

# Gata2 specifies serotonergic neurons downstream of sonic hedgehog

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Accepted 9 December 2003

Development 131, 1165-1173

Published by The Company of Biologists 2004

doi:10.1242/dev.01024

## Summary

Distinct classes of serotonergic (5-HT) neurons develop along the ventral midline of the vertebrate hindbrain. Here, we identify a Sonic hedgehog (Shh)-regulated cascade of transcription factors that acts to generate a specific subset of 5-HT neurons. This transcriptional cascade is sufficient for the induction of rostral 5-HT neurons within rhombomere 1 (r1), which project to the forebrain, but not for the induction of caudal 5-HT neurons, which largely terminate in the spinal cord. Within the rostral hindbrain, the Shh-activated homeodomain proteins Nkx2.2 and Nkx6.1 cooperate to induce the closely related zinc-finger transcription factors Gata2 and Gata3. Gata2 in turn is

necessary and sufficient to activate the transcription factors Lmx1b and Pet1, and to induce 5-HT neurons within r1. In contrast to Gata2, Gata3 is not required for the specification of rostral 5-HT neurons and appears unable to substitute for the loss of Gata2. Our findings reveal that the identity of closely related 5-HT subclasses occurs through distinct responses of adjacent rostrocaudal progenitor domains to broad ventral inducers.

Supplemental data available online

Key words: Gata, Serotonergic neurons, Sonic hedgehog

## Introduction

During development distinct classes of neurons are specified at stereotypic positions along the dorsoventral and rostrocaudal axes of the neural tube. This process is controlled in part by signaling proteins secreted from regional organizing centers such as the floor plate and mid-hindbrain boundary (MHB). Neural progenitors respond to different concentrations and combinations of these extracellular factors by expressing a variety of transcription factors that ultimately determine the cell fate. Increasingly, the cell-intrinsic signaling pathways that underlie the specification of unique neuronal subtypes are being elucidated (Eklund and Jessell, 1999; Jessell, 2001).

The coordinated specification of multiple neuronal cell types is best understood in the ventral spinal cord. Sonic hedgehog (Shh), secreted from the notochord and floor plate, forms a gradient within the ventral neural tube that results in the concentration dependent expression of homeodomain (HD) proteins by neural progenitors. These HD proteins are subdivided into two groups, termed class I and II. Class I HD proteins are constitutively expressed by neural progenitor cells and are repressed by Shh, whereas class II HD proteins require Shh for expression (Briscoe et al., 2000; Jessell, 2001). This mechanism establishes a HD code that, through mutual repression of adjacent class I and II HD proteins, defines five distinct ventral progenitor domains designated from ventral to dorsal as p3, pMN, p2, p1 and p0 (Briscoe et al., 2000; Muhr et al., 2001). Downstream of the HD code, neural progenitors

express subtype specific determinants that direct the differentiation of distinct neuronal cell fates (Briscoe and Ericson, 2001).

Vertebrate serotonergic (5-HT) neurons constitute multiple diverse populations along the rostrocaudal axis of the hindbrain that differ in the timing and positioning of their induction, in their requirements for inductive factors, and in their innervation targets and function (Aitken and Tork, 1988; Hynes and Rosenthal, 1999). The earliest 5-HT neurons to arise develop adjacent to the MHB at the ventral midline in rhombomere 1 (r1), and send ascending projections that modulate the function of multiple forebrain regions (Hynes and Rosenthal, 1999). Subsequent 5-HT populations develop in more caudal positions, with many sending descending projections to modulate spinal sensory and motoneurons (Hynes and Rosenthal, 1999). A number of inductive signals have been implicated in the development of 5-HT neurons, including Shh secreted from the floor plate, a Fgf activity present in the hindbrain, and, for rostral populations, Fgf8 secreted from the MHB (Ye et al., 1998). Gene ablation experiments further reveal that the HD transcription factor Nkx2.2, the ETS transcription factor Pet1, and the LIM-HD transcription factor Lmx1b are all involved in the genesis of 5-HT neurons, with the loss of each resulting in a loss of 5-HT neurons early in development (Briscoe et al., 1999; Hendricks et al., 2003; Ding et al., 2003; Cheng et al., 2003).

Gata transcription factors, of which there are six in

vertebrates, are characterized by conserved C<sub>4</sub>-type zinc-finger domains and are widely but distinctly expressed (Patient and McGhee, 2002). Diverse developmental functions have been ascribed to the Gata proteins, including cell fate specification, a role best characterized in the hematopoietic system (Patient and McGhee, 2002). Gata2 and Gata3 are expressed in largely overlapping domains in the central nervous system (CNS), where relatively little is known about their function (Nardelli et al., 1999). Expression data and evidence from gene targeting suggest an involvement in neurogenesis, neuronal migration and axon projection (Pandolfi et al., 1995; Nardelli et al., 1999; Pata et al., 1999; Karis et al., 2001); however, a role in specifying neuronal subtypes within the context of neural tube patterning is only beginning to be understood (Zhou et al., 2000; Karunaratne et al., 2002). Furthermore, the level of redundancy between Gata2 and Gata3 during CNS development remains unclear.

Using both gain- and loss-of-function experiments in chick and mouse embryos, we describe a specific and essential role for Gata2 within a Shh-initiated transcriptional cascade for the development of 5-HT neurons in r1 of the hindbrain. Furthermore, we ascertain the interaction between Gata2 and the transcription factors Nkx2.2, Nkx6.1, Gata3, Lmx1b and Pet1 to translate the Shh signal to a rostral 5-HT cell fate. Specifically, we find that the Shh-activated HD proteins, Nkx2.2 and high levels of Nkx6.1 act in combination to induce expression of Gata2 in ventral r1. In turn, Gata2 is sufficient to activate the transcription factors Gata3, Lmx1b and Pet1, and to induce 5-HT neurons at the expense of other neuronal subtypes along the dorsoventral axis of the rostral hindbrain. Conversely, whereas the early development of rostral 5-HT neurons is unaltered in *Gata3* null mice, *Gata2* null mice fail to specify 5-HT progenitors or support 5-HT development, even in the presence of Gata3. Thus a combinatorial transcriptional cascade, in which individual Gata family members play distinct roles, translates the broad inductive signal of Shh into the development of a specific subpopulation of 5-HT neurons.

