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Threshold-Dependent BMP-Mediated Repression: A Model for a Conserved Mechanism That Patterns the Neuroectoderm

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Introduction

Morphogen gradients play a central role in creating pattern during embryonic development [1,2]. Bone morphogenetic proteins (BMPs) are one of the best studied examples of morphogens and function in a conserved fashion to subdivide the early embryonic ectoderm into neural versus non-neural regions [3]. Following this role in establishing the primary ectodermal domains, BMPs and their antagonists, such as Short gastrulation (Sog)/Chordin (Chd), interact in a graded fashion to establish a series of nested gene expression domains in the non-neural ectoderm. While this BMP-mediated partitioning of the non-neural ectoderm has been analyzed in quantitative detail in Drosophila [4–6], relatively less is known about how patterning is initiated within the neuroectoderm.

The neuroectoderm in Drosophila and vertebrate embryos is similarly subdivided into three conserved dorsal-ventral (DV) domains expressing the homeobox genes ventral nervous system defective (vnd)/Nkx2.2, intermediate nervous system defective (ind)/Gsh, and muscle specific homeobox (msh)/Drosophila Msx1/2 (Figure 1). These neural identity genes are expressed in ventral, intermediate, and dorsal domains (Figure 1A–1C) [3,7–10], respectively, and are required to specify neural fates [11–17]. In Drosophila, both loss-of-function and mis-expression experiments have revealed that neural-identity genes cross-regulate each other in a ventral-dominant fashion wherein ventrally expressed genes repress expression of more dorsal ones [9,12,16,18], a mechanism likely to have been conserved in vertebrates [19–21].

An important unresolved question is whether subdivision of the neuroectoderm is accomplished by a conserved process or has arisen as a consequence of convergent evolution [7]. In the case of anterior-posterior (AP) patterning, where apparently species-specific upstream processes activate Hox genes in a conserved order along the AP axis [22], distinct pathways have been implicated in DV patterning of the neuroectoderm. The Dorsal gradient in Drosophila plays a central role in establishing the DV axis in Drosophila [23] and acts directly to initiate expression of vnd [24] and ind [25], while the Sonic Hedgehog (Shh) gradient in vertebrates patterns the ventral and lateral regions of the neural tube [19,23,26] (Figure 1A and 1C).

It has been suggested that BMP signaling might provide a conserved source of positional information along the DV axis in the neuroectoderm of both vertebrates and invertebrates. BMPs are expressed in the adjacent non-neural ectoderm, and following their early role during segregation of neural versus non-neural domains, they regulate expression of genes in the neuroectoderm. Disrupting the function or the extracellular distribution of BMPs or their antagonists such as BMP antagonists in the non-neural ectoderm has been analyzed in quantitative detail in Drosophila [4–6], relatively less is known about how patterning is initiated within the neuroectoderm.

Subdivision of the neuroectoderm into three rows of cells along the dorsal-ventral axis by neural identity genes is a highly conserved developmental process. While neural identity genes are expressed in remarkably similar patterns in vertebrates and invertebrates, previous work suggests that these patterns may be regulated by distinct upstream genetic pathways. Here we ask whether a potential conserved source of positional information provided by the BMP signaling contributes to patterning the neuroectoderm. We have addressed this question in two ways: First, we asked whether BMPs can act as bona fide morphogens to pattern the Drosophila neuroectoderm in a dose-dependent fashion, and second, we examined whether BMPs might act in a similar fashion in patterning the vertebrate neuroectoderm. In this study, we show that graded BMP signaling participates in organizing the neural axis in Drosophila by repressing expression of neural identity genes in a threshold-dependent fashion. We also provide evidence for a similar organizing activity of BMP signaling in chick neural plate explants, which may operate by the same double negative mechanism that acts earlier during neural induction. We propose that BMPs played an ancestral role in patterning the metazoan neuroectoderm by threshold-dependent repression of neural identity genes.

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Abbreviations: AP, anterior-posterior; BMP, bone morphogenetic protein; Brk, Brinker; Chd, Chordin; Dpp, Decapentaplegic; DV, dorsal-ventral; Hh, Hedgehog; ind, intermediate nervous system defective; msh, muscle specific homeobox; pMAD, phosphorylated form of Mothers against dpp; Shh, Sonic Hedgehog; Sog, Short gastrulation; st2-dpp, even-skipped stripe 2 enhancer driving expression of dpp; ush, u-shaped; vnd, ventral nervous system defective

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as Sog/Chd or Noggin leads to neuroectodermal patterning defects [27–31]. In addition, BMPs and Sog/Chd are expressed in the same relative orientation with regard to the domains of neural identity genes in the adjacent non-neural ectoderm (Figure 1A and 1C) [3,7,8,10,32].

Despite the similarities mentioned above, there are two apparent differences between vertebrates and invertebrates that argue against a common ancestral role for BMPs in patterning the neuroectoderm. First, it has been proposed that BMPs activate the expression of neural genes in the dorsal region of the vertebrate neural tube [33,34], whereas this pathway has only been reported to repress neural gene expression in Drosophila [18,27,35,36], whereas BMPs appear to function as morphogens to pattern the dorsal region of the vertebrate neural tube, there is no evidence that BMPs act in a similar dose-dependent fashion in the Drosophila neuroectoderm. In fact, existing studies suggest that BMPs play little, if any, role in establishing the order of neural identity gene expression, but function rather to consolidate cell fate choices [8,18,27,37]. It is possible that these prior studies failed to demonstrate a coherent role of BMPs in organizing the neuroectoderm due to the predominant influence of Dorsal in establishing the DV axis. Thus, it remains an open question whether BMPs play conserved or convergently evolved roles in establishing neuroectodermal cell fates in vertebrates and invertebrates.

