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DIFFERENTIAL EFFICIENCY OF THE ENDOCYTIC MACHINERY IN TONIC AND PHASIC SYNAPSES

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Abstract—Efficient synaptic vesicle membrane recycling is one of the key factors required to sustain neurotransmission. We investigated potential differences in the compensatory endocytic machineries in two glutamatergic synapses with phasic and tonic patterns of activity in the lamprey spinal cord. Post-embedding immunocytochemistry demonstrated that proteins involved in synaptic vesicle recycling, including dynamin, intersectin, and synapsin, occur at higher levels (labeling per vesicle) in tonic dorsal column synapses than in phasic reticulospinal synapses. Synaptic vesicle protein 2 occurred at similar levels in the two types of synapse. After challenging the synapses with high potassium stimulation for 30 min the vesicle pool in the tonic synapse was maintained at a normal level, while that in the phasic synapse was partly depleted along with expansion of the plasma membrane and accumulation of clathrin-coated intermediates at the periactive zone. Thus, our results indicate that an increased efficiency of the endocytic machinery in a synapse may be one of the factors underlying the ability to sustain neurotransmission at high rates.

Key words: amphiphysin, dynamin, intersectin, synapsin, synaptic plasticity, lamprey.

Synapses in the CNS and peripheral nervous system show diverse functional properties most likely as a consequence of adaptation to the specific requirements at a given synapse. One type of synaptic adaptation, which involves the presynaptic element, is the specialization to tonic and phasic synapses (Atwood and Karunanithi, 2002; Millar et al., 2005; Morgans, 2000). In synapses adapted to tonic activity, transmitter release can be sustained at high levels for extended periods of time. In phasic synapses, on the other hand, transmitter release normally occurs intermittently and when tonic stimulation is applied a fatigue of transmitter release is seen (Atwood and Karunanithi, 2002; Wickelgren et al., 1985). The capacity for neurotransmitter release over extended periods of time thus appears to differ between synapses. In part, this difference appears to be due to the properties of the transmitter synthesis machinery, which is more efficient in tonic than in phasic synapses (Shupliakov et al., 1995, 1997). In addition, other aspects of the presynaptic machinery are likely to be involved, such as ATP supply, organization of the active zone, and mode of neurotransmitter release (Atwood and Karunanithi, 2002; Brodin et al., 1999; Harata et al., 2006; Kavalali, 2006). It therefore seems plausible that the molecular machinery that controls recycling of synaptic vesicles may differ between tonic and phasic synapses. In the present study, we examined this possibility by focusing on endocytic proteins involved in vesicle recycling.

The well-characterized tonic glutamatergic synapse formed by afferent axons in the dorsal column of the lamprey spinal cord ("dorsal column axons") and the phasic glutamatergic synapse formed by giant reticulospinal axons in the ventral spinal cord column ("reticulospinal axons"; Shupliakov and Brodin, 2000) were used as model synapses in our experiments. The dorsal column axon exhibits tonic high frequency firing upon physiological stimulation *in vivo* (Christenson et al., 1988), while the larger reticulospinal axons are mostly silent and fire brief bursts during certain motor commands (Deliagina and Fagerstedt, 2000). The dorsal column synapse has a small pool of vesicles ($<$1000 vesicles) compared with the reticulospinal synapse, which has a large vesicle cluster ($\sim$10,000 vesicles; Gustafsson et al., 2002). Earlier studies have demonstrated that tonic dorsal column synapses are better equipped to sustain neurotransmission because the terminals contain more mitochondria (Brodin et al., 1999). Further, they contain more glutamate and glutamine than phasic reticulospinal synapses, enabling a more efficient synthesis and refilling of neurotransmitter in vesicles (Shupliakov et al., 1997). Our present results demonstrate that dynamin, amphiphysin, intersectin, and synapsin are more abundant in the tonic dorsal column synapses and suggest that these synapses have a more efficient vesicle recycling machinery compared with reticulospinal synapses.

