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AgRP mediated calcium Inhibition of feeding via the vagal afferent nerve-brain pathway

Aru Su[1†], Linghui Pan[1†], Qian Zhou[1], Qin Zhu[1], Ruihua Li[1], Mingming Liu[1], Ruifan Wu[1], Lina Wang[1], Songbo Wang[1], Qingyan Jiang[1], Gang Shu[1*] and Canjun Zhu[1*]

Abstract
Obesity poses serious health risks and is trending younger, developing effective strategies to prevent obesity is crucial. Calcium intake is a potential strategy to reduce weight/fat, as it generally enhances the body's energy metabolism. However, calcium's effects on appetite and its specific mechanisms remain unclear. To investigate these questions, we administered calcium orally to fasted mice and found that calcium inhibited food intake during the first 3 h. Long-term calcium supplementation in water decreased HFD intake, weight gain, and fat deposition while increasing energy metabolism in young mice. Mechanistically, calcium activated the vagal afferent nerves and inhibited ARC^{AgRP} neurons—key appetite regulation neuron. What's more, these effects are blunted by chemogenetic inhibition of gastrointestinal intestinal vagal afferent nerves or activation of ARC^{AgRP} neuronal activity. Overall, we showed that ingested calcium activates vagal afferent nerves, inhibiting the activity of ARC^{AgRP} neurons, thereby reducing food intake. This study supports calcium's role in obesity dietary therapy.

Keywords Calcium, Appetite, Vagal afferent nerve, AgRP

Introduction

Obesity has become a global health priority, as its prevalence continues to rise [1–3] and increasingly affects younger populations [4]. Food therapy has emerged as a widely accepted weight loss method due to its safety and cost-effectiveness [5–7]. Previous studies have shown that dietary calcium is used to fight obesity [5, 8–10] and calcium's anti-obesity effects are primarily achieved through promoting fat metabolism, increasing thermogenesis, and reducing fat synthesis [11, 12]. Surprisingly, study has found a high-calcium diet reduces the appetite of obese patients [13]. These results suggest that dietary calcium may counteract the development of obesity by suppressing appetite, but its specific mechanisms have not been fully elucidated.

Nutrients signals in the gastrointestinal tract, on the one hand, can cross the blood-brain barrier through the blood circulation to act on the central nervous system, on the other hand, they can also transmit signals to the central nervous system through the vagal or spinal afferent nerve [14–19]. Among these, the gut-brain neural pathway for nutrient sensing is a rapid appetite response circuit [20, 21]. When the gastrointestinal detects nutrients, hunger-sensitive agouti-related peptide/Neuropeptide Y (AgRP/NPY) neurons, a generally acknowledged orexigenic neuromodulator [22], in the arcuate nucleus (ARC) respond quickly [17]. Are AgRP neurons involved in dietary calcium-induced feeding regulation? If so,

†Aru Su and Linghui Pan contributed equally to this work.

*Correspondence:
Gang Shu
shugang@scau.edu.cn
Canjun Zhu
canjunzhu@scau.edu.cn
[1]State Key Laboratory of Swine and Poultry Breeding Industry, Province Key Laboratory of Animal Nutritional Regulation and Guangdong Laboratory for Lingnan Modern Agriculture, College of Animal Science, South China Agricultural University, Guangzhou, Guangdong, China

through which peripheral pathway does calcium in the gastrointestinal tract transmit signal to AgRP neurons?

To comprehensively elucidate the effect of dietary calcium on appetite. Firstly, we investigated the effects of exogenous calcium supplementation on food intake and body weight gain in mice under different energy states and diets. To identify neurons involved in calcium-induced appetite suppression, we specifically activated AgRP neurons in ARC (ARC^{AgRP}) or inhibited the nodose ganglion (NG) containing vagal afferent cell bodies through chemogenetic. Finally, we further verified the role of the vagal afferent nerve in calcium's inhibition of feeding by means of subdiaphragmatic vagotomy (Sdvx) and specific inhibition of gastrointestinal vagal afferent nerves. Our results show that calcium inhibits ARC^{AgRP} neurons via a gut-brain axis pathway mediated by gastrointestinal vagal afferent nerves, thereby reducing food intake and increasing resistance to high-fat diet (HFD) -induced obesity in young mice.

Results

Calcium conditionally inhibited food intake and increased energy expenditure

We examined the effects of acute oral calcium administration on food intake in chow diet-fed C57BL6/J mice under both fasting-refeed and ad libitum feeding. Calcium significantly suppressed food intake in fasting-refeed mice (Figs. 1 A-B) but showed no effect in ad libitum feeding mice (Figures S1A-B). Subsequently, we investigated the effects of long-term calcium supplementation and results showing that adding calcium to drinking water for 5 weeks significantly reduced HFD intake and body weight gain (Figs. 1 F-H) in young mice. However, it did not affect chow diet intake or body weight gain in young mice (Figures S1C-E). Even in 8-week-old adult mice fed with HFD, there was only a slight, non-significant downward trend in food consumption and body weight (Figs. 1 C-E). Changes in water consumption often affect animals' food intake [23]. To investigated whether the reduced HFD intake and body weight from long-term calcium supplementation were due to changes in water consumption. we added 0.5% NaCl, $CaCl_2$, and $MgCl_2$ to

