 8 measured at the AZ were compared by plotting these correlation coefficients against distance between ROI centers (Fig. 7E). Data were obtained from responses from 40 sequential APs. There was no relationship between correlation coefficient and distance between subregions visible in this plot. Results were similar for the other four synapses. To quantify this lack of correlation in all five recorded AZs, correlation coefficients of peak amplitudes between each subregion and the other eight were plotted as a color-coded grid (Fig. 7F). The data for the highlighted AZ are shown in the lower left triangle. Comparing each region with itself gave perfect correlation (red squares) but compared with other subregions correlations were randomly positive, absent, or negative (mean correlation coefficient = 0.03 ± 0.08; Fig. 7F). The mean values of correlation coefficients in all examined AZs were measured and the means displayed in the same way (Fig. 7F, upper right triangle; mean correlation coefficient from all subregions in all AZ = 0.008 ± 0.04, n = 5 AZs, not significantly different from zero, p = 0.83).

We also analyzed Ca\(^{2+}\) transients, encompassing the whole AZ, to determine their amplitude variation between APs. Ca\(^{2+}\) transients were measured in ROIs that encompassed the entire hotspot outlined in red in the first poststimulus image (Fig. 8A). Trains of stimuli were applied (five stimuli at 50 ms) and the resultant hotspot transients displayed as before but showing unaveraged responses (Fig. 8B). Three sequential stimulus train evoked transients are shown. In six AZs the amplitudes of these
events varied between stimuli, and the mean event amplitude reduced throughout the stimulus train (Fig. 8C). To further analyze amplitude variation, amplitudes of all 320 stimuli were plotted as an amplitude histogram that was well fit by five Gaussians that included a peak representing just signal noise (Fig. 8Da). Gaussian fits were constrained such that the amplitude ratios of sequential Gaussians were integers and the widths scaled with these amplitudes (not including failures). Gaussian peaks were observed at increments of $0.31 \Delta F/F$ (Fig. 8C, purple). These Gaussian peaks may represent quantal amplitudes following individual channel openings because their numbers correspond well to the numbers of synchronous channel openings seen in cell-attached recordings and calculated from molar Ca$^{2+}$ entry recordings. The amplitude reduction during trains was caused by a reduction in event probability. Amplitude histograms from each stimulus position in the train were plotted and again fit with multi-Gaussians. Similar peak increment values were observed but at lower increments at sequential stimuli (range from 0.33 to 0.29 $\Delta F/F$; Fig. 8Db). For all six axons tested, if each Gaussian corresponds to a channel event, then a mean number of $1.73 \pm 0.07$ channels open at each AZ with a minimum of 0 and maximum of 7. Histograms, normalized to the value of the unit amplitude from individual fits. The mean number of events per stimulus were then plotted against these normalized amplitudes giving a similar overall distribution of events.

**Figure 8.** Variation in Ca$^{2+}$ transients between APs. A, Single AZs were imaged (up to 120 APs in 80 trains) with no bleaching. Images expressed as $\Delta F/F$ are shown before stimuli, at the peak of responses to stimuli one to five and after stimulation. B, At individual whole AZs, responses were measured (5 stimuli, 3 repeats). Events are expressed at $\Delta F/F$ after whole axon diffuse Ca$^{2+}$ signals were subtracted (see Fig. 6Cb,Db). C, Mean amplitudes of responses to these five stimuli show a reduction throughout the stimulus train. Amplitudes normalized to the peak of the response to stimulus 1. D, Peak of transients from 320 stimuli ($80 \times$ trains of 5) were plotted as an amplitude histogram for all 320 APs. These show distinct peaks implying events are multiples of a unit amplitude. Gaussian fits (magenta) were made to this distribution with two constraints; amplitudes between peaks were equal, and variance scaled linearly with increase in amplitude. Db, A similar set of histograms were calculated for each of the first through the fifth stimulus in each train and Gaussians were again fit with the same constraints as Ca. While the numbers of low-amplitude responses increased later in the train the unit amplitudes (purple text) remained constant. E, The amplitude data from six analyzed AZs were normalized to the value of the unit amplitude from individual fits. The mean number of events per stimulus were then plotted against these normalized amplitudes giving a similar overall distribution of events.

