Arcuate Plan of Chick Midbrain Development

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In spinal cord and hindbrain development, neurons are generated as longitudinal cell columns aligned with the ventral and dorsal midlines. For rostral brain, however, the fundamental structure of early neuronal patterning remains poorly understood. We report here that, in the chick embryo, the ventral midbrain is remarkably regular in its cellular and molecular organization; it is arranged as a reiterative series of arcuate territories arrayed bilateral to the ventral midline. In the mantle layer of the ventral midbrain, an arcuate series of neuronal cell columns (midbrain arcs) is demonstrated by acetylcholinesterase histochemistry and gene expression for class III β -tubulin, homeodomain transcription factors, and neurotransmitter synthetic enzymes. In the ventricular layer of midbrain progenitor cells, WNT and NOTCH ligand gene expression displays arcuate periodicities that form a tight three-dimensional registration

with the arcs of the underlying mantle layer. Ventral midbrain arcuate patterning is even macroscopically visible, forming ridges along the ventricular surface. These observations establish that a single plan of arcuate organization governs the morphogenesis and cell-type specification of the ventral midbrain. Arcs are not restricted to the midbrain tegmentum but extend through the subthalamic tegmentum of the forebrain. Thus, the chick rostral brain, which is classically divided into midbrain and forebrain, can also be partitioned into the following: (1) a neuraxial region of arcs and (2) an anterodorsal cap that includes midbrain tectum and nonsubthalamic forebrain. We show that this partition of brain tissue is supported by the expression patterns of homologs of *Drosophila* gap genes.

Key words: midbrain arcs; acetylcholinesterase; oculomotor neurons; nucleogenesis; NOTCH; WNT5A; *Tailless*

Despite their complete dissimilarity in tissue cross sections, the spinal cord and hindbrain share a common plan of organization (Johnston, 1906; Herrick, 1915). In spinal cord, motor neurons and secondary sensory neurons are arrayed into interrupted longitudinal cell columns, with motor neuron columns placed ventrally and laterally and sensory relay columns situated dorsally. In hindbrain, this basic plan is elaborated by the addition of motor columns to accommodate the branchial arches and sensory columns for the sensory ganglia of the head. There is good evidence that this columnar design is laid down early in vertebrate neurogenesis. Neuroembryologists beginning with His (1904) have described a longitudinal sulcus limitans in spinal cord and hindbrain that sets off a basal plate, containing motor neurons, from an alar plate, enriched in secondary sensory neurons (Sidman and Rakic, 1982). Within these embryonic plates, frank longitudinal cell columns have long been recognized with Nissl staining and enzyme histochemistry (Herrick, 1915; Hugosson, 1958; Kallen, 1962). More recently, direct demonstration that motor neurons, secondary sensory neurons, and neuronal differentiation control

genes are arrayed into multiple longitudinal columns in early spinal cord and hindbrain development has been provided in molecular studies identifying markers of cell-type identity and developmental regulatory genes (Tsuchida et al., 1994; Chitnis et al., 1995; Myat et al., 1996; Logan et al., 1998).

The fundamental design principles for rostral brain are much less clear. Columns of secondary sensory neurons end in rostral hindbrain, and motor neurons extend only to the oculomotor complex of the midbrain. Moreover, in adult midbrain and forebrain, only a very few nuclei form frank longitudinal cell columns, and the rest appear too variegated in shape to suggest a simple mechanism of histogenesis (Nauta and Feirtag, 1986). A starting point for many studies of rostral brain embryology has been the suggestion of His (1904) that a sulcus limitans defining alar and basal plates can be traced to the preoptic recess in rostral forebrain. This trajectory, which would provide for broad longitudinal zones in rostral brain, has attracted some support recently (Puelles et al., 1987; Shimamura et al., 1995). Many workers, however, have placed the termination of the sulcus limitans more caudally, near the mammillary recess (Schulte and Tilney, 1915; Kingsbury, 1922; Coggeshall, 1964; Kuhlenbeck, 1973), or have challenged whether the sulcus can be followed into forebrain at all (Johnston, 1923; Rose, 1942). In embryonic hindbrain and spinal cord, it is not the sulcus limitans, although that provides the most compelling morphological evidence for a longitudinal organization. It is the organization of neurons into distinct longitudinal columns. For this reason, the starting point of our study was an examination of the mantle layer of postmitotic neurons during midbrain and forebrain embryogenesis.

