The size of release quanta at a mammalian ribbon synapse

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Introduction: We study the properties of the synapse in inner hair cells (IHC). It conveys auditory information from the cochlea to the brain (Fig 1A). It is required to faithfully transmit the timing and amplitude of auditory input. The hair cell synapse is special due to the synaptic body („ribbon“) that tethers vesicles above the active zone and due to the fact that information is coded by graded potentials, rather than action potentials. Jitter in the excitatory postsynaptic current (EPSC) amplitude could originate from a variable number of vesicles released. Recently it was suggested that this source of variability could be reduced at the IHC synapse due to release of large pre-fused vesicles („compound fusion“). To elucidate the interdependence of the release of vesicles we set out to estimate the apparent physical size of the released vesicles i.e. the capacitance of the release quanta.

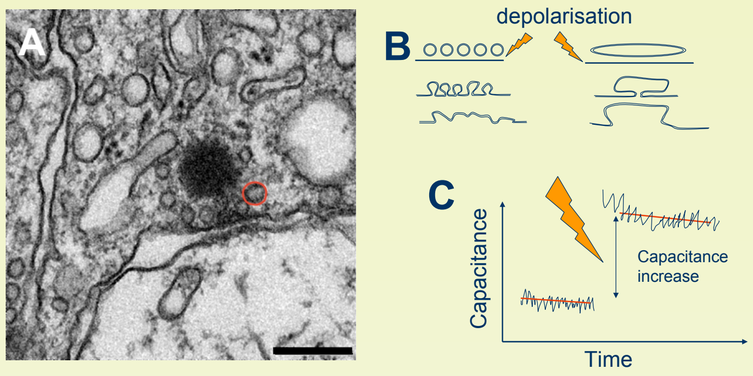


Figure 1: A Electron micrograph of a ribbon synapse in an IHC of a immature mouse (p8). From such pictures, vesicle diameters were measured – an example is shown in red (results see figure 4). **B** Two possible mechanisms of synchronous release **left**: individual vesicles fuse simultaneously – for instance because they are all driven from the same Ca^{2+}-signal; **right**: a prefused gigantic vesicle fuses. Both cases would lead to the same capacitance signal (sketched in **C**).

Method: Cochleae of NMRI-mice were isolated; the inner hair cells were patched in the perforated-patch configuration. With this little-invasive method the secretory rundown is minimized and stable recording conditions were achieved for more than one hour (see fig. 2 series conductance G_s, middle panel and membrane conductance G_m, lower panel). During this time, 80 to 480 sweeps (12 sec) were recorded with HEKA EPC-9 amplifier with an interstimulus-interval of 24 sec. We measured the membrane capacitance C_m using the Lindau-Neher technique (Lindau and Neher, 1988), implemented in the software lock-in module of Pulse v8.31 (sine+dc, f = 1 kHz, V_{rest} = -80 mV, $V_{peak\text{-}peak}$ = 70 mV). Each sweep contained one **stimulus** (10 or 20 msec depolarization to -15 mV) and two **“dummies”** (phantom pulses, no sine-wave, NO depolarization, V_{mem}=-80 mV) .

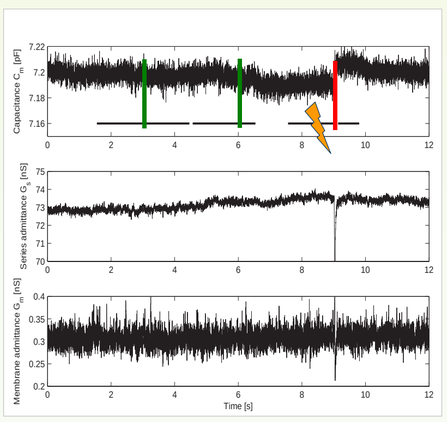


Figure 2: One sweep consists of two dummy pulses and one depolarization. Upon „blinded“ inspection reliable data stretches were selected (low noise, stable baseline: no notches etc., see horizontal bars in the in top panel). To this end each C_m sweep was split into six segments. Segments were displayed in random order but with fixed orientation to avoid subjective bias during the selection process (start: data close to stimulus/”dummy”). The step estimation was perfomed on these stretches (bars).

Fluctuation analysis: If exocytosis is assumed to be a pure Poisson point process with a constant quanta release rate λ during the stimulus interval T, then the **number n of quanta fusing** during a stimulation is distributed with mean **$\langle n \rangle = \lambda T$** and variance **$s^2(n) = \lambda T$**.

