Dynamics of Presynaptic Protein Recruitment Induced by Local Presentation of Artificial Adhesive Contacts

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ABSTRACT: **In this study, we introduce a novel approach to induce and observe the formation of presynaptic compartments in axons through a combination of atomic force microscopy (AFM) and fluorescence microscopy. First, we use a poly-D-lysine-coated bead attached to an AFM tip to induce the recruitment of two synaptic proteins, bassoon and synaptophysin, and measure their absolute arrival times to the presynaptic department. We find that bassoon arrives before synaptophysin. Second, we observe the formation of very long (several 10s of μm), structured, protein-containing membranous strings as the AFM tip was withdrawn from the axon. It is conceivable that these strings might be a novel mechanism by which new neurites or branch points along existing neurites may be generated *in situ*.** © 2012 Wiley Periodicals, Inc. Develop Neurobiol 73: 98–106, 2013

Keywords: **atomic force microscope; synaptogenesis; poly-D-lysine; presynaptic site; protein recruitment**

INTRODUCTION

Synapses are highly dynamic entities, from their initial creation and pruning to a more consolidated state (Garner et al., 2006). Studies of the initial stages of synaptogenesis are still sparse, in part due to the rarity of synapse creation in culture where single synapses are most conveniently identified and studied in detail (Ziv and Garner, 2004). Even when such events are patiently identified and scrutinized (Ahmari et al., 2000; Friedman et al., 2000; Kaether et al., 2000), the total number of events remains quite low. A method that could reliably induce synaptogenesis would therefore be highly desirable.

Another largely unresolved aspect of synaptogenesis is the required identity of synapse-inducing factors. A few specific ligand–receptor triggers have been identified, such as neuroligin/neurexin, SynCAM, and/or NGL-2 (Scheiffele et al., 2000; Biederer et al., 2002; Graf et al., 2004; Kim et al., 2006). Studies from the 80s (Burry, 1980, 1982) however indicate that no such specific ligand–receptor interactions are required and that axons are in fact quite promiscuous in their choice of postsynaptic partner. It was found that many (but not all) poly-cationic substances have synaptogenic properties whereas neutral or anionic substances do not.

Our laboratory has recently shown that presynapses induced by adhesive contact to poly-D-lysine (PDL; an artificial cationic protein) coated beads (as "stand-ins" for dendrites) are functional and share

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several characteristics with "real" presynapses, such as protein content and presynaptic vesicle recycling ability (Lucido et al., 2009). In turn, such hemi-synapses are useful in the study of synaptic development. During these studies, we concluded that a method to spatially precisely control the contact between axon and bead would be useful to study the temporal evolution of synaptic development as well as the adhesion between axon and bead. In particular, such a method would enable a systematic study of the recruitment of specific synaptic proteins rather than merely relying on chance encounters between axons and dendrites in culture.

We now introduce a novel method of combining an atomic force microscope (AFM) and fluorescence microscopy to precisely control a single contact between a hippocampal axon and a protein-coated bead in time and space. We observed the recruitment of Piccolo-Bassoon transport vesicles (PTVs) and synaptic transport vesicles (STVs) to the contact site and compare the temporal evolution to the real case (Friedman et al., 2000). In addition, we are in a unique position to observe the initial adhesion between axon and bead. We find that adhesion is immediate and point-like and that unusually long (>50 μm) membrane-bounded, protein-containing strings can be elicited from the axon. Furthermore, the adhesion points colocalize with pre-existing or recruited protein puncta, indicating the existence of special, preformed microdomains along an axon.

MATERIAL AND METHODS

Primary Cultures of Rat Hippocampal Neurons

Cultures of dissociated rat hippocampal neurons were prepared using a modification of a protocol described in Kaech and Banker (2006). Hippocampi were dissected from E17/18 embryos, treated with 0.25% trypsin at 37°C followed by Advanced DMEM-10% horse serum, washed 2x with HBSS, and mechanically dissociated with a plastic Pasteur pipette. The dissociated neurons were plated at a density of 1.8–2.3 $\times$ 10^4 cm^{-2} in PDL (Sigma)-coated glass-bottom dishes (MatTek) in serum-free Neurobasal medium supplemented with L-glutamine and B-27. One-third of the medium was replaced every 3–4 days. All culture media were purchased from Gibco (Invitrogen). The chosen cell density reflects a compromise between a lower limit that allows for long-term cell survival and a higher limit dictated by the need to locate isolated axons even on DIV10.

