

Short Communication

Eph/ephrin A- and B-family expression patterns in the leopard frog (*Rana utricularia*)

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Abstract

Eph/ephrin expression was studied in *Rana utricularia* larvae and adults with in situ receptor and ligand affinity probes. From stages TK-II (early limb bud) to VI (early foot paddle larva), tectal EphB expression is highest in a band extending transversely across the posterior optic tectum and grades off anteriorly and posteriorly. The ephrin-A expression gradient is parallel to the EphB gradient rather than being orthogonal to it. However, its high point occupies the posterior pole, and it runs from high-posteriorly to low-anteriorly. Tectal EphA expression is high anteriorly and low posteriorly, while ephrin-Bs are expressed only in a thin line at the dorsal midline. At later stages and in adults, tectal EphB expression becomes uniform.

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The EphA and EphB families of receptor tyrosine kinases have been shown to play critical roles in the formation of the retinotectal/superior collicular, visuotopic map during development in representative vertebrate species (reviewed in [8]). The former is involved in patterning the temporonasal (T-N) axis of the retina onto the anteroposterior (A-P) axis of the optic tectum/superior colliculus (OT/SC), while other evidence suggests that the EphB family contributes to organizing the projection of the dorsoventral (D-V) retinal axis onto the lateromedial (L-M) axis of OT/SC. Studies on the EphB/ephrin B-family in the chick, mouse and *Xenopus* frog have revealed expression gradients that are oriented orthogonal to the actual or expected EphA-family gradients, both in the tectum and retina. In chick and mouse, EphB receptors are graded from high ventrally to

low dorsally in the retina, while ephrin-B ligands are graded from high medially to low laterally in OT/SC [3,6]. In contrast with the patterns described in the mouse, in *Xenopus* [7], the only amphibian in which EphB-family expression has been previously studied, it is the ligand ephrin-B2 in retinal axons – in which it is graded from high dorsally to low ventrally – that appears critically to interact with receptor in OT. The OT is reported to express the receptor, EphB1, in a matching high-lateral to low-medial gradient. Disruption of B-family signaling in *Xenopus* causes a displacement of the whole retinotectal projection along a lateromedial axis. It has been assumed, in accord with the known A-P orientation of tectal ephrin-A expression patterns in other vertebrates, that this displacement would be orthogonal to the A-family gradient, although the A-family expression pattern in *Xenopus* has not been described. Our concurrent observations on A- and B-family gradients in leopard frog tadpoles suggest an alternative viewpoint.

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Larval and adult southern leopard frogs (*Rana utriculata*) were supplied by Charles Sullivan (Nashville, TN). Larvae were reared in de-chlorinated tap water and fed boiled spinach. Adults were housed in a terrarium with running tap water, equipped with dry platforms and fed liberally with an insect diet. The fusion proteins were constructed by inserting the cDNA for chick EphA3 or ephrin-A5, or mouse EphB2 or ephrin-B1, into an APtag vector, followed by transfection into either NIH 3T3 cells (for receptor expression) or 293T cells (for ligand expression).

