Mice with High FGF21 Serum Levels Had a Reduced Preference for Morphine and an Attenuated Development of Acute Antinociceptive Tolerance and Physical Dependence

Louben Dorval[a], Brian I. Knapp[a], Olufolake A. Majekodunmi[a], Sophia Eliseeva[b], and Jean M. Bidlack[a*]

[a]*Department of Pharmacology and Physiology, University of Rochester, School of Medicine and Dentistry, 14642, Rochester, NY, USA*

[b]*Department of Medicine, Pulmonary and Critical Care, University of Rochester, School of Medicine and Dentistry, 14642, Rochester, NY, USA*

**Corresponding author:* Department of Pharmacology and Physiology, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, USA

E-mail address: jean_bidlack@urmc.rochester.edu (J.M. Bidlack)

Footnote:

Abbreviations: ANOVA – analysis of variance, bpm – breaths per minute, CI – confidence interval, CPP – conditioned place preference, ELISA – enzyme-linked immunosorbent assay, FGF – fibroblast growth factor, FGF21-Tg – FGF21 transgenic, H7 – 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, IL-1β – interleukin-1β, KLB – β-Klotho, MOR – mu opioid receptor, NAc – nucleus accumbens, NFκB – nuclear factor kappa B, OUD – opioid use disorder, PKC – protein kinase C, WT– wildtype

ABSTRACT

As a result of the opioid epidemic, there is a desire to identify new targets for treating opioid use disorder. Previous studies showed that fibroblast growth factor 21 (FGF21) decreased alcohol and sweet preference in mice. In this study, FGF21-transgenic (FGF21-Tg) mice, expressing high FGF21 serum levels, and wildtype (WT) C57BL/6J littermates were treated with morphine and saline to determine if differences exist in their physiological and behavioral responses to opioids. FGF21-Tg mice displayed reduced preference for morphine in the conditioned place preference assay compared to WT littermates. Similarly, FGF21-Tg mice had an attenuation of the magnitude and rate of acute morphine antinociceptive tolerance development, and acute and chronic morphine physical dependence, but exhibited no change in chronic morphine antinociceptive tolerance. The ED_{50} values for morphine-induced antinociception in the 55°C hot plate and the 55°C warm-water tail withdrawal assays were similar in both strains of mice. Likewise, FGF21-Tg and WT littermates had comparable responses to morphine-induced respiratory depression. Overall, FGF21-Tg mice had an attenuated preference for morphine, a reduced development of morphine-induced dependence, and a reduction in the development of acute morphine antinociceptive tolerance. FGF21 and its receptor have therapeutic potential for reducing opioid withdrawal symptoms and craving, and augmenting opioid therapeutics for acute pain treatment.

Keywords:

Morphine, Reward, Antinociception, Tolerance/Dependence, FGF21, Respiratory depression

1. Introduction

Opioid use disorder (OUD) continues to be a serious problem affecting health, social, and economic welfare nationally and globally. Adverse effects of opioid misuse include analgesic tolerance development, physical and psychological dependence, respiratory depression, withdrawal symptoms, and relapse. Treatment of OUD has shifted from managing withdrawal symptoms toward medications for treating OUD (Shulman et al., 2021). Unfortunately, current treatments for OUD have shown limited effectiveness. The most common treatments for OUD are replacement therapies, which use either a full opioid agonist (methadone) or an opioid partial agonist (buprenorphine) to wean subjects off more addictive opioids and to prevent relapse. Regrettably, these therapies have a high failure rate due to poor compliance (Alho et al., 2007; Kakko et al., 2003; Roche et al., 2008).

The rewarding and reinforcing effects of morphine and other opioids lead to opioid misuse, the development of dependence, and OUD. The reinforcing effects of opioids are mediated by increases in dopamine levels in certain brain regions (Cao et al., 2021; Nisell et al., 1994; Wiss et al., 2018). In addition to opioids, sucrose, saccharin and alcohol, as well as other drugs of abuse, increase dopamine release in the nucleus accumbens (NAc) and this increase in dopamine levels has been correlated to development of preference in mice (Ma and Zhu, 2014; Yoshimoto et al., 1992).

Fibroblast growth factor 21 (FGF21) is a protein that is expressed in liver, brown adipose tissue, glia, and neurons (Potthoff et al., 2012). Unlike most fibroblast growth factors (FGFs), FGF21 is released into the bloodstream and can act throughout the body (Potthoff et al., 2012). FGF21 administration in obese diabetic mice ameliorated hyperglycemia and lowered elevated triglycerides (Coskun et al., 2008). Moreover, FGF21 administered to obese diabetic monkeys improved glucose and circulating lipids levels (Kharitonenkov et al., 2007). FGF21 analogues are now in clinical trials to treat type 2 diabetes, obesity, and nonalcoholic steatohepatitis (Geng et al., 2020). LY2405319, PF-05231023, and Efruxifermin (AKR-001)

produced a decrease in triglycerides, total cholesterol, low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol (Dong et al., 2015; Gaich et al., 2013; Stanislaus et al., 2017). Importantly, FGF21 crosses the blood-brain barrier and acts centrally to induce the sympathetic nervous system (Hsuchou et al., 2007; Owen et al., 2014) by binding to FGF receptors in complex with the obligate co-receptor β-Klotho (KLB) (Ding et al., 2012; Owen et al., 2015). In mice, elevated FGF21 levels and the FGF21 analogue PF-05231023 have been shown to attenuate sweet and alcohol preference (Talukdar et al., 2016).

In addition, mouse models have been created to better understand FGF21 signaling pathway. In this study, we used the FGF21 transgenic (FGF21-Tg) mouse model. Transgenic mice were generated and maintained on a C57BL/6J background. These mice have the FGF21 transgene under the control of apolipoprotein E promoter, which drives the expression of the gene in liver, resulting in overexpression of the FGF21 gene (Inagaki et al., 2007). FGF21-Tg mice have an extended lifespan by about 10 months and have an increased insulin-sensitivity (Zhang et al., 2012). Overexpression of FGF21 increases metabolic rate and induces weight loss in FGF21-Tg mice (Singhal et al., 2016). Moreover, female FGF21-Tg mice are infertile (Zhang et al., 2012).

Since alcohol and saccharin increase dopamine levels in areas of the brain similar to morphine and drugs of abuse, and overexpression of FGF21 has been shown to attenuate preference for alcohol and saccharin, possibly through a dopamine-dependent mechanism (Talukdar et al., 2016), we hypothesized that FGF21-Tg mice would show reduced preference for morphine. Moreover, we also characterized FGF21-Tg and wildtype (WT) mice in assays designed to examine other opioid-induced behavioral and physiological responses including analgesia, tolerance, physical dependence, locomotion, and respiratory depression. Because differences between sexes have been observed in morphine-induced responses in different strains of animals (Hopkins et al., 2004; Kest et al., 1999), we examined both male and female mice in this study.

2. Materials and methods

2.1. Animals.

FGF21-Tg mice and WT littermates (Stock number 021964) were obtained from The Jackson Laboratory, Bar Harbor, ME, USA. Mice were housed five to a cage and maintained in a temperature- and humidity-controlled room at the University of Rochester Medical Center (Rochester, NY) vivarium on a 12:12-h light/dark cycle (lights off at 18:00 h) with food and water available *ad libitum*. Breeding of hemizygous males with WT females yielded approximately 50% FGF21-Tg offspring. Male (234 FGF21-Tg and 266 WT) and female (227 FGF21-Tg and 238 WT) mice (2- to 4-month old) were used for all experiments. Different groups of mice were used for each dose of morphine administration. All procedures were pre-approved and carried out in accordance with the University Committee on Animal Resources at University of Rochester.

2.2. Drugs.

Morphine sulfate was purchased from Mallinckrodt Chemical Company (St. Louis, MO). Naloxone was purchased from Sigma-Aldrich (St Louis, Missouri, USA). Both morphine and naloxone were solubilized in sterile saline. All injections were administered through intraperitoneal (i.p.) routes in a maximum volume of 10 ml/kg.

2.3. Enzyme-linked immunosorbent assay (ELISA) to measure FGF21 protein levels.

Serum FGF21 protein levels of FGF21-Tg and WT C57BL/6J littermates were determined using a mouse/rat solid-phase FGF21 Quantikine ELISA Kit MF2100 (R&D

Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Mouse tail blood was collected in a 1.5-ml Eppendorf tube and left at room temperature for 1 h. The blood was centrifuged at 5000-*g* for 15 min at 25°C. Afterwards, the serum was collected and analyzed by ELISA for FGF21 using an EL800 microplate reader (BioTek Instruments, Winooski, VT, USA) set with a detection wavelength of 450 nm and a correction wavelength at 540 nm. Sample FGF21 concentrations were determined from a standard curve fit with a logistic 4-parameter function using SigmaPlot (Systat Software, Inc.). The mean minimum detectable concentration of FGF21 was 3.81 pg/ml according to the manufacturer.

2.4. Conditioned place preference (CPP).

The preference of FGF21-Tg and WT littermates for morphine was determined using a biased CPP protocol as described by Carey et al. (2007). Mice were placed in an apparatus divided into three compartments: two outer compartments (27.3 x 22.2 x 34.9 cm) with distinct wall striping (vertical vs. horizontal alternating black and white lines, 1.5 cm in width) and floor texture (lightly mottled vs. smooth) joined to a smaller central chamber (13.9 x 22.2 x 34.9 cm) with sliding doors (Place Preference, San Diego Instruments, San Diego, CA). Infrared beams along the walls of the apparatus, connected to a computer, allowed automated measurement of time spent in each compartment. Mice were given 30 min to move freely between all compartments to acclimate to the new environment and their initial outer chamber preference was determined. Afterwards, the mice were administered 3 or 10 mg/kg of morphine and place-conditioned in their initially non-preferred chamber for 30 min. Separate groups of mice were treated with different doses of morphine, which were chosen for their reported ability to produce CPP (Hoot et al., 2013; Orsini et al., 2005; Solecki et al., 2009). Six h later, mice were injected with an equivalent volume of saline and placed in their initially preferred chamber for 30 min. This cycle of place conditioning with morphine, followed by saline 6 h later, was repeated the

next day, and final place preference was determined on the third day (Hoot et al., 2013). CPP data are presented as the mean difference in time spent in drug- and vehicle-associated chambers ± S.E.M.

2.5. 55°C Hot plate and 55°C warm-water tail withdrawal tests to measure antinociception.

The antinociceptive responses for FGF21-Tg and WT littermates were compared using ED_{50} values in the 55°C hot plate and the 55°C warm-water tail withdrawal assays. Separate groups of mice were used for each experiment. On the day of the experiment, baseline measurements (basal latencies) were performed before each mouse was administered morphine. The 55°C hot plate test was used to determine the effect of morphine on supraspinal antinociceptive response in FGF21-Tg and WT littermates (Deuis et al., 2017). This test was performed by placing the mouse on a heated surface (55°C, Hot Plate Analgesia Meter, Model 39, San Diego Instruments, San Diego, CA) and measuring the latency for the mouse to jump or lick their paw in response to the heat stimulus. Test latencies were determined at 20, 30, 60, and 90 min after a single morphine injection (18, 20, or 23 mg/kg). A maximum antinociception score (100%) was assigned to mice that did not respond within 30 s. Thus, percentage of antinociception = 100 × [(test latency – basal latency) / (30 – basal latency)].

The effect of morphine on the spinal antinociceptive response in FGF21-Tg and WT littermates was determined using the 55°C warm-water tail withdrawal test (Drgonova et al., 2010; Mathews et al., 2008). Mice were placed in a Plexiglas mouse restrainer and positioned so the tail was immersed in a 55°C warm-water bath. Latency was recorded as the amount of time from when the mouse's tail enters the water bath to when the mouse perceives a painful stimulus, flicking its tail out of the water (Mathews et al., 2008). Test latency was determined at different time points (20, 30, 60, and 90 min) after morphine administration of 3, 5.6, and 10

mg/kg. A maximum antinociception score (100%) was assigned to mice that did not respond within 15 s to avoid tissue damage (Bidlack et al., 2002; McLaughlin et al., 2006). Percentage of antinociception = 100 × [(test latency – basal latency) / (15 – basal latency)].

