Visible Evidence for Differences in Synaptic Effectiveness With Activity-Dependent Vesicular Uptake and Release of FM1-43

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1 Department of Physiology, Medical Research Council Neural Group, University of Toronto, Toronto, Ontario M5S 1A8, Canada; 2 Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden; and 3 Life Sciences Division, University of Toronto at Scarborough, Toronto, Ontario M1C 1A4, Canada

Quigley, P. A., M. Msghina, C. K. Govind, and H. L. Atwood. Visible evidence for differences in synaptic effectiveness with activity-dependent vesicular uptake and release of FM1-43. J. Neurophysiol. 81: 356 ± 370, 1999. Activity-dependent uptake and release of the fluorescent probe FM1-43 were used to compare synaptic performance (rates of transmitter release and synaptic vesicle turnover) at different frequencies in phasic and tonic motor neurons innervating the crayfish leg extensor muscle and in the tonic motor neuron of the opener muscle. The phasic extensor motor neuron, which has a high quantal content of transmitter release, accumulated and released FM1-43 more rapidly than the tonic motor neuron, especially at low frequencies of stimulation. Individual bright spots appeared on the varicosities of the junctional terminals during stimulation in FM1-43; these spots corresponded to zones of immunostaining for the synaptic vesicle associated protein synaptotagmin, but they were larger and less numerous than synapses identified by electron microscopy and appear to represent one to several synapses with their associated clusters of synaptic vesicles. The number of bright spots observed on varicosities of the tonic terminal after stimulation at ≥20 Hz is generally similar to values for responding units (n) calculated from binomial distributions derived from quantal analysis. At frequencies of ≤10 Hz, bright spots did not usually appear on tonic extensor varicosities, and the quantal release patterns were best fitted with Poisson distributions. Another tonic motor neuron, the excitor of the opener muscle, showed individual bright spots at lower frequencies of stimulation, consistent with its higher quantal output at these frequencies and corresponding with the binomial fits for quantal release distributions. In this axon, the number of distinctive bright spots increased with frequency in the 2- to 20-Hz range, indicating increased participation of synapses during frequency facilitation. In the tonic extensor neuron terminals, the brightness and the size of the individual spots increased with frequency, and new foci of dye uptake appeared at the edges of preexisting spots. Relative intensity change varied considerably among individual spots during dye loading at different frequencies. Similarly, individual spots on a single tonic terminal remained at different rates when stimulated after previous loading with FM1-43. These results suggest differential performance of individual synapses or small groups of synapses, some being more effective in transmitter release than others, as inferred from previous ultrastructural and quantal analysis studies. The large overall differences between phasic and tonic synapses suggest differential regulation of transmitter release at individual synapses in the two neurons.

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INTRODUCTION

Synaptic effectiveness varies among neurons, even those innervating the same postsynaptic targets (Atwood 1976). Individual synapses of a single neuron may also differ in effectiveness, with some transmitting at a low rate and others transmitting more consistently with higher probability of release. This is well characterized for invertebrate neurons (Atwood 1967; Bittner 1968; Cooper et al. 1995b; Davis and Murphey 1993, 1994.), and heterogeneity of release is also prominent in vertebrate motor (Nuddell and Grinnell 1983) and central (Dobrunz and Stevens 1997; Murthy et al. 1997) neurons. Synaptic effectiveness previously has been measured usually through electrical recording of membrane potential changes or currents from postsynaptic targets. These measurements provide a good overall picture of synaptic transmission, highly resolved in time. Quantal analysis using binomial statistics also can provide estimates of the number (n) of participating units involved in transmission onto an individual postsynaptic target cell. Tentatively, these units have been postulated in some studies (Wojtowicz et al. 1991, 1994; Zucker 1973) to represent synaptic active zones. However, spatial resolution in electrical recording is often not sufficient to observe the behavior of individual synapses or active zones, especially if several occur in close proximity. In addition, it is abundantly apparent that different interpretations of the quantal release statistics can be made. To take a current example, quantal analyses of transmission at crayfish and lobster neuromuscular junctions have led to two different models based on similar data. In one model, the binomial parameter n is interpreted as the number of release sites (participating active zones) capable of transmission (Wojtowicz et al. 1994). A second model proposes that n represents instead the number of docked and primed synaptic vesicles and is not limited by the number of available release sites, which could all be equally likely to release a quantal unit (Worden et al. 1997). Additional experimental evidence obtained with another technique could help in assessing these competing models.

New optical methods based on activity-dependent uptake of fluorescent dyes (Betz and Bewick 1992, 1993; Betz et al. 1992b) provide one possibility. Although not as well resolved in the temporal domain, results from dyes
can provide spatial information about transmitter release not readily available from electrical recording and can help to define the relative activity of different regions of a nerve terminal. We have employed the fluorescent dye FM1-43, which is taken up by recycling synaptic vesicles, to examine the behavior of synapses at crayfish neuromuscular junctions. We have assessed its utility for examining nerve terminals known to release transmitter at different rates and whether different synapses of the same nerve terminal exhibit different rates of transmission.

Crustacean motor neurons provide good material for studying such differences in synaptic effectiveness. In the crayfish leg, the two excitatory motor neurons innervating the extensor muscle (phasic and tonic) exhibit a very large difference in transmission (Bradacs et al. 1997). Previously, we have shown that individual synapses of the phasic neuron are 50–1,500 times more likely than those of the tonic neuron to release transmitter in response to a nerve impulse (Bradacs et al. 1997; Msghina et al. 1998). This is a very large difference in transmission for two neurons innervating the same postsynaptic targets. Because FM1-43 accumulation depends on transmitter release and synaptic vesicle recycling, it can be predicted that more rapid changes in fluorescence will occur in nerve terminals of the phasic neuron. This prediction was tested in the present experiments.