Tadpoles were staged according to standard descriptions [9,10]. They were sacrificed for study between stages TK-II (early limb bud larva) and TK-XI (early hind foot stage) by decapitation, and the brain was dissected out ($N = 31$). Most specimens were sampled during the earlier developmental period to ensure adequate replication of results during a period of rapidly changing brain morphology. At least four larvae were sacrificed at each stage between TK-II and TK-VI; the last five stages were sampled on a spaced schedule. Adult brain tissue was also obtained by decapitation ($N = 6$). The fusion protein reactions were carried out as previously described [1,5]. Briefly, after removal of loose meningeal tissue and surface blood vessels, the fresh, unfixed brain was washed in Hepes-buffered Hank's balanced salt solution with BSA (HBAH, pH 7.0) and incubated for 90 min in the fusion protein (2–5 $\mu\text{g/ml}$ in HBAH; this concentration is in excess of the targeted ligand or receptor). It was then washed in HBAH, lightly fixed for 2 min in Hepes-buffered formalin (8%)/acetone (60%), washed in Hepes-buffered NaCl (HBS), incubated in HBS at 65 °C for 2 h to inactivate endogenous AP, washed in standard AP-staining buffer and transferred for 2–5 h to an AP coloring solution containing bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT; Roche Diagnostics, Mannheim, Germany; 0.17 mg/ml BCIP + 0.33 mg/ml NBT in AP-staining buffer). The reaction was stopped with several changes of HBS, the pia mater removed and the specimen post-fixed in 4% PFA. Reacted brains were photographed with 35 mm slide film. The developed images were digitized and manipulated with Adobe Photoshop 4 (Adobe Systems) and Canvas 7 (Deneba Systems). In some specimens, the fusion protein was omitted from the initial incubation medium to check for endogenous AP activity resistant to heat inactivation. In other specimens, an unfused product generated from the AP tag vector alone was substituted for the AP-fusion protein to test for non-specific AP binding. As a further control, the eye was removed unilaterally from a group of larvae at stage TK-III ($N = 6$) to promote degeneration of the optic axons and delete their possible contribution to the tectal staining patterns. They were sacrificed at TK-VI to VIII, and levels of EphA and EphB expression in the optic tracts and OTs were compared.

From the earliest limb bud stages studied, TK-II/III, ephrin-A is expressed in the OT in an increasing anterior to posterior gradient extending all the way to the posterior pole

(Figs. 1A and C), as also observed in the closely related species, *Rana pipiens* (unpublished observations), while tectal EphA expression (Figs. 1D and F) is confined to the anterior OT. In these early stages, only the anterior part of the OT is innervated by retinal axons [4], while more posterior regions are undergoing cell proliferation and cytoarchitectonic differentiation. Thus, the region of high ephrin-A expression extends posteriorly beyond the differentiated and innervated part of the OT into its undifferentiated zones. As in the adult [1], EphA is expressed in the optic nerve and central region of the optic tract in a stripe that was greatly reduced on the contralateral side after unilateral eye removal in each of the three larvae examined after the allowed survival period, although the level of expression in the tectum appeared unaffected, indicating that retinal axons contain EphA, and EphA expression in the tectum is to some extent endogenous.

The tectal expression pattern for EphB is initially graded but becomes more uniform at later stages. From TK-II/III (Figs. 1B and E) through TK-IV (limb bud stages), staining is most intense in a curved band located just anterior to the posterior pole and grades off both anteriorly and posteriorly. A stained band is also positioned along the posterior edge of the optic tract and the optic nerves are intensely stained. Thus, the EphB gradient observed at these early limb bud stages has essentially the same orientation as the ephrin-A gradient, but its highest level is not placed as far posteriorly. Staining is very weak or non-existent in the polar region of rapid cell proliferation [4], suggesting that EphB expression is associated with some degree of neural differentiation. In these early tadpoles, ephrin-B is expressed highly in the developing olfactory and accessory bulbs, and in the lateral pallium, but there is no significant expression in the OT, except in a narrow zone along the midline (Fig. 1G).

By TK-VI, the earliest foot paddle stage, the EphB expression gradient has flattened markedly, with an increase in the staining density in the anterior half of the OT (Fig. 2A), although the band of highest expression persists. It is readily identifiable at TK-XI (not illustrated), the latest larval stage examined, although the rest of the OT anterior and lateral to it has become uniformly stained. The least-expressing zone at the posterior pole remained visible at TK-XI ($N = 1$) and probably persists for some time afterward, but not in the mature adult (Fig. 2B). It is not known whether the elevated EphB expression in the anterior OT is due to the appearance of additional species of B-family receptors, or to a change in the expression pattern of the receptors detected earlier in development. In the larvae surviving unilateral eye-removal, EphB staining was reduced to some extent in the contralateral optic tract, but the tectal expression pattern remained unchanged. Ephrin-B expression remains confined largely to the telencephalon in adult frogs (Fig. 2B), while tectal ephrin-A expression follows the same posterior-high to anterior-low gradient present throughout development (not illustrated, but see [1]).