2.6. Acute morphine analgesic tolerance development.

After determination of morphine-induced antinociception in WT and FGF21-Tg littermates, we investigated if FGF21-Tg mice exhibited similar acute morphine-induced analgesic tolerance development as WT littermates using a method described previously (Mathews et al., 2008). FGF21-Tg and WT littermates were treated with 10, 18, or 23 mg/kg morphine in the 55°C hot plate assay and 3, 5.6, or 10 mg/kg morphine in the 55°C warm-water tail withdrawal assay. Morphine was administered at times 0, 3, 6, and 9 h. Time 0 was the first injection of morphine. Dose-response curves showing percentage of antinociception were generated for each time point. Because not every curve reached 50% antinociception, tolerance was determined by comparing the response after time 3, 6 or 9 h to time 0 h at matching doses. Antinociception was assessed 30 min after each injection because morphine produced the greatest antinociceptive response 30 min after injection in the time-course antinociception assays. Percent of antinociception was determined as described above.

2.7. Acute morphine physical dependence.

To assess the development of acute morphine physical dependence, mice received an injection of a high dose of morphine (100 mg/kg) followed 4 h later by an injection of the opioid antagonist, naloxone (10 mg/kg). This method has been shown to induce development of morphine dependence and withdrawal symptoms such as jumping, forepaw tremor, and rearing (Bilsky et al., 1996; Mathews et al., 2008; Parker and Joshi, 1998; Wang et al., 1999; Yano and

Takemori, 1977). We focused on the number of vertical jumps, as this withdrawal symptom was the most apparent. Ten min before injection of naloxone, mice were placed in a large transparent beaker (Height: 125 mm, outside diameter: 35 mm, Kimax Glassware) to habituate them to the new environment. Immediately after the naloxone injection, mice were returned to the beaker and the number of vertical jumps was recorded for 15 min.

2.8. Chronic morphine analgesic tolerance development and dependence.

In addition to acute morphine analgesic tolerance, we also explored chronic morphine analgesic tolerance development in WT and FGF21-Tg littermates. Mice were treated twice daily (every 12 h) for 5 consecutive days with 23 mg/kg morphine. On day 6, mice were administered a single dose of 23 mg/kg morphine. Analgesia was assessed every day with the 55°C hot plate assay, immediately before and 30 min after the first morphine injection of the day, and the percentage of antinociception was determined. Day 0 was the first day of morphine administration.

On the last day, withdrawal was precipitated with an injection of naloxone (10 mg/kg, 2 h after the last morphine administration). Immediately after the naloxone injection, each mouse was placed in a large transparent beaker and the number of withdrawal jumps was recorded for 15 min.

2.9. Locomotion assay.

Morphine induces locomotor activity in mice (Valjent et al., 2010). Therefore, we investigated if morphine-induced locomotion would be affected in FGF21-Tg mice in comparison to WT littermates. Saline was used as a control. Mice were administered 10 mg/kg or 23 mg/kg morphine and placed in an open-field activity apparatus (PAS-Open Field, San Diego

Instruments, San Diego, CA) for 2 h. The apparatus uses an open field Plexiglas chamber equipped with photocell emitters and receptors (Tatem et al., 2014). The distance traveled (cm) was recorded for each mouse (Fusion Software, version 3.9, Omnitech Electronics, Inc., Columbus, Ohio).

2.10. Morphine-induced respiratory depression.

Morphine and other μ opioid agonists activate the μ opioid receptor (MOR) expressed on the surface of neurons in brainstem respiratory centers (Dahan et al., 2010). Activation of these MOR leads to respiratory depression (Pattinson, 2008). To examine if FGF21-Tg mice had a different respiratory rate after morphine administration in comparison to WT littermates, a whole-body plethysmography suite (SCIREQ, Montreal, Canada) was used. This non-invasive method measures mouse respiration using differential pressure transducers connected through an interface (Emka Technologies, France) to a computer for recording and analysis of respiration parameters (Hill et al., 2018). Mice were placed in a small chamber, supplied with room air via a pump, and allowed to move freely throughout the measurements. Based on pressure changes within the chamber, we were able to analyze breathing rates. Mice were habituated to the plethysmography chambers for 3 days for 30 min. On the experimental day (day 3) after the last habituation period, baseline respiration rates, breaths per min (bpm) were measured over a 20-min period before morphine administration. Subsequently, morphine was administered (18 or 30 mg/kg) in a 10-min window and mice were returned to the plethysmograph chambers. The respiration rate was recorded at 5-min intervals for 90 min. Changes in respiration rate were used to evaluate respiratory depression following morphine injection. Data are reported as percent respiration rate relative to baseline ± S.E.M.

2.11. Statistical analysis.

Protein expression in mice was analyzed using JMP Pro software (SAS Institute, Cary, NC) with two-way analysis of variance (ANOVA). We further compared the protein expression between male and female mice of the same genotype using Student's t-test. Conditioned placed preference data were analyzed using JMP Pro software with three-way ANOVA when examining the interaction of dose, sex, and genotype in the post-conditioning. Significant effects were further analyzed using one-way ANOVA comparing morphine CPP between WT and transgenic mice at the same dose.

ED_{50} values and 95% confidence interval (CI) for morphine-induced antinociceptive dose-response curves were created using each individual data point with Prism 5.0 software (GraphPad Software, La Jolla, California). Acute and chronic morphine tolerance development data were analyzed using repeated measures one-way ANOVA. Significant results were further analyzed with paired t-test post hoc to compare the initial analgesic response (time 0 h for acute tolerance or day 0 for chronic tolerance) to responses following subsequent injections (JMP Pro). Differences in withdrawal jumps, an assessment of morphine physical dependence, were determined using two-way ANOVA followed by t-test to compare WT and transgenic mice.

Locomotor activity was analyzed with one-way, two-way, and three-way ANOVA when studying the effects of dose on the same group of mice, dose*genotype, and dose*genotype*sex; respectively. Data for respiratory depression were analyzed with two-way ANOVA when studying the effects of treatment and genotype (JMP Pro) and further analyzed by one-way ANOVA comparing two groups at a certain dose (18 mg/kg or 30 mg/kg). All data are presented as means ± standard error of the mean (SEM) with the significance set at P value < 0.05.

3. Results

3.1. FGF21 protein level in FGF21-Tg and WT mouse serum.

FGF21-Tg mice had a 2,400-fold greater level of serum FGF21 than WT littermates as measured by an ELISA ($F(1,52) = 226.5$, $p < 0.0001$, Table 1). There was no difference in FGF21 levels between male and female mice from either group ($t(19) = 0.06$, $p = 0.9503$ in FGF21-Tg, and $t(32) = 1.20$, $p = 0.2394$ in WT mice).

3.2. Morphine CPP in FGF21-Tg and WT littermates.

FGF21-Tg and WT mice had similar mean initial preferences in the CPP assay. Furthermore, no differences in mean initial preference were observed between FGF21-Tg and WT mice when separated by sex ($t(57) = 0.1242$, $p = 0.90$ and $t(45) = 1.109$, $p = 0.30$, Fig. 1a and 1b, respectively). In WT mice, morphine at 1 mg/kg did not produce CPP (Supplemental Fig. 1). Morphine CPP was observed following injection of 3 mg/kg and 10 mg/kg morphine (Fig. 1). Three-way analysis of variance indicated a difference only in genotype among male and female mice treated with 3 and 10 mg/kg (Factor *Genotype*: $F(1,103) = 15.25$, $p = 0.0002$, three-way ANOVA). Thus, we further examined differences in CPP between WT and FGF21-Tg mice treated with equal doses of morphine. Male FGF21-Tg mice had a lower preference for morphine relative to male WT at 10 mg/kg, but not at 3 mg/kg ($F(1,30) = 4.710$, $p = 0.04$ and $F(1,26) = 0.1982$, $p = 0.66$, respectively, Fig. 1a). Female FGF21-Tg mice showed lower morphine CPP compared to female WT mice at both the 10 and 3 mg/kg doses ($F(1,22) = 5.960$, $p = 0.02$ and $F(1,22) = 10.56$, $p = 0.004$, respectively). Notably, female FGF21-Tg mice showed no CPP in response to 3 mg/kg morphine (Fig. 1b). These results show that mice

expressing high serum levels of FGF21 had a reduced preference for morphine in comparison to WT littermates.

3.3. Morphine-induced antinociception in FGF21-Tg and WT littermates.

Morphine-induced antinociception was measured in FGF21-Tg and WT mice using both the 55°C hot plate and the 55°C warm-water tail withdrawal assays to assess supraspinal and spinal antinociception, respectively. In the 55°C hot plate assay, the mean basal latencies were similar for both FGF21-Tg (7.04 ± 0.08 s) and WT (7.18 ± 0.08 s) mice ($t(119) = 1.248$, $p = 0.21$). Both FGF21-Tg and WT mice administered 18, 20, or 23 mg/kg morphine reached maximal morphine-induced antinociception 30 min post-injection (Supplemental Fig. 2). Therefore, the 30-min morphine antinociceptive response was used to construct morphine dose-response curves. Morphine produced dose-dependent antinociception with ED_{50} values and 95% CI of 19.4 mg/kg (16.2 - 23.2) and 19.1 mg/kg (15.5 - 23.5) in male FGF21-Tg and WT mice, respectively (Fig. 2a). In female mice, the ED_{50} values were 19.6 mg/kg (19.5 - 19.7) for FGF21-Tg and 18.3 mg/kg (16.2 - 20.7) for WT mice (Fig. 2b). Overall, FGF21 overexpression had no effect on supraspinal morphine analgesic response in either male or female mice.

The 55°C warm-water tail withdrawal assay was used to assess whether FGF21 overexpression affects the spinal morphine analgesic response. Mice used in the tail withdrawal assay had similar mean basal latencies of 1.74 ± 0.05 s and 1.70 ± 0.05 s for FGF21-Tg and WT mice, respectively $t(124) = 0.6055$, $p = 0.55$). Maximal morphine antinociception was reached 30 min after injection in both transgenic and WT mice at every dose (data not shown). Morphine dose-response curves were constructed similarly to those from the hot plate antinociception assay. The ED_{50} values and 95% CI were 5.33 mg/kg (1.59 - 17.8) and 5.33 mg/kg (1.85 - 15.4) in male FGF21-Tg and WT mice, respectively (Fig. 2c). These values were

5.85 mg/kg (1.69 - 20.2) and 4.93 mg/kg (4.38 - 5.55) in female FGF21-Tg and WT mice, respectively (Fig. 2d). These data show that there was no difference in the potency or efficacy of morphine in the FGF21-Tg and WT littermates in both antinociceptive assays regardless of sex.

3.4. Acute morphine-induced analgesic tolerance development in FGF21-Tg and WT littermates.

The development of acute morphine analgesic tolerance in WT and FGF21-Tg mice was measured to determine if FGF21 overexpression had any effect on morphine tolerance development. To evaluate the effect of FGF21 overexpression on supraspinal morphine tolerance development in mice, using the 55°C hot-plate assay, morphine was administered repeatedly at (0, 3, 6, and 9 h) at doses ranging from 18 - 23 mg/kg. Mean baseline latencies were similar for FGF21-Tg mice and WT littermates at 7.30 ± 0.07 s and 6.97 ± 0.06 s, respectively. Moreover, basal latencies returned to pre-morphine administration levels before each subsequent injection (data not shown). There was a difference in morphine antinociception in all groups of mice (Factor *time:* $F(3,7) = 36.66$, $p < 0.0001$ in male WT; $F(3,7) = 8.86$, $p = 0.0007$ in male FGF21-Tg; $F(3,7) = 22.05$, $p < 0.0001$ in female WT; and $F(3,7) = 4.452$, $p = 0.0023$ in female FGF21-Tg). We further characterized at what point acute morphine tolerance was developed in each group of mice via paired t-test post hoc. In WT mice, acute morphine tolerance developed after the second injection (3 h, $p = 0.0011$, Fig. 3a), while FGF21-Tg male mice did not show tolerance until after the 6-h injection at the 23 mg/kg morphine dose ($p = 0.011$, Fig. 3b). WT female mice developed morphine tolerance by the second injection (3 h) at dose 23 mg/kg ($p < 0.0001$, Fig. 3c), while FGF21-Tg female mice developed tolerance to morphine by the third injection (6 h, $p = 0.043$, Fig. 3d). The decreased morphine analgesic response over 9 h was 41% and 71% in FGF21-Tg and WT mice, respectively, when

administered 23 mg/kg morphine. Overall, FGF21-Tg mice showed a reduced development to acute morphine-induced analgesic tolerance in the 55°C hot-plate assay.

