Distinct roles for \( \text{Ca}_{2.1}–2.3 \) in activity-dependent synaptic dynamics

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Ricoy UM, Frerking ME. Distinct roles for \( \text{Ca}_{2.1}–2.3 \) in activity-dependent synaptic dynamics. J Neurophysiol 111: 2404–2413, 2014. First published February 12, 2014; doi:10.1152/jn.00335.2013.—Synaptic transmission throughout most of the CNS is steeply dependent on presynaptic calcium influx through the voltage-gated calcium channels \( \text{Ca}_{2.1}–\text{Ca}_{2.3} \). In addition to triggering exocytosis, this calcium influx also recruits short-term synaptic plasticity. During the complex patterns of presynaptic activity that occur in vivo, several forms of plasticity combine to generate a synaptic output that is dynamic, in which the size of a given excitatory postsynaptic potential (EPSP) in response to a given spike depends on the short-term history of presynaptic activity. It remains unclear whether the different \( \text{Ca}_{2} \) channels play distinct roles in defining these synaptic dynamics and, if so, under what conditions different \( \text{Ca}_{2} \) family members most effectively determine synaptic output. We examined these questions by measuring the effects of calcium channel-selective toxins on synaptic transmission at the Schaffer collateral synapse in hippocampal slices from adult mice in response to both low-frequency stimulation and complex stimulus trains derived from in vivo recordings. Blockade of \( \text{Ca}_{2.1} \) had a greater inhibitory effect on synaptic transmission during low-frequency components of the stimulus train than on synaptic transmission during high-frequency components of the train, indicating that \( \text{Ca}_{2.1} \) had a greater fractional contribution to synaptic transmission at low frequencies than at high frequencies. Relative to \( \text{Ca}_{2.1} \), \( \text{Ca}_{2.2} \) had a disproportionately reduced contribution to synaptic transmission at frequencies >20 Hz, while \( \text{Ca}_{2.3} \) had a disproportionately increased contribution to synaptic transmission at frequencies >1 Hz. These activity-dependent effects of different \( \text{Ca}_{2} \) family members shape the filtering characteristics of \( \text{GABA}_\text{A} \) receptor-mediated presynaptic inhibition. Thus different \( \text{Ca}_{2} \) channels vary in their coupling to synaptic transmission over different frequency ranges, with consequences for the frequency tuning of both synaptic dynamics and presynaptic neuromodulation.

PRESYNAPTIC CALCIUM INFLUX through voltage-gated calcium channels plays multiple roles in synaptic transmission, engaging both immediate vesicle fusion and slower forms of activity-dependent short-term synaptic plasticity (reviewed in Neher and Sakaba 2008). Most, if not all, of the action potential-evoked presynaptic calcium influx is mediated by channels of the \( \text{Ca}_{2} \) family, \( \text{Ca}_{2.1} \) (P/Q-type channels), \( \text{Ca}_{2.2} \) (N-type channels), and \( \text{Ca}_{2.3} \) (R-type channels) all contribute substantially to presynaptic calcium influx as measured directly by calcium imaging (Gasparini et al. 2001; Mintz et al. 1995; Wu and Saggau 1994). However, \( \text{Ca}_{2.1} \) and \( \text{Ca}_{2.2} \) are the primary channel subtypes implicated in the local calcium microdomains at the active zone that trigger exocytosis of small synaptic vesicles (Luebke et al. 1993; Takahashi and Moyiyma 1993; Wheeler et al. 1994), with \( \text{Ca}_{2.3} \) having a smaller and more variable contribution to release (Breustedt et al. 2003; Gasparini et al. 2001; Wu et al. 1998) that may reflect a more complex range of alternative splicing in this \( \text{Ca}_{2} \) family member (Tottene et al. 1996, 2000).

In addition to triggering exocytosis, calcium influx into the presynaptic terminal also engages multiple types of short-term synaptic plasticity that operate at several different stages of vesicle trafficking and priming (reviewed in Zucker and Regehr 2002). Activity-dependent changes in calcium channel kinetics can also directly mediate short-term plasticity directly by altering the calcium influx that occurs in response to spiking (reviewed in Catterall and Few 2008). During the complex patterns of afferent activity that occur in vivo, these various forms of short-term plasticity combine so that synaptic output becomes dynamic: the size of each excitatory postsynaptic potential (EPSP) varies according to the short-term history of activity preceding that EPSP and the forms of plasticity engaged by that activity (reviewed in Zador and Dobrunz 1997).

Different calcium channel subtypes have different biophysical properties (Currie and Fox 1997, 2002; Li et al. 2007). \( \text{Ca}_{2.1} \) also couples to synaptic transmission with a higher calcium cooperativity than \( \text{Ca}_{2.2} \) (Mintz et al. 1995; Qian and Noebels 2001; Wu et al. 1999), and \( \text{Ca}_{2.2} \) is more strongly influenced by G protein-mediated inhibition than \( \text{Ca}_{2.1} \) (Colclough et al. 2000; Currie and Fox 1997). This G protein-mediated inhibition can be relieved by prior depolarization and is therefore activity dependent (Bean 1989; Currie and Fox 1997; Park and Dunlap 1998). Thus the contribution of each \( \text{Ca}_{2} \) family member to overall synaptic output may differ in a frequency-dependent way from that measured during single action potentials delivered in isolation, through a range of different mechanisms. Consistent with this idea, it has been found that blockade of \( \text{Ca}_{2.1} \) leads to a relative enhancement of paired-pulse facilitation (PPF) compared with blockade of \( \text{Ca}_{2.2} \) through the activity-dependent relief of a tonic G protein-mediated inhibition (Scheuer et al. 2004). However, the interaction between activity, calcium channel subtypes, and synaptic transmission remains poorly understood because the frequency range of physiologically relevant activity is very broad and poorly sampled by conventional stimulus protocols using one or a few frequencies to assess synaptic transmission.

