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The divergent TGF-β ligand Dawdle utilizes an activin pathway to influence axon guidance in *Drosophila*

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Axon guidance is regulated by intrinsic factors and extrinsic cues provided by other neurons, glia and target muscles. Dawdle (Daw), a divergent TGF-β superfamily ligand expressed in glia and mesoderm, is required for embryonic motoneuron pathfinding in *Drosophila*. In *daw* mutants, ISNb and SNa axons fail to extend completely and are unable to innervate their targets. We find that Daw initiates an activin signaling pathway via the receptors Punt and Baboon (Babo) and the signal-transducer Smad2. Furthermore, mutations in these signaling components display similar axon guidance defects. Cell-autonomous disruption of receptor signaling suggests that Babo is required in motoneurons rather than in muscles or glia. Ectopic ligand expression can rescue the daw phenotype, but has no deleterious effects. Our results indicate that Daw functions in a permissive manner to modulate or enable the growth cone response to other restricted guidance cues, and support a novel role for activin signaling in axon guidance.

KEY WORDS: *Drosophila*, TGF-β, Alp23B, Activin, Baboon, Smad2 (Smox), Motoneuron, Axon pathfinding, Neuromuscular system, Glia

INTRODUCTION

During nervous system development, axons are guided by attractive and repulsive cues, allowing them to navigate through a complex extracellular environment to their final destinations. The accuracy of axon guidance, target selection and synapse formation is crucial to the functioning of neuronal circuits. The precise manner in which axons contact their targets suggests that an array of unique signals may be required to direct individual growth cones. In fact, a relatively small number of guidance cues appear to be used combinatorially and reiteratively to regulate neuronal circuitry. Furthermore, late-developing axons fasciculate with pioneer axons and exit these pathways at specific sites in response to local guidance cues. Thus, axon trajectories result from a series of decisions made at choice points rather than in response to a set of global cues. Several conserved classes of molecules provide short- or long-range guidance cues in *Caenorhabditis*, *Drosophila* and vertebrates. These include the Eph receptors and Ephrin ligands; Netrins that bind the DCC/Unc-5 subfamily of immunoglobulin cell adhesion molecules (IgCAM); Slit and its receptor Robo; and Semaphorins that act through Plexin-containing receptor complexes (reviewed by Chisholm and Tessier-Lavigne, 1999; Dickson, 2002; Tessier-Lavigne and Goodman, 1996).

In *Drosophila*, embryonic motoneurons contact their synaptic targets in the bodywall musculature in a highly stereotypic manner, providing a powerful system for genetic analysis (Bate and Broadie, 1995; Keshishian et al., 1996). Extensive screens have identified additional molecules that control axon guidance and target recognition, such as the IgCAM Fasciclin 2 (Fas2) that promotes axon-axon adhesion and fasciculation (Lin and Goodman, 1994), as well as Beaten path (Beat-Ia) and Sidestep (Side) that trigger defasciculation at specific choice points (de Jong et al., 2005; Fambrough and Goodman, 1996; Pipes et al., 2001; Sink et al., 2001; Van Vactor et al., 1993). The receptor protein tyrosine phosphatase (RPTP) Lar and its homologs constitute another class of molecules that affect axon guidance, a role that appears to be conserved in *C. elegans* and mice (Desai et al., 1996; Desai et al., 1997; Krueger et al., 1996; Schindelholz et al., 2001; Sun et al., 2001). Syndecan and Dally-like function as ligands for Lar, implicating heparan sulphate proteoglycans (HSPGs) in axon pathfinding as well (Fox and Zinn, 2005; Johnson et al., 2006). The pathways that link the activity of guidance cues to changes in cytoskeletal dynamics are beginning to be resolved through the demonstration that proteins such as Rho GTPases, the GEF Trio and Ena/VASP, also affect axon extension (Huber et al., 2003; Luo, 2002). Cytoskeletal components that alter growth cone motility include Short stop (Shot), Profilin and *Drosophila* Pod1 that regulate actin and microtubule dynamics (Lee et al., 2000; Rothenberg et al., 2003; Wills et al., 1999). Interestingly, genetic screens have identified few regulators of guidance factors and signaling protein expression, although they must play an equally important role in enabling pathfinding.

In this paper, we provide evidence that Dawdle (Daw), a Transforming Growth Factor-ß (TGF-β) superfamily ligand, acts through an activin signaling pathway and controls motoneuron pathfinding during *Drosophila* embryogenesis. Activins, along with the prototypical TGF-βs and Bone Morphogenetic Proteins (BMPs), constitute three structurally and functionally distinct subfamilies of the TGF-β superfamily (Massague, 1998). TGF-β family members have multiple functions in the nervous system, such as neural induction and patterning, and regulation of neuronal survival, differentiation and synaptogenesis (Altmann and Brivanlou, 2001; Mehler et al., 1997; Stern, 2005; Unsicker and Krieglstein, 2002). However, a role for activin signaling in neuronal pathfinding has not been demonstrated previously in any system. Activins, like other TGF-β ligands, signal by binding a heteromeric receptor complex of type-I and type-II transmembrane serine-threonine kinases. Ligand binding results in type-I receptor activation and consequent phosphorylation of a receptor-specific member of the Smad family of signal-transduction proteins, which then associates with a co-Smad

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and translocates into the nucleus to directly regulate gene expression (Massague, 1998; Shi and Massague, 2003). Both *Drosophila* type-II receptors, Punt (Put) and Wishful Thinking (Wit), can bind BMPs and activins; thus, signaling specificity is dependent on recruitment of the appropriate type-I receptor to the complex (Childs et al., 1993; Letsou et al., 1995; Marques et al., 2002; Ruberte et al., 1995). The activin pathway in flies is represented by a single type-I receptor Baboon (Babo) and the signal-transducer Smad2 (Smox – Flybase), the fly homolog of activin-specific Smad2/3 (Brummel et al., 1999; Das et al., 1999; Wrana et al., 1994). The *Drosophila* genome contains four potential ligands for Babo: Activin, Myoglianin (Myg), Maverick (Mav) and Daw (reviewed by Parker et al., 2004). Both Activin and Myg bind Babo in biochemical assays, but only Activin has been shown to stimulate Smad2 phosphorylation (Lee-Hoeflich et al., 2005; Zheng et al., 2003). The absence of mutations in these four ligands has made it difficult to establish their developmental roles. However *babo* and *Smad2* mutants display phenotypes such as reduced cell proliferation in the larval brain and defects in ecdysteroid-dependent remodeling of mushroom body neurons, implicating activin signaling in neuronal development (Brummel et al., 1999; Zheng et al., 2003). Important questions that remain to be addressed are the identities of ligands that trigger *babo* activity in vivo and the contributions they make.

Here we present biochemical and genetic evidence that Daw initiates an activin signaling pathway via the receptors Put and Babo, and the transducer Smad2. Mutations in *daw* display premature stalling and defasciculation defects in embryonic motoneuron pathfinding. Interestingly, although Daw is expressed in the glia and muscles, the receptor Babo appears to be required in the motoneurons. Furthermore, our data suggest that Daw plays a permissive rather than an instructive role, potentially by enabling the response of the growth cone to other spatial cues. These results provide the first demonstration in any system that a canonical activin signaling pathway is involved in axon guidance.

MATERIALS AND METHODS

Drosophila stocks

Stocks were obtained from Bloomington Stock Center unless listed otherwise: P{EP}EP1039 (Szeged Stock Center), *Smad2^{388}/FM7c*, *FRTG13 babo^{52}/CyO*, *babo^{32}/CyO wg-lacZ*, UAS-*baboΔI* (isoforms a and b), UAS-*tkvΔI* (also known as *TkvΔGSK*), UAS-*putΔI* and OK6-Gal4 (M. B. O'Connor, HHMI, University of Minnesota, MN), dMef2-Gal4 (E. Olson, University of Texas Southwestern Medical Center, Dallas, TX), Repo-Gal4 (V. Auld, University of British Columbia, Vancouver, Canada). Maternal/zygotic *babo*-null embryos were derived from germline clones induced in *FRTG13, babo^{52}/FRTG13, ovo^{D}* females crossed to *babo^{32}/CyO wg-lacZ* males.

