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SHORT COMMUNICATION

# Role of myosin Va in neuritogenesis of chick dorsal root ganglia nociceptive neurons

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## Abstract

Myosin-Va, widely distributed throughout the developing nervous system, is involved in the transport of vesicles and other intracellular components with its globular tail domain (GTD) implicated in cargo recognition/interaction. Inactivation of myosin-Va in dorsal root ganglia (DRG) neurons of chick embryos, in vitro, decreases the rate of filopodial extension. *MYO5A* mutant mice have severe neurological defects. We have found that the overexpression of GTD in DRG cultures reduces the number of neurons with long neurites (above fourfold cell body length) and increased the number of neurons with short or no neurites. However, if transfection occurred after the onset of neuritogenesis, this was not seen. *In embryo*, we characterized the expression pattern of myosin-Va during neuritogenesis of TrkA-positive cells at different stages of chick DRG development. Myosin-Va expression was detected starting from HH25. At this stage, it was present in cells both with and without neurites. The presence of myosin-Va in DRG neurites persisted throughout the last stage analysed (HH34). The data suggest that Myosin Va can participate in embryonic DRG neuritogenesis.

**Keywords:** chicken embryo; dorsal root ganglia; myosin Va; neuritogenesis, nociceptors; TrkA

## Introduction

Myosin-Va is an evolutionarily conserved motor molecule expressed in a variety of tissues and particularly enriched in the developing nervous system (Azevedo et al., 2004). In the embryonic context, myosin-Va is expressed in the neural tube during chicken neurulation, the telencephalon, midbrain, cranial nerve fibres and superior cervical ganglia (Azevedo et al., 2004; Takagishi et al., 2005). In the chicken embryo DRG cells in vitro, myosin-Va was detected in growth cones in the interface between the actin-rich and microtubule domain (El-Husseini and Vicent, 1999; Suter et al., 1999). Inactivation of myosin-Va function through chromophore-assisted laser inactivation decreased growth cone filopodia extension and motility rate (Wang et al., 1996). The data support a role for myosin-Va in neural development.

Myosin-Va moves on actin filaments and transports neurofilaments, organelles and other components relevant to the establishment of synapses (van Diepen et al., 2009; Rao et al., 2011; Wagner et al., 2011). Myosin-Va translocates on actin filaments through its motor domain, present in the amino-terminal region. This region is followed by 6 IQ motifs that bind to calmodulin and helical domains responsible for dimerisation. GTD is present in the carboxy-terminal region of the protein and is responsible for cargo interaction (Espreafico et al., 1992; Reck-Peterson et al., 1999; Trybus, 2008).

GTD also provides sensitivity to intracellular calcium levels by regulating the equilibrium between active-inactive conformations of myosin-Va (Krementsov et al., 2004; Wang et al., 2004; Li et al., 2006; Thirumurugan et al., 2006). The current model for regulation of the activity of myosin-Va proposes that the globular domain has a dual function: in the absence of proper levels of calcium, myosin-Va assumes an inactive conformation that prevents its binding to actin and also inhibits the ATPase activity of the protein. In the presence of optimum levels of calcium, the globular domain is exposed and is able to interact with intracellular vesicular transport machinery (Wang et al., 2004; Li et al., 2006). The importance of myosin-Va GTD in the regulation of embryonic neuritogenesis was seen in *Drosophila melanogaster* neurons in vitro (Eppinga et al., 2008).

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**Abbreviations:** DRG, dorsal root ganglia; GTD, globular tail domain

Overexpression of a truncated form of the protein (without GTD) significantly reduced neurite length and the F-actin presence in neurons.

We have investigated the contribution of myosin-Va in embryonic neuritogenesis through its inactivation by an ectopic expression of the GTD. The work shows that GTD can regulate myosin-Va activity and decrease the establishment of neurites in TrkA-positive nociceptive neurons. Expression of myosin-Va in the developing DRG is shown to precede neuritogenesis.

