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Is TGF-β playing a role in ectopic neuromuscular junction formation in the nematode Caenorhabditis elegans?

Abir A. Rahman

IS TGF-β PLAYING A ROLE IN ECTOPIC NEUROMUSCULAR JUNCTION FORMATION IN THE NEMATODE *CAENORHABDITIS ELEGANS*?

by

ABIR ASHFAKUR RAHMAN

Under the Direction of Walter W. Walthall

ABSTRACT

The neuromuscular junction (nmj) is a commonly studied synapse, used often to investigate reciprocal signaling between a motor neuron and the appropriate target muscle. In *Caenorhabditis elegans*, ectopic nmjs can be created by eliminating selected embryonic muscle cells that act as guideposts for the migration of post-embryonic muscles. The ectopic muscles are required to induce sprouting from DD motor neurons, indicating the presence of a muscle derived signaling molecule that interacts with the neurons. A TGF-β homolog, *unc-129*, is reported to be transiently expressed in the dorsal body wall muscles. The timing of the expression of TGF-β coincides with the time that the DD motor neurons respecify their synapses. In this study, we show that TGF-β is expressed by the ectopic muscle and that in *unc-129* mutant animals, the ectopic muscle is unable to induce sprouting from the DD motor neurons. Therefore, we conclude that TGF-β is necessary for ectopic nmj formation in *C.elegans*.

INDEX WORDS: *Caenorhabditis elegans*, Synaptogenesis, Retrograde signaling, TGF-β, Neuromuscular junction, Netrin, Axon guidance

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DEDICATION

To Sharmin Islam, who decided to take up a career in Molecular Biology because I told her that it was a really cool field. You have always been and will continue to be one of my biggest inspirations. And to Rhea Arefin and Sanika Mahdiya, my babies. Hopefully, I can be a source of inspiration for you too.

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1. INTRODUCTION

The assembly of functional neural networks requires interactions that allow recognition among the participating cells. The developing neuron first sends processes that follow guidance cues on appropriate substrates to find appropriate target and then initiates synaptogenesis, a complex developmental process that leads to the formation of the synapse, which consists of a pre- and postsynaptic element. Both process guidance and synaptogenesis require the exchange of various signaling molecules. Molecules secreted from the target cells are collectively known as retrograde signaling molecules; whereas the signaling molecules released from the presynaptic neuron are known as orthograde signaling molecules (Fitzsimonds and Poo 1998). Reciprocal signaling has been shown to be a very important mechanism by which the events of synaptogenesis are coordinated (Sanyal, Kim and Ramaswami 2004).

The neuromuscular junction presents an intriguing location to study these phenomena. This is a synapse where the presynaptic and the postsynaptic elements arise from two distinct lineages. The ectodermally derived motor neuron must innervate appropriate target muscles derived from the mesodermal germ layer. In vertebrates, a single muscle cell is initially innervated by multiple axons. However, in the adult animal, the individual muscle cells are innervated by single motor axons. Synapses made by all but one motor axon are eliminated in an activity-dependant manner (Redfern 1970; Lichtman 1977). This high degree of specificity of pairing between the neuronal presynaptic elements and the post-synaptic elements on the muscle is achieved through reciprocal signaling.

Evidence for retrograde signaling at neuromuscular junctions is seen in *Xenopus* (Dai and Peng 1993) and in *Helisoma* (Zoran, Funte, Kater and Haydon 1993) cell cultures as well as in *Drosophila melanogaster* (Aberle et al. 2002; Keshishian and Kim 2004; Marques et al. 2002) and *Caenorhabditis elegans* (Hedgecock, Culotti and Hall 1990; Colavita et al. 1998; Macneil et al. 2009). Work on *C.elegans* has been particularly insightful because of the ability to study synaptogenesis in vivo, and the relative

ease of performing genetic manipulations. The nematode has a relatively short reproductive span, which reduces the time required to generate and maintain true breeding mutant animals. In addition, the animal is transparent and it is possible to fluorescence-label entire neurons or the presynaptic termini of the neurons of interest (Chalfie et al. 1994).

The nervous system of *C. elegans* consists of 302 neurons, out of which there are 19 GABA-ergic motor neurons that are designated the D motor neurons. There are 6 dorsal D neurons (DD) that initially innervate ventral body wall muscles, when the animal hatches, and undergo synaptic respecification at around the first larval molt, innervating dorsal body wall muscles thereafter. This change of polarity occurs without any morphological change in the neurons themselves (White et al 1978; Walthall 1990).The remaining 13 D motor neurons are designated ventral D (VD) neurons because they innervate the ventral muscles (Figure 1). They are postembryonic and are born around the same time as the DD synaptic respecification event. The DD motor neurons are seeking new muscle targets during the respecification and, presumably, are receptive at this time point, to retrograde signaling molecules from the muscles seeking innervation. We hypothesize that this molecule is TGF-β.

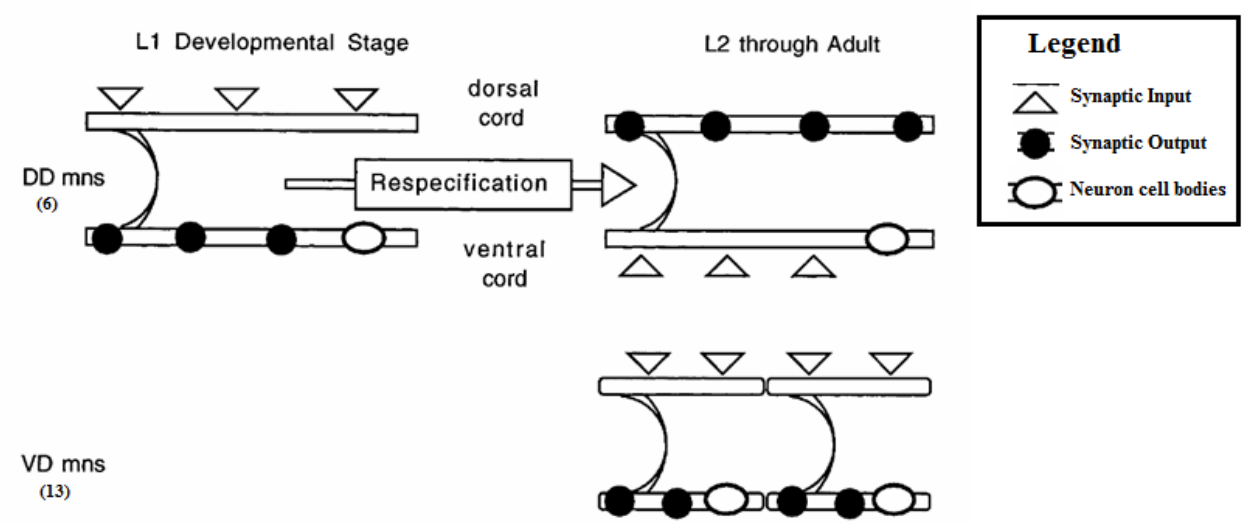

Figure 1 – The direction of information flow in DD and VD motor neurons

(Top) DD motor neurons innervate ventral muscle when the animal is born, but undergo a respecification event and innervate dorsal muscle in the later stages. (Bottom) VD motor neurons are born after the animal hatches from the egg, around the first larval molt, and innervate ventral muscle. (Figure adopted from Plunkett, Simmons and Walthall 1996)

There are 95 body wall muscles in *C.elegans,* organized into four contiguous strands running along the length of the animal (Figure 3A). Out of these 95 muscle cells, 81 are embryonic and are present when the animal hatches, and the remaining 14 are born at around the first larval molt. Since the complete lineage of the 959 somatic cells in the adult animal has been mapped out (Sulston et al. 1983), it is possible to trace each muscle cell and each neuron to their precursors. The 14 postembryonic muscle cells and some other cells are descendants of a myoblast called the M cell (born from MSapa in figure 2). A subset of the 81 embryonic body wall muscle cells are derived from the myoblasts Cap and Cpp (figure 2). This lineage map is invariant and it is, therefore, possible to eliminate specific cells from the adult animal, by performing laser ablation of the appropriate precursors in embryos. Such ablation of the Cap and Cpp cells results in the absence of the descendant body wall muscles from the animal (Plunkett, Simmons and Walthall 1996). This, in turn, creates a gap in the dorsal body wall muscle strands at hatching.

