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Neural induction in the absence of Organizer in salamanders is mediated by Ras/MAPK

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Abstract

Research on the mechanisms of embryonic induction had a great setback in the 1940s when Barth discovered and Holtfreter confirmed that ectoderm of *Ambystoma maculatum* salamander embryos could form brain tissue when cultured in a simple saline solution. We have revisited this classical experiment and found that when cultured animal cap ectoderm attaches to a glass substratum, it can self-organize to form complex organs such as brain vesicles, eyes, lens and olfactory placodes. Only anterior neural organs were generated. Under these culture conditions ERK became diphosphorylated, indicating a sustained activation of the Ras/MAPK pathway. Using sand particles as an heterologous neural inducer similar results were obtained. Addition of U0126, a specific antagonist of MEK, the enzyme that phosphorylates ERK/MAPK, inhibited neural differentiation. The closely related control compound U0124 had no effect. We conclude that neural induction in the absence of organizer in *Ambystoma maculatum* requires Ras/MAPK activation. These findings provide a molecular explanation for the activity of heterologous neural inducers that dominated thinking in amphibian experimental embryology for many decades.

Keywords

Ambystoma maculatum; *Xenopus*; Ras; MAPK; ERK; BMP; FGF; brain differentiation; Smad1; embryonic induction

Introduction

The discovery of embryonic induction was one of the most important advances in Biology during the first half of the 20th century, which culminated in the award of the 1935 Nobel Prize in Medicine to Hans Spemann (Hamburger, 1988). The key discovery was that neural induction in ectoderm required signals emitted by the underlying dorsal mesoderm, or Spemann organizer (Spemann, 1938). Following this “primary induction”, subsequent secondary inductions caused the differentiation of other tissues such as eye lens, olfactory placodes, auditory vesicles and many other organs (Holtfreter and Hamburger, 1955). The demise of this productive period of experimental embryology came with the premature pursuit of the chemical nature of the primary inducer (reviewed in De Robertis, 2006). Surprising to them at the time, it was observed that dead organizers and even cells from non-inducing tissues killed by heat or other treatments were able to induce anterior neural tissue when placed in contact with blastula ectoderm (Bautzman et al., 1932; Holtfreter, 1934, 1951). This triggered a race to purify the

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inducing factors. A bewildering number of chemical neural inducers were found, such as fatty acids, ribonucleoproteins, and even obviously unnatural inducers such as methylene blue and sand (SiO₂) particles (Holtfreter, 1951; Holtfreter and Hamburger, 1955).

In most amphibians, ectodermal animal cap explants differentiate into epidermis when cultured. However, in 1941 Lester Barth made the startling discovery that animal cap ectoderm of *Ambystoma maculatum* (a salamander related to the Axolotl that at the time was called *Amblystoma punctatum*) could give rise to massive neural differentiations in the complete absence of any inducer, simply by culturing it in saline solution (Barth, 1941). Johannes Holtfreter confirmed this observation, and argued that the induction might be caused by a relay mechanism through the influence of dying cells (Holtfreter, 1944, 1947). Holtfreter concluded that “the closer the cells were brought to the brink of death, the more pronounced was their tendency to become neural”, in a process he designated “sublethal cytolysis” (Holtfreter, 1944, 1947). He suggested that ectoderm has within it a neural tendency that is internally self-inhibited and that sublethal conditions would remove this inhibition (not unlike the current “default model” of neural induction), allowing the ectoderm to manifest the property of forming a central nervous system (CNS) morphogenetic field (Holtfreter, 1945, 1951). Thus, the early era of research on embryonic induction came to an end. Pieter Nieuwkoop revisited the problem of autoneuralization in *Ambystoma punctatum* but could not confirm the association between cell death and CNS differentiation (Nieuwkoop, 1963). Instead, he discovered that ectodermal explants of the frog *Rana pipiens*, which normally differentiates into epidermis, would form anterior CNS structures if disaggregated in Ca²⁺ free saline solution, followed by reaggregation in the presence of Ca²⁺ (Nieuwkoop, 1963). Decades later, with the advent of molecular biology, the endogenous neural inducers from Spemann’s organizer were isolated (Harland, 2000; De Robertis, 2006). However, the mechanism of autoneuralization in the absence of organizer or by heterologous inducers has remained a mystery to this day.

Modern research in amphibian neural induction is carried out in the *Xenopus* model system, which is much more resistant to autoneuralization. A key finding came with the rediscovery that when ectodermal cells are dissociated from each other for 3 hours or more, they undergo neural instead of epidermal differentiation (Grunz and Tacke, 1989; Sato and Sargent, 1989; Godsave and Slack, 1989). It was proposed that endogenous BMPs produced by ectodermal cells might diffuse and become diluted after dissociation (Wilson and Hemmati-Brinvalou, 1995; Muñoz-Sanjuan and Brivanlou, 2002), in what has become known as the “default” neural induction model (Stern, 2006). However, recently it was found that BMPs continue to signal in dissociated *Xenopus* animal cap cells. Instead, ectoderm dissociation causes a sustained activation of Mitogen-Activated Protein Kinase (MAPK) through activation of the Ras signaling pathway (Kuroda et al., 2005). This causes neuralization of the ectodermal cells by decreasing the activity of the BMP-responsive transcription factor Smad1 via phosphorylation at specific inhibitory MAPK sites (Kretzschmar et al., 1997; Pera et al., 2003; Sapkota et al., 2007).

In this study, we have re-investigated the classical problem of neuralization of *Ambystoma maculatum* ectodermal explants in the absence of organizer. We were able to reproduce, after some modifications, the anterior brain differentiations described by Barth and Holtfreter and now provide a molecular explanation for these landmark observations in embryonic induction. Experiments using U0126, a specific chemical inhibitor of MAPK ERK Kinase (MEK), the MAPKK enzyme that phosphorylates and activates the ERK/MAPK, indicate that neural induction in the absence of organizer is mediated by the activation of the Ras/MAPK signaling pathway.

