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A role for Fas II in the stabilization of motor neuron branches during pruning in *Drosophila*

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Abstract

During insect metamorphosis, the nervous system is extensively remodeled resulting in the development of new circuits that will execute adult-specific behaviors. The peripheral remodeling seen during development of innervation to the Dorsal Longitudinal (flight) Muscle (DLM) in *Drosophila* involves an initial retraction of larval neuromuscular junctions followed by adult-specific branch outgrowth. Subsequently, a phase of pruning occurs during which motor neuron branches are pruned back to reveal the stereotypic pattern of multiple contact points (or arbors) along the length of each DLM fiber. In this study, we show that the cell adhesion molecule, Fasciclin II (Fas II), is important for generating the stereotypic pattern. In Fas II hypomorphs, the number of contact points is increased, and the phenotype is rescued by targeted expression of Fas II in either synaptic partner. Arbor development has three distinct phases: outgrowth and elaboration, pruning and stabilization, and expansion of stabilized arbors. Fas II is expressed during the first two phases. A subset of branches is labeled during the elaboration phase, which is likely to initiate a stabilization pathway allowing branches to survive the pruning phase. However, since not all Fas II positive branches are retained, we propose that it primes branches for stabilization. Our data suggest that Fas II functions to restrict branch length and arbor expanse.

Keywords: Fasciclin II; Stabilization; Pruning; Arbor elaboration; Axonal branches; Innervation; Neuromuscular junction; Metamorphosis; Post embryonic remodeling; *Drosophila*

Introduction

A common theme in the formation of nervous systems is the initial exuberance of neuronal outgrowth that is seen both in the central nervous system as well as in the periphery. Outgrowth is followed by a process of refinement, which results in the mature pattern of connectivity and/or expanse of arbors. Refinement in the periphery is exemplified by the vertebrate NMJ where muscle fibers initially innervated by multiple neurons become singly innervated as a result of synapse elimination (Sanes and Lichtman, 1999). In the CNS, pruning of axonal and dendritic branches is well known. Examples of developmental axon pruning (reviewed in Kantor and Kolodkin, 2003) include the retino-tectal system, where it is necessary for establishment of topographic maps; in the visual cortex, where it is important for segregating projections of cortical neurons to appropriate target regions and in the hippocampus, where it serves to prune back mossy fiber projections to a shorter adult length.

During insect metamorphosis, the nervous system undergoes extensive remodeling. This reorganization provides useful models to study developmental pruning. One such model in *Drosophila* is exemplified by the mushroom bodies, centers for learning and memory. Prior to the onset of adult-specific outgrowth, axons of larval γ neurons are pruned back. This is a selective process, as axons of the α′/β′ neurons are not altered. The pruning is a result of degeneration that is initiated by steroid hormones and mediated by cell intrinsic mechanisms involving a ubiqui-

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tin–proteosome system acting in concert with ecdysteriod receptor BSP (Lee et al., 2000).

Another instance of pruning occurs during the development of innervation to the dorsal longitudinal muscle (Hebbar and Fernandes, 2004) and is distinct from what occurs in the mushroom bodies. In this case, following retraction of larval synapses, there is an excessive outgrowth of adult specific motor neuron branches, followed by a phase of pruning. More than 75% of the branches are eliminated through pruning, and consequently, the adult pattern of innervation emerges. Each motor neuron makes multiple contacts along the length of the muscle, and has been referred to as "multi-terminal" (Hoyle, 1983). A "terminal" refers to the higher order arbor and its collection of boutons. This innervation pattern is distinct from the single terminal innervation typical of larval muscles (Johansen et al., 1989).

When excessive branches are pruned, two aspects must be considered—the selective removal of some branches and the stabilization of those that persist. For the well-studied event of synapse elimination at the vertebrate NMJ (Sanes and Lichtman, 1999), it is proposed that a "protective signal" stabilizes some synapses, whereas an "elimination" signal initiates withdrawal. Very little is known about the mechanisms that bring about stabilization (reviewed in Zito, 2003). In the case of hippocampal pruning, there is evidence that molecules that initially promote outgrowth can also promote axon pruning at a later stage (Kantor and Kolodkin, 2003). For example, ephrins are thought to regulate both early hippocampal outgrowth and pruning of axons (Gao et al., 1999), while semaphorins and their receptors (plexins) have been implicated in axon guidance and stereotypic pruning of hippocampo-septal projections (Bagri et al., 2003). It is conceivable that in *Drosophila*, molecules initially involved in establishing synapses during the embryonic/larval stages may have distinct later roles in the context of pruning and stabilization.

The cell adhesion molecule, Fas II, is a good candidate to be involved in stabilizing branches during the formation of DLM innervation. In its classical role during insect (*Drosophila* and grasshopper) embryogenesis, it mediates selective axon fasciculation (Lin et al., 1994), guidance of growth cones (Grenningloh et al., 1991; Harrelson and Goodman, 1988), and target selection (Davis et al., 1997). In *Drosophila* it also functions to regulate post embryonic stabilization of larval NMJs (Schuster et al., 1996a) and of cholinergic inputs on the dendrites of motor neurons (Baines et al., 2002). We were interested in examining how Fas II may be re-used during the formation of adult NMJs. We focused our investigations on the formation of innervation to the Dorsal Longitudinal (flight) Muscle (DLM). Early during metamorphosis, Fas II is expressed in a subset of branches that elaborate over the muscle surface, suggesting a possible role in stabilization. Analysis of hypomorphic mutants revealed that the adult muscles have many more terminal arbors, and that this phenotype can be rescued through targeted expression of Fas II in motor neurons and muscle. Our studies demonstrate that Fas II is important for establishing the stereotypic pattern of terminal arbors on each DLM fiber, and that Fas II enables stabilization of subsets of branches during metamorphosis by influencing the length of second order branches and their expanse of higher order branches.

Materials and methods

Fly strains

Oregon R raised on standard *Drosophila* food at 25°C was used as the wild-type strain. The following Fas II alleles (described in (Grenningloh et al., 1991) were used; Fas II^{e93} (precise excision of a P-element insertion-source: R. Baines, University of Warwick), hypomorphs Fas II^{e86} (50% of wild-type levels-source: G. Davis, UCSF) and Fas II^{e76} (10% of wild-type levels-source: V. Budnik, U. Mass Med School). Since the null allele, Fas II^{EB112} is lethal by the larval stage; we generated a transhetereozygote, Fas II^{e76}/Fas II^{EB112}. Fas II overexpression was achieved by using UAS-Fas II (transmembrane Fas II) under the control of neuronal drivers elav-Gal 4 (Robinow and White, 1988; source—White, NIH) and D42-Gal 4 (Sweeney and Davis, 2002; source—S. Rao, Cornell University). Reporter gene expression for elav-Gal 4 has been observed in neuronal components up until 24 h APF. However elav-Gal 4 also drives expression in the muscles from 16 h APF onwards (Fernandes and Keshishian, unpublished observations). D42-Gal 4 drives reporter gene expression in motor neuronal branches as early as 14 h APF and continues throughout the rest of metamorphosis. However reporter expression for the early pupal stages is not as intense as with elav-Gal 4 (Hebbar and Fernandes, unpublished observations). For muscle specific overexpression of Fas II, we used MHC (Myosin Heavy Chain)-Gal 4 (source: A. Chiba, University of Illinois, Urbana-Champaign). The larval promoter for the MHC gene is active until about 10 h APF and subsequently the adult specific promoter switches on by 26 h APF (Fernandes et al., 1991). For rescue experiments, we crossed (female) homozygous Fas II^{e76}; UAS-Fas II animals to appropriate Gal 4 drivers and the male progeny heterozygous for the driver and transgene were examined. A hyperexcitable, K^+ channel double mutant, *eag^{1}Sh^{120}* (source: H. Keshishian, Yale University) was used an activity mutant. In these animals, eag^1 preferentially removes the I_K current (Wu et al., 1983) while Sh^{120} reduces I_A (Ganetzky and Wu, 1983). The double mutant synergistically increases nerve excitability and neurotransmitter release (Ganetzky and Wu, 1983).

Staging

White prepupae (0 h APF: hours after puparium formation) were collected and placed on moist filter paper on a Petri dish. They were raised at 25°C to the following stages; 18 h, 24 h, 28 h and 38 h APF. The stages were confirmed using muscle morphology (Fernandes et al., 1991). Two-day-old adults were used for analyses of adult muscle and innervation as described previously (Hebbar and Fernandes, 2004).

Immunochemistry

The general protocol followed was as described previously (Hebbar and Fernandes, 2004). Tissues were fixed with 4% paraformaldehyde (Ted Pella, Inc, CA). 10% donkey serum in 0.1% BSA and 0.3% Triton-X buffered saline was used as a blocking solution prior to primary antibody application. The following primary antibodies were used: anti HRP (1:200, raised in goat, source: Jackson ImmunoResearch Laboratories, Inc, PA), MAb1D4 (1:2 mouse anti transmembrane Fas II, source: Hybridoma Bank, Iowa), MAb 22C10 (1:25 mouse anti Futsch, source: Hybridoma Bank, Iowa), anti β-3 Tubulin (1:5000, raised in rabbit and used to label muscle outlines at 24 h APF, source: R. Pohl, Germany). Synapses were marked with anti DVGLUT (1:100, raised in rabbit and recognizes *Drosophila* vesicular glutamate transporter, source: A. DiAntonio, Washington University School of Medicine). The following secondary antibodies were used: Alexa Fluor 488 Donkey anti-goat, Alexa Fluor 555 Donkey anti-rabbit and Alexa Fluor 594 or 555 Donkey anti-mouse (all at 1:200; Molecular Probes, OR). Adult muscles were visualized with Alexa Fluor 594 phalloidin (Molecular Probes, OR).

