Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Genetic evidence for an equilibrium between docked and undocked vesicles

Jing Li and Thomas L. Schwarz

Phil. Trans. R. Soc. Lond. B 1999 **354**, 299-306
doi: 10.1098/rstb.1999.0381

References Article cited in:
http://rstb.royalsocietypublishing.org/content/354/1381/299#related-urls

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: http://rstb.royalsocietypublishing.org/subscriptions

This journal is © 1999 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

THE ROYAL SOCIETY

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Genetic evidence for an equilibrium between docked and undocked vesicles

Jing Li and Thomas L. Schwarz*

Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305-5345, USA

By using the *shibire* mutation to block endocytosis in a temperature-dependent fashion, we have manipulated the number of synaptic vesicles in a nerve terminal and have observed a remarkable proportionality of the number of quanta released to the size of the total vesicle pool. In the experiments described below we determine that approximately 0.3% of the vesicle pool is released per stimulus. The data suggest that the pool of readily releasable docked vesicles does not represent the saturation of a limiting number of release sites, but instead represents a subset of vesicles that is in equilibrium with the larger pool of vesicles. Before presenting this data and the significance of the finding for the regulation of neurotransmission, we will briefly review the use of *Drosophila* genetics as a tool for dissecting synaptic transmission.

Keywords: synaptic vesicle; release site; docking; *Drosophila*; *shibire*; endocytosis

1. THE GENETIC APPROACH TO SYNAPTIC TRANSMISSION

Genetic experiments have proven to be a potent companion to biochemistry and electrophysiology for dissecting the mechanistic basis of membrane trafficking in higher organisms. Yeast genetics has had a remarkable impact on the field and the convergence of yeast genetics and biochemical studies in higher eukaryotes has led to the clear impression that a very similar set of proteins may provide the fundamental scaffolding for membrane targeting, membrane fusion and membrane retrieval across all eukaryotic species and cell types. Also emerging from these studies is evidence that exocytosis at the cell surface shares many mechanistic similarities to membrane fusion in intracellular trafficking. However, lest we presume too quickly that all cells will be identical and all fusion processes the same, we should bear in mind other physiological processes such as muscle contraction and E–C coupling. Though highly similar proteins are involved in every cell type (e.g. actin, myosin, troponins, Ca^{2+} channels and ryanodine receptors), there are enormous differences in the regulation of those proteins and in their interactions when one compares smooth muscle to skeletal muscle. Proteins critical to contraction in one cell type may be dispensable in another.

Particularly in the highly specialized structures of neuronal synapses, we may find a great many variations played out with the now familiar components of the vesicles, plasma membrane and cytosol. Thus, while the power of yeast genetics may be unmatched for dissecting fundamental mechanisms of general membrane fusion, additional systems are necessary if we are to understand how neurons have harnessed and adapted those mechanisms for the special requirements of synaptic transmission. Those requirements include the maintenance of a stable pool of vesicles that are capable of fusing within 200 μs of an action potential, specialized active zones that restrict secretion to a small portion of the cell surface, Ca^{2+} sensors to regulate exocytosis with great precision, rapid mechanisms for the retrieval and recycling of vesicle membranes, and a capacity for subtle modulations of the amount of transmitter to be released.

In our laboratory, the use of *Drosophila* genetics has helped us to understand the synapse in three distinct manners. The most frequent approach has been to try to illuminate the function of a protein by observing the consequences of its removal. Biochemical interactions and binding properties can propose plausible hypotheses for the proteins of the synapse but, particularly when good pharmacological agents are not available, genetics can serve to put these hypotheses to the test and determine the *in vivo* significance of an individual protein. To this end, we have made and studied mutations in synaptotagmin, VAMP–synaptobrevins, and syntaxinl. In a series of papers, we demonstrated that the *synaptotagmin* gene in *Drosophila* was important for exocytosis, but not essential, and that synaptotagmin appears to be necessary for maintaining the docked pool of vesicles in the nerve terminal (Broadie *et al*. 1994; DiAntonio *et al*. 1993; DiAntonio & Schwarz 1994; Reist *et al*. 1998). Indirect evidence suggests a role for synaptotagmin in the biogenesis or recycling of vesicles as well (Reist *et al*. 1998). Our studies of syntaxin (Burgess *et al*. 1997; Parfitt *et al*. 1995) in conjunction with those of others (Broadie *et al*. 1995; Schulze & Bellen 1996; Schulze *et al*. 1995) have indicated that the same isoform of syntaxin that is essential for transmitter release, syntaxinl, is necessary for cell viability in non-neuronal cells and is necessary for the proper cellularization of the embryonic blastoderm. Thus, though in many cases there are a plethora of isoforms of trafficking proteins that appear to be

*Author for correspondence.

 © 1999 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

specialized for a particular task, in the case of syntaxin—a putative docking and fusion protein (Sollner *et al*. 1993*a*,*b*; Sollner & Rothman 1996)—the same isoform is used in very different types of exocytosis. The lack of specificity of this gene for fusion at active zones and the apparent persistence of vesicle docking in its absence (Broadie *et al*. 1995) greatly undermine the hypothesis that it serves as an address label by which a particular subset of vesicles may recognize their appropriate target.