A 55°C warm-water tail withdrawal assay was performed to determine the effect of FGF21 overexpression on spinal acute morphine analgesic tolerance development in mice. Morphine was administered with doses ranging from 3 - 10 mg/kg at (0, 3, 6, 9 h). There was no difference in baseline latencies for each group of mice used in the tail withdrawal assay with FGF21-Tg and WT littermates having basal latencies of 1.62 ± 0.04 s and 1.61 ± 0.03 s, respectively ($t(123) = 0.09014$, $p = 0.93$). Baseline latencies for every group of mice before each injection were similar (data not shown). There was a difference in morphine antinociception in all groups of mice (Factor *time:* $F(3,7) = 14.69$, $p = 0.0001$ in male WT; $F(3,7) = 2.375$, $p = 0.0289$ in male FGF21-Tg; $F(3,7) = 21.56$, $p < 0.0001$ in female WT; and $F(3,7) = 3.107$, $p = 0.0149$ in female FGF21-Tg). We further characterized at what point acute morphine tolerance was developed in each group of mice via paired t-test post hoc. Male WT mice developed acute morphine tolerance by the second injection (3 h) when administered 10 mg/kg ($p = 0.027$, Fig. 4a). Conversely, male FGF21-Tg mice did not develop acute analgesic tolerance until the 9-h injection when administered 10 mg/kg morphine ($p = 0.011$, Fig. 4b). Female WT mice became tolerant to morphine by the second injection (3 h) of 10 mg/kg morphine ($p = 0.013$, Fig. 4c). However, female FGF21-Tg mice showed tolerance development by the last injection (9 h) with 10 mg/kg morphine ($p = 0.0034$, Fig. 4d). When administered with 10 mg/kg of morphine, FGF21-Tg mice had a reduced analgesic response by 27%, while WT mice had a reduced analgesic response by 57% over the 9-h period. Overall, FGF21-Tg mice showed attenuated and slower acute morphine analgesic tolerance development compared to WT littermates in the 55°C warm-water tail withdrawal. The results between the 55°C hot plate and tail withdrawal assays were consistent.

3.5. Comparison of FGF21-Tg and WT littermates in the development of acute morphine dependence.

To understand the effect of FGF21 overexpression on morphine dependence development, an acute morphine dependence paradigm was used (Mathews et al., 2008). The number of withdrawal jumps of morphine-treated mice subsequently injected with naloxone or saline was determined. Mice treated with saline did not exhibit withdrawal jumping. Acute morphine dependence was observed in all mice and there was a significant difference based on genotype and sex ($F(3,39) = 56.01$, Sex: $p = 0.0033$ and genotype: $p < 0.0001$, Fig. 5). Naloxone-induced withdrawal jumping was similar in male and female WT mice ($t(19) = 1.080$, $p = 0.29$, Fig. 5). In FGF21-Tg mice, naloxone-induced withdrawal jumping was lower in male compared to female littermates ($t(19) = 8.833$, $p < 0.0001$, Fig. 5). Both male and female FGF21-Tg mice displayed fewer withdrawal jumps relative to their WT counterparts ($t(19) = 8.898$, $p < 0.0001$ for male FGF21-Tg and Male WT mice, and $t(19) = 8.900$, $p < 0.0001$ for female FGF21-Tg and female WT mice).

3.6. Chronic morphine-induced analgesic tolerance development in FGF21-Tg and WT littermates.

Following the acute morphine-induced analgesic tolerance, we sought to evaluate if FGF21 had any effect on chronic morphine tolerance development. Mice were administered a dose of 23 mg/kg morphine twice a day for five consecutive days. On the sixth day, mice were administered a single dose of morphine (23 mg/kg). The 55°C hot plate was used to measure antinociception 30 min before and after the first daily morphine dose. FGF21-Tg mice and WT littermates had similar baseline latencies on day 0 (7.40 ± 0.17 s and 7.42 ± 0.25 s, respectively, $t(34) = 0.0660$, $p = 0.9477$). The response latencies after morphine administration

decreased in all mice over the 6-day period, demonstrating the development of morphine tolerance after long-term morphine exposure. Morphine tolerance development was observed in all mice by the second day (Factor *time:* $F(5,2) = 213.2$, $p = 0.0116$ in male WT; $F(5,5) = 89.04$, $p < 0.0001$ in male FGF21-Tg; $F(5,4) = 116.9$, $p = 0.0003$ in female WT; and $F(5,4) = 27.28$, $p = 0.0053$ in female FGF21-Tg, Fig. 6). All mice developed chronic morphine tolerance by day 1 (male WT, $p = 0.0004$; male FGF21-Tg, $p < 0.0001$; female WT, $p < 0.0001$; and female FGF21-Tg, $p = 0.0043$). In addition, male and female FGF21-Tg mice developed chronic morphine tolerance at a similar rate to their WT counterparts (Fig. 6a and 6b). Indeed, the percent morphine antinociception was reduced from 89 ± 4% to 13 ± 1% and from 89 ± 3% to 10 ± 2% in FGF21-Tg and WT mice, respectively, from day 0 to day 5. Overall, FGF21-Tg mice showed a similar chronic morphine-induced analgesic tolerance development to WT littermates in the 55°C hot-plate assay.

3.7. Development of chronic morphine dependence in FGF21-Tg and WT littermates.

Chronic morphine dependence was evaluated after the last morphine administration of 23 mg/kg on day 5 of the morphine tolerance development model. Mice were treated with naloxone (10 mg/kg) 2 h later and the number of naloxone-induced withdrawal jumps were observed for 15 min. Chronic morphine dependence was observed in all mice and there was a significant difference based on genotype (Factor *genotype:* $F(3,34) = 15.29$, $p < 0.0001$, Fig. 7). The number of withdrawal jumps was similar in both male and female WT mice ($t(15) = 0.6138$, $p = 0.55$, Fig. 7). Likewise, male and female FGF21-Tg mice displayed similar numbers of withdrawal jumps ($t(18) = 0.6986$, $p = 0.50$, Fig. 7). However, male FGF21-Tg mice withdrawal jumps were reduced by 71% in comparison to male WT littermates ($t(16) = 5.599$, $p < 0.0001$, Fig.7). Similarly, female FGF21-Tg mice withdrawal jumps were 62% lower than female WT littermates ($t(17) = 4.048$, $p < 0.0012$, Fig. 7).

3.8. Morphine-induced locomotion in FGF21-Tg and WT littermates.

Morphine induces increased locomotor activity in mice (Hoot et al., 2013; Valjent et al., 2010). The morphine-induced locomotion (distance traveled, cm) between FGF21-Tg and WT littermates was compared. Under saline treatment, both FGF21-Tg and WT mice had similar locomotor activity travelling an average distance of 258 ± 13 cm and 256 ± 17 cm over 2 h, respectively ($t(79) = 0.1115$, $p = 0.91$). Male and female FGF21-Tg mice had similar locomotor activity under saline treatment (266 ± 14 cm and 250 ± 22 cm, respectively $t(39) = 0.5875$, $p = 0.56$). Morphine-induced locomotion was observed in all mice and there was a difference based on genotype and dose ($F(7,79) = 5.667$, genotype: $p < 0.0001$ and Dose: $p = 0.0033$, three-way ANOVA, Fig. 8). Overall, FGF21-Tg mice displayed a 6- and 7-fold increase in locomotor activity when administered 10 and 23 mg/kg morphine, respectively (Fig. 8). WT mice displayed an 8- and 14-fold increase in locomotor activity when administered 10 and 23 mg/kg morphine, respectively. There was no difference in total distance traveled by male transgenic and male WT mice at either dose of morphine (Factor *dose*genotype:* $F(3,38) = 1.629$, $p = 0.58$, two-way ANOVA, Fig. 8a). However, total distance traveled by female transgenic mice was lower compared to female WT mice at both doses of morphine ($F(3,39) = 12.13$, *dose:* $p = 0.0019$, *genotype:* $p < 0.0001$ two-way ANOVA, Fig. 8b). In addition, we further determined if there were differences in morphine-induced locomotion in WT and FGF21-Tg mice administered the same dose of morphine. Male WT and male FGF21-transgenic mice traveled similar distances when treated with 10 mg/kg morphine ($n = 10$ mice/group, $F(1,19) = 1.307$, $p = 0.27$, one-way ANOVA, Fig. 8a) and when treated with 23 mg/kg morphine ($n = 10$ mice/group, $F(1,19) = 2.059$, $p = 0.17$, one-way ANOVA, Fig. 8a). However, female FGF21-Tg mice had reduced locomotor activity relative to female WT littermates when administered 10 mg/kg morphine ($n =$

10 mice/group, $F(1,19) = 31.24$, $p < 0.0001$, one-way ANOVA, Fig. 8b) or 23 mg/kg morphine (*n* = 10 mice/group, $F(1,19) = 8.799$, $p = 0.008$, one-way ANOVA, Fig. 8b).

3.9. Comparing FGF21-Tg and WT littermates on morphine-induced respiratory depression.

Morphine produces dose-dependent respiratory depression rapidly after drug injection (Hill et al., 2018). The effect of morphine (18 and 30 mg/kg) on mouse respiratory rate was compared in FGF21-Tg and WT mice. No differences were observed between males and females for either the WT or FGF21-Tg mice. Therefore, the data were pooled. A decrease in respiratory rate, bpm, is an indicator of respiratory depression. There was no difference in percent respiration rate (relative to baseline) between FGF21-Tg and WT mice when injected with morphine (18 and 30 mg/kg). Morphine induced a dose-dependent respiratory depression 10 min after administration in both FGF21-Tg and WT mice administered with 18 mg/kg morphine. However, respiratory depression was observed at 5 and 10 min in FGF21-Tg and WT mice administered 30 mg/kg morphine (Fig.9). The respiratory rate did not change after 20 min and persisted over the 90-min observation in both FGF21-Tg and WT mice. Respiration started to recover by 80 min in the mice that received 18 mg/kg, indicative of morphine's metabolism. All mice tested developed respiratory depression, and none died during the experiments. These results show that there was no difference in morphine-induced respiratory rate changes in FGF21-Tg mice compared to WT mice.

4. Discussion

The goal of this study was to determine if there were any difference in morphine-induced physiological and behavioral responses in FGF21-Tg mice and WT C57BL/6J littermates. We studied morphine-induced events such the CPP, tolerance development, dependence, locomotion, and respiratory depression in both male and female mice.

Female FGF21-Tg mice had reduced preference for morphine at 3 and 10 mg/kg doses. However, male FGF21-Tg mice had a lower preference for morphine only at the 10 mg/kg dose. The rewarding effects of morphine are regulated by the CNS, specifically the ventral tegmental area and the NAc (Wilson-Poe and Morón, 2018). Morphine reinforcing effects have been linked to an increase in the amount of extracellular dopamine present in the NAc shells (Fadda et al., 2003). However, Talukdar et al. (2016) reported that mice treated by an osmotic mini-pump containing FGF21 for two weeks had a decrease in dopamine and its metabolites in the NAc relative to vehicle-treated mice. Additionally, FGF21 administration in mice affected the expression of dopamine-related genes, including an increase in the dopamine transporter in the NAc (Talukdar et al., 2016). These findings suggest that FGF21 has a role in the regulation of dopamine in the reward areas of the brain, which correlate with our morphine preference studies. However, additional experiments are essential to confirm if FGF21 regulates morphine-induced dopamine release and to establish the mechanism of action.

FGF21-Tg mice showed no difference in basal nociception and in morphine spinal and supraspinal antinociception compared to WT mice. While we did not observe any sex differences in morphine-induced antinociception, 10 mg/kg of oxycodone produced a greater antinociceptive effect in a 54 °C hot plate assay in male C57BL/6J mice than female mice (Collins et al., 2016). The MOR has been shown to regulate the spinal and supraspinal analgesic response of opioids such as morphine (Wang et al., 1994). Furthermore, mice lacking the MOR did not exhibit morphine-induced antinociception in either the hot plate or the tail withdrawal assays (Kieffer, 1999). FGF21-Tg mice exhibiting similar morphine antinociception

compared to WT littermates suggest that the overexpression of FGF21 has no direct effect on the MOR signaling in the spinal and supraspinal regions of the CNS that mediate analgesia.

