GLP-2 decreases food intake in the dorsomedial hypothalamic nucleus (DMH) through Exendin (9-39) in male Sprague-Dawley (SD) rats

Huiling Sun[1, 2], Kai Meng[2], Lin Hou[2], Lijun Shang[3, *], Jianqun Yan[1, 2, *]

[1] Key Laboratory of Shaanxi Province for Craniofacial Precision Medicine Research, Research Center of Stomatology College, Xi'an Jiaotong University, 98 Xi Wu Road, Xi'an, Shaanxi, 710004, P.R.China; [2] Department of Physiology and Pathophysiology, Xi'an Jiaotong University Health Science Center, 76 West Yan Ta Road, Xi'an, Shaanxi, 710061, P.R.China; [3] School of Human Sciences, London Metropolitan University, London, N7 8BD, UK

*Corresponding authors: Lijun Shang, Tel: 00-44-20-71334805; Email: l.shang@londonmet.ac.uk; ORCID: 0000-0001-5925-5903; or Jianqun Yan, Tel/fax: +86-29-82655199; Email: jqyan@mail.xjtu.edu.cn

Abstract

Glucagon-like peptide 2 (GLP-2), a member of Glucagon peptide family involved in regulating energy metabolism, can be produced and secreted by preproglucagonergic (PPG) neurons in the brain. GLP-2 reduces food intake but at which brain sites GLP-2 exerts its feeding-suppress effects are still unclear. In this study, we used the stereological microinjection technique and behavioral test to examine the functions of locally delivered GLP-2 into DMH on feeding behavior. We compared effects of different concentration of GLP-2 on the food intake behavior in free-feeding rats and fasted-refeeding rats. We found that GLP-2 inhibited food intake in fasted rats after a short-term intervention in a dose-dependent manner. Importantly, the effects of locally delivered GLP-2 can be blocked by specific GLP-1 receptor antagonist Exendin$_{(9\text{-}39)}$, but not the melanocortin-4 receptor antagonist SHU9119, indicating the involvement of specificity of GLP-2 signaling in regulating the feeding behavior. Taken together, our data revealed that GLP-2 peptide pharmacologically inhibited food intake in DMH and this effect could be blocked functionally by Exendin$_{(9\text{-}39)}$.

Key Words: Microinjection, Glucagon-like peptide 2 (GLP-2), the dorsomedial hypothalamic nucleus (DMH), Sprague-Dawley (SD) rats, Stereotaxic surgery

Introduction

Gastric emptying is a critical process for the short-term control of food intake and might be a target for appetite modulation (Holst, 2007). Glucagon like peptide-2 (GLP-2), one of proglucagon-derived peptides, decreases gastric emptying (Wojdemann, *et al.*, 1998) and inhibits gastric fundic tone leading to increasing gastric capacity (Amato, *et al.*, 2009), and therefore plays important roles in the regulation of energy absorption and maintenance of mucosal morphology, function and integrity of the gut (Drucker & Yusta, 2014). Apart from secreted from enteroendocrine L-type cells of the gut, together with glucagon like peptide-1 (GLP-1) in response to dietary nutrients (Holst, 2007; Yusta *et al.*, 2000), GLP-2 is also released from preproglucagonergic (PPG) neurons in the nucleus tractus solitarius (NTS) and adjacent medial reticular nucleus of the brain stem (Kieffer & Habener, 1999; Vrang, *et al.*, 2007). The brainstem preproglucagon neurons project predominantly rostrally with main terminal fields in hypothalamic areas involved in food intake regulation including the hypothalamic paraventricular (PVN), dorsomedial (DMH) and arcuate (Arc) nuclei (Jin *et al.*, 1988; Larsen, *et al.*, 1997; Rinaman, 1999; Vrang *et al.*, 2007). Recent studies have revealed an intriguing complexity of the brainstem-hypothalamic preproglucagon system. Whereas the GLP-1 receptor mRNA is expressed in all hypothalamic areas receiving GLP-immunoreactive fibers (Merchenthaler, *et al.*, 1999), the GLP-2 receptor expression in the hypothalamus is confined to the compact part of the DMH (Tang-Christensen, *et al.*, 2000).

In line with the anatomical location of GLP-1 and GLP-2 receptors, it has been demonstrated that central administration *i.e.* lateral ventricular injection of either GLP-

1, GLP-2 or oxyntomodulin reduces food intake (Dakin *et al.*, 2001; Tang-Christensen *et al.*, 2000; Tang-Christensen, *et al.* 1998; Tang-Christensen *et al.*, 1996; Turton *et al.*, 1996). However, regarding at which brain sites GLP-2 exerts its feeding-suppress effects are still unclear. To further address this issue, microinjection experiments by which GLP-2 can be administered directly into brain tissue are required.

Our previous studies showed that GLP-2 microinjection into the solitary tract nucleus (NTS) suppressed food intake and this effect could be mediated by the GLP-1 receptor (unpublished data). There is a report that GLP-1 receptor antagonist, $Extendin_{(9-39)}$, can block the GLP-2 suppressed-effect on food intake through the mechanism of $Exendin_{(9-39)}$ acting as a functional GLP-2 receptor antagonist (Tang-Christensen *et al.*, 2000). In the present study, we discussed whether GLP-2 microinjection into DMH also has the feeding-suppress effects and the possible regulation mechanism of the endogenous melanocortin system in the DMH using the microinjection and behavioral methods in free-feeding and fasted-refeeding rats.

Materials and Methods

Animals

Forty-two Sprague-Dawley (SD) adult male rats weighed 270±20g were obtained from the Medical Experimental Animal Center of Xi'an Jiaotong University. They were housed individually in cages with free access to a standard chow diet and tap water at 25±1°C. All experiments and protocols were approved by the ethics committee, Xi'an Jiaotong University (No 2019-1153). All protocols followed the US National Institutes

of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, 1996). We randomly set up a free-feeding group and a fasted-refeeding group (10 rats per group). Meanwhile, another two groups (11 rats per group) were set up with injection of the antagonists, SHU9119 and Exendin$_{(9\text{-}39)}$. All rats were implanted unilaterally guide cannulas into the DMH (details below). After the surgery, rats were returned to the cages for one-week habituation and recovery till their weight to reached 270±20 g. The room lights were automatically controlled with 8:30 off and 20:30 on cycles for the free-feeding group, while 18:00 off and 6:00 on for the fasted-refeeding group. There was also an overnight 16 hours' food deprivation (16:30-8:30) for the fasted-refeeding group before the start of each experiment.

Stereotaxic surgery

Rats were anesthetized with pentobarbital sodium (50mg/kg, intraperitoneally, *i.p.*) and secured on a stereotaxic apparatus (SN-2N, Narishige Group, Tokyo, Japan). The unilateral guide cannulas (23 gauge) were implanted into the DMH. The stereotaxic coordinates of the NTS were determined according to the atlas of Paxinos and Watson Lam (Lam *et al.* 2010; Walton & Paxions. 2007). The detailed settings were 0.5mm lateral to the midline, 2.8mm posterior to bregma, and 6.6mm ventral from the skull surface. The tips of the cannulas were placed 2mm above the DMH. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. 30-Gauge metal obturators were used to fill the cannulas during the intervals of experiments between tests. All rats were injected with penicillin (20,000units, *i.p.*) during the first three post-operative days to prevent infection and to recover for at least 7 days before starting the

experiments.

Experimental details

The experiment scheme was shown in Fig. 1. Every effort was made to reduce animal discomfort and the number of animals used.