In the present study, we have assessed the role of different \( \text{Ca}_{2} \) family members in synaptic transmission at Schaffer collateral synapses in hippocampal slices from adult mice, using both low stimulus frequencies typically used to assess transmission as well as stimulus patterns derived from in vivo recordings to examine synaptic dynamics over a wide and physiologically relevant range of frequencies. We find that each \( \text{Ca}_{2} \) family member contributes to synaptic transmission...
with a distinct dependence on stimulus frequency, indicating that Ca_2 family members differ in their recruitment of short-term synaptic plasticity as well as in their efficiency of coupling to exocytosis.

MATERIALS AND METHODS

The Oregon Health and Science University Institutional Animal Care and Use Committee approved all procedures for animal care according to the guidelines set forth by the National Institutes of Health, in accordance with university policy and federal law.

Hippocampal slices (500 μm thick) were prepared from 6- to 8-wk-old C57BL6 mice. Mice were deeply anesthetized with the inhalant anesthetic isoflurane and rapidly decapitated. The brain was removed, and hippocampi were bilaterally dissected. Hippocampal slices were cut with a vibratome in cooled (3–5°C) solution containing (in mM) 110 choline Cl, 26.2 NaHCO_3, 11 glucose, 2.5 KCl, 0.5 CaCl_2, 7 MgCl_2, 1.0 NaH_2PO_4, and 1.3 Na ascorbate, bubbled with 95% O_2-5% CO_2. After at least an hour in this solution for recovery, the slices were maintained until use in a holding chamber containing artificial cerebrospinal fluid (in mM: 119 NaCl, 26.2 NaHCO_3, 11 glucose, 2.5 KCl, 2.5 CaCl_2, 1.3 MgSO_4, 1.0 NaH_2PO_4, bubbled with 95% O_2-5% CO_2). They were transferred to a recording chamber, and recordings were performed with a variant of this solution that contained 50–100 μM 6,2-amino-5-phosphonovalerate (APV) and 25–50 μM picrotoxin to block NMDA and GABA_A receptors, respectively. To prevent epileptiform activity during the application of complex spike trains, the recording solution contained a higher divalent composition (4 mM CaCl_2, 4 mM MgSO_4) to enhance charge screening while retaining the Ca-to-Mg ratio of ~1 that is present in cerebrospinal fluid in vivo (reviewed in Hansel 1985).

Area CA3 was microdissected away from area CA1 to prevent recurrent excitation, and the temperature was maintained at 32–35°C. Schaffer collateral axons were stimulated with a bipolar stimulating electrode placed in stratum radiatum, with paired stimuli separated by 100 ms at a rate of 0.1 Hz between stimuli to ensure recording stability. Extracellular field potentials were recorded with patch electrodes filled with the external solution and were filtered at 1–2 kHz and digitized at 5–20 kHz with IGOR Pro software. The field EPSP (fEPSP) slope was measured during a 1- to 2 ms window following the fiber volley.

Prolonged stimulus trains (~70 s long) based on in vivo recordings (described in Ohliger-Frerking et al. 2003) were delivered with custom-written software in IGOR Pro, with a minimum of 5 min between repeated applications of the same train used to monitor baseline stability at 0.1 Hz. Trains were preceded by 30 s of constant-frequency stimulation at the same overall frequency (~1 Hz) so that the measured dynamics would represent changes in the temporal complexity of the stimulus train without changes in overall firing frequency; because the transition from 0.1 Hz to 1 Hz induces a modest but variable frequency facilitation (16 ± 2%; data not shown), the data during the train were renormalized to the last 10 stimuli during the constant-frequency stimulation so that experimental variability in synaptic dynamics could be separated from experimental variability in this facilitation. Neither backfiring nor any of the toxins used had any effect on the facilitation observed during this renormalization window (data not shown).

Toxins were delivered by bath application through the perfusion system. We note that the effect of α-agatoxina IVA (AgTx) in our hands was highly variable and on average quantitatively somewhat less than seen in prior studies. We think this likely to be attributable to a decreased penetration of this large protein into the thicker slices used here (500 μm) compared with prior studies (300 μm), leading to partial rather than complete blockade of Ca_2.1. As our experimental interpretations do not require complete inhibition by any given toxin, we did not pursue this effect further; however, we caution that the summed inhibitory effect of all three toxins is likely an underestimate of the true value.

