CHAPTER 2
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Presynaptic strength is location dependent and is not affected by brief episodes of enhanced network activity

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Abstract
The strength of presynaptic terminals is very heterogeneous in the central nervous system. Even synapses formed by the same axon release different amount of vesicles during action potential firing. The rules that determine the strength of individual terminals are incompletely understood. Previous studies have shown that presynaptic strength is dependent on the postsynaptic target cell, and that neighboring synapses have similar strength. These studies, however, were restricted to a low number of synapses per experiment. In the current study, we used SypHy, a fluorescent reporter of vesicle release, to measure presynaptic strength at hundreds of terminals simultaneously. With this method, we discovered that presynaptic strength is critically dependent on the location on the dendritic tree. This location-dependent scaling was insensitive to short periods of elevated network activity. Thus, presynaptic strength is dependent on the location of the synapse in an activity independent manner.
**Introduction**

Synapses are the primary structures in the brain at which fast communication between neurons takes place. The regulation of both pre- and postsynaptic strength is essential for proper information processing in the brain, and critical for brain function [1, 2]. The strength of the presynapse can be expressed as the chance that an incoming action potential (AP) triggers the release of a vesicle (release probability, \( p_r \)) [2-4]. The \( p_r \) of synapses in the central nervous system is generally low, but varies widely, even among synapses formed by the same axon [5-8]. Multiple factors may affect presynaptic strength simultaneously, including several secretory proteins expressed in the terminal, network activity, the activation of presynaptic signal transduction pathways and cell adhesion molecules [9-12]. \( p_r \) is furthermore strongly dependent on the number of release-ready vesicles in the terminal – the readily-releasable pool (RRP) [13, 14].

Much research in the last decades has focused on the question how the strength of individual terminals is regulated. Experiments in acute brain slices have shown that the identity of the postsynaptic cell is a major element that determines presynaptic strength [8, 15, 16]. Detailed imaging studies in culture have shown that neighboring synapses have very similar strength, especially when formed on the same dendritic branch [6, 17-19]. Interestingly, Branco et al. have shown that the local activity in individual dendritic branches determines the strength of the presynaptic terminals formed on that branch [17]. They furthermore showed that 2 hours of increased network activity is sufficient to homogenize presynaptic strength, with every synapse having a similar \( p_r \) [17]. Although other studies confirmed the localized changes in \( p_r \) after regulated activity [20, 21], they disagreed on the contribution of pre- and postsynaptic activity in the process (compare [21] and [17]). Furthermore, all these studies where performed on a relatively low number of terminals per experiment. It is thus unknown whether presynaptic strength is regulated in a cell-wide fashion, along the entire dendritic tree. The postsynaptic integration of synaptic activity is highly dependent on synapse location, with distal synapses being the strongest [22-24]. Combined with the retrograde- and activity-dependent control of presynaptic efficacy [25], one could expect a similar role for setting \( p_r \) along the dendritic arbor.

Experiments in a wide variety of preparations revealed that not all presynaptic vesicles are released during intense AP stimulation [26-28]. It has been suggested that this ‘resting vesicle pool’ may contain biochemically distinct vesicles, which preferably fuse in the absence of AP firing [28-31], but this hypothesis is heavily debated [32-34]. The resting pool can be visualized in imaging studies with the vATPase inhibitor bafilomycin, which prevents vesicle re-acidification resulting in cumulative increase in sypHy fluorescence that directly correspond to the number of exocytosed vesicles. [27, 28, 35]. Using this...
technique, it was shown that spontaneous vesicle release persists after depletion of the ‘releasable’ vesicle pool with exhaustive AP trains, suggesting that spontaneous release in drawn from the resting pool [28]. However, using similar strategies, other laboratories concluded that no such distinction between vesicle pools exists [33, 34].

In the current study, we describe a method to repeatedly measure presynaptic strength in hundreds of terminals simultaneously. Using SypHy, a fluorescent reporter of vesicle release [36, 37] we were able to measure vesicle release repeatedly from the same set of 100-500 presynaptic terminals. We discovered that resting pool vesicles can be recruited for AP-evoked release, thereby questioning the existence of separate sets of vesicles for spontaneous and activity-dependent release. In addition, we found that presynaptic strength is highly organized and based on synapse location, with the strongest terminals formed at the postsynaptic cell body. Finally, we discovered that a brief (10 minute) period of regulated network activity is insufficient to alter this location-specific regulation of presynaptic strength.