We began by staining chick brain whole mounts for acetylcholinesterase (AChE) activity. For studies of adult brain anatomy,

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AChE histochemistry rivals Nissl staining as a method for demonstrating brain nuclei in tissue sections (Graybiel and Ragsdale, 1978; Paxinos and Watson, 1986). AChE staining has also been used to identify early patterns of postmitotic neurons in CNS development (Layer, 1983; Moody and Stein, 1988; Ross et al., 1992). Unexpectedly, we found that AChE staining of embryonic brain whole mounts demonstrates an extremely regular pattern in the mantle layer of the ventral midbrain. It is organized into arcuate stripes. We describe here the anatomy of these stripes, which we call midbrain arcs. We demonstrate that the arcs differ in the cell types they contain. For example, the motor neurons of the oculomotor complex are restricted to the most medial arc, and more lateral arcuate territories are enriched in the synthetic enzyme for the neurotransmitter GABA. We show that an arcuate organization is also present in midbrain ventricular layer of progenitor cells and that these arcuate patterns lie in precise radial alignment with those of the mantle layer. We demonstrate that the arcuate organization of the ventral midbrain is even visible in unstained brain tissue. Together, our findings establish that the fundamental plan of the embryonic ventral midbrain is a quite simple pattern: a series of arcuate territories aligned with the longitudinal axis of the brain.

MATERIALS AND METHODS

Tissue preparation. Fertilized chicken eggs were placed for 2–8 d in a humidified forced-draft incubator set at 37.5°C. Embryos collected for histochemistry were submerged in 4% paraformaldehyde in a PBS solution. Cold 2.5% glutaraldehyde in PBS was used as a fixative for the scanning electron microscopy specimens. Embryo staging followed Hamburger and Hamilton (1951).

cDNAs for riboprobe generation. An 849 bp PCR product identifying chick glutamate decarboxylase (*GAD2*) (GenBank accession number AF317501; 84% nucleotide and 94% amino acid identity with the rat 65 kDa glutamate decarboxylase gene) was amplified from embryonic day 5 (E5) chick brain cDNA with forward primer 5'-RAC DGC MAA YAC BAA YAT GTT YAC-3' and reverse primer 5'-GCT GGG TTT GAG ATG ACC ATC C-3'. The *GAD2* fragment was subcloned into pCRII (Invitrogen, San Diego, CA), and riboprobes were generated with SP6 polymerase incubation of *NotI*-digested phagemid. *ACHE* riboprobes were derived from a 472 bp PCR fragment amplified from chicken genomic DNA and corresponding to bases 298–769 of GenBank accession number U03472 [T7 polymerase runoffs of an *EagI*-digested Bluescript SK(+) subclone]. *Class III β -tubulin* gene expression was detected using a 434 bp *Bst*Z1-*Sma*I subclone of the chicken β_4 -tubulin genomic clone p β G4 (Lopata et al., 1983). This fragment contains an isotype-specific sequence representing the 230 bp 3' end of the transcript, and riboprobes were generated by T7 transcription of an *EagI*-digested Bluescript SK(+) subclone. Riboprobes were also synthesized from vectors harboring chick cDNAs for *CASH1/ASCL1* (1.9 kb) (Jasoni et al., 1994), *choline acetyltransferase (CHAT)* (2.5 kb) (Yamada et al., 1993), *DEL-TAI/DLL1* (700 bp) (Myat et al., 1996), *EVX1* (1 kb) (M. Dush and G. Martin, unpublished cDNA), *NOTCH1* (1 kb) (Myat et al., 1996), *OTX2* (1.5 kb) (Bally-Cuif et al., 1995), *PAX3* (660 bp) and *PAX6* (540 bp) (Goulding et al., 1993), *PHOX2A/ARIX* (430 bp) (Ernsberger et al., 1995), *SERRATE1* (900 bp) (Myat et al., 1996), *SERRATE2* (1.6 kb) (Hayashi et al., 1996), *TAILLESS/NR2E1* (1.3 kb) (Yu et al., 1994), *WNT5A* (1.2 kb subclone), and *WNT7A* (1.2 kb subclone) (Dealy et al., 1993).

Histochemistry. AChE staining was done on whole-mount preparations with a two-step modification of the copper thiocholine method (Graybiel and Ragsdale, 1978). High-stringency whole mount *in situ* hybridization was performed with riboprobes labeled with the haptens digoxigenin and fluorescein. Labeled RNA duplexes were detected with antibody-phosphatase conjugates (Roche Molecular Biochemicals, Indianapolis, IN), and phosphatase activity was demonstrated with the distinguishable tetrazolium substrates nitro blue tetrazolium and tetranitro blue tetrazolium (Grove et al., 1998).

Figure 1. Arcs of the embryonic midbrain tegmentum are defined by AChE histochemistry. Whole-mount AChE staining of stage 28 (E6) chick embryo head reveals stripes (arcs) of dense enzyme activity in ventral midbrain. The back of the head, including the midbrain tectum and rostral roof of the hindbrain, was cutoff before staining. Caudal is to the bottom of the figure; rostral structures, including the forebrain, are in the z-axis away from the viewer. Arrow marks one of the arcs. *hb*, Hindbrain.

