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.

Keywords; Tsukushi, mouse brain, axon guidance, anterior commissure

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 teminalis (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 [5,6,7,8,9,10,11,12,13]. 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 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 hr at RT incubated with each of the primary antibodies at 4℃ overnight, washed with 0.3% PBST (3 x 10 min), and incubated with secondary antibodies for 2 h at RT. After being washed with PBST (3 x 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℃ 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, 1:10000 (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 replacing the exon with a LacZ/Neo selection cassette (Supplementary Fig. 1). We examined the expression of TSK using β-galactosidase staining of TSK+/- brains from stages E11.5 to P0 (Fig. 1). TSK was widely expressed in almost all brain regions at the embryonic stage (Fig. 1A and B). At the P0 stage, the expression of TSK was observed in the cortex and the lateral ventricle (Fig. 1C and D). The expression of TSK at the adult stage was restricted in the subventricular zone and in the lateral nucleus of the amygdala (Fig. 1E). Therefore, TSK expression was widely detected in the brain during development until the adult stage.

3.2. TSK deletion affects the anterior commissure tract

To start a phenotypic analysis of the TSK-/- brain, we first examined the brain morphology of the TSK-/- brain using HE staining in the adult stage (Fig. 2A and B). We found that the AC was

defective in the TSK-/- adult brain (Fig. 2A and B). To confirm these defects, we performed hematoxylin and KB staining, which are widely used methods for the staining of the myelin sheath of the nervous system. We prepared coronal sections of the adult mouse brains at various levels and compared the AC formation between the wild-type (WT) and TSK-/- brains (Fig. 2C-F). The AC is a bipartite tract interconnecting olfactory structures and the anterior piriform cortex and the posterior piriform cortex of the temporal lobes[4]. As shown in Fig. 2C-F, the aAC and pAC axons were recognized by KB staining in the WT coronal sections (Fig. 2C and E), but the axons of the aAC and pAC were extremely thin in the TSK-/- brain (Fig. 2D and F). Furthermore, the pAC failed to cross the midline in the TSK-/- brain (Fig. 2F). To quantify these data, we measured the area of the aAC axons (Fig. 2G and H). The area of the aAC axons in the TSK-/- brain was almost 5 times thinner than the area of the axons in the WT brain (Fig. 2I). This AC axonal absence was observed in almost all of the adult TSK-/- mouse brains (n=20). In addition to these defects in AC formation, we also found that the CC in TSK-/- brains shows a thinner axonal bundle than the WT that might be due to an enlargement of the lateral ventricle (Fig. 2E and F).

3.3. TSK is involved in AC formation

To investigate the role of TSK in AC formation, we reconstructed the commissural pathway from serial horizontal sections of WT and TSK-/- brains at the P0 and adult stages and observed the aAC and the pAC pathways (Fig. 3). The horizontal serial sections of the WT and TSK-/- adult and neonatal brains were stained with KB and an anti-neurofilament antibody, respectively. In the adult TSK-/- brain, we did not observe any thick AC axons extending from the ventral to the dorsal area compared to the WT brains (Fig. 3A and B). On P0, the AC was formed, but the bundle of AC axons was thin and did not cross the midline (Fig. 3C and D). The midline crossing AC axons in the

P0 TSK-/- brains might be the commissural component of the stria terminalis (Fig. 3D)[4]. Moreover, we analyzed AC formation at different neonatal stages, P7 and P10, and found that this thinner bundle of AC axons remained at P7 but was completely diminished at P10 (Fig. 3E and F). These data indicate that TSK is required for AC formation in the mouse brain.

3.4. aAC axons never cross the midline

To further confirm the above defects, aAC axons were traced by an injection of DiI into the olfactory bulb on P2, and DiI-labeled axons were analyzed in horizontal sections on P3. As shown in Fig. 4A, DiI-labeled aAC axons grew out of the AON, where the cell bodies of the aAC axons are located, and crossed the midline toward the contralateral side in the WT brain. Although the aAC axons of the TSK-/- brain also grew out of the AON, they never crossed the midline (Fig. 4B-D).

