Local inhibition of *Drosophila* homeobox-containing neural dorsoventral patterning genes by Dpp

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Abstract An important step in *Drosophila* neurogenesis is to establish the neural dorsoventral (DV) patterning. Here we describe how *dpp* loss-of- and gain-of-function mutation affects the homeobox-containing neural DV patterning genes expressed in the ventral neuroectoderm. *Ventral nervous system defective* (*vnd*), *intermediate neuroblast defective* (*ind*), *muscle-specific homeobox* (*msh*), and *orthodenticle* (*otd*) genes participate in development of the central nervous system and peripheral nervous system, and encode homeodomain proteins. *otd* and *msh* genes were ectopically expressed in *dpp* loss-of-function mutation, but *vnd* and *ind* were not affected. However, when *dpp* was ectopically expressed in the ventral neuroectoderm by *rho-GAL4/UAS-dpp* system, it caused the repression of *vnd*, and *msh* expressions in ventral and dorsal columns of the neuroectoderm, respectively, but not that of *ind*. The later expression pattern of *otd* was also restricted by Dpp. The expression pattern of *msh*, *vnd* and *otd* in *dpp* loss-of-function and gain-of-function mutation indicates that Dpp activity does not reach to the ventral midline and it works locally to establish the dorsal boundary of the ventral neuroectoderm.

Key words: Dorsoventral patterning; Central nervous system; Homeodomain genes; *dpp*; *Drosophila*

1. Introduction

The *Drosophila* central nervous system (CNS) arises from the bilateral ventral neuroectoderm. Single neuroectodermal cells delaminate inward from the ventral neuroectoderm, forming neural precursor cells called neuroblasts (NBs). They subsequently divide to produce the neurons and glia, generating approximately 3000 neurons and 30 glia in the mature embryonic CNS per hemisegment [1]. The formation of NB is regulated by two oppositely acting groups of genes. Proneural genes including *achaete/scute* and *lethal of scute* promote NB formation [2], whereas the neurogenic genes including *Notch* and *Delta* inhibit NB formation, whose cells remains outside and form ventral epidermis [3].

Once the NBs are formed, they are specified to unique NB identities along both the AP and DV axes. The *gooseberry*, *wingless*, *hedgehog* and *engrailed* genes are regionally expressed along the AP axis of the ventral neuroectoderm, and establish AP row identity with the neuroectoderm and NBs [4–8]. On the other hand, three signaling pathways, regulated by Dorsal, Decapentaplegic (Dpp), and epidermal growth factor receptor (Egfr), work in concert to divide the embryo into defined tissue types including mesoderm, neuroectoderm, dorsal epidermis and amnioserosa along the DV axis. The ventral side of the embryo is patterned by Dorsal protein maternally produced in a graded fashion such that the highest levels of Dorsal protein are found in the most ventral nuclei. The dorsal surface of the embryos is patterned by zygotically produced Dpp, a secreted protein of the TGF-β family. The Dpp activity gradient is high dorsally and lower ventrally due to the expression of the Dpp antagonist, *short gastrulation* (*sog*) within the neuroectoderm [9,10]. Loss of Dpp activity shows expanded neuroectoderm to the dorsal side, while ectopic Dpp activity caused expansion of dorsal tissues to the ventral side [11]. This implies that the Dpp activity gradient establishes the dorsal boundary of the neuroectoderm.

The identity of neuroectoderm along the DV domains is determined by three homeobox-containing genes, *ventral nervous system defective* (*vnd*), *intermediate neuroblast defective* (*ind*), and *muscle segment homeobox* (*msh*) [11–14]. *vnd* is expressed in the medial column where *vnd* acts as a regionalization gene that interacts with the proneural *AS-C* genes [15–17]. *ind* is expressed in the intermediate columns [14]. *msh* is expressed in the lateral column and required for their specification as demonstrated by loss- and gain-of-function mutations [13]. Intermediate levels of Dorsal can directly or indirectly activate neuroectoderm-specific genes including *vnd* and *rhomboid* (*rho*) [18]. Vnd represses *ind* in the ventral column, and Ind represses *msh* in the intermediate column. In double mutant embryos of *vnd* and *ind*, expression of *msh* expands ventrally to the midline. However, in the absence of *msh*, *ind* expression does not expand dorsally. There are two controversal reports on roles of Dpp in the patterning of neuroectoderm. Mellerick and Nirenberg [19] have proposed that Dpp

