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Dorsal–ventral patterning: a view from the top

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The generation of dorsal interneurons in the spinal cord is dependent upon specific signaling pathways and the subsequent establishment of progenitor domains mediated by cross-repressive interactions of different groups of transcription factors. These events lead to the implementation of specific differentiation programs that direct the development of distinct dorsal interneuron subtypes. Recent studies have taken advantage of complementary gain and loss-of-function studies in the chick and mouse to clarify the *in vivo* roles of transforming growth factor β signaling, basic helix-loop-helix and homeodomain transcription factors in dorsal interneuron development. The challenge now lies in identifying the precise molecular mechanisms involved and applying these insights to understanding how more ventrally located dorsal interneurons are specified.

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Introduction

The nervous system is a complex network of interacting neural circuits that utilize a vast and diverse array of neurons to integrate and disseminate information. In vertebrates, many of the specialized characteristics unique to different groups of neurons are acquired during embryogenesis [1,2]. Much of our understanding of how this occurs comes from studies of cell fate specification in the developing spinal cord [2–4].

A considerable amount of work in several organisms, in particular the chick and mouse, has led to the current model of how different spinal neurons are generated in vertebrates (Figure 1a) [2–4]. Initially, two morphologically distinct signaling centers are established at opposite ends of the dorsal–ventral axis of the developing neural tube. The floorplate is induced ventrally by axial meso-

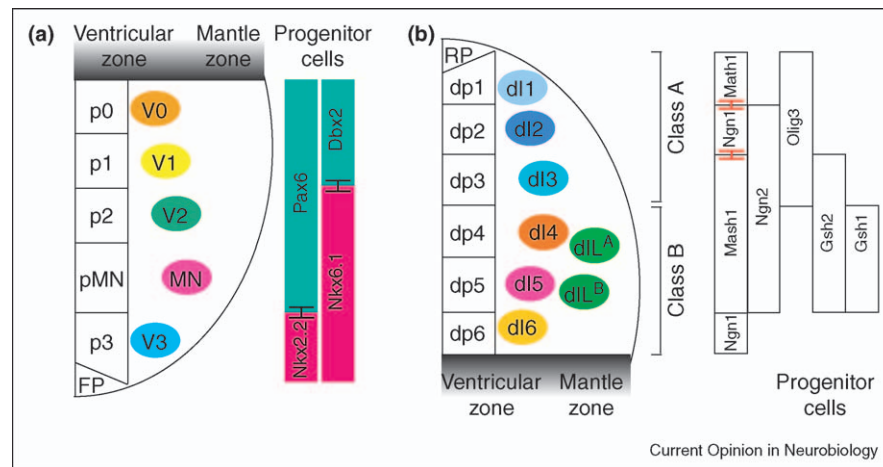
dermal structures, whereas the roofplate is generated dorsally by signals from the overlying ectoderm [2,5]. Both of these structures secrete molecules that act non cell-autonomously to regulate multiple aspects of neuronal development. The floorplate generates a gradient of *sonic hedgehog* (*shh*) that is interpreted to establish five progenitor domains (p0–p3, pMN; p, progenitor; MN, motor neuron) that express different combinations of Class I and Class II homeodomain (HD) transcription factors. The sharp boundaries between these domains are established and maintained through selective cross-repressive interactions between complementary pairs of Class I and II proteins. Progenitor cells subsequently differentiate into molecularly distinct neuronal subtypes (V0–V3, MN) and the coordination of neuronal subtype specification and differentiation is regulated by HD transcription factors and basic helix–loop–helix (bHLH) proteins. Thus, the initial location of a cell along the dorsal–ventral axis is a crucial component for the acquisition of neuronal identity.

How neuronal diversity is achieved dorsally is not as well understood. Insight gleaned from ventral patterning studies has helped to provide a framework to investigate how dorsal progenitors and dorsal interneurons (dI) are specified in the neural tube. In this review, we discuss studies during the past two years that have exploited *in vivo* functional assays in the mouse and chick that help us understand how different classes of dIs are generated during embryonic development.

