

Target recognition at the tips of postsynaptic filopodia: accumulation and function of Capricious

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While much evidence suggests that postsynaptic dynamism contributes to the formation of synapses, few studies have addressed its possible role in target selection. Do postsynaptic motile structures seek specific synaptic partner cells, as does the presynaptic growth cone? Here we studied the dynamics of myopodia, postsynaptic filopodia in *Drosophila* muscles, and the role of Capricious (CAPS) during the process of synaptic matchmaking. CAPS is a target recognition molecule with an extracellular domain containing leucine-rich repeat sequences. It is expressed in specific subsets of embryonic/larval body wall muscles, including muscle 12 (M12). We provide evidence that implicates the tips of myopodia as loci of initial neuromuscular recognition: (1) CAPS, expressed as a GFP-fusion protein in M12, accumulated at the tips of myopodia; and (2) simultaneous live imaging of presynaptic motoneurons and postsynaptic myopodia revealed that initial neuromuscular contacts occur at the tips of myopodia. The live imaging also showed that individual postsynaptic myopodia appear to be able to discriminate partner and non-partner presynaptic cells: whereas many myopodial contacts with the partner motoneurons are stabilized to form synapses, those with non-partner neurons are retracted. In *caps* mutants, or in double mutants lacking both CAPS and the closely related protein Tartan, we observed fewer contacts between myopodia of M12 and the presynaptic growth cones during the process of initial neuromuscular interaction. The nascent synaptic sites of M12 were also reduced. These results provide evidence for the sensing function of postsynaptic filopodia, and implicate Caps-mediated recognition at the tips of myopodia in synaptic matching.

KEY WORDS: Target recognition, Postsynaptic filopodia, Capricious, Synaptogenesis

INTRODUCTION

Precise partner recognition between axons and their target cells is essential for developing a functional nervous system (Tessier-Lavigne and Goodman, 1996; Garrity and Zipursky, 1995; Benson et al., 2001). A traditional view is that presynaptic growth cones explore the target region by elaborating motile filopodia, which sense and recognize specific target-recognition molecules that are expressed on the postsynaptic cells. The postsynaptic cell has generally been regarded as a static structure that plays only a passive role in this synaptic matchmaking process. Recent time-lapse studies using live-imaging techniques, however, show the dynamic nature of postsynaptic cells during periods of targeting and synaptogenesis. For example, dendritic filopodia of mammalian central neurons display rapid and extensive dynamics before synaptogenesis (Ziv and Smith, 1996; Maletic-Savatic et al., 1999; Yuste and Bonhoeffer, 2004). It has also been shown that postsynaptic motoneurons in zebrafish extend highly motile filopodia, which interact with the presynaptic Mauthner axons (Jontes et al., 2000). These studies led to the suggestion that postsynaptic dynamism may contribute to the formation and plasticity of synapses (Cohen-Cory, 2002; Goda and Davis, 2003). However, whether postsynaptic filopodia are able to select appropriate presynaptic partners has not been addressed in these previous studies. Just as the presynaptic growth cone specifically recognizes the target cells, do postsynaptic filopodia seek out appropriate presynaptic cells?

The neuromuscular connectivity of *Drosophila* is well suited for studying the molecular mechanisms of synaptic target recognition, as it is possible to follow the behavior of individual

motoneurons as they establish contacts with the target muscles (Keshishian et al., 1996). Several target recognition molecules that are expressed in specific muscles and have roles in determining synaptic specificity have previously been identified (Nose et al., 1992; Chiba et al., 1995; Winberg et al., 1998; Shishido et al., 1998). Before the discovery of myopodia, it was generally believed that active axonal growth cones find and recognize such target recognition cues expressed on muscles and establish specific synaptic contacts. Muscle cells were thought to be passive players in this process, probably due to their static appearance in fixed embryos. The discovery of myopodia, however, challenged this view, suggesting the possibility that neuromuscular recognition is a reciprocal process whereby neurons and muscles seek each other out (Ritzenthaler et al., 2000; Ritzenthaler and Chiba, 2001). Myopodia are dynamic filopodia of muscles that are most active before the arrival of the motoneuronal growth cones and progressively cluster at the nascent synaptic site. Based on their dynamic nature and on the observation that they make intimate contacts with the presynaptic growth cones, myopodia were proposed to play active roles in the guidance of motoneuronal growth cones. However, although there is evidence that clustering of myopodia depends on specific interaction between pre- and postsynaptic cells (Ritzenthaler and Chiba, 2001), the precise role of myopodia in mediating target specificity is unknown. Myopodia might simply function to increase the probability of chance encounters with presynaptic growth cones by increasing the surface area of muscles. Alternatively, protrusive activity of myopodia might be important for a local and precise interaction with the presynaptic cells, with possible mutual exchange of information.

In this study, we first investigated the subcellular localization of Capricious (CAPS), in relation to the behavior of myopodia, by live imaging of the green fluorescent protein (GFP)-tagged protein CAPS-GFP. CAPS is a transmembrane protein with leucine-rich repeats (LRRs) that is expressed in specific subsets of muscles and

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motoneurons, including ventral muscle 12 (M12) and the motoneurons that innervate it (MN12s) (Shishido et al., 1998). Previous genetic analysis implicated CAPS as an attractive target recognition molecule on M12. Here, we found that CAPS expressed in M12 localizes to the tips of myopodia before the arrival of motoneuronal growth cones. The distribution of CAPS suggested the possibility that neuromuscular interaction is initiated at the tips of myopodia, at a distance from the body of the muscles. To address this issue, we performed simultaneous live imaging of pre- and postsynaptic cells. By following the behavior of individual myopodia as they extend to and make contact with the approaching growth cones, we found that initial contacts do occur at the tips of myopodia. Although some of these contacts were stabilized to form synapses, others, including those with the non-partner motoneurons, were retracted, suggesting that myopodia are able to select specific presynaptic cells. In *caps* mutants, the number of contacts between M12 myopodia and the presynaptic growth cones is reduced. These findings are consistent with the notion that local, contact-mediated signaling at the tips of postsynaptic filopodia is crucial for target selection.