## Materials and methods

### Immunofluorescence in chicken embryo cryosections

Fertilized chicken eggs from White Leghorn (Sabor Natural farm, São Paulo, São Paulo, Brazil) were incubated at 37°C and 50% of humidity. Embryos were collected at stages HH25, HH30 and HH34 (Hamburger and Hamilton, 1992). They were fixed in PBS/paraformaldehyde 4% and cryoprotected with 20% sucrose *overnight* at 4°C and embedded in a OCT-20% sucrose mixture (1:1) prior to sectioning in cryostat at 10 μm. Immunofluorescence against myosin-Va (1:250 – produced by Espreafico and Dr. Larson), Tuj1 (1:500 – Covance, USA), TrkA (1:200 – Epitomics) and GFP (1:1,000 – Abcam), were performed on the sections. Secondary antibodies used were Alexa 488-conjugated goat anti-rabbit (1:500, Molecular Probes), Alexa 568-conjugated goat anti-mouse (1:500, Molecular Probes) and Alexa 488-conjugated goat anti-mouse (1:500, Molecular Probes). The sections were analysed either in a confocal or regular immunofluorescence microscope.

### DRG culture

The DRG neurons were obtained from Hamburger Hamilton stage HH30–HH34 chick embryos (embryonic day 7) and were cultured as described by Bronner-Fraser et al. (1996). Briefly, the embryos were dissected and the DRG were exposed by removing the spinal column. After the isolation, the ganglions were dissociated with trypsin 0.1% and the individual cells were plated in 24-well plates containing 12 mm round glass coverslips pre-coated with poly-L-lysine (BD BioCoat 354085). The coverslips were incubated for at least 12 h at 4°C with laminin 20 mg/mL (Sigma-Aldrich). The culture medium was MEM (Minimum Essential Media, Invitrogen Life Technologies) supplemented with the antibiotics penicillin (50 U/mL - Sigma) and streptomycin (50 mg/mL – Sigma), 2 mM glutamine (Sigma-Aldrich), 10% inactive horse serum (Invitrogen), 10 ng/mL NFG (Invitrogen) and 10 μM fluorodeoxyuridine-uridine solution (Sigma) to inhibit proliferation of non-neuronal cells.

### MyoVaGTD construct and DRG cell transfection

MyoVaGTD was obtained from a previous construct of chicken myosin-Va globular tail (Espreafico et al., 1992), and cMyc epitope (MEQKLISEEDL) was fused to the N-terminal GTD segment, amino acids 1425–1830 (ISP to SRV), as in NP_990631.1, and MycMyoVaGTD was subsequently inserted in pMES plasmid into *Eco*RI–*Sma*I sites. Three hours after plating, cells were transfected with the truncated protein (Myc-MyoVaGTD) or only with the pMES plasmid without insert. The pMES plasmid is a bicistronic construct that contains the eGFP gene after its IRES site. The expression of both inserts is driven by the chicken beta-actin promoter (Chen et al., 2004). Transfection was performed with Lipofectamine 2000 (Invitrogen) at a final concentration of 2 μg + 0.8 μg Myc-MyoVaGTD for 4 h in OPTI-MEM medium without antibiotics. After 4 h of transfection, the cells were washed with serum-free medium and fed with complete MEM medium containing NGF 10 ng/mL. The cells recovered for 16–18 h and then were fixed with PBS/paraformaldehyde 4% and processed for immunofluorescence.

### Immunofluorescence in DRG cultures

In cultures, immunofluorescence was left until the last step in the original culture plates to prevent excessive handling, and consequent loss of neurites. The coverslips were washed once with PBS for 10 min and then were incubated with blocking solution (PBST 0.1% with 10% NGS and 0.5% BSA) for 30 min in a moist chamber at room temperature. The blocking solution was gently removed and coverslips were incubated with the primary antibodies to the medial tail of myosin-Va (1:250 – Espindola et al., 2008); Tuj1 (1:500 – Covance, USA), TrkA (1:200 – Epitomics), GFP (1:1,000 – Abcam) and Myc (1:200 – Invitrogen) overnight. They were washed once with PBS and exposed to the secondary antibody Alexa 488-conjugated goat anti-rabbit (1:500, Molecular Probes), Alexa 568-conjugated goat anti-mouse (1:500, Molecular Probes) and Alexa 488-conjugated goat anti-mouse (1:500, Molecular Probes). DAPI was added to the secondary antibody solution (1:1,000) for nuclear staining.

### Statistics

The transfected cells were divided into three categories according to their neurite length relative to soma size (short neurites: length smaller or equal to soma, medium neurites: 1–4 times soma size and long neurites: longer than four times the soma size).