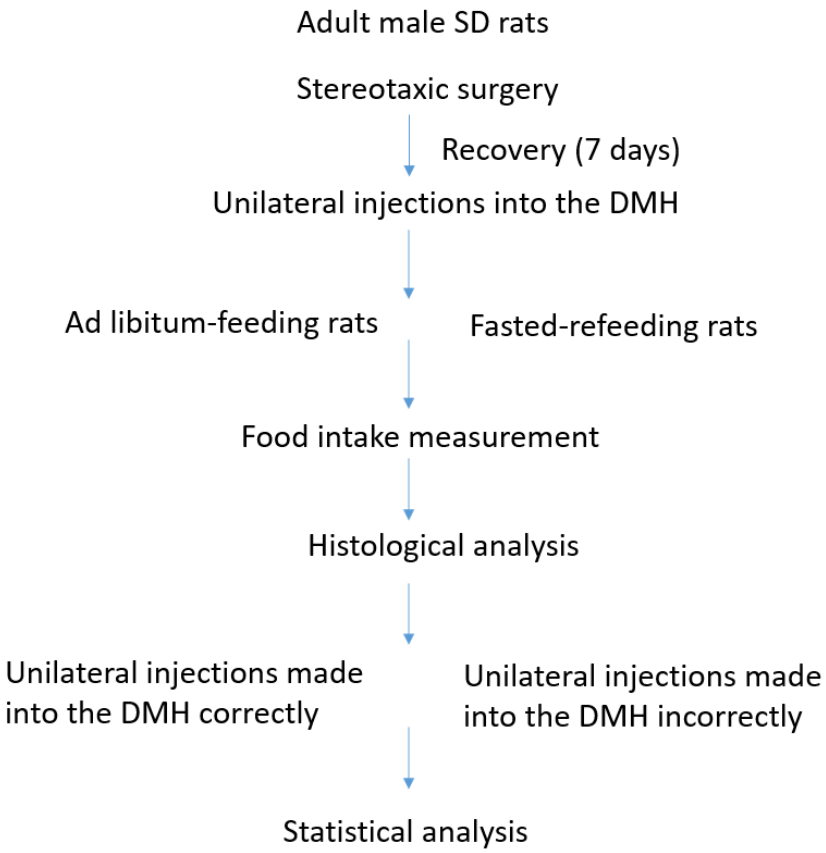


Fig. 1. The experiment scheme

A counter-balanced experiment design (Table 1) was employed to prevent any potential effect from the drug dose usages and the food intake measurement time points, *i.e.* each rat received equal treatments and served as their own control subjects. Each group in the design experiment accepted all the same procedures but carried out in different orders. GLP-2, SHU9119 and Exendin$_{(9\text{-}39)}$ in a series of dosage and with different

combinations were applied to test their effects on food intake on different feeding conditions. The free-feeding group and the fasted-refeeding group were injected into their DMH with three different doses of GLP-2 (1μg/0.5μl, 5μg/0.5μl, and 10μg/0.5μl) or vehicle (saline). In each group, 10 rats were subdivided into four subgroups of two or three rats each for the above four different dosage of GLP-2 injection, and each subgroup received these microinjections in a repeated-measures counter-balanced design. With this design, every rat received each dose in a non-sequential order, with a gap of 72h between the injections. Further experiments were designed in a similar way with another two groups of rats to test SHU9119 and Exendin$_{(9\text{-}39)}$ blocking effects on fasted-refeeding rats.

Table 1 Experimental details of groups, drug treatments and food intake measurements

Exps	Feeding condition	Drug	Counter-balanced arrangement for the dose	Measurement time points for food intake
1	free-feeding	GLP-2	0, 1, 5, 10μg	0.5, 1, 2, 3, 4, 6 and 24hr
2	Fasted refeeding	GLP-2	0, 1, 5, 10μg	0.5, 1, 2, 3, 4, 6 and 24hr
3	Fasted refeeding	GLP-2 SHU9119	saline+saline; saline+GLP-2 (10μg); SHU9119 (0.5nmol)+saline; SHU 9119 (0.5nmol)+GLP-2 (10 μg)	0.5, 1, 2, 3, 4 and 6hr
4	Fasted refeeding	GLP-2 Exendin	saline+saline; saline+GLP-2 (10μg); Exendin$_{(9\text{-}39)}$ (10μg)+saline; Exendin $_{(9\text{-}39)}$ (10μg)+GLP-2 (10μg)	0.5, 1, 2, 3, 4 and 6hrs

To be specific, rats were microinjected with GLP-2, GLP-2 combined with either SHU9119, or Exendin$_{(9\text{-}39)}$ in a series of dosage. Briefly, all rats had their food removed

at 8:00. Each group received 0.5µl injection of the GLP-2 (0, 1, 5, 10 µg) into DMH at 8:30. Rats were returned immediately to their cages after injection. Subsequent food intake was recorded at 0.5, 1, 2, 3, 4, 6 and 24hrs. For fasted-refeeding groups, rats have been fasted for 16hrs before the experiments. Each group received 0.5µl DMH injection of GLP-2 (0, 1, 5, 10 µg) before dark onset at 8:30. To testing the effects of SHU9119 and Exendin$_{(9\text{-}39)}$ on the blocking effects on GLP-2. Each group then received 0.5µl DMH injections twice with fifteen minutes gaps in between with the combination of saline, SHU9119 (0.5nmol), and GLP-2 (10µg), or saline, Exendin$_{(9\text{-}39)}$ (10µg) and GLP-2 (10µg) at 8:30 (Table 1). Rats were then returned immediately to their cages after two injections. Subsequent food intake was recorded at 0.5, 1, 2, 3, 4, 6 and 24hrs.

Delivery of the drugs

Unilateral injections into the DMH were administered using 1µL Hamilton syringes (Hamilton, Reno, NV, USA) connected by PE-10 polyethylene tubing to 30-gauge injection cannulas. At the time of experiment, obturators were removed. The injection cannula (2mm longer than the guide cannula) was carefully inserted into the guide cannula and manual injection was initiated 15s later. The injection was delivered at a flow rate of 0.5µL/min for the total volume of 0.5µL. The injection cannulas were maintained in place for 30s after delivery of the drugs or vehicle to minimize the backflow. The obturators were replaced after the injections and the rats were placed back into their cages.

Drugs

GLP-2$_{(1\text{-}33)}$ and Exendin$_{(9\text{-}39)}$ (GLP-1 receptor antagonist) were purchased from Sigma

(Sigma Chemical Company, St. Louis, MO). SHU9119 (a melanocortin receptor antagonist) was purchased from Tocris (Tocris Bioscience, United Kingdom). All drugs were dissolved in 0.9% sodium chloride immediately before the experiments. Accordingly, the 0.9% saline solution was used as a control vehicle. The drugs and vehicle solutions were prepared just before the infusion. The dose of 10μg/0.5μl Exendin$_{(9\text{-}39)}$ and 0.5nmol/0.5μl SHU9119 were chosen to test their effects on blocking GLP-2 inhibition on food intake according to previous studies (Guan *et al.*, 2012; Tang-Christensen *et al.*, 2000).

Measurements on food intake

Food intake was measured by calculating the differences of the weight of the total foods collected immediately before the starting of the experiment and after the measurement time points. Any food and spilled food were recorded to the nearest 0.1g (corrected for spillage). Food intake measurements involving overnight food deprivation consisted of removing food 30minutes prior to starting deprivation and replacing the food back immediately after any drug microinjection or treatment.

