Expression patterns of SLIT/ROBO mRNAs reveal a characteristic feature in the entorhinal-hippocampal area of macaque monkeys

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Abstract

Objective: SLITs are secreted glycoproteins that bind to Roundabouts (ROBOs) which are a family member of transmembrane receptors. SLIT signaling has well-conserved roles in mediating axon repulsion in a developing nervous system. We previously reported that SLIT1 mRNA is enriched in middle layers of the prefrontal cortex of macaque monkeys in a developmentally regulated manner. Other SLIT (SLIT2 and SLIT3) mRNAs showed preferential expressions in the prefrontal cortex with a distinct laminar pattern. To obtain further clues to the role of SLIT signaling in the organization of the primate brain, we performed ISH analysis of SLIT and ROBO mRNAs using adult macaque brain tissues.

Results: In this study, we examined the expression patterns of SLITs and ROBOs (ROBO1 and ROBO2) in other brain regions, and found intense and characteristic expression patterns of these genes in the entorhinal-hippocampal area. In situ hybridization analysis revealed that SLIT1 and SLIT2 mRNAs showed marked complementary distribution in the entorhinal cortex. SLIT and ROBO mRNAs were widely expressed in the hippocampus with modest regional preference. These findings suggest that each SLIT gene has a specialized role that is particularly important for prefrontal as well as hippocampal connectivity in the primate cortex.

Keywords: Axon guidance, Cerebral cortex, Entorhinal cortex, Hippocampus, In situ hybridization, Primates

Introduction

SLIT is a repellent guidance molecule, which is well conserved in various species [1, 2]. The repellent effect of SLIT is mediated by the receptor, Roundabout (ROBO) [3]. To date, three Slit genes, Slit1-3 [4] and four Robo genes (Robo1-4) have been identified in vertebrate genome [3, 5–7]. In mammals, SLIT/ROBO signaling is reported to have essential roles in the development of the nervous system including midline crossing, as observed in Drosophila [8, 9] and formation of major axonal tracts [10–13]. Furthermore, recent studies demonstrate expanded functional repertoires of SLITs and ROBOs, such as neurogenesis, cell proliferation/migration, angiogenesis, oncogenesis, and involvement in several diseases [2, 14–17].

We have investigated the molecular basis of differences in the architecture across neocortical areas and identified three genes, SLIT1 [18], RBP4 [19], and PNMAS [20] that are highly expressed in the higher-order association areas of macaque monkeys [21]. Among them, SLIT1 mRNA in particular is preferentially expressed in the prefrontal...
cortex compared with other association areas. Our detailed in situ hybridization (ISH) analysis demonstrates that SLIT1 mRNA is mainly distributed in the middle layers of most cortical areas, highest in the prefrontal cortex but lowest in the primary sensory areas. The prefrontal-enriched pattern was established by reduced expressions, specific for areas and layers during postnatal development. The promoter region of SLIT1 gene is hypermethylated, and it is assumed that some regulatory elements (e.g., methyl-binding proteins) are involved in the area selective expression [22]. Other SLIT (SLIT2 and SLIT3) mRNAs showed modest preference in the prefrontal cortex, whereas ROBO1 and ROBO2 mRNAs were widely detected within the cerebral cortex. Since cortical neurons, particularly those in the prefrontal cortex, simultaneously express SLIT1 and ROBO mRNAs, SLIT1 could work in either an autocrine or paracrine manner in the postnatal primate cortex, which implies that it has other functions in addition to its role as guidance cues.

In this report, to obtain further clues to the role of SLIT signaling in the organization of the primate brain, we performed ISH analysis of SLIT and ROBO mRNAs using adult macaque brain tissues. We examined the expression patterns of these genes in detail in brain regions other than the prefrontal cortex. We found unique expression patterns of these genes in the entorhinal-hippocampal area.

**Main text**

**Materials and methods**

**Experimental animals**

For ISH experiments, brains from five macaques (Macaca fuscata, juvenile to young adults, 2.6, 4.0, 5.2, 5.6, and 5.8 years old) were used. The animals were anesthetized and the brain fixed as described previously [18]. Sections from the blocks that included the entorhinal cortex and hippocampus were sliced at 35 μm thickness (Additional file 1: Figure S1).

