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Presynaptic mitochondria and the temporal pattern of neurotransmitter release

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Mitochondria are critical for the function of nerve terminals as the cycling of synaptic vesicle membrane requires an efficient supply of ATP. In addition, the presynaptic mitochondria take part in functions such as Ca^{2+} buffering and neurotransmitter synthesis. To learn more about presynaptic mitochondria, we have examined their organization in two types of synapse in the lamprey, both of which are glutamatergic but are adapted to different temporal patterns of activity. The first is the giant lamprey reticulospinal synapse, which is specialized to transmit phasic signals (i.e. bursts of impulses). The second is the synapse established by sensory dorsal column axons, which is adapted to tonic activity. In both cases, the presynaptic axons were found to contain two distinct types of mitochondria; small 'synaptic' mitochondria, located near release sites, and larger mitochondria located in more central parts of the axon. The size of the synapse-associated mitochondria was similar in both types of synapse. However, their number differed considerably. Whereas the reticulospinal synapses contained only single mitochondria within 1 μm distance from the edge of the active zone (on average 1.2 per active zone, range of 1–3), the tonic dorsal column synapses were surrounded by clusters of mitochondria (4.5 per active zone, range of 3–6), with individual mitochondria sometimes apparently connected by intermitochondrial contacts. In conjunction with studies of crustacean neuromuscular junctions, these observations indicate that the temporal pattern of transmitter release is an important determinant of the organization of presynaptic mitochondria.

Keywords: adenosine triphosphate; clathrin; exocytosis; endocytosis; lamprey; synaptic vesicle

1. INTRODUCTION

Several large synapses have recently been introduced as models in the study of presynaptic mechanisms. In addition to the 'classical' squid giant synapse (Llinas *et al*. 1992; Hunt *et al*. 1994), both mammalian and lower vertebrate synapses have proved to be useful (Von Gersdorff & Matthews 1994; Borst & Sakmann 1996; Stanley & Mirotznik 1997; Borst & Sakmann, this issue). In each case, the unusual structural features of these synapses have made it possible to address novel aspects of presynaptic mechanisms. As these synapses are adapted to specialized functions, it is likely that their properties are quite specialized. Therefore, to optimize the use of such model synapses, it is important to characterize their features in great detail. Moreover, analyses of different specialized synapses can provide general insight into adaptive mechanisms in nerve terminals.

Our laboratory is using the giant reticulospinal synapse in the lamprey to study synaptic vesicle trafficking. This synapse is formed between unbranched giant reticulospinal axons and dendrites of spinal neurons (Brodin *et al*. 1988; Shupliakov *et al*. 1992). The axons have a diameter of up to 80 μm, which makes them the fastest conducting axons in the unmyelinated lamprey central nervous system (CNS). As the release sites are located in the main trunk of the axon, they are conveniently accessible to microinjected compounds (Brodin *et al*. 1994; Pieribone *et al*. 1995; Shupliakov *et al*. 1997*a*). The reticulospinal synapses contain conventional active zones, which release glutamate from small synaptic vesicles, and gap junctions, which mediate an electrotonic component of the synaptic response. Data from previous studies indicate that this synapse is specialized to transmit phasic synaptic signals (i.e. bursts of impulses), while it is poorly equipped to transmit tonic signals (i.e. sustained high-frequency firing; Shupliakov *et al*. 1992, 1997*b*; Brodin *et al*. 1997). These properties correlate with the phasic pattern of activity recorded in reticulospinal neurons during motor activity (Kasicki *et al*. 1989).

Our previous studies have suggested that the reticulospinal synapses contain few presynaptic mitochondria, presumably reflecting a specialized feature of this synapse. In the present study, we have performed an extensive analysis of the organization of presynaptic mitochondria in serially sectioned synapses. For comparison, we have examined a different synapse, the sensory dorsal column synapse, which is adapted to tonic high-frequency firing. We show that the organization of presynaptic mitochondria differs considerably between the two types of synapse. The results are discussed in relation to

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ATP-dependent mechanisms in the nerve terminal, with emphasis on synaptic vesicle trafficking.

2. MATERIALS AND METHODS

Two adult lampreys (*Lampetra fluviatilis*) were anaesthetized by immersion in a solution of tricaine methane sulphonate (MS-222; 100 mg l^{-1} water). The spinal cord with notochord was dissected in physiological solution (Shupliakov *et al*. 1995*b*) maintained at 8 °C. Immediately after the dissection the spinal cord was fixed in 3% glutaraldehyde + 0.5% p-formaldehyde in 0.1 M phosphate buffer pH 7.4 (4 h at 4 °C). After washing in phosphate buffer (4 h) preparations were post-fixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in alcohol and embedded in Durcupan ACM, Fluka.

Series of 50–200 ultrathin sections (60–70 nm thick) were cut on an LKB Ultrotome, stained with uranyl acetate and lead citrate, and examined in a Philips CM12 electron microscope. Three-dimensional (3D) reconstructions were made with MacStereology software.

Most of the studied synapses (established by both dorsal column and reticulospinal axons) comprise a single active zone, but a smaller proportion contain two or several active zones (O. Shupliakov, unpublished data). The present analysis of mitochondria was focused on synapses with single active zones. In three cases, however, 3D analysis revealed that two active zones shared a single synaptic vesicle cluster. In these cases, the number of mitochondria per active zone was calculated by halving by two the total number of mitochondria (found within 1 μm from the edge of the vesicle cluster).

3. RESULTS

(a) *The giant reticulospinal synapse*

The glutamatergic reticulospinal synapse is formed between giant reticulospinal axons running in the ventral spinal cord and dendrites of spinal motor neurons and interneurons. Most of the synapses are mixed and contain both gap junctions and structural specializations of chemical synapses, i.e. synaptic vesicle clusters and active zones (see Shupliakov *et al*. 1992). An electron micrograph of a typical reticulospinal synapse is shown in figure 1*a*, and a 3D reconstruction is shown in figure 3*a*. Three-dimensional analysis of reticulospinal synapses revealed that mitochondria were always present in the vicinity of the synaptic vesicle cluster (figure 1*a*,*c*), although they were not visible in every ultrathin section of a serially cut synapse. We defined mitochondria located within a 1 μm distance of accumulations of vesicles in this synapse as 'synaptic mitochondria'. These mitochondria ranged from 0.8 to 1.9 μm in length (1.2 ± 0.4 μm; mean ± s.d., $n = 34$). The average number of synaptic mitochondria per active zone ranged from one to three (1.2 ± 0.2; $n = 34$).

The central region of the giant reticulospinal axons (figure 1*b*) contained a different type of mitochondrion. These organelles were densely packed in the middle of the axonal cylinder and were often observed in apposition to microtubules (figure 1*d*). As evident in longitudinal sections (figure 1*d*), these mitochondria had a similar diameter to the synaptic ones. They were, however, significantly longer and could reach up to 8 μm in length (mean 6.2 ± 1.1 μm; $n = 15$). Both types of mitochondria had transversely arranged cristae (figure 1*c*,*d*).

(b) *The dorsal column synapse*

The dorsal column in the lamprey spinal cord contains glutamatergic axons, which belong to intraspinal sensory neurons (dorsal cells; Rovainen 1979). These axons are much smaller in diameter compared with the giant reticulospinal axons (see figures 1*b* and 2*a*). The majority of these axons are derived from pressure-sensitive cutaneous afferents, which respond to skin stimulation with sustained high-frequency firing (Christenson *et al*. 1987*a*,*b*). Our previous studies have shown that the synapses in these axons are adapted to sustained transmitter release for long periods and they can be morphologically distinguished from other types of axon present in this region (Shupliakov *et al*. 1992, 1996, 1997*b*).

Representative examples of dorsal column synapses are shown in figure 2*a*–*d* and figure 3*b* (3D reconstruction). The number of mitochondria surrounding the dorsal column synapses (figures 2, 3*b*) was significantly higher as compared with reticulospinal synapses. The number of small ('synaptic') mitochondria per active zone ranged from 2 to 16 (4.6 ± 1.4; $n = 15$). The size of these mitochondria did not differ significantly from those in reticulospinal axons. The synaptic mitochondria were in several instances found to be deeply embedded in the synaptic vesicle cluster and thus located in very close proximity to the release sites (figure 2*d*). The mitochondria were often located close to each other (figures 2, 3*b*). In five cases appositions with characteristics of 'intermitochondrial contacts' (see Bakeeva *et al*. 1983, 1985; Amchenkova *et al*. 1988) were observed (figure 2*d*,*e*). This suggests that several mitochondria may function as a single electrically coupled unit in the dorsal column synapse (cf. Amchenkova *et al*. 1988).

A larger type of mitochondrion was also present in the dorsal column axons (figure 2*a*,*c*). Many of these mitochondria were located in the central part of the axon (figure 2*a*,*c*), but they also occurred close to the axonal plasma membrane and sometimes in relatively close proximity to synaptic release sites (figure 3*b*).

