Presynaptic N-type and P/Q-type Ca^{2+} channels mediating synaptic transmission at the calyx of Held of mice

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At the nerve terminal, both N- and P/Q-type Ca^{2+} channels mediate synaptic transmission, with their relative contribution varying between synapses and with postnatal age. To clarify functional significance of different presynaptic Ca^{2+} channel subtypes, we recorded N-type and P/Q-type Ca^{2+} currents directly from calyces of Held nerve terminals in α_{1A}-subunit-deficient mice and wild-type (WT) mice, respectively. The most prominent feature of P/Q-type Ca^{2+} currents was activity-dependent facilitation, which was absent for N-type Ca^{2+} currents. EPSCs mediated by P/Q-type Ca^{2+} currents showed less depression during high-frequency stimulation compared with those mediated by N-type Ca^{2+} currents. In addition, the maximal inhibition by the $GABA_B$ receptor agonist baclofen was greater for EPSCs mediated by N-type channels than for those mediated by P/Q-type channels. These results suggest that the developmental switch of presynaptic Ca^{2+} channels from N- to P/Q-type may serve to increase synaptic efficacy at high frequencies of activity, securing high-fidelity synaptic transmission.

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Transmitter release is triggered by Ca^{2+} entry through presynaptic voltage-dependent Ca^{2+} channels (Katz, 1969). In the mammalian CNS, multiple types of high-voltage-activated Ca^{2+} channels mediate synaptic transmission (Luebke *et al.* 1993; Takahashi & Momiyama, 1993). Among them N-type Ca^{2+} channels widely mediate synaptic transmission at immature synapses, but their contribution decreases with postnatal development, being replaced by P/Q-type Ca^{2+} channels (Iwasaki *et al.* 2000). To assess the functional significance of the developmental switch of presynaptic Ca^{2+} channel subtypes, it seems essential to compare properties of N-type and P/Q-type Ca^{2+} currents in the same type of nerve terminal. Compared with presynaptic P/Q-type Ca^{2+} currents, which have been characterized at the calyx of Held (Forsythe *et al.* 1998), much less is known for presynaptic N-type Ca^{2+} currents. N-type Ca^{2+} currents can be recorded from immature calyceal terminal after blocking P/Q-type Ca^{2+} channels (Wu *et al.* 1999; Iwasaki *et al.* 2000), but these remaining currents are often too small for detailed analysis. To circumvent this difficulty, we utilized mice with their α_{1A} subunit genetically ablated. These mice lack P/Q-type Ca^{2+} currents, but overexpress N-type Ca^{2+} channels in compensation (Jun *et al.* 1999; Ishikawa *et al.* 2003; Inchauspe *et al.* 2004). Using these knockout (KO) mice and wild-type (WT) littermates, we compared basic properties of N-type and P/Q-type Ca^{2+} currents directly recorded from the calyx of Held presynaptic terminals. Furthermore, we compared EPSCs mediated by N-type and P/Q-type Ca^{2+} currents. Our results indicate that P/Q-type, but not N-type, Ca^{2+} currents undergo activity-dependent facilitation (Borst & Sakmann, 1998; Cuttle *et al.* 1998; Forsythe *et al.* 1998; Tsujimoto *et al.* 2002). Synaptic strength during high-frequency stimulation was consistently higher for EPSCs mediated by P/Q-type Ca^{2+} channels than those mediated by N-type channels. Part of this study has been published in abstract form (Ishikawa *et al.* 2003).

Methods

Preparations and solutions

All experiments were performed in accordance with the guidelines of the Physiological Society of Japan. The generation and phenotype characterization of α_{1A}-deficient mice have been previously described (Jun *et al.* 1999). In the present study, both WT (+/+) and α_{1A}-deficient (−/−) mice were offspring of heterozygous (+/−) mice with the C57BL/6 J genetic background. Mice (9–12 days old) were killed by decapitation under

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halothane anaesthesia. Transverse slices (150–300 μm in thickness) containing the medial nucleus of the trapezoid body were cut from a tissue block containing the brainstem. Before recordings, slices were incubated for 1 h at 36°C in artificial cerebrospinal fluid (aCSF) containing (mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 0.5 ascorbic acid, 3 *myo*-inositol, 2 sodium pyruvate (310 mosmol kg^{-1}, pH 7.3, when saturated with 95% O_2/5% CO_2).

Recording and data analysis

Recordings were made at room temperature (24–26°C). A slice was transferred to a chamber continuously superfused with the aCSF. The giant presynaptic terminal, the calyx of Held, was visualized with a ×60 water-immersion objective (Olympus) attached to an upright microscope (Axioskop, Zeiss). To isolate Ca^{2+} currents, tetrodotoxin (1 μM), tetraethylammonium chloride (10 mM), and 4-aminopyridine (0.5 mM) were included in the aCSF. The presynaptic pipette solution contained (mM): 110 CsCl, 40 Hepes, 0.5 EGTA, 1 $MgCl_2$, 12 phosphocreatine (sodium salt), 2 ATP (magnesium salt) and 0.5 GTP (sodium salt). The presynaptic Ca^{2+} current (I_{pCa}) was evoked in calyceal nerve terminals under voltage clamp at the holding potential of −80 mV by a depolarizing pulse stepping to −10 mV every 10 s, unless otherwise noted. EPSCs were evoked by extracellular stimulation of presynaptic axons with a bipolar tungusten electrode (Forsythe & Barnes-Davies, 1993) at 0.05 Hz. For recording EPSCs, the aCSF routinely contained bicuculline methiodide (10 μM; Sigma, St Louis, MO, USA) and strychnine hydrochloride (0.5 μM; Sigma) to block inhibitory synaptic responses, and D-aminophosphonovalerate (D-APV; 50 μM; Tocris Cookson, Bristol, UK) to block NMDA receptors. During stimulation at 100 Hz (Figs 5*C* and *E*, and 6), kynurenic acid (1 mM; Tocris Cookson) was added to minimize saturation of postsynaptic AMPA receptors. The postsynaptic pipette solution contained (mM): 120 CsF, 30 CsCl, 10 Hepes, 5 EGTA, 1 $MgCl_2$ and 5 *N*-(2,6-diethylphenylcarbamoylmethyl)-triethylammonium chloride (QX-314; Alomone Laboratories, Jerusalem, Israel) 295–300 mOsm (mosmol kg^{-1}), pH was adjusted to 7.3 with CsOH. I_{pCa} and EPSCs were recorded using an Axopatch 200B or a MultiClamp 700 A amplifier, low-pass filtered at 5–6 kHz, and digitized at 50 kHz using Digidata 1200 A or Digidata 1320 (Axon Instruments). Records were also stored for back-ups on a DAT tape, digitized at 48 kHz. Off-line analysis was performed using Clampfit or Axograph (Axon Instruments) and SigmaPlot (SPSS). The electrode resistance for presynaptic and postsynaptic recording was 4–6 MΩ and 1.5–3 MΩ, respectively. The series resistance was 8–16 and 3–8 MΩ for presynaptic and postsynaptic recordings, respectively. In the I_{pCa} recording, series resistance was compensated by 70–80%. Leak currents were subtracted by the scaled pulse divided by six (P/6) protocol. The liquid junction potential between pipette and external solution was not corrected for. ω-Conotoxin GVIA (ω-CgTx) and ω-agatoxin IVA (ω-AgaTx) (Peptide Institute, Japan) were dissolved in aCSF containing 0.1 mg ml^{-1} cytochrome *c*. All values are given as means ± S.E.M., and statistical significance was evaluated by unpaired Student's *t* test, unless otherwise noted. $P < 0.05$ was considered significant.

