Proposal to determine the role of VZ-derived netrin1 in commissural axon guidance to the FP

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Abstract

Netrin1 has been proposed to act from the floor plate (FP) as a long-range diffusible chemoattractant for commissural axons in the embryonic spinal cord. However, Ntn1 mRNA and protein are also expressed in neural progenitors of the basal plate ventricular zone. In this study, I want to determine whether ventricular zone is another source of netrin-1, if yes, what is the role of VZ-derived netrin1 in commissural axon guidance to the FP.

Purpose

A proposed model for netrin activity in the spinal column of developing human embryos is that netrin emanates from the floor plate (FP) and acts as a diffusible chemoattractant to direct the ventral growth of spinal commissural axon[1](Fig.2). However, Ntn1 mRNA and protein are also expressed in neural progenitors of the basal plate ventricular zone(Fig.1). Raising a question that whether dorsal netrin-1 is produced locally in the ventricular zone, or if it diffuses from the floor plate. If it is produced locally in the ventricular zone, then which source of netrin1 promotes ventrally directed axon growth.

My hypothesis is that netrin-1 is produced locally in the ventricular zone, and FP is not the source of netrin1 directing axons to the ventral midline, while local VZ-supplied netrin1 is required for this step.

This study is important for it may overthrow the incorrect model for netrin activity established so long. And it help us to study futher about ventral growth of spinal commissural axon, to consider floor-plate-independent cellular mechanisms of ipsilateral guidance of commissural axons.

Literature review

During the establishment of neural circuits, neurons need to extend axons along precise pathways toward their synaptic targets. Axon growth cone is a specialized, highly motile structure at tip of extending axon. Growth cones explore the extracellular environment, determine the direction of growth, and then guide the extension of the axon in that direction. Axon growth cones are composed of lamellipodium, a sheetlike expansion of the growing axon at its tip, and filopodia, numerous fine processes that extend from each lamellipodium. The force to move the axon is generated by ATP-dependent modification of the actin and microtubule cytoskeletons.The dynamic polymerization and depolymerization of actin at the membrane of the lamellipodiurn, as well as within the filopodium sets the direction of growth cone movement, in part by generating local forces that orient the growth cone toward or away from substrates. The polymerization and depolymerization of tubulin into microtubules consolidates the direction of movement of the growth cone by stabilizing the axon shaft. However, the mechanisms of regulating polymerization and depolymerization was in dark for a long time. Before 30 years, people found more and more evidence and cliamed that axons navigate over considerable distances, using molecular cues in the embryonic environment to both spatially and temporally orient their growth cones[1].These guidance cues have been proposed to fall into four major categories: attractive or repulsive signals that act as either long-range diffusible molecules or short-range contact-dependent signals[2].

Netrin is one of the first families of chemoattractant molecules to be identified. They are named after the Sanskrit word "netr", which means "one who guides." Though the detailed mechanism of axon guidance is not fully understood, it is known that netrin attraction is mediated through UNC-40/DCC cell surface receptors and repulsion is mediated through UNC-5 receptors. And netrin1 is a member of the laminin superfamily first characterized in the vertebrate spinal cord. Studies in chicken and mouse led to the proposal that netrin1 emanates from the floor plate (FP) and acts as a diffusible chemoattractant to direct the ventral growth of spinal commissural axon.

The other side, commissural neurons project their axons across the midline of the nervous system to contact neurons on the opposite side, mouse commissural neurons are diverse and comprise many subtypes[3]. In the midbrain, hindbrain and spinal cord, commissural neurons transiently express the Robo3 receptor[3]. Ntn1 mRNA is also expressed in neural progenitors of the basal plate ventricular zone. Thus I want to determine whether dorsal netrin-1 is produced locally in the ventricular zone, or if it diffuses from the floor plate. If it is produced locally in the ventricular zone, which source of netrin1 promotes ventrally directed axon growth.

