Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system


ABSTRACT

The hippocampus and septum play central roles in one of the most important spheres of brain function: learning and memory. Although their topographic connections have been known for two decades and topography may be critical for cognitive functions, the basis for hippocamposeptal topographic projection is unknown. We now report for the first time that Elf-1, a membrane-bound Eph family ligand, is a candidate molecular tag for the genesis of the hippocamposeptal topographic projection. Elf-1 is expressed in an increasing gradient from dorsal to ventral septum. Furthermore, Elf-1 selectively allows growth of neurites from topographically appropriate lateral hippocampal neurons, while inhibiting neurite outgrowth by medial hippocampal neurons. Complementary to the expression of Elf-1, an Eph family receptor, Bsk, is expressed in the hippocampus in a lateral to medial gradient, consistent with a function as a receptor for Elf-1. Further, Elf-1 specifically bound Bsk, eliciting tyrosine kinase activity. We conclude that the Elf-1/Bsk ligand-receptor pair exhibits traits of a chemoaffinity system for the organization of hippocamposeptal topographic projections.

Topographic projection is a general feature of brain architecture, and appears to be critical for appropriate coding and processing of information (1). Nevertheless, little is known about the mechanisms that govern topographic organization. Among the many regions exhibiting topographic relations, the hippocampus and septum have been the focus of intense interest, since these structures play central roles in learning and memory (2-5). The hippocampus projects to the lateral septum and receives afferents from the medial septum (6-8). Moreover, hippocampal projections to the lateral septum are arranged in a precise order. Axons from the medial hippocampus project to the dorsal lateral septum, whereas axons from the lateral hippocampus project to the ventral lateral septum (6-8). Molecular mechanisms underlying these topographic projections are unknown.

The development of topographic projections is thought to require both long-range signals to guide axons to the general target area and local cues to specify individual targets precisely for each axon terminal (9-11). Long-range signals are likely to be diffusible, such as netrins, which attract selected, distant growth cones, while repelling others (12-15). In contrast, local guidance cues must match axon terminals and specific cellular targets, a requirement accommodated by matching fixed tags on afferents and corresponding targets. Complementarity of molecular tags on afferents and targets was first postulated by Sperry in his chemoaffinity hypothesis more than 50 years ago (16, 17). Only recently have specific candidate molecules been identified (18-22). The Eph family ligand and receptor, Elf-1 and Mek4, are expressed as complementary gradients in the tectum and retina, respectively (18). The repulsive axon guidance signal, a molecule closely related to Elf-1, repels the growth of retinal axons and is also expressed in an anterior-to-posterior gradient in the tectum. Repulsive axon guidance signal and Elf-1 are ligands for receptors in the Eph family (18-22), the largest group of receptor tyrosine kinases (23-32). The complementary pattern of receptor-ligand expression by presynaptic neurons fulfills a longstanding prediction of the chemoaffinity hypothesis. We now present evidence that Elf-1 and another of its receptors function as recognition tags and local guidance cues for the development of the topographic hippocamposeptal projections.

MATERIALS AND METHODS

Construction of a Ligand-Affinity Probe, Bsk-AP. The affinity probe Bsk-AP was created as follows. An Mro1 restriction site was first introduced at the junction of the extracellular and transmembrane domain by site-directed mutagenesis using an oligonucleotide 5'-GATCAAGCCGAATTCGGGACATCATCG-3' corresponding to nucleotide positions 636 to 669 (32) with a GGA addition to create a Mro1 site. An EcoRI to Mro1 DNA fragment that contained the entire coding region for the extracellular domain from the initiation codon to the last codon before the transmembrane domain of Bsk was ligated to a secreted form of human placental alkaline phosphatase in pAP-tag1 vector (33). The vector containing the fusion construct was cotransfected into NIH 3T3 cells with pSV2neo plasmid containing the aminoglycoside phosphotransferase gene, which confers G418 resistance. The transfected cells were then selected with 400 μg/ml G418 ( Gibco/BRL); neo-resistant colonies were first screened for heat-resistant alkaline phosphatase activity in the culture supernatant as described (33) and then for metabolically labeled Bsk-AP protein using immunoprecipitation with a monoclonal antibody against the human placental alkaline phosphatase (Medix Biotech). A protein with the expected size of 110 kDa was detected in both the cell lysates and the tissue culture media in all the clones secreting the phosphatase activity, but not in cells transfected with the vector alone. The highest level of alkaline phosphatase activity detected in the culture media reached 800 OD405 units/ml per hr.