Results

Pharmacological identification of presynaptic Ca^{2+} currents

Ca^{2+} channel subtypes expressed at the calyx of Held terminal were pharmacologically identified using type-specific blockers. In WT mice, as previously reported in rats of a similar age (Iwasaki & Takahashi, 1998), the N-type Ca^{2+} channel blocker ω-CgTx (2 μM) only slightly (4.9 ± 2.0%, $n = 8$) attenuated the amplitude of I_{pCa}, whereas the P/Q-type Ca^{2+} channel blocker ω-AgaTx (200 nM) blocked most of the remaining I_{pCa} (89.8 ± 2.7% of total currents, $n = 8$) (Fig. 1*A* and *B*). Small currents remaining after application of ω-CgTx and ω-AgaTx (5.3 ± 0.9%) were abolished by Cd^{2+} (100 μM). In contrast, in KO mice, ω-CgTx blocked most of the I_{pCa} (88.3 ± 2.3%, $n = 8$). The remaining currents (11.7 ± 2.3%) were not attenuated by ω-AgaTx, but they were abolished by Cd^{2+}, suggesting that these were R-type currents (Wu *et al.* 1998). Thus I_{pCa} was mainly (88%) N-type in KO mice, whereas it was mainly P/Q-type (90%) in WT mice (Fig. 1*B*). In KO mice, the total I_{pCa} amplitude (500 ± 55 pA, $n = 8$) was 52% of that in WT mice (967 ± 169 pA, $n = 8$), whereas the amplitude of N-type currents in KO mice was 9.6 times larger than that in WT mice (see Fig. 1*B* legend for values), indicating that N-type channels were overexpressed in compensation for P/Q-type channels at the calyx of Held. Similar results have recently been reported at the calyx of Held for the KO and WT mice (Inchauspe *et al.* 2004).

Activation properties of I_{pCa}

Taking advantage of predominant expression of N- and P/Q-type channels, respectively, in the calyceal terminals of KO and WT mice, we first compared the activation properties of N- and P/Q-type I_{pCa}. In the current–voltage relationship, I_{pCa} in KO mice peaked at a membrane potential more positive than that in WT mice, with no difference in their reversal potentials (Fig. 1*C*). Consistently, the activation curve obtained from tail currents (Fig. 1*D*) indicated that the half-activation

voltage was more positive, by 7.1 mV, in KO mice (−9.9 mV) than WT mice (−17.0 mV). The slope factor was similar between KO (9.4 mV) and WT (8.4 mV) mice. These results are similar to those reported by Inchauspe *et al.* (2004).

Inactivation properties of I_{pCa}

We next compared the inactivation properties of Ca^{2+} currents in KO and WT mice. As previously reported in rats (Forsythe *et al.* 1998), inactivation was observed

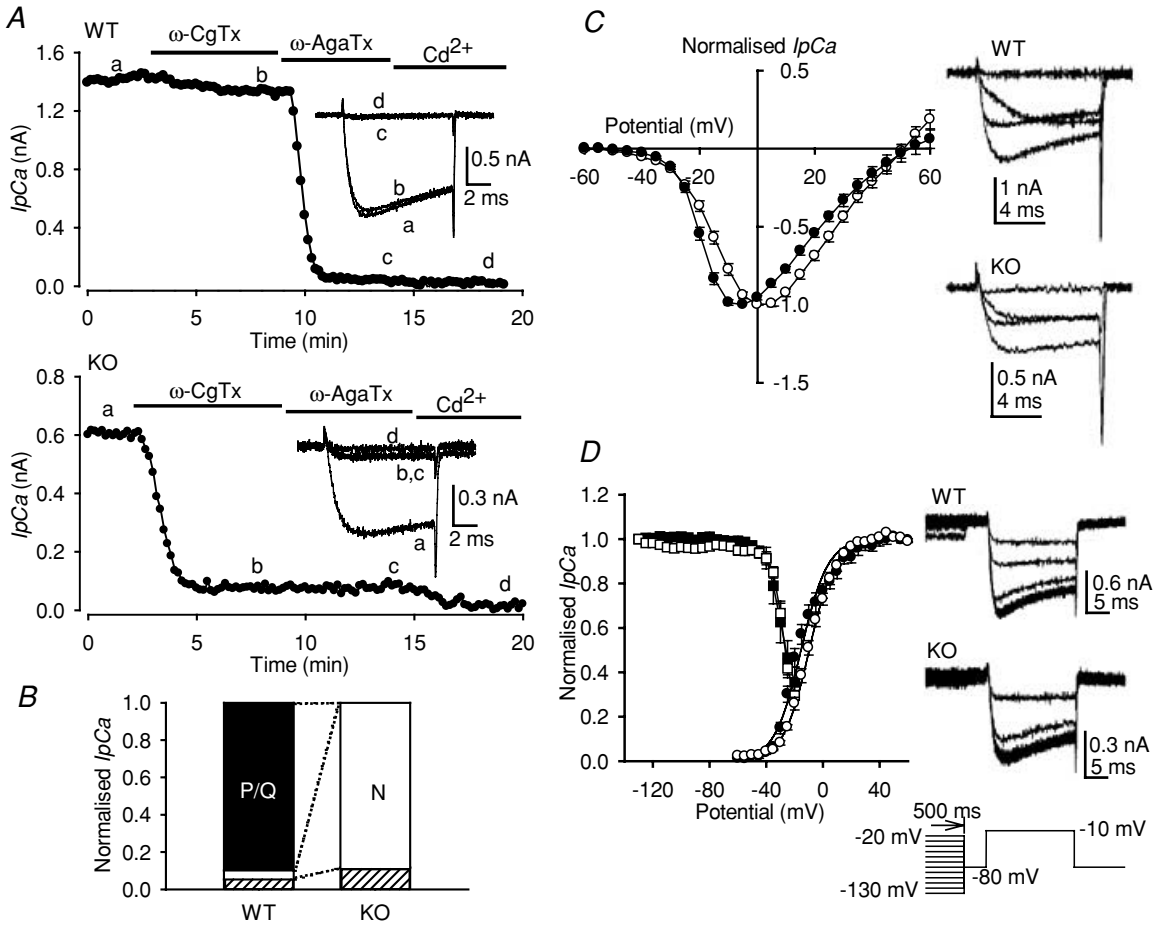

Figure 1. Presynaptic Ca^{2+} current recorded from the calyx of Held presynaptic terminal in wild-type and knockout mice

