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Tsukushi is required for anterior commissure formation in mouse brain

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ABSTRACT

The anterior commissure (AC) is one of the important commissure projections in the brain that conveys information from one side of the nervous system to the other. During development, the axons from the anterior AC (aAC) and the posterior AC (pAC) course in the same dorsoventral plane and converge into a common fascicle for midline crossing. Previously, we reported that Tsukushi (TSK), a member of the secreted small leucine rich repeat proteoglycan family, functions as a key coordinator of multiple pathways outside of cells through the regulation of an extracellular signaling network. Here, we show evidence that TSK is critical for the formation of the AC. In mice lacking TSK, the aAC and the pAC axons fail to cross the midline, leading to an almost total absence of the AC in adult mice. DiI labeling indicated that the aAC axons grew out from the anterior olfactory nucleus and migrated along normal pathways but never crossed the midline. Therefore, we have uncovered a crucial role for TSK for AC formation in the mouse brain.

1. Introduction

The mammalian brain requires neuronal interhemispheric connections to convey information from one side of the nervous system to the other. Commissural formation is highly organized and regulated by extrinsic guidance cues that direct axons into the correct path and enable the connection of specific brain areas to appropriate targets [1]. There are three major commissures in the forebrain: the corpus callosum (CC), the hippocampal commissure (HC), and the anterior commissure (AC). The AC interconnects olfactory structures and the anterior and posterior piriform cortexes of the temporal lobes [2,3]. Axons of the anterior part of the AC (aAC) grow out of the anterior olfactory nucleus (AON); axons of the posterior part of the AC (pAC) come from the lateral cortex and migrate along a pathway that crosses the midline with the stria terminalis (St), which is an another component of the AC [4]. Multiple guidance molecules are involved in the axonal projection of AC axons as evidenced by the different phenotypes of mouse mutants lacking extracellular guidance cues and their receptors, which ranges from axon defasciculation and aberrant dorsoventral trajectories to the absence of one or both limbs of the AC [5–13]. Here, we focus on the roles of Tsukushi (TSK) in AC patterning.

Previously, we reported that TSK, a member of the secreted small leucine-rich repeat proteoglycan (SLRP) family [14,15], interacts with and regulates pivotal signaling cascades. For example, TSK first functions as an organizer inducer by inhibiting Bone Morphogenetic Protein (BMP) signaling in cooperation with chordin [16]. Second, TSK interacts with Vg1 to induce primitive streak and Hensen's node formation in the chick embryo [17]. Third, TSK controls ectodermal patterning and neural crest specification in Xenopus by direct regulation of BMP4 and the activity of Notch ligand Delta-1 [18]. Finally, X-TSK modulates Xnr, fibroblast growth factor (FGF), and BMP signaling and regulates germ layer formation and patterning in the Xenopus embryo [19]. Therefore, we have demonstrated that TSK is a key coordinator of multiple pathways outside of the cell that exerts its influence through the regulation of an extracellular signaling network.

In this study, we generated TSK knockout mice and showed a defective crossing of the midline by fibers of both the aAC and the pAC using immunohistochemistry and the DiI labeling technique. We propose that TSK is an important component of the molecular pathways controlling AC development.

2. Materials and methods

2.1. Mice

Tsukushi null mutant mice were generated by inserting a LacZ/Neo cassette into the TSK coding exon (Supplementary

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Fig. 1). The mice used in these studies were backcrossed to the C57BL/6J strain for at least six generations and can be considered of an almost uniform genetic background. All experiments on mice were conducted in accordance with the guidelines of the Kumamoto University Center for Animal Resources and Development.

2.2. Tissue processing

Deeply anesthetized mice were transcardially perfused with 25 ml phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS. Mouse brains were harvested and post-fixed in the same fixative overnight at 4 °C. Brains were sliced into sections of 50–100 μm thickness using a Vibratome 2000 (Leica) for immunostaining.

2.3. Immunohistochemistry

To inactivate the endogenous peroxidase activity, the sections were treated with a solution of 3% H_2O_2/0.3% Triton-X in PBS (PBST) for 15 min at room temperature (RT). Sections were incubated with 10% normal donkey serum in PBST to block nonspecific binding for 1 h at RT incubated with each of the primary antibodies at 4 °C overnight, washed with 0.3% PBST (3 × 10 min), and incubated with secondary antibodies for 2 h at RT. After being washed with PBST (3 × 10 min), the sections were subsequently incubated with an avidin–biotin-peroxidase complex (ABC Kit Standard, Vector Laboratories) for 1 h at RT. The antibody-peroxidase complex was visualized using a VIP peroxidase substrate kit (Vector Laboratories).

2.4. Hematoxylin and Kluver–Barrera (KB) staining

For the axonal morphological analysis, adult brain sections were stained with hematoxylin/eosin (HE) using standard methods. For double staining with hematoxylin and Kluver–Barrera (KB), the sections were incubated with 0.1% Luxol fast blue at 56 °C overnight and washed in 95% ethanol for 5 min at RT. After being washed with PBS, the sections were differentiated briefly in a 0.05% lithium carbonate solution, rinsed twice with 70% ethanol, and washed with PBS. After hematoxylin staining, the sections were dried at RT and cleared with xylene.

