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A role for midbrain arcs in nucleogenesis

Seema Agarwala and Clifton W. Ragsdale*

Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, Chicago, IL 60637, USA
*Author for correspondence (e-mail: cliff@drugs.bsd.uchicago.edu)

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SUMMARY

Nuclei are fundamental units of vertebrate brain organization, but the mechanisms by which they are generated in development remain unclear. One possibility is that the early patterning of brain tissue into reiterated territories such as neuromeres and columns serves to allocate neurons to distinct nuclear fates. We tested this possibility in chick embryonic ventral midbrain, where a periodic pattern of molecularly distinct stripes (midbrain arcs) precedes the appearance of midbrain nuclei. We found that midbrain arc patterning has a direct relationship to the formation of nuclei. Both differential homeobox gene expression and diagnostic axon tracing studies established that the most medial arc contains primordia for two major midbrain nuclei: the oculomotor complex and the red nucleus. We tested the relationship of the medial arc to oculomotor complex and red nucleus development by perturbing arc pattern formation in Sonic Hedgehog and FGF8 misexpression experiments. We found that Sonic Hedgehog manipulations that induce ectopic arcs or expand the normal arc pattern elicit precisely parallel inductions or expansions of the red nucleus and oculomotor complex primordia. We further found that FGF8 manipulations that push the medial arc rostrally coordinately move both the red nucleus and oculomotor complex anlagen. Taken together, these findings suggest that arcs represent a patterning mechanism by which midbrain progenitor cells are allocated to specific nuclear fates.

Key words: Bain nuclei, Oculomotor neurons, Red nucleus, Homeobox genes, Emx2, Sonic hedgehog, FGF8, Isthmus, Chick, Mouse

INTRODUCTION

In the adult brain, many neurons are organized into well-delineated clusters of characteristic shape and size. The role of these clusters, known as nuclei, is to organize neuronal circuitry. Thus, for a brain neuron, the projections of its axons, the neurotransmitters it employs and the local and long-distance inputs it receives, are governed by its nuclear assignment. Despite major recent insights into the early patterning events of the neural tube (Jessell and Lumsden, 1997), very little is known about the cellular and molecular patterning mechanisms that generate discrete brain nuclei of appropriate size, location and cell-type composition.

The ventral midbrain provides an elegant model system for addressing the mechanisms of brain nucleogenesis. The adult midbrain tegmentum exhibits a complex structural architecture featuring spherical, ovate and plate-shaped nuclei. During embryogenesis, this nuclear organization is preceded by a more regular organization into a reiterated series of arcuate territories called midbrain arcs (Agarwala et al., 2001; Sanders et al., 2002). Arcs are known to be molecularly distinct, differing, for example, in their expression of homeobox genes (Agarwala et al., 2001). Whether the arcs simply reflect a mechanism for regulating the numbers of distinct midbrain cell-types or serve a more specific role in allocating neurons to particular nuclear fates is unknown.

The possibility that developmental periodicities might provide a patterning substrate for nucleogenesis has been addressed in connectional and fate-mapping studies of hindbrain rhombomeres. The striking finding is that most brainstem nuclei are generated from serially adjoining sets of rhombomeres with few nuclei developing from single rhombomeres (Cramer et al., 2000; Diaz et al., 1998; Lumsden and Keynes, 1989; Marin and Puelles, 1995). Thus, the extent to which anteroposterior periodicities are crucial to hindbrain nuclear specification remains uncertain (Wingate and Lumsden, 1996). Indeed, the generation of hindbrain nuclei, many of which form longitudinal columns, may be more tightly regulated in the mediolateral dimension (Clarke et al., 1998; Marin and Puelles, 1995). This observation is of particular interest because midbrain arcs, unlike hindbrain rhombomeres, are arrayed along the mediolateral axis parallel to the ventral midline.

In these studies, we explored the relationship between arc pattern formation and the generation of midbrain nuclei by focusing on the most medial arc as a prototypical arc. Using diagnostic connectional and molecular criteria we identified the anlagen for at least two midbrain nuclei within the medial arc: the oculomotor complex (OMC) and the red nucleus (RN). Both of these nuclei are part of the motor system, but their functions and connections are so dissimilar that a shared origin in the same arc was unexpected. The OMC contains motoneurons that control eye movements and the parasympathetic regulation of accommodation and pupil

contraction (Evinger, 1988). The RN, by contrast, contains no motoneurons but is a cerebellar-related nucleus mediating motor cortex and cerebellar outflow to spinal cord in the control of limb movements (Holstege and Tan, 1988; Keifer and Houk, 1994; ten Donkelaar, 1988). The two medial arc pronuclei are also molecularly distinct, differing in their expression of and dependence on homeodomain transcription factors. In this report, we show that the prospective RN is distinguished by expression of the POU domain gene *BRN3A* and the homeobox gene *EMX2*, and that Emx2 function is required for RN development.

Within the medial arc, the RN and OMC anlagen occupy distinct spatial positions in three dimensions. We reasoned that if the formation of these midbrain nuclei is in fact governed by arc patterning, then any perturbation of the medial arc should result in strictly coordinate perturbations of its constituent pronuclei. The medial arc is flanked by two signaling centers, the Sonic Hedgehog (SHH)-rich rostral floor plate and the FGF8- and WNT1-rich midbrain-hindbrain junction (Agarwala et al., 2001; Joyner et al., 2000). Manipulations of SHH and FGF8 signals are known to result in perturbations of the midbrain arcs along the mediolateral axis (SHH) (Agarwala et al., 2001) and of midbrain generally along the anteroposterior axis (FGF8) (Crossley et al., 1996). To test the relationship between arc pattern formation and the generation of midbrain nuclei, we misexpressed *SHH* and *FGF8* with in vivo electroporation technology. Results from these experiments show that ectopic *SHH* expression anywhere within the midbrain is sufficient to induce both medial arc pronuclei. Furthermore, SHH manipulations that distort the midbrain arcs elicit completely parallel distortions of the OMC and RN primordia, and FGF8 manipulations that shift the arcs forward coordinately shift both medial arc pronuclei rostrally. These results suggest that the midbrain arcs represent a patterning template for allocating midbrain progenitor cells to their correct nuclear fates.

MATERIALS AND METHODS

Expression plasmids

The *Fgf8* expression construct pXeX-*Fgf8* was engineered by inserting a 0.9 kb mouse *Fgf8b* cDNA (MacArthur et al., 1995) into the *Xenopus EF1α* promoter expression vector pXeX (Johnson and Krieg, 1994). pXeX-*SHH*, which contains a cDNA encoding the 19.2 N-terminal fragment of chick *SHH*, was constructed as described (Agarwala et al., 2001).

