
Article:
Placzek, M. and Furley, A. orcid.org/0000-0001-5549-8668 (1996) Neural development: Patterning cascades in the neural tube. Current Biology, 6 (5). pp. 526-529. ISSN 0960-9822

https://doi.org/10.1016/S0960-9822(02)00533-X

Neural development: **Patterning cascades in the neural tube**

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The vertebrate central nervous system comprises an intricate array of neurons generated in a highly organized way. Examination of the genes expressed and required at early stages of neural differentiation reveals that a coordinated signalling cascade transforms progenitor cells into discrete neuronal subsets.

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Current Biology 1996, Vol 6 No 5:526–529

During vertebrate embryonic development, a portion of the ectoderm (outer layer) is set aside to make neural tissue. These cells initially form a 'neural plate', which later folds into a tube that will form the spinal cord and brain (Fig. 1). As the folding occurs, precursor cells in the neural plate are transformed into different neuronal subsets. Surrounding non-neural tissues initially polarize and pattern the neural plate and neural tube. A key feature of these initial events appears to be the establishment of polarizing centres within neural tissue itself.

A wealth of evidence has shown that mesodermal cells of the notochord underlying the midline of the neural plate initially provide signals that 'ventralize' the developing neural tube; they induce the differentiation of cells of the 'floor plate' in the neuroepithelium immediately adjacent to the notochord, and of motor neurons in ventral neuroepithelium some distance from the floor plate (Fig. 1) [1–4]. A secreted protein, sonic hedgehog (SHH) is expressed both in notochord cells and in their precursors. SHH appears to ventralize the neural tube, mediating the induction of both floor plate cells and motor neurons [5–9]. Key amongst the molecules induced in ventral midline floor plate cells is SHH itself; it appears to confer on these cells the same ability to induce both floor plate and motor neurons, and thus establishes a continuing source of a ventralizing signal within the neural tube.

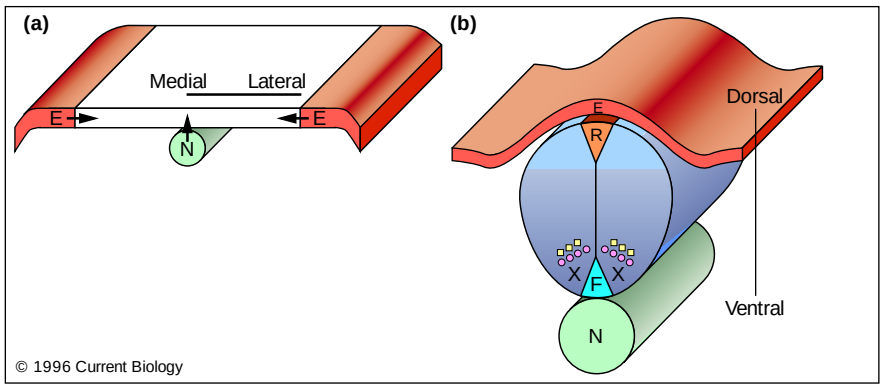
Concomitant with the ventral polarization of neural tissue, signals from the epidermal ectoderm adjacent to the lateral edge of the neural plate seem to impose dorsal pattern [10]. Members of the TGFβ superfamily of signalling proteins, including the 'bone morphogenetic proteins' BMP4 and BMP7, are expressed in epidermal ectoderm and may mediate its dorsalizing activity. Both BMP4, BMP7 and a third family member, Dorsalin-1, are subsequently expressed by cells in the dorsal aspect of the neural tube, suggesting that an initial dorsalizing stimulus is provided by non-neural tissue, but that a sustained presence is then supplied by neural cells themselves (Fig. 1) [10]. Thus, both ventralizing and dorsalizing centres may be established within neural tissue at early stages of its differentiation.