RESULTS

The midbrain arcs are defined by acetylcholinesterase histochemistry

To study pattern formation in early midbrain development, we stained chick embryo whole mounts for AChE activity (Fig. 1). Beginning on E3, AChE staining detects stripes in the midbrain tegmentum (ventral midbrain). By E5 (stage 26), four well defined AChE-rich stripes, 80–160 μ m across, are readily identified bilateral to the ventral midline (Fig. 2A). By E6, a fifth, lateralmost AChE-dense stripe is also evident (data not illustrated). At E5, the AChE-poor tissue between the stripes is narrow, except between the third and fourth most lateral AChE-rich stripes, which are well separated caudally. By E7, the other lateral AChE-poor interstripes have broadened as well. The AChE-rich and -poor stripes mark out columns of cells in the mantle layer of the neural tube, in which postmitotic neurons accumulate (Fig. 3A). Because of the arcuate shape of these columns, which is accentuated by the curve of the cephalic flexure, we give the AChE-rich stripes the name midbrain arcs, and we refer to the AChE-poor stripes as interarcs. The arcs are a transient feature of midbrain development, conspicuous in whole-mount preparations until stage 30 (E7), but are then obscured as tegmentum growth continues.

In studies of embryonic brain anatomy, AChE activity has been proposed to serve as a general marker of postmitotic neurons

Figure 2. Arcs of the midbrain tegmentum form functionally and molecularly distinguished territories. *A*, Whole-mount *in situ* hybridization of *ACHE* gene expression in midbrain tegmentum identifies arcs 1–4, numbered medial to lateral from the ventral midline. The orientation is as in Figure 1, and the view is from the ventricular surface. The ventral midbrain and adjoining hindbrain have been dissected away from the forebrain and the other head tissues of stage 26 (E5) chick embryos and flattened on a glass slide with a coverslip. The midbrain–hindbrain junction [isthmus (*is*)] and the ventral midline, including the floor plate (*fp*) of hindbrain, are unstained. *B*, Arcs are also demonstrated by gene expression for class III β -tubulin (*BTUB*), an established marker of postmitotic neurons. *C*, Two-color *in situ* hybridization experiment establishes that *CHAT*-positive motor neurons (blue, marked by open arrowhead) are confined to the first arc. Arcs demonstrated by *ACHE* gene expression appear red. Filled arrowhead notes the rostral limit of midbrain tegmentum. Asterisk indicates *CHAT*-rich trochlear motor neurons in the hindbrain (*hb*). *D*, *GAD2* gene expression marks out arc 2 (asterisk) and the more lateral arcs and interarcs but is not detected in the medial arc. *E*, Two-color, four-probe detection of regularly spaced arcuate territories of homeobox (*HX*) gene expression in ventral midbrain. 2A, *PHOX2A*; P6, *PAX6*; E1, *EVX1*; P3, *PAX3*. *F*, Two-color *in situ* hybridization demonstration that the *PAX6*-rich stripe (*P6*) lies in the *ACHE*-poor interarc between arcs 2 and 3. Scale bar: *A*, *C*, *F*, 200 μ m; *B*, *D*, 225 μ m; *E*, 250 μ m.

(Layer, 1983; Moody and Stein, 1988; Ross et al., 1992). Our findings with a second, specific neuronal marker, class III β -tubulin (Moody et al., 1989; Lee et al., 1990; Easter et al., 1993; Menezes and Luskin, 1994), support this view. The pattern of gene expression for *BTUB* in chick embryonic ventral midbrain is strikingly similar to that for *ACHE* and clearly demonstrates arcs (Fig. 2*A,B*). These observations establish that the midbrain arcs are neuronal structures and that the interarcs, as well as the arcs, contain postmitotic neurons.

The midbrain arcs have distinct neurotransmitter and transcription factor identities

To assess the relationship of the AChE-rich arcs to the motor neurons of the midbrain, we performed correlative anatomy experiments marking the midbrain oculomotor neurons by gene expression for *CHAT* (Fig. 2*C*) and by retrograde labeling of third cranial nerve axons (data not shown). These experiments established that the neurons of the oculomotor nucleus have a precise relationship to the midbrain arcs; they are restricted to the first arc. In contrast, cells expressing the GABA synthetic enzyme *GAD* are not found in the first arc but are enriched in the lateral arcs (Fig. 2*D*, *GAD2/GAD65*; *GAD1/GAD67*, data not illustrated).

Transcription factor gene expression also demonstrates that the midbrain arcs differ in their molecular identities. Our most striking findings are for a panel of homeobox genes that together identify precise, regularly spaced arcuate domains within the mantle layer of midbrain tegmentum (Agarwala et al., 2001) (Fig. 2*E,F*). The homeobox gene *PHOX2A*, which is thought to be expressed by oculomotor neurons (Pattyn et al., 1997), marks the first arc; the homeobox gene *PAX6* serves to label the interarc between arcs 2 and 3; the homeobox gene *EVX1* distinguishes the 3/4 interarc and arc 4; and the homeobox gene *PAX3*, which is expressed throughout the ventricular layer of midbrain tectum (Goulding et al., 1991), identifies the lateral edge of the midbrain tegmentum. The sequential onsets of tegmental expression for *PHOX2A* (by stage 12), *PAX6* (stage 19), and *EVX1* (stage 23) suggest that the unique molecular identities of the midbrain arcs may generally develop in a medial-to-lateral progression.

