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Correlation of Non-Uniform Protein Expression With Variation in Transmitter Release Probability

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Abstract

The strength of synaptic transmission is highly variable between different synapses. The present study examined some factors that may contribute to this variation in the strength of neurotransmission in sympathetic varicosities of the mouse vas deferens. Transmitter release was measured using a focal macropatch electrode placed over pairs of visualised varicosities. By regulating the calcium concentration of the solutions inside the recording electrode and in the bath independently of each other, transmitter release was restricted to one or two surface varicosities at each recording site. Using this technique, transmitter release probability was shown to be highly variable, even between adjacent varicosities on single axon branches. Very little variation was observed in the calcium influx following single impulse nerve stimulation between adjacent Oregon Green BAPTA-1 loaded varicosities. However, the staining intensities of three vesicular proteins, SV2, synaptophysin, and synaptotagmin 1, showed considerable variation between adjacent varicosities on single axon branches. This variation in staining intensity may be partly explained by variation in the density of synaptic vesicles. However, double staining experiments using two vesicular antigens showed some varicosities staining for one vesicular antigen, but not for the second, suggesting that the expression of these release machinery proteins is regulated locally within the varicosities. The results of the present study strengthen suggestions that synaptic strength is at least in part, regulated by variation in the expression of vesicular proteins.

Keywords

synaptic strength; sympathetic; SV2; synaptophysin; synaptotagmin 1; calcium; synaptic vesicles

INTRODUCTION

The strength of synaptic transmission is highly variable between different synapses. Some synapses produce large signals that rapidly depress upon repetitive stimulation while others produce small signals that facilitate (e.g., Dittman et al., 2000; Dobrunz and Stevens, 1997; Lavidis and Bennett, 1992, 1993a; Murthy et al., 1997; Walmsley et al., 1988). Similar variation in the strength of synaptic transmission has also been observed between different branches of single neurons in the crayfish neuromuscular junction (Hoyle and Wiersma, 1958) and more recently in mammalian CNS neurons (Gupta et al., 2000; Reyes et al., 1998; Scanziani et al., 1998). In addition to these branch specific differences, the strength of

synaptic transmission has also been observed to vary between adjacent boutons of individual axon branches (Bennett et al., 1986a; Bennett et al., 1986b; Bennett and Lavidis, 1982; D'Alonzo and Grinnell, 1985; Katz et al., 1993; Lavidis and Bennett, 1992, 1993a; Murthy et al., 1997; Staple et al., 1997).

This diversity of synaptic strength is almost certainly an important factor in the formation of complex neural networks and the laying down of memory and, as such, several studies have looked for structural and biochemical explanations for the diverse range of synaptic strengths (for review, see Atwood and Karunanithi, 2002). Proposed causes of this diversity include: the number of active zones per synapse and their relative size (Cooper et al., 1995; Msghina et al., 1999), the density of synaptic vesicles (Dobrunz and Stevens, 1997; Murthy et al., 1997), the total amount of calcium entering a terminal and the distance of calcium channels from the synaptic vesicles (Brain and Bennett, 1997; Koester and Sakmann, 2000; Rozov et al., 2001), and differences in the expression of pre-synaptic proteins affecting the calcium sensitivity of transmitter release (Dawson-Scully et al., 2000; Jeromin et al., 1999; Msghina et al., 1999; Oleskevich and Walmsley, 2000; Staple et al., 1997, 2000; Verona et al., 2000). However, as yet, there is no consensus on how the strength of synaptic transmission is regulated.

The present study has looked for factors that might contribute to variation in the strength or probability of synaptic transmission in the sympathetic varicosities of the mouse vas deferens. Through the use of focal extracellular recording techniques (Brock and Cunnane, 1987), and visualisation of living nerve terminals to facilitate the recording of transmitter release from a small number of identified boutons (Lavidis and Bennett, 1992, 1993a), the non-uniformity of transmitter release probability in the sympathetic varicosities of the vas deferens has been well characterized (Lavidis and Bennett, 1992, 1993a). Furthermore, the varicosities, spaced 3 to 5 μm apart, are thought to contain only one release site per varicosity (Bennett, 1972) making this preparation ideal for drawing correlations between structure and function.

