Different Relationship of N- and P/Q-Type Ca^{2+} Channels to Channel-Interacting Slots in Controlling Neurotransmission at Cultured Hippocampal Synapses

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Synaptic transmission at CNS synapses is often mediated by joint actions of multiple Ca^{2+} channel subtypes, most prominently, P/Q- and N-type. We have proposed that P/Q-type Ca^{2+} channels saturate type-preferring slots at presynaptic terminals, which impose a ceiling on the synaptic efficacy of the channels. To test for analogous interactions for presynaptic N-type Ca^{2+} channels, we overexpressed their pore-forming $\text{Ca}_v2.2$ subunit in cultured mouse hippocampal neurons, recorded excitatory synaptic transmission from transfected cells, and dissected the contributions of N-, P/Q-, and R-type channels with subtype-specific blockers. Overexpression of $\text{Ca}_v2.2$ did not increase the absolute size of the EPSC even though somatic N-type current was augmented by severalfold. Thus, the strength of neurotransmission is saturated with regard to levels of Ca^{2+} channel expression for both N-type and P/Q-type channels. Overexpression of Ca^{2+} -impermeable $\text{Ca}_v2.2$ subunits decreased EPSC size, corroborating competition for channel slots. Striking asymmetries between N- and P/Q-type channels emerged when their relative contributions were compared with channel overexpression. Overexpressed N-type channels could competitively displace P/Q-type channels from P/Q-preferring slots and take over the role of supporting transmission. The converse was not found with overexpression of P/Q-type channels, regardless of their C-terminal domain. We interpret these findings in terms of two different kinds of presynaptic slots at excitatory synapses, one accepting N-type channels but rejecting P/Q-type (N_{specific}) and the other preferring P/Q-type but also accepting N-type ($PQ_{\text{preferring}}$). The interaction between channels and slots governs the respective contributions of multiple channel types to neurotransmission and, in turn, the ability of transmission to respond to various stimulus patterns and neuromodulators.

Introduction

Presynaptic voltage-gated Ca^{2+} channels (VGCCs) are activated on action potential (AP) invasion to allow rapid influx of extracellular Ca^{2+} , which, in turn, triggers synaptic vesicle fusion and neurotransmitter release (Katz, 1969). Proximity of Ca^{2+} channels to the vesicle release machinery is critical for rapid and efficient synaptic transmission (Llinás et al., 1976; Stanley, 1993), suggesting an intimate relationship between the presynaptic Ca^{2+} channels and the release apparatus. Studies of hippocampal and cerebellar synapses indicate that the vast majority of single release sites are in close proximity to a mixed population of Ca^{2+} channels that jointly contribute to the local Ca^{2+} transient that triggers vesicular fusion (Mintz et al., 1995; Reuter, 1995; Reid et al., 1997). Moreover, it is well established that synaptic transmission at most CNS synapses is mediated by joint actions of multiple Ca_v2 subfamily of VGCCs, most prominently, P/Q- and

N-type (Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994; Sheng et al., 1998; Wu et al., 1999). This raises an important question as to whether P/Q- and N-type Ca^{2+} channels exhibit subtype specificity in their relationship to the release machinery.

Morphological studies have provided significant insights about the possible existence of presynaptic slots for VGCCs at transmitter release sites (Heuser et al., 1979; Robitaille et al., 1990; Haydon et al., 1994; Harlow et al., 2001). Biochemical studies have identified numerous molecular interactions between VGCCs and their binding partners that play a crucial role in coupling presynaptic VGCC to the release machinery (Sheng et al., 1998; Maximov et al., 1999; Missler et al., 2003; Spafford et al., 2003; Nishimune et al., 2004; Leenders et al., 2008). Although in most cases the putative molecular interaction does not discriminate between N- and P/Q-type channels, some degree of subtype specificity has been reported. For example, the II–III loops of N- and P/Q-type channels vary in their ability to interact with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, elements of the synaptic release machinery, and to support synaptic targeting of VGCCs (Mochida et al., 2003). Moreover, the postsynaptic density-95/Discs large/zona occludens-1 (PDZ) domain-binding motif and the proline-rich region at the cytosolic C termini of N-type VGCCs interact with CASK–Mint1–Veli complex, the modular adaptor proteins at the presynaptic active zone (Butz et al.,

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1998; Maximov et al., 1999). This interaction is both necessary and sufficient for synaptic targeting of N-type channels in cultured hippocampal neurons (Maximov and Bezprozvanny, 2002). On the contrary, the corresponding C-terminal region of P/Q-type Ca^{2+} channel is not an essential determinant for its subcellular localization, again suggesting the possibility of subtype specificity of VGCC synaptic targeting (Hu et al., 2005).

In a previous study in cultured hippocampal neurons, we demonstrated that endogenous P/Q-type channels fill up their type-preferring “slots” (Cao et al., 2004). The term “slots” refers to binding sites of limited capacity that interact with channel proteins to control their participation in neurotransmission. The channel–slot interaction ultimately plays out at presynaptic terminals, but the term leaves open whether it begins with competition for auxiliary proteins that allow exit from the endoplasmic reticulum (ER) (Saheki and Bargmann, 2009), for proteins involved in axonal sorting and trafficking (Mochida et al., 2003; Ziv and Garner, 2004), or for active zone components at the presynaptic terminal itself (Maximov and Bezprozvanny, 2002). Here, we proceed to explore the question of whether type-specific slots for presynaptic N-type Ca^{2+} channels coexist along with those that harbor P/Q-type channels. Is neurotransmission operating at a biological ceiling with respect to both channel types? Our results indicate that there exist two different kinds of functional slots for presynaptic N- and P/Q-type Ca^{2+} channels: one accepting N-type but not P/Q-type (N_{specific}) and the other accepting N-type channels but preferring P/Q-type ($PQ_{\text{preferring}}$). Both kinds of slots are filled to capacity under basal conditions. We propose that the channel–slot interactions may play important roles in determining the relative importance of the two kinds of Ca^{2+} channel and, in turn, the responsiveness of hippocampal synaptic transmission to modulation by firing pattern and G-protein-coupled receptors (GPCRs).

Portions of this manuscript have been published previously in abstract form (Cao and Tsien, 2006).

Materials and Methods

Human $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ constructs. The human $\text{Ca}_v2.1$ cDNAs used in this study encode the splice variant: $\Delta 10A$ (–V+G), 16^+17^+ , $\Delta 17A$ (–VEA), -31^* (–NP), $37a$ (Efa), 43^+44^+ , either with or without exon 47 (Soong et al., 2002). The wild-type human $\text{Ca}_v2.2$ cDNA used in this study encodes the $\text{Ca}_v2.2a$ (α_{1B-1}) isoform and can travel to synaptic terminals to mediate neurotransmitter release (Maximov and Bezprozvanny, 2002). The nonpermeable $\text{Ca}_v2.2$ subunit was generated by replacing all four pore glutamates with alanine residues (E314A, E663A, E1365A, and E1655A) in S5–6 regions of each domain. Individual mutation was generated by PCR mutagenesis (Stratagene). All cDNAs were cloned into mammalian expression vectors under the control of CMV (cytomegalovirus) promoter.

