Research article

FGF10 regulates thalamocortical axon guidance in the developing thalamus

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Thalamocortical axons (TCAs) transmit sensory information to the neocortex by responding to a variety of guidance cues in the environment. Similar to classical guidance cues (ephrins, slits, semaphorins and netrins), morphogens of FGFs can also help axons navigate to their targets. Here, expression analyses reveal that FGF10 is expressed in the chick prethalamus during the navigation of TCAs. Then, using ex vivo analyses in chick explants, we demonstrate a dose-dependent effect of FGF10 on thalamic axons: low concentration of FGF10 attracts thalamic axons, while high concentration FGF10 repels thalamic axons. Moreover, inhibition of FGF10 function indicates that FGF10 exerts a direct effect on thalamic axons. Together, these studies reveal a direct role for the member of FGF7 subfamily, FGF10, in the axonal navigation of TCAs.

1. Introduction

In the central nervous system, the thalamus is located in the dorsal diencephalon and acts to relay outside sensory information into the neocortex. Sensory signals, except olfactory signals, are transmitted to the thalamus, and then carried by thalamocortical axons (TCAs) [1]. Initially, TCAs cross the ventral prethalampus, and then make a sharp turn to the forming internal capsule in the ventral telencephalon (Fig. 1A and B); they then project dorsally into the final neocortex targets [2]. Traditionally, the thalamus was divided into two major components: the ventral thalamus and the dorsal thalamus. In fact, the ventral thalamus lies rostral to the dorsal thalamus [3]. Thus, the nomenclature now is to use terms prethalamus and thalamus to describe the ventral thalamus and dorsal thalamus respectively. TCAs pass through the prethalamus as the first step on their trajectory pathway. Although the prethalamus is the intermediate first target for thalamocortical projection formation, much less is known about the molecular mechanisms of this targeting process.

Fibroblast growth factors (FGFs) consist of a large family of polypeptide growth factors, and play various roles during embryonic development. In addition to mediating patterning activities, FGFs are found, at later stages during development, to act as axon guidance molecules. For example, FGF8, a morphogen that mediates patterning activities of the midbrain-limb boundary at earlier stages, repelled axons of midbrain dopaminergic neurons [4]. Studies also show FGF10 has an essential role in axonal navigation. FGF10 elongates the outgrowth of hypothalamic axons, and directs axons to their final targets; this study also indicates that low concentration of FGF10 attracts hypothalamic axons, while high concentration FGF10 repels hypothalamic axons [5]. FGF10 has been previously shown to be expressed in the thalamus [6]; nevertheless, it remains unclear the functions of FGF10 in the thalamus, and no reports reveal whether or not FGF10 also plays a direct role in axonal navigation in the thalamus. Here, we explore effects of FGF10, secreted from the developing prethalampus, on the innervation thalamocortical neuronal fibers. Our studies depict an important role for FGF10 signaling in directing the growth of early TCAs to the developing prethalampus. Ex vivo studies in chick explants and blockade of FGF signal pathways indicate that FGF10, expressed in the prethalampus, is crucial in directing thalamocortical innervation. Since functions of morphogens exert in a concentration dependent way, our studies also demonstrate a dose-dependent effect of FGF10 on thalamic axons. These studies reveal that low concentration of FGF10 attracts thalamic axons, while high concentration FGF10 repels thalamic axons, suggesting a mechanism for the continued extension of TCAs after they reach the prethalampus. Together, these studies reveal a direct role for the member of FGF7 subfamily, FGF10, in the axonal navigation of TCAs.

2. Materials and methods

2.1. Explant culture

All embryos were staged and dispase-isolated (1 mg/ml, Roche), and the diencephalon were then dissected out in an ‘open book’ presentation (Fig. 2A and B). According to the requirements of distinct experiments, different parts of the thalamus were dissected out

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Explants were then cultured in collagen beds (R&D #344010001) based on published techniques (Fig. 2C) [7]. When two explants were cultured together, the distance between them was around 100–300 μm. A gel Blue gel beads (BioRad #153-7302) were washed 5 times in PBS and then soaked in protein overnight at 4 °C prior to culture. FGF10 protein (R&D) was used standardly at 100 ng/ml and 500 ng/ml; its antagonist SU5402 (Calbiochem) was used at 20 μM.

