Actin Filament-Stabilizing Protein Tropomyosin Regulates the Size of Dendritic Fields

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Dendritic arbors of different neuronal subtypes cover distinct spatial territories, known as dendritic fields, to receive specific inputs in a nervous system. How the size of dendritic fields is determined by cell-intrinsic factors during development remains primarily unknown. To address this issue, we used the Drosophila embryonic peripheral nervous system. In each hemisegment, six dorsal cluster dendritic arborization (DA) neurons elaborate stereotypic dendritic branching patterns underneath the epidermis. Here we report the identification of loss-of-function mutations in the tropomyosin II gene (TmII) that result in expanded dendritic fields of DA neurons. Mosaic analysis with a repressible cell marker demonstrated that TmII functions in a cell-autonomous manner to control the formation of dendritic fields. Furthermore, we show that TmII genetically interacted with flamingo, a gene encoding a membrane receptor-like molecule that affects dendritic growth. TmII encodes multiple isoforms of a protein that stabilizes actin filaments. Our findings suggest that tropomyosin helps control the size of dendritic fields by regulating actin cytoskeletal dynamics.

Key words: dendritic field; Drosophila; size; tropomyosin; actin-binding; isoforms

Introduction

In a nervous system, different neuronal subtypes exhibit distinct dendritic morphologies (Ramón y Cajal, 1911; Masland, 2001). The size of the dendritic fields of a specific subtype of neurons can be regulated by cell–cell interactions and by both extrinsic and intrinsic factors (for review, see Whitford et al., 2002; Gao and Bogert, 2003). For instance, the dendritic field sizes of α and β ganglion cells exhibit an inverse relationship with the density of these cells in the retina (Boycott and Wäsßle, 1974). In rats (Perry and Linden, 1982) and other mammals, competitive interactions between dendrites of neighboring ganglion cells play a role in defining their dendritic field sizes. Such interactions occur primarily between the same subtype of ganglion cells (Weber et al., 1998) and are likely mediated by direct dendro-dendritic contacts (Lohmann and Wong, 2001). Similar interactions are also found between dendrites of some dendritic arborization (DA) neurons in the peripheral nervous system (PNS) of Drosophila larvae (Gao et al., 2000; Grueber et al., 2002).

One of the genes that controls dendritic field formation is flamingo (Gao et al., 1999, 2000), which encodes a G-protein-coupled receptor-like molecule with a large extracellular domain containing cadherin repeats, epidermal growth factor (EGF) motifs, and laminin A globular domains (Chae et al., 1999; Usui et al., 1999). In flamingo mutant Drosophila embryos, the dorsal dendrites of DA neurons overextend toward the dorsal midline (Gao et al., 1999, 2000). Flamingo is expressed in both dendrites and axons of DA neurons and has a cell-autonomous function in postmitotic DA neurons to control dendritic growth as shown by mosaic analysis with a repressible cell marker (MARC) (Sweeney et al., 2002). This technique allows the dendritic morphology of a single mutant neuron to be visualized in a mosaic animal (Lee et al., 1999). It remains primarily unknown what other molecules control dendritic field formation and how the signals mediated by Flamingo lead to the changes in the cytoskeleton during dendritic growth in vivo.

To further understand how the dendritic field sizes are specified during development, we performed a genetic screen to identify lethal mutations on the third chromosome. Our goal was to identify genes that regulate dendritic growth in a way similar to flamingo. Using this approach, we found that mutations in the gene tropomyosin II (TmII) increased the dendritic field sizes of DA neurons in Drosophila embryos. The gene encodes multiple isoforms of actin filament-stabilizing protein tropomyosin; some of them are expressed in neurons (Hanke and Storti, 1988; Tetzlaff et al., 1996; Cooper, 2002). Additional analysis using the MARCM technique demonstrated that TmII functions in a cell-autonomous manner to control the dendritic field sizes of DA neurons. Because of the similarities in dendritic phenotypes between flamingo and TmII mutants, we examined the genetic interaction between the two genes and found that the dendritic overextension phenotype caused by TmII mutations could be further enhanced by reducing the dosage of flamingo. The actin cytoskeleton reorganization plays an important role in neuronal morphogenesis (for review, see Luo, 2002; Gao and Bogert, 2003). Our studies demonstrate a direct role for the actin filament-stabilizing protein tropomyosin in controlling dendritic field sizes.
Materials and Methods