Materials and methods

Embryo manipulations

Ambystoma maculatum embryos collected in ponds in Tennessee were purchased from Charles D. Sullivan Co. Inc. during the breeding seasons of 2005, 2006 and 2007 (February-March). Ectodermal explants (2 mm squares) were excised from late blastula or early gastrula embryos with sharp forceps and cultured in filter-sterilized Holtfreter's standard saline solution (Holtfreter, 1945) (60 mM NaCl, 0.7 mM KCl, 2.4 mM NaHCO₃, 0.68 mM Ca₂Cl and 0.05 g/l kanamycin, pH 8.3) and cultured in sterile conditions at room temperature for 8 to 10 days until sibling larvae reached stage 39-40 (Harrison, 1969). Unoperated embryos were raised in 50% Holtfreter's solution. The specific MEK inhibitor U0126 and its negative control U0124 (Calbiochem) were applied at a concentration of 40 μ M (and 0.4% v/v dimethyl sulfoxide) in Holtfreter's solution. Explants were prepared and attached to a sterile 60 mm diameter Petri glass dish containing 5 ml of inhibitor solution. U0126 solution was replaced after 8 h and then again at 24 h of culture and, after three additional days of culture in dark conditions (inhibitor is light-sensitive), replaced with Holtfreter's standard solution. The DMSO vehicle alone did not inhibit neural differentiation. The sand used as heterologous inducer was SiO₂, 50-70 mesh particles size, from Sigma. Histology was as described in <http://www.hhmi.ucla.edu/derobertis>.

Whole mount in situ hybridization

To generate probes specific for *A. maculatum*, RNA was extracted from stage 20 embryos and reversed transcribed. Degenerate primers for nested PCR were designed by comparing the sequences of human, mouse, zebrafish and *X. laevis* Sox2: Sox forward outer (ATGAAYGCNTTYATGGTNTGG), Sox forward nested (GCGCGCAAGCTTAARATGGCNCARGARA, containing a HindIII site), Sox reverse nested (GCGCGGAATCCCCANCCRTTCATRTGNG containing an EcoRI site), Sox reverse outer (TACATNSWDATCATRTC). To clone *cytokeratin*, primers were chosen within regions conserved between *Xenopus laevis* and the salamanders *Ambystoma mexicanum* and *Ambystoma tigrinum tigrinum*: cytokeratin forward (GACAACGCCAGGCTGGC) and cytokeratin reverse (CCTCTTGGTGGTTCTTC). Sox PCR product was digested with HindIII/EcoRI and subcloned into pCS2 vector. The *cytokeratin* PCR product was introduced into pGEM-T-Easy through TA cloning. Sequences were blasted against the EST database. The Sox sequence obtained from *A. maculatum* was found to be closest to Sox3. The 227 nucleotide Sox3 and the 196 nucleotide *cytokeratin* probes were prepared by linearizing plasmids with HindIII and SalI, respectively, transcribed using T7 RNA polymerase (Ambion), and purified through a G-50 Sephadex column (Roche).

Western blots

Western blots were as described previously (Kuroda et al., 2005), except that Petri dishes were cooled on ice before detaching explants from the glass surface in order to prevent activation of MAPK. Primary antibodies used were monoclonal mouse antibody against diphospho-ERK1/2 (Sigma M8159, 1:1,000 dilution) and rabbit polyclonal antibody that detects total p44/42 MAP kinase (Erk1/2) protein (Cell Signaling Tech. #9102, 1:700 dilution).

Results

Anterior brain differentiations in *Ambystoma maculatum* ectoderm

Ambystoma maculatum ectodermal explants cultured in Holtfreter's saline solution on agarose-coated Petri dishes remained unattached to the dish and their edges curled up to enclose the unpigmented internal cell layer in a process that took one to two days. We were able to show

that some of these explants can differentiate into anterior CNS. Fig. 1A shows one such explant in which a group of strongly basophilic neuroepithelial cells differentiated at the top of the explant. This structure could be identified as an eye structure because it contained retinal pigmented epithelium (rpe, Fig. 1A inset). However, the vast majority of explants differentiated into atypical epidermis with irregular infoldings on the surface (Fig. 1B). This kind of epidermis is designated as atypical because it lacks the normal epithelial substratum of the basement membrane. Atypical epidermis can be recognized histologically by the well-spaced cuboidal cells and multiple internal cavities resulting either from surface infoldings or from nests of keratinized cells (Figure 1B).

These initial experiments indicated that CNS tissue could be obtained, although at low frequency. Holtfreter, who had obtained a higher proportion of CNS differentiations than Barth (60% versus 14%), mentioned *en passant* that most of his explants developed attached to the surface of the glass Petri dish (Holtfreter, 1944). Consequently, we next investigated whether attachment of the explants to the glass surface could increase the frequency of CNS differentiation. We compared explants cultured on agarose coated plates to those cultured on glass Petri dishes in which the cells strongly attached, shield-like, to the surface and closure of the explant was prevented. We found that attachment to the glass surface greatly facilitated CNS differentiation, which increased from 2% to 51% of the explants (Fig. 2A).

