### Cephalic Neurulation in the Mouse Embryo Analyzed by SEM and Morphometry

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ABSTRACT A detailed account of mouse neurulation is given based mostly on SEM analysis over 20 hr of development. Many observations and measurements were made on staged living embryos and on embryos prepared for scanning and light microscopy to help deduce what mechanisms may contribute to neural tube formation. Each lateral half of the early cephalic neural plate makes a convex bulge, opposite to the way it must fold to form a tube. Underlying mesenchyme and matrix are reported to have a role in forming these bulges. Processes that form the tube must overcome this opposed folding and the forces that produce it. Cranial flexure begins long before tube formation. The flexure commences at the rostral tip of the cephalic neural plate, then the apex of the flexure migrates caudally to the mesencephalic region. Early appearance of this flexure imposes a mechanical impediment to tube closure in forebrain and midbrain regions. Tube closure begins in the cervical region exactly where the neural plate is reflected dorsally by a bend in the embryo. This bend may mechanically assist closure in this region. Cells of the mouse neural plate are reported to contain organized microfilaments and microtubules, and the plate cells appear to change shape (reduce apical area and increase cell height) in the same manner as that suggested in embryos of some other species to contribute to neural tube formation. Measurements show that the lateral edges of the cephalic neural plate elongate craniocaudally more than the midline of the plate through each period. This elongation could contribute to the folding of the plate into a tube. The progress of cranial ventral flexure pauses while tube formation occurs, but edge elongation continues, presumably contributing to tube formation. There is considerable increase in volume of the neural plate during tube closure, and cell proliferation and enlargement of daughter cells seem sufficient to account for this growth. Mitotic spindles are positioned to place the majority of the daughter cells into the long axis of the neural plate, so ordered growth may be the main mechanism of elongation of the plate in the craniocaudal direction, which in turn may assist in tube formation. Mouse cephalic neural plates appear overlying already segmented cranial mesenchyme according to previous reports, and neuromeres develop precociously in the open plates, where their positions correlate exactly with the underlying segmented mesenchyme.

Some features of neurulation in the mouse embryo have already been elucidated, and there is agreement on its general course. During presomite stages, the cephalic neural plate is present and thickening, but the more caudal neural plate is yet to be formed (Waterman, 1976). Each lateral half of the early cephalic plate makes a convex bulge (Morriss and Solursh, 1978a, 1978b). This convex bilateral bulging is in the direction opposite the concave bending that later forms the tube. Neural tube formation begins in the cervical region, but fusion of the neural folds in the embryos of several rodents, including the mouse, occurs at more than one site (Waterman, 1976; Geelen and Langman, 1977; Kaufman, 1979; Morriss and New, 1979), and in this respect differs from the human embryo (Kaufman, 1979; O'Rahilly and Gardner, 1971, 1979). From the cervical region, fusion proceeds both rostrally and

Received November 11, 1981; accepted February 24, 1982.

caudally. A second point of fusion occurs in the prosencephalon and closure also proceeds both rostrally and caudally from this site. Caudal fusion from the prosencephalic region, and rostral fusion from the cervical region meet over the mesencephalon to close finally the cephalic neural tube (Geelen and Langman, 1977).

There are several electron microscope (EM) and light microscope (LM) studies of how the folds fuse in rodent embryos (Freeman, 1972; Waterman, 1975, 1976; Geelen and Langman, 1977; Sadler, 1978; Wilson and Finta, 1980). Possible roles in the fusion process of interdigitating cellular processes (Waterman, 1976) and of carbohydrate-rich surface coat materials (Sadler, 1978) have been suggested. Waterman (1975, 1976) presents many scanning electron microscope (SEM) views of mouse neurulation with a focus on details of cell behavior at the sites of fusion. Kaufman (1979) gives an SEM description of mouse cephalic neurulation with emphasis on optic vesicle formation.

Several transmission electron microscope (TEM) studies confirm that the rodent neural plate and tube have organized microfilament bundles near the apical surfaces, and oriented microtubules in the long axes of the neural cells (Freeman, 1972; Morriss and New, 1979; Wilson and Finta, 1980). An immunofluorescence study (Sadler et al., 1982) using antibodies to actin showed patterns of actin distribution that correlated with the directions of bending of the mouse neural plate. Contraction of the apical microfilament bundles, and possibly changes of cell shape relying on oriented microtubules, may thus contribute to tube formation in the mouse embryo, as has been proposed for neurulation in amphibia (Baker and Schroeder, 1967; Schroeder, 1970; Burnside, 1971, 1973; Karfunkel, 1971; Messier and Sequin, 1978) and birds (Messier, 1969; Handel and Roth, 1971; Karfunkel, 1972; Auclair and Messier, 1974; Portch and Barson, 1974; Bancroft and Bellairs, 1975; Camatini and Ranzi, 1976).

In amphibian embryos, it has been shown that craniocaudal elongation of the midline of the neural plate helps shape the early neural plate and may contribute to the folding of the plate into a tube (Jacobson and Gordon, 1976). There is a correlation between rapid elongation of the midline and tube formation (Jacobson, 1978). A similar correlation has been found in the chick embryo (Jacobson, 1980, 1981). An elastic sheet stretched along a line will fold into a tube. The neural plate is an elasticoviscous sheet and could behave similarly. Morriss and New (1979) consider the possibility that ventral midline elongation, together with a stretching of the dorsolateral edges of the plate, possibly due to continuing cranial flexure, might contribute to closure of the folds in the mesencephalic region of the rat embryo. However, in a later paper, Morriss-Kay (1981) reports that in rat embryos with 0-14 somites, the length of the midline from the anterior end of the notochord to the preotic sulcus remains at about 200  $\mu$ m. Whether there is elongation along the ventral midline or along the lateral edges of the mouse cranial plate is an open question that we address below.