Numbers of presynaptic Ca$^{2+}$ channels and primed vesicles are the same

The lamprey giant axon provides unique direct access to recording from AZs using sharp electrodes while recording from their postsynaptic targets. This enables both paired-cell recording of direct monosynaptic connection between axons and their individual targets and simultaneous manipulation of the presynaptic
biochemistry (Blackmer et al., 2001). We used this unique access to determine the numbers of primed vesicles at each AZ and their release probability (Pr). Thus, we could relate prime vesicle number and number of evoked vesicles to Ca$^{2+}$ channel opening. Paired recordings can be readily made between individual giant axons and their target ventral horn neurons. Each such reticulospinal axon makes a number of connections (up to \( \sim 12 \) synapses) onto individual target neurons in the ventral horn. We thus made paired recordings by recording reticulospinal axons with sharp microelectrodes and spinal ventral horn neurons using whole-cell patch clamp (Fig. 9A). EPSCs were recorded following evoked presynaptic APs (Fig. 9Ba). From repeated evoked EPSCs, peak evoked amplitude histograms yielded multiple events consistent with multiple AZs between the axons and target neurons (Fig. 9Bb). In this example paired recording, Gaussian fits to this histogram revealed a minimum evoked EPSC amplitude of 8.7 pA (Fig. 9Bb). In five example pairs, similar histograms were created and fitted with similar multiple Gaussian curves. The mean minimum quantal amplitude was 8.1 \( \pm \) 2.7 pA (Fig. 9Bc, \( n = 5 \)). Mean probability of release \( [Pr] \) was calculated from the Gaussian fits (Fig. 9Bb,Bc) to be 0.22 \( \pm \) 0.02. Pr was relatively uniform at these synapses and ranged from 0.16 to 0.25 (see Table 7 for summary).

We confirmed the amplitude of unitary events by recording asynchronous events between paired reticulospinal axons and their target neurons. We cannot relate raw spontaneous activity to spontaneous event amplitudes from reticulospinal axons because there are too many nonreticulospinal synapses onto the postsynaptic cell. However,
asynchronous release was enhanced from individual reticulospinal presynaptic axons by injecting high Ca\(^{2+}\) concentrations buffered with EGTA to cause a tenfold increase in total spontaneous event frequency recorded in target neurons paired to these axons. To achieve this, paired recordings were again made with microelectrodes in the axon and whole-cell recording of the ventral horn target neuron. The presynaptic microelectrode contained ~200 \(\mu\text{m}\) free Ca\(^{2+}\) at pH 7.2 in the presynaptic microelectrode. Evoked responses obtained by stimulating the axon immediately after obtaining the paired recording confirmed synaptic connectivity (Fig. 9Ca). Subsequent pressure injection (140 kPa, 200 ms repeated up to 20 times) caused a marked increase in the frequency of evoked events (Fig. 9Cb,Cc). This approach yielded a modal amplitude of these asynchronous events (6.6 \(\pm\) 1.5 pA, \(n = 4\)), not significantly different from the minimal evoked amplitude obtained from histograms (Fig. 9B; \(p = 0.58\)), supporting the hypothesis that minimal evoked amplitudes represent unitary quantal events.

To understand the relationship between total numbers of VGCCs at AZs and numbers of primed vesicles we then calculated the number of APs required to deplete the primed vesicle pool by preventing vesicle priming with botulinum toxin B (BoNT/B). BoNT/B prevents neurotransmission by selectively cleaving synaptobrevin but cannot cleave targets after formation of ternary SNARE complexes during priming (Pellegrini et al., 1994). Thus, after BoNT/B injection into presynaptic axons the stimulus dependent rate of loss of axonal response represents the exhaustion of the BoNT/B resistant primed vesicle pool. In paired recordings, again made between microelectrode recorded reticulospinal axons and whole-cell recorded targets, axons were pressure injected with light chain BoNT/B (Gerachshenko et al., 2005) in the microelectrode filling solution. After BoNT/B injection, and after 5 min to allow cleavage of unprimed synaptobrevin (Gerachshenko et al., 2005), APs were repeatedly stimulated to evoke EPSCs (Fig. 9Da). The time course (\(t\)) of EPSC amplitude decay expressed as number of APs was 100 \(\pm\) 17 (Fig. 9D). If we consider \(P\) (Fig. 9B), the mean number of primed vesicles per AZ is 22, which corresponds very closely to the mean number of Ca\(^{2+}\) channels at the AZ (23 channels).

