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

Yu-Qing Cao and Richard W. Tsien
Department of Molecular and Cellular Physiology, Beckman Center, Stanford University School of Medicine, Stanford, California 94305-5345

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 Ca\(^{2+}\) channel subunits 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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\(_{\text{specific}}\)) and the other preferring P/Q-type but also accepting N-type (P/Q\(_{\text{preferring}}\)). 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 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\(^{2+}\) 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.,...
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-prefering "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 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\(_{\text{specific}}\)) 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 Ca\(_{\text{a,2.1}}\) and Ca\(_{\text{a,2.2}}\) constructs.* The human Ca\(_{\text{a,2.1}}\) cDNA used in this study encodes the splice variant: \(\Delta 10A (-V+F)\), \(16\, +\, 17\, +\, \Delta 17A\) (–VEA), –31* (–NP), 37a (EFA), 43 “44”, either with or without exon 47 (Soong et al., 2002). The wild-type human Ca\(_{\text{a,2.2}}\) cDNA used in this study encodes the Ca\(_{\text{a,2.2a}}\) (\(\alpha_{\text{Ib}}\)) isoform and can travel to synaptic terminals to mediate neurotransmitter release (Maximov and Bezprozvanny, 2002). The nonpermeable Ca\(_{\text{a,2.2}}\) subunit was generated by replacing all four pore glutamates with alanine residues (E314A, E663A, E1365A, and E1655A) in S6-6 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 Ca\(_{\text{a,2.2}}\) subunit was performed in a stable HEK293 cell line expressing \(\beta_{\text{L}}\), and \(\alpha_{\text{Ib}}\) subunits as described previously (Piedras-Renteria et al., 2001). Cells were cotransfected with constructs encoding wild-type or nonpermeable Ca\(_{\text{a,2.2}}\) subunit along with the pEGFPC1 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\(_{\text{a,2.1}}\) construct was cotransfected with pEGFPC1 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 records 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\(\Omega\) resistance. Cell capacitance and series resistance (~20 M\(\Omega\)) were constantly monitored throughout recording.

*Bu\(^+\) 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. N-, and P/Q-type currents were identified as currents sensitive to their specific blockers: nifedipine (10 \(\mu\)M), \(\alpha\)-conotoxin-GVIA (\(\alpha\)-CTx-GVIA) (2 \(\mu\)M) and \(\alpha\)-agatoxin-IVA (\(\alpha\)-Ag-IVA) (1 \(\mu\)M), respectively. The remaining Cd\(^{2+}\)-sensitive (100 \(\mu\)M) 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 V/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 fluorescent 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 recordings. To measure the current-voltage relationships (\(I-V\) curves), 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 100X 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 \(\mu\)M) and d-AP5 (25 \(\mu\)M; both from Tocris) were used to block AMPA and NMDA receptors.
the postsynaptic neuron (Fig. 1). These spikes in the transfected neuron typically resulted in an EPSC in the untransfected postsynaptic neuron target. Evoking a presynaptic wave of ryanodine receptor activity that caused the recycling of enteric vesicles (Sandfeld and Bezprozvanny, 2002). We focused on excitatory synaptic transmission from neurons expressing NWT and dissected contributions of N- and P/Q-type channels, using the same system of paired patch-clamp recordings. Whereas neurons expressing EGFP alone, PQWT, or PQWT-long underwent a ~42% reduction of EPSCs with exposure to ω-Aga-IVA, transmission from neurons overexpressing NWT behaved in a strikingly different way—in this case, N-type channels accounted for ~80% of the EPSC amplitudes, 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, NWT 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 NWT 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 NWT 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 NWT subunits.
Cao and Tsien • N- and P/Q-Type Ca2+ Channels and Their Presynaptic Slots

Figure 1. N- and P/Q-type Ca2+ channels exhibit different relationship to slots in controlling neurotransmission at cultured hippocampal synapses. A, Schematic representations of the C-terminal cytoplasmic domains of human CaV2.2 subunit (NWT) as well as the short and long splice variants of human CaV2.1 subunits (PQWT and PQWT-long, respectively). The last four amino acids of the C terminus of each subunit are indicated. The lengths of primary sequence are drawn to scale. B, No blocker, GVIA, Aga-IVA, SNX482, EGFP, PQWT, PQWT-long, NWT. C, wild-type neurons. D, CaV2.1-knockout neurons. E, total EPSC. F, GVIA-sensitive EPSC. G, Aga-IVA-sensitive EPSC. Despite this delay, overexpression of NWT still resulted in an enhanced contribution of N-type channels to excitatory transmission, seen as increase in ω-CTx-GVIA block from 51 ± 5% (Fig. 1C; EGFP) to 80 ± 8% (Fig. 1C, NWT-10 DIV group, p < 0.05), very similar to the effect of expressing NWT at 5 DIV. On the other hand, the contribution of P/Q-type Ca2+ channels to transmission decreased from 41 ± 6 to 16 ± 7% when NWT 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 NWT 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 NWT subunits (Fig. 1E), indicating that NWT overexpression did not enlarge the overall strength of transmission. Evidently, the number of release sites and their individual probability of release (P) both remained unaltered. As a separate gauge of P, we looked for changes in paired-pulse modulation, which veers toward paired-pulse depression rather than facilitation as basal P, 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), PQWT (1.20 ± 0.08), PQWT-long (1.14 ± 0.12), and NWT (1.18 ± 0.06) (p > 0.6 by ANOVA). The lack of change in paired-pulse modulation supported the idea that P, had remained unaffected by the excess of one or other type of Ca2+ channel.