In the current study, we asked whether BMP signaling alone can provide positional information for subdividing the neuroectoderm. We created Drosophila embryos devoid of normal DV polarity and determined whether neuroectoder-
nal patterning could be restored in response to an ectopic Decapentaplegic (Dpp) gradient provided along the AP axis. These experiments reveal that BMP signaling acts in a graded fashion to preferentially repress the expression of more ventral neural identity genes within the intermediate and dorsal regions of the neuroectoderm. We employed a similar experimental strategy to generate cells of uniform DV identity in chick neural tube explants grown in culture. In this case, the patterning effect of BMPs was assessed by adding increasing doses of BMPs to the media or by coculturing BMP expressing cells with neural plate explants. As in Drosophila embryos, we observed that BMPs could act in a dose-dependent fashion to recreate DV patterning in the absence of other graded cues. Such graded BMP-mediated repression in combination with cross-regulatory repression among neural identity genes can provide information required to subdivide the neuroectoderm into discrete domains and may once have been sufficient to organize the entire ectodermal DV axis of metazoan ancestors.

Results

The neural identity genes vnd, ind, and msh are expressed in a series of non-overlapping DV domains in the Drosophila embryo (Figure 1B). These genes are expressed in a highly dynamic fashion and are activated in a ventral-to-dorsal sequence [9,18]. The BMP antagonist Sog is expressed throughout the neuroectoderm [38] prior to the activation of neural identity gene expression and fades dorsally (Figure 1D and 1E) as the Dorsal gradient collapses [38,39]. By the time msh is expressed in a single contiguous dorsal stripe, sog expression is largely lost from these dorsal-most cells (Figure 1E). During this same period, the BMP2/4 homolog Dpp is expressed in adjacent dorsal cells (Figure 1B), where it represses the expression of neural genes and acts in a graded fashion to pattern the non-neural ectoderm. It is possible that Dpp also signals to the neuroectoderm, although previous single and double mutant analyses of the dpp pathway have not resolved whether Dpp acts in a graded fashion to help establish the order of the neural domains. In none of these studies, was it possible to sort out the contribution of BMP signaling from that of the Dorsal gradient (Figure 1A). To answer whether Dpp acts as a morphogen to pattern the Drosophila neuroectoderm, we developed a system for selectively analyzing its effects in the absence of other DV cues.

Separating the Effects of Graded BMP Signaling from that of the Dorsal Gradient

In order to separate the potential patterning effect of BMP signaling in Drosophila from that imposed by the Dorsal gradient, we designed a genetic system that allowed us to replace the normal ventral-to-dorsal gradient of nuclear Dorsal with a uniform neuroectodermal level of Dorsal along the entire DV axis of the embryo (Figure 1F). These lateralized embryos were created by first eliminating polarized DV maternal patterning acting upstream of Toll signaling and then adding back uniform adjusted levels of Dorsal across the entire DV axis using activated alleles of the Toll receptor. Uniform maternal Toll signaling was adjusted to specific levels using activated Toll alleles of differing strengths and by altering the dose of maternal Dorsal [40] (see Materials and Methods for details). In such lateralized embryos, we then tested the response of neural genes to an ectopic BMP gradient formed along the AP axis. This BMP gradient was created by expressing dpp under the control of the even-skipped stripe 2 enhancer of dpp (st2-dpp) construct (Figure 1G).

In lateralized embryos, pan-neuroectodermal markers such as sog are expressed around the entire circumference of the embryo (Figure 1H). As expected from the threshold-dependent activity of Dorsal [23], mesodermal, and dorsal ectodermal markers are absent in these same embryos (unpublished data). The consistent and uniform amounts of Dorsal produced in these lateralized embryos correspond to mid-neuroectodermal levels as revealed by expression of ind (Figure 1I) along the full DV axis and the absence of vnd expression (unpublished data). The AP limits of ind expression (Figure 1I) are similar to those in wild-type embryos (Figure 1B). Within this domain, msh expression is not detectable (Figure 1I), presumably because Ind is acting in a ventral-dominant fashion to repress it [9,16]. However, in more anterior cells abutting the ind domain, where msh expression normally extends further than ind (Figure 1B), msh is expressed in a ring around the embryo (Figure 1I). These initial studies indicate that both ind and msh can be expressed in mid-neuroectodermal lateralized embryos, and that Ind efficiently excludes msh from its domain.

Dpp Represses msh and ind Expression in a Threshold-Dependent Fashion

Once we established conditions for reliably producing lateralized embryos, we tested whether it was possible to induce a graded Dpp response by crossing a st2-dpp construct into the lateralized background (Figure 2). The sole source of dpp expression in these embryos is provided by st2-dpp, except at the poles where endogenous dpp expression is independent of Dorsal regulation [41] (Figure 2A, arrows). The expected pattern of BMP pathway activation in such embryos, assessed by in situ phosphorylation of the signal transducer, phosphorylated form of Mothers against dpp (pMAD), is a broad band centered over the st2-dpp stripe [6]. We also assayed expression of the epidermal Dpp target gene u-shaped (ush) as a second marker for BMP activation. Because lateralized embryos ubiquitously express the BMP inhibitor sog, neither pMAD (Figure 2A) nor ush expression (unpublished data) could be detected near the stripe of dpp expression. However, when sog function was eliminated in st2-dpp lateralized embryos, pMAD was activated in a broad domain extending approximately eight cell diameters beyond the narrower dpp stripe (Figure 2B). In addition, ush expression was also activated in this region (Figure 2C). These results indicate that Dpp diffusing from a sharp stripe can elicit a graded response over significant distances.

