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NPY receptor subtype specification for behavioral adaptive strategies during limited food access

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The neuropeptide Y (NPY) system in the brain regulates a wide variety of behavioral, metabolic and hormonal homeostatic processes required for energy balance control. During times of limited food availability, NPY promotes behavioral hyperactivity necessary to explore and prepare for novel food resources. As NPY can act via 5 different receptor subtypes, we investigated the path through which NPY affects different behavioral components relevant for adaptation to such conditions. We tested NPY Y1 and Y2 receptor knockout mice and their wild-type littermate controls in a daily scheduled limited food access paradigm with unlimited access to running wheel. Here we show that NPY Y1 receptor deficient mice lack the expression of appetitive behavior and that NPY Y2 receptors control the level of hyperactive behavior under these conditions. Thus, receptor specificity determines the differential expression of NPY-mediated behavioral adaptations to overcome a negative energy status.

Keywords: Food anticipatory activity, gene knockout, mouse motor activity levels, NPY Y1 receptor, NPY Y2 receptor

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Introduction

Neuropeptide Y (NPY) in the brain has received broad attention for its involvement in the regulation of energy balance, especially as a potent orexigenic factor (Beck 2006; Chee & Colmers 2008; Nguyen *et al*. 2011). In addition, NPY has been implicated in the regulation of different behavioral, metabolic and hormonal homeostasis processes, such as bone metabolism (Lee & Herzog 2009; Zengin *et al*. 2010) and regulation of behavioral activity levels (Nergårdh *et al*. 2007). Brain NPY levels respond to sudden and chronic environmental situations, such as acute stressful events (Kas *et al*. 2005) and during daily scheduled food restriction (de Rijke *et al*. 2005), however, the question remains how NPY exerts its variety of functions to establish compensatory responses to these environmental challenges.

During daily scheduled limited food availability, mammalian species express distinct forms of behavioral activity that each may serve different adaptations during times of low energy status. For instance, in addition to increased behavioral activity levels, there is a strong evolutionary conserved expression of motor activity just before the time of food availability. This so-called food anticipatory activity (FAA) to the expected upcoming food can be observed in a large variety of species, including insects, fish and primates (Mistlberger 1994). As NPY can act through a set of five G-protein coupled receptors (Nguyen *et al*. 2011), we tested whether NPY receptor types underlie the expression of these different behavioral components relevant for proper adaptation to limited food access. To investigate the path via which NPY exerts its effect on these processes we compared behavioral responses of NPY Y1 and Y2 receptor knockout mice and their wild-type littermate controls in a daily scheduled limited food access paradigm with unlimited access to running wheel.

Materials and methods

Animals and housing

For this study, initial mouse breeding pairs were received from the Herzog Laboratory, The Garvan Institute of Medical Research, Australia. The NPY Y1 and Y2 germline receptor knockout mice were generated as described by Baldock *et al*. (2002). Briefly, a targeting vector for the NPY Y1 and Y2 receptor gene has been designed that allows the production of germline NPY Y1 receptor knockout ($Y1^{-/-}$) and NPY Y2 receptor knockout ($Y2^{-/-}$) mice in a 129/SvJ strain. Positive embryonic stem cells from this strain were selected and injected into blastocysts from C57BL/6J. The $Y1^{-/-}$ mice were backcrossed to a C57BL/6J background for five generations and the $Y2^{-/-}$ mice for three generations. Both homozygous $Y1^{-/-}$ and $Y2^{-/-}$, as well as $Y1^{+/+}$ and $Y2^{+/+}$ littermates, were generated by crossing the respective heterozygous animals. Genotypes of the knockout and wild-type mice were determined by polymerase chain reaction analysis of DNA extracted from ear punch.

All mice used in the experiment were bred at the Rudolf Magnus Institute of Neuroscience animal facility and were 3–4 months old at the start of the experiment. In line with our previous studies (Gelegen *et al*. 2008, 2010), test-naive female mice were used in the experiments; 10 $Y1^{-/-}$ and 10 $Y1^{+/+}$ littermates and 15 $Y2^{-/-}$ and

10 $Y2^{+/+}$ littermates. As the effects of the estrous cycle are much more subtle in mice than in rats (Kopp *et al*. 2006) and previous studies found no relationship between the variation in estrous cycle and variation in mouse behavior (Laarakker *et al*. 2011), we did not systematically monitored the different stages of estrous during this study. Following weaning at 3–4 weeks, female and male mice were separately housed in groups (2–5 mice) in cages (Macrolon Type II, Tecniplast, Milan Italy) with sawdust bedding and 1–2 tissues per cage (Kleenex®, Kimberly-Clark B.V., Ede, The Netherlands). The housing facilities were maintained on a 12:12-h dark/light cycle with an ambient temperature of $21 \pm 2^{\circ}C$ and relative humidity of $55\% \pm 10\%$. During this period, the mice were given water and food *ad lib* (Rat and Mouse Breeder and Grower diet CRM; Special Diet Services, Essex, UK). All the procedures described were approved by the Animal Experiment Committee of the Academic Biomedical Centre, Utrecht, The Netherlands.