The second potential contribution of *Drosophila* genetics, one which has yet to be exploited consistently, is the ability of genetic screens to uncover new members of the synaptic apparatus. Despite a great deal of progress in identifying proteins that are likely to contribute to the final stages of docking and fusion, there remain many unanswered questions. What keeps vesicles morphologically docked at active zones in the absence of SNARE proteins? What proteins make up the ribbons and dense bodies that are seen at many release sites? How do these structures influence secretion? What proteins keep vesicles clustered in the vicinity of an active zone? If synaptotagmin is not the major Ca^{2+} sensor for fusion, what is? Which proteins mediate the regulation of transmitter release by second messenger systems such as cAMP and protein kinase C? What mechanism aligns the presynaptic active zone directly above the postsynaptic receptors? What proteins appear to provide the filamentous links from vesicle to vesicle and how do these links influence terminal function? We cannot answer these questions with the known proteins of the nerve terminal; there are probably many more components to be identified. We and others are embarking on this task.

The third benefit of a genetic strategy, however, has been the opportunity to ask essentially physiological questions that were not originally phrased in terms of proteins and mechanisms, but in which a mutation has helped to shed light on an issue. This paper will focus on one such case in which the ability of a mutation to disrupt endocytosis has permitted an examination of the relationship of the docked pool to the rest of the readily releasable pool of vesicles in a terminal. Another example might be found in our recently published studies of the *neuronal-synaptobrevin* gene (Deitcher *et al*. 1998), in which we determined that at least a portion of the spontaneous fusions of synaptic vesicles with the plasma membrane (minis) proceed by a mechanism that is distinct from that which mediates action-potential evoked release (EPSPs); the two forms of exocytosis were genetically separable and therefore appear to have distinct mechanistic requirements.

2. VESICLE POOLS AND VESICLE DOCKING

Early studies of transmitter release invoked the presence of distinct pools of transmitter within the nerve terminal in order to explain the complexities of synaptic behaviour (Agoston *et al*. 1985; Birks & MacIntosh 1961; Zimmermann & Denston 1977; Zimmermann & Whittaker 1977). These studies were primarily electrophysiologic and biochemical in nature and soon found putative counterparts in morphological studies of the synapse (Couteaux & Pecot-Dechavassine 1973). Three pools can be distinguished. The first consists of vesicles that are ready to be released by the arrival of an action potential and can fuse with a latency of just hundreds of microseconds. Because this latency permits no time for multiple chemical reactions or for the extensive transport of vesicles, this pool has come to be equated with a pool of morphologically docked vesicles that sit immediately adjacent to the plasma membrane and may have undergone preliminary biochemical changes that are often called priming. It is generally presumed that the sites of docking are a specialization of the active zone, but the biochemical nature of docking and of those release sites remains a matter of intensive study. Depending on the probability of release at a given docking site, a greater or smaller subset of the docked vesicle pool will be released in response to an action potential. The second pool of vesicles consists of an available pool (sometimes called readily releasable) that is competent for undergoing release by stimulation, but not with the short latency of those vesicles that are already docked. These vesicles may represent the pool of vesicles that are always observed to cluster around the specializations of the active zone but which are not necessarily in direct contact with the plasma membrane. The third pool of vesicles is the reserve pool, a pool that has been detected by biochemical, anatomical and electrophysiological means, and which comprises vesicles that are held back and do not appear to be free to dock and fuse under normal circumstances. Only extreme stimuli—or perhaps neuromodulators—can activate this reserve pool so that it too can become available for release.

These pools remain largely intellectual constructs at present. We do not know which biochemical interactions constitute docking and priming, though it seems highly likely that synaptotagmin, syntaxin, VAMP–synaptobrevin and SNAP-25 are involved. Nor do we know what distinguishes reserve pool vesicles from those available for release, though the synapsin proteins are candidates for this role and interactions with cytoskeletal proteins could serve to restrain the vesicles in the reserve pool (Pieribone *et al*. 1995). In addition, some fundamental issues about vesicle docking remain unresolved. Is the process a one-way street in which vesicles move up to release sites and then remain docked until they are allowed to fuse? Do they move on and off release sites? Is there such a thing as a discrete release site or is the entire active zone paved with proteins and lipids that are competent to dock and fuse vesicles? If release sites are discrete, how many are present at an active zone? Are they normally saturated with bound vesicles or are they only partially occupied?

In the experiments described below, we took advantage of the *shibire* mutation, one of the oldest neurological mutations in *Drosophila* (Grigliatti *et al*. 1973; Ikeda *et al*. 1976; Koenig *et al*. 1983, 1989; Kuromi & Kidokoro 1998; Poodry & Edgar 1979). This mutation blocks endocytosis at the stage where vesicles pinch off from the plasma membrane. Because it is a temperature-dependent mutation, it allows us to switch on and off the recycling of synaptic vesicles at will. This in turn permits us to uncouple exocytosis from endocytosis and therefore permits us to manipulate the total number of vesicles in a terminal. From these studies, we have come to favour a model in which docking is a reversible process and in

