Brain circuitries in control of feeding behaviors

Focus on Neuropeptide Y

Gumbs, M.C.R.

Chapter IV.
Effects of a 24-hour acute fast on NPY-related gene expression in hypothalamic and mesolimbic brain regions

Abstract

Central Neuropeptide Y (NPY) promotes caloric intake in order to maintain energy balance. *Npy*-expressing neurons in the arcuate nucleus (Arc) of the hypothalamus sense peripheral signals of energy status, and during fasting and subsequent refeeding, Arc *Npy* expression and NPY levels are increased and normalized, respectively. Arc NPY neurons project to several hypothalamic- and extrahypothalamic regions, which contain neurons expressing one or more NPY receptor types (NPYR). The role of the paraventricular nucleus (PVN) in mediating NPY-induced intake has received a lot of attention. However, the lateral hypothalamus (LHA), ventral tegmental area (VTA), and nucleus accumbens (NAc) also receive Arc NPY projections. In addition, the LHA and NAc locally express NPY, whereas the VTA may only express NPY under certain physiological circumstances. Importantly, the LHA, NAc, and VTA also mediate NPY-induced behaviors, such as caloric intake and motivated behaviors. Nevertheless, the role of NPY in these regions has received much less attention. Here, we determined if a 24-hour fast, which increases *Npy* expression in the Arc, modulates *Npy-*, *Npy1r-*, *Npy2r-, Npy4r-*, or *Npy5r* expression in the LHA, VTA and/or NAc.

Npy- and *Npyr* expression was quantified by RT-qPCR in the Arc, LHA, VTA, and NAc of male Wistar rats after a 24-hour fast. Controls were not fasted. As *Npy* expression fluctuates during the light-dark cycle, mRNA expression was determined at two time points: after the onset of the light period as well as prior to the onset of the dark period.

The 24-hour fast increased Arc *Npy* expression, decreased Arc *Npy1r* expression early in the light period, and decreased Arc *Npy2r* expression just prior to the onset of the dark period. The 24-hour fast did not modulate *Npy-* or *Npyr* expression in the LHA, VTA, or NAc, regardless of time of day.

These findings indicate that the Arc acts as a primary information relay regarding energy state during a 24-hour fast, without any effects on *Npy-* or *Npyr* expression in downstream brain regions.

Introduction

The central Neuropeptide Y (NPY) system maintains energy balance through its effects on caloric intake and energy metabolism. The arcuate nucleus (Arc) of the hypothalamus contains *Npy*-expressing neurons and is a key brain region involved in the regulation of energy homeostasis. NPY projections from the Arc to the paraventricular nucleus (PVN) of the hypothalamus have received a lot of attention regarding their role in energy balance. Arc NPY neurons sense peripheral signals of energy status (Kohno & Yada, 2012), and as a result, *Npy* expression and NPY levels in the Arc fluctuate during physiological challenges, such as fasting and subsequent refeeding (Hahn, Breininger, Baskin, & Schwartz, 1998; Marks, Li, Schwartz, Porte, & Baskin, 1992). NPY levels and local release of NPY in the PVN also fluctuate accordingly (Beck et al., 1990; Dube, Sahu, Kalra, & Kalra, 1992; Sahu, Kalra, & Kalra, 1988).

Arc NPY neurons project to several hypothalamic- and extrahypothalamic regions, including the lateral hypothalamus (LHA), ventral tegmental area (VTA), and nucleus accumbens (NAc), where they signal through four NPY receptor (NPYR) subtypes (NPY1R, NPY2R, NPY4R, NPY5R; [Gumbs et al., 2019; Michel et al., 1998; Sim & Joseph, 1991; van den Heuvel et al., 2015]). In the LHA, NPY peptide levels fluctuate with changes in energy balance, and, although fasting does not affect LHA NPY peptide levels, subsequent refeeding results in increased LHA NPY levels (Beck et al., 1990). In addition, infusion of exogenous NPY into the LHA leads to a robust feeding response, which can be blocked by NPY1R- and NPY5R antagonists (M.C.R. Gumbs et al., *accepted;* Stanley et al., 1993).

NPY regulates energy balance in part by modulation of hunger- and satiety processes. However, NPY can also promote caloric intake by increasing the motivation to obtain food (Jewett, Cleary, Levine, Schaal, & Thompson, 1995). Dopamine signaling, in particular the dopamine projection from the VTA to the NAc, plays an important role in food-motivated behavior (Hernandez & Hoebel, 1988; Meye & Adan, 2014; Wise, 2004). For example, intra-NAc NPY increases the motivation to obtain a sucrose pellet, which has been proposed to occur via increased dopamine release within the NAc (Pandit, Luijendijk, Vanderschuren, la Fleur, & Adan, 2014).

Npy-expressing neurons have also been observed in the mouse LHA (Kosse & Burdakov, 2016; Marston et al., 2011), whereas in rats *Npy*-expressing neurons have been observed in the posterior LHA (Abrahamson & Moore, 2001; M.C.R. Gumbs et al., 2019; chapter III, this thesis). The NAc also contains *Npy*-expressing neurons (Chronwall et al., 1985; de Quidt & Emson, 1986), whereas the VTA does not express *Npy* during a normal physiological state (Gumbs et al., 2019). Despite the presence of NPY and NPYR in the LHA, VTA and NAc, and their role in mediating feeding behaviors, it is currently unclear if *Npy*- or *Npyr* expression in the LHA, VTA or NAc is changed similar to Arc *Npy* during changes in the physiological state.