The percentage of transfected cells (number of transfected cells (GFP-positive) in each category/total number of

transfected cells) was transformed into proportion (% of transfected cell/100). The proportion data were converted to arcsine square root [x = arc sin ($\sqrt{}$%/100)] and ANOVA was performed with two factors: plasmid transfected and NGF concentration. The level of significance was $P < 0.05$.

## Results and discussion

### GTD inhibits myosin-Va-mediated neuritogenesis in cultured nociceptors

The myosin-Va GTD inhibits the function of its motor domain in in vitro assays (Li et al., 2006). To verify if GTD can interfere with the biological functions of myosin-Va in vertebrate neuritogenesis, we investigated the effects of GTD overexpression in cultured chick nociceptive neurons.

Cultured nociceptive neurons expressed myosin-Va in the cell body and in the neurites (Figures 1A–C). In the latter, myosin-Va is present along the whole developing neurite independent of its length and stage of neuritogenesis. In neurons, which are in advanced neuritogenesis, myosin-Va is detected in the growth cone as well. The growth cone comprises of two domains, a central domain rich in microtubules and a peripheric and dynamic domain with actin filaments that participate in the formation of filopodia (Forscher and Smith, 1988; Kobova and Svitkina, 2008). In particular, the expression domain of myosin-Va in the growth cone is broader than the domain of beta III tubulin (Figures 1D–F – arrows). Myosin-Va is also expressed in glial cells present in the culture (data not shown).

To determine whether myosin-Va GTD inhibits nociceptive neuritogenesis, we transfected the cultures with Myc-MyoVaGTD, or with the plasmid pMES which encodes eGFP alone. To quantify the phenotype, the transfected neurons (identified by their expression of GFP) were divided into three categories according to their neurite length; the categories are referred to as: 0–1 – short neurites; >1–4 – medium neurites and >4 – long neurites (Figures 2B–D). The expression of Myc-MyoVaGTD was confirmed by immunostaining for the Myc epitope.

Overexpression of myosin-Va GTD significantly reduced the proportion of neurons with long neurites and increased the proportion of neurons with short neurites (Figure 2A). However, we did not detect major changes in the profile of neuritogenesis when older cultures with longer neurites were transfected (data not shown). Therefore, our results suggest firstly, that the GTD can inhibit myosin-Va biological function in vitro; and second, that myosin-Va is mainly involved in the neurite outgrowth but not maintenance.

We suggest two hypotheses might explain these results. First, the exogenous GTD could be competing with myosin-Va for binding sites within the cellular components that will