2.2. Immunofluorescence analyses

Immunohistochemical analysis of embryos (n≥6) and explants (n≥6) was performed according to standard whole-mount or cryostat sectioning techniques [8]. In chick work, antibodies used were: anti-Tuj1 (anti neuron-specific class IIIβ-tubulin; ab 78078, Abcam, USA); anti-GFP rabbit polyclonal (ab6556, Abcam, USA); anti-GFP mouse monoclonal (ab1218, Abcam); anti-Calretinin (Calb2, ab702, Abcam, USA); anti-FGF10 (bs-1326R, Bioss, China); anti-VGluT1 (ab77822, Abcam, USA); Secondary antibodies were conjugated to Alexa 647 or Alexa 488 (Jackson, USA).

2.3. Dil tracing

Retrograde and anterograde Dil labeling experiments were performed on E4 and E6 ‘open book’ thalamic explants. The lipophilic carbocyanine dye, 1, 1-dioctadecyl-3, 3', 3'-tetramethyl-indocarbocyanine perchlorate (Dil) (KeyGEN BioTECH, China) was injected into the prethalamus of diencephalon (retrograde experiments). The explants were cultured for two days, then fixed in 4 % paraformaldehyde and examined.

2.4. In situ hybridization

Embryos were processed for in situ hybridization as described previously [9]. Following development, embryos or explants (n = 5) were analyzed on cryostat sections.

2.5. RNAi electroporation

RNAi targeting vectors designed by Shanghai Genechem (China) expresses a short hairpin RNA (shRNA) directed against the sequence ACGGCTCTAAGGAGTTTAATA from a microRNA operon expression cassette. FGF10-RNAi vectors were electroporated into E4 whole-mount embryonic prethalamus. A none specific RNAi vector (against TTCTC CGAACGTGTCACGT), which has no significant homology to any known chicken gene sequences, was taken as a negative control. Electroporation procedure was as previously described [10]; the maximum DNA concentration in all electroporations was 0.5 μg/μl. After 48 h of incubation, primary analysis was performed by directly observing the location and morphology of GFP + cells. Then, the expression patterns of TUJ1 were analyzed for defects caused by RNAi targeting of FGF10.

2.6. Statistical analysis

Statistical analyses were carried out using SPSS22.0 for PC. Statistical significance of differences in means between groups was determined using a 2-tailed Student t test. P values less than or equal to 0.05 indicates a significant difference.

3. Results

3.1. FGF10 expression in the chick thalamus

During E5, the period TCAs projecting into the prethalamus, a number of FGF10 mRNA and protein expressions are found in the prethalamus of chick embryos (Fig. 3B and D). Over this period, FGF10 gene is not detected elsewhere in the chick thalamus (Fig. 3C). Retrograde Dil-labelling experiments on ‘open book’ diencephalic explants. The lipophilic carbocyanine dye, Dil, diffuses anterogradely and retrogradely specifically along neuronal processes and cell bodies. Dil was injected into the prethalamus of the E4 explants. The Dil labeled...
explants were then cultured in an ‘open-book’ flat configuration for 2 days. The retrograde analysis reveals that, over the culture period, thalamic axons had entered into the domain of the prethalamus (Fig. 3E). To further test the patterns of thalamic axon growth, immunohistochemistry was performed on frozen thalamic sections (Fig. 3F and G), which is consistent with previous studies [11]. However, TCAs do not enter into the hypothalamus, they make a sharp turn into the prospective internal capsule of the ventral telencephalon. To prove this, Dil was injected into E6 prethalamic area; after culture for 2 days, tracing with Dil showed that thalamic axons (arrows) made a sharp turn into the ventral telencephalon. Thus, the patterns of axon growth in vivo, together with the Dil tracing experiments, suggest that thalamic axons grow selectively to the prethalamus.

