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Original Article

The eyeless mutant gene (*e*) in the Mexican axolotl (*Ambystoma mexicanum*) affects *pax-6* expression and forebrain axonogenesis

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ABSTRACT This study tested the hypothesis that changes in the patterns of *pax-6* expression disrupt the anatomy and axonogenesis of the diencephalic areas of the eyeless axolotl. Proper *pax-6* expression is necessary for eye and hypothalamus morphogenesis. Since the expression boundaries of *pax-6* also provide a permissive environment for axonal outgrowth, an extensive study examining the effects of the eyeless gene (*e*) in the Mexican axolotl upon *pax-6* expression and forebrain axonogenesis was begun. This study used whole embryo *in situ* hybridization techniques to follow *pax-6* expression and whole brain immunocytochemistry to examine axonogenesis and neural differentiation. These studies demonstrated that the mutant gene *e* in the axolotl alters the response of midanterior neural-plate tissue to signals from the prechordal plate. This response was hypothesized to be a hyper-response to signals *(sonic hedgehog?)* that suppressed *pax-6* expression within the midanterior neural plate and later developmental stages. Alternatively, the affected neuroectoderm of the eyeless embryos may lack competence to express *pax-6*. Lowered *pax-6* expression inhibited eye and forebrain morphogenesis as well as neural axonogenesis and differentiation. Differentiation defects were detected as the suppression of midline dopaminergic neurons within the suprachiasmatic nucleus of eyeless animals. Thus, lowered *pax-6* expression by the midanterior neuroectoderm promotes the eyeless condition by inhibiting the role of *pax-6* in eye formation. This lowered expression also leads to concurrent alterations in the hypothalamic terrain which disrupt axonogenesis and ultimately promote sterility.

KEY WORDS: *eyeless, pax-6 expression, axonogenesis, TH immunocytochemistry*

Introduction

The eyeless mutant axolotl was discovered within an inbred white strain of *Ambystoma mexicanum* byRufus Humphrey in 1969. The eyeless mutation is inherited as a recessive gene. In the homozygous form, this gene inhibits eye formation and the animals are also sterile. Studies done by Van Deusen (1973) included reciprocal grafts of the prospective archenteron roof between normal and mutant gastrulae. Grafting mutant ectoderm (or presumptive neuroectoderm) into normal hosts inhibited eye formation, and normal ectoderm grafted to mutants produced eyed individuals. Van Deusen (1973) concluded that the prospective anterior neuroectoderm is the tissue affected by the mutant *e* gene. The affected prospective neuroectoderm area localized by Van Deusen (1973) inhibits eye formation and is localized within the presumptive neural plate eye/diencephalic field (Jacobson, 1959). Thus, sterility and eyelessness are both a result of the inability of this area to properly respond to underlying (archenteron roof) cues.

Much experimental evidence indicates that the vertebrate eye is dependent upon a coordinated series of inductive interactions between presumptive neural retina and the surface ectoderm or presumptive lens (Spemann, 1938; Jacobson, 1958; Li *et al.*, 1997). The initial sign of eye morphogenesis is observed as an evagination of the diencephalon forming the optic pits (Eagleson *et al.*, 1995). As the evaginations of the diencephalon deepen and approach the surface ectoderm, they become more extended forming the optic vesicles. Close interactions between these optic vesicles and the surface ectoderm result in a coordinated invagination of these tissue layers forming the lens vesicle and optic cup (Spemann, 1938).

A number of deficiencies or mutations directed at this process have been observed. In mice anterior deficiencies and anoph-

Abbreviations used in this paper: e, the "eyeless" gene; PCP, prechordal plate; SOT, supraoptic tract; TH, tyrosine hydroxylase; TPOC, tract of the preoptic commissure.

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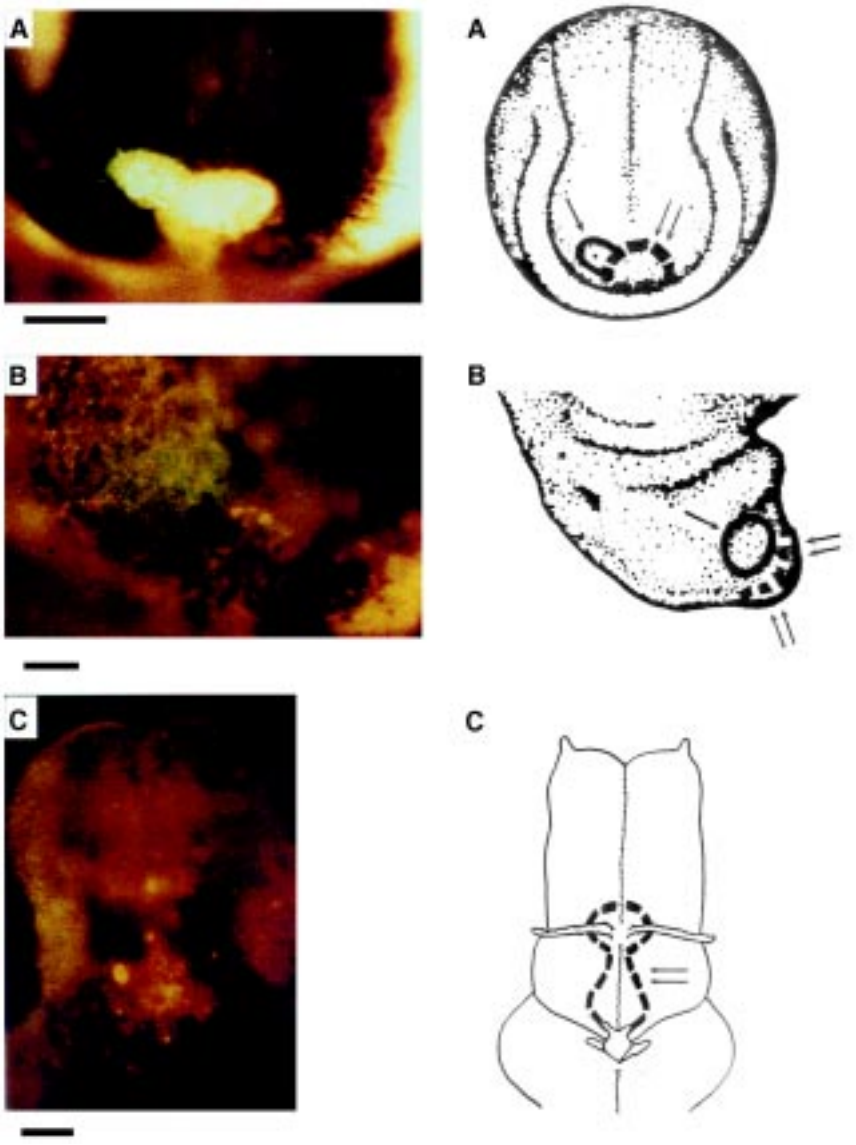

Fig. 1. Double labeling of the stage-15 neural plate embryo. **(A)** *Stage-15 Neural Plate embryo (frontal view). The neural plate embryo was labeled with DiI (yellow-red) within the midanterior region of the neural plate and DiO (green-yellow) within the lateral anterior region of the neural plate. Bar: 400 μm.* **(B)** *Stage-28 embryo (side view). The DiO (green; center of panel) label is restricted to the optic vesicle and the DiI (yellow-red) label found near the lower right of the panel is localized more rostrally within the prospective hypothalamic area. Bar: 200 μm.* **(C)** *Stage-40 brain (ventral view; anterior to the top). The DiI label is localized to the hypothalamus or rostral brain. Eyes have been removed. Bar:100 μm.*

thalmia result from null mutations in *pax 6* (Hill *et al*., 1991). *Rx* (Mathers *et al*., 1997) and *Otx2* deficiencies indicate a role for these transcription factors in eye development. And studies in the axolotl by Cuny and Malacinski (1986) indicate that proper eye development and formation require reciprocal signaling between the neurally derived optic cup tissue and the epidermal lens.