To check whether TSK influences the formation of the AC tract, we analyzed the expression pattern of TSK in the developing AC on E15. In the WT brain, the aAC fibers started to elongate toward the midline region on E13.5 and intersect with the contralateral AC fibers in the midline on E14.5 to E15 [13]. The immunostaining of horizontal sections of the E15 TSK+/- brains with anti-L1 and anti-β-galactosidase antibodies showed that TSK was expressed in the midline of the brain adjacent to the pAC and the AON on E15 (Fig. 4E and F) but not in the aAC and pAC axons themselves (Fig. 4E-H). This expression of TSK was maintained in the P0 and P7 brains (Fig. 4G and H). To address whether TSK was expressed in the cell bodies of the AON neurons, we injected DiI into the AON and traced the cell bodies of the AON retrogradely (Fig. 4J and M). As previously reported [13], the retrogradely labeled cells expressed calbindin, a marker of AON cells (Fig. 4I-L). We found that the expression of TSK overlapped with the cells labeled by DiI (Fig. 4M-P). These results confirmed TSK expression in the AON and the surrounding area in the route

of AC axons from developmental to postnatal stages and suggest the involvement of TSK in AC formation.

4. Discussion

The SLRP family are subdivided into five discrete classes based on common structural and functional properties; TSK belongs to the Class IV SLRPs [15]. We previously reported that TSK regulates the pivotal signaling cascades involved in chick and frog neurulation by interacting with BMP [16], Delta-1 [18], and FGF8 [19]. Our study reveals a new role for TSK *in vivo* during mouse neural development because TSK was expressed in the mouse brain from embryonic to the adult stages and AC formation in the TSK-/- adult brain was diminished. Our immunohistochemical study showed that the aAC and pAC axons never crossed the brain midline. Furthermore, the DiI labeling technique demonstrated the misguidance of the aAC axons, although they grew out of the AON. Therefore, our data indicate the involvement of TSK in the navigation of AC axons.

Mice deficient in several key guidance molecules (Netrin [6], Semaphorin [20], Eph [21], Draxin [12], and the FGF receptors [22]) have also shown aberrant AC formation, suggesting that the combinational guidance effect of these chemoattractants and chemorepellants plays a crucial role in the formation of the AC axonal tract. These molecules are expressed either in AC axons or in the surrounding regions of the AC axons. Our data demonstrated that TSK was expressed in strategic regions, such as the AON, the regions surrounding AC axons, and in the midline of the brain adjacent to the AC pathway, that have been implicated as the sources of known guidance molecules [1]. TSK may interact with the above guidance molecules or their receptors directly and modulate their activity.

Midline populations, including the glial wedge, glia of the indusium griseum, and midline zipper

glia, play an important role in forebrain commissure formation [1]. These glial cells secrete guidance cues, including Slits [23], Wnt [24], and draxin [12], and form cellular boundaries that surround the corpus callosum axonal tract. Similarly, glial cells form a molecular tunnel in the AC pathway and play a crucial role in AC formation [12,25]. These glial cells secrete not only guidance cues but also extracellular matrix components (ECM) proteins. Previous studies have indicated that one of the ECM proteins, heparan sulfate proteoglycans (HSPGs), interacts with multiple growth factors, such as Shh, FGF and Wnt, and contributes to cortical development [26,27,28]. Mutant mice bearing a targeted disrupted of the heparan sulfate (HS) modifying enzyme, *Ndst1*, showed severe developmental defects of the forebrain, including the AC [29]. Moreover, genetic deletion of the HS-polymerizing enzyme, *Ext1*, caused severe guidance errors in the major commissural tracts [30]. Based on these studies, it is plausible that the ECMs and proteoglycans trap and incorporate the diffusible proteins and thereby stabilize the diffusible gradients, which is essential for the proper formation of the forebrain commissure. Because TSK belongs to the SLRP family, TSK may interact with other guidance cues and/or ECMs and modulate their function, resulting in an abnormal distribution of the prominent guidance cues. Whatever the mechanisms, the results obtained in the present *in vivo* analysis emphasize the role of TSK in AC formation in the mouse brain.