Ambystoma maculatum in situ hybridization probes for the pan-neural marker *Sox3* or the epidermal marker *cytokeratin* were used to confirm neural differentiation in explants attached to a glass substratum (Figure 3A, B). We also used these probes to show that an heterologous inducer, sand particles (SiO_2 purchased from Sigma), caused neural differentiation in *Ambystoma maculatum* ectoderm in 69% of explants (n=29). Cells in direct contact with SiO_2 grains expressed the neural marker *Sox3* (Fig. 3C) and were negative for the epidermal marker *cytokeratin* (Fig. 3D). As shown in Figure 4, the results obtained by histology could be confirmed using molecular markers: whereas 68% (n=37) of explants cultured attached to the substratum were positive for *Sox3*, only 17% (n=54) of explants cultured non-attached (on agar) were positive (three independent experiments). Using the Cytokeratin probe all caps attached to glass (n=21) showed negative-staining regions, whereas non-attached caps were strongly epidermal (n=15).

Histologically, neural tubes could be readily recognized as a basophilic neural epithelium surrounding a clear lumen or ventricular cavity (Fig. 5A). In many cases, the separation of brain tissue into gray (neurons) and white (myelinated axons) matter could be distinguished, as in intact stage 39 sibling larvae (Fig. 5A, compare to 5D). Anterior sensory organs such as eyes containing retina and retinal pigmented epithelium, as well as olfactory placodes, could be observed adjoining the brain vesicles, and in one case a lens differentiated (Fig. 5A). This indicated that after the initial induction of forebrain tissue, secondary inductions such as olfactory placodes and lens did take place.

An interesting feature of these brain structures was that a neural plate was never formed on the surface of the explant, as is the case in normal development (data not shown). Thus, neural tubes must have been able to differentiate and become segregated from the epidermis that envelops them (Figs. 5A and 6A) without undergoing the normal process of neurulation, in which a tube is formed by closure of the neural folds. This mechanism of secondary cavitation of the neural tube had been previously observed to occur when ectodermal cells were dissociated and allowed to reaggregate (Townes and Holtfreter, 1955). Posterior neural structures such as auditory vesicles, rhombencephalon (which can be recognized by its thin dorsal roof, Fig. 5D), or spinal cord were never observed. This indicates that the induced CNS consisted exclusively of fore- or mid-brain and associated sensory organs, a type of

differentiation that in the older literature was designated as “archencephalic” (Holtfreter and Hamburger, 1955).

Neural tubes could be readily distinguished from epidermis (Figs. 1B, 5B), but a range of intermediate forms, designated as “neuroid” (Holtfreter, 1944) were also present. Neuroid structures lacked the keratin-containing cavities of atypical epidermis and some had a certain degree of epithelial cell organization (Fig. 5C). Neuroids comprised 15 to 35% of our explants, and while many of them represented epidermis others may have reflected low-grade neural differentiations. The difficulties in making a precise determination are exemplified in Fig. 1A, in which the non-neural part of the explant has regions both resembling epidermis (lower right) and neuroid (left, nrd) tissue. Therefore, following Holtfreter, we classified the explants as neuroid when a CNS lumen could not be identified (Holtfreter, 1944). Comparison of the frequencies observed by *Sox3* hybridizations (Figure 4) and by histological analyses (Figure 2A) suggests that neuroid tissues were positive for the neural marker gene. In two cases, neuroid explants differentiated anterior epidermal structures called balancers (Fig. 5C, ba), which normally form in tadpoles just anteriorly to the gills (Fig. 5D, inset). Balancers formed in ectodermal explants lacked mesoderm in the inner core of the structure, as is the case in the normal larva (compare Figs. 5C and 5D). In only 5 explants (out of a total of 303 serially sectioned and examined in the course of this work) did we observe formation of ventral mesoderm and blood, indicating mesodermal contamination; these explants were discarded.

Heterologous inducers activate MAPK/ERK

We next investigated whether explant attachment to glass affected the levels of Ras/MAPK activation. In *Xenopus* embryos, the simple extirpation of an animal cap, or wounding of the embryo, is sufficient to activate ERK for about 15 minutes, during which it becomes diphosphorylated by MEK (LaBonne and Whitman, 1997; Christen and Slack, 1999; Ribisi et al., 2000; Kuroda et al., 2005). A monoclonal dpERK antibody recognized the *Ambystoma* activated enzyme and showed that ERK remained active even after 3 hours of culture in saline on agarose surfaces (Fig. 2B, lane 7). Indeed, *Ambystoma maculatum* animal caps have large cells and take a very long time to curl up and cover the wound, a process that takes up to 2 days compared to less than 30 minutes in *Xenopus*. This prolonged Ras/MAPK pathway activation might explain the propensity of *A. maculatum* ectoderm to form neural tissue described by previous authors (Barth, 1941; Holtfreter, 1944; Nieuwkoop, 1963). The levels of activated ERK were increased in ectodermal sandwiches containing SiO₂, and were even higher still in animal caps cultured with the inside surface attached to the Petri dish (Fig. 2B, lanes 1-3 and 8). These elevated dpERK levels were sustained for a minimum of six hours (Fig. 2B, lane 8).

We conclude from the results presented so far that massive anterior neural inductions can be obtained in *A. maculatum* ectoderm, but preferentially in circumstances in which explants remained attached to the culture dish surface. In these conditions, explants had a sustained hyperactivity of the Ras/MAPK pathway. Sand particles acting as heterologous inducers also resulted in ERK activation, but were less effective than attachment to glass.

