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The chemorepellent draxin is involved in hippocampal mossy fiber projection

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ABSTRACT

Lamina-specific afferent innervation of the mammalian hippocampus is critical for its function. We investigated the relevance of the chemorepellent draxin to the laminar projections of three principal hippocampal afferents: mossy fibers, entorhinal, and associational/commissural fibers. We observed that draxin deficiency led to abnormal projection of mossy fibers but not other afferents. Immunohistochemical analysis indicated that draxin is expressed in the dentate gyrus and cornu ammonis (CA) 3 at postnatal day 0, when dentate granule cells begin to extend mossy fibers towards CA3. Furthermore, a neurite growth assay using dissociated cells of the neonatal dentate gyrus revealed that draxin inhibited the growth of calbindin-D28k-expressing mossy fibers *in vitro*. Taken together, we conclude that draxin is a key molecule in the regulation of mossy fiber projections.

1. Introduction

The mammalian hippocampus receives various neuronal afferents from granule cells in the dentate gyrus (DG), pyramidal cells in the cornu ammonis (CA), and layer 2/3 neurons of the entorhinal cortex, which are known as mossy, associational/commissural, and entorhinal fibers, respectively. These afferents innervate target areas of the hippocampus in a lamina-specific manner [1]. Mossy fibers project to the most proximal part of the apical dendrites of pyramidal cells in both distal (CA3ab) and proximal CA (CA3c). They innervate the most proximal part, not only of the suprapyramidal, but also the infrapyramidal side in the CA3c. However, these infrapyramidal mossy fibers gradually alter their trajectory, moving towards the suprapyramidal side, and are integrated to the suprapyramidal mossy fiber bundle in CA3ab. Conversely, associational/commissural fibers project to the inner molecular layer in the DG, the middle part of apical dendrites (the stratum radiatum: SR) and basal dendrites (the stratum oriens: SO) of CA3 pyramidal cells. In contrast, entorhinal fibers project to the outer part of the molecular layer in the DG and the most distal part of apical dendrites in CA3 (the stratum lacunosum-moleculare: SLM).

Previous studies implicate several axon guidance molecules in targeting innervation of afferents in the hippocampus [2–15]. Furthermore, it is known that semaphorins Sema6A and 6B, and their receptors plexinA2 and A4, have crucial roles in regulating mossy fiber projection [16,17]. The repulsive function of Sema6A and 6B, expressed in CA3 pyramidal neurons, prevents plexinA4-expressing mossy fibers from innervating the CA3 region. However, plexinA2, which is strongly expressed in the target area for mossy fibers in CA3, attenuates the Sema6A- and Sema6B-induced repulsion, and promotes mossy fiber extension through its neurite growth-promoting activity. Thus, it has been suggested that the balance between repulsive and attractive cues determines the trajectory of mossy fibers in CA3.

We previously isolated a diffusible repellent named draxin and demonstrated that draxin is essential for navigation of growing axons and migrating neural cells in developing embryos [7,18–27]. Further phenotypic analysis on *draxin* knockout (KO) mice

Abbreviations: DG, dentate gyrus; CA, cornu ammonis; SR, stratum radiatum; SO, stratum oriens; SLM, stratum lacunosum-moleculare; DCC, deleted in colorectal cancer; KO, knockout; PFA, paraformaldehyde; DiI, 1,1′-dioctadecyl-3, 3,3′,3′-tetramethyl-indocarbocyanine perchlorate; DiO, 3,3′-dihexyloxacarbocyanine iodide; P, postnatal day; WT, wild-type; AP, alkaline phosphatase; DSCAM, Down syndrome cell adhesion molecule.

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implicated draxin in the development of the hippocampus [28]. Loss of draxin led to a reduction in hippocampal volume and the number of dentate granule cells. Draxin, a prosurvival ligand for the dependence receptor, deleted in colorectal cancer (DCC), inhibits apoptosis of neuroblasts, thus playing a pivotal role in regulating hippocampal neurogenesis. However, it is unclear whether draxin-mediated axon guidance is necessary for the proper projection of hippocampal afferents. In this study, we investigated the effect of loss of draxin on the projections of the principal hippocampal afferents in *draxin* KO mice, and draxin-mediated axon repulsion in mossy fibers *in vitro*.

2. Materials and methods

2.1. Animals

The generation of *draxin* KO mice was described previously [18]. All animal experiments and animal care were performed according to the procedures and guidelines approved by the Committees on Animal Research at Kumamoto and Tohoku Universities.

2.2. Timm staining

Timm staining was performed as previously described [16]. Briefly, adult mice were perfused with sulfide solution, and then fixed using 10% neutral buffered formalin. Brains were cut into 18-μm horizontal sections using a cryostat (Leica), and then immersed in Arabic gum solution containing silver lactate and hydroquinone for visualization.

2.3. Measuring the length and volume of mossy fiber bundle

The length of the infrapyramidal bundle of mossy fibers was measured using ImageJ (National Institutes of Health), and shown as a percentage of the infrapyramidal length of the pyramidal cell layer through the entire CA3 (see Fig. 1C). The volume of the infrapyramidal bundle of mossy fibers in the CA3c was shown as a percentage of total volume of the suprapyramidal and infrapyramidal bundles (see Fig. 1E).

