Analysis of Multiquantal Transmitter Release From Single Cultured Cortical Neuron Terminals

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Prange, Oliver and Timothy H. Murphy. Analysis of multiquantal transmitter release from single cultured cortical neuron terminals. J. Neurophysiol. 81: 1810–1817, 1999. Application of single synapse recording methods indicates that the amplitude of postsynaptic responses of single CNS synapses can vary greatly among repeated stimuli. To determine whether this observation could be attributed to synapses releasing a variable number of transmitter quanta, we assessed the prevalence of multiquantal transmitter release in primary cultures of cortical neurons with the action potential (AP)–dependent presynaptic turnover of the styryl dye FM1–43 (Betz and Bewick 1992, 1993; Betz et al. 1996). It was assumed that if a high proportion of vesicles within a terminal were loaded with FM1–43 the amount of dye released per stimulus would be proportional to the number of quanta released and/or the probability of release at a terminal. To rule out differences in the amount of release (between terminals) caused by release probability or incomplete loading of terminals, conditions were chosen to maximize both release probability and terminal loading. Three-dimensional reconstruction of terminals was employed to ensure that bouton fluorescence was accurately measured. Analysis of the relationship between the loading of terminals and release indicated that presumed larger terminals (>FM1–43 uptake) release a greater amount of dye per stimulus than smaller terminals, suggesting multiquantal release. The distribution of release amounts across terminals was significantly skewed toward higher values, with 13–17% of synaptic terminals apparently releasing multiple quanta per AP. In conclusion, our data suggest that most synaptic terminals release a relatively constant amount of transmitter per stimulus; however, a subset of terminals releases amounts of FM1–43 that are greater than that expected from a unimodal release process.

INTRODUCTION

The action potential (AP)–dependent uptake and release of the styryl dye FM1–43 was used to evaluate the behavior of single CNS synaptic terminals (Murthy and Stevens 1998; Murthy et al. 1997, 1998; Ryan et al. 1995) and neuromuscular junction endplates (Betz and Bewick 1992; Betz et al. 1992). These studies exploited the ability of released synaptic vesicles to recycle (Heuser and Reese 1973) and be loaded with dye. Rigorous control studies by the Betz laboratory established that FM1–43 is localized to synaptic vesicles and that its turnover correlates well with more direct capacitance measurements of release (Henkel et al. 1996; Smith and Betz 1996). Use of FM1–43 in CNS studies indicates that the rate of dye release from loaded terminals is a reliable indicator of synaptic strength (Isaacs and Hille 1997; Murthy et al. 1997; Ryan et al. 1996). Furthermore, application of FM1–43 uptake and release to CNS neurons were used to provide data for statistical analyses of transmitter release (Murthy et al. 1997, 1998; Ryan et al. 1997). These studies suggest a multimodal release process in CNS neurons in which individual quantal peaks can be identified in data from FM1–43 labeling of vesicles. It was argued that these peaks reflect single-vesicle release events. One assumption made in the study by Murthy and Stevens (1998) and Murthy et al. (1997) was that CNS terminals release at most one vesicle per AP stimulus. Therefore if terminals were restricted to releasing a single vesicle apparent differences in the amount of FM1–43 turnover between terminals would reflect release probability (Murthy et al. 1997). We extended these studies and further tested these assumptions in primary cultures of cortical neurons by using conditions under which maximal loading of terminals was achieved, where release probability was high, and where analysis procedures were used to assure that bouton fluorescence was accurately measured. Our results indicate that multiquantal release occurs yet is restricted to a small but potentially significant fraction of cultured cortical neuron synapses (<20%).

