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

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Abstract

BACKGROUND: Despite strong evidence linking fibroblast growth factor 2 (FGF2) with anxiety and depression in both rodents and humans, the molecular mechanisms linking FGF2 with anxiety are not understood.

METHODS: We compare 1) mice that lack a functional *Fgf2* gene (*Fgf2* knockout [KO]), 2) wild-type mice, and 3) *Fgf2* KO with adult rescue by FGF2 administration on measures of anxiety, depression, and motor behavior, and further investigate the mechanisms of this behavior by cellular, molecular, and neuroendocrine studies.

RESULTS: We demonstrate that *Fgf2* KO mice have increased anxiety, decreased hippocampal glucocorticoid receptor (GR) expression, and increased hypothalamic-pituitary-adrenal axis activity. FGF2 administration in adulthood was sufficient to rescue the entire phenotype. Blockade of GR in adult mice treated with FGF2 precluded the therapeutic effects of FGF2 on anxiety behavior, suggesting that GR is necessary for FGF2 to regulate anxiety behavior. The level of Egr-1/NGFI-A was decreased in *Fgf2* KO mice and was reestablished with FGF2 treatment. By chromatin immunoprecipitation studies, we found decreased binding of EGR-1 to the GR promoter region in *Fgf2* KO mice. Finally, we examined anxiety behavior in FGF receptor (FGFR) KO mice; however, FGFR1, FGFR2, and FGFR3 KO mice did not mimic the phenotype of *Fgf2* KO mice, suggesting a role for other receptor subtypes (i.e., FGFR5).

CONCLUSIONS: These data suggest that FGF2 levels are critically related to anxiety behavior and hypothalamic-pituitary-adrenal axis activity, likely through modulation of hippocampal

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glucocorticoid receptor expression, an effect that is likely receptor mediated, albeit not by FGFR1, FGFR2, and FGFR3.

Keywords

Anxiety; FGF receptors; FGF2; Glucocorticoids; Hippocampus; Mouse; Stress

An important role for the regulation of the hypothalamic-pituitary-adrenal (HPA) axis by the hippocampus has been proposed in several developmental rodent models of adult emotional dysregulation caused by early life stress and manipulations of maternal care. Yet, how the hippocampal circuitry responds to local and systemic perturbations by modulating the HPA axis activity is not understood. One crucial mediator that may link responses to early life perturbations, including brain injury and stressful events, with neuroendocrine control of emotional behavior is basic fibroblast growth factor 2 (FGF2). FGF2 is a potent growth factor that regulates stem cell maintenance and neurogenesis both during embryonic development and in response to challenges such as stress or injury in the adult brain (1–6). The hippocampus expresses the highest levels of FGF2 and its receptors in the brain. FGF2 modulates hippocampal neurogenesis, neurite outgrowth, and synapse formation, and as such it affects learning and memory, long-term potentiation, and the reaction to injury (7–9). Importantly, this growth factor has also been correlated to emotional dysregulation, both in humans affected by mood disorders and in rodent models of anxiety behavior (10–15). Increased hippocampal FGF2 levels are correlated with reduced manifestations of anxiety (11) and with positive response to treatment with the antidepressant fluoxetine (12,16). In spite of this large body of circumstantial evidence, the causal link and mechanism by which FGF2 may regulate emotional behavior is not understood. Here we perform behavioral, cellular, and molecular studies in mice lacking the *Fgf2* gene, which manifest increased levels of anxiety, and rescue their phenotype with FGF2 administration in adulthood. We establish a causal link between the anxiolytic effects of FGF2 and glucocorticoid receptors (GR) and suggest that FGF2 is required in adulthood for normal neuroendocrine HPA axis activity and emotional behavior. Finally, using mice lacking FGF receptors (FGFRs), we found that FGFR1, FGFR2, and FGFR3 did not mediate the effect of FGF2 on anxiety behavior, but paradoxically, *Fgfr1* and *Fgfr2* knockout (KO) mice showed decreased anxiety behavior.

METHODS AND MATERIALS

Mice

We used the following lines: *Fgf2* exon 1 KO mice (*Fgf2* KO) (3,4,17); conditional embryonic hGFAP-cre *Fgfr2* KO (18); germline *Fgfr3* KO (19); and embryonic *Fgfr1*/*Fgfr2* conditional KO driven by *Emx1*-cre (20). For additional information related to selection of mice, number of mice cohort tested in independent experiments, and other details, see Supplemental Methods and Materials.

Drug Treatments

FGF2 Administration.—FGF2 ligand (10 ng/g) (R&D Systems, Minneapolis, MN) dissolved in 1 mol/L phosphate buffered saline with 0.1% bovine serum albumin or vehicle

control was administered by daily subcutaneous injections. This dose of FGF2 was previously established to induce recovery from injury and neurogenesis with no documented side effects (21,22).

RU486 Administration.—RU486 (25 mg/g) (mifepristone; Sigma-Aldrich, St. Loius, MO Sigma) was dissolved in sunflower oil and administered subcutaneously 40 to 45 minutes before behavioral testing.

Behavioral Analysis

All behavioral tests took place under direct bright lights (about 300 lux) with the exception of the sucrose consumption test, which took place under normal, home cage conditions. Behavior took place at the same time during the day cycle, with control and experimental mice tested together each time, rotating between groups to counterbalance for order effects. The light/dark cycle was set at 7:00 AM/7:00 PM. For details on locomotor/open field, elevated plus maze, sucrose consumption test, forced swim test, and accelerating rotarod test, see Supplemental Methods and Materials.

Immunostaining, Cell Counting, and Microscopic Analysis

Unbiased stereological estimates of cell number were obtained via a Zeiss Axioskope 2 Mot Plus attached to a motorized stage and connected to a computer running the Stereo Investigator Software (MicroBrightfield, Colchester, VT). Images presented in figures were acquired on an ApoTome equipped Axiovert 200M with Axiovision 4.5 software (Zeiss, Oberkochen, Germany). For details on tissue processing, immunostaining, and stereological cell counting procedures, see Supplemental Methods and Materials.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted with 10 μg/IP of Egr-1 antibody (SC-189) using the MAGnify ChIP system from Life Technologies (Carlsbad, CA) as per the instruction manual, with the following modifications: perfused tissue was used and thus the fixation step was omitted; primary antibody was incubated overnight at 4°C; and an increased efficiency protocol was used for de-cross linking: samples were incubated in ChIP de-cross-linking buffer at 65°C overnight and then 0.5 mol/L ethylenediaminetetraacetic acid, 1 mol/L Tris-HCl, and Proteinase K were added and incubated for 2 hours at 45°C.

