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Neuronal FGF22 signaling during development, but not in adults, is involved in anhedonia

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Abstract

Growth factor signaling in the brain is implicated in many neuropsychiatric disorders, including depression, autism, and epilepsy. Fibroblast growth factor 22 (FGF22) is a growth factor that regulates excitatory synapse development and neurogenesis in the brain. We have previously shown that adult mice in which FGF22 is constitutively inactivated in all cells throughout life (FGF22-null mice) show anhedonia, a core feature of depression in humans, suggesting that FGF22 signaling contributes to the regulation of affective behavior. Here we asked 1) whether inactivation of FGF22 specifically in neurons is sufficient to induce anhedonia in mice, and 2) whether FGF22 signaling is important during development or in adults for the regulation of affective behavior. To address these questions, we performed the sucrose preference test, which is used as an indicator of anhedonia, with neuron-specific conditional FGF22 knockout (KO) mice, in which FGF22 is inactivated in neurons at birth (neonatal-FGF22-KO mice) or in adults (adult-FGF22-KO mice). We found that neonatal-FGF22-KO mice show anhedonia (decreased preference for sucrose), while adult-FGF22-KO mice do not. Therefore, neuronal FGF22 signaling is critical during development, and not in adults, for the regulation of affective behavior. Our work also implies that defects in growth factor-dependent synapse development and/or neurogenesis may underlie depression of a developmental origin.

Keywords

Fibroblast growth factor; Synaptogenesis; Neurogenesis; Stage-specific conditional knockout mice; Anhedonia; Depression

Introduction

Fibroblast growth factors (FGFs) are a family of growth factors that regulate various brain functions [1-5]. Alterations in FGF and FGF receptor signaling have been found in many neuropsychiatric diseases, including depression [6], abnormal social behavior [7], seizures [8,9], and intellectual disability [9]. However, the manner by which FGF signaling contributes to such disorders is still largely unknown.

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We have previously shown that FGF22, a member of the FGF family, is critical for the establishment of excitatory synapses in the brain [8,10,11]. Mice in which FGF22 is constitutively inactivated (FGF22-null mice) show impaired excitatory synapse development, and these synaptic defects persist into adulthood [8]. FGF22 is also involved in neurogenesis in the brain [12,13]. FGF22-null mice show decreased neurogenesis throughout life. Thus, FGF22 contributes to multiple processes in the brain both during development and in adults. Importantly, adult FGF22-null mice exhibit depression-like behaviors including anhedonia and increased passive stress-coping behavior, without affecting anxiety-like behaviors, social cognition, and motor behaviors [14]. Therefore, FGF22 plays a unique role in the regulation of affective behaviors.

FGF22 is expressed in neurons and glia in the brain from the newborn through adult stage. In FGF22-null mice, FGF22 is constitutively inactivated in all cells throughout life. Which specific cell types and developmental periods are critical for FGF22 signaling to contribute to the depression-like phenotype remains an important open question. To address this question, we generated neuron-specific conditional FGF22 knockout (KO) mice, in which FGF22 is inactivated in neurons at birth (neonatal-FGF22-KO mice) or in adults (adult-FGF22-KO mice). Using these mice, we performed the sucrose preference test, a test that is used as an indicator of anhedonia. Here we show that neonatal-FGF22-KO mice exhibit decreased preference for sucrose, while adult-FGF22-KO mice do not. Therefore, neuronal FGF22 signaling is critical during development, and not in adults, for the regulation of affective behavior. Our work suggests that defects in FGF22-dependent synapse development and/or neurogenesis may underlie some forms of depression with a developmental origin.

Materials and Methods

Animals

Fgf22$^{flox/flox}$ mice (*Fgf22*$^{tm1a(EUCOMM)Hmgu}$) were from the Medical Research Council and European Mouse Mutant Archive [15]. Thy1-CreER mice ("SLICK-H"; Tg(Thy1-cre/ERT2,-EYFP)HGfng/PyngJ; JAX012708) were from Jackson [16]. Both males and females were used in our study. The numbers of animals used in the behavioral studies are shown in figure legends. All animal care and use was in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital.

