Relax? Don't do it!—Linking presynaptic vesicle clustering with mechanical tension

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The release of transmitter-filled vesicles from presynaptic terminals is a key step of neurotransmission. Prior to release, synaptic vesicles get clustered at a specialized patch of the presynaptic membrane, here referred to as the active zone. So far, mainly biochemical regulations at the active zone were regarded as decisive for synaptic vesicle clustering and release. However, using biophysical approaches, a recent paper [Siechen, *et al.* (2009). *Proc. Natl. Acad. Sci. U.S.A.* 106, 12611–12616] indicated also that the micromechanical regulations within axon and terminal could be crucial for proper vesicle clustering. The authors demonstrated that the synaptic vesicle accumulations vanished after axotomy but were restored after the application of physical tension. Furthermore, axons seem to be under an intrinsic tension, which could be perceived and tuned by an axon-internal tension sensing mechanism. Therefore, mechanical force could steer vesicle clustering and consequently synapse function. Here, we review this interdisciplinary study of Siechen, *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* 106, 12611–12616 (2009)] and discuss the significance of cellular mechanics on synaptic function. [DOI: 10.2976/1.3260842]

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Animals can detect external signals, integrate these with their internal state, and initiate an appropriate motor-output. To accomplish these challenges, multicellular organisms employ specialized cells termed neurons. These elementary units of the nervous system are connected to each other on tiny contact sites referred to as synapses. Chemical synapses use neurotransmitters to propagate unidirectional signal transmission. For this purpose, neurons accumulate neurotransmitter containing synaptic vesicles (SVs) at presynaptic terminals and release them by exocytosis upon arrival of action potential stimuli. Released neurotransmitter molecules diffuse through the synaptic cleft and bind to their cognate receptors on the postsynaptic density, thereby triggering a response of the postsynaptic terminal (Squire *et al.*, 2008). Notably, repeated usage of a given synapse facilitates its neuronal transmission efficiency and strengthened synapses are, in turn, believed to be a neuronal correlate of learning and memory. Beside the changes involving the postsynaptic apparatus, activity-dependent modulations of the presynaptic SV

release machinery are supposed to be critical for synaptic plasticity as well (Hawkins *et al.*, 1993). The presynaptic SV exocytosis machinery entails a protein-rich matrix termed active zone (AZ). Intense research is being performed on identifying the AZ-proteins, AZ-protein interactions, and activity-dependent changes in AZ composition (Owald and Sigrist, 2009). Nevertheless, the exact mechanisms of presynaptic vesicle clustering and regulation of activity-dependent SV release remain largely elusive.

Recent experiments by Siechen *et al.* (2009) introduced a novel, unexpected jigsaw piece to this scene. In an innovative study, the authors demonstrated that the mechanical tension within axons is essential for effective vesicle clustering in the presynaptic terminal. At first glance, a key role of tension on synaptic formation and function might sound somewhat bizarre. Nonetheless, increasing data support the concept that cells are capable of sensing physical aspects of their environment (i.e., force and geometry) at a nano-to-micrometer scale (Vogel and Sheetz, 2006). Knowing their

Figure 1. Experimental procedure and stretch/tension data. (a) The embryonic nervous system of *Drosophila* comprises two brain hemispheres and a ventral nerve cord. The motoneuron axons originate from the VNC and innervate their target muscles in a stereotypic manner. The fifth motoneuron axon (from the posterior end) was severed with a laser and the SytI staining (as SV marker) in the axon terminal compared to the neighboring, nonsevered axon terminals. Apart from significantly reduced SV clustering, severed axons showed a gap development and lacked physical tension. Notably, the size of the gap between the proximal and distal axon stub depended on the embryonic stage as nonsynapsing axons lacked tension and thus extensive gap formation. (b) To study the relation between the tension and the vesicle clustering in greater detail, a micromechanical force sensor was engaged. The probe was broad in contact with the severed axon end to induce nonspecific adhesion. Subsequently, the sensor was moved to impose axonal tension/stretch. The force $F=kx$ applied on the point of contact could be calculated from the deflection x of the probe reference. (c) Using this force probe, a linear correlation of axonal length and tension was revealed in embryonic *Drosophila* motoneuron axons. Extrapolation of stretch/tension pointed to an intrinsic axonal rest tension (intersection with the y-axis). (d) An axon stretch led to an immediate increase in axonal tension. During this fixed stretch, the axonal tension declined exponentially to the rest tension. (e) When an axon was released from an induced stretch, it shortened to original length with a constant velocity.

