Central IGF1 improves glucose tolerance and insulin sensitivity in mice

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Abstract
Insulin-like growth factor 1 (IGF1) is a key factor for tissue growth and fuel metabolism. The potential function of central IGF1 remains unclear. We previously observed that IGF1 expression is increased in the hypothalamus of obese mice lacking STAT5 in the central nervous system (CNS). In this study, we explored the potential metabolic function of central IGF1 by intracerebroventricular (ICV) injection of IGF1, over-expression of central IGF1 by administering an adeno-associated virus (AAV), and ICV injection of an anti-IGF1 antibody. Mice that over-expressed central IGF1 displayed increased appetite, improved glucose tolerance and insulin sensitivity, decreased Pomc levels in the hypothalamus, and increased UCP1 expression in brown fat tissue. This is the first study demonstrating that central IGF1 regulates several important metabolic functions.

Introduction
Insulin-like growth factors (IGFs) regulate a very large number of important physiological processes. Insulin-like growth factor 1 (IGF1) regulates somatomedin C, insulin-like growth factor 2 (IGF2) regulates a very large number of important physiological processes. IGF1 has multiple biological effects, including promoting cell growth and proliferation and regulating fuel metabolism peripherally. IGF1, which acts through IGF1 receptors and/or hybrid insulin/IGF1 receptors, has significant amino acid sequence homology with insulin, and enhances insulin sensitivity in both animal models and human subjects. IGF1 is primarily secreted by the liver and mediates the endocrine actions of growth hormone (GH). GH, the main regulator of circulating IGF1 level in mammals, has an intricate regulatory relationship with IGF1. Mice lacking IGF1 in the liver have shown to display enhanced insulin sensitivity and glucose homeostasis. A recent study has found that IGF1 is produced by all cell types in the brain. IGF1 plays an important role in childhood growth and continues to have a role in brain growth hormone (GH). GH, the main regulator of IGF1 production, can be retarded by under-nutrition, growth hormone insensitivity, lack of growth hormone receptors, or failure of downstream signaling pathways including SHP2 and STAT5B (signal transducer and activator of transcription 5B). Low serum IGF1 levels are associated with reduced insulin sensitivity, metabolic syndrome, glucose intolerance, and the development of type 2 diabetes.

The potential role of central IGF1 in regulating whole body energy homeostasis has not been investigated. To address this issue, we sought to study the effects of central IGF1 in mice. Mice that lack Stat5a/b in the central nervous system (Stat5NKO mice) develop severe obesity, accompanied by hyperphagia, hyperleptinemia, impaired thermal response to the cold, and insulin resistance. We recently completed RNA-seq studies showing increased expression of members of the Igf family, including Igf1, Igf2, Igfals, and Igfbps, in the hypothalamus of Stat5NKO mice. Igf1 expression was very low in wild-type mice but elevated 8-fold in the Stat5NKO mice (Figure S1A). To determine the potential function of central IGF1 in energy metabolism, we subjected wild-type mice to ICV injection of IGF1...
injections of IGF1 and an anti-IGF1 antibody. We found that IGF1 increased food intake while the anti-IGF1 antibody decreased appetite. We also over-expressed IGF1 long-term in the brain of wild-type mice by administering ICV injections of a recombinant adenovirus 2 (AAV2). The phenotypes that we observed with the virus-treated mice were similar to those observed after acute ICV IGF1 injection.

Materials and methods

Generation of the IGF1 over-expression construct

The AAV2 construct encoding Igf1 (AAV-Igf1; Figure S2A) was generated by using a genomic 7.4 kb fragment containing all Igf1 exon sequences of the murine Igf1 gene. The flag-tag followed the IGF1-promoter fragment after the restriction enzyme was cut. AAV packaging was performed by the University of Pennsylvania Vector Core (Lot: V4738MI-S).