MEK activity is required for CNS induction

We next asked whether autoneuralization requires Ras/MAPK activation. A potent and highly specific inhibitor of MEK, the MAPKK that phosphorylates ERK, is available. The U0126 chemical inhibitor blocks activation of the Ras/MAPK pathway in *Xenopus* (Kuroda et al., 2005) and in *A. maculatum* (Fig. 2B, lanes 4, 6 and 9). In these experiments, 5% of explants formed epidermis and 60% differentiated neural tubes when cultured in Holtfreter's solution on glass surfaces (n=40) (Fig. 6A, C). Remarkably, treatment with U0126 increased epidermal differentiation to 61% and decreased neural tubes to 15% (n=33, two independent experiments)

(Fig. 6B, C). The few neural tubes formed in the presence of MEK inhibitor were smaller and had a much lower degree of differentiation, as indicated by the absence of eyes or olfactory placodes (data not shown). One possible explanation for this residual low-grade neuralization could be that some explants adhere completely to the glass, preventing accessibility of the inhibitor (the outer layer of amphibian embryos is impermeable to most molecules). The specificity of inhibition by Erk pathway by U0126 was confirmed by using the closely related negative control compound U0124 (Favata et al., 1998). When animal caps were cultured attached to a glass surface in the presence of U0124 69% (n=35, two independent experiments) were Sox3 positive, whereas with the U0126 inhibitor 11% (n= 18) were positive. We conclude from these results that autoneuralization of *A. maculatum* ectoderm requires an active Ras/MAPK pathway.

Discussion

In this study we could reproduce the neuralization of *Ambystoma maculatum* ectoderm in the absence of organizer 65 years after its original discovery (Barth, 1941). We found that the incidence of neuralization was higher in animal caps cultured attached to a glass substratum and that brain formation required an active Ras/MAPK pathway, since it could be inhibited by the MEK chemical antagonist U0126. Ectoderm attached to glass had increased levels of active diphosphorylated ERK, which were sustained for at least 6 hours. Many cellular stimuli are known to activate ERK/MAPK, such as receptor tyrosine kinase (RTK) ligands (Blume-Jensen and Hunter, 2001), 7-transmembrane G protein-coupled receptors (Luttrell et al, 1999, 2002), wound healing (Christen and Slack, 1999), cell dissociation (Kuroda et al., 2005), cell injury (Neary et al., 2003) and integrin-mediated cell adhesion (Aplin et al., 2001). Here we did not investigate which of these mechanisms causes the Ras/MAPK activation observed in *Ambystoma* ectoderm.

MAPK and neural induction

Since the cause of the sustained ERK/MAPK activation in our experiments is unknown, it could be argued that the results are irrelevant to the course of normal brain formation and only of academic interest. However, there is ample evidence for a crucial role for MAPK in normal vertebrate neural differentiation. One of the most striking is the discovery in zebrafish of a highly localized MAP kinase phosphatase 3 (MKP3) on the dorsal (future neural) side of the early embryo, which modulates ERK/MAPK signals (Tsang et al., 2004). Fibroblast growth factors (FGFs), in particular FGF8, play an important role in neural induction and dorsal-ventral patterning in most vertebrates (Stern, 2001; Wilson and Edlund, 2001; Fürthauer et al., 2004; Delaune et al., 2005). In addition, insulin-like growth factor (IGF) signaling also causes neural induction, in particular anterior brain structures (Pera et al., 2001; Pera et al., 2003). Presumably other growth factors signaling through RTKs can also induce neural tissue via activation of MAPK (De Robertis and Kuroda, 2004). The requirement for Ras/MAPK signals is also seen when neural induction is triggered by the BMP antagonist Chordin, since it can be inhibited by dominant negative FGF or IGF receptors (Pera et al., 2003); these results suggest that MAPK activation may decrease residual Smad1 signaling even in the absence of BMP signals.

In *Xenopus*, there is some debate as to whether FGF induces anterior or posterior neural tissue (Uzgare, 1998; Hongo et al., 1999; Ribisi et al., 2000; Pera et al., 2003; Kuroda et al., 2004; Delaune et al., 2005). The present results indicate that the levels of activated Ras/MAPK attained during autoneuralization of ectoderm are sufficient and required for the formation of forebrain and its derivatives, such as eyes and olfactory placodes (Fig. 4). Our work does not explain why in some experiments FGFs induce anterior CNS and in others posterior neural tissue. The variation in *Xenopus* experiments might depend on the type of FGF used (Hardcastle et al., 2000) or the doses of FGF used (Haremake et al., 2003). Anterior neural tissue induced

by low levels of MAPK might become transformed into more posterior ones at high levels of FGF, for example via the activation of posterior genes, such as *Xcad3*, through MAPK phosphorylation of Ets transcription factors on promoter elements (Haremakei et al., 2003).

BMP promotes epidermal differentiation and the “default model” of neural induction states that ectoderm differentiates into neural tissue only when BMP signaling is inhibited (Wilson and Hemmati-Brivanlou, 1995; Muñoz-Sanjuan and Brivanlou, 2002; Stern, 2006). That BMP signaling plays a crucial role in amphibian neural induction is demonstrated by the catastrophic loss of CNS observed when three dorsal BMP antagonists - Chordin, Noggin and Follistatin - are knocked down (Khokha et al., 2005) and by the observation that the entire ectoderm becomes CNS when four BMPs are knocked down simultaneously (Reversade and De Robertis, 2005). However, neural induction by the BMP antagonist Chordin in *Xenopus* animal caps also requires activity of the FGF and IGF signaling pathways, which activate Ras/ERK (Pera et al, 2003). MAPK phosphorylates Smad1 in the linker region, which targets Smad1 for degradation or cytoplasmic retention, decreasing its transcriptional activity (Kretzschmar and Massagué, 1997; Sapkota, 2007). Therefore FGF and IGF signaling ensure a more complete inhibition of Smad1 activity than can be achieved by only inhibiting BMP (Pera et al., 2003). This inhibition of Smad1 by MAPK phosphorylation explains the neuralization observed in dissociated *Xenopus* animal cap cells in which low levels of endogenous BMPs continue to signal (Kuroda et al., 2005). In addition, FGFs, Wnts, and presumably other molecules can regulate neural development through Smad1-independent pathways as well (Wilson and Edlund, 2001; Delaune et al., 2005; Stern, 2006). For example, in ascidians activation of the *Otx* neural enhancer involves FGF-induced ERK phosphorylation of Ets and GATA transcription factors (Bertrand et al., 2003). The present study does not address the nature of the endogenous “neuralizing principle”, but shows that the molecular mechanism of autoneuralization in *Ambystoma maculatum* requires Ras/MAPK activation, explaining a classical experiment that dominated embryological thinking on CNS formation for many years.

