Vesicle Pool Heterogeneity at Hippocampal Glutamate and GABA Synapses

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Glutamate and GABA are the major fast excitatory and inhibitory neurotransmitters, respectively, in the CNS. Although glutamate and GABA have clearly distinct postsynaptic actions, we are just beginning to appreciate that presynaptic differences between glutamatergic and GABAergic neurons may contribute to distinct functions of these transmitter systems. We therefore probed possible differences between the functional synaptic vesicle populations of glutamatergic and GABAergic neurons. We examined superecliptic synaptophluorin (SpH) fluorescence during 20 Hz electrical stimulation in transfected hippocampal neurons and identified the phenotype of SpH-fluorescent synapses with *post hoc* immunostaining. With 200 stimuli (10 s), individual glutamate synapses displayed considerably more variability in peak SpH fluorescence than GABA synapses, without a strong difference in the mean SpH fluorescence increase. This spatial heterogeneity could not be accounted for by differences in endocytosis, which was nearly constant over these short time periods across glutamate and GABA synapses. Instead, variability in vesicle exocytosis correlated with variability in total vesicle staining and in measures of the total recycling pool size. Differences were also evident using FM1-43 [*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide] uptake. These data support the idea that the population of glutamate synapses exhibits more heterogeneity in release properties than the population of GABA synapses, possibly correlated with glutamatergic synaptic malleability.

Key words: synaptophluorin; exocytosis; endocytosis; recycling pool; synaptic strength; transfection

Introduction

At a simplistic level, glutamate and GABA serve opposing fast excitatory and inhibitory functions in the vertebrate CNS, but more subtle differences in glutamate and GABA function likely contribute to a diversity of functions executed by these transmitter systems. Of course, differences in the ion selectivity of ligand-gated channels gated by the two transmitters are responsible for the gross differences in polarity of postsynaptic responses. An enormous amount of work has investigated kinetic and other properties of the postsynaptic channels that are responsible for more subtle sculpting of postsynaptic responses, but less is known about presynaptic differences.

The bulk of presynaptic investigation has been performed on glutamate synapses, under the general assumption that primary mechanisms driving transmitter release are similar between glu-

tamate and GABA synapses. We are beginning to realize, however, that important differences in presynaptic function differentially sculpt glutamate and GABA release. For instance, we found recently that hippocampal glutamate and GABA vesicles differ in the degree of “reluctance” to release during action potential trains (Moulder and Mennerick, 2005). Glutamate and GABA presynaptic terminals also differ in their adaptive response to strong depolarization (Moulder et al., 2004). In other examples, different isoforms of vesicle and active zone proteins govern vesicle availability at glutamate and GABA synapses (Feng et al., 2002; Varoqueaux et al., 2002; Gitler et al., 2004). The subtype(s) of Ca²⁺ channel driving release can differ between glutamate and GABA synapses, with most glutamate release controlled by a combination of P/Q- and N-type calcium channels (Luebke et al., 1993; Reid et al., 1997; Wu and Borst, 1999; Cao et al., 2004) and many GABA synapses governed by a single class of Ca²⁺ channel (Poncer et al., 1997; Brager et al., 2003). Glutamate and GABA release can also be differentially affected by neuromodulators (Yoon and Rothman, 1991; Scanziani et al., 1992; Thompson and Gahwiler, 1992). Finally, glutamate and GABA synapses tend to exhibit different forms of frequency-dependent short-term plasticity (Davies et al., 1990; Meeks and Mennerick, 2004).

Conventional electrophysiology can be problematic when examining putative differences between glutamate and GABA presynaptic function, because the readout uses postsynaptic receptors. Therefore, postsynaptic differences can easily produce

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misleading inferences about presynaptic function. For instance, optical and time-resolved capacitance methods of examining frequency-dependent depression of transmitter release often register less depression than conventional postsynaptic current readouts (Sun and Wu, 2001; Brager et al., 2003), suggesting that postsynaptic factors confound investigation of presynaptic function. Therefore, in this work, we focus on a genetically encoded optical sensor of presynaptic function, synaptopHluorin (SpH) (Miesenböck et al., 1998). We studied presynaptic differences between glutamatergic and GABAergic hippocampal neurons at the level of GABAergic and glutamatergic functional vesicle pools, which have not been compared previously. We conclude that, although mean vesicle pool size does not differ between hippocampal glutamate and GABA synapses, both the size of the recycling pool and the size of the total vesicle pool are more variable at glutamate synapses. This may promote a wider dynamic range of presynaptic output at glutamatergic synapses.

Materials and Methods

Materials. Unless otherwise indicated, reagents were purchased from Sigma (St. Louis, MO). Folimycin was obtained from EMD Biosciences (San Diego, CA), and FM1-43FX [fixable version of *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide] was from Invitrogen (Gaithersburg, MD).

Cell culture. Detailed methods for postnatal dissociated cultures have been published previously (Mennerick et al., 1995). In brief, postnatal day 1–3 rat hippocampal neurons were dissociated by papain treatment followed by mechanical dispersion and were plating onto collagen-coated #0 thickness glass coverslips at ~ 650 cells/mm² (SpH imaging) or at ~ 100 cells/mm² (electrophysiology and FM1-43FX imaging).