In ovo electroporation

Fertilized chicken eggs were incubated in a humidified forced-draft chamber until the embryos reached stage 8-15 (Hamburger and Hamilton, 1951). Fifty to 250 nl of endotoxin-free plasmid DNA solution (1 μg/μl in 10 mM Tris, 1 mM EDTA, 0.02% Fast Green) was injected through a glass capillary needle into the midbrain vesicles of chick embryos displayed by windowing. For conventional macroelectroporations (Muramatsu et al., 1998; Sakamoto et al., 1998), two platinum electrodes ~2 mm apart were positioned to straddle the midbrain, and a series of eight electric pulses (50 mseconds, 9V) were delivered with a square wave electroporator (BTX 820, Genetronics, CA). Microelectroporation techniques (Momose et al., 1999) were employed to elicit more restricted midbrain transfections. A 500 μm tungsten wire was etched in 10% KOH to a tip diameter of 40 μm. The tungsten electrode was inserted into the lumen of the midbrain after plasmid injection and served as the negative electrode. The positive terminal was a platinum electrode placed outside the midbrain above the eye. The electrodes were held parallel at a distant of 1 mm and three electric pulses (25 mseconds, 7V) were delivered. Electroporated embryos were returned to the incubator for 3-4 days and on harvesting were submerged in 4% paraformaldehyde in a phosphate-buffered saline (PBS) solution. Embryos that showed some sign of injury from electroporation were excluded from further study. Our analyses are based on whole-mount and section in situ hybridization of 164 embryos successfully electroporated with the *SHH* (*n*=54) or *Fgf8* (*n*=110) constructs.

Axon tracing

Neural connections were studied in embryonic day (E) 5 and E6 chick brains (*n*=94) with fluorescein-conjugated lysinated dextrans (fluoro-emerald, 10 kDa, Molecular Probes). To label the OMC, the brain neurectoderm and the third cranial nerve were dissected away from adhering mesenchymal tissues in chilled Tyrode's solution and pinned to a SYLGARD dish. Tracer resuspended in water was dried to a slurry, picked up on a pair of fine forceps and applied to the dissected end of the nerve. For RN labeling, the tracer was dried on a minuten pin and stuck into dissected brainstem. Labeled embryo tissue was incubated for 30 minutes in chilled Neurobasal Medium (Life Technologies) with 5% fetal calf serum and 30 mM glucose, and placed for 2-4 hours in a 6% CO_2 incubator at 37°C. The tissue was then immersion-fixed overnight in 4% paraformaldehyde-PBS. The fluoresceinated dextrans were detected with anti-fluorescein Fab fragments conjugated to alkaline phosphatase in modified in situ hybridization protocols.

Midbrain explants

Chick embryos were collected at stage 12-15 and dissected in cold Tyrode's saline. Ventral midbrain explants with little or no adjoining dorsal midbrain tissue were kept briefly at 4°C in L-15 medium containing 10% heat-inactivated fetal bovine serum. The explants were then placed on a Millipore filter in a drop of Neurobasal medium containing 20 mM glucose, antibiotics and 10% fetal bovine serum, and were incubated in a 6% CO_2 incubator at 37°C for 2-3 days. Cultures were fixed in 4% paraformaldehyde-PBS for analysis by in situ hybridization.

Mice

All mice were cared for according to animal protocols approved by the IACUC of the University of Chicago. Outbred CD-1 timed pregnant mice were obtained from the University of Chicago Cancer Research Center Transgenic Facility. *Emx2* heterozygote mice provided by P. Gruss (Pellegrini et al., 1996) were bred and genotyped by PCR. Noon of the day of vaginal plug discovery was considered E0.5. Homozygous *Emx2* mutant embryos (*n*=25) between E12.5-E18.5 were recovered for gene expression analyses and compared with equal or greater numbers of wild-type and heterozygote littermate mice (*n*=27).

In situ hybridization

Two-color whole-mount in situ hybridization was carried out on chick brain with digoxigenin- and fluorescein-labeled riboprobes synthesized from chick cDNA plasmids for *ACHE*, *BRN3A*/*POU4F1*, *EMX2*, *EVX1*, *FGF8*, *FOXA2*, *GBX2*, *ISL1*, *OTX2*, *PAX3*, *PAX6*, *PHOX2A*, *SHH*, *TH* and *WNT1*. Whole-mount and section in situ hybridization was carried out on E12.5-E18.5 mouse neurectoderm with riboprobes for mouse class III β-tubulin/*Tubb6*, *Emx2* and *Fgf8*, and rat *Brn3a*, *Isl1* and *Th*. Riboprobe-mRNA duplexes and fluoresceinated dextrans were detected with antibody-phosphatase conjugates (Roche Molecular Biochemicals) and demonstrated by phosphatase histochemistry employing the distinguishable tetrazolium chromagens NBT and TNBT.

RESULTS

The medial arc is composed of multiple pronuclei: the oculomotor complex, red nucleus and a subset of midbrain dopaminergic neurons

Midbrain arcs are identified as acetylcholinesterase (ACHE)-rich arcuate cell-columns arrayed bilateral to the midbrain floor plate (Fig. 1A) (Ragsdale and Lumsden, 1995; Sanders et al., 2002). Two lines of evidence establish that motoneurons of the oculomotor complex (OMC) are embedded in the most medial *ACHE*-rich arc (arc 1). Gene expression for motoneuron-specific transcription factors *PHOX2A* and *ISL1* (Pattyn et al., 1997; Pfaff et al., 1996) is confined to the medial arc (Fig. 1A,B; data not shown), and retrograde tracer delivery to the third cranial nerve labels the medial arc *PHOX2A*+territory (Fig. 2A,B).

OMC neurons are not the sole constituents of the medial arc. Situated deep to the OMC, near the pial surface of the midbrain, is a second group of cells, identified as dopaminergic (DA) neurons by tyrosine hydroxylase (*TH*) gene expression (Fig. 1C,D). In the mouse, midbrain DA neurons have been reported to be migratory (Marchand and Poirier, 1983), raising the possibility that chick medial arc *TH*+ cells have migrated in from the adjoining ventral midline. This observation, combined with the late onset of *TH* gene expression in the chick and the lack of availability of early markers of chick DA neurons (Hynes et al., 1995; Smidt et al., 1997; Zetterstrom et al., 1997), leads to uncertainty about the origin of midbrain DA cells. We therefore confined our analyses of the medial arc to the development of its other constituents.

Overlapping the rostral end of the OMC along the anteroposterior axis and completely segregated from the OMC neurons along the ventricular-pial axis, is a third population of medial arc cells. This third territory is selectively enriched in mRNA for the homeodomain transcription factor EMX2 and for the POU domain transcription factor BRN3A (Fig. 1E-H; data not shown).

Connectional criteria establish that this *EMX2*+ and *BRN3A*+ territory of the medial arc contains neurons of the prospective red nucleus (pRN), a major brainstem nucleus involved in the control of limb movement. In tetrapod vertebrates, the only exclusively contralateral projection from midbrain tegmentum to lower brainstem and spinal cord originates in the RN (ten Donkelaar, 1988). Thus, this projection is diagnostic of the RN. Small tracer deposits of fluoresceinated dextrans centered in the medial arc at E6 elicit anterograde labeling of multiple fiber-tracts descending into the hindbrain, one of which crosses the midline (Fig. 2C). In reciprocal experiments, tracer injections placed in E5 or E6 hindbrain produce retrograde labeling of a single population of exclusively contralaterally projecting cells (Fig. 2D). *PHOX2A* (Fig. 2E) and *BRN3A* (Fig. 2F) gene expression combined with retrograde tract-tracing demonstrates that these identified RN neurons lie in the *BRN3A*+/*EMX2*+ territory of the medial arc.