A and *B*, pharmacological dissection of presynaptic Ca^{2+} current (I_{pCa}). *A*, ω-conotoxin GVIA (ω-CgTx; 2 μM), ω-agatoxin IVA (ω-AgaTx; 200 nM) and Cd^{2+} (100 μM) were applied in this sequence to block I_{pCa} (evoked by a 10 ms depolarizing pulse) in wild-type (WT) and knockout (KO) mice. Sample records are I_{pCa} (average of 6 records) before (a) and after (b) application of ω-CgTx, after ω-AgaTx application (c), and after Cd^{2+} application (d) (superimposed). *B*, percentage of P/Q-type (filled bar) and N-type (open bar) current components in WT and KO mice. Data are derived from 8 calyces each. The N-type current in KO mice was 443 ± 52 pA, whereas that in WT was 46 ± 21 pA. *C*, current–voltage relationships of I_{pCa} in WT (●, $n = 9$) and KO (○, $n = 11$) mice. Sample records show I_{pCa} evoked by depolarizing steps to −40, −20, 0 and +20 mV (superimposed). *D*, activation and inactivation curves. Activation curves (circles) were obtained from tail currents evoked by a 10 ms depolarizing pulse stepping to various membrane potentials. Data obtained from WT (●, $n = 9$) and KO (○, $n = 11$) mice were normalized to the maximal value, plotted against voltage, and fitted by the Boltzmann function $I = 1/(I_{max} + \exp(V_{1/2} - V)/k)$, where $V_{1/2}$ and k represent the half-activation voltage and slope factor, respectively. Five data points between −5 and +15 mV from WT mice were excluded from the activation curve fit because inactivation, proceeding during 10 ms pulse, attenuated the tail current amplitude. Resting inactivation of I_{pCa} was assessed using a protocol (right bottom) comprising conditioning prepulses of various amplitudes (500 ms in duration) followed by a test pulse (10 ms, to −10 mV). To formulate inactivation curves, amplitudes of I_{pCa} evoked by the test pulse were normalized to that evoked after a −130 mV conditioning pulse, and plotted against the conditioning voltage, both in WT (■, $n = 6$) and KO (□, $n = 8$) mice. Sample records show I_{pCa} evoked by the test pulse after conditioning prepulses (10 mV incremental steps from −130 to −20 mV, 12 records superimposed for both WT and KO mice). Lines in *A* and *C* were eye-fitted. Error bars indicate S.E.M.

only in association with activation, indicating no resting inactivation both in KO and WT mice (Fig. 1*D*). During a sustained depolarization, I_{pCa} decayed with a double exponential time course (Fig. 2*A*). The fast and slow time constants of inactivation were 77 ± 22 ms ($71 \pm 5\%$) and 775 ± 187 ms, respectively, in KO mice ($n = 6$), and 28 ± 4 ms ($41 \pm 6\%$) and 609 ± 88 ms, respectively, in WT mice ($n = 5$). There was no significant difference in the weighted mean decay time constant between KO (282 ± 86 ms) and WT (364 ± 50 ms) mice. The recovery time course of I_{pCa} from inactivation largely determines the late phase (>5 s) of recovery of EPSCs from post-tetanic depression (Forsythe *et al.* 1998). We compared the recovery time course of I_{pCa} in KO and WT mice by monitoring I_{pCa} elicited by a 10 ms depolarizing test pulse every 5 s, after inactivating I_{pCa} with a 1.9 s depolarizing conditioning pulse (Fig. 2*B*). During the first 5 s after the conditioning pulse, the amplitude of I_{pCa} rapidly recovered to $45 \pm 6\%$ ($n = 5$) in WT mice and $39 \pm 4\%$ ($n = 5$) in KO mice (no significant difference). Thereafter, the I_{pCa} amplitude recovered more slowly with mono-exponential time course (Fig. 2*B*), with a similar time constant in WT mice (14.7 ± 1.7 s, $n = 5$) and KO mice (16.2 ± 2.3 s, $n = 5$). As I_{pCa} recovered from inactivation, its rise time tended to become faster in WT mice, but not in KO mice (Fig. 2*B*).

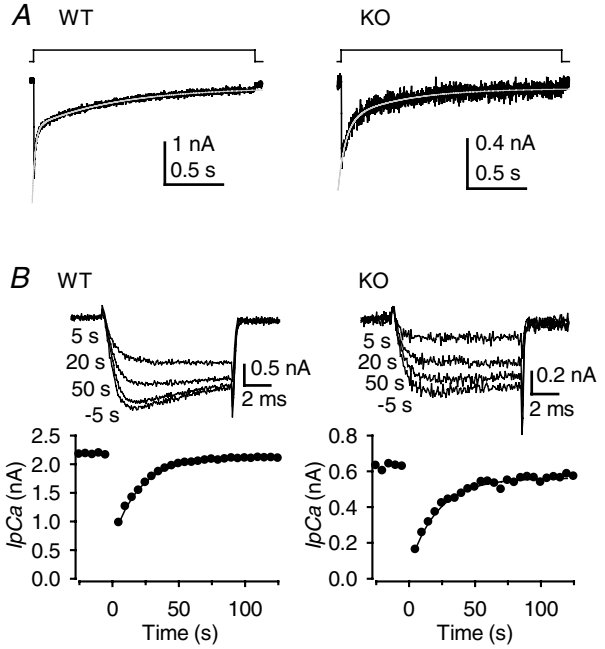

Figure 2. Inactivation and recovery time courses of I_{pCa}
A, inactivation of I_{pCa} during a 1.9 s depolarizing pulse in WT and KO mice. The inactivation time courses were fitted with double exponential curves (superimposed on records). *B*, the time course of recovery from inactivation. Ordinates indicate the amplitude of I_{pCa} elicited by a 10 ms test pulse every 5 s before and after application (at time 0) of a 1.9 s conditioning pulse. Sample records show I_{pCa} evoked at 5 s before, and 5, 20 and 50 s after the conditioning pulse (superimposed).

Activity-dependent facilitation of I_{pCa}

At the calyx of Held presynaptic terminal in WT mice, a pair of brief depolarizing pulses, given at a short interval, facilitated the second I_{pCa} (Fig. 3*A*) as in rats (Borst & Sakmann, 1998; Cuttle *et al.* 1998; Forsythe *et al.* 1998; Tsujimoto *et al.* 2002). The magnitude of facilitation at 5 ms interstimulus interval (ISI) was $19 \pm 4\%$ ($n = 10$), similar to that in rats (Cuttle *et al.* 1998). When activated repetitively at high frequency (100 Hz), I_{pCa} in WT mice underwent a marked facilitation (Fig. 3*B*), as in rats (Cuttle *et al.* 1998; Forsythe *et al.* 1998). These I_{pCa} facilitations result from a Ca^{2+}-dependent increase in activation kinetics (Borst & Sakmann, 1998; Cuttle *et al.* 1998), which is mediated by the Ca^{2+}-binding protein neuronal calcium sensor 1 (NCS-1; Tsujimoto *et al.* 2002). In contrast, in KO mice, I_{pCa} showed no facilitation in the paired-pulse protocol (Fig. 3*C*) (Inchauspe *et al.* 2004) or during high-frequency activation (Fig. 3*D*). These results indicate that I_{pCa} facilitation is a unique property of P/Q-type Ca^{2+} channels (Ishikawa *et al.* 2003; Inchauspe *et al.* 2004).

