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Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling

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During vertebrate development, the specification of distinct cell types is thought to be controlled by inductive signals acting at different concentration thresholds[1]. The degree of receptor activation in response to these signals is a known determinant of cell fate[2], but the later steps at which graded signals are converted into all-or-none distinctions in cell identity remain poorly resolved. In the ventral neural tube, motor neuron and interneuron generation depends on the graded activity of the signalling protein Sonic hedgehog (Shh)[3–5]. These neuronal subtypes derive from distinct progenitor cell populations that express the homeodomain proteins Nkx2.2 or Pax6 in response to graded Shh signalling[6,7]. In mice lacking *Pax6*, progenitor cells generate neurons characteristic of exposure to greater Shh activity[6,8]. However, Nkx2.2 expression expands dosally in *Pax6* mutants[6], raising the possibility that Pax6 controls neuronal pattern indirectly. Here we provide evidence that Nkx2.2 has a primary role in ventral neuronal patterning. In *Nkx2.2* mutants, Pax6 expression is unchanged but cells undergo a ventral-to-dorsal transformation in fate and generate motor neurons rather than interneurons. Thus, Nkx2.2 has an essential role in interpreting graded Shh signals and selecting neuronal identity.

We analysed the pattern of neurogenesis in the ventral spinal cord and hindbrain of mice containing a mutation in the *Nkx2.2* gene[9], focusing on two ventral progenitor cell populations. The ventralmost neuronal progenitor domain, defined as the region dorsolateral to HNF3β floorplate cells[10] and ventral to Pax6[on] progenitors, is initially characterized by expression of *Nkx2.2* and the related *Nkx2.9* gene[11–13] (Fig. 1a–e). This domain corresponds to the 'x' region[14]. From e10.5, high Nkx2.2 expression persists but in the spinal cord the expression of *Nkx2.9* by 'x'-domain progenitors is almost extinguished[13] (Fig. 1b, c, e, f). More dorsal progenitor cells express low levels of Pax6 but not of Nkx2.2 or Nkx2.9 (Fig. 2a, b). Both progenitor cell populations express Nkx6.1 (Fig. 2c, h)[15].

To test whether Nkx2.2 helps to establish or maintain the 'x' progenitor domain, we monitored the expression of Nkx2.9, Nkx6.1, HNF3β and Pax6 in the spinal cord of mice lacking *Nkx2.2*. The pattern of expression of Nkx2.9 and Nkx6.1 over the period e9.5 to e12.5 was similar in wild-type, heterozygote and *Nkx2.2* mutant mice (Figs 1d, e, g, h and 2c, h; also data not shown). In addition, the ventral boundary of the Pax6 progenitor cell domain was unchanged in *Nkx2.2* mutants (Fig. 2a, b, f, g), as were the domains and amount of expression of HNF3β and Shh (Figs 2b, g and 4a, g; also data not shown). Moreover, the number of progenitor cells within the 'x' domain was not altered in *Nkx2.2* mutants (Fig. 2b, c, g, h, and data not shown). Thus, Nkx2.2 function is not required to establish or maintain the 'x' progenitor domain, although Nkx2.2 and Nkx2.9 may have redundant activities. As Shh signalling is required for the establishment of the 'x' domain[6], we investigated whether Shh might also maintain the 'x' domain, even in the absence of Nkx2.2 function, by culturing ventral neural tube/floorplate (vf) explants derived from e10.5 *Nkx2.2* mutant or wild-type embryos in the presence of an anti-Shh antibody[16]. The 'x' progenitor domain was maintained in wild-type (Fig. 2l, m, q, r) and *Nkx2.2* mutant [vf] explants (Fig. 2n, o, s, t) in the presence of anti-Shh IgG (Fig. 2k, p). Thus, the 'x' progenitor domain, once established, can be maintained without Shh signalling or Nkx2.2 activity.

We next tested whether neurons that derive from 'x'-domain progenitors are affected by the loss of *Nkx2.2*. In the spinal cord,

