Effects of glucagon as a neurohormone on the central nervous system and glucose homeostasis

K. TANBEK[1], U. YILMAZ[2], S. GUL[3], A. KOÇ[4], M. GUL[5], S. SANDAL[1]

[1]Department of Physiology, Faculty of Medicine, Inonu University, Malatya, Turkey
[2]Department of Physiology, Faculty of Medicine, Karabuk University, Karabuk, Turkey
[3]Department of Histolology and Embriology, Faculty of Medicine, Malatya Turgut Ozal University, Malatya, Turkey
[4]Department of Medical Genetics, Faculty of Medicine, Inonu University, Malatya,Turkey
[5]Department of Histolology and Embriology, Faculty of Medicine, Inonu University, Malatya, Turkey

Abstract. – **OBJECTIVE:** This study aimed to elucidate the possible effects of the acute/long-term infusion of glucagon in the brain as the regulatory role on the endocrine secretions of the pancreas.

MATERIALS AND METHODS: Ninety male Wistar albino rats were divided as Control, artificial Cerebrospinal Fluid (aCSF) (120 min), Glucagon (120 min), pancreatic denervation (PD)+aCSF (120 min), PD+Glucagon (120 min), aCSF (7 days), Glucagon (7 days), PD+aCSF (7 days) and PD+Glucagon (7 days). Glucagon and solvent (aCSF) were administered after pancreatic denervation (PD) by Hamilton syringe and osmotic mini pump (1 µg/10 µl/min) in the third ventricle of the brain.

RESULTS: Acute intracerebroventricular (icv) administration of glucagon resulted in an elevation of glucagon levels and a concurrent reduction in blood glucose levels. Furthermore, in both the PD+aCSF (7 days) and PD+Glucagon (7 days) groups, there was a notable decrease in propiomelanocortin (POMC) and agouti-related protein (AgRP). Significant changes were observed in feed consumption and body weight, as well as pancreatic glucagon levels, with a simultaneous decrease in insulin levels in the PD (7 days), Glucagon (7 days), and PD+Glucagon (7 days) groups. These alterations were statistically significant when compared to the control group ($p<0.05$).

CONCLUSIONS: The research outcomes established that pancreas-secreted glucagon functions as a neurohormone within the brain, activating central pathways linked to blood glucose regulation. The presence of glucagon led to a decrease in POMC levels. Surprisingly, this reduction in POMC resulted in the suppression of AgRP. Contrary to expectations, the suppression of AgRP led to an increase in food intake rather than a decrease. As already highlighted in the results section, it was emphasized that POMC may play a more significant role than AgRP in influencing feeding behavior.

Introduction

In healthy individuals, at any time of the day, during periods of satiety and short or long-term fasting, blood sugar levels remain stable within certain limits. It is known that the active players in glucose homeostasis are insulin and glucagon. Glucagon and insulin exert their effects under opposite physiological conditions (blood glucose level). While hypoglycemia leads to stimulation of glucagon secretion and hyperglycemia to inhibition, the situation is the opposite for insulin secretion. The main activity of glucagon is to increase glucose production in the liver by stimulating the glycogenolysis and gluconeogenesis pathways, thereby raising the blood glucose level[1]. It is well known that insulin is secreted in response to increased blood glucose. Insulin increases the uptake of glucose into fat and muscle tissues, thereby bringing the increased blood glucose back to normal levels[2]. For many years after the discovery of insulin, glucose homeostasis was thought to be regulated only by peripheral processes[3]. Since glucose is the main energy source of the brain and it is vital to strictly regulate circulating glucose levels, it seems possible that the brain should take part in glucose homeostasis[4]. Claude Bernard provided the first evidence that the brain plays an active role in blood sugar homeostasis by determining that neuronal degeneration in the 4th ventricle causes glucose uria in rabbits[5]. In

Corresponding Author: Suleyman Sandal, Ph.D; e-mail: suleyman.sandal@inonu.edu.tr

a following study[6], it was reported that cerebral ischemia caused an increase in insulin secretion in mice, and the underlying mechanism was the stimulation of insulin synthesis from pancreatic beta cells by increasing the amount of nerve growth factor (NGF) and its receptor TrkA for the survival of damaged neurons in the brain. Thus, it was deduced that increased insulin levels increase the uptake of glucose, which is necessary for the survival and differentiation of damaged neurons in brain tissue. Recent studies have shown that the neuronal control system has an effect on the maintenance of hormonal balance depending on changes in blood sugar, and treatment approaches designed by considering neuronal effects in treatment processes will be more efficient. Insulin hormone acts as a neurotransmitter, stimulating appetite-related neurons in the brain and playing a role in blood glucose homeostasis by affecting food intake[7]. In another study, it was shown that rats centrally administered Brain Derived Neurotrophic Factor (BDNF) stimulated pancreatic neurons and pancreatic tissue, reducing the amount of glucagon and glucose production without changing the amount of insulin[8].

Glucagon receptors have been detected[9] in areas of the brain such as the olfactory tubercle, hippocampus, anterior pituitary, amygdala, septum, medulla, thalamus, olfactory bulb, and hypothalamus. Intracerebroventricular (icv) administration of glucagon is likely to affect appetite centers in the brain and play a role in blood glucose homeostasis by altering food intake[10]. Multiple studies[5,7] have documented the ability of intracerebroventricular (icv) glucagon administration to modulate peripheral glucose levels in different species of animals. Most intriguing, however, is the earliest study[9,11] in dogs where a high dose icv injection of 10 ng of glucagon transiently produced hypoglycemia followed by hyperglycemia. The hypoglycemic effect was abolished in vagotomized dogs, suggesting the involvement of a brain-liver axis in the glucose-lowering effect of central glucagon, whereas pancreatectomy prevented the hyperglycemic effect, attributing a pancreatic role to the rise of glucose from icv glucagon injections[11]. In experiments where icv glucagon was administered, relatively high doses of glucagon were used; however, in a recent study[10], lower doses of glucagon were specifically applied to the mediobasal hypothalamus. The study evaluated whether the intracerebroventricular (icv) application of low-dose glucagon to the mediobasal hypothalamus has a glucose-lowering or hyperglycemic effect. The results of the study reported an impact on blood glucose levels but indicated that it alone did not cause hyperglycemia.

The brain not only regulates hormonal stimuli against changes in blood glucose but also changes food intake. Peptides that stimulate food intake in the brain are melanin-concentrating hormones (MCH) and orexins, which are produced in the perifornical region of the lateral hypothalamus[12,13]. Other hormones that have been included in this family in recent years are Neuropeptide Y (NPY) and agouti-related protein (AgRP), which is produced in the arcuate nucleus. Neuropeptides that suppress food intake are propiomelanocortin (POMC)-derived hormones produced in the arcuate nucleus and the alpha-melanocyte stimulating hormone[14]. The role of AgRP in the normal physiological control of food intake is unknown; however, overnutrition and obesity in humans and mice are associated with excessive AgRP formation due to gene mutations. When the body's energy stores decrease, appetitive neurons of arcuate nuclei are activated, and NPY is released. At the same time, due to the decrease in the discharge of POMC neurons, the activity of the melanocortin pathway decreases; thus, appetite is stimulated further[15]. Therefore, it is thought[16] that the brain tissue plays an active role in maintaining this balance by detecting the changes in blood glucose and changing the amount and activities of neuronal and endocrine signals and other hormones.