We next examined the effect of graded Dpp activity on the relative patterns of ind and msh expression. We used multiplex in situ hybridization methods [42] to examine the simultaneous expression of msh, ind, and ush, while scoring for the sog+ versus sog− genotype of the embryos. These experiments revealed a clear dose-dependent repression of ind expression characterized by strong repression near the source of dpp and graded reduction in expression extending approximately 20 cell diameters posteriorly (Figure 2D, bracket). In contrast, the opposite effect was observed with regard to msh expression, resulting in its activation in cells expressing the
lowest levels of ind (Figure 2E and 2F). In control sog- lateralized embryos, where BMP signaling is blocked, st2-dpp had no discernable effect on the pattern or intensity of either msh or ind expression (Figure 2G and 2H; compare with Figure 1I). These results can be understood if Dpp signaling preferentially represses expression of ind in sog-; st2-dpp lateralized embryos, thereby relieving ind-mediated repression of msh in cells near the Dpp source. The induction of msh expression near the Dpp stripe followed by a zone of ind expression mimics the wild-type configuration of gene expression and provides the first evidence that BMP signaling can influence the pattern of neuroectodermal gene expres-
Figure 3. BMP Signaling Can Also Repress Expression of vnd

(A) Scheme for generating ventro-lateralized embryos with a uniform level of Dorsal adjusted to that present in the ventral neuroectoderm (e.g., vnd expressing cells). These embryos were collected from gd^sog+/-; Tl^+/mothers (B and C) or sog^{sed} brk^{mo3}/FM7; Tl^{wv}/Tl^{w} mothers (D and E).

(B and C) ush (yellow) and vnd (cyan) expression in a sog--; st2-dpp ventro-lateralized embryo. Note that while ush expression is induced in response to dpp expression in this embryo (bracket), the pattern of vnd expression remains unaffected.

(D and E) ush (yellow) and vnd (cyan) expression in a brk--; sog--; st2-dpp ventro-lateralized embryo.

(D) The level and width of ush expression (bracket) is greater than in sog-- single mutants (compare with [B]).

(E) Note the broad domain of reduced vnd expression (bracket), which extends anterior to the st2-dpp expression domain.

(F) Expression of msh (red), ind (green), and vnd (blue) in a st2-brk embryo that has a normal Dorsal gradient.

(F–H) Expression of msh (red), ind (green), and vnd (blue) in a st2-brk embryo that has a normal Dorsal gradient. The inset shows higher magnification of the ind/vnd border in the region of st2-brk expression, which is consistently shifted dorsally by 1–2 cells within the stripe of brk expression. This shift is most clearly revealed by a consistent flattening of what is normally a continuous arc in the ind/vnd border at the position of st2-brk expression. The inset also shows that vnd expression extends up to the ind border and that there is no gap between these gene expression domains. We quantitated the shift in the ind/vnd border in nine st2-brk and nine wild-type embryos by counting the number of ind negative cells above a line spanning the ventral border of ind expression in the head and abdomen comprising a zone four cells wide centered within the st2 domain (or its approximate corresponding position in wild-type embryos). ind is better than vnd for performing this measurement since the ventral ind border is sharper (i.e., more all-or-none) than the dorsal vnd border. This analysis reveals that in st2-brk embryos there is an average of 5.4 ± 2.65 ind negative (vnd positive) cells above the line (which corresponds to an average shift of the ind/vnd border of 5.4/4 = 1.35 cells dorsally). In contrast, for control wild-type embryos we counted an average of 0.44 ± 0.88 cells above the line corresponding to an average of 0.11 cells. This represents a 10-fold difference between wild-type and st2-brk embryos, which is highly significant in a student’s t-test (p < 0.0003).

(G) Lateral view of a slightly older embryo than that shown in (F) showing a significant dorsal shift of the msh/ind border and a smaller shift of the ind/vnd border. The carets in (F and G) indicate the approximate trajectories of the ind/vnd and msh/ind borders in wild-type embryos.

(H) Dorsal view of the same embryo shown in (G) revealing that msh expression expands to the dorsal midline.

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sion in the absence of other DV cues such as the Dorsal gradient. Similar long-range inhibition of ind and short-range induction of ectopic msh expression can be observed in sog−/−; eve2-dpp embryos with an intact Dorsal gradient (see Figure S1), indicating that ind is also likely to be more sensitive than msh to BMP-mediated repression in wild-type embryos (see also Figure S2). The fact that the zone of ind repression extends considerably further from the dpp stripe than the region of msh activation indicates that msh is not responsible for ind repression, consistent with existing evidence that msh does not regulate ind [9,16]. It seems likely, therefore, that BMP signaling acts directly to repress ind expression, as has been proposed previously [18]. These data support the prevailing ventral-dominant model for cross-regulation of neural identity genes, and exclude an alternative model in which Dpp signaling activates msh, which in turn inhibits ind.

vnd Expression Is Also Sensitive to BMP-Mediated Repression

Previous studies of the ventral-most neural identity gene, vnd, reported only a mild expansion of its expression domain in dpp−/− mutants [37], or no consistent effect [18]. We exploited our sensitive lateralized system to re-examine the BMP response of vnd in order to resolve these existing ambiguities (Figure 3). We expressed st2-dpp in embryos with uniform levels of Dorsal corresponding to the ventral neuroectoderm, which are sufficient to induce ubiquitous expression of vnd (Figure 3A). In such “ventro-lateralized” embryos, both ind and msh expression are absent, presumably due to repression by vnd. Elimination of sog function in these embryos resulted in activation of BMP signaling as judged by the localized activation of the epidermal marker ush (Figure 3B); however, vnd expression remained unaltered (Figure 3C). When we eliminated the function of both sog and the transcriptional repressor of BMP signaling, brinker (brk), we observed stronger and expanded expression of ush and potent repression of vnd in a broad zone centered over st2-dpp (Figure 3D and 3E). These results indicate that vnd is indeed sensitive to BMP-mediated repression and that Brk can block the repressive as well as activating [35] functions of BMP signaling. In analogy to what was observed in mid-lateralized embryos, it might have been expected that relief of Vnd repression in ventro-lateralized embryos would result in activation of ind in cells lacking vnd expression. However, we did not detect expression of either ind or msh in these embryos, even near the edges of the vnd repression domain (unpublished data). These data suggest that the high levels of Dpp signaling generated under these experimental conditions are sufficient to repress vnd, as well as ind and msh. Such strong BMP signaling, which is similar to that acting in the non-neural ectoderm of wild-type embryos, may obscure potential differences in the relative sensitivities of these genes to BMP-mediated repression by repressing expression of all neural genes. Although it remains to be determined what the relative sensitivity of vnd is to BMP repression (see Discussion), the fact that vnd is subject to such repression raises the possibility that Dpp might also regulate vnd expression along its dorsal border in wild-type embryos, despite the low levels of Dpp that diffuse into that region. Since the concentration of Dorsal is limiting with regard to activating vnd in cells along this border, these cells would be expected to be the most susceptible to BMP-mediated repression (see below).

