Antidepressant Effects of Fibroblast Growth Factor-2 in Behavioral and Cellular Models of Depression

Supplemental Information

Supplemental Materials and Methods

Rats were administered intraperitoneal fluoxetine hydrochloride (5 mg/kg; Lilly, Indianapolis, IN) or saline for 21 days.

Cannula Implantation and Microinjection

During surgery day, rodents were anesthetized with a ketamine (LLOYD Laboratories, Shenandoah, IA) & xylazine (Fort Dodge Animal Health, Overland Park, KS) cocktail (100 mg/kg, 10 mg/kg for mice and 80 mg/kg and 6 mg/kg respectively for rats). *For experiments involving central infusions of fibroblast growth factor-2 (FGF2)*, mice were implanted with a mini-osmotic pump (Plastics1, Roanoke, VA; model 2002, Brain infusion Kit3) that infused recombinant mouse FGF2 (R&D Systems, Minneapolis, MN; 0.5 µl/hour, 14 days; 240 ng/day) or saline (vehicle) into one of the randomly assigned lateral ventricles (-0.2 mm anterior/posterior; ±1 medial/lateral; 2.5 mm depth). *For experiments involving central infusions of SU5402 (Calbiochem, San Diego, CA) or DMSO* (vehicle), mice were implanted with a 20-gauge guide cannula into one of the lateral ventricle (-0.2 mm anterior/posterior; ±1 mm medial/lateral; depth 2.25 mm). After 1-2 days of recovery, mice were infused with SU5402 (2.5 µg/day; 1 µl; 0.25 µl/min) or DMSO using an infusion cannula connected by polyethylene tubing to 5 µl Hamilton microsyringes (Hamilton Company, Reno, NV) with a 0.25 mm projection (for details see diagrams in Figures 2A and C). The infusion cannula remained in place for 2-3 min

before being pulled out to allow for enough time for the drug to diffuse into the tissue. The dose of SU5402 chosen was founded on its ability to block FGF2-induced increase in cell proliferation *in vivo* in the subventricular zone (1). *For experiments involving infusions of FGF2 into the prefrontal cortex:* A 22-gauge guide cannula was implanted bilaterally in the prelimbic cortex of rats (Depth -3.5 mm from dura; anterior/posterior +3.2 mm; medial/lateral ±1 mm). After a 2-week recovery, cannulated rats were infused with recombinant human FGF2 (95% homology to rat FGF2; 200 ng/μl; R&D Systems) or saline (vehicle) via an infusion cannula connected by polyethylene tubing to 5 μl Hamilton microsyringes (Hamilton Company). The infusion cannula protrudes 0.5 mm beyond the guide cannula. An infusion volume of 1 μl (0.5 μl/side) was delivered at a rate of 0.1 μl/min. Again, we waited 2-3 minutes before removing the infusion cannula. *For infusions of FGF2 into the dorsal striatum:* A 22-gauge guide cannula was implanted bilaterally in the caudate putamen of rats (Depth -5 mm from dura; anterior/posterior +1.6 mm; medial/lateral ±1.9 mm). After a 10-day recovery, cannulated rats were infused with recombinant human FGF2 (200 ng/μl; R&D Systems) or saline (vehicle) via an infusion cannula connected by polyethylene tubing to 5 μl Hamilton microsyringes (Hamilton Company). The infusion cannula protrudes 0.5 mm beyond the guide cannula. An infusion volume of 1 μl (0.5 μl/side) was delivered at a rate of 0.1 μl/min. Again, we waited 2-3 minutes before removing the infusion cannula.

Chronic Unpredictable Stress (CUS) procedure

Rats were exposed to a variable sequence of mild and unpredictable stressors for 35 days, a procedure that we have found to produce depressive-like behavioral changes (2,3). Rats received two stressors/day. The stressors included rotation on a shaker, placement in a 4°C

ambient temperature, lights off for 3 hours (10 AM – 1 PM), lights overnight, 45°C tilted cages, isolation, food deprivation, odor, stroboscope, crowding, wet bedding and swimming. For a sequence of stressors see Table S1.