FGF21-Tg mice displayed reduced development of acute morphine tolerance but similar chronic morphine tolerance compared to WT littermates. FGF21 may modulate acute morphine tolerance development; findings from the following studies may elucidate the mechanisms responsible. Inflammatory cytokines and microglial activation have been linked to morphine tolerance (Wang et al., 2009). Furthermore, blocking interleukin-1 receptor or inhibiting interleukin 1β (IL-1β), a major pro-inflammatory cytokine, was effective in blocking the development of morphine tolerance (Chen et al., 2012). Recombinant human FGF21 suppressed the expression levels of major pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-α (Wang et al., 2020). FGF21 also inhibited microglial activation in mouse hippocampus and the signaling pathway of nuclear factor-κB (NF-κB), another regulator of inflammatory cytokines (Wang et al., 2020). Inhibitors of nitric oxide synthase reduced the development but not the magnitude of acute morphine antinociceptive tolerance (Wolińska et al., 2021; Xu et al., 1998). Under certain circumstances, such as ischemia, FGF21 activated endothelial nitric oxide synthase (Kawanishi et al., 2020). FGF21 effects on acute morphine tolerance may be occurring through these pathways.

Phospholipid systems have been associated with opioid tolerance development. Protein kinase C (PKC) inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) and calphostin C, prevented or reversed acute antinociceptive tolerance (Bilsky et al., 1996; Narita et al., 1995). Moreover, PKC knockout mice displayed a decreased morphine tolerance (Zeitz et al., 2001). Mice on a high-fat diet treated with FGF21 via mini-osmotic pumps had reduced PKCε activation in liver and PKCθ in skeletal muscle (Camporez et al., 2013). Likewise, FGF21 inhibited the activation of stress-related kinases including NF-κB and PKC in human skeletal muscle (Lee et al., 2012). Although we do not know if the effects of FGF21 on PKC in skeletal muscle and liver

also occur in the CNS, FGF21 effects on PKC are an important finding and another potential mechanism by which FGF21 overexpression may affect acute morphine tolerance development.

Chronic morphine tolerance differs from acute tolerance with regards to the length of morphine exposure and possibly the differences in the mechanisms of intracellular modifications of opioid receptors and their second messengers. Chronic activation of the MOR leads to desensitization and downregulation of the MOR. If FGF21 affects acute morphine tolerance through second messenger systems, these effects are conceivably subdued over time and no longer significant during chronic exposure to morphine. Thus, the changes in the number of MOR and signaling efficacy during chronic stimulation by an agonist would not be affected by FGF21 overexpression, explaining the similarity in chronic morphine tolerance development of FGF21-Tg and WT littermates.

FGF21-Tg mice displayed reduced naloxone-precipitated morphine withdrawal symptoms compared to WT mice after being exposed to morphine both acutely and chronically. Acute morphine exposure induces changes in gene expression through decreased expression of coding transcription factors such as c-fos, c-jun, and zif268, which persists with chronic opiate administration (Nestler, 1992). Moreover, Hayward et al., (1990), reported that expression of c-fos is increased during naltrexone-induced opioid withdrawal. These results suggest that c-fos and related transcription factors may regulate neuronal plasticity during morphine exposure. FGF21 has been shown to increase the expression of c-fos in regions associated with opioid dependence (von Holstein-Rathlou et al., 2016). The fact that FGF21-Tg mice had reduced morphine withdrawal symptoms compared to WT mice after being exposed to morphine both acutely and chronically suggests that FGF21 regulation of transcription factors may be involved in morphine physical dependence pathways.

Locomotor activity in response to morphine varied with sex. Female FGF21-Tg mice had reduced morphine-induced locomotor activity compared to WT littermates, but male FGF21-Tg mice had similar morphine-induced locomotor activity to their WT counterparts. In addition, 10

mg/kg morphine produced the maximal effect on locomotor activity in male FGF21-Tg mice but not in female FGF21-Tg mice. The CNS plays an important role in mediating locomotion (Frigon, 2017). The precise site of action of FGF21 in the CNS is unknown. However, its co-receptor, KLB, is expressed in areas such as the suprachiasmatic nucleus, cortex, striatum, hippocampus, and hypothalamus (Lein et al., 2007). Thus, overexpression of FGF21 affects many pathways and hormones, which in turn leads to different phenotypes and genotypes in male and female mice. For example, FGF21-Tg female mice are infertile, but FGF21-Tg male mice are fertile. This phenomenon is the result of FGF21 suppressing vasopressin and kisspeptin signaling (Owen et al., 2013). The difference in morphine-induced locomotion in FGF21-Tg male and female mice may be caused by FGF21 effects on hormonal responses upstream of the locomotion pathway.

Additional work is necessary to address the mechanism through which FGF21 regulates morphine actions in the CNS. The use of FGF21 or its analogs is fundamental to ensure the effects seen in the FGF21-Tg mice are due to the overexpression of the gene. Unfortunately, FGF21 has a short half-life (<2 h) across multiple species limiting its utility (Yie et al., 2009). FGF21 analogs are an excellent way to study the effect of FGF21. PF-05231023, LY2405319, and AKR-001 are three FGF21 analogs currently in clinical trials (Kaufman, 2020; Lee et al., 2016; Xu et al., 2009). PF-05231023 was developed by covalent conjugation of two molecules of modified FGF21 to a CovX-200 antibody scaffold resulting in two free FGF21 C- and N-termini, respectively (Huang et al., 2013). Both C- and N-termini are required for the formation of the FGF21/KLB/FGFR complex (Kharitonenkov and Larsen, 2011). FGF21 was conjugated to CovX body at the mid-region of the FGF21 protein (Foltz et al., 2012) and the conjugation site has no significant impact on the *in vitro* potency of the protein (Xu et al., 2009). PF-05231023 has a 22 h half-life (Weng et al., 2015). LY2405319 is identical to native human FGF21 regarding potency, selectivity, and efficacy in many studies (Lee et al., 2016). The drawback of using this analog is that the half-life of LY2405319 is only 2 h like the native FGF21 (Reitman,

2013). AKR-001, formerly Fc-FGF21(RGE), AMG 876, is a human immunoglobulin Fc-FGF21 fusion protein with a half-life of 3 - 3.5 days (Kaufman, 2020). These and additional FGF21 analogs are being pursued in clinical trials for treating obesity, type 2 diabetes mellitus, and nonalcoholic steatohepatitis (Geng et al., 2020; Tillman and Rolph, 2020).

The present study showed that morphine preference, acute antinociceptive tolerance and physical dependence were decreased in mice expressing high levels of FGF21 while chronic morphine tolerance was intact. These effects were observed without any impact on analgesia or respiratory depression.

5. Conclusions

In summary, we found that mice expressing high FGF21 serum levels had a reduced preference for morphine, less severe morphine withdrawal symptoms, and slower acute antinociceptive tolerance development than WT littermates. High FGF21 levels did not affect morphine-induced antinociception, chronic antinociceptive tolerance, and respiratory depression. Our results suggest that FGF21 is altering signaling pathways, possibly dopamine, involved in mediating reward and physical dependence. FGF21 and its receptor are therapeutic targets for treating opioid-mediated withdrawal symptoms and craving, and acute opioid tolerance development in patients receiving opioids acutely for pain management.

Funding and disclosure

This work was supported by the National Institutes of Health/National Institute on Drug Abuse (DA044766), the John R. Murlin Graduate Fellowship, the Harold C. Hodge Memorial Fund, and the Margo Cleveland Fund. Authors have no conflict of interest.

CRediT authorship contribution statement

Louben Dorval: Conceptualization and design of experiments, data curation, Writing - original draft and revision, was primarily responsible for the collection, interpretation, and analysis of data. **Brian I. Knapp:** Conceptualization and design of experiments, primarily responsible for ELISA and tail-withdrawal assays, Writing - interpretation of the data, review & editing. **Olufolake A. Majekodunmi:** Helped with acute morphine dependence and morphine-induced locomotion. **Sophia Eliseeva:** Helped with morphine-induced respiratory depression. **Jean M. Bidlack:** Conceptualization and design of experiments, Writing - interpretation of the data, reviewing and editing of the manuscript. Funding acquisition. All authors contributed to, and have approved, the final manuscript.

Acknowledgements

We thank Dr. Steve N. Georas for assistance with the respiratory experiments and Dr. Deborah A. Cory-Slechta for her assistance with statistical analysis.

References

Alho, H., Sinclair, D., Vuori, E., Holopainen, A., 2007. Abuse liability of buprenorphine-naloxone tablets in untreated IV drug users. Drug Alcohol Depend 88, 75-78.

Bidlack, J. M., Cohen, D. J., McLaughlin, J. P., Lou, R., Ye, Y., Wentland, M. P., 2002. 8-Carboxamidocyclazocine: a long-acting, novel benzomorphan. J Pharmacol Exp Ther 302, 374-380.

Bilsky, E. J., Bernstein, R. N., Wang, Z., Sadee, W., Porreca, F., 1996. Effects of naloxone and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 and the protein kinase inhibitors H7 and H8 on acute morphine dependence and antinociceptive tolerance in mice. J Pharmacol Exp Ther 277, 484-490.

Camporez, J. P., Jornayvaz, F. R., Petersen, M. C., Pesta, D., Guigni, B. A., Serr, J., Zhang, D., Kahn, M., Samuel, V. T., Jurczak, M. J., Shulman, G. I., 2013. Cellular mechanisms by which FGF21 improves insulin sensitivity in male mice. Endocrinology 154, 3099-3109.

Cao, D. N., Li, F., Wu, N., Li, J., 2021. Insights into the mechanisms underlying opioid use disorder and potential treatment strategies. Br J Pharmacol.

Chen, M. L., Cao, H., Chu, Y. X., Cheng, L. Z., Liang, L. L., Zhang, Y. Q., Zhao, Z. Q., 2012. Role of P2X7 receptor-mediated IL-18/IL-18R signaling in morphine tolerance: multiple glial-neuronal dialogues in the rat spinal cord. J Pain 13, 945-958.

Collins, D., Reed, B., Zhang, Y., Kreek, M. J., 2016. Sex differences in responsiveness to the prescription opioid oxycodone in mice. Pharmacol Biochem Behav 148, 99-105.

Coskun, T., Bina, H. A., Schneider, M. A., Dunbar, J. D., Hu, C. C., Chen, Y., Moller, D. E., Kharitonenkov, A., 2008. Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 149, 6018-6027.

Dahan, A., Aarts, L., Smith, T. W., 2010. Incidence, Reversal, and Prevention of Opioid-induced Respiratory Depression. Anesthesiology 112, 226-238.

Deuis, J. R., Dvorakova, L. S., Vetter, I., 2017. Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci 10, 284.

Ding, X., Boney-Montoya, J., Owen, B. M., Bookout, A. L., Coate, K. C., Mangelsdorf, D. J., Kliewer, S. A., 2012. betaKlotho is required for fibroblast growth factor 21 effects on growth and metabolism. Cell Metab 16, 387-393.

Dong, J. Q., Rossulek, M., Somayaji, V. R., Baltrukonis, D., Liang, Y., Hudson, K., Hernandez-Illas, M., Calle, R. A., 2015. Pharmacokinetics and pharmacodynamics of PF-05231023, a novel long-acting FGF21 mimetic, in a first-in-human study. Br J Clin Pharmacol 80, 1051-1063.

Drgonova, J., Zimonjic, D. B., Hall, F. S., Uhl, G. R., 2010. Effect of KEPI (Ppp1r14c) deletion on morphine analgesia and tolerance in mice of different genetic backgrounds: when a knockout is near a relevant quantitative trait locus. Neuroscience 165, 882-895.

Fadda, P., Scherma, M., Fresu, A., Collu, M., Fratta, W., 2003. Baclofen antagonizes nicotine-, cocaine-, and morphine-induced dopamine release in the nucleus accumbens of rat. Synapse 50, 1-6.

Foltz, I. N., Hu, S., King, C., Wu, X., Yang, C., Wang, W., Weiszmann, J., Stevens, J., Chen, J. S., Nuanmanee, N., Gupte, J., Komorowski, R., Sekirov, L., Hager, T., Arora, T., Ge, H., Baribault, H., Wang, F., Sheng, J., Karow, M., Wang, M., Luo, Y., McKeehan, W., Wang, Z., Veniant, M. M., Li, Y., 2012. Treating diabetes and obesity with an FGF21-mimetic antibody activating the betaKlotho/FGFR1c receptor complex. Sci Transl Med 4, 162ra153.