Figure legends

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, telencephlic 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.

Fig. 2. AC formation is diminished in the adult TSK-/- brain. (A, B) HE staining of sagittal sections in a 2 month old WT (A) and TSK -/- brain (B). (C-F) KB staining for myelinated axons in the adult stage. Coronal section at the levels of the aAC (C, D) and pAC (E, F). Note that the axons of the aAC (arrows) and the pAC (arrowheads) in the TSK-/- brain (D, F) were thinner than the WT (C, E). (G, H) High-magnification of the square areas in (C) and (D). (I) Measurement of the aAC area in the WT and TSK-/- brains. Graph showing the area of the aAC of WT (n=4) and TSK-/- (n=15) mouse brains. Asterisk, *p<0.001 based on a two-tailed Student's *t*-test. Scale bars = 1 mm in A-F, 250 μm in G and H.

Fig. 3. TSK is required for normal AC formation. (A, B) AC axons in serial horizontal sections were stained with KB in WT (A) and TSK-/- (B) adult brains. Fibers of the aAC (arrow) elongate from the AON and cross the midline with pAC (arrowhead). A thin aAC was observed in the TSK-/- brain (arrow in B). (B) Midline crossing axons were not observed in the TSK-/- brain. (C, D) Immunostaining for neurofilament in adjacent slices of WT and TSK-/- brains at P0. In the TSK-/- brain, the aAC was not visible and the pAC (arrowhead) was formed but did not cross the midline. Midline crossing fibers were of the stria teminalis (St) (asterisk). (E, F) Coronal section of TSK-/-

brains from P7 and P10. Note the abnormal formation of the aAC and the pAC at both stages. Arrows and arrowheads indicate the aAC and pAC axons, respectively. Note that the aAC and pAC axons in the TSK -/- adult brain are grossly diminished. Scale bars = 1 mm in A-D, 250 μm in E and F.

Fig. 4. The aAC axons never cross the midline in the TSK-/- brain. (A-D) DiI was injected into the olfactory bulb on P2 and the aAC axons were observed horizontally on P3 in WT brains (A) and in three different samples of TSK-/- brains (B-D). DiI-injected sites in the olfactory bulb are indicated by asterisks. Arrows point to the misguided axons (B-D). Note that the pattern of misrouting varied among the mutant mice. (E, F) immunostaining with antibodies against β-galactosidase (red) and L1 (green) was performed on horizontal sections in E15 TSK+/- brains. (E) TSK-expressing cells were located in the midline (white arrowhead). (F) TSK expression was observed in the AON (white asterisk). (G, H) β-galactosidase staining of the TSK +/- brain was performed in horizontal vibratome sections on P0 (G) and P7 (H). The expression of TSK was observed in the AON area (asterisk) and the midline (arrowhead). (I-L) The population of calbindin-positive cells in the DiI retrogradely labeled AON. (J) DiI was injected into the olfactory bulb on P5 and the aAC axons were observed horizontally on P7 in the TSK+/- brain. (K) Calbindin positive cells overlapped with the retrograde DiI labeled cells. (L) High magnification of the white square area in K. (M-P) TSK expressing cells in the DiI retrogradely labeled AON (red). (M) TSK was widely expressed in the AON (asterisk) and in the surrounding area of the AC axon (green). (N) Cells in the AON (asterisk) were retrogradely labeled with DiI. (O) Some population of TSK expressing cell overlapped with DiI positive cells. (P) High magnification of the white square area in O. Scale bars = 500 μm in A-D, 100 μm in E and F, 300 μm in G-P.