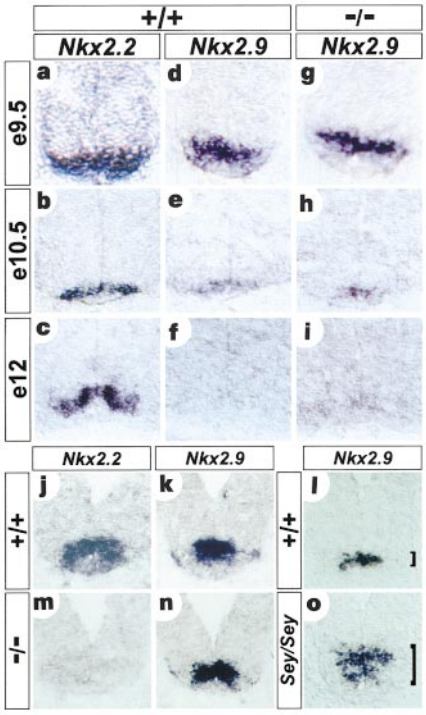

Figure 1 *Nkx2.2* and *Nkx2.9* are expressed in the 'x' domain of the neural tube. **a**–**c**, *Nkx2.2* expression in the 'x' domain of wild-type e9.5 (**a**), e10.5 (**b**) and e12 (**c**) mouse spinal cord. **d**–**i**, *Nkx2.9* is expressed in the 'x' domain of the spinal cord of wild-type (**d**–**f**) and *Nkx2.2* mutant (**g**–**i**) embryos at e9.5 (**d**, **g**) and downregulated by e10.5 (**e**, **h**). No *Nkx2.9* expression is detected at e12 (**f**, **i**). **j**, **k**, In the hindbrain *Nkx2.2* (**j**) and *Nkx2.9* (**k**) are expressed in the 'x' domain of wild-type e10.5 embryos. **m**, *Nkx2.2* expression is not detected in *Nkx2.2* mutant embryos, whereas in the hindbrain *Nkx2.9* (**n**) expression persists. **l**, **o**, The domain of *Nkx2.9* expression in the hindbrain of *Sey/Sey* embryos (**o**) expands dorsally with respect to the wild-type embryos (**l**).

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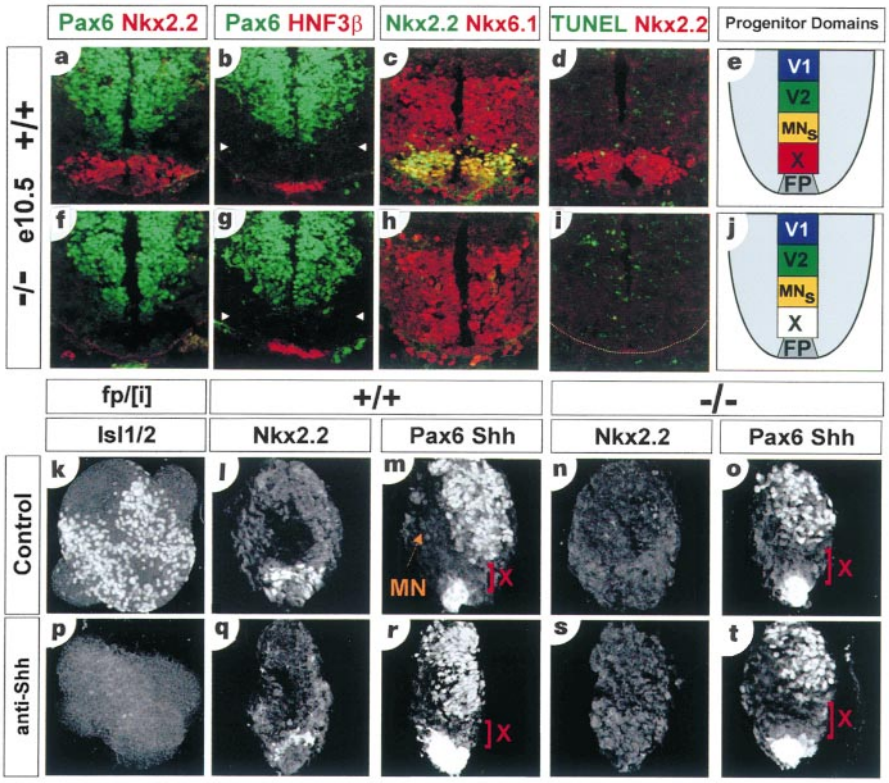

Figure 2 Nkx2.2 activity is not required for the establishment of progenitor cell domains in the ventral neural tube. **a**–**j**, The 'x' domain is present in embryos lacking *Nkx2.2* activity. Expression of transcription factors in e10.5 wild-type (**a**–**c**) and *Nkx2.2* mutant (**f**–**h**) embryos. Pax6 but not Nkx2.2 is expressed by mutant embryos (compare **a** and **f**). The lack of *Nkx2.2* activity does not result in a ventral expansion in the expression of Pax6 (**b**, **g**) (arrowheads indicate ventral boundary of Pax6 expression). Nkx6.1 expression is similar in wild-type (**c**) and *Nkx2.2* mutant (**h**) embryos. The expression of Shh is similar in *Nkx2.2* mutant and wild-type embryos (Fig. 4a, g). The ventral limit of Pax7 is unchanged in *Nkx2.2* mutant embryos (data not shown), as is the number of V1 and V2 neurons (Fig. 3, and data not shown). **d**, **i**, The number of TUNEL-labelled cells in the 'x' domain of *Nkx2.2* mutant embryos (**i**) is similar to wild-type controls (**d**). **e**, **j**, Summary diagram showing progenitor domains in the ventral neural tube of wild-type and *Nkx2.2* mutant embryos. **k**–**t**, The 'x' domain is maintained in the absence of Shh signalling or Nkx2.2 expression. **k**, **p**, Floorplate from an e10.5 mouse embryo induces Isl1/2^{on} motor neurons in conjugates with [i] regions of stage-10 chick neural tube (**k**). This activity is blocked by anti-Shh IgG (25 μg ml^{-1}) (**p**). Anti-Shh IgG also blocks the induction of Nkx2.2 in neural plate explants from e8.5 mouse embryos (not shown). Ventral neural tube/floorplate explants from 33–36 somite wild-type (**l**, **m**, **q**, **r**) or *Nkx2.2* mutant (**n**, **o**, **s**, **t**) embryos were cultured in the presence (**l**–**o**) or absence (**q**–**t**) of anti-Shh IgG for 24 h. Explants were labelled for expression of Nkx2.2 (**l**, **q**, **n**, **s**) or Pax6 and Shh (**m**, **r**, **o**, **t**). All explants are orientated with the floorplate ventral. Motor neurons do not express Pax6 (orange arrow in **m**). The 'x' domain, defined by the region between Shh^{on} floor plate cells and Pax6^{on} progenitors (red brackets), is evident in both wild-type and *Nkx2.2* mutant embryos, irrespective of the presence of anti-Shh IgG.

