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Distribution of EphA5 receptor protein in the developing and adult mouse nervous system

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Abstract

The EphA5 receptor tyrosine kinase plays key roles in axon guidance during development. However, the presence of EphA5 protein in the nervous system has not been fully characterized. To better examine EphA5 localization, mutant mice, in which the EphA5 cytoplasmic domain was replaced with β -galactosidase, were analyzed for both temporal and regional changes in the distribution of EphA5 protein in the developing and adult nervous system. During embryonic development, high levels of EphA5 protein were found in the retina, olfactory bulb, cerebral neocortex, hippocampus, pretectum, tectum, cranial nerve nuclei, and the spinal cord. Variations in intensity were observed as development proceeded. Staining of pretectal nuclei, tectal nuclei, and other areas of the mesencephalon became more diffuse after maturity whereas the cerebral neocortex gained more robust intensity. In the adult, receptor protein continued to be detected in many areas including the olfactory nuclei, neocortex, piriform cortex, induseum griseum, hippocampus, thalamus, amygdala, hypothalamus and septum. In addition, EphA5 protein was found in the claustrum, stria terminalis, barrel cortex, striatal patches, and along discrete axon tracts within the corpus callosum of the adult. These observations suggest that EphA5 function is not limited to the developing mouse brain and may play a role in synaptic plasticity in the adult.

Keywords

receptor tyrosine kinase; β -galactosidase; embryogenesis; axon guidance; hippocampus

INTRODUCTION

The Eph family of molecules comprises the largest group of receptor tyrosine kinases. These molecules have been shown to play important roles in many physiological functions including tissue segmentation, angiogenesis, axon guidance, and neural plasticity (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; O'Leary and Wilkinson, 1999; Pasquale, 2005; van der Geer et al., 1994; Wilkinson, 2001; Zhou, 1998). The first member of the family, EphA1, was identified in a hepatic carcinoma cell line (Hirai et al., 1987). A total of sixteen members have been identified since and are divided into two subclasses

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consisting of 10 EphA and 6 EphB receptor types (Pasquale, 2005). The division is based upon the preferential binding of the GPI-linked ephrin-A ligands to the A receptors and the single transmembrane domain-containing ephrin-B ligands to the B receptors. However, there are exceptions to this general rule; for example, ephrin-A5 can also interact with EphB2 and ephrin-B ligands can bind to EphA4 (Brambilla et al., 1995; Gale et al., 1996; Himanen et al., 2004). Eph receptor activation leads to tyrosine phosphorylation followed by the regulation of several downstream signaling pathways including the Erk/MAP Kinases and the recruitment of several adaptor proteins such as Nck, Grb2, Abl/Arg, and guanine nucleotide exchange factors for the Rho family of small GTPases such as Ephexin, Tiam, and Vav2 (Cowan et al., 2005; Kullander and Klein, 2002; Miao et al., 2001; Panayotou and Waterfield, 1993; Sahin et al., 2005; Shamah et al., 2001). The ephrins are also capable of reverse signaling back into the ligand expressing cells upon receptor binding (Davy and Soriano, 2005; Holmberg et al., 2005; Konstantinova et al., 2007; Lim et al., 2008a; Lim et al., 2008b).

Ephrins and Eph receptors were first recognized as repulsive axon guidance cues when the ligand, ephrin-A5, was identified as the molecule responsible for the growth cone collapse of retinal ganglion cells by posterior tectal membranes (Drescher et al., 1995). The Eph receptors and the ligands are often expressed in inversely complementary gradients across discrete regions of the CNS (Cheng et al., 1995; Zhang et al., 1996). Within the retinotectal system for example, temporal retinal axons which express higher levels of EphA receptors, terminate in the anterior tectum, a region with low ephrin expression (Cheng et al., 1995; Drescher et al., 1995; Dutting et al., 1999; Marcus et al., 1996). In contrast, the nasal axons, which have low EphA receptor expression, terminate in the posterior tectum where ephrin-A expression is high. Similarly, the development of the hippocamposeptal system is also organized by gradients of Eph receptors and ligands (Gao et al., 1996; Yue et al., 2002). Although regarded mainly for their properties of contact inhibition, it has since been shown that ligand dosage can modulate the attractive or repulsive response by the receptors (Hansen et al., 2004).

Of the Eph family receptors, EphA5 is one of the few expressed almost exclusively in the nervous system. The receptor expression appears to be highly dynamic and is continuously observed in the adult, indicative of distinct functions in different brain regions at different developmental stages. For example, high levels of EphA5 in the adult brain suggest a role in regulating animal behavior of which little is known. A better characterization may provide insight into these different functions. Although mRNA expression has been observed during development and in the adult brain in the mouse, detailed analysis of the spatial and temporal dynamics of the receptor protein is still lacking (Zhou et al., 1994). To better understand EphA5 functions in the developing and adult nervous system, we have utilized mice heterozygous for a β -galactosidase-tagged EphA5 receptor to extensively map the protein localization. In these EphA5^{+LacZ} mice, the intracellular kinase domain was replaced with β -galactosidase. Embryonic, neonatal, and adult specimens were sectioned and reacted with the chromogenic substrate, X-gal, to analyze protein levels. These analyses allowed us to present a thorough survey of EphA5 protein localization.

MATERIALS AND METHODS

EphA5^{LacZ/LacZ} Mice

The generation of the EphA5^{LacZ/LacZ} mice has been previously reported briefly (Feldheim et al., 2004). Genomic DNA encoding the EphA5 receptor was isolated from a 129SV mouse genomic library screen and then cloned into the pKOVpLacZ vector. The LacZ coding sequence was fused in frame to exon 8 of EphA5 at the endogenous Bam HI site (Fig. 1). The final construct included a phosphoglycerate kinase promoter-driven neomycin

resistance gene (PGK-Neo) in order to generate targeted ES cells which were then implanted into female mice (Fig. 1). Heterozygous mice were generated through regular breeding with wild type and mutant mice within the colony. Polymerase chain reaction was used to genotype the mice. Primers to detect the wild type allele were 5'GCCCCGTTATGAAAGTGCATCTTTTCC3' and 5'ACTGGCATGGAAATTGGCTCTGG3', yielding a 180 base pair product. Primers for the EphA5^{LacZ} allele were 5'GCCCCGTTATGAAAGTGCATCTTTTCC3' and 5'GCTGGCGAAAGGGGGATGTGC3', which generated a 300 base pair fragment.

Mice were housed under standard conditions and treated in accordance with the Guidelines for the Care and Use of Laboratory Animals of Rutgers University.

Histology and Microscopy

Early embryos at embryonic day 9 (E9), E11, E13, and E15 were removed from the mother and lightly fixed in a 2% paraformaldehyde/0.5% glutaraldehyde solution in PBS for 30 minutes prior to reaction with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Wholmount embryos were permeabilized in 0.02% NP-40 for 30 minutes and then allowed to develop for 18 hours in a reaction buffer containing 1 mg/mL X-Gal (Invitrogen, Carlsbad, CA), 5 mM Potassium Ferricyanide, 5 mM Potassium Ferrocyanide, 2 mM Magnesium Chloride, 0.01% Sodium Deoxycholate, and 0.02% NP-40. Wholmount embryos were first imaged intact, and then sectioned at 10 μ m on a Jung Histocut 820 microtome (Leica Instruments, Nussloch, Germany). For generating sections, embryos were first dehydrated in stepped concentrations of ethanol solution from 50% to 100% and then placed in xylene before being embedded into paraffin. For the analysis of older animals [E17, postnatal day 0 (P0), P6, 1 month, and 3 month], brains were dissected and quickly frozen in Tissue-tek O.C.T. Compound (Sakura Finetek, Torrance, CA). Brains were then cryosectioned into 10 μ m thick coronal or sagittal slices and collected onto plus-charged glass slides (Fisher Scientific, Pittsburgh, PA). After drying, sections were fixed in a 2% paraformaldehyde/0.5% glutaraldehyde solution in PBS for 1 minute. After fixation, sections were permeabilized with a 0.02% NP-40 in PBS, then allowed to develop at 37 °C in the reaction buffer for 18 hours. Sections were dehydrated with ethanol and placed into xylene before being mounted with Permount (Fisher Scientific, Pittsburgh, PA). Brightfield images were then obtained with a Zeiss microscope using Image Pro Plus and Axiovision software. Images were stored in .tif format and modified with Adobe Photoshop 7.0 software. Brightness and contrast were adjusted in order to even the background before converting to grayscale. Anatomical identification was verified using several different published resources (Franklin and Paxinos, 1997; Jacobowitz and Abbott, 1998; Kaufman, 1992; Lau et al., 2006; Lein et al., 2007; Rugh, 1990; Schambra et al., 1992).