## Materials and methods

### DNA constructs

cDNAs for electroporation were cloned into either pCIE, a transient expression vector with a chick  $\beta$ -actin promoter and GFP expressed from an internal ribosomal entry site (IRES), or replication-competent retrovirus RCASBP (A) and (B) vectors (Hughes et al., 1987; Morgan and Fekete, 1996). Full-length cDNAs for chick Gata2 and Gata3, and constitutively active human smoothened, Smo-M2, were described previously (Yamamoto et al., 1990; Hynes et al., 2000). Mouse Nkx2.2 and Nkx6.1 were a gift from T. M. Jessell, rat Pet1 was a gift from E. S. Deneris, and chick Lmx1b was a gift from C. Tabin. Full-length mouse Dbx2 and Dbx1, and chick Dbx2, were cloned by RT-PCR from total hindbrain RNA into pCR II-TOPO, sequence verified, and then cloned into RCASBP. In situ probes for chick Nkx2.2 and Nkx6.1 were a gift from J. L. R. Rubenstein. In situ probes for chick Pet1, Evx2, Chx10 and Lim1 were cloned by RT-PCR into pCR II-TOPO (Invitrogen). The dominant-negative Gata2 construct DN-G2 was generated by PCR amplification of the repressor domain of *Drosophila* Engrailed, amino acids 1-298 (a gift from D. S. Kessler), placed in-frame upstream of a PCR amplified N-terminal deletion of Gata2 amino acids 1-246, leaving the zinc-finger DNA binding domain intact.

### Morpholinos

The sequence of morpholino-modified antisense oligonucleotide with lissamine (Gene Tools, LLC) targeted against a splice junction of chick Nkx6.1 was 5'-ATCGAGCCTTGATCTGCCGGAGGG-3'. A morpholino with the sequence inverted was used as a control. The splice junction was identified by comparison with the mouse Nkx6.1 genomic sequence, and the intron was amplified by PCR of chick genomic DNA with primers based on the chick cDNA Nkx6.1 sequence (5' primer sequence, 5'-CCGCCCTGGAGGGACGCCCG-3'; and 3' primer sequence, 5'-GAAAATCTGCTGCCCGGAGACGTG-3').

### In ovo electroporation

Chick embryos were unilaterally electroporated at HH stage 8-10, as previously described (Hynes et al., 2000). DNA at 3-6 mg/ml or morpholinos at 1 mM were microinjected into the central canal of the neural tube, and platinum electrodes flanking the neural tube delivered 6 square pulses of 28V with a duration of 40 ms and an interpulse interval of 45 milliseconds. Chicks were harvested 2-4 days later.

### Explant culture

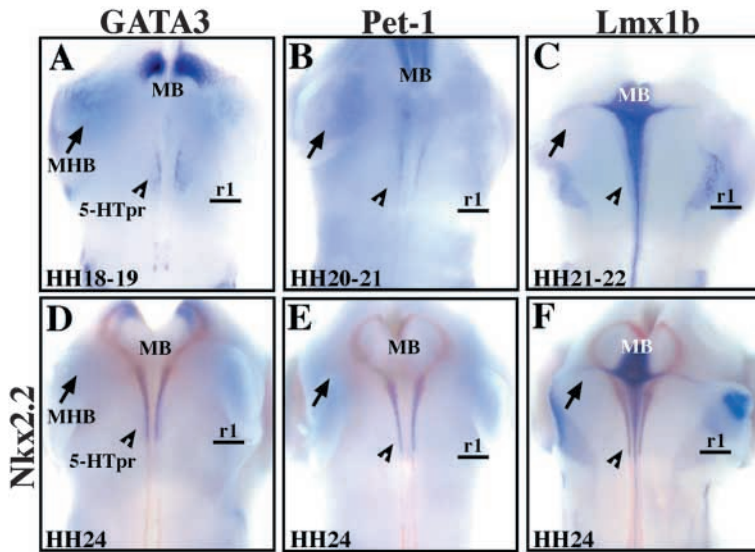
The ventral mid- and hindbrains of E8 embryos from *Gata2* heterozygous matings were dissected and cultured in collagen as described (Hynes et al., 1995; Ye et al., 1998). Explants were cultured for 5 days prior to fixation with 4% paraformaldehyde, and then processed for immunofluorescence or in situ hybridization as described below. Yolk sac tissue was collected for subsequent genotyping.

### In situ hybridization and immunofluorescence

Immunofluorescence on sections and wholemounts was performed as described (Hynes et al., 1995; Hynes et al., 2000), using rabbit anti-5-HT (Serotec), rat anti-5-HT (Chemicon), mouse anti-3C2 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-islet1 (Developmental Studies Hybridoma Bank, University of Iowa), mouse anti-Gata2 (Santa Cruz Biotech), mouse anti-Gata3 (Santa Cruz Biotech), mouse anti-Nkx2.2 (Developmental Studies Hybridoma Bank, University of Iowa) and rabbit anti-Nkx6.1 (Jensen et al., 1996). Single and double in situ hybridization were carried out as described (Ye et al., 1998).

## Results

5-HT neurons first develop in rhombomere 1 (r1) of the hindbrain adjacent to the ventral midline at embryonic day 11 (E11) in mouse and E4 in chick (Aitken and Tork, 1988; Okado et al., 1992). Expression of Gata2, Gata3, Lmx1b and Pet1 precede the appearance of 5-HT positive neurons (Nardelli et al., 1999; Bell et al., 1999; Hendricks et al., 1999; Ding et al., 2003) (Fig. 1). The Gata transcription factors are first expressed in r1 around Hamburger and Hamilton stage 17-18 (HH17-18 or E2.5-2.75) in the chick embryo (Fig. 1A). Gata2 expression precedes that of Gata3, but once expressed each Gata protein can positively regulate expression of the other (Karunaratne et al., 2002) (data not shown), and by HH24 expression of both Gata2 and Gata3 in r1 is prominent and localizes to the ventral Nkx2.2-positive p3 domain (Fig. 1D). Lmx1b expression is detected at HH18-19 within the floor plate and MHB, but by HH20-22 a second Lmx1b expression region adjacent to the floor plate in r1 becomes evident (Fig. 1C), which by HH24 co-localizes with Gata2 and Nkx2.2 (Fig. 1F and data not shown). Pet1 is localized specifically to 5-HT neurons in rodents (Hendricks et al., 1999; Pfaar et al., 2002). Similarly in chick, Pet1 expression is restricted to the ventral



**Fig. 1.** Endogenous expression of Gata, Pet1 and Lmx1b. Whole-mount in situ hybridization of chick hindbrain showing expression of (A) Gata3 at HH18-19, (B) Pet1 at HH20-21, (C) Lmx1b at HH21-22, (D) Gata3 (blue), (E) Pet1 (blue), and (F) Lmx1b (blue) with Nkx2.2 (red) at HH24. The midbrain (MB), mid-hindbrain boundary (MHB, arrow), and the 5-HT progenitors (5-Htpr, arrowhead) are marked; the boundary between rhombomere1 (r1) and r2 is indicated by a horizontal black line.

midline in r1 of the hindbrain starting at HH20-21 (Fig. 1B), with expression prominent by HH24 within the Nkx2.2 expression domain (Fig. 1E).