Self-organization of brain organs

A remarkable aspect of these experiments is the anatomical complexity that can be attained by activating MAPK activity in *A. maculatum* ectoderm. Not only brain vesicles were induced, but also eyes, olfactory placodes, balancers and lens, all in the absence of organizer. This illustrates the self-organizing nature of vertebrate development, which had been discovered during the search for the chemical nature of the primary neural inducer in the 1930s (Holtfreter and Hamburger, 1955; Nieuwkoop, 1963; De Robertis, 2006). Activation of the Ras/MAPK pathway probably explains the action of most heterologous neural inducers identified during that period of experimental embryology. We tested sand particles as an example of heterologous inducer in *A. maculatum* animal cap explants and observed that MAPK was elevated, and that neural tissue developed in the immediate proximity of the inorganic particle. Studies in *Ambystoma maculatum* are difficult given its short breeding season and limited number of embryos that can be collected in the wild. The present investigations represent the results obtained during the breeding seasons of 2005, 2006 and 2007. If extensive archencephalic inductions could be experimentally obtained in the much more tractable *Xenopus* system, for which so many molecular markers exist, this would open an excellent model system for studying the molecular mechanisms by which brain organogenesis is regulated. From an intellectual point of view, the present work has the attraction of providing a molecular explanation for experiments that played a historical role in the rich heritage of Developmental Biology (Hamburger, 1988; De Robertis, 2006).

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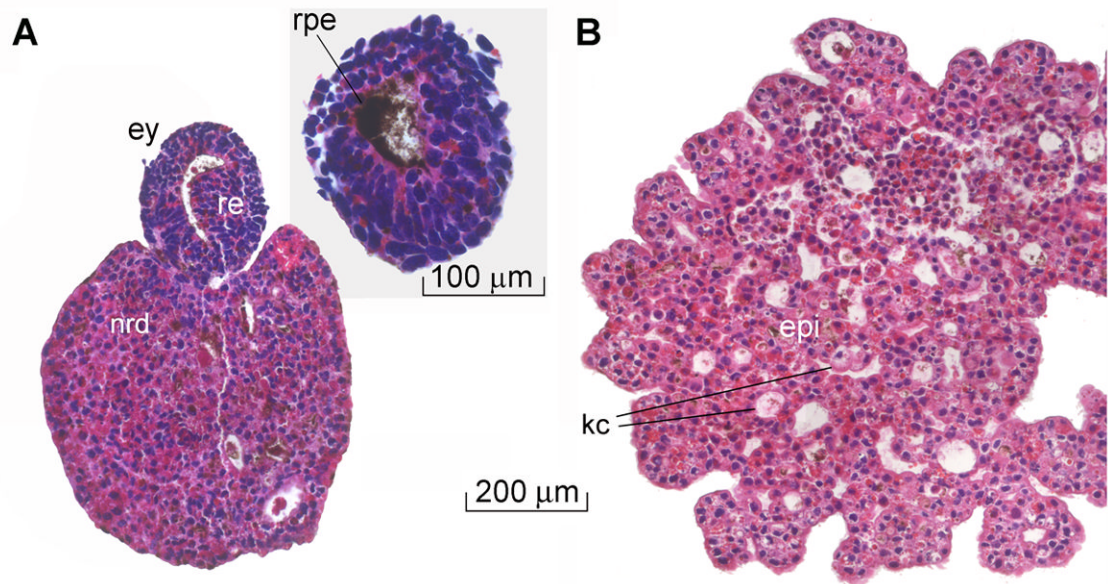


Fig. 1.

Ambystoma maculatum animal cap explants can differentiate into neural tissue after 9 days of culture in sterile Holtfreter's saline solution. (A) Neural differentiation with eye (ey) formation, retina (re) and retinal pigmented epithelium seen in a nearby section (rpe, inset). This eye developed without any CNS tissue and was not covered by epidermis (naked). The rest of the explant had neuroid (nrd) characteristics. (B) Atypical epidermis histotype with small cavities containing keratinized cells (kc). Keratinized cells can be identified by Mallory's trichrome staining (data not shown).