Molecular and genetic characterization of *daw*

pCS2+Daw contains an *Ssp*I-*Stu*I fragment from GH14433; pUAS-Daw contains a 2.5 kb *Eco*RI-*Not*I fragment from pCS2+Daw. Daw-XmnI contains a 7.8 kb fragment from P1 clone DS07149 in pCaSper. *daw^{3}* and *daw^{4}* were generated in a *b, pr, cn, wx, bw* background. Deletion end-points and lesions were determined by sequencing PCR-amplified DNA from homozygous *daw* mutant embryos. The isogenized parental stock and both EMS alleles contain two polymorphisms as compared with the *Drosophila* genome sequence: a His143→Tyr substitution and an in-frame 9 bp deletion corresponding to Ser173-Pro174-Leu175. The parental stock is viable and does not show pathfinding defects, indicating that these substitutions do not have functional consequences.

Lethal phase analysis

Egglays from *daw^{-}/CyO, Kr-Gal4, UAS-GFP* stocks were collected on agar plates at 25°C in 70% humidity. Hatched larvae were counted and homozygous mutants transferred to a fresh plate. Dead larvae were staged by mouth hook morphology. Pupae were left on plates and hatching adults transferred to fresh vials. Data represent averages from 3 trials (n=600).

Immunohistochemistry and in situ hybridization

DIG-labeled *daw* and *babo* riboprobes were used for in situ hybridization (Tautz and Pfeifle, 1989). For histochemistry, embryos were stained with 1D4 (1:100), BP102 (1:200) or α-Repo (1:200) antibody (Developmental Studies Hybridoma Center) and α-mouse biotin-conjugated secondary antibody, and visualized using the Vectorstain ABC Kit. *daw^{-}* and *babo^{-}* embryos were distinguished by the absence of *CyO, wg-lacZ* marker. *Smad2* and *put* mutants were identified by the absence of *Twist-GFP*. Embryos were analyzed using Nomarski optics.

Signaling assays

Cell signaling assays were carried out in S2 cells as previously described (Zheng et al., 2003). Smad2-FLAG, Babo and BaboΔI cDNAs were cloned into pPac. Daw, Put and PutΔI cDNAs were cloned into the inducible vector pRmHA-1Mt. BaboΔI and PutΔI were obtained from M. B. O'Connor. Proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose. Phosphorylated and total Smad2-FLAG were detected using rabbit α-PS2 (Faure et al., 2000) and mouse α-FLAG (Sigma), respectively, and the ECL Plus Kit (Amersham).

RESULTS

daw encodes an activin-related ligand expressed in embryonic muscle and glia

A BLAST search of the BDGP database for sequences with homology to human activin identified a genomic clone DS07149, and two cDNA clones GH14433 and RE17443. GH14433 contains a 1762 bp ORF flanked by 543 bp of 5′ sequence and 405 bp of 3′ untranslated region (UTR) (*daw-A* in Fig. 1A). RE17443 represents an alternate isoform with a unique first exon derived from a distinct promoter, resulting in a transcript that is shorter by 146 bp (*daw-B*, Fig. 1A). Both transcripts contain common coding exons and encode an identical predicted protein of 586 amino acids. This gene was initially called *Activin Like Protein at 23B* (*Alp23B*, CG16987). To avoid confusion with *Abnormal leg pattern* (*Alp*) and to better reflect the mutant phenotype, we and our colleagues (Serpe and O'Connor, 2006) henceforth refer to the locus as *daw*. The *daw* ORF shows several features characteristic of TGF-β superfamily ligands (Fig. 1B). The first in-frame methionine is followed by a signal sequence indicating that the protein is likely to be secreted. Two consensus cleavage sites for furin-like proteases, RRRK (464-467) and RQKR (469-472), would generate a mature ligand of 114 (or 119) amino acids. Within the ligand domain, Daw shares 35% identity with human activinC, TGF-β3 and BMP3 (Fig. 1C). However, the presence of nine rather than seven cysteines at stereotypic positions characteristic of activin and TGF-β subfamilies, suggests that Daw represents a divergent activin/TGF-β ligand in *Drosophila* (Fig. 1C).

Analysis of *daw* expression revealed maternally-provided mRNA in presyncitial stage embryos (Fig. 2A). Zygotic *daw* transcripts were seen in the mesoderm from stage 6 to 8, and at higher levels after stage 9 (Fig. 2B,C). At stage 13, expression was also detected in the visceral mesoderm and oenocytes (Fig. 2D,E). Somatic muscle expression is significantly reduced by stage 15. Instead, at stage 16, prominent expression was seen in the ventral nerve cord (VNC), in median, intermediate and lateral groups of cells in a segmental pattern (Fig. 2F,G,I,I′). Absence of *daw* expression in the VNC of *glial cells missing* (*gcm^{N7-4}*) mutants that lack glia (Fig. 2J) (Jones et al., 1995), as well as double staining with α-Repo (data not shown), indicated that the *daw*-positive cells in the VNC correspond to glia. Additional sites of transcription were the fat body (Fig. 2G), the ring gland, cells in the maxillary segment, hindgut and the posterior spiracles (Fig. 2H). In larvae, *daw* was expressed in the