Gaich, G., Chien, J. Y., Fu, H., Glass, L. C., Deeg, M. A., Holland, W. L., Kharitonenkov, A., Bumol, T., Schilske, H. K., Moller, D. E., 2013. The effects of LY2405319, an FGF21 analog, in obese human subjects with type 2 diabetes. Cell Metab 18, 333-340.

Geng, L., Lam, K. S. L., Xu, A., 2020. The therapeutic potential of FGF21 in metabolic diseases: from bench to clinic. Nat Rev Endocrinol 16, 654-667.

Hill, R., Dewey, W. L., Kelly, E., Henderson, G., 2018. Oxycodone-induced tolerance to respiratory depression: reversal by ethanol, pregabalin and protein kinase C inhibition. Br J Pharmacol 175, 2492-2503.

Hoot, M. R., Sypek, E. I., Reilley, K. J., Carey, A. N., Bidlack, J. M., McLaughlin, J. P., 2013. Inhibition of Gbetagamma-subunit signaling potentiates morphine-induced antinociception but not respiratory depression, constipation, locomotion, and reward. Behav Pharmacol 24, 144-152.

Hopkins, E., Rossi, G., Kest, B., 2004. Sex differences in systemic morphine analgesic tolerance following intrathecal morphine injections. Brain Res 1014, 244-246.

Hsuchou, H., Pan, W., Kastin, A. J., 2007. The fasting polypeptide FGF21 can enter brain from blood. Peptides 28, 2382-2386.

Huang, J., Ishino, T., Chen, G., Rolzin, P., Osothprarop, T. F., Retting, K., Li, L., Jin, P., Matin, M. J., Huyghe, B., Talukdar, S., Bradshaw, C. W., Palanki, M., Violand, B. N., Woodnutt, G., Lappe, R. W., Ogilvie, K., Levin, N., 2013. Development of a novel long-acting antidiabetic FGF21 mimetic by targeted conjugation to a scaffold antibody. J Pharmacol Exp Ther 346, 270-280.

Inagaki, T., Dutchak, P., Zhao, G., Ding, X., Gautron, L., Parameswara, V., Li, Y., Goetz, R., Mohammadi, M., Esser, V., Elmquist, J. K., Gerard, R. D., Burgess, S. C., Hammer, R. E., Mangelsdorf, D. J., Kliewer, S. A., 2007. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab 5, 415-425.

Kakko, J., Svanborg, K. D., Kreek, M. J., Heilig, M., 2003. 1-year retention and social function after buprenorphine-assisted relapse prevention treatment for heroin dependence in Sweden: a randomised, placebo-controlled trial. Lancet 361, 662-668.

Kaufman, A., Abuqayyas, L., Denney, W.S., Tillman, E.J., and Rolph, T., 2020. AKR-001, an Fc-FGF21 analog, showed sustained pharmacodynamic effects on insulin sensitivity and lipid

metabolism in type 2 diabetes patients. Cell Reports Medicine https://dpo/prg10.1016/j.xcm.2020.100057

Kawanishi, H., Ohashi, K., Ogawa, H., Otaka, N., Takikawa, T., Fang, L., Ozaki, Y., Takefuji, M., Murohara, T., Ouchi, N., 2020. A novel selective PPARα modulator, pemafibrate promotes ischemia-induced revascularization through the eNOS-dependent mechanisms. PLoS One 15, e0235362.

Kest, B., Wilson, S. G., Mogil, J. S., 1999. Sex differences in supraspinal morphine analgesia are dependent on genotype. J Pharmacol Exp Ther 289, 1370-1375.

Kharitonenkov, A., Larsen, P., 2011. FGF21 reloaded: challenges of a rapidly growing field. Trends Endocrinol Metab 22, 81-86.

Kharitonenkov, A., Wroblewski, V. J., Koester, A., Chen, Y. F., Clutinger, C. K., Tigno, X. T., Hansen, B. C., Shanafelt, A. B., Etgen, G. J., 2007. The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. Endocrinology 148, 774-781.

Kieffer, B. L., 1999. Opioids: first lessons from knockout mice. Trends Pharmacol Sci 20, 19-26.

Lee, J. H., Kang, Y. E., Chang, J. Y., Park, K. C., Kim, H. W., Kim, J. T., Kim, H. J., Yi, H. S., Shong, M., Chung, H. K., Kim, K. S., 2016. An engineered FGF21 variant, LY2405319, can prevent non-alcoholic steatohepatitis by enhancing hepatic mitochondrial function. Am J Transl Res 8, 4750-4763.

Lee, M. S., Choi, S. E., Ha, E. S., An, S. Y., Kim, T. H., Han, S. J., Kim, H. J., Kim, D. J., Kang, Y., Lee, K. W., 2012. Fibroblast growth factor-21 protects human skeletal muscle myotubes from palmitate-induced insulin resistance by inhibiting stress kinase and NF-kappaB. Metabolism 61, 1142-1151.

Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A. F., Boguski, M. S., Brockway, K. S., Byrnes, E. J., Chen, L., Chen, L., Chen, T. M., Chin, M. C., Chong, J., Crook, B. E., Czaplinska, A., Dang, C. N., Datta, S., Dee, N. R., Desaki, A. L., Desta, T., Diep, E., Dolbeare, T. A., Donelan, M. J., Dong, H. W., Dougherty, J. G., Duncan, B. J.,

Ebbert, A. J., Eichele, G., Estin, L. K., Faber, C., Facer, B. A., Fields, R., Fischer, S. R., Fliss, T. P., Frensley, C., Gates, S. N., Glattfelder, K. J., Halverson, K. R., Hart, M. R., Hohmann, J. G., Howell, M. P., Jeung, D. P., Johnson, R. A., Karr, P. T., Kawal, R., Kidney, J. M., Knapik, R. H., Kuan, C. L., Lake, J. H., Laramee, A. R., Larsen, K. D., Lau, C., Lemon, T. A., Liang, A. J., Liu, Y., Luong, L. T., Michaels, J., Morgan, J. J., Morgan, R. J., Mortrud, M. T., Mosqueda, N. F., Ng, L. L., Ng, R., Orta, G. J., Overly, C. C., Pak, T. H., Parry, S. E., Pathak, S. D., Pearson, O. C., Puchalski, R. B., Riley, Z. L., Rockett, H. R., Rowland, S. A., Royall, J. J., Ruiz, M. J., Sarno, N. R., Schaffnit, K., Shapovalova, N. V., Sivisay, T., Slaughterbeck, C. R., Smith, S. C., Smith, K. A., Smith, B. I., Sodt, A. J., Stewart, N. N., Stumpf, K. R., Sunkin, S. M., Sutram, M., Tam, A., Teemer, C. D., Thaller, C., Thompson, C. L., Varnam, L. R., Visel, A., Whitlock, R. M., Wohnoutka, P. E., Wolkey, C. K., Wong, V. Y., Wood, M., Yaylaoglu, M. B., Young, R. C., Youngstrom, B. L., Yuan, X. F., Zhang, B., Zwingman, T. A., Jones, A. R., 2007. Genome-wide atlas of gene expression in the adult mouse brain. Nature 445, 168-176.

Ma, H., Zhu, G., 2014. The dopamine system and alcohol dependence. Shanghai Arch Psychiatry 26, 61-68.

Mathews, J. L., Smrcka, A. V., Bidlack, J. M., 2008. A novel Gbetagamma-subunit inhibitor selectively modulates mu-opioid-dependent antinociception and attenuates acute morphine-induced antinociceptive tolerance and dependence. J Neurosci 28, 12183-12189.

McLaughlin, J. P., Land, B. B., Li, S., Pintar, J. E., Chavkin, C., 2006. Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. Neuropsychopharmacology 31, 787-794.

Narita, M., Narita, M., Mizoguchi, H., Tseng, L. F., 1995. Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to an intrathecally administered mu-opioid receptor agonist in the mouse. Eur J Pharmacol 280, R1-3.

Nisell, M., Nomikos, G. G., Svensson, T. H., 1994. Systemic nicotine-induced dopamine release in the rat nucleus accumbens is regulated by nicotinic receptors in the ventral tegmental area. Synapse 16, 36-44.

Orsini, C., Bonito-Oliva, A., Conversi, D., Cabib, S., 2005. Susceptibility to conditioned place preference induced by addictive drugs in mice of the C57BL/6 and DBA/2 inbred strains. Psychopharmacology (Berl) 181, 327-336.

Owen, B. M., Bookout, A. L., Ding, X., Lin, V. Y., Atkin, S. D., Gautron, L., Kliewer, S. A., Mangelsdorf, D. J., 2013. FGF21 contributes to neuroendocrine control of female reproduction. Nat Med 19, 1153-1156.

Owen, B. M., Ding, X., Morgan, D. A., Coate, K. C., Bookout, A. L., Rahmouni, K., Kliewer, S. A., Mangelsdorf, D. J., 2014. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. Cell Metab 20, 670-677.

Owen, B. M., Mangelsdorf, D. J., Kliewer, S. A., 2015. Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21. Trends Endocrinol Metab 26, 22-29.

Parker, L. A., Joshi, A., 1998. Naloxone-precipitated morphine withdrawal induced place aversions: effect of naloxone at 24 hours postmorphine. Pharmacol Biochem Behav 61, 331-333.

Pattinson, K. T., 2008. Opioids and the control of respiration. Br J Anaesth 100, 747-758.

Potthoff, M. J., Kliewer, S. A., Mangelsdorf, D. J., 2012. Endocrine fibroblast growth factors 15/19 and 21: from feast to famine. Genes Dev 26, 312-324.

Reitman, M. L., 2013. FGF21 mimetic shows therapeutic promise. Cell Metab 18, 307-309.

Roche, A., McCabe, S., Smyth, B. P., 2008. Illicit methadone use and abuse in young people accessing treatment for opiate dependence. Eur Addict Res 14, 219-225.

Shulman, M., Weiss, R., Rotrosen, J., Novo, P., Costello, E., Nunes, E. V., 2021. Prior National Drug Abuse Treatment Clinical Trials Network (CTN) opioid use disorder trials as background and rationale for NIDA CTN-0100 "optimizing retention, duration and

discontinuation strategies for opioid use disorder pharmacotherapy (RDD)". Addict Sci Clin Pract 16, 15.

Singhal, G., Douris, N., Fish, A. J., Zhang, X., Adams, A. C., Flier, J. S., Pissios, P., Maratos-Flier, E., 2016. Fibroblast growth factor 21 has no direct role in regulating fertility in female mice. Mol Metab 5, 690-698.

Solecki, W., Turek, A., Kubik, J., Przewlocki, R., 2009. Motivational effects of opiates in conditioned place preference and aversion paradigm--a study in three inbred strains of mice. Psychopharmacology (Berl) 207, 245-255.

Stanislaus, S., Hecht, R., Yie, J., Hager, T., Hall, M., Spahr, C., Wang, W., Weiszmann, J., Li, Y., Deng, L., Winters, D., Smith, S., Zhou, L., Li, Y., Véniant, M. M., Xu, J., 2017. A Novel Fc-FGF21 With Improved Resistance to Proteolysis, Increased Affinity Toward β-Klotho, and Enhanced Efficacy in Mice and Cynomolgus Monkeys. Endocrinology 158, 1314-1327.

Talukdar, S., Owen, B. M., Song, P., Hernandez, G., Zhang, Y., Zhou, Y., Scott, W. T., Paratala, B., Turner, T., Smith, A., Bernardo, B., Muller, C. P., Tang, H., Mangelsdorf, D. J., Goodwin, B., Kliewer, S. A., 2016. FGF21 Regulates Sweet and Alcohol Preference. Cell Metab 23, 344-349.

Tatem, K. S., Quinn, J. L., Phadke, A., Yu, Q., Gordish-Dressman, H., Nagaraju, K., 2014. Behavioral and locomotor measurements using an open field activity monitoring system for skeletal muscle diseases. J Vis Exp, 51785.

Tillman, E. J., Rolph, T., 2020. FGF21: An Emerging Therapeutic Target for Non-Alcoholic Steatohepatitis and Related Metabolic Diseases. Front Endocrinol (Lausanne) 11, 601290.