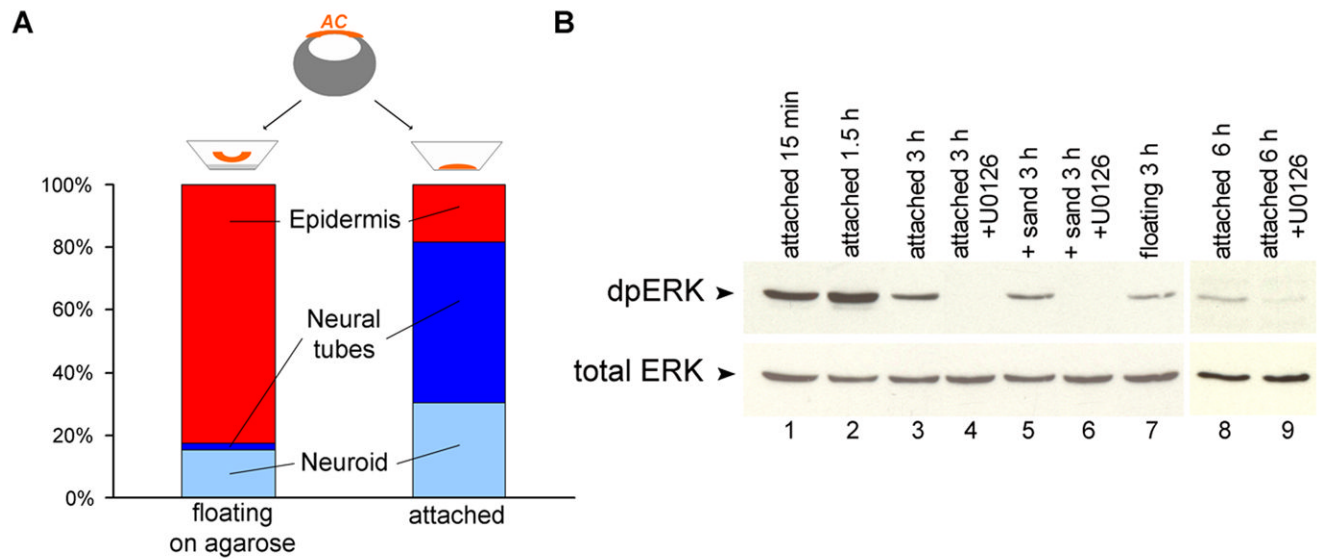


Fig. 2. Effect of culture conditions on CNS differentiation and MAPK/ERK activation in *Ambystoma maculatum* ectodermal explants. (A) Differentiated histological phenotypes observed by culturing explants on an agarose surface (n=50) or attached to a glass substratum (n=46). (B) Western blots showing diphospho ERK and total ERK in animal caps cultured attached to glass, with sand particles, or floating on an agarose surface.

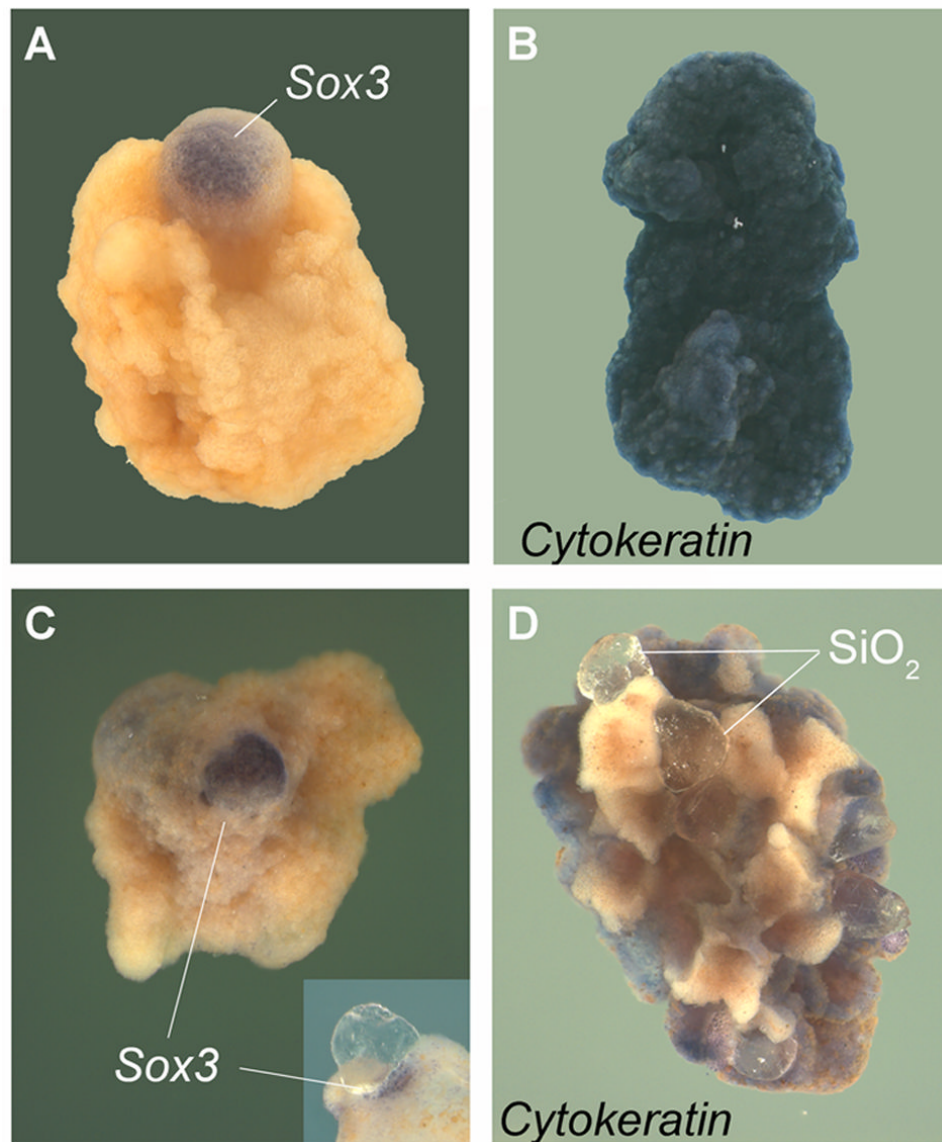


Fig. 3. Attachment to glass substratum or sand particles serve as heterologous neural inducers in *Ambystoma maculatum*. (A) Sox3 whole-mount in situ hybridization of neuralized explant cultured attached to glass for 8 days. (B) Cytokeratin staining of epidermal explant cultured on agarose, which prevents attachment. (C) Animal cap sandwiches containing grains of sand (SiO₂) stained with Sox3 showing localized neural differentiation. The inset shows an explant with a protruding SiO₂ grain particle. (D) Cells in close contact with sand particles do not stain for cytokeratin, presumably because they become neuralized by the heterologous inducer.