'x'-domain progenitors give rise to a class of ventral neurons that we term V3 neurons (Fig. 3a–d) and which express the basic helix–loop–helix (bHLH) genes *Sim1* and *Ngn3* (refs 17, 18) (Fig. 3b, e). The expression of *Ngn3* and *Sim1* is first detected at ~e10.5 (Fig. 3e, f), a time when the expression of *Nkx2.9* has been markedly downregulated by 'x'-domain progenitors in the spinal cord (Fig. 1e). To determine whether *Nkx2.2* is required for the generation of V3 neurons, we analysed the expression of *Ngn3* and *Sim1* in *Nkx2.2* mutants between e10.5 and e12.5. At e10.5, the number of *Ngn3*^{on}, *Sim1*^{on} cells in *Nkx2.2*-mutant embryos was markedly reduced (Fig. 3e, f, i, j). By e12.5, there was a virtual absence of *Ngn3*^{on}, *Sim1*^{on} neurons (Fig. 3g, k; data not shown). The transient expression of *Nkx2.9* by 'x'-domain progenitors in *Nkx2.2* mutants (Fig. 1d–i) may account for presence, initially, of a small number of V3 neurons (see below). We detected no increase in the number of TUNEL^{+} cells in the 'x' domain (Fig. 2d, i), indicating that the loss of V3 neurons is not due to apoptosis. Thus, *Nkx2.2* activity is required for the generation and possibly also maintenance of V3 neurons (Fig. 3h, l).

In wild-type mice, the generation of somatic motor neurons is excluded from Nkx2.2^{on} 'x'-domain progenitors[6]. The dorsal expansion of Nkx2.2 expression in *Pax6* mutants is accompanied by the repression of somatic motor neuron differentiation[6], suggesting that the expression of Nkx2.2 within 'x'-domain progenitors suppresses their potential for somatic motor neuron generation. We therefore examined whether somatic motor neurons are generated from 'x'-domain progenitors in *Nkx2.2* mutants. The generation of somatic motor neurons was defined by co-expression of the homeodomain proteins Isl1, Isl2, Lim3 and HB9 (refs 6, 19–21). At e10.5, there was a 21% increase in the number of somatic motor neurons (Fig. 4a, g; see legend for quantification) and many motor neurons were located next to the floorplate, within the 'x' domain (Fig. 4a–m). The dorsal boundary of somatic motor neuron generation (Fig. 4a–c, g–i) and the number of V2 neurons (Fig. 4d, j; data not shown) were unchanged.

The detection of somatic motor neurons adjacent to the floorplate could reflect either their generation from 'x'-domain progenitors or their ventral migration from a more dorsal position of origin. To resolve this, we analysed the expression of Lim3, a LIM homeodomain protein that defines motor neuron progenitors as well as post-mitotic motor neurons[6,19–21]. In *Nkx2.2* mutants examined at e10.5–e12.5, Lim3^{on} and Isl1/2^{off} motor neuron progenitors were located medially, within the ventricular zone of the 'x' domain (Fig. 4j, o) but were excluded from this domain in wild-type embryos (Fig. 4d, n). In *Nkx2.2* mutants, there was also an increase in the number of somatic motor neurons located at the border of the ventricular zone within the 'x' domain (Fig. 4n–p), a position that appears to reflect their migration laterally from the ventricular

