Antagonism for NPY signaling reverses cognitive behavior defects induced by activity-based anorexia in mice

Natasya Trivena Rokot[a#], Koji Ataka[b#*], Haruki Iwai[c], Hajime Suzuki[d], Homare Tachibe[e], Timothy Sean Kairupan[f], Kai-Chun Cheng[b], Haruka Amitani[a], Akio Inui[b], Akihiro Asakawa[a]

a. Department of Psychosomatic Internal Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

b. Pharmacological Department of Herbal Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

c. Department of Oral Anatomy and Cell Biology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

d. Department of Oral and Maxillofacial Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

e. Institute of Laboratory Animal Science Research Support Center, Kagoshima University, Kagoshima, Japan.

f. Faculty of Medicine, Sam Ratulangi University, Manado, Indonesia.

* Corresponding author:

Koji Ataka, PhD

Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1, Sakuragaoka, Kagoshima, 890-8544, Japan.

Email: kataka@m3.kufm.kagoshima-u.ac.jp

Tel: +81 99 275 5752

Fax: +81 99 275 5749

#Authorship note: Natasya Trivena Rokot and Koji Ataka contributed equally to this work.

ABSTRACT

Patients with AN often express psychological symptoms such as body image distortion, cognitive biases, abnormal facial recognition, and deficits in working memory. However, the molecular mechanisms underlying the impairment of cognitive behaviors in AN remain unknown.

In the present study, we measured cognitive behavior using novel object recognition (NOR) tasks and mRNA expressions in hypothalamic neuropeptides in female C57BL/6J mice with activity-based anorexia (ABA). Additionally, we evaluated the effects of antagonists with intracerebroventricular (icv) administration on the impairment of cognitive behavior in NOR tasks. Our results showed that NOR indices were lowered, subsequently increasing mRNA levels of agouti-related peptide (AgRP) and neuropeptide Y (NPY), and c-Fos- and AgRP- or NPY-positive cells in the hypothalamic arcuate nucleus in ABA mice. We also observed that icv administration of anti-NPY antiserum (2 μl), anti-AgRP antibody (0.1 μg), and Y5 receptor antagonist CPG71683 (15 nmol) significantly reversed the decreased NOR indices. Therefore, our results suggest that increased NPY and AgRP signaling in the brain might contribute to the impairment of cognitive behavior in AN.

Keywords: Anorexia nervosa; Cognitive behavior; Agouti-related peptide; Neuropeptide Y; Y5 receptor.

1. Introduction

Anorexia nervosa (AN) is a serious eating disorder that often occurs in adolescent women and has a higher comorbidity rate with psychiatric disorders and suicide attempts compared to other psychiatric disorders (Kask et al., 2016, Udo et al., 2019).

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorder (DSM-5) lists the following diagnostic criteria: 1) restriction of energy intake, 2) intense fear of gaining weight or becoming fat, 3) body image distortion. Additionally, the severity of AN is categorized by on body mass index (BMI). Despite many available treatments, such as medication, behavioral therapy, cognitive-behavioral therapy, and family therapy, among others, AN continues to be a refractory disorder because of its unknown etiology. Body image distortion, including negative feelings and estimations of body shape and body size, is a hallmark risk factor for development of AN (Dalhoff et al., 2019; Stice and Shaw, 2002). The disturbance of cognitive function has been reported in many studies (Frantz, 1981; Hamsher et al., 1981; Maxwell et al., 1984; Witt et al., 1985; Strupp et al., 1986; Palazidou et al., 1990; Pendleton et al., 1991; Szmukler et al., 1992; Hamatani et al., 2016; Tamiya et al., 2018; Olivo et al., 2019). It has been reported that AN patients have specific cognitive biases, such as negative interpretation biases to eating-related stimuli (Brockmeyer et al., 2018; Shafran et al., 2007). They also exhibit the abnormalities in the recognition of emotional expressions on the faces of people around

69 them, as well as their own (Hirot et al., 2016; Sfärlea et al., 2018). Further, AN patients
70 exhibit a deficit of working memory, which is an important function regulating cognition
71 functions (Kemps et al., 2006). Koyama et al. reported that working memory was not
72 recovered, and overall IQ scores were restored after weight gain in AN patients (Koyama
73 et al., 2012). Patients with AN show deficits in cognitive function, including alterations
74 in attentional styles, perceptual processing, working memory, cognitive flexibility, and
75 decision making (Reville et al., 2016). Overcoming these impairments of cognition-
76 related functions is one of the critical aims of AN treatment.

77 An activity-based anorexia (ABA) model in female rodents has been used as a well-
78 validated animal model for AN research (Carrera et al., 2014; Pierce et al., 1994;
79 Routtenberg and Kuznesof, 1967). AN is characterized by the restriction of calorie intake
80 and excessive physical exercise to avoid weight gain, leading to severe weight loss
81 (Lamanna et al., 2019). ABA rodents, which receive the scheduled feeding with free
82 access to running wheels for appropriate periods, exhibit body weight loss, reduction in
83 food intake, and hyperactivity, which are similar to features observed in AN (Schalla and
84 Stengel, 2019). Although it is difficult to assess cognitive behavior impairments in
85 animals as it is possible in AN patients, a previous study showed that female adolescent
86 rats in the ABA condition have poor performance in the novel object recognition (NOR)
87 task, which indicated impairments in object recognition or contextual memory (Boersma

et al., 2016). On the other hand, spatial memory measured with the Barnes maze was not altered (Boersma et al., 2016). However, the mechanisms involved in the impairment of cognitive functions in the ABA model remain unclear. The NOR task has been used to investigate cognitive paradigms based on working memory, attention, anxiety, and preference for novelty in rodents in the absence of reward or punishment (Antunes and Biala, 2012; Webster et al., 2014). Rodents approach and explore novel objects more frequently when they are exposed to familiar and novel objects. Therefore, cognitive function can be evaluated by results of the NOR task. Defects in cognitive function, as evaluated by the NOR task, have been observed in various animal models, including Alzheimer's disease, traumatic brain injury, schizophrenia, Parkinson's disease, autism spectrum disorder, and aging (Grayson et al., 2015; Traschütz et al., 2018).

Feeding behaviors are controlled by various neuropeptides, including orexigenic peptides: agouti-related peptide (AgRP) and neuropeptide Y (NPY), anorexigenic peptides: proopiomelanocortin (POMC), amphetamine-regulated transcript (CART), oxytocin (OXT), corticotropin-releasing factor (CRF), urocortin1 (Ucn1), and brain-derived neurotrophic factor (BDNF) in the hypothalamus (Sohn, 2015). AgRP and NPY are upregulated under hunger conditions and lead to food intake (Loh et al., 2015; Stütz et al., 2005). High AgRP and NPY levels are reported in AN patients (Moriya et al., 2006; Kaye, 1996) and ABA rats (Stütz et al., 2005). However, the reasons behind upregulated

AgRP and NPY in AN remain unclear. In animal experiments, AgRP has been shown to induce stereotypic behavior (Dietrich et al., 2015), and alter spatial navigation in probe trials and spontaneous alteration behavior in the Y-maze test (Zimmer et al., 2019). It is well known that NPY can regulate learning, memory, and anxiolysis (Gøtzsche and Woldbye, 2016; Reichmann and Holzer, 2016). This study aimed to investigate the relationship between central neuropeptides associated with feeding behavior and the deficiency of cognition behaviors in NOR tasks in ABA model mice.

2. Materials and methods

2.1. Animals

Female C57BL/6J mice at 6-8 weeks of age and 15-20 g body weight were purchased from Charles River Laboratories Japan, Inc. (Tokyo, Japan). Mice were individually maintained in a pathogen-free facility under standard conditions at 24 ± 2°C and 50 ± 10% humidity with a 12-h/12-h light-dark cycle (light-dark phase reversal: dark phase from 11:00 AM to 11:00 PM) and ad libitum access to sterile standard chow (3.4 kcal/g; CE-2, CLEA Japan Inc., Tokyo, Japan) and water in the animal facility of Kagoshima University. All animal protocols for this study were approved by the Kagoshima

University Committee for Animal Experiments, (MD17118 and MD19004). Experiments were performed in accordance with the relevant guidelines and regulations.

