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Review article

Presynaptic active zones of mammalian neuromuscular junctions: Nanoarchitecture and selective impairments in aging

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ABSTRACT

Neurotransmitter release occurs at active zones, which are specialized regions of the presynaptic membrane. A dense collection of proteins at the active zone provides a platform for molecular interactions that promote recruitment, docking, and priming of synaptic vesicles. At mammalian neuromuscular junctions (NMJs), muscle-derived laminin β2 interacts with presynaptic voltage-gated calcium channels to organize active zones. The molecular architecture of presynaptic active zones has been revealed using super-resolution microscopy techniques that combine nanoscale resolution and multiple molecular identification. Interestingly, the active zones of adult NMJs are not stable structures and thus become impaired during aging due to the selective degeneration of specific active zone proteins. This review will discuss recent progress in the understanding of active zone nanoarchitecture and the mechanisms underlying active zone organization in mammalian NMJs. Furthermore, we will summarize the age-related degeneration of active zones at NMJs, and the role of exercise in maintaining active zones.

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1. Introduction

A neuromuscular junction (NMJ) is a chemical synapse formed between a presynaptic motor neuron and a postsynaptic muscle cell. These synapses ensure efficient signaling between the two cells through the regulated release of neurotransmitters. The exocytotic release of neurotransmitters occurs at specific sites on the presynaptic membrane that are termed active zones. At the ultrastructural level, active zones appear as electron-dense projections on the presynaptic membrane where synaptic vesicles fuse for subsequent exocytosis (Couteaux and Pecot-Dechavassine, 1970; Tsuji, 2006). Electron microscope tomography has revealed that active zones in frog NMJs are elongated structures, and the way synaptic vesicles attach to the active zone makes them appear as "pearls on a string" (Harlow et al., 2001). Active zones in mammalian NMJs are much smaller than those of frog NMJs and are distributed in a discrete and scattered pattern within each presynaptic terminal (Nagwaney et al., 2009; Rowley et al., 2007). Presynaptic active zones perform the following functions involved in accurate and efficient neurotransmitter release: (1) dock and prime synaptic vesicles; (2) recruit voltage-gated calcium channels (VGCCs); (3) contribute to the precise, exactly opposite locations of pre- and postsynaptic specializations; and (4) mediate presynaptic plasticity (Südhof, 2012). The first three concepts are relevant for the active zones of NMJs. This review will focus on recent findings regarding

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active zones at mammalian NMJs. We will briefly describe what has been shown at the NMJs of other species and at other synapses. Presynaptic active zones of the central nervous system (CNS) have been reviewed extensively elsewhere (Fejtova and Gundelfinger, 2006; Gundelfinger et al., 2016; Südhof, 2012). Our goal is also to highlight recent insights into the distribution patterns of active zone proteins in mammalian NMJs using findings from super-resolution microscopy and the selective degeneration of active zone proteins in aged animals. Analysis of active zones using super-resolution microscopy at *Drosophila* NMJs will be described in a separate review article by Robert Kittel in this special issue.

2. Analysis of active zone nanoarchitecture and ultrastructure

Active zones were first described using transmission electron microscopy. They were characterized as electron-dense projections to which synaptic vesicles attach (Couteaux and Pecot-Dechavassine, 1970). Electron micrographs of rodents and frogs revealed that NMJ active zones appear as triangular electron-dense projections that extend from the presynaptic membrane into the cytosol (Couteaux and Pecot-Dechavassine, 1970; Hirokawa and Heuser, 1982; Nishimune et al., 2004; Rowley et al., 2007). Three-dimensional reconstructions of serial electron micrographs of rat NMJs revealed the location of synaptic vesicle pools that were clustered in close proximity to the active zones (Rowley et al., 2007). Transmission electron microscopy of *Drosophila* NMJs also showed electron-dense projections at the presynaptic membranes, which appear as T-shaped structures. These projections, named T-bars, also recruit vesicles to the active zones (Kittel et al., 2006). Electron micrographs of CNS synapses from an *en face* view show active zones as hexagonal grids of dense projections with a web-like pattern (Gray, 1963). These projections found in CNS synapses are formed by an array of electron-dense, cone-shaped particles that project into the cytoplasm from the presynaptic membranes (Gray, 1963; Limbach et al., 2011; Pfenninger et al., 1972). The authors suggest that these dense projections represent an underlying molecular organization of the active zone material and are not artificially induced by fixation (Limbach et al., 2011; Pfenninger et al., 1972). However, whether the exact ultrastructural appearance and quantitative parameters are influenced by specimen processing remains unknown (Limbach et al., 2011).