These data establish that the medial arc contains multiple cell types distinguished by unique axonal connections and patterns of transcription factor gene expression. These cell types are not, however, spatially intermixed, but form discrete territories. Along the anteroposterior axis, the pRN, the OMC and the medial arc *TH* neurons appear in succession, with the OMC overlapping both the pRN and the *TH* neurons when

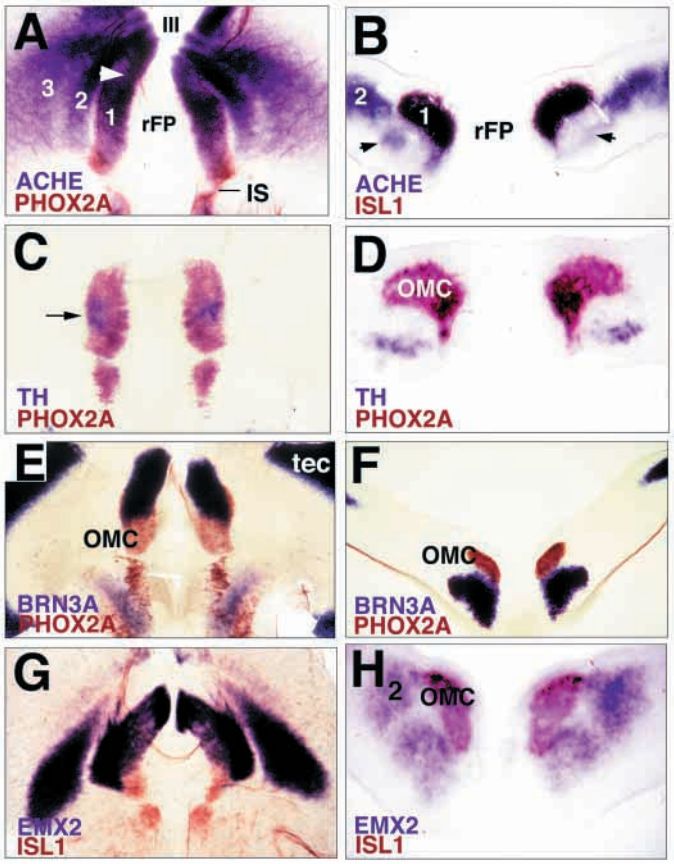

Fig. 1. The medial arc contains three molecularly distinct territories. In each row, pairs of E5 embryos are shown in whole-mount preparations (left column) and in cross-sections (right column). In the left panel, rostral is towards the top and the ventricular surface faces the viewer. In the right panel, the ventricular surface lining the midbrain vesicle is towards the top. Markers are shown in color-coded text at the bottom of each panel. (A,B) *ACHE* gene expression (purple) distinguishes arcs (labeled 1-3, medial to lateral). Two-color demonstration of *ACHE*, *PHOX2A* and *ISL1* gene expression establishes that the *PHOX2A*+ and *ISL1*+ cells are restricted the medial arc (arc 1), but do not fill it completely. Arrowhead in A indicates the rostral limit of *PHOX2A* labeling. Arrows in B indicate *ISL1*-negative territories within the *ACHE*-rich medial arc (see below). (C) *TH* (arrow) and *PHOX2A* gene expression demonstrates that some midbrain DA neurons are also found in the medial arc. (D) The *PHOX2A*+ and the *TH*+ neurons occupy discrete ventricular-pial positions within the medial arc. (E,F) A population of *BRN3A*+ cells is detected in the medial arc rostrally. (E) These cells overlap the *PHOX2A* labeling caudally and conform to the *ACHE*+ medial arc territory that lies rostral to the arrowhead in A. (F) When viewed in cross-section, the *BRN3A*+ cells lie pial to the *PHOX2A*+ territory. (G,H) *EMX2* gene expression also identifies this rostral medial arc territory. Relationship of medial arc *EMX2* labeling to the *ISL1*+ OMC is illustrated. Within the medial arc, *BRN3A* and *EMX2* are expressed exclusively within this rostral territory. *BRN3A* and *EMX2* expression is not, however, restricted to the medial arc in midbrain development. Both genes are expressed in developing tectum (E, and data not shown), *EMX2* identifies arcs 2-4 (G,H) and by E7 *BRN3A* marks out other ventral midbrain structures as well (not illustrated) (Fedtsova and Turner, 2001; Fedtsova and Turner, 1995). 1,2,3, arcs 1, 2 and 3; III, third ventricle; IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; tec, tectum.

viewed from the ventricular surface. Along the ventricular-pial axis, the three cell-groups are fully segregated, with OMC lying closest to the ventricular layer. Thus, at E5 when midbrain neurogenesis is still under way (T. A. Sanders, PhD thesis, University of Chicago, 2000), the mantle layer of the ventral midbrain is patterned in all three dimensions.

Ventral midbrain origin of pRN neurons

Previous studies have suggested that neurons of the RN, like the neurons of hindbrain precerebellar nuclei, are produced by dorsal neural tube (Cooper, 1946; Hamilton et al., 1959; Sidman and Rakic, 1982; Streeter, 1912). Indeed, modern textbooks illustrate red nucleus neurons that originate in dorsal midbrain and translocate to ventral midbrain in migratory streams (Haines, 2002; Sadler, 1995). In light of our connectional findings indicating that RN neurons are found in the medial arc of the ventral midbrain by E5, we further investigated the origin of the RN in an explant culture system. Ventral midbrain tissue was harvested for organotypic culture at stage 12-15, a time when postmitotic neurons detected in dorsal midbrain are massed in the prospective mesencephalic trigeminal nucleus adjoining the roof plate (not illustrated) (Easter et al., 1993). After 3-4 days in vitro, the ventral midbrain cultures displayed strong expression of the pRN marker *BRN3A* (Fig. 3). Moreover, based on *PHOX2A* mRNA expression, the explant pRN formed in a medial arc-like territory whose three-dimensional architecture closely matched that observed in vivo, with a *BRN3A*+ pRN lying anterior and pial to a *PHOX2A*+ OMC (Fig. 3C,D; data not shown). We detected a medial arc pRN in all explants examined, even when no *PAX3*+/*BRN3A*+ dorsal midbrain tissue remained attached to the ventral midbrain explant (Fig. 3A,C, residual tectum attached, n=54; Fig. 3B,D, no residual tectum, n=5). We conclude that *BRN3A*+ pRN neurons originate in ventral midbrain, and that the generation of a medial arc properly organized in three dimensions is an autonomous property of E3 ventral midbrain.