Verification of injections into the DMH

To verify microinjection into the DMH was precise, at the end of the experiments, the rats received unilateral injections of a 0.5μl 2% Pontamine Sky Blue dye solution into the DMH. The rats were then given a high dose of chloral hydrate and perfused transcardially with saline followed by 10% buffered formalin. The brains were removed, fixed in 10% formalin, frozen, cut into 40μm serial coronal sections on a freezing microtome, and analyzed under a light microscope to exam the sites of microinjections

in the DMH according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007). Figure 2 showed the representative image of microinjection into the DMH after 18 days (7 days' post-operative recovery and 11 days' experiments test).

Data Analysis

Data were analyzed with Prism 6.0 software (GraphPad Software) and presented as the MEAN±SEM. Two-way RM ANOVA followed by Bonferron's tests post hoc multiple comparisons were used to analyze the cumulative food intake in different groups and at the different measurement times. The significance value was set at $p<0.05$.

Results

Histological analysis of microinjections in the DMH and GLP-2 delivery into the DMH

Figure 2 showed the representative image of microinjection into the DMH. Here, the right cannula placement showed that microinjection was correctly delivered into DMH. All injections were localized within DMH areas. The histological analysis showed that 72% had unilateral injections correctly made into the DMH and the rest were outside DMH either too deep, in lateral or too shallow. Details for all microinjection was listed on Supplement Table1. Therefore, we are confident that 72 % of successful injection rate was still statistically satisfactory to analyze the relevant experiment data.

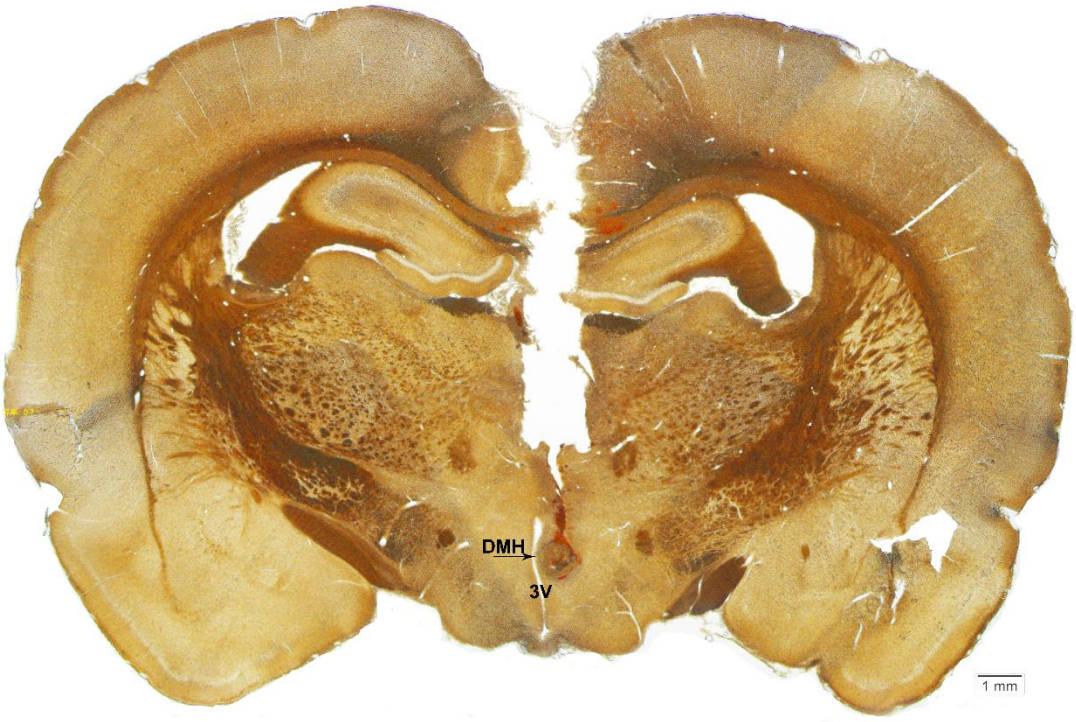


Fig.2. Representative image of unilateral injection in the DMH. Arrow pointing to the injection position into DMH. DMH, dorsomedial nucleus of the hypothalamus. 3V, the third ventricle. Scale bar: 1mm.

GLP-2 inhibits food intake in fasted-refeeding rats but not in free-feeding rats

Firstly**,** we compared the cumulative food intake with different dose of GLP-2 microinjection at different post injection time points for free-feeding rats and fasted-refeeding rats properly in-placed. For free-feeding rats (n=7), GLP-2 had no effect on food intake across the measurement time points for all doses of GLP-2 used (Figure 3A). For the fasted-refeeding rats (n=9), GLP-2 showed inhibition effects on food intake in a dose and time dependent manner (Figure 3B). There was no difference on food intake at the initial 3hrs of post-injection for all dose of GLP-2 used (Figure 3B). But there was significant decrease of food intake for the concentration of 10µg of GLP-2 compared to the control at 4 h (33.1% less) and 6 h (29.7% less) post-injection (4h: 10µg *vs* 0µg: 5.722±0.263g *vs* 8.55±1.331g, p=0.0002; 6h: 10µg *vs* 0µg: 7.611±0.701g *vs* 10.833±0.854g, p=0.0015, respectively), (Figure 3B). While 1µg and 5µg of GLP-2

microinjection had no effect on food intake when compared to the control at same time point of measurements (4h: 1µg *vs* 0µg: 7.744±0.532g *vs* 8.55±1.331g, $p>0.05$; 5µg *vs* 0µg: 7.867±0.864g *vs* 8.55±1.331g, $p>0.05$; 6h: 1µg *vs* 0µg: 9.767±0.642g *vs* 10.833±0.854g, $p>0.05$; 5µg *vs* 0µg: 9.889±0.814g *vs* 10.833±0.854g, $p>0.05$, respectively), (Figure 3B).

