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# **The role of the actin cytoskeleton in oxytocin and vasopressin release from rat supraoptic nucleus neurons** V. A. Tobin and M. Ludwig *J. Physiol.*, August 1, 2007; 582 (3): 1337-1348.

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#### Three-Dimensional Architecture of Presynaptic Terminal Cytomatrix

L. Siksou, P. Rostaing, J.-P. Lechaire, T. Boudier, T. Ohtsuka, A. Fejtova, H.-T. Kao, P. Greengard, E. D. Gundelfinger, A. Triller and S. Marty *J. Neurosci.*, June 27, 2007; 27 (26): 6868-6877. [Abstract] [Full Text] [PDF]

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# The Architecture of the Active Zone in the Presynaptic Nerve Terminal

Active zones are highly specialized sites for release of neurotransmitter from presynaptic nerve terminals. The architecture of the active zone is exquisitely designed to facilitate the regulated tethering, docking, and fusing of the synaptic vesicles with the plasma membrane. Here we present our view of the structural and molecular organization of active zones across species and propose that all active zones are organized according to a common principle in which the structural differences correlate with the kinetics of transmitter release.

Synapses are specialized cell-cell contacts where signals are reliably transduced from a neuron to its target cell in a regulated manner. In chemical synapses, signal transduction is achieved by converting the electrical signal into a chemical signal that diffuses between cells. This signal conversion occurs at active zones, highly specialized sites of the presynaptic nerve terminal. The term "active zone" was coined in 1970 by Couteaux and Pecot-Dechavassine (14) during their ultrastructural studies of partially contracted frog muscles, in which they observed that profiles of open synaptic vesicles occurred immediately adjacent to the presynaptic dense bands, and consequently they designated these dense bands "les zones actives." Subsequently, similar observations were made in other types of synapses.

Ultrastructural studies of synapses in different organisms have revealed the following conserved morphological features among active zones, regardless of their size, location, or types of neurons and their targets. First, the plasma membrane of the active zone appears to be electron dense. suggesting its proteinaceous nature. Second, synaptic vesicles cluster, tether, and fuse at the active zone (14, 36). Third, the active zone is closely and precisely aligned with a postsynaptic density (PSD) area, such that the active zone spans the same width as the PSD and the extracellular space between the two membranes (synaptic cleft) is as narrow as 30 nm (51). The latter two morphological characteristics suggest that active zones function as sites of synaptic vesicle exocytosis and neurotransmitter release.

Over the past three decades, these hypotheses have been supported by many neuroscientists through studies of synaptic vesicle exocytosis and postsynaptic neurotransmitter receptor function. Here we discuss the structural organization of active zones in different types of synapses found in a variety of organisms, summarize recent advances in the molecular characterization of the assembly

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of active zones, and propose that all active zones are organized according to the same pattern.

#### The Structure of Active Zones

Morphologically, active zones are defined as sites of synaptic vesicle docking and fusion, and physiologically they are defined as sites of neurotransmitter release. On the basis of these definitions, we dissect the active zone into three morphologically and functionally distinct components:

- 1. the plasma membrane juxtaposed to the PSD where synaptic vesicle fusion occurs,
- 2. the cytomatrix immediately internal to the plasma membrane where synaptic vesicles dock, and
- 3. the electron-dense projections extending from the cytomatrix into the cytoplasm on which synaptic vesicles are tethered.

All active zones have these three components, although they vary in appearance, especially in size and shape of dense projections. FIGURE 1 illustrates the ultrastructure of active zones found in nine different types of synapse as well as the schematic representation of the three components in each type of active zone. Below we will discuss the molecular and functional properties of each component of the active zone.

# The Plasma Membrane of the Active Zone

Besides separating the cytosol from the extracellular environment, the plasma membrane of the active zone has two "gates" essential for neurotransmission: one for  $Ca^{2+}$  entry, which is the voltage-gated  $Ca^{2+}$  channel, and the other for neurotransmitter exit, which is the synaptic vesicle fusion site. These two gates are thought to be in