Downloaded from rstb.royalsocietypublishing.org

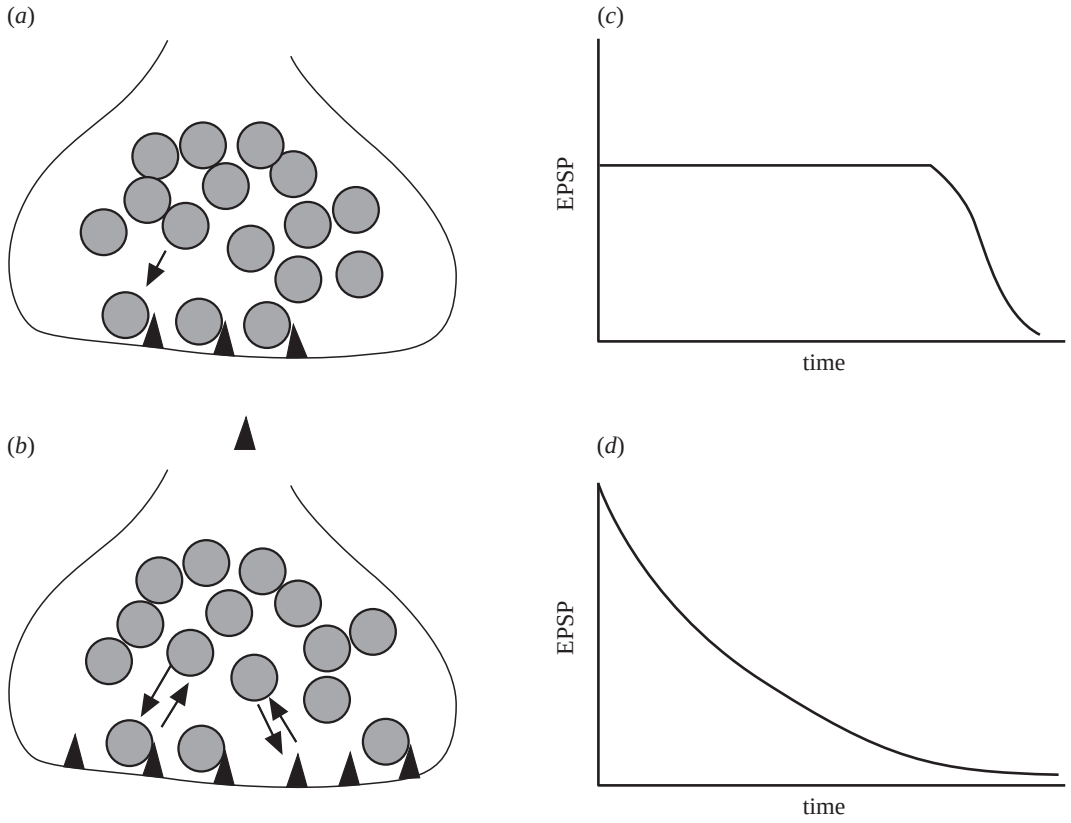

Figure 1. Models of the relationship of the vesicle pool to docking sites. (*a*) A model in which docking sites are few and saturated with docked vesicles. This saturation can arise either because the docking reaction is in essence irreversible or because there are so few docking sites and so many vesicles that every docking site has a bound vesicle almost all the time. (*b*) An alternative model in which a large number of release sites is found in a terminal and in which an equilibrium between docked and undocked vesicles determines that a percentage of the release sites will be occupied. In this model, docking is a reversible process and the number of release sites occupied will be determined by vesicle number as well as by the rate constants for docking and undocking. (*c*) A diagram of the predicted effect on transmitter release of a gradual reduction in the number of vesicles in a terminal such as that in (*a*). Because the small number of docking sites are saturated with bound vesicles, the EPSP would remain constant despite the shrinking vesicle pool until a drastic reduction in vesicle number had occurred. At that point, docking sites would outnumber available vesicles and the number of vesicles fusing per stimulus would drop sharply. (*d*) As in (*c*), the effect of a gradual reduction in vesicle number is depicted but this time for a synapse as shown in (*b*). Because of the equilibrium between the docked and undocked population, a slow reduction in total vesicle number would be reflected in a gradual decline in the docked population as well and an increase in the number of vacant release sites.

which an equilibrium is established between the docked pool of vesicles and the remainder of the vesicles in the terminal.

3. THE RELATIONSHIP OF DOCKED VESICLES TO THE POOL OF UNDOCKED VESICLES

A hypothetical experiment may serve as a good introduction to our experiments to probe the nature of the docked state. Let us first consider a synapse in which docking was either an irreversible process or in which the vast oversupply of releasable vesicles relative to docking sites insured that the docking sites were saturated (as cartooned in figure 1*a*). What would happen to the amplitude of the EPSP at such a synapse if the number of vesicles was gradually reduced? Because docking sites at this synapse would always be occupied and because the number of docking sites has not changed, there would be no change in the number of docked vesicles. Because the amount of release is proportional to the number of docked, fusion-ready vesicles, there should be no change in the amplitude of the EPSP. Only when a very large decrease in the number of vesicles in the terminal had been made would empty docking sites be found, either because the number of docked sites now exceeded the number of vesicles available to occupy them or because the numbers of vesicles and docking sites became sufficiently comparable that saturation no longer occurred. If one plotted EPSP amplitude at such a synapse as a function of time during which the vesicle number declined, one would predict a curve like that in figure 1*c* in which the amplitude is constant for an extended period during which the excess vesicles are released and then falls off suddenly once this excess has been exhausted. Now let us consider the alternative synapse in which docking sites are numerous and a true equilibrium exists between the docked pool and undocked pool as vesicles move on and off the release sites (figure 1*b*). At such a synapse, a gradual decline in total vesicle number would immediately be mirrored with a gradual decline in the fraction that were occupying docking sites. As the total fell, so too would the number of docked