Here, we determined if a 24-hour fast changes the expression of *Npy*, *Npy1r*, *Npy2r, Npy4r* or *Npy5r* in the Arc, LHA, VTA, or NAc of male Wistar rats compared to *ad libitum*-fed controls. Because Arc *Npy* expression and NPY levels fluctuate during the light-dark cycle (Akabayashi, Levin, Paez, Alexander, & Leibowitz, 1994; Jhanwar-Uniyal, Beck, Burlet, & Leibowitz, 1990), we assessed gene expression early in the light period and just prior to the onset of the dark period.

Experimental procedures

Animals and housing

Male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany), weighing 270-300 grams at arrival, were housed in temperature- (21 ± 2 ˚C), humidity- (60 ± 5%) and light-controlled (12h:12h light/dark cycle; lights on between 07:00 and 19:00) rooms with background noise (radio) during the entire experiment. Rats had *ad libitum* access to a container with a standard high-carbohydrate diet (Teklad global diet 2918; 24% protein, 58% carbohydrate, and 18% fat, 3.1 kcal/g, Envigo, Horst, The Netherlands) and a bottle of tap water. The animal ethics committee of the Netherlands Institute for Neuroscience approved all experiments according to Dutch legal ethical guidelines.

Effects of a 24-hour fast on NPY-related mRNA levels

After seven days of acclimatization to the animal facility, naïve rats were fasted for 24 hours starting either at 10:00 (i.e. ZT3) or at 18:00 (i.e. ZT11), while still having access to water. Controls had free access to the diet and water during this period. The experiment was therefore divided into two experimental groups. The first group was fed or fasted for 24 hours between 10:00 and 10:00 the next day (ZT3, N = 8 per group). The second group was fed or fasted between 17:00 and 17:00 the next day (ZT10, N = 8 per group). Animals were anesthetized using 33%CO_2/66%O_2 and then quickly decapitated. Brains were rapidly dissected, frozen on dry ice and stored at -80 ˚C.

RNA isolation and RT-qPCR procedures have been published previously (M. C. R. Gumbs et al., 2019). Sections (250 µm) were cut on the cryostat to obtain punches of the Arc (Bregma -1.72 till -3.48 mm), and bilaterally of the LHA (Bregma -1.20 till -3.00 mm), VTA (Bregma -4.68 till -6.24 mm), and NAc (Bregma 3.00 till -0.84 mm) according tot the rat brain atlas (Paxinos & Watson, 2007). Sections were placed in RNAlater (Ambion, Waltham, MA, USA), and punched with a 1 mm-diameter blunt needle. Punches were stored in 300 µL (Arc) or 500 µL (LHA, VTA and NAc) TriReagent. After homogenization using an Ultra Turrax homogenizer (IKA, Staufen, Germany), total RNA was isolated by a chloroform extraction, followed by RNA purification using the Machery Nagel nucleospin RNA clean-up kit. RNA quality was checked on Agilent RNA nano chips, using manufacturer's kit and instructions and analyzed with a Bioanalyzer (Agilent, Santa Clara, USA). Only RIN values larger than 8.50 were

included. cDNA synthesis was carried out using equal RNA input (124.44 ng; measured with Denovix DS11; Denovix, Wilmington, USA) and the transcriptor first-strand cDNA synthesis kit with oligo d(T) primers (04897030001; Roche Molecular Biochemicals, Mannheim, Germany). Genomic DNA contamination was controlled for by cDNA synthesis reactions without reverse transcriptase.

Gene expression was measured using RT-qPCR with the SensiFAST SYBR no-rox kit (Bioline, Londen, UK) and Lightcycler® 480 (Roche Molecular Biochemicals); 2 µL cDNA was incubated in a final reaction volume of 10 µL reaction containing SensiFAST and 25 ng per primer. Primer sequences for *Npy*, *Npy1r*, *Npy2r*, *Npy4r*, and *Npy5r*, and the reference genes *Ubiquitin-C, Hypoxanthine guanine phosphoribosyl transferase* (*HPRT*), and *Cyclophilin-A* are listed in Table 1. PCR products were analyzed on a DNA agarose gel for qPCR product size.

Table 1. Primer sequences.

Gene	NCBI reference number	Forward primer 5'- 3'	Reverse primer 5'- 3'
Npy	NM_012614.2	GACAATCCGGGCGAGGACGC	TCAAGCCTTGTTCTGGGGGCA
Npy1r	NM_001113357.1	TCTCATCGCTGTGGAACGTC	CCGCCAGTACCCAAATGACA
Npy2r	NM_023968.1	TGGTCCTTATACTGGCCTAT	CAGGGTGTTCACCAAAAGAT
Npy4r	NM_031581.2	CATGGACTACTGGATCTTCG	AATGAACCAGATGACCACAA
Npy5r	NM_012869.1	GCCGAAGCATAAGCTGTGGAT	TTTTCTGGAACGGCTAGGTGC
Ubiq-C	NM_017314.1	TCGTACCTTTCTCACCACAGTATCTAG	GAAAACTAAGACACCTCCCCATCA
HPRT	NM_012583.2	CCATCACATTGTGGCCCTCT	TATGTCCCCCGTTGACTGGT
Cyclo-A	NM_017101.1	TGTTCTTCGACATCACGGCT	CGTAGATGGACTTGCCACC

