Glucagon signalling in the dorsal vagal complex is sufficient and necessary for high-protein feeding to regulate glucose homeostasis \textit{in vivo}

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Abstract

High-protein feeding acutely lowers postprandial glucose concentration compared to low-protein feeding, despite a dichotomous rise of circulating glucagon levels. The physiological role of this glucagon rise has been largely overlooked. We here first report that glucagon signalling in the dorsal vagal complex (DVC) of the brain is sufficient to lower glucose production by activating a Gcgr–PKA–ERK–K<sub>ATP</sub> channel signalling cascade in the DVC of rats \textit{in vivo}. We further demonstrate that direct blockade of DVC Gcgr signalling negates the acute ability of high- vs. low-protein feeding to reduce plasma glucose concentration, indicating that the elevated circulating glucagon during high-protein feeding acts in the brain to lower plasma glucose levels. These data revise the physiological role of glucagon and argue that brain glucagon signalling contributes to glucose homeostasis during dietary protein intake.

Keywords brain; glucagon; glucose homeostasis; protein-feeding

Introduction

The fact that dietary protein ingestion does not increase blood glucose concentration, despite the ability of amino acids to be converted to glucose through hepatic gluconeogenesis, has been known for over a century [1,2]. Since then, the ability of a high-protein meal to acutely lower postprandial glucose compared to a low-protein meal has been established in humans [3–5] and rodents [6–8], but the underlying mechanisms remain unclear. The reduced amount of carbohydrates provided by the diet may account for this phenomenon. However, dietary protein is effective in reducing the rise in glucose regardless of whether it is given as an alternative to carbohydrates [4] or rather in addition to carbohydrates [3]. Increased insulin levels may also mediate the glucose-lowering effects of high-protein feeding, since adding protein to ingested glucose has a positive synergistic effect on insulin secretion [3,9,10]. However, increasing dietary protein content at the expense of carbohydrates actually reduces the post-feeding increase in insulin levels while still lowering glucose [4], and protein intake alone only marginally stimulates insulin [7,9], suggesting the ability of high-protein feeding to reduce the plasma glucose response does not necessarily rely on the contribution of insulin. Interestingly, a factor that has been largely overlooked is the acute elevation of blood glucagon concentration after a high-protein meal [4,6]. Given that glucagon is classically known to stimulate glucose production from the liver, increased glucagon would be expected to correlate with increased glucose levels. This apparent discrepancy therefore suggests the existence of another mechanism to counteract the effect of peripheral glucagon action during high-protein feeding to maintain glucose homeostasis.

In fact, we have recently discovered a negative feedback system of glucagon action in the brain, in which glucagon acts in the medio-basal hypothalamus (MBH) to suppress glucose production and improve glucose tolerance [11]. Glucagon crosses the blood–brain barrier [12], and the MBH is situated adjacent to the median eminence, a circumventricular organ which links CNS and peripheral circulation. Thus, peripheral glucagon would presumably reach the MBH to exert its glucose-lowering effect. Accordingly, we found that disrupting the MBH glucagon signalling pathway enhances the ability of intravenous glucagon administration to increase plasma glucose levels [11].

In the light of these findings for which a direct physiological role has yet to be identified, we postulated that the physiological rise of circulating glucagon seen after high-protein feeding may trigger glucagon signalling mechanisms in the brain to contribute to the glucose-lowering effect of high-protein feeding compared to low-protein feeding. Physiologically, glucagon entry into the CNS would not be restricted to the MBH and would likely activate...
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signalling mechanisms in other highly permeable regions of the brain as well. Thus, to examine whether widespread brain glucagon action plays a physiological role in peripheral glucose regulation, it became important to examine whether glucagon elicits analogous actions in hypothalamic and extra-hypothalamic gluco-regulatory brain structures. We therefore targeted the dorsal vagal complex (DVC), a region in the brainstem which also contains a circumventricular organ termed the area postrema, as well as the nucleus of the solitary tract and the dorsal motor nucleus of the vagus. The DVC senses insulin to regulate glucose homeostasis [13] and both insulin and leptin to inhibit food intake [14,15]. Glucagon receptors (Gcgr) have also been located in the brainstem [16], though a role of glucagon signalling in this region has not yet been identified. Thus, we here investigated whether glucagon acts in the DVC to lower glucose production and to contribute to glucose homeostasis during high-protein feeding in vivo (Fig 1A).