Downloaded from rstb.royalsocietypublishing.org

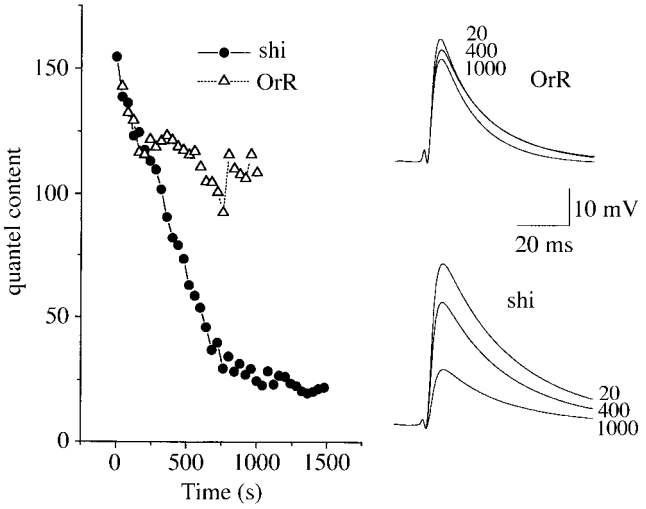

Figure 2. Repetitive stimulation at *shibire* neuromuscular synapses causes a progressive decrease in quantal content. Just prior to the onset of recording, both wild-type (OrR) and *shibire* (shi) flies were shifted to 30 °C. Intracellular recordings were made from muscle 6 or 7 of the abdominal body wall muscle (DiAntonio & Schwarz 1994) in HL3 saline (Stewart *et al*. 1994), and the nerve to that hemisegment was stimulated at 0.5 Hz. In the wild-type synapse, only a brief decline is observed at the start of the stimulation, apparently in response to the temperature shift. Thereafter, the EPSP remains constant in amplitude until the recording was terminated. In contrast, in the *shibire* mutation in which recycling is blocked, a progressive decline in the amplitude is observed throughout the experiment. Each point is the average of 20 responses and for each EPSP, the amplitude was corrected for non-linear summation (Martin 1955). Quantal content was determined by dividing the corrected peak amplitude by the mean amplitude of spontaneous miniature synaptic potentials. The traces at right illustrate averages of ten events at the time points indicated (in seconds).

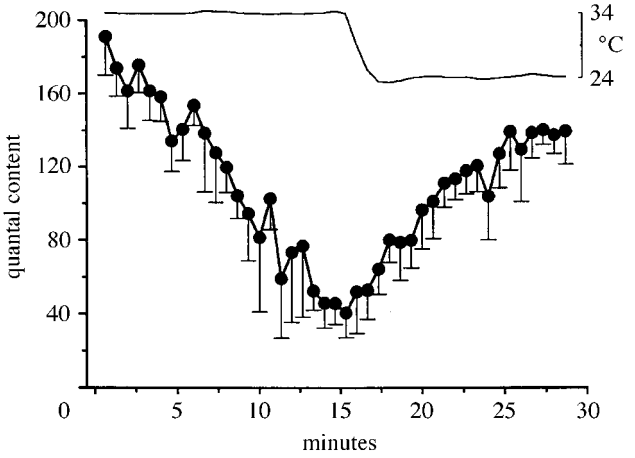

Figure 3. The progressive decline in EPSP amplitude in *shibire* is reversible. A neuromuscular preparation was stimulated at 0.5 Hz and the temperature of the preparation was altered as shown in the upper tracing, a monitor of the chamber temperature. While at 34 °C, quantal content progressively declines as the vesicle pool is depleted. Once the preparation is restored to the permissive temperature of 24 °C, however, recycling can resume and the synaptic response is observed to recover over a period of 15 min.

vesicles and, consequently, so too would the amplitude of the EPSP (figure 1*d*).

At most synapses, this experiment cannot be done because there is no way in which the vesicles can gradually be removed. The endocytotic pathway is so efficiently matched to fusions that only the most drastic of stimuli will deplete a terminal of vesicles. In *Drosophila*, however, the *shibire* mutation can be used to completely shut down endocytosis in a temperature-dependent fashion (Koenig *et al*. 1983, 1989; Kuromi & Kidokoro 1998; Poodry & Edgar 1979). The blockade of endocytosis appears to be complete; prolonged stimulation has been shown by electron microscopy to completely deplete synaptic terminals of vesicles (Koenig & Ikeda 1989). At a neuromuscular synapse in *shibire* at the non-permissive temperature, each action potential will deplete the vesicle pool by the number of quanta that have been released.

