

Fibroblast Growth Factor 2 Modulates Hypothalamic Pituitary Axis Activity and Anxiety Behavior Through Glucocorticoid Receptors

Supplemental Information

Supplemental Methods and Materials

Mice

fgf2 exon 1 homozygous knock out mice (genetic background, Black Swiss) (1, 2) (FGF2 KO) and wild type controls were bred and offspring genotyped as described previously (3). We previously described the conditional embryonic hGFAP-cre *fgfr2* KO (4), the germline *fgfr3* KO (5); and the double embryonic *Emx1-cre fgfr1* and *fgfr2* conditional KO (6). Only male mice were used in experiments. All groups of transgenic mice included mice originating from at least two different litters from different parents, in order to control for litter or maternal effects. All FGF2 KO mice in experiment 1 were injected with saline every day for 25 days prior to the behavioral analysis, as they were the control arm of a larger, ongoing study. In order to verify that the described anxiety phenotype was not due to this prior experience, we tested an independent group of naïve male animals on the open field test and forced swim test ($n = 6$ FGF2 KO and $n = 6$ wild type mice) as described in the results section. Corticosterone assays at baseline and at the end of a 60-minute restrain stress were conducted in an independent cohort of naïve FGF2 KO ($n = 5-6$ per group). All subsequent basal corticosterone experiments had at least 5 mice/group and behavioral experiments had between 6-11 mice per treatment group, as explained in figure captions. All injections, behavioral tests and blood collection experiments were conducted during the light cycle at the same time of day, across all studies. All animal experiments comply with Institutional and National policies and guidelines.

Behavioral Analysis

Locomotor/Open Field. Activity levels were assessed by placing mice in a plastic open field (26 x 48 cm) for twenty minutes. Total distance traveled and average speed were calculated using Any-Maze software (version 4.7.1). Anxiety levels in the open field task were assessed by examining the percent time and distance traveled in the center versus periphery of the open field during the first ten minutes of the locomotor test. Because differences in anxiety behavior were only observed in the first five minutes of testing, in subsequent experiments, open field tasks examined anxiety behavior for five minutes only.

Elevated Plus Maze. Mice were tested on the elevated plus maze (Mouse EPM, purchased from Stoelting, USA) for five minutes. Mice were placed in the center of the maze and monitored for five minutes.

Sucrose Consumption Test. Mice were first habituated to 1% sucrose solution (Sigma-Aldrich, USA) for approximately 48 hours. Sucrose consumption was then measured by presenting 1% sucrose bottles in home cages for one-hour the next morning (24 hours later). All sucrose and water tests were done between 9 and 11 AM and were preceded by an overnight fluid deprivation. The amount of sucrose intake was measured by weighing the bottles before and after the one-hour test. Sucrose consumption tests were conducted 1 week apart, before and after other behavioral tests to determine if an increase in stress as a result of behavioral testing would influence sucrose intake in mice with differences in their genotype. One-hour water consumption test was done the day after the last test as a control measure.

Forced Swim Test. Briefly, mice were placed in a glass cylinder (12 cm diameter) filled to a depth of 10 cm with water (23-25°C). A 10-minute swim test session was videotaped, and the time spent immobile during the swim session was recorded blindly.

Accelerating Rotarod Test. Motor learning was measured by the rotarod task on three consecutive days, for six trials per day (AccuRotor, AccuScan Instruments, Columbus, OH). The time required for mice to fall from the rotating apparatus was recorded.

Corticosterone Assays

Corticosterone assays were performed using blood from corticosterone responsivity assays in experiment 1 or using trunk blood collected at time of sacrifice in experiment 2.

Experiment 1: Retroorbital blood was collected from awake, naïve, male *fgf2* knockout and wild type mice (baseline). Two weeks later, mice were placed in clear plastic restrainers for 60 minutes and blood was collected at 60 minutes (peak) as they were removed from restrainers. The lag time for blood collection was on average 2 minutes and always less than 5 minutes.

Experiment 1: Blood samples were collected in EDTA coated Eppendorf tubes, spun for 10 minutes at 4°C and the serum collected and stored at -80°C until processing. Corticosterone levels were determined by ELISA using the Assay Design kit (Corticosterone #900-097, Lot# D1260724), and quantified on a microplate reader set to 405 nm and to 570-590 nm for correction.

Immunostaining

All mice were given an overdose of ketamine/xylazine and transcardially perfused with phosphate buffered saline (PBS) followed by 25 ml of 4% paraformaldehyde. Brains were harvested and post-fixed at 4°C for 48 hours in 4% paraformaldehyde containing 30% sucrose.

Brains were sectioned (50 µm) on a cryostat and series of sections (1 in every 8) were stored in Watson's cryoprotectant (7) at -20°C.

Free-floating sections were washed three times for 5 minutes in PBS and then incubated for 1 hour at room temperature in PBS containing 0.3% Triton (PBS-T) and 10% goat serum (10% GS/PBS-T) or 10% donkey serum (10% DS/PBS-T). Sections were then incubated in a solution of primary antibody in 10% GS/PBS-T or DS/PBS-T overnight at 4°C (except for GR staining, which was incubated at room temperature). Primary antibodies were neuronal nuclei (NeuN, 1:500, Millipore); glucocorticoid receptor (GR; 1:50, Santa Cruz); corticotropin releasing hormone (CRF; 1:100, Thermo-Scientific; 1:5000, a kind gift of Dr. Wylie Vale); glial fibrillary acidic protein (GFAP, mouse monoclonal; 1:500, Sigma). Sections were washed thoroughly and then reacted with the secondary antibody of the appropriate species: Alexa 488 or 594 (Molecular Probes), Cy3 (Jackson labs), all at a dilution of 1:500 and Dylight 649 (Jackson Labs) at 1:300.

Cell Counting and Microscopic Analysis

Unbiased stereological estimates of cell number were obtained via a Zeiss Axioskope 2 Mot Plus (Carl Zeiss, Thornwood, NY, USA) attached to a motorized stage and connected to a computer running the StereoInvestigator Software (MicroBrightfield, Colchester, VT, USA). Serial coronal sections (one every 400 μm) were used for all counts. Contours of the hippocampus and paraventricular nucleus (PVN) were drawn based on DAPI staining. Immunoreactive cells were counted using the optical fractionator probe with a 40X oil-immersion objective. Sampling grids sized 459.4 μm x 156.4 μm were used for the CA1, 300 μm x 135 μm for the DG, and 53.1 μm x 55.5 μm for the PVN and automatically placed by StereoInvestigator at each grid intersection point. Images presented in figures were acquired on an ApoTome equipped Axiovert 200M with Axiovision 4.5 software (Carl Zeiss, Thornwood, NY, USA).

Quantitative Real Time PCR

For quantitative real time PCR experiments, mice were sacrificed and brains rapidly harvested and stored at -80°C until the amygdala and hippocampus were dissected and RNA was extracted using the STRATAGENE RNA isolation kit (Agilent). GR, FGFR1, FGFR2, FGFRL1, and Egr-1 levels were assessed using TAQMAN assays (Life Technologies) and the exon17 GR promoter region was amplified using SYBRGreen (Life Technologies) using forward primer CTCCCGAGCGGTTCCAAG and reverse primer CTGGGAGGGAAAGCGAGTTT all conducted and analyzed using the Applied Biosystems STEP ONE machine and software.

Supplemental References

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