The amphibian embryo accomplishes neurulation without growth (Gillette, 1944; Jacobson and Gordon, 1976; Jacobson, 1978), but embryos of both birds and mammals definitely grow during neurulation, and growth could thus contribute to the neurulation process. Jelínek and Freibová (1966) emphasize that growth ensuing from cell proliferation most likely has a large role in neural tube formation in the chick embryo.

The object of this paper is to describe better the development of the cephalic neural plate and tube of the mouse embryo, and to use these observations, together with measurements from living embryos, sectioned material, and scanning electron micrographs to evaluate possible mechanisms of tube formation.

#### MATERIALS AND METHODS

Female CF-1 strain mice were paired with males on the day of estrus. After pairing overnight, or for a period limited to 2 or 3 hr in the early morning, females were checked for the presence of vaginal plugs. The ages of the embryos were calculated as days post coitum (p.c.), assuming that mating occurred between midnight to 2 A.M. for overnight matings, and within the 2- to 3-hour period for the more precisely timed matings. Embryos at various stages of neurulation were obtained from pregnant females at 8.0-9.25 days p.c. Females were killed by cervical dislocation and embryos were dissected out from the decidua into phosphate-buffered saline (PB-1 medium; Whittingham and Wales, 1969). The investing extraembryonic membranes were then removed with fine watchmaker's forceps, and dissected embryos were transferred to fresh PB-1 medium.

Camera lucida drawings of fresh, unfixed

embryos were made at  $55 \times$  magnification using a drawing tube fitted onto a Wild M5 stereomicroscope. The embryo was placed on its side and the silhouette of the cephalic neural folds was drawn. The position of the neural groove, which is discernible as an opaque line, was also marked on the drawing. The position of the anterior boundary of the first somite, or the position of the otic placode in older embryos, was also noted. The somite number of the embryo was then counted and all embryos were fixed in Bouin's fixative for histologic studies. Some embryos at the ages 8.5-11.0 days were photographed through a Zeiss stereomicroscope equipped with a phototube. The length of the midaxis along the neural groove from the junction of the prosencephalon and mesencephalon to the first somite, or to the otic placode, was measured on both the camera lucida drawings and on the photographic prints. The length of the neural plate along its lateral edge between the same two landmarks was also measured. Altogether, about 130 embryos with 3-45 somites were studied in this way. We also examined younger embryos of 6.5, 7.0, and 7.5 days p.c. from our library of serially cross-sectioned mouse embryos stained with hematoxylin and eosin.

Twenty-eight embryos having 5-6 somites, 10 somites, 13 somites, 16-17 somites, or 20-21 somites were selected for morphometric analysis. The embryo was oriented in the paraffin block in such a way that the plane of section was exactly transverse through the long axis of the midmesencephalon, or of the rhombencephalon at the level of the otic placode. Serial sections 8  $\mu$ m thick were cut and stained with hematoxylin and eosin. Camera lucida drawings of sections of the neural epithelium were made, at linear magnifications of  $34 \times$  or  $47 \times$ , with a drawing tube fitted onto a Zeiss research microscope. For each specimen, two consecutive sections were drawn and the positions and orientations of the metaphase-anaphase figures were noted. In two selected areas of the section, all the nuclei present were scored and the smaller diameter of the oblong nucleus was measured with a scale calibrated with a stage micrometer. The area of the sections was estimated with a compensating planimeter and the tissue volume for the two sections was computed from this area measurement and the section thickness. After correcting the nuclear score with Abercrombie's formula (1946), the apparent cell volume of the neuroepithelial cells was computed from the adjusted score of nuclear points and the volume of the selected areas. The number of cells in each pair of 8- $\mu$ m sections was then calculated from the total tissue volume and the apparent cell volume. Mitotic index was expressed as the percentage of the incidence of metaphase-anaphase in the total cell population. The neural plate was further subdivided into three compartments (see Fig. 10) and the mitotic index for each of these compartments was estimated. The height of the epithelium at three locations in the neural plate (see Fig. 10) was also measured from the camera lucida drawings.

Embryos ranging in age from presomite to 19 somites, and also at 24 somites, were collected from pregnant females at 8.0-9.5 days p.c., and were fixed in half-strength Karnovsky fixative (1965). After fixation, embryos were washed in cacodylate buffer (0.1 M, pH 7.4). With a pair of fine orthodontic wire needles, the extraembryonic membranes were removed. For one or two embryos at each somite number, the entire cephalic neural plate was preserved for an overview of the neural plate. For other embryos, a sagittal cut along the neural groove was made with the needles so that the cephalic neural plate was bisected into lateral halves. The specimens were then processed for scanning electron microscopy as previously described by Meier and Tam (1982), and were examined with an ISI super IIIA SEM operated at 15 kV. Stereo-pair pictures were taken with a tilt angle of 10° and are mounted for viewing with standard stereo glasses in this paper. Three reference points along the midline of the long axis of the neural plate were selected for the measurement of the angle of flexure of the neural plate (see Fig. 16). From SEM dorsal views of the neural plate, the distance between the two edges of the neural plate at the midpoint of the mesencephalon along the long axis was measured to give an indication of the extent of neural tube closure. Over 190 embryos were examined by SEM, about half of them in stereo. To describe neural plate and tube development, we examined with light microscopy serial sections of mouse embryos from 7.5-9.0 days p.c., and SEMs of 8.0-9.0-day embryos (0 somites to 19 somites). Since somites are added about one per hour, the time between zero and 19 somites is about 20 hr. To analyze the SEM series, we examined 8- by 10-inch prints in stereo of each dorsal view and most medial views. The examples illustrated in Figures 2 and 3 are the untilted members of the stereo pairs. Some selected stereo pairs of the same material are illustrated in Figures 4–7.