The paired homebox transcription factor *pax 6* is known to be very critical for eye development (Chow *et al*., 1999). This has been demonstrated by mutations in *pax 6* that cause aniridia syndrome in humans (Glaser *et al*., 1992), the small eye (*Sey*) mutation in mouse (Hill et al., 1991) and the eyeless phenotype in *Drosophila* (Quiring *et al*., 1994). Both the semi-dominant inheritance patterns of Aniridia and the small eye mutation in mouse (Hill et al., 1992) indicate that the concentration or levels of *pax 6* expression are important for proper eye development. Glaser (personal communication) has isolated the *pax 6* gene in the axolotl, and a sequence analysis indicated little or no change in the exons for this gene in eyeless compared to eyed individuals.

Not only does *pax 6* have a role in eye development, but its expression is also essential for the development of other diencephalic areas including the hypothalamus (Warren and Price, 1997). Gener-

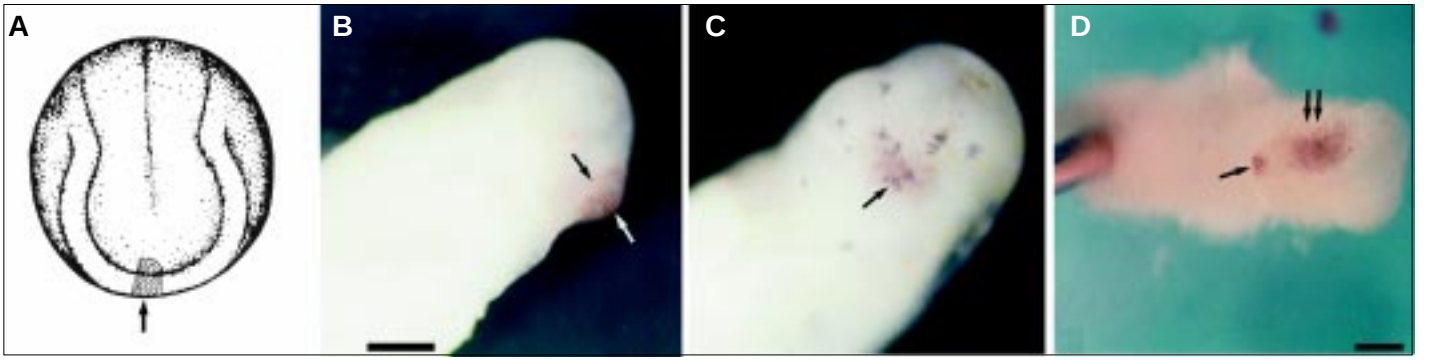

Fig. 2. Fate mapping using pigmented tissue exchange. (A) *The stage-15 albino embryo had pigmented tissue from the anterior neural plate and neural ridge of a pigmented embryo transplanted to it. Tissues were interchanged to their respective, exact positions.* **(B)** *Stage-28 embryo. Arrows denote pigmented area.* **(C)** *Stage-35 embryo. Note some pigmented ridge cells became melanophores.* **(D)** *Stage-40 brain. The labeled areas (denoted by arrows) included pituitary, ventral hypothalamus and a portion of the ventral telencephalon. The bar in panel B pertains to panels A, B, and C and denotes 500 μm. The bar in panel D denotes 100 μm.*

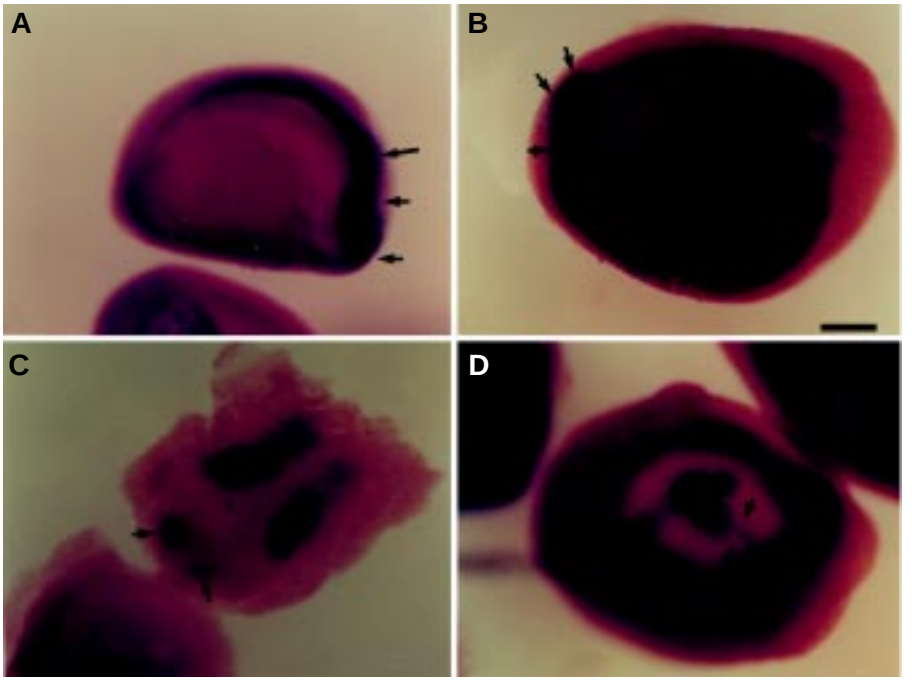

Fig. 3. *Pax-6* expression in the (A,B) normal and (C,D) eyeless axolotl stage-15 neural-plate embryo. (A) *A side view of a stage-15 normal axolotl embryo. Pax-6 expression is found within the most anterior neural plate, the hindbrain and the ventral neural cord. The brain area lacking pax 6 expression is the midbrain (Between large and small arrows).* **(B)** *A dorsal view of a stage 15 embryo stained for pax-6 expression. The anterior area stained with pax-6 includes the eye-hypothalamic field. The two eye fields are connected by a band of expression along the anterior neural plate (indicated by the far left, center arrowtip).* **(C)** *A dissected eyeless stage-15 neural plate. Note the low amount of staining within the eye field (arrowtips that indicate two smaller spots) and the lack of a band of pax-6 expression "bridging" the eye fields. Midbrain does not stain, but hindbrain and prospective spinal cord stain as segmented spots to the right behind the indicated eye fields.* **(D)** *A frontal view of a stage-15 eyeless neural plate embryo. This embryo had the greatest extent of pax-6 staining for eyeless embryos, but still lacked the anterior bridge of staining that connected the eye fields (indicated between the arrows). Bar in B represents 400 μm for all plates.*