Upon stimulation, the sympathetic varicosities release both noradrenaline (NA), which induces a slow metabotropic contraction (Bolton and Large, 1986; Minneman, 1988), and ATP, which causes depolarization of the smooth muscle and a fast ionotropic contraction via P2X₁ purinoceptors (Burnstock, 1985; Knight et al., 2003; Liang et al., 2000, 2001; Sneddon and Burnstock, 1984; Sneddon and Westfall, 1984; Westfall et al., 1990). While variations in the post-synaptic specializations have been shown to affect synaptic strength in other terminals (e.g., Casanovas et al., 1999; Levenes et al., 2001; Lim et al., 1999), in the vas deferens only a small proportion of the P2X₁-purinoceptors, which are evenly distributed over the entire surface of the smooth muscle (Knight et al., 2003; Lee et al., 2000; Liang et al., 2001; Vulchanova et al., 1996), are responsible for generating the spontaneous excitatory junction currents (Liang et al., 2001). This suggests that the variation in the strength of synaptic transmission in this preparation is pre-junctional in origin.

In the present study, we have re-examined the variation in transmitter release probability, investigated the calcium influx into sympathetic varicosities following single action potentials, the expression of release machinery proteins and the density of synaptic vesicles in adjacent sympathetic varicosities. The findings of these studies are then discussed in relation to the contribution of these factors to the non-uniform probability of transmitter release observed in the sympathetic varicosities.

MATERIALS AND METHODS

Tissue collection

Six-week-old male Balb/C mice were killed by cervical fracture and both vasa deferentia were dissected free of surrounding connective tissues in accordance with animal ethics guidelines of the University of Queensland. Vasa deferentia for electrophysiology, calcium imaging, electron microscopy, and immunohistochemistry were prepared as described below.

Electrophysiology

Vasa deferentia to be used for electrophysiology were prepared as previously described (Bennett et al., 1986b; 1992; Macleod et al., 1994). Briefly, the tissue was bathed in Tyrode's solution (NaCl, 123.4 mM; KCl, 4.7 mM; NaH₂PO₄, 1.3 mM; NaHCO₃, 16.3 mM; glucose, 7.8 mM; MgCl₂, 1 mM; and CaCl₂, 0.5–2 mM) kept at 32–34°C. The reservoir was gassed with 95% O₂ and 5% CO₂ and maintained at pH 7.4. Varicosities were exposed to the fluorescent dye 3, 3 diethyloxardicarbocyanine iodide (DiOC2 (5) (1 μM) Sigma-Aldrich Australia), for 30 seconds followed by a 3-minute wash. Labelled varicosities were excited at 540 nm and viewed using a rhodamine filter set from Olympus (Australia). These DiOC2 (5) fluorescent structures have been shown to stain positively for amines (Lavidis and Bennett, 1993b).

Visualised varicosities and the position of the recording electrode relative to them were recorded on video (S-VHS) and displayed on a video monitor (National VW-5410) to aid in positioning the tip of the electrode over specific sets of varicosities. Drawing the position of the varicosities on the monitor aided in placement of the recording electrode over surface varicosities. Nerve action potentials, EJCs, and spontaneous EJCs were recorded using glass micro-electrodes with tip diameters of 5–10 μm, internally perfused with modified Tyrode's solution (containing 2 mM CaCl₂) at a rate of 0.5 ml/hour while the bathing solution contained 0.5 mM CaCl₂. Suction was applied through the recording electrode forming a slight seal between the smooth muscle and the rim of the electrode (<1 MΩ). A water manometer monitored the effectiveness of the seal.

Using this technique, EJCs were only recorded from surface varicosities directly beneath the recording electrode. All signs of EJCs were abolished when the recording electrode was placed in a position adjacent to the visualised varicosities such that no surface varicosities were visible inside the rim of the recording electrode. The recording electrode was placed over single or pairs of visualised varicosities and the position of the electrode was recorded for analytical purposes. At each recording site, 200 stimuli were delivered at a frequency of 0.1 Hz and the average EJC amplitude and the percentage of successes and failures were measured. Successes were defined as EJCs that were greater than 2 times the amplitude of the noise with a rise time > 3 ms.