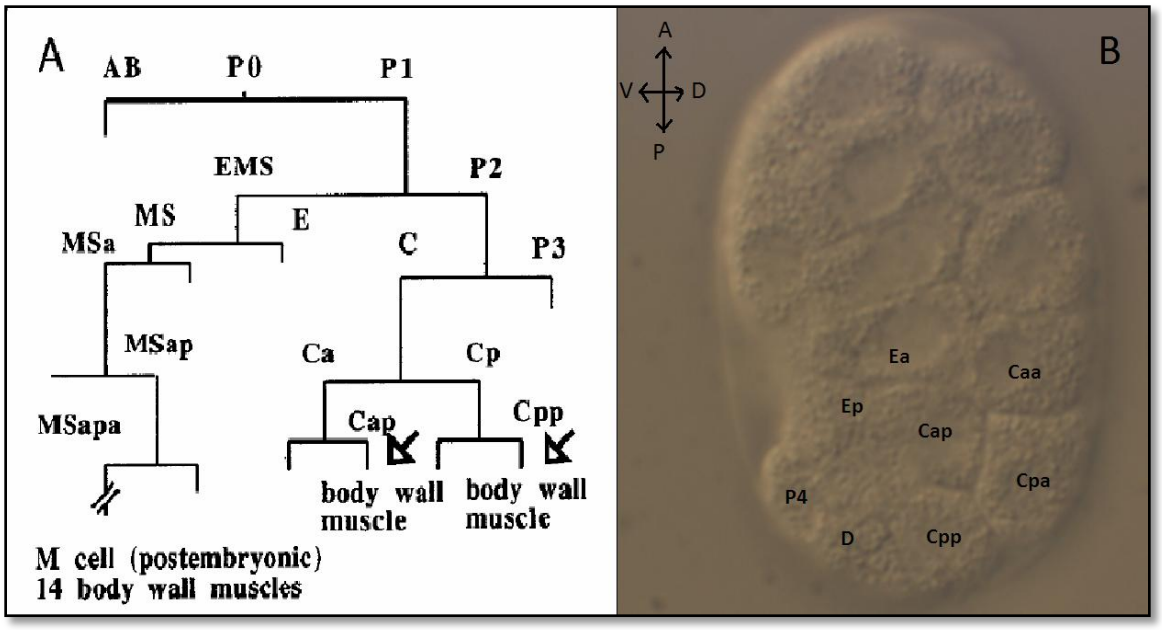

Figure 2 – The lineage and a Nomarski image of the Cap and the Cpp cells

(A) Partial lineage of *C.elegans* showing the body wall muscle precursors Cap, Cpp and MSapa. Anterior daughters are shown to the left and posterior to the right (Adopted from Plunkett, Simmons and Walthall 1996). (B) Nomarski image of a *C.elegans* egg approximately 100 minutes post fertilization. The Cap and the Cpp have just been born.

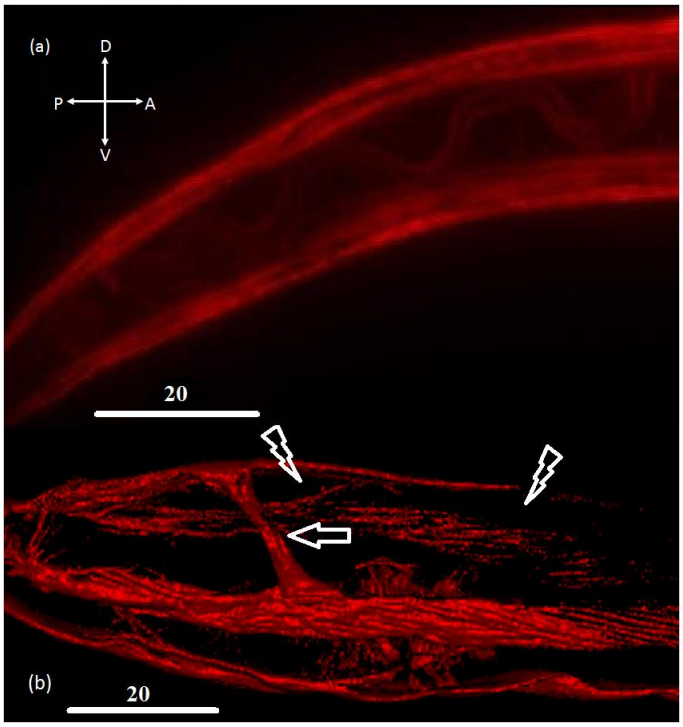

Figure 3 – Ectopic muscle generated when Cap/Cpp are ablated

Body wall muscles in *C.elegans* (a) in control animals, and (b) following ablations. The arrow indicates the location of ectopic muscle strands and the lightning bolts mark the gap region in the dorsal body wall muscle strand.

As the animal develops, ectopic strands are set up by the postembryonic muscle cells (Figure 3) presumably because the embryonic muscles act as guideposts that are now absent due to the ablation. Figure 4 shows a schematic description of the process. These ectopic muscles are then innervated by processes from the DD motor neurons that have sprouted from the dorsal nerve cord (Plunkett, Simmons and Walthall 1996). Ablation of the MSapa (post-embryonic muscle precursor, figure 2) along with Cap and Cpp leads to a gap in the dorsal muscle strands as well as absence of all ectopic muscle. In such a case, the neurite sprouting from the DD neurons was no longer seen (Plunkett, Simmons and Walthall 1996). We hypothesize that the ectopic muscles are secreting a signaling molecule that might be responsible for inducing these innervations.

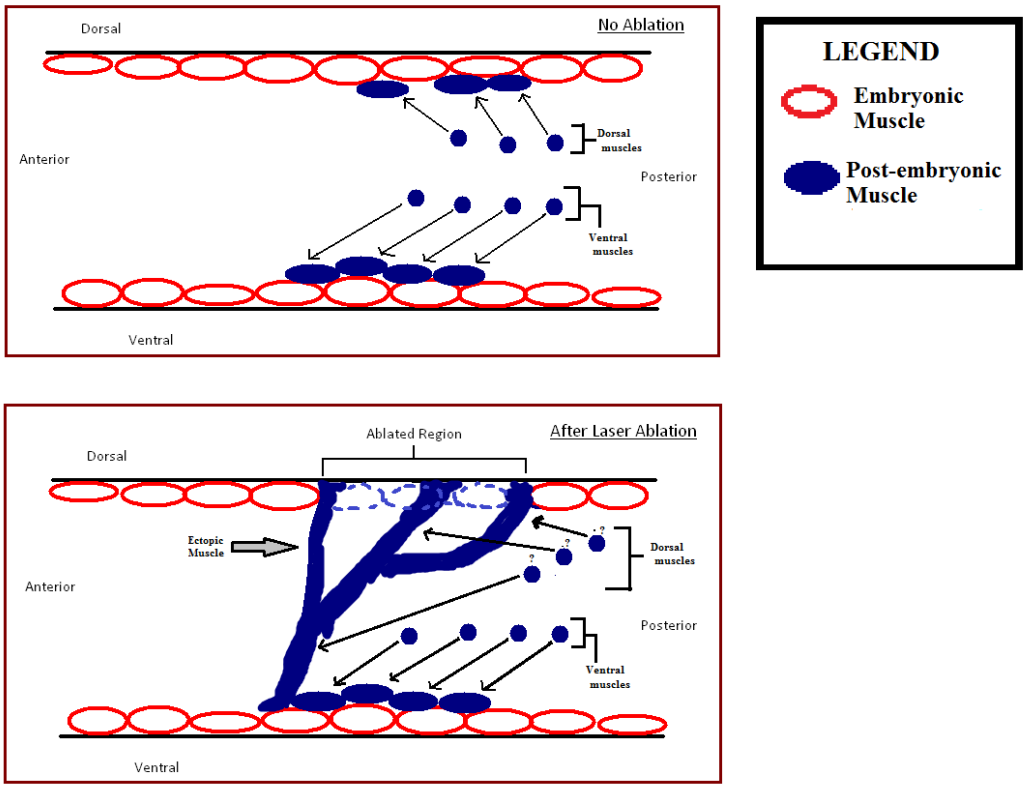

Figure 4 – Schematic explanation of ectopic muscle formation

The blue cells are M cell descendants (postembryonic) and the red cells are embryonic muscle cells. (Top) Embryonic muscles that act as guideposts are present and post-embryonic muscles are able to integrate themselves at their designated locations. (Bottom) When selected embryonic muscle precursors were ablated, the embryonic muscles were no longer present. As a consequence, the postembryonic muscle cells often lost their way and formed ectopic strands (shown using a gray arrow). The black arrows represent the fate of the post-embryonic muscles under the two conditions.