Cell culture and transfection. Characterization of the nonpermeable $\text{Ca}_v2.2$ subunit was performed in a stable HEK293 cell line expressing β_{1C} and $\alpha_2\delta$ subunits as described previously (Piedras-Renteria et al., 2001). Cells were cotransfected with constructs encoding wild-type or nonpermeable $\text{Ca}_v2.2$ subunit along with the pEGFP1 plasmid with Lipofectamine 2000. Transfected cells were identified by enhanced green fluorescent protein (EGFP) fluorescence and were recorded 2–3 d after transfection.

Mouse hippocampal neurons were cultured from 1-d-old pups at low density (~5000 cells per coverslip) as described previously (Cao et al., 2004). All animal handling was in accordance with guidelines of the Animal Care and Use Committee. Individual human Ca_v2 construct was cotransfected with pEGFP1 plasmid (1:1 molar ratio) into 5 or 10 d *in vitro* (DIV) culture using calcium phosphate method (Cao et al., 2004).

Electrophysiology. Transfected neurons were identified by EGFP fluorescence with Nikon TE-2000 microscope. Whole-cell patch-clamp recordings were performed at room temperature with Axopatch 200A or MultiClamp 700B amplifiers (Molecular Devices). pClamp 10 (Molecular Devices) was used to acquire and analyze data. Signals were filtered at 1 kHz and digitized at 10 kHz except for the current-clamp experiments (see below). Recording pipettes had <3.5 M Ω resistance. Cell capacitance and series resistance (<20 M Ω) were constantly monitored throughout recording.

Ba^{2+} current recording. Transfected neurons were recorded between 7 and 11 DIV. The recording chamber was perfused with extracellular solution (0.5 ml/min) containing the following (in mM): 140 tetraethylammonium (TEA)-Cl, 10 BaCl_2 , 10 HEPES, 20 glucose, pH 7.3 with TEA-OH, 310 mOsm. The pipette solution contained the following (in mM): 110 CsCl, 10 EGTA, 4 ATP-Mg, 0.3 GTP-Na, 25 HEPES, 10 Tris-phosphocreatine, 20 U/ml creatine phosphokinase, pH 7.3 with CsOH, 290 mOsm. Series resistance was compensated by 80%. Current traces were corrected for linear capacitive leak with on-line P/6 trace subtraction. Neurons were voltage clamped at –80 mV, and test pulses (+10 mV for 35 ms or as described in Results) were applied every 10 s. L-, N-, and P/Q-type currents were identified as currents sensitive to their specific blockers: nifedipine (10 μM), ω -conotoxin-GVIA (ω -CTX-GVIA) (2 μM) and ω -agatoxin-IVA (ω -Aga-IVA) (1 μM), respectively. The remaining Cd^{2+} -sensitive (100 μM) current was taken as R-type current. For each neuron, the peak current was normalized by the membrane capacitance (a measure of cell surface area) to reflect current density.

To measure the current–voltage relationships (*I*–*V* curves), transfected HEK293 cells were held at –80 mV and were depolarized from –60 to +100 mV (10 mV increments) for 35 ms and then repolarized back to –80 mV. *I*–*V* curves in neurons were generated by a ramp depolarization from –80 to +100 mV at 1.8 mV/ms.

Excitatory synaptic transmission. Neurons were recorded between 12 and 18 DIV to study neurotransmission. The recording chamber was perfused with Tyrode's solution (0.5 ml/min) containing the following (in mM): 125 NaCl, 2 KCl, 4 CaCl_2 , 4 MgCl_2 , 25 HEPES, 30 glucose, pH 7.3 with NaOH, 310 mOsm. The pipette solution contained the following (in mM): 100 KCl, 2 EGTA, 4 ATP-Mg, 0.3 GTP-Na, 30 HEPES, 10 Tris-phosphocreatine, 20 U/ml creatine phosphokinase, pH 7.3 with KOH, 290 mOsm. A nonfluorescent neuron in close proximity to the transfected, EGFP-positive neuron was chosen as its possible postsynaptic target. Both neurons were voltage clamped at –70 mV. The transfected, presynaptic neurons were depolarized to 0 mV for 1 ms to elicit APs once every 10 s. Correspondingly, an EPSC could be recorded at the untransfected, postsynaptic neuron. Peak EPSC amplitude under each pharmacological condition was measured as the average of 10–20 responses. N-, P/Q-, and R-type Ca^{2+} channels were blocked by ω -CTX-GVIA (2 μM), ω -Aga-IVA (1 μM), and SNX482 (0.5 μM), respectively. When studying paired-pulse modulation, presynaptic neurons were given paired depolarization at 50 ms intervals.

Characterization of AP properties. The external and pipette solutions were the same as those in studying excitatory synaptic transmission. AMPA and NMDA receptor blockers 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX) (10 μM) and AP5 (25 μM) were added to the external solution to prevent recurrent firing. Transfected hippocampal neurons (12–18 DIV) were held under current-clamp mode. Somatic APs were evoked by a 0.5 ms, 1 nA depolarizing current injection every 10 s. Signals were filtered at 10 kHz and digitized at 100 kHz.

Drug application. Nifedipine (Sigma-Aldrich) was dissolved in 100% ethanol to generate 10 mM stock solution. All peptide Ca^{2+} channel blockers were purchased from Peptides International, reconstituted in 1 mg/ml cytochrome *c* (Sigma-Aldrich) at 100 $\times$ concentration, and stored at –80°C in aliquots. Before the application of Ca^{2+} channel blockers, the perfusion to the recording chamber was stopped and cytochrome *c* was added to the bath to 0.1 mg/ml final concentration. Subsequently, the blockers were added to the bath sequentially and cumulatively as described in Results. In some experiments, NBQX (10 μM) and D-AP5 (25 μM ; both from Tocris) were used to block AMPA and NMDA receptors.

Statistical analysis. All data are reported as mean $\pm$ SEM or cumulative percentage histogram. Statistical significance between multiple experimental groups was assessed by ANOVA with *post hoc* Bonferroni's test. The nonparametric Kolmogorov–Smirnov test was used to compare EPSC amplitudes between two experimental groups.

Results

Long and short isoforms of $\text{Ca}_v2.1$ both occupy type-specific presynaptic slots

Both $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$, the pore-forming subunits of P/Q- and N-type Ca^{2+} channels, undergo extensive alternative splicing at their cytosolic C termini (Soong et al., 2002). Our previous study focused on the “short” isoform of human $\text{Ca}_v2.1$ subunits (Fig. 1A, PQ_{WT}) and demonstrated that their expression in hippocampal neurons from $\text{Ca}_v2.1$ knock-out mice fully restored the contribution of P/Q-type Ca^{2+} channels to neurotransmission (Cao et al., 2004). This left open questions about the “long” isoform of $\text{Ca}_v2.1$. Biochemical studies suggest that, unlike the short isoforms, the long isoforms of $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ subunits (Fig. 1A, $\text{PQ}_{\text{WT-long}}$ and N_{WT}) use their PDZ domain-binding motifs and the proline-rich regions to interact with CASK–Mint1–Veli complex (Maximov et al., 1999). Because this interaction may be both necessary and sufficient for synaptic targeting of N-type channels (Maximov and Bezprozvanny, 2002), we wondered whether synaptic targeting of P/Q-type channels would also differ according to the nature of their C termini. Accordingly, we directly compared the synaptic effects of overexpressing short and long isoforms of $\text{Ca}_v2.1$ (PQ_{WT} and $\text{PQ}_{\text{WT-long}}$) in cultured hippocampal neurons.

