

## The ontogenesis of cranial neuromeres in the rat embryo I. A scanning electron microscope and kinetic study

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### SUMMARY

The development of the cranial neuromeres, rostral to the level of the first somite, in rat embryos during neurulation is described. Examination of scanning electron micrographs of 4-, 8-, 12- and 16-somite-stage embryos has shown that the neuromeres develop in a strictly ordered pattern but the sequence is not ordered in either a rostrocaudal or caudorostral direction. The two mesomeres are shown to develop by subdivision of a previously formed mesomere, whilst the rhombomeres develop independently of each other. At the completion of cranial neurulation two mesomeres and nine rhombomeres (including the preotic sulcus) but no prosomeres are identifiable.

A direct correlation of the position of peaks in mitotic index along the embryonic axis with the position of sulci on the surface of the neural epithelium, was not possible. However, it is suggested that the mitotic index peak is the initial means of identifying a neuromere, and once the neuromere is expressed morphologically as a surface sulcus the mitotic index returns to its basal level.

### INTRODUCTION

During the embryonic period of development, a series of transverse neural segments has been recognized in all vertebrate groups. Three primary swellings or vesicles of the neural tube can be identified at its cranial end representing the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). The prosencephalon later divides to form the telencephalon and diencephalon, and the rhombencephalon divides to form the metencephalon and myelencephalon. Further minor subdivisions of the secondary brain vesicles occur, and these are referred to as neuromeres (or encephalomeres). In the hindbrain the neuromeres are more specifically referred to as the rhombomeres and in the midbrain, mesomeres. In the forebrain, the presence of neuromeres (prosomeres) in mammals is uncertain, and in lower animals (fish and amphibians) Bergquist (1952) found that if prosomeres existed they were of a transitory nature around the optic sulcus and had disappeared before the development of the optic vesicle proper. However, Vaage (1969) identified eight prosomeres in the forebrain of the chick embryo.

*Key words:* mitosis, neuromeres, neural tube, rat embryo, cranial development.

Neuromere formation precedes the outgrowth of cranial nerve motor fibres and the ingrowth of cranial nerve sensory fibres. The neuromeres are closely associated with the pattern of underlying cranial nerve motor nuclei, and in general the pattern is a direct one-to-one relationship. The neural crest cells and placodes which give rise to the sensory afferents are not directly related to individual neuromeres, although the afferent fibres themselves grow into specific neuromeres: the trigeminal nerve enters rhombomere 2; the facial nerve enters rhombomere 4; the glossopharyngeal nerve enters rhombomere 5; and the vagus nerve enters rhombomeres 6 and 7 (Adelmann, 1925; Bartelmez & Dekaban, 1962; Bartelmez & Evans, 1926).

Adelmann (1925) regarded neuromeres as an expression of different growth patterns: rapid forward growth in a confined space, and localized expansion preceding the development of sensory mechanisms within the hindbrain. Bartelmez (1923) and Bartelmez & Evans (1926) suggested that neuromere formation was preceded by focal proliferation within the neural epithelium. Kallen (1953) found that proliferation centres existed within the neural epithelium and that these coincided with the position of neuromeres; further evidence for this was obtained from colchicine-treated chick embryos (Kallen, 1961, 1962).

We report here the first part of a study designed to elucidate the nature of neuromeres, their mode of development and the means by which their structure is maintained. We have used scanning electron microscopy to observe the pattern of the neuromeres in rat embryos, and the sequence of their appearance and disappearance. This technique can reveal subtle morphological differences between embryos of different stages which would be difficult to detect by the more traditional methods of reconstruction from histological sections. We have also studied the pattern of mitosis along the longitudinal axis, and attempted to correlate this pattern with the development of neuromeres as observed in the scanning electron micrographs. The observations were confined to levels rostral to the first somite; at somitic levels the neuromeres are intersomitic, and there is general agreement on this among previous authors.

