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Chronic intracerebroventricular administration of anti-neuropeptide Y antibody stimulates starvation-induced feeding via compensatory responses in the hypothalamus

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***Abbreviations*:** NPY, neuropeptide Y; NPY-ab, neutralizing NPY antibodies; ICV, intracerebroventricular; AGRP, agouti-related protein; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; LHA, lateral hypothalamic area; α-MSH, α -melanocyto-stimulating hormone; MC4Rs, melanocortin-4 receptors; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; TPBS, Triton X-100 in PBS.

Abstract

To investigate how compensatory responses develop after the onset of inhibition of NPY signaling, we examined the effect of continuous intracerebroventricular (ICV) injection of neutralizing NPY antibodies (NPY-ab) on daily and fast-induced food intake in mice. A single ICV injection of NPY-ab reduced food intake in fasted mice. In contrast to a single injection, continuous ICV injection of NPY-ab for 13 d increased fast-induced food intake, although daily food intake was unaffected by continuous administration of NPY-ab. Immunohistochemistry indicated that the expression of NPY protein increases in the arcuate nucleus, lateral hypothalamic area, and paraventricular nucleus 7 d after onset of continuous NPY-ab infusion and remains at an elevated level, whereas the expression of the NPY Y1 receptor transiently increases in the same areas for 3 d and then gradually decreases. Similar results were obtained for the expression of NPY and NPY Y1 receptor mRNA. The mRNA level of agouti-related protein, another orexigenic neuropeptide, also increased in parallel with NPY, whereas that of pro-opiomelanocortin did not change over the 13 d of the NPY-ab administration. These results suggest that chronic central inhibition of NPY immediately activates orexigenic signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes the regulation of feeding and energy expenditure to maintain energy homeostasis. On the other hand, in mice that have acquired this compensation, fast-induced food intake further increases even after the energy deficit is corrected because of the dominant orexigenic signal.

Section: Regulatory Systems.

Keywords: NPY, feeding, food intake, compensation, hypothalamus, central NYP deficiency.

1. Introduction

The arcuate nucleus (Arc) of the hypothalamus, adjacent to the third ventricle, receives central and peripheral signals related to energy stores and contains at least two distinct populations of neurons involved in the regulation of feeding and body weight (Clark et al., 1984; Woods et al., 1998; Marsh et al., 1998). Orexigenic neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons in the Arc are first-order neurons involved in the hypothalamic response to circulating satiety and hunger signals, including glucose, leptin, insulin, ghrelin, and peptide YY (Schwartz et al., 2000; Schwarz and Porte, 2005). Populations of these first-order neurons in the Arc are regulated by leptin and insulin and project to other hypothalamic areas, including the paraventricular nucleus (PVN), perifornical area, and the lateral hypothalamic area (LHA) (Elmquist et al., 1998, 1999), which contain second-order neuropeptide neurons involved in the regulation of food intake and energy homeostasis (Schwartz et al., 2000).

Activation of POMC neurons reduces food intake via the release α-melanocyte-stimulating hormone (α-MSH), a degradation product of POMC, which activates melanocortin-4 receptors (MC4Rs) (Cone et al., 1996). NPY/AGRP neurons have direct inhibitory inputs onto POMC cells (Cowley et al., 2001) and also antagonize the action of α-MSH on MC4R-bearing neurons via the release of AGRP, a natural antagonist of α-MSH receptor (Ollmann et al., 1997). Moreover, both POMC and NPY/AGRP neurons express autoreceptors for some of their respective neuropeptides (Cowley et al., 2001). Thus, these two types of neurons in the ARC exert an inhibitory factor on each other.

Although intracerebroventricular (ICV) injection of NPY stimulates robust feeding (Stanley et al., 1986), surprisingly, genetic disruption of NPY signaling was found to have little effect on feeding and body weight (Marsh et al., 1998; Erickson et al., 1996; Kushi et al.,

1998). It was also reported that neonatal ablation of NPY/AgRP neurons had minimal effects on feeding, whereas that their ablation in adults caused rapid starvation (Luquet et al., 2005). The chronic absence of NPY, therefore, may elicit compensatory mechanisms that mask the effect of its deficiency in NPY-null mice. In this study, to investigate how the compensatory responses develop after the onset of inhibition of NPY signaling, we analyzed the change in expression of neuropeptides in first-order hypothalamic neurons and daily and fast-induced food intake in mice during the continuous ICV administration of NPY-neutralizing antibodies (NPY-ab). NPY We found that compensatory responses to the inhibition of NPY signaling develop in first-order hypothalamic neurons via activation of orexigenic signaling rather than inhibition of anorexigenic signaling. In this way, the regulation of food intake and energy expenditure is normalized in the absence of NPY.

2. Results

A single ICV injection of NPY-ab inhibits fast-induced but not non-fast-induced food intake

Stanley et al. (1992) reported that a single ICV injection of NPY antisera suppresses the eating induced by mild food deprivation in rat. We also examined the effect of a single ICV injection of NPY-ab on fast-induced food intake using varying NPY-ab dilutions. The increase in food intake caused by a 24-h fast was significantly inhibited by a single ICV injection of NPY-ab diluted 1:1500 (52% inhibition), 1:4000 (48% inhibition), and 1:8000 (33% inhibition) but not 1:12000 (Fig.1A). Thus, immunoneutralization of endogenous NPY by NPY-ab markedly inhibits fast-induced food intake in a dose-dependent fashion and the inhibitory effect by a 1:4000 NPY-ab dilution seems to be sub-maximal. Moreover, significant inhibition of fast-induced food intake was observed first 2 h after NPY-ab

(1:4000) injection (Fig.1B). On the other hand, mice that received a single ICV injection of NPY (0.5 nmol) showed a significant increase in food intake compared to saline-treated control mice. This NPY-induced increase in food intake was blocked by pretreatment with NPY-ab (1:4000), but there was no significant difference in *ad libitum* food intake for up to 4 h following the injection (Fig. 1C). These results suggest that NPY is involved in fast-induced hyperphagia but has less of a role in normal spontaneous food intake.

Effect of continuous ICV infusion of NPY-ab on food intake and body weight

We examined the effect of continuous ICV infusion of NPY-ab on daily food intake and body weight. Neither daily food intake nor body weight in NPY-ab-infused mice were significantly different from those in saline-infused control mice (Figs. 2A and B). We next examined the effect of chronic NPY-ab infusion on fasting-induced food intake. Food intake was measured for 3 h after a 24-h fast. In contrast to a single injection of NPY-ab, fasting-induced food intake was significantly increased in mice infused for 13 d with NPY-ab compared to mice infused with saline (Fig. 3). The different effects of a single and chronic ICV injection of NPY-ab on fasting-induced food intake may be due to compensatory mechanisms that are engaged upon the chronic inhibition of NPY signaling. On the other hand, this further stimulation of fasting-induced food intake in chronic NPY-ab-infused mice was significantly blocked by a single peritoneal injection of leptin (Fig. 3). Furthermore, 90 min after refeeding, the amount of food intake in chronic NPY-ab-infused mice was much less after treatment with leptin than with saline. This may be due to an increase in plasma leptin to nearly normal levels because fasting lowers the plasma leptin concentration.

Analysis of mRNA expression levels for NPY, NPY Y1 receptor, AGRP, and PONC during continuous infusion of NPY-ab

Seven and 13 d after the onset of continuous NPY-ab infusion, the level of Arc NPY mRNA was higher in NPY-ab infused mice than control mice (Fig. 4A). The mRNA level for AGRP, another orexigenic neuropeptide in the Arc, was also higher in NPY-ab-infused mice than in control mice after 13 d of infusion with NPY-ab (Fig. 4B). In contrast, the mRNA level of Arc POMC, a precursor of melanocortins such as α-MSH that promotes a negative energy balance, did not change during the 13 d of administration of NPY-ab (Fig. 4C). The level of NPY Y1 receptor mRNA showed a different pattern: its level transiently increased in the Arc and PVN and then decreased over time (Fig. 5A and B). Similar results, although not statistically significant, were obtained for the mRNA levels of the LHA NPY Y1 receptor (Fig. 5C).