Several extrinsic signals are necessary for dI specification

dIs are divided into six early born (dI1–6) and two late born (dIL^A and dIL^B) neuronal subtypes based on their birthdate, dorso–ventral position and HD protein expression profile (Figure 1b) [5]. They are broadly categorized into two groups; Class A neurons consist of the dorsal most dI1–3 subtypes and rely on roofplate-derived signals for their development, whereas the more ventrally located Class B neurons comprise dI4–6 and dIL^{A/B} subtypes and their generation is dependent on alternative sources of signals [6–10]. The roofplate produces at least two major classes of signaling molecules: members of the transforming growth factor β (TGF β) family (activin, bone morphogenetic protein 4 [BMP4], BMP5, BMP7, dorsalin1 and growth differentiation factor7 [GDF7]) and wingless-related mouse mammary tumor virus integration site proteins (Wnt1 and Wnt3a) [6,9–11]. Loss-of-function studies in the mouse have shown that GDF7 and Wnt proteins are required for generating Class A neurons but not Class B neurons, which is consistent with the

Figure 1



Neuronal cell types in the spinal cord. **(a)** In the ventral spinal cord, five progenitor domains (p0–3 and pMN) located in the ventricular zone generate five distinct mature neuronal subtypes (V0–3 and MN) occupying the mantle zone. The boundaries of progenitor domains are maintained and refined by selective cross repression between pairs of Class I and Class II homeodomain (HD) transcription factors. Shown here are two examples. Class I HD proteins Pax6 and Dbx2 cross repress complementary Class II HD proteins Nkx2.2 and Nkx6.1, respectively. **(b)** In the dorsal spinal cord, six progenitor domains (dp1–6) generate six early born (dl1–6) and two late born (dlL^A and dlL^B) dorsal interneurons. These eight subtypes are categorized into two classes: roofplate-dependent Class A (dl1–3) and roofplate-independent Class B (dl4–6 and dlL^{A/B}) neurons. Unlike the ventral spinal cord, basic helix–loop–helix (bHLH) transcription factors have a predominant role in patterning progenitor domains. Cross repression between Math1, Ngn1 and Mash1 are essential for delineating boundaries of Class A dorsal progenitors (dp1–3). Other progenitor markers are also shown: Ngn2 in dp2–5, Olig3 in dp1–3, Gsh2 in dp3–5 and Gsh1 in dp4–5. Abbreviations: FP, floorplate; RP, roofplate.

development of Class B neurons being independent from roofplate-derived signals [6,9]. However, recent work suggests that Wnts might have a mitogenic function rather than a role in patterning [12,13^{*}].

Investigating the role of BMPs in dI generation by similar means has not been as informative because of the problems of redundancy and of early lethal phenotypes [14]; however, alternative approaches have recently shed light on how BMPs contribute to dI development *in vivo*. In the chick, overexpression of constitutively active BMP type 1 receptors (Bmpr1) by *in ovo* electroporation was found to increase dI1 progenitors and dI1 interneurons at the expense of other classes of dIs, in a process that might be mediated by the transcription factor Msx3 [15^{*}]. Lower amounts of activated BMP receptor, however, induced a more ventral dI3 subtype [16], consistent with a requirement for graded BMP signaling in Class A neuronal generation. Conversely, inhibition of BMP signaling by expression of the BMP antagonist noggin or by neural tube-specific inactivation of both Bmpr1a and Bmpr1b receptors resulted in reduced numbers of dI1 and dI2 neurons, underscoring a role for BMPs in Class A neuronal development [13^{*},17^{*}]. Interestingly, dI3 neurons are still generated in the receptor knockout mice, raising the possibility that other signals might operate to impose dI3 subtype identities. Given that activin signaling can promote dI3 interneuron differentiation independently of BMPs, one possibility is that both BMPs and activin signaling might contribute to the specification of

dI3 neurons *in vivo* [18^{*}]. This is consistent with the observation that short interfering RNA (siRNA)-dependent knockdown of Smad4, which can mediate both BMP4 and activin signaling, results in the decrease of dI3 neuronal subtypes [13^{*}]. Taken together, these studies demonstrate a function for BMPs in Class A neuronal generation. However, they also highlight the inherent complexity of the signaling mechanisms involved in roofplate-dependent specification of Class A neurons, which is in stark contrast to the central role occupied by floorplate-derived *shh* in patterning the ventral spinal cord [2–4].

The signals involved in Class B neuronal development remain undetermined. Class B neurons are prevented from developing in the dorsal most regions of the spinal cord by the action of TGFβ signaling [16,19,20]. This has provoked discussion as to whether a Class B fate is a default state in the dorsal spinal cord or if instructive signals are required for Class B neuronal specification. What other molecules might contribute to dI generation? Candidates include retinoic acid (RA), which is required for the generation and specification of V0 and V1 ventral interneurons that neighbor the Class B neurons and of more ventrally located motor neurons [21–25]. Studies of vitamin A deficient quails show a reduction of dorsal and ventral markers, suggesting a role for RA signaling in dorsal patterning. However, the basis for the RA-dependent dorsal phenotypes remains unclear and further investigation is required to define specific roles for RA signaling in dI generation [26^{*}].

