Role of the neural pathway from hindbrain to hypothalamus in interaction of GLP1 and leptin in rats

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Abstract

Glucagon-like peptide-1 (GLP1) and leptin are anorectic hormones. Previously, we have shown that i.p. coadministration of subthreshold GLP1 with leptin dramatically reduced food intake in rats. In this study, by using midbrain-transected rats, we investigated the role of the neural pathway from the hindbrain to the hypothalamus in the interaction of GLP1 and leptin in reducing food intake. Food intake reduction induced by coinjection of GLP1 and leptin was blocked in midbrain-transected rats. These findings indicate that the ascending neural pathway from the hindbrain plays an important role in transmitting the anorectic signals provided by coinjection of GLP1 and leptin.

Key Words

- ▶ food intake
- ▶ GLP1
- ▶ leptin
- ▶ midbrain transection

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Introduction

Leptin, an anorectic hormone produced by white adipose tissue, plays an important role in the maintenance of energy balance (Schwartz *et al.* 2000). The absence of leptin results in severe obesity in both rodents and humans (Pelleymounter *et al.* 1995, Farooqi *et al.* 1998). Leptin directly binds to its obesity receptor b (ObRb) receptors, which are located mainly in the hypothalamus and hindbrain; it promotes energy expenditure by stimulating JAK/STAT3 signaling pathway (Bjorbaek & Kahn 2004). Leptin also inhibits AMP-activated protein kinase (AMPK) activity in the arcuate nucleus and in the paraventricular nucleus of the hypothalamus (Minokoshi *et al.* 2004).

Glucagon-like peptide-1 (GLP1), a gastrointestinal hormone produced by L cells in the distal small intestine and colon, is released in response to meal intake and ingested nutrients (Mojsov *et al.* 1986, Holst 2007). Because GLP1 and its receptors are present in both the CNS and peripheral tissues such as pancreatic β cells and vagal afferents, the effect of GLP1 on energy metabolism

could be mediated at central sites, peripheral sites, or both (Holst 2007, Hayes *et al.* 2010). Indeed, GLP1 reduces food intake after either central or peripheral administration (Williams *et al.* 2006).

Several gastrointestinal hormones associated with feeding and energy metabolism are not only transported to the brain via the circulation – they also modulate vagal afferent pathways (Date *et al.* 2006). The effects of peripheral GLP1, cholecystokinin (CCK), peptide YY (PYY), or ghrelin on feeding are blocked in vagotomized rats (Date *et al.* 2002, Koda *et al.* 2005). Vagal afferents reach the nucleus of the solitary tract (NTS) in the hindbrain, which contains the area postrema where the blood–brain barrier is circumvented. Thus, the hindbrain is an important site for integrating information from both the vagal afferent pathway and the circulation.

Recently, we have shown that i.p. coinjection of subthreshold GLP1 and leptin, which individually have no effect on feeding, dramatically reduced food intake by

acting on the proopiomelanocortin (POMC)/melanocortin-4-receptor (MC4R) system. We have also found that coinjection of GLP1 and leptin decreases phosphorylated AMPK levels in the hypothalamus (Poleni *et al.* 2012). However, the site at which information from subthreshold GLP1 and that from subthreshold leptin become primarily integrated remains unclear.

A bilateral transection of the midbrain surgically cuts the neural pathways from the hindbrain to the hypothalamus (Date *et al.* 2006). Midbrain-transected rats are therefore useful for investigating whether information from subthreshold doses of GLP1 and leptin is integrated in the hypothalamus or hindbrain. In this study, by using midbrain-transected rats, we examined the effect of coinjection of GLP1 and leptin on food intake, *Pomc* mRNA levels, and phosphorylation of AMPK. Our findings provide insights into the physiological control of feeding systems by peripheral hormones.

Materials and methods

Experimental animals

Male Wistar rats (10 weeks old; Charles River Japan, Shiga, Japan) weighing 300–350 g were used for all experiments. Rats were given standard laboratory chow and water and allowed to feed and drink *ad libitum*. They were housed individually in plastic cages at a constant room temperature in a 12 h light:12 h darkness cycle (0800–2000 h light).

After the rats were anesthetized with an i.p. injection of sodium pentobarbital (40 mg/kg; Abbot Laboratories), bilateral midbrain transections were performed as described previously (Crawley *et al.* 1984). In brief, the head was fixed in a stereotaxic instrument in a 2.4-mm-nose-down position. A steel knife blade of 1.5 mm wide was used to penetrate the brain in a coronal plane at two points: 0.5 mm on either side of the midline and 1 mm in front of the lambdoid suture. At each incision, the blade penetrated to a depth of 7.7 mm below the dura. In the sham operation, the skull was exposed but the brain was left intact. Only rats that exhibited increased weight gain and food intake were selected for subsequent feeding experiments. To confirm that the transection surgery had been successful, we removed the brains after the feeding tests and histologically verified the exact locations of the lesions.