Animals and metabolic measurements

Male C57BL/6J mice (8–12 weeks old) were handled and housed in the Experimental Animal Center, located in building 14C at the National Institutes of Health, Bethesda, Maryland. This study was approved by the Institutional Animal Care and Use Committee of National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, K005-LBC-15), and all procedures were conducted in accordance with the guidelines of the National Institute of Health Animal Care and Use Committee. Mice were randomly (using a computerized, random number generator through the block-randomization method of Statistics Analysis System version 9.2.) divided into six groups: saline and IGF1 group, saline and anti-IGF1 group, AAV-empty and AAV-Igf1 injection group. Food intake (averaged per day), and body weight were measured daily after injection in each group. A total number of animals should be able to show a desired effect as few or many as possible (five animals in each group is required at least 11). As our research was a pilot study, so the number of animals should be able to show a desired effect as few or many as possible (five animals in each group is required at least 11). As our research was a pilot study, so the number of animals used in experiment from U.S. law and animal experimenta tion: 10 mice were included in each group for our experiment to avoid unnecessary suffer. In vivo metabolic tests were performed using standard procedures. To measure glucose tolerance (IGTT), mice were fasted overnight for 12 h, and blood was collected from the tail vein immediately before and 15, 30, 60, 90, and 120 min after i.p. injection of glucose (2g/kg) to determine blood glucose concentrations. To obtain a measure of peripheral insulin sensitivity (ITT), mice were fasted for 4 h, and blood glucose levels were measured before and at the indicated time points after i.p. injection of human insulin (0.75 U/kg). Blood glucose levels were determined using an automated blood glucose reader (Glucometer Elite Sensor; Bayer). Plasma insulin concentrations were determined by using an ELISA kit (Crystal Chem Inc. Cat. No.:#90080).

AAV-Igf1, IGF1 & anti-IGF1 injection

Pre-experiments were carried out for each injection before the formal study. Only those mice that recovered from the surgery could be allocated to experimental groups, others were euthanized according to the guidelines of the National Institute of Health Animal Care and Use Committee.

Mice were injected with 2 ul (1 ug/ml) of IGF1 (Pepro tech, Catalog #:100-11) or an anti-IGF1 antibody (Abcam, ab9572), or with 1011 GC/mouse of the AAV-Igf1 virus into the arcuate nucleus of the hypothalamus (ARC). Following injections, mice were individually housed, and body weight and food intake were determined as described above.

Mice were anesthetized with ketamine and xylazine (100–200 mg/kg and 5–10 mg/kg, respectively, given i.p.) and placed on a heating pad during anesthesia, surgery, and recovery. The mice were placed in a small-animal stereotactic instrument (head held with ear bars and incisor holder). The surface of the skull were exposed with sterile surgical instruments then opened with a small drill over the appropriate stereotaxic coordinates. The dura was opened with a fine needle. A 30 gauge stainless steel needle attached to a 5-ul Hamilton syringe was lowered into the burr hole and 500 to 2 ul of the preparation was administered over 3–5 min per site (ARC). The animal was returned to its cage and monitored carefully twice daily for the first 48 h. Post-operative analgesia consisted of ketoprofen (5 mg/kg i.m. or s.c.) given every 24 h. A plastic cap was placed at the top of the cannula and removed for each injection after a 1-week recovery.

Brain immunofluorescence staining and liver histology

Twelve days after injection, the mice brains were collected and stored in 4% PFA overnight. Brains were sectioned at 40 um in thickness with Leica VT1000S. Sections were collected for the entire hypothalamus. Three sets of brain sections were stored at 4 °C in paraformaldehyde/phosphate-buffered saline (PBS). Flag antibodies: flag (F1804 sigma, 1:300, Secondary antibodies: Life Technology, A11017 Goat anti mouse 488; NeuN (MAB377, 1:1000); Secondary antibodies: Life technology, A11020, Goat anti Mouse 594; GFAP(C9205, 1:1000), Fluor conjugated 594 antibody, Igf1(abcam ab9572, 1:1000), secondary antibody Life technology, A11008, (1:1000)), Goat anti rabbit 488, and abcam Ab150064 (1:1000), donkey anti rabbit 594. Data analysis: fluorescent images were taken with NIDDK ALMIAC Keyence Digital Microscope and captured under exactly the same
condition for both AAV-GFP (empty) control virus-injected and AAV-Igf1 virus-injected mice. Fiji free software (formerly image J, NIH) and Fiji program were used to run “MeanSigPerPixel_ROI-bg.ijm” Macro (For details, see word format text in 4th supplementary Material). Images were processed as original TIFF images after background deduction. Average total pixels were obtained for AAV-GFP (empty) control images and AAV-Igf1 images.

