Neuromuscular target recognition by a homophilic interaction of Connectin cell adhesion molecules in *Drosophila*

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SUMMARY

*Drosophila* Connectin (CON) is a cell surface protein of the leucine-rich repeat family. During the formation of neuromuscular connectivity, CON is expressed on the surface of a subset of embryonic muscles and on the growth cones and axons of the motoneurons that innervate these muscles, including primarily SNa motoneurons and their synaptic targets (lateral muscles). In vitro, CON can mediate homophilic cell adhesion. In this study, we generated transgenic lines that ectopically expressed CON on all muscles. In the transformant embryos and larvae, SNa motoneurons often inappropriately innervated a neighboring non-target muscle (muscle 12) that ectopically expressed CON. Furthermore, the ectopic synapse formation was dependent on the endogenous CON expression on the SNa motoneurons. These results show that CON can function as an attractive and homophilic target recognition molecule in vivo.

Key words: neuromuscular connection, Connectin, *Drosophila*, target recognition, cell adhesion molecule

INTRODUCTION

During neural development, the basic pattern of synaptic connections is generated in a stepwise fashion by a series of growth cone recognitions (reviewed by Garrity and Zipursky, 1995; Keynes and Cook, 1995; Goodman, 1996). First, the growth cones traverse long distances along specific pathways towards their correct target region. They then recognize their appropriate targets and transform into synapses. Although growth cones are often confronted with a number of different cells in the target region, they find and select specific cells as synaptic partners (Garrity and Zipursky, 1995). One simple mechanism for the generation of the target specificity, as initially postulated by Sperry (1963), is differential adhesion, where the target selection is mediated by the recognition of distinct molecular labels on the target cells.

The neuromuscular system in *Drosophila* provides a simple model system for dissecting the cellular and molecular mechanisms of neuronal target recognition (reviewed by Bate and Broadie, 1995; Keshishian et al., 1996). In each abdominal hemisegment of *Drosophila* embryos and larvae, approx. 40 motoneurons innervate 30 individually identified muscles in a highly specific and stereotypic manner. Motoneurons extend their axons through five major peripheral nerves, called the intersegmental nerve (ISN) and segmental nerves a, b, c and d (SNa-d), to reach their target region. Once within the target region, the motoneuronal growth cones appear to possess the ability to choose their target(s) among a group of muscles within the filopodial reach. The high degree of precision and previous cellular manipulation of the target muscles (Sink and Whitington, 1991; Chiba et al., 1993) suggest the presence of recognition molecules that match specific motoneurons and their target muscles. This view is further supported by the identification of a number of secreted and surface molecules expressed on specific motoneuronal growth cones and/or muscles (reviewed by Bate and Broadie, 1995; Keshishian et al., 1996). Recent ectopic expression experiments implicated several of these molecules, including Semaphorin II, Fasciclin III (Fas III) and Connectin (CON), in motoneuronal guidance and target recognition (Nose et al., 1994; Chiba et al., 1995; Matthes et al., 1996).

*Drosophila* Connectin (Con) encodes a glycosylphosphatidylinositol (GPI)-linked cell surface protein (CON) of the leucine-rich repeat family (Nose et al., 1992; Gould and White, 1992). In vitro, CON can promote homophilic cell adhesion in transfected S2 cells (Nose et al., 1992; Meadows et al., 1994). During motoneuron guidance, CON protein is expressed on the surface of a subset of embryonic muscles and on the growth cones and axons of the motoneurons that innervate these muscles, including primarily SNa motoneurons and their targets, the lateral muscles. During synapse formation, CON protein becomes localized on SNa synaptic sites. The dynamic pattern of expression of CON on SNa motoneurons and on their target muscles, and its role as a homophilic cell adhesion molecule in vitro, strongly suggest that CON functions as an attractive target recognition molecule for SNa. Previous studies on *Con* loss-of-function and gain-of-function mutations, however, failed to show any evidence for an attractive function of CON during neuromuscular development (Nose et al., 1992, 1994). In the latter study (Nose et al., 1994), a heterologous enhancer from the Toll gene was used to ectopically express CON on a subset of ventral muscle fibers normally innervated...
by SNb and SNd motoneurons (Toll-connectin). The ectopic CON expression dramatically altered the trajectory and synaptogenesis of SNb, suggesting instead a repulsive function for CON during motoneuron growth cone guidance and synapse formation. On the other hand, no abnormality was found in SNa projection and innervation. The SNa growth cones were never observed to innervate inappropriately the ectopic CON-expressing ventral muscles. However, it was possible that these muscles are located too distantly from the SNa target region to be recognized as alternate targets.