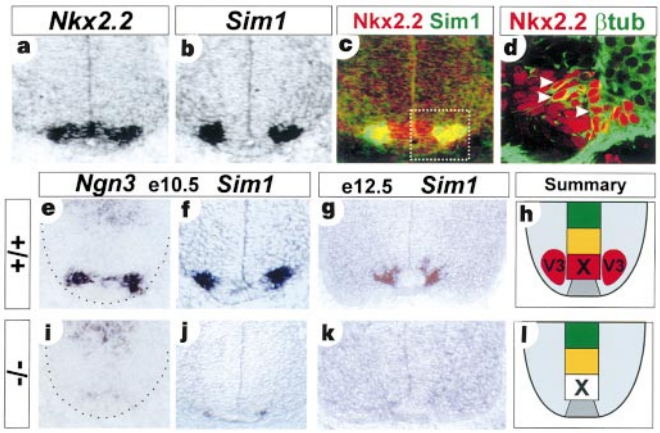

Figure 3 Nkx2.2 activity is required for the generation of V3 neurons. **a**–**c**, *Sim1*^{on} neurons are generated from 'x' domain progenitors. Expression of *Nkx2.2* (**a**) and *Sim1* (**b**) in adjacent sections of e10.5 spinal cord. **c**, Superimposition of images **a** and **b** shows that 'x'-domain cells lateral to the ventricular zone co-express Nkx2.2 and *Sim1*. **d**, Lateral Nkx2.2^{on} cells also express the neuronal specific β-tubulin isoform (arrowheads). Box in **c** shows the approximate position of the image in **d**. These cells are defined as neurons by expression of the neuronal specific β-tubulin isoform (**d**) and as V3 neurons by the selective expression of the genes encoding the bHLH transcription factor Ngn3 (**e**); and the PAS bHLH protein Sim1 (**f**). **e**–**l**, Expression at e10.5 of *Ngn3* (**e**, **i**) and *Sim1* (**f**, **j**) in the spinal cord of wild-type (**e**, **f**) and *Nkx2.2* mutant (**i**, **j**) embryos. The expression of *Ngn3* and *Sim1* by cells in the 'x' domain is markedly reduced in *Nkx2.2* mutant embryos. However, dorsal expression of *Ngn3* is unaffected. By e12.5 (**g**, **k**), *Sim1* expression is lost in *Nkx2.2* mutant embryos (compare **g**, **k**). The progressive decrease in V3 neuron number between e11 and e12.5 argues that Nkx2.2 may also be required for the maintenance of the V3 neuron phenotype. **h**, **l**, Summary diagrams showing the requirement for Nkx2.2 in the generation of V3 neurons.

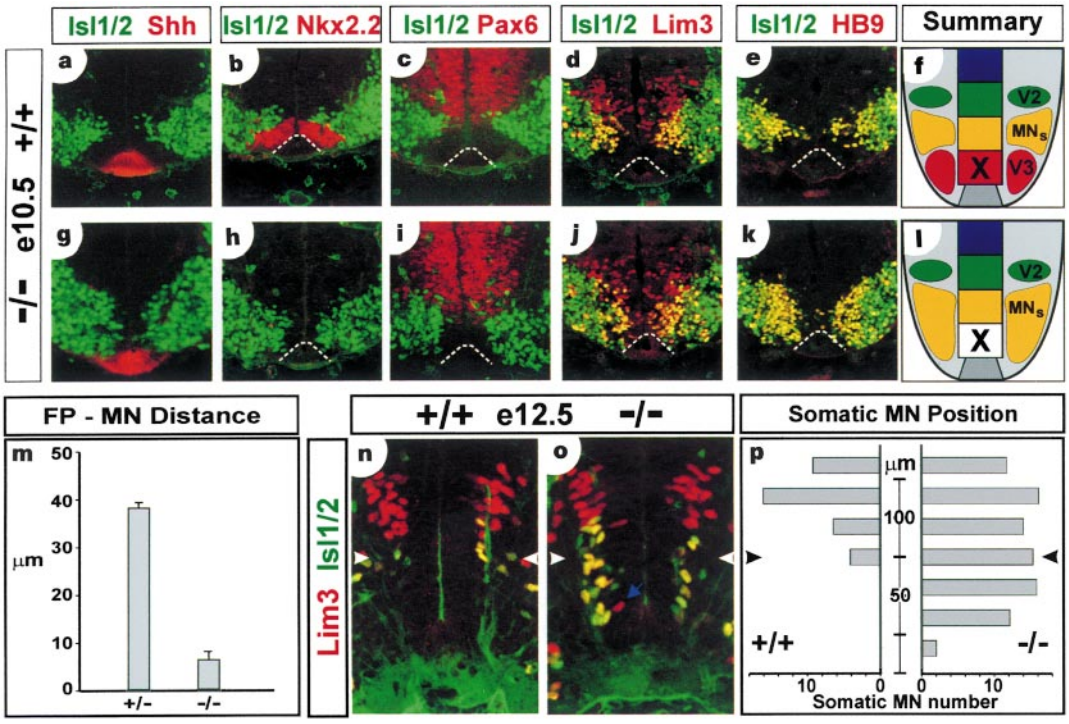

Figure 4 Nkx2.2 represses somatic motor neuron generation in the 'x' domain of the spinal cord. **a**–**l**, Locations of somatic motor neurons at forelimb levels of wild-type (**a**–**f**) and *Nkx2.2* mutant (**g**–**l**) embryos at e10.5. Dotted line indicates the extent of the floorplate. Nkx2.2^{on} progenitors do not generate Isl1/2^{on} motor neurons (**b**, **h**). The gap between the floorplate and somatic motor neurons in wild-type mice (**a**) is missing in *Nkx2.2* mutant embryos (**g**). The ventral expression in somatic motor neurons results in the generation of motor neurons from Pax6^{off} progenitors (**c**, **i**). Ectopic motor neurons express Lim3 (**d**, **j**), HB9 (**e**, **k**) and Isl1/2 (**c**, **i**). Number of somatic MNs: *Nkx2.2*+/–: 160 ± 4; *Nkx2.2*–/–: 202 ± 6 neurons per ventral quadrant $n = 6$ sections; mean + s.e.m. Number of V2 neurons: *Nkx2.2*+/–: 45 ± 9; *Nkx2.2*–/–: 46 ± 13; neurons per ventral quadrant; $n = 6$ sections. **f**, **l**, Summary diagram showing the ventral expansion in somatic motor neuron generation in *Nkx2.2* mutant embryos. In *Nkx2.2* mutants examined at e9.5, there was no change in the number or position of generation of somatic motor neurons (data not shown). This may reflect the overlap in expression of *Nkx2.9* and *Nkx2.2* (Fig. 1). Alternatively, the small dorsoventral extent of the 'x' domain at this early stage may obscure the ventral expansion of the domain of somatic motor neuron generation. **m**, Mean distance (μm) between Shh^{on} floorplate cells and the most ventral Isl1/2^{on} motor neurons in wild-type and *Nkx2.2* mutant embryos at e10.5. At e12.5, Lim3^{on}/Isl1/2^{on} somatic motor neurons are present adjacent to the ventricular zone in wild-type embryos (**n**). There is a marked ventral expansion in the production of Lim3^{on}, Isl1/2^{on} motor neurons in *Nkx2.2* mutant embryos (**o**). Arrowheads mark the ventral limit of Pax6 expression; the blue arrow indicates a Lim3^{on} motor neuron progenitor in the 'x' domain. **p**, Newly generated motor neurons as a function of distance from the ventral midline in e12.5 embryos.