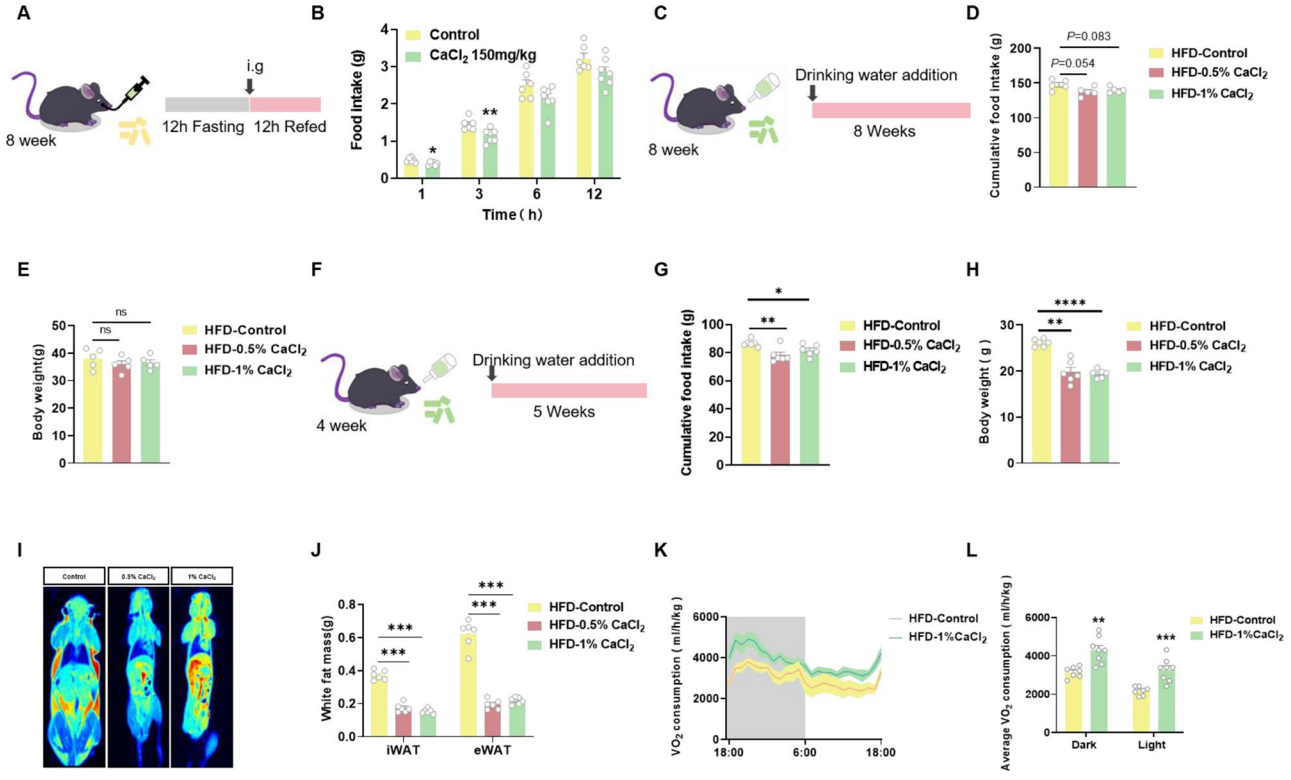

Fig. 1 Calcium reduced food intake in mice. (**A**) Diagram illustrates the experimental process: C57BL/6J mice were deprived of food for 12 h before receiving oral calcium. (**B**) Dark-cycle food intake of chow fedz z C57BL/6J mice after oral 150 mg/kg calcium or saline ($n=6$ or 7 per group). (**C**) Diagram illustrates the experimental process: calcium was added to the drinking water of 8-week-old C57BL/6J mice that were fed with HFD. (**D**) Cumulative food intake and (**E**) body weight for 8 weeks of HFD-fed adult mice with 0.5% and 1% calcium in drinking water ($n=5$ per group). (**F**) Diagram showing calcium added to the drinking water of 4-week-old mice fed with HFD. (**G**) Cumulative food intake and (**H**) body weight for 5 weeks of HFD-fed young mice with 0.5% and 1% calcium in drinking water ($n=6$ per group). (**I**) Representative images of fat and lean mass in HFD-fed young mice after 5 weeks of 0.5% and 1% calcium added to drinking water. (**J**) White fat mass of HFD-fed young mice after 0.5% and 1% calcium added in drinking water for 5 weeks ($n=6$ per group). (**K**-**L**) O_2 consumption was measured in HFD-fed C57BL/6J mice treated with 1% calcium in drinking water for 5 weeks ($n=8$ per group)

drinking water of HFD-fed mice. The results showed that all three compounds—NaCl, $CaCl_2$, and $MgCl_2$ increased water consumption compared to the control group, with NaCl having the strongest effect (Figure S1F). Notably, only $CaCl_2$ significantly reduced both cumulative food intake and body weight (Figures S1G-H). Mice with calcium added to drinking water still showed increased lean mass and decreased white fat mass as we observed in HFD-fed mice (Figures S1I and 1I-J). To investigate the effects of calcium on metabolism, we proceeded control and 1% calcium-treated mice in Comprehensive Lab Animal Monitoring System (CLAMS). And found that O_2 consumption was significantly higher in the 1% calcium-treated mice than control mice (Figs. 1K-L). Therefore, our findings support a model in which calcium inhibits feeding and promotes energy expenditure, leading to weight and fat loss.

Calcium significantly inhibited ARC AgRP neurons

Hypothalamus serves as the appetite control center, to investigate whether calcium reduces food intake through the ARC, we first examined c-Fos (a neuronal activation marker) in the ARC through immunofluorescence and found that acute calcium administration significantly suppressed c-Fos expression in the ARC of fasting mice (Figures S2A-B). There are multiple types of neurons that regulate feeding in the ARC, such as AgRP, Proopiomelanocortin (POMC), basonuclin 2 (BNC2) and tyrosine hydroxylase (TH), and these neurons have regulatory relationships with each other [24–26]. To determine which of these neuronal types mediates calcium's anorexic effects, we used well-studied NPY-GFP and POMC-GFP mice expressing green fluorescent protein to label AgRP/NPY and POMC neurons respectively. Through co-localization with c-Fos, we discovered that calcium mainly inhibits the activity of ARC^{AgRP} neurons (Figs. 2 A-B) while having no effect on POMC neuron activity (Figures S2C-D). We identified the specific regulatory role of AgRP neurons in calcium-induced food intake suppression. To further prove that AgRP neurons are involved in calcium-induced feeding inhibition, we delivered adeno-associated vectors (AAV-DIO-hM3Dq-mCherry/AAV-DIO-hM4Di-mCherry) to the ARC of AgRP-Cre mice (Figs. 2 C). As expected, targeted expression of hM3D in ARC^{AgRP} neurons significantly increased c-Fos expression and food intake (Figures S2E-G). Expression of hM4D had the decreased effect on c-Fos expression, but food intake was not affected (Figures S2H-J). As shown in Fig. 2D-F, when ARC^{AgRP} neurons were activated by targeted expression of hM3D in neurons, calcium lost its effect on neuronal excitability and food intake. Meanwhile, when inhibiting the excitability of ARC^{AgRP} neurons by targeted expression of hM4D in neurons, we also observed no difference in c-Fos expression and food intake between calcium-treated mice and control (Fig. 2G-I). These findings confirm that calcium intake reduces the activity of AgRP neurons in ARC, thereby promoting food-suppressive behavior.

Vagal afferent nerve mediated the anorexia effects of calcium

Multiple studies have shown that receptors in the vagal nerve endings can sense appetite signal in the gastrointestinal tract and transmit them to the central nervous system, thereby affecting appetite [27, 28]. To confirm that calcium signal in the GI tract can be transmitted to the brain via the vagal afferent nerve, we expressed HSV-tdTomato, an anterograde polysynaptic tracer, in the NG of C57BL6/J mice (Figs. 3 A and S3A) and found that the vector expression was validated in multiple nuclei, such as the area postrema (AP), nucleus tractus solitarius (NTS), and ARC (Fig. 3B). These results indicate an anatomical link between the NG and ARC. Thoroughly, we delivered AAV-cFos-ERT2creERT2-WPRE and AAV-CAG-Flex-tdTomato to the NG of C57BL6/J mice, labeling calcium-sensitive neurons with tdTomato [15], by employing the 4-Hydroxytamoxifen-administration approach (Fig. 3C), and found that acute oral calcium administration significantly increased c-Fos expression in NG (Figs. 3D-E). Meanwhile, the expression of p-ERK, one of indicator of neuronal activation [29], demonstrated similar increasing trend after oral calcium administration (Figs. 3F-G). These results suggest that vagal afferent nerve is indispensable for calcium-induced anorexia. To further define the role of the vagal afferent nerve in calcium appetite signaling, we constructed a model of Sdvx (Fig. 3H and Figure S3B). As shown in Figure S3C, the stomach showed extreme enlargement, consistent with the typical clinical features of Sdvx mice. Meanwhile, CTB-555, a retrograde tracer [30], was injected into the gastrointestinal wall (Figure S3D), $CTB\text{-}555^+$ neurons dispersed throughout the dorsal root ganglion (DRG) but not in NG (Figures S3E-F), confirming successful model construction. When we performed Sdvx in mice, the activation of vagal afferent nerves by acute oral administration of calcium was disappeared (Figs. 3I-J), there was also no effect on ARC (Figs. 3K-L) and ARC^{AgRP} (Figs. 3M-N) neurons activity or food intake (Fig. 3O). Together, these results suggest that calcium signal in the GI tract are perceived and transmitted by the vagal afferent nerve and ultimately to the ARC, thereby inhibiting feeding. We next investigate whether the activity of NG neurons is necessary for calcium-suppressive appetite regulation. Towards this target, AAVPHPeB-hSyn-DIO-hM4D(Gi)-mCherry vector was injected into bilateral NGs to achieve acute inhibition neurons activity in Vglut2-cre mice, which represent the largest class of neurons in NG (Fig. 4A). Two weeks later, intraperitoneal (i.p.) injection of clozapine N-oxide