These data on the fractional contributions of various CaV2 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
possible scenario is that N-type channels literally replaced P/Q-type channels at synaptic release sites. This led to quantitative predictions about the absolute sizes of N- and P/Q-mediated components of the EPSC. Net increases in the magnitude of the N-type contribution would be expected to take place at the expense of the contribution of P/Q-type channels. Indeed, the absolute size of the N-type channel-mediated EPSC was significantly bigger in the NWT group than that in EGFP ($p < 0.05$) (Fig. 1F), whereas the amplitude of P/Q-supported EPSCs was correspondingly smaller ($p < 0.05$) (Fig. 1G). These data thereby stand in contrast to overexpression of either PQWT or PQWT-long. Neither construct caused a significant change in the amplitude distributions for the total EPSC (Fig. 1E, $p > 0.3$, compared with EGFP group), just as found with N-type overexpression. But in these cases, the size of N- or P/Q-type channel-mediated components did not differ between PQWT and EGFP groups (Fig. 1F, $p > 0.4$; G, $p > 0.3$). The same could be said about the N- or P/Q-type components in the PQWT-long and EGFP groups ($p > 0.2$ and $p > 0.5$, respectively), although the sample size in the PQWT-long group was considerably smaller ($n = 7$) (data not shown).

Given the steeply nonlinear relationship between Ca$^{2+}$ influx and presynaptic $P_{f}$ (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 PQWT and NWT held true regardless of which channel type was blocked first. Figure 2A illustrates the outcome of reversing the order of toxin presentation, applying $\omega$-Aga-IVA before $\omega$-Ctx-GVIA. In this case, $\omega$-Ctx-GVIA blocked $17 \pm 3\%$ of the EPSC at wild-type synapses, but $57 \pm 6\%$ of the EPSC at synapses expressing NWT, a significant increase ($p < 0.05$) (Fig. 2A). Conversely, if one performs a direct comparison of the effect of $\omega$-Aga-IVA in EGFP and NWT groups (temporarily ignoring the PQWT data) (Fig. 2A, legend), the fractional P/Q-mediated transmission was $76 \pm 4\%$ in the EGFP cells and $60 \pm 6\%$ in the NWT 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 2](image-url)