EXPERIMENTAL PROCEDURES

Experimental animals and tissue processing

Trunk segments of lamprey (*Lampetra fluviatilis*) spinal cords were dissected out as described before (Gad et al., 2000) and incubated either in low Ca^{2+} Ringer's solution (0.1 mM $CaCl_2$, 4 mM $MgCl_2$) for 2 h at 4 °C to reduce the level of spontaneous synaptic activity in the preparation (four animals) or in high K^+ Ringer's solution (30 mM KCl) for 30 min at 4 °C to induce synaptic activity and vesicle cycling (three animals). To inhibit ATP production at synapses 40 mM NaN_3 was added to the solutions.

Specimens for immunogold immunocytochemistry were incubated in low Ca^{2+} Ringer's solution only. To preserve immunoreactivity of synaptic proteins they were fixed in fixative containing low glutaraldehyde concentration 4% paraformaldehyde/0.5% glu-

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Abbreviations: CLAP, clathrin-AP2 binding; svc, synaptic vesicle cluster.

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Table 1. Densities of immunogold labeling of synapses in reticulospinal (RS) and dorsal column (DC) axons

	n	SVC		Axoplasmic matrix particle density	Dendrite particle density
		Vesicle density	Particle density		
Synapsin RS	6	530.3 (82.1)	351.4 (49.8)	4.2 (3.9)	13.4 (17.8)
Synapsin DC	11	256.4 (93.9)	314.0 (71.9)	10.6 (8.2)	24.3 (21.7)
SV2 RS	19	466.3 (134.1)	182.9 (55.1)	1.5 (2.0)	0.9 (2.3)
SV2 DC	17	208.5 (65.5)	60.4 (32.8)	2.7 (4.4)	0.3 (1.4)
Dynamin RS (MC60)	8	318.7 (49.5)	16.2 (9.1)	1.0 (3.0)	12.1 (8.6)
Dynamin DC (MC60)	18	292.0 (75.0)	176.9 (71.3)	1.2 (6.0)	15.4 (15.9)
Dynamin RS (DG-1)	16	253.1 (71.2)	20.2 (16.6)	1.8 (2.8)	14.3 (14.7)
Dynamin DC (DG-1)	31	179.4 (49.7)	55.6 (31.6)	2.5 (4.8)	17.5 (22.3)
Amphiphysin RS	7	672.2 (79.4)	53.7 (15.0)	1.7 (1.7)	12.7 (5.2)
Amphiphysin DC	7	343.5 (73.7)	107.9 (25.2)	2.1 (3.5)	7.9 (3.2)
Intersectin RS	19	466.4 (96.8)	40.6 (14.8)	2.4 (4.9)	25.8 (10.4)
Intersectin DC	16	221.9 (62.9)	34.4 (18.6)	3.1 (2.3)	24.3 (10.2)

For each of the immunogold labelings densities of gold particles are given as average number of gold particles per μm^2 and its standard deviation in parentheses. The density of vesicles was calculated over the synaptic vesicle cluster and is represented as mean number of vesicles per μm^2 and its standard deviation in parentheses. The number of synaptic profiles is represented as *n*.

taraldehyde/4% tannic acid in cacodylate buffer (0.1 M, pH 7.4), for 1 h at 4 °C. Fixation was continued in fixative without tannic acid for 3 h at 4 °C. Pieces of the spinal cord were stained en bloc with 1% aqueous uranyl acetate (except preparations embedded in Lowicryl), dehydrated in ethanol and embedded in LR Gold (TAAB, Berks, UK) or Lowicryl resin (Fluka, Buchs, Switzerland) at low temperature (−25 °C).

Specimens for morphometrical analysis were fixed in 3% glutaraldehyde/0.5% paraformaldehyde in (0.1 M, pH 7.4) for 4 h at 4 °C and processed for electron microscopy as earlier described (Gustafsson et al., 2002).