The subset of experiments in which patch-clamp recording was followed by FM1-43FX imaging (see Fig. 6) used coverslips prepared for microisland cultures (Mennerick et al., 1995). To prepare substrate islands, coverslips were precoated with 0.15% agarose and dried. We then microstamped the coverslips with 150- to 200- μ m-diameter circular islands of collagen adsorbed onto a polydimethylsiloxane (PDMS) stamp. The microstamp was fabricated by molding PDMS on thick photoresist patterned using photolithography and microcontact printing techniques (Quist et al., 2005; Falconnet et al., 2006). In brief, SU-8 2015 photoresist (MicroChem, Newton, MA) was dispensed on a silicon wafer and spun into a thin film using a spin coater (WS-400B-6NPP-Lite; Laurell Technologies, North Wales, PA) at 3000 rpm for 1 min to produce a film thickness of ~ 15 μ m. Prebake of photoresist by heating to 95°C for 3 min on a hotplate was followed by exposure to UV light (US-KVB30D; Computronics Corporation, Bentley, Australia) through a 5080 dpi transparency photomask. Postbake of the photoresist was performed by heating to 95°C for 3 min. The photoresist film was developed using SU-8 developer solution for 2–3 min. After that, the PDMS monomer was mixed with the curing agent (10:1) and poured on the top of the photoresist master. PDMS was cured by baking for ~ 8 h at 60–80°C in a vacuum to remove bubbles. The PDMS stamp was then peeled off the mold and exposed to air plasma (PDC-32G Basic Plasma Cleaner; Harrick Scientific, Ossining, NY) for several minutes immediately before use. Collagen solution was then poured onto the PDMS device, dried by blowing clean air, and stamped onto the agarose-coated coverslip.

Plating medium consisted of Eagle's medium (Invitrogen) supplemented with heat-inactivated horse serum (5%), fetal bovine serum (5%), 17 mM glucose, 400 μ M glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cultures were developed at 37°C in a humidified incubator with 5%CO₂/95% air. Cytosine arabinoside (6.7 μ M) was added 3–4 d after plating to inhibit cell division. At 4–5 d after plating, a half-volume medium replacement was conducted using Neurobasal medium (Invitrogen) plus B27 supplement. At 6–7 d, cells were transfected with superecliptic SpH construct (kind gift from Venkatesh Murthy, Harvard University, Cambridge, MA) using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Transfection medium included 25 μ M D-2-amino-5-phosphonovalerate (D-APV)

and 1 μ M 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX). SpH imaging was conducted 11–14 d after plating (6–8 d after transfection). Patch-clamp recording and FM1-43FX imaging were also conducted 11–14 d after plating. A subset of immunostaining experiments was performed at 6–8 and 18–20 d after plating.

Imaging and immunostaining. Field stimulation was performed using an RC-21BRFS chamber (Warner Instruments, Hamden, CT) and a model S4K stimulator (Grass-Telefactor, West Warwick, RI). Cells were stimulated with 1 ms current pulses, at 10 V/cm. D-APV at 25 μ M, 1 μ M NBQX, and 50 μ M bicuculline were present during stimulation in a bath solution containing the following (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.3 (25°C). Images during stimulation were acquired at 10 s (512 $\times$ 512 image), 1 s (256 $\times$ 256 image), or 386 ms (160 $\times$ 160 image) intervals using Z-C1 software (Nikon, Melville, NY) with a C1 laser scanning confocal microscope using an inverted Nikon TE300 or TE2000S platform.

Controls were performed for bleaching of SpH fluorescence with the three laser scanning protocols used in actual image acquisition. With all three protocols, fluorescence bleached <10% over 10 s ($n = 50$ puncta each; data not shown), and we therefore did not correct for bleaching in our analyses. In experiments that used folimycin, cultures were incubated in the presence of 67 nM folimycin for 10 min before stimulation. This time was sufficient to decrease miniature EPSC frequency by $77.3 \pm 10.1\%$ ($n = 6$; data not shown), consistent with the idea that vesicle refilling was blocked and similar to previous reports (Sara et al., 2005). Spontaneous increases in SpH fluorescence have been reported during blockade of vesicle reacidification (Sankaranarayanan and Ryan, 2001). There was no detectable spontaneous change in SpH fluorescence over 10 s ($n = 10$; data not shown), so spontaneous alkalization was not corrected for during 10 s stimulus trains. For longer stimulus trains (i.e., 2 min of stimulation) (see Fig. 5), we used previously published methods to correct for spontaneous alkalization (Sankaranarayanan and Ryan, 2001); five measurements made during the prestimulus period were used to calculate the slope of spontaneous fluorescence change, and this was extrapolated over the period of stimulation. Note that, for both short and long stimulus trains, occlusion of stimulus-evoked fluorescence change by spontaneous alkalization during the 10 min incubation in folimycin could result in a small underestimation of total exocytosis and of endocytosis.

Experiments in which SpH fluorescence increases were measured during 1 Hz stimulation were conducted in the presence of folimycin. Sixty minute washout of folimycin was conducted before stimulating the same field at 20 Hz, again in the presence of folimycin (Sankaranarayanan and Ryan, 2001). Baseline SpH fluorescence values returned to within 10% of initial baseline values before 10 Hz stimulation was begun.

In a subset of experiments, brief exposure to 50 mM NH₄Cl was used to alkalinize all vesicles within synaptic terminals. The surface fraction of SpH, as determined via Equation 7 of Sankaranarayanan et al. (2000), was 0.134 ± 0.009 for glutamate synapses and 0.126 ± 0.004 for GABA synapses, assuming an α of 20.7. Our surface fraction values are very close to the previously reported surface expression of SpH [0.12 ± 0.001 (Sankaranarayanan et al., 2000)].

After stimulation, cultures were fixed with cold 100% MeOH in the stimulation chamber. After exposure to blocking solution (10% normal goat serum/0.1% Triton X-100 in PBS), cells were simultaneously incubated with vesicular glutamate transporter 1 (vGluT-1) antibody (Ab) (1:2500; Chemicon, Temecula, CA) and vesicular GABA transporter (vGAT) Ab (1:500; Chemicon). An Alexa Fluor-555 secondary Ab (Invitrogen) was used to identify glutamatergic synapses, and an Alexa Fluor-633 secondary Ab (Invitrogen) was used to identify GABAergic synapses. Only SpH-positive synapses that conclusively labeled with vGluT-1 or vGAT were used for analysis. Immunostaining experiments performed in the absence of SpH imaging used identical antibody concentrations, but cultures were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 10 min. For developmental studies (see Fig. 8), vGluT-1 and vGAT images were taken from costains on the same coverslips to compare synapse size most appropriately.