3.2. FGF10 from the prethalamus directs TCAs

Studies have shown that FGFs can direct motor and hypothalamic axons [5,12]. Based on FGF10 expression within the prethalamic, we hypothesized that FGF10 may function as a guidance cue for growing thalamic axons. To test this hypothesis, three-dimensional collagen matrices were used to identify axonal guidance effects of FGF10 from the prethalamic. When thalamic explants cultured alone, few thalamic axons are examined in the collagen matrices; immunolabelling reveals that TUJ1+ TCAs extend to the edge of the lateral explant, then circle around the peripheral area inside the explant, instead of extending into the collagen matrices (Fig. 4A and B). However, when thalamic explants were co-cultured with prethalamic explants, our studies show that explants of the prethalamus attract TCAs, axons project out of gels and straight towards FGF10+ prethalamic explants (Fig. 4D and E). Given this, we examined whether FGF10-soaked beads mimic the chemotropic effect of the prethalamus. In comparison to lateral neurons grown alone (Fig. 3A and B), FGF10 beads (100 ng/ml), but not PBS beads (Fig. 4C) can significantly promote TUJ1+ axonal outgrowth from the thalamic explants (Fig. 4F-G). The number of thalamic axons is significantly greater in proximal section (towards FGF10 beads) than in distal section (away from FGF10 beads) (Fig. 4H; Table 1). Moreover, our results show that thalamic axons inside thalamic explants also attracted by FGF10 beads (Fig. 4I). Thus, our analyses suggest a role for FGF10 in the guidance of thalamic axons at a distance. To test whether this effect of FGF10 is long-range concentration-dependent, we cultured thalamic explants in media with 100 ng/ml FGF10 evenly distributed. In the presence of FGF10 uniformly distributed, thalamocortical projections fail to extend axons into the gel (Fig. 4J). To address whether FGF signaling is involved in the axon guidance effects, explants are exposed to SU5402, a small molecule inhibitor that suppresses tyrosine kinase activity of FGF receptors [8]. FGF10 beads with SU5402 in the media (Fig. 4K) do not show any significant ability to promote axon outgrowth (Table 1). Taken together, these results support and extend the qualitative observations, indicating a role for FGF10 signaling in the long-range thalamic axonal guidance.

Previous studies show that VGluT1 are important molecules in glutamatergic transmission, and VGluT1 mRNA is expressed strongly in the dorsal thalamus[13]. Our studies show at E5 glutamatergic (VGluT1+) progenitor thalamic neurons have already been present in thalamic explants (Fig. 4L). After culture of another 3 h, many thalamic axons, labelled by the neuron-specific antibody TUJ1, were biased in their growth towards the FGF10-soaked beads (Fig. 5A). However, in PBS groups, thalamic axons extend from different directions, and no apparent thalamic axons deviated towards PBS beads (Fig. 5B). Together, these data illustrate that FGF10 is one of the chemotropic candidates released from the developing prethalamus to direct the growth of thalamic axons.

3.3. Concentration dependent functions of FGF10 in axon guidance of TCAs

Previous studies reveal roles of FGF10 in the guidance of hypothalamic axons at distinct concentrations [5]. As described above, we noted that as some thalamic axons approach the prethalamic explants, they frequently re-orient their growing pathway (18.1 % axon bundles, n = 6), turning away from the prethalamic explants (Fig. 6A arrow). To test whether this is a direct effect of high concentrations of FGF10, thalamic explants were dissected out from E4 embryos, and then cultured with FGF10-soaked beads (high concentration 500 ng/ml) in collagen gels. Co-culture of thalamic explants with high concentration...
Fig. 4. Low concentration FGF10 directs thalamic axons in chick. (A) No outgrowth is detected from thalamic explant cultured alone. (B) Immunolabelling in (A) shows thalamic axons circle around the peripheral component, instead of extending into the collagen gel. (C) Minimal numbers of hypothalamic axons grew out when lateral explants were cultured with control beads soaked in PBS. (D) Axons extend from thalamic explant co-cultured with FGF10+ prethalamic explant. (E) Immunolabelling in D shows FGF10 expression in the prethalamic explant. (F–H) Chick thalamic explants cultured with FGF10 protein-soaked beads shown in whole-mount view, right-hand panel in (F) shows high-powered view of boxed region; (G) Whole-mount immunolabelling shows TUJ1+ axons project from thalamic explants towards FGF10 soaked beads; (H) Significantly more (**P < 0.0001) axons extend from the proximal (p) versus distal (d) face of thalamic explants. Error bars represent standard error of the mean. (I) Section immunolabelling shows intrinsic TUJ1+ axons (arrow) projecting from thalamic explants towards FGF10 soaked beads. (J) With FGF10 uniformly distributed in media, few thalamic were examined in the collagen gel. (K) In the presence of the inhibitor SU5402 (20 μM), axon responsiveness to the chemoattractive FGF10 beads was abolished. (L) VGluT1 positive neurons are present within the E4 thalamic explants. Scale bars represent 100 μm in A, C, D, F, I, J and K. Scale bars represent 50 μm in B and L.
FGF10 soaked beads shows that extensive axon outgrowth from the thalamic explant is observed, but these TUJ1+ thalamic axons appear to be repelled by high concentration FGF10 sources (Fig. 6B and C). Quantitative analyses reveal that significantly greater number of axons in distal section than in proximal section (P < 0.001) (Fig. 6E; Table 1). Furthermore, immunofluorescence results show thalamic axons inside thalamic explants are repelled by high concentration FGF10 before they project into collagen gels (Fig. 6D). These results further confirm our hypothesis that FGF10 works on thalamic axons in a concentration dependent way that high concentration FGF10 exerts an opposite effect on thalamic axons in contrast to low concentration FGF10.