Transfection was performed on DIV6 using a standard Lipofectamine 2000 (Invitrogen) protocol. Three different plasmids were used; the Bassoon-GFP plasmid, which is a fusion of the amino acids 95–3938 to the C terminus of EGFP (refer to Dresbach et al., 2006), the mouse synaptophysin-GFP plasmid, and the actin-YFP construct were gifts from Dr. Edward Ruthazer (McGill University). Ten to twenty neurons were expressing the corresponding fluorescent proteins after 24 h. High expression levels persisted for up to 1 week.

Labeling of tubulin in axons was performed with TubulinTracker^{TM} Green (Invitrogen) as described in the product information sheet except that the cell culture was kept in Neurobasal medium during the imaging procedure. The final concentration of TubulinTracker^{TM} Green used was 100 nM.

Time-Lapse Protein Recruitment and Adhesion Experiments

Latex beads (7.5 μm, Bangs Laboratories) were glued to AFM cantilevers (Veeco, model: MSCT-AUHW) with UV-curable adhesive (Smith et al., 2007). The beaded cantilevers were incubated in 50 μg/ml PDL solution overnight at 4°C or used without coating for control experiments. Gluing the bead onto the cantilever prior to coating rather than *vice versa* prevents drying of the coated bead.

The prepared cantilevers were mounted on a Bioscope AFM (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) mounted over an inverted optical microscope (Axiovert S100TV, Zeiss) that facilitates bright-field or fluorescence imaging. We used a white-light excitation source sent through a filter cube suitable for GFP imaging. The white-light source was equipped with a mechanical shutter triggered by the image-acquisition program. The shutter was found to be absolutely necessary to avoid bleaching and enable long-term (several hours) image acquisition.

Bead-axon contact was achieved by positioning the cantilever next to a fluorescent axon and moving the cantilever laterally. Before initial contact, it was found to be necessary to leave the cantilever in the sample medium for 30–45 min to allow the system to equilibrate thermally, thus minimizing unwanted drift. When the contact occurred (observed with the inverted optical microscope), a movie was acquired with a CCD camera (cascade:1k, photometrics) at a rate of six frames per minute. The temperature of the sample was kept at 37°C by using a heated stage (DH-35 Culture Dish Heater, Warner Instruments) and the pH was adjusted to 7.2 by keeping the sample in a 5% CO_2/air mixture.

Image Analysis and Vesicle Speed Calculation

Movies taken with the CCD camera were processed with the NIH ImageJ software. To calculate the speed of vesicles, a linear region of interest (ROI) was defined along the length of an axon followed by a reslicing of the images along the ROI as a function of time (kymographs). A kymograph thus represents a distance on the vertical axes (i.e., the ROI) as a function of time (the horizontal axis). Diagonal traces represent vesicle movement, with the slope equals to the velocity, whereas horizontal lines are immobile vesicles. Vesicle speed was calculated as the rate of traveled distance [*y*-axes in Figs. 3(B) and 4(B)] over the duration of the vesicle movement

(*x*-axes). Calibration of the pixels per unit length for the CCD camera used in the experiments was performed using a silicon sample of known dimensions observed in bright field with the 63× and 100× oil immersion objectives.

RESULTS

Procedure for Successful Axon/Bead Contacts

For the experiments described below, we used an instrument that is designed for simultaneous AFM topography imaging and fluorescence microscopy. We did however not make use of the topography feature but rather used the AFM to contact a cantilever-mounted bead (see Fig. 1) to an axon expressing a synaptic protein of interest with nanometer accuracy. The contacting proceeds as follows:

1. Locate a hippocampal neuron expressing the synaptic protein of interest. Observation of small features (such as "strings," see below) requires a relatively high expression level.
2. In bright field, find an isolated length of axon.
3. Approach cantilever-mounted bead to surface, making sure to touch down close to, but not onto, the previously identified length of axon.
4. Using AFM controls, move the bead laterally to contact the axon.