Fly stocks and reagents. All the genetic crosses were performed at 25°C with standard food medium. Mutant fly lines with P-element insertions on the third chromosome were obtained from the Szeged Drosophila Stock Center (Szeged, Hungary). Monoclonal antibody against the N terminus of Flamingo was kindly provided by Dr. T. Uemura (Kyoto University, Kyoto, Japan). The following stocks were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN) or other laboratories: (1) GALA 109(2)80, UAS-GFP (Gao et al., 1999); (2) TM3, Sb, Kriüppel-Gal4, UAS-GFP (kindly provided by T. Kornberg, University of California, San Francisco, San Francisco, CA); (3) GALA 109(2)80, UAS-GFP, flamingo53/Gyg, Kriüppel-Gal4, UAS-GFP (Gao et al., 1999); (4) TmIIe/TM3, Sb, Ser (Erdélyi et al., 1995); (5) cTmII/TM3, Sb, Ser (Tetzlaff et al., 1996); and (6) GALA4155, UAS-mCD8::GFP, hs-FLP1, and FRT82B, tubP-GAL80/Cyo (Bloomington Stock Center).

Genetic screen for mutations affecting the sizes of dendritic fields. Mutant fly lines with P-element insertions on the right arm of the third chromosome were crossed with GALA 109(2)80, UAS-GFP; +/TM3, Sb, Kriüppel-Gal4, UAS-GFP. In the F1 generation, GALA 109(2)80, UAS-GFP/+; Pl[anZ, w1]/TM3, Sb, Kriüppel-Gal4, UAS-GFP flies were selected and self-crossed. Staged embryos from this cross were collected on grape agar plates and processed as described previously (Gao et al., 1999). Briefly, embryos were collected at 25°C overnight, dechorionated with 2.5% sodium hypochlorite, and mounted in 90% glycerol in PBS. The dendritic morphology of dorsal cluster DA neurons was assessed by confocal microscopy (Radiance 2000; Bio-Rad, Hercules, CA).

Plasmid rescue. Genomic DNA sequences flanking the sites of P-element insertions were isolated by plasmid rescue. Genomic DNA was digested with EcoRI or HindIII, self-ligated, and recovered by transforming Escherichia coli competent cells. For each plasmid rescue experiment, three independently generated clones were sequenced to determine the genomic DNA sequences adjacent the P-element insertions.

Semiquantitative reverse transcription-PCR. Messenger RNA from embryos of the desired genotypes was used to generate cDNA in reverse transcription (RT) reactions, which served as the template for PCR. PCR was performed with oligonucleotide primers corresponding to exons specific for different TmII isoforms. The PCR products were collected after 22 cycles and analyzed by Southern blotting with digoxigenin-labeled DNA probes.

Quantitative analysis of ddaE neuron dendritic fields. Images of green fluorescent protein (GFP)-labeled dorsal cluster DA neurons in wild-type or TmII mutant embryos were obtained with a Bio-Rad confocal microscope (Radiance 2000). The dendrites of ddaE neurons were traced, scanned, and converted into Photoshop (Adobe Systems, San Jose, CA) images. For statistical analyses, one ddaE neuron per larva was analyzed, and ANOVA and Student’s t-test were used. Dendritic branch length was calculated with Photoshop software (6.0).