#### RESULTS

#### Presomite embryos

During the initial phase of gastrulation, between 6.5 and 7.5 days p.c., the mouse embryo maintains a uniformly cylindrical shape. However, by the late-primitive-streak stage, differential growth of different parts of the embryo transforms it into a more open bowl-shaped structure. By 7.5-8.0 days p.c., at presomite stages, the anterior portion of the epiblast is organized into a simple columnar epithelium, the cephalic neural plate (Figs. 1A, 2-0S, 3-0S). The cranial and lateral borders of the neural plate are demarcated by the transition of the columnar neural epithelium to a more cuboid or squamous nonneural ectoderm. Viewed from the dorsal aspect of the embryo, the neural plate of the presomite embryo appears as a thickened tissue sheet that is divided into lateral halves by a deep medial neural groove (Fig. 2-0S).

Cross sections through the neural plate show that the plate is a widely open V-shaped structure by 7.5 days p.c. (Fig. 1A). Further development during presomite stages involves a folding of the edges of the plate, beginning at the most rostral border and spreading caudally along each lateral edge of the plate. This folding tucks the plate edge ventrally and slightly medially under the plate, putting the edge out of sight in dorsal view, and the plate becomes elevated above the nonneural ectoderm. Concomitantly, the middle portion of each lateral half of the plate buckles dorsally as primary mesenchyme and matrix accumulate beneath the plate halves. These conditions have begun in the presomite 8.0-day p.c. embryo (Figs. 2-0S, 3-0S, 4) and are well established in embryos with one somite (Figs. 1B, 2-1S, 3-1S).

#### Embryos with 1 somite

By the time the first somite has formed, a ventral flexure has begun in the prospective prosencephalon (Fig. 3-1S). This flexure simultaneously is involved in foregut formation and ventral displacement of the heart. The apex of the angle formed by this ventral flexure is initially at the most rostral reaches of the prosencephalon, then in subsequent stages through the 13-somite stage the apex displaces progressively more caudally. The ventral flexure takes increasing amounts of the prosencephalon out of sight in dorsal views (Fig. 2).

In these curious bihemispherical early stages, prospective brain compartments bulge dorsally, the direction opposite that which

they must eventually assume to form and close the tube. Boundaries between prospective brain parts appear in dorsal or medial views as constrictions (Figs. 2-1S, 3-1S). These bulges and furrows are the earliest indications of the proneuromeres and neuromeres, some of which are the primary brain parts (see Källén and Lindskog, 1953; Källén, 1953). Prosencephalon, mesencephalon, and metencephalon are clearly defined in the one-somite embryo, and some of these parts are even discernably foreshadowed in the 8.0-day presomite embryo (Figs. 2-0S, 3-0S), especially if viewed in stereo (Fig. 4). At the one-somite stage, the dorsal bulging of each lateral half of the neural plate is continuous from mesencephalon into metencephalon, the preotic sulcus between the two being only a slight indentation (Fig. 3-1S). The hindbrain is bent back over the more rostral parts of the cephalic neural plate (Fig. 3-1S), and abuts the primitive streak at the midline. The apex of the angle made by the bend is just posterior to the metencephalic-myelencephalic boundary. In subsequent stages, this apex is displaced caudally.

#### Embryos with 2-4 somites

Dorsal bulging of the brain plate halves increases. Continued caudal displacement of the point of ventral flexure takes additional parts of the prosencephalon out of sight in dorsal view (Figs. 2-2S to 2-4S), while caudal displacement of the point of dorsal reflection brings more posterior cranial plate into view. In embryos with three somites, the apex of the ventral flexure is about halfway between the anterior and posterior borders of the prosencephalon (Fig. 3-3S), whereas the point of dorsal reflection is located toward the caudal end of the myelencephalon. Both apices have moved even farther caudal in embryos with four somites (Fig. 3-4S), the apex of the ventral flexure being at or near the boundary between prosencephalon and mesencephalon.

In dorsal views (Figs. 2-2S to 2-4S), the broad, flat myelencephalic plate appears to narrow as the plate halves rise and their edges begin to approach one another (compare hindbrain regions of Figures 2-2S, 2-3S, and 2-4S). The width of the mesencephalon, on the other hand, continues to increase through these stages (measurements of mesencephalic width are given for all stages in a later section). By the time embryos have three somites, there is a right-angle bend in the myelencephalon and another, in the opposite direction, in the mesencephalon. A slight indentation in the prosencephalon marks the beginning of the optic evagination (Fig. 3-3S).

#### Embryos with 5-6 somites

An embryo with five somites, viewed dorsally in stereo (Fig. 5), presents a high plateau that is mostly mesencephalon. Rostrally, the prosencephalon drops away as a straight cliff, whereas caudally the long steep hindbrain drops to a lower plateau that consists of the most caudal myelencephalon and the cervical neural plate (cf. Fig. 3-5S). The distance between the neural edges of the mesencephalon becomes maximal in embryos with six somites (see Fig. 18). Furthermore, the neural edges can now be seen in dorsal view, having risen from beneath the plate (Fig. 2-6S).