Experimental procedure

During the experiment, all mice were individually housed in cages with voluntary access to a running wheel. The wheel is made of a metal grid with a circumference of 44.5 cm, a diameter of 14 cm and a width of 8.5 cm. The distance between the grid wires is approximately 1 cm. The mice were left to adapt to the conditions for 7 days before the experiment (with food, water and running wheel access *ad lib*). After this period, food was available *ad lib* only in the first 2 h of the dark phase for four consecutive days, which is the phase that the mice consume most of their food. The food was presented in pellets, five pellets were introduced in the cage at the beginning of the dark phase. After 2 h, the food and each small part were collected and the cage was carefully checked that there were no food pieces left. We were not able to collect and weigh some food left over in the form of powder as these are generally very small amounts. The experiment ended on the fifth day before the dark phase (and food access). Body weight and food intake were measured daily before and after food administration. Baseline data of food intake and body weight were collected on experiment day 1, as the mice had food *ad lib* before the first 2 h of the limited food access episode. Individual running wheel revolutions (RWR) were continuously registered by a magnet activated counter (also during the adaptation phase) using Cage Registration Software version 5.5 (Department of Biomedical Engineering, University Medical Center Utrecht, The Netherlands). The average RWR of 2 days before the experiment was taken as the baseline RWR level. The activity level during the experiment was calculated as the average RWR of 2 days before the end of experiment or individual animals last day (based on the humane end-points). During the experiment we also calculated the FAA as the sum of the RWR during the 4 h prior to food intake as defined by Mistlberger (1994). This coincided with the last 4 h of the light phase, because food was given during the first 2 h of the dark phase. Total FAA was calculated for days 2, 3 and 4 of the experiment correcting for the corresponding total day activity.

At the end of the experiment (day 5), all mice were sacrificed within 3 h prior to the dark phase. We collected truncal blood from 8 $Y1^{-/-}$ and 8 $Y1^{+/+}$ and from 8 $Y2^{-/-}$ and 5 $Y2^{+/+}$ mice; and adrenal glands from 9 $Y1^{-/-}$ to 10 $Y1^{+/+}$ and from 8 $Y2^{-/-}$ to 5 $Y2^{+/+}$ mice. The weight of the right plus left adrenal glands was calculated for each mouse. The blood, collected in eppendorf tubes with 80 μM disodium ethylenediaminetetraacetate dihydrate and 1 mg aprotonin, was spun for 10 min at a relative centrifugal force of 1520 ***g*** at 4°C. The supernatant was collected and stored until the time of the assay at −20°C. Corticosterone levels in plasma were assayed using the protocol of the radioimmunoassay ^{125}I-labeled kit (MP Biomedicals, Orangeburg, NY, USA).

Statistical analysis

In this experiment, daily body weight levels just before the 2 h of limited food access, food intake, plasma corticosterone levels and the weight of the adrenal glands were compared between the gene knockout mice and their corresponding wild-type controls. We also assessed running wheel activity levels of the mice; the change in running wheel activity levels relative to baseline levels and the expression of FAA, as absolute values and relative to their total daily wheel running activity levels (TDA). The data were expressed as means with standard error of the mean unless otherwise specified. One sample Kolmogorov–Smirnov test was used to check the Gaussian distribution and Levene's test for the homogeneity of variances of the data. Two-tailed Student's *t* test or Mann–Whitney *U* test, if the data were nonparametric, was used for analysis between the knockout and wild-type mice. Pearson's correlation test was used for the correlation analysis. Significance level was set at $P < 0.05$. The statistical analysis was carried out using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

Results

The results are shown separately for each receptor as the genetic background of the different receptor gene knockouts and corresponding wild-type controls were different (i.e. the NPY Y1 mice were backcrossed five times while the NPY Y2 mice were backcrossed three times to a C57BL/6J genetic background). Therefore, each knockout is compared with its own wild-type littermate and the wild-type mice of each receptor are not compared with each other.

NPY Y1 receptor

Body weight and food intake

During *ad lib* food conditions, $Y1^{-/-}$ mice were, on average, slightly heavier than their $Y1^{+/+}$ littermate controls [$\text{median}_{(\text{min-max})}$, $Y1^{-/-}$: 22.8 g (19.5–29.3 g) and $Y1^{+/+}$: 20.93 g (19.8–23.2 g)] but not significant (Mann–Whitney *U* test, $z = -1.965$, $P = 0.052$). This difference in body weight increased during the following 2 days of the scheduled feeding paradigm [Mann–Whitney *U* test, $z = -2.041$, $P = 0.043$ and Student's *t* test, $t_{(18)} = -2.390$, $P = 0.028$, respectively] (Fig. 1a). One $Y1^{-/-}$ and four $Y1^{+/+}$ mice reached the human end-point at day 3 of the experiment (indicating that they lost 15% body weight relative to their individual *ad lib* body weight) and were not included in further analysis. After these mice were taken out, the difference in body weight was no longer present. The difference in body weight of the $Y1^{-/-}$ and the $Y1^{+/+}$ mice was associated with differences in food intake. In general, the $Y1^{+/+}$ mice tended to consume more food than the $Y1^{-/-}$ mice, the cumulative (days 2, 3 and 4) food intake was significantly different [Student's *t* test, $t_{(14)} = 2.862$, $P = 0.013$; $Y1^{-/-}$: 3.8 ± 0.2 g and $Y1^{+/+}$: 4.7 ± 0.2 g]. Food intake differences were statistically significant during the scheduled feeding paradigm on day 1 [Student's *t* test, $t_{(18)} = 2.811$, $P = 0.012$] and on day 4 [Student's *t* test, $t_{(14)} = 2.793$, $P = 0.014$] (Fig. 1b).