For FM1-43FX dye uptake, solitary neurons were stimulated through whole-cell patch clamp as described to elicit an autaptic response (using

the above bath solution in the absence of postsynaptic blockers) and thereby define the cell as glutamatergic or GABAergic (Bekkers and Stevens, 1991; Mennerick et al., 1995). The whole-cell pipette was gently withdrawn to ensure cell survival, cell location was marked with a micro-scribe, and cells were subsequently challenged with a 2 min depolarization with 45 mM K^+ (equimolar substitution for Na^+ in the bath solution) in the presence of 10 μ M FM1-43FX plus 50 μ M D-APV and 1 μ M NBQX (Moulder et al., 2006). This protocol was designed to promote fusion and endocytosis of the entire vesicle recycling pool (Mozhayeva et al., 2002). Cells were washed for 10 s with bath saline containing 500 μ M Advasep-7 (CyDex, Overland Park, KS) and then with saline alone for 10 min. Cultures were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 10 min. FM1-43FX fluorescence was quantified as described previously (Moulder et al., 2006).

Acquired images were analyzed with MetaMorph software (Molecular Devices, Downingtown, PA). All SpH images from the same time series in an experiment were thresholded uniformly. Regions of interest (5×5 pixels) were centered on SpH puncta, and average fluorescence intensity values were obtained. Corresponding puncta from thresholded images of vGluT-1/vGAT counterstains were defined by freeform regions of interest, and area and intensity values were used to yield integrated intensity measurements. No SpH field from any experiment contained fewer than eight puncta. Typically, all puncta exhibiting $>10\%$ increase in fluorescence during stimulation (5 or 10 s) were included in analysis. However, in the two experiments in which >15 puncta in a field met the 10% criterion, analysis was restricted to 15 representative puncta, so that no individual field exercised undue influence on overall averages. In experiments analyzing SpH increases during 1 Hz stimulation followed by 10 Hz stimulation, some puncta did not exhibit $>10\%$ increase in fluorescence with 1 Hz stimulation but did with 10 Hz stimulation, and thus were included for analysis.

For vGluT-1/glutamate receptor 2 (GluR2) colabeling, GluR2 antibody (Chemicon) was used at 1:100. To determine whether presynaptic vGluT-1 puncta had apposed postsynaptic GluR2 puncta, defined thresholded regions from vGluT-1 images were transferred to corresponding GluR2 images. If a cluster of ≥ 10 pixels that met threshold criteria in the GluR2 image was overlapped by any of the vGluT-1 defined region, then these presynaptic sites were defined as having an apposed postsynaptic receptor cluster. Selection and analysis of puncta for vGluT-1/GluR2 experiments were performed by a rater naive to the experimental hypotheses.

Distance measurements, to address whether the full range of staining intensity of glutamate terminals was represented along short segments of axon, were made from z-stacks using the XYZ distance tool in MetaMorph. Distances along an individual axon were measured from montages of several adjacent fields in which a defined landmark was used to align images of an individual neurite segment up to 120 μ m in length. Puncta along the neurite were analyzed for integrated intensity values. Montages were made from six neurons.

Statistics. Data are presented in the text and in figures as mean $\pm$ SEM. Statistical tests used Student's *t* test for mean testing, linear regressions for coefficient of determination (r^2) analysis, and a variance ratio test (*F* test) for comparing the variances of GABA versus glutamate synapses.

Results

We imaged SpH fluorescent puncta in hippocampal neurons during 5 s field stimulation at 20 Hz. Consistent with previous studies, we found that SpH fluorescence increased over the course of stimulation at discrete puncta, presumably representing presynaptic terminals (Fig. 1). Excellent correspondence with *post hoc* immunostaining for either vGluT-1 or vGAT1 immunofluorescence (but never both) (Fig. 1C) verified SpH puncta as presynaptic elements (Fig. 1A,B). Furthermore, the immunostaining allowed us to separate SpH puncta into GABAergic and glutamatergic terminals. Figure 2, A and B, shows SpH fluorescence at glutamatergic and GABAergic terminals. In this relatively small sample, the mean stimulus-induced increase in SpH fluorescence was greater in GABAergic synapses ($\Delta F/F_0 = 0.78 \pm$

Figure 1. *Post hoc* immunolabeling identifies phenotype in SpH-positive neurons. **A**, **B**, Representative difference images of SpH fluorescence after 5 s stimulation at 20 Hz (green, left), paired with corresponding vGluT-1 (red, **A**) or vGAT (magenta, **B**) immunostains (middle). These images illustrate glutamatergic (**A**) and GABAergic (**B**) neurons expressing SpH. Scale bar, 10 μ m. **C**, Representative image of synapses from a glutamatergic (vGluT-1 stain, red), SpH-positive neuron (green; shown combined as yellow), with surrounding vGAT puncta (magenta) from nontransfected neurons. Scale bar, 5 μ m.

0.03; $n = 23$) than in glutamatergic terminals ($\Delta F/F_0 = 0.51 \pm 0.07$; $n = 27$; $p < 0.01$). Despite a smaller mean fluorescence intensity in this sample, glutamatergic terminals exhibited significantly more variability in fluorescence increases than GABA terminals, as determined with a one-tailed variance ratio test ($p < 0.001$) (Fig. 2C).

