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Uncoupling Histogenesis From Morphogenesis in the Vertebrate Embryo by Collapse of the Transneural Tube Potential

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ABSTRACT **We have shown that unidirectional pumping of Na^+ out of the neural tube's luminal fluids in amphibian embryos produces a large potential difference (40–90 mV, lumen negative to the abluminal surface). This transneural tube potential (TNTP) is analogous to the Na^+ dependent transepithelial potential (TEP) that exists across surface ectoderm. This TEP is retained in ectoderm after it is internalized when the neural folds fuse to form the neural tube. The TNTP can be markedly reduced for several hours by injection of the Na^+ channel blockers amiloride or benzamil into the lumen by iontophoresis through microelectrodes. Here we describe the effect of TNTP modification on developmental anatomy. Axolotl embryos possessing a fused and closed neural tube (stage 21–23) were injected with either amiloride or benzamil and allowed to continue development for 36–52 hr. These were compared to control embryos injected with vehicle alone, or to embryos in which amiloride or benzamil was iontophoresed just beneath surface ectoderm. All embryos in which the TNTP was reduced were grossly defective. These were characterized by a disaggregation of the cells comprising the structures that had already begun to form (otic primordia, brain, spinal cord, notochord) as well as a failure in the development of new structures. Remarkably, some of these embryos displayed continuing development of external form in the complete absence of concomitant internal histogenesis. We discuss the ways in which a large endogenous voltage gradient associated with an epithelial potential difference (the TNTP) may be required both for the structural integrity of the early neuroepithelium, and a prerequisite for normal morphogenesis.** © 1995 Wiley-Liss, Inc.

Key words: Neural tube, Electric fields, Morphogenesis, Cranial development, Sodium potential

INTRODUCTION

Well before the differentiation of glia and neurons, the vertebrate brain and spinal cord are established as a closed tube of embryonic epithelium, enlarged on the rostral end. This tube of presumptive neuroepithelium is formed by a progressive infolding and dorsal fusion of surface ectoderm. The neural tube forms a functional epithelial syncytium, clearly possessing an energy dependent polarized ion transporting capability (specifically moving Na^+ across itself from inside to outside). This same unidirectional movement of Na^+ also produces a potential difference across embryonic surface ectoderm of about 50 mV (inside positive with respect to the outside). The embryonic transepithelial potential (TEP) resulting from the unidirectional ion transport is established very early in development, as early as the blastula stage (Regen and Steinhardt, 1986; Slack and Warner, 1973; McCaig and Robinson, 1982; Robinson and Stump, 1984; Metcalf et al., 1994; Robinson et al., 1991). The TEP is retained in surface ectoderm after fusion of the neural folds to form the neural tube in both frog, *Xenopus laevis,* and salamander, *Ambystoma mexicanum,* embryos (Hotary and Robinson, 1991; Shi and Borgens, 1994). We have further demonstrated that the polarity and Na^+ dependence of the potential expressed across the wall of the internalized neural tube is retained from its predecessor. This is reflected as a striking transneural tube potential (TNTP) ranging from 40–90 mV, inside (luminal domain) negative with respect to the extracellular environment of the embryo (Shi and Borgens, 1994). Moreover the TNTP is sensitive to the same specific Na^+ channel blocking agents (amiloride and benzamil) as surface ectoderm (Sariban-Sohraby and Benos, 1986). Application of these agents only to the apical surface of surface ectoderm results in a marked, yet temporary, suppression of the TEP. Since the lumen of the neural tube was once the external environment of the embryo, the former apical surface of the ectoderm becomes the luminal surface of the neural tube. Amiloride or benzamil iontophoresed into the lumen through microelectrodes (final concentration in CSF, approximately 5 μM) also resulted in a temporary reduction (or collapse) of the TNTP. Na^+ transport through epithelia can also be facilitated by application of the antibiotic

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novobiocin (Johnson and Hoshiko, 1971; Rick et al., 1988) to the apical surface. This results in a modest increase in TEP, and in our studies, a modest ($\leq$ 10%) boost in the TNTP (Shi and Borgens, 1994). Modulation of Na^+ transport through the use of these agents reveals the general physiological properties of this presumptive neuroepithelium.

Our laboratory has previously reported the existence of a three-dimensional coordinate system of extracellular voltages that appear at the beginning of neurulation and disappear at its climax (Shi and Borgens, 1995; see also Metcalf et al., 1994). These voltage gradients are produced by ionic currents driven through the embryo in predictable patterns by the TEP of surface ectoderm. Furthermore, these gradients not only partition the embryo into domains, but are required for normal morphogenesis. Interruption of internal electric fields by precisely applied voltages causes predictable, and marked, abnormalities in later development (Metcalf and Borgens, 1994). We have further investigated if the voltage retained across the walls of the newly formed neural tube is required for subsequent morphogenesis and differentiation (Shi and Borgens, 1994). In this study we reduced the TNTP in order to test its relevance to ontogeny. Experimentally treated embryos were compared to controls that underwent iontophoresis of vehicle alone, or in which these agents were injected beneath the embryo's surface ectoderm. Our preliminary evaluation revealed that severe developmental abnormality resulted from reduction or collapse of the TNTP (Shi and Borgens, 1994; Fig. 1). Here we describe the developmental anatomy of experimental and control embryos in detail and show that not only is the TNTP required for normal histogenesis, morphogenesis, and development, but that its collapse can lead to the uncoupling of the global controls of pattern formation from the differentiation of the embryo's internal structures. Furthermore, we suggest that the defective developmental anatomy results from a disaggregation of cells—not their demise—and that this loss in neuroepithelial structure interrupts an early step in morphogenesis, undermining the fundamental organization of the developing embryo.