#### MATERIALS AND METHODS

Wistar strain rat embryos were obtained by killing the pregnant dam at the appropriate times on either day 9 or day 10 of pregnancy (day 0 = day of sperm-positive vaginal smear); the uteri were dissected out in phosphate-buffered saline, the embryos freed from the decidual swellings, and their extraembryonic membranes removed. The number of somites present in each embryo were counted: only embryos with 4, 8, 12 or 16 somites were used in this study. The embryos were then fixed in 2.5 % glutaraldehyde in 0.1 M-cacodylate buffer containing 0.03 %  $\text{CaCl}_2$  for 3 h, and washed for at least 3 h in 0.1 M-cacodylate buffer. Subsequent treatment was dependent on whether the embryos were to be embedded and cut for light microscopy (LM) or were to be processed for scanning electron microscopy (SEM).

#### *Scanning electron microscopy*

After washing, the embryos were sagittally bisected with a cataract knife and dehydrated through graded acetones before being critically point dried using a Polaron Critical Point Drying

Apparatus E3000 with liquid CO<sub>2</sub>. The embryos were mounted on aluminium stubs with double-sided adhesive tape, and coated with gold using a Polaron series II Cool Sputter Coater before viewing with the Jeol scanning electron microscope JSM-T20.

### *Light microscopy*

After washing, the embryos were postfixed in 1 % osmium tetroxide in 0.1 M-cacodylate buffer for 30 min. After thorough washing in distilled water, the embryos were dehydrated through graded alcohols and embedded in Spurr resin. Serial 1  $\mu$ m thick transverse sections were obtained and stained with either equal parts of 1 % methylene blue and 1 % azure II in 1 % borax, or 1 % toluidine blue in 1 % borax. Under the light microscope, the number of mitotic cells and the total number of cells/transverse section were counted at 5  $\mu$ m intervals along the neural tube to the level of the first somite.

### *Analysis of mitotic index data*

From the light microscope study, graphs were plotted of the mitotic index (MI = % cells in mitosis/transverse section) against the distance down the neural tube from the first cutting of the prosencephalon to the level of the first somite. In order to analyse the data a series of computer programs were written in FORTRAN. The initial program takes the raw MI data obtained from the transverse sections and computes the three-point running medians; this was to "smooth" the data and to eliminate any high frequency oscillations. The next stage of the analysis was to define a method for recognizing a peak in the MI; the obvious way to do this was to define a specific line which could be drawn across each graph, and above which all MI values could be said to be peak values. Two lines were possible candidates; either a line representing the mean plus one standard deviation (or even the mean itself), or the regression line. The first line does not allow for any variation in the mean MI that may exist within different brain regions, and hence peaks occurring in a region with a lower mean MI would be excluded. The least-squares regression line is the line calculated to "best-fit" all the data and therefore does consider all those values which might lie in a region with a lower mean MI. For this reason the regression line was used as the boundary for the determination of the MI peaks. In order to be able to compile a table of peak values and their precise location along the neural tube, several further criteria were imposed upon the data:

- (i) to allow up to a maximum of four points to lie on the regression line within any single range of peak values;
- (ii) to amalgamate any range of peak values that were separated by not more than 10  $\mu$ m (2 values);
- (iii) to eliminate any single or double values lying above the regression line which had not been incorporated by criteria (i) and (ii).

These criteria were applied to the results for each embryo individually. An example is shown in Fig. 1. The broken lines indicate the absolute mitotic index (MI) data from the cell counts, whilst the solid lines represent the data which had been "smoothed" to remove high frequency oscillations. The peaks identified by these criteria are numbered, and are traversed by the oblique least-squares regression line. Peaks 4 and 5 contain subpeaks. Peak 4 includes a total of four "smoothed" values lying on the regression line; values 'a' are included within subpeak 4(i) due to criterion (i), whilst 'b' link subpeaks 4(i) and 4(ii) due to criteria (i) and (ii). Peak 5 also includes four "smoothed" values lying on or just below the regression line. Values 'c' lie on the regression line and link subpeaks 5(i) and 5(ii) by criterion (i); values 'd' lie below the regression line but they combine peaks 5(ii) and 5(iii) as they only represent a distance of 10  $\mu$ m and are thus included in criterion (ii). No values in this example were eliminated by criterion (iii).