To examine the effects of vesicle depletion on the amplitude of the EPSP, we examined the neuromuscular junction of wild-type (OrR) and *shibire* larvae. An example is shown in figure 2 in which the temperature has been shifted to 30 °C just before the recordings were made. The nerves were stimulated at 0.5 Hz. In the OrR control, there is a small initial decrease in the amplitude of the EPSP but thereafter the amplitude is constant for the duration of the experiment, sometimes for as long as 45 min. In contrast, the *shibire* flies show a steady decline in the amplitude of the EPSP throughout the experiment such that by 15 min the EPSP has been reduced to about 15% of its original amplitude. Such a decline was never observed in either wild-type controls or *shibire* larvae at the permissive temperature where endocytosis could occur. If this phenomenon were due to an unrelated deleterious effect of the temperature on the viability of these mutant synapses, it should not be reversible. On the other hand, if the reduction is due to the decline in the vesicle pool during the experiment, EPSP amplitude should recover if the temperature is lowered and endocytosis is allowed to refill the pool. An example of such an experiment is shown in figure 3. The nerve was stimulated at 0.5 Hz at 34 °C to cause the reduction and was then returned to a permissive temperature (24 °C). The decrease in EPSP amplitude was indeed largely reversible and recovered within 15 min to 75% of its initial amplitude. This time-course of recovery is comparable to what has been observed for the restoration of synaptic vesicles in similar conditions, most recently as monitored by incorporation of FM1-43 (Kuromi & Kidokoro 1998). Thus this phenomenon of a declining EPSP may be a genuine reflection of the changes in the vesicle pool in that it is specific for the *shibire* genotype, occurs only at the non-permissive temperature, is reversible and reverses with an appropriate time-course. The observed gradual decline is consistent with the model presented in figure 1*b*,*d*.

If the decreasing EPSP amplitude reflects an equilibrium between the declining vesicle pool and the subpool of docked vesicles, the decline should depend on the rate at which vesicles are released from the terminal. We tested this prediction in several ways. When extracellular Ca^{2+} was reduced and the quantal content of each EPSP was consequently decreased, we found that the rate of decline of the EPSP was appropriately slowed (data not shown). We also predicted that the extent of the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

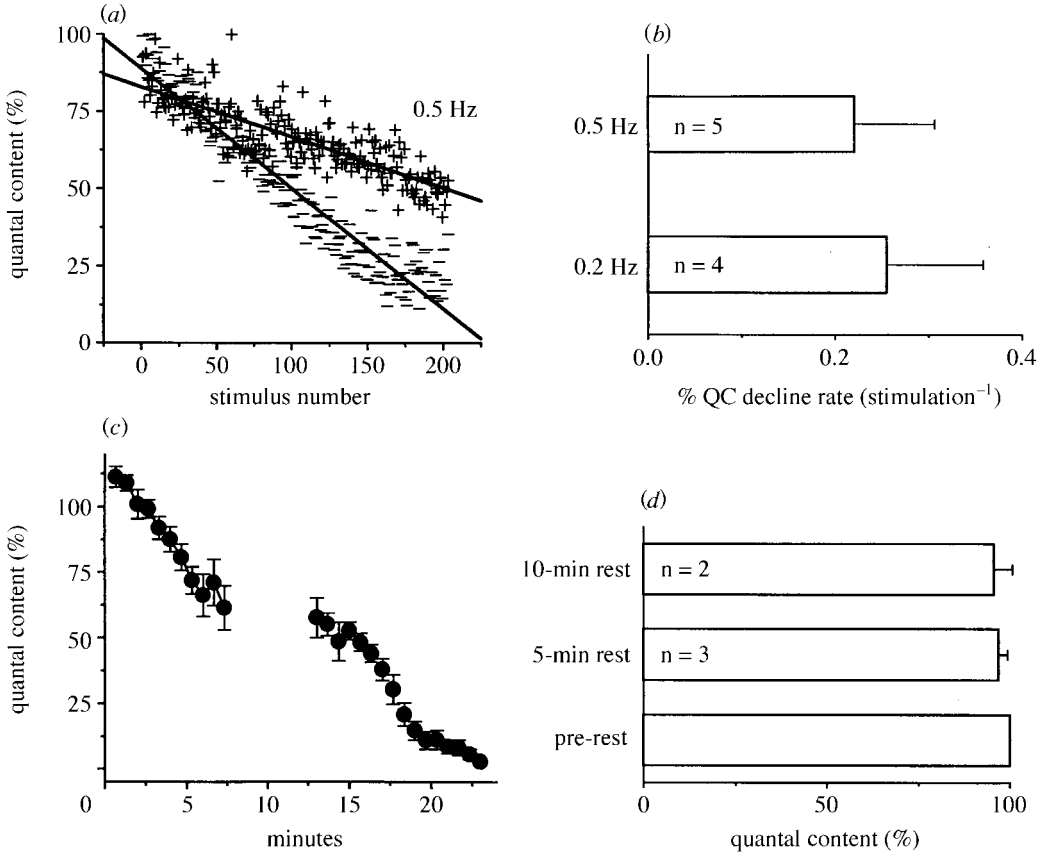

Figure 4. Decline of transmitter release in *shibire* correlates with and is dependent on stimulation. (*a*,*b*) To determine if the decline in quantal content was due to a stimulus-dependent phenomenon and to determine if a slow docking rate might explain the decline, the rate of decline was compared for preparations stimulated at 0.2(−) and 0.5(+) Hz. An individual pair of examples is shown in (*a*) and the results of all the experiments are graphed in (*b*). The rate of decline was not significantly different when expressed as a per cent decrement per stimulus. (*c*,*d*) The hypothesis that the decline was due to a decline in available vesicles was tested more stringently by stopping the stimulus altogether and determining if the synapse could recover with a 5 or 10 min rest at the non-permissive temperature. A preparation was stimulated at the non-permissive temperature for 7 min, until the quantal content had declined to 50% of its initial value (*c*). When stimulation was resumed after a 5 min gap in stimulation, the amplitude of the response had neither recovered nor further declined from the value before the rest. This finding was consistently observed after both 5 and 10 min pauses in stimulation (*d*).

