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(Citation)
Neuroscience Letters,768:136362

(Issue Date)
2022-01-18

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90008855

Comparison of the effects of intracerebroventricular administration of glucagon-like peptides 1 and 2 on hypothalamic appetite regulating factors and sleep-like behavior in chicks

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Abstract

Glucagon-like peptide (GLP)-1 and GLP-2, proglucagon-derived brain-gut peptides, function as anorexigenic neuropeptides in mammals. We previously showed that central administration of GLP-1 and GLP-2 potently suppressed food intake in chicks. GLP-1 and GLP-2 specifically activate their receptors GLP-1 receptor (GLP1R) and GLP-2 receptor (GLP2R), respectively in chickens. In adult chickens, GLP1R and GLP2R are expressed in different brain regions. These findings raise the hypothesis that both GLP-1 and GLP-2 function as anorexigenic peptides in the chicken brain but the mechanisms underlying the anorexigenic effects are different between them. In the present study, we compared several aspects of GLP-1 and GLP-2 in chicks. GLP1R mRNA levels in the brain stem and optic lobes were significantly higher than in other parts of the brain, whereas GLP2R mRNA was densely expressed in the telencephalon. Intracerebroventricular administration of either GLP-1 or GLP-2 significantly reduced the mRNA levels of corticotrophin releasing factor and AMP-kinase (AMPK) α1. The mRNA level of proopiomelanocortin was significantly increased, and those of AMPKα2 and GLP2R were significantly decreased by GLP-2, whereas the mRNA level of pyruvate dehydrogenase kinase 4 was significantly increased, and that of GLP1R was significantly decreased by GLP-1. Intracerebroventricular administration of either GLP-1 or GLP-2 induced sleep-like behavior in chicks. Our findings suggest that the anorexigenic peptides GLP-1 and GLP-2 induce similar behavioral changes in chicks, but the mechanism may differ between them.

Keywords: appetite, brain, chicken, feed intake, posture, sleep

1. Introduction

Glucagon-like peptide (GLP)-1 and GLP-2, proglucagon-derived brain-gut peptides, function as anorexigenic neuropeptides in mammals and chickens. For example, central administration of GLP-1 and GLP-2 suppressed food intake in rats [1, 2] and chicks [3]. GLP-1 and GLP-2-producing neurons in the nucleus of the solitary tract (NTS) in the medulla oblongata project to several food-regulating areas in the hypothalamus [4, 5]. We previously showed that proglucagon mRNA levels in the chicken medulla oblongata were reduced by fasting [6]. mRNA levels of the GLP-1 receptor (GLP1R) [7] and GLP-2 receptor (GLP2R) [8] were distributed in different brain regions in adult chickens. GLP-1 and GLP-2 specifically activate GLP1R and GLP2R, respectively, in chickens [7, 8]. These findings raise the hypothesis that both GLP-1 and GLP-2 function as anorexigenic peptides in the chicken brain but the mechanisms underlying the anorexigenic effects are different between them.

In mammals, AMP-kinase (AMPK) regulates feeding behavior by modulating orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and anorexigenic proopiomelanocortin (POMC) neurons [9]. AMPK activity was reduced by re-feeding in the mouse hypothalamus [10]. Central GLP1R activation suppresses food intake via inhibition of hypothalamic AMPK [11]. Hypothalamic POMC neurons are involved in anorexigenic pathways induced by GLP-1 [4] and GLP-2 [5]. It is therefore likely that AMPK and POMC are involved in the GLPs-induced anorexigenic pathway in the hypothalamus in mammals.

In chickens, fasting induced phosphorylation of AMPKα in the hypothalamus [12]. POMC neurons play important roles in the central regulation of food intake [13]. Tachibana et al. suggested that the anorexigenic effects of GLP-1 are mediated by CRF in chicks [14]. Fang et al. [16] proposed that hypothalamic pyruvate dehydrogenase kinase 4 (PDK4) is the key factor in appetite regulation in chicks and possibly influences the gene expression of NPY, AgRP, and POMC. It is therefore possible that AMPK, POMC, CRF, and PDK4 in the hypothalamus are involved in the anorexigenic pathway of GLPs in chicks.

Bungo et al. [17] reported that ICV administration of GLP-1 induced sleep-like behavior in chicks. They also showed that ICV administration of fusaric acid, an inhibitor of a noradrenaline synthesis rate limiting enzyme, attenuated the anorexigenic effects of GLP-1 in chicks [18]. In addition, noradrenaline induces sleep-like behavior and suppresses food intake in chicks [19]. All these findings suggest interactive relationships between GLP-1-induced sleep-like behavior and anorexia in chicks. However, the effects of GLP-2 on sleep-like behavior have not yet been examined.