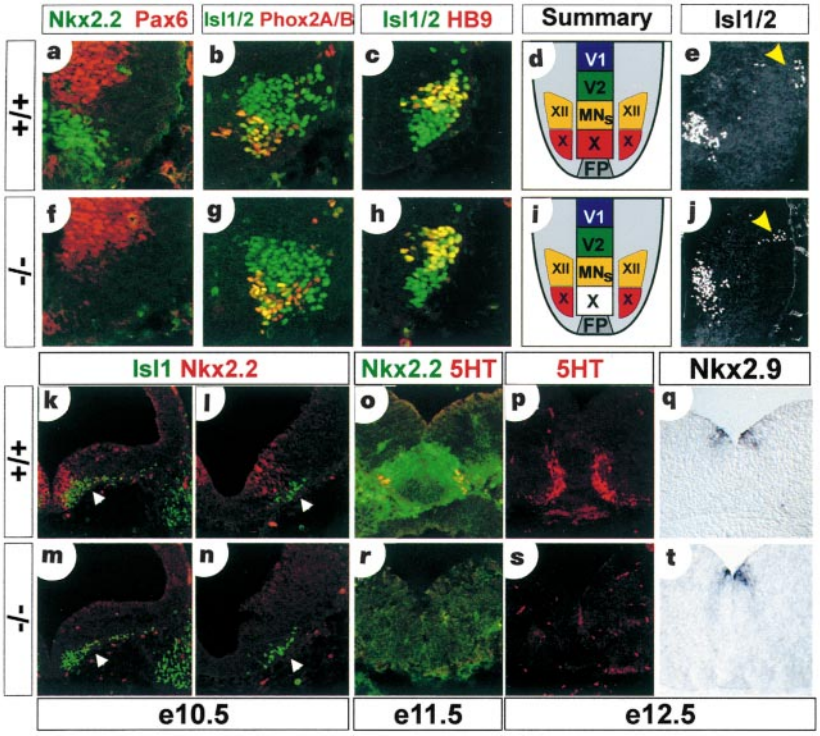

Figure 5 Nkx2.2 is required for the generation of hindbrain serotonergic neurons but not visceral motor neurons. **a**–**j**, Position of generation of motor neurons at the r8 level of the hindbrain. In wild-type e10.5 embryos (**a**–**c**) Nkx2.2^{on} progenitors (**a**) generate Isl1/Phox2A/B^{on} visceral motor neurons (**b**), whereas Pax6^{low} progenitors generate Isl1/2^{on} HB9^{on} somatic motor neurons (**c**). In *Nkx2.2* mutant embryos, 'x'-domain progenitors do not express Nkx2.2 (**f**) but visceral (**g**) and somatic (**h**) motor neurons are generated in normal numbers and position. **d**, **i**, Summary diagram of visceral and somatic motor neuron generation in wild-type and *Nkx2.2* mutant embryos. **e**, **j**, Vagal and hypoglossal motor neurons migrate to their correct positions (yellow arrowheads) in wild-type (**e**) and *Nkx2.2* mutant (**j**) embryos. Additionally, whole-mount labelling of e11 wild-type and *Nkx2.2* mutant embryos using anti-neurofilament 2H3 antibody[6] revealed that the hypoglossal motor nerve (XII) and vagus (X) and spinal accessory (XI) nerves are present (data not shown). The position of origin, transcription factor profile, and migratory pattern of facial/glossopharyngeal, abducens, trochlear and oculomotor neurons is unchanged in *Nkx2.2* mutant embryos (data not shown). **k**–**n**, Trigeminal and trochlear Isl1^{on} motor neurons (arrowheads) are generated similarly in wild-type (**k**, **l**) and *Nkx2.2* mutant (**m**, **n**) embryos. **o**, **p**, **r**, **s**, Serotonin (5HT) expression in e11.5 (**o**, **r**) and e12.5 (**p**, **s**) wild-type (**o**, **p**) and *Nkx2.2* mutant (**r**, **s**) embryos. From e11.5, serotonin neurons are generated from Nkx2.2^{on} progenitors and are subsequently lcoated adjacent to the floorplate (**p**). In *Nkx2.2* mutant embryos, the generation of these serotonin neurons is virtually eliminated. At e12.5, *Nkx2.9* expression is markedly downregulated in wild-type (**q**) and mutant (**t**) embryos. Serotonergic neurons of the dorsal raphe nuclei and dopaminergic neurons neurons of the A9 (substantia nigra) and A10 (ventral tegmental area) nuclei are generated in normal numbers and positions in *Nkx2.2* mutant embryos. These monoaminergic neurons do not derive from Nkx2.2^{on} progenitor cells (data not shown).