Cyclo-A = Cyclophilin-A, *HPRT* = Hypoxanthine guanine phosphoribosyl transferase, *Npy* = Neuropeptide Y, *Npy1r* = Neuropeptide Y receptor 1, *Npy2r* = Neuropeptide Y receptor 2, *Npy4r* = Neuropeptide Y receptor 4, *Npy5r* = Neuropeptide Y receptor 5, *Ubiq-C* = Ubiquitin-C

Statistics and analyses

RT-qPCR quantification was performed using LinReg Software (Ramakers et al., 2003). Samples deviating >5% from the mean PCR efficiency and outliers (Grubb's test) were excluded. Values were normalized against the geometric mean of *Cyclophilin*, *Ubiquitin-c*, and *HPRT* expression levels. To assess the effect of fasting on gene expression levels, unpaired parametric t-tests were performed. All statistical analyses were performed using Graphpad Prism 8 (version 8.0.2 [263], January 30, 2019), and p-values < 0.1 are reported exactly. Full statistical details and gene expression data are summarized in supplemental Table 1 (Table S1). All data are presented as mean ± *SEM*.

Results

Body weight was lower in the 24 hour-fasted rats compared to the *ad libitum-fed* rats when measured at ZT3 (fasted: 313.9 ± 2.5 g; *ad libitum*: 324.9 ± 2.4 g; $t_{7,7}$ = 3.17, p = 0.007). Body weight did not differ significantly between fasted and *ad libitum*-fed control rats when measured at ZT11 (fasted: 343.5 ± 4.1 g; *ad libitum*: 352.6 ± 4.1 g; $t_{7,7}$ = 1.55, p > 0.05).

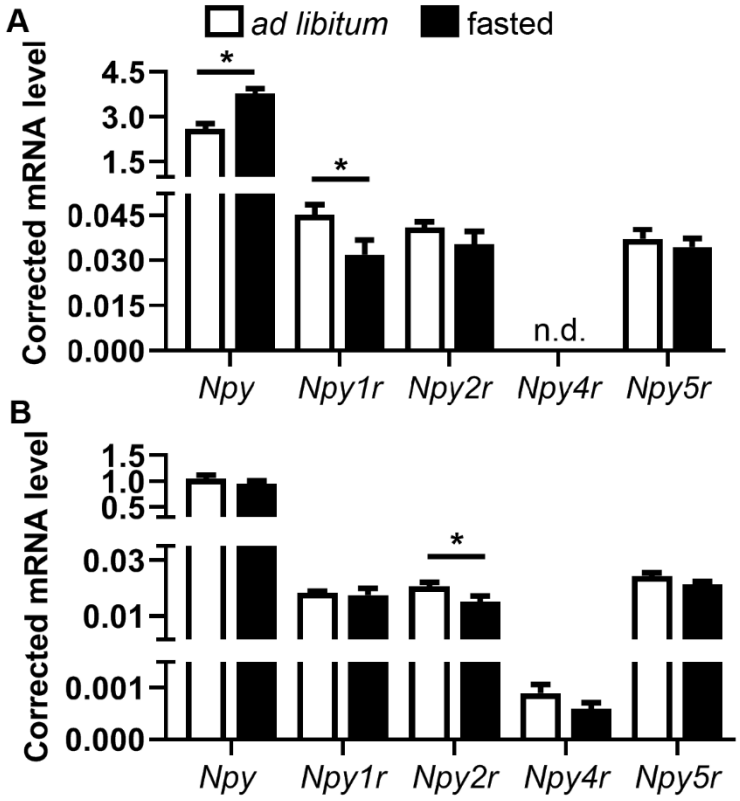

Figure 1. Time of day-dependent effects of a 24-hour fast on *Npy*- and *Npyr* expression in the Arc. A) At ZT3, Arc *Npy* expression was increased ($t_{5,5}$ = 4.83, *p = 0.0007), whereas Arc *Npy1r* expression was decreased ($t_{7,7}$ = 2.30, *p = 0.04) in 24 hour-fasted rats compared to *ad libitum*-fed controls. **B)** At ZT10, Arc *Npy2r* expression was decreased in 24 hour-fasted rats compared to *ad libitum*-fed controls ($t_{7,7}$ = 2.22, * p = 0.04). n.d. = not detected.

Time of day-dependent effects of a 24-hour fast on *Npy*- and *Npyr* expression in the Arc

When measured at ZT3, unpaired t-test analyses revealed that the 24-hour fast increased Arc *Npy* expression ($t_{5,5}$ = 4.83, p = 0.0007), and decreased Arc *Npy1r* expression ($t_{7,7}$ = 2.30, p = 0.04; see Figure 1A, and Table S1) compared to *ad libitum*-fed controls. The 24-hour fast did not alter *Npy2r*- or *Npy5r* expression in the Arc (both p > 0.05; see Figure 1A, and Table S1). Expression of *Npy4r* was not detectable in the Arc at ZT3.

When measured at ZT10, unpaired t-test analyses revealed that the 24-hour fast decreased Arc *Npy2r* expression ($t_{7,7}$ = 2.22, p = 0.04; see Figure 1B, and Table S1) compared

to *ad libitum*-fed controls, yet did not alter Arc *Npy-*, *Npy1r-*, *Npy4r-*, or *Npy5r* expression (all p > 0.05; see Figure 1B, and Table S1).