	Abbre	eviation	s
AL	Allantois	NG	Neural groove
AM	Amnion	NO	Notochordal plate
AN	Anterior neuropore	NP	Neural plate
FG	Foregut	OP	Optic evagination
FU	Fusion of neural	OT	Otic invagination
	folds	$\mathbf{PM}$	Prosencephalic-
HT	Heart		mesencephalic
MM	Metencephalic-		boundary
	myelencephalic	POS	Preotic sulcus,
	boundary		boundary between
MS	Mesencephalon		mesen- and
MS1	First mesencephalic		metencephalon
	neuromere	$\mathbf{PR}$	Prosencephalon
MS2	Second	$\mathbf{PS}$	Primitive streak
	mesencephalic	Rh	Rhombomere
	neuromere	YS	Yolk sac
MT	Metencephalon		
MY	Myelencephalon		
NE	Neural edge,		
	boundary between		
	neural and		
	non-neural		
	ectoderm		

Fig. 1. Light micrographs of transverse sections through the cephalic neural plate (prospective mesencephalon and prosencephalon), all at the magnification indicated by the bar. A) The neural plate (NP) in a 7.5-day p.c. mouse embryo with zero somites. Arrowheads indicate the approximate edges of the thickened plate, and an arrow the midline. B) An embryo with one somite, about 8.3 days p.c. The plate is now biconvex with a deep neural groove and plate edges reflected ventrally. Primary mesenchyme and matrix have accumulated beneath the plate halves. C) An embryo with eight somites (about 8.5 days p.c.). The biconvex plate halves have risen dorsally and greater amounts of mesenchyme and matrix are beneath them. The configuration of the section gives an impression of turgidity. The plate edges are more dorsally and laterally placed than before. D) An embryo with about 16 somites (nearing 9 days p.c.). The neural plate is very much thicker, and is closing into a tube. The curvature of the lateral parts of the plate halves has been reversed as the tube forms.





Fig. 2. Scanning electron micrographs of dorsal views of mouse neural plates and tubes, dissected free of enveloping tissues. Examples begin at zero somites (0S) at 8.0 days p.c., and are shown for each additional somite from one (1S) to

19 (19S). All are at the same magnification. The scale bar is 0.1 mm. Since a somite is added about every hour, the sequence illustrates about 20 hr of development. See text for description.

In the prosencephalon, the optic evaginations have begun to assume a long slit-like appearance. What appears to account for this is the rising and reversal of curvature of the rostral and rostrolateral edges of the prosencephalic plate (this process is completed by seven somites). The reversal of curvature of the edges of the prosencephalon places the optic evagination at the bottom of a deepening trench that runs most of the length of the prosencephalon as seen in medial view (Figs. 3-6S, 3-7S).

#### Embryos with 7 somites

By the time the embryo has seven somites, fusion of the neural folds commences in the cervical region and spreads into the caudal myelencephalon (Figs. 2-7S, 3-7S). The region of fold



 $Fig. \ 2. \quad Continued. \ Eleven \ somites \ (11S) \ to \ 19 \ somites \ (19S).$ 



Fig. 3. Scanning electron micrographs of medial views of mouse embryos hemisected along the long axis and dissected free of enveloping tissues. Each example is about an hour older than the preceding one, beginning with zero somites

(0S) at 8.0 days p.c. The whole embryo is shown in 0S to 3S, but only the cephalic parts in 4S on. All are at the same magnification. The scale bar is 0.1 mm. These examples parallel those shown in Figure 2. See text for description.

fusion coincides with the position of dorsal reflection of the nervous system.

The reversal of curvature of the edges of the prosencephalic neural plate has progressed almost to the boundary with the mesencephalon, giving the appearance of a horn-like projection anterior to that boundary in dorsal view (Fig. 2-7S). Although a similar reversal of the dorsal bulging of the plate has not yet begun in the mesencephalon and metencephalon, the plate edges have unfolded and now come into dorsal view.



Fig. 3. Continued. Eleven somites (11S) to 19 somites (19S).



Figs. 4-7. Stereo-pair scanning electron micrographs of dorsal views of mouse neural plates at presomite (Fig. 4), fivesomite (Fig. 5), ten-somite (Fig. 6), and 13-somite stages (Fig. 7). Scale bars are 0.1 mm. Stereo pairs are mounted for viewing with a cartographer's stereo viewer. See Figures 2 and 3 for labels.

### Embryos with 8-10 somites

During this period, the fusion of neural folds that was initiated at the cervical level proceeds rostrally in the myelencephalon (Figs. 2-8S to 2-10S). Progressive elevation of the neural edges of the prosencephalon and the mesencephalon occurs concomitantly with the narrowing of the breadth of the neural plate (in dorsal views, e.g., Figs. 2-8S to 2-10S). The reversal of the dorsal convex curvature of the mesencephalon first occurs at the rostral and



caudal portions of the mesencephalic neural plate (at eight somites); this is followed later by the rest of the mesencephalon (at nine somites; compare Figs. 2-8S and 2-9S). The reversal process also occurs lateromedially, so that in cross section, the dorsolateral portions of the plate are concave, but the more medial parts retain a dorsal bulge (see Figs. 1D, 6, 7).

At eight somites, the optic evagination still occupies the bottom of a slit that runs the length of the prosencephalon (Figs. 3-8S), but by ten somites (Fig. 3-10S) the dorsal bulging of the medial-caudal portion of the prosencephalon is reversing direction so the optic evagination henceforth protrudes from the bottom of a bowl (seen from medial view) and the slit-like appearance has disappeared.

### Embryos at 11-16 somites

During the period of formation of the 11th to 16th somites, the cephalic neural edges rapidly approach one another and the bulging in the half plates becomes predominantly concave as the neural tube nears completion (Figs. 2-11S to 2-16S, 3-11S to 3-16S, 1D, and 7). Fusion of the plate edges in the prosencephalon is imminent at 15 somites and this is presaged by the appearance of ruffles at the plate edge (Fig. 2-15S) as described by Waterman (1976). By 16 somites, fusion of the neural edges has occurred in the prosencephalon near the mesencephalic border, and is spreading both caudally and rostrally (Figs. 3-16S and 2-16S). Two anterior openings into the neural tube now exist, they being the anterior neuropore and the more persistent opening centered over the mesencephalon (Fig. 3-16S). At 16 somites, the cells at the plate edges around both these openings already show the cellular changes that precede closure (Figs. 1-16S, 3-16S).