The complex spike trains used here to assess synaptic dynamics over a broad bandwidth of frequencies take a long duration of time to apply, with a minimum of 6 min between the start of repeated iterations. As a result, it is infeasible to improve the signal-to-noise level of the fEPSP by iterating the same sweep 20–30 times in a given experimental condition, as is done with typical stimulus protocols that can be repeated at 0.1 Hz. Field recordings circumvent this need for temporal averaging because this technique has more extensive temporal averaging of a large number of synapses simultaneously, allowing highly reproducible measurement of the synaptic dynamics with single applications of a complex spike train (see Dobrunz and Stevens 1999; Ohliger-Frerking et al. 2003; repeated iterations of the same train produce correlated response patterns with r² ~0.9). To ensure that fEPSP slope measurements were an accurate representation of the synaptic signal, care was taken to ensure that the fEPSP amplitude was well below saturation and that the slope measurement window was free of contamination by population spikes.

Data analysis was performed with IGOR Pro and SigmaPlot software. Data were compared with Student’s t-test, rank sum test, and one- or two-way ANOVA as appropriate for pairwise or multiple-group comparisons; the Holms-Sidak test was used for post hoc correction of multiple comparisons after ANOVA. Significance was assessed at P < 0.05. All data are presented as means ± SE.

RESULTS

To examine the role of different calcium channel subtypes in synaptic transmission, we recorded extracellular fEPSPs in response to stimulation of the Schaffer collateral synapses between CA3 and CA1 pyramidal cells and selectively blocked different Ca_2 family members with channel-selective toxins (1 μM): AgTx to block Ca_2.1 (Mintz et al. 1992), α-conotoxin GVIA (CTX) to block Ca_2.2 (Kerr and Yoshikami 1984), and SNX-482 (SNX) to block Ca_2.3 (Newcomb et al. 1998). Consistent with previous reports that Ca_2.1 and Ca_2.2 are major contributors to synaptic transmission at this synapse (Reid et al. 1997; Scheuber et al. 2004; Wheeler et al. 1994; Wu and Saggau 1994), we found that basal synaptic transmission at 0.1-Hz stimulation was substantially blocked by bath application of either CTx (Fig. 1A; n = 16, 40 ± 6% inhibition, P < 0.001) or AgTx (Fig. 1B; n = 9, 44 ± 7% inhibition, P < 0.001). SNX also had a small but significant effect on basal transmission (Fig. 1C; n = 8, 18 ± 6% inhibition, P ≤ 0.001), consistent with prior reports (Gasparini et al. 2001) indicating the involvement of Ca_2.3 in transmitter release at this synapse.

To confirm that the effects of these toxins were presynaptic, we also examined whether toxin application elicited changes in PPF that occurs in response to two stimuli with 100-ms interstimulus interval (ISI). CTx and AgTx both led to a substantial increase in PPF, as expected (Fig. 2, A, B, and D; CTx: n = 16, 16 ± 2% increase in PPF, P < 0.001; AgTx: n = 9, 13 ± 3% increase in PPF, P < 0.005), while SNX caused a smaller increase in PPF that was consistent with the smaller inhibition of the fEPSP caused by this toxin (Fig. 2, C and D; n = 8, 5 ± 2% increase in PPF, P < 0.01).

Thus all three members of the Ca_2 family contribute to generation of the fEPSP and blockade of any of them increases PPF, consistent with the previously reported contributions of these channels to presynaptic calcium influx. To examine the effects of different Ca_2 family members over the wide range of firing frequencies that the synapse experiences in vivo, we
recorded the fEPSPs in response to a stimulus train derived from in vivo single-unit recordings of CA3 pyramidal cells (Fig. 3A, top) that encompasses a wide range of ISIs (Fig. 3A, bottom). The same stimulus train was delivered to the slice in control conditions and again after the addition of CTx, and the resulting fEPSPs in each condition were recorded and compared (Fig. 3B).

Overall, CTx led to an inhibition of fEPSPs during the stimulus train, as expected; however, upon closer examination, the inhibitory effect was not uniform but instead depended on the ISI between the spike generating the fEPSP and that immediately preceding it (Fig. 3, C and D). This could be readily visualized by measuring the ratio fEPSP<sub>CTx</sub>/fEPSP<sub>ctl</sub> for the longest ISIs during the train (Fig. 3C1). That ratio was then used to scale the fEPSP in control conditions at shorter ISIs, to generate the expected fEPSP if the inhibition was independent of ISI. As can be seen in Fig. 3C2, the observed fEPSP in CTx at short ISIs is larger than the scaled fEPSP expected based on the inhibition at long ISIs. A similar relationship was evident when comparing the entire range of data during the train, plotted as a function of ISI (Fig. 3D). The fractional inhibition by CTx was calculated as a function of ISI by use of the equation

\[
\% \text{inhibition} = 100 \times \left(1 - \frac{\text{fEPSP}_{\text{CTx}}}{\text{fEPSP}_{\text{ctl}}}\right)
\]

In this manner, the inhibition as a function of ISI could be readily observed as a high-pass filter, in which short ISIs are subject to considerably less inhibition than long ISIs (Fig. 3E).

Similar experiments with AgTx revealed a high-pass filtering by AgTx similar to that seen for CTx, although the relief of inhibition at short ISIs was apparently weaker for AgTx than for CTx (Fig. 4, A and B). The effect of SNX was more complicated, with SNX causing an increased inhibition at intermediate ISIs compared with long ISIs and a decreased inhibition at short ISIs compared with intermediate ISIs (Fig. 4, B and D).