In the present study, we compared the distribution of the GLP1R and GLP2R in the chicken brain and the effects of GLP-1 and GLP-2 on hypothalamic appetite regulating factors and sleep-like behavior in chicks. Our findings suggest that GLP-1 and GLP-2 are produced in the same region in the chicken brain, but the mechanism underlying GLP-1 and GLP-2-induced behavioral changes may differ between them.

2. Materials and methods

2.1 Animals and diet

This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation Regulations (24-03-06, 26-09-07). One day old male layers (White leghorn, Julia strain) were purchased from local hatcheries (Japan Layer K. K., Gifu, Japan). Fifty chicks were reared in electrically heated battery cages (1,725 mm×850 mm×320 mm). They were given free access to water in four waterers (ϕ 170 mm×140 mm) and a commercial chick starter diet (NICHIWA SANGYO Co., Ltd., Kobe, Japan) in four feeders (75 mm×407 mm×49 mm) under a 23-h/1-h light - dark cycle (23:00-24:00 dark). The temperature was kept at 31 ± 2℃. We previously investigated the effects of ICV administration of GLPs on food intake in 8-day-old chicks, but GLP-1-induced sleep-like behavior has been reported only in 3-day-old chicks [17]. Therefore, distribution of GLPs-related genes in

the brain and the effects of GLPs on hypothalamic appetite-regulating factor were examined at 7 and 8 days of age, while the effects of GLPs on sleep-like behavior were examined at a younger age (4 days of age).

2.2 Experiment 1: Distribution of GLP1R, GLP2R, and proglucagon mRNA in the brains of chicks.

Four 7-day-old chicks were euthanized by decapitation by a skilled person at 13:00. The whole brains were divided into six regions: telencephalon, optic lobe, cerebellum, rostral part of the brainstem (diencephalon), middle part of the brainstem, and caudal part of the brainstem, as described previously [20]. Total RNA was extracted from the tissues using Sepazol-RNA-I Super G (Nakalai Tesque, Inc., Kyoto, Japan). Real-time PCR analysis was performed as described previously [21]. Complementary DNA of GLP1R (GenBank accession no. EU770586.1), GLP2R (GenBank accession no. FJ899744.1), and proglucagon (GenBank accession no. NM_205260.3) were amplified using the following primers: GLP1R sense, 5'-CCC CGC CAG GCG TAGT-3'; GLP1R antisense, 5'-GTA CTC CTT CCA CTT CTG CAC AAC-3'; GLP2R sense, 5'-TCT CGT CTG CGG GCA AGT-3'; GLP2R antisense, 5'-GAT CTT TTG AAA TAC TGT GGC TGT TG-3'; proglucagon sense, 5'-GCA CTA AAA GAA ATG GCC AAC AAG-3'; proglucagon antisense, 5'-GCT GAT CCG GGA ATT TGT CA-3'. Complementary DNA of ribosomal protein S17 (internal standard) was amplified using primers as described previously [20].

2.3 Experiment 2: Effects of central administration of GLP-1 and GLP-2 on the mRNA levels of hypothalamic appetite-regulating factors in chicks

We have already reported that ICV administration of either 30 pmol of GLP-1 or GLP-2 in chicks strongly suppressed food intake for 120 min after administration [3]. Therefore, we used a dose of 30 pmol in the present study. Forty-two 8-day-old chicks were weighed and allocated to three groups based on body weight (14 birds

in each group). Chicken GLP-1 and GLP-2 were dissolved in 0.85% (w/v) saline solution containing 0.1% (w/v) Evans Blue. The peptides were ICV-administered according to the method of Davis et al. [22] at a volume of 10 μl after three hours of fasting. Chicks were administered with either a vehicle (control), 30 pmol GLP-1, or 30 pmol GLP-2 at 13:00. At 60 minutes after the ICV injection, the chicks were euthanized by decapitation by a skilled person. Verification of injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. Samples from chicks without Evans Blue dye in the lateral ventricle were excluded. Diencephalons were collected and preserved in RNAlater tissue storage reagent (Sigma-Aldrich, St. Louis, Mo, USA) for 2 days. The hypothalamus was excised as described previously [23].