**In situ hybridization**

The cDNA fragments were obtained by RT–PCR using the primers listed in Table 1, and subcloned into the pBlueScriptII vector. The concentrations of all the ribo-probes used in this study were adjusted to 0.1 μg/μl and

| Table 1 ISH probes used in this study |
|--------------------------------------|
| **Gene name** | **Probe name** | **Species** | **Accession No.** | **PCR primer set** | **Length** |
| SLIT1 | Slit1-1 | macaque | NM_003061 | cttccaggacctgcagaacc ccggtctcgatctcgagca 552 |
| | Slit1-2 | macaque | NM_003061 | aagtttgaatgcgaagttgc actgqcccctggttgacat 448 |
| | Slit1-3 | macaque | NM_003061 | ccggctccctccagagtctga gtcacagtttccggatcaac 822 |
| | Slit1-4 | macaque | NM_003061 | cctggtcgactccaaacgg ccatctgcgcctcaaggt 647 |
| SLIT2 | Slit2-1 | macaque | NM_00478 | ccaaggaatatcccccgcgga gagaccatcagagaattacg 770 |
| | Slit2-4 | macaque | NM_00478 | gaccccctgtgataatttg cagccccctgtgataatttg 866 |
| SLIT3 | Slit3-3 | macaque | NM_003062 | ttgaccctgagcaacaacacgccatcattcag 838 |
| ROBO1 | Robo1-1 | macaque | NM-022188 | gacagggccttgagccacaa tcttgtaacagcaagcttgag 942 |
| | Robo1-3 | macaque | NM-022188 | tgtgattttctgahtaggac taccagtgccccagtgcct 877 |
| | Robo1-4 | macaque | NM-022188 | ctcgtatccctggctgtaaac gctccattcaggacagcc 868 |
| ROBO2 | Robo2-1 | macaque | NM_002942 | aggactactctttggtgaagc ggaaccaagcagcctgtg 700 |
| | Robo2-4 | macaque | NM_002942 | ccagggccaaagggtatagc gcctcagctttgataagc 673 |

We confirmed that the multiple probes for one gene exhibit the same distribution pattern. After the initial confirmation, these multiple probes were mixed to enhance the ISH signals.
The riboprobes were stored at –30 °C. Single-color ISH was carried out essentially as previously described [18, 23]. We used more than two types of probe for each of \( SLIT1 \), \( SLIT2 \), \( ROBO1 \), and \( ROBO2 \), and confirmed that each probe exhibits the same pattern of signal distribution (data not shown). After the initial confirmation, multiple probes were mixed together to enhance ISH signals. We also confirmed that the sense probes detected no more signals above the background level. The layer positions of entorhinal cortex and the boundaries with hippocampal fields were determined on the basis of Cresyl violet staining of adjacent sections. Cresyl violet staining shows clear differences in cell size and packing density among the layers in the entorhinal cortex and the four CA fields in hippocampus [16, 24, 25].

**Image analysis**

Eight-bit gray scale color images were obtained using the digital color camera DP 70 (Olympus, Tokyo, Japan) attached to a BX-51 microscope (Olympus). The background image was subtracted using Image-Pro Plus image analysis software (Media Cybernetics, Silver Springs, MD). The laminar distribution patterns of different ISH signals (Fig. 1j, n) were analyzed as previously described [18]. Signals were extracted from the background component by converting the eight-bit gray-scale images into the binary images. The threshold used here was set to the standard deviation (SD) beyond the average intensity of each cortical section. Then, we calculated average values with respect to each row to obtain the line profile in regions of interest (ROIs: 100 µm bin, which is the height of the cortex from layer I to WM) using Image Pro Plus.

**Results and discussion**

SLITs/ROBOs are reported to be involved in the formation of hippocampal connections during the rodent development [26]. We examined the \( SLIT \) and \( ROBO \) mRNA expression patterns in the hippocampus and its surrounding areas in the adult macaques (Additional file 1: Figure S1).

In and around the hippocampus, all the \( SLIT \) and \( ROBO \) mRNAs were widely expressed (Fig. 1a–f). Each gene showed moderate regional preference. For example, the most intense signals of \( SLIT1 \) and \( ROBO1 \) mRNAs were observed in the granule cell layer of the dentate gyrus (DG, Fig. 1a, d), followed by the expression in the pyramidal cell layer of cornus ammon (CA) 2-CA4. The expression levels of \( SLIT1 \) and \( ROBO1 \) mRNAs were rather low in CA1 and the subiculum. \( SLIT2 \) and \( ROBO2 \) mRNAs were also highly expressed in the DG and uniformly distributed in CA1-CA4 (Fig. 1b, e). \( SLIT3 \) mRNA
was mainly expressed in the DG and relatively weak in other regions (Fig. 1c).