Image acquisition

All immunostained tissues were visualized using an Olympus FV500 confocal microscope. Fluorescent dyes were excited using Ar 488 and HeNe 543 nm lasers. Optical sections of 1 μm thickness (for pupal preps) and 1–3 μm (for adult preps) were taken and stacked using Fluoview Software to obtain a 2D projection. These stacked images were used for further analysis. Image panels were prepared using Adobe Photoshop® 6.0 (Adobe Systems Incorporated, CA).

Data analysis

All morphometric measurements were carried out on 2D projections. For adult muscle lengths, hemithoracic preparations of 2-day-old female flies were used. Phalloidin staining was used to outline the muscle and measurements were made in Image-Pro Plus 4.5 (Media Cybernetics®, MD). In the pupal stages, our analysis focused on primary branches that innervate dorsal muscles, DLMs a and b. Both these muscles are innervated by one motor neuron, MN5 (Ikeda and Koenig, 1988). The axon of MN5 divides into 2–3 longitudinal branches (each defined as a primary branch). These include the anterior (a), medial (b) and posterior (c) branch (Ikeda et al., 1980). Secondary branches are transverse outgrowths off a primary branch. Secondary branch length was defined as length of the branch up to the first tertiary branch outgrowth. Lengths were measured in Image-Pro Plus 4.5 (see schematic in Fig. 5 for representative traces). Density of second order branching is expressed as number of secondary branches per 10 μm of a primary branch. In addition, at 24 h APF, secondary branches with tertiary and higher order branches on DLMa were quantified. The area occupied by the secondary branch arbor on DLMa was outlined and measured in Image-Pro Plus 4.5 (see schematic in Fig. 5 for representative traces). Intensity analyses were done as reported for larval NMJs (Albin and Davis, 2004; Mathew et al., 2003). Briefly, 5 regions along an axon within the main nerve trunk (at 24 h APF) were highlighted and these areas were used to measure anti HRP and anti Fas II intensity on double labeled (anti HRP and anti Fas II) samples using Flouview software. All values represent mean ± SEM. Basic statistical functions such as mean, standard error of mean and two sample Student's *t* test were performed using Microsoft Excel. Chi Square test was performed and interpreted using Minitab program (Minitab Inc, State College, PA).

Results

Each DLM fiber is innervated by a single motor neuron, which makes multiple contacts along the length of the muscle. This innervation pattern is characteristic of insect DLMs and has been referred to as "multi-terminal" (Hoyle, 1983). We refer to each "terminal" as a "contact point" (CP), which includes the axon entry point as well as the arbor of higher order branches that emanates from it (Fig. 1A and Hebbar and Fernandes, 2004). It is the higher order branches that bear presynaptic swellings or boutons, and can be visualized by the presence of *Drosophila* vesicular glutamate transporter, DVGLUT (Daniels et al., 2004), see Fig. 1B). The characteristic DLM innervation pattern is established by developmental events that occur during the first 2 days of metamorphosis (Fernandes and VijayRaghavan, 1993; Hebbar and Fernandes, 2004). These include outgrowth of adult specific branches (14–24 h APF), and the subsequent pruning of exuberant outgrowths (24–38 h APF). To examine a role for Fas II during this period, we have followed its expression pattern and analyzed innervation patterns under conditions of increased and decreased Fas II levels.

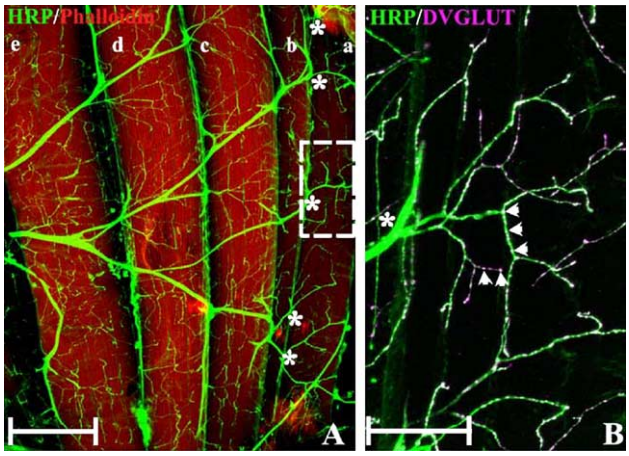

Fig. 1. Adult DLM innervation pattern is multi-terminal. (A) The innervation pattern of the adult DLMs revealed by staining with anti-HRP (green) and labeled for the muscle with Phalloidin (red) reveals multiple contacts spaced along the muscle length. The most dorsal fiber, DLMa receives 5 points of contact (asterisks). The box represents one contact point (*) on DLMa with its arbor of higher order branches. Scale bar = 100 μm (B) An arbor visualized with anti-HRP (green) at a higher magnification. Higher order branches bear presynaptic swellings or boutons (arrowheads) that are visualized by anti-DVGLUT (magenta). Scale bar = 20 μm.

Fas II is expressed in developing neuronal branches on the DLMs

At 14 h APF, Fas II is detected in the posterior dorsal mesothoracic nerve (PDMN), where it labels axons of the DLM motor neurons. Fas II expression is also detectable in the primary branches that project along the length of the dedifferentiated larval muscle scaffolds (Fig. 2). At this time, higher order branches do not display detectable levels of Fas II. At 18 h APF, when the larval scaffolds are in the process of splitting into DLM fibers (Fernandes et al., 1991), Fas II is detected along the primary branches that have now increased in length. Fas II is additionally detected in the secondary and higher order branches that elaborate over the muscle surface (Fig. 2). By 24 h APF, muscle splitting is complete and the primary branching pattern is established (Fernandes and VijayRaghavan, 1993; Fernandes et al., 1991). At this time, Fas II continues to be present along the entire length of the primary branch, in secondary branches and in higher order branches (Fig. 2). It is the second order branch that prefigures the "contact point" seen in the adult, and as early as 38 h APF (Hebbar and Fernandes, 2004).

A closer examination of Fas II expression between 18 and 24 h APF revealed some interesting features. We focused our analysis on DLMs a and b, since this dorsal pair of muscles is innervated by a single motor neuron (Ikeda and Koenig, 1988). First, Fas II is expressed in a subset of second order branches. At 18 h APF, 22.5% (n = 4) of the total pool of second order branches is Fas II positive, while at 24 h APF Fas II is expressed in 38.0% of branches (n = 6). Secondly, the number of Fas II positive branches increases approximately three-fold during this time period. At 18 h APF, there is an average of 8.5 ± 2.3 (n = 4) Fas II positive branches, whereas by 24 h, this number increases to 30.0 ± 3.8 (n = 6), indicating that additional branches become Fas II positive. The pool of Fas II positive branches is largely made up of those that bear a higher order arbor (86%). We have previously reported that 50% of branches with higher order arbors undergo pruning (Hebbar and Fernandes, 2004; see also Table 5). Consistent with this observation, we find that not all Fas II positive second order branches that bear an arbor are stabilized to give rise to the adult pattern. At 24 h APF, there are 8.3 ± 1.25 Fas II positive branches on DLMa, whereas at 38 h APF, 5 branches are seen. These five branches correspond to the stabilized "contact points" that emerge after pruning, and are therefore prefigured by second order branches during the 14–24 h period (Hebbar and Fernandes, 2004). This pattern of 5 CPs comprises the stereotypy of the adult innervation pattern.

At 38 h APF, Fas II is detected in the axon of each CP (Fig. 3A). It can also be detected in tertiary and higher-order branches of the arbor. Fas II is subsequently downregulated from the arbors after 48 h APF (data not shown). Consistent with these observations, at the adult stage, Fas II is not detected in motor neuron branches or at the terminals (Fig. 3B). This is in contrast to the larval NMJ where Fas II is detected in boutons (Schuster et al., 1996b). When a pan-neuronal driver, elav-Gal 4 (Robinow and White, 1988) is used to overexpress Fas II, the protein is seen in individual CPs (Fig. 3C), but is still undetectable at the boutons.

An interesting aspect of Fas II expression at 38 h APF is that in addition to being present in the DLM motor axons, it is also present in a compartment that extends around them (Fig. 3A′). This is likely to be a glial compartment due to the lack of anti HRP staining. Thus, Fas II expression is detected in secondary and higher order branches from 18 h APF onward and is coincident with the period of branch elaboration. It becomes downregulated after the pruning phase and is absent from the adult NMJs.

