Published in final edited form as:
Science. 2011 August 5; 333(6043): 769–773. doi:10.1126/science.1204144.

Cartilage Acidic Protein–1B (LOTUS), an Endogenous Nogo Receptor Antagonist for Axon Tract Formation

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Abstract

Neural circuitry formation depends on the molecular control of axonal projection during development. By screening with fluorophore-assisted light inactivation in the developing mouse brain, we identified cartilage acidic protein–1B as a key molecule for lateral olfactory tract (LOT) formation and named it LOT usher substance (LOTUS). We further identified Nogo receptor–1 (NgR1) as a LOTUS-binding protein. NgR1 is a receptor of myelin-derived axon growth inhibitors, such as Nogo, which prevent neural regeneration in the adult. LOTUS suppressed Nogo-NgR1 binding and Nogo-induced growth cone collapse. A defasciculated LOT was present in *lotus*-deficient mice but not in mice lacking both *lotus*- and *ngr1*. These findings suggest that endogenous antagonism of NgR1 by LOTUS is crucial for normal LOT formation.

Spatially represented information in specific regions is a common feature of the nervous system and is essential for higher brain function. Specificity of neural connections that ensures the precise relay of information is required for establishing such spatial representation of information. In these processes, various types of repulsive and attractive guidance molecules and their receptors have crucial roles (1).

The olfactory bulb (OB) is the first relay for olfactory information. The axons of early-generated mitral cells emerge from the OB at embryonic day (E) 12.5 in the mouse; thereafter, they grow laterally and then elongate caudally at the surface of the telencephalon (2, 3). This projection of mitral cells, termed the lateral olfactory tract (LOT), is

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Supporting Online Material www.sciencemag.org/cgi/content/full/333/6043/769/DC1 Materials and Methods Figs. S1 to S15 References (*23*–*33*)

stereotypically organized in the higher olfactory centers (4, 5). The LOT is a good experimental system for analysis of the molecular mechanisms underlying neural circuit formation, because LOT formation can be visualized by anterograde tracing with the lipophilic dialkylcarbocyanine (DiI) or by immunohistochemistry of the LOT marker protein neuropilin-1 (Nrp1) in whole-mount samples of brain and its developmental process can be observed in an organotypic culture system. Therefore, we used these techniques to screen for molecules that function in LOT formation.

We established a molecular targeting method by modifying the fluorophore-assisted light inactivation (FALI) technique (6, 7) (fig. S1). FALI, generally known as chromophore-assisted light inactivation, is an acute protein-ablation technique directed by binding of fluorescein iso-thiocyanate (FITC)–labeled antibodies. Light irradiation at a wavelength of 490 nm induces local generation of oxygen radicals (singlet oxygen) from the fluorophore, which react chemically with the nearby target antigen and inactivate it (fig. S1A). We modified the technique to perform continuous protein inactivation during periods of 24 hours and longer (fig. S1B). The protein inactivation can be achieved with nonneutralizing antibodies. To identify the molecules that function for LOT formation, we generated monoclonal antibodies (mAbs) produced with a homogenate of protein extract from the developing LOT and the surrounding tissues as an immuno-antigen in hamsters (8). We immunohistochemically selected antibodies that recognized membrane surface molecules on the developing LOT. An organotypic culture technique was used for in vitro FALI experiments in the developing telencephalon (9–12). FALI with FITC-labeled H24G11-mAb resulted in defasciculation (separation of axons from a bundle of axons) of the LOT and a significantly wider LOT, whereas control FALI without mAb, with unlabeled H24G11-mAb, or with FITC-labeled nonspecific hamster immunoglobulin (IgG) produced no effects (Fig. 1A and fig S2). Because the antigen recognized by H24G11-mAb was expressed in the axon tract of OB neurons, we used a mouse cDNA expression library of OB to identify the target antigen (8). By screening about 57,000 clones, we identified cartilage acidic protein–1B (Crtac1B) as the H24G11 antigen (fig. S3A). Crtac1 is a marker protein used to discriminate human chondrocytes from osteoblasts and mesenchymal stem cells in culture (13, 14). Human Crtac1B is an alternative splice variant of Crtac1 and is specifically expressed in brain tissue (14). Crtac1A, another splice variant, is a major Crtac1 protein produced by human chondrocytes and localizes in the extracellular matrix of articular cartilage (14). No Crtac1A-specific 3′–rapid amplification of cDNA ends (RACE) products are found in several mouse tissues, suggesting that only Crtac1B is expressed in mouse (14). No previous report has described the biological function of Crtac1. We designated mouse brain–specific Crtac1B as lateral olfactory tract usher substance (LOTUS). Mouse LOTUS contains four FG (phenylalanyl-glycyl)–GAP (glycyl-alanyl-prolyl) consensus sequence (FG-GAP) repeats, which are found in the N-terminal region of α-integrin (15), and an epidermal growth factor (EGF)–like calcium-binding domain (EGF-CA) in the C-terminal region (fig. S3A). LOTUS also contains hydrophobic amino acid sequences corresponding to a primary transmembrane helix of 627 to 646 residues, as estimated by Sosui software (16) (fig. S3A).

