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ORIGINAL ARTICLE

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Morphogenetic movements during cranial neural tube closure in the chick embryo and the effect of homocysteine

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Abstract In order to unravel morphogenetic mechanisms involved in neural tube closure, critical cell movements that are fundamental to remodelling of the cranial neural tube in the chick embryo were studied in vitro by quantitative time-lapse video microscopy. Two main directions of movements were observed. The earliest was directed medially; these cells invaginated into a median groove and were the main contributors to the initial neural tube closure. Once the median groove was completed, cells changed direction and moved anteriorly to contribute to the anterior neural plate and head fold. This plate developed into the anterior neuropore, which started to close from the 4-somite stage onwards by convergence of its neural folds. Posteriorly, from the initial closure site onwards, the posterior neuropore started to close almost instantaneously by convergence of its neural folds. Homocysteine is adversely involved in human neural tube closure defects. After application of a single dose of homocysteine to chick embryos, a closure delay at the initial closure site and at the neuropores, flattening of the head fold and neural tube, and a halt of cell movements was seen. A possible interference of Hcy with actin microfilaments is discussed.

Keywords Chick · Neurulation · Neuropores · Cell movements · Homocysteine

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Introduction

Neurulation involves the formation of a flat neural plate and its subsequent transformation into a hollow neural tube. This process is complex and regulated by many genetic and environmental factors. The high frequency of closure defects in human embryos (Nakatsu et al. 2000) indicates the vulnerability of this process, and has triggered numerous investigations into the fundamental aspects of neurulation (reviewed in Gordon 1985; Sadler 1998; Colas and Schoenwolf 2001; Copp et al. 2003).

In general terms, one of the steps in the process of neurulation involves the formation of a ventral furrow in the neural plate, which appears as a median hinge point (MHP) in a transverse section. This hinge point likely facilitates elevation of the neural walls, to form a neural groove. The dorsal part of the neural walls, the neural folds, bend inwards during convergence. The bending sites are referred to as dorsolateral hinge points (DLHPs). The converging neural folds subsequently fuse to form a closed neural tube. Neurulation commences independently at several points along the neuraxis. Finally, the remaining apertures, the neuropores, come to closure.

However, variations to this general pattern exist along the neuraxis and between various species. In the mouse embryo, closure initiates at the cervical level (referred to as closure 1), and subsequently three distinct Modes of spinal closure, depending on the presence or absence of MHP and DLHPs, occur along the cranio-caudal axis (Fig. 1, Modes 1–3); these Modes occur successively in time and in cranio-caudal order (Shum and Copp 1996). In the cranial region, closure 2 commences at the pros-mesencephalic boundary and closure 3 at the rostral site (Juriloff et al. 1991). Progression of closure from these sites onwards occurs like Mode 2.

Closure in the spinal region in the chick embryo essentially follows the pattern of closure in the mouse, but with several species-specific variations (van Straaten et al. 1993, 1996; Colas and Schoenwolf 2001). For instance, in contrast with the mouse, in the cranial

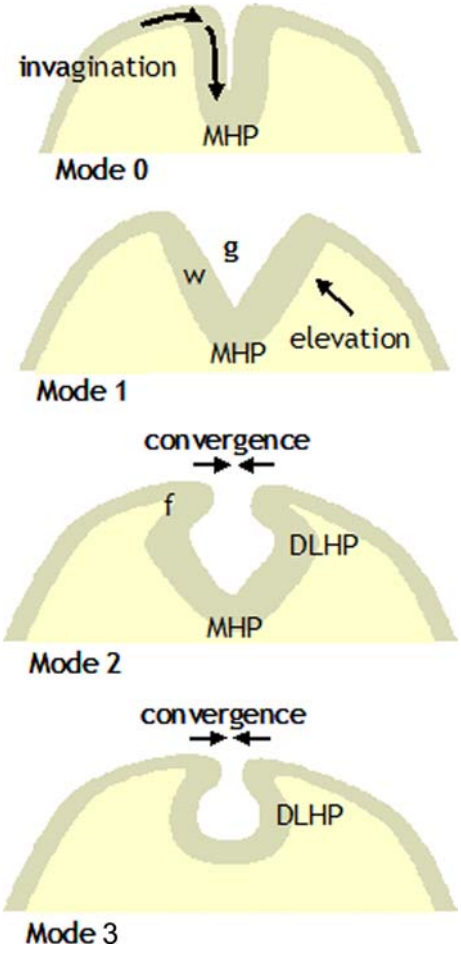

Fig. 1 Modes of neurulation as presented in transverse sections. Mode 0: initial closure at the mesencephalic level in the chick embryo. Neuroepithelial cells move medially and invaginate. Modes 1–3: spinal neurulation in the mouse embryo. Mode 1: initial closure at the upper cervical level and subsequent progression of closure in caudal direction are characterized by the presence of a MHP, but a lack of DLHPs. Mode 2: both MHP and DLHPs are present during subsequent closure. Mode 3: the DLHP only is present during the final closure phase. The transition from Mode 2 to Mode 3 is gradual. *g* neural groove, *w* neural wall, *f* neural fold. Mode 0 after van Straaten et al. 1997; Modes 1–3 after Shum et al. 1996

region of the chick embryo only one initial closure site is recognized, which is at the level of the prospective mesencephalon. Moreover, this appears the first site to close, and the cervical region being the second.