Diabetes, which is the most common of the metabolic diseases and is increasing, paves the way for the development of many other metabolic diseases, which develop as a result of the impairment in blood glucose homeostasis[17]. The increase in the number of diabetic patients worldwide underlines the importance of the studies on its treatment. Insulin hormone plays a role in blood glucose homeostasis by affecting food intake by acting on neurons related to appetite in the brain[8]. How glucagon has an opposite effect on insulin in the peripheral areas of the brain where insulin is active has not yet been fully elucidated[18].

This study aimed to obtain evidence about the possible effects of glucagon on the central nervous system (CNS) and insight into the possible mechanism of action. Therefore, pathways regarding the effect of insulin on appetite centers and how it reflects this effect to the periphery following central glucagon administration, the

neural implications of insulin and glucagon release from the pancreas, and the effects of glucagon released from the pancreas in the nervous control of the pancreas on the CNS were investigated.

Materials and Methods

Animals and Drugs

A total of 90 adult male Wistar Albino rats (8 weeks old, 300-350 g) were purchased from the Experimental Animal Research Center of Inonu University, Malatya, Turkey. All the animals were handled in accordance with the Guidelines for the Care and Use of Experimental Animals. All animals were randomly assigned to cohorts and approved by the Animal Research Ethics Committee of Inonu University Faculty of Medicine (License No.: 2019/A-42). The rats were housed at 21±2°C with a 12/12-hour light-dark cycle with free access to water and food *ad libitum* with a standard laboratory chow diet. The number of groups and rats in each group (sample size) was determined according to the power analysis based on the values specified. Accordingly, the amount of Type I error (α) was 0.05, the power of the test (1-β) was 0.8, and the effect size was 0.82 (large). While the number of groups was 9, the minimum sample size required to find a significant difference between the groups was at least 5 in each group for Western Blot analyses and at least 5 different rat tissue from each group for immunofluorescence analyses. With 10 animals in each group, the total number was determined to be 90 rats[19]. All the weight-matched male rats were randomly divided into nine groups (n=10) as Control, aCSF (120 min), Glucagon (120 min), PD+aCSF (120 min), PD+Glucagon (120 min), aCSF (7 days), Glucagon (7 days), PD+aCSF (7 days), PD+Glucagon (7 days). Body weight and food consumption were monitored daily in all groups. Random blood glucose levels were assessed with test strips (On Call, G113-11, Acon Labs, USA). The control group did not receive any treatment. Glucagon (HOR-286 ProSpec-Tany Techno Gene Ltd. Hamada, Israel) was dissolved in aCSF for icv injections, and a dose of 1 μg/10 μl was chosen based on a previous study[20] which verified glucagon with vehicle (10 μl aCSF).

Intracerebroventricular Cannulation and Administration of Glucagon

The animals were placed in individual cages 3 days before the implantation of brain infusion kits and were accustomed to cage stress. The rats were anesthetized with the combination of ketamine/xylazine (80 mg/kg; Richter Pharma AG, Australia/12 mg/kg; Alfasan International B.V., Holland). The depth of anesthesia in the rats was determined by finger pinch responses and physiological responses. After the scalps of the rats had been shaved, they were placed in a stereotaxic device (David Kopf Instruments), and the bregma was reached by cutting the scalps. Referencing Paxinos and Watson's rat brain atlas, the injection coordinates were established using bregma as the reference point, 2.2 mm posterior and 8 mm vertical from the dura mater in the third ventricle. The specified point was perforated using a drill without damaging the dura mater[21]. The specified point was perforated using a drill without damaging the dura mater.

Using a Hamilton syringe, 1 μg/10 μl glucagon and 10 μl aCSF were administered in the third ventricle for aCSF (120 min), Glucagon (120 min), PD+aCSF (120 min) and PD+Glucagon (120 min) groups.

The 21-gauge sterile guide cannula (PlasticsOne, 3280 OP-SPC OP Connector Cannulas 28-21GA Cut 8 mm) was inserted. It was attached to the skull by dental acrylic. The cannula of brain infusion kits was filled with aCSF for the aCSF (7 days), PD+aCSF (7 days) vehicle group[22,23], disallowing air bubble formation, and then the open end was dampened and closed.

Placement of Osmotic Minipumps

Then, the glucagon and sham group received aCSF for 7 days (240 μl/day) with the help of the osmotic mini pump (DURECT Corporation, ALZET Osmotic mini pump 2ML1, CA, USA). The animals were anesthetized a second time 7 days after the implantation of the brain guide cannula. The cannula under the neck skin was manually identified. The tip of the cannula was pulled out through a small skin incision. The closed end of the cannulas was cut. A Hamilton injector was used to check if the cannulas were blocked. The prepared osmotic minipumps were attached to the cannula of the brain infusion kit without air bubbles. They were placed under the neck skin of animals. Then, the infusion was started.

Pancreatic Denervation

The model of selective pancreatic denervation has been previously described in detail[24]. Under anesthesia, the splenic artery, the superior pancreaticoduodenal artery, and the inferior pan-

creaticoduodenal artery were identified under a microscope. The nerves innervating the pancreas lie in close association with these vessels. The nerves were stained with one or two drops of toluidine blue solution. Pancreatic tissue norepinephrine levels were measured by the ELISA (SunRed Biotechnology, Shanghai, China) method to evaluate the accuracy of the surgical model in the pancreatic denervation groups[25,26].

Termination of Experiment and Collection Tissues

At the end of the required period, the rats were sacrificed under anesthesia, and blood, brain, and pancreas tissues were collected. Serum, brain, and pancreas tissues were frozen on dry ice and stored at -80°C under suitable conditions until the day of the analysis for ELISA, Immunofluorescence (IF) and Western blot (WB) analyses.

ELISA Analyses

Serum insulin, glucagon, and epinephrine levels were measured using an ELISA kit for rats (SunRed Biotechnology Company, Shanghai, China) according to the manufacturer's instructions. Insulin (Cat No.: 201-11-0708) results are expressed in mlU/L. Glucagon (Cat No.: 201-11-1673) results are expressed in ng/L. Epinephrine (Cat No.: 201-11-0546) results are expressed in ng/L.

IF Analyses

Anatomical landmarks such as the median eminence and third ventricle were used to identify paraventricular nucleus (PVN) and arcuate nucleus (ARC) sections of the hypothalamus, as well as the rat brain atlas[27]. In another subset designated for western and immunohistochemical experiments, 8 μm coronal sections were generated and divided into five equal series using a freezing microtome (Leica CM 1520) as previously described. Brain and pancreas sections were washed in phosphate-buffered saline (PBS) and in PBS+0.03% Triton (PBST) for 1 h at room temperature. Brain sections were then incubated overnight at room temperature in blocking solution containing primary antiserum AgRP (Abcam, Cambridge, UK; ab228495) and POMC (Abcam; ab254257), both 1:500. Pancreas sections were then incubated overnight at room temperature in blocking solution containing primary antiserum insulin (Abcam; ab7842), and Glucagon (Abcam; ab92517), both 1:50. The next morning sections were extensively washed in PBS and then incu-

bated in Alexa-fluorophore secondary antibody (AgRP-insulin; Green ab150185, POMC-Glucagon; Red ab150079, both 1:500) for one hour at room temperature. After 3 washes with PBS, sections were mounted on glass slides. The brain and pancreas sections were stained with 4,6-diamidino-2-phenylindole (DAPI). The sections were photographed digitally using an upright optical microscope (NIS-Elements Documentation 5.02 Nikon Instruments Inc., Melville, NY, USA). Objectives of 40X were used to image either the left or the right hemisphere in the ARC of the hypothalamus. POMC-positive neurons throughout the image were counted using the ImageJ Cell Counter plug-in function for marking and numbering positive cells. Once positive cells were marked, ImageJ software was used to overlay images to quantify colocalization. Double-blinded analyses were performed on three serial sections of each rat (n=3-5 rats per group).