Given that I_{pCa} facilitation is Ca^{2+} dependent (Cuttle *et al.* 1998) and that the mean I_{pCa} amplitude in KO mice was 52% of that in WT mice, it might be argued that the lack of I_{pCa} facilitation in KO mice resulted from a lower Ca^{2+} channel density in KO mice. We examined this possibility in WT mice by partially blocking Ca^{2+} channels with incremental concentrations of ω-AgaTx (2–200 nM, Fig. 4*A*). When I_{pCa} was reduced to 40–60%, the I_{pCa} facilitation ratio (3 ms ISI, 1.26 ± 0.08, $n = 4$) remained similar to control before ω-AgaTx application (1.30 ± 0.10, $P > 0.2$, paired t test, Fig. 4*C*), although further reduction of I_{pCa} decreased the facilitation ratio (Fig. 4*B*). These results suggest that the lack of I_{pCa} facilitation in KO mice did not result from their low Ca^{2+} channel density, but from the absence of activity-dependent facilitation in N-type Ca^{2+} channels.

Short-term plasticity of synaptic transmission in WT and KO mice

We next compared EPSCs mediated by P/Q-type Ca^{2+} channels in WT mice with those mediated by N-type Ca^{2+} channels in KO mice. Despite significantly smaller I_{pCa} amplitude in KO mice, the EPSC amplitudes in WT mice (6.52 ± 0.86 nA, $n = 12$) and KO mice (6.87 ± 1.01 nA, $n = 12$) were surprisingly similar. This did not result from the postsynaptic receptor saturation, because the EPSC amplitude in the presence of the low affinity glutamate antagonist kynurenate (1 mM) was also similar between WT (1.37 ± 0.26 nA, $n = 12$) and KO (1.70 ± 0.45 nA,

$n = 12$) mice. The rise and decay kinetics of EPSCs were also similar between WT and KO mice, with 10–90% rise time being 0.33 ± 0.01 ms ($n = 12$) in WT mice, and 0.32 ± 0.02 ms ($n = 12$) in KO mice, with the weighted mean decay time constant being 1.54 ± 0.08 ms ($n = 12$) in WT mice, and 1.75 ± 0.26 ms ($n = 12$) in KO mice.

Might the differential occurrence of I_{pCa} facilitation between WT and KO mice be reflected in the short-term plasticity of EPSCs? During 100 Hz stimulation, EPSCs in both WT and KO mice underwent a depression (Fig. 5*A*), as previously reported in rats (von Gersdorff *et al.* 1997; Iwasaki & Takahashi, 2001) and mice (Wang & Kaczmarek, 1998). This depression is caused mainly by depletion of synaptic vesicles (Wang & Kaczmarek, 1998), although other factors such as inactivation of I_{pCa} (Forsythe *et al.* 1998; Xu & Wu, 2005), desensitization of postsynaptic AMPA receptors (Wong *et al.* 2003) and inhibition of transmitter release mediated by presynaptic G-protein-coupled autoreceptors (von Gersdorff *et al.* 1997; Kimura *et al.* 2003) are also involved. The magnitude of depression was less in WT mice than KO mice, although the difference was statistically insignificant. At the calyx of Held, like other synapses many, synaptic facilitation is masked by stronger synaptic depression in normal aCSF, whereas lowering $[Ca^{2+}]_o$ reduces vesicle depletion and postsynaptic receptor desensitization, thereby unmasking synaptic facilitation. In 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution, during a train of 100 Hz stimulation, EPSCs in WT mice underwent a transient facilitation (Fig. 5*B*). In the same condition, however, no significant facilitation was observed for EPSCs in KO mice. Lowering $[Ca^{2+}]_o$ also reduces I_{pCa} facilitation because of its Ca^{2+} dependence (Cuttle *et al.* 1998). In WT mice, in 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution, where I_{pCa} was reduced to 73% ± 2% ($n = 9$), I_{pCa} facilitation (ISI, 10 ms) was still significant ($6.7 \pm 0.5\%$, $P < 0.001$, $n = 10$; Fig. 5*C* and *D*), although it was less than that in a normal solution ($12 \pm 0.6\%$, $n = 14$). We also tested 0.6 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution (Inchauspe *et al.* 2004). In this solution I_{pCa} was reduced to $57 \pm 8\%$ ($n = 6$), and I_{pCa} facilitation was $4.1 \pm 0.7\%$ ($n = 6$; Fig. 5*C* and *D*).

At synapses with higher release probability, synaptic responses generally undergo stronger depression during repetitive stimulation (Otsuka *et al.* 1962; Iwasaki & Takahashi, 2001). We examined whether different release probabilities underlie the different synaptic strength during repetitive stimulation (Fig. 5*B*) between KO and WT mice. During 10 Hz stimulation in 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution, EPSCs underwent a similar magnitude of depression in both WT and KO mice (Fig. 5*E*), implying that the release probability may be similar between WT and KO mice. In the same condition, in WT mice, I_{pCa} did not show facilitation (Fig. 5*F*) as expected from the short-lasting effect of a conditional pulse on I_{pCa} facilitation in WT mice (Fig. 3*A*) and rats (Cuttle *et al.* 1998) in normal aCSF.

We further compared release probability at the calyx of Held between WT and KO mice using a high-frequency stimulation protocol (Schneggenburger *et al.* 1999). In this

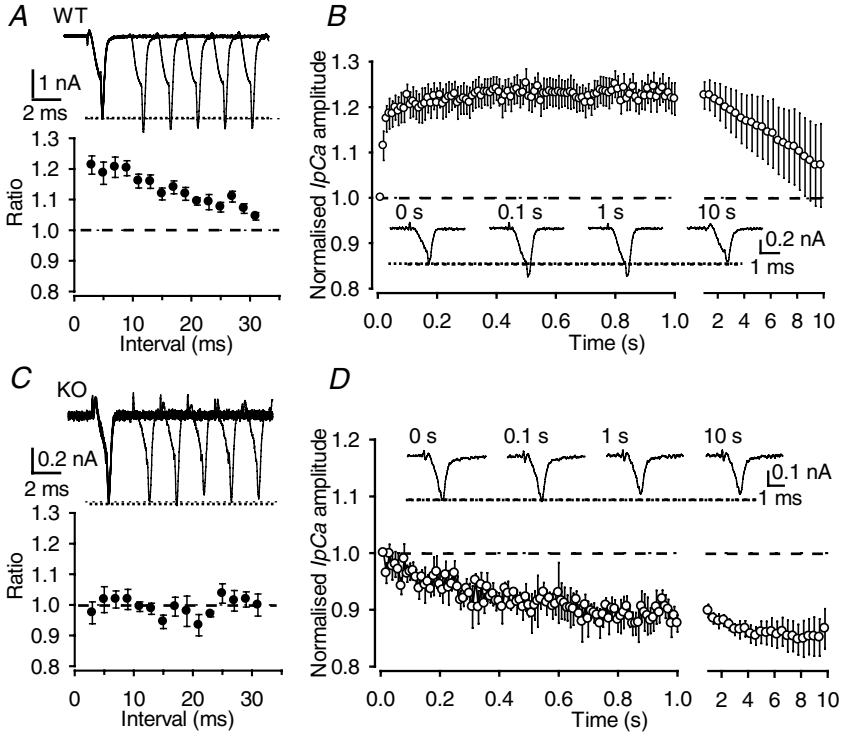

Figure 3. Activity-dependent facilitation of I_{pCa}
A and *C*, I_{pCa} evoked by a pair of 1 ms depolarizing pulses at different interpulse intervals (superimposed in sample records, dashed lines indicate the first I_{pCa} amplitude). The paired-pulse ratio (the second amplitude relative to the first one) is plotted against interpulse intervals in WT (*A*, $n = 10$) and KO (*C*, $n = 7$) mice. Paired-pulse facilitation of I_{pCa} was observed only in WT mice with interpulse intervals shorter than 30 ms. The results were essentially the same when the depolarizing pulse was stepped to 0 mV instead of −10 mV ($n = 12$, data not shown). *B* and *D*, I_{pCa} evoked by a 100 Hz train of 1 ms depolarizing pulses (10 s). Ordinates indicate the relative I_{pCa} amplitude normalized to the first amplitude (dashed line) in WT (*B*, $n = 7$) and KO (*D*, $n = 6$) mice. The time scale (abscissa) is squeezed between 2 and 10 s.