If the **size of individual fusion quanta C_q** is distributed with mean **$\langle C_q \rangle$** and variance **$s^2(C_q)$**, then the **total capacitance jump ΔC_m** (sum of n individual vesicles) is distributed with mean **$\langle C_m \rangle = \langle n \rangle \langle C_q \rangle$** and variance **$s^2(C_m) = \lambda T (s^2(C_q) + \langle C_q \rangle^2)$**. The ratio between variance and mean of the capacitance jump ΔC_m is the apparent vesicle size C_{app}.

$$\frac{s^2(\Delta C_m)}{\langle \Delta C_m \rangle} = \langle C_q \rangle \cdot \left(1 + CV_q^{\,2}\right) = C_{app} \qquad \text{Equation 1}$$

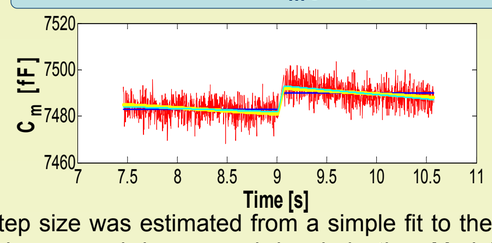
where CV_q is the coefficient of variation: $CV_q = \frac{\sqrt{s^2(C_q)}}{\langle C_q \rangle}$

Analysis flow chart

Blinded epoch selection

Exclude data stretches with excessive noise (Fig.2)

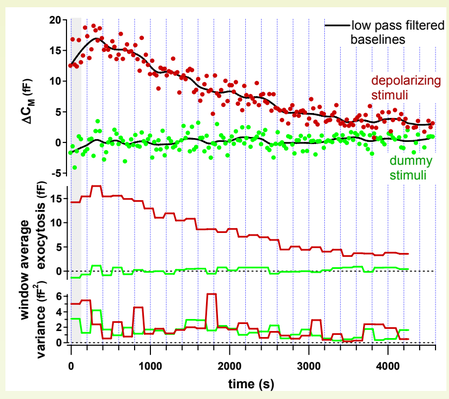
Estimate C_m jumps

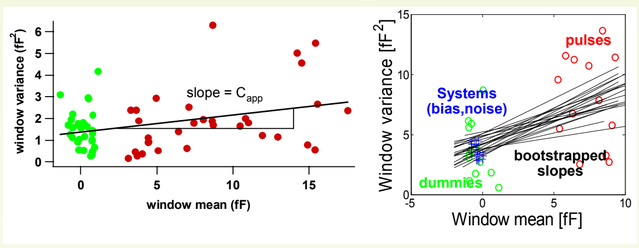


C_m step size was estimated from a simple fit to the selected stretches around dummy and depolarization. Model: overall linear trend plus jump ΔC_m at stimulus time

Non-stationary noise analysis

We approximated the temporal trend in the stimulus-evoked ΔC_m either with the sum of two exponentials or with a low pass filter (shown). For each analysis window (5 measurements wide) both, mean $\langle \Delta C_m \rangle$ and variance $s^2(\Delta C_m)$ are calculated. This is done for “depolarization” and “dummy”.





The slope $\frac{s^2(\Delta C_m^{depol}) - s^2(\Delta C_m^{dummy})}{\langle \Delta C_m^{depol} \rangle - \langle \Delta C_m^{dummy} \rangle} = C_{app}$

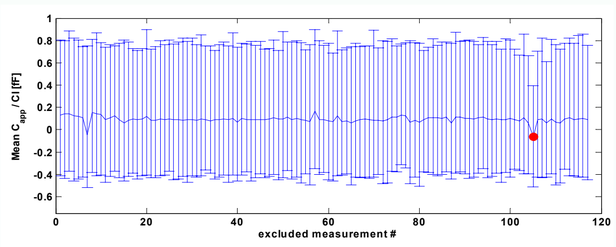
is the apparent quantal size C_{app}. Equation 2

Bootstrap with Jack- knife

The conditions for ordinary regression analysis are not fulfilled (error in x, non-normal heteroscedastic error in y). Hence a direct calculation of the slope’s confidence interval (CI) is not possible.

We relied on **bootstrap** (resampling with replacement) **and jack-knife** instead. Each slope estimate was resampled 500 times from the original set resulting in 500 values of C_{app} for each of the 5 different “reading frames”. The cumulative distribution function (CDF) of these slopes is used to reflect the confidence interval of the apparent vesicle size estimator C_{app}.

To identify outliers, bootstrap was done several times, each time excluding one of the measurements (see figure). If the exclusion, of a given measurement reduced the CI of C_{app} by more than 20%, This point was considered atypical and excluded from the analysis. In total only 1% (40 out of 3827 stimuli were Jack-knifed this way). In the example below exclusion of measurement 105 will reduce the size of the CI (indicated by bars with caps) by 25% compared to the average over all CIs obtained under inclusion of measurement 105.



Result: It has been possible to estimate the quantal size of a fusion event at the IHC synapse from whole cell capacitance noise analysis.

