

ORIGINAL**Serotonergic regulation of galanin-induced selective macronutrient intake in self-selecting rats**

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Abstract : We investigated macronutrient intake after intraventricular injection of galanin (GAL, 3 nmol/5 μ l/rat) and/or serotonin (5-HT, 50 nmol/5 μ l/rat) in self-selecting fasted rats with preferences for either carbohydrates or fats. GAL injection significantly increased carbohydrate and total intake in all rats irrespective of macronutrient preference, whereas 5-HT alone did not affect macronutrient intake. The GAL-induced increase in total intake decreased to the level of saline controls when GAL was coinjected with 5-HT. The ratio of kilocalories of carbohydrates, fats and proteins (macronutrient energy ratio) after injections of GAL and/or 5-HT was similar to the saline control. In carbohydrate-preferring rats, GAL increased carbohydrate, protein and fat intake as well as total intake. Coinjection of GAL and 5-HT tended to decrease carbohydrate intake, but increase protein and fat intake. The macronutrient energy ratio after injection of GAL did not change, but the carbohydrate energy ratio decreased after 5-HT was injected, with or without GAL. In contrast, in fat-preferring rats, GAL significantly increased carbohydrate intake. Injection of 5-HT with or without GAL did not change total macronutrient intake. The macronutrient energy ratio did not change after GAL injection with or without 5-HT. These differences suggest that macronutrient preferences should be considered in any macronutrient intake study, and that serotonergic neurons play a part in the regulation of GAL-induced macronutrient intake. *J. Med. Invest.* 55 : 196-203, August, 2008

Keywords : galanin, serotonin, macronutrient selection, carbohydrate-preferring, fat-preferring, fasted rats

INTRODUCTION

The brain regulates food intake and quality and quantity of nutrients based on information via tongue, stomach, intestine and liver. There are many neurologically active molecules in the brain involved with regulation of food intake.

Galanin (GAL) is a human neuropeptide that is widely distributed in the central and peripheral nerv-

ous system (1) and is involved in regulation of food intake (1-4). Tempel, *et al.* (4) reported that injection of GAL into the hypothalamic paraventricular nucleus (PVN) significantly increases carbohydrate intake but does not affect protein intake in satiated rats fed macronutrient diets containing carbohydrate, protein or fat. In recent experiments, this self-selecting feeding paradigm has successfully revealed similar patterns of effects of neurotransmitters and drugs on diet selection, while differentiating the effects of drugs expected to act through different neurochemical systems (5). The neuromonoamine serotonin (5-HT) decreases macronutrient intake upon injection into the third ventricle and PVN (6, 7). Additionally, macronutrient

Received for publication March 4, 2008 ; accepted April 23, 2008.

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intake decreases following injection of 5-HT (8).

It has been shown that animals' baseline macronutrient preferences can significantly affect macronutrient selection in response to various feeding stimuli, thus these preferences may confound results unless accounted for through experimental design or statistical controls (9-13). The aim of this investigation was to elucidate the relationship between GAL and 5-HT in the regulation of post-fast macronutrient self-selection in carbohydrate- and fat-preferring rats.

MATERIALS AND METHODS

This study was performed in accordance with the guidelines for animal experimentation of Kyoto Prefectural University.

Animals

Male Sprague-Dawley rats were purchased from SLC Japan (Shizuoka, Japan) and housed individually in hanging wire-mesh cages (35 × 22 × 15 cm) with lights on from 22 : 00 to 10 : 00. The ambient room temperature was maintained at 22-24°C. After fasting 16 h, the animals were allowed to select among foods of known carbohydrate, protein and fat content between 10 : 00 and 18 : 00. The animals were allowed *ad libitum* access to tap water.

Experimental diets

A three-choice macronutrient diet paradigm was used ; that is, rats self-selected from three food glass jars, each containing a single macronutrient source supplemented with vitamins and minerals : carbohydrate (corn starch and sucrose), fat (vegetable shortening and salad oil), and protein (casein) (Table 1). The glass jars were placed in the wire-

mesh cages, and their positions were alternated daily.