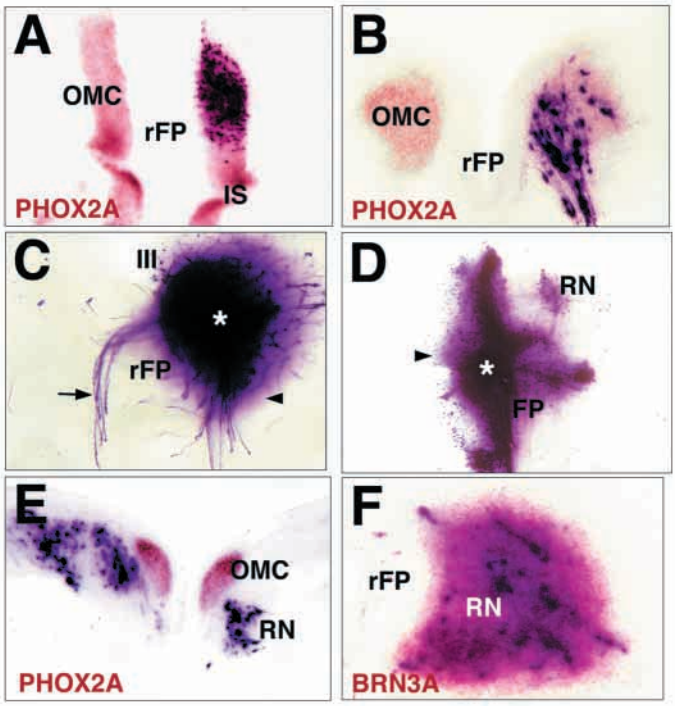

Fig. 2. Axonal projections distinguish red nucleus and oculomotor complex territories within the medial arc. (A,B) Tracer deposits delivered to the third cranial nerve establish that OMC neurons lie within the *PHOX2A*-rich territory of the medial arc. Retrogradely labeled neurons identified by anti-fluorescein alkaline phosphatase histochemistry appear as dark purple cells in E5 midbrain whole-mount (A) and cross-section (B) preparations. (C,D) Whole-mount preparations of E6 explants injected with fluoresceinated dextrans and cultured for 4 hours. (C) Anterograde labeling from a deposit in ventromedial midbrain (asterisk) identifies a crossed projection from the medial arc to the contralateral hindbrain (arrow). (D) Retrograde labeling from a hindbrain tracer deposit (asterisk) identifies red nucleus (RN) neurons in contralateral midbrain. Arrowheads in C,D indicate the midbrain-hindbrain junction. Control deposits placed in more caudal and lateral hindbrain (n=54) failed to label contralateral ventral midbrain, suggesting that at E6 RN axons have not reached the spinal cord. (E,F) Cross-sections of E6 explants where *PHOX2A* (E) and *BRN3A* (F) gene expression were combined with retrograde labeling from tracer deposits in contralateral (left) hindbrain. The retrogradely labeled RN neurons lie pial to the *PHOX2A*+ OMC (E) and are contained within the *BRN3A*+ territory of the medial arc (F). III, third ventricle; FP, floor plate of hindbrain; IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; RN, red nucleus.

Emx2 is essential for the development of the red nucleus in mice

The homeobox gene *EMX2* identifies the RN anlage in the medial arc. We sought genetic evidence for an *EMX2* role in

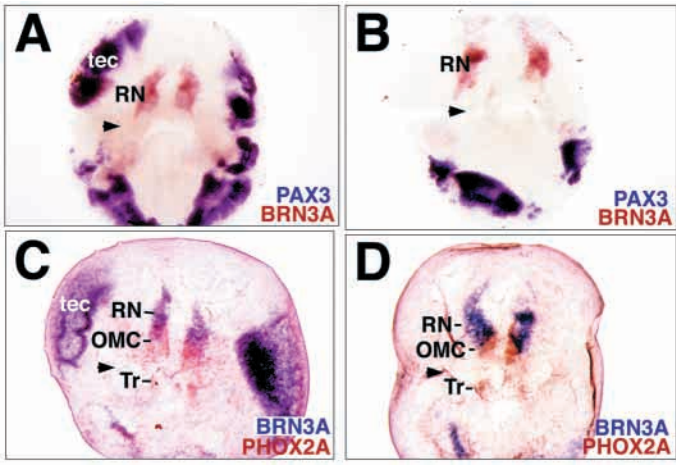

Fig. 3. The pRN originates within the ventral midbrain. Explant cultures of ventral midbrain prepared at E3, a day before a *BRN3A*+ pRN can be identified, and collected 2-3 days later. (A,B) *BRN3A*+ pRN is detected in ventral midbrain explants grown in the presence (A) or absence (B) of adjoining dorsal midbrain tissue (*PAX3*+/*BRN3A*+). (C,D) Expression of *BRN3A* (blue) in the ventral midbrain overlaps the rostral half of the *PHOX2A* (brown) gene expression territory, showing that an organotypic medial arc with appropriate three-dimensional organization is formed in midbrain explants grown with (C) or without (D) tectal tissue. Tectal tissue in cultured midbrain explants is distinguished by two markers: *PAX3*, which is expressed in dorsal midbrain at the time of culturing; and *BRN3A*, which is detected in dorsal midbrain by E5. OMC, oculomotor complex; RN, red nucleus; tec, tectum; Tr, trochlear nucleus.

RN development by analyzing mice with targeted deletions of the *Emx2* gene (Pellegrini et al., 1996). *Emx2* gene expression establishes that, as in the chick, the mouse ventral midbrain is organized into arcuate stripes (Fig. 4A). The most medial *Emx2+* arcuate stripe, when viewed in whole-mount preparations, overlaps with *Isl1* and *Brn3a* gene expression territories, indicating the presence of both an OMC (*Isl1+*) and a pRN (*Emx2+*, *Brn3a+*) within the mouse medial arc (Fig. 4B-D).

In homozygous *Emx2* mutant mice, *Brn3a* gene expression in ventromedial midbrain is retained between E12.5-E16.5, suggesting that pRN neurons are generated in the absence of Emx2 function (Fig. 5A,B). At E18.5, however, no RN can be identified with *Brn3a* or Class III β-tubulin gene expression (Fig. 5C-F), whereas *Isl1+* and *Th+* cells appear unaffected (Fig. 5G,H). Thus, within the medial arc, *Emx2* is specifically expressed by the RN pronucleus and appears to be essential for RN development.

The OMC and pRN are coordinately regulated by SHH

The first arc is flanked medially by the rostral floor plate, a source of the signaling molecule SHH. As illustrated in Fig. 6, the relationship between the *SHH* source and the medial arc is dynamic between E2, the onset of arc pattern formation, and E5, when both the pRN and the OMC can be readily identified by their signature transcription factors. At E2, *SHH* gene expression in the midbrain is confined to the midline (Fig. 6A), but by E5 it has fanned out into the ventricular region overlying the medial arc (Fig. 6B-D). Previous work has shown that SHH can induce motoneurons and dopaminergic neurons in the ventral midbrain (Agarwala et al., 2001; Hynes et al., 1995; Wang et al., 1995; Watanabe and Nakamura, 2000). Given the proximity of the *BRN3A+* cells to the *SHH* source, we tested by in ovo electroporation whether *SHH* can also elicit pRN neurons. We were particularly interested in the spatial relationship between any induced pRN and OMC neurons. As SHH is thought to specify multiple cell-types in a concentration- and position-dependent manner (Agarwala et al., 2001; Ericson et al., 1997), the observation that the pRN and the OMC are equidistant from the ventral midline SHH source at E2 would appear to predict that any induced pRN neurons would be intermixed with OMC neurons. What we found, though, was very different.