Dpp Activity Helps Establish Normal Borders of Neural Identity Gene Expression

Our analysis of BMP signaling in lateralized embryos showed that Dpp can regulate the expression of ind and msh in a dose-dependent fashion along the AP axis, and can also repress vnd expression. To test whether Dpp plays a similar dosage-sensitive role in the regulation of neural identity genes along the DV axis in the presence of an intact gradient of nuclear Dorsal, we devised an experiment to locally inhibit the response of neural genes to Dpp within the neuroectoderm of embryos with normal DV polarity. Because Brk can suppress BMP-mediated repression of vnd (Figure 3D and 3E), we reasoned that mis-expression of brk with the eve-st2 enhancer might also relieve BMP repression of ind and msh. This localized expression of the st2-brk construct has the advantage of providing an internal comparison of gene expression domains within the same embryo. In embryos carrying the st2-brk construct, all three neural domains shifted dorsally at the site of brk over-expression (Figure 3F–3H). msh expression was de-repressed in a stripe dorsally (Figure 3H) as has been observed previously in dpp−/− mutants [8,18], and the border between msh and ind shifted dorsally by approximately 4–6 cells (Figure 3F and 3G). The dorsal shift in ind expression was observed prior to initiation of msh expression (Figure 3F), consistent with their normal ventral-to-dorsal sequence of activation [18]. In addition, we observed a modest but consistent dorsal shift of 1–2 cells in the ind/vnd border within the zone of st2-brk expression (Figure 3F, carets, see legend for quantification). The domains of msh and ind expression also shift in other situations where BMP signaling is altered in the context of an intact Dorsal gradient (see Figures S1 and S2), which reinforces the view that BMP signaling plays a role in determining the positions and extents of these expression domains in wild-type embryos.

The results described above indicate that graded Dpp activity normally plays an important role in establishing the position of the border between the msh and ind domains, and to a lesser degree influences the indicated border, which forms 10–12 cells from the dorsal source of Dpp. The co-ordinate shifts in the borders of neural identity gene expression in st2-brk embryos are consistent with the known ventral-dominant chain of repression among vnd, ind, and msh. This analysis also provides additional support for cis-acting vnd sequences being sensitive to BMP repression and suggests that the dorsal border of vnd expression is normally determined by balancing the opposing influences of Dorsal activation [18,29,37] and BMP-mediated repression. We note that the dorsal expansion of vnd expression in st2-brk embryos does not necessarily imply that vnd is more sensitive to BMP-mediated repression than ind or msh, but instead that at limiting levels of Dorsal, even low levels of BMP signaling can exert a repressive effect on vnd expression.

BMPs Act in a Dose-Dependent Fashion in Apolar Chick Neural Explants

Since Dpp was able to create elements of the neuroectodermal pattern in absence of other sources of DV polarity in Drosophila embryos, we wondered whether BMPs might have a similar organizing capacity in the vertebrate
neural tube. Previous work has revealed that BMP signaling can act in a threshold-dependent fashion to pattern neural identity gene expression in vertebrates, however, the ability of BMPs to create pattern along the full DV axis in the absence of other graded cues has not been tested. As in *Drosophila*, a confounding problem in studying patterning in the vertebrate neural tube is to isolate the threshold-dependent effect of BMP signaling from that of a ventral gradient of Shh, which contributes to establishing the ordered expression of ventral and intermediate genes such as Nksx2.2 and Pax6 (Figure 1C) [30].

To isolate the patterning effect of BMPs from that of Hedgehog (Hh) we used a strategy similar to that devised in *Drosophila* for creating a field of cells with uniform DV identity. Naive chick neural plate explants were treated with 5 nM Shh, which induces ventral cell fates (Figure 4). In this ventralized background, we added BMPs over a range of concentrations and determined the percentage of cells expressing Nksx2.2, Pax6, and Msx1/2 (Figure 4A). We assayed Pax6 expression in these experiments rather than the *ind* ortholog Gsh, since Gsh expression is initiated after neurogenesis in the chick, and because Pax6 may fulfill a similar role in specifying cell fates in the intermediate region of the neural tube [17]. In the absence of added BMPs, the explants only expressed the ventral marker Nksx2.2, which represses Pax6 expression [21]. As the BMP4 concentration increased, the percentage of cells expressing Nksx2.2 declined at the same time the fraction of cells expressing intermediate (Pax6) and dorsal (Msx1/2) markers increased (Figure 4A). Concomitant with the initial decrease in Nksx2.2-expressing cells, we observed an increase in the number of motor neurons, which differentiate in the ventral-intermediate neural tube, as measured by expression of Isl1/2 (unpublished data). At the highest levels of BMP4 tested, the great majority of cells expressed only the dorsal-most marker Msx1/2. In addition, a significant number of neural crest cells, which derive from the dorsal-most region of the neural tube, migrated from the explants as revealed by HNK-1 staining (unpublished data). These results demonstrate that normal patterning in the neural tube can be recapitulated in ventralized neural tissue by altering BMP4 levels alone. Although we have not observed repression of Msx1/2 at the highest dose of BMP tested, in zebrafish it has been suggested that high levels of BMP signaling may repress these genes [43]. It is unclear whether these differences reflect experimental design or inherent differences between organisms.