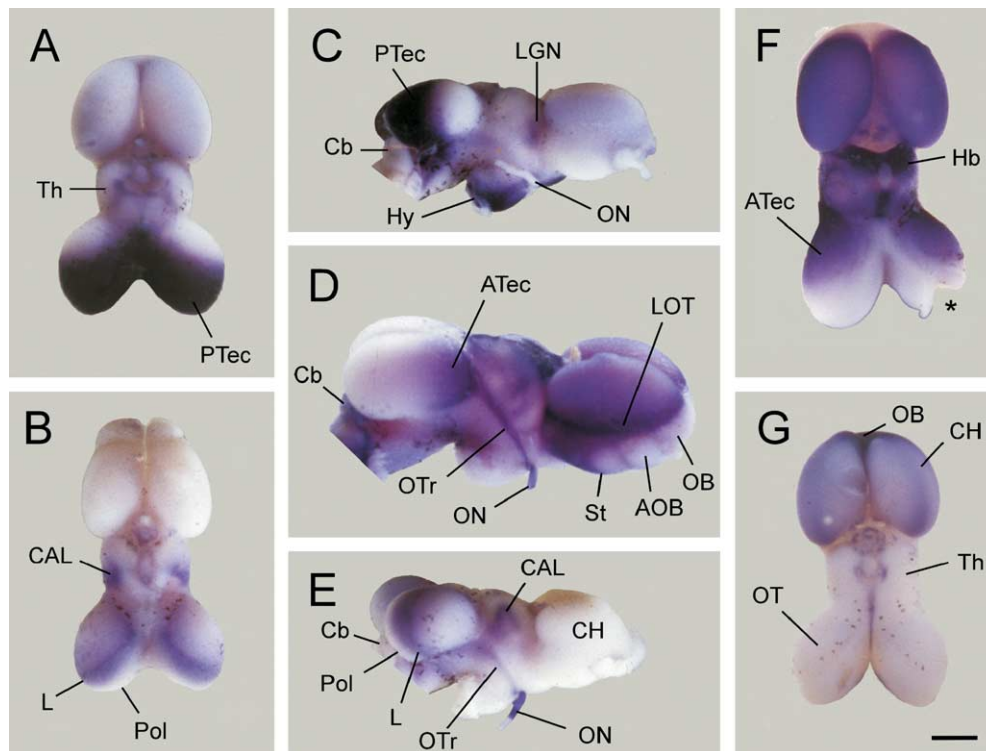


Fig. 1. A series of overhead and side view photomicrographs of *R. utricularia* tadpoles showing the patterns of A- and B-family Eph/ephrin expression in representative cases at early and later larval stages, as revealed by means of receptor- or ligand-AP fusion proteins used as affinity probes (see text). (A) Stage TK-III larva, showing the anterior-low to posterior-high expression gradient for ephrin-As detected with chicken EphA3-AP. Ephrin-A expression extends to the tectal midline and all the way through the epithelial, polar zone of the posterior OT (PTec). (B) Stage TK-III larva, showing the anterior-low to posterior-high expression gradient for EphBs detected with mouse ephrin-B1-AP. EphB expression does not extend to the tectal midline or posterior pole (Pol) but reaches its maximum in a prepolar belt or limbus (L). The area of the principal tectorecipient nucleus of the thalamus (CAL) is prominently stained. (C) Same specimen as shown in panel A. Note the positive staining in the lateral geniculate region (LGN) and hypothalamus (Hy). The optic nerve (ON, folded back) and cerebellum (Cb) are unstained. (D) Stage TK-VII larva, showing the anterior-high to posterior-low expression gradient for EphAs detected with chicken ephrin-A5-AP. The optic nerve (ON) and a central stream of the optic tract (OTr) leading to the anterior tectal pole (ATEc) are densely stained, as is the cerebellum (Cb) and the tissue along the pallial–subpallial border. The olfactory (OB) and accessory olfactory bulbs and anterior striatum (St) are negative. (E) Same specimen as shown in panel B. The densely stained limbus (L) extends to the ventrolateral margin of the posterior OT, where it meets the dorsal end of a stripe of stained axons located along the posterior margin of the optic tract (OTr). The polar region of the OT (Pol), forming a rim just posterior and concentric with the stained limbus, remains unstained. The optic nerve (ON) is prominently stained, while the hypothalamus and cerebellum (Cb) are unstained. (F) Stage TK-III larva, showing the anterior-high to posterior-low expression gradient for EphAs. EphA expression does not extend to the tectal midline, where ephrin-A expression would be prominent (see panel A). Certain features of the dorsal midline of the thalamus are stained including the habenular nuclei (Hb). Asterisk (*) indicates damaged tissue. (G) Stage TK-III larva, showing the intense expression of ephrin-Bs in the olfactory bulbs and cerebral hemisphere (CH) detected with mouse EphB2-AP. Most of the thalamus and the OT is negative, except for a thin streak along the midline. Panels A, B, F and G are dorsal views; anterior to the top. Panels C, D and E are right side views; anterior to the right. Scale bar in panel G = 0.5 mm applies to all panels.