MATERIALS AND METHODS

Fly strains

Gene expression in M12 or in all neurons was driven by *GAL4* driver lines, *GAL4-5053A* (Ritzenthaler et al., 2000) and *elav-GAL4-3E1* (Davis et al., 1997), respectively. For vital visualization of cell morphology, we used *UAS-myristylated-GFP* (a gift from Dr Chiba, University of Illinois) (Ritzenthaler et al., 2000), *UAS-myristylated-CFP* and *elav-myristylated-YFP* (Kohsaka et al., 2007). For muscle expression of intact CAPS, *UAS-caps1a5* (Taniguchi et al., 2000) was crossed with *GAL4-5053A*. To express an intracellular deletion form of CAPS in muscles or in motoneurons, *UAS-caps1D4* (Taniguchi et al., 2000) was crossed with *GAL4-5053A* or *elav-GAL4-3E1*, respectively. The *pros* mutant allele used was *pros^{m4}* (Broadie and Bate, 1993). The *caps* null mutant alleles used were *caps^{C28fs}* and *caps^{L253fs}* (Sakurai et al., 2007). *Df(3L)Ly* is a deficiency that lacks the entire *caps* gene (Shishido et al., 1998). The *trn* null mutant allele used was *trn^{25/4}* (Chang et al., 1993; Milan et al., 2001). The *caps*, *trn* double mutant alleles used were *caps^{C28fs}, trn^{delta17}* (null for both *caps* and *trn*) (Sakurai et al., 2007) and *caps^{65.2}, trn^{S064117}* (strong hypomorphic alleles for *caps* and *trn*) (Kurusu et al., 2008). The *caps^{C28fs}, trn^{delta17}* chromosome is deficient for two neighboring genes, CG33262 and CG11281, in addition to *caps* and

tartan. However, the involvement of CG33262 and CG11281 is unlikely because similar phenotypes were observed in *caps^{C28fs}, trn^{delta17}* and in *caps^{65.2}, trn^{S064117}*. *caps^{C28fs}, trn^{delta17}* embryos also display mild phenotypes in axon guidance within the ventral nerve cord and muscle attachment site formation of M12 (see Kurusu et al., 2008). We therefore restricted our analyses to segments with normal motor axon projection and muscle attachment sites.

Generation of UAS transgenic lines

The 3' end of the *caps* open reading frame (ORF) was fused with the 5' end of *GFP* (or *YFP*) ORF via a six amino acid linker. The resultant *caps-GFP* (or *caps-YFP*) cDNA was cloned into the *pUAST* vector. Individual constructs were introduced into γ , w flies by germline transformation according to a standard protocol. Two independent transformant lines, *UAS-caps-GFP 25* (an insertion on the third chromosome) and *UAS-caps-YFP B1* (an insertion on the second chromosome), were used in this study. CAPS-GFP/YFP expressed on M12 using the two lines showed the same distribution pattern.

Immunohistochemistry

Dissection and immunohistochemistry of embryos and larvae were performed as described previously (Nose et al., 1997). The following antibodies were used: monoclonal antibody (mAB) 22C10 (Fujita et al., 1982), mAB1D4 (Van Vactor et al., 1993), rabbit serum antibodies (sABs) against intracellular domains of CAPS (Shishido et al., 1998) and goat anti-HRP antibodies (Jackson Laboratories). Vital labeling of motor axons was performed by incubating dissected embryos with anti-HRP antibodies conjugated with Cy5 (Jackson Laboratories) for 10 minutes before visualization (Ritzenthaler et al., 2000). For developmental analysis of embryos, we collected eggs laid for 30 minutes and verified the developmental stage based on both the actual time passed before the time of observation or fixation, and the morphology of the midgut.

Live visualization

We captured the images of myopodia and growth cones of dechorionated whole embryos in insect saline using an LSM 510 confocal system (Zeiss) or FV1000 confocal system (Olympus). We confirmed that interruption of synaptogenesis by photodamage was very minor if any: ISNb axons made normal T-shaped terminals on muscles 12, 13, 6 and 7 in a normal time window under our visualization condition. Typically, 10-20 optical sections of 0.5-1 μm were recorded over the course of 2 hours, starting every 1-2 minutes. Dual-color time-lapse images of a whole embryo are despeckled once by ImageJ software (NIH).

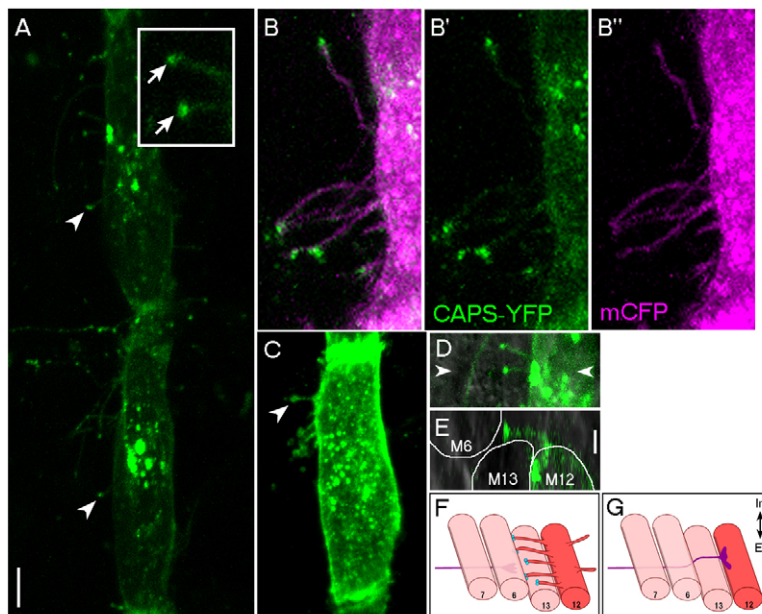


Fig. 1. CAPS accumulates at the tips of myopodia.

(A) CAPS-YFP expressed on M12 strongly accumulates at the tips of myopodia (arrowheads) at 13:00 hours AEL. Inset is a higher magnification view of myopodia. (B-B'') Membrane-bound CFP (B,B''; mCFP; magenta) distributes uniformly along the length of myopodia of M12, in contrast to the clustering of CAPS-YFP (B',B''; green) at the tips. (C) Immunohistochemical visualization of intact CAPS expressed on M12 at 14:30 hours. Whereas most myopodia have vanished due to the fixation process, there is an accumulation of CAPS at the tips of a remaining myopodium (arrowhead). (D) Concurrent visualization of CAPS-GFP by fluorescence and muscle contours by DIC. (E) Cross-section of the muscle and a myopodium cut along the plane indicated by arrowheads in D. The myopodia extend along the interior side of M13. (F,G) Schematic of the trajectories of M12 myopodia in relation to the path of MN12s at 13:00 (F) and 15:00 (G) hours. Blue circles represent CAPS at the tips. In, interior; Ex, exterior side of an embryo. Scale bars: 10 μm in A,C; 4 μm in inset of A,B; 5 μm in D,E.

Quantification of interaction between myopodia and growth cones

To analyze the dynamics of myopodia and growth cones, we used 4D confocal time-lapse images. We defined ‘contact’ between myopodia and growth cones as positions at which there was no gap in fluorescence intensity at our level of resolution. We manually counted points of contact (or no contact) using LSM510 or FV1000 software.