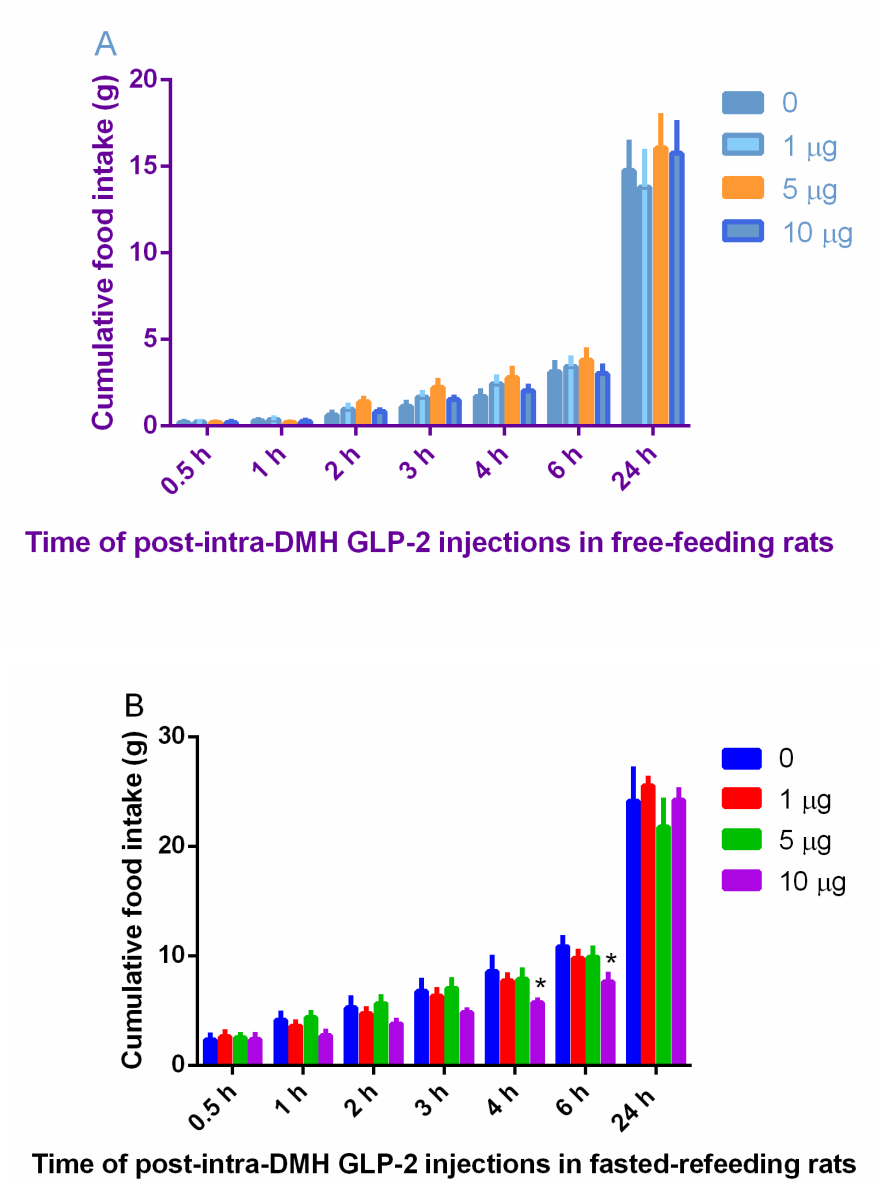


Fig 3. Effects on food intake with GLP-2 microinjection (0, 1, 5, and 10µg) unilaterally into DMH at post-injection of 0.5h, 1h, 2h, 3h, 4h, 6h and 24h for free-feeding rats (A) (n=7) and fasted-refeeding rats (B) (n=9). *compared to 0µg of GLP-2, $p<0.05$

Secondly, we compared the cumulative food intake on all rats when injections were mis-placed. There was no significant difference on food intake of GLP-2 with different dose and at different measurement time points for both free-feeding rats and the fasted-refeeding rats (Supplement Figure1).

The inhibition effect of GLP-2 on food intake in fasted-refeeding rats could be blocked by Exendin$_{(9-39)}$ but not SHU 9119

To understand the potential mechanism of inhibition effect of GLP-2 on food intake in DMH, we used GLP-1 receptor antagonist, Exendin$_{(9-39)}$ (Tang-Christensen *et al.*, 2000) and Melanocortin-4 receptor antagonist, SHU9119 (Guan *et al.*, 2012), as central GLP-2 receptor antagonist to test if this inhibition effect can be blocked. We used the same experimental design with combination of 10μg GLP-2 and Exendin$_{(9-39)}$ or SHU9119.

Firstly, we compared the cumulative food intake at different time points on all fasted-refeeding rats with combination of 10μg GLP-2 and SHU9119 microinjections into DMH properly in-placed. As expected, we found no difference on food intake at initial 3hrs of post injection for all combination of drugs, but at 4h and 6h of post-injection, there were significant decrease of both food intake with 10μg of GLP-2 compared to the control (0μg of GLP-2), (Figure 4A). (4h: saline+GLP-2 *vs* saline+saline: 5.775±0.996g *vs* 7.95±0.198g, p=0.0058; 6h: saline+GLP-2 *vs* saline+saline: 7.4±1.251g *vs* 9.9±0.334g, p=0.0009). This is in line with our above results (Figure 3B), but this inhibition effect cannot be blocked by pre-applying SHU9119 at 4h and 6h post microinject measurement time point. Specifically, SHU9119 did not block GLP-2 inhibition effect on food intake at 4h and 6h after injection (4h: saline+GLP-2 *vs* SHU+GLP-2: 5.775±0.996g *vs* 5.15±0.844g, p>0.05; 6h: saline+GLP-2 *vs* SHU+GLP-

2: 7.4±1.251g *vs* 6.55±1.095g, $p>0.05$) (Figure 4A). SHU9119 and GLP-2 combination decreased food intake at 4h and 6h after injection (4h: SHU+GLP-2 *vs* saline+saline: 5.15±0.844g *vs* 7.95±0.198g, $p=0.0001$; SHU+GLP-2 *vs* SHU+saline: 5.15±0.844g *vs* 8.0±0.042g, $p=0.0001$; 6h: SHU+GLP-2 *vs* saline+saline: 6.55±1.095g *vs* 9.9±0.334g, $p<0.0001$; SHU+GLP-2 *vs* SHU+saline: 6.55±1.095g *vs* 9.75±0.031g, $p<0.0001$; respectively) (Figure 4A). These results further confirmed that the inhibition role of GLP-2 on food intake in DMH in fasted-refeeding rats could not be blocked by SHU9119.

We also test the blocking effects of Exendin$_{(9-39)}$ on the inhibition effect of GLP-2 in a parallel experiment by replacing SHU9119 with Exendin$_{(9-39)}$. Similarly, our data showed that there was no difference on food intake at initial 3hrs of post injection. But when we compared the cumulative food intake at 4h and 6h post-injection, there were significant decrease of both food intake with 10μg of GLP-2 compared to the control (0μg of GLP-2, at4h and 6h), (Figure 4B). (4h: saline+GLP-2 *vs* saline+saline: 6.033±0.148g *vs* 7.567±0.542g, $p=0.0379$; 6h: saline+GLP-2 *vs* saline+saline: 7.333±0.27g *vs* 9.7±0.701g, $p=0.0002$). This was in agreeable with the previous results (Figure 3B) but this inhibition effect can be nearly completely blocked by pre-applying Exendin$_{(9-39)}$. This blocking effect can be seen at 4h and 6h post microinjection measurement time point. (4h: Extendin$_{(9-39)}$ +GLP-2 *vs* saline+GLP-2: 7.567±0.253g *vs* 6.033±0.148g, n=6, $p=0.0379$; Exendin$_{(9-39)}$+GLP-2 *vs* Exendin$_{(9-39)}$+saline: 7.567±0.253g *vs* 7.767±0.451g, $p>0.05$; Exendin$_{(9-39)}$+saline *vs* saline+GLP-2: 7.767±0.451g *vs* 6.033±0.148g, $p=0.0128$; 6h: Extendin$_{(9-39)}$+GLP-2 *vs* saline+GLP-2: 10.1±0.161 *vs* 7.333±0.27g, $p<0.0001$; Exendin$_{(9-39)}$+GLP-2 *vs* Exendin$_{(9-39)}$+saline: 10.1±0.161g *vs* 10.233±0.652g, $p>0.05$; Exendin$_{(9-39)}$+saline *vs* saline+GLP-2:

10.233±0.652g *vs* 7.333±0.27g, $p<0.0001$). Exendin$_{(9-39)}$ and the combination of Exendin$_{(9-39)}$ and GLP-2 showed no effects on food intake at all post-injection measurement time points compared to the control (saline+saline group, Figure 4B). These results further confirmed that the inhibitory role of GLP-2 on food intake could be blocked by Exendin$_{(9-39)}$ in fasted-refeeding rats in DMH.