#### Gata2 plays an essential role in the specification of rostral 5-HT neurons

To examine the role of the Gata transcription factors in the development of 5-HT neurons, we misexpressed either Gata2 or Gata3 in the chick neural tube using in ovo electroporation. Strikingly, either Gata protein is sufficient to induce ectopic 5-HT neurons throughout r1 of the hindbrain (Fig. 2A-C). The induction of 5-HT neurons by the Gata factors requires the presence of both the zinc-finger DNA-binding domain and the N-terminal activation domain (Yang et al., 1994) (data not shown), and substituting the activation domain with that of VP16 mimics the activity of wild-type Gata protein (data not shown). First detectable at HH25, slightly after endogenous rostral 5-HT neurons, ectopic neurons extend well-developed processes and morphologically resemble their endogenous counterparts (Fig. 2D). Furthermore they are generated cell-autonomously, as only cells misexpressing Gata become 5-HT positive (Fig. 2E-G). Spatially ectopic 5-HT neurons develop along the entire dorsoventral axis, expanding from endogenous neurons bordering the ventral midline to near the dorsal roof plate (Fig. 2C). Induction, however, is limited along the anteroposterior axis to r1 of the hindbrain, where the earliest developing 5-HT neurons reside, and no ectopic 5-HT neurons are generated in the forebrain, the midbrain, or caudal to r1 of the hindbrain (Fig. 2A,B and data not shown). Even at HH30 (E6.5), well after the development of endogenous caudal 5-HT populations in r2-7, Gata2 is insufficient to generate ectopic 5-HT neurons within these rhombomeres (data not shown).

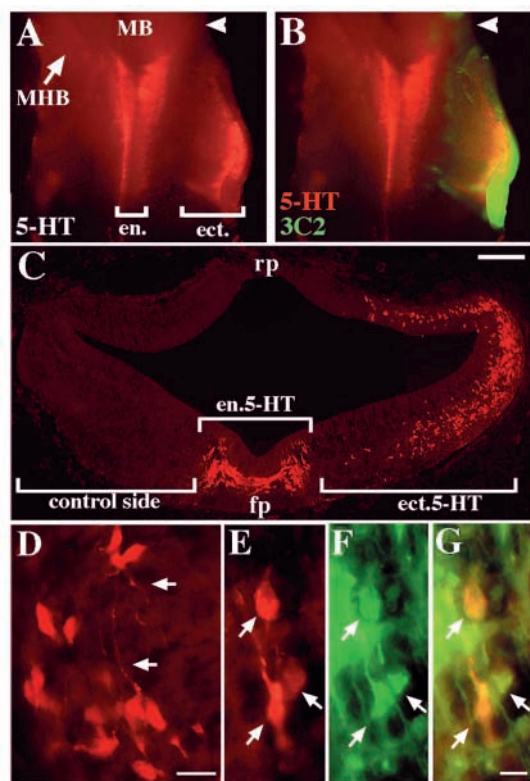
Prior to the detection of 5-HT neurotransmitter, Gata2 and Gata3 induce expression of one another (data not shown), as well as Lmx1b and Pet1 (Ding et al., 2003). Ectopic expression of these transcription factors is found throughout r1, but not in r2-7 on the electroporated side of the neural tube (Fig. 3A and data not shown). To determine whether Lmx1b and/or Pet1 are sufficient to specify 5-HT neurons independently of the Gata factors, we electroporated each factor individually, or in

combination, into the hindbrain. However, under no circumstance did we observe ectopic 5-HT neurons (data not shown), indicating that although Pet1 and Lmx1b are necessary for the development of 5-HT neurons (Hendricks et al., 2003; Ding et al., 2003; Cheng et al., 2003) they are not sufficient.

The sufficiency of the Gata transcription factors to induce 5-HT neurons along the dorsoventral axis of r1 prompted us to examine in parallel the fate of more dorsally localized neuronal populations. As described above, molecularly distinct neural subtypes arise along the dorsoventral axis of the spinal cord (Briscoe and Ericson, 2001), and some of these populations are also present in the rostral hindbrain (Fig. 3B,C and data not shown). We found that misexpression of Gata2 severely reduces the numbers of Chx10-positive V2 interneurons that develop adjacent to the p3 domain in the rostral hindbrain (Fig. 3B). Similarly, Lim1-positive neurons that occupy the p2, p1 and p0 domains, and Evx2-positive V0 interneurons are all decreased or eliminated in response to exogenous Gata2 (Fig. 3C and data not shown). Furthermore, Gata2 does not alter cell fate by affecting homeodomain (HD) protein expression, as the class I HD proteins Dbx2 and Pax7 are not repressed, and the class II HD proteins Nkx2.2 and Nkx6.1 are not activated in response to ectopic Gata2 (Fig. 3D,E and data not shown). Thus Gata2 is sufficient to re-specify neural progenitors along the dorsoventral axis of r1 to a 5-HT cell fate downstream of the HD code.

To examine the requirement of Gata2 and Gata3 in specifying endogenous 5-HT neurons, we analyzed the development of these neurons in knock-out mice. Mice homozygous for a targeted deletion in *Gata3* die between E11 and E12 with massive internal bleeding, gross abnormalities in the nervous system and disruption of fetal hematopoiesis (Pandolfi et al., 1995). Despite this, *Gata3* null embryos possess similar numbers of 5-HT positive neurons in r1 at E11 as their heterozygous littermates (Fig. 4A,B), indicating that Gata3 is not required for the proper specification of these 5-HT neurons. *Gata2* null mice die in utero between E9.5 and E10.5 from pan-hematopoietic failure (Tsai et al., 1994). As 5-HT immunoreactivity is not yet detectable at E10.5, we examined the expression of the early 5-HT neuron specific

transcription factor *Pet1* (Hendricks et al., 1999). In *Gata2*<sup>-/+</sup> embryos, *Pet1* is present bordering the ventral midline of the rostral hindbrain (Fig. 4C). By contrast, *Gata2*<sup>-/-</sup> embryos lack