RESULTS

CAPS accumulation at the tips of myopodia

As myopodia are largely lost in fixed specimens (Ritzenthaler et al., 2000; Kohsaka et al., 2007), we examined the subcellular localization of CAPS by live imaging of the dissected embryos expressing CAPS-GFP. We generated a C-terminal CAPS-GFP fusion construct, which appears to be functional according to the following criteria. First, CAPS-GFP retains its ability to induce ectopic synapse formation by MN12s when expressed in all muscles (Shishido et al., 1998) (see Fig. S1 in the supplementary material). Second, CAPS-GFP retains its ability to induce the pathfinding defect of a motor nerve ISNb, when misexpressed in all neurons (Taniguchi et al., 2000) (data not shown).

We expressed CAPS-GFP in M12, a CAPS-positive muscle, and investigated the subcellular distribution of CAPS-GFP during target selection. To express CAPS-GFP, we used an M12-specific driver, 5053A-GAL4, which confers expression of UAS transgenes before the arrival of MN12 growth cones. This allowed us to examine CAPS-GFP distribution within muscle 12 during the period of time in which it is normally innervated.

At 13:00 hours after egg-laying (AEL), when the growth cones of MN12s are approaching M12, a number of myopodia protrude from the muscles (Ritzenthaler et al., 2000). We observed strong accumulation of CAPS-GFP at the tips of myopodia (Fig. 1A, arrowheads). This was in contrast to the distribution of myristylated GFP (mGFP), which is uniform along the length of myopodia (Fig. 1B). Immunohistochemistry of intact CAPS (without GFP) expressed on muscles also revealed its presence at the tips of short myopodia that

survived fixation, indicating that the localization is not an artifact caused by the addition of GFP (Fig. 1C). Although endogenous *caps* is expressed in M12 at this stage, CAPS protein expression is below detection by our immunohistochemical procedures (Shishido et al., 1998). However, the data described above suggest that endogenous CAPS would also be localized to myopodia during this time period. When CAPS-GFP was expressed in neurons, we did not observe concentration of CAPS-GFP at the tips of growth cone filopodia (see Fig. S2 in the supplementary material).

The accumulation of a target recognition molecule such as CAPS at the tips of myopodia suggests that activity of far-reaching myopodia might function to efficiently present the target markers to presynaptic motoneurons that are approaching the target. To pursue this possibility further, we examined the trajectory of myopodia in relation to the paths of motoneuronal growth cones. Concurrent visualization of CAPS-GFP by fluorescence and contours of muscles by differential interference contrast (DIC) showed that some of the myopodia of M12 extended along the interior side of a neighboring muscle 13 (M13) and reached as far as the proximal (closer to the central nervous system) edge of M13 (Fig. 1D,E). This trajectory of myopodia corresponds to the path that the growth cones of MN12s will later take (shown schematically in Fig. 1F,G). These observations are consistent with the idea that M12 can guide neuronal growth cones toward itself by extending myopodia.

The accumulation of CAPS-GFP at the tips of myopodia appears to be an autonomous process in muscles that is independent of innervating motor axons. First, CAPS-GFP is concentrated at the tips of myopodia more than 30 minutes before the arrival of motoneuronal growth cones. Second, concentration of CAPS-GFP at the tips of myopodia took place normally in *prospero* (*pros*) mutants, in which extension of motoneuronal axons is severely delayed (Ritzenthaler et al., 2000; Broadie and Bate, 1993) (Fig. 2). These observations suggest that accumulation of CAPS at the tips of myopodia is not dependent on signals from the motoneurons.

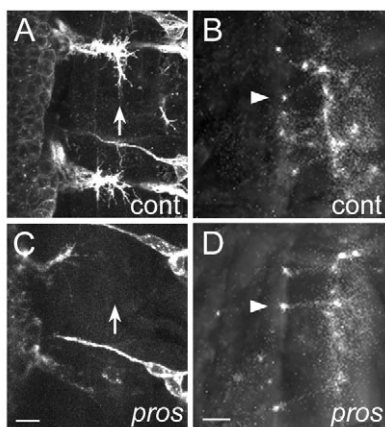


Fig. 2. CAPS-GFP accumulation at the tips is independent of motoneuronal innervation. (A,C) Staining with the axonal marker anti-Fasciclin 2 in wild type (A) and *pros* mutants (C) at 14:00 hours. Growth cones of motoneurons arrive at ventral muscle field in wild-type embryos (A, arrow). By contrast, motoneurons fail to extend to the target region in *pros* mutants (C, arrow). (B,D) CAPS-GFP expressed on M12 in wild type (B) and *pros* mutants (D). CAPS-GFP accumulates at the tips of M12 myopodia in *pros* mutants as in wild type (B,D; arrowheads). Scale bars: 10 μ m in A,C; 5 μ m in B,D.

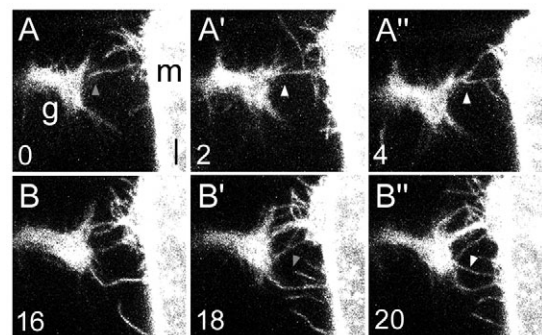


Fig. 3. Contacts between muscles and innervating growth cones at the tips of myopodia. (A-A'') The first contact between the muscle and the growth cones occurs at the tips of myopodia. (B-B'') Later interaction between muscles and growth cones also takes place at the tips of myopodia. Time-lapse imaging of axons (as visualized with *elav-GAL4: UAS-myristylated-GFP*) and myopodia (as visualized with *Gal4-5053A: UAS-myristylated-GFP*) in an intact embryo, showing three images at 2-minute intervals beginning at 13:00 (A-A'') and 13:16 (B-B'') hours. Time elapsed from the first image is shown in minutes. Arrowheads: myopodia that did (white) or did not (gray) contact the growth cones. g, growth cones; m, M12. Scale bar: 5 μ m.

Neuromuscular contacts at the tips of myopodia

Accumulation of CAPS-GFP at the tips implies that initial recognition events between presynaptic motoneurons and postsynaptic muscles might occur at the tips of myopodia rather than at the muscle fiber itself. Previous time-lapse analysis of myopodia and immunohistochemical visualization of motor axons at the end of the live imaging showed that myopodia extensively interact with the presynaptic filopodia and become clustered at the future synaptic site (Ritzenthaler et al., 2000). However, where the initial contact between the pre- and postsynaptic cells occurs and how these initial contacts are stabilized to form the synaptic sites have remained unknown. To analyze the dynamics of myopodia and presynaptic filopodia in intact embryos as they initiate their first contact and eventually intermingle to form synapses, we conducted time-lapse imaging of both structures by genetically expressing mGFP. We captured time-lapse images that span the period of target selection and early synaptogenesis (see Movie 1 in the supplementary material). By following the behavior of individual myopodia, we could locate the contacts between myopodia and motoneurons and trace the final fate of these initial contacts.