To explore further the possible role of CON as an attractive signal for SNa, in the present study, we ectopically expressed CON on all muscles by using a Myosin heavy chain (Mhc) promoter. In the transformants, SNa motoneurons often projected to and formed synaptic endings on muscle 12, a non-target muscle that immediately flanks the target region. Furthermore, the ectopic synapse formation was dependent on the endogenous CON expression on the SNa motoneurons, suggesting that it was induced by a homophilic interaction of CON molecules on the motoneurons with ectopic CON on the muscle. These results provide evidence that CON can function as an attractive and homophilic target recognition molecule in vivo.

MATERIALS AND METHODS

Expression construct and germline transformation
An expression construct, pCaSper-Mhc'-con, containing the promoter region of the Myosin heavy chain gene (Mhc'), Con cDNA and the SV40 polyadenylation site in a pCaSper vector, was constructed in the following way. A 3.0-kb Smal-EcoRV fragment of Con cDNA containing the entire open reading frame was inserted into the EcoRV site of BLSC-Mhc' (Mhc', containing 456 bp upstream of the start of transcription, the entire 5' untranslated region in the first and second exon, and the first intron of the Mhc gene, in XbaI-EcoRV sites of Bluescript; Chiba et al., 1995) to make BLSC-Mhc'-con. A NotI-KpnI Mhc'-con fragment was then inserted into the NotI-KpnI sites of pCaSper2/17 (Nose et al., 1994), 5' to the SV40 polyadenylation site, to form pCaSper-Mhc'-con.

The pCaSper-Mhc'-con construct was introduced into w1118; Dr/TM3, Δ2-3 embryos by P-element transformation (Spradling and Rubin, 1982). After crossing the Δ2-3 chromosome, over 20 transformant lines were established and examined immunohistochemically for ectopic CON expression. MC4, with an insert on the second chromosome, and MC12 and MC50, each with an insert on the third chromosome, which showed high levels of ectopic CON expression, were used as homozygotes for further study.

Genetics
ConFve238 is a viable loss-of-function Con mutation, and expresses about 5% of the normal level of CON mRNA that contains the intact protein coding region (Nose et al., 1994). Df[3L]10H1 (64B10-12; 64C5-7) uncovers the Con gene. MC4; ConFve238 and MC4; Df[3L]10H1 / TM6, Tb lines were established by standard genetic crosses and the validity of the genotype was confirmed by anti-CON staining. MC4; ConFve238/Df[3L]10H1 larvae were obtained by crossing MC4; ConFve238 and MC4; Df[3L]10H1 / TM6, Tb adults.

Fasciclin II and Fasciclin III misexpression
To direct increased levels of Fasciclin II (Fas II) expression on all muscles, transgenic flies carrying a UAS-fas II transgene on the second chromosome (Lin and Goodman, 1994, obtained from Goodman's laboratory) were crossed to a GAL4 enhancer trap line 24B (Brand and Perrimon, 1993; Luo et al., 1994). Fas II protein expression in the progeny 24B-fas II starts in the mesoderm from embryonic stage 11 and continues throughout the larval life.

The degree of muscle adhesion in 24B-fas II as compared to Toll-connectin was estimated in the following way. Since ectopic CON expression decreased towards the end of embryogenesis in Toll-connectin, direct comparison of muscle adhesion at the larval stage was impossible. We therefore first compared the degree of muscle adhesion in 24B-fas II to that in Mhc'-con at late embryonic or third instar larval stage. 24B-fas II displayed a similar or somewhat stronger level of muscle adhesion as compared to Mhc'-con. We then studied the relative level and timing of CON or Fas II expression in Toll-connectin, Mhc'-con and 24B-fas II embryos. The level of CON expression on muscles in Toll-connectin stage 14-16 embryos compares to that in Mhc'-con stage 16-17 embryos roughly from 0.5 to 1.0. In 24B-fas II embryos, the peak level of ectopic Fas II expression was reached very early during muscle development (by stage 13) and maintained throughout the embryonic and larval period. From these observations, we reasoned that the degree of muscle adhesion during stage 15 (at which time SNb axons begin to enter the ventral muscle field) in 24B-fas II embryos is comparable to or stronger than that in Toll-connectin.

A transgenic line carrying a Mhc'-fasciclin III transgene on the second chromosome (Chiba et al., 1995) was obtained from A. Chiba. In Mhc'-fasciclin III, Fasciclin III (Fas III) protein is expressed by all muscles from embryonic stage 15 and throughout larval life.

Immunocytochemistry
Embryos and larvae were dissected as previously described (Nose et al., 1992, 1994; Patel, 1994). Dissected embryos and larvae were stained with the following antibodies by standard protocols: SAb against CON (Nose et al., 1992), mAb 22C10 (Fujita et al., 1982) and mAb 1D4 against Fas II (Van Vactor et al., 1993). Intracellular dye filling was performed as described previously (Nose et al., 1994).