Results

SypHy is a reliable marker for synaptic vesicle release

To measure synaptic vesicle release at many individual terminals simultaneously, we made use of the genetically encoded fluorescent reporter SypHy. Previous studies have demonstrated that SypHy and related constructs can be used to measure presynaptic strength at individual terminals [36-39]. We tested whether we could measure presynaptic strength in large sets of terminals simultaneously. SypHy was transfected using Ca2+ phosphate [40], which yields a high expression level in a small population of neurons (typically ~1%, data not shown), and thereby ensures that all SypHy+ synapses in the field of view are formed by the same axon (see also Experimental Procedures). Under our experimental conditions, we were able to measure changes in fluorescence from 188.2 ± 17.4 regions per field of view (mean ± standard error of the mean (SEM), n = 17 cells). We first measured vesicle release during a robust stimulation protocol of 500AP at 20Hz (Figure 2.1A), which leads to a large increase in fluorescence (ΔF) in SypHy-expressing terminals (Figure 2.1B-C). The ΔF during this AP train was used as a measure of presynaptic strength. Note that during such a stimulation protocol, some vesicles will be endocytosed, after which their fluorescence is lost [27]. The measured presynaptic strength is thus likely an underestimation of the true amount of vesicles released from the terminal. ΔF varied widely between synapses in the same field of view, likely reflecting heterogeneity in presynaptic strength (Figure 2.1C-D). Indeed, the variability in ΔF is in good agreement with previous studies on variability in p_r [6, 7, 20, 38]. Compared to these studies, we did observe a relatively large fraction of synapses with little to no change in fluorescence upon stimulation (discussed below).
The changes in fluorescence could be due to other phenomena than synaptic vesicle release (e.g. changes in the pH of endosomes). We therefore tested whether ΔF was sensitive to known modulators of presynaptic strength. Elevated levels of extracellular Ca\(^{2+}\) are well known to increase presynaptic vesicle release [41]. After measuring ΔF upon stimulation with 50 000 AP at 20 Hz in 2 mM Ca\(^{2+}\) (control), cells were superfused with solution containing 10 mM Ca\(^{2+}\), and presynaptic strength was measured again using the same stimulation protocol. 10 mM Ca\(^{2+}\) increased ΔF during the AP train in ~75% of the synapses, indicating that the observed changes are indeed due to synaptic vesicle release (Figure 2.1E-F). The average ΔF per cell was significantly larger compared to control (171.1 ± 17.4 % of control, P < 0.05, paired t-test). We furthermore tested the effect of the diacylglycerol analogue PDBu, which is known to facilitate synaptic vesicle release [42-44]. As expected, PDBu increased ΔF during the AP train (average ΔF was 145 ± 17.4 % of control, P < 0.05), again indicating that SypHy reports the fusion of synaptic vesicles (Figure 2.1E-F). Thus, SypHy is a sensitive and reliable method to measure presynaptic strength.

**Figure 2.1** SypHy is a reporter of synaptic vesicle release. A. Experimental design. A SypHy transfected cell (pre) was stimulated with a field stimulation electrode to induce APs. The cell forms synapses on neighboring, untransfected cells (post). B. Typical example of 4 SypHy expressing synapses at rest (top) and after stimulation (bottom). C. Example traces of ΔF of SypHy signals induced by AP stimulation. Individual regions are shown in grey, average of all regions in one field of view (n = 323) is shown in black. Note that individual regions differ widely in amplitude and kinetics of the fluorescent signal. D. Histogram of ΔF induced by 500 AP at 20 Hz at terminals formed by 1 axon (n = 323 regions). E. Average traces of SypHy signals induced by 500 AP at 20 Hz, in control conditions (left), in the presence of 1 μM PDBu (middle), or in the presence of 10 mM Ca\(^{2+}\) (right). F. Average ΔF induced by 500 AP in 20 Hz, in the presence of 1 μM PDBu or 10 mM Ca\(^{2+}\). All values are expressed as percent change compared to control.
Unresponsive regions are not presynaptic terminals