Electron microscopy

The prostatic end of the vas deferens was cut transversely into segments 1–2 mm in length. Sections were fixed in 4% glutaraldehyde and 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, post fixed in 2% osmium tetroxide, and stained with 1% uranyl acetate. The tissue was dehydrated in acetone, and infiltrated with a 50/50 araldite/acetone mix and embedded in 100% araldite resin. Cured blocks were sectioned using an ultramicrotome (Leica Ultracut-R). Serial thin sections (60 nm, silver interface) were cut with a Microstar diamond knife stained with Reynolds' lead citrate stain (Reynolds, 1963) and uranyl acetate (4 g/L). Single varicosities in the longitudinal muscle layer were located with a JOEL 1010 transmission electron microscope (TEM) and micrographs were taken at 40,000×

magnification, using an accelerating voltage of 80 kV. Stereological analysis of the micrographs was performed as described in Griffiths (1993). Vesicle density was measured using stereological methods and calculated as the number of vesicles/ μm^2 . The study was confined to single, outer surface varicosities found on isolated axons since they have been shown to frequently come into close apposition with the smooth muscle (Cottee et al., 1996; Luff et al., 1995).

Immunohistochemistry

The proximal third of the vas deferens was removed and frozen in a cryosectioning-embedding medium. Longitudinal sections 15 μm thick were cut from the frozen tissue and preblocked with 1% Bovine Serum Albumin (BSA) in phosphate buffered saline (PBS: NaCl, 135 mM; Na_2HPO_4 , 10 mM; NaH_2PO_4 , 1.5 mM; pH 7.4) with 0.05% Triton-X 100. Primary antibodies against SV2 (DSHB, see Acknowledgments), synaptophysin (DAKO), and synaptotagmin 1 (Alamone Labs, Jerusalem, Israel) and secondary antibodies conjugated to fluoro-isothiocyanate (FITC) or CY-3 were diluted with 1% BSA in PBS (without Triton-X 100) and incubated overnight at 4°C. Stained sections were viewed with a Bio-Rad (Richmond, CA) MRC 600 scanning confocal microscope. Images were collected as a Z-series and compiled as a “brightest point” projection for later analysis using NIH image software (available from <http://rsb.info.nih.gov/nih-image/>). Only varicosities in the longitudinal muscle layer (usually about 10–20 μm thick) were chosen for analysis in accordance with electrophysiological experiments. A region of interest was selected for each varicosity in the proximal longitudinal muscle in one channel (e.g., FITC channel). A region of interest of the same size and geometry was then measured at the appropriate coordinates in the opposing channel such that the intensity of staining for both antigens was measured for each varicosity. The same process was repeated for varicosities in the second channel that were not found in the first channel. More than 500 varicosities from 5 mice were analysed, measuring the mean fluorescence intensities.

Calcium imaging

The cut prostatic end of a vas deferens was gently sucked into a glass pipette containing a saturated solution of Oregon Green 488 BAPTA-1 dextran with a relative molecular weight of 10 kDa (Molecular Probes Inc., Eugene, OR), in 2.5% Triton X-100. Vasa deferentia were loaded for 5 hours in the dark at room temperature to allow time for the indicator to migrate along axons, followed by a further 3 hours wash. All experiments were carried out in the presence of Krebs gassed with 95% O_2 and 5% CO_2 . The prostatic end of the vas deferens was then mounted in a 2-mL organ bath and placed on the stage of Leica TCS NT laser scanning confocal microscope whilst being continually perfused with Krebs and maintained at 35°C. The vas deferens was securely positioned with parallel platinum electrodes and stimulated using an isolated stimulator (Digitimer DS2). The pulse width was set between 0.06–0.6 ms and the applied voltage was adjusted to give a reliable supramaximal response following a single stimulus. The stimuli were electronically synchronized with the confocal microscope scans. The 488-nm wavelength of an argon-ion laser was used for excitation of fluorescence, which was filtered by a 515-nm long pass emission filter. When detecting Ca^{2+} transients, images (256×256) were captured for 28 seconds every 3 minutes (128 frames, 218 ms apart). This protocol prevents excessive photobleaching and phototoxicity. Colour changes in the look-up table (LUT) from orange to yellow to white to blue represents the increasing amount of Ca^{2+} binding to the indicator. The blue colour indicates pixel saturation and was, therefore, eliminated from data analysis. All experiments were carried out in the presence of nifedipine (10 μM), prazosin (1 μM), and α , β -methylene ATP (1 μM) to abolish or reduce the movement artefacts. Images were analysed using Scion Image (available from u.r.l. <http://www.scioncorp.com/>), using custom-written macros (kindly supplied by Keith Brain, Australia). As Oregon Green 488