Fluorescent *in situ* hybridization followed by immunostaining

Fluorescent *in situ* hybridization was performed with an RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics). Mm-*Fgf22* (Advanced Cell Diagnostics; No. 590561, custom made) was used as the RNAscope probe. Mice (8–9 months old) were decapitated, and the brains were removed and immediately frozen in Neg-50 frozen section medium (Thermo Fisher Scientific). Brains were sectioned at 16-μm thickness using a CM3050S cryostat (Leica Microsystems), and sections were mounted onto Diamond White Glass Charged Microscope Slides (Globe Scientific). Sections were fixed with pre-chilled 4% PFA in PBS for 15 min at 4°C, rinsed in PBS, dehydrated in an ethanol dilution series,

and air-dried for 5 minutes. A hydrophobic barrier was drawn around each section with an ImmEdge barrier pen (Vector Laboratories). Sections were then treated with hydrogen peroxide for 10 min at room temperature, rinsed in MilliQ water, treated with Protease IV for 15 minutes at room temperature, and rinsed in PBS. Subsequently, sections were incubated with the Mm-*Fgf22* probe for 2 hours at 40°C in a HybEZ oven (Advanced Cell Diagnostics) and treated with the amplifier series (AMP1 for 30 min at 40°C; AMP2 for 30 min at 40°C; AMP3 for 15 min at 40°C). Next, sections were treated with the HRP-C1 reagent for 15 min at 40°C, followed by the Opal 520 fluorophore reagent (Akoya Biosciences) for 30 min at 40°C. Finally, sections were treated with an HRP blocker for 15 min at 40°C. Sections were washed with 1X Wash Buffer between all incubation steps.

Immediately after the fluorescent *in situ* hybridization steps, immunostaining was performed. Sections were incubated with blocking buffer (2% BSA/0.1% Triton X-100 in PBS) for 30 min at room temperature, incubated with anti-NeuN antibody (1:250; EMD Millipore, Cat# MAB377) and anti-GFAP antibody (1:400; Synaptic Systems, Cat# 173002) in blocking buffer for 90 min at room temperature, and then incubated with fluorescent secondary antibodies (Donkey anti-Mouse IgG, Alexa Fluor 568; 1:500, Thermo Fisher Scientific; and Donkey anti-Rabbit IgG, Alexa Fluor 647, 1:500, Jackson ImmunoResearch) in blocking buffer for 45 min at room temperature. Sections were mounted with Fluoromount-G (Electron Microscopy Sciences).

Imaging

Fluorescent images were taken on a confocal microscope (Zeiss LSM700). 8-bit images at a 1,024 x 1,024 pixel resolution were obtained using a 25x objective lens with 0.5x zoom or a 40x objective lens with 1x zoom. Images were acquired as a z-stack. For images in Figure 2B, 10 optical sections (step size: 1.0 μm, total z-stack thickness 9.0 μm) taken with a 25x objective lens were stacked. For images in Figure 2C, 4 optical sections (step size: 0.5 μm, total z-stack thickness 1.5 μm) taken with a 40x objective lens were stacked. Images in the same set of experiments were acquired with the identical acquisition settings regarding the exposure time, laser power, detector gain, or amplifier offset. The intensity of stained signals was quantified and analyzed using MetaMorph software.

Sucrose preference test

Mice were subjected to the sucrose preference test at ages 4–10 months (ages 8–10 months for neonatal-KO and ages 4–10 months for adult-KO). Sucrose preference was assessed using a two-bottle choice experiment [13,14]: one bottle containing water and the other containing 2% (v/v) sucrose for 4 days. One day before the test, mice were singly housed in their housing room and given two bottles filled with 2% sucrose to expose them to its flavor. On the morning of day 1, a sucrose bottle and a water bottle were weighed and placed in the cage. Twenty-four hours later, these bottles were weighed and the bottle positions were switched to control for any side position preferences in the mice. The bottle positions were switched every twenty-four hours during the 4-day test, weighing the bottles each day before switching. Data are presented as the amount of sucrose consumed as a percentage of the total liquid consumed per day.