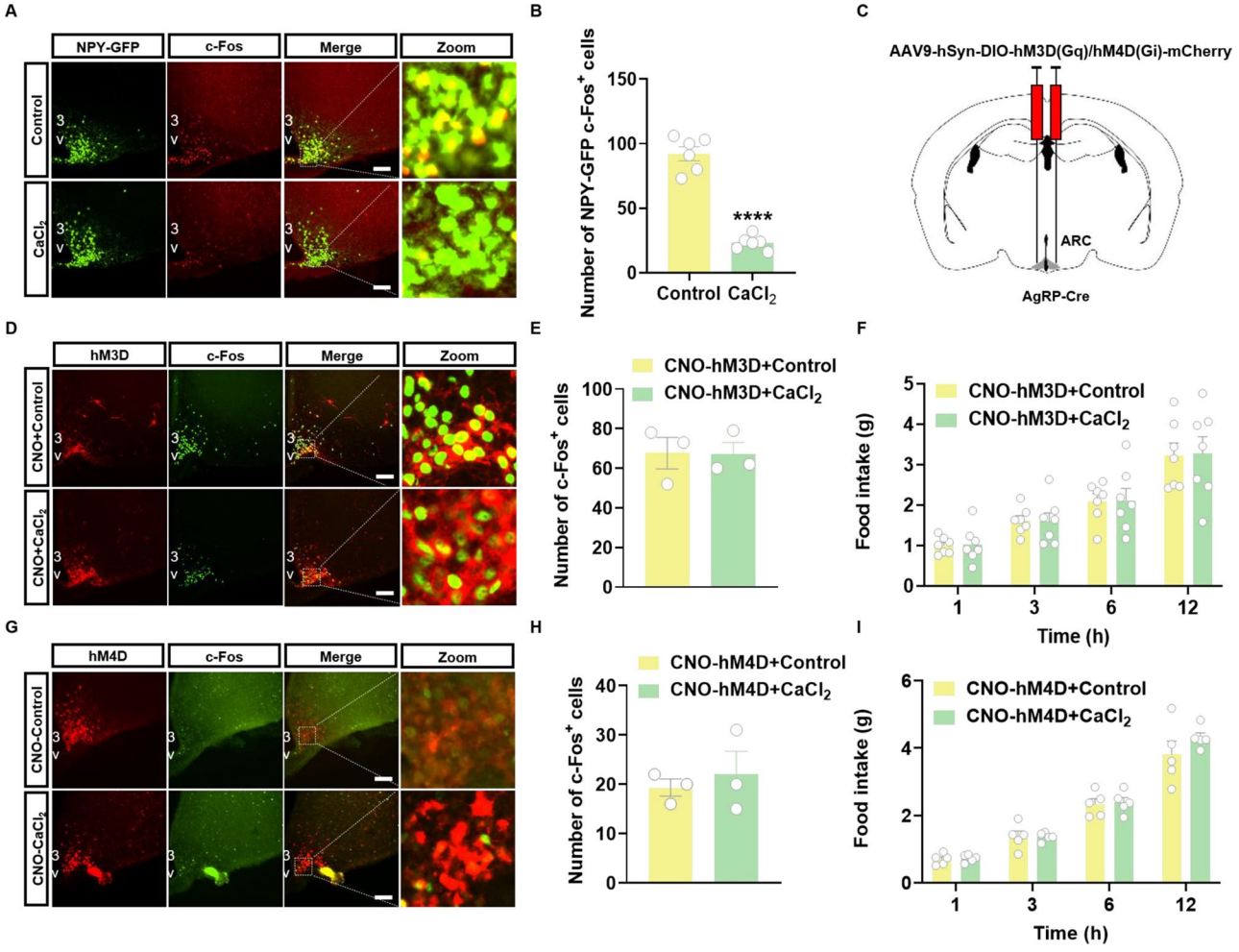

Fig. 2 Calcium reduced food intake by inhibiting the activity of AgRP neurons. (**A**-**B**) Representative immunohistochemical staining (**A**) and quantification (**B**) showing c-Fos expression in the ARC of 150 mg/kg calcium treated-NPY-GFP mice ($n = 6$ per group). (**C**) Diagram showing hM3D(Gq)/hM4D(Gi) viral injections into the bilateral ARC of AgRP-Cre mice. (**D**) Representative immunohistochemical images for data showed in (**E**), presenting c-Fos in AgRP neurons activated mice given oral with saline or calcium (150 mg/kg, $n = 3$ per group). (**F**) Effects of CNO (0.3 mg/kg) co-treated with saline or calcium on food intake monitored in male AgRP-Cre mice receiving activated AAV-hM3Dq-mCherry infection in the ARC ($n = 7$ per group). (**G**) Representative immunohistochemical images for data shown in (**H**), presenting c-Fos in AgRP neurons inhibited mice given oral administration with saline or calcium (150 mg/kg, $n = 6$ per group). (**I**) Effects of CNO (0.3 mg/kg) co-treated with saline or calcium on food intake monitored in male AgRP-Cre mice with inhibitory AAV-hM4Di-mCherry infection in the ARC ($n = 5$ per group). Scale bars:100 μm

(CNO, 0.3 mg/kg) in $Vglut2^{NG-hM4D}$ mice significantly decreased the expression of p-ERK (Figs. 4B-C). Simultaneously, oral administration of calcium had no effect on NG (Figs. 4D-E), ARC (Figs. 4 F-G) and ARC^{AgRP} (Figs. 4 H-I) neurons activity or food intake (Fig. 4J) in $Vglut2^{NG-hM4D}$ mice. We also bilaterally injected NGs with AAVPHPeB-hSyn-DIO-hM3D(Gq)-mCherry (Figure S4A), which successfully enhanced neuron activity (Figures S4B-C). The results showed when the vagal afferent nerve is in a highly excited state, calcium treatment is insufficient to affect NG neuron excitability (Figures S4D-E), ARC neuron activity (Figures S4F-G), or food intake (Figure S4H). The findings suggest that vagal afferent nerves are essential for mediating calcium-inhibitory appetite signaling in the gastrointestinal tract.

While activation of ARC^{AgRP} or inhibition of NG through chemogenetics was able to block acute oral calcium-induced reductions in food intake. it was unclear whether long-term calcium supplementation in drinking water would similarly affect food intake and body weight in mice on a HFD through these neurons. We studied how long-term calcium supplementation in drinking water affected c-Fos and p-ERK expression in the ARC and NG of mice on both chow and HFD. Consistent with the changes in food intake and body weight, long-term calcium supplementation significantly increased p-ERK expression in the NG (Figs. 5E-F) and inhibited c-Fos expression in ARC (Figs. 5G-H) of mice on a HFD diet but showed no effect on a chow diet (Figs. 5A-D). To investigate whether the resistance to HFD-induced