In general, the bead-axon approach is a relatively simple procedure. However, we did notice that the important factor to get successful protein recruitment is to have isolated axons with high expression levels and vesicle mobility. (either synaptophysin or bassoon containing vesicles).

We note that the cells expressing fluorescent protein constitute a rather heterogeneous sample, both in terms of expression level and developmental stage of a given cell. This is why before even approaching the bead to the sample, we made sure that the chosen axon had a sufficiently high expression level and that the fluorescent protein was being transported along the length of the axon. On average, we found a 50% success rate for recruiting fluorescently labeled proteins, independent of the nature of the protein (i.e., Basoon or Synaptophysin).

Figure 2 shows an example of a contacting procedure (step 4 above) where at the end the bead is seen to slightly perturb the axon; this slight bending was taken as a sign of successful contact (see also Supporting Information Movie S1). Care was taken to avoid perturbing the axon more than necessary, thus limiting potential damage and/or constriction to the axon.

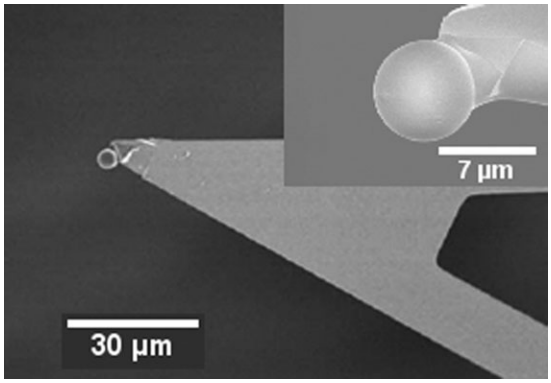

Figure 1 SEM image of an AFM cantilever with a micro-bead. A 7-μm polystyrene bead was glued at the cantilever tip and subsequently the whole assembly was coated with poly-D-lysine. Image in the upper right shows a close up of the glued bead. Uncoated beads were used in control experiments.

Synaptic Constituents are Transported in Vesicles

We observed the PDL-induced recruitment of synaptic constituents bassoon and synaptophysin (Figs. 3 and 4; see Supporting Information Movies S2 and S3). Bassoon is a scaffolding protein that is actively transported to the synaptic site in so-called PTVs (Zhai et al., 2001; Shapira et al., 2003). Synaptophysin (p38) is a classical presynaptic marker, which is located in the membrane of presynaptic neurotransmitter-containing vesicles and is transported to and from the synapse in a different vesicular carrier named STVs.

Consistent with earlier studies (e.g., Kaether et al., 2000) we observe bidirectional transport of both bassoon and synaptophysin in PTVs and STVs, respectively. Figure 4(B) is a kymograph that shows the movement of vesicles along a linear ROI as a function of time of a representative time series of synaptophysin transport along an axon. Diagonal tracks are the signatures of moving protein-containing vesicles. Note how the diagonal tracks all have the same slope. This is to be expected since the vesicles are being actively transported along microtubules as the cargo of motor proteins. Calculated speeds computed from the tracks show that the vesicles move at about 0.4 μm/s.

Bassoon is Recruited Before Synaptophysin

We observed the dynamics of bassoon and synaptophysin recruitment. Figure 4(B) shows an example of the time course of synaptophysin recruitment. In this case, the observed recruitment time of a stable punc-