Statistical Analyses

All data were analyzed using StatView 4.51 software (Abacus Concepts, Calabasas, CA). Analysis of variance was employed with genotype and time or treatment as independent variables, wherever appropriate. Probability values were considered significant when $p \leq .05$. Fisher's least significant difference post hoc tests were used with a Bonferroni correction for nonorthogonal comparisons.

RESULTS

Assessment of the FGF2 Knockout Phenotype

Behavioral Measures.—Results from the elevated plus maze in male wild-type and *Fgf2* KO mice showed that knockout mice had a remarkably higher latency to enter the open arm ($F = 80.013$, $p < .0001$) and spent significantly less time on the open arms than their wild-type littermates ($F = 14.659$, $p < .001$) (Figure 1A). No significant differences were found between genotypes in tests associated with depressive behavior: the sucrose consumption test (Figure 1B) ($F = 0.144$, $p > .05$) and the forced swim test ($F = 1.269$, $p > .05$) (Figure 1C). Finally, no differences were observed on the rotarod test (Figure 1D), suggesting that *Fgf2* KO mice do not show deficits in motor abilities and motor learning.

The elevated plus maze data were further supported by results of the locomotor activity test in the open field. Mice showed no significant differences in baseline locomotor activity regardless of genotype, including total distance traveled in the field ($F = 1.530$, $p > .05$) or average speed ($F = 1.661$, $p > .05$) (Figure 1F). However, *Fgf2* KO mice spent significantly less time in the center of the open field ($F = 5.505$, $p < .05$) (Figure 1E), another indication of increased anxiety. The difference in anxiety behavior in the open field test was observed predominantly during the first 4 minutes of the test; by 6 minutes, there were no significant differences in center time between groups (Figure 1E). In addition, there was a trend toward significance on the distance traveled in the center ($p = .08$), such that knockout animals traveled approximately half of the distance measured in wild-type control animals within the center; however, these two groups did not differ on distance traveled in the perimeter of the open field ($F = 1.768$, $p > .05$) (Figure 1G). Results of the open field were confirmed in a second group of mice ($n = 6$ *Fgf2* KO and $n = 6$ wild-type mice) (Table 1).

Brain Anatomy and Cellular Phenotypes.—The hippocampus exhibits the highest levels of FGF2 and FGFR in the brain. This region also contains the highest concentration of GR in the brain, which makes it highly responsive to serum corticosterone, the rodent analog of glucocorticoids. In particular, the hippocampus is thought to exert an inhibitory tone on the HPA axis (23–25) (Figure 2A). To understand whether the increased anxiety behavior in *Fgf2* KO mice was accompanied by a change in responsivity of the hippocampus and HPA axis to negative feedback by corticosterone (26,27), we examined GR immunoreactivity (ir) levels within the hippocampus in *Fgf2* KO mice and wild-type control mice. GRir was present in granule neurons of the dentate gyrus (DG) and pyramidal cells of the hippocampal cornu ammonis (CA) regions. We found a significant decrease in GRir in both the DG and the CA1/CA3 of *Fgf2* KO mice as compared with wild-type littermates ($F = 10.686$, $p < .01$) (Figure 2B–F). Previous work has shown that there is an age-related increase in localization of GR on glial fibrillary acidic protein $(GFAP)^+$ cells instead of neurons (28); however, co-staining with GFAP showed no differences in the percentages of $GFAP^+$ cells that were also GRir throughout the hippocampus (wild-type = 19.15% ± 5.3; *Fgf2* knockout = 17.07% ± 3.9). Thus, the lack of a functional *Fgf2* gene resulted in a strong decrease in GR expression in granule neurons and in CA1/CA3 neurons, without changing the expression of GR in $GFAP^+$ cells.

Because we conducted our behavioral analysis and histology in the same animals, we were also able to correlate the number of hippocampal GRir cells with time spent on the open arms of the plus maze and the center of the open field. Total numbers of hippocampal GRir cells were significantly and positively correlated to both of these anxiety measures across all animals (Table 2), suggesting that decreased levels of hippocampal GR are associated with the increased anxiety behavior in mice.

To better characterize HPA axis activity in the *Fgf2* KO animals, we examined GR and corticotropin releasing hormone (CRH) ir cells within the paraventricular nucleus of the hypothalamus (PVN), which contains the CRH cell bodies important for the hypothalamic regulation of the HPA axis. No group differences were observed in GRir in this region ($F = 0.019$, $p > .05$) (Figure 2G). Interestingly, however, we found an increased number of CRHir neurons within the PVN of *Fgf2* KO mice ($F = 14.554$, $p < .01$) (Figure 2H). Finally, because the central nucleus of the amygdala contains CRH cell bodies and regulates the HPA axis, we examined amygdalar CRHir. There was no difference in density of CRH protein level between wild-type and *Fgf2* KO mice (mean optical density/area in arbitrary units, wild-type: 60.1 ± 8.04; *Fgf2* KO: 54.61 ± 16.2; $F = 0.110$, $p > .05$). Together, the data suggest that FGF2 is crucial for maintenance of GR expression in differentiated neurons of the hippocampus, which, in turn, affects CRH protein expression within neurons of the PVN but not in the amygdala, consistent with the inhibitory role of the hippocampus on the HPA axis.

Corticosterone Responsivity.—Previous studies have shown that the profile observed in the current phenotype of *Fgf2* KO mice, i.e., decreased hippocampal GR expression together with increases in PVN CRH, is associated with increased corticosterone response to an anxiogenic stimulus (29). Therefore, we also compared corticosterone blood levels in wild-type control mice and *Fgf2* KO male mice in response to restraint stress. Corticosterone levels were assessed by enzyme-linked immunosorbent assay at baseline (2 weeks before) and at the end of a 60-minute restraint stressor. Results showed a main effect of genotype and time, such that *Fgf2* KOs had higher corticosterone levels at baseline and 60-minute (peak) postrestraint stress (main effect of genotype at these time points: $F = 6.02$, $p < .05$; main effect of time: $F = 362.83$, $p < .00001$) (Figure 2I). Together, the data demonstrate abnormal HPA axis response to stress in *Fgf2* KO mice.

Adult Rescue of *Fgf2* KO Mouse Anxiety Phenotype

While we have observed a strong anxiety phenotype in response to the *Fgf2* gene deletion, it remains to be determined whether this is due to irreversible developmental events consequential to the absence of *Fgf2* or whether the FGF2 ligand itself is involved in the expression of anxiety behavior. Therefore, we performed a rescue experiment by administering either vehicle or FGF2 ligand daily for 1 week to adult *Fgf2* KO or wild-type control mice and then assessing anxiety behavior, hippocampal GR levels, and circulating baseline corticosterone levels.