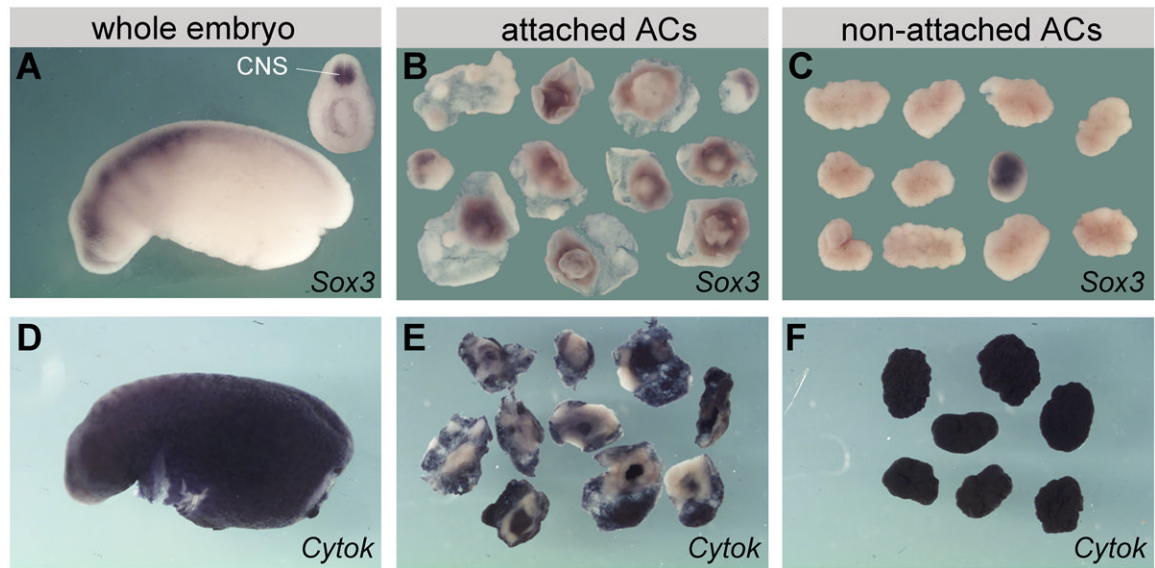
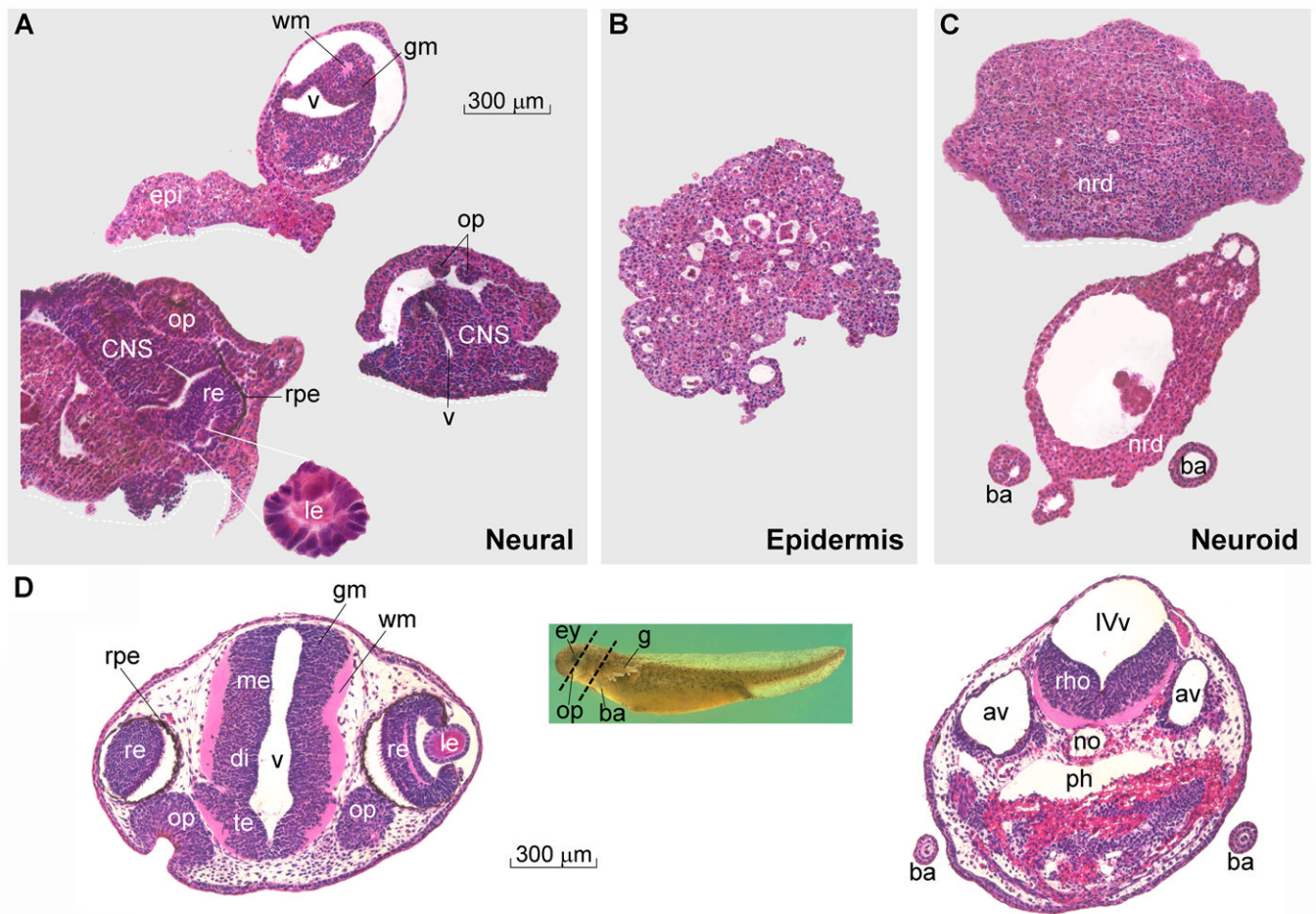


Fig. 4.

