Short-Hairpin RNA Silencing of Endogenous Fibroblast Growth Factor 2 in Rat Hippocampus Increases Anxiety Behavior

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Background: The fibroblast growth factor system has been implicated in the pathophysiology of mood disorders in humans and in affective behavior in animal models. However, the studies have been either correlative or involved exogenous administration of fibroblast growth factor 2 (FGF2). None of them have directly linked endogenous FGF2 to changes in emotional responses. Therefore, we began a series of studies to knockdown FGF2 by RNA interference to examine the role of brain FGF2 in emotional responsiveness.

Methods: We assessed the efficacy of short-hairpin RNA (shRNA) sequences targeted to FGF2 in COS7 cells transfected with a plasmid vector containing the full-length FGF2 sequence. We then sought to assess the effects of knocking down FGF2 gene expression in vivo on behavior. We microinjected a lentiviral vector containing either a shRNA targeting FGF2 or a nonsilencing sequence bilaterally into the dentate gyrus of the rat.

Results: In a reporter assay system, three different shRNA sequences resulted in significant FGF2 knockdown in vitro. Five weeks following a single microinjection of one of those sequences in vivo, we observed a significant decrease in FGF2 gene expression by messenger RNA in situ hybridization in the hippocampus. The FGF2 knockdown increased the time spent in the closed arms of the elevated-plus maze, a test of anxiety behavior.

Conclusions: The FGF2 knockdown in the hippocampus resulted in an anxiogenic effect. Together with our findings of an inverse correlation between anxiety and FGF2 expression levels, these results implicate FGF2 in the genesis and expression of anxiety disorders.

Key Words: Dentate gyrus, elevated plus-maze, fibroblast growth factor, gene, lentiviral, mRNA

The fibroblast growth factor system modulates several important functions in the central nervous system, including neurogenesis. The role of fibroblast growth factor 2 (FGF2) in ischemia, as well as neurodegenerative disorders, has been well studied (1-4). However, its role in mood disorders has only been investigated within the last decade. In postmortem brains of individuals with major depressive disorder, FGF2 gene expression was downregulated in several cortical and limbic structures, including the hippocampus (5,6).

Stress exposure or glucocorticoid administration increased FGF2 expression in the hippocampus in animal models (7-13). Specifically, hippocampal FGF2 gene expression was increased in response to anxiogenic stimuli, such as acute restraint (9). Moreover, acute corticosterone administration upregulated FGF2 in the hippocampus (11). The acute response of FGF2 upregulation in response to stress activation is thought to be neuroprotective. Under chronic conditions, such as repeated stress, adrenalectomy, and chronic administration of corticosterone, FGF2 gene expression was decreased (7-13). We previously have shown that repeated social defeat stress, an animal model of depression, decreased FGF2 gene expression in the hippocampus (14). We have also observed a positive correlation between the peak in gene expression of FGF2 in the hippocampus and the peak of corticosterone secretion (C.A.T. *et al.*, unpublished observations, March 2010). However, glucocorticoid modulation or secretion has not yet been studied following FGF2 administration or in FGF2 knockout mice. Taken together, these findings lend support for chronic knockdown of FGF2 in the hippocampus, resulting in an animal that may be more vulnerable to a stressor.

The hippocampus plays a variety of critical roles in processing emotionally salient information and controlling behavior (15–17). More recent evidence suggests that the hippocampus may play a functional role in modulating anxiety-like behavior. For example, hippocampal lesions and direct intrahippocampal injections have been shown to alter anxiety-like behavior (15,18–20). Furthermore, the role of the hippocampus in modulating individual differences in anxiety-like behavior has previously been documented (21). Here, animals that naturally differ in anxiety-like behavior exhibit differences in hippocampal glucocorticoid receptor (GR) expression. Specifically, high-anxiety animals exhibit higher gene expression of GR compared with low-anxiety animals. Moreover, the differences in hippocampal GR expression are likely responsible for the individual differences in anxiety-like behavior, as hippocampal microinjections with a GR antagonist disrupt the individual differences.

Further support for the role of FGF2 in mood disorders was found following pharmacological manipulations. For example, chronic antidepressants increased hippocampal FGF2 (22,23). Studies from our laboratory have revealed antidepressant and anxiolytic effects of chronically administered FGF2 (24,25). Interestingly, Perez *et al.* (24) reported an increase in the survival of adult-born neurons and glia in the dentate gyrus (DG) after chronic FGF2 administration. The effect on hippocampal neurogenesis was observed in animals that were genetically more prone to anxiety and exhibited an anxiolytic response to chronic FGF2 administration. Fibroblast growth factor 2 administration more than likely decreased the vulnerability to anxiety by increasing the survival of adult stem cells, particularly the generation of new astrocytes. This led us to the hypothesis that basal levels of FGF2 and the modula-

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Received Jul 13, 2010; revised Nov 9, 2010; accepted Nov 17, 2010.

tion of those levels, particularly in the hippocampus, play a role in vulnerability or resilience to anxiety.

The body of work described above, while highly suggestive, never directly established a causal relationship between endogenous levels of FGF2 in the hippocampus and anxiety behavior. To address this issue, it is critical to demonstrate two features of FGF2 in relation to affective behavior: 1) that basal levels of FGF2 messenger RNA (mRNA) in the hippocampus correlate with a behavioral index of emotionality; and 2) that direct manipulation of endogenous FGF2 expression has an impact on emotional responsiveness. In this study, we address the causal relationship between hippocampal FGF2 and anxiety behavior by correlating FGF2 expression with responsiveness in a test of anxiety and then knocking down its expression in the hippocampus and ascertaining the results.

We relied on RNA interference (RNAi) as a method to selectively decrease FGF2 expression, as RNAi represents an efficient and specific tool to suppress gene expression in mammalian cells (26). In this study, we coupled RNAi to a viral vector, which would allow us to inject it once and allow recovery before any behavioral testing. RNA interference in vivo, using transgenic approaches or viral transfer, has recently proven to be useful in elucidating the neural function of a number of specific transcripts (27–32). In particular, when combined with lentiviral vectors, short-hairpin RNAs (shRNAs) have been shown to be stably expressed in the central nervous system in vivo for several months (27). Thus, in the present study, we describe the development of a targeted RNA interference system using a lentiviral vector expressing an shRNA sequence targeted to FGF2, and we test the hypothesis that its microinjection into the dentate gyrus increases anxiety-like behavior.

Methods and Materials

shRNA and Lentivirus Construction

Target sites for rat FGF2 mRNA (accession number: NM_019305) were selected using RNAi design sites (Dharmacon, Chicago, Illinois; and Invitrogen, Carlsbad, California). Three shRNAs targeting different regions of FGF2 mRNA and a scrambled nonsilencing (NS) control shRNA that does not correspond to any mammalian mRNA were generated as nucleotide inverse repeats separated by a nine-nucleotide loop sequence (UUCAAGAGA). The target sites were as follows: FGF2 shRNA-1 (784–804), FGF2 shRNA-2 (862–882), and FGF2 shRNA–3 (797–817) (Table 1). The shRNA sequences were inserted downstream of the U6 promoter in the lentiviral vector pLL3.7, which also expressed green fluorescent protein (GFP), and the resultant knockdown efficacy was tested in vitro in COS7 cells. All shRNA constructs in the vector were verified before use by sequencing.

Table 1. Nonsilencing and FGF2 shRNA Sequences Used in the In Vitro Experiment

shRNA	Sequence
NS shRNA	UUCUCCGAACGUGUCACGUUU
	UUAAGAGGCUUGCACAGUGCA
FGF2 shRNA-1	GAAGGAAGAUGGACGGCUGUU
	UUCUUCCUUCUACCUGCCGAC
FGF2 shRNA-2	CUACAACACUUACCGGUCAUU
	UUGAUGUUGUGAAUGGCCAGU
FGF2 shRNA-3	CGGCUGCUGGCUUCUAAGUGU
	UUGCCGACGACCGAAGAUUCA

FGF2, fibroblast growth factor 2; NS, nonsilencing; shRNA, short hairpin RNA.

Based on its high efficacy and low toxicity in vitro, FGF2 shRNA-1 was chosen for use in vivo. Sequences with high toxicity (FGF2 shRNA-2 and FGF2 shRNA-3) typically exhibited a confluency of less than 40%, whereas confluency was 90% to 100% in nontoxic conditions. Lentiviral vectors containing either FGF2 shRNA-1 or NS shRNA were sent to the University of Michigan Vector Core and lentiviruses expressing FGF2 shRNA-1 (LVshFGF2-1) and NS shRNA (LVshNS) were generated (5×10^8 transducing units).

Cell Culture Studies

The COS7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Invitrogen). Because COS7 cells do not express FGF2 constitutively, rat FGF2 complementary DNA (cDNA) was introduced into the cells by a plasmid vector. The psiCHECK2 vector (Promega, Madison, Wisconsin) was chosen for this purpose because of its dual reporter specificity. Briefly, full-length rat FGF2 cDNA (a generous gift from Dr. Peter Cattini) was subcloned downstream of the *Renilla* luciferase (RL) reporter, enabling expression of a fused mRNA to which shRNAs bind and cleave, resulting in the degradation of both products. The *Firefly* luciferase (FL) reporter enabled normalization of RL expression. The ratio of RL/FL was used as a measure of knockdown efficiency.

One day after the cells were plated onto six-well plates, the cells were co-transfected with the psiCHECK2 vector containing rat FGF2 cDNA and the pLL3.7 vector containing either the NS shRNA or one of the FGF2 shRNA sequences ($n = 3$ wells/group). The concentration of plasmid transfected was 1 ng/μL. For a description of the lentivirus, readers are referred to Mahairaki *et al.* (30). T-2 transfection reagent was used to facilitate transfection (Dharmacon). Forty-eight hours after transfection, the cells were lysed with passive lysis buffer and RL and FL of the lysates were determined according to the manufacturer's instructions (Promega).

Animals

Eleven male Sprague-Dawley rats from generation five of an inhouse breeding colony weighing 385 to 450 g were used for the initial assessment of FGF2 gene expression and anxiety-like behavior, some of which had undergone 21 days of an enriched environment paradigm (for methods, see [18]). Twenty-three male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts) weighing 350 to 400 g were used for the shRNA microinjection study. These animals were habituated to the housing conditions for 1 week before any manipulations. All animals were housed under a 12-hour light/dark cycle with food and water available ad libitum. The animals were singly housed for the first 3 days following surgery. For the remainder of the experiment, the animals were housed two per cage, and the weight of the animals was monitored carefully throughout the experiment. All the procedures were performed in accordance with the National Institutes of Health Guidelines on Laboratory Animal Use and Care and in accordance with the guidelines set by the university committee on use and care of animals at the University of Michigan.

Surgeries

Under isoflurane anesthesia, 33-gauge microinjectors were lowered bilaterally into the DG (coordinates from bregma: anteroposterior −5.0, mediolateral ±3.5, dorsoventral −2.6). Microinjectors were connected to Hamilton syringes mounted on a syringe pump (Harvard Apparatus, Holliston, Massachusetts) by PE-50 tubing. Animals were microinjected with 1 μL of either LVshFGF2-1 or LVshNS (Lentivirus; University of Michigan Vector Core, Ann Arbor, Michigan) over 4 minutes at a rate of .25 μL/minute. After 5 minutes, the