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# EPHRIN-A5 MODULATES THE TOPOGRAPHIC MAPPING AND CONNECTIVITY OF COMMISSURAL AXONS IN MURINE HIPPOCAMPUS

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**Abstract—Entorhinal and commissural/associational projections show a non-overlapping distribution in the hippocampus proper and the dentate gyrus. The expression of Ephrins and their Eph receptors in the developing hippocampus indicates that this family of axonal guidance molecules may modulate the formation of these connections. Here we focused on the role of the ephrin-A5 ligand in the development of the main hippocampal afferents. *In situ* hybridization showed that *ephrin-A5* mRNA was detected mainly in the principal cells of the hippocampus proper and in the dentate gyrus throughout postnatal development. Immunocytochemical analyses revealed prominent expression of the EphA3 receptor, a putative receptor for ephrin-A5, in the main cells and the neuropil of the developing hippocampus. Tracing experiments in *ephrin-A5*(−/−) mice showed that commissural projections were transiently altered in the hippocampus proper at P5, but they were mistargeted throughout the postnatal development in the dentate gyrus.**

**Immunocytochemistry with anti-calbindin antibodies revealed that the dentate mossy fiber projection was not altered in *ephrin-A5*(−/−) mice. Electron microscopy studies showed alterations in the density of synapses and spines in commissural/associational layers, but not in entorhinal layers, and in the mossy fibers in these animals. Taken together, these findings indicate that ephrin-A5 signaling is involved in the formation and maturation of synapses in the hippocampus.**


**Key words: neuronal tracers, development, synaptogenesis, hippocampal afferents, mice.**

The development of neuronal connections requires the action of specific guidance cues. Diffusible and membrane-anchored proteins, including Semaphorins, Netrins, Slits, and Ephrins, are involved in axonal guidance during the formation of the connections of the CNS (for review see Culotti and Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996; Wilkinson, 2001; Huber et al., 2003).

Ephrins belong to a family of cell surface molecules that are ligands of the Eph family of tyrosine kinase receptors. Eph receptors and Ephrins are divided into subclasses A and B, on the basis of their amino acid sequence conservation and binding affinities (for review see Kullander and Klein, 2002). The eight EphA receptors (Eph A1–A8) interact with six ephrin-A ligands (ephrin-A1–A6), which are attached to the cell membrane via a glycosylphosphatidylinositol (GPI) linkage. EphB receptors (EphB1–B6), on the other hand, bind to three distinct ligands (ephrin-B1–B3) that are transmembrane proteins with a cytoplasmic domain. It has been proposed that Ephrin/Eph interactions mediate cell-contact-dependent signaling and growth-cone behavior by repulsion at a boundary or in a gradient-dependent manner (Wilkinson, 2001; Menzel et al., 2001). Thus, graded expression of Eph receptors and Ephrins may be involved in the formation of topographic maps in many regions of the nervous system, including the retinotectal and retinocollicular connections (Cheng et al., 1995; Drescher et al., 1995, 1997; Monschau et al., 1997; Frisen et al., 1998; Feldheim et al., 2000; Sakurai et al., 2002) and the formation of thalamocortical (Gao et al., 1998; Vanderhaeghen et al., 2000; Uziel et al., 2002; Dufour et al., 2003), vomeronasal (Knoll et al., 2001) and hippocamposeptal (Brownlee et al., 2000; Yue et al., 2002) projections. In most of these systems, ephrin-A5 plays a crucial role. Furthermore, this protein has been proposed to regulate the formation of axonal branches at appropriate locations (Castellani et al., 1998; Gao et al., 1999; Mann et al., 2002). Moreover, recent evidence indicates that Ephrin/Eph signaling may participate in the formation of synapses and dendritic spines (Feng et al., 2000; Dalva et al., 2000; Ethell et al., 2001; Takasu et al., 2002; Murai et al., 2003; Martínez et al., 2005).

Recent data have implicated several guidance molecules in the development of hippocampal connections. For instance, distinct members of the Semaphorin family are involved in the pathfinding of entorhino-hippocampal, septo-hippocampal and intrahippocampal connections (Chedotal et al., 1998; Steup et al., 1999, 2000; Chen et al., 2000; Giger et al., 2000; Cheng et al., 2001; Pozas et al., 2001; Gu et al., 2003; Sahay et al., 2003). In addition, Netrin-1 plays a crucial role in the development of

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*Abbreviations:* BDA, biotinylated dextran-amine; bp, base pair; BSA, bovine serum albumin; CALB, calbindin 28k; CALR, calretinin; DAB, diaminobenzidine; DiI, 1,1′-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine perchlorate; GAPDH, glycerol-alpha-phosphate dehydrogenase; IMLa, lower part of the inner molecular layer; IMLb, upper part of the inner molecular layer; NGS, normal goat serum; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; SSC, sodium saline citrate.

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commissural afferents in the hippocampus (Steup et al., 2000; Barallobre et al., 2000).

Here we examined the contribution of ephrin-A5 to the development of both the lamina-specific pattern of hippocampal connections and the dentate mossy fiber projections *in vivo*. Using the stripe assay, Stein et al. (1999) proposed the involvement of ephrin-A3, but not ephrin-A5, in the formation of entorhino-hippocampal axons. We found that *ephrin-A5* mRNA is expressed in the hippocampus during postnatal development. Furthermore, we analyzed the development and distribution of entorhinal and commissural afferents, and the distribution of mossy fibers in the hippocampus of *ephrin-A5*(−/−) mice. Finally, using electron microscopy, we addressed the possible role of *ephrin-A5* in hippocampal synaptogenesis and synaptic maturation.

## EXPERIMENTAL PROCEDURES

### Mutant mice

Homozygous *ephrin-A5*(−/−) mice were generated by mating heterozygous mice (Frisen et al., 1998). Tail polymerase chain reaction (PCR) analyses were used to genotype *ephrin-A5* knockout mice and control *wt* littermates (Frisen et al., 1998). The animals were treated respecting the approved protocols of the Institutional Animal Care and Use Committee and in accordance with the European Community Council Directive and the National Institute of Health guidelines for the care and use of laboratory animals. Only the minimum necessary number of animals was used in this study. Care was taken to avoid unnecessary suffering or pain during animal experimentation.

### *In situ* hybridization

The *ephrin-A5* probe was obtained by reverse-transcriptase (RT)-PCR reaction from the mRNA of E19 embryonic brains, using an upstream primer, 5′-ATTCCAGAGGGGTGACTACCACATT-3′, and a downstream primer 5′-GGAGGAGACTGTGCTATAATGTCA-3′. Two anti-sense riboprobes, which measured 523 pb and 574 pb, were obtained according to the $\alpha$- and $\beta$-*ephrin-A5* splicing variants described previously (Lai et al., 1999).

Postnatal *wt* mice (strain: C57/Bl6; P0, P5, P10, P15, P21) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, cryoprotected in 30% sucrose, and frozen in dry ice. Coronal sections (30–40 $\mu$m thick) were obtained and hybridized as described elsewhere (de Lecea et al., 1997). Briefly, free-floating sections were deproteinized with 0.2 N HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0), and post-fixed for 10 min in 4% paraformaldehyde. Sections were then hybridized to mouse $\alpha$- and $\beta$-*ephrin-A5* (500–1000 ng/ml each probe) overnight at 60 °C with a mixture of digoxigenin-labeled antisense RNA probes. The probes were dissolved in a solution containing 50% formamide, 20 mM PIPES, 5× Denhardt's solution, 10% dextran sulfate, 250 $\mu$g/ml yeast tRNA, 250 $\mu$g/ml salmon sperm DNA, 50 mM dithiothreitol, 0.62 M NaCl, and 10 mM EDTA solution. Sections were then digested with RNase A (37 °C) and washed in 0.5× sodium saline citrate (SSC) plus 50% formamide (55 °C) and in 0.1× SSC plus 0.5% sarcosyl (60 °C). Sections were blocked with 10% normal goat serum (NGS), incubated overnight with an alkaline phosphatase-labeled anti-digoxigenin antibody (1:2000) and developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-idolyl phosphate (BCIP; Life Technologies, Gaithersburg, MD, USA). After several washes, sections were mounted onto slides and coverslipped with Mowiol. Neither the *wt* sections hybridized with sense riboprobes nor those from *ephrin-A5*(−/−) mice hybridized with anti-sense riboprobes gave signals above background levels.

### PCR assay used for ephrin-A5 mRNA detection

Total RNA from several tissue samples (cerebral cortex and hippocampus of newborn mice, and cerebral cortex, hippocampus and soleus muscle of P15 mice) was purified using SV Total RNA purification kit (Promega, Madison, WI, USA), following the manufacturer's protocols. Two micrograms of this RNA was retrotranscribed using Moloney's Murine Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions but adding only the antisense oligonucleotides of use for the PCR assays (see below).

Three PCR assays were performed with our samples; 2 $\mu$l of first strand cDNA (10% of retrotranscribed RNA, approx. 200 ng of total RNA) was used in each assay, which included 0.6 units of Amershams Biosciences' *Taq* polymerase (Amersham Biosciences, Buckinghamshire, UK), 8 pmol of each oligonucleotide used (synthesized by Invitrogen, Eugene, OR, USA) and 5 nmol of each dNTP plus 1.5 $\mu$Ci of $^{33}$P-dATP (Amersham Biosciences) added as tracer for each reaction mixture. The *Total EA5* assay consisted of 3 min at 94 °C plus 20 cycles of amplification (30 min at 94 °C, 30 min at 57 °C, 1 min at 72 °C) and 7 min at 72 °C using the oligonucleotides mEA5-636 and mEA5+218 as templates. This assay gave rise to the amplification of a 418 base pairs (bp)-band from both the long and the short form of ephrin-A5. The *EA5* $\alpha/\beta$ assay consisted of 3 min at 94 °C plus 20 cycles of amplification (30 min at 94 °C, 30 min at 55 °C, 1 min at 72 °C) and 7 min at 72 °C using the oligonucleotides mEA5-718 and mEA5+218 as templates, and gave rise to two bands of 564 bp (corresponding to the $\alpha$ form of ephrin-A5) and 483 bp (corresponding to the $\beta$ form). The GAPDH assay was used as a loading control by adding the same oligonucleotides and applying the program used a previous study (Álvarez-Dolado et al., 1999). In this case the oligonucleotides were called mG-1043 and mG+608, and the amplification produced a 435 bp-band. Only 15 cycles of amplification were required for a quantitative amplification of GAPDH cDNA.

Equal volumes of each amplification were run in 4% acrylamide gels, fixed for 20 min in 7% methanol-5% acetic acid solution and dried on Whatman paper 2 h at 60 °C in a vacuum dryer. The resulting membranes were then exposed to Phosphoimager plates (Molecular Dynamics; distributed by Amersham Biosciences), analyzed in a Typhoon 8600 scanner (Molecular Dynamics), and processed and quantified using an ImageQuant 5.2 software (Molecular Dynamics).

*Oligonucleotides.* Antisense oligonucleotides were noted as "−," while forward oligonucleotides were noted "+." Their sequences were as follow: mEA5-782: A TGG CTC GGC TGA CTC ATG; mEA5-636: GA CCT TCT TCC GTT GTC TGG; mEA5+218: TTT CTG GTG CTC TGG ATG TG; mG-1043: CCT TGG AGG CCA TGT AGG CCA T; mG+608: GGC CCC TCT GGA AAG CTG TGG.

### Immunohistochemistry

Control postnatal mice (P0, P5, P10, P15 and P21) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. After dissection, brains were postfixed, cryoprotected in 30% sucrose, frozen and sectioned at 30–40 $\mu$m. Free-floating sections were blocked with 10% NGS and 4% bovine serum albumin (BSA) for 2 h, and incubated overnight with rabbit polyclonal antibodies against the EphA3 (1:100) receptor (sc-919, from Santa Cruz Biotechnology, Santa Cruz, CA, USA) or against the calcium-binding protein calbindin 28k (CALB) (diluted 1:5000; Swant Antibodies, Bellinzona, Switzerland). Primary antibodies were visualized using biotinylated goat anti-rabbit antibodies (diluted 1:200, Vector Laboratories, Burlingame, CA, USA) and streptavidin-bio-