All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. Our experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Faculty of Medicine, University of Miyazaki, Japan.

Feeding experiments

Feeding experiments were conducted in midbrain-transected rats 1 week after surgery. Rats were sufficiently habituated to handling and i.p. injections before the experiments. To investigate the effect of GLP1 and leptin on food intake, we used a dosage of 10 nmol/kg GLP1 (7–36) amide (Peptide Institute, Osaka, Japan) and 18.8 nmol/kg rat recombinant leptin (Sigma Aldrich), neither of which decreased food intake when administered alone (Poleni *et al.* 2012). We allocated the sham-operated and midbrain-transected rats to four treatment groups: single injection of saline, single injection of GLP1 (10 nmol/kg), single injection of leptin (18.8 nmol/kg), and coinjection of GLP1 (10 nmol/kg) and leptin (18.8 nmol/kg). The hormones were administered i.p. at 0930–1000 h to rats that had been fasted overnight. We then measured food intake at 0.5, 1, and 2 h after injection.

Immunohistochemistry

Saline or GLP1 and leptin was injected i.p. into the rats in which a bilateral midbrain transection or sham surgery was performed ($n=3$ per group) 2 h before transcardiac perfusion with a fixative containing 4% (w/v) formaldehyde. The brains were removed and sectioned into 40- μ m-thick samples. The regions of the hypothalamus were identified according to the information provided in the rat brain stereotaxic atlas of Paxinos & Watson (2005). Immunohistochemistry was carried out with an anti-cFos antibody (Ab-5; Calbiochem, La Jolla, CA, USA; 1:10 000), as described previously (Date *et al.* 1999).

Some sections were used for double-labeling immunohistochemistry. They were first incubated with rabbit anti-cFos antibody (9F6; Cell Signaling Technology, Danvers, MA, USA; 1:500) and stained with Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:200). We used 9F6 for the immunofluorescence staining because staining of cFos with Ab5 antibody produced weak and unclear results. Subsequently, these sections were incubated with anti- α -melanocyte-stimulating hormone (MSH) antibody (Millipore, Tokyo, Japan; 1:10 000) and pre-labeled by using commercially available labeling kits (Fluorescein Labeling Kit-NH₂; Dojindo, Kumamoto, Japan). Anti- α -MSH antiserum was used to identify POMC-like immunoreactive neurons, as POMC is a precursor of α -MSH. Images were viewed and captured with a Zeiss AX10 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Fos-positive cells were counted in five sections each of the hypothalamic arcuate

nucleus and paraventricular nucleus from each animal (Koda *et al.* 2005); Fos-expressing α -MSH-positive cells in the hypothalamic arcuate nucleus were enumerated in the same manner.

Quantitative PCR for POMC

The hypothalami were rapidly removed 1.5 h after sham-operated or midbrain-transected rats received injections of saline or of GLP1 and leptin. Total RNAs were extracted with TRIzol Reagent (Invitrogen) and reverse-transcribed by using a Superscript III First-Strand Synthesis System Kit (Invitrogen). We have previously demonstrated that *Pomc* mRNA expression significantly increases after coinjection of GLP1 and leptin (Poleni *et al.* 2012). Therefore, quantitative PCR was conducted for *Pomc* by using the hypothalami of midbrain-transected and sham-operated rats. Real-time PCR was conducted with a LightCycler system (Roche Diagnostics) and an SYBR Premix Ex Taq II Kit (Takara Bio, Otsu, Japan). The primer sequences used have been described elsewhere (Date *et al.* 2010). The relative abundances of all reaction products were normalized against the level of glyceraldehyde 3-phosphate dehydrogenase mRNA.

Western blotting

We have previously demonstrated that phosphorylation of AMPK in the hypothalamus significantly decreases after coinjection of GLP1 and leptin (Poleni *et al.* 2012). Therefore, in this study, we evaluated the level of phosphorylation of AMPK after coinjection of GLP1 and leptin into midbrain-transected rats. The hypothalami were rapidly removed 1.5 h after i.p. injection of saline or of GLP1 and leptin into sham-operated or midbrain-transected rats. Thirty micrograms of total protein per sample were analyzed by SDS-PAGE (8% acrylamide gel) and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were blocked for 1 h at room temperature with Blocking One-P (Nacalai Tesque, Kyoto, Japan), probed overnight at 4 °C with primary antibody against AMPK or phosphorylated AMPK (Cell Signaling Technology), and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (Epitomics, Burlingame, CA, USA). Specific proteins were detected by chemiluminescence with Western BLoT Quant HRP substrate (Takara Bio) in accordance with the manufacturer's instructions, and then exposed to X-ray film (Fuji Film, Tokyo, Japan). Western blotting was quantified by densitometry relative to AMPK by using

NIH Image J software (National Institute of Health, Bethesda, MA, USA).