2.2. ABA procedure

The ABA procedure was performed as previously described (François et al., 2015; Jésus et al., 2014). Briefly, mice were randomly assigned to four experimental groups: *ad libitum* feeding (Normal group), free access to running wheel with scheduled feeding (ABA group), scheduled feeding (food restriction, FR group), and free access to running wheel with *ad libitum* feeding (Wheel group). All mice were housed individually and had free access to food and water for 1 week, and mice in the ABA and Wheel groups were able to freely access a running wheel for 3 days before the start of the experiments. Food access was progressively limited in the ABA and FR groups to 6 h on Day 1, 5 h on Day 2, 4 h on Day 3, and 3 h on Days 4 to 8. Food was provided at the beginning of the dark phase. The schematic diagram of ABA schedule is presented in Fig. 1.

2.3. Measurements of body weight, food intake, and running wheel activity

Body weight and the number of wheel cycles were measured at the end of the light

phase (11:00 AM). Food intake was measured at the end of scheduled feeding in the ABA and FR groups, and at the end of the light phase (11:00 AM) in the Normal and Wheel groups. Running wheel activity (km/day) was calculated.

2.4. NOR task

On Day 8, a NOR task was performed 6 h after the end of scheduled feeding (8:00 PM) with partial modifications to previously described methods (De Rosa et al., 2005; Leger et al., 2013). Briefly, mice were placed in an empty box (black walls with open top to record video footage, 60 cm × 60 cm × 70 cm) to habituate to the environment for 10 min (habituating phase). Then, the mice were returned to their home cages. Two objects of same color, shape, and size were placed on opposite sides of the box. Mice were placed in the box to allow free exploration for 10 min (Phase I). Mice were returned to their home cages, and the two objects were removed from the box. Mice were placed in the cleared box without objects for 10 min (resting phase). Mice were returned to their home cages, and the same objects used in Phase I were placed in the box: one object was placed in the same position (familiar object), but the other object was placed in a different position. Mice were placed in the box and allowed to explore for 10 min (Phase II). The mice were returned to their home cages, and the two objects were removed from the box.

Mice were placed in the cleared box without objects for 10 min (resting phase). The same object (familiar) and a novel object were placed in the same position as in Phase I. Mice were placed in the box and allowed to explore for 10 min (Phase III). All objects and the box were cleaned with 70% ethanol to remove any odor after previous phase. Object exploration was defined as follows; the nose of the mouse touched the object, and climbing onto the object (unless the mouse sniffs the object it has climbed on) or chewing the object did not qualify as exploration. The NOR index was calculated using the following formula: (exploration time to the new object - exploration time to the familiar object) / (exploration time to the new object + exploration time to the familiar object). The NOR task procedure is presented in Fig. 3A.

2.5. Tissues sampling

Tissues were obtained from the other mice who did not receive the NOR task. Mice were deeply anesthetized by isoflurane inhalation and perfused with 0.1 M phosphate buffer 6 h after the end of scheduled feeding (8:00 PM) on Day 8. After euthanasia brain tissues were isolated for real-time quantitative PCR (qPCR) analysis. For immunohistochemistry, mice were perfused in the same way as described above, and were perfused with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer.

183 *2.6. Real-time qPCR analysis*

184 Total RNA was extracted from resected hypothalamus tissue using an RNeasy Plus
185 Mini Kit (74134; QIAGEN, Hilden, Germany), and cDNA was synthesized using a
186 SuperScript III First-Strand Synthesis System (18080-051; Invitrogen, Carlsbad, CA,
187 USA) according to the manufacturer's protocol. The real-time qPCR analysis was
188 performed with SYBR Green Master Mix (Roche Inc., Basel, Switzerland) according to
189 the manufacturer's protocol. Relative mRNA levels were quantified using the $2^{-\Delta\Delta CT}$
190 method. Changes in mRNA expression were defined as significant if the $2^{-\Delta\Delta CT}$ value
191 increased by > 2-fold or decreased by < 0.5-fold. The primers used for real-time qPCR
192 are shown in Table 1.

193

194 *2.7. Immunohistochemistry*

195

196 Hypothalamus sections were incubated with anti-c-Fos antibody (sc-52-G, Santa
197 Cruz Biotechnology, Inc., Dallas, TX, USA; 1:500) and anti-NPY antiserum (Y061,
198 Yanaihara Institute, Shizuoka, Japan, 1:500), or anti-c-Fos antibody (ABE457, Merck
199 Millipore, Belize, MA, USA; 1:500) and/or anti-AgRP antibody (ab32882, Abcam,
200 Cambridge, UK; 1:500) for 48 h at 4°C, and then incubated with the following secondary
201 antibodies: Alexa Fluor 488-conjugated anti-goat IgG (705-545-147, Jackson

ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:800) and Alexa Fluor 594-conjugated anti-rabbit IgG (711-295-152, Jackson ImmunoResearch Laboratories Inc.; 1:800) for 4 h at room temperature. Hypothalamus sections were observed using a confocal laser scanning microscope (LSM TCS SP8, Leica Microsystems, Wetzlar, Germany). Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride solution (DAPI, D523; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The number of c-Fos-positive cells was counted on one side of 2-5 species of hypothalamus tissues of each mouse, and the averages were calculated. These number were used in subsequent analysis.

2.8. Cannula implantation

Mice were anesthetized by intraperitoneal (ip) administration of a mixture of 0.3 mg/kg of medetomidine (Domitor; Meiji Seika Pharma, Tokyo, Japan), 4.0 mg/kg of midazolam (Sandoz, Tokyo, Japan), and 5.0 mg/kg of butorphanol (Vetorphale; Meiji Seika Pharma, Tokyo, Japan). A guide cannula (25-guage; Eicom, Kyoto, Japan) was implanted into the right lateral ventricle using a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Stereotaxic coordinates were 0.6 mm posterior to the bregma, 1.5 mm right lateral to the midline, and 1.5 mm below the outer surface of the

skull. The guide cannula was secured with dental cement and anchored by two stainless steel screws fixed to the dorsal surface of the skull. A dummy cannula (Eicom) was placed into each guide cannula and fixed with a screw cap (Eicom) to prevent occlusion. When intracerebroventricular (icv) delivery was administered to conscious animals, the dummy cannula was replaced by a microinjection cannula (AMI-5; Eicom), 1 mm longer than the guide cannula, and connected to a polyethylene tube (PE-50, Clay Adams, Parsippany, NJ, USA). After cannula implantation mice were recovered from anesthesia with ip administration of 0.3 mg/kg of atipamezole (Antisedan; Nippon Zenyaku Kogyo, Fukushima, Japan). At the end of the experiments, animals were euthanized by inhalation of carbon dioxide gas. After euthanasia, the correct location of the icv cannula was verified by administration of 10 µl dye (0.05% cresyl violet).

2.9. Drug administration

The following antiserum, antibodies, and chemicals were intracerebroventricularly administered: anti-NPY antiserum (2 µl/mouse, Y061, Yanaihara Institute), anti-AgRP antibody (0.1 µg/mouse, ab32882, Abcam), CPG 71683A hydrochloride, a NPY 5 (Y5) receptor antagonist (15 nmol/mouse, 2199, Tocris Bioscience, Bristol, UK), and BIBO 3304 trifluoroacetate, a Y1 receptor antagonist (30 nmol/mouse, 2412, Tocris Bioscience,

Bristol, UK). Anti-NPY antiserum and anti-AgRP antibody were dissolved in water, and CPG 71683A and BIBO 3304 were dissolved in 5% DMSO and 5% Tween-80 in water, and each solvent was administered (2 μl) as vehicle. These drugs were administered intracerebroventricularly at the end of scheduled feeding on Days 4 to 8.