Valjent, E., Bertran-Gonzalez, J., Aubier, B., Greengard, P., Herve, D., Girault, J. A., 2010. Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. Neuropsychopharmacology 35, 401-415.

von Holstein-Rathlou, S., BonDurant, L. D., Peltekian, L., Naber, M. C., Yin, T. C., Claflin, K. E., Urizar, A. I., Madsen, A. N., Ratner, C., Holst, B., Karstoft, K., Vandenbeuch, A., Anderson,

C. B., Cassell, M. D., Thompson, A. P., Solomon, T. P., Rahmouni, K., Kinnamon, S. C., Pieper, A. A., Gillum, M. P., Potthoff, M. J., 2016. FGF21 Mediates Endocrine Control of Simple Sugar Intake and Sweet Taste Preference by the Liver. Cell Metab 23, 335-343.

Wang, X., Zhu, L., Hu, J., Guo, R., Ye, S., Liu, F., Wang, D., Zhao, Y., Hu, A., Wang, X., Guo, K., Lin, L., 2020. FGF21 Attenuated LPS-Induced Depressive-Like Behavior via Inhibiting the Inflammatory Pathway. Front Pharmacol 11, 154.

Wang, Z., Bilsky, E. J., Porreca, F., Sadee, W., 1994. Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sci 54, PL339-350.

Wang, Z., Bilsky, E. J., Wang, D., Porreca, F., Sadee, W., 1999. 3-Isobutyl-1-methylxanthine inhibits basal mu-opioid receptor phosphorylation and reverses acute morphine tolerance and dependence in mice. Eur J Pharmacol 371, 1-9.

Wang, Z., Ma, W., Chabot, J. G., Quirion, R., 2009. Cell-type specific activation of p38 and ERK mediates calcitonin gene-related peptide involvement in tolerance to morphine-induced analgesia. FASEB J 23, 2576-2586.

Weng, Y., Chabot, J. R., Bernardo, B., Yan, Q., Zhu, Y., Brenner, M. B., Vage, C., Logan, A., Calle, R., Talukdar, S., 2015. Pharmacokinetics (PK), pharmacodynamics (PD) and integrated PK/PD modeling of a novel long acting FGF21 clinical candidate PF-05231023 in diet-induced obese and leptin-deficient obese mice. PLoS One 10, e0119104.

Wiss, D. A., Avena, N., Rada, P., 2018. Sugar Addiction: From Evolution to Revolution. Front Psychiatry 9, 545.

Wolińska, R., Kleczkowska, P., de Cordé-Skurska, A., Poznański, P., Sacharczuk, M., Mika, J., Bujalska-Zadrożny, M., 2021. Nitric oxide modulates tapentadol antinociceptive tolerance and physical dependence. Eur J Pharmacol 907, 174245.

Xu, J., Lloyd, D. J., Hale, C., Stanislaus, S., Chen, M., Sivits, G., Vonderfecht, S., Hecht, R., Li, Y. S., Lindberg, R. A., Chen, J. L., Jung, D. Y., Zhang, Z., Ko, H. J., Kim, J. K., Veniant, M.

M., 2009. Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. Diabetes 58, 250-259.

Xu, J. Y., Hill, K. P., Bidlack, J. M., 1998. The nitric oxide/cyclic GMP system at the supraspinal site is involved in the development of acute morphine antinociceptive tolerance. J Pharmacol Exp Ther 284, 196-201.

Yano, I., Takemori, A. E., 1977. Inhibition by naloxone of tolerance and dependence in mice treated acutely and chronically with morphine. Res Commun Chem Pathol Pharmacol 16, 721-734.

Yie, J., Hecht, R., Patel, J., Stevens, J., Wang, W., Hawkins, N., Steavenson, S., Smith, S., Winters, D., Fisher, S., Cai, L., Belouski, E., Chen, C., Michaels, M. L., Li, Y. S., Lindberg, R., Wang, M., Veniant, M., Xu, J., 2009. FGF21 N- and C-termini play different roles in receptor interaction and activation. FEBS Lett 583, 19-24.

Yoshimoto, K., McBride, W. J., Lumeng, L., Li, T. K., 1992. Alcohol stimulates the release of dopamine and serotonin in the nucleus accumbens. Alcohol 9, 17-22.

Zeitz, K. P., Malmberg, A. B., Gilbert, H., Basbaum, A. I., 2001. Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. Pain 94, 245-253.

Zhang, Y., Xie, Y., Berglund, E. D., Coate, K. C., He, T. T., Katafuchi, T., Xiao, G., Potthoff, M. J., Wei, W., Wan, Y., Yu, R. T., Evans, R. M., Kliewer, S. A., Mangelsdorf, D. J., 2012. The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. Elife 1, e00065.

Table 1. FGF21 serum protein levels from FGF21-Tg and WT littermates. FGF21 protein levels were determined by a FGF21 ELISA assay as described in Materials and Methods (section 2.3.). Data are the mean FGF21 protein levels ± S.E.M. from 10 male and 10 female FGF21-Tg mice and 15 male and 18 female WT mice. There were no differences in FGF21 serum levels between male and female mice of the same strain.

Mouse Strain	FGF21 (ng/ml) ± S.E.M.
Male FGF21-Tg	640 ± 46
Female FGF21-Tg	650 ± 44
Male WT	0.30 ± 0.040
Female WT	0.23 ± 0.045

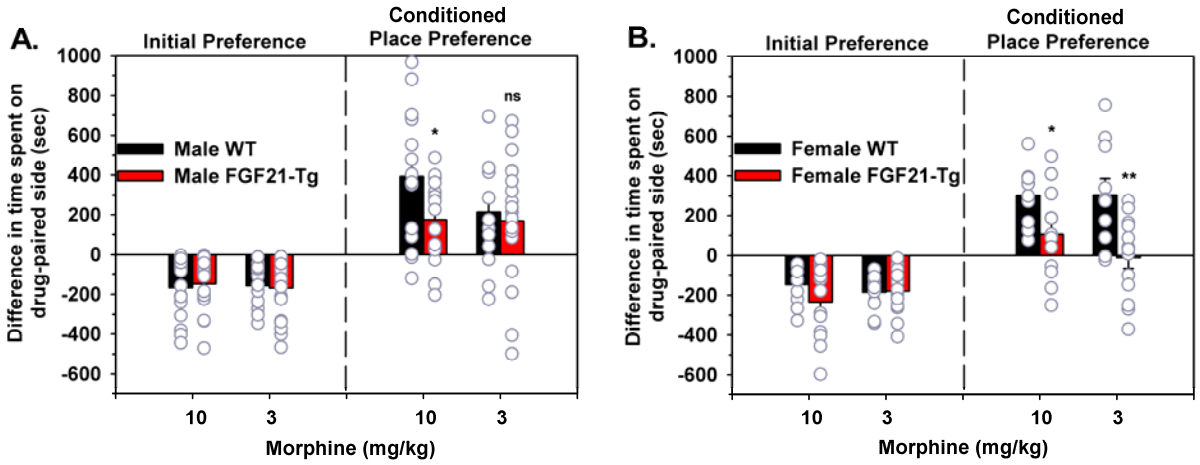

Fig. 1. Effect of FGF21 overexpression on morphine CPP**.** A three-day biased CPP was used in this experiment. Different groups of mice were used for each morphine dose. FGF21-Tg and WT mice had similar initial preference. A) Male FGF21-Tg mice had a lower morphine CPP than male WT mice at 10 mg/kg (*n* = 15-16 mice/group, $F(1,30) = 4.710$, $p = 0.04$, one-way ANOVA). However, both groups of mice had similar morphine CPP at 3 mg/kg (*n* = 12-15 mice/group, $F(1,26) = 0.1982$, $p = 0.66$, one-way ANOVA). B) Female FGF21-Tg mice exhibited preference for morphine at 10 mg/kg but not at 3 mg/kg. In addition, female FGF21-Tg morphine CPP was lower than female WT at both doses (*n* = 10-13 mice/group in both doses, $F(1,22) = 5.960$, $p = 0.02$ for 10 mg/kg morphine and $F(1,22) = 10.56$, $p = 0.004$ for 3 mg/kg morphine, one-way ANOVA). Ns, not significant, *$p < 0.05$ and **$p < 0.01$ for FGF21-Tg mice compared with WT littermates at equal dose.

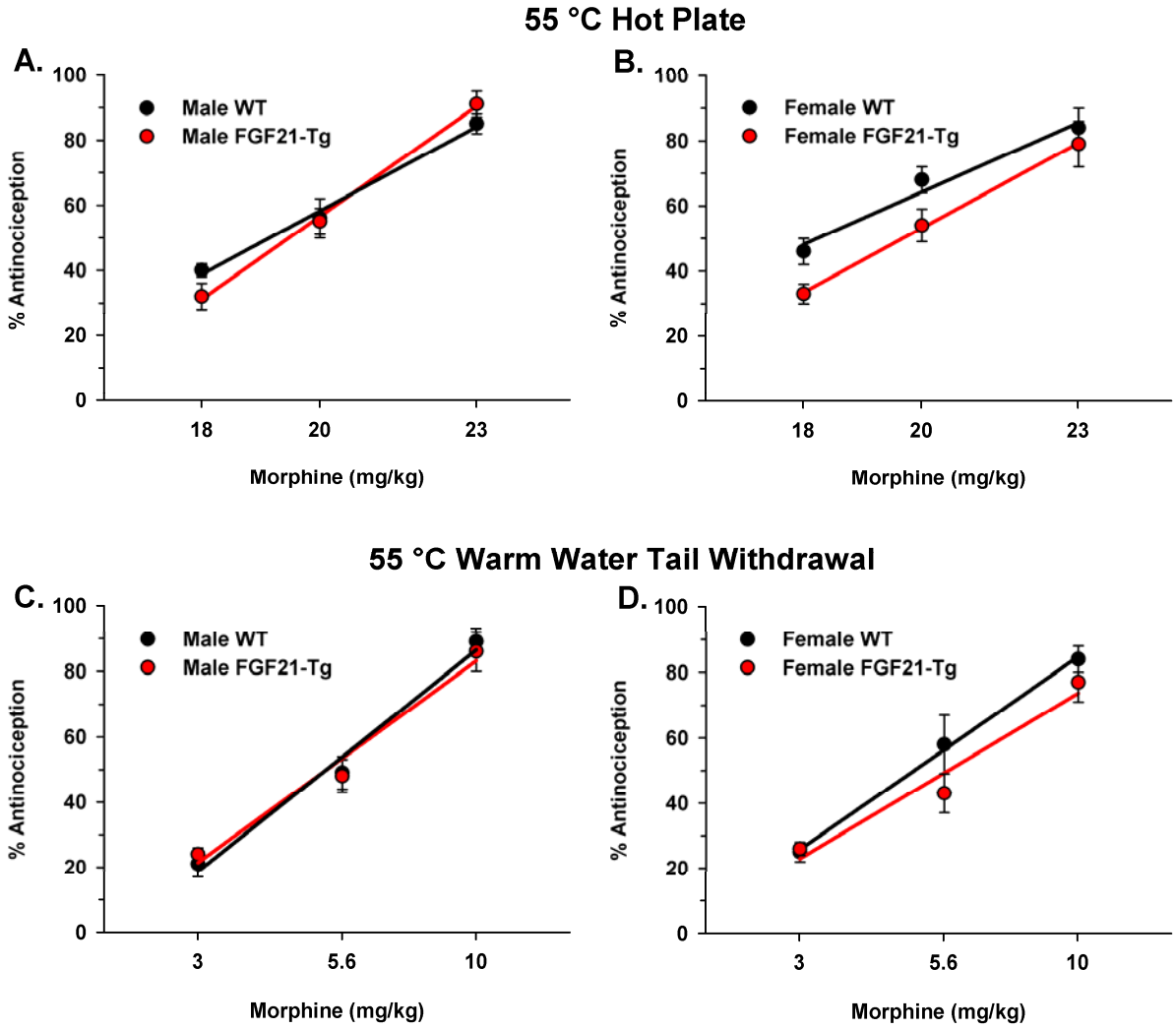

Fig. 2. Morphine antinociception in 55°C hot plate and 55°C warm-water tail withdrawal tests. Morphine produced a peak antinociception 30 min after administration in both the hot plate assay (A, B) and tail withdrawal tests (C, D). Thus, all measurements were performed 30 min after morphine administration. A - B) In the hot plate test, ED_{50} values from dose response curves of morphine administration (18, 20 and 23 mg/kg) showed that morphine antinociception was similar in FGF21-Tg and WT littermates (*n* = 10 mice/group). C - D) The tail withdrawal test revealed that ED_{50} values from dose response curves of morphine administration (3, 5.6 and 10 mg/kg) were also similar in FGF21-Tg and WT mice (*n* = 10-15 mice/group).