Statistical Analysis

Data were prepared and analyzed using Microsoft Excel or GraphPad Prism. The statistical test performed was two-tailed Student's t-test. The p values are indicated in the figure legends. The results were considered significant when $p < 0.05$. All data are expressed as mean ± s.e.m.

Results

FGF22-null mice in which FGF22 is constitutively inactivated in all cells throughout life show anhedonia [14], a core feature of depression in humans. Our goal in this study was to identify the specific cell types and developmental periods that are critical for FGF22 signaling to regulate affective behavior. For this, we utilized stage-specific FGF22 conditional KO mice. We mated *Fgf22*$^{flox/flox}$ mice [15] with Thy1-CreER mice (SLICK-H, which expresses tamoxifen-dependent Cre [CreER] only in neurons; almost all neurons in the brain express CreER postnatally [16]) to generate *Fgf22*$^{flox/flox}$::Thy1-CreER (inducible FGF22KO) mice. To inactivate *Fgf22*, we injected tamoxifen at P0 (0.1 mg/mouse to inactivate FGF22 neonatally = neonatal-FGF22-KO) or in adults (4–9 months of age; 0.5 mg/mouse for 5 days to inactive FGF22 in adults = adult-FGF22-KO) (Figure 1). We have previously shown that these tamoxifen injection methods effectively inactivate the target gene [8,10].

We first verified that *Fgf22* is indeed inactivated in neurons in the brain of FGF22-KO mice. For this, we performed *in situ* hybridization for *Fgf22* using the RNAscope technology, followed by immunostaining for NeuN (neuronal marker) and GFAP (astrocytic marker). We confirmed that the expression of *Fgf22* mRNA was significantly decreased in the brain in both neonatal-FGF22-KO and adult-FGF22-KO mice. In the CA3 region of the hippocampus, where *Fgf22* is highly expressed [8], only 8.73 ± 3.60% (neonatal-KO) or 10.00 ± 2.71% (adult-KO) of *Fgf22* mRNA was detectable in FGF22-KO mice relative to Control mice (Figure 2A and B). The remaining *Fgf22* mRNA was not expressed in NeuN-positive neurons, but in GFAP-positive astrocytes (Figure 2C, arrowheads). Thus, neonatal FGF22-KO mice have FGF22 inactivated specifically in neurons from P0, while adult-FGF22-KO mice have FGF22 inactivated in neurons in adults. By comparing these two animals, we can identify critical periods (during development vs. adulthood) for FGF22 signaling in the regulation of affective behavior. In addition, in these animals, FGF22 signaling was inactivated only in neurons. Thus, we can identify the role of FGF22 signaling specifically in neurons. As controls, we used *Fgf22*$^{flox/flox}$ mice (i.e., no Cre) injected with tamoxifen, *Fgf22*$^{flox/flox}$::Thy1-CreER without tamoxifen, and *Fgf22*$^{flox/flox}$ mice without tamoxifen.

Using the FGF22 conditional KO and control mice we have generated, we examined whether or not they display anhedonia. The sucrose preference test is a reward-based behavioral test to assess preference for sucrose-sweetened water over regular water. This test is used as an indicator of anhedonia [17]. Normally, mice prefer to drink sucrose far more than regular water, but mice with anhedonia show less preference for sucrose.

We first tested neonatal-FGF22-KO mice. We found that neonatal-FGF22-KO mice show a significantly lower preference to sucrose over water than control littermates (Figure 3). This result is consistent with anhedonia, indicating that FGF22 signaling in neurons is critical for regulating affective behavior between P0 and adulthood.

We next tested adult-FGF22-KO mice. In contrast to neonatal-FGF22-KO mice, we found that adult-FGF22-KO mice show a similar preference to sucrose over water relative to control littermates (Figure 4). Therefore, FGF22 signaling in adults does not contribute to the emergence of anhedonia.