Fas II alleles exhibit altered adult innervation patterns

Immunostaining studies indicated that Fas II is expressed in a subset of higher order branches during the period of branch elaboration (Fig. 2). To test if this restricted pattern of expression has a bearing on branch elaboration and/pruning, innervation patterns in hypomorphic alleles of Fas II were examined (Grenningloh et al., 1991). The following alleles were used: Fas II^{e86} (50% of wild-type levels) and Fas II^{e76} (10% of wild-type levels). Since the null Fas II^{EB112} is lethal at the larval stages (Grenningloh et al., 1991), a transhetereozygote, Fas II^{e76}/Fas II^{EB112}, was generated which has the least amount of Fas II in a viable allele (less than 5%). Fas II^{e93} (precise excision of a P-insert which was used to derive the above lines) and Oregon-R were used as controls.

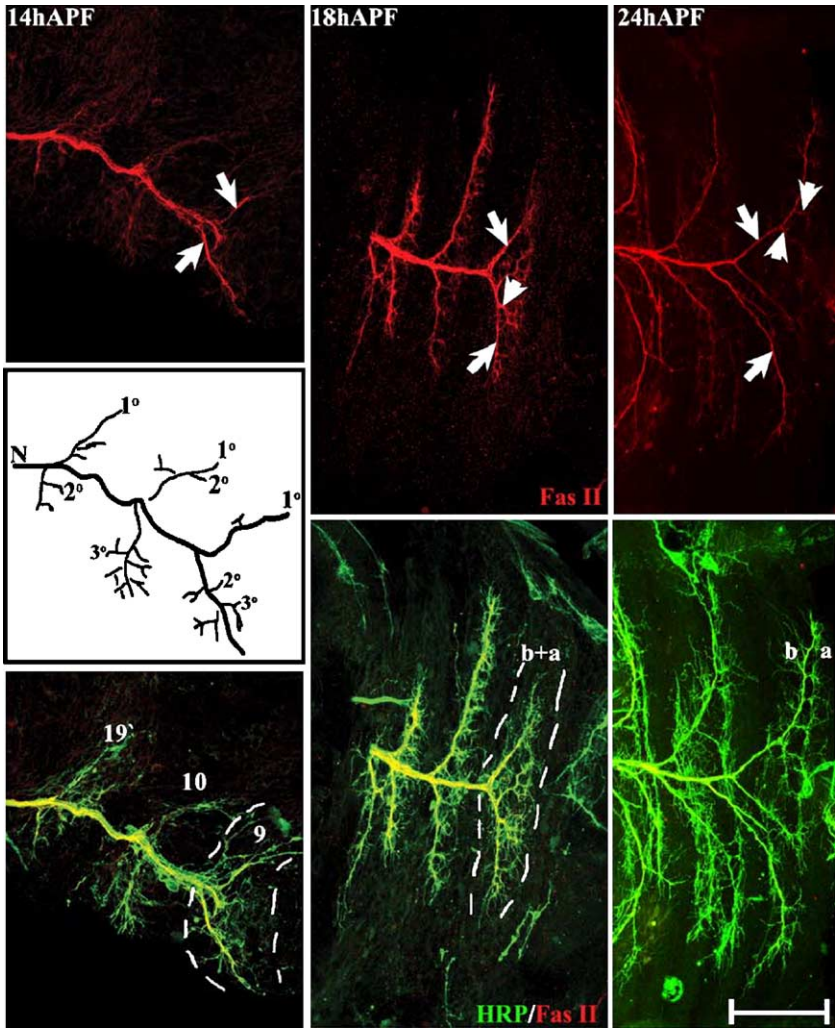

Fig. 2. Transmembrane Fas II localization during development of DLM innervation (18–24 h APF). Top panel: Fas II (red) localization. Primary branches are indicated with an arrow; secondary outgrowths with an arrowhead. Bottom panel: double label with anti Fas II (red) and general nervous system marker, anti HRP (green). Middle panel: schematic depicting the organization of DLM innervation between 14 and 24 h APF; nerve trunk (N), longitudinal primary branches (1°), transverse secondary branches (2°) and the tertiary branches (3°). At 14 h APF, Fas II is clearly evident along the main nerve trunk. It is also seen along the primary branches (arrows in top panel) that run along the length of the larval scaffolds (MFs 9, 10 and 19′). Dashed line indicates the outline of MF 9, which later gives rise to DLMs a and b that are innervated by a single motor neuron. By 18 h APF, Fas II is prominent in a subset of secondary branches and their arbors (arrowhead in top panel). This trend continues at 24 h APF. Scale bar = 50 μm.

Adult innervation patterns were observed in these mutants by immunostaining for anti-HRP (Fig. 4). We focused on examining the number of CPs on the dorsal-most muscle, DLMa. In the wild-type, DLMa displays an average of 5.0 ± 0.23 CPs. Each CP originates from a stabilized second order branch (Hebbar and Fernandes, 2004). The number of CPs in Fas II^{e93} and Fas II^{e86} was not significantly different from the wild-type (Fig. 4). However, both Fas II^{e76} and Fas II^{e76}/Fas II^{EB112} exhibited an increase in the number and range of CPs (Table 1, Fig. 4). The mean number of CPs in Fas II^{e76} and Fas II^{e76}/Fas II^{EB112} were 5.8 ± 0.16 and 6.0 ± 0.17 respectively, an increase that was statistically significant. The increased number of CPs is also evident when the range of CPs is examined (Table 1, Fig. 4). In the wild-type, the number of CPs ranged from 4 to 6 with 69% of animals exhibiting 5 CPs (Table 1). By contrast, Fas II^{e76} animals display 5–7 CPs with 36% exhibiting 5 CPs. In Fas II^{e76}/Fas II^{EB112} animals, the range of CPs remains the same as in Fas II^{e76}, however, fewer animals (25%) exhibit 5 CPs. Thus, decreasing Fas II levels causes an increase in the number and range of CPs (Table 1 and Fig. 4). Associated with the increase in number of CPs, an increase in muscle length is observed (Fig. 4, top panel and Table 2). This trend is statistically significant only in the case of the most severe hypomorph, Fas II^{e76}/Fas II^{EB112}. In these animals there is an 11% increase in muscle length. More importantly, the increase in length occurs after the pruning event as there is no significant alteration at 24 h APF (data not shown). It is of interest to note that CPs are uniformly spaced along the length of the muscle (Figs. 1 and 4). This has a bearing on the occurrence of rapid local depolarizations, ensuring that

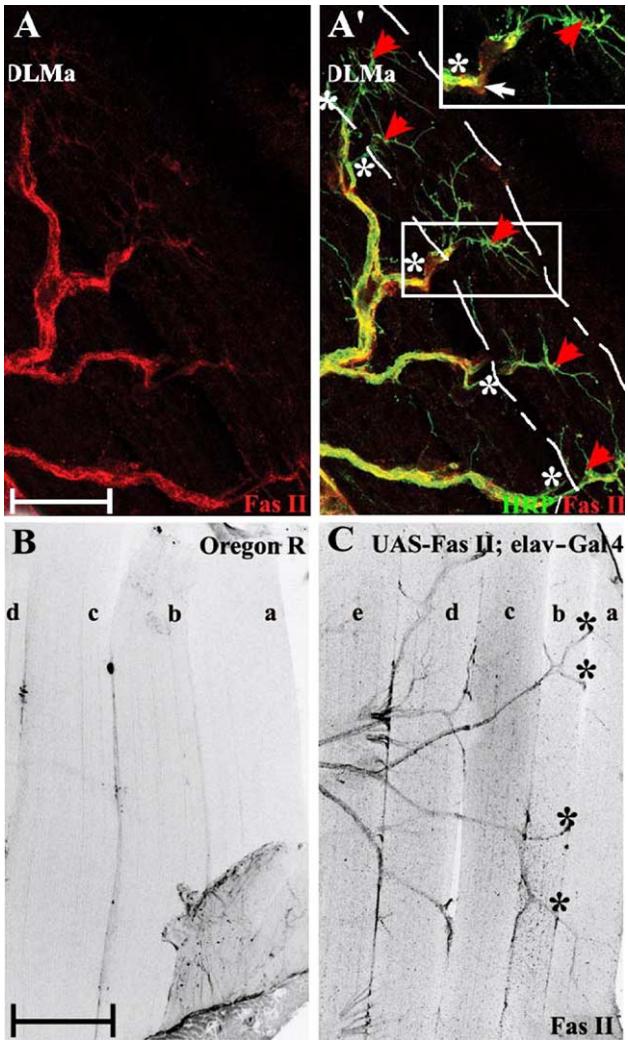

Fig. 3. Fas II expression after pruning. Top panel: (A) Fas II (red) expression at 38 h APF on DLMa. (A′) Double label with general nervous system marker, anti HRP (green). Inset in A′ (same as boxed area) shows the partial arbor of a CP (*). Fas II expression in branches of the motor neuron that innervates DLMa. The neuron makes multiple points of contact (CP, *) along the length of the fiber (dashed outline) and elaborates arbors (red arrowheads) on the muscle surface. Each CP is prefigured by a secondary branch seen at 24 h APF. The double labeling reveals that in addition to labeling axons (yellow indicates co-localization of HRP and Fas II), Fas II is also present in a compartment not marked by anti-HRP (white arrow in A′ inset), which is likely to be glial in nature. Scale bar = 25 μm. Bottom panel: Fas II localization in adult hemithoracic preparations. (B) In the wild-type, Fas II is not detectable in adult DLM motor axons. DLMs (a–d) are shown. (C) Upon overexpression of Fas II using pan-neuronal driver, elav-Gal 4, it is detectable in the DLM axons as well as in the contact points (*). Scale bar = 100 μm.

the muscle membrane is isopotential through the entire length (Hoyle, 1983). This direct relationship between muscle length and number of contact points indicates that a homeostatic event that involves neuron–muscle communication must occur along with or subsequent to the pruning process to ensure co-ordinate expansion of terminal arbors and muscle length (Hebbar and Fernandes, 2004).