In Situ Hybridization

In situ hybridization was performed as previously published (Zhang et al., 1997). Brains were quickly dissected, frozen on powdered dry ice, and then stored at -80 °C. Brains were then later cryosectioned at 14 μ m and the sections were mounted onto glass slides coated with 2% triethoxy-3-aminopropyl silane (Sigma-Aldrich, St. Louis, MO) in acetone. Thawed slides were post-fixed for 20 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and then permeabilized with Proteinase K (40 ng/mL) for 30 minutes at 37 °C, refixed with 4% paraformaldehyde, followed by acetylation in 50 mM triethanolamine in acetic anhydride solutions (100 mM) for 10 minutes and dehydrated. The slides were then hybridized to EphA5 riboprobes (described below) at 2,500,000 cpm/mL under stringent conditions (50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 0.2 mg/mL herring sperm DNA and 10 mM dithiothreitol) overnight at 55 °C. Slides were then washed in 5 $\times$ SSC (saline – sodium citrate) at 65 °C for 20 min, followed by a wash in 50%

formamide in $2 \times \text{SSC}$ for 30 min at the same temperature. The sections were then washed twice in RNase buffer (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5 mM EDTA) for 20 min each and incubated for 30 min at 37 °C. Finally, the sections were washed in 50% formamide, $2 \times \text{SSC}$ at 65 °C for 30 min and in $2 \times \text{SSC}$ and $0.1 \times \text{SSC}$ at room temperature for 15 minutes. After the washes, sections were dehydrated in stepped concentrations of ethanol and exposed to X-ray film for 3–6 days. Following film development, the sections were coated with Kodak NTB-2 photographic emulsion, diluted 1:1 with distilled water. The sections were exposed for 2–3 weeks at 4 °C, developed and counterstained with thionin.

EphA5 ribroprobes were generated from the pGEM4-EphA5 plasmid containing a 452 base pair DNA insert from the mouse EphA5 cDNA (nucleotides 1818–2270, flanked by BamHI and Sph I restriction sites). The sequence encodes a 150 amino-acid portion of the intracellular domain of the mouse EphA5 receptor and does not exhibit significant homology to other members of the Eph family. For the antisense probe, the plasmid was linearized with BamHI and transcribed with T7 RNA polymerase. The sense riboprobe, used as a negative control, was obtained by linearizing pGEM4 EphA5 plasmid with Sph I. This portion of EphA5 encodes the transmembrane domain and the extracellular domain immediately upstream. All sense and anti-sense probes were synthesized by *in vitro* transcription with [^{35}S]-UTP ($>1000\text{Ci/mmol}$).

Antibody Characterization and Western Blot Analysis

The affinity purified polyclonal anti-EphA5 antibody (L-15) used for western blot analysis was produced in rabbit against the C-terminal peptide of human EphA5. The immunogen used to create the antibody was from the Hek7 (EphA5, accession number P54756) amino acid sequence 977 to 991 (FASTA sequence – IKMGRYTEIFMENGY), which is different from the corresponding sequences of other Eph receptors. The antibody was purchased from Santa Cruz Biotechnology, Inc (sc-1014, lot # E291) and is recommended for detection of mouse, rat, human, and chicken EphA5 by western blot analysis, and recognizes a 130 kDa band. Only a single band of the appropriate size was detected using this antibody. Erk-1 antibody (K-23) was also commercially available through Santa Cruz Biotechnology, Inc (sc-94, lot # J245). The affinity-purified, rabbit polyclonal antibody was raised against a peptide located in subdomain XI of rat Erk 1, accession number P21708. Amino acids 305 to 327 were used as the immunogen to create the antibody. The antibody detects the 44 kDa band and to a lesser extent the 42 kDa band of Map Kinases from mouse, rat, human, chicken, frog, and zebrafish. The antibody is recommended for use in western blot detection, and did not detect non-specific bands. The secondary antibody used for Western blot primary antibody detection was peroxidase conjugated anti-rabbit IgG (whole molecule) from Sigma (A 0545, lot # 032K4882). The antiserum against purified rabbit IgG was produced in goat and then affinity purified and absorbed with Human IgG before cross-linking with peroxidase.

For western blot analysis, mouse brains from developmental stages E11, E15, P0, P6, and adult were dissected and immediately frozen in liquid nitrogen. Using a polytron homogenizer, the brains were then homogenized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Igepal (Sigma-Aldrich, St. Louis, MO) with $1 \times$ proteinase inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The brain lysates were cleared by centrifugation at 14,000 rpm for 20 minutes at 4 °C. Sixty micrograms of protein from each sample were fractionated by SDS-PAGE (Bio-Rad 10% Tris-HCl gel), and transferred to nitrocellulose paper. The membrane was first probed with rabbit anti-EphA5 (L-15) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and then stripped and reprobed with rabbit anti-Erk-1 antibody for loading control (1:1000, Santa Cruz Biotechnology). Peroxidase conjugated anti-rabbit IgG antibody (Sigma-Aldrich, St. Louis, MO) was used as

a secondary antibody and the proteins were detected with a chemiluminescence kit (Roche Diagnostics, Basel, Switzerland).

RESULTS

To investigate the distribution of the EphA5 receptor in the nervous system during development and in the adult, we first examined EphA5 protein levels in brain extracts from different developmental stages of wild type mice using western blot analysis. EphA5 protein was detected in the brain at all stages of development and in the adult (Fig. 2A), indicating that the receptor functions both during development and in the adult. In comparison to total Erk-1/2 levels (used as a protein loading control), EphA5 receptor protein levels appeared to be highest at E15 and then decreased slightly thereafter (Fig. 2C). However, the striking aspect of this graph is that the level of protein was remarkably constant at all ages examined from E11 through adult.

EphA5 protein distribution during mouse embryogenesis

We next examined the localization of EphA5 protein directly, utilizing EphA5^{LacZ} mice (Feldheim et al., 2004). The heterozygous EphA5^{+/LacZ} mice used in the following study did not show any gross morphological or behavioral deficiencies. The patterns of EphA5 protein distribution revealed by the LacZ marker was first validated using *in situ* hybridization analysis of wild type mice to identify mRNA expression. A comparison showed that these two methods yielded similar patterns both during development and in the adult (Fig. 2D–G, Fig. 12, Fig. 14).

E9 – E11—LacZ staining in early stage embryos revealed very limited levels of EphA5 protein overall. At E9, there was robust staining in the roof of the anterior mesencephalon (Fig. 3A, Fig. 4C) comprised of the pretectal and tectal regions. Additionally, faint staining could be seen along the hindbrain (Fig. 3A, Fig. 4D). Other notable areas of β -galactosidase activity at E9 were within the somites and the caudal tip of the embryo (Fig. 3A). EphA5 receptor protein was also evident in the optic vesicles (Fig. 3A). No staining was observed in the organs or the forebrain (Fig. 3A, Fig. 4A–C) at this stage.

The distribution of EphA5 protein was dramatically broadened by E11 (Fig. 3B, Fig. 5). Initial staining in the forebrain was detected in the lateral ganglionic eminence, primarily along the neuroepithelium (Fig. 5A, F). The lateral ganglionic eminence contains progenitors destined to become part of the olfactory cortex as well as the striatum (de Carlos et al., 1996; Jimenez et al., 2002). The medial ganglionic eminence remained free of protein (Fig. 5A). Likewise, the receptor was still absent from the neocortex (Fig. 3B, Fig. 5A, B, F). The high temporal to nasal gradient within the optic cup also became evident (Fig. 5C, G). Near the eye lies the semilunar ganglion of cranial nerve V (Fig. 3B, Fig. 5F) which had moderate levels of protein. Further interior, EphA5 protein began to appear dorsally at low levels in the primordial thalamus (Fig. 5C). As the midbrain developed, specific staining began to arise in the mesencephalon in the pretectal area, the anterior ventral tegmentum, and the tectum (Fig. 3B, Fig. 5B, C, F). More caudal structures, such as the pons and the cerebellar plate were stained for the receptor protein at moderate levels (Fig. 3B, Fig. 5D, E, F). LacZ staining in cranial nerve ganglia V, VIII, and X was also identifiable (Fig. 3B, Fig. 5D, E, F, Table 1). In addition, EphA5 receptor protein was observed in the ventral spinal cord, along with the spinal ganglia (Fig. 3B, Fig. 5A–F). However, organs and limb buds were still lacking any observable EphA5 protein levels (Fig. 3B, Fig. 5D, E).

E13 – E15—By embryonic day 13, staining revealed EphA5 protein in the neocortex at robust levels in differentiated neurons (Fig. 6A–D). The receptor began to appear in the

olfactory lobes just as the bulbs became apparent (Fig. 6B). In addition, the emerging hippocampus also showed EphA5 protein staining, evident as the dark blue wispy lines in Fig. 6C. Within the telencephalon, the medial and lateral ganglionic eminence became further distinguishable however staining was still limited to the neuroepithelium on the lateral ganglionic eminence (Fig. 6C). The amygdala was generally devoid of EphA5 protein staining (Fig. 6D). In addition, the receptor was also absent from the thalamus at these stages (Fig. 6C). Several structures just superior to the thalamus, including the pretectum and superior colliculus exhibited high protein levels (Fig 6D). In the developing cerebellum, the staining was quite robust (Fig. 3C, Fig. 6E). Strong levels of staining were maintained in cranial nerve ganglia V, VIII, X, and cranial nerve nucleus XII (Fig. 3C, Fig. 6, Table 1). At this stage of development, isolated patches of EphA5 protein in all four limb buds began to develop but other non-neuronal organs still remained negative (Fig. 3C).