Changes in NPY and NPY Y1 receptor protein levels during continuous infusion of NPY-ab

We used immunohistochemistry to assess how NPY and NPY Y1 receptor protein levels are affected by continuous infusion by NPY-ab. The level of NPY protein was significantly higher in the Arc at 7 d and 13 d, and in both the LHA and PVN at 13 d after onset of continuous NPY-ab infusion, whereas the level of NPY Y1 receptor protein transiently increased in the same areas after 3 d and then gradually decreased (Fig. 6A and B). Interestingly, the number of NPY-positive cells in the Arc increased, but the number of NPY fibers was much lower during the first 3 d after the onset of NPY-ab infusion (Fig. 7). In addition, some NPY-positive neuronal cell bodies and fibers in the Arc were also positive for NPY Y1 receptor protein (Fig. 7).

3. Discussion

The results of the present study demonstrate that chronic central inhibition of NPY immediately activates orexigenic signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes feeding and energy expenditure to maintain energy homeostasis. Enhancement of fasting-induced hyperphagia by chronic administration of NPY-ab may therefore be due to activation of a dominant orexigenic signal after acquisition of this compensatory mechanism.

Food intake by the mice was significantly increased by a single ICV injection of NPY compared to injection of saline. Although this increase in food intake by NPY was prevented by pretreatment with NPY-ab, a single ICV injection of NPY-ab did not affect *ad libitum* food intake in non-fasted mice. This suggests that NPY does not play a positive role in spontaneous food intake in mice. On the other hand, a single ICV injection of NPY-ab after a 24-h fast significantly inhibited the fasting-induced increase in food intake. Therefore, it appears that NPY participates in fast-induced hyperphagia but much less to normal spontaneous food intake.

NPY gene expression and secretion of NPY in the hypothalamus are increased during the depletion of body fat stores (Kalra et al., 1991; White and Kershaw, 1990) in conjunction with reduced leptin/insulin signaling to the brain (Wilding et al., 1993). Moreover, leptin inhibits Arc NPY gene expression (Schwartz et al., 1996; Stephens et al., 1995), and the genetic knockout of NPY reduces hyperphagia and obesity in ob/ob mice (Erickson et al., 1996), indicating that the full response to leptin deficiency requires NPY signaling. The hyperphagic response in insulin-deficient diabetes is accompanied by increased hypothalamic synthesis and release of NPY (Williams et al., 1989), and these responses are blocked by either systemic administration or direct injection of insulin into the brain (Sipols et al., 1995).

Therefore, NPY may not have an important role in spontaneous feeding responses when leptin or insulin levels are normal.

We also examined the effect of continuous ICV infusion of NPY-ab on daily food intake and body weight. Both daily food intake and body weight in NPY-ab-infused mice were not significantly different from those in saline-infused control mice. We further examined the effect of chronic NPY-ab infusion on fasting-induced food intake. In contrast to the effects of a single NPY-ab injection, fasting-induced food intake was significantly increased in the mice infused for 13 d with NPY-ab compared to mice treated with saline. This further stimulation of fasting-induced food intake in chronic NPY-ab-infused mice was partially blocked by a single peritoneal injection of leptin. The chronic inhibition of NYP signaling in NPY-ab-infused mice might induce mechanisms to compensate for the decreased NPY signaling, and low leptin and/or insulin levels induced by fasting might therefore result in the activation of anabolic neural pathways, enhancing hyperphagia.

Orexigenic NPY and AGRP are colocalized in Arc neurons (Hahn et al., 1998), and anorexigenic POMC also exist in a distinct, but adjacent, subset of Arc neurons (Elias et al., 1998). A majority of NPY/AGRP and POMC neurons express leptin receptors (Cheung et al., 1997; Baskin et al., 1999), and both types of neurons are regulated by leptin but in an opposing manner: NPY/AGRP neurons are inhibited by leptin and consequently are activated when leptin levels are low (Schwartz et al., 1996; Hahn et al., 1998), whereas POMC neurons are activated by leptin, and are therefore inhibited when leptin levels are low (Schwarz et al., 1996; Thornton et al., 1997). Furthermore, there is a high concentration of insulin receptors in the Arc (Baskin et al., 1988), and a deficiency of insulin seems to activate NPY/AGRP neurons (Williams et al., 1989; Sipols et al., 1995). Compensatory mechanisms to decrease NPY signaling caused by chronic NPY-ab administration seem to occur in the first-order

neurons in the Arc, orexigenic NPY/AGRP neurons, and anorexigenic POMC neurons because they are the neurons that respond to the circulating satiety and hunger signals, including glucose, leptin, and insulin (Schwartz et al., 2000).

To examine this hypothesis, we measured mRNA levels for NPY, NPY Y1 receptor, AGRP, and PONC during continuous infusion of NPY-ab. The levels of Arc NPY and AGRP mRNA increased in a time-dependent manner, and their levels were significantly different between saline- and NPY-ab-infused mice 7 and 13 d after the onset of the NPY-ab infusion, respectively. In contrast, the level of Arc POMC mRNA did not change during 13 d of NPY-Ab infusion. The level of NPY Y1 receptor mRNA showed a different pattern: it transiently increased in the Arc and PVN and then gradually decreased during the infusion of NPY-ab. Although not statistically significant, similar results were obtained for the level of LHA NPY Y1 receptor mRNA. These results suggest that the first compensatory response to normalize the NPY-ab-induced inhibition of NPY signaling is a transient increase in the mRNA level for the NPY Y1 receptor. Thereafter, the mRNA level for the NPY Y1 receptor increases but recovers to the basal level within 7 d. Finally, the levels of both NPY and AGRP mRNAs increase. Such compensatory responses were also observed by immunohistochemical analysis for NPY and the NPY Y1 receptor. On the other hand, although anorexigenic POMC neurons are first-order neurons, the mRNA level for POMC in the Arc did not change over the 13 d of infusion with the NPY-ab. These results suggest that chronic central inhibition of NPY immediately activates orexigenic NPY signaling without affecting anoexigenic POMC signaling in first-order hypothalamic neurons and that this compensatory mechanism normalizes feeding and energy expenditure to maintain energy homeostasis. On the other hand, when mice that have acquired this compensation are

compelled to fast, fast-induced food intake is further increased even after the energy deficit is corrected because of the dominant orexigenic signal.

MC4R antagonism leads to hyperphagia, reduced energy expenditure and ultimately obesity (Lu et al., 1994). Thus, it was suggested that α-MSH acts tonically to limit food intake. In the present study, the mRNA level of AGRP, which antagonizes the action of α-MSH on MC4Rs, increased in the Arc after chronic central inhibition of NPY. Therefore, the compensatory change in AGRP expression may lead to not only suppression of anorexigenic signaling but also alternation of set point to limit food-intake, which consequently results in enhancement of fasting-induced hyperphagia. A single ICV injection of NPY-ab inhibited the fasting-induced increase in food intake but did not affect food intake in non-fasted mice. Moreover, continuous ICV infusion of NPY-ab stimulated fasting-induced food intake but did not affect daily *ad libitum* food intake and body weight. However, the stimulation of fasting-induced food intake caused by continuous ICV infusion of NPY-ab was blocked by leptin. These results imply that the circulating satiety and hunger signals such as glucose, leptin, and insulin could be importantly involved in the different effect of NPY-ab on food intake observed in fasted and non-fasted conditions.

4. Experimental Procedures

Animals and ICV infusion of NPY-Ab

Rabbit whole antisera to NPY were purchased from Sigma-Aldrich (Tokyo, Japan). A certificate of analysis obtained from the manufacturer shows that a feeble cross-reactivity (less than 0.01%) is observed with NPY fragments including NPY (18-36), NPY (13-36) and peptide YY (PYY) conjugated to BSA by dot blot analysis. We purified the IgG from the whole antisera by using protein G-Sepharose columns and concentrated it using

Slide-A-Lyzer dialysis kit (PIERCE, Rockford, IL). The IgG fractions from the NPY antisera were reconstituted to original volume with saline and then used as NPY-ab in this experiment.