To perform a quantitative comparison of the filtering effects generated by each toxin, we averaged together fEPSPs recorded throughout the train that had similar ISIs. We also normalized the filtering curve for each experiment to the mean value in the bin associated with the longest ISIs, to limit the effect of variability in the maximal inhibition that was observed across experiments using the same toxin. When the averaged data across experiments were compared, each toxin had an effect that was significantly different from the others (Fig. 5A; \(P < 0.001\)) in which the magnitude of the difference was dependent on the ISI \(\) (\(P < 0.001\); see Fig. 5A and Table 1).

For both CTx and AgTx, the inhibition became progressively smaller as the ISI became shorter. Relative to the inhibition seen during the longest ISIs (\(>3\) s), the inhibition of release at the shortest ISIs (\(<50\) ms) was significantly smaller for both

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Fig. 1. Blockade of any of the 3 members of the Ca<sub>v</sub>2 family inhibits the field excitatory postsynaptic potential (fEPSP). fEPSPs were elicited at a baseline frequency of 0.1 Hz and compared before and after application of different subtype-selective calcium channel toxins (each bath-applied at 1 μM): ω-conotoxin GIVA (CTx) to block Ca<sub>v</sub>2.2 (A), ω-agatoxin IVA (AgTx) to block Ca<sub>v</sub>2.1 (B), and SNX-482 (SNX) to block Ca<sub>v</sub>2.3 (C). Representative fEPSPs before and after application of each toxin are shown (A<sub>1</sub>–C<sub>1</sub>), as are the averaged data displaying the time course of effect of each toxin (A<sub>2</sub>–C<sub>2</sub>).
One possible explanation for the differences in filtering across toxins is that different toxins might inhibit the basal release probability to different mean levels, causing different levels of overall vesicle depletion during the train. This seems unlikely to explain the difference between CTx and AgTx because the inhibition of basal transmission was not significantly different (40 ± 6% vs. 44 ± 7%; see Fig. 1) but could explain the difference between SNX and the other two toxins. To test this possibility, we averaged together six experiments from the CTx and AgTx data sets in which the level of basal inhibition was smaller than average but similar in magnitude to that seen in SNX (20 ± 7% vs. 18 ± 6%; Fig. 5, A and B). We found that the high-pass filter for this low inhibition in CTx/AgTx subset of the data was intermediate to that seen for the full CTx data set and that seen for the full AgTx data set and was still clearly distinguishable from the filtering effect generated by SNX (P = 0.006; Fig. 5, A and B). Thus the biphasic filtering caused by SNX is specific to inhibition of CaV2.1–2.2. We also infer that the shapes of the filtering curves in CTx and/or AgTx cannot be strongly dependent on the degree of basal inhibition, as the filtering curve from experiments selected for a modest inhibitory effect was similar to that expected from averaging the full data sets for each toxin.

There are two general mechanistic explanations for why each toxin might have a filtering curve that differs from the others. It might be the case that calcium influx through a different channel subtype is differentially affected by each toxin. CTx (−29 ± 3% relative to the mean inhibition at long ISIs, n = 10; P < 0.001) and AgTx (−14 ± 2% relative to the mean inhibition at long ISIs, n = 9; P < 0.001). At the shortest ISIs (ISI < 50 ms), the inhibition by CTx became significantly less effective than that of AgTx (P < 0.001). The filtering curve of SNX was significantly different from those of both CTx and AgTx over most of the frequency range and was biphasic. Relative to the SNX-induced inhibition at the longest ISIs (>3 s) the inhibition at intermediate ISIs (200–500 ms) was modestly but significantly larger (+6 ± 2% relative to the mean inhibition at long ISIs, n = 6, P < 0.05), while the inhibition at short ISIs (<50 ms) was significantly less than that seen at intermediate ISIs (−6 ± 3% relative to the mean inhibition at long ISIs, n = 6, P < 0.05 compared with the increase at intermediate ISIs) and statistically indistinguishable from that seen at long ISIs (P > 0.1).

One possible explanation for the differences in filtering across toxins is that different toxins might inhibit the basal release probability to different mean levels, causing different levels of overall vesicle depletion during the train. This seems unlikely to explain the difference between CTx and AgTx because the inhibition of basal transmission was not signifi-
particular channel subtype preferentially elicits forms of short-term plasticity that extend over a particular frequency range. In this case, blockade of that channel would preferentially remove that form of plasticity while blockade of the other channels would preferentially spare it. Alternatively, the channels might be differentially coupled to basal transmitter release, and short-term plasticity might alter the calcium occupancy of the release process so that channels that are poorly coupled to release become irrelevant. In the former case, summation of the three independent toxin effects would remain constant, as the opposing effects of each individual toxin cancels out the effects of the others (see, for example, Wu and Saggau 1995). In the latter case, summation of the three independent toxin effects would lead to an overall decrease in the summed effect of the toxins as the occupancy of the calcium sensor increases (see, for example, Wheeler et al. 1996).