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signaling represses *vnd* expression and thus establishes the dorsal border of the *vnd* domain. In contrast, Ohlen and Doe [20] find that embryos with severely reduced Dpp activity showed no change in the pattern of *vnd* expression.

In this study, we investigated how the expression of homeobox-containing neural genes are affected by the Dpp activity gradient. *dpp* null mutation and ectopic expression from *rho-GAL4/UAS-dpp* were used to see the regulation of *vnd*, *ind*, *msh* and *otd*. We report that Dpp can repress *msh*, *vnd*, and *otd* in the short range.

2. Materials and methods

2.1. Drosophila stocks and culture

Marker mutations and balancer chromosomes used are as described in [33]. Files were reared in vials containing a standard cornmeal/yeast medium seeded with live yeast. Fly stocks were maintained at 19°C, and all crosses and egg collection were performed at 25°C. *dppH46* was used as a null mutant. *rho-Gal4* and P[W+; UAS-*dpp*] were used to express ectopically *dpp* in the neuroectoderm.

2.2. Embryonic cuticle preparation

Eggs were collected with 6–12-h intervals and incubated for 24 h at 25°C. Embryos were collected and transferred to double-sided cellophane tape for manual dechorionation. Embryos were then mounted in 1:1 mixture of Hoyer's mountant and lactic acid, and devitellinized with a fine tungsten needle. Embryonic internal structures were cleared at 60°C on a slide warmer for several days [21]. The embryos were examined by dark field microscopy.

2.3. In situ hybridization

dpp expression was monitored by whole-mount in situ hybridization using digoxigenin-labeled antisense RNA probes. The probes were prepared according to the manufacturer's directions (Boehringer Mannheim). The prehybridization procedure and hybridization conditions are based on the protocol of [22] as modified by [23].

2.4. Gal4/UAS strains

The *Gal4/UAS* system allows genes to be expressed ectopically in specific cell types or tissues [24]. In this study, to drive ectopic expression of *dpp* in the neuroectodermal region where *rho* (*rho*) is expressed, *rho-Gal4* was used. P[w+; UAS-*dpp*]/GAL4-*rho* embryos were collected for in situ hybridization.

3. Results and discussion

3.1. Expression pattern of dpp gene and its ectopic expression with UAS/GAL4 system

During early embryonic development, *dpp* is normally expressed along the dorsal 40% of embryo, where it specifies the formation of dorsal surface (Fig. 1A). *dpp*H46, which is a haplo-insufficient null mutant, was used to examine whether loss of *dpp* caused an expansion of neural DV patterning genes to the dorsal side [25]. While wild-type embryos have fine hairs in dorsal side (Fig. 1D), *dpp* loss-of-function mutant embryo shows ventral structures on the whole surface (Fig. 1E).

Ectopic expression of *dpp* from an *UAS-dpp* transgene was ectopically driven in the neuroectodermal cells under control of the *rho-GAL4* gene (Fig. 1C). *rho* is normally expressed in the neuroectoderm, but not expressed in mesoderm because it is repressed by Snail [26]. *rho-GAL4/UAS-dpp* embryos die before eclosing to larva. Largely hooked ventral hairs of *rho-GAL4/UAS-dpp* embryos almost disappeared, indicating the dorsalization of ventral cells (Fig. 1F).

