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Embryonic Neuroepithelial Sodium Transport, the Resulting Physiological Potential, and Cranial Development

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We have shown that the amiloride/novobiocin-sensitive sodium transport system of adult animal integuments is first observed in embryonic surface ectoderm and show here that this physiology is retained in this ectoderm following the closure of the neural folds. Unidirectional transport of Na^+ out of the neural tube lumen results in a potential difference on the order of 40–90 mV, negative with respect to the abluminal surface. This transneural tube potential can be collapsed by iontophoresis of Na^+ channel blockers amiloride or benzamil into the lumen, leading to severe cranial defects and incomplete morphogenesis. Modestly increasing the transneural tube potential with injection of novobiocin into the lumen also produces a lesser degree of developmental abnormality. We discuss the ways in which this physiology may help control the organization of the early nervous system. © 1994 Academic Press, Inc.

INTRODUCTION

It is both convenient and instructive to think of ectoderm as the embryo's "skin"—but not just in the sense that animal integument is derived from this germinal layer. The term defines not only that ectoderm is a physical barrier protecting the inside of the embryo from its external environment, but should as well convey the concept of an established polarity and physiology. The skin of adult vertebrates is polarized in the apical/basal dimension and can be characterized by particular physiological properties that are likewise polarized, such as the unidirectional transport of ions across itself (Kirschner, 1983; Borgens, 1982; Vanable, 1989). The neural tube is formed from such internalized "skin." Its lumen is filled with the water that a short time before bathed the neurula. In this report we are concerned with the physiological properties and polarity of the newly formed neural tube and if these properties play a role in cranial development and morphogenesis.

Previously it has been shown that the ectoderm of amphibian gastrulae and neurulae is physiologically polarized by a unidirectional transport of Na^+ from outside to inside producing an inwardly positive potential difference across itself (McCaig and Robinson, 1982; Metcalf *et al.*, 1994; Metcalf and Borgens, 1994). This is made possible by the early establishment of tight seals between cells of the ectoderm's apical domain. The development of tight junctions between embryonic cells forming an epithelial syncytium is an important step in development. Tight seals between cells usually appear coincident with a unidirectional transport of ions through them producing ionically distinct fluid compartments within the embryo and steady transepithelial potentials (TEPs). For example, in vertebrate embryos, development of high resistance (ca. 3 MΩ) tight seals in the apical domains between blastomeres coupled with extrusion of Na^+ from their basal membranes (carrying with it water) osmotically swells the blastocoele, "inflating" the blastula (Slack and Warner, 1973). This Na^+ transport results in a blastocoele about 20 mV positive with respect to the exterior (Regen and Steinhardt, 1986). During amphibian gastrulation, the surface ectoderm continues to display inwardly directed ionic currents, an internally positive TEP of about 20–50 mV which increases in magnitude with stages of development, and is temporarily dependent on external Na^+ concentration (McCaig and Robinson, 1982; Robinson and Stump, 1984; Metcalf *et al.*, 1994; Metcalf and Borgens, 1994).

Using axolotl embryos, we show that: (1) the TEP of the embryonic urodele increases in magnitude with stage of development in a manner similar to that of the anuran embryo (McCaig and Robinson, 1982), (2) this ectodermal TEP is maintained across the neuroepithelium of the neural tube following closure of the neural folds, (3) the resultant transneural tube potential (TNTP) retains the polarity and Na^+ dependence of surface ectoderm, (4) the TNTP is sensitive to apically applied Na^+

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channel blocking agents amiloride and benzamil and to novobiocin (which is known to increase the TEP of epidermal syncytia) in a similar manner to surface ectoderm, and (5) this TNTP is required for normal cranial development.

METHODS

Most of the relevant details concerning embryo use and husbandry, physiological measurement, and microscopy have been detailed in previous reports in this series (Metcalf *et al.*, 1994; Metcalf and Borgens, 1994). We direct the reader to this description and only provide abbreviated details here.

Embryos and Husbandry

Axolotl (*Ambystoma mexicanum*) embryos were obtained from the Indiana University Axolotl Colony as early gastrulae and staged according to Bordzilovskaya *et al.* (1989). They were housed at 5°C until use when they were moved to finger bowls at room temperature in 25% Holtfreter's medium (15 m*M* NaCl, 0.17 m*M* KCl, 0.23 m*M* $CaCl_2$, and 0.6 m*M* $NaHCO_3$; pH 7.4, and resistivity 600–650 Ω/cm). The jelly coat and vitellin membrane were mechanically removed prior to physiological measurement.

Physiological Recordings

Embryos, stripped of their extraembryonic membranes, were positioned dorsal surface up in an indentation made for them in the substrate of the measurement chamber (2% agar) and bathed in 25% Holtfreter's solution. Unbeveled 2 MΩ microelectrodes (100 m*M* NaCl filling solution, ca. 2 mV tip potentials) were pulled on a David Kopf 700 C and connected to standard bridge circuitry as described elsewhere (Metcalf *et al.*, 1994; Metcalf and Borgens, 1994). Chart recordings were made on a Fisher 5000 Recorder. TRITC-concanavalin A (2 mg/ml) was mixed with the microelectrode filling solution and iontophoreised into the lumen of the neural tube to confirm the placement of the electrode in five embryos (Fig. 1C). Since the marker is positively charged, this was accomplished using positive current pulses (30 nA/sec) at 0.5 Hz for 20 min.

Mean TEP measurements for each developmental stage were obtained in the following manner: TEPs were sampled at three to five locations along the dorsal midline (in neurula, this would be within the neural plate). (In embryos, peak TEPs are always observed at the dorsal surface; however, their magnitude varies with location at any one stage of development (Metcalf *et al.*, 1994)). The peak TEP was recorded for each of 8–12 embryos and pooled generating a mean value for each stage.

Pharmacological Agents

Amiloride, benzamil, and novobiocin (1 m*M*) were all purchased from Sigma Chemical Corp. and made up in the standard 100 m*M* NaCl microelectrode filling solution for iontophoresis. For the injection of pharmacological agents, 30 nA/sec current pulses were used at a frequency of 0.5 Hz for 10 min.