The initial mesencephalic closure in the chick embryo as well as the subsequent progression of closure have been described to occur according to Mode 2, thus by MHP formation, DLHP formation and neural fold convergence (Bancroft and Bellairs 1975; Schoenwolf 1982; Nagele and Lee 1987). However, by studying neurulation in chick embryos under the least disturbing conditions possible, using *in ovo* imaging and transverse sections, we showed a new and unique mechanism for the initial phase of neural tube closure (van Straaten et al. 1997): instead of neural wall elevation, the neural groove arises by invagination. While this groove deepens, its walls remain apposed over their ventro–dorsal extension. Then, beginning ventrally, the walls gradually separate in dorsal direction. Finally, only the most dorsal part, which develops into neural folds, remains apposed and here adhesion and fusion occur, while the deeper part of the groove widens to form the neural tube lumen. We will use the term "Mode 0" for this specific closure process (Fig. 1).

In the above study, it was suggested that the invagination was driven by surface neuroepithelial cells, which move medially, topple over the edge of the groove and contribute to the height of its walls. Movies focusing on the dorsal cells strengthened the suggestion that dorsal cells indeed moved towards the neural groove (van Straaten et al. 2002). In the current paper, we will delineate critical cell movement vectors that are fundamental to the morphogenetic remodelling of the neural tube. We will therefore mark individual surface cells and quantify the rate and direction of the cellular movements. We will use these data to specifically address the question, to what extent the medial cell movements contribute to the deepening of the median neural groove. Since the cell movements appear not to halt after the groove is formed, but merely change direction in rostral direction, we also address the question to what extent these rostrally directed cells contribute to the development of the anterior neural plate. Finally, the morphogenetic movements of neural walls and folds during closure of the anterior and posterior neuropore are determined.

In our studies on neurulation mechanisms, the role of vitamins in neural tube closure is the subject of interest. It is now generally accepted that the occurrence and recurrence rate of neural tube defects (NTDs) can be reduced when folic acid is supplemented periconceptionally (MRC-Vitamin-Study-Research-Group 1991; Czeizel and Dudas 1992). When intracellular 5-methyltetrahydrofolate levels are reduced, e.g. by reduced availability of methylenetetrahydrofolate reductase (MTHFR) (van der Put et al. 1997), this results in a reduced remethylation of homocysteine (Hcy) to methionine with a subsequent rise in homocysteine levels. This fits the finding that in mothers of children with NTD, elevated plasma Hcy levels were detected (Steegers-Theunissen et al. 1994; Van der Put et al. 1997); it was suggested that these levels are teratogenic to the embryo. The effect of Hcy was tested on various mammals but, apart from many developmental disturbances, neural tube closure defects were not found (Van Aerts et al. 1994; Kubova et al. 1995; Hansen et al. 2001; Greene et al. 2003). On the contrary, administration of Hcy to chick embryos *in ovo* did result in an increased frequency of NTDs (Rosenquist et al. 1996; Epeldegui et al. 2002). We studied this effect in more detail and in narrow time windows of defined developmental stages in an in vitro chick embryo model. We found that Hcy induced a transient, dose-dependent widening of the anterior neuropore at the 4 and 5 somite stages, resulting in a temporary delay of neural tube closure (Afman

et al. 2003). Here we show that the divergence and closure delay also occurs at the initial closure site and at the posterior neuropore. Moreover, the head fold and neural tube flatten, while cell movements come to a temporary halt. The possibility that Hcy disturbs neurulation by interfering actin microfilament functioning via methylation is discussed.

Material and methods

Preparation of chick embryos

Eggs of white leghorn chickens were incubated at 39°C and 55% humidity in a roller incubator for ~30 h. The eggs were gently cracked in a bowl. The embryonic disc was dissected free of egg white and was centred within a filter paper ring (Chapman et al. 2001). The embryo, including the overlying vitelline membrane and paper ring, was dissected and placed in a 35 mm Petri culture dish on an agar:egg white (1:1) substrate. Embryos ranging from Hamburger and Hamilton stages 6 to 8+ (Hamburger and Hamilton 1951), corresponding to 0–5 somites, were used.

Time-lapse video microscopy and homocysteine application

Time-lapse video microscopy of the dorsal cell layer in the cranial region was performed as follows. The culture dish, covered by the lid, was placed between two thermo-stages on a Leica MZFLIII stereomicroscope. The lower plate was set at 39°C; the upper plate was set at 45°C to prevent condensation. Digital recordings were made with a computer-linked 3CCD camera, using an interval period of 2 min between frames. With the objective magnifications used (3,2× to 8×), the size of one pixel corresponded to 4.8 to 1.9 μm. After an adaptation period of ~15 min, the upper thermo-stage and the dish lid were removed. One droplet of 40 μl of saline (control) or 10 or 30 mM L- homocysteine-thiolactone (Hcy) (Sigma Aldrich, Steinheim, Germany), dissolved in saline and adjusted to a pH of 7.4, was applied on top of the vitelline membrane, in the centre of the paper ring, after which the lid and thermo-stage were replaced. This operation occurred within one frame interval. Images were collected for an additional 2–3 h and merged into a movie. During that period, the embryo acquired ~2 additional somites since the formation of a somite in the chick embryo requires ~100 min (van Straaten et al. 1996). For presentation in this paper, movies were reduced in length to show essential events only and they were reduced in size; scale factors as indicated above and in figure legends are adapted to these reduced sizes.