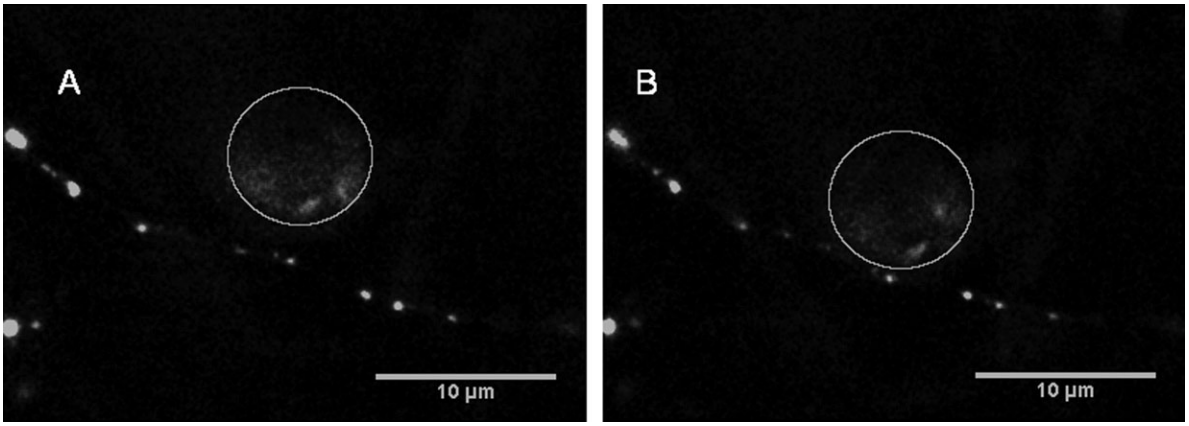

Figure 2 Microbead-axon approach. Precise (nm) control of the bead-axon approach and further contact is achieved by using an AFM. After the bead is brought into contact with the substrate close to a chosen axon (A), it is moved laterally until it gently touches the axon (B). The contact is observed trough an inverted optical microscope (see also Supporting Information Movie S1).

tum was 36 min. We measured an average recruitment time of 43 ± 9 min [see histogram in Fig. 5(B)].

Figure 3(G) shows an example of the dynamics of bassoon recruitment. We measured an average recruitment time of 23 ± 10 min [see histogram in Fig. 5(A)]. PTVs, and thus bassoon is clearly recruited before synaptophysin in STVs.

Adhesion Occurs Before Protein Recruitment

We systematically investigated the onset of adhesion between a PDL-coated bead and an axon simply by laterally contacting an axon, waiting a prescribed time and bringing the bead back to its starting position. The observation that the axon remains attached to the bead was taken as a sign of adhesion. We consistently found that adhesion is almost instantaneous and certainly has already occurred after 5 min. With uncoated bead contacts, we routinely found no evidence of adhesion.

The adhesion event does not seem to be correlated (in time) with recruitment although once recruitment has occurred, the focal adhesion point colocalizes with a recruited punctum (Sabo et al., 2006).

Membrane-Bound Strings Can Be Pulled Out of an Axon

Unexpectedly, upon laterally moving a PDL-coated bead away from an axon, we observed the formation of one or more thin strings between axon and bead (see Fig. 6; see Supporting Information Movie S4). We originally intended to measure the adhesion strength between the PDL-coated bead and the axon by mean of AFM force spectroscopy. However, this purpose was defeated by the fact that the strings can be pulled to such great lengths (>50 μm) that we reach the maximum dynamic range of our AFM instrument before the strings break. It indicates that the adhesion was so strong that the membrane (as well as other components of the cytoskeleton as described in the next section) is being pulled before the structures responsible for the bead-axon adhesion collapse. This behavior resulted in force spectroscopy curves that show a continuous adhesion at the retraction part of the force-distance curves (a maximum of 2.5–3 μm ramp was employed), which short-circuited our intentions of measuring adhesion strength.

One or two focal adhesion points are observed along the length of the contact area. Adhesion is thus point-like as opposed to along the whole contact area. This behavior is reminiscent of that observed in Sabo et al. (2006) where it is reported that synapses form at "special" sites along the axon where STVs have been observed to pause intermittently. These pause sites are spaced by several μm, the same minimum distance we observe between focal adhesion points.

Properties of Axon Strings

We observe that the bead-axon focal adhesion points colocalize with the synaptic protein puncta (distinguished as a fluorescent protein accumulation under the optical microscope) which appears after the protein recruitment has occurred. Upon pulling, the recruited protein puncta can stay at the axon or follow the bead (which occurred in the majority of the cases; an example of a protein puncta following the bead

