Vagal Blocking for Obesity Control: A Possible Mechanism-of-Action

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Helene Johannessen[1], David Revesz[2], Yosuke Kodama[1], Nikki Cassie[3], Karolina P Skibicka[4], Perry Barrett[3], Suzanne L Dickson[4], Jens J Holst[5], Jens F Rehfeld[6], Geoffrey van der Plasse[7], Roger AH Adan[7], Bård Kulseng[8], Elinor Ben-Menachem[2], Chun-Mei Zhao[1], Duan Chen[1,8]

[1]Department of Cancer Research and Molecular Medicine, NTNU - Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Clinical Neuroscience and Rehabilitation, The Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden; [3]Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland; [4]Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden; [5]Department of Biomedical Sciences, the Panum Institute, University of Copenhagen, Copenhagen, Denmark; [6]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark; [7]Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, Netherlands; [8]Center for Obesity Research, Department of Surgery, St. Olavs Hospital, Trondheim University Hospital

Correspondence to Professor Duan Chen, MD, PhD, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel: +47 725 73320. Fax: +47 725 76612. Mob: +47 984 09 675. E-mail: duan.chen@ntnu.no.

Abstract

Purpose: Recently, the US FDA has approved "vagal blocking therapy or vBLoc® therapy" as a new treatment for obesity. The aim of the present study was to study the mechanism-of-action of "VBLOC" in rat models.

Materials and Methods: Rats were implanted with VBLOC, an intra-abdominal electrical device with leads placed around gastric vagal trunks through an abdominal incision and controlled by wireless device. Body weight, food intake, hunger/satiety, and metabolic parameters were monitored by a comprehensive laboratory animal monitoring system. Brain-gut responses were analyzed physiologically.

Results: VBLOC reduced body weight and food intake, which was associated with increased satiety but not with decreased hunger. Brain activities in response to VBLOC included increased gene expression of leptin- and CCKb-receptors, interleukin-1β, tumor necrosis factor and transforming growth factor β1 in the brainstem, increased CCK, somatostatin and tyrosine hydroxylase in the hippocampus, increased NPY, AgRP and Foxa2 in the hypothalamus, and reduced CCKb receptor, melanocortin 4 receptor and insulin receptor in the hypothalamus. Plasma concentrations of CCK, gastrin, glucagon, GLP-1 and PYY and gastric acid secretion were unchanged in response to VBLOC.

Conclusion: Based on the present study, we suggest that VBLOC induces satiety through vagal signaling, leading to reduced food intake and loss of body weight.

Key Words: Body weight, Food intake, Gut-brain axis, Rats, Vagus nerve

Introduction

The number of obese individuals is increasing rapidly, leading to a global obesity epidemic. So far, only bariatric surgery has demonstrated long-term therapeutic effects, and therefore the use of surgery to treat obesity is increasing (1, 2). Considering the large number of obese patients, the risk of surgical complications and high surgery-related cost, the development of minimal invasive procedures to treat obesity is urgently needed (3).

The gut-brain axis is known to play an important role in the pathogenesis of obesity. Nutritional and metabolically relevant information is conveyed to the brain by gut-produced hormones and the vagus nerve (4). The vagus nerve responds to mechanical and chemical stimuli from the gastrointestinal tract and transmits satiety signals to sites in the central nervous system that regulate eating behavior (5-7). Through the nucleus of the tractus solitarius (NTS) in the brainstem, afferent vagal signals may be relayed to reach several parts of the brain including the parabrachial nucleus, reticular formation, hippocampus, amygdala and hypothalamus (8). The hypothalamus is regarded as a key regulatory component of a central network for food intake where orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons are thought to play a central role (5). However, the brainstem also contributes in food intake and body weight regulation (9). In addition to the regulation of food intake, the vagus nerve also regulates gastric acid production and pancreatic secretion (10, 11). The central role of the vagus in the regulation of food intake and energy expenditure makes it an ideal target for new less or non-invasive procedures to treat obesity.

Recently, the US FDA has approved “vagal blocking therapy or vBLoc® therapy” by which an intra-abdominal electrical device with leads is placed laparoscopically around the vagus nerve as a new treatment for obesity (12, 13). The term VBLOC has been often used in

clinical trials, patent applications and the US FDA approval documents. VBLOC was initially believed to be of "vagotomy effect" but in fact this is unlikely as pointed out by the FDA approval letter, i.e. "the specific mechanisms for weight loss due to the use of the device are unknown". Hence, the aim of this study was to study the mechanism-of-action (MOA). To this end, we utilized animal models to examine how VBLOC impacted upon body weight, food intake, hunger/satiety, eating behavior, metabolic parameters, gut hormone concentrations, gastric acid secretion and the expression profile of candidate energy balance-regulating genes in the hypothalamus, brainstem and hippocampus.

Materials and methods

Animals

Rats (adult, male, Sprague-Dawley) were purchased from Taconic (Ejby, Denmark) and Janvier Labs (France). Rats (adult, female, Long Evan) were obtained at NTNU, Trondheim, Norway. They had free access to tap water and standard rat pellet food (RM1 811004, Scanbur BK AS, Sweden). Three to four animals were housed together in individually ventilated cages on wood chip bedding with a 12-hour light/dark cycle, room temperature of 22ºC and 40-60% relative humidity. The standard housing conditions were specific pathogen free and in agreement with FELASA (Federation of European Laboratory Animal Science Association) recommendations. All animals were euthanized between 0800-1000 A.M. Thus, the plasma concentrations of gut hormones measured at sacrifice could be considered as post prandial levels. Animal experiments were performed according to the guidelines for the design and statistical analysis of experiments using laboratory animals after being approved by the Norwegian National Animal Research Authority (Forsøksdyrutvalget, FDU). All applicable institutional and national guidelines for the care and use of animals were followed.

In all studies regarding eating behaviour the animals were acclimatized to the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH USA) for 24h before data collection. At data collection the animals were kept in CLAMS for 48h, and data from the last 24h were used for analysis. Before CLAMS the rats were habituated to their normal food as powder for three days, as the food in CLAMS is in powder form. Body weight development was followed throughout the study period (three times per week).

Experimental design

Pilot experiment

The paradigm of VBLOC between 0.5mA – 2.0 mA with 30 Hz, 500 μs, 30 s ON and 5 min OFF was chosen according to the safety, tolerance and possible efficacy assessments in response to the long-term VBLOC.

Short-term VBLOC

In the first experiment, rats (Sprague-Dawley) received VBLOC (2 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) while they were subjected to *in vivo* electrophysiological recording in the hippocampus. In the second experiment, rats (Sprague-Dawley) were acclimatized to Bollman cages for 3 hours at 3 separate occasions before and after VBLOC and gastric fistula implantation, and then subjected to gastric acid secretion measurement (baseline and pentagastrin-stimulation). After gastric acid output measurement, VBLOC was turned off. Three days after, rats received VBLOC (2 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) for 48h while eating behaviour and metabolic parameters were measured in CLAMS (14, 15). The animals were randomized into VBLOC and control groups (6 rats per group). The control group received the same implantations without any stimulation. Immediately after

48h of VBLOC, the rats were euthanized and brain samples were collected for *in situ* hybridization, and plasma was collected for radioimmunoassay.

