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FGF-2 deficiency causes dysregulation of Arhgef6 and downstream targets in the cerebral cortex accompanied by altered neurite outgrowth and dendritic spine morphology

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ABSTRACT

Fibroblast growth factor 2 (FGF-2) is an abundant growth factor in the brain and exerts multiple functions on neural cells ranging from cell division, cell fate determination to differentiation. However, many details of the molecular mechanisms underlying the diverse functions of FGF-2 are poorly understood. In a comparative microarray analysis of motor sensory cortex (MSC) tissue of adult knockout (FGF-$2^{-/-}$) and control (FGF-$2^{+/+}$) mice, we found a substantial number of regulated genes, which are implicated in cytoskeletal machinery dynamics. Specifically, we found a prominent downregulation of Arhgef6. Arhgef6 mRNA was significantly reduced in the FGF-$2^{-/-}$ cortex, and Arhgef6 protein virtually absent, while RhoA protein levels were massively increased and Cdc42 protein levels were reduced. Since Arhgef6 is localized to dendritic spines, we next analyzed dendritic spines of adult FGF$2^{-/-}$ and control mouse cortices. Spine densities were significantly increased, whereas mean length of spines on dendrites of layer V of MSC neurons in adult FGF-$2^{-/-}$ mice was significantly decreased as compared to respective controls. Furthermore, neurite length in dissociated cortical cultures from E18 FGF-$2^{-/-}$ mice was significantly reduced at DIV7 as compared to wildtype neurons. Despite the fact that altered neuronal morphology and alterations in dendritic spines were observed, FGF-$2^{-/-}$ mice behave relatively unsuspicious in several behavioral tasks. However, FGF-$2^{-/-}$ mice exhibited decreased thermal pain sensitivity in the hotplate-test.

1. Introduction

Fibroblast growth factor-2 (FGF-2) is a prominent member of the FGF family and abundantly expressed in brain (for reviews, see Bieger and Unsicker, 1996; Reuss and von Bohlen und Halbach, 2003; Unsicker et al., 2006). Multiple neural functions have been assigned to FGF-2, mostly based on studies, in which FGF-2 was administered exogenously *in vitro* and *in vivo*. For example, early studies have documented the mitogenic and differentiative effects of FGF-2 on astrocytes, the survival and differentiation promoting effects of FGF-2 on cultured and lesioned peripheral and CNS neurons, and mitogenic and differentiative regulation of neural progenitor cells (Bieger and Unsicker, 1996; Unsicker et al., 1987, 2006; Otto and Unsicker, 1990). However, similar to postulated functions of other growth factors, the majority of proposed neural functions of FGFs are extrapolations from pharmacological experiments and applications of exogenous FGFs.

Analyses of mice lacking FGF-2 have failed to support many of the functions originally proposed for FGF-2. For example, loss of about 20% of cortical neurons in FGF-2 deficient mice are likely due to impaired migration rather than lack of "trophic" support (Dono et al., 1998). Loss of calbindin- and parvalbumin-positive cells in the cortex has been described, but not explained mechanistically (Ortega et al., 1998). The proposed role of FGF-2 in adult neurogenesis is still controversial: although the phenotype of the FGF-2 knockout implies a significant decrease in the numbers of

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newly generated neurons in the adult dentate gyrus, the number of proliferating progenitors is not decreased. Most importantly, however, exogenous administration of FGF-2 does not normalize adult hippocampal neurogenesis, suggesting that FGF-2 may regulate adult neurogenesis in cooperation with other growth factors (Werner et al., 2011).

Beyond the overt discrepancies in the pharmacological actions of FGF-2 and its knockout phenotype, there are significant gaps in our understanding of the molecular and cellular mechanisms underlying the FGF-2 knockout phenotype. Thus, neither the migratory deficit and neuron loss in the developing cortex (Dono et al., 1998; Ortega et al., 1998) nor alterations in hippocampal spine morphology visualized in FGF-2 knockout mice are understood in molecular terms (Zechel et al., 2009).

In an attempt towards providing an understanding of the molecular mechanisms that shape the FGF-2 knockout phenotype, we have conducted a comparative microarray analysis of the adult mouse motor sensory cortex (MSC) isolated from FGF-2 knockout and wildtype animals. In addition to a multitude (67) of >2-fold regulated genes, this analysis discovered a substantial number of genes implicated in the regulation of the cytoskeletal machinery. These included, *inter alia*, Arhgef6, Palladin (Palld), Tubulin beta6 (Tubb6), and Myosin 3a (Myo3a). Neuronal cytoskeleton and several of its key molecular regulators are important *physiological* targets of FGF-2 *in vivo*, possibly underlying impaired synaptic structure and function seen in FGF-2 deficient mice (Zechel et al., 2009, 2010). Based on the microarray results, we also investigated whether structural alterations (beside the known reduction in neuron numbers) were found in cortical tissue derived from FGF-2 deficient mice. Using Golgi-impregnation we could demonstrate that FGF2-deficient mice display alterations in spine densities and spine morphology in the MSC. Furthermore, we investigated whether the alterations seen in the cortex of FGF-2 deficient mice translate into altered behavior. However, except for decreased thermal sensitivity revealed in the hot plate test, all other behavioral parameters tested were inconspicuous.

2. Material and methods

2.1. Animals

Adult (9–11 week-old) male wildtype and FGF-2$^{-/-}$ mice used for the study were of the strain generated by Rosanna Dono and Rolf Zeller (Dono et al., 1998). FGF-2$^{-/-}$ mice and their wildtpye littermates were obtained by breeding of heterozygous FGF-2-deficient mice. All animal experiments reported here were conducted in accordance with the guidelines of the European Community Council.

2.2. RNA isolation and microarray analysis

RNA was extracted using RNeasy Mini-Kit. The RNA quality was assessed using the Agilent 2100 Bioanalyzer and all RNA samples used for analysis showed a 260/280 ratio between 1.8 and 2.0. Five micrograms of RNA were used for microarray experiments. Microarray analysis was performed with tissue from 3 animals per condition at the Center for Biosystems Analysis at the University of Freiburg using the Agilent SingleColor 14868 chip.

2.3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Tissues were homogenized and RNA was extracted using the TRIzol Reagent (Lifetechnologies, Darmstadt, Germany). Primer sequences were picked from Primerbank (http://pga.mgh.harvard.edu/primerbank/) and controlled for efficiency (between 90%–110%). Their sequences are shown in Table 1. Amplification was monitored by measuring the fluorometric intensity of SYBR Green I at the end of each elongation phase and the expression of the housekeeping gene 18S was quantified for normalization in each sample. The change threshold cycle number (ΔCt) was normalized to the mean of the reference gene 18S, by subtracting $\Delta Ct_{referencegene}$ from $\Delta Ct_{targetgene}$. Fold induction was determined by calculating $2^{\Delta\Delta Ct}$. Data is given as fold change of target cDNA relative to the control.

Table 1
Primers used for Real Time-PCR.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Arhgef6	ATGAATCCAGAAGAACGCCT	GGTTCCAGACAATACTTCTCCA
Cdc42	TCCACTTACTGTGGAGGCCTG	CAGGGAAGAGGAACAGAATGG
Cofilin	TCTGGGCCCCCGAGAAT	TTGATGGCATCCTTGGAGC
Ctnnb1	CCCAGTCCTTCACGCAAGAG	CATCTAGCGTCTCAGGGAACA
Erk1	GAGCACTTGGGTTACTCCACG	TCAGATGAATTTTCGTTGGCAGA
Erk2	TCCGCCATGAGAATGTTATAGGC	GGTGGTGTTGATAAGCAGATTGG
Jnk1	CACCATTGCATGTCCATCTG	GGCTTGATAACTCATGGCTG
Jnk2	TCAGTGGGTTGCATCATGGG	GGATGGTGTTCCTAGCTGTTCA
Jnk3	CCCGGAAGGGACTATATTGAC	CTCCACGTAGTTTCTGACTG
Moesin	GGTCCTTGTTGAGTTTGTGCT	TCTTATGCCGTCCAGTCTAAGT
Rock2	TGTTGGCAAAGGCCATAATATCT	TTGGTTCGTCATAAGGCATCAC
RhoA	AGCTTGTGGTAAGACATGCTTG	GTGTCCCATAAAGCCAACTCTAC

2.4. Western blotting

Protein levels were quantified by Western blotting. In brief, the homogenized proteins from mouse MSC cortices were subject to SDS-PAGE and transferred to nitrocellulose membrane or polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in a buffer solution, containing 0.1% Triton X and 5% milk powder, for one hr at room temperature (RT). Subsequently, the membrane was incubated overnight at 4 °C with primary antibodies directed against Arhgef6, phosphorylated Jnk1/2/3, Moesin, Rock2 (each 1:1000; Santa Cruz, Heidelberg, Germany), Rac1, Cdc42, RhoA (each 1:500; Cellbiolabs, Heidelberg, Germany), β-Catenin, phosphorylated Cofilin and Erk1/2, Gapdh (each 1:1000; Cell Signaling, Frankfurt/Main, Germany) and β-Actin (1:1000; Sigma-Aldrich, Taufkirchen, Germany). After incubation and three washing steps with phosphate buffer saline and 0.1% Triton X-100 (PBS-T) the membrane was probed with horse-radish peroxidase-linked secondary antibodies for 1 h at RT. Blots were processed using an Imagequant LAS 4000 (GE Healthcare, Solingen, Germany). Signal levels were normalized to ß-actin or Gapdh, and quantifications of the immunoblots were performed using the ImageJ Imaging Program (NIH, USA).

2.5. Primary cortical cell culture and neurite outgrowth assay

FGF-2$^{-/-}$ and wildtype mice (n = 3, each; tissues from 2 animals pooled per n) at E18 were used to establish dissociated cell cultures from the MSC cortex. Cortices were isolated, dissociated using 0.25% trypsin with 0.025% DNAse and triturated with fire-polished Pasteur pipettes. Cell cultures were plated at a density of 12,000 cells onto Poly-ornithine/Laminin-coated (Sigma-Aldrich, Germany) glass coverslips (12 mm diameter), which were maintained in a 24-well culture plate with Neurobasal A medium (Life Technologies) supplemented with B27 (2%; Life Technologies), 200 mM α-glutamine (0.25%; Life Technologies), GlutaMAX 100 (1%; Life Technologies), and streptomycin/penicillin (1%; Life Technologies). Starting at DIV3, 50% of the medium was changed every 3 days. Cultures were maintained until DIV7. To test for phenotype rescue, cultures from FGF-2$^{-/-}$ mice (n = 3) were treated with FGF-2 (20 ng/ml, Life Technologies) starting at DIV1. Cultures were fixed at DIV7 in 4% PFA/PBS for 20 min (RT), permeabilized