Neural pathways controlling homeostatic and hedonic feeding in rats on free-choice diets
van den Heuvel, J.K.

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Effect of a choice and non-choice high-fat high-sugar diet on hypothalamic neuropeptide Y and nucleus accumbens enkephalin mRNA expression

José K. van den Heuvel
Mieneke C. Luijendijk
Maike Brans
Eric Fliers
Andries Kalsbeek
Roger A.H. Adan
Susanne E. la Fleur
ABSTRACT
Rats subjected to a free-choice high-fat high-sugar (fcHFHS) diet become overtly obese and show sustained hyperphagia, which correlates with increased arcuate nucleus (ARC) NPY mRNA levels after 7 days on the diet. On the other hand, we showed that rats on a non-choice HFHS (ncHFHS) diet, which contains similar amounts of fat and sugar as the fcHFHS diet, but in pelleted form, showed initial overconsumption, but did not remain hyperphagic, pointing to an important role for choice per se in mediating hyperphagia when consuming excess fat and sugar. As NPY mRNA was correlated to feeding behavior in rats on the fcHFHS diet, we hypothesized that in rats on the ncHFHS diet NPY mRNA would be significantly lower compared to rats on regular chow or fcHFHS diet.

We therefore subjected rats to the fcHFHS, ncHFHS or a chow control diet (CHOW) for 7 days and measured adiposity, plasma leptin levels and ARC NPY mRNA. After 7 days, caloric intake, leptin, adiposity and ARC NPY mRNA were significantly increased in rats on the fcHFHS diet compared to rats on the CHOW diet. Also in rats on the ncHFHS diet caloric intake after 7 days diet was significantly increased compared to rats on the CHOW diet, yet significantly lower compared to rats on the fcHFHS diet. Adiposity, leptin and NPY mRNA levels were not significantly increased in rats on the ncHFHS diet compared to rats on CHOW. As we previously showed that after 2 days on the diet, caloric intake in rats on the ncHFHS diet is still similar to that of rats on the fcHFHS diet, we also investigated NPY mRNA expression after 2 days. After 2 days diet, caloric intake in rats on the ncHFHS and the fcHFHS diet were equally increased and so were the ARC NPY mRNA levels. Lastly, we determined whether enkephalin expression in the nucleus accumbens (Acb) may be involved in the hyperphagic behavior in rats on the fcHFHS diet. We determined Acb preproenkephalin (ppENK) mRNA in CHOW, ncHFHS and fcHFHS-fed rats after 2 and 7 days diet and showed that ppENK mRNA levels were significantly decreased in rats on the fcHFHS diet at both time points, but unaffected in rats on the ncHFHS.

In conclusion, our data suggest that the increased NPY mRNA levels in rats on the fcHFHS diet are not determined by the choice component per se, but rather by the combination of fat and sugar in the diet. Moreover, our data suggest that the sustained hyperphagia observed in rats on the fcHFHS diet is characterized not only by increased ARC NPY mRNA levels, but also by altered downstream signals including downregulation of ppENK mRNA in the Acb.
INTRODUCTION

We previously showed that rats on a choice diet with saturated fat and chow (fcHF) diet or a choice diet of saturated fat, chow and liquid sugar (fcHFHS) for 1 week are both hyperphagic. However, NPY mRNA expression in the arcuate nucleus of the hypothalamus (ARC) responded differentially, with decreased levels in rats on the fcHF diet and increased levels in rats on the fcHFHS diet (12). NPY mRNA levels at 1 week of diet exposure were predictive of subsequent feeding behavior as fcHF-fed rats decreased food intake to levels similar to rats on a chow control diet (CHOW), whereas fcHFHS-fed rats remained hyperphagic (11,12). The increased NPY mRNA expression levels in rats on the fcHFHS diet raised the question whether the combination of saturated fat AND sugar causes the activation of the NPY system or whether this is due to the multiple choice component of the fcHFHS diet.

To investigate the effect of diet choice, we developed a diet that contains similar amounts of the different diet components as chosen spontaneously when on the fcHFHS diet, but combined in one pellet. We call this the non-choice HFHS (ncHFHS) diet (129). Using this diet, we previously showed that the choice component is important in mediating sustained hyperphagia as ncHFHS-fed rats, in contrast to fcHFHS-fed rats, did not remain hyperphagic (129). However, whether the choice component is also important for mediating the increased NPY mRNA levels observed in rats on the fcHFHS diet remained to be determined. As ncHFHS-fed rats show similar caloric intake and feeding patterns (with respect to meal frequency and meal size) as fcHF-fed rats (129) and since fcHF-fed rats show decreased NPY mRNA levels after 1 week diet and do not remain hyperphagic (12), we hypothesized that NPY mRNA levels in ncHFHS-fed rats would also be downregulated. We previously showed that rats on the ncHFHS diet initially increase caloric intake to levels observed in rats on the fcHFHS diet, but adjust their caloric intake after 2 days, as opposed to rats on the fcHF diet that also initially increase their intake but adjust caloric intake after 7 days diet. Therefore, in the present study we also determined NPY mRNA levels after 2 days on the diet in rats on CHOW, ncHFHS and fcHFHS diet.