method (Fig. 6*A*), the zero time intercept of the slope, fitted to a cumulative amplitude histogram of EPSCs evoked at 100 Hz, provides an estimate of the size of the readily releasable pool of synaptic vesicles (N) multiplied by the mean quantal amplitude (q). The release probability (p) can then be estimated by dividing the first EPSC amplitude by Nq. These estimations revealed no significant difference between KO and WT mice in both Nq and p (Fig. 6*B*), suggesting further that the short-term facilitation of EPSCs observed predominantly in WT mice (Fig. 5*B*) did not arise from a difference in release probability. Therefore, these results strongly suggest that the activity-dependent facilitation of P/Q-type Ca^{2+} currents underlies synaptic facilitation in WT mice in 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution.

Presynaptic inhibition mediated by GTP-binding-protein-coupled receptor in WT and KO mice

In neuronal and secretory cells, the inhibitory effect of GTP-binding (G) proteins on N-type Ca^{2+} channels is stronger than that on P/Q-type Ca^{2+} channels (Bourinet *et al.* 1996; Zhang *et al.* 1996; Currie & Fox, 1997). At central synapses, the G-protein-coupled receptor (GPCR)-mediated presynaptic inhibitions are selectively blocked by the N-type-channel-specific blocker ω-CgTX (Umemiya & Berger, 1994; Momiyama & Koga, 2001; Liang *et al.* 2003). The calyces of Held in WT and KO mice provide an ideal preparation to test the subtype selectivity in the coupling between GPCRs and Ca^{2+} channels in the nerve terminal. At the calyx of Held, the $GABA_B$ receptor agonist baclofen inhibits I_{pCa} (Takahashi *et al.* 1998) via G protein $\beta\gamma$ subunits, thereby presynaptically inhibiting EPSCs (Kajikawa *et al.* 2001). As illustrated in Fig. 7*A*, bath applications of baclofen in incremental concentrations attenuated EPSCs in a concentration-dependent manner in both WT and KO mice. The concentration–response relationship of baclofen (Fig. 7*B*) indicated that the maximal inhibition of EPSCs by baclofen (200 μM) in KO mice (92 ± 2%, $n = 4$) was significantly higher ($P < 0.05$) than that in WT mice (83 ± 3%, $n = 5$), whereas the IC_{50} was similar between WT (0.41 μM) and in KO (0.38 μM) mice. Consistently, inhibition of I_{pCa} by 20-μM baclofen in KO mice (53 ± 2%, $n = 6$) was significantly higher ($P < 0.05$) than that in WT mice (37 ± 4%, $n = 3$; Fig. 7*C*). These results suggest that, at the calyx of Held, presynaptic $GABA_B$ receptors couple with N-type Ca^{2+} channels more strongly than with P/Q-type Ca^{2+} channels.

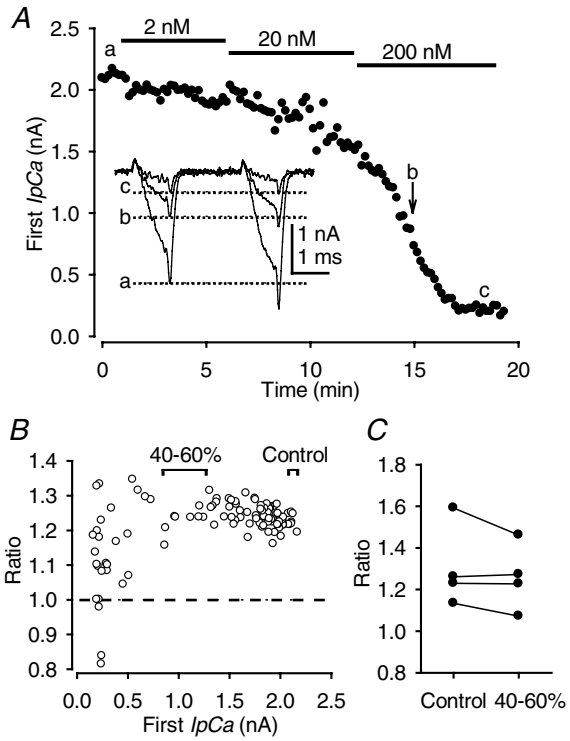

Figure 4. The effect of Ca^{2+} influx on I_{pCa} facilitation in WT mice
A, the first I_{pCa} amplitude of a pair during application of incremental concentrations of ω-AgaTx (2–200 nM). Ca^{2+} currents were evoked by a pair (3 ms interval) of 1 ms depolarizing pulses (from −80 to 0 mV) applied every 10 s. Sample records show pairs of I_{pCa} evoked at different epochs (a–c, superimposed). The first I_{pCa} amplitudes are indicated by dashed lines. *B*, the paired-pulse ratio of I_{pCa} (ordinate) plotted against the first I_{pCa} amplitude (abscissa). *C*, the paired-pulse ratio of I_{pCa} when ω-AgaTx blocked I_{pCa} to 40–60% was not significantly different (paired *t* test) from that before ω-AgaTx application (control).

Discussion

Common and different properties of N-type and P/Q-type Ca^{2+} currents recorded from calyceal nerve terminals

Using α_{1A}-deficient mice and their WT littermates, we compared characteristics of N-type and P/Q-type Ca^{2+} currents directly recorded from the calyx of Held presynaptic terminals. Presynaptic N-type and P/Q-type Ca^{2+} currents shared common properties, with both lacking resting inactivation and both having similar kinetics in inactivation and recovery from inactivation. A small difference was found for the voltage range of activation, which was more negative in the P/Q-type than in N-type channels. The most striking difference was found for the activity-dependent facilitation, which was predominantly observed for P/Q-type Ca^{2+} currents. This Ca^{2+} current facilitation is dependent upon Ca^{2+} influx (Cuttle *et al.* 1998) and mediated by the Ca^{2+}-binding protein NCS-1 (Tsujimoto *et al.* 2002), but independent of G proteins. It is therefore distinct from the depolarization-induced disinhibition of G proteins (Ikeda, 1991; Kasai, 1992; Brody & Yue, 2000), and also from the calmodulin-dependent Ca^{2+} current facilitation (Lee *et al.* 2000; DeMaria *et al.* 2001) (for a review, see Takahashi, 2005). Another difference between N- and

P/Q-type Ca^{2+} currents in the calyceal nerve terminal was found for the heterotrimeric G-protein-mediated inhibition. The magnitude of inhibition of N-type I_{pCa} by maximal concentration of baclofen was significantly greater than that of P/Q-type I_{pCa}, as reported for Ca^{2+} currents in neuronal and secretory cells (Bourinet *et al.* 1996; Zhang *et al.* 1996; Currie & Fox, 1997).