For ligand detection, cultured neurons were tested for Bsk-AP binding as described (33). Frozen sections of mouse brain were also stained using a similar procedure to detect Bsk ligands.

Neuron Culture. Neurons from different regions of the brain of embryonic day 18 rats were dissected in phosphate-buffered saline (PBS), dissociated, and cultured in a 1:1 (vol/vol) mixture of Ham’s F-12 and Eagle’s minimum essential medium supplemented with insulin (25 μg/ml), transferrin (100 μg/ml) putrescine (60 μM), progesterone (20 nM), selenium (30 nM), glucose (6 mg/ml), penicillin (0.5 unit/ml), and streptomycin (0.5 μg/ml). Cells were plated in a poly(D-lysine) substrate.

Abbreviation: PI-PLC, phosphatidylinositol-specific phospholipase C.

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In Situ Hybridization. Elf-1 mRNA was detected with two different anti-sense oligonucleotide probes. Probe Elf-1-04 (5'-TTGAAAGCCTCGGTGCGG-TGGTACAGGAG-GCTGGCCCTCCATT-3') is a 48-mer oligonucleotide in Elf-1 coding region (nucleotide position 280–327) (34). Probe Elf-1-06 (5'-ACCTCATCCCTGTGGCTTGTGTCCTC-TCCAGTGTCACCAGCAATGT-3') is a 48-mer oligonucleotide in the 3' noncoding region of mouse Elf-1 gene (nucleotide position 926–971) (34). These probes had no significant homology to any known sequence.

Bsk transcripts were detected using a 373-bp anti-sense RNA probe that include part of the extracellular domain, the transmembrane domain, and a small region immediately after the transmembrane domain (nucleotide position 1445 to 1818) (32). These regions had little homology to other eph family receptors. In situ hybridization was performed basically as described (32) with the modification that hybridization and posthybridization washes were carried out at 42°C and 65°C, respectively, for oligonucleotide probes.

Expression of Elf-1 Ligand. Mouse Elf-1 was cloned using polymerase chain reaction. The upstream primer is 5'-GGATCCGCCGCGCCGGCCATGGGCCGCGGACG-3' corresponding to nucleotide position 6 to 25 (34) with a BamHI and a NotI restriction site added to the 5' end of the primer. The downstream primer is 5'-GGGAGCTCTGAGTGGTGTCTTCGCGCCTGAC-3' (nucleotide position 639 to 660) with XhoI and SacI sites. The PCR fragment was 680-bp long and included the entire coding region of Elf-1. The fragment was then cloned into a retroviral vector pLIG*, which contains a β-galactosidase gene fused to a aminoglycoside phosphotransferase for G418 resistance (35). The construct was then transfected into NIH 3T3 cells. G418-resistant colonies were then screened for Elf-1 expression using Bsk-AP binding as described (33). Bsk-AP binds to Elf-1-expressing NIH 3T3 cells (Elf-1-3T3) strongly. In contrast, no significant staining was observed in parental or vector-transfected NIH 3T3 cells.

Neurite Outgrowth Assay. For assaying growth inhibition of axons, neurons (4 x 10^5 cells per well) from different regions of the brain were dissected and plated in 12-well dishes preseated with a confluent monolayer of Elf-1-expressing or control NIH 3T3 cells transfected with the vector in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), penicillin (0.5 unit/ml), and streptomycin (0.5 μg/ml). Cells were grown for 48 hr. Neurons form aggregates under these conditions. After treatment, neurons were fixed in 4% paraformaldehyde in PBS and stained with anti-neuron-specific enolase with Vectastain ABC kit (Vector Laboratories). The number of neurites longer than five cell body-lengths (~100 μm) on the aggregates were scored. Random fields were selected and all the aggregates in the fields were scored. At least 300 aggregates were surveyed for each sample assayed and the experiments were repeated at least three times.