Finally, we examined sucrose preference of additional control animals without tamoxifen injections. Without tamoxifen injection, *Fgf22*$^{flox/flox}$::Thy1-CreER and *Fgf22*$^{flox/flox}$ mice showed normal sucrose preference (Figure 5). Taken together, the results from these behavioral tests suggest that FGF22 signaling in neurons is critical during development, but not in adults, for the regulation of affective behavior.

Discussion

Using stage-specific conditional KO mice for FGF22, we showed developmental stage-specific roles of FGF22 for the manifestation of anhedonia: FGF22 signaling is critical during development, but not in adults. We also showed that FGF22 in neurons plays important roles for the anhedonia phenotype. Since FGF22 regulates excitatory synapse formation and neurogenesis during development [8,13], our results suggest that FGF22-dependent synapse development and/or neurogenesis may be regulating affective behavior.

The onset of human depression is primarily in adults; however, some forms of depression have a developmental origin [18]. For example, childhood abuse and trauma can directly predict the severity of depression [19]. Our study may suggest that defects in growth factor-dependent signaling during development may underlie depression of a developmental origin.

Interesting next questions include how factors known to influence risk for depression, such as childhood abuse and trauma, might affect FGF22-dependent signaling during development, and whether appropriate regulation of FGF22 signaling can alleviate depression that begins in childhood. Our study raises a possibility to target FGF22 as a possible treatment of certain forms of depression with a developmental origin.

Conclusion

We found that neonatal-FGF22-KO, but not adult-FGF22-KO, mice show anhedonia, a core feature of depression in humans. Our results indicate that FGF22 in neurons is critical during development for the regulation of affective behavior. Since FGF22 regulates synapse development and neurogenesis, defects in growth factor-dependent synapse development and/or neurogenesis may underlie depression of a developmental origin.

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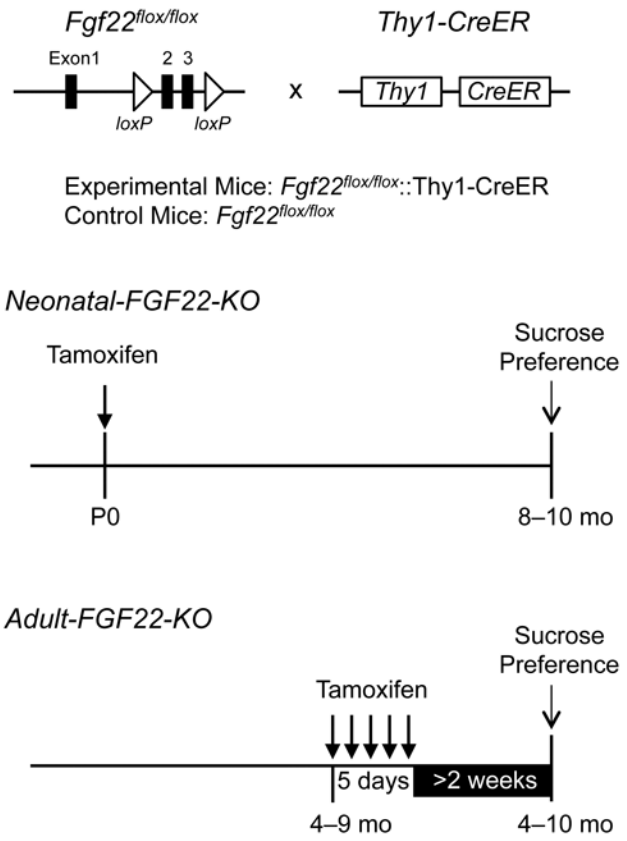

Figure 1.

Generation of neonatal- and adult-FGF22-KO mice. *Fgf22*$^{flox/flox}$ mice were crossed with mice carrying *Thy1*-promoter-driven CreER (*Thy1-CreER*). *Fgf22*$^{flox/flox}$::Thy1-CreER (inducible FGF22KO) mice were injected with tamoxifen at P0 (0.1 mg/mouse; "neonatal-FGF22-KO") or at 4–9 months of age (0.5 mg/mouse for 5 days; "adult-FGF22-KO"). Littermate *Fgf22*$^{flox/flox}$ mice injected with tamoxifen were used as controls. Sucrose preference tests were performed at the age of 4–10 months.