Neural or epidermal differentiation of *A. maculatum* ectodermal explants analyzed by molecular markers. (A). *Sox3* staining of whole embryo at Harrison stage 27 with head facing to the left, the inset shows a section showing CNS-specific staining. (B, C). *Sox3* expression in sibling animal caps cultured attached or non-attached to the glass petri dish, respectively. Note that attachment to glass promotes neural differentiation. (D). *Cytokeratin* staining of *A. maculatum* embryo. (E, F). *Cytokeratin* expression in animal caps cultured attached or non-attached to the glass petri dish, respectively. See text for quantitation.

**Fig. 5.**

Organs and histotypes that differentiate in *Ambystoma maculatum* ectoderm cultured in Holtfreter's saline solution. (A) Sampling of neural structures developed in explants. Dotted lines indicate region of attachment to the dish. An enlargement of the lens from a nearby section is also shown. (B) Atypical epidermis phenotype. Note the irregular surface and small cavities containing keratinized cells. (C) Neuroid differentiations. One is accompanied by balancer-like epidermal tubular structures. (D) Sibling larvae sectioned at the level of the forebrain (left) and hindbrain (right). Inset shows an external view of *A. maculatum* larva at Harrison stage 39, with planes of sections and position of the balancers indicated. Abbreviations: av, auditory vesicle; ba, balancer; CNS, central nervous system; di, diencephalon; epi, epidermis; ey, eye; g, gills; gm, gray matter; le, lens; me, mesencephalon; no, notochord; nrd, neuroid; op, olfactory placode; ph, pharynx; re, retina; rho, rhombencephalon; rpe, retinal pigmented epithelium; te, telencephalon; v, brain ventricle; wm, white matter; IV v, fourth ventricle.

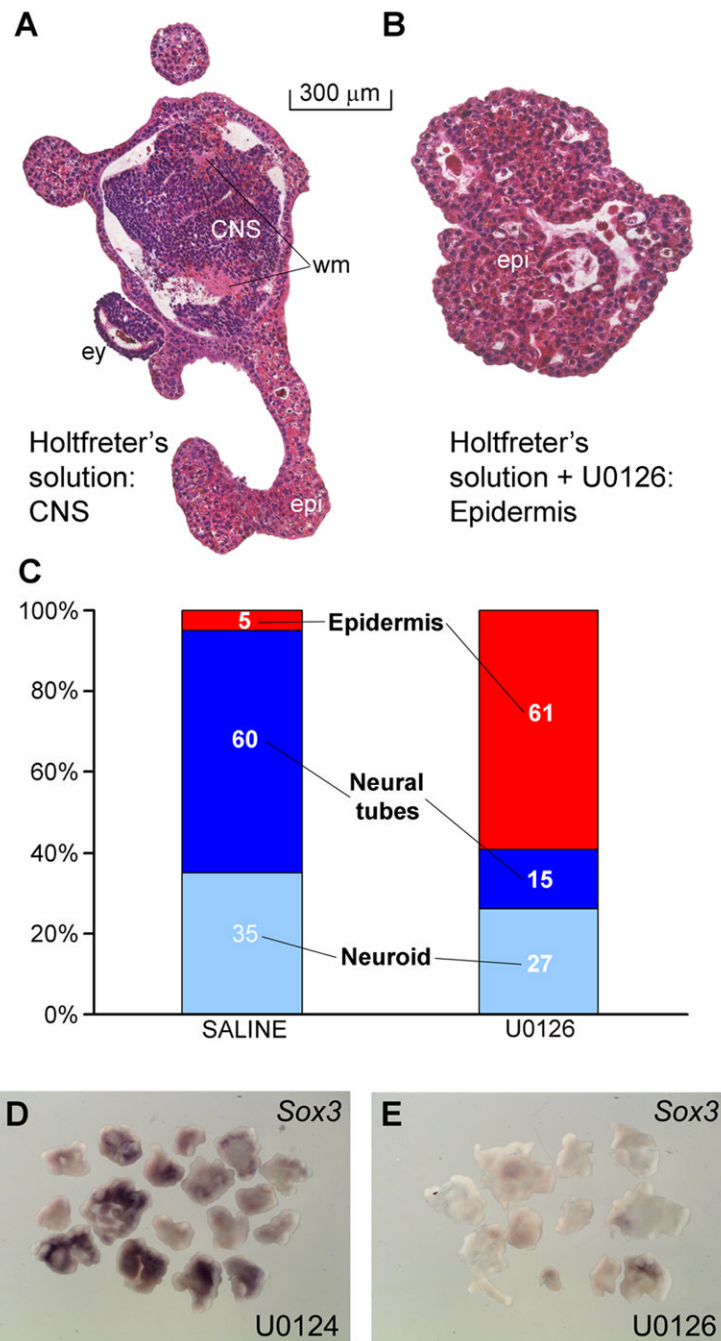


Fig. 6. Ras/MAPK pathway activity is required for CNS differentiation in *Ambystoma maculatum* ectodermal explants. (A) Section of animal cap cultured for 9 days in Holtfreter's saline solution showing neural differentiation in the form of brain structures (CNS) containing myelinated white matter (wm) enveloped in epidermis (epi), and a naked eye (ey) and its retina (re). (B) Animal cap from sibling embryo cultured in the presence of 40 μ M U0126 (MEK-specific inhibitor) showing epidermal differentiation. (C) Quantitation of results from serial sections of two experiments in which animal caps were cultured attached to glass on saline (n=40) or in saline containing 40 μ M U0126 (n=33). (D, E) Sox3 in situ hybridizations of

animal caps cultured attached to glass in the presence of the negative control compound U0124 or the Erk pathway inhibitor U0126, respectively. See text for quantitation.