To study synaptic transmission, we used paired patch-clamp recordings from an EGFP-fluorescent, transfected neuron and its untransfected postsynaptic neuron target. Evoking a presynaptic spike in the transfected neuron typically resulted in an EPSC in the postsynaptic neuron (Fig. 1B, no blocker). Subtype-specific VGCC blockers ω -CTx-GVIA (2 μM), ω -Aga-IVA (1 μM), and SNX482 (0.5 μM) were sequentially and cumulatively introduced into the recording chamber to block N-, P/Q-, and R-type Ca^{2+} channels, respectively. The addition of each toxin produced a stepwise reduction in EPSC size that was readily quantified (Fig. 1B). The EPSC was completely blocked with all three toxins present, as confirmed by the absence of any additional effect of AMPA receptor blocker NBQX (data not shown). This allowed us to calculate the relative contributions of N-, P/Q-, and R-type Ca^{2+} channels to synaptic transmission. Under this experimental paradigm, we found that the contributions of endogenous N- and P/Q-type channels were each reflected by a $\sim 45\%$ reduction of the transmission at synapses expressing EGFP alone (Fig. 1C, EGFP). The contributions of N- and P/Q-type channels to transmission in the PQ_{WT} group were similar to that of EGFP group. Thus, an excess of the short isoform of $\text{Ca}_v2.1$ did not alter the relative contributions of N- and P/Q-type channels to neurotransmission, confirming our previous results (Cao et al., 2004). We went on to express the long isoform, testing the notion that the C-terminal PDZ domain-binding motif of $\text{PQ}_{\text{WT-long}}$ might confer the ability to displace N-type channels. In fact, overexpressing $\text{PQ}_{\text{WT-long}}$ did little to alter the pharmacology of transmission (Fig. 1C). The relative contribution of P/Q-type channels was not significantly greater than that in the EGFP or PQ_{WT} groups, nor was the contribution of N-type channels diminished. To verify that $\text{PQ}_{\text{WT-long}}$ was fully capable of expression and support of neurotransmission at nerve terminals, we transfected cultured hippocampal neurons from $\text{Ca}_v2.1$ knock-out mice with constructs encoding EGFP and $\text{PQ}_{\text{WT-long}}$ (Fig. 1D). $\text{PQ}_{\text{WT-long}}$ channels were able to reconstitute pharmacological responsive-

ness to ω -Aga-IVA, just as previously shown for PQ_{WT} (Cao et al., 2004) (data repeated for ease of comparison in Fig. 1D). After previous application of ω -CTx-GVIA, exposure to the P/Q-type channel blocker caused a $50 \pm 14\%$ drop in EPSC size in the $\text{PQ}_{\text{WT-long}}$ -transfected $\text{Ca}_v2.1$ knock-out neurons (Fig. 1D), no different ($p > 0.6$) than the $41 \pm 4\%$ decrease seen in wild-type mouse neurons with endogenous P/Q-type channels (Fig. 1C, EGFP group). The cumulative distribution of overall EPSC size remained unaltered, regardless of whether long or short forms of $\text{Ca}_v2.1$ were overexpressed (Fig. 1E). Given that $\text{PQ}_{\text{WT-long}}$, like PQ_{WT} , is fully capable of expression at nerve terminals, it is striking that expression of either long or short forms did not increase synaptic strength.

Together, these observations suggest that both isoforms of P/Q-type channels occupy limited number of presynaptic slots with similar overall efficacy. The C-terminal PDZ domain-binding motif does not alter P/Q-type channel–slot interaction as defined in our previous study. These results are in line with a previous report (Hu et al., 2005) indicating that, unlike N-type channels, P/Q-type channels do not use their C-terminal PDZ domain-binding motif as an essential determinant for synaptic localization.

Overexpression of N-type Ca^{2+} channel significantly increases its contribution to excitatory transmission

The lack of significant reduction in the contribution of N-type channels (Fig. 1C, open bars) to synaptic transmission in PQ_{WT} and $\text{PQ}_{\text{WT-long}}$ groups implies that endogenous N-type channels likely occupy additional slots, different from those that P/Q-type channels are able to access. This raises several additional questions: for example, if additional slots exist, are they specific to N-type channels, and are they filled to capacity? When N-type channels are overexpressed, can they displace P/Q-type channels from their slots? We approached these questions by expressing human $\text{Ca}_v2.2$ (Fig. 1A, N_{WT}), the pore-forming subunit of N-type Ca^{2+} channel, in cultured hippocampal neurons. The isoform of N_{WT} we used contains the PDZ domain-binding motif and the proline-rich region at C terminus and can travel to synaptic terminals to mediate neurotransmitter release (Maximov and Bezprozvanny, 2002). We focused on excitatory transmission from neurons overexpressing N_{WT} and dissected contributions of N-, P/Q-, and R-type channels, using the same system of paired patch-clamp recordings. Whereas neurons expressing EGFP alone, PQ_{WT} , or $\text{PQ}_{\text{WT-long}}$ underwent a $\sim 42\%$ reduction of EPSCs with exposure to ω -CTx-GVIA, transmission from neurons overexpressing N_{WT} behaved a strikingly different way—in this case, N-type channels accounted for $\sim 80\%$ of the EPSC amplitude, significantly higher than in any of the other groups ($p < 0.05$). Evidently, the contribution of endogenous N-type channels was not at a biological ceiling, in sharp contrast to P/Q-type channels.

In the preceding experiments, N_{WT} subunits were overexpressed by transfection of low-density hippocampal cultures at 5 DIV, with the goal of achieving high transfection efficiency. Because 5 DIV is about the time of increased synaptogenesis and expression of P/Q-type channels, it seemed plausible that such early overexpression of N_{WT} might affect the development or maturation of synapses, to the detriment of proper assembly of slots and of P/Q-type channel participation. Accordingly, we performed additional experiments wherein overexpression of N_{WT} was postponed to 10 DIV, thus allowing synaptogenesis to be well advanced and endogenous P/Q-type channel expression to approach a plateau before the introduction of excess N_{WT} subunits.