Behavioral activity levels

General running wheel activity levels of the $Y1^{-/-}$ mice during the baseline (5903.35 ± 1540.13 RWR or 2.63 ± 0.69 km) and experiment (8952.35 ± 2622.65 RWR or 3.98 ± 1.17 km) were not different from the $Y1^{+/+}$ mice (baseline 8082.35 ± 1738.04 RWR or 3.60 ± 0.77 km and experiment $11\,814.6 \pm 2493.41$ RWR or 5.26 ± 1.11 km) (Fig. 1c,d, respectively). Both showed a relative increase in wheel running activity levels during the restriction phase when compared with their baseline levels ($Y1^{-/-}$: $206.2\% \pm 61.8\%$ and $Y1^{+/+}$: $170.8\% \pm 36.6\%$; Fig. 1e). However, there was a significant

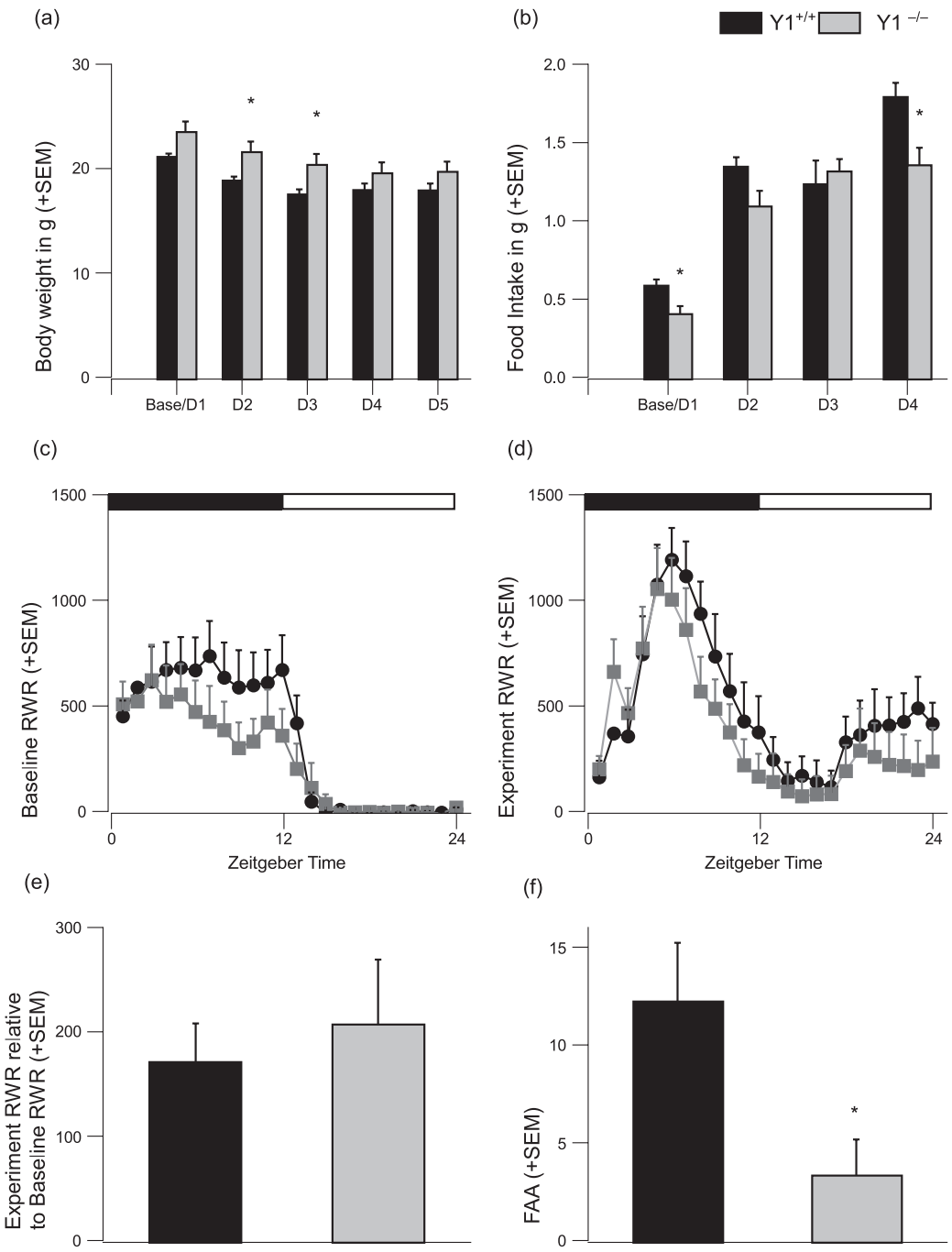

Figure 1: NPY Y1 receptor results. (a) Body weight of $Y1^{-/-}$ and $Y1^{+/+}$ mice on day 1/baseline $P = 0.052$, and on subsequent restriction day 2 ($P = 0.043$) and day 3 ($P = 0.028$). (b) Food intake during the 2 h of the first (baseline) through fourth day of the daily scheduled limited food access paradigm. In this paradigm, $Y1^{-/-}$ and the $Y1^{+/+}$ mice showed significant differences in food intake on day 1/baseline and on day 4. Removal of the four $Y1^{+/+}$ mice with accelerated body weight loss on day 4 likely explains the change in genotype effect in body weight and food intake on the subsequent days. (c) and (d) Hourly activity levels during the baseline and experiment (the average RWR of 2 days before the last experimental day), respectively. Zeitgeber time has been indicated on the *x*-axis. The corresponding dark and light cycle has been indicated with a dark and white bar on top of the graph, respectively. Note that the FAA shown in (d) represents FAA levels during 2 days before the end of the experiment. (e) Average activity levels of the experiment as percentage of the baseline levels. (f) Average FAA during the experiment. The data are presented as mean (+ standard error of mean) and (*) indicates genotype effect.

