Short communication

Synaptotagmin-like expression in the motor nerve terminals of crayfish

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Abstract

Synaptotagmin-like immunoreactivity was shown to be localized at crayfish neuromuscular junctions by whole mount immunocytochemistry. Synaptotagmin-like immunoreactivity was present in both phasic and tonic excitatory terminals and inhibitory nerve terminals. Immunoblots indicated that the antibody DSYT-2 raised against *Drosophila* synaptotagmin, labelled proteins at relative molecular weights of 87 kDa and 107 kDa in crayfish ganglion and neuromuscular preparations.

Keywords: Synaptotagmin; Vesicle; Synapse; Neuromuscular junction; Crustacean

Synaptotagmin is an abundant integral protein of synaptic vesicles at motor nerve terminals of *Drosophila* and other species (for review see [10]). Parts of the primary structure are highly conserved from *Drosophila* to humans [16,17]. Various domains of the protein are believed to have functions related to other similar proteins. For example, there are two copies of a internal repeat which is homologous to the regulatory region of protein kinase C [17], arachidonic acid-specific phospholipase A2, a phospholipase C and GTPase-activating protein [3]. It has been shown that the C2 repeats are similar to those of other proteins which show a calcium-dependent membrane interaction [18] indicating that synaptotagmin may be a Ca^{2+} sensor for modulating neurotransmitter release. Another indication that this molecule has a regulatory effect on transmission is that the COOH terminus region is likely to be the binding site for neurexins which implicates it as a mediator for docking of the synaptic vesicles to the presynaptic membrane [16]. The synaptotagmin protein itself has been shown to bind calcium in the presence of acid phospholipids [2] at a concentration range that is consistent with its proposed role in neurotransmitter release [1]. More direct evidence of its role in transmission comes from mutations which substantially alter neurotransmission [5,11–13,15].

Since synaptotagmin is highly conserved and is believed to be a key protein involved with vesicular docking and a possible calcium sensor which leads to the induction of vesicular fusion, we tested for its presence in the presynaptic terminals of crayfish motor neurons. The terminals of these neurons are large, extensively used for physiological studies [4,7], and of different types: a single muscle may be innervated by both tonic and phasic excitatory neurons as well as by inhibitory neurons. The tonic terminals consist of strings of varicosities, whereas phasic terminals have thin filiform processes [14]. In addition, synapses within the presynaptic terminal show a range in structural complexity [19]. Crayfish synapses show the characteristic pre- and post-synaptic membrane specialization along with a pronounced dense structure raised from the presynaptic membrane. This enhanced presynaptic dense structure (dense body or bar) is normally surrounded by closely associated vesicles that appear to be docked there [7,9]. This site is referred to as the synaptic active zone. Some synapses of tonic terminals demonstrate no active zones while others may show one or more active zones [4]. The presence of synapses with multiple active zones may result in punctate staining of synaptic vesicle proteins as compared to synapse with one or no active zones.

We hypothesized that since a number of the synaptic vesicle docking proteins are conserved in structure, antibodies raised against *Drosophila* synaptotagmin [10] would be likely to react with conserved proteins of crustacean

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nerve endings, since *Drosophila* and crustaceans are in the same arthropod subphylum (*Mandibulata*), and share features of peripheral innervation [20].

The in situ immunocytochemistry was performed on the opener and extensor muscles in the first walking leg of specimens of the crayfish, *Procambarus clarkii*, measuring 5–6 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA). Preparations were dissected in a modified Van Harreveld's crayfish solution [4] and processed for immunocytochemistry as described by Littleton et al. [11]. The localization of the reactive protein within various nerve terminals is demonstrated in Fig. 1. Fig. 1A is from the central region of the opener muscle where strings of varicosities arising from both the inhibitory and the single tonic excitatory neuron occur. It is evident that both excitatory and inhibitory varicosities stain, whereas the bottleneck regions between the varicosities stain much less intensely. The majority of the synapses occur in these varicose regions with relatively few in the bottleneck regions [6]. It was also apparent that the main axon branches of the two neurons and muscle fibers show little