To see which of these predictions is correct, we added the basal level of inhibition for each toxin back to the filtering curve for each toxin and then summed the effects of all three toxins together (Fig. 5C). The summed effect of the three independent toxin effects would remain constant, as the opposing effects of each individual toxin cancels out the effects of the others (see, for example, Wu and Saggau 1995). In the latter case, summation of the three independent toxin effects would lead to an overall decrease in the summed effect of the toxins as the occupancy of the calcium sensor increases (see, for example, Wheeler et al. 1996).

To determine whether the filtering characteristics associated with different calcium channels are relevant to endogenous neuromodulatory changes in the complement of calcium channels, we examined the effects of presynaptic inhibition by the GABA_B receptor agonist baclofen (3 μM), which is thought to preferentially inhibit Ca_{2.2} through a direct interaction with G protein βγ-subunits (Brody and Yue 2000; Scheuber et al. 2004; Wu and Saggau 1995). In a previous study done in rats, we found that inhibition by baclofen was associated with a high-pass filtering effect during complex spike trains that was reminiscent of the high-pass filtering observed with CTx in the present study (Ohliger-Frerking et al. 2003). We therefore repeated these experiments in the present study for purposes of direct comparison to the effects of the toxins in the present study. We found that, as expected, baclofen induced a rapid and strong inhibition of basal synaptic transmission (baclofen-induced inhibition of fEPSP slope, 74 ± 4%, n = 7, P < 0.001; Fig. 6, A and B) while enhancing PPF (PPF in baclofen relative to control, with a 100-ms ISI: 134 ± 3%, n = 7, P < 0.001; not shown). We generated responses to the same complex train used for the toxins, before and after application of baclofen, and calculated the filtering curve generated by GABA_B receptor activation. Consistent with reports that GABA_B receptor activation preferentially inhibits Ca_{2.2}, we found that the filtering curve generated by baclofen was remarkably similar to that generated by CTx (Fig. 6C); there was no statistically resolvable difference between the two filtering curves when they were compared directly (P > 0.8).

However, we also noted that the basal level of inhibition by baclofen significantly exceeded that of CTx (baclofen inhibi-
Inhibition by CTx at short ISIs.

but decreases at short ISIs, primarily because of the disproportionately reduced
experiments in either CTx or AgTx in which the inhibitory effect was modest.

the difference persisted when the effect of SNX was compared with a subset of
toxins by 2-way ANOVA: *significant differences between SNX and the other
3 toxins by 2-way ANOVA: #significant differences between SNX and the other
toxins cannot be explained by the comparatively smaller overall inhibition by SNX, as the
difference persisted when the effect of SNX was compared with a subset of
experiments in either CTx or AgTx in which the inhibitory effect was modest.

In marked contrast, SNX becomes more effective at intermediate ISIs, returning to
basal level of effectiveness at shorter ISIs. *Significant differences between SNX and the other
2 toxins.

A: the average filtering curves from multiple experiments in each toxin are shown on the same scale for comparison purposes after normalization to the mean level of inhibition (μ) elicited by each toxin during low-frequency components of the train. CTx and AgTx are both less effective at shorter ISIs, although this effect is significantly stronger for CTx than for AgTx.

In marked contrast, SNX becomes more effective at intermediate ISIs, returning to a basal level of effectiveness at shorter ISIs. *Significant differences between all 3 toxins by 2-way ANOVA; #significant differences between SNX and the other
2 toxins.

B: the difference between the effect of SNX and the other toxins cannot be explained by the comparatively smaller overall inhibition by SNX, as the
difference persisted when the effect of SNX was compared with a subset of
experiments in either CTx or AgTx in which the inhibitory effect was modest.

C: the summed effect of all 3 toxins remains constant at long and intermediate ISIs
but decreases at short ISIs, primarily because of the disproportionately reduced
inhibition by CTx at short ISIs.

Fig. 5. Each Ca_{2.2} family member has a distinct contribution to synaptic dynamics during complex spike trains. A: the average filtering curves from multiple experiments in each toxin are shown on the same scale for comparison purposes after normalization to the mean level of inhibition (μ) elicited by each toxin during low-frequency components of the train. CTx and AgTx are both less effective at shorter ISIs, although this effect is significantly stronger for CTx than for AgTx. In marked contrast, SNX becomes more effective at intermediate ISIs, returning to a basal level of effectiveness at shorter ISIs. *Significant differences between all 3 toxins by 2-way ANOVA; #significant differences between SNX and the other 2 toxins.

B: the difference between the effect of SNX and the other toxins cannot be explained by the comparatively smaller overall inhibition by SNX, as the difference persisted when the effect of SNX was compared with a subset of experiments in either CTx or AgTx in which the inhibitory effect was modest. C: the summed effect of all 3 toxins remains constant at long and intermediate ISIs but decreases at short ISIs, primarily because of the disproportionately reduced inhibition by CTx at short ISIs.

fEPSP. Previous reports have implicated a variety of other mechanisms for this residual effect, including other presynaptic calcium channels (Dittman and Regehr 1996; Wu and Saggau 1995), exocytosis downstream of calcium influx (Blackmer et al. 2001; Dittman and Regehr 1996; Scanziani et al. 1992), and presynaptic G protein-coupled inwardly rectifying potassium channels (GIRKs) (Ladera et al. 2008, but see Luscher et al. 1997). We note that postsynaptic hyperpolarization by baclofen-activated somatodendritic GIRKs would be expected to increase the driving force for the fEPSP rather than decrease it, so activation of postsynaptic GIRKs is unlikely to account for this residual inhibitory effect.