Sucrose Consumption Test

Mice were tested on sucrose consumption test to examine whether antidepressants or SU5402 influence sucrose intake. Mice were first habituated to 1% sucrose solution for 48 hours. Sucrose consumption was then measured by presenting 1% sucrose bottles in home cages for 1 hour the next morning. This was followed by 1 hour water test the day after as a control measure. 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 1 hour test and then the difference was normalized to body weight of the respective animal.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

After sacrifice, frontal cortex (enriched with prefrontal cortex) was dissected at 2.2 mm anterior from bregma on ice and stored at -80°C until processed. Total RNA was extracted, 500 ng was reverse-transcribed into complementary DNA and then underwent real-time PCR, for details refer to (4). We used the Primer3 (v.0.4.0) program to design forward & reverse primers for specific genes and specificity was verified using nucleotide blast software (BLAST Interface, NCBI). Relative concentrations of specific genes were normalized against house-keeping gene cyclin D; then fold change was calculated as 2^ of the difference between the concentration of

the normalized gene for each animal and the average of the control. The primers used are listed below:

FGF2:

Forward primer 5’-gcgacccacacgtcaaactacagc-3’

Reverse primer 5’-gaagccagcagccgtccatcttc-3’

Fibroblast growth factor receptor-1 (FGFR1):

Forward primer 5’-gcatggttgaccgttctggaagc-3’

Reverse primer 5’-cgctcttcttggtgccgctcttc-3’

In situ **Hybridization**

In situ hybridization procedure was conducted according to standard protocols used in our laboratory (4, 5). FGFR1 primers were designed using the Primer3 website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers and PCR product were blasted to ensure specificity. The primers used are listed below. Reverse primer contained a T7-binding promoter (sequence shown below in capital letters).

Forward primer 5’-ccctgaggcgttgtttgacc -3’

Reverse primer

5’CCAAGCCTTCTAATACGACTCACTATAGGGAGAggtccagcggtatggacagg-3’

A template PCR product was generated with the above primers in PCR reaction and was verified by sequencing to ensure specificity to FGFR1. Using MAXIscript kit T7 polymerase (Ambion, Austin, TX), we generated FGFR1 specific antisense complementary RNA probes by an in vitro transcription reaction using a PCR product-derived template. Probes were radiolabeled with ^{35}S-rCTP and two million counts (per slide) of FGFR1 were used to hybridize

unto fixed brain sections (16 μm) at 55°C overnight. On day 2, following a series of different washes, slides were exposed to Kodak MR autoradiographic film. Images were captured using a computer-controlled digital camera (Cohu, Poway, CA) and imported into Image J (Scion Corp, Frederick, MD) for densitometric analysis. Using the manufacturer's calibration scale, raw densitometry data were converted to nCi of 14C per gram of tissue, which are linearly related to the tissue levels of the specific messenger RNA.

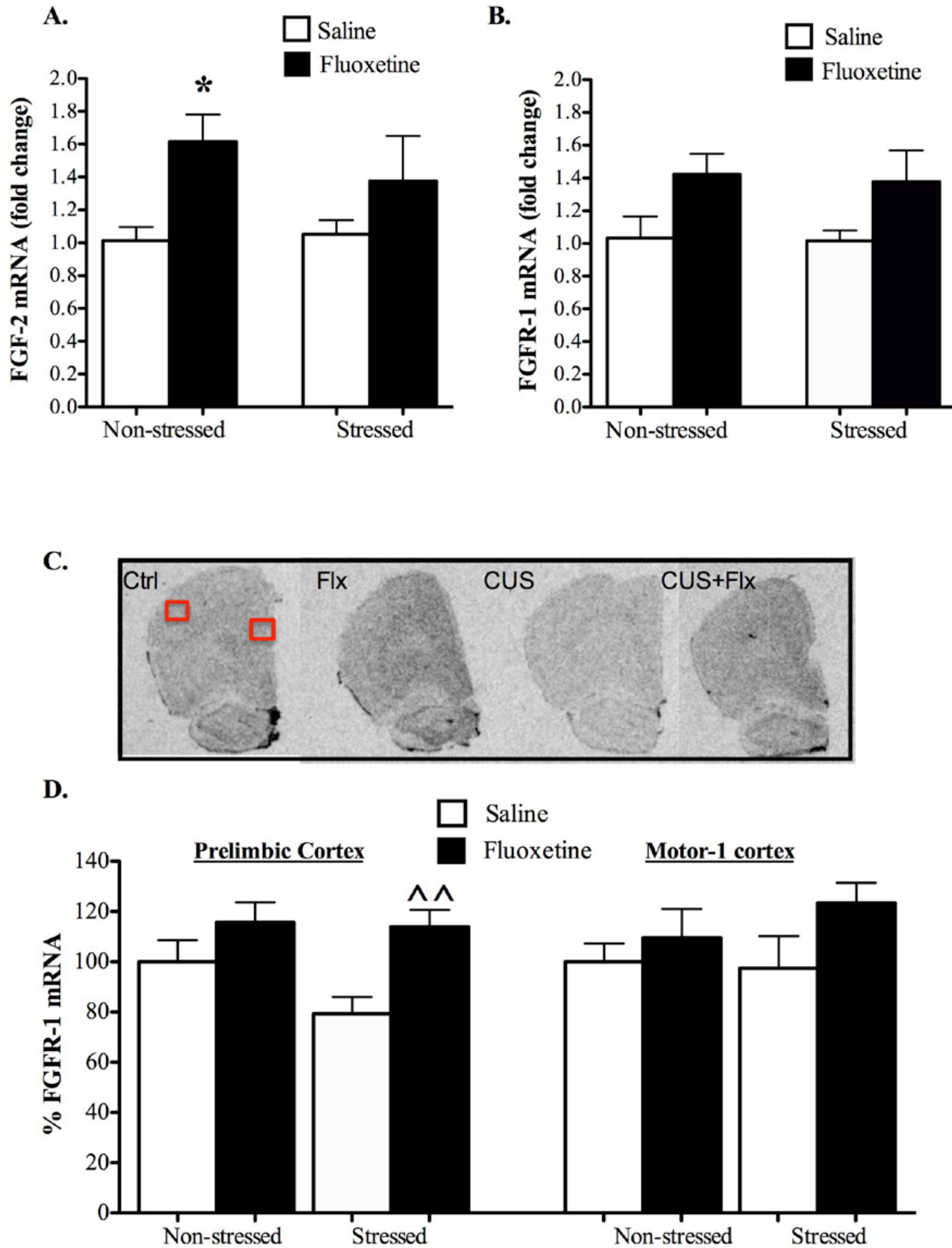

Figure S1. Fluoxetine treatment results in regulation of expression of fibroblast growth factor-2 (FGF-2) and FGF receptor-1 (FGFR-1) within the frontal cortex (FC) of rats. Rats were exposed to chronic unpredictable stress (CUS) or control conditions for 35 days, and on day 15 they were injected with either saline or fluoxetine (Flx, 5 mg/kg) for 20 days. **(A-B)** Quantitative real-time

polymerase chain reaction of the FC tissue (enriched in prefrontal cortex) was analyzed for FGF2 and FGFR-1. Significant differences were observed between saline and fluoxetine treated animals in the non-stressed group. There was no change in expression for any of them in the stressed group. Data are normalized to cyclin-D and are expressed as mean fold change ± SEM ($n = 5$ or $n = 6$); *$p \leq 0.05$ compared to saline-treated non-stressed group (analysis of variance and Fisher's protected least significant difference (PLSD) post hoc test). **(C)** A representative image of the *in situ* hybridization of FGFR-1 in the different groups of animals. **(D)** Quantitative analysis of *in situ* hybridization shows significant increase of FGFR-1 messenger RNA (mRNA) in the prelimbic (PrL) cortex of CUS rats treated with fluoxetine compared to those treated with saline. This effect was specific to the PrL cortex since there was no significant change in the primary motor cortex following fluoxetine treatment. Data are expressed as percentage from control group ± SEM ($n = 4$ or $n = 5$); ^^$p < 0.01$ compared to CUS saline treated-group (analysis of variance and Fisher's PLSD post hoc test). Ctrl, control.