Results

High-protein feeding reduces the plasma glucose rise and increases plasma glucagon levels compared to low-protein feeding

To first confirm the findings of previous studies which reported that increasing the protein content of a meal leads to a reduction in plasma glucose levels accompanied by a rise in glucagon concentration [3–6], we performed fasting–refeeding experiments on rats that were maintained on a regular chow diet, then fasted for 24 h and refed with isocaloric high-protein/low-carbohydrate/low-fat (“high-protein”; HP) or low-protein/high-carbohydrate/low-fat (“low-protein”; LP) purified diets which contained 65.4% and 21.5% kilocalories from protein, respectively (Fig EV1A). Rats also underwent DVC cannulation surgery and DVC saline infusion during the experiment to ensure a comparable status to those in later experiments in which the role of glucagon was to be examined via DVC infusions of compounds. As expected, intake of low-protein diet (Fig 1C) increased plasma glucose (Fig 1B) and insulin levels (Fig 1C) compared to baseline. Consistent with previous literature, we found that, compared to low-protein feeding, high-protein feeding lowered plasma glucose levels at 30 min onwards and reduced the area under the curve (AUC) of plasma glucose (Fig 1B). This effect was independent of changes in cumulative food intake and plasma insulin (Fig 1C) but correlated with a rise in plasma glucagon (Fig 1D) compared to low-protein feeding. Though a lowering of insulin after high-protein versus low-protein feeding has been reported [4], we here observed no change, albeit with a non-significant trend of insulin to be lowered at 30 min post-high-protein refeeding (Fig 1C).

Glucagon infusion into the DVC activates glucagon receptors to lower glucose production and stimulate PKA

Before beginning to assess whether the rise in plasma glucagon during high-protein feeding acts in the DVC, we first aimed to determine whether DVC glucagon action per se regulates glucose homeostasis (Fig 1A). The Gcgr is expressed in several brain regions including the brainstem [11,16,17]. Western blot analyses revealed the presence of Gcgr in rat DVC tissue (Fig 1E), similar to the MBH [11], while the liver and muscle were used as positive and negative controls, respectively. To address whether glucagon action in the DVC regulates glucose metabolism, rats underwent a pancreatic euglycemic clamp procedure (Fig EV1B). Compared to an infusion of saline, DVC glucagon administration increased the glucose infusion rate required to maintain euglycemia (Fig 1F) due to a suppression of glucose production (Figs 1G and EV2A) without altering glucose uptake (Fig EV2B). We secondly confirmed that the effects of DVC glucagon are specific to Gcgr signalling through the infusion of the Gcgr antagonist (GRA), des-His3 [Glu9]glucagon amide. This antagonist also attenuated the effect of DVC glucagon administration to lower glucose production without independently affecting glucose metabolism (Figs 1F and G, and EV2A and B).

Next, we sought to identify the signalling mechanisms through which DVC Gcgr activation regulates glucose production (Fig 1A). Given that the Gcgr couples to the Gαs protein which signals through protein kinase A (PKA), we investigated whether DVC glucagon signalling activates PKA. We observed via Western blot analysis that infusion of glucagon into the DVC promoted PKA activation, represented by the phosphorylation of the downstream PKA substrate, CAMP response element-binding protein (CREB), as compared to a saline infusion (Fig 1H). Notably, the co-infusion of GRA negated the glucagon-induced increase in p-CREB. Taken together, glucagon–Gcgr signalling in the DVC stimulates PKA activity and lowers glucose production.

