Commissureless endocytosis is correlated with initiation of neuromuscular synaptogenesis

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SUMMARY

We show that the Commissureless (COMM) transmembrane protein is required at neuromuscular synaptogenesis. All muscles in the Drosophila embryo express COMM during the period of motoneuron-muscle interaction. It is endocytosed into muscles before synaptogenesis. In comm loss-of-function mutants, motoneuron growth cones fail to initiate synaptogenesis at target muscles. This stall phenotype is rescued by supplying wild-type COMM to the muscles. Cytosplastically truncated COMM protein fails to internalize. Expressing this mutant protein in muscles phenocopies the synaptogenesis defects of comm mutants. Thus, synaptogenesis initiation is positively correlated with endocytosis of COMM in postsynaptic muscle cells. We propose that COMM is an essential part of the dynamic cell surface remodeling needed by postsynaptic cells in coordinating synaptogenesis initiation.

Key words: Adaptin, Axon, Drosophila, Endocytosis, Growth cone, Internalization, Neuromuscular, Synaptogenesis, Commissureless

INTRODUCTION

Initiation of synaptogenesis depends on the compatibility between a neuronal growth cone and its target (Haydon and Drapeau, 1995). Often underappreciated is the fact that the majority of the cell-cell contacts that a growth cone experiences earlier in development lead to no synaptogenesis. It seems reasonable to propose that synaptic compatibility depends, at least in part, on aspects of molecular profiles surrounding the growth cones that are under specific temporal controls. Consistent with this, heterochronic coculture experiments indicate that the likelihood of synaptogenesis depends on the age of the target cell (Fletcher et al., 1994). It is not clear if this reflects earlier presence of synapse inhibition factors or later appearance of synapse initiation factors in the postsynaptic cells. However, these observations suggest that postsynaptic cells have an ability to coordinate synaptogenesis initiation by temporally modulating their membrane-associated molecules. The molecular mechanisms for such molecular coordination are not known.

In the Drosophila neuromuscular system, individual cellular contacts of motoneuron growth cones before reaching appropriate target muscles are fairly well characterized (Chiba, 1998; Keshishian and Chiba, 1993). In each body wall hemisegment, a set of 30 muscle cells are arranged in specific arrays along the proximal-distal (ventral-dorsal) axis, while motoneurons, estimated to be about 40 in total number, extend their axons into these muscle arrays in successive waves. Those motoneuron growth cones that exit the CNS earliest travel farthest and reach the distal-most (dorsal) muscles. Others that extend later travel less distance and stop at more proximal (lateral and ventral) muscles. Thus, except for those located most distally, each muscle is contacted by many motoneuron growth cones before being finally innervated by the specific growth cone(s) that are destined to innervate it. Under normal conditions, the timing of synaptogenesis initiation on each muscle is reproducible. These observations raise a possibility that the muscles (postsynaptic cells) may be regulating their cell surface molecular profiles in temporally dynamic manners in order to regulate synaptic compatibility.

Commissureless (COMM) is a type I transmembrane protein possessing largely uncharted domains (Tear et al., 1996). COMM’s relatively large cytoplasmic domain contains a short Adaptin (AP-2) recognition sequence commonly found among endocytosing molecules (Fig. 1A). Previous studies have identified COMM as a molecule expressed by the midline glia and involved in midline signaling of the Drosophila embryonic CNS (Seeger et al., 1993; Tear et al., 1996).

In this study, we show that, in addition to the midline glia, the COMM protein is also expressed in muscles. We also show that motoneurons in the comm loss-of-function mutants fail to initiate synaptogenesis on target muscles. Furthermore, we demonstrate a correlation between internalization of COMM protein in muscles and neuromuscular synaptogenesis initiation. We propose a novel mechanism of rapid cell surface remodeling of the postsynaptic cells that dynamically regulates the time frame of specific cell-cell recognition.
MATERIALS AND METHODS

Drosophila lines

Commissureless null (comm5) and loss-of-function (comm1) mutants are as described by Tear et al., 1996. comm1 truncates the cytoplasmic domain at amino acid 217 and continues for 26 novel amino acids (Fig. 1B). For the rescue experiment (see below), we generated a transformant line carrying a wild-type COMM coding sequence under the control of the UAS promoter (UAS-commvt-Δ2). Similarly, for the misexpression experiments, a line that has a cytoplastically truncated COMM (UAS-commcytor-Δ3-1) was generated. The commcyt lacks the entire cytoplasmic domain after amino acid 185 (Fig. 1C). We used the GAL4/UAS conditional gene activation system (Brand et al., 1994) to express the wild-type or modified COMM in the musculature of either wild-type or comm null embryos. Both muscle-specific enhancer trap ‘GAL4 driver’ lines GAL4b24 (P element is on the third chromosome) and Stock Center, Bloomington, Indiana) and GAL4005 (P element is on the second chromosome, and the misexpression starts at hour 12; source: David Teague and A. Chiba) were used. Canton S strain served as a wild-type control. Genotypes of the experimental animals are given in Table 1.