SHH misexpression in the ventral midbrain of stage 8 to 15 chick embryos produced by E5 expanded expression of *SHH* (Fig. 7A) and its transcriptional targets *FOXA2*/*HNF3B* and *PTC1* (Fig. 7B) (Agarwala et al., 2001). *SHH* overexpression also expanded the population of *PHOX2A+* oculomotor neurons and the pRN territory identified by *BRN3A* and *EMX2* labeling (Fig. 7C-F). Remarkably, the normal spatial relationship between the OMC and the pRN territories, seen in the control (left) hemitegmentum, was maintained in the enlarged territories along all three dimensions. The expanded OMC and pRN were coextensive along the mediolateral axis (Fig. 7D,F), partially overlapping along the anteroposterior axis (Fig. 7C,E) and completely segregated along the ventricular-pial axis (Fig. 7D,F). These results demonstrate

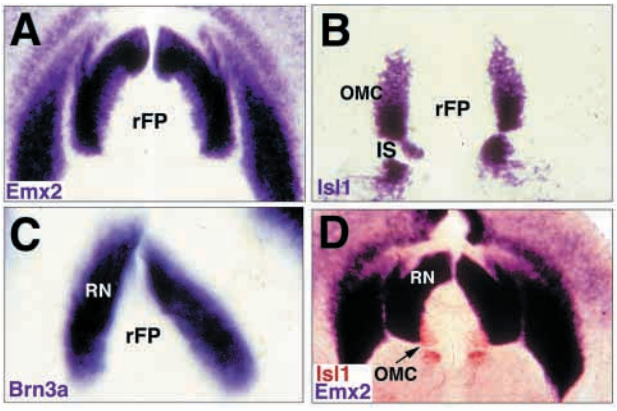

Fig. 4. The mouse ventral midbrain has a medial arc expressing molecular markers of the OMC and the pRN. (A) *Emx2* labeling identifies arcs in mouse E12.5 midbrain wholemounts, oriented as in Fig. 1. (B,C) *Isl1* (B) and *Brn3a* (C) labeling of the OMC and the pRN in the mouse medial arc. (D) Combined *Isl1* and *Emx2* labeling confirms that the organization of the mouse medial arc is similar to that of the chick. IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; RN, red nucleus.

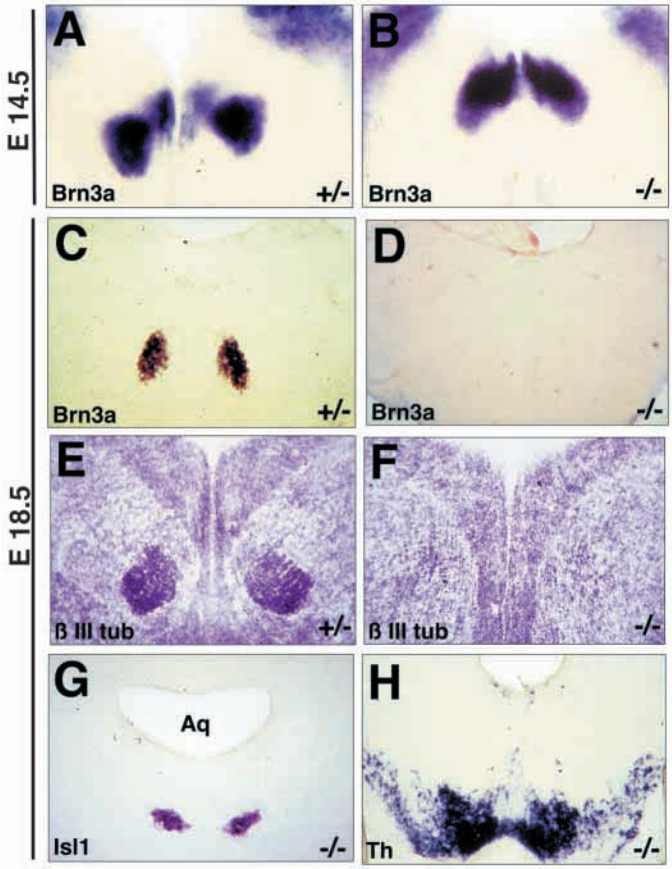

Fig. 5. *Emx2* is required for the formation of the RN in mice. (A,B) *Brn3a+* cells are present in midbrain wholemounts of E14.5 heterozygous (A) and homozygous (B) mutant littermates, demonstrating that pRN neurons are initially specified in *Emx2* mutants. (C-F) *Brn3a* (C,D) and class III β-tubulin (E,F) gene expression fail to identify a RN at E18.5 in homozygous *Emx2* mutant mice. (G,H) Normal *Isl1* (G) and *Th* (H) gene expression in *Emx2* homozygous mice at E18.5 suggests that among medial arc derivatives, the RN is selectively disrupted by mutations in the *Emx2* gene. Aq, cerebral aqueduct.

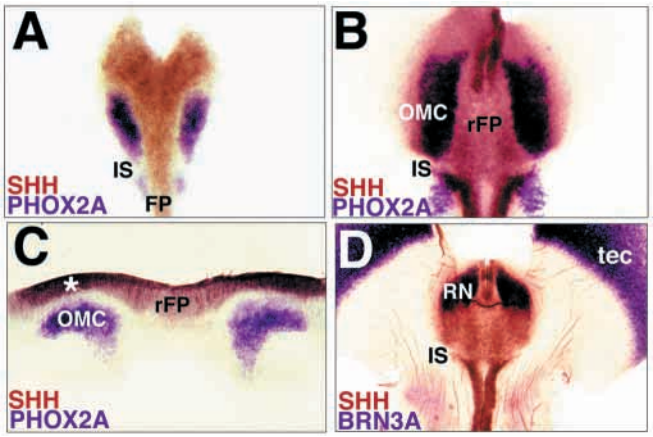

Fig. 6. Relationship of *SHH* gene expression to the developing chick OMC and pRN. (A-C) *PHOX2A* and *SHH* gene expression at E 2.5 (A) and E5 (B,C), shown in whole-mount preparations (A,B) and in transverse section (C). The *SHH* source is confined to the midline at E2 (A), but from E3 expands within the ventricular layer (asterisk in C) to overlie the OMC (B,C). (D) E5 midbrain wholemount demonstrating that ventricular *SHH* expression also overlies the *BRN3A*+ pRN. Midbrain *BRN3A* labeling is first detected at E4 (data not shown). FP, floor plate of hindbrain; IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; RN, red nucleus; tec, tectum.

that *SHH* misexpression does not produce an intermixture of OMC and pRN cell-types, but rather a coordinated expansion of discrete OMC and pRN territories.