In SypHy imaging experiments, we observed a fraction of regions (14.6 ± 4.2 %, n = 18 cells) that did not respond to the action potential train. These regions, however, did respond to superfusion of 50 mM NH4, which increases the pH in all acidic compartments [37], indicating that these regions do contain SypHy. These unresponsive regions might resemble silent synapses [20, 45], other acidic organelles like endosomes or trafficking vesicles [27, 46]. To test whether unresponsive regions are synapses, we correlated SypHy signals with the expression of the synapse marker Bassoon. Since Bassoon is almost exclusively expressed in synapses at DIV14 [47], regions positive for Bassoon can be considered presynaptic terminals. SypHy transfected cells were stimulated twice with 900 AP at 20 Hz, which is reported to deplete the pool of releasable vesicles [36, 39], and subsequently superfused with NH4 (Figure 2.2A-B). Cells where fixed, and stained for the presynaptic active zone protein Bassoon. We then examined whether regions unresponsive to AP stimulation were positive for Bassoon. ~90% of the unresponsive regions did not colocalize with Bassoon, indicating that these regions are not presynaptic terminals (Figure 2.2A-B, n = 3). The remaining 10% likely are silent synapses or extrasynaptic vesicles. Since most unresponsive regions are not synapses, these regions are excluded from analysis for the remainder of the study.

Figure 2.2. Regions that do not respond to AP stimulation do not colocalize with Bassoon. A. Typical example of colocalization of SypHy and Bassoon puncta. Top image shows live SypHy signal in the presence of 50 mM NH4+. Arrows indicate 5 regions that were presumed to be synapses, selected based on increase in ΔF upon NH4+. Bottom images show the same region after fixation and immunostaining for Bassoon and MAP. Regions 1, 2 and 5 do not co-localize with Bassoon and hence are not synapses. Scale bar = 5 µm. B. ΔF traces of the regions in A, during stimulation of 900 AP at 20 Hz and addition of 50 mM NH4+. Images were acquired at 1 Hz with 100 ms exposure. Note that regions 1, 2 and 5 did not respond to AP stimulation, and did not express Bassoon.
Vesicles are recruited from the resting pool during recruitment

Several studies suggested that a specific pool of vesicles preferably fuses at rest, and that these vesicles are not released upon AP stimulation [28, 48]. A key observation supporting this hypothesis was, that spontaneous release still occurs after depletion of the releasable vesicle pool in the presence of bafilomycin [28]. In this study, however, it was not tested if any recovery of AP evoked release occurs after depletion of the releasable pool. We tested if such recovery occurs, which can be visualized with SypHy (Figure 2.3). After addition of 0.5 µM bafilomycin, the releasable vesicle pool was depleted with 900 AP at 20 Hz (train 1). As shown previously [27, 28], SypHy signals reach a plateau during this protocol, indicating that the releasable pool has been depleted (Figure 2.3A-B). After a waiting period of 2 minutes, another 900 AP were evoked (train 2) to see if any recovery of evoked release occurred. Surprisingly, we found that train 2 evoked the release of previously unreleased vesicles (Figure 2.3A-C). On average 39 ± 0.07 % of the total vesicle

![Figure 2.3. Vesicles are recruited from the resting pool after intense stimulation. A. Average ΔF of all regions from one neuron (n = 416 regions), stimulated twice with 900 AP at 20 Hz (train 1 and train 2) with 2-minute interval in the presence of 0.5 µM bafilomycin, and subsequently washed with 50 mM NH4. The total increase in F during this protocol is defined as the total vesicle pool. B. Example ΔF traces of individual regions subjected to the protocol in A. C. Average fraction of the total vesicle pool released during the first and second train of 900 AP at 20Hz, and the remaining, unreleased fraction (NH4). Data derived from 7 cells, N = 3.](image-url)
pool was release during the train 1, while another 8 ± 0.01% was released during train 2 (n = 7, N = 3; Figure 2.3C). Thus, after depletion of the releasable vesicle pool, resting vesicles can mature to be released upon AP stimulation.