A 24-hour fast does not alter *Npy*- or *Npyr* expression in the LHA

When measured at ZT3, unpaired t-test analyses revealed that a 24-hour fast did not alter *Npy-*, *Npy1r-*, *Npy2r-*, *Npy4r-*, or *Npy5r* expression in the LHA of 24 hour-fasted rats compared to *ad libitum*-fed controls (see Figure 2, and Table S1). Gene expression was not tested at ZT10.

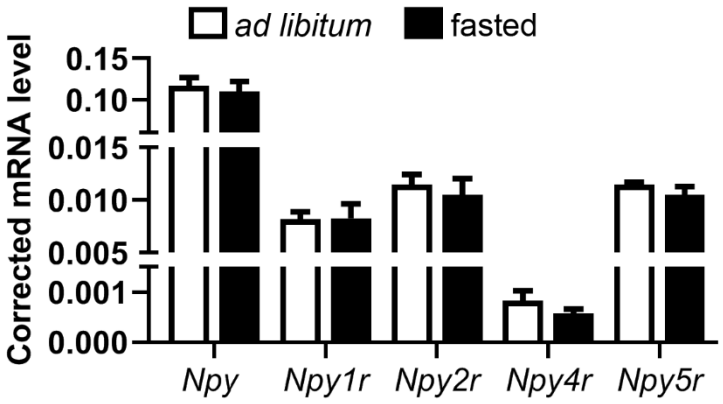

Figure 2. A 24-hour fast does not alter *Npy*- or *Npyr* expression in the LHA. At ZT3, *Npy*- and *Npyr* expression in the LHA are not altered by a 24-hour fast compared to *ad libitum*-fed controls (all p > 0.05).

A 24-hour fast does not alter *Npy*- or *Npyr* expression in the VTA

When measured at ZT3, unpaired t-test analyses revealed that a 24-hour fast did not alter VTA *Npy-*, *Npy1r-*, *Npy2r-*, *Npy4r-*, or *Npy5r* expression in 24 hour-fasted rats compared to *ad libitum*-fed controls (see Figure 3A, and Table S1).

When measured at ZT10, unpaired t-test analyses revealed that a 24-hour fast also did not alter VTA *Npy-*, *Npy1r-*, *Npy2r-*, *Npy4r-*, or *Npy5r* expression in 24 hour-fasted rats compared to *ad libitum*-fed controls (see Figure 3B, and Table S1).

A 24-hour fast does not alter *Npy*- or *Npyr* expression in the NAc

When measured at ZT3, unpaired t-test analyses revealed that a 24-hour fast showed a trend to decrease NAc *Npy5r* mRNA ($t_{7,7}$ = 1.98, p = 0.07; see Figure 4A, and Table S1). A 24-hour fast did not affect NAc *Npy-*, *Npy1r-*, or *Npy2r* mRNA expression (all p > 0.05; see Figure 4A pg. 75, and Table S1). Expression of *Npy4r* was not detectable in the NAc at ZT3.

When measured at ZT10, unpaired t-test analyses revealed that a 24-hour fast did not affect NAc *Npy-*, *Npy1r-*, *Npy2r-*, or *Npy5r* mRNA expression (all p > 0.05; Figure 4B pg. 75, and Table S1). Expression of *Npy4r* was not detectable in the NAc at ZT10.

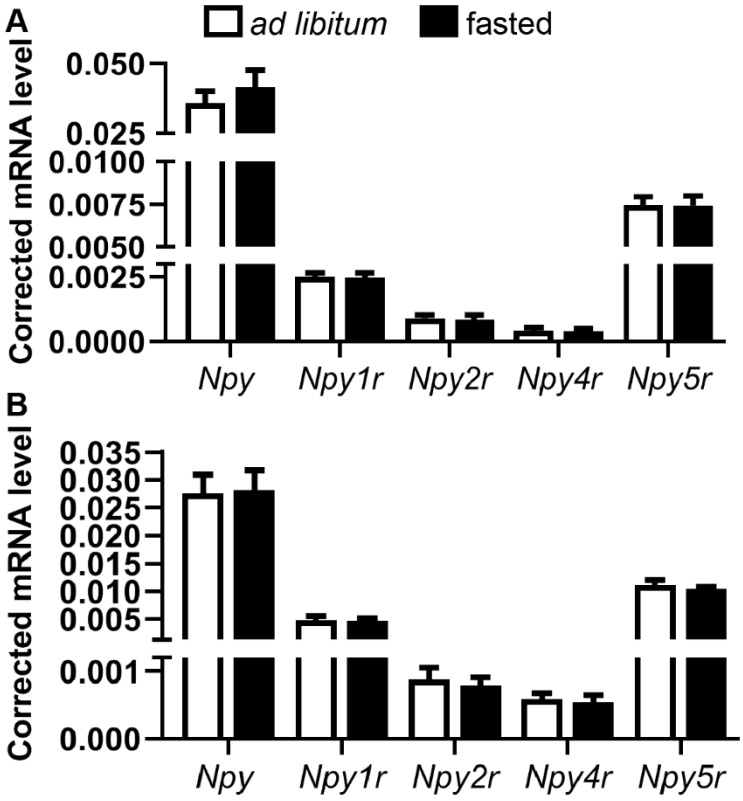

Figure 3. A 24-hour fast does not alter *Npy*- or *Npyr* expression in the VTA. A) VTA *Npy*- and *Npyr* mRNA expression are unaffected by a 24-hour fast at ZT3, nor **B)** at ZT10. All $p > 0.05$.