During the period from 11 to 16 somites, the neuromeres have all assumed the characteristic appearance seen in lower vertebrate embryos, that is, they bulge outward rather than inward as some did in earlier stages of the mouse embryo, and they are separated by sulci protruding into the prospective brain lumen. Neuromeres that can be readily identified include prosencephalic, mesencephalic-1, mesencephalic-2, metencephalic, and other rhombomeres (Fig. 3-15S).

#### Embryos at 17-19 somites

During this time, closure of the cephalic neural tube is completed. The anterior neuropore is closing in the 18-somite embryo (Fig. 3-18S). The neural edges of the mesencephalon rapidly approximate one another along their full lengths during the period between 16 and 18 somites (Figs. 2-16S to 2-18S, 3-16S to 3-18S), and are completely fused by 19 somites (Figs. 2-19S, 3-19S). This is accompanied by increasing concavity of the mesencephalic half-plates (Figs. 3-17S to 3-19S).

## Growth of the cephalic neural plate during neural tube formation

It is apparent that the prospective brain grows considerably from the presomite embryo to the 19-somite embryo. Examination of cross sections (Fig. 1), dorsal SEM views (Figs. 2 and 4–7), and medial SEM views of hemisected embryos (Fig. 3) readily confirms this.

Examples of this growth were measured in the midmesencephalon and in the myelencephalon at the level of the otocyst from cross sections cut normal to the axis of each brain part as described in Materials and Methods. The results (Fig. 8) show little increase in volume to 10 somites, then about a 3.5-fold increase in volume in midmesencephalon in embryos with between 10 somites and 20 somites. During the same period, myelencephalon also increased its volume very little to 10 somites, then about doubled its volume by 20 somites.

Counts of the cell numbers in the same cross sections (Fig. 9) showed a similar pattern of increase, suggesting that the growth in tissue volume is likely a consequence of cell division followed by enlargement of the daughter cells.

#### Spindle orientation of dividing cells, and height of neuroepithelium

Since growth of the neuroepithelium is probably a consequence of cell division and subsequent enlargement of the daughter cells, the orientation of the spindles of dividing cells could greatly influence the shape of the growing epithelium. To evaluate spindle orientation, cross sections of the neural plate in the myelencephalon, and in the mesencephalon, were subdivided into three equal-sized compartments-lateral, medial, and intermediate – as shown in Figure 10. In each of these compartments, the numbers of division figures oriented to place the daughter cells either along the axis or transverse to the axis were determined (Tables 1 and 2).

At the midline and at the boundaries between each of the compartments (Fig. 10), measurements of neuroepithelial heights were made (Figs. 11 and 12).



Fig. 8. Tissue volumes were measured in midbrain (top) and in hindbrain at the level of the otocyst (bottom) in two adjacent transverse sections, each 8  $\mu$ m thick, cut normal to

the long axis of the brain part. Each point represents the volume of a 16- $\mu$ m slice of the brain in an embryo with the number of somites indicated.

# Lengths of ventral midline and of lateral edges of the neural plate

Since elongation of either the ventral midline or of the lateral edges of the plates (ultimately, the dorsal midline of the neural tube) could contribute to buckling of the plate into a tube, measurements were made as shown in Figure 13. The results of these measurements are given in Figure 14. Both the ventral midline and the lateral edges increase in length during the period of closure of the tube in embryos with 7–19 somites. During this period the lateral edges increase in length by 67% and the ventral midline by 40%.

Since the plots of length increase resemble parts of growth curves, we replotted the data on semilog paper and found that straight lines do fit the data well and the lengths in each case are doubling about every 15 hr (Fig. 15).

# Measurements of the ventral cranial flexure

As described above and illustrated in Figure

3, the cranial flexure begins as a ventral bend in the most rostral prosencephalon in embryos with one somite. The bending then increases and the apex of the angle of the flexure migrates caudally until it is beneath the midmesencephalon by 10–13 somites. The apex then migrates rostrally in embryos with 14–19 somites, finally to occupy the prosencephalicmesencephalic boundary. Because the apex of the flexure migrates, quantitative evaluation of the progress of the flexure is complicated.

We have chosen three points that can be identified at each stage and measured the angle of flexure formed by these three points. The points are the prosencephalon floor directly beneath the optic evagination (or prospective evagination), the mesencephalon floor midway along its anterior-posterior extent (the apex of the angle of flexure for much of the time the tube is closing), and the brain floor at the boundary between metencephalon and myelencephalon. By choosing these points, we are measuring mostly the effect of flexure on the mesencephalon. The angles formed by



Fig. 9. The cell numbers were calculated (see Materials and Methods) in the same  $16\text{-}\mu\text{m}$  slices of midbrain (top) and

hindbrain (bottom) as were used to determine volumes (see Fig. 8).



Fig. 10. Light micrographs of transverse sections through the myelencephalon illustrating the three equalsized compartments (m, medial; i, intermediate; l, lateral), separated by dotted lines in B, in which the orientation of mitotic spindles was evaluated for Tables 1 and 2. A spindle oriented to add daughter cells in the long axis is at the end of the top arrow in A, and a spindle that will add daughter cells transverse to the long axis is at the end of the lower arrow. At the higher magnification of A, the outlines of nuclei that were counted in such sections can be seen. Scale bar in A is 0.01 mm, scale bar in B is 0.1 mm. these points are illustrated in Figure 16. Measurements of these angles from embryos of various stages are given in Figure 17.