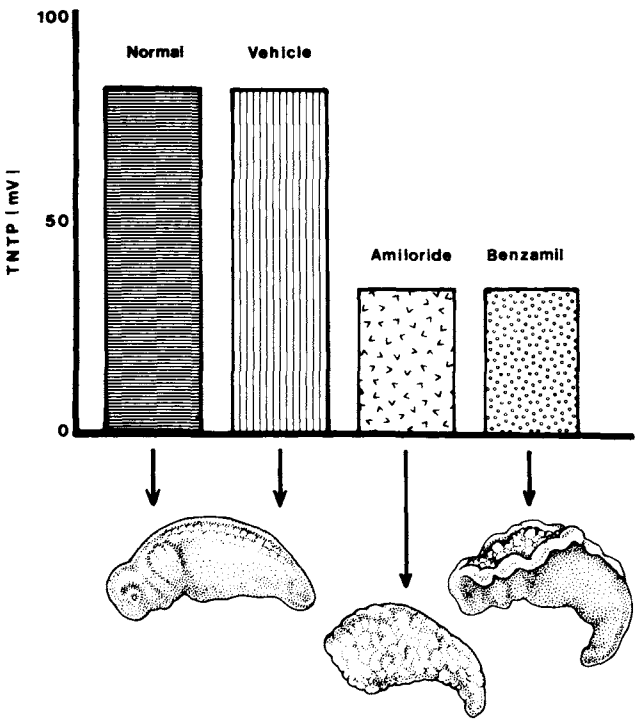

Fig. 1. The effect of TNTP reduction on embryonic development. The first two vertical bars show the normal TNTP (of about 80 mV) was unaffected by iontophoresis of vehicle (microelectrode filling solution: 100 mM Na^+Cl) into the lumen of stage 21–23 axolotl embryos. A typical stage 34 embryo is illustrated directly below these bars to show that such manipulation had no effect on subsequent embryonic development. Bars three and four show that the TNTP was reduced by more than 50% following iontophoresis of the Na^+ channel blockers amiloride or benzamil into the lumen of the neural tube shortly after its fusion. The result of these manipulations produced grossly abnormal forms, many that remained viable for at least as long as control embryos which were killed at stage 34–36. Beneath the amiloride bar, a headless form is illustrated. Such embryos were devoid of any cranial development. Beneath the benzamil bar an embryo is shown in which typical cranial structures were observed to be missing (optic, otic, olfactory, or gill primordia) and the neural tube had exvaginated along its dorsal surface. Both of these forms were extreme examples of teratogenesis and were not viable. (This summary figure reconstructs data presented in Shi and Borgens, 1994.)

RESULTS

Experimental and Control Groups

All embryos were manipulated experimentally following complete closure of the neural folds forming the neural tube (stages 21–22; Bordzilovskaya et al., 1989). Three control populations were used for histological comparison: animals in which (1) benzamil or (2) amiloride was injected beneath the rostral surface ectoderm, and (3) a group in which vehicle (see Methods) was iontophoresed into the lumen of the neural tube. These were compared to two experimental groups where either benzamil or amiloride was injected into the lumen of the neural tube. The methodology used for iontophoresis was identical in all cases. The procedures used for the iontophoresis of pharmacological agents or vehicle lasted approximately 10 min, after which embryos were studied for varying times (36–52 hr) until they were killed and evaluated histologically (Table 1). Equal populations of embryos in which benzamil and amiloride were iontophoresed into the lumen of the neural tube were studied. Amiloride treated embryos were affected most severely, however, and 4 of the 9 embryos viable past stage 30/31 are described here, as well as 10 of 13 viable benzamil treated embryos. The balance were either grossly deformed individuals (many times completely lacking a cranial enlargement) or were not viable to stage 30/31 (refer to their description in Shi and Borgens, 1994).

It is important to emphasize that at the time of microelectrode impalement of the neural tube, all embryos possessed a stable TNTP. This TNTP indicated the formation of a distinct neural tube, closed and iso-

TABLE 1. Developmental Responses to Reduction of the Transneural Tube Potential

Application	Embryo number	Abnormal/ total[b]	Internal structures[a]									
			Optic	Otic	Olfac.	Brain	Neural tube	Notochord	Cardiac	Brachial	Gut	Somites
Amiloride within neural tube[c]	4	4/4	1/4	0/4	2/4	0/4	2/4	0/4	0/4	0/4	2/4	2/4
Benzamil within neural tube[c]	10	10/10	1/10	1/10	1/10	1/10	2/10	1/10	1/10	2/10	2/10	3/10
Vehicle within neural tube[d]	4	0/4						Normal				
Amiloride beneath ectoderm (control)[e]	8	0/8						Normal				
Benzamil beneath ectoderm (control)[e]	8	0/8						Normal				

[a]Within this category, the numbers of embryos possessing these recognizable primordia are given above the total number evaluated. Olfac., olfactory.
[b]Number of abnormal embryos over the total number that were evaluated histologically.
[c]The pharmacological agent was iontophoresed into the lumen of the neural tube at the cranial enlargement (refer to Experimental Procedures).
[d]Vehicle within the microelectrode tip was subject to iontophoresis in control embryos using the same procedures as in [c].
[e]The pharmacological agent was iontophoresed beneath the surface ectoderm in these *control* embryos.

lated from the external milieu. Histological differentiation of some internal primordia has already begun at this stage of development. For example, stage 21/22 axolotl embryos already possess a notochord, some somites, optic vesicles, hyomandibular furrowing (with demarcation of the future gill region), and a downward curvature of the head delineating the fore, mid, and hind brain regions (refer to Bordzilovskaya et al., 1989).

Significant development in the axolotl occurs by stage 32–36, the developmental stages reached by all control embryos. All control embryos demonstrated internal organization typical of this stage of development. Here we limit our catalog of developing primordia to easily observed "landmark" structures such as the compartmentalization of the brain, spinal cord anlagen, optic, otic, and olfactory anlagen, notochord, somites, gill, gut, and cardiac primordia. These were used as an index of normal internal development, while the external form was noted and recorded. Figure 2 provides examples of such normal development. Table 1 provides the proportions of the population demonstrating the occurrence of internal differentiation for these structures. In every case, reduction of the TNTP resulted in markedly abnormal development.

Morphogenesis Without Histogenesis: The Formation of "Pseudoembryos"

One obvious effect of TNTP reduction was the lack of externally visible sensory primordia (such as the eye or otic structures), while the general form of the embryos appeared relatively normal as they continued to increase in length and girth. We have referred to these as "pseudoembryos" and have provided a preliminary description of their form (Shi and Borgens, 1994). Out of 14 embryos whose TNTP was collapsed, 5 retained a recognizable and normal external form. They were later found to be comprised of a mass of cells that was not organized into any discrete internal structures. Cavities were present in these embryos, but none of these were bounded by an epithelium and did not form any recognizable lumina, not even a gut. Figures 3 and 4 provide examples of such embryos. Figure 3 shows that densities of cells that existed in positions where organ rudiments were to form (or had begun to form) did not possess recognizable characteristics and were not delimited from surrounding cells. Importantly, the cells forming some primordia (such as neural tube, notochord, and somites) appeared to loose their attachments one to another and to disaggregate; they did not appear to disintegrate (see Epithelial Potential Difference and Epithelial Organization, below). Occasionally "ghosts" of these discrete primordium could be visualized, where definition of their structure was barely apparent (Fig. 4). A second group of five embryos was similar to that group just described in that a reasonable normal (though bloated) external form continued to develop, while some internal differentiation was apparent, though grossly hypomorphic. These embryos could not easily be staged. In these cases a recognizable, yet incomplete, brain, spinal cord, and somites were present. Other structures such as a notochord, gut, cardiac primordia, etc. were absent (Table 1, Fig. 5).