Consistent with the idea that inhibition of Ca_{2.2} has a dominant but not exclusive role in GABA_B receptor-mediated presynaptic inhibition, we found that the baclofen-induced inhibition of the fEPSP was not abolished by preincubation of the slices in CTx (Fig. 7, A and D; baclofen in CTx: 57 ± 8% inhibition, n = 10, P < 0.001). In contrast, baclofen had a much larger inhibitory effect in slices preincubated in AgTx (Fig. 7, B and D; baclofen in AgTx: 81 ± 5% inhibition, n = 9, P < 0.01 comparing the inhibition in AgTx to that in CTx) or SNX (Fig. 7, C and D; baclofen in SNX: 84 ± 1% inhibition, n = 7, P < 0.01 comparing the inhibition in SNX to that in CTx).

If the residual inhibitory effect of baclofen on the fEPSP is mediated by inhibition of Ca_{2.2} and/or Ca_{2.3}, then the addition of baclofen in the presence of CTX should elicit a filtering curve during the complex stimulus train that has a modestly decreased inhibition at short ISIs (similar to AgTx), a modestly increased inhibition at intermediate ISIs (similar to SNX), or an additive combination of the two weighted in proportion to the inhibitory effect on each channel subtype. Compared with the filtering curve generated by baclofen in the absence of CTx (Fig. 7E; n = 7), the filtering curve generated by baclofen in CTX-preincubated slices was significantly altered (Fig. 7E; n = 6, P < 0.05 comparing baclofen in control conditions to baclofen in CTX), with a decreased inhibition at short ISIs and a significantly increased inhibition at intermediate ISIs, qualitatively similar to the filtering expected based on an additive combination of both AgTx and SNX. We conclude that the frequency characteristics of the baclofen-

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Mean inhibition (in %) of fEPSP. Previous reports have implicated a variety of other mechanisms for this residual effect, including other presynaptic calcium channels (Dittman and Regehr 1996; Wu and Saggau 1995), exocytosis downstream of calcium influx (Blackmer et al. 2001; Dittman and Regehr 1996; Scanziani et al. 1992), and presynaptic G protein-coupled inwardly rectifying potassium channels (GIRKs) (Ladera et al. 2008, but see Luscher et al. 1997). We note that postsynaptic hyperpolarization by baclofen-activated somatodendritic GIRKs would be expected to increase the driving force for the fEPSP rather than decrease it, so activation of postsynaptic GIRKs is unlikely to account for this residual inhibitory effect.

Consistent with the idea that inhibition of Ca_{2.2} has a dominant but not exclusive role in GABA_B receptor-mediated presynaptic inhibition, we found that the baclofen-induced inhibition of the fEPSP was not abolished by preincubation of the slices in CTx (Fig. 7, A and D; baclofen in CTx: 57 ± 8% inhibition, n = 10, P < 0.001). In contrast, baclofen had a much larger inhibitory effect in slices preincubated in AgTx (Fig. 7, B and D; baclofen in AgTx: 81 ± 5% inhibition, n = 9, P < 0.01 comparing the inhibition in AgTx to that in CTx) or SNX (Fig. 7, C and D; baclofen in SNX: 84 ± 1% inhibition, n = 7, P < 0.01 comparing the inhibition in SNX to that in CTx).

If the residual inhibitory effect of baclofen on the fEPSP is mediated by inhibition of Ca_{2.2} and/or Ca_{2.3}, then the addition of baclofen in the presence of CTX should elicit a filtering curve during the complex stimulus train that has a modestly decreased inhibition at short ISIs (similar to AgTx), a modestly increased inhibition at intermediate ISIs (similar to SNX), or an additive combination of the two weighted in proportion to the inhibitory effect on each channel subtype. Compared with the filtering curve generated by baclofen in the absence of CTx (Fig. 7E; n = 7), the filtering curve generated by baclofen in CTX-preincubated slices was significantly altered (Fig. 7E; n = 6, P < 0.05 comparing baclofen in control conditions to baclofen in CTX), with a decreased inhibition at short ISIs and a significantly increased inhibition at intermediate ISIs, qualitatively similar to the filtering expected based on an additive combination of both AgTx and SNX. We conclude that the frequency characteristics of the baclofen-
GABABR agonist baclofen (bac) leads to a reduction in the size of the fEPSP, comparable to that induced by CTx. As expected, the time course of baclofen inhibition is shown, as observed between the application of 2 complex spike trains to compare synaptic dynamics in control conditions and in the presence of baclofen. C: the filtering curve elicited by baclofen (light blue diamonds) is remarkably similar to that elicited by CTx (red circles; CTx data from Fig. 5).