Western Blot Analyses

We examined the expressions of AgRP and POMC in the brain and insulin and glucagon in the pancreas of the rats by Western Blot. Tissue samples were homogenized using steal beads (Next Advance BBY24M, Inc. Innovative Lab Products for the Life Sciences, USA) in ice-cold lysis buffer at pH 7.4. Samples were homogenized in RIPA (Abcam; ab156034) buffer supplemented with protease inhibitors (Abcam; ab201111). BCA assays (Abcam; BCA Protein Quantification Kit ab102536) were used to quantify protein concentration. The same amount of protein was loaded into each well, which, depending on the antibody, ranged from 15 to 20 μg. Proteins were resolved by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (1620177, Biorad Life Sciences Research, USA) as described. Following the transfer with a transblot turbo transfer system (Thermo Scientific, Waltham, MA, USA), the membrane was blotted for 1 hour in a 5% milk powder solution prepared with TBS (Tris-Buffer-Saline) containing Tween 20 and washed 3 times with TBS/T 10 minutes apart. Then, membranes were incubated with the following primary antibodies: Insulin (Abcam; ab7842), Glucagon (Abcam; ab92517), AgRP (Abcam; ab228495), POMC (Abcam; ab254257) and β-Actin (Abcam; ab8226) in 5% BSA powder overnight at +4°C. Following primary antibody incubation, membranes were washed 3 times with TBS/Tween 20 for 10 minutes. Then, incubated

with peroxidase-conjugated secondary antibody in a 5% milk powder solution prepared with TBS/Tween 20 for 1 hour at room temperature. Following the washing of the membrane, it was treated with luminol and peroxide mixed in a 1:1 ratio and viewed under the UVP Syngene GBox Chemi-XRQ Gel Documentation System. The β-actin antibody was used as a loading control and viewed following washing steps after treatment with a secondary antibody. Densitometry analyses were performed using ImageJ. Arbitrary densitometry units were assigned; data is presented as mean±SEM. The expression of the target protein was normalized to that of the β-Actin protein expression. The western blot data in the figures and supplemental figures represent more than three independent experiments.

Statistical Analysis

The data obtained from the study were made using the program developed by the faculty members of Inonu University Faculty of Medicine, Department of Biostatistics and Medical Informatics, and made available as an open access program[28]. In data analysis, first of all, controls were made to prevent missing and erroneous data and excessive variable/outlier problems, and corrections were made if necessary. Quantitative data were summarized as mean±SD, and qualitative data were summarized as numbers (percentage). In order to compare the examined variables between groups with a one-way analysis of variance, conformity to normal distribution and homogeneity of variances (Levene) were checked. When these assumptions were met, the difference between the group means, one-way analysis of variance, and multiple comparisons were made with the Tukey HSD when the variances were homogeneous and with the Tamhane T2 when they were not. In cases where the normality assumptions were not met, the Kruskal-Wallis H was used, and the Conover was used for multiple comparisons. A $p<0.05$ was considered statistically significant.

Results

Evaluation of the Body Weights and Blood Glucose Levels of the Rats

According to our findings (Table I), the increase in blood glucose levels from the 30th minute in PD+aCSF (120 min), aCSF (120 min) and PD+Glucagon (120 min) was statistically significant compared to the initial blood glucose. In the icv glucagon administered group, blood glucose levels were statistically significantly higher compared to the pre-application in the 30th and 120th minutes ($p<0.05$).

In groups where glucagon was administered centrally for long durations, feed consumption, and body weight changes were followed for one week subsequent to glucagon administration. The body weight changes are given in Table II, as a % change proportional to the initial weights of the rats in each group. In addition, the weight in the PD+Glucagon (7 days) group was statistically significant compared to the Glucagon (7 days) group ($p<0.05$). The average feed consumption in centrally glucagon-administered groups for 7 days is given in Table II. The increase in feed consumption in the Glucagon (7 days) group is statistically significant compared to the aCSF (7 days) group. The weekly average feed consumption of the PD+Glucagon (7 days) group was also statistically significantly higher than the Glucagon (7 days) and PD+aCSF (7 days) groups ($p<0.05$).

Evaluation of the Blood Insulin, Glucagon, and Epinephrine Levels

The results for serum insulin, glucagon, and epinephrine levels are given in Table III. According to our findings, the decrease in serum insulin levels in Glucagon (120 min), PD+aCSF (120 min), and PD+Glucagon (120 min) groups in the study model, in which acute effects were examined with 120 min administration were statistically significant compared to the Control and aCSF (120 min) groups ($p<0.05$). When the PD+Glucagon (120 min) group was compared to the Glucagon (120 min) group, the decrease in insulin level was statistically significant ($p<0.05$). In the study protocol examining the long-term effects of central Glucagon administration, the increase in serum insulin level in the Glucagon (7 days), PD+aCSF (7 days), and PD+Glucagon (7 days) groups was statistically significant compared to the Control and aCSF (7 days) groups.

According to our findings, in the study model in which acute effects were examined with 120 minutes of administration, a decrease in serum glucagon levels in Glucagon (120 min), PD+aCSF (120 min) and PD+Glucagon (120 min) groups, Control and aCSF (120 min) group was statistically significant ($p<0.05$). In the study model examining the long-term effects of central glucagon administration, the increase in serum gluca-

Table I. Blood glucose concentration (mg/dL) after icv-glucagon administration during 0-120 min for 7 days.

	0 min	30 min	60 min	90 min	120 min	0 day	7 days
Control	75.9 ± 4.7	83 ± 5.8	79 ± 6.2	82 ± 8.7	86.4± 7.5		
aCSF (120 min)	77.9 ± 5.1	91.55 ± 6.4	83.3 ± 5.6	74.37 ± 9.3	80.22 ± 8.4		
Glucagon (120 min)	93.87 ± 16.7	118.75 ± 20.6	139.62[a,b] ± 8.3	126.55 ± 11.2	166.66[a,b,c,d] ± 33.7		
PD+aCSF (120 min)	115.12 ± 12.4	156[a] ± 13.4	175.4[a] ± 9.7	161.42[a] ± 17.6	219[a,b,c,d] ± 30.4		
PD+Glucagon (120 min)	109.5 ± 16.7	117.22 ± 21.7	165.66[a,b] ± 26.8	132 ± 11.4	116 ± 12.6		
aCSF (7 days)						85.57 ± 5.6	89.28 ± 12.6
Glucagon (7 days)						87.44 ± 18.3	105.66[e] ± 1 8.9
PD+aCSF (7 days)						93.66 ± 11.5	87 ± 5.21
PD+Glucagon (7 days)						92.4 ± 20.63	88 ± 16.82

Data are given as mean ± SD and comparison between groups was made with Kruskal-Wallis. [a]statistically different compared to 0 min, [b]statistically different compared to 30 min, [c]statistically different compared to 60 min, [d]statistically different compared to 90 min, [e]statistically different compared to 0 day. $p < 0.05$ n = 10 (PD; pancreatic denervation, aCSF; artificial cerebrospinal fluid, min; minutes).

