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Linx Mediates Interaxonal Interactions and Formation of the Internal Capsule

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SUMMARY

During the development of forebrain connectivity, ascending thalamocortical and descending corticofugal axons first intermingle at the pallial-subpallial boundary to form the internal capsule (IC). However, the identity of molecular cues that guide these axons remains largely unknown. Here, we show that the transmembrane protein Linx is robustly expressed in the prethalamus and lateral ganglionic eminence-derived corridor and on corticofugal axons, but not on thalamocortical axons, and that mice with a null mutation of *Linx* exhibit a complete absence of the IC. Moreover, regional inactivation of *Linx* either in the prethalamus and LGE or in the neocortex leads to a failure of IC formation. Furthermore, Linx binds to thalamocortical projections, and it promotes outgrowth of thalamic axons. Thus, Linx guides the extension of thalamocortical axons in the ventral forebrain, and subsequently, it mediates reciprocal interactions between thalamocortical and corticofugal axons to form the IC.

INTRODUCTION

The internal capsule (IC) is a major brain tract comprised of thalamocortical and corticofugal projections that reciprocally connect the neocortex and subcortical structures. How ascending thalamocortical and descending corticofugal projections are guided to their appropriate cortical and subcortical targets, respectively, is a major unanswered question. In the mouse, ascending thalamocortical axons reach the diencephalic-telencephalic boundary (DTB) by embryonic day (E) 12.5 (López-Bendito and Molnár, 2003). Upon reaching the DTB, these axons change their trajectory, extend through the ventral forebrain and, by E13.5, reach the pallial-subpallial boundary (PSPB). Guidepost cells of the prethalamus and corridor cells derived from the lateral ganglionic eminence (LGE) serve to direct thalamocortical projections through the DTB, into the medial ganglionic eminence, and toward the PSPB (López-Bendito et al., 2006; Métin and Godement, 1996). On the other hand, descending corticofugal axons emanating from deep layers of the neocortex reach the PSPB at ~E13.5, when they transiently pause prior to invasion of the subpallium. This waiting period is regulated by Sema3E/PlexinD1 signaling (Deck et al., 2013). At the PSPB, thalamocortical and corticofugal axons intermingle and then extend in close proximity to each other and in opposite directions en route to cortical and subcortical targets, respectively (Hevner et al., 2002; McConnell et al., 1989; Molnár et al., 1998a). According to the "handshake hypothesis" (Blakemore and Molnár, 1990), thalamocortical and corticofugal axons associate at the PSPB (Molnár et al., 1998a, 1998b), and this association between ascending and descending axons serves to guide their distal projections beyond the PSPB to appropriate target regions. Indeed, recent findings indicate that descending corticofugal axons are required to guide ascending thalamic axons across the PSPB (Chen et al., 2012; Molnár et al., 2012). The identity of cues that mediate interactions (i.e., the handshake) between thalamocortical and corticofugal axons at the PSPB and that guide ascending and descending axonal projections across this boundary has been elusive (Molnár et al., 2012).

Linx is a *LIG* gene family transmembrane protein originally described as a mediator of axonal extension, branching, and guidance of somatosensory and spinal motor neurons (Mandai et al., 2009). Here, we demonstrate that Linx is robustly expressed on corticofugal axons, but not on thalamocortical axons, and that mice with a null mutation of *Linx* exhibit a complete absence of the IC, although layer V cortical neurons and thalamic neurons are otherwise intact. Moreover, regional inactivation of Linx either in the prethalamus and LGE or in the neocortex leads to a failure of IC formation. Furthermore, Linx binds to thalamocortical projections and promotes their outgrowth. Thus, Linx guides the extension of thalamocortical axons in the ventral forebrain, and it mediates reciprocal interactions between thalamocortical and corticofugal axons at the PSPB and guidance and extension of all ascending and descending projections of the mammalian neocortex.

RESULTS

Linx Expression in the Developing Brain

To determine whether Linx controls axonal projections in the mammalian brain, we first assessed the spatial and temporal