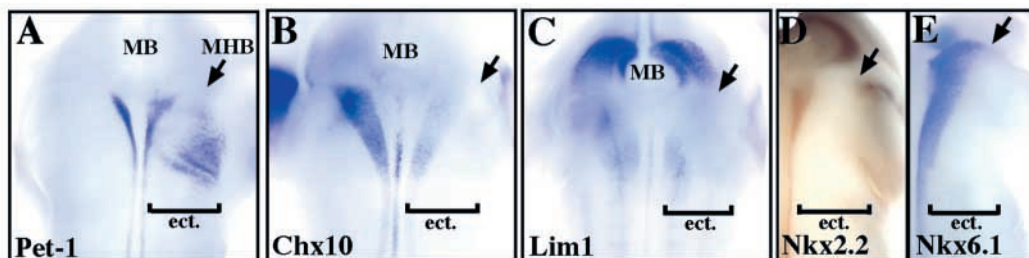
**Fig. 2.** Ectopic generation of 5-HT neurons by *Gata2* in r1. (A,B) Whole-mount immunofluorescence of chick hindbrain 4 days after electroporation of *Gata2*, with antibodies against 5-HT (red, A,B) and 3C2, a viral envelope marker (B, green). Endogenous 5-HT neurons (en.) versus ectopic 5-HT neurons (ect.) are bracketed (A). An arrowhead indicates the lack of 5-HT neurons in the midbrain (MB) despite the presence of exogenous *Gata2* (B, green). The midbrain (MB) and mid-hindbrain boundary (MHB, arrow) are marked. (C) Immunofluorescence for 5-HT on thin (10–12  $\mu$ m) sections through r1 of the hindbrain. The dorsal roof plate (rp) and ventral floor plate (fp) are marked; endogenous 5-HT neurons (en. 5-HT) versus ectopic 5-HT neurons (ect. 5-HT), and unelectroporated side (control side) are marked by brackets. (D–G) Higher magnification of ectopic 5-HT positive neurons (red; D,E,G) from C, showing cell bodies and processes (D, arrows) and co-localization (E–G, arrows) with 3C2 (F,G, green). Scale bars: C, 75  $\mu$ m; D, 12.5  $\mu$ m; G, 8  $\mu$ m.

expression of *Pet1*, even though *Nkx2.2*, and thus p3, neural progenitors are present (Nardelli et al., 1999) (Fig. 4D, left). Furthermore, expression of *Gata3* is still found throughout much of the hindbrain, including r1, as previously reported [(Pata et al., 1999) but contrary to the findings of Nardelli et al. (Nardelli et al., 1999)] (Fig. 4D, right), and partially overlaps with *Nkx2.2* (Fig. 4D, bottom right), suggesting that both hindbrain 5-HT and interneuron progenitors initiate expression of *Gata3* in the absence of *Gata2*.

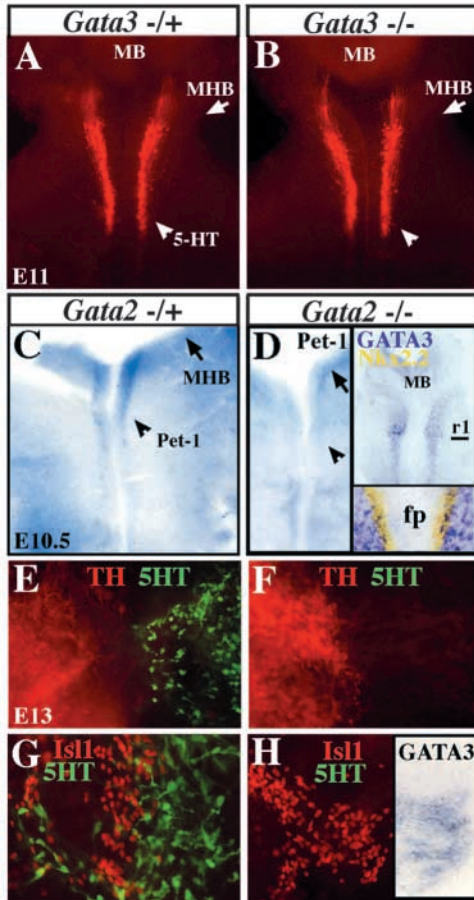
To examine 5-HT neuron development in the absence of *Gata2* directly, explant cultures of E8 ventral midbrain and hindbrain were analyzed at the equivalent of E13. In explants from wild-type and *Gata2*<sup>-/+</sup> embryos, 5-HT positive neurons are consistently detected along with tyrosine hydroxylase-positive dopaminergic neurons and *Isl1*-positive motoneurons (Fig. 4E,G). However, 5-HT neurons are completely absent in explants from *Gata2*<sup>-/-</sup> embryos, although the development of dopaminergic neurons and motoneurons is unaffected (Fig. 4F,H). Furthermore, 5-HT neurons fail to develop despite the continued expression of *Gata3* in the explants (Fig. 4H), although expression by 5-HT progenitors versus interneuron populations could not be confirmed. Thus *Gata3* appears unable to substitute for the loss of *Gata2*, and *Gata2*, although only sufficient in r1, is required for the correct specification of all 5-HT populations.

### **Shh induces 5-HT neurons in r1 through *Gata2***

The morphogen *Shh* patterns the neural tube along the dorsoventral axis and is required for the development of 5-HT neurons (Ye et al., 1998; Hynes et al., 2000), suggesting that *Gata2* may function as its downstream mediator. To investigate this, we used a constitutively active form of the *Shh* co-receptor smoothened (*Smo-M2*) that mimics *Shh* signaling (Xie et al., 1998; Hynes et al., 2000). Similar to *Gata2*, electroporation of *Smo-M2* in the hindbrain results in the cell-autonomous generation of 5-HT neurons throughout r1 at HH24 (Fig. 5A–D). Importantly, the induction of ectopic 5-HT neurons by *Smo-M2* is preceded by approximately 12–15 hours by the activation of both *Gata2* and *Gata3* (Fig. 5E and data not shown), followed by the activation of *Lmx1b* and *Pet1* (Fig. 5F and data not shown). The induction of *Gata2* occurs along the entire dorsoventral axis of r1 (Fig. 5G), and only cells that express *Smo-M2* ectopically express *Gata2* (Fig. 5H). Furthermore only cells that ectopically express *Gata2* become 5-HT positive (Fig. 5I), indicating that *Gata2* is correctly expressed to serve as a mediator between *Shh* and 5-HT development.