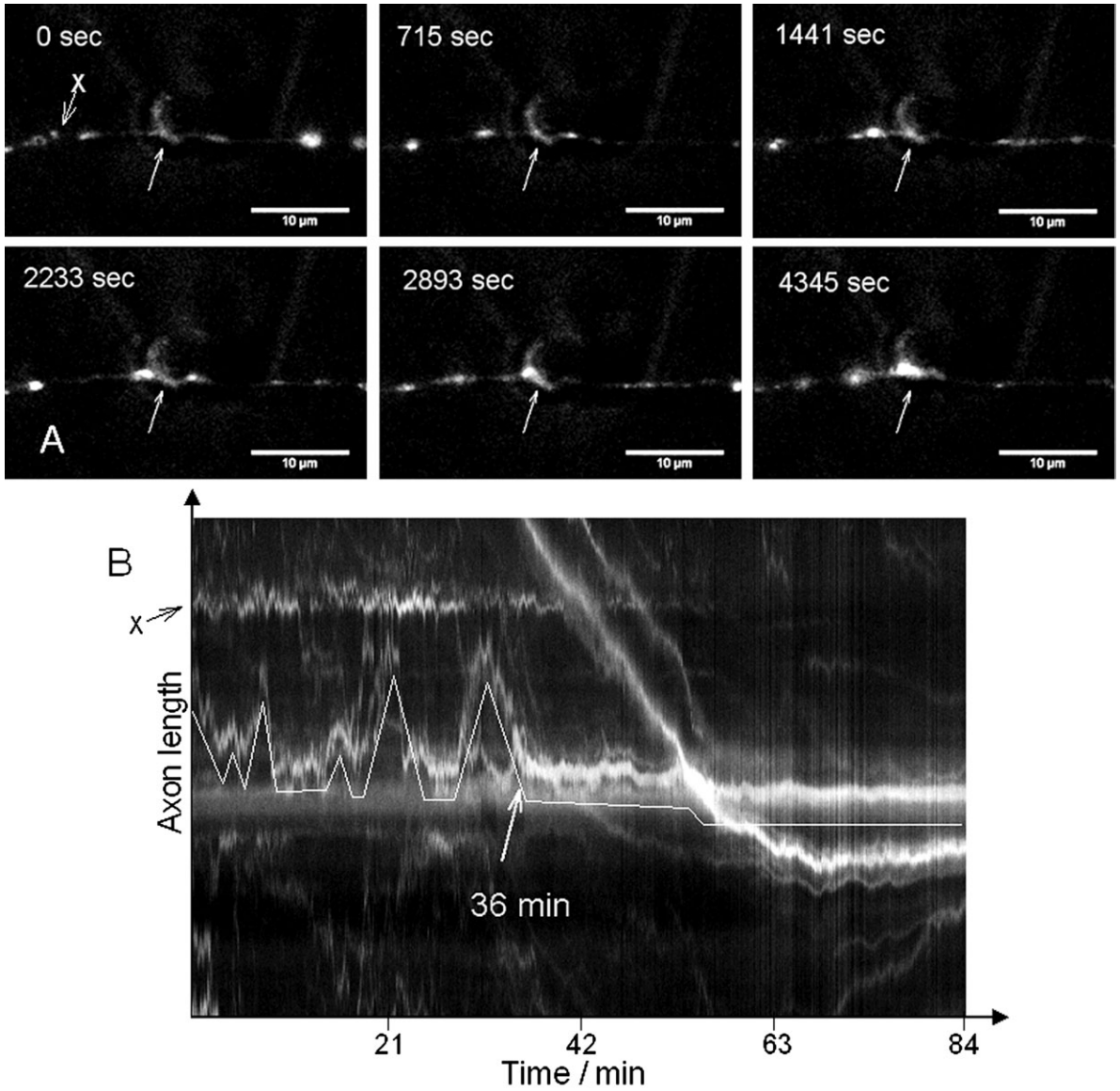

Figure 3 (A) Recruitment of Piccolo-Bassoon Transport Vesicles at the bead-axon contact site. The arrow indicates the area of axon-bead contact and hence the position of the recruited bassoon punctum, (B) For the analysis of vesicular movement and presynaptic recruitment, a linear region of interest (ROI) was defined along the length of an axon and a kymograph was produced. Localized punctum (mark X in A) shows up as horizontal lines in (B). Vesicles moving to the right in (A) correspond to negative slopes (slopes = velocity) in (B). Anterograde and retrograde vesicular movement are shown in accordance with previous observations. Vesicular recruitment is depicted as an increase in fluorescence intensity at the bead-axon contact site. The highlighted line shows the movement of a vesicle that becomes stable at the bead-axon contact site after 36 min. The average stable recruitment time for nine experiments was 23 ± 10 min of contact although vesicles transiently stopped at the bead-axon contact site at earlier times (see also Supporting Information Movie S2).

can be seen in the Supporting Information Movie S4). The colocalization of adhesion points and protein puncta, be it those from preformed synapses or new synapses induced by the contacting of a PDL-coated bead, suggests that only the focal adhesion points have the ability to form synapses. Presumably, the appearance of the initial adhesion is mediated by heparin sulfate proteoglycans (Lucido et al., 2009) that in turn trigger the formation of a protein puncta at the site of adhesion.