**RRP size is spatially organized**

Recent work has shown that synapses formed on the same dendritic branch have a similar $p_r$ [17]. To test whether presynaptic strength is organized in a similar fashion on the entire dendritic tree we performed dual whole-cell recordings on SypHy transfected cultures (Figure 2.4A). We recorded from one SypHy transfected cell (referred to as the presynaptic cell), and a neighboring, untransfected cell (referred to as the postsynaptic cell), which was filled with Alexa to observe its morphology (Figure 2.4A-B). The presynaptic cell was stimulated with 40 AP at 20 Hz, a protocol that is routinely used by others to probe the RRP [14, 39, 49]. This protocol was chosen because the RRP is closely correlated with $p_r$ [14, 49], and is much shorter than the $\tau_{\text{decay}}$ of endocytosis ($\tau_{\text{decay}} \approx 14-15$ s, [36, 50]). Thus, the measured peak $\Delta F$ is entirely caused by vesicle exocytosis and endocytosis is only initiated after the end of the AP train. When we created intensity maps of $\Delta F$ of all terminals in the field of view, we found that these maps were highly organized, with localized clusters of synapses having a larger RRP (n = 12 cell pairs, N = 2; Figure 2.4C-E). When the RRP was measured repeatedly with 5 minute interval, we did not observe significant changes in the average RRP size or the spatial distribution of RRP size (Figure 2.4 C-F), illustrating that RRP size is stable over time. Thus, the amount of vesicles released during AP firing depends on the location of the synapse.

**Spatial organization of RRP size is altered by gabazine**

Electric activity of the pre- and postsynaptic cell is a well-described determinant of presynaptic strength [9, 10, 17, 21]. Branco et al. showed that increased network activity ablates the dendrite-specificity of $p_r$ [17]. We therefore hypothesized that activity might play an important role in the spatial distribution of presynaptic strength. To test this hypothesis, we altered network activity using the GABA-A receptor antagonist gabazine (GZ). We first measured the RRP twice in control solution, by stimulating the SypHy transfected cell with 40 AP at 20 Hz via the recording pipette. The sample was then superfused with 20 μM gabazine for 10 minutes, which strongly increases network activity (Figure 2.5A-B). During this period, the presynaptic cell was held in current clamp, so that the cell took part in the activity of the network. GZ-induced activity would usually manifest in bursts of APs, although continuous spiking did also occur (Figure 2.5B). After GZ treatment, the RRP was again probed at 5 minute intervals, to see the effect on the spatial distribution of RRP size. GZ differentially affected RRP size, depending on synapse location. In the example in Figure 2.5, synapses formed close to the soma of a postsynaptic cell (region 2) showed
Figure 2.4. RRP size is stable over time. A. Experimental design. A SypHy transfected cell (pre) was stimulated with 40 AP at 20 Hz to measure the RRP size. A neighboring, untransfected cell (post) was filled with Alexa to visualize the dendritic arbor. B. Typical example of an Alexa-filled neuron that receives presynaptic input from a SypHy transfected cell. The transfected cell body was not in the field of view. Scale bar = 20 µm. C. RRP size of all synapses in the field of view of the cell pair in B. Every dot represents a synapse, and the color indicates the RRP size. Background image is the Alexa signal. D. RRP size of the cell pair in B-C, after 15 minutes. E. Typical examples of ΔF in 4 individual synapses. Images were acquired at 60 Hz. F. Changes of average RRP size over time. Numbers indicate the number of observations for each data point. Data from 2 independent cultures.
Figure 2.5. GZ induces localized changes in RRP size. A. Experimental design. A SypHy positive neuron was stimulated twice with 40 AP at 20 Hz to measure the RRP size. After 10 minutes of 10 µM GZ, the RRP was measured another 3 times at 5 minute interval. During GZ, the cell was held in current clamp. B. Typical example of AP firing induced by GZ. Inset shows a zoom of one burst of APs. C. Example image of SypHy in the presence of 50 mM NH4+. Rectangles indicate regions that were analyzed in D-E. The SypHy transfected cell body is not in the field of view. Scale bar = 20 µm. D. Example traces of ΔF induced by 40 AP 20 Hz in the regions indicated in C. Images were acquired at 10 Hz with 100 ms exposure. E. Effect of GZ on RRP size for all regions of the cell in C. Every square represents a synapse, and the color indicates the RRP size. Left is RRP size before GZ, middle RRP size after GZ, and right the difference between both experiments.
an increased RRP (region 3). These differences were stable over a period of 10 minutes (Figure 2.5D). Similar results were obtained in 5 cells (N = 2), whereas in control cells, no differences were found (n = 4, N = 2, data not shown). These results suggest that network activity can differentially tune RRP size in different regions of the cell.