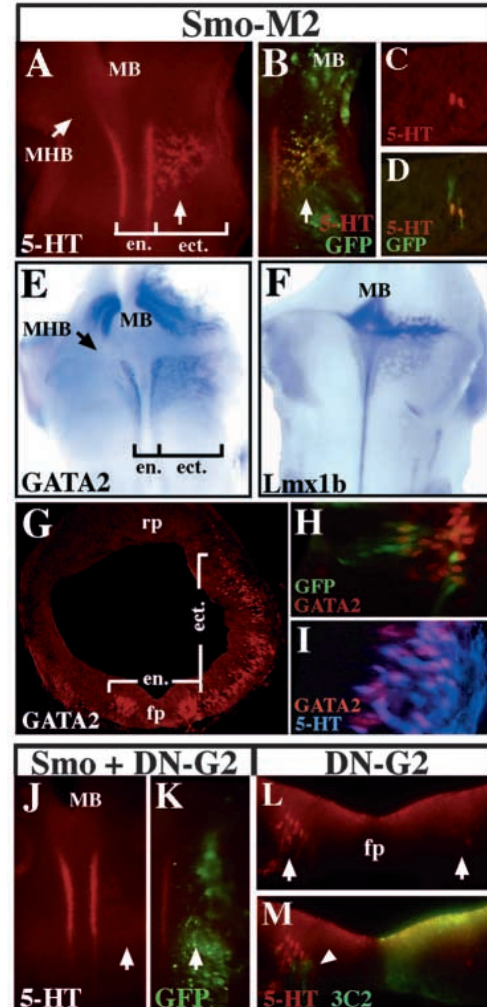


**Fig. 3.** *Gata2* re-specifies dorsal neural progenitors independently of HD proteins. (A–E) Whole-mount in situ hybridization of chick hindbrains 3–4 days after electroporation of *Gata2*, showing (A) *Pet1* with ectopic (ect.) expression bracketed, (B) *Chx10*, (C) *Lim1*, (D) *Nkx2.2*, and (E) *Nkx6.1* in which the electroporated (ect.) side is bracketed. The midbrain (MB) and mid-hindbrain boundary (MHB, arrow) are marked.



**Fig. 4.** *Gata2* is necessary, and *Gata3* is dispensable, for the development of rostral 5-HT neurons. (A,B) Whole-mount immunofluorescence for 5-HT of (A) *Gata3*<sup>+/+</sup> and (B) *Gata3*<sup>-/-</sup> embryos at E11. (C,D) Whole-mount in situ hybridization for Pet1 on *Gata2*<sup>+/+</sup> (C), and Pet1 (D, left) and *Gata3* (D, top right) on *Gata2*<sup>-/-</sup> (D) embryos at E10.5. A close up of double in situ hybridization for *Gata3* (blue) and *Nkx2.2* (yellow) in r1 at the ventral midline is shown on the bottom right of D. The floor plate (fp), midbrain (MB), mid-hindbrain boundary (MHB, arrow), 5-HT immunoreactivity (A,B; arrowheads), and the presence (C) versus absence (D) of Pet1 transcript (arrowheads) are marked. (E-H) Immunofluorescence staining on explant cultures from *Gata2*<sup>+/+</sup> (E,G) versus *Gata2*<sup>-/-</sup> (F,H) embryos, at the equivalent of E13, with antibodies against tyrosine hydroxylase (E,F; TH, red) or *Isl1* (G,H, red), compared with 5-HT (E-H, green). (H) In situ hybridization for *Gata3* on *Gata2*<sup>-/-</sup> explant cultures (inset, blue).

To provide further confirmation that *Gata2* acts downstream of *Shh*, we determined whether the induction of 5-HT neurons by the *Smo-M2/Shh* signal depends on a functional *Gata2* protein. To this end, we generated a dominant-negative protein in which the zinc-finger DNA-binding domains of *Gata2* were fused with the transcriptional repressor domain of *Drosophila* Engrailed (DN-G2) (Sykes et al., 1998). Consistent with our hypothesis, cells that express *Smo-M2* fail to develop into 5-HT neurons in the presence of co-electroporated DN-G2 (Fig. 5J,K). By contrast, co-expression of *Smo-M2* with the Engrailed repressor domain alone has no effect on 5-HT neuron



**Fig. 5.** *Shh* induces 5-HT neurons via *Gata2*. (A-I) Whole-mount immunofluorescence, thin section immunofluorescence or whole-mount in situ hybridization on chick hindbrain 3-4 days after electroporation of *Smo-M2.IRES.GFP*. (A,B) Whole-mount immunofluorescence for 5-HT (A,B; red), merged with GFP (B, green); only the electroporated side is shown in B. (C,D) Immunofluorescence on thin (10-12  $\mu$ m) sections through r1 for 5-HT (C,D; red), merged with GFP (D, green). Arrow (A,B) shows corresponding point in both panels. (E,F) Whole-mount in situ hybridization for *Gata2* (E) and *Lmx1b* (F). Endogenous 5-HT neurons (en.) versus ectopic 5-HT neurons (ect.) are bracketed (A,E). (G-I) Immunofluorescence on thin (25  $\mu$ m) sections through r1, with antibodies against *Gata2* (red); higher magnification through the width of the neural tube shows co-localization of *Gata2* with GFP (H, green) and 5HT (I, blue). (J-M) Immunofluorescence 3 days after electroporation of dominant-negative *Gata2* (DN-G2), either with *Smo-M2* (J,K) or alone (L-M). (J,K) Whole-mount immunofluorescence for 5-HT (J,K; red), merged with GFP (J, green). Only the electroporated side of the hindbrain is shown in J and an arrow indicates the absence of ectopic neurons. (L,M) Immunofluorescence on thin (10-12  $\mu$ m) sections through the ventral neural tube at r1 of the hindbrain for 5-HT (L,M; red), merged with 3C2 (M, green). Arrows indicate the population of 5-HT neurons on the control (left) side and their absence on the electroporated (right) side. An arrowhead indicates the absence of 5-HT (L, red) in cells expressing DN-G2 (L, green) on the control side. The midbrain (MB), mid-hindbrain boundary (MHB, arrow) are indicated on wholemounts. fp, floor plate; rp, roof plate.

induction (data not shown). Moreover, electroporation of DN-G2 in ventral r1 inhibits the development of endogenous 5-HT neurons (Fig. 5L,M), further demonstrating the essential role of Gata2 during 5-HT neuron specification.