We also approached the questions of nonuniformity of synaptic performance, linked to the interpretations of binomial statistical analyses applied to tonic crustacean neurons. According to the models in which \( n \) represents the number of release sites, there ought to be a relationship between the values for \( n \) and the number of fluorescently labeled synapses. In contrast, the model in which \( n \) represents primed, releasable vesicles at any available release site, all equally likely to release vesicles on stimulation, would not predict a correspondence between \( n \) and fluorescently labeled synapses; all synapses ought to become labeled if all can participate in release and linked vesicle recycling.

In addition, frequency-dependent changes can be assessed with fluorescent dyes. Tonic crustacean motor neurons exhibit pronounced frequency facilitation, with much more transmitter being released at higher frequencies of activation (Bittner 1968; Bradacs et al. 1997). Thus it can be predicted that the amount of fluorescence at individual synapses, and possibly the number of active synapses revealed by FM1-43 uptake, will vary with frequency of impulses, especially if the number of release sites is limited, as in the models relating binomial \( n \) to a limited number of release sites. We investigated frequency-dependent alterations in FM1-43 uptake and found evidence for differences in behavior of individual synapses or small groups of synapses, supporting the notions of nonuniform synaptic behavior and a limited number of release sites.

Preliminary results with FM1-43 and crustacean neuromuscular synapses have been reported previously in abstracts at scientific meetings (Msghina et al. 1995; Quigley et al. 1996). Recently, a complementary study using FM1-43 with high-frequency stimulation to investigate the effects of serotonin in crayfish opener muscle terminals was reported by Wang and Zucker (1998).

**Methods**

**Animals and solutions**

Freshwater crayfish (*Procambarus clarkii*) were obtained from Atchafalaya Biological Supplies (Raceland, LA) and maintained in dechlorinated tap water at 15°C with a diet of carrots and lentils. Only crayfish measuring 4–6 cm in length from telson to rostrum were used. The leg extensor muscle was dissected from one of either the first or second pairs of walking legs as described by Bradacs et al. (1997). Supplementary observations were made on the opener muscle of the first walking leg, prepared as described in Wojtowicz and Atwood (1984, 1985). Preparations were bathed in modified van Harreveld’s crayfish solution, which contains the following (in mmol L\(^{-1}\)): 205.3 NaCl, 5.3 KCl, 13.5 CaCl\(_2\)\(\cdot\)2H\(_2\)O, 2.5 MgCl\(_2\)\(\cdot\)6H\(_2\)O, 10 glucose, and 0.5 N-2-hydroxyethylpiperazine-\(N^\prime\)-2-ethanesulfonic acid buffer, adjusted to pH 7.4. The dye, FM1-43 (Molecular Probes, Eugene, OR), was dissolved in this solution at a concentration of 2 \(\mu\)M and stored at 4°C until used. All experiments were conducted at room temperature.

**Electrophysiology**

The nerve bundle containing both the tonic and phasic extensor axons was dissected out in the proximal segment and drawn into a tightly fitting suction electrode for stimulation using a Grass Instruments S48 stimulator and SIU5 stimulus isolation unit. First nerve impulse (Bradacs et al. 1997; Msghina et al. 1998). Supplementary observations were made on the opener muscle of the first walking leg, prepared as described in Wojtowicz and Atwood (1984, 1985). Preparations were bathed in modified van Harreveld’s crayfish solution, which contains the following (in mmol L\(^{-1}\)): 205.3 NaCl, 5.3 KCl, 13.5 CaCl\(_2\)\(\cdot\)2H\(_2\)O, 2.5 MgCl\(_2\)\(\cdot\)6H\(_2\)O, 10 glucose, and 0.5 N-2-hydroxyethylpiperazine-\(N^\prime\)-2-ethanesulfonic acid buffer, adjusted to pH 7.4. The dye, FM1-43 (Molecular Probes, Eugene, OR), was dissolved in this solution at a concentration of 2 \(\mu\)M and stored at 4°C until used. All experiments were conducted at room temperature.

**Anterior motor neurons**

Animals and solutions are described above. The nerve bundle containing both the tonic and phasic extensor axons was dissected out in the proximal segment and drawn into a tightly fitting suction electrode for stimulation using a Grass Instruments S48 stimulator and SIU5 stimulus isolation unit. First nerve impulse (Bradacs et al. 1997; Msghina et al. 1998). Supplementary observations were made on the opener muscle of the first walking leg, prepared as described in Wojtowicz and Atwood (1984, 1985). Preparations were bathed in modified van Harreveld’s crayfish solution, which contains the following (in mmol L\(^{-1}\)): 205.3 NaCl, 5.3 KCl, 13.5 CaCl\(_2\)\(\cdot\)2H\(_2\)O, 2.5 MgCl\(_2\)\(\cdot\)6H\(_2\)O, 10 glucose, and 0.5 N-2-hydroxyethylpiperazine-\(N^\prime\)-2-ethanesulfonic acid buffer, adjusted to pH 7.4. The dye, FM1-43 (Molecular Probes, Eugene, OR), was dissolved in this solution at a concentration of 2 \(\mu\)M and stored at 4°C until used. All experiments were conducted at room temperature.

**Electrophysiology**

The nerve bundle containing both the tonic and phasic extensor axons was dissected out in the proximal segment and drawn into a tightly fitting suction electrode for stimulation using a Grass Instruments S48 stimulator and SIU5 stimulus isolation unit. First the tonic axon, which has a lower stimulus threshold, was excited selectively by adjusting the amplitude or duration of the stimulus intensity. The phasic axon could be recruited by raising the stimulus intensity. To minimize muscle contractions during the recordings, muscle fibers were stretched by extending the muscle until contractions were no longer evident. Excitatory postsynaptic potentials (EPSPs) from the distal muscle fibers were recorded using intracellular electrodes filled with 3 M KCl with tip resistances between 10 and 16 MΩ. Electrical signals were amplified using both a Dagan 8500 preamplifier and an Intrionix Technologies 2004-F signal conditioner with a high-pass filter of 0.5 Hz and a low-pass-filter of 5 kHz. The signals were digitized using an Axon Instruments TL-1 DMA Interface and stored on tape for later analysis with AVGPLT software on a pentium computer.

