FGF21 maintains glucose homeostasis by mediating the crosstalk between liver and brain during prolonged fasting

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**Running Title:** FGF21 mediates the crosstalk of liver & brain

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Abstract

Hepatic gluconeogenesis is a main source of blood glucose during prolonged fasting, and is orchestrated by endocrine and neural pathways. Here we show that the hepatocyte-secreted hormone Fibroblast Growth Factor 21 (FGF21) induces fasting gluconeogenesis via the brain-liver axis. Prolonged fasting induces activation of the transcription factor PPARα in the liver and subsequent hepatic production of FGF21, which enters into the brain to activate the Hypothalamic-Pituitary-Adrenal (HPA) axis for release of corticosterone, thereby stimulating hepatic gluconeogenesis. Fasted FGF21 knockout (KO) mice exhibit severe hypoglycemia and defective hepatic gluconeogenesis due to impaired activation of the HPA axis and blunted release of corticosterone, a phenotype similar to that observed in PPARα KO mice. By contrast, intracerebroventricular injection of FGF21 reverses fasting hypoglycemia and impairment in hepatic gluconeogenesis by restoring corticosterone production in both FGF21 KO and PPARα KO mice, whereas all these central effects of FGF21 were abrogated by blockage of hypothalamic FGF receptor-1. FGF21 acts directly on the hypothalamic neurons to activate the Mitogen-Activated-Protein kinase ERK1/2, thereby stimulating the expression of corticotropin-releasing-hormone by activation of the transcription factor cAMP Response Element Binding protein (CREB). Therefore, FGF21 maintains glucose homeostasis during prolonged fasting by fine-tuning the inter-organ crosstalk between liver and brain.
Hepatic gluconeogenesis is tightly controlled by counter-regulatory hormones such as glucagon, cortisol, and insulin, via regulating the expression of key gluconeogenic enzymes, including glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). Fibroblast growth factor 21 (FGF21), a metabolic regulator mainly secreted from the liver in response to fasting and starvation under the control of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α, plays a critical role in maintaining energy homeostasis and insulin sensitivity in both rodents and nonhuman primates (1-6). Therapeutic dose of FGF21 decreased blood glucose in diabetic animals without causing hypoglycemia (4). FGF21 has also been shown to act as a key downstream effector of PPARα mediating several metabolic adaptation responses to starvation, including hepatic fatty acid oxidation, ketogenesis and growth hormone resistance (1,2,7). In addition, FGF21 is implicated in hepatic gluconeogenesis, although it remains controversial whether hepatocytes are a direct action site of FGF21 (8,9). There is an obvious dichotomy between the effects of endogenous FGF21 and pharmacological actions of the recombinant peptide with respect to hepatic metabolism (4,6,9).

FGF21 can cross the blood-brain barrier (10), and is detectable in both human and rodent cerebrospinal fluid (10,11). Continuous intracerebroventricular injection of FGF21 into obese rats increases energy expenditure and insulin sensitivity (12). More recently, FGF21 has been shown to act on the central nervous system to increase systemic glucocorticoid levels, suppresses physical
activity and alters circadian behavior (13). Furthermore, FGF21 acts on the hypothalamus to suppress the vasopressin-kisspeptin signaling cascade, thereby mediating starvation-induced infertility of female mice (14). However, the physiological roles of FGF21 and its central actions in regulating glucose metabolism during adaptive starvation responses remain unknown.

In this study, we show that FGF21 is a key metabolic regulator essential for maintaining glucose homeostasis, by sending the starvation signal from the liver to the brain, where it acts on the hypothalamic neurons to induce phosphorylation of the MAP kinase ERK1/2, which in turn stimulate the production of corticotropin-releasing hormone (CRH) and subsequent release of adrenal corticosterone by activation of cAMP response element binding protein (CREB), thereby leading to enhanced hepatic gluconeogenesis.

RESEARCH DESIGN AND METHODS

Animal Studies

All animal experimental protocols were approved by the Animal Ethics Committee of the University of Hong Kong. PPARα-KO mice in C57BL/6N background were originally obtained from Jackson Laboratories (Sacramento, CA). FGF21-KO mice in C57BL/6J background were described previously (15). The mice were housed in a room under controlled temperature (23°C ± 1°C) with free access to water and standard chow. Male mice at the age of 12-14 weeks were used in all animal experiments. Mice were studied by hyperinsulinemic euglycemic clamp to assess endogenous glucose production as previously described (16), except that mice were fasted 24 hours
before the clamp and somatostatin was not infused. Bilateral or sham adrenalectomy was conducted under isoflurane anesthesia one week before various fasting experiments. Plasma corticosterone levels were measured to confirm the completeness of adrenalectomy.

**Icv and Intra-PVN Injection in Mice**

Male FGF21-KO, PPARα-KO mice and their respective WT littermates at the age of 12-14 weeks were chronically implanted with 26G stainless cannula (Plastics One, Inc., Roanoke, VA) in the cerebral ventricle or the PVN region of the brain as described (17). A neutralizing monoclonal antibody against FGFR1 (Novus, NBP2-12308, 0.2 µg/g body weight) or CRH antagonist (α-helical CRH$_{9-41}$, Sigma-Aldrich C2917, 0.2 µg/g body weight) were infused by intra-PVN injection before central administration of endotoxin-free rmFGF21 (18) (0.02 µg/g body weight).