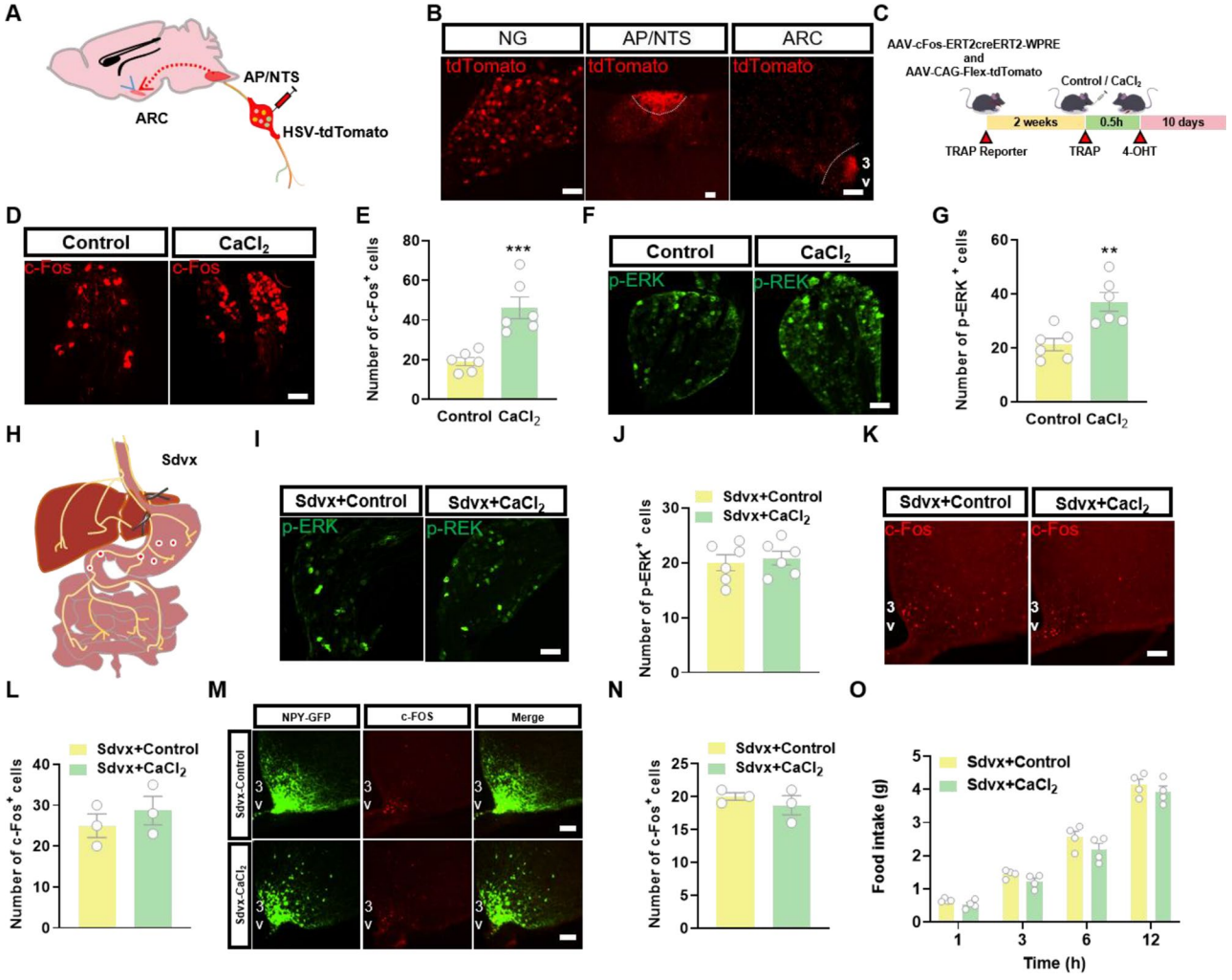

Fig. 3 Calcium activated vagal afferent nerve. (**A**) Diagram illustrates HSV-tdTomato viral injections to the bilateral NGs of C57BL/6J mice. (**B**) Representative images showing virus infections in some nuclei (AP/NTS and ARC). (**C**) c-Fos-creERT2 and flex-tdTomato viral were injected into bilateral NGs of C57BL/6J mice to label calcium-induced c-Fos neurons. (**D**) Typical images for data showed in (**E**) presented c-Fos-tdTomato-labeled neurons of nodose ganglia in mice which were oral administration with saline or calcium (150 mg/kg), after tamoxifen induced (100 mg/kg) ($n=6$ per group). (**F**) Typical pictures for data showed in (**G**) presented p-ERK$^+$ neurons of nodose ganglia in mice which were oral administration with saline or calcium (150 mg/kg) ($n=6$ per group). (**H**-**I**) Sdvx model: (**H**) Diagram showing where the vague nerve was blocked; (**I**) Pictures show p-ERK$^+$ in NGs that oral saline/calcium administration (150 mg/kg) in Sdvx-mice. (**J**) Statistical data of p-ERK$^+$ cell in (**I**) ($n=6$ per group). (**K**) Representative immunohistochemical staining shows c-FOS expression in the ARC of Sdvx-WT mice after treatment with saline or calcium ($n=3$ per group). (**L**) Statistical data of c-Fos$^+$ cell in (**K**). (**M**) Representative immunohistochemical staining shows c-FOS expression in the ARC of Sdvx-NPY-GFP mice after treatment with saline or calcium ($n=3$ per group). (**N**) Statistical data of c-Fos$^+$ cell in (**M**). (**O**) Food intake of Sdvx-mice that were oral administration with calcium in 12 h ($n=4$ per group). Scale bars:100 μm

obesity through long-term calcium supplementation in drinking water similarly depends on NG and ARC^{AgRP}. Subsequently, we selected Cre-dependent sodium channel AAV-Flex-mNaChBac-WPRE-GFP virus to chronically activate ARC^{AgRP} neurons (Figs. 5I) and potassium channel AAV-EF1a-DIO-Kir 2.1-tdTomato to chronically inhibit vagal afferent neurons (Figs. 5N). The results showed that injection of Flex-NachBac virus into ARC significantly increased c-FOS expression in AgRP neurons (Figs. 5J-K), while in this state, 0.5% calcium supplementation in drinking water had no effect on cumulative food intake and body weight (Figs. 5L-M). Similarly, when vagal nerves were chronically inhibited (Figs. 5O-P), 0.5% calcium supplementation in drinking water also had no effect on cumulative food intake and body weight (Figs. 5Q-R).

Specific Inhibition of Gastrointestinal vagal afferent nerve blocked calcium-induced anorexia

The vagal nerve regulates multiple organs in the abdominal cavity, such as the lungs, liver, and gastrointestinal [31]. To investigate whether specifically inhibiting the gastrointestinal vagal afferent nerve could block calcium-induced appetite suppression, we used a retrograde