METHODS

Embryonic cortical neurons and glial cells (from day 18 rat fetuses) were grown 3–4 wk in vitro on poly-D-lysine–coated glass coverslips before use in imaging experiments. Coverslips were cut into two pieces, placed into a customized perfusion chamber (volume ~500 μl), and fixed by platinum weights to prevent drifting. Continuous perfusion was supplied by a Hanks balanced saline solution (HBSS) medium containing (in mM) 137 NaCl, 5.0 KCl, 0.34 Na2HPO4 (7H2O), 10.0 Na+–Hepes buffer, 1.0 NaHCO3, and 22.0 glucose at pH 7.4 and ~315 mosm. CaCl2 and MgSO4 were altered as indicated. To stimulate activity-dependent synaptic uptake (loading) and release (unloading) of FM1–43, constant current stimulation (30 mA) was delivered via two platinum electrodes fixed on opposite sides of the perfusion chamber (distance ~8 mm). This field stimulation reliably induced AP generation in single neurons (see Fig. 1). All experiments were conducted at room temperature (~23°C).

Bouton loading was achieved by application of 1.200 stimuli at 10 Hz in the presence of 10 μM FM1–43; CaCl2 and MgSO4 were supplemented at 2.5 and 1.0 mM, respectively. We expected that this number of stimuli would result in complete loading of vesicle pools, as FM1–43 experiments (Liu and Tsien 1995; Murthy et al. 1997) and ultrastructural analysis (Harris and Sultan 1995) estimate that on average, cortical synapses contain ~500 synaptic vesicles. During AP-evoked FM1–43 loading, synaptic activity was blocked with a cocktail of glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 3 μM) and 2-amino-5-phosphono-valeric acid (APV; 60 μM). The time of FM1–43 exposure coinciding with
2,3-dione (CNQX); 60 relative position within the confocal fluorescence intensity in the focal plane was corrected based on its experiment with a computer-controlled focus motor. Each bouton’s was CaCl₂-free and contained 5 mM MgSO₄ to minimize synaptic loading, the preparation was washed for 15 min. The washing medium for completion of endocytosis (Ryan and Smith 1995). After bouton m (spaced at 0.54 APs (Fig. 3). A series of 13 consecutive confocal images consisting of either 21 paired (10-ms interpulse interval; 40 trials are overplotted. B: example of a neuron after 10-Hz field stimulation with single APs in 2.5 mM [Ca²⁺], and 1 mM [Mg²⁺]; the stimulus artifacts were removed from the trace. AP trains was ~2 min and continued for 60 s after the last AP to allow for completion of endocytosis (Ryan and Smith 1995). After bouton loading, the preparation was washed for 15 min. The washing medium was CaCl₂-free and contained 5 mM MgSO₄ to minimize synaptic FM1–43 release attributed to spontaneous APs and miniature synaptic activity. Bouton unloading was performed in a medium expected to result in maximal release probability (5 mM CaCl₂ and 1 mM MgSO₄), CNQX (3 μM) and APV (60 μM) were also supplemented to block recurrent synaptic stimulation.

Confocal imaging with a Bio-Rad MRC 600 system attached to a Zeiss upright (Axioskop) microscope with an Olympus 0.9 NA ‘60 water immersion objective was used for all experiments. Laser intensity was attenuated to 1%, and the confocal pinhole was set to 3.5 (Bio-Rad units). To improve signal-to-noise properties, the slow scan mode (without averaging) was used. Corrections in image intensity were made for field inhomogeneity (signal attenuation on edges) associated with high NA objectives by dividing data sets by a control image (carboxyfluorescein solution).

For data acquisition a field of 128 × 128 μm (400 × 400 pixels) was scanned every 3 s during baseline and periods of AP trains (see Fig. 3). Imaging during the first 45 s (baseline) was used to calculate signal-to-noise properties at each synapse and was followed by continuous 1-Hz field stimulation (21 s) to determine synaptic FM1–43 unloading responses. After this first train stimulus, a second baseline without stimulation (30 s) was established to allow reloading of the readily releasable pool of vesicles. Finally, a second 1-Hz train (21 s) consisting of either 21 paired (10-ms interpulse interval; n = 18 experiments) or unpaired stimuli (n = 4 experiments) was applied, and presynaptic FM1–43 fluorescence intensity was monitored. This acquisition was followed by a 10-Hz stimulus train (120 s) to determine the total amount of FM1–43 fluorescence that was releasable by APs (Fig. 3B). A (vertical) z series of 13 consecutive confocal images (spaced at 0.54 μm) over the area of interest was acquired for each experiment with a computer-controlled focus motor. Each bouton’s fluorescence intensity in the focal plane was corrected based on its relative position within the confocal z section (Fig. 2). Additionally, boutons contaminated by signals from stained structures above or below their focal plane were eliminated from further analysis (e.g., Fig. 2, boutons 2 and 3).

