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The Beat generation: a multigene family encoding IgSF proteins related to the Beat axon guidance molecule in *Drosophila*

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SUMMARY

A previous genetic screen led to the identification of the *beaten path* (*beat Ia*) gene in *Drosophila*. Beat Ia contains two immunoglobulin (Ig) domains and appears to function as an anti-adhesive factor secreted by specific growth cones to promote axon defasciculation. We identify a family of 14 *beat*-like genes in *Drosophila*. In contrast to *beat Ia*, four novel Beat-family genes encode membrane-bound proteins. Moreover, mutations in each gene lead to much more subtle guidance phenotypes than observed in *beat Ia*. Genetic interactions between *beat Ic* and *beat Ia* reveal complementary functions. Our data suggest a model whereby Beat Ic (and perhaps other membrane-bound family members) functions in a pro-adhesive fashion to regulate fasciculation, while Beat Ia (the original secreted Beat) functions in an anti-adhesive fashion to regulate defasciculation.

Key words: Axon, Guidance, Beat, Beaten Path, Attraction, Repulsion, *Drosophila*, Cell adhesion molecule

INTRODUCTION

The regulation of axon outgrowth during neuronal development is a complex process that involves many different types of guidance molecules. Multiple guidance systems impinge simultaneously on an individual growth cone. The growth cone integrates these diverse signals at specific choice points, measures the relative balance of attractive and repulsive signals, and makes the appropriate guidance decision.

Guidance signals can be divided into four main categories: chemoattractants, which attract growth cones from a distance; chemorepellents, which repel growth cones from a distance (thus steering them in a different direction); contact repellents, which are expressed on surfaces that discourage axon extension; and contact attractants, which are expressed on surfaces that encourage axon extension. Many contact attractants are cell adhesion molecules (CAMs) of several different gene families. CAMs help to promote growth, keep axons fasciculated and, in some cases, to control steering and turning (Tessier-Lavigne and Goodman, 1996).

The diversity of molecules required for axon guidance has been provided in part by gene duplication. Vertebrates and fruit flies both have multiple netrin genes that appear to have been independently duplicated in their respective evolutionary lineages (Serafini et al., 1994; Mitchell et al., 1996). While the fruit fly genome contains five genes encoding Semaphorins (repulsive guidance ligands) and two genes encoding Plexins (their repulsive receptors), the human genome appears to contain 22 semaphorin genes and 9 plexin genes (Adams et al., 2000; Venter et al., 2001).

We present evidence on the expansion of the family of *beat*-like genes in *Drosophila*. A genetic screen for mutations that perturb the pattern of motor axon projections in the fruit fly *Drosophila melanogaster* led to the discovery of the *beaten path* (*beat*) gene (VanVactor et al., 1993). The Beat protein appears to function as an anti-adhesive factor secreted by motoneuron growth cones at defasciculation choice points. In *beat* loss-of-function mutants, motor axons extending along the major motor nerves in the periphery are unable to overcome axon-axon adhesion to other motor axons, and fail to defasciculate at their appropriate branch points, instead continuing to extend along the motor nerves and growing past their target muscles. Reducing the levels of the homophilic axonal cell adhesion molecule (CAM) Fasciclin II (Fas II) on motor axons partially suppresses the *beat* phenotype and allows specific motor axons to exit at the appropriate choice point and contact their target muscles (Fambrough and Goodman, 1996). Beat protein is a secreted member of the immunoglobulin superfamily (IgSF) (Bazan and Goodman, 1997).

The fruit fly genome sequence reveals that the *beaten path* gene (now called *beat Ia*) is actually just the first of a 14-member family of *beat*-like genes in *Drosophila*. Of the four novel Beat family genes for which we have full-length cDNAs, all are membrane-bound, possessing either GPI-links or transmembrane domains. The new Beat-like proteins thus resemble other IgSF CAMs expressed on subsets of axons.

We examine the expression and mutant phenotype of *beat Ic*, the closest relative of the original *beat* (*beat Ia*). We examine a subset of the other *beat*-family genes in a more limited fashion. Mutations in each gene lead to much more

subtle guidance phenotypes than observed in *beat Ia*. Genetic interactions between *beat Ic* and *beat Ia* reveal complementary functions, with Beat Ic appearing to function in a pro-adhesive fashion and Beat Ia appearing to function in an anti-adhesive fashion. These results suggest a model whereby most members of the Beat family (the membrane-bound proteins) function as cell adhesion molecules to regulate axon fasciculation, while Beat Ia functions as a negative regulator of the other Beats to regulate axon defasciculation.

MATERIALS AND METHODS

Bioinformatics

tBLASTn searches of the *Drosophila* genomic sequence were performed at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). The query sequence that provided the most meaningful results was Beat Ia predicted protein sequence from residues 27-248 (including the cystine knot domain in the query found no strong matches). Strong matches were compared with predicted protein sequences for the novel Beat-like proteins created using *Drosophila* genomic sequence at Gadfly (http://flybase.bio.indiana.edu/annot). These sequences were aligned using Lasergene software (DNASTAR), and refined to minimize gapping. Where possible, predicted protein sequences from cDNAs or partial cDNAs were used. Sequences were then aligned again using the Macintosh PPC version of CLUSTALW (Higgins et al., 1992) and output according to alignment.

Genetic stocks

Chromosomal deficiency stocks that delete *beat Ic* were identified by PCR on genomic DNA prepared from adult flies transheterozygous for various combinations of deficiencies (flies lacking *beat Ic* are viable). Either Df(2L)RM5 or Df(2L)RA5 was used in all analyses, as their breakpoints had previously been mapped to the *beat I* locus (Fambrough and Goodman, 1996; Ashburner et al., 1999). Generation of *beat Ia* alleles was described previously (Fambrough and Goodman, 1996).