Rats were categorized into two groups according to macronutrient preference under self-selection conditions. Carbohydrate-preferring rats were those whose ratio of carbohydrate intake to fat caloric intake (C/F ratio) was greater than 1. Those whose C/F ratio was less than 1 were considered fat-preferring rats, in accordance with Bray, *et al.* (2, 3). In the present study, we measured each rat's C/F ratio in the 30 min after intraventricular saline injection because we found that the C/F ratio up to 480 min after saline injection was not significantly different.

Stereotaxic surgery

Rats were anesthetized intraperitoneally with pentobarbital sodium (1 ml/kg body weight, Nembutal™, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and implanted with a chronic, unilateral, 24-gauge, stainless steel guide cannula (length 11 mm). With the incisor bar placed 3.3 mm below the interaural line, the coordinates used were : lateral 1.4 mm ; anterior -0.8 mm from bregma ; depth 3.4 mm from the skull surface. These coordinates were chosen to allow the cannula and the injector needle (31-gauge) into the lateral ventricle. Correct positioning was verified by emergence of spinal fluid into the guide cannula. The guide cannula was then anchored to the skull with four stainless steel screws and dental cement (Nissin Dental Products Inc., Kyoto, Japan) and closed with a polyethylene tube cap. All animals were allowed to recover for at least 7 d before experimental testing.

Materials

Galanin (Peptide Institute, Inc., Osaka, Japan) and serotonin (Research Biochemicals, Natick, MA, USA) were dissolved in sterile, normal saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) prior to injection.

Experimental procedures

Seven-week old male Sprague-Dawley rats implanted with guide cannulae into the lateral ventricle were injected with GAL (3 nmol) and/or 5-HT (50 nmol) dissolved in 5 µl saline, or injected with 5 µl saline only (control). GAL and/or 5-HT was injected intraventricularly at 10 : 00 to rats previously fasted for 16 h. These treatments were followed by injection of saline (5 µl/rat) for 2 d control period. The ambient room light was turned off from

Table 1 Composition of experimental diets.

	(g/kg)	Protein diet	Carbohydrate diet	Fat diet
Casein ¹⁾		935	-	-
α-Starch ²⁾		-	623	-
Sucrose ³⁾		-	312	-
Shortening ⁴⁾		-	-	770
Salad oil ⁵⁾		-	-	100
Mineral mixture ^{1,6)}		35	35	70
Vitamin mixture ^{1,6)}		10	10	20
Cellulose ¹⁾		20	20	40

¹⁾ Oriental Yeast Co., Ltd. ²⁾ Matsutani Chemical Industry Co., Ltd. ³⁾ Itochu Seito Co., Ltd. ⁴⁾ Nippon Lever k.k. ⁵⁾ Fuji Oil Co., Ltd. ⁶⁾ AIN-76™

10:00 to 22:00. Rats were simultaneously allowed access to the three macronutrient sources (carbohydrate, protein and fat) (Table 1) from 10:00 to 18:00. Food intake was measured 30 min after intraventricular injections.

Statistical analysis

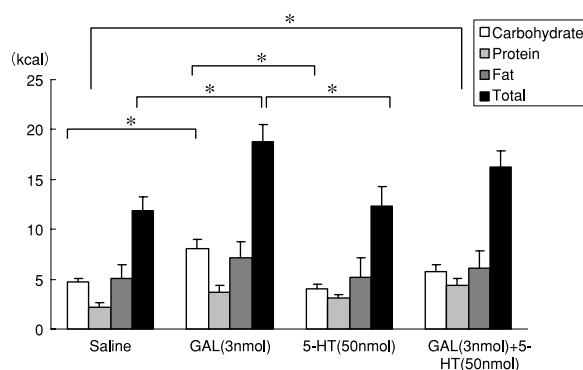
Macronutrient intake (kcal) in response to GAL and/or 5-HT was compared to those obtained after saline vehicle injection (control) using one-way analysis of variance (ANOVA) with repeated measures on diets (three-choice) and materials (GAL and 5-HT). Secondary comparisons were made using the Tukey-Kramer adjustment. A one-way ANOVA was used to evaluate differences in macronutrient intake between pre- and post-treatment. Proportions of macronutrient intake compared to total intake following GAL and/or 5-HT injection were analyzed using two-factor ANOVA. Secondary comparisons were done using the Dunnett adjustment. SPSS 11.0 J for Windows (SPSS Inc.) was used for analyzing the data. A P-value of less than 0.05 was considered to be significant.