### 3.4. FGF10 is required in the correct targeting of TCAs

To determine whether FGF signaling may exert a direct effect on thalamic axons, we employed RNA interference (RNAi) to silence FGF10 gene expression and to investigate guidance effects of FGF10. The FGF10-RNAi plasmids express GFP markers, allowing simple in vivo tracking of vectors directly (Fig. 7A). Compared to the un-electroporated side (Fig. 7B), the microRNA expression system works significantly, achieving the downregulation of FGF10 in the developing prethalamus (Fig. 7C), confirming the efficiency of this knockdown approach. In comparison to negative controls with none specific RNAi vector expression (Fig. 7D), electroporation of the FGF10-RNAi plasmid induces chaotic axon growth, and precocious turning of thalamic axons in a whole mount view (Fig. 7E). However, electroporation of FGF10-RNAi does not change the expression pattern of thalamic neurons (Fig. 7G and H), indicating that the guidance effects of FGFs are independent of its earlier role in thalamic development. Moreover, in addition of SU5402 in negative controls significantly reduces thalamic axonal extension, and few axon fascicles can be detected but are significantly shorter than negative control groups (Fig. 7F).

### Discussion

Recently, morphogens with evolutionarily-conserved roles in patterning embryonic tissues, such as Hedgehogs (Hhs), bone morphogenetic proteins (BMPs), Wnts and fibroblast growth factors (FGFs) have been reported to act as long-range guidance molecules [14,15]. Here, we investigated whether FGF10 might display such a role during the trajectory formation of TCAs. To avoid potential confounding environment in vivo, we carried out in vitro experiments to determine guidance effects of FGF10 in the thalamus. Our studies reveal a direct effect of FGF10 in thalamic axonal guidance. FGF10 exerts striking orienting effects on thalamic axons in chick explant cultures. Furthermore, analyses of blockaded FGF signaling reveal a direct and indispensable effect of FGF10 on developing thalamic axons.

![Fig. 5. Tropic effects of FGF10 on thalamic axons.](image-url)
4.1. Thalamic-derived FGF10 mediates a direct guidance effect on developing thalamic axons

Morphogens are diffusible proteins that provide patterning information by producing long-range concentration gradients. The patterning function of such morphogens begins at very early stages of development, prior to the differentiation of neurons, so it is reasonable to speculate that the remaining gradient of these morphogens might provide directional information for the axonal navigation at later stages [15]. Early reports, moreover, proved a role for FGF signaling in pathfinding of hypothalamic axons, with compromised FGF signal transduction leading to defects in hypothalamic axonal projection [5,16]. Consistent with these studies that FGFs are potent axon guidance cues, our studies demonstrate that the FGF7 subfamily member FGF10 found in the thalamus directs pioneering thalamic axons towards their target area, the forming internal capsule.

Co-culture experimental results indicate that the FGF10+ prethalamus can stimulate the growth of developing thalamic axons at a distance, raising the possibility that the prethalamus might be a source of an attractive cue(s) for neurosecretory axons in the thalamus. Our results reveal, moreover, that FGF10 expression is restricted to the forming prethalamus during nascent extension of thalamic axons. This suggests that FGF10 might also act as a long range diffusible chemotactant cue in the thalamus.

Within the diencephalon, FGF7 subfamily member FGF3 acts synergistically with FGF8 to pattern the ventral thalamus; however, analyses of the guidance effects of FGFs in the later developing thalamus were not included in these studies [17,18]. Our initial experiments in vitro, showing an outgrowth and re-orientation of thalamic axons, suggested an attractive influence by FGF10 beads. In electroporation experiments in which FGF10-RNAi was majorly transfected throughout the prethalamus, silencing of prethalamic derived FGF10 results in significant defects in the ordered growth of thalamic axons. Moreover, FGF10 knockdown induces chaotic turning of TCA axons through premature prethalamus. Intriguingly, addition of SU5402 results in failure outgrowth of the TCA axons, which not only suggests the necessity of FGFR signaling in TCA projection, but also indicates other FGFs may play a role in thalamic axonal guidance, thus causing much more severe defects than merely FGF10 signaling knockdown. GABAergic Calb2+ neurons and glutamatergic VGluT1+ neurons are present in RNAi thalamic explants, while the innervation defects are apparent, making it unlikely that innervation defects are a secondary consequence of compromised thalamic development. These results indicate that FGFR signalling is required for the long-range chemotactant effect of the developing prethalamus, and FGF10 mediates a direct chemotropic effect in guiding thalamic axons.

Fig. 6. Effects of high concentration FGF10 on the innervation of thalamic axons. (A) Thalamic neurons extend axons in response to prethalamic explants, but some axons (arrow) appear turn away from prethalamic explants. (B) High concentration FGF10 soaked beads repel thalamic axons away from the source of FGF10. (C) In high powered views, TUJ1+ axons repelled by high concentration FGF10. (D) Section immunolabelling shows intrinsic TUJ1+ axons (arrow) projecting from thalamic explants away from high concentration FGF10 soaked beads. (E) Significantly more (**p < 0.0001) axons extend from distal (d) versus proximal (p) face of thalamic explants. Scale bars represent 100 μm.
4.2. A dose-dependent dual function for FGF10 in TCA innervation

Morphogens provide positional information by producing long-range concentration gradients and induce distinct responses in a concentration-dependent manner [19]. For example, only low concentration FGF10 can significantly promote statoacoustic ganglion (SAG) neurite outgrowth [20]. Furthermore, studies reveal that low concentration FGF10 attracts hypothalamic axons, while high concentration FGF10 repels hypothalamic axons [5]. FGF10 is expressed in a graded fashion, with highest levels in the prethalamus, anticipating the caudal-to-rostral innervation of TCAs. Here, by 3-dimensional gel culture system, our studies in chick embryos suggest a concentration-dependent role for FGF10 in regulating thalamic axonal projections, attracting thalamic axons at lower concentrations, but stalling/repelling thalamic axonal growth at high concentrations. Our data suggest that, as Slits in hypothalamus [21], high concentration FGF10 in the hypothalamus also acts as a chemorepellent, steering TCAs away from the hypothalamus, and then into the ventral telencephalon.

Consistent with the emerging idea that classical morphogens are potent axon guidance cues at later developing stages, our studies provide significant insight into the mechanisms of axon guidance within the developing chick thalamus. Taken together, our data suggest a model of thalamic innervation that TCA growth cones integrate FGF signaling over space and time, with low levels of FGF signaling initially attracting TCAs towards the prethalamus, while higher FGF10 levels, from the prethalamus and the hypothalamus, stalling their growth and then repelling TCAs into the developing internal capsule of the ventral telencephalon.

Authors’ contribution

Fang Liu designed the experiment and wrote the manuscript. Kuan Liu, Zhongsheng Lv, Hong Huang and Mingyue Li performed the experiment and collected the data. Li Xiao, Xiang Li and Gang Li statistically analyzed the data.
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