Discussion

The aim of this study was to determine if the expression of *Npy* and the *Npyr* in the LHA, VTA, and NAc are altered following a 24-hour fast. We found that Arc *Npy* expression is increased, whereas Arc *Npy1r* expression is decreased after a 24-hour fast compared to controls when measured early in light period. We also found a trend for NAc *Npy5r* expression to decrease compared to controls at this time point. When measured just prior to the onset of the dark period, a 24-hour fast lowered Arc *Npy2r* expression compared to controls, whereas no effects were observed in the NAc. Finally, a 24-hour fast did not alter *Npy*- or *Npyr* expression in the LHA or VTA either early in the light period, or just prior to the onset of the dark period. Thus, our findings show that a 24-hour fast can alter *Npy*- and *Npyr* expression in the Arc, and possibly the NAc, without any effects in the LHA and VTA.

Effects of the 24-hour fast on Arc NPY-related gene expression

The 24-hour fast altered *Npy*-, *Npy1r*- and *Npy2r* expression, but not *Npy4r*- or *Npy5r* expression in the Arc. The responsiveness of Arc *Npy* expression and Arc NPY levels to fasting at a time point when *Npy* levels are generally low (i.e. after the dark phase when rats generally have been eating; [Akabayashi et al., 1994]). The factors involved in mediating the

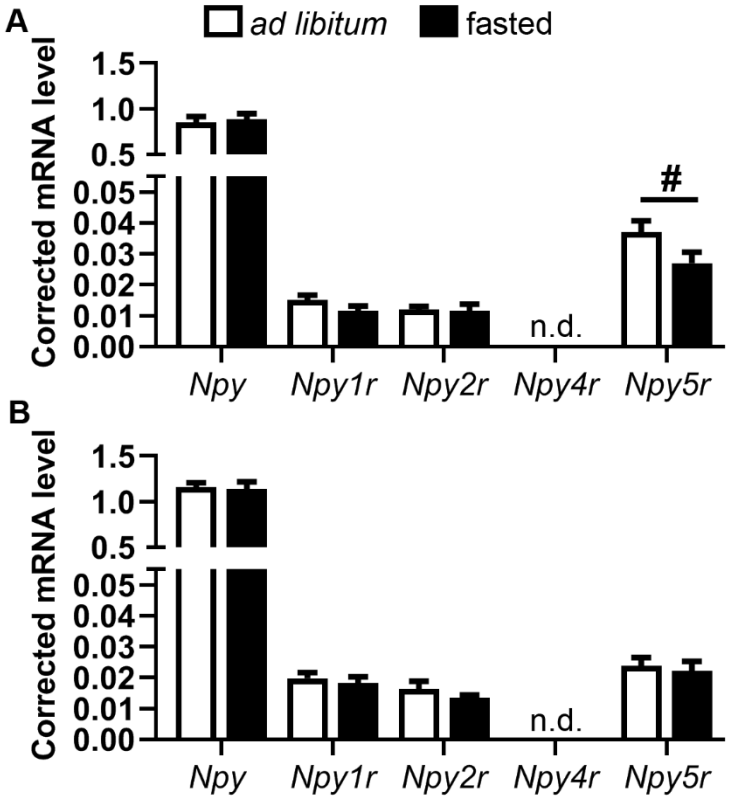

Figure 4. A 24-hour fast does not affect *Npy*- or *Npyr* expression in the NAc. A) At ZT3, there is statistical trend for decreased NAc *Npy5r* mRNA ($t_{7,7}$ = 1.98, p = 0.07), and no effect of a 24-hour fast on NAc *Npy-, Npy1r-*, *Npy2r-*, or *Npy4r* gene expression. **B)** At ZT10, Nac *Npy-* and *Npyr* expression are not affected by a 24-hour fast. # = statistical trend, n.d. = not detected.

effects of fasting on Arc NPY neurons have been described in detail. For example, energy status-relaying hormones, such as insulin, leptin and ghrelin, and metabolic signals, such as glucose, can modulate activity of Arc NPY neurons (Kohno & Yada, 2012). have been previously described (Hahn et al., 1998; Marks et al., 1992). Here, we confirm the effects of a 24-hour fast on Arc *Npy* expression. Because intracerebral administration of exogenous NPY promotes caloric consumption (Clark, Kalra, Crowley, & Kalra, 1984; Stanley, Chin, et al., 1985), it is likely that elevated *Npy* expression in the Arc drives caloric consumption during refeeding following a fast. Unexpectedly, Arc *Npy* expression just prior to the onset of the dark period was not altered by a 24-hour fast. We hypothesize that this can be explained by the day-night rhythm in Arc *Npy* expression. Just prior to dark phase onset, Arc *Npy* levels are generally elevated (Akabayashi et al., 1994), which prepares the nocturnal animal for dark time feeding and behavioral activity. This natural elevation in Arc *Npy* expression might potentially mask the 24-hour fast-mediated elevations in Arc *Npy* expression. In addition, there is a difference in the relative deprivation state of the fasted groups versus their respective controls. Rats feed predominantly during the dark period; therefore, control rats

that are sacrificed just after the dark period will be more satiated than control rats that are sacrificed just before the onset of the dark period.