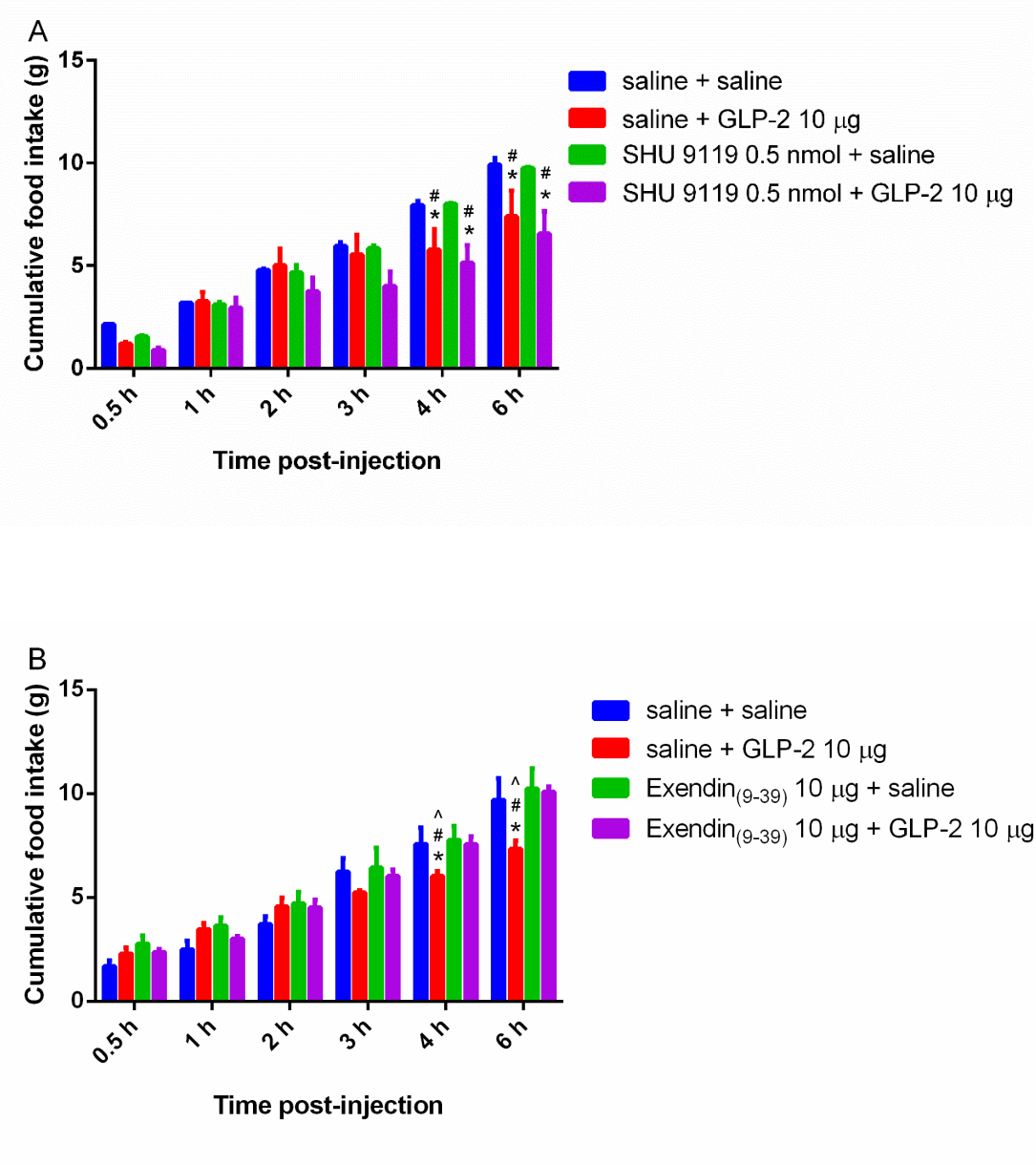


Fig.4. Unilaterally microinjection of SHU9119 (0.5nmol)/Exendin$_{(9-39)}$ (10μg) , respectively, into the DMH 15min prior to GLP-2 (10μg) injection. The intra-DMH GLP-2-induced inhibition in fasted-refeeding rats at 4 h and 6 h were not blocked by SHU9119 (A); however, the effects were abolished by Exendin$_{(9-39)}$ (B) (n=6).

*compared to saline+saline; #compared to Exendin$_{(9-39)}$+GLP-2; ^compared to Exendin$_{(9-39)}$+saline. Values as MEAN±SEM, $p<0.05$. Note: Microinjection of SHU9119 (*i.e.*, SHU9119+saline) (A)/Exendin$_{(9-39)}$ (*i.e.*, Exendin$_{(9-39)}$+saline) (B) alone into to the DMH had no effect on food intake as compared to saline+saline group ($p>0.05$), SHU9119+GLP-2 group/Exendin$_{(9-39)}$+GLP-2 group.

Above all, the fact that the inhibition effect of GLP-2 on food intake could be blocked by Exendin$_{(9-39)}$ but not SHU9119 indicated that this inhibition effect of GLP-2 on food intake might function through the GLP-1 receptor but not the melanocortin-4 receptor in DMH. Because the GLP-2 receptor antagonist has relatively high partial agonistic activity (Thulesen *et al.*, 2002), and there is as yet no ideal known potent GLP-2 receptor antagonist. GLP-1 receptor antagonist, Exendin $_{(9-39)}$ (Tang-Christensen *et al.*, 2000) and MC4-R antagonist, SHU9119 (Guan *et al.*, 2012) were normally chosen to validate the blocking effect of GLP-2.

Secondly, we compared the cumulative food intake at different time point on all fasted -refeeding rats with combination of 10μg GLP-2 and SHU9119 or Exendin$_{(9-39)}$ injections into mis-placed DMH. There was no significant difference shown in all groups (Supplement Figure 2).

Discussion

The roles of GLP system in the regulation of feeding behavior have been intensively investigated previously. However, the importance and the mechanism of action responsible for the GLP-2 dependent modulation of feeding remain largely uncertain (Tang-Christensen *et al.*, 2000; Lovshin *et al.*, 2001; Sørensen *et al.*, 2003; Schmidt *et al.*, 2003). It has been demonstrated that central administration *i.e.* lateral ventricular injection of either GLP-1, GLP-2 or oxyntomodulin reduces food intake (Dakin *et al.*, 2001; Tang-Christensen *et al*., 2000; Tang-Christensen, *et al.*, 1998; Tang-Christensen

et al., 1996; Turton *et al.*, 1996). However, regarding at which brain sites GLP-2 exerts its feeding-suppress effects are still unclear. In this study, we directly administered microinjection of GLP-2 into brain tisuue. Our results indicated that unilaterally injection of GLP-2 into DMH could suppress food intake only in fasted-refeeding rats but not in free-feeding rats. This inhibition could be blocked by pretreatment with Exendin$_{(9–39)}$ but not SHU9119. The results from rats with misplaced injections also confirmed that the GLP-2 effect on food intake is specific to the DMH.

The DMH is an integrative center receiving food intake related information from a variety of sources (Crosby, *et al.* 2011; Zhu, *et al.* 2007). GLP-2-immunoreactive fibers in the DMH may originate in the NTS (Vrang *et al.*, 2007), but GLP-2 receptors are present predominantly in the compact not the ventral subdivision of the DMH (Tang-Christensen *et al.*, 2000). To our best knowledge, there is not any other study which has directly examined GLP-2 injection into DMH and its effect on food intake. It was debated that intracerebroventricular GLP-2 suppresses food intake, but peripheral GLP-2 does not (Lovshin, *et al.*, 2001; Tang-Christensen *et al.*, 2000; Tang-Christensen, *et al.* 2001). Our results indicated that unilaterally injected GLP-2 into DMH could suppress food intake and this inhibition could be blocked by Exendin$_{(9-39)}$. However, in an *in vitro* assay, GLP-2 has been shown not to bind to the GLP-1 receptor, and GLP-2 receptors are insensitive to GLP-1 (Yusta *et al.*, 1999). While, it seems unlikely that Exendin$_{(9-39)}$ binds directly to the GLP-2 receptor and a more likely explanation is that GLP-1 and GLP-2 receptors act in parallel requiring both to be fully operational in order to induce anorexia. Glucagon-like peptide-2 actions on feeding are dependent on intact central GLP-1 receptors because pharmacological antagonism of GLP-1 receptors by prior administration of Exendin$_{(9-39)}$ abolishes GLP-2 induced anorexia (Tang-

Christensen *et al.*, 2000). A pharmacological and behavioral experiment confirmed that this effect was *via* a mechanism insensitive to taste aversion (Tang-Christensen *et al.*, 2000). These data suggest that by activating DMH neurons a short-term reduction in food intake can take place.