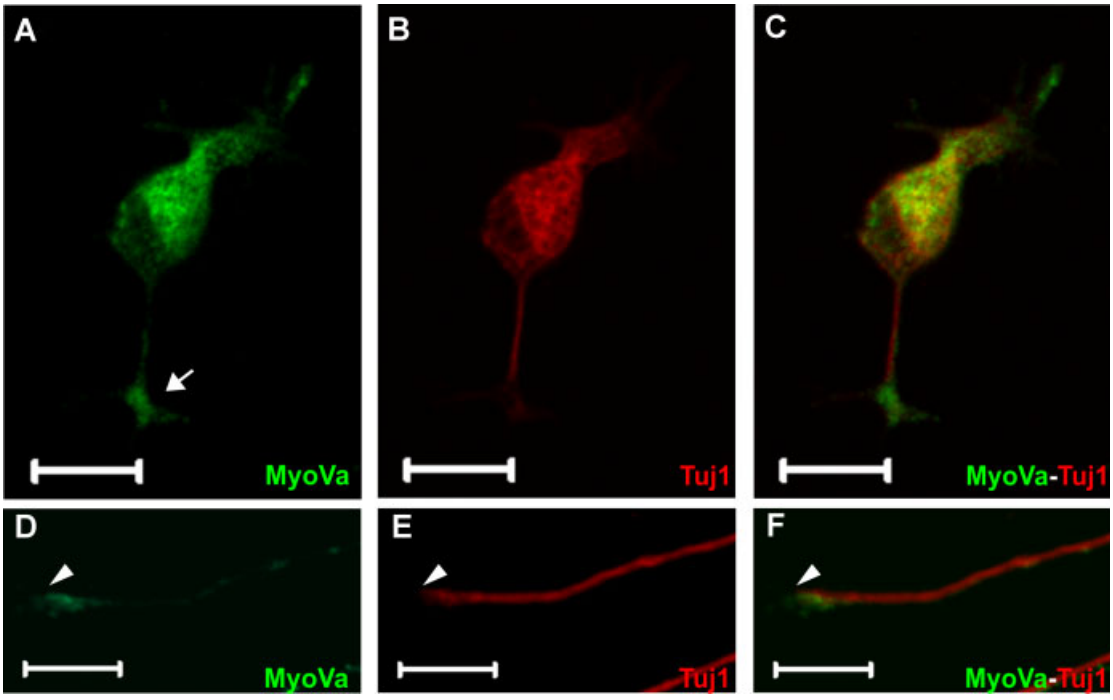


**Figure 1 Myosin-Va is expressed in cultured nociceptive neurons.** Myosin-Va is expressed in the cell body, along the developing neurite (A–C) and in the growth cone (A – arrow, D – arrowhead). In the growth cone, the expression domain of myosin-Va is broader than the domain of beta III tubulin (TUJ1, compare D and E). Cells were plated with 200 ng/mL NGF for about 24 h. Scale: 20 μm.

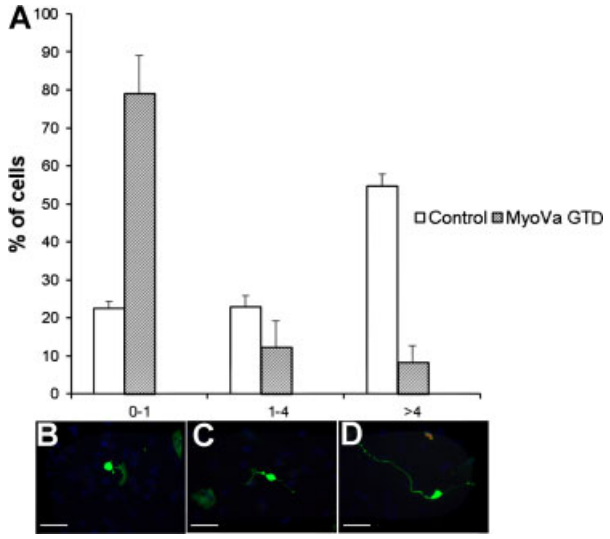


**Figure 2** Overexpression of myosin-Va GTD interferes with neuritogenesis. (A) The transfection of Myc-MyoVaGTD significantly increases the proportion of cells with short/no neurites (Category 0–1) and reduces the proportion of cells with long neurites (Category >4), when compared to control pMES. No statistical difference was observed in the proportion of medium neurites (Category >1–4). To identify the effect of myosin-Va GTD in the neuritogenesis, the transfected cells were divided into three categories according to the neurite length. Category 0–1–short neurites (B), >1–4–medium neurites (C), and >4 – long neurites (D). Test: ANOVA, $^{*}P < 0.05$. Scale: 20 μm.

be transported. In other words, the ectopic myosin-Va GTD prevents the interaction of the endogenous myosin-Va GTD with different cargos whose transport are relevant for neurite formation, such as cytoskeletal proteins, endoplasmic reticulum and the lipid phosphatase PTEN (van Diepen et al., 2009; Rao et al., 2011, Wagner et al., 2011). Our second hypothesis is that GTD overexpression may inactivate endogenous protein activity because GTD binds to the motor domain simulating the inactive conformation of the protein and inhibiting full-length myosin-Va ATPase activity (Li et al., 2006). But note, these possibilities are not mutually exclusive.

### Myosin Va and neuritogenesis in embryo

We investigated whether myosin-Va is actually associated with neurite emission in the developing embryo by correlating the expression pattern of myosin-Va with different phases of neuritogenesis in the DRG. Myosin-Va is found in the cytoplasm of most cells in the DRG at HH25. To correlate the presence of myosin-Va with neurite outgrowth, its expression was characterised together with that of Beta III tubulin. Beta III tubulin is consistently associated with growing neurites and its presence is required for proper neuritogenesis (Joshi and Cleveland, 1989; Guo et al., 2011). At embryonic stage HH25, all the cells undergoing neuritogenesis (beta III tubulin/TUJ1-positive) express myosin-Va (Figures 3A and D). However, some DRG cells are myosin-Va-positive, but did not start to emit neurites yet (TUJ1-negative) (Figure 3G – arrowhead). This suggests that myosin-Va is expressed prior to the onset of neuritogenesis.