Subsequent freeze-fracture electron microscopy studies revealed the membrane-embedded structure of active zones at a macromolecular resolution in an *en face* view orientation. In human, mouse, rat, and lizard NMJs, parallel rows of large intramembranous particles (10 − 12 nm in diameter) were seen on the cytosolic half of the plasma membrane (the P-face of freeze-fracture electron micrographs) (Ellisman et al., 1976; Fukunaga et al., 1982; Fukuoka et al., 1987b; Walrond and Reese, 1985). Based on these data, an active zone unit of mammalian NMJs has been proposed as consisting of a parallel array of 20 intramembranous particles arranged in four rows (Ellisman et al., 1976). These active zone units were observed in large numbers and were distributed in discrete locations within the presynaptic membrane of NMJs. However, the organization of the large intramembranous particles on the P-face was different at frog NMJs (Heuser et al., 1979; Heuser et al., 1974). At frog NMJs, an active zone was identified as a continuum of the intramembranous particles that nearly spanned the width of a nerve terminal branch (Ko, 1985). The existence of these active zone units defined by these intramembranous particles has been further supported by the tomography analyses described below.

Electron microscope tomography revealed the first three-dimensional structure of the cytoskeletal matrix of the active zones (CAZ) of frog NMJs (Harlow et al., 2001). The CAZ is a collection of proteins that constitute an active zone (see "NMJ active zone assembly and organization" for details) (Dresbach et al., 2001). The macromolecule components of the active zones identified by electron microscope tomography were named "beams," "ribs," and "pegs," and they were connected to each other and arranged along the midline of the presynaptic ridge (see Fig. 5 of reference (Harlow et al., 2001)). Based on the frequency and distribution of the pegs, the authors suggested that pegs are putative VGCCs embedded in the presynaptic membrane. A recent electron microscope tomography analysis of frog NMJs further revealed that an active zone has three sublayers: the superficial layer and the intermediate layer, both of which are approximately 15 nm thick, and the deepest layer, which is up to 45 nm thick (Szule et al., 2012). The superficial layer contains the previously described beam-rib-peg assembly and is followed by the intermediate layer, which contains macromolecules named steps and spars. The deepest layer includes macromolecules termed masts, booms and topmasts (Szule et al., 2012). These macromolecules are arranged in a highly ordered ultrastructure that interacts with synaptic vesicles at multiple points on an active zone (see Fig. 2 of reference (Szule et al., 2012)).

An electron microscope tomography analysis of mouse NMJs identified a similar highly ordered ultrastructure with the beam-rib-peg assemblies and two docked synaptic vesicles (Nagwaney et al., 2009). The macromolecules of mouse active zones showed a bilateral arrangement relative to the two docked synaptic vesicles (Fig. 1). The numbers and distribution patterns of the pegs were very similar to the parallel rows of the large intramembranous particles that were identified as an active zone unit by freeze-fracture electron microscopy analysis of mouse NMJs. The macromolecules of mouse active zones show a bilateral arrangement, whereas the macromolecules of frog active zones show a unilateral arrangement relative to the docked vesicles (see Fig. 8 of reference (Nagwaney et al., 2009) for a comparison of the arrangement of macromolecular connections in the active zones of mouse versus frog NMJs). This bilateral arrangement in mice may favor more efficient vesicle docking and/or fusion (Nagwaney et al., 2009). The authors observed only two primary docked vesicles per active zone and further predicted that there are approximately 450 primary docked vesicles per mouse NMJ. The beam-rib-peg complexes of frog and mouse NMJs are significantly different from that of CNS synapses.

Electron microscope tomography analyses of neocortical synapses revealed an array of polyhedral cages approximately 60 nm in diameter at active zones (Zampighi et al., 2008). Previously, filamentous proteins protruding from presynaptic membranes were observed using rapid freezing combined with freeze-etched electron microscopy (Landis et al., 1988). Synaptosomes prepared from adult rat cortices were stained with ethanolic phospho-tungstic acid (EPTA) and analyzed using electron microscopy. These micrographs clearly showed a presynaptic particle web formed by electron-dense protrusions from the membrane that were connected with thin linkers (Phillips et al., 2001). This ultrastructure of CNS active zones detected using EPTA staining was somewhat similar to the distribution pattern of polyhedral cages revealed by the tomography method (Fig. 8 of reference (Phillips et al., 2001) and Fig. 10 of reference (Zampighi et al., 2008)). Hair cell ribbon synapses analyzed using electron microscope tomography revealed a ribbon attached to a presynaptic density, which are located in the center of two rows of docked synaptic vesicles (Frank et al., 2010). This structure was closer to, but not the same as, the frog NMJ active zone. There seem to be ultrastructural differences between the active zones of CNS synapses, hair cell synapses, and NMJs (see Fig. 1 of (Zhai and Bellen, 2004) or of (Ackermann et al., 2015) or Figs. 6 and 7 of (Slater, 2015) for descriptions of the different active zone morphologies in var-