**Biochemical and Immunological Analysis**

Serum levels of insulin and FGF21 were quantified with immunoassays according to the manufacturer’s instructions (Antibody & Immunoassay Services, University of Hong Kong). Mouse FGF21 assay was based on an affinity-purified rabbit polyclonal antibody specific to mouse FGF21, and did not cross-react with other members of the FGF family. The intra- and interassay variations were 4.2 and 7.6%, respectively. Mouse insulin assay was based on a monoclonal antibody pair raised using recombinant mouse insulin as an antigen, with intra- and inter-assay variations of 5.3% and 6.2% respectively. Serum levels of corticosterone (Enzo Life Sciences) and glucagon
(Millipore), and plasma levels of ACTH (MD Bioproducts) and CRH (Phoenix Pharmaceuticals) were determined using immunoassays according to the manufacturer's instructions. For analysis of adrenaline and noradrenaline in the liver, frozen tissue was extracted with 0.1 N HCl and analyses performed according to the supplier's instructions (Alpco Diagnostics, Windham, NH).

**Ex vivo Studies**

The livers of male 12-week-old C57BL/6 mice were isolated and primary hepatocytes were prepared from male Wistar rats (200 g) and used to determine glucose production as described (19). The mouse hypothalamic explants were prepared as previously described (20). The hypothalamic slices were pretreated without or with PD98059 for 30 minutes, followed by treatment with rmFGF21 for different periods.

**RNA Extraction and Real-time PCR**

Total RNA was extracted from mouse tissues and rat hepatocytes, cDNA was synthesized and the mRNA expression levels were quantified with real time PCR as described previously (21).

**Western Blot**

Total cellular proteins in liver and hypothalamic lysates were separated by SDS-PAGE, and probed with a rabbit polyclonal antibody against PGC-1α (abcam), FGF21 (18), p-ERK, t-ERK, p-CREB,
t-CREB (Cell Signaling Technology), FGFR1, βKlotho (Santa Cruz) or β-actin (Sigma).

Immunohistochemistry

Paraformaldehyde-fixed brains were frozen in liquid nitrogen and stored at -80 °C until sectioning with a cryostat (Leica CM1950) at -20 °C. Slides were washed and then blocked in 10% goat serum with 3% BSA in TBS for one hour at room temperature, followed by incubation with primary antibody overnight at 4°C. On the second day, the slides were further incubated with Alexa Fluor 488 or 594-labeled mouse-specific secondary antibodies (invitrogen) for another one hour at room temperature. Images were captured with a fluorescence microscope (QImaging, Olympus IX71).

Statistical Analysis

All analyses were performed with Statistical Package for Social Sciences version 11.5 for windows (SPSS, Chicago, IL). Comparison between groups was performed using 1-way ANOVA followed by least-significant difference post hoc test. In all statistical comparisons, a p value < 0.05 was used to indicate a statistically significance difference.

RESULTS

FGF21 knockout (KO) mice exhibit hypoglycemia during prolonged fasting

Blood glucose levels were comparable between FGF21 KO and WT mice in the fed state and within
six hours of food deprivation (Fig. 1A). However, during the prolonged fasting period (24 to 48 hours), FGF21 KO mice exhibited a much more rapid decline in blood glucose levels and more severe hypoglycemia as compared to WT mice (Fig. 1A). Moreover, both the mRNA and protein expression levels of hepatic peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, a transcriptional coactivator that plays a key role in mediating the fasting-induced expression of gluconeogenic genes (22), were comparable between FGF21 KO mice and WT littermates in the fed state (Fig. 1A-B). However, the amplitude of fasting-induced elevation in PGC-1α expression was significantly decreased as compared to WT mice, and this change was accompanied by a marked impairment in fasting-induced expression of G6pase and PEPCK (Fig. 1A-B). The rate of glucose production in FGF21 KO mice, as estimated by pyruvate tolerance test, was much lower than that in WT littermates in fasted state (Fig. 1C). We further assessed whole-body glucose homeostasis in euglycemic glucose clamp studies. During constant hyperinsulinemia in mice after fasting, a higher glucose infusion rate was required to maintain normal glucose level in KO mice than in WT mice (Fig. 1D). This difference was primarily accounted for by a significant reduction in glucose production in KO mice, though whole-body glucose disposal was similar in WT and KO mice (Fig. 1D). Treatment of mouse liver explants with recombinant mouse FGF21 (rmFGF21) for up to 24 hours had no obvious effect on expression of gluconeogenic genes (PGC-1α, PEPCK, G6Pase) and glucose production, although the glucocorticoid receptor agonist dexamethasone led to a significant induction of the gluconeogenic program (Supplemental Figure 1A-B). Similarly, dexamethasone, but not FGF21, increased the expression of the gluconeogenic genes and glucose production in
primary rat hepatocytes (Supplemental Figure 1C-D). Furthermore, there was no difference in circulating levels of insulin and glucagon, nor the levels of hepatic adrenaline and noradrenaline between FGF21 KO mice and WT littermates in either fed or fasted state (Supplemental Figure 2A-D), suggesting that insulin, glucagon and hepatic sympathetic nerves are not the downstream mediators of FGF21 in the maintenance of fasting glucose homeostasis.