Cloning and molecular analyses

beat Ic, *beat Ib*, *beat IIa* and *beat VI* cDNAs were isolated from a Berkeley Drosophila Genome Project lambda ZAPII cDNA library prepared from adult heads. For the rest of the *beat* genes, primer pairs were designed from genomic sequence that would amplify a short (300~500 bp) fragment of each gene and used for PCR on aliquots of the BDGP head library. cDNAs and PCR fragments were sequenced on an ABI Prism 377 using Dye-terminator chemistry. Sequences were analyzed using the Macintosh version of Lasergene software (DNASTAR.)

beat Ic gain-of-function constructs were prepared using PCR with primers engineered to incorporate an *Eco*RI site into the 5′ end and a *Kpn*I site into the 3′ end of the ORF. This PCR product was subcloned into the corresponding sites of pUAST, sequenced across the entire insert and injected with pACΔ23 helper plasmid at a ratio of 5:1 into W1118 embryos. Insertions from resulting transformant lines were then amplified by PCR, gel-purified and sequenced.

Immunohistochemistry and microscopy

Antibody labeling of embryonic structures was performed as described previously (Patel, 1994). In situ hybridization was performed as described by Kopczynski and Muskavitch (Kopczynski and Muskavitch, 1992) with minor modifications (C. Kopczynski, unpublished). Briefly, antisense RNA probes were synthesized containing digoxigenin (Boehringer), hybridized to wild-type embryos overnight at 52°C, washed extensively in 50% formamide/Tween20/2×SSC, stained with alkaline phosphatase-conjugated anti-digoxigenin (Boehringer), washed and developed for alkaline phosphatase activity. Stained embryos were cleared in 70% glycerol/PBS.

Accession numbers

The GenBank Accession Numbers for cDNAs or cDNA fragments for the genes described in this paper are: *beat Ib*, AF32511; *beat Ic*, AF32512; *beat IIa*, AF32513; *beat IIb*, AY052378; *beat IIIa*, AY052379; *beat IIIb*, AY052380; *beat IIIc*, AY052381; *beat IV*, AY052382; *beat Va*, AY052383; *beat Vb*, AY052384; *beat Vc*, AY052385; *beat VI*, AY052386; and *beat VII*, AY052387.

RESULTS

The Beats are a large multi-gene family in *Drosophila*

The degree of primary amino acid sequence identity among the Beats ranges from 24% to 72% across the Ig domains. In general, homology is highest at the N-termini and drops significantly towards the C-termini. A phylogenetic tree constructed from CLUSTALW alignments (Fig. 1) of the homologous regions of the Beats reliably divides the family into seven distinct classes (Fig. 2A). The original *beat* is renamed *beat Ia* for clarity.

Eleven of the Beats reside in four chromosomal clusters of two to three genes each. Without exception, Beats that are more closely related to each other at the level of primary amino acid sequence are also located in the same cluster. Therefore, it is likely that Beats located in a local cluster are the products of local gene duplications.

All of the Beats are expressed in the adult head: fragments of each of them can be amplified by PCR out of a head-specific cDNA library (data not shown). Therefore, none of the Beats is a pseudogene.

We have isolated full-length cDNAs for *beat Ib*, *beat Ic*, *beat IIa* and *beat VI*. The predicted protein structures of these new Beats are significantly different from that of Beat Ia, which is predicted to be a dimerized, secreted molecule: these new Beats are predicted to be membrane-anchored (Fig. 2B).

Many of the Beats are expressed in different subsets of CNS neurons

In situ hybridization of embryos shows that most of the Beats are transcribed in subsets of neurons in the embryonic CNS (Fig. 3). *beat IIIa*, *beat IIIb*, *beat IV* and *beat VII* do not show detectable embryonic expression. Interestingly, the class II Beats, *beat IIa* and *beat IIb*, are also expressed in a subset of the ventral oblique muscles, making them the only Beats with detectable embryonic expression in a non-neural tissue. Of course, these muscles are targets for specific motor axons. The class V Beats all appear to be transcribed in large, ventral neurons in the CNS, possibly the VUM neurons.

Many of the Beats that are expressed in the embryo show highly restricted and specific expression patterns; none of them is expressed as broadly as *beat Ia*, which is expressed in all motoneurons during the stages of axon outgrowth (Fambrough and Goodman, 1996).

Single-gene mutations in several other Beats have only subtle phenotypes or no phenotype

