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Ninjin-yoeito activates ghrelin-responsive and unresponsive NPY neurons in the arcuate nucleus and counteracts cisplatin-induced anorexia

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ABSTRACT

Reduced appetite or anorexia substantially deteriorates quality of life in various diseases including cancer, depression and heart failure. Furthermore, reduced appetite may stand upstream of sarcopenia and frailty. All these diseases are heavy burdens in the modern medicine and society. Therefore, the means that counteracts reduced appetite has been awaited, however, effective and well evidenced substance is not currently available. Ninjin-yoeito, a Japanese kampo medicine comprising twelve herbs has been used to treat anorexia. However, underlying mechanism is little known. Neuropeptide Y (NPY) and ghrelin are the most potent central and peripheral inducers of appetite, respectively. This study sought to determine whether Ninjin-yoeito influences NPY and/or ghrelin-responsive neurons in the hypothalamic arcuate nucleus (ARC), a feeding center. We isolated single neurons from ARC of mice and measured cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura-2 fluorescence imaging, followed by immunocytochemical identification of NPY neurons. Ninjin-yoeito (1–10 μg/ml) increased $[Ca^{2+}]_i$ in ARC neurons, the majority (80%) of which was immunoreactive to NPY. One fraction of these Ninjin-yoeito-responsive NPY neurons also responded to ghrelin, while another fraction did not. Furthermore, oral administration of Ninjin-yoeito (1 g/kg/day) counteracted the reductions in food intake and body weight by cisplatin, an anti-cancer drug, in mice. These results demonstrate that Ninjin-yoeito directly targets both ghrelin-responsive and unresponsive NPY neurons in ARC and preserves food intake and body weight in cisplatin-treated anorectic mice. Ninjin-yoeito's signaling through ghrelin-responsive and ghrelin-unresponsive NPY pathways may provide strong mechanistic basis for this medicine for treating anorectic conditions associated with cancer, depression, heart failure, sarcopenia, frailty and aging.

1. Introduction

Reduced appetite/food intake and body weight, which deteriorate the quality of life (QOL) (Hiura et al., 2012; Landi et al., 2016), is associated with cancer chemotherapy, stress-related diseases, sarcopenia and frailty in the elderly (Morley, 2013; Raghavendran et al., 2011). Hence, effective tools to promote appetite have been awaited. However, substances that effectively and safely promote appetite have not been available in the clinic and daily life.

Ninjin-yoeito, a Japanese traditional Kampo medicine, has been used clinically and shown to be effective to treat anorexia, fatigue, anemia, cold limbs, persistent cough, mental disequilibrium and to promote recovery from disease (Suzuki et al., 2015; Takaku et al., 2017; Yamasaki et al., 2008). Its ability to ameliorate this variety of symptoms may partly result from counteraction of anorexia. Ninjin-yoeito is composed of 12 crude drugs, some of which are known to pass through the blood brain barrier (BBB). Hence, oral administration of Ninjin-yoeito could act on the brain. In fact, central administration of ginsenoside Rg1, a component of Ninjin-yoeito, attenuated anorexia produced by elevated environmental temperature (Fujimoto et al., 1989). However, the effect of Ninjin-yoeito on the hypothalamic feeding-regulatory neurons has been little studied.

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In the hypothalamic feeding center (Schwartz et al., 2000), the first order neurons in the arcuate nucleus (ARC) receive the peripheral metabolic signals including ghrelinn, and inform the second order centers in the paraventricular nucleus (PVN), parabranchial nucleus (PBN) and lateral hypothalamic area (LHA), where the metabolic and central information are integrated to control feeding (Chabot et al., 2014; Luquet et al., 2005; Schwartz et al., 2000; Tung et al., 2008). Thus, appetite/feeding is regulated by complex neural circuits. Despite this complexity, recent studies have shown that the ARC neuron coexpressing AgRP and NPY (AgRP/NPY neuron) is indispensable and sufficient for producing appetite. The ARC NPY neuron senses ghrelin and blood glucose lowering (Kohno et al., 2007; Muroya et al., 1999; Zhang et al., 2012), the essential peripheral hunger signals. NPY injection into the brain (Glenn Stanley et al., 1986) and activation of ARC NPY/AgRP neurons increase food intake (Kohno et al., 2011; Tanaka and Kurosumi, 1992). Selective deletion of ARC AgRP/NPY neurons in adult mice rapidly reduces feeding and body weight, often leading to death (Gropp et al., 2005; Luquet et al., 2005). Inversely, selective activation of AgRP/NPY neurons with optogenetics triggers the orchestrated feeding behavior (Aponte et al., 2011). A combined method of measuring cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent immunostaining (Yada et al., 1993) revealed that 80% of the ARC neurons that respond to ghrelin (ghrelin-responsive neurons) are NPY immunoreactive (IR) (Kohno and Yada, 2012; Yada et al., 2012). Ghrelin stimulates feeding primarily by activating NPY neurons (Kohno et al., 2007; Nakazato et al., 2001; Zhang et al., 2012). However, clinical use of ghrelin for anorexia remains unrealized (Méquinion et al., 2013; Morley, 2013). The present study aimed to explore whether Ninjin-yoeito directly activates NPY neurons and/or ghrelin-responsive neurons in ARC, and whether it counteracts the anorectic and weight lowering effects of cisplatin, a chemotherapy drug for cancer patients. We examined the effect of Ninjin-yoeito on $[Ca^{2+}]_i$ in the single neurons isolated from ARC of mice, and subsequently immuno-stained them for NPY. We found that Ninjin-yoeito increased $[Ca^{2+}]_i$ preferentially in NPY-IR neurons in ARC, which did or did not respond to ghrelin. Oral administration of Ninjin-yoeito upwardly altered food intake and body weight in cisplatin-treated anorectic mice. The present data demonstrate that Ninjin-yoeito activates ghrelin-responsive and unresponsive NPY neurons in ARC, and restores feeding and weight in cisplatin-treated anorectic mice.