4. DISCUSSION

(a) *Organization of presynaptic mitochondria*

Studies of crustacean neuromuscular junctions have demonstrated a correlation between the content of presynaptic mitochondria and the pattern of motor neuron activity (Atwood & Wojtowicz 1986; Nguyen *et al*. 1997). The fact that a similar correlation applies to central synapses in the lamprey strongly support the possibility of a coupling between the mitochondrial capacity and the temporal pattern of neurotransmitter release. In crustaceans, the individual mitochondria are generally larger in tonic than in phasic synapses (Atwood & Wojtowicz 1986), whereas in lamprey only the number appears to differ. In both systems, however, the properties of the individual mitochondria appear to differ between tonic and phasic synapses. Thus, studies in crustaceans indicate that the metabolic activity is higher in the mitochondria of tonic than of phasic synapses (Nguyen *et al*. 1997). Studies in lamprey indicate that the conversion of glutamine to glutamate is correspondingly more efficient in mitochondria of tonic synapses (Shupliakov *et al*. 1997*b*; see also Shupliakov *et al*. 1995*a*).

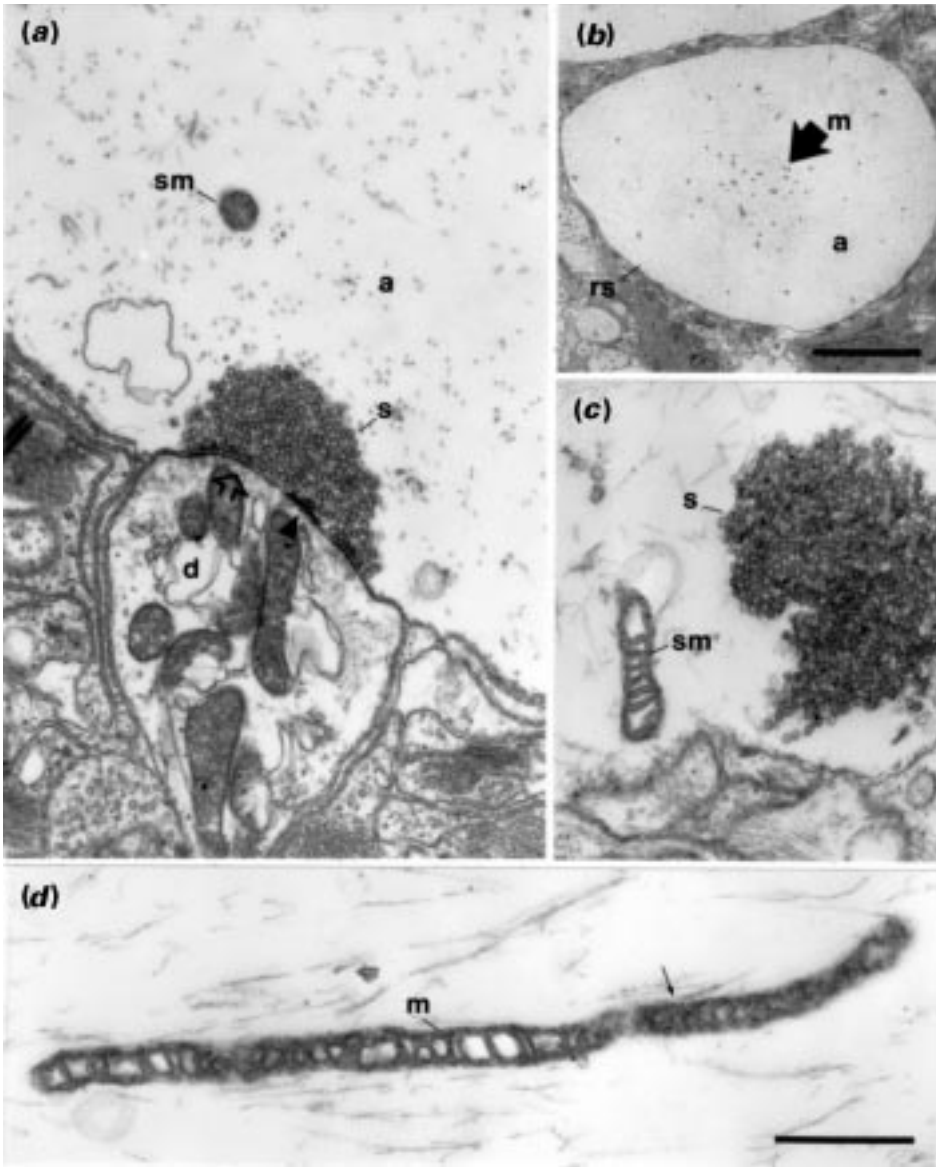

Figure 1. Organization of mitochondria pools in the giant reticulospinal axon in lamprey. (*a*) and (*b*) Electron micrographs from transversal sections of the spinal cord. (*a*) A mixed reticulospinal synapse established on a dendrite (d) of a spinal neuron. The active zone of the chemical synapse and the gap junction are indicated by an open arrow and an arrowhead, respectively. A single profile of a synaptic mitochondrion (sm) is present at the side of the synaptic vesicle cluster (s). (*b*) Low power electron micrograph of a reticulospinal axon (rs). The thick arrow indicates a pool of elongated mitochondria (m) located in the middle of the axonal cylinder. a, axoplasmic matrix. (*c*) and (*d*) Electron micrographs of longitudinal sections through a synaptic mitochondrion (*c*) and an elongated mitochondrion (*d*). The arrow in (*d*) points to a close apposition between the mitochondrion and a microtubule. Scale bar: *a*, *c*, *d*: 1 µm; *b*: 10 µm.

Studies in non-neuronal tissues have shown that mitochondria may be connected by intermitochondrial contacts (Bakeeva *et al*. 1983, 1985; Amchenkova *et al*. 1988). Evidence for a functional coupling between connected mitochondria has been obtained in studies of cultured fibroblasts and cardiomyocytes. It was found that mechanical or laser-induced damage of a single mitochondrion resulted in energy loss in the neighbouring mitochondria (Amchenkova *et al*. 1988). The contacts between synaptic mitochondria in dorsal column synapses were morphologically similar to those described in fibroblasts and cardiomyocytes. It therefore seems likely that groups of mitochondria in the dorsal column synapse may function as a single unit.

(b) *Role of ATP in the cycling of the synaptic vesicle membrane*

Presynaptic mitochondria appear to serve a number of important functions. They provide energy to the synaptic release machinery (see below) and to transporters, including both plasma membrane transporters and those that load synaptic vesicles with neurotransmitter (Erećinska & Silver 1994; Ozkan & Ueda 1998). They also participate in the synthesis of neurotransmitters (Shupliakov *et al*. 1997*c*) and in the buffering of intracellular Ca^{2+} (Grohovaz *et al*. 1996; Melamedbok & Rahamimoff 1998). Each of these functions obviously needs to be efficient in frequently active terminals, but less so in infrequently active terminals. Thus, a coupling