Table II. Role of the icv-glucagon administration on weight change and food intake during a week.

	Food intake (g)	Weight change (%)
Control	160.72 ± 8.379	5.23 ± 8.66
aCSF (7 days)	67.6a ± 1.93	5.14 ± 4.06
Glucagon (7 days)	77.38[a,b] ± 7.13	10.91[a] ± 2.78
PD+aCSF (7 days)	68.58[a,c] ± 6.99	7.47 ± 6.42
PD+Glucagon (7 days)	89.06[a,b,c,d] ± 5.56	14.41[a,b] ± 6.44

Data are given as mean ± SD and comparison between groups was made with Kruskal-Wallis. [a]statistically different compared to Control, [b]statistically different compared to aCSF (7 days), [c]statistically different compared to Glucagon (7 days), [d]statistically different compared to PD+aCSF (7 days) groups. $p < 0.05$ n = 10 (PD; pancreatic denervation, aCSF; artificial cerebrospinal fluid, min; minutes).

gon level in the PD+Glucagon (7 days) group was statistically significant compared to the Control and aCSF (7 days) and Glucagon (7 days) and PD+aCSF (7 days) groups ($p<0.05$). In the serum glucagon levels following short (120 min) and long (7 days) term central glucagon administration, the difference between PD+aCSF (120 min) with PD+aCSF (7 days) and PD+Glucagon (120 min) and PD+Glucagon (7 days) groups was statistically significant ($p<0.05$).

According to our findings, in the study model in which acute effects were examined, a decrease in serum epinephrine levels in Glucagon (120 min), PD+Glucagon (120 min) groups was statistically significant compared to the Control, aCSF (120 min), and PD+aCSF (120 min) groups ($p<0.05$).

Evaluation of the Insulin and Glucagon Levels in Pancreatic Tissues

Islet cells of pancreatic tissue were examined with triple immunofluorescence staining technique (Figure 1), by which protein concentrations of glucagon produced in α islet cells (Figure 1A), insulin produced in β cells (Figure 1B) and were compared (Figure 1C). It was observed that pancreatic denervation and glucagon administration were effective in insulin and glucagon secretion in the model in which short-term effects were examined in pancreatic denervation groups. It was observed that the amount of insulin and glucagon in the PD+aCSF (120 min) group was decreased compared to the Control group, and insulin was increased in the PD+Glucagon (120 min) group. It was determined that the one-week effect of the application of pancreatic denervation decreased the amount of insulin and glucagon. Glucagon administration decreased the insulin and glucagon levels. Again, insulin and glucagon levels in the PD+Glucagon (7 days) group were similar to the findings of the control group.

Pancreatic insulin protein levels in the glucagon-administered groups are given in Figure 2A. According to our findings, in the study model in which the acute effects were examined following 120 minutes of administration, the increase in insulin levels in the PD+Glucagon (120 minutes) group and the differences between the other groups were statistically significant ($p<0.05$). The difference between Glucagon (120 min) and Glucagon (7 days), PD+aCSF (120 min) and PD+aCSF (7 days), and PD+Glucagon (120 min) and PD+Glucagon (7 days) groups were also statistically significant ($p<0.05$). Pancreatic tissue

Table III. Role of the icv-glucagon administration on blood insulin, glucagon and epinefrine levels.

	Insulin (mIU/L)	Glucagon (ng/L)	Epinefrine (ng/L)
Control	2.65 ± 1.65	94.36 ± 53.64	209.27 ± 102.33
aCSF (120 min)	1.79 ± 0.52[a]	62.86 ± 15.79	129.68 ± 37.76
Glucagon (120 min)	0.49 ± 0.14[a,b]	33.74 ± 6.11[a,b]	91.22 ± 44.75[a]
PD+aCSF (120 min)	0.87 ± 0.52[a,b]	45.07 ± 6.70[a,b]	182.82 ± 145.05[c]
PD+Glucagon (120 min)	0.92 ± 0.46[a,b]	56.70 ± 14.07[a,c]	138.78 ± 57.73[a]
aCSF (7 days)	2.63 ± 0.51[a]	73.26 ± 5.23[a]	210.84 ± 18.38
Glucagon (7 days)	3.14 ± 0.86[a,d]	77.09 ± 21.41	198.6 ± 42.46
PD+aCSF (7 days)	3.13 ± 0.23[a,d]	81.58 ± 5.35[d]	216.32 ± 26.46
PD+Glucagon (7 days)	3.22 ± 0.60[a,d]	93.65 ± 19.29[d,e]	189.68 ± 55.44

Data are given as mean ± SD and comparison between groups was made with Kruskal-Wallis. [a]statistically different compared to Control, [b]statistically different compared to aCSF (120 min), [c]statistically different compared to glucagon (120 min), [d]statistically different compared to aCSF (7 days) [e]statistically different compared to Glucagon (7 days) groups. $p < 0.05$ n=10 (PD; pancreatic denervation, aCSF; artificial cerebrospinal fluid, min; minutes).
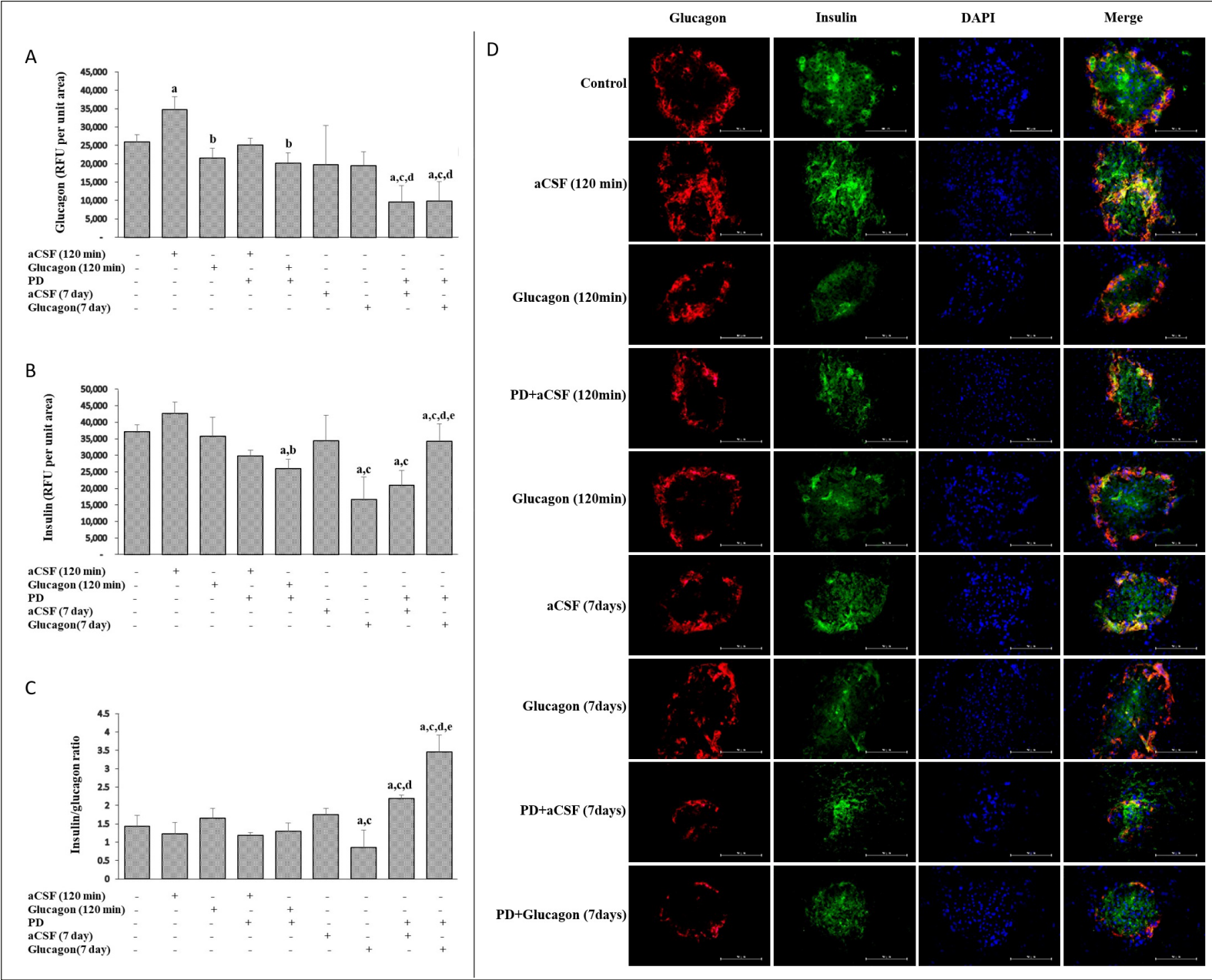
Figure 1. Immunostaining for insulin and glucagon. Quantitative fluorescence measurements as relative fluorescence units (RFU) for (**A**) glucagon, (**B**) insulin, and (**C**) insulin to glucagon ratio. Immunolocalization of islets (**D**) for ß-cell marker insulin (green) and α-cell marker glucagon (red), counterstained with 4,6-diamidino-2-phenylindole (DAPI) (nuclear stain) (*blue*) Scale 50 μm.. Letter (a) indicates significance compared to Control and (b) indicates significance compared to aCSF (120 min), (c) indicates significance compared to aCSF (7 days), (d) indicates significance compared to Glucagon (7 days), (e) indicates significance compared to PD+ aCSF (7 days). In panels (**A-C**), one-way ANOVA was used (*p*<0.05).
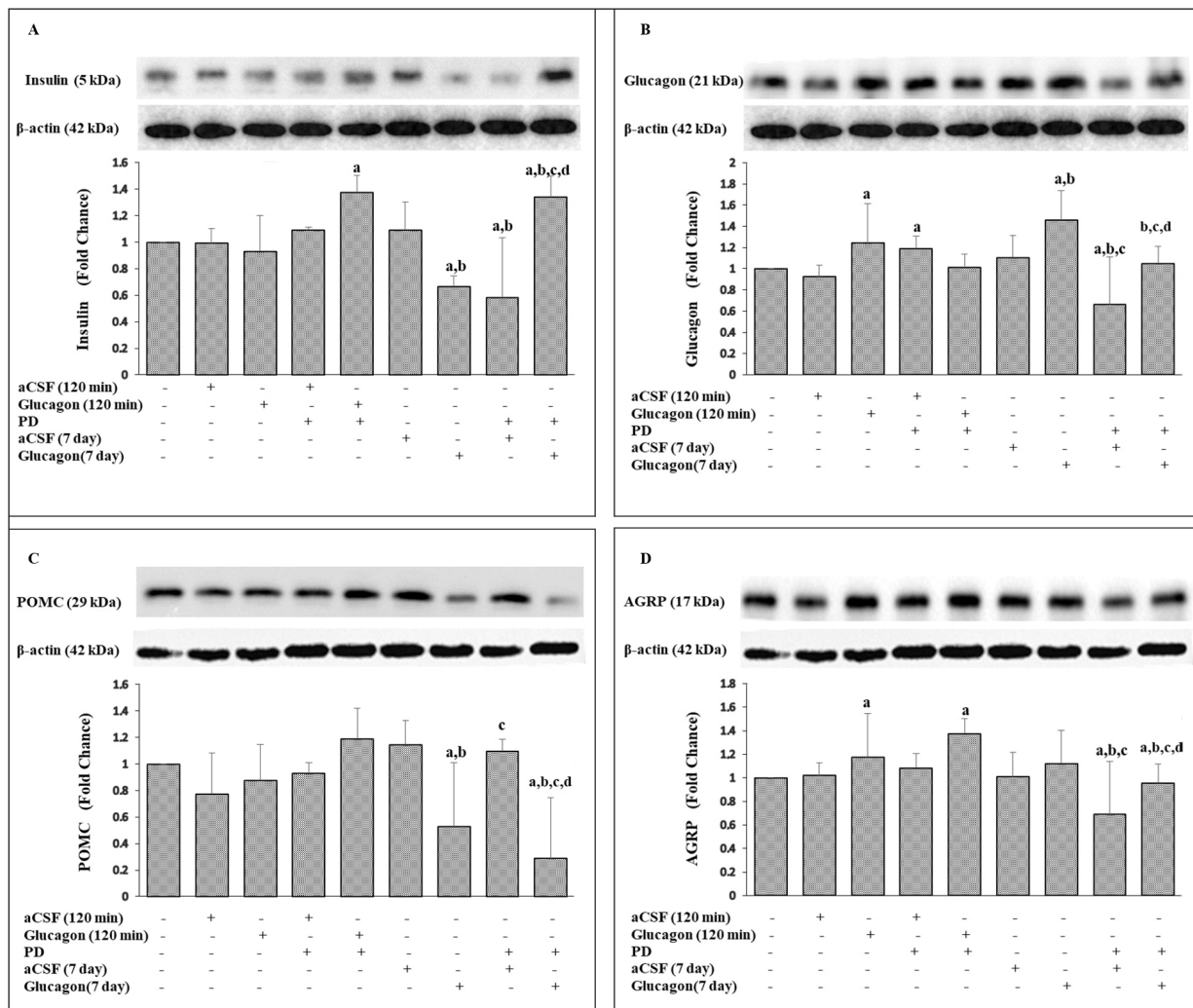
Figure 2. Effect of glucagon icv injection on POMC and AgRP expression in the brain; insulin and glucagon in the pancreas. Protein content of insulin (**A**), glucagon (**B**) in the pancreas and POMC (**C**), AgRP (**D**) in the hypothalamus of male rats. Letter (a) indicates significance compared to Control, (b) indicates significance compared to aCSF (7 days), (c) indicates significance compared to Glucagon (7 days), (d) indicates significance compared to PD+ aCSF (7 days). In panels (**A-D**), one-way ANOVA was used ($p < 0.05$).

glucagon protein levels in the groups in which glucagon was administered centrally for 120 minutes and for 7 days are given in Figure 2B. In the study model in which acute effects were examined with 120 minutes of administration, the differences in the increase in glucagon levels in the Glucagon (120 min) and PD+aCSF (120 min) groups were statistically significant ($p<0.05$) In the study protocol examining the long-term effects of central glucagon administration, the increase in glucagon level in the Glucagon (7 days) group and the decrease in the glucagon level in the PD+aCSF (7 days) group were statistically significant compared to the control, aCSF (7 days) and PD+Glucagon (7 days) groups ($p<0.05$).