In further contrast to *beat Ia*, loss-of-function mutations in

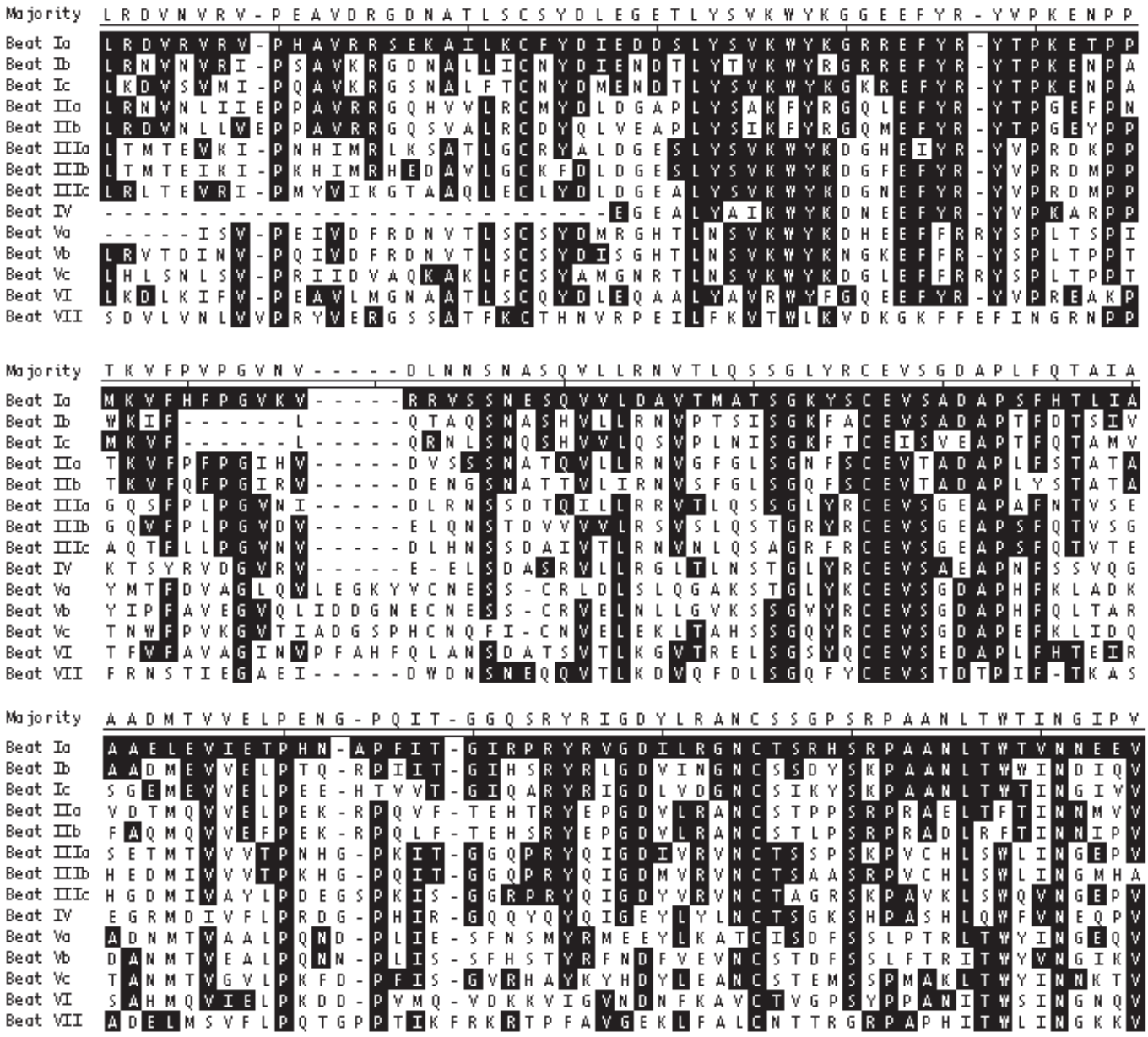

Fig. 1. Alignment of predicted protein sequences of the Beats. CLUSTALW alignment of the best predicted protein sequences for all of the Beats through the Ig domains, generated using a combination of gene predictions from genomic sequence, homology to Beat Ia and cDNA or partial cDNA sequences. Highlighting denotes residues that are identical to Beat Ia. Sequences not shown could not be reliably predicted and are omitted.

several of the other Beats have only partially expressive defects in axon guidance (Table 1). We used the extensive genetic reagents available to study genes in the *Adh* region of chromosome arm 2L (Ashburner et al., 1999), where the class I Beats reside, to generate animals that lack *beat Ib* and *beat Ic* (Fig. 3). Extensive analysis of animals lacking *beat Ib* failed to reveal any significant defect in embryonic or larval neural development, and altering the levels of Beat Ib does not appear to modify the severity of other axonal phenotypes (not shown). *beat Ic* mutants display a strong phenotype in the development of the transverse nerve (see below) and a mild phenotype in the development of the ISNb.

We also used double-stranded (ds) RNA interference to

Table 1. Loss-of-function lesions in many of the Beats lead to only partially expressive phenotypes

Genotype	*n*	Abnormal ISNb (%)
Wild type	69	1
beat Ic$^{-/-}$	74	19
beat Ib$^{-/-}$	78	5
beat IIa + *beat IIb* RNAi	65	17
beat VI RNAi	90	13
Buffer control RNAi	52	10

Embryos that lack *beat Ic* and *beat Ib* were generated using chromosomal deficiency overlaps: for *beat Ic*, Df(2L)TE35D-GW19/Df(2L)RM5; for *beat Ib*, Df(2L)TE35D-GW11/Df(2L)RA5 (see Fig. 4).

examine the loss-of-function phenotypes of several other Beats. Embryos injected with *beat VI* ds RNA have abnormal ISNb development in ~13% of hemisegments, which is similar to the 10% of abnormal hemisegments observed in buffer-injected control embryos. Similarly, only 17% of hemisegments in embryos co-injected with *beat IIa* and *beat IIb* ds RNAs display defective ISNb phenotypes (Table 1). None of these treatments caused significant defects in the development of any other major motor nerve.

beat Ia and *beat Ic* interact in the development of the ISN and its branches

The lack of a strong phenotype in single-gene mutants of genes that belong to large families is hardly surprising. There may well be functional redundancy between the members of the *beat* gene family, or it may be that each of the new Beats plays only a small role in generating axonal specificity, secondary to stronger mechanisms such as Fas II-mediated axon adhesion or Semaphorin-mediated repulsion. If the latter is true, then we may be able to observe an effect of the loss of one of the new Beats if we look in a sensitized genetic background. For example, loss of the homophilic cell adhesion molecule Connectin from a subset of motor axons has no effect on their development, but removing *connectin* activity in a *beat Ia* mutant background partially suppresses the *beat Ia* mutant phenotype (Fambrough and Goodman, 1996), revealing the wild-type role of Connectin in providing specific axonal adhesion. As a further example, *semaII* is an axonal repellent expressed on all muscles that prevents inappropriate synaptic arborizations. Loss-of-function mutations in *semaII* result in only partially expressive innervation defects, but these mutations suppress a repulsive phenotype of NetB overexpression on SNa motor axons (Winberg et al., 1998), thus revealing a role for SemaII as a repellent for guidance of the SNa motor axons.