**Quantal recording**

Focal extracellular recordings of synaptic currents were selectively made from either phasic or tonic terminals with macro-patch electrodes (Dudel 1981) of 10–15 \(\mu\)m ID connected to an amplifier obtained from Zeitz Instruments Vertriebs GmbH (Augsburg, Germany). The evoked responses from each recording site of the tonic axon met the requirements of stationarity in activity for the time analyzed (see Smith et al. 1991; Wojtowicz et al. 1994); 500–1,000 sweeps were collected for averaging. For the phasic axon, transmitter output during maintained stimulation always declines slowly with time due to depression, even at relatively low frequencies (Msghina et al. 1998); accordingly, stability of the recording conditions was judged by monitoring the seal resistance of the macro-patch electrode at the site and rejecting sites where it changed. Estimations of quantal content at low frequencies were obtained by counting quanta (tonic axon) or by measuring the ratio of areas of evoked responses to unitary quantal events (Cooper et al. 1995c). The procedures for determining best fits (Poisson or binomial) for the data sets from the tonic axon were as described previously (Cooper et al. 1995b,c; Msghina et al. 1998; Wojtowicz et al. 1994).

**Staining protocol**

The exposed inner side of the extensor muscle was superfused for 2–3 min with crayfish solution containing 2 \(\mu\)M FM1-43 before...
To check the status of synaptic transmission and action potential conduction during the stimulation regimes used for loading terminals with FM1-43, we monitored changes in fluorescently labeled spots. Differences in the rate of FM1-43 uptake and release, and synaptic transmitter release, were imaged. Higher frequency stimulation (10 or 15 Hz) then was applied for 5 min, and the same regions imaged again to observe changes in fluorescently labeled spots.

Facilitation experiments

Either the tonic or phasic axon of the leg extensor preparation was loaded by stimulation with 9,000 pulses at 20 Hz. After the selected area had been imaged, the procedure was repeated at 30 Hz and then at 50 Hz, imaging the same area each time. Intensity measurements from three to four individual bright spots per preparation were made from composites of the z series. The data were normalized to the intensity measured after 20-Hz stimulation.

In the opener muscle, stimulation was first applied at a low frequency (5 Hz for 10 min or 2.5 Hz for 20 min) and terminals were imaged. Higher frequency stimulation (10 or 15 Hz) then was applied for 5 min, and the same regions imaged again to observe changes in fluorescently labeled spots.

Destaining experiments

The tonic extensor axon was selectively stimulated at 50 Hz for 3 min to load with FM1-43. Selected terminals were imaged, and the preparation then was destained by restimulating (without dye in the bath) at 20, 30 or 50 Hz for 9,000 pulses. The bath was replaced with fresh saline after each stimulation period. The destaining procedure was repeated four times for a total of 36,000 pulses. Analysis was performed on composite images of the z series. Intensity measurements from three to four bright spots were taken using C-focal software and normalized to the initial stained value. A total of 18–24 spots was averaged from six preparations for each destaining frequency.

**RESULTS**

**Synaptic transmission of phasic and tonic neurons**

We addressed the question of the relationship between FM1-43 uptake and release, and synaptic transmitter release, through observations on a pair of well-differentiated neurons: the phasic and tonic motor neurons innervating the main extensor muscle of the crayfish leg. Previous work has shown that there is a large difference in transmitter release per impulse at nerve terminals of the extensor motor axons, and this is largely responsible for the much larger EPSPs recorded in single fibers of the extensor muscle (Bradacs et al. 1997). A major goal of the present study was to determine whether the differences in quantal release are correlated with differences in the rate of FM1-43 uptake and release at active synapses. Accordingly, we stimulated the phasic and tonic axons at various frequencies to induce uptake of FM1-43.

To check the status of synaptic transmission and action potential conduction during the stimulation regimes used for loading terminals with FM1-43, we monitored changes in the EPSP and action potential during the stimulation in several preparations to see whether transmission and conduction were sustained during the loading period and how they changed with frequency. These experiments also provided a comparison with previous work on the crayfish opener muscle (Bittner 1968).

Transmission persisted undiminished throughout the stimulation period for the tonic axon but not for the phasic axon.
and Atwood 1985a; Pahapill et al. 1987) persisted for many minutes after tetanic stimulation. For the phasic axon, the loading stimulation was probably effective for FM1-43 uptake mainly during the initial period of stimulation when transmitter release was intense.

In contrast, EPSPs of the tonic axon were sustained throughout the period of stimulation with no diminution (Figs. 1 and 2). When phasic and tonic EPSP amplitudes were compared during trains of 9,000 impulses at frequen-

(Figs. 1 and 2). At frequencies of 10–30 Hz, transmission declined rapidly and continuously for the phasic axon and was succeeded by a period of very pronounced enhanced transmission for single impulses which was evident when stimulation was resumed after a pause (Fig. 1). At 50 Hz, there was sometimes evidence of a more abrupt decline in transmission (Fig. 2). The enhancement of EPSPs due to posttetanic potentiation and long-term facilitation (Lnenicka

![Fig. 1. Monitoring phasic and tonic synaptic potentials periodically during dye-loading. Tonic and phasic excitatory postsynaptic potentials (EPSPs) from crayfish leg extensor muscle monitored periodically (at the times shown) under the same stimulus regime used for loading of nerve terminals with FM1-43 dye with 20-Hz stimulation. An average of the first 10 responses in each stimulus series is shown in A1, B, C, and D1. After the 7.5-min record (A3), the stimulation was discontinued for 1 min to scan the preparation with confocal microscopy. Stimulation then was resumed at 8.5 min (B), and continued until 16 min, at which time it was again interrupted for 1 min and resumed at 17 min (C). Stimulation of the tonic axon continued until 34 min, with two 1-min interruptions and resumptions (D, 1 and 2.) Tonic EPSPs persist for the entire period of stimulation (A–D), while in this example, the phasic EPSP rapidly fatigues (A 1–3) and has almost completely disappeared by 3 min (A2). With stimulus resumption after scanning (B), an enhanced phasic EPSP is seen due to posttetanic potentiation and long-term facilitation, and a still larger EPSP appears at 17 min (C). Phasic EPSP was not recorded beyond 17 min. Bars: vertical, 2 mV tonic, 10 mV phasic; horizontal, 20 ms.