POMC and AgRP Findings in the Brain Tissues

POMC and AgRP concentrations in brain tissues were compared with the dual immunofluorescence staining technique (Figure 3); no difference was observed in POMC (Figure 3A) and AgRP levels (Figure 3B), following short-term pancreatic denervation and glucagon administration. In the long-term experimental protocol, the AgRP levels were higher in the Glucagon (7 days) and PD+Glucagon (7 days) groups compared to the groups that did not receive any glucagon.

Brain POMC protein levels following short and long-term glucagon administration are given, and there is no significant difference between groups
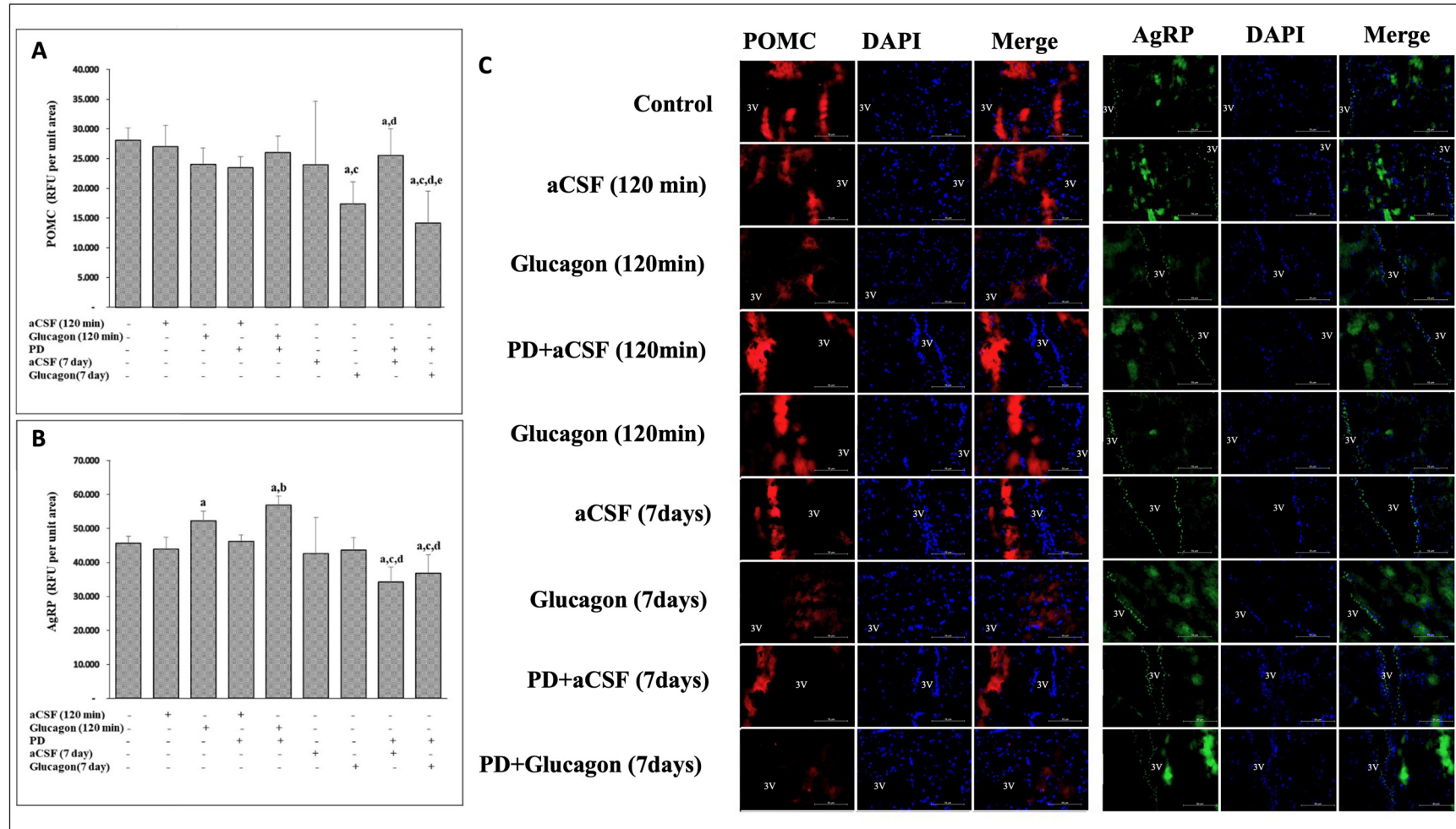
Figure 3. Immunostaining for POMC and AgRP. Quantitative fluorescence measurements as relative fluorescence units (RFU) for (**A**) POMC, (**B**) AgRP. Immunolocalization of the third ventricle (**C**) for marker AgRP (*green*) and marker POMC (red), counterstained with 4,6-diamidino-2-phenylindole (DAPI) (nuclear stain) (*blue*). Letter (a) indicates significance compared to Control and (b) indicates significance compared to aCSF (120 min), (c) indicates significance compared to aCSF (7 days), (d) indicates significance compared to Glucagon (7 days), (e) indicates significance compared to PD+ aCSF (7 days). In panels (**A-B**), one-way ANOVA was used ($p < 0.05$). Scale 50 μm.

following short-term administration (Figure 2C). On the other hand, the decrease in POMC in the Glucagon (7 days) group was statistically significant compared to the Control and aCSF (7 days) groups. The difference between the Glucagon (7 days) group and the PD+aCSF (7 days) group and the PD+Glucagon (7 days) group and the difference between the PD+aCSF (7 days) group and the PD+Glucagon (7 days) group is statistically significant ($p<0.05$).

When the effects of short and long-term central glucagon administration on AgRP protein levels were compared, it was observed that the AgRP levels were even higher in the Glucagon (7 days) group than in the Glucagon (120 min) group. When the PD+Glucagon (7 days) group and the PD+Glucagon (120 min) group were compared, an increase in AgRP level was observed. Brain AgRP levels in the groups in which glucagon was administered centrally for 120 minutes and 7 days, the differences between the groups were not statistically significant following both short and long-term administration (Figure 2D).