BAPTA-1 is a non-ratiometric dye, values for the change in fluorescence following stimulation (ΔF Signal) were normalized against the resting values using the equation:

$$\Delta F \text{ Signal} = \left(\frac{S}{S_0} \right) - 1$$

where S is the mean fluorescence signal for a varicosity in a given frame and S_0 is the average resting fluorescence intensity from all frames of the varicosity captured prior to stimulation. Data are presented as means $\pm$ standard error of the mean (SEM; the n value referring to the number of varicosities).

RESULTS

Variation in the probability of transmitter release from sympathetic varicosities

The fluorescent dye, DiOC2 (5) was used to visualise surface sympathetic varicosities on single axons as previously described by Lavidis and Bennett (1992, 1993a). Figure 1A shows a field approximately 45 by 35 μm at the prostatic end of the vas deferens stained with DiOC2 (5). A macropatch electrode was placed over pairs of visualised surface varicosities (Fig. 1B) to record signals like those shown in Figure 1C. Recording sites were rejected if they did not meet both of the following criteria: (1) the nerve terminal impulse (prejunctional action potential, NTI) was invariably present, and (2) at least one spontaneous EJC was observed during the recording period. Despite the presence of the NTI in each sweep shown in Figure 1C (marked with an asterisk), only half of the ten stimuli evoked an excitatory junction current (EJC).

Two hundred stimuli were delivered to each recording site at a frequency of 0.1 Hz. The probability of release at each recording site was gauged by the percentage of stimuli that resulted in a success where successes were defined as EJCs having an amplitude more than 2 times the noise level with a rise time < 3 ms. If EJCs had a rise time greater than 3 ms, they were assumed to have originated from deeper varicosities rather than surface varicosities and were, therefore, ignored (unpublished observation). Examples of rejected EJCs can be seen in Figure 1C, traces 5, 6, and 7. In accordance with previous findings (Lavidis and Bennett, 1992), the probability of transmitter release varied considerably from one site to the next as reflected in the percentage of stimuli that successfully evoked an EJC at different recording sites. Figure 1D shows the percentage of stimuli that successfully produced an EJC at each of the ten recording sites shown in Figure 1B. The success rate of recording EJCs varied noticeably between the different recording sites, ranging from 0% (i.e., 100% failures, sites 5, 8, and 9) up to 50% (site 4). The success rate also varied between consecutive varicosities on single axon branches (e.g., sites 5, 6, and 7), suggesting that the factors that regulate transmitter release probability must also vary between contiguous varicosities. The transmitter release probability was examined from a total of 508 varicosities. Of these, 318 (~63%) showed an EJC success rate of less than 1% (less than one success in 100 stimuli). The remaining 37% of varicosities showed variable success rates with the highest success rate observed being 55%.

Variation in the magnitude of the calcium transients

Variation in stimulus induced calcium entry into nerve terminals may play a significant role in regulating the probability of transmitter release (Bennett and Lavidis, 1979; Bennett and Lavidis, 1989; Fernandez-Chacon et al., 2001; Heidelberger et al., 1994; Kuno and Takahashi, 1986; Macleod et al., 1994; Schneggenburger and Neher, 2000). A previous study examined the stimulus induced calcium influx into sympathetic varicosities (Brain and

Bennett, 1997), yet the extent of variation in the calcium transients between adjacent varicosities was not fully examined. In the present study, sympathetic varicosities were loaded with excess Oregon Green 488 BAPTA-1 and stimulated in the presence of 2 mM calcium. Figure 2A shows a string of surface varicosities at the prostatic end of the vas deferens at rest and following nine individual stimuli delivered to this string of varicosities at 2-minute intervals. Upon stimulation, all varicosities examined showed a transient increase in the fluorescence intensity. Panels s1 to s9 in Figure 2A show the increased fluorescence in four consecutive varicosities of a single axon after each of nine stimuli (s1–s9).