Long-term VBLOC

In the first experiment (Sprague-Dawley rats), VBLOC was started 4 weeks after implantation of the device which was constantly ON while the current (mA) was gradually increased for 6-8 weeks. Each rat was placed in CLAMS at 4 time points for measurements of eating behavior and metabolic parameters; 3 weeks after VBLOC implantation (before stimulation, baseline), at 0.5 mA stimulation, at 1 mA stimulation and at 2 mA stimulation. The settings were 30 Hz, pulse width 500 μs and the ON and OFF time were 30 s and 5 min. The VBLOC and control groups consisted of 9 and 4 rats per group, respectively. The control group had the same implantation with no stimulation. At euthanization brain samples were taken for Taqman array analysis and RNA Sequencing. Plasma was collected for radioimmunoassay. In the second experiment (Long Evan rats), smaller VBLOC devices (μVBLOC) were developed due to device-size-related irritation. The animals were randomized into VBLOC and control groups (4 rats per group). VBLOC was continuously ON at 2 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF for 2 weeks. At euthanization plasma was collected for radioimmunoassay.

Statistical Analysis

The results are expressed as mean ± SEM. Statistical comparisons were performed using independent *t*-test between the surgical groups. ANOVA with Sidak test was performed for energy expenditure statistics, while ANOVA with Tukey's test was performed to determine eating behavior and metabolic parameters. A p-value of <0.05 (two-tailed) was

considered statistically significant. The data analysis was performed in SPSS version 15.0 and 20.0.

Supplementary Materials and Methods

Information regarding animal surgery, determination of eating behavior and metabolic parameters, gastric acid output measurement, taqman array, *in situ* hybridization, RNA sequencing, radioimmunoassay, *in vivo* electrophysiology and statistical analysis are provided in the Supplementary Materials and methods.

Results

In vivo electrophysiology tetrodes recorded VBLOC-induced activity in the brain (Fig. 1a). In response to the short-term VBLOC (48h at 2 mA current), gene expression of NPY and AgRP in the arcuate nucleus (ARC) of the hypothalamus as well as NPY gene expression in dorsomedial hypothalamic nucleus (DMH) were up-regulated, but NPY gene expression in the brainstem, and plasma concentrations of gut hormones were unchanged (Fig. 1b-e). On the other hand, basal and pentagastrin-stimulated gastric acid secretions were unchanged in response to the short-term VBLOC (Fig. 1f).

During the short-term VBLOC (48h at 2 mA current), there were no changes in body weight, food intake, eating behavior, and metabolic parameters including energy expenditure (Table 1). The animals showed no signs of discomfort stemming from the VBLOC.

In response to the long-term VBLOC in which the current was gradually increased (from 0.5 mA to 2 mA), the body weight and food intake (g) were reduced as the current was increased, eventually reaching reductions of 10% ($p>0.05$) and 30% ($p<0.05$), respectively (Fig. 2a-c). Energy expenditure (kcal/h/body weight) was reduced compared to baseline values (Fig. 2d). Satiety ratio particularly during nighttime was increased, but the number of

meals ("hunger index") was unchanged (Fig. 2e,f). When the current was started at 2 mA, a 10% body weight reduction was achieved within 1 week ($p<0.05$) (Fig. 2a). Additional parameters of eating behavior and metabolism were unchanged after the long-term VBLOC (Table 2).

Analysis of the hypothalamus showed a significant increase in gene expression of NPY and forkhead box protein A2 (Foxa2), a decrease for cholecystokinin b (CCKb) receptor and a tendency for decreased expression of the melanocortin 4 receptor (MC4R) and insulin receptor (Insr) (Fig. 3a). In the brainstem, the gene expression of leptin- and CCKb -receptors, and interleukin-1β (IL1b), tumor necrosis factor (Tnf) and transforming growth factor β1 (Tgfb1) was increased (Fig. 3b). In the hippocampus, VBLOC increased expression of CCK, somatostatin and tyrosine hydroxylase (crucial for the production of dopamine) (Fig. 3c,d). The plasma concentrations of CCK, gastrin, glucagon, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) were unchanged (Fig. 3e,f).

Discussion

The brain-gut axis functions in both afferent and efferent directions via the vagus nerve. To examine the effects of VBLOC on either efferent or afferent or both directions, the gut responses (gastric acid secretion and gut hormones) and the brain responses (electrical activity and gene expression) were analyzed. VBLOC was originally derived from vagus nerve stimulation. In the neuromodulation spectrum, it has been postulated that low frequency electrical impulses increases the neurophysiologic activity, resulting in neurostimulation. The converse occurs with higher frequency electrical impulses, resulting in neuroblocking. In the present study the two electrodes of VBLOC were wrapped around both the anterior and posterior nerve branches together with extra tissue from the esophagus. The cathode was placed on the proximal part and anode on the distal part (Fig. 4a). During VBLOC the cathode

induced depolarization, leading to a stimulation of the afferent fibers. The afferent fibers are believed to be involved in the sensory feedback on satiety, satiation and energy metabolism. As shown in the present study, VBLOC induced satiety signaling in the brainstem and satiety ratio (but not hunger) in eating behavior. During VBLOC the distal anode hyperpolarized the membrane and imposed an anodic block on the efferent fibers (Fig. 4b). The stimulation of vagus nerve is known to induce gastric acid secretion via the efferent fibers on the parietal cells and the gastrin-producing G cells in the stomach (16, 17). As shown in the present study, VNS did not affect the acid secretion and gut hormones (particularly gastrin, CCK, GLP-1 and PYY), indicating a functional blockade of vagal efferent fibers, named as VBLOC. The long-term application of VBLOC (6-8 weeks), particularly with the application of the highest current for 2 weeks, resulted in reduced food intake and body weight in the rat model.

Previously, several animal studies reported reduced body weight gain and food intake in response to VNS that was applied mostly at the left vagal branch in the neck. However, the results reported were of low quality due to large variations, and no SD or SEM values were given in the figures (18-23). In the present study, the short-term (48h) VBLOC was unable to alter eating behavior. However, the long-term (6-8 weeks) VBLOC, particularly when started with a high current (2 weeks), reduced food intake and body weight. This is in line with a report that subdiaphragmatic VNS attenuated weight gain in obese minipigs during 14 weeks (24).

