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The role of TGFβ signaling in the formation of the dorsal nervous system is conserved between *Drosophila* and chordates

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SUMMARY

Transforming growth factor β signaling mediated by Decapentaplegic and Screw is known to be involved in defining the border of the ventral neurogenic region in the fruitfly. A second phase of Decapentaplegic signaling occurs in a broad dorsal ectodermal region. Here, we show that the dorsolateral peripheral nervous system forms within the region where this second phase of signaling occurs. Decapentaplegic activity is required for development of many of the dorsal and lateral peripheral nervous system neurons. Double mutant analysis of the Decapentaplegic signaling mediator Schnurri and the inhibitor Brinker indicates that formation of these neurons requires Decapentaplegic signaling, and their absence in the mutant is mediated by a counteracting repression by Brinker. Interestingly, the ventral peripheral neurons that form outside the Decapentaplegic signaling domain depend on Brinker to develop. The role of Decapentaplegic signaling on dorsal and lateral peripheral neurons is strikingly similar to the known role of Transforming growth factor β signaling in specifying dorsal cell fates of the lateral (later dorsal) nervous system in chordates (*Halocythia*, zebrafish, *Xenopus*, chicken and mouse). It points to an evolutionarily conserved mechanism specifying dorsal cell fates in the nervous system of both protostomes and deuterostomes.

Key words: *Drosophila*, Decapentaplegic, Peripheral nervous system, BMP, TGFβ, Schnurri, Brinker, Evolution

INTRODUCTION

The transforming growth factor β (TGFβ) super-family of proteins is involved in many developmental processes in higher eukaryotes (reviewed by Affolter et al., 2001; Massagué and Chen, 2000; Newfeld et al., 1999). The dynamics among TGFβ family members, their receptors and their inhibitors control cellular processes through the transcriptional regulation of target genes. In vertebrates, TGFβ homologs act during the development of the nervous system in two different steps, initially defining the border of the embryonic neurogenic anlage (neural plate) and later engaging in detailed pattern formation by specifying within the anlage the dorsal neural structures (reviewed by Lee and Jessell, 1999; Streit and Stern, 1999).

Early in *Xenopus* development, the TGFβ molecule bone morphogenetic protein 4 [BMP4, which belongs to the BMP2/4/Decapentaplegic (Dpp) subfamily] defines the non-neurogenic region of the embryo, while its inhibitor Chordin (Chd) defines the neurogenic anlage. Similarly, in zebrafish the specification of non-neurogenic and neurogenic regions appears to be governed by BMP2 and CHD, respectively (Hammerschmidt et al., 1996; Holley and Ferguson, 1997; Kishimoto et al., 1997; Mullins et al., 1996; Schulte-Merker et al., 1997).

Later in vertebrate development, BMP/TGFβ signaling is important for specification of dorsal cell fates in the nervous system. It is involved in the formation of the roof plate, neural crest and dorsal sensory interneurons of the neural tube (Lee and Jessell, 1999). In chicken explant assays, the epidermal ectoderm that harbors BMPs, or the BMPs themselves (BMP4 and BMP7), induce neural plate tissue to express dorsal neural markers for the roof plate, neural crest and dorsal interneurons (D1 and D2), while secreted antagonists (Noggin + Follistatin) can inhibit expression of these markers (Dickinson et al., 1995; Liem et al., 1997; Liem et al., 1995). Genetic experiments in zebrafish support the role of BMP signaling in pattern formation within the neural tube. Analysis of *swirl/bmp2b*, *snailhouse/bmp7* and *somatibun/smad5* mutants, as well as injection of the BMP antagonist CHD, reveal that BMP signaling is essential for establishing neural crest, dorsal sensory neurons (Rohon Beard neurons) and interneurons of the neural tube (Barth et al., 1999; Nguyen et al., 2000). In mouse, a number of candidate TGFβ molecules are expressed in the dorsal neural tube and the overlying ectoderm (Lee and Jessell, 1999; Liem et al., 1997). These include BMP4 of the BMP2/4/Dpp subfamily; BMP5 and BMP7 of the BMP5-8/60A subfamily; Activin B, which is closely related to *Drosophila* DmActivin; and growth/differentiation factor 7 (GDF7), which is equally related to *Drosophila* Dpp, 60A and Screw (Scw) (Newfeld et al., 1999). After neurulation, the

dorsal epidermal ectoderm, which later separates from the neural tube, and the roof plate are sources of these TGFβ molecules. Ablation of the roof plate produces defects in dorsal neural fate specification very similar to defects generated by blocking TGFβ/BMP signaling (Liem et al., 1997), strongly suggesting that TGFβ signals emanating from the roof plate specify dorsal neural cell types (Lee et al., 2000; Millonig et al., 2000). Consistent with this, mice that lack GDF7, which is expressed in the roof plate, lack specific dorsal interneurons (D1A), directly showing a role for this TGFβ in the generation of dorsal neural cell types (Lee et al., 1998). However, this mutation does not reflect all the aspects of TGFβ signaling shown in other experiments in which TGFβ inhibitors have been added or roofplate has been deleted. It is very likely that GDF7 and other BMP family members expressed by the roofplate hold non-redundant functions (Lee et al., 1998). Finally, in the ascidian *Halocynthia roretzi* BMP signaling affects neural plate patterning. At the time of sensory organ fate decision (tailbud stage) BMPb (belonging to the BMP2/4/Dpp subfamily of BMPs) is expressed in the dorsal neural plate (Miya et al., 1997). Overexpression of BMPb and Chd affect the choice of cell fates between sensory structures forming at the edge of the neural plate, consistent with a role for BMP signaling in specification of cell fates in the dorsal nervous system (Darras and Nishida, 2001).

During the development of the nervous system in the *Drosophila* embryo, TGFβ signaling is biphasic (Dorfman and Shilo, 2001). In the first phase, the two BMP class molecules Dpp and Scw and the secreted inhibitor Short gastrulation (Sog) have an initial role strikingly similar to the role of homologous proteins in zebrafish and *Xenopus* (de Robertis and Sasai, 1996; Holley and Ferguson, 1997). Through broad dorsoventral patterning of the blastoderm, they define the borders of the ventral neurogenic region (VNE) (Arora et al., 1994; Biehs et al., 1996; Neul and Ferguson, 1998; Nguyen et al., 1998; Podos and Ferguson, 1999). The VNE gives rise to the ventral nerve cord (VNC) and some ventral peripheral nervous system (PNS) neurons (Campos-Ortega, 1993; Schmid et al., 1999; Schmidt et al., 1997). Later in development, localized Dpp signaling appears in the dorsal ectoderm where the dorsolateral PNS is forming (Dorfman and Shilo, 2001; Jackson and Hoffmann, 1994), raising the possibility of a role for Dpp in specification of dorsal cell fates in PNS development.

We have explored the possibility of an evolutionarily conserved role of TGFβ family members in specification of the dorsal nervous system by investigating the function of this second phase of Dpp signaling for PNS development in *Drosophila*. We report that this signaling indeed promotes formation of the dorsal and lateral PNS neurons, apparently mediated by activation of proneural gene expression. The inhibitor Brinker (Brk) comes into play when Dpp signaling is compromised and is in addition involved in the formation of the ventral PNS neurons. These results indicate that TGFβ signaling is important for detailed patterning of the dorsal nervous system in protostomes as well as in deuterostomes.