Confocal images were exported as byte arrays by removal of data headers and analyzed with custom routines written with the IDL (Research Systems, Boulder, CO) programming language. For each experiment, 300 putative synaptic boutons were analyzed, and fluorescence changes over time were averaged over ~3.7 μm² at each site. Nonreleasable FM1–43 fluorescence (defined as background fluorescence remaining after the 1,200-pulse stimulus train) was subtracted at each bouton before further analyses were performed. FM1–43 release in response to AP-inducing field stimulation was averaged over the 21-s stimulus train (7 images), and an automated response criterion was used to select responsive from nonresponsive putative boutons (Fig. 3A). To be considered for further analysis, boutons had to meet the following criteria: 1) the decrease in FM1–43 fluorescence in response to two 1-Hz trains of stimulation had to be >2.5 SD of the baseline fluorescence, and 2) the baseline variation (SD) had to be <10% of the bouton’s total releasable fluorescence. For experiments in which the effect of changes in [Ca²⁺], on paired-pulse modulation (PPM) were examined, the first selection criterion was modified (the decrease in FM1–43 fluorescence in response to the paired 1-Hz train stimulation alone had to be >2.5 SD of the baseline fluorescence). This more strict criterion was used to counteract effects of low [Ca²⁺], on the signal-to-noise properties of FM1–43 release values caused by low release probability and to more accurately measure PPM by avoiding selection of boutons exhibiting high release during both stimulus trains.

To determine the degree of PPM at each bouton, the relative FM1–43 release (normalized to baseline period 2) during the second (paired) stimulus train was divided by the relative FM1–43 release (normalized to baseline period 1) during the first (unpaired)

FIG. 1. Electric field stimulation results in reliable action potential generation. Recordings were made with whole cell, current clamp under conditions used for FM1–43 loading and unloading [2 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); 60 μM dL-APV]. A: paired action potentials (APs) were induced by 1-ms field pulses applied at 10-ms interpulse interval in 5 mM [Ca²⁺] and 1 mM [Mg²⁺]; 40 trials are overplotted. B: example of a neuron after 10-Hz field stimulation with single APs in 2.5 mM [Ca²⁺], and 1 mM [Mg²⁺]. The stimulus artifacts were removed from the trace.

FIG. 2. Analysis of synaptic FM1–43 fluorescence data. Bouton loading corrected for the relative position of the bouton within the focal plane. By using a focus motor, serial optical sections were collected, and the fluorescence z profile of each bouton was established off-line. An automated procedure was employed to correct FM1–43 fluorescence of out-of-focus boutons based on their position within the z section. For example, the intensity of bouton 1 was multiplied by its maximal fluorescence (4 μm position) divided by that observed at the focal plane. Boutons that had complex z profiles such as 2 and 3 were not analyzed as a significant amount of signal may be contained in boutons above or below the bouton of interest. On the basis of the expected size of synaptic vesicle clusters and the apparent point spread function for the microscope, we estimated that presynaptic vesicle clusters would be within an apparent 1- to 2-μm z section (measurements defined at full-width, half-maximal intensity).
stimulus train (Figs. 3, A and B). Analysis was restricted to boutons with PPM values between 0 and 10 (98% of all sites). For control experiments, both stimulus trains consisted of unpaired stimuli (Fig. 4A), and the FM1–43 release ratio was calculated accordingly (relative FM1–43 release during second/train 1).