Range of action of ventralizing and dorsalizing signals

Both SHH and members of the TGFβ superfamily have features characteristic of secreted molecules; nevertheless, the extent of their diffusion through the neuroepithelium remains unclear. SHH has been localized by immunolabelling, but to date it has been detected only on the surface of floor plate cells and on cells a very short distance from the floor plate [8,11]. There is evidence, however, to suggest the long-range action of a ventralizing signal within the neuroepithelium, affecting even intermediate regions of the neural tube. Studies in which the expression of three genes — *msx1*, *pax3 and dsl1* (encoding Dorsalin-1) — have been monitored over time show that all three are initially expressed in broad domains but are gradually restricted to dorsal domains by the action of a ventralizing signal that can be simulated by either notochord or purified SHH [10]. The extinction of expression of these genes, first from ventral and subsequently from intermediate regions of the neural tube, provides evidence for the action of a ventralizing factor that spreads over time to affect cell fate progressively more and more distantly. Indeed, it is possible that a ventralizing signal spreads throughout the entire neural tube and is counteracted dorsally by locally acting signals provided by, for example, TGFβ family members, which have been shown to inhibit the differentiation of ventral cell groups (Fig. 1) [10,12]. A question then arises: is this ventralizing signal SHH itself?

SHH appears to function as a morphogen, as it induces the differentiation of floor plate and motor neurons at different and distinct concentration thresholds. Exposure of neural explants to concentrations of the active amino-terminal domain of SHH (SHH-N) between 0.5 and 1.6 nm results in the appearance of motor neurons but not floor plate cells, whereas at ten-fold higher concentrations SHH-N induces floor plate cells [6,8]. Two models could account for this ability of SHH-N to mediate the induction of distinct types of ventral cells. In the first, most of the active portion of SHH is associated with the surface of notochord and floor plate cells but small amounts diffuse through the neural tube and ventralize it directly, so inducing motor neuron differentiation and the expression of the general ventral marker *Nkx2.2* [13] while repressing expression of *pax3*, *msx-1* and *dsl1*. The second model proposes that SHH is not a direct morphogen; rather, concentrations of SHH-N that are insufficient to induce floor plate act on adjacent cells to induce

Figure 1



(a) The neural plate is made up of simple columnar epithelial cells (white area). Cells at the midline are contacted directly by notochord (N), while the most lateral edges of the neural plate contact epidermal ectoderm (E); both provide signals that change the fate of the neuroepithelial cells (arrows). **(b)** The neural tube is formed as the neural plate folds and fuses at its dorsal (previously lateral) edges. Floor plate cells (F) are induced at the ventral midline. Cells of unknown phenotype (X) differentiate in spinal regions immediately next to the floor plate; adjacent to these, motor neurons (pink) and interneurons (yellow) differentiate. A ventralizing signal appears to spread dorsally and may establish a morphogen gradient (blue shading). Roof plate cells (R) are induced by epidermal ectoderm and express molecules of the TGFβ superfamily, which may counteract ventralizing signals.

an unknown intermediary long-range signalling molecule(s) that has motor neuron-inducing and ventralizing activity. At present, little evidence distinguishes between these models; however, the finding that ventral midline neural tube cells that have not yet begun to differentiate into floor plate, despite being underlain by notochord, are unable to induce motor neurons [4] appears to argue against the second.

The ability of SHH-N to act as a morphogen raises the possibility that the neural tube is patterned along its entire ventro-dorsal aspect as a result of a single morphogenic gradient that may be limited or counteracted in dorsal regions by the action of short-range dorsalizing signals. In this scheme, progenitor cells occupying distinct positions along the ventro-dorsal axis of the neural tube would differentiate differently in response to small changes in concentration of the morphogen. However, recent work by Pfaff *et al.* [14] suggests that this model may be too simplistic. Instead, the SHH-mediated induction of motor neurons appears to set in motion a second cascade that elaborates pattern within the dorso-ventral neural tube.