zone[22]. The aberrant ventral position of motor neurons in *Nkx2.2* mutants appears therefore to be a consequence of their generation from 'x'-domain progenitors and not from ventrally directed migration. Moreover, most ventral somatic motor neurons are now generated from progenitor cells that do not express Pax6 (Fig. 4c, i). This result provides further evidence that Pax6 is not required directly for specification of somatic motor neuron fate but acts through repression of Nkx2.2. Together, these observations indicate that Nkx2.2 expression in 'x'-domain progenitors suppresses their potential for somatic motor neuron generation (Fig. 4f, l).

In the hindbrain, 'x'-domain progenitors give rise to visceral motor neurons rather than to V3 neurons, but somatic motor neurons still derive from Pax6^{on} progenitors[6,19]. The switch from V3 neuron to somatic motor neuron generation in the spinal cord of *Nkx2.2* mutant embryos suggested that 'x'-domain progenitors at hindbrain levels might undergo a corresponding ventral-to-dorsal switch, and generate somatic rather than visceral motor neurons. However, we found that the generation of somatic and visceral motor neurons in the hindbrain of *Nkx2.2* mutants was unchanged (Fig. 5b, c, g, h, k–n). In addition, the later pattern of migration of visceral (vagal) motor neurons (Fig. 5e, j) and the trajectory of the axons of hypoglossal and vagal motor neurons was similar in *Nkx2.2* mutant and wild-type embryos (data not shown). Thus, in the hindbrain, *Nkx2.2* activity is not required for the generation of visceral motor neurons, nor does the inactivation of *Nkx2.2* result in a ventral expansion of the domain of somatic motor neuron generation (Fig. 5d, i).

What might account for the absence of a switch in motor neuron identity in the hindbrain? In the spinal cord, the extinction of *Nkx2.9* by 'x'-domain progenitors is virtually complete by e11.0 (Fig. 1e, h). Within the hindbrain, however, *Nkx2.9* and *Nkx2.2* expression overlaps until e11.0 (Fig. 1k). The pattern of expression of *Nkx2.9* is similar in *Nkx2.2* mutant embryos from e9.5 to e11.0 (Fig. 1n), and the expression of *Nkx2.9* is expanded dorsally in a manner similar to that of *Nkx2.2* in mice lacking *Pax6* (Fig. 1l, o). As Nkx2.9 is closely related to Nkx2.2 (ref. 13), it is likely that the normal generation of somatic and visceral motor neurons in the hindbrain of *Nkx2.2* mutants reflects the overlap in expression and redundant activity of *Nkx2.9*.

In the hindbrain, the period of visceral motor neuron generation persists until ~e11.0 (refs 6, 23) but later 'x'-domain progenitors appear to generate other cell types, notably serotonergic neurons[24]. These neurons occupy a ventromedial position, adjacent to the floorplate (Fig. 5o, p). Although the expression of Nkx2.2 persists during the period over which these serotonergic neurons are generated (Fig. 5o), by this time the expression of *Nkx2.9* is markedly downregulated (Fig. 5q). These observations raise the