Behavior.—Adult male wild-type mice and *Fgf2* KO mice were injected daily for 7 days with vehicle or FGF2 (see timeline, Figure 3A) and then tested on the elevated plus maze,

open field test, and forced swim test. Results showed that again, vehicle-injected *Fgf2* KO mice had similar increased levels of anxiety behavior on both the open field and the elevated plus maze; however, FGF2 treatment reinstated normal anxiety behavior such that there were no differences between wild-type control mice and FGF2-treated, *Fgf2* KO mice (elevated plus maze: $F = 0.075$, $p > .05$; open field: $F = 0.073$, $p > .05$) (Figure 3B, C). No effect of FGF2 treatment on anxiety behavior was seen in control wild-type mice (EPM: $F = 0.003$, $p > .05$; open field: $F = 6.849E\text{-}5$, $p > .05$) (Figure 3B, C). Interestingly, as in our earlier experiments, no significant differences were observed between wild-type and *Fgf2* KO mice on the forced swim test; however, a small decrease in the percentage of time spent immobile and increase in latency to become immobile were observed in the FGF2-injected versus vehicle-injected wild-type control mice, suggesting a mild antidepressant effect in wild-type mice (Figure 3D) (omnibus analysis of variance interaction $F = 1.9$, $p \geq .05$; planned comparison in wild-type mice (t test $p = .03$).

HPA Axis Phenotype.—To assess whether FGF2 treatment had a corresponding corrective effect on the neuroendocrine measures of anxiety, basal serum corticosterone levels at sacrifice were assessed by enzyme-linked immunosorbent assay. *Fgf2* KO mice again showed a significant increase in basal corticosterone levels, with FGF2 treatment inducing a complete reversal of the chronically elevated corticosterone levels in *Fgf2* KO mice (Figure 3E). Hippocampal GR messenger RNA (mRNA) levels were assessed by quantitative polymerase chain reaction (qPCR), and a decrease in GR mRNA was observed in *Fgf2* KO mice, with a reversal to baseline levels following FGF2 administration (Figure 3F). No significant effect of FGF2 was observed in either corticosterone or GR levels in wild-type mice. Immunostaining for GR showed that FGF2 ligand administration increased GR in both the CA and DG regions of the hippocampus (Figure 3H).

Because the amygdala plays a crucial role in the positive modulation of HPA axis activity, we also examined GR mRNA levels of the amygdala and found a significant GR mRNA increase in *Fgf2* KO mice, regardless of adult FGF2 administration (Figure 3G), suggesting a different role of FGF2 in the amygdala and that only those changes in the hippocampus are consistent with the behavioral phenotype we have observed in the adult rescue.

FGF2 Modulates GR Expression Through Egr-1

A potent regulator of *Gr* expression in the hippocampus is the immediate early gene and transcription factor EGR-1 (also known as zif-268 and NGF1A). GR expression increases in response to increased binding of EGR-1 to the exon 1_7 promoter region of the *Gr* gene, and increased EGR-1 activity has been associated with decreased anxiety behavior and decreased corticosterone release (30). To understand if EGR-1 could mediate the effect of FGF2 on *Gr* expression, we used qPCR to examine levels of *Egr-1* mRNA expression in the hippocampus of wild-type and *Fgf2* KO mice with or without FGF2 rescue treatment. Consistent with prior data suggesting that *Egr-1* is downstream of FGF signaling (31), *Egr-1* mRNA was significantly decreased in the hippocampus of *Fgf2* KO mice and was reinstated to normal level with FGF2 administration (Figure 4A). Therefore, we hypothesized that FGF2 positively modulates *Egr-1* transcription, leading to changes in binding activity of EGR-1 to the exon 1_7 promoter region of the *Gr*. To test this, we performed ChIP using an EGR-1

antibody in wild-type and *Fgf2* knockout mice and then used qPCR to amplify the EGR-1 binding domain of the *Gr* exon 1_7 promoter region of both the input and IP samples. We analyzed the data by comparing the absolute difference between the cycle thresholds (ΔCT) of the input and the IP samples (Figure 4B), and additionally, we ran the qPCR product on a southern gel and analyzed the band intensities using National Institutes of Health ImageJ software (Bethesda, MD) (Figure 4C). Results showed a greater difference in ΔCT between input and IP samples in the hippocampus of *Fgf2* KO mice as compared with wild-type control mice, indicating that there was less binding of EGR-1 to the exon 1_7 promoter region of the *Gr* in the absence of FGF2 (Figure 4B). This was verified by a decrease in the amount of quantifiable product using gel band analysis ($F = 8.706$, $p < .05$) (Figure 4C). The data suggest that FGF2, presumably acting through its receptor-mediated tyrosine kinase activity, regulates EGR-1 levels and binding activity at the *Gr* promoter region.

GR Is Necessary for Anxiolytic Effects of FGF2.—To ascertain whether the upregulation of hippocampal GR in response to FGF2 treatment is necessary to exert FGF2's anxiolytic effects, we treated *Fgf2* KO mice with vehicle or FGF2 and then injected them with RU486 (mifepristone), a glucocorticoid receptor antagonist, 40 minutes before testing them on the elevated plus maze (Figure 4D). GR blockade completely blocked the anxiolytic effects of FGF2 (open arms: $F = 5.923$, $p < .05$; closed arms: $F = 7.877$, $p < .01$) (Figure 4E, F). RU486 also showed a paradoxical anxiolytic effect in *Fgf2* KO, vehicle-injected mice, although this was variable and did not reach statistical significance ($F = 2.379$, $p > .15$) (Figure 4E, F).

Role of *Fgfr* in Anxiety Behavior.—To ascertain if FGFRs are necessary for changes in anxiety behavior, we examined mice that conditionally lacked *Fgfr1*, *Fgfr2*, or both receptors in the dorsal forebrain. The conditional knockout of *Fgfr1* and the double knockout of *Fgfr1* and *Fgfr2* were driven by the *Emx1*-cre line and the conditional knockout of *Fgfr2* by the *hGFAP*-cre line. We also examined mice lacking *Fgfr3* constitutively. Baseline behavioral tests on the elevated plus maze showed no changes in anxiety behavior in *Fgfr3* knockout mice (Figure 5A). Interestingly, we observed a decrease in anxiety behavior in both *Fgfr1* and *Fgfr2* conditional knockout mice when compared with their respective control mice ($F_{2,30} = 11.8546$, $p < .001$) (Figure 5A, B), which is the opposite effect than what we observed with *Fgf2* KO mice. We then administered FGF2 ligand to double *Fgfr1*/*2* conditional knockout mice (driven by *Emx1*-cre) using the same protocol as in previous experiments and found that both FGF@ administration and *Fgfr* knockout showed a main effect of decreased anxiety behavior.