Induction of interneurons by motor neurons

During their differentiation, motor neurons express a series of LIM homeodomain proteins, thought to be transcription factors involved in specifying cell fate. One of these, Islet-1 (encoded by *Isl1*), provides the earliest known marker of differentiating motor neurons and appears to be expressed by all motor neuron subsets. Other LIM genes, such as *Lhx3* and *Gsh4*, are expressed after *Isl1* and appear to specify different motor neuron subsets [15,16]. In a beautiful series of experiments, Pfaff *et al.* [14] have examined neuronal differentiation in the spinal cord and hindbrain of mice with a targeted ablation of the *Isl1* gene [14]. Mice homozygous for this mutation (*Isl1*$^{-/-}$) die *in utero* at embryonic day 11–12, apparently as a result of abnormal development of the dorsal aorta. Examination of the mice at earlier stages, however, reveals that motor neurons also fail to differentiate. The role of Islet-1 in motor neuron determination is unclear, in part because it is not known whether commitment to a motor neuron fate occurs before or after precursor cells have undergone their final mitotic division. Islet-1, which is expressed in motor neurons only after their final division, does not appear to commit a cell to a motor neuron fate. Nevertheless, cells lacking Islet-1, which would normally differentiate to a motor neuron fate, instead die by apoptosis [14].

Despite the absence of motor neurons in the *Isl1*$^{-/-}$ mutant mice, the neural tube as a whole seems to have normal dorso-ventral polarity. The profile of general pattern markers remains intact, suggesting that the neural tube is ventralized (expressing *Nkx2.2*) and dorsalized (expressing *msx1/2* and *pax3*) as normal. Floor plate cells also differentiate with an apparently normal profile and express *shh*. Some neurons, at least, are generated normally: *lim1/2*-expressing interneurons differentiate in an apparently undisrupted fashion. However, in addition to the lack of differentiated motor neurons, two further effects are observed as a result of lacking *Isl1* or motor neurons. First, *Isl1*$^{-/-}$ mice have a decrease in the number of mitotic cells in their ventral spinal cord, which cannot be accounted for solely by their general reduction in size. Second, a class of interneurons which normally differentiates in a position immediately dorsal to motor neurons, and which can be identified by their expression of the homeodomain protein Engrailed1, is absent.

Two mechanisms could account for the lack of Engrailed1-expressing interneurons. First, Engrailed1-expressing cells could require Islet-1 in their precursors. In experiments that elegantly exploit the *Isl1*$^{-/-}$ mutant mice, neural tube explants from *Isl1*$^{-/-}$ mice were cultured *in vitro* adjacent to explants containing largely motor neurons from quail neural tube (Fig. 2a,b). The quail explant rescued the differentiation of Engrailed1-expressing cells in the mutant tissue, showing that these cells do not require the expression of

Islet-1 autonomously for their differentiation. The rescue of Engrailed1-expressing interneurons raises the alternative possibility that their differentiation is dependent on a motor neuron-derived signal. Support for this suggestion is provided in experiments in which similar quail explants were cultured adjacent to intermediate regions of chick neural tube that do not normally express either Islet-1 or Engrailed1. In this case, Engrailed1-expressing interneurons differentiated in the wild-type chick neural tissue, suggesting a motor neuron-dependent step in the differentiation of Engrailed1-expressing interneurons.