Despite this delay, overexpression of N_{WT} still resulted in an enhanced contribution of N-type channels to excitatory transmission, seen as increase in ω -CTx-GVIA block from $51 \pm 5\%$ (Fig. 1C, EGFP) to $80 \pm 8\%$ (Fig. 1C, $N_{WT-10 \text{ DIV}}$ group, $p < 0.05$), very similar to the effect of expressing N_{WT} at 5 DIV. On the other hand, the contribution of P/Q-type Ca^{2+} channels to transmission decreased from 41 ± 4 to $16 \pm 7\%$ when N_{WT} was overexpressed (Fig. 1C, filled bars, $p < 0.05$).

We performed additional tests to determine whether these results can be attributed to a genuine difference between N- and P/Q-type channels in their disposition with respect to the presynaptic release machinery. One possibility to consider is that the excess of N_{WT} channels drives the creation of ectopic release sites, thus expanding the impact of the N-type contribution to transmission by augmenting neurotransmission as a whole. However, we observed that overall EPSC size did not differ between synapses expressing EGFP or N_{WT} subunits (Fig. 1E), indicating that N_{WT} overexpression did not enlarge the overall strength of transmission. Evidently, the number of release sites and their individual probability of release (P_r) both remained unaltered. As a separate gauge of P_r , we looked for changes in paired-pulse modulation, which veers toward paired-pulse depression rather than facilitation as basal P_r rises. The ratio of the EPSC amplitudes elicited by pairs of stimuli 50 ms apart was determined. This paired-pulse ratio (PPR) proved to be no different among the various groups, EGFP (1.16 ± 0.04), PQ_{WT} (1.20 ± 0.08), $PQ_{WT-long}$ (1.14 ± 0.12), and N_{WT} (1.18 ± 0.06) ($p > 0.6$ by ANOVA). The lack of change in paired-pulse modulation supported the idea that P_r had remained unaffected by the excess of one or other type of Ca^{2+} channel.

These data on the fractional contributions of various Ca_v2 channels pointed to a marked asymmetry: overexpressed N-type channels were able to gain dominance over P/Q-type channels but not vice versa. Given that the overall strength of transmission did not increase (Fig. 1E), a

of N-type-dependent EPSC ($p < 0.05$ vs EGFP group). No significant difference was found between PQ_{WT} and EGFP groups ($p > 0.4$). **G**, Cumulative distribution of ω -Aga-IVA-sensitive EPSC amplitude of EGFP, N_{WT} , and PQ_{WT} groups of neurons as in **C**. The P/Q-component of the EPSC was significantly smaller in the N_{WT} group ($p < 0.05$ vs EGFP group; $p < 0.01$ vs PQ_{WT} group). No significant difference was found between PQ_{WT} and EGFP groups ($p > 0.3$).

Given the steeply nonlinear relationship between Ca^{2+} influx and presynaptic P_r (Rahamimoff and Dodge, 1969), the effect of toxins on synaptic transmission depends on their order of application (Takahashi and Momiyama, 1993; Wheeler et al., 1994). With this in mind, we determined whether the asymmetry between the effects of PQ_{WT} and N_{WT} held true regardless of which channel type was blocked first. Figure 2A illustrates the outcome of reversing the order of toxin presentation, applying ω -Aga-IVA before ω -CTx-GVIA. In this case, ω -CTx-GVIA blocked $17 \pm 3\%$ of the EPSC at wild-type synapses, but $37 \pm 6\%$ of the EPSC at synapses expressing N_{WT} , a significant increase ($p < 0.05$) (Fig. 2A). Conversely, if one performs a direct comparison of the effect of ω -Aga-IVA in EGFP and N_{WT} groups (temporarily ignoring the PQ_{WT} data) (Fig. 2A, legend), the fractional P/Q-mediated transmission was $76 \pm 4\%$ in the EGFP cells and $60 \pm 6\%$ in the N_{WT} group, significantly less ($p < 0.03$). These data provided additional evidence that overexpression of N-type Ca^{2+} channel significantly increased their contribution to excitatory transmission while concomitantly reducing the participation of P/Q-type.

Figure 3. Characterization of nonpermeable human $\text{Ca}_v2.2$ subunits (N_{imperm}). **A**, Topology of human $\text{Ca}_v2.2$ subunit. The locations of four concerted glutamate-to-alanine mutations in the pore-forming region of N_{imperm} are indicated. **B**, Representative AP traces from hippocampal neurons expressing EGFP alone or EGFP with N_{imperm} . Somatic APs were evoked by a depolarizing current injection (0.5 ms, 1 nA). Expression of N_{imperm} did not change AP waveform (N_{imperm} vs EGFP group). Furthermore, blocking both endogenous N-type Ca^{2+} channels and exogenous N_{imperm} channels with $2 \mu\text{M}$ ω -CTx-GVIA did not significantly alter the AP shape (N_{imperm} + GVIA vs other groups). See Table 1 for comparisons of AP properties. **C**, The current–voltage relationships (I – V curves) of N_{WT} and N_{imperm} in HEK293 cells ($n = 5$ in each group). The N_{imperm} group did not show any inward Ba^{2+} current but displayed an outward current when depolarized beyond 0 mV. **D**, The I – V curves of the N-type (ω -CTx-GVIA-sensitive) Ba^{2+} current in hippocampal neurons expressing EGFP alone (EGFP, $n = 6$), EGFP with N_{imperm} (N_{imperm} , $n = 5$), or EGFP with both N_{imperm} and P/Q_{WT} subunits (N_{imperm} + P/Q_{WT} , $n = 8$). Transfected neurons were voltage clamped at -80 mV. I – V curves were generated by applying a ramp depolarization from -80 to $+100$ mV at 1.8 mV/ms. The ω -CTx-GVIA-sensitive current densities were obtained by subtraction of I – V relationships in the absence and presence of toxin. Inward currents were consistently seen over the voltage range between -20 and $+20$ mV. At more depolarizing voltages, I – V curves from N_{imperm} -expressing neurons exhibited a negative shift of reversal potential and an increase of outward current compared with the I – V curve of EGFP-expressing neurons, as expected for a combination of endogenous N-type channels and exogenous N_{imperm} channels operating in parallel. **E**, Peak whole-cell Ba^{2+} current density through P/Q-type Ca^{2+} channels at $+10$ mV from hippocampal neurons expressing EGFP alone (EGFP, $n = 12$), EGFP with N_{imperm} (N_{imperm} , $n = 8$), or EGFP with both N_{imperm} and P/Q_{WT} Ca_v2 subunits (N_{imperm} + P/Q_{WT} , $n = 7$). The expression of N_{imperm} channels did not alter the current density from endogenous P/Q-type channels (N_{imperm} vs EGFP groups), nor did it affect the increase of P/Q-current density as a result of overexpression of P/Q_{WT} ($***p < 0.001$, N_{imperm} + P/Q_{WT} vs other groups). **F**, Peak whole-cell Ba^{2+} current density through N-type Ca^{2+} channels at -10 mV from the same neurons in **D**. At this voltage, the N_{imperm} channels did not exhibit inward or outward current. The expression of N_{imperm} (and further addition of P/Q_{WT}) did not alter the current density from the endogenous N-type Ca^{2+} channels. Error bars indicate SEM.