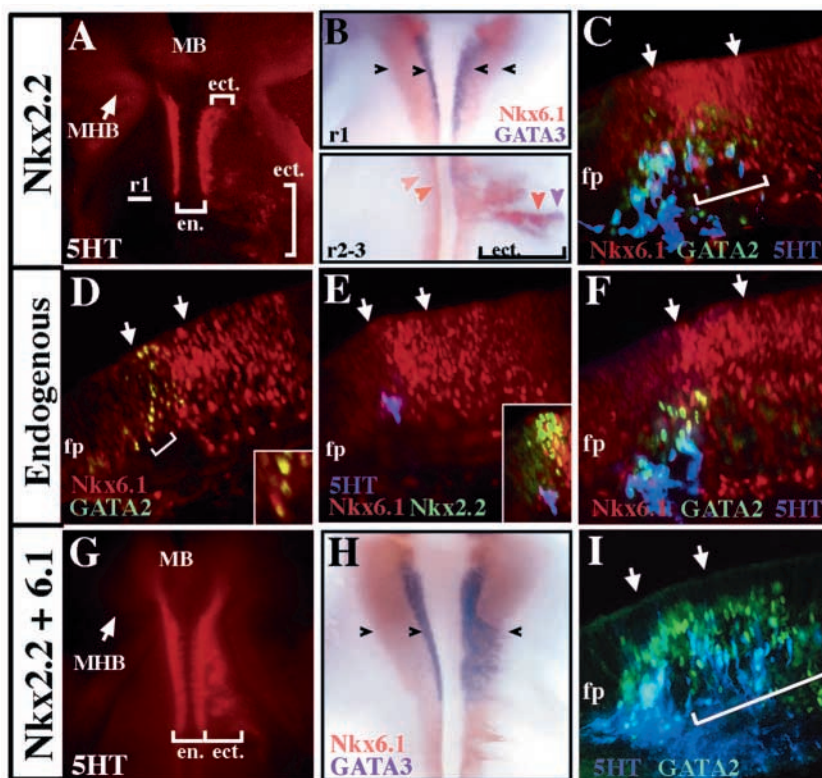
### Nkx2.2 and Nkx6.1 mediate induction of Gata by Shh in r1

To place Gata2 within a Shh-dependent pathway for the specification of rostral 5-HT neurons, we undertook to identify the HD protein code that controls Gata2 expression within r1. 5-HT neurons develop within the ventral Nkx2.2-positive p3 domain in r1 (Fig. 1), and are largely absent in *Nkx2.2* knock-out mice (Briscoe et al., 1999). Thus we first tested whether Nkx2.2 is sufficient to induce Gata2 and 5-HT neurons. When Nkx2.2 is misexpressed in the chick hindbrain, ectopic Gata2, Gata3, and 5-HT neurons are induced in r1, but only within a narrow ventral domain immediately lateral to endogenous 5-HT neurons (Fig. 6A) and endogenous Gata expression (Fig. 6B, top). This suggests that Nkx2.2 is sufficient in the most ventral regions of the rostral hindbrain, but that additional Shh-activated transcription factors are required for the induction of 5-HT neurons in more lateral positions.

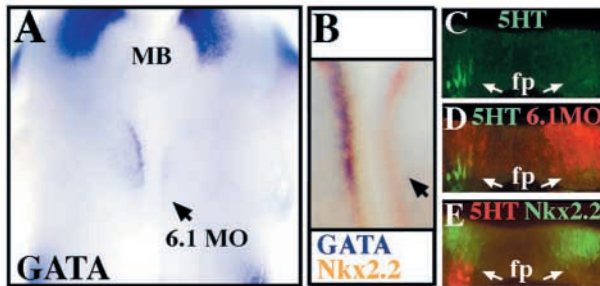
Nkx6.1 is a Shh-inducible HD transcription factor similar to Nkx2.2 but with a broader expression domain (Briscoe et al., 2000). We find that in the rostral hindbrain, transcripts for Nkx6.1 along the dorsoventral axis are not uniform, but rather a region of higher expression is detected near the ventral midline (Fig. 3E; Fig. 6B, bottom, orange arrows). This domain of higher Nkx6.1 expression is seen at the protein level in the ventricular zone at HH20 (Fig. 6D,E, white arrows) and is maintained at HH25 (Fig. 6F, white arrows). High Nkx6.1 expression partially overlaps with the Nkx2.2-positive p3 domain (Fig. 6E, inset), and within this overlap co-localizes with Gata2 expression (Fig. 6D). These high Nkx6.1 expressing, Gata2-positive progenitors localize increasingly more ventrally in deeper layers (Fig. 6D, bracket and inset), where they begin to express 5-HT (Fig. 6E). More mature 5-HT neurons continue to express Gata2 and localize even further ventrally in the mantle layer (Fig. 6F). This suggests that Shh regulates Gata2 and 5-HT expression in r1 through a combination of Nkx2.2 and high Nkx6.1 signals. Consistent with this, induction of Gata2 and 5-HT neurons by ectopic Nkx2.2 is confined within the region of high Nkx6.1 expression (Fig. 6C). Furthermore, whereas electroporation of Nkx6.1 alone has no effect (data not shown), co-electroporation of Nkx6.1 with Nkx2.2 results in the induction of Gata-positive neurons in a wide domain within r1 of the

hindbrain (Fig. 6H,I), and subsequently the induction of 5-HT neurons extends more laterally compared with Nkx2.2 alone (Fig. 6G,I).

Further support for the involvement of Nkx6.1 in rostral 5-HT development is revealed by ectopic expression of Nkx2.2 caudal to r1. In contrast to in r1, in r2-7 misexpression of