Whole cell, current-clamp experiments (Hamill et al. 1981) were conducted with an Axon Instruments Axopatch 200B amplifier and 7 MΩ electrodes pulled from 1.5-mm glass capillaries. The patch pipettes were filled with a solution containing (in mM) 0.05–0.3 fluo-3 K⁺ salt, 122 K⁺MeSO₄, 20 NaCl, 5 Mg-ATP, 0.3 GTP, and 10 HEPES (pH 7.2); in some cases EDTA was substituted for fluo-3 as a Ca²⁺ buffer.

For statistical testing of normality, the Kolmogorov-Smirnov test was used. Nonparametric tests were applied for comparisons of medians (Mann-Whitney test). For correlation analysis, the nonparametric Spearman test was used over the Pearson test when it resulted in a better fit to a linear model. One-way analysis of variance (ANOVA) was used to confirm that data acquired in different experiments could be pooled.

**FIG. 4.** Distributions of FM1–43 release ratios at synaptic boutons during 2 consecutive trains of APs separated by 30 s (in 5 mM [Ca²⁺]o, 1 mM [Mg²⁺]o). The release ratio for each bouton was obtained by dividing the fractional FM1–43 release during stimulus train 2 by that during stimulus train 1. A: in 4 control experiments (n = 448 boutons) both stimulus trains were comprised of 21 single stimuli at 1 Hz. The distribution is positively skewed caused by 25% of the boutons showing apparent facilitation during stimulus train 2 [paired-pulse modulation (PPM) values >2]. These boutons are excluded from further analysis; 75% of all boutons (n = 1,292) do not exhibit paired-pulse facilitation in 5 mM [Ca²⁺]o (PPM values <2).
RESULTS

Activity-dependent turnover of the synaptic vesicle probe FM1–43 was used in primary cultures of cortical neurons to estimate the number of transmitter quanta released from a single terminal in response to AP stimulation. To ensure that FM1–43 bouton loading and release were accurately measured, several control experiments were performed. By using whole cell, current-clamp recordings it was established that electric field stimulation (1 ms; 30 mA) reliably resulted in single APs (n = 8 cells; data not shown). Paired field stimuli (10-ms interpulse interval; 5 mM Ca$^{2+}$, 1 mM Mg$^{2+}$) applied every 1 s for ≤50 s also reliably induced AP pairs (Fig. 1A; n = 4 cells). Thus whole cell, current-clamp records suggest that the number of field stimuli given is indicative of the number of APs produced. For studies in which field stimuli were employed, a stimulus intensity of ~50% above threshold was used to ensure that most terminals were responsive. This threshold was established three ways: direct measurement of the relationship between FM1–43 release and field voltage, current-clamp recordings, and the use of fluo-3–loaded cultures (data not shown) as described by others (Ryan and Smith 1995).

The goal of our experiments was to quantitate the amount of FM1–43 released from terminals to determine if single or multiple vesicles were released per AP. Stained boutons could potentially be out of focus and were also subject to signal contamination from neighboring boutons or other stained structures. Therefore we corrected each bouton’s fluorescence intensity based on its relative position within the vertical (z-) axis of the specimen (Fig. 2; see METHODS) and excluded boutons contaminated by signals from stained structures above or below their focal plane (e.g., Fig. 2, boutons 2 and 3). To establish whether presumed boutons generated a significant FM1–43 unloading response during field stimulation, automated software procedures were used that selected responsive boutons based on their individual signal-to-noise properties (Fig. 3A; see METHODS). FM1–43 fluorescence that could not be released during a 10-Hz field stimulation train (1,200 APs; Fig. 3B) was subtracted at each site to ensure that fluorescence values obtained accurately represent a pool of releasable synaptic vesicles (Murthy et al. 1997; Ryan and Smith 1995). Analysis of the relative release rates indicated that on average little depression occurred over the 21 stimuli applied at 1 Hz (Fig. 3C).