It is conceivable that the seesaw relationship between contributions of N- and P/Q-type channels could arise because of a competition for a limited supply of auxiliary subunits. To test for this, we went on to examine the effect of overexpressing P/Q_{WT} , $\text{P/Q}_{\text{WT-long}}$, or N_{WT} subunits on whole-cell Ca^{2+} channel activity

(Fig. 2*B,C*). P/Q- and N-type components were defined as Ba^{2+} currents sensitive to their specific blockers ω -Aga-IVA ($1 \mu\text{M}$) and ω -CTx-GVIA ($2 \mu\text{M}$), respectively. In P/Q_{WT} or $\text{P/Q}_{\text{WT-long}}$ groups, whole-cell P/Q current densities were approximately fourfold greater than that in the EGFP group (Fig. 2*B1*, $p < 0.001$), whereas the level of P/Q-current density in P/Q_{WT} and $\text{P/Q}_{\text{WT-long}}$ groups was similar (Fig. 2*B1*). In contrast, overexpressing P/Q_{WT} or $\text{P/Q}_{\text{WT-long}}$ in neurons did not alter the somatodendritic N-type Ca^{2+} channel activity, as indicated by similar ω -CTx-GVIA-sensitive current densities in EGFP, P/Q_{WT} , and $\text{P/Q}_{\text{WT-long}}$ groups (Fig. 2*C1*). Neither L- and R-type current density was significantly altered (data not shown). These results indicated that hippocampal neurons were able to synthesize, assemble, and insert both isoforms of P/Q-type channels into somatodendritic membranes at approximately four times their normal level without compromising the activity of other high VGCCs. The same set of data provided reassurance that transfected neurons (as indicated by the EGFP fluorescence) in the P/Q_{WT} and $\text{P/Q}_{\text{WT-long}}$ groups consistently expressed exogenous P/Q-type channels. In both cases, the smallest P/Q-type current density was no less than the largest P/Q-type current density in neurons expressing EGFP alone (Fig. 2*B2*), suggesting that all neurons in the P/Q_{WT} and $\text{P/Q}_{\text{WT-long}}$ groups reliably expressed exogenous P/Q-type channels.

In the converse experiment, we found that overexpression of N_{WT} resulted in an approximately threefold increase of ω -CTx-GVIA-sensitive current density compared with that of EGFP group (Fig. 2*C1*, $p < 0.001$). Once again, the distributions of current were strikingly different; the smallest N-type current density in the N_{WT} group exceeded the largest N-type current density in the EGFP group (Fig. 2*C2*). Importantly, the magnitude of ω -Aga-IVA-sensitive current remained unaltered (Fig. 2*B1*). Based on these comparisons, we conclude that the interplay between N- and P/Q-type channels is not merely a matter of competition for a limited supply of ancillary Ca^{2+} channel subunits. Instead, N-type channels appear to compete with P/Q-type channels in some another way.

Using nonpermeable $\text{Ca}_v2.2$ subunits to probe for N-type slots

Our experiments uncovered a striking asymmetry between N- and P/Q-type channels. Although even massive overexpression of P/Q-type channels was unable to drive down the synaptic contri-

Table 1. N_{imper} channels do not alter AP properties in hippocampal neurons

	EGFP	N_{imper}	N_{imper} + GVIA
AP amplitude (mV)	99.9 ± 2.50	90.8 ± 2.66	96.3 ± 0.93
AP half-width (ms)	1.35 ± 0.08	1.58 ± 0.11	1.65 ± 0.20
Depolarization slope (mV/ms, 10–90%)	75.2 ± 3.22	71.8 ± 12.1	75.4 ± 11.2
Repolarization slope (–mV/ms, 10–90%)	86.1 ± 8.13	77.3 ± 5.28	70.1 ± 8.57

$n = 3\text{--}4$ in each group.

bution of N-type channels, the converse was not the case: N-type channels were capable of displacing P/Q-type. The finding of an irreducible contribution of N-type channels suggested the existence of slots specific for N-type, impervious to competition from P/Q-type.

To probe further for the possibility of N-type slots, we turned to the strategy of overexpressing Ca^{2+} -impermeable N-type channels (N_{imper}) and testing for dominant-negative effects. Human $\text{Ca}_v2.2$ subunits incapable of Ca^{2+} -permeation were generated by replacing all four pore glutamates with alanine residues (Fig. 3A), and their effects were tested in recordings of neuronal spikes (Fig. 3B) and synaptic transmission (see below). As a preliminary step, electrophysiological comparisons of N_{WT} and N_{imper} channels were performed in HEK293 cells (Fig. 3C). Unlike cells expressing N_{WT} , HEK293 cells expressing N_{imper} failed to display any inward Ba^{2+} current but only showed an outward current when depolarized beyond 0 mV. The outward current through N_{imper} channels was blocked by 2 μM ω -CTx-GVIA (data not shown), indicating that N_{imper} and N_{WT} exhibited similar responsiveness to the toxin blockade.

APs in both cell bodies and axons of excitatory neurons in hippocampus are dominated by large currents generated by Na^+ and K^+ channels (Schmidt-Hieber et al., 2008). Activation of VGCCs lags behind that of Na^+ channels (Bean, 2007), leading to opening of only a small fraction of Ca^{2+} channels for the narrow APs of hippocampal neurons (King and Meriney, 2005). To be sure that the outward current through N_{imper} channels would not exert confounding effects on AP generation, we expressed N_{imper} subunits along with EGFP or EGFP alone in hippocampal culture and measured somatic APs evoked by a 0.5 ms, 1 nA depolarizing current injection in transfected neurons. Expression of N_{imper} did not change AP properties (Fig. 3B); neither amplitude, half-width, or AP waveform were altered to any significant degree (Table 1). That key properties of spiking in the presynaptic neuron remained essentially unchanged was as expected if Na^+ and K^+ currents dwarfed currents through Ca^{2+} channels (either inward or outward). This is a worst-case analysis of what would occur in axons, in which Na^+ channels are denser than in cell bodies and would be even more dominant over Ca^{2+} channels. In an additional test, we exposed neurons expressing N_{imper} to ω -CTx-GVIA (2 μM), which, as already mentioned, acutely blocks outward as well as inward currents through N-type Ca^{2+} channels (McCleskey et al., 1987). The N-type specific blocker did not significantly alter AP properties (Fig. 3B, Table 1). This result further confirmed that N-type currents exerted little or no influence on AP shape in cultured hippocampal neurons.

As additional control experiments, we turned back to hippocampal neurons to find out whether N_{imper} interferes with the whole-cell Ba^{2+} current through the endogenous N- and P/Q-type Ca^{2+} channels. N-type current was isolated as the ω -CTx-GVIA-sensitive component of membrane currents evoked by a ramp depolarization from -80 to $+100$ mV. Hippocampal neurons expressing EGFP alone displayed the I - V relationship expected for endogenous N-type channels, first activating near

-20 mV, a milder depolarization than required in HEK293 cells, possibly because of differences in surface charge. In neurons expressing $\text{Ca}_v2.2$ subunits rendered incapable of Ca^{2+} permeation, the ω -CTx-GVIA-sensitive component consisted of clear inward currents at weaker depolarization levels, giving way to pronounced outward currents beyond $+20$ mV. This was just as expected for the summation of mostly inward currents carried by endogenous N-type channels (Fig. 3D, green symbols) and solely outward currents supported by N_{imper} channels (Fig. 3C, maroon symbols). Additional expression of PQ_{WT} channels (N_{imper} + PQ_{WT}) left the composite N-type I - V curve unchanged, suggesting that all three channel types operate independently and in parallel.