2.4. Labeling of mossy and entorhinal fiber trajectories

Brains were dissected from adult mice perfused with 4% paraformaldehyde (PFA) in PBS, and then cut into 200-μm horizontal slices using a vibratome. Small crystals of lipophilic carbocyanines 1,1′-dioctadecyl-3, 3,3′, 3′-tetramethyl-indocarbocyanine perchlorate (DiI, Invitrogen) and 3,3′-dihexyloxacarbocyanine iodide (DiO, Invitrogen) were placed into the infrapyramidal and suprapyramidal sides of the granule cell layer in the DG. Specimens were then incubated for three days in 4% PFA solution at 37 °C. To label entorhinal fibers, small DiI crystals were introduced into the entorhinal cortex of adult brains perfused with 4% PFA in PBS. After a 2-week incubation at 37 °C, DiI-injected brains were cut into 100-μm-thick horizontal slices using a vibratome (Leica).

2.5. Immunostaining

Brains were fixed with 4% PFA in PBS, and cut into horizontal sections (18 μm) using a cryostat. In the case of immunocytochemistry, cultured cells were fixed with 4% PFA in PBS. Sections or cells were blocked in PBS containing 5% normal donkey serum (Millipore) and 0.1% Triton X-100. Samples were then incubated overnight at 4 °C with the appropriate primary antibody in PBS containing 5% normal donkey serum. This was followed by incubation with the appropriate secondary antibody. The VECTASTAIN Elite ABC system (Vector Laboratories) was used to enhance immunoreactive signals where required. Visualization was performed using VECTOR VIP Peroxidase Substrate Kit (Vector

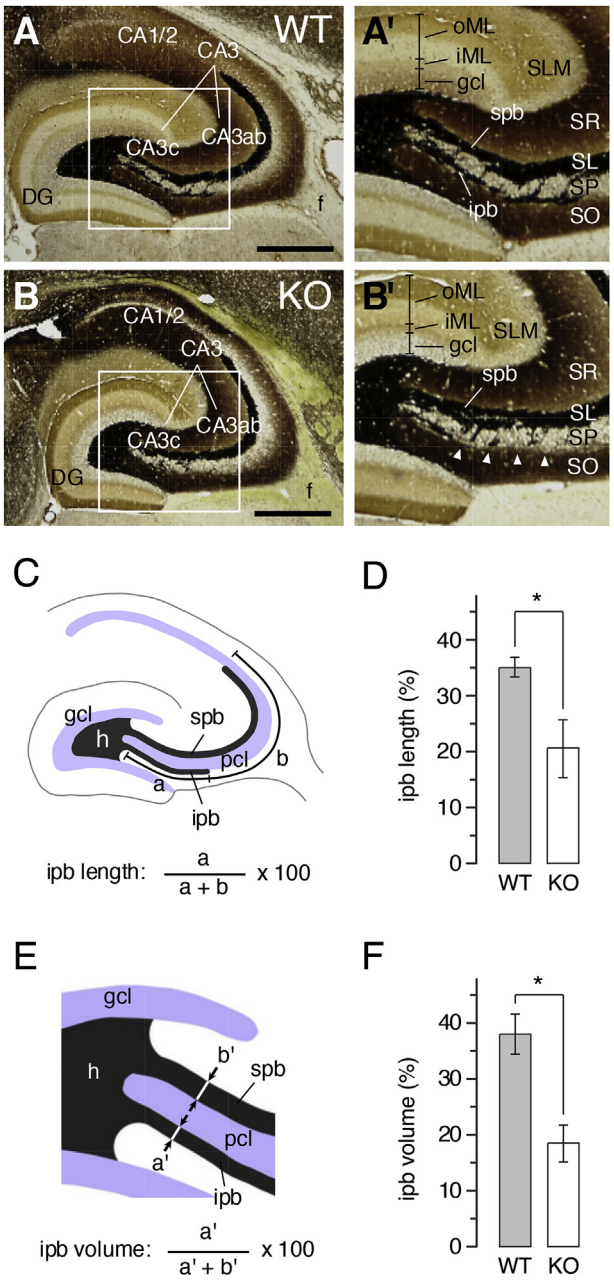

Fig. 1. Projection pattern of mossy fibers. **A**, **B**, Mossy fiber projection detected by Timm staining in wild-type (WT; **A**) and *draxin* knockout (KO; **B**) mice. Boxed regions in A and B are magnified in A′ and B'. **C**, Schematic for calculation of the length of infrapyramidal mossy fiber bundle. **D**, Length of the infrapyramidal mossy fiber bundle calculated by the formula shown in **C**. **E**, Schematic for calculation of the volume of infrapyramidal mossy fiber bundle. **F**, The volume of the infrapyramidal mossy fiber bundle calculated by the formula is shown in **E**. The average length and volume of infrapyramidal bundles in **D** and **F** were calculated from 5 independent brains per group, respectively. Error bars indicate standard error of mean (s.e.m) *$p < 0.01$ (Student's t-test). Arrowheads in **B′** indicate the normal trajectory of the infrapyramidal bundle of mossy fibers. CA: cornu ammonis; DG: dentate gyrus; f: fimbria; gcl: granule cell layer; h: hilus; pcl: pyramidal cell layer; iML: inner part of the molecular layer; oML: outer part of the molecular layer; SLM: stratum lacunosum moleculare; SR: stratum radiatum; SL: stratum lucidum; SP: stratum pyramidale; SO: stratum oriens; spb: suprapyramidal bundle; ipb; infrapyramidal bundle. These abbreviations also apply to the other figures in this manuscript. Scale bars: **A** and **B**, 500 μm.