3.2. Effects of dpp null and gain-of-function mutation on the expression of vnd, ind and msh

vnd, *ind*, and *msh* are expressed in ventral, intermediate, and dorsal columns of neuroectoderm, respectively (Fig. 2A,D,G). *vnd* is detected first, followed by *ind* and lastly *msh*. *vnd* expression is set by Dorsal and maintained by Egfr signal in the ventral domain of neuroectoderm. Vnd represses *ind* expression and thus establishes the ventral boundary of the Ind domain [14]. Vnd and Ind keep the expression of *msh* in the dorsal columns of neuroectoderm [20].

Dpp has been proposed to inhibit *vnd* expression from the long distance [19], indicating that Dpp directly controls the expression of *vnd*. However, we did not see any ectopic expression of *vnd* in the *dpp* null mutant embryo (Fig. 2B). As embryos of Dorsal and Dpp double mutation do not show *vnd* expression, lack of *vnd* in dorsal mutant embryos does not

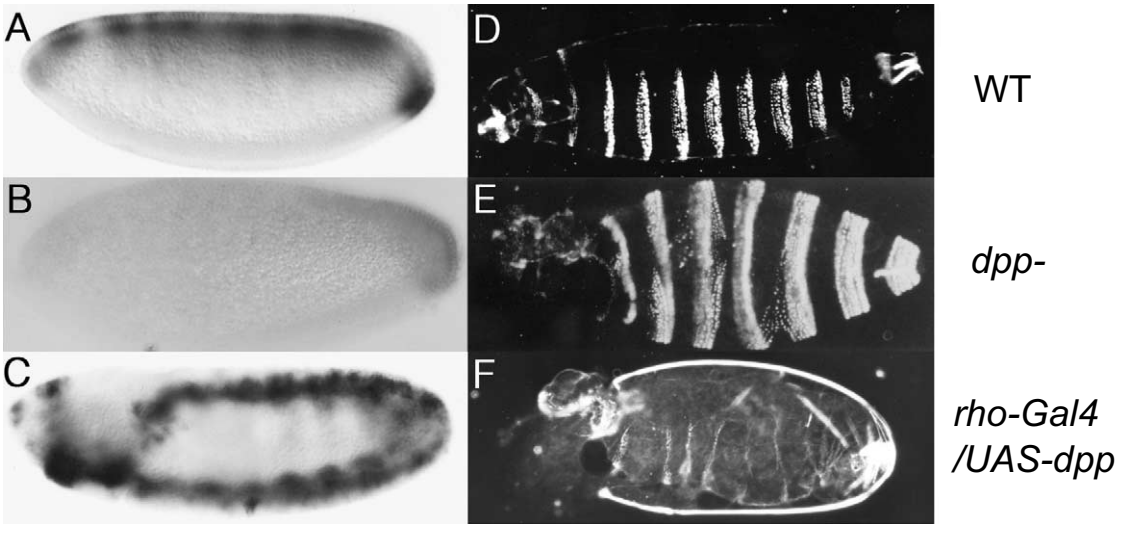

Fig. 1. *dpp* expression and ventral cuticular structures. A: A wild-type embryo. *dpp* is expressed in dorsal 40% of embryo. B: *dpp* null mutant embryo. *dpp* is not expressed in the doral side. C: Ectopic expression of *dpp*. *UAS-dpp* expression was driven under the control of *rho-GAL4*. *dpp* is ectopically expressed in the ventral neuroectoderm. D: A wild-type first instar larva. Dorsal epidermis has mostly fine hairs, while the ventral epidermis has largely hooked hairs. E: *dpp* null mutant embryo. Ventral denticle belts cover the whole epidermis. F: *rho-GAL4/USA-dpp* embryo. Ectopic expression of *dpp* causes dorsalization of the ventral epidermis.