Statistical analysis

All data are expressed as mean $\pm$ S.E.M. Statistical significance was evaluated by using Student's *t*-test, one-way ANOVA (for group), or two-way repeated-measures ANOVA (for group and time). When the ANOVA indicated a significant effect of a variable of concern, differences between groups were analyzed by using the Tukey-Kramer *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Food intake

Coinjection of GLP1 and leptin significantly decreased food intake 1 and 2 h after coinjection in sham-operated rats compared with sham-operated rats that had received injections of saline, whereas it did not reduce food intake in midbrain-transected rats (Fig. 1).

Fos expression

In sham-operated rats, coinjection of GLP1 and leptin significantly increased the number of Fos-positive cells relative to that after injection of saline in the

Figure 1

Effects of i.p. administration of saline or of GLP1 (10 nmol/kg) and leptin (18.8 nmol/kg) on food intake in sham-operated rats and midbrain-transected rats. Data represent means and S.E.M. ($n = 8$ or 9 each). A significant interaction between group and time was determined by a two-way ANOVA for repeated measures ($P < 0.05$; $F = 2.74$; $df = 6$). The Tukey-Kramer *post-hoc* test revealed significant differences in food intake between the sham-operated saline and sham-operated GLP-1 and leptin groups 1 and 2 h after injection. * $P < 0.05$ vs sham-operated saline; ** $P < 0.01$ vs sham-operated saline.

hypothalamic arcuate nucleus (Fig. 2A and B) and paraventricular nucleus (Fig. 2C and D). In contrast, in both areas of midbrain-transected rats, the number of Fos-positive cells after coinjection of GLP1 and leptin did not differ from that after saline injection (Fig. 2A, B, C, and D). Double immunohistochemistry for Fos and α -MSH demonstrated that Fos was expressed in $62.2 \pm 5.5\%$ of α -MSH-immunoreactive neurons of the arcuate nucleus in the sham-operated rats after coinjection of GLP1 and leptin. In contrast, Fos was expressed in only $6.1 \pm 0.5\%$ of α -MSH-immunoreactive neurons in the midbrain-transected rats after coinjection of GLP1 and leptin (Fig. 2E and F). There were no significant differences in the number of α -MSH-immunoreactive cells between sham-operated rats and midbrain-transected rats (29.7 ± 2.7 and 33.5 ± 2.6 per section, respectively; data not shown).

Pomc mRNA levels

To examine the contribution of the neural pathway from the hindbrain to the hypothalamus to the increase in *Pomc* mRNA levels, we measured *Pomc* mRNA levels in the hypothalami of midbrain-transected rats. The *Pomc* mRNA level after coinjection of GLP1 and leptin into sham-operated rats was significantly higher than that after injection of saline, whereas no increase in *Pomc* mRNA expression after coinjection was found in the midbrain-transected rats (Fig. 3).

Phosphorylation of AMPK

Phosphorylation of AMPK in the hypothalami of sham-operated rats was dramatically and significantly reduced after coinjection of GLP1 and leptin (Fig. 4). However, no reduction in AMPK phosphorylation occurred in the hypothalami of midbrain-transected rats after coinjection (Fig. 4).

Discussion

Food intake and energy metabolism are finely regulated by the complicated interaction of many factors. Recently, we have shown that a single i.p. injection of GLP1 at 10 nmol/kg or leptin at 18.8 nmol/kg never induces food intake reduction in rats, whereas coinjection of the two at these subthreshold doses dramatically reduces food intake (Poleni *et al.* 2012). Several hormones, including ghrelin, CCK, PYY, and GLP1, at least in part, use the vagal afferent pathway to transmit starvation or satiety signals to the brain (Stanley *et al.* 2005). The NTS of the hindbrain is the