**DISCUSSION**

In the present study, we have found that the different Ca,2 family members contribute to synaptic transmission in ways that vary as a function of prior activity, with each channel subtype having a distinct activity dependence. Consistent with prior reports, we found that Ca,2.1 and Ca,2.2 are the major sources of calcium influx underlying synaptic transmission at low-frequency stimulation (1 Hz), but at intermediate frequencies the contribution of calcium influx through Ca,2.3 (assessed by blocking these channels with SNX) disproportionately increases and at high frequencies the contribution of calcium influx through Ca,2.2 (assessed by blocking these channels with CTx) disproportionately decreases. These different activity-dependent effects are substantial compared with the overall contribution of each channel to synaptic transmission; when the effects of blocking Ca,2.1 and Ca,2.2 with AgTx and CgTx are compared, the difference in synaptic inhibition caused by these toxins at high frequencies is about one-third the size of the synaptic inhibition itself at low frequencies. The different activity-dependent effects associated with each channel also lead to predictable changes in the filtering characteristics associated with GABABR receptor-mediated presynaptic inhibition, both in control conditions and after the toxin-induced blockade of Ca,2.2.

What is the reason for the different activity dependencies expressed by the different Ca,2 family members? Two general explanations could underlie such effects. First, different calcium channels could have distinct frequency-dependent effects on the calcium occupancy of the exocytotic calcium sensor, either through differential calcium influx or differential coupling to exocytotic calcium sensors. Second, the calcium influx through different calcium channels could differentially engage forms of short-term synaptic plasticity that are independent of calcium-exocytosis coupling, such as changes in the number of releasable vesicles or the maximal release probability.

One way to differentiate between these possibilities is to sum the individual effects of toxins that each block a distinct Ca,2 channel subtype, to determine whether there is a frequency-dependent change in their summed contribution to synaptic transmission. At the highest-frequency components of the train (>10 Hz), the summed effects of the toxins decreased, indicating a higher occupancy of the calcium sensor for the exocytotic process. This was accompanied by a disproportionate reduction in the contribution of Ca,2.2 to synaptic transmission. This suggests that as the release process saturates with respect to calcium, a closer association of Ca,2.1 and Ca,2.3 over Ca,2.2 near the vesicle docking sites preferentially renders the calcium influx through Ca,2.2 redundant. Similar results have been observed during synaptic transmission at low frequencies during spike broadening with the potassium channel blocker 4-AP to enhance calcium influx during the spike (Wheeler et al. 1996). However, we caution that we cannot rule out alternative scenarios such as different proteins involved in calcium-release coupling of vesicles docked near Ca,2.2 relative to those docked near Ca,2.1 and Ca,2.3 (Müller et al. 2010).

At intermediate frequencies in the range of 2–5 Hz, calcium influx through Ca,2.3 had an increased contribution to synaptic transmission that was accompanied by a decrease in the contribution of calcium influx through Ca,2.1 and Ca,2.2 so that the summed contribution of all three channels was unaffected. This argues that calcium influx through Ca,2.3 at this frequency range is preferentially coupled to a calcium sensor that affects short-term plasticity rather than directly influencing release. It remains unclear why Ca,2.3 should selectively play this role; one possibility is that Ca,2.3 channels in the bouton may be disproportionately located outside the active zone, making them more important in setting the residual calcium levels that define short-term plasticity than in the calcium microdomains that directly couple the presynaptic spike to exocytosis. We also note with interest that the frequency range of this effect is relatively narrow and has not been examined in previous studies of Ca,2.3 in synaptic transmission and plas-
ticity (Breustedt et al. 2003; Dietrich et al. 2003); the improved resolution of such narrow bandwidth effects is a major advantage of the complex stimulus protocols used here.

Although the stimulus protocol used here allows for greater coverage of the physiologically relevant frequency range than conventional stimulus protocols, one potential concern is that the activity-dependent effects seen during a particular complex spike train might be specific to the particular temporal characteristics of that train. However, we have found in a prior study that the GABAB receptor-mediated presynaptic inhibition measured during one complex spike train can be used to accurately predict the effects of GABAB receptor-mediated inhibition during a different complex spike train (Frerking and Ohliger-Frerking 2006), suggesting that synaptic filtering measured with this technique is not strongly dependent on the particular spike pattern used in a given spike train.

The inhibitory interaction between G protein βγ-subunits and CaV2 channels is widely observed to be relieved by prior depolarization (reviewed in Tedford and Zamponi 2006), and it has been suggested that this prepulse relief of inhibition might be a mechanism underlying the high-pass filtering of synaptic transmission induced by presynaptic inhibitors (Brody and Yue 2000; Scheuber et al. 2004; but see Kreitzer and Regehr 2000). Relief from a tonic G protein-mediated inhibition is also thought to be the underlying mechanism behind a prior observation that PPF elicited with paired stimuli at 20 Hz is disproportionately enhanced in the presence of AgTx and reduced in CTx, through a mechanism that requires intact G protein function (Scheuber et al. 2004).

However, several of our observations are incompatible with this idea. We did not observe this differential effect with paired stimuli at 10 Hz, and we observed the opposite during complex spike trains in frequency ranges; in our hands, the inhibition caused by CTx was subject to a greater activity-dependent relief than the inhibition caused by AgTx. We also found that CTx elicits a high-pass filtering similar to that seen with baclofen. As the time constant for CTx unbinding is 100 s and not enhanced by depolarization (Mould et al. 2004), it is not subject to rapid use-dependent relief.