We assumed that the amount of FM1–43 release from a single bouton is proportional to release probability, the fraction of the vesicular pool loaded with FM1–43, and the number of quanta released with a single AP (see Eqs. 1 and 2 and DISCUSSION). Because release probability (Prel.) is dependent on [Ca$^{2+}$]o (Dodge and Rahamimoff 1967; Katz and Miledi 1968; Mintz et al. 1995), we elevated [Ca$^{2+}$]o to 5 mM to maximize Prel. and thus ensure that potential differences in FM1–43 release reflect the number of quanta released and not Prel.. To confirm a high release probability under these conditions, we used paired-pulse stimulation (Castro-Alamancos and Connors 1997; Debanne et al. 1996; Dobrunz and Stevens 1997; Stevens and Wang 1995). In all experiments (n = 18) we applied a second paired stimulus train (21 paired stimuli at 1 Hz; interpulse interval 10 ms) after the initial 1-Hz stimulus train and calculated the degree of PPM at each bouton (n = 1,772) by dividing the relative FM1–43 fluorescence change (% change) observed during the second train by that observed during the first train (Figs. 3B and 4B). To allow sufficient time for reloading the readily releasable pool of synaptic vesicles (Dobrunz and Stevens 1997; Stevens and Sullivan 1998; Stevens and Tsujimoto 1995), we established a second baseline period (30 s) between the two stimulus trains. As a control we conducted experiments (n = 4) in which both first and second stimulus trains were comprised of only single stimuli. In these control experiments, the ratios of the FM1–43 fluorescence changes during stimulus train 2 versus stimulus train 1 were distributed around a median of 0.96 (n = 448 boutons; Fig. 4A). The slightly higher mean release ratio of 1.13 ± 0.83 was attributed to only 5% of values (outside the median release ratio ± 2 SD); exclusion of these potentially spurious values resulted in a mean release ratio of 1.00 ± 0.52. This result demonstrated a constant rate of FM1–43 release at single boutons in response to identical consecutive stimulation protocols. This control is important as it establishes that the system is stable and exhibits little run-down or facilitation.

The values for PPM (1 Hz paired/1 Hz single stimuli) at single synaptic boutons were distributed around a median of 1.36 (mean 1.77 ± 1.46; n = 1,772; Fig. 4B). A PPM ratio of 2.0 would be predicted if both the first and second stimuli were successful. Comparison of the release ratios obtained with the two different stimulation paradigms indicated greater release with paired stimuli than with single stimuli (Mann-Whitney test: P < 10$^{-4}$). However, the majority of synapses (>75%) showed PPM values <2, confirming that most synapses in these cultured preparations possess a high Prel. in 5 mM [Ca$^{2+}$]o. Additional control experiments (n = 4; 1 mM [Ca$^{2+}$]o) indicated that evoked FM1–43 could be elevated by 60% (difference in median release rate; Mann-Whitney test: P < 10$^{-4}$) when increasing [Ca$^{2+}$]o from 1 to 5 mM (n = 51/691 boutons). Paralleling this increase in FM1–43 release rates, we found a significant decrease in PPM (25% difference in median PPM; Mann-Whitney test: P < 0.05) when increasing [Ca$^{2+}$]o from 1 to 5 mM. These findings are in agreement with studies that show maximal Prel. and paired-pulse depression in cortical and hippocampal synapses in 5 mM [Ca$^{2+}$]o (Castro-Alamancos and Connors 1997). To further rule out that differences in FM1–43 release amounts between synaptic boutons were caused by differences in Prel., we restricted further analyses to boutons with a high initial Prel. (PPM ratio <2; n = 1,292 boutons).