For labeling specific subsets of motoneurons, we used two transformant lines: RK20-lacZETIC-10/Cyo line which carries a transgene RK20-lacZ that consists of the promoter of a neuronal type-specific gene RK20 driving the expression of lacZ gene encoded β-galactosidase (P element on the second chromosome; see Rose et al., 1997 for its use; source: John Thomas and Stefan Thor, Salk Institute), and eve-tr/lacZXR1 line which carries a transgene eve-tr/lacZ that consists of ~7.8–9.2 kb EcoRI fragment of the neurogenic enhancer of even skipped gene driving the expression of a fusion protein that combines microtubule-binding protein τ and β-galactosidase (P element on the first chromosome; source: Xiaomao Zhu, Hui Dou, Chris Doe and A. Chiba), prospero mutant (pros17; Doe et al., 1991; source: C. Doe, University of Illinois) was used to alter the timing of motoneuron-muscle contact.

Immunocytochemistry

Methods for whole embryonic immunocytochemistry and fillet dissection are as described and followed a standard protocol of fixation, membrane permeabilization (with 0.1% Triton-100) and preincubation with BSA before incubation with primary antibodies (Chiba et al.,

Table 1. Rates of failing synaptogenesis (%) by the five motoneuron groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>ISN</th>
<th>SNa</th>
<th>SNb</th>
<th>SNc</th>
<th>SNd</th>
</tr>
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<tbody>
<tr>
<td>Wild typea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Canton S</td>
<td>55</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (7)</td>
<td>7 (0)</td>
<td>4 (4)</td>
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<tr>
<td>comm mutationb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>comm1/comm1</td>
<td>83</td>
<td>14 (0)</td>
<td>33 (26)</td>
<td>63 (52)</td>
<td>40 (30)</td>
<td>46 (45)</td>
</tr>
<tr>
<td>comm1/comm5</td>
<td>57</td>
<td>9 (0)</td>
<td>23 (23)</td>
<td>62 (51)</td>
<td>44 (40)</td>
<td>43 (38)</td>
</tr>
<tr>
<td>Rescue of comm mutationc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAL4b0/GAL4b0; comm1/comm1</td>
<td>54</td>
<td>15 (0)</td>
<td>37 (33)</td>
<td>67 (56)</td>
<td>46 (33)</td>
<td>46 (44)</td>
</tr>
<tr>
<td>UAS-comm1/UAS-comm1 (parental line 1)</td>
<td>57</td>
<td>8 (0)</td>
<td>25 (20)</td>
<td>63 (58)</td>
<td>30 (22)</td>
<td>40 (40)</td>
</tr>
<tr>
<td>UAS-comm1/UAS-comm1 (parental line 2)</td>
<td>82</td>
<td>5 (0)</td>
<td>16 (10)</td>
<td>59 (56)</td>
<td>29 (27)</td>
<td>36 (32)</td>
</tr>
<tr>
<td>GAL4b2/GAL4b2 (parental line 1)</td>
<td>55</td>
<td>0 (0)</td>
<td>7 (5)</td>
<td>5 (3)</td>
<td>5 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>GAL4b2/GAL4b2 (parental line 2)</td>
<td>51</td>
<td>0 (0)</td>
<td>2 (2)</td>
<td>6 (6)</td>
<td>6 (6)</td>
<td>4 (4)</td>
</tr>
</tbody>
</table>

Methods: Motoneurons were grouped into five groups according to the specific peripheral nerves through which they extend their axons: ISN, SNa, SNb, SNc, and SNd. The rates (%) in each of the five motoneuron groups were scored at hour 18 of embryogenesis, using mAb 1D4 immunocytochemistry (Greenfield et al., 1991; Van Vactor et al., 1993). The rates in ( ) indicate the cases where the motoneuron growth cones stall short of the target muscles. In all the remaining cases, the growth cones extended beyond the normal stopping points but without apparently innervating alternative muscles. Sample sizes (n) are indicated as the number of abdominal hemisegments (pooled from 5-9 embryos) examined for each data set.

Genotypes: (a) Wild-type control is based on the Canton S strain. (b) Three allelic situations of comm mutants show similar neuromuscular phenotypes. comm1/comm1 is the null mutant, while commvt/commvt is a loss-of-function mutant with truncation of COMM protein at its distal portion of the cytoplasmic domain (Fig. 1B). commvt/commvt is the transheterozygote between the two. (c) The neuromuscular phenotype in the comm null mutants can be rescued by supplying wild-type COMM in muscles at the time of motoneuron-muscle contacts (GAL4b0/GAL4b0; commvt/commvt). The expression of the wild-type COMM is controlled by a myotopic enhancer (see Materials and Methods). The two parental lines serve as controls. (d) Misexpression of cytoplastically truncated COMM in muscles (UAS-comm1/comm1; GAL4b2/GAL4b2) phenocopies the comm mutant phenotype at the neuromuscular system. The misexpression uses a myotopic enhancer driver (see Materials and Methods). The truncated COMM (commcyt) lacks the entire cytoplasmic domain (Fig. 1C). The two parental lines serve as controls.
COMM is present in 1993. The only modification was that we used a two-step fixation method: 5 minute primary fixation at low pH (3% paraformaldehyde, 7.6 mM monobasic sodium phosphate, 32 mM dibasic sodium phosphate, with pH adjusted to 6.7), followed by another 5 minute secondary fixation at high pH (3% paraformaldehyde, 0.2 M sodium borate, with pH adjusted to 11.0). This improved tissue preservation.