Previous studies on the effects of acute fasting on *Npyr* have been limited to the Arc *Npy1r* and *Npy2r*. A severe acute fast of 48 hours, decreased NPY1R peptide and *Npy1r* mRNA, and did not affect NPY2r peptide or *Npy2r* mRNA levels in the Arc (X. Cheng et al., 1998). Accordingly, we found that Arc *Npy1r* mRNA was decreased after 24-hours of fasting at the beginning of the light period. Lower Arc *Npy1r* levels combined with lower NPY1R levels has been suggested to indicate a mechanism to protect against increased NPY signaling as seen after fasting (X. Cheng et al., 1998). However, studies also indicate that the NPY system seems relatively insensitive to desensitization as repeated intracerebral infusions of NPY elicit orexigenic responses of the same magnitude (Paez & Myers, 1991; Stanley et al., 1986). As NPY1R are found predominantly on proopiomelanocortin (POMC) neurons in the Arc, which is a separate population from the Arc NPY neurons (Broberger, Landry, Wong, Walsh, & Hokfelt, 1997; R. D. Cone, 2005), and activation of NPY1R inhibits POMC neurons (Ghamari-Langroudi et al., 2005; Roseberry et al., 2004), a downregulation of *Npy1r* after fasting seems paradoxical. Knock-down of the *Npy1r* specifically in the Arc by siRNA was shown not to affect feeding behavior (Higuchi, 2012). However, the effects on fasting-induced refeeding were not examined (Higuchi, 2012). Future studies could investigate the role of Arc NPY1R specifically in fasting-induced refeeding to provide insight into the functional implications of this downregulation.

In addition, Arc *Npy2r* mRNA decreased with fasting when measured just prior to the onset of the dark period. The NPY2R is found predominantly as a pre-synaptic receptor on Arc NPY neurons, and is thought to function as an auto-receptor to inhibit NPY release (Broberger et al., 1997). Accordingly, NPY2R knockdown transiently increases fasting-induced refeeding in mice (Qi, Fu, & Herzog, 2016). Lowered Arc *Npy2r* mRNA at a time when a rat's physiology is preparing for dark-time feeding, may indicate increased turnover of NPY2R to respond to increased NPY release. However, in the absence of NPY2R peptide data, a different explanation cannot be ruled out. Indeed, the study by X. Cheng et al. (1998), did not find a difference in Arc NPY2R levels after a 48-hour fast, and Arc siRNA knockdown of *Npy2r* Arc did not affect feeding (Higuchi, 2012). However, it is likely that the NPY2R levels were measured at the beginning of the light period as their results on NPY1R/*Npy1r* correspond to our data at the beginning of the light period. In addition, the effect of Arc *Npy2r* knockdown on fasting-induced refeeding was not examined. Future studies could assess NPY2R peptide levels prior to the start of the dark period, or receptor turnover rates as well as the effects of Arc *Npy2r* knockdown on fasting-induced refeeding to ascertain our hypothesis on NPY2R turnover.

Prior studies have not reported on the effects of fasting on expression of other NPYR in the Arc. We show that Arc *Npy4r*- and *Npy5r* mRNA expression are not altered after a short acute fast when measured at two time points. Accordingly, Arc-specific siRNA knockdown of

Npy4r did not affect feeding (Higuchi, 2012), whereas the behavioral effect of activation of NPY4R specifically in the Arc has not been investigated. We here show that the NPY4R is expressed in very low quantities in the Arc, but may be regulated as expression was undetectable at ZT3, and detectable at ZT10. The NPY4R is more sensitive to pancreatic polypeptide (PP) than it is to NPY, which is a member of the PP-fold family of peptides like NPY, and released by the pancreatic islets of Langerhans in response to a meal (Bard, Walker, Branchek, & Weinshank, 1995; Gerald et al., 1996; G. Katsuura et al., 2002; Lundell et al., 1995). Together, these findings may indicate that the NPY4R is less likely to be involved in mediating the effects of fasting in the Arc. However, future studies should investigate if Arc-specific NPY4R activation or -knockdown affects fasting-induced refeeding. Lastly, the NPY5R is implicated in mediating the orexigenic effects of NPY, but NPY5R knockout mice do not show altered fasting-induced refeeding (Maclean, Bergouignan, Cornier, & Jackman, 2011; Marsh et al., 1998; Nguyen et al., 2012). Arc-specific siRNA knockdown of NPY5R has not been reported on (Higuchi, 2012), however intracerebroventricular infusion of antisense deoxynucleotides for *Npy5r* inhibits fasting-induced refeeding (Schaffhauser et al., 1997). Therefore, a role of Arc NPY5R in fasting-induced refeeding cannot be excluded, and should be addressed by future studies.