The higher variability in $\Delta F/F_0$ values did not result from higher variability in initial (F_0) values because mean and variance of F_0 values were similar between glutamate and GABA synapses (583.6 ± 40.8 arbitrary units for glutamate, 650.1 ± 40.9 for GABA terminals; $p > 0.05$, one-tailed variance ratio test). Additionally, there was no difference in the variability of autofluorescence between the cell types, derived from antibody-labeled nontransfected terminals ($p > 0.05$, one-tailed variance ratio test; $n = 50$ terminals each for glutamate and GABA). As will be shown subsequently, the difference in mean SpH fluorescence evident in Figure 2C was not apparent in all datasets (see below). However, the difference in heterogeneity between glutamate and GABA terminals was highly reproducible; it is this feature that we explore in detail.

SpH increases under basal conditions reflect the combination of exocytosis and endocytosis/vesicle reacidification (Sankaranarayanan and Ryan, 2001). Therefore, differences between glutamate and GABA synapses could reflect differences in either exocytosis or endocytosis (or both). To isolate exocytosis, we examined SpH fluorescence changes in the presence of 67 nM folimycin, an inhibitor of the vacuolar ATPase responsible for vesicle reacidification (Sara et al., 2005), during 10 s field stimulation at 20 Hz (Fig. 3). Endocytosis was isolated by digitally

Figure 2. Glutamate synapses exhibit more heterogeneity in SpH fluorescence increases than do GABA synapses. **A, B**, Five representative examples of SpH fluorescence increases at puncta induced by 20 Hz stimulation of a field containing a transfected glutamatergic neuron (**A**) and a field containing a transfected GABAergic neuron (**B**). SpH fluorescence was monitored with laser scans taken every 1 s. **C**, Histograms of peak SpH fluorescence ($\Delta F/F_0$) from glutamatergic (black) and GABAergic (gray) puncta. Bin size was 0.1 arbitrary units. $n = 27$ (glutamate) and 23 (GABA).

Figure 3. Endocytosis does not account for higher variance of SpH fluorescence at glutamatergic terminals. **A**, Representative difference image of SpH fluorescence after 10 s stimulation at 20 Hz in the presence of 67 nM folimycin. Numbers indicate puncta detailed in **B1–B3**. Scale bar, 5 μ m. **B**, SpH fluorescence of the indicated puncta from **A** during stimulation in the presence (solid lines) and absence (dashed lines) of folimycin. **C, D**, Five representative examples of SpH fluorescence increases induced by 20 Hz stimulation in the presence of folimycin in a glutamatergic (**C**) and a GABAergic (**D**) neuron. SpH fluorescence was monitored with scans performed every 386 ms.

subtracting responses in the presence of folimycin from preceding responses in the absence of folimycin (Sankaranarayanan and Ryan, 2001).

Figure 3B shows examples of individual SpH puncta (illustrated in Fig. 3A) analyzed with and without folimycin present. We found that both GABA and glutamate synapses exhibited

only limited endocytosis during these short trains and that the rates of endocytosis did not differ between synaptic types (20.1 ± 2.3 arbitrary fluorescence units/s for glutamate, $n = 75$ and 21.6 ± 3.9 arbitrary fluorescence units/s for GABA, $n = 69$; $p > 0.05$). These rates correspond to $17.3 \pm 2.0\%$ (glutamate) and $15.8 \pm 2.8\%$ (GABA) of the total fluorescence increases in the presence of folimycin over the 10 s of stimulation. Although we are confident that the impact of endocytosis was minimal in these experiments, accurate measures of endocytic rates are difficult to determine within short time periods given the rate of vesicle reacidification at these synapses [~4 s (Atluri and Ryan, 2006; Granseth et al., 2006)].

When we examined the SpH responses of glutamate versus GABA terminals in the presence of folimycin, thereby isolating a measure of exocytosis, we again observed greater glutamate fluorescence variability, similar to that observed in experiments without folimycin (Fig. 3C,D). Again, variability in initial fluorescence (F_0) was similar between glutamate and GABA terminals (589.4 ± 27.8 arbitrary units for glutamate, 640.8 ± 30.8 for GABA terminals; $p > 0.05$, one-tailed variance ratio test). Overall, folimycin-treated glutamate terminals showed a fluorescence change of $1.23 \pm 0.22 \Delta F/F_0$, and GABA terminals showed a change of $1.45 \pm 0.07 \Delta F/F_0$. Although the trend was toward a smaller mean fluorescence increase at glutamate synapses, similar to the experiment represented in Figure 2, the difference in means was not statistically significant in this dataset ($p > 0.17$). However, the greater variability of glutamate terminals was robust in this independent sample ($p < 0.01$, one-tailed variance ratio test). These results suggest that variability in the number of exocytosing vesicles explains the difference in distributions of SpH fluorescence at hippocampal glutamate and GABA synapses. Interestingly, our data failed to suggest any difference in endocytosis over short stimulus trains for GABA versus glutamate synaptic terminals.