2.10. Data Analysis

The data are presented as means ± standard error of the mean (SEM). Comparisons between two groups were performed using two-tailed Student's *t*-tests. One- or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to compare three or more groups. Differences were considered statistically significant at $p < 0.05$. All statistical analyses were performed using Prism 6 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Changes in body weight, food intake, and wheel activity

Food intake and body weight of the ABA ($n = 8$) and FR groups ($n = 7$) animals were

259 significantly decreased from Day 1 and Day 2, respectively, compared to the Normal (n
260 = 8) and Wheel groups (n = 8, Fig. 2A and B, $F_{27, 216} = 23.25$, $p < 0.0001$, $\eta^2 = 0.48$ in
261 food intake and $F_{27, 216} = 103.71$, $p < 0.0001$, $\eta^2 = 0.52$ in body weight; two-way ANOVA,).
262 Wheel activity of the ABA group significantly increased at Day 2; however, there were
263 no significant differences at other days during the experiments compared to the Wheel
264 group (Fig. 2C, $F_{14, 98} = 4.85$, $p < 0.0001$, $\eta^2 = 0.40$; two-way ANOVA).
265
266 *3.2. ABA induces the impairment of NOR behaviors*
267
268 There were no significant differences in the NOR index among all groups in Phase I
269 (Fig. 3B). NOR index in the ABA group (n = 5) was significantly lower than the Normal
270 group (n = 5), whereas the ABA group was not significantly different from the FR (n = 6)
271 and Wheel groups (n = 5) in Phase II (Fig. 3C, $F_{3, 17} = 4.546$, $p = 0.0163$, $\eta^2 = 0.45$; one-
272 way ANOVA). NOR index in ABA group was significantly lower than the Normal, FR,
273 and Wheel groups in Phase III (Fig. 3D, $F_{3, 17} = 9.686$, $p = 0.0006$, $\eta^2 = 0.54$; one-way
274 ANOVA). There were no significant differences between the Normal, FR, and Wheel
275 groups in all Phases.
276
277 *3.3. ABA increases the mRNA levels of NPY and AgRP in hypothalamus*

278

279 The mRNA levels of NPY and AgRP in the ABA group (n = 6) were significantly
280 higher than those in the Normal (n = 5), FR (n = 6), and Wheel groups (n = 6, Fig. 4,
281 NPY: $F_{3, 19}$ = 14.29, $p < 0.0001$, η^2 = 0.70, AgRP: $F_{3, 18}$ = 6.081, p = 0.0048, η^2 = 0.50;
282 one-way ANOVA). The mRNA levels of POMC in the Wheel group were significantly
283 higher than those in the ABA and FR groups (Fig. 4, $F_{3, 19}$ = 7.796, p = 0.0014, η^2 = 0.55;
284 one-way ANOVA). The mRNA levels of arginine vasopressin (AVP) in the ABA group
285 were significantly higher than those in the Normal group, and those in the Wheel group
286 were significantly higher than the Normal and FR groups (Fig. 4, $F_{3, 19}$ = 7.796, p = 0.0105,
287 η^2 = 0.51; one-way ANOVA). There were no significant differences among all groups for
288 the mRNA levels of cocaine- and CART, OXT, CRF, Ucn1, and BDNF (Fig. 4; one-way
289 ANOVA).

290

291 *3.4. ABA increases the number of c-Fos-positive cells in arcuate nucleus of hypothalamus,*
292 *which are NPY- or AgRP-positive cells*

293

294 The number of c-Fos-positive cells in the arcuate nucleus of the hypothalamus was
295 significantly higher in the ABA group (n = 7) than in the Normal (n = 6), FR (n = 6), and
296 Wheel groups (n = 6, Fig. 5A, $F_{3, 21}$ = 13.52, p = 0.1234, η^2 = 0.66; one-way ANOVA). c-

297 Fos-positive cells were also NPY- or AgRP-positive (Fig. 5B and C).

298

299 *3.5 Anti-NPY antiserum, anti-AgRP antibody, and Y5 receptor antagonist reverse the*
300 *impairment of NOR induced by ABA*

301

302 The icv administration of anti-NPY antiserum (n = 4), anti-AgRP antibody (n = 5),
303 and the Y5 receptor antagonist CGP71683 (n = 4) reversed the decrease in the NOR index
304 induced by ABA in Phase III (n = 4, n = 6, and n = 5, Fig. 6A to C, t(6) = 4.500, p =
305 0.0041, t(9) = 3.246, p = 0.0101, and t(7) = 3.406, p = 0.0114, η^2 = 0.77, 0.54, and 0.62),
306 whereas icv administration of the Y1 receptor antagonist BIBO 3304 trifluoroacetate (n
307 = 4) did not reverse the decrease in the NOR index induced by ABA in Phase III (n = 4,
308 Fig. 6D). There were no differences of NOR indices between mice with drug
309 administration and vehicle in Phase I (Supplementary fig. 1A-D). The NOR indexes in
310 Phase II of ABA mice with icv administration of anti-AgRP antibody and Y5 receptor
311 antagonist were significantly increased (Supplementary fig. 1B and C, t(9) = 2.851, p =
312 0.0191, η^2 = 0.47, and t(7) = 3.087, p = 0.0176, η^2 = 0.68). The icv administration of anti-
313 AgRP antibody and a Y1 receptor antagonist BIBO 3304 trifluoroacetate significantly
314 decreased the food intake and wheel activity (anti-AgRP antibody: $F_{8, 72}$ = 96.11, p <
315 0.0001, η^2 = 0.49 in food intake, and $F_{7, 63}$ = 13.37, p < 0.0001, η^2 = 0.23 in wheel activity.

316 Y1 receptor antagonist: $F_{8, 48} = 35.38$, $p < 0.0001$, $\eta^2 = 0.95$ in food intake and $F_{7, 42} =$
317 2.45, $p = 0.0334$, $\eta^2 = 0.18$ in wheel activity; two-way ANOVA), whereas icv
318 administration of anti-NPY antiserum and Y5 receptor antagonist did not alter the food
319 intake and wheel activity (Supplementary fig. 2).
320
321 **4. Discussion**
322 AN is a complex and serious motivated behavioral situation with high morbidity and
323 mortality, including suicide especially in adolescent women, leading to self-induced
324 weight loss, immoderate food intake restriction, and elevated physical activity (Guarda et
325 al., 2015). Although it is difficult to reproduce the negative motivation against eating food
326 in AN patients in animals, the ABA model is used as a model mimicking hunger and
327 starvation conditions in AN patients. ABA model rodents have unlimited access to
328 running wheels despite food restrictions, namely self-induced hyperactivity under hunger,
329 which causes body weight loss (Schalla and Stengel, 2019). Although only ABA rats, not
330 Normal, FR, and Wheel rats, exhibit no significant differences in terms of the time spent
331 interacting with familiar and a novel objects (Boersma et al., 2016), the mechanisms of
332 action remain unclear. Previous studies have shown that mRNA levels of AgRP and NPY
333 in the hypothalamus of ABA rats are higher (de Rijke et al., 2005). Elevated levels of
334 AgRP in plasma and NPY in cerebrospinal fluid have been observed in AN patients (Kaye,

1996; Moriya et al., 2006). Higher levels of AgRP and NPY were observed in the present study. These increases in orexigenic peptides such as AgRP and NPY might reflect the hunger situation in AN, in which the body may seek the food intake to dispel hypoalimentation. However, the negative motivation to eating (as AN patients would not like to eat) makes it hard to treat AN.

The effects of excess levels of orexigenic peptides in AN patients have been little known. Animal studies have demonstrated that the activation of AgRP neurons in the absence of food induces repetitive/stereotypic behaviors in the marble-burying test, and digging and grooming in home cages, which are related to obsessive behaviors (Dietrich et al., 2015). Furthermore, activation of AgRP neurons has been reported to reduce behavioral flexibility in modified Barn's maze tests, in which food rewards are not required, and diminish performance in the Y-maze test in mice (Zimmer et al., 2019). These results suggest that AgRP is connected with memory-related cognitive process. In addition, it has been reported that these AgRP functions are canceled by an NPY 5 receptor antagonist (Dietrich et al., 2015; Zimmer et al., 2019).