In situ hybridization revealed that LOTUS mRNA was expressed in several specific brain areas, including the OB, hippocampus, cerebral cortex, thalamus, and spinal cord in E15.5 mouse embryos (Fig. 1B). In particular, LOTUS mRNA was strongly expressed in the mitral cell layer of the ventromedial OB (Fig. 1B). LOTUS was detected at around 85 kD by immunoblot analysis in E14 mouse OB (fig. S3B). In cultured OB neurons, LOTUS was immunocytochemically detected in growth cones and neurite shafts by the addition of mAb to LOTUS (H24G11-mAb) to the culture medium without fixation (Fig. 1C), suggesting that LOTUS is a membrane protein.

To examine the in vivo function of LOTUS, we next generated *lotus*-deficient mice ($LOTUS^{-/-}$) by a conventional gene-targeting method (fig. S4). We analyzed LOT development as visualized by DiI, which was injected into the OB, at E18 when LOT formation was developmentally established. In $LOTUS^{-/-}$ brains, OB axons projected caudally in a defasciculated manner, resulting in significantly wider expansion of DiI staining in the LOT, whereas they projected in a fasciculated form in wild type (Fig. 1D and fig. S5). This phenotype was confirmed by observation of each DiI-positive axon fiber at a distal portion inside the LOT at higher magnification (Fig. 1D). Thus, LOTUS appears to function in axonal bundling of the OB neurons and LOT development. In vitro axonal bundling was decreased in cultured OB axons of $LOTUS^{-/-}$ compared with that of wild-type mice (fig. S6).

Because LOTUS is considered to be a membrane protein lacking an intracellular domain, it may interact with certain proteins expressed on the cell surface to functionally act on LOT development. To identify possible binding partner(s) of LOTUS, we produced alkaline phosphatase (AP)–fused LOTUS (AP-LOTUS) and examined the localization of its binding sites in mouse forebrain (whole mount from E13) by visualization of AP. AP-LOTUS bound to the ventrolateral area of the forebrain, mainly including the LOT and its projection areas (fig. S7A). When AP-LOTUS was added to the culture medium, AP-LOTUS was detected on growth cones and distal neurites of cultured E13 OB neurons (fig. S7B). We therefore conducted systematic cDNA expression screening to isolate candidate genes from the mouse cDNA expression library of OB (8). One cDNA clone whose product might interact with LOTUS was isolated that generated AP-LOTUS binding activity in human embryonic kidney cell line 293 (HEK293) cells. The cDNA clone encoded the Nogo66 receptor, also known as Nogo receptor–1 (NgR1) (17) (Fig. 2A). NgR1 is a glycosylphosphatidy-linositide-anchored protein and the common receptor of at least three myelin components that inhibit axonal growth and prevent nerve regeneration (18–20). The binding of LOTUS to overexpressed NgR1 was concentration-dependent, with a dissociation constant (K_d) of about 6.8 nM (Fig. 2B). LOTUS did not bind to other Nogo receptor family members, such as Nogo receptor–2 (NgR2) and Nogo receptor–3 (NgR3) (fig. S8). LOTUS was also immunoprecipitated with NgR1 in HEK293 transient (HEK293T) cells overexpressing both LOTUS and NgR1 (fig. S9). Moreover, AP-LOTUS did not bind to LOT in *ngr1*-deficient mice ($NgR1^{-/-}$), suggesting that NgR1 may be a physiological binding molecule of LOTUS in the developing LOT (Fig. 2C).