The brains processed as AP tag vector controls were stained uniformly in a pink coloration, while the AP-inactivation controls were white.

The identities of the specific within-family receptors and ligands detected by the affinity probes cannot be determined since the fusion proteins bind to many A- and B-family members. For example, EphA3-AP fusions could be expected to bind ephrin-A2 and ephrin-A5, or other A-family ligands that may exist in the frog. However, the possibility of cross-family reactivity is mooted by the distinctively different patterns of binding obtained with the four probes. For example, if the EphA3-AP reagent had significant affinity for some ephrin-B ligand present in the frog brain, in addition to the ephrin-A ligands it was designed to detect, such hypothetical B-ligand would have to be highly expressed in the posterior part of OT, along

with the ephrin-As localized there. However, the hypothetical B-ligand would have to have the unlikely property of having little or no binding affinity with EphB2-AP, since the use of that probe revealed no ephrin-B expression anywhere in OT. Similar arguments rule out or make unlikely other cross-family binding permutations.

Tectal EphB/ephrin-B expression has been previously analyzed in *Xenopus* [10], although only at one early stage of development (stage 39). EphA/ephrin-A expression patterns have not been described in that species. At stage 39, a pre-limb bud stage in which the brain of *R. utricularia* embryos would still be highly pigmented and unsuitable for whole-mount analysis, tectal EphB1 mRNA in *Xenopus* embryos is distributed in a ventral (lateral)-high to dorsal (medial)-low gradient in cross sections of the midbrain. Ephrin-B1 and ephrin-B2 mRNAs are expressed at and