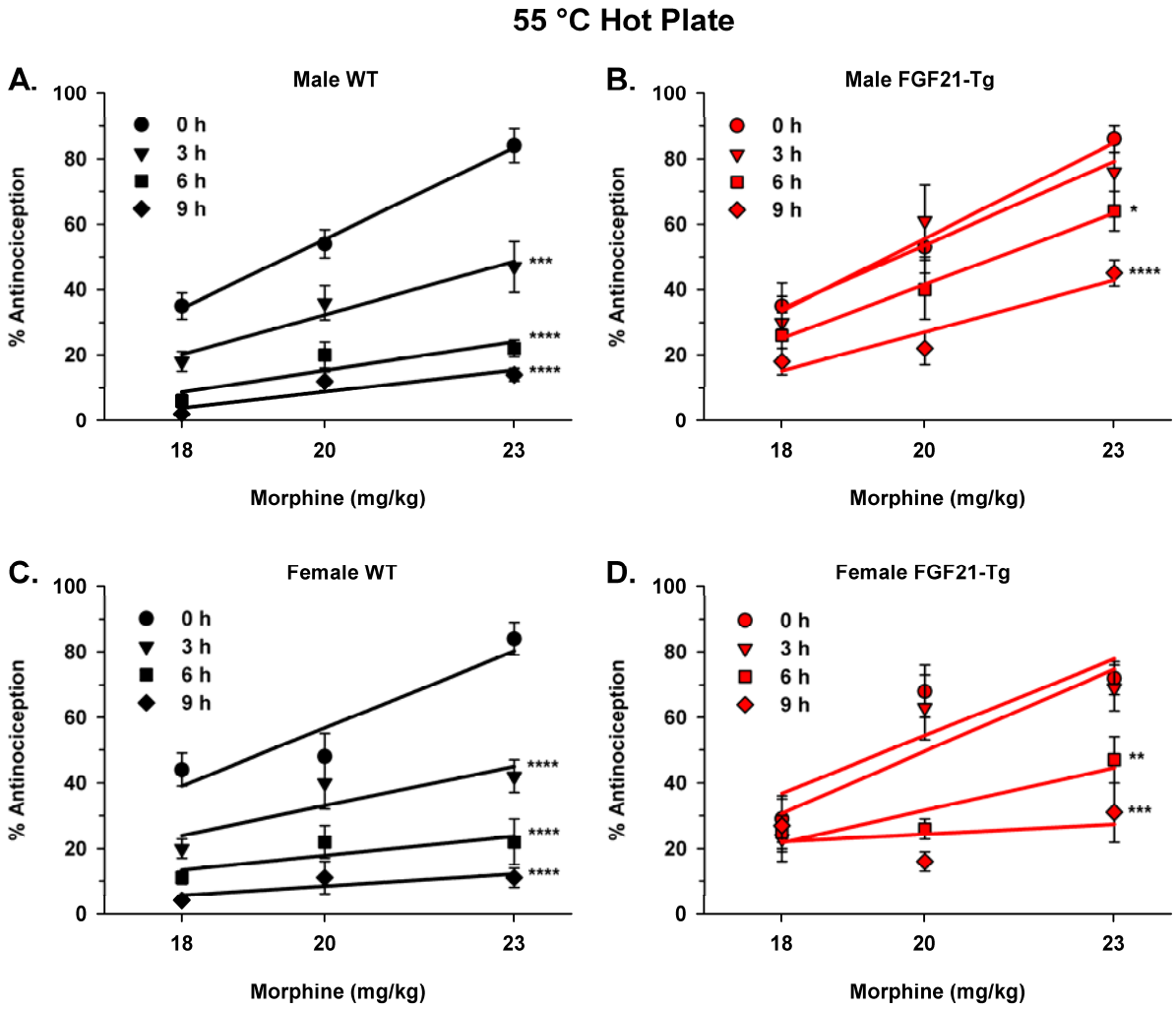

Fig. 3. Effect of FGF21 overexpression on acute morphine analgesic tolerance development in the 55°C hot-plate test. Mice were administered morphine (10, 18, or 23 mg/kg) at time 0, 3, 6, and 9 h. Antinociception was assessed 30 min after each injection. A) Male WT mice started to develop tolerance by the second injection (3 h) when administered morphine (*n* = 10, 0 h vs 3 h, p = 0.0011 at 23 mg/kg, paired t-test). B) Male FGF21-Tg mice developed morphine tolerance by the third injection (6 h) when administered 23 mg/kg (*n* = 10, 0 h vs 6 h, p = 0.011, paired t-test). C) Female WT mice became tolerant to morphine by the second injection (3 h) at 23 mg/kg (*n* = 10, 0 h vs 3 h, p < 0.0001, paired t-test). D) Female FGF21-Tg mice developed tolerance to morphine by the third injection at 23 mg/kg (*n* = 10, 0 h vs 6 h, p = 0.043, paired t-test). *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 for injection times compared to time 0 h at 23 mg/kg morphine.

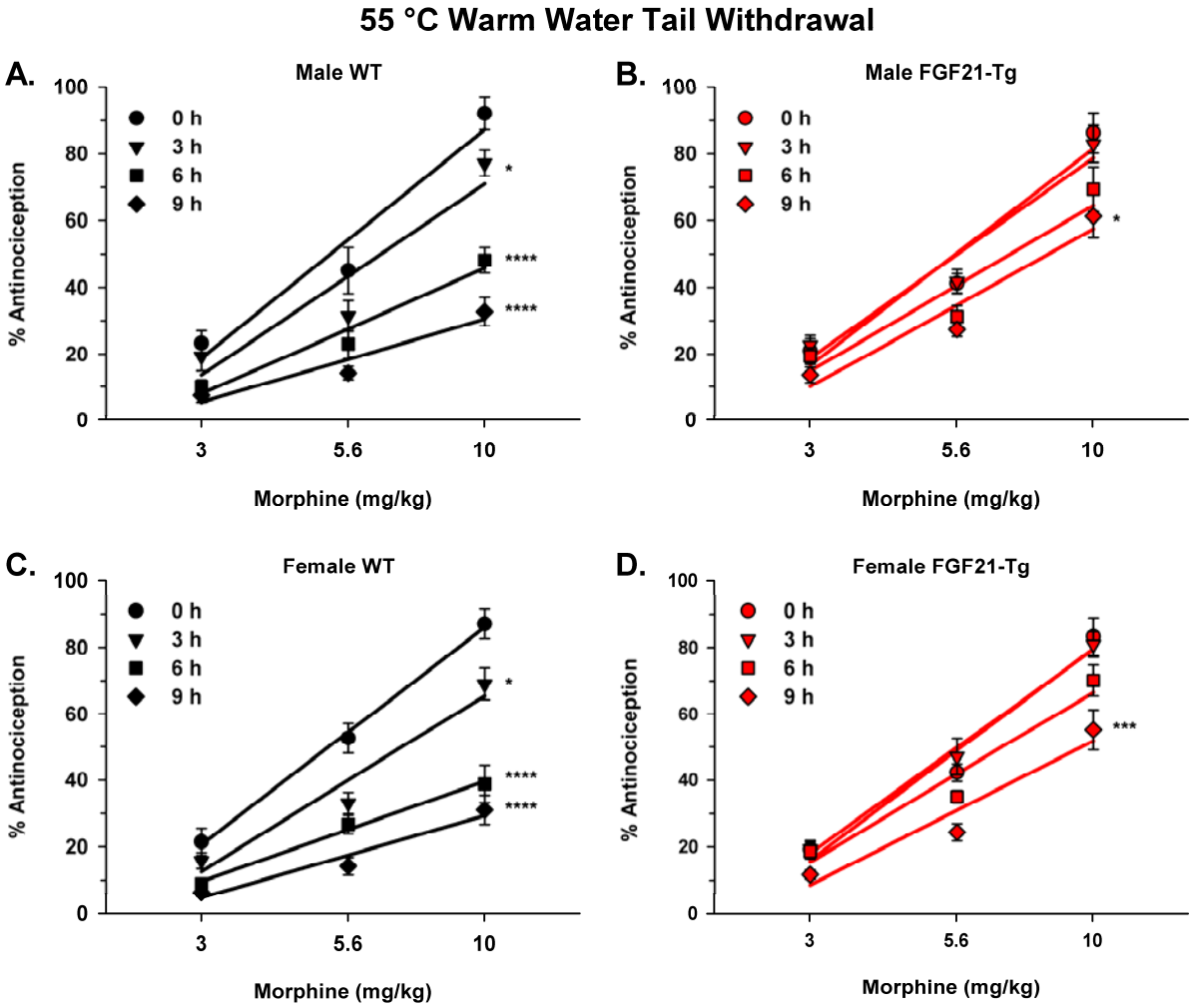

Fig. 4. Effect of FGF21 overexpression on acute morphine analgesic tolerance development in the 55°C warm-water tail withdrawal test. Mice were administered morphine (3, 5.6, or 10 mg/kg) at time 0, 3, 6, and 9 h. Antinociception was assessed 30 min after each injection. A) Male WT mice developed tolerance by the second injection when administered with 10 mg/kg morphine (n = 10, 0 h vs 3 h, p = 0.027, paired t-test). B) Male FGF21-Tg mice developed morphine tolerance only after the last injection when administered 10 mg/kg (n = 10, 0 h vs 9 h, p = 0.011, paired t-test). C) Female WT mice became tolerant to morphine by the second injection (3 h) at 10 mg/kg (n = 10, 0 h vs 3 h, p = 0.013, paired t-test). D) Female FGF21-Tg mice showed tolerance after the last injection at 10 mg/kg (n = 10, 0 h vs 9 h, p = 0.0034 paired t-test). *p < 0.05, ***p < 0.001 and ****p < 0.0001 for injection times compared to time 0 h at 10 mg/kg morphine.

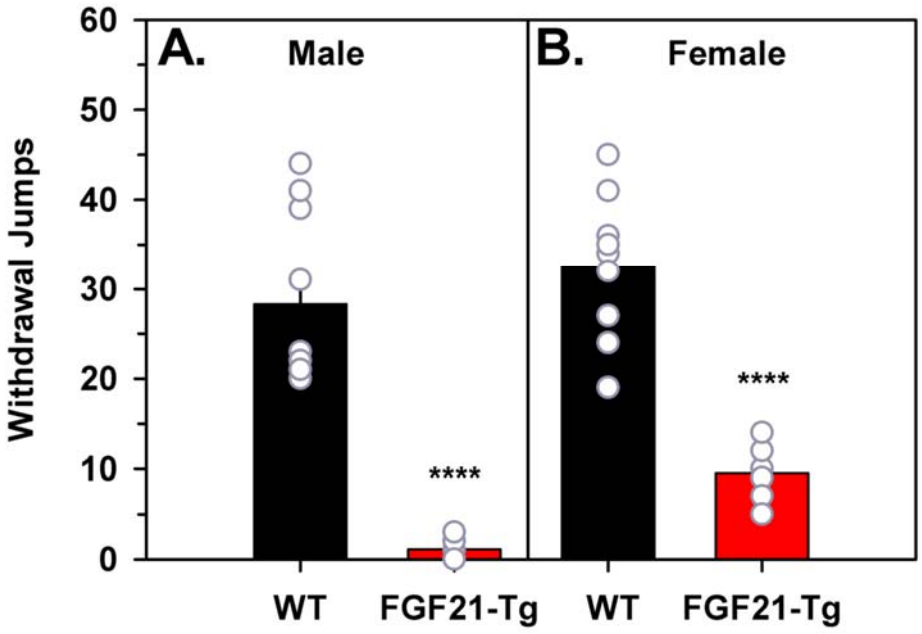

Fig. 5. Acute morphine dependence. Mice received an injection of morphine (100 mg/kg) followed by naloxone (10 mg/kg) 4 h later. Subsequently, mice were observed for 15 min and naloxone-induced withdrawal jumps were recorded. A) Male FGF21-Tg mice had lower withdrawal jumps than male WT mice (n = 10 mice/group, $t(19) = 8.898$, $p < 0.0001$, Student's t-test). B) Female FGF21-Tg had fewer withdrawal jumps relative to female WT mice (n = 10 mice/group, $t(19) = 8.900$, $p < 0.0001$, Student's t-test). ****$p < 0.0001$ for FGF21-Tg mice compared with WT littermates.