Fig. 2. Immunoblot showing reaction products of antibody with material homogenized from 70 *Drosophila* heads (A), from 12 crayfish ganglia (B), and from crayfish tonic superficial lateral abdominal extensor muscle (C). Electrophoresis and immunoblotting was carried out as described previously [8].
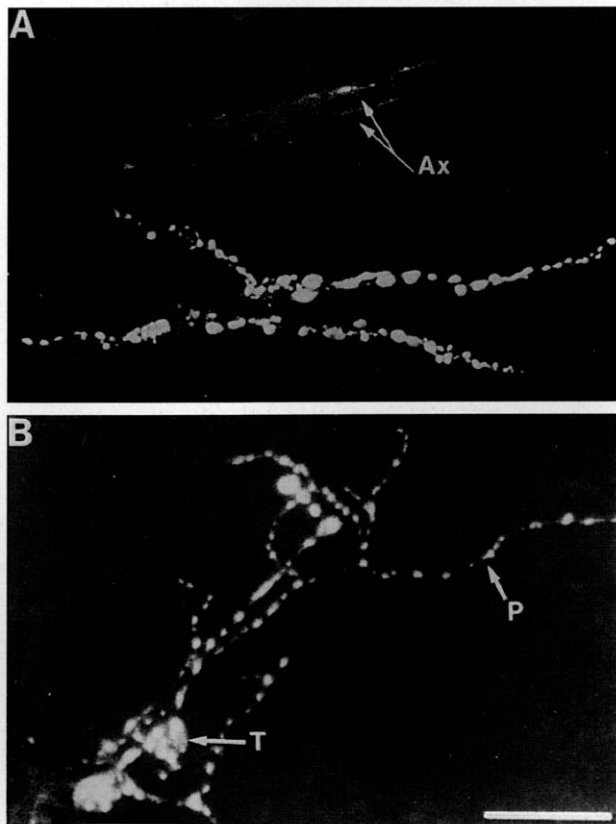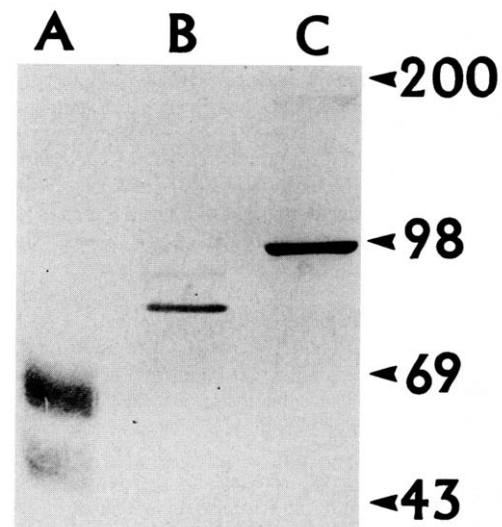

Fig. 1. Synaptotagmin staining pattern in whole mounts of the neuromuscular junction. A: both the tonic excitor and inhibitor terminal varicosities stain in the opener muscle. Note that bottlenecks between varicosities and the main axons (Ax) show little reactivity. B: on the leg extensor muscle, tonic (T) and phasic (P) excitatory terminals and inhibitory terminals show immunoreactivity. Note the punctate staining in the large tonic varicosities and along the phasic filiform processes. (Scale bar: 25 μm for A and B).

staining reaction compared to the terminals. In the leg extensor preparation (Fig. 1B), both the tonic and phasic excitatory terminals as well as the inhibitory terminals stain. The phasic terminals appear filiform and the tonic appear varicose [14]. Some of the tonic varicosities (Fig. 1B) have a punctate staining pattern which may be indicative of the underlying complex synaptic regions.

To determine if the antibody DSYT-2, which was raised against *Drosophila* synaptotagmin recognizes a homologous protein in the crayfish terminals, immunoblots were preformed on homogenized ganglia (nervous and glia tissue) and on a muscle which contained tonic and inhibitory motor nerve terminals. For a control, we repeated the procedures outlined by Littleton et al. [13] in *Drosophila* (wild type, Canton-S) with whole mount preparations and immunoblots of homogenized adult fly heads. Our results obtained in the whole mounts of *Drosophila* neuromuscular junctions are as those reported earlier [13]. The immunoblots indicate that proteins within the crayfish material also show immunoreactivity to this *Drosophila* synaptotagmin antibody (Fig. 2). However, whereas the *Drosophila* material showed the expected band at 67 kDa and a degradation product at 54 kDa [13], the crayfish material extracted from ganglia showed a band of relative molecular weight of 87 kDa, while the muscle material had a band at 107 kDa.

In summary, crayfish ganglionic and neuromuscular preparations contain proteins which reacts with an antibody for *Drosophila* synaptotagmin, and are larger than the insect protein. The whole mount staining shows that this protein is localized to the terminal regions of excitatory and inhibitory motor neurons. The punctate staining pattern observed in the phasic terminals and in regions of

the large varicosities of the tonic terminals indicates that the immunoreactive protein may be associated with vesicle populations localized at synapses. Thus, the antibody provides an excellent marker for crustacean neuromuscular synapses. Current work is underway to further examine this punctate staining pattern at the ultrastructural level with electron microscopy, and to determine whether or not higher molecular weight isoforms or homologs of synaptotagmin are present in the crayfish.

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