Male *ddY* mice were maintained under controlled temperature and lighting conditions with a 12-h light/12-h dark cycle (lights on at 06:00). Seven-wk-old mice were anesthetized with Avertin® (0.36 g kg^{-1}), and permanent 30-gauge stainless steel infusion cannulae (Alzet Brain infusion Kit 3; Durect Corp., Cupertino, CA) that had been connected to Alzet osmotic pumps (model 2002; Durect Corp.) filled with either NPY-Ab or saline were stereotactically placed 0.8 mm posterior to the bregma, 0.1 mm lateral to the midsagittal suture, and 3 mm below the surface of the skull. The osmotic pump was implanted into a subcutaneous pocket in the midscapular area of the back of the mice as described by the manufacturer. Using this osmotic pump, solutions were infused continuously at a rate of 0.5 μl/h for 14 d. For a single injection, mice were unilaterally implanted with 26-gauge stainless steel cannulae (Plastics One; Roanoke, VA, USA) into the ICV under anesthesia with Avertin® (0.36 g kg^{-1}) 2 wk before experiments. The NPY-ab was diluted 1:4000 with saline, dilution of which was determined by the dose-dependent data shown in Fig.1A and also considering product information from the manufacturer. A single ICV injection of the diluted NPY-ab (1:4000) successfully blocked the 24-h-fast-induced food intake (Fig.1A and B) and also the ability of NPY to stimulate food intake (Fig.1C).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

After the mice were anesthetized with ether and decapitated, their brains were removed and cut into 1-mm thick coronal sections including the mid-hypothalamus. The isolated sections extended rostrally to just behind the optic chiasma and caudally just anterior to the

mammilary bodies, occupying the central region of the hypothalamus. The tissues were transferred to cold phosphate-buffered saline (PBS) and sectioned into the Arc, LHA, and PVN regions with the aid of prominent landmarks (fornix, third ventricle, and optic tract) under a dissecting microscope. The tissue sections of these hypothalamic areas were frozen individually in liquid nitrogen and used for RT-PCR. Total RNA isolated from the tissue sections using TRIZOL Reagent (Invitrogen, Carlsbad, CA) was quantified by measuring the absorbance at 260 nm, and its integrity was confirmed by denaturing agarose gel electrophoresis. The mRNA expression levels of NPY, NPY Y1 receptor, AGRP, POMC, and the housekeeping gene GAPDH were quantified by RT-PCR. Total RNA (25 ng) was reverse-transcribed using an oligo (dT) primer and AMV reverse transcriptase with a Takara RNA LA PCR^{TM} Kit (AMV) v.1.1 (Takara Shuzo Co., Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA products were amplified using primers for mouse NPY, NPY Y1 receptor, AGRP, POMC, and GAPDH (Table 1). The PCR reaction was carried out in a Bio-Rad I cycler (Bio-Rad, Tokyo, Japan).

Analysis of cDNAs of NPY, NPY Y1 receptor, AGRP, POMC, and GAPDH

Amplified cDNAs were separated on 3.0% agarose gels, stained with SYBR Green (Takara Shuzo Co., Kyoto, Japan), and quantified using an Epi-Light UV FA500 analyzer (Aishin Seiki, Tokyo, Japan) and NIH imaging software. The mRNA levels were determined as the ratio of the fluorescence intensity to that for GAPDH cDNA.

Immunohistochemistry

Mice were anesthetized with Avertin® (0.36 g kg^{-1}) and transcardially perfused with heparinized PBS, followed by 15 ml of 4% neutral-buffered paraformaldehyde solution. The

brains were dissected out and post-fixed with 10% neutral-buffered paraformaldehyde solution. After post-fixation, the brains were cut on an oscillating tissue slicer throughout the mid-hypothalamus into 40-mm thick sections. The sections were permeabilized with 0.5% (v/v) Triton X-100 in PBS (TPBS) for 1 h and then blocked in TPBS containing 2.0% normal goat serum for 1 h. After blocking, the sections were incubated for 24 h at 4°C in rabbit anti-NPY polyclonal antibody (Sigma-Aldrich; 1:3000 in TPBS) and sheep anti-NPY Y1 Receptor polyclonal antibody (Biogenesis; 1:2000 in TPBS). After rinsing in TPBS, the sections were incubated for 24 h in AlexaFluor® 488 goat anti-rabbit IgG (Molecular Probes; 1:1000 in TBST) and AlexaFluor® 594 donkey anti-sheep IgG (Molecular Probes; 1:1000 in TBST). After washing with PBS, sections were mounted onto slides and coverslipped with fluorescent mounting medium (Vectashield; Vector laboratories, Burlingame, CA, USA). Images were obtained by confocal laser scanning microscopy (Nikon, Tokyo, Japan).

Statistical methods

To evaluate the changes of food intake, data were analyzed by repeated measures analysis of variance (ANOVA) after Bartlett test. The Tukey-Kramer test was used as a post hoc test. For comparisons of mRNA expression levels, data were analyzed by either Student's *t*-test or Welch's *t*-test following an *F*-test. A *P* value of less than 0.05 was considered to indicate statistical significance.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee in Obihiro University of Agriculture and Veterinary Medicine and were conducted under the Guidelines for Animal Experiments in Obihiro University of

Agriculture and Veterinary Medicine and the Guiding Principles in the Use of Animals in Toxicology that were adopted by the Society of Toxicology in 1989. The animals were humanely killed at the end of the experiment by an overdose of anesthetic ether.

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References

Baskin, D.G., Wilcox, B.J., Figlewicz, D.P., Dorsa, D.M., 1988. Insulin and insulin-like growth factors in the CNS. Trends Neurosci. 11, 107-111.

Baskin, D.G., Breininger, J.F., Schwartz, M.W., 1999. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. Diabetes 48, 828-833.

Cheung, C.C., Clifton, D.K., Steiner, R.A., 1997. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 138, 4489-4492.

Clark, J.T., Kalra, P.S., Crowley, W.R., Kalra, S.P., 1984. Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology 115, 427-429.

Cone, R.D., Lu, D., Koppula, S., Vage, D.I., Klungland, H., Boston, B., Chen, W., Orth, D.N., Pouton, C., Kesterson, R.A., 1996. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. Recent Prog. Horm. Res. 51, 287-317.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., Low, M.J., 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411, 480-484.

Elias, C.F., Lee, C., Kelly, J., Aschkenasi, C., Ahima, R.S., Couceyro, P.R., Kuhar, M.J., Saper, C.B., Elmquist, J.K., 1998. Leptin activates hypothalamic CART neurons projecting to the spinal cord. Neuron 21, 1375-1385.

Elmquist, J.K., Bjorbaek, C., Ahima, R.S., Flier, J.S., Saper, C.B., 1998. Distributions of leptin receptor mRNA isoforms in the rat brain. J. Comp. Neurol. 395, 535-547.

Elmquist, J.K., Elias, C.F., Saper, C.B., 1999. From lesions to leptin: hypothalamic control of food intake and body weight. Neuron 22, 221-232.

Erickson, J.C., Clegg, K.E., Palmiter, R.D., 1996. Sensitive to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature 381: 415-418.

Erickson, J.C., Hollopeter, G., Palmiter, R.D., 1996. Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. Science 274, 1704-1707.

Hahn, T.M., Breininger, J.F., Baskin, D.G., Schwartz, M.W., 1998. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat. Neurosci. 1, 271-272.

Kushi, A., Sasai, H., Koizumi, H., Takeda, N., Yokoyama, M., Nakamura, M., 1998. Obesity and mild hyperinsulinemia found in neuropeptide Y-Y1 receptor-deficient mice. Proc. Natl. Acad. Sci. USA 95, 15659-15664.

Kalra, S.P., Dube, M.G., Sahu, A., Phelps, C.P., Kalra, P.S., 1991. Neuropeptide Y secretion increases in the paraventricular nucleus in association with increased appetite for food. Proc. Natl. Acad. Sci. USA 88, 10931-10935.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Woychik, R.P., Wikison, W.O., Cone, R.D., 1994. Agouti protein is an antagonist of the melanocyte stimulating-hormone receptor. Nature 371, 799-802.

Luquet, S., Perez, F.A., Hnasko, T.S., Palmiter, R.D., 2005. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. Science 310, 683-685.