2. Materials and methods

2.1. Materials

Ninjin-yoeito, an herbal supplement composed of 12 crude drugs, was supplied by Kracie Pharma Ltd. (Tokyo, Japan) as dried powder. Ninjin-yoeito powder was mixed with distilled water to prepare the stock solution. For $[Ca^{2+}]_i$ imaging, Ninjin-yoeito solution was diluted at the concentrations used for superfusion in HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) solution composed of (in mM) 129 NaCl, 5.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgSO_4$, and 10 HEPES with pH adjusted at 7.4 using NaOH supplemented with 5 mM glucose. Fresh solution of Ninjin-yoeito was prepared before each experiment.

2.2. Animals

Male C57BL/6J mice aged 4–12 weeks were obtained from Japan SLC (Shizuoka, Japan) and housed for at least 1 week under conditions of controlled temperature (23 °C ± 1 °C), humidity (55% ± 5%) and lighting (light phase 7:30–19:30). Food and water were available ad libitum. Animal experiments were carried out after receiving approval from the Institutional Animal Experiment Committee and in accordance with the Institutional Regulation for Animal Experiments at Jichi Medical University and Kobe University.

2.3. Preparation of single neurons from ARC

The ARC was isolated from the brain of mice aged 5–7 weeks and single neurons were prepared as reported previously (Kohno et al., 2003). Briefly, mice were anesthetized with intraperitoneal injection of urethane (ethyl carbamate; 1 g/kg, ip) and decapitated, and their brain was removed. Brain slices containing ARC were prepared, and the whole ARC of the left and right sides was punched out. The dissected tissues were incubated in HKRB supplemented with 20 units/ml papain (Sigma Aldrich, St. Louis, MO), 0.015 mg/ml deoxyribonuclease, and 0.75 mg/ml BSA for 16 min at 36 °C in a shaking water bath, followed by gentle mechanical trituration for 5–10 min. The cell suspension was centrifuged at 100 × *g* for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips. The cells were kept at 30 °C in moisture-saturated dishes till $[Ca^{2+}]_i$ measurements for up to 10 h.

2.4. Measurements of $[Ca^{2+}]$ in single ARC neurons

At 2–10 h after cell preparation, $[Ca^{2+}]_i$ was measured by ratiometric fura-2 fluorescence imaging as previously reported (Kohno et al., 2003). Briefly, following incubation with 2 μM fura-2 AM (DOJINDO, Kumamoto, Japan) for 30 min at 30 °C, the cells were mounted in a chamber and superfused at 1 ml/min with HKRB containing 2.5 mM glucose at 30 °C. Data were taken from the single cells that were identified as neurons by the criteria reported previously (Kohno et al., 2003); relatively large diameter ($\geq$10 μm), clear and round cell bodies on phase-contrast microscopy, and $[Ca^{2+}]_i$ responses to KCl (55 mM). Ninjin-yoeito (1–10 μg/ml) and ghrelin (10^{-8} M) were administered under superfusion conditions. Fluorescence ratio images were produced by Aquacosmos ver. 2.5 (Hamamatsu Photonics, Shizuoka, Japan). When $[Ca^{2+}]_i$ changed within 5 min after addition of agents and their amplitudes were at least twice larger than fluctuations of baseline, they were considered responses. In all experiments, neurons from at least three separate preparations were analyzed.