Real-time PCR analysis was performed as described in Experiment 1. Complementary DNA of CRF (GenBank accession no. NM_001123031.1), PDK4 (GenBank accession no. NM_001199909.1), AMPK α1 (GenBank accession no. DQ302133.1) and AMPK α2 (GenBank accession no. DQ340396.1) were amplified using the following primers: CRF sense, 5'-CAT CTC CCT GGA CCT GAC TTT C-3'; CRF antisense, 5'-CCG ATG ATT TCC ATC AGT TTC C-3'; PDK4 sense, 5'-AGTCTG CTT CCA AAC ATT ACC AAA C-3'; PDK4 antisense, 5'-CAG TCT GCT TTG GAC CTT TAC TTG-3'; AMPK α1 sense, 5'-CGG CGG CAG ATA AAC AGA A-3'; AMPK α1 antisense, 5'-CAG AAT GTA ATG CCC AAT CTT CAC-3'; AMPK α2 sense, 5'-CGC CTT TTC CAG CAG ATT CT-3'; AMPK α2 antisense, 5'-GAC AAC CAT GTG T CG GTG ACA-3'. Complementary DNA of NPY, AgRP, and POMC were amplified using primers as described previously [24]. Complementary DNA of GLP1R and GLP2R was amplified using primers as described in Experiment 1.

2.4 Experiment 3: Effects of central administration of GLP-1 and GLP-2 on AMPK protein levels in the chick hypothalamus

Either a vehicle, 30 pmol GLP-1, or 30 pmol GLP-2 was administered as described in Experiment 2. At 60 minutes after administration, the chicks were euthanized by decapitation by a skilled person, and the

hypothalamus was excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

Western blot analysis was performed as described previously [24]. Anti-AMPK (#2532), anti-phospho-AMPK (pAMPK) (Thr172) (#2531), anti-β-actin (#8457), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) were purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-β-actin antibody was used to detect a loading control.

2.5 *Experiment 4: Effects of central administration of GLP-1 and GLP-2 on sleep-like behavior in chicks.*

Forty-two 4-day-old chicks were weighed and allocated to three groups based on body weight (14 birds in each group). Either a vehicle, 30 pmol GLP-1, or 30 pmol GLP-2 was administered at 13:00 as described in Experiment 2. The postures were observed at 30 minutes after the ICV injection. As described in the previous study [17], four postures were categorized: (1) active wakefulness; (2) standing/sitting with eyes open; (3) standing motionless with eyes closed; (4) sitting motionless with head drooped (sleeping posture). After observation, chicks were euthanized by decapitation. Verification of injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. Data from chicks without Evans Blue dye in the lateral ventricle were excluded.

2.6 Data analysis

Data from Experiment 1 were analyzed by the Tukey-Kramer test. Data from Experiments 2 and 3 were analyzed by one-way analysis of variance, and Fisher's protected least significant difference test. Data from Experiment 4 were analyzed by the Games-Howell test. All statistical analyses were performed using a commercial software package (StatView version 5, SAS Institute, Cary, NC, USA, 1998).

2. Results

GLP1R mRNA levels in the brain stem and optic lobes were significantly higher than in other parts of the brain (Fig. 1a), whereas GLP2R mRNA was densely expressed in the telencephalon (Fig. 1b). Proglucagon mRNA levels in the caudal part of the brain stem were significantly higher than in other parts of the brain (Fig. 1c).

As shown in Fig. 2, ICV administration of 30 pmol of either GLP-1 or 2 significantly reduced the mRNA levels of CRF and AMPKα1, whereas that of POMC was significantly increased, and those of AMPKα2 and GLP2R were significantly decreased by ICV injection of GLP-2, but not GLP-1. On the other hand, only GLP-1 significantly increased the mRNA level of PDK4 and decreased that of GLP1R. However, neither GLP-1 nor GLP-2 affected the protein levels or phosphorylation of AMPK (Supplementary file).

ICV administration of 30 pmol of either GLP-1 or 2 significantly induced sleep-like behavior in chicks, and there was no significant difference between the GLP-1 and GLP-2 groups (Fig. 3).