The entorhinal cortex is the interface between the hippocampus and the neocortex [27, 28]. It contains grid cells with their unique firing discharge pattern, and was reported as the key area for spatial representation in mammals [29, 30]. Abnormalities in the cells of layer II of the human entorhinal cortex have been implicated in the pathophysiology of schizophrenia and Alzheimer’s disease [31, 32]. In the entorhinal cortex, Densitometric analysis indicated conspicuous laminar preference of SLIT and ROBO mRNAs. Most strikingly, SLIT1 and SLIT2 mRNAs showed complementary distribution (Fig. 1). Intense signal of SLIT1 mRNA was observed in layers II, V, and VI (Fig. 1g and j, blue arrowheads) with only a low signal intensity of SLIT1 mRNA in layer III. Large cells that constitute the characteristic cell islands in layer II [16] showed a particularly high signal intensity of SLIT1 mRNA. On the other hand, SLIT2 mRNA was predominantly expressed in layer III (Fig. 1j), with only a weak expression in other layers (Fig. 1h). This complementarity is reminiscent of the pattern in area TE except that SLIT1 and SLIT2 mRNAs are expressed in different layers [18]. Owing to the different laminar preferences of these genes, we observed a sharp border between the entorhinal cortex and the perirhinal cortex (data not shown). SLIT3 mRNA was expressed similarly across layers in the entorhinal cortex, although intense signals were observed in the layer II and the upper part of layer V. ROBO1 and ROBO2 were also expressed widely across layers, which is similarly observed in other cortical areas. Interestingly, ROBO1 and ROBO2 mRNAs were abundant in the upper and lower parts of layer VI, as indicated by blue and red arrowheads in Fig. 1n, respectively, but the pattern not in neocortical areas. The differences in the laminar distribution among the SLITs in both the entorhinal cortex and neocortex might reflect different functions of these genes in the cerebral cortex of primates.

Although the roles of SLITs/ROBOs in the guidance of neurons during development may be expected, their functions in postnatal brains, where no major guidance events occur, remain unclear. A possible role of these molecules is in the control of regeneration in response to injury. All Slits are reported to be expressed in reactive astrocytes at the injury site portion of the rat brain [33]. Since ROBO mRNAs are expressed in substantial cell populations in the cerebral cortex, SLITs secreted around the injury site may inhibit irrelevant axonal regeneration. Another plausible possibility is that the SLIT-ROBO system has a role in maintaining certain neuronal morphologies and circuits [34–37]. Lines of evidence suggest that axon guidance molecules are implicated as critical regulators in synaptogenesis and synaptic plasticity [37–39]. Numerous studies have shown the dynamics of neuronal processes in the postnatal cortex [40–42]. In this regard, we note that SLIT mRNAs were abundant in the brain regions where high neuronal integration and/or plasticity plays roles, such as the entorhinal cortex, dentate gyrus, and prefrontal cortex, where the other higher-order association area enriched genes, RBP4 and PNMA5 are also highly expressed [19, 20]. These genes may subserve neural plasticity and cognitive function. Further research is needed to examine these possibilities.

Limitations

In this study, we found the characteristic expression pattern of the axon guidance molecule SLITs and its receptor ROBOs in the hippocampus and entorhinal cortex of adult macaque monkeys. We did not perform other methods such as qPCR or immunohistochemistry. Gene manipulation analysis including overexpression and suppression of gene expression will be needed to test our hypothesis that these groups of molecules are involved in structural plasticity of postnatal primate brains.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13104-020-05100-7.

Additional file 1: Figure S1. A coronal section of macaque brain containing hippocampus and entorhinal cortex for cresyl violet staining. Scale bar = 5 mm. EC, entorhinal cortex; HC, hippocampus; LGN, lateral geniculate nucleus; TEd, dorsal inferotemporal cortex; TEv, ventral inferotemporal cortex; sst, superior temporal cortex; PC, perirhinal cortex; D, dorsal; V, ventral; L, lateral; M, medial.

Abbreviations

CA: Cornu ammon; DG: Dentate gyrus; ISH: In situ hybridization; ProS: Prosubiculum; PrS: Presubiculum; ROBO: Roundabouts; Sub: Subiculum.

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Authors’ contribution

TS and YK performed the experiments. TS and TY designed the study, and wrote the draft of the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets, which were used and/or analyzed in the current study, are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

All the experiments described here were performed in compliance with the guidelines for animal experiments of the National Institutes of Natural Sciences, Japan and the National Institutes of Health, USA. All efforts were made to minimize animal suffering and the number of animals used.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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