Functional Dissection of *Drosophila* Capricious: its Novel Roles in Neuronal Pathfinding and Selective Synapse Formation.

Hiroki Taniguchi

Doctor of Science

Department of Molecular Biomechanics

School of Life Science

The Graduate University for Advanced Studies

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ABSTRACT

Drosophila Capricious (CAPS) is a transmembrane protein with leucine-rich repeat (LRR) motifs, expressed on small subsets of neurons and muscles, including muscle 12 and the motoneurons that innervate it (muscle 12 MNs). Pan-muscle ectopic expression of CAPS alters the target specificity of muscle 12 MNs, indicating that CAPS can function in muscles as a target recognition molecule. In this study, I first examined the effect of ectopic pan-neural expression of CAPS on the motoneuronal circuit. I found that pan-neural expression of CAPS alters the pathfinding of muscle 12 MNs. The defect appeared to be caused by changes in the steering behavior of muscle 12 MNs at a specific choice point along their pathway to the target muscle. These results revealed a novel function of CAPS in axon pathfinding. I then performed deletion analyses of CAPS. I expressed CAPS lacking the intracellular domain in all neurons or in all muscles, and studied their ability to induce the pathfinding and targeting phenotypes. I found that the function of muscularly expressed CAPS in target recognition is intracellular domain dependent, whereas the function of neurally expressed CAPS in pathfinding is not, suggesting that CAPS may function in neurons and muscles in a different manner. The requirement of the intracellular domain for the function of muscularly expressed CAPS suggests the presence of a signaling event within muscle cells that is essential for selective synapse formation.

CHAPTER I

Functional Analysis of Neurally Expressed Capricious in Axon Pathfinding

INTRODUCTION

The nervous system consists of a large number of neurons, which together form a neural network by making synaptic connections with each other. Although synaptic contacts are seen throughout the nervous system, they are formed specifically, not randomly, between certain regions or cells, thus making the neural circuits functional. For example, the cortical neurons in motor area involved in the motion of the right hand specifically synapse with motorneurons that innervate muscles in the right hand or with interneurons that in turn innervate the motor neurons (which synapse with the muscles), by extending their axons through stereotyped pathways. If these axons incorrectly made synapses with motorneurons innervating left hand muscles, we would hold up our left hand when we try to raise our right hand. In this way, the formation of specific neural connections underlies the correct function of the nervous system.

During the formation of the nervous system, the wiring of neural circuits is mainly performed by two steps, axon pathfinding and target recognition (Fig. 1). During the first process, neuronal growth cones traverse long distances along stereotyped pathways to their appropriate target regions. Then during

the latter process, growth cones find and synapse with their specific target(s), by searching over many neighboring cells in the target region (Garrity and Zipursky, 1995; Tessier-Lavigne and Goodman, 1996).

In these processes, neuronal growth cones are guided by a variety of different environmental cues expressed on their pathway and in the target region, that act either as contact-mediated signals or as diffusible factors (Fig. 1, Fig. 2). These cues have been shown to have attractive or repulsive effects on the growth cones mediated by their corresponding receptors. Recent extensive studies on Netrins and Semaphorins have provided basic insights into the role of chemotropic factors in axon guidance. Furthermore, several lines of evidence have demonstrated the importance of cell adhesion molecules in axon pathfinding and target recognition (reviewed in Tessier-Lavigne and Goodman, 1996). However, how these molecules orchestrate to generate the precise pattern of neural connectivity *in vivo* remains largely unknown. The *Drosophila* neuromuscular system provides an excellent model to address this problem. In each abdominal hemisegment of *Drosophila* embryos and larvae, ~40 motoneurons innervate 30 muscles in a specific and stereotypic manner (Fig. 3) (reviewed in Keshishian et al., 1996). Motor axons exit the CNS in two different motor nerves, the intersegmental nerve (ISN) and the segmental nerve (SN). They then make appropriate pathfinding decisions along their pathway within five peripheral motor branches (ISN, ISNb, ISNd, SNa, and SNc) that innervate distinct groups of muscle fibers. Once the motor axons reach their target region, they transiently probe the surface of many neighboring muscles, withdraw most of these contacts and form stable synapses with their own target(s). Recent studies have revealed a number of molecular components required for precise motor axon

pathfinding in this system (e.g., Lin and Goodman, 1994; Desai et al., 1996; Krueger et al., 1996; Fambrough and Goodman, 1996; Yu et al., 1998). Many of these molecules play roles in specific fasciculation/defasciculation events in the periphery, a key process in the formation of discrete motor nerves. Recent studies have also identified several molecules that are implicated in the process of target recognition, including secreted molecules, such as Netrin A, Netrin B and Semaphorin II, and cell surface molecules, such as Connectin, Fasciclin III, Toll and Capricious (Nose et al., 1992, 1997; Chiba et al, 1995; Matthes et al., 1995; Mitchell et al., 1996; Rose et al., 1997; Kose et al., 1997; Winberg et al., 1998; Shishido et al., 1998).

Connectin (Con) is a homophilic cell adhesion molecule of the leucine-rich repeat (LRR) family (Fig. 4), the gene of which was identified as a P-element insertion site of the enhancer trap line expressing the reporter gene in small subsets of muscle fibers (Fig. 3; Nose et al., 1992). Con is expressed on the surface of a subset of embryonic muscles and neurons, including lateral muscles and the motoneurons that innervate them (SNa motoneurons) . *In vitro*, Con can mediate homophilic cell adhesion in transfected S2 cells. The specific expression pattern of Con on SNa motoneurons and their target muscles together with its ability to promote homophilic cell adhesion strongly suggests that Con functions as an attrractive target recognition molecule *in vivo*. This possibility was ascertained by the misexpression study in which Con is expressed on all muscles. In the animals pan-muscularly expressing Con, ISNa motoneurons often extended collaterals to a neighboring non-target muscle (muscle12) (Nose et al., 1997). This phenotype was also shown to be dependent on neuronal Con expression, consistent with the ability of Con to facilitate a homophilic adhesion *in vitro*. These results provides the

evidence that Con can function as an attractive target recognition molecule through its homophilic cell adhesion property. However, in the *con* loss of function mutants, no defects were found in the neuromuscular connectivity, suggesting that the other molecules act on lateral muscles to keep them properly innervated (Nose et al., 1992).

Such a situation that the loss-of-function mutant shows only mild or no defects in target selection, while its specific expression in subsets of muscle fibers and the drastic effects of their ectopic expression strongly implicate its role in neuromuscular target recognition is also true of the other candidates for target recognition molecules described above. This fact is interpreted as suggesting that neuromuscular target specificity is determined by a combination of functionally overlapping muscle cues. This notion is further supported by a recent finding that motoneuronal growth cones can assess the balance of multiple cues, both attractive and repulsive, in selecting their targets (Winberg et al., 1998). However, is the potency of each cue as a target recognition molecule completely equivalent? There may be molecules that function predominantly in the balance of several cues. It is Capricious (CAPS) identified in our laboratory that suits this condition.

Capricious (CAPS), encoded by the gene *capricious* (*caps*), is a cell-surface protein of the LRR family (Fig. 4) which was identified as a candidate target recognition molecule by analyzing an additional enhancer trap line expressing a reporter gene in small subsets of muscle fibers. (Fig. 3; Shishido et al., 1998). During the formation of neuromuscular connectivity, CAPS is expressed in four dorsal (1, 2 ,9 and 10) and six ventral (12, 14-17 and 28) muscles. It is also expressed in a small subset of motoneurons including identified motoneurons, aCC, RP2, RP5 and U, all of which innervate *caps*

positive muscles. Thus, *caps* expression pattern strongly implies neuromuscular target specificity. During the innervation of a ventral muscle 12, CAPS is expressed on the surface of the muscle and also on the axons and synaptic sites of the motoneurons that innervate the muscle (muscle 12MNs, including RP5). On the other hand, CAPS is not expressed on the neighboring muscle 13 and the motoneurons that innervate it (RP1 and RP4). Loss of function of the *caps* alters the target specificity of muscle 12 MNs; the MNs often form synaptic endings on a neighboring nontarget muscle, muscle 13, in addition to its normal target (Fig. 5). A similar and more robust phenotype was observed when CAPS is ectopically expressed on all muscles (Fig. 5) (Shishido et al., 1998). These results clearly showed that endogenous CAPS on muscle 12 is required to restrict the muscle 12MNs axons in a target muscle and ectopically expressed CAPS on muscle 13 is sufficient to attract them to a non-target cell. However, in the previous study, the role played by neurally expressed CAPS was not examined. Since CAPS is expressed not only on muscles but also on the motoneurons during the targeting of muscle 12 MNs, neurally expressed CAPS is also likely to play a role in this process. Neurally expressed CAPS may also play a redundant role in earlier events of motoneuronal pathfinding, that was not revealed by the analysis of the loss-of-function mutants.

To assess the possible function of neurally expressed CAPS, in this study, I examined the effect of pan-neural ectopic expression of CAPS on the formation of motoneuronal circuits. I found that when CAPS was overexpressed on all neurons, muscle 12 MNs often diplayed specific pathfinding defects in the periphery although the neuromuscular connectivity remained unchanged. The results showed that changes in the level of CAPS

expression on the motoneurons can influence their steering behavior at a specific choice point. Based on these results, possible role of neurally expressed CAPS in axon pathfinding will be discussed.

MATERIALS AND METHODS

Pan-neural Expression by GAL4-UAS System

To direct ectopic and increased expression of CAPS on all neurons, I employed the GAL4-UAS system (Fig. 6). To drive pan-neural expression, *elav-GAL4^{3E1}* lines (Davis et al., 1997) was crossed with *UAS-caps* lines. The progenies were bred at 29 °C and 25 °C to drive high and moderate level of ectopic expression, respectively.

UAS-caps-Ia+Ib was generated by recombining two *UAS-caps* inserts, namely, *UAS-caps-Ia* (on the X chromosome) and *caps-Ib* (on the third, Shishido et al., 1998).

Immunocytochemistry

Horseradish peroxdase (HRP) immunocytochemistry was carried out essentially as previously described (Nose et al., 1992; Lin and Goodman, 1994).

Embryos were dechorionated in 50% bleach for 3 min, rinsed with distiled water several times, and fixed in heptane saturated with phosphate-buffered saline (PBS) containing 4% formaldehyde for 20 min. They were then devitellinized and washed several times with PTw (PBS and 0.1% Tween-20) before blocking in PTwN (PTw plus 5% normal goat serum) for 30 min. Primary antibody incubations were performed overnight at 4°C in 1.5 ml Eppendorf tubes with mild agitation. mAb1D4 (Van Vactor et al., 1993) was used at a dilution of 1:5 to visualize all motor nerves. sAbs against the

intracellular domain of CAPS (Shishido et al., 1998) was used at 1:300 to detect CAPS expression. Embryos were washed with PTw four times for 15 min each. Secondary antibody was added and incubated for 2hr at room temperature. After washing with PTw three times for 30 min, embryos were stained by using a Vector Elite ABC kit for enhanced detection. The stained embryos were washed several times with PTw , rinsed with PBS, and cleared in 80% glycerol. For detailed analysis, embryos were dissected on glass slides using tungsten needles. They were coverslipped and sealed by manicure.

Third instar larvae were collected, washed with PBS, and soaked in cold PBS. Larvae were immobilized onto a silicone plate using insect pins and cut along their lateral or dorsal surface with spring scissors for microsurgery under a stereomicroscope. Gut and brains were pulled away from larvae with a pincette. The larvae were then fixed for 1 hour in Bouin's fixing fluid. Fixed preparations were washed in PTw four times for 15 min each. Immunohistochemistry was performed as above. mAb22c10 (Fujita et al., 1982) and mAb1D4 (Van Vactor et al., 1993) were used as primary antibodies to visualize all motor nerves. Finally, larvae were cleared in 80% glycerol.

The preparations were observed under a Zeiss Axiophoto microscope. Staging of embryos was according to Hartenstein (1993).

RESULTS

Ectopic and Increased Expression of CAPS on All Neurons Causes Pathfinding Defects by Muscle 12 MNs

caps is normally expressed in a subset of CNS neurons including aCC, RP2, RP5 and U motoneurons (Shishido et al., 1998). To study the possible role of neuronal CAPS *in vivo*, I induced ectopic and increased expression of CAPS in all neurons using the GAL4-UAS system (Fig. 6). The GAL4 is a yeast transcription factor that binds to the specific upstream activation sequence (*UAS*) and promotes the transcription of the downstream gene. There are many GAL4 lines generated by randomly inserting a P element containing a *GAL4* gene fused to a minimal promotor into the genome. The GAL4 protein is produced by the enhancer control sequences near the P element insertion site in a stage and tissue specific manner. Thus, by crossing the appropriate GAL4 line with the UAS line which bears the exogenous gene that is to be analyzed downstream of the *UAS*, one can express any genes in a specific temporal and spatial pattern.

A GAL4 line, *elav-GAL4*^{3E1}, which expresses GAL4 in all neurons (Davis et al., 1997), was crossed with the *UAS-caps-Ia+Ib* line, which contains two copies of *UAS-caps*. The progeny (*elav-GAL4*^{3E1}*/UAS-caps-Ia+Ib*) was raised at 29 °C during embryonic and larval development to induce maximal ectopic expression. As expected, CAPS was ectopically expressed on all neurons, starting from embryonic stage 12, in *elav-GAL4*^{3E1}*/UAS-caps-Ia+Ib* individuals (Fig. 7). CAPS protein was detected in all major axon tracts in the CNS and in

the periphery suggesting that ectopically expressed CAPS was properly transported to axons.

To analyze the effect of pan-neural CAPS expression on the formation of the nervous system, I first examined axonal processes of the motor neurons in the third instar larvae by mAb 1D4 (anti-Fasciclin II) and mAb 22C10 staining. No gross morphological defects were seen in the CNS and musculature, suggesting that their overall development proceeded normally. However, I detected a highly specific change in the trajectory of motoneurons that innervate muscle 12 (muscle 12 MNs). In wild-type larvae, axons of muscle 12 MNs, that fasciculate to form the terminal branch of the intersegmental nerve b (ISNb), project along the internal surface of muscle 13 before reaching their final target, muscle 12 (Fig. 8A). In contrast, in *elav-GAL4^{3E1}/UAS-caps-Ia+Ib* larvae, they passed along the exterior of muscle 13 in 29% of segments (Fig. 8B, quantitative data summarized in Table 1). Such a phenotype was not observed in control larvae (*elav--GAL4^{3E1}/+* raised at 29 °C, Table 1). Despite the abnormality in their trajectory, the axons of muscle 12 MNs reached their normal target by turning interiorly at the cleft between muscles 12 and 13, and established normal synapses on muscle 12. Thus, ectopic and increased CAPS expression on all neurons affects axon pathfinding of muscle 12 MNs, but not their synapse formation.

I next examined the dose dependency of the axon guidance phenotype (Table 1). To induce reduced level of ectopic CAPS expression, I either used the line *UAS-caps-Ia5*, which carries one copy of *UAS-caps*, or bred the embryos and larvae at a lower temperature (see Material and Methods for details). In *elav-GAL4^{3E1}/UAS-caps-Ia5* larvae, the percentage of the misrouting phenotype of muscle 12 MNs decreased to 23% . Furthermore,

when *elav-GAL4*^{3E1}*/UAS-caps-Ia+Ib* individuals were raised at 25 °C, the frequency of the misrouting phenotype decreased dramatically to 10%. Thus, the penetrance of the misrouting phenotype is dependent on the level of expression of ectopic CAPS on neurons.

In larvae that express CAPS pan-neurally, I also observed defects in the formation of the transverse nerve (data not shown). The nerve was often split and the cell body of a neuron associated with the nerve (lateral bipolar cell) was occasionally mislocated. With the exception of the transverse nerve phenotype, the effect of ectopic CAPS on the formation of motor nerves is highly specific to muscle 12 MNs. Notably, the sub-branches of ISNb that terminate on ventral muscles other than muscle 12 displayed no abnormalities in their trajectory or targeting. The other motor nerves, the intersegmental nerve (ISN), intersegmental nerve d (ISNd), segmental nerve a (SNa) and segmental nerve c (SNc) also retained their normal morphology, although it remained possible that there were subtle abnormalities that could not be detected with the marker tested in this study.