Given that the new Beats, as predicted membrane-anchored IgCAMs, most closely resemble other proteins that function as axonal CAMs, we decided to use the *beat Ia* mutant as a sensitized background to test *beat Ic* for adhesive activity in vivo. If Beat Ic functions in an adhesive fashion, then the loss of *beat Ic* should suppress the axonal hyper-adhesivity phenotype in *beat Ia* mutants. We thus examined motor axon defasciculation phenotypes in *beat Ic*, *beat Ia* double mutants. Using a chromosomal deficiency overlap that deletes both *beat Ic* and *beat Ia* (Fig. 4), we found that loss of *beat Ic* does indeed improve the *beat Ia* mutant phenotype, from only 42% ISNb defasciculation in a *beat Ia* null background to 63% ISNb defasciculation in a *beat Ic*, *beat Ia* double mutant (Table 2). Furthermore, the ISNd defasciculation phenotype in *beat Ia* mutants is improved from 14% to 32% in this double mutant combination. Significantly, other nerves that exhibit defasciculation errors in the absence of Beat Ia do not appear to be suppressed in the same way.

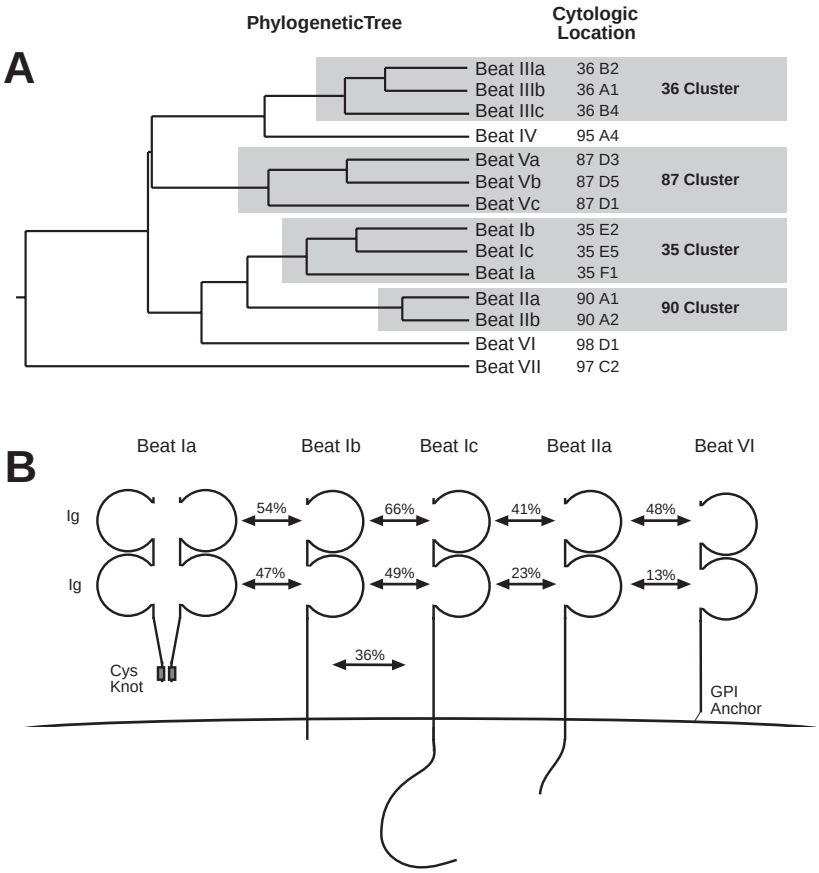

Fig. 2. Phylogenetic tree and predicted protein structures of the Beats. (A) Phylogenetic tree constructed from the alignment in Fig. 1, noting cytological locations and clustering. (B) Predicted protein structures of all the Beats for which the entire ORF has been sequenced. Beat Ia is predicted to be a dimerized secreted protein, while the rest are predicted to be membrane anchored, either by transmembrane domains or a GPI link.

Restricted expression of *beat Ic* is required for normal defasciculation of motor axons in the ISNb

In wild type, *beat Ic* mRNA is first transcribed in the early (stage 9) embryo in a subset of cells that invaginate to form the cephalic furrow and germ band (not shown). This expression ceases by the end of germ band retraction, and no *beat Ic* transcript is visible in embryos of stage 12/1. Transcription of

Table 2. Interaction between *beat Ia* and *beat Ic* in the development of the ISNb and ISNd

	ISNb			ISNd		
Genotype	*n*	Wild type (%)	Bypass (%)	*n*	Wild type (%)	Bypass (%)
beat Ia$^{-/-}$	66	5	58	65	9	86
beat Ic$^{-/+}$, *beat Ia*$^{-/-}$	52	4	46	48	17	83
beat Ic$^{-/-}$, *beat Ia*$^{-/-}$	65	20	37	41	29	68

beat Ia$^{-/-}$ embryos were generated using a homozygous protein-null allele of *beat Ia*. *beat Ic*$^{-/+}$, *beat Ia*$^{-/-}$ embryos were generated using a protein-null allele of *beat Ia* over a chromosomal deficiency that deletes both *beat Ia* and *beat Ic*: *beat Ia*^{466}/Df(2L)RM5. Embryos doubly mutant for *beat Ia* and *beat Ic* were generated using a chromosomal deficiency overlap that deletes both genes: Df(2L)ScoR+18/Df(2L)RM5.