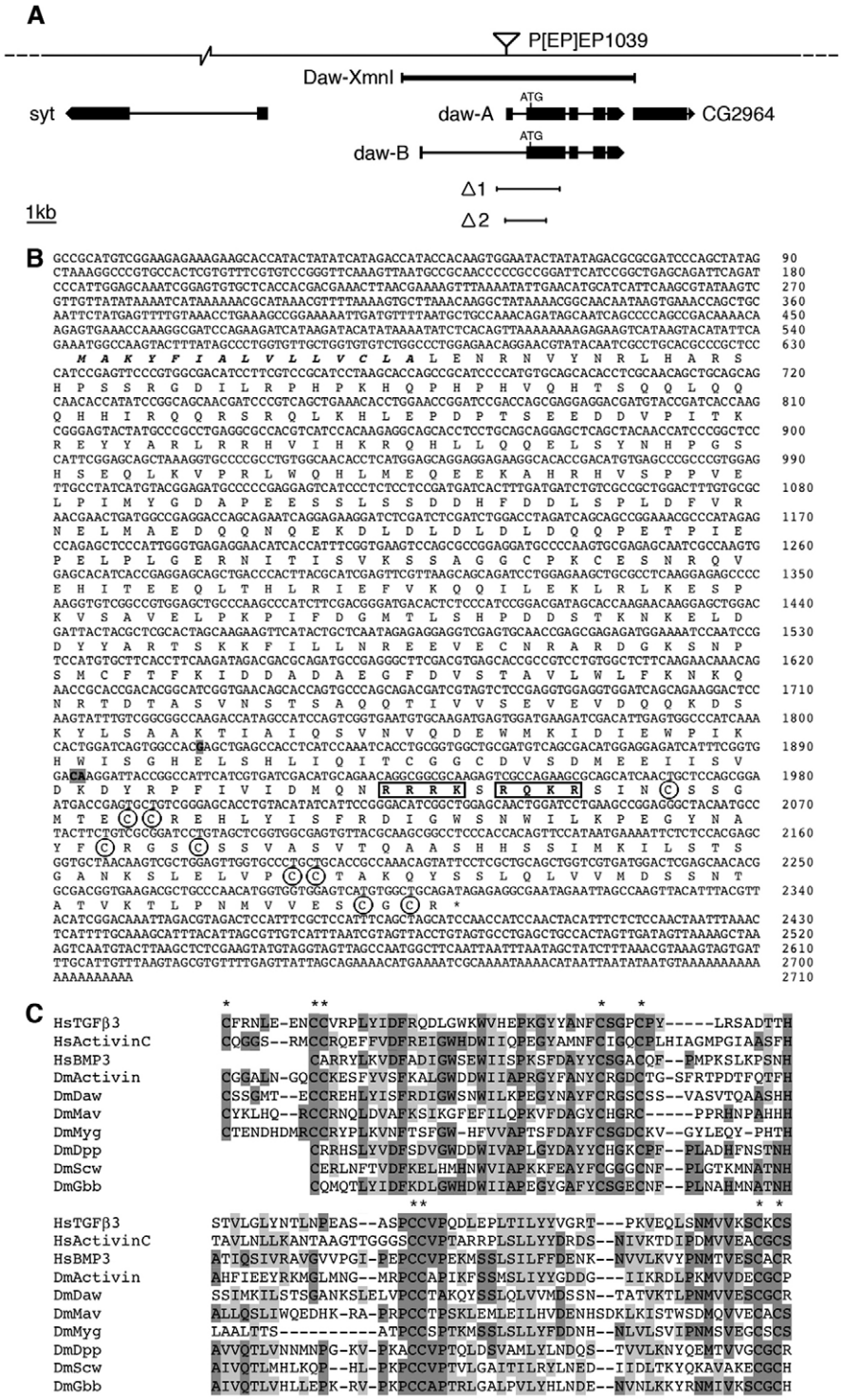

Fig. 1. Molecular characterization of *daw*. (**A**) Genomic organization of the *daw* region. The *synaptotagmin* (*syt*) gene is located 5.5 kb centromere distal of *daw* and CG2964 is located 300 bp proximal of *daw*. Alternate isoforms *daw-A* and *daw-B*, which share common coding exons, are shown. The P{EP}EP1039 insertion site, genomic rescue fragment Daw-XmnI and deletions $daw^{\Delta 1}$ and $daw^{\Delta 2}$ are indicated. (**B**) Sequence and conceptual translation of *daw* (Genbank accession no. AY051485). Italics indicate signal sequence, boxes are proteolytic cleavage sites, and circles mark cysteine residues conserved in TGF-β and activin ligands. Lesions in daw^{3} (deletion of CA) and daw^{4} (G→T substitution) are highlighted in gray. (**C**) Clustal-W alignment of ligand domains of human TGF-β3, activinC, BMP3, and *Drosophila* TGF-β superfamily members. Identities are in dark gray, similarities in light gray, and asterisks mark conserved cysteines.

outer proliferative center of the optic lobe and in the central brain (Fig. 2K), the wing and leg imaginal discs (Fig. 2L,M), and in larval bodywall muscles (Fig. 2N). Northern blots detected a 2.7 kb transcript at high levels in late embryonic and larval stages and in adult males and females (data not shown).

daw mutants display multiphasic lethality

Imprecise excision of *P{EP}EP1039*, a viable *P*-element inserted 77 bp upstream of the *daw-A* transcription start site, generated two small deletions $daw^{\Delta 1}$ and $daw^{\Delta 2}$ that remove 2.1 kb and 1.4 kb of DNA, respectively (Fig. 1A). Both deletions eliminate the first exon of *daw-A* and the translation start site in the second exon. In a screen for lethal loci uncovered by *Df(2L)C144* (Littleton and Bellen, 1994), we isolated two EMS alleles daw^{3} and daw^{4} that fail to complement $daw^{\Delta 1}$. daw^{3} contains a 2 bp deletion at 1893-1894 (Fig. 1B), leading to a frame shift in the prodomain and production of 66 altered residues before termination. A G→T change at position 1819 in daw^{4} introduces a premature termination codon, also in the prodomain. In both cases, the resulting mutant proteins (Met1-Leu515 and Met1-His425, respectively) would lack the ligand

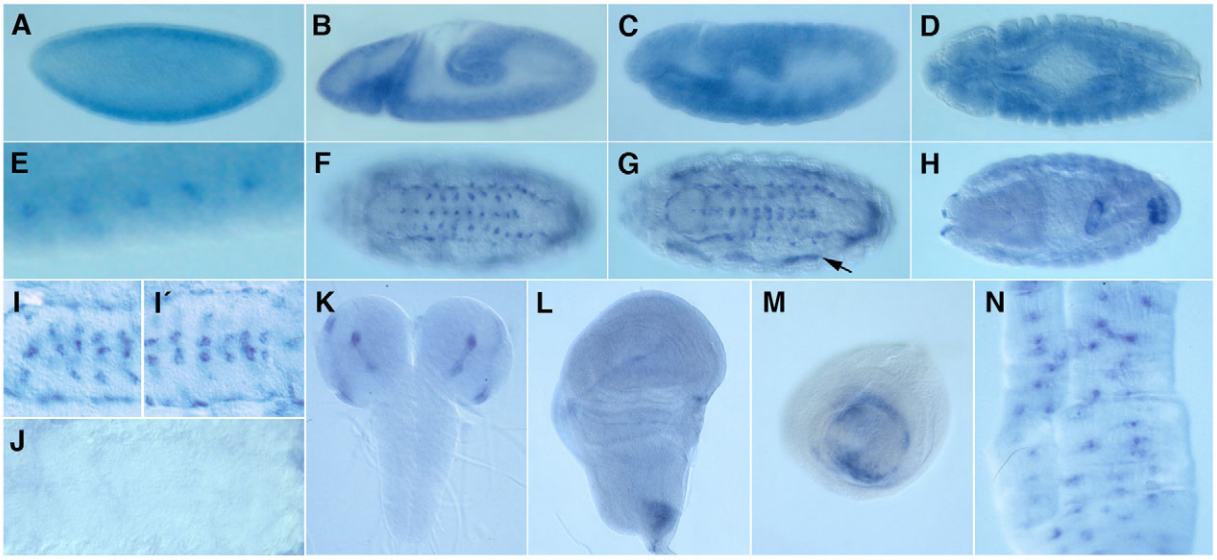

Fig. 2. Expression profile of *daw*. (**A-N**) Embryos, larval imaginal discs and brains were hybridized with *daw* probe. (A) Stage-5 embryos showing uniform maternal transcript. Sites of zygotic expression include: mesoderm at stage 7 (B) and stage 11 (C); visceral and somatic mesoderm (D) and oenocyte (magnified view E) at stage 13; VNC (optical sections F,G), fat body (arrowhead in G), posterior spiracles, hindgut and the maxillary segment (H), median, intermediate and lateral clusters of glial cells in the VNC (magnified views I,I′), at stage 17. (J) VNC of *gcm*$^{-}$ embryos. Larval expression is in the optic lobe and central brain (K), wing (L) and leg (M) imaginal discs, and bodywall muscles (N). A-C are lateral views; D,E,H are dorsal views; F,G,I,I′,J are ventral views; A-J, anterior is to left.

domain. The phenotype of *daw*^{3} and *daw*^{4} in trans to *Df(2L)C144* is indistinguishable from homozygous mutants, suggesting that they represent strong loss-of-function or null alleles. A 7.8 kb genomic rescue construct that includes both *daw* isoforms (Daw-XmnI, Fig. 1A) rescues all four alleles, indicating that the observed lethality results from loss of *daw* activity.

Characterization of the *daw* lethal phase showed 9-25% embryonic lethality (Table 1). Mutant larvae showed reduced motility but no significant lethality. The majority of mutants (59-68%) died either as white prepupae or as pharate adults that did not eclose despite rupturing the operculum (Table 1). Surprisingly, when animals were reared at low density on nutritive agar plates, 7-24% of homozygous mutants eclosed. Mutant adults were uncoordinated and died shortly after eclosion, but rare animals that did survive longer were fertile. Adult escapers were never encountered in standard vial conditions suggesting that *daw* function is influenced by population density. The multiphasic lethality of *daw* mutants is consistent with its broad expression profile and is suggestive of multiple or reiterative roles in development.

Daw is required for motoneuron pathfinding during embryonic development

Expression of *daw* in glia and muscles and the behavioral defects observed in mutants are consistent with a role in the developing nervous system. Visualization of the longitudinal and commissural axons in the VNC of *daw* mutants using mAbs BP102 and 1D4 (α-Fas2) revealed occasional breaks and fusions in the longitudinal axon fascicles at a low incidence (5%) (Fig. 3A,B). Examination of the glial cell population in *daw* mutants with α-Repo antibodies showed no overt defects (Fig. 3C). In addition, neuronal cell fate markers including *even skipped*, *engrailed*, *achaete*, *eagle*, *Fasciclin 3* and *gooseberry* were unaltered in mutant embryos, suggesting that *daw* is not involved in cell fate specification (data not shown).