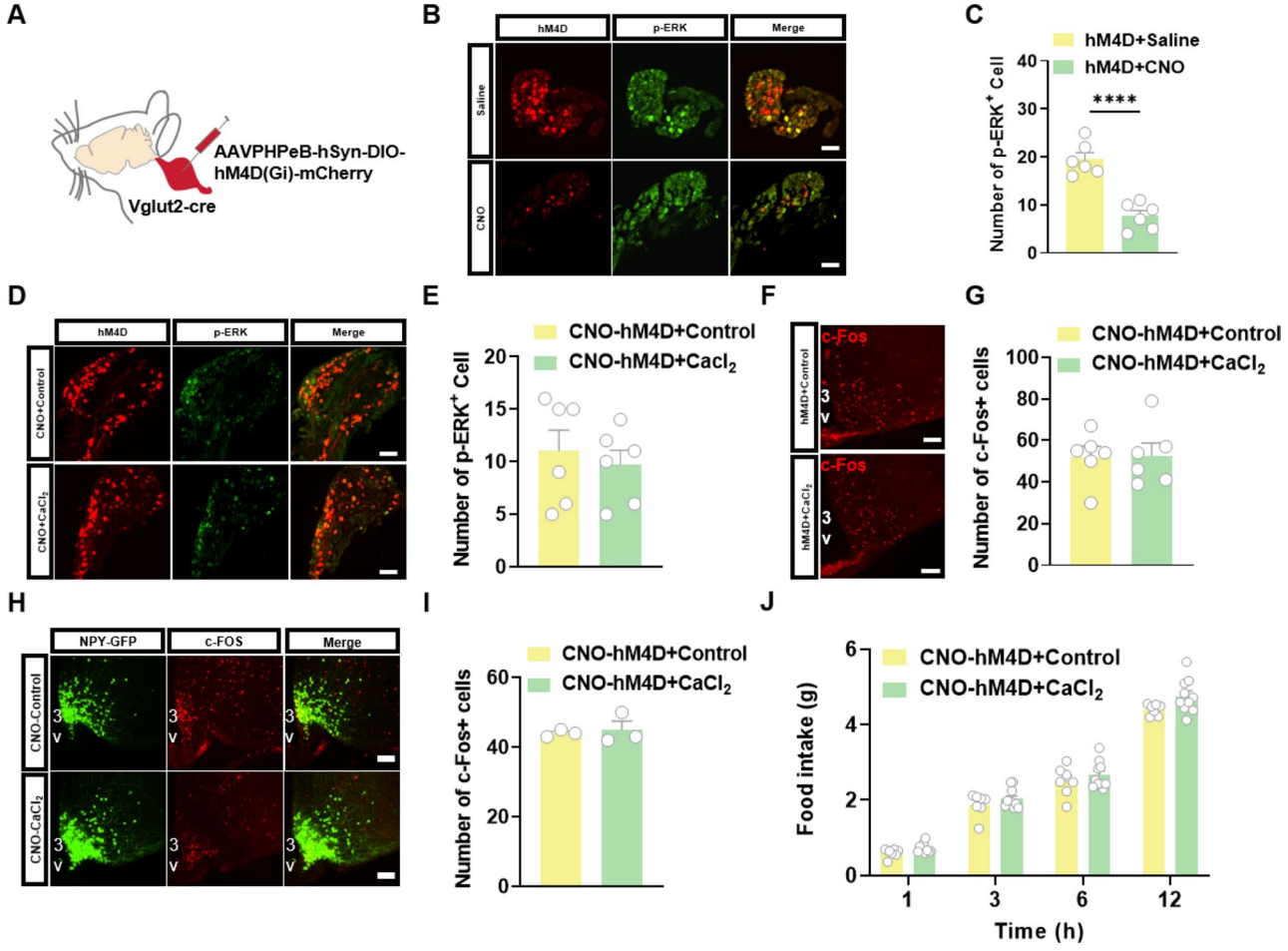

Fig. 4 Inhibition of vagal afferent nerve activity blocked calcium-induced anorexia. (**A**) Diagram showing injections of hM4Di vectors to the bilateral NGs of Vglut2-Cre mice. (**B**) Representative expression pattern of hM4Di in the NG (red in the first column), p-ERK expression (green in the second column), and merged images of red and green (the third column). (**C**) Statistical data of p-ERK$^+$ cells in (**B**) ($n=6$ per group). (**D**) Representative immunohistochemical pictures for data showed in (**E**), depicting p-ERK$^+$ in NGs of glutaminergic neurons activated-mice which were administration with saline or calcium (150 mg/kg) ($n=6$ per group). (**F**) Typical pictures for data show in (**G**), presenting c-Fos in ARC of WT mice which were oral calcium administration ($n=6$ per group). (**H**) Typical pictures for data show in (**I**), presenting c-Fos in ARC of Vglut2-cre::NPY-GFP mice which were oral calcium administration ($n=3$ per group). (**J**) Effects of CNO (0.3 mg/kg) co-treated with saline or calcium (150 mg/kg) on food intake in male Vglut2-Cre::NPY-GFP mice with inhibitory AAVPHPeB-hSyn-DIO-hM4Di-mCherry infection in the NGs ($n=7$ or 10 per group). Scale bars: 100 μm

adeno-associated virus (AAV-Retro-hSyn-Cre-WPRE-hGH) with an optimized multiple injection protocol targeting the gastric and anterior intestinal walls. We then performed bilateral injections of AAVPHPeB-DIO-hM4Di-mCherry into the NG (Fig. 6A). Results showed that the functional vector virus successfully infected NG neurons while inhibiting p-ERK expression (Fig. 6B and C). Consistent with our hypothesis, when calcium was oral administered to NG$^{\text{Retro-hM4D}}$ mice, we observed no differences in the activity of NG (Figs. 6D-E), ARC (Fig. 6F and G) and ARC$^{\text{AgRP}}$ (Fig. 6H and I) neurons activity or food intake (Fig. 6J) between groups. Collectively, our observations support a model in which calcium activates vagal afferent nerve to restrain AgRP activity, thereby decreasing food intake.

Discussion

The effect of calcium on mouse food intake appears to be related to the energy homeostasis and dietary energy density. We found that acute oral calcium administration had no effect on food intake under ad libitum feeding conditions but reduced short-term food intake under fasting conditions. Similarly, long-term calcium supplementation in drinking water does not affect food intake with a chow diet but suppresses food intake only with HFD feeding. This selective effect may be due to disrupted leptin and insulin signaling pathways during fasting or long-term HFD feeding, which differs from ad libitum feeding or chow diet feeding. As some studies have shown that calcium can effectively alleviate leptin/insulin signal resistance [32, 33]. Consequently, calcium's suppressive effect on food intake becomes more