Marsh, D.J., Hollopeter, G., Kafer, K.E., Palmiter, R.D., 1998. Role of the Y5 neuropeptide Y receptor in feeding and obesity. Nat. Med. 4, 718-721.

Ollmann, M.M., Wilson, B.D., Yang, Y.K., Kerns, J.A., Chen, Y., Gantz, I., Barsh, G.S., 1997. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. Science 278, 135-138.

Schwartz, M.W., Baskin, D.G., Bukowski, T.R., Kuijper, J.L., Foster, D., Lasser, G., Prunkard, D.E., Porte, D. Jr., Woods, S.C., Seeley, R.J., Weigle, D.S., 1996. Specificity of

leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. 45, 531-535.

Schwartz, M.W., Woods, S.C., Porte, D. Jr., Seeley, R.J., Baskin, D.G. 2000. Central nervous system control of food intake. Nature 404, 661-671.

Schwartz, M.W., Porte, D. Jr., 2005. Diabetes, obesity, and the brain. Science 307, 375-379.

Sipols, A.J., Baskin, D.G., Schwartz, M.W., 1995. Effect of intracerebroventricular insulin infusion on diabetic hyperhagia and hypothalamic neuropeptide gene expression. Diabetes 44, 147-151.

Stanley, B.G., Kyrkouli, S.E., Lampert, S., Leibowitz, S.F., 1986. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. Peptides 7, 1189-1192.

Stanley, B.G., Magdalin, W., Seirafi, A., Nguyen, M.M., Leibowitz, S.F., 1992. Evidence for neuropeptide Y mediation of eating produced by food deprivation and for a variant of the Y1 receptor mediating this peptide's effect. Peptides 13, 581-587.

Stephens, T.W., Basinski, M., Bristow, P.K., Bue-Valleskey, J.M., Burgett, S.G., Craft, L., Hale, J., Hoffmann, J., Hsiung, H.M., kriauciunas, A., Mackellar, W., Rosteck, P.R.Jr., Schoner, B., Smith, D., Tinsley, F.C., Zhang, X., Heiman, M., 1995. The role of neuropeptide Y in the antiobesity action of the obese gene product. Nature 377, 530-532.

Thornton, J., Cheung, C.C., Clifton, D.K., Steiner, R., 1997. Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. Endocrinology 138, 5063-5066.

White, J.D., Kershaw, M., 1990. Increased hypothalamic neuropeptide Y expression following food deprivation. Mol. Cell. Neurosci. 1, 41-48.

Wilding, J.P., Gilbey, S.G., Bailey, C.J., Batt, R.A., Williams, G., Ghatei, M.A., Bloom, S.R., 1993. Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased

neurotensin mRNA in hypothalamus of the obese (ob/ob) mouse. Endocrinology 132, 1939-1944.

Williams, G., Gill, J.S., Lee, Y.C., Cardoso, H.M., Okpere, B.E., Bloom, S.R., 1989. Increased neuropeptide Y concentrations in specific hypothalamic regions of streptozocin-induced diabetic rats. Diabetes 38, 321-327.

Woods, S.C., Seeley, R.J., Porte, D. Jr., Schwartz, M.W., 1998. Signals that regulate food intake and energy homeostasis. Science 280, 1378-1383.

Figure legends

Fig. 1. Effects of a single ICV injection of NPY-ab on the stimulation of food intake by fasting (A) (B) and NPY (C). (A) Mice were administered NPY-ab (5 μl/mouse) (1:1500, 1:4000, 1:8000, or 1:12000 antibody dilution) or vehicle (saline) alone (5 μl/mouse) by ICV injection after a 24-h fast and then fed *ad libitum*. After injection, total food consumption for 4 h was measured. Results represent means ± SD (n=3). (B)Mice were administered NPY-ab (1:4000; 5 μl/mouse; n = 4) or vehicle (saline) alone (5 μl/mouse; n = 4) by ICV injection after a 24-h fast and then fed *ad libitum*. After injection, food consumption was measured at 1-h intervals for 4 h. Results represent means ± SEM. (C) Mice were treated with vehicle (saline) alone (5 μl /mouse; n = 3), NPY (0.5 nmol/5 μl /mouse; n = 3), or NPY-ab (1:4000; 5 μl /mouse; n = 3) by ICV injection 5 min after ICV injection of saline (5 μl /mouse) and then fed *ad libitum*. Another group (NPY + NPY-ab) received NPY (0.5 nmol/5 μl /mouse; n = 3) by ICV injection 5 min after ICV injection of NPY-ab (1:4000; 5 μl /mouse) and then fed *ad libitum*. After injection, food consumption was measured at 1-h intervals for 4 h. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. control (saline-treated) mice.

Fig. 2. Effect of chronic ICV administration of NPY-ab on daily food intake (A) and body weight (B). Mice (7-wk-old) were continuously infused (0.5 μl/h) for 13 d with NPY-ab (1:4000; n = 7) or vehicle (saline) alone (n = 7) using Alzet osmotic minipumps that were implanted on day 0. Results represent means ± SEM.

Fig. 3. Effects of chronic ICV administration of NPY-ab on food intake after a 24-h fast. Mice (7-wk-old) were continuously infused (0.5 μl/h) for 13 d with NPY-ab (1:4000; n = 5)

or vehicle (saline) alone (n = 5) using osmotic minipumps. Leptin (50 μg/mouse; n = 3) was injected intraperitoneally to NPY-ab-infused mice 2 h before measuring food consumption. Mice were fed *ad libitum* after a 24-h fast, and food consumption was measured at 0.5-h intervals for 2 h. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. saline-infused mice; ^{+}P<0.05 and ^{++}P<0.01 vs. NPY-ab-infused mice.

Fig. 4. Effect of chronic ICV administration of NPY-ab on the expression of NPY (A), AGRP (B), and POMC mRNA (C) in the Arc. NPY, AGRP, and POMC mRNA levels in NPY-ab- and saline-infused mice were measured by RT-PCR 3 (n = 3), 7 (n = 5), and 13 d (n = 5) after the onset of infusion. The mRNA levels of these peptides are shown as the signal relative to that for GAPDH. Results represent means ± SEM. **P*<0.05 vs. saline-infused control mice.

Fig. 5. Effect of chronic ICV administration of NPY-ab on the expression of NPY Y1 receptor mRNA in the Arc (A), PVN (B), and LH (C). The NPY Y1 receptor mRNA level in the Arc, PVN, and LH in NPY-ab- and saline-infused mice was measured by RT-PCR 3 (n = 5), 7 (n = 5), and 13 d (n = 5) after starting infusion. The mRNA levels of these peptides are shown as the signal relative to that for GAPDH. Results represent means ± SEM. **P*<0.05 and ***P*<0.01 vs. saline control mice.

Fig. 6. Changes in the levels of NPY and NPY Y1 receptor proteins in the hypothalamus during ICV infusion of NPY-ab. (A) NPY and NPY Y1 receptor expression in the hypothalamus was analyzed in sections immunostained for NPY and NPY Y1 receptor before and 3, 7, and 13 d after starting ICV infusion of NPY-ab. Images were obtained by confocal laser scanning microscopy. Confocal images of cells stained for both NPY (green) and NPY

Y1 receptor (red) are shown. As shown in the merged images, these two proteins colocalize. The scale bar represents 1 mm. (B) Expression levels of NPY Y1 receptor (Left) and NPY (Right) protein in the Arc, PVN, and LHA were estimated by quantification of the average fluorescent intensity of those areas using Nikon software (EZ-C1). The data were determined as the ratio of the average fluorescent intensity of the Arc, PVN and LHA areas to that of the ventral posteromedial area with the background fluorescent intensity. Results represent means ± SD. *P<0.05, $^{\#}P$<0.05, and ^{+}P<0.05 vs. before ICV infusion of NPY-ab in the Arc, PVN, and LHA, respectively.

Fig. 7. Higher magnification of fluorescent double-immunocytochemical staining of NPY and NPY Y1 receptor in the Arc. NPY and NPY Y1 receptor expression in the Arc was analyzed in sections stained for NPY and NPY Y1 receptor before and 3 and 13 d after starting ICV infusion of NPY-ab. Images were obtained by confocal laser scanning microscopy. The merged images show the colocalization of NPY (green) and NPY Y1 receptor (red). The scale bar indicates 50 μm.