Since the concentration of dye may have varied between consecutive varicosities, the fluorescence signals were normalized against the fluorescence intensity at rest such that calcium influx was measured as a change in fluorescence (ΔF Signal; described in Materials and Methods). Only varicosities with stable resting calcium levels were chosen for this study (see Fig. 2B). Figure 2B shows a plot of the normalized fluorescence intensity versus time for the four varicosities shown in Figure 2A during rest (Fig. 2B, open circles) and following the application of a single impulse. In total, 63 varicosities from a total of 9 different preparations were examined in the present study. Figure 2C shows another string of varicosities loaded with Oregon Green 488 BAPTA-1 before and after stimulation (single impulse). The average and individual ΔF Signal values for each of the varicosities shown in Figure 2C are shown in Figure 2D. As with the varicosities shown in Figure 2A, relatively little variation was observed between consecutive varicosities in this string. This relatively small variation in stimulus-induced calcium influx compared to that seen in transmitter release probability suggests that transmitter release probability might be regulated by factors downstream of calcium influx, such as the calcium sensitivity.

Variable co-localisation of the synaptic release machinery at different varicosities

One factor that might affect the calcium sensitivity of release is the expression and localisation of release machinery proteins. It has been suggested that the heterogeneous expression levels and patterns of many release machinery proteins in different synapses may explain the differences in release probability and plasticity (for review, see Staple et al., 1997, 2000). Such variation in the expression of release machinery proteins between consecutive boutons of single cultured neurons was shown to correlate to the variation in the release probability between those boutons (Staple et al., 1997, 2000). In the present study, the intensity of staining and the co-localisation of three release machinery proteins involved in regulating transmitter release were examined along strings of sympathetic varicosities.

Figure 3 shows examples of the longitudinal muscle layer at the prostatic end of the vas deferens stained with antibodies against the vesicular proteins SV2, synaptophysin, and synaptotagmin 1. Unlike the stimulus-induced calcium influx, the staining intensity of these antigens was highly variable between the varicosities with no consistent pattern. The staining intensity was even seen to vary noticeably between contiguous varicosities on single axon branches. The extent of variation between consecutive varicosities can be seen clearly in the graphs below each staining panel. Figure 3A and B illustrate the variation observed in SV2 staining, Figure 3C and D illustrate the variation in synaptophysin staining, and Figure 3E and F illustrate the variation in synaptotagmin 1 staining.

Such variation in the staining intensities of vesicular proteins may reflect variation in the density of synaptic vesicles between consecutive varicosities. To test this possibility, thin (60 nm) transverse sections of the vas deferens were viewed using transmission electron microscopy. As observed in previous studies (Klemm, 1995; Luff et al., 1995), the varicosities lacked clearly defined active zones and small synaptic vesicles (SSV) were generally dispersed evenly throughout the varicosity. However, in cases where the

apposition distance between the nerve and muscle was small (less than 50 nm), vesicles appeared to be clustered towards the muscle cell. Due to the lack of clearly defined active zones in the sympathetic varicosities, our analysis was confined to a general measure of vesicle density within the varicosities. The density of vesicles was measured by counting vesicle numbers in 200- × 200-nm squares placed randomly over the micrographs of the varicosities. This stereological technique, while giving a value of vesicles/ μm^2 , allowed us to accurately estimate the overall vesicle density of the varicosities (Griffiths, 1993). As observed in other studies (Klemm, 1995; Luff et al., 1995), the vesicle density was highly variable between sympathetic varicosities. Figure 4A and B illustrate the variation in vesicle density observed in this study. The average vesicle density was 141 ± 7 vesicles/ μm^2 (mean $\pm$ SEM, $n = 144$ varicosities).

If the variation in the staining intensity of vesicular antigens is due exclusively to variation in the vesicle density, then varicosities double stained for two vesicular antigens should show similar relative staining intensities for both of the antigens. However, in double-staining experiments, the relative staining intensities of both antigens were not always matched. For example, Figure 5A shows two strings of varicosities double stained for SV2 and synaptophysin. Most of the varicosities showed positive staining for both SV2 and synaptophysin. However, in some varicosities, the staining of one of the two the antigens was so low as to be virtually indistinguishable from the background staining while the other antigen showed a staining intensity comparable to other varicosities in the string. Similarly, double-staining experiments involving SV2 and synaptotagmin 1 also revealed the presence of varicosities apparently stained for only one antigen (Fig. 5B). The percentage of varicosities examined showing detectable levels of one or both antigens is shown in Figure 5C (over 500 varicosities were examined for each double staining experiment).

Due to the thickness of the sections (15 μm) and the presence of Triton-X 100 (0.05%) in the blocking solution, the differences in staining intensity between consecutive varicosities are unlikely to be the result of a penetration artefact (Morris et al., 2000). Furthermore, the absence of staining seen in these varicosities was not restricted to any of the primary or secondary antibodies, since staining controls in which the primary-secondary antibody combinations were switched also showed a similar pattern. Finally, since positive staining for synaptic vesicle antigens does not mean that the stained structure is a functional release site, only strings of fluorescent puncta were examined. The average distance between puncta was the same as the distance between varicosities visualised with DiOC₂ (5) in the present study, which conforms to previously published estimates of the intervaricosity distance (Lavidis and Bennett, 1992). In this way, the immunofluorescent puncta examined in the present study can be identified as sympathetic varicosities. These results suggest that the protein composition of synaptic vesicle membranes is variable, and, in some cases, vesicle membranes may be nearly or even completely devoid of one or more specific vesicular proteins.

DISCUSSION

Variance in the probability of transmitter release

The synaptic strength and plasticity of neurons forms the basis of our ability to learn. The importance of this variance in synaptic strength is highlighted by studies showing disparity in the synaptic strengths of different branches of single neurons and between adjacent boutons of single axon branches (Bennett et al., 1986a,b; Bennett and Lavidis, 1982; D'Alonzo and Grinnell, 1985; Gupta et al., 2000; Katz et al., 1993; Lavidis and Bennett, 1992, 1993a; Murthy et al., 1997; Reyes et al., 1998; Scanziani et al., 1998; Staple et al., 1997). The processes underlying the regulation of synaptic strength and plasticity however, are poorly understood. In agreement with previous work (Blakeley and Cunnane, 1979;

Lavidis and Bennett, 1992), the present study showed the probability of transmitter release from contiguous varicosities of single sympathetic neurons to be highly variable. However, previous studies (Lavidis and Bennett, 1992, 1993a; Macleod et al., 1994) have reported the average probability of release to be much higher than was observed in the present study. The difference between these studies and the present study likely arose from differences in the number of varicosities recorded from when the extracellular electrode was placed on the surface of the vas deferens. Previous studies bathed the tissue in 2 mM calcium while recording release probability. Due to the dense sympathetic innervation of the vas deferens (Bennett, 1972, 1973), it is likely that release recorded in this way is the sum of multiple release sites. In the present study, transmitter release was restricted to a smaller number of surface varicosities due to the tissue being bathed in a lower calcium concentration while the recording electrode was internally perfused with solution containing 2 mM calcium. The increased resolution of the recording technique used in this study has allowed us to gain a more accurate estimate of the probability of release at individual release sites than has previously been possible.