We have estimated the volume of the neural tube in ca. 3-mm-long embryos (2.5×10^{-8} L), the total charge injected by 50% duty cycle iontophoresis (6×10^{-6} C) and have applied Faraday's constant to estimate the molarity of amiloride within the neural tube at equilibrium (approximately 2.5–5 μ*M*). It is reasonable to assume higher concentrations in a region local to the electrode tip during iontophoresis. The concentration attained for all injected agents is within an order of magnitude (1–100 μ*M*) of bath concentrations effective in reducing the TEP (or ionic currents generated by it) in other studies (refer to Metcalf *et al.*, 1994; Metcalf and Borgens, 1994; and Robinson and Stump, 1984).

Iontophoresis of the microelectrode filling solution alone (referred to as vehicle) was performed as a control measure and with the same regimen as described above.

Histology

Approximately 48 hr following either experimental or control applications of benzamil, amiloride, novobiocin, or vehicle, viable embryos were immersion fixed in 4% glutaraldehyde, dehydrated through ascending grades of alcohol, and embedded in either paraffin or medcast resin by conventional techniques. These embryos included 5 from the amiloride-, 5 from the benzamil-, and 4 from the novobiocin-injected experimental groups and a total of 9 control embryos. These control embryos included those in which vehicle was iontophoreised into the neural tube lumen and those in which amiloride or benzamil was injected just beneath the surface ectoderm. Control embryos had developed to stage 31–33 when sacrificed; however, most age-matched experimental embryos could not be staged due to their aberrant morphology.

Paraffin-imbedded embryos were sectioned at 10 μm on a rotary microtome and were stained with a Masson's trichrome procedure. Plastic-imbedded embryos were sectioned at 1 μm on a Nova thermal feed ultramicrotome. All plastic-imbedded embryos were cut in cross section and stained with toluidine blue. Alternate paraffin-imbedded embryos were sectioned sagitally or in cross section. Photography was performed on an Olympus VAN OX Universal microscope.

RESULTS

The Neural Tube and Transneural Tube Potential

When a microelectrode is advanced through the ectoderm of the amphibian embryo, the sharp positive (with respect to the bath) deflection that is observed is the TEP (McCaig and Robinson, 1982; Metcalf *et al.*, 1994; Metcalf and Borgens, 1994) (Fig. 1). We have determined the ontogeny of the TEP in axolotls as has been reported for *Xenopus* (McCaig and Robinson, 1982) where the magnitude of TEPs climbs with developmental stage (Fig. 2A). As the microelectrode is advanced further into the lumen of the neural tube, a second and marked potential shift is observed, the transneural tube potential (TNTP). In the axolotl embryo, a neural tube potential (NTP) is strikingly negative with respect to the bath, and the sum of the TEP and the NTP represents the TNTP (Fig. 1B). We have made 69 microelectrode penetrations of the neural tube in stage 22 to 28 axolotl embryos recording TNTPs ranging from 11 to 124 mV. In every case the lumen was negative to the abluminal surface. Immediately following closure of the neural folds, the NTP is barely detectable, since the TNTP is roughly of the same magnitude as the TEP (Fig. 2B). In the embryonic stages following neural tube formation, both the TEP and the TNTP increase in magnitude steadily (Fig. 2), suggesting that the ectoderm, now internalized as an ion-transporting neuroepithelium, may retain some of the qualitative characteristics of surface ectoderm.

The Inside-Out Neural Tube

If the walls of the neural tube indeed function in a way similar to surface ectoderm, then the unidirectional movement of Na^+ which subserves the ectodermal TEP should be retained. Once neural fold closure is complete, the apical domain of surface ectoderm becomes the luminal domain of the neural tube (the fluid filling the lumen of the neural tube was a short time before the medium bathing the outside of the embryo). Amiloride or benzamil applied to the luminal (apical) surface of the neural tube should collapse the TNTP. Using microelectrodes containing amiloride, we first determined the TNTP of 14 embryos and subsequently iontophoresed amiloride into these lumens. This resulted in a fall in TNTP by 30–40% immediately following (even during) iontophoresis, and by 80% at 15 min following iontophoresis (Figs. 3 and 4; Table 1). We further confirmed the Na^+-dependent character of the TNTP by iontophoresing benzamil (Cuthbert and Fanelli, 1978; McGinnis and Vanable, 1986) into the lumen of another 14 stage 23–25 axolotl embryos. The drop in TNTP was similar to that of amiloride, as well as the continued gradual fall in potential for over 15 min of recording (Fig. 4A; Table 1). In 5 amiloride-treated embryos we followed the time course of this suppression and observed a gradual return to near normal values by 12 hr postinjection (Fig. 4B). Injection of vehicle into the lumens of 11 embryos had no effect on the TNTP (Fig. 4 and Table 1).

This amiloride-sensitive Na^+ channel of the apical membranes in epithelial syncytia is also known to be responsive to novobiocin, which facilitates Na^+ movement into these cells and boosts the TEP (Sariban-Sohraby and Benos, 1986; Rick *et al.*, 1988). Iontophoresis of novobiocin into the lumen of the neural tube moderately increased the TNTP in every case but one (Table 1).

Cranial Differentiation Following TNTP Collapse

The temporary suppression of the TNTP by amiloride and benzamil allows a test of the relevance of the TNTP to embryonic development. Though substantial suppression may only last for several hours (Fig. 4B), developmental change is correspondingly rapid during the early stages following neural tube formation. Both amiloride and benzamil were iontophoresised into the lumens of separate experimental groups of stage 22–23 embryos. Three control populations were studied as well. In one, the iontophoresis of vehicle into the lumen of the neural tube was performed in 11 embryos. In a second control group, amiloride was iontophoresed just beneath the surface ectoderm in 10 embryos and in the third group, benzamil was injected just beneath the surface ectoderm in 10 embryos. All embryos in which iontophoresis of vehicle into the neural tube lumen was performed (using a regimen identical to that used for drug injection as in Fig. 3) not only survived the injection but developed through stage 31–33, at which time embryos were killed and prepared for microscopy. All but 1 of the 20 control embryos in which amiloride and benzamil was injected beneath the surface ectoderm also developed normally through stage 33. All 14 embryos in which amiloride was injected into the neural tube failed to develop normally as did 13 of the 14 benzamil-injected embryos. Table 2 summarizes these effects of TNTP suppression as well as the responses to control applications and statistically compares the group responses to iontophoresis of these agents to control applications. By external observation the locus of most extreme developmental abnormality was the cranial enlargement near or at the region of iontophoresis. In most of these cases embryos went on to develop headless or with substantive deletions of CNS