Together, these findings with neurotransmitter-specific and homeobox gene expression suggest that the midbrain arcs identify a

patterning system by which functionally and molecularly distinct assemblies of midbrain neurons are formed in early development.

Periodicities in the midbrain ventricular layer

The acetylcholinesterase-rich arcs are restricted to the mantle layer of postmitotic neurons (Fig. 3*A*), but arcuate patterns in midbrain gene expression are not. In the ventricular layer of tegmental progenitor cells, there are arcuate heterogeneities in gene expression for components of the NOTCH and WNT signaling pathways (Figs. 3*B–F*, 4). The NOTCH ligand *DELTA1* is distributed in a series of arcuate bands arrayed bilaterally to the ventral midline (Fig. 3*B*). Similar arcuate territories are identified in whole-mount preparations for the related proneural gene *CASH1/ASCL1* (Fig. 3*E,F*). In the embryonic hindbrain, *DELTA1* gene expression demonstrates longitudinal bands that interdigitate with stripes of messages for a second NOTCH ligand, *SERRATE1* (Myat et al., 1996). We found a similar complement in ventral midbrain. *SERRATE1* and *SERRATE2* mRNAs are expressed in arcuate bands (Fig. 3*B,C*), and these fall within the periodic gaps in *DELTA1* expression (correlation not documented).

Of the three *WNT* genes reported to be expressed in developing mouse ventral midbrain (Parr et al., 1993), one of them, *WNT1*, is restricted to the midbrain–hindbrain junction during chick tegmentum embryogenesis (Fig. 4*A*) (Bally-Cuif and Wassef, 1994). The other two, however, are expressed in developing ventral midbrain, in which they form clear arcuate periodicities (Fig. 4*B,C*). *WNT5A* is expressed in a strikingly regular pattern of three uniformly spaced stripes bilateral to the ventral midline (Fig. 4*B*). The distribution of *WNT7A* message is more complex but is also arranged into arcuate periodicities (Fig. 4*C*).

We tested in two-color, whole-mount *in situ* hybridization experiments how the periodicities in progenitor layer signaling molecules relate to the pattern of arcs and interarcs in midbrain mantle layer. These correlative experiments established a strict three-dimensional registration between gene expression patterns in the two embryonic layers (Figs. 3*E,F*, 4*D–F*). The precision of this registration is best illustrated by the interdigitation of the ventricular layer *WNT5A* bands with the mantle layer homeobox genes *PHOX2A*, *PAX6*, and *EVX1*, when viewed in whole-mount midbrain preparations. The medial gap in *WNT5A* expression

Figure 3. Gene expression for NOTCH signaling components identifies stripes in midbrain ventricular layer. *A*, Cross section through ventral midbrain illustrating the mantle (*ml*) and ventricular (*vl*) layers, which are readily distinguished by *ACHE* (*ml*) and *NOTCH1* (*vl*) gene expression. Note that *ACHE*-rich arcs are less conspicuous in tissue sections than in whole-mount preparations. Arrowhead indicates ventral midline. *B*, *C*, Whole-mount gene expression for the NOTCH ligands *SERRATE1* (*B*) and *SERRATE2* (*C*) demonstrates ventricular layer stripes. *D*, A series of ventricular layer arcuate bands bilateral to the ventral midline is seen with *in situ* hybridization for the NOTCH ligand *DELTA1*. Asterisks mark *DELTA1* mRNA-poor zones. Comparison of the fiducial asterisks in *B–D* illustrates that the *SERRATE* and *DELTA1* patterns interdigitate, with the lateral gap in *DELTA1* expression being particularly dense in *SERRATE1* message and the two more medial *DELTA1*-poor zones being enriched in *SERRATE2* mRNA. *E*, *F*, Two-color whole mounts document that the arcuate bands in the mantle and ventricular layers lie in spatial register. In these experiments, the whole mounts serve to “project” the expression pattern of one embryonic layer onto another. Despite the distortions created by flattening, which include some shear between the ventricular and mantle layers, the interdigitation between the mantle layer homeobox genes *PHOX2A* (*E*, brown) and *PAX6* (*F*, brown) and the ventricular layer proneural gene *CASH1* (blue) is clear. At this age, *CASH1* gene expression closely follows that of *DELTA1* (see *D*). Orientation of E5 whole mounts (*B–F*) as in Figure 2. Scale bar: *A*, *C*, *D*, 300 μ m; *B*, 400 μ m; *E*, 350 μ m; *F*, 325 μ m.

overlies the *PHOX2A*-positive first arc (Fig. 4*D*), the lateral *WNT5A* stripes straddle the *PAX6*-rich interarc (Fig. 4*E*), and the lateral edge of *WNT5A* expression is hugged by the *EVX1* band (Fig. 4*F*). The schematic diagram of Figure 5 summarizes this spatial architecture for the ventral midbrain, describing the relationship of the *ACHE*-rich arcs and the homeobox gene territories of the mantle layer to the *WNT5A* and *DELTA1* periodicities of the ventricular layer.