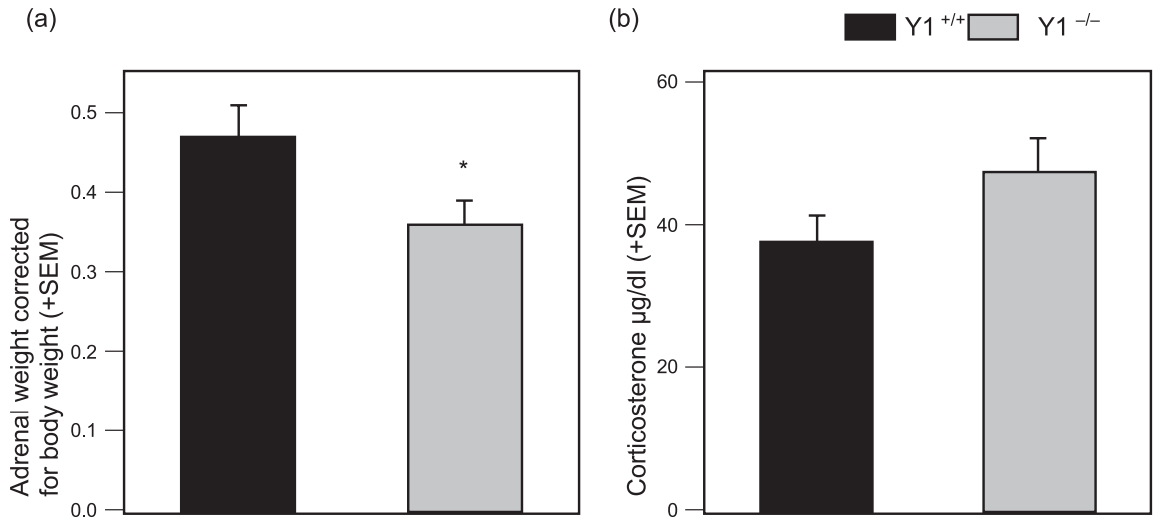

Figure 2: The weight of the adrenal glands, corrected for body weight, graph (a), and the corticosterone levels, graph (b), for $Y1^{-/-}$ and $Y1^{+/+}$ mice. The data are presented as mean (+ standard error of mean) and (*) indicates a significant genotype effect.

difference in the total levels of FAA. $Y1^{-/-}$ mice showed less FAA when compared with their $Y1^{+/+}$ littermates ($Y1^{-/-}$: 3.4% ± 1.8% and $Y1^{+/+}$: 12.2% ± 3%), relative to their TDA [Student's *t* test, $t_{(18)} = 2.529$, $P = 0.021$] (Fig. 1f). The absolute values of the total FAA were also significantly different (Mann–Whitney *U* test, $z = -2.117$, $P = 0.035$, data not shown).

Adrenal glands and corticosterone levels

The weight of the adrenal glands was corrected for body weight at the time of tissue collection. The $Y1^{+/+}$ mice had significantly larger adrenal glands than the $Y1^{-/-}$ mice [$Y1^{-/-}$: 0.36% ± 0.03% and $Y1^{+/+}$: 0.47% ± 0.04%; Student's *t* test, $t_{(17)} = 2.373$, $P = 0.030$] (Fig. 2a). The absolute weight was not correlated with plasma corticosterone levels (data not shown). The plasma corticosterone levels were not significantly different ($Y1^{-/-}$: 47.3 ± 4.6 µg/dl and $Y1^{+/+}$: 37.5 ± 3.6 µg/dl; Fig. 2b).

NPY Y2 receptor

Body weight and food intake

During the scheduled feeding paradigm $Y2^{-/-}$ mice and their $Y2^{+/+}$ littermates did not differ in body weight throughout the experiment (Fig. 3a). Consistent with these body weight similarities, $Y2^{-/-}$ and $Y2^{+/+}$ mice consumed similar levels of food during *ad lib* and experimental conditions (Fig. 3b), the cumulative (days 2, 3 and 4) food intake was not significantly different ($Y2^{-/-}$: 4.7 ± 0.1 g and $Y2^{+/+}$: 4.6 ± 0.2 g, data not shown).

Behavioral activity levels

In contrast to the $Y1^{-/-}$mice, $Y2^{-/-}$ mice showed a robust effect on the general running wheel activity levels during the scheduled feeding paradigm (baseline 11 990.5 ± 1618.33 RWR or 5.34 ± 0.72 km and experiment 12 359.3 ± 1834.87 RWR or 5.50 ± 0.82 km) (Fig. 3c,d) when compared with their wild-type controls (baseline 8822.95 ± 2107.92 RWR or 3.93 ± 0.94 km and experiment 6373.45 ± 2274.2 RWR or 2.84 ± 1.01 km). Under these conditions, the $Y2^{+/+}$ mice decreased and maintained their reduced wheel running activity levels, while the $Y2^{-/-}$ mice, after an initial decrease, significantly increased their wheel running activity levels [$Y2^{-/-}$: 101.7% ± 10.4% and $Y2^{+/+}$: 63.9% ± 8.3%; Student's *t* test, $t_{(23)} = -2.604$, $P = 0.016$] (Fig. 3e). In contrast to $Y1^{-/-}$ mice, there was no difference between $Y2^{-/-}$ mice and their $Y2^{+/+}$ littermate controls in FAA levels, either as absolute values (data not shown) or relative to the TDA ($Y2^{-/-}$: 4.3% ± 1.5% and $Y2^{+/+}$: 3.7% ± 1.2%; Fig. 3f). Note that the FAA shown in Fig. 2d shows FAA during 2 days prior to the end of the experiment. Absolute FAA levels of $Y2^{-/-}$ appear slightly higher than $Y2^{+/+}$, however, there is no significant difference in these FAA levels [$\text{median}_{(\text{min}-\text{max})}$, $Y1^{+/+}$: 94.5 (8–442.5) RWR and $Y1^{-/-}$: 195.5 (39–5114.5) RWR; Mann–Whitney *U* test, $z = -1.609$, $P = 0.112$].