Further analysis of P/Q-type and N-type currents is documented in Figure 3, E and F. The ω -Aga-IVA-sensitive P/Q-type current density, gauged at a test potential of $+10$ mV, was similar in N_{imper} -expressing neurons to that of the control group, confirming that the number of somatodendritic P/Q-type channels remained unaltered (Fig. 3E). In contrast, P/Q-type current was increased approximately fivefold by additional expression of PQ_{WT} . We also assessed the magnitudes of ω -CTx-GVIA-sensitive current through endogenous N-type channels (Fig. 3F), using a test potential of -10 mV to avoid the outward current through N_{imper} channels (Fig. 3D). Neither overexpression of N_{imper} nor that of N_{imper} + PQ_{WT} in combination caused a significant alteration in currents through endogenous N-type Ca^{2+} channels.

Effects of N_{imper} channels on synaptic transmission

Having confirmed that endogenous N- and P/Q-type channels display normal activity in neurons expressing N_{imper} , we proceeded to test how N_{imper} affects the relative contribution of N- and P/Q-type channels to synaptic transmission as well as overall synaptic strength (Fig. 4). The expression of N_{imper} channels significantly reduced the size of EPSCs (Fig. 4A, C, D, $p < 0.05$, N_{imper} vs EGFP group), indicating that the nonpermeable N-type channels were properly expressed in the presynaptic neuron and somehow competed with endogenous channels. We again measured PPR as an indication of P_r changes. The ratio of the EPSC amplitudes elicited by pairs of stimuli 50 ms apart was significantly increased for presynaptic partners expressing N_{imper} (1.40 ± 0.07) compared with neurons expressing EGFP alone (1.16 ± 0.04 ; $p < 0.05$) (Fig. 4E), suggesting that impermeant N-type channels reduce P_r at presynaptic terminals. However, the clear reduction in synaptic strength was not accompanied by a shift toward P/Q-dominated transmission (Fig. 4B, N_{imper} vs EGFP group). The implication is that N_{imper} channels displaced both endogenous N- and P/Q-type channels from slots that they would otherwise occupy, leaving their relative contributions unchanged while diminishing transmission overall.

To substantiate this interpretation, we coexpressed PQ_{WT} along with N_{imper} , on the idea that this should ensure that P/Q-preferring slots are filled up with permeable P/Q-type channels, while leaving N_{imper} to compete with endogenous N-type channels. Indeed, we observed that coexpression of PQ_{WT} and N_{imper} resulted in a marked reduction of the N-type contribution to synaptic transmission, from 51 ± 4 to $21 \pm 3\%$ (Fig. 4B, open bars) (N_{imper} + PQ_{WT} vs EGFP group, $p < 0.05$). Correspondingly, the contribution of P/Q-type Ca^{2+} channels to transmission increased from 41 ± 4 to $70 \pm 4\%$ when PQ_{WT} and N_{imper} were overexpressed (Fig. 4B, filled bars) ($p < 0.05$). At the same time, the absolute strength of transmission was no less than the EGFP control (Fig. 4C, D), nor did values of PPR differ be-

Figure 4. Effects of N_{imperm} channels on synaptic transmission. **A**, Averaged EPSC traces (5–7 traces at the end of each condition) elicited by neurons expressing EGFP alone (EGFP), or EGFP with human Ca_v2 subunits (N_{imperm} , $N_{imperm} + \text{PQ}_{WT}$, and PQ_{WT} , respectively) as well as the effect of toxins on EPSC amplitude. Neurons were exposed to the N-, P/Q-, or R-type Ca^{2+} channel blockers ω -CTx-GVIA (2 μM), ω -Aga-IVA (1 μM), and SNX482 (0.5 μM) sequentially and cumulatively as indicated. **B**, Relative contributions of N-, P/Q-, and R-type channels to EPSC amplitude in neurons expressing EGFP alone (EGFP, $n = 20$), or EGFP with human Ca_v2 subunits (N_{imperm} , $N_{imperm} + \text{PQ}_{WT}$, and PQ_{WT} ; $n = 10$, 10, and 18, respectively). Ca^{2+} channels were blocked as shown in **A**. Overexpression of N_{imperm} did not alter the relative contributions of N-, P/Q-, and R-type channels to excitatory transmission. However, coexpression of PQ_{WT} and N_{imperm} resulted in a significant reduction of N-type contribution to synaptic transmission ($*p < 0.05$, $N_{imperm} + \text{PQ}_{WT}$ vs EGFP group). Correspondingly, the contribution of P/Q-type channels to transmission was significantly increased in $N_{imperm} + \text{PQ}_{WT}$ group ($\#p < 0.05$, compared with EGFP group). **C**, Cumulative distribution of overall EPSC amplitude at synapses expressing EGFP alone ($n = 70$), or EGFP with human Ca_v2 subunits (N_{imperm} , $n = 22$; $N_{imperm} + \text{PQ}_{WT}$, $n = 18$; PQ_{WT} , $n = 26$). Presynaptic expression of N_{imperm} channels alone significantly reduced the size of EPSCs ($p < 0.05$, N_{imperm} vs EGFP group). In contrast, the synaptic strength was well maintained when PQ_{WT} and N_{imperm} were coexpressed at presynaptic terminals ($p > 0.17$, compared with EGFP group). **D**, Ensemble average of overall EPSC amplitude from the same groups in **C**. **E**, PPR at synapses expressing EGFP alone or EGFP with various human Ca_v2 subunits ($n = 12$ –70). Pairs of EPSCs were elicited by stimuli 50 ms apart, and the ratio of the EPSC amplitudes was determined. PPR was significantly increased at synapses expressing N_{imperm} compared with that at synapses expressing EGFP alone ($*p < 0.05$, one-way ANOVA *post hoc* Bonferroni). PPR values were not altered in other experimental groups. Error bars indicate SEM.

tween the two groups (Fig. 4E). Evidently, the excess of $\text{Ca}_v2.1$ subunits protected P/Q-prefering slots from being occupied by N_{imperm} . However, N_{imperm} replaced some endogenous N-type channels and prevented them from coupling to release machinery.