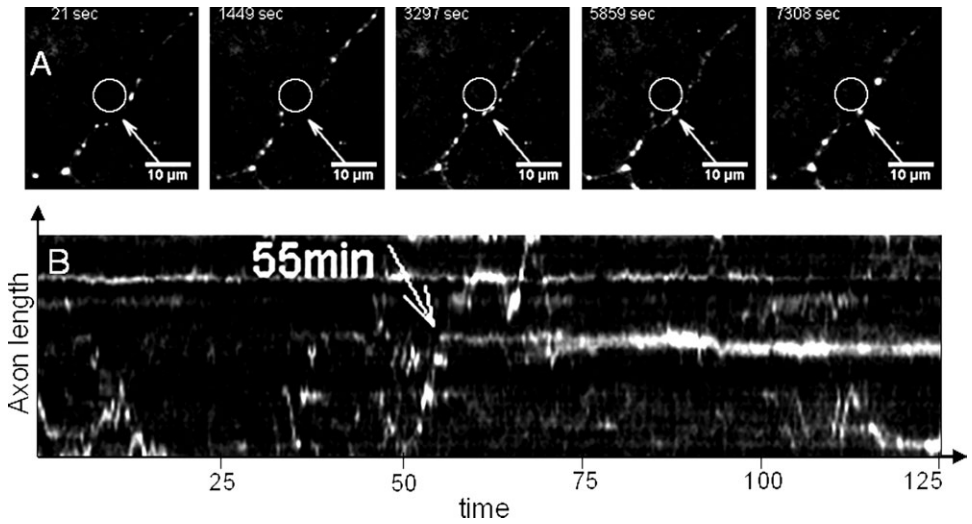

Figure 4 A: Recruitment of synaptic transport vesicles at the bead-axon contact site. The arrow indicates the area of axon-bead contact and hence the position of the recruited synaptophysin punctum. B: Kymograph demonstrates anterograde and retrograde vesicular movement as previously observed. In this experiment vesicular recruitment is observed after 55 min. On average (eight trials) the recruitment time was observed to be 43 ± 9 min (see also Supporting Information Movie S3).

We created strings from axons in which three different fluorescent proteins were expressed, one at a time: synaptophysin-GFP, bassoon-GFP and actin-YFP; as well as in axons where tubulin was fluorescently labeled with the dye Tubulin Tracker™ Green (Invitrogen). In all four cases, we readily observed the strings to fluoresce. Hence, at least these four proteins must be present in the strings.

Whether the protein inside the strings is structured in a functional way similar to than of true axons is uncertain but we did occasionally observe the motion of a proteinaceous punctum along a string (see Fig. 7; see also Supporting Information Movie S5). We cannot readily distinguish between F- and G-actin but when pulling a string, we are effectively creating a large membrane fluctuation.

Another curious observation is the fact that when moving a bead-attached string parallel to an axon, the string initially stretches further but then quickly slides along the axon in order to minimize string length (see Supporting Information Movie S4).

Previous studies by Bernal et al. (2007) showed that the initial axon response to a quick stretching resembles that of an elastic material but it is immediately followed by a viscoelastic behavior with a relaxation time of $\sim$10 s. Our results show that the