The *C.elegans* TGF-β homolog, *unc-129*, is secreted by the dorsal body wall muscles (Colavita et al. 1998). Its expression in the dorsal body wall muscles is transient such that at L1, all dorsal body wall muscles are expressing *unc-129* but by around the L1/L2 molt, only a subset of the muscles is expressing *unc-129,* gradually reaching the point when, in the adult animal, none of the dorsal muscles are expressing *unc-129*. In this study, the ectopic muscles were seen to express *unc-129* at around the L1/L2 molt. We suggest that this TGF-β is functioning as a retrograde signaling molecule, inducing innervation of the ectopic muscles when the DD motor neurons are seeking new targets, and is then no longer expressed once the synaptogenesis is complete.

According to Macneil et al. (2009), UNC-129 interacts with two non-canonical receptors, UNC-5 and UNC-40, to mediate pioneer axon guidance via the UNC-6/Netrin pathway. UNC-129 is thought to switch the signal transduction mechanism of the developing neuron from UNC-5 alone signaling to UNC-5+UNC-40 signaling, which in turn, increases the sensitivity of the growth cone to UNC-6. This becomes important in cases where UNC-6 acts as a repulsive signal and neural processes extend away from regions of high concentrations of UNC-6, such as the case of DD motor neuron axon migration. As the growth cone migrates from UNC-6 rich regions to UNC-6 deficient regions, an increase in sensitivity takes place in the growth cone, in order to compensate for the decrease in UNC-6 concentration. UNC-129 is graded opposite to UNC-6 and is thought to promote this compensatory increase in sensitivity (Colavita and Culotti 1998; Macneil et al. 2009). In this paper, we show that the ectopic muscle, generated by the Cap/Cpp ablation process, expresses *unc-129*. The mechanism described by Macneil et al. (2009) might therefore be the mechanism for ectopic muscle innervation via retrograde signaling mediated by the UNC-129, UNC-5 and UNC-40. We, therefore, tested a hypothesis that *unc-129* is necessary for the ectopic neurite branching and subsequent innervation of ectopic muscle.

2. MATERIALS AND METHODS

Strain maintenance: All strains of *C. elegans* were grown and maintained at 22°C on NGM agar plates as described by Brenner (1974). Mating plates were set with 6 males and 2 L4/adult hermaphrodites per plate. Table 1 shows a list of the genotypes of the different strains used and the respective tissues that expressed GFP. The N2 Bristol strain was used as wild type for crossing purposes.

Table 1 - Genotypes of the animals used.

Transgenic animals expressing GFP under neuron-specific promoters were used to visualize the respective neurons.

Genotype	Locomotion Phenotype	Fluorescence label
pflp11::gfp[1]	Wild Type	DD, VD and a few DA motor neurons
pflp13::gfp[1]	Wild Type	DD motor neurons
punc129::gfp[2]	Wild Type	DA and DB motor neurons, and dorsal body wall muscles
unc129:pflp11::gfp (ER 151)	Mild kinker (*unc-129* mutant)	DD, VD and a few DA motor neurons
unc5:pflp11::gfp (ER 152)	Severe coiler (*unc-5* mutant)	DD, VD and a few DA motor neurons
unc40:pflp11::gfp (ER 153)	Variable kinker (*unc-40* mutant) (dumpyish)	DD, VD and a few DA motor neurons

1. Christine Li, personal communication
2. J. G. Culloti, personal communication

Genetic crossing: Hermaphrodites from transgenic strains expressing the desired GFP transcriptional reporter were first crossed with N2 male animals to generate heterozygous males. These heterozygous males were then mated with homozygous hermaphrodites containing the desired mutation (recessive). The progeny were screened for heterozygous, GFP-positive hermaphrodites, with wild-type locomotion. Approximately 6 of these hermaphrodites were then placed on culture plates together and allowed to reproduce by self-fertilization. The progeny of these six worms were screened for individuals that were homozygous for the mutation, as indicated by the locomotion defective phenotype, and expressed the GFP transgene. Strains were then maintained under positive selection for GFP expression.

Laser Ablations: Healthy, gravid hermaphrodites were cut open to obtain eggs. The eggs were then mounted on 0.1% agarose gel pads and visually monitored at 1000x magnification until the embryonic precursors to the dorsal body wall muscles, Cap and Cpp, were born, around 100 minutes post-fertilization (Sulston et al. 1983). These cells were then ablated using a nitrogen laser. They were shot 3 times for 10 seconds each at the firing rate of approximately 4 shots per second, under the 100x objective lens set at a numerical aperture setting of 1.6. Eggs were then recovered in a culture plate at 22°C. Ablations were performed in animals from each of the genotype groups from table 1. Successfully ablated animals were then identified, fixed and stained with RITC-Phalloidin by a procedure modified from Plunkett, Simmons and Walthall (1996), and analyzed using deconvolution microscopy (Deltavision Olympus IX70 microscope). The images thus obtained were then color coded green for GFP and red for RITC-phalloidin. Images were taken only of the posterior third of the animal because the Cap/Cpp ablation only affects that part of the animal. The rest of the animal remains mostly unaffected.
Scoring criteria: Ectopic muscle was defined as any muscle strand outside the four muscle quadrants and that did not form part of the gut of the animal. Ectopic neurite branches were counted only if they appeared to terminate in close proximity to an ectopic muscle strand. Otherwise, they were considered aberrant commissures and not included in the count.

3. RESULTS

To determine whether the findings of Plunkett, Simmons and Walthall (1996), could be reproduced in embryos carrying transgenic GFP reporters we used the promoter of *flp-13 (pflp13::gfp).* This GFP construct is expressed in the DD motor neurons (Kim and Li 2004). In unablated animals, this allowed observation of the ventrally located cell bodies of the neurons, ventral postsynaptic processes, commissures projecting to the dorsal nerve cord where they branched. Four body wall muscle strands (shown in red) flanked the two nerve cords (Figure 5). Images were always taken of the posterior third of the animal because the Cap/Cpp descendants are normally located in this region of the animal and the ablation procedure only affects this part of the animal. The rest of the animal remains mostly unaffected.

In ablated animals, the tail was severely deformed because the missing muscles normally act as girdles and help maintain the shape of the worm. Ectopic muscle was observed in the ablated regions as described by Plunkett, Simmons and Walthall (1996). Any muscle strand observed outside the four contiguous body wall muscle strands, and not contributing to the gut, was considered ectopic. Ectopic muscle was observed in 8 of preps out of 10 (Table 2). In addition, ectopic GFP-positive branches emanating from the DD motor neurons extended from the dorsal nerve cord to the ectopic muscle strands as shown figure 6 (shown with white arrow). This was consistent with the observations made by Plunkett, Simmons and Walthall (1996) that the ectopic muscle is able to attract innervation.

This finding was further confirmed by performing ablations in *pflp11::gfp* transgenic animals. The animals in this background looked similar to the *pflp-13* animals, with GFP being present in the DD, VD and DA motor neurons (Kim and Li 2004). In the unablated animals, GFP was observed in the cell bodies and the processes of these neurons, including those forming the dorsal and ventral nerve cords. The muscle strands, like the *pflp-13* animals, were flanking the nerve cords (Figure 7, a-c). Ectopic

muscles were also seen after ablations performed in these animals, along with ectopic neural processes that innervated the muscles (Figure 7, d-f).