Table 1. Lethal phase of *daw* alleles

Genotype	Embryo	L1	L2	L3	Pupa	Adult
daw$^{\Delta 2}$/*daw*$^{\Delta 2}$	9	6	0	9	61	15
daw^{3}/*daw*^{3}	25	0	0	0	68	7
daw^{4}/*daw*^{4}	13	0	0	4	59	24
Oregon R	0	0	0	0	0	100

Numbers indicate the % lethality observed at each phase for each genotype.

We next analyzed motoneuron guidance in *daw*$^{-}$ embryos stained with 1D4. During late embryogenesis, approximately 40 motoneurons in each abdominal hemisegment innervate bodywall muscles in a stereotypic fashion (reviewed by Bate and Broadie, 1995; Keshishian et al., 1996; Landgraf et al., 1997). Motoneurons exit the VNC via two routes, the segmental nerve (SN) and the intersegmental nerve (ISN), and project into the muscle field through five major pathways: the main ISN, ISNb, ISNd, SNa and SNc (Fig. 3D). In *daw*$^{-}$ embryos, ISNb and SNa axons exited the VNC correctly and extended into their target field, but failed to advance completely and innervate the appropriate muscles (Fig. 3E). In wild-type embryos, ISNb fasciculates with the ISN for a short distance, before it enters the ventral muscle domain and innervates the ventral longitudinal muscles (VLMs). Axons in ISNb innervate muscles 6/7, or extend dorsally to muscle 13, while a subset bypass muscle 13 to innervate muscle 12 (Fig. 3D,F) (for details, see Landgraf et al., 1997). Analysis of ISNb defects in different *daw* alleles showed that in rare instances (1-6% hemisegments) the axons stalled at muscle 6/7 and failed to form synapses on muscles 13 and 12. More commonly, ISNb axons terminated at muscle 13 (11-21%, open arrowhead in Fig. 3G), or reached muscle 12 but were unable to form a synapse (6-17%, arrow in Fig. 3G, data for individual alleles in Fig. 3J). In total, 23-38% hemisegments in *daw*$^{-}$ embryos displayed some defect in ISNb pathfinding.

Similar defects were seen in the context of SNa pathfinding. In wild type, the SNa extends dorsally past the VLMs to the lateral muscles, where it bifurcates to form a dorsal branch that innervates muscles 21-24, and a lateral branch that innervates muscles 5 and 8

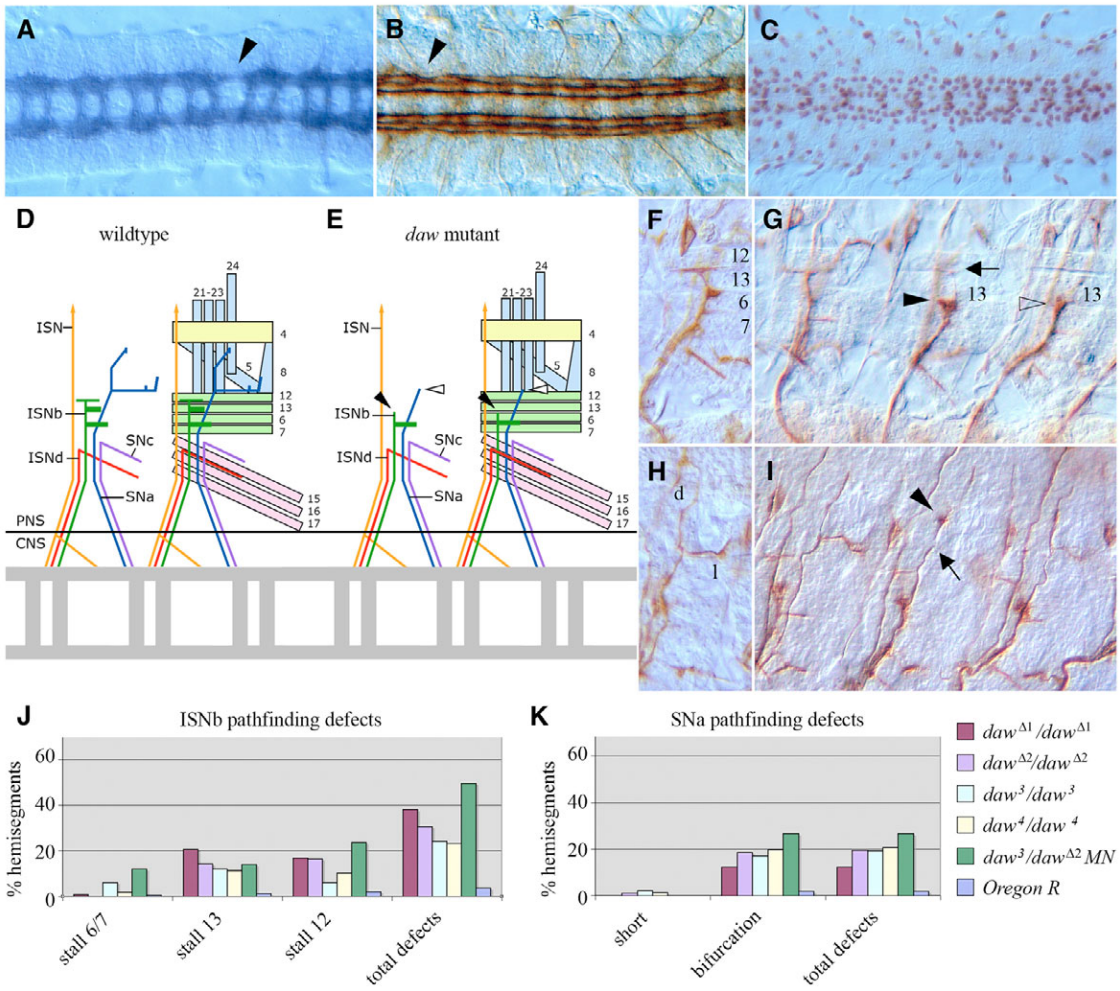

Fig. 3. Aberrant pathfinding in *daw* mutants. (**A-C**) Late stage 16/17 *daw*^{3} embryos labeled with (A) BP102, (B) 1D4 or (C) α-Repo antibodies. Arrowheads mark rare CNS defects. (**D**,**E**) Schematic representation of motoneuron pathfinding in wild-type and *daw*$^{-}$ embryos. Anterior is left, dorsal is up. Axon trajectories are depicted on the left and muscle targets are included on the right. (D) Five main nerve branches are shown that innervate correspondingly colored muscles. ISNb axons defasciculate to form three branches that innervate the muscle 6/7 cleft, muscle 13 and muscle 12. SNa forms a dorsal branch that innervates muscles 21-24 and a lateral branch that innervates muscles 5 and 8. For detailed description see Landgraf et al. (Landgraf et al., 1997). (E) In *daw*$^{-}$ mutants the ISNb and SNa extend into their correct target fields, but terminate prematurely (black and white arrowheads, respectively). (**F-I**) Motoneuron projections in late stage 16/17 filleted embryos stained with 1D4. (F) Wild-type ISNb synapses on muscles 6/7, 13 and 12. (G) In *daw*^{3} embryos, ISNb stalls at muscle 13 (black and white arrowheads), or reaches muscle 12 but fails to form a synapse (arrow). (H) Wild-type SNa dorsal (d) and lateral (l) branches. (I) *daw*^{3} embryos displaying failure of SNa defasciculation and loss of the lateral branch (arrow). Arrowhead marks stalled axons. (**J**,**K**) Incidence of ISNb and SNa pathfinding defects in *daw*$^{-}$ and *Oregon R* embryos. ISNb defects in J were scored as: stalling at muscles 6/7, muscle 13, or muscle 12 and failure to form a synapse. SNa defects in K were scored as: failure to reach target (short), or defasciculate at branchpoint (bifurcation). Hemisegments scored: *daw*$^{\Delta 1}$ *n*=161/115, *daw*$^{\Delta 2}$ *n*=300/233, *daw*^{3} *n*=222/221, *daw*^{4} *n*=238/200, *daw* maternal and zygotic nulls (MN) *n*=93/93, *Oregon R* *n*=155/178 for ISNb/SNa pathfinding.