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close proximity to each other on the basis of several observations. First, the time delay between Ca2+ entry and synaptic vesicle fusion is only 0.2 ms (69, 87). Second, theoretical analyses of Ca<sup>2+</sup> diffusion dynamics and quantal secretion have shown that the probability of secretion of a synaptic vesicle decreases threefold with a doubling of the distance between the Ca<sup>2+</sup> channel and the synaptic vesicle from 25 to 50 nm (4). It is thus likely that in most synapses the space between  $\mathrm{Ca}^{\scriptscriptstyle 2+}$  channels and docked synaptic vesicles at the active zone is <50 nm (3, 87). Numerous immunohistochemical studies have demonstrated the localization of Ca<sup>2+</sup> channels near the active zone (46, 75, 104); however, the organization and arrangement of  $\rm Ca^{2+}$ channels at active zones are best suggested by freeze-fracture studies on frog, lizard, and mammalian neuromuscular junctions (NMJs) (19, 37, 92). On freeze-fracture replicas of NMJ boutons, double rows of prominent intramembranous particles of 10–12 nm in diameter can be seen with occasional synaptic vesicles clustering closely beside them (FIGURES 1N AND 2C). These rows of particles are in exact register with the postsynaptic folds in the underlying muscle (37) and are postulated to be voltage-gated Ca<sup>2+</sup> channels (13, 72, 75). The most direct evidence supporting this view comes from atomic force microscopy on the large calyciform synapse of the chick ciliary ganglion, showing a row-like arrangement of the immunolabeled Ca<sup>2+</sup> channels (35).

If the calcium channels are organized in orderly arrays at active zones, one would expect the other gate (the synaptic vesicle fusion site) to be organized in a corresponding manner. The vesicle fusion sites are specialized to allow the lipid bilayers of synaptic vesicles and active zone plasma membrane to come together and form a hydrophilic fusion pore. The SNARE complex has been thought to be the driving force for bringing the membranes together, facilitating lipid bilayer mixing and subsequent membrane fusion (43, 74). The t-SNAREs syntaxin and SNAP-25 have been localized to the presynaptic plasma membrane, although their distribution is not restricted to the active zone and the precise positioning and organization of t-SNAREs at the active zone is not clear (25, 38, 82). Biochemical analyses have demonstrated the direct interaction between the t-SNAREs syntaxin and SNAP-25 and Ca<sup>2+</sup> channels (11, 44, 57, 90). This may be the possible mechanism for the close positioning of the fusion machinery and Ca2+ channels required for Ca2+-dependent exocytosis. Interestingly, a study in PC-12 cells, a neurosecretorv cell line, has shown that syntaxin and SNAP-25 form cholesterol-dependent clusters in the plasma membrane, and these clusters seem to define sites at which secretory vesicles dock and fuse with high preference. Cholesterol removal causes dispersion of clusters and the subsequent inhibition of exocytosis (52). This study provides a clue for the potential role of lipids in active zone plasma membrane specification.

Other important components of the plasma membrane are the adhesion molecules by which the precise alignment of the active zone with the PSD is most likely mediated. Several classes of adhesion molecules have been shown to be present at the active zone: cadherins (85, 99), protocadherins (23), nectins (64, 88), neural cell adhesion molecule (77)/fasciclin II (15)/aplysia cell adhesion molecule (60), Down syndrome adhesion molecule (80), syndecans (42), L1/neuroglian (93), integrins (12), neurexins (63), and sidekicks (100, 101). All adhesion molecules share common protein motifs: an extracellular domain that mediates binding with the postsynaptic counterparts or extracellular matrix, a single-pass transmembrane domain or membrane anchor, and often an intracellular domain that binds to the cytoskeleton or the intracellular scaffolding proteins (28, 86). All of these adhesion molecules except neurexin, which is expressed presynaptically and binds its postsynaptic receptor neuroligin (100), are expressed in both pre- and postsynaptic terminals, and adhesion is formed through homophilic interactions. Adhesion molecules at the active zone are more than just "glue." They also mediate signaling within and between nerve terminals and modulate neurotransmission. Numerous reviews have commented on the detailed mechanisms of these molecules in synapse adhesion and regulation (22, 68, 79, 88).

In summary, the primary function of the plasma membrane at the active zone during neurotransmitter release is to mediate fusion of synaptic vesicles upon calcium entry. This is achieved by an array-like organization of the Ca2+ channels and the localization of the fusion machinery at the membrane.

#### The Cytomatrix Underlying the Plasma Membrane of the Active Zone

When viewed by electron microscopy, the cvtomatrix of the active zone is electron dense and displays a web-like pattern, which was first noticed by Bloom and Aghajanian (5) and subsequently by Pfenninger and colleagues (70). Recently, elegant studies have provided the first three-dimensional views of the cytomatrix at the active zone of the frog NMJ and the mammalian central nervous system (CNS) synapse. Using electron microscope tomography, Harlow and colleagues (32) revealed a striking array-like structure at the frog NMJ consisting of "beams" and "ribs" that connect

docked synaptic vesicles with putative Ca<sup>2+</sup> channels (FIGURE 2, A–D). The beams run along the midline of the presynaptic ridge parallel to the ridge's long axis, and the ribs extend from the beams and connect the synaptic vesicles near the vesicle-plasma membrane interface. In addition, the ribs are connected to the intramembrane macromolecules resembling the putative Ca<sup>2+</sup> channels seen in freeze-fracture studies (see above,

FIGURES 1N, 2C, AND 2D). With this organization, each docked vesicle is perfectly aligned with at least one Ca<sup>2+</sup> channel, which would allow high release fidelity.