**Discussion**

Evoked neurotransmission is triggered by Ca\(^{2+}\) influx within 60 \(\mu\text{s}\) of AP firing (Sabatini and Regehr, 1996) close to the fusion machinery (Adler et al., 1991). However, how many VGCCs open to trigger fusion of single vesicles remains unclear. While this has previously proven impossible to visualize in simple low probability central synapses with single AZs, recordings from larger, specialized calyceal synapses (Stanley, 1991; Sheng et al., 2012) reveal complex AZs with mixed properties. Single VGCCs (Ca\(^{2+}\) nanodomains) may gate release (Stanley, 1993; Bucurenciu et al., 2010; Weber et al., 2010; Eggermann et al., 2012; Scimemi and Diamond, 2012), and in ciliary ganglion synapses are tethered to docked vesicles (Wong et al., 2014). At other synapses multiple VGCCs (Ca\(^{2+}\) microdomains) drive release (Llinas et al., 1992; Oheim et al., 2006), with a developmental shift to nanodomain-evoked release at the calyx of Held (Borst and Sakmann, 1996; Nakamura et al., 2015). Indirect recordings have suggested few VGCCs gate release in other synapses, such as frog neuromuscular junction (Shahrezaei et al., 2006), parallel fiber/Purkinje synapses (Kusch et al., 2018), excitatory and inhibitory cortical (Bucurenciu et al., 2008; Schmidt et al., 2013) and ribbon synapses (Brandt et al., 2005). While distances between VGCCs and the release machinery vary (Schneggenburger and Neher, 2000), a striking parallel is the requirement for little Ca\(^{2+}\) for release (Stanley, 2016; Hamid et al., 2019). Universical release at simple lamprey reticulospinal AZs (Schwartz et al., 2007), allows determination of Ca\(^{2+}\) requirements where an AP evokes release of one vesicle (Brodin and Shupliakov, 2006).

Our cell-attached recordings demonstrate a small pool of VGCCs (14–68, mean of 33, N, P/Q, R, and L-type) at each AZ. Channel openings were only recorded at sites of stimulated vesicle turnover, supporting earlier imaging experiments (Takahashi et al., 2001; Photowala et al., 2005; Bleckert et al., 2012) demonstrating localization of presynaptic Ca\(^{2+}\) channels to AZs. Of these channel subtypes, we found 11–51 (mean of 23) of N-type, P/Q-type, and R-type channels. It is these channels that contribute to Ca\(^{2+}\) signals for release (Wheeler et al., 1994; Gasparini et al., 2001), including in lamprey synapses (Krieger et al., 1999; Büschges et al., 2000; Photowala et al., 2005). Similar observation of multiple channel subtypes was reported by direct recordings at rat calyx of Held terminals (Sheng et al., 2012) and through imaging calcium transients or analysis of neurotransmission at other terminals (Wheeler et al., 1994; Poncer et al., 1997; Wu et al., 1999; Fox et al., 2008; Cao and Tsien, 2010). In contrast, at chick ciliary terminals, N-type channels are the primary source of calcium driving release (Stanley, 1991, 1993).

Our observation of L-type channels at AZs is intriguing, as they are not implicated in synaptic transmission (Krieger et al., 1999; Photowala et al., 2005), suggesting a novel role. The presence of multiple channel subtypes suggests potential differential occupancy of slots with varying distance from the primed vesicle (Cao and Tsien, 2010) and differential requirements for multiple channel subtypes in neurotransmission. We have previously found that blocking R-type channels most strongly reduced synaptic transmission (Photowala et al., 2005). This is supported by our analyses of channel numbers at single terminals where we find greater numbers of R-type channels 4–32 (mean of 12). Importantly, even with all channel subtypes available, at each AZ, few channels (1–7, mean of 4) from the total pool of channels (mean of 23) open during depolarization. That few channels open during APs is confirmed by quantitative imaging of the molar amount of Ca\(^{2+}\) that enters each AZ revealing Ca\(^{2+}\) entry of \(<\sim4\) ns per AP. Thus, we confirmed in intact axons in intact spinal cords that up to just four channels open during single APs at single AZs.

We show similar average numbers of presynaptic channels to those reported at rat calyceal terminals (Sheng et al., 2012). However, calyceal terminals were more heterogenous, and demonstrated greater variance in channel numbers (5–218, mean of 42). This represents a more complex synaptic architecture in calyceal terminals where many AZs form around a complex synaptic cleft. More notably our findings that, of the available pool, very few (1–7, mean of 4) channels open, resemble observations at rat calyceal terminals (1–35, mean of 7; Sheng et al., 2012). Nevertheless, the range of available channels varies less in lampreys. This may reflect the simplicity of the lamprey single AZs, where \(P\) also varied little (\(P\) range in lamprey axons, 0.16–0.25). The low variance in size of lamprey single synapses (Gustafsson et al., 2002) and of \(P\) in this study, compared with calyceal synapses (Sheng et al., 2012), corresponds to a lower variance in Ca\(^{2+}\) channel numbers. Possible differences between stronger and weaker synapses, in terms of coupling between VGCCs and
Docked vesicles (Rebola et al., 2019), could explain our observed variability in channel openings.