The DMH cells also express the Melanocortin-4 receptor (MC4R) (Harrold, *et al.*, 1999). MC4R signaling in the brain is required partially for intracerebroventricular GLP-2-mediated suppression of food intake and this effect in an MC4R-dependent manner (Guan *et al.,* 2012). However, our results showed that SHU9119, as MC4R antagonist, could not block the effect of GLP-2 on food intake in DMH. We inferred that the reason might be due to different animals used (mice *vs* rat).

Some studies have collectively shown that the major target of the brainstem preproglucagon neurons is the hypothalamus (Larsen *et al.,* 1997; Merchenthaler *et al.*, 1999; Rinaman, 1999; Vrang *et al.,* 2007). Preproglucagon projections constitute the predominant input from the nucleus of the solitary tract to the dorsomedial hypothalamic nucleus. While approximately 65% of NTS-neurons projecting to the DMH co-stored the preproglucagon-derived peptide GLP-2, only 25% of the NTS neurons projecting to the PVN were found to be GLP-ergic (Vrang *et al.*, 2007).

In this study, we examined the effects of microinjection of GLP-2 (1, 5, 10μg) into the DMH on food intake in free-feeding rats and fasted-refeeding rats. Unexpectedly, we found that GLP-2 microinjections did not significantly affect cumulative food intake in free-feeding rats (Figure 3A). This observation is not in agreement with previous reports in rodents (Rinaman *et al.*, 1999; Lovshin *et al.*, 2001; Tang-Christensen *et al.*,

2000; Dalvi *et al.*, 2012). Tang-Christensen *et al.* discovered that central injection of 10 μg of GLP-2 caused a significant decrease in 2-h food intake than vehicles in free-feeding rats (Tang-Christensen *et al.*, 2000). Lovshin *et al.* demonstrated that the central administration of pharmacological doses of GLP-2 powerfully inhibited short-term food intake in free-feeding mice (Lovshin *et al.*, 2001). The data from Dalvi *et al.* also showed that *icv* 5 μg of GLP-2 remarkably suppressed food intake in free-feeding mice (Dalvi *et al.*, 2012). It is somewhat difficult to explain the discrepancy of their results with ours from the free-fed animals. The difference might be due to the administration route (*icv vs* intra-DMH.) and, subsequently, different sites of action of the GLP-2. Notably, all three studies mentioned above-injected GLP-2 into either the lateral ventricle or the third cerebral ventricle to focus on the interactions of GLP-2 with the major hypothalamic nuclei that lie in the vicinity of the third or lateral ventricle. However, our data derived from the anorexigenic action of GLP-2 was injected directly into the DMH.

It is worth noting that the expression of appetite regulatory peptides/hormones is known to be changed by fasting or food deprivation (Yuan *et al.*, 2014). Thus, the condition that influences of anorectic or orexigenic hormones and nutritional signals can be reduced or increased, respectively. In the present study, the anorectic effect was only detected in the fasted rats suggesting that the effect of intra-DMH injection of GLP-2 may be related to the nutritional state of animals.

Our results showed the reduction of food intake observed four-six hours after the injection instead in the first hours (Baldassano *et al.*, 2012) and 2-hours (Tang-Christensen *et al.*, 2000). The reason for this could be complex as mentioned above.

These might due to different animal species (C57BL/J mice, Wistar rats,), different route of GLP-2 application (*i.p.* centrially administered, lateral ventricle ICV), and other conditions. In this study, we used SD rats, GLP-2 injected directly into DMH. However, similar observation was also observed where the reduction of food intake observed from first-four hours after an intracerebroventricular injection of h[Gly^2] GLP-2 into in mice (Lovshin *et al.,* 2001). Further investigation is therefore needed to investigate how these differences occur.

In conclusion, our study indicated that GLP-2 pharmacologically inhibited food intake in DMH and this effect could be blocked functionally by $Exendin_{(9-39)}$. This was the first study on the effect of direct administration of GLP-2 and its antagonist in the medial hypothalamic nucleus on feeding behavior, and preliminarily explained the interaction between GLP-2 and the dorsal medial hypothalamic nucleus. Our results would provide useful information on the regulation mechanism of food intake and may provide a new target for the treatment of obese patients.

Acknowledgements

This study was supported by the Health Research Fund Project of Shaanxi Province (No.2018D049) and by the Science and Technology Plan Project of Shaanxi Province (No.2020SF-138).

Author contributions

Huiling Sun, Lijun Shang and Jianqun Yan designed the experiment; Huiling Sun and Lin Hou performed experiments; Huiling Sun and Jianqun Yan analyzed the data; Huiling Sun, Jianqun Yan, Lijun Shang and Kai Meng interpreted the results of

experiments; Huiling Sun and Lijun Shang drafted and revised the manuscript; all authors approved the final version of the manuscript.

Declaration of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Reference

Amato, A., Baldassano, S., Serio, R., & Mule, F. (2009). Glucagon-like peptide-2 relaxes mouse stomach through vasoactive intestinal peptide release. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 296*(3), G678-G684. doi:10.1152/ajpgi.90587.2008

Baldassano, S., Bellanca, A.L., Serio, R., & Mule, F. (2012). Food intake in lean and obese mice after peripheral administration of glucagon-like peptide 2. Journal of Endocrinology, 213(3): 277–284

Crosby, K. M., Inoue, W., Pittman, Q. J., & Bains, J. S. (2011). Endocannabinoids Gate State-Dependent Plasticity of Synaptic Inhibition in Feeding Circuits. *Neuron, 71*(3), 529-541. doi:10.1016/j.neuron.2011.06.006

Dakin, C. L., Gunn, I., Small, C. J., Edwards, C. M. B., Hay, D. L., Smith, D. M., Bloom, S. R. (2001). Oxyntomodulin inhibits food intake in the rat. *Endocrinology, 142*(10), 4244-4250. doi:DOI 10.1210/en.142.10.4244

Dalvi PS, Belsham DD. Glucagon-Like Peptide-2 Directly Regulates Hypothalamic Neurons Expressing Neuropeptides Linked to Appetite Control in Vivo and in Vitro. Endocrinology. 2012; 153(5):2385-97.

Drucker, D. J., & Yusta, B. (2014). Physiology and Pharmacology of the Enteroendocrine Hormone Glucagon-Like Peptide-2. *Annual Review of Physiology, Vol 76, 76*, 561-583. doi:10.1146/annurev-physiol-021113-170317

Guan, X. F., Shi, X. M., Li, X. J., Chang, B., Wang, Y., Li, D. P., & Chan, L. (2012). GLP-2 receptor in POMC neurons suppresses feeding behavior and gastric motility. *American Journal of Physiology-Endocrinology and Metabolism, 303*(7), E853-E864. doi:10.1152/ajpendo.00245.2012

Harrold, J. A., Widdowson, P. S., & Williams, G. (1999). Altered energy balance causes selective changes in melanocortin-4(MC4-R), but not melanocortin-3 (MC3-R), receptors in specific hypothalamic regions: further evidence that activation of MC4-R is a physiological inhibitor of feeding. *Diabetes, 48*(2), 267-271. doi:10.2337/diabetes.48.2.267

Holst, J. J. (2007). The physiology of glucagon-like peptide 1. *Physiological Reviews, 87*(4), 1409-1439. doi:10.1152/physrev.00034.2006

Jin, S. L. C., Han, V. K. M., Simmons, J. G., Towle, A. C., Lauder, J. M., & Lund, P. K.