Polyclonal anti-Commissureless antibodies (1:500 dilution) recognizes the extracellular (N terminus) domain of COMM protein and labels both wild-type and truncated forms of COMM used in this study (Tear et al., 1996). Other primary antibodies used were: mAb 7G10 (anti-Fasciclin III, 1:10 dilution; Patel et al., 1987; source: Hybridoma Center, Iowa City, Iowa), mAb ID4 (anti-Fasciclin II, 1:10 dilution; Grenningloh et al., 1991; source: Corey Goodman, University of California at Berkeley), mAb CL.427 (anti-Connectin, 1:4 dilution; Meadows et al., 11994; source: Rob White, Cambridge University), mAb TollCD (anti-Toll, 1:10 dilution; Hashimoto et al., 1991; source: Carl Hashimoto, Yale University), Ab DSYT2 (anti-Synaptotagmin, 1:100 dilution; Littleton et al. 1993; source: Hugo Bellin, Baylor College of Medicine), anti-BSA (1:100 dilution; source: Vladimir Gelfand, University of Illinois), anti-β-galactosidase (1:5000 dilution; source: Promega), and TRITC-conjugated anti-HRP (1:100 dilution; source: Jackson Immuno Research Lab, West Grove, PA). These primary antibodies were visualized either with fluorescently (FITC or TRITC) tagged secondary antibodies or histochemically using ABC kit (Vector Lab, Burlingame, CA).

Endosome visualization
We dissected hour 6-13 embryos live in insect saline (Johansen et al., 1989) and incubated them in the presence of 1-2% bovine serum albumin (BSA) for 5 hours at 25°C. Following washes with saline, the embryos were fixed and processed for double immunolabeling with anti-BSA and anti-COMM antibodies. They were visualized through fluorescent confocal microscopy. For each developmental stage (hours 11, 14, 16 and 18), the data set consisted of 30-40 abdominal hemisegments pooled from 4-7 dissected embryos. To digitally reconstruct the cross sectional views from the confocal Z series images, we used NIH Image software.

Functional assessment of neuromuscular synapses
We assessed whether or not the neuromuscular synapses were functional based on individual embryos' ability to contract body wall muscles upon physical stimulation. Embryos, initially collected on a standard ‘collection’ juice plate, were transferred to a new juice plate and placed in fixation at high pH (3% paraformaldehyde, 0.2 M sodium borate, with pH adjusted to 11.0). This improved tissue preservation.

Their chorion and vitelline membranes intact, and periodically examined under a dissecting microscope for morphological development as well as movement. All embryos that passed gastrulation stages and developed both pigmented segmental denticles and pigmented jaws were subjected to movement test, which consisted of gentle rubbing repeated up to three times on the dorsal surface of the embryo with a pulled blunt-ended capillary tube. Positive movement was scored if both clear jaw movement and peristalsis (coordinated muscle contractions occurring in waves through body segments) occurred. We interpreted such movements as a sign for functional neuromuscular synapses.

RESULTS
Embryonic muscles express COMM protein
Tear et al. (1996) reported that COMM mRNA is present in

Fig. 2. COMM in embryonic muscles. (A) COMM protein is expressed in all 30 muscles in each hemisegemnt of hour 18 wild-type embryos. Some muscles are out of focus in A1. The tracing in A2 is based on A1 and shows the identities of some muscles. The dorsalmost muscles express somewhat lower levels of COMM than the rest of the musculature. (B) A higher magnification view of a muscle (muscle 14, outlined by dotted line) shows prominent COMM enrichment in microsomes inside the muscle (arrowheads). In contrast, muscle surface has relatively low levels of COMM. The view corresponds roughly to the region of another preparation indicated with box in A1. (C) The combination of BSA vital labeling of endosomes and COMM immunocytochemistry demonstrates that the COMM-positive microsomes represent a subset of endosomes that form during hours 11-18. This hour 18 embryo has been vitally labeled for endosomes during hours 11-18. The three panels in C1 show the endosomal and COMM stains each separately (BSA and COMM, respectively) as well as both together (BSA + COMM) in two muscles (muscles 6 and 7, outlined by dotted lines). A reconstructed double-channel cross-sectional view (C2) of one of the muscles (muscle 7, outlined by dotted line) clearly reveals both COMM-positive and COMM-negative endosomes that appear, respectively, yellow (red plus green channels, white arrowhead) and red (red channel alone, open arrowheads). They are not distinguishable in terms of size and distribution pattern in the muscles. This cross section was based on the confocal Z series images of the region indicated with white bar (with a ‘scissors mark’) in C1. Scale bars (vertical bars), 10 μm.
mesodermal tissue in early embryos. We examined the COMM protein expression pattern in mesoderm-derived tissues using immunocytochemistry.