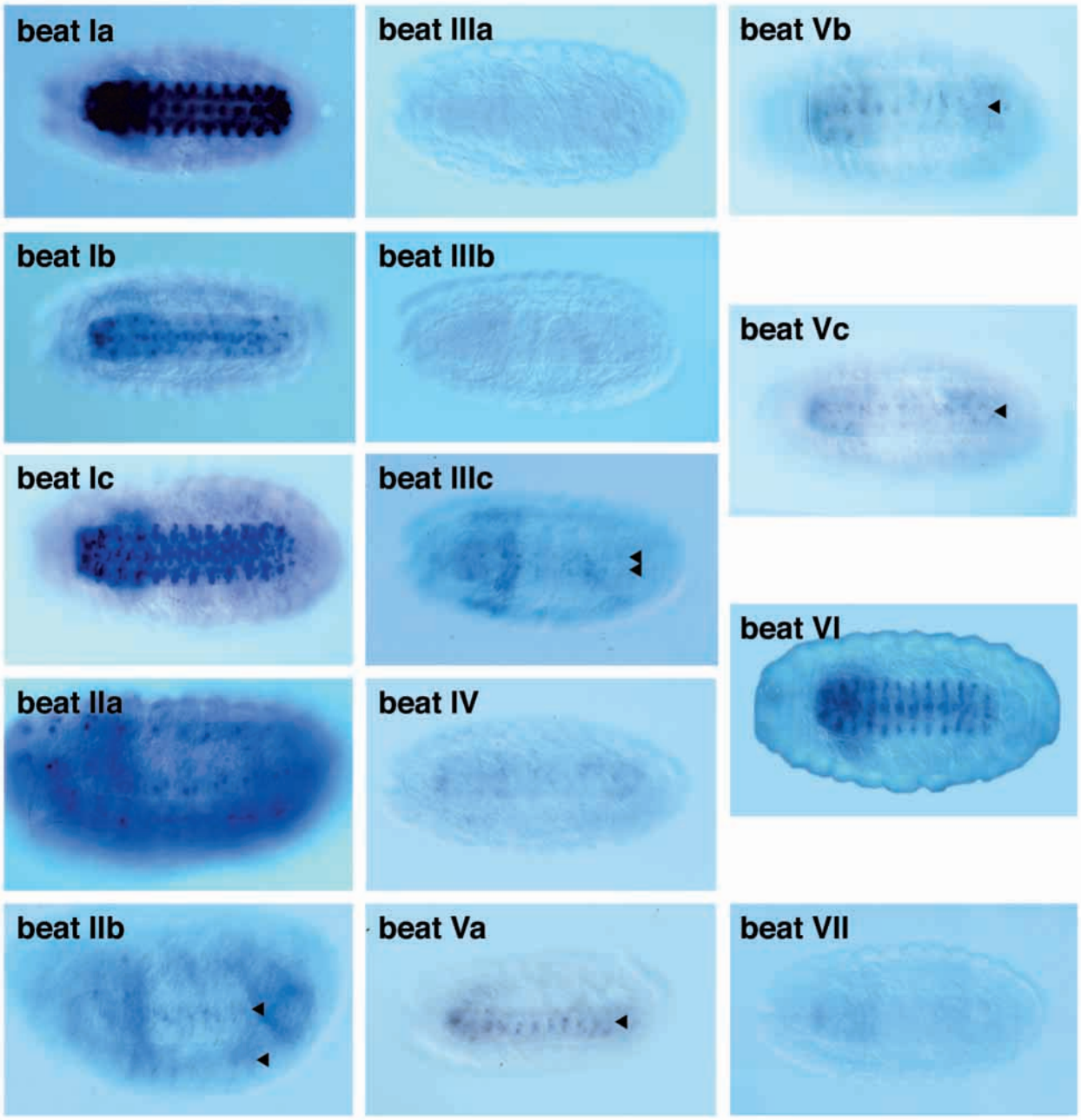

Fig. 3. Expression patterns of the Beats. Ventral or ventral oblique views of embryonic mRNA in situ using probes constructed from cDNAs or PCR fragments of the Beats noted. Anterior is towards the left. Arrowheads indicate faint expression.

beat Ic commences again in a small subset of cells at stage 13, shortly after the onset of *beat Ia* expression (Fig. 5B). It is impossible to identify these cells definitively at this stage because of cell movements, but some of the *beat Ic*-positive cells at this stage also stain with mAb 1D4 (anti-Fas II), which labels all motoneurons. The number of cells expressing *beat Ic* mRNA increases gradually but steadily to include a subset of the identified motoneurons. This restricted expression pattern is in contrast to that of *beat Ia*, which appears to be expressed in all embryonic motoneurons (Fambrough and Goodman 1996) (Fig. 5A).

We used the *elav*-GAL4 enhancer, which drives expression in all neurons, in conjunction with a UAS-*beat Ic* transgene to alter the restricted pattern of Beat Ic expression found in wild type. Using two copies of driver and two copies of reporter to misexpress Beat Ic in all neurons causes defects in ISNb development. Of the ISNb axons that show a phenotype in this treatment, many can be seen stalling in tangles as they enter their target muscle field (Fig. 5D). Some ISNb motor axons appear to be more sensitive to Beat Ic levels than others. The RP3 motor axon stalls 23% of the time in this treatment, while RP1 only stalls 5% of the time. This phenotype appears to be dose sensitive, as reducing the levels of misexpressed Beat Ic by using only one copy of the *elav*-GAL4 driver reduces the frequency of these errors from 23% to 11%.