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Supplementary Figure and Legend

Fig. S1. Construction of TSK-/- mice.

A targeting vector for TSK was constructed, and the TSK-/- mice (Acc, No, CDB0547K: http://www.cdb.reken.jp/arg/mutant%20mice%20list.html) were generated as described (S1).

TSK-/- mice were viable and fertile. C. PCR amplification for genotyping was performed on templates of unpurified tail lysate with the following set of primers: 5’-cccagcagtagcaacaacaa-3’ (TSK-S), 5’-gagcttgtaagtcccttgga-3’ (TSK-AS) and 5’-gatccccatcaagattatcg-3’ (LacZ). TSK-S and TSK-AS amplified a 267-bp fragment identifying the WT allele, whereas the lacZ and TSK-AP amplified a 428-bp fragment corresponding to the null allele.

Figure1

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Figure 1

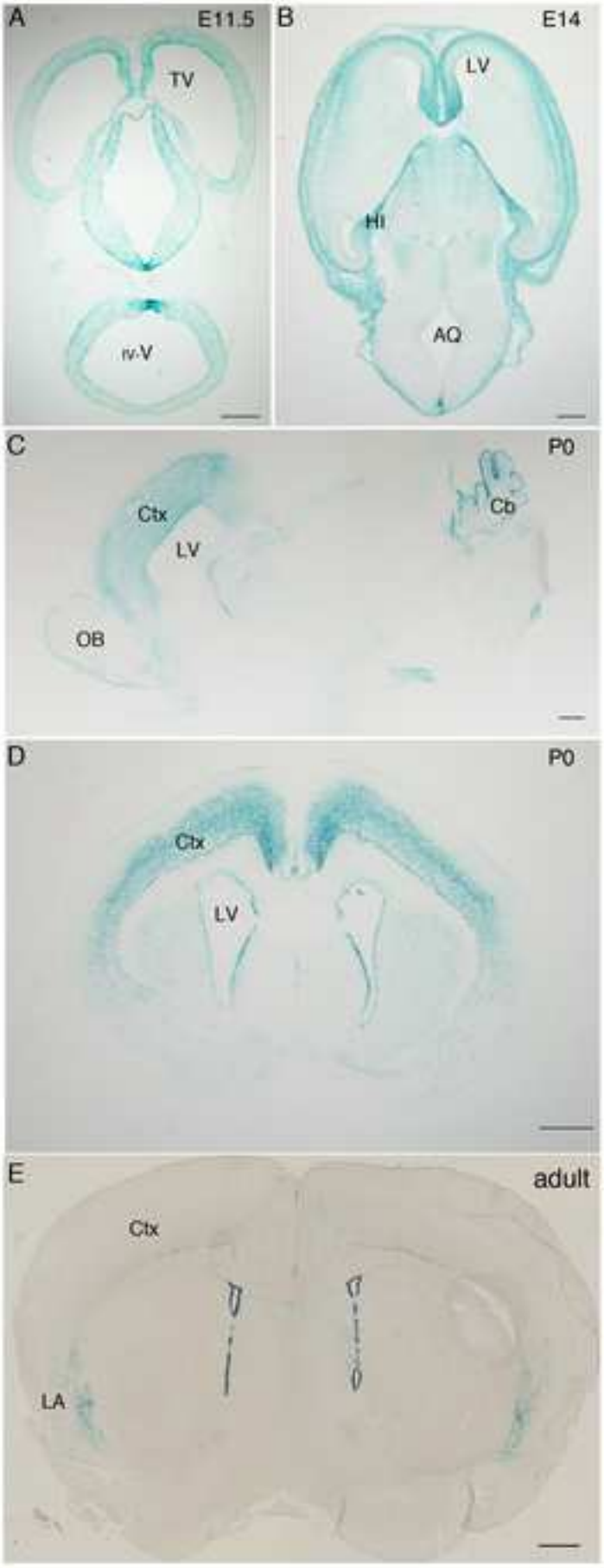

Figure2

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Figure 2