MARCM. Single-cell analysis of the Drosophila larval PNS was performed as described by Sweeney et al. (2002). Briefly, FRT82B, TmII130510, TM3, Ser male flies were crossed with GALA155, UAS-mCD8::GFP, hs-FLP1/FM7, P[anZ, w1]/TM3, Sb, Kriüppel-Gal4 virgin flies. Then, GALA155, UAS-mCD8::GFP, hs-FLP1/FM7, P[anZ, w1]/FRT82B, TmII130510 male flies were crossed with GALA4155, UAS-mCD8::GFP, hs-FLP1/FM7, P[anZ, w1]/FRT82B, tubP-GAL80/Cyo virgin flies. Embryos from this cross were collected at 25°C for 3 hr. At 3–6 hr after egg laying (AEL), embryos were heat shocked in a 37°C water bath for 40 min to induce mitotic recombination. Vials were then kept at 25°C for 3–4 d. Third instar larvae were collected and examined for the presence of a single mCD8::GFP-labeled dorsal cluster PNS neuron, and images of dendritic morphology were obtained as described above.

Results

Identification of mutations that affect the size of dendritic fields

In the late stages of Drosophila embryogenesis, the dorsal dendrites of DA neurons cease to extend, whereas the lateral dendrites grow toward the adjacent segment boundaries. Therefore, the dendritic fields of DA neurons in contralateral dorsal clusters do not overlap and stop short of the dorsal midline (Gao et al., 1999, 2000) (Fig. 1A). To understand how the size of dendritic fields is specified during development, we performed a genetic screen to identify lethal mutations caused by P-element insertions on the third chromosome that altered the size of DA neuron dendritic fields. In line S130510, the dorsal dendrites failed to stop at the correct length, resulting in enlarged dendritic fields of dorsal cluster DA neurons (Fig. 1A). The dendritic over-
Effects of TmII isoforms on dendritic field formation

To assess the function of different TmII isoforms in dendritic field formation, we obtained mutant lines that were allelic to TmII<sup>el4</sup>/TmII<sup>el4</sup> (Fig. 1B). In the TmII<sup>el4</sup> line (Tetzlaff et al., 1996), a P-element inserted in the first exon of cTmII abolished cTmII expression but did not affect the expression of muscle isoforms (Fig. 1C). In the TmII<sup>el4</sup> mutant line (Erdélyi et al., 1995), a small deletion at the TmII locus abolished the expression of all known TmII isoforms (Fig. 1C).

We measured the size of the dendritic fields of dorsal cluster DA neurons in the A1 segment of embryos at 20 hr AEL. Dendritic field size increased by 37% in the absence of cTmII isoform and by 51% in TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos. Similar phenotypes were observed in A2 and A3 abdominal segments (data not shown).

The dorsal cluster contains six DA neurons that develop distinct dendritic fields. To further characterize the dendritic phenotype caused by TmII mutations, we traced and quantitatively analyzed the dendrites of ddaE neurons in 20 hr AEL embryos, which have a relatively simple dendritic branching pattern and do not develop spine-like fine structures (Fig. 2A). In the A1 segment, the average length of ddaE dorsal dendrites was 32.2 ± 0.8 μm (n = 34) in wild-type embryos and 47.3 ± 1.4 μm (n = 22; p < 0.0001) in TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos, in which the expression of all known TmII isoforms was greatly reduced (Fig. 2B). The cTmII-specific mutation also caused significant increase in dorsal dendrite length, 40.3 ± 0.9 μm (n = 34; p < 0.0001) in TmII<sup>el4</sup> homozygous mutant embryos and 41.8 ± 0.8 μm (n = 28; p < 0.0001) in TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos, suggesting that cTmII contributes to the dendritic overextension phenotype. ddaE neurons in other abdominal segments (such as A2 and A3) in mutant embryos with different combinations of TmII alleles exhibited a similar dendritic phenotype (data not shown). The phenotypes in TmII<sup>el4</sup>/TmII<sup>el4</sup> were less severe than those in TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos (p < 0.005), suggesting that other TmII isoforms also regulate the size of dendritic fields.

