Developmental upregulation of presynaptic NCKX underlies the decrease of mitochondria-dependent post-tetanic potentiation at the rat calyx of Held synapse

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Running title:
Developmental changes in Ca²⁺ clearance and short-term plasticity

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Abstract

The sensitivity of post-tetanic potentiation (PTP) to high frequency stimulation (HFS) steeply decays during the first two postnatal weeks. We investigated the underlying mechanisms for the developmental change of PTP induced by HFS (100 Hz, 2 sec) at postnatal days 4-6 and 9-11 at the rat calyx of Held synapse. Low concentration tetraphenylphosphonium (2 μM), an inhibitor of mitochondrial Na+/Ca2+ exchanger, suppressed the amount of post-tetanic residual calcium and PTP to a larger extent at the immature calyx synapse, indicating a developmental reduction of mitochondrial contribution to PTP. The higher amount of mitochondrial Ca2+ uptake during HFS and consequent post-tetanic residual Ca2+ at the immature calyx of Held was associated with higher peak of HFS-induced Ca2+ transients, most likely because the mitochondrial Ca2+ uptake during HFS was supra-linearly dependent on the presynaptic [Ca2+] level. Probing into the contribution of Na+/Ca2+ exchangers to calcium clearance, we found a specific upregulation of the K+-dependent Na+/Ca2+ exchanger (NCKX) activity in the mature calyx of Held. We conclude that the upregulation of NCKX limits the Ca2+ buildup and inhibits mitochondrial Ca2+ uptake during HFS, which in turn results in the reduction of post-tetanic residual Ca2+ and PTP at the mature calyx of Held synapse.

Keywords: Ca2+ clearance mechanisms, post-tetanic potentiation, K+-dependent Na+/Ca2+ exchanger, mitochondria, presynaptic terminal
Introduction

Post-tetanic potentiation (PTP), a transient enhancement of EPSC after high-frequency stimulation (HFS) which lasts for tens of seconds, has been reported not only in the neuromuscular junction (NMJ) (Tang and Zucker 1997) but also in central synapses including calyx of Held synapses (Habets and Borst 2005; Korogod et al. 2005), hippocampal Schaffer collateral (Brager et al. 2003) and mossy fiber synapses (Lee et al. 2007a). The causal relationship is well established between PTP and the post-tetanic transient elevation of presynaptic resting [Ca$^{2+}$], so-called post-tetanic residual Ca$^{2+}$ (Ca$_{res}$) at the NMJ and the calyx of Held synapse (Habets and Borst 2005; Kamiya and Zucker 1994; Korogod et al. 2005; Lee et al. 2008; Tang and Zucker 1997). Previously, we have reported that mitochondrial Ca$^{2+}$ uptake during HFS is a prerequisite for the generation of post-tetanic Ca$_{res}$ at the calyx of Held (Lee et al. 2008).

The calyx of Held synapse undergoes a variety of structural and functional changes during postnatal development (Felmy and Schneggenburger 2004; Taschenberger et al. 2002; Wang et al. 2008; Wimmer et al. 2006). The immature calyx of Held synapses exhibit higher sensitivity for PTP induction compared to more mature ones (Korogod et al. 2005), but the underlying mechanisms are not well defined. Previously, we have reported that Na$^+$/Ca$^{2+}$ exchanger (Na/CaX) clears Ca$^{2+}$ loads more readily than mitochondria at the calyx of Held and that mitochondria take part in Ca$^{2+}$ clearance only when Na/CaX is inhibited or its function is saturated (Kim et al. 2005). Ca$^{2+}$ clearance becomes faster as the calyx of Held matures (Chuhma and Ohmori 2001), but it has not been investigated whether developmental changes in Ca$^{2+}$ clearance are causally related with developmental changes in PTP. Given that presynaptic mitochondria compete with Na/CaX for incoming Ca$^{2+}$ (Kim et al. 2005), we hypothesized that mitochondrial Ca$^{2+}$ uptake during HFS could be reduced by a developmental increase in the activity of other Ca$^{2+}$ clearance mechanisms such as Na/CaX or plasma membrane Ca$^{2+}$-ATPase (PMCA). Such developmental decrease in the mitochondrial Ca$^{2+}$ uptake may in turn
suppress post-tetanic Ca\textsubscript{res} and thus PTP in mature calyx of Held synapses.

Here, we studied whether the developmental change of Ca\textsuperscript{2+} clearance mechanisms underlies that of PTP at the calyx of Held synapse. To this end, we compared PTP, the peak of HFS-induced Ca\textsuperscript{2+} transients (HFS-CaT), mitochondrial Ca\textsuperscript{2+} uptake during HFS and post-tetanic Ca\textsubscript{res} at the calyx of Held synapse at two developmental stages, post-natal day 4-6 and 9-11. We found that the peak Ca\textsuperscript{2+} levels ([Ca\textsuperscript{2+}]\textsubscript{peak}) of HFS-CaTs are closely correlated with the amount of mitochondrial Ca\textsuperscript{2+} uptake and post-tetanic Ca\textsubscript{res}, and that the developmental upregulation of the K\textsuperscript{+}-dependent Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCKX) activity limits the intracellular [Ca\textsuperscript{2+}] buildup during HFS to reduce mitochondrial Ca\textsuperscript{2+} uptake during HFS, resulting in the reduction of post-tetanic Ca\textsubscript{res} and PTP.
Methods

Preparation of brain stem slices.

Calyx of Held synapse preparations were prepared from transverse 200 µm thick brainstem slices containing the medial nucleus of trapezoid body. Postnatal day of 4 to 6 or 9 to 11 Sprague Dawley rats were decapitated, and brainstems were chilled in ice-cold low- Ca\(^{2+}\) aCSF, which contained (in mM) 125 NaCl, 25 NaHCO\(_3\), 2.5 KCl, 1.25 NaH\(_2\)PO\(_4\), 2.5 MgCl\(_2\), 0.5 CaCl\(_2\), 25 glucose, 0.4 Na ascorbate, 3 myo-inositol, and 2 Na pyruvate [pH, 7.4 when saturated with carbogen (95% O\(_2\), 5% CO\(_2\)); osmolarity, ~320 mOsm]. Slices were made using a vibratome slicer (VT1200, Leica, Germany), and slices were incubated at 37°C for 30 min in normal aCSF, the constituents of which are the same as low- Ca\(^{2+}\) aCSF except 1 mM MgCl\(_2\) and 2 mM CaCl\(_2\), and thereafter maintained at room temperature (23 - 25°C) until required. Protocols were approved by the Animal Care Committee of Seoul National University.

Electrophysiological recording and Reagents.

Slices were transferred to a recording chamber in an upright microscope (BX50WI; Olympus, Tokyo, Japan). Whole-cell patch-clamp recordings from calyces of Held were made under visual control using differential interference illumination. Presynaptic whole-cell patch abolishes the PTP and reduces the Ca\(_{\text{res}}\) at the calyx of Held synapse (Korogod et al. 2005; Lee et al. 2010). Thus, for estimation of PTP and presynaptic [Ca\(^{2+}\)], we preload Ca\(^{2+}\) indicator dye to calyx terminals by a brief presynaptic whole-cell patch-clamp recording for about 90 s with the K-gluconate-containing pipette solution composed of (mM): 120 K-gluconate, 30 KCl, 20 HEPES, 4 Na\(_2\)-phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP and 0.2 fura-4F (Fig. 2, 4 and 5) or fura-FF (Fig. 3) (pH 7.3 adjusted with KOH). Postsynaptic recordings were made in the voltage-clamp mode at a holding potential of -70mV. Series resistance (R\(_s\); range 4 - 15 MΩ) was regularly checked and partially compensated by up to 75%
for remaining uncompensated $R_s$ value of 3 - 4 MΩ. Experiments were discarded when uncompensated $R_s$
106 exceeded 15 MΩ. Patch pipettes with a resistance of 3.5 – 4.5 MΩ were used for recordings. Postsynaptic patch
107 pipettes contained (in mM): 110 Cs-gluconate, 20 TEA-Cl, 10 NaCl, 20 HEPES, 10 EGTA, 4 Mg-ATP, 5 Na₂-
108 phosphocreatine, 5 lidocaine N-ethyl bromide (QX314; an intracellular blocker of Na⁺ currents), pH 7.3
109 (adjusted with CsOH). For measuring presynaptic Ca²⁺ transients under presynaptic whole-cell patch-clamp
110 recording conditions, K-gluconate-containing pipette solution was used with 0.05 mM fura-4F instead of 0.2
111 mM fura-4F. For a K-free pipette solution, K-gluconate and KCl in the K-gluconate pipette solution were
112 replaced with equimolar tetramethylammonium (TMA)-gluconate and TEA-Cl, respectively. Presynaptic Ca²⁺
113 current was recorded in the presence of 1 μM TTX and 10 mM TEA-Cl using Cs-gluconate pipette solution
114 containing (in mM): 120 Cs-gluconate, 30 TEA-Cl, 20 HEPES, 0.05 fura-4F, 4 Mg-ATP, 0.3 Na-GTP, 5 Na₂-
115 phosphocreatine, pH 7.3 (adjusted with CsOH). Recordings were made at room temperature (23 – 25 °C) except
116 experiments in Fig. 1 B (32 – 34 °C) with an EPC10 amplifier (HEKA, Lambrecht, Germany). EPSCs were
117 evoked by stimulating presynaptic axons (0.1 - 0.2 ms, 10 - 20 V) with a bipolar stimulating electrode
118 (TM53CCINSI; World Precision Instruments, FL) placed at the midline of the brainstem. To enhance spatial
119 voltage-clamp of a postsynaptic neuron, EPSCs were reduced to 40 ± 4.6% or 43 ± 4.5% by bath application of
120 1 mM kynurenic acid (KYN, Fig. 1) or 100 nM 2,3-dioxo-6-nitro-1,2,3,4,-tetrahydrobenzoquinoxaline-7-
121 sulfonamide (NBQX, Fig. 2). KYN, a competitive antagonist of AMPA receptors, is a better AMPA receptor
122 antagonist for monitoring presynaptic activity, because it inhibits AMPA receptor desensitization. KYN,
123 however, could not be used in combination with micro-fluorometry, because KYN had significant fluorescence
124 at UV excitation light (max. at 352 nm), which appreciably interferes with the Ca²⁺-indicator signal. Fura-4F
125 and fura-FF were purchased from Molecular Probes (Eugene, OR, USA). Ru360 was purchased from
126 Calbiochem (Darmstadt, Germany). KYN, QX314 and TTX were purchased from Tocris (Bristol, UK). Other
127 reagents including tetraphenylphosphonium (TPP⁺) were purchased from Sigma (St. Louis, MO, USA).
Presynaptic $[Ca^{2+}]$ imaging.

Intracellular $[Ca^{2+}]$ at the calyx of Held was measured as previously described (Kim et al. 2005). Briefly, excitation light from a monochromator (Polychrome-II; TILL Photonics, Graefelfing, Germany) was delivered to an upright microscope (BX50; Olympus). Imaging was performed using a 60x water immersion objective (NA, 0.9; LUMPlanFl; Olympus) and an air-cooled slow-scan CCD camera (SensiCam; PCO, Kelheim, Germany) using on-chip binning (8 x 16 pixels). The fluorescence ratio ($R = F_{iso}/F_{380}$) at the isosbestic wavelength (360 nm; $F_{iso}$) to that at 380 nm ($F_{380}$) was converted to $[Ca^{2+}]$, according to the equation: $[Ca^{2+}] = K_{eff} \cdot (R - R_{min}) / (R_{max} - R)$.

Calibration parameters were determined by “in-cell” calibration. $R_{min}$ values were measured using a $Ca^{2+}$-free internal solution containing 10 mM BAPTA. $R_{max}$ values were obtained from in vitro measurement, because calyces of Held did not endure internal dialysis with high $CaCl_2$ (10 mM). The values for the calibration ratio at intermediate $[Ca^{2+}]$, were measured in the calyx using a pipette solution containing 8 mM BAPTA and 6 mM $CaCl_2$ ($[Ca^{2+}] \approx 660$ nM) for fura-4F, 10 mM $N$-(2-Hydroxyethyl) ethylenediamine-$N,N',N''$-triaceitic acid (HEDTA) and 4 mM $CaCl_2$ ($[Ca^{2+}] \approx 3.04$ μM) for fura-FF. The effective dissociation constants of fura-4F and fura-FF ($K_{eff}$) were calculated by measuring the fluorescence ratio at these intermediate $[Ca^{2+}]$, and by re-arranging the equation for $K_{eff}$. The $K_d$ values of fura-4F and fura-FF were calculated as 0.75 μM and 3.14 μM, respectively, from $K_d = K_{eff} \cdot (α + R_{min}) / (α + R_{max})$, where $α$ is the isosbestic coefficient.

The decay phase of a $Ca^{2+}$ transient evoked by a train of two or twenty pulses was fitted with mono- or bi-exponential function having the form of $A_0 + A_1 \exp(-r_1 \cdot t)$ or $A_0 + A_1 \exp(-r_1 \cdot t) + A_2 \exp(-r_2 \cdot t)$, respectively (Fig. 4). The decay rate constant ($r_c$) of a bi-exponential fit is defined as the decay rate at the peak of a CaT divided by the peak amplitude, which is $(A_1 \cdot r_1 + A_2 \cdot r_2) / (A_1 + A_2)$ (Lee et al. 2007b).

Estimation of mitochondrial calcium uptake and post-tetanic calcium extrusion into cytosol.

Consider a HFS-CaT comprised of $n$ action potential-induced CaTs (AP-CaTs), each of which occurs with a
uniform inter-pulse interval (Δt). Because the calcium buildup during HFS is the difference between the sum of action potential (AP) -induced calcium rises and the sum of calcium cleared during HFS, the peak amplitude of the HFS-CaT [ΔCa(n)] can be described as:

\[
\Delta Ca(n) = \Sigma_i^n A_i - \Sigma_i^n [(dCa/dt)_i \times \Delta t]
\]

where \(A_i\) is the amplitude of \(i\)-th AP-CaT and the second term with a minus sign represents the Ca\(^{2+}\) decay exerted by Ca\(^{2+}\) clearance mechanisms during the interval of Δt. Equations (1) can be re-written in terms of total calcium (free plus bound form, CaT) as

\[
\Delta Ca_T(n) = \Delta Ca(n) \times (1 + \Sigma \kappa) = \Sigma_i^n A_{Ti} - \Sigma_i^n [(dCa_T/dt)_i \times \Delta t]
\]

where \(A_{Ti}\) is an increment of total calcium induced by \(i\)-th pulse, and \(\Sigma \kappa\) represents the linearized calcium binding ratio of exo- and endogenous calcium buffers. The linearized \(\kappa\) value of a buffer, B, between two calcium concentrations \(x_1\) and \(x_2\), is defined by

\[
K_d \times [B]_{total} / [(K_d + x_1)(K_d + x_2)]
\]

where \(K_d\) is a dissociation constant of B. The \(K_d\) values of fura-FF was assumed as 3.14 μM, and the endogenous calcium binding ratio was assumed to be 40 (Helmchen et al. 1996).

The second term of the right-hand side of equation (2) can be split into contributions of mitochondria and other Ca\(^{2+}\) clearance mechanisms under control conditions. Thus,

\[
\Delta Ca_T(n)_{\text{control}} = \Sigma_i^n A_{Ti} - \Sigma_i^n [(dCa_T/dt)_i, \text{others} \times \Delta t].
\]

Assuming that Ru360 blocks only the mitochondrial calcium uptake with no off-target effect, \(\Delta Ca(n)\) in the presence of Ru360 [\(\Delta Ca(n)_{Ru360}\)] is:

\[
\Delta Ca_T(n)_{Ru360} = \Sigma_i^n A_{Ti} - \Sigma_i^n [(dCa_T/dt)_i, \text{others} \times \Delta t].
\]

Subtraction of \(\Delta Ca_T(n)_{\text{control}}\) from \(\Delta Ca_T(n)_{Ru360}\) yields

\[
\Delta Ca_T(n)_{Ru360} - \Delta Ca_T(n)_{\text{control}} = \Sigma_i^n [(dCa_T/dt)_i, \text{mito} \times \Delta t] + \varepsilon
\]

where \(\varepsilon = \Sigma_i^n [(dCa_T/dt)_i, \text{others} - (dCa_T/dt)_i, \text{others}].\) Since the activity of other Ca\(^{2+}\) clearance mechanisms is saturated at high [Ca\(^{2+}\)], (See Discussion), the error term, \(\varepsilon\), would not be large. If the error term is negligible, the cumulative total amount of mitochondrial calcium uptake (Φ\(_{\text{mito}}\)) at the \(n\)-th pulse can be calculated as:
Two terms in the righthand side of equation (6) were measured at the same calyx terminal preloaded with fura-FF before and after the 2nd patch with the pipette solution containing fura-FF and Ru360. The change in fura-FF concentration was estimated from fluorescence at 360 nm, which was typically increased by about 20-50% after re-patch. Such increase in [fura-FF] caused the change of the value for \((1 + \Sigma \kappa)\) by less than 5 - 10%.

The post-tetanic calcium extrusion from mitochondria \((J_{mito})\) can be roughly estimated by the following equation (Lee et al. 2007a):

\[
J_{mito}(t) \equiv \gamma \int_{0}^{t} [Ca^{2+}]_{res}(t') dt'
\]

where \([Ca^{2+}]_{res}(t')\) is the time course of the \(Ca_{res}\), and \(\gamma\) is calcium clearance of the calyx of Held. The \(\gamma\) value was estimated as follows:

\[
\gamma = rw \times (1 + \Sigma \kappa)
\]

where \(rw\) is the rate constant of a CaT induced by paired APs (Fig. 4). The \(\kappa\) value for fura-4F was calculated assuming that \(K_d\) of fura-4F is 0.75 μM.

Statistics and data presentation.

Data are presented as mean ± s.e.m, with \(n\) indicating the number of synapses analyzed. In figures, the number of synapses or calyceal terminals that we studied is indicated as a number in parentheses on each bar. Statistical analyses on data measured at the same synapse and on data at different synapses were performed using the Student's paired t-test and the Student's t-test, respectively. The statistical significance for the difference of time courses of PTP (Fig. 1-2) or HFS-CaTs (Fig. 5) was determined using two-way analysis of variance (ANOVA). Statistical values are presented as mean ± SEM.
Results

*Developmental changes of mitochondria-dependent PTP at the calyx of Held synapse.*

The amplitude of PTP linearly depends on the level of post-tetanic Ca\textsubscript{res} (Habets and Borst 2005; Korogod et al. 2005; Lee et al. 2008). The majority of post-tetanic Ca\textsubscript{res} is caused by Na\textsuperscript{+}-dependent Ca\textsuperscript{2+}-efflux from mitochondria subsequent to Ca\textsuperscript{2+} uptake during HFS (Lee et al. 2008). It has been reported that the amplitude of PTP evoked by the same HFS becomes smaller at the calyx of Held synapses from rats of postnatal days 8-10 (P8-P10) than those from P4-P6 rats, when the length of HFS (100 Hz) is 2 seconds or shorter (Korogod et al. 2005). To investigate whether the mitochondrial contribution to PTP undergoes a development change, we examined the effect of mitochondrial Na\textsuperscript{+}-dependent Ca\textsuperscript{2+}-efflux inhibition on PTP induced by HFS (100 Hz, 2 s) at the calyx of Held synapse from rats of two age groups, P4-P6 (denoted by P5) and P9-P11 (P10). We determined the baseline EPSCs amplitude evoked by stimulation of afferent fibers every 10 s. The stimuli at 0.1 Hz were resumed 10 s after the end of HFS to monitor the change in synaptic strength. The baseline EPSC amplitude was not different between P5 (1.24 ± 0.17 nA, n = 7) and P10 (1.94 ± 0.47 nA, n = 6, p = 0.17, Fig. 1Ae). Consistent with the previous observation (Korogod et al. 2005), the amplitude of PTP at P10 was significantly smaller than that at P5 (Fig. 1Af, p = 0.010) under control conditions.

Low concentration TPP\textsuperscript{+} (IC\textsubscript{50}, 0.2 μM) is a selective inhibitor of mitochondrial Na\textsuperscript{+}-dependent Ca\textsuperscript{2+}-efflux, although it dissipates mitochondrial membrane potential at higher concentration (> 10 μM) (Aiuchi et al. 1985; Karadjov et al. 1986; Wingrove and Gunter 1986). Previously, we have shown that 2 μM TPP\textsuperscript{+} abolishes the post-tetanic Ca\textsubscript{res} with little effect on the peak of HFS-CaT (100 Hz, 4 s) at the calyx of Held (Lee et al. 2008). We assessed the effects of TPP\textsuperscript{+} on PTP induced by HFS (100 Hz, 2 s). The amplitude of the baseline EPSC was not altered by TPP\textsuperscript{+} treatment (P5, 1.33 ± 0.19 nA, n = 7, p = 0.19; P10, 1.80 ± 0.40 nA, n = 6, p = 0.13, paired t-test). In both age groups, the peak amplitude of PTP was significantly reduced by bath application of 2 μM
TPP+, from 123.8 ± 18.9% to 64.4 ± 6.7% in P5 (n = 7, p = 0.014, paired t-test, Fig. 1Aa and Ab) and from 55.4 ± 8.3% to 38.8 ± 5.9% in P10 (n = 6, p = 0.012, paired t-test, Fig. 1Ac and Ad). As the calyx synapse matured, the TPP+-sensitive component of PTP (denoted by PTPmito) was reduced to a larger extent (59.4 ± 17.4% at P5, 16.6 ± 4.3% at P10, p = 0.048, Fig. 1Ag) than the TPP+-insensitive component (PTPnon-mito, 64.4 ± 6.7% at P5 and 38.8 ± 5.9% at P10, p = 0.017, Fig. 1Ah). In addition to the peak of PTP, the time courses of PTP were compared between control conditions and in the presence of 2 μM TPP+ using two-way ANOVA test. Whereas the time course of PTP at P5 was significantly different between these two conditions (F₁,₃₀₀ = 19.22, p < 0.001), that at P10 was not different (F₁,₂₅₀ = 2.409, p = 0.122). These results indicate that the reduced contribution of mitochondria to PTP is primarily responsible for the different magnitude of PTP at P5 and P10. The [Ca2+] elevation at axon terminals during HFS is essential for the induction of PTP, and critically depends on temperature (David and Barrett 2000). We tested whether the developmental change of PTPmito also occurs in near physiological temperature. We did the same experiments at the bath temperature of 32 – 34 ºC. The baseline EPSC amplitudes were not different between P5 (3.55 ± 0.31 nA, n = 7) and P10 (3.79 ± 0.84 nA, n = 6, p = 0.78, Fig. 1Be). The peak amplitude of PTP was significantly lower at 32 – 34 ºC than at room temperature in both age groups (P5, 31.84 ± 3.91%, n = 7, p < 0.01; P10, 14.81 ± 4.14%, n = 6, p < 0.01). Nonetheless, the PTP amplitude at P5 was significantly higher than at P10 (p = 0.012, Fig. 1Bf). Furthermore, bath-applied TPP+ significantly reduced the PTP amplitude at P5 (17.07 ± 2.29%, n = 7, p < 0.01, Fig. 1Ba-b), but not at P10 (15.91 ± 3.07%, n = 6, p = 0.836, paired t-test, Fig. 1Bc-d). Accordingly, PTPmito was significantly higher at P5 than at P10 (P5, 14.76 ± 2.49%; P10, -1.10 ± 2.31%, p < 0.01, Fig. 1Bg), but PTPnon-mito was not different between two age groups (P5, 17.07 ± 2.29%; P10, 15.91 ± 3.07%, p = 0.76, Fig. 1Bh). Comparing the time course of PTP, we found that it was significantly affected by TPP+ at P5 (F₁,₃₀₀ = 5.29, p = 0.022) but not at P10 (F₁,₂₅₀ = 0.292, p = 0.59). Such different effects of TPP+ on PTP between two age groups indicate the developmental decrease in the mitochondrial contribution to PTP at physiological temperature. TPP+ had no effect on the baseline EPSC amplitude in both age groups (P5, 3.51 ± 0.31 nA, n = 7, p = 0.19; P10, 3.85 ± 0.76
nA, n = 6, p = 0.13). Next, we focused on the developmental changes of Ca\(^{2+}\) dynamics responsible for the developmental decrease of PTP\(_{\text{mito}}\) at postnatal calyx of Held synapse. All subsequent experiments were performed at room temperature.

**Developmental changes in post-tetanic residual Ca\(^{2+}\) and PTP**

The smaller effect of TPP\(^+\) on PTP at the P10 calyx of Held implies a smaller amount of post-tetanic Ca\(_{\text{res}}\) at P10 than at P5. To test this prediction, we examined presynaptic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and EPSC simultaneously at the same synapse. We loaded the calyx axon terminal with a Ca\(^{2+}\)-indicator dye via a whole-cell patch pipette containing 200 \(\mu\)M fura-4F for a brief period (90 s) and pulled off the pipette (referred to as preloading technique; Korogod *et al.*, 2005). The amount of post-tetanic Ca\(_{\text{res}}\) is represented by the time integration of the Ca\(^{2+}\) signal two seconds after the cessation of HFS (Lee *et al.* 2007a; Lee *et al.* 2008), and this estimate is independent of Ca\(^{2+}\) buffer (Lee *et al.* 2007a).

Preloading the calyx terminal with fura-4F significantly reduced the baseline EPSC amplitude (to 0.61 ± 0.08 nA, n = 5, p = 0.017) and enhanced PTP induced by HFS (100 Hz, 2 s) (180.8 ± 5%, n = 5, p = 0.03) at the P5 calyx synapses, whereas it had no significant effect on the baseline EPSC amplitude (0.96 ± 0.26 nA, n = 6, p = 0.1) and on PTP (58.9 ± 5.9%, n = 6, p = 0.74) at the P10 synapses (Fig. 2A, B). These differential effects of preloading on the baseline EPSC amplitude and PTP are consistent with the idea that the spatial coupling between synaptic vesicles and Ca\(^{2+}\) channels becomes tighter as the calyx synapse matures (Wang *et al.* 2008).

As expected from the smaller PTP\(_{\text{mito}}\) at P10 synapses, the amount of post-tetanic Ca\(_{\text{res}}\) was significantly smaller at P10 synapses (0.40 ± 0.07 \(\mu\)M·s, p < 0.01) than at P5 (2.48 ± 0.18 \(\mu\)M·s) (Fig. 2A, B).

We used 2 \(\mu\)M TPP\(^+\) to inhibit the mitochondrial Na\(^{+}\)-dependent Ca\(^{2+}\) efflux without disturbing mitochondrial membrane potential and Ca\(^{2+}\) uptake (Lee *et al.* 2008). As expected, TPP\(^+\) did not alter the peak of HFS-CaTs (P5, p = 0.54; P10, p = 0.80, Fig. 2Ae and 2Be), but it significantly suppressed PTP and post-tetanic Ca\(_{\text{res}}\) in both age groups (PTP, p < 0.01 for P5, p = 0.014 for P10; post-tetanic Ca\(_{\text{res}}\), p < 0.01 for P5, p = 0.019 for P10,
paired t-test, Fig. 2A and 2B). For both age groups, the time courses of PTP was significantly different between control and in the presence of \( \text{TPP}^+ \) \((P5, F(1, 200) = 33.55, p < 0.001; P10, F(1,250) = 7.70, p = 0.006)\). The developmental reduction of PTP_{mito} paralleled mitochondria-dependent Ca_{res} amount (Fig. 2C), indicating that the reduced PTP_{mito} at P10 can be ascribed to the smaller amount of mitochondria-dependent Ca_{res}.

Previously, we have reported that mitochondrial Ca\(^{2+}\) uptake occurs at high [Ca\(^{2+}\)] levels, where Na/CaX is saturated (Kim et al. 2005). Thus, we compared the peak of HFS-CaT at P5 and at P10. The peak of HFS-CaTs were distinctly higher at P5 \((3.94 \pm 0.54 \mu M, n = 5, \text{Fig. 2Ae})\) than at P10 \((1.19 \pm 0.12 \mu M, n = 6, p < 0.01, \text{Fig. 2Be})\). Furthermore, a higher peak of HFS-CaTs (denoted by [Ca\(^{2+}\)]_{peak}) was associated with a higher amount of post-tetanic Ca_{res} (Fig. 2D). We further investigated the relationship between [Ca\(^{2+}\)]_{peak} and mitochondrial Ca\(^{2+}\) uptake.

Developmental change in mitochondrial Ca\(^{2+}\) uptake during HFS

The smaller amount of post-tetanic Ca_{res} at P10 suggests a lower mitochondrial Ca\(^{2+}\) uptake during HFS. To test this idea, we compared HFS-CaTs in each age group before and after inhibition of mitochondrial Ca\(^{2+}\) uptake using Ru360. It has been shown that Ru360 specifically inhibits the mitochondrial Ca\(^{2+}\) uniporter with little effect on L-type Ca\(^{2+}\) current and other Ca\(^{2+}\) clearance mechanism including PMCA and Na/CaX (Matlib et al. 1998). The presynaptic terminal was preloaded with a K-gluconate internal solution with 200 µM fura-FF, a low-affinity Ca\(^{2+}\) indicator dye. After withdrawal of the pipette, we recorded Ca\(^{2+}\) transients evoked by HFS of afferent fibers (100 Hz for 2 s). The [Ca\(^{2+}\)]_{peak} of HFS-CaT was 3.85 \pm 0.47 \mu M in control conditions at P5 \((n = 5)\). Subsequently, we re-patched the same axon terminal using a patch pipette containing 200 µM fura-FF and 20 µM Ru360. After a brief (~30 s) whole-cell patch, we pulled off the pipette and recorded the HFS-CaT again. After intracellular application of Ru360, an inhibitor of the mitochondrial Ca\(^{2+}\) uniporter, the [Ca\(^{2+}\)]_{peak} was significantly elevated to 21.2 \pm 2.0 \mu M at same axon terminal \((n = 5, p < 0.01, \text{paired t-test, Fig. 3A})\). In contrast, at P10 the [Ca\(^{2+}\)]_{peak} increased from 1.42 \pm 0.1 \mu M to 3.5 \pm 0.3 \mu M \((n = 5, p < 0.01, \text{paired t-test, Fig. 3B})\).
increment of the \([\text{Ca}^{2+}]_{\text{peak}}\) caused by Ru360 was significantly higher at P5 than at P10 (17.4 ± 1.70 µM and 2.1 ± 0.15 µM, respectively, p < 0.01). We plotted the \([\text{Ca}^{2+}]_{\text{peak}}\) values of HFS-CaTs in the presence of Ru360 as a function of the control \([\text{Ca}^{2+}]_{\text{peak}}\) in Figure 3C, showing the high correlation between these two parameters.

Fig. 3C implies that the higher mitochondrial \(\text{Ca}^{2+}\) uptake may be related to the higher elevation of \([\text{Ca}^{2+}]\), during HFS. To test this idea, we estimated the cumulative mitochondrial \(\text{Ca}^{2+}\) uptake (denoted by \(\Phi_{\text{mito}}\)) during HFS according to equation (6), and plotted these values as a function of \([\text{Ca}^{2+}]\), under control conditions in Fig. 3D. This plot shows a supralinear dependence of mitochondrial \(\text{Ca}^{2+}\) uptake on the presynaptic \(\text{Ca}^{2+}\) level. Furthermore, we found that the mitochondrial \(\text{Ca}^{2+}\) uptake at P5 is not different from that at P10 as long as the \([\text{Ca}^{2+}]\) level is the same (Fig. 3D), arguing against the possibility that the lower post-tetanic \(\text{Ca}_{\text{res}}\) at P10 is caused by lower mitochondrial function. These results indicate that the higher \([\text{Ca}^{2+}]_{\text{peak}}\) of a HFS-CaT leads to a higher mitochondrial \(\text{Ca}^{2+}\) uptake (Fig. 3C), which in turn results in a higher mitochondria-dependent \(\text{Ca}_{\text{res}}\) (Fig. 2D) and \(\text{PTP}_{\text{mito}}\) (Fig. 2C).

Finally, we tested whether the amount of post-tetanic mitochondrial \(\text{Ca}^{2+}\) extrusion (\(J_{\text{mito}}\)) estimated by equation (7) is close to the mitochondrial \(\text{Ca}^{2+}\) uptake estimated at the end of HFS according to equation (6). To estimate the former (\(J_{\text{mito}}\)), the time integral of \(\text{TPP}^-\)-sensitive \(\text{Ca}_{\text{res}}\) was calculated from the data in Fig. 2, and the value for \(\text{Ca}^{2+}\) clearance (\(\gamma\)) was calculated according to equation (8) using the \(\text{Ca}^{2+}\) decay rate constant (\(r_u\)) measured from CaTs evoked by double APs (inter-spike interval = 5 ms, 4.1/s for P5 and 4.9/s for P10, Fig. 4). The two estimates were not significantly different from each other in both age groups (Fig. 3E), suggesting that most of the \(\text{Ca}^{2+}\) taken up by mitochondria during HFS is extruded after the end of HFS.

The estimates for mitochondrial \(\text{Ca}^{2+}\) uptake are valid when Ru360 has no effect on the calcium current, but bath-applied Ru360 has been reported to reduce the amplitude of an AP-induced CaT in motor nerve terminal (David 1999). To test if intracellularly applied Ru360 has any effect on \(\text{Ca}^{2+}\) current, we compared \(\text{Ca}^{2+}\) currents induced by HFS (short depolarizing pulses at 100 Hz for 2 s) with or without 20 µM Ru360 in the pipette solution at the calyx of Held terminals of P10. Fig. 3F shows that Ru360 has no significant effect on \(\text{Ca}^{2+}\). 

14
current (p > 0.4 at all time points). The total amounts of charge influx elicited by the HFS was not different between the two conditions either (411.8 ± 39.8 pC, n = 7; 394.3 ± 23.5 pC, n = 6, p = 0.724). Despite little effect of Ru360 on Ca\(^{2+}\) influx, higher elevation of [Ca\(^{2+}\)] in the presence of Ru360 may enhance diffusion of Ca\(^{2+}\) from the terminal to the attached axon, resulting in underestimation of mitochondrial Ca\(^{2+}\) uptake. Nevertheless, such Ca\(^{2+}\) clearance by diffusion may be limited because most afferent axon fibers are truncated during slice preparation.

**Developmental upregulation of Ca\(^{2+}\) clearance mechanisms**

The lower peak of HFS-CaTs at P10 can be ascribed to a smaller AP-induced Ca\(^{2+}\) influx or a higher Ca\(^{2+}\) clearance. The amount of AP-induced Ca\(^{2+}\) influx at P10 is thought to be only lower by about 5% than that at P5 because of the shorter AP duration at P10 (Taschenberger et al. 2002), implying that faster Ca\(^{2+}\) clearance rather than smaller Ca\(^{2+}\) influx is responsible for the lower peak of HFS-CaTs at P10. We investigated developmental changes of Ca\(^{2+}\) clearance at the calyx of Held. We have previously reported that Na/CaX is a major Ca\(^{2+}\) clearance mechanism whereas sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase contributes little to Ca\(^{2+}\) clearance at the calyx of Held (Kim et al. 2005). The Na/CaX activity can be further separated into K\(^+\)-independent form of Na/CaX (NCX) and NCKX according to the dependence on intracellular K\(^+\) (Lee et al. 2002). NCX and NCKX can be specifically inhibited by intracellular perfusion of exchanger inhibitory peptide (XIP) and K\(^+\)-free TMA\(^+\) solution, respectively (Kim et al. 2005; Lee et al. 2007b; Lee et al. 2002; Li et al. 1991), while both of them (denoted by Na/CaX) can be inhibited by lowering the extracellular [Na\(^+\)]. We investigated the developmental changes of NCX and NCKX activities at the calyx of Held loaded with 50 \(\mu\)M fura-4F via a whole cell patch pipette. Because the Na/CaX activity is activated at higher [Ca\(^{2+}\)], than PMCA (Kim et al. 2005), we examined the inhibitory effects on CaTs of two different amplitudes evoked by 2 or 20 repetitive depolarizing pulses at 200 Hz (denoted by CaT2 or CaT20, respectively) (pulse duration, 2 ms). From a holding potential of -70 mV, the depolarization step was adjusted between -20 mV and 0 mV such that the peak of the resulting CaT20 was
near 2 µM (in the range of 1.5 - 2.5 µM). The statistical means of the decay rate constants under various conditions are summarized in Table 1.

Under control conditions, that is, in the presence of normal concentrations of intracellular K⁺ ([K⁺]ᵢ) and extracellular Na⁺ ([Na⁺]ₒ), the decay rate constant of CaT2 was not different between P5 and P10 (Fig. 4Aa). However, the decay rate constant (rₛ) of CaT2₀ was significantly faster at P10 than at P5 (p < 0.01, Fig. 4Ab). Next, we compared the decay rate constant using the TMA-internal solution for selective inhibition of NCKX. Under this condition, both decay rate constants, CaT2 and CaT2₀, were not significantly different between P5 and P10 (Fig. 4B), suggesting that NCKX is responsible for the developmental acceleration of the decay rate. To study the contribution of NCX, we intracellularly perfused the calyx terminal with K⁺-gluconate internal solution containing 10 µM XIP. Under this condition, the decay rate constant of CaT2₀ was faster at P10 than at P5 (Fig. 4Cb), whereas that of CaT2 was not affected at either age (Fig. 4Ca). Furthermore, the difference in the decay rate constant of CaT2₀ between P5 and P10 in the presence of XIP (4.7/s) was comparable to that under control conditions (5.3/s), suggesting that NCX is not developmentally upregulated. To exclude possible developmental changes of Ca²⁺ clearance mechanisms other than NCX and NCKX, we compared CaTs at the two age groups after suppression of the Na/CaX by replacement of external 125 mM Na⁺ with equimolar Li⁺. Under this condition, neither the decay rate constant of CaT2 and nor that of CaT2₀ was significantly different between P5 and P10 (Fig. 4D), indicating little developmental changes of Ca²⁺ clearance mechanisms other than the Na/CaX. Furthermore, within the same age group, the decay rate constant of CaT2 was not affected by any of the above inhibitors (p > 0.05), implying no developmental change of PMCA.

Finally, we estimated contributions of NCKX and NCX to Ca²⁺ clearance from difference in decay rate constants between K⁺ and TMA⁺ (for NCKX) and between K⁺ and XIP (for NCX) at P5 and P10 (Fig. 4E – F). The contribution of NCKX was significantly increased during development (P5, 2.13 ± 0.52; P10, 6.32 ± 0.83, p < 0.01), whereas the NCX activity was not different between P5 and P10 (P5, 3.82 ± 0.65; P10, 4.37 ± 1.33, p = 0.71). These results suggest that the developmental upregulation of the NCKX activity is responsible for the
faster Ca\textsuperscript{2+} clearance at mature calyx of Held terminals.

*Shaping of a HFS-induced Ca\textsuperscript{2+} transient by Ca\textsuperscript{2+} clearance mechanisms*

We tested how such developmental changes of NCX and NCKX shape the HFS-CaT evoked by a train of depolarizing pulses (100 Hz for 4 s; denoted by “CaT400”) under whole-cell patch-clamp recording conditions. We used the same step depolarizing pulse as in Fig. 4. First, we confirmed that the [Ca\textsuperscript{2+}]\textsubscript{peak} of CaT400 under whole-cell patch-clamp recording conditions is not different from that evoked by afferent fiber stimulation without a presynaptic whole-cell patch at each age group (Fig. 5A). Next, the averaged trace of control CaT400s was compared with that of CaT400s recorded under each condition that inhibits NCKX or NCX (Fig. 5B for P5 and Fig. 5C for P10). In addition, averaged traces of CaT400s at P5 and P10 were compared under different conditions in Fig. 5D. When either NCKX or NCX was inhibited using the TMA-based internal solution (for NCKX) or XIP (for NCX), the averaged CaT400 at P5 was significantly different from that at P10 (TMA, $F_{(1, 1600)} = 34.64, p < 0.001$, Fig. 5Da; XIP, $F_{(1, 2000)} = 1771.8, p < 0.001$, Fig. 5Db). Despite the overall difference in the Ca\textsuperscript{400} trace between P5 and P10 under the TMA condition, the second half of the Ca\textsuperscript{2+} rising phase plus the decay phase were not different between two age groups ($F_{(1, 912)} = 0.88, p = 0.35$, Fig. 5Da). The Ca\textsuperscript{2+} charge transfer elicited by the same train of depolarizing pulses (100 Hz, 4 s) was not significantly different between the two age groups (Fig. 5E). These results together with those of Fig. 4 support the idea that upregulation of NCKX is primarily responsible for the increased Ca\textsuperscript{2+} clearance at P10. We concluded that such a developmental increase of Ca\textsuperscript{2+} clearance prevents mitochondria from absorbing Ca\textsuperscript{2+} during HFS, resulting in the reduction of post-tetanic Ca\textsubscript{res} and PTP.
Discussion

In the present study, we found that the lower amplitude of PTP at P10 is caused in part by the decrease in mitochondria-derived \( \text{Ca}_{\text{res}} \) (Fig. 2C). The lower post-tetanic \( \text{Ca}_{\text{res}} \) at P10 is closely related to the lower \( [\text{Ca}^{2+}]_{\text{peak}} \) of the HFS-CaT (Fig. 2D) because a lower \( [\text{Ca}^{2+}] \) buildup leads to lower mitochondrial \( \text{Ca}^{2+} \) uptake and thus lower post-tetanic \( \text{Ca}_{\text{res}} \) (Fig. 3C-D). This finding is consistent with our previous reports that mitochondrial \( \text{Ca}^{2+} \) uptake is activated at high \( [\text{Ca}^{2+}] \), where Na/CaX is saturated (Kim et al. 2005). We showed that NCKX is most prominently upregulated at P10 (Fig. 4) and that the higher NCKX activity results in the lower \( [\text{Ca}^{2+}]_{\text{peak}} \) of HFS-CaTs at P10 (Fig. 5). Therefore, we conclude that the reduced mitochondrial \( \text{Ca}^{2+} \) uptake during HFS at P10 can be ascribed to developmental up-regulation of NCKX.

During the second postnatal week, the calyx of Held undergoes drastic developmental changes not only in \( \text{Ca}^{2+} \) clearance but also in its morphology, electrophysiological properties, short-term plasticity of ESPCs and synaptic ultrastructures (Chuhma and Ohmori 2001; Lee et al. 2012a; Taschenberger et al. 2002; Taschenberger and von Gersdorff 2000; Wang et al. 2008), implying that the developmental increase in NCKX occurs in parallel with other changes which optimize the calyx of Held for precise synaptic transmission. Similar rapid changes in calcium clearance have been reported in other parts of the mammalian brain during early postnatal weeks. For example, SERCA and NCX activities greatly increase in supraoptic magnocellular neurons within a few days during the weaning period (Lee et al. 2007c). The mRNA expression levels of NCKX2, NCKX3 and NCX2 are strongly upregulated during the second week in cultured hippocampal neurons (Kip et al. 2006).

Mechanisms for PTP at the calyx of Held synapse

Although the present study focused on mitochondrial \( \text{Ca}_{\text{res}} \) as a mechanism for PTP at the calyx of Held synapse, other mechanisms for PTP have been reported: post-tetanic increase in \( \text{Ca}^{2+} \) influx (Habets and Borst...
2006), increase in quantal size mediated by compound vesicles (He et al. 2009; Xue and Wu 2010) and Ca$^{2+}$-dependent activation of protein kinase C (PKC) (Fioravante et al. 2011; Korogod et al. 2007). Non-mitochondrial mechanisms are responsible for about half of the PTP at P5 and the majority of the PTP at P10 (Fig. 1).

Previously, we have reported that the post-tetanic increase in release probability was mediated by mitochondria-dependent Ca$_{\text{res}}$ (Lee et al. 2008). Consistent with this view, Fioravante et al. (2011) showed that the post-tetanic release probability increase in the calyx synapses of PKC k/o mice, where post-tetanic Ca$_{\text{res}}$ was not altered, is not different from that in the wild-type synapses. The present study shows that mitochondria-dependent mechanisms play a minor role in PTP at the mature calyx of Held. The experiments in Fioravante et al. (2011; P11-14) and Korogod et al. (2007; P7-10) have been performed in the relatively mature calyx of Held (P11-14) compared to Lee et al. (2008, P6-8). The minor role of mitochondrial Ca$_{\text{res}}$ in PTP at the mature calyx of Held may explain why most but not all of the PTP was suppressed at the calyx of Held in PKC k/o mice, given that PKC is involved in the post-tetanic increase in the readily releasable vesicle pool size.

The postnatal calyx of Held synapses are developmentally heterogeneous. Numerous parameters involving synaptic transmission are developmentally regulated (Taschenberger et al. 2002). Therefore, synaptic mechanisms underlying synaptic plasticity may be heterogeneous to result in seemingly inconsistent findings at the early postnatal calyx of Held synapses.

**Interpretation of presynaptic Ca$^{2+}$-buildup during HFS**

A Ca$^{2+}$ buildup during HFS depends on the net flux of Ca$^{2+}$ into and out of the cytosolic compartment. In Fig. 3D, we estimated the cumulative mitochondrial Ca$^{2+}$ uptake during HFS from the difference of Ca$^{2+}$ buildup between the control and in the presence of Ru360. This estimation is valid when the activity of other Ca$^{2+}$ clearance mechanisms and Ca$^{2+}$ influx are not altered by internal application of Ru360 (Matlib et al. 1998) (Fig. 3F). Even if Ru360 has no direct effect on other Ca$^{2+}$ clearance mechanisms, higher [Ca$^{2+}$] buildup itself can
increase the activity of other Ca\(^{2+}\) clearance mechanisms (or dCa\(_t\)/dt), and thus the error term in equation (5) may not be negligible, which can invalidate equation (6). But this concern is unlikely. The slope of the [Ca\(^{2+}\)] buildup during HFS depends on Ca\(^{2+}\) influx and clearance. If calcium clearance increases linearly as [Ca\(^{2+}\)], increases in the presence of Ru360, the slope of calcium buildup should become diminished, because calcium influx is rather reduced at the late period of HFS (Fig. 5E). Fig. 3A-B show that the slope of the [Ca\(^{2+}\)] buildup did not lessen during the [Ca\(^{2+}\)] buildup phase in the presence of Ru360, implying that calcium clearance might be saturated at such high [Ca\(^{2+}\)] level. Therefore, dCa\(_t\)/dt may be little dependent on [Ca\(^{2+}\)], at least in the range where mitochondrial Ca\(^{2+}\) uptake is active, and thus the error term in equation (5) may not seriously affect the \(\Phi_{\text{mito}}\) estimates calculated according to equation (6) (Fig. 3D). This argument is further supported by the statistical similarity between estimates for mitochondrial Ca\(^{2+}\) uptake during HFS and TPP\(^+\)-sensitive post-tetanic Ca\(^{2+}\) extrusion from mitochondria (Fig. 3E and F).

This way of estimation of Ca\(^{2+}\) clearance, however, cannot be used for blockers of Ca\(^{2+}\) clearance mechanisms other than mitochondria. Because the higher buildup of [Ca\(^{2+}\)] caused by inhibition of NCX or NCKX invoke new participation of mitochondria in Ca\(^{2+}\) clearance (Fig. 3D), the [Ca\(^{2+}\)] buildup in the presence of a Ca\(^{2+}\) clearance mechanism blocker can be alleviated by mitochondria. This idea can explain why the increase in the peak Ca\(^{2+}\) of CaT400s caused by XIP is larger in P10 than that in P5 (Fig. 5B and C), despite that the NCX activity estimated from CaT20s was not different between at P5 and at P10 (Fig. 4). Given that NCX is the major Ca\(^{2+}\) clearance mechanism in the moderate [Ca\(^{2+}\)] range and that the [Ca\(^{2+}\)] dynamic range of the control CaT400 at P5 is higher than that of P10, the higher Ca\(^{2+}\) buildup caused by XIP may recruit mitochondrial Ca\(^{2+}\) uptake more readily at P5 than P10, which in turn can alleviate excessive rise of [Ca\(^{2+}\)], during HFS at P5. In contrast, inhibition of NCKX may invoke little such mitochondrial Ca\(^{2+}\) uptake at P5 because NCX is still active and intrinsic NCKX activity is low at P5.

Finally, it should be noted that the dependence of mitochondrial contribution to Ca\(^{2+}\) clearance on [Ca\(^{2+}\)], in the present study is different from our previous results (Kim et al. 2005). When the CaT was induced by a short
depolarizing pulse (50 ms), mitochondrial Ca\(^{2+}\) uptake started at \([\text{Ca}^{2+}] > 2.5 \text{ \(\mu\)M. In contrast, it started at \([\text{Ca}^{2+}] > 1 \text{ \(\mu\)M when CaTs were induced by HFS (Fig. 3D). Assuming that more homogeneous Ca\(^{2+}\) elevation is induced by HFS than by a short depolarizing pulse, this discrepancy may result from difference in the spatial distances from Ca\(^{2+}\) channels to mitochondria or NC(K)X. The second possibility is suppression of NC(K)X during HFS. It is well known that Na/CaX activity is steeply dependent on the membrane potential (V_m) (Earm et al. 1990). The V_m of the calyx of Held is not completely repolarized during repetitive firing at 100 Hz, but the trough of V_m is kept depolarized by approximately 20 mV (Kim et al. 2007). Such 20 mV depolarization can profoundly reduce the Na/CaX activity (Earm et al. 1990). Given that NC(K)X competes with mitochondria for Ca\(^{2+}\) (Kim et al. 2005), such suppression of NC(K)X may provide more chance for mitochondria to take up cytosolic Ca\(^{2+}\). Thirdly, a shift of the threshold for the mitochondrial Ca\(^{2+}\) uptake in Kim et al. (2005) may be caused by a wash-out of cytosolic factors that facilitate mitochondrial Ca\(^{2+}\) uptake such as polyamines under the whole-cell configuration (Jensen et al. 1987).

**Ca\(^{2+}\) clearance mechanisms in the axon terminal**

The present study showed that NCX is the major calcium clearance mechanism, and NCKX plays a minor role at P5 (Fig. 4E-F). As the calyx matures, the NCKX activity increases by a factor of three, whereas the NCX activity is relatively stable, resulting in NCKX and NCX contributions to total calcium clearance of 42% and 29% at P10. These values are comparable to 41% and 28% that we have previously reported for contributions of NCKX and NCX (Kim et al. 2005). It should be noted, however, that these estimates are valid for the \([\text{Ca}^{2+}]\), elevation of about 2 \(\mu\)M. As shown in Fig. 4, neither NCKX nor NCX contributes to Ca\(^{2+}\) clearance in the decay phase of lower amplitude CaTs induced by two APs. Furthermore, NCKX might be saturated at lower cytosolic \([\text{Ca}^{2+}]\) than NCX. The comparison of Ca\(^{2+}\) buildup phases of CaT400s under TMA and XIP conditions implies that the Ca\(^{2+}\)-buildup is more effectively suppressed by NCKX than NCX at the early phase of the CaT400 where \([\text{Ca}^{2+}]\) is approximately 2 \(\mu\)M (red trace in Fig. 5C), consistent with higher contribution of NCKX in this
[Ca^{2+}] range (Fig. 4E-F). The Ca^{2+} buildup under the XIP condition (red trace), however, crossed over that under the TMA condition (blue trace in Fig. 5C) at the time point when the 300th AP invaded and the [Ca^{2+}] was approximately 4 μM, implying that the NCKX activity might be not so strong as NCX at such high [Ca^{2+}] range.

The present study demonstrates that the major developmental change in Ca^{2+} clearance is the increase in the NCKX activity at calyx of Held axon terminal. Among the NCKX family, NCKX2 may mediate the NCKX activity at axon terminals because NCKX at axon terminals exhibits low affinity for K^+ (Lee et al. 2002) and PKC-dependent enhancement (Lee et al. 2006), which are characteristics of NCKX2. More importantly, NCKX2 has been shown to be transported to the axon terminal via KIF21A, whereas it undergoes endocytosis in somatodendritic regions (Lee et al. 2012b). These results are consistent with functional studies that have shown a lack of the NCKX activity in somatodendritic regions (Kim et al. 2003; Lee et al. 2009; Lee et al. 2007b).

Fast Ca^{2+} clearance enables an axon terminal to keep the presynaptic global [Ca^{2+}] low during HFS. High Ca^{2+} buildup promotes asynchronous neurotransmitter release (Chuhma et al. 2001; Otsu et al. 2004), which in turn dissipates the reluctant synaptic vesicles that can be readily recruited for synchronous release (Lee et al. 2012a). Furthermore, presynaptic axon terminals, which have a higher surface-to-volume ratio, undergo much higher [Ca^{2+}] elevation during HFS than soma, which renders involvement of mitochondria inevitable without other efficient Ca^{2+} clearance mechanisms. As shown in the present study, mitochondrial calcium uptake is closely associated with PTP. Therefore, efficient calcium clearance is essential for precise synaptic transmission and avoiding excessive depression. The developmental upregulation of NCKX might be one of the mechanisms that optimize the calyx of Held for fast and accurate synaptic transmission.
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Figure legends

Figure 1. Developmental changes in post-tetanic potentiation (PTP). Aa, Averaged time courses of relative EPSC amplitude before (open circles) and after the bath application (> 10 minutes) of 2 μM tetraphenylphosphonium (TPP⁺) (filled circles) at post natal day 4-6 (P5) rats. High frequency stimulation (HFS, 100 Hz for 2 sec) was applied at the zero time. Ab, Mean values for PTP before and after bath application of TPP⁺. Data points measured from the same synapse are connected with a dotted line. Ac-d, The same experiment as in Aa-b in postnatal day 9-11 (P10) rats. Ae, Mean amplitude for baseline EPSCs at P5 and P10. On the bar graph, each data point was measured from individual synapses. Af, Mean amplitude for PTP at P5 and P10 under control conditions. Ag, Mean values for the difference of PTP amplitudes between the control condition and after application of TPP⁺ (TPP⁺-sensitive PTP, denoted by PTP_{mito}). Ah, Mean values for the TPP⁺-insensitive PTP amplitude (denoted by PTP_{non-mito}). B, The same experiments as in A except at 32 - 34 °C. Mean ± SEM; n.s., not significant (p > 0.05); *p < 0.05; **p < 0.01; Ab, Ad, Bb and Bd, paired t-test; Ae-Ah and Be-Bh, unpaired t-test.

Figure 2. Developmental correlative changes of presynaptic Ca²⁺ transients and PTP. Aa, Averaged time courses of the relative EPSC amplitude before (open circles) and after (filled circles) the bath application of TPP⁺ at P5. Ab-c, Mean amplitude of EPSC (Ab) or PTP (Ac) under control conditions and after bath application of TPP⁺. Ad, Averaged traces of post-tetanic residual Ca²⁺ (Ca_{res}) evoked by HFS of afferent fibers (100 Hz, 2 s) before (black) and after (gray) bath application of TPP⁺. Inset shows the Ca²⁺ transients induced by HFS (HFS-CaT) before (black) and after (gray) bath application of TPP⁺. Ae-f, Mean values for the [Ca²⁺]_{peak} of HFS-CaTs (Ae) and the time integral of post-tetanic Ca_{res} (Af) under control condition and after bath application of TPP⁺. Ba-f, The same experiments as in A at P10. C, Plot of TPP⁺-sensitive PTP (PTP_{mito}) as
a function of the amount of TPP\(^{-}\)-sensitive Ca\(_{\text{res}}\). D, Plot of the amount of post-tetanic Ca\(_{\text{res}}\) under control conditions as a function of the [Ca\(^{2+}\)]\(_{\text{peak}}\) of HFS-CaT. In C-D, mean values (filled symbols) are superimposed on individual data of P5 (open circles) and P10 (open triangles). Data are mean ± SEM; n.s., not significant (p > 0.05); *p < 0.05; **p < 0.01; Ab-f and Bb-f, paired t-test; C and D, unpaired t-test.

**Figure 3.** Differential mitochondrial Ca\(^{2+}\) uptake during HFS at P5 and P10. A, Averaged traces of HFS-CaT before (black) and after (gray) application of Ru360 via patch pipette at P5 (left) and mean values for [Ca\(^{2+}\)]\(_{\text{peak}}\) of HFS-CaT before and after application of Ru360 (right). Inset, the initial part of averaged HFS-CaT in expanded coordinates. B, The same experiment as in A at P10. C, Plot of the [Ca\(^{2+}\)]\(_{\text{peak}}\) of HFS-CaT in the presence of Ru360 as a function of the [Ca\(^{2+}\)]\(_{\text{peak}}\) of HFS-CaT under control conditions (circles, P5; triangles, P10). The data were fitted by linear regression and the correlation coefficient was 0.94. D, Plot of the cumulative amount of mitochondrial [Ca\(^{2+}\)] uptake as a function of cytosolic [Ca\(^{2+}\)] under control conditions. Plots of P5 (open circles) and P10 (gray dots) were superimposed. A polynomial function, \(k_0 + k_1x + k_2x^2\), was fitted to the plot (\(k_0 = 9.52, k_1 = -45.81, k_2 = 70.01\)). The abscissa was truncated at 3 \(\mu\)M because the estimate is too noisy at higher [Ca\(^{2+}\)] range (Inset). E, Mean values for the amount of mitochondrial Ca\(^{2+}\) uptake during HFS and post-tetanic mitochondrial Ca\(_{\text{res}}\) at P5 and P10. F, Averaged traces of Ca\(^{2+}\) influx elicited by 1 ms short depolarizing pulses at 100 Hz for 2 s under control conditions (solid line) and with 20 \(\mu\)M Ru360 included in the pipette solution (broken line). Calcium currents were recorded at the P10 calyx of Held terminals. Shading indicates the SEM of each averaged trace (control, black; Ru360, gray). Mean ± SEM; n.s., not significant (p > 0.05); **p < 0.01; unpaired t-test.

**Figure 4.** Developmental changes in Na/Ca exchangers. Aa, Averaged traces of Ca\(^{2+}\) transients evoked by double depolarizing pulses (pulse duration, 2 ms; inter spike interval, 5 ms) (CaT2) under presynaptic whole-cell voltage clamp with K\(^{+}\)-gluconate internal pipette solution at P5 (black) and P10 (gray) (left) and mean
values for amplitudes (Δ[Ca^{2+}], middle) and decay rate constant (r_w) of CaT2s (right). Ab, Averaged traces of Ca^{2+} transients evoked by a train of 20 depolarizing pulses at 200 Hz (CaT20) under the same conditions as in Aa (left). Mean values for Δ[Ca^{2+}] (middle) and decay rate constant (r_w) of CaT20s (right). B-C, The same experiment as in A except that the whole-cell voltage clamp was used with TMA^{-}-gluconate internal pipette solution (B) or with K^{+}-gluconate internal pipette solution including exchanger inhibitor peptide (XIP) (C). D, The same experiment as in A except that external Na^{+} was replaced with Li^{+}. E-F. Estimations of NCKX and NCX activities at P5 and P10. The NCKX activity was calculated from the difference of Ca^{2+} decay rate constants between CaT20s under K^{+} and TMA-internal conditions. The NCX activity was calculated from the difference between K^{+} and XIP conditions. Mean ± SEM; n.s., not significant (p > 0.05); **p < 0.01; unpaired t-test.

Figure 5. Shaping of the HFS-induced Ca^{2+} buildup by block of NCKX or NCX. Aa, Averaged traces of Ca^{2+} transients evoked by HFS (100 Hz for 4 s) of afferent fibers (left) and by a similar train of depolarizing pulses in whole-cell voltage clamp mode (right). The calyx of Held at P5 (black) or P10 (gray) was loaded with fura-4F using preloading techniques (left) or via whole-cell patch pipette (right). Ab, Mean values for the peak [Ca^{2+}] of HFS-CaTs shown in Aa. B-C, Averaged traces of CaTs evoked by a train of short depolarizing pulses at 100 Hz for 4 s (CaT400) under the presynaptic whole-cell voltage clamp condition at P5 (B) and P10 (C). The presynaptic whole-cell mode utilized K^{+}-gluconate internal solution as the control condition (black traces) or TMA^{-}-gluconate pipette solution for inhibition of NCKX (left column, blue) or K^{+}-gluconate pipette solution including 10 μM XIP for inhibition of NCX (middle column, red). In the rightmost panel in B and C, averaged CaT400 traces under the three conditions are superimposed. D, Comparison of averaged traces of CaT400 at P5 (black) and P10 (gray) under the conditions of TMA^{-} internal solution (Da) or K^{+} internal solution including XIP (Db). E. Averaged traces of presynaptic Ca^{2+} influx elicited by a train of depolarizing pulses at 100 Hz for 4 s at P5 (black) and P10 (gray). Shading indicates the SEM range of each averaged trace (A-E). Mean ± SEM;
n.s., not significant (p > 0.05); **p < 0.01; unpaired t-test.
**Figure 5**

**Aa**
- Fiber stimulation (stim.)
- Graphs showing peak calcium ([Ca^{2+}]_p) for P5 and P10 stages with a 2 μM bar.

**Ab**
- Graphs showing peak calcium ([Ca^{2+}]_p) for different stages with statistical analysis (n.s., **p**).
- P5 vs. P10 comparison for fiber stimulation and depolarization pulses.

**B**
- TMA+ (TMA+): Graphs for P5 and XIP with control (ctrl.) and TMA+ treatment.
- XIP: Graphs for control (ctrl.) and XIP treatment.

**C**
- P10: Graphs for control (ctrl.) and TMA+ treatment.
- XIP: Graphs for control (ctrl.) and XIP treatment.

**Da**
- TMA+ for P5 and P10 stages.

**Db**
- XIP for P5 and P10 stages.

**E**
- Graph showing pC values over the number of stimulations (0 to 400)
**Table 1.** The statistical mean values of the decay rate constant and the peak Ca\(^{2+}\) amplitude (Δ[Ca\(^{2+}\)]) of CaT2 and CaT20 under various conditions.

|        | P5       |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        |        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