主論文の要旨

# Essential Role of Linx/Islr2 in the Development of the Forebrain Anterior Commissure

（脳中枢神経での前交連の形成におけるLinx/Islr2の重要な役割）

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**Introduction**

Linx, also referred to as immunoglobulin superfamily containing leucine-rich repeat 2, is a member of the leucine-rich repeat and immunoglobulin family of proteins (**Fig. 1A**). A previous study showed that Linx is expressed in projection neurons in the cortex and in cells that comprise the passage to the prethalamus that form the internal capsule, indicating the involvement of Linx in axon guidance and cell-cell communication. This study was designed to complement the above previous study. We analyzed the phenotypes of Linx-deficient mice, and check the downstream signal pathways of Linx.

**Materials and methods**

Linx-deficient mice were generated using the Targeted embryonic stem (ES) cell clone which was purchased from the KOMP Repository (www.komp.org). The targeting construct was designed to replace the whole Linx ORF (exon 3) with the LacZ-loxP-neo-loxP cassette. We injected the Evans dye into the LV to visualize the circulation of cerebral spinal flow in vivo. The defect in AC formation was evident in horizontal section by fluorescent labelling of AON neurons. We generated Linx knockout cells using the CRISPR/Cas9 genome editing. To identify new Linx interacting proteins, we generated 293FT cells stably expressing Linx. We immunopurified a protein complex by IP, then performed additional analysis of the immunocomplex using mass spectrometry.

**Results**

We first examined the tissue distribution of Linx and its expression in cell lines. As expected, Linx expression was specifically found in the brain with an exception in the testes of male mice (**Fig. 1B, C**). Linx expression was abundant in the cerebrum and the olfactory bulb (OB), indicating a role for Linx in the function or development of the forebrain (**Fig. 1D**). Linx is expressed throughout postnatal and adult stages, suggesting multifaceted roles in development as well as homeostasis, neurogenesis, or physiological functions of the nervous system (**Fig. 1E**). Linx was also detected in several mouse and human neuroblastoma cell lines (**Fig. 1F**).

Next, We generated Linx-deficient mice and found that Linx-deficient mice die soon after birth at P0 (**Fig. 2A, B, C**). The expression of Linx was observed in the dentate gyrus, CA3 and CA1 regions of the hippocampus and in the region near the apical surface of the cortex (**Fig. 2D**). One of the phenotypes we observed in the Linx-/- mice was the development of severe hydrocephalus (**Fig. 2E**). Hydrocephalus and the enlargement of the lateral ventricle (LV) were confirmed by observations of coronal sections using transmitted light microscopy (**Fig. 2F**). Injection of Evans dye into the LV to visualize the circulation of cerebral spinal flow in vivo did not show any impediment in either intra- or extra-ventricular CSF flow (**Fig. 2G**). Further histological analyses showed that Linx is not expressed in the ependymal cells of the choroid plexus, and the expression and localization of Na+/K+-adenosine triphosphatase (Na+/K+-ATPase) and E-cadherin were not affected in Linx+/- cells, suggesting that Linx is not involved in the function of the choroid plexus (**Fig. 2H, I**). As previously reported, we found that Linx-/- mice displayed a severe defect in the development of the internal capsule (**Fig. 3A**). In addition, the formation of the anterior commissure

was completely defective in Linx+/- mice (**Fig. 3B**). These observations were further confirmed by Nissl and Klüver-Barrera (KB) staining (**Fig. 3C, D**). The defect in AC formation was also evident in horizontal section from Linx+/- brains (**Fig. 3E**). The defect in AC formation was also evident in horizontal section from Linx+/- brains by fluorescent labeling of AON neurons (**Fig. 3F**). Our study suggests that Linx is essential for commissural and longitudinal projections of specific neurons in the forebrain (**Fig. 3G**).

To gain insight into Linx function, we examined the subcellular localization of endogenous Linx in cultured N1E-115 neuroblastoma cells. In undifferentiated N1E-115 cells, Linx localizes to the tips of short neurites (**Fig. 4A**). In differentiated N1E-115 cells after serum starvation and in primary cultured hippocampal neurons, Linx also preferentially localizes to the tips of neurites and growing axons (**Fig. 4B, C**). Furthermore, we also localized Linx to the leading processes of neuroblasts that migrated out from explants isolated from the rostral migratory stream (RMS) of the forebrain (**Fig. 4D**). We next compared morphology as well as neurite extension in control and Linx-depleted differentiated N1E-115 cells, and found that Linx-depleted cells exhibited impaired neurite extension (**Fig. 4E**). In primary cultured hippocampal neurons, axonal projections from neurons isolated from the brain of Linx-/- mice were defective (**Fig. 4F**). Intriguingly, hippocampal neurons isolated from Linx-/- brains showed increase in growth cone and cell body areas compared to those from Linx+/+ brains. Linx-depleted N1E-115 cells also exhibited an increase in cell body size in both undifferentiated and differentiated cells, which could be rescued by the expression of exogenously added Linx (**Fig. 4E, G, H**). These data suggest a role for Linx in multiple cellular processes that involve neurite elongation and regulation of cell size.

A previous study showed the interaction between Linx and RTKs. In the present study, we confirmed the interaction between Linx and Ret or TrkA in N1E-115 cells (**Fig. 5A, B**). However, we did not observe the effect of Linx overexpression on ERK signaling in N1E-115 cells stimulated with GDNF, the ligand for Ret (**Fig. 5C**).

Then we identified new Linx interacting proteins using mass spectrometry (**Fig. 6A**). One of the proteins was Rho-kinase (**Fig. 6B, C**). We also observed that the phosphorylation (Ser19) of myosin light chain (MLC) was elevated in neurons isolated from Linx+/+ compared to Linx-/- mice (**Fig. 6D**). Furthermore, the inhibition of Rho-kinase activity by its inhibitor Y-27632 resulted in an increase in cell size including growth cone and cell body areas (**Fig. 6E, F**). Finally, we transduced GFP or the catalytic domain of Rho-kinase, the increase in cell body area and the defect in neurite extension in Linx-depleted cells were rescued (**Fig. 6G, H**).

**Discussion**

This study complements a previous study by providing additional evidence for the involvement of Linx in the development of commissural and longitudinal projections in the forebrain. At present, the mechanism underlying the development of hydrocephalus in Linx-deficient and Linx+/- mice remains unclear; however, it could partly be explained by our data demonstrating that Linx is involved in Rho-kinase-mediated regulation of brain cytoarchitecture.

In the present study, by monitoring MLC phosphorylation we found that Linx interacts with and regulates

the activity of Rho-kinase. We believe this is consistent with the effects of Linx depletion on neurite extension in N1E-115 cells and hippocampal neurons. How Linx controls the activity of Rho-kinase and the Linx/Rho-kinase complex is involved in the development of brain architecture including the AC, however, remain to be determined and will be the subject of future studies.

**Conclusion**

We demonstrated that Linx-deficient mice develop severe hydrocephalus and defects in the development of the anterior commissure. In the other hands, we identified Rho-kinase as an interacting protein with Linx, further suggesting the involvement of Linx in cytoskeletal organization and other various cellular processes during brain development.