**FGF21 deficiency impairs fasting-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and release of corticosterone in mice**

Glucocorticoids are fasting-inducible hormones that play a central role in activating gluconeogenic genes and sustaining glucose homeostasis during starvation. Our recent clinical study showed that FGF21 and cortisol display similar circadian rhythm in humans (23). Therefore, we next investigated the functional relationship between FGF21, corticosterone and its upstream regulators in mice. In WT mice, serum levels of corticosterone were progressively increased in response to fasting (Fig. 1E). However, the magnitude of fasting-induced elevation of serum corticosterone in FGF21 KO mice was much smaller than WT mice (Fig. 1E). Likewise, fasting for 24 hours caused a marked induction in hypothalamic expression of CRH (Fig. 1F) and pituitary release of adrenocorticotropic hormone (ACTH) (Fig. 1H), whereas such fasting-induced expression of these two corticotropic hormones was severely impaired in FGF21 KO mice (Fig. 1F and H), suggesting that FGF21 deficiency causes defective fasting-induced release of corticosterone possibly by impairing the activation of the HPA axis. On the contrary, the expression of arginine vasopressin
(AVP) was comparable between WT and FGF21 KO mice under both fed and fasted states (Fig. 1G), suggesting that these hormones do not contribute to the hypoglycemia phenotypes in FGF21 KO mice. Moreover, the difference in fasting blood glucose between WT and KO mice totally disappeared after the production of corticosterone was blocked by adrenalectomy in mice (Fig. 1I), suggesting that corticosterone mediates the effects of FGF21 in regulating fasting glucose homeostasis.

We next investigated whether the fasting hypoglycemia phenotype in FGF21 KO mice can be reversed by supplementation with corticosterone. Injection of the synthetic glucocorticoid dexamethasone into FGF21 KO mice reversed fasting hypoglycemia to a level comparable to WT mice (Supplemental Figure 3A). The dexamethasone-mediated normalization of fasting glucose homeostasis was associated with significantly elevated expression of PGC-1α and its target genes PEPCK and G6pase (Supplemental Figure 3B-D).

**FGF21 acts in the brain to induce corticosterone production and hepatic gluconeogenesis**

Consistent with a previous report (10), we found that FGF21 was present in the hypothalamus and its hypothalamic level was markedly elevated in response to fasting (Fig. 2A), despite that there was no any detectable FGF21 mRNA expression in this tissue even in fasted state (Fig. 2A). Furthermore, intraperitoneal injection of exogenous rmFGF21 led to its marked accumulation in the hypothalamus region (Fig. 2B), confirming that FGF21 can cross the blood-brain-barrier into the
hypothalamus.

To investigate whether FGF21 induces fasting gluconeogenesis through its central actions, recombinant mouse FGF21 (rmFGF21) was infused directly into the cerebroventricle of FGF21 KO mice by intracerebroventricular (icv) injection via a cannula pre-implanted one week before the treatment. The results showed that the central infusion of rmFGF21 at 0.02µg/g body weight, a dose at which there was still no detectable FGF21 in the peripheral blood of FGF21 KO mice, led to a significant elevation of blood glucose levels as compared to the vehicle-treated mice (Fig. 2C). The central administration of rmFGF21 also induced the expression of hepatic PGC-1α, PEPCK and G6pase (Fig. 2C), and increased the hypothalamic level of CRH (Fig. 2D), pituitary release of ACTH (Fig. 2F) and serum corticosterone (Fig. 2G), but had no effect on hypothalamic expression of AVP (Fig. 2E). Furthermore, direct incubation of mouse hypothalamic slices with rmFGF21 was sufficient to induce the CRH expression in a dose-dependent manner (Fig. 2H), whereas the central effects of FGF21 on stimulation of corticosterone release and elevation of blood glucose levels were blunted by intra-paraventricular nucleus (intra-PVN) administration of the CRH antagonist α-helical CRH$_{9-41}$ (Fig. 2I-J). On the contrary, intravenous injection of a low dose of rmFGF21 (0.02µg/g), which was insufficient to cause any notable elevation of FGF21 in the brain of FGF21 KO mice, had no obvious effect on either blood glucose level, or the expression of the gluconeogenic genes in the liver or the hormones of the HPA axis (CRH, ACTH and corticosterone) (Supplemental Figure 4A-E).
Central injection of rmFGF21 did not increase the levels of noradrenaline or adrenaline in the liver (Supplemental Figure 5A-B), suggesting that sympathetic nerves is not involved in FGF21-mediated hepatic glucose production. Plasma ketone bodies, hepatic expression of key ketogenic genes and several genes governing lipid metabolism in the liver were not altered by central treatment of rmFGF21 (Supplemental Figure 5C-D), indicating that the effects of FGF21 on ketogenesis and lipid metabolism are not attributed to its central actions.

To further confirm the role of the HPA axis in FGF21-mediated gluconeogenesis, FGF21 KO mice were pretreated with the glucocorticoid receptor antagonist RU486 or subjected to adrenalectomy prior to icv injection of rmFGF21. The results showed that the central effects of rmFGF21 on elevation of blood glucose and induction of the hepatic gluconeogenic genes were largely abrogated by either pharmacological blockage of the glucocorticoid receptor or depletion of endogenous corticosterone by adrenalectomy (Fig. 2K-M).

**The central effects of FGF21 on activation of the HPA axis and hepatic gluconeogenesis are mediated by hypothalamic FGFR1**

The metabolic actions of FGF21 are mediated by FGF receptor (FGFR) 1 in complex with βKlotho (24). FGFR1 is highly expressed in hypothalamic areas such as paraventricular nuclei (PVN) and supraoptic nuclei (25). Both real-time PCR and Western blot analysis showed that the expression level of FGFR1 in the hypothalamus was comparable to that in white adipose tissue, a major
peripheral target of FGF21 (Fig. 3A). FGFR1 is abundantly expressed in the PVN of the hypothalamus (Fig. 3B) and a large portion of FGFR1 colocalized with CRH neurons (Fig. 3B). In addition, both mRNA and protein expressions of βKlotho were detectable in the hypothalamus, although its expression level was much lower than that in adipose tissues and livers (Fig. 3C). Specifically, βKlotho was detectable in the PVN of the hypothalamus (Fig. 3D).