#### Closure of the mesencephalic neural folds

The mesencephalon is the last part of the brain to complete tube formation, and its involvement in cranial flexure before closure complicates the mechanics of its closing. We have measured the distance between the plate edges at the level of the midmesencephalon to give a quantitative record of the progress of tube formation in the mesencephalon (Fig. 18).

#### DISCUSSION

By the time embryos have formed the first six somites, the cephalic neural plate has formed, thickened, tucked its edges beneath itself, made a deep medial groove, formed convex lateral halves, formed a cranial flexure, and begun formation of the proneuromeres and neuromeres. In the cervical region, the plate has commenced to fold into a tube. The region of initial tube closure exactly correlates with the



Fig. 11. Height of the neuroepithelium of the midbrain midway along its length was measured at the positions indicated by the dotted lines in Figure 10, in embryos with different numbers of somites.

region of dorsal reflection of the neural plate, and this upward bending of the plate could most likely assist in the mechanical buckling of the plate to make a tube. In embryos with seven somites, the cervical neural plate has closed into a tube and the neural edges (folds) of the rest of the cephalic plate have begun to rise, but the entire cephalic plate, except for the optic evaginations, still bulges dorsally, contrary to the way it must fold to become a tube. The elongated, bihemispherical shape of the early mammalian neural plate appears to result from accumulation of mesenchyme cells and extracellular matrix beneath each lateral half of the neural plate (Morriss and Solursh, 1978a,b). In vitamin A-induced exencephaly, fewer mesenchyme cells and matrix are found beneath the brain plate, and the plate becomes irregular and "floppy," and fails to form a tube (Marin-Padilla, 1966; Morriss, 1972; Morriss and Steel, 1974). Synthesis of GAG (glycosaminoglycans, mostly hyaluronic acid) correlates with increased primary mesenchyme and extracellular spaces beneath the plate (Solursh and Morriss, 1977). Our observations are consistent with the interpretations of Morriss and Solursh explaining the biconvex shape of the cephalic plate in embryos with zero to eight somites. If the mesenchyme and matrix it produces are to exert an actual force on the plate, it seems most likely that that force would be fluid pressure resulting from the hydration of the matrix. The turgid appearance of a section such as our Figure 1C seems to support this idea.

In the 12-hr period during which embryos have from seven to 19 somites, the cephalic plate reverses its previous bihemispherical dorsally directed bulging to evert in the opposite direction and the tube closes. The processes of tube closure must, therefore, counter whatever forces are causing the dorsal bulging. In addition, closure in the mesencephalon and posterior prosencephalon must proceed counter to the mechanical impediment of the ventral cephalic flexure. The cephalic flexure



Fig. 12. Height of the neuroepithelium of the hindbrain at the level of the otocyst was measured at the positions indicated by the dotted lines in Figure 10, in embryos with different numbers of somites.



Fig. 13. Camera lucida drawing of the cephalic neural plate of a mouse embryo showing the landmarks used in measuring the lengths of the neural plate along its ventral midline (B-D), and along its lateral edges (future dorsal midline) (A-C). Line A-B is at the boundary of the prosencephalon and mesencephalon. Line C-D is at the level of the anterior boundary of the first somite. In older embryos in which the first somite is not readily discernible, line C-D was placed at the level of the otic placode, but the variation in the length measured is within 120  $\mu$ m out of a total length of about 3 mm (i.e.,  $\pm 4\%$ ).

should tend to keep the edges of the plate below the level of the plate, yet the neural folds rise and the tube is formed in the flexure region. It may be that mesencephalon closes into a tube last because it must overcome these impediments. Fusion of the plate edges at the second site of tube closure (in the prosencephalon) occurs only after the dorsal bulge is reversed at that site. Reversal of dorsal bulging occurs earlier in the prosencephalon than in the mesencephalon.

We measured the progress of ventral cephalic flexure (Fig. 17) and found that the angle of flexure drops rapidly from over  $200^{\circ}$  to less than  $90^{\circ}$  during the time the embryo goes from zero to seven somites. In embryos with seven to 19 somites, during which time the cephalic plate is closing into a tube, the change in angle of flexure pauses and even may increase for a time. In embryos with seven to 16 somites, the angle of flexure rises slightly (Fig. 17), then falls back to the starting value. In embryos with 16 somites, closure is almost complete and is completed by 19 somites. During this time, the angle of flexure declines by 21%. After closure is complete, the angle of flexure



Fig. 14. Lengths of the lateral edges (top) and the ventral midline (bottom) of neural plates are shown for embryos with different numbers of somites. Each point is one case. The limits of the lines measured are shown in Figure 13.

again begins to decline rapidly. The correlation of cessation of angle change in the flexure with the period of closing of the plate suggests that these processes are arranged so that active flexure stops during closure of the plate, thereby reducing the effect of flexure as a mechanical impediment to tube formation.