From stage HH30 onwards, all cells undergoing neuritogenesis in DRG express both myosin-Va and beta III tubulin (Figures 3B and E). At stage HH34, the intensity of staining for myosin-Va is more pronounced in the ventrolateral area of the DRG (Figure 3C). This expression pattern may occur due to the difference in the diameter of DRGs cell after HH30. During development, the different neuronal subtypes in the DRG neurons differ in cell body size. Nociceptive neurons (TrkA-positive) have smaller cell bodies, whereas mechanoceptive (TrkB-positive) and proprioceptive (TrkC-positive) neurons have larger cell bodies. Initially, the TrkA-positive neurons populate the entire DRG. After stage HH30, these smaller neurons segregate to the dorsomedial region (Rifkin et al., 2000) and represents >70% of the total DRG neurons (Patapoutian, 2001). Therefore, the stronger ventrolateral staining seen in HH34 could be a visual bias generated by the larger cell bodies of the TrkB/C-positive neurons.

Alternatively, it is possible that neuritogenesis in dorsomedial TrkA-positive cells is delayed relative to the ventrolateral population. Indeed, the projection of small-diameter nociceptive afferents to the dorsal horn is delayed relative to other sensory projections (Eide and Glover, 1997). In this scenario, TrkA-positive cells would be expected to emit beta III tubulin-positive neurites at later embryonic stages.

### Nociceptive neuritogenesis

To distinguish between these two possibilities, we explored the temporal dynamics of neurite appearance in nociceptive (TrkA-positive) neurons in vivo. TrkA-positive cells in the DRG arise during the second neurogenic wave that starts around stage HH25 (Rifkin et al., 2000). In the embryo, the addition of new TrkA-positive neurons in the DRG lasts 48 h from the stage HH25.

Initially, TrkA-positive cells were uniformly distributed in the DRG and few cells had beta III tubulin-positive neurites (Figure 4A, HH25 and Figure 4B). At stage HH25 some neurons have already begun the process of neuritogenesis (Figure 4B). From stage HH30 onwards, most TrkA-positive neurons present beta III tubulin in structures similar to neurites. At stage HH34, the vast majority of TrkA-positive cells are concentrated in the DRG dorsomedial region and most cells are beta III tubulin positive (Figures 3D–F and 4A; Guan et al., 2003).

Sensory neurons have distinct timing for neurite entry in central. The DRG ventrolateral neurons, composed of