In about half of the cases, a transient slight contraction of the embryo occurred after the application of the droplet, both in saline and Hcy-treated embryos. This is illustrated in the Movies 7–10, where the application of the droplet is within the presented frames: in the former two, no contraction is seen; while in the latter two, a transient effect occurs.

Selection criteria for movies

Movies were considered informative when the embryos met the following criteria. (1) The embryo should not float outside the frame window during the video session. (2) The dorsal side of the embryo should remain in focus throughout the video session. (3) The embryo should remain viable and should not deform throughout the session. (4) The embryo should show at least one of the morphogenetic processes of interest with sufficient quality in order to describe or measure that process. Especially due to the last criterium, the number of animals reported for each process varied.

Thirty two out of 51 movies (63%) with saline-treated embryos were informative, and 22 out of 36 movies (61%) with Hcy-treated embryo were informative. Of the latter, 7 embryos received 10 mM Hcy and 15 received 30 mM. Both groups were used for morphological descriptions, but only the 30 mM group had sufficient statistical power to be used for quantitative purposes.

Determination of velocity of cell movements

Cell movements at the level of the initial closure site, both in medial and anterior direction were determined measuring the movement of individual cells (Fig. 2). These cells had to meet two requirements: (1) they should remain recognizable in a continuous series of frames during at least 30 min, (2) they should stay in pace with neighboring cells. Per movie, 2 to 8 cells were selected, depending on the number of suitable cells. Only movies with sufficient resolution, i.e. magnifications corresponding to 1.9 or 2.4 μm per pixel, were suitable for this purpose. The cells were digitally marked with a dot on individual frames. For convenience, in some cases, an outstanding particle which accompanied a moving cell served this purpose. The movement of these dots with respect to reference points (Fig. 2) was measured, using a Leica Quantimet Image Analysis System with Qwin 500 software. Measurements were performed on individual frames with an interval of 10 min, within the period of 1 h after application of saline or Hcy. The average velocity and standard deviation (SD) was calculated and expressed in μm/h.

Apart from these cell movements, anterior movement of the rostrum proper, anterior movement of the (anterior point of the) initial closure, narrowing of the posterior neuropore and narrowing of the of the anterior neuropore were also determined (Fig. 2). Most morphogenetic processes measured lasted a few somite stages only; therefore, specific measurements were

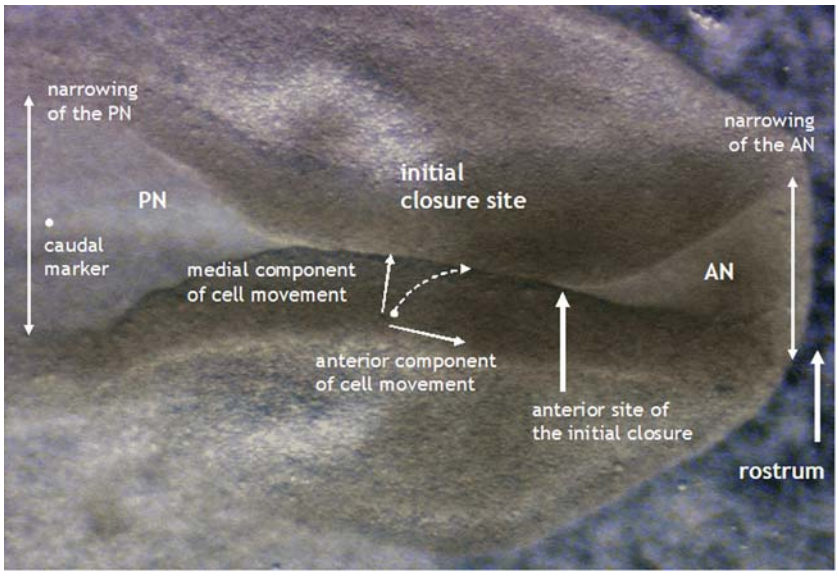

Fig. 2 Marker points and distances used in this study. Dorsal view of the cranial region of a 3-somite chick embryo. Neural walls and folds are recognizable as dark bands. At the initial closure site, the dashed arrow indicates the coarse of a particular cell, while the associated arrows indicate the two movement vectors of that cell. As a reference for the medial movement of the cell, the medial border of the neural wall was chosen. As a reference point for the movement in anterior direction the caudal marker was used. The anterior shift of the anterior site of the initial closure and of the rostrum proper were also related to the caudal marker. The narrowing of the PN and AN were determined at the inner linings of the neural walls. From these movements, velocities were calculated. *AN* anterior neuropore; *PN* posterior neuropore.

performed on embryos at specific developmental stages only (see Table 2, Fig. 6).

Results

During development of the cranial neural tube in the chick embryo, we distinguished various morphogenetic processes as indicated in Table 1. These included dorsal cells moving in medial direction during initial closure, anterior cell movements resulting in rostrum extension, anterior neuropore (AN) formation and closure and narrowing and closure of the posterior neuropore (PN). These processes will be separately described, mostly qualitatively, in the following section. Next, these processes were quantified to provide reference data for the effects of Hcy on these processes.

Medial cell movements during initial closure

At the stages of 0–2 somites in the cranial region of the chick embryo, dorsal neuroepithelial cells moved in medial direction (Movies 1, 2; Fig. 3). These cells subsequently rolled into, and deepened the median furrow of the neural plate into a groove at the future mesencephalic level, determining the initial phase of the closure of the neural tube. We designated this specific closure mechanism as Mode 0 (Fig. 1).

Remarkably, in 16% of saline-treated embryos between 0 and 2 somites, this process reversed, resulting in unrolling of the initial neural groove (Movie 3). Either by such unrolling, or by widening of the initial neural groove (in Hcy treated embryos, see below), the groove was reshaped into the original, relatively flat neural

Table 1 Occurrence of various anterior morphogenetic processes in control and Hcy-treated chick embryos

		Medial cell movement		Anterior cell movement		AN formation		AN closure		PN narrowing		PN closure	
Somite range		0–2		0–3		0–3		3–5		0–5		3–5	
Application		Saline	Hcy	Saline	Hcy	Saline	Hcy	Saline	Hcy	Saline	Hcy	Saline	Hcy
Process	Present	10	1	15	2	16	2	11	0	27	5	13	5
	Absent	4	6	7	11	7	9	0	10	2	5	0	3
	Reversed	3	0	0	0	0	2	2	4	1	12	0	6
Total		17	7	22	13	23	13	13	14	30	22	13	14

Assessments were made during 1 h after application of saline or Hcy. The somite range during which a given process occurred is indicated. Only those embryos which fitted within that somite range at the beginning of the movie were evaluated. Depending on the relevant somite numbers and the suitability of the movies, the total number of embryos screened per process varied.
AN anterior neuropore; *PN* posterior neuropore

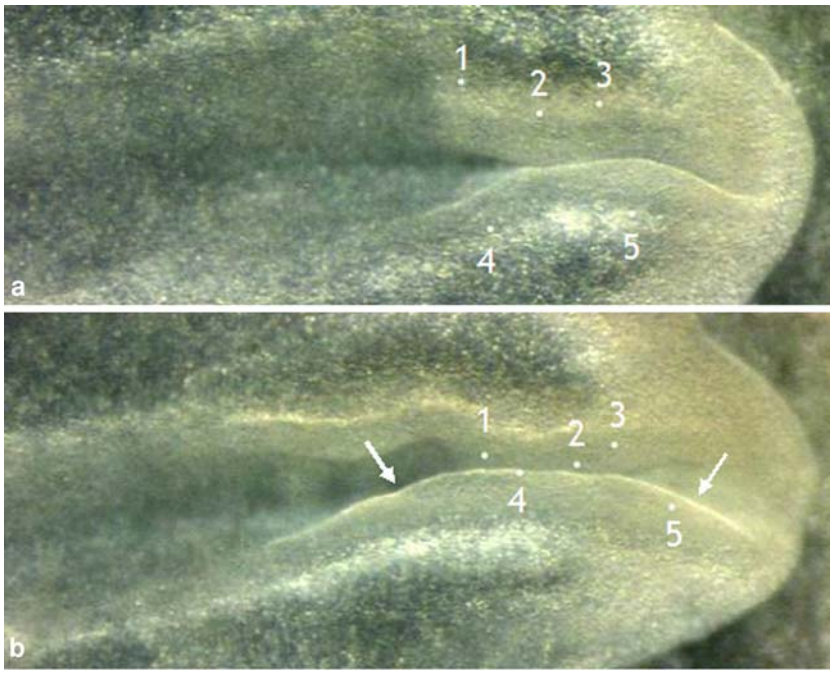

Fig. 3 Relative movement of five marked cells in two successive stages in a 2-somite chick embryo. Cells in the region of the initial closure site move medially and anteriorly. The neural walls of the AN (*right arrow*) and the neural folds of the PN (*left arrow*) develop. Frames#1 and#51 of Movie 2; interval 100 min. Frames aligned on a caudal marker

plate. Subsequently (1–2½ h after application of saline or Hcy), Mode 0 did not resume, but closure commenced alternatively, according to Mode 3: from the flattened neural plate neural folds arose bilaterally, which converged, gained momentum and finally approached their counter partner at high velocity (Movie 4). By measuring the decrease in width between both folds, a velocity of 200–300 μm/h was calculated (determined in 4 embryos). The convergent movements lasted 1/2–2½ h, depending on the initial distance between the folds. Thus, while the initial mechanism, Mode 0, appears a unique mechanism, a conventional alternative, Mode 3, appeared when Mode 0 failed.

Anterior cell movements, rostrum extension, anterior neuropore formation and closure

Movement of the dorsal cells gradually changed course from a medial into an anterior direction up to the 3-somite stage (Fig. 2; Movies 2, 5). It appeared that these cells were the chief contributors to the anterior extension of the embryo, since the rostrum proper displaced at an identical rate as the anteriorly moving cells (Table 2). The anterior wave of cells extended the neural plate in anterior direction. Neural walls developed, diverged in anterolateral direction at stages of 2–3 somites and formed the borders of a triangularly shaped AN, which increased in length concomitantly (Movie 5).

Surprisingly, during this AN elongation, the initial closure site moved backwards, or to put it differently, the AN reopened in caudal direction (Movies 2, 5). The relative caudal shift of this site appeared from morphometrical data: while the anterior point of the initial closure site moved anteriorly at a velocity of 20 ± 55 μm/h ($n = 6$) relative to a caudal reference point (Fig. 2), the flanking cells and the rostrum moved anteriorly at a velocity of 49 ± 18 μm/h ($n = 6$). However, this process of AN reopening was not consistent, as indicated by the high standard deviation.

During further development, the AN triangle continued to enlarge and to widen at its anterior pole (Movie 6, Fig. 4) to a maximum at the stage of 4 somites. Once a distinct AN was present, its neural walls elevated and

Table 2 Velocities (mean ± standard deviation, in μm/h) of various anterior morphogenetic movements in control and Hcy-treated embryos

	Medial movement of cells			Anterior movement of cells			Anterior movement of rostrum			PN narrowing		
	n	*m*	SD	*n*	*m*	SD	*n*	*m*	SD	*n*	*m*	SD
Saline	6	50	31	6	49	18	6	51	26	5	122	53
Hcy	6	1	9	5	40	13	5	87	11	5	−34	33

Velocities were determined in 2-somite embryos (PN narrowing) or 1–2 somite embryos (others). The numbers of embryos which were suitable for velocity determination are indicated (*n*). Velocities were determined during 1 h after application of Hcy or saline. Significance of differences between Hcy and saline groups ($P < 0.05$, Student's *t*-test) for all parameters except for anterior movement of cells ($P = 0.19$)
PN posterior neuropore

developed neural folds, which subsequently converged, from the initial closure site onwards, up to final closure of the AN. Meanwhile, the AN enlarged to form the prosencephalon (Movies 6, 7).

Narrowing and closure of the posterior neuropore

At the stage of ~3 somites, neural folds became visible caudally to the initial closure site. These folds subsequently converged (Mode 2 closure), but further caudally the neural walls appeared to be pushed upwards by the developing somites, resulting in apposition of the neural walls without convergence (Mode 1 closure). At this stage, the PN closed fast (Movies 1, 5, 6, 7), which is due to the almost parallel orientation (in longitudinal direction) of its neural walls.

Effects of Hcy on morphogenetic movements

After application of a single dose of Hcy to embryos in culture, many of the above-described processes were disturbed. Effects were seen within 20 min, and mostly lasting for ~1 h, thereafter normal closure was restored, indicating a transient effect of Hcy. In order to quantify the effects of Hcy, the occurrence of the above processes (Table 1) as well as velocities of cells and structures (Table 2) were determined in Hcy-treated embryos and in saline-treated controls during 1 h after application. After Hcy application, the walls of the initial neural groove diverged to varying degrees in half of the cases, resulting in a flattened neural plate.

The medial cell movements halted in most cases (Movies 8, 9), whereas these movements continued in the majority of the saline-treated embryos (Table 1). The average velocity of the medial cell movements indeed amounted ~0 μm/h in Hcy-treated embryos, while this was ~50 μm/h in saline-treated embryos (Table 2).

The rostrum extended at almost double velocity (Table 2) after Hcy application, and the width of the head fold also increased (Movie 9). This circumferential enlargement of the head fold can be explained by dorsoventral flattening of the cranial region, as suggested by Movies 8 and 9.

Like the medial cell movements, the cell movements in anterior direction seemed to halt as well (Movies 8, 9), but when their velocity was measured (relative to a caudal

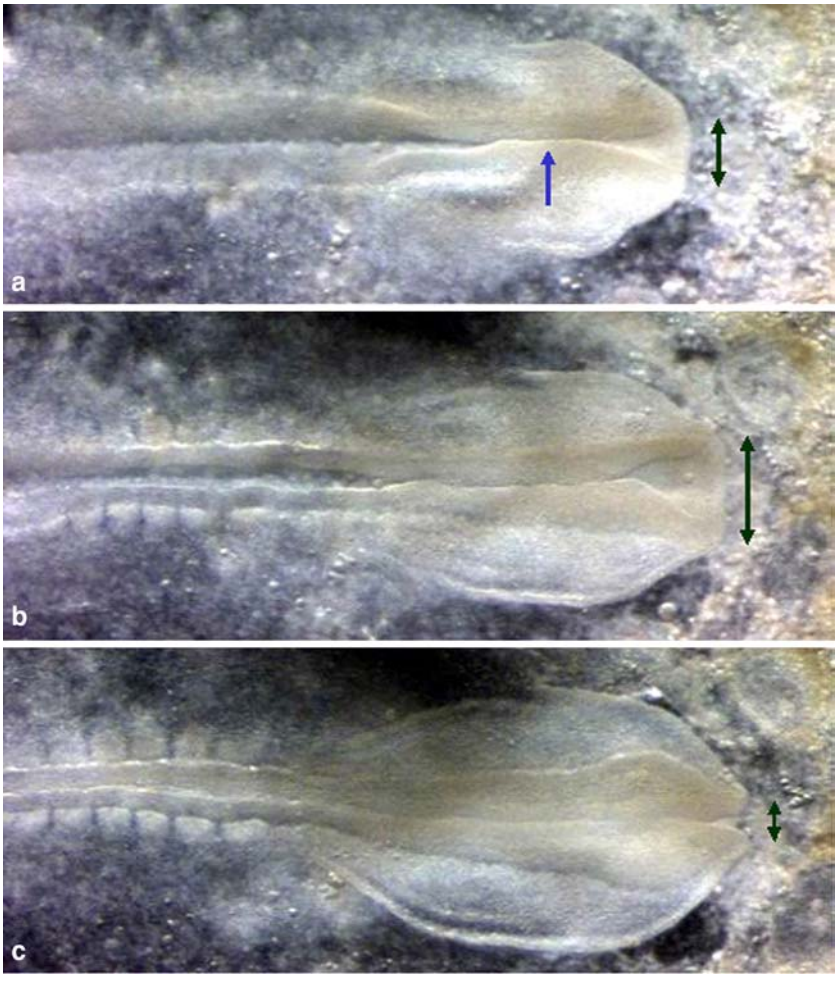

Fig. 4 Neurulation in three successive stages in a 3-somite chick embryo. The AN widens (arrow intercept) with prominent neural walls (figure *a* > *b*) and subsequently closes (*b* > *c*). The neural groove narrows, especially between *a* and *b*. Closure of AN and PN proceeds from the initial closure site (blue arrow) onwards by convergence of neural folds (Mode 2), but in between somites (left half of the images) mostly by apposition of the neural walls (Mode 1). Frames#10,#60 and#120 (20, 120 and 240 min) of Movie 6.

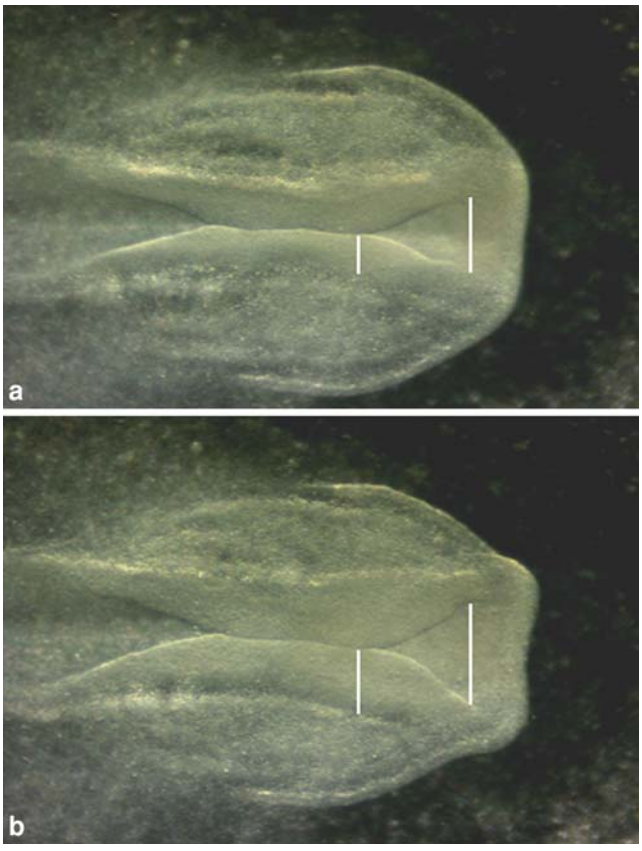

Fig. 5 Effect of Hcy on the cranial region in a 4-somite chick embryo as shown in frame#12 (**a**) and frame#24 (**b**) of Movie 10. Hcy was applied after frame 10. Time interval between frames a and b: 24 min. The AN widens (*right bars*) and the closed part of the neural tube seems to flatten (*left bars*)

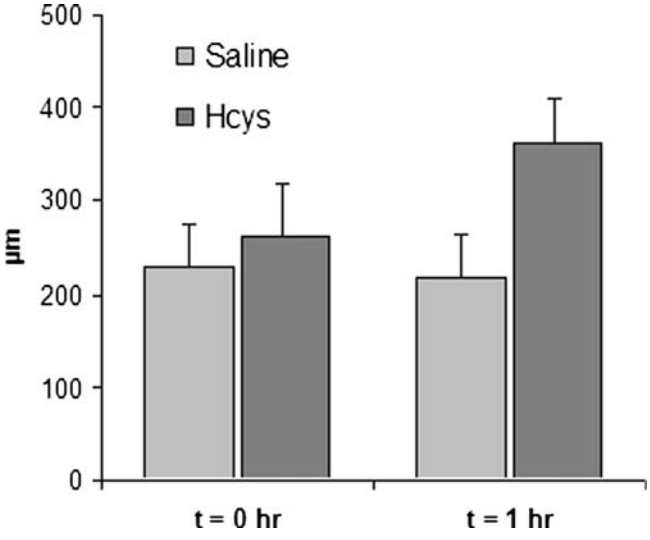

Fig. 6 Width of the anterior neuropore in 4- and 5 somite embryos just before and 1 h after treatment with saline or Hcy. Numbers of embryos used: saline 7, Hcy 5. Significance of difference at $t = 1$ h: $P = 0.008$ (Student's t-test)

reference point), it amounted to ~40 µm/h, which was only slightly less than in the saline-treated group (Table 2). This discrepancy is explained as follows. As a result of the flattening, all cranial structures move into anterolateral directions simultaneously, including the cells at the initial closure site. It is therefore likely that the actual contribution of these cells to the formation of the AN is halted. The flattening effect of Hcy seems to occur in the closed part of the neural tube as well, as seen in Movie 10 and Fig. 5, and occurred rapidly.

After Hcy application, proper AN formation did not occur in most embryos (Movie 9), while it was seen in the majority of the saline-treated embryos (Table 1). The subsequent step in AN closure, elevation and convergence of the neural folds, was absent or reversed into divergence in all cases (Movie 10), while it did occur normally in most saline-treated embryos (Table 1). The divergence resulted into a significantly wider AN (Fig. 6), which increased 1.47-fold during the first hour after Hcy application, while in saline-treated embryos this amounted to 0.94. The slight reduction in the latter is due to the fact that at these developmental stages, the AN is at its widest and just begins to close.

Hcy affected narrowing of the PN especially at the younger stages. In most cases, narrowing did not occur or was reversed into widening (Movie 9), while it did occur in most saline-treated cases (Table 1). Narrowing was quantified at the stage of 2 somites, when this process had a relatively high rate, and amounted to ~120 µm/h in saline-treated embryos, while it was ~30 µm/h in reverse direction in the Hcy-treated embryos (Table 2).

Closure of the PN was halted or reversed in most Hcy-treated embryos (Table 1, Movies 9, 10), while it was seen in all saline-treated embryos. However, resumption of PN closure was among the first processes being restored after Hcy had evoked effects, followed by resumption of most previously described processes.

Discussion

Medial cell movements drive initiation of closure

By following individual cells, we validated the previously postulated (van Straaten et al. 1997) and illustrated (van Straaten et al. 2002) medially directed roll-in movements of dorsal neuroepithelial cells during the process of initial closure at the level of the prospective mesencephalon in the chick embryo. These invaginating cells appeared to be the major contributors to the initial neural groove. Invagination lasted about 5 h (3 somite stages) and once fully developed, the neural groove measured ~300 µm in depth (van Straaten et al. 1997). Cells contributing to the groove moved with a medial velocity of 50 µm/h (this study), which indicates that 250 µm of the groove height is a consequence of invagination. With a cell cycle time of 8–10 h (Smith and Schoenwolf 1987), the remainder of the groove height is likely obtained by proliferation.

The initial closure mechanism is unique but has a backup

The invagination process during initial closure, Mode 0, seems to be a unique mechanism of neurulation that is not observed elsewhere along the neuraxis. Due to the permanent contact of both neural walls during groove formation, the chance of mismatch of the tips of the groove is avoided and correct closure thus warranted. However, in some saline-treated embryos the invagination reversed, and after Hcy treatment the neural groove widened in half of the embryos, both situations resulting in a relatively flat neural plate. In these cases, where Mode 0 failed, an alternative closure process appeared to commence. This involved the development of neural folds, which subsequently converged, according to a Mode 3 closure. It is possible that these convergent forces are identical to those, which do occur normally once the neural groove is fully formed and a lumen arises (van Straaten et al. 2002). In previous *in ovo* studies we did not observe this conversion to Mode 3 in the chick embryo, indicating that it is an artefact, likely due to the experimental condition. However, the fact that the cranial neural plate is able to recourse to another neurulation mechanism, once the Mode 0 mechanism fails, suggests a built-in backup mechanism for initial closure, which ascertains that initiation of neural tube closure is at lowest failure risk possible

Anterior cell movements contribute to the anterior neural plate

The cell movements in the cranial region of the chick embryo did not only have a medial, but also an anterior vector, which gained importance during development. This anterior wave of cells contributed to the developing anterior neuropore and thereby to the head fold.

Cell movements are well known to contribute to tissue remodelling during embryogenesis. For example, the process of convergent extension appears to be a main contributor to the reshaping of an embryo from an ovoid into an elongated discoid shape, as was studied extensively in amphibian embryos (Keller et al. 1992; Davidson and Keller 1999; Wallingford and Harland 2001; Ezin et al. 2003). During convergent extension, a sheet of cells narrows in transverse direction by intercalation in the medial region and consequently elongates in cranio-caudal direction. In the caudal chick embryo, convergent extension occurs as well and results in an extension of at least three times in 24 h (Schoenwolf and Alvarez 1989). Such an elongation was also seen using a dye labelling technique; after local application of dye to the prospective caudal region of the chick embryo, an extended craniocaudal trail was found after 24 h of culture (Lawson et al. 2001). However, in the latter study hardly any spreading of dye was registered when applied to the region of the prospective forebrain or hindbrain, indicating lack of cell movement. This seems to contradict our results, but the discrepancy may be explained as follows: in our movies a group of cells remained together (thus no spreading occurred), indicating that medial intercalation of cells (resulting in convergent extension) did not take place. The movements in medial or anterior directions in our study were detected only because measurements were referred to a ''fixed'' point. Such a reference was not included in Lawson's study.

The movies presented in our study clearly imply that the change in cell movements from medial into anterior direction resulted in anterior extension of the embryo, and that this specifically concerned development of the head fold and neural plate. In the rat embryo, anterior movement during formation of the forebrain has been suggested as well, since forebrain expansion could not be explained by proliferation only (Tuckett and Morriss Kay 1985). We therefore state that the cranial region in the chick embryo also uses the process of cell movements to extend longitudinally.

The anterior neuropore expands at the cost of initial closure site

The anterior neuropore (AN) expanded in anterolateral direction, resulting in a triangular shape. In addition, the AN expanded in caudal direction at the cost of the initial closure site. Although this caudal expansion could not be validated by morphometry in our study, it was seen in most movies, like in Movies 1, 5 and 6. They showed that the neural walls at the initial closure site moved anteriorly, but that the apposition zone rolled backwards, relative to these walls.

How to explain this phenomenon? The anterior neural plate develops into the prosencephalon, which expands to become the largest brain vesicle. Movie 6 and Fig. 4 showed that the beginning of this expansion was already visible at the stage of 4 somites, when the AN was most extended before it was closed by elevation and convergence. We therefore explain the backward movement of the initial closure as a consequence of the prosencephalic expansion, which already started before the AN closes.

Homocysteine might interfere with actin dynamics

After application of a single dose of Hcy, three major morphogenetic effects were seen: (1) divergence and closure delay at the initial closure site, at the AN and at the PN; (2) flattening of the head fold and neural tube, and (3) halting of cell movements. The closure delay at the AN confirms our previous results (Afman et al. 2003).

The concentration of Hcy necessary to evoke an effect on various neurulation processes seems relatively

high. However, from our previous study (Afman et al. 2003), it appeared that the uptake efficiency by a chick embryo in culture is rather low; Hcy levels of 12 and 19 μM were detected after supplementation with 10 and 30 mM Hcy respectively. These values were below detection level of 2 μM in control embryos, and comparable to the slightly elevated levels of 15 μM as found in mothers of NTD children. From that previous study it also appeared that the Hcy effect was specific and not due to osmotic changes, since identical concentrations of cysteine or leucine did not evoke any effect. Also, general development was unaffected as indicated by a normal gain of somites.

How to explain the various effects of Hcy on cranial neurulation? Divergence of the neural walls and folds is a counteracting movement to normal neurulation. Flattening of the head fold and neural tube indicates that the rigidity of these structures is diminished. Both divergence and flattening were also found after application of cytochalasin-D, which is a polymerization-inhibiting factor for actin (van Straaten et al. 2002). Actin is ubiquitously present in the neural plate and tube and is suggested to generate morphogenetic movements in neurulation by reshaping neural plate cells from columnar into a wedge shape through apical constriction of actin microfilaments; as a result, the neural plate bends inwards. An active role for actin microfilaments is at least evident in cranial neurulation. Apart from this morphogenetic function, actin was suggested also to be involved in maintaining rigidity of neural walls and folds (Morriss Kay and Tuckett 1985; Ybot Gonzalez and Copp 1999; van Straaten et al. 2002).

The similarity in effect of Hcy and cytochalasin-D: divergence of neural walls/folds and flattening, suggests that Hcy interferes with actin, which is corroborated by the third effect of Hcy in our study: halting of cell movements. Movement of cells, specifically cell rearrangements, imply that cells switch neighbours. In order to do so, cells need to express protrusive activity (Keller et al. 1992). Crawling cells displace themselves by a similar overall mechanism of protrusion through a dynamic turnover of actin (Rafelski and Theriot 2004). Furthermore, interference of Hcy with actin was shown in endothelial cells in culture, where Hcy mediated actin cytoskeleton reorganization and the integrity of cell–cell junctions (Dardik et al. 2002). It is thus possible that both halting of cell movements, widening of neural walls and flattening of the head fold and neural tube are effects of Hcy on actin dynamics.

The mechanism of Hcy interference with actin dynamics is not clear. Elevated levels of Hcy, as an intermediate in the methylation cycle, might interfere with methylation of proteins or genes. Data from rat embryo studies suggest that the methylation of actin and tubulin is required for proper neural tube closure (Moephuli et al. 1997). Whether Hcy exerts its effect through direct methylation of actin, through methylation of actin-related proteins or genes or through an alternative mechanism remains to be determined.

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