We further tested the organizing activity of BMPs by asking whether an ectodermal graft, which acts as a localized source of BMPs, could induce patterned expression of neural identity genes in chick neural plate explants. We placed ventralized Nksx2.2-expressing explants into contact with BMP-expressing ectodermal cells along one edge of the explant (Figure 4B). This experimental scheme, which is similar in concept to expressing a stripe of *dpp* in *sog*-lateralized *Drosophila* embryos, resulted in loss of Nksx2.2 expression and induction of concentric domains of Msx1/2 and Pax6 expression. In these grafted co-cultures, Msx1/2 was expressed in a band of cells in the neural plate explant closest to the ectodermal tissue (Figure 4B, top left), while strong Pax6 expression was excluded from cells near the source of BMPs, but was observed in a broad zone further away from the graft (Figure 4B, lower left). These results reveal that in

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**Figure 4. Dose-Dependent BMP-Mediated Repression in Neural Plate Explants**

(A) Chick intermediate neural plate explants were grown in 5 nM Shh and a range of BMP4 from 0 nM to 2.4 nM. Cells were stained with antibodies for Nksx2.2, Pax6, or Msx1/2 and number of positive cells per explant was counted and the percentage of positive cells was calculated and graphed. Error bars indicate standard error of the mean. The number of explants assayed was as follows, starting at 0 nM and ending at 2.4 nM BMP4: Nksx2.2 (25, 12, 15, 18, 12), Pax6 (18, 6, 18, 24, 30, 12, 24, 28), and Msx1/2 (12, 6, 16, 11, 17, 11, 17, 17).

(B) Intermediate chick neural plate explants (NP) with (left) or without (right) BMP-expressing non-neural ectodermal tissue (EC) placed on the top edge, and cultured in the presence of 5 nM Shh. The fraction of Msx1/2-expressing cells is highest near the BMP source (top, left), and diminishes as a function of distance from the ectoderm. In a separate neural plate ectoderm co-culture (bottom, left), Pax6 is expressed at a greater distance from the ectoderm, but not in nearby neural plate cells, which presumably express Msx1/2. These results mimic the relative expression domains of Msx1/2 and Pax6 in a stage 20 chick neural tube (far right).

(C) Simplified summary model indicating the proposed similarities in BMP-mediated patterning of the vertebrate and invertebrate neuroectoderm. Two processes collaborate to establish the pattern of neural identity gene expression in *Drosophila* and vertebrates: graded BMP signaling preferentially represses expression of ventral neural identity genes (left), which then engage in a chain of ventral-dominant repression wherein more ventral genes prevail in repressing the expression of more dorsal genes (right). The indicated inhibition of Msx1/2 by Pax6 remains hypothetical. Not indicated on this scheme are additional levels of cross-inhibition (e.g., Vnd inhibition of msh, late Ind repression of vnd, and Pax6 repression of Nksx2.2) [9,10,16,21,61,62], which are likely to help sharpen and refine the pattern created by the core mechanism of threshold-dependent BMP repression coupled to ventral dominance.

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the absence of other DV patterning cues, BMPs can diffuse over a long range into the neural plate explant and act in a dosage-sensitive fashion to pattern expression of neural identity genes in the same DV order as in the endogenous neural tube. We conclude that diffusion of BMPs alone can establish pattern along the full DV axis of the vertebrate neural tube.

Discussion

It is generally assumed that divergent upstream regulatory processes activate orthologous sets of neural identity genes in the same DV order [7,18]. This model is analogous to that for convergent regulation of HOX genes in a conserved order along the AP axis [22]. Two primary considerations have lead to the view that the conserved DV pattern of neural gene expression may have evolved independently in these two lineages, rather than reflecting a common ancestral state. First, until the current study, there has been no evidence that BMPs act in a dose-dependent fashion in Drosophila. Second, in vertebrates, where BMPs have been shown to act in a dose-dependent fashion to pattern the neural tube [33,34,43–49], a common view is that they promote expression of neural identity genes such as Msx1/2 in dorsal regions of the neural tube [33]. In flies, however, BMPs have only been observed to repress expression of neural genes, suggesting that the underlying mechanisms of neural gene regulation by BMPs may be fundamentally different [7,18].

An alternative hypothesis for neuroectodermal patterning that reconciles existing data is that BMPs act by a mechanism analogous to that operating in Drosophila, namely by preferentially repressing expression of more ventral neural identity genes. An attractive feature of this simple unified model is that the mechanism of BMP action is the same during both neural induction and neuroectodermal patterning. The only difference is that during the latter process, BMPs diffusing into the neuroectoderm are present at much lower levels, with the effect that the repression of neural gene expression becomes dependent on BMP dosage.

Dpp Mediates Dose-Dependent Repression of Neural Identity Genes in Drosophila

In this study, we present two lines of evidence that BMPs act in a dosage-dependent fashion to help pattern the Drosophila neuroectoderm. First, we find that a localized source of Dpp is capable of creating patterned expression of msh and ind in lateralized embryos with no other known source of DV patterning. Second, we show that localized inhibition of BMP signaling in embryos with a normal Dorsal gradient shift all three borders of neural identity gene expression dorsally. The simplest explanation for these results is that BMP signaling represses neural gene expression in the neuroectoderm, as it does earlier during neural induction. However, in contrast to neural induction, where the high levels of BMP signaling present in the epidermal ectoderm completely abolish the expression of all neural genes, the considerably lower levels of BMPs present in the neuroectoderm act in a threshold-dependent fashion to create separate domains of neural gene expression. Neural identity genes expressed more ventrally are most susceptible to this graded BMP repression, whereas those expressed in more dorsal positions are progressively less sensitive. Ventral-dominant cross-repression among neural identity genes, which is a well-established phenomenon in Drosophila akin to posterior dominance among HOX genes along the AP axis, helps refine the pattern into mutually exclusive domains of neural identity gene expression. These findings demonstrate that as in vertebrate embryos, BMPs act in a dosage-sensitive fashion to contribute to neuroectodermal patterning.