MATERIALS AND METHODS

Drosophila melanogaster strains

Flies were raised on standard *Drosophila* medium at 25°C. The P-element-induced allele shn^{k00401} and the strong hypomorphic EMS-induced allele shn^{1} created by a premature stop codon (Arora et al., 1995) were obtained from the Bloomington Stock Center. The P-element induced allele shn^{k04412} was kindly provided by H. Bellen. The brk^{m68} loss of function allele was kindly provided by J. Urban (Jazwinska et al., 1999a). The UAS-*brk* was described previously (Jazwinska et al., 1999a). The 4×HS-*ssog* transformed flies were kindly provided by E. Bier (Yu et al., 2000). To activate miss-expression of genes in the neuroectoderm, as well as in early SOPs, we used the previously reported *Kr*-Gal4 line (Castelli-Gair et al., 1994). For analysis of double mutant embryos, brk^{m68};shn^{k04412} and brk^{m68};shn^{1}, we generated the stocks brk^{m68}/*FM7*, *grh-lacZ*;shn^{k04412}/*CyO*, *wg-lacZ*, brk^{m68}/*FM7*, *grh-lacZ*;shn^{1}/*CyO* and *wg-lacZ*. Double mutants were identified by the lack of β-galactosidase expression. Information about strains not described in the text and balancer chromosomes can be found elsewhere (Lindsley and Zimm, 1992).

Immunohistochemistry

Immunohistochemistry was performed in whole-mount embryos using the following primary antibodies: rabbit anti-β-galactosidase antiserum (1:1000 dilution; Cappel); anti-Ato rabbit antiserum (1:1000 dilution) (Jarman et al., 1994); anti-p-Mad rabbit antisera (1:50 dilution) (Tanimoto et al., 2000); and anti-Ac monoclonal antibodies (1:10 dilution) (Skeath et al., 1992); and 22C10 monoclonal antibody (1:20 dilution) (Fujita et al., 1982) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Fluorescent Cy2- and Cy3-conjugated secondary antibodies (Jackson Immunoresearch), and Alexa488-conjugated secondary antibodies (Molecular Probes) were used at 1:1000 dilution. HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (Promega) were used at 1:250 dilution. AP-conjugated goat-anti rabbit antibodies were used at 1:2000 dilution.

Embryos were staged according to Campos Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997), and were fixed and processed for whole-mount antibody staining using standard techniques (Patel, 1994). Stained embryos were cleared in 80% glycerol, mounted and examined on a Zeiss Axiophot. Alternatively, fluorescent embryos were analyzed by confocal microscopy using a Zeiss LSM 510 microscope.

Heatshock experiments

Embryos were collected on apple-juice agar plates for 1 hour at 25°C. They were allowed to age for 3 hours at 25°C before administering 1 hour heat shock at 37°C by immersion of the agar plates in a water bath. Embryos were allowed to recover at room temperature for 30 minutes and thereafter kept at 25°C until the correct stage was achieved before fixation and phenotypic analysis.

Cell death staining

Embryos were stained with Acridine Orange to reveal apoptotic cells according to the previously described protocol (Abrams et al., 1993).

RESULTS

Part of the PNS develops within the region of active Dpp signaling in the dorsal ectoderm

The second phase of Dpp signaling, covering most if not all the dorsal ectoderm, starts at stage 9 and lasts until stage 10/11 [3.40 to 5.20 hours after egg laying (AEL)] (Dorfman and Shilo, 2001). Initially proneural clusters (PNCs) and later sensory organ precursors (SOPs), singled out within each PNCs, can be visualized by the expression of the proneural genes *achaete* (*ac*, 4.20-7.20 hours AEL), *atonal* (*ato*, 5-6.30 hours AEL) and *amos* (*amo*, 5.20-6 hours AEL) (Campos-Ortega and Hartenstein, 1997; Huang et al., 2000; Lage et al.,

1997; Ruiz-Gomez and Ghysen, 1993; Younossi-Hartenstein and Hartenstein, 1997). Thus, the second wave of Dpp signaling precedes and overlaps with the development of the PNCs and SOPs. We examined the domain of Dpp signaling using an enhancer trap *lacZ* line inserted in the gene *daughters against dpp* (*dad*), a target of Dpp (Tsuneizumi et al., 1997). Double immunofluorescence staining shows that dorsally located Ac and Ato positive PNCs and SOPs originate inside the *dad-lacZ* positive region (Fig. 1), suggesting that they have received, or still receive, Dpp signaling. A subset of PNCs and SOPs are, however, ventral to the *dad-lacZ* domain (Fig. 1). As the PNS neuronal precursors differentiate close to the position where they originate, we can conclude that a part of the dorsal PNS forms within an active Dpp signaling region.

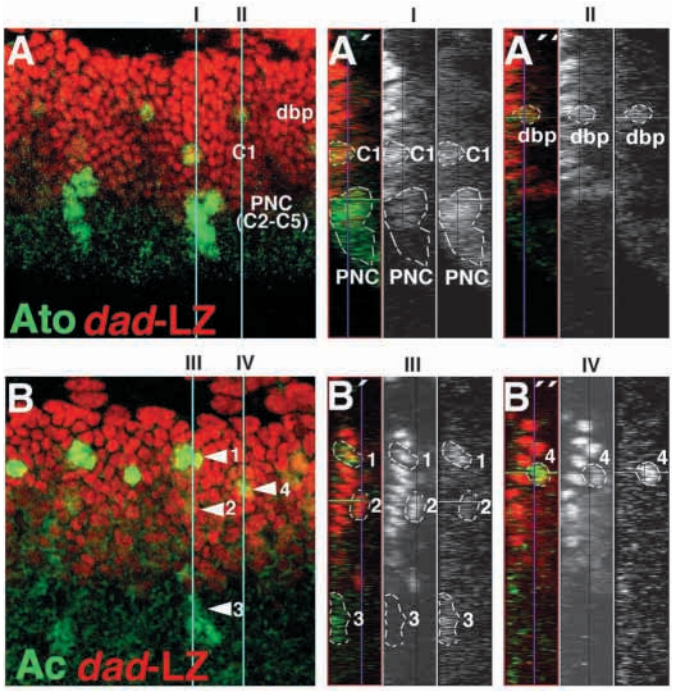

Fig. 1. Dorsal sensory neurons develop within the Dpp signaling region in the dorsal ectoderm. (A,B) Merged confocal images of PNCs (cell clusters) and SOPs (single cells) visualized by immunostaining using anti-Ato (green in A) or anti-Ac (green in B) antibodies in two consecutive abdominal segments of staged 11 embryos. Anterior is towards the left and dorsal is upwards. The Dpp signal receiving cells in a *dad-lac*Z enhancer trap line are visualized by anti-β-galactosidase antibodies (red). Orthogonal sections are indicated by the blue lines (I and II in A, and III and IV in B) and shown in A′, A″ and B′, B″, respectively. Merged images are shown in color; black and white images represent the separate red (middle) and green (right) channels. In A, the C1 SOP, which contributes to one of the chordotonal organs, is marked by a stippled line (A′) and originates within the *dad-lac*Z domain (red). The SOP giving rise to the dorso-bipolar neuron (dbp) is in the process of delamination (A″) and also originates within the *dad-lac*Z domain. The more ventrally located PNC cluster, from which the C2-C5 chordotonal SOPs will be singled out, is only partially within the *dad-lac*Z domain. In B, the Ac-positive PNCs 1 and 3 originate within and outside the *dad-lac*Z domain, respectively (B′). The delaminating SOPs 2 and 4 form within the *dad*-lacZ domain (B′,B″).

