Supplementary Discussion

Normalization of data with poor signal–to–noise introduces severe artifacts.

Our FM data reinstate and extend an earlier study\(^1\), which has been criticized\(^2\) for restricting fractional release rate analysis to spontaneously labeled boutons with high release probability, i.e. boutons that showed a destaining response of at least two times the baseline standard deviation. To escape this criticism we therefore copied in our destaining analysis of spontaneous loading ‘blindly’ the binary mask computed for AP loading. But, while we in general acknowledge the validity of the criticism (as do the authors of the criticized study by attributing their finding of even faster fractional release rates for spontaneously loaded boutons to this selection procedure), we would like to note the following.

Determination of the fractional release rates from FM dye destaining experiments requires normalization, i.e. subtraction of background after full destaining followed by division by initial fluorescence. This, however, is only allowed, if some destaining actually did occur, i.e. a significant destaining signal is measured, e.g. larger than two times the baseline standard deviation\(^1\). Otherwise normalization of flat profiles will artificially produce traces with linear decline from 1 to 0. For boutons, where no release occurred – or even no dye uptake during spontaneous loading — this would result in a linear decay or ‘destaining’ not due to real release, but merely due to normalization. The mean fluorescence decay is then a weighted average composed of 1) real monoexponential release and 2) an artificial linear decay component (Supplementary Figure 1b). To illustrate this point we mimicked poor signal–to–noise by offsetting the binary detection mask by ten pixels before extracting the ‘destaining’ profiles for spontaneously loaded boutons (Supplementary Figure, 1a). Now the slowly and monotonically declining profile is remarkably similar to those reported recently\(^2\). By using the binary mask for AP load also ‘blindly’ for destaining analysis of spontaneous loading, we also and on purpose (see above) normalized some traces with poor signal–to–noise, where no release occurred. But in contrast to the recent study we took two important measures to minimize the occurrence of such traces and thus the shown normalization artifact: instead of FM 2–10 we used FM 1–43 with much better signal–to–noise, and we measured simultaneously instead of sequentially, thereby eliminating the problem of lateral bouton movement that often is substantial for time periods > 30 min, as required for sequential measurements\(^2\). In fact it is the small contribution of FM 1–43 traces, showing minimal or no destaining, but therefore a linear decay after normalization, that account for the slightly
slower overall release rate (Fig. 1e) – if omitted, both average destaining profiles perfectly match (data not shown). Generally, this illustrates that one has to caution against the improper and excessive use of normalization in imaging analysis which can easily produce profound artifacts and misleading results.

**Reduction of evoked EPSCs and mEPSC frequency by vATPase blockers**

In our experiments folimycin had little effect on mEPSC frequency in the absence of stimulation (Supplementary Figure 2) while in recent studies\(^2,3\) a more drastic reduction of mini frequency was reported. This apparent difference, however, can be attributed to differences in temperature during folimycin application, type and concentration of application, and type of neuronal preparation.

Our mean interval between the application of folimycin and the beginning of recording was 20 min. If the mEPSC rate per synaptic site was around 0.011 s\(^{-1}\) (miniature event every 90 s) one would expect a reduction of at 25–40 % assuming a total cycling pool of 30 – 50 vesicles\(^4\). We have measured a decrease in mEPSC rate of about 15%. Given the high variability of miniature frequencies in continental neuronal cultures we may have underestimated this decrease. While our experiments were performed at room temperature folimycin was applied previously\(^2\) for 10 min at 37°C in the incubator prior to recording at room temperature. Since miniature postsynaptic currents are known to be very temperature sensitive — a Q10 value of around 9 has been shown for cortical neurons in rat slice preparations\(^5\) — a 10 to 15 °C temperature increase should yield an over 10–fold increase in mEPSC frequency and allow folimycin to act very efficiently on the cycling pool. In fact, when applying folimycin at 37°C in the incubator for 10 min, we also observed a marked (p = 0.10) reduction of mEPSC frequency but not amplitude in response to folimycin treatment (Supplementary Figure 2), in good agreement with the previous work\(^2\).

In contrast to our finding of a small effect of folimycin on mEPSC frequency at room temperature a clear reduction already after 10 min of bafilomycin treatment has been reported\(^3\). In these experiments, however, the vATPase blocker bafilomycin was dialyzed intracellularly through the patch pipette at high concentration into autaptic hippocampal neurons. Also the exemplar autaptic neurons recorded from may have had very high release probability per bouton, which would have accelerated the folimycin action. In this same experiment\(^3\) both, EPSC amplitude and mEPSC frequency were recorded in parallel from the same cell during folimycin application; both declined with the same time course and to the
same degree over 70 minutes. Such measurement configuration is certainly less prone to errors than recording and comparing EPSC amplitude and mEPSC frequency from different cells of confluent neuronal cultures, given the notoriously large cell-to-cell variability. Also when using global field stimulation different subsets of synapses for evoked and spontaneous release will be monitored, which may increase variability.