This model of threshold-dependent BMP-mediated repression is also consistent with the previous observation that Screw, another BMP that acts synergistically with Dpp in embryos, is required to repress msh expression in dorsal cells (C. M. Mizutani, unpublished data). Interestingly, other neural genes such as those of the Achaete-Scute complex are derepressed in dpp− embryos, but not in sav− mutants [27], providing a further indication that higher levels of BMP signaling are required to repress msh expression than other neural genes.

A Conserved Ancestral Role of BMPs in Patterning the Neuroectoderm?

The results we obtained in lateralized chick neural plate explants are strikingly similar to those observed in lateralized Drosophila embryos. We observed that increasing concentrations of BMPs progressively favors the expression of dorsal over ventral neural identity genes. We also found that a graft of BMP-expressing cells could organize graded spatial expression of dorsal and lateral markers in lateralized neural plate explants in much the same way that a localized source of Dpp can create pattern in the context of uniform levels of Dorsal in lateralized Drosophila embryos. These findings are consistent with prior evidence that BMPs act in a dose-dependent fashion to pattern gene expression in dorsal and lateral regions of the vertebrate neural tube [33,34,43–49]. Previous studies also provided evidence that BMPs extend their influence into ventral regions of the neural tube and in conjunction with graded levels of Shh can cause a ventral-to-dorsal shift in the identities of neural progenitor cells [32,50–52]. In our current study we extend these findings by showing that BMPs can act in the absence of other patterning cues to mediate normal patterning of the neural tube, including the induction of motorneuron-specific markers. In addition, we show that a localized source of BMPs from the ectoderm is capable of establishing a graded response in apolar neural plate explants over long range. These data, in principle, could be explained by a model similar to that proposed above for Drosophila, in which BMP signaling acts by repressing neural gene expression (Figure 4C).

The view that BMPs may repress neural gene expression in vertebrates runs contrary to the currently favored model in which BMPs activate expression of target genes [33,34]. Evidence cited in favor of the “activation” model include the fact that over-expression of BMPs in Xenopus can induce Msx1 expression in the presence of cyclohexamide, suggesting a direct activation of Msx1 by BMPs [53]. Also, Msx1 and Msx2 promoter elements have been identified that mediate BMP-dependent activation of reporter gene constructs [54–56].

There are important caveats, however, to the interpretation that BMPs activate expression of Msx genes in the neural ectoderm, which stem from the fact that the Msx1/2 genes are expressed in complex patterns that include non-neural as well as neural components. For example, in Xenopus and zebrafish embryos, Msx1 is expressed in two stripes of cells, one in ventral ectodermal cells and the other dorsally at the
border between the ectoderm and neural ectoderm. Since the strongest expression is detected ventrally, it was not possible in the original experiments to distinguish between Msx1 activation by BMPs in ventral versus dorsal cells [53]. The observation that the dorsal domain of Msx1 expression is graded ventrally and is lost in more central regions of the non-neural ectoderm [53] suggests that high levels of BMP signaling found in those regions may actually play a role in repressing Msx1/2 expression. Consistent with this possibility, when BMP signaling was blocked selectively in cells giving rise to the dorsal Msx1 domain in zebrafish, Msx1 expression expanded [43]. This finding suggests that the Msx1/2 and msh genes may be similarly regulated by BMPs in both vertebrates and flies, wherein high levels of BMP signaling repress rather than activate expression of these orthologous genes. Interestingly, BMPs have also been proposed to repress the expression of Pax6 in the forebrain [57–59] and to potently repress the lateral expression of Dbx1/2 genes in the neural tube [60].

It is also noteworthy that while minimal Msx enhancer elements have been described to respond positively to BMP signaling and drive parts of the endogenous gene expression profiles, none of these elements drive expression in the dorsal region of the neural plate [54–56]. Thus, it remains to be determined whether Msx1/2 genes are activated directly by BMP signaling in the dorsal neural tube or indirectly by relieving cross-inhibition mediated by more ventrally expressed transcription factors such as Gsh, Pax6, or Dbx1,2. Further analysis of regulatory elements directing faithful expression of Msx1/2 and other neural identity genes in the neuroectoderm will be required to address this question.

Based on the considerations raised above, we favor a parsimonious model in which BMPs function similarly during patterning of the neuroectoderm in vertebrates and invertebrates. According to this hypothesis, an ancestral cascade of sensitivity of neural identity genes to BMP-mediated repression has been preserved intact in vertebrates whereas in Drosophila, only the two dorsal-most genes (ind and msh) may have remained differentially sensitive to BMP repression. Although the relative sensitivity of vnd and ind to BMP repression in Drosophila is unresolved, it is nonetheless apparent that along the dorsal border of vnd expression, where levels of the Dorsal activator are limiting, BMP signaling plays a role in opposing Dorsal activation of vnd. In the absence of BMP repression, vnd expression expands dorsally 1–2 cells, where it presumably represses ind expression by virtue of ventral dominance. As mentioned above, it may also be the case that in invertebrates the Msx1/2 genes have become relatively less sensitive to BMP repression since Msx1 is expressed in neural crest forming cells of the non-neural ectoderm immediately adjacent to the neural plate [53].

Mechanisms for Refining the Graded BMP Response

The graded positional information created by BMP signaling in the neuroectoderm is subsequently refined by cross-inhibitory interactions among neural identity genes. The result of these interactions is to segregate gene expression patterns into non-overlapping adjacent territories. In Drosophila, cross-regulatory interactions follow a strict ventral-dominant hierarchy in which ventral genes repress more dorsal ones. In vertebrates, neural identity genes also cross-inhibit each other [21,61,62], although it has not been resolved whether these interactions are biased along the DV axis. There is some evidence that also favors ventral dominance in vertebrates, however. For example, Nkx2.2, can inhibit the ectopic expression of Pax6 in ventral neural tube cells in which Hh signaling has been blocked (e.g., see Figure S3). In addition, in Xenopus tropicalis, it has recently been observed that mis-expression of the Gsh2 gene in the neural plate represses dorsal expression of Msx1, but not ventral expression of Nkx6.1 (H. Isaacs, unpublished data), consistent with these transcription factors acting in a ventral-dominant fashion as they do in flies. While additional studies will be required to establish whether mutual cross-inhibition among vertebrate neural identity genes follows a clear ventral hierarchical order, such interactions, even if symmetrical, would nonetheless act to resolve biases in gene expression created by a graded response to BMPs. Consistent with a conserved function of BMP signaling in the neuroectoderm, we find that BMPs act in a dose-dependent fashion in lateralized apolar chick neural plate explants to create patterned expression of neural identity genes in much the same way they do in lateralized Drosophila embryos.