**Network activity does not affect presynaptic strength**

The experiments described above suggest that network activity regulates RRP size in a synapse specific manner. To test whether this affects synaptic transmission, we performed paired whole-cell recordings, and repeated the GZ protocol (Figure 2.6A-B). Control cell pairs were subjected to the same protocol, but superfused with normal extracellular solution. GZ significantly increased the average AP frequency (control: 13.9 ± 5.3 min-1, n=13; GZ: 83.4 ± 36.3 min-1, n = 14; p = 0.04, unpaired t-test), the number of AP bursts (control: 0.3 ± 0.1 min-1; GZ: 5.4 ± 1.8 min-1; p = 0.007) and the number of APs per burst (control: 4.7 ± 0.7; GZ: 9.5 ± 1.5, p = 0.005). Presynaptic efficacy was measured using a paired-pulse protocol with 50 ms interval before GZ (before), 1 minute after GZ (early) and 10 minutes after GZ (late, Figure 2.6A-D). GZ significantly affected the EPSC amplitude of evoked responses of both early and late PP trials (Figure 2.6E). However, paired pulse ratio was not affected by GZ (Figure 2.6F), suggesting that the change in EPSC amplitude was entirely due to a reduced postsynaptic response.

GZ could have unintended side effects, e.g. increased excitability of the presynapse by blocking presynaptic GABA-A receptors or off-target effects. We therefore repeated the experiment, but now induced activity in one of the cells via the recording pipette (Figure 2.6 G-I). 60 bursts of 10 AP at 10, 50 or 100 Hz were induced in the presynaptic cell (cell A). This protocol closely mimics the AP firing pattern that was induced by GZ (see above). The other cell (cell B) was kept at -70 mV in voltage clamp. This experimental design enabled us to distinguish between the effect of presynaptic activity (synapse A->B, ‘pre’) and postsynaptic activity (synapse B->A, ‘post’). As illustrated in Figure 2.6H, presynaptic activity significantly reduced the EPSC amplitude, whereas postsynaptic activity increase EPSC amplitude, most prominently at intermediate frequencies. However, both pre- and postsynaptic activity had no effect on PPR (Figure 2.6I). Thus, 10 minutes of increased activity does not affect presynaptic efficacy, or differences are too small to be detected using electrophysiological approaches.

**AP-induced activity does not affect spatial distribution of RRP size**

The results of electrophysiological experiments (Figure 2.6) seem in conflict with the results from SypHy experiments (Figure 2.5), as we did not detect any change in presynaptic efficacy after GZ- or AP-induced activity. We therefore tested the effect of increased AP firing on the spatial distribution of RRP size (Figure 2.7). We first measured RRP size, and then induced activity in the pre- or postsynaptic cell in bursts of 10, 50 or
2

Figure 2.6. 10 minute-increase in network activity does not affect presynaptic efficacy. A-C Experimental design. A. Presynaptic efficacy was measured with paired pulses of 50 ms inter-event interval, after which 10 µM GZ was superfused for 10 minutes. PP was measured immediately after (early) and 10 minutes after GZ (late). Both cells were held in voltage clamp (VC), except during GZ (current clamp, CC). B. Graphical representation of a cell pair. C. Typical example of a cell pair. D. Typical example of PP responses in control and GZ experiments. E. GZ significantly reduced EPSC amplitude compared to control. E. GZ had no effect on PP ratio (PPR). Data from 11 (control) and 12 (GZ) cell pairs, 2 independent cell cultures. G. Experimental design of AP-evoked activity in a pair of cells. Bursts of APs were evoked for 10 minutes in cell A, while cell B was held in VC. Changes in PPR in synapse A->B report the effect of presynaptic activity, whereas the PPR of synapse B->A measures the effect of postsynaptic activity. H. Presynaptic bursting activity significantly reduced EPSC size compared to postsynaptic activity. I. Presynaptic nor postsynaptic activity had a significant effect on PPR. Data from 4-9 cell pairs for each data point, from 2 independent cell cultures. *p < 0.05, ***p < 0.001.