The picture of the mammalian CNS synapse was revealed by Phillips and colleagues (71). In this study, they purified a presynaptic "particle web" consisting of ~50 nm pyramidally shaped particles interconnected by ~50- to 100-nm-spaced fibrils

Replica from freeze fractured frog NMJ terminal

С

#### A Frog NMJ terminal



FIGURE 2. Models of the active zone structure in frog NMJ and mammalian central nervous system synapses A–D: models of the active zone structure in frog NMJs (32). A: transmission electron micrograph of frog NMJ, where synaptic vesicles are docked to the plasma membrane through dense projections. B: 3-dimensional reconstructed and surface-rendered view of the active zone material and docked vesicles. C: replica from a freeze-fractured frog NMJ showing a series of particles/macromolecules on each slope of a ridge with surface-rendered rib and beam assemblies. D: arrangement of ribs, beams, and synaptic vesicles at frog NMJs. E–G: models of the active zone structure in mammalian central nervous system synapses (71). E–F: transmission electron micrographs of purified active zones from mammalian central nervous system in side view (E) and top view (F). Thin fibrils can be seen (arrow in F). G: active zone structure of mammalian central synapses.

50 µm

50 µm

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Thin fibrils

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(FIGURE 2, E–F). The particles are evenly spaced by the fibrils, and this web therefore forms 50- to 100-nm slots for synaptic vesicles to dock and fuse (FIGURE 2G).

The electron-dense nature of the cytomatrix underlying the plasma membrane suggests that a large number of proteins are localized there and that the cytomatrix at the active zone is important in regulating vesicle docking and fusion. In searching for the building blocks of this specialized cytomatrix, a number of protein components have been identified. On the basis of their function or putative function, the proteins identified in the active zone cytomatrix can be classified into three categories. First, the classical cytoskeletal proteins corresponding to actin, tubulin, myosin, spectrin  $\alpha$ -chain and  $\beta$ -chain, and  $\beta$ -catenin (9, 40, 71) are the fundamental elements of the framework of active zone cytomatrix. Second are the known scaffolding proteins, including SAP90/PSD95/Dlg, SAP97, and CASK/LIN-2 (34, 47, 48, 65). These proteins are not restricted to active zones because they also participate in clustering of postsynaptic receptors and are involved in the organization of a variety of cell junctions (20, 27, 67). If the cytoskeleton proteins form a grid-like structure at the active zone, these scaffolding proteins probably link the ion channels and the fusion machinery onto the grid to ensure proper active zone function. For example, CASK interacts with  $\beta$ -neurexin, syndecan 2, Ca2+ channels, the cytosolic protein Veli/LIN-7, and the Munc18/n-Sec1-interacting protein Mint1 (10, 34, 41, 59). Third, there are the active zone-specific proteins including RIM1, Munc13/ unc13, Bassoon, Piccolo/Aczonin, and CAST/ERCs (8, 17, 21, 94, 95, 96). Their active zone-specific localization and their multidomain structure allow them to participate in modulating synaptic vesicle docking, priming, and fusion, as well as the initiation of the assembly of the active zone structure. Physiological studies indicate that some of these proteins are involved in vesicle priming as well as synaptic transmission regulation (18, 76, 89).

In summary, the primary function of the cytomatrix at the active zone is to mediate docking of synaptic vesicles. The cytoskeletal and scaffolding proteins form a web-like structure consisting of slots for synaptic vesicle docking, and components of the cytomatrix regulate vesicle priming and fusion.

#### The Electron-Dense Projections Extending from the Cytomatrix of the Active Zone: Synaptic Ribbons

Some active zones have very prominent electrondense projections extending from the cytomatrix into the cytoplasm. They were first described and characterized in vertebrate sensory synapses involved in vision, hearing, and balance (50, 55, 91). These dense projections, or synaptic ribbons, are ribbon-like or spherical, extend 0.5-1 µm into the cytoplasm, and always have a "halo" of synaptic vesicles tethered to their surface [FIGURE 1, H-] (55)]. Because of their remarkable appearance, it has been thought that ribbon synapses are different from all other synapses, and the synaptic ribbons are exclusive to ribbon synapses to mediate the graded and sustained neurotransmitter release of these synapses (55, 91). However, we propose that electron-dense projections are not unique to ribbon synapses but rather are an integral part of the active zone and have an evolutionarily conserved structure. They have different sizes and shapes in different types of synapses but perform the same function of tethering synaptic vesicles at active zones.