Morphological demonstration of the midbrain arcs

We found that histochemistry is not required to demonstrate the arcuate patterning of the ventral midbrain. Arcuate periodicities are visible in freshly dissected, unstained midbrain whole mounts (Fig. 6*A*). At least part of these refractive variations may be caused by a series of delicate ridges that furrow the ventricular surface of the ventral midbrain (illustrated in the scanning electron photomicrograph of Fig. 6*B*). These ridges, which are visible

Figure 4. Periodic patterns of *WNT* gene expression demonstrated in midbrain ventricular layer. *A*, *WNT1* message in the E5 chick is detected at the isthmus but not in the ventral midbrain. *B*, *C*, *WNT5A* (*B*) and *WNT7A* (*C*) mRNAs are expressed in the ventral midbrain ventricular layer, in which they form periodic arcuate bands. *D–F*, The *WNT5A* periodicities interdigitate with the homeobox genes expressed in the mantle layer when viewed in whole-mount preparations. Two-color *in situ* hybridization experiments detect *WNT5A* expression in blue and *PHOX2A* (*D*), *PAX6* (*E*), and *EVX1* (*F*) expression in brown. Midbrain whole mounts at stage 26 (*A*, *B*, *D–F*) and stage 27 (*C*), oriented as in Figure 2. *hb*, Hindbrain; *is*, isthmus. Scale bar: *A*, 175 μ m; *B*, *D*, *E*, 200 μ m; *C*, 275 μ m; *F*, 230 μ m.

beginning at approximately stage 22 and persist through stage 30, overlie the AChE-rich arcs of the mantle layer (correlation not illustrated).

The relationship of the arcs to domains of gap gene expression

By AChE staining, the midbrain arcs extend in reduced numbers into the rostrally adjoining subthalamic region of the forebrain (Fig. 7*A*). There, at the level of the intrathalamic zona limitans (*zli*), the medial subthalamic arc abruptly terminates, and the lateral arcs appear to be deflected dorsally along the posterior border of the *zli*. A similar trajectory is observed in the patterns of ventricular layer developmental control genes (Fig. 7, *B*, *CASH1*, *C*, *OTX2*). These observations suggest a division of rostral brain into a posteroventral domain containing arcs aligned with the ventral midline (embryonic midbrain and subthalamic tegmentum) and an anterodorsal domain comprising midbrain tectum and nonsubthalamic forebrain. This division of rostral brain falls orthogonal to the transverse boundary between midbrain and forebrain, which is taken to be primary in current models of forebrain development (Shimamura et al., 1995). If the arc-based division of rostral brain is also fundamental, then it may be reflected in the expression patterns of developmental control genes involved in brain regionalization.

Molecular genetic studies of *Drosophila* development have identified a small set of terminal and head gap genes that control the development of broad overlapping domains of the fly brain (Cohen and Jurgens, 1990; Finkelstein and Perrimon, 1991; Hirth

Figure 5. Schematic summary of the three-dimensional spatial registrations in ventral midbrain among the developmental signaling molecules (*WNT5A*, *DELTA1*) of the ventricular layer (*top tier*) and the arcs (*ACHE*) and the homeobox genes (*HX*) of the mantle layer (*bottom tier*). Arcs are numbered 1–5 on both sides of the ventral midline. *HX* genes, Compendium of expression patterns for homeobox genes *PHOX2A*, *PAX6*, *EVX1*, and *PAX3*, ordered sequentially from the midline as in Figure 2*E* and distinguished by hue. Expression throughout the midbrain tectum of *DELTA1* and the *HX* gene *PAX3* is indicated by arrows, the base of which identifies the lateral edge of the tegmentum. This summary is based on pairwise comparisons of gene expression in two-color *in situ* hybridization whole-mount preparations.

Figure 6. Arcs of the embryonic midbrain tegmentum have gross morphological correlates. *A*, Dissected, fixed but unstained whole mount of E5 ventral midbrain shows arcuate periodicities under transmitted light illumination. *B*, Scanning electron micrograph of a stage 26/27 chick midbrain demonstrates a series of arcuate ridges, one of which is marked by an arrow, along the ventricular surface of the ventral midbrain. The dorsal half of the isthmus (*is*) is intact, but the view is otherwise as in Figure 1. *fb*, Forebrain. Scale bar, 250 μ m.

et al., 1995; Schmidt-Ott et al., 1995). Among these genes, *tailless* (*tll*) is required for the development of the protocerebrum, the most anterodorsal brain region, whereas *orthodenticle* regulates an overlapping domain that includes part of the protocerebrum and extends into the adjoining brain region, the deutocerebrum (Younossi-Hartenstein et al., 1997). We reexamined the expression patterns of *TAILLESS/NR2E1*, the only reported vertebrate homolog of *tll* (Yu et al., 1994; Monaghan et al., 1995), and the *orthodenticle* homolog *OTX2* (Simeone et al., 1992), in light of our finding of arcs in embryonic midbrain and forebrain. As is true of their homologs in flies, *TAILLESS* is expressed throughout the most anterodorsal part of embryonic brain, and the *OTX2* expres-

sion domain overlaps that of *TAILLESS* (Fig. 7*C,D*). The posterior border of *OTX2* expression coincides with the midbrain–hindbrain junction (Fig. 5*C*) (Millet et al., 1996). The *TAILLESS* domain does not, however, correspond to any classic division of brain, such as forebrain. Instead, *TAILLESS* gene expression identifies a specific domain in the midbrain and forebrain: the dorsal midbrain and forebrain dorsal and anterior to the subthalamic region. Thus, arcs and *TAILLESS* gene expression together divide midbrain and forebrain into two nonoverlapping domains: the *TAILLESS*-negative arcs of midbrain and subthalamic tegmentum and a *TAILLESS*-positive cap comprising midbrain tectum and nonsubthalamic forebrain.

DISCUSSION

Three lines of evidence establish that the fundamental embryonic plan of the ventral midbrain is a series of arcuate territories arrayed bilateral to the ventral midline. First, this organization is macroscopically visible in dissected brain as refractive differences in whole-mount histology and as longitudinal ridges along the ventricular surface. Second, the neurons in the ventral midbrain, as demonstrated by histochemistry for the neuronal markers AChE and class III β -tubulin, are not homogeneously distributed across the mantle layer but are arrayed into arcs. Third, in the midbrain ventricular layer, arcuate periodicities in midbrain progenitor cells are detected by gene expression for developmental regulatory molecules, including ligands for NOTCH and WNT signaling pathways and the proneural gene *CASH1*. The finding of spatial registration among the arcuate patterns we observed shows that a common arcuate plan underlies the cellular and molecular organization of the embryonic midbrain tegmentum. More particularly, the three-dimensional registration of the ventricular layer periodicities and the mantle layer arcs strongly suggests a developmental plan in which the arcuate periodicities of the midbrain tegmentum are initiated among the progenitor cells of the ventricular layer, and the initial migration of postmitotic neurons from the ventricular layer into the mantle layer is radial. Radial migration away from the ventricular layer has been described in many regions of the brain, including the dorsal midbrain (Rakic, 1971, 1988; Gray and Sanes, 1991). In ventral midbrain, strict radial migration would account for the registration we found and provides for a straightforward mechanism preserving an arcuate distribution of cells as they travel from the ventricular layer into the mantle layer.

Figure 7. The embryonic chick brain rostral to the isthmus comprises two domains: one of the arcs aligned with the ventral midline (A–C) and the other enriched in the gap gene homolog *TAILLESS* (D). Illustrated are left half brains of stage 26 embryos, processed for whole-mount *in situ* hybridization and mounted for a sagittal view from the ventricular surface. The cephalic flexure places the hindbrain in the *bottom left quadrant* of each *panel*, the ventral midbrain near the *center*, and the forebrain to the *right*. A, *ACHE*-rich arcs of the midbrain tegmentum extend into the subthalamic region of forebrain. The midbrain–forebrain junction that the arcs traverse is marked by a *dotted line*. The position of the rostral limit of the arcs in subthalamus is noted by a *filled arrowhead* (A–D), and the *filled arrowhead* is pointed toward the zli, which is indicated by an *open arrowhead* in A and B. B, Arcuate stripes in midbrain ventricular layer, here demonstrated by expression of the proneural gene *CASH1*, also continue into the embryonic subthalamus. In rostral subthalamus, the lateral *CASH1* bands appear not to stop but to deflect from their arcuate course to align with the zli. This trajectory may reflect that of Sonic Hedgehog, which in rostral subthalamus withdraws from the ventral midline and tracks the zli dorsally (Ericson et al., 1995; Marti et al., 1995). C, D, Gap gene homologs *OTX2* and *TAILLESS* identify overlapping expression domains in the ventricular layer of rostral embryonic brain. The arc domain of midbrain and subthalamic tegmentum (A) is enriched in *OTX2* expression (C) but is almost free of *TAILLESS* expression (D). The *OTX2* hybridization also demonstrates arcuate heterogeneities in ventral midbrain ventricular layer and an isolated clump of *OTX2*-positive cells at the caudal end of rhombomere 1 in hindbrain. 1–4, Arcs 1–4; hb, hindbrain; is, isthmus; tc, midbrain tectum; tg, tegmentum. Scale bar: A–C, 500 μ m; D, 440 μ m.