We acquired 3D time-lapse images in 2-minute intervals, from 12:45 hours AEL, when myopodia and the approaching growth cones make their initial contact, to 15:00 hours, when the clustering of myopodia is complete. By following the dynamics of myopodia in relation to that of the growth cones, we located the contacts formed between them (for the definition of ‘contacts’, see Materials and methods). We first analyzed contacts formed during the early phase of the imaging period (12:45-13:30 hours). M12 formed, on average, 5.9 myopodial contacts with the growth cones during this period (65 contacts found in 11 hemi-segments examined). All of these contacts occurred at the tips of myopodia, indicating that the tips are the major meeting sites between the synaptic partners (Fig. 3).

We next followed the final fate of the contacts between myopodia and motoneuronal growth cones. We found that 45% (29/65) of the contacts were stabilized and contributed to the formation of synapses. The remaining 55% of contacts were eventually eliminated. For example, in the time-lapse imaging shown in Fig. 4A, we followed the behavior of two myopodia as they encountered the growth cones. The contact made by the upper myopodium was stabilized. Stabilization of the contact was accompanied by the rapid recruitment of additional myopodia and neuronal filopodia to that site, leading to much thicker bundles of microprocesses (Fig. 4A, 88 minutes). By contrast, contact made by the lower myopodium was eliminated. We analyzed the duration of such lost contacts and found that 78% (28/36) of them were transient contacts that lasted less than 10 minutes (4.0 minutes on average) and the remaining 22% were semi-stable contacts that were maintained for 10-60 minutes. Thus, myopodial contacts can be categorized into three groups: transient contacts that last less than 10 minutes, semi-stable contacts that last 10-60 minutes, and stable contacts that contribute to the formation of synapses.

Partner recognition by myopodia

The concentration of CAPS and occurrence of the initial neuromuscular contact at the tip of the myopodium strongly suggests that it is a major site of neuromuscular recognition. It might be the site at which myopodia present their target marker(s) to the presynaptic cell by direct cell-cell contact. At the same time, it might also be the site at which myopodia ‘sense’ the information provided by the presynaptic cells. If such mutual recognition process takes place, then one would expect the behavior of myopodia to differ depending on which cells they encounter. For example, myopodia

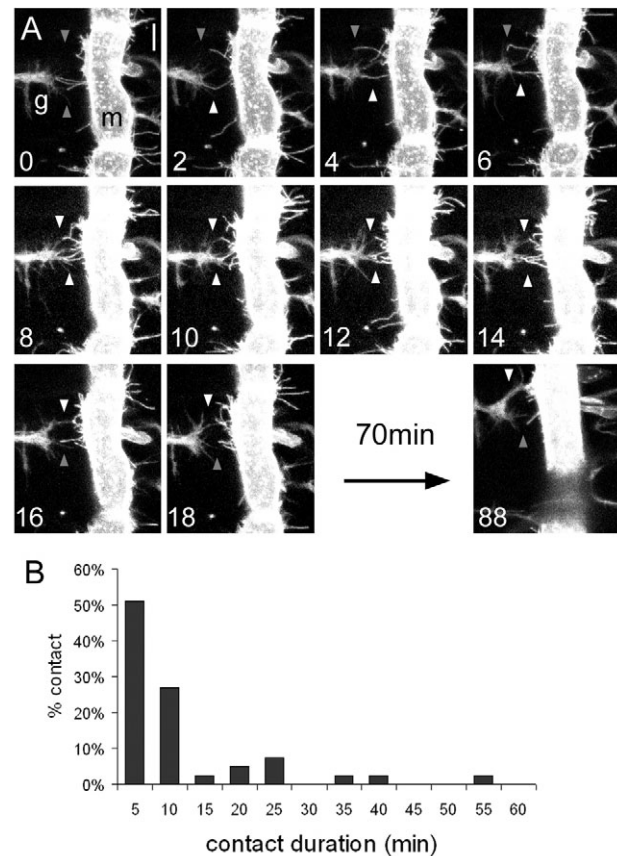


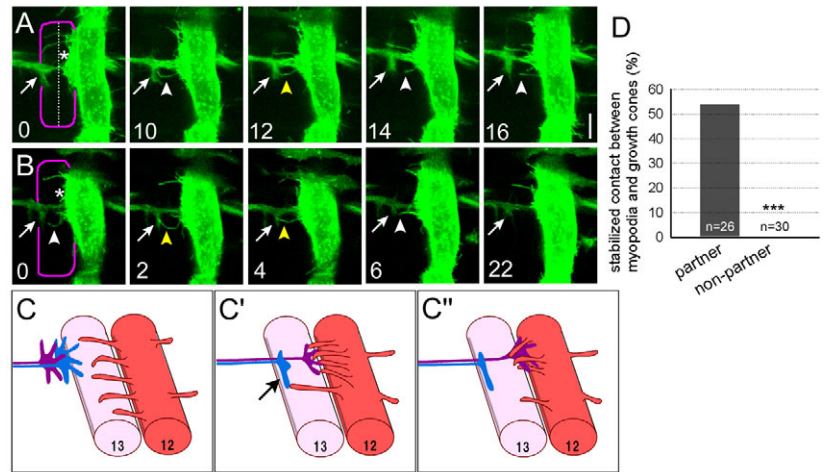
Fig. 4. Stabilized and lost contacts between myopodia and growth cones. (A) Fate of contacts between myopodia and growth cones is traced by time-lapse imaging with 2-minute intervals that spans the entire period of synaptogenesis (13:17-14:45 hours). Time elapsed from the first image is shown in minutes. The fate of two contacts was traced (arrowheads). The upper one was established after 8 minutes and was finally stabilized (88 minutes). The lower one, which formed after 2 minutes, was later destabilized (16 minutes). Arrowheads: myopodia that did (white) or did not (gray) contact the presynaptic growth cones. (B) Distribution of the duration of lost contacts between myopodia and growth cones. g, growth cones; m, M12. Scale bar: 10 μ m.

of M12 might distinguish between neurites of partner (MN12s) and non-partner motoneurons, such as those that innervate the neighboring muscle 13 (MN13s). During the early phase of our time-lapse imaging described above (12:45-13:30 hours), the growth cone of MN12s and MN13s were too close to be discerned by morphology (Fig. 5C). To determine whether the behavior of myopodia differs depending on whether they encounter partner or non-partner neurons, we next studied contacts made during a later phase of the imaging period (13:30-14:00 hours), when growth cones of MN12s and MN13s are mostly discernable because of distal movement of the MN12 growth cones (Fig. 5C'). By this stage, MN13s (including RP1 and RP4 neurons) had begun to arborize along the proximal edge of M13 (Sink and Whittington, 1991; Halpern et al., 1991). By contrast, the growth cones of MN12s (including RP5) had moved to a more distal region. We therefore compared the behavior of myopodial contacts with the neural processes situated on the distal half of muscle 13 (putative MN12 growth cones) to the behavior of contacts with those situated on the

Fig. 5. Myopodial contacts with non-partner motoneurons are eliminated.

(A,B) Time-lapse imaging of myopodia and growth cones in intact embryos beginning at 13:45 (A) and 13:55 (B) hours. By this time, the growth cones of MN12s (asterisks) have moved to the proximal edge of M12 and can be largely distinguished from those of MN13s (arrows) that are situated near the proximal edge of M13 (contours of M13 are shown by purple lines). Here, we define neural processes situated on the proximal half of M13 (the boundary of the proximal and distal halves of M13 is indicated by a dotted line in A) as putative MN13 growth cones. The fate of two contacts between M12 myopodia and putative MN13 growth cones is followed. Arrowheads indicate myopodia that did (yellow) or did not (white) contact the growth cones. Both of these contacts are eventually eliminated. Time elapsed from the first image is shown in minutes.

(C-C'') Schematic of the positions of growth cones of MN12s and MN13s. (C) At 13:00 hours, growth cones of MN12s and MN13s cannot be distinguished because both are situated near the proximal edge of M13. (C') Slightly later, the distal movement of MN12 growth cones makes it possible to discern them from those of MN13s (arrow). MN13s have begun to arborize along the proximal edge of M13 by this stage. (C'') Myopodia and growth cone filopodia intermingle to form the nascent synapse at 14:00 hours. (D) Quantification of myopodia contact stability. None of the myopodial contacts with the presumed non-partner neurons was stabilized. *** $P=1.7\times 10^{-6}$; Fisher's test. Scale bar: 10 μm .



proximal half of M13 (putative MN13 growth cones) (see Fig. 5A). Although discrimination of MN12s and MN13s is not unambiguous because these neurons have yet to withdraw all of the ectopic filopodia projecting to non-target muscles at this stage, we hoped to be able to detect some tendency by this analysis. Indeed, we found that while 54% of the contacts with the putative partner neurons were stabilized ($n=26$ myopodia in 21 hemisegments), none of the contacts with the putative non-partner neurons was stabilized ($n=30$ myopodia in 21 hemisegments; the difference in stabilization rate was statistically significant, $P=1.7\times 10^{-6}$, Fisher's test) (Fig. 5A,B,D). These results support the notion that myopodia are able to discriminate between partner and non-partner motoneurons.

Dual-color imaging of pre- and postsynaptic structures

In the time-lapse analyses described above, it was difficult to follow individually the dynamics of pre- and postsynaptic structures once they intermingle and form a cluster of multiple filopodia and myopodia. We therefore next visualized motor axons and muscles in different colors. We did this by imaging dissected embryos that expressed mGFP in M12 and were live-stained for anti-HRP (Fig. 6) or by time-lapse imaging of embryos that expressed mYFP in neurons and mCFP in M12 (see Fig. S3 in the supplementary material). During 13:00-14:00 hours, MN12 growth cones and myopodia were often seen to adhere laterally (Fig. 6B). This suggests that following contact at the tip of myopodia, growth cones are guided to M12 by extending along the surface of myopodia. During this period, myopodia cluster at the site of innervation and subsequently fuse to form a lamellipodia-like structure (Ritzenthaler et al., 2000). While interacting with the clustered myopodia, growth cones transform into a bifurcated terminal-like structure (Fig. 6C,D, arrowheads). The nascent presynaptic terminals were often observed within the lamellipodia-like structure of myopodia (Fig. 6C, asterisk). These results suggest that filopodial interaction between neurons and the target muscle is important not only for myopodia clustering but also for the formation of presynaptic terminals. The filopodia-myopodia complex may provide a substrate upon which the initial pre- and postsynaptic differentiation takes place.

We also studied CAPS localization during this period by triple labeling of CAPS-YFP, myopodia and growth cones (Fig. 7). As described above, CAPS is concentrated at the tip of myopodia just before contact with the growth cone (Fig. 7A). Interestingly, CAPS-YFP concentration remained at the tips even after the growth cones contacted and extended along the myopodia (Fig. 7B). When myopodia clustering is completed, strong concentration of CAPS-YFP was no longer observed in myopodia (Fig. 7C), even though CAPS-YFP continued to be expressed by M12. The specific and temporal concentration of CAPS is consistent with the idea that CAPS is involved in the initial recognition between muscles and neurons at the tip of myopodia.

Synaptogenesis in *caps* mutants

The existence of CAPS at the tips of myopodia suggests its possible involvement in early recognition events mediated by myopodia. We therefore performed live imaging to study the interaction between myopodia of M12, a *caps*-expressing muscle, and the innervating motoneuronal growth cones, in *caps* null mutants. There are no gross developmental defects in the differentiation of motoneurons in *caps* mutants (Shishido et al., 1998). Axon projection of MN12s to the vicinity of the target muscle also appeared normal, as assessed by the distance between the growth cones and M12 at the beginning of the time-lapse imaging (13:00 hours) (see Fig. S4 in the supplementary material). There was no overt abnormality in the differentiation of muscles (see Fig. S5 in the supplementary material) or in the morphology of myopodia. The number of myopodia was also normal [7.2 per muscle ($n=21$) in wild type compared with 6.1 ($n=24$) in *caps* mutants; $P>0.05$, two-tailed *t*-test]. Therefore, in *caps* mutants, myopodia protrude and initiate their contacts with the growth cones as in the wild type. However, subsequent stabilization of the contacts appeared to be affected. At 13:30 hours, there was a reduced number of contact points between myopodia and growth cones (Fig. 8A,B) [4.7 in cont ($n=17$); 3.0 in *caps* mutant ($n=22$); $P<0.01$, two-tailed *t*-test]. The number of free myopodia that do not contact the growth cones was unchanged, suggesting that formation of myopodia was not affected [5.7 per muscle ($n=17$) in wild type compared with 5.7 ($n=22$) in *caps*

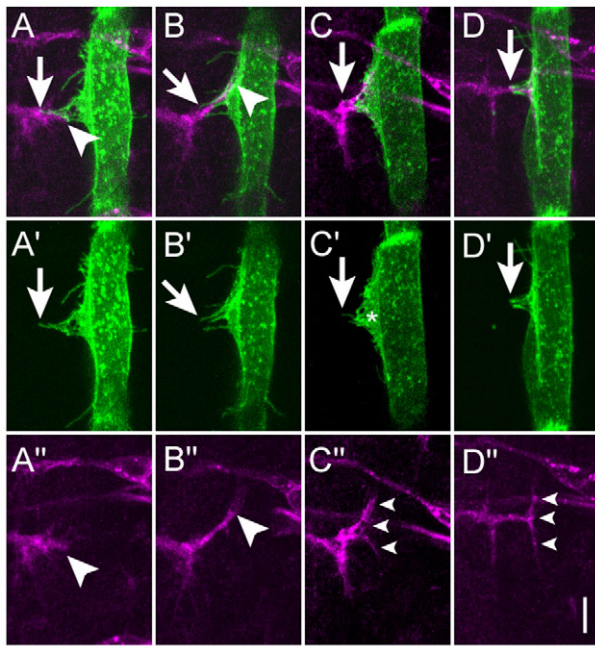


Fig. 6. Dual-color imaging of pre- and postsynaptic structures. (A-D'') Images of myopodia (A-D, A'-D'; mGFP, green) and motor axons (A-D, A''-D''; anti-HRP: Cy5, magenta) in dissected embryos during 13:30-14:00 hours. Tips of myopodia (arrows) and ends of axons (arrowheads) are indicated. The asterisk indicates the lamellipodia-like structure (see text). Scale bar: 10 μ m.

mutants; $P > 0.05$, two-tailed t -test]. These observations imply that fewer contacts between the myopodia and growth cones are stabilized in *caps* mutants. Thus, *caps* may be involved in the initial recognition between myopodia and their partner motoneurons.

In *caps* mutants, there was also a delay in the formation of presynaptic terminals on M12 (Fig. 8C). Furthermore, although the terminals were eventually formed by 15:00 hours in all segments, their sizes were significantly smaller (as assessed by ratio of the length of MN12 axon terminals to that of M12; 0.30 in *caps*^{C28fs}/*Df(3L)Ly* and 0.29 in *caps*^{L259fs}/*Df(3L)Ly*, compared with 0.36 in $+/+$ and 0.36 in $+/Df(3L)Ly$ embryos; $P < 0.001$) (Fig. 8D,E). Thus, synapses are formed in the absence of *caps* but in a less robust manner. As the dual-color imaging described above (Fig. 6) suggests that the filopodia-myopodia complex provides a substrate upon which the nascent presynaptic terminal is formed, the defects in terminal formation observed in *caps* mutants may result from defects in contact formation between the myopodia and growth cones. The defects in terminal formation appear to be specific to M12, as no abnormality was found in *caps*-negative muscles M6 and M7 (Fig. 8F).

As described in the Introduction, CAPS is expressed both in presynaptic MN12 and postsynaptic M12. To assess if *caps* is required pre- and/or postsynaptically, we studied the effects of expressing a putative dominant-negative form of CAPS (CAPS-ID, a deletion mutant lacking an intracellular domain) (Taniguchi et al., 2000) in either motoneurons or M12s. We found that expression in muscles but not in motoneurons causes the same terminal defects as seen in *caps* mutants (Fig. 8G). These results further support the notion that *caps*-mediated recognition at the tip of myopodia is important for the formation of specific synaptic connections.

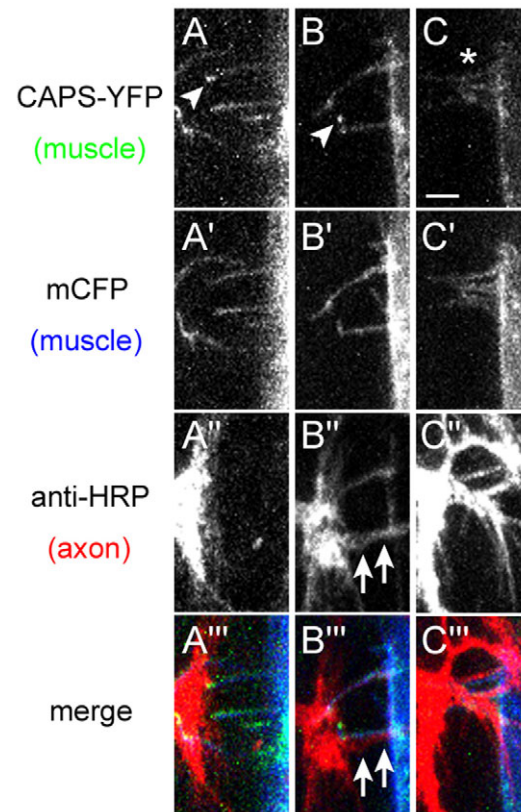


Fig. 7. Distribution of CAPS during pre- and postsynaptic interaction. Triple-color imaging of CAPS-YFP in muscle (A-C, A'''-C'''; green), mCFP in muscle (A'-C', A'''-C'''; blue) and axon (A''-C'', A'''-C'''; anti-HRP: Cy5, magenta) in dissected embryos at 13:00-14:00 hours. (A-A''') CAPS-YFP accumulation at the tips of myopodia (arrowhead) before innervation. (B-B''') CAPS-YFP concentrates at the tips of myopodia (arrowhead) even after growth cones contact and extend along myopodia (arrows). (C-C''') No concentration of CAPS-YFP is seen in assembled myopodia (asterisk). Scale bar: 5 μ m.

The phenotype observed in *caps* mutants described above is relatively mild, suggesting that there are redundant molecules that can contribute to target recognition in the absence of CAPS. The best candidate for such a redundant target recognition molecule is Tartan, which is the closest relative of CAPS in the *Drosophila* proteome (65% identical to CAPS within the extracellular domain) and is encoded by a nearby gene. Caps and Tartan are clearly the product of a gene duplication, suggesting that an ancestral organism may have used only one CAPS/Tartan homolog for target recognition.

Indeed, Kurusu et al. (Kurusu et al., 2008) recently reported that double mutants for *caps* and *tartan* have stronger axon guidance phenotypes than the single mutants, and some segments have ISNBs with 'looped' terminals, suggesting a defect in recognition of M12 (Kurusu et al., 2008). We therefore analyzed filopodia-myopodia interaction in *caps tartan* double mutants. As in *caps* mutants, axon elongation towards the target region was relatively normal in the double mutants (see Fig. S4 in the supplementary material). However, the following target recognition and terminal formation were severely affected. The number of contact points between growth cones and M12 was greatly reduced (Fig. 8A,B). The length of axon terminals on M12 was also much shorter (Fig. 8D,E), with some muscles completely

Fig. 8. Synaptogenesis in *caps* and *caps trn* mutants.

(A) Interaction between myopodia and motoneuronal growth cones in control, *caps*^{C28fs}/*Df(3L)Ly, trn*^{25/4}/*caps*^{C28fs} *trn*^{delta17} and *caps*^{C28fs} *trn*^{delta17} at 13:30 hours. Scale bar: 10 μ m.

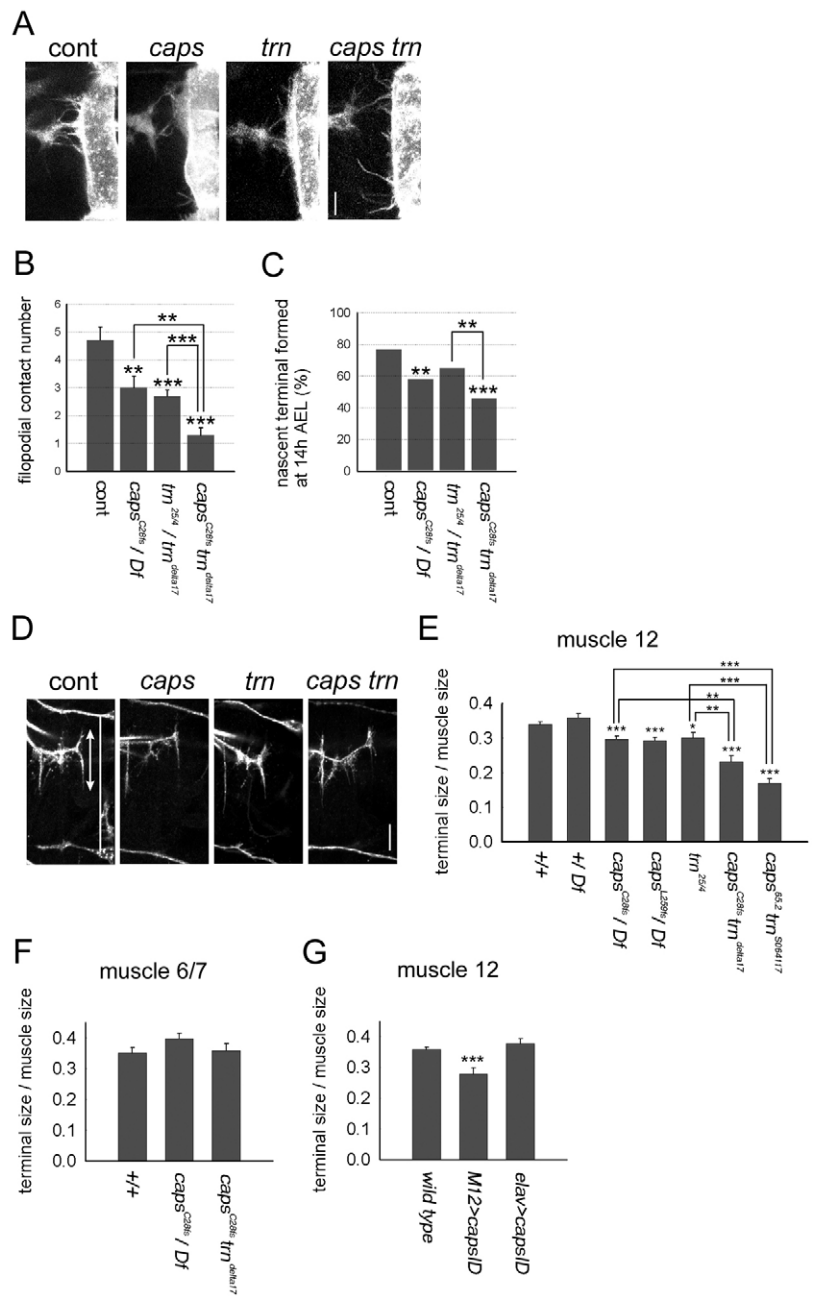
(B) Quantification of numbers of contact points between myopodia and growth cones at 13:30 hours AEL. Numbers of contacts are reduced in *caps*^{C28fs}/*Df(3L)Ly, trn*^{25/4}/*caps*^{C28fs} *trn*^{delta17} and *caps*^{C28fs} *trn*^{delta17}. ***P*<0.01, ****P*<0.001; two-tailed *t*-test.

(C) Fraction of hemisegments that have formed a nascent terminal at 14:00 hours AEL. ***P*<0.01, ****P*<0.001; χ^2 test. We define a nascent terminal as a bifurcated structure formed on lamellipodia-like myopodial cluster (as shown in Fig. 6C) or on the body of M12 (as shown in Fig. 6D). The analysis was undertaken in a genotype-blinded manner.

(D-G) Size of the M12 axon terminal at 15:00 hours is reduced in *caps* and *caps trn* mutants.

(D) Axon terminals visualized with anti-HRP immunostaining in wild-type, *caps*^{C28fs}/*Df(3L)Ly, trn*^{25/4} and *caps*^{C28fs} *trn*^{delta17} embryos at 15:00 hours. The white arrow and line indicate the length of the axon terminal and muscle, respectively, that were used for the quantitative analyses in E. Scale bar: 10 μ m. (E) Terminal size is reduced in *caps* and *caps trn* mutants. The ratio of the length of MN12 axon terminals to that of M12 in controls (+/+ , *n*=185 segments; +/*Df(3L)Ly*, *n*=77), *caps* mutants (*caps*^{C28fs}/*Df(3L)Ly*, *n*=98; *caps*^{L259fs}/*Df(3L)Ly*, *n*=123), *trn* mutants (*trn*^{25/4}, *n*=62) and *caps trn* mutants (*caps*^{C28fs}, *trn*^{delta17}, *n*=51; *caps*^{65.2}, *trn*^{S064117}, *n*=92). The ratio is significantly smaller in two alleles each of *caps*, *trn* and *caps trn* mutants as compared with +/+ controls (**P*<0.05, ***P*<0.01, ****P*<0.001; two-tailed *t*-test). The same statistical results are obtained when +/*Df(3L)Ly* line was used as a control instead of +/+ for the *t*-test (not shown). The ratios in *caps trn* double mutants (*caps*^{C28fs}, *trn*^{delta17} or *caps*^{65.2}, *trn*^{S064117}) are significantly smaller than in *caps* (*caps*^{C28fs}/*Df(3L)Ly*) or *trn* (*trn*^{25/4}) single mutants. The same statistical results are obtained when the double mutants are compared with *caps*^{L259fs}/*Df(3L)Ly*, another *caps* single mutant line (not shown). (F) Terminal formation is normal in *caps*-negative muscles, M6 and M7. No significant difference in the size of M6/7 terminals in *caps*^{C28fs}/*Df(3L)Ly* (0.40; *n*=47) and *caps*^{C28fs}, *trn*^{delta17} (0.36; *n*=47) compared with controls (0.35; *n*=47) (*P*>0.05; two-tailed *t*-test).