100 Hz for 10 minutes. RRP size was then measured again to see if any change occurred in the distribution of RRP size. In none of the measured cell pairs, increased presynaptic activity lead to obvious changes in RRP size. Neither presynaptic (Figure 2.7 A-D) nor postsynaptic activity (Figure 2.7 E-H) had an overall effect on the spatial distribution of RRP size. Changes in individual synapses did occur, but no coordinated changes in a population of synapses (as induced before by GZ, Figure 2.5) were observed. Thus, brief periods of AP firing do not affect the location specificity of RRP size.
Discussion
The strength of presynaptic terminals varies widely at many types of synapses in the brain
[4, 7, 8, 51, 52], which is thought to have an important role in computational processing of
information [1]. Previous studies have shown that neighboring synapses have similar $p_r$
[6, 17, 18], which depends on the postsynaptic target [8, 15, 16], but how $p_r$ is regulated
on the entire dendritic tree has not been explored in detail so far. In the current study,
we described an imaging method using SypHy, which allows the reliably measurement
of presynaptic strength of large population of synapses at a high spatial and temporal
resolution (Figure 2.1 and 2). With this technique, we observed that presynaptic strength
is highly dependent on the location of the synapse (Figure 2.4 and 5). This location
specificity is stable over time, and insensitive to short episodes of elevated neuronal
activity (Figure 2.7). Finally, we found that vesicles from the resting pool can be recruited
for AP evoked release (Figure 2.3). In conclusion, SypHy imaging proves to be a powerful
technique to repeatedly measure presynaptic strength in a large set of synapses,
investigate location-specific regulation of presynaptic strength and measure recruitment
of unreleased vesicles.

SypHy is a reliable reporter of presynaptic strength
Previous studies on (location specific) rules for presynaptic strength made use of
electrophysiological [4, 53] or FM dye methods [6, 17]. Although these methods proved
powerful in describing heterogeneity in $p_r$ and the relationship between neighboring
synapses, they were limited to a relatively small number of terminals (typically 5-30).
Furthermore, with FM dye it is virtually impossible to measure $p_r$ at the same synapse
repeatedly, since for every new measurement the sample has to be reloaded with FM dye
[54]. To measure presynaptic strength at a large population of synapses, we employed
the genetically encoded construct SypHy, which was shown by others to reliably report
multiple rounds synaptic vesicle fusion at individual boutons [36-38]. We found that
SypHy responses can be potentiated by PDBu and high extracellular Ca2+ (Figure 2.1E-F),
two treatments known to increase synaptic vesicle release [41, 42, 45], and that responsive
regions colocalize with the presynaptic marker Bassoon (Figure 2.2). The amplitude of
the responses showed a wide heterogeneity (Figure 2.1C-D), much like heterogeneity in
presynaptic strength reported by others [6, 7, 20]. However, compared to other studies,
we observed a relatively large fraction of SypHy positive regions that showed little to no
response to AP trains (Figure 2.1D). Virtually all unresponsive regions did not colocalize
with Bassoon, suggesting that these regions are not presynaptic terminals, and were
not included in the analysis (Figure 2.2). The relatively large population of unresponsive
NH4 positive regions under our experimental conditions is likely due to the method used
for selecting these regions for analysis. Regions of interest were selected based on the
response to 50 mM NH4+ [36, 37, 39], and is thus independent of vesicle release. Previous
**Figure 2.7.** Pre- or postsynaptic AP burst firing does not affect RRP size. A-D. Typical example of the effect of presynaptic burst firing. A. Live image of SypHy in the presence of 50 mM NH4+, and Alexa. * indicates the location of another, unrecorded cell body. Scale bar = 20 µm. B. Typical example of ∆F in individual synapses before and after presynaptic burst firing. C. RRP size of all synapses in the field of view in B before burst firing. Every dot represents a synapse, and the color indicates RRP size. D. RRP of all the synapses in C, after burst firing. E-H. Effect of postsynaptic burst firing. E. Live image of SypHy in the presence of 50 mM NH4+, and Alexa. * indicates the location of the SypHy transfected cell body. Scale bar = 20 µm. B. Typical example of ∆F in individual synapses before and after postsynaptic burst firing. Images were acquired at 60 Hz. C. RRP size of all synapses in the field of view in B before burst firing. Every dot represents a synapse, and the color indicates RRP size. D. RRP of all the synapses in C, after burst firing.
studies selected synapses based on vesicle release measured by electrophysiology [4, 7] or FM dye loading [6, 17, 20], which makes it more difficult to detect very weak or silent synapses. Taken together, SypHy is a reliable method to repeatedly measure presynaptic strength in a large population of synapses, and especially allows for analysis of very weak synapses.