(Fig. 3D,H). In *daw*$^{-}$ embryos, SNa extended into the lateral muscle field correctly but frequently exhibited loss of one or both branches (12-21% of hemisegments, Fig. 3E, arrow in Fig. 3I). In a few instances, the SNa stopped short of the bifurcation choice point (2-4%, Fig. 3K). In both the ISNb and SNa pathways, we frequently observed a thickening at or in the vicinity of the affected branchpoints, suggesting that axons stalled prematurely (open and closed arrowheads in Fig. 3G,I). We also observed minor defects in the ISN, but the ISNd and SNc trajectories appeared unaltered (data not shown).

In embryos from *daw*^{3}/*daw*^{3} mothers mated with *daw*$^{\Delta 2}$/*daw*$^{\Delta 2}$ males, the range of axon guidance defects was qualitatively similar to zygotic nulls, but the incidence increased to 50% ISNb and 27% SNa defects (Fig. 3J,K), indicating that maternally-provided Daw contributes to neuronal pathfinding. By comparison, wild-type embryos displayed only 4% defects in ISNb and 2% in SNa (Fig. 3J,K). Taken together, these results indicate that *daw* is required for axon guidance or outgrowth of ISNb and SNa axons.

Daw signals via the activin type-I receptor Babo and Smad2

The presence of nine conserved cysteines in the Daw ligand domain suggests that it belongs to the activin/TGF-β subfamily. In *Drosophila*, activin signaling is mediated by the type-I receptor Babo, which can associate with either Put or Wit type-II receptors

(Brummel et al., 1999; Lee-Hoeflich et al., 2005; Wrana et al., 1994). In both cases, complex formation results in phosphorylation of Smad2 but not of the BMP-specific Smad Mothers against dpp (Mad). Furthermore, both *babo* and *Smad2* are involved in axon remodeling in the larval mushroom body, linking their activities in vivo (Zheng et al., 2003). *wit* null embryos show no axon guidance defects (Marques et al., 2002), making Put a more likely type-II receptor for Daw. To establish the Daw signaling pathway, we assayed its ability to mediate Smad2 phosphorylation in transiently transfected *Drosophila* S2 cells (Fig. 4A). Cells transfected with Smad2 alone showed no basal phosphorylation when probed with α-PS2 antisera (Faure et al., 2000) that recognizes phosphorylated Smad2. However, low levels of activation could be seen in the presence of Babo, without addition of ligand (Fig. 4A, lanes 1, 2). In cells challenged with Daw-conditioned media, we observed a significant increase in Smad2 phosphorylation that was further enhanced upon cotransfection with Babo (lanes 4, 5). The response of S2 cells to ligand alone suggests that endogenous receptor levels are sufficient to allow Daw signaling to occur (see lane 2), consistent with the finding that S2 cells express *babo* and *put* (http://flight.licr.org/). To resolve the identity of the endogenous receptor, we cotransfected a dominant-negative form of Babo (BaboΔI) that retains the ligand-binding domain but lacks the intracellular kinase domain required for signaling. Similar dominant-negative Tkv and Sax receptors are known to disrupt BMP signaling in a ligand-specific manner (Haerry et al., 1998; Neul and Ferguson, 1998; Nguyen et al., 1998). Expression of BaboΔI blocked the response to Daw (Fig. 4A, compare lanes 4, 6), suggesting that the endogenous Daw signaling complex includes Babo.

Likewise, we found that cotransfection of Put also resulted in activation of Smad2 (Fig. 4B, lane 8). Significantly, phosphorylation increased when cells were additionally challenged with Daw-conditioned media (compare lanes 8, 11). Furthermore, the response to Daw was reduced when S2 cells were cotransfected with dominant-negative Put-ΔI (Fig. 4B, compare lanes 10, 12). Thus, our data demonstrate that Daw signal-transduction requires the activin pathway components Put and Babo, and triggers phosphorylation of Smad2.

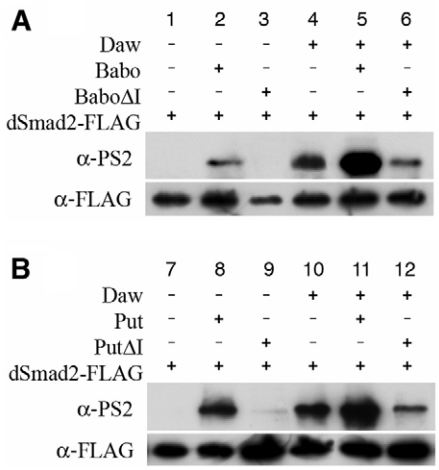

Fig. 4. Daw induces Smad2 phosphorylation via activin pathway receptors. S2 cells were transiently transfected with Smad2 alone or with wild-type and dominant-negative receptors as marked. (**A**) Response to Babo; (**B**) response to Put. Cells were exposed to control or Daw-conditioned media and western blots probed with α-PS2 to detect phosphorylated Smad2. Smad2 expression was monitored using α-FLAG. Smad2 phosphorylation was detected only in the presence of receptor (lanes 2, 8), or ligand (lanes 4, 10), but not in their absence (lanes 1, 7). Exposure to Daw enhanced Smad2 phosphorylation in Babo- and Put-expressing cells (lanes 5, 11). Coexpression of dominant-negative receptors (lanes 6, 12) reduced phosphorylation levels in response to ligand, demonstrating receptor specificity.

Activin pathway mutants show similar axon guidance defects to *daw* mutants

To determine whether Daw utilizes a canonical signaling pathway to regulate growth cone guidance, we assayed whether mutations in *put*, *babo* and *Smad2* disrupt motoneuron pathfinding. Both *put* and *Smad2* are enriched in the embryonic mesoderm and nervous system (Brummel et al., 1999; Childs et al., 1993), making them plausible candidates for mediating Daw signaling in vivo. We found that *babo* transcripts were ubiquitously distributed and highly enriched in the brain and the VNC during stages 13-17 (Fig. 5A,B). *daw*, *babo* and *Smad2* expression also overlapped at later stages; for example, in the optic lobe, leg and wing discs (Fig. 2) (Brummel et al., 1999; Das et al., 1999).

Analysis of motoneuron pathfinding in $babo^{-}$ embryos revealed defects similar to those observed in *daw* mutants. In zygotic null $babo^{32}$ animals, both ISNb and SNa branches entered their muscle fields correctly but failed to extend correctly. In 24% of hemisegments, ISNb axons stalled prematurely (Fig. 5C,D), and in 20% of hemisegments the SNa failed to defasciculate resulting in loss of either dorsal or lateral branches (Fig. 5G, arrows). Both ISNb and SNa pathways showed a thickening of the nerve in the vicinity of the disruption, consistent with a stalling defect (Fig. 5G, arrowhead). We generated *babo* germline clones, and found that the penetrance of defects increased to 58% in ISNb and 31% in SNa pathfinding (Fig. 5C).

Determining the contribution of the type-II receptor Put to motoneuron pathfinding is complicated by its requirement for dorsal cell fate specification during early embryogenesis in response to the BMP ligands Decapentaplegic and Screw (Letsou et al., 1995; Ruberte et al., 1995). Consequently, embryos lacking *put* function are ventralized. To circumvent this problem, temperature-sensitive put^{88} embryos (Simin et al., 1998) were raised at the permissive temperature (18°C) until stage 14, then shifted to restrictive temperature (25°C). These embryos displayed no overt patterning defects, but did show motor axon guidance defects. ISNb axons were stalled in 31% of hemisegments (Fig. 5C,E), and 32% of SNa axons failed to branch or extend completely (Fig. 5C,H). Whereas the penetrance of SNa pathfinding defects was comparable to *daw* or *babo* nulls, the lower incidence of ISNb defects may reflect the fact that our assay conditions do not completely eliminate *put* function.