DISCUSSION

In the current study, we established for the first time that a primary role of FGF2 is to maintain appropriate levels of hippocampal (but not amygdalar) *Gr* gene expression, which, in turn, regulates HPA axis activity. These results tie together a body of literature that has demonstrated hippocampal regulation of HPA axis function with emerging data correlating FGF2 to mood and anxiety behavior. There were two domains where neuroendocrine function was altered in *Fgf2* KO mice. First, decreased *Gr* expression was observed in both the DG and CA fields of the hippocampus of *Fgf2* KO mice, and this correlated with

increased levels of anxiety in both the open field and elevated plus maze. Secondly, there was overactivity of the HPA axis, as shown by increased corticosterone levels and increased CRH levels in the PVN.

The hippocampus, which contains high concentrations of GR, is thought to be a crucial link in the negative feedback regulation of the HPA axis (24,25,32) (Figure 2A). Transgenic mice that overexpress *Gr* in the hippocampus show enhancement of such feedback inhibition, and mice that lack one copy of the *Gr* alleles show, similar to *Fgf2* KO mice, HPA axis hyperactivity (33). Those reports suggest that a primary function of *Fgf2* gene expression is to increase *Gr* gene expression in the hippocampus. Conversely, the absence of FGF2 drives hyperactivity of the HPA axis and increased anxiety behavior. In support of this hypothesis, we were able to rescue both the hippocampal GR levels and the anxiety behavior in *Fgf2* KO mice by administering FGF2. Pharmacologic antagonism of GR was sufficient to prevent the effects of FGF2, which together suggest that ongoing acute modulation of hippocampal GR expression by FGF2 is critically involved in this anxiety phenotype.

While the hippocampus regulates HPA axis function through negative feedback, the amygdala exerts positive feedback on the HPA axis through amygdalar GR receptors (34). In the current study, *Gr* mRNA levels were significantly increased within the amygdala of *Fgf2* KO mice, consistent with *Fgf2* KO mice having an upregulation of the HPA axis and increased baseline corticosterone levels. However, while increases in amygdalar GR may also play a contributory role to the increased HPA axis activity observed in *Fgf2* KO mice, amygdalar GR changes were not affected by adult FGF2 rescue administration, suggesting that GR changes in the amygdala are not crucial for the anxiolytic action of FGF2 in adulthood.

Interestingly, *Fgf2* KO mice injected with the GR antagonist RU486 that were not FGF2 treated showed mild anxiolytic responses to RU486 on the elevated plus maze, which may be a consequence of antagonizing the increased GR levels within the amygdala of *Fgf2* KO mice. The dose of RU486 used in the current study typically does not have effects on anxiety behavior in rodents unless an animal has undergone repeated stress before its administration (35,36). Therefore, it could be that the elevated baseline anxiety levels in *Fgf2* KO mice may, in part, recapitulate an animal that has undergone repeated stress, i.e., stress might induce increased GR expression in the amygdala, suggesting that the mild anxiolytic response of RU486 might be attributable to a decrease of amygdalar GR–mediated positive feedback on the HPA axis. It is important to note that RU486 can also act as a progesterone receptor blocker, and therefore the effects of RU486 on anxiety behavior reported may also be mediated through progesterone receptors. However, because the current study was conducted in male mice, progesterone levels are typically low throughout early adulthood and similar to the lowest levels observed in female mice across the estrous cycle. Nevertheless, further studies using different methods to attenuate GR receptor activity (i.e., using small hairpin RNAs) would be needed to assess the contribution of progesterone receptors, if any, to the anxiolytic effects of FGF2.

While, indeed, there may well be developmental effects of FGF2 on the amygdala, these data suggest that only the hippocampal changes are consistent with the behavioral phenotype

we have observed in the adult rescue. The expression of FGF2 ligand and its receptors (FGFR1–3) in adulthood is highest in the hippocampus, a region that also exhibits a neuronal expression of these molecules (37,38), which might explain why neuronal GR expression may be uniquely modulated in neurons of the hippocampus by FGF2. In addition, *Gr* exon 1_7 activity, which is modulated by the immediate early gene and transcription factor EGR-1, which binds to the exon 1_7 promoter region of the *Gr* gene, is high within hippocampal neurons and relatively low in other regions (39,40), therefore placing the hippocampus in a position where glucocorticoid receptor expression may be uniquely sensitive to changes in FGF2 and EGR-1 levels.

A strikingly similar phenotype associating hippocampal GR levels and HPA axis function has been reported in the offspring of models of early adversity and high- and low-licking and grooming mothers (29,41–43). For example, mice raised by low-licking and grooming mothers show decreases in hippocampal GR levels, increased stress responsivity, and interestingly, lower levels of FGF2 expression within the hippocampus (29,44). This parallel suggests that hippocampal FGF2 levels may be perturbed by maternal behavior during the early postnatal period and may be responsible for the development of the abnormal HPA axis in animals reared under maternal deprivation. While the current studies demonstrate that acute restoration of FGF2 levels in adulthood is sufficient to normalize anxiety behavior, these experiments do not rule out additional roles for FGF2 in anxiety behavior and HPA axis reactivity during development.

While a role for FGF2 in anxiety behavior has been previously suggested, the FGFR involved in modulating anxiety behavior has not been previously assessed. The current study found no role of FGFR3 in modulating anxiety and a paradoxical decrease in anxiety in *Fgfr1* and *Fgfr2* knockout mice. *Fgfr* KO mice (45–47) have extensive behavioral deficits, including hyperactivity and cognitive alterations. Because FGFRs bind all 23 FGF ligands in a promiscuous way, their knockouts are likely disrupting a much larger system than FGF2 alone, making it a difficult model to use in this line of investigation. Furthermore, *Fgfr* KO mice are lacking FGFR throughout development. For all of these reasons, it is possible that the current results reflect specific interactions of FGFRs with any of the other 20 or so FGF ligands that could be modulating development and behavior. For example, FGF9 has recently been suggested to increase both anxiety- and depression-like behavior (48), and unlike FGF2, FGF9 is upregulated in the hippocampus of patients with major depression (48), implicating an action opposite to that of FGF2. Further studies are needed to understand the role of different FGFRs in anxiety behavior, including exploring the role of the newly discovered decoy receptor FGFR5.

While the current studies show that knockout of *Fgf2* conduces to an anxiety phenotype and apparently not to depressive-like behaviors, it is important to note that we have only examined tests related to learned helplessness, and FGF2 may be involved in other aspects of depressive behaviors such as social, sexual, or other motivated behaviors.