In the chick embryo, cephalic flexure occurs mostly after the brain has already formed into a tube. A bend in the tube, of course, requires that the roof, which occupies the outside of the curve, elongate more than the brain floor. During flexure of the chick brain, the percentage increase in length of mesencephalon roof is 5-25 times that of mesencephalon floor (Goodrum and Jacobson, 1981). In the mouse embryo, cephalic flexure starts in the very early plate, so the comparative elongation of the outer plate edges (future dorsal midline) and the ventral midline cannot be predicted. We measured these elongations and found that both the midline and the edges of the plate (future dorsal midline) elongate at steadily increasing paces, the lateral edges being ahead of the midline in amount of elongation. Thus, through any period, the elongation in the



Fig. 15. The same and some additional data for lengths of lateral edges and ventral midlines of the neural plates shown in Figure 14 are replotted here on a semilog scale (length is the logarithmic scale).

lateral edges is greater and more rapid than in the midline (Fig. 14). To raise the neural edges and form a tube, the lateral edges must elongate more than the midline, but in early stages of the flexure, when the edges are reflected below the plate, more elongation of the edges could help unfold the reflected plate edges and widen the plate. Our measurements show (Fig. 18) that the width of the mesencephalon increases until the embryo has six somites, and by then the plate edges have sufficiently unfolded that they become visible from above. In embryos with seven or more somites, elongation of the lateral edges may contribute to tube closure and appears to do so. In embryos with seven to 19 somites, when the cephalic tube is closing, the plate edges increase length by 67% while the midline increases length by 40%. This differential appears to be used up in lifting the neural edge as the plate folds, and during this period flexure pauses.



Fig. 16. On this scanning electron micrograph of a medial view of a hemisected mouse embryo, the three points chosen to define the angle of cephalic flexure are shown. One point is in the brain floor directly beneath the optic evagination, one (the apex of the angle) is in the brain floor midway along the length of the mesencephalon, and the third point is in the brain floor at the boundary between metencephalon and mesencephalon (or their precursors). Scale bar is 0.1 mm.

Both midline and plate edge elongation plot a straight line on a semilog graph (Fig. 15). This suggests that exponential growth is behind the elongation, and indeed, both midline and edges are doubling length every 15 hr, but midline starts at a lesser length (Fig. 15). If this elongation is due to growth from cell division and subsequent enlargement of daughter cells, then there must be sufficient mitoses and there must be preferential placement of the daughter cells into the long axis. In such early neuroepithelia, spindle orientation and cytokinesis almost entirely place cells so they expand the plate surface rather than increase its height. Our observations on mitotic index and spindle orientation (Tables 1 and 2) found average mitotic indices between 4.5 and 8.4 during the closure period, and spindle orientation was such that 59%-64% of the daughter cells would increase the length of the long axis. Cell division and increase of daughter cell volume may be sufficient to drive neural plate



Fig. 17. Angles of cephalic flexure, measured as shown in Figure 16, are given for embryos with different numbers of somites.



Fig. 18. Distances between the edges of the neural plates at the level of the midmesencephalon are given for embryos with different numbers of somites. This records plate widening, then closure into a neural tube.

	Metaphase-anaphase index %			Orientation of spindles %		
Number of somites in embryos	Overall	Lateral <sup>1</sup>	Intermediate	Medial	Along the long axis	Transverse
6/7	6.4	12.3	5.5	3.6	80	20
	4.3	4.6	3.6	4.9	61	39
	5.5	8.4	4.1	3.8	58	42
	$(5.4)^2$	(8.4)	(4.4)	(4.1)	(66)	(34)
10	8.3	9.8	11.6	2.2	53	47
	8.5	8.3	10.0	6.4	74	26
	(8.4)	(9.0)	(10.8)	(4.3)	(64)	(36)
13	6.3	5.8	5.9	7.2	66	34
	5.3	5.5	5.1	5.2	53	47
	4.9	6.8	5.4	2.7	58	42
	(5.5)	(6.0)	(5.5)	(5.0)	(59)	(41)
16/17	3.8	4.5	5.3	2.0	56	44
	4.5	3.2	6.4	3.7	60	40
	5.1	6.3	5.4	3.3	43	57
	4.8	5.0	4.6	5.9	64	36
	4.3	4.2	4.4	4.6	71	29
	(4.5)	(4.6)	(5.2)	(3.9)	(59)	(41)
20/21	3.7	2.7	3.8	4.0	69	31
	4.6	3.7	4.1	6.1	63	37
	(4.2)	(3.2)	(3.9)	(5.0)	(66)	(33)

TABLE 1. Mitotic indices and orientations of spindles in sections cut normal to the long axis at the midpoint of the mesencephalon.

 $^1Lateral,$  intermediate, and medial are equal-sized compartments as illustrated in Figure 10.  $^2($  ) = mean of values in group.

TABLE 2. Mitotic indices and orientation of spindles in sections cut normal to the long axis of the myelence	ohalon at				
the level of the otic placode.					

	Metaphase-anaphase index %			Orientation of spindles %		
Number of somites in embryos	Overall	Lateral <sup>1</sup>	Intermediate	Medial	Along the long axis	Transverse
6/7	13.4	8.3	19.1	12.4	94	6
	8.0	6.9	11.6	9.6	85	15
	6.3	6.0	8.3	4.3	63	37
	$(9.2)^2$	(7.0)	(13.0)	(8.7)	(81)	(19)
10	10.1	15.4	8.2	6.4	62	38
	6.3	6.4	6.5	6.0	69	31
	11.4	11.9	9.0	14.2	63	37
	(9.3)	(11.2)	(7.9)	(8.8)	(65)	(35)
13	4.4	3.2	8.1	1.8	64	36
	5.5	8.8	6.8	1.9	68	32
	8.1	7.1	7.7	9.7	67	33
	(5.9)	(6.4)	(7.5)	(4.4)	(66)	(34)
16/17	3.5	3.9	3.6	3.0	87	23
	7.5	6.1	7.8	8.4	66	34
	6.5	4.9	11.2	3.9	44	56
	4.7	2.6	4.7	6.9	57	43
	(5.5)	(4.4)	(6.8)	(5.5)	(64)	(36)

 $^iLateral,$  intermediate, and medial are equal-sized compartments as illustrated in Figure 10.  $^2($  ) = mean of values in group.

elongation in the mouse embryo, but rearrangement of cells to increase nervous system length is not ruled out. Cell rearrangement appears to be the mechanism by which the amphibian neural plate elongates (Jacobson, 1978).