Another likely contributor to the low probability of release observed in this study is the inclusion of release sites that showed little or no stimulus evoked release (~63% of recording sites). It has been suggested that low probability transmitter release from sympathetic varicosities may sometimes be due to the absence of postsynaptic P2X receptor clusters beneath the varicosities (Hansen et al., 1999a,b). Growing evidence, however, suggests that P2X₁-purinoceptors are uniformly distributed over the entire surface of smooth muscle cells and are, thus, evenly co-localized under each varicosity (Knight et al., 2003; Lee et al., 2000; Liang et al., 2001; Vulchanova et al., 1996). Since the present study was only concerned with pre-junctional regulation of release probability, rather than post-junctional factors, each recording site was monitored for the presence of spontaneous transmitter release. If no such release was observed, then the recording site was rejected from further analysis. In this way, our results directly reflect variation in the pre-junctional regulation of transmitter release.

Variance in calcium dynamics in sympathetic varicosities

Stimulation of varicosities loaded with Oregon Green 488 BAPTA-1 produced a transient increase in fluorescence intensity in every varicosity examined without fail. This increase in the fluorescence intensity has been shown to be dependent on calcium influx through voltage-gated calcium channels (Brain and Bennett, 1997). Very little variation was observed in the calcium influx between adjacent varicosities, especially compared to the large variation observed in transmitter release probabilities between varicosities. It should be noted that the calcium signals measured in the present study do not reflect calcium concentrations within individual micro-domains, but more closely reflect the total amount of calcium entering the synaptic bouton. As such, the published relationship between cytosolic calcium concentration and transmitter release probability (Dodge and Rahamimoff, 1967; Macleod et al., 1994) may not apply to the signals measured in this study. However, the relatively consistent nature of the calcium signals measured in this study suggests that calcium influx into each micro-domain may be relatively constant. Such a non-intermittent influx of calcium upon arrival of the action potential suggests that transmitter release failure occurs at some point downstream of calcium entry. These results are in agreement with findings in the tonic and phasic terminals of the crayfish (Msghina et al., 1999). As suggested by Msghina et al., (1999), transmitter release probability appears to rely more heavily on the calcium sensitivity of the terminal than on the amount of calcium entering during an action potential.

Release machinery proteins and transmitter release efficacy

A recent review by Atwood and Karunanithi (2002) suggested that variable expression of release machinery proteins may be a significant contributor to variation in the strength and plasticity of transmitter release. The present study looked at the staining levels of three vesicular proteins: SV2, synaptophysin, and synaptotagmin 1. Extensive variation was observed in the staining intensity of these three vesicular proteins between adjacent varicosities. Given the thickness of the sections (15 μm) and the presence of Triton X-100 (0.05%) in the blocking solution, this variation in the staining intensity between adjacent varicosities in a single section is unlikely to be a penetration artefact (Morris et al., 2000), suggesting that the variation in staining intensities reflects variation in the expression levels of these vesicular proteins within the varicosities. This suggestion is supported by the wide range of vesicle densities observed in electron micrographs.

Given the variation in vesicle densities observed in the present study, it is tempting to suggest that the variation in staining intensities of vesicular proteins is entirely consistent with and, therefore, due to the variation in vesicle density. However, double-staining experiments with two vesicular antigens showed that the individual vesicular protein levels are regulated independently of each other. The relative staining intensity of pairs of vesicular antigens was highly variable between consecutive varicosities. In extreme cases, some varicosities showed detectable staining for only one of the two antigens, suggesting the presence of synaptic vesicles that either completely lacked or at least possessed undetectable levels of one of the vesicular proteins. This suggests that some vesicles may not possess a full complement of the necessary release machinery proteins, or if the proteins are present at imperceptibly low levels, then the stoichiometric ratio of important interaction partners (e.g., SV2 and synaptotagmin 1; Pyle et al., 2000; Schivell et al., 1996) might be non-uniform.

As suggested by Atwood and Karunanithi (2002), such variation in the ratio of these vesicular proteins might feasibly affect the probability and or plasticity of release. Recent analyses of neuronal compartments have revealed the presence of mRNAs in axons and dendrites of neurons suggesting that protein translation may occur locally within sub-compartments of neurons (for review, see Job and Eberwine, 2001). Work by Staple et al. (1997, 2000) showed that the staining intensity of some vesicular proteins to be variable between adjacent boutons in cultured hippocampal neurons and that the variation in staining intensity was correlated with FM1-43 fluorescence (Staple et al., 1997, 2000). Together these findings provide a strong argument to suggest that adjacent release sites within a single neuron do not express uniform levels of each and every vesicular protein and that variation in the strength of synaptic transmission between adjacent release sites within a single terminal is affected by the levels of protein expression.