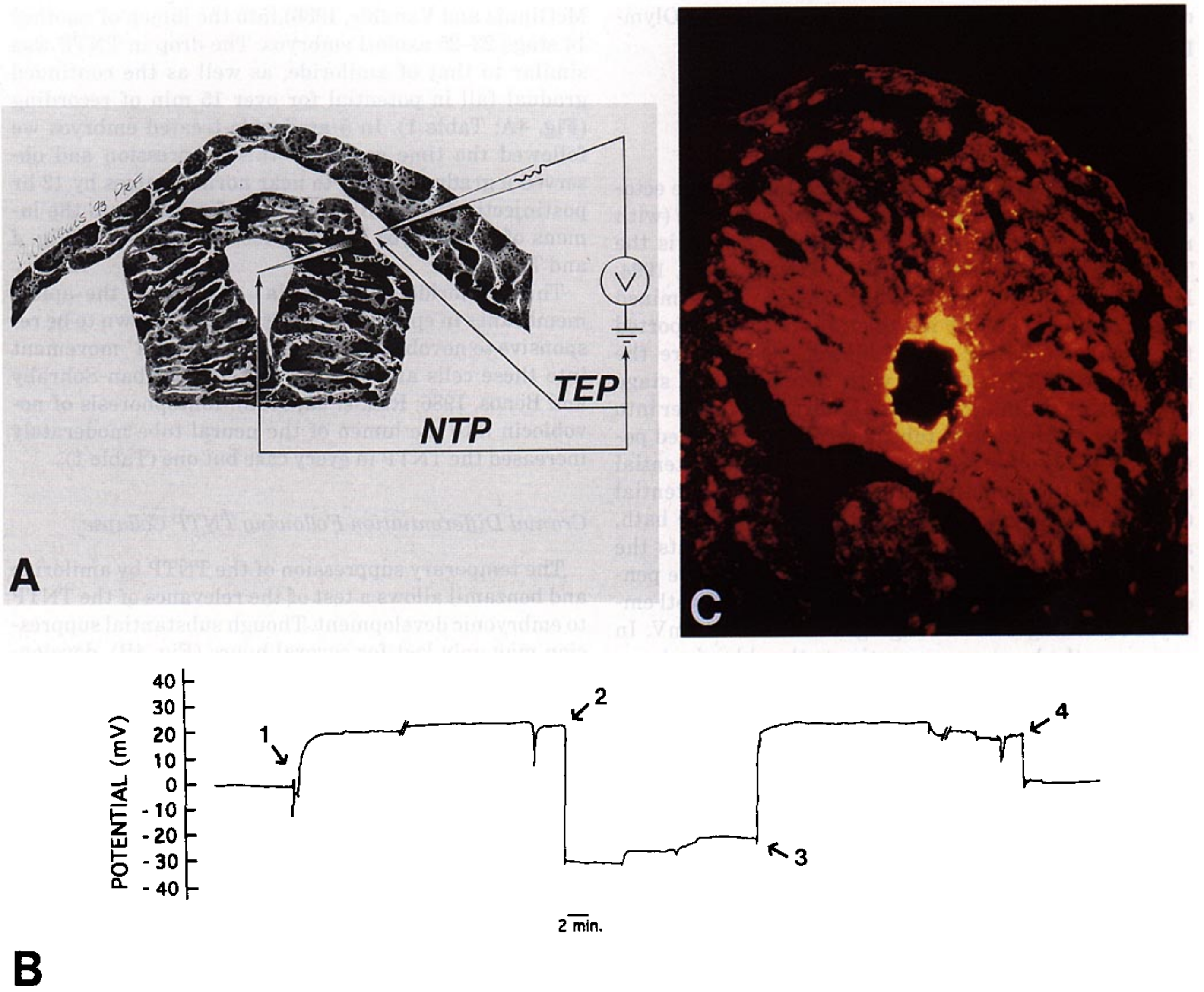

FIG. 1. (A) Microelectrode detection of TEP, NTP, and TNTP in the axolotl embryo. Steady advancement of the microelectrode through the ectoderm, and subsequently the neural tube registers voltage differences (TEP and NTP, respectively) with respect to the bath ground. (B) Electrical record of the TEP, NTP, and TNTP. At the beginning of the record, the microelectrode was advanced through the dorsal ectoderm of a stage 24 embryo, the positive deflection (with respect to bath ground) is the transepithelial potential (TEP) of about 20 mV (first arrow; break in record = 10 min of recording not shown). Deeper penetration into the lumen of the neural tube resulted in a sharp negative deflection (second arrow), a neural tube potential (NTP) of about 30 mV. The sum of the TEP and NTP represents the transneural tube potential (TNTP) which reflects an internally negative, 50-mV difference between luminal and abluminal surface of the neural tube. Further advancement of the electrode (about 50 μm), through the adjacent wall of the neural tube again revealed the TEP (third arrow). Break in this portion of the record represents another 10 min of recording not shown. Finally, at arrow 4, the microelectrode is withdrawn. Note the stability of the baseline. (C) Fluorescence photomicrograph of a cross section of a TRITC-concanavalin A-injected axolotl embryo. TRITC-con A was iontophoresed into the neural tube of this stage 23 embryo from the same electrode used to measure the NTP. The adhesion of the fluorescent marker to the inner surface of the neural tube confirmed the placement of the electrode tip within the lumen of five embryos.

structure (Table 2, Fig. 5). The modest *increase* in TNTP produced by novobiocin appeared to have no obvious effect on development by external observation (Table 2), *but did produce abnormalities detected by histology* (see below).

Control injection of either benzamil or amiloride under the ectoderm served to modestly and temporarily hyperpolarize the TEP of surface ectoderm (by less than 10%) *since both compounds are positively charged* (Table 1), but had no effect on development.