To measure the AP-induced FM1–43 release from single boutons, we averaged the change in FM1–43 fluorescence over seven images (recorded every 3 s) during 1-Hz stimulation (21 stimuli; Fig. 3, B and C). Data were pooled from 18 separate experiments that were conducted under comparable conditions (constant confocal gain and pinhole setting, laser intensity, and FM1–43 concentration). To determine that these experiments were comparable, we conducted a one-way ANOVA on both baseline variation and FM1–43 release data. This analysis demonstrated that for the 18 experiments analyzed both the stimulated FM1–43 release and the baseline noise values were drawn from the same distributions and thus could be pooled (one-way ANOVA: P < 0.05). Similarly, one-way ANOVA found that the FM1–43 release ratios ob-
tained in four control experiments were also drawn from the same distribution and thus could be pooled (P < 0.05). In the analysis of the pooled data we found that the relative FM1–43 fluorescence decrease over the 21 s of stimulation was linear (linear regression: r = −1.0; P < 10⁻⁵; average of n = 1,292 boutons; Fig. 3C), indicating that on average little depression of release occurred during this period. Within this population of synapses we found that the degree of FM1–43 unloading during the 21 APs train varied considerably (mean 5.80 ± 3.21 pixel value). Accordingly, we also found a high CV (SD/mean: 0.44) for FM1–43 unloading after subtraction of the baseline variance. Furthermore, we found that the amount of FM1–43 loading into boutons after 1,200 APs, a measure of the vesicular pool and thus synapse size (Henkel et al. 1996), exhibits a similar high degree of variability (mean 39.7 ± 18.4 pixel value; CV baseline variance subtracted: 0.46). When comparing the amount of FM1–43 loading with the amount of FM1–43 fluorescence released per AP we observed a significant positive correlation (r = 0.65; P < 10⁻⁵; n = 1,292 boutons) between these two parameters (Fig. 5A). However no significant correlation (r = −0.03; P = 0.50) was found between the amount of FM1–43 unloading during 21 APs and the degree of baseline variation measured over an identical time period (Fig. 5B). Hence factors such as FM1–43 bleaching or dye loss from nonvesicular pools contributed little to the observed positive correlation between synaptic FM1–43 loading and release.

As our data indicated that the amount of FM1–43 released per AP and the total FM1–43 loading of boutons were not constant (Fig. 5A), we analyzed the distribution of FM1–43 release amounts across boutons. This distribution demonstrated a positive skew when data sets comprising most boutons (PPM <10; skew = 2.17 pixel value; n = 1,772 boutons) or only those boutons with high P_rel (PPM <2; skew = 2.43 pixel value; n = 1,292 boutons) were examined (Fig. 6, A and B). Hence the positive skew toward higher FM1–43 release amounts was not attributed to sites with low initial P_rel (480 sites with PPM >2). Furthermore, the degree of PPM (a measure of P_rel) contributed little to the observed positive skew in FM1–43 release amounts at synaptic boutons with high P_rel (PPM <2; n = 1,292 boutons) as we find a poor correlation (r = −0.25) between the amount of FM1–43 release and the degree of PPM at single sites (Fig. 6D). In contrast, when considering the full range of PPM values (0–10; n = 1,772 boutons) we find a better correlation (r = −0.54) between PPM and FM1–43 release amounts, mostly caused by boutons with low initial release amounts (Fig. 6C).

As we observed that data sets with a broad range of PPM values (<10) could contain boutons with low initial P_rel (high PPM values), we restricted further analysis to data sets in which boutons possessed PPM values of <2 (Fig. 6B). To examine how the distribution of the FM1–43 release amounts compared with the noise within this data set (caused by bleaching, background dye loss, and instrument noise), we calculated the FM1–43 fluorescence variation at each bouton during a baseline period identical to the stimulus train duration (Fig. 6B; baseline noise as shaded histogram). The baseline noise data were not positively skewed (median: −0.07 pixel value; mean: −0.11 ± 1.93 pixel value; skew: −0.13 pixel value) and hence could not account for the positive skew observed in the of FM1–43 release amounts. Additionally, the negative skew in the baseline noise distribution was attributed to only 0.2% (3/1,292) of the values; exclusion of these values resulted in a normally distributed baseline noise population (Kolmogorov-Smirnov test vs. a Gaussian distribution: P > 0.05).