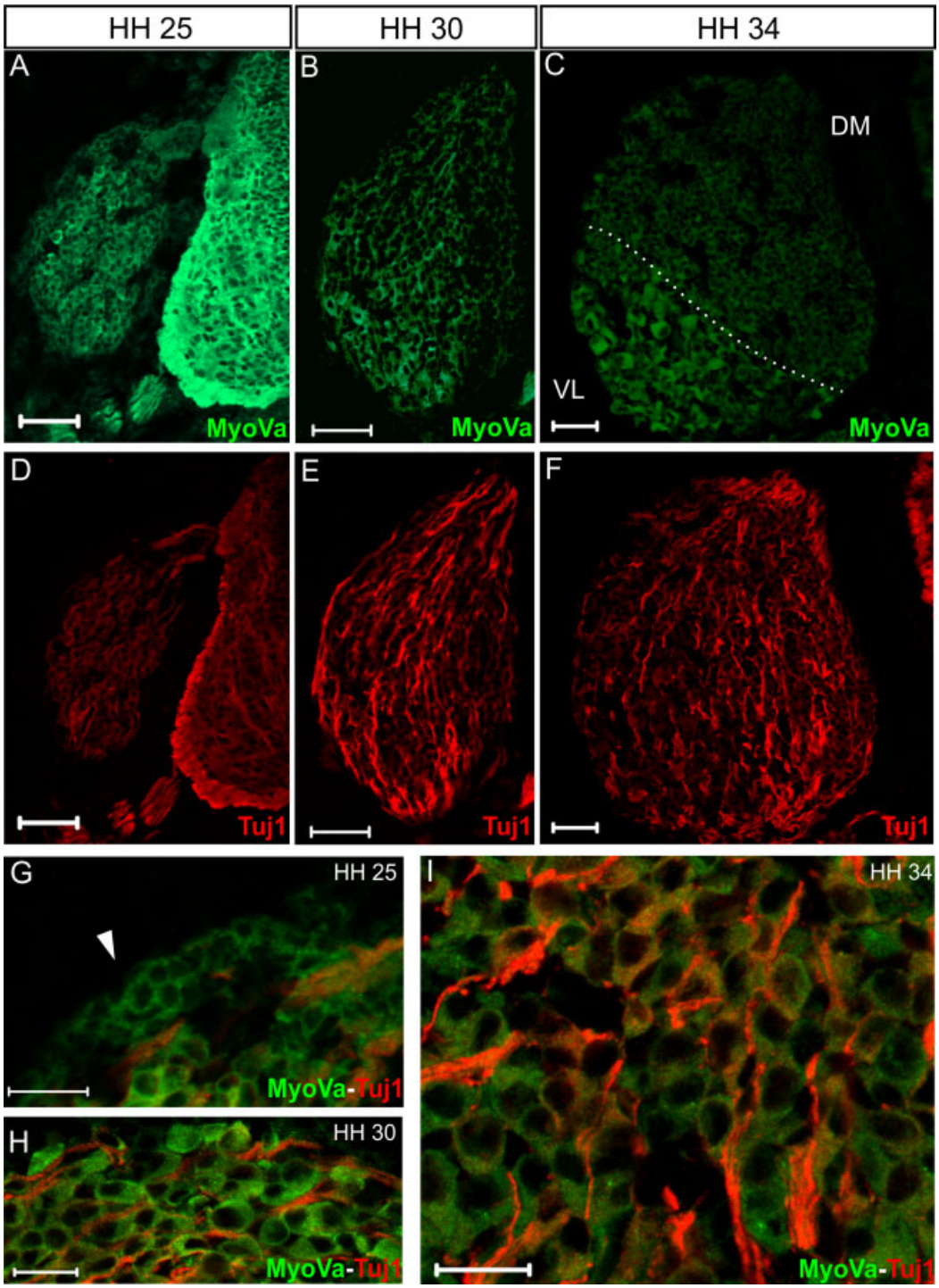


**Figure 3** Myosin-Va expression pattern in DRG cells at stages HH25, HH30 and HH34. Myosin-Va expression precedes that of beta III tubulin (G – arrowhead). Thereafter it is expressed in most of cells which are undergoing neuritogenesis (Tuj1-positives) at stage HH25 (A and D). At stage HH30, all cells in the DRG express both myosin-Va and beta III tubulin (B, E and H). At stage HH34, the intensity of staining for myosin-Va becomes more pronounced in the ventrolateral (VL) area of the DRG (C). The boundary between these two domains is marked by a dotted line. Beta III tubulin labelling remains homogeneous throughout the DRG (F). (I) High magnification of cells undergoing neuritogenesis at stage HH34. DM, dorsomedial; VL, ventrolateral. Scale: (A–F) 50 μm, (G–I) 20 μm.