Unexpectedly, after 2 days diet, NPY mRNA expression was equally increased in rats on the fcHFHS and ncHFHS diet. An additional factor, downstream of NPY, should therefore be involved in mediating the sustained hyperphagia in rats on the fcHFHS diet. Enkephalin is part of the opioid system, highly expressed in the Acb and has been demonstrated to have a clear role in palatable feeding (208). Enkephalin has been shown to be affected by palatable diets such as the liquid containing fat and sugar Ensure (38). We hypothesized that although the diet composition between ncHFHS and fcHFHS diet is similar, the choice component between the diets might result in differential Acb enkephalin mRNA expression levels. Therefore, we also examined preproenkephalin (ppENK) mRNA levels in the Acb in rats on the ncHFHS, fcHFHS and CHOW diet for 2 and 7 days.
MATERIAL AND METHODS

Animals and dietary intervention

Male rats (Wistar, Charles River, Germany) weighing 250±10 g were individually housed in Plexiglas cages at a temperature of 21–23 °C in a light-controlled room (lights on 0700–1900). Rats were allowed to adapt to their environment for 5 days. All experiments were approved by the Committee for Animal Experimentation of the University Medical Center Utrecht, the Netherlands.

Three groups (n=8) were established, two experimental groups and a control group: 1) a free-choice high-fat high-sugar (fcHFHS) diet group in which a dish of saturated fat (Beef tallow (Ossewit/Blanc de Boeuf), Vandemoortele, Belgium) and a bottle of 30% sugar water (1.0M sucrose mixed from commercial grade sugar and tap water) were present in the cage in addition to regular chow (special diet service (SDS), England) and tap water; 2) a non-choice high-fat high-sugar (ncHFHS) diet group offered tap water and a diet that was custom-made using the same ingredients as the fcHFHS diet and containing the same percentages of fat, sugar and chow as consumed previously by rats on the fcHFHS diet (10-12). The three components were mixed and made into a pellet (Ab Diets, Woerden, the Netherlands) containing 15% kcal sugar, 35% kcal fat and 50% kcal chow (% total amount of calories); 3) the control group, which remained on ad libitum regular chow and tap water (CHOW). Rats were subjected to the CHOW, ncHFHS or fcHFHS diet for either 2 days or 7 days.

Fat mass analysis and statistics

At the end of the experiment, rats were killed between 0900 and 1000 hours within 10 seconds after they were removed from their home cages. Brains were quickly frozen and stored at -80°C. Individual mesenteric-omental, epididymal, subcutaneous (inguinal), and perirenal white adipose tissues were dissected, cleaned, and weighed. Trunk blood was centrifuged and plasma was stored at -20°C. Plasma concentrations of leptin were determined as previously described (158). Analysis of variance (ANOVA) was performed for overall effects between groups. If significant, post hoc tests were performed for individual group differences (Tukey).

In situ hybridization

Coronal sections of 20 µm were labelled with 33P antisense RNA probes for NPY and ppENK mRNA according to the protocol previously described (49). The films were developed and NPY and ppENK expression levels in ARC were quantitatively analysed using an Epson-Perfection 4990 Photo-flatbed-scanner. All images (800 dpi) were analysed using ImageJ (Rasband, WS, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/, 1997–2005). Grey values were determined in regions of interest and measured bilaterally. Specific signal was calculated by the subtraction of the background value.
RESULTS

After 7 days diet, caloric intake was significantly increased in rats on the fcHFHS and the ncHFHS diet, compared with rats on the CHOW diet. Moreover, caloric intake in rats on the fcHFHS diet was significantly higher compared to rats on the ncHFHS diet (Fig. 1A). Fat mass accumulation and plasma concentrations of leptin were increased significantly in rats on the fcHFHS diet, but not in rats on the ncHFHS diet, compared with rats on the CHOW diet (Fig. 1B,C). As published previously (12) ARC NPY mRNA levels were significantly increased in rats on the fcHFHS diet compared to rats on the CHOW diet. ARC NPY mRNA levels in rats on the ncHFHS diet showed intermediate values as compared to the rats on the CHOW and fcHFHS diet, but did not differ significantly from either of them (Fig. 1D).