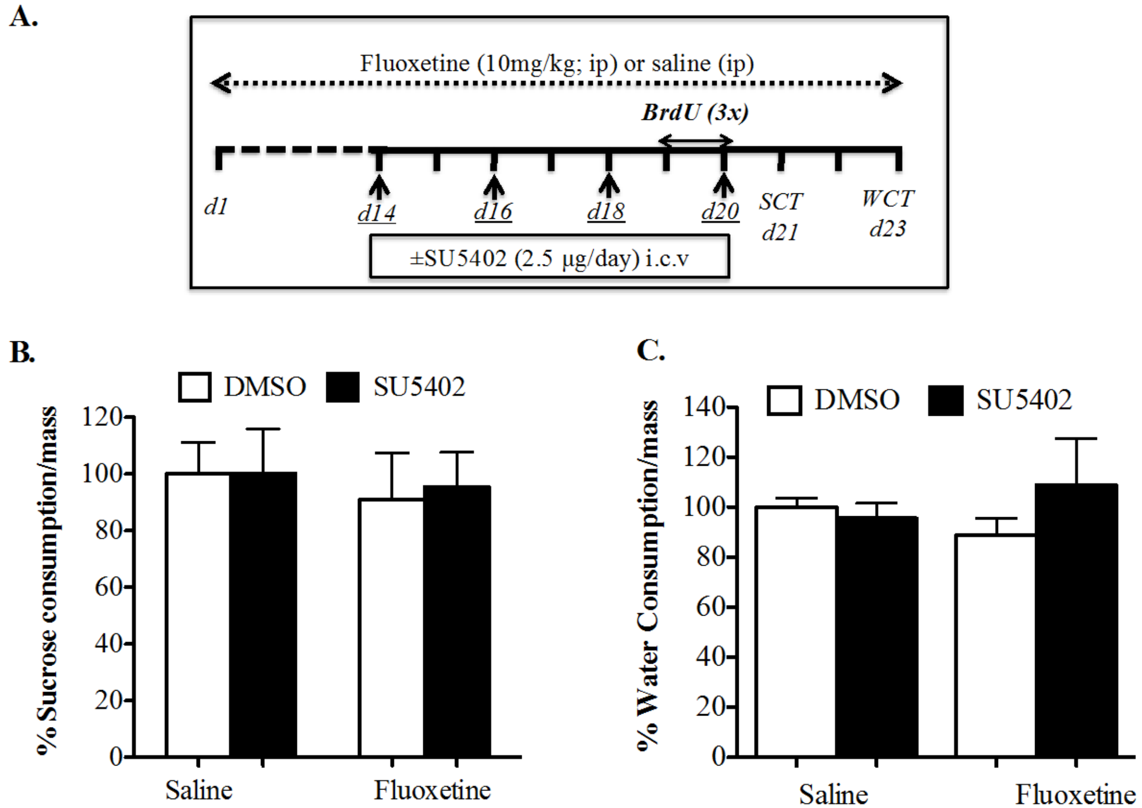

Figure S2. Fluoxetine and FGF receptor inhibitor (SU5402) do not influence sucrose intake in baseline conditions in mice. **(A)** Schematic diagram indicating the timeline for antidepressant treatment, days of infusions of SU5402 (underlined) and sucrose and water consumption tests. **(B)** Sucrose consumption did not change following fluoxetine or SU5402 administrations in non-stressed conditions. **(C)** Water consumption is measured as a control test. Fluoxetine treatment and SU5402 infusions did not influence water intake in baseline conditions. Fluid intake is normalized to the respective body weight (average sucrose intake/mass for controls = 0.069 ± 0.007; average water intake/mass for controls = 0.109 ± 0.004) and the data are expressed as mean percentage from control group (n = 9-11 per group ± SEM). i.c.v., intracerebroventricular; ip, intraperitoneal.