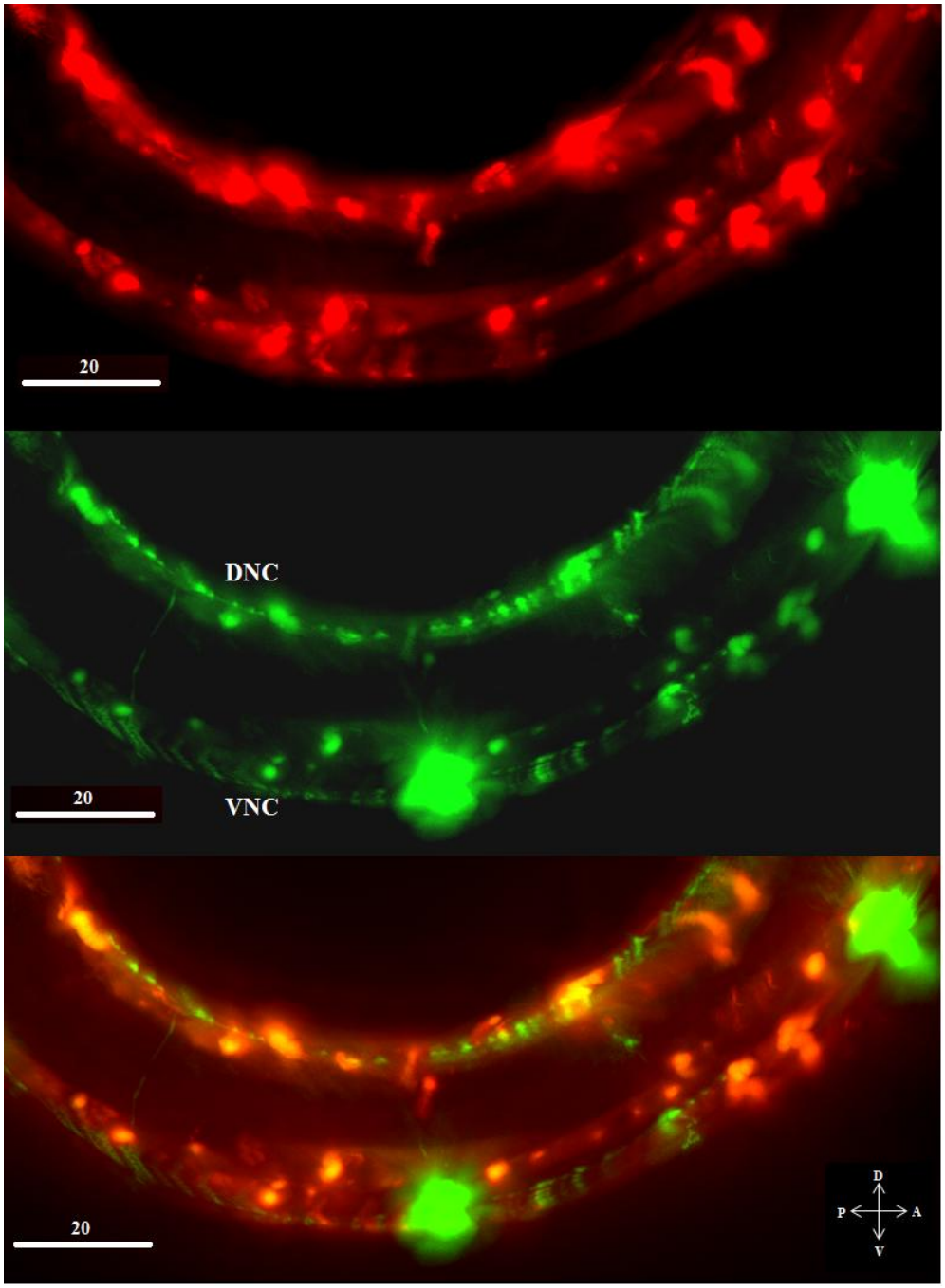

Figure 5 - Neuron and muscle morphology in an unablated *pflp-13::gfp* animal.

DD motor neurons and their processes are shown in green and muscles are shown in red. Some red signal from the muscles was also detected through the green filter.

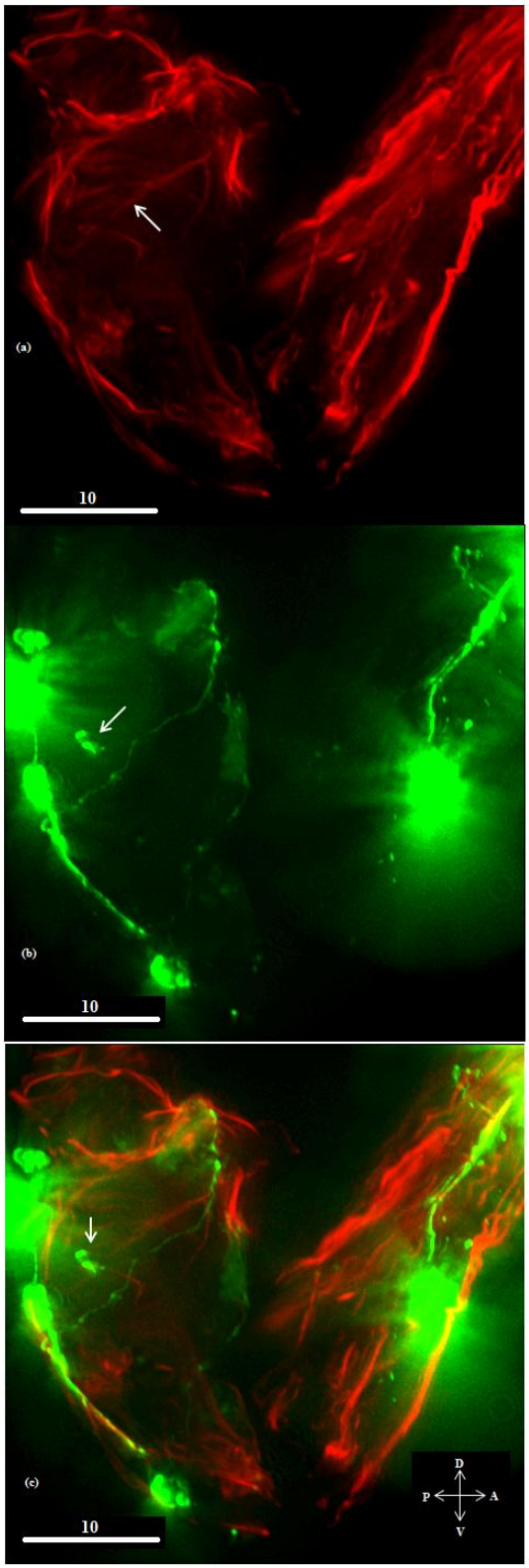

Figure 6 – Ectopic muscle innervated by DD motor neurons.

Body wall muscles are shown in red and DD motor neurons in green (*pflp13::gfp)*. Arrow points to an ectopic nmj.