ally, the diencephalon of *Sey* mice exhibits reduced proliferation rates and altered patterns of downstream gene expression events when compared to normal mice (Warren and Price, 1997). Warren and Price (1997) also observed altered gene-expression boundaries within the diencephalon of *Sey* mice. It is known that gene-expression boundaries along the rostral brain provide important cues for initial axonogenesis (Wilson et al., 1990; Matsuo et al., 1997). Therefore, altered *pax 6* expression or its misexpression could not only affect eye morphogenesis but also disrupt diencephalon development and morphogenesis.

It is possible that faulty neuroectodermal/mesendodermal communication could lead to alterations in *pax 6* expression. With altered presumptive diencephalic expression patterns, these malfunctions could also result in the anatomical and neural tract differences observed in eyed vs. eyeless axolotls (Eagleson and Malacinski, 1986). The present study was begun to map the presumptive rostral areas of the axolotl neural plate embryo, to determine areas where *pax 6* expression might be altered in the eyeless compared to the eyed axolotl, and to follow early axonogenesis of the rostral forebrain of eyeless compared to eyed axolotl. We tested the hypothesis that changes in the patterns of *pax 6* expression disrupt the morphogenesis and axonogenesis of the diencephalic areas of the eyeless axolotl.

Results

Mapping of the forebrain areas of the Axolotl

The presumptive optic vesicle and eye regions mapped to the anterior lateral regions of the neural plate embryo (Figs. 1 A,B). The rostral diencephalon (including presumptive hypothalamus) mapped to the midanterior regions of the neural-plate (Figs. 1 A,B,C). The presumptive areas of the forebrain were consistent with the neural plate areas mapped by Jacobson (1959). There were a few forebrain areas that did consistently map differently from Jacobson's (1959) neural-plate fate map. Using both fluorescent labeling and pigmented tissue-transfer methods, we consistently found that a portion of the anterior diencephalon, the ventral telencephalon and the pituitary were derived from stage-15 anterior neural-ridge tissues (Figs. 2 A,B,C,D).

The prospective eye region (lateral anterior neural plate) label of (6) eyeless neural plate embryos became part of the lateral and ventral diencephalon.

Temporal and spatial expression of pax-6 in eyed and eyeless Ambystoma mexicanum

In stage-15 neural plate embryos (Fig. 3), the region expressing *pax 6* was located within the anterior edge of the neural plate

TABLE 1

TYROSINE HYDROXYLASE (TH) IMMUNOCYTOCHEMISTRY IN STAGE 40 EYED, EYELESS AND PCP-EXTIRPATED AXOLOTLS

Group	n	TP (+TH cells)	SC cells (+TH cells)
Eyed	5	37.6 + 2.07	15.6 + 2.97
Eyeless	4	34.4 + 5.77	0.4 + 0.89
-PCP	7	34.0 + 2.45	1.0 + 0.70

Eyeless and -PCP groups were significantly different ($P<0.001$; t-test) than from control (eyed) group with regard to number of cells in the SC. SC denotes suprachiasmatic nucleus dopaminergic clusters (midline cells). TP represents the cells of the ventrolateral tuberculum posteriori.

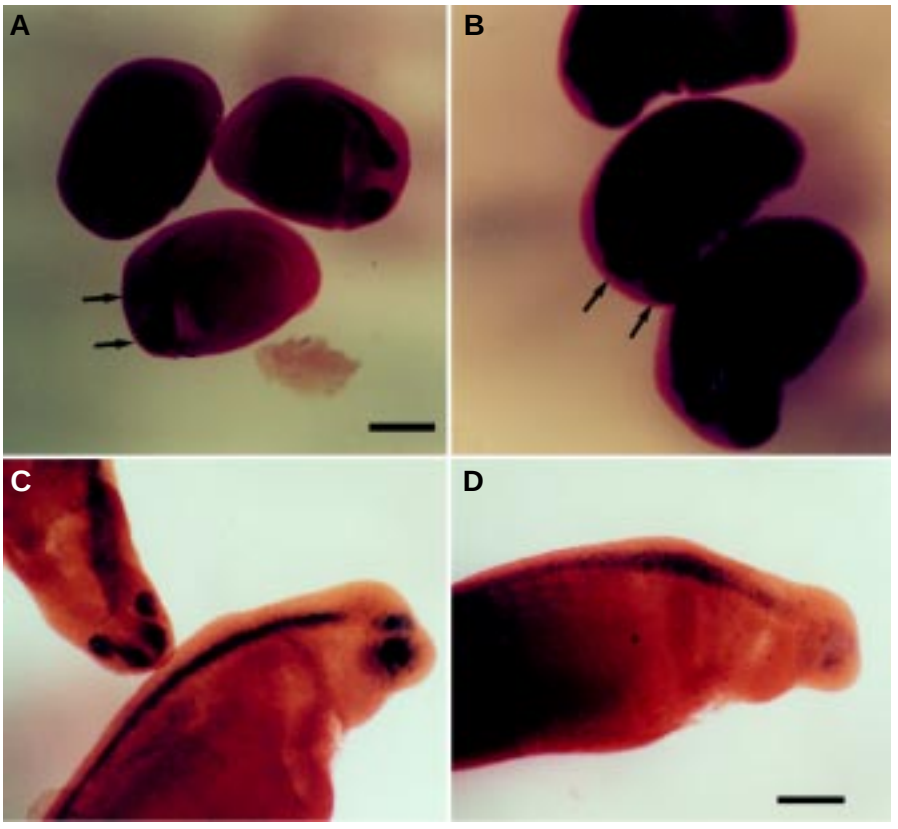

Fig. 4. (A,B) Normal neural tube axolotl embryos together with (C,D) normal and eyeless stage 30731 embryos stained for *pax-6*. (A) *Side views of stage 22 embryos stained forpax-6. Note the staining within the forebrain and hindbrain (indicated by arrows) and ventral spinal cord, but the lack of staining within the midbrain.* **(B)** *Side views of stage 27/28 embryos stained for pax-6. Note the staining within the forebrain and hindbrain (indicated by arrows) and ventral spinal cord, but the lack of staining within the midbrain.* **(C)** *Stage-30/31 normal embryos stained for pax-6. Two embryos are arranged such that a dorsal (upper left) and side view can be observed. The dorsal view indicates that the eyes, hypothalamus and telencephalon stain for pax-6. The side view demonstrates that ventral hindbrain and spinal cord stain for pax-6.* **(D)** *A Stage-30/31 eyeless embryo stained for Pax 6. Note the lack of pax 6 staining within the forebrain areas (except for a small amount of staining within the nasal region). Posterior to the midbrain, pax 6 staining is comparable to normal stage-30/31 embryos. Bars: A,B, 1 mm; C,D, 400 μm.*

and along the presumptive spinal areas. The midbrain area lacked *pax-6* expression, but hindbrain and spinal cord exhibited *pax 6* expression (Fig. 3A). The *pax-6* stained area of the anterior neural plate curves into a U-shaped area with a small connecting band (single arrow, Fig. 3B) that was attached to two circular areas. This bridge of attachment was observed in all (12) stage-15 embryos we observed.