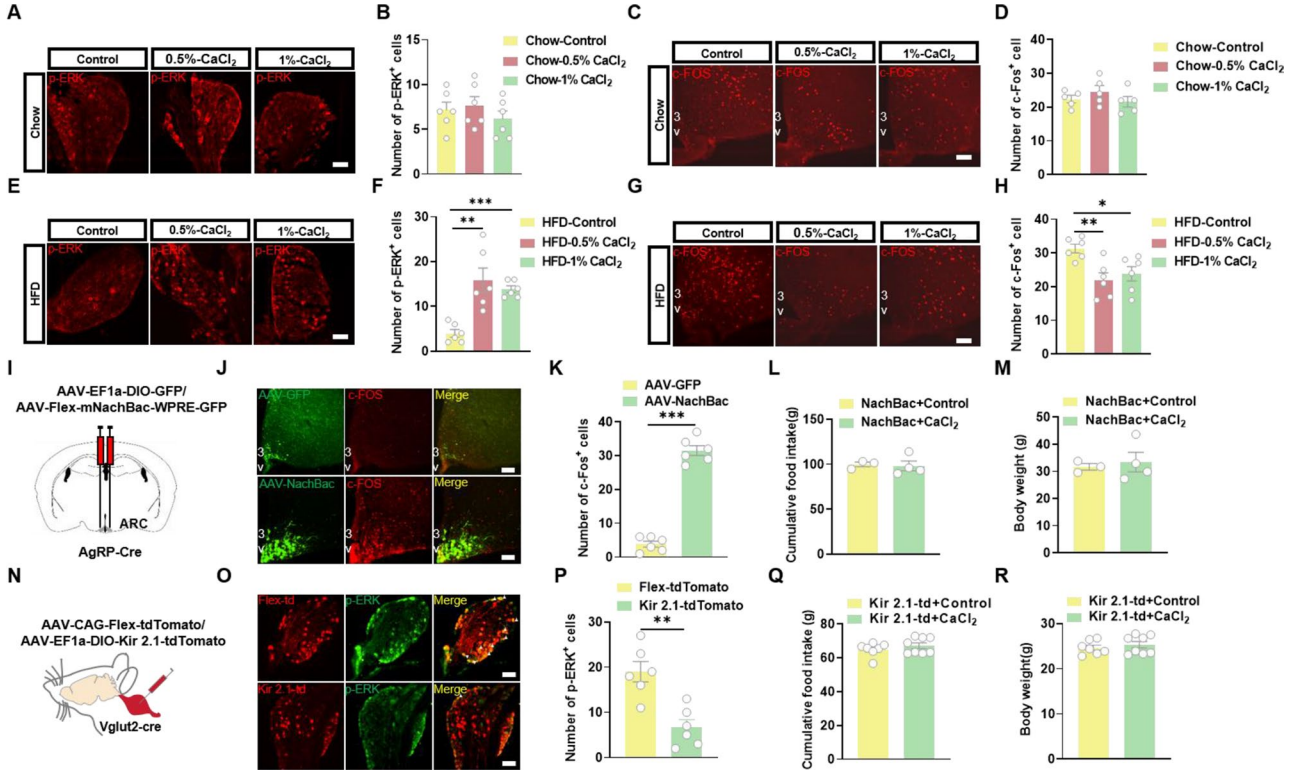

Fig. 5 Long-term activation of AgRP or inhibition of vagal nerves blocks calcium-induced anorexia on HFD. (**A**) Representative immunofluorescence pictures for data showed in (**B**), presenting p-ERK$^+$ in NGs of Chow-fed C57BL/6J mice after 5 weeks of 0.5% and 1% calcium added to drinking water ($n=6$ per group). (**C**) Representative immunofluorescence pictures for data showed in (**D**), presenting c-FOS$^+$ in ARC of Chow-fed C57BL/6J mice after 5 weeks of 0.5% and 1% calcium added to drinking water ($n=6$ per group). (**E**) Representative immunofluorescence pictures for data showed in (**F**), presenting p-ERK$^+$ in NGs of HFD-fed C57BL/6J mice after 5 weeks of 0.5% and 1% calcium added to drinking water ($n=6$ per group). (**G**) Representative immunofluorescence pictures for data showed in (**H**), presenting c-FOS$^+$ in ARC of HFD-fed C57BL/6J mice after 5 weeks of 0.5% and 1% calcium added to drinking water ($n=6$ per group). (**I**) Diagram showing AAV-EF1a-DIO-GFP/AAV-Flex-mNaChBac-WPRE-GFP viral injections into the bilateral ARC of AgRP-Cre mice. (**J**) Representative expression pattern of DIO-GFP and Flex-NachBac in the ARC (green in the first column), c-Fos expression (red in the second column), and merged images of red and green (the third column) in fast-refeeding AgRP-Cre mice. (**K**) Statistical data of c-Fos$^+$ cells in (**J**) ($n=6$ per group). (**L**-**M**) Effects of 0.5% calcium supplementation in drinking water on cumulative food intake (**L**) and body weight (**M**) in HFD-fed Vglut2-Cre mice with activating Flex-NachBac injection ARC for 4 weeks ($n=3$ or 4 per group). (**N**) Diagram showing AAV-CAG-Flex-tdTomato/AAV-EF1a-DIO-Kir 2.1-tdTomato viral injections into the bilateral NG of Vglut-Cre mice. (**O**) Representative expression pattern of flex-tdTomato and kir 2.1-tdTomato in the NG (red in the first column), p-ERK expression (green in the second column), and merged images of red and green (the third column). (**P**) Statistical data of p-ERK$^+$ cells in (**O**) ($n=6$ per group). (**Q**-**R**) Effects of 0.5% calcium supplementation in drinking water on cumulative food intake (**Q**) and body weight (**R**) in HFD-fed Vglut2-Cre mice with inhibitory kir 2.1-tdTomato injection in the nodose ganglia for 4 weeks ($n=7$ or 8 per group). Scale bars: 100 μm

pronounced during fasting or long-term HFD feeding. We investigated developmental timing's role in calcium's metabolic effects by conducting long-term calcium supplementation experiments through drinking water under high-fat feeding conditions in both young and adult mice. Our findings showed that calcium supplementation had a significantly stronger effect on HFD-induced obesity in young mice than in adult mice, indicating age-dependent calcium sensitivity. This aligns with established literature showing that dietary composition during puberty is crucial for growth and metabolic programming [34, 35]. This enhanced calcium sensitivity during puberty helps explain why young animal models are prevalent in studies of calcium's metabolic effects under high-fat feeding conditions [10, 36]. The age-dependent sensitivity also explains our observation that calcium strongly inhibits HFD intake and enhances energy metabolism, leading to significant reduction in adipose tissue development and low body fat percentages. We added calcium to drinking water, this might affect the water consumption of mice, thereby influencing their food intake and body weight [23]. To address this possibility, we examined the effects of long-term supplementation of 0.5% NaCl, $CaCl_2$, and $MgCl_2$ in drinking water on HFD intake and body weight in mice. Results showed that compared to the control group, all three compounds increased water consumption, with NaCl showing the strongest effect. Notably, only $CaCl_2$ significantly suppressed food intake and body weight. Furthermore, under chow diet feeding, $CaCl_2$ did not affect food intake and body weight, confirming that $CaCl_2$'s reduction effect on HFD intake and body weight depends on calcium. This finding illustrated that a

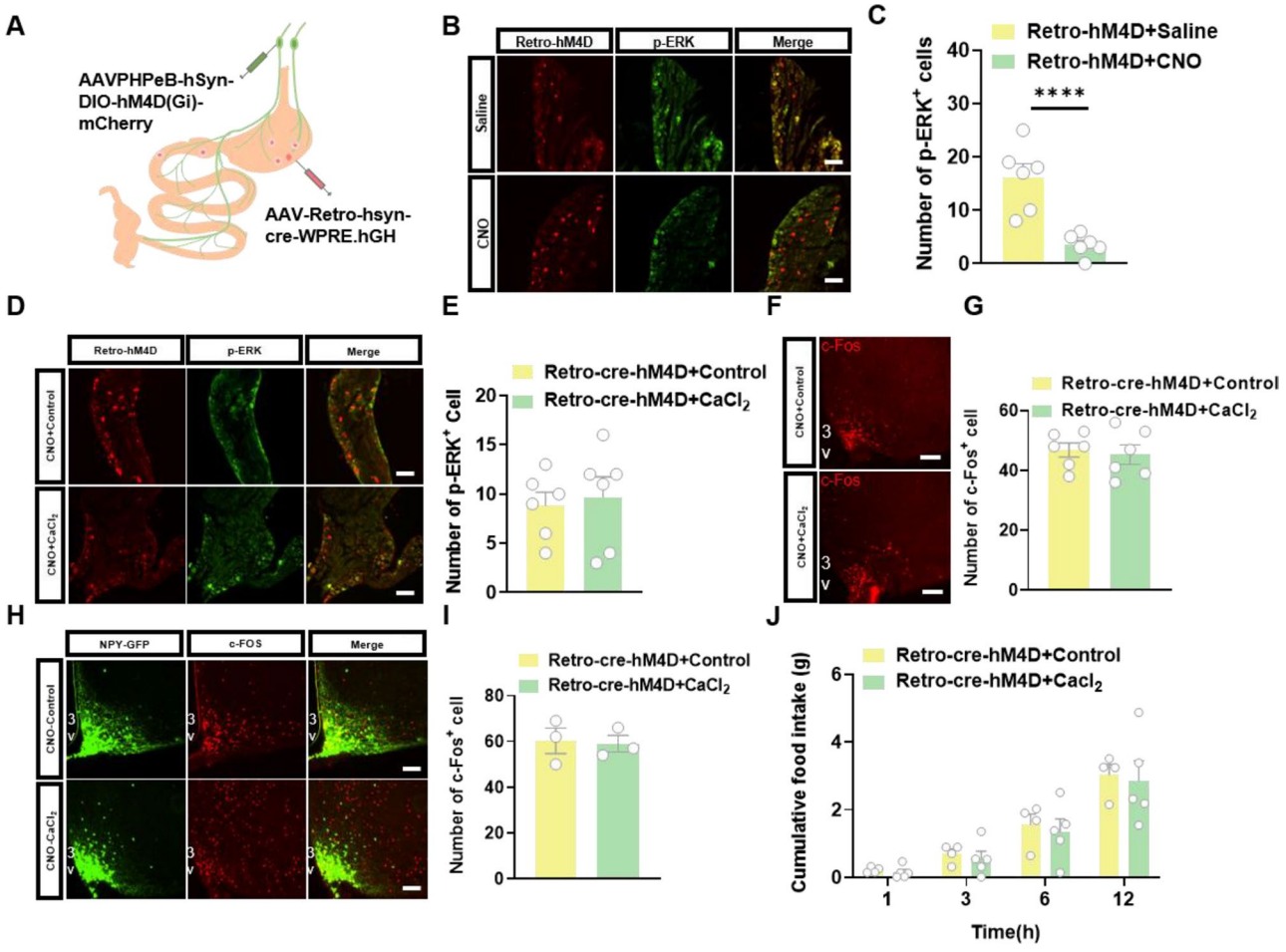

Fig. 6 Calcium induced anorexia by activating the gastrointestinal branch of vagal afferent nerve. (**A**) Diagram showing AAV-Retro-hsyn-cre viral multi-site injections to gastrointestinal wall and PAAVPHPeB-hSyn-DIO-hM4D(Gi)-mCherry viral injections to the bilateral NGs of WT mice. (**B**) Representative expression pattern of hM4D(Gi) in the NG (red in the first column), p-ERK expression (green in the second column), and merged images of red and green (the third column). (**C**) Statistical data of p-ERK$^+$ cell in (**B**) ($n=5$ or 6, per group). (**D**) Representative immunofluorescence pictures for data showed in (**E**), presenting p-ERK$^+$ in NGs of gastrointestinal neuronal activation mice which were oral administration with saline or calcium (150 mg/kg, $n=6$ per group). (**F**) The typical pictures for data show in (**G**), presenting c-Fos in ARC of WT mice which were administration with calcium or saline and co-inhibited gastrointestinal vagal nerve ($n=6$ per group). (**H**) The typical pictures for data show in (**I**), presenting c-Fos in ARC of NPY-GFP mice which were administration with calcium or saline and co-inhibited gastrointestinal vagal nerve ($n=3$ per group). (**J**) Effects of CNO (0.3 mg/kg) co-treated with saline or calcium on food intake in male WT mice receiving conditional AAV-Retro-hsyn-cre infection in gastrointestinal and inhibitory AAVPHPeB-hSyn-DIO-hM4Di-mCherry infection in the NG ($n=4$ or 5, per group). Scale bars: 100 μm

calcium diet has weight/fat loss effects, which are attributed not only to inhibited fat synthesis and increased thermogenesis [11, 12] but are also closely related to reduced energy intake.

Through c-Fos immunofluorescence co-localization with the well-studied POMC and AgRP neurons, we found that calcium does not affect POMC neuron activity but can inhibit AgRP neurons. This finding is consistent with our observation of reduced activity across the ARC, suggesting that calcium may decrease food intake by specifically inhibiting AgRP neurons. While POMC cannot regulate acute feeding [37], it's important to note that ARC^{AgRP} activity can be inhibited by BNC2 neurons [24]. Therefore, the calcium-induced reduction in food intake may be mediated through BNC2 neurons. Furthermore, we found that when AgRP neurons were activated or inhibited by chemogenetics, the calcium-induced inhibition of feeding was disappeared. Chemogenetics is the gold standard for regulating neuronal activity, as confirmed by our c-Fos expression. Both activation and inhibition of AgRP neurons can block calcium-induced anorexia because the NTS, which receives vagal afferent nerve projections, inhibits less than 15% of ARC^{AgRP} through GABA neurons [38] but we are activating or inhibiting all, and given the redundant characteristics of AgRP neurons in promoting feeding [39], this leads to hM3D activation of AgRP effectively blocking calcium-induced anorexia. On the other hand, hM4D has already

inhibited AgRP neuron activity, thus masking calcium's inhibitory effect, which explains why calcium cannot further reduce c-Fos expression in the ARC, thereby blocking calcium's anorexic effect. Conversely, if calcium does not regulate feeding through ARC^{AgRP}, then neither chemogenetic activation nor inhibition of AgRP would be able to block calcium-induced anorexia. Taken together, these results suggest that calcium's inhibition of feeding mainly depends on the inhibition of AgRP neurons activity.

The vagal afferent nerve provides the primary sensory innervation of the gastrointestinal tract and mediates basic physiologic functions of feeding regulation and gastrointestinal motility [40]. Gastrointestinal hormones such as CCK, ghrelin, and cannabinoid can activate their respective receptors on vagal nerve endings, altering afferent firing and modulating feeding [41–43], activate Glp1r and Oxtr neurons in NG significantly inhibit AgRP neurons and reduce food intake [14], and fat or macronutrients in the gastrointestinal tract cannot decrease AgRP neuron activity in the absence of vagal signaling [17]. Those studies highlight the crucial role of vagal afferent nerve in the transmission of nutritional signals. Our viral tracer results revealed anatomically connections between the vagal afferent nerve and the ARC. Meanwhile, oral calcium increased the expression of c-Fos and p-ERK in NG, suggesting that the vagal afferent nerve may mediate gastrointestinal calcium signal. After Sdvx or inhibited the most abundant of glutamatergic neurons in NG [31], the effects of calcium on food intake were disappeared, demonstrates that the instantaneous firing of vagal afferent nerves is an important and indispensable part of gastrointestinal calcium-triggered feeding inhibition.

Although chemogenetic-specific activation of ARC^{AgRP} or inhibition of NG can effectively reverse the suppressive effect of acute oral calcium on short-term food intake, further verification is needed to determine whether long-term calcium supplementation in drinking water's resistance to HFD-induced obesity similarly depends on these neurons. Therefore, we selected Cre-dependent Na^+ channel AAV-Flex-mNachBac-WPRE-GFP virus and K^+ channel AAV-EF1a-DIO-Kir 2.1-tdTomato virus to achieve long-term activation of AgRP and vagal afferent nerves [39]. Immunostaining of c-Fos/p-ERK in ARC^{AgRP}/NG demonstrated that expression of NachBac/Kir 2.1 significantly activated or inhibited ARC^{AgRP}/NG. More importantly, consistent with chemogenetic acute activation of ARC^{AgRP} or inhibition of NG, long-term activation of AgRP or inhibition vagal afferent nerves both blocked calcium's reduction of food intake and body weight on HFD. This further proves that calcium's resistance to HFD-induced obesity works through activating vagal afferent nerves, which in turn inhibits ARC^{AgRP}.

The endings of the vagal afferent nerve are widely distributed in many important organs of the abdominal cavity. This complex anatomical structure makes the information sensed by the vagal afferent nerve extremely diverse and large [14, 44, 45], thus single activation or inhibition of an entire NG neurons to explore calcium feeding effects is imperfect. Instead, we performed specific inhibition of gastrointestinal vagal afferent nerves with AAVrg-cre and DIO-hM4D(Gi) vectors in mice. Our results suggested that when gastrointestinal vagal signaling is blocked, calcium is unable to exert an inhibitory effect on AgRP neurons, leading to the disappearance of the foraging inhibitory effect. These findings imply that gastrointestinal vagal afferent nerve play an essential role in calcium-induced feeding suppression.

In summary, this study demonstrated that ingested calcium activates gastrointestinal vagal afferent nerves, inhibiting the activity of ARC^{AgRP} neurons, thereby reducing food intake, and enhancing the resistance of young mice to HFD-induced obesity. Our findings reveal a gut vagal afferent-brain axis for dietary calcium-induced anorexia.

Limitations of study

Although we have revealed that gastrointestinal calcium signal is transmitted to the central nervous system via the vagal afferent nerve, this study has not yet explored how calcium signals in the intestine are sensed by the vagal afferent nerve. Previous study has preliminarily elucidated that gastrointestinal calcium exerts an inhibitory effect on food intake by acting on calcium-sensing receptor (CaSR) and oral administration of CaSR agonists can significantly reduce food intake in mice [46]. What's more, vagal afferent nerve endings contain multiple receptors that sense intestinal signals [41, 43]. These studies providing a valuable research direction for further elucidation of this mechanism.

Materials and methods

Animals

Mice were housed under controlled conditions: temperature of 23 ± 3 °C, humidity of 50 ± 10%, and a 12-hour light/dark cycle. Unless otherwise stated, mice had free access to chow diet and drinking water. NPY-hrGFP (stock no. 006417), POMC-hrGFP (stock no. 009593), Vglut2-ires-Cre (stock no. 028863), and AgRP-ires-Cre (stock no. 012899) mice were purchased from the Jackson Laboratory. C57BL6/J male mice were purchased from the Zhuhai BesTest Biotech company (Zhuhai, Guangdong, China). All mice used for injections were 8–12 weeks old and had a postoperative recovery period at least 2 weeks.

Calcium (sodium, magnesium) treatment

In the acute test, mice under either 12-hours food deprivation or ad libitum feeding were orally administered calcium chloride (150 mg/kg). For the chronic test, mice fed either a standard chow diet (18.0% protein, 58% carbohydrate, 4.5% fat) or HFD (26.2% protein, 26.3% carbohydrate, 34.9% fat) received 0.5% or 1% calcium (sodium, magnesium) chloride in their drinking water. It's important to note that the mice in the acute trial were 8 weeks old, while those in the chronic trial were 4 and 8 weeks old respectively.

Food and water intake

Hourly and daily food intake was measured after at least 3 days of acclimation, ensuring that the start time of feeding measurement was 6:30 pm. For chronic tests, food and water intake were measured at the same time each week.

Body weight studies

Mice received drinking water containing varying concentrations of calcium. Their body weights were monitored weekly throughout the study period. The MesoQMR system measured the lean mass of the mice.

Energy expenditure analysis

Energy expenditure is determined by oxygen consumption. Subjects were individually housed in metabolic cages with food and water freely available. Metabolic cages (Promethion Metabolic Screening Systems, Sable Systems International, North Las Vegas, NV, USA) were used to measure oxygen consumption. Mice were acclimatized in the metabolic cages for at least 12 h before data collection began. Valid data were then collected for 48 h per mouse.

Subdiaphragmatic vagotomy

We performed subdiaphragmatic vagotomy on C57BL6/J and NPY-hrGFP mice. After disinfection, we made a 1.5 cm incision in the upper abdomen and used cotton swabs to gently separate the liver tissue from the stomach. The gastric tube was exposed from the abdominal cavity. We then separated and clipped the vagal trunks attached to both sides of the gastric tube.

Stereotaxic injection

Mice were anesthetized with isoflurane. After shaving their heads and creating holes in the skull, a small volume (200 nl) of AAV-DIO-hM3Dq-mCherry (#44361-AAV1, Addgene, 1×10^{13}V.G/ml), AAV-DIO-hM4Di-mCherry (#111397-AAV1, Addgene, 1×10^{13}V.G/ml) or AAV-Flex-mNaChBac-WPRE-GFP (XT499, Taitool, 1×10^{13}V.G/ml) was bilaterally injected into the ARC of AgRP-Cre mice at coordinates X = ± 0.20 mm, Y = − 1.45 mm, Z = − 5.9 mm respectively. Following surgery, the mice were placed on a temperature-controlled heating pad until they regained consciousness.

Nodose ganglia injection

For viral injections into the nodose ganglion, anesthetized mice were placed in a supine position and an incision ($1.5 cm) was made along the ventral surface of the neck. After, stripping the surrounding muscle and fat tissue, along with the side of the carotid artery, the vagal afferent nerve was exposed. The nodose ganglia is located at the end of the vagal afferent nerve near the skull. Pulled glass pipette ($20 mm tip diameter) was filled with virus AAVPHPeB-hSyn-DIO-hM3D(Gq)-mCherry (#44361-PHPeB, Addgene, 1×10^{13}V.G/ml, 200 nl), AAVPH-PeB-hSyn-DIO-hM4D(Gi)-mCherry (#44362-PHPeB, Addgene, 1×10^{13}V.G/ml, 200 nl), PT-7939 rAAV-cfos-ERT2creERT2-WPRE-hGH polyA (PT-7939, BrainVTA, 1×10^{13}V.G/ml, 200nl) and AAV-CAG-Flex-tdTomato (#122501 Addgene, 1×10^{13}V.G/ml, 200 nl), AAV-EF1a-DIO-Kir 2.1-tdTomato (XT-0770, Taitool, 1×10^{13}V.G/ml) respectively. and inserted into the target areas. Post-surgery, care was taken to maintain the mice's body temperature until they were awake.

Peripheral organs viral injections

Pre-operation can be referred to as Sdvx surgery. First, the stomach and duodenal antrum were exposed, separately. Then, AAV-hSyn-Cre-WPRE-hGH (AAV Retrograde, #105553-AAVrg, Addgene, 500 nl) or CTB-555 (BrainVTA, 1 ug/ul, 200 nl) were inserted with 35G microliter syringes (Hamilton, Shanghai). It's crucial to maintain moisture in the exposed gastrointestinal tract using thermostatic tissue buffer.

Tissue preparation, immunohistochemistry, imaging, and post hoc.

When finished the behavioral experiment, mice were anesthetized with isoflurane. Mice were transcranial perfused with PBS and 4% paraformaldehyde for 3 min each. The freshly fixed brains, white fat (no perfusion), NGs, and DRG were then immediately collected. After the subjects were post-fixed in 4% PFA for more than 24 h, 30% sucrose was used to dehydrate. And brains were processed for more than one day, while ganglia were processed for at least 2 h. Brains were frozen and sectioned into 35-μm slices using a sliding microtome (Leica SM2010 R, Germany), while ganglia were sectioned into 15-μm slices using a cryostat (Leica 3050 S, Germany). The sections were washed three times in PBS with 1% Triton X-100 (0.1% PBST, pH 7.4). Primary antibodies against c-Fos (1:2000, #2250, CST) or p-ERK (1:500, #9101, CST) were added to the sections and incubated overnight at 4 °C on a shaker. The next day, the sections underwent three 10-minutes 0.1% PBST rinses, then

were incubated with a secondary goat anti-mouse FITC antibody (1:2000, bs-0296G-FITC, Bioss, China) at room temperature for 2 h on a shaker. Sections with reporter expression and/or immunostained fluorescein were visualized using an immunofluorescence imaging system (Nikon, Japan). For c-Fos and p-ERK counts, each group included at least three different mice, and neurons with clear and strong immunostaining signals were counted.

Data analysis

Statistical analysis and graphical presentation were performed using SPSS 26 software (IBM, Chicago, IL, USA) and GraphPad Prism 8.0 (San, Diego, CA, USA). After verification of the normal distribution of the data, one way ANOVA followed by Dunnett's for multiple comparison with the single control were performed. Differences between two groups were analyzed using unpaired Student's t test. Results are presented as means ± SEM of more than three replicates, the differences between the groups are indicated by $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

Supplementary Information

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Supplementary Material 1

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Author contributions

A.S. conducted the research with help from L.P., Q.Z.; Qin Zhu, R.L. M.L. provided essential reagents; J.L. provided the necessary experimental assistance; R.W., S.W., Q.J., and L.W., provided essential guidance and stipend support for A.S.; C.Z., G.S. and A.S conceived and designed the experiments, and wrote the manuscript.

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Data availability

All data and materials are available upon request from the corresponding authors Canjun Zhu (canjunzhu@scau.edu.cn) or Gang Shu (shugang@scau.edu.cn).

Declarations

Ethics approval and consent to participate

All animal procedures complied with South China Agricultural University's institutional animal care and use committee guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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