The Fas II phenotype is rescued using neuronal and muscle drivers

To confirm that the observed Fas II phenotypes were due to decreased levels of Fas II, the Gal4AS system of targeted expression (Brand and Perrimon, 1993) was used to deliver Fas II in a tissue specific manner. In these rescue experiments, a neuronal driver, elav-Gal 4 was used to drive UAS-Fas II in the Fas II^{e76} background. In the mutant, we have shown that the number of CPs is increased (Table 1). This is also evident in the number of animals displaying more than the mean number of wild-type CPs (more than 5). In Fas II^{e76}, 64% of animals displayed the phenotype, whereas in the wild-type, only 23% of animals display more than 5 branches, a difference that is statistically significant (P = 0.04). The mutant phenotypes were rescued by targeted Fas II expression (Table 1). Two genetic controls were also examined: Fas II^{e76}; UAS-Fas II (homozygotes) which showed a phenotype similar to the mutant alone, and Fas II^{e76}/+, which exhibited a wild-type phenotype (data not shown). A second neuronal driver, D42-Gal 4, was unable to rescue the Fas II^{e76} phenotype (Table 1).

Interestingly, the elav-Gal 4 driver is expressed in DLM fibers during metamorphosis (Fernandes and Keshishian, unpublished observations). We therefore considered the possibility that muscle expression of elav-Gal 4 may contribute to the rescue. When a muscle driver MHC-Gal 4 was used to overexpress UAS-Fas II in the background of Fas II^{e76}, the mutant phenotype was rescued, as determined by the decrease in number of animals that displayed more than 5 CPs (Table 1). However, the mean number of CPs was significantly reduced in comparison to wild-type (Table 1). Thus, it seems likely that a balance between muscle and neuronal Fas II may exist to promote appropriate branch development. We have not been able to detect muscle Fas II to the extent that we can detect it in the neuronal component, and this may simply indicate that there are low levels of muscle Fas II. Associated with the decrease in the number of CPs, the muscle length is also significantly reduced (Table 2).

How are the additional CPs generated in Fas II hypomorphs?

The adult DLM innervation pattern is established within the first 2 days of metamorphosis (0–38 h APF) as a result of adult-specific neuronal outgrowth followed by the subsequent pruning of more than 75% of second order branches (Hebbar and Fernandes, 2004). The additional contact point seen in Fas II hypomorphic adults could be a result of excessive initial outgrowth or due to the stabilization of a greater number of secondary branches. In order to determine the developmental origins of the extra CP in the hypomorph, we analyzed representative pupal stages in Fas II^{e76}/Fas II^{EB112}.

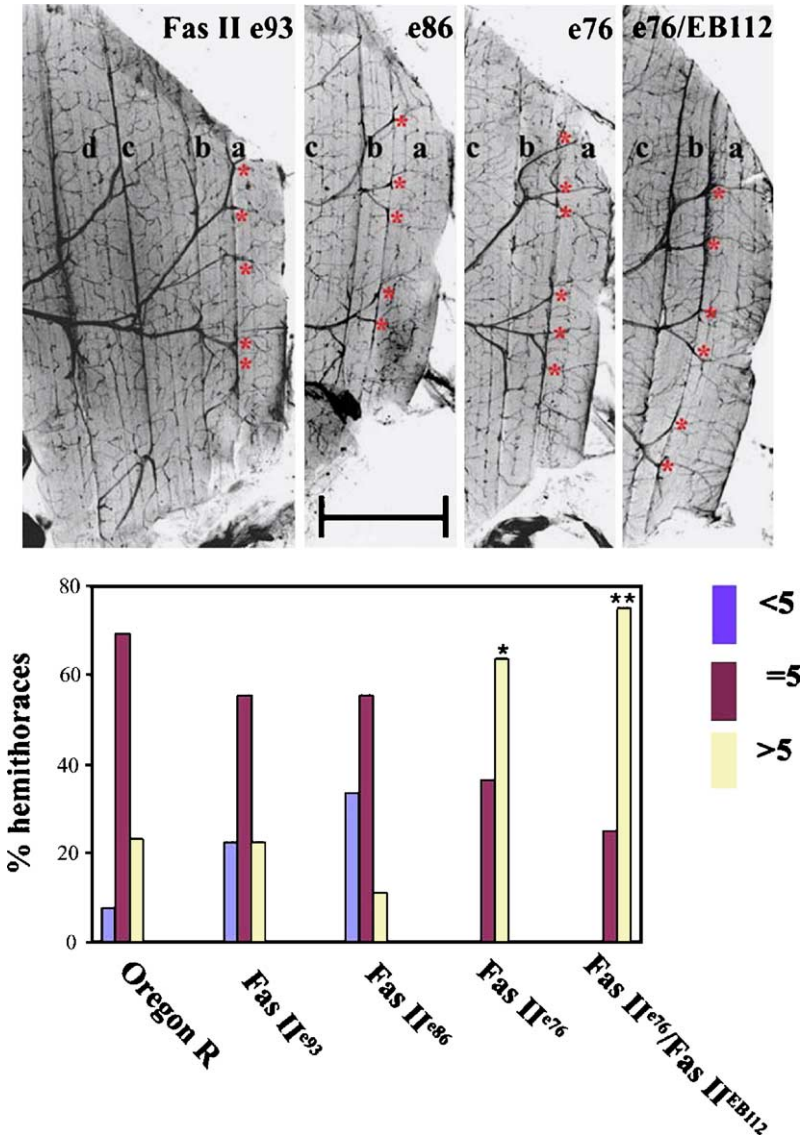

Fig. 4. Adult Fas II hypomorphs exhibit an altered DLM innervation pattern. Top panel: anti HRP reveals innervation pattern on adult DLMs (a–d shown for Fas II^{e93} and (a–c) for the remaining alleles). In the genetic control, Fas II^{e93}, there are 5 contact points (*) on dorsal most fiber DLMa. This is unchanged in the mild hypomorph, Fas II^{e86}. As many as 6–7 contact points are seen in severe hypomorphs Fas II^{e76} (10% wild-type Fas II levels) and Fas II^{e76}/Fas II^{EB112} (<5% wild-type Fas II levels). Scale bar = 100 μm. Bottom panel: representation of the range of contact points on DLMa within each genotype depicted in the top panel. * Indicates significance at $P < 0.05$ and **significance at $P < 0.025$ (χ^2_1 test). Numbers are as indicated in Table 1.

In order to better characterize outgrowth and expanse of secondary branches, morphometric measurements such as number and density of second order branches (for outgrowth), length of secondary branches and areas occupied by arbors (for expanse) were carried out (Table 4). We quantified the number of secondary branches that bear higher order arbors as another measure of developmental progression. Our measurements focused on primary branches that innervate DLMa. At 18 h APF, second order branches are well defined and it is at this time that Fas II expression is evident in second order branches (Fig. 2). At this stage, the innervation pattern seen in Fas II^{e76}/Fas II^{EB112} shows no significant differences in secondary branch density or in the number or lengths of secondary branches (Table 3), suggesting that decreased Fas II levels do not impact early events. Next, the innervation pattern was examined at 24 h APF (Fig. 5), when second order branching is maximal (Fernandes and VijayRaghavan, 1993). There is no statistical difference in the numbers or density of second-order branches between the wild-type and the mutant. Likewise, there is no change in the number of secondary branches that bear higher order branches. However, in Fas II^{e76}/Fas II^{EB112}, the average length of secondary branches on DLMa is significantly increased. In addition, the area occupied by higher order arbors of a secondary branch on the muscle is enhanced in the hypomorph (Table 4). Taken together, these data suggest that a reduction in Fas II does not affect outgrowth of

Table 1
Range of CPs as displayed by various genotypes

Genotype	No. of CPs (mean ± SEM)	Range of CPs	<5 CPs %	5 CPs %	>5 CPs %
Oregon R	5.2 ± 0.12 (20)	4–6	8	69	23
Fas II^{e76}	5.8 ± 0.16 (14)***	5–7	0	36	64*
Fas II^{e76}/Fas II^{EB112}	6.0 ± 0.17 (17)***	5–7	0	25	75**
Fas II^{e76}; UAS-Fas II; elav-Gal 4	5.1 ± 0.26 (16)	4–7	31.5	31	37.5
Fas II^{e76}; UAS-Fas II; MHC-Gal4	4.6 ± 0.18 (13)**	4–6	46*	46	8
Fas II^{e76}; UAS-Fas II; D42-Gal4	5.7 ± 0.14 (11)**	5–7	0	36	64
UAS-Fas II; elav-Gal 4	4.4 ± 0.15 (19)***	3–6	58***	37	5
UAS-Fas II; MHC-Gal 4	4.3 ± 0.20 (11)***	3–5	54*	46	0

Number in parenthesis represents sample size.
* $P < 0.05$.
** $P < 0.025$.
*** $P < 0.005$.

second order branches, but that during the period of branch elaboration (18–24 h), the reduced Fas II levels promote an increase in the secondary branch length and expanse of the developing arbor, which in turn has a bearing on branch stabilization.