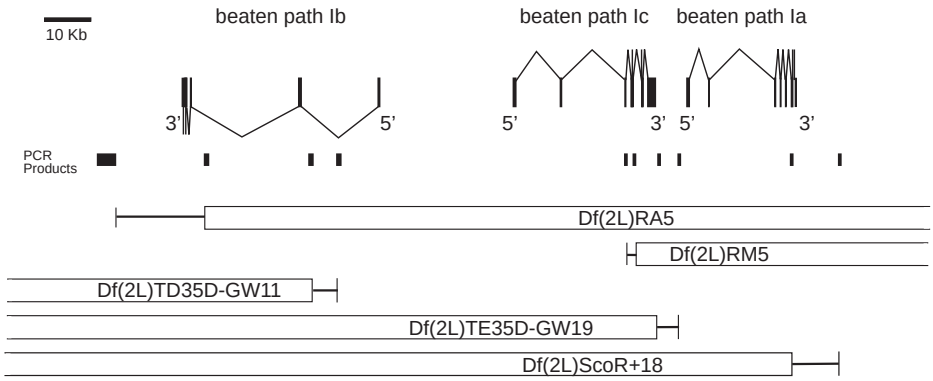

Fig. 4. Structure of the genomic locus of the class I Beats. Shown are the locations and direction of transcription of the class I Beats at chromosome band 35 on 2L; distal regions are to the left. Each of the genomic loci of the Beats appears to be roughly as large (~15-20 kb) and complex as this. Intervening genes are omitted. Chromosomal deficiency stocks used in this study are depicted below. The breakpoints for Df(2L)RA5 and Df(2L)TE35D-GW11 were mapped originally for this study; the breakpoints for the other Dfs listed are from Fambrough and Goodman (Fambrough and Goodman, 1996).

Restricted expression of *beat Ic* is required for normal development of the transverse nerve

In normal development, the transverse nerve forms from the fasciculation of the axons of a sensory nerve (the lateral bipolar dendrite or LBD neuron) and a motor nerve (the transverse motor nerve or TMN). These two growth cones grow along the process of a specialized glial cell called the dorsal median cell (DMC), thought to be crucial for the proper development of the transverse nerve (Gorczyca et al., 1994). At stage mid- to late 15, these two growth cones find each other on the surfaces of the ventral interior muscles, fasciculate (Fig. 6A), and then both continue growing – the sensory axon following the efferent motor axon projection into the CNS and the motor axon following the LBD dorsal projection to a dorsal muscle target.

Embryos that lack *beat Ic* often display errors in the development of the TN. Whereas in wild type, the TMN and LBD axons are tightly fasciculated by stage late 16, *beat Ic* mutants of the same age are often bifurcated and exploring the surface of the ventral muscles (Fig. 6B,C). This effect appears to be due to the loss of *beat Ic* in the TMN, as *beat Ic* mRNA is not detected in the LBD cell body, but can be seen in a cell located just outside the longitudinal axon tracts, below the level of the axons, the location of the TMN cell body.

Alterations in Beat Ic expression in these two neurons also result in TN fasciculation defects. The *elav*-GAL4 driver drives relatively high levels of transgene expression in both the TMN and the LBD. Using this driver to misexpress a UAS-*beat Ic* transgene results in an increase in the level of Beat Ic in the TMN and simultaneously misexpresses Beat Ic in the LBD neuron. This manipulation results in increased stalling and ectopic exploration of the ventral muscle surfaces by the TN (Table 3).

We attempted to rescue the TN phenotype in *beat Ic*$^{-/-}$ mutants by re-supplying Beat Ic protein to all neurons using the *elav*-GAL4 driver described above. If the *elav*-GAL4 driver perfectly recapitulates the *beat Ic* expression pattern, then one would expect complete rescue of the *beat Ic* mutant phenotype. However, *elav*-GAL4 drives expression in both the TMN and in the LBD neuron, whereas *beat Ic* is normally only expressed in the former. In spite of this limitation, TN fasciculation is improved in *beat Ic* mutants by *elav*-GAL4-driven misexpression of UAS-*beat Ic*: from 44% of wild type to 56% of wild type (Table 3).

