INTRODUCTION

The final step of axon guidance is marked by mutual recognition between a growth cone and its synaptic target. This synaptic target recognition triggers a rapid reorganization of cytoskeletal and vesicular transport systems within a growth cone, converting it into a presynaptic terminal specialized for regulative neurotransmission (Haydon and Drapeau, 1995; Igarashi et al., 1997). Large arrays of molecules guide growth cones through their complex pathways and many of them are thought to also contribute to synaptic target recognition (Chiba and Keshishian, 1996; Garrity and Zipursky, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). However, less well understood are the molecules that serve as growth cone receptors for target recognition, as well as the mechanisms that promote cytoskeletal and vesicular reorganization in the growth cones.

A simple hypothesis, derived from Roger Sperry’s ‘chemoaffinity theory’ (Sperry, 1963), is that the first step of presynaptic differentiation is mediated by homophilic cell adhesion molecules shared by synaptic partner cells at the time of contact. A series of studies on Connectin, a cell-attached molecule containing leucine-rich repeats, provided the first in vivo evidence for the homophilic synaptic target recognition hypothesis (Nose et al., 1992, 1994, 1997). Large arrays of molecules guide growth cones through their complex pathways and many of them are thought to also contribute to synaptic target recognition (Chiba and Keshishian, 1996; Garrity and Zipursky, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996). However, less well understood are the molecules that serve as growth cone receptors for target recognition, as well as the mechanisms that promote cytoskeletal and vesicular reorganization in the growth cones.

SUMMARY

We demonstrate that the cell adhesion molecule Fasciclin III (FAS3) mediates synaptic target recognition through homophilic interaction. FAS3 is expressed by the RP3 motoneuron and its target muscles during synaptic target recognition. The RP3 growth cone can form synapses on muscles that ectopically express FAS3. This mistargeting is dependent on FAS3 expression in the motoneurons. In addition, when the FAS3-negative aCC and SNa motoneuron growth cones ectopically express FAS3, they gain the ability to recognize FAS3-expressing muscles as alternative targets. We propose that homophilic synaptic target recognition serves as a basic mechanism of neural network formation.

Key words: cell adhesion, chemoaffinity, Fasciclin III, growth cone, homophilic, Ig-CAM, synaptogenesis, target recognition, Drosophila

MATERIALS AND METHODS

Drosophila stocks

Wild type

Both Canton S strain and white strain, the genetic background for all the transgenic lines used, served as 'wild-type' controls. Fig. IA shows normal FAS3 expression patterns.
No expression of FAS3
FAS3 null mutant (FAS3E25; Elkins et al., 1990) was used to examine growth cone targeting in FAS3-free environment (Fig. 1B).

Muscle misexpression of FAS3
We established new lines of muscle FAS3 misexpressor transgenic flies based on one of the previously generated MF lines (Chiba et al., 1995). These consist of the promoter from the muscle-specific Muscle myosin heavy chain gene (Mhc) (Wassenberg et al., 1987) and FAS3 coding sequence. After random mobilization of the original MF transgene to new locations, five homozygous viable lines were established. They carry the transgene on either the first chromosome (MF10), the second chromosome (MF29 and MF42), or the third chromosome (MF27 and MF50). These lines all showed very similar FAS3 misexpression patterns and normal development of embryonic CNS and muscles (Fig. 1C). They also served as controls for each other for potential insertional mutagenesis and positional effects of transgene insertions. Despite FAS3 misexpression, muscles retain normal expression patterns of other cell surface molecules such as Toll, Connectin and Fasciclin II (not shown).

Muscle misexpression but no neuron expression of FAS3
Genetic crosses between FAS3 null and FAS3 muscle misexpressor (FAS3E25; MF), homozygous viable lines, show no FAS3 expression other than in muscles (Fig. 1D).