RESULTS

Ectopic expression of Connectin by the Mhc promoter
We used the promoter region from the muscle-specific Myosin heavy chain (Mhc) gene to ectopically express CON on all muscles in the P-element-mediated transformants. A 2.5-kb fragment in the 5' portion of the Mhc gene (Mhc') was previously shown to be sufficient for muscle-specific expression (Hess et al., 1989). We used a modified version of this fragment, which was previously used to misexpress Fas III (Chiba et al., 1995), to ectopically express CON on all muscles. A Con cDNA containing the entire open reading frame was transcriptionally fused to the Mhc promoter, and was inserted into a P-element vector, Casper 2/17 (for details, see Materials and methods). Over 20 independent Mhc'-con transformant lines were isolated, three of which (MC4, MC12 and MC50) were analyzed in this study.

As expected, CON protein was ectopically expressed on the surface of all muscles in Mhc'-con embryos (Fig. 1). The ectopic expression started at stage 15, when SNa motoneurons normally reach their target region and elaborate filopodia processes over lateral muscles, reached its highest level by mid-stage 16, and continued throughout the larval life. The level of ectopic CON protein expression in the Mhc'-con lines was approximately two to three times higher than the en-
dogenous CON expression on the lateral muscles 21-24, thus making all muscle fibers equivalent in terms of CON expression.

Ectopic CON expression did not result in gross developmental defects of the CNS, PNS and musculature of the Mhc'-con embryos or larvae. Major motor nerves projected normally in the CNS and in the periphery. All muscles formed in their correct locations with normal insertion sites. However, close examination of the Mhc'-con embryos and larvae showed that SNa motoneurons often inappropriately innervated a neighboring non-target muscle, muscle 12. As will be described later, we also noted increased muscle-muscle adhesion in subsets of ventral muscles, including muscles 6 and 7. With the exception of the synapse formation on muscles 6 and 7, the

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**Fig. 1.** Ectopic Connectin expression on all muscles in Mhc'-con transgenic embryos. Two abdominal hemisegments of stage 16 wild type (A) and Mhc'-con (B) embryos stained with anti-CON SAb and immunocytochemistry. In this and the following figures (Figs 1-5), anterior is to the left and dorsal is up. (A) In wild-type embryos, CON is expressed on lateral muscles 21-24 (white arrow), ventral muscle 27 (black arrow) and on the motor axons innervating these muscles (white arrowheads, slightly out of focus). Note the lack of staining on muscle 12. (B) In Mhc'-con transgenic embryos, CON was expressed at a high level on all somatic muscles including muscle 12. Scale bar, 20 μm.

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**Fig. 2.** Neuromuscular connectivity and expression of Connectin in wild type and Mhc'-con. Schematic diagrams of the muscles, motoneuron projections and synaptic endings in each abdominal hemisegment of Drosophila embryos in relation to CON expression. Only the lateral portion of the body wall (boxed in A) is shown in B-D, focusing on the SNa target region. (A) Wild-type pattern of muscles and motor nerves. CON is expressed on the surface of subsets of muscles (dark gray), as well as on the axons (black lines) and synaptic terminals (black circles) of the motoneurons that innervate these muscles. CON-negative muscles are shown in lighter gray, and CON-negative motoneurons by white lines and circles. (B) Enlarged view of SNa projection and synaptogenesis, and the relevant muscles in the lateral region of the body wall in wild-type embryos. The SNa projects to the lateral region of the body wall and then bifurcates at a position (arrow) distal to muscle 12. CON is expressed on the axons and synapses of SNa motoneurons and on their target muscles, with the exception of muscle 8. Muscle 12 that immediately flanks the SNa target region does not express CON and is innervated by the SNb, a CON-negative nerve. (C) In Mhc'-con embryos, CON was ectopically expressed on all muscles (dark gray). An ectopic axon branch diverged from the SNa at or near the bifurcation point and projected to muscle 12 (arrow). The innervation of the normal SNa target muscles was normal, as well as the formation of the native endings on muscle 12 by the SNb. (D) In Mhc'-con; Con-embryos, CON expression on SNa is absent because of the lack of the endogenous Con gene, while ectopic CON expression on all muscles, driven by the transgene, is present. In this situation, no ectopic innervation was seen, indicating that the formation of the ectopic synapse is dependent on the CON expression on the SNa.
increase in muscle adhesion had no effects on motoneuron projection and synaptogenesis.