Variability in SpH fluorescence during stimulation might be explained by dynamic changes in exocytosis at individual terminals during activity. If so, then we might expect more dynamic change in release rate during stimulation at glutamate synapses. For instance, perhaps the glutamate terminals yielding the weakest SpH fluorescence increases are terminals that rapidly deplete available vesicles and therefore exhibit a high rate of release early but a low rate near the end of a 10 s stimulus train. We examined the slope of fluorescence changes over the course of 10 s stimulation (386 ms imaging interval), normalized to the peak fluorescence increase at each synapse, to determine whether dynamic changes in vesicle release accounted for the differences between glutamate and GABA synaptic behavior. Figure 4A shows that the normalized slopes of mean fluorescence changes in the presence of 67 nM folimycin did not differ between glutamate and GABA synapses. The average slopes from fluorescence increases at individual puncta (without normalization) also did not differ between the two synapse types ($0.12 \pm 0.021 \Delta F/F_0$ per second for glutamate, $0.14 \pm 0.007 \Delta F/F_0$ per second for GABA; $p > 0.05$), although the increased variability in exocytosis is again apparent for glutamate synapses by this measure ($p < 0.005$, one-tailed variance ratio test). Furthermore, to examine whether the variance in glutamate synapses could be explained by differences in

Figure 4. Dynamic changes in exocytosis do not account for spatial heterogeneity. **A**, Average normalized exocytosis, as determined using SpH fluorescence increases in the presence of folimycin during 20 Hz stimulation in glutamate (filled symbols; $n = 75$) and GABA (open symbols; $n = 69$) synapses. Fluorescence increases were normalized to the final value at 10 s to highlight any changes in the slope approaching the final fluorescence value. Both glutamate and GABA SpH fluorescence increases were relatively linear over this time frame. The slope of the normalized mean fluorescence change in glutamate synapses (solid line) was $0.11 \pm 0.01 \Delta F/F_0$ per second; the slope in GABA synapses (dashed line) was $0.10 \pm 0.01 \Delta F/F_0$ per second. **B**, Average exocytosis of the top (filled symbols; $n = 19$) and bottom (open symbols; $n = 19$) quartiles of glutamate synapses. Again, both subsets were well described as a linear increase in fluorescence over this range, suggesting no differences in the dynamics of presynaptic function at low-fluorescence glutamate synapses versus high-fluorescence glutamate synapses. The slope of the mean fluorescence change was 0.11 for both the top (solid line) and bottom (dashed line) quartiles. **C**, **D**, Two hundred stimuli were delivered at 20 Hz (filled bars) and at 1 Hz (open bars) in the presence of 67 nM folimycin, and SpH fluorescence increases were measured at the same synapses. **C**, Peak fluorescence values at glutamate and GABA synapses after 200 stimuli. **D**, Coefficient of variation for exocytosis values at glutamate and GABA synapses after stimulation at 20 or 1 Hz. For **C** and **D**, $n = 32$ (glutamate) and 34 (GABA).

the dynamics of release within this population, we separated the quartile of glutamate synapses exhibiting the strongest SpH change from the quartile of synapses exhibiting the weakest SpH change and examined normalized slopes of fluorescence increase. We found that the normalized slopes did not differ between the bottom quartile of responders versus the top quartile of responders (Fig. 4B). Over this stimulus length, we detected no deviation from an essentially linear release rate (Fig. 4).

We also examined release under conditions designed to maximize release probability. We used 1 Hz stimulation to induce synaptic vesicle exocytosis, in which vesicle depletion is not limiting and in which release probability is only indirectly related to the recycling pool size (Waters and Smith, 2002). Under these conditions, both glutamate and GABA synapses exhibited greater variance in the amount of exocytosis (isolated using folimycin) than observed in the same synapses with 20 Hz stimulation (Fig. 4C,D), similar to observations by Waters and Smith (2002). However, glutamate synapses still showed greater variability than GABA synapses at either frequency of stimulation ($p < 0.001$ for both 1 and 20 Hz data, one-tailed variance ratio test). Therefore, although release probability will always depend to some extent on the available vesicle pool, it appears that dynamic changes in transmitter release during stimulation are unlikely to account for the high variability among glutamate synapses.

Instead, it seems that differences inherent to individual terminals likely account for the more varied behavior of glutamate terminals. SpH fluorescence increases during activity reflect recruitment of the recycling pool of vesicles, those vesicles capable of entering into the exocytosis/endocytosis pathway. In addition, synapses contain a large number of “resting” vesicles that appear not to enter into the recycling pool (Murthy and Stevens, 1999; Sudhof, 2000; Harata et al., 2001; Rizzoli and Betz, 2005). We were interested in determining whether the high variability of glutamate synapses arises from differences in the fraction of total vesicles in the recycling pool or whether variability in the total vesicle number itself might explain the differences.

In a first experiment, we used previously described stimulation protocols to examine the recycling vesicle pool explicitly, measured by SpH fluorescence. By 2 min of 20 Hz stimulation in the presence of folimycin, the fluorescence increase had clearly saturated (Fig. 5A), as expected if all functionally competent vesicles had been released and consistent with previous studies (Fernandez-Alfonso and Ryan, 2004). The degree of this maximum fluorescence increase correlated strongly with the degree of fluorescence increase observed with briefer stimulus trains for both glutamate and GABA synapses (Fig. 5B,C). When we expressed the ratio of the fluorescence difference obtained during short trains (10 s) to the fluorescence difference during long trains (2 min), we observed smaller, but still statistically significant, differences in the variability of release rate at the two synaptic types (0.47 ± 0.03 , $n = 40$ for glutamate and 0.49 ± 0.02 , $n = 32$ for GABA; $p < 0.05$, one-tailed variance ratio test). These results indicate that the factors governing the SpH fluorescence variability at glutamate synapses are evident in recycling pool size (and possibly total vesicle number), as expected from our earlier results (Fig. 4) suggesting that dynamic changes during stimulation do not participate strongly.

As an independent means of assessing recycling pool size variability in glutamatergic versus GABAergic neurons, we performed FM1-43FX labeling in solitary “microisland” neurons. Neurotransmitter phenotype in solitary neurons was first confirmed using patch-clamp recording of autaptic currents (Fig. 6A,B), then the recycling pool of vesicles was labeled with FM1-43FX (Fig. 6C,D). Although the average FM1-43FX integrated intensity was not statistically different in synapses from glutamatergic versus GABAergic neurons, glutamate synapses exhibited considerably more variability in the recycling pool size ($p < 0.005$, one-tailed variance ratio test) (Fig. 6E). Therefore, both FM1-43FX and SpH imaging techniques indicated that glutamate synapses display more spatial heterogeneity in functional vesicle pool size.