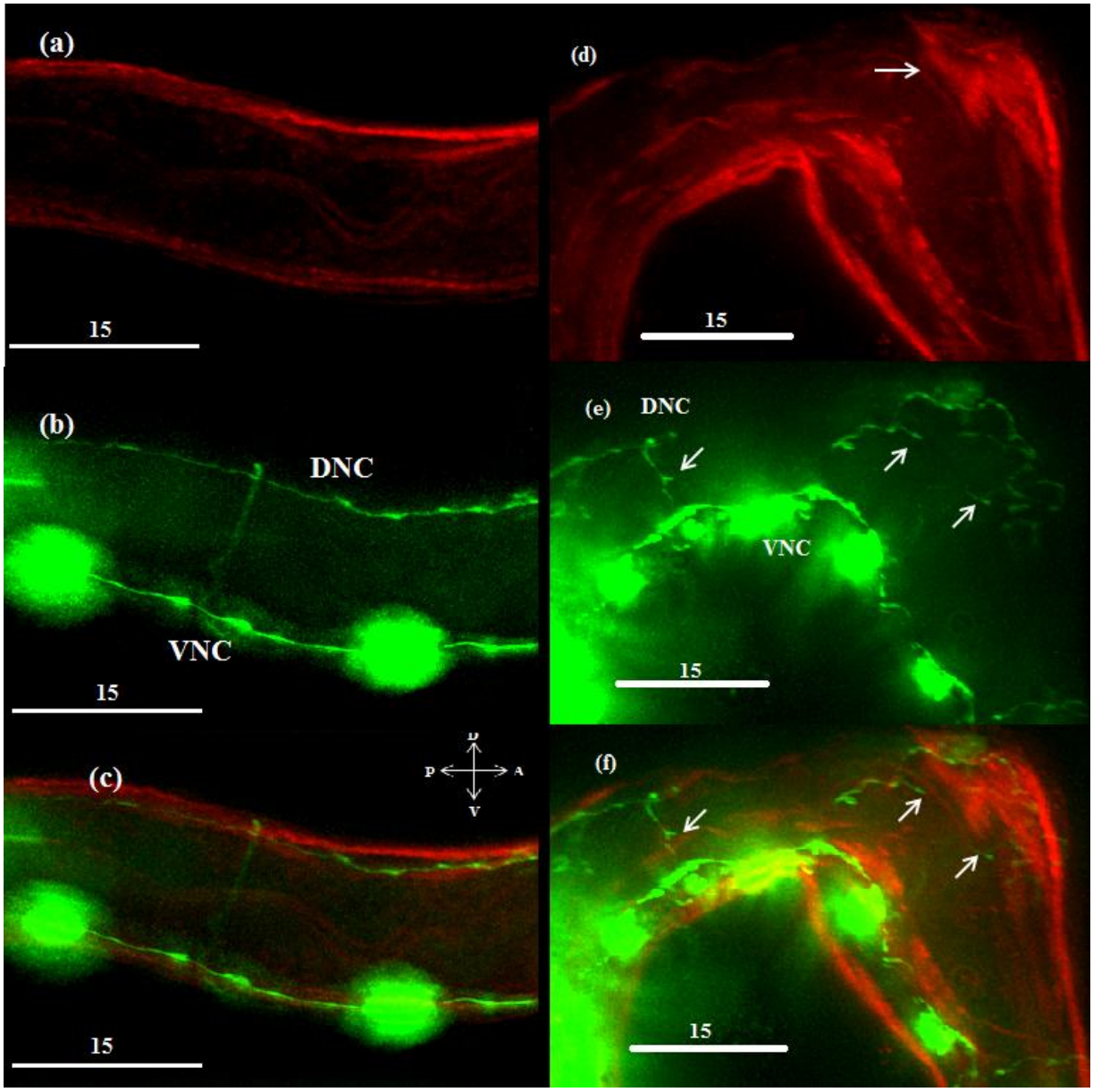

Figure 7 – Neuron and muscle morphology in a *pflp11::gfp* animal

Body wall muscles are shown in red while DD and VD motor neurons are shown in green. (a-c) Unablated specimen. (d-f) Ablated specimen. The white arrow in (d) shows an ectopic muscle strand. The arrows in (e) and (f) indicate associated neurite sprouting.

Table 2 - The number of animals with ectopic nmjs in the tested genetic backgrounds

Animal	Total number of specimens with ectopic muscle	Total number of specimens that displayed ectopic neurite sprouting associated with the ectopic muscle
Wild Type	10	8
***unc-129* mutant**	9	0

Next, we wanted to test if TGF-β was the signal from the ectopic muscles that was necessary in order to induce sprouting from the DD motor neurons. First, ablations were performed in animals expressing the *punc-129::gfp* transgene (ER80). This construct allowed the visualization of the cells that expressed *unc-129*, which are the DA and DB motor neurons and dorsal body wall muscles (Colavita and Culotti 1998). This was to see if the ectopic muscle strands were, indeed, expressing TGF-β. If TGF-β is indeed the suspected signal, then it should be expressed in the ectopic muscles at the time of DD respecification. In the unablated animals, expression of *unc-129* in the dorsal body wall muscles is transient such that at around the time of the DD motor neuron respecification, only a subset of the muscle cells expresses *unc-129* (Figure 8). We hypothesized that this subset consisted primarily of post-embryonic muscle cells that are seeking neuronal innervation.

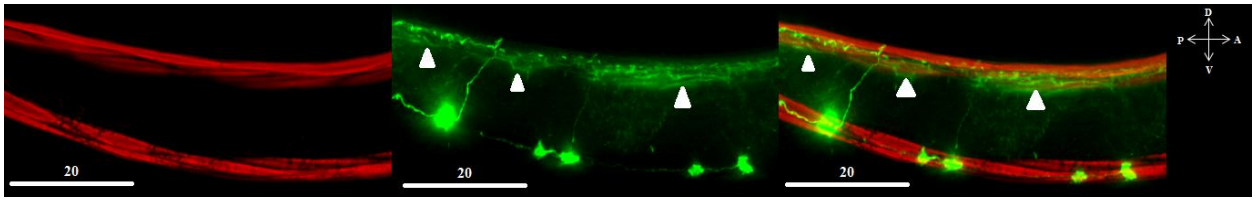

Figure 8 – The dorsal body wall muscles and the DA and DB motor neurons expressing TGF-β

Unablated ER80 animal. Body wall muscles are shown in red and cells expressing *unc-129* are shown in green. Muscle strands expressing *unc-129* are shown using white pointers.

In the ablated ER80 animals, the ectopic muscle fluoresced green (n=3), indicating that *unc-129* is expressed by the ectopic muscle cells (Figure 9). Thus ectopic muscles express TGF-β coincident with DD motor neuron respecification.