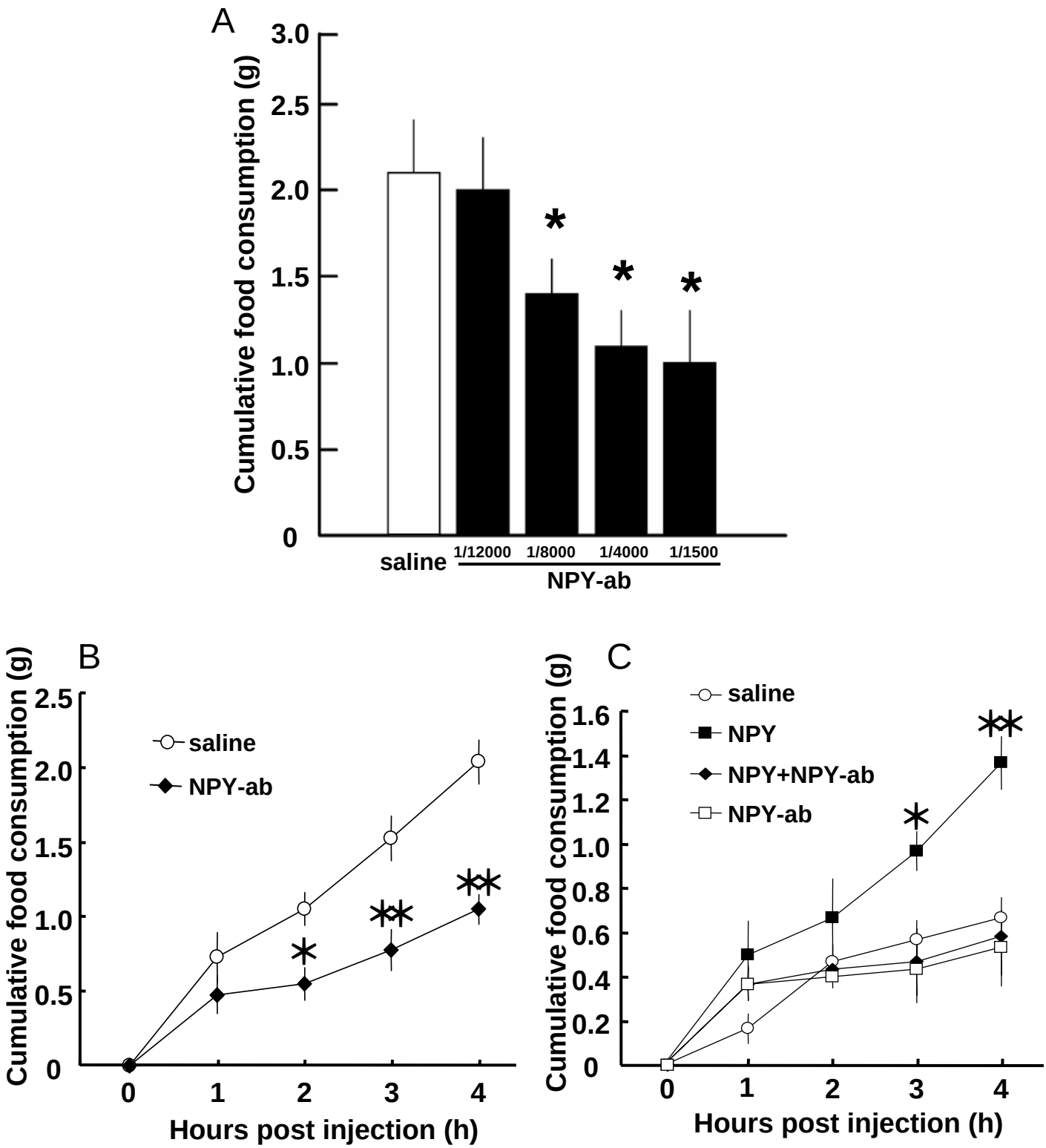

A
Cumulative food consumption (g)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
*
saline
1/12000
1/8000
1/4000
1/1500
NPY-ab
B
Cumulative food consumption (g)
saline
NPY-ab
Hours post injection (h)
C
Cumulative food consumption (g)
saline
NPY
NPY+NPY-ab
NPY-ab
Hours post injection (h)

Fig. 1

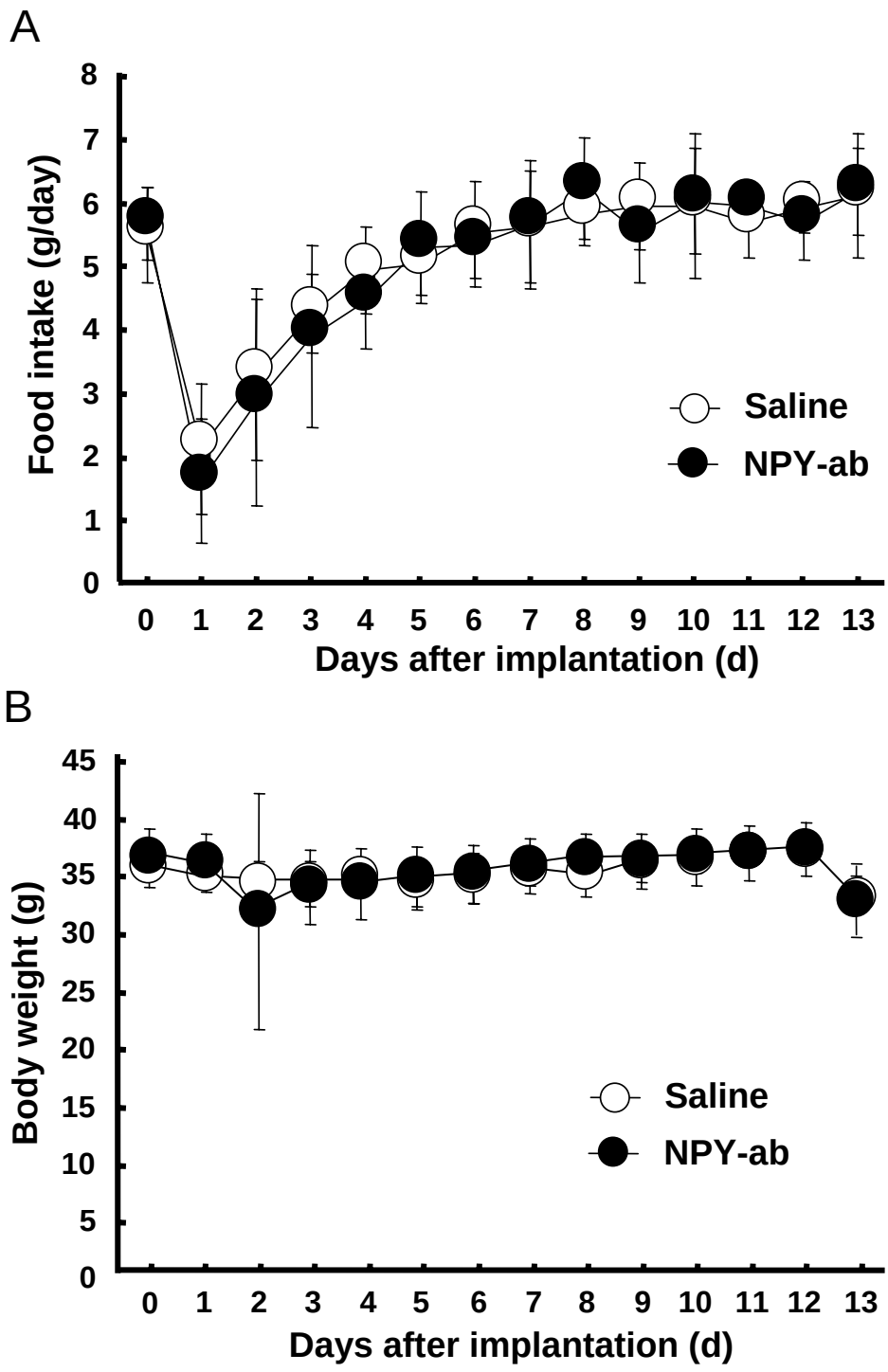

A
Food intake (g/day)
0
1
2
3
4
5
6
7
8
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days after implantation (d)
Saline
NPY-ab
B
Body weight (g)
0
5
10
15
20
25
30
35
40
45
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days after implantation (d)
Saline
NPY-ab