We examined the expression and distribution of LOTUS and NgR1 in embryonic mouse forebrains at E13. Immunohistochemistry in whole-mount forebrain samples revealed that LOTUS and NgR1 were distributed in the LOT (Fig. 2D). Immunohistochemistry using serial sections of embryonic mouse brain revealed that LOTUS and NgR1 showed an overlapped distribution in the LOT (fig. S10, arrows). LOTUS was coexpressed with NgR1 in growth cones of cultured mouse OB neurons (Fig. 2E). Thus, LOTUS and NgR1 appear to be present together in the LOT and on the same cell surface in growth cones of cultured neurons.

Nogo-A (NgA), an NgR1 ligand, is expressed in the LOT (21). We immunohistochemically confirmed the distribution of NgA in the LOT in mouse embryos at E13 and detected NgA localized with LOTUS in growth cones of cultured mouse OB neurons (fig. S11). Nogo66, the 66–amino acid functional domain for repulsive action in the C-terminal region of NgA (22), inhibits axonal growth and prevents nerve regeneration through its binding to NgR1 (19, 20). Because NgR1 was expressed in LOT axons (Fig. 2, D and E, and fig. S10), we questioned why the repulsive action induced by Nogo66-NgR1 binding did not affect LOT development. We therefore examined whether the overexpression of LOTUS together with NgR1 affected Nogo66 binding to NgR1 in COS7 cells. The binding of AP-fused Nogo66

(AP-Nogo66) was detected in COS7 cells expressing NgR1 alone in a dose-dependent manner, whereas overexpression of LOTUS with NgR1 suppressed the binding of AP-Nogo66 to NgR1 (Fig. 3A and fig. S12). These results indicate that LOTUS overexpressed with NgR1 inhibits Nogo66 binding to NgR1 and may have an antagonistic action on Nogo66 binding to NgR1.

We next examined whether LOTUS physiologically exerts an antagonistic action on the Nogo66-induced growth cone collapse in cultured dorsal root ganglion (DRG) neurons from E13 chick embryos in which NgR1 is expressed (17, 22). Nogo66-Fc, which is fused to the Fc portion of mouse IgG at the C terminus, induced growth cone collapse, which was attenuated after overexpression of Myc-tagged LOTUS (Myc-LOTUS) (Fig. 3B and fig. S13A). Cultured OB neurons from wild-type mice exposed to exogenously added Nogo66-Fc did not show inducible growth cone collapse, whereas Nogo66-Fc did induce growth cone collapse in neurons from LOTUS$^{-/-}$ animals (Fig. 3C and fig. S13B). Furthermore, the binding of exogenously added AP-Nogo66 was increased in the LOT and its projection area in brains of LOTUS$^{-/-}$ animals (fig. S14), suggesting that LOTUS may prevent Nogo66 (or NgA)–NgR1 interaction in the LOT. Thus, LOTUS may exert an antagonistic action on Nogo66-induced repulsive signaling.