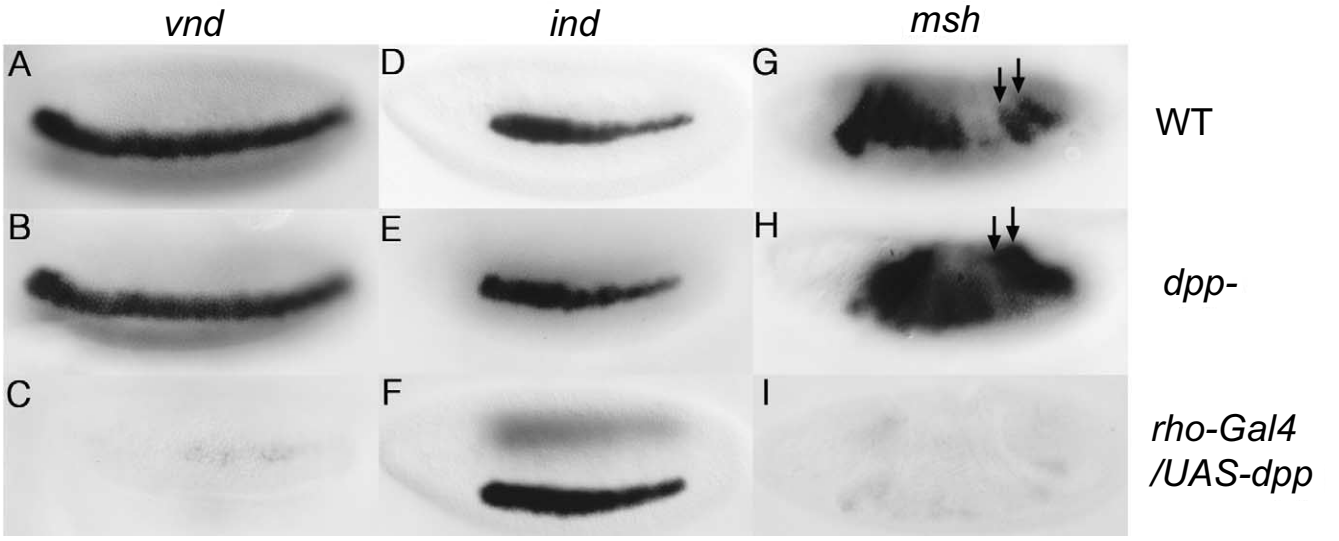

Fig. 2. Expression of *vnd*, *ind*, and *msh* during early embryogenesis. *vnd* (A), *ind* (D), and *msh* (G) expression in wild-type embryos. *vnd*, *ind* and *msh* are expressed in ventral, intermediate, and dorsal columns of neuroectoderms, respectively. *vnd* (B), *ind* (E) and *msh* (H) expression in *dpp* loss-of-function mutant embryos. *vnd* and *ind* expression were not affected, but *msh* expression was expanded to the dorsal side. *vnd* (C), *ind* (F), and *msh* (I) expression in the *dpp* gain-of-function mutant embryos. *vnd* and *msh* expression was severely reduced, but *ind* expression was not affected.

seem to be due to derepression of Dpp activity in the neuroectoderm [20]. Ohlen and Doe [20] did not observe change of *vnd* expression from the ectopic expression of four copies of *dpp*, either. We used a different approach to drive *dpp* expression in the neuroectoderm. As *dpp* was ectopically expressed in the neuroectoderm under control of *rho-GAL4*, *vnd* expression was severely reduced (Fig. 2C). This suggests that gene products from four copies of *dpp* might not reach the *vnd* domain so that *vnd* was not affected. Our result provides that *vnd* can be directly regulated by Dpp signal transduction if Dpp works at proper distance.

ind showed a different result from *vnd*. According to [20], *dpp* null mutation does not affect the *ind* expression, but ectopic expression of *dpp*, which was produced by four copies of *dpp*, significantly reduced *ind* expression. However, in our study, loss- and gain-of-function mutation of *dpp* did not change the expression of *ind* (Fig. 2E,F). Unlike the method of [20], we ectopically produce *dpp* using UAS/GAL4 system in the neuroectoderm. This different result may be due to the regulation of *ind* by Egfr. Dorsal and Egfr act together to activate *ind* expression, and the dorsal boundary of the Ind domain is set by the dorsal boundary of Egfr signaling.

msh is not detected until stage 7, which is observed last among three of *vnd*, *ind* and *msh*. Dorsal activates the expression of *msh* [20]. Unlike *vnd* and *ind*, *msh* expression was expanded to more dorsal side in *dpp* null mutant embryos (Fig. 2H), indicating that Dpp normally represses *msh* and maintains the dorsal boundary of the neuroectoderm. Ectopic expression of *dpp* in the neuroectoderm strongly repressed *msh* expression (Fig. 2I). As overexpression of *vnd*, *ind*, and *dpp* also represses *msh* expression ([20], and our data), the DV border of *msh* expression is defined by repression of Dpp.