Mouse liver was harvested and fixed in 4% paraformaldehyde/phosphate-buffered saline for overnight and embedded in paraffin. For each liver, 5 um-thick sections were mounted on slides and stained with hematoxylin and eosin (HE) staining. Image acquisitions were performed using a BZ-II Viewer.

**RNA and protein analyses**

Total RNA was isolated using Trizol reagent (Cat. No.: 15596018) and QIAGEN RNase kit (Cat. No.: 74104). RNA concentrations were determined by a Qubit bioanalyzer (Qubit2.0, Invitrogen, Q32866) prior to real-time PCR for gene expression. Western blotting was carried out with lysates prepared from isolated mouse liver or BAT by using standard techniques. Protein bands were quantitated using Image J software. The primers and antibodies used are listed below (See supplemental Tables 1 & 2).

**Measurement of hepatic glycogen and triglyceride levels**

Liver samples were homogenized in PBS, and glycogen and triglyceride contents were detected by using commercially available kits (Sigma; Cat. No.: MAK016 and

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**Fig. 1 Immunostaining to detect AAV-GFP(empty) and AAV-IGF1 expression.**

- **A.** Representative immunofluorescence staining of GFP fluorescence and IGF1 to show viral expression in the hypothalamus of AAV-GFP (empty) injected mice.
- **B.** Representative immunofluorescence staining to detect IGF1 expression and GFAP (Glial marker) expression in the hypothalamus of AAV-Igf1 injected mice.
- **C.** Representative immunofluorescence staining to determine IGF1 and NeuN (neuron marker) expression in the hypothalamus of AAV-Igf1 injected mice. Scale bar indicates 100 um, n = 10.
Cat. No.: TR0100, respectively), strictly following the manufacturer’s instructions.

**Statistical analysis**

Statistical analyses were performed using SPSS 18.0. Multiple group comparisons were made with ANOVA, followed by the Tukey HSD test for multiple comparisons. Comparisons between two groups were performed using unpaired 2-tailed Student’s t test, and rank sum test was used for more than two time points. Data are shown as means ± SD, \( P < 0.05 \) was considered statistically significant.

**Results**

**Hypothalamic IGF1 expression is increased in obese mice**

We first examined IGF1 protein expression in the hypothalamus of the Stat5NKO mice. We found a higher IGF1 expression in Stat5NKO mice compared to their Stat5fl/fl lean control littermates (Figure S1B). To test the hypothesis that IGF1 expression is increased in other obese mouse-models, we also studied mice that had been maintained on a high-fat diet. In this model of diet-induced obesity (DIO), hypothalamic IGF1 expression was also significantly increased, compared to mice fed a standard chow diet (CD) (Figure S1C).