Midbrain nucleogenesis

For hindbrain and spinal cord development, it is accepted that particular neuronal cell types, such as motor neurons, are specified initially in longitudinal columns and subsequently migrate to

take up their correct position in mature CNS (Harkmark, 1954; Tan and Le Douarin, 1991; Hemond and Glover, 1993; Clarke et al., 1998). For motor neurons and secondary sensory neurons in hindbrain, these migratory cell populations, as they form distinct nuclei, retain their organization into longitudinal cell columns. In contrast, the nuclei of the mature midbrain are not described as longitudinal columns, the exception being the oculomotor complex, which we found to arise in the first arc. Our data, however, show that the midbrain arcs differ in their molecular identities, suggesting the general hypothesis that the arcs and interarcs act as staging territories for assigning neurons to nuclear fates. What then becomes of this arcuate plan in mature midbrain? One possibility is that the arcuate territories migrate, as do columns in hindbrain, but lose their longitudinal organization and acquire different shapes. A second possibility is that there is a covert organization of at least parts of midbrain tegmentum into longitudinal zones. Recent work on the midbrain periaqueductal gray, which appears fairly homogeneous in Nissl-stained material, suggests that this structure may in fact be functionally organized into longitudinal columns (Bandler and Shipley, 1994). Fate-mapping studies of the arcs, combined with a reexamination of adult midbrain systems neuroanatomy, will be needed to address the specific relationships between arcs and their descendents.

Arcs extend into the subthalamus

On the basis of fiber-connection data and histology, neuroanatomists since Forel (1877) have viewed the subthalamus of the adult vertebrate forebrain as a rostral extension of midbrain tegmentum (Kuhlenbeck, 1939; Herrick, 1948; Nauta and Haymaker, 1969). Our finding that midbrain arcs continue rostrally into the subthalamic region indicates that there may be an embryological basis for this similarity. Indeed, one view might be that the subthalamic tegmentum is simply displaced midbrain tegmentum. Both classical and recent models of vertebrate brain embryology, however, place the territory of the subthalamic arcs within diencephalon (Herrick, 1948; Figdor and Stern, 1993; Rubenstein et al., 1994). Moreover, midbrain and diencephalic arc patterns are clearly distinct. At the transition from midbrain to forebrain, the number of arcs is abruptly reduced, and of the midbrain homeobox genes *PHOX2A*, *PAX6*, and *EVX1*, only *PAX6* is expressed in the subthalamus (data not illustrated). These observations indicate that the midbrain and subthalamic arcs share the property of being columns of neurons aligned with the ventral midline but differ in their cell-type composition.

Arcs and the epichordal nervous system

The arcuate plan of the midbrain and subthalamic tegmentum shares many features with the longitudinal organization of the embryonic hindbrain and spinal cord. Most prominently, both tissues contain within their mantle layers columns of neurons aligned with the ventral midline (this study; Kallen, 1962; Chitnis et al., 1995) and within their ventricular layers interdigitating stripes of gene expression for the NOTCH ligands *DELTA* and *SERRATE* (this study; Lindsell et al., 1996; Myat et al., 1996; Matisse and Joyner, 1997). Moreover, just as ventricular ridges identify the arcs of the midbrain, so too do ventricular ridges reflect the presence of longitudinal cell columns in the vertebrate hindbrain, being evident in the embryos of many species and particularly prominent in adult fish (Herrick, 1915). At the molecular level, there are many details of gene expression that differ between the midbrain arcs and the columns of caudal CNS. For example, *PAX6* gene expression in ventral midbrain identifies a

Figure 8. Diagram of a chick embryo illustrating the proposed division of vertebrate embryonic CNS into the following: (1) a neuraxial region containing multiple neuronal columns, including arcs, aligned with the ventral midline and (2) an anterodorsal cap distinguished by its expression of the gap gene homolog *TAILLESS*. The columns illustrated identify midbrain and subthalamic arcs (red), secondary sensory neurons (light blue), and motor neurons (dark blue). The *TAILLESS* cap (beige) consists of dorsal midbrain and nonsubthalamic forebrain. Chick embryo sketch adapted from Lumsden (1990). b1–b4, Branchial arches 1–4; d, diencephalon; m, midbrain; r1–r8, rhombomeres 1–8; sc, spinal cord; t, telencephalon.

restricted territory in the mantle layer clearly lateral to the motor neurons of arc 1, but in hindbrain and spinal cord, *PAX6* is expressed in a broad domain of the ventricular layer, including the progenitor territory for some motor neurons (Jessell, 2000). Nonetheless, at a synoptic level, our finding of an arcuate plan to midbrain and subthalamus suggests a fundamental division of embryonic CNS into the following: (1) a neuraxis from spinal cord to subthalamus containing frank cell columns, including arcs, aligned with the ventral midline and (2) an anterodorsal cap distinguished by gene expression for *TAILLESS* (Fig. 8).