Vesicles from the resting pool can be recruited for AP-evoked fusion
It has been suggested that presynaptic terminals contain several pools of biochemically distinct vesicles, each responsible for separate modes of fusion [28, 29, 31, 48]. A key observation supporting this hypothesis is that spontaneous release still occurs after depletion of the releasable vesicle pool, suggesting that spontaneous release is driven by the resting pool [28]. In contrast, we found that after intense stimulation, the releasable vesicle pool can be replenished by previously unreleased (e.g. ‘resting’) vesicles (Figure 2.3). It is therefore not unlikely that the observed spontaneous release after pool depletion is also mediated by vesicles that are newly recruited for AP-evoked release. Indeed, studies using various optical techniques concluded that spontaneous and AP-evoked vesicle release is driven by the same pool of vesicles [32-34]. Our results corroborate these conclusions. It is possible that the apparent separation of vesicles in ‘resting’ and ‘releasable’ is due to different stages in vesicle maturation. During intense stimulation, the rate of vesicle fusion might exceed the rate at which the molecular machinery can prime vesicles for release, and therefore, the remaining vesicles appear to be ‘resting’.

RRP size is dependent on synapse location in an activity insensitive manner
Our results demonstrate that the size of the RRP is determined by synapse location (Figure 2.4). Previous studies demonstrated that heterogeneity in $p_r$ could be reduced by 2 hours of enhanced network activity [17, 21]. These studies furthermore demonstrated that local synaptic activity is essential for (location-specific) regulation of presynaptic strength [17, 21]. They did, however, disagree on whether pre- or postsynaptic activity is instructive for inducing these changes [17, 21]. Our initial experiments with GZ suggested that 10 minutes of activity is sufficient to affect the location specificity of RRP size (Figure 2.5). However, in subsequent electrophysiological and imaging experiments we did not find any effect on presynaptic strength during such short periods of elevated activity. Although GZ and AP bursts strongly decreased EPSC amplitude, PPR was not affected by both protocols (Figure 2.6). The observed decreased EPSC amplitude is most likely due to desensitization or endocytosis of AMPA receptors, possibly part of a homeostatic response to elevated activity [10]. Thus, the spatial distribution of RRP size seems to be insensitive to brief elevation of pre- or postsynaptic activity, and the effect of GZ reported in Figure 2.5 is likely due to aspecific effects of the compound, or incomplete washout of
the drug. Alternatively, the differences induced by GZ could be too small to be detected using electrophysiology, or could be cancelled out by facilitation and depression of different populations of synapses. However, we did not observe any effect of pre- or postsynaptic AP burst firing on the location specificity of RRP size in SypHy experiments (Figure 2.7). 10 minutes of increased network activity is thus not sufficient to induce localized changes in RRP size.

The burst firing protocol used here did induce a potent reduction in postsynaptic response (Figure 2.6). Remarkably, previous studies observed a change in both pre- and postsynaptic response after altered network activity, and these effects were contributed to homeostatic mechanisms [10, 17, 21]. Although our data does not seem to fit with these observations, it is not unlikely that these are two independent mechanisms. A homeostatic response requires prolonged alterations in network activity, and is thought to involve the synthesis of retrograde signaling molecules [21, 55]. The 10 minute burst protocol used here might be too short to induce such changes. However, burst protocols can under some circumstances be used to induce (postsynaptic) long-term depression (LTD) [56-58]. Thus, the potent change in postsynaptic response with unaltered presynaptic strength might be due to induction of LTD, and not by homeostatic mechanisms. To which extent these forms of plasticity are expressed by independent molecular pathways is not fully understood.

An important open question is how location-specific regulation of synaptic strength affects information processing by the neuronal network. The dendritic integration of synaptic activity has been shown to be critically dependent on synapse location [24]. In CA1 pyramidal cells, postsynaptic strength increases at more distal synapses, due to a higher abundance of AMPA receptors [23, 53] and surpralinear summation of EPSPs [22, 59]. In view of the bidirectional coupling of pre- and postsynaptic strength [15, 17, 25], it is well possible that presynaptic strength is tuned in accordance to the local postsynaptic strength, to optimize the computational performance of the neuronal circuit.
References