Aggregates of medial hippocampal neurons exposed to Elf-1 lacked extensive neurites. These neurites that stained positively for neuron-specific enolase are Trypan blue exclusive, indicating that they are living cells. In addition, a small percentage of hippocampal neurons plated on the 3T3-Elf-1 cells grew neurites at comparable length as on the control cells. Cerebellar neurons grew on 3T3-Elf-1 as well as on control cells. Thus, the lack of neurites is most likely due to the specific function of Elf-1, and not to a nonspecific toxicity to neurons.

RESULTS

Detection of Bsk Ligand in the Septum. We previously isolated an eph family receptor named Bsk from a mouse brain cDNA library, and documented high expression in the hippocampus (32). To examine whether the Bsk receptor and its ligand(s) potentially regulate hippocampal topographic projection, we studied expression of Bsk ligands in target fields.

For ligand detection, an affinity probe, Bsk-AP, was constructed by tagging the Bsk ligand-binding domain with a human placental alkaline phosphatase. To test for the presence of ligand, we cultured cells from the septum, cortex, and hippocampus itself, which are regions that receive hippocampal projections, and the olfactory bulb and cerebellum, which do not receive projections. The cultured neurons were assayed for Bsk-AP binding. Significant numbers (37.8%) of the cultured septal neurons exhibited Bsk-AP binding, indicating expression of a Bsk ligand (Table 1 and Fig. 1). In contrast, a lower proportion of neurons was labeled in the olfactory bulb cultures, and no binding was detected in cortical, hippocampal, or cerebellar cultures (Table 1). To localize ligand in the brain in situ, embryonic and adult brain mouse sections were stained with Bsk-AP. The septum of E18 and adult brain stained positively, consistent with the observations in culture, and staining was restricted to the lateral septum, the hippocampal

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Positive neurons (%)*</th>
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<tbody>
<tr>
<td>Olfactory bulb</td>
<td>13.6 (7.3–20)*</td>
</tr>
<tr>
<td>Cortex</td>
<td>0</td>
</tr>
<tr>
<td>Septum</td>
<td>37.8 (27.4–48.6)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>&lt;1 (0.0–9.0)</td>
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*Average of three experiments. Total of 600 neurons in about 15 to 20 random fields were scored for each region in each experiment. The lowest and the highest percentage of positive neurons in the three experiments performed. The percentage of neurons positive for Bsk-AP staining varied in different dissections.

Fig. 1. Expression of a ligand of Bsk in septal neurons. Neurons from different regions of embryonic rat brain (gestation day 18) were dissected, dissociated, and cultured on poly(L-lysine)-coated dishes under serum-free conditions. After 2 days in culture, the neurons were stained with a ligand affinity probe, Bsk-AP, which consists of the extracellular domain of Bsk and human placental alkaline phosphatase fused in frame. Septal (A), cortical (B), and hippocampal (C) neurons stained with Bsk-AP. (Bar = 100 μm.)
target area, with highest staining ventrally (data not shown; see below for in situ hybridization). In addition to the septum and olfactory bulb, the ligands were also detected in the hypothalamus and in the nasal epithelium (data not shown), indicating potential roles in multiple systems.

**Elf-1 mRNA Gradient in the Lateral Septum.** Since the ligand, Elf-1, is known to bind a chicken analogue of Bsk in vitro, and is known to be expressed in areas stained by Bsk-AP in our study (18, 34), it appeared likely that Elf-1 may function as a Bsk ligand in vivo. We performed in situ hybridization analysis on mouse brain to determine whether Elf-1 was responsible for the Bsk-AP binding in the lateral septum. Since hippocampo-septal topographic projection develops postnatally, probably after the first week of birth (36), we examined Elf-1 expression on postnatal day 14. High levels of Elf-1 transcripts were detected in the lateral septum (Fig. 2). Moreover, the expression exhibited a dorsoventral gradient with highest levels in the ventral portion of the lateral septum (Fig. 2).