**Fig. 6.** Nkx2.2 and Nkx6.1 mediate Gata expression and 5-HT development in r1. (A,B) Chick hindbrain 3-4 days after electroporation of Nkx2.2, showing whole-mount immunofluorescence for 5-HT (A, red) and in situ hybridization (B) divided into r1 versus r2-3 for Gata3 (purple) and Nkx6.1 (orange). Ectopic (ect.) versus endogenous neurons (en.) are bracketed and rhombomere 1 (r1) is marked (A). Black arrowheads delineate the extent of Gata3 to Nkx6.1 expression on the electroporated (ect) versus control side (B, top). Orange arrowheads indicate the gradient of Nkx6.1 expression, whereas purple and orange arrowheads indicate the ectopic expression (ect. bracketed) of Nkx6.1 and Gata3, respectively (B, bottom). (C) Immunofluorescence on thin (25  $\mu$ m) sections through r1 on the side of the neural tube electroporated with Nkx2.2 or Nkx6.1 (red), Gata2, (green) and 5-HT (blue), showing the higher expression of Nkx6.1 (arrows) where endogenous and ectopic (bracketed) Gata2 expression and 5-HT neurons develop. (D-F) Immunofluorescence on thin (25  $\mu$ m) sections through r1 showing endogenous expression of Nkx6.1 (D-F, red), Gata2 (D,F, green), Nkx2.2 (E, inset green) and 5-HT (E,F, blue) at HH20 (D,E) and HH25 (F), with the higher Nkx6.1 expressing ventral region marked by white arrows. (D, inset) Co-localization between high Nkx6.1 and Gata2 in bracketed region. (E, inset) Overlay of Nkx2.2 with the Nkx6.1 and 5-HT expression shown in the main panel. (G,H) Whole-mount immunofluorescence for 5-HT (G, red), or in situ hybridization for Gata3 (purple) and Nkx6.1 (orange) (H), on chick hindbrain performed 3-4 days after electroporation of Nkx2.2 and Nkx6.1. Ectopic (ect.) versus endogenous neurons (en.) are bracketed (G); black arrowheads delineate the extent of Gata3 to Nkx6.1 expression on the electroporated (right) versus control side in (H). (I) Immunofluorescence on thin (25  $\mu$ m) sections through r1 on the electroporated side of the neural tube, with antibodies against Gata2 (green) and 5-HT (blue). The approximate boundaries of the endogenous high expressing Nkx6.1 domain are marked by arrows, and the ectopic Gata2 and 5-HT positive neurons are bracketed. The midbrain (MB) and mid-hindbrain boundary (MHB, arrow) are indicated on wholemounts; the floor plate (fp) is indicated on sections.



**Fig. 7.** Nkx6.1 is necessary for the development of 5-HT neurons in r1. (A,B) Whole-mount in situ hybridization of chick hindbrain 3 days after electroporation with morpholino-modified antisense oligonucleotides targeted against Nkx6.1 (6.1 MO), for (A) Gata2 and Gata3, or (B) Gata (purple) and Nkx2.2 (orange). Arrows indicate the loss of Gata expression of the electroporated side. The midbrain (MB) and mid-hindbrain boundary (MHB, arrow) are indicated (A). (C-E) Immunofluorescence on thin (10–12  $\mu$ m) sections through the ventral neural tube in r1 of the hindbrain three days after electroporation with 6.1 MO (D, red), with antibodies against 5-HT (C,D, green; E, red) or Nkx2.2 (E, green). The location and absence of 5-HT neurons is indicated by arrows; the floor plate (fp) is marked.

Nkx2.2 activates expression of Nkx6.1 in the ventricular zone (Fig. 6B, bottom; and data not shown). Concurrent with this ability, Gata factors and subsequently 5-HT neurons are induced in regions of high Nkx6.1 expression (Fig. 6A,B, bottom; and data not shown). Thus there is a strict dependence on the combined expression of Nkx2.2 and Nkx6.1 for the activation of Gata2 and specification of early born 5-HT neurons.

As a requirement for Nkx6.1 in 5-HT neuron development has not previously been described, we examined whether Nkx6.1 is necessary for endogenous 5-HT development within r1 of the hindbrain by misexpressing the class I HD protein Dbx2. Dbx2 inhibits Nkx6.1 expression in the spinal cord (Briscoe et al., 2000); however, neither Dbx2 nor the closely related Dbx1 represses Nkx6.1 expression in r1, and 5-HT neuron development is unaffected (data not shown). Interestingly, the Nkx6.1 and Dbx2 expression domains are not adjacent in r1 of the hindbrain, and neither are the expression domains of Nkx2.2 and its corresponding class I HD protein Pax6 (data not shown). This suggests that the mutual repression between class I and II HD protein pairs that are adjacent in the spinal cord (Briscoe et al., 2000; Muhr et al., 2001) may not act in r1 to establish progenitor domains, or is lost at later stages of development.

Thus we used morpholino-modified antisense oligonucleotides to interfere with Nkx6.1 expression (Kos et al., 2001; Heasman, 2002). Electroporation of fluorescently-labeled morpholinos targeted against a slice junction in chick Nkx6.1 (6.1 MO; see Fig. S1 at <http://dev.biologists.org/supplemental/>) results in the loss of both Gata and Pet1 expression on the electroporated side of the rostral hindbrain (Fig. 7A and data not shown). The disruption of downstream transcription factors is specific as Nkx2.2 expression is maintained in cells that fail to activate Gata (Fig. 7B and data not shown). Subsequently, 5-HT neurons fail to develop in the presence of the Nkx6.1 morpholino despite the continued expression of Nkx2.2 (Fig. 7C–E). An inverted control morpholino has no effect on Gata expression or 5-HT development (data not shown). Thus evidence from both gain- and loss-of-function studies support the hypothesis that Nkx2.2 and high levels of Nkx6.1 make up the HD code for the specification of 5-HT neurons within r1.

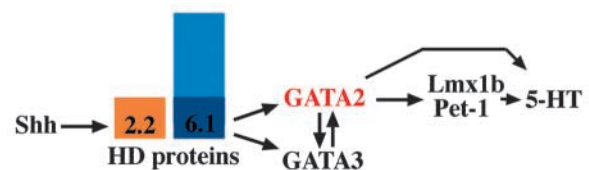
## Discussion

The zinc-finger transcription factors Gata2 and Gata3 are expressed in the ventral hindbrain subsequent to the establishment of HD-positive progenitor domains but preceding the consolidation of a 5-HT phenotype (Fig. 1). Both

are expressed in neural precursors and postmitotic neurons (Nardelli et al., 1999), suggesting a potential role in specifying neural cell fate. We find that ectopic expression of either Gata protein is sufficient to induce 5-HT neurons in r1 of the hindbrain (Fig. 2), and that this occurs downstream of the Shh-regulated HD protein code, and upstream of Lmx1b and Pet1 (Figs 3, 8).