Adrenal glands and corticosterone levels

$Y2^{-/-}$ mice and their $Y2^{+/+}$ littermates showed no significant differences for adrenal glands weight corrected for body weight (Fig. 4a) nor for their corticosterone levels [$\text{median}_{(\text{min}-\text{max})}$, $Y2^{-/-}$: 31.3 (9–69) µg/dl and $Y2^{+/+}$: 38.9 (28–46) µg/dl] (Fig. 4b). As in $Y1^{-/-}$ mice, there was no correlation between the absolute adrenal glands weight and the plasma corticosterone levels (data not shown).

Discussion

The data obtained show that NPY receptors have different roles in regulating behavioral adaptive strategies in mice during daily scheduled food restriction. Mice with a genetic

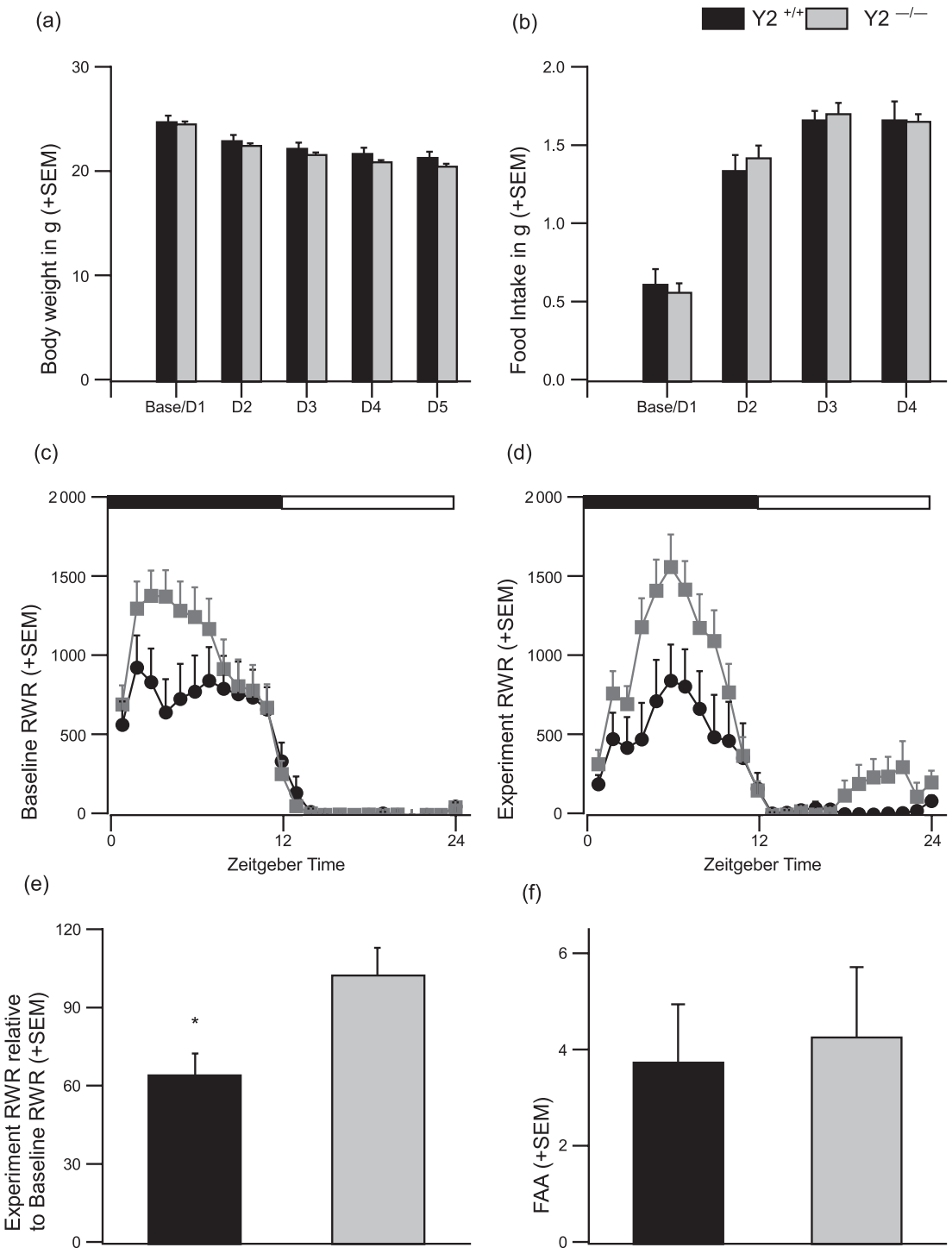

Figure 3: NPY Y2 receptor results. $Y2^{-/-}$ and the $Y2^{+/+}$ mice showed similar body weight levels (a), as well as food intake (b) during both the baseline and food restricted phase. (c) and (d) Hourly activity levels during the baseline and experiment (the average RWR of 2 days before the last experimental day), respectively. Zeitgeber time has been indicated on the *x*-axis. The corresponding dark and light cycle has been indicated with a dark and white bar on top of the graph, respectively. Note that the FAA shown in (d) represents FAA levels during 2 days before the end of the experiment. (e) Average daily running wheel activity levels during the restriction phase relative to baseline levels. (f) Average FAA during the experiment. The data are presented as mean (+ standard error of mean) and (*) indicates a significant genotype effect.