**Selective Inhibition of Hippocampal Neurite Outgrowth by Elf-1.** Since the dorsal and ventral lateral septums are innervated by the medial and lateral hippocampal neurons, respectively, the differential distribution in the lateral septum was consistent with Elf-1 function as an identification tag. This suggests that Elf-1 interacts with Bsk, or another eph family receptor in the hippocampal neurons, to specify topographic projection to the septum. One critical prediction of this hypothesis is that medial and lateral hippocampal neurons respond differentially to Elf-1. To examine this prediction, dissociated medial and lateral hippocampal neurons from E18 rat brain were plated on a confluent monolayer of NIH 3T3 cells expressing Elf-1 (3T3-Elf-1) or transfected with vector alone. Neurite outgrowth from medial hippocampal neurons was inhibited by 3T3-Elf-1, but not by control cells, after 2 days in culture (Fig. 3). In contrast, Elf-1 did not significantly inhibit neurite outgrowth from lateral hippocampal neurons, consistent with the Elf-1 chemoaffinity hypothesis (Fig. 3C). Moreover, Elf-1 had no effect on outgrowth of cerebellar neurites and only limited inhibition on cortical neuritogenesis (Fig. 3C).

Since Elf-1 can be removed from the cell surface with phosphatidylinositol-specific phospholipase C (PI-PLC) (34), we characterized the effect of PI-PLC treatment on Elf-1 inhibition of medial hippocampal neurite outgrowth. Removal of Elf-1 from 3T3 cell surfaces restored neurite outgrowth from overlying medial hippocampal neurons (Fig. 3D). Taken together, our observations suggest that Elf-1 can specifically and selectively inhibit medial hippocampal neurons, while allowing lateral axons to project to the ventrolateral septum, thereby contributing to the generation of the topographic map.

![Fig. 2. Elf-1 gradient in the lateral septum. (A and B) Bright- and dark-field photomicrographs, respectively, of a coronal section of a P14 mouse brain through the septal region hybridized with an Elf-1 anti-sense oligonucleotide. (C and D) Bright- and dark-field photomicrographs, respectively, of a higher magnification of the lateral septal region positive for Elf-1 expression shown in A and B. Hybridized brain sections were counterstained with thionin to identify the histological patterns. The silver grains cannot be seen clearly at these magnifications. (D) The image was enhanced with an image analysis program (IMAGE PRO, Nikon) for quantitation in E. The boxed areas in D indicate regions quantitated in E for levels of Elf-1 expression in dorsal, medial, and ventral lateral septum. (E) Gradient of Elf-1 expression in the lateral septum. The boxed areas in D were quantitated for percent area of cells covered by silver grains using the IMAGE PRO analysis program. Cell-free areas were not included in the quantitation to avoid bias due to cell density differences. Results shown were averages of measurements of all the cells in the boxed areas (± SEM). The difference between Elf-1 levels in the dorsal and ventral lateral septum is significant (P < 0.05). LS, lateral septum; MS, medial septum. (A and B, bar = 1 mm; C and D, bar = 100 μm.)](image-url)
Bsk mRNA Gradient in the Hippocampus. Since Elf-1 is known to bind and activate a chicken homologue of Bsk (37), we examined the possibility that Bsk mediates the effect of Elf-1 in our mammalian system. We specifically examined the prediction, based on Elf-1 selective inhibition of medial hippocampal neuritogenesis, that the receptor is expressed at high levels medially and at low levels laterally in the hippocampus. In fact, Bsk transcripts were expressed abundantly in medial hippocampus, but were undetectable laterally by in situ hybridization (Fig. 4 A–D). Expression exhibited a striking gradient: coronal and sagittal serial sections revealed maximal Bsk mRNA levels medially and a progressive decrease to the lateral pole (Fig. 4 E and F). In contrast, mRNA for a control limbic marker protein, LAMP (38), was equally distributed in medial and lateral hippocampus (data not shown). Thus, Bsk and Elf-1 were expressed in complementary gradients in afferent and target fields; medial hippocampal neurons, which express high Bsk levels, project to dorsal lateral septum, which exhibit low levels of Elf-1. Conversely, lateral hippocampal neurons, expressing low levels of Bsk mRNA project to ventral lateral septum, which expresses high levels of Elf-1 transcripts.