To estimate the fraction of boutons exhibiting multiquantal release, we used two different methods: determination of the number of release values that 1) cause the positive skew of the distribution and 2) are outside the median release value ±2 SD of the baseline noise. By using the first approach, we found that the skew in the distribution of evoked FM1–43 release could
be attributed to 17% (n = 221/1,292) of all boutons with the highest FM1–43 release amounts (Fig. 6B), as exclusion of these boutons from the analysis resulted in a population that was not different from the baseline noise distribution (Kolmogorov-Smirnov test vs. baseline noise: P > 0.05). By using the second approach, we found that 13% (n = 167/1,292) of the values were greater than the median release value + 2 SD of the baseline noise. With the use of a computer simulation that modeled the signal-to-noise properties of our system, we observed that the Kolmogorov-Smirnov test could reliably detect a skewed population (n = 1,292 release values) attributed to even <10% of boutons exhibiting multiquantal release. Thus during stimulation most of the boutons released FM1–43 amounts that could be described by a single gaussian peak, whereas release amounts of the remaining sites (13–17%, depending on the analysis) were outside a unimodal distribution and were apparently the result of multiquantal release.

**DISCUSSION**

We observed a significant positive correlation between loading of single boutons (with FM1–43) and the amount of FM1–43 they release in response to APs generated with field stimuli (Fig. 5A). A similar result was previously observed by Betz and Bewick (1993) at the neuromuscular junction. These authors found that despite variability in the intensity of single FM1–43 spots most sites appeared to release a relatively constant percentage of loaded FM1–43 per stimulus. Their conclusion was that larger release sites release proportionally more transmitter. Studies also indicate a similar relationship in central neurons as Isaacson and Hille (1997) and Ryan et al. (1997) reported that, whereas the percentage of FM1–43 release from loaded terminals varies little, the size of terminals varies greatly. These observations indicated that CNS synaptic terminals do not release a constant amount of transmitter per impulse. Ryan et al. (1997), with FM1–43 endocytosis in response to single APs, reported that multiquantal release may occur from hippocampal terminals. However, it is possible that the apparent differences in the number of quanta released may reflect differences in release probability among boutons (Hessler et al. 1993; Murthy and Stevens 1998; Murthy et al. 1997; Rosenmund et al. 1993) or conceivably incomplete loading of vesicle pools. Murthy et al. (1997) reported that larger terminals, those with greater FM1–43 loading, had a higher release probability. An assumption made by Murthy and Stevens (1998) and Murthy et al. (1997) and (for some experiments) by Ryan et al. (1997) was that endocytosis and exocytosis were matched so that the degree of FM1–43 loading (endocytosis) would be a measure of release probability. We extended these findings by directly measuring FM1–43 release (and not endocytosis) with conditions in which two complicating variables, vesicle pool loading and release probability, were fixed at saturating levels. Additionally, to prevent synaptic depression we used brief and low-number stimulation protocols (21 APs at 1 Hz) that result in the release of only a small fraction (on average 15%) of the dye-loaded vesicle pool. As vesicle repriming (availability of vesicles once released) was estimated to have a t1/2 of ~20–30 s (Ryan et al. 1993, 1996), we did not expect significant dilution by unlabeled vesicles during our dye unloading measurements. By using this experimental approach, we confirm the findings of Korn et al. (1993), Trussell et al. (1993), Tong and Jahr (1994), Vincent and Marty (1996), Ryan et al. (1997), and Auger et al. (1998), which indicate that multiquantal transmitter release can occur from single synaptic terminals, albeit at a relatively small proportion of terminals (~20%). Presumably multiquantal release could account for a portion of the apparent variability in the amplitude of synaptic responses that are recorded from single boutons (Forti et al. 1997; Liu and Tsien 1995; Murphy et al. 1995).