physical surroundings, cells initiate appropriate responses. Mechanosensation, mechanotransduction, and mechanore-sponse constitute such force-feedback loops. Remarkably, several studies have emerged over the past years that argue for a strong influence of the mechanical microenvironment on cellular functions such as gene expression (Chien, 2007), cytoskeletal dynamics (Nelson *et al.*, 2005), and induction of apoptosis (Cheng *et al.*, 2009). So why not also on synapse formation and SV release?

SYNAPTIC VESICLE CLUSTERING IS DEPENDENT ON MECHANICAL TENSION

The *Drosophila* NMJ as a model system to study synapses

For their experiments, Siechen *et al.* (2009) employed the neuromuscular junction (NMJ) of *Drosophila melanogaster* embryos. The NMJ is the synaptic connection between motoneuron axons and their target muscles [see Fig. 1(a)]. It is a genetically and experimentally easy to access sys-

tem, characterized by stereotypic relationships between innervating motoneuron axons and target muscle cells (Collins and DiAntonio, 2007; Keshishian *et al.*, 1996). After the initial contact of the axon and the target muscle in an approximately 14 h into embryogenesis, a sequence of maturation events takes place including presynaptic SV clustering during the early stage of synaptogenesis (Ackley and Jin, 2004; Featherstone and Broadie, 2000).

Presynaptic SV clusters are lost after axotomy—a key role of tension

First, Siechen *et al.* (2009) asked whether the connection between the presynaptic terminal and the neuronal soma was mandatory for proper synapse formation and SV clustering. For this purpose, they chemically and physically isolated the axon terminal from the cell body by severing the axons with a laser [see Fig. 1(a) inset]. Two hours after axotomy, the embryos were dissected and stained for a SV marker [Synaptotagmin-I (SytI)] to reveal SV recruitment at presynaptic terminals (Littleton *et al.*, 1993). While the neighboring, nonsevered control axons featured SytI accumulations on presynaptic terminals, axotomized neurons lacked presynaptic SV clusters as well as mechanical tension. To study whether the loss of physical force by itself was causally related to SV clustering at all, the severed axon end was subjected to tension in a subsequent experiment. Surprisingly, pulling severed axons with the help of a micropipette resulted in a significantly higher level of SytI staining at presynaptic terminals when compared to nonpulled, severed axons. This indicated either a restoration of SV clusters or a prevention of their loss if severed axons were pulled immediately after axotomy. Therefore, the lack of physical tension seemed to be crucial for impaired SV clustering in non-pulled, severed axons and not the interrupted biochemical connection to the cell body.

A micromechanical force sensor to study the tension within axons

To gain further insight into the biological impact of mechanical tension on presynaptic vesicle recruitment, Siechen *et al.* (2009) made use of a previously described micromechanical force sensor (Yang and Saif, 2005). In order to induce non-specific binding of an axon to the probe, the latter was placed in contact with the axon for 10 min. By moving the force sensor with a piezoelectric actuator, the intact axon was stretched subsequently. This, in turn, led to tension in the axon [see Fig. 1(b)].

Embryonic *Drosophila* motoneuron axons are under rest tension

Using this force sensor, it was feasible to relate applied force (tension) with induced axon stretch and vice versa. In these experiments, both parameters were found to be in linear correlation in embryonic *Drosophila* motoneuron axons. Of

note, extrapolation of the tension-stretch data pointed toward an intrinsic stretch component and a mechanical rest tension in the axons [see Fig. 1(c)]. However, innate stretch and tension were only present in matured, already synapsing axons: the laser treatment of these axons led to a fast and extensive gap formation between proximal and distal axon stubs [see Fig. 1(a) inset]. When, in contrast, the axons had been severed prior to NMJ synapse formation, they only showed a slow and small gap development upon laser-induced injury. Hence, the process of tension development appears to be connected to the initiation of synapse formation.