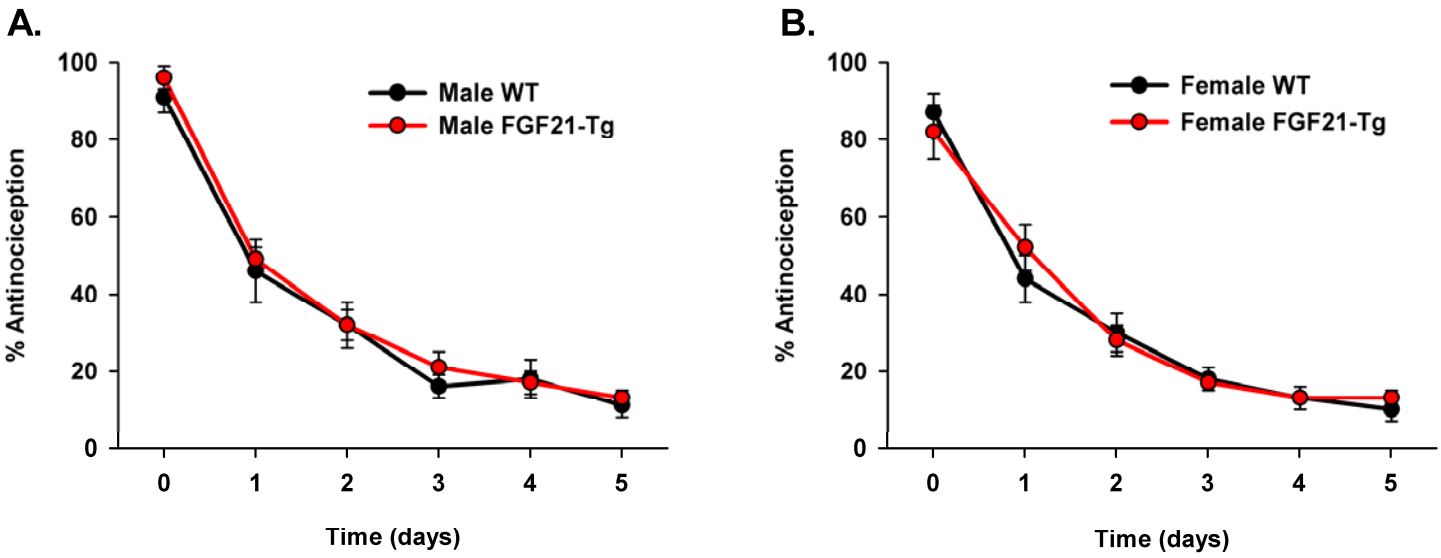

Fig. 6. Effect of FGF21 overexpression on chronic morphine analgesic tolerance development in the 55°C hot plate test. Mice were treated twice a day for 5 consecutive days with 23 mg/kg morphine. Day 0 was the first day of morphine administration. Analgesia was assessed every day, immediately before and 30 min after the first morphine injection. On day 6, morphine-induced analgesia was measured after a single administration of 23 mg/kg morphine. A) Male WT and transgenic mice developed tolerance by the second day (day 0 vs 1, n = 7, p = 0.0004 and n = 10, p < 0.0001, respectively, paired t-test). B) Female WT and transgenic mice became tolerant by the second day (day 0 vs 1, n = 9, p < 0.0001 and n = 9, p = 0.004, respectively, paired t-test). There was no difference in tolerance development between WT and FGF21-Tg mice.

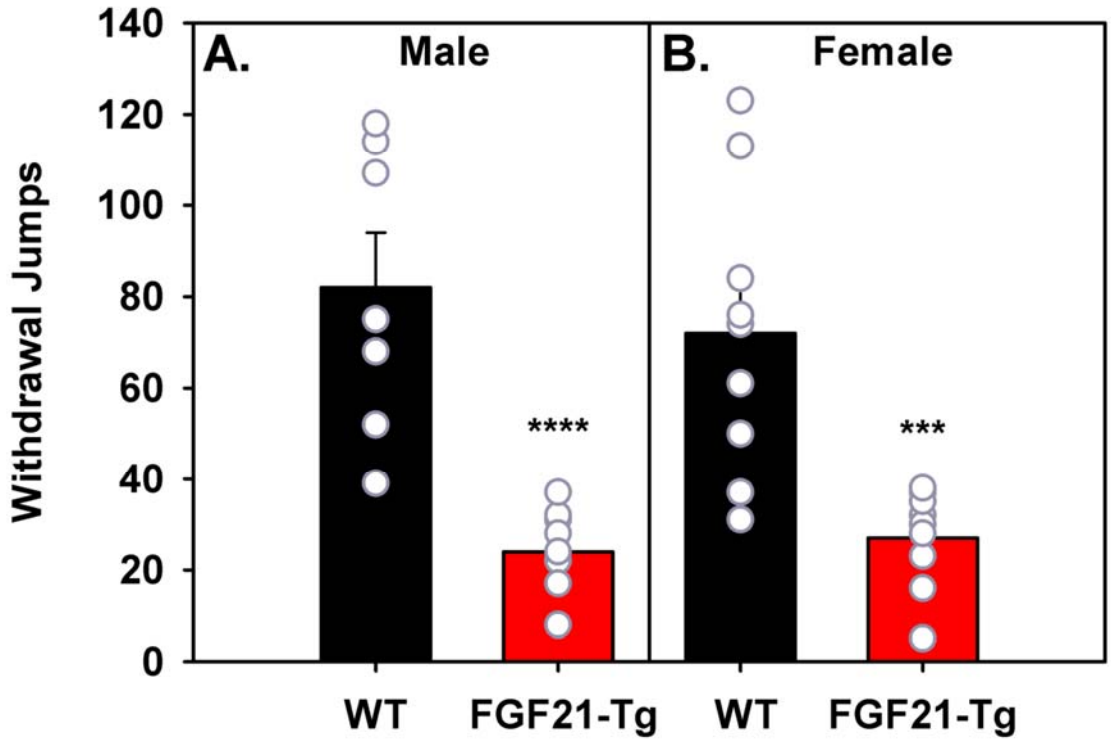

Fig. 7. Chronic morphine dependence. Following the development of tolerance, mice were administered with naloxone (10 mg/kg, 2 h after the last administration of morphine) and observed for 15 min. A) Male FGF21-Tg mice had fewer withdrawal jumps than male WT mice (*n* = 7-10 mice/group, t(16) = 5.599, p < 0.0001, Student's t-test). B) Female FGF21-Tg had fewer withdrawal jumps relative to female WT mice (*n* = 9 mice/group, t(17) = 4.048, p < 0.0012, Student's t-test). ***p < 0.001 and ****p < 0.0001 for FGF21-Tg mice compared with WT littermates.

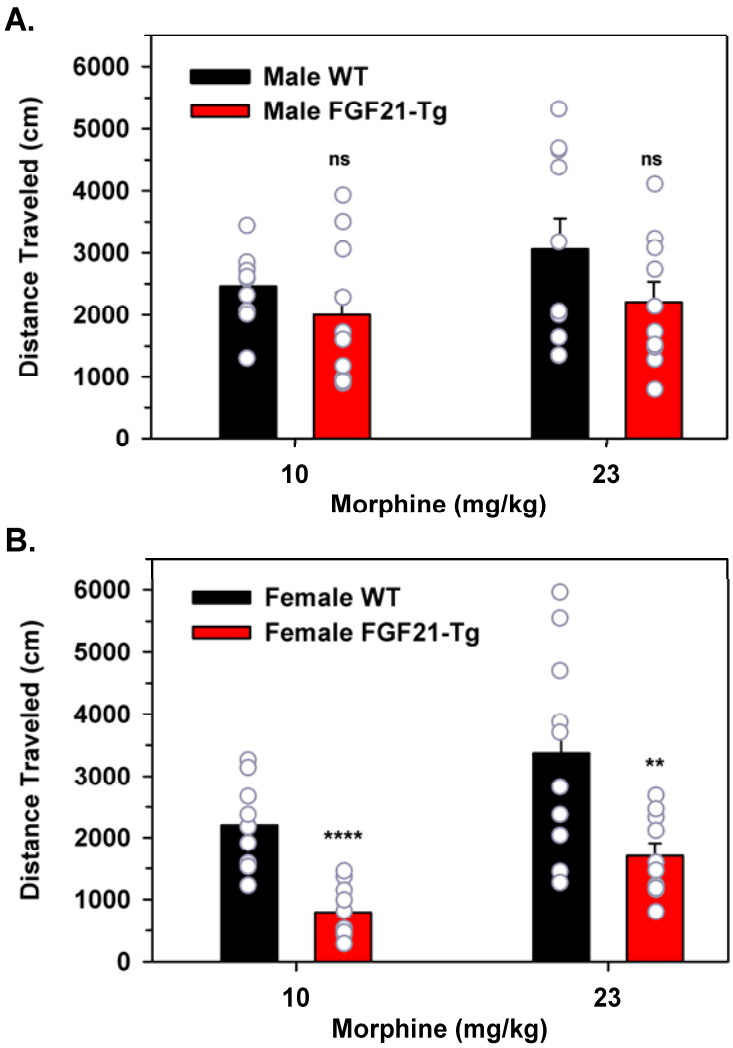

Fig. 8. Effects of FGF21 overexpression on morphine-induced locomotion. Mice were administered with 10 mg/kg or 23 mg/kg morphine and placed in an open-field activity apparatus for 2 h. A) There was no difference in total distance traveled by male FGF21-Tg and WT mice treated with 10 mg/kg morphine (*n* = 10 mice/group, p = 0.27, one-way ANOVA) and 23 mg/kg morphine (*n* = 10 mice/group, p = 0.17, one-way ANOVA). B) Female FGF21-Tg mice had a lower locomotor activity than female WT littermates when administered with 10 mg/kg morphine (*n* = 10 mice/group, p < 0.0001, one-way ANOVA) and 23 mg/kg morphine (*n* = 10 mice/group, p = 0.008, one-way ANOVA). Ns, not significant, **p < 0.01 and ****p < 0.0001 for FGF21-Tg mice compared with WT littermates.

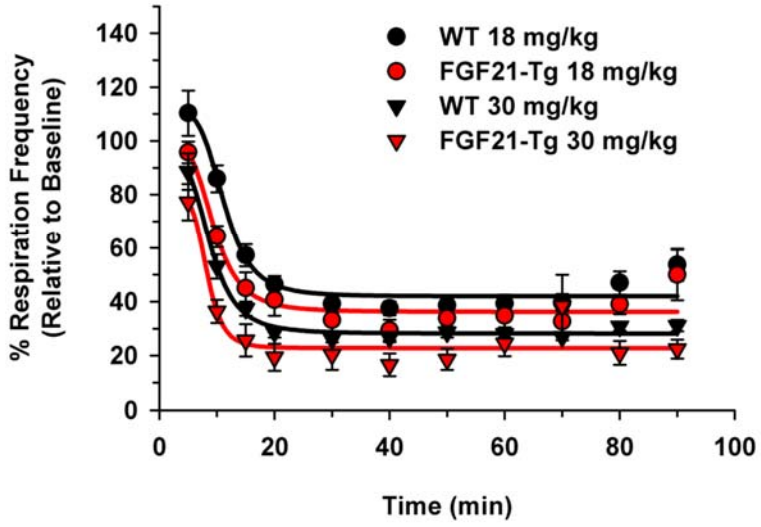

Fig. 9. Effects of FGF21 overexpression on morphine-induced respiratory depression. There was no difference in respiratory rates between male and female mice of each group. Thus, the male and female mice were pooled. Mice were placed in a chamber of a whole-body plethysmography suite before and after administration of morphine. Mice breathing rates were analyzed based on pressure changes within the chamber. The baseline rate of respiration over 20 min was 391 ± 20 and 475 ± 22 breaths per min (bpm) in WT and FGF21-Tg mice, respectively, for the mice tested at 18 mg/kg morphine. The baseline bpm was 408 ± 16 and 468 ± 14 bpm for WT and FGF21-Tg mice, respectively, for mice tested with 30 mg/kg morphine. FGF21-Tg and WT mice showed a similar decrease in respiratory rate relative to baseline after administration of morphine doses of 18 mg/kg and 30 mg/kg (n = 10-12 mice/group).