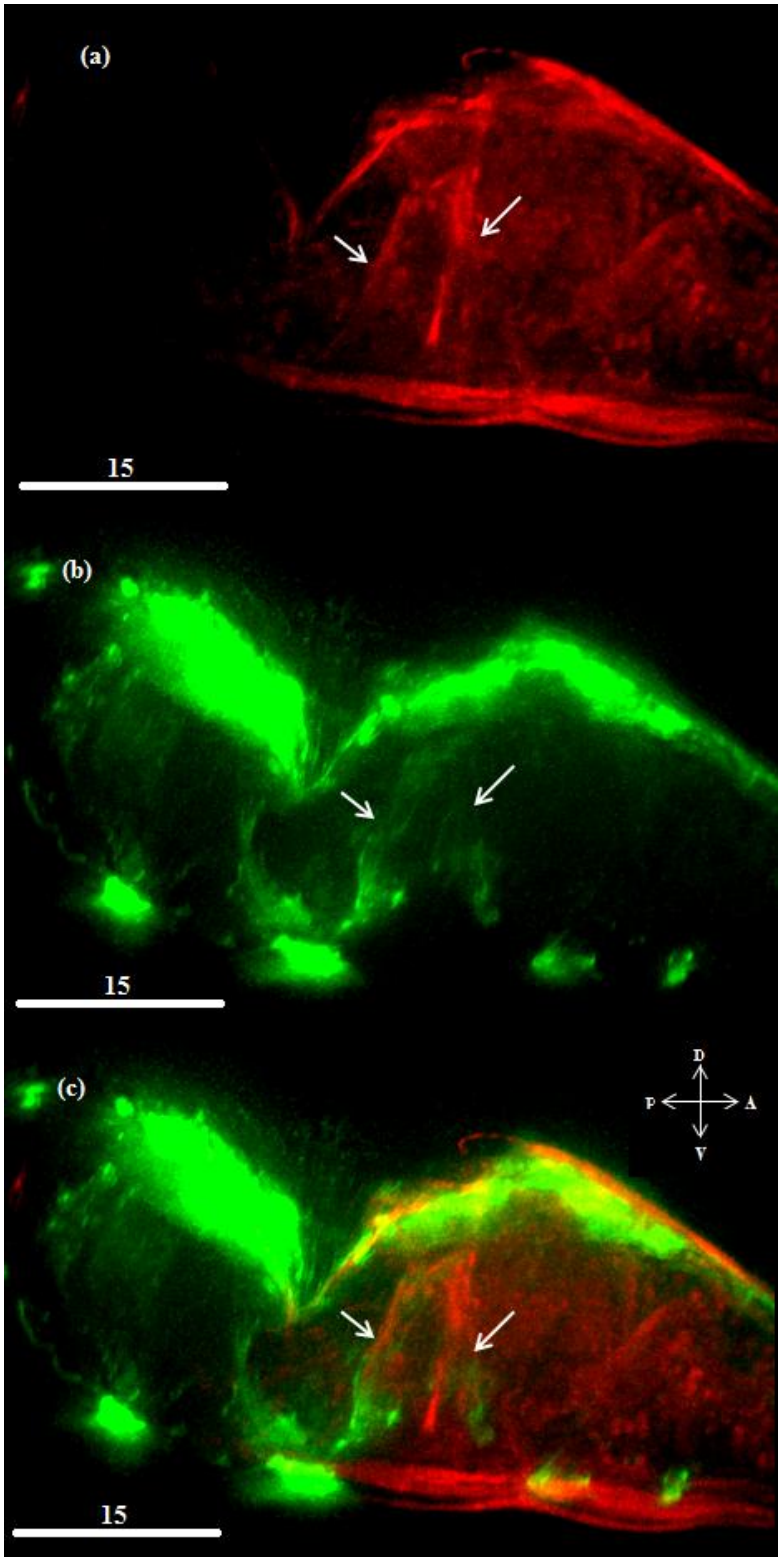

Figure 9 – The ectopic muscle is expressing TGF-β

Body wall muscles are shown in red and cells expressing *unc-129* (*punc129::gfp*) are shown in green. Two muscle strands expressing *unc-129* are shown using white arrows.

To determine whether TGF-β is necessary for the induction of ectopic neurites, ablations were then performed in *unc-129* mutant embryos (*ev554*) carrying the *pflp11::gfp* transgene (*pflp11::gfp; unc-129(ev554*)). If TGF-β is necessary, then mutant animals should not display ectopic neural processes associated with ectopic muscles in ablated animals. The unablated animals in this background often displayed axon guidance defects. The dorsal nerve cord was often absent or formed laterally below the two dorsal strands. Colavita et al (1998) reported that mutations in *unc-129* show axon guidance defects similar to *unc-6, unc-5* and *unc-40* mutations, which includes commissures that misguided and fail to reach the dorsal nerve cord. Therefore, aberrant commissures were seen in the ablated as well as unablated animals. Figure 10 shows the morphology of the neurons in an unablated specimen (top) and an ablated specimen (bottom). As suspected, the single commissure visible in the ablated specimen is not close to the ectopic muscle strand (shown by the white arrow), and is therefore not considered to have formed an ectopic nmj. Out of the 9 animals that displayed ectopic muscle strands, none displayed ectopic muscle-associated neurite sprouting (Table 2). Therefore, we concluded that TGF-β, was, in fact, necessary for ectopic muscle induced neurite branching.

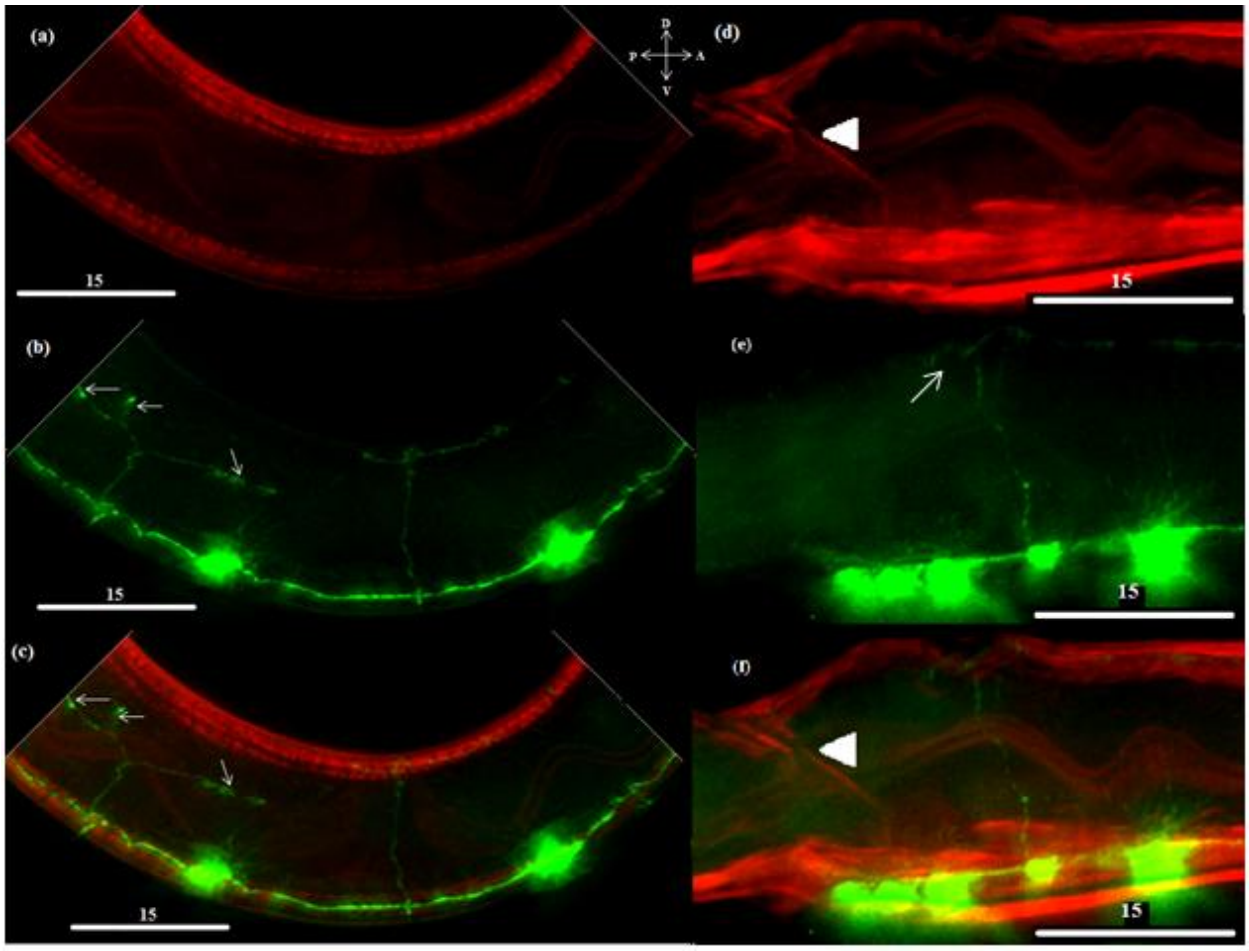

Figure 10 – Aberrant neurite growth in *unc-129* mutant animals.

(a-c) An unablated individual. The neural processes indicated by the white arrows have only reached halfway across the animal. (d-f) An ablated individual. White pointer in (d) and (f) shows ectopic muscle strand. The white arrow in (e) shows the only commissure visible. There are no neural processes seen to be innervating the ectopic muscle. Body wall muscles and gut muscles are depicted in red and DD, VD and DA motor neurons are shown in green (*pflp11::gfp*).

4. DISCUSSION

Synapses play a key role in regulating the information flow in nervous systems. It is, therefore, important for pre-synaptic elements to locate appropriate post-synaptic partners. The neuromuscular junction (nmj) is an interesting synapse. Given the ectodermal developmental origin of neurons and mesodermal origin of muscles, the involved cells face a challenge to locate each other and establish a functional connection. This is achieved through various signaling mechanisms (Fitzimonds and Poo 1998; Sanyal, Kim and Ramaswami 2004), many of which have already been characterized in vertebrates. Despite all that is known about nmjs, many more pathways remain to be discovered in this complex process. In this research, *C.elegans* neuromuscular junctions were used to study the genes important for neuron-muscle communication and subsequent nmj development. Specifically, we used a technique where selected embryonic myoblasts that act as guideposts for the postembryonic muscles, are laser-ablated. This, then, resulted in the postembryonic muscle setting up ectopic strands that induced neurite sprouting from DD motor neurons.