SHH misexpression in lateral and dorsal midbrain can elicit ectopic midbrain arcs (Agarwala et al., 2001). We tested whether *SHH* misexpression in lateral tegmentum or in tectum would also produce pRN cell-types. *SHH* electroporation at the tectal-tegmental border (Fig. 7G) or in dorsal midbrain (Fig. 7H), where arcs are normally never seen, reproduced the entire pattern of midbrain arcs, including the *EMX2*+/*BRN3A*+ pRN and the *PHOX2A*+ OMC territories of the medial arc. The ectopic OMC and pRN occupied discrete positions within the ectopic medial arc, such that their spatial relationship always resembled that of the normal OMC and pRN in all three dimensions (Fig. 7H insert). Thus, each of a variety of SHH manipulations in midbrain development coordinately affect the size and shape of the pRN and the OMC, indicating that the patterning element regulated by SHH is the medial arc and not simply the induction of its constituent cell types.

Distance from the isthmus also regulates medial arc nucleogenesis

An ectopic SHH signal in dorsal or ventral midbrain produces OMC and RN anlagen that are offset in the anteroposterior axis. This finding suggests that midbrain cells, in interpreting the SHH signal, rely on independent positional information regarding their anteroposterior position [see also Fedtsova and Turner (Fedtsova and Turner, 2001)]. We have tested the

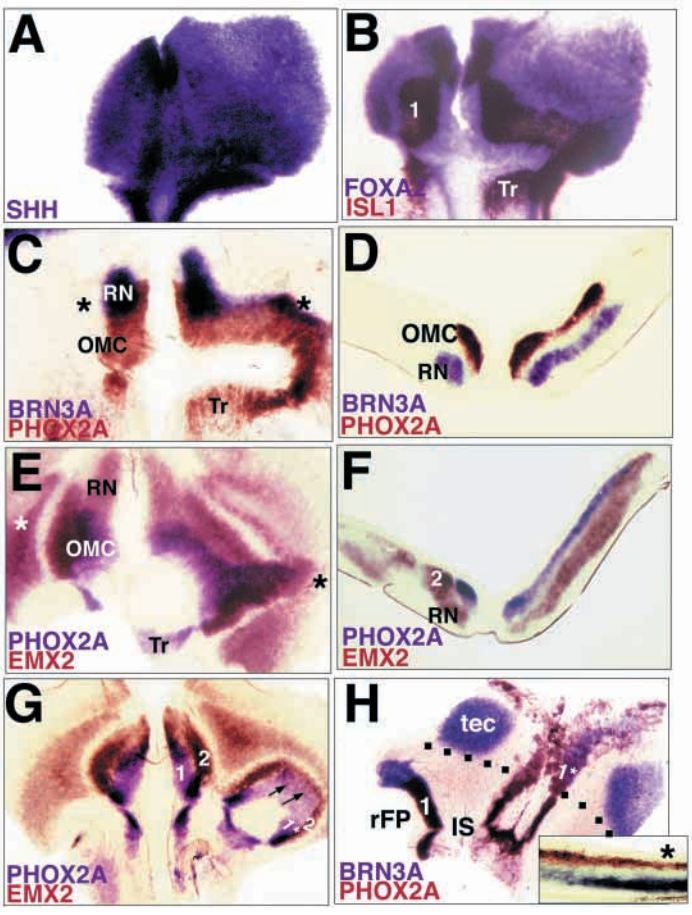

Fig. 7. Coordinate regulation of medial arc pronuclei by SHH manipulations. (A,B) Expanded gene expression for *SHH* (A) and its downstream transcriptional targets *FOXA2* and *ISL1* (B) is seen at E5, after electroporation of pXeX-*SHH* into chick E2 ventral midbrain. In this example, the electroporated side of the brain is the right side; the left side serves as an internal control. In the hindbrain the expanded *ISL1* gene expression identifies the trochlear (Tr) nucleus. (C-F) An enlarged *SHH* source elicits a strictly coordinate expansion of the OMC and the pRN, as demonstrated by two-color gene expression for *PHOX2A* and *BRN3A* (C,D), and *PHOX2A* and *EMX2* (E,F). The E5 wholemounts shown in C and E were cut at the level of the asterisks to prepare the sections illustrated in D and F. The OMC and pRN territories, although expanded, maintain their spatial relationship in all three dimensions: co-extensive along the mediolateral axis (D,F), partially overlapping along the anteroposterior axis (C,E) and completely segregated along the ventricular-pial axis (D,F). (G) E5 wholemount of *EMX2* and *PHOX2A* gene expression shows that *SHH* in lateral tegmentum elicits a properly patterned ectopic medial arc (1*). Arrows indicate *EMX2* labeling of the ectopic pRN, which partially overlaps the *PHOX2A* labeling of the ectopic OMC and lies pial to it. (H) Wholemount of E5 midbrain hemitegmentum (below dotted line) and tectum (above dotted line) demonstrating that *SHH* misexpression in dorsal midbrain also produces an ectopic medial arc (1*) containing an ectopic *PHOX2A*+ OMC and an ectopic *BRN3A*+ pRN. Note that *SHH* misexpression in dorsal midbrain results in the loss of *BRN3A* labeling of the tectum, which is found in the inner part of the mantle layer and is thereby readily distinguished from pRN-like *BRN3A* labeling, which is found more pially. (H, inset) Cross-section through an ectopic medial arc in dorsal midbrain documents the ventricular-pial segregation of the *PHOX2A*+ (brown) and *BRN3A*+ (blue) territories. The insert is oriented with the tectal ventricular surface marked by an asterisk. 1 and 2, arcs 1 and 2; IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; RN, red nucleus; tec, tectum; Tr, trochlear nucleus.

role of the isthmus in supplying this positional information by shifting its location rostrally, into prospective midbrain. To shift the isthmus forward, we drew on previous findings that ectopic expression of FGF8 can transform prospective midbrain into hindbrain tissue (Irving and Mason, 1999; Liu et al., 1999; Sato et al., 2001) and that the juxtaposition of hindbrain and midbrain tissue, even at ectopic sites, induces a new isthmus (Irving and Mason, 1999). This manipulation, which contracts the size of the midbrain tegmentum, allowed us to ask whether it is the position of the isthmus caudally or the distance from the forebrain rostrally that controls the anteroposterior patterning of the medial arc. If it is distance from the isthmus, then rostral midbrain cell-types should be lost as the isthmus is moved rostrally. If it is distance from the forebrain, then moving the isthmus forward should delete caudal midbrain territories. If position relative to both boundaries is important, then rostral advance of the isthmus may delete central elements.