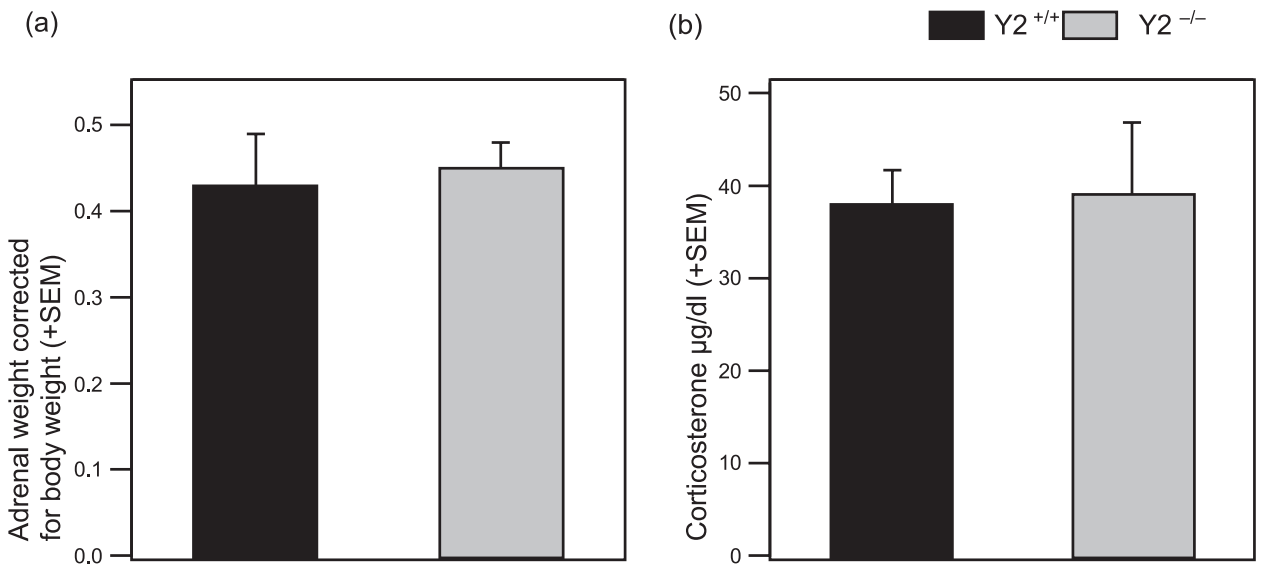

Figure 4: The weight of the adrenal glands, corrected for body weight, graph (a), and the corticosterone levels, graph (b), for $Y2^{-/-}$ and $Y2^{+/+}$ mice. The data are presented as mean (+ standard error of mean).

deletion of Y1 receptors expressed significant lower levels of FAA when compared with their wild-type littermate controls. The reduced expression of this appetitive behavior is consistent with pharmacological studies in various rodent species showing that NPY Y1 receptor ligands affect the appetitive phase of food intake (Keen-Rhinehart & Bartness 2007; Lecklin *et al*. 2002, 2003). In contrast to $Y1^{-/-}$ mice, $Y2^{-/-}$ mice showed increased daily motor activity levels specifically during the daily scheduled feeding paradigm when compared with wild-type littermate controls. These data indicate that, during scheduled limited food access, NPY Y1 receptors promote appetitive behaviors, such as FAA, whereas NPY Y2 receptors are involved in regulating daily energy expenditure levels during a negative energy balance. On the second and third day of the scheduled food restriction paradigm, we observed that $Y1^{-/-}$ mice had a significantly higher body weight than their $Y1^{+/+}$ littermates. Furthermore, four $Y1^{+/+}$ mice needed to be taken out of the experiment before the intended end of the daily feeding schedule because of accelerated body weight loss. While we did not observe high body weights in NPY $Y1^{-/-}$ mice under baseline conditions (Kushi *et al*. 1998), our data suggest that the NPY Y1 receptor in mice affect body weight maintenance during limited food access. Alternatively, the reduced levels of FAA in NPY $Y1^{-/-}$ mice during limited food access may have contributed to the body weight changes, because the final hour of FAA coincided with the time of our daily body weight measurements.

The induction of behavioral hyperactivity levels during limited food access that was observed in $Y2^{-/-}$ mice resembles the behavioral response following NPY injections under similar food limiting conditions (Nergårdh *et al*. 2007). As NPY Y2 receptors have been considered auto receptors (Chen *et al*. 1997; King *et al*. 1999) that are located on NPY producing neurons (Caberlotto *et al*. 2000), the genetic deletion of the NPY Y2 may have resulted in behavioral hyperactivity via reduced inhibition on NPY release. Further studies are necessary to confirm this and to answer the question how NPY subsequently stimulate behavioral hyperactivity, however, our results support the notion that this is not via the NPY Y1 receptor. Alternatively, behavioral hyperactivity during limited food availability may be a compensatory behavior to deal with altered anxiety levels that NPY Y2 receptor deficient mice are known to express (Aydin *et al*. 2011; Heilig 2004). Indeed, a study in eating disorder patients indicated that anxiety symptoms and food restriction synergistically contribute to increased levels of physical activity in the acute phase of anorexia nervosa (Holtkamp *et al*. 2004). It would, therefore, be of interest to study the relationship between increased physical activity and anxiety levels, in the acute phase, in anorexia nervosa patients as a function of the occurrence of NPY and NPY Y2 receptor mutations in this human eating disorder population.