It should be noticed that in response to VBLOC, the gene expression, especially NPY/AgRP, in the hypothalamus, was unexpectedly changed with a drive for increased food intake and a reduction in energy expenditure (5, 25-27). However, the gene expression in the brainstem and the hippocampus was changed, which was compatible with an anorexigenic drive in response to VBLOC, being consistent with the weight loss. The brainstem is the first target for vagal afferent fibers in the central nervous system. VBLOC-induced afferent signals

can be sent from the brainstem to the hippocampus, which has also been observed in previous studies (28). In fact, the hippocampus is not only involved in memory, but also has a role in incentive motivation (e.g. for food), in the processing of hormonal signals (CCK, ghrelin, and motilin), and in the regulation of food intake (29-33).

Gut hormones are thought to be central in appetite regulation in the gut-brain axis, and satiety hormones such as CCK, glucagon, GLP-1, and PYY are known to reduce food intake (4). However, these gut hormones were unaltered after both short and long-term VBLOC. Thus, we speculate that there are the two pathways, i.e. gut hormone – hypothalamus and vagus nerve – brainstem, within the gut-brain axis, and that these are independent each other. Ghrelin levels were not included due to a technical problem. However, it is unlikely that ghrelin plays a role in VBLOC-induced weight loss, because the hunger index was not changed during both daytime and nighttime.

Taken together, we suggest that the VBLOC, by activating the vagal signaling to the brainstem and hippocampus and blocking the vagal signaling to the gut, leads to increased satiety, reduced food intake and eventually the loss of body weight. It is our hope that this study will serve as the basis for many more experimental studies to better understand the mechanism-of-action of VBLOC therapy in the future.

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Conflict of Interest Disclosure Statement

Authors 1-7, 9-15 have nothing to disclose in connection with this study.

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Ethical Approval

All applicable institutional and/or national guidelines for the care and use of animals were followed.

Informed Consent

Does not apply

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Figure Legends

Fig. 1: **The effects of short-term VBLOC. A.** *In vivo* electrophysiological recordings of stimulation-induced activity in the brain. The graph depicts the average waveform of measured activity, the insert show the raw data from which the average waveform was constructed. **B**. Effects of short-term VBLOC (48h) on mRNA expression of NPY, AgRP and POMC in the arcuate nucleus (ARC) and dorsomedial hypothalamic nucleus (DMH). *:$p<0.05$ between sham-VBLOC (white) and VBLOC (black). **C**. Effect of short-term VBLOC (48h) on NPY expression in the brainstem. Note: no significant difference between sham-VBLOC (white) and VBLOC (black). **D&E**. Effects of short-term VBLOC (48h) on plasma concentrations of gut hormones. Note: no significant difference between sham-VBLOC (white) and VBLOC (black). **F**. Effects of VBLOC on baseline and pentagastrin (PG)-stimulated gastric acid secretion. Note: no significant difference between any VBLOC currents with or without PG injection.

Fig. 2: The effects of long-term VBLOC on body weight, eating behavior and metabolic parameters. A. Effects of different currents of VBLOC on body weight. Note: when current was started at 2 mA a weight reduction was accomplished instantly ($p<0.05$) (white). **B.** Effects of long-term VBLOC on food intake (g/24h). *:$p<0.05$ between sham-VBLOC (white) and VBLOC (black). **C.** Effects of long-term VBLOC on food intake (g/100g body weight). *:$p<0.05$ between values before VBLOC and at 2 mA. Note: white columns represent sham-VBLOC, black columns represent VBLOC. **D.** Effect of long-term VBLOC on energy expenditure (kcal/h/body weight). **:$p<0.01$ between values before VBLOC and at 2 mA (ANCOVA with Sidak test). Note: white columns represent sham-VBLOC, black columns represent VBLOC. **E.** Effects of long-term VBLOC on satiety ratio (min/g) during nighttime. Note: *$p<0.05$ (one-tailed) between sham-VBLOC (white) and VBLOC (black) at

2 mA. **F.** Effects of long-term VBLOC on number of meals (24h). Note: no significant difference between sham-VBLOC (white) and VBLOC (black).

Fig. 3: **The effects of long-term VBLOC (2 mA) on brain gene expression and gut hormones. A.** Effects of long-term VBLOC (2 mA) on hypothalamic mRNA expression of neuropeptide Y (NPY), forkhead box protein A2 (Foxa2), CCKb receptor, melanocortin 4 receptor (Mc4r) and insulin receptor (Insr). *:p<0.05 between sham-VBLOC (white) and VBLOC (black). Note: p=0.067 on Insr and p=0.086 on Mc4r (two-tailed). **B.** Effects of long-term VBLOC (2 mA) on mRNA expression of leptin receptor (Lepr) and CCKB/2 receptor (Cckbr), interleukin-1β (IL1b), tumor necrosis factor (Tnf) and transforming growth factor β1 (Tgfb1) in the brainstem. *:p<0.05 between sham-VBLOC (white) and VBLOC (black). **C&D.** Effects of long-term VBLOC (2 mA) on mRNA expression of CCK, somatostatin and tyrosine hydroxylase in hippocampus. *, **, ***:p<0.05, 0.01, 0.001 between sham-VBLOC (white) and VBLOC (black). **E&F.** Effects of long-term VBLOC (2 mA) on plasma concentrations of gut hormones. Note: no significant difference between sham-VBLOC (white) and VBLOC (black).

Fig. 4: Overview of VBLOC device position and function. A. Photograph showing the position of the implanted VBLOC electrodes around the gastric vagus nerve in a rat. **B.** Drawing illustrating how the cathode induces depolarization and stimulation of the afferent fibers, while the anode hyperpolarizes the membrane and imposes an anodic block on the efferent fibers.

Table 1: Responses of body weight, food intake, eating behavior and metabolic parameters to short-term VBLOC at 2 mA.