Fgf8 delivery by electroporation to midbrain at E2 (HH stages 8-15) produced by E5 a rostral expansion of the *GBX2*+ hindbrain territory, a rostral regression of the *OTX2*+ midbrain territory and a concomitant rostral shift of the isthmus, identified by *WNT1* and *FGF8* gene expression (Fig. 8B,D; data not shown). In addition, the electroporated midbrain often took on the tubular appearance of rostral hindbrain (Fig. 8D). In the *Fgf8* electroporated hemitegmentum, the hindbrain trochlear nucleus (a rhombomere 1 derivative) was elongated and the midbrain arcs, identified by *PHOX2A*, *PAX6* and *EVX1* labeling, appeared compressed (Fig. 8A,E). Within the medial arc, *PHOX2A* and *BRN3A* gene expression territories were both shifted rostrally (Fig. 8E-H). The extent of this rostral shift was related to the anteroposterior location of the ectopic isthmus. Thus, progressively more rostral *Fgf8* electroporations resulted in progressively more rostrally located isthmi, which in turn produced more rostrally located OMC and RN pronuclei (Fig. 8E-H).

Remarkably, the positional relationships of the shifted *PHOX2A*+ and *BRN3A*+ territories to the ectopic isthmus remained constant: the OMC territory always abutted the isthmus, the pRN territory appeared at a distance, and the rostral OMC and the caudal pRN territories overlapped. However, with very rostral *Fgf8* electroporations that converted the midbrain tegmentum entirely to *GBX2*+/*OTX2*– hindbrain, *BRN3A* gene expression was severely reduced (n=4) or could not be detected (n=2; data not shown), with *PHOX2A* labeling entirely restricted to the subthalamus of caudal forebrain. These results demonstrate that manipulations that shift the medial arc rostrally produce corresponding rostral shifts in the OMC and the pRN that invariably maintain the spatial relationships between these pronuclei. In addition, our results show that the pronuclei of the medial arc are specified according to their distance from the caudally located isthmus.

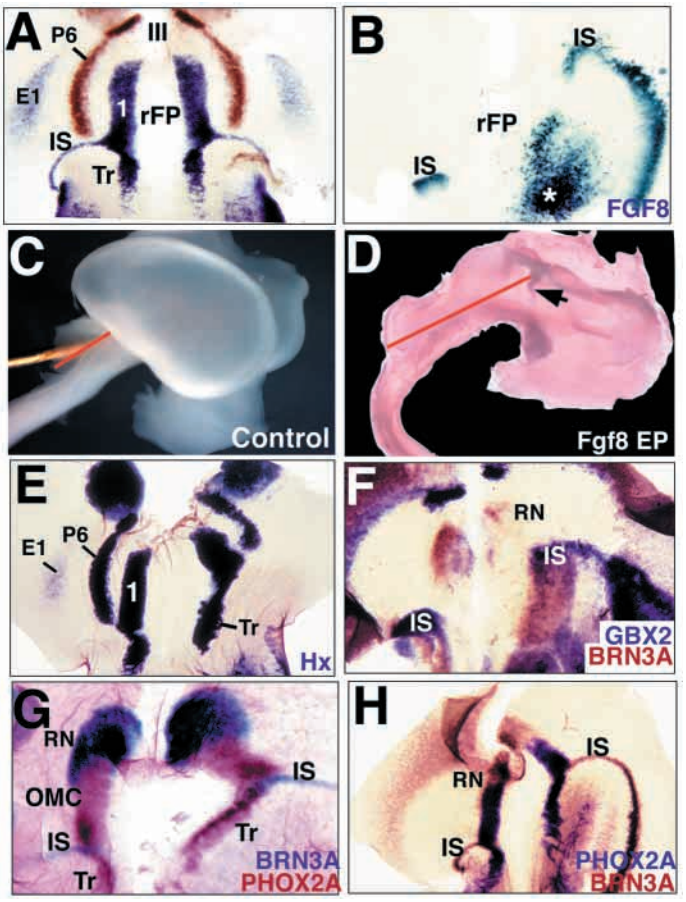

Fig. 8. The OMC and the pRN maintain their anteroposterior relationship, even when the isthmus is shifted rostrally and the midbrain is reduced in size. Rostral movement of the isthmus is induced by mouse *Fgf8* misexpression in chick midbrain, which converts midbrain into hindbrain and thereby creates a more rostral midbrain-hindbrain junction. (A) Relationship of the isthmus (IS), identified by *WNT1* labeling (blue), to midbrain arc patterning, identified with probes for the homeobox genes *PHOX2A* (arc 1, blue), *PAX6* (P6, brown) and *EVX1* (E1, blue). (B) *FGF8* gene expression at E5 after unilateral mouse *Fgf8* misexpression at E2 documents the rostral shift of the isthmus on the electroporated (right) side, with the left side serving as a control. Asterisk indicates expression of the mouse *Fgf8* transgene. Chick *FGF8* gene expression, detected by cross-hybridization of the mouse *Fgf8* riboprobe, identifies the normal (left) and ectopic (right) isthmi (IS). (C,D) Side views of unelectroporated (C, Control) and *Fgf8*-electroporated (D, Fgf8 EP) E5 brainstems show that the rostral shift of the isthmus elicits a transformation of the dorsal midbrain from the globose shape characteristic of the tectum into a tubular shape similar to that of the hindbrain. Dorsal is towards the top, and rostral is towards the lower right. Red lines in C and D mark the extent of rhombomere 1. Arrow in D indicates location of the ectopic isthmus induced by *Fgf8* misexpression. (E) Elongated trochlear nucleus (*PHOX2A*+) and compressed midbrain arcs (Hx indicates homeobox genes *PHOX2A*, *PAX6* and *EVX1*) illustrate the expansion of rhombomere 1 at the expense of ventral midbrain tissue after *Fgf8* misexpression. (F) *BRN3A* gene expression demonstrates rostral shift of the pRN territory after *Fgf8* misexpression. Expanded *GBX2* labeling demonstrates the concomitant rostral shift in the isthmus (IS) and a transformation of midbrain to hindbrain. (G,H) The *PHOX2A*+ and *BRN3A*+ territories of the medial arc maintain their relative anteroposterior positions whether the rostral shift of the isthmus, as identified by *WNT1* gene expression (blue in G, brown in H), is moderate (G) or substantial (H). To demonstrate the rostral shift of BRN3A labeling in these wholemounts, the right brainstem has been kinked (G) or the left forebrain has cropped (H). 1, arc 1; III, third ventricle; IS, isthmus; OMC, oculomotor complex; rFP, rostral, or midbrain, floor plate; RN, red nucleus; Tr, trochlear nucleus.

DISCUSSION

We have found that the medial arc contains the anlagen of two principal nuclei of the midbrain: the OMC and the RN. The OMC was localized to the medial arc by retrograde labeling of third cranial nerve motoneurons and gene expression for motoneuron-specific transcription factors. We identified the RN anlage by the diagnostic criterion of a projection from midbrain tegmentum towards lower brainstem and spinal cord that is exclusively contralateral (Lawrence and Kuypers, 1968; Massion, 1967; ten Donkelaar, 1988). Anterograde and retrograde tracing experiments established the presence of such a crossed projection from a medial arc territory that is spatially and molecularly distinct from the OMC. A crossed rubrospinal projection has been found in all birds and mammals studied (ten Donkelaar, 1988), and is thought to originate from magnocellular RN neurons (Burman et al., 2000; ten Donkelaar, 1988). In this study, we have not distinguished between magnocellular and parvocellular subdivisions of the chick RN because in the adult bird neurons of all sizes appear throughout the RN (Wild et al., 1979).