COMM protein is expressed in all 30 muscles in each abdominal hemisegment of late stage embryos (Fig. 2A). Slightly lower levels of expression are seen in the most distal (dorsal) set of muscles that are targeted by the ISN motoneurons whose axons grow past all the other more proximal muscles that have high levels of COMM expression. The muscle-supplied protein is visible as early as hour 11 of embryogenesis. It precedes the contacts between motoneuron growth cone and muscles by at least 1 hour. COMM expression persists until hour 18, by which time cuticular deposition begins to block antibody penetration into whole embryonic tissues.

Within muscles, COMM protein becomes localized in punctate microsome structures of approximately 0.5-1.0 μm diameter (Fig. 2B arrowheads). These microsomes are not stained unless the plasma membrane is pretreated with a permealizing reagent such as Triton-100 (data not shown). Through a combination of vital labeling of endosomes and COMM immunocytochemistry, we found that these COMM-positive microsomes are a subset of endosomes that undergo endocytosis during hours 11-18 (Fig. 2C). About 25% of the newly formed endosomes are COMM positive. They are similar in size and distribution pattern to those that are COMM negative. We did not detect any COMM immunoreactivity in muscle nuclei. The presence of COMM in endosomes, combined with the presence of an Adaptin recognition site within the cytoplasmic domain of COMM, is consistent with its cytoplasmic domain associating with the Adaptin/Clathrin complex.

At hour 11, COMM is mostly found on the cell surface membrane (Fig. 3A). However, by hour 18 the majority of COMM immunoreactivity shifts to the endosomes (Fig. 3B). The COMM-positive endocytic activity peaks around hour 14, coinciding with the onset of neuromuscular synaptogenesis on the majority of muscles. As a result, when COMM protein is largely on muscle surfaces, the motoneuron growth cones that contact them extend past these muscles. However, as the bulk of the protein internalizes into muscles, those growth cones that are the normal synaptic partners of these muscles approach them and initiate synaptogenesis on respective muscles.

Motoneuron growth cones fail to initiate synaptogenesis in comm mutants

The first hint that muscle-supplied COMM protein plays a role during interactions between motoneuron growth cones and muscles came from analysis of neuromuscular development in three comm mutant alleles: comm1/comm1 (null), comm5/comm1 (loss of function due to truncation of the distal cytoplasmic domain that includes the putative Adaptin recognition site; see Fig. 1B), and comm7/comm1 (transheterozygous). As reported previously, these mutant embryos exhibit major axon pathway defects at the CNS midline, presumably due to the loss of the normal COMM expression in the midline glia (Seeger et al., 1993; Tear et al., 1996).

The morphological development of muscles proceeds normally in all comm loss-of-function alleles (Fig. 4). Muscle cell surface molecules Toll, Fasciclin II, Fasciclin III, and Connectin are all found on the surface of muscles that normally express them (data not shown). In comm1/comm1, the mutant COMM with partial deletion of its cytoplasmic domain fails to internalize (Fig. 4C). In all three alleles, COMM-independent endocytosis is still observed in muscles (Fig. 4B).

Despite the axon pathway defects at the CNS midline, the nerves containing motoneuron axons extend out the CNS normally in all mutant alleles (Fig. 5). Subsequent pathway selections by the axons are indistinguishable from wild type until their growth cones reach respective target muscles.

To further confirm this point at the level of single cells, we traced the pathways of two sets of motoneuron growth cones in the comm mutants using specific neuronal marker constructs: eveΔ/lacZ, which labels the aCC and RP2 motoneurons, and RK20Δ/lacZ, which labels the RP3 and RP5 motoneurons (see Materials and Methods). The aCC and RP2 growth cones in wild type do not cross the CNS midline and extend ipsilaterally through ISN (Fig. 6A,C). This remains true in the comm mutants (Fig. 6D,F). In contrast, the RP3 and RP5 growth cones, which normally cross the CNS midline and extend contralaterally through SNb (Fig. 6B,C), fail to cross the CNS midline and extend through ipsilateral SNb (Fig. 6E,F). Both RP3 and RP5 growth cones apparently reach their respective target muscles and then stall (Fig. 6E, arrow). This clearly shows that, after exiting the CNS, the individual motoneuron growth cones exhibit no pathfinding errors before arriving at their respective target regions. Thus, outside the CNS, COMM does not function as an axon guidance molecule.

However, upon contacting the target muscles, the

Fig. 3. COMM internalization. (A) At hour 11, COMM protein is predominantly associated with the muscle surface. (B) By hour 18, however, COMM is virtually absent from the surface and, instead, localized in endosomes within the muscles. Dotted lines outline the muscles of the confocal images. White bars in A1 and B1 indicate the regions from which the cross-sectional views were reconstructed for, respectively, A2 and B2. Schematics in A3 and B3 illustrate the temporal correlation between the COMM internalization and the arrival of innervating motoneuron growth cones. Scale bar, 10 μm.
motoneuron growth cones fail to initiate synaptogenesis. In the hour 18 wild-type embryos, Synaptotagmin, a synaptic vesicle protein (Littleton et al., 1993), accumulates as punctate bodies at the axon terminals undergoing synaptogenesis (Fig. 5D arrowheads). In contrast, the motor axon terminals in the COMM null mutants at the same stage merely show low levels of diffused Synaptotagmin staining (Fig. 5I arrowheads). The latter condition is similar to when the axons are still navigating through the non-target areas (data not shown; see Yoshihara et al., 1997). In the majority of the cases, the growth cones stall at or just short of their normal targets (Fig. 5F,G arrows). In the remaining cases, they extend beyond the normal stopping points without apparently settling on alternative targets (Fig. 5H circle). These phenotypes are evident at frequencies of 14-63% in all five nerves (ISN, SNa, SNb, SNe, SND) and persist even past hour 18, the normal
period of synaptogenesis (Table 1 for summary). ISN, SNc and the posterior branch of SNa normally contain motoneuron axons that do not cross the CNS midline (Fig. 5E; Landgraf et al., 1997; A. Schmid, C. Doe, A. C., unpublished data). In the mutants no motoneuron axons apparently cross the CNS midline. Yet the growth cones in ISN, SNa and SNc all exhibit the defects similar to those of the other motoneuron groups (Fig. 5J). The three allelic situations show little variation (Table 1). Therefore, the neuromuscular synaptogenesis defects are likely to be a direct effect of disrupting muscle-derived COMM protein.

**Synaptogenesis is rescued by adding wild-type COMM protein in muscles**

To further test that the synaptogenesis defects results directly from the loss of normal COMM protein in muscles, we attempted to rescue the phenotype by misexpressing wild-type COMM in the muscles of COMM null mutant embryos (see Materials and Methods). We used a myotopic genomic enhancer to deliver the wild-type COMM in all muscles starting around hour 12, after the midline defect has occurred but before motoneuron growth cone-muscle interaction. Misexpressed COMM protein shows distribution patterns in muscles very similar to wild type, increasing in endosomes by hour 14 (Fig. 7A).

With the COMM misexpression in the muscles, motoneuron growth cones resume synaptogenesis (Fig. 7B). The rescue of synaptogenesis is observed in all five nerves (Fig. 7D) and the rates of failed synaptogenesis goes down by approximately 60-80% in each nerve (Table 1 for summary). SNb contains a number of motoneuron growth cones that normally cross the CNS midline (Landgraf et al., 1997; Sink and Whitington, 1991). In this rescue experiment, even with the persistent CNS midline phenotype, the SNb motoneuron endings that are formed on the muscles look similar to wild type (compare Figs 5A, 7B).

Further evidence indicated that resupplying COMM in muscles is sufficient to rescue the synaptogenesis defects caused by the COMM null mutation. First, similar to the wild-type situation, the motor axon endings accumulate Synaptotagmin as punctate bodies after the muscle rescue (Fig. 7C arrowheads). Second, at hours 20-24, just before the end of embryogenesis, these rescued embryos are seen to contract muscles in coordinated manners in 78% of the cases (n=289), similar to the wild-type embryos though with somewhat reduced vigor (see Materials and Methods). This clearly contrasts to the null mutant embryos which, when they survive until the same stage, show externally visible contraction of their body wall muscles only in 16% of the cases (n=96).

Therefore, these results establish that initiation of neuromuscular synaptogenesis requires the COMM protein in muscles.

**Fig. 6.** Identified motoneuron axons in comm mutants. (A-C) In wild-type hour 18 embryos, the aCC and RP2 motoneurons (A1) extend their axons through the ISN pathway (A2), while the RP3 and RP5 (B1), RP5 whose cell body lies directly beneath that of RP3 is out of focus) extend their axons across the CNS midline (dotted line) and through the SNb pathway in the periphery (B2), the out-of-focus axons are traced with a fine broken line). The first set of motoneurons represents those that do not extend their axons across the CNS midline (dotted line) and through the SNb pathway in the periphery (B2), the out-of-focus axons are traced with a fine broken line). The first set of motoneurons represents those that do not extend their axons across the CNS midline, and the second set represents those that do cross the midline. These two sets of motoneuron axon pathways were visualized by using, respectively, the eve−/lacZ and RK20−/lacZ constructs (see Materials and Methods). (D-F) In comm null mutant (comm+/comm+) embryos, the cell bodies in the mutant embryos are displaced slightly laterally (D1 and E1). However, the axon pathways of the aCC and RP2 motoneurons remain normal both within the CNS and periphery until the axons reach near the normal target muscles (D2). Similarly, the RP3 and RP5 motoneuron axon pathways are identical except for one difference: in wild type, these motoneuron axons cross the midline, but in the mutant they extend ipsilaterally. Nevertheless, their subsequent axon pathways within the CNS as well as in the periphery (SNb) remain intact until the axons reach near the normal target muscles. Therefore, the axon growth cones fail to initiate synaptogenesis on the target muscles (E2 arrow). Schematics in C and F summarize the data. Scale bars, 10 μm.
Cytoplasmically truncated COMM acts as a ‘dominant negative’ factor during synaptogenesis initiation

How does COMM control synaptogenesis initiation? Is its internalization necessary, as hinted by the situation with the \( \text{comm}^{lof}/\text{comm}^{lof} \) loss-of-function mutant allele (see Fig. 4C)? A complication with this mutant allele is that the modified COMM protein contains a novel amino acid sequence with potentially novel functions (Fig. 1B). Noting that COMM’s cytoplasmic domain contains a putative Adaptin recognition sequence (Fig. 1A), we hypothesized that COMM endocytosis would stop without its cytoplasmic domain. We set out to test the significance of COMM internalization by misexpressing cytoplasmically truncated COMM in muscles of wild-type embryos (Fig. 1C; see Materials and Methods). Following the misexpression of truncated COMM, which lacks nearly the entire cytoplasmic domain, high levels of COMM immunoreactivity are seen on the muscle surfaces, while the number of COMM-positive endosomes drops to roughly 25% of the normal level (Fig. 7E). It therefore appears that, not only does this truncated COMM fail to internalize in muscles, but it also acts as a dominant negative form of the protein that prevents internalization of most of the endogenous COMM (compare Figs 4C, 7E).

Interestingly, the motoneuron growth cones fail to initiate synaptogenesis and often stall just short of the target muscles (Fig. 7F,G). Except for SNa, which for an unknown reason exhibits a less severe case, the defects are virtually identical in both rates and appearance to the \( \text{comm} \) loss-of-function mutants (compare Figs 5F, 7F; see Table 1 for summary). In a control experiment where wild-type COMM is misexpressed in the muscles through the same misexpression system, motoneuron innervation proceeds normally despite the oversupplied COMM on the muscle surface (data not shown). Thus, the cytoplasmically truncated COMM apparently works as a ‘dominant negative’ factor for synaptogenesis initiation. Furthermore, lack of COMM-mediated endocytosis is once again correlated with a lack of synaptogenesis initiation.

COMM internalization is independent of growth cone contact

How is the timing of COMM endocytosis regulated? A likely candidate seems to be the arrival of motoneuron growth cones into the muscle fields. To determine if the motoneuron growth cones provide cues, we used the \textit{prospero} mutation to delay motoneuron growth cone extension (Broadie and Bate, 1993; Doe et al., 1991).

At hour 18, even in the absence of growth cone-muscle contacts, the COMM endocytosis proceeds normally (compare Fig. 8A,B). This suggests that the timing of COMM-mediated endocytosis is independent of motoneuron growth cones. COMM is likely to bind to factor(s) closely associated with muscles prior to internalization. This...
process of COMM activation is regulated temporarily so that it would, under the normal condition, coincide with synaptogenesis initiation between appropriate pairs of motoneuron growth cones and muscle targets (see Fig. 3).

DISCUSSION

We studied the role of Commissureless (COMM) transmembrane protein in the Drosophila embryonic neuromuscular system. COMM is expressed on the surface of all muscles when inappropriate motoneuron growth cones extend past non-target muscles, and is internalized just before appropriate motoneuron growth cones initiate synaptogenesis on correct target muscles (Fig. 3). Genetic manipulations demonstrate that endocytosis of COMM is a positive correlate to synaptogenesis initiation at all muscles (Figs 5E,J, 7D,G for summary). These findings establish the unique role of COMM in synaptogenesis initiation. They also suggest a molecular mechanism with which the timing of synaptogenesis may be specifically coordinated in a developing nervous system.

Two roles of COMM

The previous studies have revealed that COMM plays a crucial role in CNS midline signaling (Kidd et al., 1998; Seeger et al., 1993; Tear et al., 1996). We showed that the COMM protein is essential for normal neuromuscular synaptogenesis. Furthermore, we demonstrated that the two events are experimentally separable. Thus, COMM provides an example where a single protein influences the development of motoneuron axons in two different contexts, earlier at the CNS midline and later at synaptogenesis initiation in the periphery. It has been proposed that, at the CNS midline, COMM’s main role is to control expression of the specific growth cone receptor Robo (Kidd et al., 1998). However, at present, it is not clear if the COMM-Robo interaction exists during neuromuscular development, and whether or not the two roles of COMM rely on the same molecular pathways needs to be determined in the future.