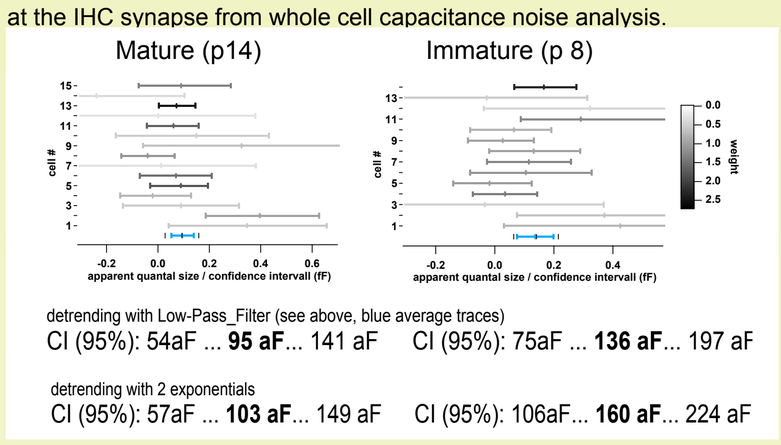


Figure 3: Results for C_{app} from 15 mature and 14 immature IHCs. Estimators for C_{app} from individual cells are given with mean and 95% CI. Not all CIs are shown completely. The line color indicates how much weight the respective cell got in the average. For the calculation of the weights see below.

To relate the estimated sizes of the release quanta to the capacitance of single vesicles at this synapse, we measured in mature and immature IHCs respectively, the size of more than 600 vesicles by electron microscopy. Independent experiments indicated, that the used fixation method does not induce shrinkage, compared to Cryo-EM.

Entering the values for C_q and CV_q obtained from the morphology into equation 1, a C_{app} of 53 aF (mature) and 53.2 aF is predicted. These values are outside the measured CI, i.e. the measured distribution of vesicle sizes cannot explain the measured C_{app}, if independent fusion of individual vesicles is assumed. This suggests that vesicles fuse in a coordinated manner. Figure 4 B shows two distributions which could explain the data.

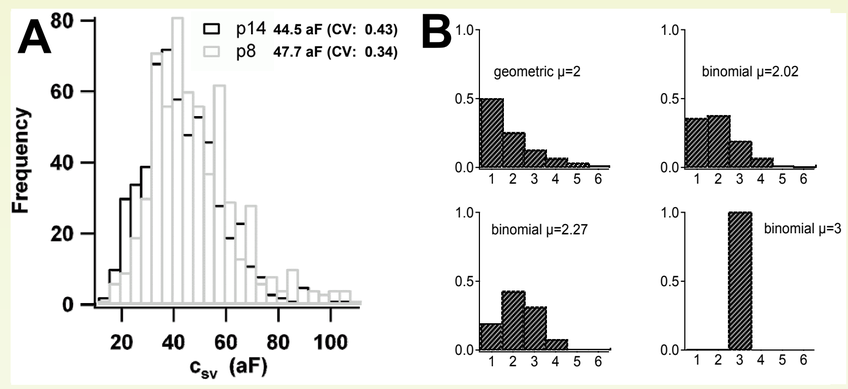


Figure 4: A Using size measurements from EM and assuming a specific membrane capacitance of 1μF/cm² we obtained average vesicle capacitances of 44.5 aF and 47.8 aF for mature and immature IHCs respectively .**B** Different distributions of “coordination numbers” that comply to C_{app}=3*$\langle C_{ves} \rangle$ - the result for immature IHCs

Reliability of the estimator – can we trust the result of this analysis?: To test if this complex analysis is robust under the typical noise in the C_m signal we simulated capacitance traces as closely as possible. Capacitance step sizes were generated using a point process to simulate the stochasticity in the number of fusing vesicles and different distributions (geometric, constant size) for the vesicle sizes. To simulate random fluctuations we combined Gaussian noise with a slow drift process similar to an Ornstein-Uhlenbeck process. The parameters of these two components were chosen to precisely match the power spectrum of the simulated noise and the noise actually measured in our real recordings. **In all simulations the noise analysis obtained the expected C_{app}** (according to equation 1) **with little bias** (<5%) and little dependence on the simulation parameters (amount and time course of rundown, noise parameters). An additional indication for the practicality of the analysis is the fact that both methods that were used to remove the temporal trend from the stimulus-evoked ΔC_m (low-pass-filter and double exponential) lead to very similar estimates for C_{app}.

Conclusion: The statistical analysis of the whole cell data allows to estimate the apparent quantal size C_{app} of a fusion event at the ICH ribbon synapse with relatively narrow margins: immature IHCs C_{app}= 90 – 210 aF; mature IHCs C_{app}= 55 – 145 aF. Using simulated C_m traces we showed that the applied noise analysis introduces little bias and is robust.

Combining estimates of the single vesicle capacitance from EM (C_{ves}≈ 46 aF) with the measurements of C_{app}, we have strong indication for statistically correlated release of several vesicles. Especially at ribbon synapses of immature IHCs the release of quanta larger than 1 vesicle is the normal (most frequent) case.

The result could be caused by different distributions of quantal sizes, but assuming a smooth, single peak distribution, the limiting cases are basically the geometric distribution with p=0.5 and the singular distribution where all quanta have the same size of $3C_{ves}$. The larger the average quantal size is, the more rare are quanta which are larger i.e. distributions for larger average quanta are more narrow.