The second wave of embryonic Dpp signaling is necessary for the correct formation of the dorsal and lateral PNS

The embryonic abdominal (A) PNS of *Drosophila* consists of three bilateral clusters of neurons (ventral, lateral and dorsal) per segment, which can be most especially appreciated in the serially homologous segments A1-A7 (Fig. 2) (Ghysen et al., 1986). In order to investigate whether the second phase of Dpp signaling is necessary for patterning the PNS, we analyzed mutant alleles for a gene involved in the Dpp signaling pathway, *schnurri* (*shn*). This gene encodes a zinc-finger transcription factor that is necessary for the transcription of some Dpp target genes and binds directly to the main Dpp mediator Mothers against Dpp (Mad) (Arora et al., 1995; Dai et al., 2000; Grieder et al., 1995; Staehling-Hampton et al., 1995; Udagawa et al., 2000). Unlike the zygotic mutants of *dpp*, *scw*, *tolloid* (*tld)* or *mad*, *shn* mutants have no effect on the initial *dpp/scw* governed dorsoventral patterning of the blastoderm. They express normally the early Dpp target genes, such as *pannier* (*pnr*, stage 7), *dpp* itself in the dorsal ectoderm (stage 9) and *Krüppel* (*Kr*) (which is a marker for the amnioserosa), showing that the dorsal ectoderm is correctly specified (Arora et al., 1995; Grieder et al., 1995; Torres-Vazquez et al., 2001). By contrast, several Dpp target genes that are expressed following the second phase of Dpp signaling are affected in *shn* zygotic mutants: at stage 11, the expression of genes responsive to Dpp signaling, such as *dad*, *pnr*, *spalt* or *dpp* itself is reduced or lost (Grieder et al., 1995; Marty et al., 2000; Torres-Vazquez et al., 2001; Tsuneizumi et al., 1997). Thus, any failures in PNS formation, which are observed in *shn* mutant embryos, must originate from the second rather than the first phase of Dpp signaling and are likely to be mediated by Shn. We searched for PNS malformations in strong *shn* zygotic mutant embryos using the ubiquitous PNS neuronal marker 22C10 (Fujita et al., 1982). Homozygous shn^{1} and shn^{k00401} fail to undergo dorsal closure and show severe defects of PNS development (Fig. 2B and data not shown). A strong reduction in number of neurons is observed, especially in the dorsal and lateral PNS clusters, although it is difficult to determine exactly which neurons are affected because of the dorsal closure failure. Therefore, we also analyzed another allele, shn^{k04412}, which does undergo dorsal closure (Kania et al., 1995). In these embryos, position and identity of PNS neurons could be more clearly assigned. In homozygosity (Fig. 2C,E), as well as in transheterozygosity over shn^{1}, this mutant shows a reduction in the number of dorsal and lateral neurons, similar to the other mutants analyzed. These results are consistent with a role for Shn-mediated Dpp signaling in the formation of the dorsal and lateral PNS.

A different way to interfere with the second phase of Dpp signaling is to express specific inhibitors once the initial dorsoventral patterning is accomplished. Brk is a nuclear protein that negatively regulates Dpp-induced genes and is expressed ventrally in a complementary pattern to Dpp in the embryo (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Jazwinska et al., 1999b; Minami et al., 1999). Sog is a secreted protein that can bind to Dpp and inhibit it from signaling, and Supersog (Ssog) is a hyperactive inhibitory fragment of Sog (Yu et al., 2000). In order to avoid interference with the first wave of Dpp signaling (stage 5 to 7, 2.10-3.10 hours AEL), we misexpressed *brk* and *ssog* from stage 8 (3.10 hours AEL) to interfere with the second phase (stage 9 to

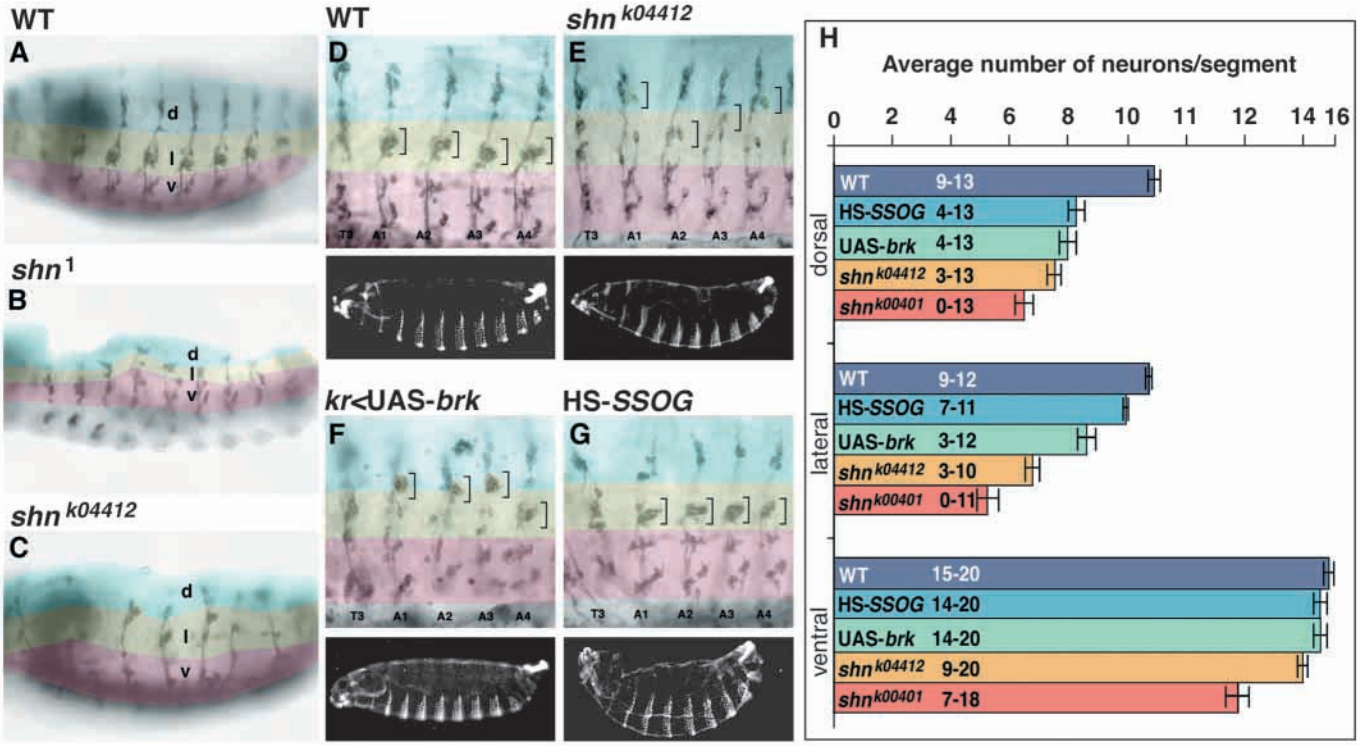

Fig. 2. The second phase of Dpp signaling is required for normal PNS formation. For clarity, in A-G the dorsal (d), lateral (l) and ventral (v) regions encompassing PNS clusters are colored blue, yellow and pink, respectively. (A-C) Whole-mount stage 16 embryos stained with the 22C10 antibody that reveals PNS neurons. (A) Wild type (WT) embryo. (B,C) Homozygous *shn*^{1} (B) and *shn*^{k04412} (C) mutants have fewer neurons and present serious disorganization in the dorsal and lateral PNS clusters. *shn*^{1} mutants fail to undergo dorsal closure. As a result of this failure, the gut is extruded through the top, the embryo is twisted and the ventral nerve cord is pulled inwards. Neurons belonging to the other side of the embryo are visible most ventrally. (D-G) Flat preparations showing one thoracic (T3) and four abdominal (A1-A4) segments (upper panels). Cuticle preparations of late stage embryos (lower panels) show no clear expansion of the ventral denticles in any genotype. Fewer neurons can be observed in *shn*^{k04412} (E), *Kr*-Gal4;UAS-*brk* (F) and HS-*ssog* (G) embryos, especially in the dorsal and lateral clusters. In alleles of *shn* and overexpression of *brk*, the pentascolopodial organ (brackets and yellow in D-G) frequently has fewer neurons and is positioned close to the dorsal cluster. (H) Mean values, standard error (bars) and range (numbers to the right of the genotype) of the number of neurons per dorsal, lateral and ventral clusters in abdominal segments (A1-A7) in different genetic backgrounds. Abdominal segments A1-A7 on both sides of 10 embryos were quantified for each genotype. The inhibition of Dpp signaling results in the reduction in number of neurons, principally in the dorsal and lateral PNS clusters. WT, *n*=63; *shn*^{k04412}, *n*=116; *shn*^{k00401}, *n*=90; *Kr*-Gal4;UAS-*brk*, *n*=53; HS-*ssog*, *n*=182.