## RESULTS

### *Scanning electron microscopy*

It was necessary to bisect sagittally the embryos once neural tube closure had begun in order to observe the sulci on the luminal surface of the neural epithelium;

the bisection was unnecessary in the 4-somite-stage embryos, but it was done for consistency within the study.

*4-Somite-stage embryos (Fig. 2A)*

The most prominent feature of the cranial neural folds is the preotic sulcus. Anterior (rostral) to the preotic sulcus the neural folds are smooth and reveal no indentations or sulci. Posterior to the preotic sulcus there was another main sulcus and a further sulcus was beginning to appear close to the level of the first somite. The sulci at this stage of development were much easier to observe in dorsal views of the embryo than in the sagittally bisected halves. The distance of the sulci from the top of the embryo were measured along the same axis as the serial transverse sections were obtained, in order for comparisons to be made. The measurements showed quite a large degree of variation between the embryos; the preotic sulcus

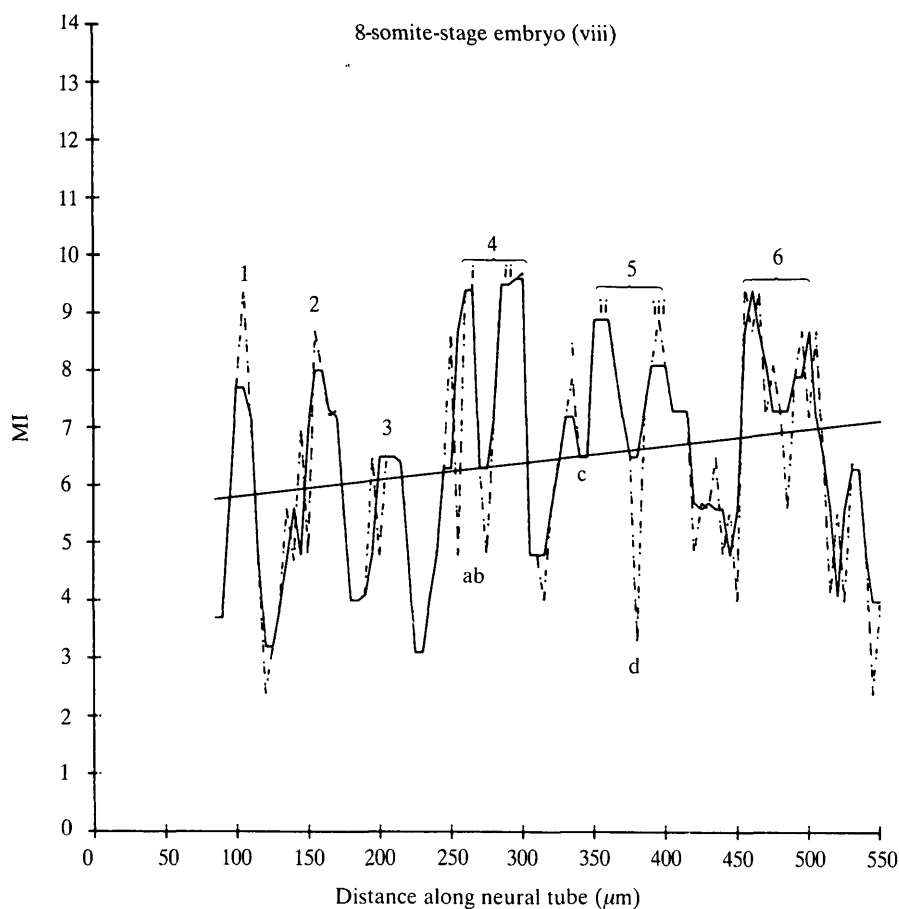


Fig. 1. An example of the computer output following the analysis. A full explanation of the figure is given in the text.

occurred at a distance between 150 and 200  $\mu\text{m}$ , and the second and third sulci occurred at a further 20–60  $\mu\text{m}$  along the neural tube.