Research plan

Determine whether ventricular zone is another source of netrin-1

Use genetic approaches in mice to selectively remove netrin1 from different regions(FP or VZ) of the spinal cord, then observe the distribution of netrin1. First, conditionally ablated netrin1 from the FP (netrin1 delta FP) using the Shh::cre driver line[4] in combination with a netrin1 flox/flox allele[5]. For Sonic hedgehog, has been shown to be both necessary and sufficient for the induction of floor plate. Thus in these mice, the presence of cre in the FP results in the specific loss of netrin1 protein from the FP. Then observe the presence of netrin-1 in the netrin1 delta FP using Ntn1 hypomorphs (Ntn1 $^{\beta\ geo}$) in which netrin-1 is fused to β -galactosidase (β -gal) and trapped into endosomes[6]. In contrast, removed netrin1 from all dorsal spinal progenitors (netrin1 delta VZ) by recombining the netrin1 flox/flox allele with a Pax3::cre driver line, because Pax3 is active all dorsal spinal progenitors[7]. Then observe the presence of netrin-1 in the netrin1 delta VZ using Ntn1 hypomorphs (Ntn1 β geo). Set a wide type mice as control, observe the presence of netrin-1 in the control group.

Methods and Materials

Here I should generate mutant mice. Netrin1 conditional knockout embryos would like to generated by crossing Shh::cre or Pax3::cre drivers with netrin1 flox/flox mice. Netrin1 flox/flox mice (ref.5), Shh::cre (ref.4) and Pax3::cre (ref.7) would like to maintained in C57BL/6 backgrounds. Ntn1 $^{\beta\ geo}$ knockout lines (ref.6)would like to previously be described and

genotyped by PCR. In addition, in order to observe the distribution of netrin1, Immunohistochemistry is necessary. Mouse embryonic spinal cords (E10.5-E12.5) were fixed in 4% paraformaldehyde for 2 hr at 4 ℃, cryoprotected in 30% sucrose in PBS overnight and thin-sectioned to yield 30 mm transverse sections. Antibody staining was performed by incubating the sections with primary antibodies at 4 ℃ overnight, followed by fluorescently-labeled secondary antibodies at room temperature for 2 hr[8]. Antibodies against netrin1 and b-galactosidase would like to be used for immunostaining. Images would like to be acquired by Vutara 352 Super Resolution Microscope, which based on single molecule localization techniques (PALM, STORM, etc.).Vutara 352 enables quantitative imaging at the nanoscale. Vutara’s proprietary Biplane technology provides 3-D imaging during all acquisitions. The methods, mutant mice generation, immunohistochemistry and super resolution microscope are important in the entire experiment not only this part.

Results

My expected result is that netrin1 is expressed in both of ventricular zone and floor plate in control group. Netrin1 is expressed in ventricular zone in netrin1 delta FP, netrin1 is expressed in floor plate in netrin1 delta VZ. Therefor it can be confirmed that netrin-1 is also produced locally in the ventricular zone. If the result is not as expected, netrin1 is absent in ventricular zone in netrin1 delta FP,netrin1 is expressed in floor plate in netrin1 delta VZ. I can get a conclusion that dorsal netrin-1 is not produced locally in the ventricular zone but diffuses from the floor plate. And there is no need to do later experiments.

Whether FP-Derived Netrin1 or VZ-Derived Netin1 Is Required for Commissural Axon Guidance to the FP

To resolve the role of FP- versus VZ-derived netrin1, I would like to use genetic approaches to determine the spatial requirement for netrin1 in the developing spinal cord. Removing netrin1 expression from either the VZ or the FP, then observe the growth of commissural axons. Generate Gli2 $^{-/-}$ mutants [9], because Gli2 is a key transcriptional regulator that transduces sonic hedgehog (Shh) signaling . And Sonic hedgehog has been shown to be both necessary and sufficient for the

induction of floor plate. The FP is ablated in Gli2 $^{-/-}$ mutants, resulting in the loss of FP-derived netrin1. In contrast, generate netrin1 double mutant, in which netrin1 expression is lost from the VZ in Gli2. Also, set a wide type mice as control. Then compare the phenomenon of three groups, include Netrin1 expression distribution, the growth of commissural axons (including NF+ axons, Robo3 + and Tag1 + commissural axons).