site of the first synaptic contact for vagal afferent projections from the gastrointestinal tract. The hindbrain also contains the area postrema, where the blood–brain barrier is circumvented. Thus the hindbrain is accessible to feeding signals not only through the vagal afferents but also directly via circulating hormones, including leptin. Indeed, we have confirmed the induction of phosphorylated STAT3 in the hindbrain after peripheral injection of leptin (data not shown). Some reports have also shown that peripheral leptin administration results in rapid induction of STAT3 phosphorylation in the NTS (Elias *et al.* 2000, Hosoi *et al.* 2002, Munzberg *et al.* 2003). In addition, the NTS has particularly high levels of ObRb expression (Leshan *et al.* 2006, Myers *et al.* 2009). These observations indicate that the hindbrain is involved in the interaction of GLP1 and leptin, which reduces food intake. In a previous study, we have also shown that coinjection of subthreshold doses of GLP1 and leptin significantly increased *Pomc* mRNA levels and decreased phosphorylation of AMPK in the hypothalamus (Poleni *et al.* 2012). These findings indicate that the signals caused by coinjection of GLP1 and leptin are ultimately integrated, at least in part, in the hypothalamus via the hindbrain and are responsible for reduced food intake.

In this study, we used midbrain-transected rats, in which the rostral pathway to the NTS was disrupted to investigate the role of the neural pathway from the hindbrain to the hypothalamus in the reduction of food intake induced by the interaction of GLP1 and leptin. Coinjection of subthreshold doses of GLP1 and leptin in midbrain-transected rats did not reduce food intake. This finding indicates that the neural pathway from the hindbrain to the hypothalamus is an important route involved in the food intake reduction induced by the interaction of GLP1 and leptin. Thus, satiety signals produced by coinjection of these hormones may reach the hindbrain and then be transmitted to the hypothalamus via certain neurotransmitters. A more complicated and sophisticated system is likely to integrate various signals in the hypothalamus. Further studies are needed to elucidate the relationship between neural circuits and the peripheral signals involved in feeding.

To investigate the hypothalamic nuclei that are activated by coinjection of GLP1 and leptin, we evaluated the expression pattern of Fos, an immediate early gene product associated with functionally activated neural circuits (Sagar *et al.* 1988, Chan *et al.* 1993). Fos expression in the hypothalamic arcuate nucleus or paraventricular nucleus was induced by coinjection of GLP1 and leptin in sham-operated rats, but not by a single administration of

Figure 2

Localization of Fos expression following administration of saline, GLP1 (10 nmol/kg), leptin (18.8 nmol/kg), or GLP1 (10 nmol/kg) and leptin (18.8 nmol/kg) to rats receiving midbrain-transection or sham surgery. (A and C) Fos expression was determined in the hypothalamic arcuate nucleus (A) and paraventricular nucleus (C) of sham-operated rats and midbrain-transected rats after i.p. administration of saline or GLP1 + leptin. 3V, third ventricle; scale bar, 100 μ m. (B and D) Number of Fos-positive cells in the arcuate nucleus (B) and paraventricular nucleus (D) of sham-operated rats and midbrain-transected rats after i.p. administration of saline or GLP1 + leptin. Data represent means $\pm$ s.e.m. ($n=5$ each).

* $P<0.05$ vs sham-operated saline; ** $P<0.01$ vs sham-operated saline (one-way ANOVA followed by Tukey-Kramer test). (E) Double immunostaining with Fos (red) and α -melanocyte-stimulating hormone (MSH, green) in the arcuate nucleus of sham-operated rats and midbrain-transected rats after i.p. administration of saline or GLP1 + leptin. Scale bar: 50 μ m. (F) Number of Fos-expressing α -MSH cells in the arcuate nucleus in sham-operated rats and midbrain-transected rats after i.p. administration of GLP1 and leptin. Data represent means $\pm$ s.e.m. ($n=5$ each). *** $P<0.001$ vs sham-operated GLP1 and leptin (Student's t -test).

Figure 3

Quantitative polymerase chain reaction analysis of proopiomelanocortin (POMC) in the hypothalami of sham-operated rats and midbrain-transected rats after i.p. administration of saline or of GLP1 (10 nmol/kg) and leptin (18.8 nmol/kg). Data were normalized against the amount of glyceraldehyde 3-phosphate dehydrogenase. Data represent means $\pm$ S.E.M. ($n=5$ each). *** $P<0.001$ vs sham-operated saline (one-way ANOVA followed by Tukey-Kramer test).

saline, GLP1, or leptin alone in sham-operated or midbrain-transected rats (data not shown). In contrast, coinjection of GLP1 and leptin in midbrain-transected rats did not induce Fos expression in either the arcuate nucleus or the paraventricular nucleus. Our double-immunostaining study showed that most of the α -MSH-positive neurons of the arcuate nucleus in the sham-operated rats expressed Fos protein after coadministration of GLP1 and leptin, but this was not the case in midbrain-transected rats. These findings imply that some signals produced by coinjection of GLP1 and leptin affect POMC neurons located in the arcuate nucleus via neural pathways from the hindbrain to the hypothalamus.

