

Short Communication

Partial nucleotide sequences and expression patterns of frog (*Rana pipiens*) ephrin-A2 and ephrin-A5 mRNA

Yoshiaki Yagita^a, Isaac Barjis^b, Michael Hecht^c, Helene Bach^{d,e},
David A. Feldheim^f, Frank Scalia^{c,*}

^aDepartment of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita City, Osaka 565-0871, Japan

^bDepartment of Physical and Biological Sciences, New York City College of Technology, 300 Jay Street, Brooklyn, NY 11201, USA

^cDepartment of Anatomy and Cell Biology, State University of New York, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, USA

^dDepartment of Neuroscience, New York State Psychiatric Institute, Columbia University, New York, NY 10032, USA

^eDepartment of Psychiatry, Columbia University, New York, NY 10032, USA

^fDepartment of Molecular, Cellular, and Developmental Biology, University of California Santa Cruz, Santa Cruz, CA 95064, USA

Accepted 21 June 2005

Available online 9 August 2005

Abstract

We have generated 362 bp and 547 bp partial sequences for *Rana pipiens* ephrin-A2 and ephrin-A5 mRNA, respectively. Translation homologies for the comparable segments of cDNA of chicken, mouse and human are 90.8, 86.9 and 84.4% for the ephrin-A2 sequence and 85.7, 85.0 and 85.0% for the ephrin-A5 sequence. Digoxigenin-labeled riboprobes were prepared and applied by means of in situ hybridization to whole-mounts of the brains of mature adults and expression patterns in tadpoles were also explored. The RNA probes revealed similar posterior (high) to anterior (low) expression gradients in the adult tectum, demonstrating that both ephrin-As are expressed in the adult *Rana pipiens* tectum. Only the ephrin-A2 probe was tested on tadpole brain, yielding an appropriately graded expression pattern similar to the adult.

© 2005 Elsevier B.V. All rights reserved.

Theme: Development and regeneration

Topic: Pattern formation, compartments and boundaries

Keywords: Efn2; Efn5; Optic tectum development; Retinotopic mapping; Gene expression

The EphA family of receptor tyrosine kinases and their ligands, the ephrin-As has been shown to be crucial for the proper development of the retinotectal/collicular topographic map (reviewed in [11]). EphA/ephrin-A signaling patterns the temporo-nasal axis of the retinal map into the antero-posterior axis of the midbrain optic tectum/superior colliculus [3]. Considering the ability of anamniote vertebrates to regenerate an optic nerve postnatally, the question has been raised whether the EphA/ephrin-A family plays a role in the regeneration of the retinotectal projection in such species. Evidence has been presented [9,12] indicating that

expression of tectal ephrin-A2 and retinal EphA3/A5 is upregulated in goldfish during optic nerve regeneration, and interference with the EphA/ephrin-A interaction disrupts the proper formation of the regenerated map [13]. In zebrafish and leopard frog, there is a persistent expression of ephrin-As in the adult, but evidence of an effect of optic nerve injury was not obtained [1,2]. Different methods were used in the cited studies to detect expression of the ephrin-As: immunocytochemistry (goldfish), RNA in situ hybridization (zebrafish), and in situ binding analysis (leopard frog) with receptor and ligand affinity probes (RAP). The analysis carried out on the leopard frog (*Rana pipiens*) by means of the RAP methodology, employed an EphA3-alkaline phosphatase fusion protein (EphA3-AP; [7]) to probe for

* Corresponding author. Fax: +1 718 270 3732.

E-mail address: fscalia@downstate.edu (F. Scalia).

expression in the adult tectum of the ligands, ephrin-A2 and ephrin-A5, for which EphA3 has a high affinity (ephrin-A5 > ephrin-A2; [6]), although other ephrin-A ligands that might be present in the frog tectum might be detected. However, with such an affinity probe, it could not be known with certainty which ligands are present in the adult frog brain. Concerning ephrin-A2 and ephrin-A5, specifically, if expression of both ligands persists in the adult, the possibility would still exist that the greater affinity of the probe for ephrin-A5 might obscure small changes in the expression level of ephrin-A2, in the case that ephrin-A5 expression is unaffected by optic nerve injury. Furthermore, it could be argued that a coordinated upregulation of tectal EphAs might compete with the binding of the fusion protein probe to available ephrin-As [15]. In view of such issues, it seemed desirable to develop frog-specific RNA probes to differentially target frog ephrin-A2 and -A5 mRNA expression for further study of the potential roles of the ephrin-A family in optic nerve regeneration in the adult.