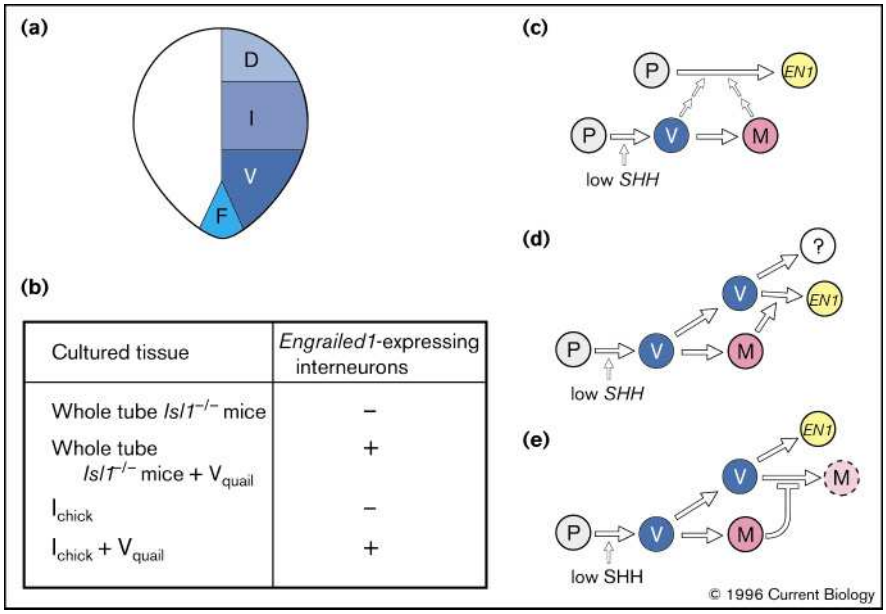
The source of the differentiation signal for Engrailed1-expressing interneurons is unknown. The interneurons begin to differentiate very soon after the differentiation of motor neurons [14], raising the possibility that motor neuron precursors, rather than motor neurons themselves, establish an early signalling source. Likewise, it remains unclear whether the signal operates directly or indirectly through intermediary cells. At least one other class of interneurons are absent from *Isl1*$^{-/-}$ mice. Although this class, which express *Gsh4* and *Lhx3*, differentiate after Engrailed1-expressing interneurons, and are therefore unlikely to relay the signal, it is possible that their progenitors may fulfil this function. It is also possible that an undefined class of interneurons is also absent and that these cells normally act to mediate the differentiation of Engrailed1-expressing interneurons directly.

Morphogens and cascades in the ventral neural tube

The difficulty of assessing the source and action of the interneuron differentiation signal reflects, at least in part, uncertainty as to the lineage relationship of motor neurons and interneurons. A corollary of this uncertainty is the question of how the motor neuron-derived signal interacts with a proposed ventralizing gradient established by SHH. As outlined above, previous studies have raised the possibility that a ventralizing signal may spread to dorsal regions of the neural tube. The question arises as to whether Engrailed1-expressing interneurons have and must receive ventralizing signals for their differentiation. A number of models can be proposed to explain the apparent dependence of Engrailed1-expressing interneuron differentiation on motor neurons (Fig. 2c–e). Firstly, motor neurons, their ventralized precursors, or both, may provide a source of factor that directly or indirectly induces other progenitors to adopt an Engrailed1-expressing fate (Fig. 2c). Alternatively (Fig. 2d,e), a common ventralized progenitor may exist for both motor neurons and interneurons, with the progenitor obliged to make motor neurons before progressing to make interneurons. In one model (Fig. 2d), the motor neurons act as a source of an inducing signal. The other model (Fig. 2e) reflects the fact that differentiating neurons in other parts of the nervous system provide lateral signals to nearby progenitors that restrict their ability to adopt equivalent fates [17]. This raises the possibility that motor neurons normally inhibit adjacent progenitors from assuming the motor neuron fate, so permitting them to adopt other fates, such as becoming interneurons. The disproportionate decrease in mitotic cells in the ventral neural tube of *Isl*$^{-/-}$ mice could imply that, in the absence of a motor neuron signal, progenitor cells continue to embark on a non-productive pathway of motor neuron differentiation (Fig. 2e).

Two observations support the view that Engrailed1-expressing cells derive from cells that have not been

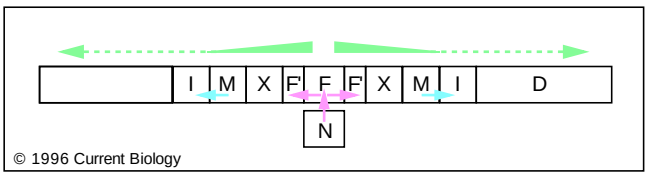
Figure 2



Cultured tissue	*Engrailed1*-expressing interneurons
Whole tube *Isl1*$^{-/-}$ mice	–
Whole tube *Isl1*$^{-/-}$ mice + V_{quail}	+
I_{chick}	–
I_{chick} + V_{quail}	+