After 2 days diet, caloric intake was significantly increased in rats on the fcHFHS and the ncHFHS diet, compared with rats on the CHOW diet (Fig. 2A). Fat mass accumulation was not different between diet groups and plasma concentrations of leptin were increased significantly in rats on the fcHFHS diet, but not on the ncHFHS diet, compared with rats on the CHOW diet (Fig. 2B,C). ARC NPY mRNA levels were significantly increased in rats on the fcHFHS and ncHFHS diet compared to rats on CHOW diet (Fig 2D).

Figure 1. Caloric intake (A), total white adipose tissue (WAT) weight (B), leptin (C) and arcuate nucleus neuropeptide Y (NPY) mRNA levels (D) in rats on the CHOW, free-choice high-fat high-sugar (fcHFHS) or non-choice high-fat high-sugar (ncHFHS) diet for 7 days. Data are mean ± SEM. Different letters represent significant differences between bars (P<0.05). AU = arbitrary units.
Acb ppENK mRNA levels were significantly decreased in rats on the fcHFHS diet compared to rats on either CHOW or ncHFHS diet, after both 2 and 7 day on the diet (Fig 3A,B).

**DISCUSSION**

In this study we show that NPY mRNA levels were increased in rats on the fcHFHS diet for 7 days, consistent with our previous studies (12), as well as after 2 days diet. Unexpectedly, in rats on the ncHFHS diet, NPY mRNA levels were not significantly different from those in rats on the fcHFHS diet at either day 2 or day 7. On the other hand, only at day 2 but not at day 7, did NPY mRNA levels in ncHFHS-fed rats differ significantly from that in rats on the CHOW diet. In contrast, ppENK levels in the Acb were downregulated in rats on the fcHFHS diet, but not in rats on the ncHFHS diet, compared to rats on the CHOW diet after both 2 and 7 days diet. These data suggest that the increased NPY mRNA levels in rats on the fcHFHS diet are not determined by the choice component per se, but rather by the combination of fat and sugar in the diet. Moreover, our data suggest that the sustained hyperphagia observed in rats on

![Figure 2. Caloric intake (A), total white adipose tissue (WAT) weight (B), leptin (C) and arcuate nucleus neuropeptide Y (NPY) mRNA levels (D) in rats on the CHOW, free-choice high-fat high-sugar (fcHFHS) or non-choice high-fat high-sugar (ncHFHS) diet for 2 days. Data are mean ± SEM. Different letters represent significant differences between bars (P<0.05). AU = arbitrary units](image-url)
the fcHFHS diet is characterized not only by increased ARC NPY mRNA levels, but also by altered downstream signals, such as altered Acb enkephalin levels.

Previous studies suggested that increased ARC NPY levels are invariably related to increased caloric intake (71), which may be consistent with data in the current study as rats on the ncHFHS diet have lowered their caloric intake levels at 7 days diet compared to 2 days diet and this is associated with lowered NPY mRNA levels as well. On the contrary, we previously showed that at 7 days diet fcHF-fed rats are equally hyperphagic as fcHFHS-fed rats, yet show decreased NPY mRNA levels. Therefore, the results from our free-choice diets indicate that not the caloric intake or the choice component, but rather the diet composition, i.e. the combination of fat and sugar in the diet, is an important factor for the increase in ARC NPY mRNA levels.

We observed a significant decrease in Acb ppENK mRNA levels in rats on the fcHFHS diet at 2 and 7 days diet, but not in rats on the ncHFHS diet. These data indicate that the choice element might be an important factor regulating Acb ppENK expression. However, in addition to the choice element, rats on the fcHFHS diet also drink the sugar component (in contrast to rats on the ncHFHS diet who get the sugar from the pellet). An effect of liquid palatable food on Acb ppENK mRNA is in line with...
reports by others as a daily 3h exposure to a single bottle of Ensure (a liquid diet form with fats and sugars) resulted in a downregulation of ppENK expression (38). Moreover, a decrease in Acb ppENK was also found upon palatable snacking of a sucrose solution (209). Therefore, the presence of a sucrose solution in the fCHFHS diet, which leads to increased snacking behavior (129), may lead to adaptations in the brain reward circuitry, such as the Acb, resulting in decreased ppENK levels and long term hyperphagia.

The differential regulation of ppENK between nCHFHS and fCHFHS diet suggests that either the choice component or the liquid component, or both of them, are an important factor in the regulation of ppENK expression and may reflect different sensations of the palatability of the diet. Still the pelleted nCHFHS diet, rich in fat and sugar, is also highly palatable as it instantly induces overconsumption ((129) and Fig. 2). Therefore, it remains to be determined whether the choice component on its own is sufficient to trigger the ppENK response, or whether additional factors, such as a liquid component, are needed.

In conclusion, our data suggest that the combination of fat and sugar induces changes in NPY signaling and that additional downstream factors are needed for the induction of hyperphagia. One of these factors may comprise opioid signaling in the Acb.