RESULTS

Effects of GAL and 5-HT on macronutrient intake in all rats

GAL (3 nmol/5 μ l/rat) was intraventricularly injected. All rats irrespective of macronutrient preferences significantly increased total food intake after injection of GAL. Carbohydrate, protein and fat intake increased, with carbohydrate intake increasing significantly after GAL injection (Fig. 1a). When 5-HT (50 nmol/5 μ l/rat) was intraventricularly injected, macronutrient intake did not change significantly compared with the saline control (Fig. 1a). Moreover, when GAL and 5-HT were simultaneously injected, carbohydrate and fat intake did not increase significantly but protein intake increased significantly. Carbohydrate intake following injection of GAL alone was significantly higher than the saline control level, although carbohydrate intake following coinjection of GAL and 5-HT did not change compared with the saline control level (Fig. 1a). The ratio of kilocalories of carbohydrates, fats and proteins consumed (the macronutrient energy ratio) following treatment did not change regardless of whether GAL was injected with or without 5-HT (Fig. 1b).

(a) Macronutrient energy intakes



(b) Macronutrient energy ratio

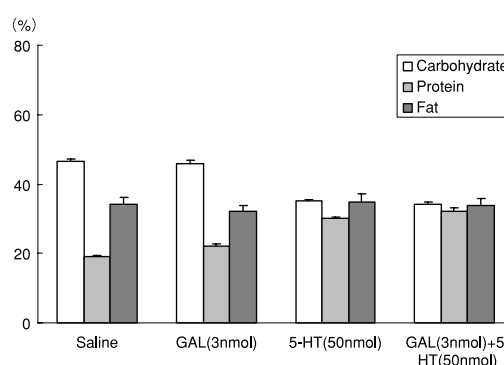


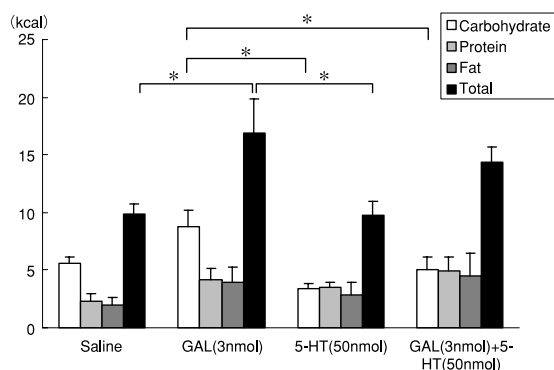
Fig. 1 Effects of intraventricular injections of saline, GAL (3 nmol), 5-HT (50 nmol), GAL (3 nmol) and 5-HT (50 nmol) in all rats. (a) The carbohydrate, protein, fat and total food intake (kcal) in the first 30 min following the injections is shown. (b) Intake (kcal) of carbohydrate, protein and fat as a percent of the total food intake (kcal) 30 min after injection are shown. Values represent mean \pm S.E.M. *) $p < 0.05$ ($n = 14$)

Effects of GAL and 5-HT on macronutrient intake in carbohydrate-preferring rats

After GAL (3 nmol/5 μ l/rat) was injected intraventricularly into carbohydrate-preferring rats, their carbohydrate, protein and fat intake increased proportionately, and total food intake increased significantly (Fig. 2a). The macronutrient energy ratio did not change significantly (Fig. 2b); the fraction of kilocalories from carbohydrate (carbohydrate energy ratio) was still 60% of the total consumed (Fig. 2b).