How then do we explain the discrepancy between our observations and those of Scheuber et al.? There are a number of technical differences between our studies, including difference in recording methods, stimulation protocol, normalization methods, animal species, developmental stage, and recording temperature; it is beyond the scope of the present study to determine which of these differences, if any, underlie our different observations. However, we note that the synaptic dynamics are known to be sensitive to both developmental

Fig. 7. The filtering effect of baclofen depends on the complement of CaV2 channels supporting synaptic transmission. A: baclofen still had an inhibitory effect even when applied in the presence of CTx, indicating that CaV2.2 cannot be the only effector for GABAβγ-mediated presynaptic inhibition. B and C: however, baclofen almost entirely abolished transmission in slices preincubated in AgaTx (B) or SNX (C). D: summary data from the experiments in A–C are shown, indicating that the effect of baclofen during low-frequency transmission depends on the complement of CaV2 channels supporting synaptic transmission. E: the filtering induced by baclofen in CTx-preincubated slices (red diamonds) is significantly different from the filtering induced by baclofen in control conditions (black circles). In CTx, there is a disproportionately weaker inhibition at low frequencies and a disproportionately stronger inhibition at high frequencies, similar to the filtering expected if the residual baclofen-induced inhibition were due to a combined effect on CaV2.1 and CaV2.3 (see Fig. 5A for comparison).

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stage (Dekay et al. 2006) and recording temperature (Klyachko and Stevens 2006), which was part of the rationale underlying our decision to use adult mice, recordings at near-physiological temperatures, and stimulus patterns similar to the activity seen in vivo. We note that the relief of prepulse inhibition that underlies the results seen in Scheuer et al. (2004) is steeply dependent on the duration of the depolarization, requiring a relatively slow spike waveform (Park and Dunlap 1998); one possible explanation for the discrepancy is that the faster kinetics of spiking at near-physiological temperatures may be unable to recruit this effect, allowing us to more cleanly assess other mechanisms by which each channel subtype contributes to synaptic dynamics.

Although Ca_{2.2} is clearly the dominant calcium channel subtype inhibited by baclofen, previous reports have also indicated an inhibitory effect of GABA_B receptors on synaptic transmission elicited by other calcium channel subtypes (reviewed in Wu and Saagau 1997). Consistent with this conclusion, we found that the filtering characteristics of baclofen change depending on the calcium channels available to support synaptic transmission. The baclofen-induced filtering seen in CTX-treated slices is qualitatively similar to that expected if the remaining inhibitory effect of baclofen is a combined inhibition of Ca_{2.1} and Ca_{2.3}, with a disproportionately weak inhibition at high frequencies and a disproportionately strong inhibition at low frequencies. This resemblance is striking and suggests that calcium channel inhibition is a major contributing factor to the inhibitory effects of baclofen on stimulus-evoked release, a conclusion that is apparently at odds with previous reports that GABA_B receptor activation inhibits calcium-independent spontaneous quantal release (Blackmer et al. 2001; Dittmann and Regehr 1996; Scanziani et al. 1992). However, we cannot exclude the possibility that the baclofen-induced filtering in CTX-treated slices is due to a calcium-independent inhibition of synaptic transmission, and that the resemblance of this filtering to the combined filtering seen in AgTx and SNX is coincidental. We note that a straightforward resolution to the apparent discrepancy is that the faster kinetics of spiking at near-physiological temperatures may be unable to recruit this effect, allowing us to more cleanly assess other mechanisms by which each channel subtype contributes to synaptic dynamics.

The reason why Ca_{2.2} is more susceptible to inhibition than Ca_{2.1} and Ca_{2.3} remains unknown but may be related to the recent finding that Ca_{2.2} is preferentially associated with syntaxin 1A while Ca_{2.1} and Ca_{2.3} are preferentially associated with syntaxin 1B (Müller et al. 2010); of these two genes, syntaxin 1A more effectively facilitates G protein-coupled inhibition of calcium channels (Lü et al. 2001). In conclusion, we have found that G protein-coupled inhibition of Ca_{2.2} is more pronounced than for other Ca_{2.2} family members (reviewed in Tedford and Zamponi 2006). The reason why Ca_{2.2} is more susceptible to inhibition than Ca_{2.1} and Ca_{2.3} remains unknown but may be related to the recent finding that Ca_{2.2} is preferentially associated with syntaxin 1A while Ca_{2.1} and Ca_{2.3} are preferentially associated with syntaxin 1B (Müller et al. 2010); of these two genes, syntaxin 1A more effectively facilitates G protein-coupled inhibition of calcium channels (Lü et al. 2001). In conclusion, we have found that although all three Ca_{2.2} family members contribute to synaptic transmission at the Schaffer collateral synapse, each one has a unique contribution to the synaptic dynamics that are engaged by the complex patterns of afferent activity seen in vivo. As a result, neuro-modulatory changes in the complement of available calcium channels alter the synaptic dynamics in a predictable way; we expect that the synaptic dynamics will be similarly affected by channelopathies that influence the composition of Ca_{2.2} channels contributing to synaptic transmission (Cao et al. 2004). As changes in synaptic dynamics affect the types of behaviorally relevant activity transmitted across the synapse (Frerking and Ohliger-Frerking 2006), we anticipate that changes in the complement of synaptic calcium channels may significantly alter the types of signals effectively transmitted by the synapse in vivo.

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