Factors mediating Class A versus Class B differentiation

An obvious first step in deciphering the molecular mechanisms that direct the development of Class A and Class B neurons is in the identification and characterization of molecules that are differentially expressed in these neuronal groups. Previous studies have shown that the HD protein Lbx1 is expressed specifically in postmitotic Class B neurons. Loss and gain of function studies in the mouse and chick, respectively, indicate that Lbx1 acts dually to repress Class A neuronal specification programs while promoting Class B fates in postmitotic dIs [19,20]. These studies have led to the idea that dIs remain plastic in their fates even after they have exited the cell cycle, a property that is shared by ventrally located motor neurons that depend on HD proteins for their specification [2]. They also raise the possibility that similar postmitotic determinants might exist for Class A neurons, although such factors have not been identified to date. Furthermore, are there factors that act earlier to distinguish progenitor cells that will give rise to Class A and Class B neurons? Such factors have not been isolated for Class B progenitors but a recent study has identified a gene that imposes Class A character on dorsal cells [27**].

Expression analyses combined with genetic lineage tracing experiments demonstrate that the bHLH protein Olig3 is expressed in dorsal progenitors that give rise to all dI1–3 Class A neurons (Figure 1b). Targeted deletion of Olig3 in the mouse results in severe deficits in Class A neurons that are accompanied by the dorsal generation of ectopic dI4-like neurons (Figure 2a,b) [27**]. These findings suggest that Olig3 is required for Class A neuronal generation and might function by suppressing the emergence of Class B neurons. Analysis of Lbx1 and

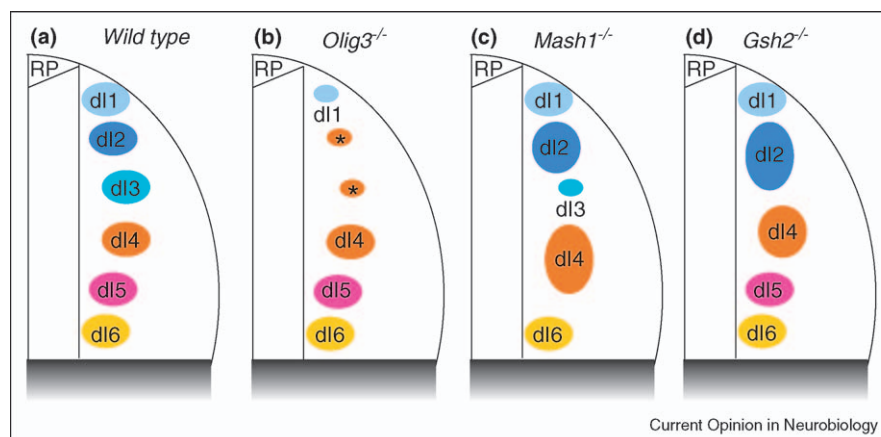
Olig3 double mutants showed rescue of dI1 and dI2 but not dI3 neurons, suggesting that Olig3 does not solely suppress Class B differentiation programs dorsally, but can direct the generation of dI3 subtypes. Consistent with this hypothesis, overexpression of Olig3 in the chick dorsal spinal cord resulted in an increase of dI3s over other Class A neuronal subtypes and at the expense of Class B neurons [27**].

How might Olig3 function in progenitors to influence the generation of Class A neurons? Dorsal progenitors can be partly classified by the non-overlapping expression of bHLH transcription factors that act as crucial determinants of dI identity; specifically, Math1 expression marks progenitors that will give rise to dI1 subtypes [28], whereas expression of Ngn1/2 and Mash1 delineate progenitor domains that will generate dI2 and dI3–5 subtypes, respectively (Figure 1b) [29]. In Olig3 mutants, expression of Math1, Ngn1 and Ngn2 was reduced in progenitors located within the dI1 and dI2 domains, thus explaining the dearth of dI1 and dI2 subtypes in these mice (Figure 2a,b). Overexpression of Mash1 and Olig3 together greatly increases the number of dI3s compared with Mash1 or Olig3 overexpression alone. This suggests that Olig3 and Mash1 function together in progenitor cells to impose subsequent dI3 fates [27**], but whether these effects are additive or synergistic in nature remains unclear. Furthermore, ectopic dI3s are generated predominantly in the dorsal rather than the ventral spinal cord, indicating that other signals are acting to constrain their development dorsally.

bHLH and HD proteins in dI3–dI5 generation

Superimposed upon the regulatory mechanisms specifying Class A and Class B neuronal fates are distinct dI

