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The rodent Four-jointed ortholog Fjx1 regulates dendrite extension

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Abstract

The extrinsic and intrinsic factors that regulate the size and complexity of dendritic arborizations are still poorly understood. Here we identify Fjx1, the rodent ortholog of the *Drosophila* planar cell polarity (PCP) protein Four-jointed (Fj), as a new inhibitory factor that regulates dendrite extension. The *Drosophila* gene *four-jointed* (*fj*) has been suggested to provide directional information in wing discs, but the mechanism how it acts is only poorly understood and the function of its mammalian homolog *Fjx1* remains to be investigated. We analyzed the phenotype of a null mutation for mouse *Fjx1*. Homozygous *Fjx1* mutants show an abnormal morphology of dendritic arbors in the hippocampus. In cultured hippocampal neurons from *Fjx1* mutant mice, loss of Fjx1 resulted in an increase in dendrite extension and branching. Addition of Fjx1 to cultures of dissociated hippocampal neurons had the opposite effect and reduced the length of dendrites and decreased dendritic branching. Rescue experiments with cultured neurons showed that Fjx1 can act both cell-autonomously and non-autonomously. Our results identify Fjx1 as a new inhibitory factor that regulates dendrite extension.

Keywords: Four-jointed; Dendrite; Hippocampus; Planar cell polarity

Introduction

The shape and complexity of dendritic arborizations is an important determinant of their function to receive and process information. Despite recent progress in identifying intrinsic and extrinsic factors that regulate dendrite architecture (Jan and Jan, 2003; Whitford et al., 2002a), the signaling pathways that determine the pattern of dendritic arborizations are not well understood. Genetic approaches and in vitro systems allowed the identification of contact-dependent and secreted signals that regulate the development of dendritic arbors. These include signals that stimulate dendrite growth or act as chemotropic guidance cues like Sema3A, Slit1, neurotrophins, BMPs, or Wnt7b (Polleux et al., 2000; Rosso et al., 2005; Whitford et al., 2002a,b). In addition, proteins were identified that mediate contact-dependent homotypic repulsion and limit the extension of dendrites (Whitford et al., 2002a).

A screen for genes that determine the size and shape of dendritic fields in *Drosophila* led to the identification of Flamingo (Fmi) as a signal that mediates homotypic repulsion between dendrites (Gao et al., 1999, 2000; Grueber et al., 2002; Reuter et al., 2003). *Fmi* encodes an atypical cadherin with a large extracellular domain and seven transmembrane segments (Usui et al., 1999). Fmi and its three mammalian homologs (Celsr1–3) are core components of the PCP pathway that determines the polarity of cells in the plane of an epithelium, e.g. in the *Drosophila* eye and wing imaginal discs (Curtin et al., 2003; Fanto and McNeill, 2004; Klein and Mlodzik, 2005). A set of core components is required for PCP in multiple tissues and is highly conserved between species (Seifert and Mlodzik, 2007; Wang and Nathans, 2007; Zallen, 2007). The available evidence suggests that the atypical cadherins Dachsous (Ds) and Fat (Ft) control PCP upstream of the integral membrane protein Frizzled (Fz) and the cytosolic protein Dishevelled (Dsh) to transmit the positional information for the orientation of wing hairs (Klein and Mlodzik, 2005; Tanoue and Takeichi, 2005). In vertebrates, the PCP pathway is required for convergent extension during gastrulation and the development of the sensory epithelium in

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the inner ear. In addition, Celsr2 and -3 have been implicated in neuronal differentiation (Shima et al., 2007; Takeichi, 2007; Wada et al., 2006). Celsr2 is involved in the development of dendritic trees of cortical and cerebellar neurons and is thought to maintain the size and complexity of dendritic arbors by homophilic interactions (Shima et al., 2004). Deletion of *Celsr3* in mice disrupts the formation of major axon fascicles (Tissir et al., 2005).

Genetic experiments suggest that the type II transmembrane protein Four-jointed (Fj) acts upstream of Ds and Ft (Amonlirdviman et al., 2005; Ma et al., 2003). Fj, Ds, and Ft are thought to provide global directional information in the wing disc that aligns the activity of Fz/Dsh in different cells (Amonlirdviman et al., 2005; Ma et al., 2003). The role of Fj is not as well understood as that of other PCP proteins. Proteolytic cleavage of Fj in its transmembrane domain releases the extracellular C-terminal fragment (Buckles et al., 2001; Strutt et al., 2004). Both, a function as a secreted ligand and a role in the maturation of other proteins in the Golgi have been proposed (Buckles et al., 2001; Strutt et al., 2004). It was reported recently that Fj can be found mainly in the Golgi of *Drosophila* S2 cells and imaginal discs (Strutt et al., 2004). Cleavage and secretion apparently are not required for its function since a Fj protein fused to a Golgi localization signal has increased activity compared to the wild type protein (Strutt et al., 2004). However, the mammalian Fj ortholog Fjx1 (Ashery-Padan et al., 1999) is secreted efficiently when expressed in cell lines (Rock et al., 2005a).

Mouse *Fjx1* is expressed in the developing brain, like the mammalian orthologs of *Fmi*, *Ft*, and *Ds* (Ashery-Padan et al., 1999; Rock et al., 2005b; Shima et al., 2002; Tissir et al., 2002). An AP fusion protein of Fjx1 revealed the presence of Fjx1 binding sites in the cortex and hippocampus, supporting the possibility that Fjx1 acts as a ligand for an unknown receptor (Rock et al., 2005a). Here we analyzed the phenotype of *Fjx1* knockout mice and show that Fjx1 is required for the normal development of dendritic arbors in the hippocampus. Our results identify Fjx1 as a new signal that directs the development of dendrites.

Materials and methods

Fjx1 knockout mice

The *Fjx* gene is encoded by a single exon that contains the entire coding sequence. The exon was replaced by the *LacZ* gene followed by a PGK-*neo* resistance cassette. A 4.1 kb *Eco*RI/*Sma*I fragment containing the promoter region and 56 bp of 5′ UTR was cloned in front of a lacZ/PGK-*neo* cassette in pHM2. A 1.7 kb *Bam*HI/*Xba*I fragment containing 632 bp of the 3′ UTR was inserted downstream and the entire insert was then transferred into pPNT (a gift of R.C. Mulligan) to add the HSV-tk negative selection marker. 129/SVJ ES cells were electroporated with the linearized plasmid. Positive clones were identified by Southern blot using a 1.3 kb *Eco*RI (5′) and a 2 kb *Xba*I (3′) probe to detect a 8.5 kb (6.5 kb wt) and a 13.0 kb (9.3 kb wt) *Sac*I fragment, respectively. One positive clone was injected into C57/B6 blastocysts. Germ line transmission was confirmed by Southern blot and PCR. Genotyping by PCR was done using the primers 4J5A (AGGGC TGTCT TCTCT GCCACG), 4J3A (TCCCA AAGAG ACTGC CATTCC) and 4JNeoA (CAGGA CATAG CGTTG GCTACC) for 36 cycles of 30 s at 93 °C, 30 s at 59 °C, and 1 min at 72 °C resulting in fragment of 336 bp and 690 bp for the wild type and mutant alleles, respectively. The *B6.Cg-TgN(Thy1-YFPH)2Jrs* transgenic line that expresses YFP in a subset of neurons (Feng et al., 2000) was obtained from the Jackson Laboratory (Bar Harbor).

Histology and delivery of DiI-coated particles

0.6 μm gold particles (BioRad) were coated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) based on published procedures (Gan et al., 2000). Briefly, 15 mg gold particles were mixed with 5 mg DiI dissolved in 500 μl methylene chloride. Gold particles were coated with DiI by evaporation of methylene chloride, resuspended in 3 ml distilled water and 0.75 μl polyvinylpyrrolidone (20 mg/ml) was added to the sonicated solution. The suspension was used to coat Tefzel tubing (Biorad) according to the manufacturer's recommendation. P15 Fjx1 mouse brains were fixed with 4% PFA in PBS overnight at 4 °C. The hippocampus was dissected and DiI-coated gold particles were delivered using the Helios Gene Gun System (Biorad) with a pressure of 160–180 psi. A membrane filter with a 3-μm pore size and 8.0×10^5 pores/cm^2 (Falcon) was inserted between the Gene Gun and the tissue (Gan et al., 2000). The hippocampus was incubated for 5–7 days in PBS at 30 °C. 100 μm coronal sections were prepared using a vibratome (Leica) and analyzed by confocal microscopy (Zeiss Axiovert 200 M) and the Zeiss AIM LSM510 Image Browser software. Care was taken that the analyzed neurons were fully contained within the z stack collected.

Neuronal culture and transfection

Cultures of dissociated hippocampal neurons were prepared as described previously (Schwamborn et al., 2006). Briefly, the hippocampus was dissected from E18 rat or mouse embryos in ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (Invitrogen), incubated with papain (Sigma) for 15 min at 37 °C, and dissociated by trituration in DMEM (Invitrogen) containing 10% fetal calf serum (Biochrom), 0.5 mM glutamine and 100 U/ml penicillin/streptomycin (Invitrogen). Neurons were plated onto glass coverslips coated with poly-ornithine (Sigma) at a density of 50,000 cells per coverslip and cultured at 37 °C and 5% CO_2. After neurons attached to the substrate (around 4 h after plating) the medium was changed to neurobasal medium with B27 supplement, 0.5 mM glutamine and 100 U/ml penicillin/streptomycin (Invitrogen). At 3 d.i.v., neurobasal medium was changed to OptiMEM (Invitrogen) and neurons were transfected by calcium phosphate coprecipitation using 2.5 μg of DNA per coverslip at 37 °C and 3% CO_2 for 1 h (Schwamborn et al., 2006). After washing with neurobasal medium, the culture was continued in the presence of 3 μM AraC (Sigma). At 7 d.i.v., neurons were fixed in 4% paraformaldehyde and 15% sucrose in phosphate-buffered saline (PBS) for 20 min at room temperature and processed for immunohistochemistry. Expression vectors containing the complete coding sequence of mouse Fjx1 or of Fjx1 fused to alkaline phosphatase have been described before (Rock et al., 2005a). pEGFP-N1 (Clontech) was used as control vector.

Immunofluorescence

The mouse monoclonal Tau-1 and anti-MAP2 antibodies were obtained from Chemicon. Polyclonal rabbit anti-Fjx1 was described before (Rock et al., 2005a). Alexa-594, Alexa-488, and Alexa-350 conjugated secondary antibodies were obtained from Molecular Probes. For staining with the anti-Fjx1 antibody, neurons were fixed with methanol/acetone (1:1) for 20 min at −20 °C.

Neuronal morphology was analyzed using a Zeiss Axiophot microscope equipped with an Orca CCD camera (Hamamatsu) and the WASABI software (Hamamatsu), Imagetool, and Adobe Photoshop. Neurites that were at least twice as long as the other processes and showed Tau-1 immunoreactivity in their distal segments were counted as axons. MAP2-positive neurites longer than the diameter of the cell soma were counted as dendrites.

Generation of recombinant Fjx1 protein

A stable HEK293 cell line expressing a fusion protein of Fjx1 and placental alkaline phosphatase (AP) (Flanagan and Leder, 1990) was used to obtain recombinant Fjx1 (Rock et al., 2005a). 293-fjx1-AP cells were maintained in