Next, we studied the effects of TmII mutations on the lateral dendrites of ddaE neurons. The total length of all lateral branches that extended from the dorsal dendrite was significantly greater in TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos than in wild-type embryos (91.6 ± 3.9 vs. 59.6 ± 3.3 μm; n = 24; p < 0.0001) (Fig. 2C). TmII<sup>el4</sup>/TmII<sup>el4</sup> mutant embryos also had significantly more lateral branches than wild-type embryos (13.0 ± 0.5 versus 9.3 ± 0.3; n = 24; p < 0.0001) (Fig. 2D). TmII mutations did not affect the average length of lateral branches (data not shown), presumably attributable to the “stop signals” at the segment boundaries. These findings suggest that TmII mutations result in enlarged...
dendritic fields by increasing both the length of dorsal dendrites and the number of lateral branches of ddaE neurons.

**TmII functions cell autonomously to regulate dendritic fields**

To determine whether TmII regulates dendritic fields in a cell-autonomous manner, we used the MARCM technique to generate single TmII<sup>el4</sup> mutant DA neurons in third instar larvae (Lee et al., 1999; Sweeney et al., 2002). Because most GFP-labeled single neurons were ddaC neurons, quantitative analysis on ddaC is presented here. In most cases, only one ddaC neuron was labeled by GFP in individual third instar larvae. The dendritic field of ddaC covers the hemisegment between the adjacent segment boundaries and from the dorsal midline to the lateral cluster (Sweeney et al., 2002). Because the width of ddaC neuron dendritic fields is restricted by the width of each segment, we calculated the dorsolateral dimension of ddaC neurons relative to the anteroposterior dimension of the segment. We found that the average dendritic field of TmII mutant ddaC neurons was 19% larger than those of wild-type ddaC neurons (p < 0.0001) (Fig. 3).

**Dendritic overextension phenotype in TmII mutants was enhanced by reduced dosage of flamingo**

Our previous studies indicate that Flamingo, a putative G-protein-coupled receptor that contains a large N-terminal domain with cadherin repeats and EGF-like domains, controls dendritic field formation in a cell-autonomous manner (Gao et al., 2000; Sweeney et al., 2002). Flamingo also affects the size of dendritic territories of mushroom body neurons (Reuter et al., 2003). To investigate potential genetic interactions between flamingo and TmII, we examined the TmII<sup>s130510</sup>/TmII<sup>el4</sup> mutant embryos that were also heterozygous for a flamingo mutation. Both TmII<sup>s130510</sup> and TmII<sup>el4</sup> mutations greatly reduce the expression of all known TmII isoforms (Fig. 1). For this experiment, we identified a Flamingo protein-null allele, flamingo<sup>51</sup>. In flamingo<sup>51</sup> homozygous mutant embryos, no Flamingo was detectable by immunostaining analysis with a monoclonal antibody specific for the N terminus of Flamingo (data not shown). In addition, a point mutation was identified in the coding region of flamingo that resulted in a stop codon at amino acid 1393, shortly after the ninth cadherin repeat. In TmII<sup>s130510</sup>/TmII<sup>el4</sup> mutants, reducing the dosage of flamingo enhanced the dendritic overextension phenotype (Fig. 4), suggesting a genetic interaction between the two genes in regulating dendritic field formation. We did not see an effect in embryos that were heterozygous for both TmII and flamingo (data not shown), which is probably attributable to the function of TmII being less sensitive to dosage.

**Figure 3.** TmII has a cell-autonomous function in controlling the size of ddaC neuron dendritic fields as demonstrated by the MARCM technique. A, The distance between the most dorsal and the most lateral dendritic boundaries was measured as the dorsolateral dimension of ddaC dendritic fields. The ratio of the dorsolateral dimension to the width of the segment reflects the relative size of ddaC dendritic fields. The dendritic fields of ddaC mutant neurons (n = 22) are significantly larger than those of wild-type (WT) neurons (n = 15) (Student’s t test; p < 0.0001). B, A wild-type ddaC neuron. C, A TmII<sup>s130510</sup> mutant ddaC neuron. An epithelial cell is labeled with GFP in C.