Synapsing axons are able to sense their tension and to adjust themselves to a certain basal tension

To explore whether synapsing axons are capable of sensing this internal tension and physically adjust themselves accordingly, intact axons were subjected to a sustained stretch while tension was monitored over time. In this experiment, axonal tension rose instantaneously after the stretch imposition and then declined exponentially to the original rest tension within less than half an hour [see Fig. 1(d)]. After the stretched axon was released from the stretch, it shortened to original length with constant velocity [see Fig. 1(e)]. Taken together, both experiments strongly support the idea that axons can adjust their internal tension to a basic, fixed value in an autoregulative manner.

Induced axon stretch leads to enhanced SV clustering

Considering these findings, the authors hypothesized that modulating the axon-intrinsic tension might have a physiological function to tune SV clustering and thereby the SV release. To address whether an imposed force affects SV clustering in an intact axon as well, innervated muscles were clamped to the force probe and then pulled as described previously. If the hypothesis held true, increased SV clustering in the presynaptic junction should be expected. Indeed, the extent of presynaptic SytI staining at the terminals was more than twofold higher when axons had been stretched by 5%. These enhanced SytI clusters appeared within half an hour after the stretch imposition and sustained for at least 90 min. Notably, the enhanced SV accumulations were still observed after 30 min although the axon had already released its tension to the basic value [see Fig. 1(d)].

Thus, the stretching seems to initiate a cellular response that counteracts the tension increase with the aim reaching the rest tension. Yet this regulation obviously produces a cellular memory effect, which in turn, induces the clustering of additional SVs to the presynaptic terminal.

F-ACTIN MAY LINK VESICLE CLUSTERING TO MECHANICAL FORCE RESPONSE

However, many questions remain open—as is typically the case for interesting discoveries. To start with, of which nature is this cellular response downstream of the mechanical

Figure 2. Model for tension-dependent SV clustering. The motoneuron axon senses mechanical tension by an unknown mechanism. Changes in axonal tension trigger the extent of F-actin polymerization, while increased tension promotes F-actin polymerization. Actin filaments recruit SV to the presynaptic terminal, and thus, enhanced F-actin polymerization leads to enhanced SV clustering.

force? The authors already delivered a first hint at a possible mechanism within one of their experiments. When the constriction velocity of axons released from stretch was monitored, the axons showed a constant shrinkage speed of 5 nm/s [see Fig. 1(e)]. This rate is strikingly similar to the walking pace described for single, unimpeded actomyosin complexes (Yildiz *et al.*, 2003).

While on the one hand, the induction of actin polymerization by mechanical stress is well established (Vogel and Sheetz, 2006); F-actin, on the other hand, has been implicated in various synaptic functions including SV clustering (Cingolani and Goda, 2008). Thus, the authors favor an interaction between actin polymerization and vesicle clustering in the following manner (see Fig. 2).

Actin filaments cluster SVs to the presynaptic terminal. To guarantee F-actin polymerization, a basal tension is required that is established during early synaptogenesis and maintained by actomyosin interactions. The absence of tension favors F-actin depolymerization, which results in vesicle dispersion. On the other hand, applying stretch leads to a momentarily increased tension, which subsequently returns to the rest value [see Fig. 1(d)]. However, the stretch/tension history promotes actin polymerization, which in turn enhances F-actin scaffolding and thus SV clustering.

But which evidence places F-actin in context of synapse function and physical force?

F-ACTIN AND SYNAPSES

Along with microtubules and intermediate filaments, actin is the major cellular scaffold protein. It exists in two distinct states: either in a monomeric gel-state (G-actin) or as an asymmetric two-stranded helical filament (F-actin) of polymerized G-actin modules. The assembly and disassembly of

F-actin can be rapid, due to various actin-binding proteins that modulate actin polymerization (Pollard *et al.*, 2000). Therefore, F-actin is employed for both static and dynamic biological tasks, including intracellular protein trafficking (Vale, 2003), cell motility (Pollard and Borisy, 2003), cellular adhesion (Geiger and Bershadsky, 2002), and more.

F-actin has several functions in developing and matured neurons

F-actin is highly enriched in both the pre- and postsynaptic apparatus (Drenckhahn and Kaiser, 1983). In developing neurons, it is required for neurite formation, extension and branching, as well as for synaptogenesis (Cingolani and Goda, 2008).