**Ectopic synapse formation by SNa motoneurons in Mhc<sup>-con</sup>**

During normal development of the neuromuscular system, the SNa axons exit the CNS in the segmental nerve root and form a distinct fascicle that projects to the lateral region of the body wall by stage 15. The SNa then bifurcates at the distal edge of muscle 12; one branch innervates lateral transverse muscles 21-24 and another extends posteriorly and innervates muscles 5 and 8 by the end of embryogenesis (Fig. 2A,B, Fig. 4A). This pattern of SNa branching and innervation persists throughout larval life (see Fig. 3A). The axons and growth cones in the SNa express CON, as they project towards and arborize on the lateral muscles 5 and 21-24 that also express CON (Fig. 1A, Fig. 2A,B; Nose et al., 1992). During synaptogenesis, strong CON expression can be seen localized on the synaptic sites of these lateral muscles. CON is also expressed on ventral muscles 27 and 29 and on dorso-lateral muscle 18, as well as on the axons in the SNc and ISN that innervate these muscles (Fig. 2A). On the other hand, muscle 12, which immediately flanks the SNa target region, and more ventral muscles, i.e. 6, 7, 12, 14, 28 and 30, do not express CON and are innervated by the SNb motoneurons, which do not express CON (Fig. 2A; see also Fig. 6A).

To study the effects of ectopic CON expression on SNa targeting, we first examined the anatomy of the neuromusculature in the third instar larvae. The body wall of dissected fillets of Mhc<sup>-con</sup> third instar larvae were stained with mAb 22C10 to visualize motoneuron projections and synaptic endings. In these larvae, we observed an axon branch emanating from the SNa that formed an ectopic nerve ending on muscle 12 in 45-63% of segments, depending upon which line was examined (Fig. 2C, Fig. 3B-D, summarized in Table 1). Most of the ectopic axon branches (39/50 cases in the MC4 line) diverged from the SNa at or shortly proximal to the SNa bifurcation point, and turned back to innervate muscle 12 (Fig. 3B,C). The ectopic axon was also occasionally (11/50 cases) observed to diverge at a position posterior to the bifurcation point, in the terminal branch projecting to muscles 5 and 8 (Fig. 3D). In either case, the ectopically placed nerve endings were mostly seen in the posterior region of muscle 12.

The formation of the ectopic axon branch innervating muscle 12 was not accompanied by a denervation of muscles normally innervated by SNa; synapses normally formed on muscles 5, 8 and 21-24 in Mhc<sup>-con</sup>. Thus, the ectopic axon branch projecting to muscle 12 is likely to be formed by collateral sprouting of SNa axons. Such expansion of the motoneuronal terminal arbor has also previously been reported to occur when the target muscles were duplicated (Chiba et al., 1993).

It is known that in the absence of the native innervation, a muscle can induce other motoneurons to form ectopic branches (Keshishian et al., 1994; Halfon et al., 1995). However, the

**Fig. 3.** Ectopic SNa synaptic endings on muscle 12 in Mhc<sup>-con</sup> transgenic larvae. Body wall fillet preparations of wild-type (A) and Mhc<sup>-con</sup> (B-D) third instar larvae, stained with mAb 22C10 to visualize motoneuron projection and synaptic endings. In wild-type larvae (A), SNa motor nerve projects to the lateral region of the body wall distal to ventral muscle 12, then bifurcates to extend one branch distally to muscles 22-24 and another posteriorly to muscles 5 and 8. The SNa never innervate muscle 12 that immediately flanks the target region. In contrast, in Mhc<sup>-con</sup> larvae (B-D), the SNa extended an axonal branch that formed a synaptic ending on muscle 12 (large arrows). Despite the presence of the ectopic endings, formation of the native synapses on muscle 12 by the SNb motoneurons was largely normal (small arrows in B and C, out of focus in D). Arrowheads indicate the SNa bifurcation points. Scale bars, 140 μm (A,B), 200 μm (C,D).

**Table 1. Formation of the ectopic synaptic ending on muscle 12 by SNa motoneurons in Mhc<sup>-con</sup> larvae, and its dependence on the endogenous CON expression**