#### *8-Somite-stage embryos (Fig. 2B)*

By the 8-somite stage of development the spinal and myelencephalic neural folds have begun to fuse and sagittal bisection was necessary to view the surface sulci. The preotic sulcus was no longer a deep and prominent sulcus. Around the optic vesicle, and more prominently just posterior (caudal) to it, there was an indentation of the epithelial surface; this may have been a prosomere but more likely was associated with the outer edge of the optic vesicle. The mesencephalon contained one prominent sulcus which was elongate in a rostrocaudal direction and spanned the width of the neural tube. Within the base of the mesencephalic sulcus a gyrus was beginning to form (not visible in all embryos) and this was apparently subdividing the sulcus into two smaller sulci. Either side of the preotic sulcus there were two smaller sulci, and in the myelencephalon there were two more round indentations on the epithelial surface. The preotic sulcus was found at 120–150  $\mu\text{m}$  down the neural tube, the two minor sulci occurring 10–30  $\mu\text{m}$  either side of the preotic sulcus. The two myelencephalic indentations occurred approximately 200 and 250  $\mu\text{m}$  along the axis of measurement. The decreased distance measured to the preotic sulcus compared with the 4-somite-stage embryos was presumably due to the developing cranial flexure, some of the 'length' of the embryonic axis having become incorporated into the flexure.

#### *12-Somite-stage embryos (Fig. 2C)*

By the 12-somite stage, the second zone of neural fold fusion had developed between the midbrain and forebrain; the forebrain had continued to enlarge together with the degree of flexion. The degree of flexing that had occurred between the 8- and 12-somite stages was nowhere near as great as between the 4- and 8-somite stages; this was reflected by the preotic sulcus lying at a similar level to that of the 8-somite stage embryos, i.e. at approximately 120–140  $\mu\text{m}$  along the axis of measurement. The large elongate mesencephalic sulcus had fully subdivided into two separate sulci. The sulcus immediately anterior to the preotic sulcus was not always visible; its disappearance may be as a result of its inclusion in the lengthening of the second mesencephalic sulcus due to the fluid nature of the epithelium in this region (Tuckett & Morriss-Kay, 1985a). The preotic sulcus was no longer a sharp indentation on the epithelial surface and its appearance would suggest that regression had occurred when compared with 4- and 8-somite-stage embryos. Posterior to the preotic sulcus four new sulci had developed; three of these sulci lying anterior to the two myelencephalic sulci of the 8-somite-stage embryo, and the fourth new sulcus appearing posteriorly close to the level of the first somite. The actual position of these sulci was highly variable between embryos although the order of their development was consistent.

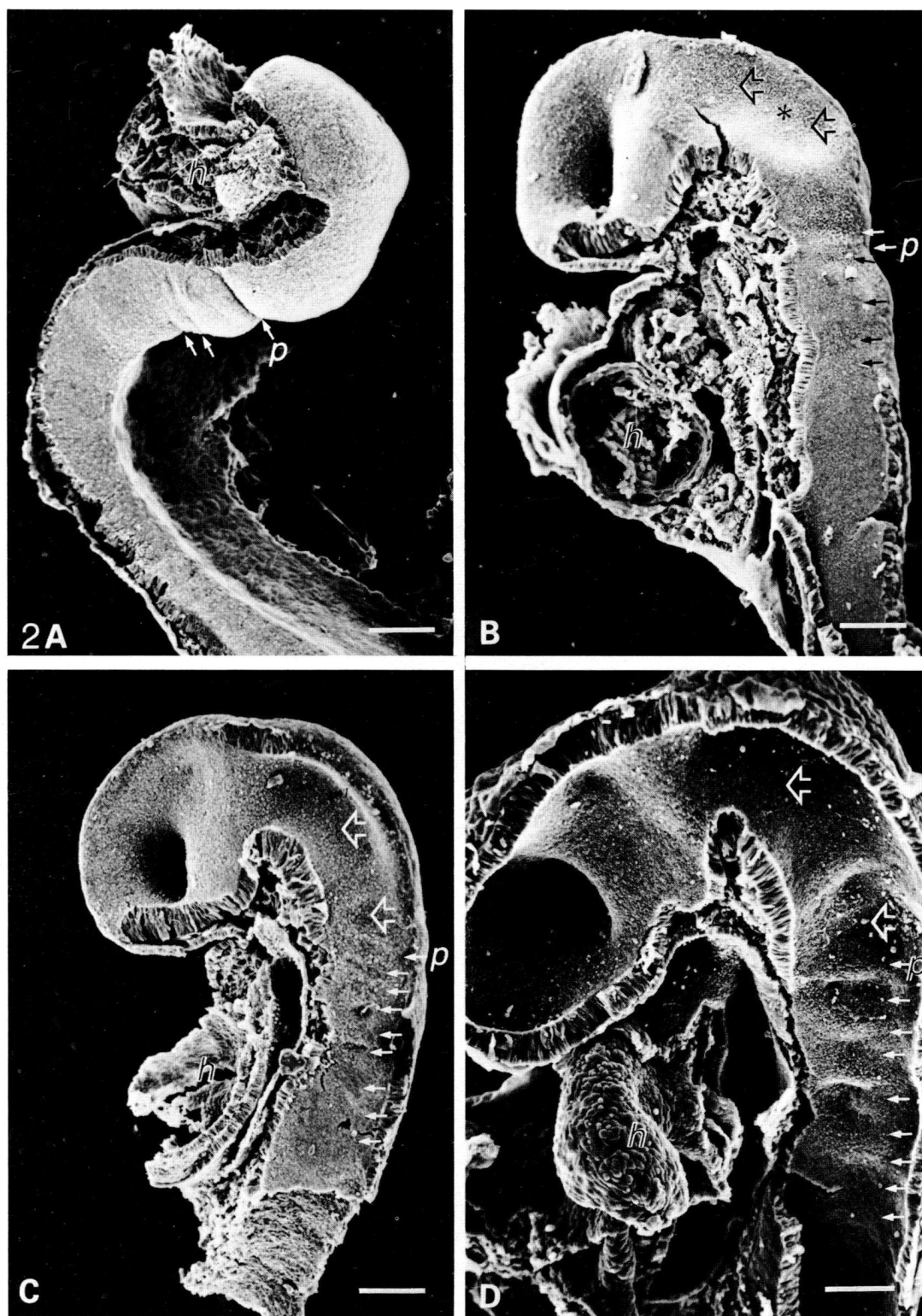


Fig. 2

Table 1. *Summary of the ontogenesis of cranial neuromeres*

Somite Stage	Brain Region			
	Mesencephalon	Rostral metencephalon	POS	Caudal metencephalon + myelencephalon
4				( )
8	( )			
12		( )*	( )*	
16			( )*	

(|) = indefinite sulcus, appearing or regressing\*  
POS = preotic sulcus

*16-Somite-stage embryos (Fig. 2D)*

By the 16-somite stage, the neural folds had fused continuously between the forebrain and the spinal regions. The forebrain had continued to elongate in the transverse plane; the degree of flexion had remained almost the same as in the 12-somite-stage embryos, and was reflected by the preotic sulcus lying at approximately 150  $\mu\text{m}$  along the axis of measurement. The preotic sulcus had continued to regress and its appearance was now no different from the other hind-brain sulci.

Comparing the number of sulci and their position along the embryonic axis in 12- and 16-somite-stage embryos, there was no difference in the total number of sulci nor in their distribution within each brain compartment. The actual position of the sulci along the axis of measurement was variable, both between the two different somite stages and within each somite stage.

Table 1 summarizes the distribution of sulci within the different brain regions.