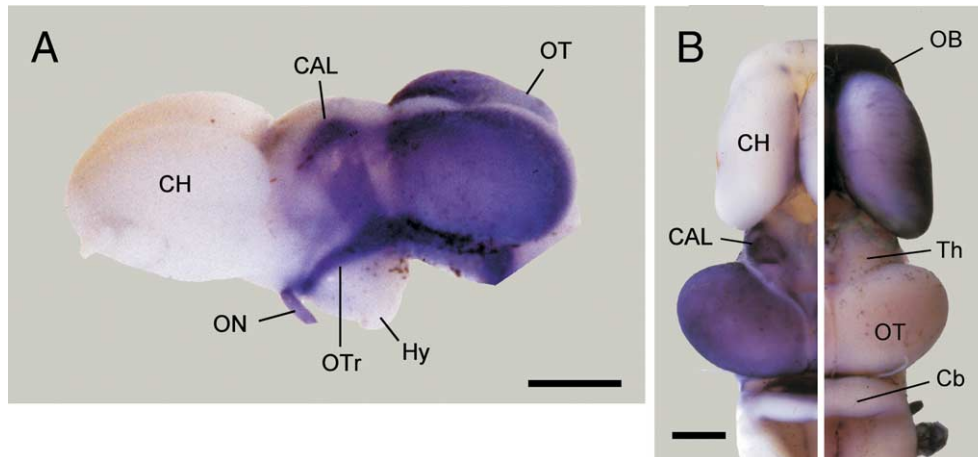


Fig. 2. Representative specimens illustrating the altered expression of EphB in later larval stages and the adult. (A) Expression of EphB in a stage TK-VI *R. utricularia* tadpole. While still graded, the tectal expression pattern is more uniform than at earlier stages, showing increased expression in the anterior OT. The polar zone remains only lightly stained. The posterior margin of the optic tract (OTr) is prominently stained, as is the midthalamic zone (CAL) to which the OT projects. Anterior is to the left. Scale bar = 0.5 mm. (B) In the adult leopard frog, tectal EphB expression (left panel) becomes uniform throughout and remains elevated in the middle region of the thalamus (CAL). Ephrin-B is highly expressed in the olfactory bulb (OB) and cerebral cortex, but as in the tadpole, not detectable in the OT (right panel). Scale bar = 1 mm.

around the dorsal midline in a relatively undifferentiated region that is not innervated by the retina at that stage. While there are methodological differences, it appears that the gradient of EphB expression we have observed in the limb bud stages of *Rana* would be orthogonal to the EphB1 gradient described for *Xenopus*, since it is oriented in the A-P, rather than the D-V direction. The EphB gradient in *Rana* is higher at the posterior end of the OT, while in *Xenopus* the high end of the gradient is ventral (lateral). These observations may reflect species differences among amphibians. However, such a conclusion may be premature since the available data allow only comparison of the sectional views of the EphB1 gradient in *Xenopus*, with the global patterns obtained in *Rana* from whole-mount preparations. Whole-mount preparations for *Xenopus*, serial section reconstructions or sectioning in different planes might provide a more readily comparable image of the alignment of the gradients in the two species. Moreover, the expression patterns may be stage-dependent. Nevertheless, one conclusion stemming from our whole-mount material is clear – contrary to expectations based on reports of the expression patterns in chick and mouse, the A- and B-family gradients observed in a Ranid frog are not orthogonal to each other, but are aligned together parallel to the A-P or longitudinal axis of the OT during early development. A parallel alignment of the A- and B-family gradients is consistent with observations that in zebrafish, ephrin-As [2] and ephrin-B2a [11] are highly expressed in the posterior OT. Since, in the developing zebrafish, ephrin-B2a is expressed in recently postmitotic tectal cells, it has been suggested that the ephrin-B ligand may function as a target recognition or stop signal. However, in *Rana*, it is the receptor EphB that is expressed in the posterior tectum, where, if it is also preferentially co-localized with newly postmitotic cells, it may be associated with early stages of synaptogenesis and/

or dendrite maturation, as observed in other systems [5,6,8], rather than in mapping per se.

At present, there has been no direct analysis of Eph/ephrin expression in the leopard frog retina, although the presence of EphA and EphB in the optic nerve and tract appears predictive of their expression in the retina. On the question whether EphB receptor may serve a guidance function in the tectum in a reverse signaling paradigm, ephrin-B ligand staining was not observed in the optic nerve and tract, nor in the innervated part of the tectum, where it would presumably be present in retinal axons. However, it is possible that ephrin-B is expressed in the retina but is masked in the optic pathway by potentially higher concentrations of EphB in neighboring axons. If it can be shown that ephrin-B is indeed expressed in the retina, one would expect it to be graded across the temporonasal axis, rather dorsoventrally, if it is to interact with the tectal EphB gradient in topographically meaningful way. In that case, assuming EphA is also expressed across the temporonasal axis of the retina, it may be reasonable to suggest that the A- and B-families act synergistically in Ranid frogs to pattern one map axis, while some other molecular agent is responsible for the orthogonal axis. Further study is needed to identify the particular EphB receptors expressed in the OT, to examine their distribution at earlier stages, to analyze the A- and B-family expression patterns in the retina and to investigate the functional role of these molecules in the Ranid frogs.

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