In the past few years, several genes have been implicated in the regulation of FAA, Orexin receptor (Akiyama *et al*. 2004; Kaur *et al*. 2008), Mu Opioid receptors (Kas *et al*. 2004) and Ghrelin (Lesauter *et al*. 2009; Verhagen *et al*. 2011), among others. A recent study in NPY knockout mice showed a delayed onset of FAA when compared with controls (Gunapala *et al*. 2011). Our data suggest that this NPY effect in the development of FAA may be modulated via the Y1 receptor.

Reduced FAA in $Y1^{-/-}$ mice was associated with reduced adrenal glands weight, but not with release of the corticosterone hormone, suggesting that FAA levels are not related to acute responses of the hypothalamus–pituitary–adrenal axis at this time of day (Krieger & Hauser 1978). Although several discussions are ongoing about the existence of a food entrainable oscillator that is driving these scheduled induced behavioral responses to limited food access

(Gooley *et al*. 2006; Mistlberger 2006; Mistlberger 2009; Saper & Fuller 2007), none of gene knockout mouse strains that affect FAA showed a complete ablation of FAA during scheduled feeding. This suggests that various gene products can have a modulating effect on this anticipatory behavior rather than that of being necessary and required for the generation of FAA. Interestingly, some of these systems identified seem to functionally interact, providing some more ground for the underlying neurobiological mechanisms of FAA. For example, different studies have shown that the ghrelin-stimulating effects on foraging behavior and food intake can be blocked by a NPY Y1 receptor antagonist (Chen *et al*. 2004; Keen-Rhinehart & Bartness 2007). These findings indicate that more emphasis should be put toward the functional and neuroanatomical integration of neuropeptide systems that each are known to modulate FAA levels.

Taken together, it is expected that different receptors of NPY have different functions in behavioral processes regulating energy balance. Here we show that NPY Y1 receptors promote the expression of appetitive behavior prior to daily scheduled limited food access and that NPY Y2 receptors control behavioral hyperactivity levels under these conditions.

References

Akiyama, M., Yuasa, T., Hayasaka, N., Horikawa, K., Sakurai, T. & Shibata, S. (2004) Reduced food anticipatory activity in genetically orexin (hypocretin) neuron-ablated mice. *Eur J Neurosci* **20**, 3054–3062.

Aydin, C., Oztan, O. & Isgor, C. (2011) Vulnerability to nicotine abstinence-related social anxiety-like behavior: molecular correlates in neuropeptide Y, Y2 receptor and corticotropin releasing factor. *Neurosci Lett* **490**, 220–225.

Baldock, P.A., Sainsbury, A., Couzens, M., Enriquez, R.F., Thomas, G.P., Gardiner, E.M. & Herzog, H. (2002) Hypothalamic Y2 receptors regulate bone formation. *J Clin Invest* **109**, 915–921.

Beck, B. (2006) Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. *Philos Trans R Soc Lond B Biol Sci* **361**, 1159–1185.

Caberlotto, L., Fuxe, K. & Hurd, Y.L. (2000) Characterization of NPY mRNA-expressing cells in the human brain: co-localization with Y2 but not Y1 mRNA in the cerebral cortex, hippocampus, amygdala, and striatum. *J Chem Neuroanat* **20**, 327–337.

Chee, M.J. & Colmers, W.F. (2008) Y eat? *Nutrition* **24**, 869–877.

Chen, X., DiMaggio, D.A., Han, S.P. & Westfall, T.C. (1997) Autoreceptor-induced inhibition of neuropeptide Y release from PC-12 cells is mediated by Y2 receptors. *Am J Physiol* **273**, H1737–H1744.

Chen, H.Y., Trumbauer, M.E., Chen, A.S., Weingarth, D.T., Adams, J.R., Frazier, E.G., Shen, Z., Marsh, D.J., Feighner, S.D., Guan, X.M., Ye, Z., Nargund, R.P., Smith, R.G., Van der Ploeg, L.H., Howard, A.D., MacNeil, D.J. & Qian, S. (2004) Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* **145**, 2607–2612.

Gelegen, C., Pjetri, E., Campbell, I.C., Collier, D.A., Oppelaar, H. & Kas, M.J. (2010) Chromosomal mapping of excessive physical activity in mice in response to a restricted feeding schedule. *Eur Neuropsychopharmacol* **20**, 317–326.

Gelegen, C., van den Heuvel, J., Collier, D.A., Campbell, I.C., Oppelaar, H., Hessel, E. & Kas, M.J. (2008) Dopaminergic and brain-derived neurotrophic factor signalling in inbred mice exposed to a restricted feeding schedule. *Genes Brain Behav* **7**, 552–559.

Gooley, J.J., Schomer, A. & Saper, C.B. (2006) The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. *Nat Neurosci* **9**, 398–407.

Gunapala, K.M., Gallardo, C.M., Hsu, C.T. & Steele, A.D. (2011) Single gene deletions of orexin, leptin, neuropeptide Y, and ghrelin do not appreciably alter food anticipatory activity in mice. *PLoS One* **6**, e18377.

Heilig, M. (2004) The NPY system in stress, anxiety and depression. *Neuropeptides* **38**, 213–224.

Holtkamp, K., Hebebrand, J. & Herpertz-Dahlmann, B. (2004) The contribution of anxiety and food restriction on physical activity levels in acute anorexia nervosa. *Int J Eat Disord* **36**, 163–171.

Kas, M.J., Bruijnzeel, A.W., Haanstra, J.R., Wiegant, V.M. & Adan, R.A. (2005) Differential regulation of agouti-related protein and neuropeptide Y in hypothalamic neurons following a stressful event. *J Mol Endocrinol* **35**, 159–164.

Kas, M.J., van den Bos, R., Baars, A.M., Lubbers, M., Lesscher, H.M., Hillebrand, J.J., Schuller, A.G., Pintar, J.E. & Spruijt, B.M. (2004) Mu-opioid receptor knockout mice show diminished food-anticipatory activity. *Eur J Neurosci* **20**, 1624–1632.

Kaur, S., Thankachan, S., Begum, S., Blanco-Centurion, C., Sakurai, T., Yanagisawa, M. & Shiromani, P.J. (2008) Entrainment of temperature and activity rhythms to restricted feeding in orexin knock out mice. *Brain Res* **1205**, 47–54.

Keen-Rhinehart, E. & Bartness, T.J. (2007) NPY Y1 receptor is involved in ghrelin- and fasting-induced increases in foraging, food hoarding, and food intake. *Am J Physiol Regul Integr Comp Physiol* **292**, R1728–R1737.

King, P.J., Widdowson, P.S., Doods, H.N. & Williams, G. (1999) Regulation of neuropeptide Y release by neuropeptide Y receptor ligands and calcium channel antagonists in hypothalamic slices. *J Neurochem* **73**, 641–646.

Kopp, C., Ressel, V., Wigger, E. & Tobler, I. (2006) Influence of estrus cycle and ageing on activity patterns in two inbred mouse strains. *Behav Brain Res* **167**, 165–174.

Krieger, D.T. & Hauser, H. (1978) Comparison of synchronization of circadian corticosteroid rhythms by photoperiod and food. *Proc Natl Acad Sci U S A* **75**, 1577–1581.

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 U S A* **95**, 15659–15664.

Laarakker, M.C., van Lith, H.A. & Ohl, F. (2011) Behavioral characterization of A/J and C57BL/6J mice using a multidimensional test: association between blood plasma and brain magnesium-ion concentration with anxiety. *Physiol Behav* **102**, 205–219.

Lecklin, A., Lundell, I., Paananen, L., Wikberg, J.E., Mannisto, P.T. & Larhammar, D. (2002) Receptor subtypes Y1 and Y5 mediate neuropeptide Y induced feeding in the guinea-pig. *Br J Pharmacol* **135**, 2029–2037.

Lecklin, A., Lundell, I., Salmela, S., Mannisto, P.T., Beck-Sickinger, A.G. & Larhammar, D. (2003) Agonists for neuropeptide Y receptors Y1 and Y5 stimulate different phases of feeding in guinea pigs. *Br J Pharmacol* **139**, 1433–1440.

Lee, N.J. & Herzog, H. (2009) NPY regulation of bone remodelling. *Neuropeptides* **43**, 457–463.

LeSauter, J., Hoque, N., Weintraub, M., Pfaff, D.W. & Silver, R. (2009) Stomach ghrelin-secreting cells as food-entrainable circadian clocks. *Proc Natl Acad Sci U S A* **106**, 13582–13587.

Mistlberger, R.E. (1994) Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neuroscience & Biobehavioral Reviews* **18**, 171–195.

Mistlberger, R.E. (2006) Circadian rhythms: perturbing a food-entrained clock. *Curr Biol* **16**, R968–R969.

Mistlberger, R.E. (2009) Food-anticipatory circadian rhythms: concepts and methods. *European Journal of Neuroscience* **30**, 1718–1729.

Nergårdh, R., Ammar, A., Brodin, U., Bergström, J., Scheurink, A. & Södersten, P. (2007) Neuropeptide Y facilitates activity-based-anorexia. *Psychoneuroendocrinology* **32**, 493–502.

Nguyen, A.D., Herzog, H. & Sainsbury, A. (2011) Neuropeptide Y and peptide YY: important regulators of energy metabolism. *Curr Opin Endocrinol Diabetes Obes* **18**, 56–60.

de Rijke, C.E., Hillebrand, J.J., Verhagen, L.A., Roeling, T.A. & Adan, R.A. (2005) Hypothalamic neuropeptide expression following chronic food restriction in sedentary and wheel-running rats. *J Mol Endocrinol* **35**, 381–390.

Saper, C.B. & Fuller, P.M. (2007) Inducible clocks: living in an unpredictable world. *Cold Spring Harb Symp Quant Biol* **72**, 543–550.

Verhagen, L.A., Egecioglu, E., Luijendijk, M.C., Hillebrand, J.J., Adan, R.A. & Dickson, S.L. (2011) Acute and chronic suppression of the central ghrelin signaling system reveals a role in food anticipatory activity. *Eur Neuropsychopharmacol* **21**, 384–392.

Zengin, A., Zhang, L., Herzog, H., Baldock, P.A. & Sainsbury, A. (2010) Neuropeptide Y and sex hormone interactions in humoral and neuronal regulation of bone and fat. *Trends Endocrinol Metab* **21**, 411–418.

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