Looking at the spatial pattern, eyeless stage-15 embryos exhibited lower amounts of *pax 6* expression. Eventhough hindbrain and spinal cord *pax-6* expression was normal within eyeless embryos, the anterior areas of *pax-6* expression were diminished (Fig. 3 C,D). The diminished *pax 6* expression in stage-15 embryos varied (compare Fig. 3C with Fig 3D) among eyeless embryos, but in all cases of stage-15 eyeless embryos (8) examined, the anterior *pax-6* expression was completely separated into two anterior circular arrays that lacked an anterior connecting band of *pax 6* expression.

Pax 6 expression within later-staged normal axolotl embryos (Fig. 4) was typically observed to be within the prosencephalon, the eyes, the hindbrain and the ventral spinal cord, but lacking within the midbrain (Fig. 4 A,B). Stage-30/31 embryos also exhibited this pattern of expression (Fig. 4C), whereas eyeless stage-30/31 embryos lacked the diencephalic and eye *pax 6* expression patterns and had only faint *pax 6* expression in the telencephalon and nasal placodes (Fig. 4D).

Axolotl forebrain axonogenesis detected by acetylated-Tubulin immunocytochemistry

Within the axolotl fore- and midbrain the initial, most prominent tract first detected was the medial longitudinal fasciculus as well as the ventral-most longitudinal tract within the brain in stage-32 normal axolotls (Fig. 5 A,B). By stage 33, connections were observed to this tract with the postoptic commissure (t-poc; Fig. 5 C,D,E) of the diencephalon. By stage 35, most of the various forebrain tracts had extended out and interconnected (Figs. 5 E,F and 5G).

Within the eyeless axolotl, the initial midbrain/hindbrain tracts of the medial longitudinal fasciculus and the ventral-most longitudinal tracts (vlt) formed, but the tpoc did not form (Fig. 5H; empty arrowtip). The supraoptic tract (SOT) to the telencephalon developed, but was much reduced in the eyeless (Fig. 5H) compared to the eyed axolotl (Fig. 5G).

Effects of the removal of the Prechordal Plate (PCP) upon navigation of forebrain axons

Removal of the prechordal plate in normal stage-15 embryos resulted in loss of eyes or a cycloptic condition (not shown) for stage-35 embryos. Whole-brain immunocytochemistry for acetylated- tubulin indicated that forebrain axons (especially from the postoptic commissure) were disoriented and failed to form discernable tracts (Fig. 6A) in forebrains that had the PCP removed, whereas the tract of the postoptic commissure (tpoc) was evident in operated controls (arrowtips; Fig. 6B).

Detection of TH-positive neurons within the brains of normal, eyeless and experimental Mexican axolotls

Abundant tyrosine hydroxylase (TH)-positive tracts (large arrowtip; Fig 7A) and neurons (small arrowtips; Fig. 7A) were observed within normal stage-40 axolotl brains. TH-positive neurons were abundant in the lateral forebrain within the tuberculum

posteriori (TP) and along the midline of the forebrain within the suprachiasmatic nucleus (SC). TH-positive cells were also observed within the olfactory bulb and along the spinal cord (small arrowtip; Fig. 7A). No TH-positive cells were observed within the midbrain.

Stage-40 eyeless axolotls (Table 1) and axolotls with the PCP removed had a similar number of dopaminergic (TH-positive cells) neurons within the lateral TP of the diencephalon, but they had significantly fewer dopaminergic cells within the SC (Fig 8 B,C; Table 1) when compared to eyed normals.

Discussion

Analysis of *pax 6* -expression in the eyed axolotl embryos followed the same patterning as seen in mice (Walther and Gruss, 1991; Matsuo *et al*., 1993; Grindley et al., 1995), zebrafish (Krauss et al., 1991; Puschel et al., 1992) and chick embryos (Li *et al*., 1997). In each case, during neural plate and later stages, *pax 6* was localized to the presumptive eye and forebrain tissue and to a lesser extent the hindbrain and spinal cord (Figs. 3 C,D and 4 C,D).

Within the eyeless axolotl neural-plate embryos, hindbrain and spinal cord *pax 6*-expression was unaffected by the eyeless gene (Fig. 4 C,D). But within eyeless compared to normal embryos, a consistently different pattern of forebrain *pax 6* expression occurred during early neural plate and later stages. In the regions that give rise to eyes (Fig. 1), *pax 6*-expression was less abundant and appeared lateral to the midline of the anterior neural plate (Fig. 3 C,D). In the eyeless neural-plate embryos, the midline area that lacked normal *pax 6*-expression was underlain by prechordal plate (PCP) tissue. Previous studies by Van Deusen (1973) indicated that this eyeless neural-plate area fails to respond to underlying PCP tissue, and the eyeless defect revolves around competence of this neuroectoderm to respond to PCP signals. Since *sonic hedgehog* (*shh)* inhibits *pax 6*-expression, one would hypothesize that the overlying neuroectoderm is more sensitive to *shh* in eyeless embryos compared to normals. This would result in the larger area lacking *pax-6* expression along the anterior midline observed in eyeless stage-15 neural-plate embryos (Fig. 3 C,D). *In vitro* studies by Cuny and Malacinski (1986) support this concept that the midanterior neural-plate tissue is defective with regard to eye formation. The present studies further indicated that this "failure" to respond might be reflected in an altered spatial expression pattern of *pax-6* at this neural-plate stage of development. Such a defect could be due to oversecretion of the patterning molecule *sonic hedgehog* (*shh*) by the PCP or an exaggerated competence (or sensitivity) by the neuroectoderm to respond to *shh*. We tested two aspects of this *pax-6* misexpression, to see if later developmental defects in the eyeless axolotl could be attributed to this altered spatial expression of *pax-6*.