To further investigate relationships between primed vesicles and Ca\(^{2+}\) channels, we compared numbers of primed vesicles with available channels to show a remarkably similar number (23 channels and 22 primed vesicles). There is substantial evidence that primed vesicles form as part of a protein complex that tether, or is tethered by, fusion competent Ca\(^{2+}\) channels (Stanley, 1993; Kaeser et al., 2011; Wong et al., 2014). Thereby, entry of small amounts of calcium can trigger vesicle fusion (Stanley, 1993, 2016). Our findings from multiple approaches indicate that few channels, perhaps even one, open during single APs. That there are such small numbers of channels with near 1:1 stoichiometry to primed vesicle numbers suggests direct coupling between primed vesicle complexes and activation of Ca\(^{2+}\) channels. This is supported by the opening of small numbers of channels during APs. High-speed LLS imaging of single AP-evoked presynaptic Ca\(^{2+}\) transients demonstrated that channel openings are stochastic. Random variations in amplitudes of evoked Ca\(^{2+}\) transients in subregions of the AZ indicate that the position of individual channel openings within the AZ varies between APs. These findings strongly support the nanodomain model of evoked release, where few, or just one channel causes release (Stanley, 1993; Brandt et al., 2005; Gentile and Stanley, 2005; Shahrezaei et al., 2006; Bucurenciu et al., 2010).

LLSM imaging also confirmed that APs evoked few channel openings. By imaging the same AZs repeatedly we resolved Ca\(^{2+}\) signals that showed a unitary quantum of amplitude. Because this distribution resolves as 0–6 peaks above background noise and corresponds to numbers of channels from cell-attached recording and dye/buffer analysis, we conclude that these peaks and corresponds to numbers of channels from cell-attached recording and dye/buffer analysis, we conclude that these peaks correspond to individual channel openings. From these data in the six AZs studied typically in one AP a mean of just two channels opened with a range from 0 to 6. These results imply that variance in channel opening may partly explain quantal variation in evoked release of a limited number of primed vesicles paired with few channels.

Prior results in lamprey synapses favor nanodomains between Ca\(^{2+}\) channels and the release machinery, with synaptic transmission resistant to the slow Ca\(^{2+}\) buffer, EGTA (Schwartz et al., 2007). This is consistent with other synapses where Ca\(^{2+}\) channels responsible for fusion are extremely close to primed vesicles, including at central terminals (Stanley, 1991, 1993; Sheng et al., 2012; Hamid et al., 2019). Such close association even from one channel is sufficient to elevate Ca\(^{2+}\) to hundreds of micromolar required for Ca\(^{2+}\)-synaptotagmin interaction (Radhakrishnan et al., 2009) and fusion (Augustine, 2001). It is possible that sequential APs activate up to 20 available channels distributed across the AZ with low probability. We have used multiple methods to confirm that at each AZ, small numbers or single Ca\(^{2+}\) channels open at each AP. Our LLS data emphasize this, at a resolution not previously obtainable at synapses, showing distinct amplitude bins consistent with single channel data obtained from cell-attached patch recordings and even failures of events at single terminals. These data also indicate that the events occur stochastically across the AZ.

Our results are consistent with fusion competent primed vesicle complexes that colocalize with one channel. The number of primed vesicles and available Ca\(^{2+}\) channels is equal at AZs (a stoichiometry of 1:1). Ca\(^{2+}\) channel openings are dispersed across the AZ rather than clustered, and few channels open (approximately four). Thus, we propose that one channel is sufficient to evoke fusion because the channels are part of a fusion competent complex. Nevertheless, there remains a low probability of such an opening channel evoking release following any single AP. The overall effect implies a minimal Ca\(^{2+}\) load, that might otherwise be toxic, and minimized metabolic energy required for Ca\(^{2+}\) clearance. Nevertheless, ~20 primed vesicles with P\(_0\) of 0.22 allows 100 APs to fire before exhausting the primed pool. The reticulospinal axons can maintain up to ~50-Hz firing rates (Sirota et al., 2000) leaving 2 s for this exhaustion. This time frame, as in most synapses, allows replenishment of the primed pool to maintain signaling over long periods. Our findings present a model for efficient synaptic transmission at central synapses that achieves rapid neurotransmission with high temporal precision, while permitting maintenance of this transmission.

References


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