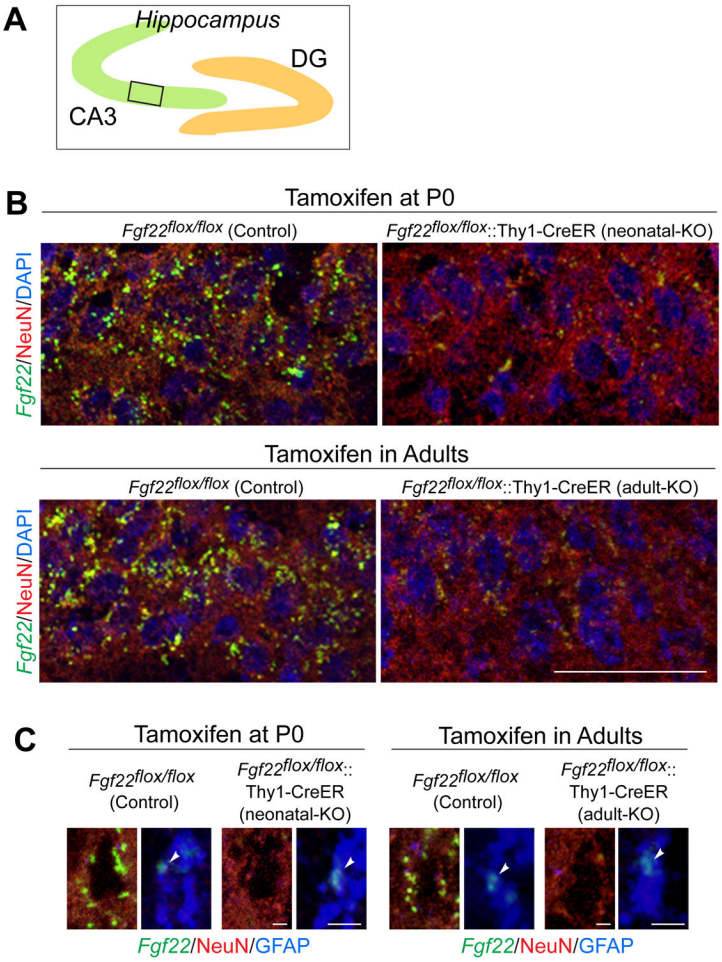

Figure 2.

Verification of *Fgf22* inactivation in FGF22-KO mice. Neuron-specific inactivation of *Fgf22* in neonatal- and adult-FGF22-KO mice was assessed by RNAscope *in situ* hybridization for *Fgf22* followed by immunostaining for NeuN (a neuronal marker) and GFAP (an astrocytic marker).

(A) Illustration showing the pictured area in (B). Images were taken from the CA3 pyramidal cell layer (boxed) in the hippocampus. DG, dentate gyrus.

(B) *Fgf22* mRNA (green) is highly expressed in the CA3 pyramidal cell layer in control mice. In contrast, *Fgf22* mRNA expression is substantially decreased in both neonatal- and adult-FGF22-KO mice. The *Fgf22* signal intensity is 8.73 ± 3.60% (neonatal-KO) and 10.00 ± 2.71% (adult-KO), respectively, relative to that in controls (n = 8 images per genotype). Red: NeuN, Blue: DAPI.

(C) Representative images showing *Fgf22* mRNA expressions in neurons and astrocytes in controls, neonatal-KO, and adult-KO mice. *Fgf22* mRNA in neurons is undetectable in both neonatal- and adult-FGF22-KO mice. In contrast, *Fgf22* mRNA in astrocytes remains in both neonatal- and adult-FGF22-KO mice (arrowheads). Red: NeuN, Blue: GFAP.

Scale bars, (B) 50 μm, (C) 2 μm.