Discussion

Similar to the brain, nervous tissue, erythrocytes, leukocytes, and renal medulla cells can only meet the energy needs from glucose. Therefore, it is important to keep circulating glucose levels in balance at certain intervals[29]. Glucagon is a hormone that increases blood glucose levels through hepatic glucose production, which is synthesized from pancreatic α cells and released into the blood[30]. At the most basic level, as circulating blood sugar levels increase, glucagon secretion decreases; when blood sugar levels decrease, glucagon secretion increases, helping to maintain blood sugar levels. The primary focus of the majority of studies investigating glucagon control has been on understanding the response of glucagon secretion to glucose variations, especially in cases of hypoglycemia. Although it is thought that the brain may play a role in the changes in glucagon levels through various stressors (hypoglycemia, leptin, etc.), there is insufficient data on the mechanism[31].

In our study, while examining the CNS interaction of icv-administered glucagon in pancreatic denervation experimental groups, we investigated whether the effects on pancreatic tissue were regulated by a neuronal or hormonal interaction. In addition, we aimed to clarify whether the possible CNS activity of icv-administered glucagon is manifested by changing the sympathetic nervous system activity and affecting the extrapancreatic tissues or by altering the release of these hormones from the pancreas.

The increase in blood glucose in denervated groups indicates that central control plays a role in the endocrine secretions of the pancreas[25]. In addition, fluctuations in the blood glucose levels in the group receiving icv glucagon play a role in blood glucose change by acting on the CNS as well as its peripheral effects[32]. When the effects of short and long-term central glucagon administration on serum insulin levels were examined, it was observed that it was effective in reducing the insulin level in the short term. It was determined that long-term central glucagon administration had an effect on increasing the amount of insulin. Insulin secretion of the pancreas is controlled by the CNS, and it decreases the acute effect of the glucagon hormone and increases the chronic effect. It is known[33] that the pancreas is controlled by many hormones, peptides, and neurotransmitters. While the first studies[34] on the pancreas were generally aimed at elucidating endocrine or exocrine functions, they focused on other metabolic functions of the pancreas over time. Activation of the parasympathetic tone to the endocrine function of the pancreas can be increased by lesions of the ventromedial hypothalamus, and this leads to increased beta cell proliferation and mass[35,36] indicating that not only the secretion capacity but also the total number of beta cells can be regulated by this nervous control. CNS is an important sensor of ambient glucose levels. In the case of hypoglycemia, it directs nerve signals that increase glucagon secretion. It has been shown[37] that neurons in multiple regions containing glucose transporter 2 (GLUT2) in the solitary tract region of the hypothalamus and medulla in the CNS contribute to the stimulation of glucagon secretion by increasing parasympathetic impulses during hypoglycemia[38]. The increase in blood glucose in the glucagon-administered group may be related to the decrease in insulin and the increase in glucagon in the pancreatic tissue. Depending on the decrease of insulin, the blood glucose concentration increases due to the decrease in the uptake of glucose from the portal circulation into cells. Unless there is a metabolic disorder, the central effect of glucagon can be improved by the coordinated interaction of liver, pancreas, muscle and adipose tissue.

Multiple stimuli contribute to the regulation of glucagon release from the α-cells. Changes in blood glucose levels are the main regulator of insulin secretion. Islet cells rapidly detect and respond to changes in glucose in the environment. However, the direct sensitivity of α-cells to glucose change is not clear. Many studies[34] have shown that glucagon secretion is modulated by the paracrine signals from β and δ cells. It has been suggested that neural stimuli have a modulating rather than an initiating function on the secretion of pancreatic endocrine secretions. It has been reported[39] that neurohormonal interactions have specific and different effects for each cell type in the islets of Langerhans.

However, in addition to these local factors, glucagon release is directly controlled by changes in circulating catecholamines in addition to sympathetic and parasympathetic innervation. It is mainly circulating epinephrine that stimulates glucagon release. Autonomic factors originating from preganglionic sympathetic neurons in the brain act on chromaffin cells in the adrenal medulla and increase catecholamine release through cholinergic nicotinic stimulation[40]. Increased circulating catecholamines prevent hypoglycemia by increasing glucagon release. In a study[41] examining the effective stimulatory pathways in the regulation of pancreatic endocrine secretion, it was suggested that pancreatic denervation in dogs does not have a measurable role in the amount and efficiency of fasting and stimulated insulin secretion. In a study[42] claiming that post-transplant glucose and insulin abnormalities cannot be attributed to denervation, it was shown that evoked blood glucose and insulin levels did not differ between the innervated and denervated groups. Furthermore, it has been suggested that neurotransmission from the pancreas is not required for cyclic insulin release in response to enteral or parenteral glucose loading and physiological and pharmacological hyperglycemia. When continuous infusion of glucose, insulin, epinephrine and propranolol were administered to inhibit endogenous insulin secretion and thus to assess insulin sensitivity, no significant change in insulin sensitivity was detected. They[43] suggested that denervation induces an insulin response (not glucagon) to increased glucose, possibly by interrupting the catecholaminergic negative stimulus in the β-cell.

When insulin and glucagon levels were evaluated in the pancreatic tissue in the short-term (120 min) experimental protocol, it was determined that icv-glucagon administration increased the glucagon protein level and there was a decrease in the insulin level. While a decrease was observed in blood glucagon and insulin levels in all groups, an increase in blood glucose levels occurred. Present and previous studies' results[44] suggest that the possible effect may be that the glucagon signal from the center increases the level of glucagon from the pancreas, and this increase leads to the suppression of insulin synthesis, which also causes a decrease in blood insulin levels, resulting in an increase in blood glucose levels. The increase in blood glucose level causes decreases in blood glucagon levels.

It is stated[41] that the main control in a structurally intact pancreas is alterations in blood glucose levels. Our findings suggest that there may be a neural effect in the short-term control of pancreatic endocrine secretions (insulin and glucagon). However, it is very difficult to clearly state which of these neural controls has a more dominant role in the insulin-glucagon-blood glucose triangle. In the pancreatic tissue that was evaluated following the long-term (7 days) experimental protocol, icv glucagon administration induced an increase in glucagon levels and a decrease in insulin levels. The decrease in insulin and glucagon levels in the pancreatic tissue one week after PD administration can be evaluated as an indicator of neural control in the synthesis and autoregulation of pancreatic islet cells mentioned previously[34]. The results suggest that the balance in blood glucose levels may be achieved through an increase in insulin secretion. Similar to the PD group, it appears that maintaining blood glucose balance is linked to a more substantial increase in insulin secretion in the pancreas compared to glucagon.

AgRP and POMC are effective in the neural control of weight changes due to nutritional behaviors. While a POMC increase suppresses appetite, an AgRP increase increases food consumption with appetite. Studies[45] have shown that many endocrine signals regulate feeding behaviors by affecting these pathways in a coordinated manner. It is suggested that POMC and AgRP are compatible by interacting with each other, and when this harmony is disrupted, a metabolic syndrome such as obesity develops. POMC is known to be active in the firing of AgRP neurons[46,47]. In one[48] of the studies evaluating the relationship between eating behaviors and basal metabolism, it was suggested that exercise induced an increase in POMC and that an increase in POMC increased leptin receptors. It has been stressed as an

indication that POMC may play a more active role in the regulation of appetite. Appetite suppression occurs through the heightened inhibiting effect of the hormone leptin on AgRP and NPY in this metabolic pathway. Our findings and previous studies[47] suggest that POMC is effective in the firing of AgRP neurons together, showing that the decrease in POMC level caused by glucagon provides evidence that POMC abolishes the firing effect on AgRP and thus causes a decrease in AgRP levels. The central administration of glucagon-suppressed POMC and increased feed intake of the animals is consistent with the known mechanism of appetite regulation, yet it is in contrast with the reduction of AgRP[49]. Therefore, the fact that the decrease of AgRP does not suppress appetite can be accepted as evidence[48] that POMC may have a more effective role in the regulation of appetite than AgRP.