10/11, 3.40-5.20 hours AEL). No expansion of ventral denticles was observed confirming the absence of interference with the early TGFβ-driven dorsoventral patterning. UAS-*brk* expression in segments T2-A3, which is driven by the *Kr*-Gal4 driver, and ubiquitous expression of *ssog* in the entire embryo, produced using a HS-*ssog* construct, lead to reduced number of neurons in the dorsal and lateral PNS (Fig. 2F-H). The effects are less severe for Ssog misexpression than for UAS-*brk* misexpression and notable for *shn* mutations (Fig. 2). Approximately 20% of the embryos expressing ubiquitous *ssog* do not undergo dorsal closure, similar to the phenotype observed when strong alleles of *shn* are analyzed. The HS-*ssog* produces a manifest decrease in phosphorylated-Mad (p-Mad) in the dorsal region (Fig. 3J,K) (Tanimoto et al., 2000). This indicates a reduction in Dpp signaling responsible for the phenotype. The residual p-Mad staining observed in some embryos (Fig. 3K) might be the reason why Ssog misexpression leads to less severe effects than UAS-*brk* misexpression or *shn* mutations.

In all the mutant backgrounds mentioned above, the dorsal and lateral PNS clusters show a severe reduction in the number of neurons (Fig. 2H). No major differences are found depending on the neuronal type: the percentage of external sensory organ neurons lost is similar to the loss of neurons in the chordotonal organs (Rusten et al., 2001). The penetrance of this effect, as measured in the differentiated PNS clusters, varies among abdominal segments (Fig. 2). The average reduction in neuronal number ranges from 25% (HS-*ssog*) to 41% (*shn*^{k00401}) in the dorsal cluster and 8% (HS-*ssog*) to 52% (*shn*^{k00401}) in the lateral cluster. By contrast, the ventral cluster is less affected because it shows 2% (HS-*ssog*) to 18% reduction (*shn*^{k00401}). The lateral pentascolopodial organ shows migration defects in these embryos (Rusten et al., 2001), but the other sensory organs are located in their expected relative positions.

The second wave of Dpp signaling is necessary for correct expression of proneural genes

The reduced number of neurons observed in the dorsal and lateral PNS when Dpp signaling is impeded could result from lack of proneural gene expression, which is known to be necessary for PNC and SOP formation (Jan and Jan, 1993). We

analyzed the expression of *ato* and *ac* to examine the specification of progenitor cell subclasses in mutant backgrounds defective for Dpp signaling. The development of the serially homologous abdominal segments A1 to A7 is similar and very synchronous. Thus, in the wild type, whenever a specific number of PNCs and SOPs appear in one abdominal segment, a similar pattern is observed in the other abdominal segments as well (Fig. 3A,B). This is not true for *shn*^{k04412} mutants and for embryos expressing ubiquitous *ssog*, where the numbers of Ac and Ato positive SOPs and PNCs vary among the abdominal segments (Fig. 3D,F and Fig. 3C,E, respectively). This is consistent with the variably penetrant phenotypes observed in differentiated PNS among abdominal segments (Fig. 2). In embryos expressing *Kr*-Gal4;UAS-*brk*, we observed loss of Ato- and Ac-positive PNCs and SOPs specifically in the abdominal segments A1-A3 where *brk* was misexpressed, when compared with abdominal segments A4-A7 that served as an internal reference. The reduced numbers of Ato- and Ac-positive neuronal progenitors appear to result from failure of PNC formation rather than an increase in cell death ratio: apoptosis does not appear to increase in segments expressing *brk* compared with the other abdominal segments (Fig. 3I). Taken together, these results suggest that reduction in the number of neurons is produced by failure in proneural gene expression.

The absence of Brk counteracts the neuronal loss produced by compromised Dpp signaling

The transcriptional activation of target genes mediated by Dpp signaling is known to be regulated in different ways. While some target genes appear to be activated directly by Mad, Shn and the mediator Medea (Med), probably working as a complex, other target genes are regulated indirectly by relieving the repressive activity of Brk (Affolter et al., 2001). Dpp signaling and *brk* expression are mutually inhibitory. In particular, Dpp signaling represses *brk* expression through the action of Shn. In *shn* mutant embryos, *brk* expression expands dorsally during the germ band elongation stage, when the PNS forms (Marty et al., 2000; Torres-Vazquez et al., 2001). Conversely, in *brk* mutant embryos, *dpp* expression expands ventrally, as does expression of Dpp target genes like *pnr* and *dad* (Jazwinska et al., 1999b; Torres-Vazquez et al., 2001). The question arises as to whether the reduced number of neurons, in the experiments documented in Fig. 2, is a direct effect of the loss of Dpp signaling via Shn or, alternatively, is the result of the dorsally expanded Brk expression in *shn* mutant embryos. This question was addressed by comparing the numbers of neurons in wild type, single mutant and double mutant embryos (Fig. 4). The numbers of dorsal and lateral neurons are comparable in wild type and *brk* mutant embryos, suggesting that Brk is normally not involved in the formation of these neurons. By contrast, these numbers are substantially reduced in *shn* mutants, confirming that Dpp signaling is indeed involved in this process. Importantly, the number of neurons observed in *brk;shn* double mutant embryos is higher than in *shn* single mutants, approaching the number seen in the wild type. However, the recovery in *brk*^{m68}*;shn*^{1} embryos is incomplete, suggesting an additional role of Shn independent of Brk, as shown previously (Torres-Vazquez et al., 2001). These conclusions are consistent with a putative regulatory diagram presented on the left in Fig. 5A (see Discussion).

When the number of ventral (rather than dorsal or lateral) PNS neurons is analyzed in single or double mutants, the results are dramatically different (Fig. 4), revealing a different underlying genetic regulatory mechanism (Fig. 5A). In this case, *brk* mutants show a 30% reduction in neuronal numbers,