Histogenesis Without Morphogenesis

In addition to pseudoembryos we detected a third class of abnormality. In these individuals (4 of 14, Table 1) development after TNTP collapse resulted in completely chaotic external and internal development. These forms lacked any recognizable structure whatsoever, did not resemble controls *in any way,* and could not be staged. In many cases it was impossible to determine a dorsal or ventral surface, or rostral or caudal

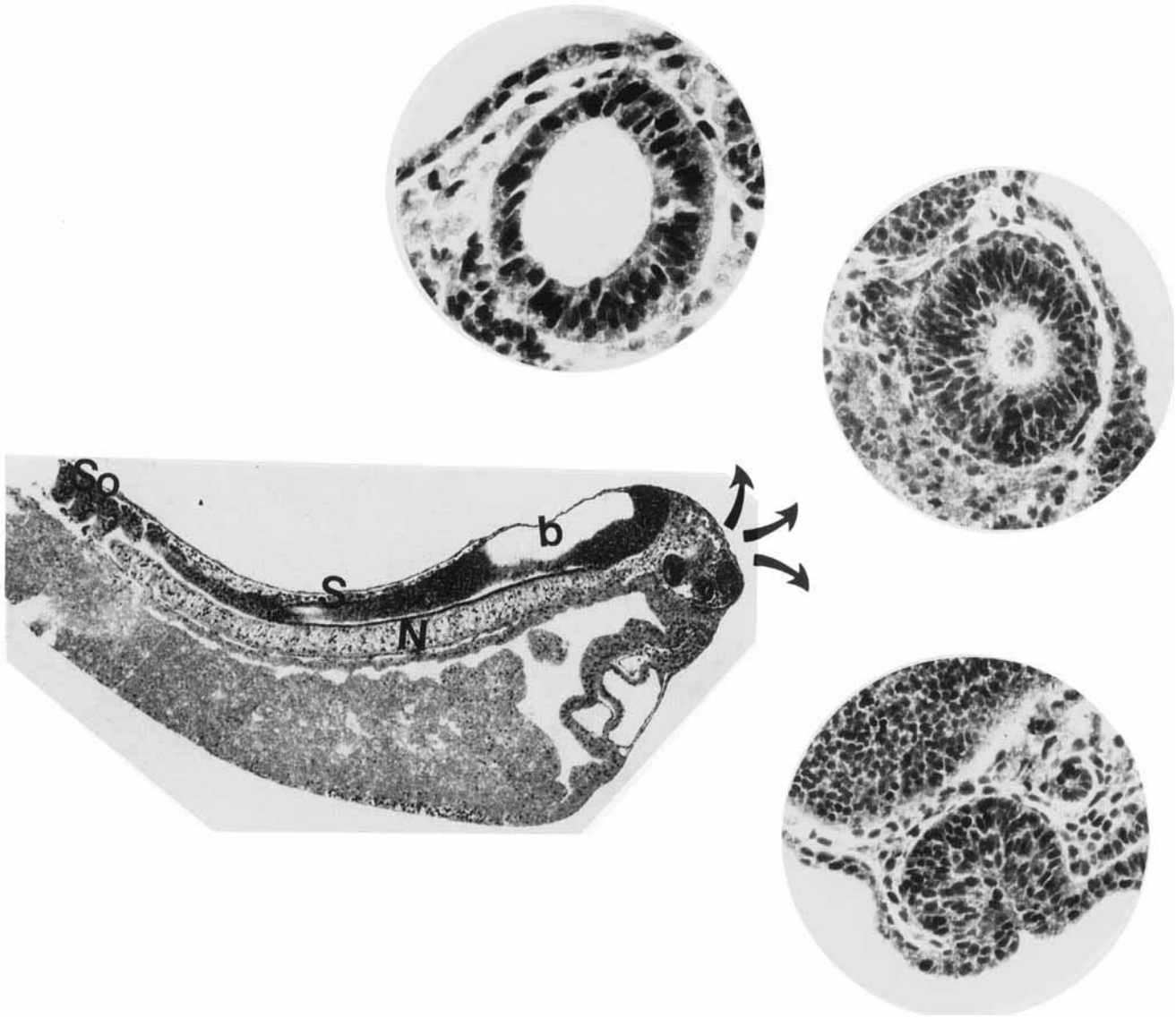

Fig. 2. Normal development in a stage 34 axolotl. These photomicrographs demonstrate developing anatomies typical of all control embryos. The mid sagittal section in the center is taken from control embryo 138, in which iontophoresis of amiloride beneath the surface ectoderm was carried out (refer to Experimental Procedures and Table 1). N = notochord, S = spinal cord, So = somites, b = lumen of brain, a portion of all three CNS accessory structures can be seen in this one section as well. Going clockwise, the adjacent high magnification photomicrographs show detail of the otic and optic vesicle (and lens) and the olfactory pit at this stage of development. These three magnifications were taken from another control embryo, 132, which underwent iontophoresis of benzamil beneath its surface ectoderm at stage 22. The longitudinal section (tail incomplete) was 2.7 mm in length.

end on the basis of histology. Individuals in this group were slightly oblong, but lacked a cylindrical tapered body or down-turned cranial enlargement. The external shape was characterized by local swellings and protuberances (Shi and Borgens, 1994; see also Metcalf and Borgens, 1994). Internally these protuberances (or blisters) were either empty of cells, filled with isolated stellate-shaped mesenchymal cells or clusters of cells, or filled with whorls of disorganized epithelia extending into the blister from internal (and likewise disorganized) epithelia (Fig. 6). Examples of one or all of the above could be found on any one individual (as is the case in the example provided in Fig. 6). None of these "embryos" contained ordinary primordia. They did contain densities of cells aggregated into what might be mistaken as anlagen, but these did not appear in any characteristic location which would allow identification. Cavities that were bounded by an epithelium were common; however, none of these possessed a typical shape, location, or any other identifying characteristic. Ectodermal thickenings similar to placodes that would normally identify a locus of differentiation (such