A 24-hour fast does not affect *Npy*- or *Npyr* expression in the LHA, VTA or NAc

We investigated if the LHA NPY system would be responsive to the effects of an acute fast. A previous study reported no difference in NPY peptide after an acute fast when measured just after the start of the light period (Beck, Jhanwar-Uniyal, et al., 1990). Here, we show that a similar acute fast does not affect *Npy* mRNA levels in the LHA. As NPY peptide increased after refeeding in the same study (Beck, Jhanwar-Uniyal, et al., 1990), it is, however, clear that the NPY system in the LHA is responsive to energetic status. *Npy* mRNA levels are quite low in the LHA, but observations from our lab and others have placed NPY neurons in a posterior region of the LHA (Abrahamson & Moore, 2001; Grove, Brogan, & Smith, 2001; Chapter III, this thesis). Though *Npy*-expressing glucose-sensing neurons have been reported in the mouse LHA (Marston et al., 2011), no studies to date have investigated if they constitute a glucose-sensing population in the rat. A different source for LHA NPY comprises the Arc NPY neurons (Broberger, De Lecea, et al., 1998; Elias et al., 1998). NPY peptide release in the LHA may thus be altered corresponding to the changes observed in Arc NPY expression after changes in energetic status (Hahn et al., 1998; Marks et al., 1992). Though NPY release has not been measured in the LHA after a physiological challenge to energy balance, it has been shown that the density of LHA NPY-immunoreactive fibers is affected by scheduled-feeding (Ramirez-Plascencia, Saderi, Escobar, & Salgado-Delgado, 2017). We did not find a difference in *Npyr* mRNA expression in the LHA, indicating that if NPY release in the LHA is changed after an acute fast, changes in NPYR are not found at the gene expression level. Future studies should

assess LHA NPY peptide release in response to physiological challenges as well as assess if NPYR are changed at the peptide level.

We also hypothesized that the VTA NPY system would be responsive to the effects of an acute fast. In the VTA, *Npy* expression is low or non-detectable under standard physiological circumstances (M. C. R. Gumbs et al., 2019). Here, we extend those observations and show that *Npy* expression is not affected by an acute fast of 24 hours. We have previously reported that the low level of *Npy* mRNA measured in the VTA is probably due to contamination of the VTA brain tissue punch, which may contain a part of the posterior LHA or supramammillary nucleus (M. C. R. Gumbs et al., 2019). NPY neurons in the Arc and hindbrain project to the VTA, and both populations are responsive to alterations in the physiological state (M. C. R. Gumbs et al., 2019; Hahn et al., 1998; A. J. Li & Ritter, 2004; Marks et al., 1992). Increased NPY levels in the Arc are accompanied by increased NPY release in the PVN, one of the Arc NPY neuronal output structures, after an acute fast (Dube et al., 1992; Hahn et al., 1998; Marks et al., 1992). In addition, it has been shown that NPY can affect local neurotransmission and motivational behaviors when infused into the VTA (Pandit et al., 2014a; K. S. West & Roseberry, 2017). Therefore, we also hypothesized that *Npyr* expression would be altered in the VTA of fasted rats. However, we did not find any changes in *Npyr* expression in the VTA. It may be that the effect of fasting on the VTA NPY system is not directly apparent in gene expression levels, and rather that NPY- or NPYR peptide levels or NPY release are altered. Alternatively, the effects of fasting on motivation may be mediated directly in the VTA, for instance via changes in dopamine neurons or changes in hormone levels such as corticosterone, insulin, ghrelin or leptin that can directly affect dopamine neurons, thereby circumventing the NPY system (Bruijnzeel, Corrie, Rogers, & Yamada, 2011; J. J. Cone, McCutcheon, & Roitman, 2014). Future studies could address these possibilities.

Lastly, it was expected that fasting would affect the NAc NPY system based on similar reasoning as for the LHA and VTA. Indeed, NAc NPY infusion can increase palatable feeding and the motivation to obtain food (Pandit et al., 2014a; van den Heuvel et al., 2015), which may be mediated by NPY release from local neurons as well as release from an NPY Arc->NAc projection (van den Heuvel et al., 2015). However, our data indicate that fasting does not affect NAc *Npy*- or *Npyr* expression. Only a trend for a decrease in *Npy5r* mRNA expression was observed. It may, however, be that changes in *Npy*- or *Npyr* expression are occluded by inclusion of the entire structure in brain punches. Indeed, the NPY system in the NAc interacts reciprocally with the mesolimbic dopaminergic system (Midgley et al., 1994; Quarta et al., 2011; Sorensen et al., 2009), and dopamine modulation of NAc NPY expression can be region-specific along the rostro-caudal axis (Midgley et al., 1994). Techniques allowing a higher resolution to address region-specific changes may thus observe differences in the NAc NPY system after fasting. In addition, as with the LHA, and VTA, measuring NPY peptide levels and/or -release should also be explored.

Summary

This is the first study to report expression for *Npy* as well as all *Npyr* after an acute fast in hunger/satiety-related and reward-related areas in the brain. Arc NPY-related gene expression was altered by energetic state, whereas in the LHA as well as in the VTA and NAc, NPY-related gene expression was not altered by energetic state. Our data thus indicate that the Arc is the primary region where the energetic physiological state after fasting affects NPY-related gene expression. These data therefore provide evidence that the Arc NPY system is mainly concerned with sensing physiological state directly, which fits with the Arc's location near the partial blood-brain-barrier and its function in sensing energy. Future studies that determine NPY peptide levels or -release in the LHA, and in the VTA and NAc of the mesolimbic dopamine system, will help to elucidate if the Arc NPY neurons convey NPYergic information on energetic state to these downstream regions to promote adaptive behaviors such as increased intake and/or motivation.