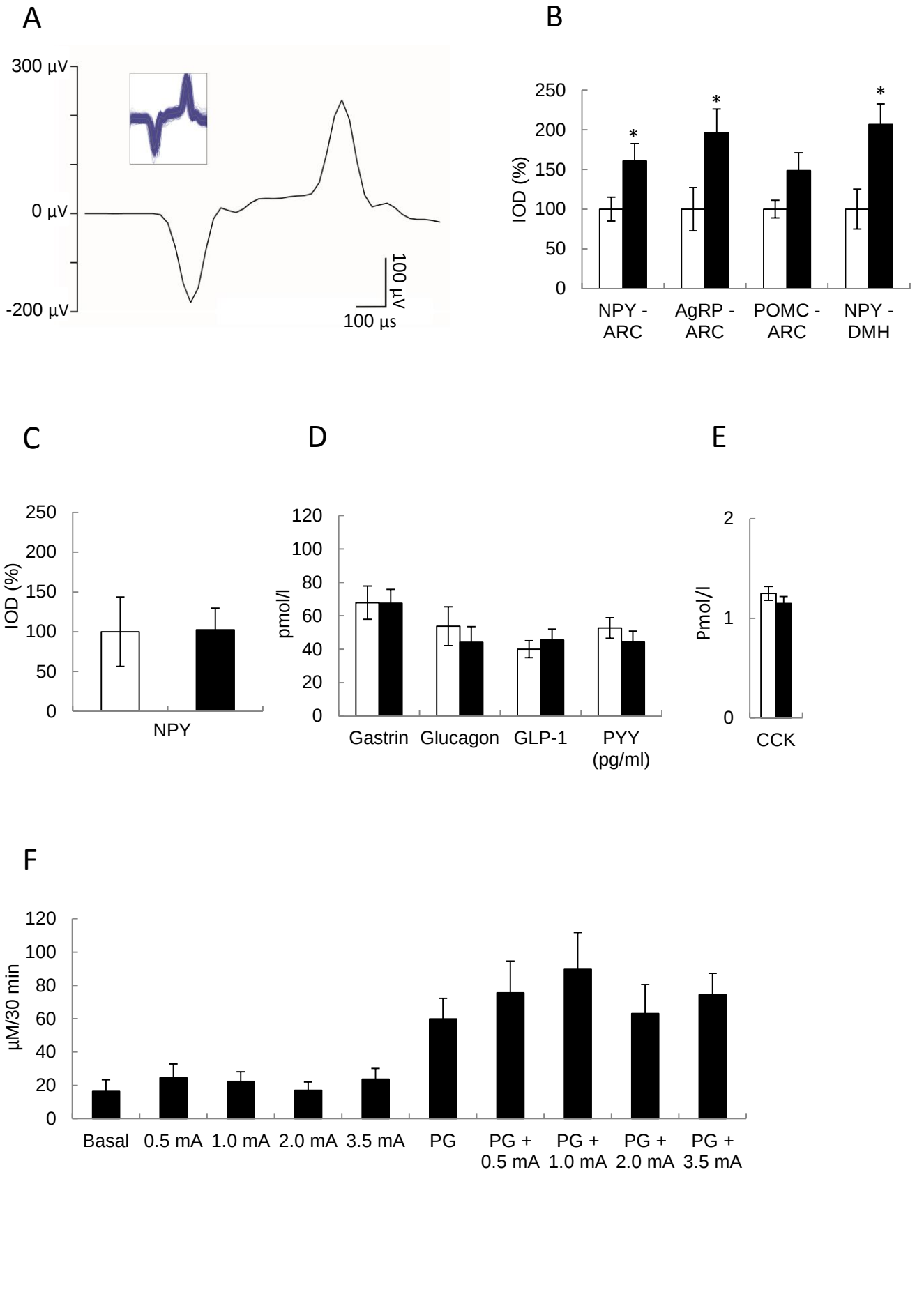
	Parameters	Sham-VBLOC (n=6)	VBLOC 2 mA short-term (n=6)	*p*-value
	Body weight	401.87±10.05	371.88±10.63	0.068
24 hours	**Food intake (g)**	25.58±1.69	25.48±1.51	0.966
	Food intake (g/100g body weight)	6.6±0.44	6.99±0.37	0.517
	Calories intake (kcal)	65.37±4.35	65.47±3.89	0.966
	Calories intake (kcal/100g body weight)	16.95±1.14	17.95±0.96	0.517
	Number of meals	25.5±6.43	24±8.25	0.889
	Meal size (g/meal)	1.24±0.19	1.47±0.27	0.490
	Meal size (kcal/meal)	3.18±0.5	3.79±0.69	0.490
	Satiety ratio (min/g)	51.37±3.91	50.66±3.58	0.895
	Rate of eating (g/min)	0.29±0.03	0.25±0.02	0.286
	Energy expenditure (kcal/h)	2.24±0.04	2.03±0.09	0.072
	Energy expenditure (kcal/h/100g body weight)	0.58±0.01	0.56±0.02	0.357
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.005±0	0.187
	RER	1.13±0.01	1.1±0.03	0.260
Daytime	**Food intake (g)**	9.41±0.92	9.91±0.99	0.715
	Food intake (g/100g body weight)	2.43±0.24	2.72±0.26	0.429
	Calories intake (kcal)	24.17±2.37	25.48±2.54	0.715
	Calories intake (kcal/100g body weight)	6.23±0.62	6.99±0.67	0.429
	Number of meals	10.33±3.03	11±5.05	0.912
	Meal size (g/meal)	1.2±0.21	1.55±0.38	0.432
	Meal size (kcal/meal)	3.09±0.54	3.99±0.96	0.432
	Satiety ratio (min/g)	68.12±7.09	63.27±6.7	0.629
	Rate of eating (g/min)	0.3±0.02	0.28±0.02	0.506
	Energy expenditure (kcal/h)	1.92±0.07	2.09±0.04	0.074
	Energy expenditure (kcal/h/100g body weight)	0.54±0.01	0.53±0.01	0.490
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.004±0	0.201
	RER	1.11±0.02	1.08±0.02	0.305
Nighttime	**Food intake (g)**	16.17±1.02	15.56±1.67	0.763
	Food intake (g/100g body weight)	4.17±0.27	4.27±0.45	0.857
	Calories intake (kcal)	41.56±2.63	39.99±4.3	0.763
	Calories intake (kcal/100g body weight)	10.72±0.69	10.96±1.14	0.857
	Number of meals	15.17±3.49	13±3.32	0.662
	Meal size (g/meal)	1.27±0.19	1.49±0.27	0.522
	Meal size (kcal/meal)	3.28±0.48	3.84±0.7	0.522
	Satiety ratio (min/g)	38.46±2.81	40.66±4.83	0.701
	Rate of eating (g/min)	0.28±0.03	0.23±0.03	0.217
	Energy expenditure (kcal/h)	2.38±0.05	2.13±0.12	0.093
	Energy expenditure (kcal/h/100g body weight)	0.61±0.01	0.58±0.03	0.316
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.005±0	0.192
	RER	1.15±0.02	1.11±0.03	0.257

Data are expressed as means ± SEM. No significant difference between sham-VBLOC and VBLOC (two-tailed).

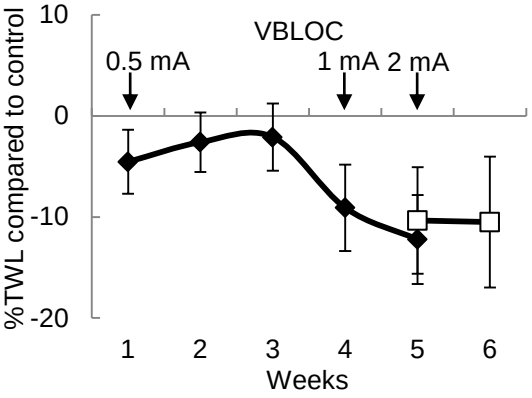
Table 2: Responses of food intake, eating behavior and metabolic parameters to long-term VBLOC at 2 mA.

