Dear Editor,

Mammalian central nervous system neurons show asymmetry during early brain development that defines the elaboration of neural circuitry (Kriegstein and Noctor, 2004). Many intracellular signaling pathways, which are important for the transition to the polarized state and the development of axons and dendrites, have been identified (Barnes and Polleux, 2009). How these pathways are initiated during neuronal development in vivo remained elusive until Yi et al. (2010) found that transforming growth factor-β (TGF-β) is essential for the differentiation and growth of axons. JNK is also crucial for neuronal polarity and axon formation (Oliva et al., 2010). However, whether and how JNK is activated by TGF-β signaling during brain development is not clear.

Previously, we have identified several components of the JNK pathway that play important roles during brain development (Yang et al., 2012; Xu et al., 2014). Based on these findings, we screened for MAPKKKs, the upstream kinases of the JNK pathway that are involved in neuronal development as well as TGF-β signaling. We have discovered that one of the candidates, TGF-β-activated kinase 1 (TAK1), is expressed at high level in the brain at the embryonic stage, but its expression level declines after birth (Figure 1A). Our results show that, in the neocortex of embryonic day 18.5 (E18.5) mice, TAK1 is expressed mainly in the subventricular zone, intermediate zone (IZ), and cortical plate (CP), but weakly in the ventricular zone, which is a similar expression profile to that of TGF-β receptor 2 (TβR2; Supplementary Figure S1A) (Yi et al., 2010). More strikingly, both the active, phosphorylated forms of TAK1 and JNK were detected along the same TuJ1-rich fasciculations within the IZ of neocortex as that of TβR2 (Figure 1B and Supplementary Figure S1B) (Yi et al., 2010). This implies that TAK1 is expressed in the axons, a prediction that was confirmed by immunostaining of cultured neurons (Figure 1C).

To investigate whether TAK1 is involved in axon outgrowth, we knocked down TAK1 expression with shRNAs in hippocampal neurons. As shown in Figure 1D and Supplementary Figure S2A, the axon length of TAK1 knockdown neurons was much shorter than that of controls, and was similar to those cultured in the presence of the JNK inhibitor, SP600125. The decrease of axon length caused by TAK1 knockdown could be rescued by co-expression of TAK1, which was suppressed by SP600125. This indicates that TAK1 regulates axon outgrowth through the activation of JNK.

To confirm the role of TAK1 in axon outgrowth, we knocked out TAK1, specifically in the brain, by crossing TAK1<sup>Flox</sup>/<sup>Flox</sup> mice with nestin-Cre mice, because conventional TAK1 knockout mice are embryonic lethal (Jadrich et al., 2006). Interestingly, brain-specific TAK1 knockout (cKO) mice were also embryonic lethal at around E18.5 with anencephaly, indicating that TAK1 is essential for brain development (Supplementary Figure S2). Even at E15, cKO mice were alive, but were smaller than control mice (Figure 1E). We therefore isolated and cultured hippocampal neurons from E14.5 mice and found that the average axon length of neurons from cKO mice was substantially shorter than that from wild-type littermates (Figure 1E and Supplementary Figure S3). More importantly, we knocked out TAK1 by transfection of Cre in TAK1<sup>Flox</sup>/<sup>Flox</sup> brain via in utero electroporation at E14.5 and found that axons were absent in ~65% of neurons in the CP compared with ~30% in controls at E17.5, implicating that TAK1 regulates axon specification (Figure 1F). Interestingly, axon outgrowth was largely normal at P7 (Supplementary Figure S4). Therefore, TAK1 knockout leads to delayed axon specification and outgrowth.