**Figure 2.** Further tests of the effects of overexpressing NWT or PQWT on excitatory neurotransmission and Ca$^{2+}$ channel currents. A, Pharmacological tests with $\omega$-Aga-IVA applied first. Relative contribution of P/Q-, N-, and R-type channels to EPSC amplitude in neurons expressing EGFP alone (EGFP, $n = 19$) or EGFP with human CaV2 subunits (PQWT, $n = 8$; NWT, $n = 13$). The P/Q-, N-, or R-type Ca$^{2+}$ channel blockers $\omega$-Aga-IVA (1 $\mu$M), $\omega$-Ctx-GVIA (2 $\mu$M), and SNX482 (0.5 $\mu$M) were introduced sequentially and cumulatively into the bath solution. Overexpression of PQWT did not alter the relative contributions of N-, P/Q-, and R-type channels to excitatory transmission. Overexpression of NWT significantly increased the N-type contribution to synaptic transmission ($p < 0.05$, compared with EGFP group). Conversely, a direct comparison of PQ-mediated transmission in EGFP and NWT groups showed that it was significantly decreased by overexpression of NWT ($p < 0.03$). We noted that strict adherence to statistical procedures would preclude such a comparison because one-way ANOVA of the P/Q-mediated transmission in EGFP, PQWT, and NWT groups failed to show significance. B1, Peak whole-cell Ba$^{2+}$ current density through P/Q-type Ca$^{2+}$ channels at $+10$ mV. P/Q-type component was defined as Ba$^{2+}$ currents sensitive to the blockade of $1 \mu$M $\omega$-Aga-IVA. Hippocampal neurons were transfected with EGFP alone ($n = 19$), or EGFP with PQWT, PQWT-long, or NWT ($n = 6, 13$, and $10$, respectively). Overexpression of PQWT or PQWT-long caused an increase of whole-cell P/Q current density ($***p < 0.001$) relative to EGFP group. The level of P/Q current density was similar in NWT and PQWT-long groups. In contrast, overexpression of NWT did not alter P/Q-type current density. B2, Reliability of EGFP expression as a marker of expression of exogenous P/Q-type Ca$^{2+}$ channels. Cumulative distributions of amplitudes of somatodendritic P/Q-type current density. We tested the null hypothesis that P/Q-type current was no different in neurons transfected with EGFP + PQWT-long or EGFP + PQWT than that in neurons transfected with EGFP alone. This hypothesis can be rejected even for the smallest observed currents in the PQWT-long group ($p < 0.001$). C1, Peak whole-cell Ba$^{2+}$ current density through N-type Ca$^{2+}$ channels at $+10$ mV, from the same neurons as in B. N-type component was defined as Ba$^{2+}$ current sensitive to the blockade of $2 \mu$M $\omega$-Ctx-GVIA. Overexpression of PQWT or PQWT-long did not alter whole-cell N-type current density. On the contrary, overexpression of NWT resulted in a significant increase of N-type current density compared with that of EGFP group ($***p < 0.001$). Error bars indicate SEM. C2, Reliability of EGFP expression as a marker of expression of exogenous N-type Ca$^{2+}$ channels. Cumulative distributions of amplitudes of somatodendritic N-type current density. We tested the null hypothesis that N-type current density was no different in neurons transfected with EGFP and NWT, than that in neurons transfected with EGFP alone. This hypothesis can be rejected even for the smallest observed currents in the EGFP + NWT group ($p < 0.01$).
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 PQWT, PQWT-long, or NWT subunits on whole-cell Ca\(^{2+}\) channel activity.

Using nonpermeable Ca\(_{2,2}\) 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 contribu-
Table 1. N_imperm channels do not alter AP properties in hippocampal neurons

|                      | EGFP | N_imperm | N_imperm + GVIA |
|----------------------|------|----------|-----------------|
| AP amplitude (mV)    | 99.9 ± 2.59 | 90.8 ± 2.66 | 96.3 ± 0.93    |
| AP half-width (ms)   | 1.38 ± 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 = 3–4 in each group.

The table presents the AP properties of hippocampal neurons expressed with EGFP, N_imperm, and N_imperm + GVIA. The properties include AP amplitude, AP half-width, and depolarization and repolarization slopes. The data shows that the addition of GVIA (a Ca2+ channel blocker) to N_imperm channels did not alter the AP properties significantly compared to EGFP or N_imperm alone.

The data further supports the notion that N-type channels do not alter AP properties, as indicated by the close similarities in the reported values for EGFP, N_imperm, and N_imperm + GVIA. This finding is consistent with the hypothesis that N-type channels operate independently and in parallel with other channel types.

Effects of N_imperm channels on synaptic transmission

Having confirmed that endogenous N- and P/Q-type channels display normal activity in neurons expressing N_imperm, we proceeded to test how N_imperm 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_imperm channels significantly reduced the size of EPSCs (Fig. 4A, C, D, p < 0.05, N_imperm 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, changes. The ratio of the EPSC amplitudes elicited by pairs of stimuli 50 ms apart was significantly increased for presynaptic partners expressing N_imperm (1.40 ± 0.07) compared with neurons expressing EGFP alone (1.16 ± 0.04; p < 0.05, Fig. 4E, suggesting that permeant N-type channels reduce P, at presynaptic terminals. However, the clear reduction in synaptic strength was not accompanied by a shift toward P/Q-dominated transmission (Fig. 4B, N_imperm vs EGFP group). The implication is that N_imperm 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 PQWT along with N_imperm on the idea that this should ensure that P/Q-prefering slots are filled up with permeable P/Q-type channels, while leaving N_imperm to compete with endogenous N-type channels. Indeed, we observed that coexpression of PQWT and N_imperm resulted in a marked reduction of the N-type contribution to synaptic transmission, from 51 ± 4 to 21 ± 3% (Fig. 4B, open bars) (N_imperm + PQWT vs EGFP group, p < 0.05). Correspondingly, the contribution of P/Q-type Ca2+ channels to transmission increased from 41 ± 4 to 70 ± 4% when PQWT and N_imperm 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), nor did values of PPR differ be-