	Parameters	Sham-VBLOC (n=4)	VBLOC 2 mA long-term (n=9)	*p*-value
24 hours	**Food intake (g)**	23.03±1.1	15.22±2.74	0.024*
	Food intake (g/100g body weight)	5.44±0.65	3.93±0.65	0.192
	Calories intake (kcal)	59.17±2.83	39.13±7.04	0.024*
	Calories intake (kcal/100g body weight)	13.99±1.66	10.11±1.68	0.192
	Number of meals	17.5±2.6	21.11±9.14	0.803
	Meal size (g/meal)	1.38±0.15	1.51±0.43	0.782
	Meal size (kcal/meal)	3.54±0.38	3.87±1.12	0.782
	Satiety ratio (min/g)	56.6±2.7	164.02±61.51	0.119
	Rate of eating (g/min)	0.36±0.04	0.28±0.04	0.229
	Energy expenditure (kcal/h)	2.26±0.08	1.92±0.12	0.095
	Energy expenditure (kcal/h/100g body weight)	0.53±0.06	0.49±0.02	0.417
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.004±0	0.218
	RER	1.18±0.01	1.08±0.03	0.063
Daytime	**Food intake (g)**	5.94±1.7	5.15±1.04	0.692
	Food intake (g/100g body weight)	1.5±0.55	1.38±0.29	0.836
	Calories intake (kcal)	15.26±4.38	13.25±2.68	0.692
	Calories intake (kcal/100g body weight)	3.85±1.4	3.55±0.74	0.836
	Number of meals	5.75±1.18	7.56±3.89	0.770
	Meal size (g/meal)	0.99±0.17	1.43±0.43	0.369
	Meal size (kcal/meal)	2.54±0.44	3.67±1.11	0.369
	Satiety ratio (min/g)	129.53±35.36	4180.21±3966.1	0.520
	Rate of eating (g/min)	0.35±0.05	0.28±0.05	0.447
	Energy expenditure (kcal/h)	2.1±0.1	1.83±0.09	0.103
	Energy expenditure (kcal/h/100g body weight)	0.5±0.06	0.47±0.02	0.532
	Energy expenditure (kcal/h/cm^2 body surface)	0.004±0	0.004±0	0.285
	RER	1.15±0.03	1.06±0.03	0.080
Nighttime	**Food intake (g)**	17.09±2.02	10.07±2.02	0.061
	Food intake (g/100g body weight)	3.94±0.35	2.55±0.46	0.087
	Calories intake (kcal)	43.91±5.2	25.88±5.19	0.061
	Calories intake (kcal/100g body weight)	10.14±0.9	6.56±1.18	0.087
	Number of meals	11.75±3.3	13.56±5.41	0.837
	Meal size (g/meal)	1.64±0.23	1.66±0.52	0.983
	Meal size (kcal/meal)	4.22±0.59	4.26±1.34	0.983
	Satiety ratio (min/g)	36.85±3.52	103.58±32.29	0.073
	Rate of eating (g/min)	0.37±0.04	0.27±0.04	0.137
	Energy expenditure (kcal/h)	2.41±0.07	2.02±0.14	0.098
	Energy expenditure (kcal/h/100g body weight)	0.57±0.06	0.52±0.03	0.346
	Energy expenditure (kcal/h/cm^2 body surface)	0.005±0	0.004±0	0.190
	RER	1.21±0.01	1.11±0.03	0.015*

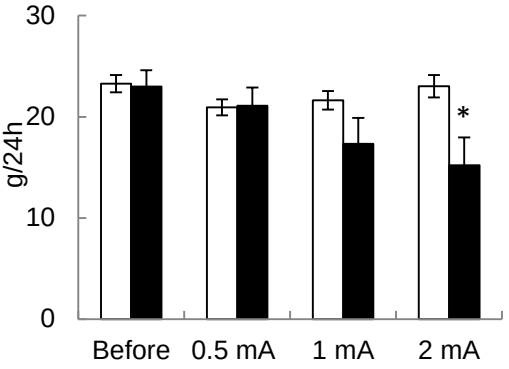
Data are expressed as means ± SEM. *:$p<0.05$ between sham-VBLOC and VBLOC (two-tailed).


A
300 μV
0 μV
-200 μV
100 μV
100 μs
B
IOD (%)
250
200
150
100
50
0
*
*
*
NPY - ARC
AgRP - ARC
POMC - ARC
NPY - DMH
C
IOD (%)
250
200
150
100
50
0
NPY
D
pmol/l
120
100
80
60
40
20
0
Gastrin
Glucagon
GLP-1
PYY (pg/ml)
E
Pmol/l
2
1
0
CCK
F
μM/30 min
120
100
80
60
40
20
0
Basal
0.5 mA
1.0 mA
2.0 mA
3.5 mA
PG
PG + 0.5 mA
PG + 1.0 mA
PG + 2.0 mA
PG + 3.5 mA