Another mechanism for shaping the BMP activity and refining the neural domains may be ventral expression of BMP antagonists. In Drosophila, Sog initially occupies the entire neuroectoderm (Figure 1D) and then fades progressively from dorsal cells as msh expression is initiated (Figure 1D and 1E) [38,39]. This trend continues until sog expression is confined to the ventral midline. As sog expression fades dorsally, it may create a broader domain of elevated BMP signaling sufficient to repress ind and to permit msh activation. In sog—lateralized embryos, the separation of msh and ind domains is less defined than in wild-type embryos, which may result from the lack of ventral Sog and Brk gradients that normally contribute to refining these expression domains. In addition, some cells co-express both msh and ind, albeit few cells express these genes at comparable levels. It may be that a gradient of Dorsal is required to establish full ventral dominance under these conditions. Alternatively, ventral dominance may normally depend in part on the progressive temporal activation of neural identity genes in a ventral-to-dorsal pattern, which may help more ventral genes consolidate their expression domains. In vertebrates, BMP antagonists such as Chd and Noggin emanating from the notochord [32,63] (Figure 1C) may likewise help sharpen the BMP gradient ventrally.

Secondary Evolution of Species-Specific DV Patterning Systems

One marked difference in the integrated systems acting to pattern the neuroectoderm in flies and vertebrates is that distinct pathways have evolved in addition to the proposed conserved core of BMP-mediated patterning. While BMPs appear to play a major if not predominant role in specifying cell fates in dorsal regions of the fly and vertebrate neuroectoderm, the relative importance of these signaling systems is reversed in ventral regions where Dorsal and Shh play primary patterning roles in flies and vertebrates respectively. In the lateral column, a combination of BMPs and ventral patterning cues (Dorsal and Shh) act in concert to determine the position and extent of gene expression.

The deployment of Shh in patterning the ventral neural tube appears to be a vertebrate innovation since no ventral source of Hh signaling is observed in Drosophila [7] or in basil
arthropods such as millipedes [64]. Graded Hh signaling may have arisen during the course of chordate/vertebrate evolution to supplement a pre-existing long range BMP signaling system. The secondary recruitment of Hh signaling to DV patterning is further supported by the finding that in the complete absence of Hh signaling, ventral and intermediate neural cell types still form. Interestingly, Nkx2.2 expression is not observed in such embryos, which may be due to the loss of notochord-derived BMP antagonists [65–69]. It has been suggested that the restoration of ventral pattern in the absence of Hh signaling could be provided by the remaining BMP gradient [19]. Furthermore, the notochord-derived BMP antagonists Noggin and Chd can act synergistically with Shh [30]. It has been suggested that the restoration of ventral pattern in the absence of Hh signaling could be provided by the remaining BMP gradient [19]. Furthermore, the notochord-derived BMP antagonists Noggin and Chd can act synergistically with Shh in the neural tube to promote ventral cell fates, including Nkx2.2 expression [31,32]. These observations are consistent with evidence that BMPs exert an influence along the entire dorsal-ventral axis of neural tube [34,46].

The NFXβ-related transcription factor Dorsal may also have been co-opted to assume a prominent role in patterning the ventral portion of the Drosophila neuroectoderm, leading eventually to a reduction in the peak sensitivity of vnd to BMP repression. In addition, Dorsal is likely to act in conjunction with its target genes Sog and Brk to refine the ind and msh expression domains. Perhaps these later lineage-specific adaptations evolved to help sharpen and reinforce the effect of BMP signaling, particularly at the low end of the concentration gradient. One potential evolutionary pressure in creating a secondary ventral organizer may have been an increase in body size. It has been proposed that early bilateralia may have been only a few millimeters or less in size (for reviews see [70,71]). In organisms of such small dimension, a single morphogen may have formed a sufficiently steep concentration gradient over short distances to reliably subdivide the entire DV axis into discrete domains. As larger organisms evolved during Precambrian radiations, however, a single morphogen may no longer have been sufficient to provide the same detailed patterning information. Although, as mentioned above, BMPs are apparently able to diffuse substantial distances into ventral regions of the vertebrate neural tube, such gradients may be too flat in distant regions to produce sharp patterns. Indeed, in animals lacking all Hh signaling, while ventral genes are expressed in approximately normal domains, these expression patterns are not as sharp as in wild-type embryos.

In view of the fact that BMP signaling plays an important role in patterning the non-neural ectoderm as well as the neuroectoderm of Drosophila and vertebrates, we propose that graded BMP signaling created by opposing sources of BMPs and BMP-inhibitors such as Sog/Chd (Figure 3C) may once have been sufficient to establish ordered domains of gene expression along the full DV axis of the ectoderm. According to this model, high BMP levels in the non-neural ectoderm were sufficient to suppress expression of all neural genes. Diffusion of Sog/Chd into this region created graded high-level BMP signaling, which lead to threshold-dependent activation of BMP target genes and subsequent partitioning of cells into discrete territories. In the neuroectoderm, where graded levels of BMPs became limiting, threshold-dependent repression of gene expression provided the spatial information for subdividing that region into three primary domains. Thus, by a combination of threshold-dependent activation and repression of target genes in epidermal versus neural regions of the embryo, BMPs may have once organized the entire DV axis of ancestral metazoa.