At E15, there was little change in EphA5 protein levels from that observed at E13. However, continued growth in the embryo expanded the regions of protein levels. For example, the level of protein staining in the neocortex grew thicker and more robust over time (Fig. 3D, Fig. 7A). Deep within the telencephalon, the lateral ganglionic eminence continued to stain for the presence of the receptor while the medial ganglionic eminence stayed negative (Fig. 7B, Fig. 8C). Interestingly, protein levels in the ganglionic eminence remained limited to the neuroepithelium. The level of EphA5 protein staining in the hippocampus also became more evident with further development (Fig. 8C, D). The epithalamus was also positive for the receptor protein although the other thalamic nuclei were either negative or weakly expressive (Fig. 8D). The staining in the pretectum and tectum remained strong at this stage (Fig. 7D, Fig 8D). In the spinal cord, EphA5 protein appeared prominently in the ventral spinal cord (Fig. 3D, Fig 8C). Likewise, the staining within the spinal ganglia became more evident (Fig. 3D, Fig 8D). Most of the major non-neuronal organs lacked any staining with the exception of the ventral glans penis which had very robust signals (Fig. 8D). Staining could also be observed in the hind and forelimbs to a greater extent than at E13 (Fig. 3C, D)

E17—By embryonic day 17, the distribution of EphA5 protein began to match what later appeared in the adult. Within the olfactory bulb, differences in protein levels began to be delineated in select regions (Fig. 9A). In the eye, the temporonasal gradient was less defined than what was seen in previous stages (Fig. 9A). However, a high level of EphA5 protein was evident in the retinal ganglion cell layer which became better defined (Fig. 9A). The cerebral cortex also showed a high level of protein (Fig. 9). Above the genu of the corpus callosum, a thin line of staining for EphA5 receptor protein can be observed which marks the subplate (Fig. 9A, B). Along the neocortex, there are several areas of intensely strong protein staining, the induseum griseum (Fig. 9B), the piriform cortex (Fig. 9A, B), and the claustrum (Fig. 9A). The striatum and septum had mild levels of staining with the lateral septum being the strongest (Fig. 9B). The hippocampus had also undergone further differentiation and still continued to show receptor protein levels in a decreasing gradient from the medial to the lateral areas (Fig. 9C–E). Although the thalamus had been nearly devoid of protein in the earlier embryonic stages, EphA5 protein staining began to appear in several nuclear groups at significant levels. The epithalamus, the reunion nucleus, and several dorsal thalamic nuclear groups had mild levels of protein (Fig. 9C, D). However, hypothalamic staining appeared weak (Fig. 9C, D). In the midbrain, the pretectal nucleus and the superior colliculus had very low protein levels in contrast to levels previously observed at E15 (Fig. 9E, F). Likewise, by late embryogenesis, EphA5 protein in the hindbrain was nearly absent although the ventral spinal cord still had high levels (Washburn et al., 2007).

Early postnatal protein levels of EphA5 receptor in the CNS

At postnatal day (P) 0, more refined staining of EphA5 protein was observed in several of the areas previously discussed. In the olfactory bulbs, rings of EphA5 receptor protein in mitral and glomerular cell layers were differentiable from the surrounding tissue (Fig. 10A). Along the neocortex, the intensity of staining varied with lower protein levels in the frontal and cingulate cortices medially but higher levels of protein in the insular and parietal cortices laterally (Fig. 10B). The piriform cortex also had robust staining (Fig. 10B). Signals in the basal ganglia remained weak. The dorsal striatum appeared devoid of protein but the nucleus accumbens in the ventral striatum has higher levels of the receptor (Fig. 10C). More posteriorly, staining was weak in the caudate putamen while absent from the globus pallidus which is derived from the medial ganglionic eminence (Fig. 10C). In the induseum griseum, EphA5 signals were maintained at very high levels (Fig. 10B, C). In the hippocampus, EphA5 protein staining still remained at the highest levels observable but no protein in the dentate gyrus was observed (Fig. 10D, E). The staining in the lateral septum, a target of hippocampal fibers, was evident at moderate levels (Fig. 10B). In the thalamus, the reunions nucleus and most dorsal nuclei such as the anterior, intralaminar, and lateral nuclear groups showed EphA5 staining. The ventrolateral thalamus remained unstained (Fig. 10C–E). The hypothalamic area had only weak signals (Fig. 10C–E). The level of EphA5 staining in the midbrain was low at P0. Although some signals were detected around the ventricle, in the central gray region, staining was no longer observed in the pretectal and tectal nuclei (Fig. 10F).

At P6, the most anterior region with evidence of EphA5 protein was still within the olfactory lobes (Fig. 11A). The mitral and glomerular layers both had strong signals (Fig. 11A). Within the neocortex, signals in the piriform cortex were the most robust (Fig. 11C). Upon close examination, layers II/III and V of the neocortex had higher levels of staining than other layers (Fig. 11E). Similar to P0, the parietal and insular cortices at P6 had similar protein levels which were still higher than the more medially located frontal and cingulate cortices (Fig. 11B–D). In the barrel cortex, although not well defined at this stage, high levels of EphA5 protein were observed (Fig. 11D). Additionally in the telencephalon, the receptor protein could be seen in the striatal patches in the caudate putamen. Striatal protein levels generally had been upregulated from earlier developmental stages (Fig. 11C). The staining of EphA5 protein in the hippocampus was maintained thus far and was the area with the highest levels (Fig. 11D, E). The dentate gyrus also began to stain for EphA5 receptor protein at this stage but the signals appeared only as a thin line (Fig. 11D, E). Within the thalamus, there appeared to be clear differences in protein levels of various nuclei (Fig. 11D, E). The anterior, lateral and intralaminar nuclear groups, as well as the reunion nuclei had strong staining (Table 2). EphA5 protein was distinctly lacking from the mediodorsal thalamic nucleus. In addition, the receptor protein was absent in the posterior and ventral thalamic nuclei such as the ventrolateral and ventromedial thalamic nuclei (Fig. 11E, Table 2). In the mesencephalon, the ventral dopaminergic nuclei, the substantia nigra and the ventral tegmental area, both had distinct but low levels of staining (Table 2). Signals in the pretectal and tectal regions appeared more diffuse and weak at this stage (Fig. 11F).

EphA5 protein levels in the adult CNS

In the adult mouse, intense signals of EphA5 protein were widespread throughout the brain. Robust protein levels were seen primarily in the olfactory bulb, neocortex, hypothalamus, amygdala, and the hippocampus (Fig. 12, Fig 13, Fig 14). In contrast, the cerebellum and many thalamic nuclei were nearly devoid of staining while intermediate levels of protein were found in the striatum (caudate putamen and nucleus accumbens) and pretectal region (Fig. 12, Fig 13). Several thalamic nuclei appeared to have greater levels of protein such as the reunion and habenular nuclei (Fig. 12, Table 2). The relative levels of protein observed

generally correlated well with the mRNA levels as determined by *in situ* hybridization analysis. For example, both high protein and mRNA levels were found in the olfactory bulb, septum, hippocampus, amygdala, and entorhinal cortex (Fig. 12, Fig 14).

Most regions with EphA5 protein staining in the adult brain were similar to the signal pattern observed at earlier stages; however, the regions became anatomically well defined. Within the olfactory lobe, EphA5 protein was high along the mitral cell layer, the internal plexiform layer, and within the individual glomerules of the glomerular layer (Fig. 14A, A'). These regions stood out against the already robust protein levels seen throughout the granular and external plexiform layers. The accessory nucleus also had strong staining (Fig. 13D). Within the anterior nucleus of the olfactory lobes, the lateral and ventral lobes had the strongest signals (Fig. 12A). Further posterior, EphA5 receptor staining in the piriform and endopiriform cortices became well delineated (Fig. 12B, Fig 13B). The claustrum also had high levels of staining (Fig. 12A). The olfactory tubercle and lateral olfactory tract however remained relatively free of protein (Fig. 12B, Fig 13C). Although the cerebral cortex in general bore high signals of EphA5 protein, the somatosensory cortex, primarily the barrel cortex, was more robust than elsewhere (Fig. 12B, C, Fig 13A–C). Within the telencephalon, the globus pallidus had little to no staining while the caudate putamen had relatively moderate staining (Fig. 12C, Fig 13C) with stronger signals in the striatal patches. Within the hippocampus, an area of intense EphA5 protein staining, varying levels could be observed throughout the region (Fig. 14C, C'). The pyramidal layer of the hippocampus was the most robust region of protein staining. The stratum oriens layer just dorsal to the pyramidal cells had high levels of staining when compared to the molecular layer ventral to CA1. Within the dentate gyrus, EphA5 staining was weak overall although the granule cell layer was still prominent. Both the hypothalamus and the amygdaloid nuclei also had robust protein levels (Fig. 12D, Fig 13, Fig 14B, B'). The highest levels of staining in the amygdala were in the lateral amygdaloid nucleus followed by the basoamygdaloid and central amygdaloid nuclei (Fig. 14B). However, the cortical amygdaloid nuclei (Fig. 12E) had slightly less protein. The outer nuclei of the thalamus, the zona incerta and specific dorsal thalamic nuclei were positive for EphA5 receptor protein (Fig. 12D, Table 2). Lateral and anterior thalamic nuclear groups had moderate levels in particular. Staining was absent in the medial dorsal, ventromedial, ventroposterior, and the posterior thalamic nuclei (Table 2). The reunion nucleus also had high protein levels (Fig. 2D, Table 2). Sagittal sections through the adult brain demonstrated that although the thalamus overall expressed EphA5 protein weakly, the dorsal thalamus had higher levels than the ventral thalamus (Fig. 13D). Epithalamic nuclei, such as the habenular nuclei appeared to have had moderate protein levels whereas hypothalamic nuclei had quite robust EphA5 signals (Fig. 12C, D, Fig 13D). Staining was absent in the cerebral peduncles (Fig. 12D, E). In the midbrain, dorsally, the pretectal nuclei had mild staining along with the periaqueductal gray (Fig. 12F, Fig 13D). Further posterior, juxtaposed to the unstained inferior colliculus, the superior colliculus was observed to have mild levels of EphA5 receptor protein (Fig. 12F, 13D). Along the ventral midbrain region, areas corresponding to the substantia nigra and the ventral tegmental areas had fair levels of protein (Fig. 12F, Fig 13D). The ventral midbrain also showed signals for EphA5 receptor protein in the interpeduncular nuclei. The medial lemniscus was free of staining, as was the red nucleus (Fig. 12F, Table 2). In the hindbrain, the pons showed only weak signals for EphA5 protein (Fig. 13C, D). The cerebellum, although nearly completely devoid of EphA5 protein, showed scattered staining in the purkinje cell layer (Fig. 13A).