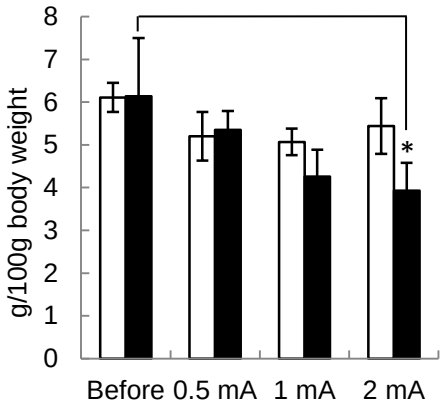
A


%TWL compared to control
10
0
-10
-20
VBLOC
0.5 mA
1 mA
2 mA
1
2
3
4
5
6
Weeks

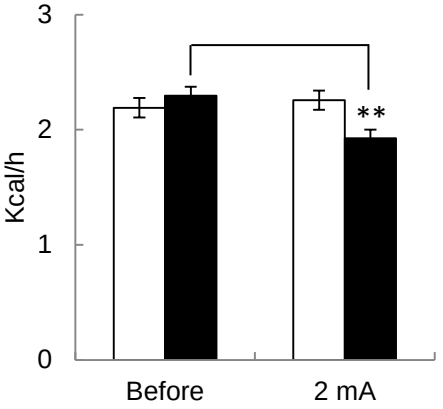
B


g/24h
30
20
10
0
*
Before
0.5 mA
1 mA
2 mA

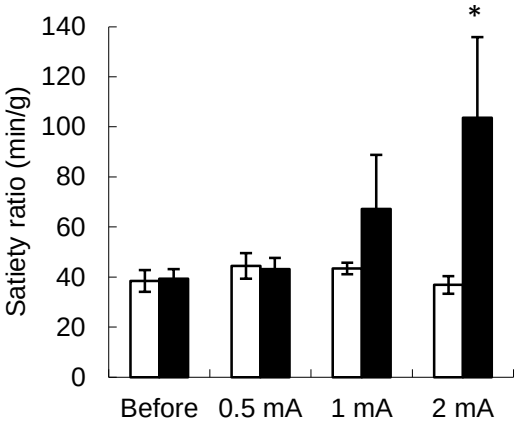
C


g/100g body weight
8
7
6
5
4
3
2
1
0
*
Before
0.5 mA
1 mA
2 mA

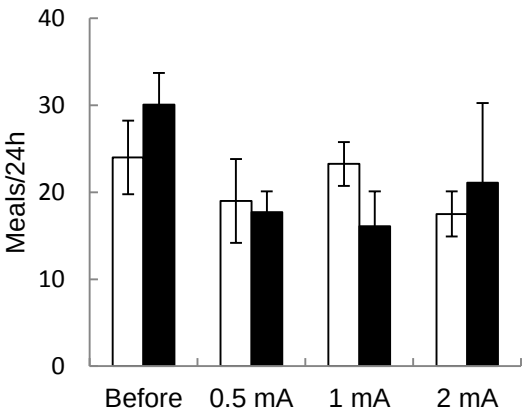
D

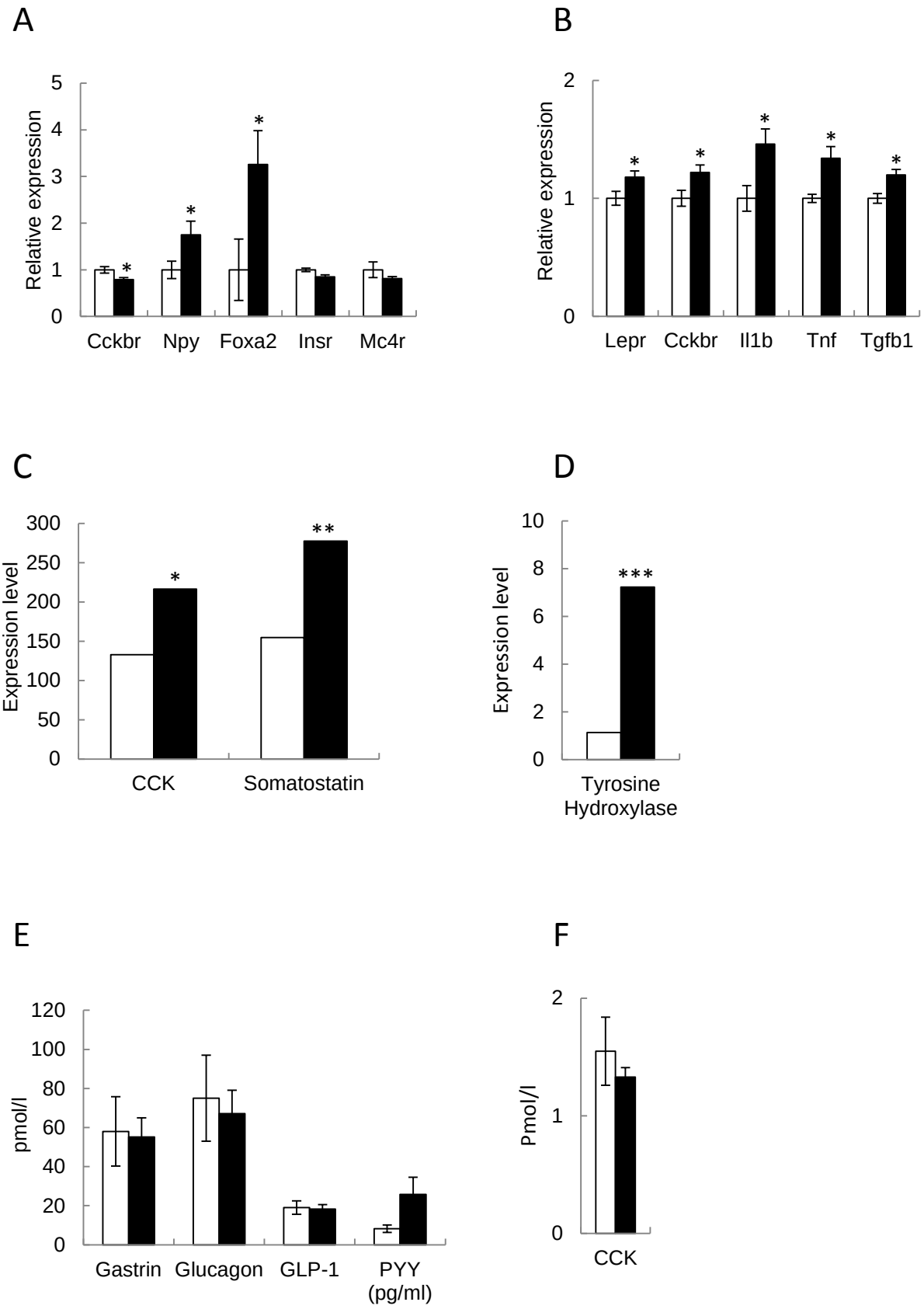

Kcal/h
3
2
1
0
**
Before
2 mA

E

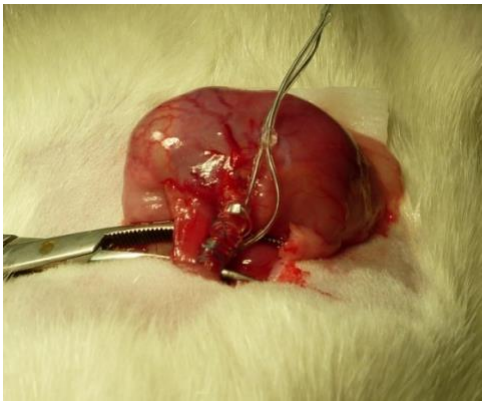

Satiety ratio (min/g)
140
120
100
80
60
40
20
0
*
Before
0.5 mA
1 mA
2 mA

F

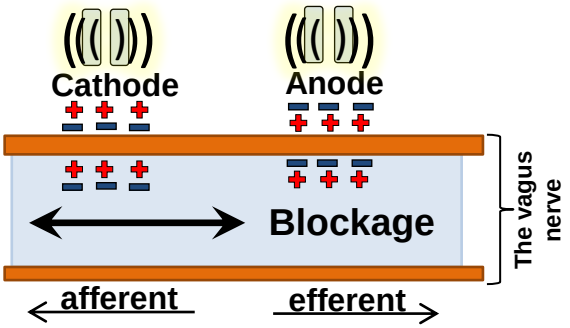

Meals/24h
40
30
20
10
0
Before
0.5 mA
1 mA
2 mA


A
Relative expression
5
4
3
2
1
0
*
*
*
Cckbr
Npy
Foxa2
Insr
Mc4r
B
Relative expression
2
1
0
*
*
*
*
*
Lepr
Cckbr
Il1b
Tnf
Tgfb1
C
Expression level
300
250
200
150
100
50
0
*
**
CCK
Somatostatin
D
Expression level
10
8
6
4
2
0
***
Tyrosine
Hydroxylase
E
pmol/l
120
100
80
60
40
20
0
Gastrin
Glucagon
GLP-1
PYY
(pg/ml)
F
Pmol/l
2
1
0
CCK