Not all secondary branches with an arbor are stabilized (Hebbar and Fernandes, 2004), and this is reflected in the case of Fas II positive branches as well. At 24 h APF, DLMa has as many 10 secondary branches that bear arbors. A subset of these (8.3) is positive for Fas II (Table 5), and only 5 are incorporated into the adult pattern. This suggests a role for Fas II in priming branches for stabilization during metamorphosis. The earliest evidence that specific branches will be stabilized prior to the pruning phase, comes from studies that examined the labeling of 22C10, an antibody that binds to a microtubule associated protein, Futsch (Hummel et al., 2000). In the wild type at 24 h APF (Fig. 6A), 3.8 secondary branches with arbors are positive for Futsch/22C10 ($n = 9$). By 28 h APF (Fig. 6B) when pruning is in progress, more secondary branches, 4.4 ($n = 7$) are labeled by futsch/22C10. By the completion of pruning at 38 h, all stabilized secondary branches express Futsch/22C10 ($n = 4$, Table 5). Thus, the onset of Futsch/22C10 between 24 and 38 h AP in secondary branches is a signature of their stabilization. When Futsch/22C10 localization was examined at 24 h in Fas II^{e76}/Fas II^{EB112}, many more secondary branches (4.7; $n = 9$) are seen to be positive for Futsch/22C10 (Fig. 6C).

Table 2
Adult muscle length is increased in Fas II^{e76}/Fas II^{EB112} animals

Genotype	DLMa muscle length (μ)
Oregon R (females)	555.13 ± 16.84 (7)
Fas II^{e93} (females)	590.93 ± 10.23 (6)
Fas II^{e86} (females)	583.58 ± 20.11 (5)
Fas II^{e76} (females)	593.48 ± 8.6 (5)
Fas II^{e76}/Fas II^{EB112} (females)	615.03 ± 10.93* (6)
Fas II^{e76}; UAS-Fas II (males)	574.94 ± 22.39 (5)
Fas II^{e76}; UAS-Fas II; MHC-Gal4 (males)	497.71 ± 23.41[a] (6)

Values are mean + SEM (n).
[a] $P < 0.05$ between Fas II^{e76}; UAS-Fas II and Fas II^{e76}; UAS-Fas II; MHC-Gal4.
* $P < 0.05$ between Oregon R and Fas II^{e76}/Fas II^{EB112}.

Overexpression of Fas II using Gal 4 drivers

Our analysis of Fas II hypomorphs has shown that lowering Fas II levels results in more CPs. Does the converse hold true—i.e., when Fas II levels are increased, will it result in fewer CPs? We were able to create a "hypermorphic" condition by using the UAS/Gal4 system (Brand and Perrimon, 1993) to overexpress Fas II in the wild-type background. A neuronal driver, elav-Gal 4 and a muscle driver MHC-Gal 4 were used.

In UAS-Fas II; elav-Gal 4 adults, the mean number of CPs is lower than the wild type (4.4 ± 0.15), a difference that is statistically significant when compared to the wild-type (Table 1). The range of CPs in these animals has shifted (3–6; Table 1), also an indicator of the decrease in CPs. A majority of animals, 58%, exhibit less than the typical 5 CPs at the adult DLMs ($P = 0.005$; Table 1). Thus, as expected, increased Fas II expression with elav-Gal 4 results in fewer CPs. What brings about the generation of fewer CPs? From our results with the hypomorphic mutants, it can be predicted that increasing Fas II levels would restrict axonal outgrowth and arbor elaboration. When branching patterns were examined at 24 h APF, we found that the number of secondary branches along a primary is decreased. In the wild type, there are typically 22.75 ± 1.7 branches along a primary branch. In UAS-Fas II; elav-Gal 4 pupae, there are fewer second order branches along a primary (19.30 ± 1.70; Table 4). This reduction is statistically significant ($P = 0.013$). As a

Table 3
Morphometric characteristics at 18 h APF

Morphology	Oregon R (11)	Fas II^{e76}/Fas II^{EB112} (8)
Density of second-order branching	3.85 ± 0.34	3.54 ± 0.33
No. of secondary branches	22.72 ± 2.35	23.87 ± 2.01
Length of secondary branch	4.90 ± 0.38	5.09 ± 0.43

Values are mean ± SEM. Number in parenthesis represents sample size.

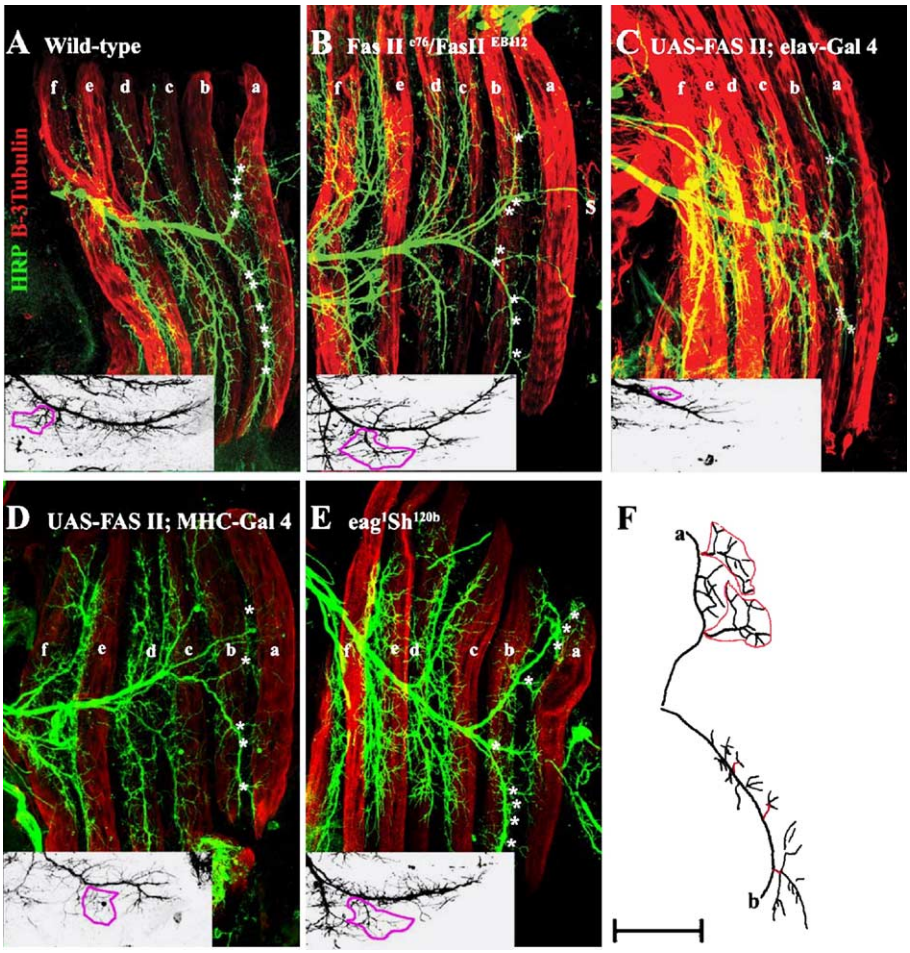

Fig. 5. Fas II restricts secondary branch elaboration and numbers at 24 h APF. A–E: Innervation (visualized with anti HRP) on DLMs at 24 h APF. Muscle profiles are visualized with anti β-3 tubulin (red). Insets: A portion of the primary branch with all of the secondary outgrowths visualized with anti HRP and inverted to a black and white image. Magenta outlines represent area of a representative secondary branch arbor. By 24 h in the wild type (A), the six DLM fibers are visible, and primary branches have extended along the length of the entire muscle. Secondary branches with higher order arbors are labeled (*) on DLMa. Fas II^{e76}/Fas II^{EB112} (B) and eag^{1}Sh^{120b} (E) display a similar number of second order branches (Table 1). When Fas II is overexpressed in the wild-type background, as in the case of UAS-Fas II; elav-Gal 4 (C) and UAS-Fas II; MHC-Gal 4 (D), fewer secondary branches are observed. (F) Schematic of wild-type innervation at 24 h APF on DLMa. There are 2 primary branches, one directed anteriorly (a) and the other posteriorly (b). Short red lines indicate secondary branch length measurements. Red outlines indicate measurements of area occupied by secondary branch arbor. Scale bar = 50 μm.

result, the pool of secondary branches that can elaborate into higher order branches is also reduced (Table 4, Fig. 6) and consequently by 38 h APF fewer branches are stabilized. This is reflected in the decreased number of CPs in the adults.