Synaptic transmission mediated by P/Q-type or N-type Ca^{2+} channels

EPSCs mediated by P/Q-type and those mediated by N-type Ca^{2+} channels showed different short-term synaptic plasticity in low Ca^{2+} aCSF (1 mM $[Ca^{2+}]_o$ 2 mM $[Mg^{2+}]_o$), with the former undergoing a transient

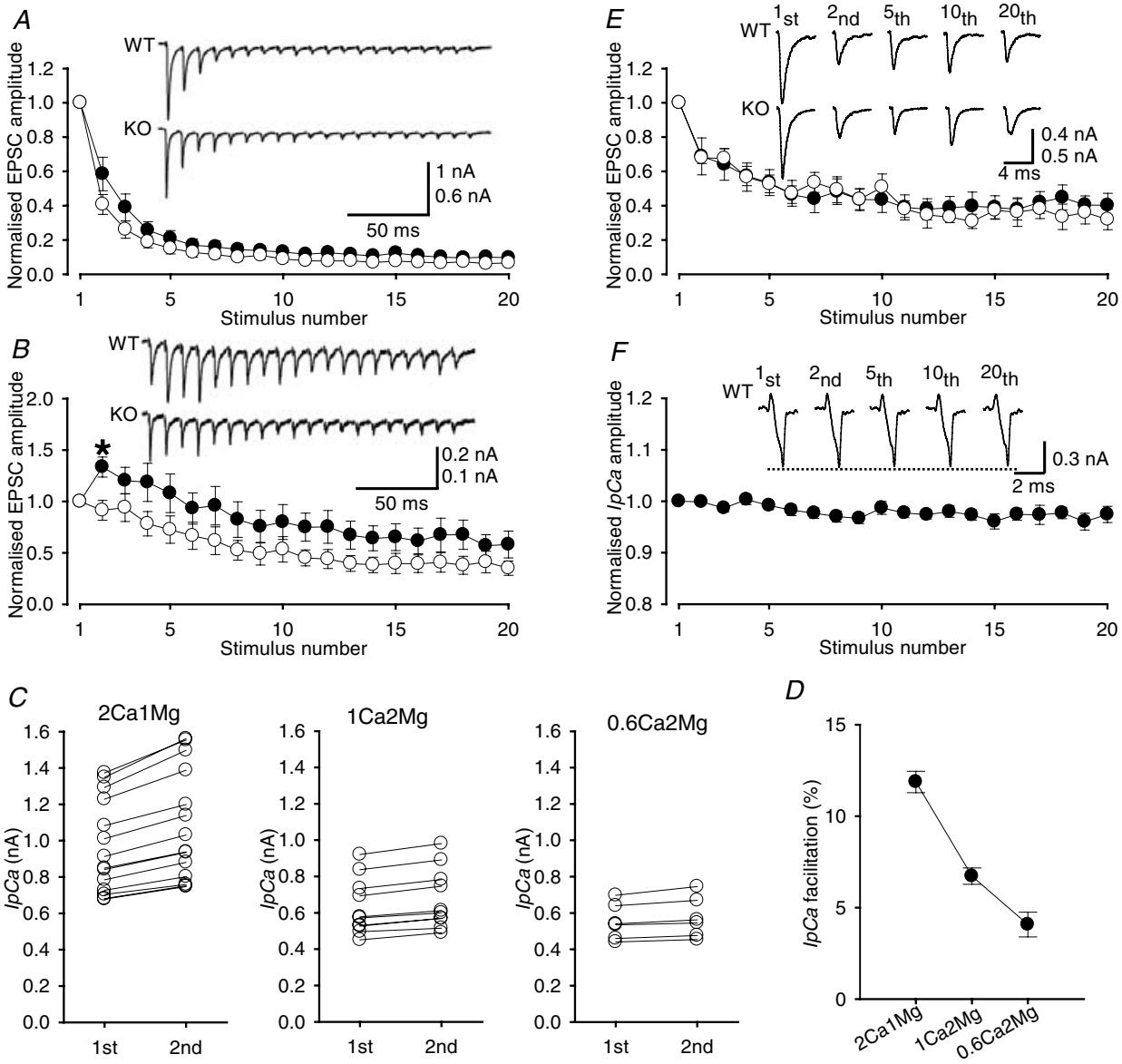

Figure 5. Activity-dependent changes in I_{pCa} and EPSCs in KO and WT and mice
A, EPSCs evoked by a train of 20 stimuli at 100 Hz in normal artificial cerebrospinal fluid (aCSF) in WT (upper sample trace, ●) and KO (lower sample trace, ○) mice. *B*, EPSCs evoked by a train of 100 Hz stimulation in 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution in WT (●) and KO (○) mice. The asterisk indicates the point at which the EPSC amplitude in WT mice was significantly larger than that in KO mice ($P < 0.05$). *C*, the first and second amplitude of I_{pCa} in WT mice evoked at 100 Hz (10 ms interstimulus interval) in normal aCSF (left panel), 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution (middle panel) and 0.6 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution (right panel). The second I_{pCa} amplitudes were significantly larger than the first one in all solutions ($P < 0.01$ in paired *t* test). *D*, the mean magnitude of I_{pCa} facilitation (%) in WT mice in three different solutions. *E*, EPSCs evoked by a train of 20 stimuli at 10 Hz in 1 mM $[Ca^{2+}]_o$ (2-mM $[Mg^{2+}]_o$) solution in WT (●) and KO (○) mice. *F*, normalized amplitudes of I_{pCa} ($n = 5$) in WT mice evoked by a train of 20 stimuli at 10 Hz in 1 mM $[Ca^{2+}]_o$ (2 mM $[Mg^{2+}]_o$) solution. No I_{pCa} facilitation was observed. Error bars indicate S.E.M.

facilitation during a 100 Hz train of stimulation, whereas the latter showed a depression throughout the train. Such a difference can, in principle, be caused by different release probability; however, this was not the case between WT and KO mice. Given that the activity-dependent I_{pCa} facilitation was observed only for P/Q-type channels in WT mice, this is the most likely cause for the difference in short-term plasticity between KO and WT mice. In the low Ca^{2+} aCSF solution, the second I_{pCa} following the first one at the 10 ms interval, was facilitated by 6.7%. Assuming fourth power for the I_{pCa}–EPSC relationship, an increase of the EPSC amplitude caused by the I_{pCa} facilitation is estimated to be 30%. This magnitude is close to the difference in the magnitude of the second EPSCs between WT and KO mice during 100 Hz stimulation in 1 mM $[Ca^{2+}]_o$/2 mM $[Mg^{2+}]_o$ solution (42%, Fig. 5*B*),