2.5. Immunocytochemistry and identification of NPY neurons

After $[Ca^{2+}]_i$ measurements, cells were fixed with 4% paraformaldehyde. They were pretreated with 3% H_2O_2 for 1 h and blocked in 10% normal goat serum and in 0.1 M PBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with primary antiserum to NPY (DiaSorin, Stillwater, MN) diluted 1:10,000 in PBS containing 1.5% normal goat serum. After rinsing, cells were incubated with biotinylated secondary antibody raised against rabbit IgG (Vector Laboratories Inc., Burlingame, CA; diluted 400-fold) for 1 h at room temperature. After rinsing, the sections were labeled with avidin-peroxidase complex (ABC kit, Vector) for 1 h and color-developed with 3, 3′-diaminobenzidine (DAB).

$[Ca^{2+}]_i$ and immunocytochemical data were correlated to each other, based on the photographs of the single neurons subjected to $[Ca^{2+}]_i$ measurements in the microscopic field (Kohno et al., 2003). To help identify the location of single neurons, the single neurons were plated on coverslips whose reverse sides were marked with cross scratches produced by diamond knife.

2.6. Measurement of food intake

Mice were housed in individual cages and habituated to standard pellet diet (CE-2, 3.45 kcal/g, CLEA Japan, Tokyo, Japan) and to handling at least 1 week before experiments. Intraperitoneal (ip) injection of cisplatin (Wako Pure Chemical, Osaka, Japan) at 8 mg/kg was performed on day 0 and 4 of feeding experiments. From day 0, mice were orally injected with Ninjin-yoeito (1 g/kg) or distilled water once daily for 10 consecutive days. Food intake and body weight were measured each day.