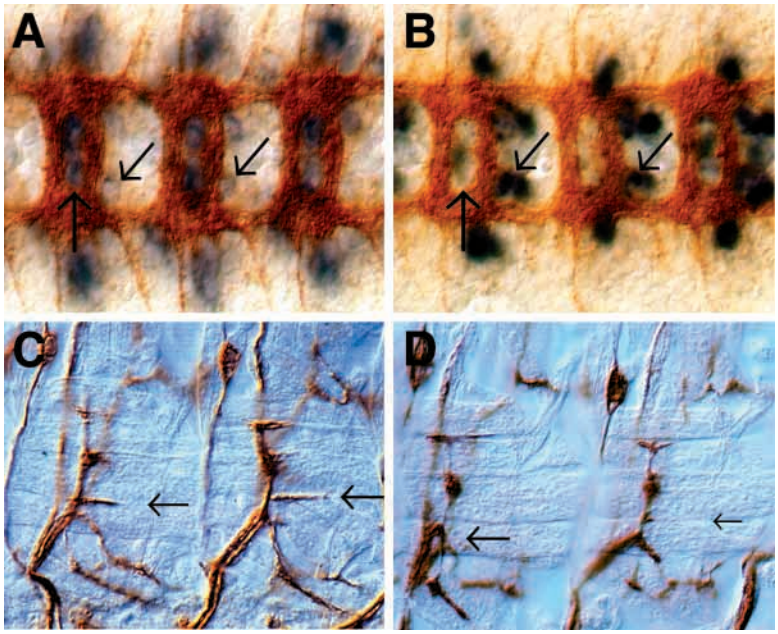

Fig. 5. Restricted expression of *beat Ic* mRNA is required for normal ISNb development. (A,B) Embryonic mRNA in situ using *beat Ia* or *beat Ic*, double-labeled with mAb BP102, which labels all CNS axons. Anterior is towards the left. At stage embryonic 13, *beat Ia* is expressed strongly in all motoneurons (A), while *beat Ic* is expressed in a much more restricted subset of neurons (B). In particular, very weak *beat Ic* expression can be seen in the aCC motoneuron (diagonal arrow) and no *beat Ic* expression can be detected in the RP motoneurons (vertical arrow). (C) Two abdominal segments of a wild-type stage 16 embryo, stained with anti-FasII mAb 1D4 to visualize motoneurons. At this stage, the axons that compose the ISNb have separated and spread out to innervate their appropriate muscle targets. In particular, the RP3 axon can be seen innervating the cleft between muscles 6 and 7 (arrows). (D) Two abdominal segments from a similarly-prepared *elav*-GAL4; UAS-*beat Ic* embryo. Increasing the levels of Beat Ic causes axons to tangle and stall as they enter their target muscle field (large arrow). Motoneuron RP3 is often seen to be delayed in growing into the cleft between muscles 6 and 7 (small arrow).

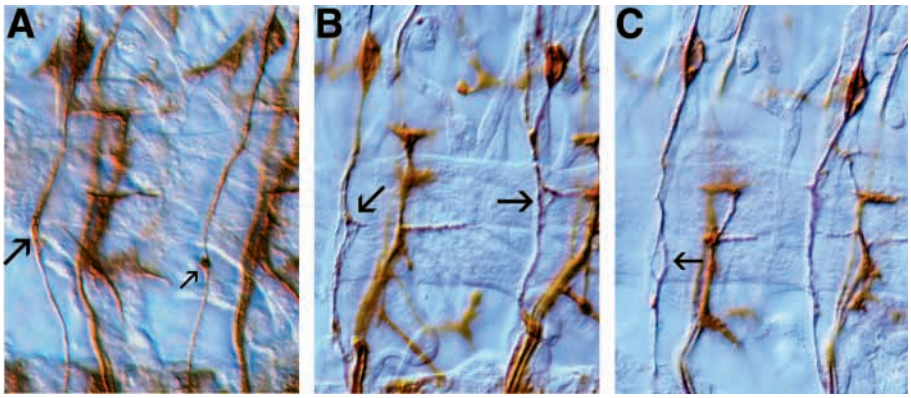

Fig. 6. Transverse nerve phenotypes in *beat Ic* mutants. (A) An abdominal segment in a wild-type stage late 16 embryo that shows the TNs growing on the segment boundaries on either side. The TN is fully developed by this stage, and the LBD axon has tightly fasciculated with the TMN axon (large arrow). The TMN axon can sometimes be seen innervating muscle 25 at this stage (small arrow). (B) An abdominal segment in a *beat Ic* mutant (Df(2L)TE35D-GW19/Df(2L)RM5) embryo at stage late 16, showing TN fasciculation defects (arrows). The TMN axon and the LBD axon are both wandering onto the surface of the ventral muscles instead of fasciculating with each other. (C) Another abdominal segment in a *beat Ic* mutant embryo. The TMN and LBD axons appear to be growing right past each other (arrow). The TN growing on the adjacent segment boundary is relatively normal.

DISCUSSION

Evolution of the Beats

The expansion in gene number that gave *Drosophila* 14 members of the Beat gene family must have been fairly recent, as searches of the completed nematode and human genomic sequence find no strong matches. The vertebrate gene that shows the most homology to Beat is the BL1 gene of mice and humans, but this gene is predicted to encode a protein with a significantly divergent domain structure: three Ig domains, followed by a transmembrane domain and a cytoplasmic tail. The worm gene with the greatest homology to Beat is F12F3.3, the protein product of which is predicted to resemble titins, with dozens of tandem repeats of FN3 and Ig domains. Hence, it seems that the Beat Ig domains predate the arthropod divergence, but since that time these domains have been swapped around as discrete modules onto many different proteins in both lineages. It will be interesting to examine the genomic sequences of other dipterans to see if the expansion in *beat* gene number was an invention of *Drosophila*.

Why have the Beats undergone such tremendous expansion in the *Drosophila* lineage? It may be that some Beat family-regulated developmental process required very fine modulation of signal over time and space. One way to provide this fine modulation would be by duplicating a *beat* gene and giving it a slightly different signaling or expression property. In support of this notion, we note that many of the Beats are expressed in similar patterns in the embryo: class I Beats are all broadly expressed in ventral nerve cord cells and motoneurons late in embryogenesis; class II Beats are expressed in the ventral oblique muscles and are the only Beats expressed in muscles; class III Beats are all weakly expressed in the embryo; and class IV Beats are all expressed at similar levels by large, ventral cells in the CNS. All of these Beats may play critical roles during some later developmental stage, such as the larval or the adult nervous system.

The fact that the homology among the Beats is highest at the N termini may reflect a binding interaction common to all of the Beats that takes place primarily at the N-terminal immunoglobulin domain.

