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The eye in the brain: retinoic acid effects morphogenesis of the eye and pathway selection of axons but not the differentiation of the retina in *Xenopus laevis*

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We have analyzed the effects of all-trans retinoic acid (RA) on the morphogenesis, differentiation and projection of the eye of *Xenopus*. RA was applied in concentrations of 10^{-5}, 5×10^{-6} and 10^{-6} M at stages 9–17. Animals were reared until stages 40–48. RA applied before stage $11^1/_2$, abated completely formation of an eye or a retina, at later stages it led to the formation of microphthalmic eyes. Even in the absence of an eye parts of the forebrain had characteristics of the retina, but rods and cones reached then into the lumen of the third ventricle. The projection of eyes of RA-treated animals was revealed with rhodamine dextran amine. Ganglion cell axons projected bilaterally to the tectum, to the hindbrain, the contralateral retina and, occasionally, to the olfactory bulb. RA affects both morphogenesis of the eye and pathway selectivity of ganglion cell axons but not differentiation of the neural retina.

Biologically active retinols, i.e. 'vitamin A', and specific intercellular and cytoplasmic retinol and retinoic acid binding proteins are essential for normal function of the adult vertebrate retina [1]. Retinols apparently play also a crucial role during development of the eye [1] and vitamin A deficiency causes microphthalmia in pigs [10]. Retinoic acid (RA), well known for its teratogenic effects [8], and also as a putative morphogen of the limb [19], was recently shown to abate formation of the eyes in *Xenopus laevis* [3] presumably through repatterning of the mesoderm [15]. Thus, both artificially low and artificially high levels of retinoids may result in malformations of the eyes. In order to obtain further insights into the effects of retinoic acid, we studied the concentration dependent and age dependent effects of all-trans retinoic acid (RA) on the morphogenesis and differentiation of the eye and the retina and the projection patterns of the retina in RA treated animals.

We analyzed a total of 148 *Xenopus laevis*. Forty-eight embryos were treated at stages 9, 10, 11, $11^1/_2$, 12, 13, 15 and 17 with 5×10^{-6} M all-trans retinoic acid (RA) in a modified Holtfreter solution for 30 min and were subsequently reared in tap water. When controls were at stage 40–41 six embryos of each batch were fixed in Smith's fixative, dehydrated and embedded in histowax. The embryos were cut transversely at 12 μm and stained with haematoxylin–eosin as described elsewhere [3]. Hundred additional embryos were treated at stages $11^1/_2$ and 12 either with 10^{-6} or 10^{-5} M RA in aged tap water for 30 min. When controls were at stages 46–48 six embryos of each batch were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated and embedded in epoxy resin. Serial 10 μm transverse sections were counterstained with Stevenel's blue, coverslipped and examined.

Seventy-six animals treated at stage 12 with 10^{-5} or 10^{-6} M RA were anesthetized in tricaine methanesulfonate at stages 46–48 and rhodamine dextran amine [7] was applied to the retina or the cut optic nerve. After two hours transport time the animals were anesthetized and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected, cut along the dorsal midline of the midbrain and forebrain, mounted flat on a gelatinized slide and viewed with an epifluorescence microscope. In 30 of these brains dextran amine coupled to fluorescein was applied one hour prior to the rhodamine dextran amine application to the cut rostral spinal cord to reveal the reticulo-spinal cells as an internal reference for the position of target areas of the retinal fibers.

Depending on the stage of application and the concentration the experimental animals either developed no

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recognizable eye structures or had eyes which were completely or partly incorporated in the brain. When present, eyes were only partially surrounded by pigment. Complete suppression of eyes was obtained with treatment in stages 9–11½, complete suppression of morphogenesis was achieved at stage 12 and decreasing suppression of morphogenesis occurred in older stages. Concentration-dependent effects at stage 12 showed complete suppression of morphogenesis with 10^{-5} M RA.