Previously, we have also shown that coinjection of subthreshold doses of GLP1 and leptin did not affect the mRNA expression of neuropeptide Y, agouti-related protein, or cocaine-and amphetamine-regulated transcript in the hypothalamic arcuate nucleus, whereas it significantly increased *Pomc* expression (Poleni *et al.* 2012). POMC is a precursor of α -MSH, which, when released from the axon terminals of POMC neurons, binds to and activates MC4R, thereby reducing food intake and body weight (Huszar *et al.* 1997). In the adult rodent brain, the majority of POMC neurons are located in the hypothalamic arcuate nucleus (Cone 2005, Padilla *et al.* 2012). Therefore, although we did not investigate POMC expression here in our micro-dissection of the arcuate nucleus, we assumed that our data for the whole

hypothalamus approximately reflected *Pomc* mRNA levels in the arcuate nucleus. We have also previously shown that central preadministration of MC4R antagonists blocks the effect of coinjection of GLP1 and leptin on food intake (Poleni *et al.* 2012). These findings indicate that coinjection of GLP1 and leptin ultimately reduces food intake via the POMC/MC4R system. We have shown here that the increase in *Pomc* mRNA expression after coinjection of GLP1 and leptin was blocked in midbrain-transected rats. This result indicates that the satiety signals produced by coinjection of GLP1 and leptin affect the POMC/MC4R system in the hypothalamus via first-order neurons located in the hindbrain.

AMPK acts as a general energy sensor and integrator of nutrient or hormonal signals in the hypothalamus (Kahn *et al.* 2005, Ramamurthy & Ronnett 2006). Hypothalamic AMPK activity is decreased by the action of anorectic hormones such as leptin (Andersson *et al.* 2004, Minokoshi *et al.* 2004), insulin (Kola 2008), or GLP1 (Seo *et al.* 2008). Furthermore, MC4R is involved in AMPK activity in the hypothalamus (Minokoshi *et al.* 2008). In this study, we have demonstrated that coinjection of subthreshold doses of GLP1 and leptin dramatically

Figure 4

Western blot analysis of phosphorylated AMP-activated protein kinase (pAMPK) in the hypothalami of sham-operated rats and midbrain-transected rats after i.p. administration of saline or of GLP1 (10 nmol/kg) and leptin (18.8 nmol/kg). The band intensity ratio of pAMPK to AMPK in sham-operated rats after saline administration was arbitrarily set as 1.0. Data represent means $\pm$ S.E.M. ($n=4$ each). ** $P<0.01$ vs sham-operated saline (one-way ANOVA followed by Tukey-Kramer test).

decreased the phosphorylation of AMPK in the hypothalamus of sham-operated rats, whereas this effect was blocked in midbrain-transected rats. Taken together, these findings show that the satiety signals provided by coinjection of GLP1 and leptin are transmitted, at least in part, via the neural pathway from the hindbrain to POMC neurons present in the arcuate nucleus, which in turn decrease the phosphorylation of AMPK through the POMC/MC4R system and thereby reduce food intake. Because MC4R is expressed in various brain areas, including the paraventricular nucleus, lateral hypothalamus, and ventromedial nuclei, AMPK phosphorylation needs to be evaluated in these hypothalamic areas. However, some reports have shown that MC4R located in the paraventricular nucleus is essential for food intake regulation (Balthasar *et al.* 2005, Cone 2006). Therefore, we surmise that our data regarding levels of AMPK phosphorylation in the whole hypothalamus mainly reflect the levels in the paraventricular nucleus. To confirm this conjecture, we will need to evaluate the levels of phosphorylation of AMPK in a future micro-dissection study of the paraventricular nucleus.

We have shown here that various interactive effects of GLP1 and leptin in the reduction of food intake were blocked in midbrain-transected rats. Our findings imply that identifying the transmitters involved in conveying the interactive signals of GLP1 and leptin from the hindbrain to the hypothalamus, as well as elucidating the interactive mechanism of GLP1 and leptin in influencing AMPK, will advance our interpretation of the physiological roles of feeding-related hormones. Investigating the effect of repeated coinjections of GLP1 and leptin on food intake reduction or weight loss will be important as well. Our data will help to further understand the mechanisms of feeding and energy balance from the perspective of multifactorial regulatory systems that underlie functions such as neurohormonal integration.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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