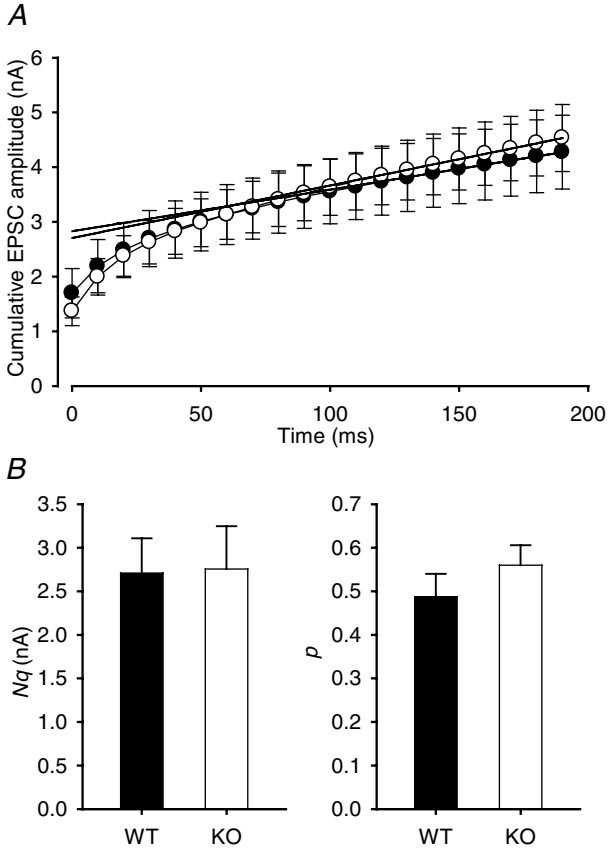

Figure 6. Release probability in WT and KO mice
A, cumulative amplitudes of EPSCs during a 100 Hz train in WT (●) and KO (○) mice (data taken from Fig. 5*A*). Data points in the range of the 16–20th EPSC amplitudes were fitted by linear regression and extrapolated to time zero to estimate the readily releasable pool size of synaptic vesicles (*Nq*). *B*, left panel, *Nq* estimated in individual cells; right panel, release probability (*p*) estimated from the first EPSC amplitude divided by *Nq*. In WT and KO mice, *Nq* was 2.71 ± 0.40 nA ($n = 12$) and 2.76 ± 0.49 nA ($n = 12$), respectively, whereas *p* was 0.49 ± 0.05 ($n = 12$) and 0.56 ± 0.05 ($n = 12$), respectively. Neither *p* nor *Nq* was significantly different between WT and KO mice. Error bars indicate S.E.M.

supporting further that the activity-dependent facilitation of P/Q-type channels significantly contributes to the facilitation of P/Q-type channel-mediated synaptic transmission. Inchauspe *et al.* (2004) reported that EPSCs at the calyx of Held evoked by the paired-pulse stimulation protocol in 0.6 mM $[Ca^{2+}]_o$/2–3 mM $[Mg^{2+}]_o$ solution show facilitation in WT mice, but not in KO mice, and they assumed that this difference was caused by the difference in I_{pCa} facilitation, although they did not measure I_{pCa} facilitation in their study conditions. We tested the I_{pCa} facilitation in their study conditions (0.6 mM $[Ca^{2+}]_o$/2 mM $[Mg^{2+}]_o$) and our results (Fig. 5*D*) generally support their conclusion. At the calyx of Held in rats, before postnatal day 10, synaptic transmission is mediated by N-type, P/Q-type and R-type Ca^{2+} channels (Iwasaki & Takahashi, 1998; Wu *et al.* 1998). After this period, however, P/Q-type Ca^{2+} channels become predominant in mediating synaptic transmission (Iwasaki & Takahashi, 1998; Iwasaki *et al.* 2000). Such a developmental switch of presynaptic Ca^{2+} channel subtypes is also observed at thalamic and cerebellar inhibitory synapses (Iwasaki *et al.* 2000). Our finding of P/Q-type-specific Ca^{2+} current facilitation suggests that the switch of Ca^{2+} channel subtypes from N- to P/Q-type in developing nerve terminals may strengthen reliability of transmission by raising synaptic efficacy during high-frequency transmission. At synapses with low release probability, synaptic facilitation is prominent, and it plays an important role in the generation of postsynaptic action potentials (Henze *et al.* 2002). At such synapses, P/Q-type Ca^{2+} current facilitation may play a critical role in the signal flow through neuronal circuits.

G-protein-coupled receptor-mediated inhibition of transmitter release triggered by P/Q-type or N-type Ca^{2+} channels

It is generally believed that N-type Ca^{2+} channels are more tightly linked to heterotrimeric G proteins. N–type Ca^{2+}currents are selectively inhibited by adenosine A1 receptor agonists in brainstem neurones (Umemiya & Berger, 1994), and by ATP (Currie & Fox, 1996) or GTPγS (Currie & Fox, 1997) in chromaffin cells. Compared with recombinant α_{1A} Ca^{2+} currents, α_{1B} Ca^{2+}currents are more strongly inhibited by opioids (Bourinet *et al.* 1996), somatostatin (Zhang *et al.* 1996) and ATP (Currie & Fox, 2002). Consistently A_1 receptor agonist selectively blocks glycinergic transmission mediated by N-type Ca^{2+} channels in brainstem slices (Umemiya & Berger, 1994). However, at the calyx of Held, an A_1 receptor agonist inhibited EPSCs mediated by N-type or P/Q-type Ca^{2+} channels to a similar extent (Kimura *et al.* 2003), suggesting that the selective coupling of N-type Ca^{2+} channels with heterotrimeric G proteins may not be general. In the present

study, we re-examined this issue at the calyx of Held for the presynaptic inhibitory effect of baclofen on I_{pCa} and EPSCs mediated by P/Q-type Ca^{2+} channels in WT mice and N-type Ca^{2+} channels in KO mice. Our results indicate that the maximal inhibition by baclofen of N-type channel-mediated EPSCs is by 8% greater than that of P/Q-type channel-mediated EPSCs, whereas the IC_{50} values of the baclofen effect on EPSCs were similar between those mediated by N-type and P/Q-type Ca^{2+} channels. Thus, compared with P/Q-type Ca^{2+} channels, N-type Ca^{2+} channels are more strongly coupled with $GABA_B$ receptors at the calyceal nerve terminal, although the difference is smaller than those previously reported (Umemiya & Berger, 1994; Currie & Fox, 1996, 1997, 2002; Bourinet *et al.* 1996; Zhang *et al.* 1996).

At the calyx of Held, EPSCs were not affected by a $GABA_B$-receptor-selective antagonist (Yamauchi *et al.* 2000), suggesting that presynaptic $GABA_B$ receptors are not normally activated by endogenous GABA, at least in slices. Metabotropic glutamate receptors (von Gersdorff *et al.* 1997) and A_1 adenosine receptors (Kimura *et al.* 2003) in the calyceal terminals are activated during repetitive transmission at the immature calyx of Held, but their contribution to synaptic depression is small. Nevertheless, given that less tight coupling of GPCRs with P/Q-type channels is widely reported at many synapses (Umemiya & Berger, 1994; Momiyama & Koga, 2001; Liang *et al.* 2003), the N-to-P/Q-type switch of presynaptic Ca^{2+} channels during postnatal development (Iwasaki *et al.* 2000) might potentially attenuate the GPCR-mediated presynaptic inhibition at various CNS synapses.