If LOTUS expressed in the LOT functionally inhibits the molecular function of NgR1 in vivo, loss of LOTUS function would permit NgR1 signaling that may give rise to defasciculation of LOT axonal bundles, as seen in LOTUS$^{-/-}$ animals (Fig. 1E). Thus, the LOT develops normally in NgR1$^{-/-}$ animals without signaling inputs from NgR1 ligands (fig. S15). LOT defasciculation induced by loss of LOTUS appears to be caused by NgR1-mediating signaling, because LOT defasciculation was attenuated in LOTUS$^{-/-}$ animals together with NgR1$^{-/-}$ (Fig. 4). The relative width of the LOT was decreased in LOTUS$^{-/-}$; NgR1$^{-/-}$ animals when compared to that of LOTUS$^{-/-}$ animals (Fig. 4B). Antagonistic action of LOTUS to NgR1 provides insight into neural development mechanisms and may contribute to therapeutic approaches for brain injury and degenerative brain damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by grants from the Core Research for Evolutionary Science Technology (CREST) of the Japan Science and Technology Agency (to Y.S., T.H., Y.G., and K.T.); grants-in-aid from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (to T.H. and K.T.); grants from the Dr. Ralph and Marion Falk Medical Research Trust and the NIH (to S.M.S.); and a grant for Research and Development Project II (No. S2107) of Yokohama City University, Japan (to Y.G. and K.T.). The authors thank T. Takeyama and Y. Saito at the Olympus Corporation (Tokyo, Japan) for technical assistance with FALI; H. Sekiguchi-Kaneko, T. Okada, M. Ogawara, and S. Chen for technical assistance; and Y. Sasaki, K. Ogura, K. Nishiyama, H. Ito, and T. Takahashi for helpful discussions and comments. K.T. and Y.S. designed the study, and K.T. and Y.G. supervised the experiments, data analysis, and interpretation. The experiments were carried out by Y.S., M.I., Y.K., M.Y., N.Y., F.N., Y.A., and K.T. T.K. and T.H. generated the mouse cDNA expression library of the olfactory bulb and collaborated on the production of monoclonal antibodies. T.A. and H.K. collaborated on the generation of *crtac1B (lotus)*-deficient mice. S.M.S. collaborated on experiments using *ngr1*-deficient mice and NgR2-3. K.T. wrote the paper, and Y.S., F.N., N.Y., and Y.G. edited the manuscript. S.M.S. is a cofounder and consultant of Axerion Therapeutics. Yale University holds a patent licensed to Axerion Therapeutics related to Ngr1 receptor blockers for recovery from central nervous system damage. Yokohama City University has a patent pending on Crtac 1B(LOTUS) function in neuronal cell growth. Materials transfer agreements restrict the use of Crtac1B(LOTUS) knockout mice and NgR1 knockout mice and expression constructs of NgR1, NgR2, and NgR3 proteins.

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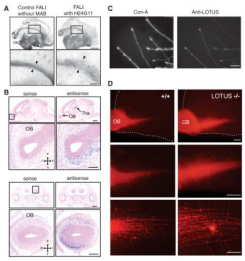

Fig. 1.

Identification of LOTUS. (**A**) LOT phenotypes caused by FALI using H24G11-mAb. Control FALI with light irradiation in the absence of mAb (MAB), FALI of antigen recognized by H24G11-mAb. Boxes in top images correspond to bottom images. Arrowheads, width of LOT axonal bundles. Scale bars, 400 μm (top) and 100 μm (bottom). (**B**) In situ hybridization of LOTUS in E15.5 mouse brain. Saggital (top four) and frontal (bottom four) sections are shown. Boxes correspond to the lower images. Scale bars, 1 mm (top) and 100 μm (bottom). Tha, thalamus; d, dorsal; v, ventral; r, rostral; c, caudal. (**C**) Immunocytochemistry of LOTUS with H24G11-mAb in cultured OB neurons. Lectin staining using concanavalin-A (Con-A) was used for visualizing entire OB neurons. Scale bar, 20 μm. (**D**) Phenotypes of *lotus*-deficient mice in LOT development at E18. Lateral views of whole-mount brain show DiI staining (red) in *lotus*-deficient mice (LOTUS$^{-/-}$, right), compared with the LOT in wild-type mice (+/+, left). Distal portion of each DiI-positive axonal fiber at higher magnification (bottom row). Scale bars, 500 μm in top and middle images, 100 μm in bottom.

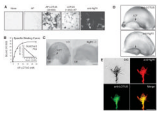

Fig. 2.