We next examined the effects of overexpressing Fas II in the muscle. In UAS-Fas II; MHC-Gal 4 animals, the number of CPs is significantly reduced (4.3 ± 0.2; Table 1). The reduction in the number of CPs is reflected in the range of

Table 4
Morphometric characteristics at 24 h APF

Morphology	Oregon R (20)	Fas II^{e76}/Fas II^{EB112} (16)	UAS-Fas II; elav-Gal4 (13)	UAS-Fas II; MHC-Gal4 (11)	eag^{1}Sh^{120b} (10)
Density of second-order branching	2.26 ± 0.17	2.44 ± 0.211	1.75 ± 0.67	4.14 ± 2.45	4.14 ± 0.38***↑
No. of secondary branches	25.75 ± 1.7	29.8 ± 2.59	19.30 ± 1.70*↓	17.09 ± 1.77*↓	40.5 ± 5.0*↑
Length of secondary branch	6.49 ± 0.31	10.17 ± 0.716***↑	5.58 ± 0.67	10.29 ± 1.38	6.22 ± 0.28
No. of secondary branches with arbors	4.55 ± 0.47	4.5 ± 0.83	2.76 ± 0.62*↓	2.90 ± 0.5*↓	4.0 ± 0.96
Average area occupied by arbors of a secondary branch (μm^2)	131 ± 20.46	346 ± 39.73***↑	185.31 ± 49.98	419 ± 81.26**↑	280.5 ± 63.2*↑

Values are mean ± SEM. Number in parenthesis represents sample size.
* $P < 0.05$.
** $P < 0.025$.
*** $P < 0.005$.

Table 5
Secondary branches with arbors on DLMa before and after pruning

Age	No. of secondary branches with arbors	Fas II positive branches	22C10 positive branches
24 h APF	10.6 ± 0.88 (6)	8.33 ± 1.25 (6)	3.88 ± 0.30 (9)
38 h APF	4.86 ± 0.14 (7)	5.0 ± 0.0 (3)	4.75 ± 0.25 (4)

Values are mean ± SEM. Number in parenthesis represents sample size.

CPs in these animals (3–5; Table 1). Thus, increasing Fas II levels in muscles alone has an effect on the eventual number of CPs in the adult. Does muscle overexpression of Fas II also result in fewer branches at 24 h APF? This is indeed the case. At 24 h, UAS-Fas II; MHC-Gal 4 animals display reduced numbers of secondary branches (Table 4). This overall reduction is further reflected in the reduced number of secondary branches that bear tertiary branches (Table 4 and Fig. 5). Thus, increasing Fas II levels either in muscle alone or in both neuron and muscle, results in fewer secondary branches. Our morphometric analyses also revealed that Fas II overexpression in muscle results in an increase in the expanse of the secondary branch in terms of its length and area occupied on muscle surface (Table 4). This is different from observations with the elav-Gal 4 driver and reflects the onset of MHC promoter activity and/or the need for a balance between pre- and post-synaptic Fas II (see Discussion).

Investigating the relationship between Fas II and electrical activity for the patterning of adult innervation

Studies on the development of larval NMJs have demonstrated that hyperactive mutants have excessive branches and boutons (Budnik et al., 1990) and that this is due to a lowering of Fas II levels (Schuster et al., 1996b). Our own studies have shown that the hyperactive mutant, $eag^{1}Sh^{120}$ has fewer CPs at the DLM (Hebbar and Fernandes, 2004), a phenotype distinct from the larva. This raises two relevant questions. First is branch length and expanse reduced in hyperactive mutants during development? Second are Fas II levels altered in the hyperactive mutant?

$eag^{1}Sh^{120}$ displays an increase in the number and density of second order branching during pupal development. 75% of animals exhibit less than the typical 5 CPs in the adult; at 24 h APF, in $eag^{1}Sh^{120}$ animals, there is an

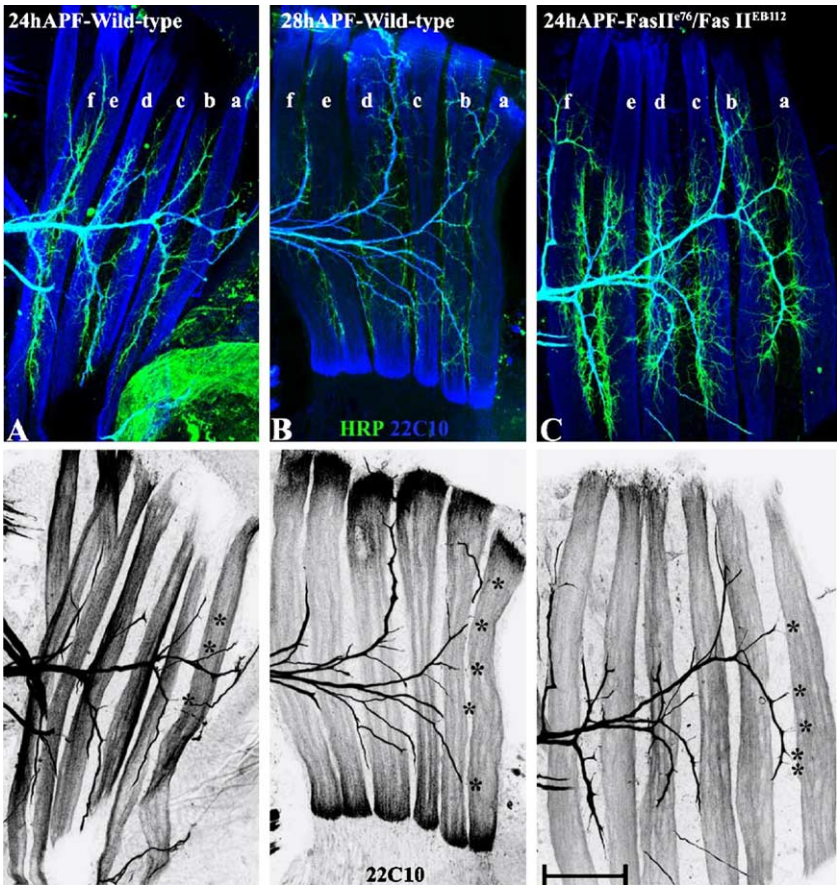

Fig. 6. Futsch/22C10 localization at 24 h and 28 h APF. Top panel: double labeling with Anti HRP (green) and Anti Futsch/22C10 (blue). Bottom panel: Futsch immunoreactivity from top panels visualized in black and white. (A) At 24 h APF, Futsch/22C10 localizes to a small number of secondary branches (asterisks in bottom panel) that bear higher order arbors. (B) By 28 h APF, when pruning is underway, a greater number of secondary branches are labeled. (C) In the Fas II hypomorphs, Fas II^{e76}/ Fas II^{EB112} at 24 h APF, many more secondary branches are labeled than the wild-type. Scale bar = 50 μm.

increase in the number of secondary branches (Hebbar and Fernandes, 2004) and in the density of branching (Fig. 5; Table 4). The secondary branches are as long as their wild-type counterparts (Table 4). Interestingly the expanse of the secondary branch arbor measured as area occupied by the arbor is significantly greater than the wild-type (280 ± 63 μm^2 vs. 131 ± 20.46 μm^2 in the wild type, $P = 0.03$).

Since secondary branch length is increased in Fas II hypomorphs (with respect to wild-type) and remains unchanged in *eag^{1}Sh^{120}* animals, the prediction would be that Fas II levels in *eag^{1}Sh^{120}* are similar to the wild-type. In studies of the larval NMJ, intensity of immunostaining has been used as a measure of Fas II levels (Schuster et al., 1996b; Mathew et al., 2003). Intensity analyses were carried out along axons at 24 h APF. Fas II levels in *eag^{1}Sh^{120}* were within 10% of the wild type levels. The hypomorph, Fas II^{e76}/Fas II^{EB112} exhibited a 44% decrease in intensity, indicating reduced Fas II levels. In elavGal4; UAS-Fas II, animals a 79% increase in staining intensity was detected (Fig. 7). The staining intensity of anti-HRP was measured as an independent control. Anti HRP staining intensity remained constant in the genotypes that were tested for levels of Fas II intensity (Fig. 7). Thus, unlike in the larva, Fas II levels along the axons of DLM motor neurons remain unchanged in *eag^{1}Sh^{120}* pupae, indicating that activity and Fas II may act in different pathways leading to establishment of the DLM innervation pattern.