3. Discussion

In the present study, we showed different distributions of proglucagon, GLP1R, and GLP2R mRNAs in the chick brain. This is the first evidence showing statistical differences in the mRNA levels of these genes between brain parts. Kuenzel reported that three neural pathways, including the trigeminal sensorimotor system, visual system/basal ganglia pathway, and olfactory pathway, play different roles in controlling food intake in birds [28]. The telencephalon is involved in these pathways and optic lobes are involved in the visual system/basal ganglia pathway. In mammals, the NTS receives peripheral signals, such as vagal afferent activation, and GLPs producing neurons in the NTS convey these signals to several areas, including the hypothalamus, in mammals [4]. We demonstrated that the caudal part of the brain stem, which includes NTS, may be the major production area of GLP-1 and GLP-2 in chicken brains and various

expression sites of GLP1R and GLP2R mRNAs throughout the brain. These findings raise the hypothesis that GLPs in NTS may convey peripheral signals to several areas in the brain, which in turn results in anorexia in chickens as well as in mammals. Our findings suggest the importance of determining the distribution of GLP-producing neurons throughout the brain.

Guan reported that ICV administration of GLP-2 in mice significantly suppressed food intake and significantly increased POMC mRNA levels in the ARC in the hypothalamus [5]. In addition, the anorexigenic effects of GLP-2 were abolished in melanocortin receptor-4 knockout mice. They also found that POMC neuron-specific GLP2R knockout mice exhibited hyperphagia [5]. In the present study, we showed that ICV administration of GLP-2 significantly increased POMC mRNA levels in the hypothalamus, suggesting that POMC neurons are involved in the anorexigenic pathway of GLP-2 in chicks. We also showed that proglucagon mRNA levels are densely expressed in the caudal part of the brain stem. GLP-2 producing neurons in the NTS project to the hypothalamus and directly influence POMC neurons in mice [5]. Therefore, further study is needed to clarify whether GLP-2-producing neurons in the NTS directly project to the POMC neurons in the infundibular nucleus in the chicken hypothalamus.

ICV administration of GLP-1 and GLP-2 significantly reduced the mRNA levels of GLP1R and GLP2R, respectively, suggesting the desensitization of these receptors. It is well known that agonist exposure downregulates G protein coupled receptor activity via several mechanisms, including transcriptional regulation [29]. GLP-1 significantly suppresses GLP1R expression in rat insulinoma cells [30]. In the present study, ICV administration of GLP-1 and GLP-2 differently affected the mRNA levels of POMC and PDK4 in the hypothalamus. These findings suggest that both GLP-1 and GLP-2 regulate the transcription of different genes via their receptor.

Fang et al. [16] found that fasting increased the mRNA levels of PDK4 in the chicken hypothalamus. They also showed that ICV administration of alpha-lipoic acid, a PDK4 inhibitor, suppressed food intake in chicks [16]. These findings suggest that PDK4 functions as an orexigenic factor in chicks. In

the present study, GLP-1 upregulated the gene expression of PDK4. It is therefore likely that GLP-1-increased PDK4 mRNA is not the cause of the anorexigenic action of GLP-1. The mechanism underlying the upregulation of PDK4 expression in the hypothalamus in GLP-1 group is not clear. However, in the mammalian brain, glucocorticoids upregulate PDK4 transcription in astrocytes but not in neurons [31]. ICV administration of GLP-1 in chicks elevates plasma corticosterone at 30 min after administration [14], but not at 60 min [15]. Therefore, the elevation of the mRNA levels of PDK4 in the GLP-1 group may be due to the acute and temporal elevation of plasma corticosterone in chicks. It is not clear whether the appetite-regulating role of PDK4 is different between astrocytes and neurons. Further study are required to clarify the relationships among astrocytes, PDK4, and GLP-1-induced anorexia in chicks.

Previous studies suggest that AMPK is involved in the appetite regulating pathway in chicks. For example, central administration of ghrelin, an anorexigenic peptide in chickens, suppressed the phosphorylation of AMPK in both high and low weight strains of chicks [25]. Glucocorticoids cause hyperphagia via the AMPK-NPY signaling pathway [26]. However, in the present study, neither total AMPK nor phosphorylated AMPK were affected by GLPs, although hypothalamic AMPKα mRNA levels were reduced by them. It is therefore likely that AMPK may not play an important role in the anorexigenic action of GLPs in chicks, at least in this experimental condition. Further study is needed to clarify whether GLP-1 and GLP-2 regulate AMPK activity in each hypothalamic nucleus, which in turn regulates food intake in chickens.