Anxiety disorders are currently the most prevalent psychiatric illnesses in the United States (49); despite these alarmingly high rates, the available pharmacotherapy is far from optimal. The current study lends further support for necessary investigation of FGF2 (or a

downstream target) as a potential HPA axis modulator and anxiolytic treatment in human populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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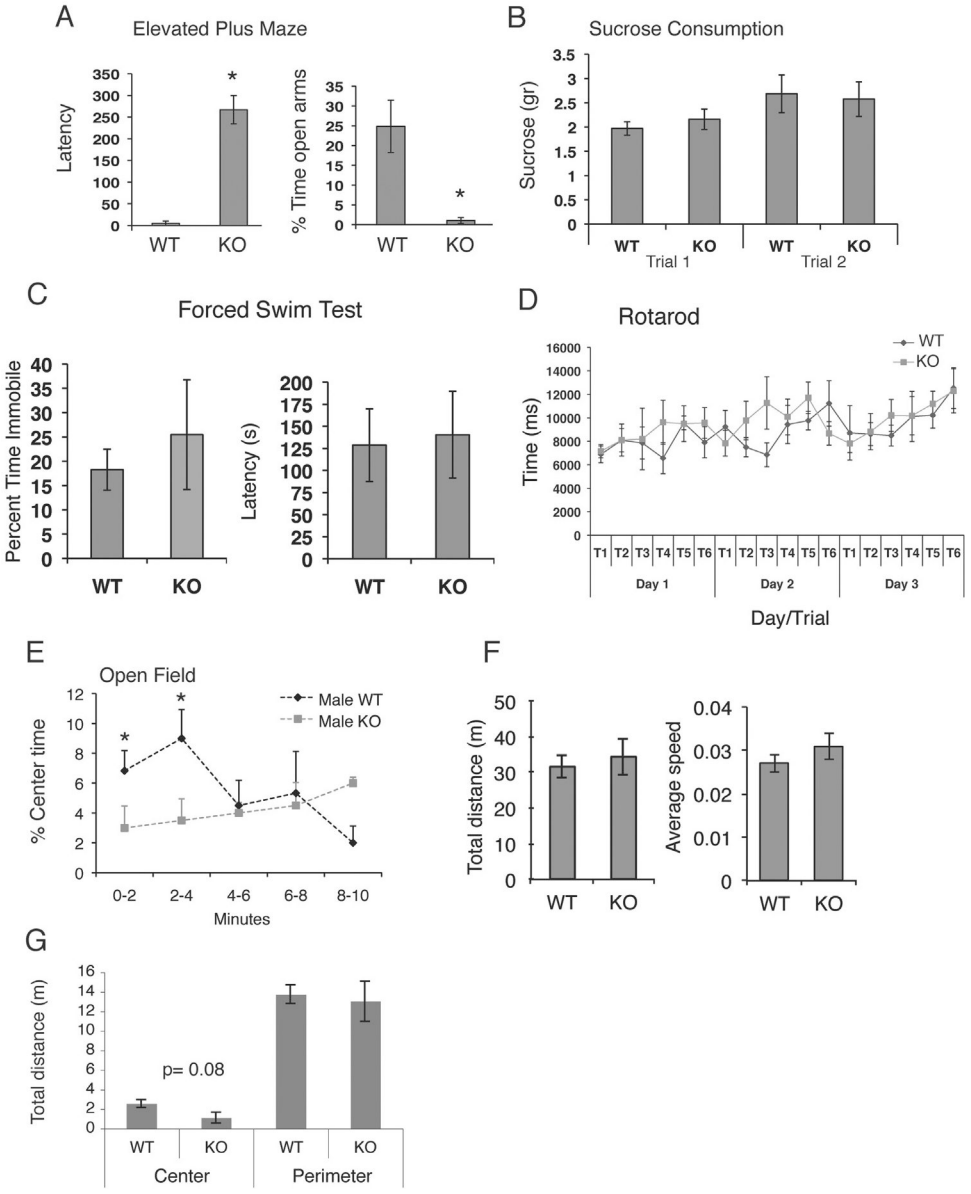

Figure 1.

Increased anxiety in fibroblast growth factor 2 (*Fgf2*) knockout (KO) mice as compared to wild-type (WT) mice. Latency to enter the open arm and the percent time spent on the open arms of the elevated plus maze **(A)**. KO male mice took longer to enter the open arms and spent significantly less time in the open arms. No significant differences were observed on the sucrose consumption test at either an early time point, trial (T) 1, or in trial 2, a time point at the end of all behavioral treatments **(B)**. No significant group differences were found on the forced swim test on percent time immobile and the latency to immobility **(C)**, and finally, there were no significant differences on the accelerating rotarod test **(D). (E–G)** Performance of WT and *Fgf2* KO mice on the locomotor test. KO male mice spent significantly less time in the center of the open field during the first 4 minutes of the task **(E)**, suggesting increased anxiety. No significant differences, however, were observed

between WT and KO male mice on total distance and speed **(F)** or on total distance in the perimeter region over the entire duration of the test (20 minutes) **(G)**. There was a trend ($p = .08$) for decreased distance travelled in the center region over the entire duration of the task **(G)**. *Denotes significant differences ($p < .05$). Error bars denote SEM. Wild-type mice, $n = 6$; *Fgf2* KO mice, $n = 6$.