![Fig. 2. Comparison of phasic and tonic EPSPs during continuous stimulation at several frequencies. Phasic EPSPs (▲) and tonic EPSPs (△) recorded from the same extensor muscle fiber at 5 and 10 Hz for 9,000 pulses. For 20-, 30-, and 50-Hz stimulation, the tonic and phasic EPSPs were recorded from separate preparations. EPSP values were obtained by averaging 10 responses for each point. Phasic EPSPs are larger than the tonic at low stimulation frequencies but depress rapidly at higher frequencies. Tonic EPSPs are small at low frequencies but show facilitation at higher frequencies with no depression even at 50 Hz.](http://jn.physiology.org/content/163/4/359)
cies ranging from 5 to 50 Hz (Fig. 2), it was apparent that while the EPSP of the phasic axon was maintained with gradual decline at the lower frequencies and faded rapidly at frequencies >20 Hz, there was no corresponding depression of the tonic EPSP even at the highest frequency. The resistance of the tonic EPSP to depression exceeds that of the opener muscle excitor axon (OE), for which comparable plots (Bittner 1968) exhibit depression at frequencies of \( \leq 40 \) Hz. Thus loading of the tonic terminals with FM1-43 is likely to occur throughout the period of stimulation. Because dye loading conducted at frequencies >10 Hz probably decreases for the phasic axon during the second half of the train despite continuation of action potentials, the phasic-tonic difference in dye loading per impulse is greatest during the initial phase of stimulation and reverses as the stimulation is continued.

Action potentials were recorded from the major axon branches with intracellular or extracellular electrodes during \( \geq 20 \) Hz stimulation to assess their ability to conduct without impulse failure during loading stimulation. Action potential conduction continues in both axons over the periods of time used for loading. Thus the decline of the phasic EPSP is not likely due to impulse failure of the major axon branches. The decline of the EPSP at frequencies \( \leq 30 \) Hz can be attributed mainly to synaptic depression (see also Nguyen et al. 1997). At frequencies >30 Hz, the more abrupt transition of EPSP amplitude to a lower value (Fig. 2) could be due in part to failure of propagation in one or more preterminal branches.

Quantal analysis of transmission

Transmitter release statistics were followed at single recording sites of the tonic axon as frequency increased, extending preliminary results of Msghina et al. (1998). Pooled results from 11 experiments are summarized in Table 1. Typically, the release pattern at \( \leq 10 \) Hz could be fitted best by a Poisson distribution in contrast to results for the more extensively studied crayfish opener axon (Cooper et al. 1995a–c; Johnson and Wernig 1971; Wernig 1972; Wojtowicz et al. 1994; Zucker 1973) but as the frequency increased >10 Hz, binomial distributions provided the best fits for most of the sites (Table 1). The number of releasable units \( (n) \) in the binomial distributions varied from 4 to 16 in different varicosities. Fits of distributions for phasic terminals were not attempted because the large number of quantal units being released made discrete counts difficult, and accurate fits for distributions were not feasible with our methods (Cooper et al. 1995c). At single recording sites including both phasic and tonic terminals, quantal content at low frequencies of stimulation is invariably 50–1,300 times larger for the phasic axon (Bradacs et al. 1997; Msghina et al. 1998).

Transmitter release statistics for the opener axon have been reported in several previous studies (Cooper et al. 1995a–c; Hatt and Smith 1976; Johnson and Wernig 1971; Wernig 1972; Wojtowicz et al. 1991, 1994; Zucker 1973). In general, quantal output is higher at low frequencies than for the tonic extensor axon, and a majority of the quantal release distributions are best described by binomial rather than Poisson statistics, even at frequencies of 1–5 Hz. There is a clear difference in the statistical picture for the two tonic axons.

Labeling of phasic and tonic terminals with FM1-43

Comparison of FM1-43 uptake in phasic and tonic terminals was made at several different frequencies. Less than 20 Hz, a low level of FM1-43 labeling occurred in the tonic terminals while robust labeling of phasic terminals was apparent (Fig. 3). At higher frequencies, labeling of tonic terminals became more evident, and individual fluorescent spots were clearly defined (Fig. 4).

Labeled phasic and tonic terminals (Figs. 4–6) exhibited bright spots, usually on varicosities. The identity of the two terminals has been established in previous work on their morphological differences (Bradacs et al. 1997) and was confirmed in several experiments by injecting one of the axons with Texas Red dextran after physiological identification (Figs. 3, 4, and 6). For the phasic axon, the small varicosities were uniform in size and all were brightly labeled at frequencies of 5 or 10 Hz after 5–10 min of stimulation or less. In the tonic axon, the varicosities varied in size along the terminal, and the number of bright spots increased with the size of the varicosity (Figs. 4 and 5). Demonstration of well-defined discrete bright spots in tonic varicosities typically required stimulation at \( \geq 20 \) Hz for several minutes (Fig. 4, B1, C, and D). Thus comparison of FM1-43 uptake in phasic and tonic terminals at several different frequencies revealed weak labeling of the tonic terminals and strong labeling of the phasic terminals at frequencies <20 Hz. At higher fre-

### Table 1. Statistical analysis of quantal release

<table>
<thead>
<tr>
<th>Frequency, Hz</th>
<th>Poisson</th>
<th>Binomial</th>
<th>Quantal Content ( m )</th>
<th>Facilitation of ( m ) (normalized to 1 Hz)</th>
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<tr>
<td></td>
<td></td>
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<td>( n )</td>
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<tr>
<td>1</td>
<td>11/11</td>
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<td>2</td>
<td>10/10</td>
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<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>10/11</td>
<td></td>
<td>6.750 ± 1.377</td>
<td>0.046</td>
</tr>
<tr>
<td>10</td>
<td>6/10</td>
<td></td>
<td>(4, 5, 8, 10)</td>
<td>0.085 ± 0.033</td>
</tr>
<tr>
<td>20</td>
<td>3/10</td>
<td></td>
<td>(4, 5, 6, 8, 12, 16)</td>
<td>0.159 ± 0.026</td>
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Poison: fraction of the experiments in which a Poisson distribution provided the best fit for the data. Binomial: individual integer values of \( n \) are shown in brackets for the experiments in which binomial distributions best fitted the data. Facilitation is estimated by normalizing all values to the value at 1 Hz in each experiment; the means ± SE of 10 experiments are shown.
funquencies, labeling of tonic terminals became more evident and individual bright spots were more clearly defined.

Correspondence of the bright spots with regions containing synapses was established by staining after FM1-43 labeling experiments with an antibody for the synaptic vesicle associated protein synaptotagmin (Cooper et al. 1995a). As shown in Fig. 6, synaptotagmin labeling occurred in the same regions in which FM1-43 uptake had been observed. Both procedures produced punctuate fluorescent spots, especially on tonic terminals. This strengthens the case that FM1-43-labeled bright spots represent locations at which active synapses occur.

In the OE and opener inhibitor axon terminals, individual fluorescent bright spots were clearly evident after stimulation at 2–5 Hz for 10–15 min (Fig. 7). Much less stimulation was required to produce discrete spots in the OE axon than in the tonic extensor axon, reflecting the difference in transmitter release at low frequencies. At higher frequencies (10–20 Hz), individual spots brightened and additional spots appeared in locations where initial intensity values were low (Fig. 7).

**Comparison of confocal and electron microscopy data**

In an attempt to provide a structural correlate for the fluorescent spots revealed by confocal microscopy, we examined varicosities and synapses obtained via serial thin section electron microscopy (cf. Lnenicka et al. 1986). The latter entailed analyzing tissue prepared for this study as well as reexamination of tissue prepared for a previous study (King et al. 1996). Serial reconstruction of individual varicosities limited the number of varicosities analyzed by electron microscopy compared with those analyzed by confocal microscopy (Table 2). Despite this limitation, the comparison is particularly instructive in that the fluorescent bright spots are several-fold larger than individual synapses in both phasic and tonic varicosities. This comparison shows that individual fluorescent spots likely represent one to several (closely grouped) synapses with their associated clouds of synaptic vesicles rather than single synapses. This is indicated by the elongated shapes of some individual spots, which suggest staining of two or more closely adjacent synapses. Data on the number of bright spots and synapses per varicosity indicate that for the phasic axon, there are overall about six times as many synapses as bright spots, while for the tonic axon, this ratio is ~5 (Table 2). Serial reconstructions in which all synapses on a varicosity were counted showed that the number of synapses increases with varicosity size (Fig. 5) and that for larger varicosities, there are many more synapses than individual bright spots. Also, the bright spots often occur only in limited regions of the tonic varicosity (Fig. 4), whereas the synapses examined in three-dimensional reconstructions of varicosities invariably are widely distributed over the surface of a varicosity, both in the leg extensor tonic axon (King et al. 1996; Msghina et al. 1998) and in the crayfish opener muscle’s excitatory axon (Cooper et al. 1995b, 1996b; Wojtowicz et al. 1994). This suggests that not all synapses on a tonic varicosity are equally likely to take up FM1-43, which in turn indicates nonuniform probability of transmitter release and the occurrence of “silent” synapses.

**Differential loss of FM1-43 from phasic and tonic terminals**

Further observations were made during staining and destaining of individual bright spots. Phasic and tonic terminals were compared in destaining experiments, in which stimulation was applied after previous labeling with FM1-43 followed by washout of the dye. As with the loading experiments, changes occurred more rapidly and at lower frequencies in the phasic terminals. Thus at 5 or 10 Hz, phasic terminals rapidly destained, whereas tonic terminals retained much of their label (Figs. 3, 6, and 8). At higher frequencies, destaining of tonic terminals could be produced, but in many preparations it was clearly evident only at frequencies >30 Hz (Fig. 9). We detected a stimulation- and illumination-independent loss of fluorescence in several experiments for which we could not readily account; it was apparently not due to bleaching per se because it occurred without any illumination (Fig. 9). Significant activity-dependent loss of fluorescence only >30 Hz (with 9,000-impulse trains) for the tonic axon contrasts strikingly with the rapid destaining observed at 5 and 10 Hz (even with fewer impulses) for phasic terminals (Figs. 3, 6, and 8).
FIG. 4. Loading of phasic and tonic terminals with FM1-43. A, 1–3: stimulation for 10 min at 50 Hz in 2 μM FM1-43 resulted in loading the phasic and tonic terminals (A1) after which the tonic axon was injected with Texas Red, revealing more completely the outlines of the terminals in the same area (A2). Merged images reveal the punctate nature of the FM1-43 uptake in both axons (A3). B, 1 and 2: selective stimulation of the tonic axon at 50 Hz for 3 min in 2 μM FM1-43 loads tonic nerve terminals (B1). When both phasic and tonic axons were stimulated, the phasic terminals were loaded and seen to run beside the tonic ones (B2). C: selective loading of the tonic axon at 40 Hz for 20 min in 2 μM FM1-43 dye reveals a tonic nerve terminal comprising a string of several varicosities. Larger varicosities have more defined foci of FM1-43 uptake (bright spots) than the smaller varicosities. D: tonic nerve terminals loaded as in C showing punctate appearance of dye in some of the varicosities. Larger varicosities show several bright spots, some of which appear to be partially fused. Scale bars: A–C, 10 μm; D, 5 μm.
For the opener muscle, initial stimulation at low frequencies (1–5 Hz) resulted in several well-defined fluorescent spots on many of the varicosities. After imaging this result, the frequency was increased to 10–15 Hz (sufficient to produce a large facilitation of the EPSP) (Bittner 1968). Reimaging showed that additional spots appeared on many of the varicosities, often at the edges of preexisting spots, in regions where initial intensity of fluorescence had been low. Furthermore preexisting spots became much brighter (Fig. 7).

In the extensor muscle, the tonic terminals did not exhibit individual bright spots at frequencies of 1–10 Hz (Fig. 3), so we could not study changes at individual foci at low frequencies in this axon. At higher frequencies (>20 Hz), individual spots were established and could be followed. Pronounced increases in brightness of individual labeled spots occurred when the number of impulses was kept constant while the frequency was increased, indicating dependence of FM1-43 uptake on frequency as well as on the number of impulses (Figs. 8 and 10). The relative increase was less at these higher frequencies for the phasic axon (Fig. 8), in accordance with its smaller frequency facilitation and the depression of transmission (Figs. 1 and 2). Mean size of the bright spots increased with frequency in both axons. For selected tonic terminals, mean bright spot areas were 10.19 ± 1.98, 10.63 ± 1.73, and 13.57 ± 3.9 (SD) μm² when produced by 20, 30, and 50 Hz stimulation, respectively. In selected phasic terminals, the bright spots were much smaller, with mean areas of 3.74 ± 0.52, 4.79 ± 0.61, and 5.61 ± 0.72 μm² after stimulation at 20, 30, and 50 Hz, respectively. The change in brightness could reflect more transmitter turnover within the same group of synapses, addition of reactive synapses to the group, or both. Resolution was not sufficient to differentiate between these possibilities.

Detailed examination of individual bright spots along tonic terminals at frequencies of ≥20 Hz showed that in general as the frequency increased, the brightness of the

**Frequency dependence of staining and destaining**

In view of the large frequency facilitation of the tonic extensor and opener muscle EPSPs and the smaller frequency facilitation of the phasic extensor EPSP, staining and destaining were investigated at several different frequencies in terminals of all three axons.

![Graph showing relationship between size of varicosity and number of FM1-43-labeled spots](image)

**Fig. 5.** Relationship between size of varicosity and number of FM1-43-labeled spots, and ultrastructurally identified synapses for the tonic axon. Larger varicosities have a greater number of FM1-43-labeled spots. Planar area was measured from confocal images of varicosities labeled with 2 μm FM1-43 (50 Hz stimulation for 3 min). A total of 353 bright spots were counted from 93 varicosities. Ultrastructurally identified synapses of 5 serially sectioned varicosities increased in number with varicosity size and exceeded the number of bright spots for similar-sized varicosities examined with confocal microscopy.

**Fig. 6.** Comparison of staining with FM1-43 and synaptotagmin antibody. A: stimulation of both tonic and phasic axons for 10 min at 50 Hz in 2 μM FM1-43 served to load both tonic and phasic nerve terminals. B: stimulation of both axons in the absence of FM1-43 for 25 min at 10 Hz selectively destained the phasic terminals, whereas the tonic terminals appeared to be little affected. C: injection of the tonic axon with 10 kDa Texas Red dextran and viewing with a red filter confirmed the nondestaining terminals as tonic ones. D: preparation then was fixed and immunostained for the synaptic protein synaptotagmin to show synaptotagmin immunopositivity of synaptic regions (corresponding closely to FM1-43 labeled spots) for these same phasic and tonic nerve terminals. Note the appearance of numerous fluorescent beads used to identify the site (some marked by arrows) which may partially mask the correspondence between nerve terminals here to those in A. Scale bar: 10 μm.
FIG. 7. Changes in FM1-43 uptake in opener motor axon terminals as frequency increases. Stimulation of opener excitor (A) and excitor and inhibitor terminals (B and C) at 2 different frequencies (5 and 15 Hz in A and B; 2.5 and 10 Hz in C) leads to spot formation at the lower frequencies (A, 1 and 3, B1, and C1) and to differential brightening of individual spots at the higher frequencies (A, 2 and 4, B2, and C2). Plots of relative intensity increase for individual foci (indicated by lower case letters, a–m) show a wide range in the relative increase of spot brightness at the higher frequency (A5, B3, and C3). Highly visible spots emerge in locations in which the initial fluorescence was very weak (e–g). Scale bars: 5 μm.

individual spots increased, and they often became larger. In many of the varicosities, some of the initially dim regions brightened strongly at higher frequencies (Fig. 10). Frequency-dependent changes in transmission took place within or around spots that had been already established at 20 Hz, and there was evidence that new regions adjacent to those apparent at the lower frequency became brighter at a higher frequency. Thus enlargement of existing spots could involve addition of new foci to a fused cluster. Individual spots did not show uniform behavior: the relative changes in brightness varied among them when stimulation frequency was increased, as illustrated in Fig. 10.

Similar evidence for nonuniformity among identified bright spots of the tonic extensor terminals appeared in destaining experiments in which individual spots were followed (Fig. 11). Bright spots in previously loaded terminals lost fluorescence at different rates during restimulation. Some spots disappeared completely during the destaining cycle (Fig. 11, F and G). These observations indicate that synapses associated with separate bright spots on the terminal emit vesicle-associated dye at different rates, supporting the hypothesis that synapses on a terminal are not uniform in their physiological behavior.