In the present study, both 30 pmol of GLP-1 and GLP-2 induced sleep-like behavior in chicks. We previously showed that both 30 pmol of GLP-1 and GLP-2 potently suppressed food intake in chicks [5]. Sleep induction is one of the causes of food intake suppression of food intake in chicks [17]. These findings suggest that GLPs-induced sleep-like behavior is one of the causes of reduced food intake in chicks. GLP-1 induced sleep-like behavior may be induced by the noradrenergic system in chicks [17,19]. Interestingly, central administration of noradrenalin in chicks suppressed CRF-induced vocalization and locomotion and induced

sleep-like behavior [27]. Tachibana et al. reported that central administration of a CRF receptor antagonist in chicks attenuated the anorexigenic effects of GLP-1 at 30 min after injection [14]. However, in the present study, ICV administration of GLP-1 in chicks reduced the mRNA levels of CRF at 60 min after injection and induced sleep-like behavior. Thus, the acute anorexigenic effects of GLP-1 may be partly mediated by CRF, but noradrenergic system-induced sleep-like behavior plays a more important role in decreased food intake in chicks. Further study are required to clarify whether noradrenalin suppresses hypothalamic CRF expression in GLPs-injected chicks.

4. Conclusion

In the present study, we compared the effects of central administration of GLP-1 and GLP-2 on sleep-like behavior and appetite-regulating factors in the chick hypothalamus. Our findings suggest that both GLP-1 and GLP-2-induced behavioral changes in chicks may be expressed partly through different pathways.

Acknowledgments

The author Ahmed Kewan is funded by a full scholarship [PD27] from the Ministry of Higher Education of the Arab Republic of Egypt. This work was partly supported by JSPS KAKENHI (grant number 19K06353).

Author contributions

Ahmed Kewan: Conceptualization, Investigation, Writing-Original draft preparation, Methodology. Hitomi Shimatani: Investigation. Takaoki Saneyasu: Varidation, Writing-reviewing & editing. Hiroshi Kamisoyama: Funding acquisition, Writing-reviewing & editing, Kazuhisa Honda: Conceptualization,

Writing-reviewing & editing.

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Figure captions

Fig. 1. Distribution of GLP-1 receptor, GLP-2 receptor, and proglucagon mRNAs in the chick brain. Data represent means ± SEM of four birds. Groups with different letters are significantly different ($P < 0.05$).

Fig. 2. Effects of central administration of GLP-1 and GLP-2 on the mRNA levels of hypothalamic appetite-regulating factors in chicks. NPY, neuropeptide Y; POMC, proopiomelanocortin; AgRP, agouti-related protein; CRF, corticotrophin releasing factor; AMPKα1, AMP-activated protein kinase alpha 1;

AMPKα2, AMP-activated protein kinase alpha 2; PDK4, pyruvate dehydrogenase kinase; GLP1R, glucagon-like peptide-1 receptor; GLP2R, glucagon-like peptide-2 receptor. Data are the means ± S.E.M. of eight birds in each group and are expressed as a percentage of the mean in the control group. * Significant with respect to the control group ($P < 0.05$).

Fig. 3. Central administration of GLP-1 and GLP-2 on chick posture. Numbers of chicks were as follows: saline, 12; GLP-1, 11; GLP-2, 12. *Significance with respect to the control group ($P < 0.05$). (1) active wakefulness, (2) standing/sitting with eyes open, (3) standing motionless with eyes closed, (4) sitting motionless with head drooped.