To interrogate the roles of hypothalamic FGFR1 in FGF21-induced hepatic gluconeogenesis via the HPA axis, we performed bilateral intra-PVN injection to directly deliver the neutralizing antibody into PVN. The intra-PVN injection of anti-FGFR1 antibody largely blocked rmFGF21-induced activation of ERK1/2 in the hypothalamus (Fig. 4). The central actions of rmFGF21 on elevation of blood glucose and induction of hepatic gluconeogenic gene expression were abrogated by a single intra-PVN administration of a neutralizing monoclonal antibody against FGFR1 (Fig. 5A-B). Furthermore, blockage of hypothalamic FGFR1 by its neutralizing antibody also negated the stimulatory effects of central administration with rmFGF21 on hypothalamic CRH expression and serum levels of ACTH and corticosterone (Fig. 5C-F). On the other hand, intra-PVN administration of the anti-FGFR1 neutralizing antibody had no obvious effect on glucose levels, hepatic gluconeogenic gene expression and the HPA activity in FGF21 KO mice without FGF21 treatment (Fig. 5A-F). Physiologically, a single intra-PVN administration of the anti-FGFR1 antibody lowered fasting blood glucose and reduced the expression of hepatic gluconeogenic genes in WT mice to a level comparable to those in KO mice (Fig. 5G-H). Furthermore, fasting-induced elevations in
hypothalamic CRH expression and serum levels of corticosterone were significantly diminished by the neutralizing antibody-mediated blockage of hypothalamic FGFR1 (Fig. 5I-J).

**FGF21 mediates PPARα-induced corticosterone production and hepatic gluconeogenesis in mice via central nervous system**

During fasting, the transcription factor PPARα is activated in the liver, which in turn promotes hepatic expression of FGF21 and its release into the circulation (1-3). Therefore, we investigated whether PPARα activation induces corticosterone production by induction of FGF21. Chronic treatment of WT mice with the PPARα agonist fenofibrate led to a progressive elevation in serum levels of FGF21 as well as corticosterone (Fig. 6A-B). However, the magnitude of fenofibrate-induced increase of serum corticosterone was significantly attenuated in FGF21 KO mice (Fig. 6B). Likewise, fenofibrate-induced hypothalamic expression of CRH was also impaired in FGF21 KO mice as compared to WT controls (Fig. 6C). Furthermore, fasting-induced elevation of serum corticosterone levels and hypothalamic expression of CRH in PPARα KO mice were markedly reduced as compared to WT littermates (Fig. 6B-C). On the other hand, the impairments in fasting-induced production of corticosterone and CRH were partially reversed by icv injection of rmFGF21 (Fig. 6D-E).

Consistent with previous reports (26), we found that PPARα KO mice exhibited similar blood glucose levels with WT controls in fed state, but developed severe hypoglycemia after 16 hours of
fasting (Fig. 6F). The hypoglycemic phenotype of PPARα KO mice further deteriorated after prolonged fasting (24 and 48 hours). On the other hand, icv administration of rmFGF21 into PPARα KO mice not only led to a significant alleviation of fasting hypoglycemia, but also reversed the impairment of fasting-induced expression of several gluconeogenic genes (PGC-1α, PEPCK and G6pase) (Fig. 6F-G). Likewise, replenishment of PPARα KO mice with dexamethasone also resulted in a partial reversal of hypoglycemia and decreased gluconeogenic gene expression in fasted PPARα KO mice, suggesting that PPARα maintains fasting glucose homeostasis via activation of the FGF21-glucocorticoid axis (Fig. 6F-G).

**FGF21 stimulates CRH production by activation of the ERK1/2-CREB signaling axis**

CREB is a key transcription factor responsible for transactivation of the CRH gene in PVN neurons (27). The MAP kinase ERK1/2 activates CREB by phosphorylating the transcription factor at Serine-133 (28). Notably, FGF21 induces phosphorylation and activation of ERK1/2 both in adipocytes (4) and in whole hypothalamus of mice (29). Therefore, we next investigated the roles of ERK1/2 and CREB in mediating the central actions of FGF21 in mice. Consistent with previous reports (30), we found that phosphorylation of both ERK1/2 and CREB was significantly enhanced by fasting for 24 hours in WT mice (Fig. 7A). However, fasting-induced phosphorylation of ERK1/2 and CREB was impaired in FGF21 KO mice (Fig. 7A).

Injection of rmFGF21 into the PVN region of FGF21 KO mice induced phosphorylation of both
ERK1/2 and CREB, whereas such a stimulatory effect of rmFGF21 was completely blocked by pretreatment with the ERK inhibitor PD98059 in the hypothalamic PVN region (Fig. 7B & Supplemental Figure 6). Likewise, the central effects of rmFGF21 on induction of hypothalamic CRH expression, elevation of serum corticosterone and blood glucose levels, and stimulation of hepatic gluconeogenic gene expression were all abrogated by intra-PVN injection of PD98059 (Fig 7C-F). Furthermore, direct incubation of mouse hypothalamic slices with rmFGF21 was sufficient to induce the phosphorylation of ERK and CREB (Fig. 7G) and to increase the mRNA expression and protein release of CRH (Fig. 7H-I). However, all these effects of rmFGF21 on hypothalamic slices were blocked by PD98059 (Fig. 7G-I). Taken together, these findings suggest that FGF21 stimulate hypothalamic CRH production via the ERK1/2-CREB signaling cascade, which in turn triggers the release of corticosterone for induction of gluconeogenesis (Fig. 8).

**DISCUSSION**

Appropriate counter-regulatory hormone responses to hypoglycemia are critical for maintaining blood glucose levels within a narrow range. However, the molecular events that coordinate this process remain poorly defined. In both rodents and humans, fasting-induced hepatic production of FGF21 is mediated by PPARα, which is a master regulator coordinating metabolic adaptions to fasting and starvation (1-3). In addition to its central role in controlling hepatic fatty acid metabolism, PPARα is also a critical mediator of fasting-induced hepatic gluconeogenesis in mice.
PARα KO mice exhibit severe fasting hypoglycemia despite normoglycemia in the fed state (26). However, PARα is not a direct transcriptional activator of the key gluconeogenic genes, suggesting that it induces hepatic gluconeogenesis via an indirect mechanism(s) (32). In the present study, we found that both PARα and FGF21 KO mice exhibited a similar degree of hypoglycemia due to defective hepatic gluconeogenesis during prolonged fasting, whereas hypoglycemia in fasted PARα KO mice and FGF21 KO mice can be rectified by central administration of FGF21, suggesting an obligatory role of the PARα-FF21 axis in inducing hepatic gluconeogenesis during metabolic adaptation to prolonged fasting. In support of this notion, transgenic expression of FGF21 was sufficient to induce hepatic gluconeogenesis in the fed state to a level normally attained during prolonged fasting, while FGF21 KO mice showed impaired hepatic gluconeogenesis after 24-hour fasting (9). By contrast, Badman and colleagues found that fasting glucose levels were comparable between WT and FGF21 KO mice generated in their laboratory (33). This discrepancy may be due to different ages of mice or different fasting schedules. Nevertheless, they also showed impaired gluconeogenic gene induction (PGC-1α and PEPCK) in their FGF21 KO mice, which is consistent to our present study.

The hypothalamus has emerged as a key player in the regulation of glucose homeostasis, by sensing hormones and nutrients to initiate metabolic responses (34). Hormones such as insulin, glucagon and leptin have been shown to regulate hepatic glucose metabolism through both central and peripheral actions (34,35). In this study, we provide several lines of evidence demonstrating that the
stimulatory effect of FGF21 on hepatic gluconeogenesis is mainly mediated by its central actions on hypothalamic neurons. First, fasting hypoglycemia and impaired expression of hepatic gluconeogenic genes in both FGF21 KO and PPARα KO mice can be reversed by icv injection of FGF21 at a low dose that is not detectable in the peripheral blood. Second, FGF21-induced corticosterone release and hepatic gluconeogenesis can be completely blocked by central administration of a neutralizing antibody against its receptor FGFR1 or a pharmacological inhibitor of ERK1/2. Furthermore, both our animal and ex vivo studies showed that FGF21 stimulates CRH expression in the hypothalamus via ERK1/2-mediated activation of CREB, suggesting that CRH neurons are the direct target of FGF21.

Consistent with our observations in PPARα KO mice, administration of the PPARα agonist fenofibrate has been shown to elevate fasting serum levels of corticosterone, enhance hepatic gluconeogenesis and increase blood glucose levels (36). These findings support the notion that PPARα coordinates metabolic adaptation responses to starvation through a bipartite mechanism: direct transactivation of the genes involved in hepatic fatty acid oxidation and ketogenesis (26,31) and indirect activation of the HPA axis via induction of FGF21. The synergic effect of this dual action of PPARα is to provide glycerol, fatty acids, ketones, and alanine as sources for gluconeogenesis to maintain glucose homeostasis.

In peripheral tissues, adipocytes have been identified as a main target that confers the pleiotropic
metabolic actions of FGF21 (4, 19). In white adipocytes, adiponectin expression and secretion is induced by FGF21 and mediates several metabolic benefits of FGF21 treatment (18, 37). These findings together with our present report further support the notion that FGF21 exerts its diverse metabolic effects indirectly, by modulating the production of other hormones such as adiponectin and corticosterone. The central and peripheral actions of FGF21 on activation of the HPA axis and its peripheral actions on promoting adipose secretion of adiponectin appear to have opposite effects on hepatic glucose production. However, these two actions of FGF21 may occur under different physiological conditions. FGF21 secretion in adipose tissue is elevated in the fed state, which in turn serves as an autocrine signal to induce PPARγ activity and adiponectin secretion, thereby leading to enhanced hepatic insulin sensitivity (18). Conversely, the central effect of FGF21 on hepatic glucose production consequent to increased release of corticosterone occurs only during prolonged fasting. Such a dual effect of FGF21 may enable the control of blood glucose within a narrow range in different nutritional states.

In conclusion, our results demonstrate FGF21 as a critical hormonal regulator of glucose homeostasis during prolonged fasting, by coupling hepatic PPARα activation to corticosterone release via stimulation of the HPA axis, thereby leading to enhanced hepatic gluconeogenesis (Fig. 8). This novel liver-PPARα-FGF21-brain-corticosterone circuit can fine-tune the crosstalk between brain and liver to avoid hypoglycemia during nutrient deprivation or other adverse clinical events. Interestingly, a novel “hepato-adrenal syndrome” has recently been suggested demonstrating a
defect in HPA activation in patients with severe liver disease (38). Hypoglycemia is a common clinical problem in patients with a defect in HPA regulation such as hypopituitarism and Addison’s disease. It is also frequently seen in diabetic patients treated with insulin or other medications improperly. Unravelling the liver-adrenal circuit with FGF21 as key player may open new avenues for the treatment of these metabolic diseases with hypoglycemia unawareness.

Author contributions

Q.L. designed, performed the experiments, analyzed the data and wrote the manuscript. A.X. conceived the concept, supervised the project and wrote the manuscript. L.Z. and J.Z. performed the experiments. Y.W., S.R.B., C.R.T. and H.D. provided technical assistance, reagents and valuable advice for this study. K.S.L.L. supervised the study and edited the manuscript.

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Conflicts of interest

No potential conflicts of interest relevant to this article were reported.

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FIGURE LEGENDS

Fig. 1: Fasting-induced gluconeogenesis and activation of the HPA axis are impaired in FGF21 KO mice. A. Blood glucose levels and the mRNA abundance of hepatic gluconeogenic genes PGC-1α, PEPCK and G6Pase in FGF21 KO mice and WT littermates in either fed state or after fasting. B. The protein abundance of PGC-1α determined by Western blot analysis and was quantified using scanning densitometry. The results were expressed as fold changes relative to internal control β-actin. C. Pyruvate tolerance test (PTT) was performed in 24 hours-fasted FGF21 KO mice and WT littermate mice by using 2 g pyruvate per kg body weight. D. Glucose infusion rate, glucose disposal rate and glucose production during a hyperinsulinemic euglycemic clamp experiment. E-H. The amount of hormones of HPA axis in FGF21 KO mice and WT littermates either in fed or fasted state: the serum levels of corticosterone (E) was determined by ELISA, the mRNA abundance of CRH (F) and AVP (G) in the hypothalamus was determined by qRT-PCR, plasma ACTH levels (H) was determined by ELISA. I. Blood glucose in FGF21 KO mice and WT littermates receiving adrenalectomy (ADX) or sham surgery in either fed state or various time points after fasting. n=8 for WT mice and n=10 for KO mice. *p<0.05, ** p<0.01; NS, not significant. Data were expressed as mean ± SEM.

Fig. 2: FGF21 acts in the brain to activate HPA axis and induce hepatic gluconeogenesis.

A. The protein levels of FGF21 in the hypothalamus, the mRNA abundance of FGF21 in the livers
and hypothalamus of C57BL/6J mice in fed state or fasted for 24 hours. B. FGF21 protein levels in the hypothalamus, which was collected at 30 minutes after intraperitoneal injection of rmFGF21 (1 mg/kg) or vehicle in FGF21 KO mice. The mice were perfused with 1xPBS by transcardiac perfusion before the collection of hypothalamic tissues. Representative western blotting analysis and qRT-PCR assays from two independent cohorts run in duplicate are shown, n=4. C-G: FGF21 KO mice were treated with rmFGF21 (0.02µg/g, icv) or vehicle. (C) Blood glucose levels and the mRNA abundance of hepatic gluconeogenic genes PGC-1α, PEPCK and G6Pase in the livers. (D) CRH and (E) AVP mRNA in the hypothalamus, plasma levels of ACTH (F) and serum levels of corticosterone (G) were determined at three hours after icv injection. *p<0.05, **p<0.01 versus vehicle control (n=6). Data were expressed as mean ± SEM. H. The hypothalamic slices from FGF21 KO mice were incubated with different concentrations of rmFGF21 for 2 hours. The culture medium was collected for determination of CRH by ELISA. *p<0.05, **p<0.01 versus vehicle control (n=6). Data were expressed as mean ± SEM. I-J. FGF21 KO mice were pretreated with CRH antagonist α-helical CRH9-41 (anti-CRH, 0.2µg/g, intra-PVN) for 30 minutes, followed by injection with rmFGF21 (0.02µg/g). Serum levels of blood glucose (I) and corticosterone (J) were detected at three hours after injection. *p<0.05, n=6. Data were expressed as mean ± SEM. K-L. 12-week-old FGF21 KO mice were pretreated with RU 486 by intraperitoneal injection (20 mg/kg) for one hour, followed by icv infusion of rmFGF21 (0.02 µg/g) or vehicle for three hours. K. Blood glucose levels were determined at various time points after treatment. *p<0.05, **p<0.01 versus vehicle. #p<0.05 versus rmFGF21 group. (n=6). Data were expressed as mean ± SEM. L. The
mRNA abundance of hepatic PGC-1α, PEPCK and G6Pase were determined by qRT-PCR. *p<0.05, **p<0.01 (n=6). Data were expressed as mean ± SEM. **p<0.01 versus vehicle+sham surgery; #p<0.05 versus rmFGF21+sham surgery. (n=6). Data were expressed as mean ± SEM.

**Fig. 3: FGFR1 and βklotho are expressed in the hypothalamus of mice.** The abundance of FGFR1 mRNA and protein (A), βklotho mRNA and protein (C) in liver, white adipose tissue (WAT) and hypothalamus (hypo) in 12-week-old male C57BL/6J mice, as determined by real-time PCR and Western blot analysis respectively. Representative images of mouse hypothalamic slices immunostained for FGFR1 (B), βklotho (D) and CRH (B & D). Note that the merged image shows the colocalization between FGFR1 and CRH (B) and the specificity of anti-CRH antibody (abcam, CAT # number ab11133) was validated by using the CRH blocking peptide (sigma, CAT # C3042) to neutralize the antibody. No positive fluorescent signal was detected when the hypothalamic slices were co-incubated with anti-CRH antibody and blocking peptides (data not shown). Representative western blotting analysis and immunostained figures from three independent cohorts are shown, n=6.

**Fig. 4: FGF21 signaling in hypothalamus is blocked by FGFR1 neutralizing antibody.**

12-week-old FGF21 KO mice were treated with an anti-FGFR1 monoclonal antibody (0.2 µg/g
body weight, AF) or mouse non-immune IgG (NI) by a single hypothalamic paraventricular (intra-PVN) injections, followed by another intra-PVN injection with rmFGF21 (0.02µg/g body weight) or vehicle control. Hypothalamic protein levels of p-ERK and t-ERK were determined by western blotting analysis after 30 minutes of injection and was quantified using scanning densitometry. Representative western blotting analysis from three independent cohorts run in duplicate are shown, n=6. *p<0.05. Data were expressed as mean ± SEM.

**Fig. 5: Hypothalamic FGFR1 is obligatory for FGF21-induced activation of the HPA axis and hepatic gluconeogenesis in mice.** 12-week-old FGF21 KO mice were treated with an anti-FGFR1 monoclonal antibody (0.2 µg/g body weight, AF) or mouse non-immune IgG (NI) by a single hypothalamic paraventricular (intra-PVN) injection, followed by another intra-PVN injection with rmFGF21 (0.02µg/g body weight) or vehicle control. (A) Blood glucose levels. (B) the mRNA abundance of PGCU1α, PEPCK and G6Pase in the livers. C-F. The amount of hormones of HPA axis: (C) The mRNA abundance of CRH in the hypothalamus of mice collected three hours after treatment. (D) Representative images of immunofluorescent staining with anti-CRH antibody in the PVN region of the hypothalamus (scale bar, 200µm). (E) Plasma ACTH levels and (F) serum corticosterone levels were determined at different time intervals. *p<0.05, ** p<0.01 versus vehicle+NI group; #p<0.05, ##p<0.01 versus rmFGF21+NI group (n=5). Data were expressed as mean ± SEM. G-J. 12-week-old WT mice were treated with AF (0.2 µg/g body weight) or NI by intra-PVN injections, followed by fasting for 24 hours. Fed WT mice and FGF21 KO mice treated
with NI were used as control. (G) Blood glucose levels and (H) the mRNA abundance of PGC-1α, 
PEPCK and G6Pase in the livers. (I) The mRNA abundance of CRH in the hypothalamus and (J) 
serum corticosterone levels were determined. *p<0.05, ** p<0.01, n=5. Data were expressed as 
mean ± SEM.

**Fig. 6:** FGF21 mediates PPARα-induced activation of HPA axis and hepatic gluconeogenesis in 
mice.

A-C. FGF21 KO mice and WT littermates were treated with fenofibrate (Feno, 200 mg/Kg) or 
vehicle by daily intraperitoneal injection for seven days. (A) Serum levels of FGF21 and (B) serum 
corticosterone and (D) the mRNA abundance of CRH in the hypothalamus were determined at day 
3 and day 7 after treatment. **p<0.01 versus WT mice treated with vehicle. #p<0.05 versus 
WT+Feno group (n=6-8). D-E. PPARα KO mice and WT littermates were treated with rmFGF21 
(0.02µg/g) or vehicle by icv injection in every six hours during 48 hours of fasting. (D) Serum 
levels of corticosterone were measured at different time points after treatment. (E) The mRNA 
abundance of CRH in the hypothalamus was determined by real-time PCR analysis after 24 hours 
of fasting. *p<0.05, ** p<0.01 versus WT+vehicle group. #p<0.05 versus KO + vehicle group (n=8). 
F-G. 12-week-old PPARα KO mice and WT littermates were treated with rmFGF21 (0.02 µg/Kg) or 
vehicle by icv infusion in every six hours during fasting, or dexamethasone (DEX, 2 mg/Kg) via 
intraperitoneal injection, followed by fasting for 48 hours. (F) Blood glucose levels were measured 
at different time intervals. *p<0.05, **p<0.01 versus KO+vehicle (n=5). Data were expressed as
mean ± SEM. (G) The mRNA abundance of PGC-1α, PEPCK and G6Pase in the livers of mice collected at fed state or 24 hours after fasting. *p<0.05, **p<0.01 (n=5). Data were expressed as mean ± SEM.

**Fig. 7: FGF21 increases CRH production via ERK-CREB-CRH signaling pathways.**

**A.** The hypothalamic tissue lysates of 12-week-old FGF21 KO mice and WT littermates in either fed or fasted (24 hours) state were subjected to immunoblotting analysis with antibodies against phosphorylated ERK1/2 (p-ERK), total ERK1/2 (t-ERK), phosphorylated CREB (p-CREB) or total CREB (t-CREB). **B-F.** FGF21 KO mice were treated with the ERK inhibitor PD98059 (2µg/mouse) or vehicle control (DMSO) by a single intra-PVN injection, followed by another intra-PVN injection with rmFGF21 (0.02µg/g body weight) or vehicle control. **(B)** Hypothalamic protein levels of p-ERK, t-ERK, p-CREB and t-CREB were determined by western blotting analysis after 30 minutes of injection. **(C)** Blood glucose and **(D)** the mRNA abundance of PGC-1α, PEPCK and G6Pase in the livers, **(E)** the mRNA abundance of CRH in the hypothalamus and **(F)** serum corticosterone levels were measured after three hours of injection. **G-I.** The hypothalamic slices from FGF21 KO mice were pre-incubated with PD98059 (20µM) for 30 minutes, followed by treatment with rmFGF21 (1µg/ml). **(G)** Hypothalamic protein levels of p-ERK, t-ERK, p-CREB and t-CREB were determined by western blotting analysis after 15 minutes of incubation with rmFGF21. **(H)** The mRNA abundance of CRH in the hypothalamus and **(I)** the CRH levels in culture medium was determined after three hours of incubation with rmFGF21. Representative
western blotting analysis and qRT-PCR assays from three independent cohorts run in duplicate are shown, *p<0.05 (n=6). Data were expressed as mean ± SEM.

Fig. 8: A working model by which FGF21 maintains glucose homeostasis during fasting via mediating the cross-talk between brain and liver.
Fig. 1
Fig. 2
Fig. 3

A

Hypo Liver  WAT

β-tubulin

FGFR1

Merged

Mouse anti-FGFR1

Mouse IgG

CRH

3V

DAPI

Merged

FGFR1 Relative mRNA level

Liver  WAT  Hypo

0  10  20  30  40  50

β-tubulin

Hypo Liver  WAT

C

β-klotho

Merged

Mouse anti-β-klotho

Mouse IgG

CRH

DAPI

Merged

Liver  WAT  Hypo

0  0.05  0.10  0.15  0.20

β-tubulin

Scale bar  200μm  20μm
Fig. 4
Glycerol
HSL
TG
Fasting
Glucose homeostasis
Hypothalamus
Pituitary
ACTH
Adrenal cortex
CRH
FFA
PPARα
FGF21
FGFR1
β
Klotho
Cortisol
PGC-1α
GR
PEPCK
G6pase
Gluconeogenesis
Glucose homeostasis

Fig. 8
Supplemental Figure 1: FGF21 does not exert direct effects on hepatic glucose production. The liver explants from C57BL/6J mice (A, B) or primary rat hepatocytes (C, D) were incubated with rmFGF21 (2 μg/ml), dexamethasone (DEX, 1μM) or vehicle for 24 hours in glucose free DMEM medium. (A, C). The glucose concentration in the conditioned medium. (B, D). The mRNA abundance of PGC-1α, PEPCK and G6Pase expressed as fold relative to vehicle-treated group. A similar effect of FGF21 and dexamethasone on expression of the three gluconeogenic genes was also observed in primary hepatocytes cultured in different glucose levels (0, 5 and 15 mM). *p<0.05 (n=5). Data were expressed as mean ± SEM.
Supplemental Figure 2: FGF21 deficiency does not alter serum levels of insulin, glucagon or hepatic levels of noradrenaline and adrenaline in mice. Serum levels of insulin (A), glucagon (B), hepatic noradrenaline (C) and adrenaline (D) in 10-week-old male FGF21-KO mice and WT littermates were measured at fed state or 24 hours after fasting. Data were expressed as mean ± SEM (n=8).
Supplemental Figure 3: Dexamethasone reversed the impaired hepatic glucose production in FGF21 KO mice. 10-12-week-old FGF21 KO mice were treated with DEX (2mg/kg) or vehicle by intraperitoneal injection, followed by fasting for 48 hours. (A) Effects of dexamethasone (DEX) on blood glucose levels in FGF21 KO mice. **p<0.01 versus KO+vehicle group; #p<0.05 versus WT+vehicle group (n=6). (B-D) The hepatic mRNA abundance of PGC-1α, PEPCK and G6Pase as determined by real-time PCR. The livers were collected from mice treated with DEX or vehicle and fasted for 24 hours. *p<0.05; **p<0.01 (n=6). Data were expressed as mean ± SEM.
Supplemental Figure 4: Peripheral administration of low-dose FGF21 does not affect the HPA axis or hepatic gluconeogenesis. FGF21 KO mice were treated with rmFGF21 (0.02µg/g) by intraperitoneal injection. (A) Blood glucose levels measured at different time points. (B) The mRNA abundance of PGC-1α, PEPCK and G6Pase in the liver, (C) the mRNA abundance of CRH in the hypothalamus, (D) plasma ACTH levels and (E) serum corticosterone levels were determined at three hours after treatment. N.S: not significant. Data were expressed as mean ± SEM (n=4).
Supplemental Figure 5: Central administration of rmFGF21 has no effects on hepatic noradrenaline and adrenaline, plasma ketone body and expression of several genes involved in hepatic ketogenesis and lipid metabolism. 10-week-old male FGF21 KO mice were treated with rmFGF21 (0.02µg/g) or vehicle by intracerebroventricular (icv) injection. WT mice treated with vehicle were used as control. (A) noradrenaline levels and (B) adrenaline levels in livers. (C) Serum levels of β-hydroxybutyrate. (D and E) The hepatic mRNA abundance of ketogenic genes including peroxisome proliferator activated receptor alpha (Ppara), acyl-CoA oxidase 1 (Acox1), carnitine palmitoyl transferase 1 (Cpt1) and 3-hydroxy-3-methylglutaryl CoA synthase 2 (Hmgcs2); Genes involved in regulation of hepatic lipid metabolism, including lipoprotein lipase (Lpl), pancreatic lipase (Pnlip), pancreatic lipase-related protein 2 (Pnliprp2) and carboxyl ester lipase (Cel) were determined by real-time PCR. Data were expressed as mean ± SEM. * p<0.05, (n=6).
Supplemental Figure 6: FGF21 activates the ERK1/2-CREB signaling pathway to stimulate CRH production. The protocol of treatment for mice is the same as described in Figure 6. Mouse hypothalamic slices (paraventricular nucleus, adjacent to the third ventricle) were immunostained for p-ERK1/2, p-CREB and CRH as indicated. The samples were taken at 30 minutes after the injection of rmFGF21 for p-ERK1/2 and p-CREB, and 3 hours for CRH. Representative images of immunostained slices are shown, n=6.