decline should be a function of the number of stimuli and independent of the rate of stimulation. In contrast, if an equilibrium did not exist and instead the decline reflected a slow rate for the refilling of emptied release sites, we would anticipate that less of a reduction would occur if the stimuli are presented at a lower rate. Therefore, to determine if the decline in EPSP amplitude correlates well with the decrease in pool size, we compared the rate of decline during stimulation at 0.2 Hz and 0.5 Hz (figure 4*a*,*b*) and plotted the EPSP size against the stimulus number. The increased interval between stimuli at 0.2 Hz did not slow the decline by permitting more vesicles to dock. To put the hypothesis to a more stringent test, we determined if extended periods of rest would allow the EPSP to recover as vesicles moved to release sites. A representative experiment is shown in figure 4*c*, in which a preparation was stimulated at 0.5 Hz until the EPSP amplitude had declined to half of its original value and the stimulation was then suspended for 5 min before resuming stimulation. The amplitude of the EPSP when stimulation was resumed was just what it had been before the rest period and indeed even 10 min of rest (at 34 °C) failed to restore the EPSP (figure 4*d*). Thus the behaviour of the synapse closely matched expectations for a synapse in which the docked sites were not saturated and in which docking was a reversible phenomenon that created an equilibrium between docked and undocked vesicles.

To test this hypothesis further, we reanalysed the data from the *shibire* synapse in figure 2 to determine if the number of vesicles released was proportional to the number of releasable quanta in the synapse at any given time. We determined the number of releasable vesicles that had been present at the start of the experiment by summing the quantal content of all the EPSPs that occurred during the experiment. Because the mini frequency is only a few per second at these synapses and the evoked responses were 20–150 quanta, we ignored the minor contribution of spontaneously released vesicles. As the synapse had not been completely emptied by the end of the experiment, this number is probably an underestimate. At each subsequent time-point in the experiment, the number of remaining vesicles could be calculated by

Downloaded from rstb.royalsocietypublishing.org

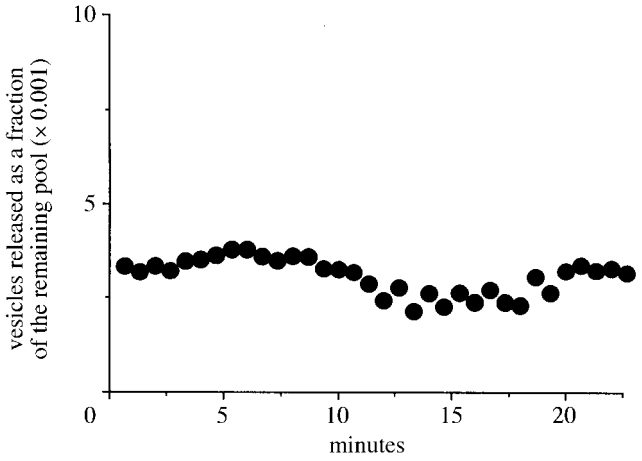

Figure 5. The quantal content of the synapse is proportional to the releasable vesicles remaining in the terminal. The data from the *shibire* neuromuscular synapse in figure 2 were replotted to describe the relationship of the quantal content to the total releasable vesicles in the terminal and this value was found to be remarkably constant over a period in which the quantal content of the response declines by a factor of 8. To determine the initial total releasable pool, the number of quanta released by each impulse was summed for the duration of the experiment. Because recycling was blocked by the mutation, each releasable vesicle can be released only a single time. To determine the remaining releasable pool at a given subsequent time-point, the number of quanta released by all preceding stimuli was subtracted from the initial value. The quantal content of each event was divided by the size of the remaining releasable pool and plotted against time.

subtracting from the initial value all of the quanta that had been released up to that point. The quanta released at each time-point were then plotted as a function of the estimated remaining vesicles at that time (figure 5). This relationship proved to be remarkably constant. While the amplitude of the EPSP declined eightfold over a 22 min period, the fraction of the releasable pool that was released by a single impulse remained fixed at 0.3%.

4. CONCLUSIONS

At a glutamatergic synapse in *Drosophila*, we have taken advantage of a mutation that permits us to conditionally suspend endocytosis in order to investigate the relationship of transmitter release to the vesicle population of the synapse. The blockade of endocytosis gave rise to a progressive decline in the amplitude of the EPSP that began immediately upon onset of the blockade. These findings can best be explained by hypothesizing an equilibrium between the releasable pool of vesicles in the terminal and the docked vesicles that are available for release by a given action potential. Were docking sites the limiting factor in the terminals (due to saturating numbers of surplus vesicles or the irreversibility of docking), the terminal would have maintained a constant amplitude EPSP until the depletion of vesicles was severe. Alternative explanations of the data, such as an irreversible run-down of the synapse or a slow rate of refilling of the sites, are not compatible with the reversibility of the response at the permissive temperature, the requirement for stimulation to see the decline and the complete absence of recovery when the preparation is rested at the non-permissive temperature.