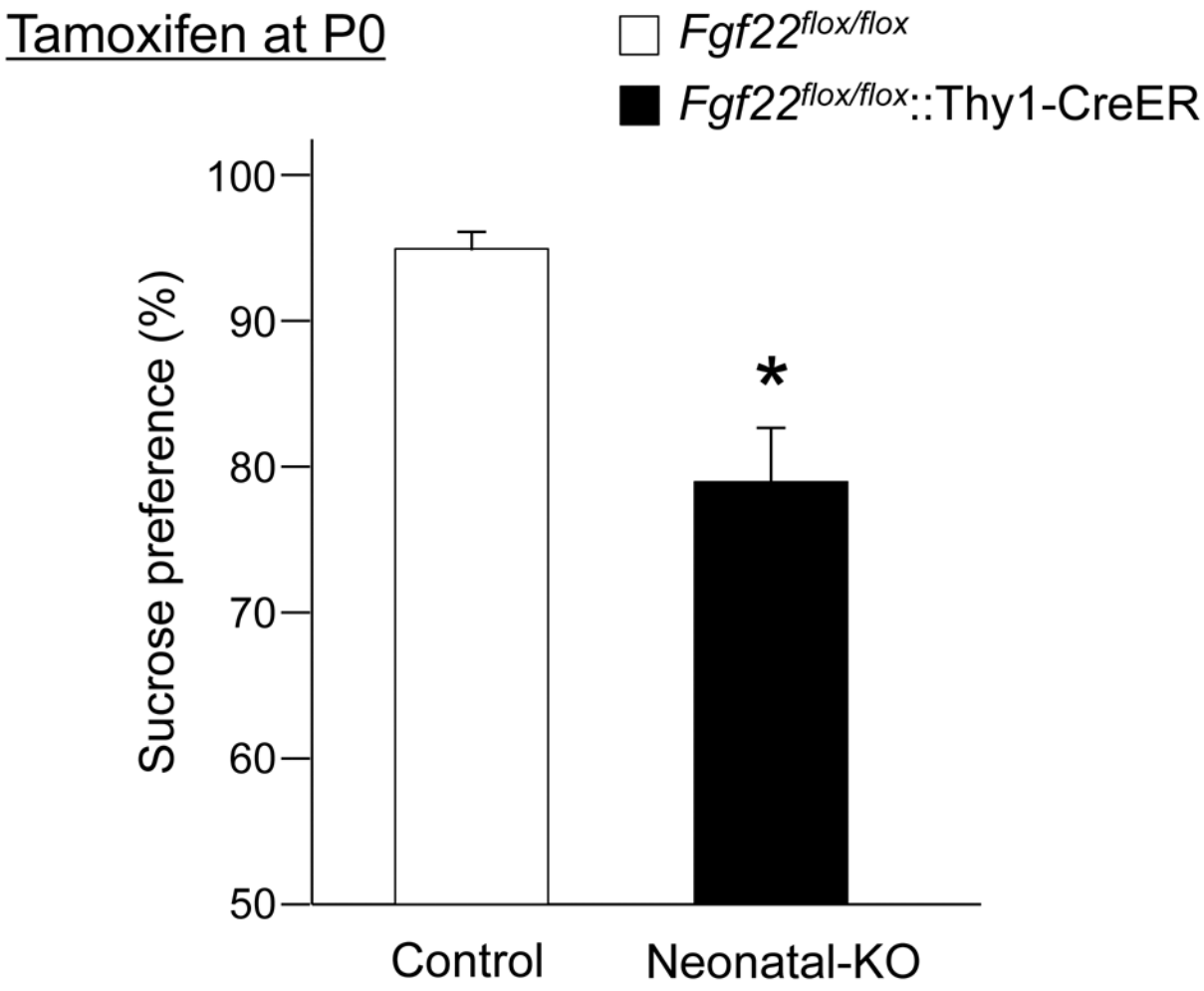

Figure 3.

Sucrose preference test with neonatal-FGF22-KO mice. Neonatal-FGF22-KO mice show a significantly lower preference to sucrose over water than control littermates. n = 5 mice per genotype. *p = 0.0442 (Student's t-test).