Table 3. Manipulation of Beat Ic levels in the transverse nerve affects fasciculation

Genotype	*n*	Fasciculated TN (%)	Bifurcated TN (%)
Wild type	67	93	0
beat Ic$^{+/-}$	64	84	2
beat Ic$^{-/-}$	71	34	20
GOF *beat Ic*	52	88	6
Control for *beat Ic* rescue	68	44	10
beat Ic rescue	68	56	7

beat Ic$^{+/-}$ embryos were of the genotype Df(2L)RM5/+. *beat Ic*$^{-/-}$ null mutants: Df(2L)TE35D-GW19/Df(2L)RM5. GOF *beat Ic*: *elav*-GAL4; UAS-*beat Ic*. The *elav*-GAL4 control for *beat Ic* rescue was Df(2L)TE35D-GW19/Df(2L) RM5; *elav*-GAL4/+. *beat Ic* rescue embryos were Df(2L)TE35D-GW19/Df(2L) RM5; *elav*-GAL4 / UAS-*beat Ic*.

Potential role of Beat family members in heterophilic adhesion

Loss-of-function mutations in *beat Ic* partially suppress the hyper-adhesive motor axon phenotype in *beat Ia* mutants. In a *beat Ia* mutant, motor axons fail to defasciculate. Simultaneously removing *beat Ic* allows some of them to defasciculate. Beat Ic therefore appears to function in wild type to promote motor axon adhesion and fasciculation. But Beat Ic does not appear to be a homophilic adhesion molecule: S2 cells expressing high levels of Beat Ic do not aggregate (Q. Lin and G. C. T. Pipes, unpublished), and *beat Ic* is only expressed in a limited subset of neurons, not broadly enough to mediate adhesion among all of the axons in the ISN, ISNb and ISNd. Therefore, the function of Beat Ic in axon adhesion is presumably mediated by heterophilic binding to an unknown Beat receptor protein.

This Beat receptor could, in turn, bind to all the various members of the Beat family with differing avidities. In this model, a broadly expressed Beat receptor could provide varying levels of adhesion among different subsets of axons, depending on which membrane-anchored Beat they express.

A role for Beat Ic in heterophilic adhesion could explain the loss- and gain-of-function phenotypes of *beat Ic* in the formation of the transverse nerve (TN). The TN forms as two axons meet around the ventral muscles: the TMN axon extends out from the CNS, and the LBD sensory axon extends in towards the CNS. *beat Ic* is expressed in the TMN but not in the LBD. In the absence of Beat Ic, these axons do not properly fasciculate. However, when extra Beat Ic is transgenically

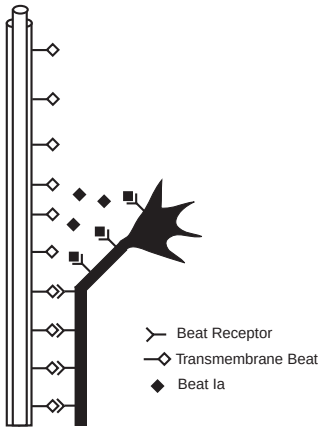

Fig. 7. Model for Beat Ia-mediated regulation of membrane-anchored Beat adhesion complexes. In this model, a subset of axons expresses a membrane-anchored Beat (white diamonds) that forms heterophilic adhesion complexes with an unknown Beat receptor in *trans*. Beat Ia (black diamonds) is secreted by a different subset of growth cones at defasciculation choice points and binds to the Beat receptor, displacing the adhesion complexes and locally reducing axonal adhesivity.

expressed by both neurons, we also observe a defasciculation phenotype. This result is most easily explained by the presence of a putative Beat Ic receptor on either the LBD axons, or some other guiding cell. Saturating this receptor with excess ligand might lead to a gain-of-function phenotype that appears in certain respects similar to the loss of function. These data lead us to propose a model in which Beat Ic and its putative receptor are part of a guidance system that normally drives the TMN axon to fasciculate with the LBD axon.

Control of adhesion during axon outgrowth

Despite extensive mutant screens of much of the *Drosophila* genome, a defasciculation phenotype has never been described for the motor axon projection (VanVactor et al., 1993). No mutant has been described in which the motor axons prematurely defasciculate in the periphery. Even in embryos with severe reductions in the axonal levels of the homophilic cell adhesion molecule Fas II, motor nerves adhere to each other properly as they grow out into the periphery. We infer from this that what keeps motor axons fasciculated as they extend into the periphery are multiple adhesion molecules, possibly from a redundant family of multiple genes. Whatever the nature of this non-Fas II adhesion mechanism, it is regulated in large part by secretion of Beat Ia at defasciculation choice points. Embryos that lack Beat Ia show severe phenotypes in which motor axons fail to defasciculate and instead continue to extend along the major motor nerves, tightly fasciculated with other motor axons. The other Beat genes in the fly have single-gene phenotypes that are much less severe, ranging from no observable phenotype (*beat Ib*) to moderate TN fasciculation defects (*beat Ic*). These other Beat genes are also all membrane anchored, either by a transmembrane domain or a GPI-linkage.

These other Beats represent obvious candidates for being the Beat Ia-regulated axonal adhesion proteins. Given that Beat Ia is a secreted protein that resembles cell adhesion molecules, we propose that Beat Ia functions as a competitive inhibitor of axon adhesion mediated by transmembrane Beat family members binding to a Beat receptor. Secretion of Beat Ia at specific points would thus allow fine alterations in the level of axonal adhesivity (Fig. 7). Testing this model in the future will require the identification of the putative Beat family receptor(s).

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