**Figure 4.** Genetic interaction between TmII and flamingo. All embryos were at the 20 hr AEL stage, and DA neurons were labeled by GFP under the control of GAL4 109(2)80. The length of dorsal dendrites of ddaE neurons in wild-type (WT) (n = 34), flamingo<sup>51/+</sup> (n = 22), TmII<sup>s130510</sup>/TmII<sup>el4</sup> (n = 22), and flamingo<sup>51</sup>/+; TmII<sup>s130510</sup>/TmII<sup>el4</sup> (n = 32) embryos was measured as described above. There is no significant difference between wild-type and flamingo heterozygous embryos (Student’s t test; p > 0.2). The dendritic overextension phenotype is significantly enhanced in TmII<sup>s130510</sup>/TmII<sup>el4</sup> mutants by reducing the dosage of flamingo (Student’s t test; p < 0.005).

**Discussion**

Dendritic morphogenesis can be controlled by a number of factors, such as extrinsic signaling molecules or cell-specific transcription factors (for review, see Whitford et al., 2002; Gao and Bogert, 2003). Here we provide in vivo evidence that tropomyosin, a protein that directly binds to and stabilizes actin filaments, functions cell autonomously in postmitotic neurons to regulate the size of dendritic fields.

**Role of tropomyosin in dendrite development**

Tropomyosin is a rod-shaped, coiled-coil protein that binds along the side of the actin filament (Araya et al., 2002). In non-muscle cells, tropomyosin is not associated with newly polymerized actin filaments at the leading edge of motile cells (DesMarais et al., 2002). Instead, it stabilizes actin filaments by inhibiting Arp2/3 complex-mediated nucleation in the deeper cortex (Blanchard et al., 2001). Tropomyosin prevents the dissociation of
actin subunits from actin filaments and increases the physical strength of actin filaments (for review, see Cooper, 2002). In addition, tropomyosin regulates the association between actin filaments and other actin-binding proteins, such as Myo 1b, an unconventional myosin that localizes to the leading edge of motile cells (Tang and Ostap, 2001).

Little is known about the dynamics of actin filaments in dendritic growth and branching in developing organisms. Our findings raise the possibility that the absence of tropomyosin reduces the stability of actin filaments, resulting in increased dendritic growth. Alternatively, increased association of motor proteins with actin filaments in the absence of tropomyosin may contribute to dendritic extension. The latter possibility is consistent with the finding that a kinesin-related motor protein, CHO1/MKLP1, is required for dendrite formation in cultured neurons (Yu et al., 1997).

Both TmII and flamingo mutations caused overextension of dorsal dendrites of DA neurons (Gao et al., 2000; this study). The genetic interaction between the two genes (Fig. 4) raises the possibility that the Flamingo pathway may somehow affect the actin filaments. How the signal mediated by membrane receptor-like molecule Flamingo influences the reorganization of the actin cytoskeleton still remains primarily unknown. The identification of factors that link Flamingo and actin-binding proteins, such as tropomyosin, will be of great interest.

**Different effects of tropomyosin isoforms**

In *Drosophila*, multiple TmII isoforms arise as a result of alternative promoters and alternative splicing events (Hanke and Storti, 1988; Erdélyi et al., 1995). cTmII is expressed in neurons, and at least three isoforms are specifically expressed in muscles. Although cTmII affects dendritic field size, TmII<sup>1306510</sup>/TmII<sup>el4</sup> mutants, in which all isoforms are affected by the P-element inser-tion, exhibit a more severe dendritic phenotype than TmII<sup>el4</sup> mutants. Thus, other TmII isoforms may also contribute to the regulation of dendritic growth. It is unlikely that muscle-specific TmII isoforms have non-cell-autonomous effects on neurons. Myosin heavy chain mutations also result in the paralysis of embryos; however, the dendrites in these mutant embryos appear to develop normally (our unpublished observation). We speculate that one or more TmII isoforms that have not been characterized (Hanke and Storti, 1988; Erdélyi et al., 1995) have functions similar to those of cTmII.

Mammalian tropomyosins also have multiple isoforms, some of which are specifically expressed in neurons (Weinberger et al., 1993). Dimerization between different isoforms further increases the complexity of tropomyosin function (Araya et al., 2002). It is likely that the combinatorial action of different tropomyosin isoforms contributes to the generation of diverse dendritic morphologies in the nervous system.

**References**


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