Morphologically, dense projections have been observed in different types of synapses in different species. At Caenorhabditis elegans NMJs, dense projections in the shape of a plaque have been described [FIGURE 1B (31)]. In Drosophila, Tshaped dense projections can be seen in NMJs, the tetrad synapses of the visual system (FIGURE 1, F AND G), and in CNS synapses (61, 102). In crustacean NMJs, dense projections appear to be cylindrical [FIGURE 1D (29)]. In vertebrate NMJs, dense projections have been described in frog, lizard, and mammals [FIGURE 1, N AND P (19, 36, 92)]. In mammalian CNS synapses, dense projections were also noticed in electron microscopic studies as early as the 1960s and recently have been purified and visualized in great detail [FIGURE 2, E-F (5, 71)]. On the basis of the size of dense projections, we can divide different types of active zones into two groups: those with prominent dense projections, including invertebrate synapses with T bars and vertebrate ribbon synapses, and those with less-prominent dense projections, including vertebrate NMJs and CNS synapses. The dense projections in these latter active zones are not very prominent and project <100 nm into the cytoplasm and therefore have generally been considered to be part of the cytomatrix at the active zone (18, 26, 51).

Physiologically, numerous studies have demonstrated the tethering function of dense projections. In vertebrate sensory synapses, the motor protein KIF3A, which is a component of ribbons, is likely to mediate the tethering of synaptic vesicles (66). By tomography study, the synaptic vesicles in frog NMJs are clearly shown to be tethered through ribs to the beams, corresponding to the dense projections (32). In *Drosophila* and crayfish, synaptic vesicles cluster around T bars, although the mechanism of tethering is not known (FIGURE 1, A–G). Recently the dense projections of mammalian CNS active zones have been biochemically purified and molecularly characterized (71). These dense projections are ~50 nm in size, are pyramidlike (FIGURE 2, E AND F), and contain synaptic vesicle binding proteins such as synapsin and RIM (39, 71, 96). Therefore, the pyramidlike dense projections of mammalian CNS synapses are thought to tether and cluster synaptic vesicles to the active zone. Despite their proposed function of tethering synaptic vesicles, dense projections do not seem to be essential for neurotransmitter release, as suggested by a series of knockout studies. In the mammalian retina, Bassoon has been shown to be a component of photoreceptor ribbon synapse (6). In homozygous bassoon mutant mouse retinas, photoreceptors have a greatly reduced number of ribbons and the existing ribbons are freely floating in the cytoplasm with synaptic vesicles attached to them. Electroretinogram recordings from these photoreceptors showed that neurotransmission is maintained at low-intensity light stimulation but dramatically reduced at high-intensity light stimulation (16). This suggests that synaptic vesicles can fuse without the ribbon anchored to the plasma membrane. However, strong and continuous stimulation cannot be sustained. Knockout studies of RIM1 in C. elegans suggest that RIM1 is not required for morphological docking of synaptic vesicles or calcium-regulated fusion (49). In addition, RIM1 knockout mice have very mild synaptic phenotypes, suggesting that RIM1 may not be essential for neurotransmitter release. Alternatively, there is genetic redundancy, and other proteins can perform a similar function to that of RIM (81). In addition, knockout studies of synapsin suggest that it may not be required for the vesicle fusion step but rather for the synapsin-dependent cluster of vesicles. This clustering is apparently required to sustain release of neurotransmitter in response to high levels of neuronal activity (39).

If the function of dense projections is tethering synaptic vesicles, what is the physiological advantage of varying their size and shape? One possibility is that larger dense projections greatly increase the number of synaptic vesicles tethered at the active zone and therefore increase the size of the readily releasable pool. This is quite evident in ribbon synapses. For example, the ribbon in frog saccular hair cells is a sphere ~0.5  $\mu$ m in diameter, and on average there are 400 vesicles attached to the surface of the spherical projection, whereas 124 vesicles are attached to the active zone membrane. All vesicles attached to the ribbon can be released upon strong stimulation [FIGURE 1L (53, 54, 58)]. Therefore, these large, dense projections allow an increase in the size of the readily releasable pool without increasing the size of the active zone fusion area and PSD field. This feature is particularly important in sensory synapses, because sustained release upon continuous stimulation requires a huge readily releasable pool and a large synaptic vesicle replenish capacity, and the defined space representation of an individual sensory neuron in the visual or auditory field restricts the size of each terminal. In contrast, at many NMJs, where stimulations are not continuous, the size of nerve terminals is not restricted and the active zone must expand as the muscle grows, so dense projections are relatively small. Interestingly, in Drosophila and crustacean NMJs, active zones with prominent T bars can be seen adjacent to those without T bars within the same presynaptic nerve terminal (FIGURE 1, D AND F). It has been proposed that the active zones with prominent T bars have a stronger output, possibly because more synaptic vesicles are released upon stimulation. Supporting evidence comes from crustacean studies showing that high-output NMJ terminals have a threefold higher density of dense projections than the low-output terminals arising from the same excitatory motor axon, although no difference was observed in total synaptic area (29, 30).