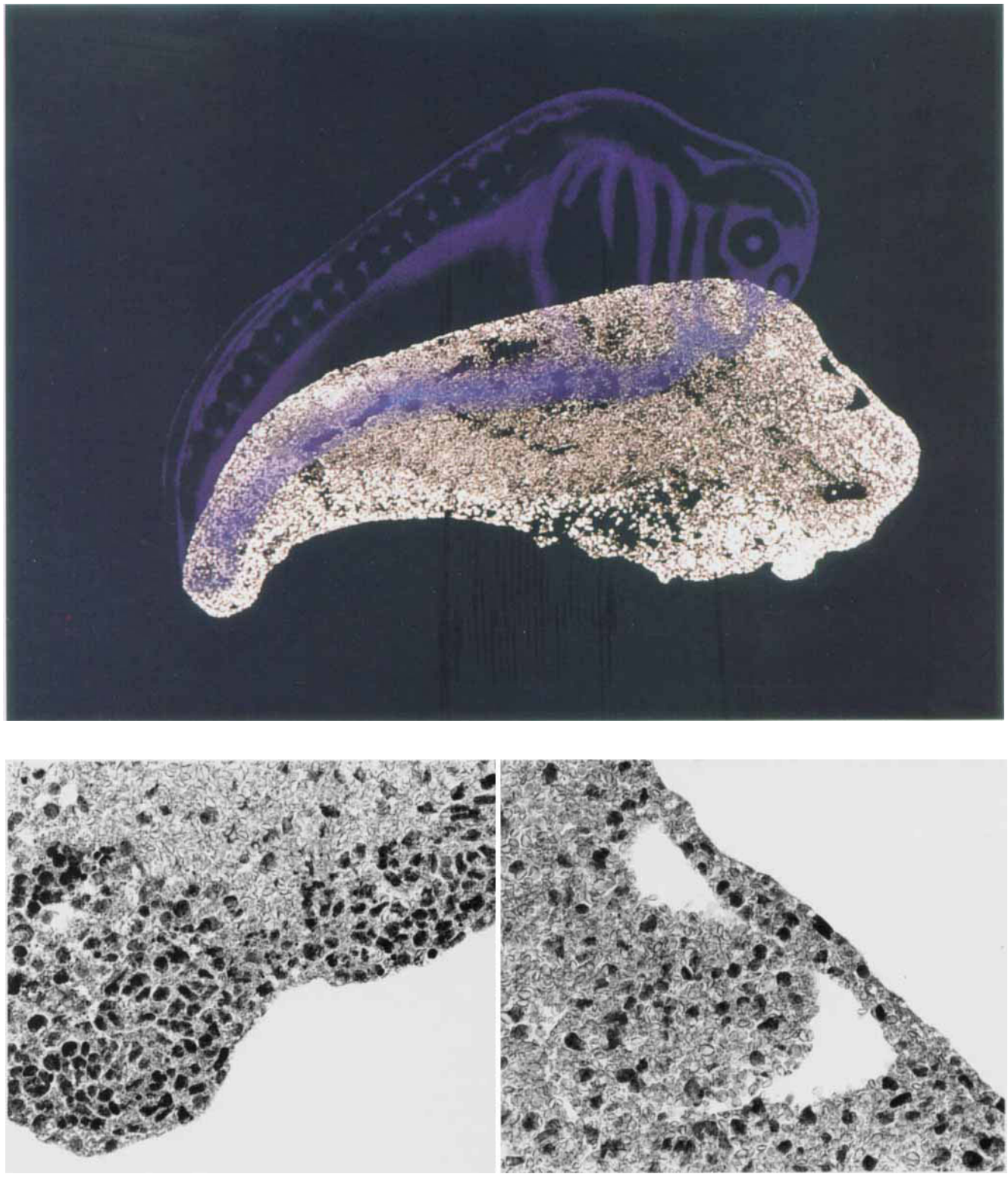

Fig. 3. General features of a "pseudoembryo" resulting from TNTP suppression with benzamil. An artist drawing of a typical stage 34 axolotl (see Bordzilovskaya et al., 1989) is superimposed on the mid-sagittal section of embryo 149; note that in spite of TNTP collapse, and the lack of internal histogenesis of structure, *the external shape* of this embryo is normal. Such embryos were as viable as control embryos, continuing to elongate and developing a (sometimes down-turned) cranial enlargement in spite of the lack of a developing brain. The small cavities at the rostral end (in a region where the brain should be), as well as what appeared to be cell aggregations ventral to this are enlarged directly below. Note the diminutive cavity is not bounded by any epithelium typical of developing brain or spinal cord, and the aggregations (compare with Fig. 2) of some pigmented cells are not defined into any distinct rudiment, placode, or vesicle. The embryo was 2.5 mm long at sacrifice.

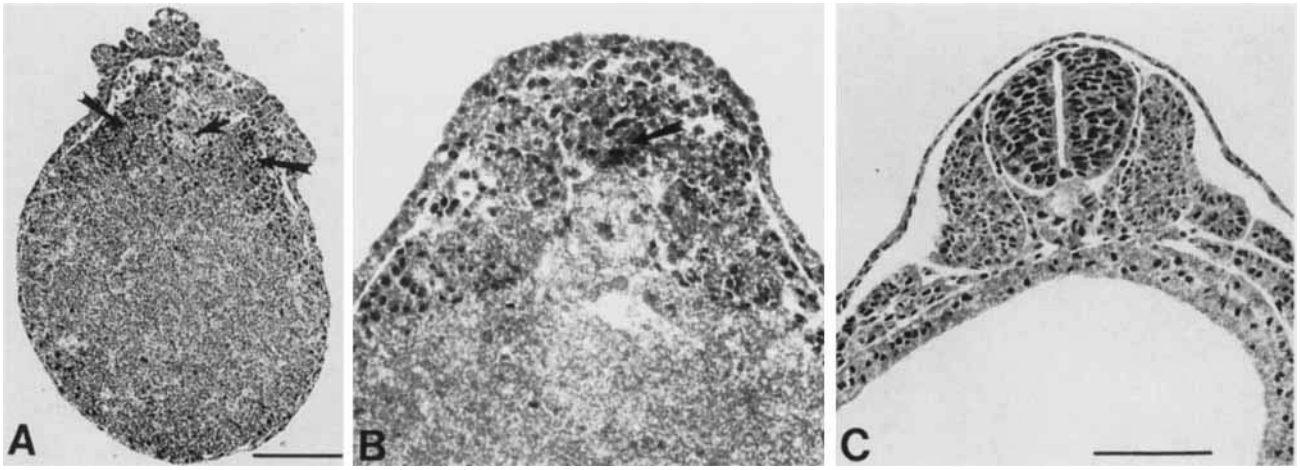

Fig. 4. Effect of benzamil induced TNTP collapse on embryonic development. **A:** A cross section from the mid rostral level, experimental embryo 120. Note the indistinct notochord (arrowhead) and undeveloped region normally occupied by somites (arrows). The overall form of this embryo was relatively normal. Trichrome, calibration line = 160 μm. **B:** Cross section at a similar plane in experimental embryo 150. Note the lack of a well-defined neural tube, somites, and notochord (arrow). The overall shape of this embryo (as in A) was relatively normal. **C:** Cross section of control (vehicle injected) embryo 143 for comparison. This cross section was taken from an *approximate* region of the embryos shown in A and B (although these did not possess a pharyngeal cavity or gut). Note the well-defined notochord, somite structure, gut, and presumptive spinal cord. Trichrome, calibration line for C and B = 180 μm.

as the otic vesicle or lateral line organ rudiments) were scattered over these embryos at many locations as part of the surface ectoderm. In summary, the general appearance of this subgroup suggested histogenesis was proceeding following TNTP collapse, but in a random, chaotic, and uncontrolled way.

Embryonic Epithelia Following TNTP Collapse

As described above, all abnormally developing embryos possessed an external ectoderm, and in some cases, whorls of internalized ectoderm that may or may not have delimited internal cavities. The gross structure of both surface ectoderm and internal developing epithelia was abnormal, however. Normally the surface ectoderm of stage 31–36 embryos is smooth, comprised of cuboidal epithelial cells, and is usually two cell layers thick (except at dense thickenings where CNS accessory structures are forming such as the olfactory pits, otic, and optic primordia) (Fig. 7A). In experimental embryos, the individual cells comprising surface ectoderm were sometimes bulbous, protruding away from the surface singly or in clusters. This ectoderm was uneven, lobulated, and in many places on the embryo lacked any definition from the mass of internal cells (compare Fig. 7C and D). Focused regions of ectodermal plaquing were evident, including the formation of ectodermal appendages which extended away from the body in curious shapes (Fig. 7E). The thickness of surface ectoderm and internal neuroepithelial primordia was measured at four locations (for each histological cross section), and these were obtained at five levels along the embryo's rostral/caudal axis (see Experimental Procedures). Only four experimentally treated embryos (of a total of 14) possessed recognizable CNS primordia at any level allowing this evaluation. These data were compared to similar measurements on 6 control embryos. Morphometric evaluation of surface ectoderm demonstrated that there was no significant difference in the thickness of the ectoderm between experimental and control embryos in spite of the curious and uneven character of the ectoderm of experimentally treated embryos. In addition, the thickness of the neuroepithelium at mid embryo to rostral measurement levels was not different between the two groups. In two of four experimental treated embryos, however, an enlarged caudal neural tube was noted. The thickness of this neuroepithelium (range 204–214 μm, luminal surface to abluminal surface) was significantly thicker than controls (50–67 μm) ($P \leq 0.005$, Mann-Whitney).

DISCUSSION

What Is the Role of the Transneural Tube Potential?

This laboratory has long been interested in the probable dual function of potential differences (TEPs) existing across adult skin and its predecessor, embryonic surface ectoderm. We have argued that the TEP is not only involved in the organism's general physiologies (such as osmoregulation and the maintenance of skin hydration), but as well in wound healing, regeneration, and pattern formation (Borgens, 1982, 1984, 1989; see also Vanable, 1989; Rajnicek et al., 1988, Chiang et al., 1989). We have recently offered evidence that polarized subectodermal voltage gradients associated with the inhomogenous distribution of TEPs helps to control emerging pattern in the amphibian embryo (Metcalf et al., 1994, Metcalf and Borgens, 1994). Here we discuss

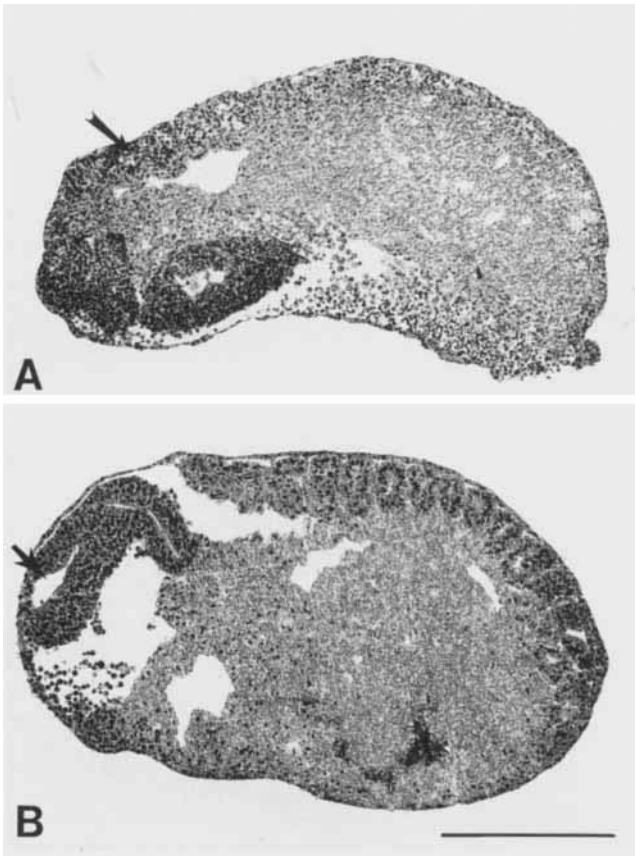

Fig. 5. These longitudinal sections show two examples of embryos (TNTP collapsed) whose internal organization was both deficient and abnormal, but that possessed some recognizable and appropriate structure. **A:** A benzamil injected embryo (128) showing reasonably normal (yet somewhat bloated) external form. However, the brain had extended unnaturally into the ventral region, and there was no caudal remnant of it or the spinal cord in any other section. Somites had begun to form dorsally, but occupied an abnormally rostral position (arrow). Trichrome. **B:** Another benzamil injected embryo (22) showing similar overall conformation, and a presumptive neuroepithelium surrounding a lumen that is recognizable as pro/mesencephalon (arrow). A central cavity in this embryo was unlined, however, and there were no gill or cardiac primordium. The developing brain was truncated, and extended abruptly downward, at about the level of the rhombencephalon. There was no extension of a neural tube, and a notochord was not present. However recognizable somite development had occurred. Trichrome, calibration line, for A and B = 600 μm.

the possible developmental role of a particular potential difference (the TNTP) which exists across presumptive neuroepithelium. First, however, it might be helpful to consider the role a TNTP might play in the general physiology of the developing organism.

The transport of ions against a concentration gradient is the means by which the differing ionic compositions of fluid compartments can be maintained in the adult or embryo (De Loof, 1992; Gumbiner, 1990). The luminal domain of the neural tube is filled with a fluid that will be cerebrospinal fluid (CSF) which is distinct in its ionic composition from body fluids. Bulk movement of Na^+ from luminal fluids is indicated by our data (Shi and Borgens, 1994), suggesting that early CSF might be depleted in Na^+ relative to body fluids, which helps to produce the negative potential difference (pd) between luminal fluids and the extracellular environment outside of the neural tube.

In the mammal, CSF is largely a secretion product of the choroid plexus combined with the exchange of electrolytes and other moieties across meningeal and ependymal linings as well as across the blood vessels of the brain and spinal cord. Under normal conditions, mammalian CSF is *positive* to body fluids and slightly *elevated* in Na^+ content, however, both the polarity of the pd between the CSF and body fluids, and the concentration of various solutes, may be greatly influenced by several factors including the pH and oxygen content of arterial blood (Held et al., 1964; Loeschcke, 1971; see also Guyton, 1991; Berne and Levy, 1993). Thus comparison between the amphibian embryo and the adult mammal in this regard may not be instructive.

In the dogfish, the pd between the CSF and body fluids is luminally negative in polarity (Hogben et al., 1960). These authors suggest the polarity of this pd may arise from the transport of Na^+ out of the CSF, but emphasized the pumping of Cl^- into CSF (recall that the specific amiloride sensitive Na^+ transport was not known to exist in neuroepithelia for another 30 years). Their data, and their interpretations, are in general agreement with the measurements we report, suggesting that at least in the non-mammalian vertebrate, the amiloride/novobiocin sensitive Na^+ transport system may be a means to produce at least *presumptive* CSF.

The amphibian "pond water" within the recently closed domain of the neural tube is already quite dilute in Na^+, and one might reasonably suggest would soon become completely depleted of Na^+. On deeper reflection, however, this is unlikely given the fact that epithelia can be quite leaky through cell junctions to transported ions and still maintain a sizable pd. Moreover, there would be a tendency for Na^+ to move back down its concentration gradient into presumptive CSF through this paracellular pathway. Furthermore, there are several classes of amiloride sensitive channels in epithelia which differ in their selectivity for Na^+ and can likewise move other ions (such as K^+) in the same direction contributing to the TEP (Palmer, 1992; Smith and Benos, 1991). We have not yet determined the type of amiloride sensitive channel expressed in the wall of the early neural tube. In summary, there is little available data to formulate a clear hypothesis relating the early physiology of the embryonic neural tube to the production of CSF in the adult animal. Alternately (or additionally) one might focus on the effect of a neuroepithelial pd on its developing cells per se. The data presented here raise three important and related questions: Is the potential across the early neuroepithelium critical to the maintenance of its own structure? Is the

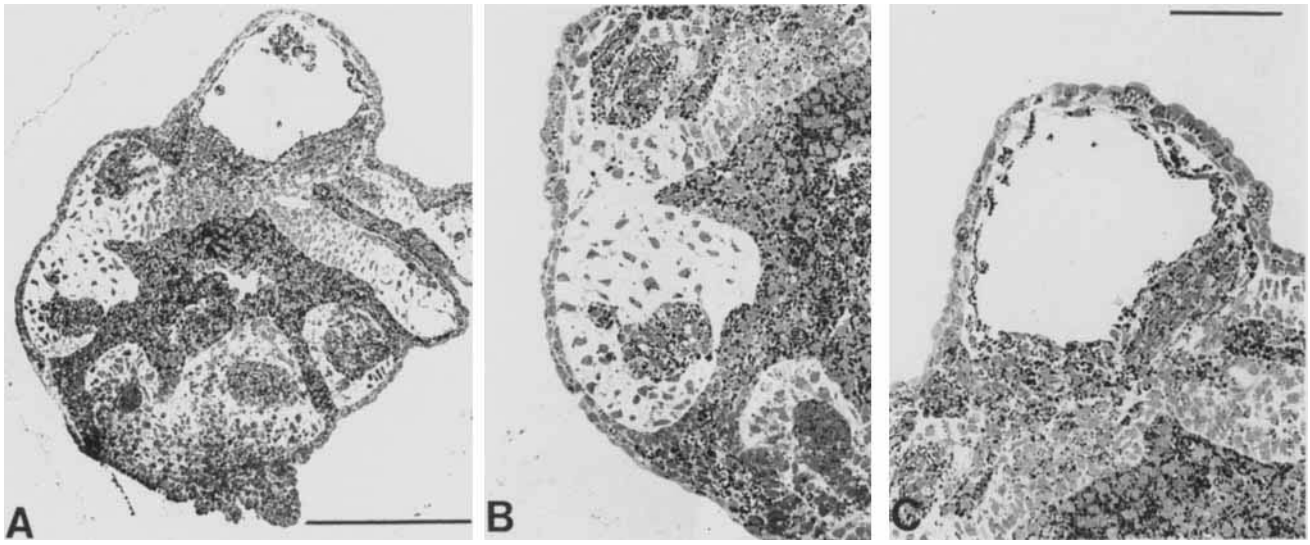

Fig. 6. Effect of an amiloride induced TNTP collapse. **A:** Low power magnification of experimental embryo 61. Note the complete absence of recognizable external or internal structure, and the chaotic development of epithelium bounded cavities that occurred throughout this embryo. There was no way to define rostral or caudal, dorsal or ventral, on this "embryo." **B:** High magnification view of an externally protruding fluid filled "blister" containing both individual and aggregates of stellate mesenchyme cells. **C:** Another protuberance similar to that depicted in B, however this contains only fluid. Note the presence of epithelial structures within the embryo bounding these abnormal cavities (in both A and B). The index line in A = 500 μm; in C and B = 150 μm.

general structure or physiology of the epithelial walls of the early neural tube critical to morphogenesis and differentiation? How does the shaping of the embryo's overall form continue in the absence of internal differentiation?

Epithelial Potential Difference and Epithelial Organization

All of the cells that comprise epithelial syncyntia share certain characteristics including the establishment of distinct and polarized apical/basal domains and the ability to vectorally transport ions across these domains (Gumbiner, 1990). These characteristics provide the cytological basis for epithelial function in separating distinct fluid compartments that differ in their ionic concentration and composition. The precise mechanisms leading to the generation of epithelial cell polarity during early development is unclear. Cell polarization during the formation of an epithelium is known to involve the localization of extracellular proteins, especially the CAM Uvomolin (which influences the location of cell-to-cell contacts) and laminin, influencing cell to substrate contacts to specific domains, among other influences (Rodriguez-Boulan and Nelson, 1989; Gumbiner, 1990). The self-amplified generation of epithelial cell polarity probably involves the segregation of ionic pumps within the membrane which may also control cell-to-cell, and cell-to-substrate adhesion through various Ca^+ dependent signaling pathways (Jaffe, 1981; Rodriguez-Boulan and Nelson, 1989). It is also probable that the pd generated by the basic asymmetry of the epithelium *itself* may be responsible for *maintaining* the structural asymmetry of the epithelium. This is quite consistent with the anatomical findings presented here; however, we know of no direct experimental test of the notion that a TEP is indeed critical to *epithelial* structure in other systems. Moreover, this requirement could be more characteristic of early neuroepithelia since a dissolution of *surface* ectoderm is not observed when intact amphibian embryos (anuran and urodele) are bathed in a pond water containing the same Na^+ channel blockers amiloride and benzamil (in much greater concentration) or bathed in Na^+ depleted media. All of these manipulations would serve to reduce or collapse the TEP (Metcalf et al., 1994; Rajnicek et al., 1988).

How then, might a TNTP help maintain the structural integrity of the neural tube? First, the TNTP is sufficiently large to produce extracellular gradients of voltage already know to determine the *gross alignment, and even migration, of a variety of embryonic cells and their cellular processes*. The TNTP in both Xenopus and Ambystoma embryos would be associated with an extracellular electric field of about 0.5 to more than 1 V/mm (Hotary and Robinson, 1990; Shi and Borgens, 1994). A bipolar organization of many cells in culture (including fish epidermal cells, the keratocyte; Cooper and Keller, 1984) and astrocytes (Borgens et al., 1994) can be induced *and* predictably oriented by extracellular voltages of lesser magnitude than this (reviewed by Borgens, 1992; Robinson, 1985; Nuccitelli, 1988). Second, extracellular voltage gradients in the mV/mm range can also induce and maintain, asymmetric distributions of integral membrane components within

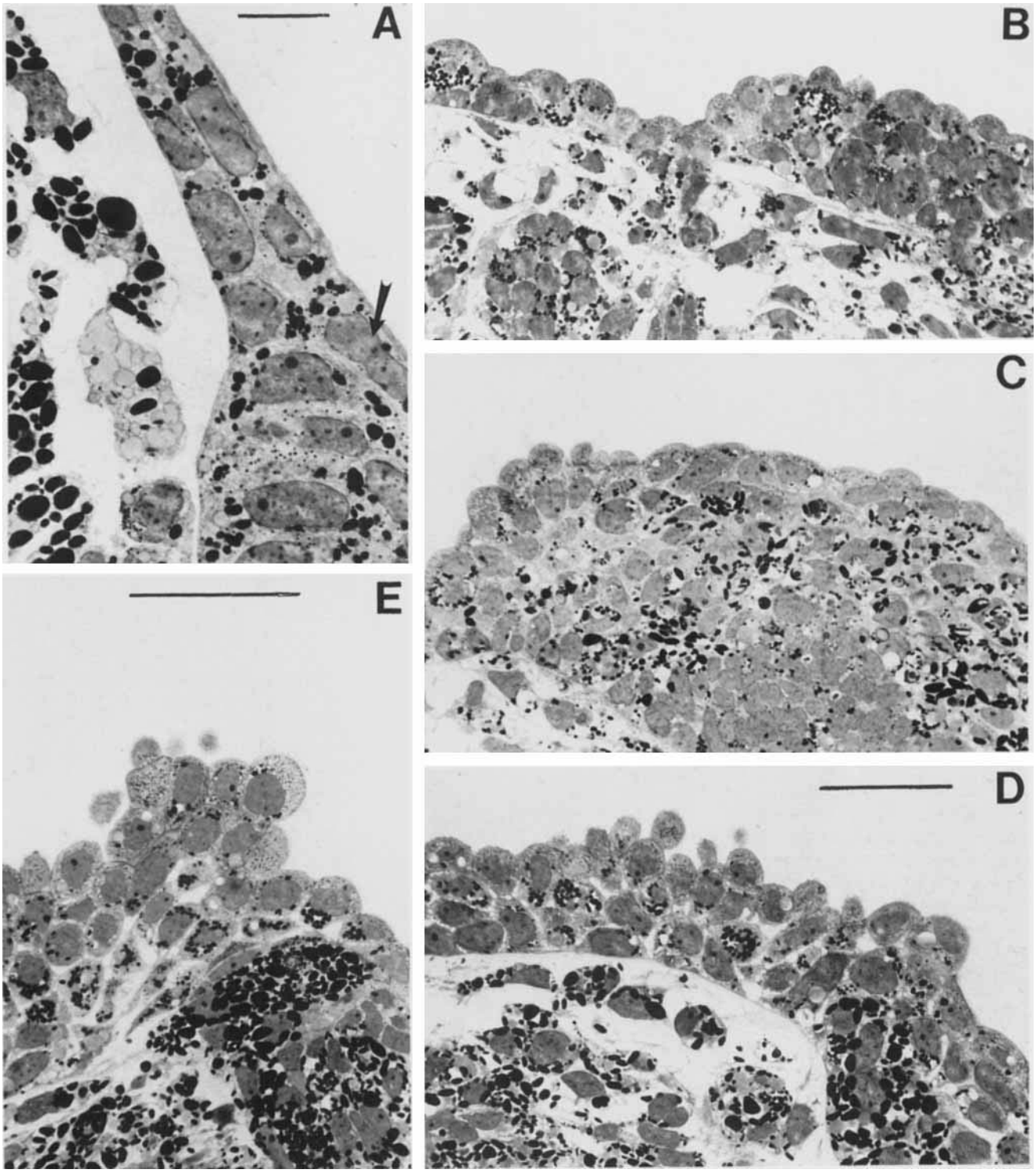

Fig. 7. Abnormal epithelialization in response to TNTP collapse. Normal surface ectoderm (**A**) is usually cuboidal in character, smooth, and two layered except at specific thickenings such as that covering this otic plaque (arrow). Control embryo 6, iontophoresis of vehicle into the neural tube lumen, Toluidine blue, calibration line = 25 μm. Surface ectoderm in experimentally treated embryos was very uneven in thickness (**B**) and may or may not be distinct from underlying cells. Compare B, **C,** and **D.** Individual cells were bulbous, not cuboidal, and often appeared to be protruding or extruding away from the uneven surface (D and **E**). Calibration in D (for B and C) = 60 μm; in E = 80 μm.

cell membranes (such as many different types of membrane receptors) by mechanisms of lateral electrophoresis/electrosmosis (Jaffe, 1977; McLaughlin and Poo, 1981; Metcalf et al., 1994). These voltage mediated effects on the general organization of cells—or on the molecular components of their membranes—are fully reversible. Since an epithelium is characterized by restriction of some integral membrane components to basal, basolateral, and apical domains, it is reasonable to expect that large extracellular voltages, produced by the functioning syncytia, *may act back on the epithelium to help maintain these asymmetries.*

It follows then, that a reduction or collapse in the TNTP could lead to a loss in the structural polarity of epithelial cells and perhaps overall epithelial organization. It is clear from the data presented here, that a precipitous reduction in the TNTP *does not cause cell death in the neural tube* at least in this sample of embryos viable to stage 30/31. We have described many regions of cell death in embryos (such as localized "necrotic zones" in the amphibian limb bud) at both the LM and EM level (Borgens et al., 1987). We saw no evidence of dead, or even desquamating neural tube cells in this study. To the contrary, we observed evidence that the neural tube wall simply lost its definition; the cells of which it was comprised graded into the adjacent and nondescript cellular fabric of the embryo (Fig. 8). Furthermore, the control injections of amiloride or benzamil beneath surface ectoderm did not adversely effect development. This argues against the presumption of a non-specific "toxic" effect of these drugs on embryonic cells. More specifically these control injections show that a direct effect of the drug on the cells themselves *unrelated to the polarity of the potential difference* is unlikely. Amiloride and benzamil was infused just beneath surface ectoderm; however, neither the TEP was grossly modified (the drugs must be applied to the apical surface to collapse or reduce the TEP) nor the structure of the surface ectoderm affected. These data provide evidence for the notion that the pd across the presumptive neuroepithelium of the CNS is required for its structural integrity.

Neural Tube Structure and Morphogenesis

We believe the teratology presented here and in Shi and Borgens (1994) relates directly to interruption in an early cascade of developmental events that require both temporal and structural conformity to achieve normal development. It does not require much imagination to posit that a loss in neural tube structure alone would have enormous negative affects on subsequent development. For example, the out pocketing of the neural tube wall is necessary to induce eye vesicle formation. The floorplate region of the neural tube contains vital cues governing pattern formation within the CNS (Pownall, 1994). Substrate cues guide neural crest migration from their location on the dorsolateral wall of the neural tube. The various inductive relationships between the underlying neuroepithelium and overlying ectoderm are well known, and need not be cataloged here. Many secondary and tertiary outcomes in development (both structural and perhaps chemical) depend on the presence of a neural tube.

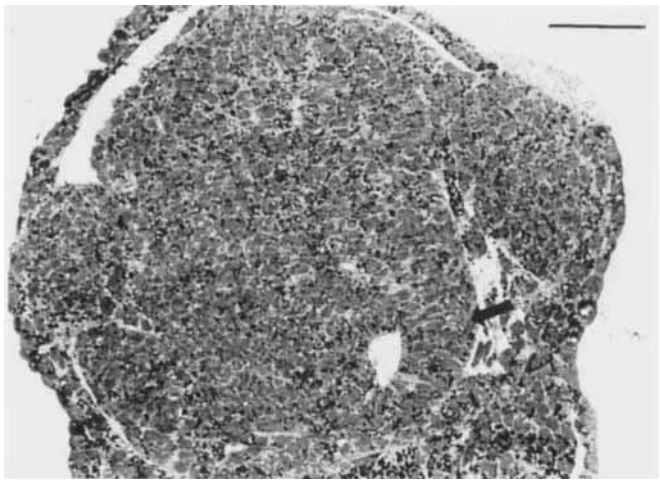

Fig. 8. In this amiloride treated (TNTP collapsed) individual (embryo 59), significant portions of the neural tube had either failed to develop or had dedifferentiated along its length. Dedifferentiation, or disaggregation, of the neural tube neuroepithelium in the absence of cell death is clearly shown in this view. Note a reasonably well formed tube on the right (arrow) comprised of columnar epithelium. On the left side, this epithelial organization has completely disappeared and cells appeared to fan out into adjacent regions. Toluidine blue, calibration line = 240 μm.

Secondly, the formation of "pseudoembryos" challenges our conventional paradigms concerning the generation of body form. It is usually conceived that the shape of an organism, or its parts, is largely a function of the shape of the components of which it is constructed. We know of three other instances where the global control of pattern formation can be uncoupled from local controls of form or histological differentiation. This can occur during the formation of experimentally produced doublet cells in ciliated protozoans (Frankel, 1984), in the electrically induced regeneration of "pseudolimbs" in adult *Xenopus* (Borgens et al., 1979) and in the embryos of higher plants (Goldberg et al., 1994). There is no trivial explanation for the genesis of "embryos" whose external form continued to develop following collapse of the TNTP while dissolution of internal structures occurred simultaneously with a failure of organogenesis.

EXPERIMENTAL PROCEDURES

The general husbandry of axolotl embryos is well described in previous publications in this series and we direct the interested reader to these (Metcalf and Borgens, 1994; Metcalf et al., 1994; Shi and Borgens, 1994). Embryos for the experiment were provided by the Indiana University Axolotl colony, and staged according to Bordzilovskaya et al. (1989).

The impalement of both surface ectoderm and the neural tube using microelectrodes has also been de-

scribed previously (Metcalf et al., 1994; Shi and Borgens, 1994). The measurement of the TNTP with microelectrodes was accomplished using conventional bridge circuitry, while iontophoresis of drugs or vehicle was carried out using a WPI microprobe system (model 707) and grass stimulator (model S44). A TNTP was followed for 15–30 min and determined to be stable prior to the injection of agents into the neural tube lumen from the recording microelectrode. Amiloride (1 mM in 100 mM NaCl) or benzamil (1 mM in 100 mM NaCl) was iontophoresed out of the microelectrode tip (30 nA/sec, 0.5 Hz for 10 min). In all embryos, iontophoresis of these agents into the neural tube lumen produced an immediate 30–40% reduction in the magnitude of the TNTP, which continued its collapse (approximately 80% of initial values) by 15 min of iontophoresis. In 4 amiloride-injected embryos we followed a partial recovery of the TNTP to 12 hr after the initiation of iontophoresis. In no case did the TNTP regain its initial magnitude—or its *expected* magnitude—during this time, given that both the TEP and TNTP increase in size with developmental stage (Shi and Borgens, 1994). We have previously described estimates of the concentration of benzamil and amiloride near the electrode tip to be on the order of 5 μM. This is within the concentration range known to inhibit Na^+ uptake and the TEP of a wide variety of epithelium, and specifically the TEP of embryonic surface ectoderm (Shi and Borgens, 1994; Metcalf et al., 1994). Control injections (referred to as "vehicle") were carried out using the same iontophoresis parameters; however, the microelectrode contained only filling solution (100 mM NaCl; see also Shi and Borgens, 1994). All injections were carried out on stage 21–23 embryos (about 1.5 mm in length) possessing a closed, well-formed neural tube. All embryos were stripped of their jelly coat and vitelline membrane before being placed into special chambers for physiological measurement and iontophoresis (Shi and Borgens, 1994). Following iontophoresis, embryos were allowed to develop in their media for 36–52 hr until they were killed and fixed for histological processing.

Embryos were fixed in 4% glutaraldehyde, dehydrated in ascending concentrations of alcohol by conventional techniques, and embedded in either paraffin or medcast (plastic) resin. In the latter, semithin (1 μM) sections were cut using glass knives on a Sorvall Ultratome ultramicrotome. Plastic embedded sections were stained with Toluidine blue and paraffin embedded sections (approximately 10 μM) were stained using Masson's trichrome. All photography was performed on a Olympus Vanox Universal microscope.

Specific sections for histomorphometry were captured to a Macintosh Quadra 800 computer using RastorOps MediaGrabber™ software. Calibration lines for each magnification were obtained by using the index score of a hemacytometer at the same magnification. Morphometry was performed using IP Lab Spectrum software in which the thickness of both external and internal epithelial structure was derived at five predetermined sampling positions at 5 levels along the embryo's rostral/caudal axis (the level of olfactory, optic, auditory, cardiac, and hepatic primordia).

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