**Over-expression efficiency of Igf1 in the arcuate nucleus of hypothalamus**

After injecting recombinant AAV virus into the arcuate nucleus (ARC, \( 10^{11} \) genome copies/mouse) of wild-type mice, IGF1 over-expression in the ARC was confirmed via immunofluorescence (Fig. 1a-c). Both A and B are stained sections of the arcuate nucleus to show virus expression through GFP fluorescence and IGF1 antibody staining in AAV-GFP (empty) injected mice (Fig. 1a). We then applied flag tag antibody-staining to show AAV-Igf1 virus-
expression after AAV-Igf1 injection into the mouse brain. Both IGFl and GFAP (Gial marker) expressions were detected via immunostaining in AAV-Igf1-injected mice. The stained astrocytes showed only GFAP expression, and no IGFl (Fig. 1b). Furthermore, both IGFl and NeuN (neuron marker) expression in AAV-Igf1-injected mice were observed via immunostaining (Fig. 1c). These results showed that most IGFl-positive cells were NeuN-positive as well, indicating the expression of injected AAV-Igf1 in neurons. We also examined for IGFl over-expression efficiency after AAV-GFP and AAV-Igf1 virus injection in the hypothalamus of the mice using immunostaining and western blot. Our experiment showed that IGFl expression increased significantly in the hypothalamus of AAV-Igf1 mice than that in the AAV-GFP-injected mice. Total pixel-calculation that followed also verified that IGFl-expression level was higher in the AAV-Igf1 group than in the AAV-GFP group (Figs. 2a, b, Figure S2B). These results indicated that AAV-Igf1 injected mice had increased IGFl expression in the brain, indicating a successful IGFl over-expression model.

Central IGFl increases food intake

To explore the potential function of central IGFl, we injected IGFl or an anti-IGFl antibody into the lateral cerebral ventricle (ICV) of wild-type mice. The IGFl-injected mice displayed a significant increase in food intake (Fig. 3a), where as the mice injected with the anti-IGFl antibody showed reduced food intake (Fig. 3b). Neither treatment affected body weight during the 48-hour observation period (Figs. 3c, d). To study the effects of long-term IGFl over-expression in the brain, we injected the wild-type mice with a recombinant AAV coding for IGFl. Specifically, the virus was injected into the ARC, which plays a key role in the central regulation of food intake and energy homeostasis. We found that the virus-treated mice displayed enhanced appetite but unchanged body weight (Figs. 3e, f), mimicking the effects of acute central IGFl administration.

Central IGFl improves glucose homeostasis and insulin sensitivity

We then measured serum insulin and blood glucose levels in mice fasted for 4 or 16 (o/n) hours. We found that acute central IGFl treatment (Figs. 4a, b) or prolonged IGFl over-expression in the ARC (Figs. 4c, d) significantly increased serum insulin and reduced blood glucose levels. Glucose and insulin tolerance tests showed that both treatments led to improved glucose tolerance (Figs. 4e, f) and enhanced insulin sensitivity (Figs. 4g, h). In contrast, ICV injection of the anti-IGFl antibody caused decreased serum insulin and elevated blood glucose levels (Figs. 4i, j). Somewhat surprisingly, these mice displayed normal glucose tolerance and insulin sensitivity (Figs. 4k, l).

To explore the mechanism by which central IGFl improved glucose homeostasis and insulin sensitivity, we examined the levels of Akt and the insulin receptor beta subunit (ISR-beta) in the liver, which are predicted to play a key role in IGFl signaling12-14. Akt activation is associated with its auto-phosphorylation at Thr-47315. Western blotting studies demonstrated that the abundance of total ISR-beta and Akt did not differ between control and AAV-Igf1 mice, whereas the expressions of the phosphorylated (active) forms of Akt and ISR-beta were significantly increased in the AAV-Igf1 mice (Figs. 4m, n). These results provide further evidence that enhanced central IGFl is critical for insulin responsiveness through activation of Akt and ISR-beta.

The presence of UCP1 in brown adipose tissue (BAT) directs its oxidative metabolism almost entirely to thermogenesis16, 17. UCP1/BAT activity is a well-known regulator of energy homeostasis. Western blotting studies showed that UCP1 expression in BAT from AAV-Igf1 mice was significantly increased, compared to control mice (mice injected with empty AAV) (Fig. 4o, p). Strong neuroanatomical and functional evidence has shown that WAT and BAT are innervated and regulated by the sympathetic nervous system (SNS) for thermoregulation and beige adipocyte formation18. Since UCP1 is the hallmark protein responsible for cold- and diet-induced thermogenesis in BAT19, its expression can also be regulated by SNS activity. These data suggest that central IGFl action enhances central sympathetic outflow.