A



B



Supplementary Materials and Methods

Vagal Blocking for Obesity Treatment: A Possible Mechanism-of-Action

Manuscript type I: Original Contribution

Helene Johannessen[1], David Revesz[2], Yosuke Kodama[1], Nikki Cassie[3], Karolina P Skibicka[4], Perry Barrett[3], Suzanne L Dickson[4], Jens J Holst[5], Jens F Rehfeld[6], Geoffrey van der Plasse[7], Roger AH Adan[7], Bård Kulseng[8], Elinor Ben-Menachem[2], Chun-Mei Zhao[1], Duan Chen[1,8]

[1]Department of Cancer Research and Molecular Medicine, NTNU - Norwegian University of Science and Technology, Trondheim, Norway; [2]Department of Clinical Neuroscience and Rehabilitation, The Sahlgrenska Academy at Gothenburg University, Gothenburg, Sweden; [3]Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, Scotland; [4]Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden; [5]Department of Biomedical Sciences, the Panum Institute, University of Copenhagen, Copenhagen, Denmark; [6]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark; [7]Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht, Utrecht, Netherlands; [8]Center for Obesity Research, Department of Surgery, St. Olavs Hospital, Trondheim University Hospital

Correspondence to Professor Duan Chen, MD, PhD, Erling Skjalgssons Gate 1, Eastside 3rd Floor, Laboratory Centre of St. Olav's Hospital, 7006 Trondheim, Norway. Tel: +47 725 73320. Fax: +47 725 76612. Mob: +47 984 09 675. E-mail: duan.chen@ntnu.no.

Running head: Vagal Blocking for Obesity

Animal surgery

All surgeries were performed under general anesthesia with isofluran (4% for induction, 2% for maintenance). Atropin was given at a dose of 0.04 mg/kg subcutaneously 20 min before anesthesia. Buprenorphine was injected subcutaneously (0.05 mg/kg) immediately after surgery in all animals, and one day postoperatively when needed. Physiological saline (0.9% NaCl) was given subcutaneously at 10 mL after surgeries to keep the animals hydrated.

VBLOC implantation was performed through a midline abdominal incision. The subdiaphragmatic truncal vagus nerve was dissected from the esophagus and two electrodes (Lead Model 302, Cyberonics, Houston, TX) were wrapped around both the anterior and posterior nerve (Fig 4a). The wire from the electrodes was attached to fat tissue around the stomach and to the muscular layer using 6-0 and 4-0 absorbable sutures (Vicryl, Ethicon Inc., Sommerville, NJ, USA), respectively. On the back of the rat a subcutaneous pocket for the stimulator (model 102 Pulse Generator, Cyberonics, Houston, TX) was made and here it was connected to the wire. The abdomen was closed in two layers using 4-0 absorbable sutures and the back was closed using the same sutures (Ethicon). The control and VBLOC rats received the same procedure. Implantation of the μ-VBLOC device followed the same procedure. In the gastric acid secretion experiment a gastric fistula was in addition implanted in the stomach. Before this procedure the animals were fasted overnight, and after surgery they were given water immediately and food the same evening.

Determination of Eating Behavior and Metabolic Parameters

Rats were placed in the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments International, Columbus, OH, USA) with free access to standard rat powder food (RM1 811004, Scanbur BK AS, Sweden) and tap water. This system

is composed of a four-chamber open circuit indirect calorimeter designed for continuous monitoring of individual rats. Eating behavior and metabolic parameters were recorded automatically. High-resolution feeding data was generated by monitoring all feeder balances every 0.5 s. The end of an eating event (meal) was determined when the balances were stable for more than 10 s and a minimum of 0.05 g of food were eaten. An air sample was withdrawn every 5 min. Energy expenditure (EE) (kcal/h) was calculated according to the equation: $(3.815+1.232\ RER) \times VO_2$, where RER (respiratory exchange ratio) was the volume of CO_2 produced per volume of O_2 consumed. VO_2 was the volume of O_2 consumed per h per kilogram of mass of the animal. Parameters that were obtained during daytime (7 am–7 pm) and nighttime (7 pm–7 am) for each individual rat included number of meals, meal size, meal duration, accumulated food intake, intermeal interval, rate of eating, satiety ratio, drinking activity, energy expenditure and ambulatory activity. The intermeal interval was defined as the interval in minutes between two meals. Rate of eating was calculated by dividing the average meal size by the average duration of a meal, and satiety ratio, an index of the non-eating time produced by each gram of food consumed, was calculated by dividing the average intermeal interval by the average meal size.

In all studies performed food intake was higher and satiety ratio lower during nighttime than daytime for all animals at every timepoint.

Gastric acid output measurement

The rats were fasted for 24h before measurement. Gastric acid output was collected for 30 min at baseline, 0.5 mA, 1.0 mA, 2.0 mA, 3.5 mA and at the same settings with pentagastrin subcutaneous injection (10 μg/kg). Between each collection the stimulation was turned off and there was a break of 90 min. The rats received 1 ml physiological saline

subcutaneously every second hour. The gastric acid was investigated for pH, H^+-secretion and amount (ml).

Taqman array

Hindbrain (medulla and pons) and hypothalamus were dissected at euthanization and stored in RNAlater (Qiagen) at -80°C. Brain samples were then homogenized in Qiazol (Qiagen, Hilden, Germany) using a TissueLyzer. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit (Qiagen), both with additional DNAse treatment (Qiagen). RNA quality and quantity were assessed by spectrophotometric measurements (Nanodrop 1000, NanoDrop Technologies, USA). For cDNA synthesis, total RNA was reversed transcribed using random hexamers (Applied Biosystems, Sundbyberg, Sweden), and Superscript III reverse transcriptase (Invitrogen Life Technologies, Paisley, UK), according to the manufacturer's description. Recombinant RNaseout® Ribonuclease Inhibitor (Invitrogen) was added to prevent RNase-mediated degradation. Samples were run in duplicates. Real-time RT PCR was performed using TaqMan®Low Density Array (LDA) custom-made platforms. Platfoms were designed with TaqMan probe and primer sets for target genes chosen from an on-line catalogue (Applied Biosystems). The sets were factory-loaded into the wells of TaqMan® LDAs. Each port on the LDA card was loaded with cDNA corresponding to 100 ng total RNA, combined with nuclease free water and 50 µl TaqMan® Gene Expression Master Mix (Applied Biosystems) to a final volume of 100 µl. The LDA cards were analyzed using the 7900HT system with a TaqMan LDA Upgrade (Applied Biosystems). Thermal cycling conditions were: 50°C for 2 min, 94.5°C for 10 min, followed by 40 cycles of 97°C for 30 s, and 59.7°C for 1 min. Gene expression values were calculated based on the Ct method (Livak 2001), where the saline-treated group was designated the calibrator. Hydroxymethylbilane synthase (hmbs) was used as reference gene. Target genes as well as

the primer sets were Hmbs, Foxa2, Npy, Lepr, Insr, Cckbr, Mc4r, Il1b, Tnf, Tgfb1 (catalogue number Rn00565886_m1, Rn01415600_m1, Rn01410145_m1, Rn01433205_m1, Rn00567070_m1, Rn00565867_m1, Rn01491866_s1, Rn00580432_m1, Rn01525859_g1 and Rn00572010_m1, respectively).