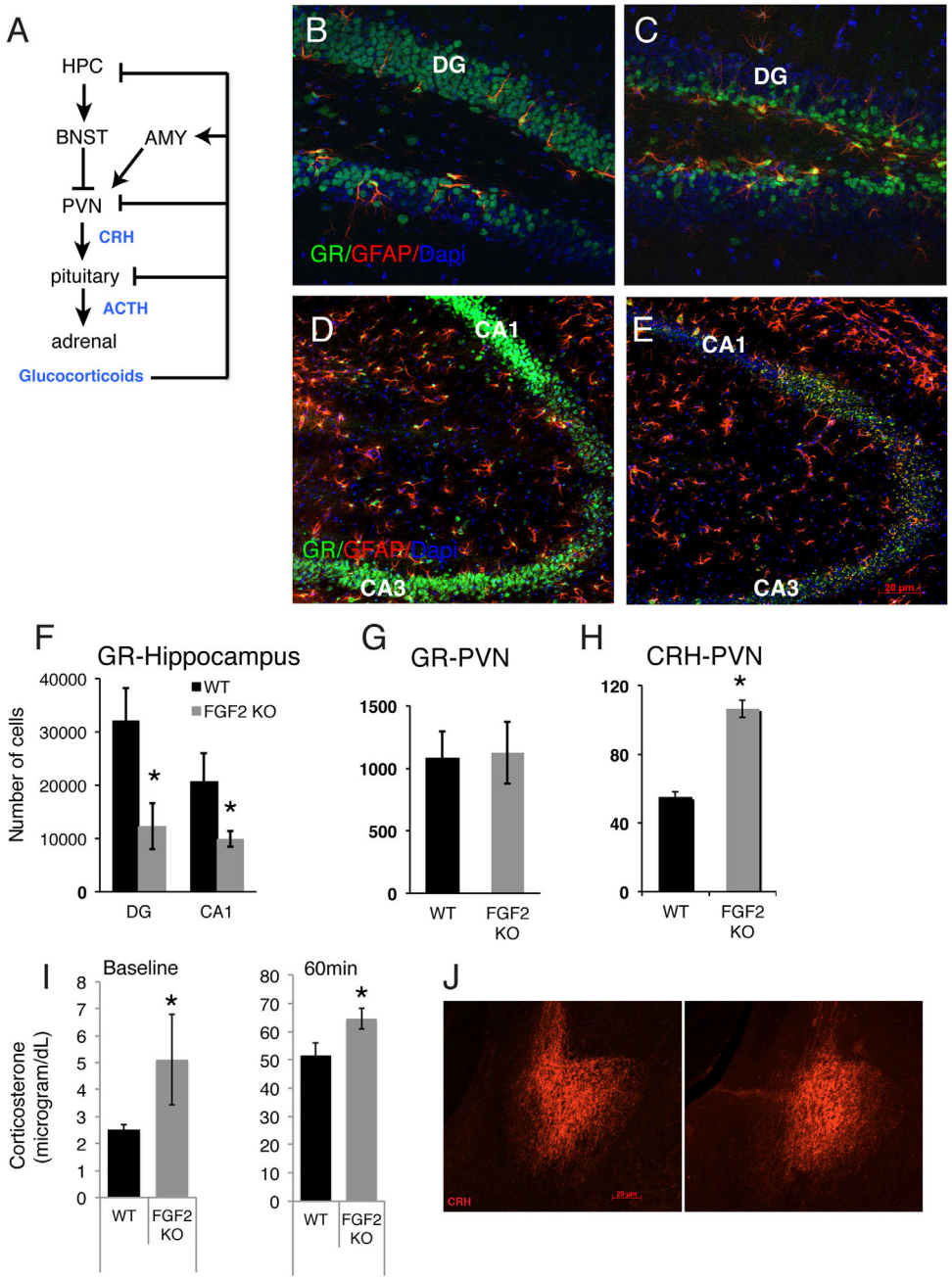

Figure 2.

Dysregulated hypothalamic-pituitary-adrenal axis in fibroblast growth factor 2 (*Fgf2*) knockout (FGF2 KO) mice. **(A)** Schematic outline of hypothalamic-pituitary-adrenal axis and feedback loops. **(B–E)** Representative images of glucocorticoid receptor (GR) and glial fibrillary acidic protein (GFAP) co-immunostaining within the dentate gyrus (DG) **(B, C)** and cornu ammonis (CA) fields **(D, E)** of wild-type (WT) **(B, D)** and *Fgf2* KO mice **(C, E)**. Scale bar = 40 μm in **(B)** and **(C)** and 20 μm in **(D)** and **(E). (F–H)** Quantitative analyses of the immunocytochemical data carried out by unbiased stereology. Total number of GR^+ cells in the DG, CA1 **(F)**, and in the paraventricular nucleus of the hypothalamus (PVN) **(G)**, and

total number of corticotropin-releasing hormone $(CRH)^+$ cells in the PVN **(H)** (WT, $n = 4$; *Fgf2* KO, $n = 3$). **(I)** Mean corticosterone blood concentration at baseline and after 60 minutes of restraint stress. Significantly higher levels were observed in KO mice at the baseline and poststress time points (WT, $n = 5$; *Fgf2* KO, $n = 6$). No differences in CRH staining intensity were observed in the amygdala (AMY) **(J)**. *Denotes significant differences between groups ($p < .05$). Error bars denote SEM. ACTH, adrenocorticotropic hormone; BNST, bed nucleus of the stria terminalis; Dapi, 4′,6-diamidino-2-phenylindole dihydrochloride; HPC, hippocampus. [Adapted with permission from Brown *et al.* (34).]

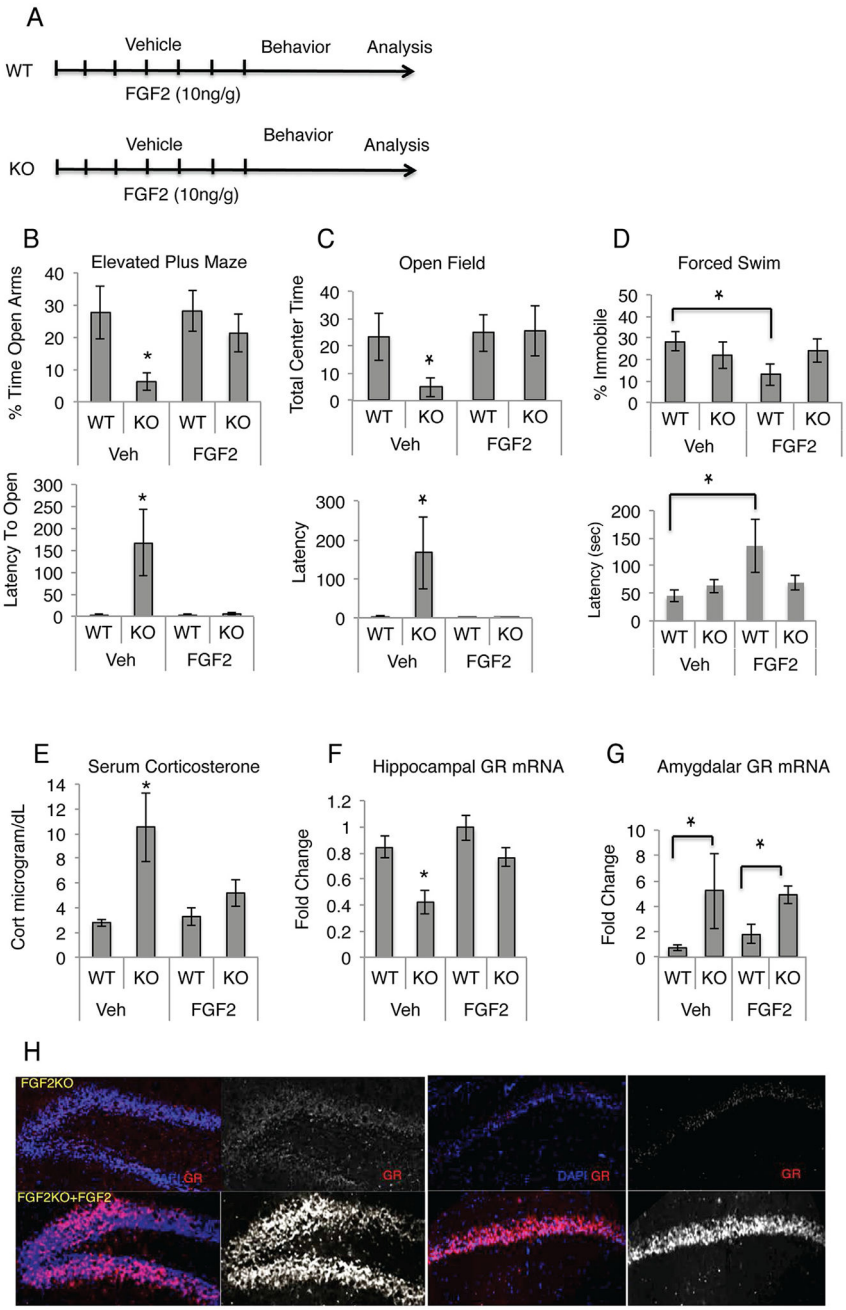

Figure 3.