In normal embryos, Mellerick and Nirenberg [19] showed that intermediate levels of Dorsal is sufficient to activate *vnd*.

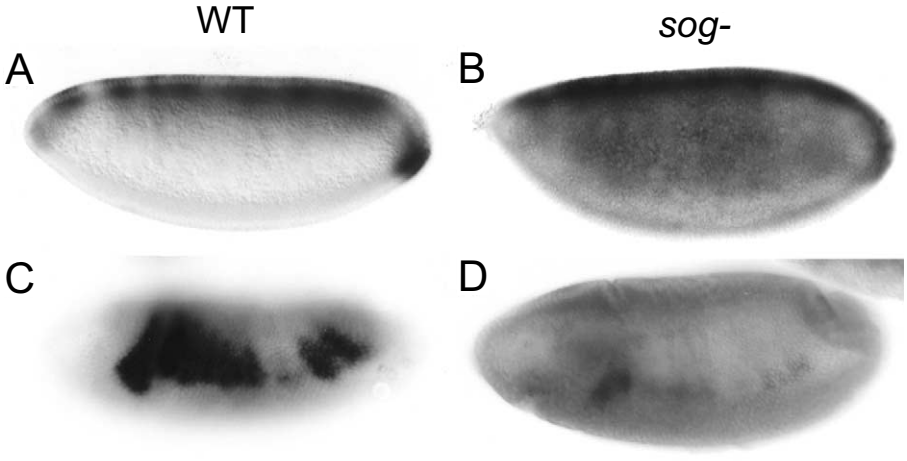

Fig. 3. Effects of *sog* mutation on *msh* expression. A: *dpp* expression in a wild-type embryo. *dpp* is expressed in dorsal 40% of embryo. B: *dpp* expression in a *sog* mutant embryo. *dpp* is expanded almost to the ventral region of the neuroectoderm. C: *msh* is expressed in the dorsal columns of neuroectoderm. D: *msh* expression in a *sog* mutant embryo. *msh* expression is greatly reduced in the neuroectoderm.