Neuron misexpression of FAS3
A newly generated, homozygous viable ’neuron misexpressor’ line (HFET) carries a transgene (insertion on the third chromosome) consisting of a heat-shock promoter (hsp70) and FAS3 coding sequence. The transgene expression is under the control of an enhancer element which, without heat shock, causes ectopic expression of FAS3 early in aCC and a few other neurons (Fig. 1E), as well as SNa motoneurons late in development (Fig. 1F). Neuronal misexpression of FAS3 is down-regulated by third larval stage. The line is also capable of overexpressing FAS3 in all tissues upon heat shock. Analyses of the HFET line were done in a wild-type genetic background. Note, however, in Fig. 1E,F the HFET line was crossed to FAS3 null background (FAS3E25; HFET) in order to show unambiguously the pattern of FAS3 misexpression.

Overexpression of FAS3
Heat-shock-induced overexpression was used to supplement FAS3 misexpression in some experiments. Heat-shock treatment consisted of a 30 minute 37°C pulse from the base line temperature of 22-23°C. Embryos on a juice-agar collection plate were sealed with paraffin and immersed in a 37°C water bath. Activation of the heat-shock promoter (hsp70) induced overexpression of FAS3 in all tissues within 2 hours.

Growth cone visualization
Dye injection
Lucifer yellow (Molecular Probe, Eugene) injection was done according to the method described (Halpern et al., 1991; Chiba et al., 1993). The dye-filled growth cones were visualized using a cooled CCD camera as described (Rose et al., 1997).

Definition of ‘synaptic target recognition’
Neuronal growth cones in Drosophila embryos exhibits characteristic morphological changes during initiation of synaptogenesis, herein referred to collectively as synaptic target recognition (Halpern et al., 1991; Sink and Whitington, 1991a,b). The changes apparent at the light microscopic level are well correlated with electrophysiological onset of synaptic transmission (Broadie and Bate, 1993a,b) and have been used to assess the state of growth cone differentiation in these motoneurons (Nose et al., 1994; Kopczynski et al., 1996; Matthes et al., 1995).

Definition of ‘mistargeting’
The following morphological criteria were applied to detect ‘mistar-

geting’ by specific growth cones (RP3 and aCC) in late stage embryos (based on Chiba et al., 1995; Rose et al., 1997). The dye-labeled growth cone (a) has retracted most of its long fine filopodia, (b) aligns along either a muscle cleft or an edge of a muscle other than its normal target(s), (c) bends its tip in relation to the axon shaft, (d) exhibits a
Homophilic target recognition

varicose appearance and (e) does all of these by the time (hour 18) that the particular growth cone would have normally initiated synaptogenesis on its targets. In addition, (f) growth cone morphology must be different from its normal state and not indicative of a delayed outgrowth.

Immunocytochemistry

Immunocytochemistry method was as described (Chiba et al., 1993). Primary antibodies used are: mAb 7G10 (anti-FAS3, 1:100 dilution; Patel et al., 1987; source: Hybridoma Center, Iowa City), mAb 1D4 (anti-Fasciclin II, 1:5 dilution; Grenningloh et al., 1991; source: Corey Goodman, University of California at Berkeley), mAb CL427 (anti-Connectin, 1:4 dilution; Meadows et al., 1994; source: Rob White, Cambridge University), mAb TollCD (anti-Toll, 1:10 dilution; Hashimoto et al., 1991; source: Carl Hashimoto, Yale University), mAb DSYT2 (anti-synapto- tagmin, 1:100 dilution; Littleton et al., 1993; source: Hugo Bellen, University of Texas), and anti-peroxidase antibodies (Jackson Immunoresearch Laboratory, Pennsylvania).

RESULTS

Removing FAS3 from the RP3 growth cone

In order to test the idea that FAS3 mediates homophilic synaptic target recognition, we removed FAS3 from the RP3 growth cone. We asked if this growth cone could still respond to ectopically expressed muscle FAS3. Transgenic muscle misexpressor lines were crossed into the FAS3 null mutant line (see Materials and Methods). Embryos thus generated had RP3 growth cones lacking FAS3 in a background in which all muscles express FAS3 during motoneuron-muscle interaction (Fig. 1D).