Analysis of mutations in *Smad2* also revealed pathfinding defects reminiscent of zygotic loss of *daw* and *babo*. In $Smad2^{388}$ mutants, 21% of hemisegments had ISNb defects and 7% showed loss of lateral or dorsal SNa branches (Fig. 5C,F,I). The milder phenotype, as compared with *babo* and *daw* nulls, is likely to reflect perdurance of maternal product, as maternal/zygotic *Smad2*-null embryos have a phenotype equivalent to loss of ligand or type-I receptor (Serpe and O'Connor, 2006).

To further establish that Daw and components of the activin signaling pathway regulate axon guidance, we examined genetic interactions between *daw*, *babo* and *put*. In put^{88}/+ and $babo^{32}$/+ single heterozygotes, less than 4% of hemisegments showed abnormal ISNb pathfinding. In each case, heterozygosity for *daw* further enhanced the phenotype to 14% and 20%, respectively (Table 2), which is equivalent to zygotic loss of Daw signaling. We conclude that Put, Babo and Smad2, three components necessary for Daw signal-transduction in biochemical assays, are also required for

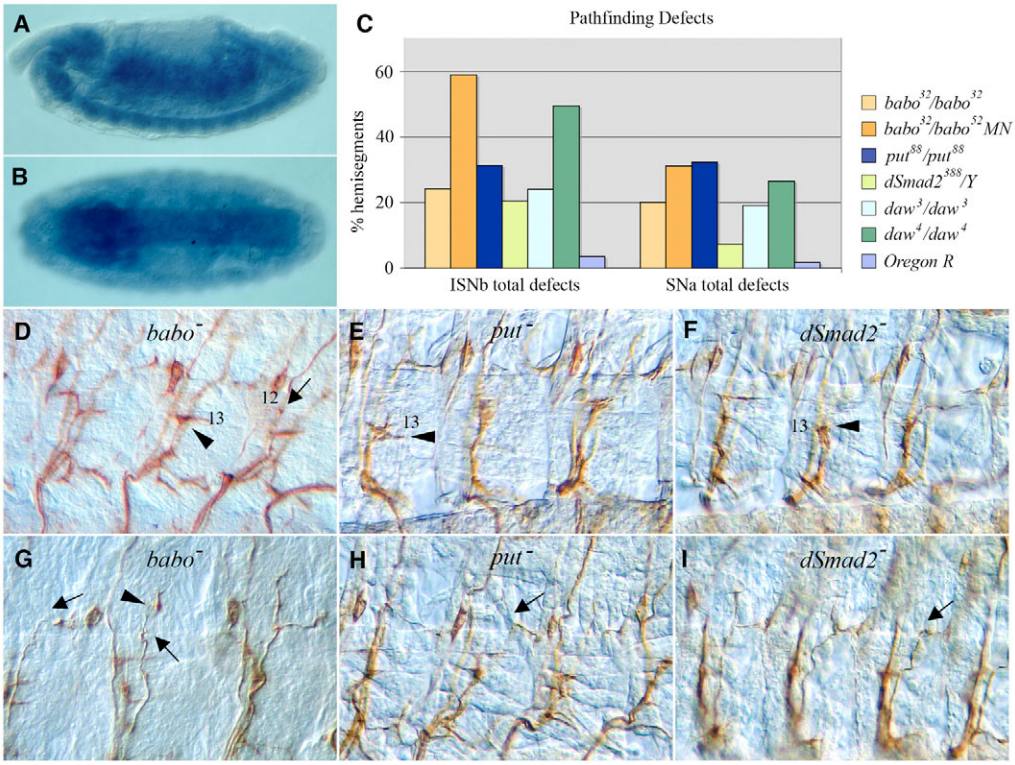

Fig. 5. Activin signaling pathway components have similar expression and mutant phenotypes. (**A**,**B**) *babo* mRNA is enriched in the VNC and brain in stage 13 (A, lateral view), and stage 15 (B, ventral view) embryos. (**C**) Incidence of total pathfinding defects in *babo*, *put* and *Smad2* mutants, as compared with *daw*⁻ and *Oregon R*. MN refers to maternal/zygotic nulls. Hemisegments scored: $babo^{32}/babo^{32}$ *n*=187/120, *babo* MN *n*=211/116, put^{88}/put^{88} *n*=208/174, $Smad2^{388}/Y$ *n*=137/138. (**D-F**) ISNb and (**G-I**) SNa guidance defects. Motoneuron projections in late stage 16/17 filleted embryos stained with 1D4. Arrowheads in D-G indicate stalled axons. Arrow in D marks ISNb axons that extend to muscle 12 but fail to form a synapse. In G-I, arrows indicate defasciculation failures and loss of SNa branches.

ISNb and SNa axons to extend and form correct synapses. Furthermore, the close correlation of the phenotypic defects encountered in *daw* mutants and components of its signaling pathway, as well as the genetic interactions observed between these genes, suggest that the effect of Daw on axon guidance is mediated through a canonical activin signaling pathway.

Daw signals to motoneurons to regulate pathfinding

All three genes required to mediate Daw signaling, *put*, *babo* and *Smad2*, are transcribed in the VNC and in embryonic muscles. To determine whether Daw-receptor activity is required in the motoneurons or in the target tissue, we used the Gal4-UAS system to block the response to Daw in a tissue-restricted and cell-autonomous manner, and assayed ISNb and SNa pathfinding. Dominant-negative UAS-*baboΔI* is expressed in muscles (dMef2-Gal4), glia (Repo-Gal4), neurons (Elav-Gal4), or specifically in motoneurons (OK6-Gal4), from stages 13, 12, 12 and 15, respectively (Aberle et al., 2002; Marques et al., 2002; Ranganayakulu et al., 1996; Sepp et al., 2001). We found that only expression in motoneurons, but not in muscles or glia, was effective in disrupting ISNb and SNa pathfinding. Embryos expressing a single copy of UAS-*baboΔI* in motoneurons showed 35% ISNb pathfinding defects and 5% SNa branching defects (Fig. 6A, Table 3). Using four copies of UAS-*baboΔI* increased the incidence of SNa pathfinding defects to 22% (Table 3), suggesting that the ISNb and SNa pathways are differentially sensitive to Daw signaling, or express different levels of Babo protein. Essentially similar results were obtained when four copies of UAS-*baboΔI* were expressed in post-mitotic neurons using Elav-Gal4 (Table 3). Dominant-negative receptors corresponding to

Table 2. Genetic interactions between *daw*, *babo* and *put*

Genotype	Stall 6/7	Stall 13	Stall 12	Total	*n*
Oregon R	1	1	2	4	180
daw^{3}/+	0	2	0	2	162
daw^{3}/daw^{3}	6	12	6	24	169
put^{88}/+	0	0	0	0	226
daw^{3}/+; put^{88}/+	0	6	8	14	216
$babo^{32}$/+	0	1	3	4	278
daw^{3}/+; $babo^{32}$/+	0	14	6	20	174

Shown are the % of hemisegments with ISNb defects; for a description of classes of defects see legend to Fig. 3.

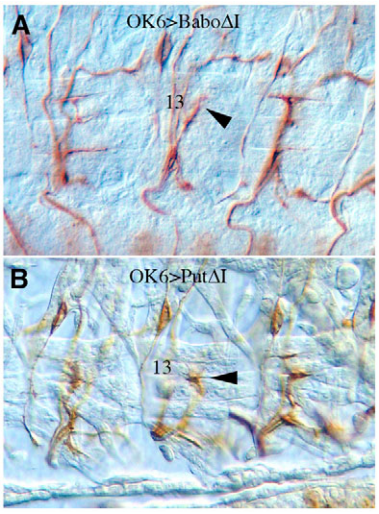

Fig. 6. Disruption of Babo and Put signaling induces pathfinding defects. UAS-*baboΔI*, UAS-*putΔI* or UAS-*tkvΔI* were expressed using different Gal4 drivers at 29°C. Motoneuron projections in late stage 16/17 filleted embryos stained with 1D4. OK6-Gal4 driven expression of BaboΔI (**A**) and PutΔI (**B**) results in stalling of ISNb axons and thickening of axon bundles (arrowheads).