DISCUSSION

We show in this study that the patterns of EphA5 protein levels are dynamic and complex. EphA5 protein begins to appear in a limited number of regions such as the dorsal midbrain as early as E9, the earliest age examined in this study. Analysis using *in situ* hybridization

revealed that mRNA expression was lacking in E8 mouse embryos in the same region (Zhang et al., 1997), suggesting that the earliest expression occurs between E8 and E9. However, the distribution of EphA5 protein in the midbrain is greatly diminished on and after E17. The transient presence of EphA5 protein correlates with early neurogenesis and migration of differentiated cells in the midbrain region, and may play a role in midbrain morphogenesis. In contrast, the appearance of LacZ staining in the cerebral cortex starts around E13, and continues into adult. The distribution of EphA5 protein in the adult cerebral cortex is especially high, implicating a role for EphA5 in cortical plasticity. The widespread and dynamic patterns of EphA5 protein levels reported in this study clearly indicate multiple functions in the nervous system, possibly from neurogenesis at the embryonic stages to plasticity in the adult. Our results also reveal several unique aspects of EphA5 protein distribution that are discussed in the following sections.

Differential patterns of EphA5 protein levels

Several general patterns of EphA5 protein levels are apparent in the mouse nervous system. There is a clear dorsoventral differentiation in the levels of EphA5 protein. As the mouse embryo develops, EphA5 protein appears to spread throughout the dorsal-most structures within the brain with only weak levels in the ventral regions. The most superficial and dorsal structure, the neocortex, is one of the most robust regions of protein in the developing mouse and in the adult. This dorsoventral differentiation also appears in the basal ganglia, the dorsally situated lateral ganglionic eminence is positive for EphA5 protein but the ventrally located medial ganglionic eminence is not. Additionally, the septal nuclei exhibit the same pattern whereby there is a high dorsal and low ventral gradient of protein signal which is maintained through adulthood. The trend also extends to the midbrain where there are higher protein levels in the superior colliculus and little protein in the inferior colliculus.

The emergence of EphA5 protein appears to also follow a caudal to rostral axis. At E9, the strongest signal was found in the dorsal midbrain in the tectal neuroepithelium. Over time, as EphA5 protein signals moved anteriorly into the neocortex and other more caudal structures, the pretectum and tectal nuclei, began to lose protein signal. A similar caudal to rostral progression was also observed in the spinal cord. The protein levels begin in the caudal tail and advance along the spinal cord rostrally as the embryo develops. However, in the spinal cord, the dorsoventral differences in staining were the opposite to that of the brain. EphA5 protein is observed at the highest levels in the ventral regions [Fig. 5 and (Washburn et al., 2007)]. Our previous studies have revealed that ephrin-A5, a ligand of EphA5, is present in a complementary fashion in the dorsal regions of the spinal cord (Washburn et al., 2007; Yue et al., 1999; Zhang et al., 1997), suggesting a role in regulating cell-cell interactions between the dorsal and ventral spinal cord.

EphA5 receptor protein is distributed over several major regions

The EphA5 receptor has been implicated in several vital roles in the development of the nervous system (Feldheim et al., 2004; Yue et al., 2002). The role of EphA5 in mapping axon terminals upon the target field topographically is the best understood. Inhibition of EphA receptor functions leads to targeting defects in several different pathways. For example, overexpression of a truncated EphA5 receptor in mouse led to hippocampal axon projection errors to the septum (Yue et al., 2002). In another well-characterized pathway, the retinotectal tract, interactions between EphA receptor expression gradients in the retina and the ephrin-A ligand gradients in the retinal axon target region, the tectum, regulate the formation of the retinotectal axon projection map (Cheng et al., 1995; Drescher et al., 1995; Marcus et al., 1996). When the EphA receptor function is inactivated, disruption of the map occurs (Feldheim et al., 2004). The present study indicates that EphA5 protein is present in

several major neural systems, suggesting that EphA5 plays a widespread role in regulating axon connections and neural plasticity.

Cerebral cortex—EphA5 protein can be detected in the cerebral cortex around E11 (Fig. 5), the time neurogenesis starts (Angevine and Sidman, 1961). The early protein signals are more concentrated in the intermediate zone and the cortical plate, where differentiated neurons are located. Studies of EphA5 mRNA transcription showed similar results (Yun et al., 2003; Zhang et al., 1997). Only low levels of EphA5 protein were found in the ventricular zone where mitotic neuronal precursors are located (Fig. 6), suggesting that the onset of EphA5 protein detection accompanies neuronal differentiation. The presence of EphA5 protein in differentiated neurons is consistent with a function in cortical axon guidance. As embryogenesis advances to later stages, EphA5 protein staining becomes more widespread and can also be found in the ventricular zone (Fig. 6). In the adult brain, there is extensive EphA5 protein staining throughout the different layers, with higher levels in layers 2/3 and 5 (Fig. 11). Particularly high protein levels were detected in the barrel cortex. In tangential cortical sections, each barrel is clearly marked by EphA5 staining (Table 2). The strong protein signals in the adult cerebral cortex suggests a possible function of EphA5 in neural plasticity.

The olfactory - limbic system—Consistent with earlier studies (St John et al., 2000), the current analysis shows that EphA5 is expressed at high levels in many areas of the developing olfactory system, for example, the glomeruli, the anterior and accessory olfactory nuclei, as well as the piriform and endopiriform cortices. Existing data indicate that normal axon outgrowth from olfactory epithelial explants requires EphA5 (St John et al., 2000). The olfactory system is anatomically connected to the amygdala, lateral septum, hypothalamus, and the hippocampus, regions that are part of the limbic system (Isaacson, 1982). The limbic areas express high levels of EphA5, as noted in a previous study (Zhou et al., 1994). The protein levels of EphA5 in these related systems possibly reflect a common evolutionary origin (Isaacson, 1982). The function of EphA5 in the limbic system has just begun to be analyzed. In the hippocampus, EphA5 is expressed in a dorsomedial (high) to a ventrolateral (low) gradient (Gao et al., 1996; Yue et al., 2002), and regulates the formation of the topographic hippocamposeptal projection map (Gao et al., 1996; Yue et al., 2002). Within the hippocampus, protein levels are found primarily within the CA1–CA3 region of the pyramidal cell layer. There is evidence that EphA and ephrin-A also regulate intra-hippocampal circuit formation (Stein et al., 1999). Functions of EphA5 in other limbic circuits remain to be identified.

Thalamus—Although thalamic protein levels are weak overall, the staining is not uniform. EphA5 has been shown to play a role in ensuring precise thalamocortical projections to the barrel cortex (Gao et al., 1998; Uziel et al., 2002). Within the thalamus, several nuclei distinctly lack any staining. However, those nuclei that are positive for EphA5 protein, primarily the anterior dorsal group, contain the relay nuclei responsible for memory and learning in humans (Martin, 1996). Major inputs into the anterior thalamic group are from the hypothalamus, the hippocampus, and the tectum. Output from this region is mainly to the cingulate gyrus (Martin, 1996). The intralaminar nuclei also bear moderate levels of EphA5 protein levels. This group contains the centromedian and parafascicular nuclei which are responsible for the regulation of cortical activity (Martin, 1996). The lateral group, where EphA5 protein was distinctly lacking, contains inputs mostly from the cerebellum, brain stem, and spinal cord (Martin, 1996). The output of this region to the premotor and primary motor cortex is responsible for movement planning and proprioception. EphA5 protein in the thalamus is likely to regulate both intrathalamic connections among different thalamic nuclei and the efferent and afferent axon connections with other parts of the brain.

Striatum—Although not completely understood, EphA5 may have a role in the development of the mesostriatal pathway. Protein levels in the putative striatum can be seen as early as E11 along the neuroepithelium of the lateral ganglionic eminence. Also notable are positive islands of staining in the matrix compartment in the striatum of the adult mouse. A previous study has shown that EphA receptors are indeed found in the striatal matrix compartment using affinity binding of ephrin-A-Fc fusion protein, although the identity of the receptors were not known (Janis et al., 1999). Thus, the current study shows that at least one of the EphA receptors identified previously is EphA5. From the ventral midbrain, where dopaminergic neurons lie, axons course through mesostriatal tracts which synapse onto the patches within the striatal region. The midbrain dopaminergic neurons and the striatum have been shown to have trophic effects on each other (Emgard-Mattson et al., 1997; Ohtani et al., 2003). Ephrin-A ligands have been detected in the patch compartment in striatum (Janis et al., 1999), suggesting regulation of interaction between neurons in the patch and matrix compartments. EphA5 protein signals can also be found in the ventral midbrain early in embryogenesis. In late embryogenesis, the main midbrain dopaminergic nuclei, the substantia nigra and the ventral tegmental area, both have significant levels of EphA5 protein (Table 2). Stimulation of these neurons with ephrin-A5 promotes neurite outgrowth, and loss of EphA5 abrogates this effect (Cooper et al., 2008). In addition, a decrease in dopaminergic projections from the midbrain into the striatum occurs when EphA receptor activity is blocked (Seiber et al., 2004). Together these observations indicate that EphA5 may play a key role in regulating the formation of the nigrostriatal dopaminergic pathway.