Pathfinding Defects of Muscle 12 MNs Caused by Pan-neural CAPS Expression Occur during Late Embryogenesis

To determine when the axon guidance errors of muscle 12 MNs begin to be seen in individuals that pan-neurally express CAPS, I analyzed the development of the motor nerves in the embryos by mAb 1D4 staining. In wild-type embryos, motor axons exit the CNS through the ISN or segmental nerve (SN) roots. They then divide into five peripheral motor branches (ISN, ISNb, ISNd, SNa, and SNc) that project to different groups of muscle fibers.

The axons of muscle 12 MNs follow the ISNb pathway. During embryonic stage 15, the axons of ISNb, including those of muscle 12 MNs, separate from the ISN and enter the ventral muscle field at muscle 28 (Fig. 9A). They then extend between the external surface of muscles 6 and 7 and the internal surface of muscle 14. At a choice point near muscle 30, muscle 12 MNs and motoneurons that innervate muscle 13, shift their trajectory by turning to a more internal muscle layer. Muscle 12 MNs then extend along the internal surface of muscle 13, forming the terminal branch of ISNb. They reach their final target (muscle 12) by late stage 16, and begin to form functional synapses by early stage 17.

In *elav-GAL4^{3E1}/UAS-caps-Ia+Ib* embryos, no abnormality was seen in the development of ISNb until mid-stage 16; it defasciculated from ISN at the normal branching point, extended along the internal surface of muscle 14, and reached at muscle 30. However, specific defects in the ISNb trajectory were observed at its distal edge during late stage 16 to early stage 17 (Fig. 10B-E, summarized in Fig. 9B). The terminal branch of ISNb formed by muscle 12 MNs normally extend along the internal surface of muscle 13 and start to establish synaptic contacts with muscle 12 by this stage (Fig. 10A). In 18 % of hemisegments in *elav-GAL4^{3E1}/UAS-caps-Ia+Ib* embryos, a misrouting phenotype similar to that seen in the larvae was observed. Instead of traveling along the internal surface of muscle 13, the terminal branch of ISNb took an abnormal path along the external surface of muscle 13 to reach muscle 12 (right segment in Fig. 10B, C, quantitative data summarized in Table 1). In 13 % of hemisegments in *elav-GAL4^{3E1}/UAS-caps-Ia+Ib* embryos, the ISNb stalled near muscle 30, failing to extend to more interior muscle layers (Fig. 10D, E). Occasionally, thin axonal processes were seen to emanate from the

stalled nerve terminal, which stopped prematurely and failed to innervate muscle 12 (Fig. 10D, black arrowhead). These results suggest that ectopic expression of CAPS in all neurons changes the behavior of muscle 12 MNs at their specific choice point near muscle 30.

DISCUSSION

CAPS is a LRR transmembrane protein (Fig. 4) which was originally characterized as a target recognition molecule on muscles (Shishido et al., 1998). In this study, I reveal a novel function of CAPS in neuronal pathfinding by pan-neural ectopic expression experiments.

The anatomical analysis of motoneuronal circuits in *elav-GAL4^{3E1}/UAS-caps-Ia+Ib* larvae revealed specific pathfinding defects in muscle 12 MNs. Axons of muscle 12 MNs extended along the external instead of the internal surface of muscle 13 before reaching the target muscle 12. This finding in the larvae led me to analyze the developmental processes of axon extension of muscle 12 MNs in the embryos. In embryos that express CAPS pan-neurally, axons of muscle 12 MNs appeared to extend normally until mid-stage 16. However, from late stage 16 to early stage 17, striking defects were seen in the trajectory of the most distal part of the ISNb , indicative of the abnormal steering behavior of muscle 12 MNs. In addition to the misrouting phenotype (18%) as seen in the larvae, a stall phenotype (13%), in which the terminal branch of the ISNb stopped prematurely near muscle 30 was observed. Since the summation of the frequencies of the misrouting (18 %) and stall (13 %) phenotypes in the embryos was equivalent to that of the misrouting phenotype in larvae (31 %), I assume that many of the stalled axons would later take the abnormal path to reach their target muscle. These results strongly suggest that ectopic pan-neuronal expression of CAPS affects the behavior of muscle 12 MNs at their specific choice point near muscle 30.

By what mechanism does pan-neurally expressed CAPS alter the behavior of muscle 12 MNs at this specific choice point? Axons have to complete two

steps, defasciculation from the main nerve tract and steering into the specific target region, in order to successively change their trajectory at discrete choice points along the motor pathway (Fig. 11; e.g., Lin et al., 1994; Desai et al., 1996, 1997; Krueger et al., 1996; Fambrough and Goodman, 1996; Yu et al., 1998). The defasciculation event would mainly relate to changes in axon-axon interactions, while the steering event relate to changes in axon-target region interactions. Recent loss-of-function and gain-of-function study have revealed many molecules involved in these steps.

Fasciclin II (Fas II) is a homophilic cell adhesion molecule belonging to immunoglobulin super family, which is normally expressed on motor axons. When the levels of Fas II on all motor axons are raised transgenically, the ISNb axons fail to defasciculate from the ISN at their first choice point, and instead form a bundle within the ISN (Grenningloh et al., 1991; Lin et al., 1994). Beat, which is encoded by the *beaten path* (*beat*) gene, is a novel secreted protein expressed by motor neurons. Loss-of-function *beat* mutants show ISNb defasciculation defects similar to those observed in gain-of-function *fas II* mutants. However, this phenotype in *beat* mutants are suppressed by genetically removing Fas II function, suggesting that Beat function as a negative regulator of axon fasciculation by modulating axon-axon adhesive interactions (Fambrough and Goodman, 1996). Mutations in the gene *semaphorin I* (*sema I*), a member of the *semaphorin* family that mediates repulsive axon guidance events (Yu et al., 1998), and the gene *dptp69D* and *dptp99A*, which encode receptor protein tyrosine phosphatases (RPTPs) (Tian et al., 1991; Desai et al., 1996, 1997) also cause the defasciculation defects of the ISNb from the ISN. These data indicate that axon fasciculation versus defasciculation is regulated by a balance of attractive (adhesive) and repulsive

(antiadhesive) forces between axons. In contrast with the loss-of-function and gain-of-function mutants described above, the mutants of *dlar* which encodes another RPTP show similar but subtly different phenotype (Krueger et al., 1996). The ISNb axons in *dlar* mutants do appear to leave the ISN and then extend dorsally as a discrete bundle parallel to the ISN, suggesting that DLAR is required for the steering into the ventral muscle field of the ISNb axons rather than defasciculation from the ISN. Thus, defasciculation and steering behavior of developing axons at the choice points may be genetically separable events.

Although CAPS does not promote cell adhesion when expressed on S2 cells (Shishido et al., 1998), it is conceivable that overexpressed CAPS on motor axons changes their behavior by increasing axon-axon interactions. However, I consider this unlikely. If pan-neurally expressed CAPS increased the axon-axon interactions in a general manner, one would expect to see defasciculation defects at many other choice points along the motor pathway as has been seen in overexpression mutants of *fasciclin II* or in loss-of-function mutants of *dptp69D, dptp99A, beat* and *semaphorin I* (Lin and Goodman, 1994; Desai et al., 1996; Fambrough and Goodman, 1996; Yu et al., 1998). Instead, the phenotype in individuals that express CAPS pan-neurally is highly restricted to the most distal branch of the ISNb. Furthermore, although the stall phenotype was seen in some of the ISNb terminals in the embryos, these axons appear to eventually separate from the main ISNb. These observations suggest that pan-neurally expressed CAPS affects the pathway choice of muscle 12 MNs by influencing their proper steering events rather than defasciculation.

In this model, ectopically expressed CAPS on muscle 12 MNs affects their behavior by changing their affinity for the neighboring cells on their pathway. Since CAPS is normally expressed on muscle 12 MNs, the effect found in this study is likely to be due to an increase in the quantity of CAPS protein on the motoneurons. This notion is supported by the dose dependency of the phenotype. In wild-type embryos, at the specific choice point near muscle 30, ISNb terminals must be more attracted by the cues from muscle 13 than the external muscle layer (e.g. muscles 28, 14 and 30) to steer into the interior surface of muscle 13 (Fig. 12A). On the contrary, in embryos that express CAPS pan-neurally, they may increase their affinity for the external muscle layer, so that they could not navigate into more interior muscle layer (e.g. muscles 12 and 13). There are two kinds of phenotypes, misrouting and stall, in the embryos that express CAPS in all neurons. What makes the difference between them? In the case of misrouting phenotype, ISNb terminals may be much more attracted by the cues on muscle 30 than muscle 13, so as to be guided along the external surface of muscle 13 to reach their target, muscle 12 (Fig. 12B). On the other hand, in the case of stall phenotype, the extent of the ISNb terminals response to the attractive signals from muscle 13 and the external muscle layer may be nearly equal, so that they are trapped around at the choice point near muscle 30 (Fig. 12C). This is supported by the observation in which thin processes sometimes extend on the interior surface of muscle 13 in embryos displaying the stall phenotype. The difference between the two phenotypes may reflects the phenotypic severity. To test this idea, it is required to analyze the embryos that express much more CAPS in all neurons than in embryos used in this study. During normal development, CAPS on muscle 12 MNs could function as a receptor that

interacts with molecular cues expressed on their pathway along the external muscle layer (Fig. 13). Since CAPS is normally expressed on muscles 14 and 28 (Shishido et al., 1998, see Fig. 9A, Fig. 12), this may involve a homophilic interaction between the CAPS on motoneurons and on muscles. Alternatively, CAPS on the motoneurons may interact with an unknown ligand expressed on the muscles. To examine these possibilities, it will be required to create the lines that express CAPS in all neurons in the *caps* mutant background. In any event, the results indicate that the steering of muscle 12 MNs at the choice point near muscle 30 is highly sensitive to the amount of CAPS protein on the motoneurons. To further understand how muscle 12 MNs are properly guided along their normal pathway by CAPS mediated molecular mechanisms, it is important to identify the molecules interacting with CAPS extracellular domain.

CHAPTER II

Functional Analysis of the Intracellular Domain of Capricious in Axon Pathfinding and Selective Synapse Formation

INTRODUCTION

Neuronal growth cones are thought to be a specialized structure to sense a number of environmental cues required for axon pathfinding and target selection (Garrity and Zipursky, 1995; Tessier-Lavigne and Goodman, 1996). Several cell adhesion molecules, such as Connectin, Fasciclin III, and *D*N-cadherin, and receptor molecules, such as receptor protein tyrosine phosphatases (*D*LAR, *D*PTP69D and *D*PTP99A), Roundabout (Robo) (a receptor for Slit), and Frazzled (a receptor for Netrins), are shown to be expressed on them and play an essential role in the formation of *Drosophila* nervous system (Nose et al., 1992; Chiba et al., 1995; Iwai et al., 1997; Krueger et al., 1996; Desai et al., 1996; Kidd et al., 1998; Kolodziej et al., 1996) However, how these cell surface molecules transduce extracellular signals into the growth cones is largely unknown.

On the other hand, target cells (postsynaptic cells) are also shown to receive a variety of signals including differentiation and proliferation factors and receptor clustering factors from growth cones (presynaptic cells) during neural development. For example, in the *Drosophila* visual system, Hedgehog and the EGF-like ligand Spitz which are transported to retinal axon termini

act as anterograde signals that regulate the proliferation and differentiation of target cells in the brain (Huang et al., 1998) During synapse formation at the vertebrate neuromuscular junction, Agrin and AREA produced by motoneurons are required for the accumulation of postsynaptic components such as acetylcholin receptors (reviewed in Burden, 1998). These observations suggest that signals from axons to target cells are also important for the generation of precise neural circuits. However, little is known about the active role of target cells in the process of selective synapse formation.

CAPS is a transmembrane protein with 14 leucine-rich repeat motifs in its extracellular domain. Although its intracellular domain contains no known motif, the first 28 amino acids are highly homologous to the corresponding region of Tartan, another LRR protein in *Drosophila*. To study the function of the intracellular domain of CAPS and its possible link to the cytoskeltal and/or signal transduction machineries, I performed a deletion analysis of CAPS in this study. I ectopically expressed CAPS lacking the intracellular domain in neurons and muscles, and examined if the modified CAPS could induce the pathfinding and targeting phenotypes described in chapter I. I found that the function of muscularly expressed CAPS in target recognition is intracellular domain dependent whereas that of neurally expressed CAPS in pathfinding is not, suggesting that CAPS may function in neurons and muscles in different manners. The requirement of the intracellular domain for the function of muscularly expressed CAPS suggests the presence of a signaling event in muscles that is essential for selective synapse formation

Possible implication of these findings described above in the role of postsynaptic recognition molecules will be discussed.

MATERIALS AND METHODS

Ectopic Expression by GAL4-UAS System

To direct ectopic and increased expression of intact CAPS , $CAPS^{ID}$, and $CAPS^{ED}$, I employed the GAL4-UAS system (Fig. 6). To drive pan-muscle expression and pan-neural expression, *24B-GAL4* (Luo et al., 1994) and *elav-GAL4*^{3E1} lines (Davis et al., 1997), respectively, were crossed with individual lines carrying UAS constructs. The progenies were bred at 29 °C and 25 °C to drive high and moderate level of ectopic expression, respectively.

To obtain additional *UAS-caps* lines that induce varying levels of expression of intact CAPS, *caps-Ia* was remobilized by hybrid dysgenesis. Ten lines were established, crossed with *24B-GAL4*, and examined immunohistochemically for ectopic CAPS expression. One line that exhibited high level of expression, *UAS-caps-Ia5*, with an insertion on the third chromosome, was used in this study. The levels of ectopically expressed CAPS in the progeny generated by crossing different UAS lines with the same GAL4 driver were, *UAS-caps-Ia=UAS-caps-Ib <UAS-caps-Ia5.*

Deletion Constructs and Germline Transformation

Expression constructs, p*UAS-caps*^{ID} (a construct to express CAPS with an intracellular deletion) and p*UAS-caps*^{ED} (a construct to express CAPS with an extracellular deletion), were generated as follows. For the construction of

p*UAS-caps*^{ID}, two fragments were prepared. The NotI-PstI fragment which encodes the first 393 amino acids of CAPS was excised from a *caps* cDNA (Shishido et al., 1998). The PstI-XhoI fragment, which encodes amino acids 394-470 and a stop codon, was amplified by PCR so that PstI and XhoI sites were introduced into the 5′ and 3′ terminals, respectively. These fragments were coligated into the NotI/XhoI sites of the pUAST vector (Brand and Perrimon, 1993). The resultant construct contained the amino terminal 470 amino acids of CAPS without the intracellular domain downstream of the UAS promoter.

For the construction of p*UAS-caps*^{ED}, the following two fragments were generated by PCR. The BamHI-NotI fragment, which encodes amino acids 450-532 and a stop codon of CAPS was amplified so that the BamHI and NotI sites were introduced into the 5′ and 3′ terminals, respectively. The EcoRI-BamHI fragment, which encodes the first 71 amino acids of CAPS and a glycine, was amplified so that the EcoRI and BamHI sites were added to the 5′ and 3′ terminals, respectively. These fragments were coligated into the EcoRI/NotI sites of the pUAST vector. The resultant construct encoded CAPS lacking the region extending from the second LRR domain to the amino acid just preceding the transmembrane domain.

The individual constructs were introduced into *y, w* by germline transformation according to a standard protocol (Spradling and Rubin, 1982). Over twenty transformants for each construct were obtained. Two lines, *UAS- caps*^{ID}*-4* and *UAS- caps*^{ED}*-20*, which could induce the highest levels of ectopic expression, were used in this study.

Immunocytochemistry

Dissection and immunohistochemical staining of third instar larvae were performed as described in chapter I. mAb22c10 (Fujita et al., 1982) and mAb1D4 (Van Vactor et al., 1993) were used to visualize all motor nerves. sAbs against the intracellular and extracellular domains of CAPS (Shishido et al., 1998) were used to detect CAPS expression on muscles. Ectopically expressed intact CAPS, $CAPS^{ID}$, and $CAPS^{ED}$ on muscles were detected by fluorescence labeling. The preparations were observed under a Zeiss Axiophoto microscope.

RESULTS

Function of Neurally Expressed CAPS in Pathfinding Is Independent of its Intracellular Domain

CAPS is a transmembrane protein with a short intracellular domain which has no known functional motif (Fig. 4; Shishido et al., 1998). To examine the function of CAPS in neurons and muscles, I performed additional ectopic expression experiments using modified forms of the CAPS protein (Fig. 14). I generated two forms of CAPS deletion; $CAPS^{ID}$, which lacks the entire intracellular domain, and $CAPS^{ED}$, which lacks most of the extracellular domain extending from the second LRR domain to the amino acid just preceding the transmembrane domain (amino acids 72 to 449). Ectopic expression of these deletion constructs was induced by the GAL4-UAS system. I utilized the UAS lines, *UAS-caps^{ID}-4* and *UAS-caps^{ED}-20*, to ectopically express $CAPS^{ID}$ and $CAPS^{ED}$, respectively, in the following experiments. When crossed with GAL4 drivers, these lines induced similar levels of ectopic CAPS expression to the *UAS-caps-Ia5* line with intact CAPS. The results were also confirmed using other UAS lines for each construct (data not shown).

I first crossed the UAS lines with *elav-GAL4* line to study the ability of the deleted forms of CAPS to induce the pathfinding defects of muscle 12 MNs described above. Ectopically expressed $CAPS^{ID}$ and $CAPS^{ED}$ were detected on all major nerve tracts indicating that the modified forms of CAPS were processed and transported properly (data not shown). The effects of pan-

neuronal expression of $CAPS^{ID}$ and $CAPS^{ED}$ were studied in third instar larvae. Misexpression of $CAPS^{ED}$ caused no defects compared to controls in the trajectory of muscle 12 MNs, indicating that the extracellular domain of CAPS is essential for the induction of the misrouting phenotype (Table 1). On the other hand, when $CAPS^{ID}$ was expressed, the misrouting phenotype of muscle 12 MNs was observed to the same extent as when the intact CAPS was expressed (19 % in *elav-caps^{ID}-4* compared to 23 % in *elav-caps-Ia5* , Fig. 15A, B; Table 1). Thus, the intracellular domain of CAPS is not required for the ability of neurally expressed CAPS to cause pathfinding defects of muscle 12 MNs.

Function of Muscularly Expressed CAPS in Target Recognition Is Dependent on its Extracellular and Intracellular Domain

I next examined the effects of pan-muscle expression of the CAPS deletion constructs. When intact CAPS was ectopically expressed on all muscles, muscle 12 MNs extended their axons to and established aberrant synapses with the neighboring muscle 13, indicating that CAPS can function on muscles as a target recognition molecule (Shishido et al., 1998). I studied the requirement of the extracellular and intracellular domains of CAPS in this process by crossing the UAS lines for $CAPS^{ID}$ and $CAPS^{ED}$ with *24B-GAL4* drivers. Pan-muscle expression of $CAPS^{ID}$ and $CAPS^{ED}$ was confirmed by staining with sAbs against CAPS (Shishido et al., 1998). When intact CAPS was ectopically expressed in all muscles, the protein was not uniformly distributed on the muscle surface but was strongly concentrated at

neuromuscular synaptic sites. $CAPS^{ID}$ and $CAPS^{ED}$ were similarly localized at synaptic boutons when expressed in muscles, indicating that the deleted regions play no role in the synaptic localization of the protein (Fig. 16A-D).

I examined if the deletion constructs can cause the mistargeting phenotype of muscle 12 MNs by staining the third instar larvae of *24B-caps^{ID}-4 , and 24B-caps^{ED}-20.* In the control larvae that misexpressed intact CAPS at a similar level (*24B-caps-Ia5*), terminals of muscle 12 MNs formed ectopic synapses on muscle 13 in 40% of segments (Fig. 15C, Table 2). In contrast, in *24B-CAPS^{ID}-4* and *24B-caps^{ED}-20* larvae, the extent of targeting errors by muscle 12 MNs was dramatically decreased to the background level (Fig. 15D, Table 2). Thus, both extracellular and intracellular domains are required for muscle CAPS to mediate target recognition. This is in contrast with the observations in relation to pan-neurally expressed CAPS, where the intracellular domain is not essential for inducing the misrouting phenotype (The effects of neurally and muscularly expressed $CAPS^{ID}$ and $CAPS^{ED}$ are summarized in Fig. 17A-C). These results suggest that neurally expressed CAPS and muscularly expressed CAPS may function in a different manner.

DISCUSSION

CAPS is a transmembrane protein with a short intracellular domain which has no known functional motif (Shishido et al., 1998). In this study, I examined the requirement of the intracellular domain of CAPS in newly discovered function of CAPS in axon guidance (chapter I) and in its previously described function in target recognition (Shishido et al., 1998).

The data from the deletion analysis showed that pan-neural expression of $CAPS^{ID}$ is as potent as that of the intact CAPS in inducing the pathfinding defects, indicating that the intracellular domain is not required for this function of the molecule. Thus, if CAPS functions as a receptor in muscle 12 MNs as proposed in chapter I, it must transduce the signal by interacting with other molecules on the membrane (Fig. 18). The possible signals mediated by neuronal CAPS appear to be different from those by Dlar, a receptor protein tyrosine phosphatase, which is required for the proper axon pathfinding, because overexpressed CAPS has a dose dependent effect as described in chapter I, but overexpressed Dlar no effect. This suggests that the receptor complex including CAPS may use other signaling systems than phosphorylation.

In a previous study, Shishido et al. showed that when CAPS is expressed in all muscles, muscle 12 MNs form ectopic synapses on muscle 13 (Shishido et al., 1998). Since CAPS is normally expressed on muscle 12 but not on muscle 13, it was proposed that CAPS functions as a specific molecular label on muscle 12 to be recognized by muscle 12 MNs. Three other candidate target recognition molecules, Connectin, Fasciclin III and Toll are similarly expressed on the surface of a subset of muscles (Nose et al., 1992, 1997; Chiba

et al, 1995; Kose et al., 1997; Rose et al., 1997). How do these molecules mediate selective synapse formation? Are they simply presented by muscles as ligands that are passively recognized by motoneurons? Or, do they play more active roles in the initiation of synapse formation? To obtain insights on CAPS function in selective synapse formation, in this study, I studied the effect of deletion of its intracellular domain. I found that the ability of pan-muscularly expressed CAPS to induce targeting errors by muscle 12 MNs was completely abolished when the intracellular domain was deleted. This is not due to nonspecific conformational change of the molecule, because the $CAPS^{ID}$ retained its activity when ectopically expressed on neurons. Furthermore, pan-muscularly expressed $CAPS^{ID}$ was found to be present at synaptic sites as was intact CAPS, suggesting that the deletion does not affect the transportation or localization of the protein. Taken together, the results suggest that some molecular event(s) in muscles mediated by intracellular domain of CAPS are required for the ectopic synapse formation.

In what way might the intracellular domain of CAPS be critical for its function? One possible role played by the intracellular domain could be its interaction with cytoplasmic components necessary for synaptic localization. Such a role was shown for the intracellular domain of Fasciclin II, a homophilic cell adhesion molecule essential for the growth and maintenance of neuromuscular junctions (Thomas et al., 1997; Zito et al., 1997). Indeed, there is evidence for the existence of synaptic targeting mechanisms for CAPS. The CAPS protein is normally localized on newly formed synaptic boutons in subsets of muscles in the first instar larvae (Shishido et al., 1998). I found in this study that ectopically expressed CAPS is concentrated at neuromuscular synaptic sites of third instar larvae. However, the intracellular domain

appears to be unnecessary for this process, since $CAPS^{ID}$ is normally concentrated at synaptic sites. Thus, the essential role played by the intracellular domain in selective synapse formation is not in the synaptic targeting of the molecule. Interestingly, $CAPS^{ED}$ which lacks most of the extracellular domain was also normally localized at synapses. Thus, the functional domain necessary for synaptic targeting may be in the transmembrane domain. Alternatively, there may be multiple targeting systems that act both through the intracellular and extracellular domains of CAPS.

Another possible role of the intracellular domain could be signaling events required for selective postsynapse formation. Recently, cell biological evidence is accumulating to support the notion that postsynaptic cells play active roles in synapse formation. During axo-dendritic synaptogenesis in vertebrates, dendritic filopodia and their protrusive motility are proposed to be essential for the initiation of synaptic contacts (e.g., Ziv and Smith, 1996; Saito et al., 1997). Similarly during neuromuscular synaptogenesis in *Drosophila*, muscles extend fine processes (called myopodia) that actively interact with motoneuronal growth cones (Akira Chiba, personal communication). It is an interesting possibility that CAPS on muscles participate in such processes by interacting with receptors on specific motoneurons via its extracellular domain and by transmitting the signal into muscles via its intracellular domain (Fig. 19). Although the exact nature of the signal mediated by the intracellular domain of CAPS is still unknown, the present study supports the notion that molecular events occurring in postsynaptic cells are essential for the formation of specific synapses (Fig. 20).

If this is the case, muscular CAPS could acts as a bidirectional signaling molecule, serving both as a ligand for the MN and a receptor (Fig. 20). Interestingly, in the developing vertebrate nervous system, there is also biochemical evidence that shows the presence of bidirectional signaling . Ephrins and Eph receptors are receptor-ligand systems well known for their involvement of neural development (reviewed in Brückner and Klein, 1998; Flanagan and Vanderhaeghen, 1998). The EphB2 extracellular domain can cause tyrosine phosphorylation of Ephrin-B class transmembrane ligands in vitro through unknown protein tyrosine kinases (Holland et al., 1996; Brückner et al., 1997). Moreover the possibility that Ephrin transduces signals in vivo is also supported by a genetic study. Mice without EphB2 show axon guidance errors in the anterior commissure, but this errors are never seen in the mice lacking only its kinase domain, suggesting that Ephrin functions as a receptor for Eph (Henkemeyer et al., 1996). Thus, it has been speculated that Ephs and Ephrins carry signals mutually when topographic connections between retina and tectum are formed. It is an interesting possibility that muscular CAPS and its neuronal receptor function in a similar manner during the formation of *Drosophila* neuromuscular connectivity.

Future studies about CAPS signaling including the identification of the molecules that interact with the CAPS intracellular domain might lead to the general understanding for the molecular mechanisms of the selective synapse formation.

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Figure 1 Basic mechanisms of axon pathfinding (A) and target recognition (B). During the formation of the neural circuits, neuronal growth cones traverse long distances along stereotyped pathways to their appropriate target regions . Then, they find and synapse with their specific target(s), by searching over many neighboring cells in the target region. In these processes, growth cones are guided by repulsive cues (shown in -) from the extra-pathway, non-target region, and non-target cells and attractive cues (shown in +) from the pathway, target region, and target cells.

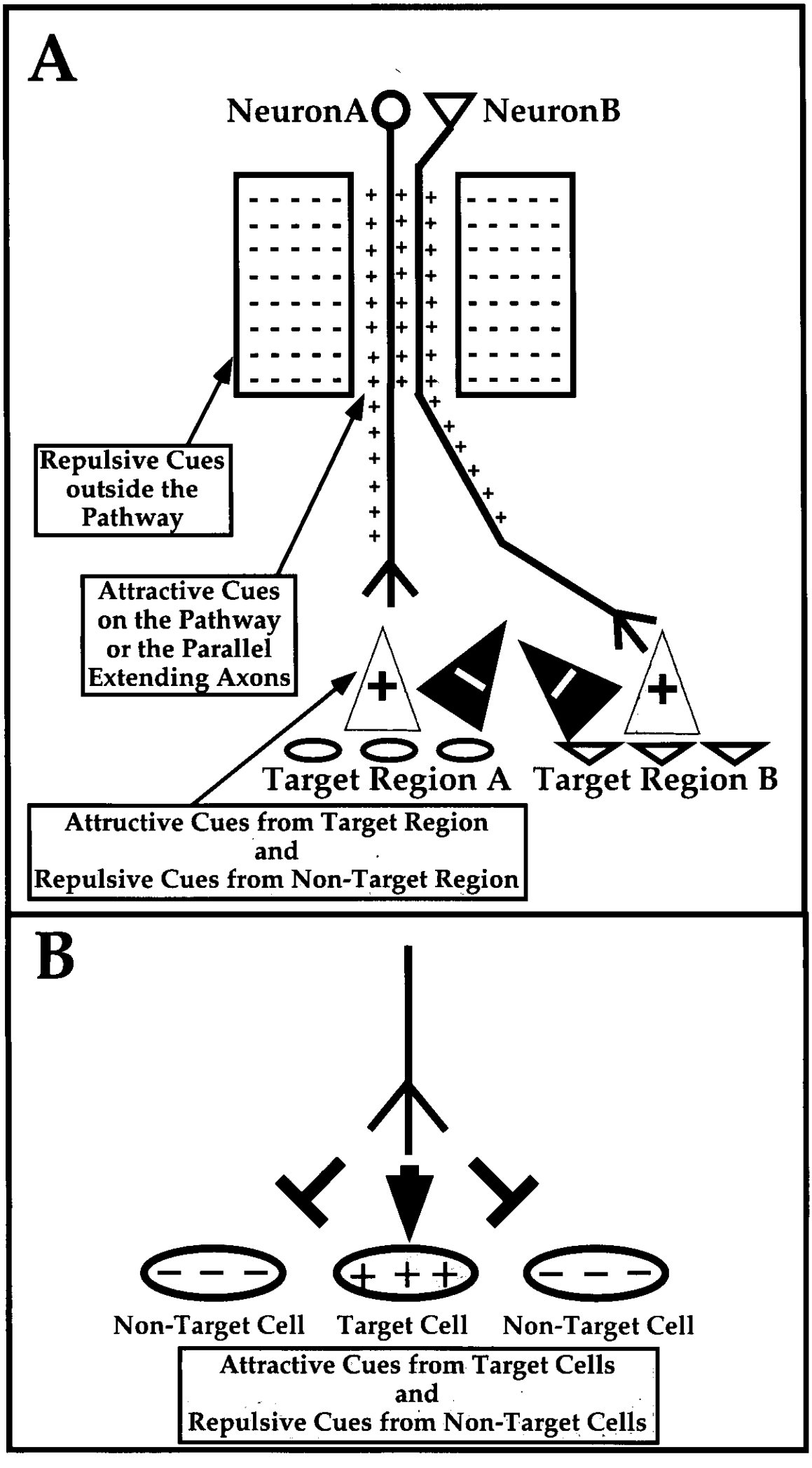
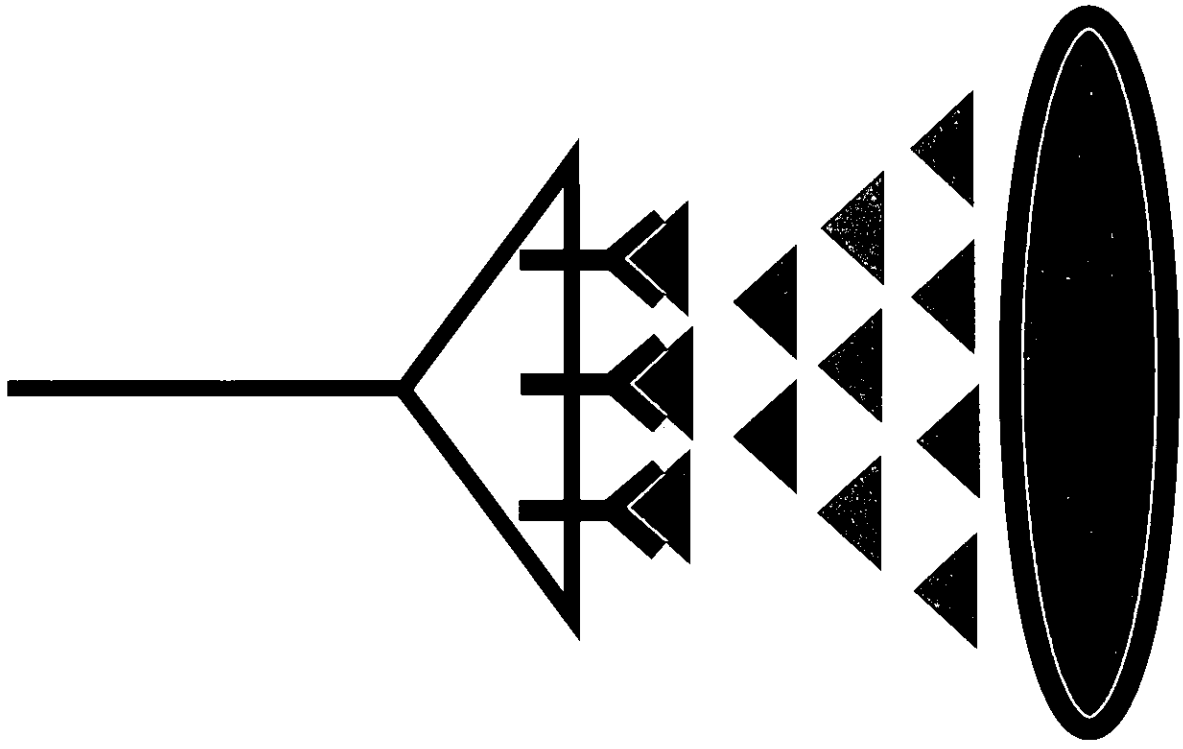

A
NeuronA
NeuronB
Repulsive Cues outside the Pathway
Attractive Cues on the Pathway or the Parallel Extending Axons
Target Region A
Target Region B
Attructive Cues from Target Region and Repulsive Cues from Non-Target Region
B
Non-Target Cell
Target Cell
Non-Target Cell
Attractive Cues from Target Cells and Repulsive Cues from Non-Target Cells

Figure 2 Schematic diagrams showing two modes of action of axon guidance molecules. Guidance molecules can act either as long range cues in the form of secreted proteins (A) or as short range cues in the form of membrane-bound proteins (B).

A



B

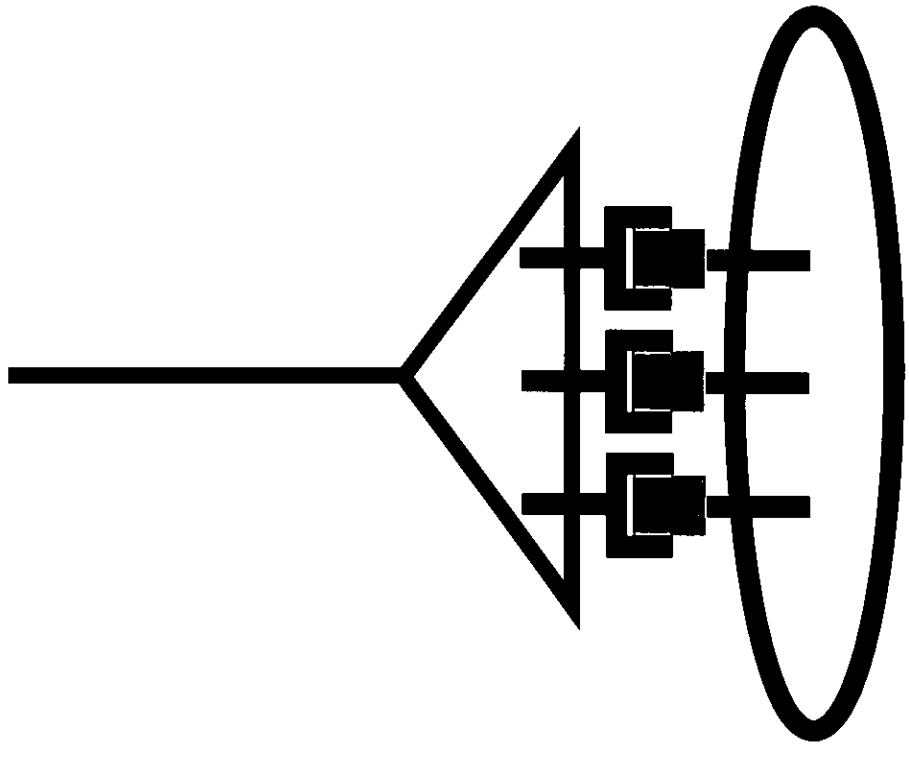


Figure 3 Schematic diagrams showing expression of Con and CAPS in the neuromuscular system of *Drosophila* embryos. In each abdominal hemisegment of *Drosophila* embryos and larvae, ~40 motoneurons innervate 30 muscles in a specific and stereotypic manner (reviewed in Keshishian et al., 1996). Motor axons exit the CNS in two different motor nerves, the intersegmental nerve (ISN) and the segmental nerve (SN). They then make appropriate pathfinding decisions along their pathway within five peripheral motor branches (ISN, ISNb, ISNd, SNa, and SNc) that innervate distinct groups of muscle fibers. Con is expressed on the surface of muscles 18, 21-24, 27, and 29 (gray) and a subset of neurons (black), including the motoneurons that innervate them (SNa and SNc motoneurons). CAPS is expressed in four dorsal (1, 2 ,9 and 10) and six ventral (12, 14-17 and 28) muscles (gray). It is also expressed in a small subset of motoneurons (black) including identified motoneurons, aCC, RP2, RP5 and U, all of which innervate *caps* positive muscles.

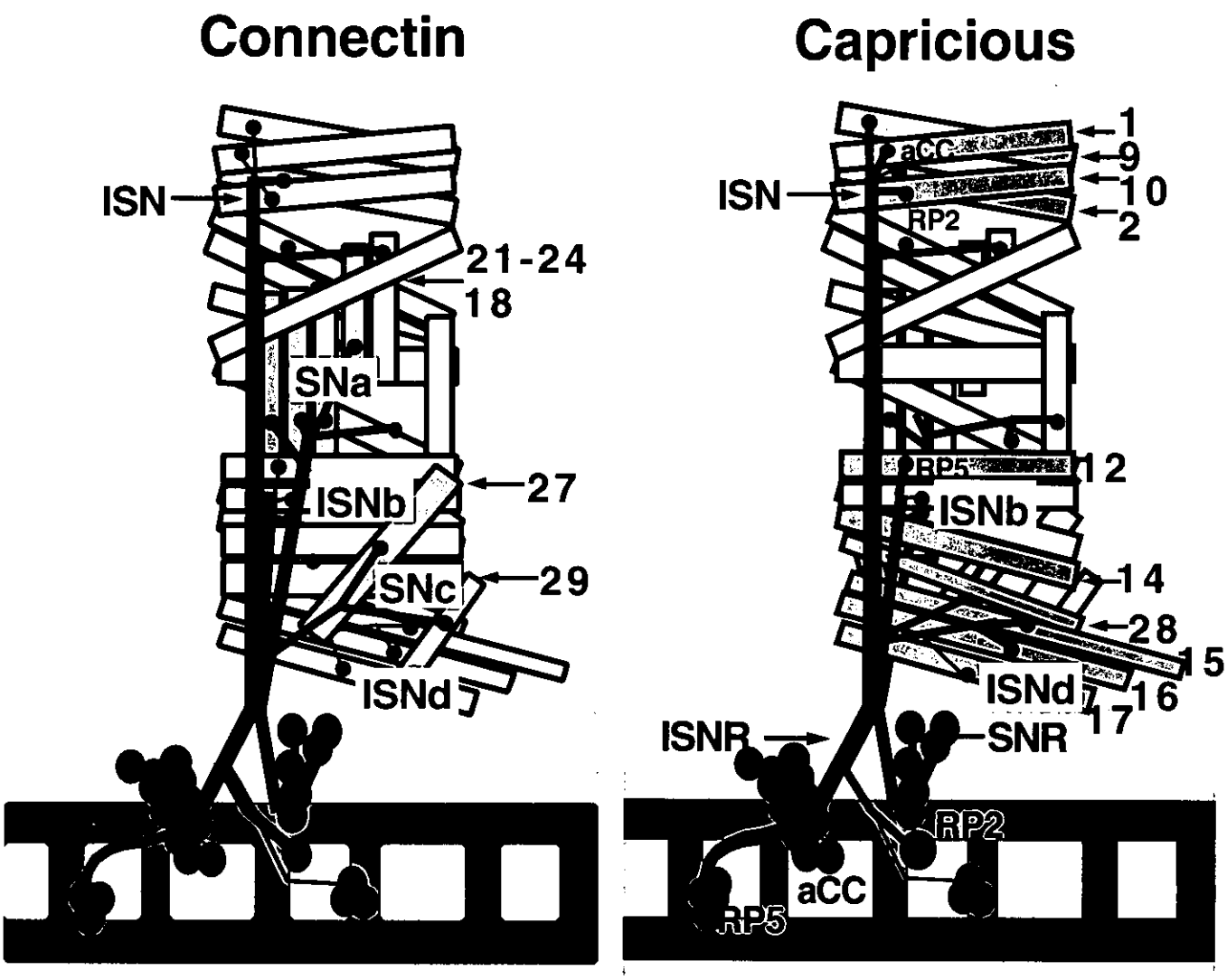

Connectin
ISN
21-24
18
SNa
ISNb
27
SNc
29
ISNd
Capricious
ISN
aCC
RP2
1
9
10
2
RP5
12
ISNb
14
28
15
ISNd
16
17
ISNR
SNR
RP2
aCC
RP5

Figure 4 Schematic diagram showing the structure of *Drosophila* LRR proteins. The black boxes denote signal peptides and gray boxes indicate amino- and carboxi- flanking regions. The gray ovals show LRR motifs. CAPS is highly homologous to Tartan.

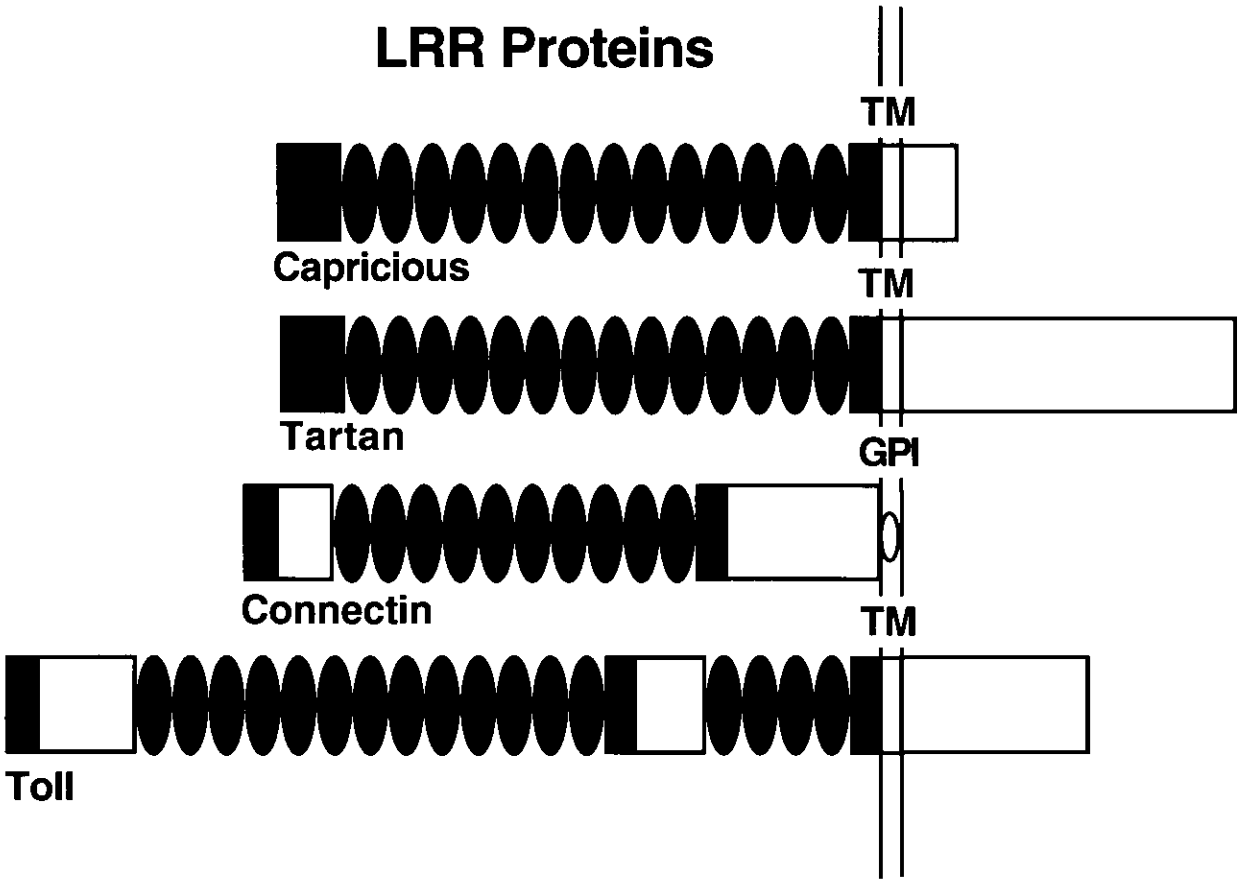
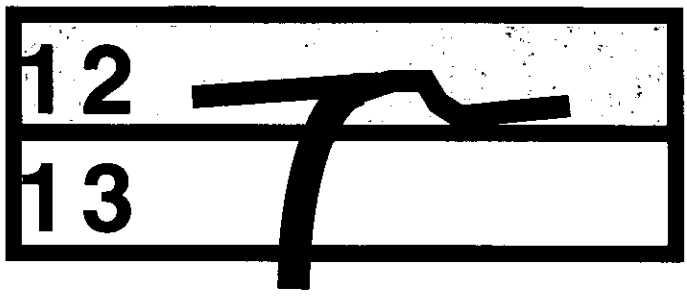
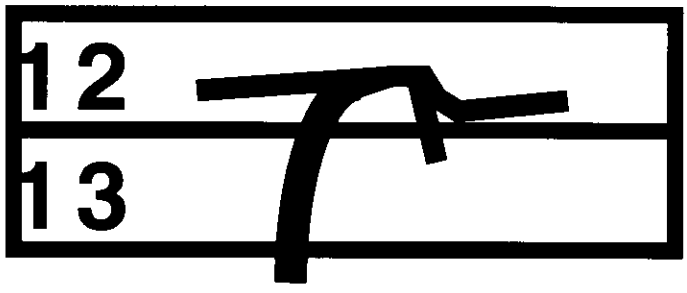

LRR Proteins
TM
Capricious
TM
Tartan
GPI
Connectin
TM
Toll

Figure 5 Schematic diagrams summarizing the phenotypes of *caps* loss-of-function and gain-of-function mutants in the neuromuscular specificity. In wild type, *caps* is expressed on muscle 12 (gray), but not muscle 13 (white) and the synapses formed by the neurons that innervate muscle 12 (muscle 12 MNs) are restricted on the surface of muscle 12 (A). On the other hand, in loss-of-function mutant, the muscle 12MNs often form synaptic endings on a neighboring nontarget muscle, muscle 13, in addition to its normal target (B). A similar and more robust phenotype was observed when CAPS is ectopically expressed on all muscles (C).

A Wild-Type



B Loss-of-Function



C Gain-of-Function

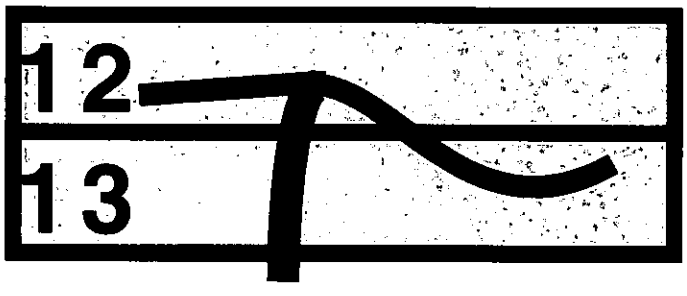


Figure 6 Schematic diagram showing GAL4-UAS system. The GAL4 is a yeast transcription factor that binds to the specific upstream activation sequence (*UAS*) and promotes to transcribe its downstream gene. There are many GAL4 lines generated by randomly inserting a P element containing a *GAL4* gene fused to a minimal promotor into the genome. The GAL4 protein is produced by the enhancer control sequences near the P element insertion site in a stage and tissue specific manner. For instance, the GAL4 lines, 24B and elav, express GAL4 protein in all muscles and all neurons, respectively. Thus, by crossing the appropriate GAL4 line with the UAS line which bears the exogenous gene we analyze combined downstream to the *UAS*, we can express any genes in a specific temporal and spatial pattern.

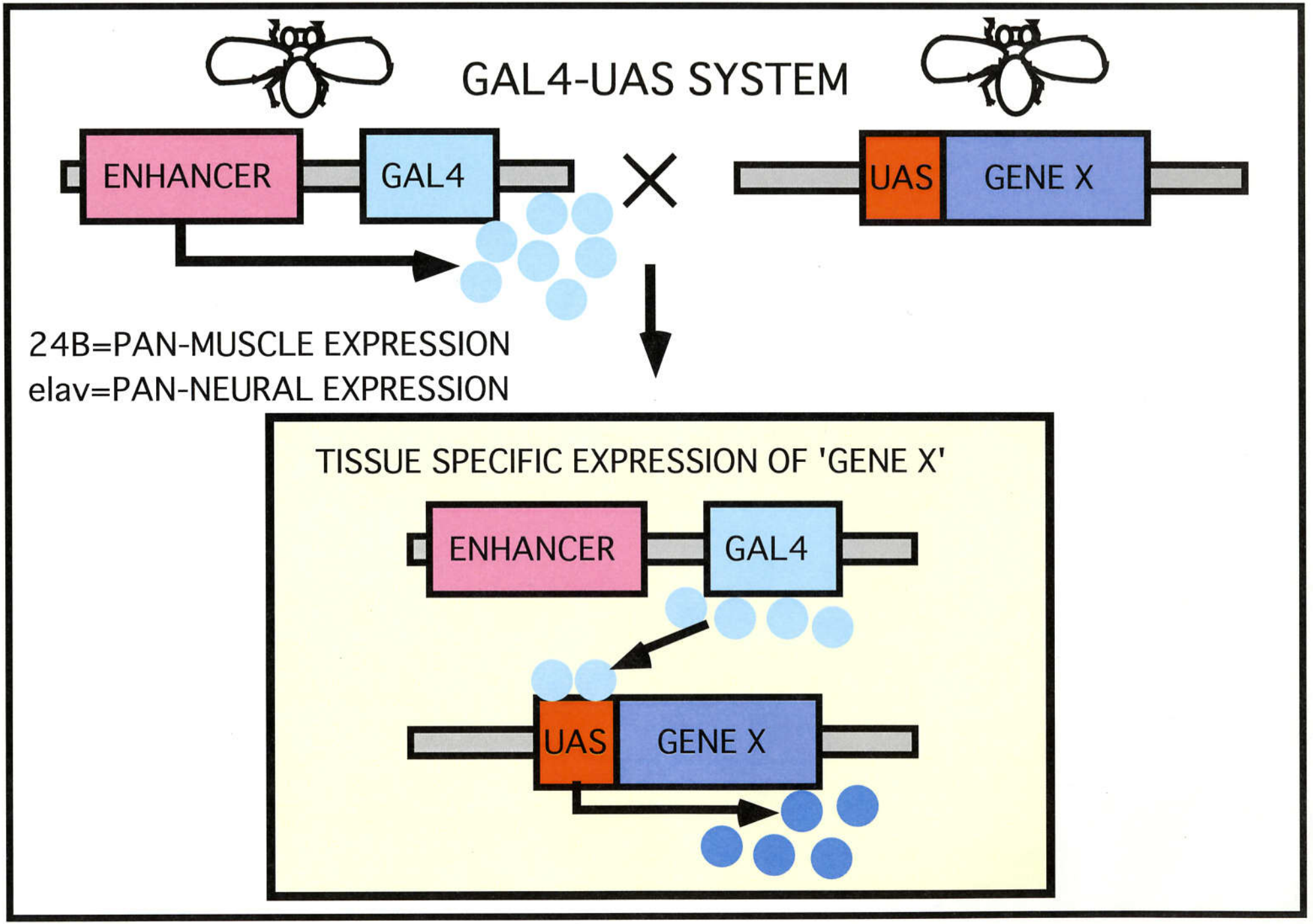

GAL4-UAS SYSTEM
ENHANCER
GAL4
UAS
GENE X
24B=PAN-MUSCLE EXPRESSION
elav=PAN-NEURAL EXPRESSION
TISSUE SPECIFIC EXPRESSION OF 'GENE X'
ENHANCER
GAL4
UAS
GENE X

Figure 7 Pan-neural CAPS expression by the GAL4-UAS system. Expression of CAPS protein in stage 16 wild-type (A) and *elav-caps-Ia+Ib* (B, C) embryos, detected by staining with a sAb against the intracellular domain of CAPS. (A, B) ventral views; (C) a lateral view. (A) In a stage 16 wild-type embryo, CAPS protein is expressed on subsets of CNS axons. (B, C) In an *elav-caps-Ia+Ib* embryo, strong CAPS protein expression is detected in all major axonal tracts in the CNS (B) and in the periphery (C). Scale bar, 175 μm.

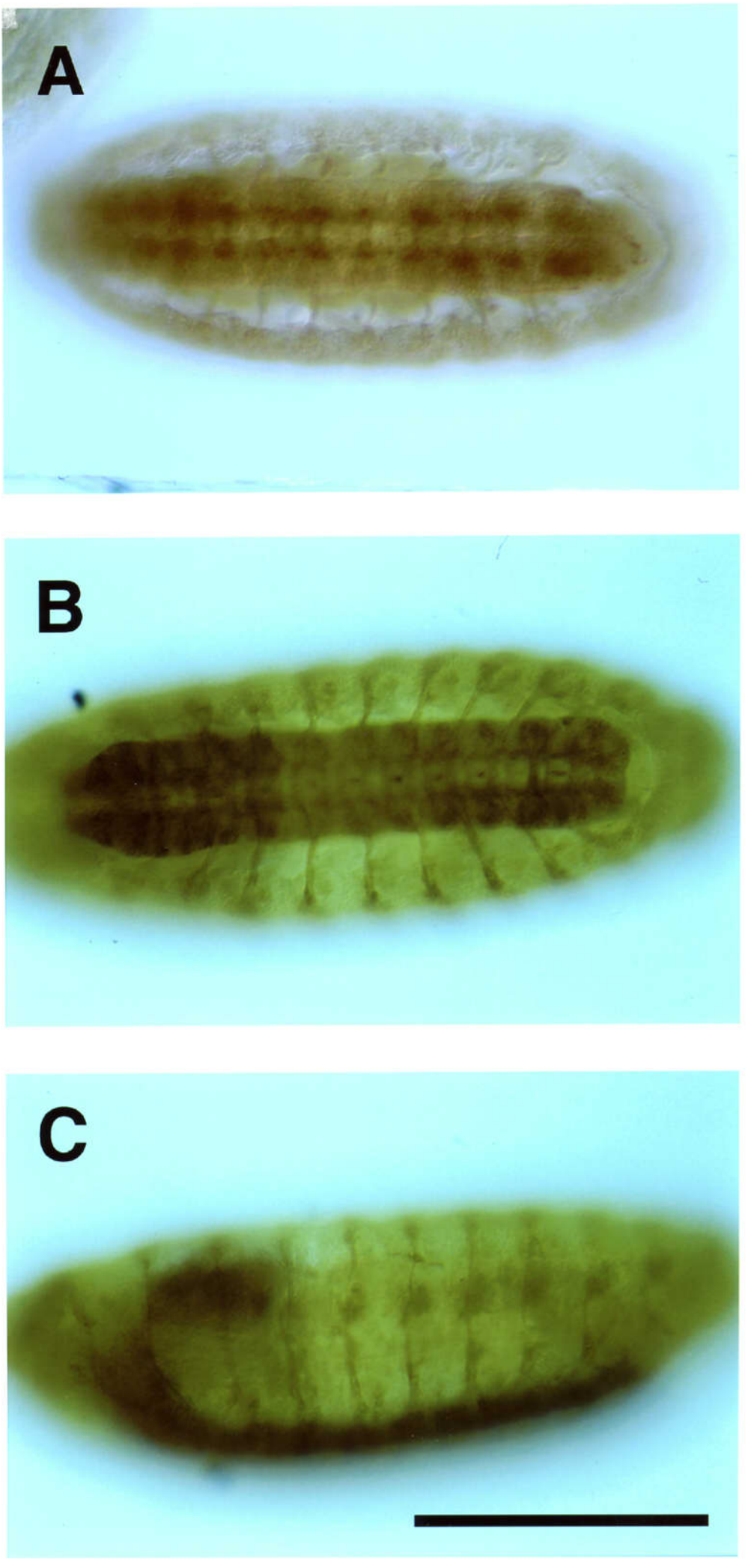

A
B
C

Figure 8 Abnormal motor nerve projection in third instar larvae caused by pan-neuronal expression of CAPS. Body-wall fillets of wild-type (A) and *elav-caps-Ia+Ib* (B) third instar larvae, stained with mAb 1D4 and 22C10 to visualize motoneuron projections and synaptic endings. In a wild-type larva (A), the terminal branch of ISNb containing axons of muscle 12 MNs passes along the internal surface of muscle 13 (arrow) and establishes synapses with muscle 12 (arrowheads). In contrast, in a *elav-caps-Ia+Ib* larva (B), the nerve branch passes along the exterior of muscle 13 (arrow). In spite of the altered pathway, the nerve branch establishes morphologically normal synapses with muscle 12 as in wild-type larvae (arrowheads). Scale bar, 140 μm.

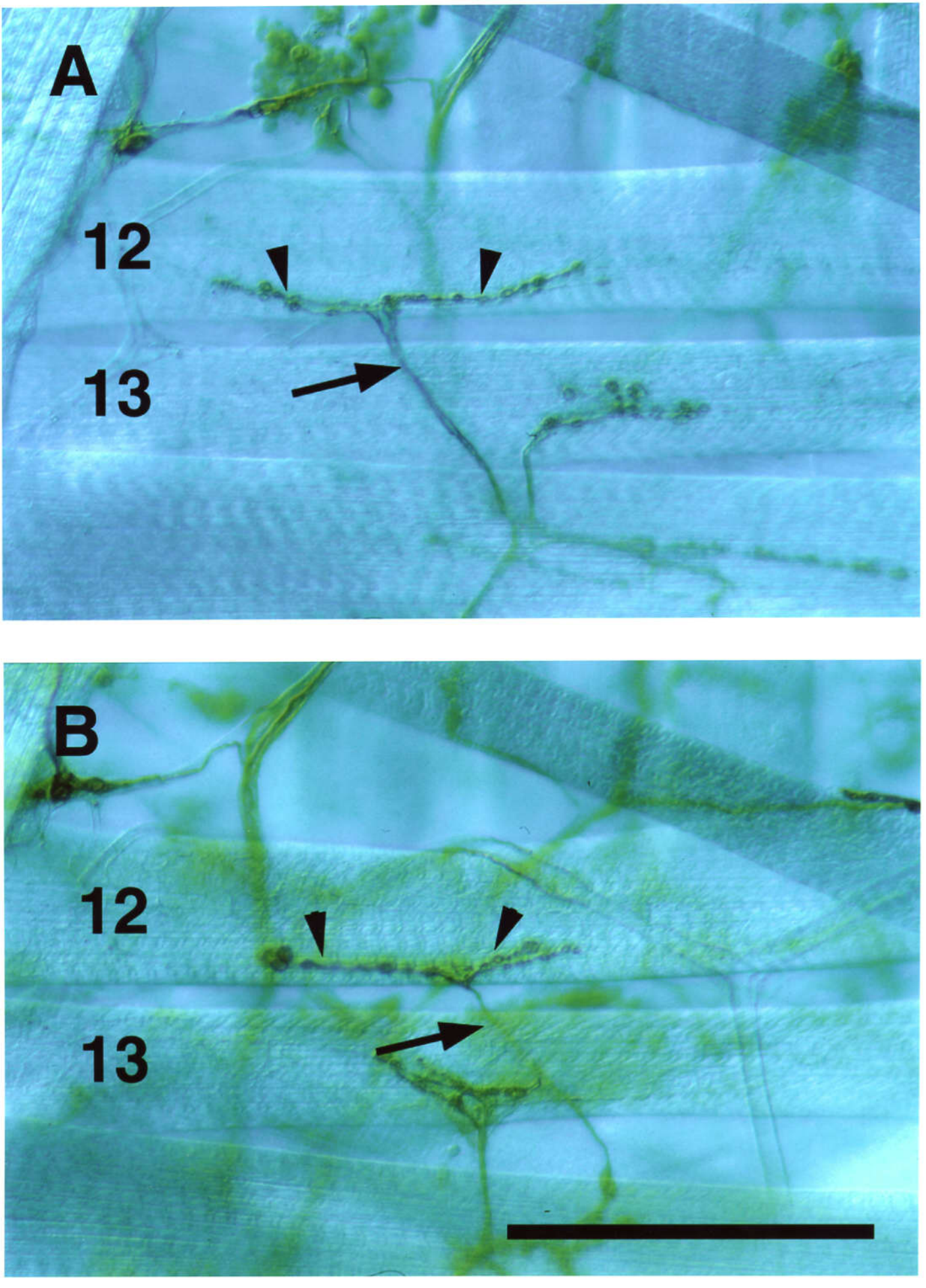

A
12
13
B
12
13

Figure 9 Schematic diagrams summarizing the effect of pan-neural CAPS expression on ISNb projection in embryos. Cross-sectional schematic diagrams showing the pathway and innervation pattern of ISNb in early stage 17 wild-type (A) and *elav-caps* (B) embryos. The interior of an embryo is to the left, dorsal is to the top. In wild-type embryos (A), ISNb axons separate from ISN and enter the ventrolateral muscle field near muscle 28. They then extend between the external surface of muscle 6 and 7 and the internal surface of muscle 14. At muscle 30, the ISNb moves towards the internal surface of muscle 13. By early stage 17, the distal end of ISNb reaches muscle 12 and begins to form synapses. (B) In *elav-caps* embryos, the distal branch of ISNb often shows a misrouting phenotype (misroute) in which it travels along the external surface of muscle 13, or a stall phenotype (stall), in which it stalls prematurely near muscle 30. Muscles that endogenously express CAPS are shaded light gray.

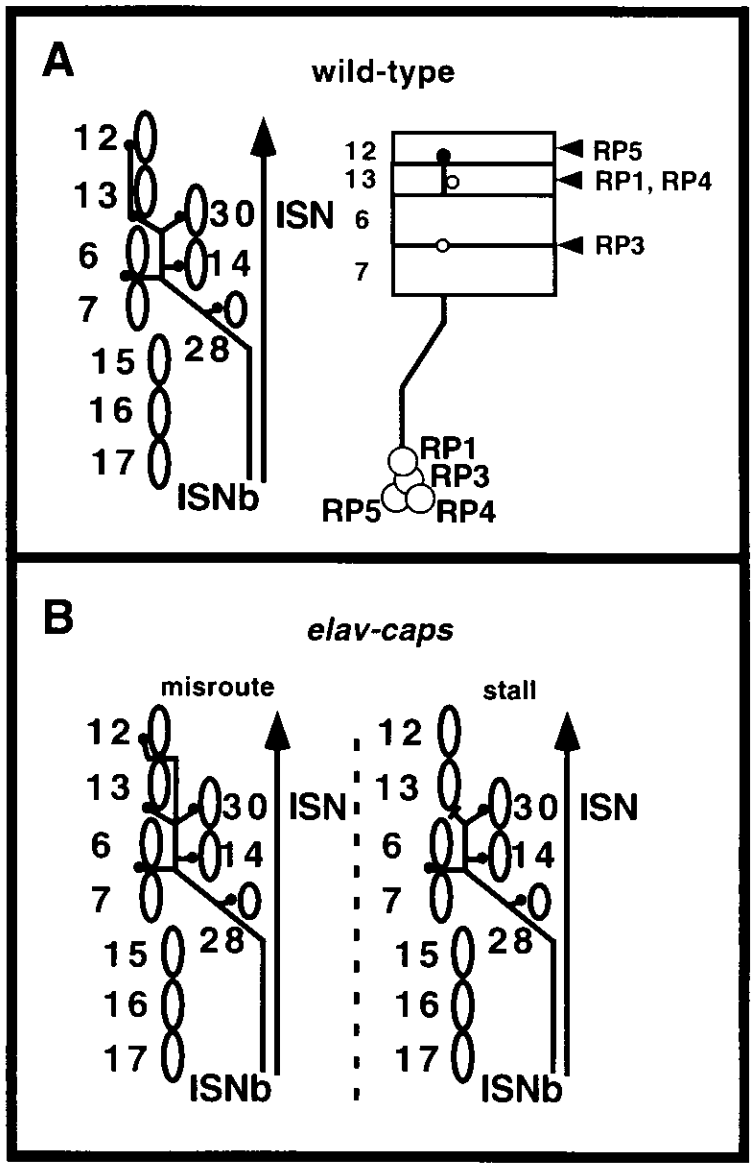

A
wild-type
12
13
6
7
15
16
17
30
14
28
ISN
ISNb
12
13
6
7
RP5
RP1, RP4
RP3
RP1
RP3
RP5
RP4
B
elav-caps
misroute
stall
12
13
6
7
15
16
17
30
14
28
ISN
ISNb
12
13
6
7
15
16
17
30
14
28
ISN
ISNb

Figure 10 Abnormal ISNb development in *elav-caps-Ia+Ib* embryos. Body-wall fillets of early stage 17 wild-type (A) and *elav-caps-Ia+Ib* (B-E) embryos, stained with mAb 1D4 to visualize the anatomy of the peripheral motor axon pathways and terminals. Muscles 12, 13, 6, 7, 30 and 14 are numbered on each panel. (A) Normal pattern of ISNb trajectory and terminals. The terminal branch of the ISNb passes along the internal surface of muscle 13. By this stage, the nerve forms bifurcated endings along the proximal edge of muscle 12 (arrows). (B,C) Three segments of an *elav-caps-Ia+Ib* embryo at different focal planes. The focus is on muscles 12, 13, 6 and 7 in (B), and on muscles 30 and 14 in (C). In the right segment, the ISNb passes along the external surface of muscle 13 (asterisks in B, C). Note that despite this abnormal trajectory, the nerve forms morphologically normal endings on muscle 12. (D, E) Three segments of another *elav-caps-Ia+Ib* embryo, focused on muscles 12, 13, 6 and 7 (D) and muscles 30 and 14 (E). In the left segment, the ISNb completely stalls at muscle 30 (white arrowheads in D, E) with no axonal processes extending to muscle 12. In the middle segment, the main ISNb branch appears to stall. However, a thin axonal process is seen to extend towards muscle 12 (black arrowhead in D). In the left and middle segments of B and C, and in the right segment of D and E, normal pattern of ISNb projection and termination is seen. Scale bar, 15 μm.

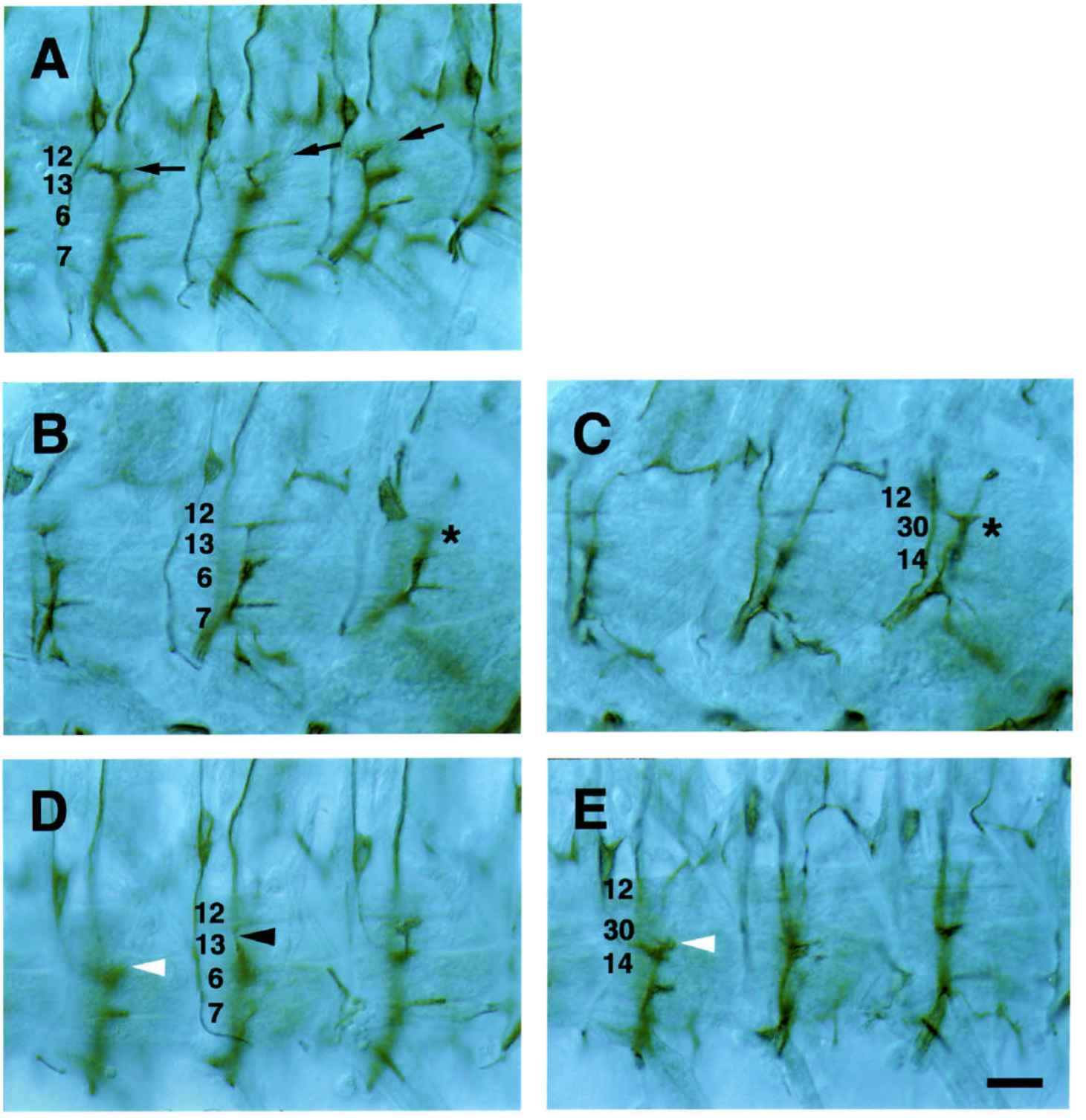
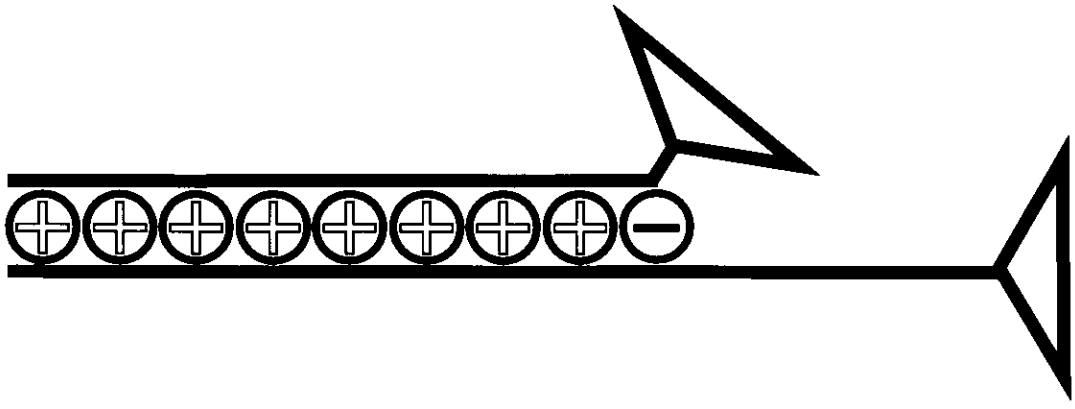

A
12
13
6
7
B
12
13
6
7
*
C
12
30
14
*
D
12
13
6
7
E
12
30
14

Figure 11 Schematic diagrams showing the mechanisms for changing axonal trajectory at specific choice points. Axons have to complete two steps, defasciculation from the main nerve tract (A) and steering into the specific target region (B), in order to successively change their trajectory at discrete choice points along the motor pathway (e.g., Lin et al., 1994; Desai et al., 1996, 1997; Krueger et al., 1996; Fambrough and Goodman, 1996; Yu et al., 1998). The defasciculation event would mainly relate to changes in axon-axon interactions, while the steering event relate to changes in axon-target region interactions.

A. Defasciculation
(Related to Axon-Axon interactions)



B. Steering
(Related to Axon-Target Region interactions)

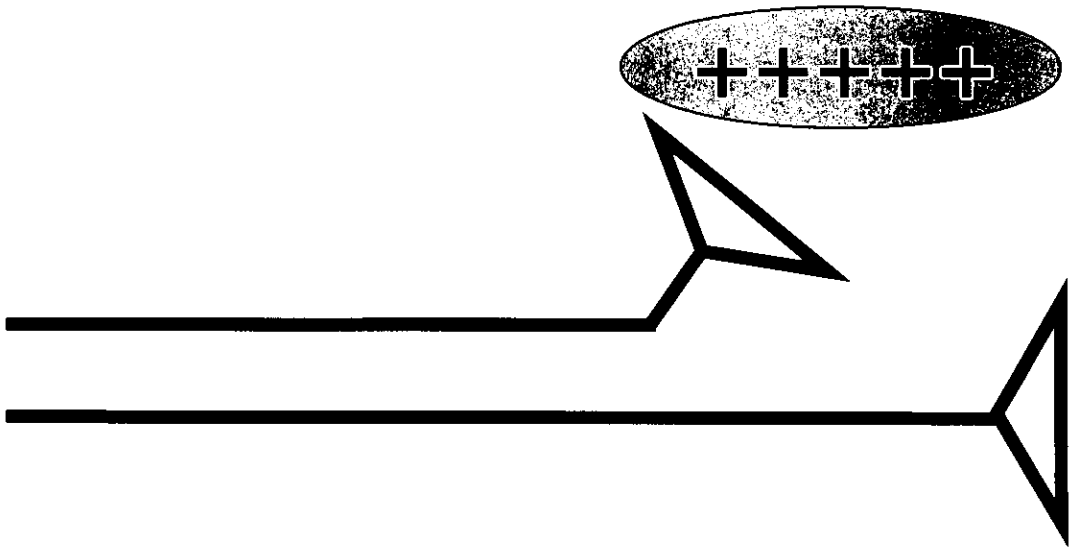


Figure 12 Schematic diagrams showing putative cause for the defects in axonal navigation of gain-of-function mutants that express *caps* in all neurons. In wild-type embryos, at the specific choice point near muscle 30, ISNb terminals must be more attracted by the cues from muscle 13 than the external muscle layer (e.g. muscles 28, 14 and 30) to steer into the interior surface of muscle 13 (A). On the contrary, in the case of misrouting phenotype, ISNb terminals may be much more attracted by the cues on muscle 30 than muscle 13, which enables them to extend externally to muscle 13 to reach their target, muscle 12 (B). In the case of stall phenotype, the extent of the ISNb terminals response to the attractive signals from muscle 13 and the external muscle layer may be nearly equal, so that they are trapped around at the choice point near muscle 30 (C). The difference between two phenotypes may reflects the phenotypic severity.

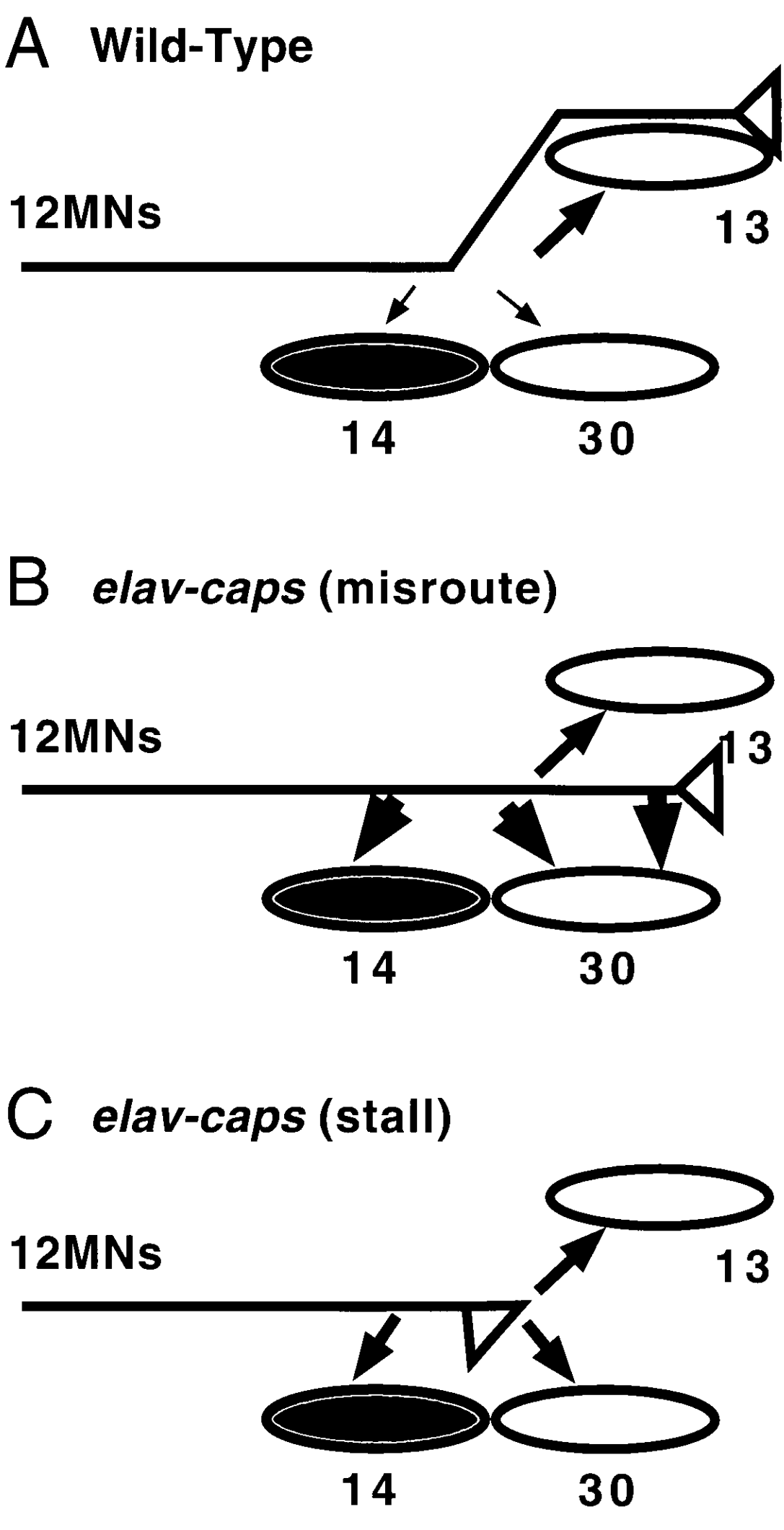

A Wild-Type
12MNs
13
14
30
B elav-caps (misroute)
12MNs
13
14
30
C elav-caps (stall)
12MNs
13
14
30

Figure 13 Schematic diagram showing the possible role of neuronal CAPS in axon pathfinding during normal development. CAPS on muscle 12 MNs could function as a receptor that interacts with molecular cues expressed on their pathway along the external muscle layer (e.g. muscles 14 and 30).

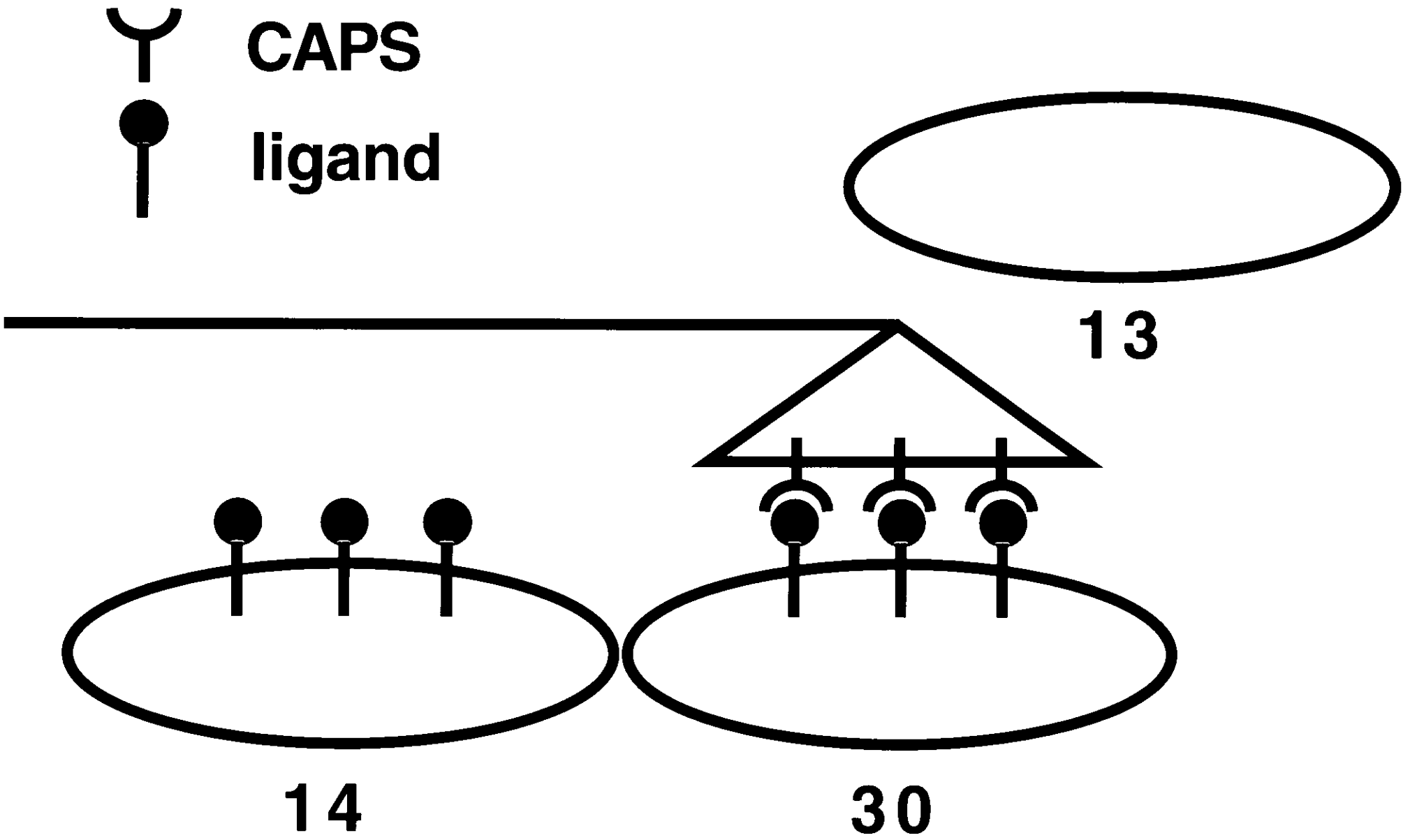

CAPS
ligand
13
14
30

Figure 14 Schematic diagram of the structure of the deletion constructs. Hatched rectangles, LRR motifs; gray boxes, carboxy-terminal- and amino-terminal-flanking regions; black boxes, signal peptides; horizontally lined boxes, transmembrane domains.

Deletion Constructs

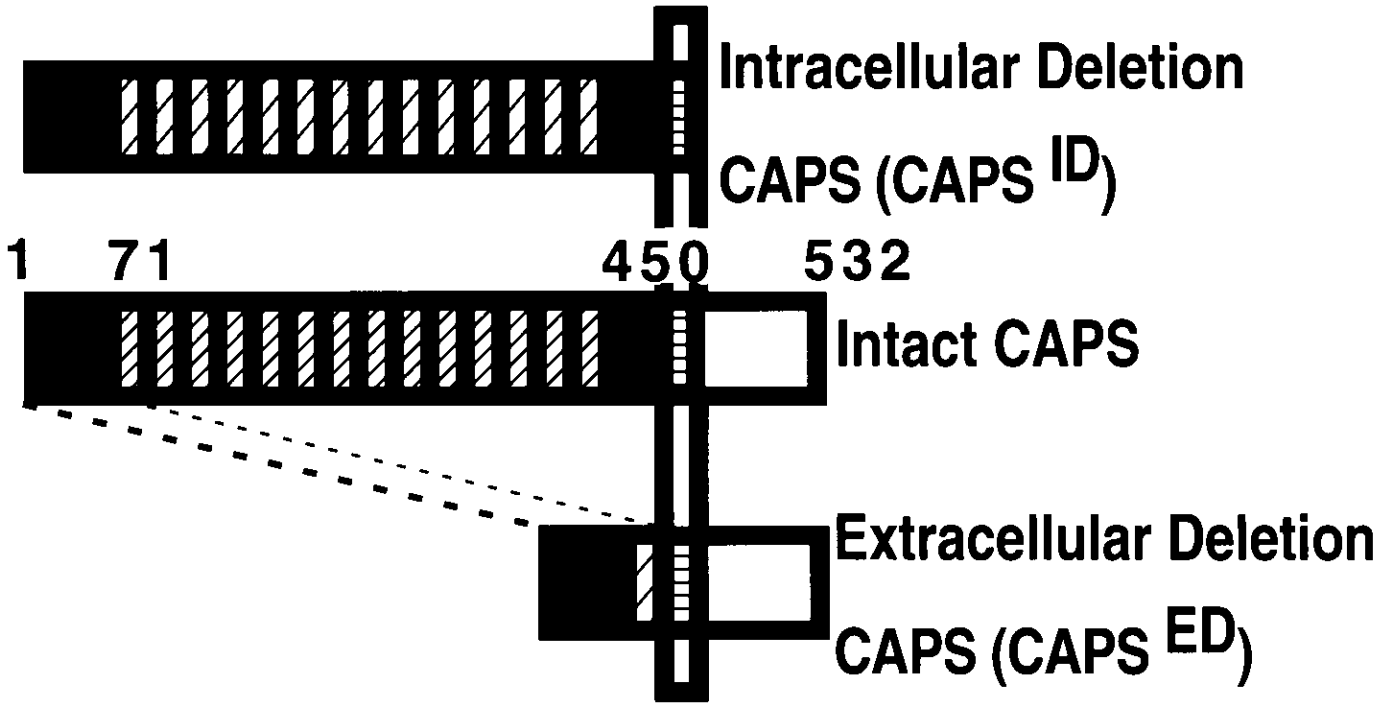


Figure. 15 Pan-neural and pan-muscle expression of modified CAPS and their effects on the pathway and targeting of the distal branch of ISNb. Body wall fillets of *elav-caps -Ia5* (A), *elav-ID-caps -4*(B), *24B-caps -Ia5* (C) and *24B-ID-caps-4* (D) larvae, stained with mAb 1D4 and 22C10. (A, B) Both intact CAPS (A) and ID-CAPS (B) can cause the misrouting phenotype of muscle 12 MNs (arrows) when overexpressed on the nerve. (C, D) Pan-muscle expression of intact CAPS (C) induces the mistargeting phenotype in which muscle 12 MNs establish ectopic synapses with muscle 13 (arrow). Such a phenotype is not observed when ID-CAPS is expressed in the same manner (D). Scale bar, 100 μm.

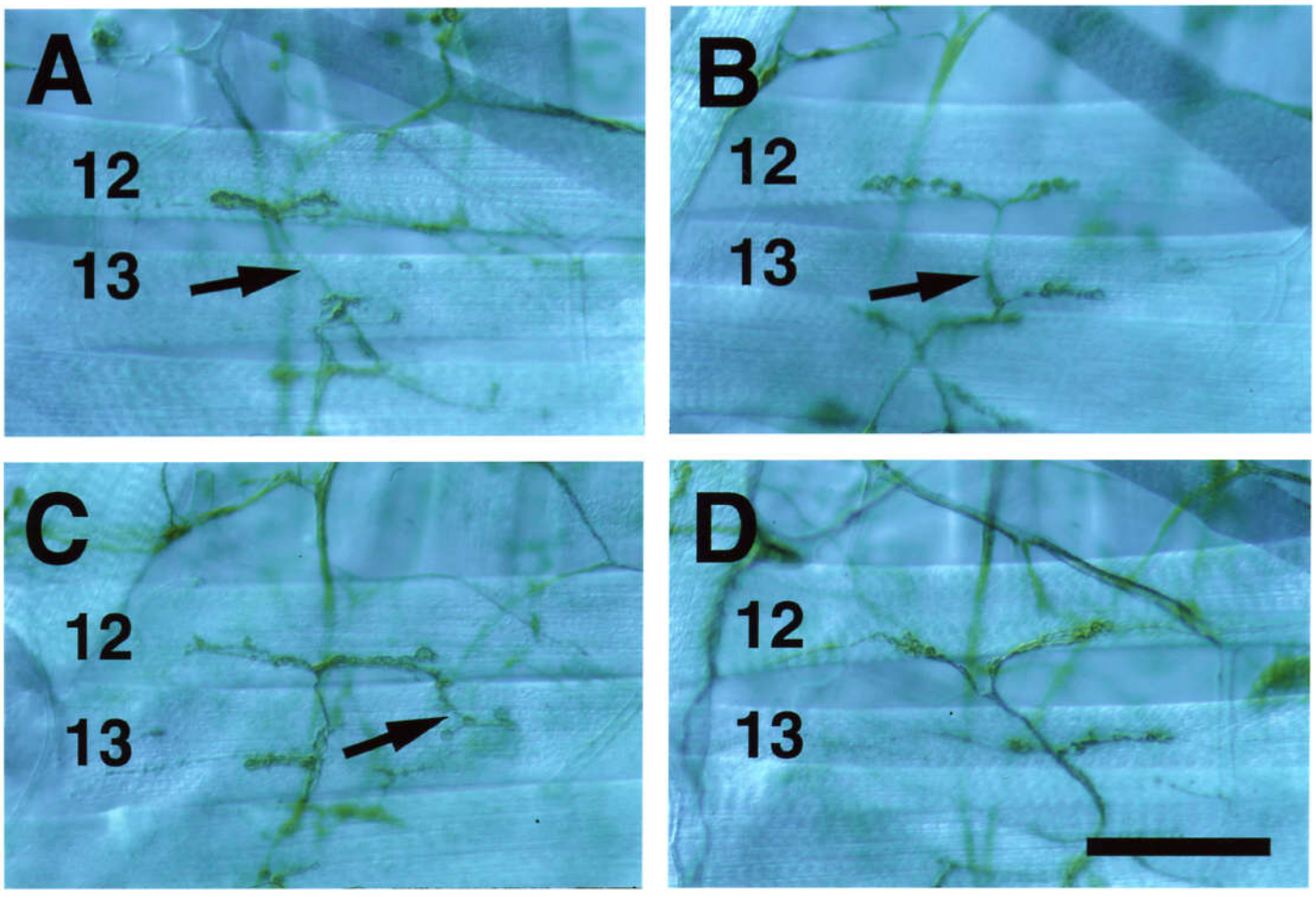

A
12
13
B
12
13
C
12
13
D
12
13

Figure 16 Localization of ectopically expressed CAPS (A, C), ID-CAPS (B) and ED-CAPS (D) on muscles. Body wall fillets of *24B-caps -Ia5* (A, C), *24B-ID-caps-4* (B), and *24B-ED-caps-20* (D) larvae, stained with Sabs against the extracellular (A, B) and intracellular (C, D) domains of CAPS. Note that intact CAPS, ID-CAPS and ED-CAPS are all concentrated at synaptic sites (white arrowheads). Scale bar, 100 μm.

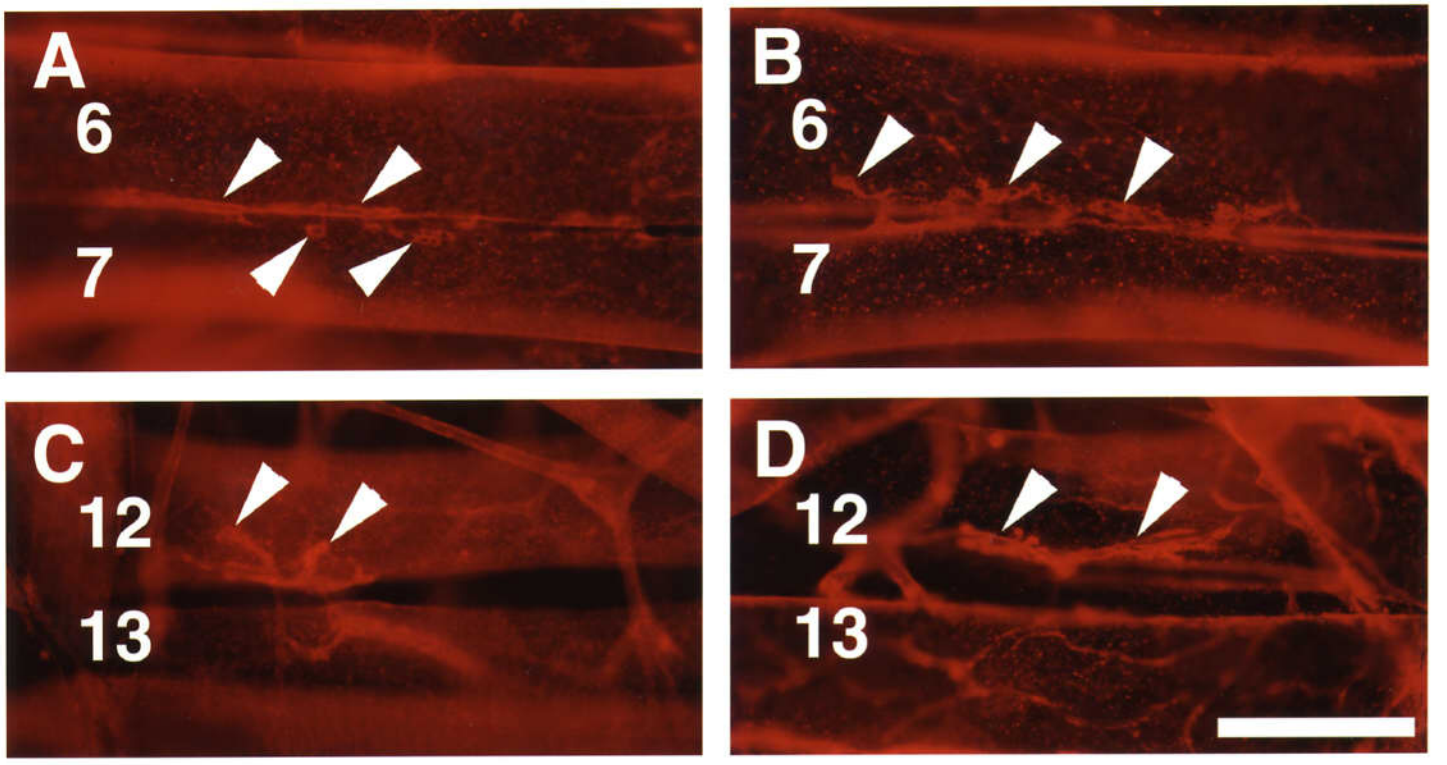

A
6
7
B
6
7
C
12
13
D
12
13

Figure. 17 (A-C) Schematic diagrams of trajectory and targeting of the distal branch of ISNb in wild-type larvae (A), in larvae, pan-neurally (B), and pan-muscularly (C), expressing various forms of CAPS. Endogenously and ectopically expressed CAPS on muscles are shown in light and dark gray, respectively. The endogenous and ectopic expression of CAPS on the ISNb nerve branch is shown in gray and black, respectively.

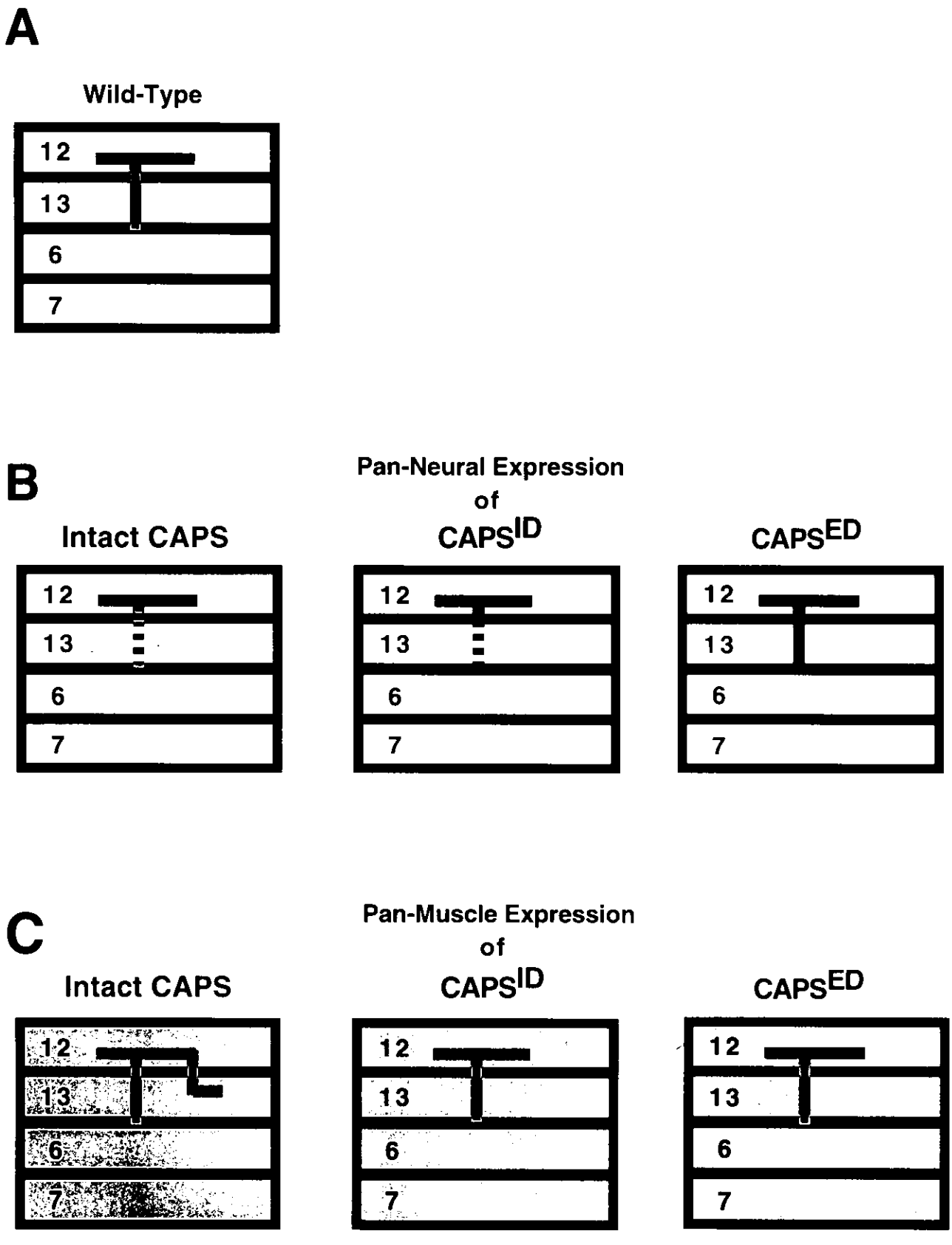

A
Wild-Type
12
13
6
7
B
Intact CAPS
Pan-Neural Expression of CAPS^ID
CAPS^ED
C
Intact CAPS
Pan-Muscle Expression of CAPS^ID
CAPS^ED

Figure 18 Schematic diagrams showing models for CAPS signaling in neurons. CAPS transmits the signals into neurons in its intracellular domain independent manner. (A) CAPS signals may be transmitted by other molecule(s) that don't directly associate with CAPS. (B) Alternatively, CAPS may transduce its signals via other molecule(s) that directly associate with CAPS.

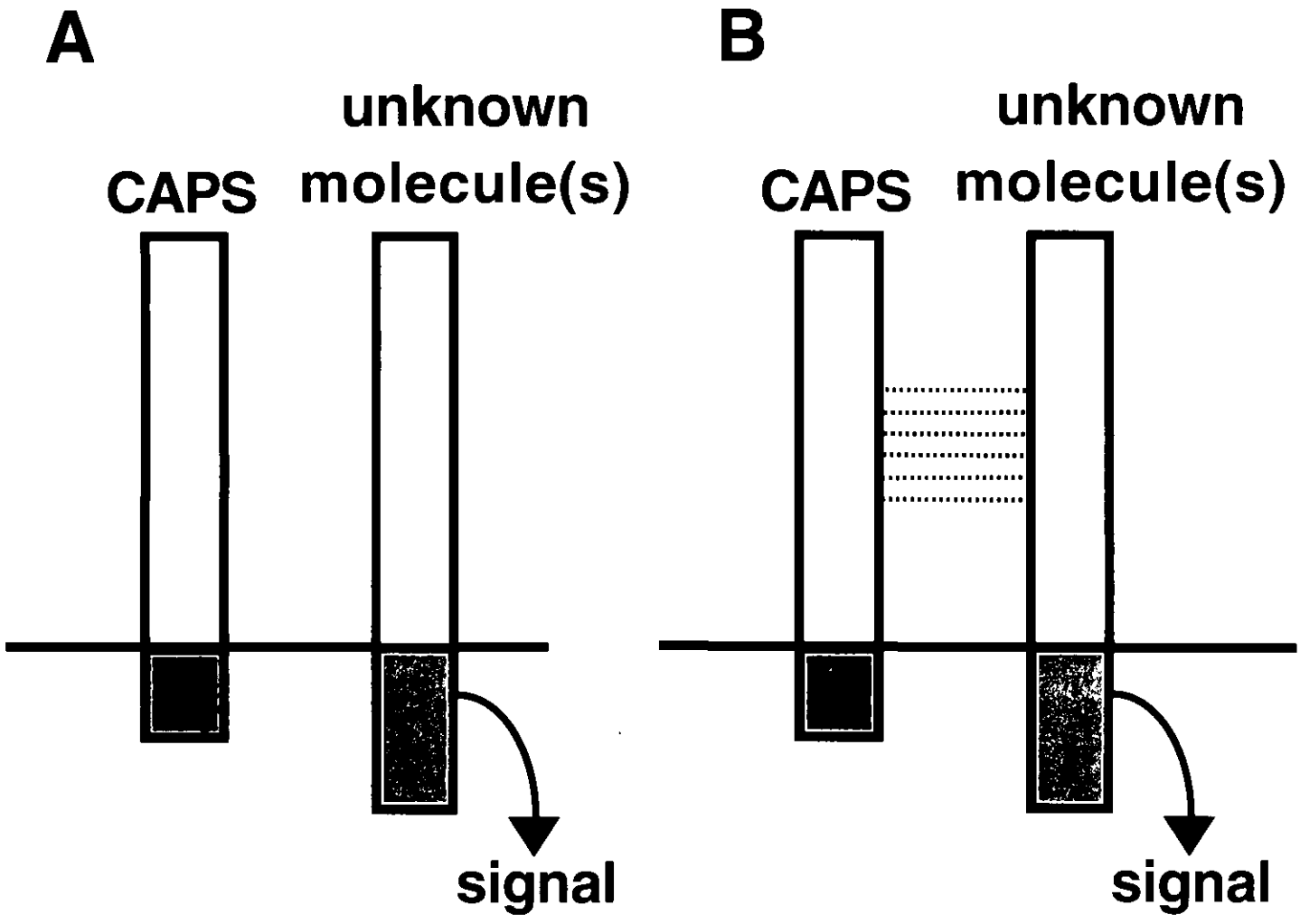

A
unknown
molecule(s)
CAPS
signal
B
unknown
molecule(s)
CAPS
signal

Figure 19 Schematic diagrams showing models for CAPS signaling in muscles. CAPS transmits the signals into muscles in its intracellular domain dependent manner. (A) CAPS may transduce its signals by itself. (B) Alternatively, CAPS may transduce its signals via other molecule(s) that directly associate with CAPS intracellular domain.

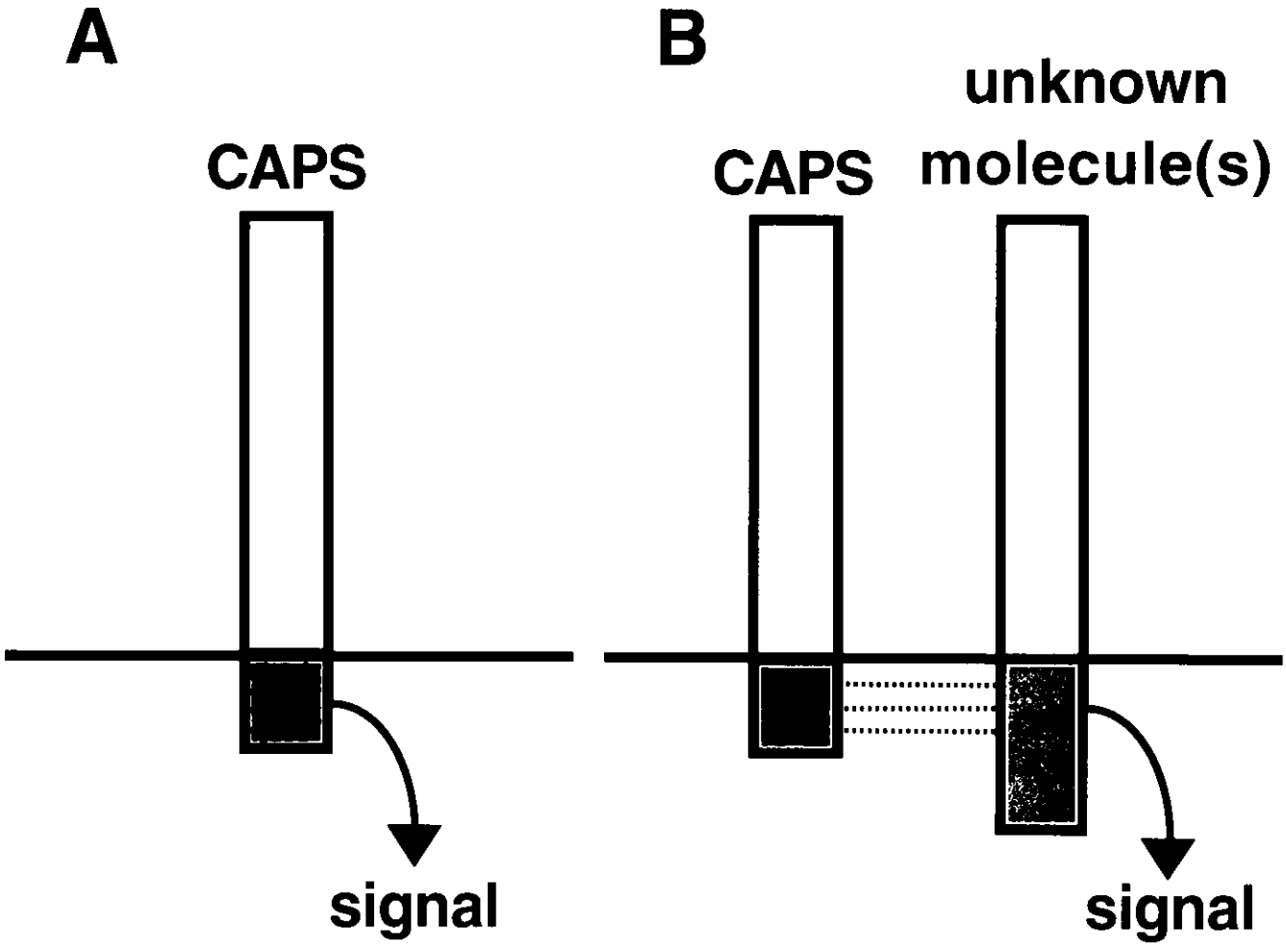

A
CAPS
signal
B
unknown
molecule(s)
CAPS
signal

Figure 20 Schematic diagram showing the possible role of muscular CAPS in selective synapse formation. Muscular CAPS could play an active role in selective synapse formation by interacting with receptors on specific motoneurons via its extracellular domain and by transmitting the signal into muscles via its intracellular domain. The signal into muscles may drive signaling cascade required to make specific synapses or regulate the motility of myopodia.

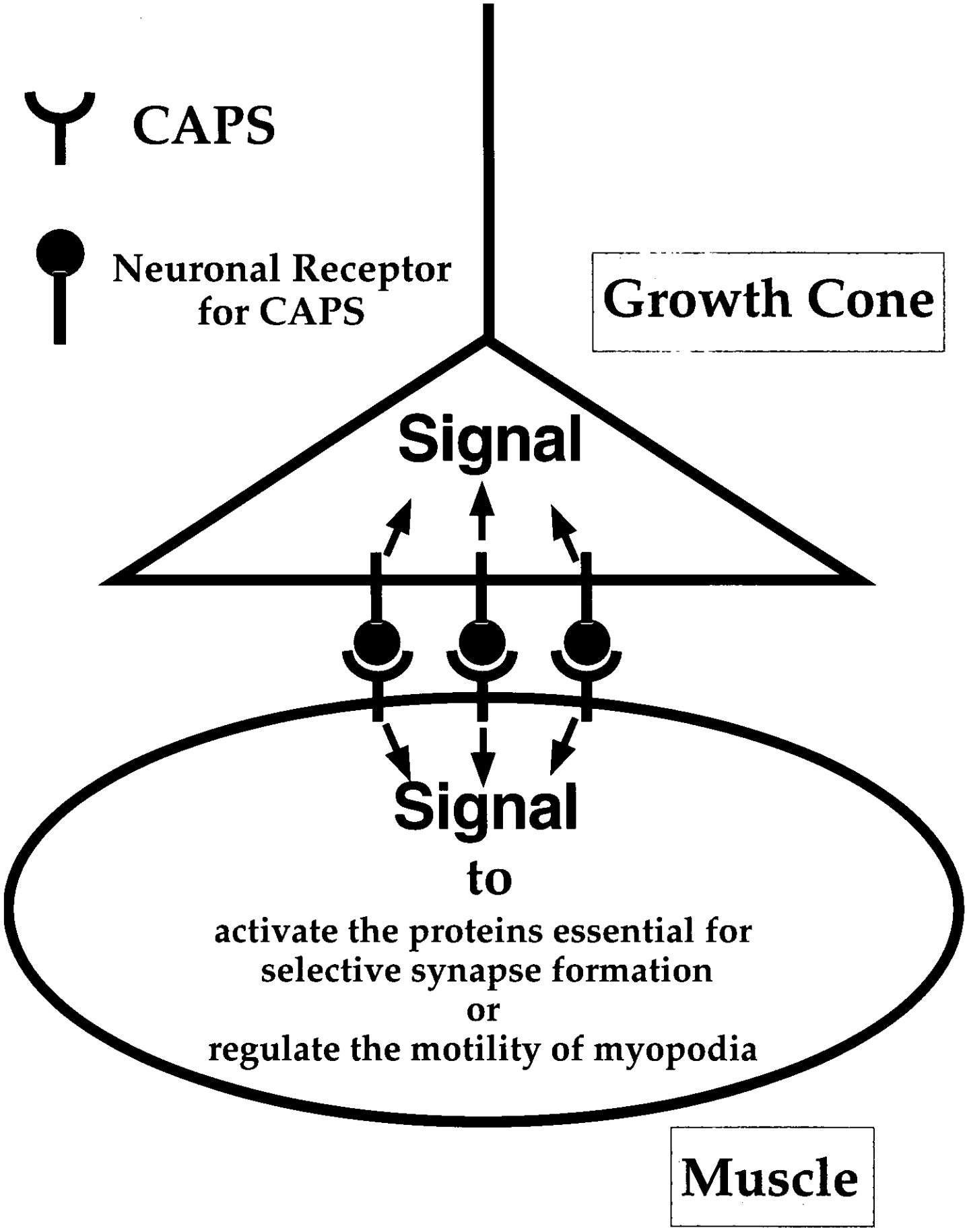

CAPS
Neuronal Receptor for CAPS
Growth Cone
Signal
Signal
to
activate the proteins essential for selective synapse formation
or
regulate the motility of myopodia
Muscle

Table 1 Effects of Pan-neural Expression of Intact and Modified Forms of CAPS on ISNb Pathfinding

	Third Instar Larvae		Embryos		
Genotype[a] (temperature)[e]	Misroute[b]	(n)[d]	Misroute[b]	Stall[c]	(n)[d]
elav-GAL4 (control) (29°C)	0%	(112)	0%	1%	(102)
elav-caps-Ia+Ib (29°C)	29%	(112)	18%	13%	(100)
elav-caps-Ia5 (29°C)	23%	(141)	-	-	
elav-caps-Ia+Ib (25°C)	10%	(118)	-	-	
elav-ID-caps-4 (29°C)	19%	(113)	-	-	
elav-ED-caps-20 (29°C)	2%	(101)	-	-	

[a]For details of the genotypes, see Materials and Methods.

[b]% of hemisegments displaying the misrouting phenotype in which the terminal branch of ISNb projected along the external surface of muscle 13.

[c]% of hemisegments displaying the stall phenotype in which the terminal branch of ISNb stalled near muscle 30.

[d]number of hemisegments examined.

[e]temperature at which the individuals were raised.

Table 2 Effects of Pan-muscle Expression of Modified Forms of CAPS on ISNb targeting

Genotype[a] (temperature)[d]	Ectopic Synapse Formation[b]	(n)[c]
24B-GAL4 (control) (25°C)	4%	(112)
24B-caps-Ia5 (25°C)	40%	(150)
24B-ID-caps-4 (25°C)	5%	(114)
24B-ED-caps-20 (25°C)	9%	(102)

[a]For details of the genotypes, see Materials and Methods.

[b]% of hemisegments of third instar larvae in which muscle 12 MNs formed ectopic synaptic endings on muscle 13.

[c]number of hemisegments examined.

[d]temperature at which the individuals were raised.