Fig. 2

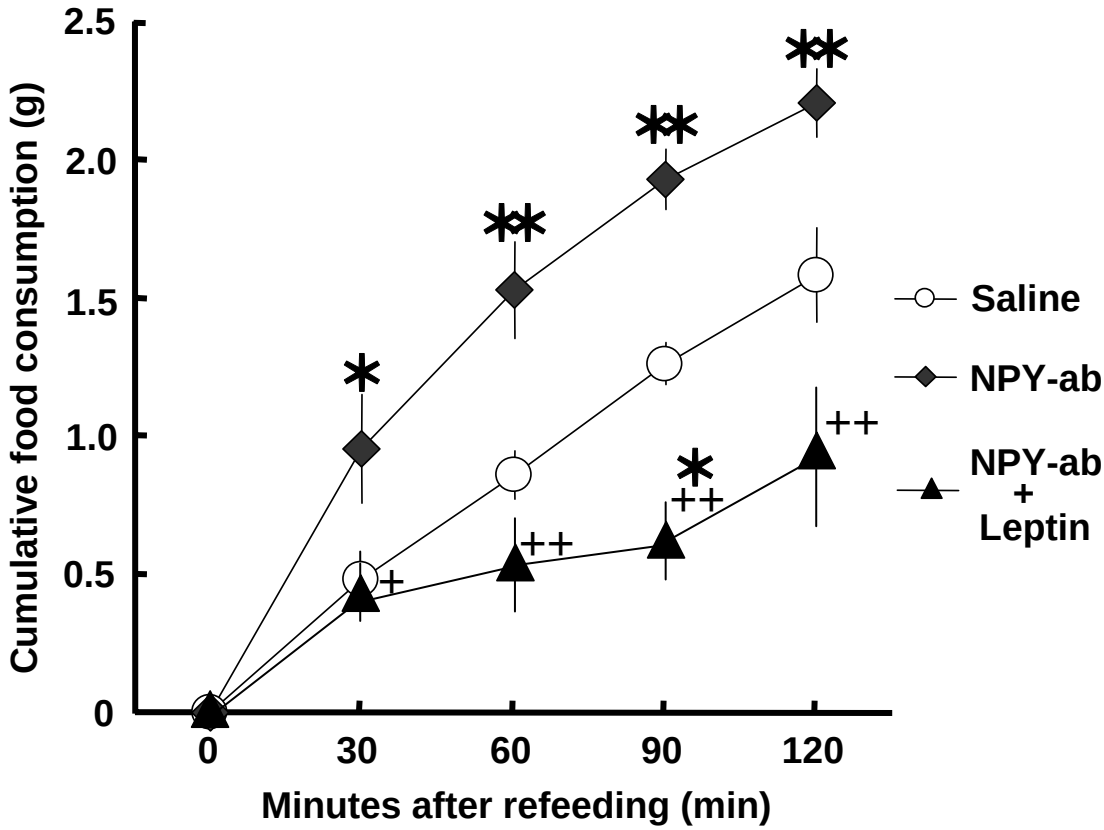

2.5
2.0
1.5
1.0
0.5
0
Cumulative food consumption (g)
0
30
60
90
120
Minutes after refeeding (min)
*
**
**
**
+
++
++
++
Saline
NPY-ab
NPY-ab
+
Leptin

Fig. 3

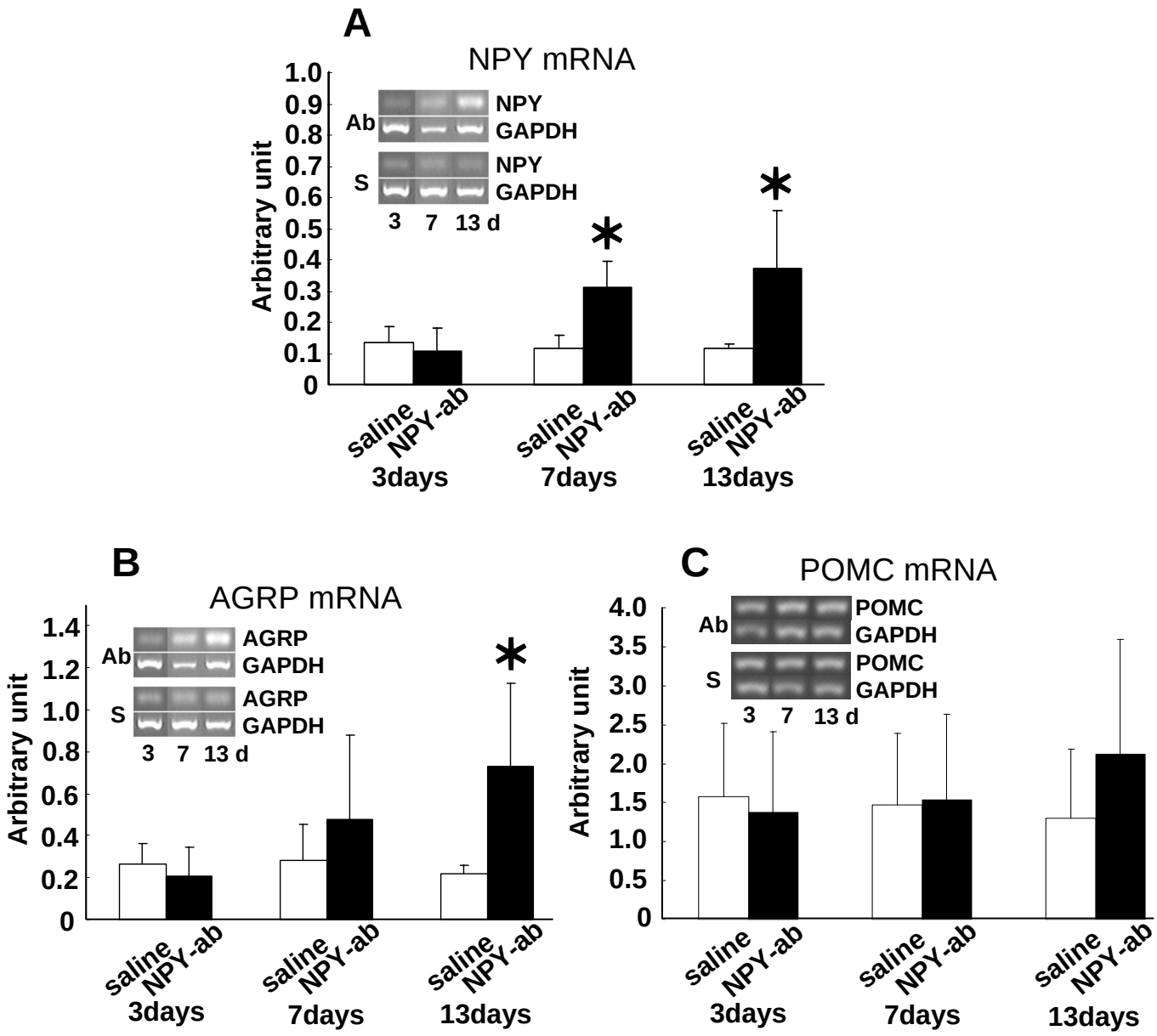

A
NPY mRNA
Arbitrary unit
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Ab
NPY
GAPDH
S
NPY
GAPDH
3 7 13 d
saline
NPY-ab
3days
7days
13days
B
AGRP mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
AGRP
GAPDH
C
POMC mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
POMC
GAPDH

Fig. 4

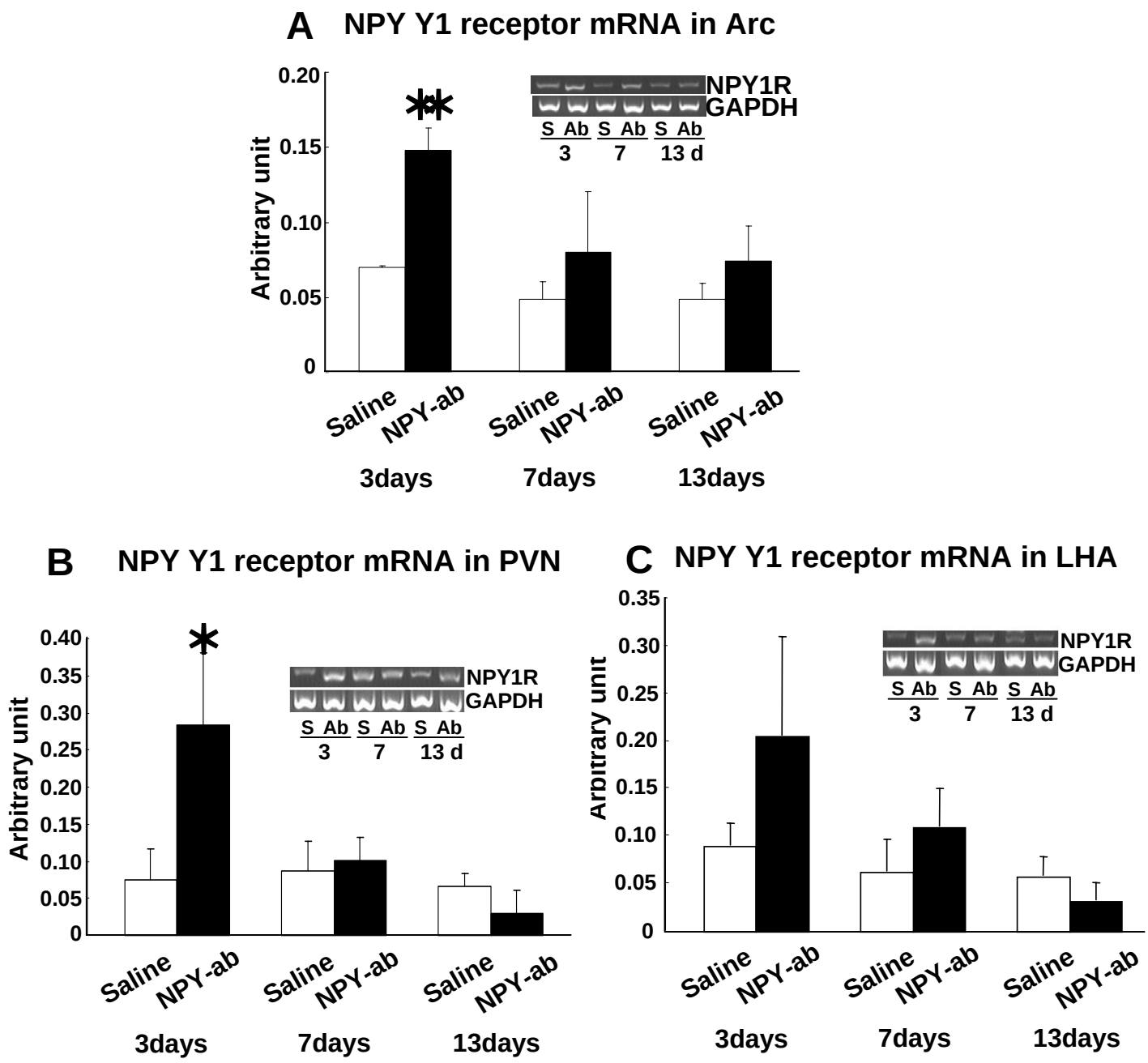
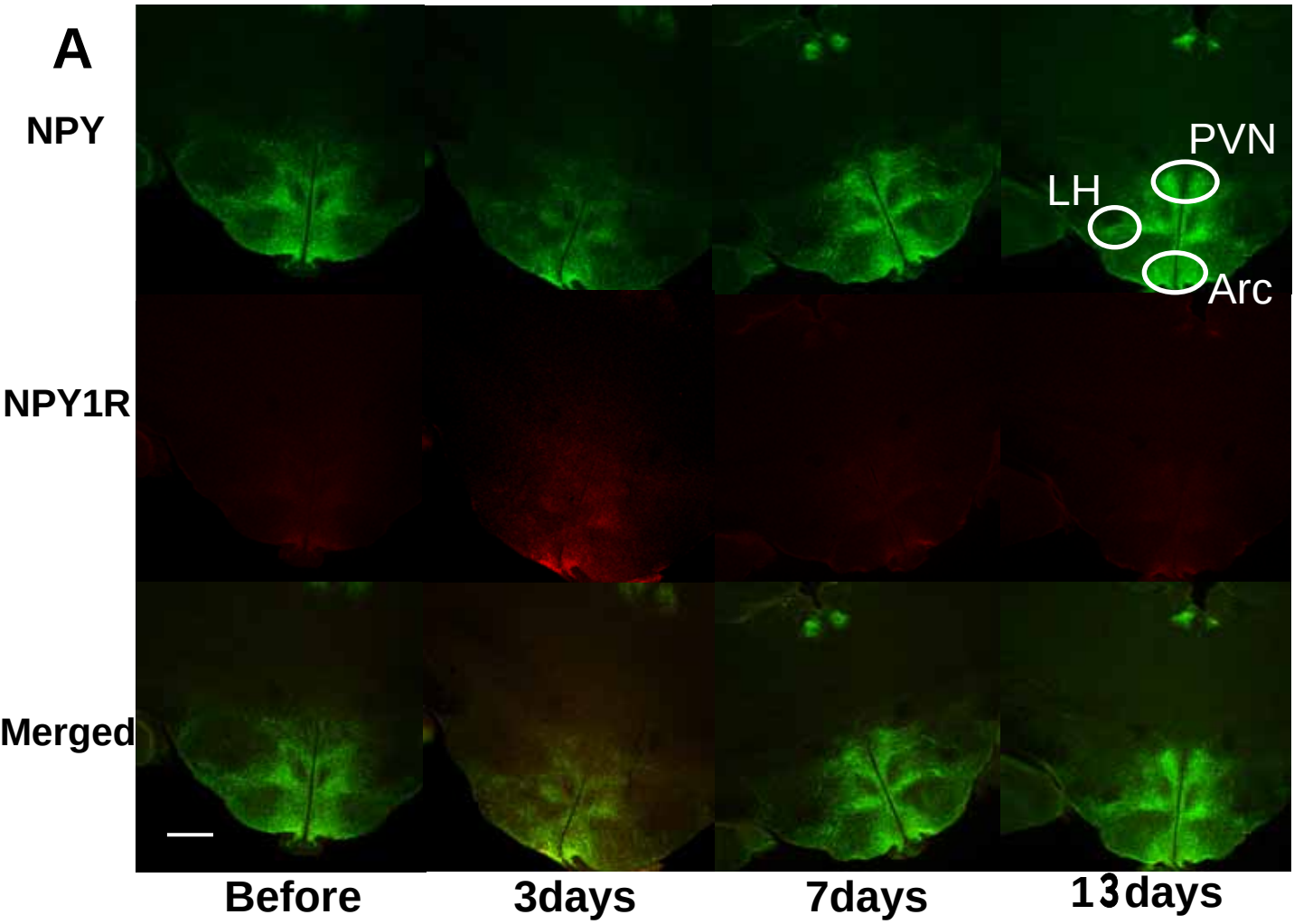

A
NPY Y1 receptor mRNA in Arc
Arbitrary unit
0.20
0.15
0.10
0.05
0
**
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days
B
NPY Y1 receptor mRNA in PVN
Arbitrary unit
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
*
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days
C
NPY Y1 receptor mRNA in LHA
Arbitrary unit
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
NPY1R
GAPDH
S Ab S Ab S Ab
3 7 13 d
Saline
NPY-ab
3days
7days
13days

Fig. 5


A
NPY
NPY1R
Merged
PVN
LH
Arc
Before
3days
7days
13days

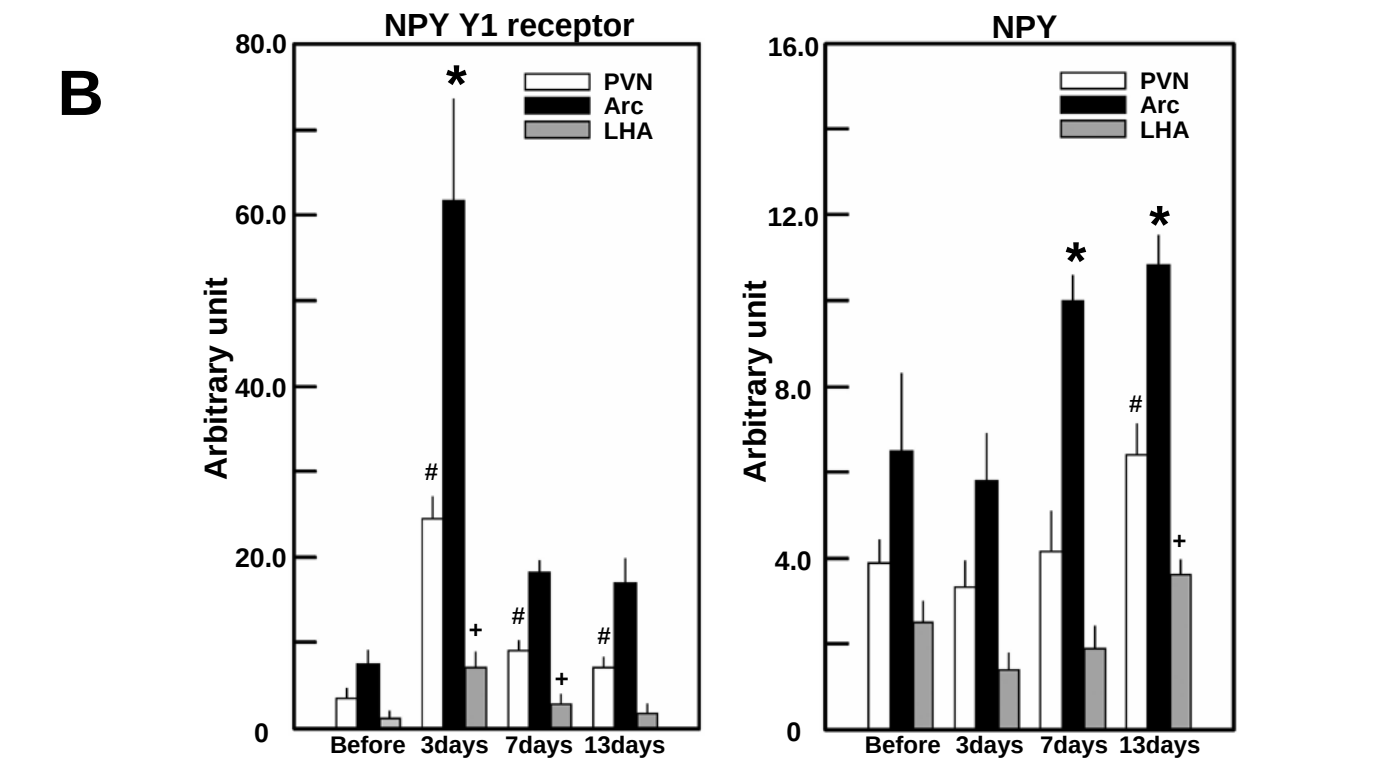

B
NPY Y1 receptor
NPY
PVN
Arc
LHA
Arbitrary unit
80.0
60.0
40.0
20.0
0
16.0
12.0
8.0
4.0
Before
3days
7days
13days
*
#
+

Fig. 6

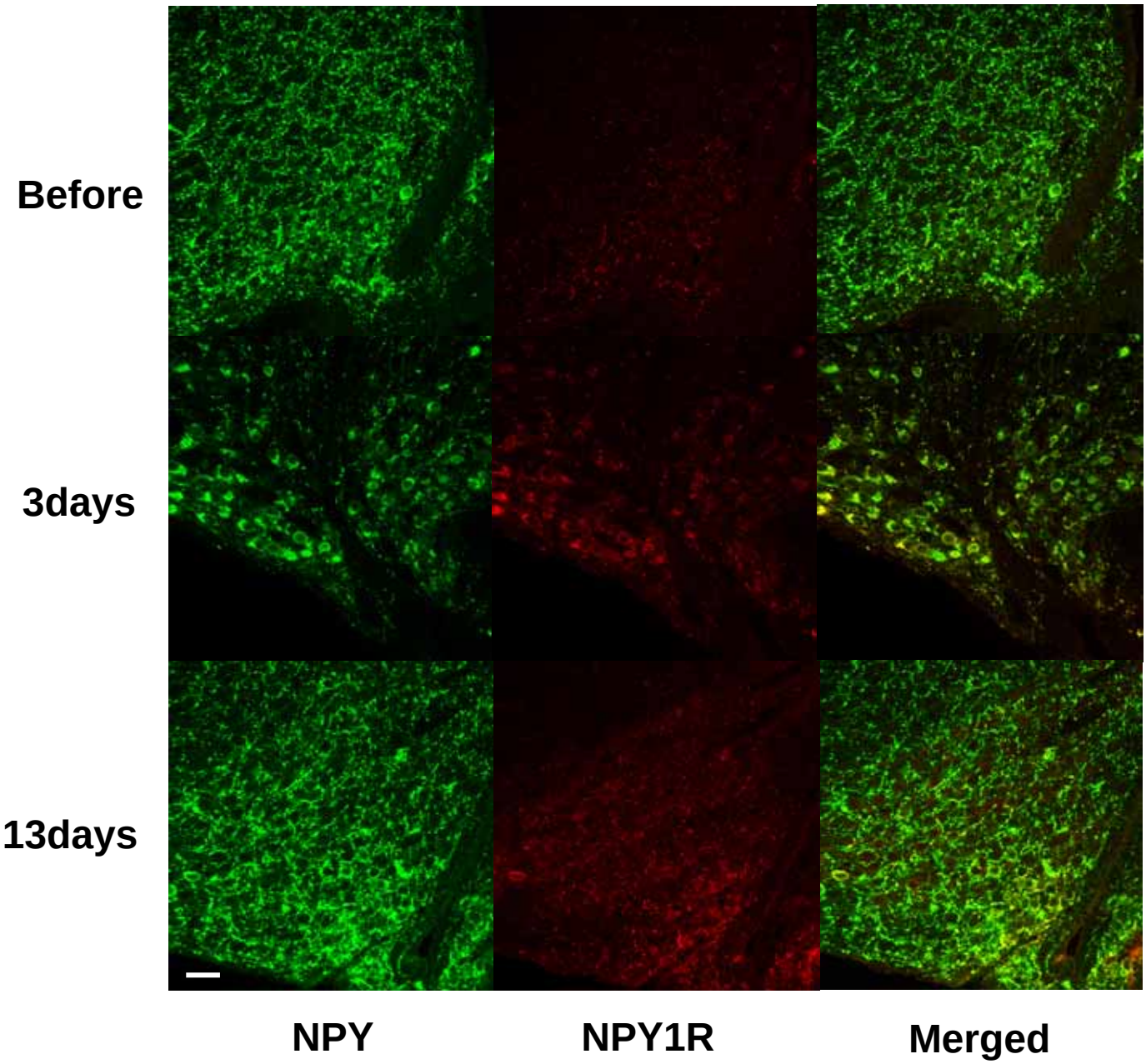


Fig. 7

Table 1. PCR primers in 5'- 3' direction

Transcript	Primers		Product size (bp)
GAPDH	Sense	GGGTGGAGCCAAACGGGTC	**532**
	Antisense	GGAGTTGCTGTTGAAGTCGCA	
NPY	Sense	ACTCTCACAGGCTGTCTTAC	**103**
	Antisense	ATAGTCTCGTAGTCGTCGTC	
NPY1R	Sense	TCAGACCTCTTAATGAAGGAAAGCA	**436**
	Antisense	GAGAACAAGTTTCATTTCCCATCA	
AGRP	Sense	CAGAAGCTTTGGCGGAGGT	**80**
	Antisense	AGGACTCGTGCAGCCTTACAC	
POMC	Sense	CTGCTTCAGACCTCCATAGATGTG	**120**
	Antisense	CAGCGAGAGGCGAGTTTGC	