Adult fibroblast growth factor 2 (FGF2) administration rescues the anxiety phenotype. **(A)** Timeline of FGF2 rescue experiment. **(B, C)** *Fgf2* knockout (FGF2 KO) mice show increased anxiety on the elevated plus maze **(B)** and the open field (5 minutes) **(C)**, and FGF2 ligand administration returns anxiety behavior to that observed in wild-type (WT) mice. No differences were observed on the forced swim task between genotypes; however, a small antidepressant effect of FGF2 was observed in WT mice **(D)** (WT, vehicle [Veh], n = 8; *Fgf2* KO, Veh, n = 8; WT, FGF2, n = 10; *Fgf2* KO, FGF2, n = 8). **(E)** FGF2 administration restored the increased basal corticosterone level to that of WT mice (WT,

Veh, $n = 4$; *Fgf2* KO, Veh, $n = 4$; WT, FGF2, $n = 3$; *Fgf2* KO, FGF2, $n = 3$). **(F)** FGF2 administration restored decreased hippocampal glucocorticoid receptor (GR) messenger RNA (mRNA) found in *Fgf2* KO mice to levels observed in WT mice. **(G)** *Fgf2* KO mice showed a significant increase in amygdalar GR mRNA, regardless of FGF2 administration (WT, Veh, $n = 4$; *Fgf2* KO, Veh, $n = 4$; WT, FGF2, $n = 3$; *Fgf2* KO, FGF2, $n = 3$). **(H)** *Fgf2* KO also showed an increase in GR protein in both the cornu ammonis and dentate gyrus regions of the hippocampus (WT, Veh, $n = 4$; *Fgf2* KO, Veh, $n = 4$; WT, FGF2, $n = 4$; *Fgf2* KO, FGF2, $n = 4$). Asterisks denote significant differences from all other groups, $p < .05$. Lines indicate significant differences between two groups, $p < .05$. Error bars denote SEM. Cort, corticosterone; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

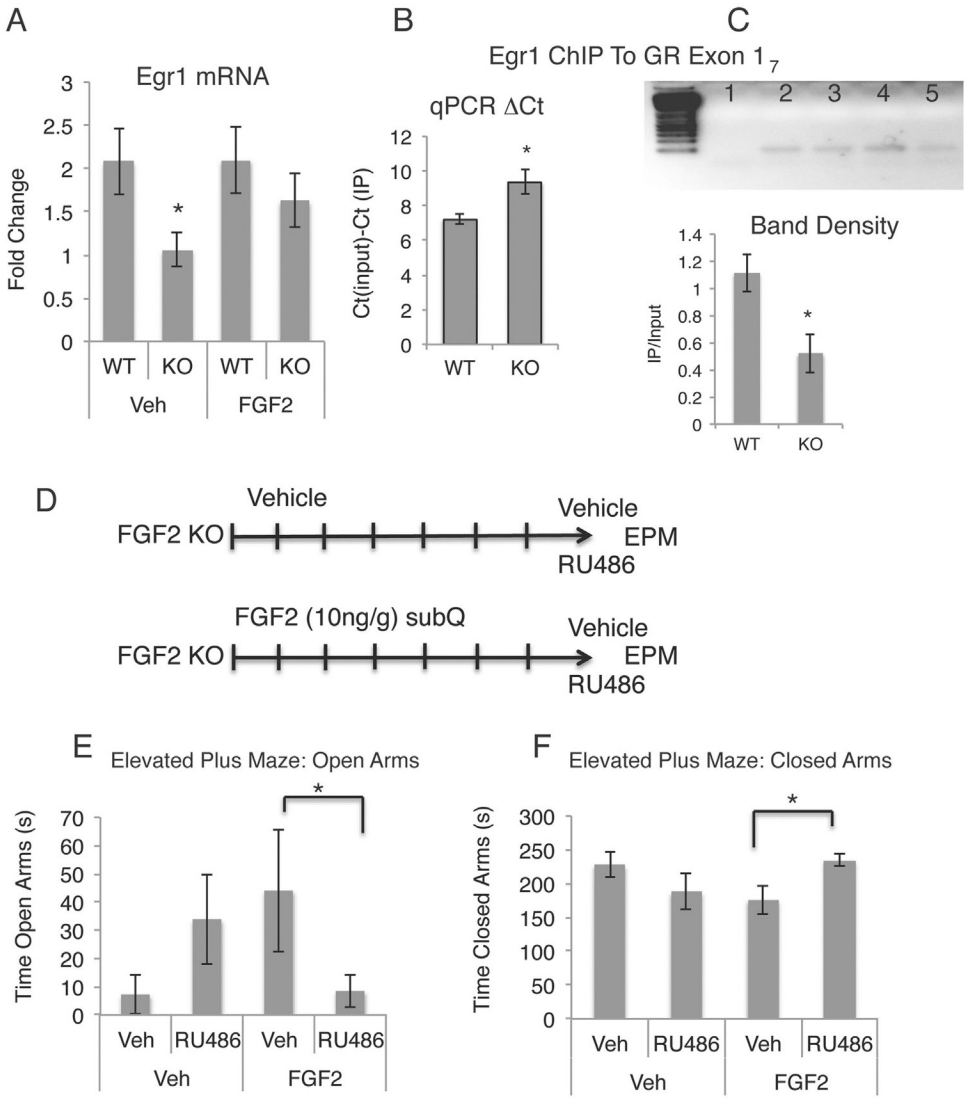

Figure 4.