Author contributions and acknowledgements

Myrtille Gumbs designed the RT-qPCR experiment, Ewout Foppen performed the animal experiments, Myrtille Gumbs and Unga Unmehopa performed the laboratory techniques, Myrtille Gumbs wrote the manuscript, and all authors including Joram Mul, Jan Booij and Susanne la Fleur critically reviewed the manuscript.

Supplemental data

Table S1. Normalized gene expression levels and statistical details.

			Normalized gene expression level		Statistics
			Ad libitum	**Fasted**	
Arc	**ZT3**	*Npy*	2.60 ± 0.17	3.77 ± 0.17	$t_{5,5}$ = 4.83, p = 0.0007*
		Npy1r	0.05 ± 0.003	0.03 ± 0.005	$t_{7,7}$ = 2.30, p = 0.04*
		Npy2r	0.04 ± 0.002	0.04 ± 0.004	$t_{7,7}$ = 1.20, p > 0.05
		Npy4r	n.d.	n.d.	-
		Npy5r	0.04 ± 0.003	0.03 ± 0.003	$t_{7,7}$ = 0.64, p > 0.05
	ZT10	*Npy*	1.05 ± 0.07	0.95 ± 0.06	$t_{7,7}$ = 1.06, p > 0.05
		Npy1r	0.02 ± 0.0005	0.02 ± 0.0002	$t_{7,7}$ = 0.39, p > 0.05
		Npy2r	0.02 ± 0.001	0.01 ± 0.0002	$t_{7,7}$ = 2.22, p = 0.04*
		Npy4r	0.0009 ± 0.0002	0.0006 ± 0.0001	$t_{6,6}$ = 1.50, p > 0.05
		Npy5r	0.02 ± 0.001	0.02 ± 0.001	$t_{7,7}$ = 1.90, p > 0.05
LHA	**ZT3**	*Npy*	0.12 ± 0.01	0.11 ± 0.01	$t_{6,6}$ = 0.46, p > 0.05
		Npy1r	0.008 ± 0.0007	0.008 ± 0.001	$t_{6,7}$ = 0.06, p > 0.05
		Npy2r	0.01 ± 0.001	0.01 ± 0.002	$t_{7,7}$ = 0.54, p > 0.05
		Npy4r	0.0008 ± 0.0002	0.0006 ± 0.00009	$t_{5,7}$ = 1.29, p > 0.05
		Npy5r	0.01 ± 0.0003	0.01 ± 0.0008	$t_{7,7}$ = 1.17, p > 0.05
VTA	**ZT3**	*Npy*	0.004 ± 0.004	0.04 ± 0.0006	$t_{5,6}$ = 0.78, p > 0.05
		Npy1r	0.003 ± 0.0002	0.002 ± 0.0002	$t_{6,6}$ = 0.12, p > 0.05
		Npy2r	0.0009 ± 0.0001	0.0008 ± 0.0002	$t_{6,6}$ = 0.29, p > 0.05
		Npy4r	0.0004 ± 0.0008	0.0004 ± 0.00008	$t_{6,6}$ = 0.02, p > 0.05
		Npy5r	0.007 ± 0.0005	0.007 ± 0.0006	$t_{7,6}$ = 0.03, p > 0.05
	ZT10	*Npy*	0.04 ± 0.004	0.04 ± 0.006	$t_{5,6}$ = 0.78, p > 0.05
		Npy1r	0.005 ± 0.0007	0.005 ± 0.0005	$t_{7,7}$ = 0.14, p > 0.05
		Npy2r	0.0009 ± 0.0002	0.0009 ± 0.0001	$t_{7,7}$ = 0.41, p > 0.05
		Npy4r	0.0006 ± 0.00008	0.0005 ± 0.0001	$t_{6,6}$ = 0.37, p > 0.05
		Npy5r	0.01 ± 0.0009	0.01 ± 0.0003	$t_{6,7}$ = 0.68, p > 0.05
NAc	**ZT3**	*Npy*	0.85 ± 0.006	0.89 ± 0.06	$t_{7,6}$ = 0.39, p > 0.05
		Npy1r	0.02 ± 0.002	0.01 ± 0.002	$t_{7,7}$ = 1.65, p > 0.05
		Npy2r	0.01 ± 0.001	0.01 ± 0.002	$t_{7,7}$ = 0.18, p > 0.05
		Npy4r	n.d.	n.d.	-
		Npy5r	0.04 ± 0.004	0.03 ± 0.004	$t_{7,7}$ = 1.98, p = 0.07
	ZT10	*Npy*	1.16 ± 0.05	1.14 ± 0.07	$t_{3,4}$ = 0.25, p > 0.05
		Npy1r	0.02 ± 0.002	0.02 ± 0.002	$t_{4,4}$ = 0.52, p > 0.05
		Npy2r	0.02 ± 0.003	0.01 ± 0.0008	$t_{4,3}$ = 0.95, p > 0.05
		Npy4r	n.d.	n.d.	-
		Npy5r	0.02 ± 0.003	0.02 ± 0.003	$t_{4,4}$ = 0.39, p > 0.05

Arc = arcuate nucleus of the hypothalamus, LHA = lateral hypothalamic area, NAc = nucleus accumbens, n.d. = not detectable, *Npy1r/Npy2r/Npy4r/Npy5r* = Neuropeptide Y receptor subtype, VTA = ventral tegmental area, * p < 0.05.