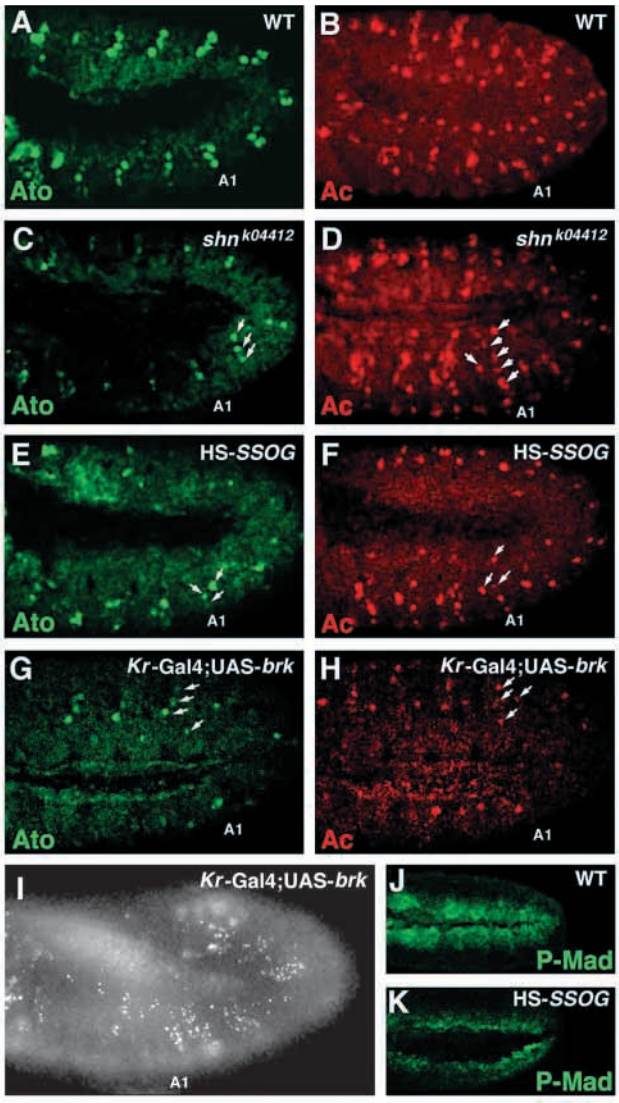

Fig. 3. Proneural gene expression is reduced when Dpp signaling is impeded. Expression of the proneural gene products, Ato (green) and Ac (red), in PNCs and SOPs at stage 11 (A-H). (A,B) The number and position of serially homologous PNCs and SOPs are similar in A1-A7 wild-type abdominal segments. (C-H) Proneural gene expression is lost in some segments in *shn*^{k04412} homozygous embryos (C,D), embryos expressing HS-*ssog* (E,F) and embryos overexpressing *brk* in segments A1-A3 (G,H). Arrows point to SOPs and PNCs that are not present in certain other segments.
(I) Visualization of apoptotic cell death with Acridine Orange reveals no preferential cell death in segments A1-A3 versus other abdominal segments in embryos overexpressing UAS-*brk* under *Kr*-Gal4.
(J,K) Active TGFβ signaling is visualized by anti-p-Mad antibody at stage 9 embryos (Tanimoto et al., 2000). While WT embryos show a broad expression domain of p-Mad (J), embryos where *ssog* is expressed under heatshock show a severe reduction in p-Mad levels and expression domain **(**K**).** The effect varies among embryos: some embryos have no p-Mad after the heat shock treatment.

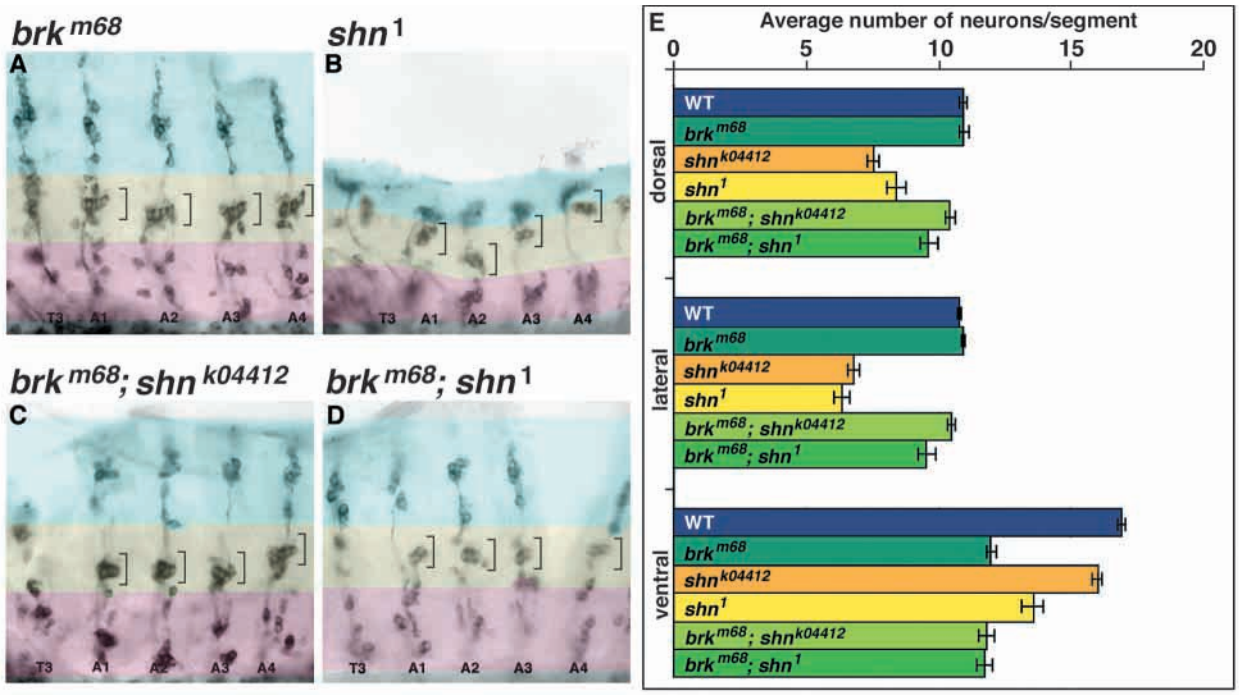

Fig. 4. Opposing genetic roles of *shn* and *brk* in formation of PNS neurons. (A-D) Flat preparations of stage 16 embryos showing one thoracic (T3) and four abdominal (A1-A4) segments with PNS neurons visualized with the 22C10 antibody. Regions that harbor neurons that belong to the dorsal, lateral or ventral clusters are color-coded blue, yellow and red, respectively. (A) brk^{m68} homozygous embryo with normal numbers of abdominal PNS neurons in dorsal and lateral clusters, but fewer in ventral clusters (compare with Fig. 2D). (B) Homozygous shn^{1} embryos display a dramatic reduction in neuronal numbers and disorganization in the dorsal and lateral clusters, but less so in ventral clusters (compare with Fig. 2B). The compact appearance of the PNS is due to a lack of dorsal closure (dorsal extension of the epidermis) in these animals. Double homozygous embryos for brk^{m68};shn^{k04412} (C) and brk^{m68};shn^{1} (D) display a clear rescue in neuronal numbers relative to the single *shn* mutant (A); this rescue can be observed in dorsal and lateral but not in ventral clusters. The correct positioning of the pentascolopodial organ and dorsal closure are also restored in these double mutants (brackets; compare with Fig. 2E,H). (E) Mean values and standard error of the number of neurons in the dorsal, lateral and ventral clusters in abdominal segments with different genetic backgrounds. *shn* and *brk* have opposite roles in the dorsal and lateral clusters as *brk;shn* double mutants have more neurons than *shn* mutants alone. In the ventral cluster, *brk* appears to act independently of *shn* as *brk;shn* double mutants have comparable numbers of neurons to *brk* single mutants. WT, n=63; shn^{k04412}, n=116; brk^{m68}, n=121; brk^{m68};shn^{k04412}, n=74; shn^{1}, n=37; brk^{m68};shn^{1}, n=27.

most often affecting the ventralmost vmd5 sensory organ (Ghysen et al., 1986), while *shn* mutants show a less severe reduction (2-18%; Fig. 2H). Importantly, the reduction in the number of neurons in double mutants *brk;shn* is indistinguishable from that in the *brk* mutant alone. This indicates that in the ventral region the formation of some PNS neurons requires *brk* activity, and that this requirement is only marginally modulated by *shn* (Fig. 5A; see Discussion). In addition, the double mutant *brk;shn* do not show additive effects in the loss of neurons respective to the single mutants. Therefore, we conclude that both factors are important for the formation of the same subpopulation of ventral neurons.

In summary, apparently two populations of neurons exit. The dorsal and lateral clusters form one population that is mainly dependent on Dpp signaling, while the ventral cluster forms another population largely independent of Dpp signaling.

DISCUSSION

We have investigated the role of the second phase of TGFβ signaling in *Drosophila* embryos, which entails the BMP2/4 ortholog Dpp (Dorfman and Shilo, 2001). We show that at least some dorsal and lateral neurons (henceforth the dorsal/lateral PNS population) appear within a domain of Dpp signaling, and require this signaling to develop. For this population Dpp signaling acts in a positive manner, and Brk is normally of no significance. However, if Dpp signaling through Shn is impaired, Brk evidently represses this developmental process. By contrast, for a second population of ventral PNS neurons (henceforth the ventral PNS population), the developmental regulation is quite different. In this case, Brk acts in an effectively positive manner, whereas Shn is less important. Interestingly, this second population develops in a domain where no Dpp signaling is detectable by its usual readout, *dad* expression. Whether in this case Shn reflects a minor, non-Dpp dependent, mechanism remains to be determined.