To determine whether either of the Gata proteins is essential for the development of 5-HT neurons we analyzed Gata null embryos. Knock-out analysis revealed that 5-HT neurons in r1 are specified normally in Gata3 null embryos, but are completely absent in mid-hindbrain explants derived from Gata2 null mice (Fig. 4). Furthermore, in Gata2 null embryos, Gata3 expression is maintained in the r1 Nkx2.2-positive domain at E10.5 and in explant cultures at the equivalent of E13, suggesting that Gata3 is unable to specify 5-HT neurons in the absence of Gata2 (Fig. 4). By contrast, the loss of 5-HT neurons in caudal raphe nuclei of adult chimeric Gata3<sup>-/-</sup> mice (van Doorninck et al., 1999) indicates that Gata3 has a role in the development of caudal 5-HT populations. Thus Gata2 and Gata3, despite their overlapping CNS expression domains and similar structures, have distinct roles in 5-HT development. This is reminiscent of the distinct roles played by different Gata family members during hematopoiesis (Patient and McGhee, 2002), and by LIM-HD proteins in determining motoneuron subtype identity (Shirasaki and Pfaff, 2002).

Gata2 is sufficient to induce 5-HT neurons only in r1 (Fig. 2), despite being induced by Nkx2.2 in r2–7 preceding the induction of 5-HT neurons (Fig. 6), and despite being necessary for the proper specification of all 5-HT neurons in explants from Gata2 null embryos. Unlike 5-HT neurons in r1, the development of caudal populations follows that of visceral



**Fig. 8.** The specification of rostral 5-HT neurons in the vertebrate hindbrain. Shh signaling in the ventral midline activates the type II HD protein Nkx2.2 and high levels of Nkx6.1 in the p3 domain of the rostral hindbrain. Nkx2.2 and Nkx6.1 are sufficient to activate expression of Gata2 and Gata3, which can positively regulate each other. Gata2, in turn, is necessary and sufficient to activate Lmx1b and Pet1, and to specify 5-HT neurons. Gata2 may activate additional transcription factors and/or may be required to directly cooperate with Lmx1b and Pet1 in 5-HT specification. By contrast, Gata3 is dispensable for the specification of rostral 5-HT neurons.

motoneurons (vMN) from common progenitors (Pattyn et al., 2003). Underlying the switch between early born vMN and later born 5-HT neurons is the downregulation of Phox2b expression, and Gata2, though not sufficient, may be required for this process. Thus, a key factor for the development of caudal 5-HT neurons that is regulated by Nkx2.2 is suggested but not identified by this study. Interestingly, in the spinal cord, Gata2 has been implicated in the development of Chx10-positive V2 interneurons, which are reduced in number in *Gata2* null mice (Zhou et al., 2000). However, in r1 of the hindbrain, Gata2 is not sufficient to specify V2 interneurons, but rather transforms them to a 5-HT cell fate.

Preceding the induction of ectopic 5-HT neurons, Gata2 activates *Lmx1b* and *Pet1* (Fig. 3), consistent with the timing of their developmental expression (Fig. 1). Both *Lmx1b* and *Pet1* are required for the specification of 5-HT neurons (Hendricks et al., 2003; Ding et al., 2003; Cheng et al., 2003), yet we find that they are not sufficient to activate one another or to generate 5-HT neurons. Thus Gata2 may activate additional transcription factors and/or cooperate directly with *Lmx1b* and/or *Pet1* to specify these neurons (Fig. 8).

Upstream of Gata2, the HD proteins Nkx2.2 and Nkx6.1 act together to mediate the Shh signal for the induction of 5-HT neurons in r1 (Figs 6, 7). In the ventral spinal cord, Nkx6.1 is uniformly expressed and is not required for the development of V3 interneurons (Briscoe et al., 1999; Briscoe et al., 2000; Sander et al., 2000). Yet in r1 of the hindbrain, expression of Nkx6.1 is more complex, and we detect a region of higher expression near the ventral midline that overlaps with the Nkx2.2-positive p3 domain, and from which Gata2-positive 5-HT progenitors arise (Fig. 6). Furthermore, high levels of Nkx6.1 expression are required to induce 5-HT neurons, and Nkx2.2 only generates ectopic 5-HT neurons in conjunction with high expression levels (Fig. 6). Conversely, a reduction in functional Nkx6.1 disrupts 5-HT development (Fig. 7). Thus, we identify Nkx6.1 as an essential component in the p3 domain of r1, and identify graded expression along the dorsoventral axis as a novel mechanism of HD code diversity.

In contrast to the importance of Nkx6.1 in rostral 5-HT neuron development in the chick, work by others suggests that Nkx6.1 is not essential for their development in the mouse (Pattyn et al., 2003). In *Nkx6* null embryos, in which both Nkx6.1 and the closely related Nkx6.2 are disrupted, *Pet1* expression is maintained throughout the hindbrain, suggestive of normal 5-HT development (Pattyn et al., 2003). Rostrocaudal patterning of the hindbrain diverges between species in many respects, including Hox gene expression, neuronal migration, axonal projections, and the rhombomeric development of neuronal populations including 5-HT neurons (Glover, 2001). For example, 5-HT neurons do not develop in r4 in the mouse, where their development appears to be inhibited by Nkx6 via Hox1b (Pattyn et al., 2003), but 5-HT neurons do develop in chick r4 where both Nkx6 and Hox1b are expressed (Fig. 3) (S.E.C., unpublished) (Glover, 2001). Furthermore, the Nkx6.2 expression domain differs between chick and mouse (Vallstedt et al., 2001), and no Nkx6.1 protein gradient is detected in the mouse hindbrain (S.E.C., unpublished). Together, these differences suggest the possibility that an altered HD code with respect to Nkx6 and 5-HT development occurs across species.

To conclude, we have identified a Shh-activated

transcriptional cascade that controls the specification of a specific subset of 5-HT neurons: early born 5-HT neurons in r1 that send ascending projections into the brain. This cascade is only sufficient in r1, but because these neurons also depend on Fgf8 secreted from the MHB (Ye et al., 1998), this cascade may be spatially limited by the requirement for this Fgf signal. Although components of this cascade, including Gata2, are required for the specification of most or all 5-HT neurons, there appears to be differences in their specific roles in distinct 5-HT populations (Briscoe et al., 1999; Hendricks et al., 2003; Ding et al., 2003), and additional factors that contribute to the development of caudal populations and affect the switch between Phox2b-positive vMN and 5-HT development remain to be identified. Though it is clear that Gata3 has a specific role in caudal 5-HT neuron development (van Doorninck et al., 1999), it alone does not explain the difference in specification requirements. Thus, overlapping but distinct signaling cascades are implicated in the development of rostral versus caudal 5-HT neurons and may underlie the individual functions of these related neuronal populations.

We thank Scott Heller for providing the Nkx6.1 antibody, and Leon Parker, T'Nay Pham and Maike Schmidt for helpful discussion and support.

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