Origin of the red nucleus

Although many populations of neurons remain within the region of brain where they are generated, some migrate great distances to reach their final locations (Rakic, 2000). Notable examples include dorsal hindbrain neurons that migrate ventrally to populate precerebellar nuclei in the pons and medulla (Harkmark, 1954; Rodriguez and Dymecki, 2000; Wingate and Hatten, 1999). Previous studies have held that neurons of the midbrain RN, like neurons of the hindbrain cerebellar system, are initially generated in dorsal neural tube (Cooper, 1946; Haines, 2002; Hamilton et al., 1959; Sidman and Rakic, 1982; Streeter, 1912). These conclusions were primarily based on histological descriptions of streams of cells that appeared to be migrating from dorsal to ventral midbrain (Sadler, 1995). In this study, we found that SHH, a ventral midline positional signal, can induce a RN anlage and precisely regulate its size. In addition, we found that a *BRN3A*+ pRN could develop in ventral midbrain explant cultures that had no dorsal midbrain attached. These observations lend strong support to the idea that, unlike many other cerebellar system neurons, neurons of the RN are ventral neural tube derivatives.

The homeobox gene *Emx2* is required for the maturation of the red nucleus

Two homeobox genes, *BRN3A* and *EMX2*, identify the pRN territory of the medial arc. Analysis of mutant mice indicates that both genes are needed for RN development. In Emx2-deficient mice, the *Brn3a*-positive neurons of the pRN are generated, but are not detected beyond E16.5. By E18.5, RN neurons are also lost in mice lacking *Brn3a* (McEvilly et al., 1996; Xiang et al., 1996). Thus, both *Emx2* and *Brn3a* are essential for the proper maturation of the red nucleus, although they appear not to be required for its initial specification (this report) (McEvilly et al., 1996). That *Emx2* and *Brn3a* are both expressed in the pRN suggests that the genetic requirement for these transcription factors is autonomous to pRN neurons, but it will be important to test this possibility directly in chimeric mouse experiments (Alvarez-Bolado et al., 2000).

Previous studies of Emx2 function have indicated a role for this gene in the growth and initial areal patterning of the cerebral cortex (Bishop et al., 2000; Cecchi and Boncinelli, 2000; Mallamaci et al., 2000). Our findings suggest a broader role for *Emx2* in brain development, one that includes the regulation of neuronal survival and the development of nuclei. In addition, the finding that the development of the RN requires at least two homeodomain transcription factors, Emx2 and Brn3a, coupled with previous work indicating that multiple homeodomain proteins are needed for the generation of oculomotor neurons and midbrain dopamine neurons (Pattyn et al., 1997; Simon et al., 2001; Smidt et al., 2000), suggests the general hypothesis that the development of distinct nuclei within specific arcs is regulated by multiple homeodomain transcription factors.

Regulation of medial arc nucleogenesis by SHH and FGF8

Our in ovo electroporation experiments establish that *SHH* misexpression eliciting an expansion or induction of the medial arc produces both oculomotor and pRN cell-types. With all SHH manipulations, the spatial relationships of the ectopic OMC and pRN neurons remain constant and resemble those within the unmanipulated medial arc: the OMC and pRN form distinct pronuclei that are co-extensive in the mediolateral axis and partially separated along the anteroposterior axis. We tested whether this anteroposterior segregation of the medial arc would be maintained if the medial arc were moved rostrally within the ventral midbrain. We found that as an ectopic isthmus is induced at progressively more anterior positions by ectopic FGF8 delivery, the medial arc is shifted rostrally as well. In these brains, the OMC and the pRN appear at progressively more anterior sites, until first the pRN, and eventually both the pRN and OMC territories are lost, and the entire electroporated half of the midbrain is converted to hindbrain. These results demonstrate that rostral shifts in the location of the medial arc result in corresponding shifts in the OMC and the pRN. They also show that anteroposterior patterning of medial arc pronuclei is controlled by caudal positional signals emanating from the midbrain-hindbrain junction. These signals may include FGF8 itself, as well as other isthmus-specific FGF and WNT proteins (Carl and Wittbrodt, 1999; Crossley et al., 1996; Wurst and Bally-Cuif, 2001). Interestingly, whatever the molecular character of these anteroposterior patterning cues, our results indicate that they are present throughout the midbrain, as *SHH* misexpression anywhere in midbrain results in the production of an ectopic medial arc within which the ectopic pRN and the OMC retain appropriate anteroposterior polarity.

Patterning of midbrain nuclei along the ventricular-pial axis

An emerging view in studies of neural development is that a two-dimensional Cartesian coordinate system specifies cell fates within the developing neuroepithelium. According to this ‘grid’ model, orthogonal signaling centers provide positional information for the medial-lateral and anteroposterior patterning of the nervous system (Jessell and Lumsden, 1997; Simon et al., 1995; Wolpert, 1969; Ye et al., 1998). However, this model is not sufficient to account for the segregation we have found of the midbrain pronuclei along the ventricular-pial axis. What might be the mechanism for patterning midbrain

nuclei along this third axis? In the rat, although the OMC and the RN are born between E12-E14, the peaks of their neurogenesis are staggered by 0.5-1.0 day (Altman and Bayer, 1981; Marchand and Poirier, 1982). Thus, cells that leave the ventricular zone could be specified successively to be OMC neurons (born E12-E13) then RN neurons (born E13-14). The mechanism underlying such a temporal switch in cell-type generation could be the length of time a progenitor cell spends in the proliferative zone. Alternatively, the switch may be mediated by specific signaling molecules (Desai and McConnell, 2000).

One possibility, prompted by our observations on the changing profile of midbrain *SHH* gene expression, is that SHH itself could participate in ventricular-pial patterning. As Fig. 6 illustrates, although the source of *SHH* in the ventral midbrain is a strip at its onset, by E3 it fans out to assume a wedge-shaped profile (Ericson et al., 1995; Marti et al., 1995). Based on the rat midbrain neurogenesis studies and the onset of *PHOX2A* in the chick OMC by E2 and *BRN3A* in the pRN by E4, *SHH* gene expression overlies the medial arc at a time when the fates of medial arc neurons are still being specified (Altman and Bayer, 1981). That the same signaling systems can be involved sequentially in the control of patterning along different axes has been shown in studies of early *Drosophila* development (Gonzalez-Reyes et al., 1995).

Conclusions

We have examined the relationship between midbrain arc pattern formation and the specification of midbrain nuclei, using the medial arc as a prototypical arc. Our results indicate that a single arc can harbor multiple spatially segregated neuronal territories that by connectional and molecular criteria correspond to specific midbrain nuclei. These findings suggest that one role for the arcuate patterning of the ventral midbrain is the generation of nuclei.

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