At matured synapses, F-actin could be linked to the maintenance and regulation of vesicle pools—either by serving as a scaffold to restrict SV mobility or as a conduit for vesicle transfer between the pools (Dillon and Goda, 2005). For this purpose, F-actin associates with filaments formed by the protein synapsin in the presynaptic bouton core. The synapsin filaments are linked to SVs. If, due to increased neuronal transmission demand of SVs is high, synapsin is phosphorylated and, in consequence, sequesters SVs from F-actin in order to replenish available vesicles. Furthermore, enhanced synaptic activity seems to promote reversible F-actin polymerization at the presynaptic terminal (Colicos *et al.*, 2001).

Besides, there is also a biochemical and morphological body of evidence that implies actin as a major component of the AZ (Phillips *et al.*, 2001). Here, F-actin could have a dual function: on the one hand, it could be a negative regulator by forming a molecular or physical barrier for vesicle priming in order to prevent facile SV fusion. On the other hand, it could promote vesicle docking and thus play a positive role. For both aspects experimental evidence exists. It is conceivable that the function of F-actin might depend on synapse type and synaptic activity (Dillon and Goda, 2005).

Notably, at the *Drosophila* NMJ, F-actin is supposed to be involved in recruiting SVs to the readily releasable pool (Kuromi and Kidokoro, 1998) and the depolymerization of F-actin seems to provoke a dissemination of SVs (Delgado *et al.*, 2000).

F-actin is particularly important to allow stabilization of immature synapses

Siechen *et al.* (2009) observed the impact of tension on presynaptic SV clustering at immature synaptic connections. It is noteworthy that, in cultured hippocampal neurons, the effect of F-actin depolymerization on synaptic structure and SV clustering is related to the maturation state of synapses (Zhang and Benson, 2001). While, especially, the development and maintenance of immature synapses greatly depends on F-actin function, matured synapses generally appear less vulnerable toward a loss of F-actin. In the aforementioned cell culture study (Zhang and Benson, 2001),

F-actin depolymerization during the first week in culture resulted in a near complete loss of synapses as defined by synaptophysin-labeled vesicle clusters, FM-dye labeling, and electron microscopy. During the second week, the basic synaptic structure was no longer disrupted but the number and the size of vesicle clusters were reduced. By 18 days in culture, synaptophysin clusters and SV recycling were largely resistant to F-actin depolymerization. This decrease in the synapse dependency on F-actin might be mediated by AZ-proteins, which are acquired and retained during synaptogenesis and therefore could partially compensate the loss of F-actin function.

In summary, localized production of actin filaments is in fact a prime candidate process for the support of vesicle clustering at assembling immature synapses. What remains an open question is how F-actin formation could be coupled to the sensation of physical force.

F-ACTIN AND PHYSICAL FORCE

In the last 2 decades, studies began to decipher the mechanical impact on cellular processes such as motility, morphogenesis, proliferation, apoptosis, and more (Geiger and Bershadsky, 2002). A cellular mechanosensation device is necessary to recognize forces. In this context, various mechanisms have been described including mechanosensitive ion channels, force-dependent enzymatic activities, or conformational changes in protein structure (Vogel and Sheetz, 2006).

Mechanosensitive ion channels allow stretch-induced influx of calcium ions

Mechanosensitive ion channels open their gate upon stretch and allow ion influx to trigger intracellular signaling cascades. Whereas some channels respond to stress in the lipid bilayer, others are physically tethered to the cytoskeleton and/or the extracellular matrix in order to transmit forces to the channel. Notably, calcium influx was shown to trigger F-actin polymerization (Forscher, 1989; Oertner and Matus, 2005). Thus, a stretch could be utilized to open a force-gated calcium channel to eventually promote actin filament generation (Glogauer *et al.*, 1997; Vogel and Sheetz, 2006).

Focal adhesions can trigger mechanical stress-induced actin polymerization

Focal adhesions (FAs) are another resource for mechanosensation. FAs are cell junctions, which link the extracellular matrix via integrin-family proteins to the actin cytoskeleton. FAs form dynamically in response to mechanical force and trigger local F-actin polymerization. Conformational changes in cell adhesion molecules or other proteins of the cell adhesion membrane plaque could sense mechanical force either by modifications in enzyme activity or by exposure of cryptic peptide sequences. Tension is essential for both growth and maintenance of FAs but not for assembly

of the FA precursors (focal complexes). Furthermore, inhibition of myosin II by blebbistatin leads to relief from tension and rapid disassembly of FAs. In short, cells can engage FAs not only as physical anchors but also as mechanosensors (Geiger and Bershadsky, 2002).