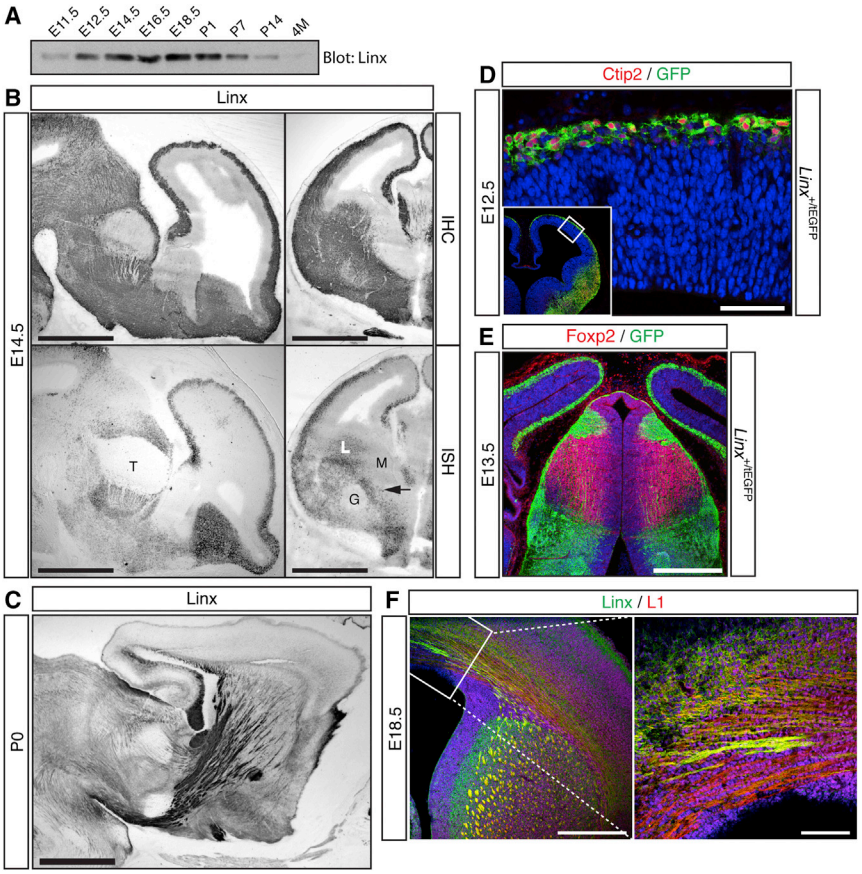

Figure 1. Expression of Linx in the Developing Brain

(A) Western blot probed with anti-Linx. An aliquot of 40 μg protein of brain homogenate was loaded into each lane. E: embryonic day; P: postnatal day; M: month.

(B) Immunohistochemistry (IHC) with anti-Linx and in situ hybridization (ISH) probed for *Linx*. Sagittal (left) and coronal (right) sections of wild-type mouse brain at E14.5 are shown. Adjacent sections were used for analyses. The specificity of anti-Linx used for these IHC experiments is documented in findings shown in Figure S1 and elsewhere (Mandai et al., 2009). Arrow: lateral ganglionic eminence (LGE)-derived corridor; L: LGE; M: medial ganglionic eminence; T: thalamus; G: globus pallidus.

(C) A sagittal section of P0 wild-type mouse brain stained with anti-Linx.

(D) Coronal sections of the developing neocortex of a $Linx^{+/tEGFP}$ embryo stained with GFP (green) and Ctip2 (red) antibodies and counterstained with TO-PRO-3 (blue) at E12.5. The boxed area shown in the inset is magnified.

(E) A coronal section of the developing thalamus of a $Linx^{+/tEGFP}$ embryo stained with GFP (green) and Foxp2 (red) antibodies and counterstained with TO-PRO-3 (blue) at E13.5.

(F) Coronal sections of the neocortex of an E18.5 wild-type embryo stained with Linx (green) and L1 (red) antibodies and counterstained with TO-PRO-3 (blue).

Bars: 1.0 mm ([B] and [C]), 50 μm (D), 0.4 mm ([E] and [F] left), and 0.1 mm ([F] right). These results are representative of three independent experiments.

patterns of Linx expression in brains of embryonic and neonatal mice. Linx protein is detected in the developing brain as early as E11.5 and is most abundant during late gestation, while levels are relatively low in the adult brain (Figure 1A). At E14.5, Linx protein is robustly expressed in the cerebral cortex, prethalamus (nomenclature described elsewhere [Puelles and Rubenstein, 2003]), LGE, and the LGE-derived corridor that penetrates the medial ganglionic eminence and provides a permissive substrate for extension of thalamocortical axons (Figure 1B) (Bielle et al., 2011). On the other hand, Linx is undetectable in the thalamus and globus pallidus at this stage. In situ hybridization for *Linx* mRNA confirms these expression patterns (Figure 1B). At postnatal day (P) 0, Linx protein is most highly associated with the IC (Figure 1C). We also assessed the cellular pattern of Linx expression in the developing cortex utilizing a *Linx* knockin allele ($Linx^{tEGFP}$) in which the coding determinants of a tau-enhanced green fluorescent protein (EGFP) fusion protein were introduced into the *Linx* locus (Mandai et al., 2009). At E12.5, *Linx* is expressed in virtually all Ctip2 (alias, Bcl11b)-positive cortical projection neurons in layer V of the pallium (Arlotta et al., 2005) (Figure 1D). At E13.5, *Linx* is expressed in the pretectum but not in the thalamus, which expresses Foxp2 (Ferland et al., 2003; Suzuki-Hirano et al., 2011) (Figure 1E). Additionally, analysis of older embryos revealed that *Linx* is highly expressed in corticofugal neurons in deep cortical layers, including layers V and VI and the subplate, as well as neurons of the marginal zone (Figures 1F, 3C, and 3D). Thus, although absent from axons of thalamocortical neurons, Linx is highly expressed both in the prethalamus and within the corridor of the developing striatum through which thalamocortical axons project. Linx is also highly expressed on the majority of L1-positive axons of the cortex, with the exception of those in the deepest cortical layer (Figure 1F). Thus, Linx is highly expressed on most axons of the deep cortical neurons that give rise to corticofugal projections, which associate via the handshake with $Linx^{-}$ thalamocortical axons at the PSPB. This pattern of Linx expression, together with its previously defined role as a mediator of axonal growth and guidance in the peripheral nervous system (Mandai et al., 2009), prompted us to examine its role in both the initial trajectory of corticofugal axons and as a mediator of reciprocal interactions between corticofugal and thalamocortical axons.

Requirement of Linx for Formation of the IC

To assess the involvement of Linx during establishment of forebrain neuronal circuits, and of corticofugal and thalamocortical projections in particular, we analyzed brains of E18.5 *Linx* null mice. Strikingly, *Linx* mutant embryos exhibit a complete absence of the IC, and thalamocortical projections that normally form the ascending component of the IC are markedly aberrant in these mutant embryos (Figures 2A and 2B). Remnants of the