(G) Postsynaptic expression of a deletion form of CAPS lacking the intracellular domain affects axon terminals on M12. Graph shows ratio of the length of MN12 axon terminals to that of M12 in control embryos and in embryos expressing a deletion mutant form of CAPS (CAPS-ID) in postsynaptic muscles (M12>*capsID*) or in presynaptic neurons (*elav*>*capsID*). Terminal size is reduced in M12>*capsID* (0.28; *n*=30) but not in *elav*>*capsID* (0.38; *n*=35) compared with controls (0.36; *n*=134; ****P*<0.001; two-tailed *t*-test). Error bars in E-G represent the mean \pm s.e.m.



lacking the terminals (7 out of 51 hemisegments compared with 0 out of 47 hemisegments in control; *P*=0.0084, Fisher's test) (see Kurusu et al., 2008). These phenotypes were qualitatively similar to those of *caps* single mutants and *trn* single mutants (Fig. 8) but were much stronger. These results are consistent with the idea that the function of CAPS in mediating recognition between M12 and its presynaptic motoneurons is partially redundant with that of Tartan. In the absence of both CAPS and Tartan, most M12s still became innervated, suggesting that there are additional cell surface proteins that also contribute to target recognition of M12, but the synapse on M12 is much reduced relative to wild type.

Whether the synapse could repair itself and grow to a normal size in the absence of CAPS and Tartan could not be determined, because *tartan* mutants are embryonic lethal.

DISCUSSION

Filopodia are thin cellular protrusions that are thought to function as sensors of the local environment (Rørth, 2003). Sensory function of filopodia has been best illustrated in neuronal growth cones (Henley and Poo, 2004; Gallo and Letourneau, 2004). For example, a single filopodium of a neuron in culture is able to steer the entire growth cone when it contacts a more adhesive substrate (Letourneau, 1975).

Growth cones of grasshopper pioneer axons in vivo change their direction upon the contact of a single filopodium with guidepost cells (O'Connor et al., 1990). Filopodia are also found both pre- and postsynaptically during the process of synaptogenesis and are believed to actively participate in cellular interactions (Cohen-Cory, 2002; Goda and Davis, 2003). Although there is evidence for the sensory function of presynaptic filopodia, few studies have addressed the question of whether postsynaptic filopodia are able to select specific partners. In this study, we took advantage of the fact that highly specific and stereotyped synaptogenesis occurs during a relatively short period of time at the neuromuscular junction of *Drosophila*. We present several lines of evidence that support the active role of myopodia, postsynaptic filopodia of muscles, in synaptic partner recognition.

We found a striking concentration of CAPS at the tips of myopodia. If myopodia have a passive role and merely help increase the target area, then it would seem to be more efficient for CAPS to be distributed uniformly along the length of myopodia. There would be more chance for the target marker to be presented to the presynaptic cells. Concentration of CAPS at the tips instead supports the idea that myopodia play an active role in directing growth cones to their targets. Filopodia and other cellular extensions have been observed in a variety of cells that send and/or receive signals, and implicated in contact-mediated long-distance communication (Rørth, 2003). By presenting molecules such as CAPS at the tips, myopodia might be able to efficiently send signals to presynaptic motoneurons. At the same time, myopodia might receive signals from the presynaptic cells by using molecules such as CAPS as sensors. Such bidirectional signaling occurring at the tips of myopodia might be a trigger for synaptic matchmaking. As CAPS is also expressed in MN12s and can function as a homophilic cell adhesion molecule in vitro (Shinza-Kameda et al., 2006), the signaling might be mediated by homophilic interaction between CAPS on pre- and postsynaptic cells.

It has been postulated that the tips of filopodia are the signaling center that regulate filopodial extension, retraction and adhesion from the following reasons. First, it is often the distal part of the filopodium that makes contact with the environmental cues (Letourneau, 1975; O'Connor et al., 1990). Second, actin monomers are added to the barbed ends of filaments at the tips of filopodia (Mallavarapu and Mitchison, 1999). Third, several signaling and adhesion molecules, including Mena, integrins and the tyrosine-phosphorylated proteins, are concentrated at the tips of filopodia (Bear et al., 2002; Grabham and Goldberg, 1997; Wu and Goldberg, 1993). Therefore, filopodia tips are well situated to link information from the environment to the dynamics of filopodia. Concentration of CAPS suggests a possibility that signaling events at the tips of myopodia are crucial in selective synapse formation. Consistent with this idea, we found, by live imaging of neuromuscular interaction in vivo, that many of the initial contacts between motoneuronal growth cones and muscles do occur at the tips of myopodia. By tracing the dynamics and final fate of individual myopodia, we also found that some of these contacts are stabilized to form the synaptic site, whereas others are eliminated. These results suggest that protrusive activity of myopodia actively contributes to neuromuscular interaction. Furthermore, we provide evidence that the behavior of myopodia differs depending on whether or not contact was made with a partner motoneuron. These results suggest that myopodia search for appropriate synaptic partners. Taken together with the localization of CAPS, these observations provide strong evidence for the sensory function of myopodia, and also highlight the role of myopodial tips as a

possible signaling center for synaptic matchmaking. As the dynamic behavior of the postsynaptic cell is just as important as that of the presynaptic cell, future studies of axon guidance and target recognition during the formation of synapses should focus on the postsynaptic cell as much as the presynaptic cell.

What kind of signaling is taking place at the tips of myopodia? How is the signal transmitted to other regions of muscles to affect the process of synaptogenesis? Recognition by cell adhesion molecules such as CAPS might allow for stabilization of specific myopodial contacts. Signaling at the tips might be transmitted to neighboring regions of the muscle to induce myopodia clustering and postsynaptic differentiation. Our data indicate that signaling in muscles through the cytoplasmic domain of CAPS is likely to be required for target recognition, as CAPS-ID, which lacks the cytoplasmic domain, acts as a dominant negative when expressed in muscle.

The reduction in the number of contacts between myopodia and growth cones and size of the nascent synaptic sites observed in *caps* and *caps, tartan* double mutants are consistent with this model. Myopodia-filopodia interaction appears also to be important for the differentiation of the presynaptic terminals. We observed that presynaptic terminals form while interacting with the surrounding myopodia. Reduction in the size of nerve terminals observed in *caps* and *caps, tartan* double mutants also suggests that stabilization of contacts between growth cones and myopodia is crucial for presynaptic differentiation. As myopodia-like structures and their clustering have been reported in vertebrates (Misgeld et al., 2002), signaling events that regulate myopodia-growth-cone interaction might be a common mechanism for neuromuscular synaptogenesis. Future studies on the molecular events occurring at the tips of myopodia might shed light on the very beginning of synaptogenesis.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/7/1127/DC1>

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