In addition to the major areas discussed above, EphA5 protein was also found in the eye, in numerous cranial nerve ganglia, and in the spinal cord, primarily the ventral regions (Washburn et al., 2007). Nevertheless, EphA5 protein is restricted mostly to the nervous system with only a few exceptions. The appearance of EphA5 protein in a wide variety of neural systems at different developmental times points to multiple roles in regulating the ontogenesis and functions of the nervous system.

Similarities and differences of EphA5 mRNA and protein levels

The results of our analysis are generally consistent with the findings of past surveys. There is a consensus that the EphA5 receptor is limited primarily to the nervous system in both mRNA and protein studies (Maisonpierre et al., 1993; Taylor et al., 1994; Zhang et al., 1997; Zhou, 1997). In both the embryonic and adult mouse, β -galactosidase activity was not detected in the organs examined including the lung, liver, spleen, heart, kidney, thymus gland and skeletal muscles, with the exception of the male gonads (Fig. 8D, and data not shown), consistent with previous *in situ* hybridization studies (Maisonpierre et al., 1993; Taylor et al., 1994). Reports on peak protein levels in the brain differ slightly but generally fall between E15 and E18 before decreasing to intermediate levels in the adult (Taylor et al., 1994; Zhou, 1997). Additionally, in both EphA5 protein and mRNA studies, levels in the spinal cord, hindbrain, and midbrain eventually decrease, leaving the bulk of EphA5 signals in the forebrain of the adult (Taylor et al., 1994; Washburn et al., 2007; Zhang et al., 1997; Zhou, 1997). Early embryonic EphA5 mRNA in the distal tip of the tail and mesencephalon reported earlier (Zhang et al., 1997) correlates with the protein levels shown in the current study. Expression of EphA5 mRNA has also been reported in the embryonic cortex, hippocampus, ganglionic eminence, amygdala, hypothalamus, cerebellum, retina, tectum, cranial nerve nuclei and dorsal thalamus (Zhang et al., 1997), regions which were also found with high protein signals. In the adult brain, the levels of EphA5 protein observed in this study also agrees with previous *in situ* analysis that the highest levels of EphA5 receptor are to be found in the hippocampus followed by the cortex and that the lowest level is found in the cerebellum (Maisonpierre et al., 1993; Taylor et al., 1994; Zhang et al., 1997).

Despite the many similarities, there are some differences between the current protein study and the past mRNA analyses. In the adult, mRNA studies detected only moderate levels of expression in the cerebral cortex [Fig. 12, also see (Maisonpierre et al., 1993; Zhou et al., 1994)]. However, the current study showed robust EphA5 protein levels using both western blot and β -galactosidase staining. The high level of EphA5 protein reported is also confirmed by a previous study using an affinity purified anti-EphA5 antibody (St John et al., 2000). It is possible that *in situ* hybridization has difficulty detecting less than robust levels of expression, indicating superior sensitivity of the LacZ staining method used in the current analysis.

Using both western blot and LacZ histological detection methods, the current study provides a thorough analysis of EphA5 protein levels throughout development and in adult. Receptor protein was visualized directly on the tissue, an improvement which complements previous examinations of EphA5 mRNA and receptor protein. The current study revealed a number of general patterns, including the preferred dorsal protein levels in the early developing brain, the preferred ventral protein levels in the spinal cord, as well as the dynamic changes in several brain regions. These data provide a foundation for future analysis of the biological functions of this receptor in development and adult organisms. EphA5 protein has been detected in the mouse, rat and human brain (Liebl et al., 2003; Maisonpierre et al., 1993; Olivieri and Miescher, 1999; Taylor et al., 1994; Zhang et al., 1997), suggesting that roles during developing and adult stages are generally well conserved across mammalian species.

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LITERATURE CITED

- Angevine JB Jr, Sidman RL. Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature*. 1961; 192:766–768. [PubMed: 17533671]
- Brambilla R, Schnapp A, Casagrande F, Labrador JP, Bergemann AD, Flanagan JG, Pasquale EB, Klein R. Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases. *Embo J*. 1995; 14(13):3116–3126. [PubMed: 7621826]
- Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell*. 1995; 82(3):371–381. [PubMed: 7634327]
- Cooper MA, Kobayashi K, Zhou R. Ephrin-A5 regulates the formation of the ascending midbrain dopaminergic pathways. *Dev Neurobiol*. 2008 Nov 10. [Epub ahead of print].
- Cowan CW, Shao YR, Sahin M, Shamah SM, Lin MZ, Greer PL, Gao S, Griffith EC, Brugge JS, Greenberg ME. Vav family GEFs link activated Ephs to endocytosis and axon guidance. *Neuron*. 2005; 46(2):205–217. [PubMed: 15848800]
- Davy A, Soriano P. Ephrin signaling in vivo: look both ways. *Dev Dyn*. 2005; 232(1):1–10. [PubMed: 15580616]
- de Carlos JA, Lopez-Mascaraque L, Valverde F. Dynamics of cell migration from the lateral ganglionic eminence in the rat. *J Neurosci*. 1996; 16(19):6146–6156. [PubMed: 8815897]
- Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell*. 1995; 82(3):359–370. [PubMed: 7634326]
- Dutting D, Handwerker C, Drescher U. Topographic targeting and pathfinding errors of retinal axons following overexpression of ephrinA ligands on retinal ganglion cell axons. *Dev Biol*. 1999; 216(1):297–311. [PubMed: 10588880]

- Emgard-Mattson M, Karlsson J, Nakao N, Brundin P. Addition of lateral ganglionic eminence to rat mesencephalic grafts affects fiber outgrowth but does not enhance function. *Cell Transplant*. 1997; 6(3):277–286. [PubMed: 9171160]
- Feldheim DA, Nakamoto M, Osterfield M, Gale NW, DeChiara TM, Rohatgi R, Yancopoulos GD, Flanagan JG. Loss-of-function analysis of EphA receptors in retinotectal mapping. *J Neurosci*. 2004; 24(10):2542–2550. [PubMed: 15014130]
- Flanagan JG, Vanderhaeghen P. The ephrins and Eph receptors in neural development. *Annu Rev Neurosci*. 1998; 21:309–345. [PubMed: 9530499]
- Franklin, KBJ.; Paxinos, G. *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press; 1997.
- Gale NW, Holland SJ, Valenzuela DM, Flenniken A, Pan L, Ryan TE, Henkemeyer M, Streibhardt K, Hirai H, Wilkinson DG, Pawson T, Davis S, Yancopoulos GD. Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron*. 1996; 17(1):9–19. [PubMed: 8755474]
- Gao PP, Yue Y, Zhang JH, Cerretti DP, Levitt P, Zhou R. Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections. *Proc Natl Acad Sci U S A*. 1998; 95(9):5329–5334. [PubMed: 9560275]
- Gao PP, Zhang JH, Yokoyama M, Racey B, Dreyfus CF, Black IB, Zhou R. Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system. *Proc Natl Acad Sci U S A*. 1996; 93(20):11161–11166. [PubMed: 8855326]
- Hansen MJ, Dallal GE, Flanagan JG. Retinal axon response to ephrin-as shows a graded, concentration-dependent transition from growth promotion to inhibition. *Neuron*. 2004; 42(5):717–730. [PubMed: 15182713]
- Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, Jeffrey PD, Vearing C, Geleick D, Feldheim DA, Boyd AW, Henkemeyer M, Nikolov DB. Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci*. 2004; 7(5):501–509. [PubMed: 15107857]
- Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F. A novel putative tyrosine kinase receptor encoded by the eph gene. *Science*. 1987; 238(4834):1717–1720. [PubMed: 2825356]
- Holmberg J, Armulik A, Senti KA, Edoff K, Spalding K, Momma S, Cassidy R, Flanagan JG, Frisen J. Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. *Genes Dev*. 2005; 19(4):462–471. [PubMed: 15713841]
- Isaacson, RL. *The Limbic System*. New York: Plenum Press; 1982. 327 p.
- Jacobowitz, DM.; Abbott, LC. *Chemoarchitectonic Atlas of the Developing Mouse Brain*. Boca Raton: CRC Press; 1998.
- Janis LS, Cassidy RM, Kromer LF. Ephrin-A binding and EphA receptor expression delineate the matrix compartment of the striatum. *J Neurosci*. 1999; 19(12):4962–4971. [PubMed: 10366629]
- Jimenez D, Lopez-Mascaraque LM, Valverde F, De Carlos JA. Tangential migration in neocortical development. *Dev Biol*. 2002; 244(1):155–169. [PubMed: 11900465]
- Kaufman, MH. *The Atlas of Mouse Development*. San Diego: Academic Press; 1992. 512 p.
- Konstantinova I, Nikolova G, Ohara-Imaizumi M, Meda P, Kucera T, Zarbalis K, Wurst W, Nagamatsu S, Lammert E. EphA-Ephrin-A-mediated beta cell communication regulates insulin secretion from pancreatic islets. *Cell*. 2007; 129(2):359–370. [PubMed: 17448994]
- Kullander K, Klein R. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol*. 2002; 3(7):475–486. [PubMed: 12094214]
- Lau, C.; Ng, L.; Pathak, S.; Kuan, L.; Sutram, M.; Youngstrom, B.; Kawal, R.; Hawrylycz, M. *Allen Brain Atlas - Brain Explorer*. Version 1.2 Build 20. Seattle: Allen Institute for Brain Science; 2006.
- Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramée AR,

- Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivilay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sot AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wahnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007; 445(7124):168–176. [PubMed: 17151600]
- Liebl DJ, Morris CJ, Henkemeyer M, Parada LF. mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous system. *J Neurosci Res*. 2003; 71(1):7–22. [PubMed: 12478610]
- Lim BK, Matsuda N, Poo MM. Ephrin-B reverse signaling promotes structural and functional synaptic maturation in vivo. *Nat Neurosci*. 2008a; 11(2):160–169. [PubMed: 18193042]
- Lim YS, McLaughlin T, Sung TC, Santiago A, Lee KF, O'Leary DD. p75(NTR) mediates ephrin-A reverse signaling required for axon repulsion and mapping. *Neuron*. 2008b; 59(5):746–758. [PubMed: 18786358]
- Maisonpierre PC, Barrezaeta NX, Yancopoulos GD. Ehk-1 and Ehk-2: two novel members of the Eph receptor-like tyrosine kinase family with distinctive structures and neuronal expression. *Oncogene*. 1993; 8(12):3277–3288. [PubMed: 7504232]
- Marcus RC, Gale NW, Morrison ME, Mason CA, Yancopoulos GD. Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. *Dev Biol*. 1996; 180(2):786–789. [PubMed: 8954746]
- Martin, JH. *Neuroanatomy: text and atlas*. Martin, JH., editor. Stamford: Appleton & Lange; 1996. 578 p.
- Miao H, Wei BR, Peehl DM, Li Q, Alexandrou T, Schelling JR, Rhim JS, Sedor JR, Burnett E, Wang B. Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway. *Nat Cell Biol*. 2001; 3(5):527–530. [PubMed: 11331884]
- Ohtani N, Goto T, Waeber C, Bhide PG. Dopamine modulates cell cycle in the lateral ganglionic eminence. *J Neurosci*. 2003; 23(7):2840–2850. [PubMed: 12684471]
- O'Leary DD, Wilkinson DG. Eph receptors and ephrins in neural development. *Curr Opin Neurobiol*. 1999; 9(1):65–73. [PubMed: 10072375]
- Olivieri G, Miescher GC. Immunohistochemical localization of EphA5 in the adult human central nervous system. *J Histochem Cytochem*. 1999; 47(7):855–861. [PubMed: 10375373]
- Panayotou G, Waterfield MD. The assembly of signalling complexes by receptor tyrosine kinases. *Bioessays*. 1993; 15(3):171–177. [PubMed: 8387783]
- Pasquale EB. Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol*. 2005; 6(6):462–475. [PubMed: 15928710]
- Rugh, R. *The Mouse: Its Reproduction and Development*. New York: Oxford University Press; 1990. 430 p.
- Sahin M, Greer PL, Lin MZ, Poucher H, Eberhart J, Schmidt S, Wright TM, Shamah SM, O'Connell S, Cowan CW, Hu L, Goldberg JL, Debant A, Corfas G, Krull CE, Greenberg ME. Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. *Neuron*. 2005; 46(2):191–204. [PubMed: 15848799]
- Schambra, UB.; Lauder, JM.; Silver, J. *Atlas of the Prenatal Mouse Brain*. San Diego: Academic Press; 1992. 327 p.
- Seiber B-A, Kuzmin A, Canals JM, Danielsson A, Paratcha G, Arenas E, Alberch J, Ogren SO, Ibanez CF. Disruption of EphA/ephrin-A Signaling in the Nigrostriatal System Reduces Dopaminergic Innervation and Dissociates Behavioural Responses to Amphetamine and Cocaine. *Molecular and Cellular Neuroscience*. 2004; 26:418–428. [PubMed: 15234346]
- Shamah SM, Lin MZ, Goldberg JL, Estrach S, Sahin M, Hu L, Bazalakova M, Neve RL, Corfas G, Debant A, Greenberg ME. EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell*. 2001; 105(2):233–244. [PubMed: 11336673]

- St John JA, Tisay KT, Caras IW, Key B. Expression of EphA5 during development of the olfactory nerve pathway in rat. *J Comp Neurol.* 2000; 416(4):540–550. [PubMed: 10660883]
- Stein E, Savaskan NE, Ninnemann O, Nitsch R, Zhou R, Skutella T. A role for the Eph ligand ephrin-A3 in entorhino-hippocampal axon targeting. *J Neurosci.* 1999; 19(20):8885–8893. [PubMed: 10516308]
- Taylor V, Miescher GC, Pfarr S, Honegger P, Breitschopf H, Lassmann H, Steck AJ. Expression and developmental regulation of Ehk-1, a neuronal Elk-like receptor tyrosine kinase in brain. *Neuroscience.* 1994; 63(1):163–178. [PubMed: 7898646]
- Uziel D, Muhlfriedel S, Zarbalis K, Wurst W, Levitt P, Bolz J. Miswiring of limbic thalamocortical projections in the absence of ephrin-A5. *J Neurosci.* 2002; 22(21):9352–9357. [PubMed: 12417660]
- van der Geer P, Hunter T, Lindberg RA. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol.* 1994; 10:251–337. [PubMed: 7888178]
- Washburn CP, Cooper MA, Zhou R. Expression of the tyrosine kinase receptor EphA5 and its ligand ephrin-A5 during mouse spinal cord development. *Neurosci Bull.* 2007; 23(5):249–255. [PubMed: 17952132]
- Wilkinson DG. Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci.* 2001; 2(3):155–164. [PubMed: 11256076]
- Yue Y, Chen ZY, Gale NW, Blair-Flynn J, Hu TJ, Yue X, Cooper M, Crockett DP, Yancopoulos GD, Tessarollo L, Zhou R. Mistargeting hippocampal axons by expression of a truncated Eph receptor. *Proc Natl Acad Sci U S A.* 2002; 99(16):10777–10782. [PubMed: 12124402]
- Yue Y, Su J, Cerretti DP, Fox GM, Jing S, Zhou R. Selective inhibition of spinal cord neurite outgrowth and cell survival by the Eph family ligand ephrin-A5. *J Neurosci.* 1999; 19(22):10026–10035. [PubMed: 10559410]
- Yun ME, Johnson RR, Antic A, Donoghue MJ. EphA family gene expression in the developing mouse neocortex: regional patterns reveal intrinsic programs and extrinsic influence. *J Comp Neurol.* 2003; 456(3):203–216. [PubMed: 12528186]
- Zhang JH, Cerretti DP, Yu T, Flanagan JG, Zhou R. Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction. *J Neurosci.* 1996; 16(22):7182–7192. [PubMed: 8929427]
- Zhang JH, Pimenta AF, Levitt P, Zhou R. Dynamic expression suggests multiple roles of the eph family receptor brain-specific kinase (Bsk) during mouse neurogenesis. *Brain Res Mol Brain Res.* 1997; 47(1–2):202–214. [PubMed: 9221918]
- Zhou R. Regulation of topographic projection by the Eph family receptor Bsk (EphA5) and its ligands. *Cell Tissue Res.* 1997; 290(2):251–259. [PubMed: 9321686]
- Zhou R. The Eph family receptors and ligands. *Pharmacol Ther.* 1998; 77(3):151–181. [PubMed: 9576626]
- Zhou R, Copeland TD, Kromer LF, Schulz NT. Isolation and characterization of Bsk, a growth factor receptor-like tyrosine kinase associated with the limbic system. *J Neurosci Res.* 1994; 37(1):129–143. [PubMed: 8145300]

Native Locus

Fig. 1.

EphA5 gene targeting strategy. The EphA5 gene targeting construct is comprised of a 1.9 kb 5' homology region containing part of exon 8 and the upstream intron sequences, a LacZ gene fused in frame at the Bam HI site, a neomycin (PKG-Neo) cassette, the 3' homology sequence from the intron region downstream of exon 13, and a HSV thymidine kinase (HSV-tk) gene. The targeted EphA5 locus is depicted in the third line with characteristic Bam HI restriction digestion sites which can be used to differentiate it from the wild type. Exact nucleotide and amino acid sequences at the EphA5-LacZ fusion site are shown at the bottom. The fusion site is located 36 amino acid residues down stream of the transmembrane domain, and the two critical juxtamembrane domain tyrosine residues that are conserved among Eph receptors are further downstream from this site and thus not present in the fusion protein.

Fig. 2.

High levels of EphA5 protein are detected in both the developing and the mature mouse brain. **A:** Western blot analysis of EphA5 protein from different stages of brain development. Endogenous EphA5 receptor protein was detected at several developmental stages and in the adult of the wild type mouse brain. Sixty micrograms of protein extract from the whole head (E11 and E15) or from the brain (P0, P6, and Adult) were loaded and analyzed with an anti-EphA5 antibody. The same blot was reprobed with anti-Erk-1 antibody as a protein loading control. **B:** EphA5 antibody blocking with the immunizing peptide. Preadsorption with a blocking peptide severely reduced the ability of the EphA5 antibody to detect the receptor, indicating high specificity. **C:** Relative levels of EphA5

expression at different developmental stages. The pixel values of the EphA5 signals were corrected by the pixel values of the Erk-1 signals. **D, F:** In situ hybridization with an anti-sense EphA5 probe of wild type brain sections from E17 and adult mice, respectively. **E, G:** EphA5 protein detected with β -galactosidase histochemical staining in E17 and adult heterozygous mice. EphA5 protein analysis mirrors the mRNA expression patterns. ctx, cortex; Hip, hippocampus; Th, thalamus. Scale bars = 1 mm.