Compensatory changes associated with the α_{1A}-subunit knockout

Despite significantly smaller I_{pCa} in KO mice compared with WT mice, mean amplitude of EPSCs was surprisingly similar between KO and WT mice. This was not secondary to saturation of postsynaptic AMPA receptors because mean amplitudes of EPSCs in KO and WT mice were

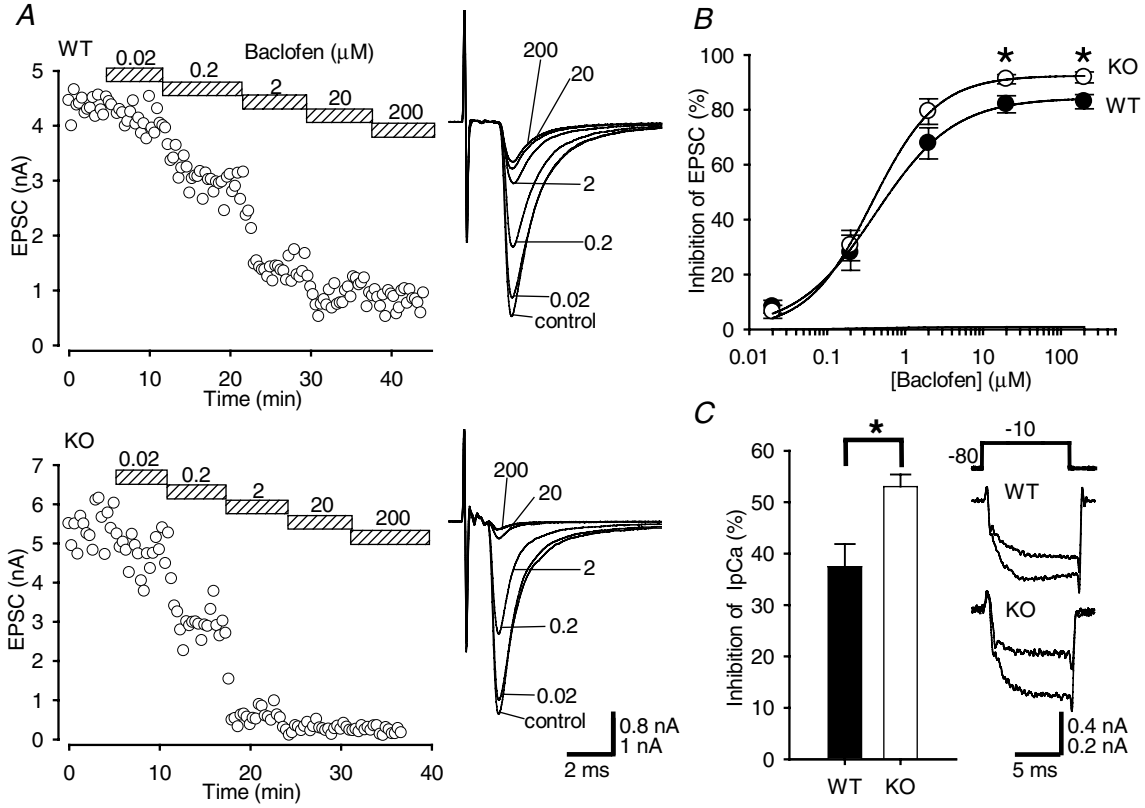

Figure 7. Inhibitory effects of baclofen on EPSCs and I_{pCa} in WT and KO mice
A, cumulative inhibitory effects of baclofen in WT (upper panel) and KO (lower panel) mice with sample traces of EPSCs (right panels, superimposed). *B*, concentration–response curves of inhibitory effect of baclofen on EPSCs in WT (●) and KO (○) mice. The magnitudes of inhibition (ordinate) at 20 and 200 μM were significantly larger in KO than in WT mice ($^*P < 0.05$). Data were derived from 5 and 4 cells in WT and KO mice, respectively. A curve fit to the data points represents the equation: inhibition (%) = [maximal inhibition]/[1 + (IC_{50}/baclofen concentration)^n], where maximal inhibition was 84% in WT and 93% in KO mice, IC_{50} was 0.42 μM in WT and 0.39 μM in KO mice, and *n* values were 0.86 and 1.04 in WT and KO mice, respectively. *C*, inhibition of I_{pCa} by baclofen (20 μM) in KO mice was significantly stronger ($P < 0.05$) than that in WT mice. Error bars indicate S.E.M.

still similar after reducing the AMPA receptor occupancy with kynurenate. Analysis using the tetanic stimulation protocol indicated that neither the release probability nor the number of readily releasable synaptic vesicles is different between WT and KO mice. Inchauspe *et al.* (2004), however, reported that EPSCs in KO mice are on average by 33% smaller than those in WT mice. The main difference between their results and ours lies in the smaller EPSC amplitude in their KO mice (3.9 nA, $n = 7$) compared with ours (6.9 nA, $n = 12$). Although the reason for the difference is not entirely clear, it might arise from general conditions of KO mice, because their KO mice (11–15 days old) are older than ours (9–12 days old), and the α_{1A}-subunit KO mice become increasingly weak after 12 postnatal days until they eventually die 3 weeks after birth (Jun *et al.* 1999).

In KO mice, a compensatory mechanism(s) seems to maintain release probability, despite reduced presynaptic Ca^{2+} influx. A possible mechanism may be that N-type Ca^{2+} channels overexpressed in KO mice are more efficiently coupled to the release machinery than P/Q-type channels in WT mice. This, however, is unexpected from previous reports. At the normal calyx of Held of rats, N- and R-type Ca^{2+} channels are distantly located from release sites, and Ca^{2+} influx through them is less effective in triggering transmitter release compared with that through P/Q-type Ca^{2+} channels (Wu *et al.* 1999). Furthermore, at the neuromuscular junction of α_{1A}-KO mice, overexpressed N-type Ca^{2+} channels are distantly located from release sites (Urbano *et al.* 2003). Another possible mechanism for the compensatory increase in release probability would be that the release machinery might acquire higher Ca^{2+} sensitivity in KO mice. Activation of presynaptic protein kinase C directly facilitates transmitter release (Hori *et al.* 1999) by increasing Ca^{2+} sensitivity of the release machinery (Lou *et al.* 2005). Also, an increase in cAMP in the nerve terminal facilitates transmitter release without affecting I_{pCa} (Sakaba & Neher, 2001; Kaneko & Takahashi, 2004). It remains to be seen whether these second messengers are involved in this compensatory mechanism.

Whilst the amplitude of I_{pCa} in KO mice was about one half of that in WT mice, blocking P/Q-type Ca^{2+} currents in WT mice by 50% with ω-AgaTx had no significant effect on the magnitude of I_{pCa} facilitation. In contrast, a reduction of I_{pCa} to 57% by lowering external $[Ca^{2+}]_o$ to 0.6 mM reduced I_{pCa} facilitation from 12 to 4.1%. These results suggest that the magnitude of P/Q-type Ca^{2+} current facilitation is highly dependent upon the amount of Ca^{2+} influx through individual Ca^{2+} channels, but it is relatively independent of the Ca^{2+} channel density. Presumably the Ca^{2+}-dependent I_{pCa} facilitation occurs immediately around the site of Ca^{2+} entry.

Given that native presynaptic N-type Ca^{2+} currents in WT animals are too small for detailed analysis, our study cannot entirely exclude a possibility that the difference in I_{pCa} observed between WT and KO mice might arise from an unexpected secondary effect induced by gene knockout. It should also be noted that, despite a clear correlation between I_{pCa} facilitation and synaptic facilitation shown here, a causal relationship between them still remains to be established. Despite these reservations, results obtained here will provide a new step toward understanding the meaning behind different Ca^{2+} channel subtypes triggering transmitter release at CNS synapses.

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