The height or thickness of the neuroepithelium does increase during the closure period (Fig. 11), but this is due to change of cell shape, not to piling up of cells in that dimension. The changes of cell shape redistribute cell volume to increase neuroepithelial thickness and to reduce the surface area of the neural plate.

Given the observed elongation of the mouse cephalic plate, how could this contribute to tube formation? In amphibian and chick embryos, midline elongation correlates with folding of the neural plate and tube formation (Jacobson, 1978, 1981). If the plate were behaving like an elastic sheet, midline elongation would fold the plate out of the plane and help raise the edges and close the tube. In the cephalic plate of the mouse embryo, the lateral halves of the neural plate are bent in the wrong direction when the plate edges begin to rise. In the mesencephalon, the progress of reversal of this bend is from lateral toward medial (see Figs. 1-3). An elastic sheet will form half a tube if stretched along its edge. Elongation of the lateral edges of the cephalic plate could, therefore, contribute to tube formation if the mouse plate behaves as an elastic or elasticoviscous sheet. The changing rate of increase in length of the lateral edges is consistently ahead of the changing rate of increase in length of the midline (slopes of lines in Fig. 14). Therefore, in the mouse embryo, the observed elongation of the lateral edges of the mesencephalic plate could contribute to the observed folding of the plate halves near the edges as tube formation occurs.

Other mechanisms also appear to contribute to tube formation in the mouse embryo. Cells of the mouse neural plate have the usual subcellular machinery of organized microfilaments and microtubules. Contraction of the apical surfaces of the plate cells, using microfilaments, and elongation of the plate cells perpendicular to the surface, involving microtubules, could contribute to tube formation as described for other species. Our measurements of changes in neuroepithelial height give some indication of how important this mechanism may be (Figs. 11 and 12). The time of greatest increase in cell heights correlates well with the time of reversal of plate curvatures in each region of the cross sections. The greatest rate of height increase, mostly during the period when embryos have seven to 13 somites, is also the time during which plate elongation is minimal (Fig. 14), and changes in tissues volume (Fig. 8) and cell number (Fig. 9) are low in embryos with seven to 10 somites. It seems likely that microfilament contraction may principally drive early tube formation with plate elongation having an increasing role during final stages of tube formation.

Bulges and furrows that define emerging neuromeres (or proneuromeres) (Källén, 1953) are apparent in the wide-open neural plate of mouse embryos with one somite and thereafter (Figs. 2-1S, 3-5S, 3-15S). It is helpful to have a complete series, such as our Figures 2 and 3, to follow through the subtle changes in neural plate morphology and identify the prospective brain parts and neuromeres. Tamarin and Boyde (1976) studied the 8-day mouse embryo with SEM. One point they made is that "five basic brain segments" are discernible on the basis of surface contours of the neural plate. However, they consistently mislabel the brain parts, calling the prosencephalon the telencephalon (on which they place the optic vesicle; their Figure 29), and calling the anterior mesencephalon the diencephalon (e.g., their Figure 24).

The first and most rostral bulge that forms is the prosencephalon and it remains a single entity through the course of our study. Behind it, a large bulge forms that is the prospective mesencephalon (Fig. 2-1S), and this bulge soon subdivides into two mesencephalic neuromeres (Fig. 2-10S). Four bulges appear in the prospective rhombencephalon, the most rostral being prospective metencephalon, and the more caudal three being in the anterior myelencephalon. These neuromeres are wedge-shaped (Fig. 3-15S).

The attention given to neuromeres came, in part, from the hope that these segments reflected the primordial metamerism of the head (cf. Kingsbury and Adelmann, 1924; Adelmann, 1925). The neuromeres change through time and various names and interpretations have been given to them (Berquist and Källén, 1954). The primordial metamerism of the head, in the form of somitomeres composed from the primary mesenchyme, is known now, and it has been related to neuromeres in the chick head (Anderson and Meier, 1981) and in the head of the mouse embryo (Meier and Tam, 1982). In the mouse embryo, the first somitomere underlies the prosencephalon, the second and third somitomeres are under mesencephalic neuromeres 1 and 2, the fourth is under the metencephalon, and the fifth through seventh underlie rhombomeres in the myelencephalon (see Fig. 3-15S). Meier and Tam point out that the one-to-one relationship between somitomeres and neuromeres is transitory. While initially the boundaries between somitomeres match boundaries between neuromeres, new neuromeres arise by subdivision of old ones, and these new boundaries fall along the midpoints of the somitomeres. The seven cranial somitomeres are already formed in the 7.5-day p.c. mouse embryo (Meier and Tam, 1982), and the neuromeres begin to appear in the neural plate only after 8.0 days. It is reasonable to assume that neuromere formation in the neural plate reflects interactions with the already formed somitomeres.

In chick embryos, most neuromeres appear after brain tube closure, and cerebrospinal fluid pressure may have some role in their shaping (Desmond and Jacobson, 1977). This cannot be true of mouse embryos, since most neuromeres have appeared before the brain closes into a tube. One prominent shape change, elongation of the prosencephalon, was shown experimentally to require cerebrospinal fluid pressure in the chick embryo (Goodrum and Jacobson, 1981). It is noteworthy that in the mouse embryo prosencephalic elongation follows completion of brain tube closure (compare Figs. 3-18S and 3-19S).

#### ACKNOWLEDGMENTS

This work was supported by grant NS 16072 from the National Institutes of Health to A.G. Jacobson. We thank Dr. Frank Bronson for providing animal facilities, and Dr. Stephen Meier, Prof. D.J. Riches, and Dr. David T. Yew for discussions and critical evaluation of the manuscript.

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