NPY is a 36 amino acid peptide found in the central and peripheral nervous systems (Beck, 2006). The arcuate nucleus of the hypothalamus is an NPY-abundant area, in which NPY neurons co-synthesize another orexigenic peptide, AgRP (Hahn et al., 1998). AgRP neurons, as well as NPY, are well-known to promote food intake, and activation of

AgRP neurons induces hunger-associated behaviors such as consumption of food, working at operant tasks for food, and attending to food cues (Andermann and Lowell, 2017).

NPY/AgRP neurons in the arcuate nucleus project to the paraventricular nucleus, dorsomedial nucleus, lateral hypothalamus, and ventromedial nucleus (Beck, 2006). Central NPY neurons participate in various biological actions, including cardiovascular regulation, cognition, memory, and appetite, which are mediated by six receptors, and Y1, Y2, Y4, Y5, and Y6 have been cloned from humans and mice (Blomqvist and Herzog, 1997). NPY secreted from NPY/AgRP neurons in the arcuate nucleus binds to the Y1 and Y2 receptors on POMC/CART neurons, leading to the inhibition of their firing activities (Mercer et al., 2011). In addition, NPY binds Y5 receptors on POMC/CART neurons and inhibits release of melanocortin from POMC/CART neurons, in which melanocortin can inhibit NPY/AgRP neurons through melanocortin 3 receptors (MC3Rs) and MC4Rs on NPY/AgRP neurons (Mercer et al., 2011). In the present study, we found that ABA mice induced the activation of AgRP and NPY neurons in the arcuate nucleus and defective cognitive behaviors in the NOR task. Furthermore, these impairments to cognitive behaviors were reversed by the central administration of anti-AgRP antibody, anti-NPY antiserum, and Y5 receptor antagonist. The present study is the first report demonstrating that AgRP, NPY, and Y5 receptors are related to defects in cognitive behaviors induced

by ABA.

In the present study repeated central administration of a Y1 receptor antagonist, but not the Y5 receptor antagonist, decreased food intake in ABA mice (Supplementary fig. 2D). A double blocker for both Y1 and Y5 is required as an anti-obesity medication (MacNeil, 2007); however, the function of Y1 receptor in terms of food intake might be predominant in starvation conditions such as AN. The repeated central administration of anti-AgRP antibody decreased food intake in ABA mice, with an accompanying decrease in wheel activity; however, the body weights of these mice were not altered (Supplementary fig. 2B). Wheel running activity in ABA models indicates self-motivated behavior for rodents in absence of any rewards (Sherwin, 1998). AgRP and Y5 receptors might contribute to voluntary exercise in ABA mice. The present study has shown that the mRNA level of AVP was higher in ABA and Wheel mice than in Normal and FR mice. Both ABA and Wheel mice had free access to the running wheel. AVP in the suprachiasmatic nucleus of the hypothalamus is reported to be associated with voluntary behaviors (Cormier et al., 2015). Cormier et al. demonstrated that microinjection of AVP into the suprachiasmatic nucleus reduces wheel running activity in hamsters (Cormier et al., 2015). However, plasma AVP levels in humans have been reported to increase after prolonged endurance exercise, such as a 56-km ultramarathon (Hew-Butler et al., 2008). The higher mRNA levels of AVP in ABA and Wheel mice in the present study might have

been prompted by exercise. On the other hand, mRNA levels of POMC in ABA and FR mice were lower than those in Wheel mice. Chronic food restriction has been reported to decrease mRNA levels of POMC in the arcuate nucleus in rats (Kim et al., 1996). Continuous voluntary exercise and food restriction could induce such alterations to AVP and POMC levels in the ABA model.

This study had certain limitations. We indicated the inhibitory effects of central administered antagonists; however, we did not perform gene silencing to specifically remove the target mRNA and use knockout mice in the present study; this should be addressed in the future experiments. The ABA model recreate the conditions of AN, such as reduced food intake and hyperactivity. Although, ABA shares various similarities with AN in humans (Schalla and Stengel, 2019), our results have to be reconfirmed by clinical studies in the future.

In conclusion, the present study has shown that ABA induces defective recognition behaviors in NOR tasks and activation of AgRP and NPY neurons in the arcuate nucleus. These defects were reversed by the central administration of anti-NPY antiserum, anti-AgRP antibody, and Y5 receptor antagonist. Overall, AgRP and NPY signaling, including the Y5 receptor, might represent valuable and novel targets for the treatment of cognitive behavior impairments in AN, which has become increasingly prevalent globally.

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Author Contribution

N. T, R., K. A. and A. A. conceived and designed the study.

N. T, R., K. A., H. I., H. S., H. T., T. S. K. and K. C. C. performed the experiments.

N. T. R. and K. A. performed the data analysis.

N. T. R., K. A., H. A. and A. A. wrote the paper.

N. T. R., K. A., H. I., H. S., H. T., T. S. K., K. C. C., H. A., A. I. and A. A. reviewed draft of the paper.

Competing Interests

The authors have declared that no conflicts of interest exist.

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612 Figure legends

613 Fig. 1. Timeline for ABA procedures. Mice were divided into four groups: ad libitum
614 feeding (Normal), free access to running wheel with scheduled feeding (ABA), scheduled
615 feeding (food restriction, FR), and free access to running wheel with ad libitum feeding
616 (Wheel) groups

617

618 Fig. 2. Time courses of food intake, body weight, and wheel activity. Food intake (A),
619 body weight (B), and wheel activity (C) were measured during the ABA procedure (n =
620 7 - 8). The values are presented as means ± SEM. Differences were considered significant
621 at ** $p < 0.01$ compared with the Normal group and $^{\delta}p < 0.05$ and $^{\delta\delta}p < 0.01$ compared
622 with the Wheel group.

623

624 Fig. 3. NOR indices. (A) Schema of the NOR task. NOR indices of Phase I (B), II (C),
625 and III (D) were calculated 6 h after the end of scheduled feeding on Day 8 (n = 5 - 6).
626 Values are presented as means ± SEM. Differences were considered significant at ** $p <$
627 0.01.

628

629 Fig. 4. mRNA levels of neuropeptides in the hypothalamus of ABA mice. mRNA levels
630 of neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC),

631 cocaine- and amphetamine-regulated transcript (CART), arginine vasopressin (AVP),
632 oxytocin (OXT), corticotropin-releasing factor (CRF), urocortin1 (Ucn1), and brain-
633 derived neurotrophic factor (BDNF) were measured in hypothalamus tissues obtained
634 from mice 6 h after the end of scheduled feeding on Day 8 (n = 5 - 6). Values are presented
635 as means ± SEM. Differences were considered significant at * $p < 0.05$, or ** $p < 0.01$.
636
637 Fig. 5. Immunostaining for c-Fos, AgRP or NPY. Brain tissues were isolated and fixed
638 with 4% PFA and 0.5% GA in 0.1 M PB 6 h after the end of scheduled feeding on Day 8
639 (n = 6 - 7). Coronal sections of the arcuate nucleus were stained with an anti-mouse c-Fos
640 and/or anti-NPY antiserum or anti-AgRP antibody. (A) Representative images of c-Fos-
641 positive cells in the arcuate nucleus using immunofluorescent staining (white arrowheads).
642 The number of c-Fos-positive cells was counted. Values are presented as means ± SEM.
643 Differences were considered significant at ** $p < 0.01$. (B) Representative images of c-
644 Fos- and NPY-, c-Fos- and DAPI-, or NPY- and DAPI-positive cells (white arrow heads)
645 in the arcuate nucleus cells. (C) Representative images of c-Fos- and AgRP-, c-Fos- and
646 DAPI-, or AgRP- and DAPI-positive cells (white arrow heads) in the arcuate nucleus cells.
647
648 Fig. 6. NOR indices in Phase III of ABA mice with central administration of anti-NPY
649 antiserum, anti-AgRP antibody, Y5 receptor antagonist, and Y1 receptor antagonist. Anti-

650 NPY antiserum (2 µl/mouse), anti-AgRP antibody (0.1 µg/mouse), Y5 receptor antagonist
651 CPG 71683A hydrochloride (15 nmol/mice), Y1 receptor antagonist BIBO 3304
652 trifluoroacetate (30 nmol/mice), and each vehicle (2 µl/mouse) were administered
653 intracerebroventricularly at the end of scheduled feeding on Days 4 to 8 ($n = 4 - 6$). Values
654 are presented as means ± SEM. Differences were considered significant at * $p < 0.05$ or
655 ** $p < 0.01$.

Figure 1

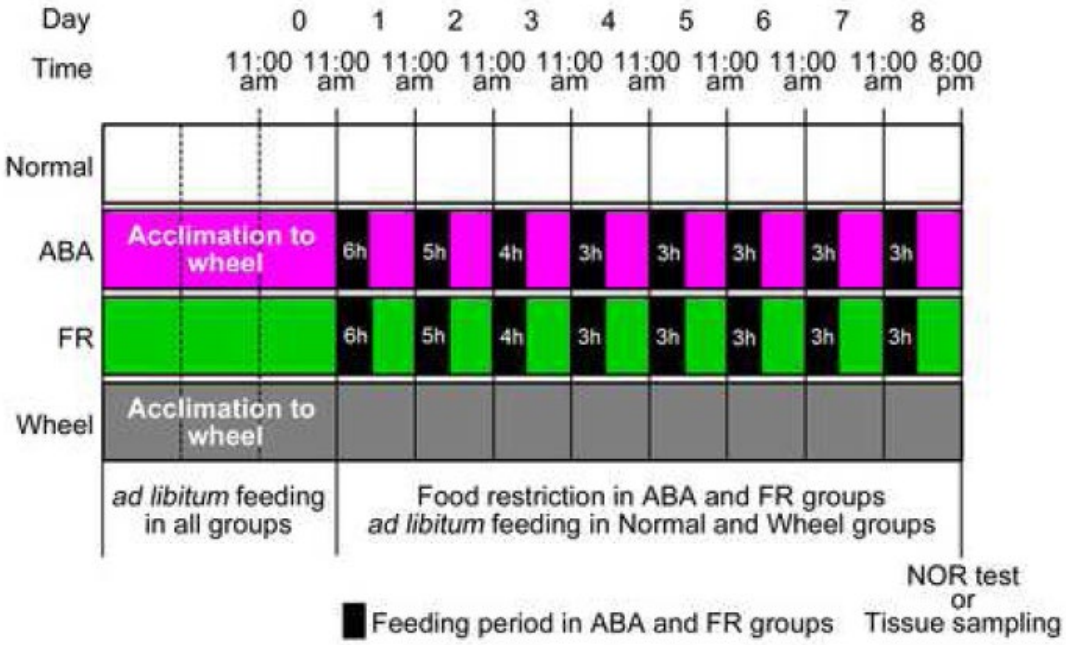
Day
0
1
2
3
4
5
6
7
8
Time
11:00 am
11:00 am
11:00 am
11:00 am
11:00 am
11:00 am
11:00 am
11:00 am
11:00 am
8:00 pm
Normal
ABA
Acclimation to wheel
6h
5h
4h
3h
3h
3h
3h
3h
FR
6h
5h
4h
3h
3h
3h
3h
3h
Wheel
Acclimation to wheel
ad libitum feeding in all groups
Food restriction in ABA and FR groups
ad libitum feeding in Normal and Wheel groups
NOR test
or
Tissue sampling
Feeding period in ABA and FR groups

Figure 2

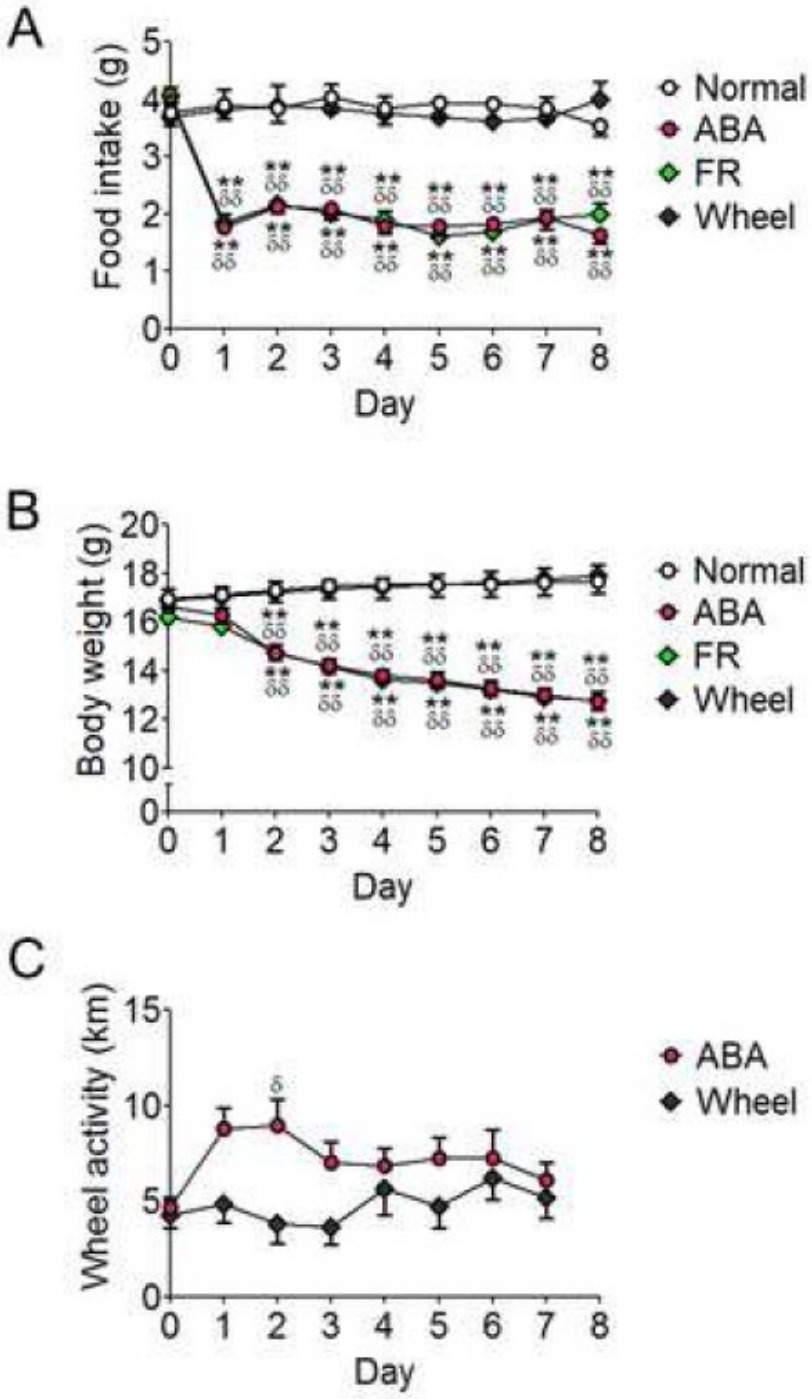
A
Food intake (g)
Day
Normal
ABA
FR
Wheel
B
Body weight (g)
Day
Normal
ABA
FR
Wheel
C
Wheel activity (km)
Day
ABA
Wheel

Figure 3

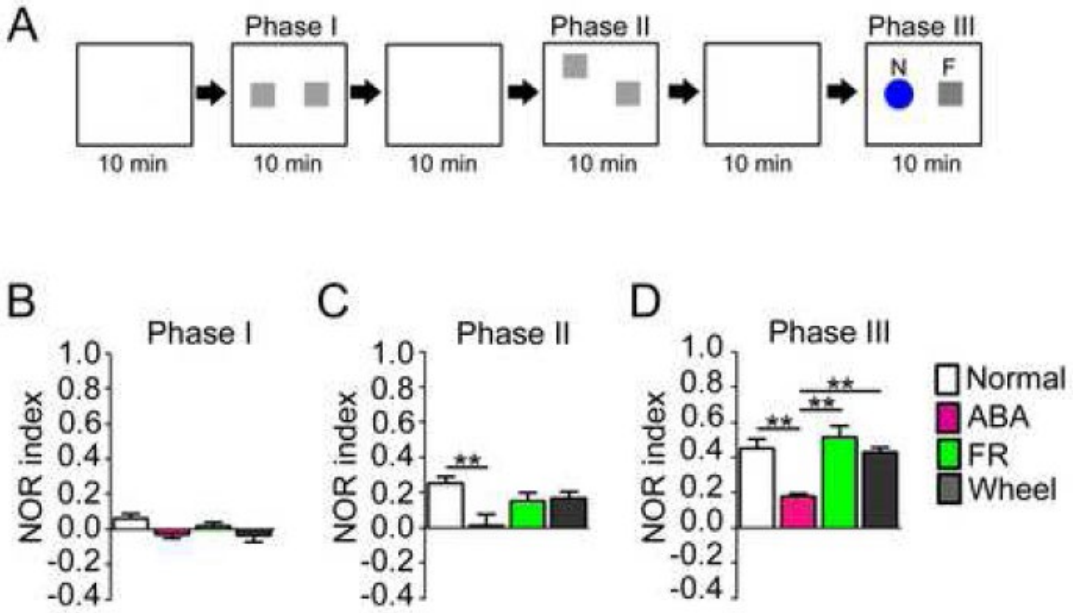
A
Phase I
Phase II
Phase III
N
F
10 min
10 min
10 min
10 min
10 min
10 min
B
Phase I
C
Phase II
D
Phase III
NOR index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
**
**
**
Normal
ABA
FR
Wheel

Figure 4

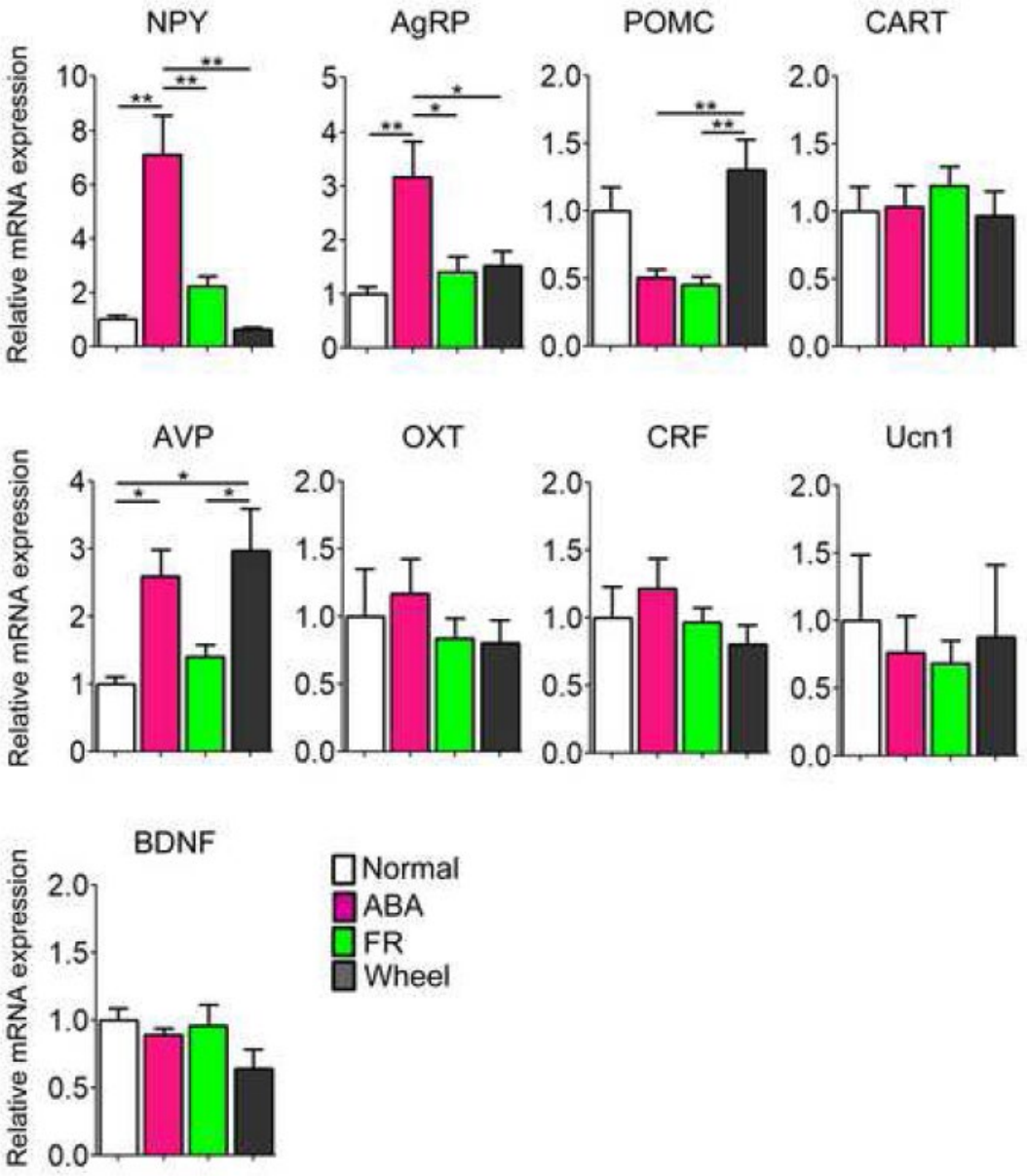
NPY
AgRP
POMC
CART
AVP
OXT
CRF
Ucn1
BDNF
Relative mRNA expression
Normal
ABA
FR
Wheel

Figure 5

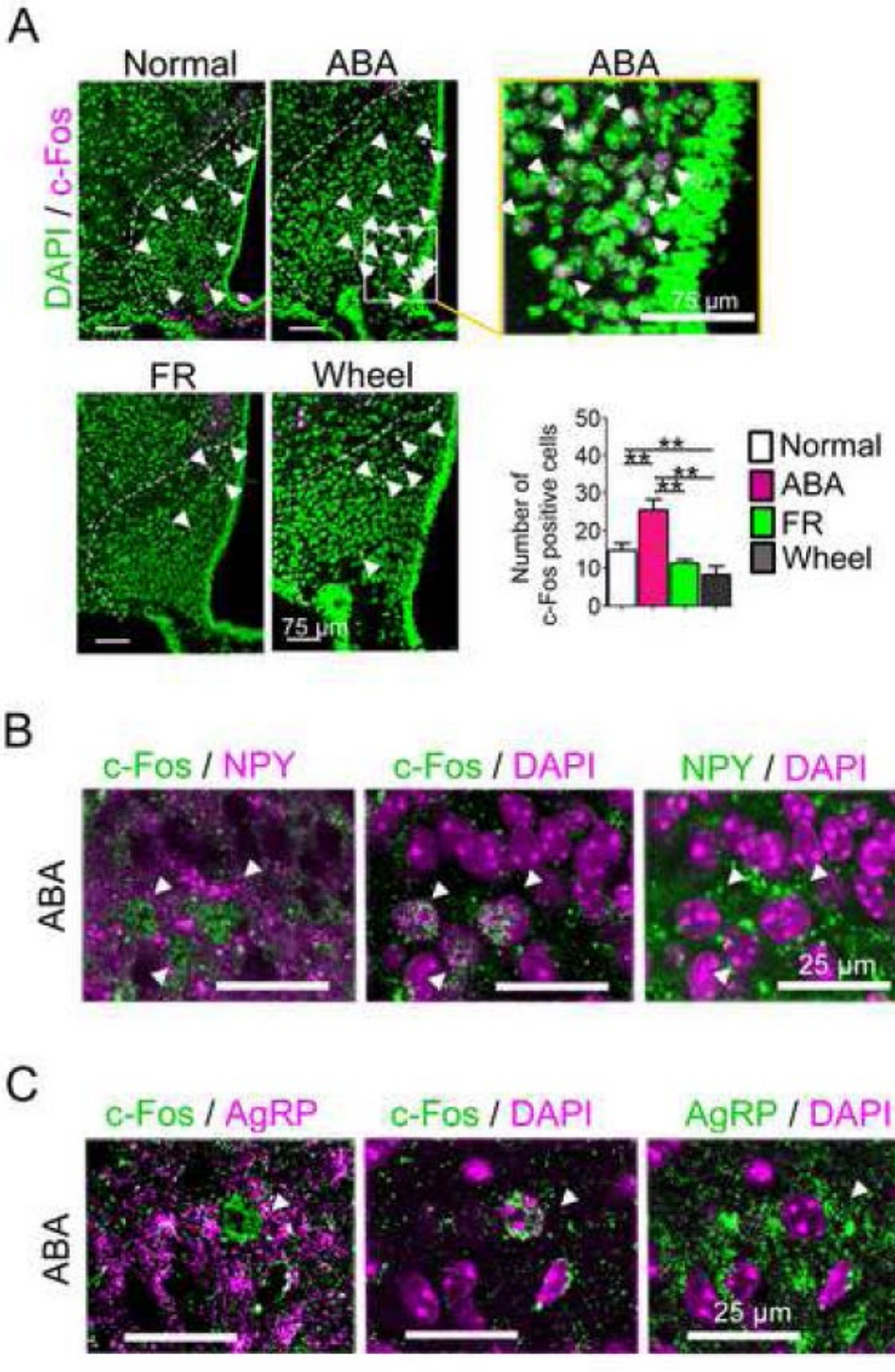
A
Normal
ABA
ABA
DAPI / c-Fos
75 μm
FR
Wheel
75 μm
Number of c-Fos positive cells
50
40
30
20
10
0
**
**
**
Normal
ABA
FR
Wheel
B
c-Fos / NPY
c-Fos / DAPI
NPY / DAPI
ABA
25 μm
C
c-Fos / AgRP
c-Fos / DAPI
AgRP / DAPI
ABA
25 μm

Figure 6

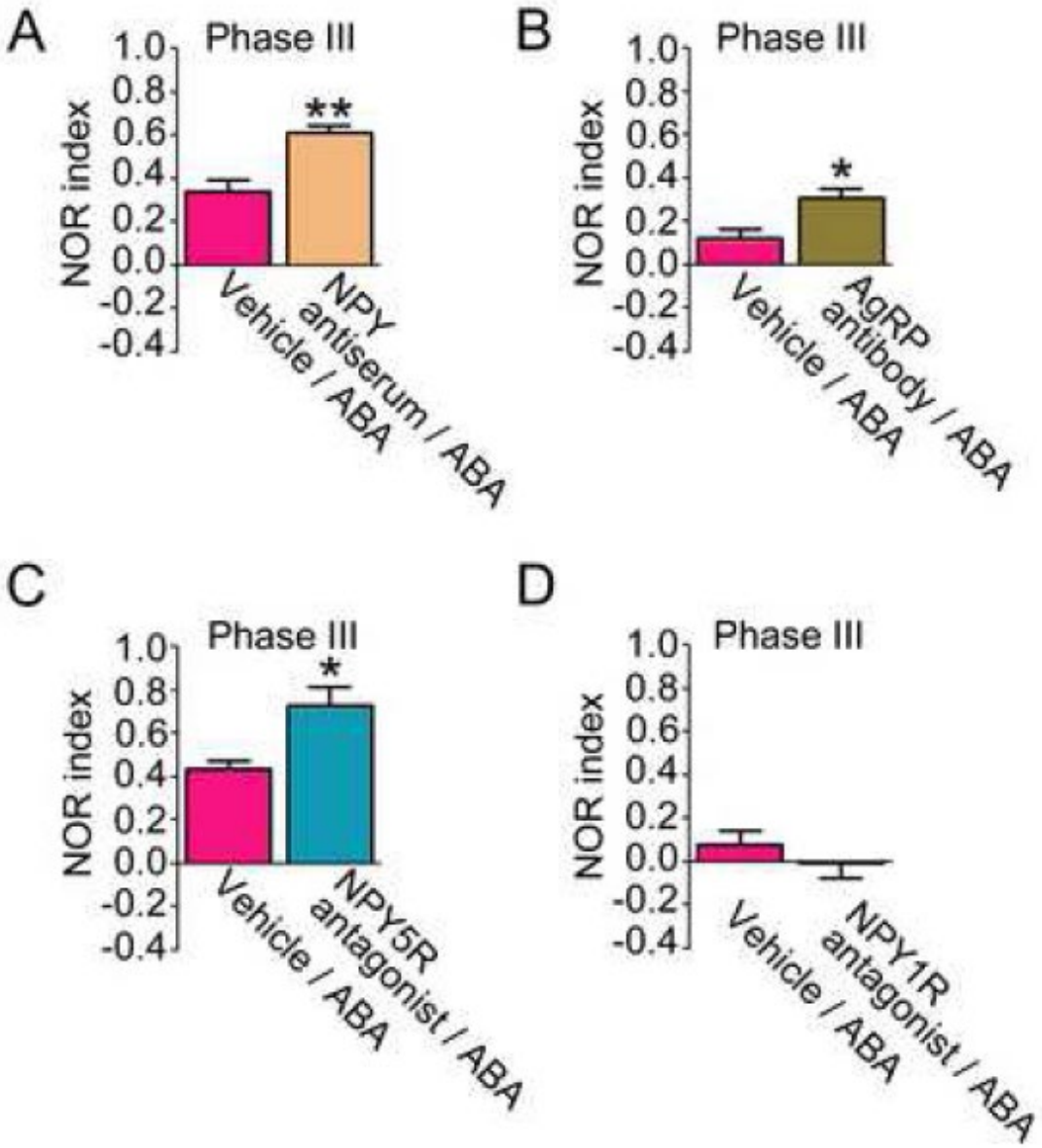
A
Phase III
NOR index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
**
Vehicle / ABA
NPY antiserum / ABA
B
Phase III
NOR index
*
Vehicle / ABA
AgRP antibody / ABA
C
Phase III
NOR index
*
Vehicle / ABA
NPY5R antagonist / ABA
D
Phase III
NOR index
Vehicle / ABA
NPY1R antagonist / ABA

Figure S1

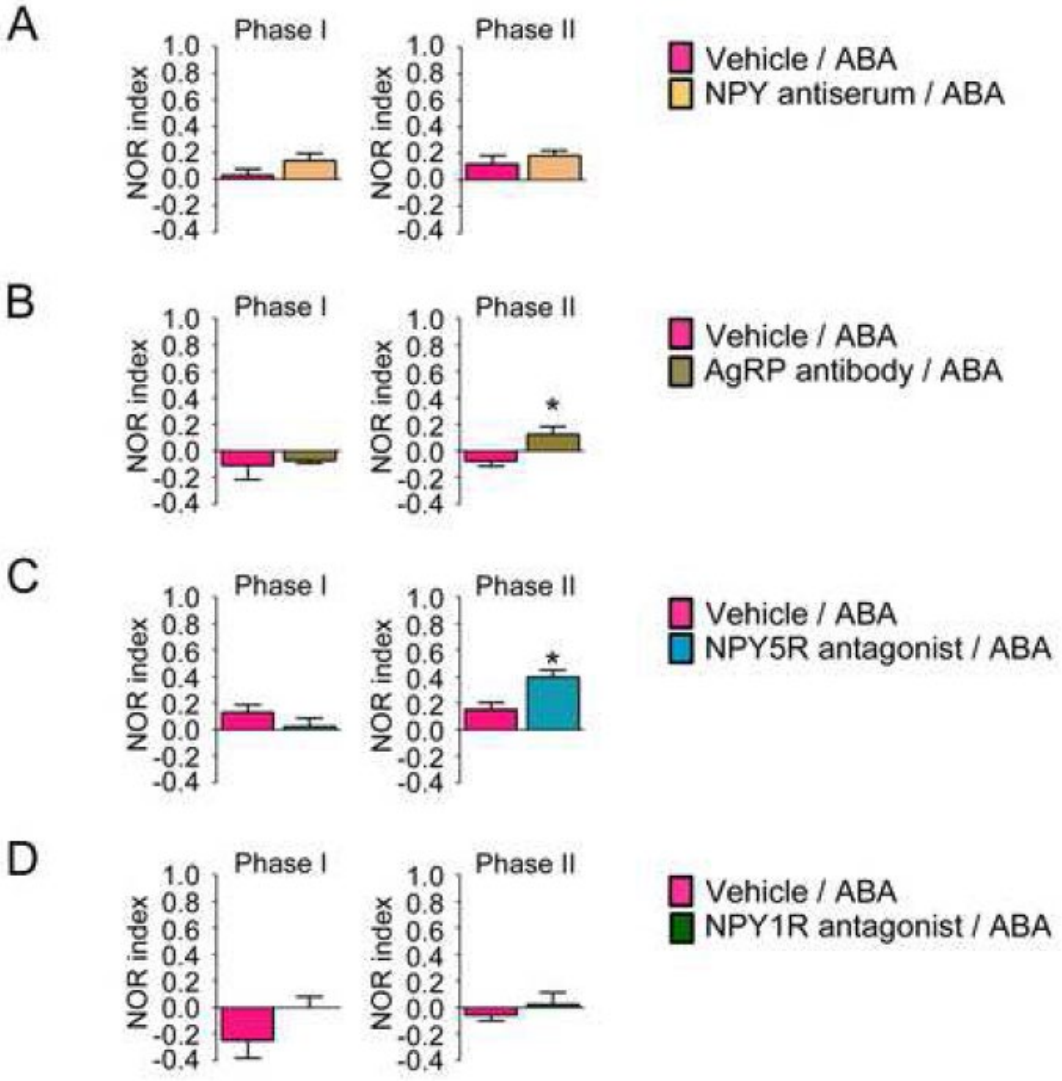
A
Phase I
Phase II
NOR index
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
Vehicle / ABA
NPY antiserum / ABA
B
Phase I
Phase II
NOR index
*
Vehicle / ABA
AgRP antibody / ABA
C
Phase I
Phase II
NOR index
*
Vehicle / ABA
NPY5R antagonist / ABA
D
Phase I
Phase II
NOR index
Vehicle / ABA
NPY1R antagonist / ABA

Figure S2

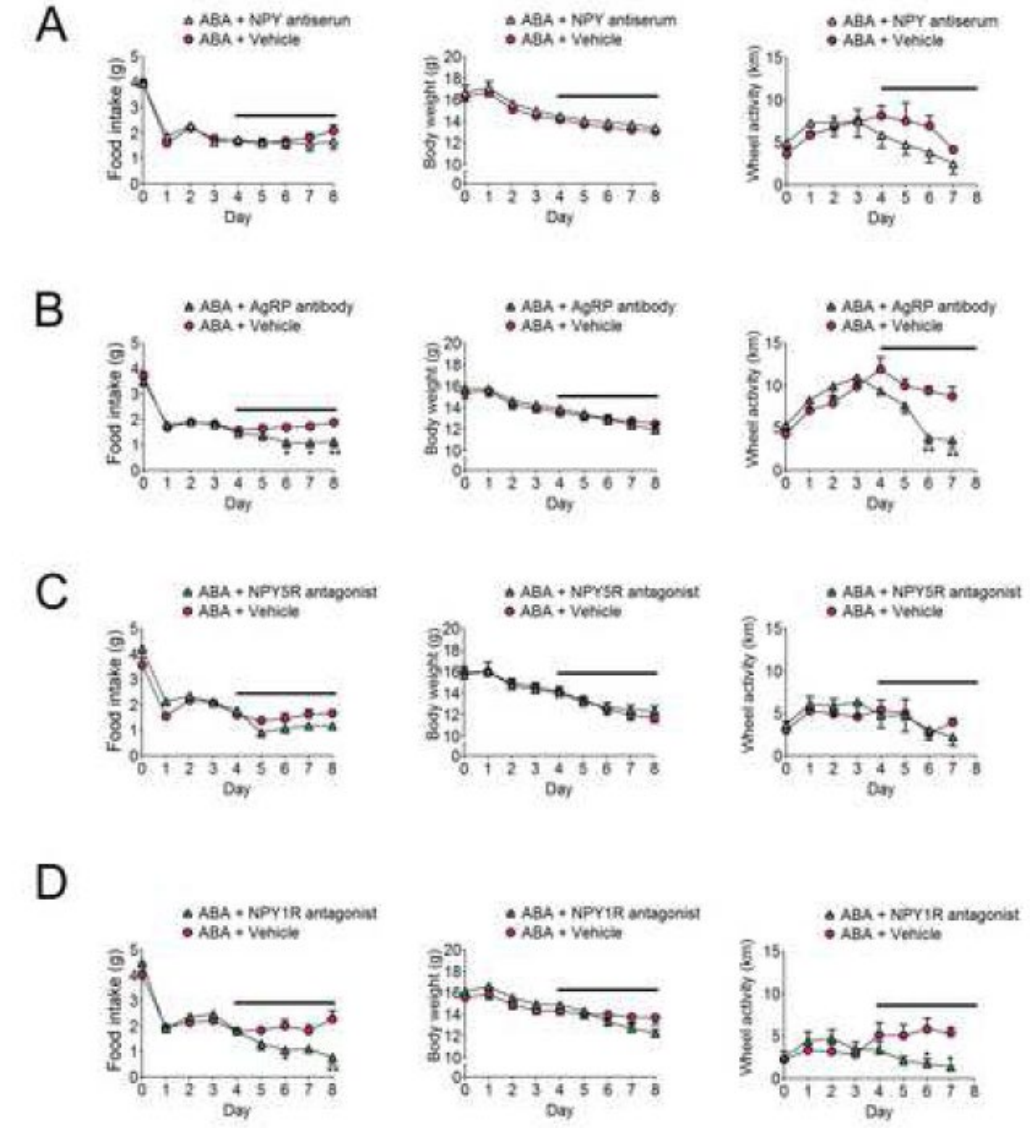
A
ABA + NPY antiserum
ABA + Vehicle
Food intake (g)
Body weight (g)
Wheel activity (km)
Day
B
ABA + AgRP antibody
ABA + Vehicle
C
ABA + NPY5R antagonist
ABA + Vehicle
D
ABA + NPY1R antagonist
ABA + Vehicle

Table 1.

		Forward	Reverse
neuropeptide Y (NPY)	NM_012614	CGCTCTGCGACACTACATCAAT	TGAGATGAGGGTGGAAACTTGG
agouti-related peptide (AgRP)	NM_033650	GCGGCCTGAAAGCTTTGTC	TCCTGTAGCCAGGGCATGAG
proopiomelanocortin (POMC)	NM_012625	AGAGGCCACTGAACATCTTTGTC	ATCTATGGAGGTCTGAAGCAGGAG
cocaine- and amphetamine-regulated transcript (CART)	NM_017110	TCAAGAGTAAACGCATTCCGATCTA	TCCTCACTGCGCACTGCTCT
arginine vasopressin (AVP)	NM_016992	ACCTCTGCCTGCTACTTCCAGA	ACACTGTCTCAGCTCCATGTCG
oxytocin (OXT)	NM_012996	TGCCAGGAGGAGAACTACCTG	TATTCCCAGAAAGTGGGCTCAG
corticotropin-releasing factor (CRF)	NM_031019	CAGAGCCCAAGTACGTTGAGAG	GCTCTCTTCTCCTCCCTTGGTA
urocortin1 (Ucn1)	NM_021290	CATCTTGCACTGGGCAGACACT	AAGCTGTGCCAAGAGCAGCAAC
brain-derived neurotrophic factor (BDNF)	NM_001048139	TCAAGTTGGAAGCCTGAATGAATG	CTGATGCTCAGGAACCCAGGA
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_017008	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG