Synaptic vesicle endocytosis at a CNS nerve terminal: Faster kinetics at physiological temperatures and increased endocytotic capacity during maturation

Robert Renden and Henrique von Gersdorff
The Vollum Institute, Oregon Health and Science University, Portland OR 97239

Running title: Endocytotic capacity during early development.

Keywords: Auditory brainstem, exocytosis, endocytosis, presynaptic Ca currents, vesicle pools, calyx of Held, MNTB, postnatal development, physiological temperatures, capacitance measurements.

Correspondence: Henrique von Gersdorff, The Vollum Institute, L-474, Oregon Health and Science University, 3181 Southwest Sam Jackson Park Road, Portland, OR 97239. E-mail: vongersd@ohsu.edu

Abstract

Synaptic vesicle membrane must be quickly retrieved and recycled after copious exocytosis to limit the depletion of vesicle pools. The rate of endocytosis at the calyx of Held nerve terminal has been measured directly using membrane capacitance measurements from immature postnatal day P7-P10 rat pups at room temperature (RT: 23-24°C). This rate has an average time constant of tens of seconds and becomes slower when the amount of exocytosis (measured as capacitance jump) increases. Such slow rates seem paradoxical for a synapse that can operate continuously at high input frequencies. Here we perform time-resolved membrane capacitance measurements from the mouse calyx of Held in brainstem slices at physiological temperature (PT: 35-37°C), and also from more mature calyces after the onset of hearing (P14-P18).

Our results show that the rate of endocytosis is strongly temperature-dependent, while the endocytotic capacity of a nerve terminal is dependent on developmental stage. At PT we find that endocytosis accelerates due to the addition of a kinetically fast component (time constant: $\tau = 1-2$ s) immediately following exocytosis. Surprisingly, we find that at RT the rate of endocytosis triggered by short (1-5 ms) or long ($\geq$10 ms) depolarizing pulses in P14-P18 mice are similar ($\tau \approx 15$ s). Furthermore, this rate is greatly accelerated at PT ($\tau \approx 2$ s). Thus, endocytosis becomes faster and less saturable during synaptic maturation, making the calyceal terminal more capable of sustaining prolonged high frequency transmitter release.

Introduction

Synaptic vesicles are reused following exocytosis in order to sustain chemical transmission at synapses (Rizzoli and Jahn 2007; Schweizer and Ryan 2006;
Südhof 2004). Studies using membrane capacitance have determined the rate of endocytosis in neuroendocrine cells (Betz and Angleson 1998; Smith and Neher, 1997), in sensory neurons (Moser and Beutner 2000; Rieke and Schwartz 1996) and in large nerve terminals (Hallermann et al. 2003; Sun and Wu 2001; von Gersdorff and Matthews 1994). For small central bouton-type synapses optical measurements have been used to probe the vesicle cycle (Aravanis et al. 2003; Ferguson et al. 2007; Gandhi and Stevens 2003). These studies have generally found that activity-dependent endocytosis is composed of at least two kinetically distinct components: one that occurs after brief stimuli with fast rates of hundreds of milliseconds to seconds, and another slower form that occurs after stronger stimulation with slower rates of tens of seconds (LoGiudice and Matthews, 2007; Wu 2004). However, the physiological relevance of these two separate modes of endocytosis is still under debate, as well as their molecular mechanisms and possible modulation.

The calyx of Held, a large CNS presynaptic terminal, is amenable to direct patch clamp recordings as well as dye imaging studies (de Lange et al. 2003; Sun and Wu 2001). Endocytosis at this synapse occurs with a time constant of several seconds, becoming slower as the stimulus duration or strength increases (Sun and Wu, 2001; Yamashita et al. 2005). It is GTP- and dynamin-dependent (Yamashita et al. 2005), but independent of free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\); Sun et al. 2002). In addition, the rate of endocytosis is dependent on the magnitude of previous exocytosis (Sun et al. 2002; Yamashita et al. 2005). However, repetitive strong stimulation recruits an additional calmodulin-sensitive fast component of endocytosis, dependent on high [Ca\(^{2+}\); elevations (Wu et al. 2005).

These previous studies used P7 to P11 rat pups. However, rats and mice are deaf before postnatal day P12 (Blatchley et al. 1987), and their calyx of Held synapse is developmentally immature (Taschenberger et al. 2002). Additionally, most experiments using this synapse were performed at room temperature. Previous reports have shown that the rate of endocytosis is very sensitive to temperature, with up to a three-fold increase in the rate at physiological temperatures (Fernandez-Alfonso and Ryan 2004; Johnson et al., 2005; Micheva and Smith 2005; Teng and Wilkinson 2003). It is thus likely that the kinetics of endocytosis at this calyceal synapse has been underestimated.

Here we report measurements of endocytosis from the young murine calyx of Held (P7-P10) at physiological temperature (PT, 35-37°C), and from older mice where the calyx is more functionally mature (P14-P18). In immature mouse calyces at RT (23-24°C), our results agree well with previous reports from the rat calyx; however, we see a two- to three-fold increase in the rate of endocytosis at PT, regardless of stimulation intensity. Unexpectedly, in more mature mouse terminals endocytosis rates were similar for short (single action-potential-like) pulses and long depolarizing pulses, consistent with the hypothesis that retrieval capacity is increased during synaptic maturation, perhaps due to more abundant endocytotic proteins. In these terminals, physiological temperature further
accelerated membrane retrieval two-fold, even for long (20-30 ms) depolarizations that triggered copious exocytosis. The ability to faithfully follow high frequency firing increases with age and temperature (Taschenberger and von Gersdorff 2000), and we thus propose that this may be partly due to a greater capacity to quickly retrieve fused vesicular membrane and recycle vesicles in mature synapses.

Methods

Slice Preparation. C57/bl6J mice (Jackson Labs, Bar Harbor ME) postnatal day P7 to P18 old were used in this study. Young (P7-P10) mice had closed eyes, and lacked a startle response to brief auditory stimulus (e.g. hand clap). Mice in the older age group (P14-P18) had open eyes, and showed a response to auditory stimuli. After decapitation, the brainstem was quickly removed from the skull, and submerged in ice-cold saline, containing the following (in mM): 125 NaCl, 2.5 KCl, 3 MgCl2, 0.2 CaCl2, 25 glucose, 25 NaHCO3, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol, and 2 Na-pyruvate, pH 7.3–7.5 when bubbled with carboxen (95% O2, 5% CO2); osmolality was 310–315 mOsm. Transverse slices were made 150-160 μm thick for presynaptic recordings, and 200 μm thick for postsynaptic recordings, containing the medial nucleus of the trapezoidal body (MNTB) using a vibratome (VT1000; Leica, Bannockburn, IL). Slices were then transferred to an incubation chamber containing normal saline bubbled with carboxen (95% O2, 5% CO2), maintained for 60 min at 35°C and thereafter at room temperature (22-25°C) until used for recording. Normal saline was the same as slicing saline, but with 1 mM MgCl2 and 2 mM CaCl2.

Electrophysiology. Slices were transferred to a recording chamber, and perfused at 1-3 mL/min with a normal saline bath solution. Solution was warmed to 35-37°C in some experiments using an in-line heater (Warner Instruments, Hamden CT). Recordings at physiological temperature were initiated 10 min to 1 hr after the slice was re-equilibrated at 35-37°C, unless otherwise noted (e.g. Figure 2). Slices were visualized using infrared differential interference contrast microscopy (Leica) and a 40x or 63x water-immersion objective, and observed on a television screen using a CCD camera (Hamamatsu Photonics, Bridgewater, NJ). Ionotropic glutamate receptors (AMPA and NMDA) were blocked by bath perfusion of 50 μM D-APV, and 10 μM NBQX or CNQX, respectively; glycine receptors by 0.5 μM strychnine and GABA receptors by 10 μM bicuculline. This treatment eliminated the possibility of capacitatively coupled postsynaptic receptor conductances (Borst et al. 1995; Forsythe 1994; Wolfel and Schneggenburger 2003). Ca2+ currents were isolated by blocking INa with 1 μM TTX and IK with 5 mM tetraethyl ammonium (TEA) in the bath solution. The pipette internal solution for presynaptic calcium current and capacitance recordings contained the following (in mM): 130 Cs-gluconate, 15 CsCl, 5 Na2-phosphocreatine, 10 HEPES, 0.2 EGTA, 20 TEA-Cl, 4 Mg-ATP, and 1 GTP, pH adjusted to 7.3 with CsOH, and osmolality of 305-310 mOsm.

Pipettes were pulled from thick-walled borosilicate glass (Sutter Instruments, Novato, CA) with a Sutter P-97 electrode puller to open tip resistances of 4-8 MΩ
for presynaptic recordings. Pipette tips were coated with dental wax to reduce pipette capacitance. Pipette pressure was monitored with a manometer, but was not adjusted; hydrostatic pressure was zero, or slightly negative (Heidelberger et al. 2002). Data were acquired at 10–25 μs sampling rate, using an EPC-9 patch clamp amplifier controlled by Pulse 8.4 software (HEKA Elektronik, Lambrecht/Pfalz, Germany), filtered on-line at 2.9 kHz, and run by a Power Macintosh G3 computer (Apple Computers, Cupertino, CA). Step depolarizations were to 0 mV, unless stated otherwise.

Presynaptic terminals were voltage clamped at -80 mV, and access resistance was compensated up to 75%, such that residual series resistance ($R_s$) was usually 7-10 MΩ. Terminals with membrane resistance ($R_m$) < 1 GΩ were discarded from analysis. Membrane capacitance was calculated from a 1 kHz, 30 mV amplitude sine wave on the holding potential, using the software lock-in capability of the EPC-9 amplifier (Gillis 1995). The reversal potential was assumed to be 0 mV. Membrane capacitance ($C_m$) was not measured during step depolarizations (Sun and Wu 2001; Taschenberger et al. 2002), and a minimum of 30 s was allowed between depolarizations to allow for complete recovery from synaptic depression (Kushmerick et al. 2006).

Calyces of young animals can be fit by a single or two-compartmental passive model, due to the presence of the afferent axon, but this does not significantly alter their exocytosis or endocytotic properties, as reported previously (Taschenberger et al. 2002; Wu et al. 2005). However, during development, the presynaptic calyx morphology becomes more complex (Taschenberger et al. 2002), which could result in significant filtering and loss of adequate voltage clamp in the terminals of older animals. In animals older than P14, passive membrane capacitance properties nearly always indicated a two-compartmental terminal, which could be adequately fit by a bi-exponential function (Supplemental Figure 1A). Mean time constants ($\pm$ SEM) were $t_{\text{fast}}$ = 0.19 ± 0.01 ms, which carried 82% of the current, and $t_{\text{slow}}$ = 2.1 ± 0.1 ms ($N = 56$ calyx terminals). These numbers match well with those reported for passive $C_m$ of P12-14 rat calyces (Taschenberger et al. 2002). In addition, the sine wave used for calculation of membrane capacitance was not significantly filtered by the complex morphology, because similar exocytotic increases in $C_m$ were observed for depolarizations when the sine wave frequency was varied between 500 Hz and 2 kHz (Supplemental Figure 1B). Thus, accurate measurement of membrane capacitance in the presynaptic terminal of older animals could still be achieved using a 1 kHz sine wave, even in the presence of complex terminal morphology.

**Drugs and reagents.** All salts, as well as NBQX, strychnine, kynurenic acid, and cypermethrin were purchased from Sigma (St. Louis, MO). Tetrodotoxin was purchased from Alomone Labs (Jerusalem, Israel). All other pharmacological agents (APV, bicuculline, CNQX) were purchased from Ascent Scientific (Weston-Super-Mare, UK).

**Analysis.** Baseline $C_m$ was linearly corrected for drift 10-20 sec before a step depolarization (Horrigan and Bookman 1994). For exocytosis, baseline $C_m$ was measured at 0.5 to 2 sec prior to step depolarization. Exocytosis (or $C_m$ jump) was evaluated as the difference in baseline just prior and 0.5 to 1 sec following a
depolarization, beginning >250 ms following the end of depolarization to avoid depolarization-induced changes in membrane conductance and capacitance artifacts (Yamashita et al. 2005). Presynaptic calcium currents were calculated by P/5 leak subtraction with leak traces acquired just prior to depolarization.

Long traces that tracked C_m for endocytosis were made using ‘snapshots’ of the membrane capacitance, as reported previously (Sun et al. 2002; Yamashita et al. 2005). Briefly, a 1 KHz sine wave was recorded for 20 ms at a high sampling rate (20 μsec) every 500 ms before and after the step depolarization. The calculated C_m was then averaged for each 500 ms time point. This allowed us to accurately track C_m over the course of 2-3 minutes after a step depolarization. Endocytosis (or C_m decay) sweeps from a single cell were averaged, and fit with a single or double-exponential starting immediately (20 ms) following the step depolarization, until the trace returned to baseline values or the end of the sweep (up to 2 min). This approach could result in contamination of the first C_m data point following the depolarization step, due to capacitance artifacts that occur <400 ms following the depolarization (Yamashita et al. 2005). However, eliminating the first data point (C_m at 20-40 ms following the step depolarization) from decay fits had only a marginal effect on the time constant at RT. Endocytosis at PT was only affected for long (30 ms) depolarizations, decreasing the weight and increasing the value of the time constant by less than 1 sec for fast endocytosis, but dramatically reduced the measured capacitance jump. The weighted mean decay rate (τ_m) was calculated from the individual exponential time constants (τ_fast and τ_slow) and their respective amplitude components (A_1 and A_2) as:

$$\tau_m = \tau_{fast} \times (A_1/(A_1+A_2)) + \tau_{slow} \times (A_2/(A_1+A_2)).$$

Most cells used for analysis had 2-5 sweeps of capacitance at a given step depolarization. Endocytosis was analyzed only in sweeps obtained <10 min following break-in to avoid run-down of responses (Hull and von Gersdorff 2004; Parsons et al. 1994). Ideally, perforated-patch techniques would be implemented for recording capacitance (Price and Trussell 2006). However, this mode of recording typically produces high access resistance (R_s=30-50 MΩ), which precludes reliable and low-noise C_m recordings (Gillis 1995).

Retrieval rates were calculated by converting the exo-end relationship from fF/sec to SV/sec, using a single vesicle capacitance of 61 aF (Wu et al. 2007). Thus, ΔC_m (fF) / [τ_m (sec) / C_m vesicle (fF/SV)] = SV/sec. If a linear dependence of endocytosis rate to exocytosis (ΔC_m) was shown, as for immature calyces, values for 1/slope from a linear fit were used. In the case of mature calyceal terminals, where the capacity for endocytosis is increased and there is no relationship between the magnitude of exocytosis and the speed of endocytosis, we used ΔC_m and τ_m values due to short (2ms) and long (30 ms) depolarizations to estimate retrieval rates for these two conditions.

Data were analyzed off-line and presented using Igor Pro (Wavemetrics, Lake Oswego, OR). Single exponential values are reported for passive cell membrane properties, unless a biexponential fit returned a 50% smaller relative χ² value,
defined as \((\chi_1^2 - \chi_2^2)/\chi_2^2\), where \(\chi_1^2\) is from a monoexponential fit, and \(\chi_2^2\) is from a biexponential fit. For endocytosis, single exponential values are reported, unless the relative \(\chi^2\) value was 15% smaller for a biexponential function. Statistical analyses were performed using Prism 4.0 (Graphpad, San Diego CA). Mean ± SEM are reported, unless otherwise noted. Significance is reported as * = p<0.05, ** = p<0.01, and *** = p<0.001, using appropriate tests.

Results
Previously, we reported that capacitance jumps and the refilling rate of the readily releasable pool of vesicles at the calyx of Held increases dramatically at physiological temperature (PT), but we did not discern whether endocytosis was also accelerated at PT (Kushmerick et al. 2006). Here, we examined endocytosis at PT to gain further insight into the factors that may rate limit synaptic vesicle recycling and reuse.

Capacitance measurements at the mouse calyx terminal
Capacitance jumps reflecting Ca\(^{2+}\)-dependent exocytosis of synaptic vesicles due to depolarizations lasting 1 to 30 ms were measured in immature calyces (P7-P10), both at room temperature (RT, 22-25 °C) and physiological temperature (PT, 35-37°C). The \(\Delta C_m\) jumps correlate well with cumulative EPSCs and total synaptic vesicle release at RT in the rat calyx of Held using deconvolution analysis (Sakaba 2006). Our exocytosis data from mice (Supplemental Figure 2) also shows that longer depolarizations lead to larger Ca\(^{2+}\) influx and increasingly larger \(\Delta C_m\) jumps (Kushmerick et al. 2006). In older mice (P14-P18), exocytosis at RT was larger than in younger animals for similar Ca\(^{2+}\) influx, indicating a larger releasable pool of vesicles and/or increased Ca\(^{2+}\) sensitivity, similar to previous reports in more mature rats (Taschenberger et al. 2002). We thus suggest that the basic excitation-secretion coupling parameters, as well as developmental changes in exocytosis capacity, are likely conserved and similar between rats and mice.

EPSCs show significant variability from trial to trial at the calyx of Held (Scheuss et al. 2002). Likewise, exocytosis measured as changes in \(\Delta C_m\), also shows inter-trial variability. Figure 1A shows this and an example of the even more substantial variability observed in endocytosis rate from trial to trial (see also Wu et al. 2005). The coefficient of variation (Cv) for the \(Q_{Ca}\) (total Ca\(^{2+}\) influx charge, measured as integral of the Ca\(^{2+}\) current) of the sweeps shown in Figure 1A was 0.039, while the Cv for \(\Delta C_m\) was 0.149 (Figure 1B). In addition, this figure also illustrates the substantial run-down of membrane retrieval (capacitance decay) after about 10 minutes following establishment of the whole-cell recording configuration, presumably due to dilution of soluble endocytotic proteins from the cytosol by the intracellular patch-pipette solution (Parsons et al., 1994). Endocytosis is thus more labile than exocytosis, perhaps due to the large number of proteins necessary for membrane fission (Koh et al., 2007). Therefore, endocytosis rates reported here were evaluated as the mean of several trials per
cell within the first 10 minutes following break-in, similar to other endocytosis protocols at this synapse (Yamashita et al. 2005).

We next determined that a change in membrane capacitance due to a 1 ms step depolarization to +10 or 0 mV is similar to that of a fiber-evoked action potential (AP) at RT in young (P7-P10) and older (P16-P18) mouse calyx, respectively. Previous work has shown that the quantal content of an EPSC at the calyx terminal is independent of temperature (Kushmerick et al. 2006). In young terminals (P7-P10), low frequency stimulation at 0.1 Hz resulted in an average EPSC charge (area under the current waveform) of 17.1 ± 3.0 pC (N = 8), and an average quantal (mEPSC) charge of 56.7 ± 5.1 fC (amplitude: 39.9 ± 4.1 pA) from the same cells (240 events per cell, on average). These values give an estimated quantal content (or exocytosis) of 310 ± 51 synaptic vesicles (SVs) per AP. Using an average capacitance of 61 aF per synaptic vesicle (Wu et al. 2007), this corresponds to a capacitance increase of 19.0 ± 3.1 fF for a single AP. In calyces from this age range, a step depolarization of 1 ms to +10 mV resulted in a capacitance increase of 23.3 ± 2.4 fF (N = 40). Depolarization of 1 ms to +10 mV, also resulted in similar Ca2+ influx as previous studies which estimated AP-equivalent stimulation parameters (Fedchyshyn and Wang 2005; Yang and Wang 2006). In P16-P18 animals, the average EPSC charge was 10.1 ± 1.8 pC (N = 11), and the average quantal (mEPSC) size charge from the same cells was 26.9 ± 2.4 fC (55.2 ± 3.1 pA, 123 events per cell, on average). This estimates a quantal content of 367 ± 44 quanta at RT, which corresponds to a capacitance increase of 22.4 ± 1.7 fF for a single AP, again assuming 61 aF per vesicle. Depolarization for 1 ms to 0 mV in older terminals (P14-P18) resulted in a capacitance increase of 33.5 ± 5.8 fF (N = 17). We thus considered a 1 ms depolarizing pulse as an approximately AP-like stimulus at the mouse calyx.

**Endocytosis rate increases at physiological temperature**

In about 80% of all calyces recorded from P7-P10 mice, membrane capacitance following the capacitance jump triggered by a depolarizing pulse decayed back towards baseline. This suggests the retrieval of fused vesicle membrane from the plasma membrane (Figure 2A and 2B). This decay could usually be accurately fit by a single or double exponential, and returned to baseline in most cases, though an overshoot beyond baseline membrane capacitance (Cm) was occasionally observed (Artalejo et al. 1995). Notably, capacitance decay (endocytosis) was only seen when we included 1 mM GTP in the intracellular solution (Sun et al. 2002; Yamashita et al. 2005). The rate of membrane retrieval occurred with a time constant of 10 to 50 sec at RT, and was linearly dependent on the magnitude of previous exocytosis for depolarizing pulses of 1 to 10 ms (Figure 4B).

At PT, the rate of endocytosis sped up substantially both for short (AP-like) and longer (depleting) pulses, due to the addition of a second kinetically distinct exponential decay component (Figure 2A and 2C). This second component (τ ~ 1 sec) was roughly one order of magnitude faster than that seen at RT. The slower
component of endocytosis at PT (τ ~ 20-30 sec) was similar to the rate seen at RT. A double exponential function was thus necessary to produce a good fit of the C_m decay (see Materials and Methods). The fast rate of endocytosis was responsible for 12-50% of the mean endocytosis over the stimulus durations examined. Specifically, for 1-5 ms depolarizations at PT, 9 of 20 terminals showed a fast component of decay, which accounted for ~30% of the total amplitude of C_m decay. By contrast, at RT, only 2 of 42 terminals showed a fast component of endocytosis for pulse durations of 1-10 ms.

One possible mechanism underlying the faster membrane retrieval rates seen at PT could be increased Ca^{2+} influx for a given depolarization duration, due to faster activation kinetics of Ca^{2+} channels at PT (Kushmerick et al. 2006). To test this hypothesis, we empirically matched Ca^{2+} influx at RT and PT, by reducing depolarization at PT to match the charge measured for a 2 ms depolarization at RT in the same cell (Figure 3; N = 6 at RT, N = 9 at PT). Cells were recorded at RT, then the temperature quickly ramped to PT (N = 4 terminals); or at PT first, then temperature lowered to RT (N = 4 terminals). When Ca^{2+} influx and exocytosis were matched at RT and PT, endocytosis was still significantly faster at PT, on average. Exponential fits to endocytosis from individual cells resulted in mean time constants of 15.9 ± 2.6 sec at RT (N = 6 cells), and 8.0 ± 2.3 sec for stimulus-matched terminals at PT (N = 7 cells). This increase in endocytosis rate at PT was significant (P = 0.045, Student’s t-test). Thus, the faster endocytosis observed at PT for a short depolarizing voltage-clamp step is independent of Ca^{2+}-influx, indicating it is likely not due solely to a Ca^{2+}-dependent effect.

When mean endocytosis rate (τ_m) was plotted against the size of capacitance jump for 1-10 ms pulses, the data were fit well by a linear function (Figure 4B). This result is consistent with previous reports (Sun et al. 2002; Yamashita et al. 2005). This slope, an exocytosis-dependent slowing of endocytosis rate, was not significantly different between RT and PT, (P = 0.5040 Student’s t-test); though overall rates were substantially faster. While the source of this endocytotic inhibition is still not clear, it may indicate a saturation of compensatory endocytotic capacity of the terminal with increasing exocytosis.

At PT, the average endocytosis rate for 1 ms depolarizing pulses (AP-like) in immature terminals was τ = 7.6 ± 2.2 sec (N = 6; Figure 4B), which is significantly faster than that observed at RT (τ = 20.3 ± 3.7 sec (N = 25); P = 0.0064 using Student’s t-test corrected for unequal variances). So the rate of endocytosis is more than twice as fast at PT than at RT, even though there is much more exocytosis at PT. Clearly, the capacity to retrieve large amounts of vesicular membrane is greatly enhanced at PT.

**Acceleration of endocytosis after prolonged depolarizations**

For depolarizing steps >10 ms we observed an acceleration of endocytosis at RT (open symbols; Figures 4B). For repetitive 20 ms depolarizations, recruitment of a Ca^{2+}-dependent, kinetically distinct form of fast endocytosis occurs in young
rats at RT (Wu et al. 2005). We assume our results represent a similar Ca\textsuperscript{2+}-dependent acceleration of endocytosis due to strong stimulation, but now occurring at a lower threshold. We also observed a similar increase in endocytosis rate for depolarizations of 20 to 30 ms at PT in terminals of similar age animals, relative to the retrieval rates observed for 10 ms depolarization at PT (Figure 4B). We assume that the mechanism underlying this acceleration in endocytosis rate is similar to that at RT.

**Endocytosis capacity increases in mature synapses**

Next, we investigated endocytosis in more mature synapses. Endocytosis rates for young and mature terminals were similar in response to a 1 ms depolarization (Figure 5A and 5B). Mature calyces had $\tau = 14.5 \pm 3.6$ sec (N = 5), and endocytosis in immature calyces had $\tau = 20.3 \pm 3.7$ sec (N = 25; P = 0.4992, Student's t-test). However, the amount of exocytosis increased dramatically in older animals (Figure 7A), especially for short depolarizations, due to an increased pool size and more efficient excitation-secretion coupling in older animals (Taschenberger et al. 2002; Fedchyshyn and Wang 2005).

We depolarized the terminal in older animals over a variety of durations (1-30 ms) at RT, and noticed a clear lack of dependence of endocytosis rate on the magnitude of $\Delta C_m$ or exocytosis (Figures 5B and 7). In more mature calyces, endocytosis was fit almost exclusively by a single exponential at RT. Bi-exponential endocytosis was seen in 1 of 6 cells at 5 ms (contributing to 50% of mean endocytosis in that calyx), and 1 of 5 cells at 30 ms (contributing 50% of mean endocytosis in that calyx). Across the depolarization range examined here, the rate of endocytosis had a time constant of $\tau = 16$ seconds, regardless of the magnitude of exocytosis or depolarizing pulse duration.

Evaluation of endocytotic capacity in terms of maximum retrieval rates immediately following depolarization (synaptic vesicles per sec; SV/sec) shows that the capacity for vesicle retrieval increases in older calyceal terminals proportionally to exocytosis, and does not saturate. For a 1 ms depolarization ($\tau_m = 14.5$ sec), maximum retrieval rate at RT for older terminals can be approximated as $\Delta C_m / \tau_m / (C_m/\text{vesicle}) = 68.1 \text{ fF} / 14.51 \text{ sec} / 0.061 \text{ fF/SV} = 77 \text{ SV/sec}$. For a 30 ms depolarization, the maximum retrieval rate increases to 534 SV/sec, while the time constant for endocytosis stays nearly constant ($\tau = 15.7$ sec). We thus propose that mature synapses have a greater capacity to retrieve membrane due to more abundant set of endocytotic proteins and/or efficient endocytotic machinery. A developmental increase in the number of retrieval sites or development of a stronger interaction between the endocytotic machinery and newly exocytosed vesicles could both be responsible.

We also directly measured endocytosis rates in older terminals at PT for depolarization lengths of 2 ms and 30 ms, to estimate the maximum vesicle retrieval rate immediately following exocytosis in mature functional terminals at PT (Figure 6). Capacitance decay in these recordings was very fast, and
Data from calyx terminals for 2 ms pulses were well fit by a single exponential function \((\tau = 3.3 \pm 0.8 \text{ sec}, N = 6 \text{ for 2 ms depolarization})\). For 30 ms depolarizations, \(\tau_m = 7.6 \pm 2.8 \text{ sec}\), with two of four terminals returning a biexponential fit. For these two terminals, \(\tau_{\text{fast}} = 2.3 \pm 0.3 \text{ sec}\), carrying 66 \pm 1% of the total time constant, and \(\tau_{\text{slow}} = 31.5 \pm 6.3 \text{ sec}\). Thus, at PT maximum retrieval rates can be estimated as 921 SV/sec and 1045 SV/sec, for a 2 ms and 30 ms depolarization, respectively. We measured the quantal content of an evoked EPSC as ~295 SVs in P16-18 mice at PT (N = 5 terminals, data not shown). This result suggests that the mature calyx could operate indefinitely at frequencies of ~3.3 Hz without depression, solely by local recycling of SV membrane. Since this calyx synapse can actually follow much higher stimulation frequencies, we propose that it additionally taps into a large reserve pool of vesicles in order to support sustained high frequency synaptic transmission at PT.

**Ca\(^{2+}\)**-dependent acceleration of endocytosis is developmentally regulated

In order to investigate further the Ca\(^{2+}\) dependence of endocytosis, we plotted the endocytotic rate constant against Ca\(^{2+}\) charge influx elicited by depolarization (Figure 8). As expected, a curve similar to that for exocytosis versus endocytosis was generated. For similar Ca\(^{2+}\) charge \((Q_{\text{Ca}})\) influxes produced by short depolarizations (AP-like), endocytosis at PT was significantly faster than at RT: \(Q_{\text{Ca}}\) for 1 ms at PT was similar to that for 2 ms depolarization at RT \((1.9 \pm 0.4 \text{ pC for 1 ms at PT, N = 6; 1.9 \pm 0.1 \text{ pC for 2 ms at RT, N = 18}; P = 0.9388 \text{ by Student’s t-test})\); however, endocytosis was significantly faster at PT \((\tau = 7.6 \pm 2.3 \text{ sec at PT}; 25.0 \pm 4.1 \text{ sec at RT}; P = 0.0132 \text{ by Mann-Whitney nonparametric U test})\). A similar trend was seen when 2 ms depolarization at PT was compared to 5 ms at RT \((Q_{\text{Ca}} = 3.9 \pm 0.4 \text{ pC for 2 ms at PT, N = 5; 5.1 \pm 0.6 \text{ pC for 5 ms at RT, N = 7}; P = 0.1473})\), with faster endocytosis at PT \((\tau = 11.3 \pm 3.0 \text{ sec at PT, and 32.6 \pm 5.7 \text{ sec for RT}; P = 0.0041 \text{ by Mann-Whitney U test})\). Likewise, comparing 5 ms at PT to 10 ms at RT yielded similar \(Q_{\text{Ca}}\) \((10.0 \pm 2.9 \text{ pC for 5 ms at PT, N = 4; 12.6 \pm 2.2 \text{ pC for 10 ms at RT, N = 5}; P = 0.5343})\), but significantly faster endocytosis at PT \((\tau = 10.3 \pm 3.4 \text{ sec at PT, and 53.6 \pm 17.2 \text{ sec at RT; P = 0.0159 \text{ by Mann-Whitney U test}}})\). These results further support the premise that endocytosis at PT is not faster strictly due to increased Ca\(^{2+}\) influx.

Endocytosis due to longer depolarizations was accelerated in a Ca\(^{2+}\)-dependent manner in younger terminals, both at RT and PT. In younger animals, endocytosis was slowest for a 10 ms depolarization, both at RT and PT (Figure 8A). For 20 and 30 ms pulses at either RT or PT, endocytosis accelerated to rates similar to those observed for brief stimuli (Figure 4A, B). We assume that this result indicates a “threshold” for Ca\(^{2+}\)-dependent acceleration of endocytosis at about 10-12 pC Ca\(^{2+}\) influx in the immature calyx of Held terminal (see also Wu et al. 2005).

A Ca\(^{2+}\)-dependent acceleration of endocytosis for 20-30 ms pulses was not present in more mature terminals (P14-P18), where endocytosis was well
represented by similar monoexponential rates for both short and long depolarizations (Figure 8B). These data can be fit by a linear function (slope = 66.4 ± 151.2 ms/pC). At RT, Ca\(^{2+}\) influx was similar at P7-P10 and P14-P18 terminals at 5 and 10 ms depolarizations, but endocytosis was faster in older terminals. For 5 ms depolarization, Q\(_{\text{Ca}}\) was not significantly different (5.0 ± 0.6 pC for P7-P10, N = 7; 8.2 ± 1.7 pC for P14-P18, N = 6; P = 0.0632), but endocytosis rate was significantly faster in older terminals (τ = 32.6 ± 5.7 sec for P7-P10, and 12.2 ± 1.9 sec for P14-P18; P = 0.0082 by Mann-Whitney U test). For a 10 ms depolarization, similar Q\(_{\text{Ca}}\) were observed (12.6 ± 2.2 pC for P7-P10, N = 5; 14.6 ± 1.5 pC for P14-P18, N = 7; P = 0.4755), but endocytosis rate was also significantly faster in P14-P18 terminals (τ = 53.6 ± 17.2 sec for P7-P10, and 14.3 ± 3.1 sec for P14-P18; P = 0.0177, Mann-Whitney U test). This surprising lack of slowing of endocytosis in older animals may be due to an increased accumulation of endocytotic proteins and/or sites.

We thus suggest that the endocytotic machinery in older animals is not saturated even when a majority of the synaptic vesicles in the readily releasable pool are exocytosed (vesicle pool depletion), perhaps due to a larger supply of active endocytotic sites. Though the exact mechanism remains unclear, the mature terminal seems capable of supporting an increased releasable pool size and an increased rate of pool refilling in older animals (Kushmerick et al. 2006), perhaps in part due to this increased capacity for sustained endocytosis.

**Discussion**

We used membrane capacitance measurements to directly assay the rate of endocytosis of the calyx of Held terminal at RT and PT in young mice. We also examined the speed of endocytosis in older mice (P14-P18) when the calyx is more functionally mature. Increasing the amount of exocytosis slows down endocytosis at RT in immature animals, consistent with previous reports for pulse durations <10 ms (Sun et al. 2002; Yamashita et al. 2005). However, we find that for longer depolarizing pulses (20-30 ms) endocytosis accelerates. At PT, endocytosis rate is augmented by a second, faster rate, which is responsible for about 25% of the mean endocytosis time constant in the young calyx, and closer to 67% in older terminals. Additionally, in older animals the endocytotic rate was independent of stimulation intensity (or depolarizing pulse duration). The capacity to quickly endocytose relatively large amounts of exocytosed membrane is thus substantially enhanced in more mature synapses.

**Exocytosis and endocytosis in young calyces at RT**

Previous reports showed a linear dependence of endocytosis rate on capacitance jump (exocytosis) for depolarizing steps up to 10 ms (Yamashita et al. 2005). Surprisingly, when we fully exhausted the readily releasable pool in young animals at RT (depolarization >10 ms), the rate of endocytosis increased. Recently, it has been shown that endocytosis speeds up after prolonged stimulation trains in a Ca\(^{2+}\)-dependent manner (Wu et al. 2005), and [Ca\(^{2+}\)], and
calmodulin accelerate the refilling of a fast releasable pool (Sakaba and Neher 2001a; Wang and Kaczmarek 1998). A 20-30 ms stimulation may induce the release of a reluctant pool of vesicles, which are then preferentially retrieved via a fast pathway. Heterogeneity in the releasable pool at the calyx of Held has been shown previously, consisting of a quickly releasing pool of vesicles which are slowly recycled, and a slowly releasing pool that is quickly refilled (Sakaba 2006; Sakaba and Neher 2001a; Trommershauser et al. 2003). Refilling of quickly releasable vesicles is modulated by cAMP and calmodulin (Sakaba and Neher 2001a, 2001b). The identities of these pools may be linked to differential sensitivity to Ca²⁺, to sub-maximal release rates, or distance to Ca²⁺ sources (Wadel et al. 2007; Wolfel et al. 2007). It is possible that these differences are conveyed to vesicle retrieval rates, as well.

What is the source of the synaptic vesicles responsible for refilling the releasable vesicle pool at the calyx of Held? Postsynaptic studies in immature rats (P8-P10) at RT have shown that recovery from synaptic depression is frequency dependent. Moderate stimulation (10-100 Hz) recovers with a τ = 4 sec (von Gersdorff et al. 1997), while strong stimulation (300 Hz) invokes two phases of recovery: fast recovery (τ < 100ms), and a slower form (τ = 4-5 sec; Wang and Kaczmarek 1998). Furthermore, refilling of the vesicle pool requires polymerized actin (Sakaba and Neher 2003), and presynaptic inhibition of dynamin abolishes endocytosis, and eventually inhibits exocytosis (Yamashita et al. 2005). These results indicate that reuse of synaptic vesicles is partially responsible for the refilling of the pool. Moreover, studies using lipophilic dyes to label recycling of vesicles have shown that with rather low frequency stimulation, only a subset of vesicles are used (de Lange et al. 2003). However, these studies cannot fully discriminate between partial replenishment of the releasable pool from intracellular reserves, or immediate refilling and reuse of recently fused vesicles (Schneggenburger and Forsythe, 2006). Our results support the conclusion that local vesicle recycling plays an important role in the maintenance of fusion competent synaptic vesicles, and helps to determine the possible maximal rates of local reuse of vesicles at the calyx of Held, under immature and more mature conditions, and at physiological temperatures.

The adult calyx of Held is capable of entrained synaptic transmission at 600 Hz in mature mouse terminals for short periods of afferent fiber stimulation (Wu and Kelly 1993). However, we estimate that the maximal rate of transmission, based solely on local vesicle reuse, is only ~3 Hz at PT in terminals from P16-P18 animals. This apparent discrepancy requires clarification. While the calyceal terminal is capable of following high stimulation frequencies, it is not capable of doing so indefinitely, and undergoes substantial depression, even at relatively low frequencies (e.g. 10 to 100Hz; von Gersdorff and Borst 2002). At stimulation frequencies above 100 Hz at RT, depression seems to be mediated principally by vesicle pool depletion. We thus propose that the calyx of Held relies on a ready supply of vesicles from a reserve pool in order to follow higher stimulation frequencies.
Unfortunately, we cannot fully account for the fate of excess synaptic vesicle membrane during high frequency stimulation. Bulk endocytosis has been shown at other large presynaptic terminals (Holt et al. 2003; LoGiudice and Matthews, 2007). There is also evidence of bulk endocytosis at the calyx of Held, though its relative contribution (10% of all endocytosis) probably does not fully account for the complete retrieval of exocytosed membrane following strong stimulation (Wu and Wu 2007).

**What does faster endocytosis at PT reflect?**

At the calyx of Held, Ca\(^{2+}\) channel activation and recovery from short-term depression is faster at PT, and the recruitment of vesicles during steady-state synaptic depression is also accelerated at PT, while initial vesicle pool size and excitation-secretion coupling are relatively unaffected (Kushmerick et al. 2006; Postlethwaite et al. 2007). Here we saw a significant increase in the mean endocytosis rate at PT, due to the addition of a kinetically distinct fast form of endocytosis. Two previous studies have also examined the effect of temperature on vesicle retrieval in neuronal presynaptic terminals, using optical methods, and report an increase in the speed of endocytosis. In hippocampal cultured synapses, Fernandez-Alfonso and Ryan (2004) report a Q\(_{10}\) for endocytosis of 1.4, allowing endocytosis to sustain vesicle exocytosis at PT for stimulation frequencies up to 10 Hz (i.e. a frequency that would normally deplete the releasable pool at RT); and Micheva and Smith (2005) estimate that the endocytosis rate increases 3-fold at PT. Here we observed an average 1.44-fold faster endocytosis at PT (for a 12°C increase). This faster endocytosis at PT is compelling but still circumstantial evidence that the retrieval of vesicle membrane may be a rate limiting factor for continued high frequency transmission at room temperature (von Gersdorff and Borst 2002).

This fast component of endocytosis at PT could reflect the preferential activation of a modulatory pathway, or an increase in the rate of clathrin-mediated endocytosis, or the addition of a distinct temperature-sensitive mechanism not seen at RT. Alternatively, an additional, faster component of endocytosis may be activated at PT simply due to increased enzymatic activity of GTPases involved in membrane fission, and/or increased ease of vesicle budding from the cell membrane due to increased fluidity of the lipid membrane.

Increased intraterminal Ca\(^{2+}\), either due to prolonged depolarization or ionophore application, has been shown to facilitate endocytosis at RT (Teng and Wilkinson 2005; Wu et al. 2005), and could represent a pathway that may be activated at lower threshold at PT. Though Ca\(^{2+}\) influx is significantly increased at PT, especially for short pulses (Kushmerick et al. 2006), we show here that the temperature-dependent effect seems to be independent of Ca\(^{2+}\) influx or depolarization length (Figures 3 and 8A). In addition, we note that the rate of Ca\(^{2+}\) extrusion is dramatically increased at PT (Helmchen et al. 1997; Kimura et
Thus, we propose that the increased endocytosis rate at PT is not due to activation of an additional Ca$_{2+}$-dependent endocytotic pathway.

Studies at the vertebrate NMJ support the hypothesis that the increase in endocytotic rate at PT is due to a speeding of the clathrin-mediated mode of retrieval (Teng and Wilkinson 2000). These studies show that the uncoating of clathrin from retrieved vesicles following endocytosis is strongly temperature-dependent, and may be a rate-limiting step for up to 50% of endocytosis following prolonged stimulation (Teng and Wilkinson 2000). The activity of clathrin-mediated endocytotic proteins (e.g. auxilin, synaptojanin, endophilin) may thus be rate limiting for endocytosis at RT (Jung and Haucke 2007). We thus suggest that an increased activity of proteins that catalyze endocytosis, specifically the fission reaction measured here using C$_m$ measurements, may be responsible for the faster endocytosis rates observed at PT.

The fast endocytosis we observe at PT could alternatively represent rapid non-clathrin mediated endocytosis (e.g. ‘kiss and run’ exo-endocytosis a la Ceccarelli et al. 1973). Indeed, the existence of this fast form of exo-endocytosis has recently been shown to occur for a small subset (about 5%) of single vesicle events at the calyx of Held at RT (He et al. 2006). It is therefore possible that PT increases the contribution of fast clathrin-independent endocytosis to a detectable level using whole-cell capacitance measurements.

**Endocytotic rates in more mature nerve terminals**

We observed a constant endocytosis rate over a wide range of depolarizing pulse durations in calyces from older animals at RT (Figure 7 and 8B). This differs greatly from what has been observed in younger animals (Figure 7B; Sun et al. 2002; Yamashita et al. 2005). Moreover, the time constant we observed ($\tau \approx 15$ s) is nearly identical to that recently reported for hippocampal synapses using optical imaging methods (Granseth et al. 2006). It is possible that the “threshold” for a Ca$_{2+}$-dependent acceleration of endocytosis is lower in older animals (Gad et al. 1998), or more closely coupled to Ca$_{2+}$ influx, analogous to developmental changes seen for excitation-secretion coupling at the calyx (Fedchyshyn and Wang 2005). Interestingly, dynamin expression levels increase during postnatal synaptic maturation (Cnops et al. 2007). Development also induces the expression of a calcineurin-dependent form of endocytosis in isolated cortical synapses, 2-4 weeks after birth (Smillie et al. 2005). A similar effect could be present at the calyx of Held, though we found no effect of the calcineurin inhibitor cypermethrin (200 nM to 2 μM) on endocytosis in P16 terminals (data not shown). We also note that intraterminal Ca$_{2+}$ is extruded faster from older calyces (Chuhma and Ohmori 2001). So [Ca$_{2+}$], changes may be more tightly regulated in older calyces (Felmy and Schneggenburger 2004).

If endocytosis is no longer saturating in older animals, this may indicate that endocytosis hotspots increase supralinearly to the number of active zones during maturation. A 30 ms depolarization resulted in a capacitance jump of 510 fF in
older animals at RT (Figure 6B), which on average represents the exocytosis of about 8000 synaptic vesicles (SV). There are only ~600 active zones (AZs) in the P14 rat calyx (Taschenberger et al. 2002), but the retrieval rate in older terminals varies from 77 to 534 SV/sec, showing no apparent saturation, even for a depolarizing pulse that probably depletes the releasable pool of synaptic vesicles.

Endocytotic proteins, and presumably sites for vesicle retrieval, are localized near - but separate from – active zones within other large presynaptic terminals (Fergestad and Broadie 2001; Roos and Kelly 1999; Teng et al. 1999; Koh et al., 2007). While the number of AZs in the calyx remains similar between the two ages studied here (500 AZs at P9; Satzler et al. 2002; Taschenberger et al., 2002), apparently there is substantial refinement in the mechanisms underlying synaptic vesicle retrieval. If we assume that the capacitance of individual SVs and the retrieval rate per site remain constant during development, then an apparent increase in endocytotic capacity predicts a dramatic increase in endocytotic hot spots in older animals, 36-fold between P7-P10 and P14-P18 (from 0.2 SV/sec/AZ to 7.2 SV/sec/AZ; see Methods). Interestingly, the rate of endocytosis we report for mature calyces at RT is roughly similar to that seen at the mossy fiber bouton of rats at a similar age, around 4 SV/sec/AZ (Hallermann et al. 2003). Concomitant increases in the number of endocytotic sites, and thus the capacity for endocytosis, while reducing the probability for synaptic vesicle release at AZs (Taschenberger et al. 2002), would increase the capacity of this more mature synapse for sustained high frequency activity.

In conclusion, we find that akin to other aspects of the synaptic vesicle cycle, the rate of endocytosis at the calyx of Held changes dramatically at physiological temperature and over the course of postnatal development. Our findings suggest that the calyx acquires a substantial capacity for fast and complete membrane retrieval following copious exocytosis after the onset of hearing. This likely adds further to its ability to sustain high-frequency transmission for prolonged periods, as suggested by in vivo recordings from more mature animals (Guinan and Li 1990) and by in vitro recordings following a period of extended synaptic rest or inactivity (Hermann et al. 2007).

Acknowledgements
The authors thank Dr. Christopher Kushmerick (Universidade Federal de Minas Gerais, Belo Horizonte, Brasil) for helpful comments and discussions. R.R. was supported partly by a NRSA-NIDCD Fellowship (DCD-06768) and H.v.G. was supported by grants from the Human Frontier Science Program and National Institute of Deafness and Communication Disorders (NIDCD).
References:


Johnson SL, Marcotti W, Kros CJ. Increase in efficiency and reduction in Ca\textsuperscript{2+} dependence of exocytosis during development of mouse inner hair cells. J Physiol (Lond) 563:177-191, 2005.


**Figure Legends:**

**Figure 1:** Exocytosis and endocytosis are variable between trials.
Sample recording of Ca2+ currents and resultant changes in Cm from a P10 mouse calyceal terminal at RT.

A. Ca2+ currents recorded from a calyx of Held presynaptic terminal show minimal rundown over a 20 min period after break-in. Depolarizations were 10 ms in duration to 0 mV.

B. Exocytosis (ΔCm jumps) and endocytosis (Cm decay) show significant inter-trial variability, and endocytosis tends to run down after 15 minutes. Shown are single Cm sweeps of the same calyceal terminal as in A (color-matched to ICa). Endocytosis from this terminal is initially reliable, but tends to run down after 10-15 min following break-in. Resting Cm also showed an activity-independent drift, and was 16.5 pF and 14 pF for this cell at the first and last sweep shown, respectively. The average trace (dotted black line) is for the first 5 sweeps (from 0 to 12 min after break-in).

**Figure 2:** A fast component of endocytosis appears at PT.

A. Membrane capacitance of the calyx of Held terminal was monitored before and after a 2 ms depolarization to 0 mV. Depolarization at RT (black symbols) increased membrane capacitance by 110 fF, followed by a monoexponential return to baseline. When raised to PT, this same calyceal terminal responded to a 2 ms depolarization with a much larger capacitance jump (270 fF, red symbols), and endocytosis was fit by a double exponential composed of a fast and slow component. Average resting Cm for the cell shown was 18.6 pF at RT (7 sweeps) and 18.8 pF at PT (4 sweeps); average resting Rs was 1.4 MΩ at RT and 2 MΩ at PT; and average resting Rm was 9.3 GΩ at RT and 1.5 GΩ at PT. Ca2+ currents from the step depolarizations are shown in the inset.
B. In young terminals at RT, examples of endocytosis following exocytosis are shown due to either an short pulse (1 ms to +10 ms) or a 20 ms depolarization to 0 mV. Endocytosis was relatively slow and well fit by a single or double exponential (red lines). Ca$^{2+}$ currents are shown in the inset. Capacitance increased by 32 fF for the 1 ms depolarization and 396 fF for the 20 ms depolarization in these examples. Resting C$_m$ was 18.5 pF for both terminals.

C. At PT, a kinetically distinct fast component was added to endocytosis, for both short and long depolarizations. The C$_m$ decay was fit by a double exponential. The time constants ($\tau$) of the fit and the relative contribution of the fast component are shown. Ca$^{2+}$ currents are shown in the inset. Capacitance increased 162 fF for a 1 ms pulse and 293 fF due to a 20 ms depolarizing pulse in these terminals. Resting C$_m$ was 31.5 pF and 15.6 pF, respectively.

Figure 3: Endocytosis rate at RT and PT with similar Ca$^{2+}$ influx.
A. A step depolarization (2 ms to 0 mV) in P7-P10 terminals at RT was matched at PT by reducing depolarization step to -17 to -23 mV, resulting in similar average Ca$^{2+}$ influx (integral of the Ca$^{2+}$ current: 1.9 ± 0.2 pC at RT; 2.1 ± 0.2 pC at PT; average currents from 6 terminals at RT, and 9 terminals at PT; P = 0.6, Student’s t-test).

B. Average capacitance measurements due to matched Ca$^{2+}$ currents shown in panel A result in similar exocytosis (45.5 ± 5.8 fF at RT, and 71.5 ± 13.0 fF at PT; P = 0.16, Student’s t-test), but endocytosis was significantly accelerated almost twofold at PT. Average R$_m$ was 1.8 ± 2.7 GΩ at RT and 1.7 ± 0.3 GΩ at PT. Average R$_s$ was 17.2 ± 2.5 MΩ at RT and 15.3 ± 2.1 MΩ at PT. Values from each calyx terminal were the average of at least 3 individual sweeps (6 terminals at RT and 9 terminals at PT).

Figure 4: Endocytosis and exocytosis coupling at RT and PT.
Summary data for membrane capacitance decay (endocytosis) in P7-P10 calyces at RT (black) and PT (red) at a variety of depolarizing pulse durations.

A. PT dramatically increases exocytosis, especially for shorter duration pulses (1-5 ms) relative to RT. The rate of endocytosis speeds up at PT and becomes better estimated by a double-exponential function, with a significant fraction occurring in the first 10 sec following depolarization. Time scale is shared for all three panels in A.

B. Rate of endocytosis is proportional to exocytosis in immature calyces for pulses of 1 ms to 10 ms. Mean endocytosis rate (weighted time constant $\tau_m$; see Methods) was plotted against mean capacitance jump due to depolarizing pulses of 1-30 ms duration. For 1-10 ms pulses at RT or PT (closed symbols), a line fits well the data (slope = 164.1 ± 46.8 ms/fF for RT; slope = 110.6 ± 35.9 ms/fF for PT). For step depolarizations >10 ms, the mean endocytosis rate at RT accelerated to rates similar to that seen at PT (open symbols). At RT, N = 25 calyx terminals for 1 ms, N = 21 for 2 ms, N = 7 for 5 ms, N = 5 for 10 ms, N = 21 for 20 ms, and N = 6 for 30 ms depolarizations. At PT, N = 6 terminals for 1 and 2 ms, and N = 5 for 5, 10, 20, and 30 ms depolarizations.
Figure 5: Monoexponential time course for endocytosis in calyx terminals at RT during development.
A. Examples of immature terminals that show a rate of endocytosis that was fit well by a single exponential (red line). An 1 ms depolarization resulted in a capacitance increase of 45 fF (left). Longer depolarization (20 ms, right) gave rise to a much larger capacitance jump (365 fF) but similar capacitance decay. Resting $C_m$ for the cells shown are 19.4 pF (left) and 17.4 pF (right).
B. Examples of more mature terminals (P14-P16), where a 1 ms depolarization resulted in a capacitance jump (55 fF; left panel), that decayed with the time course of a single exponential. Longer depolarization (20 ms) induced larger exocytosis (360 fF), but similar rates of endocytosis, which were fit by a monoexponential function. Resting $C_m$ for the cells shown here are 18 pF (left) and 12.9 pF (right).

Figure 6: Endocytosis in more mature terminals speeds up at PT
Sample traces from P14-P16 terminals recorded at RT or PT.
A. Short 2 ms depolarizations resulted in a capacitance jump of 114 fF at RT (black traces; P14 animal, resting $C_m = 14.1$ pF). At PT (red traces), a 2 ms depolarization resulted in a jump of 202 fF (P15 animal, resting $C_m = 18.4$ pF). Endocytosis was fit by a monoexponential function both at RT and PT. The inset shows the respective Ca$^{2+}$ currents.
B. Longer 30 ms depolarization resulted in jumps of 294 fF at RT (P16 animal, resting $C_m = 11.1$ pF), and 446 fF at PT (P14 animal, resting $C_m = 19.9$ pF). Endocytosis was fit by a monoexponential function at RT, but required a biexponential fit function at PT. The weighted time constant ($\tau_m$) for endocytosis due to a 30 ms jump at PT was 10.1 sec (see Methods). The inset shows the respective Ca$^{2+}$ currents. The Ca$^{2+}$ current inactivation was fit by a monoexponential function at RT ($\tau = 15$ ms), and a biexponential function at PT ($\tau_{fast} = 2.2$ ms, carrying 59% of the decay; $\tau_{slow} = 18.6$ ms; and $\tau_m = 8.9$ ms).

Figure 7: Synaptic maturation increases the capacity for endocytosis.
Compared to immature calyces, endocytosis is faster in more mature calyces for a given depolarizing pulse, even though $\Delta C_m$ jumps are substantially larger for more mature terminals.
A. Summary capacitance decay data for 1-30 ms depolarizations are shown for immature terminals at RT (P7-P10; black traces), for mature terminals at RT (P14-P18; blue traces), and mature terminals at PT (P14-P18; red traces). N = 4-25 calyx terminals for each depolarization length.
B. In mature calyces, the mean endocytosis rate versus exocytosis at RT was fit by a line with a slope that was not significantly different from zero ($3.8 \pm 10.1$ ms/fF) for step depolarizations of 1-30 ms in duration. N = 5 terminals for 1 and 2 ms, N= 6 for 5 ms, N = 7 for 10 ms, N = 6 for 20 ms, and N = 5 for 30 ms depolarization at RT. For mature terminals at PT, endocytosis was faster than at RT for both short and longer depolarizations (N = 6 for 2 ms, N = 4 for 30 ms depolarization). Data for P7-P10 terminals at RT is the same as in Figure 4.
Figure 8: Endocytosis: Dependence on Ca influx, temperature, and age.
A. Plotting the mean endocytosis rate against calcium influx illustrates the Ca$^{2+}$-dependence of endocytosis in younger animals. Endocytosis rate was slowest at around 10 ms depolarization at RT (black symbols). At PT (red symbols), endocytosis was similarly slowed at 10 ms depolarization, though $Q_{\text{Ca}}$ (integral of $I_{\text{Ca}}$) was larger and endocytosis rate was faster. Lines are Gaussian fits to the data used to guide the eye. For similar $Q_{\text{Ca}}$, endocytosis at PT was significantly faster than at RT, as indicated by the asterisks.

B. Endocytosis rate showed no significant dependence on Ca$^{2+}$ influx in mature terminals (blue open symbols), and was faster at all depolarizations examined (1-30 ms pulses). Endocytosis in older animals was significantly faster than for immature terminals at 5 and 10 ms, though $Q_{\text{Ca}}$ was similar at these depolarizing pulse durations.

Supplemental Data:

Supplemental Figure 1: Passive properties of mature calyces and $C_m$ measurements.
A. Passive membrane properties due to a 10 mV hyperpolarization were well fit by a biexponential function in a majority of terminals in older mice (P14-P18). Left panel shows stimulation protocol (-10 mV hyperpolarization step from holding potential), resulting current transient from a P16 calyx (black line) with biexponential fit (red line). Left panel shows the same sample cell with a monoexponential fit. Note the increased amplitude of residual values (difference of observed value and fit function). Scale bars are shared between left and right panels.

B. Similar jumps in $C_m$ were seen for more mature calyceal terminals for command sine waves between 500 Hz and 2 kHz. Shown is a sample cell from a P17 mouse calyx at RT. Capacitance jumps were evoked by a 2 ms step depolarization to 0 mV and $C_m$ was calculated from sine waves with frequencies at 500 Hz (black traces), 1 kHz (red traces), and 2 kHz (blue traces). $C_m$ was monitored continuously before and after the depolarization. No differences were seen in the amplitude of exocytosis ($\Delta C_m$ jumps) due to changes in lock-in frequency in this age range (N=3 calyx terminals).

Supplemental Figure 2: $\Delta C_m$ jumps in the mouse calyx of Held
Size of exocytic response is plotted against Ca$^{2+}$ charge for a variety of step depolarizations (1-30 ms) in immature (P7-P10) terminals at RT (black squares) and PT (red squares), as well as mature (P14-P18) mouse calyces (open blue circles). Error bars represent SEM. Results agree with previous reports, showing that exocytosis is relatively efficient for short depolarizations, but tends to saturate for pulses longer than 10 ms in immature terminals (P7-P10). Saturation of the releasable pool of vesicles is greatly reduced in terminals from older (P14-P18) animals.
A. Exocytosis as a function of depolarizing pulse duration in younger and older terminals, both at RT and PT.

B. Individual data points of depolarization-evoked exocytosis as a function of Ca$^{2+}$ influx over two orders of magnitude in all the conditions studied.

C. Global summary of exocytosis data, revealing that exocytosis tends to saturate above ~10 pC of $Q_{Ca}$ in younger animals (P7-P10), both at RT and PT. In older mouse terminals (P14-P18) exocytosis does not saturate.
Figure 1

200 pA
5 ms

A

20:00
12:45
10:45
7:30
5:00
2:30

\( I_{\text{Ca}} \)

B

\[ \tau = 42 \text{ sec} \]

---

98x103mm (600 x 600 DPI)
Figure 2

106x179mm (600 x 600 DPI)
A

-80 mV

0 mV

-23 mV

RT $Q_{Ca} = 1.9$ pC

PT $Q_{Ca} = 2.08$ pC

2 ms

500 pA

B

○ RT $\tau = 6.5$ sec (N=6)

+ PT $\tau = 3.8$ sec (N=8)

Cm

Rm

Rs

50 fF

0.5 GΩ

2 MΩ

10 sec

Figure 3
Figure 4
Figure 5
106x87mm (600 x 600 DPI)
Figure 6

100x231mm (600 x 600 DPI)
Figure 8