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Fig. 1.

The probability of transmitter release from sympathetic varicosities is highly non-uniform.

A: A field of varicosities fluoresced with the mitochondrial marker DiOC₂ (5). Single sympathetic axons can be seen with varicosities spaced 3–5 μm apart. **B:** A sketch of the varicosities in A indicating the positions of the recording electrode (tip diameter $\sim 6 \mu\text{m}$). **C:** Ten consecutive traces recorded from one electrode placement. Each trace has a stimulus artefact (SA) and a nerve terminal impulse (NTI). Excitatory junction currents (EJC) appear intermittently. Traces 3, 5, 6, 7, and 8 are defined as failures due to the presence of a NTI and absence of an EJC with a rise time ≥ 3 ms. **D:** A graph of the percentage of successful stimuli (stimuli that generated an EJC) for each of the ten recording sites depicted in B. Two hundred stimuli were delivered to each recording site at a frequency of 0.1 Hz. A value of 0%, as seen at sites 5, 8, and 9, indicates a failure to produce any EJCs at this recording site despite the presence of both NTIs and spontaneous EJCs.

Fig. 2.

Sympathetic varicosities loaded with Oregon Green 488 BAPTA-1 show an increase in fluorescence intensity following stimulation. **A:** A field of 4 varicosities loaded with Oregon Green at rest, and following nine single stimuli (15 V, 0.1 ms) delivered at 2-minute intervals. The increase in fluorescence indicates the cytosolic calcium concentration has increased. Scale bar = 5 μm . **B:** The average change in fluorescence (ΔF Signal) following a single impulse is plotted against the time for varicosities 1–4 in A (filled diamonds, mean $\pm$ SEM). The normalised resting calcium is also plotted (open circles). When the tissue was stimulated, the fluorescence intensity increased rapidly to a peak and then declined slowly over 1 to 2 seconds. The individual peak ΔF Signal values for varicosities 1–4 following the same stimulus are indicated. **C:** A different field of varicosities loaded with Oregon Green at rest, and following a single stimulus (15 V, 0.1 ms). Scale bar = 5 μm . **D:** The average ΔF Signal following a single impulse is plotted against the time for varicosities 1–4 in C (filled diamonds, mean $\pm$ SEM). The normalised resting calcium is also plotted (open circles). The individual peak ΔF Signal values for varicosities 1–4 are indicated.

Fig. 3.

The staining intensity of vesicular antigens varies between consecutive varicosities. **A,B:** Two strings of varicosities labelled for SV2. **C,D:** Two strings of varicosities stained for synaptophysin. **E,F:** Two strings of varicosities stained for synaptotagmin 1. The difference in staining intensities between adjacent varicosities is shown in the graph below each panel. SV2 staining intensities showed the least variation, while synaptotagmin 1 staining intensities showed the greatest variation. Scale bars = 10 μ m.

Fig. 4.

Two transverse micrographs of sympathetic varicosities. Both micrographs were captured at 40,000 $\times$ magnification. Small synaptic vesicles, large dense cored vesicles and mitochondria are all clearly visible at this magnification. **A:** A varicosity with a relatively high vesicle density. **B:** A varicosity with a relatively low vesicle density. Scale bar = 200 nm.

Fig. 5.

Double-staining experiments showed non-uniform variation in the staining intensities between two antigens within single varicosities. **A:** Two strings of varicosities double stained for SV2 (red) and synaptophysin (green). The co-localisation of these two vesicular antigens is shown in the bottom frame of each panel. Arrowheads indicate varicosities with little or no apparent staining of one of the two antigens. **B:** Two strings of varicosities double stained for SV2 (red) and synaptotagmin 1 (green). The co-localisation of the two antigens is shown in the bottom frame of each panel. Arrowheads indicate varicosities with little or no apparent staining of one of the two antigens. Scale bars = 10 μ m. **C:** Graphs showing the percentage of varicosities with detectable levels of one or both of the vesicular antigens. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]