PKA activation in the DVC is required for glucagon action

To investigate whether the observed increase in PKA activation mediates the effect of DVC glucagon infusion to lower glucose production (Fig 1A), we next co-infused the PKA inhibitor Rp-cAMPS with glucagon into the DVC during a pancreatic clamp (Fig EV1B). While Rp-cAMPS itself had no effect on glucose metabolism, it negated the ability of a DVC glucagon infusion to increase the glucose infusion rate (Fig 2A) and suppress glucose production (Figs 2B and EV3A) with no effect on glucose uptake (Fig EV3B). We confirmed by Western analysis that PKA activity was negated in the DVC tissues of rats receiving co-infusion of glucagon with Rp-cAMPS (Fig 2C). Thus, PKA is a downstream effector of glucagon–Gcgr signalling in the DVC to lower glucose production.

Activation of Erk1/2 and KATP channels in the DVC are required for glucagon action

Gcgr–PKA signalling activates extracellular signal-regulated kinase 1 and 2 (Erk1/2) in pancreatic islets as well as MIN6 [18] and HEK293
Figure 1. High-protein feeding lowers glucose via brain glucagon signalling

A Schematic representation of the working hypothesis: High-protein feeding elevates plasma glucagon levels, triggering a Gcgr→PKA→Erk1/2→KATP channel signalling pathway in the DVC to regulate glucose production and stimulate PKA.

B Plasma glucose concentrations during fasting–refeeding experiments in low-protein diet plus DVC saline infusion (LP/saline; n = 6) and high-protein diet plus DVC saline infusion (HP/saline; n = 7) groups with area under the curve (AUC; mg·h/dl) shown. Values are shown as mean ± SEM. *P < 0.05 and ***P < 0.001 compared to low-protein diet determined by repeated-measures ANOVA and Bonferroni post hoc test.

C Cumulative food intake (top) and plasma insulin concentration (bottom) during fasting–refeeding experiment. Values are shown as mean ± SEM.

D Plasma glucagon concentration during fasting–refeeding experiments. Values are shown as mean ± SEM. *P < 0.05 and **P < 0.01 compared to low-protein diet determined by repeated-measures ANOVA and Bonferroni post hoc test.

E Western blot showing glucagon receptor protein expression in rat liver, muscle, DVC and MBH.

F, G Glucose infusion rates (F) and rates of glucose production (G) during pancreatic clamp experiments with DVC infusion of saline (sal; n = 5), glucagon (gcg; n = 6), glucagon-specific monoclonal antibody (mAb; n = 5), gcg plus mAb (n = 5), glucagon receptor antagonist (GRA; n = 5) or gcg plus GRA (n = 6). Values are shown as mean ± SEM. **P < 0.01 and ***P < 0.001 compared to all other groups as determined by ANOVA and Tukey’s post hoc test.

H Representative Western blot (top) and quantified level of phosphorylation of CREB in DVC tissues obtained at the end of clamps with DVC infusion of sal (n = 6), gcg (n = 5) or gcg plus GRA (n = 5), expressed as fold increase over saline. Values are shown as mean ± SEM. **P < 0.01 compared to all other groups as determined by ANOVA and Tukey’s post hoc test.

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ATP-sensitive potassium (KATP) channel signalling [13], while MBH suppresses glucose production through Erk1/2 and subsequent [19] cell lines. Furthermore, insulin administration into the DVC suppresses glucose production through Erk1/2 and subsequent [20]. We therefore investigated whether DVC glucagon infusion lowers glucose production via a PKA–Erk1/2–KATP channel-dependent pathway (Fig 1A). We first observed increased phosphorylation of Erk1/2 in the DVC tissues of rats that received a glucagon infusion compared to saline (Fig 3A). This effect was nullified by a co-infusion of Rp-cAMPS with glucagon (Fig 3A), indicating that glucagon activates Erk1/2 via a PKA-dependent mechanism.