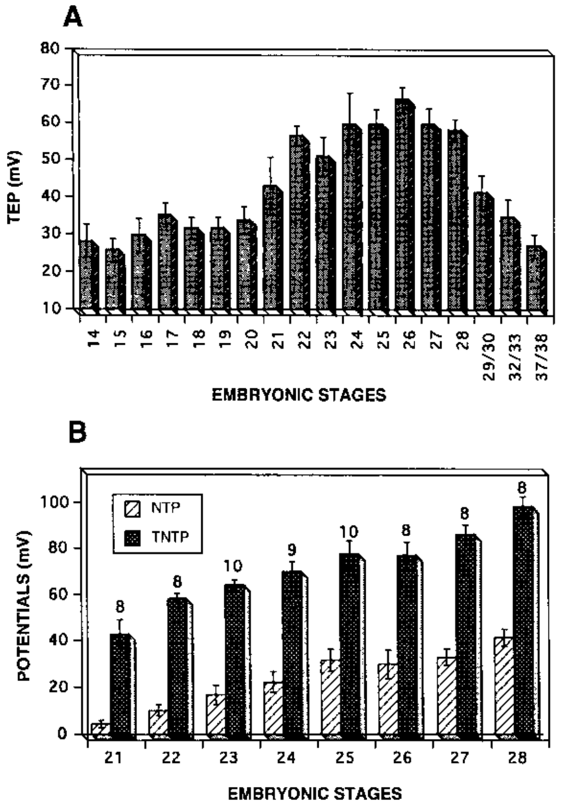

FIG. 2. (A) Ontogeny of the ectodermal TEP in *Ambystoma* embryos. Each data point represents measurements made on 8–12 separate embryos at each developmental stage. A total of 159 embryos constituted the sample population and mean values and SEM are presented for each stage. Note the steady increase in the magnitude of the TEP to stage 26 and its subsequent decline. (B) The average neural tube potential (NTP) and transneural tube potential (TNTP) in axolotl embryos by stage. Each paired bar indicates mean TNTP and NTP and the SEM. The number of embryos sampled is given above the paired bars. Measurement protocol as in Fig. 1. Note that the NTP was low immediately following the formation of the neural tube. Both NTP and TNTP increase steadily following sealing of the neural folds at stage 20/21.

Histology

All control embryos showed evidence of typical development relative to the stage at which they were sacrificed. This included all embryos in which vehicle was iontophoresed into the neural tube lumen and those embryos in which either amiloride or benzamil was iontophoresed just beneath the surface ectoderm. Easily identified differentiating structure included the formation of the various compartments of the early brain, the presumptive spinal cord and notochord, morphogenesis of olfactory and otic primordia, either optic vesicles or the formation of the eyecup and lens, differentiation and segmentation of somites along the long axis of the body, cardiac and brachial primordia, etc. (refer to Figs. 6A–6C). We detected no evidence of abnormal development, which included an inspection of the neural tube at the location of iontophoresis of vehicle. The presumptive spinal cord and brain at all locations was delineated by a well organized neuroepithelium, and their respective lumens appeared typical. The overall shape of the embryo body was, as well, typical for the respective stage of development (Bordzilovskaya *et al.*, 1989).

All embryos in which either amiloride or benzamil was injected into the lumen of the neural tube displayed grossly defective development. In 7 of the 10 benzamil-treated embryos analyzed, the interior of the embryo was comprised of a formless mass of cells. There was no evidence of any developing structure in these embryos, in spite of the fact that at the stage they were injected they would already have developed some early primordia (optic vesicle, brachial archs, pronephrous, etc.) and a well-developed neural tube. What differentiation had begun to occur in these embryos had not only become arrested, but had apparently been reversed by the experimental treatment. These embryos possessed some body cavities; however, none were bounded by any epithelium and these cavities were in no way similar to the early forming gut or nervous system. Curiously however, the external shape of some of the elongate embryos appeared relatively normal, with a clear tail and "down-turned" cranial enlargement (Bordzilovskaya *et al.*, 1989) in spite of the complete absence of internal structure (Fig. 6D). In the balance of benzamil-treated experimental embryos, some evidence of development was apparent. This included some differentiation of the brain (possessing grossly undersized lumens) but

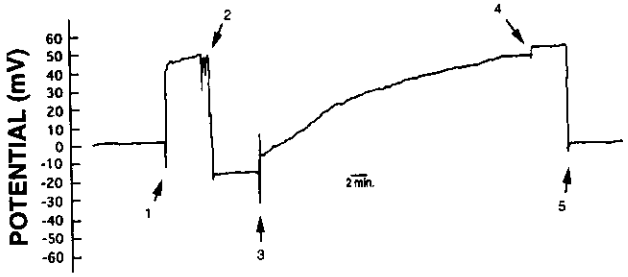

FIG. 3. TNTP response to iontophoresis of amiloride. Following penetration of dorsal ectoderm (first arrow), a ca. 65-mV TNTP was measured in this stage 24 axolotl embryo (second arrow, refer to Fig. 1). Five minutes later, amiloride was iontophoresed into the neural tube from the measurement electrode (third arrow). Note the slow steady fall in the TNTP. The elimination of the TNTP reveals the NTP to be dominated by the potential expressed only across the ectoderm itself (the TEP), as shown by passing the microelectrode completely through the neural tube (fourth arrow). At the fifth arrow, the microelectrode was withdrawn.

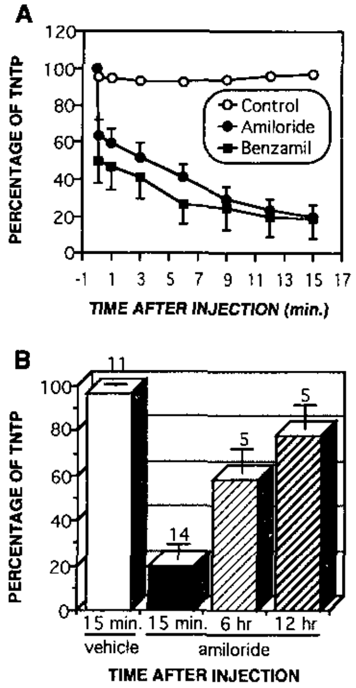

FIG. 4. (A) TNTP decrease in response to amiloride and benzamil. Top curve (○) represents the TNTP plotted against time after vehicle (100 mM NaCl) iontophoresis. Filled circles follow the decrease in TNTP after the injection of benzamil into the neural tube. Filled squares, follow the TNTP at time points after the injection of amiloride into the neural tube. Error bars, SEM. TNTPs are expressed as a percentage of the TNTP prior to the injection of either drug or vehicle into the neural tube. Eleven embryos were injected with vehicle, 14 with amiloride, and 14 with benzamil. (B) Recovery of the TEP from amiloride suppression. Decrease and recovery of the TNTP in the presence of amiloride. The average TNTPs (and their SEM) following injection of amiloride are plotted against three time points (15 min, 6 hr, and 12 hr). The average TNTP response to iontophoresis of vehicle is shown after 15 min of exposure time. Note the marked decrease in TNTP at 15 min postinjection and the significant recovery of the TNTP by 12 hr. The numbers above each bar show the total number of embryos measured at each time.

without accompanying development of an early spinal cord or notochord. The only other recognizable tissue or organ primordia within these embryos was sparse development (or remnants?) of somites. Only 1 of 5 amiloride-treated embryos developed without internal structure similar to that of the benzamil-treated embryos just described. The other 4 showed various amounts of defective morphogenesis, including unrecognizable ectodermal plaques, unrecognizable internal condensations of cells, some development of abnormal epithelial lined body cavities, and a degree of recognizable brain structure. Four novobiocin-treated embryos were evaluated histologically. These all appeared normal by external observation and 2 of these showed normal internal development typical for their stage of development at sacrifice. The other 2 possessed developmental abnormalities—indicated by the abnormal positioning of sensory primordia or vesicles and placodes that were exaggerated in size or otherwise unrecognizable. Other normal organ and tissue rudiments were present in these embryos.

DISCUSSION

The skins of most animals studied, including man, possess inwardly positive integumentary potentials ranging from about 20 to 60 mV (Kirschner, 1973, 1983; Barker *et al.*, 1980; Vanable, 1989). These potentials arise by a multistep process first analyzed in the classic investigations of Koefed-Johnson and Ussing (1958) using isolated frog skin. The apical membranes of the outer living layers of epidermis posses specific Na^+ channels facilitating diffusion of this ion into these cells to be actively pumped out of their lateral membranes and conjoined cells of the basal layers. These channels appear in three classes differing in their selectivity for Na^+, and their properties have been recently reviewed and summarized (Palmer, 1992, and Smith and Benos, 1991). Apical tight junctions limit the paracellular movement of Na^+, and so the transintegumentary TEP is relatively dependent on the concentration of external Na^+ as it moves down this electrochemical gradient. The TEP is rapidly (and reversibly) responsive to increases or decreases in the concentration of Na^+ bathing the apical surface (Helman and Fischer, 1977). Amiloride, benzamil, ethylisopropyl amiloride (EIPA), and the methyl ester of lysine (MEL) are known to specifically block the Na^+ channel of apical membranes in epithelial syncytia, resulting in temporary suppression or elimination of the transcutaneous voltage (Kirschner, 1973, 1983; Sariban-Sohraby and Benos, 1986; Robinson *et al.*, 1991; Palmer, 1992). Novobiocin, on the other hand, facilitates specific entry of Na^+ through apical epithelial membranes increasing both the short circuit Na^+ current and TEP in isolated epithelia (Johnson and Hoshiko, 1971; Rick *et al.*, 1988). These general physiological properties of adult epidermis are also manifest in the ectoderm of the embryo. Ectodermal TEPs (inwardly positive, 15–40 mV) and/or inwardly directed transcutaneous currents have been recorded in the embryos of frogs (McCaig and Robinson, 1982; Robinson and Stump, 1984), salamanders (Metcalf and Borgens, 1994; Metcalf *et al.*, 1994), birds (Jaffe and Stern, 1979; Stern and MacKenzie, 1983), and mammals (Winkel and Nuccitelli, 1989, see also Robinson *et al.*, 1991, for single cell studies). In amphibians, an ionic current leaving the blastopore driven by the ectoder-

TABLE 1

Number of embryos	Injection	Injection site[a]	TNTP or TEP[b] before injection (mV)	TNTP or TEP[b] after injection (mV)	Statistical comparison[c]	Comments
14	Amiloride	Neural tube lumen	61 ± 2.6 (49–81)	11 ± 3.2 (0–46)	$P < 0.0001$	Collapse of TNTP
10	Amiloride	Beneath surface ectoderm	41 ± 7.5 (17–82)	44 ± 6.4 (17–78)	$P = 0.7$	Slight increase in TEP[d]
10	Benzamil	Neural tube lumen	58 ± 2.8 (36–72)	16 ± 4.9 (0–57)	$P < 0.0001$	Collapse of TNTP
10	Benzamil	Beneath surface ectoderm	36 ± 2 (26–45)	39 ± 2.8 (25–51)	$P = 0.3$	Slight increase in TEP[e]
8	Novobiocin	Neural tube lumen	42 ± 6.1 (18–66)	47 ± 5.9 (24–68)	$P = 0.5$	Slight increase in TNTP[f]
11	Vehicle	Neural tube lumen	76 ± 7.1 (39–100)	73 ± 6.5 (39–95)	$P = 0.7$	Slight decrease in TNTP[g]

[a] Iontophoresis into lumen of neural tube or beneath ectoderm using microelectrode (see text).

[b] TNTP values given for injection into neural tube; TEP values given for injection under surface ectoderm. Mean and SEM in mV's; range in parentheses.

[c] TNTP and TEP comparisons used two-tail Student's *t* test.

[d] 7/10 embryos showed a 7% increase in mean potential.

[e] 7/10 embryos showed an 8% increase in mean potential.

[f] 7/10 embryos showed a 12% increase in mean potential.

[g] 8/11 embryos showed a decrease in the mean potential of less than 4%.

mal TEP is rapidly and reversibly responsive to changes in the external concentration of Na^+ and to the drug amiloride dilute (50 μM) in the bathing medium (Metcalf and Borgens, 1994; Metcalf *et al.*, 1994). Curiously then, the physiological characteristics of adult animals' skins are already present in the earliest ectodermal covering of the embryo. Furthermore, Hotary and Robinson (1990) also report an inwardly negative TNTP in *Xenopus* embryos. However, they were uncertain of the physiological polarity of the neural tube wall, as no test to determine this was performed. The TNTP of the anuran embryo appears to

TABLE 2

				Developmental response					
Number of embryos	Injection	Injection site[a]	Died	Severe cranial[b] defects—nonviable	Severe cranial[c] defects—viable	Modest cranial[d] defects—viable	Statistical evaluation[e]	Number of viable embryos sectioned	Number with histological defects
14	Amiloride	Neural tube	0	5	7	2	$P < 0.0001$	5	5
10	Amiloride	Beneath surface ectoderm	0	0	0	0	NP[f]	1	0
14	Benzamil	Neural tube lumen	0	0	11	2	$P < 0.0001$	5	5
10	Benzamil	Beneath surface ectoderm	0	0	0	0	NP[f]	4	0
8	Novobiocin	Neural tube lumen	0	0	1	0	$P < 0.035$	4	2
11	Vehicle	Neural tube lumen	0	0	0	0	NP[f]	4	0

[a] Iontophoresis into lumen of neural tube or beneath ectoderm using microelectrode (see text).