We have cloned frog specific cDNA fragments having high nucleotide and translation sequence homology with corresponding segments of the published sequences for ephrin-A2 and ephrin-A5 mRNA in various species. Our cDNA fragments were generated by RT-PCR using frog (*R. pipiens*) mRNA isolated from normal adult brain and both specific and degenerate primers designed from regions of the zebrafish and chicken sequences for ephrin-A2 and -A5 mRNA. The upstream primer for the ephrin-A2 product (GenBank accession no. AY762617), GTGAGCATCAACGACTACCTGG, is located at position 946–967 in the published zebrafish sequence (GenBank accession no. Y09668). The downstream primer, AAGGGCTCTGGAGACTCGTA, corresponds to the sequence located at 1285–1304, and is identical to it except for the three underlined base-pair substitutions. The upstream primer for the ephrin-A5 product (GenBank accession no. AY910530) corresponds to AGACTACCACATGACGTGTG, located at 162–182 of the chicken sequence (GenBank accession no. NM205184), differing at the four underlined sites. The downstream primer, TAATATCAAAGCATTGC, is located at 691–708 in the chicken sequence. The PCR products were subsequently inserted into a pGEM-3Zf plasmid (Promega, Madison, WI) between *Eco*R1 and *Hind*III restriction sites. The plasmids were grown in DH5alpha cells, subsequently linearized with *Eco*R1, and sequenced for validation. After linearizing the plasmids with *Eco*R1, the DNA sequence was transcribed in vitro with SP6 polymerase for construction of antisense digoxigenin labeled riboprobes (DIG RNA Labeling Kit, Roche Diagnostics, Mannheim). Linearization with *Hind*III and subsequent in vitro transcription with T7 polymerase provided the sense RNA probes.

For ephrin-A2, the 362 bp product obtained by RT-PCR has 66.8% homology (Wilbur-Lipman DNA Alignment, DNASTAR, Madison, Wisconsin) with the target locus in the zebrafish (*Danio rerio*, GenBank accession no. Y09668) and 77.2% homology with the comparable locus for chicken

(*Gallus gallus*, GenBank accession no. NM204983) ephrin-A2 mRNA. Translation homologies (Fig. 1, top) yield a 76.2% alignment match (Lipman-Pearson Protein Alignment, DNASTAR, Madison, Wisconsin) with the targeted zebrafish ephrin-A2 amino acid sequence, 90.8% with chicken, 86.9% with mouse (*Mus musculus*, GenBank accession no. NM007909) and 84.4% with human (*Homo sapiens*, GenBank accession no. NM004105). For ephrin-A5, our 547 bp product has 85.7% homology with the comparable locus for chicken ephrin-A5 mRNA (*G. gallus*, GenBank accession no. NM205184), 85.0% homology with mouse (*M. musculus*, GenBank accession no. NM010109) and 85.0% homology with human (*H. sapiens*, GenBank accession no. BC075055). Translation homologies (Fig. 1, bottom) yield a 91.5% match with chicken ephrin-A5, 90.4% match with mouse and 90.4% match with human. Comparison of the ephrin-A2 and ephrin-A5 partial sequences with each other yields only a 55.0% homology in their region of overlap (Fig. 2). The high degree of nucleotide and translation homology with other tetrapod species strongly suggests that our cDNA fragments are authentic replicas of *R. pipiens* mRNA coding for ephrin-A2 and ephrin-A5 in the internal, targeted loci, while the relatively lower similarity between the two sequences provides a measure of their mutual differentiability.

Expression patterns in the fully mature adult brain were studied by applying the RNA probes to whole-mount preparations after formaldehyde fixation, and the results were compared with our earlier observations on the protein expression patterns. In addition, to check whether the ephrin-A expression pattern during tectal development follows the expected anterior-low to posterior-high gradient observed in other species [4,11], and as also observed in southern leopard frog (*R. utricularia*) tadpoles [1,14] by means of RAP analysis, larval *R. pipiens* brains of developmental stages 3–21 [17] were analyzed with the ephrin-A2 riboprobe after formaldehyde fixation. Ephrin-A mRNA expression in Ranid frogs is also of interest in across-species comparisons since, while mRNA expression patterns have been described for members of the B-family of receptors and ligands in the developing *Xenopus* visual system [8,10], nothing is known of the A-family expression patterns in the *Xenopus* frog. Tadpoles were obtained by artificial fertilization.

Hybridization was detected by means of an alkaline phosphatase (AP) labeled DIG antibody, histochemically visualized with a BCIP/NBT (5-bromo-3-chloro-indolyl phosphate, nitroblue tetrazolium) reaction system (Roche Diagnostics, Mannheim). In situ hybridization was carried out essentially following an SDS-based modification of the Wilkinson method [18,19]. On completion of the final AP reactions, the brains were photographed, embedded in paraffin, sectioned and reexamined under the light microscope. Proteinase K treatment, hybridization and stringency wash temperatures were optimized for the diencephalon and midbrain.

Ephrin-A2

V S I N D Y L D I Y C P H Y E I P	-	L P A D R M E R Y I L Y	Rana
V S I N D Y L D I Y C P H Y E E P	-	L P A E R M E R Y V L Y	Gallus
V S I N D Y L D I Y C P H Y G A P	L P P A E R M E R Y I L Y		Mus
V S I N D Y L D I Y C P H Y G A P	L P P A E R M E H Y V L Y		Homo
V S I N D Y L D V Y C P Y Y E S P	- Q P H S R M E R Y I L F		Danio

M V N Y D G H A S C D H R Q K G F K R W E C N R P D S P N G	Rana
M V N Y E G H A S C D H R Q K G F K R W E C N R P D S P S G	Gallus
M V N G E G H A S C D H R Q R G F K R W E C N R P A A P G G	Mus
M V N G E G H A S C D H R Q R G F K R W E C N R P A A P G G	Homo
M V N H D G Y L T C E H R M R G F K R W E C N R P Q S P D G	Danio

P L K F S E K F Q L F T P F S L G F E F R P G H E Y Y Y I S	Rana
P L K F S E K F Q L F T P F S L G F E F R P G H E Y Y Y I S	Gallus
P L K F S E K F Q L F T P F S L G F E F R P G H E Y Y Y I S	Mus
P L K F S E K F Q L F T P F S L G F E F R P G H E Y Y Y I S	Homo
P L R F S E K F Q L F T P F S L G F E F R P G H E Y Y Y I S	Danio

S T P P N L V D K P C L R L K V Y V R P T N D S L Y E S P E	Rana
A S P L N V V D R P C L K L K V Y V R P T N D S L Y E S P E	Gallus
A T P P N L V D R P C L R L K V Y V R P T N E T L Y E A P E	Mus
A T P P N A V D R P C L R L K V Y V R P T N E T L Y E A P E	Homo
S P H P N H A G K P C L K L K V Y V K P T S S G - Y E S P E	Danio

P	Rana
P	Gallus
P	Mus
P	Homo
P	Danio

Ephrin-A5

V C I N D Y L D V F C P H Y E D S V P E D K T E R Y V L F M	Rana
V C I N D Y L D V F C P H Y E D S V P E D K T E R Y V L Y M	Gallus
V C I N D Y L D V F C P H Y E D S V P E D K T E R Y V L Y M	Mus
V C I N D Y L D V F C P H Y E D S V P E D K T E R Y V L Y M	Homo
V C I N D Y L D V Y C P H Y M D T V P E E R T E R Y V L Y M	Danio

V N F D G Y S S C D H T S K G F K R W E C N R P H S S N G P	Rana
V N F D G Y S S C D H I S K G F K R W E C N R P H S P N G P	Gallus
V N F D G Y S A C D H T S K G F K R W E C N R P H S P N G P	Mus
V N F D G Y S A C D H T S K G F K R W E C N R P H S P N G P	Homo
V N Y D G Y S S C D H T A K G F K R W E C N R P H S P N G P	Danio

L K F S E K F Q L F T P F S L G F E F R P G R E Y Y Y I S S	Rana
L K F S E K F Q L F T P F S L G F E F R P G R E Y F Y I S S	Gallus
L K F S E K F Q L F T P F S L G F E F R P G R E Y F Y I S S	Mus
L K F S E K F Q L F T P F S L G F E F R P G R E Y F Y I S S	Homo
L K F S E K F Q L F T	Danio

A I P D N G R R S C L K L R V Y V R P A N S C M K T I G V H	Rana
A I P D N G R R S C L K L K V F V R P A N S C M K T I G V H	Gallus
A I P D N G R R S C L K L K V F V R P T N S C M K T I G V H	Mus
A I P D N G R R S C L K L K V F V R P T N S C M K T I G V H	Homo

D R V F D V N D K V E N S L E P A D D T I H E S A E P S R G	Rana
D R V F D V N D K V E N S L E P A D D T V R E S A E P S R G	Gallus
D R V F D V N D K V E N S L E P A D D T V H E S A E P S R G	Mus
D R V F D V N D K V E N S L E P A D D T V H E S A E P S R G	Homo

E N S A P I L R I P N W L L T T L L F L L A M L L I L	Rana
E N A A Q T P R I P I R L L A T L L F L L A M L L I L	Gallus
E N A A Q T P R I P S R L L A I L L F L L A M L L T L	Mus
E N A A Q T P R I P S R L L A I L L F L L A M L L T L	Homo

Fig. 1. High levels of translation homology suggest authenticity of the partial sequences. Amino acid translations for the ephrin-A2 (top) and ephrin-A5 (bottom; frame-shifted to GTGTGC) sequences are compared with the orthologous sequence fragments for chicken, mouse, human and zebrafish. The areas of homology are boxed.

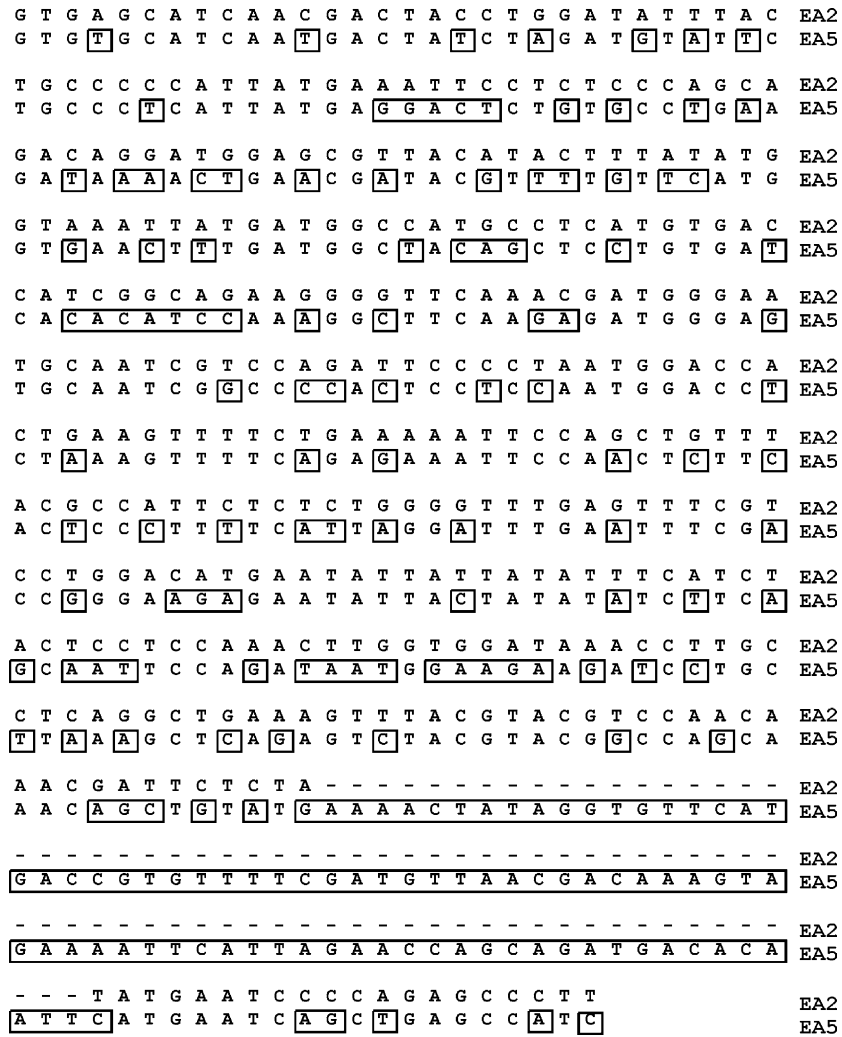


Fig. 2. Broad areas of mutual mismatch in the partial sequences promote selectivity in hybridization. Sequence homology between the frog ephrin-A2 (EA2) and ephrin-A5 (EA5) PCR products in their area of overlap. Mismatched base-pairs in the ephrin-A5 sequence are boxed. Note the total mismatch of 81 bp in the last four lines.

In the tadpole, the ephrin-A2 antisense riboprobe hybridized strongly in the posterior part of the developing optic tectum (Figs. 3A,B). In the sectioned material, the cellular labeling observed in the tectum (Figs. 3D,E) extended decrementally from its polar epithelial generative zone [5], anteriorly, part of the way into the definitive tectum. Since the invading retinal axons do not enter the proliferative zone, even at these early developmental stages [5], it is clear that ephrin-A2 expression is maximal well posterior to the part of the tectum in which the retinal axons are synapsing. Where the tectal cells are closely aggregated, as in the zone of cell generation, all cells appeared to be labeled. Strong cellular staining was also observed in the underlying torus semicircularis (Tor), a homologue of the inferior colliculus (Fig. 3D), in the nucleus isthmi (NI), in the pretectum (P), in the cell mass associated with the “nucleus” of Bellonci and “corpus” geniculatum thalamicum (LGN) and in the hypothalamus (Hyp) and preoptic area (PA). The staining of the cellular component of the

nucleus of Bellonci and corpus geniculatum shows through as the blue spot appearing in the anterior thalamus in Fig. 3B (LGN).

These patterns of RNA expression are similar to the distribution of ephrin-A protein previously observed in *R. utricularia* (southern leopard frog) tadpoles [1]. Although *R. pipiens* and *utricularia* are closely related species of leopard frog, we wanted to compare the patterns of ephrin-A2 RNA and ephrin-A protein expression directly in *R. pipiens*. Therefore, brains of *R. pipiens* tadpoles of the same developmental stages were subjected to RAP analysis with the EphA3-AP probe. The fusion protein reactions and controls were carried out as previously described [1,7]. As indicated in Figs. 3B and C, the RNA and protein expression patterns are closely similar.

In the adult frog (*R. pipiens*), the antisense probes hybridized in tectal neurons and ependymoglia in a posterior-high to anterior-low gradient (Fig. 4). The topography of the expression gradients for the two ephrin-As

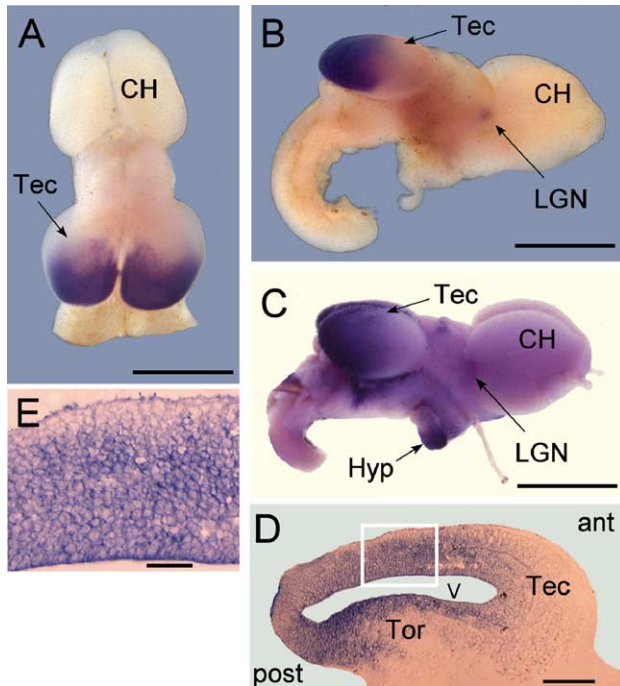


Fig. 3. Pattern of expression of ephrin-A in the tadpole brain. (A) Dorsal view of the brain of a stage 6 leopard frog (*R. pipiens*) tadpole after RNA in situ hybridization with a DIG-labeled probe for frog-specific ephrin-A2 mRNA, showing strong staining in the posterior tectum (Tec). Anterior to the top. Scale bar = 1 mm. (B) Lateral aspect of the same specimen (in panel A), showing additionally a positive staining in the LGN complex, which is located below the surface in the anterior thalamus in the frog. Anterior to the right; dorsal up. Scale bar = 1 mm. (C) Lateral aspect of the brain of a stage 6 *R. pipiens* tadpole stained for alkaline phosphatase after incubation in an Eph3-AP fusion protein designed to detect expression of ephrin-A2 and or ephrin-A5 protein. The protein expression gradient in the tectum (Tec), running from high, posteriorly, to low, anteriorly, is identical to the RNA expression gradient shown in panel B. Positive staining also appears in the LGN and hypothalamus (Hyp). In comparing panels B and C, it can be noted that certain surface features stained by the RAP method are not stained by the riboprobe. In particular, certain fibers in the optic tract contain ephrin-A protein, but there are no neuronal cell bodies in the optic tract. Similarly, the region of the hypothalamus staining positively for ephrin-A protein is practically all neuropil. The periventricular, cellular layer of the Hyp, which is stained in sectioned material, lies well deep to the superficial neuropil and does not show through in panel B. Anterior to the right; dorsal up. Scale bar = 1 mm. (D) Parasagittal section of the tectum of a stage 3 *R. pipiens* tadpole, showing posterior-high to anterior-low, graded expression of ephrin-A2 mRNA after hybridization with the frog-specific probe. The positive staining extends below the tectal ventricle (V) into the torus semicircularis (Tor). The rectangular frame, magnified in panel E, lies approximately at the anterior end of or just anterior to the zone of cellular proliferation. Anterior to the right; dorsal up. Scale bar = 350 μ m. (E) Higher magnification of the region of the tectal plate outlined in panel D (anterior to the right), showing cellular localization of stain in the thin shells of perikaryal cytoplasm. Scale bar = 90 μ m. CH = cerebral hemispheres.

was similar, and cells in all layers in the posterior tectum were stained. Cell masses in the periventricular zones of PA, Hyp and P were labeled, as well as in the anterior thalamus (LGN), Tor and NI. The sense probes did not hybridize appreciably with any area of adult or tadpole brain (Fig. 4C). The topography of this pattern of expression is closely comparable to the patterns previously described in adult *R.*

pipiens and *R. utricularia* [1,14] by means of RAP analysis. However, since most of the neuronal cell bodies in the tectum and diencephalon lay well beneath the surface of the brain, being separated from the pia by a superficial neuropil, the ability to observe the topographic distribution of cellular staining in the whole-mount RNA-probed material depends upon the ability of the colored cell masses to show through to the surface. The technique may not be sensitive enough to detect such mRNA that may be in the dendrites. In contrast, the brain tissue is not rendered translucent during the RAP processing, and it is only the binding to protein associated with the terminal ends of the neuronal dendrites and radial glial processes that is observed. Thus, a precise comparison of the cellular, as opposed to the topographic specificity of the staining obtained with the two types of probes cannot be readily made. Nevertheless, the fact the apical dendrites of the cells of the deeper layers pass through the superficial zone of termination of the retinal axons as these dendrites extend perpendicularly toward the tectal surface [16], binds together the two staining patterns by mapping one onto the

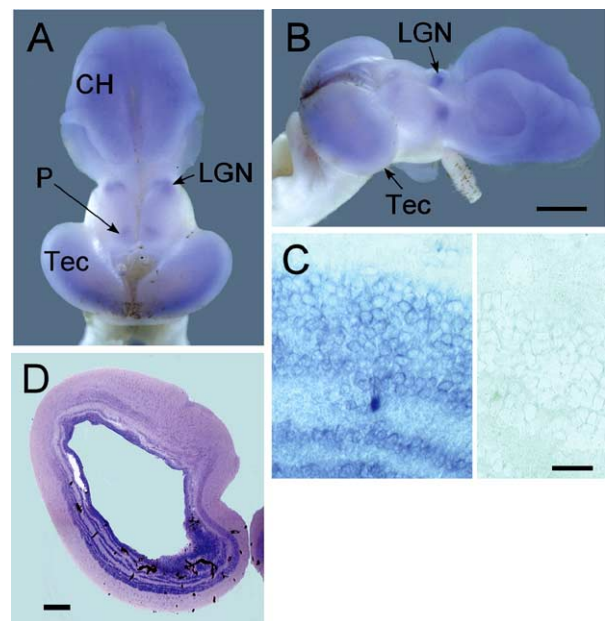


Fig. 4. Ephrin-A expression pattern in the adult frog. (A) Dorsal view of the brain of an adult Northern leopard frog (*R. pipiens*) after RNA in situ hybridization with a DIG-labeled probe for frog-specific ephrin-A2 mRNA. The posterior poles of the cerebral hemisphere (CH) were removed prior to hybridization to allow direct viewing of the lateral geniculate nuclei (LGN), which are located in the anterior thalamus in the frog. Positive staining of the probe is evident in LGN, pretegmentum (P) and posterior tectum (Tec). Anterior to the top. (B) The specimen in panel A viewed from above and to the right. Labeling as in panel A. Anterior to the right. Scale bar = 1 mm for panels A and B. (C) Tectal neurons are labeled by the antisense ephrin-A2 probe in the principal cell layers of the tectum (left panel), but not by the sense control (right panel). The intensely stained element in the center of the left panel is associated with a blood vessel. Such objects are randomly present throughout the tissue. Scale bar = 50 μ m. (D) Horizontal section of the tectum (left hemisphere) at a mid-dorsoventral level, showing the gradation (anterior-low to posterior-high) of cellular labeling obtained with the DIG-labeled ephrin-A5 RNA antisense probe. Anterior to the top; lateral to the left. Scale bar = 200 μ m.

other. Thus, leopard frogs appear to retain a degree of ephrin-A expression into adulthood, as now confirmed in *R. pipiens* by two different methodologies, and both ephrin-A2 and ephrin-A5 are expressed in the adult tectum.

All procedures involving live animals were carried out in accordance with IACUC guidelines.

Acknowledgments

We thank Vincent Garofalo for his help in photography, and Wei Quan and Inna Rozenberg for their technical assistance. Grant sponsors: FS was supported by SUNY Downstate Medical Center. YY was supported by NIH NS20147.

References

- [1] H. Bach, D.A. Feldheim, J.G. Flanagan, F. Scalia, Persistence of graded ephrin-A expression in the adult frog visual system, *J. Comp. Neurol.* 467 (2003) 549–565.
- [2] C.G. Becker, R.L. Meyer, T. Becker, Gradients of ephrin-A2 and ephrin-A5b mRNA during retinotopic regeneration of the optic projection in adult zebrafish, *J. Comp. Neurol.* 427 (2000) 469–483.
- [3] A. Brown, P.A. Yates, P. Burrola, D. Ortuno, A. Vaidya, T.M. Jessell, S.L. Pfaff, D.D.M. O’Leary, G. Lemke, Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling, *Cell* 102 (2000) 77–88.
- [4] H.J. Cheng, M. Nakamoto, A.D. Bergemann, J.G. Flanagan, Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map, *Cell* 82 (1995) 371–381.
- [5] J. Currie, W.M. Cowan, Some observations on the early development of the optic tectum in the frog (*Rana pipiens*), with special reference to the effect of eye removal on mitotic activity in the larval tectum, *J. Comp. Neurol.* 156 (1974) 123–142.
- [6] J.G. Flanagan, P. Vanderhaeghen, The ephrins and Eph receptors in neural development, *Annu. Rev. Neurosci.* 21 (1998) 309–345.
- [7] J.G. Flanagan, H.-J. Cheng, D.A. Feldheim, M. Hattori, Q. Lu, P. Vanderhaeghen, Alkaline phosphatase fusions of ligands or receptors as in situ probes for staining of cells, tissues and embryos, in: J. Thorne, S.D. Emr, J.N. Abelson (Eds.), *Methods in Enzymology*, Academic Press, New York, 2000, pp. 19–210.
- [8] T.L. Jones, I. Karavanova, M. Maeno, R.C. Ong, H.F. Kung, I.O. Daar, Expression of an amphibian homologue of the Eph family of receptor tyrosine kinases is developmentally regulated, *Oncogene* 10 (1995) 1111–1117.
- [9] C.E. King, A. Wallace, J. Rodger, C. Bartlett, L.D. Beazley, S.A. Dunlop, Transient up-regulation of retinal EphA3 and EphA5, but not ephrin-A2, coincides with re-establishment of a topographic map during optic nerve regeneration in goldfish, *Exp. Neurol.* 183 (2003) 593–599.
- [10] F. Mann, S. Ray, W.A. Harris, C.E. Holt, Topographic mapping in dorsoventral axis of the *Xenopus* retinotectal system depends on signaling through ephrin-B ligands, *Neuron* 35 (2002) 461–473.
- [11] T. McLaughlin, R. Hindges, D.D.M. O’Leary, Regulation of axial patterning of the retina and its topographic mapping in the brain, *Curr. Opin. Neurobiol.* 13 (2003) 57–69.
- [12] J. Rodger, C.A. Bartlett, L. Beazley, S.A. Dunlop, Transient up-regulation of the rostrocaudal gradient of ephrin A2 in the tectum coincides with reestablishment of orderly projections during optic nerve regeneration in goldfish, *Exp. Neurol.* 166 (2000) 196–200.
- [13] J. Rodger, P.N. Vitale, L.B. Tee, C.E. King, C.A. Bartlett, A. Fall, C. Brennan, J.E. O’Shea, S.A. Dunlop, L.D. Beazley, EphA/ephrin-A interactions during optic nerve regeneration: restoration of topography and regulation of ephrin-A2 expression, *Mol. Cell. Neurosci.* 1 (2004) 56–68.
- [14] F. Scalia, D.A. Feldheim, Eph/ephrin A- and B-family expression patterns in the leopard frog (*Rana utricularia*), *Dev. Brain Res.* (in press), doi:10.1016/j.devbrainres.2005.05.002.
- [15] D. Sobieszczuk, Masking of Eph receptors and ephrins, *Curr. Biol.* 9 (1999) R469–R470.
- [16] G. Szekely, G. Setalo, G. Lazar, Fine structure of the frog’s optic tectum: optic fiber termination layers, *J. Hirnforsch.* 14 (1973) 189–225.
- [17] A.C. Taylor, J.J. Kollros, Stages in the normal development of *Rana pipiens* larvae, *Anat. Rec.* 94 (1946) 7–23.
- [18] D.G. Wilkinson, Whole mount in situ hybridization of vertebrate embryos, in: D.G. Wilkinson (Ed.), *In situ Hybridization: A Practical Approach*, IRL Press, Oxford, 1992, pp. 75–83.
- [19] M.X. Zuber, D.H. Shain, Sodium dodecyl sulfate (SDS)-based whole-mount in situ hybridization of *Xenopus laevis* embryos, *J. Biochem. Biophys. Methods* 31 (1996) 185–188.