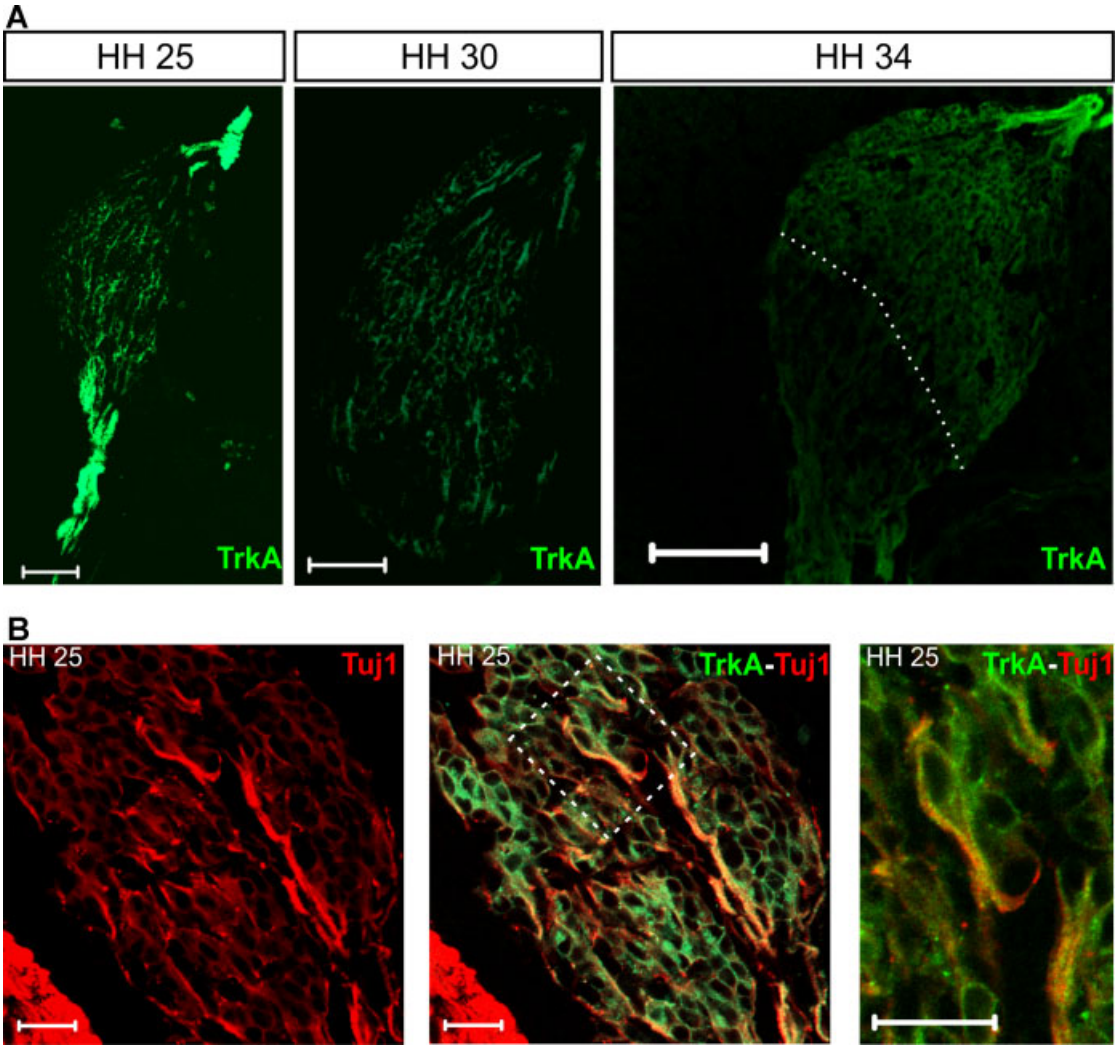


**Figure 4** The onset of TrkA-positive neurons neuritogenesis is at stage HH25. Initially, (stages HH25 and HH30), TrkA-positive neurons are uniformly distributed along the DRG (A). At stage HH25, some cells have already started neuritogenesis (B). At stage HH34, the TrkA-positive cells segregate and populate the DM region of DRG (A). The boundary between the DM and VL domains is marked by a dotted line. VL, ventrolateral; MD, dorsomedial. Scale: (A) 50, 50 and 100 μm; (B) – 20 μm.

mechanoreceptors and proprioceptors, emit axons that enter the spinal cord by dorsal horn medial region towards the deeper layers of the grey matter. The nociceptive neurons present in the dorsalmedial region of DRG send out axons that enter the spinal cord by the dorsal horn lateral region and synapse at the more superficial lamina I and II (Eide and Glover, 1997; Brown and Fyffe, 1981). The difference between dorsalmedial neurons and ventrolateral neurons in the embryonic stage for establishment of central projection is ∼5 days (Eider and Glover, 1997). However, we found that at HH34 TrkA-positive cells already emit neurites. In fact, neuritogenesis in TrkA-positive neurons had already occurred at HH25. Thus, the higher intensity of staining for myosin-Va in the VL domains at HH34 are probably due to soma size differences between the sensory neurons and not to the absence of neuritogenesis from TrkA-positive neurons at this stage.

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