<table>
<thead>
<tr>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ectopic CON expression&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Endogenous CON expression</th>
<th>% Ectopic endings&lt;sup&gt;c&lt;/sup&gt; (n)</th>
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<tr>
<td>w1118</td>
<td>–</td>
<td>+</td>
<td>0 (83)</td>
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<tr>
<td>MC50</td>
<td>++</td>
<td>+</td>
<td>45 (53)</td>
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<tr>
<td>MC12</td>
<td>++</td>
<td>+</td>
<td>54 (37)</td>
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<tr>
<td>MC4</td>
<td>++</td>
<td>+</td>
<td>63 (80)</td>
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<tr>
<td>MC4; Con&lt;sup&gt;rev238&lt;/sup&gt;</td>
<td>++</td>
<td>–</td>
<td>1 (83)</td>
</tr>
<tr>
<td>MC4; Con&lt;sup&gt;rev238&lt;/sup&gt;/Df(3L)&lt;sup&gt;10H&lt;/sup&gt;</td>
<td>++</td>
<td>–</td>
<td>2 (93)</td>
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<sup>a</sup>For details on the transgenic status and background genotype, see Materials and methods.
<sup>b</sup>Visual estimate of the relative level of ectopic CON expression on muscle 12 as compared with the endogenous CON expression on lateral muscles 21-24.
<sup>c</sup>In Con<sup>rev238</sup> and Con<sup>rev238</sup>/Df(3L)<sup>10H</sup> genetic backgrounds, the endogenous CON is expressed at 2-5% of the normal level.
ectopic innervation on muscle 12 observed in Mhc<sup>-</sup>-con is unlikely to be caused by such mechanism, because formation of the native endings on muscle 12 by SNb motoneurons appeared largely normal in Mhc<sup>-</sup>-con, regardless of the presence of the ectopic innervation (see Fig. 3B,C). The native endings were found at their normal position and had their processes in the normal orientations in the anterior region of muscle 12.

In Mhc<sup>-</sup>-con, ectopic CON expression started at the late embryonic period and persisted throughout the larval period. To study if the ectopic endings on muscle 12 are induced during the embryonic period when the neuromuscular connectivity is initially formed or later during the larval period, we then analyzed the SNa morphology in stage 17 Mhc<sup>-</sup>-con embryos with mAb 1D4 (anti-Fas II). In these embryos, SNa axons often (53%, 51/96 segments in MC4) projected to and arborized on muscle 12 (Fig. 4B-D). Such SNa ectopic arborizations on muscle 12 were rarely seen in wild-type embryos (1%, 1/102 segments in w<sup>1118</sup>). These results suggest that the ectopic innervation of muscle 12 occurred during the late embryonic period when CON is normally expressed on SNa motoneurons and on the target muscles.

**Formation of the ectopic innervation is dependent on the Connectin expression on SNa**

The results described above showed that CON expression on muscle 12 is sufficient to elicit the synapse formation by SNa motoneurons. As CON is also expressed on the SNa, the formation of the ectopic synapse is most likely to be induced by a homophilic interaction between CON molecules on the motoneurons and CON on the muscle. To test this hypothesis, we introduced the Mhc<sup>-</sup>-con transgene into a Con mutant background that retained only 2-5% of the normal level of CON (Mhc<sup>-</sup>-con ; Con). In this genetic situation, CON expression on the SNa (driven by the endogenous gene) is nearly absent, while ectopic CON expression on muscles (driven by the transgene) is present (see Fig. 2D). In Mhc<sup>-</sup>-con; Con<sup>-</sup> larvae, ectopic innervation of muscle 12 was only rarely observed, indicating that CON expression on SNa is necessary for the formation of the ectopic synapse (Table I). These results are most consistent with the idea that the ectopic synapse formation is mediated by the recognition of ectopic CON on muscle 12 by CON molecules on the SNa motoneurons.

Previous analysis of the Con loss-of-function mutations revealed no gross abnormality in the guidance and synaptogenesis of the SNa, suggesting that the function of CON for SNa development at least in part overlaps with the function of other gene product during normal development (Nose et al., 1992, 1994; see Discussion). Like in Con<sup>-</sup>mutants without the transgene, in Mhc<sup>-</sup>-con ; Con<sup>-</sup> the innervation of the native SNa target muscles was normal.

**Increased muscle adhesion by ectopic Connectin**

We noted an increase in muscle-muscle adhesion in certain pairs of ventral muscle fibers in Mhc<sup>-</sup>-con larvae. The increase in muscle adhesion was most apparent in the larval dissections that were pulled slightly towards the dorsal midline when flattened. In such preparations, there are normally gaps between adjacent ventral longitudinal muscles (Fig. 5A). However, in Mhc<sup>-</sup>-con larvae, extensive muscle-muscle contact was seen along the entire length of the apposing muscles (Fig. 5B). Such increase in muscle-muscle adhesion appeared to be confined to specific pairs of ventral muscles that form in close proximity, run in parallel and insert to neighboring epidermal insertion sites (between muscles 6 and 7, 12 and 13, and 15 and 16).