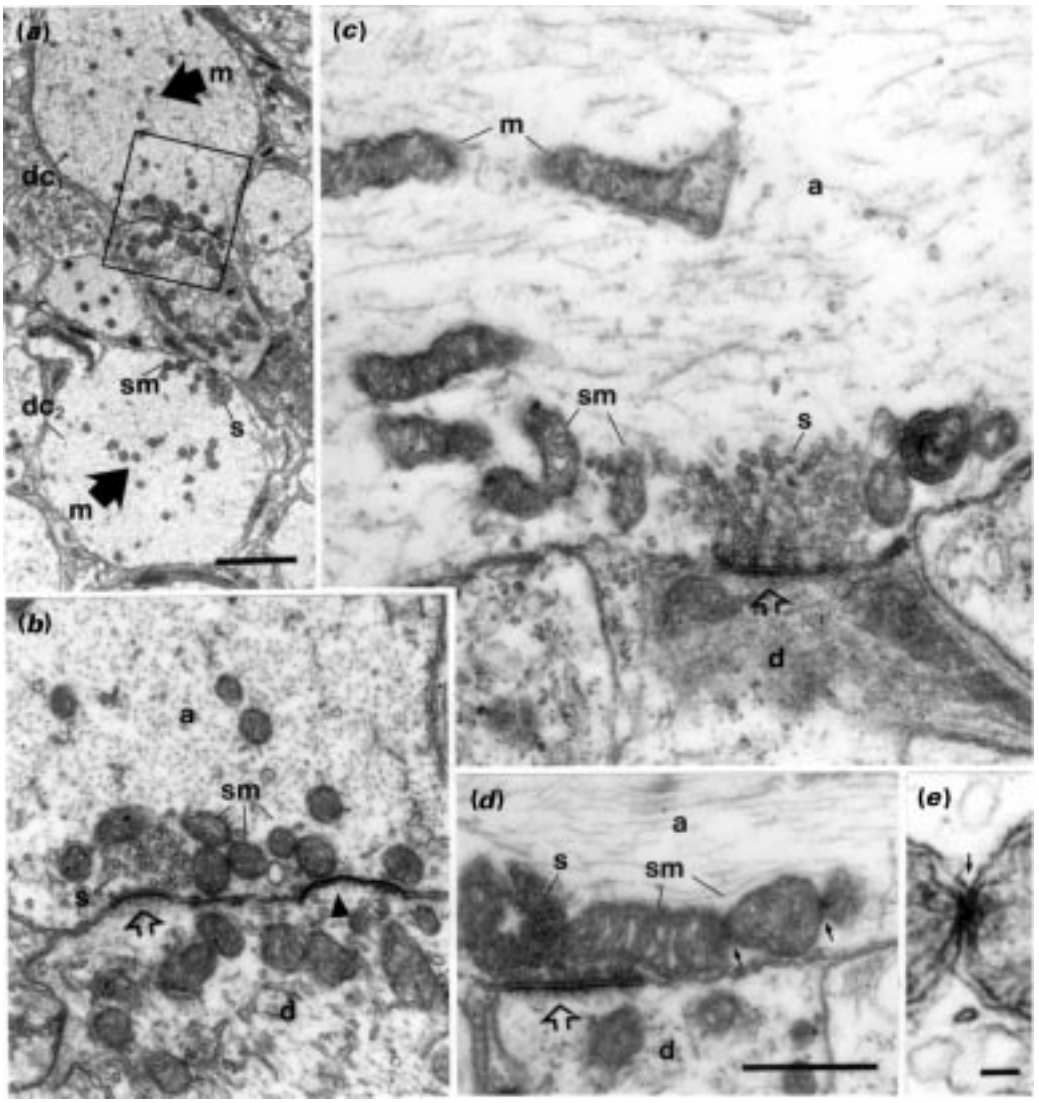

Figure 2. Mitochondria pools in dorsal column axons. (*a*) Low power electron micrograph from a transverse section of two dorsal column axons (dc1 and dc2) forming synaptic contacts on the same dendritic shaft. A separation between synaptic mitochondria (sm) located in the vicinity of the synapses and mitochondria (m, thick arrows) in the central part of the axons can be seen. The area in the rectangle, containing one of the synapses, is shown in (*b*) at higher magnification. Specializations of chemical and electrical junctions are indicated as in figure 1(*d*): dendritic shaft. (*c*)–(*e*) Electron micrographs of longitudinal sections. An accumulation of synaptic mitochondrial profiles around dorsal column synapses is seen both in the transverse (*a* and *b*) and longitudinal (*c* and *d*) sections. (*d*) and (*e*) Electron micrographs of intermitochondrial contacts (thin arrows) between synaptic mitochondria. Scale bar: *a*: 2 µm; *c*–*d*: 1 µm; *e*: 0.1 µm.

between mitochondrial organization and synaptic activity is not unexpected.

For the sake of brevity, we will focus the remainder of this discussion on one aspect of presynaptic mitochondrial function: the requirement of ATP in synaptic vesicle membrane trafficking. Regarding other functions of presynaptic mitochondria, we refer to other articles (Erecinska & Silver 1994; Nguyen *et al*. 1997; Shupliakov *et al*. 1997*c*; Melamedbok & Rahamimoff 1998; Ozkan & Ueda 1998; Scotti *et al*., this issue).

A problem with defining the role of ATP in synaptic vesicle trafficking is that the exact route taken by a synaptic vesicle (and its possible variability in different types of terminal) has not been established. To consider the role of ATP in the vesicle cycle we will use one out of several possible models (figure 4). This model implies that, after fusion, the synaptic vesicle is retrieved by clathrin-mediated endocytosis (Cremona & De Camilli 1997; Gonzáles-Gaitan & Jäckle 1997; Shupliakov *et al*. 1997*b*). The clathrin-coated vesicle is then uncoated and turns directly into a synaptic vesicle (without passing through an endosomal step (Takei *et al*. 1996; Schmidt *et al*. 1997; Prior & Clague 1997; Murthy & Stevens 1998). Regarding alternative recycling pathways, see Ceccarelli & Hurlbut (1980), Fesce *et al*. (1994), Koenig & Ikeda (1996), Betz & Angleson (1998), Faundez *et al*. (1998), Kavalali *et al*. (this issue).