DISCUSSION

The major new findings of the present study are as follows: synaptic physiology of three different motor axons (2 tonic and 1 phasic) is well correlated with their patterns of uptake and release of the fluorescent indicator FM1-43; terminals of a single tonic motor neuron possess
TABLE 2. Quantitative analysis of phasic and tonic varicosities via confocal and electron microscopy

<table>
<thead>
<tr>
<th></th>
<th>Phasic</th>
<th>Tonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confocal microscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of varicosities analyzed</td>
<td>45</td>
<td>93</td>
</tr>
<tr>
<td>Number of spots</td>
<td>54</td>
<td>353</td>
</tr>
<tr>
<td>Number of spots per varicosity*</td>
<td>1.2 ± 0.08</td>
<td>3.84 ± 0.034</td>
</tr>
<tr>
<td>Planar area of spot, (\mu m^2)*</td>
<td>1.46 ± 0.12</td>
<td>2.14 ± 0.08</td>
</tr>
</tbody>
</table>

| Electron microscopy  |        |       |
| Number of varicosities analyzed | 9      | 5     |
| Number of synapses     | 59     | 96    |
| Number of synapses per varicosity | 6.56   | 19.20 |
| Contact area of synapse, \(\mu m^2\)* | 0.14 ± 0.02 | 0.38 ± 0.03 |

* Values are means ± SE.

Two axons of the tonic type (the tonic extensor and OE axons) differ in transmission properties. The OE terminals have a higher rate of transmitter release at low frequencies and show depression sooner when stimulated at high frequencies. These differences correlate with lower in vivo operating frequency of the OE axon (\(~5–10\) Hz) (Wilson and Davis 1965) compared with the higher average frequency of the tonic extensor neuron (\(~20–30\) Hz) (Bradacs et al. 1997). The results suggest that within the tonic neuron category, synaptic properties are adjusted appropriately to meet the activity required for locomotion. Such adjustment (long-term adaptation) was shown experimentally for phasic neurons (Lnenicka and Atwood 1985a,b).

Dye uptake and release related to synaptic output

The differences in transmitter release are well correlated with the markedly different rates of FM1-43 uptake and release observed in this study. Phasic extensor terminals were labeled at relatively low frequencies of stimulation (Fig. 3), whereas tonic extensor terminals did not develop well-defined fluorescent spots at frequencies \(>10–20\) Hz. The OE terminals, known to have higher quantal content than the tonic extensor terminals at low frequencies, were labeled at frequencies of \(2–10\) Hz. Qualitatively, observations with FM1-43 can be used to compare synaptic efficacy of different nerve terminals. The observations are in accord with previous studies showing that FM1-43 changes reflect vesicle turnover at synapses (Betz and Bewick 1993; Betz et al. 1992a, 1994).

**Fig. 8.** Comparison of FM1-43 uptake and loss in tonic and phasic extensor terminals. A: comparison of relative dye intensity in tonic (●) and phasic (○) terminals after uptake in 2 \(\mu M\) FM1-43 at 20 Hz for 7.5 min, 30 Hz for 5 min, and 50 Hz for 3 min while maintaining a constant number of pulses (9,000) at each frequency. Data were normalized to the initial intensity value after 20-Hz stimulation and corrected for dye loss from vesicles. For each point, 3–4 spots were measured in each of the 6 preparations for a total of 18–24 bright spots. B: destaining of tonic terminals (●) and phasic terminals (○) at 10-Hz stimulation over 25 min. Terminals were loaded in 2 \(\mu M\) FM1-43. Data were obtained from 4 tonic and 5 phasic preparations and were normalized to the values measured just after the loading stimulation.
Nature of individual bright spots

The number of bright spots on varicosities is less than the number of synapses revealed by serial electron micrographs of similar sized varicosities (Table 2, Fig. 5). Both synapses and bright spots increase in number with the size of the varicosity (Figs. 4 and 5), but individual synapses exceed bright spots in number. Phasic varicosities usually show one or two bright spots after stimulation, whereas electron micrographs reveal on average six synapses (Table 2). For the tonic axon, one or two spots were seen on small varicosities with an approximately linear increase in the number of bright spots as varicosity size increases (Fig. 5). On average, four spots were seen. The number of synapses on reconstructed varicosities is greater, averaging 19 (Table 2), and is scaled to the size of the varicosity (Fig. 5). Moreover, the size of the bright spots (1.46 ± 0.12 \( \mu m^2 \) for phasic terminals, 2.14 ± 0.08 \( \mu m^2 \) for tonic terminals) is 9–10 times larger than for reconstructed synapses (mean size, 0.2–0.4 \( \mu m^2 \) for both axons). Thus a single bright spot likely represents one or more synapses with associated synaptic vesicles (see also Wang and Zucker 1998). Earlier work with vesicular uptake of the electron-dense marker horseradish peroxidase has shown that clouds of vesicles near synapses are larger than the synapses themselves and may merge or overlap (Thompson and Atwood 1984). Furthermore, mixing of labeled and unlabeled vesicles occurs in the pools of vesicles near synapses (Holtzman et al. 1971; Thompson and Atwood 1984). With sufficient stimulation, the entire pool of vesicles near an active synapse is likely to contain vesicles labeled by FM1-43. This behavior was evident in several earlier studies on frog motor nerve terminals (Betz and Bewick 1992; Betz et al. 1992b; Henkel et al. 1996a).

Relationship between labeled spots and binomial parameters

A test of the interpretations to be drawn from the Poisson and binomial statistical descriptions of quantal release is provided by observations of the number of fluorescent spots in tonic terminals and comparison with calculations of the binomial parameter \( n \). At stimulation frequencies of \( \leq 10 \) Hz, most recording sites on tonic extensor terminals gave quantal release patterns best described by Poisson distributions; correspondingly, bright spots were not apparent. Both observations suggest generally low release from an undefined number of synapses. At 20 Hz, bright spots are reliably induced, and most of the recording sites produced quantal release best described by binomial distributions (Table 1). At this frequency, the number of bright spots on tonic varicosities coincides approximately with the values for binomial \( n \) calculated from the best-fitting distributions (Table 1). This suggests that bright spots, consisting of one or more synapses participating in release, correspond reasonably well to the estimated val-

**Fig. 9.** Frequency dependence of destaining of tonic terminals loaded with FM1-43. A: decline of fluorescence intensity of tonic nerve terminal varicosities after loading in 2 \( \mu M \) FM1-43 for 3 min at 50-Hz stimulation. Measurements made every 5 min reveal the curve for normal decline in staining intensity due to dye loss from synaptic vesicles. Best-fitting second-order curve was generated by Sigma Plot. . . . fluorescence lost after 20 min without imaging. B: tonic terminals were destained at 20-Hz (●), 30-Hz (●), or 50-Hz (●) stimulation (using 9,000 pulse trains at each frequency) after FM1-43 dye loading. Loading was done by bathing the preparation in 2 \( \mu M \) FM1-43 and stimulating for 3 min at 50 Hz. After loading, the dye was washed out for 30 min before destaining began. Destaining with no stimulation (●) is provided for comparison. C: destaining data from B corrected for decline of fluorescence due to activity-independent loss of dye. Dye loss due to stimulation alone is significant only at 50 Hz in this group of terminals.
FIG. 10. Frequency-dependent changes in uptake of FM1-43 and nonuniform behavior of individual foci in tonic terminals. A, 1–3: stimulation of the tonic axon at 20, 30, and 50 Hz, respectively (in 2 μM FM1-43) shows more intense staining of the same nerve terminal varicosities (a–e) with increasing stimulus frequency. A4: measurement of relative intensity for varicosities a–e from A, 1–3 above shows the increase in intensity with increasing stimulus frequency. Individual varicosities vary in their relative intensity changes at higher frequency. B and C: different subregions of varicosities (a) and (e) exhibit differential changes as frequency is increased from 20 Hz (B1 and C1) to 50 Hz (B2 and C2). Graphs of the relative intensity changes (B3 and C3) illustrate different rates of brightening at the higher frequencies for subregions of varicosities (a) and (e) respectively. Additional subregions of fluorescence (h, j, and m) are added to preexisting spots at the higher frequency. Scale bar: A, 1–3, 10 μm; B, 1 and 2, and C, 1 and 2, 5 μm.

ues of n. To demonstrate this more clearly, it will be necessary to match the statistical calculations and the dye labeling for the same varicosities, and in addition to obtain correlated electron microscopic images of labeled active synapses, using a technique such as that described by Henkel et al. (1996a) to make FM1-43 labeled vesicles visible in the electron microscope. This experiment is technically difficult but not impossible: however, our preliminary attempts with the method of Henkel et al. (1996a) have not been successful in crustacean terminals. As indicated above, in many cases the binomial parameter n and the bright spots may represent a group of associated synapses rather than a single synapse. If this is the case, synaptic transmission among nearby synapses may be coupled rather than independent. This is suggested also by the observation that bright spots expand at higher frequencies, possibly by recruiting additional synapses at their edges (Figs. 7 and 10). The mechanistic basis for possible coupling of nearby synapses and synaptic recruitment is not presently apparent.

Both fluorescent labeling and statistical results were different for the OE axon. Overall quantal content at low frequencies is generally higher and binomial distributions usually fit the data best (Cooper et al. 1995b; Johnson and Wernig 1971). Correspondingly, our present observations show that varicosities of the crayfish opener muscle consistently develop discrete bright spots at lower frequencies than in the tonic extensor neuron. Clearly, the two axons are adapted to operate over different frequency ranges and their overall patterns of transmitter release differ (Fig. 1) (Bittner 1968; Bradacs et al. 1997). For both axons, frequencies giving rise to binomial distributions of quantal release correspond with frequencies at which discrete bright spots can be induced reliably.

Formation of small numbers of fluorescent spots in restricted regions of tonic varicosities (Fig. 4), taken in conjunction with the more widespread distribution and larger number of synapses found in serial reconstructions from electron micrographs, supports the view that synapses do not participate equally in transmitter release (Wojtowicz et al. 1994). The alternative model based on vesicle mobilization to any available release sites (Worden et al. 1997), implying participation of all synapses and no limitation on release sites, is less well supported by the present observations on FM1-43 labeling. If only some of the available synapses participate, as implied by
the present results, the distinction between the two alternatives may disappear, because an active synapse must of necessity possess docked and primed vesicles, and preferential docking and priming at a subset of synapses may impart a higher probability of release to that subset.

**Frequency-dependent effects**

Phasic and tonic terminals differ in their degree of frequency facilitation (Bradacs et al. 1997), and this is reflected in the larger changes in intensity of FM1-43 spots of tonic terminals as frequency of stimulation is increased. Loading of FM1-43 is dependent on frequency as well as on the number of impulses. In the crayfish opener muscle, we found that the number of bright spots increased when the frequency was raised from 1–5 to 10–20 Hz, indicating recruitment of additional active synapses to the response pool. In the extensor tonic axon, studied at higher frequencies, initially dim regions of the varicosities brightened when the frequency was raised from 20 to 50 Hz, indicating frequency-dependent increase of release in locations of initially low activity. Thus it is possible that additional synapses associated with preexisting bright spots may be recruited at higher frequencies. However, some synapses on the terminal probably remain “silent,” because many regions of the large tonic varicosities remain blank (Fig. 4) even though synapses would be expected to occur all over the varicosities as judged from serial reconstructions from electron microscopy (Cooper et al. 1995b; Msghina et al. 1998). Evidence for recruitment of initially “silent” synapses by serotonin application has been reported recently for the crayfish OE neuron by Wang and Zucker (1998).

Evidence of nonuniform behavior of individual bright spots was also evident from their different rates of staining and destaining (Figs. 9–11). Nonuniform staining and destaining of individual spots in the frog neuromuscular junction was shown by Betz et al. (1992b). The observation supports the general hypothesis that synapses on a nerve terminal operate with different release probabilities (Smith et al. 1991). Morphological counterparts of more active and less active synapses have been proposed: “complex” synapses, with more than one active zone, could have a higher probability of transmitter release (Cooper et al. 1996b). To prove this point more conclusively, it will be necessary to match FM1-43 spots with electron microscopic observations of the same region.

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