Activation of Bsk Tyrosine Kinase Activity by Elf-1. To further investigate the potential interaction between Elf-1 and Bsk, COS-7 cells transfected with Elf-1 cDNA under a cytomegalovirus promoter, as well as 3T3-Elf-1 cells, were tested for Bsk-AP binding directly. Bsk-AP stained Elf-1-transfected cells intensely, while control cells exhibited no binding (data not shown), indicating direct interaction between ligand and receptor. Moreover, the interaction was biologically functional, since coculture of 3T3-Elf-1 and 3T3-Bsk (NIH 3T3 cells expressing Bsk) elicited Bsk tyrosine kinase activity (Fig. 4G). In contrast, cells transfected with Lerk2, an Elf-1-related ligand of the eph family, showed no binding to Bsk-AP, indicating that the interaction between Bsk and Elf-1 was specific (data not shown).

DISCUSSION

Our observations suggest that Elf-1 and its receptor serve as chemoaffinity labels for topographic projection in the hippocampal system. The differential inhibition of hippocampal neuritic growth by Elf-1 suggests that the fixed ligand may exclude medial hippocampal axons while permitting lateral axons to innervate the ventrolateral septum (Fig. 5). These differential effects of Elf-1 on hippocampal neurons correlates with the gradient of hippocampal Bsk expression, suggesting that Bsk may, indeed, mediate the Elf-1 effect. The demonstration that Elf-1 and Bsk directly interact in vitro, eliciting tyrosine kinase activity, is entirely consistent with biologically significant interaction in vivo. Finally, the complementary gradients of ligand and receptor exist in the septum and hippocampus from E18 through adulthood (data not shown).
shown), suggesting ongoing involvement in synaptic plasticity during maturity, as well as in the generation of topography during development.

However, our observations do not exclude the possibility that other receptors of Elf-1, such as Mek4, sek, and members of the transmembrane eph ligand subfamily, are also involved in the genesis of the hippocamposeptal map. Other ligands and/or receptors may indeed play a role because Elf-1/Bsk interaction alone may not explain why lateral hippocampal neurons do not project to the dorsal lateral septum. The combinatorial expression of multiple ligands and receptors may play critical roles in the genesis of topographic projections in the brain.

Our findings in the hippocamposeptal system are consistent with the recent report of complementary gradients for Elf-1 and Mek4, another member of the eph receptor family, in the retinotectal system, in which topographic projection is prominent (18). This extended family of ligands and receptors, consequently, may play important roles in the generation of topographic projection in multiple brain systems. Our observations provide support for Sperry's chemoaffinity theory (16, 17), but emphasize the importance of chemorepulsion of neurites in the genesis of topographic projections. In the retinotectal system, as well, chemorepulsion appears to constitute a predominant mechanism (19, 20, 39-41). Future studies, presumably, will determine the relative contributions of repulsion and attraction in the generation of topographic projections.

The present paper is the first study of the molecular signals that generate the topography of hippocampal projections to the septum. Understanding how the output pathways of the hippocampus are organized may shed light on mechanisms of learning and memory, as well as diseases affecting these processes, including Alzheimer disease and schizophrenia.

We thank B. Cullen for providing a human placenta alkaline phosphatase plasmid, L. Lillian for providing pLIG* vector, and P. Levitt for stimulating discussions of the project. This work was partially
FIG. 5. Schematic representation of the complementary expression of Bsk and Elf-1 and the topographic projection in the hippocampal-Septal system. Neurons in the hippocampus (H) project to the lateral septal target (LS) in a gradient, with neurons in the medial (M) hippocampus projecting to the dorsal lateral septum (D) and neurons in the lateral hippocampus (L) projecting to the ventral lateral septum (V). Complementary gradients of Bsk (red) and Elf-1 (blue) were observed in the hippocampus and the septum, respectively. Elf-1 inhibited the growth of medial but not lateral hippocampal neurons, consistent with a function of turning away medial axons from the ventral lateral septum. Other members of the Eph family receptors and ligands such as Mek4 and AL-1/RAGs may also be involved in the development of the topographic projection.

Supported by National Science Foundation Grant NSF IBN-9409930, by National Institutes of Health Grant HD23315, by Johnson & Johnson Discovery Fund, and by a grant from Triphor Pharmaceuticals, Inc.