After injection of 5-HT (50 nmol/5 μ l/rat) into carbohydrate-preferring rats, carbohydrate intake decreased to the same level as protein and fat intake (Fig. 2a), causing the macronutrient energy ratio to be significantly different from saline controls (Fig. 2b). The carbohydrate energy ratio after injection of 5-HT decreased from 59% to 37% of the kilocalories consumed compared with the sa-

(a) Macronutrient energy intakes



(b) Macronutrient energy ratio

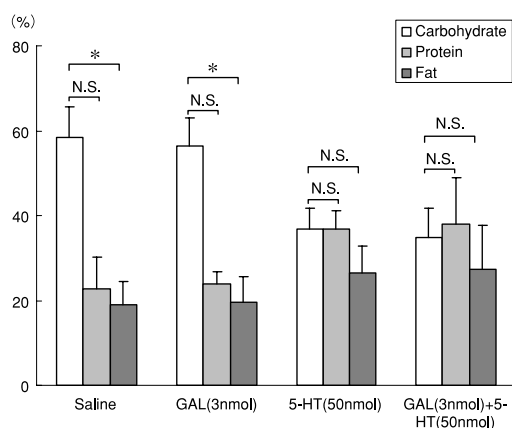


Fig. 2 Effects of intraventricular injections of saline, GAL (3 nmol), 5-HT (50 nmol), GAL (3 nmol) and 5-HT (50 nmol) in carbohydrate-preferring rats. (a) Intake (kcal) of carbohydrate, protein, fat and total intake (kcal) 30 min after injection is shown. (b) Intake (kcal) of carbohydrate, protein and fat are shown as a percent of the total food intake (kcal) 30 min after injection. Values represent mean \pm S.E.M. *, $p < 0.05$ ($n = 7$)

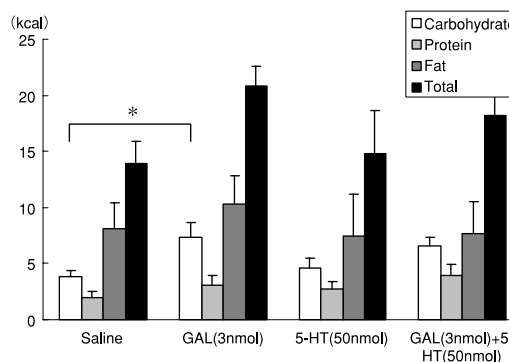
line control. In contrast, the ratio of protein kilocalories to total intake increased from 23% to 37%, and the ratio of fat kilocalories to total intake increased from 19% to 27%. Total intake was the same after coinjection of GAL and 5-HT as after 5-HT injection alone (Fig. 2a). Coinjection of GAL and 5-HT changed the ratio of macronutrients consumed compared with the saline control, and exhibited the same pattern as 5-HT injection alone (Fig. 2b).

Effects of GAL and 5-HT on macronutrient intake in fat-preferring rats

After injection of GAL into fat-preferring rats, carbohydrate intake increased significantly compared with the saline control but not after simultaneous injection of GAL and 5-HT (Fig. 3a). There were no significant effects on protein, fat and total intake (Fig. 3a). The macronutrient energy ratio

did not change significantly after injection of GAL alone, 5-HT alone, or GAL and 5-HT into fat-preferring rats (Fig. 3b).

(a) Macronutrient energy intakes



(c) Macronutrient energy ratio

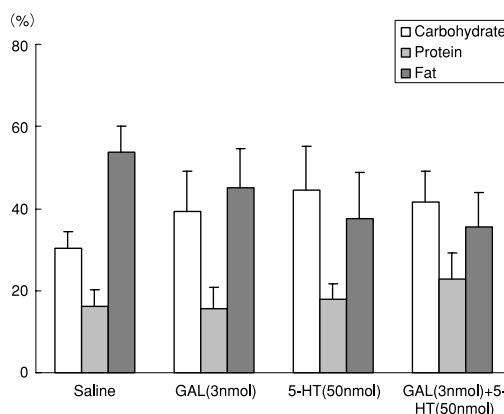


Fig. 3 Effects of intraventricular injections of saline, GAL (3 nmol), 5-HT (50 nmol), GAL (3 nmol) and 5-HT (50 nmol) in fat preferring-rats. (a) Intake (kcal) of carbohydrate, protein, fat and total intake (kcal) 30 min after injection is shown. (b) Intake (kcal) of carbohydrate, protein and fat as a percentage of the total food intake (kcal) 30 min after injection are shown. Values represent mean \pm S.E.M. *, $p < 0.05$ ($n = 7$)