Concomitant with the increase in muscle adhesion, we also observed abnormalities in the innervation of muscles 6 and 7. These muscles are normally jointly innervated by the SNb motoneurons (including RP3) that project internally through the cleft between these muscles and form synapses on their internal surfaces (Fig. 5A, Fig. 6A). However in Mhc<sup>-</sup>-con, the synaptic terminals were often missing on the internal surfaces or were greatly reduced in size (Fig. 5B). Instead the SNb motoneurons were often seen to innervate these muscles on their external surface (Fig. 5B, inset). It is currently unknown whether the synapses are formed by the motoneurons that normally innervate muscles 6 and 7, or by other motoneurons.

![Fig. 4. SNa projection in Mhc<sup>-</sup>-con embryos. Fillets of stage-17 wild-type (A) and Mhc<sup>-</sup>-con (B-D) embryos stained with mAb 1D4 and immunocytochemistry. (A) Normal pattern of SNa projection and branching (arrow). As in the third instar larvae, two branches of SNa, one projecting to the lateral transverse muscles and another projecting to muscles 5 and 8, are seen. (B-D) In Mhc<sup>-</sup>-con embryos, SNa axons are seen to project to and arborize on muscle 12 (arrows). In B and C, in addition to the two normal axon branches, an ectopic SNa axon branch that forms a nerve ending on muscle 12 can be seen. In D, an axon branch arborizes on muscle 12 and then further projects toward muscles 5 and 8. Scale bar, 20 μm.](image-url)
In any case, these observations suggest that the increased muscle adhesion between muscles 6 and 7 interferes with the normal development of the synapses (see Fig. 6D).

We previously reported that ectopic expression of CON on subsets of ventral muscles, including muscles 6 and 7, in Toll-Connectin embryos, changed the morphology and trajectories of the SNb motoneurons (Nose et al., 1994). First, during the early phase of SNb pathfinding (stage 15-16, see Fig. 6A), ectopic CON prevented the entrance of the SNb growth cones towards the target region (Fig. 6B). Second, later during development, ectopic CON on muscles 6 and 7 prevented the formation of synapses by RP3 motoneurons. In these studies, the increase in muscle adhesion was not noticed due to the small size of muscles in the embryos. In Toll-Connectin, the ectopic expression decreased towards the end of the embryogenesis and the morphology of the muscles appeared normal in the third instar larvae. The results were thus interpreted to suggest a repulsive function of CON during SNb pathfinding and synaptogenesis. However, the observations made in our current study raise the possibility that the phenotypes were instead caused by increased muscle-muscle adhesion.

To test whether the alterations in SNb guidance and synaptogenesis in Toll-Connectin and Mhc-con were due to a specific effect of CON or were in fact caused by an indirect influence of increased muscle adhesion, we examined the morphology of SNb motoneurons in the embryos and third instar larvae that expressed Fasciclin II (Fas II) and Fasciclin III (Fas III) at a high level on all muscles (24B-fas II, Lin and Goodman 1994; Mhc-fas III, Chiba et al. 1995; see Materials and methods for details). Both Fas II and Fas III can function as a homophilic cell adhesion molecule in vitro (Snow et al., 1989; Grenningloh et al., 1990), and promote muscle-muscle adhesion in vivo when ectopically expressed at a high level (see below). We reasoned that if the same SNb phenotype is observed by ectopic expression of Fas II or Fas III, it is likely to be caused by non-specific effects of muscle adhesion, but that, on the other hand, if the phenotype is only seen in response to the ectopic CON, it would be the consequence of the specific function of CON.

We first examined the effect of increased muscle adhesion on SNb synaptogenesis, by examining the 24B-fas II and Mhc-fas III third instar larvae. As in Mhc-con larvae, we observed extensive muscle-muscle adhesion between subsets of ventral muscles in these larvae (Fig. 5C,D). We also observed similar abnormalities in the innervation of muscles 6 and 7 to those seen in Mhc-con (Fig. 5C,D). Although the SNb axons reached the target region normally (see below, see also Chiba et al., 1995), the terminal arbors on the internal surface of muscles 6 and 7 were greatly reduced (Fig. 5C,D). These results further support the notion that increased muscle adhesion can interfere with the normal development of SNb synapses on muscles 6 and 7 in a non-specific manner.