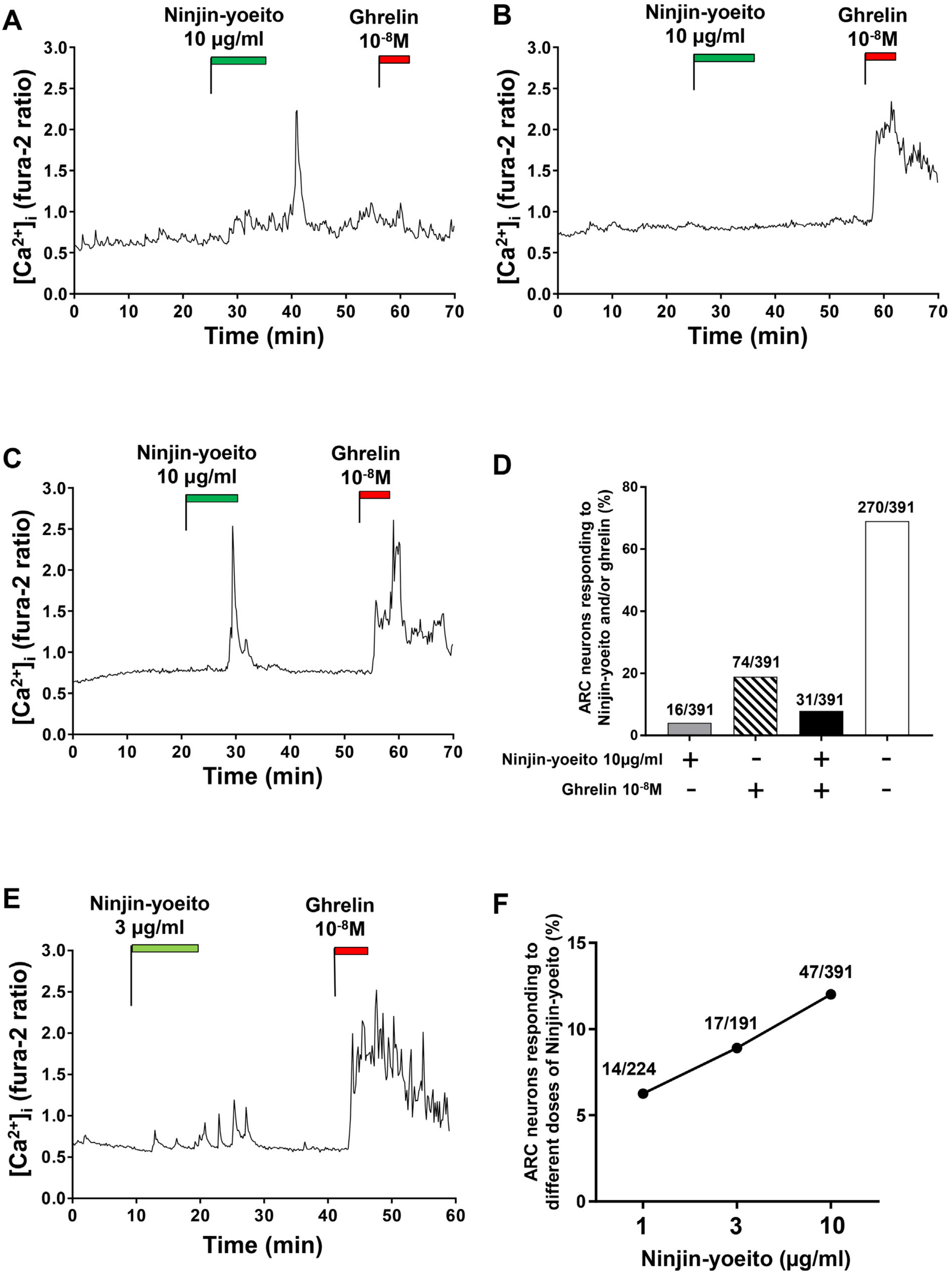

Fig. 1. Effects of Ninjin-yoeito and ghrelin on $[Ca^{2+}]_i$ in single neurons isolated from ARC. A: An ARC neuron responded to Ninjin-yoeito (10 μg/ml), but not ghrelin (10^{-8} M), with increases in $[Ca^{2+}]_i$. B: An ARC neuron responded to ghrelin, but not Ninjin-yoeito, with $[Ca^{2+}]_i$ increases. C: An ARC neuron responded to both ghrelin and Ninjin-yoeito with $[Ca^{2+}]_i$ increases. D: Incidence of ARC neurons that responded to Ninjin-yoeito, ghrelin, both and none of them. Among 47 ARC neurons that responded to Ninjin-yoeito, 31 neurons (66.0%) also responded to ghrelin. E: An ARC neuron responded to Ninjin-yoeito at lower dose (3 μg/ml) with $[Ca^{2+}]_i$ increases. F: Incidence of the ARC neurons with $[Ca^{2+}]_i$ response to 1, 3 and 10 μg/ml Ninjin-yoeito. The numbers above each bar/point indicate the number of neurons that responded over that examined.

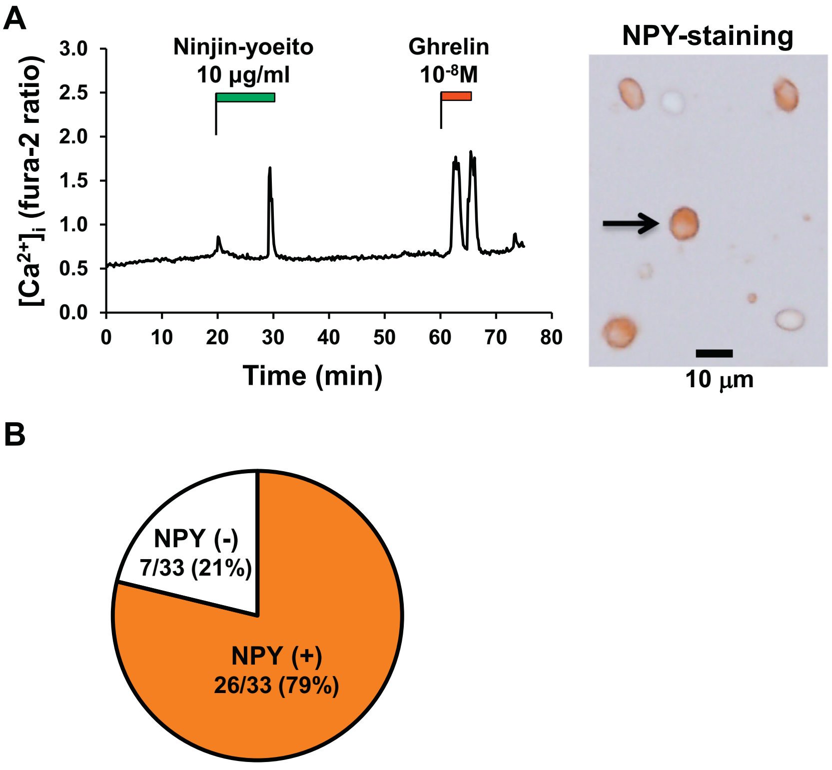

Fig. 2. Ninjin-yoeito increases $[Ca^{2+}]_i$ in NPY neurons of ARC. A: Ninjin-yoeito (10 μg/ml) increased $[Ca^{2+}]_i$ in an ARC neuron that was subsequently shown to be immunoreactive (IR) to NPY by immunocytochemistry, as indicated by an arrow. Ghrelin also increased $[Ca^{2+}]_i$ in this neuron. There were four NPY-IR and two non NPY-IR neurons in this immunostaining field. Scale bar indicates 10 μm. B: Incidence of NPY-IR neurons in ARC neurons that responded to Ninjin-yoeito. Twenty six of 33 Ninjin-yoeito-responsive neurons (79%) were NPY-IR neurons.

2.7. *Statistical analysis*

Data are presented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni multiple comparison tests using Prism 5 (GraphPad Software, CA). $P < 0.05$ was considered significant.

3. Results

3.1. *Ninjin-yoeito increases $[Ca^{2+}]_i$ in single neurons isolated from ARC*

$[Ca^{2+}]_i$ in single neurons isolated from ARC was measured under superfusion conditions. After $[Ca^{2+}]_i$ was stabilized at baseline, administration of Ninjin-yoeito (10 μg/ml) for 10 min induced an increase in $[Ca^{2+}]_i$ in a single neuron (Fig. 1A). In this neuron, subsequent administration (5 min) of ghrelin (10^{-8} M), at 20 min after washing out Ninjin-yoeito, failed to increase $[Ca^{2+}]_i$ (Fig. 1A). The second type of neuron responded to ghrelin, but not Ninjin-yoeito, with increases in $[Ca^{2+}]_i$ (Fig. 1B), confirming previous reports that ghrelin increases $[Ca^{2+}]_i$ in ARC neurons (Kohno et al., 2007; Kohno et al., 2003). The third type of neuron responded to both Ninjin-yoeito and ghrelin with increases in $[Ca^{2+}]_i$ (Fig. 1C). In total, among 391 single ARC neurons examined, 16 neurons (4.1%) responded to Ninjin-yoeito only (Fig. 1A and D), 74 (18.9%) responded to ghrelin only (Fig. 1B and D), 31 (7.9%) responded to both Ninjin-yoeito and ghrelin (Fig. 1C and D), and 270 (69.1%) responded to none of them (Fig. 1D). The $[Ca^{2+}]_i$ increases took place at 2–10 min after administration of Ninjin-yoeito, exhibiting relatively long time lag till the onset of responses. Among 47 neurons that responded to Ninjin-yoeito, 31 neurons (66.0%) responded to ghrelin. These results indicate that Ninjin-yoeito directly activates ARC neurons, including ghrelin-responsive (66.0%) and unresponsive (34.0%) neurons. Ninjin-yoeito at 1, 3 and 10 μg/ml increased $[Ca^{2+}]_i$ in 14 (6.3%), 17 (8.9%), and 47 (12.0%) of 391 neurons, respectively, showing a concentration-dependent effect (Fig. 1E and F). Since 10 μg/ml Ninjin-yoeito exerted the greatest effect, this concentration was used in the rest of $[Ca^{2+}]_i$ studies.

3.2. *Ninjin-yoeito increases $[Ca^{2+}]_i$ in NPY neurons of ARC*

To examine whether Ninjin-yoeito activates NPY neurons, $[Ca^{2+}]_i$ measurements were followed by immunocytochemistry with an anti-NPY antibody. Administration of Ninjin-yoeito (10 μg/ml) increased $[Ca^{2+}]_i$ in a single ARC neuron that was subsequently shown to be IR to NPY by immunocytochemistry (Fig. 2A). Ghrelin (10^{-8} M) also increased $[Ca^{2+}]_i$ in this neuron. Among 33 neurons that had responded to Ninjin-yoeito, 26 neurons (79.0%) were IR to NPY (Fig. 2B).

3.3. *Ninjin-yoeito increases $[Ca^{2+}]_i$ in ghrelin-responsive and unresponsive NPY neurons*

The incidence of responses to Ninjin-yoeito (10 μg/ml, 10 min) and ghrelin (10^{-8} M, 5 min) in NPY-IR neurons were examined. Ninjin-yoeito (10 μg/ml), but not ghrelin (10^{-8} M), induced an increase in $[Ca^{2+}]_i$ in a single ARC neuron that was subsequently shown to be IR to NPY (Fig. 3A). The second type of ARC NPY-IR neuron responded to ghrelin (10^{-8} M), but not Ninjin-yoeito (10 μg/ml), with $[Ca^{2+}]_i$ increases (Fig. 3B). The third type of ARC NPY-IR neuron responded to Ninjin-yoeito (10 μg/ml) and ghrelin (10^{-8} M) with $[Ca^{2+}]_i$ increases (Fig. 3C). Among 81 single ARC NPY-IR neurons examined, 8 neurons (9.9%) responded to Ninjin-yoeito only (Fig. 3A and D), 30 (37.0%) responded to ghrelin only (Fig. 3B and D), 18 (22.2%) responded to both (Fig. 3C and D) and 25 (30.9%) responded to none of them (Fig. 3D). Among 26 NPY neurons that responded to Ninjin-yoeito, 18 neurons (69.2%) responded to ghrelin, indicating that Ninjin-yoeito activates both ghrelin-responsive (69.2%) and ghrelin-unresponsive (30.8%) NPY neurons.

3.4. *Ninjin-yoeito counteracts reduced food intake and body weight in cisplatin-treated mice*

The effect of Ninjin-yoeito on food intake and body weight was examined in mice receiving an anorectic cisplatin, a chemotherapy drug for cancer patients. Mice were injected with cisplatin (8 mg/kg, ip) on

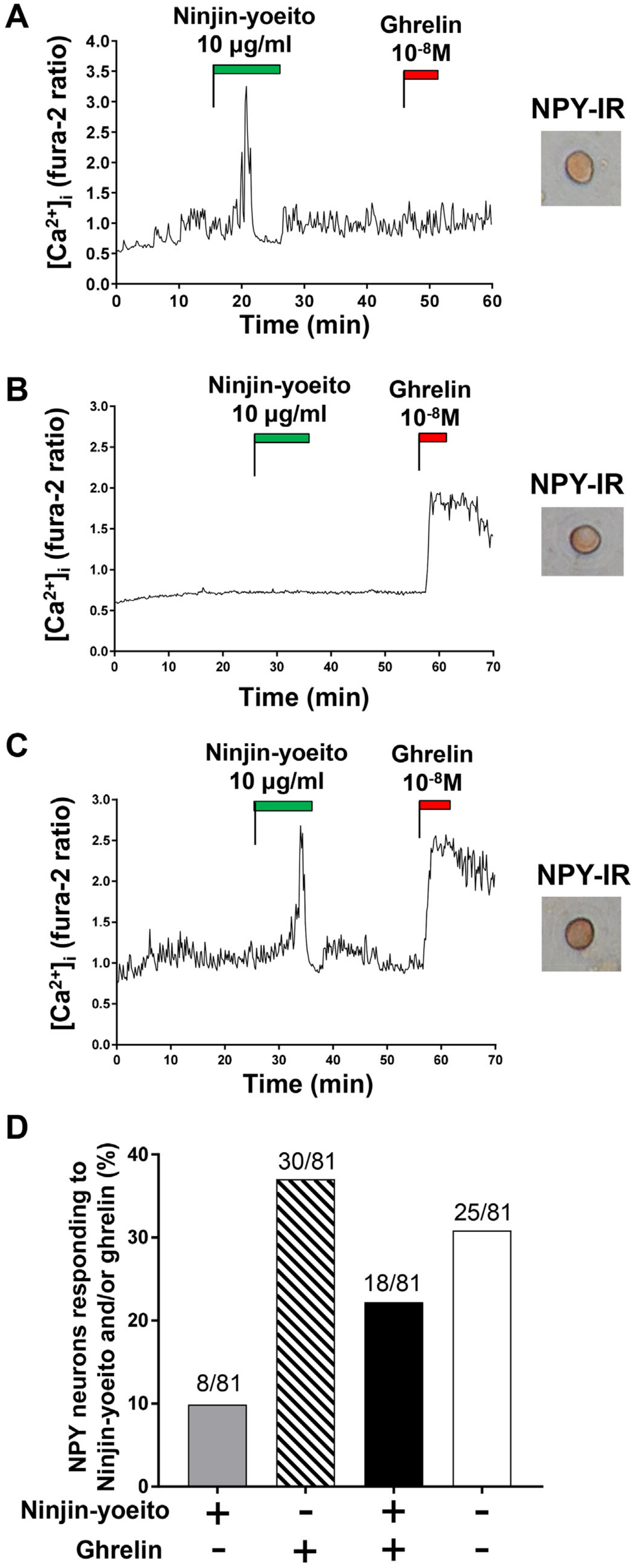

Fig. 3. Effect of Ninjin-yoeito and ghrelin on $[Ca^{2+}]_i$ in ARC NPY neurons. A: Ninjin-yoeito (10 μg/ml), but not ghrelin (10^{-8} M), increased $[Ca^{2+}]_i$ in a single ARC neuron that was subsequently shown to be IR to NPY. B: An ARC NPY neuron responded to ghrelin, but not Ninjin-yoeito, with $[Ca^{2+}]_i$ increases. C: An ARC NPY neuron responded to both ghrelin and Ninjin-yoeito with $[Ca^{2+}]_i$ increases. D: Incidence of ARC NPY neurons that responded to Ninjin-yoeito, ghrelin, both and none of them. Among 26 ARC NPY neurons that responded to Ninjin-yoeito, 18 neurons (69.3%) also responded to ghrelin. The numbers above each bar indicate the number of neurons that responded over that examined.

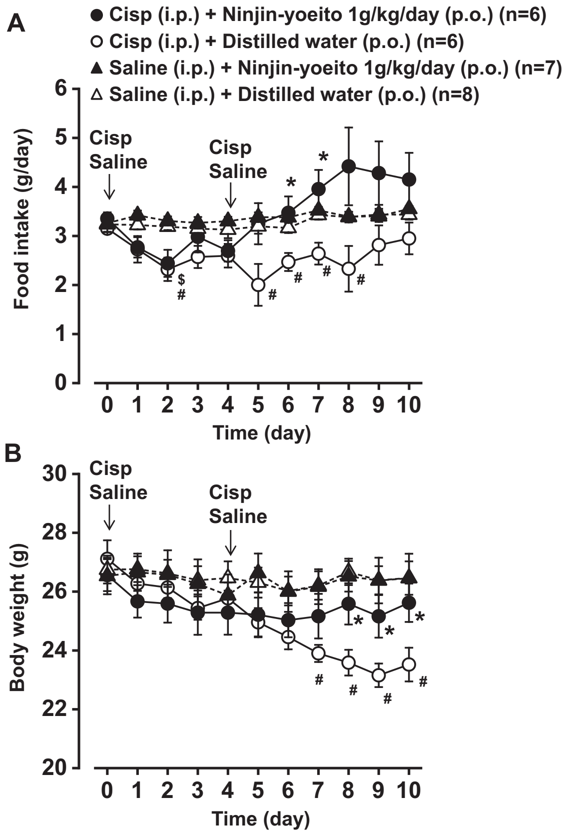

Fig. 4. Oral administration of Ninjin-yoeito counteracts reduced food intake and body weight in cisplatin treated mice. A and B: Cisplatin (8 mg/kg; Cisp) was ip administered on day 0 and 4. Ninjin-yoeito at 1 g/kg/day (filled circle) or distilled water (open circle) was orally administered at 8:00 AM daily from day 0 through day 10. Food intake and body weight were measured daily just before administrating Ninjin-yoeito. The first administration of cisplatin reduced food intake and tended to reduce body weight in the period of day 0 to 4, on which Ninjin-yoeito had little effect. The second administration of cisplatin reduced food intake at days 5–8 (A) and body weight at days 7–10 (B), and these reductions were counteracted by Ninjin-yoeito, compared to control distilled water, with statistical significance observed on days 6 and 7 for food intake (Fig. 4A) and on days 8–10 for body weight. All data were presented as mean ± SEM. $^{*}P < 0.05$; Cisp + Ninjin-yoeito vs. Cisp + Distilled water, $^{\#}P < 0.05$; Cisp + Distilled water vs. Saline + Distilled water, $^{\$}P < 0.05$; Cisp + Ninjin-yoeito vs. Saline + Ninjin-yoeito by one-way ANOVA followed by Bonferroni's multiple comparison tests.

day 0 and day 4, while subjected to oral administration of Ninjin-yoeito (1 g/kg/day) daily from day 0 through day 9. The first administration of cisplatin (8 mg/kg, ip) reduced food intake at day 2 and tended to reduce body weight at day 3, on which Ninjin-yoeito had little effect (Fig. 4A and B). The second administration of cisplatin on day 4 reduced food intake at days 5–8 (Fig. 4A) and body weight at days 7–10 (Fig. 4B). These reductions were counteracted by Ninjin-yoeito (1 g/kg/day), compared to control distilled water, in which statistical significance was observed on days 6 and 7 for food intake (Fig. 4A) and on days 8–10 for body weight (Fig. 4B). These results indicate that Ninjin-yoeito counteracts the anorectic and weight-reducing effects of the second cisplatin dosing in mice.

4. Discussion

Ninjin-yoeito dose-dependently increased $[Ca^{2+}]_i$ in single neurons isolated from ARC in mice. Immunocytochemistry following $[Ca^{2+}]_i$ measurements showed that as much as 79% of the neurons that responded to Ninjin-yoeito were IR to NPY. These data identified the NPY neuron as the major target for Ninjin-yoeito in ARC. Ghrelin also increased $[Ca^{2+}]_i$ in single NPY neurons of ARC, confirming previous reports (Kohno et al., 2007; Kohno et al., 2003). In the present study, of 48 NPY neurons that exhibited $[Ca^{2+}]_i$ responses to ghrelin, 18 (37.5%) also responded to Ninjin-yoeito with $[Ca^{2+}]_i$ increases. In addition, out of 33 NPY neurons that failed to respond to ghrelin, 8 (24.2%) responded to Ninjin-yoeito with $[Ca^{2+}]_i$ increases. These data revealed that Ninjin-yoeito activates two distinct subpopulations of NPY neurons, one ghrelin-responsive and the other ghrelin-unresponsive. In parallel, treatment with Ninjin-yoeito significantly counteracted reductions in food intake and body weight induced by second cisplatin dosing in mice. These results demonstrate that Ninjin-yoeito directly activates ghrelin-responsive and ghrelin-unresponsive NPY neurons in ARC and relieves anorexia and weight loss by a chemothrapy drug cisplatin.

Several studies have reported the effect of Ninjin-yoeito in experimental animals under different physiological conditions (Suzuki et al., 2017; Suzuki et al., 2015). Only a few studies reported the effect of Ninjin-yoeito on food intake in a descriptive manner, leaving the underlying mechanism unknown. The present study, for the first time, identified the ARC NPY neurons as the direct primary target of Ninjin-yoeito. The ARC NPY neuron is considered the principal neuron for initiating feeding behavior (Schwartz et al., 2000; Yada et al., 2012). Selective activation of ARC AgRP/NPY neurons acutely and robustly triggers feeding (Aponte et al., 2011; Krashes et al., 2011), while their selective deletion in adult mice markedly reduces feeding (Gropp et al., 2005; Luquet et al., 2005), placing the ARC AgRP/NPY neuron as the necessary and adequate inducer of feeding. Taken together, we speculate that the ability of Ninjin-yoeito to activate ARC NPY neurons underlies its orexigenic action shown in the present and previous studies (Fujimoto et al., 1989).

We found that Ninjin-yoeito activates two distinct subpopulations, ghrelin-responsive and unresponsive NPY neurons in ARC. Ghrelin is the sole orexigenic hormone of the peripheral origin, which is released under fasted states and potently stimulates feeding primarily through activating ARC NPY neurons (Kohno et al., 2007; Nakazato et al., 2001). Hence, the ghrelin-NPY pathway serves as the core physiological route for appetite (Zhang et al., 2012). We found that Ninjin-yoeito activated as much as 38% (18/48 cells) of the ghrelin-responsive NPY neurons in ARC. The Ninjin-yoeito activation of ghrelin-responsive NPY neurons may be linked to promotion of feeding behavior. Another notable finding of the current study is that Ninjin-yoeito activated 24% of ghrelin-unresponsive NPY neurons. This interaction may serve as an additional orexigenic mechanism unique for Ninjin-yoeito. It has been reported that the ghrelin resistance occurs with age and is implicated in reduced appetite (Amitani et al., 2017; Miyazaki et al., 2014; Takeda et al., 2010). Hence, the Ninjin-yoeito activation of ghrelin-unresponsive NPY neurons may have a therapeutic potential to restore the appetite in the elderly subjects with ghrelin resistance including those with frailty.

Our finding that Ninjin-yoeito counteracted the anorexigenic effect of cisplatin is in consistent with several previous reports. Central administration of ginsenoside Rg1, a component of Ninjin-yoeito, attenuates anorexia produced by elevated environmental temperature to 30 °C (Fujimoto et al., 1989). It was reported that cisplatin decreases expression of orexin in the hypothalamus and that elevating orexin-A signaling in ARC alleviates cisplatin's side effects on food intake and/or gastric motility via NPY-dependent pathway (Guo et al., 2018). Orexin-A activates NPY neurons in ARC to promote food intake (Muroya et al., 2004). Taken together, Ninjin-yoeito's signaling through ARC NPY neurons may counteract anorexia under toxic conditions including cisplatin treatment and elevated temperature.

Our data demonstrated that Ninjin-yoeito activates ghrelin-responsive and ghrelin-unresponsive NPY neurons in ARC and increases food intake in mice treated with cisplatin. Activation of the ARC NPY neuronal pathway could serve to intervene the anorexia and weight loss during cancer chemotherapy. This neuronal pathway is stimulated by ghrelin and orexin in experimental animals, however, these peptides cannot be used clinically. The present finding of particular relevance is that the ARC NPY neuronal pathway is activated by Ninjin-yoeito, a Japanese medicine commercially available to treat human subjects with depressed appetite and weight loss. Hence, Ninjin-yoeito may provide an approach to reduce depressed appetite, a major side effect of cancer chemotherapy. Furthermore, a role of NPY family peptides and Y receptors in modulating the tumor growth has emerged (Zhang et al., 2011). NPY reportedly acts against cancer (Chiba et al., 2014), though underlying mechanisms are elusive. Hence, the ability of Ninjin-yoeito to promote NPY system could act as anti-cancer via restoring appetite and another mechanism(s).

Reduced appetite/feeding is associated with, and even placed upstream of, frailty. Hence, the ability of Ninjin-yoeito to activate orexigenic NPY neurons and increase feeding may act against frailty. In addition, aging is deeply related to frailty (Landi et al., 2016). The effect of calorie restriction to prolong the life is mediated by the upregulated NPY system (Chiba et al., 2014). Hence, the Ninjin-yoeito-stimulated NPY system potentially promotes life span. This anti-aging and the appetite-restoring abilities of NPY pathway could underlie the anti-frailty effect of Ninjin-yoeito.

In this study, we found that Ninjin-yoeito activates NPY neurons and restores food intake and weight gain in anorectic mice treated with cisplatin. This appetite-promoting action of Ninjin-yoeito may help to attenuate pathological conditions and improve QOL of a wide variety of patients with anorexia, which include cancer, frailty, heart failure and stress disorders. However, further study is definitively required to elucidate the mechanism underlying the activation of ARC NPY neurons by Ninjin-yoeito.

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