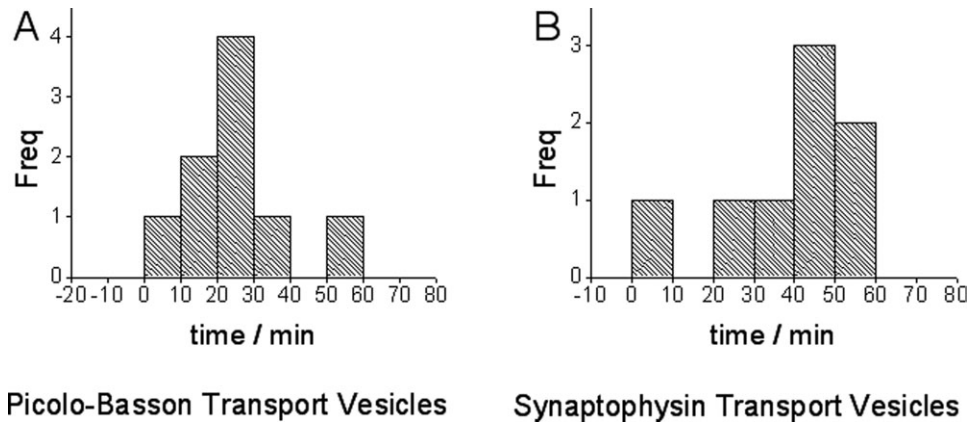

Figure 5 Histograms of recruitment times of (A) Piccolo-Bassoon transport vesicles and (B) synaptic transport vesicles. The average bead-axon contact time before vesicular recruitment occurred was 23 min for PTV while that for STV's was 43 min. This difference in recruitment time reflects the order in which proteins are required to assemble to form a presynaptic site.

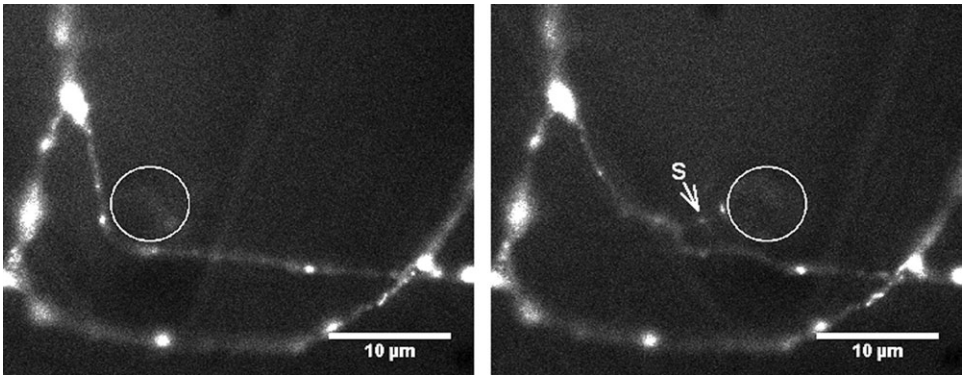

Figure 6 String formation observed upon pulling an adhesive bead away from an axon. The movie shows the axon of a cell expressing synaptophysin-GFP. A long thin fluorescent string (S in fig B) was formed between the site of protein recruitment in the axon and the adhesion point in the bead. After stretching the string, it tends to minimize its length by displacing its initial point of contact along the axon. We have observed strings as long as 50 μm, limited by the AFM scan range. In separate experiments, we demonstrated that such strings contain tubulin, bassoon and actin as well as synaptophysin (see also Supporting Information Movie S4).

labeled and thus identifiable components present in the axon are also present in the strings which would suggest that potentially similar bio-mechanical characteristics are elicited by the strings.

DISCUSSION

A number of techniques to study the protein content at synaptic sites have been available to researches in the past (Ichtchenko et al., 1996; Benson and Tanaka, 1998; Dieck et al., 1998; Garner et al., 2000; Kennedy, 2000; Scannevin and Huganir, 2000; Dresbach et al., 2001; Murthy and Camilli, 2003). Many of those techniques are widely used in biology studies. However, time lapse studies of protein recruitment at presynapses are scarce in part due to the lack of a technique capable of inducing the presynaptic array at will. The few studies that have addressed the issue relied upon random dendrite-axon encounters where no control of either the contact site or contact time were possible (Ahmari et al., 2000; Friedman et al., 2000; Kaether et al., 2000).