We then studied the potential effect of muscle adhesion on the early phase of SNb guidance at the entry point to the ventral muscles, by examining the trajectories of SNb axons in 24B-fas II embryos that expressed Fas II on all muscles from an...
Fig. 6. Increased muscle adhesion and the development of the SNb in response to ectopic Connectin, Fas III and Fas II. (A) Cross-sectional schematic diagrams showing the development of SNb in wild-type embryos. SNb target muscles are shown in light gray. Interior side of the embryo is to the left, dorsal is up. At stage 15, SNb growth cones leave the ISN and begin to enter the ventral muscle region at the choice point near muscle 28 (thick arrow). They then extend along the inside of muscles 14 and 30 during stage 16. By stage 17, the mature pattern of innervation, which persists throughout larval life, can be seen. RP3 motoneuron innervates muscles 6 and 7 at the cleft between these muscles, and RP1 innervates muscle 13. (B) In Toll-connectin embryos, CON was expressed on a subset of ventral muscles (dark gray) during the early phase of SNb development (stage 15-16). RP1, RP3 and other SNb growth cones often failed to enter the ventral muscle region (Nose et al., 1994). They instead either extended along the ISN to the dorsal region (bypass), or took a detour around the ventral muscles to reach the target region (detour). (C) In contrast with ectopic CON, ectopic Fas II (shaded) in 24B-fas II did not alter the trajectories of the SNb growth cones. This diagram shows the results of intracellular dye fills of RP1 (n=7) and RP3 (n=6) motoneurons. In all cases, the SNb motoneurons entered the ventral muscle field normally and projected towards the correct target region. These results point to the specificity in the action of ectopic CON during SNb pathfinding. (D) In Mhc' -con larvae, some of the ventral muscles displayed extensive muscle-muscle contact (between 6 and 7, 12 and 13, and 15 and 16). The innervation of muscles 6 and 7 was also abnormal. The SNb axons failed to extend through the cleft and arborize on the internal surfaces of these muscles. They instead formed synapses on the external surfaces. A similar phenotype was seen in the larvae that ectopically expressed Fas II (24B-fas II) or Fas III (Mhc' -fas III).

DISCUSSION

Connectin as a homophilic and attractive target recognition molecule

The ability of growth cones to respond to molecular guidance cues in their microenvironment is a fundamental feature of the formation of neural connectivity. We showed in this study that Drosophila CON can function both as a target recognition molecule on the surface of a subset of muscles, and as a receptor on the motoneuronal growth cones, to mediate the generation of neuromuscular specificity.

We used a muscle-specific Myosin heavy chain promoter to generate transgenic lines that expressed CON ectopically on all muscles, when SNa motoneurons normally initiate contact with their target muscles bearing CON on their surface. In the transformants, SNa axons were observed to innervate muscle 12, a non-target muscle that immediately flanks the target region. The results showed that ectopic CON expression is sufficient to transform muscle 12 into a synaptic target of SNa motoneurons, thus indicating a role of CON as an attractive target recognition molecule. Since CON is also expressed on the growth cones of SNa motoneurons, and can function as a homophilic cell adhesion molecule in vitro, the ectopic synapse formation was most likely to be mediated by the homophilic interaction between CON on the SNa growth cones and CON on muscle 12. To test the validity of this prediction, we generated a situation in which CON was expressed on muscle 12 but not on the SNa motoneurons by introducing the Mhc' -con transgene into a Con' genetic background. SNa axons were rarely observed to innervate muscle 12 in this
situation, indicating that CON expression on SNa axons is necessary for the formation of the ectopic synapses. Taken together, these results provide evidence that CON can function as an attractive and homophilic target recognition molecule during the formation of neuromuscular synapses.

Several other features of these results further support this conclusion. First, the ectopic synapse formation on muscle 12 by SNa motoneurons was observed in several independent Mhc'-con lines examined. Second, except for the increased adhesion of some ventral muscle fibers, all other aspects of muscle differentiation appeared to be normal in Mhc'-con embryos and larvae. The ectopic CON expression thus does not appear to affect general properties of muscle differentiation or specification. Third, the overall organization of motor nerves appeared unchanged in Mhc'-con. Importantly, the formation and morphology of the native SNb synaptic endings on muscle 12 appeared normal, arguing against a possibility that the formation of the ectopic innervation was elicited by the absence of the native endings. Finally and most importantly, the ectopic innervation on muscle 12 was not observed in the case of a Con'-background, even when CON was ectopically expressed on all muscles. If the ectopic synapse formation was caused by some change in the general properties of muscles themselves, such as increased adhesion, we should have seen the same phenotype in this genetic background, but we did not. These results are most consistent with the idea that direct interaction of CON protein on muscle 12 and CON on SNa growth cones elicited the formation of the ectopic synapses.

Repulsive function of Connectin

Previous analysis of Toll-connectin embryos (Nose et al., 1994) revealed SNb phenotypes that suggested a repulsive function of CON during SNb growth cone guidance and synaptogenesis. The present study, however, showed that the SNb phenotype during synaptogenesis (but not during pathfinding) was more likely to be caused by the increased muscle adhesion and not by a repulsive effect of CON.