No spare N-type-specific slots

To test whether N-type-specific slots are normally filled to capacity by endogenous N-type channels, we compared normal transmission with that found when N_{WT} and PQ_{WT} subunits were both coexpressed in great excess of normal levels. The P/Q-prefering slots would be filled up by PQ_{WT} and/or the endogenous P/Q-type channels. If there were spare N-type specific slots, overexpression of N_{WT} would still increase the relative contribution of N-type channels to synaptic transmission; if not, N_{WT} would only displace endogenous N-type channels and the percentage of N-supported transmission would remain unaltered. Indeed, expression of extra PQ_{WT} reduced the ω -CTx-GVIA-sensitive fraction from $81 \pm 8\%$ back to $48 \pm 5\%$, not significantly different from the control (EGFP group) and in clear contrast to the findings with overexpression of N_{WT} alone (Fig. 5A, N_{WT} + PQ_{WT} vs N_{WT}). Remarkably, overall synaptic strength was not increased (Fig. 5B), despite the fact that whole-cell P/Q- and N-type currents both increased severalfold (Fig. 5C,D). The values of PPR were not different either: EGFP (1.16 ± 0.04), N_{WT} (1.18 ± 0.06), and $N_{WT} + \text{PQ}_{WT}$ (1.16 ± 0.07). These data indicated that, like endogenous P/Q-type channels, endogenous N-type channels fully engaged the presynaptic slots preferentially available to them. The apparent contribution of R-type channels remained relatively minor under the conditions of these experiments.

Discussion

This study defined some key features of the slots that determine which kinds of presynaptic Ca^{2+} channels trigger excitatory neurotransmission in cultured hippocampal neurons. The functional roles of N- and P/Q-type channels were probed by overexpression of both permeable and impermeable channels. Although some of the results fulfilled predictions arising from our previous work (Cao et al., 2004), most of our findings were unexpected. In summary, (1) neurotransmission is normally saturated with regard to Ca^{2+} channel expression and cannot be increased by strong overexpression of exogenous N- and P/Q-type channels; (2) N- and P/Q-type Ca^{2+} channels differ markedly in their relationships to slots that impose a ceiling on their presynaptic roles; (3) P/Q-type Ca^{2+} channels maximally fill a subset of slots ($\text{PQ}_{prefering}$) that restrict their contribution to transmission; they can be displaced from the $\text{PQ}_{prefering}$ slots by overexpression of N-type Ca^{2+} channels,

they can be displaced from the $\text{PQ}_{prefering}$ slots by overexpression of N-type Ca^{2+} channels,

but put back by additionally overexpressing P/Q-type channels; (4) N-type Ca^{2+} channels normally occupy an additional set of slots (N_{specific}) that cannot be populated by P/Q-type channels; these slots are maximally occupied by N-type channels.

Our findings lead to significantly different predictions for how deletion of P/Q- or N-type channels would affect global synaptic strength and readjustment of the burden on the channels that remain. Deletion of P/Q-type channels should merely shift the burden of transmission to N-type channels, which can replace P/Q at $PQ_{\text{preferring}}$ slots. Indeed, at the calyx of Held, EPSC size is preserved relative to wild-type at $\text{Ca}_v2.1$ knock-out synapses (Inchauspe et al., 2004, 2007; Ishikawa et al., 2005). Conversely, deletion of N-type channels should weaken neurotransmission because the remaining P/Q-type channels would not interact with $N_{\text{selective}}$ slots and would thus fail to compensate fully. Indeed, in $\text{Ca}_v2.2$ knock-out mice, basal excitatory transmission is substantially reduced (Jeon et al., 2007). Thus, observations with channel overexpression and deletion are mutually consistent in supporting the hypothesis in Figure 5E. The central feature is the mutual interaction of (1) functionally different kinds of Ca_v2 channels and (2) multiple, operationally distinct kinds of slots. The scheme features a pronounced asymmetry. $PQ_{\text{preferring}}$ slots are not wholly selective, displaying greater proclivity for P/Q-type channels but also accepting N-type channels if these are particularly abundant or if P/Q-type channels are absent. On the other hand, N_{specific} slots reject P/Q-type channels even when these are greatly overexpressed or N-type channels are eliminated.

Functional implications of multiple types of Ca^{2+} channel slots

The existence of two kinds of slot might offer functional advantages if the two types of channel differ substantially in how they are regulated or how they trigger transmitter release. Studies at large nerve terminals indicate that presynaptic P/Q- and N-type channels are fairly similar in their voltage and time dependence of activation and deactivation (Li et al., 2007). Ca^{2+} currents supported by P/Q-type channels are more apt to facilitate during a train of brief stimuli (Inchauspe et al., 2004, 2007; Ishikawa et al., 2005). N-type channels are more susceptible to modulation by GPCRs for neurohormones (Bourinet et al., 1996; Currie and Fox, 1997; Zhou et al., 2003; Cao and Tsien, 2005). Because Ca_v2 family members differ in responsiveness to repetitive activity and neuromodulators, controlling their contribution to presynaptic

Figure 5. N-type-specific slots are filled to capacity by endogenous N-type channels. **A**, Relative contributions of N-, P/Q-, and R-type channels to EPSC amplitude in neurons expressing EGFP alone (EGFP, $n = 20$), or EGFP with human Ca_v2 subunits (N_{WT} , $n = 23$; $N_{\text{WT}} + PQ_{\text{WT}}$, $n = 11$). The N-, P/Q-, or R-type Ca^{2+} channel blockers ω -CTX-GVIA ($2 \mu\text{M}$), ω -Aga-IVA ($1 \mu\text{M}$), and SNX482 ($0.5 \mu\text{M}$) were applied sequentially and cumulatively into the bath solution. In the presence of extra PQ_{WT} , overexpression of N_{WT} could no longer increase the N contribution to synaptic transmission, indicating that N- and P/Q-type channels compete for a common subset of slots (* $p < 0.05$, N_{WT} vs EGFP group; no significant difference between $N_{\text{WT}} + PQ_{\text{WT}}$ and EGFP groups). **B**, Cumulative distribution of overall EPSC amplitude at synapses expressing EGFP alone ($n = 70$), or EGFP with human Ca_v2 subunits (N_{WT} , $n = 61$; $N_{\text{WT}} + PQ_{\text{WT}}$, $n = 18$). The synaptic strength was comparable across all groups ($p > 0.33$, Kolmogorov–Smirnov test). **C**, Peak whole-cell Ba^{2+} current density through P/Q-type Ca^{2+} channels at $+10$ mV from hippocampal neurons expressing EGFP alone (EGFP, $n = 19$), or EGFP with human Ca_v2 subunits (N_{WT} , $n = 10$; $N_{\text{WT}} + PQ_{\text{WT}}$, $n = 7$). Overexpression of PQ_{WT} , either alone or with N_{WT} , resulted in significant increase of whole-cell P/Q current density (*** $p < 0.001$, relative to EGFP group). The magnitude of P/Q-current density was not significantly different between $N_{\text{WT}} + PQ_{\text{WT}}$ (50.1 ± 3.8 pA/pF) and the PQ_{WT} groups (58.0 ± 13.3 pA/pF) (Fig. 2B) ($p > 0.5$). Overexpressing N_{WT} in neurons did not alter P/Q-type current density. **D**, Peak whole-cell Ba^{2+} current density through N-type Ca^{2+} channels at $+10$ mV from the same neurons in **C**. Overexpression of N_{WT} , either alone or with PQ_{WT} , significantly increased the whole-cell N-type current density (** $p < 0.01$, relative to EGFP group). The magnitude of N-type current density was comparable between the N_{WT} (32.8 ± 3.3 pA/pF) and the $N_{\text{WT}} + PQ_{\text{WT}}$ (35.1 ± 4.7 pA/pF; $p > 0.6$) groups. Error bars indicate SEM. **E**, Different relationship of N- and P/Q-type Ca^{2+} channels to slots in controlling neurotransmission at cultured hippocampal synapses. Our data indicate that there are at least two different kinds of presynaptic slots at excitatory hippocampal synapses. The N_{specific} slots accept N-type channels selectively but reject P/Q-type Ca^{2+} channels. The $PQ_{\text{preferring}}$ slots prefer P/Q-type Ca^{2+} channels but also accept N-type when P/Q-type channels are missing or N-type are overexpressed. Note that terminology for slots does not consider R-type channels.