The variability in recycling pool size could reflect differences in the fraction of total vesicles resident in the recycling pool, implying similar distributions of total vesicle number at the two synaptic types, or the variability could stem from the total vesicle number itself. In the next set of studies, we examined whether the number of total vesicles at synaptic puncta exhibit more variability at glutamate synapses than at GABA synapses. We took vesicular transporter immunostaining intensity as a measure of total number of vesicles. We discovered that the same difference in distributions observed in SpH fluorescence (Figs. 2, 3) also applied to staining intensity for vesicular transporters. The main difference between the synaptic types lay in the range over which staining intensity extended. Both low and high ends of the distributions of vesicular transporter staining were extended in glutamate synapses compared with GABA synapses (Fig. 7A–C). When we compared vesicular transporter fluorescence with the

Figure 5. Recycling pool size correlates with differences in exocytosis. **A**, Average SpH fluorescence increases in the presence of folimycin during 20 Hz stimulation ($n = 20$; 10 glutamate and 10 GABA terminals). Time to reach saturation was similar for glutamate and GABA synapses. Images were acquired every 10 s. **B**, **C**, Peak exocytosis ($\Delta F/F_0$) with 10 s of 20 Hz stimulation correlates with the maximal amount of exocytosis ($\Delta F/F_0$), measured after 2 min of stimulation, in both glutamate (**B**) and GABA (**C**) synapses. For **B**, $n = 40$; $r^2 = 0.734$. For **C**, $n = 32$; $r^2 = 0.754$.

Figure 6. Recycling pool size as measured with FM1-43FX labeling is more variable in glutamatergic neurons. **A–D**, Evoked postsynaptic currents were recorded in solitary microisland cultures from glutamatergic (**A**) and GABAergic (**B**) neurons. The presynaptic stimulus has been blanked. The slower kinetics of response in **B** are diagnostic of GABA_A receptor kinetics; high Cl^- concentration in the patch pipette was used to amplify GABA current amplitude at -70 mV and yielded inward currents for both glutamate and GABA responses (Mennerick et al., 1995). Synapses from these neurons were then labeled with FM1-43FX (**C**, **D**). The field in **C** was obtained from the neuron whose excitatory postsynaptic current is shown in **A**; the field in **D** was obtained from the neuron whose postsynaptic current is shown in **B**. Scale bar, $4 \mu\text{m}$. **E**, Box plots of FM1-43FX integrated intensities from synapses in electrophysiologically identified glutamatergic or GABAergic neurons. Dotted lines indicate mean values, solid lines indicate median values, box edges represent 25–75% values, and whiskers represent 10–90% values. $n = 120$ synapses each. AU, Arbitrary units.

change in SpH fluorescence intensity measured in live cells at the same terminals, we found that the two measures correlated remarkably well in both cell types (Fig. 7A,B).

As another measure of total vesicle pool size, we returned to SpH-transfected cells and examined the fluorescence increase in response to NH_4Cl stimulation, which alkalinizes all synaptic vesicles (Sankaranarayanan et al., 2000). As expected, NH_4Cl -induced fluorescence increases were more variable at glutamate synapses than at GABA synapses ($\Delta F = 3665 \pm 146$ for glutamate synapses and 3924 ± 85 for GABA synapses; $p < 0.001$, one-tailed variance ratio test; $n = 48$ and

47 for glutamate and GABA, respectively). Furthermore, fluorescence increases induced by NH_4Cl exposure correlated with exocytosis over short time periods with 20 Hz stimulation ($r^2 = 0.842$ for glutamate and 0.739 for GABA). These results suggest that glutamate synapses differ from GABA synapses at the level of variability in total vesicle number. Because the same variability found in SpH fluorescence is also evident in vGluT-1 staining, we conclude that glutamate synapses do not necessarily differ from GABA synapses in the fraction of the total vesicles resident within the recycling pool.

We considered the possibility that the variability in vesicle number at vGluT-1-positive puncta represents the presence of “transport packets” (Ahmari et al., 2000), postsynaptically silent synapses, or other nonsynaptic clusters of glutamate vesicles. To test this idea, we colabeled synapses with vGluT-1 and with GluR2 antibodies to label presynaptic and postsynaptic elements. We defined synaptic vGluT-1 aggregations as vGluT-1-positive puncta with clearly apposed postsynaptic AMPA receptor subunit staining (Fig. 8A). The distribution of vGluT-1 staining at puncta apposed to a GluR2-positive postsynaptic element did not differ from the total population of vGluT-1 puncta (the coefficient of variation was 73.4% for GluR2-apposed puncta and 76.3% for all puncta) (Fig. 8B). This result suggests that the high variability in function and vesicle number at glutamate synapses pertains to synaptic elements.

We also considered the possibility that high variability in vesicle pool size at glutamate synapses might represent a general property of immature synapses. We examined synapses both later [days *in vitro* (DIV) 18–20] and earlier (DIV 6–8) than our previous experiments and still observed the same variability differences between glutamate and GABA synapses as under our standard DIV 11–14 conditions ($p < 0.01$ at 6–8 DIV and $p < 0.025$ at 18–20 DIV, one-tailed variance ratio test) (Fig. 8C). Although these results do not completely exclude the possibility of developmental causes of heterogeneity, the data suggest that these features of glutamate and GABA synapses persist over a considerable time window.