Identification of NgR1 as a LOTUS binding partner. (**A**) AP staining of AP-LOTUS(29-602) and LOTUS(1-602)-AP in COS7 cells expressing NgR1. Scale bars, 50 μm. (**B**) AP-LOTUS saturation binding curve to NgR1-expressing COS7 cells (mean for three separate experiments). Scatchard analysis of NgR1-expressing COS7 cells to increasing concentration of AP-LOTUS (inset). (**C**) AP-LOTUS binding in E13 whole-mount forebrain. AP-LOTUS is detected in LOT axons (arrow) in wild-type (+/+) but not *ngr1*-deficient mice (right). Scale bar, 500 μm. (**D**) Distribution of LOTUS (top) and NgR1 (bottom) in developing mouse forebrain from E13. Arrows, immunostaining in the LOT. Scale bar, 500 μm. (**E**) Cellular distribution of NgR1 and LOTUS in cultured OB neurons from E13 mouse embryos. Differential interference contrast (DIC) images correspond to fluorescence images of indirect immunocytochemistry with antibodies against LOTUS (anti-LOTUS) and NgR1 (anti-NgR1). Scale bar, 10 μm.

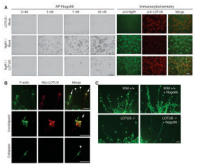

Fig. 3.

Antagonistic action of LOTUS on Nogo66 binding to NgR1 and Nogo66-induced growth cone collapse. (**A**) AP staining of AP-Nogo66 (3, 7, 20 nM) in cells expressing LOTUS alone (LOTUS/mock), NgR1 alone (NgR1/mock), or both proteins (NgR1/LOTUS). Scale bar, 100 μm. Cell surface expression of NgR1 (green) and LOTUS (red) was confirmed by double immunocytochemistry with anti-NgR1 or anti-LOTUS applied to unfixed COS7 cells under non-permeabilizing conditions (right images). (**B**) Growth cone morphology is visualized by double staining of F-actin with fluorescence-conjugated phalloidin and Myc-LOTUS with anti-Myc in cultured E13 chick DRG neurons. Myc-LOTUS–expressing growth cones (arrows) and growth cones collapse (arrowheads) are shown. Scale bars, 20 μm. (Middle and bottom rows) Magnified images of growth cone. Scale bar, 10 μm. (**C**) Nogo66-Fc–induced growth cone collapse visualized by F-actin staining with fluorescence-conjugated phalloidin. Cultured OB axons in wild-type (+/+) (top) or lotus-deficient mice ($LOTUS^{-/-}$) (bottom). Scale bar, 20 μm.

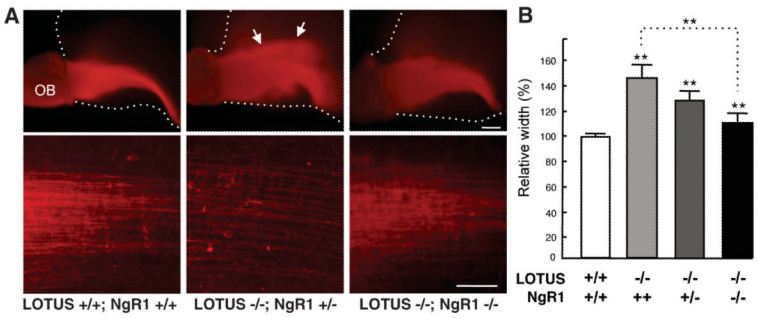

Fig. 4.

LOTUS and NgR1 functions in LOT axonal bundling in vivo. (**A**) Lateral views of whole-mount brain from *lotus* and *ngr1*-deficient mice at E18. Defasciculation of the LOT axonal bundle seen as wider expansion of DiI staining (arrows) in LOTUS$^{-/-}$; NgR1$^{+/-}$ mice (middle images) but not in double homozygous mutants of *lotus* and *ngr1* (LOTUS$^{-/-}$; NgR1$^{-/-}$) (right images). The rescue of the defasciculation is confirmed by observation of a distal portion of DiI-positive axonal fibers at higher magnification (bottom row). Scale bars, 500 μm (top) and 100 μm (bottom). (**B**) Quantitative analysis of width in LOT axonal bundles. The data are calculated as described in fig. S2. $^{**}P < 0.01$ by one-way analysis of variance test with all pairwise multiple comparison test (Tukey test), $N > 3$ littermates.