Discussion

The stereotypy of the DLM innervation pattern is established within the first 48 h of metamorphosis (Hebbar and Fernandes, 2004). 8 h after the larva turns into an immobile pupa, the larval NMJs are completely withdrawn and the nerve maintains contact with the larval scaffolds that will give rise to the DLMs (Fernandes and VijayRaghavan, 1993). A phase of adult-specific outgrowth then begins. Primary branches grow along the dedifferentiating larval scaffolds (12 h APF) and even as they are extending, second order branches begin appearing. This coincides with the onset of myoblast fusions that initiate fiber formation (Fernandes et al., 1991). By 18 h APF, third order branches are visible, and by the end of the first day (24 h APF), higher order branching is maximal (Hebbar and Fernandes, 2004). Only a small subset of the pool of second order branches contributes to the adult innervation pattern; the remainder are pruned back between 24 and 38 h APF, revealing the adult pattern of multiple contact points (CPs). The stereotypy of the adult pattern of innervation lies in the number of stabilized CPs along the length of the muscle fiber. Interestingly, boutons first become visible after the pruning event. For the remainder of metamorphosis, arbors corresponding to each contact point grow in size and match the growth of the muscle fiber. Thus, there are three major phases of arbor development (summarized in Fig. 8): outgrowth and elaboration when adult specific outgrowth

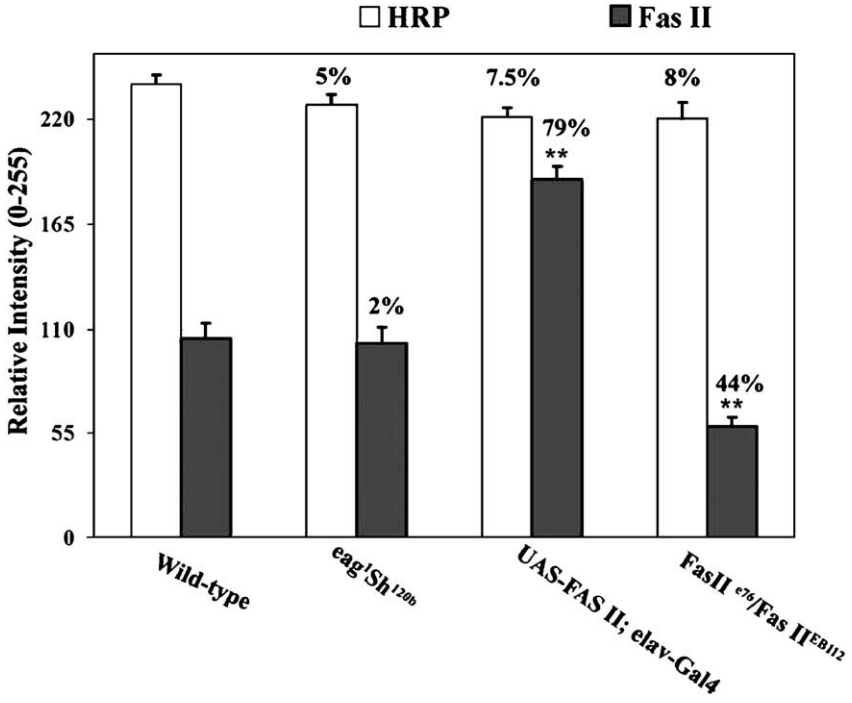

Fig. 7. Neuronal levels of Fas II are not different in eag^{1}Sh^{120b} at 24 h APF. Quantification of fluorescence intensity of anti Fas II and anti HRP staining along the main nerve trunk in wild-type (Oregon R), hyperactive mutant eag^{1}Sh^{120b}, UAS-Fas II; elav-Gal 4 and Fas II^{e76}/Fas II^{EB112} hypomorphs. The average anti HRP level varies between 5 and 8% across all genotypes. As expected, Fas II levels are altered significantly ($P < 0.000005$) in Fas II^{e76}/Fas II^{EB112} animals and UAS-Fas II; elav-Gal 4 animals. Fas II levels in eag^{1}Sh^{120b} animals are similar to wild-type ($P = 0.7$). Numbers on the bars indicate the percent change in intensity as compared to the wild-type. The level of significance is indicated by (*).

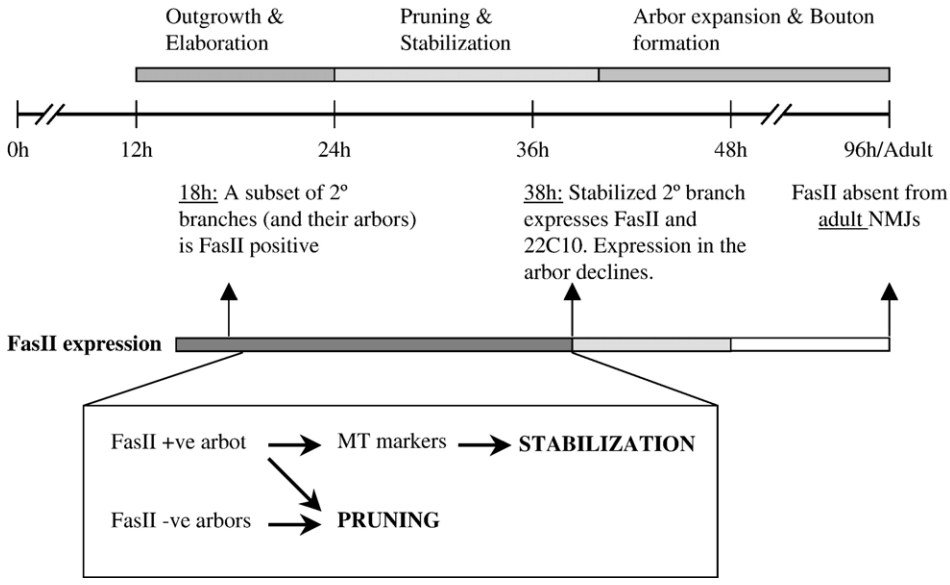

Fig. 8. A schematic representation of the role of Fas II in patterning DLM innervation. There are 3 phases of arbor development that result in the adult pattern of DLM innervation: outgrowth and elaboration of adult-specific motor neuron branches, pruning of excessive branches, and the expansion of stabilized arbors. Fas II is expressed during the first two phases. Between 14 and 18 h APF, secondary branches grow out and begin elaborate higher order branches on the muscle surface. By 18 h APF, a subset of branches with higher order arbors is positive for Fas II. At 24 h APF, some branches that bear arbors also express Microtubule (MT) associated protein, Futsch/22C10, and are the ones that will be stabilized to generate the stereotypical multi-terminal innervation at the adult DLMs. The remainder of branches are either Fas II (+)/Futsch/22C10 (−) or Fas II(−) /Futsch/22C10 (−). Since not all Fas II (+) branches are retained, we propose that Fas II primes branches for stabilization, and that it initiates a series of events that result in the expression of molecules such as 22C10, which complete the stabilization process. These stabilized branches survive the pruning phase. The downregulation of Fas II during the last phase (decline is indicated by the lighter bar, and absence by the open bar) is likely to have a bearing on the expansion of stabilized arbors during the remainder of metamorphosis. Fas II is absent at the adult stage, and another cell adhesion molecule is likely to be involved in maintenance of the terminal arbor.

of branches occurs, pruning, when excessive branches and their arbors are removed to reveal the adult pattern of CPs, and the expansion phase, during which the stabilized arbors expands in tandem with the muscle, which is known to grow three times in size (Finlayson, 1975). In this study, we have investigated the role of Fas II in stabilizing second order branches to thereby generate the adult pattern of multiple CPs. We present a model (Fig. 8) wherein Fas II regulates the length of second order branches and the expansion of arbors during the outgrowth and elaboration phase. These are important first steps in the pathway that leads to branch stabilization, a process that is initiated prior to the pruning phase. Fas II levels begin to decline subsequent to the pruning phase, and we propose that this may have a bearing on the co-ordinate expansion of the stabilized arbor and the muscle fiber during the final phase of arbor development.

The dynamic nature of Fas II expression

Although second order branches are first seen as early as 12 h APF (Fernandes and VijayRaghavan, 1993), they do not express Fas II until about 18 h APF. It is likely that during this span of 6 h a neuron–muscle communication or an interaction among neuronal branches may result in the onset of Fas II expression. Each second order branch prefigures a potential "contact point" for the adult pattern of innervation. Thus, the expression of Fas II in subsets of second order branches may be significant for stabilization. Interestingly, a large fraction (86%) of Fas II branches is comprised of those that have elaborated an arbor. Of these, 60% are incorporated into the adult innervation pattern. The fact that not all Fas II arbors survive the pruning phase indicates that the expression of Fas II initiates a "stabilization pathway" that must involve other molecules that act together to regulate branch properties. It is likely that the muscle may be a participant in the stabilization process, in a manner likely to vertebrate NMJs, where it has been proposed to be the source of "protective/survival factors" that may be limiting (Nguyen and Lichtman, 1996; Chang and Balice-Gordon, 1997). As the pool of arbors marked by Fas II goes through the pruning phase, 22C10, which detects a microtubule associated protein, begins to appear in branches that will be stabilized into the adult CPs. The number of 22C10 positive branches gradually increases from 24 h APF to 28 h APF, and by 38 h APF (completion of pruning) all the retained branches express Fas II as well as 22C10.

Once branches are stabilized, and the adult pattern of multiple CPs emerges, Fas II expression in the stabilized CPs begins to decline, a feature that is evident by 48 h APF (data not shown). The downregulation of Fas II may be significant for continued expansion of the arbors, which occurs through the remainder of metamorphosis, and presumably matches muscle growth (Hebbar and Fernandes, 2004). Fas II is absent from the adult NMJ, a feature that distinguishes the synapses of the adult DLM from its larval

counterpart. Fas II is an important component at the mature larval synapse (Schuster et al., 1996a). Thus, Fas II plays a role during development of the adult innervation pattern of the DLM, and it is likely that other cell adhesion molecules or a different form of Fas II may be present at the mature NMJ. In *Manduca*, it has been reported that during development of leg muscle innervation, a transmembrane Fas II is present, whereas a GPI linked Fas II later becomes localizes to the adult NMJs (Knittel et al., 2001).