***In situ* hybridization**

Brain samples were taken at euthanization and snap-freezed in isopentane on dry ice before stored at -80°C wrapped in alufolie. The frozen brains were cut (14 µm) in the region spanning the hypothalamus between Bregma –0.10 to –2.54 mm and brainstem (NTS) according to the Mouse Brain Atlas of Franklin & Paxinos 1997 and sections were mounted onto poly-L-lysine-coated slides. Briefly, sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), washed in 0.1 M PB, acetylated in 0.25% acetic anhydrate in 0.1 M triethanolamine, and washed again in PB. Sections were dehydrated using graded ethanol. DNA templates for the generation of anti-sense riboprobes for AgRP and POMC were generated by PCR. Briefly, primers for the amplification of AgRP were based on Genbank sequence U89484 to amplify the sequence between bases 113-341 (forward primer 5'-TGTTCCCAGAGTTCCCAGGTC-3', reverse primer 5'-GCATTGAAGAAGCGGCAGTAGCAC-3'). Primers for the amplification of POMC were based on Genbank sequence J00162 to amplify the sequence between bases 263-665 (forward primer 5'-GGGCAAGCGCTCCTACTCCAT-3', reverse primer 5'-GCCCTTCTTGTRSRCGTTCTTGA-3'). The DNA sequence for NPY was a full-length cloned rat NPY gene sequence. ^{35}S antisense probes were made using 150ng amplified cloned insert bearing transcription factor binding sites using T7 (AgRP), T3 (NPY) or SP6 (POMC) RNA polymerase as stated. The radioactive probes were applied to the slides in 70 µl hybridization mixture (0.3 M NaCl, 10 mM Tris-HCL (pH 8), 1 mM EDTA, 0.05% transfer

RNA, 10 mM dithiothreitol, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA and 10% dextran sulphate) and hybridized overnight at 58°C. Post-hybridization, slides were rinsed in 4x SSC and treated with ribonuclease A (20 µg/µl) at 37°C before being washed in decreasing concentrations of SSC and dehydrated using graded ethanol. Slides were dried and exposed to Kodak Biomax MR film for various lengths of time. Autoradiographic films were scanned at 600 dpi on an Epson scanner linked to a computer running Image-Pro PLUS version Autoradiographic films were scanned at 600 d.p.i. and analysed using Image Pro Plus v.7.0 (Media Cybernetics UK, Marlow, Bucks, UK), analysis software (Media Cybernetics UK, Wokingham, UK). Integrated optical density was obtained by reference to the ^{14}C microscale. NPY, POMC and AgRP mRNA expression was measured in three sections NPYmRNAs were quantified from 3 sections containing the NTS. Values were averaged for each animal.

RNA sequencing

Hippocampus was dissected at euthanization and stored in RNAlater (Qiagen) at -80°C. Total RNA was isolated using an Ultra-Turrax rotating-knife homogenizer and the *mir*Vana™ miRNA Isolation Kit (AM1560, Ambion, USA) according to the manufacturer's instructions. Concentration and purity of total RNA were assessed using a NanoDrop (NanoDrop Technologies, Inc., Wilmington, DE, USA) photometer. The A260/280 ratios were 2.09 ± 0.02 (mean ± SEM). RNA integrity was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and found satisfactory with RIN values 9.1±0.3. High-throughput cDNA sequencing (RNA-seq) was performed at Norwegian Sequencing Centre, Dept. of Medical Genetics Ullevål, Oslo University Hospital, using HiSeq2000. Differential expression analysis on the data was performed, where reads for all samples were aligned to the rat genome and differential expression was calculated using cuffcompare in the Cufflinks package.

Radioimmunoassay

Plasma samples were collected at euthanization and kept at -80°C. Radioimmunoassay was performed to analyze GLP-1, PYY, gastrin, CCK and glucagon using specific antibodies with commercially available kets. Results were expressed as pmol/l (GLP-1, gastrin, CCK, glucagon) and pg/ml (PYY).

***In vivo* electrophsyiology**

Rats were anaesthetized with 0.1ml/100gr i.m. Hypnorm and 0.05ml/100gr i.p. Midazolam. Anesthesia was maintained for the duration of surgery and recordings through additional administration of Hypnorm (i.e. 10% every half hour). Eye drops (CAF; ceva Sante Animale b.v., Naaldwijk, The Netherlands) were given for the duration of the experiment. Body temperature was maintained at 37.5°C by means of a heating pad. Following placement of the VBLOC, as previously described, the animals were mounted in a stereotaxic frame (Kopf Instruments) and the cranium was exposed. At this time a local anesthetic was applied to the skull (Xylocaine spray; 10%, Astra, Hässle AB, Mölndal, Sweden). A hole was drilled over the hippocampal area (coordinates AP -3.2, ML 2.2; Paxinos and Watson, 2005), the dura was removed, and a bundle 4 tetrodes (0.005", Pt/Ir, Fine Wire, California, US) were lowered into the brain (DV -3.2) until stable neuronal activity was measured. The drill hole was subsequently filled with mineral oil (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and a surgical screw was placed into the scull to serve as ground. Following measurements animals were sacrificed with an overdose of sodium pentobarbital. During VBLOC (at 2.0 mA, 30 Hz, 500 μs, 30 s ON and 5 min OFF) data was recorded at 40Khz using a Multineuron Acquisition Processor (MAP) recording system (Plexon, Dallas, USA). Signals were passed through a unity-gain amplifier (20×), amplified and filtered with a Plexon 16-channel preamplifier (PBX3 /16sp-r-G50, 50x amplification, 150-8000 Hz filtered).

Subsequently, a 1200μs digitized data sample was stored by the MAP system whenever the signal crossed a preset voltage threshold. The data were subsequently analyzed with offline cluster cutting procedures based on the average waveforms across the four leads of each tetrode (Offline Sorter x64 V3; Plexon).