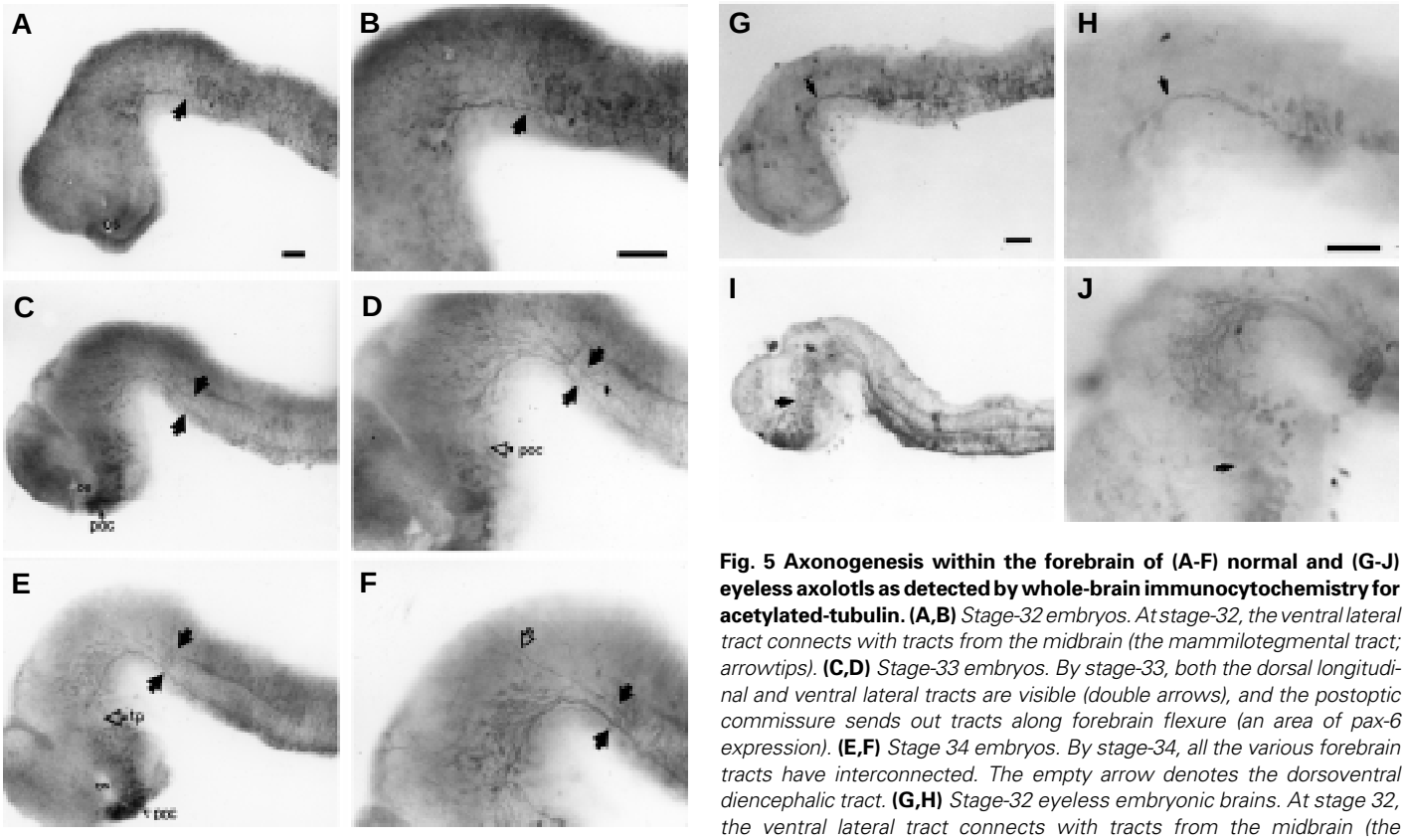

Fig. 5 Axonogenesis within the forebrain of (A-F) normal and (G-J) eyeless axolotls as detected by whole-brain immunocytochemistry for acetylated-tubulin. (A,B) *Stage-32 embryos. At stage-32, the ventral lateral tract connects with tracts from the midbrain (the mammilotegmental tract; arrowtips).* **(C,D)** *Stage-33 embryos. By stage-33, both the dorsal longitudinal and ventral lateral tracts are visible (double arrows), and the postoptic commissure sends out tracts along forebrain flexure (an area of pax-6 expression).* **(E,F)** *Stage 34 embryos. By stage-34, all the various forebrain tracts have interconnected. The empty arrow denotes the dorsoventral diencephalic tract.* **(G,H)** *Stage-32 eyeless embryonic brains. At stage 32, the ventral lateral tract connects with tracts from the midbrain (the mammilotegmental tracts; arrowtips).* **(I,J)** *Stage-34 brains from eyeless embryos. At stage 34, extensive tracts are observed throughout the midbrain, but forebrain tracts of the postoptic commissure are lacking (an area where pax-6 expression is observed in normals, but lacking in eyeless embryos). The arrowtip denotes where these tpoc tracts are usually observed at this stage. Abbreviations: os, optic stalk; the postoptic commissure; tpoc, the tract of the postoptic commissure. Scale bars: in A, 100 μm for A, C and E; in B, 100 μm for B, D and F; in G, 100 μm for G and I; in H, 100 μm for H and J.*

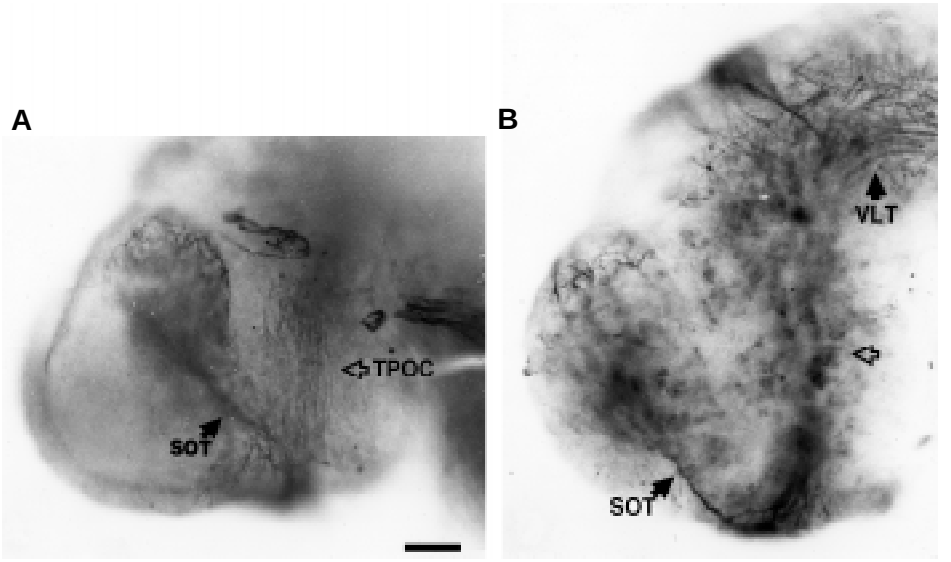

Fig. 6. A comparison of eyed and eyeless stage-35 forebrains. *Axonogenesis in the* **(A)** *eyed and* **(B)** *eyeless forebrain differs in a number of features. The most obvious difference is the lack of development of a tract of the preoptic commissure (TPOC) within the eyeless forebrain (B, empty arrow). This area is a border area of pax-6 expression in the ventral diencephalon in normal stage-30/31 axolotls (Fig. 4C) that is lacking pax-6 expression in eyeless stage-30/31 embryos (Fig. 4D). In addition, the supraoptic tract (SOT) is less extensive within the stage 35 eyeless (B) compared to eyed embryos (A). Bar: 100 μm for both plates.*

It has recently been demonstrated that dopaminergic neurons are induced by *shh* (Hynes et al., 1995). The PCP acts as a source of *shh* in amphibians (Ekker *et al.*, 1995). It was of interest to determine if dopaminergic neurons within the eyeless axolotl are affected by this mutant condition. We, therefore, did studies looking at dopaminergic neuron development in the normal, mutant, and PCP-extirpated axolotl.