Integrin binding is crucial for synaptic function

Interestingly, several lines have also implicated integrins—the cell adhesion molecules of FAs—in synaptic function. For instance, application of RGD-peptides, which are known to interfere with integrin binding, inhibits induction of hippocampal long-term potentiation (Staubli *et al.*, 1990). At the *Drosophila* NMJ, the integrin Volado was shown to be important for synaptic morphology, transmission efficacy, and functional synaptic plasticity (Rohrbough *et al.*, 2000). Hence, in an interesting and feasible experiment, it could now be addressed whether integrins are involved in mediating the tension effects, as described by Siechen *et al.* (2009).

Muscle stretch has acute effects on synapse function as well

Apart from the long-term regulations of synaptic architecture and function by physical tension (Siechen *et al.*, 2009); more acute effects of mechanical stretch on spontaneous and evoked SV release have previously already been described (Chen and Grinnell, 1995; Fatt and Katz, 1952). In these studies, stretching muscles led to more than doubled spontaneous and evoked transmitter release within 10–20 ms. In fact, the time observed by Siechen *et al.* (2009) for the recruitment of additional vesicles (minutes time scale) was several orders of magnitude larger than the time needed for increased transmitter release, as described in the experiments showing acute effects of mechanical stretch (several milliseconds). In the latter studies, a critical role of actin function is unlikely, as its dynamics are too slow. Furthermore, this mode of synapse tuning is not adaptive; it bypasses the calcium triggering step and mainly depends on integrin binding as well as dephosphorylation of one or more proteins in the link between integrin binding and vesicle fusion (Grinnell *et al.*, 2003). In contrast, the process of Siechen *et al.* (2009) appears to be a result of slow, localized cellular changes that involve synaptic assembly steps, for instance F-actin polymerization. However, its precise molecular mechanism remains still to be uncovered.

PERSPECTIVE

Siechen *et al.* (2009) reinforced the role of mechanical force, a so far neglected player in this context, among the mechanisms involved in shaping synaptic structure and function. As mentioned above, to date studies concerning SV release have mainly focused on the biochemical properties of the AZ (Owald and Sigrist, 2009). Apart from that, a series of experiments showed an enhanced neurotransmitter release after muscle stretch (Chen and Grinnell, 1995; Fatt and Katz,

1952; Grinnell *et al.*, 2003). Now, to our knowledge, Siechen *et al.* (2009) provide the first evidence that recognition of axon and target alone is not sufficient for proper synapse development and function but that, in addition, tension is crucial. However, studies on the exact relation between the sensing of mechanical force and synaptic differentiation events (e.g., vesicle clustering) are challenging. In particular, not all preparations will be as amenable for effective manipulation of mechanical force as the one used by Siechen *et al.* (2009).

Notably, in this study, SVs labeled by SytI essentially constituted the only marker used for synaptic differentiation. Therefore, not only the nature of the additional SVs (or the synaptic pool they belong to) remains elusive but it is also unclear whether the ultrastructure of the AZ per se is changed. For this, electron microscopic analysis and/or high-resolution light microscopy should be appropriate (Fouquet *et al.*, 2009). Furthermore, it is of special interest how axonal tension is perceived. In this commentary we disclosed two possibilities, namely, mechanosensitive ion flux and focal adhesion contacts. In future studies, it will be important to gain direct information about the very local coupling of mechanical stress and biochemical reactions (e.g., F-actin polymerization). In this context, combining the application of FRET-based mechanical force-sensors (Papusheva *et al.*, 2009) with *in vivo* live imaging of synapse differentiation (Rasse *et al.*, 2005) might be warranting. But even beyond these basic issues, many more general questions arise. To name a few: do NMJ axons rely on information about changes in mechanical tension *in vivo*, in order to tune synaptic transmission? If this is the case, are synapses in the CNS subject to similar influences as well? Is this phenomenon also dependent on the age of individual synapses, i.e., do immature and mature synapses share the same properties? Actin is highly enriched in dendritic spines: are there also tension-induced changes in spine morphology that contribute to synaptic plasticity?

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