TGF-β can activate TAK1 in non-neuronal cells (Derynck and Zhang, 2003); we therefore investigated the role of TGF-β in TAK1 and JNK activation in cultured neurons. We isolated neuronal progenitor cells from both E14.5 TβR2 and TAK1 brain-specific knock-out mice and treated them with either TGF-β2 or TGF-β3. As shown in Figure 1G and H, both TGF-β2 and TGF-β3 were able to induce the phosphorylation of TAK1 and JNKs in wild-type cells, but not in TβR2 cKO cells. This indicates that TGF-β induces the activation of TAK1 and JNK through TβR2. Intriguingly, TGF-β2 and TGF-β3 were only able to induce a very low level of JNK phosphorylation in TAK1 cKO cells. This indicates that TAK1 is essential for TGF-β-induced JNK activation in neuronal cells.

TGF-β acts through type I (TβR1) and type II (TβR2) receptors to exert its diverse functions, through Smad-dependent and independent signaling pathways, in a wide array of cellular processes (Derynck and Zhang, 2003). In addition to TGF-β, TAK1 acts in a signaling nexus that responds to various upstream signals, to influence
Figure 1 TAK1-mediated TGF-β signaling regulates axonal growth. (A) Expression of TAK1 during brain development. Cell lysates from E14.5 to P21 mouse neocortex were analyzed by western blotting. (B) Images of E18.5 mouse coronal neocortex sections stained with p-TAK1 (Tyr187 phosphorylated form), Tuj1 (premature neuronal marker), and DAPI. Scale bar, 50 μm. (C) Primary hippocampal neurons from an E18.5 mouse were cultured for 4 days and labeled with TAK1 antibody and DAPI. (D) Primary hippocampal neurons from E14.5 mouse were transfected with either control or TAK1 shRNAs (shCtrl or shT) in pLentiLox 3.7 at 12 h after culture and inspected 3 days later. Scale bar, 25 μm. Lower panel: quantitation of neuronal axon length in the presence or absence of SP600125 (10 μM, treated for 24 h). (E) Images of E15 control and TAK1 brain-specific (crossed with nestin-Cre mice) knockout embryos. Right panel: quantification of axon length of primary hippocampal neurons from E14.5 control and TAK1 cKO embryos cultured for 3 days. (F) TAK1 is required for axon formation during brain development. Coronal sections of the cortical plate (CP) from TAK1<sup>Flox/Flx</sup> brains were electroporated with control or Cre in pCAGIG at E14.5 and inspected at E17.5. Tracings of representative transfected neurons are shown in the middle panel. Scale bar, 5 μm. Right panel: quantification of cells with axons. (G and H) Neural progenitor cells from E14.5 control, TβR2 (G) or TAK1 (H) brain-specific (crossed with nestin-Cre mice) knockout mouse cortices (cultured for 3 days) were treated or untreated with TGFβ2 (50 ng/ml) or TGFβ3 (50 ng/ml) for 30 min and analyzed by western blotting for levels of TGFβR2, TAK1, p-TAK1, p-JNK, and GAPDH. All data are means ± SEM (n = 100 – 150 in D and E; Control, n = 360 and Cre, n = 256 in F), *P < 0.05, **P < 0.01, t-test.
downstream processes via different pathways, including JNK signaling (Delaney and Mlodzik, 2006). In this study, we show that TGF-β induces the activation of JNK via TβR2 and TAK1. In addition, TAK1 plays an important role in axon development. Intriguingly, we found that TAK1 brain-specific cKO mice are embryonic lethal at E18, whereas TβR2 cKO mice are largely normal. There are two possibilities for this discrepancy. First, we used TAK1 phosphorylation at Tyr187 as an indicator for TAK1 activation; however, other phosphorylation sites in TAK1 are also essential for TAK1 activity toward JNK activation and functions (unpublished data). Therefore, the apparent incapacity of TGF-β-induced Tyr187 phosphorylation of TAK1 in TβR2 cKO cells may not indicate void TAK1 activation. Second, TAK1 may regulate additional signaling pathways involved in brain development, such as the NF-κB pathway, which may control different aspects of brain development including cell proliferation and/or survival.

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References