Internalization of COMM

COMM-positive endosomes constitute a subpopulation of endosomes in muscles (Fig. 2C). Coexisting endosomal subpopulations in a single cell suggests that internalization of different cell-associated molecules can be independently controlled (Marks et al., 1997). COMM’s cytoplasmic domain has a putative Adaptin (AP-2) recognition site (Fig. 1A). The same sequence is frequently found in rapidly endocytosing, or ‘cargo receptor’, molecules such as transferrin receptor or

![Fig. 8. COMM internalization in prospero mutant. (A) COMM protein in 18 hour wild-type embryo. COMM has increased in endosomes (arrowheads). (B) In prospero mutant embryos of the same stage, motoneuron axon outgrowth is much delayed. COMM protein distribution pattern in the muscles (arrowheads) remains indistinguishable from wild type. This indicates that COMM endocytosis occurs independent of the contact with motoneuron axons. Scale bar, 10 μm.](image)

![Fig. 9. Two models of COMM’s role. (A) ‘Janitor’ model proposes a three-step mechanism that involves COMM endocytosis. Molecule ‘X’ symbolizes unidentified COMM activation/synapse inhibition factors. COMM’s main role is to quickly translocate, or ‘sweep away’, synapse inhibition factors from the target membrane in time to guarantee the compatibility between the growth cone and the target. (B) A simple ‘signaling’ model proposes a similar three step mechanism that does not involve COMM endocytosis. Molecule ‘Y’ symbolizes factor(s) that promote synaptogenesis. COMM’s main role is to mediate signal transductions across the muscle membrane that accommodate synaptogenesis. See text for details.](image)
M6P/insulin-like growth factor receptor (Kirchhausen et al., 1997; Marks et al., 1997). The site is thought to associate with Adaptn/Clathrin complex, a major promoter of endocytosis. When either the entire cytoplasmic domain or the portion that contains the Adaptn recognition site is missing (Fig. 1B,C), COMM does not internalize into the muscles (Figs 4C, 7E). Thus, it is possible that COMM’s internalization depends on its cytoplasmic domain being recognized by the Adaptn/Clathrin complex. A muscle cell, with its relatively large size, is an excellent model cell system in which such intracellular vesicle dynamics can be examined in detail.

What initiates COMM endocytosis at such a timely manner during the motoneuron-muscle interactions? The evidence points to activation of COMM’s extracellular domain. The cytoplasmically truncated COMM, when misexpressed on the muscle surface along side with endogenous COMM, prevents even the wild-type COMM from internalizing (Fig. 7E). We interpret this to mean that the outnumbered wild-type COMM is outcompeted by the truncated COMM for its binding partners and thus remains inactivated. We suggest that activation of COMM’s extracellular domain is an important step for initiating COMM internalization.

COMM’s extracellular binding partners are not known. Candidates include any cell surface molecules concurrently present on the muscles, component molecules of the extracellular matrix surrounding the muscles (Yoshihara et al., 1997), as well as hormonal and other diffusible molecules. Factors closely associated with motoneuron growth cones can be excluded from the list, since COMM endocytosis persists without muscles being contacted by the growth cones (Fig. 8B).

**COMM as a coordinator of synaptogenesis initiation**

Recent studies with the *Drosophila* nervous system have identified a number of synaptic target recognition molecules that provide either positive or negative cues to specific motoneuron growth cones (Chiba et al., 1995; Kose et al., 1997; Matthes et al., 1995; Mitchell et al., 1996; Nose et al., 1994, 1997; Rose et al., 1997; Winberg et al., 1998). However, how the postsynaptic muscle cells regulate both the cell specificity and timing for expressing these recognition molecules in timely manners remains poorly understood.

COMM’s role differs from those of the target recognition molecules such as Connectin, Fasciclin III, Semaphorin II, Netrin and Toll in the *Drosophila* neuromuscular system (Chiba and Rose, 1998; Kose et al., 1997; Matthes et al., 1995; Mitchell et al., 1996; Nose et al., 1997; Rose et al., 1997). Unlike these molecules, the role of COMM is not specific to a small set of muscles nor needed on the muscle surface during synaptogenesis. The role of COMM in coordinating synaptogenesis initiation in the neuromuscular system is a novel one, providing a hint for how such dynamic regulations may be accomplished.

**COMM endocytosis and synaptogenesis initiation**

The most significant conclusion from our genetic analysis is that there is a positive correlation between COMM endocytosis and synaptogenesis initiation in the embryonic neuromuscular system. Before synaptogenesis initiation, the COMM protein is expressed on the muscle surface. During this time, motoneuron growth cones that contact the muscles extend past them without stopping to innervate. This could be explained by some muscle-associated factors that inhibit synaptogenesis initiation by the growth cones. As the growth cones that are destined to innervate the muscles approach, COMM endocytosis reaches its peak.