It is known from previous studies that Dpp signaling and Brk are mutually antagonistic. For example, in the second phase of Dpp signaling, *brk* is negatively regulated by Dpp signaling itself via Shn (Marty et al., 2000; Torres-Vazquez et al., 2001). Conversely, in the first phase of Dpp signaling Brk directly or indirectly counteracts Dpp signaling: in *brk* mutant embryos, the domain of *dpp* expression expands while the VNE (from which the ventral nerve cord develops) is reduced (Jazwinska et al., 1999b; Skeath et al., 1992). Cell lineage

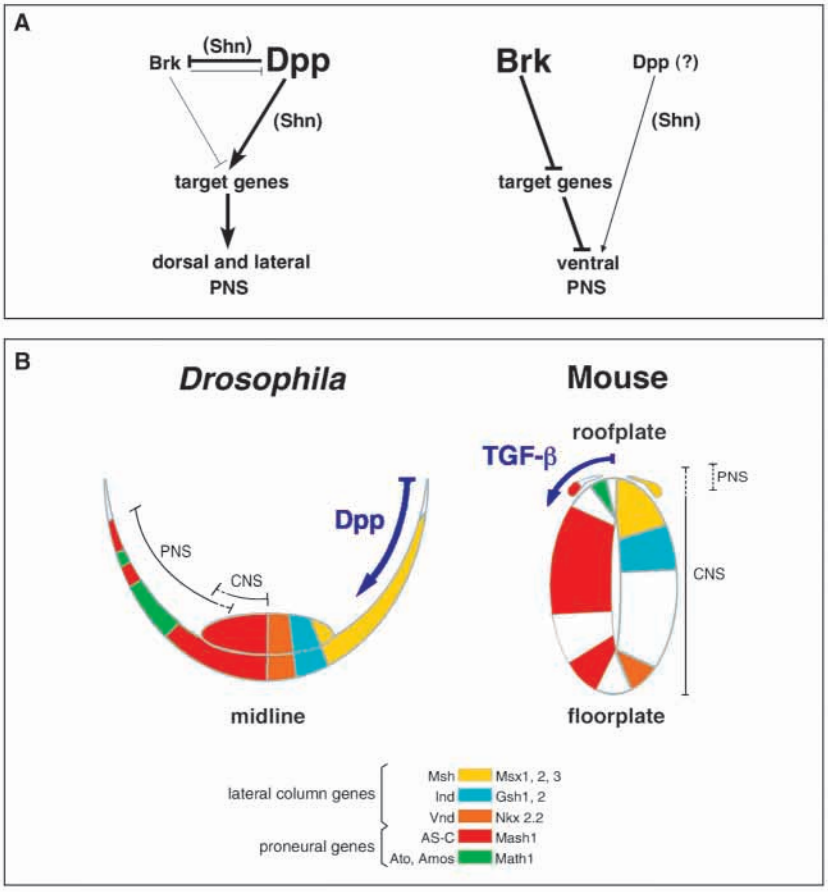

Fig. 5. (A) Summary of genetic data for the roles of TGFβ signaling in *Drosophila* PNS development. Dpp signaling in the dorsal ectoderm promotes the formation of the PNS and requires the co-factor Shn to keep Brk expression low in this domain. Thus, Shn and Brk have opposing effects on the formation of dorsal and lateral PNS clusters. In the ventral cluster, Brk expression is high, while Dpp signaling is low or absent. Brk promotes neuronal formation, probably by a double-negative mechanism that is independent of Shn. (B) Schematic representation of the similarities between the *Drosophila* nervous system (summarizes stages 8-11 of development) and the developing neural tube in mouse, in terms of the key molecules involved in their patterning (Arendt and Nubler-Jung, 1999; D'Alessio and Frasch, 1996; Gowan et al., 2001; Tanabe and Jessell, 1996). Regions of proneural gene expression are shown on the left half of the schemes while lateral column genes are shown on the right half. The proneural and lateral column genes are largely expressed in the neural progenitors. For simplicity, regions containing differentiating neurons are also colored according to their progenitors, although frequently they have lost the expression of the corresponding genes by this stage. Further references can be found in the main text. Similar proneural genes and lateral column genes specify the dorsoventral pattern of the nervous system. The dorsal nervous system fates are induced by TGFβ signaling in both *Drosophila* and chordates. TGFβs expressed in the roof plate in mouse include BMP4, BMP7 and BMP5, Dorsalin, Activin B and GDF7 (reviewed by Lee and Jessell, 1999; Liem et al., 1997). Neural crest originates from a cell population in the roof plate (Echelard et al., 1994; Lee et al., 2000; Selleck and Bronner-Fraser, 1995). Homologous gene pairs are shown in the same color. Achaete-scute complex (AS-C)/Mash1; Absent solo-MD neurons and olfactory sensilla (Amo)/Math1; Atonal (Ato)/Math1; Ventral nervous system defective (Vnd)/Nkx-2.2; Intermediate neuroblasts defective (Ind)/Gsh-1; and Muscle segment homeobox Msh/Msx1/2/3.

tracing studies have shown that some ventral PNS neurons originate from the VNE (Brewster and Bodmer, 1996; Schmid et al., 1999; Schmidt et al., 1997). The similar genetic requirements for *brk* in the ventral PNS population and in the ventral nerve cord arising from the VNE strongly suggest that this population represents the part of the PNS that originates within the VNE.

Both Dpp signaling and Brk are thought to act by transcriptional regulation. For simplicity, in the diagram of Fig. 5A, we show them as acting antagonistically on the same target genes in the dorsolateral population of neurons. However, in the ventral population, no antagonistic effect is observed in *brk;shn* double mutants: Brk promotes the formation of neurons and Shn also has a positive albeit secondary effect. This suggests that the major 'double negative' Brk-dependent mechanism and the minor positive Shn-dependent mechanism work in parallel on this population. Two distinct, dorsolateral and ventral mechanisms are feasible, because specific proneural clusters can be regulated by different proneural genes (Jarman et al., 1993; Ruiz-Gomez and Ghysen, 1993) or by different enhancers of the same gene. The diagrams in Fig. 5A are not unique explanations, alternative circuitries may be identified in further work. The diagrams serve to illustrate our main conclusion, that different genetic mechanisms, which are driven predominantly by Dpp or Brk, are regulating PNS neuronal formation in the two populations.

Whatever the detailed mechanism of PNS formation, it is now clear that TGFβ signaling is important for two independent phases in *Drosophila* embryonic nervous system development. First, it is necessary for defining the border of the VNE. In the second phase, TGFβ signaling by Dpp is necessary for development of the dorsalmost nervous system, the dorsal and lateral PNS, while the ventral PNS population is regulated primarily by the inhibitor Brk. This biphasic role has parallels in vertebrates, as summarized in the Introduction, and has important implications about the evolutionary history of the development of the nervous system.

A conserved role for late TGFβ signaling in nervous system formation

In a current hypothesis, the insect ventral side corresponds to the dorsal side of vertebrates, because of an inversion of the dorsoventral axis during evolution (Arendt and Nubler-Jung, 1994; de Robertis and Sasai, 1996; Holley and Ferguson, 1997). Furthermore, the central nervous system (CNS), which consists of the brain and nerve cord, although located ventrally in insects and dorsally in chordates, presents striking similarities and has been suggested to be evolutionarily

conserved among Bilateria (Arendt and Nubler-Jung, 1999). In both insects and vertebrates the anteroposterior regionalization of the neuroectoderm is regulated by orthologous homeotic genes, the cephalic gap genes, *engrailed* and *eyeless/Pax6* genes (Arendt and Nubler-Jung, 1996; Reichert and Simeone, 1999). Both the ventral midline cells of *Drosophila* and the floor plate of vertebrates function as centers for mediolateral regionalization. One difference is that in vertebrates, the main morphogen emanating from the midline is Sonic hedgehog (Shh), while in *Drosophila* it is a TGFα-related ligand (Spitz) signaling through the dEGF-receptor (Arendt and Nubler-Jung, 1999). Despite the fact that non-homologous molecules serve to pattern the mediolateral axis, striking similarities exists in the pattern of genes that specify a series of longitudinal columns in the neuroectoderm (Arendt and Nubler-Jung, 1999; Jurata et al., 2000). In both *Drosophila* and vertebrates, homologous genes specify the proneural columns from the midline outwards: *vnd/Nkx-2.2, ind/Gsh-1* and *msh/Msx1/2/3* (Fig. 5B), indicating that the mediolateral regionalization of the neural axis is evolutionary conserved (Weiss et al., 1998).