We observed an increase in muscle–muscle contact in some ventral muscles in Mhc'-con larvae. Although CON protein was expressed on all muscle fibers in Mhc'-con, we saw the phenotype only in certain pairs of ventral muscle fibers that normally form in close proximity, such as muscles 6 and 7. Even in normal embryos and larvae, these muscles show extensive muscle–muscle contact, and the ectopic expression of CON appeared to increase further the degree of the muscle adhesion. This increase in muscle adhesion appears to be a general consequence of increased expression of cell adhesion molecules, since similar phenotypes were seen in the larvae that ectopically expressed Fas II or Fas III on all muscles. These results suggest that increased muscle adhesion can interfere with the normal development of the synapses on muscles 6 and 7 in a general manner, and that the SNb phenotype seen previously in Toll-connectin embryos was also caused, at least in part, by the increased muscle–muscle adhesion, and not by a repulsive effect of CON. It should, however, be noted that recent electron microscopic analysis showed that initial embryonic innervation on muscles 6 and 7 forms on their outer surface, and movement into the cleft and then on the inner surface is only secondary to this (Schuster et al., 1996). It is thus unclear at present if and how the increase in muscle–muscle adhesion can interfere with this early phase of synaptogenesis.

We also reexamined the repulsive function of CON during SNb pathfinding, by studying the possible effect of muscle adhesion on SNb growth cone guidance at the entrance to the ventral muscle fields. Since we were unable to determine by morphological studies if the ectopic expression of CON or other cell adhesion molecules at this stage increases adhesion between ventral muscles in such a manner as to prevent the entry of SNb axons, we tested these possibilities by examining the behavior of SNb growth cones in response to ectopic Fas II. The SNb axons extended to the ventral muscle region normally, pointing to the specificity in the effect of the ectopic CON. The results thus support the conclusion made in the previous study that CON can function as a repulsive signal during SNb growth cone guidance. However, establishment of the role of CON as a repulsive guidance molecule would require future identification of the receptor molecule(s) on SNb axons.

The role of Connectin during normal development of neuromuscular connectivity

Although the analysis of gain-of-function phenotype of CON presented in this study clearly shows that CON can function as an SNa-targeting molecule, the analysis of the loss-of-function mutations reported in previous studies failed to reveal any gross abnormality in SNa guidance and targeting (Nose et al., 1992, 1994). Similar conclusions were reached in previous studies on the in vivo function of several other putative neuronal recognition molecules in the same system (e.g. Fas II, Lin et al., 1994; Fas III, Chiba et al., 1995; Semaphorin II, Matthes et al., 1995). The lack of clear phenotypes in the loss-of-function mutations was previously interpreted as suggesting that these molecular guidance cues function in a partially overlapping and context-dependent manner during the formation of neuromuscular connectivity. Several observations made in this and other studies suggest that CON functions in a similar manner as an attractive cue for SNa targeting.

While CON was expressed on the surface of all muscles in Mhc'-con, the ectopic synapse formation by SNa was confined to muscle 12, to one immediately flanking the SNa target muscles. Thus, ectopic expression of CON does not transform all muscles into acceptable targets for the SNa motoneurons. Other factors, such as proximity to the normal target region, appear to restrict the action of ectopic CON during SNa targeting. This could explain why we did not see any evidence for an attractive function of CON in a previous study in which CON was ectopically expressed on ventralmost muscle fibers (Nose et al., 1994). A similar restriction in alternate target
selection was previously observed when one of the SNa targets, muscle 5, was deleted in certain mutants or by laser ablation (Cash et al., 1992). In those experiments, the SNa axon(s) that normally projects to the missing muscle instead formed synaptic endings on neighboring muscles. Such ectopic endings were mostly found on muscle 12. These results suggest the presence of signals, possibly along the pathway, that determine the location and timing of SNa synaptogenesis.

The experiments by Cash et al. (1992) also showed that the SNa axons that normally innervate muscle 5 can reach the lateral region of the body wall in the absence of their targets. In an independent work, we also observed that even when most or all of the lateral muscles are absent as in the apterous (Bourgouin et al., 1993) or muscle segment homeobox (msh; Robert et al., 1989; T. Isshiki and A. N., unpublished data) mutants, SNa axons can still extend to the normal target region (A. N., unpublished observation). These results suggest that cues other than those coming from the target muscles can guide SNa growth cones towards the target region, and these guidance mechanisms alone may be able to determine to a large extent the location of the synaptic endings. On the other hand, much evidence suggests that motoneuronal growth cones actively seek for cues expressed on the surface of the target muscles (Sink and Whittington, 1991; Chiba et al., 1993). It thus appears that, during normal development, multiple cues both along the pathway and on the target muscles function in a partially overlapping manner to generate the target specificity. We propose that CON functions as part of a complex network of guidance cues that determines the neuromuscular specificity.

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