The control groups are as follows (Figs 2-4). First, in FAS3 null mutants, there is no FAS3 in either motoneurons or muscles (Fig. 1B); mAb 1D4 immunocytochemistry showed that the 6/7 cleft is innervated normally in 91% of such endings at hour 18 of embryogenesis (Figs 2E, 4B1). This is slightly lower than the wild-type cases (100%), suggesting that the RP3 growth cone may occasionally fail to target the 6/7 cleft in the absence of FAS3 expression (Figs 2B, 4A1). Dye injection into RP3 at the same stage revealed that the RP3 growth cone indeed misinnervates alternative target, muscle 13, at a low rate (7%) (Figs 2F, 4B2). Thus, losing FAS3 from both growth cones and muscles causes only minor targeting inaccuracies. Second, when the wild-type FAS3-positive RP3 growth cone encounters muscles that misexpress FAS3, the frequency at which

Fig. 2. Synaptic target selection by the RP3 growth cone. Schematics (A,D,G,J) summarize both the overall motoneuron innervation patterns at muscle 6/7 cleft, as revealed with mAb 1D4 (B,E,H,K), and the RP3 growth cone target choices, as revealed through dye injection (C,F,I,L). All data are based on 18-hour fillet-dissected embryos. (A-C) Wild type (white strain). Note that the motoneuron endings at the 6/7 cleft includes those of the RP3 motoneuron and a later arriving second motoneuron MN6/7b (B). Three examples of the dye-injected RP3 ending are given, one digital overlay photography of a fluorescent and a corresponding DIC micrograph and two tracings (C). They show the typical morphology of the RP3 growth cone at this stage. The RP3 growth cone has retracted most of its previously extended filopodia and is now aligned with the muscle 6/7 cleft. Box in A corresponds to areas shown in the photographs. (D-F) No FAS3 expression in either neurons or muscles (FAS3-25 null mutant line). Innervation at 6/7 cleft, as revealed with either mAb 1D4 immunocytochemistry (E) or dye injection into the RP3 motoneuron (F), remains virtually normal. (G-I) FAS3 misexpression in muscles (homozygous MF transgenic line). innervation at the muscle 6/7 cleft is often missing and there is also extra growth cone activity in neighboring muscles such as 15 and 16 (G, circle). Dye injection revealed that RP3 often mistargets alternative muscle targets, e.g. 13, 15, or 14 and 30 (I, circles). Outlines of muscles 6 and 7 are given when these muscles are out of RP3 growth cone focal plane. (J-L) Muscle misexpression but no neuron expression of FAS3 (FAS3-25 MF). The muscle 6/7 cleft innervation is sometimes reduced (K, arrow), due, at least partially, to the failure of the RP3 growth cone to align at the cleft (L, tracings). Notice, however, that the RP3 growth cone does not readily mistarget alternative targets. This contrasts to the ‘muscle misexpression’ case (G-I). Asterisks show the points from which motoneuron axons approach target muscles (C,F,I,L). See Fig. 4 for data summary. Scale bars are 10 μm.
Innervation at the 6/7 cleft is missing increases to 49% (Figs 2H, 4C1). Dye injection demonstrated that this is largely due to RP3 innervating alternative muscles often (59% of the time) (Figs 2I, 4C2). RP3’s mistargeting has a long-lasting effect on the neuromuscular wiring since, in 43% of these animals, the 6/7 cleft remains uninnervated during larval development (Figs 3B, 4C3). Thus, consistent with the previous study (Chiba et al., 1995), the RP3 growth cone is capable of ‘recognizing’ FAS3-expressing muscles as targets.

Will a FAS3-deficient RP3 still mistarget alternative FAS3-expressing muscles? Collective visualization of all motoneuron endings revealed that 53% of the cases lack innervation at 6/7 muscle cleft, the normal RP3 innervation site (Figs 2K, 4D). However, using collective immunovisualization, we could not determine if the RP3 growth cone was mistargeting alternative muscles or simply failing to innervate its normal target site.