Fig. 1

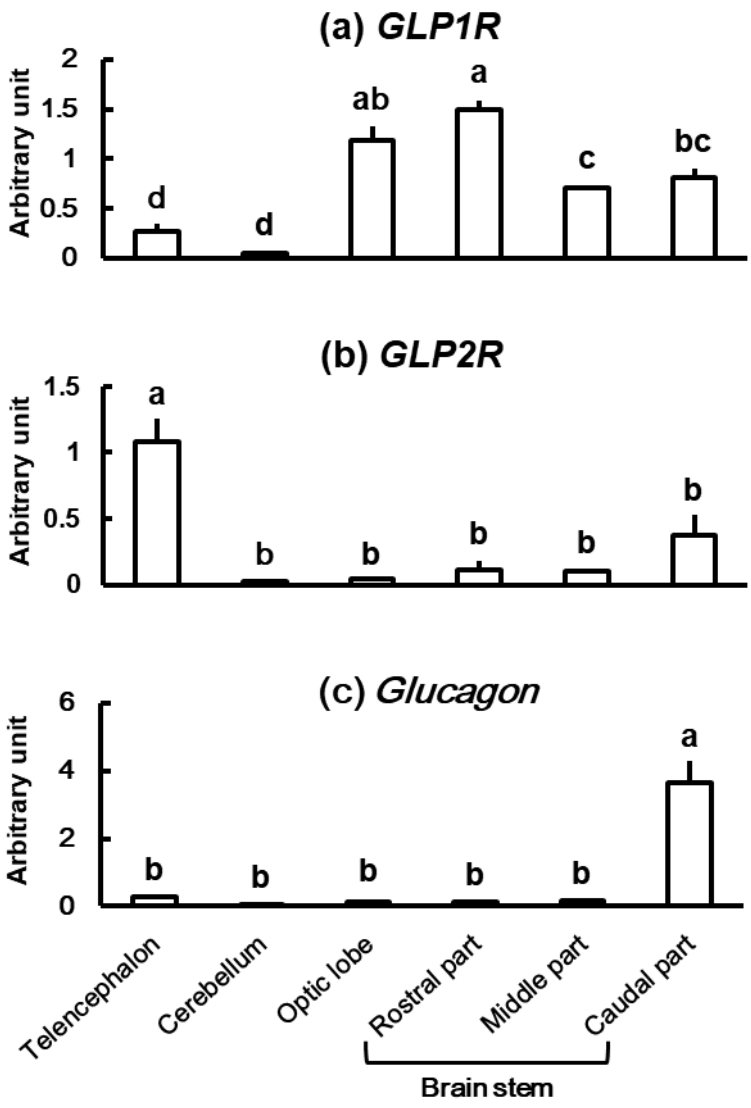
(a) GLP1R
Arbitrary unit
2
1.5
1
0.5
0
d
d
ab
a
c
bc
(b) GLP2R
Arbitrary unit
1.5
1
0.5
0
a
b
b
b
b
b
(c) Glucagon
Arbitrary unit
6
4
2
0
b
b
b
b
b
a
Telencephalon
Cerebellum
Optic lobe
Rostral part
Middle part
Caudal part
Brain stem

Fig. 2

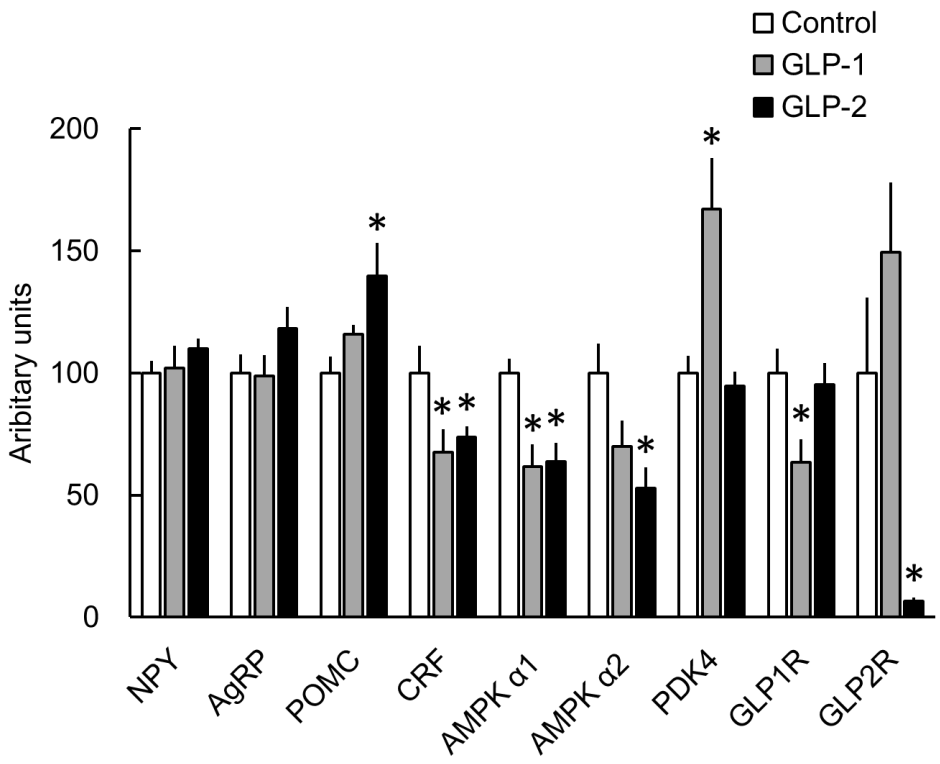
Control
GLP-1
GLP-2
Aribitary units
200
150
100
50
0
NPY
AgRP
POMC
CRF
AMPK α1
AMPK α2
PDK4
GLP1R
GLP2R

Fig. 3