The developmental time at which the ectopic (postembryonic) muscle strands are formed coincides with the respecification of the DD motor neurons. Plunkett, Simmons and Walthall (1996) found that these ectopic muscles are able to induce sprouting from the DD motor neurons. They have shown that the presence of ectopic muscle leads to neurite sprouting from the dorsal nerve cord, and when the same ectopic muscle (postembryonic) precursors were laser-ablated along with the embryonic myoblasts, the sprouting from the DD motor neurons was not observed. This indicates that the ectopic muscle is sufficient for ectopic neurite sprouting. We have shown that *unc-129* (TGF-β) is expressed by these ectopic muscles. We have also shown that in the absence of TGF-β, the ectopic muscles are no longer innervated by DD motor neurons. Therefore, we conclude that *unc-129* (TGF-β), from the postembryonic ectopic muscles, is necessary for ectopic nmj formation.

The underlying mechanism for the formation of ectopic nmjs is tied closely to the series of events presumably taking place in the DD motor neurons during the synaptic respecification event. The DD motor neurons respecify their synapses in the four hours surrounding the first larval molt (White et al. 1978; Hallam and Jin 1998). At some point before this time, the DD respecification process is most likely initiated by some form of an intrinsic trigger(s). Very little is known about this intrinsic trigger, but *lin-14* is a heterochronic gene which has been implicated in controlling the time of DD respecification (Hallam and Jin 1998).

Following the initiation of the respecification event, the DD motor neurons begin eliminating synapses from the ventral side of the animal and forming synapses on the dorsal side. Park et al. (2011) have identified *cyy-1* (cyclin-box containing protein) and *cdk-5* (cyclin-dependant kinase -5) to be playing a part in the process. Specifically, *cyy-1* is important for synapse removal and *cdk-5* is shown to be important for new synapse formation. Park et al. (2011) have also shown that materials from previous ventral pre-synaptic termini are recycled to construct the new dorsal pre-synaptic termini. This indicates a potentially critical role for cytoskeletal factors and motors, such as kinesin and dynein, because this recycling of pre-synaptic material is an important characteristic of the DD respecification event. The recycling of presynaptic materials is reduced in *unc-104/kinesin3* mutants. Park et al. (2011) concluded that CDK-5 facilitates UNC-104-mediated intracellular transport of presynaptic material. CDK-5, along with a regulatory subunit p39, has also been implicated in actin modifications and microtubule formation in mice (Humbert, Dhavan and Tsai 2000). Coincident with these cytoskeletal changes, presumable changes at the new dorsal pre-synaptic termini allow the neuron to be receptive to new post-synaptic partners, and this probably leads to ectopic neurite branching if the appropriate post-synaptic muscle are present in ectopic locations, instead of the dorsal body wall muscle strands.

Various key genes and pathways have been identified in the process of synaptic partner recognition (Shen and Scheiffele 2010) and our data suggests that TGF-β may be one such gene. During

the respecification event, the dorsal process of the DD motor neurons probably undergoes changes in the receptor profile, possibly by enrichment of UNC-5 and UNC-40 receptor proteins (see below), at their pre-synaptic (dorsal) termini so as to become sensitive to TGF-β, which then reveals the location of the appropriate post-synaptic muscles. In the case where the appropriate post-synaptic target muscles (post-embryonic) are formed in ectopic locations, due to ablation of the embryonic muscle precursors, for example, sprouting of neurites that are seeking new targets, takes place.

A possible mechanism by which TGF-β induces such sprouting was first described by Colavita et al (1998). They showed that TGF-β is expressed transiently in dorsal body wall muscles and plays a role in pioneer axon guidance. They have shown that TGF-β acts via the netrin pathway (Colavita et al 1998; Macneil et al. 2009). The netrin receptors characterized therein are UNC-5 and UNC-40. *src-1* codes for a kinase that has also been implicated in mediating *unc-5* signaling (Lee, Li and Guan 2005). Together, these genes might be part of a gene network that normally promotes ventral-to-dorsal migration of axons earlier in development and is underlying the formation of ectopic nmjs, upon the ablation procedure.

This mechanism entails that neural processes that migrate from ventral to dorsal respond initially to *unc-6/netrin* gradients via *unc-5* only mediated signaling. During their migration, they switch to *unc-5+unc-40* mediated signaling, which increases the sensitivity of the growth cones to lower concentrations of *unc-6/netrin* (Macneil et al 2009). This is consistent with observed netrin signaling patterns in *Drosophila* (Keleman and Dickson 2001). UNC-129 is reported to mediate this switch in signaling. This is probably achieved by blocking UNC-5 signaling, as is indicated by binding assays where UNC-129 was shown to be able to physically bind UNC-5. A similar interaction was also observed between BMP7 (vertebrate TGF-β homolog) and RCM (vertebrate unc-5 homolog). Presumably, it is a consequence of this increased sensitivity to *unc-6/netrin* induced repulsion that UNC-129 can attract migrating neurites.

In the absence of target muscle (embryonic and postembryonic) that would normally flank the dorsal nerve cord, the DD motor neurons send out ectopic neural processes, presumably in search of target muscle at other locations. This could be an intrinsic mechanism that is normally suppressed by the intact muscle flanking the dorsal nerve cord. The suppression may be mediated by a retrograde signal from the muscle. Aberrant sprouting has been shown to occur AVB neurons if synaptic activity is blocked (Loria et al. 2003; Hobert, Tessmer and Ruvkun 1999; Zhao and Nonet 2000), indicating that this process is very dynamic and synaptic activity is necessary for maintenance of a previously established synapse. In the absence of post-synaptic muscle (embryonic), the sprouting from the DD motor neurons may be due to the result of the activation of a similar genetic program.

Following sprouting, neurites migrate towards the ectopic muscle. This is caused by further changes in cytoskeletal structure, cell surface membrane structure, and interactions with the extracellular matrix and any secreted factors. A comparable process is that of axon regeneration (Chen et al. 2011). Following a laser axotomy procedure, some but not all *C.elegans* neurons regrow their axons. Among those that do regenerate are the PLM sensory neurons and motor neurons. Chen et al. (2011) identified several mutants that displayed defects and enhancements in this regeneration ability. The list of mutations included genes for cytoskeletal factors and extracellular matrix (ECM) proteins. Although most mutations reduced regeneration abilities, mutations in the ECM protein coding genes often caused enhancements in regeneration. This suggested that interplay, between the ECM proteins and cytoskeletal factors, was important for axon guidance. Secreted guidance molecules such as TGF-β probably interact with ECM proteins such as agrin, and ephrins that have been shown to be important in regeneration as well as axon guidance in vertebrate model systems.

In addition, parts of the Wnt signaling pathway and the slit-robo pathway have been identified by Chen et al. (2011). This suggests that regeneration is somehow reactivating the axon guidance genetic program used by the respective neurons during development. However, they also identified

several genes to be involved in regeneration that are not known to be associated with axon guidance or neuronal development. For example, genes involved in endocytosis of synaptic vesicles and in the biosynthesis of GABA were involved. This led to the conclusion that regeneration is more complex than was initially thought to be. Nevertheless, it can be speculated that at least part of the regeneration process comprises reactivation of earlier developmental programs in the neuron. This is consistent with a previous description of the laser axotomy procedure, where the proximal end of the severed axon was seen to reform the growth cone and migrate to the appropriate post-synaptic partner (Yanik et al. 2004). This, therefore, raises the possibility that the non-canonical TGF-β pathway identified by Macneil et al. (2009) works in parallel with the regeneration pathways identified by Chen et al. (2011) and to a certain extent, the regeneration process might be reactivating an axon guidance program already in place, presumably suppressed by factors in a functional synapse.