Mechanisms of fibroblast growth factor 2 (FGF2)-glucocorticoid receptor (GR) interactions. **(A)** Hippocampal *Egr-1* messenger RNA (mRNA) is downregulated in *Fgf2* knockout (KO) mice and reinstated with FGF2 administration as assessed by quantitative polymerase chain reaction (qPCR) (wild-type [WT], vehicle [Veh], $n = 4$; FGF2 KO, Veh, $n = 4$; WT, FGF2, $n = 3$; FGF2 KO, FGF2, $n = 3$). **(B, C)** Results of hippocampal chromatin immunoprecipitation (ChIP) with EGR-1 antibody followed by qPCR amplification of the *Gr* exon1_7 promoter region. Y axis in **(B)**: cycle threshold (CT) (input) 2 CT (IP) indicates the difference in cycle thresholds (ΔCT) between the input and IP samples. Increased ΔCT in *FGF2* KO mice suggests decreased EGR-1 binding. **(C)** Gel electrophoresis of qPCR amplicons. Lane 1: negative control; Lane 2: WT input; Lane 3: WT IP; Lane 4: *Fgf2* KO input; Lane 5: *Fgf2* KO IP. Below the gel, results of band densitometry showing decreased EGR-1 binding, quantified as IP/input. **(D)** Timeline of the RU486 experiment. **(E, F)** RU486 blocks the effect of FGF2 rescue administration on anxiety behavior in *Fgf2* KO mice. RU486 decreased time in open arms of the elevated plus maze (EPM) **(E)** and

increased the time spent in the closed arms **(F)** in FGF2-treated *Fgf2* KO mice (Veh, Veh, $n = 6$; Veh, FGF2, $n = 7$; RU486, Veh, $n = 6$; RU486, FGF2, $n = 11$). Error bars denote SEM. Asterisks denote significant differences from all other groups, $p < .05$. Lines indicate significant differences between two groups, $p < .05$. subQ, subcutaneously.

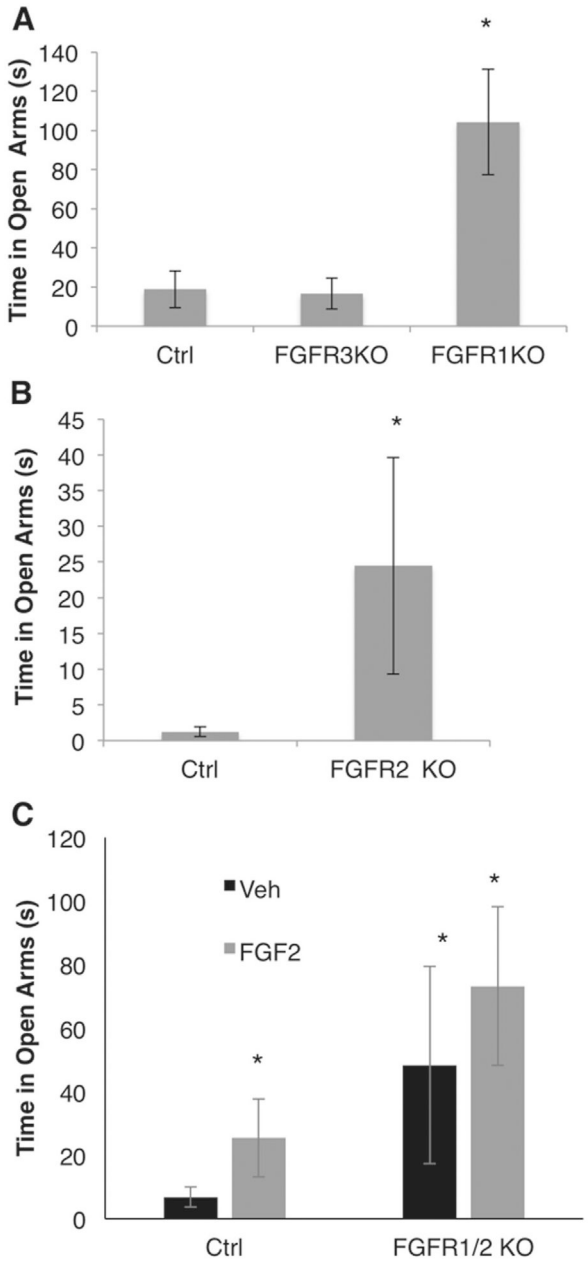

Figure 5.

The role of fibroblast growth factor receptor (*Fgfr*)1, 2, and 3 in anxiety behavior. Panel **(A)** shows time on the open arms in control (Ctrl) (cre-negative or wild type), *Fgfr3* constitutive knockout (FGFR3 KO), and *Fgfr1* conditional KO mice (FGFR1 KO) driven by *Emx1*-cre (Ctrl, *n* 5 12; *Fgfr3* KO, *n* = 13; *Fgfr1* KO, *n* = 8). Panel **(B)** shows time on the open arms of the elevated plus maze in Ctrl (cre-negative) and *Fgfr2* conditional KO mice (FGFR2 KO) driven by the hGFAP-cre line (Ctrl, *n* = 9; *Fgfr2* KO, *n* = 9). Panel **(C)** shows time on the open arms in Ctrl (cre-negative) and FGFR1/2 conditional KO mice driven by *Emx1*-cre in response to vehicle (Veh) or *Fgfr2* administration (Ctrl, Veh, *n* = 8; *Fgfr1*/*2* KO, Veh, *n* = 6;

Ctrl, FGF2, $n = 10$; *Fgfr1/2* KO, FGF2, $n = 6$). *Denotes significant differences from Ctrl. Error bars denote SEM.

Table 1.

Open Field Results From a Second Naïve Cohort, Percent Time Spent in the Center of the Open Field

Trial Day	Genotype	Minutes 1–5 Mean ± SE	Minutes 6–10 Mean ± SE	Minutes 11–15 Mean ± SE
Day 1	WT	32.7 ± 2.3	30.3 ± 5.6	31.9 ± 8.8
	KO	17.0 ± 4.9[a]	18.1 ± 3.6[a]	17.2 ± 4.5[a]
Day 2	WT	18.1 ± 4.7	14.6 ± 1.9	19.1 ± 2.8
	KO	10.1 ± 2.9	18.0 ± 4.8	17.1 ± 3.6
Day 3	WT	8.8 ± 2.1	11.1 ± 2.9	13.4 ± 3.2
	KO	11.4 ± 4.6	13.2 ± 3.7	18.1 ± 6.6

KO, *Fgf2* knockout; WT, wild-type.

[a]Denotes significant difference ($p < .05$) from WT control mice.

Table 2.

Correlation Matrix for Anxiety Behavior and Hippocampal Glucocorticoid Receptors

	Open Field	EPM
Open Field	1	–
EPM	0.412[a]	1
GR-Total Hippocampus	0.727[b]	0.403[a]
GR-DG	0.569[b]	0.353[c]
GR-CA1	0.853[b]	0.420[a]

CA1, cornu ammonis 1; DG, dentate gyrus; EPM, elevated plus maze; GR, glucocorticoid receptor.

[a] $p \leq .05$.

[b] $p \leq .01$.

[c] $p = .09$.