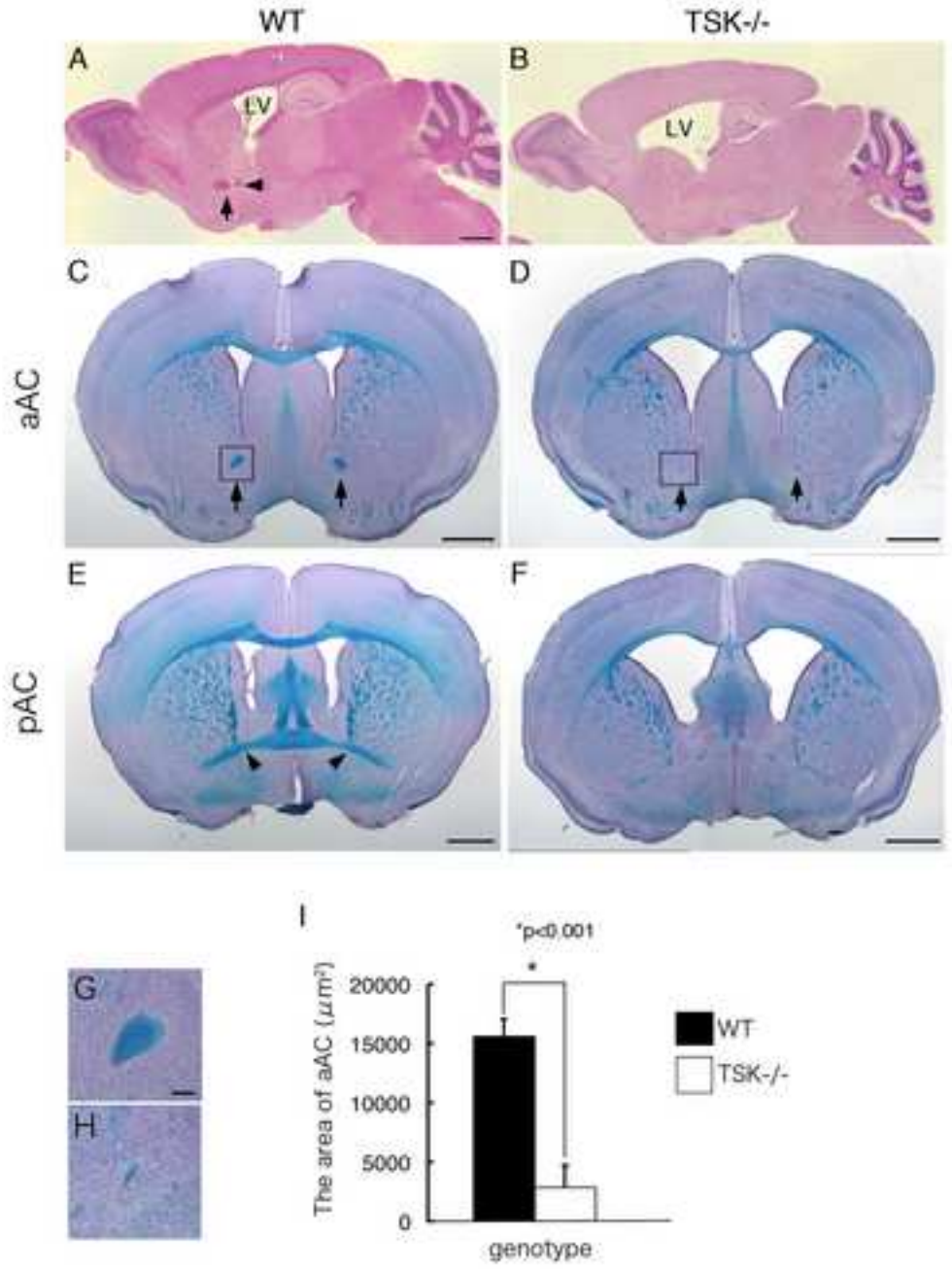

Figure3
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Figure 3

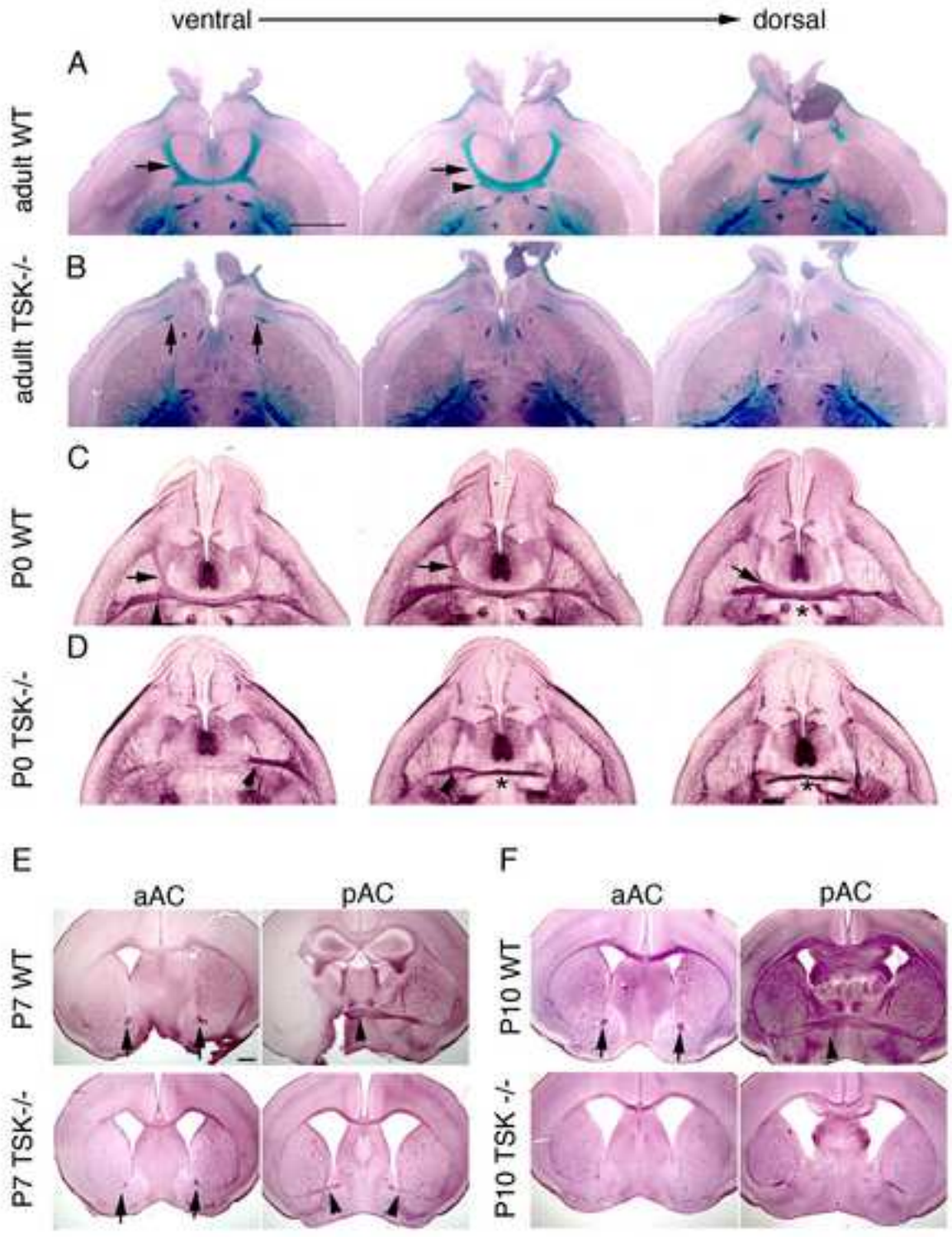

Figure4

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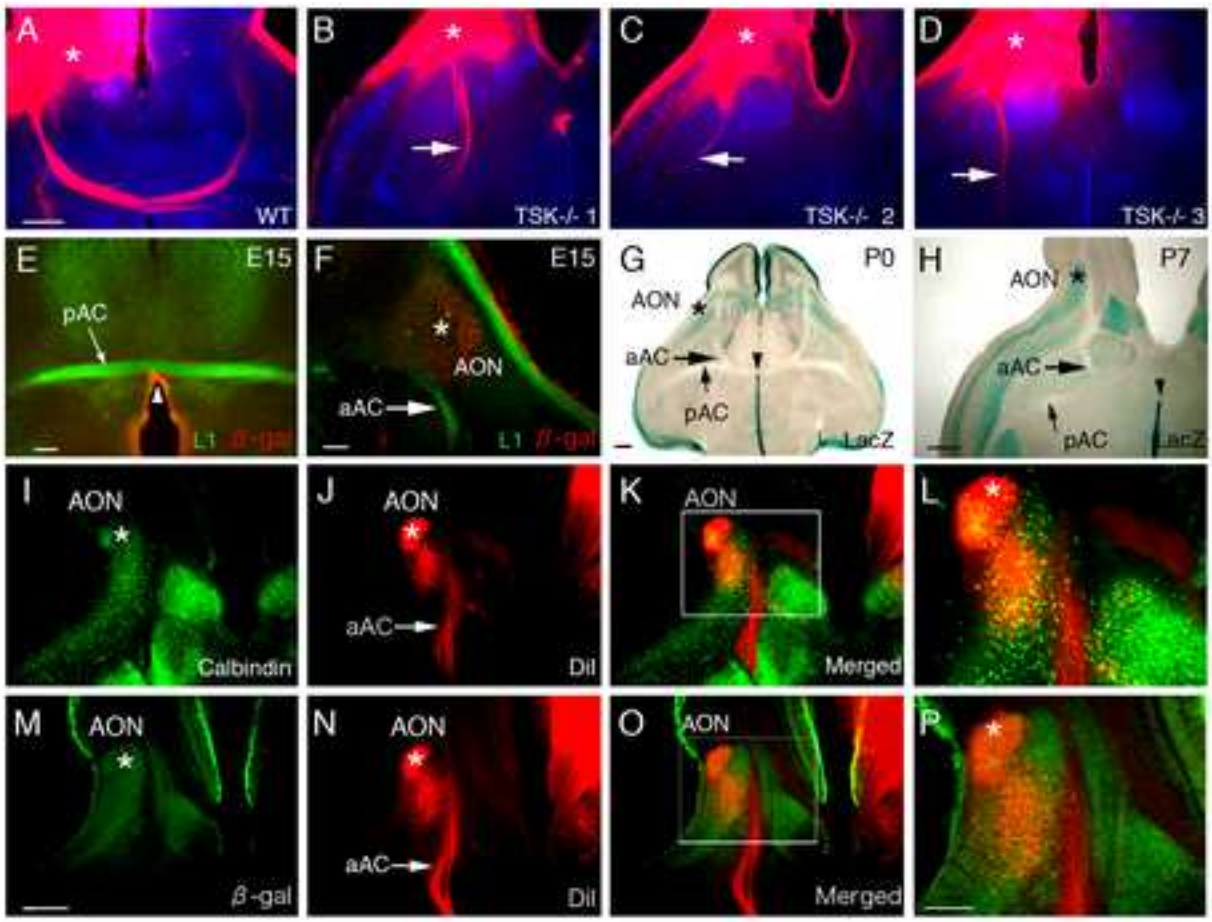
Figure 4



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