In this study, we successfully induced the formation of presynaptic sites that have several characteristics of presynapses formed at axon-dendrite contacts (Lucido et al., 2009). We made use of the AFM to put a PDL-coated bead in contact with an axon. The advantages of using the AFM include a nanometric precision in the positioning of the bead relative to the axon as well as a good control of the time and duration of the bead-axon contact. To increase the likelihood of observing the recruitment of proteins, we chose axons with relatively high fluorescent intensity and observable vesicle movement in the axon shaft (Qiang et al., 2010). Measurements of the recruitment

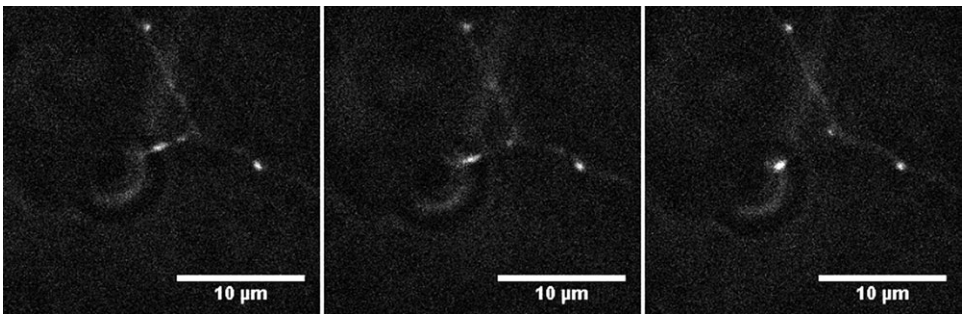

Figure 7 Proteins moving along string, still images separated by $\sim$1 s. The observation of vesicles moving along a formed string was a rare event. When observed, it resembled the usual movement of vesicles along the axons. This is not surprising given the detection of actin and tubulin, two important components of the transport apparatus, in the strings (see also Supporting Information Movie S5).

times for synaptophysin, and bassoon at the contact site showed that synaptophysin is recruited after bassoon. This marks a natural time course where bassoon, a known scaffolding protein, is recruited first, presumably to stabilize the framework needed to tether presynaptic vesicles (here marked by synaptophysin) at the synaptic site. We observe the same recruitment timing of these two particular protein-containing vesicles as happens upon natural axon/dendrite contact (Friedman et al., 2000). This demonstrates that the inductive signals from the PDL-coated beads to the axon have the same effects as the signals from a contacting dendrite.

Kymographs [see Figs. 3(B) and 4(B)] defined along the length of the axon allows the observation of vesicle movement in anterograde and retrograde directions in accordance with previous reports (Kaether et al., 2000).

Although two proteins were studied in this work, the technique presented here would allow time lapse studies of the recruitment of any other protein in the presynaptic site, the only requirement would be to get neurons expressing the fluorescently tagged protein of interest.

Bead-axon adhesion of PDL-coated beads occurs shortly after contact and definitively before recruitment. The type of interaction that occurs is believed to happen between the positively charged surface of the bead and the negatively charged motives of heparan sulfate proteoglycan (HSPG) transmembrane proteins (Lucido et al., 2009). Besides, it has been shown that the cytoskeleton is rearranged at the presynaptic site in mature synapses (Garner et al., 2002) thus the mere redistribution of the underlying cytoskeleton (which has a net negative charge, Janmey et al., 1998) due to simple charge interaction with the PDL molecules on the bead could also be a potential contributor to the formation of presynapses (Liu et al., 2006).

Bead-axon adhesion of PDL-coated beads was observed to be strong enough to pull membrane strings from the main axon. We determined that those strings contained actin, synaptophysin, piccolo-bassoon and tubulin. In few cases, we observed movement of protein punctum along the formed strings which could signify that the strings are functional axon branches.

Recent results support this idea. Yang et al. (2009) and Lan and Papoian (2008) have shown that membrane fluctuation can facilitate the formation of filopodia by creating "empty" space between the membrane and the tip of the actin filaments where actin polymerization and elongation occur (Bray and Chapman, 1985). The mechanical stress exerted on the cytoskeleton when the bead is pulled could severe some microtubule fibers generating free microtubule ends. Qiang et al. (2010) showed that the appearance of an increased number of interstitial branches in axons is related to the generation of free microtubule ends in the axon shaft. Subsequent organization and filling of the strings with cytoskeleton, vesicles and other internal components of the axon directed by either active transport (newly formed microtubule filaments) or Brownian motion could end up in a functional axon branch (Goldberg and Burmeister, 1986).

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