Several lines of evidence suggest that the *Drosophila* embryonic PNS also has a direct equivalent in vertebrate embryos: the sensory neurons in, or emerging from, the dorsal neural tube. The first evidence concerns the expression patterns of lateral column genes. In vertebrates, the most lateral part of the Msx-positive neural plate gives rise to the roof plate of the dorsal neural tube, from which the neural crest originates before it migrates out and gives rise to the sensory neurons of the PNS and other tissues (Fig. 5) (Arendt and Nubler-Jung, 1999; Echelard et al., 1994; Selleck and Bronner-Fraser, 1995). The adjacent Msx expression domain that remains in the neural tube includes precursors of dorsal sensory interneurons. In several extant vertebrates, some primary sensory neurons do not migrate out, but remain in the dorsal spinal cord, where they are identified as 'Rohon-Beard' neurons in zebrafish and *Xenopus* and 'dorsal cells' in Amphioxus (Baker and Bronner-Fraser, 1997; Clarke et al., 1984; Martin and Wickelgren, 1971). In *Drosophila*, the *msh* gene (the ortholog of *Msx*) is expressed in the lateral columns of the *Drosophila* VNE, and slightly later in the developing PNS (D'Alessio and Frasch, 1996). Thus, both in vertebrates and in *Drosophila* the Msx/Msh-positive lateral (later dorsal) nervous system includes precursors of the sensory PNS and interneuron populations.

The second evidence concerns the expression patterns of proneural genes. These genes can be grouped as *Achaete-Scute Complex* (*AS-C*) related genes and *ato*-related genes (Hassan and Bellen, 2000). The lateral and ventral motor region of the vertebrate neural tube express only the AS-C homolog, Mash1, while the dorsolateral interneuron and sensory neuron regions of the neural tube and the neural crest express both the AS-C like Mash1 and Math1, the protein most related to Ato and Amo (Gowan et al., 2001; Hassan and Bellen, 2000). Similarly, in *Drosophila* the ventral regions from which the CNS develops express only AS-C genes, whereas the PNS develops from a more dorsolateral region where both AS-C and Ato/Amo proteins are expressed.

A final parallel is the expression pattern of TGFβ molecules. By early neurulation in chordates (the ascidian *H. roretzi*, *Xenopus*, zebrafish, chicken and mouse) BMPs are strongly localized at the edges of the neural plate (Darras and Nishida, 2001; Miya et al., 1997; Streit and Stern, 1999). In *Drosophila*, the active Dpp signaling covers the dorsal part of the epidermis, including the forming dorsal PNS (Dorfman and Shilo, 2001; Jackson and Hoffmann, 1994). Thus, in all these organisms, BMP2/4/Dpp orthologs are expressed at the lateral (later dorsal) parts of the forming nervous system. Taken together, these data suggest that the dorsalmost neural tube of vertebrates, including the neural crest, is evolutionarily homologous to the most dorsal nervous tissue in *Drosophila*, the embryonic PNS (Fig. 5).

In chordates, after the neural anlage is specified, TGFβ signaling is necessary for induction of dorsal neuronal cell populations and migration of neural crest cells (Darras and Nishida, 2001; Lee and Jessell, 1999). An important contribution of the present work is the demonstration that TGFβ signaling is necessary for induction of the most dorsal nervous system in *Drosophila* (the dorsolateral PNS) as it is in vertebrates. We suggest that the induction of dorsal neuronal subtypes by TGFβ signaling is an evolutionarily conserved step in nervous system patterning in all Bilateria.

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REFERENCES

Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. *Development* **117**, 29-43.

Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. (2001). Nuclear interpretation of Dpp signaling in *Drosophila*. *EMBO J.* **20**, 3298-3305.

Arendt, D. and Nubler-Jung, K. (1994). Inversion of dorsoventral axis? *Nature* **371**, 26.

Arendt, D. and Nubler-Jung, K. (1996). Common ground plans in early brain development in mice and flies. *BioEssays* **18**, 255-259.

Arendt, D. and Nubler-Jung, K. (1999). Comparison of early nerve cord development in insects and vertebrates. *Development* **126**, 2309-2325.

Arora, K., Dai, H., Kazuko, S. G., Jamal, J., O'Connor, M. B., Letsou, A. and Warrior, R. (1995). The *Drosophila schnurri* gene acts in the Dpp/TGF-β signaling pathway and encodes a transcription factor homologous to the human BMP family. *Cell* **81**, 781-790.

Arora, K., Levine, M. S. and O'Connor, M. B. (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8**, 2588-2601.

Baker, C. V. and Bronner-Fraser, M. (1997). The origins of the neural crest. Part II: an evolutionary perspective. *Mech. Dev.* **69**, 13-29.

Barth, K. A., Kishimoto, Y., Rohr, K. B., Seydler, C., Schulte-Merker, S. and Wilson, S. W. (1999). BMP activity establishes a gradient of positional information throughout the entire neural plate. *Development* **126**, 4977-4987.

Biehs, B., Francois, V. and Bier, E. (1996). The *Drosophila short gastrulation* gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev.* **10**, 2922-2934.

Brewster, R. and Bodmer, R. (1996). Cell lineage analysis of the *Drosophila* peripheral nervous system. *Dev. Genet.* **18**, 50-63.

Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. *Cell* **96**, 553-562.

Campos-Ortega, J. A. (1993). Early neurogenesis in *Drosophila melanogaster*: Cold Spring Harbour Laboratory Press.

Campos-Ortega, J. A. and Hartenstein, V. (1997). The embryonic development of *Drosophila melanogaster*. Berlin: Springer-Verlag.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting

the temporal requirements for homeotic gene function. *Development* **120**, 1983-1995.

Clarke, J. D., Hayes, B. P., Hunt, S. P. and Roberts, A. (1984). Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of *Xenopus laevis*. *J. Physiol.* **348**, 511-525.

D'Alessio, M. and Frasch, M. (1996). *msh* may play a conserved role in dorsoventral patterning of the neuroectoderm and mesoderm. *Mech. Dev.* **58**, 217-231.

Dai, H., Hogan, C., Gopalakrishnan, B., Torres-Vazquez, J., Nguyen, M., Park, S., Raftery, L. A., Warrior, R. and Arora, K. (2000). The zinc finger protein Schnurri acts as a Smad partner in mediating the transcriptional response to Decapentaplegic. *Dev. Biol.* **227**, 373-387.

Darras, S. and Nishida, H. (2001). The BMP/Chordin antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Dev. Biol.* **236**, 271-288.

de Robertis, E. M. and Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* **380**, 37-40.

Dickinson, M. E., Selleck, M. A., McMahon, A. P. and Bronner-Fraser, M. (1995). Dorsalization of the neural tube by the non-neural ectoderm. *Development* **121**, 2099-2106.

Dorfman, R. and Shilo, B. Z. (2001). Biphasic activation of the BMP pathway patterns the Drosophila embryonic dorsal region. *Development* **128**, 965-972.

Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS. *Development* **120**, 2213-2224.

Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982). Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* **79**, 7929-7933.

Ghysen, A., Dambly-Chaudière, C., Aceves, E., Jan, L. Y. and Jan, Y. N. (1986). Sensory neurons and peripheral pathways in *Drosophila* embryos. *Roux's Arch. Dev. Biol.* **195**, 281-289.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

Grieder, N. C., Nellen, D., Burke, R., Basler, K. and Affolter, M. (1995). Schnurri is required for *Drosophila* Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* **81**, 791-800.