The lateral diencephalic (TH^+) cell numbers were comparable in eyeless and prechordal plate-extirpated axolotls (Table 1). This was indicated by the differentiation of similar numbers of tyrosine hydroxylase-positive (TH^+) neurons in the tuberculum posteriori. But the eyeless and prechordal plate-deficient axolotls had fewer midline dopaminergic suprachiasmatic (SC) neurons (Table 1). Proper prechordal plate (PCP) interactions are important for the induction of SC dopaminergic neurons, and the eyeless anterior neuroectoderm had a suppressed response to the PCP as indicated by the failure of midline dopaminergic neurons to differentiate. Therefore, the neuroectodermal area affected by the eyeless mutation is suppressed for *pax 6* -expression as well as having a reduced number of SC dopaminergic neurons. If there were a heightened response to PCP due to greater sensitivity to *shh*, this should result in decreased *pax 6* -expression and increased numbers of dopamingeric neurons along the anterior midline (SC) of the affected area. Our results indicated that tissue competence for *shh* was lacking in the eyeless condition, rather than the defect being due to hypersensitivity for *shh* by the PCP. The fewer dopaminergic neurons within the SC was a situation expected because of extirpation of the PCP and low levels of sensitivity to *shh*.

It is known that gene expression boundaries affect navigation of axons (Wilson *et al.*, 1990; Shimamura *et al.*, 1995). The expression boundary of *pax 6* is implicated in patterning the tract of the preoptic commissure (tpoc; Shimamura *et al.*, 1995). Since *pax 6* -expression boundaries are severely altered in eyeless embryos, we ascertained whether diencephalic tracts were also disrupted due to the eyeless gene. Our studies demonstrated that the eyeless diencephalon had a defective or absent tpoc (Fig. 5 G-J). In addition, we determined that the PCP played an important role in proper tpoc development (Fig. 6). Therefore, the interaction between PCP and overlying neuroectoderm was faulty, and this led to later navigational defects of tract formation within the ventral diencephalon. Hence, diencephalic alterations in axonal tract navigation in the eyeless embryo could account for the sterility and the hypothalamic tract defects observed in adult eyeless animals (Van Deusen, 1973; Eagleson and Malacinski, 1986).

In conclusion, these studies demonstrated that proper development of the eye and hypothalamus requires precise spatial and temporal signaling and tissue-response events between the PCP and overlying anterior neuroectoderm. They further indicated that the gene (*e*) suppresses the response of midline anterior neural

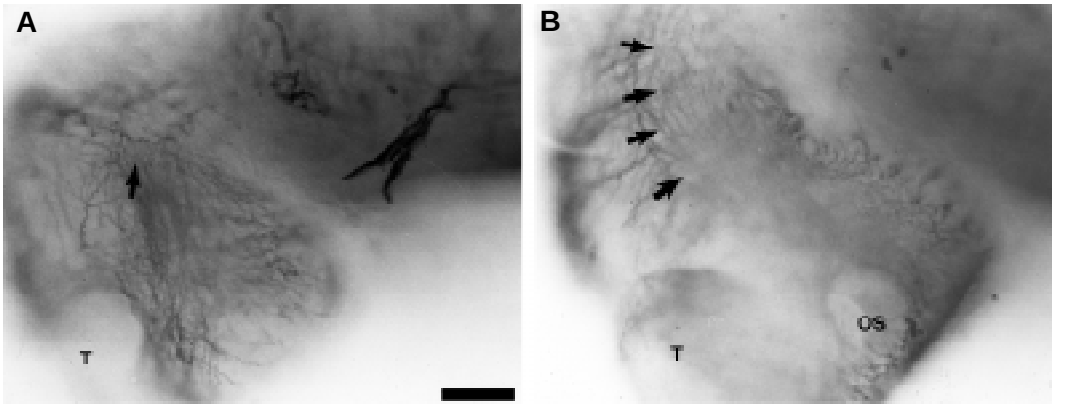

Fig. 7. Effects of prechordal plate (PCP) extirpation at stage-15 upon forebrain tract formation. **(A)** *Stage-35 axolotl brains with the PCP removed at stage-15 had disorganized tracts and lacked proper forebrain interconnections compared to* **(B)** *normal, unoperated stage-35 embryo brains. Arrowtips denote tracts that originate from the postoptic commissure. Bar in A denotes 100 μm for A and B.*

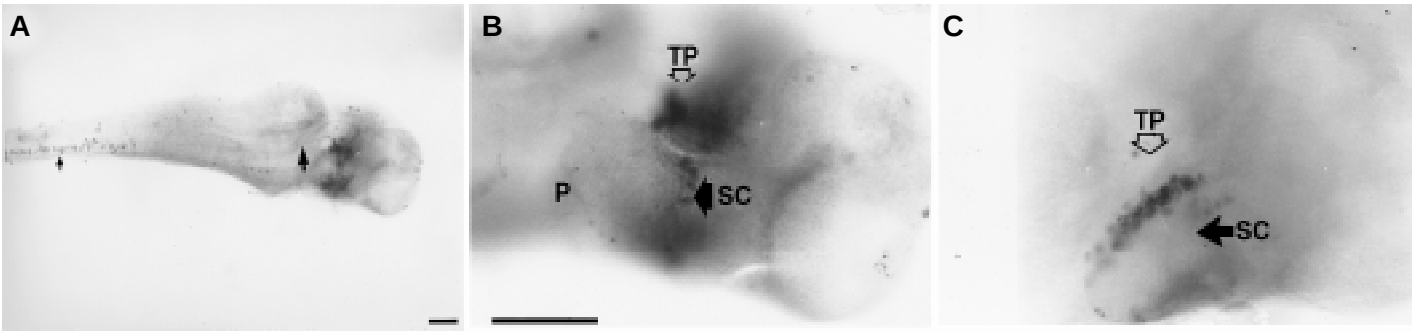

Fig. 8. Effects of prechordal plate removal at stage-15 upon dopaminergic cells in the axolotl forebrain. (A) *Whole-brain immunocytochemical analysis for tyrosine hydroxylase (TH) in a normal axolotl stage-40 brain. The large arrowtip follows the tract of the tuberculum posteriori (TP). These tracts arc along and follow the pioneer tracts of the postoptic commissure (tpoc).* **(B)** *A closer view of this forebrain indicating the TH-positive stained cells of the TP and suprachiasmatic nucleus (SC).* **(C)** *is a similar view of a brain from which the PCP was extirpated at stage 15. Note the lack of organized tracts from the TP and the lack of TH⁺ cells within the SC. Bar in A denotes 100 μm and the bar in B represents 100 μm for B and C.*

plate tissue that is part of the neural plate eye-hypothalamic field. Due to the lack of the formation of the tpoc and midline SC dopaminergic cells, this alteration of *pax 6* -expression within the eye-hypothalamic field resulted in later diencephalic defects that ultimately result in eyelessness and sterility.