[b] Major deletion of head structure, most developed nearly "headless." The embryos were unable to develop to stage 30/31.

[c] Major deletion of head structure but embryos viable past stage 30/31.

[d] Loss of at least one externally detectable primordium (i.e., optic, otic, brachial, buccal) or contorted body structure.

[e] Statistical comparison: Fisher's exact test compares abnormalities detected by external morphology in experimental embryos with control (vehicle-injected) embryos.

[f] No change, statistical evaluation not performed (NP).

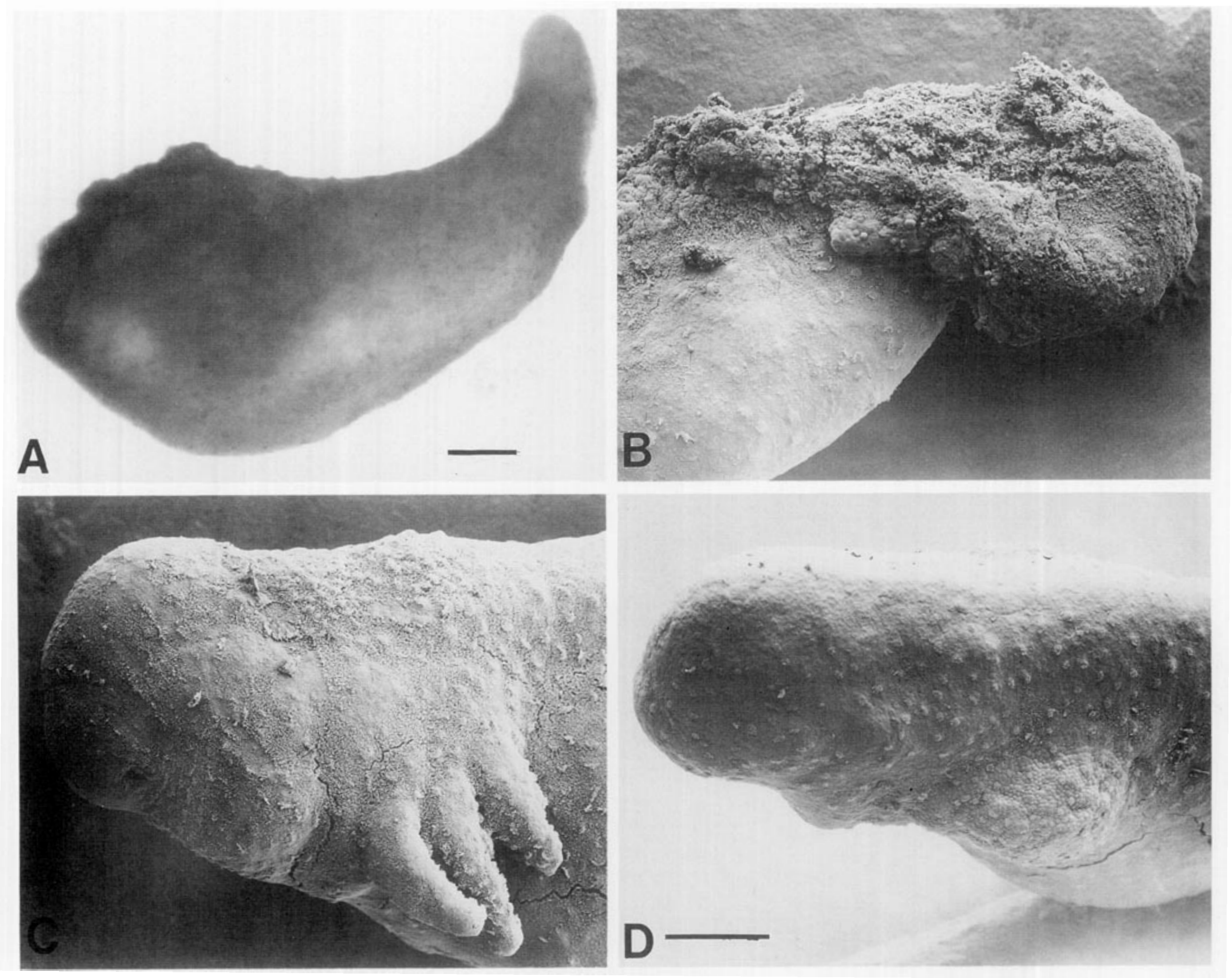

FIG. 5. Effects of amiloride and benzamil Iontophoresis on cranial development. (A) Stereomicrograph of a benzamil-treated embryo within its culture chamber. This embryo was injected at stage 23 and continued development for 28 hr in the absence of any continuing cranial differentiation. Such nearly "headless" embryos were not viable and represent the most severe structural abnormalities caused by collapse of the TNTP. (B) Scanning electron micrograph of an amiloride-injected embryo (unable to be staged.) The cranial enlargement exvaginated 48 hr after iontophoresis of amiloride into the neural tube lumen at stage 23. The body of this embryo was contorted and abnormal as well. (C) Scanning electron micrograph of an amiloride-treated embryo (unable to be staged) injected at stage 23. Note the well-developed external gills and the complete absence of externally visible optic or otic differentiation. (D) Scanning electron micrograph of a control (carrier-injected) embryo. Iontophoresis of vehicle into the neural tube lumen was completed at stage 23. At stage 33 this embryo shows normal conformation, as did all embryos in this control series. Note the well-developed otic, optic, and gill primordia. Scale bar for A, 350 μm; B–D, 300 μm.

be on the same order of magnitude and of the same polarity as that of the urodele embryo described here.

Effects of Amiloride, Benzamil, and Novobiocin

The inability of amiloride to permanently block epithelial potentials is also typical of the ectoderm of urodele embryos and the skin of adults (Metcalf and Borgens, 1994; Metcalf *et al.*, 1994; Borgens *et al.*, 1979). We believe this response to the long-term reduction in the access of external Na^+ to be achieved by the adaptive transport of another ion(s) across embryonic and adult epithelia rescuing the TEP (in this case the TNTP); however, the ionic composition of such adaption currents is unknown.