To test whether the high-protein-induced elevation of circulating glucagon levels triggers Gcgr signalling in the DVC to reduce the post-refeeding glucose response in this unclamped setting, we administered GRA into the DVC at the same concentration as in clamp experiments to inhibit Gcgr signalling. While DVC infusion of GRA had no effect compared to a saline infusion on the glucose response in low-protein diet fed rats (Fig 4A) nor in low-protein/low-carbohydrate/high-fat-fed rats (Fig EV5A), it decreased the glucose AUC during high-protein/low-carbohydrate/high-fat feeding.

Glucagon signalling in the DVC maintains glucose homeostasis during high-protein feeding

After delineating the Gcgr-specific signalling cascade that suppresses glucose production in response to DVC glucagon administration (Figs 1–3), we next sought to examine whether this mechanism plays a physiological role to mediate the previously observed relationship between elevated plasma glucagon (Fig 1D) and reduced plasma glucose (Fig 1B) induced by the high-protein diet compared to the low-protein diet in fasting–refeeding experiments.

To test whether the high-protein-induced elevation of circulating glucagon levels triggers Gcgr signalling in the DVC to reduce the post-refeeding glucose response in this unclamped setting, we administered GRA into the DVC at the same concentration as in clamp experiments to inhibit Gcgr signalling. While DVC infusion of GRA had no effect compared to a saline infusion on the glucose response in low-protein/high-carbohydrate/low-fat-fed rats (Fig 4A) nor in low-protein/low-carbohydrate/high-fat-fed rats (Fig EV5A), it increased the glucose AUC during high-protein/low-carbohydrate/low-fat feeding.

**Figure 2.** DVC PKA activation is required for glucagon action.

A, B Glucose infusion rates (A) and rates of glucose production (B) during pancreatic clamp experiments with DVC infusion of sal (n = 5), gcg (n = 7), Rp-cAMPS (n = 5) or gcg plus Rp-cAMPS (n = 5). Values are shown as mean ± SEM. **P < 0.01 compared to all other groups as determined by ANOVA and Tukey’s post hoc test.

C Representative Western blot (top) and quantified level of phosphorylation of CREB in DVC tissues obtained at the end of clamps with DVC infusion of sal (n = 6), gcg (n = 5) or gcg plus Rp-cAMPS (n = 5), expressed as fold increase over saline. Values are shown as mean ± SEM. ***P < 0.001 compared to all other groups as determined by ANOVA and Tukey’s post hoc test.

Source data are available online for this figure.
low-fat feeding and abolished the lowering of glucose concentration at 60 min post-refeeding (Fig 4A). This glucose elevation argues that the increased plasma glucagon concentration in response to high-protein intake (Fig 1D) triggered the DVC Gcgr signalling mechanism to lower plasma glucose and maintain glucose homeostasis, while occurring independently of any changes in cumulative food intake (Figs 4B and EV5B), plasma insulin (Fig 4C) or plasma glucagon (Fig 4D) at 60 min post-refeeding. In summary, these data indicate that DVC glucagon signalling mediates the acute glucose-lowering effect of high-protein feeding, presumably due to a suppression of glucose production as demonstrated in pancreatic clamp experiments.

Figure 3. DVC activation of Erk1/2 and KATP channels is required for glucagon action.
A Representative Western blot (top) and quantified level of phosphorylation of Erk1/2 in DVC tissues obtained at the end of clamps with DVC infusion of sal (n = 5), gcg (n = 5) or gcg plus Rp-cAMPS (n = 5), expressed as fold increase over saline. Values are shown as mean ± SEM. *P < 0.05 compared to all other groups as determined by ANOVA and Tukey’s post hoc test. A representative immunoblot is shown.
B Experimental procedure and protocol for pancreatic clamp experiments using genetic approaches.
C, D Glucose infusion rates (C) and rates of glucose production (D) during pancreatic clamp experiments with DVC infusion of sal (n = 6), gcg (n = 7), PD98059 (PD; n = 5), gcg plus PD (n = 5), glibenclamide (glib; n = 5) or gcg + glib (n = 7) and viral injection of GFP (n = 5), gcg plus GFP (n = 5), MEK1-DN (n = 5) or gcg plus MEK1-DN (n = 5). Values are shown as mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to all other groups as determined by ANOVA and Tukey’s post hoc test.
Source data are available online for this figure.
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Discussion

Increasing the protein content of a meal acutely lowers the post-prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly discrepant prandial glucose response while stimulating a seemingly 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randomly assigned into the various diet and treatment groups detailed below.