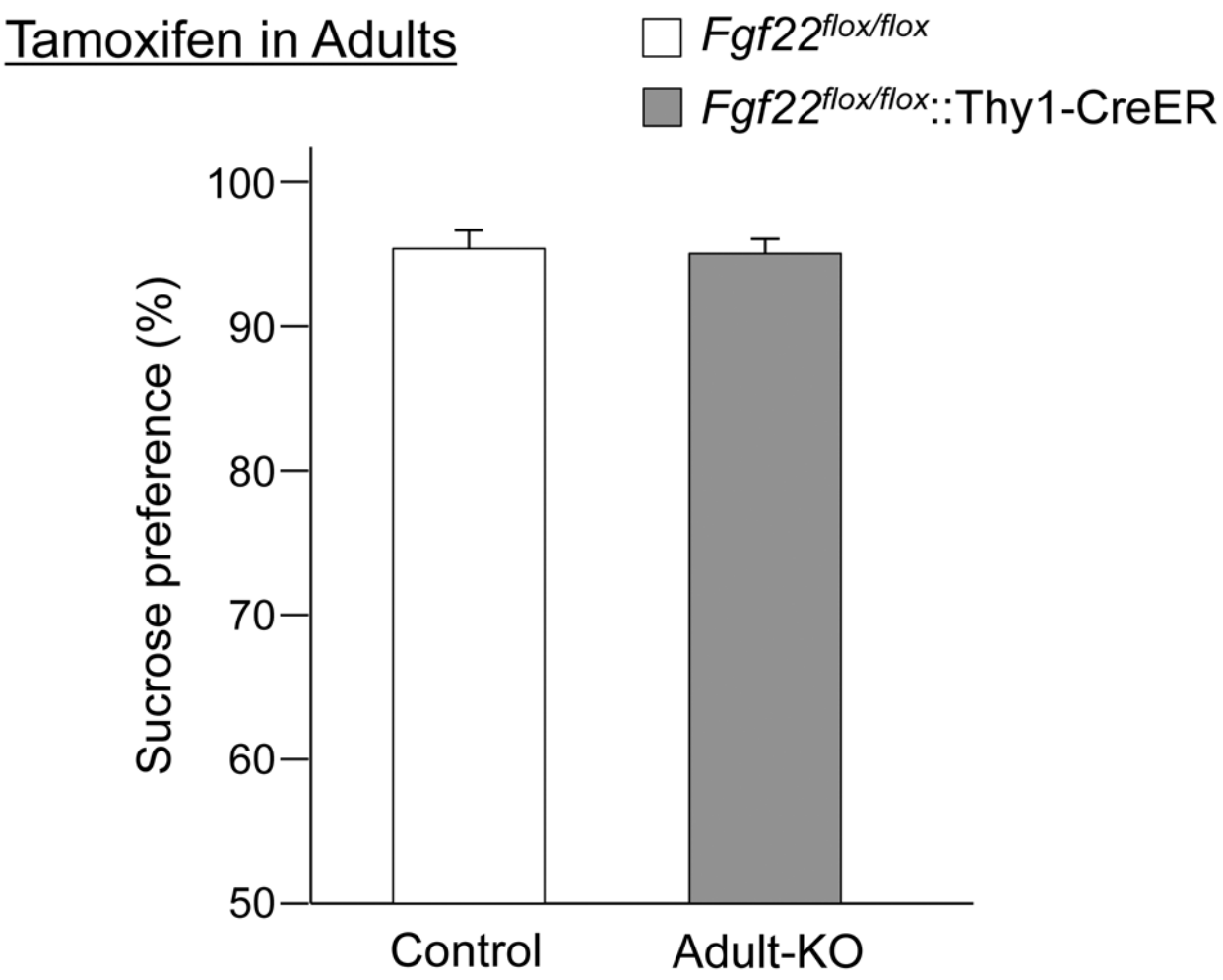

Figure 4.

Sucrose preference test with adult-FGF22-KO mice. In contrast to neonatal-FGF22-KO mice, adult-FGF22-KO mice do not show differences from control littermates in terms of sucrose preference. n = 6 (WT) and 7 (KO) mice. p = 0.893 (Student's t-test).