Fig. 3. Whole mount X-Gal staining of mouse embryos. Dark staining is indicative of EphA5 protein. **A:** E9, **B:** E11, **C:** E13, **D:** E15, EphA5^{+/LacZ} embryos. Cb, cerebellum; ctx, neocortex; e, eye; fl, forelimb; hb, hindbrain; hl, hindlimb; hr, heart; ma, mandibular arch; mes, mesencephalon; my, myelencephalon; SC, spinal cord; V, cranial nerve ganglion V (trigeminal); X, cranial nerve ganglion X (vagus). Scale bars = 1 mm.

Fig. 4.

EphA5 protein in E9 mouse embryo. **A–C:** EphA5^{+/LacZ} E9 mouse embryos were reacted with X-gal and sectioned frontally to better visualize the EphA5 protein staining. The highest level observed was in the anterior mesencephalon and pretectum (C). Organs and other nervous system tissues appeared to be devoid of expression (A, B). **D:** Higher magnification of a whole mount stained E9 embryo shows additional staining in the hindbrain region. aa, aortic arches; cl, coelom; cv, cardinal vessel; da, dorsal aorta; dien, diencephalon; hb, hindbrain; l, lung bud; lb, limb bud; ma, mandibular arch; mes, mesencephalon; mx, maxillary arch; ov, optic vesicle; SC, spinal cord; v, ventricle. Scale bars = 0.5 mm. Embryo drawings used to convey the plane of section in Figure 3–Figure 6, and Figure 8 were adapted from (Rugh, 1990).

Fig. 5.

EphA5 protein in the E11 mouse embryo as revealed by X-gal staining. **A–E:** Serial frontal sections through a stained whole mount embryo. **F:** A sagittal view of the E11 embryo. **G:** Frontal section through the eye showing the lens and the EphA5 temporonasal gradient in the retina. Cb, cerebellum; ctx, cortex; dTh, dorsal thalamus; hyp, hypothalamus; LGe, lateral ganglionic eminence; In, lens; MGe, medial ganglionic eminence; my, myelencephalon; N, nasal; p, pons; pT, pre-tectum; ret, retina; SC, spinal cord; Sg, spinal ganglion; T, tectum; T (in G), temporal; Th, thalamus; vIV, fourth ventricle; V, cranial nerve ganglion V; VIII, cranial nerve ganglion VIII; Vm, ventral midbrain; X, cranial nerve ganglion X. Scale bars = 1 mm (A–F), 100 μm (G).

Fig. 6.

EphA5 protein in the E13 brain as revealed by X-gal staining. Frontal sections were made through an E13 mouse embryo. A, amygdala; ctx, cortex; dTh, dorsal thalamus; Hip, hippocampus; LGe, lateral ganglionic eminence; ln, lens; MDR, medullary reticular formation; MGe, medial ganglionic eminence; ob, olfactory bulb; pc, posterior commissure; pT, pretectum; ret, retina; s, septum; sc, superior colliculus; V, cranial nerve ganglion V; X, cranial nerve ganglion X; XII, cranial nerve nucleus XII (hypoglossal). Scale bar = 1 mm.

Fig. 7.

Frontal views of EphA5 protein distribution in the E15 mouse brain as revealed by X-gal staining. A, amygdala; Cb, cerebellum; CP, caudoputamen; ctx, cortex; ET, epithalamus; Hip, hippocampus; ic, inferior colliculus; pT, pretectum; s, septum; sc, superior colliculus; Th, thalamus; VIII, cranial nerve VIII; XII, cranial nerve nucleus XII. Scale bar = 1 mm.

Fig. 8. Sagittal views of EphA5 protein localization in the E15 mouse embryo as revealed by X-gal reactivity. The dorsoventral patterning of EphA5 protein is evident in this plane. Cb, cerebellum; ctx, cortex; e, eye; ET, epithalamus; gp, glans penis; Hip, hippocampus; hl, hindlimb; LGe, lateral ganglionic eminence; Lv, liver; V, cranial nerve V; MGe, medial ganglionic eminence; ob, olfactory bulb; p, pons; pT, prethalamus; SC, spinal cord; sc, superior colliculus; Sg, spinal ganglion; Th, thalamus. Scale bar = 1 mm.

Fig. 9.

Coronal views of EphA5 protein staining in the E17 mouse brain. From E17 and onwards EphA5^{+/-LacZ} brains were sectioned and then reacted with X-gal. A, amygdala; cc, corpus callosum (genu); Cl, claustrum; ctx, neocortex; (m) medial-, (l) lateral- Hip, hippocampus; hyp; hypothalamus; IG, induseum griseum; LS, lateral septum; ml, medial lateral thalamic nucleus; pir, piriform cortex; pT, pretectum; pv, periventricular thalamic nucleus; ret, retina; rgl, retinal ganglion cell layer; sc, superior colliculus; sp, subplate; St, striatum; Th, thalamus; vpl, ventroposterior lateral thalamic nucleus. Scale bar = 1 mm.

Fig. 10.

X-gal staining of EphA5 protein in the P0 mouse brain. cg, central gray; CP, caudate putamen; ctx, neocortex; gl, glomerular layer; GP, globus pallidus; gr, granular layer; Hip, hippocampus; hyp, hypothalamus; IG, induseum griseum; LS, lateral septum; mt, mitral layer; NAc, nucleus accumbens; ob, olfactory bulb; pir, piriform cortex; pT, pretectum; sc, superior colliculus; (d) dorsal-, (v) ventral- Th, thalamus; V, cranial nerve V. Scale bars = 0.5 mm (A), 1 mm (B–F). Image used to denote the plane of section in figure 9–figure 11 was adapted from (Jacobowitz and Abbott, 1998).

Fig. 11.

X-gal staining of EphA5 protein in the P6 mouse brain. Ad, anterodorsal thalamic nucleus; aob, accessory olfactory bulb; aon, anterior olfactory nucleus; BC, barrel cortex; CP, caudate putamen; ctx, neocortex; ep, external plexiform layer; gl, glomerular layer; GP, globus pallidus; gr, granular layer; Hip, hippocampus; IG, induseum griseum; ip, internal plexiform layer; hyp, hypothalamus; mob, main olfactory bulb; mt, mitral layer; ob, olfactory bulb; pir, piriform cortex; pv, periventricular thalamic nucleus; rn, reunion nucleus; s, septum; sc, superior colliculus; Th, thalamus; (d) dorsal-, (v) ventral- Th, thalamus. Scale bars = 0.5 mm (A), 1 mm (B–F).

Fig. 12.

Coronal views of EphA5 protein and mRNA in the adult mouse brain. **A–F:** Coronal sections were prepared from 3 month old EphA5^{+/LacZ} mouse brains. **B', D', F':** Autoradiograph of wild type adult mouse coronal sections corresponding to panels B, D, and F to demonstrate the similarities of EphA5 mRNA and protein expression patterns as revealed by X-gal reactivity. A, amygdala; Ad, anterodorsal thalamic nucleus; APT, anterior pretectal nuclei; BC, barrel cortex; cbp, cerebellar peduncle; cc, corpus callosum; Cl, claustrum; CP, caudate/putamen; cp, cerebral peduncle; ctx, neocortex; GP, globus pallidus; Hip, hippocampus; hyp, hypothalamus; IG, induseum griseum; IPn, interpeduncular nuclei; LG, lateral geniculate; LH, lateral hypothalamus; M, mammillary nuclei; Me, mesencephalic nuclei; MG, medial geniculate; NAc, nucleus accumbens; ob, olfactory bulb; ot, olfactory tubercle; PAG, periaqueductal gray; Pir, piriform cortex; Po, posterior thalamic nuclear group; pv, periventricular thalamic nucleus; RN, red nucleus; sc, superior colliculus; SN, substantia nigra; Th, thalamus; vm, ventromedial thalamic nuclei; vp, ventroposterior thalamic nuclei; VTA, ventral tegmental nuclei; ZI, zona incerta. Scale bar = 3 mm.

Fig. 13.

Sagittal views of EphA5 protein in the adult EphA5^{+/LacZ} mouse brain. **A–D:** lateral to medial brain sections reacted with X-gal. A, amygdala; aon, anterior olfactory nucleus; BC, barrel cortex; Cb, cerebellum; cc, corpus callosum; CP, caudate putamen; DG, dentate gyrus; dTh, dorsal thalamus; GB, globus pallidus; Hip, hippocampus; hyp, hypothalamus; ic, inferior colliculus; MDN, midbrain dopaminergic neurons (A9); ob, olfactory bulb; ot, olfactory tubercle; Pir, piriform cortex; p, pons; pT, prepectum; sc, superior colliculus; sub, subiculum, vTh, ventral thalamus. Scale bar = 3 mm.

Fig. 14.

Higher magnification images of selected areas of EphA5 protein and mRNA expression in the adult brain. **A, A'**: olfactory bulb, coronal section. **B, B'**: coronal view of the amygdaloid nuclei, left hemisphere. **C, C'**: dorsomedial hippocampus, coronal section. BLA; basolateral amygdaloid nucleus; CA, central amygdaloid nucleus; CA1–CA3, regions of the hippocampus proper (cornu ammonis); cc, corpus callosum; DG, dentate gyrus; ep, external plexiform layer; FC, fasciola cinereum; gl, glomerular layer (with stained glomerules); gr, granule cell layer; hc, hippocampal commissure; ip, internal plexiform layer; IPn, interpeduncular nuclei; LA, lateral amygdaloid nucleus; LMol, lacunosum moleculare; M, mammillary nuclei; ml, medial lemniscus; mol, molecular layer of the dentate gyrus; mt, mitral cell layer; or, oriens layer; PAG, periaqueductal gray; pm, polymorph layer of the dentate gyrus; Py, pyramidal layer; RN, red nucleus; sLu, stratum lucidum; SN, substantia nigra; VTA, ventral tegmental area. Scale bars = 1 mm (A, A'), 0.5 mm (B, B', C, C').

TABLE 1

EphA5 Protein in the Developing Mouse¹

Region	E9	E11	E13	E15	E17
Telencephalon					
Olfactory Bulb			++	++	++
Retina		++	++	++	++
RGC					++
Neocortex	-	-	++	++	++
Frontal					+
Cingulate					++
Piriform					+++
Entorhinal					++
Insular					++
Parietal					++
Corpus Callosum					-
Hippocampus			+	++	++
CA1					+++
CA2					+++
CA3					++
Dentate Gyrus					+
Induseum Griseum					+++
MGE		-	-	-	
LGE		-	+	+	
Clastrum					+
Caudate/Putamen					+
Globus Pallidus					-
Lateral Septum					+
Medial Septum					-
Diencephalon					
Thalamus	-	-	-	-	+
Anterior					+
Medial					-

Region	E9	E11	E13	E15	E17
Lateral					+
Intralaminar					+
Ventral					-
Geniculate					+
Epithalamus		+	+	+	+
Hypothalamus		+	+	+	+
Mesencephalon					
Pretectum	+	++	++	++	+
Superior Colliculus	+	++	++	++	+
Inferior Colliculus	-	-	-	-	-
Ventral Midbrain		+	+	+	+
Metencephalon					
Pons	-	+	+	+	+
Cerebellum	-	+	+	+	+
Myelencephalon					
Cranial Nerve V	++	++	++	++	nd
Cranial Nerve VIII	++	++	++	++	nd
Cranial Nerve X	++	++	++	++	nd
Cranial Nerve XII	++	++	++	++	nd
Spinal Cord (proximal)	-	-	+	+	++
Spinal Cord (distal)	++	++	++	++	++

J Key: blank, structure not developed; -, no staining; +, ++, ++++, weak, moderate, robust staining; nd, not determined.

TABLE 2

EphA5 Protein in Postnatal and Adult Mice²

Region	P0	P6	Adult
Olfactory			
Olfactory Nerve Layer	–	+	+
Glomerular Layer	+	++	++
Glomerules	+	++	+++
External Plexiform Layer	–	–	++
Mitral Cell Layer	+	++	+++
Internal Plexiform Layer	+	+	+
Lateral Olfactory Tract	–	–	+
Granular Layer, Olfactory	+	+	++
Granular Layer, Accessory	+	+	++
Anterior Olfactory Nuc., external	–	+	+
Anterior Olfactory Nuc., dorsal	+	+	++
Anterior Olfactory Nuc., lateral	+	+	++
Anterior Olfactory Nuc., medial	–	+	++
Anterior Olfactory Nuc., ventral	–	+	+
Anterior Commissure	–	–	+
Tenia Tecta	+	++	+++
Piriform Cortex	+	+++	+++
Endopiriform Nucleus	+	++	+++
Cortex			
Cortical Layer I	–	+	++
Cortical Layer II–VI	+	++	++
Insular	+	++	++
Sensory	+	++	+++
Motor	+	++	++
Cingulate	+	+	++
Limbic	+	+	++
Clastrum	+	+	+++
Corpus Callosum	–	–	+
Barrel Cortex	–	++	+++
Cortical Plate	+		
Basal Ganglia			
Caudate/Putamen	–	+	+
Striatal Patches	–	+	+
Nucleus Accumbens	–	–	–
Bed Nucleus of the Stria Terminalis	+	++	++
Ventral Pallidum	+	++	++
Globus Pallidus	–	–	–
Fimbria	–	–	–

Region	P0	P6	Adult
Subventricular Zone	+	+	–
Amygdala			
Anterior Amygdaloid Area	+	+	++
Central Amygdaloid Nuc.	+	+	++
Basomedial Amygdaloid Nuc.	+	+	++
Basolateral Amygdaloid Nuc.	+	+	++
Cortical Amygdaloid Nuc.	–	+	+
Medial Amygdaloid Nuc.	+	+	++
Lateral Amygdaloid Nuc.	+	+	++
Medial Posterior Amygdaloid Nuc.	+	+	++
Posterior Cortical Amygdaloid Nuc.	–	+	+
Anterior Cortical Amygdaloid Nuc.	–	+	+
Amygdaloid Hippocampal Area	+	+	++
Amygdalostratial Transition Area	+	+	++
Septum			
Diagonal Band	+	++	++
Lateral Septum, dorsal	+	++	+
Lateral Septum, intermediate	+	+	+
Lateral Septum, ventral	–	+	+
Medial Septum	–	+	+
Septofimbrial Nuc.	–	+	+
Septohippocampal Nuc.	–	+	+
Triangular Septal Nuc.	–	–	–
Hippocampus			
CA1	+++	+++	+++
CA2	+++	+++	+++
CA3	+++	+++	+++
Lacunosum Molecular Layer	–	++	++
Oriens Layer	+	++	+++
Stratum Lucidem	–	++	+
Dentate Gyrus Molecular Layer	–	++	++
Fasciola Cinereum	+	+++	+++
Dentate Granular Layer	–	++	+++
Dentate Polymorph Cell Layer	+	++	+++
Pyramidal Cell Layer	++	+++	+++
Induseum Griseum	+++	+++	+++
Epithalamus			
Lateral Habenular Nuc.	–	+	+
Medial Habenular Nuc.	–	–	–
Thalamus			
Anterior Thalamic Nuclei	+		
Anteroventral Thalamic Nucleus		–	+

Region	P0	P6	Adult
Anterodorsal Thalamic Nucleus		+	+
Anteromedial Nuclei		–	–
Interanterodorsal		+	+
Reticular Thalamic Nuclei	–	–	+
Lateral Thalamic Nuclei	+		
Lateral Posterior Thalamic Nuc.		+	+
Lateral Dorsal Thalamic Nuc.		+	+
Ventral Thalamic Nuclei	–		
Ventral Postlateral Nuc.		–	–
Ventral Postmedial Nuc.		–	–
Intralaminar Thalamic Nuclei	+		
Central Medial Thalamic Nuc.		+	+
Paracentral Thalamic Nuc.		+	+
Parataenial Thalamic Nucleus		+	+
Post. Intralam. Thalamic Nuc.		+	+
Paraventricular Thalamic Nuc.		+	+
Medial Thalamic Nuclei	–		
Intermediodorsal Nuc.		+	+
Mediodorsal Nuc.		–	–
Reuniens Nuclei	+	++	++
Rhomboid Nuclei	–	+	+
Medial Geniculate	–	–	–
Lateral Geniculate, dorsal	–	+	+
Lateral Geniculate, ventral	–	+	+
Ethmoid	–	+	+
Submedius	+	+	+
Zona Incerta	+	+	+
Posterior Limitans	+	+	+
Hypothalamus			
Supraoptic Nuc.	–	–	–
Suprachiasmatic Nuc.	–	–	–
Paraventricular Nuc.	–	–	+
Anterior Nuc.	–	–	+
Ventromedial Nuc.	–	–	+
Dorsomedial Nuc.	–	–	+
Arcuate Nuc.	–	–	–
Lateral Hypothalamic Area	+	+	++
Posterior Hypothalamic Area	–	–	+
Supramammillary	–	–	++
Medial Mammillary, Medial	–	–	–
Medial Mammillary, Lateral	–	+	++
Lateral Mammillary	–	–	–

Region	P0	P6	Adult
Preoptic Area			
Medial Preoptic Nuc.	–	–	+
Ventromedial Preoptic Nuc.	–	–	+
Median Preoptic Nuc.	–	–	+
Lateral Preoptic Nuc.	–	–	+
Ventrolateral Preoptic Nuc.	–	–	+
Anterodorsal Preoptic Nuc.	–	–	+
Midbrain			
Anterior Pretectal Nuc.	–	–	+
Periaqueductal Gray	–	+	++
Olivary Pretectal Nuc.	–	+	++
Posterior Pretectal Nuc.	–	+	++
Red Nucleus	–	–	–
Ventral Tegmentum Nuc.	+	++	++
Substantia Nigra	+	++	++
Superior Colliculus, superficial gray	–	–	+
Superior Colliculus, intermed gray	–	+	+
Superior Colliculus, intermed white	–	+	+
Superior Colliculus, deep gray	–	+	+
Dorsal Raphe	–	+	+
Interpeduncular Nuc.	–	+	+
Edinger-Westphal Nuc.	–	+	+
Paralemniscal Nuc.	–	–	–
Lateral Lemniscus	–	–	–
Pontine Nuclei	–	–	–
Pontine Reticulotegmental Nuc.	–	+	+
Cerebellum			
External Granular Cell Layer	–	–	–
Molecular Layer	–	–	–
Medullary Layer	–	–	–
Purkinje Cell Layer	+	+	+
N. Lateral Deep, dentate	–	–	–
N. Medial Deep, fastigial	–	–	–

²Key: blank, structure absent; –, no staining; +, ++, +++, weak, moderate, robust staining; nd, not determined.