Figure 2



Phenotypes pertaining to dorsal interneuron (dI) development in wild type and knockout mouse embryos. **(a)** In wild type spinal cord, six early dI subtypes are generated. **(b)** In Olig3 mutants, dI1 neurons are reduced and dI2–3 neurons are mis-specified to a dI4-like identity (*), whereas Class B dI interneurons remain unaffected. **(c)** In Mash1 mutants, dI3 neurons are dramatically reduced and dI5 neurons are absent, whereas neighboring dI2 and dI4 neurons are increased. **(d)** In Gsh2 mutants, dI3 neurons are selectively lost, whereas dI2 and dI4 neurons are expanded.

differentiation programs controlled by bHLH determinants that are regionally expressed in progenitors (Figure 1b). Cross-repressive interactions between these proteins are thought to refine dorsal progenitor domains, reminiscent of the HD protein interactions that operate ventrally [5].

The position of dorsal progenitors along the dorsal-ventral axis largely correlates with the differentiation of specific dI subtypes before their migration, as in the case of dI1 and dI2 neurons [5]. Thus, dI3–dI5s have been largely assumed to derive from Mash1⁺ progenitor cells. However, analyses of Mash1 knockout mice reveal a requirement for Mash1 in the development of dI3 and dI5 but not dI4 neurons (Figure 2c) [30^{••},31^{••}]. Lineage studies show that dI3 and dI5 progenitors express Mash1 but that dI4 progenitors express low to undetectable levels of the protein, underscoring the value of such analyses in assigning progenitor–neuron relationships [30^{••}]. Consistent with a role for Mash1 in dI3 and dI5 specification, overexpression of Mash1 in the chick by *in ovo* electroporation resulted in increased levels of dI3 and dI5 subtypes at the expense of dI2 and dI4 neurons [30^{••},31^{••}]. Interestingly, the correct numbers of dI3 and dI5 neurons that are generated might require the later function of the bHLH protein Ngn2, the expression of which spans the dI2–dI5 region [30^{••}]. Although functionally downstream of Mash1, how Ngn2 interacts with Mash1 to generate appropriate numbers of dI3 and dI5 neurons remains unclear. A surprising feature of the Mash1 knockout phenotype is that dI4s are increased in the mutants but not as a result of the conversion of presumptive dI3 and dI5s to dI4 fates. Why this is the case is not known and awaits further identification of molecular determinants of ventrally located dI4 subtypes.

The development of dI3 and dI5 neurons is particularly interesting as they are differentially dependent upon TGFβ signals for their generation, but both derive from Mash1 expressing progenitors (Figure 1b). Expression analysis of the early patterning HD proteins Gsh1 and Gsh2 reveals that dI3 neurons express Gsh2, whereas dI4–5 neurons co-express Gsh1 and Gsh2 [31^{••}]. In Gsh2 mutants, dI3 neurons are selectively ablated with a concomitant increase in dI2s, whereas dI4–dI6 neurons develop normally (Figure 2d). The increase in dI2 subtypes at the expense of dI3s is attributed to the ventral expansion of the dI2 determinant Ngn1 [29] within the presumptive dI3 domain, given that Ngn1 is capable of promoting the differentiation of dI2 subtypes and repressing Mash1 expression [31^{••}]. Thus, Gsh2 appears to function downstream of TGFβ signals by repressing Ngn1 and, therefore, enabling the expression of the dI3 determinant Mash1 in presumptive dI3 progenitors. Analysis of Gsh1 and Gsh2 mutants suggest that Gsh1 and Gsh2 might have redundant functions in the generation of dI4 and dI5 fates but how this occurs is not clear [31^{••}]. In

addition to defining roles for bHLH and HD proteins in dI generation, these studies are beginning to uncover a possible transcription factor code for dI neuronal specification.

Conclusions and future directions

The large number of signaling molecules implicated in dorsal patterning promises an intricate and complex network of downstream events that mediate dI differentiation. These studies together with previous work highlight a recurrent theme in neuronal fate determination in the spinal cord, namely the use of cross-repressive interactions between proteins to establish progenitor domains. Here, both bHLH proteins and HD proteins are required for demarcating the expression domains of determinants of dI subtypes within progenitors and suggest a possible code for dI neuronal differentiation. Although many of these analyses focus on dI1–dI3 generation, these studies also highlight how little is known about the development of more ventral dI subtypes. The further identification and characterization of factors that mark dI4–6 progenitors and neurons will greatly assist in understanding how these classes of neurons develop.

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