DISCUSSION

Galanergic receptors exist in the nucleus of the solitary tract and the lateral parabrachial nucleus (2). It has been reported that GAL increases macronutrient intake after injection into the third ventricle, paraventricular nucleus (PVN) or amygdaloid body (1-4). Conversely, 5-HT, a neuromonoamine, decreases macronutrient intake after injection into the third ventricle and PVN (6, 15-17). It has been reported that macronutrient intake decreases after injection of 5-HT (8).

GAL has important inhibitory actions on central 5-HT neurotransmission by affecting 5-HT_{1A} receptor-mediated events (18, 19). However, there have been few reports about macronutrient intake controlled by these systems. Therefore we investigated macronutrient intake and macronutrient energy ratio after injection of GAL and/or 5-HT in self-selecting, fasted rats that showed preference for carbohydrate or fat.

We first investigated GAL and 5-HT effects on macronutrient intake in all rats irrespective of macronutrient preferences. GAL significantly increased carbohydrate intake compared with the saline control, causing total food intake to increase significantly. Koegler, *et al.* (2) reported that when injected into the third ventricle GAL reliably stimulated carbohydrate intake within 30 min, and thus total food intake also increased significantly. The results of our studies correspond with the above report. The injection of 5-HT (50 nmol/5 μ l/rat) alone did not affect macronutrient intake in our study. These results do not correspond with previous reports (20, 21), which found that intraventricular injection of 5-HT (more than 200 nmol/rat) decreased macronutrient intake. It is possible that the quantity of 5-HT injected was lower in our study (50 nmol/rat).

GAL (3 nmol/5 μ l/rat), which significantly increased carbohydrate intake, and 5-HT (50 nmol/5 μ l/rat), which did not affect macronutrient intake, were simultaneously injected into the ventricle. Coinjection of GAL and 5-HT did not cause an increase in carbohydrate intake. It has been reported that GAL and 5-HT coexist and are coexpressed in the dorsal raphe nucleus (DRN) of rats (22, 23), and that GAL modulates the activity of 5-HT neurons in the DRN via galanergic receptor 1 (GALR1) (22). These results suggest that although this amount of 5-HT did not decrease macronutrient intake, it did stimulate the 5-HT neural pathways and suppress the effects of GAL. In addition, GAL is a neurotransmitter that stimulates macronutrient intake, while 5-HT is a neurotransmitter that suppresses it. Our results suggest that GAL and 5-HT may interact in the regulation of macronutrient intake.

It has been reported that baseline macronutrient preferences are an important consideration in studies examining the effects of pharmacological or physiological treatments on macronutrient selection (3, 13, 24, 25). The present study was designed to qualify the neuromodulatory effects of GAL and

5-HT on macronutrient intake in self-selecting rats with macronutrient preferences. There have been a few studies that mention regulation of macronutrient selection or that took macronutrient preferences into account (14, 24, 26). It has been reported that rats show variation in preferences for specific macronutrients when carbohydrate, protein and fat are offered simultaneously (24).

Macronutrient preferences were characterized based on the ratio of fat intake to carbohydrate intake, e.g., carbohydrate-preferring rats were those whose fat to carbohydrate ratio was less than 1, and fat-preferring rats were those whose fat to carbohydrate ratio was greater than 1 (25). We also used these categories to divide rats into two groups by macronutrient preferences. We considered that macronutrient intake in the first 30 min after saline injection was appropriate for this categorization. The categories of macronutrient preferences used in this study could be widely used in other studies to control for the preference variable (3, 25).