Is it possible that presynaptic terminals of a given vesicle-pool size cluster together, as might happen, for instance, if small terminals characterize particularly small branches of an axon? To address this question, we first analyzed vGluT-1 puncta from SpH costains. In each field, we identified the smallest vGluT-1 punctum and then measured the distance to that punctum from all other puncta in the field (Fig. 9A). We found that linear distance between puncta did not correlate with vGluT-1 integrated intensity. Because the nearest neighbor typically represents an-

other punctum along the same axon, this result is contrary to what would have been expected if smaller synapses localized together along a neurite.

We also explicitly identified vGluT-1-positive puncta that appeared to arise from a single presumed axon (identified by contiguous puncta arrangement along a single process of up to 120 μm in length). In six examples, we found that the range of puncta integrated intensities showed a very broad distribution of fluorescence intensities (Fig. 9*B,C*), even within this restricted length of up to 120 μm . The spread of puncta fluorescence over 120 μm (and even shorter lengths) (Fig. 9*B,C*) was similar to data acquired from random fields of immunostained puncta (Fig. 7*A*); the coefficient of variation was 69.3% for puncta along 100 μm neurite segments and 65.0% for the data in Figure 7*A*. In contrast, the coefficient of variation for the GABA synapses in Figure 7*B* was 30.8%. Additionally, we did not observe any correlation between vGluT-1 integrated intensity and distance along the axon. In other words, neither large nor small vGluT-1 puncta were found to cluster together, and the entire range of vesicular marker staining intensity occurred within relatively short lengths (~ 100 μm) of axon. These results are consistent with the idea that variability in synaptic glutamate pool size can occur throughout the length of individual hippocampal axons.

Discussion

Differences between glutamate and GABA signaling have long been the subject of scrutiny, given the important roles these neurotransmitters have in functional brain circuitry. Although postsynaptic glutamate and GABA receptors have traditionally been the focus of these studies, recent work has unveiled specific presynaptic differences. Our work adds to a growing list of differences in presynaptic function. We find that glutamate synapses exhibit more variability in total available vesicles and released vesicles than GABA synapses. This broader distribution of presynaptic output does not result from more variability in exocytosis dynamics during stimulation or more variability in the fraction of vesicles resident within the recycling pool. Rather, glutamate synapses exhibit more variability in the total number of vesicles from which the functional recycling pool is drawn. We speculate that the greater variability in presynaptic output from glutamate synapses may correlate with malleability of these synapses, although we acknowledge that our work does not directly demonstrate changes in glutamate vesicle pool size with time or experience. Our results also do not distinguish whether the range of

Figure 7. Exocytosis correlates with total vesicle pool size. *A, B*, Peak Sph fluorescence with 10 s of 20 Hz stimulation in the presence of folimycin correlates with vGluT-1 (*A*) or vGAT (*B*). For *A*, $r^2 = 0.889$. For *B*, $r^2 = 0.912$. *C*, Histograms of the integrated intensities of vGluT-1 (black) and vGAT (gray) puncta. Integrated intensity was calculated as the area of a punctum (defined by a freeform region of interest molded to the punctum) multiplied by the intensity of pixels exceeding threshold in this region. Bin size was 25,000 arbitrary units. For *A–C*, $n = 75$ (glutamate) and 69 (GABA).

Figure 8. Variability differences are synaptic and persist at various developmental stages. *A*, Representative image of vGluT-1 (green) and GluR2 (red) colocalization. Scale bar, 5 μm . *B*, Histograms of the integrated intensities for all vGluT-1 puncta (black) and for only the subset of vGluT-1 puncta apposed to GluR2 puncta ("synaptic"; gray). Bin size was 25,000 arbitrary units. $n = 140$ (all) and 103 (synaptic). *C*, vGluT-1 or vGAT integrated intensity values at synapses from neurons at DIV 6–8 (Young) or DIV 18–20 (Old). Dotted lines indicate mean values, solid lines indicate median values, box edges represent 25–75% values, and error bars represent 10–90% values. $n = 100$ (glutamate, 6–8 d), 108 (GABA, 6–8 d), 108 (glutamate 18–20 d), and 98 (GABA, 18–20 d).

Figure 9. Glutamate synapse size does not correlate with distance from similarly sized puncta. *A*, Linear distance to all vGluT-1-positive puncta in a 50 $\times$ 50 μm field was measured from the smallest vGluT-1-positive punctum and the integrated intensity of those puncta plotted as a function of that distance. $n = 68$; $r^2 = 0.003$. *B, C*, Montaged images of individual neurites immunostained for vGluT-1 were used to examine the relationship between integrated intensities of vGluT-1 puncta and their distance along a contiguous length of axon. *A*, Representative neurite segment (< 30 μm) contacted by a presumed single axon is shown in *B*. In *C*, integrated intensities of vGluT-1 puncta are plotted as a function of their distance along the axon. The gray symbols represent the puncta numbered in *B*. Note that, even in this relatively short (< 30 μm) segment, puncta size spans almost the entire range of measured integrated intensities. $n = 92$; $r^2 = 0.009$.

variability might be the cause or effect of greater plasticity of glutamate synapses. Nevertheless, the greater range of output at glutamate synapses at any point in time needs to be factored into our understanding of how these different synapses respond to repetitive activation.

We used a genetically encoded optical sensor of presynaptic

function, SpH (Miesenbock et al., 1998), in combination with *post hoc* immunostaining for vGluT-1 and vGAT to examine vesicle release at individual synapses. We found that 5–10 s of 20 Hz stimulation produced greater variability in peak SpH fluorescence increases at glutamate synapses than at GABA synapses (Figs. 2, 3). This did not result from differences in endocytosis, because both types of synapses exhibited similar endocytic rates over this timescale, with endocytosis having limited impact on SpH fluorescence increases. We therefore explored whether the variability in glutamate synapses could be explained by changes in the rate of exocytosis or by a greater variability in the size of the recycling vesicle pool. Not only was the normalized rate of exocytosis similar between glutamate and GABA synapses (Fig. 4A), but it was also similar between those glutamate synapses exhibiting the greatest and least amount of exocytosis (Fig. 4B). Therefore, changes in release during stimulation do not explain the variability of glutamate synapses observed in the present study. These conclusions do not preclude differences in release dynamics during early stages of release beyond the resolution of our imaging (Moulder and Mennerick, 2005), which release mainly the small subset of “immediately” releasable vesicles.