Hammerschmidt, M., Serbedzija, G. N. and McMahon, A. P. (1996). Genetic analysis of dorsoventral pattern formation in the zebrafish: requirement of a BMP-like ventralizing activity and its dorsal repressor. *Genes Dev.* **10**, 2452-2461.

Hassan, B. A. and Bellen, H. J. (2000). Doing the MATH: is the mouse a good model for fly development? *Genes Dev.* **14**, 1852-1865.

Holley, S. A. and Ferguson, E. L. (1997). Fish are like flies are like frogs: conservation of dorsal-ventral patterning mechanisms. *BioEssays* **19**, 281-284.

Huang, M. L., Hsu, C. H. and Chien, C. T. (2000). The proneural gene *amos* promotes multiple dendritic neuron formation in the *Drosophila* peripheral nervous system. *Neuron* **25**, 57-67.

Jackson, P. D. and Hoffmann, F. M. (1994). Embryonic expression patterns of the *Drosophila decapentaplegic* gene: separate regulatory elements control blastoderm expression and lateral ectodermal expression. *Dev. Dyn.* **199**, 28-44.

Jan, Y. N. and Jan, L. Y. (1993). *The Peripheral Nervous System*. Cold Spring Harbour Laboratory Press.

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. *Cell* **73**, 1307-1321.

Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1994). *atonal* is the proneural gene for *Drosophila* photoreceptors. *Nature* **369**, 398-400.

Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999a). The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.

Jazwinska, A., Rushlow, C. and Roth, S. (1999b). The role of Brinker in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* **126**, 3323-3334.

Jurata, L. W., Thomas, J. B. and Pfaff, S. L. (2000). Transcriptional mechanisms in the development of motor control. *Curr. Opin. Neurobiol.* **10**, 72-79.

Kania, A., Salzberg, A., Bhat, M., D'Evelyn, D., He, Y., Kiss, I. and Bellen, H. J. (1995). P-element mutations affecting embryonic peripheral nervous system development in *Drosophila* melanogaster. *Genetics* **139**, 1663-1678.

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466.

Lage, P., Jan, Y. N. and Jarman, A. P. (1997). Requirement for EGF receptor signaling in neural recruitment during formation of *Drosophila* chordotonal sense organ clusters. *Curr. Biol.* **7**, 166-175.

Lee, K. J., Dietrich, P. and Jessell, T. M. (2000). Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403**, 734-740.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261-294.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes Dev.* **12**, 3394-3407.

Liem, K. F., Jr, Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.

Liem, K. F., Jr, Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGF-β-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91**, 127-138.

Lindsley, D. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. San Diego, USA: Academic Press.

Martin, A. R. and Wickelgren, W. P. (1971). Sensory cells in the spinal cord of the sea lamprey. *J. Physiol.* **212**, 65-83.

Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of *brinker* transcription. *Nat. Cell Biol.* **2**, 745-749.

Massagué, J. and Chen, Y. G. (2000). Controlling TGF-β signaling. *Genes Dev.* **14**, 627-644.

Millonig, J. H., Millen, K. J. and Hatten, M. E. (2000). The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. *Nature* **403**, 764-769.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). *brinker* is a target of Dpp in *Drosophila* that negatively regulates Dpp- dependent genes. *Nature* **398**, 242-246.

Miya, T., Morita, K., Suzuki, A., Ueno, N. and Satoh, N. (1997). Functional analysis of an ascidian homologue of vertebrate BMP-2/BMP-4 suggests its role in the inhibition of neural fate specification. *Development* **124**, 5149-5159.

Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Haffter, P., Heisenberg, C. P. et al. (1996). Genes establishing dorsoventral pattern formation in the zebrafish embryo: the ventral specifying genes. *Development* **123**, 81-93.

Neul, J. L. and Ferguson, E. L. (1998). Spatially restricted activation of the SAX receptor by SCW modulates DPP/TKV signaling in *Drosophila* dorsal-ventral patterning. *Cell* **95**, 483-494.

Newfeld, S. J., Wisotzkey, R. G. and Kumar, S. (1999) Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. *Genetics* **152**, 783-795.

Nguyen, M., Park, S., Marques, G. and Arora, K. (1998). Interpretation of a BMP activity gradient in *Drosophila* embryos depends on synergistic signaling by two type I receptors, SAX and TKV. *Cell* **95**, 495-506.

Nguyen, V. H., Trout, J., Connors, S. A., Andermann, P., Weinberg, E. and Mullins, M. C. (2000). Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. *Development* **127**, 1209-1220.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole-mount *Drosophila* embryos and larvae using antibody probes. *Methods Cell Biol.* **44**, 445-487.

Podos, S. D. and Ferguson, E. L. (1999). Morphogen gradients: new insights from DPP. *Trends Genet.* **15**, 396-402.

Reichert, H. and Simeone, A. (1999). Conserved usage of gap and homeotic genes in patterning the CNS. *Curr. Opin. Neurobiol.* **9**, 589-595.

Ruiz-Gomez, M. and Ghysen, A. (1993). The expression and role of a proneural gene, *achaete*, in the development of the larval nervous system of *Drosophila*. *EMBO J.* **12**, 1121-1130.

Rusten, T. E., Cantera, R., Urban, J., Technau, G., Kafatos, F. C. and

Barrio, R. (2001). Spalt modifies EGFR-mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila* promoting the development of oenocytes. *Development* **128**, 711-722.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G. M. (1997). The embryonic central nervous system lineages of *Drosophila melanogaster*. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev. Biol.* **189**, 186-204.

Schulte-Merker, S., Lee, K. J., McMahon, A. P. and Hammerschmidt, M. (1997). The zebrafish organizer requires Chordino. *Nature* **387**, 862-863.

Selleck, M. A. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-538.

Skeath, J. B., Panganiban, G., Selague, M. and Carroll, S. B. (1992). Gene regulaton in two dimensions: the proneural *achaetae* and *scute* genes are controlled by combinations of axis-patterning genes through a common intergenic control region. *Genes Dev.* **6**, 2606-2619.

Staehling-Hampton, K., Laughon, A. S. and Hoffmann, F. M. (1995). A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for Dpp signaling. *Development* **121**, 3393-3403.

Streit, A. and Stern, C. D. (1999). Neural induction. A bird's eye view. *Trends Genet.* **15**, 20-24.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274**, 1115-1123.

Tanimoto, H., Itoh, S.., ten Dijke, P. M. and Tabata, T. (2000). Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**, 59-71.

Torres-Vazquez, J., Park, S., Warrior, R. and Arora, K. (2001). The transcription factor Schnurri plays a dual role in mediating Dpp signaling during embryogenesis. *Development* **128**, 1657-1670.

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Koernberg, T., Christian, J. and Tabata, T. (1997). Daughters against dpp modulates Dpp organizing activity in *Drosophila* wing development. *Nature* **389**, 627-631.

Udagawa, Y., Hanai, J., Tada, K., Grieder, N. C., Momoeda, M., Taketani, Y., Affolter, M., Kawabata, M. and Miyazono, K. (2000). Schnurri interacts with Mad in a Dpp-dependent manner. *Genes Cells* **5**, 359-369.

Weiss, J. B., von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the *Drosophila* central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.

Younossi-Hartenstein, A. and Hartenstein, V. (1997). Pattern, time of birth, and morphogenesis of sensillum progenitors in *Drosophila*. *Microsc. Res. Tech.* **39**, 479-491.

Yu, K., Srinivasan, S., Shimmi, O., Biehs, B., Rashka, K. E., Kimelman, D., O'Connor, M. B. and Bier, E. (2000). Processing of the *Drosophila* Sog protein creates a novel BMP inhibitory activity. *Development* **127**, 2143-2154.