Central IGFl reduces liver weight and lowers triglyceride and glycogen levels

We also examined the effects of long-term central IGFl over-expression on liver weight and triglyceride and glycogen levels, and found that liver weight and hepatic triglyceride and glycogen levels were significantly reduced in AAV-Igf1 mice, compared to control mice (Figs. 5a, c). Liver morphology did not differ between AAV-Igf1 and control mice (Fig. 5b). These results suggest that central IGFl signaling promotes glucose and lipid metabolism in the liver. This observation is consistent with the concept that central IGFl enhances stimulates sympathetic outflow (see previous paragraph).

Effects of IGFl on gene expression of Pomc, Npy, Agrp in the hypothalamus

The ARC is a key hypothalamic nucleus in the regulation of appetite20, 21. Although the ARC contains other cell types, two main opposing types of output neurons have been defined: those expressing pro-opiomelanocortin (Pomc; Pomc neurons) and those containing Agouti-related protein (AgRP) and
neuropeptide-Y (NPY) (AgRP neurons). The Pomp neurons release α-melanocyte stimulating factor (α-MSH), which collectively promotes activity and energy expenditure, and suppresses food intake. AgRP and NPY are neuropeptides that suppress energy use and promote food intake. To study whether central IGF1 modulates the expression of these appetite-regulating peptides, we used qRT-PCR to measure their expression levels after central administration of IGF1 or an anti-IGF1 antibody, or after long-term IGF1 expression in AAV-Igf1 mice. Acute IGF1 injection and long-term over-expression of IGF1 in the mouse brain did not affect Agrp and Npy expression, but significantly reduced Pomc expression. In contrast, central administration of the anti-IGF1 antibody increased Pomc expression, but did not change Agrp and Npy expression. It is therefore likely that the orexigenic effect observed after central IGF1 administration or over-expression is caused, at least partially, by a reduced Pomc expression.

**Discussion**

The central nervous system plays an important role in the development of obesity. Loss of cytokine-STAT5 signaling in the CNS and pituitary gland alters energy balance and leads to obesity in mice. We recently identified a large number of genes with altered expression levels in the hypothalamus of Stat5NKO mice (unpublished data). Surprisingly, in the hypothalamus of Stat5NKO mice, most members of the Igf1 family were found to be up-regulated. STAT5 has been shown to positively regulate IGF1 function in peripheral organs, such as skeletal muscle and liver. Some studies showed that modulating IGF1 has a dramatic effect on glucose metabolism in mice under both normal and high-fat diet feeding. For example, whole body over-expression of IGFBP1 effectively reduced the hypoglycemic effect of endogenous IGF1 and caused elevated plasma insulin levels, yet reduced insulin-stimulated glucose transport and glycogen synthesis in skeletal muscle, indicative of...
Fig. 4 (See legend on next page.)
mild insulin resistance. Pituitary-derived GH exerts its growth effects primarily by regulating the expression of IGF-I in both hepatic and non-hepatic tissues. GH’s action is initiated upon binding to the cell-surface GH receptor (GHR), a homodimeric transmembrane protein. The GH-GHR interaction induces signal transduction through recruitment and activation of the cytosolic Janus kinase 2 (JAK2). The activated complex of signaling pathways includes four STAT pathways (STAT1, 3, 5a, and 5b), the MAPK (mitogen-activated protein kinase), and the PI3K (phosphoinositide-3 kinase) pathways. Activation of these pathways culminates in the regulation of multiple genes, including IGF1, IGFBP3, and IGFALS (acid labile subunit, ALS). One study has reported that brain-specific over-expression of IGFBP-6 under the control of glial fibrillary acidic protein promoter causes glucose intolerance, insulin resistance, and elevated weight gain both on a normal diet and on a high-fat diet. Reduction of IGF1 signaling by inactivating the IGF1R may also contribute to weight gain, glucose intolerance, and insulin resistance over time.