Table 3. ISNb and SNa defects caused by expression of dominant-negative receptors

Genotype	% Hemisegments with ISNb defects					% Hemisegments with SNa defects			
	Stall 6/7	Stall 13	Stall 12	Total	*n*	Short	Bifurcation	Total	*n*
Oregon R	1	1	2	4	180	0	2	2	180
UAS-*baboΔI*	0	3	3	6	108	0	3	3	108
OK6>BaboΔI	0	10	25	35	80	1	4	5	77
OK6>BaboΔI x4	0	17	19	36	162	4	18	22	134
Elav>BaboΔI x4	1	18	15	34	107	3	17	20	107
dMef2>BaboΔI	0	0	7	7	71	0	0	0	88
Repo>BaboΔI	0	0	4	4	102	0	2	2	104
OK6>PutΔI	1	18	14	33	122	1	2	3	120
OK6>TkvΔI	1	1	2	4	141	0	1	1	140

For a description of classes of defects see legend to Fig. 3.

babo-a and babo-b isoforms, which encode different extracellular domains and potentially display altered ligand specificity/affinity (Wrana et al., 1994), gave comparable results suggesting that Daw binds to both receptor subtypes. By contrast, expression of UAS-*baboΔI* in muscles resulted in just 7% of hemisegments showing the weakest class of ISNb defects (loss of synapse on muscle 12), and no defects in SNa branching. When Babo function was disrupted in glia, only 4% of hemisegments showed ISNb defects and 2% displayed loss of one SNa branch. Thus, loss of Daw signaling in glia and muscles resulted in defects comparable to wild-type and control UAS-*baboΔI* embryos (Table 3). A neuron-specific requirement for reception of Daw signal was further reinforced in experiments using the dominant-negative Put receptor. In animals carrying UAS-*putΔI* and OK6-Gal4, 33% of hemisegments showed disruption of ISNb pathfinding (Fig. 6B, Table 3), recapitulating the defects encountered in *daw*, *put* and *babo* mutants. We observed only a 3% incidence of pathfinding errors in SNa axons, reflecting their reduced sensitivity. Taken together, these results suggest that Babo and Put function is required in the motoneurons.

To establish the specificity of the phenotypes resulting from expression of BaboΔI and PutΔI, we assayed whether a dominant-negative BMP-receptor TkvΔI had similar consequences. Expression of a single copy of UAS-*tkvΔI* in the wing disc disrupts wing venation and growth, consistent with loss of BMP signaling (Haerry et al., 1998). By contrast, expression of UAS-*tkvΔI* in motoneurons did not result in significant pathfinding defects (4% in ISNb, 1% in SNa, Table 3). Increasing the copy number of UAS-*tkvΔI* did not increase the penetrance, suggesting that the phenotypes caused by expression of BaboΔI and PutΔI are specific and represent disruption of an endogenous activin signaling pathway in motoneurons.

Restricted transcription of *daw* is not important for its function

In order to determine which site of Daw transcription (muscle or glia) is important for axon guidance, we assayed the ability of tissue-restricted Daw expression to rescue ISNb defects in *daw*$^{-}$ embryos. We found that driving one copy of UAS-*daw* in muscles using dMef2-Gal4, or in glia with Repo-Gal4, decreased the incidence of ISNb defects in *daw*$^{\Delta 1}$ homozygous embryos, whereas two copies of UAS-*daw* provided almost complete rescue (89% and 95%, respectively), and reduced the incidence of defects to wild-type levels (Table 4). Given that *daw* encodes a secreted ligand, these data suggest that restricted transcription of *daw* is not crucial for its function. Ectopic expression of a single copy of UAS-*daw* in motoneurons also reversed the defects encountered in mutant embryos (Table 4). Interestingly, excess or ectopic Daw did not perturb motoneuron pathfinding in any way. These results support a model in which *daw* provides a permissive signal whose effect is restricted by other guidance cues. Alternatively, its function could be restricted to a subset of motoneurons through the action of other molecules that regulate Daw activity.

DISCUSSION

We have shown that Daw, an activin-related ligand, functions as a regulator of embryonic motoneuron pathfinding. In *daw* mutants, ISNb and SNa pathway axons arrest prematurely and fail to innervate their targets. Mutants for *put*, *babo* and *Smad2* recapitulate this phenotype providing the first demonstration that a canonical activin signaling pathway can be utilized for axon guidance. Our data suggest that glia- and muscle-derived Daw functions as a paracrine signal to influence the Daw-receptor Babo in motoneurons. Ectopic *daw* expression has no deleterious effects, although it rescues guidance defects in the mutant. Thus, Daw does not act as a spatially restricted signal, and instead is likely to function in a permissive manner to enable or modulate the response of the growth cone to other guidance cues.

An activin signaling pathway is involved in axon guidance in *Drosophila*

Cell signaling assays and phenotypic analyses indicate that Daw affects motoneuron pathfinding by acting through Put, Babo and Smad2. Supporting this idea, the incidence of ISNb

Table 4. Rescue of *daw*$^{-}$ pathfinding defects by ectopic expression of ligand

Genotype	Stall 6/7	Stall 13	Stall 12	Total	% Rescue	*n*
Oregon R	1	1	2	4	–	180
daw$^{\Delta 1}$/*daw*$^{\Delta 1}$	1	21	17	39	–	161
daw^{3}/*daw*$^{\Delta 1}$ UAS-*daw*	0	26	16	42	–	107
daw^{3}/*daw*$^{\Delta 1}$ OK6>Daw	0	8	7	15	62	180
daw^{3}/*daw*$^{\Delta 1}$ dMef>Daw	0	6	3	9	77	145
daw^{3}/*daw*$^{\Delta 1}$ dMef>Daw x2	0	3	1	4	89	360
daw^{3}/*daw*$^{\Delta 1}$ Repo>Daw	0	6	8	14	64	168
daw^{3}/*daw*$^{\Delta 1}$ Repo>Daw x2	0	1	1	2	95	156

Shown are the % of hemisegments with ISNb defects; for a description of classes of defects see legend to Fig. 3.

pathfinding defects increases when animals with a single copy of the receptors Put and Babo are further depleted of Daw ligand (Table 2). Mutations in Daw and its receptors result in a similar range and penetrance of phenotypes, arguing that Daw is the primary contributor to activin signaling in motoneuron pathfinding and that the canonical pathway can fully account for the ability of Daw to influence axon guidance. The slightly higher penetrance of ISNb defects in *babo* as compared with *daw* maternal/zygotic nulls (59% versus 50%, Fig. 5C), raises the possibility that an additional ligand could contribute to embryonic motor axon guidance. Both Activin and Myg can bind Babo (Lee-Hoeflich et al., 2005; Zheng et al., 2003), and are expressed in neural or muscle cells compatible with such a role (Lo and Frasch, 1999). Intriguingly, overexpression of Activin (and to a lesser extent Myg) can partially rescue daw^{-} pathfinding defects (L.P. and K.A., unpublished). However, an assessment of their roles in axon pathfinding must await the recovery of mutations in these genes. Furthermore, *daw* may have other functions in addition to embryonic pathfinding. A majority of *daw* mutants die during pupal stages despite the fact that pathfinding defects are largely corrected by the third larval instar (L.P. and K.A., unpublished).

Daw is likely to function by signaling to motoneurons

Daw could act as a paracrine signal from the muscle or glia to influence motoneurons. Alternatively, it could provide an autocrine signal that supports glial or muscle growth/function and affects axon outgrowth indirectly. Our data show that cell-autonomous disruption of activin signaling in muscles or glia does not disrupt motoneuron pathfinding, ruling out an autocrine mechanism. By contrast, expression of BaboΔI and PutΔI receptors in motoneurons effectively phenocopies daw^{-} (Fig. 6, Table 3), suggesting that axon guidance defects could arise from the inability of motoneurons to respond to a paracrine Daw signal. Interestingly, the retrograde Gbb/BMP signal transduced by Wit/Tkv and Mad that regulates synapse morphology and function in larval motoneurons (Aberle et al., 2002; Marques et al., 2002), shows minimal crosstalk despite acting in the same tissue. Disruption of BMP signaling, by expression of TkvΔI in motoneurons (Fig. 5C) or mutations in *wit* (Marques et al., 2002), does not affect axon guidance although it affects neuromuscular junction (NMJ) function.