Another potentially interesting aspect of Fas II expression is that at 38 h APF, it is present in a compartment that encompasses the axons of the PDMN. This is likely to be glia, as it is not HRP positive. Since the glial expression is evident after the pruning phase is completed, it may have a bearing on the subsequent phase of arbor expansion and synapse formation. Recent studies in vertebrates have shown that thrombospondin released from glia bring about synapse formation (Christopherson et al., 2005). This may be relevant to synapse formation at the DLM, as bouton formation is initiated only after the pruning phase is completed (Hebbar and Fernandes, 2004).

Regulating arbor development

The onset of Fas II expression is coincident with the onset of a phase of motor neuron elaboration (18–24 h APF), during which higher order branching occurs, resulting in the formation of arbors. In addition to the expansion of arbors, new secondary branches are also being added, and these presumably comprise the 20% of Fas II positive branches that have not elaborated into higher order branches. It is important to bear in mind that branch elaboration and branch addition occurs during the time that myogenesis is ongoing. The continued fusion of myoblasts serves to increase muscle surface area, which may be conducive for branch addition. During this period, both electrical activity (Hebbar and Fernandes, 2004) and the muscle surface (Fernandes and Keshishian, 1998) are known to promote branch elaboration, and therefore a role for Fas II must be considered in that context.

In Fas II hypomorphs, the numbers of CPs (or stabilized secondary branches) that make up the adult innervation pattern are significantly increased. This is brought about not by increasing the number of second order branches, but by generating longer and more elaborated secondary branches during the pupal phase. A possible explanation is that Fas II normally restricts branch outgrowth and that the loss of adhesion allows second order branches to explore and elaborate over the muscle surface. In restricting branch elaboration, it is likely that Fas II acts on secondary branches through local interactions, with Fas II on other secondary branches and/or on the muscle restricts expansion of secondary branch arbors. Similar spatio-local interactions through Ca^{2+} mediated signaling are believed to regulate the size of dendritic arbors in developing optic tectal neurons of *Xenopus* (Cline, 2001).

The increased expanse is likely to be favorable for stabilization perhaps by access to protective signals, and this is reflected in the increased number of stabilized secondary branches/CPs in the adults. Since Fas II is a homophilic cell adhesion molecule (Grenningloh et al., 1991), it is possible that two molecules on the axonal branch interact to restrict secondary branch elaboration. Alternatively, Fas II on the axon may interact with muscle Fas II to bring about the restriction. Increasing the levels of Fas II by using elav-Gal 4 and MHC-Gal4 to overexpress Fas II, results in fewer CPs. In this instance, the pool of second order branches is reduced, and changes in adhesion can be used to explain the phenotype. It is possible as a result of increased Fas II levels, there is increased adhesion between axons (or between axon and muscle) that in turn inhibits the formation of secondary branches.

Neuronal Fas II or muscle Fas II?

Fas II expression in the presynaptic compartment is obvious during the 18–38 h period, and it is not surprising that a neuronal driver (elav-Gal 4) is able to rescue the hypomorphic phenotypes. However, the ability of a muscle driver to suppress Fas II hypomorphic phenotypes was surprising. This suggested a role for muscle-derived Fas II, which is undetectable in our immunostaining experiments presumably due to low levels. Interestingly, elav-Gal 4, a reported neuronal driver is also expressed in muscle fibers starting at about 16 h APF (Fernandes and Keshishian, unpublished observations), and must be considered in explaining how the innervation phenotype is rescued. When the elav-Gal 4 driver is used to express Fas II in the hypomorphic background, there is a complete rescue of the number of CPs seen in the adult. However, when the MHC-Gal4 driver is used, the number of CPs is actually reduced. MHC driven expression is likely to increase Fas II disproportionately in one compartment, while elav-Gal 4 would increase it in both pre- and post-synaptic compartments. Thus, the difference in number of CPs observed in the two conditions of overexpression suggests that a balance between pre- and post-synaptic Fas II regulates aspects of arbor development.

A novel role for Fas II in adult NMJ formation

An important question implicit in our studies is the manner in which molecules are re-utilized in the second round of NMJ formation during metamorphosis. In embryonic/larval NMJ development, Fas II is important for growth cone guidance (Grenningloh et al., 1991), target selection (Davis et al., 1997) and subsequently in synapse growth and maintenance (Schuster et al., 1996a). Our results show that Fas II plays a role in stabilizing branches, thus influencing patterning of the adult NMJ. Two major attributes of Fas II expression suggest that the mechanism(s) by which it operates in the adult context is distinct

from the embryo/larva. This is not completely unexpected since the developmental events in the formation of the embryonic/larval and adult NMJs are distinct (Fernandes and Keshishian, 1995, 1999). First, Fas II is only present during metamorphosis, when innervation is being patterned, and is absent from the adult NMJ. This transient expression of Fas II is unlike the situation in the larval NMJ. Fas II is downregulated at the early stage of synapse formation in the late embryo but is subsequently seen at the mature larval NMJ and regulates synapse maintenance (Schuster et al., 1996a). At the adult NMJ, since Fas II is absent, it appears that other cell adhesion molecules may be involved. The absence of Fas II from the adult synapse also suggests that the molecular architecture of the adult synapse differs from its larval counterpart. Second, at the larval NMJ, reductions in Fas II levels result in a reduced NMJ (Schuster et al., 1996a), and it can be argued that the NMJ at the DLM is expanded. The extremely small size of boutons on the DLM fibers is a challenge for the purposes of obtaining bouton counts (Hebbar and Fernandes, 2004). However, since the higher order branches of each CP bear boutons (Fig. 1), and given the increase in number of CPs in Fas II hypomorphs, it is conceivable that the NMJ is expanded. Average expanse of arbors measured by muscle area occupied, as well as number of branch tips is not significantly different between controls and Fas II hypomorphs (data not shown). Although not directly related to size of the NMJ, it has been documented that axonal projections of developing adult wing sensory neurons in the CNS also display increased branching when Fas II levels are severely reduced (Whitlock, 1993).

Relationship between Fas II and electrical activity

In several allelic combinations of the hyperactive mutant, eag^{1} Sh^{120}, larval NMJs are expanded through increases in bouton number as well as through increased higher order branching (Budnik et al., 1990). It has been demonstrated that an increase in electrical activity is associated with reduced Fas II levels, which in turn cause expansion of the larval NMJ, suggesting that electrical activity and Fas II act in the same pathway (Schuster et al., 1996b). Our studies with the development of DLM innervation suggest that the relationship between electrical activity and Fas II is different. Although the establishment of stereotypic branch points of DLM motor axons (multiterminal innervation) on the muscle and the terminal arbor expanse at each branch point may not have a direct parallel with the larval NMJ (single-terminal innervation), it is nevertheless useful to dissect apart the relationship between electrical activity and Fas II in the context of patterning an adult NMJ (Table 6). The number of CPs or terminal arbors are increased in Fas II hypomorphs and decreased in electrical activity mutants. Although Fas II is absent at the adult NMJ, it is present during the patterning of innervation earlier in the metamorphic phase. At 24 h APF, Fas II is present at wild-type

Table 6
Relationship between Fas II and electrical activity during the patterning of DLM innervation

	$Fas\ II^{e76}/Fas\ II^{eB112}$	$eag^{1}\ sh^{120b}$
# CPs (adult)	↑	↓
Fas II levels (24 h)	↓	WT
2° branch length	↑	WT
Density of 2°	WT	↑
Arbor expanse	↑	↑

levels in eag^{1} Sh^{120}, as measured along second order branches of the motor axons. This is different from the larval NMJ, where Fas II levels are reduced. At 24 h APF, the length of secondary branches is normal, but branch addition (measured by density of second order branching) is altered. Thus, branch length is not regulated by electrical activity, and possibly influenced by Fas II. Each second order branch develops an arbor, and the expanse is increased in hyperactive mutants. How can this be reconciled with increased arbor expanse that is also seen in hypomorphs? We propose that elaboration of higher order branches that takes place prior to the pruning phase is regulated by two opposing forces—electrical activity expands the arbor, and Fas II serves to restrict the expansion. In Fas II mutants therefore, loss of Fas II allows unrestricted expansion; whereas in eag^{1} Sh^{120}, hyperactivity drives the expansion, overriding the effects of normal Fas II levels. Despite the presence of arbors with increased expanses, why then are fewer arbors stabilized in hyperactive mutants? As we have suggested in a previous study (Hebbar and Fernandes, 2004), this effect is likely due to enhanced pruning and resembles events at the developing vertebrate NMJ, where chronic stimulation of muscle accelerates synapse elimination (Thompson, 1983). Although pruning occurs prior to the appearance of morphologically identifiable boutons, it is likely that the terminal arbors are nascent synapses, and consistently, they do express the pre-synaptic marker, synaptotagmin (Hebbar and Fernandes, 2004).

In conclusion, we have identified a role for Fas II in patterning the DLM innervation in the context of branch stabilization. We show that manipulating Fas II levels does not grossly alter the innervation pattern, but that it affects attributes such as number of contact points, branch length and expanse of arbors. Our data indicate that Fas II primes branches for stabilization and future studies will aim to identify molecules that act in concert with Fas II to regulate branch development of adult motor neurons during metamorphosis.

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