Animals were treated according to the Swedish Animal Welfare Act SFS 1988:534, as approved by the local Animal Research Committee of Stockholm. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Antibodies

Polyclonal antibodies DG-1 (Grabs et al., 1997) and MC60 (Henley et al., 1998) raised against rat dynamin 1 were used for detection of dynamin. Amphiphysin was detected using antibodies, raised against the clathrin-AP2 (CLAP) binding region of lamprey amphiphysin (Evergren et al., 2004b), LIS-AC antibodies were raised against an amino acid sequence corresponding to the SH3 A–C domains of lamprey intersectins (manuscript in preparation; Evergren et al., 2004a), and finally, lamprey-specific D domain antibodies were used to detect synapsin (Bloom et al., 2003). The SV2 antibody has been described and characterized previously (Buckley and Kelly, 1985) and was provided by Dr. R. Jahn (Goettingen, Germany). Prior to immunolabeling experiments the specificity of all antibodies was evaluated using Western blot analysis with lamprey brain detergent extract (data not shown, see Bloom et al., 2003; Evergren et al., 2004b).

Post-embedding immunogold

Embedded blocks were trimmed to contain regions with dorsal column and reticulospinal axons (Shupliakov et al., 1992). Serial ultrathin sections were prepared in an LKB or Reichert ultramicrotome using a diamond knife (Diatome, Biel, Switzerland). The sections were collected on nickel mesh or formvar-coated nickel slot grids, and incubated with antibodies following a post-embedding immunogold protocol: primary antibody at 4 °C overnight, followed by a 2 h incubation at room temperature with a gold-conjugated secondary antibody (Amersham Biosciences, Uppsala, Sweden; dilution 1:50). Gold particles were enhanced using IntenSE Silver Enhancement kit (Amersham Biosciences). Sections were counterstained with uranyl acetate and Reynold's lead citrate and examined in a Philips CM12 or Tecnai 12 electron microscope. In control experiments the primary antibodies were omitted or preabsorbed with the respective antigen; in none of these experiments specific labeling was observed.

Synapses established by reticulospinal and dorsal column axons were photographed on film or with a CCD camera at a magnification of 22,000×. Negatives were digitized using a Flextight Precision II scanner (Imacon, Copenhagen, Denmark). Morforel (Blackstad et al., 1990) and Image J (Rasband, 2005) software were used for measurements and quantification of the images. Synapses from two animals were analyzed and they displayed a similar immunoreactivity pattern. The number of synapses for each immunogold labeling is shown in Table 1. For each synaptic profile, the densities of gold particles were quantified over defined areas, such as synaptic vesicle cluster (svc), axoplasmic matrix (a), and post-synaptic dendrite (d). Svc was defined as an area containing synaptic vesicles, and axoplasmic matrix was defined as a 0.5 μm region surrounding the svc. In svcs labeled for synapsin a 100 nm proximal pool has been shown to contain reduced levels of synapsin (Bloom et al., 2003). Therefore, the distal and proximal pools were quantified separately. Background labeling on tissue-free areas was subtracted from each profile. To calculate the number of particles per synaptic vesicle, the number of particles over the svc region was divided by the number of synaptic vesicles in the studied area of the cluster.

Morphometrical analysis of stimulated synapses

Since the trunk region of the lamprey spinal cord is about 15 cm long and has the same organization along its length, control and experimental specimens were prepared from the same animal. This allowed comparison of the morphology of synapses, deriving from the same group of axons, exposed to different experimental conditions. Two animals were used in these experiments. Synapses were photographed in serial ultrathin sections and those containing a single round active zone were selected for further analysis (Gustafsson et al., 2002). From 10 to 21 synapses were analyzed for each group in pieces of the spinal cord exposed to high K^+, and respective "control" pieces incubated in low Ca^{2+} Ringer's solution. To inhibit ATP production, synapses from the same preparation were exposed to 40 mM sodium azide (NaN_3) in low Ca^{2+} or high K^+ Ringer's solutions. The number of synaptic vesicles, of coated pits, and the curvature index was determined from middle sections of the synapses. The number of synaptic