Nevertheless, our results as well as those of Chen et al. (2011) indicate that DD motor neurons have more developmental potential during the respecification than previously characterized. The presence of ectopic muscles as well as surgery of the axon can induce neurite sprouting, followed by guidance to appropriate post-synaptic targets. In the case of ectopic nmj formation, TGF-β and the netrin pathway are presumably instrumental in promoting the sprouting of ectopic processes, possibly by the mechanism described by Macneil et al (2009).

We have demonstrated that TGF-β is necessary for ectopic nmj formation and that it is expressed by the appropriate post-synaptic cell. The findings of Colavita et al. (1998) were that motor axons are misguided in the absence of TGF-β. In this research, we were able to change the location of the TGF-β expressing cell and, in a way, demonstrated that it is sufficient to induce ectopic neurite sprouting. The downstream factors of this process still need to be elucidated. The receptors have been proposed by Macneil et al. (2009) as *unc-5* and *unc-40*. However, it remains to be seen whether there is actually binding activity. Further downstream factors represent the link between this extracellular signal,

TGF-β, and the intracellular factors such as cytoskeletal elements etc., that underlie the migration of the neurite. These are to be identified as well. TGF-β may also have an effect on synaptogenesis, which can be characterized if expression of TGF-β could be temporally manipulated. It is important in this case to make sure that axon guidance is completed and that only the effect on synaptogenesis is tested. Finally, all this information can be put together as a set of signaling cascades that operate to promote the unusual phenomenon of ectopic nmj formation. With this information, it will then be possible to formulate models of nmj formation in other organisms.

TGF-β pathways are highly conserved across species. There is evidence that TGF-β plays a role in axon guidance in *Drosophila melanogaster*. The development of the Drosophila neuromuscular junction involves the TGF-β homolog, *Gbb* (Glass-bottom boat) and the receptor *wit* (Wishful Thinking). The TGF-β homologs in vertebrates, such as the BMPs (Bone Morphogenic Proteins), have also been implicated in axon guidance (Sanyal et al. 2004). The findings of this research have considerable implications in our understanding of the development of nmjs in general. It would be interesting to see if the role played by TGF-β in nmj formation in more complex organisms is similar to that in *C.elegans*. However, the pathway described by Macneil et al (2009), is a non-canonical one. Characterization of this pathway will present us with an interesting example of how a ligand from one particular signaling pathway, can play a pleiotropic role in a second, considerably different pathway. The neuromuscular junction has been studied for a long time and there appears to be numerous signaling mechanisms at play to ensure proper formation the nmj. A better understanding of the nmj formation will generate insight into the complex signals that allow neural circuits to be formed in general. This, in turn, will shed light on how neurons assume their function as information carriers and establish a fully functional nervous system.

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APPENDIX: INCOMPLETE DATA SETS

Additional ablations were performed in *unc-5* and *unc-40* mutants carrying the *pflp11::gfp* transgene. Both of these mutants, without any ablations performed, displayed severe axon guidance defects. Similar to *unc-129* mutants, these mutants often lacked a dorsal nerve cord. Unablated *unc-40* mutants displayed a higher frequency of animals with neuronal processes that failed to complete the ventral-to-dorsal migration, when compared to *unc-129* animals (Figure A1). This mutant was more similar to the *unc-129* mutant because the misguided neurites were able to reach the midline, before getting disrupted, whereas *unc-5* mutants displayed misguided axons that got disrupted at the beginning of the migration to the ventral side. Neuronal processes were often seen to stay restricted to the ventral side in the *unc-5* mutant animals (Figure A2).

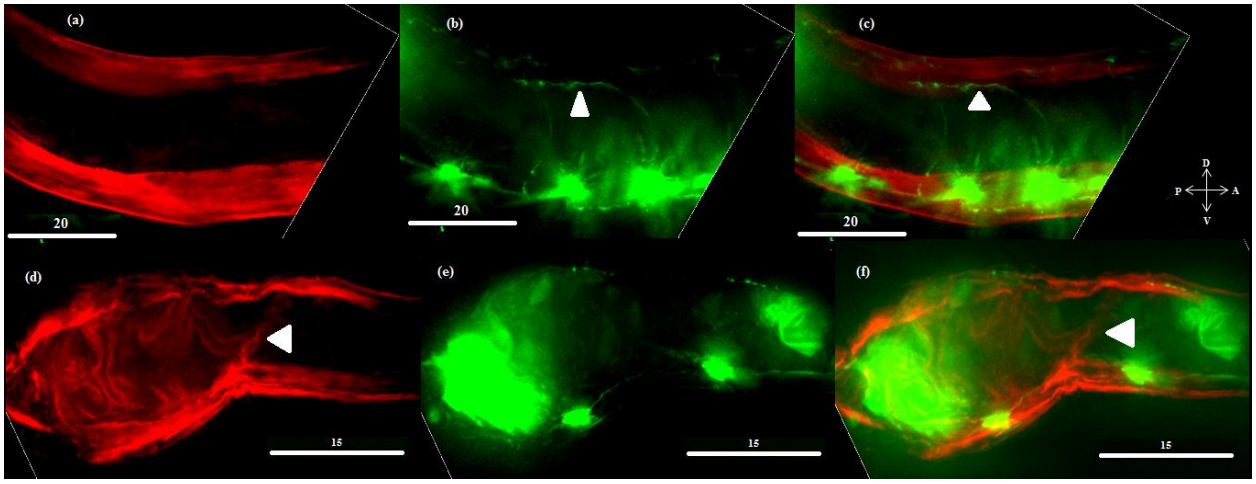

Figure A1 – Neuron morphology in unc-40 mutant animal.

(a-c) Unablated unc-40 animal. The white pointer shows an axon that got disrupted slightly before it reached the dorsal nerve cord. (d-f) Ablated unc-40 animal. The white pointer shows ectopic muscle. There are no neural processes seen to be innervating the ectopic muscle. Body wall muscles and gut muscles are depicted in red and DD, VD and DA motor neurons are shown in green (*pflp11::gfp*).

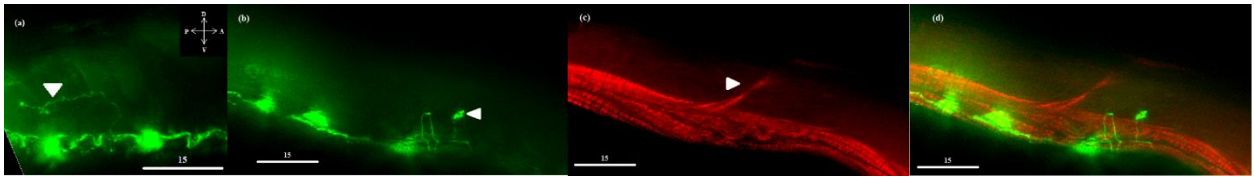

Figure A2 – Neuron morphology in unc-5 mutant animals.

(a) Unablated *unc-5* mutant animal. The arrow shows a misguided commissure. The dorsal nerve cord is absent and all but one neural processes are restricted to the ventral side of the animal. (b-d) Ablated *unc-5* mutant animal. The pointer marks the position of a probable ectopic muscle. There are no neural processes seen to be innervating it. The dorsal nerve cord is absent in this animal as well. Body wall muscles and gut muscles are depicted in red and DD, VD and DA motor neurons are shown in green (*pflp11::gfp*).

In addition, the *unc-5* and *unc-40* mutant animals displayed an increased fragility to the ablation procedure. Most of the eggs recovered from the ablations performed did not hatch. Those that did hatch were prone to early deaths. For this reason, only two ablated specimens were successfully ablated and analyzed in the *unc-40* mutant background (Figure A1) and one ablated specimen was analyzed in the *unc-5* background (Figure A2).

Although ectopic muscles were observed in these animals, there were no neural processes associated with them. Some aberrant commissures were seen in the *unc-40* mutant animals, but they occurred in regions where there was no ectopic muscle present. In addition, dorsal nerve cords were often absent. At this point, the results of the ablations in the *unc-5* and *unc-40* mutants remain inconclusive. Further experiments are to be done if necessity is to be established. However, our data indicates that *unc-129* is necessary for the formation of ectopic neuromuscular junctions.