It seems very likely that such an anterior–posterior division in CNS reflects anterior–posterior differences in the signaling properties of the ventral midline and underlying axial mesoderm. The classical floor plate of Kingsbury (1922) is restricted to spinal cord and hindbrain, but more recent authors identify a rostral floor plate in ventral midbrain and caudal diencephalon on histological and gene expression grounds (Kuhlenbeck, 1973; Dale et al., 1997; Pera and Kessel, 1997). The relationship of the rostral floor plate to underlying axial mesoderm is not clear because of

the tremendous tissue movements that take place in early embryogenesis (Vaage, 1969; Dale et al., 1999). However, detailed molecular embryological studies, including work in explant culture, indicate that the critical interactions that produce midbrain floor plate involve the underlying notochord, whereas the prechordal mesendoderm mediates the induction of the rostral diencephalic ventral midline, which is molecularly distinct from floor plate (Dale et al., 1997, 1999; Foley et al., 1997; Pera and Kessel, 1997). Together, these observations suggest that one embryological basis for the difference between the neuraxis of columns and arcs and the *TAILLESS*-rich anterodorsal cap is that the neuraxis is epichordal and has a floor plate, whereas the ventral midline of the cap overlies prechordal mesendoderm (Puelles et al., 1987; Shimamura et al., 1995; Foley et al., 1997; Dale et al., 1999).

One conspicuous feature of the floor plate in spinal cord and hindbrain and its rostral extension into midbrain and subthalamus is that it expresses the signaling molecule Sonic Hedgehog (SHH). Does ventral midline SHH, known to regulate cell-type specification in ventral spinal cord (Jessell, 2000), also control ventral midbrain patterning? We found recently that ectopic SHH in midbrain can regulate the expression pattern of three homeobox genes, *PHOX2A*, *PAX6*, and *EVX1* (Agarwala et al., 2001), which serve to mark the midbrain arcs (this study). Whether SHH can function more globally to elicit the complete program of ventral midbrain development, including the generation of ventricular layer periodicities and the formation of mature midbrain nuclei, remains to be established.

***TAILLESS* gene expression in rostral brain**

Immense molecular insight into hindbrain embryology has issued from the modern rediscovery of rhombomeres, the cellular evidence that rhombomeres form neuroectoderm segments, and the genetic evidence that specific *Drosophila HOM-C* homologs control features of rhombomere identity (Lumsden and Krumlauf, 1996). In contrast, relatively few similarities in detailed structure have been identified between the anterior ends of the brains of insects and vertebrates. The (supraesophageal) insect brain comprises the protocerebrum, the deutocerebrum, and the tritocerebrum. There is broad acceptance in the literature that the deutocerebrum and tritocerebrum, both of which express *HOM-C* genes (Hirth et al., 1998), are modified segments. The segmental status of the most anterodorsal region, the protocerebrum, remains, however, as controversial in the insect literature as do claims in the vertebrate literature for a segmental organization to the forebrain (Finkelstein and Perrimon, 1991; Rubenstein et al., 1994; Schmidt-Ott et al., 1995; Younossi-Hartenstein et al., 1996; Larsen et al., 2001).

Our findings suggest a different purchase on the problem of how rostral brain organization relates to that of more caudal CNS. Younossi-Hartenstein et al. (1997) have described in detail the pattern of gap gene expression in the neuroblasts that give rise to the fly larval brain. The protocerebrum is specifically identified by gene expression for the terminal gap gene *tl*, whereas the head gap gene *orthodenticle* is expressed in an overlapping domain of posterior protocerebrum and the anterior half of the deutocerebrum. Gene isolation and characterization studies in chick and mouse embryos showed that the vertebrate *tl* homolog is expressed in anterior CNS (Yu et al., 1994; Monaghan et al., 1995), but its expression domain, forebrain and dorsal midbrain, did not appear to correspond to any known structural division within vertebrate brain. We have now identified a morphological correlate for the *TAILLESS* expression domain in

chick brain. The arc-containing ventral midbrain and subthalamus are *TAILLESS* negative; the rest of the midbrain and forebrain is enriched in *TAILLESS* gene expression. This finding, that the homolog of a *Drosophila* protocerebrum marker identifies a novel division of chick brain, suggests that fly protocerebrum and vertebrate rostral brain will show additional similarities of morphology and gene regulation (Chang et al., 2001; Reichert and Simeone, 2001; Page, 2002). Moreover, our finding that the midbrain and subthalamic tegmentum share with more caudal CNS a common embryological plan of cell columns aligned with the ventral midline predicts that the *tlx*-positive protocerebrum will differ from ventral nerve cord and caudal fly brain in features of its longitudinal organization (Arendt and Nubler-Jung, 1999).

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