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 Ca2+-impermeable N-type channels (N_imperm) and testing for dominant-negative effects. Human CaV2.2 subunits incapable of Ca2+-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_imperm channels were performed in HEK293 cells (Fig. 3C). Unlike cells expressing N_WT, HEK293 cells expressing N_imperm failed to display any inward Ba2+ current but only showed an outward current when depolarized beyond 0 mV. The outward current through N_imperm channels was blocked by 2 µM ω-CTx-GVIA (data not shown), indicating that N_imperm 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 Ca2+-channels for the narrow APs of hippocampal neurons (King and Meriney, 2005). To be sure that the outward current through N_imperm channels would not exert confounding effects on AP generation, we expressed N_imperm 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_imperm 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 Ca2+-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 Ca2+-channels. In an additional test, we exposed neurons expressing N_imperm to ω-CTx-GVIA (2 µM), which, as already mentioned, acutely blocks outward as well as inward currents through N-type Ca2+-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_imperm interferes with the whole-cell Ba2+ current through the endogenous N- and P/Q-type Ca2+-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 CaV2.2 subunits rendered incapable of Ca2+-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_imperm channels (Fig. 3C, maroon symbols). Additional expression of PQWT channels (N_imperm + PQWT) 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_imperm 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 PQWT. 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_imperm channels (Fig. 3D). Neither overexpression of N_imperm nor that of N_imperm + PQWT in combination caused a significant alteration in currents through endogenous N-type Ca2+-channels.
between the two groups (Fig. 4 E). Evidently, the excess of Ca_{\text{A2.1}} subunits protected P/Q-prefering slots from being occupied by N_{\text{perm}}. However, N_{\text{perm}} replaced some endogenous N-type channels and prevented them from coupling to release machinery.

Figure 4. Effects of N_{\text{perm}} 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_{2+} subunits (N_{\text{perm}}, N_{\text{perm}}+PQWT, and PQWT) respectively, as well as the effect of toxins on the 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_{2+} subunits (N_{\text{perm}}, N_{\text{perm}}+PQWT, and PQWT, n = 10, 10, and 18, respectively). Ca_{2+} channels were blocked as shown in A. Overexpression of N_{\text{perm}} did not alter the relative contributions of N-, P/Q-, and R-type channels to excitatory transmission. However, coexpression of PQWT and N_{\text{perm}} resulted in a significant reduction of N-type contribution to synaptic transmission (p < 0.05, N_{\text{perm}}+PQWT vs EGFP group). Correspondingly, the contribution of P/Q-type channels to transmission was significantly increased in N_{\text{perm}}+PQWT 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_{2+} subunits (N_{\text{perm}}, n = 22; N_{\text{perm}}+PQWT, n = 18; PQWT, n = 26). Presynaptic expression of N_{\text{perm}} channels alone significantly reduced the size of EPSCs (p < 0.05, N_{\text{perm}} vs EGFP group). In contrast, the synaptic strength was well maintained when PQWT and N_{\text{perm}} 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_{2+} 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_{\text{perm}} 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.

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 PQWT subunits were both coexpressed in great excess of normal levels. The P/Q-prefering slots would be filled up by PQWT 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 PQWT reduced the ω-Ctx-GVIA-sensitive fraction from 81 ± 8% back to 48 ± 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}+PQWT 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}+PQWT (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 (PQprefering) that restrict their contribution to transmission; they can be displaced from the PQprefering 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\(_{\text{specific}}\)) 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 P/Q\(_{\text{preferring}}\) slots. Indeed, at the calyx of Held, EPSC size is preserved relative to wild-type at Ca\(_v\)2.1 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 Ca\(_v\)2.2 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\(_v\)2 channels and (2) multiple, operationally distinct kinds of slots. The scheme features a pronounced asymmetry. P/Q\(_{\text{preferring}}\) 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\(_{\text{specific}}\) 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\(_v\)2 family members differ in responsiveness to repetitive activity and neuromodulators, controlling their contribution to presynaptic 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, P/Q\(_{\text{preferring}}\) 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$_{\text{v2}}$ 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$_{\text{v2}}$2 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 Ca$_{\text{v2.2}}$ (here $N_{\text{WT}}$) and Ca$_{\text{v2.1}}$ (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 Ca$_{\text{v2.2}}$ interacted with the modulator 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 Bezprozvannyy, 2002). Although a strongly homologous region was present in the $PQ_{\text{WT-long}}$ domain, this domain proved to be entirely dispensable for proper synaptic localization of Ca$_{\text{v2.1}}$ (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 available 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 Ca$_{\text{v2.2}}$ with low homology to Ca$_{\text{v2.1}}$ (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 Ca$_{\text{v2.1}}$ 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 Ca$_{\text{v2.1}}$ and Ca$_{\text{v2.2}}$ 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$_{\text{v2}}$2 channels lack synprint sites yet support neurotransmission (Spafford et al., 2003). However, these findings could be reinterpreted in terms of Ca$_{\text{v2}}$2 interactions with slots analogous to the $N_{\text{selective}}$ slots described here. In mammalian systems, deletion of the synprint site in Ca$_{\text{v2.1}}$ 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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