Our study findings showed that the changes in POMC, AgRP, feed consumption, and body weight were statistically significant in the PD group compared to the non-PD group, which also revealed that neural stimulation is an important factor in this mechanism. It has been reported[50] that leptin keeps the blood glucose level in balance by preventing hypoglucagonemia by acting on POMC neurons to regulate the energy balance due to decreased food intake.

It has been suggested[51,52] that a single dose of icv glucagon infusion in rodents increases energy expenditure due to stimulation of brown adipose tissue thermogenesis. In studies[53-55] evaluating chronic icv administrations, the role of glucagon in energy expenditure has not been fully explained. Interestingly, central glucagon signals appear to play an important role in regulating liver glucose production. The role of the central effects of glucagon in the regulation of energy metabolism and glycemia has not been fully elucidated.

In another study[54] on rodents, it is suggested that glucagon infusion into the mediobasal hypothalamus under pancreatic clamp conditions causes normoglycemia. Since central glucose receptor signals control hepatic glucose production *via* protein kinase A and intact hepatic vagal innervation mechanisms, glucagon signaling in the mediobasal hypothalamus is predicted to prevent the development of hypoglycemia due to continuous hepatic glucagon signaling.

Our findings showed that the glucagon administered to the third ventricle region of the rats exerted effects on diet and pancreatic tissue. Our findings also provided evidence that glucagon,

known as an endocrine hormone, plays an active role in glucose homeostasis by acting like a neurohormone in the brain. Although it shows these effects on the pancreatic tissue through a nervous stimulus pathway, it is also possible that it may show its possible effects on other organs such as the liver, adrenal gland, kidney, and metabolic pathways, which were not examined in our study but are known to be effective on glucose homeostasis. Furthermore, our findings showed that 7 days of glucagon administration caused an increase in the body weights of animals, and this increase was higher, especially in pancreatic denervation groups. In light of this information, it can be said that the brain is effective on pancreatic secretion, and it may be possible that pancreatic denervation may change the secretion of insulin and glucagon even when its control over pancreatic secretions is constrained, *via* affecting the activity of different metabolic pathways. The appetite-enhancing effect of glucagon, and thus food intake, seems likely to be exerted by reducing the suppression of the actors involved in the stimulation of appetite in the brain. The fact that icv-glucagon administration caused a significant decrease in POMC levels in our study provides evidence that glucagon may be a central actor in the regulation of appetite. However, we also think that the related pathways in the liver should be examined in order to fully explain the role of AgRP and POMC in glucose homeostasis.

Western blot and IF methods showed that insulin and glucagon synthesis from pancreatic tissue were decreased when neuronal transmission between the brain and pancreas was blocked by PD in rats. Based on the information in the literature and our findings, we can say that the neural control of the brain plays a very active role, as well as the blood glucose and autocrine effects on the glucose homeostasis of the pancreas. It is stated that the evaluation of AgRP (appetite enhancing) and POMC (appetite suppressor) protein levels, hypothalamic factors effective in the regulation of nutritional behaviors, following continuous and long-term (7 days) application of icv-glucagon administration rather than a single dose and short-term (120 min) application will provide more valuable data to the literature. A single glucagon administration will not be adequate in reaching the therapeutic dose, and there will be an interruption in the process of its effect. However, with the continuous infusion, reaching the therapeutic dose and the continuity of the effectiveness will be ensured.

In our study, it was revealed that icv glucagon administration to the third ventricle, which is close to the ARC and PVN regions in the brain, where nutritional behaviors are regulated, causes a decrease in the POMC level. It was determined that this decrease in POMC caused a suppression of AgRP, but this suppression in AgRP caused an increase rather than a decrease in food intake. This supports the findings that POMC has a more dominant role than AgRP in the regulation of appetite. However, in order to demonstrate this effect of POMC on AgRP more evidently, it would be appropriate to determine the changes in melanocortin receptor-3 and NPY levels. Although not evaluating this parameter in our study creates a limitation in terms of elucidating the mechanism of action, it is very important to be able to provide information about the possible pathway with the available data. It is known that insulin has a suppressive effect on AgRP neurons. The fact that icv-glucagon administration causes a decrease in POMC levels suggests that glucagon, which has an opposite effect to insulin in the periphery, has a similar effect in the regions where nutritional behaviors are regulated in the CNS.

Our results revealed that the role of glucagon in blood glucose homeostasis is not limited to increasing blood glucose by acting on the liver after being released from the pancreas when the blood glucose level decreases. In addition, it can simultaneously be effective in the synthesis and secretion of insulin and glucagon by showing a neuroendocrine effect. It also revealed that it possibly affects many physiological pathways that develop in response to these effects. Central glucagon signaling indicates that an increase in the level of glucagon from the pancreas results in suppression of insulin synthesis, which causes a decrease in blood insulin levels and, therefore, an increase in blood glucose levels. We assume that there is a possible mechanism where this increase in blood glucose level also causes decreases in blood glucagon levels. While PD application causes a decrease in both the levels of insulin and glucagon in the pancreas and their release into the blood, PD+Glucagon administration causes an increase in blood glucose and, in response, an increase in insulin secretion and a decrease in glucagon secretion. However, the main stimulating factor on pancreatic secretion is blood glucose. The findings of this study present evidence that neural stimuli can play a role in both short-term and long-term regulation of the synthesis and secretion of endocrine

secretions, maintaining a mutual balance under changing physiological conditions. Nevertheless, it remains challenging to determine which variable or variables are primarily governed by this neural control in maintaining glucose homeostasis, identifying the key regulatory step aimed at balancing blood glucose, discerning the factors sensitive to change that elicit a response, and understanding the regulatory mechanisms underlying these responses. Future studies may provide positive reflections on treatment processes, including the application of different doses of glucagon in diet-related physiological courses (such as fasting, satiety, before and after meals) and in disorders of impaired glucose metabolism, especially diabetes.

Conclusions

Findings of this study have provided significant information that glucagon may have a much greater role in glucose homeostasis than currently known, and that it can exhibit this activity both peripherally and centrally. It also provided evidence that it may have a central role in regulating appetite.

In conclusion, we suggest that in metabolic diseases where the balance in glucose homeostasis is impaired, such as obesity and diabetes, more serious consideration of glucagon inclusion in the treatment process (by taking into account its central effect) may be beneficial in increasing the effectiveness of the treatment approach.

ORCID ID
Kevser Tanbek: 0000-0003-2099-2273
Umit Yilmaz: 0000-0003-0248-3483
Semir Gul: 0000-0002-4668-9603
Ahmet Koc: 0000-0003-3484-2137
Mehmet Gul: 0000-0002-1374-0783
Suleyman Sandal: 0000-0002-8916-3329

Data Availability
All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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