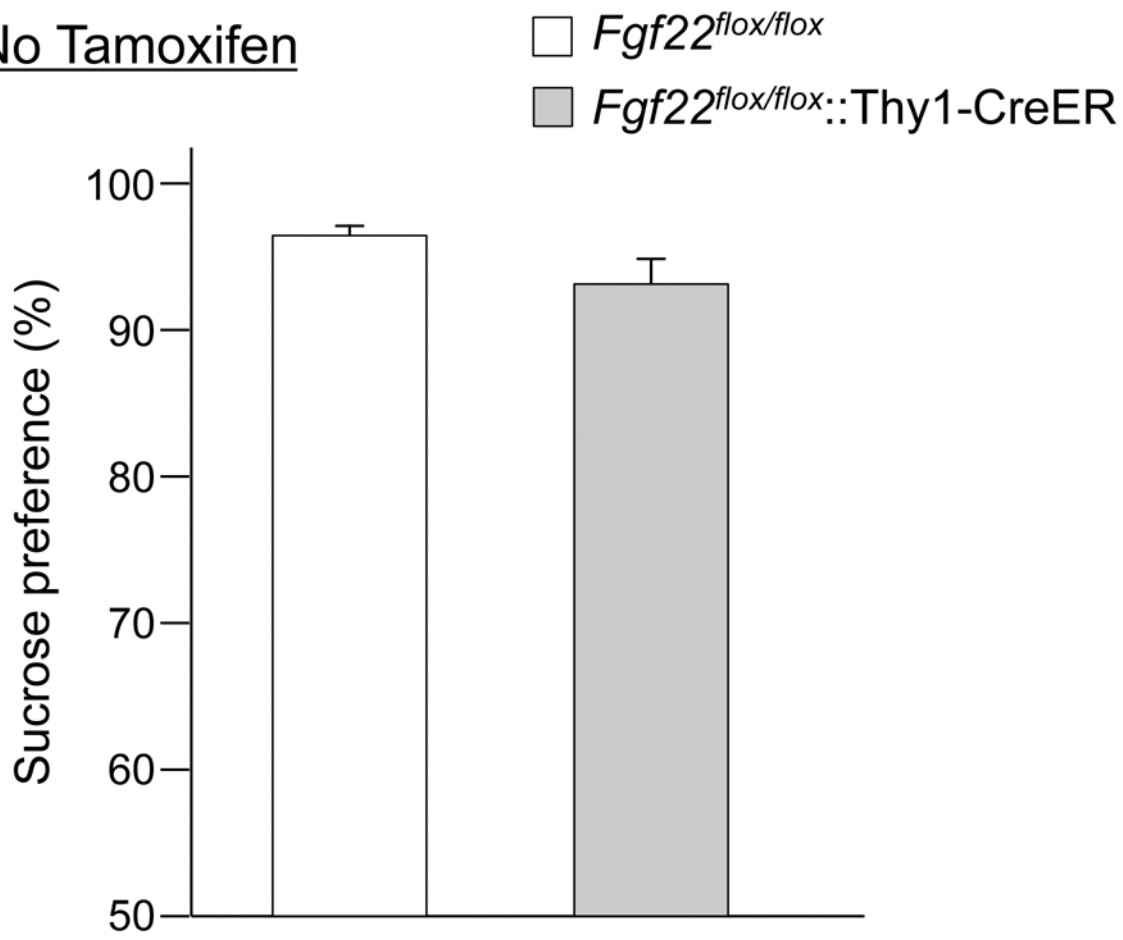

Figure 5.

Sucrose preference test with *Fgf22*$^{flox/flox}$::Thy1-CreER mice without tamoxifen injections and *Fgf22*$^{flox/flox}$ mice without tamoxifen injections. Without tamoxifen injections, *Fgf22*$^{flox/flox}$::Thy1-CreER and *Fgf22*$^{flox/flox}$ mice showed normal sucrose preference. n = 5 mice for each genotype.