Several other important possible explanations for variability differences are unlikely based on our results. Although differences in the contribution of full versus transient fusion could explain the differences in variability, existing evidence suggests that vesicle alkalization rate is rapid and is complete even during partial fusion (Tsuboi and Rutter, 2003; Granseth et al., 2006; Vardjan et al., 2007). In addition, our evidence suggests that fluorescence intensity variability differences are evident in antibody staining of resting glutamate and GABA presynaptic terminals, in which fusion differences are not relevant. Because variability differences were evident in both antibody staining of nontransfected cells and with FM1-43FX dye labeling, we conclude that transfection of SpH itself did not cause changes that might explain our results, such as alterations in vesicle diameter. Finally, we directly excluded differences in initial SpH fluorescence and in cellular autofluorescence as sources of the additional variability at glutamate synapses.

The degree of exocytosis did, however, strongly correlate with the size of the recycling vesicle pool in both glutamate and GABA synapses (Fig. 5B,C), as well as with the size of the total vesicular pool at individual synapses, determined using immunostaining for vesicular transporters (Fig. 7A–C) and by NH_4Cl alkalization of SpH fluorescence. Note that total pool size is a relatively static property of the synapses. Because total pool size exhibited the same variability differences as activity-dependent measures of vesicle pools, our results cannot be accounted for by differences in the effectiveness of stimulation to activate the different cell types or of action potentials to recruit vesicles (Moulder and Mennerick, 2005). Rather, the differences reported here are inherent to the synapses.

Glutamate terminals with weak SpH fluorescence increases and dim vGluT-1 staining intensity appear to be functional presynaptic elements. We considered whether synapses at the lower extreme of our measured distribution might reflect transport packets (Ahmari et al., 2000) or other extrasynaptic clusters of vesicles, but synaptic vGluT-1 puncta (defined by GluR2 apposition) had a similar distribution of vGluT-1 integrated intensity levels as did all vGluT-1 puncta examined (Fig. 8). In addition, we found no correlation between vGluT-1 staining intensity and either the distance to other puncta or the distance along a contiguous 100 μm stretch of axon (Fig. 9). This suggests that glutamate synapses with disparate vesicle pool sizes can make contact with

postsynaptic partners over close distances, perhaps even with the same dendrite (Fig. 9B).

The fact that functional SpH fluorescence, FM1-43FX fluorescence, and static vGluT-1 immunostaining revealed high variability in glutamate vesicular pool sizes suggests that these results are not simply attributable to variability in protein levels. Rather, the data indicate that variability in vesicle number underlies the differences we observed. Previous work using transgenic mouse lines with SpH expression primarily restricted to glutamatergic or GABAergic neurons did not describe greater variability in the vesicle pool size at glutamate synapses (Li et al., 2005), although this difference may not have been apparent from the way in which these previous data were analyzed. It is worth noting, however, that Li and colleagues do report that glutamate synapses have a larger resting pool size [compared with the size of the readily releasable pool (RRP)] than GABA synapses and that this size is quite variable (Li et al., 2005).

Electron microscopy studies have also examined the size of glutamate synapses and the number of vesicles contained therein. Both of these values have a wide distribution in glutamate synapses reconstructed from hippocampal slices and cultured hippocampal neurons (Harris and Sultan, 1995; Schikorski and Stevens, 1997). Similar measurements, to our knowledge, have not been made for GABA synapses. Importantly, these studies found that docked vesicle number (presumably representing the RRP) was highly correlated with total vesicle number per synapse (Harris and Sultan, 1995; Schikorski and Stevens, 1997) and with presynaptic bouton size (Harris and Sultan, 1995). This is consistent with our observation that exocytosis values are correlated with the size of the total vesicle pool (Fig. 7A–C) as well as with the size of the recycling pool (Fig. 5B,C).

Ultrastructural studies have also revealed the presence of presynaptic glutamate boutons with multiple active zones (Harris and Sultan, 1995; Schikorski and Stevens, 1997). We therefore considered whether this explains the variability in glutamate synapse function and staining intensity in our experiments. We believe this is unlikely for three reasons. First, a large contribution from multiple active zone synapses should result in a higher mean SpH fluorescence at glutamatergic synapses, whereas we found that average exocytosis with 10 s of 20 Hz stimulation was actually slightly lower for glutamate synapses ($1.23 \pm 0.22 \Delta F/F_0$) than for GABA synapses ($1.45 \pm 0.07 \Delta F/F_0$). Second, we found no consistent evidence for a bimodal distribution in SpH fluorescence increases or vGluT-1 integrated intensities (Fig. 7A) that would suggest two or more populations of glutamate synapses. Last, both low and high ends of the distributions of vesicular transporter staining and SpH fluorescence increases were extended in glutamate synapses compared with GABA synapses (Fig. 7A–C).

In summary, our results suggest that spatial heterogeneity is a common feature of exocytosis at glutamate synapses and that these spatial differences correlate with the size of the recycling and total vesicle pools. This variability in the size of vesicle pools provides a dynamic range of synaptic strength at glutamate synapses not present at GABA synapses. It will be of great interest to determine what governs glutamate total vesicle pool size and whether this property itself is malleable.

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