**Surgical procedures**

Vascular catheterization and DVC cannulation surgeries were performed as described [13]. In brief, rats were anaesthetised for surgeries with an intraperitoneal injection of a cocktail of ketamine (60 mg/kg body weight; Vetalar, Bioniche) and xylazine (8 mg/kg body weight; Rompun, Bayer). Ten days prior to experiments, rats underwent stereotaxic implantation (David Kopf Instruments, Tujunga, CA, USA) of a 26-gauge stainless steel bilateral guide cannula (C235G, Plastics One Inc.) into the DVC, targeting the nucleus of the solitary tract (0.0 mm on the occipital crest, 0.4 mm lateral to midline, 7.9 mm below skull surface). In rats receiving DVC adenoviral injections, 3 μl of either GFP (pfu = 3 × 10^8) or MEK1-DN (pfu = 1.8 × 10^8) adenovirus was injected into each site immediately following DVC cannulation surgery. This MEK1-DN virus has been previously demonstrated to abolish the ability of DVC insulin to lower glucose production [13]. Six days later, catheters were surgically inserted into the jugular vein and carotid artery for infusion and blood sampling. Recovery was monitored by daily food intake and weight gain. Rats that did not recover to at least 90% of their pre-surgical body weight were excluded from the study. Rats were randomly designated into groups prior to experiment, and no blinding was done during the experimental procedures described below.

**Pancreatic (basal insulin) euglycemic clamp procedure**

Rats were restricted to 15 g of chow the night before experiments to ensure comparable nutritional status. The experiments began between 10:30 AM and 11:30 AM and were conducted as described [11,20] in conscious, unrestrained rats. A primed-continuous intravenous infusion of 3-[³H]-glucose tracer (40 μCi bolus, 0.4 μCi/min; PerkinElmer) was initiated at 0 min and maintained until the end of the experiment (t = 210 min) to assess glucose kinetics through tracer dilution methodology. After a basal period of 90 min, the pancreatic clamp was initiated with a continuous infusion of somatostatin (3 μg/kg/min) and insulin (1.2 μU/kg/min) to maintain basal insulin levels, and a variable infusion of 25% glucose as necessary to maintain euglycemia. Plasma samples were collected at 10-min intervals for determination of [³H]-glucose-specific activity and plasma glucose, insulin and glucagon concentrations. At the end of the experiment, rats were anaesthetised, and then, DVC tissue samples were collected, frozen in liquid nitrogen and stored at –80°C for later analysis.

**DVC infusions during pancreatic clamp experiments**

All substances were infused at a rate of 0.33 μl/h through the bilateral DVC cannula. Saline (0.9%) or glucagon (Sigma, 5 pg/μl) was infused at t = 90–210 min. The following substances were pre-infused for 90 min prior to the clamp (t = 0–90 min) and infused alone or with glucagon during the clamp: (i) the glucagon-specific monoclonal antibody, K79bB10 (Sigma-Aldrich, 0.02 μg/μl); (ii) the Gcgr antagonist, des-His¹ [Glu⁵] glucagon amide (Tocris Bioscience, 5 ng/μl); (iii) the PKA inhibitor, Rp-cAMPS (Tocris Bioscience, 40 μM); (iv) the MEK1/2 inhibitor, PD98059 (Sigma-Aldrich, 100 μM); or (v) the K_ATP channel inhibitor, glibenclamide (Sigma-Aldrich, 100 μM).

The selected glucagon concentration was previously shown to lower glucose production when administered into the MBH, and at the specified doses, K79bB10, des-His¹ [Glu⁵] glucagon amide, Rp-cAMPS and glibenclamide all negated the effect of MBH glucagon [11,20]. The selected PD98059 concentration has been previously demonstrated to abolish the ability of DVC insulin to lower glucose production [13].