Materials and Methods

Animals

Adult axolotls that are homozygous for the eyeless gene (gene e) are sterile. Eyeless embryos can be obtained by matings between heterozygote indivduals, but with heterozygous matings the morphological aspect of eyelessness cannot be discerned in embryos morphologically until stage 29/30. Fertile eyeless adults were obtained by head transplants between eyed and eyeless stage 32 to 35 embryos (Brun, 1993). Several of these operations were performed, and six “eyed, fertile” animals with eyeless gonads were raised to adulthood. This resulted in 5 males and 1 female which were used for matings. In some cases, a genetically eyeless fertile male was mated with a female heterozygous for the eyeless (e) gene. This resulted in 50% of the offspring or embryos being eyeless. The one adult genetically eyeless female was mated once with another fertile, genetically eyeless male. These embryos were fixed in MEMFA (Minimal Essential Medium with Formaldehyde) and preserved in 100% ethanol for later study.

In many cases, embryos from heterozygote matings were used, and eyelessness was assessed by the presence or absence of optic vesicles after clearing in Murray’s solution (2 parts benzyl benzoate: 1 part benzyl alcohol) subsequent to the *in situ* hybridization experiments. These embryos from heterozygote matings were supplied by the Indiana University Axolotl Colony. Embryos were staged according to Bordzilovskaya *et al.*, (1989).

Mapping the forebrain areas of the axolotl

To localize the possible areas affected by the mutant *e* -gene, the presumptive fates of the neural plate forebrain areas were mapped by two methods. In many cases, fluorescent labeling techniques were used as described for *Xenopus laevis* in Eagleson and Harris (1990). But because the neural plate of the axolotl is a single layer thick and more susceptible to damage than *Xenopus*, we also did a number of studies in which pigmented forebrain areas were microdissected and transplanted to albino embryos. Pigmented or fluorescently labeled embryos were allowed to develop to stage 39/40, fixed in 4% paraformaldehyde and viewed under a Standard-14 Zeiss microscope equipped with epifluorescence or a dissecting microscope and photographed (Kodak, Ektachrome 200 ASA).

In situ hybridization

Whole-mount *in situ* hybridization was performed according to Harland (1991), with minor modifications. Antisense RNA probes were labeled with digoxigenin-UTP or fluorescein-UTP using the Boehringer-Mannheim (Roche) Genius RNA labeling kit. RNA localization was visualized with BCIP/NBT. The full-length axolotl *pax 6* probe was generously provided by Dr. Tom Glaser (University of Michigan). The modifications of Harland’s technique for axolotl embryos included the following: 1) Due to the larger size of axolotl embryos, fewer embryos per vial were used. One axolotl embryo was equivalent to three or four *Xenopus* embryos; 2) The axolotl embryos were much more fragile to the *in situ* procedure, and they tended to break up more frequently. For this reason, we used lower incubation and preincubation temperatures (55- 57°C instead of 60°C). This overall procedure often resulted in greater nonspecific background, but several changes of Murray’s (for up to two weeks) usually got rid of excess nonspecific staining.

Whole brain anti-acteylated Tubulin immunocytochemistry

Since *pax 6* -expression patterns are important for forebrain axonal guidance (Shimamura et al., 1995), we investigated development of the axonal tracts within the axolotl forebrain. To visualize developing neurons within the forebrain of eyed and eyeless axolotl embryos, a monoclonal antibody against acteylated tubulin was used (Sigma, St. Louis, MIssouri, USA). Embryos ranging from stages 28 to 41 (hatching) were anesthetized in MS 222 and fixed in fresh 4% paraformaldehyde buufered with 0.1 M phosphate buffer (pH 7.4). Brains were microdissected out in Sylgaard dishes and dehydrated with a series of alcohols. The brains were then postfixed in Dent’s fixative (20% DMSO/80% methanol) 2 to 6 hours at room temperature. The tissue was then rehydrated and bleached in 4% paraformaldehyde containing 7 % hydrogen peroxide (under a fluorescent lamp). The tissue was then washed several times in 0.1 M phosphate buffered saline (PBS; pH 7.4). It was then rinsed three times for 10 minutes in PBS with 0.3% Triton-X and 0.2% bovine serum albumin (PBT). Brains were then incubated 30 minutes in PBT with 5% goat serum and transferred to PBT with 5% goat serum containing the primary antibody of mouse anti-acetylated tubulin (diluted 1:50). The whole brains remained in the solution with the primary antibody overnight or over the weekend at 4°C. After antibody incubations, brains were incubated in the secondary antibody of goat antimouse immunoglobulin with attached peroxidase (Jackson Laboratories, Inc) at dilutions of 1:400 overnight at 4°C. After several washes in PBS, the brains were reacted with DAB (500 μg/ml) containing 0.03% hydrogen peroxide. The reaction was stopped with PBS, and the brains were dehydrated and cleared in benzyl benzoate/benzyl alcohol (Murrays). Brains were mounted in Permount and photographed with a Standard-14 Zeiss microscope using Kodak T Max film (ASA 200 or 400).

Whole brain Tyrosine Hydroxylase immunocytochemistry

In order to test if dopaminergic neurons were affected by the eyeless condition or the loss of the prechordal plate, whole-brain immunocytochemistry for tyrosine hydroxylase (TH) was performed. Whole brains from stage-35 to 40 axolotls were dissected out from normal, eyeless and operated animals. Whole-brain immunocytochemistry was performed in the same manner as whole-brain anti-acetylated tubulin immunocytochemistry with the exception that the primary antibody used was mouse anti-tyrosine hydroxylase (Incstar, USA).

Embryonic surgery

To produce animals lacking prechordal plate (PCP), which is the source of sonic hedgehog (*shh*), PCP-extirpation operations were performed. Stage-15 neural plate axolotl embryos were placed in Steinberg's solution (Armstrong and Malacinski, 1989) in 2% agarized petri dishes for operations. Membranes were removed manually with Dumont (#5 or #55) forceps, and the demembraned embryo was placed in a hole within the agar. An incision was made along and beneath the anterior neural ridge. The cut portion was gently lifted up with forceps, and the underlying prechordal plate (PCP) was exposed. This area was removed or scraped away from beneath the neural plate using forceps or sharpened tungsten needles. The neural-fold tissue was set back down and pushed together with the ectoderm by placing a small piece of cover slip over the area. Healing required about 5 minutes before the operated individuals could be transferred to another dish to develop. Operated controls consisted of embryos in which the cuts were made but the PCP tissue was not removed.

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References

ARMSTRONG, J.B. and MALACINSKI, G.M. (Eds.) (1989). *The Developmental Biology of the Axolotl*. Oxford University Press, New York.

BORDZILOVSKAYA, N.P. DETTLAFF, T.A., DUHON, S.T. and MALACINSKI, G.M. (1989). Developmental-stage series of axolotl embryos. In The *Developmental Biology of the Axolotl* (Eds. J.B. Armstrong and G.M. Malacinski). Oxford University Press, New York, pp. 201-219.

BRUN, R.D. (1993). Bilateral eye formation in the eyeless mutant salamander following unilateral, partial excision of neural fold tissues: A quantitative study. *J Exp. Zool*. 265: 541-548.

CHOW, R.L., ALTMANN, C.R., LANG,.A. and HEMMATI-BRIVANLOU, A. (1999). *Pax6* induces ectopic eyes in a vertebrate. *Development*.126: 4213-4222.

CUNY, R. and MALACINSKI, G. (1986). Axolotl retina and lens development: mutual tissue stimulation and autonomous failure in the eyeless mutant retina. *J. Embryol. Exp. Morphol*. 96: 151-170.

EAGLESON, G.W. and MALACINSKI, G. (1986). A scanning electron microscopy and histological study on the effects of the eyeless (*e/e*) gene upon the hypothalamus in the Mexican axolotl, *Ambystoma mexicanum* Shaw. *Anat. Rec*. 215: 317-327.

EAGLESON, G.W. and HARRIS, W.A. (1990). Mapping the presumptive brain regions in the neural plate of *Xenopus laevis*. *J. Neurobiol.* 21: 427-440.

EAGLESON, G., FERREIRO, B. and HARRIS, W.A. (1995). Fate of the anterior neural ridge and the morphogenesis of the *Xenopus* forebrain. *J. Neurobiol.* 28: 146-158.

EAGLESON, G.W., UBINK, R., JENKS, B.G. and ROUBOS, E.W. (1998). Forebrain differentiation and axonogenesis in amphibians: I. Differentiation of the suprachiasmatic nucleus in relation to background adaptation behavior. *Brain Behav Evol*. 52: 23-36.

EKKER, S.C., McGREW, L.L., CHENG-JUNG, L., LEE, J.J., VON KESSLER, D.P., MOON, R.T. and BEACHY, P.A. (1995). Distinct expression and shared activities of members of the *hedgehog* gene family of *Xenopus laevis*. *Development* 121: 2337-2347.

GLASER, T. WALTON, D.S., and MAAS, R.L. (1992). Genomic structure, evolutionary conservation and aniridia mutations in the human Pax6 gene. *Nat. Genet*. 2: 232-239.

GRINDLEY, J., DAVIDSON, D., and HILL, R. (1995). The role of pax-6 in eye and nasal development. *Development* 121: 1433-1442.

HARLAND, R.M. *In situ* -hybridization: an improved whole-mount method for *Xenopus* embryos. In *Methods of Cell Biology* (Eds. B.K. Kay and H.B. Peng) Academic Press, San Diego, pp. 685-695.

HILL, R.E., FAVOR, J., HOGAN, B.L., TON, C.C., SAUNDERS, G.F., HANSON, I.M., PROSSER, J., JORDAN, T., HASTIE, N.D. and VAN HEYNINGNEN, V. (1991). Mouse small eye results from mutations in a paired-like homeobox containing gene. *Nature* 354: 522-525.

HUMPHREY, R.R. (1969). A recently discovered mutant "eyeless" in the Mexican axolotl (*Ambystoma mexicanum*). *Anat. Rec*. 163: 306.

HYNES, M., PORTER, J.A., CHIANG, C., CHANG, D.I., TESSIER-LAVIGNE, M., BEACHY, P.A., and ROSENTHAL, A. (1995). Induction of midbrain dopaminergic neurons by *sonic hedgehog*. *Neuron* 15: 35-44.

JACOBSON, A.G. (1958). The roles of the neural and nonneural tissues in lens induction. *J. Exp. Zool.* 139: 525-557.

JACOBSON, C. (1959). The localization of the presumptive cerebral regions in the neural plate of the axolotl larva. *J. Embryol. Exp. Morph.ol*. 7: 1-21.

KRAUSS, S., JOHANSON,T., KORZH, V. and FJOSE, A. (1991). Expression pattern of the zebrafish pax genes suggest a role in early brain regionalization. *Nature* 353: 267-270.

LI, H.-S., TIERNEY, C., WEN, L., WU, J.Y., and RAO, Y. (1997). A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development* 124: 603-615.

MATHERS, P.H., GRINBERG, A., MAHON, K.A. and JAMRICH, M. (1997). The *Rx* homeobox gene is essential for vertebrate eye development. *Nature* 387: 603-607.

MATSUO, T., OSUMI-YAMASHITA, N., NOJI, E., KOYAMA, E., MYOKAI, F., MATSUO, N., TANIGUCHI, S., DOI, H., ISEKI, S., NINOMIYA, Y., FUJIWARA, M., WATANABE, T. and ETO, K. (1993). A mutation of the Pax6 gene in rat small eye is associated with impaired migration of midbrain crest cells. *Nat. Genet.* 3: 299-304.

PUSCHEL, A., GRUSS, P. and WESTERFIELD, M. (1992). Sequence and expression pattern of pax-6 are highly conserved between zebrafish and mice. Development 114: 643-651.

QUIRING, R., WALLDORF, U., KLOTER, U. and GERHING, W.J. (1994). Homology of the eyeless gene of Drosophila to the small eye gene in mice and Aniridia in humans. *Science* 265: 785-789.

SHIMAMURA, K., HARTIGAN, D.J., MARTINEZ, S., PUELLES, L. and RUBENSTEIN, J.L.R. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* 121: 3923-3933.

SPEMANN, H. (1938). *Embryonic Development and Induction*. New York: Yale University Press.

VAN DEUSEN, E. (1973). Experimental studies on a mutant gene (e) preventing the differentiation of eye and normal hypothalamus primordia in the axolotl. *Dev. Biol*. 34: 135-158.

WALTHER, C. and GRUSS, P. (1991). Pax-6, a murine paired box gene is expressed in the developing CNS. *Development* 113: 1435-1449.

WARREN, N. and PRICE, D.J. (1997). Roles of pax-6 in murine diencephalic development. *Development* 124: 1573-1582.

WILSON, S.W., ROSS, L.S., PARRETT, T. and EASTER, S.S. Jr. (1990). The development of a simple scaffold of axon tracts in the brain of the zebrafish, *Brachydanio rerio*. *Development* 108: 121-145.

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