In the present study, injection of GAL into ventricles of carbohydrate-preferring rats caused their carbohydrate, protein and fat intake to increase. Therefore total intake increased significantly although the macronutrient energy ratio remained similar to the saline control. These results suggest that GAL does not affect macronutrient selection in carbohydrate-preferring rats. However, injection of GAL into the ventricles of fat-preferring rats caused only carbohydrate intake to increase significantly. It has been shown that paraventricular GAL injection causes a significant increase in plasma noradrenaline (NA) levels (27), and that noradrenergic neurons play a role in appetite and ingestion responses to glucoprivation (11). Furthermore, sweets have been shown to stimulate the endogenous opioid system both by inducing a release of β -endorphin and by increasing the binding affinity of opioids at receptor sites (28, 29). The differences in macronutrient preferences may indicate variation in responses such as these to carbohydrate availability.

In the present study, GAL was injected intraventricularly into fasted rats, and carbohydrate-preferring rats increased intake of all three macronutrient groups, which significantly increased total intake. It has been shown that GAL concentration in the PVN is significantly higher in carbohydrate-preferring rats than in fat-preferring rats (30). GAL is also a potent modulator of 5-HT-mediated transmission, particularly 5-HT_{1A} receptor-mediated func-

tions (18, 31-33). Furthermore, paraventricular GAL injection is known to cause a significant increase in plasma NA levels (27). In the present study, intraventricular GAL injection increased carbohydrate intake in carbohydrate- and fat-preferring rats, suggesting that intraventricular GAL injection enhances endogenous release of NA and increases carbohydrate intake in both carbohydrate- and fat-preferring rats.

In the present study, intraventricular 5-HT injection in carbohydrate-preferring rats slightly decreased carbohydrate intake, but slightly increased protein and fat intake compared with controls, whereas 5-HT injection did not change macronutrient intake in fat-preferring rats. The macronutrient energy ratio in both fat-preferring and carbohydrate-preferring rats after injection of 5-HT was almost the same compared with controls. It has been shown that the urge to consume carbohydrate-rich food is regulated by serotonergic neurons and that carbohydrate appetite is triggered by cerebral 5-HT deficiency (34). Intraventricular 5-HT injection studies have confirmed the involvement of central 5-HT in reducing food intake in different species (21).

In carbohydrate-preferring rats, the increase in carbohydrate intake and total intake after injection of GAL returned to control levels upon coinjection with 5-HT; the macronutrient energy ratios in this case were similar to controls. Total intake did not change after injection of 5-HT alone because carbohydrate intake decreased while protein and fat intake increased.

Several lines of evidence (16, 26) have led to the proposal that the serotonergic system may indirectly oppose the catecholaminergic system to control feeding behavior. In addition to the observation that plasma NA levels rise upon paraventricular GAL injection (27), it has also been observed that serotonergic stimulation of the PVN inhibits the NA-elicited feeding response in this nucleus (17). In the present study intraventricular GAL injection increased not only intake of carbohydrate but also of protein and fat. These results might be due to decreased 5-HT activity. The fact that the increase in carbohydrate intake after injection of GAL returned to control levels after coinjection of GAL and 5-HT likewise suggests that serotonergic neurons play an important role in regulation of feeding behavior.

In fat-preferring rats, the increase in carbohydrate intake observed after injection of GAL was

not observed after coinjection of GAL and 5-HT. The macronutrient energy ratio observed after injection of 5-HT with or without GAL was unchanged compared to controls (Fig. 3b), indicating that 5-HT injection did not affect macronutrient intake in fat-preferring rats. These results suggest that 5-HT neurons may not be involved in macronutrient selection in fat-preferring rats.

It has been reported that GAL increases macronutrient intake in satiated rats (1, 2, 4, 35), and that the inhibitory effects of 5-HT on food intake are stronger in satiated rats than in fasting rats (17). In the present study, we investigated macronutrient selection only in fasted rats. Further studies are needed to clarify the disparate effects of fasting and satiety conditions on macronutrient intake.

In conclusion, this study suggests that macronutrient preferences might be considered in future macronutrient intake studies, and that serotonergic neurons play a role in the regulation of GAL-induced macronutrient intake.

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