**Fasting–refeeding experiments**

High-protein (Harlan Laboratories, TD.91352) and low-protein (Harlan Laboratories, TD.06220) purified diets were isocaloric with casein as the protein source. The kilocalorie distribution of the high-protein diet was 65.4% protein, 21.3% carbohydrate and 13.4% fat, while the low-protein diet consisted of 21.5% protein, 65.3% carbohydrate and 13.1% fat. Low-protein/low-carbohydrate/high-fat diet (Research Diets, D12492) consisted of 20% protein, 20% carbohydrate and 60% fat. Rats were maintained on regular chow and fasted for 24 h prior to refeeding. Continuous DVC administration of either saline or the Gcgr antagonist des-His¹ [Glu⁵] glucagon amide (Tocris Bioscience, 0.005 μg/μl) at a rate of 0.33 μl/h began 90 min prior to refeeding and was maintained until the end of the experiment (t = 60 min). The refeeding experiment began at 11:00 AM (t = 0 min) by presenting 5 g (18.75 kcal) of high-protein or low-protein diet or an isocaloric amount of low-protein/low-carbohydrate/high-fat diet to the rats. Plasma samples were obtained at 15-min intervals for determination of plasma glucose, insulin and glucagon levels. Cumulative food intake was assessed by weighing remaining food at 15-min intervals.

**Biochemical analyses**

Plasma glucose concentrations were determined by the glucose oxidase method (Glucose Analyzer GM9; Analox Instruments, Lunenburg, MA, USA). Plasma insulin and glucagon concentrations were measured by radioimmunoassay (Linco Research, St. Charles, MO, USA).

**Western blot analysis**

Western blot analysis was performed as previously described [13]. Tissues were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Nonidet P-40, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche). Protein concentration of each homogenized tissue was determined with the Pierce 660 nm protein assay (Thermo Scientific). Tissue lysates (20–60 μg) were subjected to electrophoresis on an 8%, 10% or 12% polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were incubated for 1 h with 5% (w/v) BSA in TBS-T and then immunoblotted with the indicated primary antibodies (diluted 1/1,000 in 5% BSA) for 16 h at 4°C. The blots were then washed 3 times with TBS-T and incubated with the secondary antibody diluted 1/4,000 in 5% skimmed milk for 1 h. Blots were washed 5
times with TBS-T; then, the signal was detected with an enhanced chemiluminescence commercial kit (Clarity Western ECL, Bio-Rad). Blots were imaged with GelCapture (DNR), and the phosphorylation level of CREB and Erk1/2 was quantified with GelQuant software (DNR) and normalized for the corresponding total protein level. The following primary antibodies were used: anti-glucagon receptor (Novus, cat# NLS4256), anti-CREB (Cell Signalling, #9197), anti-p-S133 CREB (Cell Signalling, #9198), anti-Erk1/2 (Cell Signalling, #4695) and anti-p-T202/Y204 Erk1/2 (Cell Signalling, #4376).

Statistical analyses

Power calculations were not performed; however, the sample size for each group was chosen based on prior knowledge of statistical power from previously published experiments and on study feasibility. In pancreatic clamp experiments, the time period 60–90 min was averaged for the basal condition, and the time period 180–210 min was averaged for the clamp condition. Our data showed normal variance, and thus, statistical analysis of two groups was performed using unpaired Student’s t-test, and where comparisons were made across more than two groups, ANOVA was performed and, if differences between groups were found significant, Tukey’s multiple comparison post hoc test was used to compare each group against all other groups. Measurements that were taken repeatedly over time were compared using repeated-measures ANOVA and, if significant, this was followed by a Bonferroni post hoc test to determine the statistical significance between groups. These statistical analyses were performed for all figures unless otherwise stated. P < 0.05 was considered statistically significant.

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Author contributions

MPL conducted and designed experiments, performed data analyses and wrote the manuscript; MAA, JTYY and BMF assisted with experiments; and TKTL supervised the project, designed experiments and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