(1988). Distribution of Glucagonlike Peptide-I (Glp-I), Glucagon, and Glicentin in the Rat-Brain - an Immunocytochemical Study. *Journal of Comparative Neurology, 271*(4), 519-532. doi:DOI 10.1002/cne.902710405

Kieffer, T. J., & Habener, J. L. (1999). The glucagon-like peptides. *Endocrine Reviews, 20*(6), 876-913. doi:DOI 10.1210/er.20.6.876

Lam, C. K., M. Chari, B. B. Su, G. W. Cheung, A. Kokorovic, C. S. Yang, P. Y. Wang, T. Y. Lai, and T. K. Lam. 2010. 'Activation of N-methyl-D-aspartate (NMDA) receptors in the dorsal vagal complex lowers glucose production', J Biol Chem, 285: 21913-21.

Larsen, P. J., TangChristensen, M., Holst, J. J., & Orskov, C. (1997). Distribution of glucagon-like peptide-1 and other preproglucagon-derived peptides in the rat hypothalamus and brainstem. *Neuroscience, 77*(1), 257-270. doi:Doi 10.1016/S0306-4522(96)00434-4

Lovshin, J., Estall, J., Yusta, B., Brown, T. J., & Drucker, D. J. (2001). Glucagon-like peptide (GLP)-2 action in the murine central nervous system is enhanced by elimination of GLP-1 receptor signaling. *Journal of Biological Chemistry, 276*(24), 21489-21499. doi:DOI 10.1074/jbc.M009382200

Merchenthaler, I., Lane, M., & Shughrue, P. (1999). Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. *Journal of Comparative Neurology, 403*(2), 261-280. doi:Doi 10.1002/(Sici)1096-9861(19990111)403:2<261::Aid-Cne8>3.0.Co;2-5

Rinaman, L. (1999). Interoceptive stress activates glucagon-like peptide-1 neurons that project to the hypothalamus. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 277*(2), R582-R590.

Schmidt PT, Naslund E, Gryback P, Jacobsson H, Hartmann B, Holst JJ, Hellstrom PM. Peripheral administration of GLP-2 to humans has no effect on gastric emptying or satiety. Regul Peptides. 2003; 116(1-3):21-5.

Sørensen LB, Flint A, Raben A, Hartmann B, Holst JJ, Astrup A. No effect of physiological concentrations of glucagon-like peptide-2 on appetite and energy intake in normal weight subjects. International Journal of Obesity and Related Metabolic Disorders. 2003; 27:450-6.

Tang-Christensen, M., Larsen, P. J., Thulesen, J., Romer, J., & Vrang, N. (2000). The proglucagon-derived peptide, glucagon-like peptide-2, is a neurotransmitter involved in the regulation of food intake. *Nature Medicine, 6*(7), 802-+. doi:Doi 10.1038/77535

Tang-Christensen, M., Vrang, N., & Larsen, P. J. (1998). Glucagon-like peptide 1(7-36) amide's central inhibition of feeding and peripheral inhibition of drinking are abolished by neonatal monosodium glutamate treatment. *Diabetes, 47*(4), 530-537. doi:DOI 10.2337/diabetes.47.4.530

Tang-Christensen, M., Vrang, N., & Larsen, P. J. (2001). Glucagon-like peptide containing pathways in the regulation of feeding behaviour. *International Journal of Obesity, 25*, S42-S47. doi:DOI 10.1038/sj.ijo.0801912

TangChristensen, M., Larsen, P. J., Goke, R., FinkJensen, A., Jessop, D. S., Moller, M., & Sheikh, S. P. (1996). Central administration of GLP-1-(7-36) amide inhibits

food and water intake in rats. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 271*(4), R848-R856.

Thulesen, J., Knudsen, L. B., Hartmann, B., Hastrup, S., Kissow, H., Jeppesen, P. B. Poulsen, S. S. (2002). The truncated metabolite GLP-2 (3-33) interacts with the GLP-2 receptor as a partial agonist. *Regulatory Peptides, 103*(1), 9-15. doi:Doi 10.1016/S0167-0115(01)00316-0

Turton, M. D., OShea, D., Gunn, I., Beak, S. A., Edwards, C. M. B., Meeran, K., Bloom, S. R. (1996). A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature, 379*(6560), 69-72. doi:DOI 10.1038/379069a0

Vrang, N., Hansen, M., Larsen, P. J., & Tang-Christensen, M. (2007). Characterization of brainstem preproglucagon projections to the paraventricular and dorsomedial hypothalamic nuclei. *Brain Research, 1149*, 118-126. doi:10.1016/j.brainres.2007.02.043

Watson, G.C. Paxinos, The Rat Brain in Stereotaxic Coordinates, 6th Edn., Academic Press, San Diego, CA, 2007.

Wojdemann, M., Wettergren, A., Hartmann, B., & Holst, J. J. (1998). Glucagon-like peptide-2 inhibits centrally induced antral motility in pigs. *Scandinavian Journal of Gastroenterology, 33*(8), 828-832.

Yusta, B., Huang, L., Munroe, D., Wolff, G., Fantaske, R., Sharma, S., Drucker, D. J. (2000). Enteroendocrine localization of GLP-2 receptor expression in humans and rodents. *Gastroenterology, 119*(3), 744-755. doi:DOI 10.1053/gast.2000.16489

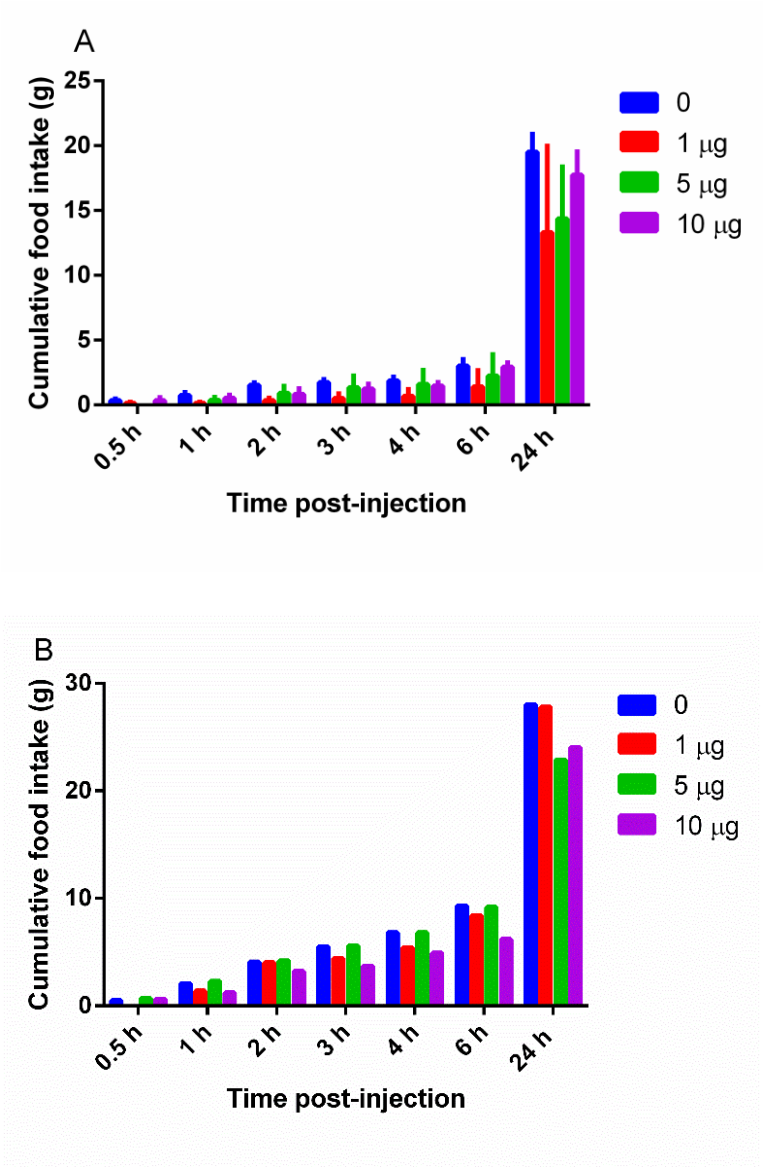
Yusta, B., Somwar, R., Wang, F., Munroe, D., Grinstein, S., Klip, A., & Drucker, D. J. (1999). Identification of glucagon-like peptide-2 (GLP-2)-activated signaling pathways in baby hamster kidney fibroblasts expressing the rat GLP-2 receptor. *Journal of Biological Chemistry, 274*(43), 30459-30467. doi:DOI 10.1074/jbc.274.43.30459