In this study, we describe the metabolic phenotypes of IGF1- and IGF1 antibody-treated mice. Gain of function studies (ICV injection of IGF1 or AAV-mediated over-expression of IGF1) as well as loss of function experiments (ICV injection of an anti-IGF1 antibody) demonstrated that central IGF1 promoted food intake and insulin secretion, reduced blood glucose levels, and led to improved glucose tolerance (Fig. 4). Moreover, over-expression of IGF1 in the brain led to enhanced phosphorylation/activation of the insulin receptor (IRb subunit) and AKT, a key mediator of signaling pathways stimulated by insulin-like peptides. Central IGF1 treatment increased central p-Akt, p-IR, and p-akt, which are downstream effectors of IGF1 signaling.

In mice, systemic IGF1 overexpression increases the expression of UCP1 in BAT, suggesting a role for IGF1 in the regulation of energy expenditure. Consistent with this notion, we also observed that central IGF1 can promote BAT activity and enhance energy expenditure. Since BAT activity is primarily under the control of the sympathetic nervous system, central IGF1 activity likely leads to an increase in central sympathetic outflow. This suggests a potential role for central IGF1 in the hypothalamus of mice.
observed reduced hepatic triglyceride and glycogen levels (Fig. 5a-c). To the best of our knowledge, this is the first study that directly examined the role of central IGF1 in regulating lipid and glucose metabolism.

The regulation of energy homeostasis is closely controlled by the central nervous system and integrated signals from the periphery, includes insulin signaling\(^5\). Insulin acts on ARC neurons to inhibit the expression of the orexigenic neuropeptides NPY and AgRP and to enhance the expression of Pomc, an appetite-suppressing peptide, resulting in reduced food intake\(^38\--41\). In contrast, we observed in the present study that over-expression of IGF1 in the ARC promoted food consumption, most likely due to an increase in Pomc expression. The receptors and neuronal pathways involved in this phenotype will be explored in the future studies. Meanwhile, the autonomic nervous system also plays a key role in the response to these signals, innervating peripheral metabolic tissues, including BAT, WAT, liver, and skeletal muscle\(^42\). Taking together, these new data shed light onto the role of central IGF1. In particular, the reported metabolic effects in BAT and in liver, caused by AAV-induced expression in brain of Igf1, are attributed mainly to a direct effect of IGF1 on neurons, and then mediated by the sympathetic nervous system. This is however highly speculative, and more experiments are required to justify this hypothesis.

Nevertheless, some limitations should be acknowledged in the interpretation of our results. One is that we only used single IGF1 and anti-IGF1 antibody doses. In addition, the detailed neuronal/cellular pathways through which central IGF1 exerts its metabolic actions remain to be explored. Also, the potential ICV IGF1 actions on other (non-hypothalamic) brain regions regulating food-intake need to be studied further. IGF2, which has similar homology with IGF1, is primarily secreted by the liver and in utero by the placenta. Igf2 has been reported to play an important role in the subventricular zone and in the subgranular zone of the hippocampus for adult-neurogenesis in mice\(^43\). Moreover, over-expression of Igf2 is associated with the intrauterine programming of adipose tissue and fetal overgrowth\(^44\). Current data about IG2 and obesity is both limited and conflicting, so the potential role of central IGF2 in regulating energy and glucose homeostasis also need to be further elucidated. Nevertheless, our findings may guide future efforts to develop novel classes of drugs useful for the treatment of obesity and diabetes.

**Conclusion**

In the present study, we systematically explored the role of central IGF1. We found that central IGF1 can promote feeding, improve insulin sensitivity and glucose tolerance, and stimulate energy expenditure via thermogenesis.

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**Authors’ contributions**

HH did experiments, analyzed data and wrote the manuscript; ZYC allocated experimental groups, performed the IGF1, anti-IGF1 and virus injections, and IF staining analysis; L2 helped generate the AAV construct; SPF did data analysis; YHC and MR helped with the physiological studies; BMZ guided the experiments, wrote and revised the manuscript.

**Competing interests**

The authors declare that they have no competing financial interests.
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