Fig. 2. Scanning electron micrographs of sagittally bisected rat embryos, showing the apical surface of the neural epithelium at four stages of neurulation. *p*, preotic sulcus; *h*, heart. Scale bars represent 40  $\mu\text{m}$ .

(A) 4-somite-stage embryo. The preotic sulcus is clearly visible, together with two neuromeres lying immediately caudal to it (arrowed).

(B) 8-somite-stage embryo. The elongate mesencephalic sulcus is marked by the open arrows, the gyrus at its centre is marked by the asterisk. The neuromeres either side of the preotic sulcus are indicated by the small closed arrows.

(C) 12-somite-stage embryo. The elongate mesencephalic sulcus has been fully subdivided by the gyrus to form two mesomeres (open arrows). The preotic sulcus has continued to regress. The sulcus immediately rostral to the preotic sulcus has been incorporated in the second mesomere. The sulci of the hindbrain are indicated by the closed arrows.

(D) 16-somite-stage embryo. The distribution of the sulci is similar to that in the 12-somite-stage embryo. The open arrows indicate the mesomeres, and the closed arrows incide the rhombomeres.

*Light microscopy: mitotic index data*

Examination of the serial transverse sections revealed a variation in the level of the mitotic index between adjacent sections; a series of peaks in mitotic activity was observed when the MI was plotted against the distance down the neural tube; the amplitude of each peak varied between the embryos of the same somite stage and there was no set periodicity. It was not possible to cumulate the data obtained from embryos within the same age group as there was a variation in the size of each peak and in the distance to the level of the first somite, thus any analysis of the data had to consider each embryo individually.

Tables 2–5 contain the peak data obtained after imposing the criteria described in the materials and methods. For each peak two values are given: the first value corresponds to the distance in  $\mu\text{m}$  along the neural tube, from the first cutting of the prosencephalon, at which the peak is defined to begin; and the second value corresponds to the last peak value before the curve drops below the regression line.

Table 2. *4-Somite-stage embryos mitotic index peak data. Peak range values in  $\mu\text{m}$  along the neural tube.*

Embryo.					
(i)	185–195				
(ii)	35– 55	175–225	245–265	335–345	
(iii)	30– 50	150–165	250–300		
(iv)	45– 70	90–110	130–140	240–280	
(v)	35– 60	140–160	190–200	255–275	315–325
(vi)	50– 60	85–120			
(vii)	85–130	155–170	210–230	255–265	
(viii)	110–125	150–160	185–195	245–255	300–315
(ix)	60– 80	215–225	265–305		
(x)	125–135	160–220	290–305		

Table 3. *8-Somite-stage embryos mitotic index peak data. Peak range values in  $\mu\text{m}$  along the neural tube.*

Embryo.								
(i)	50– 70	130–150	185–205	350–380	440–495			
(ii)	55– 95	150–160	325–340	385–460				
(iii)	30– 70	120–140	280–300	365–385	450–465	510–520		
(iv)	20– 35	110–140	160–170	195–205	260–275	295–315	445–465	515–535
(v)	105–115	150–190	275–305	325–340	535–545			
(vi)	35– 50	85– 95	115–140	210–245	265–300	375–440	480–490	
(vii)	20– 35	160–170	215–385	475–485				
(viii)	100–110	150–170	200–215	255–300	330–415	455–500		
(ix)	100–130	290–420	490–520	550–570				
(x)	70–100	270–320	355–380	400–410	435–445	495–550	575–590	





#### *4-Somite-stage embryos*

Embryos (i) and (vi) were expected to have fewer MI peaks than the other 4-somite-stage embryos as the data for those were not complete. In general there was a peak at the cranial end of the neural tube, and each embryo had a peak which occurred in a position similar to that occupied by the preotic sulcus. There was no consistent number of peaks present; the mean number of peaks/embryo was three.

#### *8-Somite stage embryos*

In the 8-somite-stage embryos the number of peaks ranged from four to eight. In general there was a peak at the cranial end of the neural tube, and a second or third peak occurring at a distance along the neural tube similar to the distance for the preotic sulcus. The position of the remaining peaks varied greatly between the embryos. The mean number of peaks/embryo was six.

#### *12-Somite-stage embryos*

The number of peaks/embryo in the 12-somite-stage embryos varied from five to twelve, with a mean of eight. The increase in number of peaks observed compared with the 8-somite-stage embryos was, in general, in the "extra" 200  $\mu\text{m}$  along the neural tube to the level of the first somite. It was difficult to find any patterning in the development of these extra peaks.

#### *16-Somite-stage embryos*

In the 16-somite-stage embryos the number of peaks ranged from six to ten with a mean of seven. The average number of peaks was one less than in the 12-somite-stage embryos; the significance of this is uncertain but a possible explanation is discussed later.

### DISCUSSION

Observation of the appearance of the sulci from the SEMs in this study has shown that they do not appear in an ordered rostrocaudal or caudorostral sequence along the neural tube, although the development of each sulcus occurred in a strict temporal sequence (Table 1). No waves of segmentation were observed during the period of cranial neurulation but this does not rule out the possibility of waves of segmentation occurring at later stages. These findings differ from those of Kallen & Lindskog (1953) and Bergquist & Kallen (1954) who identified in a variety of vertebrates, three different waves of segmentation within the cranial neural tube, each wave being associated with the development and disappearance of encephalomeres (which they called proneuromeres, neuromeres and post-neuromeres). Thus the encephalomeres were found to form three times during brain morphogenesis, each time in a rostrocaudal direction, and similarly disappear three times but in a caudorostral direction. In the developing rat brain, a simple

rostrocaudal sequence of development takes place in the differentiation of the neural plate, beginning with the shield-shaped area which subsequently forms the midbrain and forebrain regions. But in other respects early brain development in the rat deviates from the simple rostrocaudal pattern of non-mammalian vertebrates: neural tube closure begins in the myelencephalon in a caudorostral direction, followed by apposition and fusion at the midbrain-forebrain junction, then separate closure of the forebrain and midbrain/upper-hindbrain neuropores (Morris & New, 1979). Neural crest emigration begins in the midbrain, followed by the postotic region, followed by the preotic metencephalon (Tan & Morris-Kay, 1985). The order of cranial neural fold fusion, cranial neural crest cell emigration, and cranial neuromere formation thus all differ from each other in the rat, having in common only the absence of a simple longitudinal sequence.

Vaage (1969) proposed that new neuromeres form by successive subdivision of the first-formed neuromeres. In the chick embryo the final arrangement of neuromeres was found to be eight prosomeres, two mesomeres and eight rhombomeres; some of these were found to develop into the permanent intracranial sulci. Our results show that in the rat embryo, the two mesomeres form by subdivision of the elongate mesencephalic sulcus, but the rhombomeres appear as individual entities and not from the subdivision of previously formed sulci.

There was no evidence of the mesomeres disappearing during neurulation as described by Adelmann (1925). At the end of cranial neurulation it was possible to identify two mesomeres and nine neuromeres including the preotic sulcus. There was no evidence of prosomeres apart from the indentation in the forebrain epithelium immediately posterior to the optic vesicle in the 8-somite-stage embryos.

Accurate correlation of the preotic sulcus with the position of the future brain vesicles is important, since it provides such a clear indication of position in early-neural-fold-stage embryos. Meier & Tam (1982) assigned it as the division between metencephalon and myelencephalon; this is clearly incorrect since the otic region caudal to it becomes part of the metencephalon of the adult brain. Adelmann (1925) referred to it as the third rhombomere. We have only observed one sulcus between the mesencephalic sulci and the preotic sulcus, and therefore designate it as the second rhombomere, even though the first rhombomere is indefinite towards the end of cranial neurulation.

It has previously been suggested that neuromeres are centres of higher mitotic activity within the neural epithelium (Bartelmez, 1923; Bartelmez & Evans, 1926; Kallen, 1953, 1961, 1962) every centre producing an external swelling of the embryo either side of the region of fusion, and a depression or groove in the plane of the ventricular (luminal) neural epithelium. This study has shown clearly the difficulty presented in trying to correlate the mitotic index data with the position of surface sulci (neuromeres). One of the major difficulties arises from the fact that each somite stage lasts for approximately 2 h and thus there is some variation in the stage of development of the neural epithelium of embryos within the same somite

group. A second major difficulty encountered is associated with defining the axis of measurement, especially in the light microscopy part of the study; any variation in the plane of cutting is reflected by a variation in the number of sections cut to the level of the first somite. Because of these problems it was not possible to correlate directly each sulcus as identified by SEM with a peak in mitotic index. But it is also possible that each MI peak is correlated not with neuromeres *per se*, but with their formation, so that once a neuromere is fully expressed morphologically, the differences are no longer necessary and the MI may return to the basal level of its neighbouring cells. This would explain why the mean number of peaks is less than the number of sulci after the 4-somite stage of development, and why the 16-somite-stage embryo has fewer peaks than the 12-somite-stage embryo even though they have the same number of neuromeres. According to Adelmann (1925) during ontogenesis neuromeres may 'disappear'; this disappearance may be preceded by a reduction in mitotic activity which is recognized here by the loss of a MI peak.

The mitotic index is evaluated in this study on the basis of observed numbers of mitotic figures, a method which would not reveal any variation in the duration of individual phases of the cell cycle along the embryonic axis. The observed mitotic index variation is probably associated with an alteration of the duration of specific phases within the cell cycle itself, since we have shown previously (Tuckett & Morriss-Kay, 1985a) that there is no significant variation in the cell cycle time within and between the three brain regions during cranial neurulation.

Neuromeres are the first sign of morphological segmentation within the developing nervous system. Segmentation of the cranial primary mesenchyme into somitomeres has been described in chick embryos (Anderson & Meier, 1981) and in mouse embryos (Meier & Tam, 1982), and has been shown by them to precede the segmentation of the neural epithelium. The direct one-to-one relationship between cranial neuromeres and somitomeres is only transitory. In the spinal region, neuromeres are directly related to the somitic segmentation of the paraxial mesoderm, each neuromere being situated at the level of the intersomitic cleft.

Previous studies in this laboratory have shown that cell number is very strictly controlled in relation to pattern formation in the cranial neural epithelium during neurulation. Between the 4-somite and 8-somite stages the forebrain expands 20 fold in volume, apparently as a result of cells from the midbrain flowing rostrad into it to supplement its intrinsic cell division (Tuckett & Morriss-Kay, 1985a). This process is essentially complete by the 8-somite stage, correlating with the appearance of the first neuromeric sulci within the apparently fluid region rostral to the preotic sulcus (study of embryos of a greater variety of stages than reported here has shown that the first mesencephalic sulcus and the most rostral metencephalic sulcus first appear at the late 7- to early 8-somite stage). We have proposed that the preotic sulcus represents a barrier to caudal movement of the fluid region (Tuckett & Morriss-Kay, 1985a). Taken together, the observations from the previous and present studies suggest that neuromeres form in the midbrain/upper hindbrain

region only after this region has stabilized, i.e. is no longer a fluid sheet flowing towards and into the forebrain. Loss of definition of the preotic sulcus after the 8-somite stage is compatible with this interpretation. Formation of neuromeres caudal to the preotic sulcus may also represent a stabilization process.

We propose that neuromeres represent a partitioning process within the developing central nervous system which is an essential stage in pattern formation. It is possible that they represent compartments between which communication is limited, and within which subsequent development is in some ways autonomous. This hypothesis is discussed in more detail in relation to fine structural aspects of neuromere construction and maintenance in a subsequent report (Tuckett & Morriss-Kay, 1985b).

Thanks are due to Mr M. Barker for technical assistance. This study was supported by the Medical Research Council and the University of Oxford.

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(Accepted 15 January 1985)