The mechanisms involved in the synaptic vesicle cycle can be broadly divided into exocytosis, endocytosis and intraterminal transport mechanisms, each of which may involve ATP-dependent mechanisms. With regard to the ATP dependence of exocytosis, most studies of this problem have been conducted in different endocrine cells,

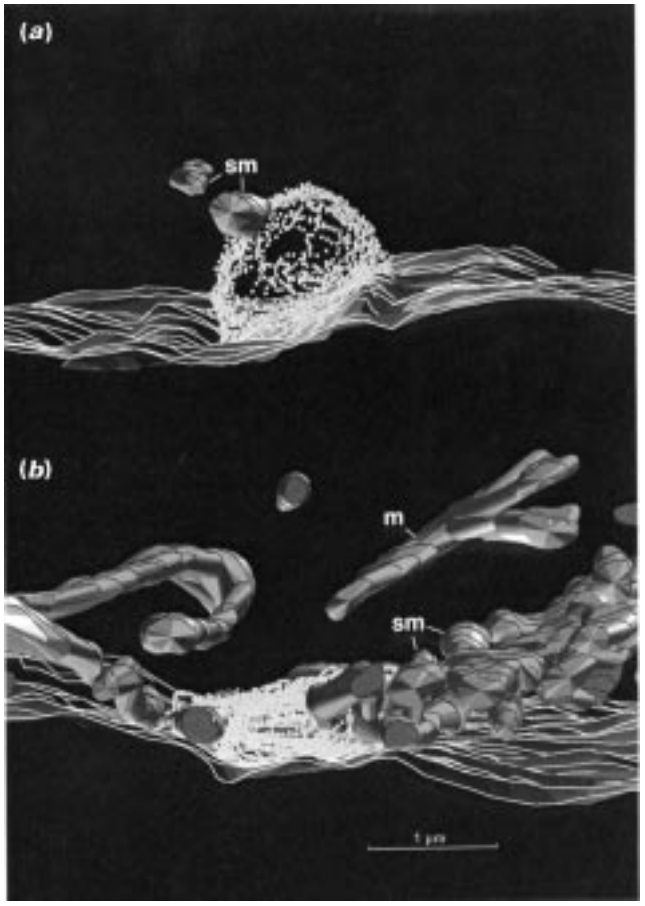

Figure 3. 3D reconstructions of mitochondria in the vicinity of a reticulospinal synapse (*a*) and of a dorsal column synapse (*b*). White dots delineate the borders of synaptic vesicle clusters. White lines through each reconstruction correspond to single ultrathin sections assembled with MacStereology software. sm, synaptic mitochondria; m, elongated mitochondrion.

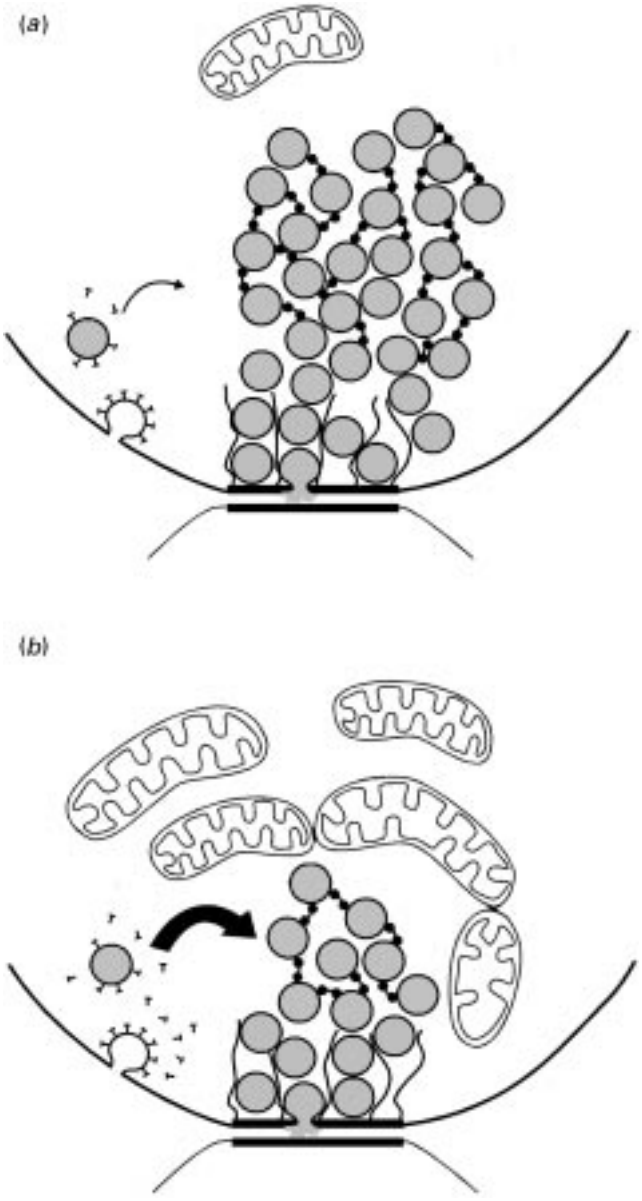

Figure 4. Schematic representation of the presynaptic element of synapses adapted to different patterns of activity. (*a*) A 'phasic' synapse with a low capacity for vesicle recycling (represented by the thin arrow) and a large reserve pool of synaptic vesicles. (*b*) A 'tonic' synapse with a high capacity for vesicle recycling (represented by the thick arrow) and a smaller reserve pool. The capacity of the vesicle recycling machinery and the dimensions of the reserve pool are, however, likely to be independent parameters, both of which may be adapted according to the physiological pattern of activity. Terminals that are tonically active need an efficient recycling machinery and, as a consequence, a large content of mitochondria (see text). Terminals that are phasically active (i.e. in bursts of action potentials) do not need an efficient recycling machinery, but may release at high rates intermittently by recruiting vesicles from the reserve pool. The small bars with two dots (in between the vesicles) represent synapsin. The thin lines represent a filamentous cytomatrix covering the 'synapsin-independent' pool of vesicles (see Brodin *et al*. 1997).

but a recent study (Heidelberger 1998) indicates that the conditions may be similar in nerve terminals. In both cases, a brief bout of exocytosis can occur in the absence of ATP. Thus, after ATP has been depleted or substituted with non-hydrolysable analogues, a limited pool of vesicles can undergo exocytosis, but sustained exocytosis is blocked (Holz *et al*. 1989; Parsons *et al*. 1995; Eliasson *et al*. 1997; Heidelberger 1998). After the ATP-independent vesicle pool has been depleted, exocytosis can be rapidly reinstalled by photorelease of ATP (Eliasson *et al*. 1997). This strict ATP dependence of sustained exocytosis is generally attributed to a 'priming' mechanism which, however, remains to be exactly defined. At the biochemical level, different ATP-dependent reactions coupled to exocytosis have been described. One is the ATP hydrolysis by N-ethylmaleimide-sensitive factor (NSF) (Söllner *et al*. 1993), which is thought to make the soluble NSF-attachment protein receptor (SNARE) proteins competent to function in fusion (Mayer *et al*. 1996; Hanson *et al*. 1997; Weber *et al*. 1998). Another is the formation of phosphatidyl-4,5-inositolbisphosphate (PtdIns-4,5P_2) by phosphatidylinositol-4-phosphate-5-kinase and phosphatidylinositol transfer protein (Martin 1997*a*,*b*). Interestingly, morphological studies in two types of endocrine cell have shown that the number of secretory vesicles that can undergo ATP-independent exocytosis is similar to the number of vesicles located in the vicinity of the plasma membrane (Parsons *et*

al. 1995; cf. Von Gersdorff *et al*. 1996; Heidelberger 1997). One interpretation of this finding is that the biochemical priming reactions (i.e. ATP hydrolysis by NSF and/or phosphorylation of inositolphospholipids) occur at about the same time as the vesicle reaches the plasma membrane. Another possibility is that the blockade of sustained exocytosis after ATP depletion is not directly related to these reactions, but rather to an inhibition of ATP-dependent transport mechanisms which bring the vesicles to the plasma membrane (see below). These different possibilities need not be mutually exclusive. For instance, PtdIns-4,5P_2 has been proposed to be important for the interaction between secretory vesicles and the cytoskeleton (De Camilli *et al*. 1996; Martin 1997*a*).

Soon after exocytosis, the synaptic vesicle membrane is retrieved by endocytosis (Henkel & Almers 1996; Cremona & De Camilli 1997; Kavalali *et al*., this issue). This process appears to be the one that requires the highest ATP levels of all the trafficking steps in the vesicle cycle. This was first suggested by early studies of synapses treated with metabolic inhibitors, which showed that synaptic vesicle recycling is perturbed, whereas transmitter release can continue until the synaptic vesicle pool is depleted (Atwood *et al*. 1972). Recent studies in lamprey synapses have suggested that two distinct processes may be affected, as two types of membrane structure appear in ATP-depleted synapses upon stimulation. They include membrane invaginations with a complex structure and which are devoid of coated intermediates, and invaginated clathrin-coated pits with constricted necks which accumulate in large numbers (O. Shupliakov, L. Zotova, L. Brodin, unpublished data). Results from *in vitro* assays of (non-synaptic) receptor-mediated endocytosis suggest that ATP may be required both during the assembly of the clathrin coat and during membrane fission (Schmid 1993). However, recent studies have shown that 'synaptic' clathrin-coated pits can be reconstituted with rat brain cytosol and synthetic lipid membranes in the absence of ATP (Takei *et al*. 1998). Different protein as well as lipid kinases and phosphatases have also been implicated in synaptic vesicle recycling but their precise roles are as yet unclear (Robinson *et al*. 1994; Südhof 1995; Cremona & De Camilli 1997; Bauerfeind *et al*. 1997; Slepnev *et al*. 1998). After (or possibly during) fission of the coated pit, the vesicle membrane is uncoated in a process involving ATP hydrolysis by the uncoating Hsc70–auxilin complex (Ungewickell *et al*. 1995).

The newly formed synaptic vesicle eventually reaches the existing synaptic vesicle pool clustered at the release site. The new vesicle may either mix randomly with this pool (Betz & Bewick 1992) or it may be preferentially targeted to a restricted part of it (Kiromi & Kidokoro 1998). The organization of the major, distal part of the vesicle pool depends on synapsins (see Pieribone *et al*. 1995; Hilfiker *et al*., this issue), which, interestingly, have been found to bind ATP (Hosaka & Südhof 1998). Under resting conditions, the synaptic vesicle pool is virtually immobile, while on stimulation, synaptic vesicles are drawn to the plasma membrane (Betz & Angleson 1998). Information about the molecular basis of these transport events is as yet lacking, but it appears likely that the transport from the site of endocytosis to the vesicle cluster uses a transport mechanism that is differently regulated as compared to that mediating the transport to the plasma membrane. In both these events, in particular the latter one, the transport needs to be rapid and tightly regulated to avoid failure of synaptic transmission. By analogy with other vesicular transport systems, it seems plausible that ATP-dependent motor proteins are involved at some point(s) of these transport events. It is therefore interesting to note that presynaptic microinjection of antibodies to myosin II have been reported to inhibit synaptic transmission (Mochida *et al*. 1994). Moreover, myosin V has been found to be associated with synaptic vesicles by interacting with VAMP-synaptophysin (Prekeris & Terrian 1997).

Thus, ATP may be essential in perhaps five to seven different processes of the synaptic vesicle cycle and different regulatory ATP-dependent mechanisms may also be involved. This indicates that the cycling of synaptic vesicle membrane accounts for a large part of the energy expenditure in the brain.

(c) *ATP supply and synaptic vesicle trafficking in specialized synapses*

In the giant reticulospinal synapse, clathrin-mediated endocytosis appears to be the main mechanism for synaptic vesicle recycling (Brodin *et al*. 1997). Under normal conditions, no depletion of the synaptic vesicle pool is seen when low to moderate rates of stimulation are used (0.2–5 Hz). After microinjection of compounds which perturb clathrin-mediated endocytosis, however, such stimulation causes a depletion of synaptic vesicles along with an accumulation of clathrin-coated endocytic intermediates in the plasma membrane around active zones (Shupliakov *et al*. 1997*b* and unpublished observations by the same authors). At high rates of repetitive stimulation (i.e. 20 Hz), a depletion of synaptic vesicles can be induced also in normal (i.e. not injected) synapses (Wickelgren *et al*. 1985; Shupliakov *et al*. 1995*b*; see also Ceccarelli & Hurlbut 1980). This indicates that the capacity of the endocytic machinery is exceeded when exocytosis occurs at high rates. By recruiting synaptic vesicles from the large cluster present at release sites, synaptic transmission can continue for a short period, but then decays (Pieribone *et al*. 1995). We assume that the limited capacity for vesicle recycling (combined with the large reserve pool of synaptic vesicles) in the reticulospinal synapse reflects an adaptation to phasic synaptic activity. As this synapse is not meant to be tonically active, an efficient recycling machinery is not required (figure 4*a*), whereas for tonic synapses like the dorsal column synapse (figure 4*b*), the reverse is true. As endocytosis is the trafficking step most sensitive to low ATP levels (see above), the number of presynaptic mitochondria is likely to be a key determinant of the capacity for vesicle recycling. That is, the larger number of vesicles that undergo exocytosis and need to be endocytosed per time unit, the larger number of mitochondria would seem to be required. The capacity for tonic versus phasic transmitter release is thus likely to depend heavily on the organization and efficiency of presynaptic mitochondria (figure 4; cf. Nguyen *et al*. 1997; Msghina *et al*. 1998). However, the capacity for vesicle recycling also depends on other factors, such as the levels of endocytic proteins. Recent studies indicate that this parameter may also vary

between tonic and phasic synapses (Shupliakov *et al.* 1996).

From the experimental point of view, the limited capacity for vesicle recycling in the lamprey reticulospinal synapse can provide an advantage. Thus, for instance, when non-physiological tonic, high-frequency stimulation is applied, endocytosis will lag behind exocytosis. This creates an artificial time-window which makes it possible to study endocytosis in the absence of Ca^{2+} influx and exocytosis (Gad *et al.* 1998).

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