Suppression of the TEP is not observed when amiloride or benzamil is injected beneath surface ectoderm since these pharmacological agents must be applied only to the outer surface of ectodermal cells to be effective in

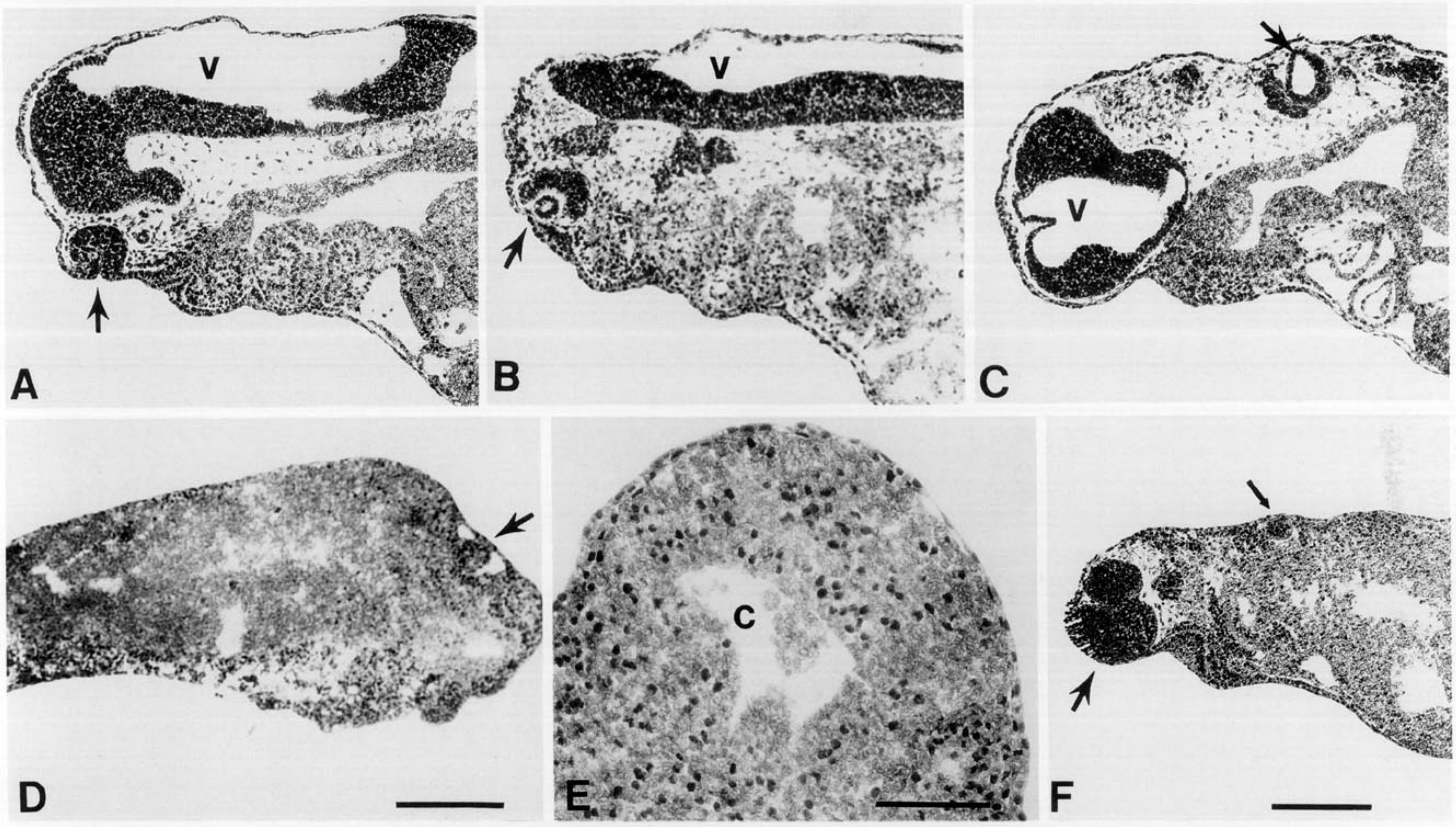

FIG. 6. Histology of embryos. (A, B, and C) Normal development in a stage 23 control axolotl. Iontophoresis of benzamil under the surface ectoderm was performed in this embryo. Saggital sections in A show a normal olfactory pit (arrow); in B, eye and lens (arrow); and in C, otic vesicle (arrow). Note as well the developed cranial enlargement, ventricles (v) of the forming brain, and heart primordia (at the mid ventral surface of each photomicrograph). This example was identical to all embryos where vehicle was iontophoresed into the neural tube lumen or to injections made beneath surface ectoderm. D and E show the lack of developing structure in experimental embryos in which the TNTP was collapsed by iontophoresis of benzamil into the neural tube lumen. In D, the body shape of the embryo was reasonably normal for a stage 30^+ embryo; however, no primordia (such as the olfactory pit, optic, otic structure, or gill) was externally visible. Upon sectioning, it was discovered that no internal differentiation had occurred at all. This embryo lacked all primordia or organ rudiments, including somites, notochord, brain, gut, or CNS accessory structures. In E, another benzamil-treated experimental embryo remained viable for about 40 hr following iontophoresis. Externally and internally, this embryo was an amorphous mass of cells. The cavity (c) extended throughout the embryo, was not bounded by an epithelium, and had no normal counterpart in normally developing controls. In F, a novobiocin-injected experimental embryo shows an intermediate level of abnormality. This embryo remained viable until sacrifice at stage 33. The olfactory and optic primordia (large arrow) were exaggerated in size and abnormally located. What appeared to be the rudiments of an otic vesicle (small arrow) was underdeveloped and located far central from its normal location. This embryo had no notochord, neural tube, or cranial enlargement (early brain) and developing somites were only found at the tail region. The scale bar for D, 0.4 mm; for E, 0.16 mm; for F, 0.25 mm. The scale in F is the same for A, B, C.