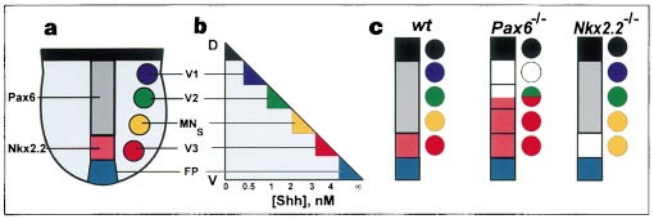

Figure 6 Distinct roles for Nkx2.2 and Pax6 in the control of ventral neuronal identity in response to graded Sonic hedgehog signalling. Progenitor cell domains, neuronal subtypes, their control by graded Shh signalling and the changes in cell identity that occur in *Pax6* and *Nkx2.2* mutants. **a**, Pax6 and Nkx2.2 progenitor cell domains and the dorsoventral postion of generation of V1, V2 and V3 neurons and somatic motor neurons (MNs) in the ventral spinal cord. The position of the floor plate (FP) is shown and dorsal progenitor cells and neurons are indicated by dark grey shading. **b**, The relationship between Shh concentration and the generation of ventral interneurons, somatic motor neurons and floorplate cells, obtained from *in vitro* induction assays (modified from ref. 3). **c**, Changes in progenitor cell domains and neuronal subtype identity in *Pax6* and *Nkx2.2* mutants. WT: ventral pattern in wild-type embryos. Nkx2.2 is expressed in a ventral progenitor domain (the 'x' domain) adjacent to the floorplate. At spinal cord levels, these progenitors give rise to V3 neurons. Pax6 is expressed by all other ventral progenitor cells, in a dorsal-to-ventral gradient. The ventral-most population of Pax6^{on} progenitors gives rise to somatic motor neurons, whereas more dorsal Pax6^{on} progenitors give rise to V1 and V2 neurons. In *Pax6* mutants, there is a dorsal expansion in the domain of expression of Nkx2.2 (and *Nkx2.9*) in the position of generation of V3 neurons, and a reduction in somatic motor neurons and V2 neurons[6]. The generation of V1 neurons is lost in the absence of Pax6. In *Nkx2.2* mutants there is no change in Pax6 expression yet the fate of neurons is switched: 'x'-domain progenitors give rise to somatic motor neurons instead of V3 neurons. Shh signalling is unchanged in *Pax6* and *Nkx2.2* mutants, and the switch between somatic motor neuron and V3 neuron fate is a reflection of the pattern of Nkx2.2 expression. For details, see text.

possibility that the production of serotonergic neurons from 'x'-domain progenitors is affected in *Nkx2.2* mutants. Few, if any, serotonergic neurons were detected at the r2 level of the hindbrain in *Nkx2.2* mutants (Fig. 5r, s), despite the normal generation of trigeminal visceral motor neurons at the same axial level at an earlier stage (Fig. 5m). Thus, *Nkx2.2* function in hindbrain 'x'-domain progenitors is required for the development of serotonergic neurons. These results support the idea that *Nkx2.2* and *Nkx2.9* have overlapping functions in controlling the fate of 'x'-domain progenitors in the hindbrain. The apparent distinction in motor neuron phenotype observed in the spinal cord and hindbrain in *Nkx2.2*-mutant embryos may therefore reflect, in part, the differential timing of extinction of *Nkx2.9* at these two axial levels of the neural tube.

Together, these findings provide insight into the mechanisms by which progenitor cells interpret graded inductive signals to generate all-or-none distinctions in cell fate. Nkx2.2 has an essential role in interpreting graded Shh signalling and specifying the subtype identity of neurons generated from 'x'-domain progenitors (Fig. 6). The loss of *Nkx2.2* activity results in a 'ventral-to-dorsal' switch in progenitor fate, and the generation of neuronal progeny characteristic of cells that have been exposed to 2–3-fold less Shh activity[6] (Fig. 6). Our results do not establish the sufficiency of Nkx2.2 in mediating the 'dorsal-to-ventral' switch in progenitor cell fate. Nevertheless in *Pax6* mutants, Nkx2.2 is expressed ectopically in more dorsal progenitors, and there is a concomitant dorsal-to-ventral switch in progenitor cell fate, leading to the generation of neuronal subtypes characteristic of exposure to 2–3-fold higher Shh concentration[6] (Fig. 6). A *Drosophila Nkx2.2* homologue, *vnd*, controls neuronal identities in the central nervous system in a manner similar to that shown here for Nkx2.2 (refs 25, 26). Loss of *vnd* function leads to a ventral-to-dorsal switch in neuronal fate and overexpression of *vnd* induces a complementary dorsal-to-ventral switch in neuronal identity[26]. Thus, although distinct inductive signals initiate ventral patterning in the vertebrate and insect central nervous systems, the *Nkx2.2/vnd* homeobox genes appear to represent an evolutionarily conserved step in this patterning process. □

Methods

Immunohistochemistry. Proteins were localized as described[6,19,20]. Nkx2.2 was detected with a monoclonal antibody (mAb) or rabbit antiserum raised against chick Nkx2.2 (ref. 6). The rabbit anti-Pax6 antibody was a gift from S. Wilson[27]. Nkx6.1 was detected with a rabbit antiserum[28]. β-Tubulin was detected with mAb TuJ1 (ref. 29). Phox2A/B was detected with a rabbit antiserum[30] provided by J. Brunet. Serotonin was detected with a mAb (Research Biochemicals); Isl1/2, Lim3, HB9, Shh and HNF3β antisera have been described[6,19].

Localization of mRNA. *In situ* hybridization was performed as described[6] using digoxigenin-labelled antisense riboprobes to *Sim1*, *Ngn3*, *Nkx2.2* and *Nkx2.9*.

Explant cultures. For *in vitro* cultures, ventral neural tube/floorplate explants were isolated from 33–36-somite (e10.5) wild-type and *Nkx2.2* mutants. Explants were dissected bilaterally at the ventral midline. Each half was cultured for 24 h either under control conditions or in the presence of mAb anti-Shh IgG 5E1 (ref. 16).

***Nkx2.2* mutation.** A null mutation in the *Nkx2.2* gene was generated by eliminating the entire coding region[9]. Genotyping of embryos was carried out by Southern-blot analysis as described[9].

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Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms

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Many biochemical, physiological and behavioural processes show circadian rhythms which are generated by an internal time-keeping mechanism referred to as the biological clock. According to rapidly developing models, the core oscillator driving this clock is composed of an autoregulatory transcription–(post) translation-based feedback loop involving a set of 'clock' genes[1–6]. Molecular clocks do not oscillate with an exact 24-hour rhythmicity but are entrained to solar day/night rhythms by light. The mammalian proteins Cry1 and Cry2, which are members of the family of plant blue-light receptors (cryptochromes) and photolyases, have been proposed as candidate light receptors for photoentrainment of the biological clock[7–10]. Here we show that mice lacking the Cry1 or Cry2 protein display accelerated and delayed free-running periodicity of locomotor activity, respectively. Strikingly, in the absence of both proteins, an instantaneous and complete loss of free-running rhythmicity is observed. This suggests that, in addition to a possible photoreceptor and antagonistic clock-adjusting function, both proteins are essential for the maintenance of circadian rhythmicity.

We were interested in identifying mammalian homologues of the DNA-repair enzyme photolyase, a protein that undoes ultraviolet-induced DNA damage in a single-step process (photoreactivation) requiring light energy captured by blue-light-collecting chromophores[11,12]. In this search, we and others have cloned two genes with strong homology to class I photolyases of lower species[7–10]. In addition to the photolyase core domain, the gene products appeared to contain a carboxy-terminal extension also found in plant blue-light receptors (cryptochromes), for which the mammalian photolyase-like genes were designated *cry1* and *cry2*. Plant Cry proteins mediate light-dependent processes such as phototropism, growth and flowering[13–15]. Since placental mammals as well as endogenous or recombinant mammalian Cry proteins lack clearly detectable photoreactivating activity, the mammalian Cry proteins may act as photoreceptors rather than photolyases[7,9,10]. The biological 'master' clock in the suprachiasmatic nucleus (SCN) of the brain controls many physiological processes, from body temperature to the sleep–wake cycle. A major question in mammalian chronobiology is how the clock is entrained to solar time, thereby keeping an organism in an exact 24-h rhythm. The absence of photoentrainment in eye-less rodents indicates that the light receptors feeding into the SCN circadian system must reside in the eye[16,17], but the process does not seem to depend on retinal photoreceptor cells and their visual pigments, as Retinal-degenerate (*Rd*) mice show a normal circadian response to light[17,18]. Since mammalian *cry* genes are specifically expressed in the ganglion and inner nuclear layer of the retina, the Cry1 and Cry2 proteins are possible candidates for circadian photoreceptors[19].

To explore the biological function of mammalian Cry1 and Cry2, we have generated *cry1* and *cry2* mutant mice through gene targeting in embryonic stem cells (Fig. 1). Analysis of the transcriptional status of the targeted *cry1* and *cry2* genes, using the reverse transcription-long-range polymerase chain reaction, revealed no detectable transcripts in the corresponding knockout animals, thus demonstrating that we have created null-mutant mice. Targeted *cry1* and *cry2* alleles both segregate at expected mendelian ratios, indicating that the absence of either Cry1 or Cry2 does not interfere with embryonic development. Moreover, *cry1* and *cry2* mutant mice are completely healthy and show no overt phenotype (the oldest animals are now 14 and 7 months, respectively). We analysed the possible role for Cry proteins in the biological clock by measuring the circadian wheel-running behaviour of *cry*-knockout mice under normal light/dark (LD) cycles and in constant darkness (dark/dark; DD). We made two unexpected observations. First, compared with wild-type mice which, when subjected to DD conditioning, have a free-running rhythm close to 24 hours ($\tau = 23.77 \pm 0.07$ h ($n = 14$)), the internal clock of *cry1* mutants runs significantly faster ($\tau = 22.51 \pm 0.06$ h ($n = 9$); $P < 0.00001$) (Fig. 2a, b). In contrast, *cry2* mutants exhibit a clear increase in period length ($\tau = 24.63 \pm 0.06$ h ($n = 5$); $P < 0.00001$) (Fig. 2c). Heterozygous animals showed wheel-running patterns comparable to wild-type mice, and there were no clear sex- or age-related differences (data not shown). These findings suggest that Cry1 and Cry2 antagonistically modulate the period length of the clock. Second, under LD conditions, both mutants show a circadian periodicity of 24 h (Fig. 2a–c), suggesting that a deficiency in either *cry1* or *cry2* does not produce a detectable loss of light entrainment of locomotor activity. However, as *cry1* mice still contain a functional Cry2 protein which may (partly) take over the function of Cry1 (and vice versa), functional redundancy may blur the phenotypic outcome. Thus, it was of interest to examine double-mutant mice.

Like *cry*-single-knockout mice, double-mutant animals are viable and show no gross phenotypic abnormalities (by 5 months old). Unexpectedly, these mice ($n = 8$) still display an essentially 24-h circadian rhythm under LD conditions (Fig. 2f, upper part). This suggests that, despite the absence of both Cry proteins, the biological clock—as reflected by locomotor activity–may still receive a light input. However, when double-mutant mice are shifted to a DD regime, they show a striking instantaneous and complete circadian arrhythmicity (Fig. 2f). This indicates that there is no internal circadian clock running with any significant momentum, although we do not exclude the possibility that there is still an ultradian component. Note also that the instantaneous arrhythmicity in double-mutant mice differs from any clock mutant analysed thus far. This includes the only mouse 'clock' mutant (*clock*) described to