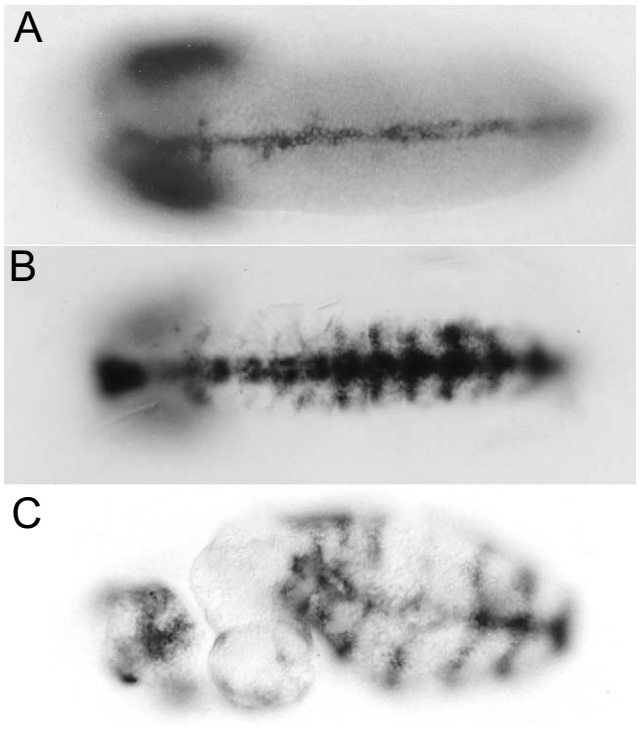

Fig. 4. Expression of *otd* in wild-type and *dpp* mutant embryos. A,B: Wild-type embryo. *otd* is expressed in one cell line of the ventral midline of the CNS per hemisegment at stage 10 (A). But at later stage, *otd* expression is largely expanded (B). C: *dpp* loss-of-function mutant. Early expression pattern of *otd* was not affected (data not shown), but at later stage *otd* was expressed in the dorsal area, indicating that dorsal boundary of *otd* expression is established by Dpp.

As *dpp* null mutation does not affect the expression of *vnd*, it is not clear how the dorsal border of *vnd* expression is determined. *vnd* expression seems to be more dependent to Dorsal protein. The ventral border of *ind* expression is established by Vnd, but the dorsal border of *ind* expression does not seem to be established by Dpp repression because the *ind* domain is normal in *dpp* mutant embryos ([14], and our data). As *ind* expression is also activated by Egfr signal transduction pathway, the determination of DV border of *ind* expression appears to be more complex [20].

3.3. Effects of dpp antagonist, Sog, on the expression of msh

We used another way to induce ectopic expression of *dpp* in more ventral neuroectoderm. *Sog* that is antagonistic to Dpp [27]. Dpp is ectopically expressed in the neuroectoderm of *sog* mutant embryo (Fig. 3B). This expansion of Dpp greatly reduced *msh* transcription in the neuroectoderm (Fig. 3D). Sog is a secreted protein that is produced in the presumptive neuroectoderm. Distribution of Sog is graded, with higher levels near the neuroectoderm and progressively lower levels dorsally [28]. Our results show that Dpp signal and its fine redistribution by Sog protein are involved in establishing *msh* domain along the DV axis. However, in *sog* mutant embryos *vnd* and *ind* were normally expressed, indicating that *Dpp* signal is not enough to inhibit *vnd* expression in the ventral region of the neuroectoderm. This suggests that there are other inhibitory molecules against spreading of Dpp activity.

Local action of Dpp appears to be mediated by the antagonistic action by Sog, Dad, Tsg and Brinker. It has been known that Sog, Tsg, and Dpp form an inhibitory complex. When Tolloid (Tld) cleaves Sog in the complex, Dpp is released and free [23,29]. If it happens near th neuroectoderm, Dpp seems to be rebound by the uncleaved Sog because there are excess amounts of Sog. However, in dorsal region, as there is little amount of Sog, free Dpp can now transduce the signal by binding to receptor. Brinker is also antagonistic to Dpp by repressing the targets of Dpp [30].

3.4. Effects of dpp null and gain-of-function mutation on the expression of orthodenticle (otd)

otd is essential for establishing the eyes, antenna and parts of brain [31], and specifying the ventral neuroectoderm in the CNS [32]. During early embryogenesis, it is expressed in one–two stripes along the ventral midline at later stage (Fig. 4A), and then its expression is expanded toward lateral side in the neuroectoderm (Fig. 4B). It has not been known yet how the dorsal boundary of *otd* expression is determined.

otd is normally expressed in early embryo of *dpp* mutant in which it is observed in a single stripe of the neuroectoderm in each hemisegment. However, *otd* expression was remarkably expanded toward the dorsal side in the *dpp* null mutant embryos (Fig. 4C). This indicates that at early embryogenesis Dpp activity does not reach up to the ventral midline, suggesting that the dorsal boundary of *otd* expression is not determined by Dpp. However, at a later stage when *otd* is expressed in the lateral side, the dorsal boundary of Dpp is now restricted by Dpp, indicating that Dpp locally works in determining the dorsal boundary of genes expressed in the ventral region.

In summary, the expression of *msh*, *vnd* and *otd* in *dpp* loss- and gain-of-function mutations indicates that Dpp activity does not reach to the ventral midline, and it locally works in determining the dorsal boundary of the neural DV patterning genes.

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