Our observation that, under conditions of high release probability, presumed larger boutons (greater FM1–43 loading) release more FM1–43 per impulse than smaller terminals suggests multiquantal release. As larger boutons possess greater release site areas and more docked vesicles (Schikorski and Stevens 1997) they provide a conceivable anatomic basis for multivesicular release. Fitting the amount of FM1–43 released...
versus bouton loading to a model where bouton volume was proportional to release site area failed to describe our data. This model would predict a curve with a slope proportional to bouton radius $^{-1}$ (bouton area/bouton volume; $\pi r^2 / 4/3 \pi r^3 = 0.75 r^{-1}$). The data were better described by a simple linear relationship between bouton loading and the amount of release. However, analysis of the correlation indicates that a relatively large fraction of the observed variance in release amounts (42%; $R^2 = 0.42$; Fig. 5A) could be accounted for by a linear relationship with bouton loading.

As synapticboutons possess a variety of parameters that control the rate of evoked vesicular release (excitability, probability of release, amount of release, stimulation induced facilitation, or depression), we used different controls to confirm that differences in the rate of FM1–43 unloading among boutons would reflect differences in the amount of vesicular release and not other parameters of synaptic variability. First, we chose stimulus parameters that result in a linear rate of FM1–43 release during a train of APs (Fig. 3C). This apparent linear rate of release was confirmed by the analysis of consecutive images of FM1–43–loaded boutons as described by Isaacson and Hille (1997). Second, we used conditions expected to result in maximal release probability at all boutons and excluded boutons from the analysis that showed the potential of further increase of release probability (Figs. 4 and 6). Additionally, factors such as AP propagation failure were unlikely to account for the variability in release amounts among terminals (Allen and Stevens 1994; Mackenzie et al. 1996).

Consistent with the idea of multiquantal release, analysis of the distribution of release amounts demonstrated a significant skew toward higher release amounts, which was in excess of the system noise. Making the assumption that the peak with the smallest amplitude reflects the fluorescence value of a single vesicle, we would expect that most terminals on average release one vesicle and that a subpopulation of boutons (<20%) releases two or more vesicles per stimulus.

$$F_{rel} = P_{rel} * n * F_{ves} * (Ves_{FM1-43} / Ves_{total})$$

(1)

$$F_{rel} = n * F_{ves}$$

(2)

Equation 1 describes the proposed relationship among the amount of FM1–43 release ($F_{rel}$) and $P_{rel}$ (release probability), $F_{ves}$ (the fluorescence of a single vesicle, a constant), the proportion of FM1–43–loaded vesicles ($Ves_{FM1-43} / Ves_{total}$), and $n$, the number of vesicles released per AP. If we assume that $P_{rel}$ approaches 1.0 (confirmed by the lack of paired-pulse facilitation) and that a high proportion of the vesicle pool is loaded so that $Ves_{FM1-43} / Ves_{total}$ approaches 1 (we use saturating loading conditions), then we can simplify Eq. 1 to Eq. 2. Equation 2 shows that under the assumptions we made the amount of FM1–43 release (measured at single boutons) is expected to be proportional to the number of quanta released ($n$) and the fluorescence of a single vesicle ($F_{ves}$, a constant).

The apparent linear relationship between release amount and the bouton size would suggest a process in which terminals can regulate the number of quanta released based on their size. However, this is not a strict relationship as additional synaptic parameters (other than terminal size) could contribute to the skew in FM1–43 unloading of boutons. Structural analysis of forebrain excitatory synapses suggests that a significant proportion, 10–20%, contains multiple release sites (reviewed by Edwards 1995). From our data it is not possible to determine whether terminals that release a larger amount of FM1–43 per impulse contain multiple release sites. Furthermore, in using mass cultures of neurons we assume that all neurons regardless of phenotype (i.e., glutamatergic or GABAergic) load a similar amount of FM1–43 into their terminals. This is most likely the case because the dye is loaded passively (Betz et al. 1996) into vesicles that possess similar sizes in different phenotypes of CNS synaptic terminals (Hamori et al. 1990). Nevertheless, regardless of the previous caveats, our data obtained by analysis of FM1–43 loading and unloading suggest that CNS terminals can release multiple quanta, adding caution to interpretation of experiments that apply quantal analysis to CNS synapses.

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