Daw functions as a permissive signal to modulate response to other guidance cues

An important question is whether Daw functions as an instructive cue that provides directional information, or as a permissive factor that promotes axon outgrowth. Several lines of evidence argue against an instructive role. First, axon pathfinding does not require restricted expression of *daw*. Guidance defects associated with *daw* mutants can be rescued by *daw* expression in sites of endogenous transcription, and ectopically in motoneurons (Table 4). Second, in *daw* mutants, axons do not extend into inappropriate areas or show ectopic branching, phenotypes typical of mutations in *Sema-2a*, *Netrin-A* and *Netrin-B* that provide spatial guidance cues or target recognition (Harris et al., 1996; Mitchell et al., 1996; Winberg et al., 1998a). Finally, misexpression of Daw did not cause mistargeting of axons, indicating no apparent spatial sensitivity to Daw. Our data are therefore consistent with a permissive role in which Daw enables and/or modulates the response of the growth cone to other restricted cues.

Potential targets of Daw signaling

Both *daw* and *Smad2* mutants display similar errors in pathfinding, suggesting that *daw* acts at the transcriptional level by altering the expression of one or more molecules that regulate growth cone response or motility. We found no evidence for cell fate changes in the embryonic nervous system of *daw* mutants; and pathfinding defects can be rescued using OK6-Gal4 that initiates expression in motoneurons at stage 15 (L.P. and K.A., unpublished), well after neuronal cell fates are specified (Table 4). Furthermore, we find no guidance errors in *daw* third-instar larvae. These results argue that motoneurons are correctly specified and that the embryonic pathfinding errors are compensated by larval stages.

Daw signaling could act on a wide range of transcriptional targets in the neuron. Mutations in several genes that function as guidance cues, such as *plexin A*, *Sema-1a* and *side*, show ISNb and SNa phenotypes similar to *daw* mutants. Thus *daw* could alter the activity of or the response to these guidance cues. Plexin A and Sema-1a are expressed in neurons and mediate local repulsion (Winberg et al., 1998b). Side, a muscle-derived attractant is unlikely to be directly regulated (de Jong et al., 2005; Sink et al., 2001), however components involved in the response to *side* could be downstream of *daw*. Interestingly, overexpression of the IgCAM Fas2 that promotes axon fasciculation also results in stalling defects reminiscent of the daw phenotype (Lin and Goodman, 1994). Thus, Daw could act on Fas2 or Beat-Ia, which potentially downregulates Fas2 in motoneurons (Fambrough and Goodman, 1996), to decrease adhesiveness at specific choice points in response to cues directing defasciculation. Other possible targets are the RPTPs that affect fasciculation and outgrowth. Whereas the *Lar*-null phenotype is significantly stronger than that of *daw*, the combinatorial loss of RPTP10D, RPTP69D and RPTP99A mimics the loss of Daw activity in ISNb and SNa pathways (Desai et al., 1996; Desai et al., 1997; Krueger et al., 1996; Sun et al., 2001). Finally, *daw* mutants show phenotypic overlap with mutations in the actin-microtubule-crosslinking proteins Pod1 and Shot, and in the actin-binding protein Profilin, which are required for axon outgrowth (Huber et al., 2003; Lee et al., 2000; Rothenberg et al., 2003; Wills et al., 1999). This raises the possibility that Daw signaling could control the expression or activity of genes involved in regulating cytoskeletal dynamics. Future epistasis studies will resolve whether Daw acts in conjunction with, or parallel to, known pathways that regulate axon fasciculation and extension.

The consequences of mutations in *daw* are often less severe and limited to a subset of axon pathways affected by the genes discussed above, suggesting that Daw could in part act redundantly with other proteins. Alternatively, *daw* activity could be spatially restricted to select axon pathways by localized expression of receptors, co-receptors or other pathway components. For example, in the *Drosophila* CNS, only axons expressing the Derailed receptor are sensitive to the Wnt5 repulsive cue, and are hence directed away from the posterior into the anterior commissure (Yoshikawa et al., 2003). Alternatively, receptor expression could be dynamically modulated, as seen in the downregulation of Robo in commissural neurons by transient expression of Commissureless, which results in local insensitivity to Slit at the midline (Keleman et al., 2002). Although mRNA for the Daw-receptors Babo and Put can be detected throughout the VNC (Fig. 5A,B) (Childs et al., 1993), it remains to be seen whether the proteins are enriched in a subset of growth cones, restricting the response to Daw. A further possibility is that the activity of the ligand itself could be spatially regulated. A recent study has shown that mutations in *tolloid-related* (*tlr*; *tolkin*

– Flybase) that encodes a metalloprotease of the BMP1/Tolloid family (Nguyen et al., 1994), display persistent pathfinding defects (Meyer and Aberle, 2006). In an accompanying manuscript (Serpe and O'Connor, 2006), *tlr* is shown to be required for activation of a latent Daw complex. Intriguingly, we find that *daw/+; tlr/+* embryos show pathfinding defects consistent with a functional link between the two genes (L.P. and K.A., unpublished). However, localized activation of Daw by Tlr appears unlikely because Tlr is present in the hemolymph and circulates throughout the embryo. Finally, Daw interaction with the extracellular matrix or HSPGs could result in localized presentation of the ligand to the extending growth cone. HSPGs are known to modulate BMP activity by affecting ligand stability and receptor interaction (Hacker et al., 2005). An intriguing possibility is that in addition to functioning as ligands for Lar (Fox and Zinn, 2005; Johnson et al., 2006), HSPGs could also function in axon guidance by enhancing Daw signaling.

TGF-β signaling and axonal pathfinding

This study provides the first evidence that an activin pathway, acting through its transcription factor Smad2, can direct axon pathfinding. The mechanism by which Daw functions stands in contrast to previous studies implicating BMP/TGF-β ligands in direct regulation of growth cone motility independent of a nuclear response. In vertebrates, BMP7/GDF7 heterodimers secreted by the spinal cord roof plate mediate repulsion of commissural axons away from the dorsal midline (Butler and Dodd, 2003). Exposure to BMP7 for as little as 30 minutes resulted in growth cone collapse in cultured neurons. BMP7 can also stimulate formation of dendritic arbors by directly regulating the cytoskeleton. This Smad-independent effect requires interaction of the BMP type-II receptor with LIMK1 that regulates the actin-depolymerising factor cofilin (Foletta et al., 2003; Lee-Hoeflich et al., 2004), and suggests a potential mechanism by which BMPs may influence growth cone motility as well. In *Drosophila*, a BMP/Wit pathway acting through LIMK1 promotes synapse stabilization at the NMJ although this is independent of ADF/Cofilin, suggesting that at least part of this mechanism is conserved (Eaton and Davis, 2005).

In *C. elegans*, the TGF-β family member UNC-129 functions as a target-derived chemoattractant for dorsally projecting motor axons (Colavita et al., 1998). UNC-129 is also likely to exploit an unconventional mechanism to direct motoneuron guidance, as mutations in the single type-II receptor DAF-4 or the Smad signal-transducer do not cause pathfinding defects. Thus TGF-β family members may utilize both canonical (as seen for Daw) and non-canonical strategies to regulate neuronal guidance, depending on context. It remains to be determined if an activin signaling pathway, comparable to Daw, plays a role in vertebrate axon guidance. The finding that axons from ventrally-derived retinal ganglion cells fail to enter the optic nerve head in *Bmpr1b*-deficient mice implicates a conventional BMP signal-transduction pathway in vertebrate growth cone guidance (Liu et al., 2003). Conversely, the recent finding that LIMK1 and cofilin have been implicated in axon outgrowth in mushroom body neurons (Ng and Luo, 2004), raises the possibility that an activin/BMP ligand acting through Wit could initiate this process in the larval brain.

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