2.5. Antibodies

The following antibodies were used for immunohistochemistry:rat anti-L1, 1:5000 (Chemicon); goat anti-β-gal, 1:500 (CAPEL); rabbit anti-β-gal, 1:10,000 (CAPEL); mouse anti-neurofilament (2H3) (Hybridoma Bank), biotin-labeled anti-rat IgG, 1:500 (Southern Biotechnology Associates); FITC-conjugated anti-rat IgG, 1:500 (Jackson); and Cy3-conjugated anti-rabbit IgG, 1:500 (Jackson).

2.6. DiI labeling

To trace the AC axonal tract, a 5% solution of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) was injected into the AON region on days P2 and P5, and the brains were analyzed on days P3 and P7, respectively. Animals were transcardially perfused with PBS and fixative, and horizontal sections of the brains (100-μm thickness) were cut on a vibratome.

3. Results

3.1. TSK is expressed in the mouse brain

Previously, we reported that TSK is involved in neural development in Xenopus [18]. To address TSK functioning *in vivo*, we established TSK−/− mice, which were viable and fertile, by

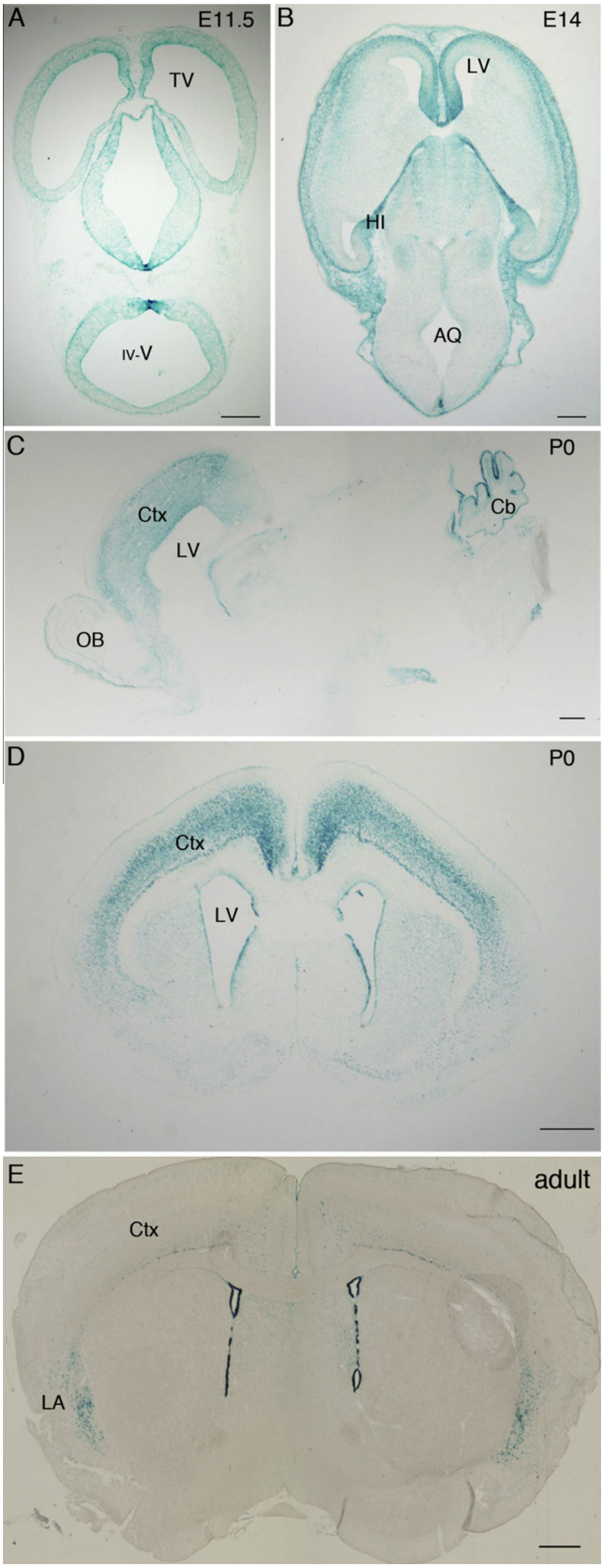

Fig. 1. TSK is expressed in different stages in the mouse brain. (A, B) β-galactosidase staining for TSK expression in a horizontal section of TSK+/− mouse embryos at E11.5 (A) and E14 (B). TSK was widely expressed in the brain at embryonic stages. (C, D) TSK expression in the TSK+/− mouse brain on P0 in sagittal (C) and coronal (D) sections. TSK was expressed in the Ctx and Cb at P0. (E) Adult expression of TSK in a coronal section. TSK expression was restricted to the SVZ. TV, telencephalic ventricle; IV–V, fourth ventricle; LV, lateral ventricle; HI, hippocampus; AQ, Aqueduct. SVZ, subventricular zone. LA, lateral nucleus of the amygdala. Scale bars = 300 μm.