release provides a powerful way to delimit the range of regulation. By putting a ceiling on the abundance of P/Q-type channels at the active zone, $PQ_{\text{preferring}}$ slots could cap the degree of Ca^{2+} channel facilitation while setting a lower bound on the extent of neuromodulation, both important aspects of presynaptic regulation. Patterns of stimulation and delivery of neuromodulators vary from moment to moment, but their range of impact could be predetermined by the makeup of presynaptic slots.

Implications for neuronal development and disease

The concept of multiple kinds of slots could be related to the staggered onsets of Ca^{2+} channel expression during neuronal development, N-type channels appearing in embryonic synapses, whereas P/Q-type emerges much later in development. Although some synapses wind up relying almost completely on P/Q-type, not N-type (Iwasaki et al., 2000), many CNS synapses do not undergo a complete N- to P/Q-type switch. For example, the P/Q-type component of transmission flattens off at ~50% at synapses in the dorsal horn (Iwasaki et al., 2000) and at somewhat higher levels at hippocampal synapses (Scholz and Miller, 1995). It may be advantageous that the channel that expresses earlier (N-type) acts as a universal donor that can fully support transmission in the absence of the other. Conversely, the delayed but abundant expression of P/Q-type channels allows them to capture a subset of the slots. Their inability to compete for $N_{\text{selective}}$ slots helps curb their contribution, while also safeguarding the participation of N-type channels and the G-protein-mediated modulation of Ca^{2+} influx. Regulating the number of slots may fine-tune neurotransmission more reliably than grading the production of the channels themselves. Ca_v2 channels support vital functions other than neurotransmission, including regulation of transcription and other forms of Ca^{2+} -dependent signaling in somatodendritic regions. Deployment of slots allows local control of presynaptic channel abundance.

Possible structural features to account for distinctions between N- and P/Q-type channels

Our findings allow us to refine the concept of slots beyond its initial, inclusive definition as binding sites of limited capacity that interact with channel proteins to control their participation in neurotransmission. This deliberately left open whether the interaction is initiated before proteins can exit from the ER, or at checkpoints upstream of axonal trafficking, or at synaptic sites themselves. The present results show that whole-cell Ca^{2+} channel currents are greatly increased by overexpression of N- or P/Q-type pore-forming subunits, whereas synaptic strength has reached a ceiling. This narrows down the possible checkpoints for mammalian Ca_v2 channels, excluding one at the exit of proteins from the ER (Saheki and Bargmann, 2009), which would jointly restrict surface expression in cell body and axon.

Recognition of the distinctness of $PQ_{\text{preferring}}$ and $N_{\text{selective}}$ slots provides fresh perspective on previous biochemical findings aimed at understanding how N- and P/Q-type channels are anchored at the active zone. Maximov et al. (1999) initially focused on common features of long forms of $\text{Ca}_v2.2$ (here N_{WT}) and $\text{Ca}_v2.1$ (here $PQ_{\text{WT-long}}$), reasoning that the most important molecular determinants would be shared by both N- and P/Q-type channels. This led to description of how specific portions of a C-terminal domain of $\text{Ca}_v2.2$ interacted with the modular adaptor proteins Mint1 and CASK. The corresponding channel fragment disrupted channel clustering at synaptic sites, exerted a dominant-negative effect on synaptic function, and drove a chimera with a marker protein to synaptic sites (Maximov and Bezprozvanny, 2002). Although a strongly homologous region was present in the $PQ_{\text{WT-long}}$, this domain proved to be entirely dispensable for proper synaptic localization of $\text{Ca}_v2.1$ (Hu et al., 2005). Likewise, inclusion or exclusion of the same domain had no impact on neurotransmission (Fig. 1). The disparity between the structural basis of N- and P/Q-type channel anchoring would be mysterious on the initial premise that both shared similar molecular determinants but is readily interpretable in light of our findings that N-type channels can access binding sites not avail-

able to P/Q-type. Recognition of the existence of an $N_{\text{selective}}$ slot directs attention to proteins that bind selectively to N-type and not P/Q-type channels. Accordingly, a possible element of $N_{\text{selective}}$ slots is the MAP1A light chain 2, which binds to a subdomain within the C-terminal of $\text{Ca}_v2.2$ with low homology to $\text{Ca}_v2.1$ (Leenders et al., 2008).

Similar lines of reasoning prompt a refined search for the structural basis of $PQ_{\text{preferring}}$ slots. Results from our electrophysiological study (Fig. 1), and parallel cytochemical findings of Hu et al. (2005), suggest that the $PQ_{\text{preferring}}$ slot interaction does not involve the distal C-terminal domain of $\text{Ca}_v2.1$ encoded by exon 47. Attention must now shift to protein–protein interactions not specific to P/Q-type channels, but shared with N-type. An obvious candidate is the synprint site, a relatively well conserved region of the II–III loop of $\text{Ca}_v2.1$ and $\text{Ca}_v2.2$ subunits, originally defined by interaction with various SNARE proteins (Catterall, 1999; Mochida et al., 2003). Skepticism about the involvement of synprint sites has arisen from study of transmission in invertebrate species whose Ca_v2 channels lack synprint sites yet support neurotransmission (Spafford et al., 2003). However, these findings could be reinterpreted in terms of Ca_v2 interactions with slots analogous to the N_{specific} slots described here. In mammalian systems, deletion of the synprint site in $\text{Ca}_v2.1$ reduced the effectiveness of P/Q-mediated neurotransmission, and insertion of N- or P/Q-synprint sites into L-type channels was sufficient to establish L-mediated synaptic transmission (Mochida et al., 2003). Likewise, splice variants of mammalian N-type channels lacking the synprint region are strongly impaired in presynaptic clustering (Szabo et al., 2006). Together with our finding that P/Q- and N-type channels compete for a common subset of slots (Fig. 5), these data suggest that both types use synprint sites for interactions with $PQ_{\text{preferring}}$ slots, whereas N-type channels can do without synprint sites for binding to $N_{\text{selective}}$ slots but rely instead on determinants near their C termini. Our interpretations may reconcile multiple studies but will require additional testing with new channel constructs.

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