In summary, although the morphology of dense projections varies greatly among different types of synapses, the primary function of dense projections is to tether synaptic vesicles at the active zone. Larger dense projections tether more synaptic vesicles and therefore increase the size of the readily releasable pool.

# Active Zone Assembly and the Regulation of Active Zone Density and Spacing

Active zone assembly occurs after the initial axontarget recognition and contact. It ends with the establishment of functional neurotransmitter release sites. In cultured hippocampal neurons, active zone assembly takes ~30 min (2, 24). According to a recently proposed unitary assembly model, active zone-specific proteins are packaged into transport vesicles for delivery to the nascent synaptic contact site. Upon fusion of such vesicles with the plasma membrane, the active zone proteins are deposited and localized. In cultured hippocampal synapses, one active zone forms from two to three transport vesicles (84, 103). Considering that on average each active zone has 10-15 synaptic vesicle release sites or "grid units," each transport vesicle should carry the building material for 4-5 synaptic vesicle release sites. This model suggests that active zone assembly occurs within an hour, which allows rapid synaptogenesis during development and synapse expansion during activity-dependent long-term potentiation. Genetic analyses in C. elegans and Drosophila

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have identified mutations of several genes that affect active zone assembly. The first gene was the C. elegans syd-2 gene. In the syd-2 loss-of-function mutants, active zones of NMJ terminals are lengthened and are less electron dense (105). The Syd-2 protein is localized to active zones and is a member of the Liprin protein family, which contains coiledcoil and sterile  $\alpha$ -motif domains (83). Liprins interact with the Lar family of receptor protein tyrosine phosphatases (RPTPs) and cluster RPTPs to focal adhesions (83). *Drosophila* Liprin- $\alpha$  is also localized to active zones at NMJs, and in flies mutant for Liprin and Dlar, the size of active zones are ~2.5-fold bigger than normal and the morphology is more irregular (45). In Drosophila, loss of wishful thinking (wit) causes a reduced number of boutons, an increased number of active zones per bouton, and freely floating T bar structures in the cytoplasm (1). Wit is a BMP type II receptor that is expressed in a subset of neurons, including motor neurons. However, the mechanism as to how Wit regulates active zone assembly is not understood (1, 56).

Active zones are not static but rather plastic structures. In tetrad synapses of the Drosophila visual system, the number of presynaptic ribbons/T bars changes with alterations in light stimulation (7, 78). In crustacean NMIs, high-frequency stimulation-induced long-term facilitation also correlates with an increase in the number of active zones and dense projections (98). In mammalian hippocampal neurons, long-term potentiation also correlates with the expansion or "division" of active zones (33, 97). Interestingly, a recent study using a transgenic calcium-imaging technique at Drosophila larval NMJs demonstrated that persistent strengthening of junctional vesicle release relies on the recruitment of additional active zones with normal spacing; increasing active zone density alone without changing bouton size only results in transient increase in evoked vesicle release on single active potentials but not consolidated enhancement of neurotransmission (73).Therefore, long-term potentiation requires assembly of new active zones and expansion of the bouton size, because the density of active zones has to be maintained. Ultrastructural observations from Drosophila and Sarcophaga have also suggested that the density of active zones is tightly regulated and that there is a minimum spacing required between neighboring active zones (62), presumably allowing each active zone to have sufficient and equal access to synaptic vesicle pools and/or recycling machinery.

#### Summary

The active zone in the presynaptic nerve terminal is a highly organized, beautifully designed, and

dynamic structure specialized for neurotransmitter release. With the identification of some of the building blocks of active zones, we are beginning to understand some of the morphological and functional properties of the active zone. Active zones consist of a plasma membrane, a cytomatrix. and dense projections. Although these three components are morphologically distinct, they are intimately connected with each other and the postsynaptic nerve terminal, ensuring the fidelity of synaptic vesicle tethering, docking, and fusion, as well as signal detection. Although the morphology of active zones and the molecular composition vary among species, tissues, and cells, the architectural design of the active zone is likely to be conserved.

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