Method and Materials

Here, Gli2 (ref.9) mice were bred into 129/Sv backgrounds, the netrin1 mutant strain stems from lacZ having been inserted into the netrin1 genomic locus and is considered to be a hypomorphic allele. Antibodies against netrin1 and b-galactosidase would like to be used for immunostaining. Images would like to be acquired by Vutara 352 Super Resolution Microscope.

Results

My expected result is that Gli2 -/- mutants, the absence of the FP has no significant effect on the trajectory of commissural axons, they continue to ubiquitously avoid the VZ in similar numbers to control littermates. In contrast, axons robustly extend into the VZ in Gli2; netrin1 $^{lacZ/lacZ}$ spinal cords. Such a expected result show that FP-derived Netrin1 is not required for commissural axon guidance to the FP. If the result is not as expected, axons extend into the VZ in Gli2 -/- mutants, and the phenomenon of axons growth in Gli2; netrin1 double mutants is similar to that of control. Then I can get a conclusion that VZ-derived Netrin1 is not required for commissural axon guidance to the FP.

Discussion

Netrin1 was first identified as a long-range diffusible chemoattractant, secreted by the FP. In this study, I want to confirm a alternative model(Fig.3), to determine whether ventricular zone is another source of netrin-1, if yes, what is the role of VZ-derived netrin1 in commissural axon guidance to the FP. If results are as expected, the netrin1 does not act as a long-range secreted chemoattractant for commissural spinal axons but instead promotes ventrally directed axon outgrowth by haptotaxi.

Figures

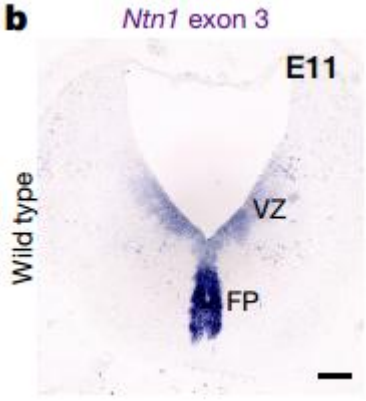

Fig.1 the mRNA encoding the third exon of Ntn1, floxed in the conditional knockout, is expressed in the ventricular zone and floor plate

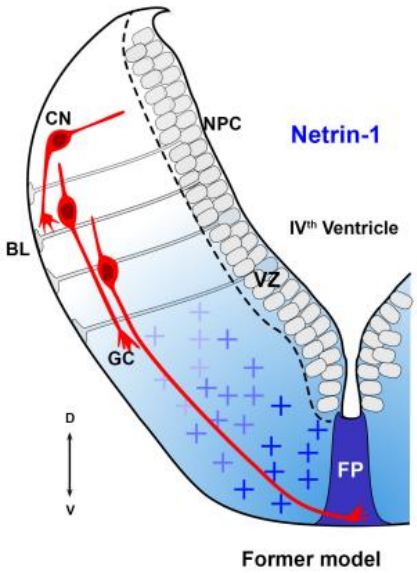

Fig.2 Former model for netrin activity in the spinal column of developing human embryos

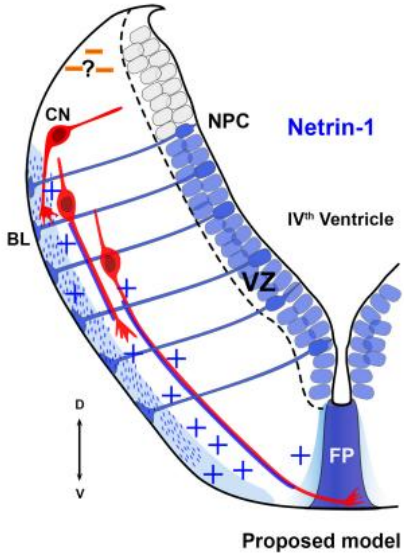

Fig.3 Alternative model for netrin activity in the spinal column of developing human embryos I want to confirmed

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