Induction of Engrailed1-expressing cells. **(a)** The regions of neural tube isolated in the *in vitro* assay [14]; D, dorsal; I, intermediate; V, ventral; F, floor plate. Motor neurons differentiate within region V. **(b)** Engrailed1-expressing cells (EN1) differentiate only when neural explants are cultured with V explants. **(c–e)** Models for the differentiation of motor neurons and interneurons (see text for details). Low concentrations of SHH induce progenitor cells (P) to a ventralized fate (V; expressing *Nkx2.2* but not *msx1* or *pax3*). Ventralized cells can still divide and their progeny include motor neurons (M). In both (d) and (e), the possibility remains that the cell shown as Engrailed1-expressing may in fact be another interneuron which acts as an intermediary to relay the signal.

Figure 3



Sequential signalling cascades operate to pattern the developing neural plate and neural tube. For simplicity, only the neural plate is depicted. High levels of SHH (pink arrows) provided by notochord (N) induce floor plate cells (F) in the overlying neuroepithelium. These, in turn, express high levels of SHH and can recruit adjacent cells to adopt a floor plate fate (F′). However, further patterning does not appear to be mediated solely by the lateral propagation of a cascade of inductive signals, as cells depicted in region X cannot induce motor neurons [3]. Instead, a long-range ventralizing signal (green arrows), which may be SHH itself, appears to spread laterally over time to ventralize the neural tube and induce motor neuron differentiation. The spread of this signal at the time of interneuron differentiation is unclear (fading green arrow). However, motor neurons can seemingly induce the differentiation of Engrailed1-expressing interneurons (I) in neural tissue that has not been exposed to a ventralizing signal. It is unclear whether the induction of Engrailed1-expressing interneurons is a direct effect (blue arrows) or whether it is mediated by secondary cell types that are also induced by motor neurons.

exposed to a ventralizing signal. *In vivo*, the overt differentiation of Engrailed1-expressing cells is dorsal to motor neurons, a caveat being that the location of their progenitors is not known. More persuasively, perhaps, intermediate explants induced *in vitro* to give Engrailed1-expressing interneurons by quail ventral explants (Fig. 2a,b), express both *pax3* and *msx1/2* at the time of isolation [10], suggesting that they have not been exposed to a ventralizing factor. It remains possible, however, that a motor neuron factor may induce the differentiation of distinct classes of interneurons depending on the extent of their prior exposure to a ventralizing signal. Whether or not any class of interneurons directly requires SHH for its differentiation will become apparent when similar rescue experiments are performed using intermediate tissue from mice in which *shh* has been 'knocked out'.

The requirement of Engrailed1-expressing interneurons for motor neurons or their precursors suggests that the patterning of cells within the ventral neural tube arises through, and is refined by, the action of a coordinated cascade of signals (Fig. 3). First, a morphogen provided by notochord — SHH — acts to induce the differentiation of floor plate cells in the most ventral region of the neural tube and of motor neurons more laterally. As part of their differentiation programme, floor plate cells themselves express *shh*, and can likewise induce cells to adopt either floor plate or motor neuron fates. Motor neurons in turn, express a factor that enables them to induce the differentiation of specific cells. Although unable to homeogenetically induce their own differentiation, motor neurons regulate the differentiation of adjacent Engrailed1-expressing interneurons. Although it is not known whether these interneurons subsequently form interconnections with the motor neurons that induced them, motor neurons and interneurons certainly do form synaptic connections, and the ability of motor neurons to induce the subsequent differentiation of neighbouring interneurons may provide a general means by which to organize local circuits. The observation that projection neurons generally differentiate before their attendant interneurons suggests that such cascades may operate widely to pattern the developing vertebrate central nervous system. Further studies using the elegant combination of assays *in vitro* and gene knockout mice are sure to shed light on the continuing problem of cell fate specification in the central nervous system as a whole.

Acknowledgements

Many thanks to T. Jessell and S. Pfaff for insightful comments on the manuscript and to J. Brockes for artwork

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