Histologic examination revealed that the eyes were in many cases in broad continuation with the third ventricle and outer segments of receptor cells protruded into the ventricle. If an eye was at all recognizable, the retina showed numerous folding (Fig. 1a). Comparable rosettes develop in the chicken retina after treatment with the mitogen blocker cytosine arabinoside [14]. RA is known to arrest mitogenesis in cranial neural crest [8] and to induce differentiation of human embryonic carcinoma stem cells in a concentration-dependent manner [16]. If RA effects mitogenesis and causes premature differentiation in the developing retina, then the above described similarities in chicken and frog retina may both reflect reduced proliferation. Although the developing retina may not be a specific target for RA, the nearby neural crest derived mesenchyme obviously accumulates RA and is rich in RA receptors [2, 20]. It is possible that RA effects primarily ectomesenchyme or normal mesoderm [15] and suppression of the eyes is a secondary phenomenon.

Cases without externally recognizable eyes had numerous receptor cells around the ventricle (Fig. 1b). The ventral and lateral wall of the ventricle showed the differentiation into a retina-like pattern rather then the typical organization of the frog forebrain: the receptor cell layer replaced the ependymal cells of the normal forebrain; the ganglion cells were in a submeningeal layer normally covered by neuropil; the axons of the ganglion cells formed a submeningeal fiber layer not found in normal forebrain. Further caudal we could sometimes identify a preoptic nucleus with its recess (Fig. 1b). Caudal to the preoptic nucleus the ganglion cell axons turned towards the midline to form an optic chiasma. Irrespective to the suppressed morphogenesis of the eye, the retina displayed the gnathostome organization. This indicates that the presumably late evolutionary event which has transformed the lamprey retinal pattern [4] into the gnathostome pattern, is not suppressed concomitantly with the eye morphogenesis.

Lenses, known to be variously dependent in amphibians on inductive interaction with the developing eye [13] are consistently absent in the more severe reductions of the eye. More differentiated eyes had two or even three small lenses instead of a single one (Fig. 1a). Despite recent suggestions of only a minor role played by the optic vesicle for the formation of the lens in *Xenopus* [9], the interaction of different parts of the variously

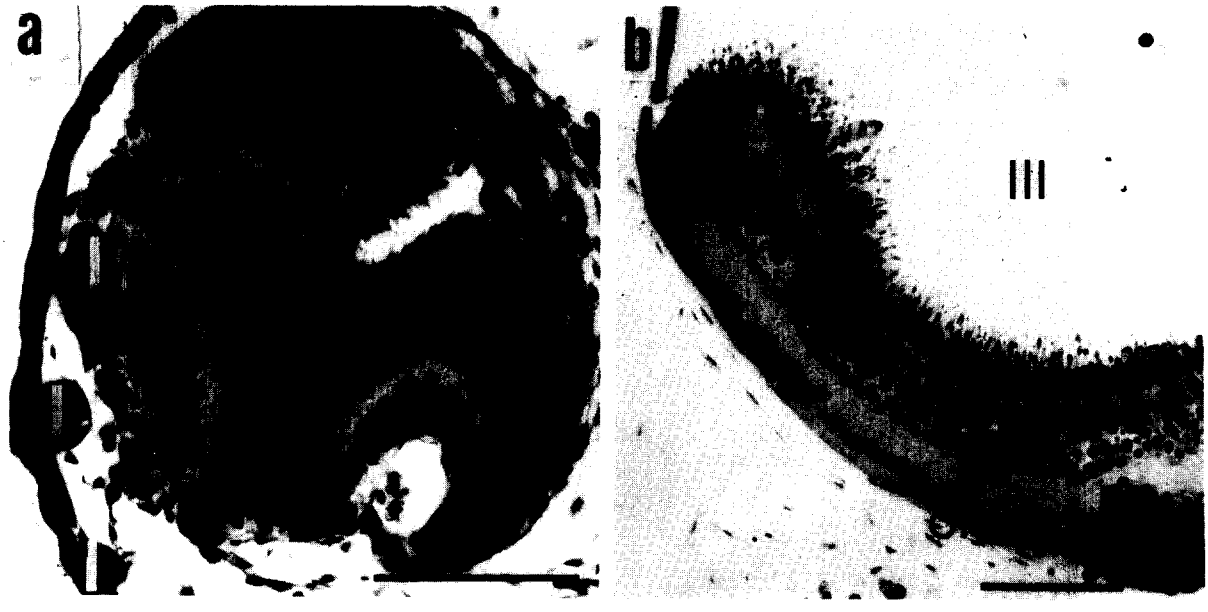

Fig. 1. 5×10^{-6} (a) and 10^{-5} M (b) retinoic acid (RA) induced transformation of the eyes, lenses and the forebrain if applied at stage 12. Histowax (a) and epoxy resin (b) embedded tissue of animals fixed at stages 41 (a) and 48 (b). Note the distorted morphogenesis of the retina and the formation of 3 small lenses but the normal organization of the retina layers (a). Even complete suppression of morphogenesis does not affect the differentiation of retinal layers (b). Abbreviations: g, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; l, lens; ofl, optic fiber layer; onl, outer nuclear layer; opl, outer plexiform layer; pn, preoptic nucleus; III, third ventricle. Bar = 100 μm.

folded eye cup with the epidermis may have led to the formation and maintenance of multiple lenses at different sites of eye-ectodermal interaction.

Retinal projections showed deviations from normal even in RA-treated animals with only moderate distortions of the eye. Rhodamine dextran amine (RDA) labelled projections showed a pronounced reciprocal connection to the contralateral retina in 60% of the cases. In some animals this projection was rather massive and the majority of fibers was found to cross to the contralateral side (Fig. 2b,c). In a few cases (6%) we found single fibers which ran into the forebrain to ramify in the olfactory bulb. In some cases the retinal projections coursed only in the basal optic tract and then towards the hindbrain (4%) or looped around the diencephalon (4%). Similar projections are only known in experimentally perturbed animals. For example, loops and direct hindbrain projections were recently described after the tectum was surgically removed [18]. These similarities are consistent with the apparent lack of a histologically identifiable tectum in our preparations with these aberrant projections.

Even in cases with an almost normal retinal projection predominantly to the contralateral tectum there was of-

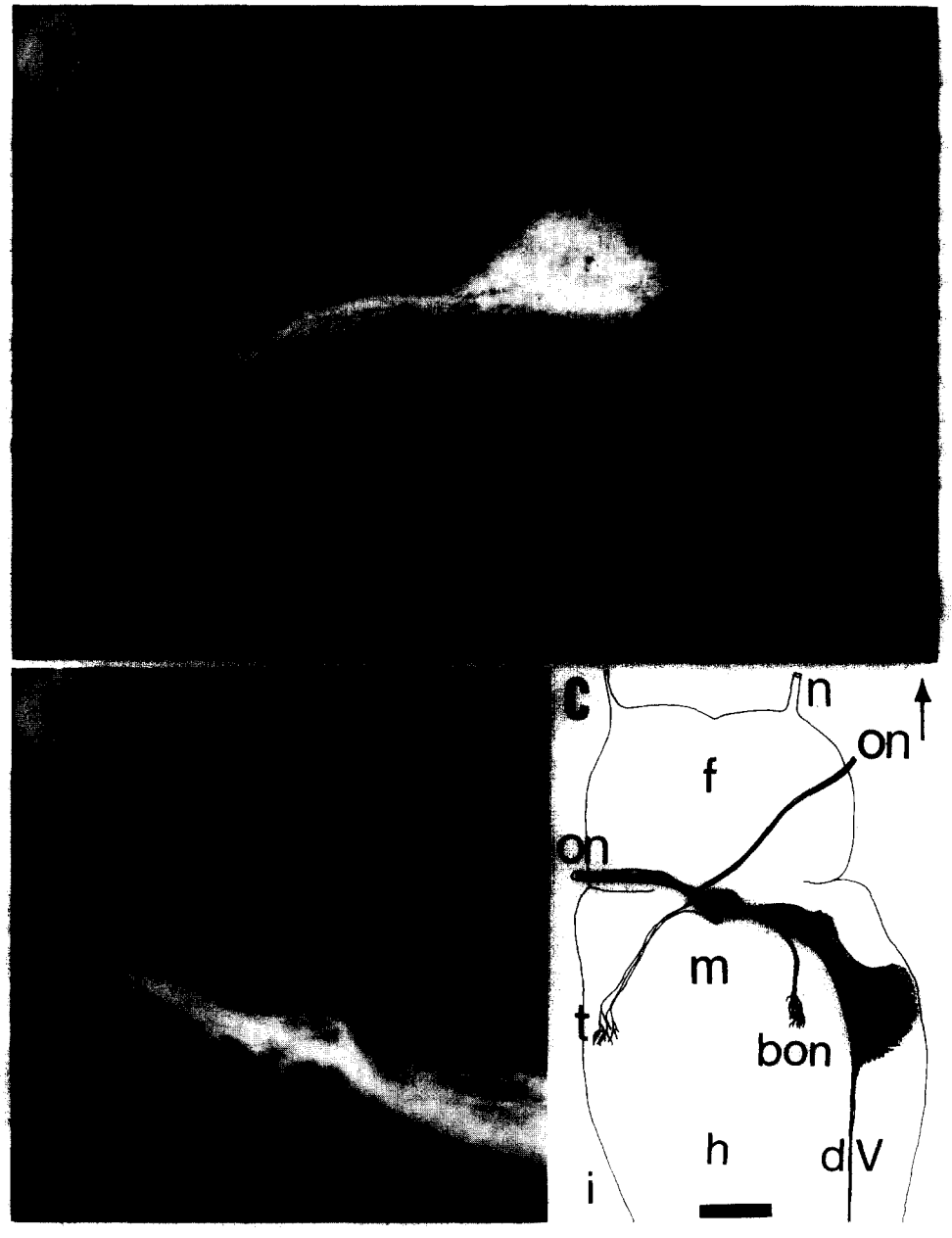

Fig. 2. Retinal projections of animals treated at stage 12 with 10^{-6} M RA. Note the projection to the contralateral retina, bilateral to the tectum and from the tectum into the hindbrain. Arrow indicates rostral. Abbreviations: bon, basal optic neuropil; c, contralateral; ch, chiasma; f, forebrain; h, hindbrain; i, ipsilateral; m, midbrain; n, olfactory nerves; nb, neuropil of Bellonci; dV, descending trigeminal tract; t, tectum; on, optic nerve. Bar = 100 μm.

ten (ca. 34%) an extension of this projection to the hindbrain (Fig. 2a,c). These fibers always run within the descending tract of the trigeminus (Fig. 3). Apparently the optic tract and the descending trigeminal tract share one epitope [17, 21]. Our data are consistent with the suggestion that this epitope may provide selection cues [11, 18] for retinal ganglion cell axons along their trajectory. The further extension of retinal axons into the hindbrain argues against an exclusive tropic role of the tectum [18], at least after RA treatment.

A number of factors may contribute to these aberrant projections. For example, the shortened optic nerve may

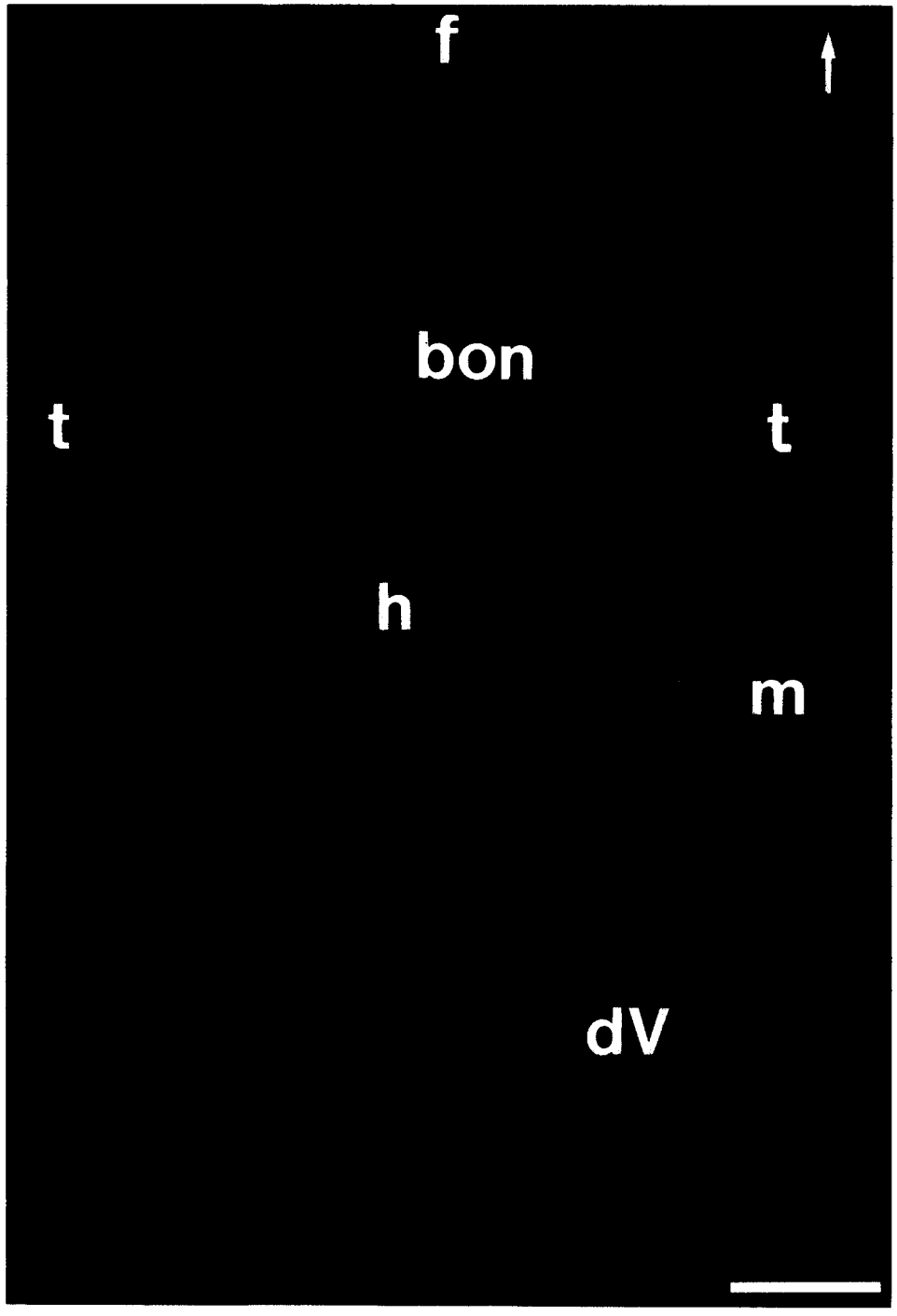

Fig. 3. Whole mount of an animal treated with 10^{-6} M RA at stage 12. The projection of the retina is revealed with rhodamine dextran amine. In addition, the spinal cord was transected and the differently colored fluorescein dextran amine was applied 3 h prior to sacrifice to fill the reticulospinal neurons. Note the projection of the retina into the hindbrain. The fibers run within the descending trigeminal tract (dV) adjacent to the morphologically modified Mauthner cell (m). Abbreviations see Fig. 2. Bar = 100 μm.

allow outgrowing fibers to interact with the chiasma before the chiasmatic sorting mechanisms are developed [5]. Size mismatch between the retina and the tectum, as well as heterochrony in developmental rates, may cause the retinal projection to expand beyond the tectum. The bilateral retinal projection may reflect a premature metamorphosis-like behavior of retinal axons which have been shown to project bilateral to the diencephalon under the influence of thyroid hormones [12]. RA may act directly on thyroid hormone receptors or RA receptors and thyroid hormones may form heterodimers [1] and may thereby cause the premature metamorphosis-like change of pathway selection.

In summary, our data show that RA suppresses the morphogenetic transformation of a part of the diencephalon [6] into the eye and also disturbs the projection of the retina. However, the differentiation of the retina is even in the absence of any eye formation normal, albeit in an unusual position within the brain.

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