Fluorescent dye injection of RP3 cell bodies allowed us to specifically examine the target preference of FAS3-deficient RP3 growth cones at the same stage. In 43% of the cases, RP3 growth cones extend normally, reach the peripheral target region and innervate the 6/7 muscle cleft (Fig. 2L photo image). However, in another 43% of the cases, after reaching the target region, RP3 growth cones stall beneath the 6/7 cleft (Fig. 2L tracings). The reason for RP3 growth cones stalling just short of the 6/7 cleft instead of innervating at the cleft may be partially attributed to an increased adhesion between muscles after FAS3 misexpression (see below). Importantly, in only 14% of the cases do FAS3-deficient RP3 growth cones appear to mistarget nearby muscles (Fig. 4D2). This rate of mistargeting is significantly lower than the previous case (59%), in which the RP3 growth cone maintains its FAS3 expression, and is approaching the rate of mistargeting (7%) in FAS3 null mutants (Fig. 4). Thus, after removal of FAS3, the RP3 growth cone loses its ability to readily recognize FAS3-expressing muscles as alternative targets.

In third stage larvae, about 4 days later, mAb 1D4 immunocytochemistry showed the 6/7 cleft lacking normal innervation 26% of the time (Fig. 4D3). In 12% of the cases, nerve terminals were seen about 10 μm ventral to the cleft, instead of being at the same focal plane as the dorsal surfaces of muscles 6 and 7, as is the case in wild-type larvae (Figs 3C, 4D3). The two muscle membranes appeared to tightly adhere to each other, unlike in wild type (Fig. 3A). These larval data are consistent with the idea that FAS3-deficient RP3, while rarely targeting alternative muscles, sometimes fails to pass through the 6/7 cleft.

We conclude that FAS3 likely mediates synaptic target recognition of the RP3 motoneuron through homophilic interaction.

Adding FAS3 to the FAS3-negative aCC growth cone
To further investigate FAS3’s ability to homophilically mediate synaptic target recognition, we tested whether the addition of FAS3 to a normally FAS3-negative motoneuron-muscle pair would be sufficient to induce de novo synaptic target recognition. We focused on the aCC motoneuron, another well-documented neuron in the Drosophila embryo (for example, Keshishian et al., 1993; Doe et al., 1988). The FAS3-negative aCC growth cone is the first motoneuron growth cone to exit the CNS. It pioneers the ISN axon pathway during a period when no muscles express FAS3. The aCC growth cone then innervates the FAS3-negative muscle 1 in the dorsal musculature by hour 18 (Fig. 5A-C).
An enhancer trap line was newly generated that misexpresses FAS3 in the aCC motoneuron growth cone during axon pathfinding and target selection (Fig. 1E; see Materials and Methods). Dye injection revealed that the FAS3-expressing aCC growth cone in this neuron misexpressor line extends normally through the ISN pathway. Moreover, the growth cone also reliably selects its normal target muscle despite FAS3 misexpression (Fig. 5D,E). In the reciprocal experiment using the muscle misexpressor lines, the FAS3-negative aCC growth cone misexpression but no neuron expression (FAS3E25 ; MF, where ‘MF’ is MF10, MF27 and MF50). Numbers (n) of abdominal (A2-A7) hemisegments (in total no. of animals) examined are indicated. Mistargeting is defined morphologically as in previous studies (see Materials and Methods). Alternative targets selected by RP3 in (C) are 6 (7%), 12 and/or 13 (31%), 15 and/or 16 (31%), and 14 and/or 30 (31%).
cone encounters muscles that express FAS3 ectopically. In this case as well, the innervation of muscle 1 remains normal (Fig. 5F,G). Thus, ectopic FAS3 expression in either the aCC growth cone or the muscles alone causes no visible effect on aCC’s target choice (Table 1).

What happens if FAS3-expressing aCC contacts FAS3-expressing muscles? Neuron and muscle misexpression lines were combined in order to express FAS3 on both the aCC growth cone and the muscles it encounters (Fig. 5H). Ectopic FAS3 expression in the musculature starts at approximately hour 14 and intensifies towards hour 18, when growth cone choices were analyzed. The FAS3-misexpressing aCC growth cone encounters FAS3-misexpressing muscles before contacting its normal target. We hypothesized that, if FAS3 in a growth cone is sufficient to make it responsive to FAS3-expressing muscles, then the aCC growth cone would misinnervate alternative target muscles.

A hint of targeting errors by aCC came from collective immunovisualization of ISN growth cones at hour 18. By this time in wild-type embryos, aCC growth cones would have not only arrived at the most dorsal (distal) muscle 1 but also initiated synaptic differentiation (Fig. 5B). In contrast to wild type, when both aCC and muscles misexpress FAS3, muscle 1 lacks innervation in 4% of the cases (Fig. 5I; Table 1A). In third stage larvae, we noted that lack of innervation at muscle 1 persists in these mutants at a 5% level, consistent with the idea that, in these cases, the mistargeted aCC growth cone never makes it to muscle 1, even days after embryogenesis (Fig. 6B). Since this muscle is normally innervated by at least one additional motoneuron, these rates (4% and 5%, respectively) may be an underestimate of the actual rate of aCC failing to innervate muscle 1. Also, whether or not the aCC growth cone mistargets alternative muscles could not be determined by immunovisualization.

More direct evidence for mistargeting came from dye injection into the aCC motoneuron at the same stage. In 25% of the cases, the FAS3-bearing aCC growth cone stops short of its normal target (muscle 1) and instead bends its head slightly posteriorly along the FAS3 bearing muscle 2, exhibiting a morphology characteristic of synaptic target recognition (Fig. 5J; Table 1B). Such morphological changes are not typical of this growth cone at earlier stages in wild-type embryos when it grows past muscle 2. Thus, the results suggest that FAS3 can homophilically mediate target recognition between growth cones and muscles that do not normally express FAS3.

An additional experiment was conducted in which we assessed potential dosage effects of misexpressed FAS3. We supplemented Mhc-driven muscle misexpression by applying a heat shock to the embryos at hour 12 during the period when the aCC growth cone contacts muscles proximal to its normal target (see Materials and Methods). This heat-shock treatment increases levels of ectopic muscle FAS3 at least two-fold by hour 14. Thus, while the timing of FAS3 misexpression remains very similar, the dosage is multiplied by heat-shock treatment. In heat-shocked embryos, the proportion of muscle 1’s missing innervation increases from 4% to 12%, as scored by immunovisualization (Table 1A). In general accordance with this, dye injection into aCC also showed that heat-shock treatment leads to a slight increase from 25% to 30% in the rate of aCC mistargeting (Table 1B). These results suggest that dosage may be a factor influencing FAS3’s homophilic role.

Adding FAS3 to the FAS3-negative SNa growth cones

To further confirm the FAS3 gain-of-function phenotype, we experimented with other motoneuron growth cones. Motoneurons of the SNa nerve are normally Connectin-positive but FAS3-negative, and they innervate Connectin-positive but FAS3-negative lateral muscles (Nose et al., 1992; Fig. 7A,B). We took advantage of the fact that the enhancer trap misexpressor line described above also misexpresses FAS3 in SNa motoneurons late in embryogenesis (Fig. 1F). SNa growth

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**Table 1. Innervation by the aCC motoneuron growth cone**

<table>
<thead>
<tr>
<th>FAS3 expression</th>
<th>(A) Target muscle</th>
<th>Do not target muscle</th>
<th>n²</th>
<th>(B) Target muscle</th>
<th>Mistarget muscle</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type a</td>
<td>100%</td>
<td>0%</td>
<td>329 (30)</td>
<td>100%</td>
<td>0%</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Neuron misexpression b</td>
<td>100%</td>
<td>0%</td>
<td>164 (15)</td>
<td>100%</td>
<td>0%</td>
<td>7 (7)</td>
</tr>
<tr>
<td>Muscle misexpression c</td>
<td>100%</td>
<td>0%</td>
<td>291 (30)</td>
<td>100%</td>
<td>0%</td>
<td>7 (7)</td>
</tr>
<tr>
<td>Neuron and muscle misexpression d</td>
<td>96%</td>
<td>4%</td>
<td>310 (30)</td>
<td>75%</td>
<td>25%</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Neuron and muscle misexpression (plus heat shock) e</td>
<td>88%</td>
<td>12%</td>
<td>361 (33)</td>
<td>70%</td>
<td>30%</td>
<td>12 (12)</td>
</tr>
</tbody>
</table>

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aWhite strain.
bHFET “enhancer trap” line.
cMF29 and MF2 transgenic lines.
dHFET, MF and HFET, MF/+ lines.
eSame as d, but a heat shock was given at hour 12 to supplement FAS3 misexpression in musculature (see Materials and Methods). No abnormal innervation by the aCC or other ISN motoneuron growth cones resulted when either wild-type or “muscle misexpression” line received the same heat-shock treatment (n=125-135).
fThe ISN sub-branch reaching muscle 1 (ISN1) was examined in fillet-dissected 18-hour embryos after mAb 1D4 immunocytochemistry.
gCases where the distal tip of ISN contact muscle 1.
hCases with clear absence of ISN1 subbranch.
iNumbers of abdominal (A2-A7) hemisegments (total no. animals) examined.
jThe aCC growth cone was examined in fillet-dissected 18-hour embryos after dye injection into aCC cell body.
kCriteria applied were similar to g (also see Materials and Methods). A part of discrepancy between this and g may be accounted for by the presence of additional motoneuron(s) for muscle 1, which are not visualized here.
lMuscle 2 is the only alternative target observed in this series. It is not known if other muscles could be also selected as alternative targets if the onset of muscle misexpression was pushed earlier.

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HF ET MF 29 MF 42 MF 135
Homophilic target recognition

cones start misexpressing FAS3 during the period of axon pathfinding and reach peak expression level just before target selection, around hour 16.

As with aCC, FAS3 misexpression in the SNa nerve alone (Fig. 7C,D) or in muscles alone (Fig. 7E,F) causes no apparent mistargeting. This again suggests that unmatched FAS3 expression does not lead to mistargeting.

However, when both growth cones and muscles are simultaneously made to express FAS3 ectopically, abnormal axon collaterals occasionally (in 17% of the cases) stem out of the SNa nerve in hour 18 embryos (Fig. 7G,H; Table 2). These collaterals are thin, probably consisting of only one or two axons, and misinnervate muscles 12, 13, 26 or 27, which are all immediately proximal to the normal targets of SNa motoneurons.

Function of mistargeted synapses

In third stage larvae, persistent ectopic collaterals from the SNa nerve are seen to innervate muscle 12, 13 or 26 in 11% of the cases (Fig. 8B,C circles). These ectopic endings form boutons similar to normal synaptic endings at this stage (Fig. 8B inset) and, just like normal endings, accumulate synaptotagmin (Fig.

Table 2. Innervation by the SNa motoneuron growth cones

<table>
<thead>
<tr>
<th>FAS3 expression</th>
<th>SNa growth cones (mAb 1D4)</th>
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<tbody>
<tr>
<td></td>
<td>No ectopic targeting</td>
</tr>
<tr>
<td>Wild type&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100%</td>
</tr>
<tr>
<td>Neuron misexpression&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99%</td>
</tr>
<tr>
<td>Muscle misexpression&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100%</td>
</tr>
<tr>
<td>Neuron and muscle misexpression&lt;sup&gt;d&lt;/sup&gt;</td>
<td>83%</td>
</tr>
</tbody>
</table>

<sup>a</sup>white strain.
<sup>b</sup>HF<sup>ET</sup> “enhancer trap” line.
<sup>c</sup>MF<sup>29</sup> and MF<sup>42</sup> transgenic lines.
<sup>d</sup>HF<sup>ET</sup>; MF line.
<sup>e</sup>The SNa nerve was examined in fillet-dissected 18-hour embryos after mAb 1D4 immunocytochemistry.
<sup>f</sup>“Wild-type” cases where SNa nerve splits into distally extending SNa21-24, which innervate muscles 21-24, and posteriorly extending SNa5/8, which innervate both muscles 5 and 8.
<sup>g</sup>Cases where, in addition to the normal SNa branches, extra axon(s) innervate muscles not normally innervated by SNa motoneurons. Alternative targets include muscles 12 (33%), 13 (12%), 26 (29%), 27 (2%), and unidentified muscle(s) in the ventrolateral musculature (23%).
<sup>h</sup>Numbers of abdominal (A2-A7) hemisegments (total no. animals) examined.

Fig. 6. The ISN nerve in third stage larvae, with mAb 1D4 immunocytochemistry. (A) In wild type (white strain), muscle 1 is innervated at its proximal edge in 100% of the cases (n=65 hemisegments in 6 animals) (A, box). (B) With FAS3 misexpression in both aCC motoneuron and muscles (HF<sup>ET</sup>; MF), muscle 1 lacks innervation in 5% of the cases (n=98 hemisegments in 10 animals). Innervations on muscle 2 are outside of view fields (A,B). Scale bars are 20 μm.

Fig. 7. Synaptic target selection by the SNa growth cones. Schematics (A,C,E,G) summarize the FAS3 expression patterns and the SNa motoneuron innervation patterns revealed with mAb 1D4 at hour 18 (B,D,F,H). (A,B) In wild type (white strain), the SNa motoneuron cell bodies (whose number is undetermined) form a cluster in the CNS (A). Their axons diverge and innervate two lateral groups of muscles. A posteriorly directed subbranch innervating 5 and 8 and a distally directed subbranch innervates 21, 22, 23 and 24 (A,B). (C,D) With FAS3 misexpression in SNa motoneurons (HF<sup>ET</sup>), SNa innervation remains indistinguishable from wild type. (E,F) With FAS3 misexpression in all muscles (MF), SNa innervation remains wild type-like. (G,H) When there is FAS3 misexpression in both SNa motoneurons and all muscles (HF<sup>ET</sup>; MF), extra axon collaterals sometimes appear from the SNa nerve and misinnervate muscles 12, 13, or 26. See Table 2 for data summary. Scale bars are 10 μm.
These results suggest that mistargeting by SNa motoneurons during embryogenesis leads to persistent and functional ectopic endings.

**DISCUSSION**

Our in vivo study tested FAS3’s role as a homophilic synaptic target recognition molecule through two complementary sets of experiments (Fig. 9). In one, a normally FAS3-positive growth cone (RP3) loses its ability to recognize FAS3-expressing alternative targets when its own FAS3 is removed (Fig. 9A). In the other, normally FAS3-negative growth cones (aCC and SNa’s) gain the ability to recognize FAS3-expressing alternative targets when they are made to express FAS3 ectopically (Fig. 9B). These reciprocal experiments have yielded strong evidence that FAS3 mediates synaptic target recognition through homophilic interaction. The results provide precedent for a homophilic synaptic target recognition mechanism derived from Sperry’s ‘chemoaffinity theory’ (Sperry, 1963).

**Biased selection of alternative targets**

When FAS3 is misexpressed, de novo synaptic targeting can be induced between motoneurons and muscles not normally known to synapse with each other. In each experiment where such ectopic synaptogenesis occurs, specific FAS3-bearing growth cones show preference for certain muscles even when all muscles misexpress FAS3 at more or less the same level. Relative proximity can be a major limiting factor, as those muscles outside of normal filopodial reach of the growth cones do not receive ectopic endings. Among those within reach of the growth cones, some muscles may be more favorable than others due to local molecular environment. Various growth cone guidance molecules, including Connectin, Toll, DPTPs, Semaphorins, Netrins, Beat and Neuroglian, are present on overlapping subsets of muscles and/or motoneurons (Desai et al., 1997; Fambrough and Goodman, 1996; Hall and Bieber, 1997; Harris et al., 1996; Matthes et al., 1995; Mitchell et al., 1996; Nose et al., 1997; Rose et al., 1997). Their collective influence on a given growth cone is thought to be partly redundant, partly combinatorial and partly antagonistic. These molecules may ‘bias’ the RP3 growth cone as it selects certain ventral muscles as alternative targets, or the FAS3 misexpressing aCC growth cone as it settles on muscle 2.

**Muscle-muscle adhesion**

Our study provides new insight into FAS3-mediated muscle-muscle interactions. It has been previously proposed that muscle-provided cell surface molecules may influence growth cone-muscle interactions indirectly by promoting muscle-muscle adhesion and blocking growth cone access to muscle clefts (Nose et al., 1997; Rose et al., 1997). This idea is difficult to test since the same molecules can also affect growth cones directly. In our experiments, when the FAS3-deficient RP3 growth cone encountered a muscle 6/7 cleft that lacks FAS3 (in FAS3 null mutants), the growth cone has no problem (0% of the cases) extending through the cleft (Fig. 4B3). In contrast, when the same FAS3-deficient RP3 growth cone faces a muscle 6/7 cleft that accumulates FAS3 (in the cross between FAS3 null and ‘muscle misexpressor’ H. Kose and others)
lines), the growth cone often (in 12% of the cases) fails to fully extend through the cleft (Figs 3C, 4D, 3). However, FAS3-positive RP3 growth cones that do not innervate ectopic targets are capable of innervating the 6/7 cleft in both wild-type and ‘muscle misexpressor’ embryos. Presumably, in this case, the attraction between the FAS3-positive growth cone and its target is robust enough to overcome any FAS3-induced muscle-muscle adhesion.

Homophilic synaptic target recognition: a mechanism for neural network formation

The most significant conclusion from this study is that FAS3, a small Ig-CAM, can mediate synaptic target recognition between motoneuron growth cones and muscles through homophilic interactions.

The role of FAS3, and other molecules sharing similar functions, is likely to initiate multistep processes of presynaptic differentiation (Haydon and Drapeau, 1995; Prokop et al., 1996; Zoran et al., 1996). Many maturing presynaptic terminals are characterized by the disappearance of actin-rich filopodial and lamellosipodial processes, as well as the dynamic appearance of a variety of cytoplasmic proteins involved in synaptic vesicle release/release machinery, such as α-adaptin, dynamin, syntaxin, synaptoophysin and synaptotagmin (Betz and Wu, 1995; Estes et al., 1996; Gonzalez-Gaitán and Jackle, 1997; Igarashi et al., 1997). Since the genes for most of these vesicle regulatory molecules are expressed widely among growing neurons, it is possible that a common pathway exists for presynaptic terminal differentiation. What remains unclear is how stereotypic molecular events that convert a growth cone into a mature presynaptic terminal are coupled with the activation of molecules such as FAS3 that mediate specific cell adhesion.

New questions also arise regarding the universality of synaptic target recognition mechanisms based on homophilic interaction. As mentioned before, Connectin, a leucine-rich repeat containing cell surface molecule with little homology to FAS3, can also mediate ectopic synaptic target recognition through homophilic interaction between motoneurons and muscles (Nose et al., 1997). This raises the possibility that a wide variety of cell surface molecules, which are specifically expressed by both growth cones and their potential targets, may mediate synaptic ‘matchmaking’ through homophilic interaction.

Homophilic interaction as a ‘matchmaking’ mechanism for synaptic partner cells is often proposed with reservation because the number of genes in an entire genome seems too little to account for all of the synaptic connections in an entire nervous system, even one as simple as Drosophila (Tessier-Lavigne and Goodman, 1996). However, the site and timing of putative recognition molecule expression can be very dynamic, as is the case for FAS3 (Keshishian et al., 1993). Thus, the same recognition molecules may be used repeatedly in different regions and/or at different times, provided they are tightly regulated both spatially and temporally. We propose a simple hypothesis that homophilic synaptic target recognition is one of the basic mechanisms leading to neural network formation.

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