Table S1. Daily schedule for the chronic unpredictable stress paradigm (CUS) in rats. Shown are the types, duration, and number (in parenthesis) of stressors per day (D) throughout the stress procedure.

Type of Stressor and Duration	Occurrence During 35 Days
Cold 4°C 1 hour	D: 2, 8, 14, 16, 26, 33; (6)
Swim Stress 18°C 10 min	D: 5, 10, 17, 22, 27, 34; (6)
Cage Rotation 1 hour	D: 1, 3, 13, 20, 28, 30, 32; (7)
Isolation overnight	D: 4, 6, 13, 18, 21, 31, 33, 35; (8)
Food/Water Deprivation overnight	D: 6, 10, 17, 22, 25,30; (6)
Light On overnight	D: 1, 8, 15, 27, 32, 34; (6)
Light Off overnight	D: 2, 11, 18, 23, 28, 31, 35; (7)
Odor overnight	D: 7, 12, 20, 24; (4)
Stroboscope overnight	D: 7, 11, 19, 26, 29; (5)
Wet Bedding overnight	D: 4, 9, 15, 21, 25, 29; (6)
Crowding overnight	D: 5, 12, 14, 19, 23; (5)
Tilt Cage 45° overnight	D: 3, 9, 16, 24; (4)

Table S2. Daily schedule for the chronic unpredictable stress paradigm (CUS) in mice (3 stressors/day; 2 weeks). Shown are the types, duration, and number (in parenthesis) of stressors per day (D) throughout the stress procedure.

Type of Stressor and Duration	Occurrence During 14 Days
Lights ON overnight	D: 1,4,6,9 (4)
Cold 4°C 1 hour	D: 2,5,10,13 (4)
Cage Tilt 45° overnight	D: 5 (1)
Lights Off 3 hours	D: 2,6,7,9 (4)
Food Deprivation	D: 2,8 (2)
Cage Rotation 1 hour	D: 3,6,12 (3)
Stroboscope overnight	D: 3,10,12,13 (4)
Restraint 1 hour	D: 4,7,9,10 (4)
Odor overnight	D: 4,8 (2)
Swim Stress 18°C overnight	D: 5,8,12 (3)
Cage Tilt 45° 3 hours	D: 2,3,7,13 (4)

Table S3. Daily schedule for the chronic unpredictable stress paradigm (CUS) in mice (2-3 stressors/day; 4 weeks). Shown are the types and duration of stressors per day (D) throughout the stress procedure.

Type of Stressor and Duration	**Occurrence During 29 Days**
Lights On overnight	D: 4, 7, 11, 13, 15, 18, 22, 24, 26, 27
Cold 4°C 1 hour	D: 1, 6, 15, 18, 21, 24, 27
Cage Tilt 45° overnight	D: 1, 16, 20
Lights Off 3 hours	D: 15, 7, 11, 15, 17, 21, 25, 26, 27, 29
Food Deprivation	D: 2, 10
Cage Rotation 1 hour	D: 2, 5, 10, 16, 20, 23, 25, 29
Stroboscope overnight	D: 3, 6, 9, 12, 14, 17, 19, 21, 23, 25, 28
Restraint 1 hour	D: 3, 8, 13, 16, 20, 22
Odor for at least 3 hours	D: 5, 8, 15, 22, 24, 28
Swim Stress 18°C overnight	D: 4, 9, 12, 17, 19
Cage Tilt 45° 3 hours	D: 7, 11, 14, 23,25, 26, 28

Supplemental References

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4. Duric V, Banasr M, Licznerski P, Schmidt HD, Stockmeier CA, Simen AA, *et al.* (2010): A negative regulator of MAP kinase causes depressive behavior. *Nat Med*. 16:1328-1332.
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