Materials and Methods

Drosophila stocks and genetic crosses. The following stocks were used in this study: Df(2L)DTD48/CyO23 (deficiency for dpp, yw; s2–dpp provided by H. Ashe), w; Fpl1, and w; s2–FRT-stop–FRT–brk (provided by S. Small). We briefly provide a summary of the maternal mutations used to generate lateralized embryos with uniform levels of Dorsal. The Tl1 mutation encodes a partially activated form of Toll receptor capable of transducing the signal for Dorsal transport into all nuclei independently from the ligand Spa¨tzle. gastrulation defective is an upstream component of the Toll pathway required for the production of Spa¨tzle. Combining gd, a null allele of gastrulation defective, with Tl1 in females leads to the production of embryos with uniform levels of Dorsal, since the remaining wild type copy of Toll is no longer activated by ligand. The levels of Dorsal in gd; Tl1/+ embryos correspond to those specifying the ventral neuroectoderm (e.g., expressing vnd uniformly). Further reduction of Dorsal levels in gd; Tl1/homozygous females results in the production of embryos with uniform levels of Dorsal, since the remaining wild type copy of Toll is no longer activated by ligand. The levels of Dorsal in gd; Tl1/homozygous females correspond to those specifying the ventral neuroectoderm (e.g., expressing vnd uniformly). An alternative way to generate ventro-lateralized embryos is to collect them from mothers homozygous from the weakly activated Tl1 allele. In some experiments it was also necessary to eliminate zygotic function of sog or sog and brk in embryos using respectively the sog2 or brk78/I1 alleles. Mutant genotypes described in text were generated according to the following crosses (recessive markers omitted for clarity): to generate gd; sog2–1/td; gd; Tl1/+; dlp/O females were crossed to gd females; Tl1/TM3, 3b males (Figure 1D, Figure 2); to generate gd; sog2–1/td; Tl1/+ females for producing mid-lateralized sog– male embryos, gd; sog2–1/td; dlp/O females were crossed to gd; Tl1/Y; Tl1/TM3, 3b males (Figure 1D, Figure 2); to generate gd; sog7/7; Tl1/+ females for producing ventro-lateralized sog– male embryos, gd; sog7/7; dlp/O females were crossed to gd; Tl1/Y; Tl1/+ males (Figure 3B and 3C), and to generate sog7/7; brk/Y; FG7; Tl1/+ females for producing ventro-lateralized sog– brk– male embryos used in the experiments described in Figure 3D and 3E, sog7/7; brk/Y; FG7; Tl1/TM3, 3b stock. Females collected from crosses described above were crossed to w males (control) or to yw; s2–dpp males (experimental), and embryos lacking sog mRNA expression were analyzed for other gene expression patterns. Other standard mutant stocks were obtained from the Bloomington Stock Center (Bloomington, Indiana, United States) and are described in Lindsey and Zimm [72] or Flybase (http://flybase.bio.indiana.edu).

Immunofluorescence and in situ hybridization. Fluorescent multiplex in situ hybridization methods used to detect multiple RNA transcripts are described in detail in Kosman et al [42]. For double protein detection and in situ staining, fixed embryos were treated with acetone at 4°C, dehydrated and with probes, followed by immunostaining with rabbit anti-phosphoMOD (1:2,000, a gift from P. ten Dijke) and antibodies were used to detect the RNA labeled probes. Detection of primary antibodies was performed either with secondary antibodies labeled with Alexa Fluor dyes (used at 1:500, Molecular Probes). A list of primary and secondary antibodies used in these experiments is available in the online supplementary material of Kosman et al [12]. Images of fluorescently labeled embryos were acquired on a Leica SP2-AOBS (Leica Microsystems, Wetzlar, Germany) scanning confocal microscope with 20x or 40x objective lenses.

Culture and immunofluorescence staining of chick neural plate explants. Chick neural plate explants were cultured in collagen pillows as described in [73] for 28 h with 5 nM Shh provided as described in [74] and in the absence or presence of varying concentrations of human recombinant BMPs (provided by S. Small). We briefly provide a summary of the BMPs used to create homogeneous or non-homogeneous BMP gradients in these embryos. BMPs were added to the embryo culture media at 100 ng/ml. In some experiments, BMPs were added at 4 h of culture to ensure that the BMP concentration is constant throughout the duration of the experiment.

Distribution of Sog/Chd into this region created graded high-level BMP signaling, which lead to threshold-dependent activation of BMP target genes and subsequent partitioning of cells into discrete territories. In the neuroectoderm, where graded levels of BMPs became limiting, threshold-dependent repression of gene expression provided the spatial information for subdividing that region into three primary domains. Thus, by a combination of threshold-dependent activation and repression of target genes in epidermal versus neural regions of the embryo, BMPs may have once organized the entire DV axis of ancestral metazoa.
Supporting Information

Figure S1. BMP Represses ind at a Longer Range than msh
A sog--; s2-dpp embryo with an intact Dorsal gradient stained for dpp (blue), ind (green), and msh (red). ind is repressed in a broad domain (bars) from the source of dpp expression. In regions of strong ind repression, msh expression invades ventrally into the r2 domain (arrows).

Figure S2. Differential Sensitivities of ind and msh Regulation to BMP Repression
A sog-beh--; Dp(2;2) DTD48 embryo which has normal DV polarity and high levels of Dpp within the neuroectoderm. Low levels of msh expression (red) can be detected within the intermediate neuroectoderm domain, where ind expression (green) is largely lost.

Figure S3. Ventral Expansion of Pax6 in Response to Inhibition of Shh Signaling is Mediated by Repression of Nkx2.2
(A) Mis-expression of the Gli3 repressor (Gli3R) causes cell autonomous expression of Pax6 as well as repression of Nkx2.2 (unpublished data).
(B) Co-expression of Nkx2.2 with Gli3R reverses Pax6 activation by Gli3R.

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