We considered two other possible explanations as well. Might the fusion of the vesicles in the absence of endocytosis prevent future fusions because the release sites are not freed for reuse? This does not appear to be a satisfactory explanation for the following reasons: (i) electron microscopy has demonstrated that all the vesicles can be cleared from a *shibire* terminal with sufficient stimulation (Koenig & Ikeda 1989). Were the first rounds of release to have blocked release sites, an unreleasable pool of residual vesicles should have been seen; (ii) if unrecycled vesicles had partially interfered with subsequent fusions, we would have expected to see these sites disinhibited over time as the membrane and proteins were allowed to move laterally from the release sites. Yet we saw no recovery of EPSP amplitude even with 10 min of rest; (iii) the amplitude of the EPSP as a fraction of remaining vesicles remained constant during the experiment. This finding was predicted by the equilibrium model but not by blockade models. If release sites were blocked every time a vesicle fuses but is not recovered, the amplitude of the EPSP should have dropped suddenly and precipitously with the very first stimulations. If the blockade develops only after a critical mass of membrane has been added to the surface, there should have been very little decrement early in the experiment. A second alternative model invokes a hypothetical cytosolic factor that might be consumed by vesicle fusions and not restored without endocytosis. This model also is unattractive as an explanation. For release to have declined from the start of the experiment, this hypothetical factor would necessarily be limiting exocytosis in the normal terminal. As it is known that all the vesicles can be released from the terminals, there nevertheless must be sufficient of this factor for every vesicle to be fusion-competent. Thus, the simplest explanation is that the limiting element is the number of vesicles themselves.

This model is consistent with observations of others at both fly and mammalian synapses. In an elegant earlier study of *shibire* (Koenig *et al.* 1989), it was noted that EPSPs declined after terminals were shifted to the non-permissive temperature and, although the time-course and properties of this decay were not studied in as great detail as the present study, the authors undertook the daunting task of correlative electron microscopy. They noted that at a given time after the temperature shift, the fall in the EPSP was indeed paralleled by a decrease in the population of synaptic vesicles. More recently, in cultured hippocampal neurons, Ryan *et al.* (1997) noted a correlation between the number of vesicles at a synapse and the number of vesicles that synapse released per impulse. This was determined by comparing the total loading of FM1-43 into a synapse in culture with the decrement seen per stimulus. Though they could not change the number of vesicles at a given synapse, they noted that across synapses in the dish, the brighter synapses (those with the most releasable vesicles) released the most FM1-43. This finding is certainly consistent with our equilibrium model, though it could also be explained by mechanisms in which the number of active zones or release sites in a bouton were correlated with the number

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

of vesicles therein. Still, their value for the fraction of total releasable vesicles that is released by a given action potential (0.47%) is in remarkable agreement with our own (0.3%) and strongly suggests that the equilibrium model may be a general property of many types of synapse.

The existence of an equilibrium between docked and undocked vesicles at the synapse may not hold at rapid rates of stimulation; under those conditions the rate of docking (vesicle mobilization) is likely to become limiting and synaptic depression will be seen that is not a consequence of a decline in total vesicles. The experiments above were conducted at 0.5 Hz or slower to avoid this complication. Nor will the equilibrium necessarily be found at every synapse; whether or not docking sites are saturated will depend on the numbers of release sites and the vesicle supply as well as on the rate constants for both docking and undocking. At vertebrate neuromuscular junctions and similarly specialized synapses it is certainly plausible that different models will apply. Similarly, recent studies on lamprey synapses lacking synapsin (Pieribone *et al*. 1995) have noted synapses in which there is a great reduction in the number of vesicles in a terminal and yet the amplitude of transmission is largely unchanged. This could of course reflect a fundamental difference in the properties of docking, but it could also indicate that the animals have increased the number of release sites or altered the docking rates in order to compensate for their shortage of vesicles. Another attractive model would posit that the remaining, tightly clustered population of vesicles near the active zone is indeed in equilibrium with the docked pool and that the missing vesicles form a reserve pool at those synapses which are not mobilized except under intense stimulation conditions. *Drosophila* neuromuscular junctions have recently been shown to have a reserve pool (Kuromi & Kidokoro 1998) that does not get mobilized for release unless the readily releasable pool has been depleted, including when *shibire* mutations have prevented the refilling of that pool, as in the present study.

Appreciating the equilibrium that appears to exist between docked and undocked vesicles is crucial to our understanding of synaptic modulation. If docking sites are limiting, then presynaptic increases or decreases in synaptic strength can only be accomplished by changing the number of functional release sites or by changing the probability of fusion of a docked, releasable vesicle (for example by modulating Ca^{2+} influx or the affinity of the Ca^{2+} sensor). But where an equilibrium exists, many other parameters become significant potential control points, including the rate of endocytosis, vesicle number, and rate constants for docking and undocking.

This work was supported by a Silvio Conti Center for Neuroscience Award from the National Institute of Mental Health.

REFERENCES

Agoston, D. V., Kosh, J. W., Lisziewicz, J. & Whittaker, V. P. 1985 Separation of recycling and reserve synaptic vesicles from cholinergic nerve terminals of the myenteric plexus of guinea pig ileum. *J. Neurochem.* **44**, 299–305.

Birks, R. & MacIntosh, F. 1961 Acetylcholine metabolism of a sympathetic ganglion. *Can. J. Biochem. Physiol.* **39**, 787–827.