reducing the TEP through blockade of apically located Na^+ channels. Since these agents are positively charged at physiological pH, the injection did cause a slight hyperpolarization of the TEP (Table 1) in most cases. Moreover, these control experiments show that amiloride and benzamil do not have a general toxic effect on embryonic development. Iontophoresis of novobiocin, which leads to a modest (12%) increase in the TNTP, had an intermediate effect on development (see below). The sometimes striking effect on the TNTP and the vitiation of cranial development by these dissimilar agents suggest that the TNTP may be important for CNS differentiation and patterning. Furthermore, any major change in the size or structure of the developing neural tube is likely to produce a cascade of secondary effects, not only on secondary CNS structures, but the general development of the embryo, which is dependent on many tissue interactions and inductive events. We believe these results show that the TNTP itself may be required for normal ontogenetic development (see below). However other possible effectors might include: (1) an induced salt imbalance via blockage of luminally transported Na^+; (2) a change in neural tube structure produced by an interruption in the bulk movement of water across the neuroepithelium, and (3) possibly subtle effects on the pH or ionic concentration of neuroepithelial cells secondary to the alteration in their Na^+ transport. (1) We believe an explanation for embryonic deficits based on a general salt imbalance highly unlikely given the minute volume of neural tube fluid and the osmoregulatory capability of the amphibian embryo, which can develop normally in media of a wide range of Na^+ concentrations. (2) It is more likely that a gross change in the morphology of the neural tube could be caused by a retention of luminal water (secondary to a reduction in outwardly transported Na^+) and this could affect subsequent development. However, this notion is not supported by the small size (or complete absence of) lumens in benzamil- or amiloride-injected neural tubes (they would be expected to swell) or the fact that some novobiocin-injected embryos displayed a similar (but less severe) teratological response to amiloride injection. This being said, we still believe changes in water movement across the early neuroepithelium per se may still be an important response to these applications and worthy of further investigation. (3) It is possible, though in our view unlikely, that interrupted development occurred in response to an increase or decrease in the transport of some other ion or pH, given the high selectivity of the amiloride-sensitive channel for Na^+. Furthermore, the concentrations of amiloride that affect Na^+/H^+ exchange is at least 10-fold higher than that required to block the Na^+ channel (Smith and Benos, 1991; Palmer, 1992). We applied concentrations at the low end of even this range, and we do not know the consequences to development of minor shifts in intracellular pH of the lining cells of the early neural tube.

The TEP, TNTP, and Morphogenesis

The preliminary morphology presented here clearly shows both an interruption of, and loss of, cellular differentiation caused by TNTP reduction or even its slight increase (a more complete morphological evaluation of this teratology will be presented in a separate publication). Altogether, we believe the most likely mechanism of action to be a requirement for the TNTP itself—both for maintaining the integrity of the neuroepithelium and its physical influence on cell structure, polarity, and possible migration. This notion receives support from a literature clearly describing both tropic and trophic responses of embryonic cells to DC electric fields much weaker than that measured to exist across the walls of the neural tube.

The electric field associated with an 80 mV p.d. across a 50 μm wide neuroepithelium of the stage 28 axolotl would exceed 1 V/mm. In *Xenopus*, the internally negative TNTP of 15 mV across the 30 μm wide neural tube of stage 22 embryos (Hotary and Robinson, 1991) would be associated with a substantial electrical field of about half this value. These very large extracellular voltage gradients would normally exist across differentiating cells of the neuroepithelium. This fact raises three interesting notions. First, the steepest drop across the syncytia would probably be across cells lining the lumen itself—since the highest electrical resistance would expected to be at this region of tight junction sealing. It is in this region of the presumptive CNS that neural cell division and differentiation begins (Purves and Lichtman, 1985). Secondly, if one considers the geometry of the neural tube, the largest field (given a uniform TNTP) would be expected across the floor plate region, since the neural tube wall is thinnest here. This region is of particular importance to CNS patterning and differentiation (Yamada *et al.*, 1991; Pownall, 1994). Finally, the numbers of amphibian neuroblasts (as well as other cells disaggregated from neural tube) that are stimulated to grow in culture is strikingly increased by the presence of a weak (100–200 mV/mm) extracellular voltage (Hinkle *et al.*, 1981). One might reasonably suggest that the culture media for developing neurons *is not complete* without a weak polarized gradient of voltage imposed across the cell's exterior.

Embryonic cells such as neurons, myoblasts, fibroblasts, neural crest, and glia are extremely sensitive to extracellular voltage gradients (reviewed by Robinson, 1985; Nuccitelli, 1988; Borgens, 1992). Extracellular voltage gradients on the order of 1-100 mV/mm are sufficient to induce (1) substantial cellular migration (usually toward the cathode or negative pole of the imposed field), (2) structural reorganization (isodiametric cells such as fibroblasts and myoblasts become bipolar and align perpendicular to the voltage gradient), (3) cell process formation and tropic growth (usually toward the cathode), or (4) cell process retraction (usually toward the anode). The very gross structural abnormalities, the lack of cellular differentiation, the apparent dedifferentiation of primordia already formed, and the production of "pseudoembryos" (a relatively normal external shape without any normal internal cellular differentiation) suggests the TNTP to be very fundamental to pattern formation, morphogenesis, and the maintenance of differentiated structure. Moreover, the possible control of neural development by unidirectional Na^+ transport and/or its associated potential difference adds to an emerging literature detailing a controlling role of ectodermal TEPs in early vertebrate development.

The ectodermal TEP drives current out of the blastopore and neural folds in *X. laevis* and *A. mexicanum* supporting steady, polarized, internal transembryo potential differences (ca. 10-20 mV/mm) prior to neural tube formation (Metcalf *et al.*, 1994). When slightly higher exogenous voltages (25-75 mV/mm) are artificially imposed across these neurulae, developmental abnormality and arrest is produced (Metcalf and Borgens, 1994). Moreover, the locus of aberrant development at the head or tail is dependent on the polarity of the external field application. Endogenous currents (producing similar extracellular voltage gradients) leave the posterior intestinal portal during tail morphogenesis in the chick embryo (Hotary and Robinson, 1990). Reduction in these natural voltages accomplished through shunting the endogenous current by mechanical means leads to aberrant tail morphogenesis (Hotary and Robinson, 1992). Therefore, we believe it probable that a steady potential difference acts back on cells of the neuroepithelium to confer some form of developmental control through its action on cell organization, migration, or structural alignment.

Furthermore, a loss or reduction in this important control leads not only to a failure in the cascade of inductive and permissive cellular interactions important to morphogenesis, but our data suggest that the potential is required for the maintenance of developing structures as well.

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