Yuan DY, Zhou CW, Wang T, Lin FJ, Chen H, Wu HW, Wei RB, Xin ZM, Liu J, Gao YD, Chen DF, Yang SY, Wang Y, Pu YD, Li ZQ. Molecular characterization and tissue expression of peptide YY in Schizothorax prenanti: Effects of periprandial changes and fasting on expression in the hypothalamus. Regul Peptides. 2014; 190:32-8.

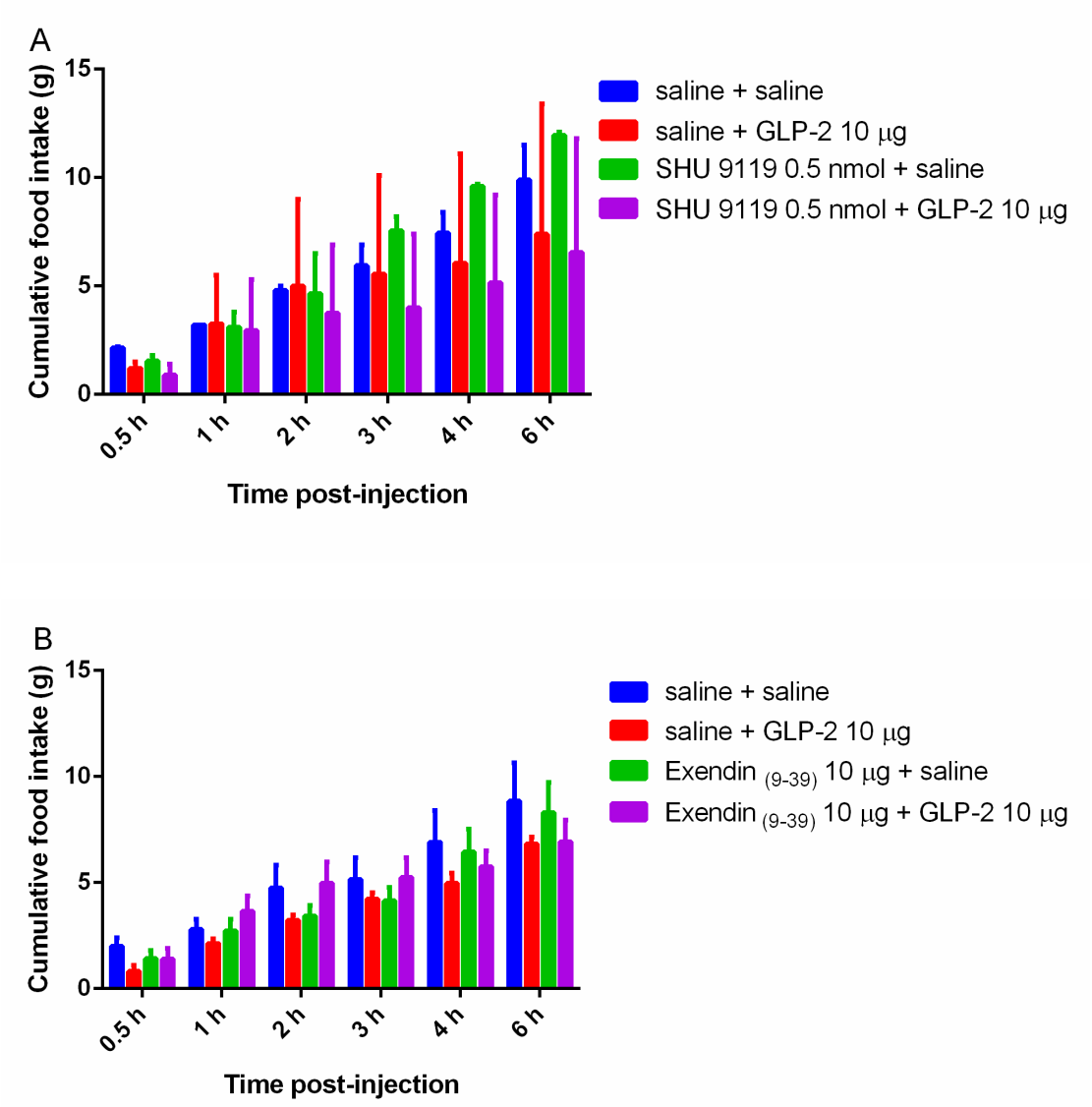
Zhu, J. N., Guo, C. L., Li, H. Z., & Wang, J. J. (2007). Dorsomedial hypothalamic nucleus neurons integrate important peripheral feeding-related signals in rats. *Journal of Neuroscience Research, 85*(14), 3193-3204. doi:10.1002/jnr.21420

Supplement Table 1 Microinjection in DMH

number of animals	microinjected in DMH in place	microinjection outside the DMH
free-feeding group, n = 10	n = 7	n = 3
fasted-refeeding group, n = 10	n = 9	n = 1
for SHU9119 experiments, n = 11, 3 died during the experiment	n = 6	n = 2
for Exendin$_{(9\text{-}39)}$ experiments, n = 11	n = 6	n = 5



Supplement Figure 1. Microinjection of GLP-2 (0, 1, 5, and 10µg) unilaterally outside DMH effects on food intake at 0.5h, 1h, 2h, 3h, 4h, 6h and 24h after injection in free-feeding rats (A) (n=3) and fasted-refeeding rats (B) (n=1). No effects of GLP-2 on both ad libitum-feeding rats and fasted-refeeding rats. Values as MEAN±S.E.M, $p<0.05$



Supplement Figure 2. Microinjection of GLP-2 (10μg) unilaterally outside DMH had no effect on food intake at 4h to 6h after injection in fasted-refeeding rats. SHU9119 (0.5nmol) (n=2) (A) Exendin(9-39) (10μg) (n=5) (B). Cumulative food intake was recorded at 0.5h, 1h, 2h, 3h, 4h, and 6h after injection. *compared to saline+saline; #compared to Exendin(9-39)+GLP-2; ^compared to Exendin(9-39)+saline. Values as Mean± S.E.M., $p<0.05$