We present two models for COMM’s role that are consistent with all the observations so far (Fig. 9). The first model, which we call the ‘janitor’ model, proposes that COMM endocytosis itself serves as a critical element of the membrane remodeling that accommodates synaptogenesis. The second model proposes that COMM’s key role is to mediate transmembrane signal transduction event.

The ‘janitor’ model proposes the following sequence of events (Fig. 9A). First, COMM’s extracellular domain binds to COMM activation factors, activating the endocytic pathway, which is likely mediated by the Adaptn/Clathrin complex (Fig. 9A step 1). The activation factors are independent of motoneuron growth cones, and it is possible that they also serve as synapse inhibition factors. Second, COMM acts as an endocytosing molecule. This endocytosis rapidly and specifically removes the synapse inhibition factors from the surface of the muscles (Fig. 9A step 2). Third, this remodeling of the muscle surface makes it compatible with synaptogenesis initiation (Fig. 9 step 3). It defines a time frame in which specific synaptic target recognitions take place. The motoneuron growth cones that arrive after COMM endocytosis can initiate synaptogenesis on appropriate muscles. The decision of individual growth cones to select specific target muscles among their neighbors and initiate synaptogenesis is made at most within a few hours after COMM endocytosis. COMM is thought to coordinate the general timing of synaptogenesis by ‘cleaning up’ the target cell surface and thereby facilitating target recognition between specific synaptic partner cells.

The ‘signaling’ model is of a more general nature. One simple scenario proposes the following (Fig. 9B). First, COMM binds to COMM activation factors (Fig. 9B step 1). Second, this leads to the transmembrane signal transduction whose exact nature remains open to speculation (Fig. 9B step 2). It could either take place locally within the cytoplasm (step 2a) or involve gene regulation (step 2b). Presumably, the distal portion of COMM’s cytoplasmic domain contains the critical functional domains, since the truncated COMM cannot promote synaptogenesis. Third, the result of the signal transduction is to resurface the muscle membrane so that it now becomes compatible for synaptogenesis (Fig. 9B step 3). This could be achieved by either insertion and/or activation of synaptogenesis promotion factors (as depicted in the diagram) or removal and/or inactivation of synaptogenesis inhibition factors.

The two models are not mutually exclusive. Each, however, makes specific predictions. On the one hand, the ‘janitor’ model predicts that the COMM-positive endosomes in muscles are enriched with molecules that inhibit synaptogenesis. It will be interesting to determine if any putative inhibitory muscle surface molecules (e.g. Toll, Semaphorin II and Netrins) are co-internalized into the COMM-positive endosomes (Matthes et al., 1995; Mitchell et al., 1996; Rose et al., 1997; Winberg et al., 1998). The model also predicts that blocking COMM’s endocytic activity during the period of motoneuron-muscle contact should be sufficient to stop its role. One version of the
"signaling" model, on the other hand, would predict that inhibiting either transcription or translation in the muscles during the same period prevents synaptogenesis.

A novel mechanism of rapid membrane surface remodeling

The principle in the ‘janitor’ hypothesis is a novel type of short-term microtransloaction of specific proteins. It is generalizable to regulation of cell-cell interactions in various contexts. The cell surface provides a platform on which diverse transmembrane cell signaling pathways converge and diverge. Evidence for dynamic changes in its cell surface molecular profiles exists for neurons especially during synaptic modulation (Bailey et al., 1997; Fredette et al., 1993). Similarly, during development neuronal growth cones are presumably capable of readjusting their responsiveness to specific cues based on previous exposure (Chiba and Keshishian, 1996). At least a part of the readjustment is thought to reflect rearrangements of surface receptors in the growth cones (Grabham and Goldberg, 1997). One frequently proposed mechanism involves protein internalization that ultimately leads to regulation of specific cell surface molecule genes. Examples include cases with Trk and Sevenless receptor tyrosine kinases (Cagan et al., 1992; Kramer and Phistry, 1996; Seeger et al., 1993). Such feedback loops, especially in a neuron with a long distance between its axon tip and cell body, require a lag time before the cell surface profile can be sufficiently remodelled. They may not be suitable when quick cell surface remodeling is called for to accommodate the next diversifying cell-cell interaction, as in axon development. Our analysis on the role of COMM during neuron-muscle interaction leads us to propose a simple alternative mechanism that also involves protein internalization. Its main significance, we propose, is rapid removal of specific molecules from the cell surface in a timely manner. This quick surface remodeling by a specific endocytosing molecule may serve as a fundamental mechanism for orchestrating synaptogenesis in a developing brain as well as for coordinating various other cell-cell recognition events in developing animals.

Conclusions

We have shown that initiation of neuromuscular synaptogenesis is positively correlated with COMM endocytosis in muscles. Our finding supports a mechanism in which a postsynaptic cell regulates cell surface compatibility prior to synaptogenesis initiation.

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