Broadie, K., Bellen, H. J., DiAntonio, A., Littleton, J. T. & Schwarz, T. L. 1994 Absence of synaptotagmin disrupts excitation–secretion coupling during synaptic transmission. *Proc. Natn. Acad. Sci. USA* **91**, 10 727–10 731.

Broadie, K., Prokop, A., Bellen, H. J., O'Kane, C. J., Schulze, K. L. & Sweeney, S. T. 1995 Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila*. *Neuron* **15**, 663–673.

Burgess, R. W., Deitcher, D. L. & Schwarz, T. L. 1997 The synaptic protein syntaxin1 is required for cellularization of *Drosophila* embryos. *J. Cell Biol.* **138**, 861–875.

Couteaux, R. & Pecot-Dechavassine, M. 1973 Données ultrastructurales et cytochimiques sur le mécanisme de libération de l'acétylcholine dans la transmission synaptique. *Arch. Ital. Biol.* **3**, 231–262.

Deitcher, D. L., Ueda, A., Stewart, B. A., Burgess, R. W., Kidokoro, Y. & Schwarz, T. L. 1998 Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the *Drosophila* gene neuronal-synaptobrevin. *J. Neurosci.* **18**, 2028–2039.

DiAntonio, A. & Schwarz, T. L. 1994 The effect on synaptic physiology of synaptotagmin mutations in *Drosophila*. *Neuron* **12**, 909–920.

DiAntonio, A., Parfitt, K. D. & Schwarz, T. L. 1993 Synaptic transmission persists in synaptotagmin mutants of *Drosophila*. *Cell* **73**, 1281–1290.

Grigliatti, T. A., Hall, L., Rosenbluth, R. & Suzuki, D. T. 1973 Temperature-sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults. *Mol. Gen. Genet.* **120**, 107–114.

Ikeda, K., Ozawa, S. & Hagiwara, S. 1976 Synaptic transmission reversibly conditioned by single-gene mutation in *Drosophila melanogaster*. *Nature* **259**, 489–491.

Koenig, J. H. & Ikeda, K. 1989 Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. *J. Neurosci.* **9**, 3844–3860.

Koenig, J. H., Saito, K. & Ikeda, K. 1983 Reversible control of synaptic transmission in a single gene mutant of *Drosophila melanogaster*. *J. Cell Biol.* **96**, 1517–1522.

Koenig, J. H., Kosaka, T. & Ikeda, K. 1989 The relationship between the number of synaptic vesicles and the amount of transmitter released. *J. Neurosci.* **9**, 1937–1942.

Kuromi, H. & Kidokoro, Y. 1998 Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive *Drosophila* mutant, *shibire*. *Neuron* **20**, 917–925.

Martin, A. R. 1955 A further study of the statistical composition of the endplate. *J. Physiol.* **130**, 114–122.

Parfitt, K., Reist, N., Li, J., Burgess, R., Deitcher, D., DiAntonio, A. & Schwarz, T. L. 1995 *Drosophila* genetics and the functions of synaptic proteins. *Cold Spring Harb. Symp. Quant. Biol.* **60**, 371–377.

Pieribone, V. A., Shupliakov, O., Brodin, L., Hilfiker-Rothenfluh, S., Czernik, A. J. & Greengard, P. 1995 Distinct pools of synaptic vesicles in neurotransmitter release. *Nature* **375**, 493–497.

Poodry, C. A. & Edgar, L. 1979 Reversible alteration in the neuromuscular junctions of *Drosophila melanogaster* bearing a temperature-sensitive mutation, *shibire*. *J. Cell Biol.* **81**, 520–527.

Reist, N. E., Buchanan, J., Li, J., DiAntonio, A., Buxton, E. M. & Schwarz, T. L. 1998 Morphologically-docked synaptic vesicles are reduced in *synaptotagmin* mutants of *Drosophila*. *J. Neurosci.* **18**, 7662–7673.

Ryan, T. A., Reuter, H. & Smith, S. J. 1997 Optical detection of a quantal presynaptic membrane turnover. *Nature* **388**, 478–482.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

Schulze, K. L. & Bellen, H. J. 1996 *Drosophila* syntaxin is required for cell viability and may function in membrane formation and stabilization. *Genetics* **144**, 1713–1724.

Schulze, K. L., Broadie, K., Perin, M. S. & Bellen, H. J. 1995 Genetic and electrophysiological studies of *Drosophila* syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell* **80**, 311–320.

Sollner, T. H. & Rothman, J. E. 1996 Molecular machinery mediating vesicle budding, docking and fusion. *Cell Struct. Funct*. **21**, 407–412.

Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. & Rothman, J. E. 1993*a* A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**, 409–418.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. & Rothman, J. E. 1993*b* SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324.

Stewart, B. A., Atwood, H. L., Renger, J. J., Wang, J. & Wu, C. F. 1994 Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J. Comp. Physiol*. A **175**, 179–191.

Zimmermann, H. & Denston, C. R. 1977 Separation of synaptic vesicles of different functional states from the cholinergic synapses of the Torpedo electric organ. *Neuroscience* **2**, 715–730.

Zimmermann, H. & Whittaker, V. P. 1977 Morphological and biochemical heterogeneity of cholinergic synaptic vesicles. *Nature* **267**, 633–635.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES